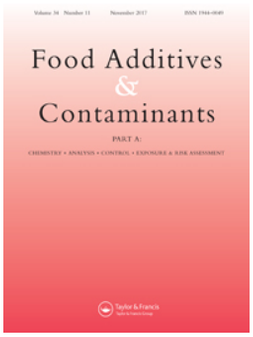

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ARTICLE

Reduction of acrylamide in whole-wheat bread by combining lactobacilli and yeast fermentation

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ABSTRACT

This study mainly focuses on a strategy for reducing acrylamide content in whole-wheat bread by combining lactobacilli and yeast in sourdough breadmaking. Combinations of sourdough (fermented dough using different *Lactobacillus* strains including *Lactobacillus plantarum* PTCC 1896 [probiotic], *L. sakei* DSM 20,017, *L. rhamnosus* DSM 20,021, and *L. delbrueckii* DSM 20,081) and yeast, in comparison with yeast alone, were used for breadmaking. The results showed that acrylamide levels in breads fermented using sourdough+yeast were in all cases much lower (6.9–20 µg/kg on a dry weight basis [d.b.]) than those in the yeast-only fermented bread (47.6 µg/kg d.b.). Significant ($p < 0.05$) correlations were also found between pH, total titratable acids (TTA) and lactic acid, and acrylamide content. Furthermore, the obtained results showed that the moisture content of dough directly influenced the formation of acrylamide in bread ($r = 0.925$, $p < 0.0001$). In addition, no significant correlations were observed between acrylamide content in breads and either the reducing sugar or free amino acid contents in dough samples. According to the different effects of *Lactobacillus* strains, it could be concluded that the acrylamide reducing potential of lactobacilli was strain-specific, with *L. rhamnosus* being the most effective. This suggests that sourdough fermentation with appropriate *Lactobacillus* strains can be used as an advantageous technology to reduce the acrylamide content of whole-wheat breads.

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Acrylamide reduction; sourdough fermentation; whole-wheat bread; *Lactobacillus plantarum*; *Lactobacillus sakei*; *Lactobacillus rhamnosus*; *Lactobacillus delbrueckii*

Introduction

Acrylamide concentration in processed food products has become a very serious health issue. Acrylamide is a food-borne toxicant that may cause gene mutation and damage the nervous system. This chemical processing contaminant is naturally formed when foods are heated at temperatures >120°C, via the Maillard reaction between a series of specific amino acids (e.g. asparagine) and compounds with carbonyl groups (e.g. glucose, fructose, maltose) (Negoita et al. 2014). Heat-treated potato, coffee, and cereal products are the major sources of the dietary exposure of acrylamide (Surdyk et al. 2004). Bakery products contribute approximately 25% of the daily acrylamide exposure through the diet (Claus et al. 2008a). Due to its high daily consumption, bread is the main source contributing to human exposure to acrylamide (Forstova et al. 2014). Therefore, it is essential to reduce acrylamide in bread even if the concentrations of acrylamide are low. The growing interest by consumers in the nutritional benefits of whole-grain products has resulted in a growing demand for whole-wheat bread. However, it has been reported that the whole-wheat flour can produce more acrylamide compared to refined wheat flour, owing to higher asparagine content of the germ and bran fractions (Capuano et al. 2009). Nevertheless, considering the nutritional benefits of the consumption of wholemeal bread, reduction of acrylamide by avoiding wholemeal flour-based foods could be considered unadvisable.

Up to now, many technological measures have been considered for acrylamide reduction in foods. These approaches are aimed at reducing the amount of reactants, that is, asparagine and reducing sugars, making conditions for the Maillard reaction unfavourable, and/or eliminating or reducing acrylamide following its formation. For more detailed data, we refer to a review study by Claus et al. (2008a). However, the most suitable methods are ones that, besides the reduction of acrylamide, cause no alterations to the other properties of food products (such

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as nutritional and organoleptic characteristics) and at the same time are safe and practicable at the industrial level (Konings et al. 2007). With due attention to the importance of acrylamide reduction without compromising the quality and financial aspects of food products, among different acrylamide reduction strategies, yeast fermentation seems to be a suitable and efficient approach. It has been demonstrated that prolonged fermentation of dough with yeast can reduce acrylamide in bread via the extensive consumption of high amounts of free asparagine (Fredriksson et al. 2004; Fink et al. 2006; Claus et al. 2008b; Mustafa et al. 2009). However, acrylamide reduction by yeast fermentation would not be so successful in fermented breads because of the increased release of fructose by yeast (Konings et al. 2007).

