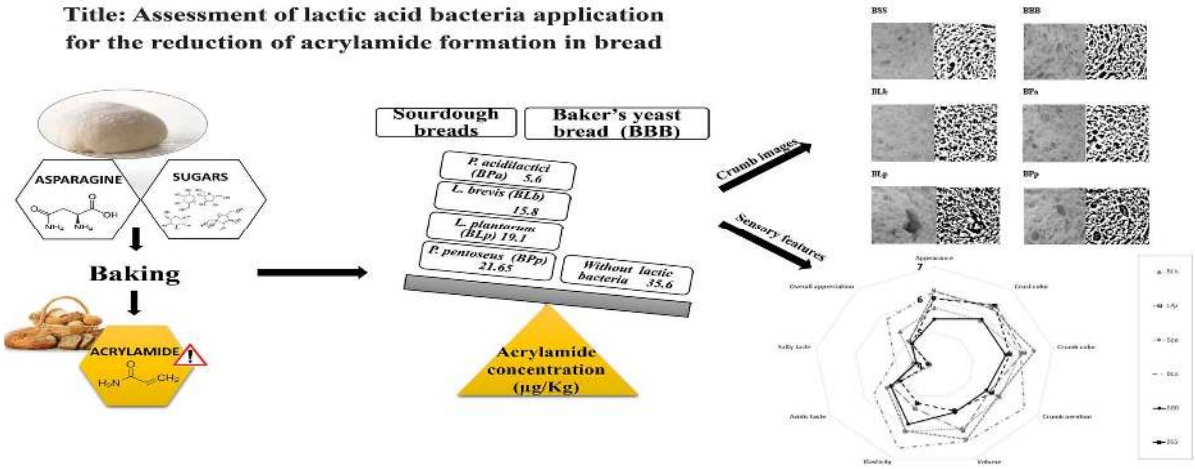
Assessment of lactic acid bacteria application for the reduction of acrylamide formation in bread

Insaf Nachi, Imene Fhoula, Imen Smida, Imen Ben Taher, Moncef Chouaibi, Janis Jaunbergs, Vadims Bartkevics, Mnasser Hassouna

Title: Assessment of lactic acid bacteria application for the reduction of acrylamide formation in bread



Assessment of lactic acid bacteria application for the reduction of acrylamide formation in bread

Insaf Nachi[a], Imene Fhoula[b], Imen Smida[c], Imen Ben Taher[a], Moncef Chouaibi[d], Janis Jaunbergs[e], Vadims Bartkevics[e] and Mnasser Hassouna[a]

[a], UR13AGR02 Unité de bio-conservation et valorisation des produits alimentaires. Université de Carthage, Ecole Supérieure des Industries Alimentaires, 58, avenue Alain Savary, 1003 Tunis, Tunisie.

[b], LR03ES03 Laboratoire Microorganismes et Biomolecules Actives**.** Université de Tunis El Manar, Faculté des Science de Tunis, 2092, Campus Universitaire, Tunis, Tunisie.

[c], INSERM U1241/Nutrition Metabolism Cancer / UFR Sciences Pharmaceutiques et Biologiques, Université de Rennes 1, 35043 Rennes, France.

[d], Laboratoire de conservation des aliments. Université de Carthage Ecole Supérieure des Industries Alimentaires, 58, avenue Alain Savary, 1003 Tunis, Tunisie.

[e], Institute of Food Safety, Animal Health and Environment, Lejupes Street 3, 1076 Riga, Latvia.

'Declarations of interest: none'

* Corresponding author. Email address: nachiinsaf@gmail.com

Abbreviations:

SS, spontaneous sourdough; SLb, sourdough inoculated with *L.brevis* S12; SLp, sourdough inoculated with *L.plantarum* S28; SPp, sourdough inoculated with *P.pentoseus* S14; Spa, sourdough inoculated with *P.acidilactici* S16; DSS, dough made with spontaneous sourdough; DLb, dough inoculated with *L.brevis* S12 sourdough; DLp, dough inoculated with *L.plantarum* S28 sourdough; DPp, dough inoculated with *P.pentoseus* S14 sourdough; DPa, dough with *P.acidilactici* S16 sourdough; DBB, Baker's yeast dough made without the

addition of sourdough; BSS, bread made by using spontaneous sourdough without LAB strains; BLb, bread made with *L.brevis* S12 sourdough; BLp, bread made with *L.plantarum* S28 sourdough; BPp, bread made with *P.pentoseus* S14 sourdough; BPa, bread made with *P.acidilactici* S16 sourdough; BBB, Baker's yeast bread; TTA, Total titratable acidity; LAB, Lactic acid bacteria; DY, dough yield.

Abstract

Four isolated lactic acid bacteria LAB strains *Lactobacillus brevis*, *Lactobacillus plantarum*, *Pediococcus pentoseus,* and *Pediococcus acidilactici* were selected and used to inoculate sourdough in order to reduce acrylamide content. After fermentation for 16 h, all of the inoculated sourdoughs showed lower pH values compared to the spontaneous sourdough. This acidification was accompanied by a significant (p<0.05) increase in the concentration of reducing sugars. The baked bread samples made with the tested LAB strains showed significantly reduced acrylamide content, in particular for the sample inoculated with *P.acidilactici* (5.64 µg/kg), compared to the bread sample prepared with baker's yeast (35.6 µg/kg). The resulting breads were also evaluated for several other quality parameters. The highest softness was registered for the breads obtained from the fermentation by *P.acidilactici* (2704 g). The different tested strains also influenced the color, the void fraction, and the cell density of the breads. The sensory evaluation indicated that the crust color, crumb aeration, as well as the salty and acidic tastes were not significantly affected by sourdough incorporation. However, breads made from LAB sourdoughs were more appreciated by the tasters. This study proved the suitability use of the selected *P.acidilactici* strain for industrial-scale bread production.

Keywords: Sourdough bread; Lactic acid bacteria; Acrylamide; Sensory evaluation

1. Introduction

Bread is one of the staple foods for human nutrition. It supplies a considerable portion of the nutrients required for growth, maintenance of health and well-being. The baker's yeast bread is the most consumed type in Tunisian diet (EI, 2014). During bread baking, some hazardous byproducts can be formed, such as acrylamide. This compound has been recognized as a neurotoxic and potentially carcinogen substance for humans (IARC, 1994). Acrylamide has been detected in a wide range of cooked and fried carbohydrate rich foods, particulary crisp bread, potato chips, breakfast cereals, and coffee (Gökmen et al., 2007). The most probable route of acrylamide formation is through Maillard reactions involving the amino acid asparagine and reducing sugars (Mottram et al., 2002). It also can be formed through several other pathways, such as the reaction between aspartic acid and reducing sugars (Yaylayan & Stadler, 2005), thermal degradation of amino acids and proteins (Keramat et al., 2011), as well as decarboxylation and deamination of asparagine (Granvogl & Schieberle, 2006).

