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Research Note

Use of Selected Sourdough Strains of *Lactobacillus* for Removing Gluten and Enhancing the Nutritional Properties of Gluten-Free Bread

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ABSTRACT

Forty-six strains of sourdough lactic acid bacteria were screened for proteolytic activity and acidification rate in gluten-free (GF) flours. The sourdough cultures consisted of *Lactobacillus sanfranciscensis* LS40 and LS41 and *Lactobacillus plantarum* CF1 and were selected and used for the manufacture of GF bread. Fermentation occurred in two steps: (i) long-time fermentation (16 h) and (ii) fast fermentation (1.5 h) using the previous fermented sourdough as inoculum (ca. 43%, wt/wt) with *Saccharomyces cerevisiae* (baker's yeast). GF bread started with baker's yeast alone was used as the control. Gluten was added to ingredients before fermentation to simulate contamination. Initial gluten concentration of 400 ppm was degraded to below 20 ppm only in the sourdough GF bread. Before baking, sourdough GF bread showed phytase activity ca. sixfold higher than that of GF bread started with baker's yeast alone. Atomic absorption spectrophotometric analysis revealed that the higher phytase activity resulted in an increased availability of free Ca^{2+}, Zn^{2+}, and Mg^{2+}. The concentration of free amino acids also was the highest in sourdough GF bread. Sourdough GF bread had a higher specific volume and was less firm than GF bread started with baker's yeast alone. This study highlighted the use of selected sourdough cultures to eliminate risks of contamination by gluten and to enhance the nutritional properties of GF bread.

Celiac disease (CD) is increasing worldwide; an estimated 0.5 to 2.0% of the population in most European countries and the United States have CD *(14).* The only accepted treatment for CD is a life-long strict gluten-free diet (GFD) *(11).* A GFD is a diet without storage proteins of wheat, rye, barley, kamut, and hybrids of these grains, such as triticale. Genes encoding HLA-DQ2/DQ8 predispose to CD by preferential presentation to mucosal $CD4^+$ T cells of epitopes contained in these grains that have undergone gastrointestinal digestion and deamidation by tissue transglutaminase *(17).* However, a GFD has drawbacks. Adherence to a strict GFD is difficult to maintain because many products are contaminated with nontolerated cereals. Overall, two threshold levels are distinguished by the Codex Alimentarius Commissions of the World Health Organization and the Food and Agriculture Organization of the United Nations: <20 ppm for foods that are naturally free of gluten or <200 ppm for foods that have been rendered gluten free (GF) *(6).* However, in several studies *(11, 12, 18)* frequent contamination of GF baked goods by concentrations of gluten exceeding the above thresholds has been found.

The use of sourdough as starter for leavened goods is considered as one of the oldest biotechnological processes. Sourdough is a mixture of flour (e.g., wheat or rye), water, and other ingredients that is fermented by naturally occurring lactic acid bacteria and yeasts *(10).* The sourdough process results in various positive effects on volume, texture, nutritional value, and shelf life of baked goods, mainly due to the metabolic activity of lactic acid bacteria *(8, 15).* However, very limited published information dealing with the use of sourdough in GF baked goods is available *(15, 16).*

In this study, sourdough lactic acid bacteria were selected based on proteolytic activity and acidification rates during fermentation of GF flours. A sourdough containing selected lactic acid bacteria was used for the manufacture of GF bread. The reduced gluten content and the nutritional properties of this bread were determined and compared with those in the GF bread started with baker's yeast alone.

MATERIALS AND METHODS

Lactic acid bacteria and culture conditions. Forty-six strains of lactic acid bacteria previously isolated from Italian wheat sourdoughs were used in this study: *Lactobacillus sanfranciscensis* (nine strains), *Lactobacillus rossiae* (nine strains), *Lactobacillus plantarum* (seven strains), *Lactobacillus brevis* (six strains), *Lactobacillus pentosus* (four strains), *Lactobacillus alimentarius* (one strain), *Lactobacillus fermentum* (two strains),

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TABLE 1. *Ingredients used for the manufacture of gluten-free breads*

Bread dough	Ingredient	Concn (%)
Dough I	Tap water[a]	41.05
	Corn starch	13.84
	Rice flour	36.81
	Buckwheat flour	7.38
	NaCl	0.92
Dough II	Tapioca starch	6.40
	Sucrose	2.40
	Corn starch	10.41
	Guar gum	1.60
	Olive oil	4.87
	Glycerine	1.20
	Sorbitol	1.12
	NaCl	0.84
	Baker's yeast[b]	2.29
	Whey milk	2.49
	Water	22.96
	Dough I	43.40

[a] When used for the manufacture of sourdough GF bread, the tap water used for the preparation of dough I contained *Lactobacillus sanfranciscensis* LS40 and LS41 and *Lactobacillus plantarum* CF1 at ca. 7.0 log CFU ml^{-1}.