Sourdough fermentation (dough fermentation with different lactic acid bacteria [LAB] [Gobbetti 1998]) versus yeast fermentation is another fermentation method in bread baking. The use of sourdough has several advantages. First, sourdough is easily accessible and harmless to humans. Secondly, the sourdough fermentation process can be easily adapted to the regular bread production line. Finally, in comparison with yeast fermentation only, mixed cultures of LAB and yeast, in the form of sourdough fermentation, have been proven to be ideal for enhancing the texture, palatability, aroma, shelf life, and nutritional value of wholemeal breads. In such mixed cultures, yeasts act primarily as leavening agents, while LAB contributes mainly to acidification and production of flavouring and other metabolic compounds of bread (Ur-Rehman et al. 2006; Plessas et al. 2008). However, there has been no comprehensive study regarding the effects of sourdough on acrylamide content in bakery products. Recently, some researchers have linked some LAB strains (including *L. casei-casei* DSM 20,011, *L. reuteri* DSM 20,016, *L. sakei* KTU05-6, *Pediococcus acidilactici* KTU05-07, and three different *Pediococcus pentosaceus* strains [KTU05-8, KTU05-9, and KTU05-10 strains]) to acrylamide reduction in bakery products (Bartkiene et al. 2013a, 2013b, 2013c; Dastmalchi et al. 2016).

The most important LAB isolated from sourdough belong to the genus *Lactobacillus*. The aim of this study was to investigate the potential for acrylamide reduction in whole-wheat bread of some new *Lactobacillus* strains in sourdough, in comparison with fermentation with yeast alone. The chemical parameters significant for acrylamide formation, including free amino acids, reducing sugars, organic acids, moisture content and acid properties, were determined in fermented doughs and the breads baked from them to demonstrate the mechanisms by which sourdough and yeast could reduce acrylamide.

Materials and methods

Sourdough fermentation

Microbial strains and culture conditions

Four *Lactobacillus* strains were used in this study to prepare sourdough. These starters were *L. delbrueckii* subsp. *bulgaricus* (DSM 20,081), *L. sakei* subsp. *sakei* (DSM 20,017), and *L. rhamnosus* (DSM 20,021), which were supplied from the Iranian Biological Resource Center (Tehran, Iran); *L. plantarum* subsp. *plantarum* (PTCC 1896), which came from the Culture Collection of Food Biotechnology and Microbiology Laboratory (Isfahan University of Technology, Isfahan, Iran), had been previously isolated from Iranian infants' faecal flora and identified as a strain with probiotic properties (Mirlohi et al. 2009). Instantly active dried yeast containing *Saccharomyces cerevisiae* was obtained from Fariman Company (Khorasan Razavi, Mashhad, Iran). In order to prepare vacuum dried lactobacilli, the strains were propagated for 48 h: *L. rhamnosus* and *L. delbrueckii* were grown anaerobically (10% CO_2) at 37°C in de Man, Rogosa, and Sharpe (MRS) broth (Merck KGaA, Darmstadt, Germany), and *L. sakei* was grown aerobically at 30°C in MRS broth adjusted to pH 6.2–6.5. These stock cultures were maintained frozen at −80°C in MRS medium containing 25% (v/v) glycerol (Sigma, St Louis, MO, USA) as cryoprotectant.

Preparation of culture filtrates for sourdoughs

For experimental use, the frozen strains were activated by two successive transfers in sterile MRS broth for 20 h using 10% (v/v) inoculums under the respective conditions mentioned in the *Microbial strains and culture conditions* section. *L. plantarum* was incubated anaerobically at 37°C. Cells were harvested by centrifugation (10,000*g*, at 4°C, 10 min), washed with sterile distilled water, and centrifuged (10,000*g*, at 4°C, 5 min). The supernatant was discarded, and the cells were resuspended in the tap water used for making the sourdoughs.

Fresh cells were added immediately to the sourdough at 10^7 colony-forming unit (cfu)/g of sourdough.

Preparation of sourdoughs

Triticum aestivum cv. Pishtaaz was purchased from Isfahan Agricultural Research Center (Isfahan, Iran) and milled on a local stone mill to obtain whole-wheat flour (10.4% moisture, 1.63% ash, 17.1% protein, and 26% wet gluten). The particle size distribution of the flour was 1.04 g/100 g > 475 µm, 26.08 g/100 g > 180 µm, 26.05 g/100 g > 125 µm, 6.00 g/100 g > 106 µm, and 39.85 g/100 g < 106 µm. The sieve analysis of the whole-wheat flour was carried out by sieving two 100 g portions of the flour for 5 min. For sourdough preparation, the inoculum of one single *Lactobacillus* strain was homogeneously dispersed in 75 g tap water and then mixed with 75 g whole-wheat flour (dough yield = weight of the dough×100/weight of the flour = 200) in plastic beakers. Sourdoughs were individually incubated for 18 h at 30 or 37°C, aerobically or anaerobically according to the optimal growth conditions of the selected *Lactobacillus*. In all samples of fermented sourdoughs, counts of *Lactobacillus* were in the range of 10^8–10^9 cfu/g and remained almost at constant levels in all sourdoughs.