A wide range of researchers representing national food safety authorities, academia, and food manufacturers have sought to better understand the mechanisms of acrylamide formation and to find ways to minimize its formation in foods (EFSA, 2011).

Previous studies have indicated that the occurrence of acrylamide in heat-processed foods may depend on specific factors, such as the initial concentrations of asparagine, reducing sugars, pH, water activity, time and temperature of the heating proces (Gökmen et al., 2007).

Over the last decades, there have been many attempts to reduce acrylamide formation by the optimization of processing conditions. Indeed, the HEATOX report by Hellenäs et al. (2005) showed that acrylamide synthesis increased at higher baking temperature (200–260°C) and with time (10–25 min). Another strategy for reducing the acrylamide content depended on the choice of appropriate raw materials. Wang et al. (2017) suggested that the generation of acrylamide can be mitigated by reducing the presence of degraded starch in flour.

Several studies indicated that fermentation processes performed with lactic acid bacteria (LAB) and yeast could reduce the acrylamide content in bread. This effect is mainly related to a decrease of pH rather than to the consumption of precursor nutrients (asparagine and reducing sugars) by microorganisms growing in sourdough (Fredriksson et al., 2004; Bartkiene et al., 2013b; Wang et al., 2017).

Moreover, the use of lactic acid bacteria in sourdough improved the properties of dough and enhanced the flavor and texture of bread (Saeed et al., 2014). It was also reported that the use of sourdough extended the microbiological shelf life of bread by controlling and inhibiting spoilage organisms during fermentation, due to the lower pH value and antimicrobial metabolites of LAB (Cizeikiene et al., 2013). Likewise, lactic acid

sourdough had a higher content of biogenic compounds, lower level of anti-nutritional factors and a better value of glycemic response, as well as improved the uptake of minerals (Gobbetti et al., 2012).

So, the challenge of this study was to find a way to reduce the acrylamide content in Tunisian bread without compromising its organoleptic properties. Thus, a lactic fermentation was conducted using four LAB strains, previously isolated from Tunisian flours and identified. They were tested to decrease the acrylamide concentration in bread. Up to our knowledge, no research has been conducted on acrylamide reduction in Tunisian food. For this purpose, the known acrylamide precursors (asparagine and reducing sugars) were measured in fermented dough. At the same time, the influence of lactic acid fermentation on sensory features of the final products was studied.

2. Materials and methods

2.1. Materials

The flour used (type PS-7 and moisture content 14.43%) was purchased from the mill SOTUMIS (Ennakhla) situated in Tunisia. Chemicals and analytical standards were mainly purchased from Sigma-Aldrich (Dublin, Ireland). All analytical standards had a purity of at least 95%.

2.2. Microorganisms

Fresh yeast was supplied by Rayen Food Industries. The strains of lactic acid bacteria (LAB) used in this study (*Lactobacillus brevis* strain S12 (MF458471), *Lactobacillus plantarum* strain S28 (MF458477), *Pediococcus pentoseus* strain S14 (MF4584872), and *Pediococcus acidilactici* strain S16 (MF458474) were previously isolated, identified by 16S ribosomal RNA (rRNA) gene from various Tunisian bakery flours and selected basing on their acidification activity according to the method described by Alfonzo and al. (2013) where LAB cells were assessed to reduce pH of a flour extract after 24 h, 48 h and 72 h of inoculation (Data not shown).

2.3. Preparation of sourdough

Experimental sourdough bread was manufactured using the previously described selected strains of LAB (*L.brevis* S12, *L.plantarum* S28*, P.pentoseus* S14 and *P.acidilactici* S16) based on their acidification capacity. The LAB strains were cultivated in MRS broth (1% maltose w/v, 1% lactose w/v, pH 5.6) at 30°C for 18 h. Bacterial cells were harvested by centrifugation (10,000 × g, 10 min, 4°C), washed twice with 50 mM sterile potassium phosphate buffer (pH 7.0) before being resuspended in tap water. Each strain was individually

144 characterized and inoculated into the dough. The sourdough was made according to Nionelli et al., (2014).
145 Briefly, for preparing 100 g of sourdough, 62.5 g of flour were mixed with 37.5 mL of tap water containing 10^9
146 CFU/g of LAB cells. The obtained sourdough was incubated at 30°C for 16 h. A spontaneous sourdough (SS)
147 prepared without inoculation with the selected LAB strains was used as a control.
148
149 **2.4. Making of bread**
150 The dough was made according to the recipe of Nionelli et al., (2014). In all of the experiments, 20% (w/w) of
151 sourdough was added to dough. Baker's yeast was added at the amount of 2% (w/w). Dough yield (DY = (dough
152 weight / flour weight) × 100) was fixed at 160. After that, dough samples were left to ferment for 1 h 30 min.
153 The control bread was made according to the same formulation, but without the addition of sourdough. All bread
154 samples were prepared in triplicates. All breads were baked at 220°C for 20 min.
155
156 **2.5. Analytical methods**
157 **2.5.1. Determination of pH and total titratable acidity**
158 The values of pH and total titratable acidity (TTA) were determined after mixing 10 g of sourdough or dough
159 with 90 mL of distilled water using a Stomacher mixer. The pH was measured using a pH meter and the
160 acidification capacity (ΔpH) was calculated as the difference between pH after fermentation and pH before
161 fermentation. The mixture was used to measure the TTA expressed as the amount (mL) of 0.1 M NaOH solution
162 needed to reach the pH value of 8.3.
163
164 **2.5.2. Determination of reducing sugars**
165 The sugar content (glucose, fructose, and sucrose) of freeze-dried doughs was determined by high-performance
166 liquid chromatography (HPLC) system (Waters 2695 Separations Module) according to the method of Bartkiene
167 et al. (2013). Five grams of each sample were mixed with 20 mL of deionized water. The filtrate was placed in a
168 water bath for 1 h at 75°C, in order to inactivate the enzymes, before being centrifuged at 13500 × g for 10 min.
169 The obtained supernatants were analyzed by a HPLC system equipped with an AKTA purification system, an
170 analytical column (Phenomenex Luna 5µm NH_2, 150 × 4.6 mm (Torrance, CA, USA)) and a refractive index
171 detector (Waters 2414). The compounds were quantified using a calibration curve of the corresponding standards
172 ranging between 50 and 500 mg/mL. Sucrose, glucose, and D-fructose standards were obtained from Sigma-
173 Aldrich.

