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Research article

Suitability of pitaya fruit fermented by sourdough LAB strains for bread making: its impact on dough physicochemical, rheo-fermentation properties and antioxidant, antifungal and quality performance of bread

Jacob Ojobi Omedi [a], Jing Huang [a], Weining Huang [a,c,*], Jianxian Zheng [b,c,**], Yongqing Zeng [b], Binle Zhang [a,c], Liyuan Zhou [c,d], Faqun Zhao [c], Ning Li [c], Tiecheng Gao [c]

[a] State Key Laboratory of Food Science and Technology, Laboratory of Baking and Fermentation Science, Cereals/Sourdough and Ingredient Functionality Research, Jiangnan University, Wuxi 214122, China
[b] College of Food and Bioengineering, South China University of Technology, Guangzhou Institute of Food Industry, Guangzhou, 510000, China
[c] Fujian Zunjin Health Science and Technology Co., Ltd., IBF International Inc., Quanzhou, Fujian 362200, China
[d] Shandong Daoxiancun Food Industry Co., Ltd., Heze, Shandong 274000, China

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ABSTRACT

The objective of this study was to investigate the suitability of incorporating pitaya fruit fermented by antifungal LAB strains *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus* at 1: 30 °C for 24h or 2: 31 °C for 19.5h as an ingredient with respect to bread making performance and bio-preservation effect. Underlying mechanisms related to gluten protein hydrolysis, starch hydrolysis, and yeast activity in dough were explored. The antioxidant activity, antifungal activity and bread making performance of the resulted breads were analyzed. Also, the antifungal phenolic acids in the breads were identified and quantified. Incorporation of fermented substrates in dough increased yeast activity and gas production capacity, but decreased gas retention capacity. This was attributed to increased dough acidity after incorporating fruit substrates. As a result, reducing sugar and free sulfhydryl (SH) groups increased in these doughs which indicated higher starch and gluten protein hydrolysis, respectively. However, SH groups increased at lower rate in presence of substrates fermented by *L. plantarum* and *P. pentosaceus* at condition 2 than 1. This could be due to improvement of gluten network as revealed by decreased α-helix (%) and increased β-turn (%) in secondary gluten structures in these doughs which subsequently resulted in more homogeneous microstructural properties than in presence of unfermented substrate compared to wheat dough. Subsequently, bread specific volume increased (6.6–20.0%) in presence of fermented substrates, especially fermented by *L. plantarum* at (2). Moreover, bread incorporated with fermented substrates (*P. pentosaceus* than *L. plantarum* at 1 than 2) had enhanced antioxidant activities, lower fungal growth rates based on challenge tests and mold free shelf life. Antifungal phenolic acids such as gallic acids, caffeic acid, protocatechuic acid were only detected in bread incorporated with fruit substrates, and their total content higher in fermented substrates.

1. Introduction

Bread is viewed globally as one of the staple foods which is generally characterized as a highly perishable product. This is mainly attributed to physicochemical changes like staling and firming, and microbiological spoilage which reduce the product shelf life (He and Hoseney, 1990; Pitt and Hocking, 2009). Fungal spoilage of bread has significantly contributed to economic losses in the baking industry subsector (Morassi et al., 2018; Suhr and Nielsen, 2004). The fungal species commonly involved in spoilage are *Penicillium*, *Aspergillus*, *Fusarium*, *Mucor*, *Cladosporium*, and *Rhizopus* (Dijksterhuis, 2017; Pitt and Hocking, 2009). Traditionally, artificial chemical preservatives such as salts of weak organic acids like propionic acids and sorbic acids have been commonly used to control fungal spoilage of bread (Dijksterhuis, 2017; Molina and Giannuzzi, 2000). In recent years, increased consumer awareness of health-related hazards of food chemical preservatives has resulted in a trend towards increased preference of natural preservative free processed foods (de Boer and

* Corresponding author.
** Corresponding author.
E-mail addresses: wnhuang@jiangnan.edu.cn (W. Huang), fejxzhen@scut.edu.cn (J. Zheng).

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Schösler, 2016). In response, the industry has increased efforts to reduce the amount of chemical preservatives in baked products through use of natural alternatives.

Natural bio-preservation techniques have been explored for use in the food industry (Mogoşanu et al., 2017). The use of microorganisms with antifungal properties and/or their metabolites have increasingly been explored as alternatives for bio-preservation of baked products (Belz et al., 2018; Mogoşanu et al., 2017). Due to their "generally regarded as safe" (GRAS) status, lactic acid bacteria (LAB) strains are the preferred choice of microorganisms for food bio-preservation (Crowley et al., 2013; Yépez et al., 2017). The bio-preservative effects were attributed to diverse antimicrobial active metabolites hydrolyzed and released in LAB fermented ingredient substrates (Gänzle et al., 2009). In a study by Axel et al. (2016), carboxylic acids produced by *Limosilactobacillus reuteri* and *Lactobacillus brevis* extended shelf-life of quinoa and rice-based sourdough bread. Similarly, Rizzello et al. (2011) found that a mixture of organic acids and peptides were synthesized in *Lactiplantibacillus plantarum* and *Lactobacillus rossiae* fermented wheat germ sourdough which consistently increased bread shelf life. LAB fermentation of fruit substrates has been shown to enhance contents of phenolic acids such as salicylic acid, and caffeic acid (Septembre-Malaterre et al., 2018). These phenolic acids could enhance the antifungal properties of the fermented substrate (Amborabé et al., 2002; Ma and Ma, 2014).

Fruits are common parts of the human balanced diet which are as sources of micronutrients, provide energy for metabolic processes and are precursors for protein synthesis (Liu, 2013). Pitaya natively grown in Central and South America, has increasingly been cultivated in other parts of the world. Due to its exotic appearance, rich nutritional value, and health properties, global interest in adding value to pitaya has significantly increased (García-Cruz et al., 2017; Hua et al., 2018). Given that fruit components have been incorporated in bakery products (Agudelo et al., 2015), this provides room for development of innovative baked products with a wide range of functional and therapeutic properties.