Bread making formulations

The dough formulations are given in Table 1. The sourdough+yeast fermented doughs (D-2: *L. bulgaricus*+yeast, D-3: *L. sakei*+yeast, D-4: *L. rhamnosus* +yeast, and D-5: *L. plantarum*+yeast) were produced by replacing 30% of the total flour with flour in the form of the prepared sourdough. The amount of water to be added to the flour was determined by a farinograph (Brabender OHG, Duisburg, Germany) to reach the 500 Brabender units consistency. In order to obtain the constant flour and water content in sourdough +yeast fermented doughs (D-2 to D-5), the total amount of water and wheat flour in the bread dough recipe was calculated by taking into consideration the corresponding amount of water and wheat flour present in sourdoughs. Yeast fermented dough (D-1) was manufactured without the addition of sourdough and used as the control dough. Bread doughs were prepared by mixing all the ingredients for 7 min with a spiral mixer. The dough was rested in bulk for 30 min in the mixer; then it was divided into 100 g loaves, shaped and placed on an oven tray for 10 min. The loaves were then proofed in a proofing cabinet at 35°C and 95% relative humidity for 40 min. Fermentation was carried out in triplicate. Dough samples were taken after fermentation and analysed immediately for pH and the total amount of titratable acids (TTA) or frozen for the later analyses of reducing sugars, organic acids, and free amino acids. Baking was carried out in a heated electric oven at 220°C for 15 min. After cooling at room temperature, loaves were stored frozen until the later measurements of pH, TTA, organic acids, and acrylamide.

Determination of acrylamide by gas chromatography electron capture detector (GC-ECD)

The analysis of acrylamide was done by gas chromatography electron capture detector (GC-ECD) using the standard addition method, similar to the study of Zhu et al. (2008). This method is sensitive enough for the analysis of acrylamide in heat-processed foods with excellent recoveries (92.5%–101.5%), excellent linearity with typical values for the correlation coefficient of 0.999–1.000, and with LOD and LOQ values of 0.6 µg/kg and 2.0 µg/kg, respectively (Zhu et al. 2008). The method entails extraction of acrylamide with water, filtration, derivatisation with hydrobromic acid and saturated bromine-water, liquid–liquid extraction with ethyl acetate, and concentration with nitrogen. The chromatographic analysis was per-

Table 1. Microbial composition and formulation of fermented doughs.

		Sourdough+yeast fermented doughs			
Ingredients	Yeast fermented dough D-1	D-2 (*L. bulgaricus*)	D-3 (*L. sakei*)	D-4 (*L. rhamnosus*)	D-5 (*L. plantarum*)
Whole-wheat flour (g)	125.0	87.5	87.5	87.5	87.5
Sourdough (g)	-	75.0	75.0	75.0	75.0
Yeast (*S. cerevisiae*) (g)	2.5	2.5	2.5	2.5	2.5
Salt (g)	2.5	2.5	2.5	2.5	2.5
Commercial improver (g)	0.375	0.375	0.375	0.375	0.375
Added water (ml)	97.5	60.0	60.0	60.0	60.0

Codes D-1 to D-5, as shown in this table, refer to yeast (D-1) and sourdough+yeast fermented doughs (D-2 to D-5).

formed on a GC-6890N chromatographic system (Agilent Technologies, Palo Alto, CA, USA) coupled with an electron capture detector, a DB-WAX capillary column (polyethylene glycol, 30 m × 0.25 mm internal diameter [i.d.], 0.5 μm film thickness, Agilent Technologies), and the 7683B automatic liquid sampler injector. Nitrogen was used as the carrier gas. Following injection, the column was held at 60°C for 1 min, then programmed at 20°C/min to 220°C and held for 3 min at 220°C, then at 30°C/min to 250°C and held for 5 min at 250°C. The GC-ECD sample injector interface temperature and detector interface temperature were both held at 260°C. Bread crust samples were separated by hand, dried at 30°C in a fan oven, and pulverised and homogenised with a model AR100 coffee grinder (Moulinex, Guangdong, China) to prepare the samples for the analysis of acrylamide concentrations. All chemicals were obtained from Sigma-Aldrich Chemie GmbH, Buchs, Switzerland and Merck KGaA, Darmstadt, Germany and were of analytical grade. A working solution was used for the quantification of acrylamide in samples by the standard addition method. For the preparation of working solution, the stock solution (acrylamide in methanol, 100 μg/ml) was diluted with 0.22 μm filtered distilled water (10 μg/ml). These solutions could be kept at 4°C in the dark for one month. The retention time of 2,3-Dibromopropionamide (2,3-DBPA) standard compound solution peak was used for the qualification of acrylamide in analytes. The 2,3-DBPA solution was prepared by dissolving 2,3-DBPA in ethyl acetate.

Determination of pH and TTA

The pH and TTA were determined on fermented doughs and breads following the Haggman and Salovaara (2008) procedure. Sample aliquots (10 g) were homogenised with 100 ml of distilled water by stirring. The pH value was recorded using a Jenway 3330 pH meter (Fisher Scientific UK Ltd, Loughborough, UK), and the acidity was determined by the titration of this suspension against 0.1 N NaOH to a final pH of 8.5. All tests were performed at least in duplicate.