174

175 **2.5.3. Analysis of asparagine**

176 Asparagine was extracted by the method reported by Rizzello et al. (2010). Each sample (2 µL) was injected into
177 HPLC coupled with a Shimadzu LC-20AD evaporative light scattering detector (ELSD). The mobile phase was
178 composed of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in 98% acetonitrile) with a
179 flow rate of 0.7 mL/ min at 30°C, and the injection volume was 10 µL. The gradient starting from 0 % B for 5
180 min, 15 % B for 7 min, 30 % B for 5 min, and finally followed by a 18 min maintained at 0% B. Asparagine was
181 quantified using a calibration curve made with L-asparagine (Sigma-Aldrich).

182

183 **2.5.4. Determination of acrylamide by LC-MS/MS**

184 The extraction of acrylamide was carried out on bread samples according to the method of Bartkiene et al.
185 (2013). It was conducted with acetonitrile and QuEChERS salt mixture (4 g anhydrous $MgSO_4$ (Sigma Aldrich)
186 and 0.5 g of NaCl (Analytika Ltd (Check Republic)). The extracts were then added to 50 mg of PCA-sorbent
187 (SSELECTRA Bulk Sorbents) and 150 mg anhydrous $MgSO_4$ to obtain pure extracts. These latter were than
188 analyzed with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The quantitative
189 analysis was performed by QTRAP 5500 LC-MS/MS instrument (AB SCIEX, Framingham, MA, USA) coupled
190 to an Acquity UPLC system (Waters, Milford, MA, USA). The separation of acrylamide was obtained with Luna
191 3µm HILIC column (100 × 3.00 mm) (Phenomenex, Torrance, CA, USA). The solvent system was composed of
192 0.1% formic acid in water and 0.1% formic acid in methanol with a flow rate of 0.3 mL/min at 30°C, and the
193 injection volume was 10 µL. The conditions selected for the MS/MS detection were as follows: curtain gas
194 (Nitrogen): 30.0 psi; ion spray: 5500 V; temperature: 400°C, nebulizer gas (Nitrogen): 40 psi; nebulizer gas
195 (Nitrogen): 50 psi. Acrylamide was quantified using a calibration curve made with internal standard (acrylamide-
196 d_3) dissolved in acetonitrile and chromatograms were acquired and processed by using the Analyst software,
197 version 1.5 (AB SCIEX).

198

199 **2.5.5. The evaluation of bread quality**

200 The hardness of bread samples was determined with TVT 6700 texture analyzer (Perten Instruments, North Ryde
201 BC, NSW, Australia). All samples were prepared and baked on the day of test. Three slices of bread (20 mm
202 thick) were compressed by using a cylindrical compression probe with 75 mm diameter. The following settings
203 were selected: test speed 5 mm/s, 50% deformation of the sample and two compression cycles with a break of 12

s. Data was analyzed using the TexCal software. Color parameters (L*, a*, b*) of the crust and crumb parts of bread samples were determined in triplicate by using a CR-300 colorimeter (Minolta, Japan).

The crumb features of breads were evaluated after 24 h of storage. Images of three slices were acquired using a Nikon D3100 digital camera (55 mm lens; level of sensitivity: ISO1600; shutter speed: 1/640 s). Two 20 mm × 20 mm square fields of view were evaluated in each image. Segmentation was performed manually, by binarization of 8-bit greyscale images into black-and-white images using the Otsu ImageJ thresholding algorithm implemented with Fiji 1.51 software package. After that, the void fraction (the fraction of the total area corresponding to the bread pores) and cell density (number of cells/mm^2) were extracted and calculated.

Sensory analysis of baked breads was accomplished after 1 hour of manufacturing by a semi-trained panel (38 panelists consisting of students (male and female) from the High School of Food Industries of Tunisia). The panelists evaluated the sensory properties of the bread samples based on their degree of acceptance (scale of 1-8, with 8 being the highest score). The sensory attributes were discussed with the panelists during the introductory sensory training sessions. Before the sensory evaluation, the loaves were cut into 1.5 cm thick slices and were then served in random order. The elasticity, crust and crumb colors, acidic and salty taste, appearance, volume and crumb aeration were considered as sensory attributes. All sensory evaluation tests were performed in accordance to the ethical and professional guidelines described in the document issued by the Institute of Food Science and Technology (IFST, 2015).

2.5.6. Statistical analysis

All experiments were repeated three times and illustrated as the mean values ± standard deviations. Statistical analyses were performed using the IBM SPSS Statistics software version 23.0. The data were analyzed using one-way analysis of variance (ANOVA), followed by the Duncan's test with the significance level set at $p<0.05$ to establish the significance of differences between the samples. Pearson correlation analysis was also performed.

3. Results and discussion

3.1. Physico-chemical analysis of sourdough

The values of pH, TTA, the content of sugars (glucose, fructose, and sucrose) and free asparagine in spontaneous and inoculated fermented sourdoughs were determined, as presented in Table 1.

After 16 h of fermentation, the pH of all sourdough samples had decreased from 6.36 ± 0.06 to 4.03 ± 0.06 in SS - 3.55 ± 0.04 in SLb. Moreover, the highest ΔpH (2.81) (p<0.05) was found in the sourdough prepared with *L.brevis* S12 (SLb), whereas the lowest ΔpH value (2.33) was measured in the spontaneous sourdough (SS). In fact, LAB are recognized to considerably decrease the pH values in sourdoughs, but the variation of pH depends on the LAB strain used (Elsanhoty et al., 2016). According to Cizeikiene et al., (2013), adding sourdough to bread could control and notably inhibit spoilage organisms through lowering the pH value during fermentation.

Besides, TTA was significantly (p<0.05) increased after fermentation, from 2.5 mL to 5.2–12.2 mL. The comparison of results highlighted that all sourdoughs inoculated with the selected LAB strains had significantly (p<0.05) higher TTA values than those observed for spontaneous sourdough (SS). Furthermore, a high negative correlation (r = -0.953) between the pH and TTA values was found after fermentation. These findings are in agreement with the results of Mamhoud et al. (2016). Concerning the analysis of reducing sugars, a significant (p<0.05) increase of their concentration (from 612 ± 38 to 966 ± 21 mg/100g dry weight (d.w.) and from 266 ± 48 to 563 ± 12 mg/100g d.w. for glucose and fructose, respectively) was shown after 16 h of fermentation. Interestingly, *P.pentoseus* S14 was the key strain for the formation of reducing sugars at concentrations reaching 1529 mg/100g d.w. after fermentation (74.15%). The use of LAB caused a significant (p<0.05) increase of the glucose concentration (718–966 g/100g d.w.) compared to spontaneous sourdough (529 g/100g d.w.) after fermentation. This observed increase of glucose concentration in sourdough could be related to the degradation of sucrose and starch by cereal amylases and LAB (Bartkiene et al., 2013).