[b] Baker's yeast was added at ca. 8.0 log CFU ml^{-1}.

Lactobacillus paracasei (four strains), *Lactobacillus casei* subsp. *casei* (three strains), and *Pediococcus pentosaceus* (one strain). Strains were routinely cultivated at 30 or 37°C for 24 h in modified deMan Rogosa Sharpe (mMRS) broth (Oxoid, Basingstoke, UK) with the addition of 5% (vol/vol) fresh yeast extract and 28 mM maltose at a pH of 5.6.

Proteolytic activities. Proteinase activity was determined by using albumins and globulins extracted from wheat flour *(3).* Peptidase activities were determined as described by De Angelis et al. *(3)* by using synthetic substrates such as Leu-*p*-NA, Pro-*p*-NA, Leu-Leu, Leu-Leu-Leu, Val-Pro, and Pro-Gly, which are relatively specific for general aminopeptidase type N (PepN), proline iminopeptidase (PepI), dipeptidase (PepV), tripeptidase (PepT), prolidase (PepQ), and prolinase (PepR), respectively.

Acidification rate. Sixty-two grams of a mixture of maize, rice, and buckwheat flours (ratio 6:13:1) and 38 ml of sterile tap water containing individual bacterial strains at ca. 8.0 log CFU ml^{-1} were used to prepare 100 g of dough (dough yield: dough weight × 100/flour weight; ca. 160). Dough was mixed manually for 5 min and fermented at 30°C for 7 h. Acidification was determined on-line with a Foodtrode electrode (Hamilton, Bonaduz, Switzerland). Acidification data were modeled according to the Gompertz equation as modified by Zwietering et al. *(20).* The same equation was used to evaluate acidification data during manufacture of GF breads. Fermentations were carried out in triplicate, and each sourdough was twice analyzed.

Manufacture of GF breads. The list of ingredients used for the manufacture of GF breads is given in Table 1. Two type of breads were manufactured: sourdough GF and baker's yeast breads. A commercial *Saccharomyces cerevisiae* concentrate brick was used for the baker's yeast. For the manufacture of sourdough GF bread, the fermentation (dough yield of 172) of dough I was allowed for 16 h at 30°C. After fermentation, an aliquot (ca. 43%, wt/wt) of dough I was used as a natural starter (sourdough) for the fermentation (dough yield of 162) of dough II at 30°C for 1.5 h. For the manufacture of baker's yeast bread, dough I was not fermented and was added directly to dough II (Table 1). Fermentation of dough II was allowed for 1.5 h at 30°C. Fermentations were carried out in triplicate, and each GF dough or bread was twice analyzed.

Sourdough GF bread was made using the recipe and protocol described at an industrial plant by Giuliani S.p.A. (Milan, Italy) and stored for 6 months at room temperature in polyethylene packaging.

Gluten degradation. Concentrations of gluten ranging from 100 to 500 ppm were added to the ingredients used for mixing dough I (Table 1). Both sourdough and baker's yeast GF breads were manufactured according to the protocol described. Before baking, protein were extracted from the dough directly by 60% ethanol to include both peptides and proteins that were hydroalcohol soluble. Immunological analysis was carried out with the R5-sandwich enzyme-linked immunosorbent assay (ELISA). The R5 monoclonal antibody and the horseradish peroxidase–conjugated R5 antibody were used for gluten analysis. The R5-sandwich ELISA analysis *(19)* was performed with a Transia Plate detection kit (Diffchamb, Västra Frölunda, Sweden) following the manufacturer's instructions.

Phytase activity and determination of free metals. Phytase activity of doughs was measured in terms of inorganic orthophosphate released from the phytic acid by phytase *(4).*

The concentrations of free Ca^{2+}, Fe^{2+}, Zn^{2+}, and Mg^{2+} in water extracts of doughs was determined at the laboratory of Redox SNC (Monza, Italy) following an inductively coupled plasma method with atomic absorption spectrophotometric (AAS; IRIS Intrepid, Thermo Elemental, Thermo Fisher Scientific, Waltham, Mass.) analysis and an air-acetylene flame.