Organic acid content

Organic acids were determined by a Shimadzu model LC-6A high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) equipped with an UV/visible detector (model SPD−6AV) at 214 nm; this was done using a Shimadzu LC-6A infusion pump with the oven temperature of 75°C and a flow rate of 0.7 ml min^{-1}. The fermented dough (5 g) and bread samples were homogenised for 30 min in 50 ml of distilled water with an ultrasonic bath and heated for 10 min at 90°C to denature the enzymes. After centrifugation (4500*g*, 15 min), the water extracts were filtered through 0.22 μm syringe filters, and aliquots of the filtrates were analysed by HPLC as described by Bartkiene et al. (2013c). A Shim-Pack SCR 101H (300 × 7.9 mm i.d.) cation exchange column (Shimadzu Corporation, Kyoto,Japan) was used as analytical column. Ultrapure water with pH adjusted to 2.5 with HPLC-grade sulphuric acid was used as the mobile phase. Certified standards of acetic (Scharlau, Barcelona, Spain), and lactic acids (Merck KGaA, Darmstadt, Germany) were used for the identification of the organic acids. External standards were used for quantification. The quantification was achieved by comparison with analytical curves using standard acids ranging between 20 and 4000 mg/kg.

Reducing sugar content

The same extraction method and chromatograph system used for the determination of organic acids were used for the analysis of reducing sugars, using a refractive index Shimadzu RID-6A detector. The sugar separation was performed using a 300 × 7.9 mm i.d., by employing 10 μm (particle size) Shimadzu Shim-Pack SCR-101N column using ultrapure water, degassed under vacuum in an ultrasonic bath, as the mobile phase. The flow rate of the eluent was 0.8 ml/min, and an injection volume of 20 μl was used. External standards were used for quantification. Reduced sugars were quantified using a calibration curve of the corresponding standards ranging between 10 and 3000 mg/kg. Glucose, D-fructose, and D-maltose standards, which were of an analytical grade, were obtained from Merck.

Free amino acid analysis

Free amino acids were quantified by the Pico-Tag method developed commercially by Waters Associates (Bidlingmeyer et al. 1984; White et al. 1986). In this method, phenylisothiocyanate is used for pre-column derivatisation, while reversed-phase HPLC separates the phenylthiocarbamyl (PTC) derivatives detected by their UV absorbance.

A Waters 1525 Binary HPLC system (Waters Assoc., Milford, MA, USA) equipped with a 2489 UV-Visible detector (Waters Assoc.) fixed wavelength at 254 nm was employed to detect PTC derivatives. The column was a reversed-phase Symmetry-C18, 150 mm × 4.6 i.d., 5 µm particle size (Waters Assoc.), at a column oven temperature of 38°C (± 1°C).

Statistical analysis

All analytical experiments were carried out in duplicate, and the average values are reported. Data were subjected to the analysis of variance to determine significant differences by completely randomised design; this was done using SAS software (SAS Institute Inc., Cary, NC, USA). Calculated mean values were compared by least significant difference (LSD) tests. To discover the relationships between the attributes of the fermented bread doughs and the bread acrylamide content, correlation coefficients (r) were also calculated by the Minitab software (Minitab Inc., State College, PA, USA).

Results and discussion

The effect of yeast and sourdough+yeast fermentations on acrylamide content

According to previous studies, more than 99% of the acrylamide is produced in the crust, where the temperature reaches much higher values than in the bread crumb, where the temperature does not exceed 100°C (Lingnert et al. 2002; Surdyk et al. 2004; Gokmen and Senyuva 2008). Therefore, in our study, bread crust samples were separated, dried, and analysed for acrylamide contents.

Acrylamide concentrations in bread crust samples are shown in Figure 1. The results showed that the acrylamide levels of breads fermented by sourdough+yeast in all cases were significantly

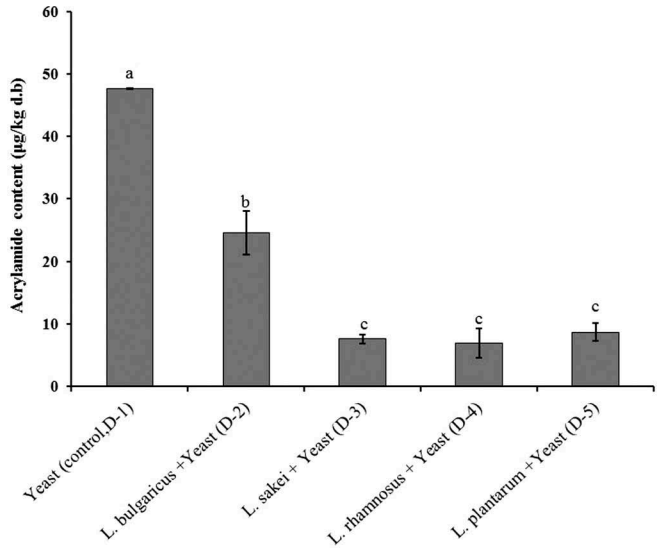

Figure 1. Acrylamide levels in bread crust samples prepared with fermented doughs. Results are mean ± SD (n = 2). Different letters mean a significant difference ($p < 0.01$).

Note: The letters a–c indicate the highest to the lowest mean values, respectively.