Before fermentation, the concentration of sucrose was estimated to be 460 ± 71 mg/100g d.w. After 16 h of fermentation, a significant (p<0.05) decrease of sucrose amount was noticed in sourdoughs, ranging from 413 ± 22 mg/100g d.w. in DSS to 173 ± 5 mg/100g d.w. in SLp). The sharpest decrease of sucrose concentration was recorded in the sourdough inoculated with *L.plantarum* S28 (62.39%), compared to the results for spontaneous sourdough, which showed only a very weak decrease (9.78%).

The fermentation of dough plays a major role in the control of the acrylamide formation rate related to the presence of asparagine (Dastmalchi et al., 2016). In this context, the content of free asparagine was determined in all sourdough samples before and after fermentation (Table 1) in order to investigate whether the quantity of this amino acid was reduced by fermentation.

Before fermentation, the content of free asparagine was found to be 16.1 ±0.6 mg/100g d.w. Nevertheless, all LAB strains were significantly (p<0.05) able to increase the asparagine content after 16h of fermentation. That

could be explained by the proteolytic activity of the selected starters (Bartkiene et al., 2013, Mamhoud et al., 2016).

3.2. Physico-chemical analysis of dough

The variation of pH, the concentrations of reducing sugars and asparagine in the dough samples were measured after fermentation and are presented in Table 2. The Baker's yeast dough (DBB), which was made without the addition of sourdough, was considered as a control.

The pH values obtained using the selected LAB strains and spontaneous sourdough are reported in Table 2. As expected, the sourdoughs made with the selected LAB strains caused a significantly (p<0.05) more pronounced decrease of pH (4.13–4.68) in comparison to DSS (4.97) and DBB (5.77). Diana et al., (2014) reported that the addition of sourdough to the dough formulation resulted in a linear increase in the acidity of the product. These results are consistent with those obtained by Suhr and Nielsen. (2004) who attested that the pH value reached 5 using yeast and 4.4 – 4.8 using sourdough in the dough formulation.

After 1h 30 min of fermentation, it was noticed that the amount of reducing sugars in the control dough (DBB) was significantly (p<0.05) higher than that of other doughs except for the DLp.

Indeed, the amount of fructose was significantly (p<0.05) higher in DBB (532 ± 11 mg/100g d.w.) compared to the other dough samples (from 230 ± 7 mg/100g d.w. to 326 ± 10 mg/100g d.w.). These results can be explained by the fact that fructose is used as an electron acceptor by LAB and thus is reduced to mannitol (Wisselink et al., 2002).

Sucrose was not detected in any of the analyzed samples. This observation can be explained by several studies that already revealed the presence of a very active invertase enzyme in yeast, which promotes rapid breakdown of sucrose to glucose and fructose (Koschwanez et al., 2011).

The concentration of asparagine in dough samples is shown in Table 2. The effects of LAB strains on the amount of asparagine depended on the particular strains. The amount of asparagine is ranged from 9.9 ± 0.4 mg/100g d.w. in DPp to 16 ± 0.9 mg/100g d.w. in DBB, and these values were similar to those reported previously (Wang et al., 2017). A significant decrease (p<0.05) of asparagine content was shown in DSS, DPp, and DPa (14.2 ± 0.2 mg/100g d.w., 9.9 ± 0.4 mg/100g d.w., and 10.5 ± 0.1 mg/100g d.w., respectively), compared to DBB (16 ± 0.9 mg/100g d.w.), which is fermented with yeast only. The results showed that fermentation of wheat dough with LAB reduced the concentration of asparagine. These results are in agreement with those of Wang et al., (2017).

In addition, Fredriksson et al., (2004) reported that during bread dough fermentation amino acids are assimilated by yeast or LAB and metabolized as a source of nitrogen.

3.3. Analysis of acrylamide

All bread samples were evaluated for their acrylamide levels and the results are presented in Figure 1. To better understand the mechanism of acrylamide formation and its relationship to dough fermentation, the physico-chemical characteristics and composition of sourdough were assessed.

The concentration of acrylamide in bread samples varied from 5.64 µg/kg to 35.6 µg/kg. The highest acrylamide content was detected in the control bread sample (BBB). According to European Commission, for this type of bread (Wheat based soft bread), the indicative value of acrylamide was estimated to 80 µg/kg (EC, 2013). However, bread is classified as the main food contributing to the daily ingestion of acrylamide which can cause a public health problem (EFSA, 2011) and Tunisians are big consumers of bread; on average 70 Kg/person/year (Benaours, 2017).

All LAB strains were able to significantly ($p<0.05$) reduce the acrylamide content of bread. Especially, the *P.acidilactici* strain S16 allowed to decrease the acrylamide levels by 84.2% (to 5.64 µg/kg) compared to the control bread (35.6 µg/kg). The other LAB strains (*L.brevis* S12*, L.plantarum* S28, and *P.pentoseus* S14) provided significantly ($p<0.05$) decreased acrylamide levels (reduction by 55.6%, 49.2%, and 39.2%, respectively) compared to the control sample. Thus, the effect of LAB fermentation on acrylamide reduction depended on the particular LAB strain used in sourdough and its adaptability or competition with yeast in the absorption of nutrients (Dastmalchi et al., 2016).

Bartkiene et al., (2013) showed that the fermentation with a commercial *L.casei* strain could reduce the acrylamide content in bread samples by 29.4% on average. In the same way, Baardseth et al. (2006) found that LAB could reduce the acrylamide level in French fries by 71% after 120 min of fermentation.

Besides, a significant decrease ($p<0.05$) of acrylamide content was noticed in bread leavened with spontaneous sourdough. This result was consistent with the findings by Bartkiene et al., (2013) and Forstova et al., (2013).

The concentration of glucose and sucrose in sourdoughs was significantly ($p<0.05$) slightly correlated to the formation of acrylamide ($r = 0.565$ and $r = -0.617$, respectively). These results were in agreement with those of Surdyk et al., (2004). Sugars seems to be the most important ingredient that influences the acrylamide formation during baking (Baardseth et al., 2006; Dastmalchi et al., 2016). Unlike sugars, the asparagine content of sourdoughs did not show a clear correlation with acrylamide concentration.

Numerous studies suggested that lowering pH values by microorganisms is one of the solutions to preventing the Maillard reaction, the main path of acrylamide formation (Bartkiene et al., 2013; Keramat et al., 2011; Dastmalchi et al., 2016). The first step in Maillard reaction is the generation of a Schiff base that can form 3-aminopropionamide, a precursor of acrylamide (Granvogl et al., 2004).