Determination of lactic acid bacteria, organic acid, and free amino acids. The level of lactic acid bacteria was estimated by culture on mMRS agar at 30°C for 48 h. The concentrations of lactic and acetic acids were determined by enzymatic methods (Diffchamb). Total and individual free amino acids were analyzed with a Biochrom 30 series amino acid analyzer (Biochrom Ltd., Cambridge Science Park, UK) as described by De Angelis et al. *(3).*

Structural, sensory, and nutritional analysis of GF breads. The specific volume and firmness of GF breads were determined after 4 and 24 h of storage, respectively, according to official American Association of Cereal Chemistry (AACC) methods 10-10 and 74-09, respectively *(1).* A laboratory panel of reviewers gave an indication of consumer acceptance of the products under study. Breads were baked the day before sensory testing and served at room temperature under normal (daylight) illumination. A serving of each bread, identified by code numbers, on a single tray was served to each panelist. Ten untrained panelists evaluated each product for quality attributes of elasticity, color, acid taste, acid flavor, sweetness, dryness, and general taste, which were rated on a scale from 0 (lowest) to 10 *(9).* Proteins were determined with the Kjeldahl method. Total carbohydrates and soluble sugars were determined by high-performance liquid chromatography. Total fat, saturated fat, and total dietary fiber were determined according to the official AACC methods 30-10, 58-19, and 33-05, respectively *(1).* Calories were determined as a summation of protein, lipids, and carbohydrates.

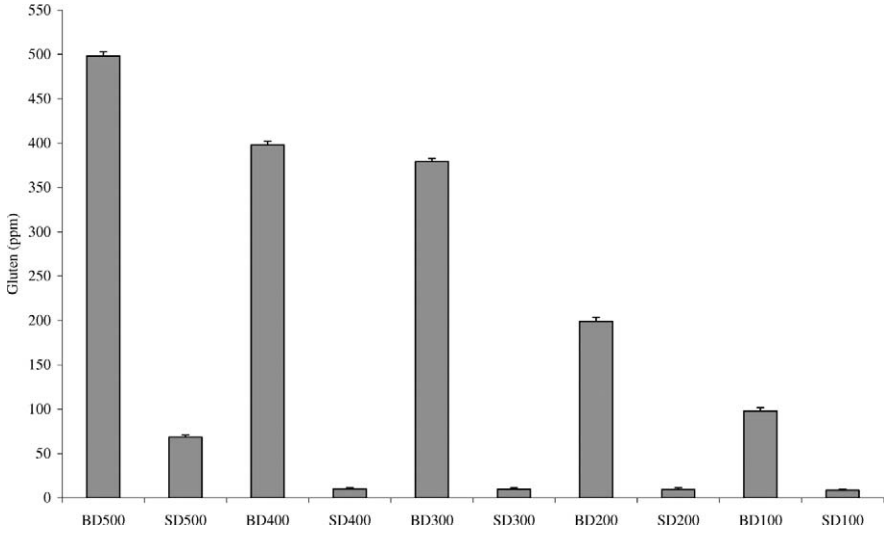

FIGURE 1. *Concentration (ppm) of gluten in dough I fermented with baker's yeasts (BD) and sourdough (SD) for 16 h. Gluten was added during mixing at 500 ppm (BD500 or SD500), 400 ppm (BD400 or SD400), 300 ppm (BD300 or SD300), 200 ppm (BD200 or SD200), and 100 ppm (BD100 or SD100). The other dough ingredients are described in the "Materials and Methods." Data are the mean ± standard deviation of three independent fermentations analyzed twice.*

Statistical analysis. Data were subjected to a one-way analysis of variance, and pairwise comparison of treatment means was achieved with Tukey's procedure at $P < 0.05$ with the statistical software Statistica 6.0 for Windows (1998; Statsoft, Tulsa, Okla.).