Bread is a baked product that is viewed globally as a staple food and priced highly for its sensory properties such as taste, texture and aroma. However, bread is a highly perishable product (Pitt and Hocking, 2009). In our previous study (Omedi et al., 2019), several antifungal phenolic acid contents were found to be enhanced after pitaya substrate fermentation by sourdough LAB strains. Moreover, in a similar study (He, 2017), typical 20% substitution rate of sourdough fermented by antifungal LAB strains were found to confer antifungal benefits through reduced proliferation of spoilage fungi in steamed cake. In this study, an initiative to incorporate LAB strain fermented pitaya substrate as a potential bio-preservative ingredient in wheat dough and bread system was taken. Therefore, the objective of this study was to investigate the suitability of incorporating pitaya substrate fermented at either 1: 30 °C, 24h or 2: 31 °C, 19.5h by two antifungal LAB strains *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus* as an ingredient with respect to bread making performance and bio-preservation effect. To ensure higher specific volume, 20% w/w (flour basis) optimum concentration was determined in preliminary experiments using unfermented pitaya substrate. The effects of LAB strain and fermentation condition of substrate on dough were studied by taking into account the underlying mechanisms related to gluten protein hydrolysis, starch hydrolysis, and yeast activity through observing changes in free sulfhydryl (SH) groups, secondary structures of gluten, reducing sugars content, rheo-fermentation properties and microstructure properties of dough. The resulted breads were characterized based on quality, antioxidant activity based on 2, 2-Azino--bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 1,1-diphenyl-2-picrylhydrazyl (DPPH), antifungal activity using mold free shelf life and challenge tests. Furthermore, the antifungal phenolic acids in breads were identified and quantified.

2. Materials and methods

2.1. Chemicals and materials

Pitaya fruit obtained from local supermarket (Wuxi, China) was used to prepare the substrate. External standards for phenolic acid quantification by HPLC method were of purity: ≥97%, and included caffeic, protocatechuic, ferulic, gallic, vanillin, syringic, chlorogenic, and p-hydroxybenzoic acids (Sinopharm Chemical Reagent Co. Ltd). Other chemicals used were of analytical grade.

2.2. Microorganisms, culture media and growth conditions

LAB strains *L. plantarum*, AS 2-11 (MW602529) and *P. pentosaceus*, BS0-14 (MW602530), and indicator mold strains *Aspergillus niger, Cladosporium sphaerospermum* and *Penicillium chrysogenum* were obtained from culture collection of Laboratory of Baking and Fermentation Science, Cereals/Sourdough and Ingredient Functionality Research, Jiangnan University. For use as inoculum, frozen stocks of LAB strains in MRS medium supplemented with 40% glycerol (w/v) at -80 °C were cultured twice on MRS broth at 30 °C for 24h, then cultured to their late exponential phase, harvested, and washed twice in sterile NaCl solution. Mold strains from frozen stocks stored at -80 °C in YPD and 60% glycerol were activated by culturing overnight in malt extract broth at 25 °C. They were further plated on malt extract agar and incubated at 25 °C for 3–7 days. For use in antifungal tests, fungal spore suspensions at known concentration were prepared by brushing the plate surface with sterile NaCl using a sterile swab. Spores were re-suspended in sterile NaCl containing 0.1% Tween 80 and stored at 4 °C until further use.

2.3. Fruit preparation and fermentation

Pitaya fruit was homogenized into a puree using a food blender. Fruit puree (100g) was weighed into 250mL Erlenmeyer flask, sealed and sterilized (121 °C, 10min), followed by cooling for 60min. The substrate was then inoculated with cultures (10^6 CFU/mL) of *L. plantarum* or *P. pentosaceus* and fermented at 30 °C for 24h (1) or 31 °C for 19.5h (2) (Design Expert, V.8.0.6).

2.4. Dough and bread preparation with LAB fermented substrate

Experimental doughs were prepared by replacing 20% wheat flour with substrates fermented by *L. plantarum*: LF1 and LF2 and *P. pentosaceus*: PF1 and PF2 at (1) or (2). Ingredients used were: 240g wheat flour (7.9% moisture content), 105g tap water, 3g yeast, 1.5g salt, 18g sugar, 48g fermented substrate (86.0% moisture content), and 12g butter. Two controls, dough prepared with all ingredients with unfermented instead of fermented substrate used (CuF), and wheat dough (Cw) prepared with wheat flour (300g), water (180g), yeast (3g), salt (1.5g), sugar (18g), and butter (12g). Straight dough method procedure was used in bread making process. All weighed ingredients, except butter, were mixed in a spiral mixer (Sinmag, Wuxi, China) at slow speed (3min), and fast speed (1min). Butter was then added and mixed at slow speed (3min) and fast speed (1min). After mixing, dough was covered with polyethylene film and rested (5min) at room temperature. Dough was then divided (90g piece), rounded, and rested (5min), followed by shaping and transfer into baking pans, then proofed (Sinmag, Wuxi, China) (90min, 38 °C, 85% RH). This was followed by baking in a preheated oven (Sinmag, Wuxi, China) (top: 190 °C and bottom: 220 °C) (Li et al., 2011) for 21min. After baking, respective breads were cooled for 2h at room temperature, then analyzed.

2.5. Dough analysis

2.5.1. Physicochemical characteristics of dough prepared with fermented substrate

LAB cell counts were determined after mixing, before and after proofing of dough. Ten grams of dough was homogenized with 90mL distilled water, followed by gradient dilution in sterile NaCl solution, and plating on MRS agar and incubated at 30 °C for 48h. Plating was done in triplicate. For pH and TTA of dough, 10g of sample was homogenized with 90mL of distilled water. The pH was recorded by a pH meter (Mettler Toledo, China), while TTA value was expressed as the titration volume of 0.1N NaOH needed to reach a final pH of 8.6. Cells counts, pH and TTA analysis was also done on the unfermented and LAB fermented fruit substrate.

2.5.2. Rheo-fermentation properties of dough

The rheo-fermentation properties of dough were determined using a rheo-fermentometer F3 (Chopin, Villeneuve-La-Garenne Cedex, France) as previously described by Tang et al. (2018) with some modification. Briefly, 300g of dough was placed in the fermentation chamber and a 2000g cylindrical weight was placed on top. The dough was evaluated at 30 °C for 3h. All tests were performed in triplicate. Gas production and dough development characteristics were recorded.

2.5.3. Determination of free sulfhydryl (SH) group content in dough

The content of free SH groups in dough were determined following Ellman's reagent (5,5′-dithiobise-2-nitrobenzoic acid, DNTB) assay as described by Beveridge et al. (1974) with modifications. Briefly, doughs (30mg) were suspended in 3mL of solution of 0.1M Sodium phosphate buffer pH 8.0 containing 0.5 mg/mL EDTA. The dispersions were oscillated at 25 °C for 30min for solubilization. After solubilization, 30μL of 4 mg/mL DNTB was incubated and oscillated for 30min in the dark at 25 °C, followed by centrifugation (10000×g, 10min). Absorbance of supernatant was measured at 412nm against the blank. For quantification, a standard curve determined with reduced glutathione was used. Each sample was measured in triplicate.