($p < 0.01$) lower (6.9–20 µg/kg bread crust on a dry weight basis [d.b.]) than those in the yeast fermented bread (47.6 µg/kg bread crust, d.b.). The acrylamide level of 47.6 µg/kg, as found in the present study in the crust of yeast fermented bread (80 min fermentation), was well in accordance with ranges reported by Fredriksson et al. (2004). Their results of crust analyses of long-time yeast fermented bread (6 h) and breads fermented for a short period of time (30 min) were 24 µg and 180 µg of acrylamide/kg bread crust (d.b.), respectively. As all acrylamide is essentially found in the crust of bread, by using the weights of crust and crumb the acrylamide content in bread can be calculated on the whole bread (including crust and crumb) weight basis. By taking this into account, the values corresponding to 180 and 24 µg of acrylamide/kg (crust weight basis) were 33 and 4 µg of acrylamide/kg (whole bread weight basis), respectively (Fredriksson et al. 2004). It has been reported that breads produced with different raw materials, manufacturing recipe, and processing conditions have acrylamide concentrations ranging from 10 to 150 µg/kg on a whole bread weight basis (Arribas-Lorenzo and Morales 2012; Serpen et al. 2012; Forstova et al. 2014; Negoita et al. 2014). These concentrations are about 4 to 5 times more than acrylamide contents reduced by yeast

fermentation. The yeast fermented wheat breads contain relatively low concentrations of acrylamide. In our study, however, all sourdough+yeast fermentations significantly reduced that low acrylamide content of the whole-wheat breads even more. Overall, the results of this study demonstrated that acrylamide levels in whole-wheat breads could be lowered by 48.5% and 85.5% using sourdough+yeast fermentation with *L. bulgaricus* and *L. rhamnosus*, respectively, as compared to yeast fermentation only (Figure 1). In contrast, 90-min fermentation with *Lactobacillus* in combination with yeast in a similar study by Dastmalchi et al. (2016) resulted in 5.7%–22.8% reduction in the acrylamide levels of the final products, in comparison to yeast fermentation only. The bread roll fermented by *L. casei-casei* and *L. reuteri* in this study contained 219.1 and 289.75 µg acrylamide/kg on a whole bread weight basis, respectively, which was much higher than our obtained results (Dastmalchi et al. 2016).

The effect of biological acidification on acrylamide content

Acidic properties of fermented dough and bread samples are presented in Table 2. Fermentation with sourdough+yeast reduced the pH of doughs D-2 to D-5, in comparison to the yeast fermented dough (D-1). This reduction was much more pronounced in the corresponding breads. Accordingly, TTA values and the amount of organic acids (lactic and acetic acid) in doughs and breads containing sourdough were significantly ($p < 0.05$) higher than those in control ones. The acidity differences between D-2 to D-5 were due to the type of fermentation (homo- and hetero-fermentation), the ability of *Lactobacillus* strains to produce acids, and the competitive or non-competitive associations between lactobacilli and yeast that could have synergistic or antagonistic effects on acid production. *L. bulgaricus* is obligately homofermentative with homolactic fermentation; it is the simplest type of fermentation for producing lactic acid exclusively. *L. sakei, L. rhamnosus*, and *L. plantarum* are facultatively heterofermentative with heterolactic fermentation, where some lactate as well as pentoses can be further metabolised yielding other acids (such as acetic acid), alcohols, and carbon dioxide. Compared to acetic acid, the pKa of lactic acid is 1 unit less (4.76 versus 3.86 at 25°C), meaning that lactic acid deprotonates 10 times more easily than acetic acid does, thereby providing higher acidity values. However, it has been reported that pH variation in fermented doughs greatly depends on both lactic and acetic acid production and slightly on CO_2 dissolution in the water phase (Palacios et al. 2006). Consequently, dough fermented only by *Saccharomyces cerevisiae* (D-1) and its respective bread, with no lactic acid production, had the highest pH (5.8 and 6.1, respectively) and the lowest TTA values (8.5 and 5.5 ml NaOH 0.1 N, respectively); this dough was followed in the order by D-3, D-2, D-4 and D-5.

The results showed significant ($p < 0.05$) correlations between pH, TTA, and lactic acid and acrylamide content. In all studies, acrylamide was decreased with increasing amounts of acids. There was a positive correlation between acrylamide content and pH ($r = 0.738$ and $r = 0.887$ for dough and bread pH, respectively) and a negative one for TTA values ($r = -0.859$ and $r = -0.827$ for dough and bread TTA, respectively). Among the analysed organic acids, lactic acid had a significant effect ($r = -0.691$ and $r = -0.724$ for dough and bread lactic acid, respectively) on the reduction of acrylamide content in breads because of its higher acidic

Table 2. Acidic characterisations of fermented doughs after proofing and their respective breads.