RESULTS AND DISCUSSION

Screening of sourdough lactic acid bacteria for proteolytic activities. As determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the hydrolysis of albumins and globulins allowed the differentiation of sourdough lactic acid bacteria into three major groups depending on the hydrolysis profiles. The first group included most of the *L. sanfranciscensis* and *L. fermentum* strains, the second included almost exclusively *L. rossiae* strains, and the third was the most heterogeneous, with all the remaining species. Peptidase activities were determined on synthetic substrates, mainly containing proline residues. Enzymatic activities varied markedly depending on the species and strains. Most of the *L. sanfranciscensis* strains had PepN activity on Leu-*p*-NA that ranged from 5.0 ± 0.39 to 9.8 ± 0.45 U. LS40 and LS41 had the highest activities (23.7 ± 0.65 and 22.5 ± 0.77 U, respectively). The median value for PepI activity was 8.85 U. LS40, LS41, and LS11 had the highest PepI activity at 16.0 ± 0.99, 14.0 ± 1.07, and 10.5 ± 1.12 U, respectively. The median PepV activity was 262.5 U. Strains LS40 and LS41 had the highest PepV activity and were in the 95th percentile of the aggregate strain data (398.0 ± 11.23 and 400 ± 16.05 U, respectively). PepT activity differed markedly from 300.2 ± 4.3 to 1,095 ± 11.2 U (median value of 870 U). The most active strains, LS14, LS40, LS41, LS4, and LS11, had activities in the range of 900.0 ± 34.12 to 1,095.0 ± 20.44 U. PepQ activity had a very broad range: 4.0 ± 0.12 to 450.0 ± 23.74 U (median value of 325.0 U). The 5th and 95th percentiles of the aggregate strain data were 3.0 ± 0.25 U (LS4) and ca. 850.0 ± 15.28 U (LS40 and LS41), respectively. Only a few strains had appreciable PepR activity; the 25th and 95th percentile of the aggregate strain data were 4.2 ± 0.98 and 75.0 ± 0.67 U, respectively. For PepR, the most active strains were LS40 and LS41 (200 ± 11.95 and 250 ± 7.88 U, respectively). Based on the same analysis, *L. plantarum* CF1, *L. rossiae* LR15 and Ci35, *L. brevis* 1Hd, and *P. pentosaceus* 2XA3 also had peptidase activities comparable to those of *L. sanfranciscensis* LS40 and LS41 (data not shown). Based on the proteolytic activities, *L. sanfranciscensis* LS4, LS40, *L. plantarum* CF1, *L. rossiae* LR15 and Ci35, *L. brevis* 1Hd, and *P. pentosaceus* 2XA3 were selected for further studies.

Acidification rate of sourdough lactic acid bacteria. *L. sanfranciscensis* strains LS4 and LS40 and *L. plantarum* CF1 produced the greatest decreases of pH (*A,* 2.38 to 2.45), the highest values of V_{max} (0.76 to 0.64 ΔpH min^{-1}), and low values of λ (0.22 to 0.12 h). Strains markedly acidified and grew well in the GF matrix, reaching the usual cell numbers (ca. 9.0 log CFU g^{-1}) for wheat sourdough processes *(8).*

L. sanfranciscensis LS40 and LS41 and *L. plantarum* CF1 were used as starter for the manufacture of sourdough GF bread. These lactic acid bacteria have hetero- or homofermentative metabolisms and are one of the most common combinations of lactic acid bacteria found in sourdoughs *(8).*

Gluten degradation during the production of bread. Two GF breads were made with the ingredients listed in Table 1. No detectable gluten was found in the mixtures (Table 1). Gluten (100 to 500 ppm) was added to the ingredients of dough I, and both sourdough and baker's yeast GF breads were made. As determined with the R5-sandwich ELISA, concentrations of gluten up to 400 ppm were degraded in sourdough GF bread to levels of ca. 10 ppm (Fig. 1). When gluten was added at 500 ppm, the residual concentration was ca. 68 ppm in sourdough GF bread. In contrast, the concentrations of gluten in baker's yeast GF bread did not differ significantly ($P < 0.05$) from those added initially. Under our experimental conditions, risks of contamination by gluten seemed to be eliminated by using selected sourdough. For instance, inclusion of oats in a GFD is not widely recommended, at least in the United States and Canada, because of concerns of unacceptable high levels of cross-contamination. Lundin et al. *(12)* found

contamination levels of <1.5 to >400 ppm in commercial oats from single bags. Wheat starch is used in some European countries as part of a GFD. Despite its accepted use in Europe, wheat starch is currently not recommended for inclusion in GFDs in North America *(11).* Because it is very difficult to remove gliadins and glutenins completely, wheat starch usually contains trace amounts of these nontolerated proteins. For companies that occasionally produce both GF and gluten-containing products, the risk of cross-contamination with flour is frequent, especially in bakeries. In recent research conducted to establish a safe threshold of gluten consumption for patients with CD *(2),* the authors concluded that such patients may tolerate 20 ppm of gluten per day and that the ingestion of contaminating gluten should be kept lower than 50 mg/day.