2.5.4. Secondary structure of gluten using Fourier transform infrared spectroscopy (FTIR)

Secondary structure of gluten in doughs was determined by FTIR spectrometer (NEXUS, Thermo., US). Lyophilized dough samples were mixed with potassium bromide at a ratio 1:100 (w/w) and grinded into powder in an agate mortar and pestle set. Spectra of each sample were recorded from 4000 to 400 cm^{-1} at 32 scans and $4cm^{-1}$ resolutions, and collected by OMNIC software (version 8.2, Thermo Nicolet Corp) and analyzed by PeakFit Software (Version 4.12, Systat Software, Inc., USA).

2.5.5. Reducing sugar content in dough

Reducing sugar content in dough was measured by DNS method (Miller, 1959) and assayed as reported by Reshmi et al. (2017) and Su et al. (2019) with some modifications. Briefly, 5g of sample was mixed with 10mL distilled water, homogenized at 200 rpm at constant temperature oscillation incubator. Supernatants of substrates were obtained after centrifugation (10000g, 15min, 4 °C), filtered (0.45μm). The supernatant (150μL) was mixed with 1mL DNS in test tubes, then placed in a boiling water bath for 10min and cooled for 10 min at room temperature. Each solution was further diluted with 2mL of distilled water, mixed thoroughly and absorbance at 540nm recorded using a spectrophotometer (TU-1810, Purkinje General Instrument Co., Ltd, Beijing, China). The percentage of reducing sugar content, based on glucose equivalents was calculated as shown in Eq. (1).

$$\text{Reducing sugar content } (\%) = \frac{\text{m x d}}{\text{M x 1000}} \text{ x 100} \quad (1)$$

where m is the reducing sugar content (mg) in the sample solution, d means dilution ratio, and M represents the mass of sample (g).

2.5.6. Microstructure analysis of dough using by scanning electron microscopy (SEM)

Microstructures of doughs were observed using SEM (Model S3400N VP, Hitachi, Japan). Doughs were freeze-dried, cut, gold sputter coated for 2min, and observed using SEM at an accelerating voltage of 3kV. Micrograph images were recorded at 600x and 1000x magnification.

2.6. Bread analysis

2.6.1. Specific volume of bread prepared with fermented substrate

Specific volume of bread was measured using the seed displacement method (AACC 10–05.01) 6h after baking. Specific volume of bread (mL/g) was calculated as shown in Eq. (2).

$$\text{Specific loaf volume} = \frac{\text{Volume of Loaf (mL)}}{\text{Weight of Loaf (g)}} \quad (2)$$

2.6.2. Textural characteristics of bread prepared with fermented substrate

Textural characteristics of bread was measured 2h after baking using a Texture Pro CT V 1.4 Build 17 (Brookfield Engineering Laboratory, Middleboro, MA, USA) in the texture profile analysis (TPA) test mode, consisting double compression test and equipped with an aluminum 36mm diameter cylindrical probe, following the procedure described by Tang et al. (2018).

2.7. Identification of phenolic acids in bread using reverse-phase (RP)-HPLC

RP-HPLC method was used to identify and quantify eight phenolic acids in methanolic extract of bread as described by Omedi et al. (2019). Data acquisition and integration was performed using Empower software package. Identification of phenolic acids was performed using external standards analyzed under same conditions.

2.8. ABTS and DPPH antioxidant activity of bread prepared with fermented substrate

The antioxidant activity of bread was determined using ABTS and DPPH assays as described by Sui et al. (2015) in methanolic extracts prepared as described by Omedi et al. (2019).

2.9. Challenge test and mold free shelf-life (MFSL) of bread

Antifungal activity of bread was determined on bread slices as described by Ryan et al. (2008) with some modifications. Uniform bread slices were sprayed on both sides with approximately 200μL of solution containing 10^6 fungal spores. Each slice was placed in a plastic bag and sealed; a small hole was drilled in each bag to ensure aerobic condition. The slices were then stored at room temperature (25 °C) for 5 days. Fungal out-growth on slice surfaces for each bread type was monitored for aerial mycelia and quantified daily. A series of 6 slices for each bread type was inoculated. Bread spoilage was evaluated based on percentage of total surface area of each slice where fungal outgrowth occurred. Bread slice was deemed positive if more than 1% of total surface area was covered with fungi.

MFSF of bread was determined on unchallenged slices sealed in plastic bags at stored at room temperature. The fungal colonies on bread surfaces were monitored daily for 5 days. Fungal contamination was calculated as described by (Sun et al., 2020). Briefly, no visible colonies (-), one colony (+), two (++), and three or more (+++) on bread surfaces were classified.

2.10. Statistical analysis

Results of three independent assays were presented as mean values. Data was compared using one-way analysis of variance (ANOVA), while multiple comparisons of data was performed by Duncan' test at $p < 0.05$ level of significance using SPSS 26.0.

3. Results and discussion

3.1. Physicochemical characteristics of doughs prepared with fermented substrate

Fruits are naturally acidic and when incorporated in a food matrix they can lower the system' pH (Agudelo et al., 2015). The physicochemical characteristics of fermented fruit substrates and doughs prepared with fermented fruit substrate were presented in Figure 1 and Figure 2, respectively. Compared with unfermented substrate (uF), pH values were significantly lower (3.58–3.74) while the TTA increased (except in PF2) (7.1–7.5mL) after LAB fermentation of the fruit substrates (Fig. 1A, B). Compared to Cw (4.96) and CuF (4.96), incorporation of fermented fruit substrate generally lowered pH of LF2, PF1, LF1, and PF2 to 4.18, 4.28, 4.30, and 4.44, respectively before proofing (Figure 2A). After proofing, dough pH increased by 4.0–19.5% in the range 4.8–5.35 with highest values observed in Cw, then CuF, PF2, PF1, LF2, and least in LF1 (Figure 2A). This showed that lower pH values were observed in doughs incorporated with substrates fermented by *L. plantarum* than *P. pentosaceus* at (1) than (2). For TTA, incorporation of fermented fruit substrate significantly increased its value in an opposite way relative to pH before proofing (Figure 2B). However, a significant increase and decrease were observed in Cw and doughs (LF2>LF1>PF2>PF1>CuF) prepared with fruit substrates, respectively, after proofing. Few known studies have investigated the effect of addition of LAB fermented fruit substrates in a bread dough system. Compared to sourdough-based dough systems, changes in pH and TTA of doughs incorporated with fermented substrates followed a similar change pattern (Arendt et al., 2007; Omedi et al., 2016). The changes in pH and TTA values before and after proofing of dough may be attributed to metabolites such as organic acids and phenolic acids naturally present or released in fruit substrates after LAB fermentation (Arendt et al., 2007; Rizzello et al., 2011). Furthermore, LAB cell counts were in the range 6.55–7.37 Log CFU/g before and after proofing of doughs with fermented fruit substrate. Higher counts were observed in LF1 and LF2 than PF1 and PF2, with no significant change observed before and after proofing (Figure 2C). This implied that incorporation of fermented fruit substrate acted as a dough acidifier and a carrier vehicle for LAB strains in dough which may enhance the techno-functional and bioprotective properties of dough and the resulting bread (Gänzle et al., 2009; Xu et al., 2019; Yépez et al., 2017).