3.4. Characteristics of the experimental breads

The color parameters (L*, a*, b*) of crust and crumb obtained from the bread samples are listed in Table 3. As it can be seen, the crust lightness (L*) of inoculated sourdough breads (BLb, BLp, and BPa) was significantly lower (p<0.05) than that of control bread (BBB). Moreover, all sourdoughs inoculated with LAB caused significant changes in the "a*" values of crusts, with BPp showing the lowest value. Statistical analysis indicated that the "L*" and "b*" parameters of crusts correlated positively (r = 0.486 and r = 0.510, respectively, p<0.05) with the acrylamide levels in breads. These results were in agreement with those of Surdyk et al., (2004). The crumbs of BLp, BLb, and BPp bread samples had significantly (p<0.05) high values of luminosity (L*) (74.21, 73.85, and 74.49) compared to the BSS sample (70.11). A few differences were registered for the b* and a* parameters of the crumb.

The highest hardness (5149 ± 119 g) was observed for the bread made with yeast only (BBB) (Table 3). The softest breads were those obtained from sourdoughs fermented with *P.pentoseus* S14 (1815 ± 216 g) and *P.acidilactici* S16 (2704 ± 42 g). As reported by Dastmalchi et al., (2016), the addition of sourdoughs reduced the firmness of bread, which depended mainly on the acidification level of sourdough.

The crumb structure of breads was evaluated by digital image analysis (Figure 2). According to Table 3, the observed void fraction and cell density were significantly (p<0.05) different from BBB. This finding confirmed that the final characteristics of the breads were influenced by the starter strains and their interactions.

3.5. Sensory attributes of breads

The results of the sensory analysis of the experimental breads are shown in Figure 3. The crust color, crumb aeration, and detection of salty or acidic taste were not significantly (p<0.05) affected by the addition of sourdough. Moreover, the appearance of bread samples showed an increasing trend towards average scores when LAB sourdoughs were added. The BPa bread sample had the higher appearance score of 6.27, compared to BSS and BBB (6.03 and 5.41, respectively). Likewise, the crumb color and the volume attributes increased when sourdoughs were used. Organic acids, alcohols, esters, carbonyl compounds, carbon dioxide, and

exopolysaccharides produced by LAB during sourdough fermentation are the probable factors resulting in the improved volume, texture, flavor, and aroma of baked bread samples (Saeed et al., 2014).

4. Conclusion

In essence, our proposed strategy provides an effective way to reduce acrylamide formation in bread by using selected lactic acid bacteria strains for fermentation of dough. The most pronounced reduction of acrylamide formation (by 84.7%) was obtained in bread made with *P.acidilactici* strain S16. At the same time, the influence of lactic acid fermentation on the sensory properties of the final bread samples was studied. The obtained results indicate that the incorporation of selected lactic acid bacteria in the bread preparation can improve the texture and flavor of bread. Selected *P.acidilactici* strains could be potentially developed for further applications in fermented products. Finally, a continued effort is recommended to investigate the effects of other lactic bacteria strains on acrylamide synthesis during the baking of bread.

Acknowledgements

This work was supported by the Tunisian Ministry of Higher Education and Scientific Research (LR10CBBC02).

References

Alfonzo, A., Ventimiglia, G., Corona, O., Di Gerlando, R., Gaglio, R., Francesca, N., Moschetti, G & Settanni, L. (2013). Diversity and technological potential of lactic acid bacteria of wheat flours. *Food microbiology*, *36*(2), 343-354.

Baardseth, P., Blom, H., Skrede, G., Mydland, L. T., Skrede, A., & Slinde, E. (2006). Lactic Acid Fermentation Reduces Acrylamide Formation and Other Maillard Reactions in French Fries. *Journal of Food Science*, *71*(1), C28–C33.

Bartkiene, E., Jakobsone, I., Juodeikiene, G., Vidmantiene, D., Pugajeva, I., & Bartkevics, V. (2013b). Study on the reduction of acrylamide in mixed rye bread by fermentation with bacteriocin-like inhibitory substances producing lactic acid bacteria in combination with *Aspergillus niger* glucoamylase. *Food Control*, *30*(1), 35–40.

Benarous, R. (2017). La fin des tensions à la production. *Le temps*. http://www.letemps.com.tn/article/102006/la-fin-des-tensions-%C3%A0-la-production

Cizeikiene, D., Juodeikiene, G., Paskevicius, A., & Bartkiene, E. (2013). Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. *Food Control*, *31*(2), 539–545.

Dastmalchi, F., Razavi, S. H., Labbafi, M., & Faraji, M. (2016). The Impact of *Lactobacillus plantarum*, *Paracasei*, *Casei–Casei*, and *Sanfranciscensis* on Reducing Acrylamide in Wheat Bread. *Journal of Agricultural Science and Technology*, *18*(0), 1793–1805.

Diana, M., Rafecas, M., & Quílez, J. (2014). Free amino acids, acrylamide and biogenic amines in gamma-aminobutyric acid enriched sourdough and commercial breads. *Journal of Cereal Science*, *60*(3), 639–644.

EC: European Commission, (2013). Commission Recommendation of 8 November 2013 on investigations into the levels of acrylamide.

EFSA, 2011. Scientific report of European Food Safety Authority, Results on acrylamide levels in food from monitoring years 2007–2009 and exposure assessment, Parma, Italy, pp. 48.

EI: Euromonitor International, (2014). Country Report: Bakery in Tunisia. http:/www.euromonitor.com/bakery-in-tunisia/report

Elsanhoty, R. M., Ghonamy, A. g., El-Adly, N. a., & Fawzy Ramadan, M. (2017). Impact of Lactic Acid Bacteria and *Bifidobacterium* on the Survival of *Bacillus subtilus* During Fermentation of Wheat Sourdough. *Journal of Food Processing and Preservation*, *41*(4).

Forstova, V., Belkova, B., Riddellova, K., Vaclavik, L., Prihoda, J., & Hajslova, J. (2013). Acrylamide formation in traditional Czech leavened wheat-rye breads and wheat rolls. *Food Control*, *38*, 221–226.

Fredriksson, H., Tallving, J., Rosén, J., & Åman, P. (2004). Fermentation Reduces Free Asparagine in Dough and Acrylamide Content in Bread. *Cereal Chemistry - CEREAL CHEM*, *81*.

Gobbetti, M. (2012). Lactic Acid Bacterium and Yeast Microbiotas of 19 Sourdoughs Used for Traditional/Typical Italian Breads: Interactions between Ingredients and Microbial Species Diversity. *Applied and Environmental Microbiology*, *78*(4), 1251–1264.

Gökmen, V., Açar, Ö. Ç., Köksel, H., & Acar, J. (2007). Effects of dough formula and baking conditions on acrylamide and hydroxymethylfurfural formation in cookies. *Food Chemistry*, *104*(3), 1136–1142.