Characterization of sourdough and baker's yeast breads. The acidification rate by the selected sourdough lactic acid bacteria was characterized during long-time fermentation (16 h) with values of *A,* V_{max}, and λ of 2.53 ± 0.12, 0.90 ± 0.08 ΔpH min^{-1}, and 0.06 ± 0.01 h, respectively. After the second step of fermentation, the resulting sourdough GF bread had a pH of 4.35 ± 0.12 and contained ca. 130 ± 5 mM lactic acid and ca. 43 ± 2 mM acetic acid, with a quotient of fermentation (molar ratio between lactic and acetic acids) of ca. 3.0. Levels of lactic acid bacteria in the dough before baking were ca. 9.0 log CFU ml^{-1}. In contrast, baker's yeast GF bread had a pH of 5.52 ± 0.10, which reflected the very slight decrease with respect to the initial pH of 5.75 before fermentation.

Because GF baked goods generally are not enriched or fortified and frequently are made from refined flour or starch, they may not contain the same levels of nutrients as the gluten-containing counterparts they are intended to replace *(7),* particularly with respect to some cations. Overall, phytic acid contained in grains acts as an antinutritional factor because it is an excellent chelator of cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+} and complexes with the basic amino acid group of proteins, thus decreasing the dietary bioavailability of these nutrients *(5).* Wheat bread made through sourdough fermentation resulted in a more suitable pH condition for the degradation of phytic acid by endogenous phytases in flour, and sourdough may be a source of microbial phytases *(4).* Before baking, phytase activity was determined in the water extracts of doughs. The phytase activity of the GF sourdough (0.053 ± 0.008 U) was significantly higher ($P < 0.05$) than that of GF baker's yeast dough (0.002 ± 0.0001 U). The water extracts of the respective breads were subjected to AAS analysis for determining the concentration of some free metals. In agreement with the findings for phytase activity, sourdough GF bread contained significantly higher concentrations of free Ca^{2+}, Zn^{2+}, and Mg^{2+} ($P < 0.05$) than did baker's yeast GF bread. Only the concentration of free Fe^{2+} remained the same between the two breads.

The concentration of total free amino acids in the sourdough GF bread (ca. 1,615 ± 49 mg kg^{-1}) was significantly higher ($P < 0.05$) than that in baker's yeast GF bread (ca. 420 ± 23 mg kg^{-1}). Free amino acids such as Glu, Leu, Lys, Arg, and Pro were characteristic of the sourdough GF bread.

Few available published studies have considered the influence of sourdoughs fermented by different strains of lactic acid bacteria on the textural quality of GF bread during storage *(13, 15).* Compared with chemical acidification or nonacidified dough, sourdough fermentation caused an increase in dough elasticity and staling was delayed *(15).* These effects were mainly attributed to the breakdown of nongluten proteins and starch components by sourdough lactic acid bacteria. The specific volume of sourdough GF bread (1.35 ± 0.04 cm^3 g^{-1}) was significantly higher ($P < 0.05$) than that of baker's yeast bread (1.25 ± 0.02 cm^3 g^{-1}), and the firmness of sourdough GF bread was significantly lower (16.62 ± 0.27 versus 22.36 ± 0.18 N) ($P < 0.05$). The sourdough GF bread was made at an industrial plant according to the protocol described. Texture characteristics were confirmed and mold contamination was inhibited during 6 months of storage under polyethylene packaging at room temperature.

GF breads were subjected to sensory analysis. Except for color and dryness attributes, the scores for the two GF breads were significantly different ($P < 0.05$). Sourdough GF bread received the highest scores for acid taste and flavor (data not shown). The overall perception of taste was markedly better for sourdough GF bread. Sweetness seemed to characterize baker's yeast GF bread. The quotient of fermentation of the sourdough GF bread was ca. 3.0, which agreed with the optimal values for sensory properties of wheat sourdough bread *(8).*

Nutritional values for 100 g of sourdough GF bread were as follows: calories, 1,022/242 kJ/kcal; protein, 4.8 g; total carbohydrates, 40.5 g; soluble sugars, 2.3 g; total fat, 6.8 g (including 0.67 g of saturated fat); and dietary fiber, 7.0 g. Compared with other GF breads (www.schaer.com; www.celiapan.it; www.ds4you.com/it) mainly marketed in Italy, the sourdough GF bread of this study had high levels of protein and dietary fiber and low total carbohydrates.

Although the exploitation of sourdough in GF systems is still in its infancy, these results indicate that sourdough may be useful as a technological tool for preventing risks associated with gluten contamination and for improving the nutritional, texture, and flavor characteristics of GF breads. A large industrial application of this biotechnology is warranted.

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