3.2. Rheo-fermentation properties of doughs prepared with fermented substrate

The rheo-fermentation properties characteristics of dough that were influenced by incorporation of fermented substrates were presented in Table 1. Dough development properties included H_m and h for maximum and final height of dough development during fermentation, respectively, and H'_m, the height of maximum gas formation, was used as a measure of yeast activity. Gaseous release properties included V_t and RC, total gas volume and retention coefficient (percentage of retention volume to the total volume), respectively. Compared to Cw and CuF, H_m and h values in PF2 and LF1 significantly declined, while H'_m values increased (Table 1). This suggested that addition of substrates fermented by *P. pentosaceus* and *L. plantarum* at condition 2 and 1, respectively significantly affected yeast fermentation and dough development. A similar and opposite trend was observed in PF1 and LF2, respectively (Table 1). This implied that stronger acids were present in fermented fruit substrates incorporated in PF2, LF1, and PF1, than LF2. Dough acidification due to these acids may have increased repulsions of positively charged side groups of gluten causing increased gluten solubility which weakened the gluten network (Takeda et al., 2001). Therefore, $(H_m\text{-}h)/H_m$, fermentation tolerance was highest in PF2, LF1, and PF1, an indicator of unstable doughs due to weakened gluten network. Similarly, gluten network was found to be weakened in doughs prepared strong acids such as 0.3% malic acid (Su et al., 2019).

However, no significant effect on gluten network and dough stability was reported when acidification was by weak acids such as acetic acid (0.1%) and lactic acid (0.4%) (Su et al., 2019). Therefore, we suggested that dough development properties were sensitive to pH and acidity changes attributed to incorporation of fruit substrates. Compared to Cw, H'_m and V_t increased in similar proportions by 4.79–20.17% and 7.04–24.51%, respectively, in doughs incorporated with fruit substrates (Table 1). Increased content of fermentable sugars from added fruit substrates may have improved yeast activity and enhanced gas production during proofing (Gobbetti et al., 1995). Reflected by higher yeast activity (H'_m) in LF1 than LF2, then PF2 than PF1, the total gas volume produced was similarly higher (Table 1). However, compared to Cw (93.4%), incorporation of fruit substrates lowered gas retention coefficient (RC) by 3.85–13.6%. An opposite but similar trend between RC and H'_m was observed in dough after incorporation of fruit substrates. For instance, LF1 and PF2 had the highest yeast activity and total gas produced but their doughs exhibited lower gas retention coefficient than Cw. This indicated that incorporation of fermented fruit substrates could acidify dough resulting in increased yeast activity and gas production, but also weaken gluten network and reduce its ability to retain the produced gas. Similar observations were reported in acidified doughs (Arendt et al., 2007; Su et al., 2019). However, RC of LF2 and CuF doughs were higher, which may be attributed to improved dough stability and gluten network structure due to lower fermentation tolerance ($(H_m\text{-}h)/H_m$) (Table 1). Other interactions involving phenolic acids may have also stabilized dough and improved gluten network (Xu et al., 2019).

3.2.1. Changes in free SH group content in dough prepared with fermented substrate

The changes in SH groups were determined to indicate changes in gluten SS bonds of dough during mixing and dough fermentation. The

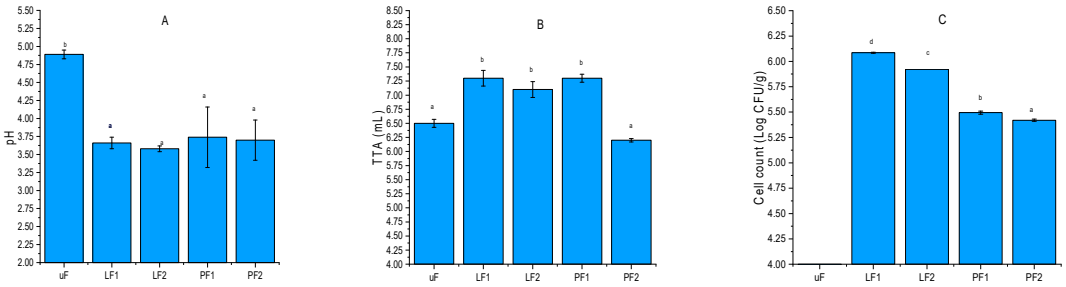


Figure 1. Physicochemical properties of fruit substrate. A) pH; B): TTA; and C: cell counts of unfermented fruit substrate (uF), fruit substrate fermented by *L. plantarum* at 30 °C for 24h (LF1), 31 °C for 19.5h (LF2) and *P. pentosaceus* at 30 °C for 24h (PF1), 31 °C for 19.5h (PF2).

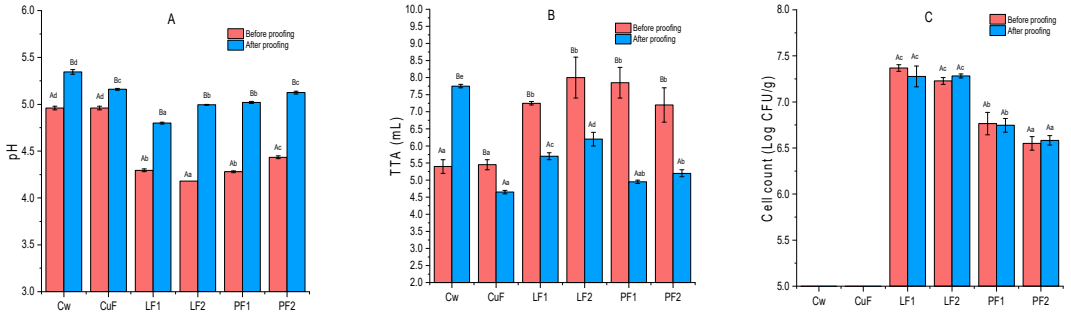


Figure 2. Physicochemical properties of doughs prepared with fermented fruit substrate. A): pH; B: TTA; and C: cell counts before and after proofing of dough. Different lowercase letters represented significant difference ($p < 0.05$) of different dough samples before and after proofing for the specific property. Different capital letters represented significant difference ($p < 0.05$) of the same dough sample before and after proofing for the specific property.