Granvogl, M., Jezussek, M., Koehler, P., & Schieberle, P. (2004). Quantitation of 3-Aminopropionamide in PotatoesA Minor but Potent Precursor in Acrylamide Formation. *Journal of Agricultural and Food Chemistry*, *52*(15), 4751–4757.

411 Granvogl, M., & Schieberle, P. (2006). Thermally Generated 3-Aminopropionamide as a Transient Intermediate
412 in the Formation of Acrylamide. *Journal of Agricultural and Food Chemistry*, *54*(16), 5933–5938.
413 Hellenäs, K.-E., Abramsson-Zetterberg, L., & Skog, K. (2005). The HEATOX Project. *Journal of AOAC*
414 *International*, *88*(1), 242–245.
415 IARC (1994) Monographs on the Evaluation of Carcinogen Risk to Humans: Some Industrial Chemicals,
416 No. 60, Lyon: International Agency for Research on Cancer.
417 IFST (2015). Guidelines for ethical and professional practices for the sensory analysis of food.
418 https://www.ifst.org/knowledge-centre-other-knowledge/ifst-guidelines-ethical-and-professional-
419 practices-sensory
420 Koschwanez, J. H., Foster, K. R., & Murray, A. W. (2011). Sucrose utilization in budding yeast as a model for
421 the origin of undifferentiated multicellularity. *PLoS biology*, *9*(8).
422 Keramat, J., LeBail, A., Prost, C., & Soltanizadeh, N. (2011). Acrylamide in Foods: Chemistry and Analysis. A
423 Review. *Food and Bioprocess Technology*, *4*(3), 340–363.
424 Mamhoud, A., Nionelli, L., Bouzaine, T., Hamdi, M., Gobbetti, M., & Rizzello, C. G. (2016). Selection of lactic
425 acid bacteria isolated from Tunisian cereals and exploitation of the use as starters for sourdough
426 fermentation. *International Journal of Food Microbiology*, *225*(Supplement C), 9–19.
427 Mottram, D. S., Wedzicha, B. L., & Dodson, A. T. (2002). Food chemistry: Acrylamide is formed in the
428 Maillard reaction. *Nature*, *419*(6906), 448–449.
429 Nionelli, L., Curri, N., Curiel, J. A., Di Cagno, R., Pontonio, E., Cavoski, I., Rizzello, C. G. (2014). Exploitation
430 of Albanian wheat cultivars: Characterization of the flours and lactic acid bacteria microbiota, and
431 selection of starters for sourdough fermentation. *Food Microbiology*, *44*(Supplement C), 96–107.
432 Rizzello, C. G., Nionelli, L., Coda, R., Cagno, R. D., & Gobbetti, M. (2010). Use of sourdough fermented wheat
433 germ for enhancing the nutritional, a for food preservation. *Pakistan Journal of Food Sciences*, *24*(4),
434 211–217.
435 Suhr, K. I., & Nielsen, P. V. (2004). Effect of weak acid preservatives on growth of bakery product spoilage
436 fungi at different water activities and pH values. *International Journal of Food Microbiology*, *95*(1),
437 67–78.
438 Surdyk, N., Rosén, J., Andersson, R., & Åman, P. (2004). Effects of Asparagine, Fructose, and Baking
439 Conditions on Acrylamide Content in Yeast-Leavened Wheat Bread. *Journal of Agricultural and Food*
440 *Chemistry*, *52*(7), 2047–2051.

441 Wang, S., Yu, J., Xin, Q., Wang, S., & Copeland, L. (2017). Effects of starch damage and yeast fermentation on
442 acrylamide formation in bread. *Food Control*, *73*(Part B), 230–236.
443 Wisselink, H. ., Weusthuis, R., Eggink, G., Hugenholtz, J., & Grobben, G. (2002). Mannitol production by lactic
444 acid bacteria: A review. *International Dairy Journal*, *12*, 151–161.
445 Yaylayan, V. A., & Stadler, R. H. (2005). Acrylamide Formation in Food: A Mechanistic Perspective. *Journal of*
446 *AOAC International*, *88*(1), 262–267.
447

Table 1. The physico-chemical parameters and composition of sourdoughs after fermentation for 16 h.

Samples	pH	Total titratable acidity (mL NaOH)	Glucose (mg/100 g d.w.)	Fructose (mg/100 g d.w.)	Total reducing sugars (mg/100 g d.w.)	Sucrose (mg/100 g d.w.)	Asparagine (mg/100g d.w.)
SS	4.03 ± 0.06^{c}	5.2 ± 0.3^{a}	529 ± 18^{a}	250 ± 3^{b}	779 ± 15^{a}	413 ± 22^{d}	18.0 ± 1^{a}
SLb	3.55 ± 0.04^{a}	12.2 ± 0.5^{d}	751 ± 26^{bc}	274 ± 4^{b}	1025 ± 22^{b}	203 ± 8^{b}	20.0 ± 0.3^{b}
SLp	3.65 ± 0.1^{ab}	11.0 ± 0.4^{bc}	718 ± 62^{b}	196 ± 5^{a}	914 ± 57^{c}	173 ± 5^{a}	17.7 ± 0.5^{a}
SPp	3.69 ± 0.04^{b}	10.3 ± 0.4^{b}	966 ± 21^{d}	563 ± 12^{d}	1529 ± 19^{d}	223 ± 19^{bc}	19.8 ± 0.4^{c}
Spa	3.57 ± 0.02^{a}	11.6 ± 0.4^{cd}	793 ± 11^{c}	356 ± 4^{c}	1149 ± 7^{e}	241 ± 10^{c}	18.7 ± 0.4^{a}

The data are presented as the mean values ± standard errors (n = 3). The mean values followed by the same letter within column are not significantly different (p< 0.05); SS: spontaneous sourdough; SLb: sourdough inoculated with *L. brevis* S12; SLp: sourdough inoculated with *L. plantarum* S28; SPp: sourdough inoculated with *P. pentoseus* S14; SPa: sourdough inoculated with *P. acidilactici* S16.

Table 2. The pH values and concentrations of glucose, fructose, total reducing sugars and asparagine in different dough samples after 90 min of fermentation.