	Fermented dough				Bread			
Sample	pH	TTA[f]	Lactic acid (g/100 g d.b.)	Acetic acid (g/100 g d.b.)	pH	TTA	Lactic acid (g/100 g d.b.)	Acetic acid (g/100 g d.b.)
D-1[g]	5.8 ± 0.1^{a}	8.5 ± 1.3^{c}	0^{d}	0.048 ± 0.002^{a}	6.1 ± 0.1^{a}	5.5 ± 0.3^{c}	0^{e}	0.018 ± 0.001^{a}
D-2	5.5 ± 0.3^{a}	12.4 ± 0.6^{b}	0.048 ± 0.000^{c}	0.025 ± 0.001^{b}	5.1 ± 0.0^{bc}	10.8 ± 0.4^{a}	0.021 ± 0.001^{c}	0.016 ± 0.002^{a}
D-3	5.6 ± 0.1^{a}	13.0 ± 0.0^{ab}	0.048 ± 0.005^{c}	0.023 ± 0.002^{b}	5.2 ± 0.0^{b}	9.3 ± 0.2^{b}	0.013 ± 0.000^{d}	0.007 ± 0.000^{c}
D-4	5.1 ± 0.1^{b}	12.2 ± 0.0^{b}	0.080 ± 0.002^{b}	0.025 ± 0.000^{b}	4.8 ± 0.1^{d}	11.6 ± 0.4^{a}	0.048 ± 0.000^{b}	0.012 ± 0.000^{b}
D-5	5.1 ± 0.0^{b}	14.4 ± 0.8^{a}	0.165 ± 0.001^{a}	0.050 ± 0.000^{a}	4.9 ± 0.1^{cd}	11.0 ± 1.1^{a}	0.051 ± 0.001^{a}	0.018 ± 0.002^{a}

[f] The TTA (total titratable acid) is expressed in ml of 0.1 N NaOH.
d.b.: on a dry weight basis.
[g] Codes used for fermented doughs, as presented in Table 1.
Different letters indicate significant ($p < 0.05$) differences in a given column. The letters a–e indicate the highest to the lowest mean values, respectively. Data are presented as mean values ± SD (n = 2).

characterisation. The explanation is that the first step in acrylamide formation in the Maillard reaction is the formation of Schiff bases from asparagine and reducing sugars (Eriksson 2005). Since only the non-protonised form of asparagine can form the Schiff base, when pH passes the isoelectric point of the asparagine amino group, the initial amino-carbonyl reaction may be hampered due to the protonation of the amino group (Lingnert et al. 2002), thereby decreasing the Maillard reaction and acrylamide content in bread. It has been reported that the optimum pH for acrylamide formation is around 8 (Blank et al. 2005). It has also been reported that the addition of consumable acids can result in acrylamide reduction in bakery products (Claus et al. 2008a; Zhang et al. 2009). On the other hand, it is noticeable that addition of organic acids, especially acetic acid, to the bread dough is only acceptable in the case of sourdough; otherwise, it may cause undesirable changes in the bread flavour and sensory attributes (Paramithiotis et al. 2006).

The effect of reducing sugars on acrylamide content

Although it has been reported that the addition of reducing sugars into bread dough may not cause acrylamide to increase (Surdyk et al. 2004), a previous study claimed that yeast increased fructose release early in the fermentation cycle and the expected reduction in acrylamide concentrations did not occur (Konings et al. 2007). Since the endogenous amylolytic activity of the flour was different in doughs with various acidity values, and because of the different exhibited amylolytic activity of studied *Lactobacillus* strains, the outcome of this microbial association with acrylamide concentration could be different from the reported one. So, reducing sugars in dough samples were analysed in order to determine the effect of our studied microbial cultures on the type and concentration of reducing sugars in doughs and a consequent influence on acrylamide content in breads.

The reducing sugars determined in doughs were maltose and fructose, while glucose was not detected (Figure 2). To our knowledge, the impact of maltose on acrylamide content in bread has not been previously studied. Doughs fermented by sourdough +yeast (D-2 to D-5) had numerically but insignificantly higher amounts of maltose, as compared to D-1. No significant differences were observed between the fructose content of yeast and sourdough+yeast fermented doughs, contrary to a previous study claiming that yeast increased fructose release early in the fermentation cycle (Konings et al. 2007). Total reducing sugars was considered as the sum of glucose, fructose, and maltose contents. Thus, it is reasonable to assume that total reducing sugar content of various doughs may not be significantly different. In the current study, no significant relation was found between acrylamide content in breads and any reducing sugar content in doughs. This was in accordance with results indicating that, because of the presence of free sugars formed during baking, the amount of reducing sugars could not be a limiting factor for acrylamide formation (Keramat et al. 2011).

The measured sugars in the dough and in the final bread were the result of a balance between flour carbohydrates hydrolysis and their consumption by lactobacilli and yeast present. Inability of the applied technique to detect glucose could be attributed to its low concentrations, which were very close to the HPLC detection limit (LOD). Glucose is an easily fermentable and preferable sugar for yeast and lactobacilli and thus can explain the low concentrations. Given that maltose is the most abundant carbohydrate in the flour and may also be released through mild acidic or enzymatic hydrolysis of starch in sourdough systems (Arendt et al. 2007), its concentration in all samples was much higher than the fructose content. The increase in the amount of maltose in sourdough+yeast fermented doughs versus yeast fermented dough could also be attributed to the hydrolytic activity of lactobacilli amylase and the mild acid hydrolysis of starch in sourdough systems.