Table 1. Effect of fermented fruit substrate on dough development and gas release parameters.

Sample (s)	Dough development parameters				Gaseous release parameters	
	H_m (mm)	h (mm)	H'_m (mm)	$(H_m\text{-}h)/H_m$ (%)	V_t (mL)	RC (%)
Cw	63.40 ± 2.47b	59.80 ± 0.85c	60.50 ± 0.71a	5.36 ± 5.04b	1265 ± 90.51a	93.40 ± 0.71e
CuF	68.90 ± 1.98c	65.10 ± 1.13d	69.70 ± 0.28c	5.50 ± 1.07b	1521 ± 84.85c	86.00 ± 0.28c
LF1	58.20 ± 0.57a	45.40 ± 0.42e	72.70 ± 1.27d	21.99 ± 0.03d	1575 ± 46.67c	83.70 ± 0.14b
LF2	74.00 ± 0.99c	74.00 ± 0.99a	63.40 ± 0.57b	0.00 ± 0.00a	1354 ± 57.98ab	89.80 ± 0.57d
PF1	60.80 ± 0.28ab	51.80 ± 0.57b	69.00 ± 0.42c	14.80 ± 1.33c	1471 ± 28.28bc	84.40 ± 0.57b
PF2	58.60 ± 0.99a	44.80 ± 0.57a	71.90 ± 0.85d	23.55 ± 0.33d	1531 ± 36.77c	80.70 ± 0.42a

Data were represented as means ± SD (n = 3), different letters in the same column indicate significant differences at $p < 0.05$. (1) and (2) attached on sample name indicate substrate fermentation condition at 30 °C for 24h and 31 °C for 19.5h, respectively. Cw: Wheat control dough. CuF: Wheat control dough containing 20% unfermented fruit substrate. LF and PF: Wheat dough containing 20% fruit substrate fermented by *L. plantarum*, and *P. pentosaceus*, respectively. H_m: Maximum dough fermentation height. h: Final dough height after fermentation. H_m: Maximum height of gas formation. $(H_m\text{-}h)/H_m$: Fermentation tolerance. V_t: Total gas volume. RC: Retention coefficient.

results were presented in Table 2. Compared with Cw (0.46 μmol/g), the free SH groups content increased in dough the range 61.62–200.85% after incorporating fruit substrates. However, compared with Cw, the rate of increase of SH groups content was higher in CuF and lower in presence of fermented substrate in dough. No statistically significant difference in free SH groups in LF2 (0.75 μmol/g), PF2 (0.85 μmol/g) and LF1 (0.88 μmol/g) was observed, which was lower than CuF (1.27 μmol/g) and PF1 (1.39 μmol/g), but higher than Cw. Therefore, incorporation of fruit substrates led to changes in SS bonds of proteins in dough in contrast to wheat dough control. This may be attributed to influence of organic acids in fruit substrates which initiated hydrolysis and disruption of SS bonds of gluten in dough (Su et al., 2019). However, addition of fermented fruit substrate reduced the rate of increase of free SH groups of dough in contrast to doughs containing unfermented fruit substrate. LAB fermentation of fruit substrate might have increased release of phenolic acids from their bound state and increased degradation of dietary fiber into soluble dietary fiber (Michlmayr and Kneifel, 2014). The changes probably favored formation of covalent and non-covalent linkages with SS bonds in dough containing fermented fruit substrate which led to less free SH groups formed and increased preservation of the gluten network integrity in dough (Xu et al., 2019).

3.2.2. Secondary structure characteristic of gluten protein in dough

Changes in secondary structure conformation of gluten proteins in dough samples was observed in the Amide I region (1600-1700 cm^{-1}) originating from C=O stretching vibrations in FTIR spectra (F. Liu et al., 2018). Five components of intermolecular β-sheet, antiparallel β-sheet, α-helix, β-turn and β-sheet were quantified and results presented in Table 2. Results showed that β-sheet (intermolecular β-sheet, antiparallel β-sheet and β-sheet) was the main secondary structure, followed by α-helix, and β-turn the least. Compared with Cw, addition of fruit substrates generally increased β-sheet (%) of dough, with the highest increase seen in PF2 (16.32%), then LF1 (11.40%), CuF (7.44%), and LF2 (4.24%). β-sheets are the main secondary structure of gluten in dough

Table 2. Effect of incorporation of fruit substrate on reducing sugars, free SH and secondary structure characteristics of gluten proteins in dough.

			Secondary structure characteristics				
Sample (s)	Reducing sugar (%)	SH content (μmol/g)	intermolecular β-sheet (%)	antiparallel β-sheet (%)	α-helix (%)	β-turn (%)	β-sheet (%)
Cw	3.28 ± 0.01a	0.46 ± 0.07a	2.53 ± 0.03d	31.68 ± 0.43c	25.19 ± 0.01c	20.17 ± 0.04f	19.37 ± 0.01b
CuF	3.44 ± 0.04b	1.27 ± 0.03c	2.29 ± 0.01c	32.25 ± 0.01d	30.73 ± 0.02e	10.43 ± 0.12b	23.02 ± 0.10f
LF1	3.21 ± 0.01a	0.88 ± 0.15b	4.06 ± 0.07f	36.62 ± 0.00e	22.74 ± 0.02b	16.97 ± 0.16d	19.00 ± 0.14a
LF2	3.47 ± 0.06b	0.75 ± 0.08b	2.88 ± 0.01e	31.11 ± 0.01b	25.86 ± 0.04d	17.32 ± 0.04e	21.85 ± 0.01d
PF1	3.46 ± 0.00b	1.39 ± 0.08c	1.49 ± 0.01a	23.65 ± 0.00a	40.04 ± 0.09f	12.10 ± 0.02a	20.17 ± 0.02c
PF2	3.42 ± 0.02b	0.85 ± 0.02b	2.00 ± 0.06b	37.87 ± 0.01f	21.76 ± 0.01a	14.66 ± 0.01c	22.44 ± 0.01e

Data were represented as means ± SD (n = 3), different letters in the same column indicated significant differences at $p < 0.05$. (1) and (2) attached on sample name indicate substrate fermentation condition at 30 °C for 24h and 31 °C for 19.5h, respectively. Cw: Wheat control dough. CuF: Wheat control dough containing 20% unfermented fruit substrate. LF and PF: Wheat dough containing 20% fruit substrate fermented by *L. plantarum* and *P. pentosaceus*, respectively.