Samples	pH	Glucose (mg/100g d.w.)	Fructose (mg/100g d.w.)	Total reducing sugars (mg/100 g d.w.)	Asparagine (mg/100g d.w.)
DSS	4.97 ± 0.01^{d}	826 ± 23^{bc}	230 ± 7^{b}	1057 ± 30^{c}	14.2 ± 0.2^{b}
DLb	4.49 ± 0.02^{b}	833 ± 20^{bc}	262 ± 6^{c}	1096 ± 21^{c}	15.7 ± 0.2^{c}
DLp	4.13 ± 0.04^{a}	1494 ± 26^{d}	258 ± 8^{c}	1752 ± 29^{e}	14.8 ± 0.4^{bc}
DPp	4.52 ± 0.03^{c}	500 ± 14^{a}	232 ± 6^{b}	732 ± 19^{a}	9.9 ± 0.4^{a}
DPa	4.68 ± 0.04^{c}	471 ± 19^{a}	326 ± 10^{d}	797 ± 14^{b}	10.5 ± 0.1^{b}
DBB	5.77 ± 0.09^{e}	748 ± 31^{b}	532 ± 11^{e}	1281 ± 39^{d}	16 ± 0.9^{c}

The data are presented as the mean values ± standard errors (n = 3). The mean values followed by the same letter within the column are not significantly different (p<0.05). DSS: dough made with spontaneous sourdough; DLb: dough inoculated with *L. brevis* S12 sourdough; DLp: dough inoculated with *L. plantarum* S28 sourdough; DPp: dough inoculated with *P. pentoseus* S14 sourdough; DPa: dough with *P. acidilactici* S16 sourdough; DBB: Baker's yeast dough made without the addition of sourdough.

Table 3. The characteristics of bread loaves.

Samples	Crust color			Crumb color			Hardness (g)	Void fraction (%)	Cell density (cells/mm^2)
	L*	a*	b*	L*	a*	b*			
BLb	60.33 ± 0.47^{b}	11.25 ± 0.64^{c}	34.64 ± 0.33^{b}	73.85 ± 0.41^{b}	-1.27 ± 0.05^{bc}	14.13 ± 0.24^{a}	4338 ± 212^{b}	35.11 ± 1.5^{b}	2888.82 ± 254.99^{a}
BLp	60.91 ± 0.39^{b}	10.62 ± 0.33^{c}	35.23 ± 0.33^{b}	74.21 ± 0.19^{b}	-1.01 ± 0.02^{cd}	15.95 ± 0.49^{b}	4188 ± 444^{b}	47.04 ± 0.6^{d}	4764.22 ± 112.03^{cd}
BPp	68.71 ± 0.21^{e}	5.94 ± 0.05^{a}	34.42 ± 0.19^{b}	74.49 ± 0.23^{b}	-1.64 ± 0.09^{a}	14.97 ± 0.64^{b}	1815 ± 216^{a}	43.99 ± 2.32^{d}	3949.54 ± 104.56^{bc}
Bpa	58.49 ± 0.15^{a}	10.33 ± 0.37^{bc}	32.73 ± 0.32^{a}	71.09 ± 0.43^{a}	-0.84 ± 0.06^{a}	14.38 ± 0.2^{a}	2704 ± 42^{a}	34.89 ± 1.23^{b}	3217.14 ± 386.02^{ab}
BSS	63.77 ± 0.44^{d}	9.56 ± 0.05^{b}	34.91 ± 0.66^{b}	70.11 ± 0.67^{a}	-1.14 ± 0.2^{cd}	13.85 ± 0.07^{a}	4060 ± 57^{b}	31.15 ± 0.90^{a}	5256.74 ± 27.67^{d}
BBB	63.09 ± 0.07^{d}	9.58 ± 0.38^{b}	35.36 ± 0.11^{b}	74.89 ± 0.30^{b}	-1.53 ± 0.1^{ab}	14.63 ± 0.18^{a}	5149 ± 119^{b}	39.57 ± 0.87^{c}	3831.8 ± 659.82^{b}

Data are presented as the mean value ± standard error (n = 3); the mean values followed by the same letter within column are not significantly different (p<0.05); BSS: bread made with spontaneous sourdough but without LAB strains; BLb: bread made with *L. brevis* S12 sourdough; BLp: bread made with *L. plantarum* S28 sourdough; BPp: bread made with *P. pentoseus* S14 sourdough; BPa: bread made with *P. acidilactici* S16 sourdough; BBB: Baker's yeast bread.

Figure captions

Fig. 1. Acrylamide content in different bread samples. BSS: bread made by using spontaneous sourdough without LAB strains; BLb: bread made with *L. brevis* S12 sourdough; BLp: bread made with *L. plantarum* S28 sourdough; BPp: bread made with *P. pentoseus* S14 sourdough; BPa: bread made with *P. acidilactici* S16 sourdough; BBB: Baker's yeast bread.

Fig. 2. Representative images of the crumb samples from experimental breads (unprocessed digital images (left) and binary images thresholded using the Otsu ImageJ algorithms (right). BSS: bread made by using spontaneous sourdough without LAB strains; BLb: bread made with *L. brevis* S12 sourdough; BLp: bread made with *L. plantarum* S28 sourdough; BPp: bread made with *P. pentoseus* S14 sourdough; BPa: bread made with *P. acidilactici* S16 sourdough; BBB: Baker's yeast bread.

Fig. 3. A spider web chart of the sensory analysis data for experimental breads. BSS: bread made by using spontaneous sourdough without LAB strains; BLb: bread made with *L. brevis* S12 sourdough; BLp: bread made with *L. plantarum* S28 sourdough; BPp: bread made with *P. pentoseus* S14 sourdough; BPa: bread made with *P. acidilactici* S16 sourdough; BBB: Baker's yeast bread.

Figure 1.

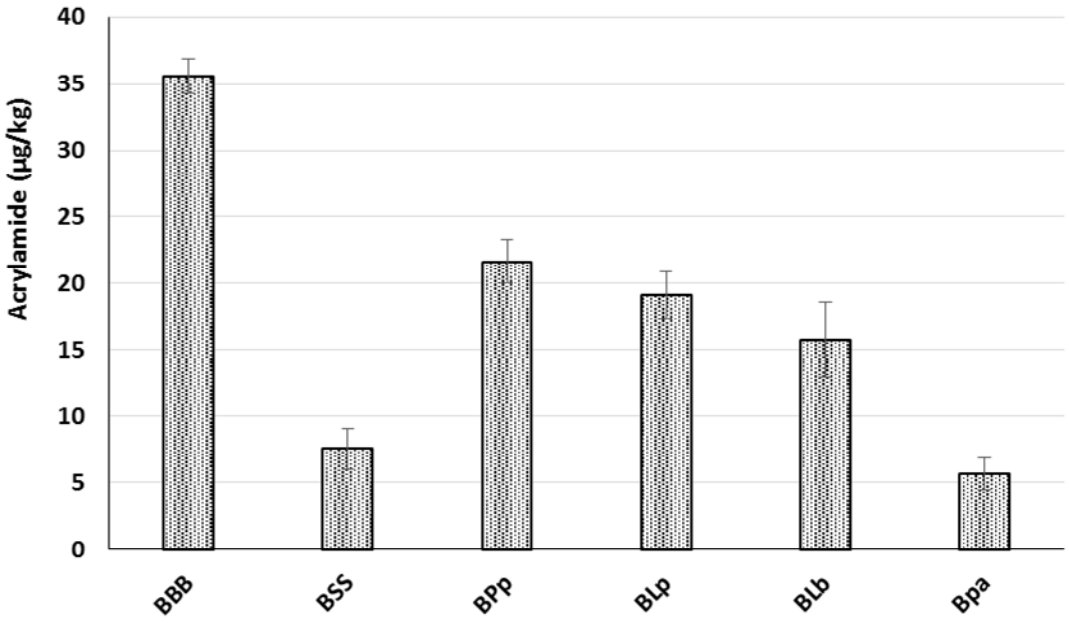


Figure 2.