The effect of free amino acids levels of fermented doughs on acrylamide content

Quantification of individual and total free amino acids in dough samples has been summarised in Table 3. Doughs fermented with sourdough+yeast showed an additive effect in the total levels and most individual amino acids. However, no correlation ($p < 0.05$) was observed between any amino acid

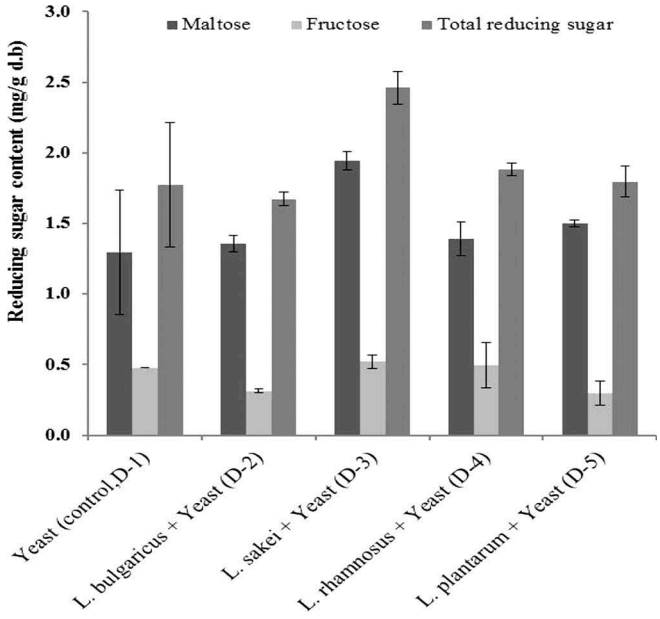

Figure 2. Reducing sugar contents of fermented doughs after proofing. Data are presented as means ± SD (n = 2). There are no significant differences between treatments ($p < 0.05$).

Table 3. Individual and total free amino acid content of fermented bread doughs after proofing.

	Content (µg/g dough, d.b.)				
Amino acid	**D-1**[a]	D-2	D-3	D-4	D-5
Aspartic acid +Glutamine	48.15	123.64	141.82	87.50	60.71
Glutamic acid +Asparagine	242.59	307.27	247.27	246.43	178.57
Serine	59.26	96.36	74.55	41.07	58.93
Glycine	70.37	83.64	83.64	57.14	71.43
Histidine	14.81	38.18	29.09	10.71	17.86
Arginine	183.33	300.00	300.00	233.93	248.21
Threonine	33.33	80.00	58.18	35.71	46.43
Alanine	192.59	352.73	334.55	239.29	250.00
Proline	105.56	210.91	167.27	141.07	110.71
Tyrosine	87.04	156.36	96.36	76.79	82.14
Valine	44.44	141.82	134.55	98.21	103.57
Methionine	n.d.	20.00	14.55	12.50	12.50
Isoleucine	n.d.	81.82	49.09	37.50	42.86
Leucine	16.67	174.55	98.18	105.36	107.14
Phenylalanine	n.d.	123.64	45.45	37.50	44.64
Lysine	59.26	107.27	89.09	89.29	66.07
Tryptophan	24.07	29.09	20.00	25.00	25.00
Total free amino acids	1181.48	2427.27	1983.64	1575	1526.79

d.b.: on a dry weight basis.
[a] Codes used for fermented doughs, as presented in Table 1.
n.d.: not detected.

or total amino acids and the acrylamide content in fermented doughs. Biochemical changes in free amino acid content during dough preparation could clearly differentiate doughs with different microbial starters. Cysteine and ornithine were not quantified in any doughs because they were present only in trace amounts. Alanine, glutamic acid+asparagine, and arginine predominated in all fermented doughs, accounting on average for 45% of free amino acids. The total free amino acid content of bread dough fermented with yeast *S. cerevisiae* alone (D-1) was 22.6%–51.3% lower than those fermented with sourdough+yeast (D-5 and D-2, respectively) (Table 3). Among other doughs, dough D-2 had the highest contents of total and free amino acids; this dough was followed by doughs D-3, D-4, and D-5.

The probable explanation for not finding the correlation is that acrylamide production in complex biological matrices such as fermented doughs is not restricted to a single factor such as free amino acids alone. It is probable that lower acidity in dough D-1 with lower amino acid content caused the higher acrylamide content, as compared to doughs D-2 to D-5. In relation to doughs D-2 to D-5, higher amounts of free amino acids, besides higher acidity, could have caused the reduced acrylamide content, probably by promoting competing reactions with the precursors and/or covalently binding the formed acrylamide, in accordance with previously reported results (Konings et al. 2007; Claus et al. 2008a; Zhang et al. 2009). However, it is believed that in flat bread the acrylamide reduction requires relatively high dosages of amino acid addition, which could result in some unpleasant odours and diminished quality properties (Konings et al. 2007). Therefore, it seems that the increased levels of amino acids in

doughs could be accepted only in the case of sourdough; this might be attributed to the improvement of bread quality and prevention of acrylamide formation by a combination of chemical factors such as acidity and sugar and moisture content.