(Yu et al., 2020). The increase in β-sheet in doughs containing fruit substrates suggested increased protein aggregation due to new linkages formed with components in the fruit substrates (Belton, 1999). On the other hand, relative to controls (Cw and CuF), α-helix (%) and β-turn (%) generally declined and increased, respectively in doughs containing fermented fruit substrates. Relative to Cw, α-helix declined in LF1 and PF2, and increased in LF2 and PF1. On the other hand, relative to CuF, the α-helix generally declined in LF1, PF2, LF2, except PF1. Moreover, β-turn declined and increased in all dough relative to CwD and CwD-uf, respectively. Structural changes and decline in α-helix content in dough have been attributed to conversion of α-helix into β-turn as proteins unfold due to acidification related deamination (Abedi and Pourmohammadi, 2021). Decrease in α-helix (%) in doughs incorporated with fermented fruits was consistent with changes in free SH groups of dough (Table 2). Low pH (Figures 1A, 2A) due to acidification from incorporated fermented fruit substrates probably weakened hydrogen and hydrophobic interactions of gluten in dough which led to increased protein unfolding and reduction of α-helix through enhanced conversion into β-turn (Abedi and Pourmohammadi, 2021).

Furthermore, the LAB strain and condition used to ferment the fruit substrate may have produced diverse metabolites which influenced gluten network stability differently. As a result, the increased β-turn (%) in doughs containing fermented fruit substrates, especially LF2, LF1, and PF2 led to an improved glutenin content and gluten quality (Abedi and Pourmohammadi, 2021).

3.2.3. Reducing sugar content in dough

As shown in Table 2, incorporation of fruit substrates generally increased the reducing sugar content of dough after proofing (Table 2). Compared with Cw (3.28%), the reducing sugar content increased by 4.84%, 5.62%, 5.58%, and 4.05% in CuF, LF2, PF1, and PF2, respectively, but declined in LF1 (−2.39%). This suggested that incorporation of substrates in dough could enhance starch hydrolysis into reducing sugars which may extend product shelf life through delayed starch retrogradation (Kapelko et al., 2013).

3.2.4. Microstructural changes of dough prepared with fermented substrate

The effect addition of fermented fruit substrate on microstructure of dough was shown in Figure 3 (A-L). A more homogenous and continuous microstructure was observed in control Cw (Fig. 3A, B). A less homogenous and less continuous dough structure was observed in CuF which suggested a lowered dough quality (Fig. 3C, D) (Goesaert et al., 2005). However, incorporation of fermented fruit substrate improved the homogeneity and continuity of the microstructure of dough in the order LF2 >LF1 >PF2 >PF1. Changes in microstructural of doughs containing fruit substrates were in agreement with changes in gluten proteins based on SH and secondary structure conformation. Incorporation of fermented fruit substrate were confirmed to enhance the microstructural properties of dough.

3.2.5. Specific volume of bread prepared with fermented substrate

Changes in specific volume of bread prepared with fruit substrates was presented in Table 3. The specific volume significantly increased (6.59–20.01%) in breads after addition of fruit substrates. Compared with Cw (4.50 mL/g), the highest specific volume was seen in LF2 (5.41 mL/g), CuF (5.38 mL/g), LF1 (4.96 mL/g), PF2 (4.85 mL/g), and PF1 (4.80 mL/g). Changes in specific volume of bread have been attributed to gluten network integrity and dough extensibility which expand and retain gas produced during proofing of dough (Bellido et al., 2009). Furthermore, in a recent study, type and concentration of organic acids added in dough were found to selectively increase yeast fermentation in dough and subsequently enhanced specific volume of bread (Su et al., 2019). In this study, incorporation of fruit substrates increased fermentation tolerance ($(H_m\text{-}h)/H_m$), an indication of weakened gluten network and unstable dough. Interestingly, yeast activity (H'_m) increased which led to increased total gas volume (V_t) produced, however the gas retained (RC) reduced by 3.85–13.6% in doughs incorporated with fruit substrates (Table 1).

Therefore, changes in specific volume of bread were attributed to the positive effect of acidification due to incorporation of 20% fruit substrates on yeast activity and gas produced during proofing of dough. In addition, LAB fermentation at the different conditions may have changed the organic acid composition and content in fruit substrates which led to the varying increase in specific volume of breads. Besides organic acids, other metabolites in the fruit substrates such as phenolic acids may have also positively contributed to increased specific volume of bread (Xu et al., 2019).

3.2.6. Textural properties of bread prepared with fermented substrate

Textural properties of bread were presented in Table 3. Compared with Cw, hardness increased ($p < 0.05$) by 22.81–72.55% in breads containing fruit substrates. Interestingly, compared with CuF, significantly lower and higher hardness values were observed in crumbs containing fruit substrates fermented by different LAB strains at condition 1 (LF1, PF1) and 2 (LF2, PF2), respectively. Organic acids and phenolic acids added in dough can increase crumb softening (Su et al., 2019; Xu et al., 2019). This suggested that fermentation condition used affected composition of metabolites such as organic acids and phenolic acids in fruit substrates which selectively had a softening effect on bread crumbs in contrast with CuF. However, at 20% addition rate, content of the metabolites was probably low to significantly lower crumb firmness of bread in contrast with Cw. Furthermore, chewiness and gumminess significantly decreased in CuF, LF1 and PF1, but increased in LF2 and PF2 (Table 3), while no statistically significant change was observed in cohesiveness and springiness of all breads. Increase in springiness and cohesiveness coupled with decrease in hardness, gumminess and chewiness values of bread have been positively correlated with good bread quality (Wronkowska et al., 2015). Therefore, addition of fruit substrates

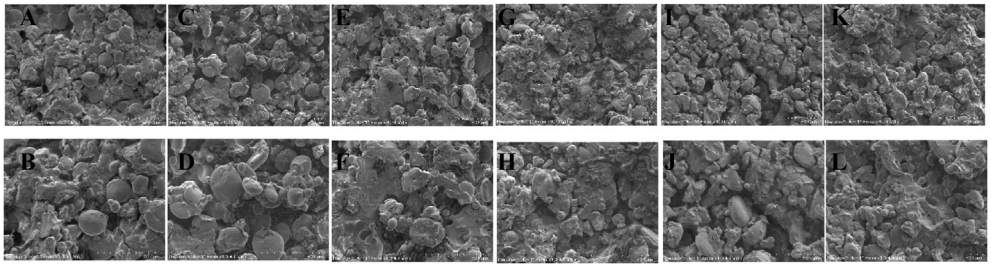


Figure 3. Scanning electron microscope (SEM) micrographs. A and B, wheat control dough (Cw); C and D, wheat control dough containing 20% unfermented fruit substrate (CuF); E and F, wheat dough containing 20% fruit substrate fermented by *L. plantarum*, at 30 °C for 24h (LF1); G and H, wheat dough containing 20% fruit substrate fermented by *L. plantarum*, at 31 °C for 19.5h (LF2); I and J, wheat dough containing 20% fruit substrate fermented by *P. pentosaceus* at 30 °C for 24h (PF1); and K and L, wheat dough containing 20% fruit substrate fermented by *P. pentosaceus* at 31 °C for 19.5h (PF2). Micrographs A, C, E, G, I and K were magnified at 600x and B, D, F, H, J and L were magnified at 1000x.