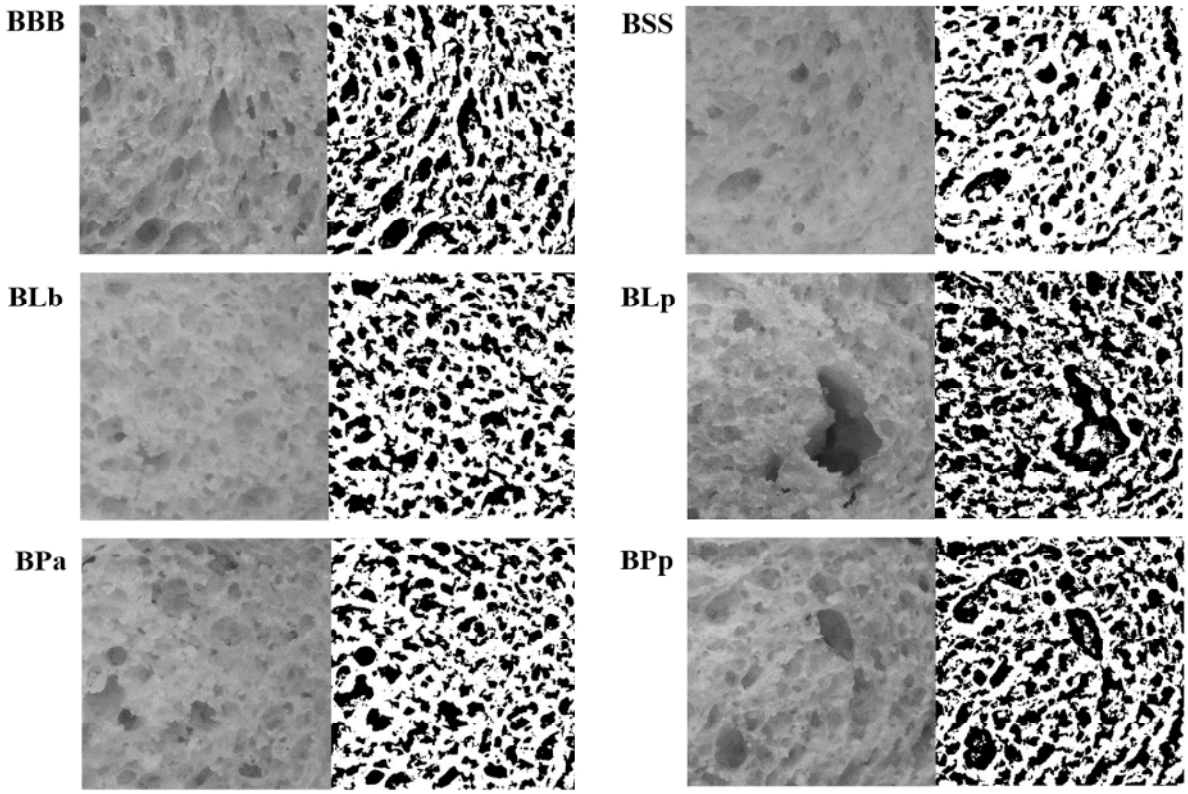
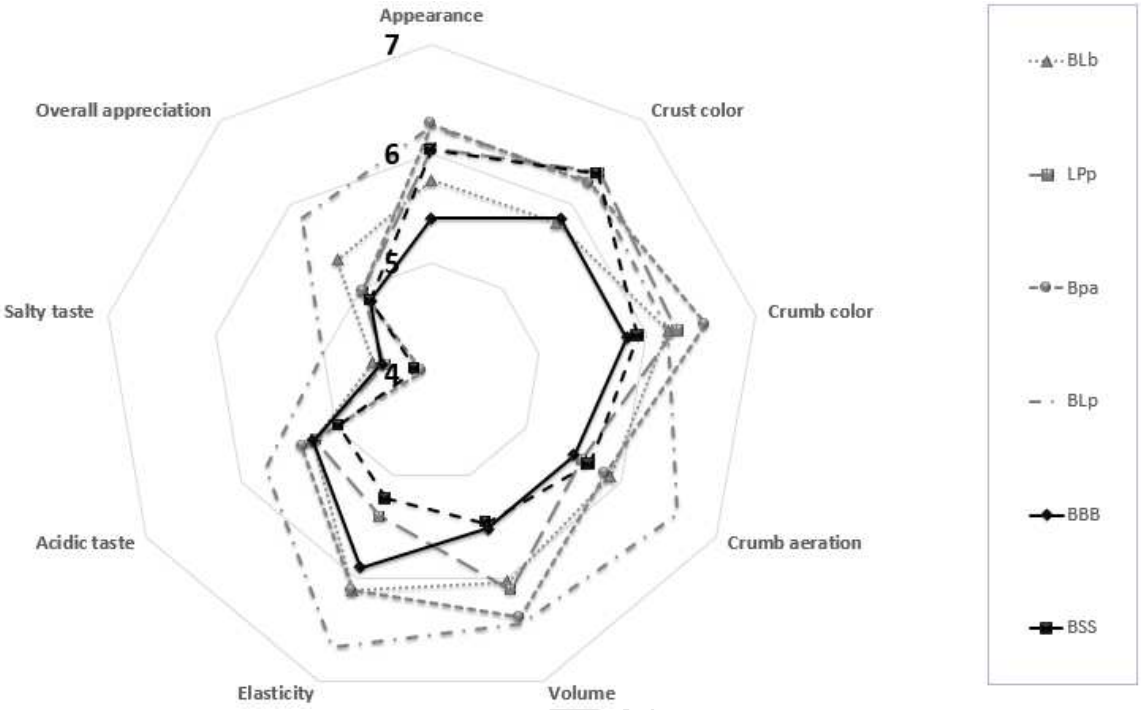


Figure 3.



Highlights

- Four lactic acid bacteria were inoculated separately in bread sourdough.
- Incorporation of lactic acid bacteria in bread reduced acrylamide content.
- Acrylamide amount is correlated to sugar concentration in sourdough.
- Incorporation of lactic acid bacteria in bread can improve its texture and flavor.