The impact of moisture amounts on the acrylamide content

Figure 3 shows the moisture content of sourdoughs, fermented doughs, and their respective breads. The results obtained showed that the moisture content of dough directly influenced the formation of acrylamide in bread (r = 0.925, p < 0.0001). The bread made with high-moisture dough had higher acrylamide concentrations. The differences between the moisture content of studied sourdoughs and that of breads were not significant. However, in spite of the constant water content in the dough recipe, the data showed that doughs fermented by sourdough+yeast (D-2 to D-5) have significantly (p < 0.05) lower moisture content, as compared to the D-1. This trend could be attributed to some biological reactions of LAB in dough (e.g. protein and starch hydrolysis), which leads to the water reduction.

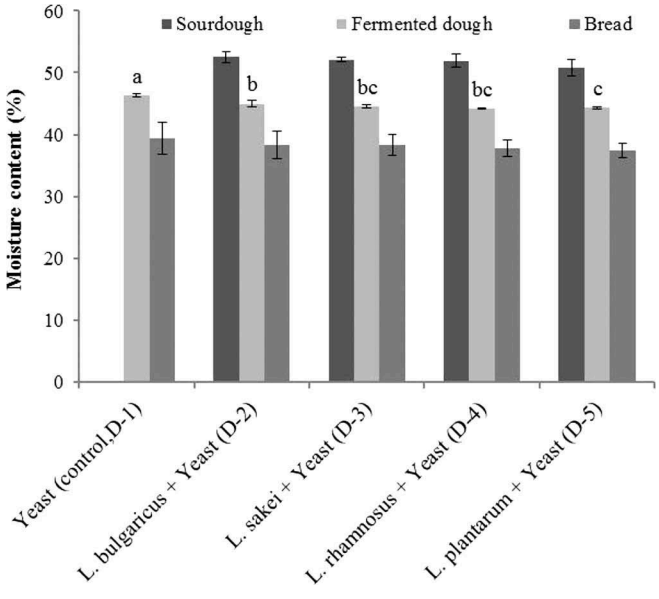

Figure 3. Moisture content of sourdoughs, fermented doughs after proofing, and their respective breads. Data are presented as means ± SD (n = 2). Only significant differences are shown on bars, with different letters (p < 0.05).

Note: The letters a–c indicate the highest to the lowest mean values, respectively.

Recently, it has been established that the water content in wheat is one of the most important factors in the formation of acrylamide (Zhang et al. 2009), and acrylamide formation is very sensitive to small changes in moisture, particularly at the lower range of moisture content (Konings et al. 2007). However, there are some complexities regarding the effect of moisture content on acrylamide formation. For instance, it is known that acrylamide formation is favoured at low moisture contents, since the low moisture content of crust enhances the Maillard reaction (Konings et al. 2007). On the other hand, acrylamide concentration has been observed to increase at higher water contents (Zhang et al. 2009). For example, it is reported that adding water to the reaction mixture of asparagine and glucose results in an up to 3-fold increase of acrylamide. In the reaction system based on fructose, the acrylamide concentration is also enhanced with increasing water content to some extent, which is in agreement with our observations. This phenomenon could be explained by the fact that the occurrence, the chemical reactivity of the precursors, and the physical state of the reaction system are important factors that should be considered to achieve reduced acrylamide amounts. In this respect, the water content may affect both the chemical reaction and the physical state of the reaction system by changing the physical state from solid to suspension (Blank et al. 2005). Consequently, to a limited extent, doughs with higher moisture content, only by having the suspension physical state, could result in the production of breads with higher acrylamide concentrations.

Conclusion

Currently, extensive fermentation with yeast is known as one of the most efficient methods for acrylamide reduction in bakery products, where the reduction is due to reduced levels of free asparagine. However, the results of the present study revealed that fermentation with sourdough +yeast is more efficient in reducing the acrylamide content in whole-wheat bread. The results also showed that the levels of acrylamide in whole-wheat breads made with sourdough+yeast were in all cases lower than those in the control sample (yeast alone). According to the average medium bound levels of 42 μg/kg, reported for acrylamide

in soft breads, and its content reduced to 7–20 μg/kg by sourdough fermentation in this study, it is estimated that daily acrylamide intake in human populations through bread consumption could be reduced 2–6 times. These results are the consequence of the combined effects of the increased levels of dough acidity and free amino acids, and the reduced levels of dough moisture content. Another possible reason for the reduction of acrylamide during fermentation could be via its adsorption by the yeast or conversion into acrylic acid by the metabolism of bacteria. It could therefore be concluded that the acrylamide-reducing potential of lactobacilli is strain-specific; *L. plantarum, L. sakei* subsp. *sakei*, and *L. rhamnosus* followed by *L. delbrueckii* subsp. *bulgaricus*, and accompanied by *S. cerevisiae* could be used to reduce acrylamide. However, besides the well-documented complexity of the associations between *S. cerevisiae* and lactobacilli in sourdoughs, sourdough and dough are also dynamic systems; in order to use this technology with various microbial strains, primary researches are required to understand and optimise processes leading to reduced acrylamide production.

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