Table 3. Quality and textural properties of bread prepared with fermented fruit substrates.

		Texture characteristics				
Sample (s)	Specific loaf volume (mL/g)	Hardness (g)	Cohesiveness	Springiness	Gumminess	Chewiness
Cw	4.5 ± 0.17a	388 ± 19.80a	0.74 ± 0.01ab	11.42 ± 0.06a	386.50 ± 7.78bc	43.30 ± 0.71ab
CuF	5.38 ± 0.13c	525 ± 0.00c	0.77 ± 0.01b	11.51 ± 0.24a	296.50 ± 19.09a	33.45 ± 1.34a
LF1	4.96 ± 0.17b	476.5 ± 4.95b	0.75 ± 0.00ab	11.235 ± 0.04a	357.50 ± 4.95b	39.40 ± 0.42ab
LF2	5.41 ± 0.11c	563 ± 18.38c	0.73 ± 0.01a	14.84 ± 5.06a	412.50 ± 4.95c	60.10 ± 21.21b
PF1	4.80 ± 0.06ab	517 ± 32.53bc	0.73 ± 0.01a	11.31 ± 0.14a	377.00 ± 18.38b	41.75 ± 1.48ab
PF2	4.85 ± 0.00b	669.5 ± 17.68d	0.75 ± 0.01ab	11.215 ± 0.02a	500.50 ± 9.19d	55.00 ± 1.13ab

Data were represented as means ± SD (n = 3), different letters in the same column indicated significant differences at $p < 0.05$. (1) and (2) attached on sample name indicate substrate fermentation condition at 30 °C for 24h and 31 °C for 19.5h, respectively. Cw: Wheat control dough. CuF: Wheat control dough containing 20% unfermented fruit substrate. LF and PF: Wheat dough containing 20% fruit substrate fermented by *L. plantarum* and *P. pentosaceus*, respectively.

affected textural properties of bread based on fermentation state of substrate, LAB strain and fermentation conditions used.

3.2.7. Antioxidant activity of bread prepared with fermented substrate

The results of antioxidant activity of bread based on ABTS and DPPH assays was presented in Table 4. Antioxidant activity values of bread based on DPPH (29.33 ± 0.51 to 31.65 ± 0.87 μmol/g) were significantly higher than those based on ABTS (2.04 ± 0.02 to 3.33 ± 0.36 μmol/g). Compared with Cw (2.04 μmol/g), the ABTS activity increased ($p < 0.05$) in breads containing fruit substrates in the order CuF > PF2>LF1>LF2 = PF1. However, relative to Cw, DPPH activity declined ($p < 0.05$) in CuF, and showed no statistical difference in breads containing fermented fruit substrates. Changes in antioxidant activity observed may be attributed to the increased release of phenolic compounds in fruit substrate after LAB fermentation (Di Cagno et al., 2011). In addition, some phenolic compounds could have been released in wheat flour during dough and bread preparation of doughs incorporated with fermented fruit substrates (Di Cagno et al., 2011). The phenolic compounds then acted as hydrogen donors with diverse ability to scavenge on the radicals as reflected by their scavenging ability in bread (M. Liu et al., 2018).

3.3. Antifungal phenolic acid content of bread prepared with fermented substrate

The results of antifungal phenolic acid content in methanolic extracts from breads incorporated with fermented fruit substrate were presented in Table 4. The results showed that none of the phenolic acids was detected in Cw, whereas they were detected in bread containing fruit substrates. In bread containing fruit substrates, gallic acid (153.09–182.10 μg/kg) was detected in all samples, with its content higher in breads containing fermented fruit substrates. On the other hand, caffeic acid was detected in all but LF1, while protocatechuic acid was detected only in CuF, vanillic and ρ-hydroxybenzoic only in detected in PF2. However, compared to CuF, total content of phenolic acids was highest in PF2, then LF1, and PF1. This suggested that new antifungal phenolic acids whose content increased after fermentation were introduced into bread due to incorporation of fruit substrate (Romero-Segura et al., 2012; Septembre-Malaterre et al., 2018). LAB bio-transformations are possible due to production of enzymes like β-glucosidase during substrate fermentation (data not shown). Furthermore, phenolic acid type and content present in bread was affected by fermentation condition and LAB strain used (Heleno et al., 2015; Septembre-Malaterre et al., 2018).

3.3.1. MFSL of bread prepared with fermented substrate

Results of MFSL of bread was presented in Table 5. MFSL of bread in this study was 3 days as the first mold growth on slices was observed after four days of storage. At 4 days of storage, two mold colonies were visible on Cw, CuF and LF2, while only one was visible on LF1, PF1 and PF2. Changes observed in latter samples were attributed to antifungal phenolic acids like gallic acid, caffeic acid, and protocatechuic acid in bread and organic acid like malic acid in fruit substrate which inhibited and reduced growth rate of molds (Rizzello et al., 2011). However, three or more colonies were visible on all bread slices after 5 days of storage.

3.3.2. Challenge test of bread prepared with fermented substrate

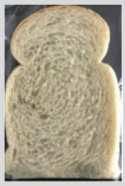
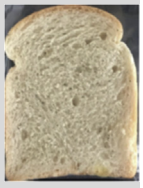
Results of antifungal activity of bread challenged singly with fungal spores of *C. sphaerosperm*, *A. niger* and *P. chrysogenum* was presented in Figure 4 (A-C). No visible growth was observed at 2 days of storage in Cw and CuF, but increased ($p < 0.05$) to 95 and 70%, respectively, with 100% growth observed on both bread slice surfaces after 4 days of storage. Similar findings were reported in wheat bread challenged against *A. niger*, *F. culmorum*, and *P. expansum* suspension, but slices were instead fully covered by mold growth after 3 days of storage (Ryan et al., 2008). In contrast, at 2 days of storage, 5-50% growth was observed on bread slices containing fermented substrate. Thereafter, relative to Cw and CuF, a gradual increase in mold growth

Table 4. Antioxidant activity and antifungal phenolic acids content in breads containing fermented fruit substrate.

	Antioxidant activity TEAC (μmol/g)		Phenolic acid content (μg/kg)							
Sample (s)	ABTS	DPPH	Gallic (RT: 5.54mi)	Protocatechuic (RT: 9.11min)	Chlorogenic (RT: 14.52min)	Caffeic (RT: 20.39min)	Syringic (RT: 21.52min)	Vanillin (RT: 33.09min)	p-hydroxybenzoic (RT: 33.44min)	Ferulic (RT: 33.84min)
Cw	2.04 ± 0.02a	31.65 ± 0.87bc	-	-	-	-	-	-	-	-
CuF	3.83 ± 0.35c	29.33 ± 0.51a	153.09 ± 0.04a	6.99 ± 0.03b	-	1.63 ± 0.02d	-	-	-	-
LF1	3.20 ± 0.28bc	31.70 ± 0.55bc	182.10 ± 0.51e	-	-	-	-	-	-	-
LF2	3.06 ± 0.27bc	31.99 ± 0.63bc	154.42 ± 0.53a	-	-	0.39 ± 0.1c	-	-	-	-
PF1	3.06 ± 0.13b	30.98 ± 0.47ab	162.64 ± 1.65bc	-	-	0.45 ± 0.01 c	-	-	-	-
PF2	3.33 ± 0.36bc	30.70 ± 0.16ab	167.97 ± 0.06d	-	-	0.45 ± 0.01c	-	0.11 ± 0.01b	113.11 ± 5.21b	-

Data were represented as means ± SD (n = 3), different letters in the same column indicated significant differences at $p < 0.05$. (1) and (2) attached on sample name indicate substrate fermentation condition at 30 °C for 24h and 31 °C for 19.5h, respectively. RT: Retention time (min). (-): Not detected. Cw: Wheat control dough. CuF: Wheat control dough containing 20% unfermented fruit substrate. LF and PF: Wheat dough containing 20% fruit substrate fermented by *L. plantarum* and *P. pentosaceus*, respectively. RT: retention time.

Table 5. Mold free shelf life of bread containing pitaya fruit substrates.

	Bread samples					
Days of storage	Cw	CuF	LF1	LF2	PF1	PF2
1	-[a]	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	++	++	+	++	+	+
5	+++	+++	+++	+++	+++	+++
Fungal infection on 5th day.						

[a] Contamination on bread surfaces was calculated as follows: no visible colonies (-), one colony (+), two (++), and three or more (+++) on bread surfaces were classified.

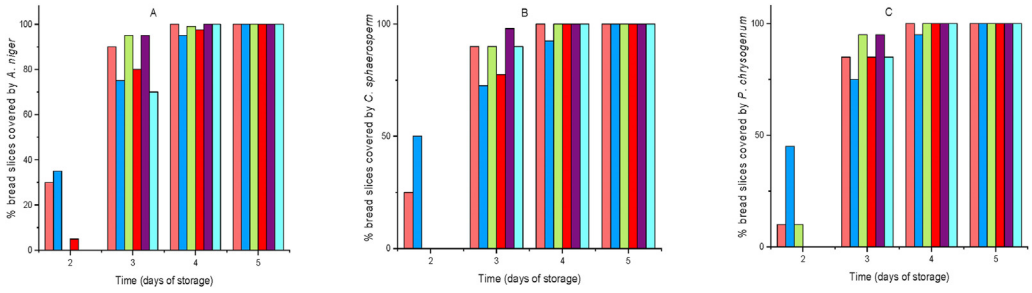


Figure 4. Antifungal activity of bread prepared by replacing 20% wheat flour with pitaya fruit substrate fermented by *L. plantarum* LF1 ( ) and LF2 ( ) and *P. pentosaceus* PF1 ( ) and PF2 ( ). Controls: wheat bread (Cw) ( ), and wheat bread containing 20% unfermented fruit substrate (CuF) ( ). (1) and (2) attached on sample name indicate substrate fermentation condition at 30 °C for 24h and 31 °C for 19.5h, respectively. Bread challenged against: *A. niger* (A), *C. sphaerosperm* (B) or *P. chrysogenum* (C) spores.

was generally observed in bread containing fermented substrates after 2 days of storage, with bread surfaces fully covered by mold after 5 days of storage. In an earlier study, surfaces of wheat bread sourdough fermented by antifungal *L. plantarum* and challenged by fungal strains of *A. niger* and *P. expansum* were fully covered by mold growth at 4 days of storage (Ryan et al., 2008). Despite high initial fungal spore concentration (10^6), fermented substrates reduced rate of mold growth in bread challenged by indicator mold strains. Lower growth was observed in slices of PF than LF when substrates were fermented at (1) than (2). Furthermore, compared to Cw and CuF, lower growth was observed in breads containing fruit substrates and challenged against *C. sphaerosperm*, then *A. niger* and least against *P. chrysogenum*. PF samples showed better antifungal activity, and were highly sensitive against *C. sphaerosperm*, then *A. niger* and least against *P. chrysogenum*. Challenge test observations positively correlated with MFSH results (Table 5). The bio-preservative effects observed were attributed to among others, antifungal phenolic acids in bread and organic acids in fruit substrate which reduced growth rate of molds.

4. Conclusion

This study demonstrated suitability of pitaya fruit substrate fermented by LAB strains as an ingredient in dough with potential bio-preservative effects in wheat bread. Incorporation of fermented fruit substrate increased yeast activity which promoted increased gas production in the doughs. The rate of sulfhydryl content increase lowered while the α-helix content decreased and β-turn content increased in the secondary structures of doughs incorporated with fermented fruit substrates. This could have resulted in the more homogenous microstructure of doughs incorporated with substrate fermented by *L. plantarum* then *P. pentosaceus* than unfermented substrate relative to wheat dough. Specific volume increased in presence of fruit substrates, while bread hardness was lower in presence of fermented substrates than unfermented relative to wheat bread. The antioxidant activity improved in breads incorporated with fermented fruit, while the rate of fungal growth based on mold free shelf life and challenge test during storage were lower. We proposed that changes observed in dough were attributed to dough acidification due to metabolites such as organic acids and phenolic acids in fermented fruit substrates which induced changes in proteins and starch of dough, improved the antioxidant activity of breads, and slightly lowered the rate of fungal growth during storage. The findings provided new insightful knowledge on bread making suitability, bio-preservative effect and potential to replace chemical preservatives in dough and bread using fruit substrate ingredients fermented by antifungal LAB strains.

Declarations

Author contribution statement

Jacob Ojobi Omedi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jing Huang, Binle Zhang, Liyuan Zhou, Faqun Zhao, Tiecheng Gao: Contributed reagents, materials, analysis tools or data.

Weining Huang, Jianxian Zheng, Yongqing Zeng, Ning Li: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data associated with this study has been deposited at NCBI under the accession numbers *L. plantarum*, AS 2-11 (MW602529) and *P. pentosaceus*, BS0-14 (MW602530).

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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