Combined Effect of Sourdough Lactic Acid Bacteria and Additives on Bread Firmness and Staling

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The effect of various sourdoughs and additives on bread firmness and staling was studied. Compared to the bread produced with *Saccharomyces cerevisiae* 141, the chemical acidification of dough fermented by *S. cerevisiae* 141 or the use of sourdoughs increased the volume of the breads. Only sourdough fermentation was effective in delaying starch retrogradation. The effect depended on the level of acidification and on the lactic acid bacteria strain. The effect of sourdough made of *S. cerevisiae* 141−*Lactobacillus sanfranciscensis* 57−*Lactobacillus plantarum* 13 was improved when fungal α-amylase or amylolytic strains such as *L. amylovorus* CNBL1008 or engineered *L. sanfranciscensis* CB1 *Amy* were added. When pentosans or pentosans, endoxylanase enzyme, and *L. hilgardii* S32 were added to the same sourdough, a greater delay of the bread firmness and staling was found. When pentosans were in part hydrolyzed by the endoxylanase enzyme, the bread also had the highest titratable acidity, due to the fermentation of pentoses by *L. hilgardii* S32. The addition of the bacterial protease to the sourdough increased the bread firmness and staling.

Keywords: *Amylase; firmness; lactic acid bacteria; pentosans; sourdough; staling*

INTRODUCTION

The retrogradation of starch toward a more crystalline form is considered the primary cause of bread staling (D'Appolonia and Morad, 1981; Kulp and Ponte, 1981). Storage temperature (Aibara and Esaki, 1998), moisture content of the bread (Rogers et al., 1988), changes in gluten structure, and water migration from gluten to starch during storage (Wilhoff, 1971) have been considered to be important factors influencing the firming rate of bread.

Attempts to retard the physicochemical changes to improve the shelf life of the bread have been made in the product formulation (Ludewig, 1988), bread-making process (Salovaara and Valjakka, 1987), and packaging conditions (Ortolá et al., 1989). Monoglycerides, diacetyl tartaric ester of monoglycerides, sodium stearoyl lactylate, carboxymethylcellulose, hydroxypropylmethylcellulose, and α-amylase have been studied as antistaling agents, and their effects depend on the type of flour and bread-making process used. Such additives influenced the crumb-firming kinetics by changing the specific volume of the bread (Armero and Collar, 1998). Various levels of treatments with α-amylase from bacterial, fungal, and cereal sources (Kuracina et al., 1987; Martin and Hoseney, 1991) were used to improve the bread baking quality by influencing the water-binding capacity of starch and/or producing large quantities of residual dextrin. New amylases from *Bacillus subtilis*, which produce maltose and maltotriose from starch, and *Streptomyces albus* were recently cloned and are considered to be effective in retarding starch retrogradation (Byoung-Cheol et al., 1998). Currently, several amylases from various sources are used in the baking industry as antistaling agents (Dziezak, 1991). The addition of gluten or proteases, which mainly influence dough machinability and bread quality, also influence bread keeping quality. Some authors reported that the increase in protein content of the dough produces a substantial increase in loaf volume and a retarding of staling. Others stated that the primary effect of proteins in reducing the staling rate is to dilute the starch (Kulp and Ponte, 1981). Fessas and Schiraldi (1998) reported an increase in crumb firmness when soluble proteins were added to the dough and also found a delay of the staling in pentosans-added breads; such opposite effects were ascribed to the different crumb structures formed in the course of leavening and baking. Nonstarch polysaccharides (pentosans), which bind large quantities of water, are important functional ingredients in bread-making (Casier et al., 1973), but their role in the shelf life of leavened baked products is somewhat disputed.

Acidification by sourdough lactic acid bacteria (LAB), microbial hydrolysis of starch, and proteolysis affect physicochemical changes throughout bread storage including a positive effect in delaying both bread firmness and staling (Corsetti et al., 1998a).

This study describes the combined effect of various selected sourdough lactic acid bacteria and various additives such as α-amylase, protease, pentosans, and pentosanases on the keeping quality of bread.

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MATERIALS AND METHODS

Microorganisms and Culture Conditions. *Lactobacillus sanfranciscensis* 57 and CB1 *Amy* (*L. sanfranciscensis* CB1 containing the *Amy* gene from *Bacillus stearothermophilus*) (Gobbetti et al., 1996), *Lactobacillus hilgardii* S32, *Lactobacillus fructivorans* DA106 (obligately heterofermentative strains), *Lactobacillus plantarum* 13 (facultatively heterofermentative strain), *Lactobacillus farciminis* CC1 (obligately homofermentative strain), and *Saccharomyces cerevisiae* 141 were from the Culture Collection of the Institute of Dairy Microbiology, Agricultural Faculty of Perugia, Italy. *Lactobacillus amylovorus* CNBL1008 (obligately homofermentative strain) was kindly provided by Prof. L. Morelli from the Institute of Microbiology, Agriculture Faculty of Piacenza, Università Cattolica del Sacro Cuore, Italy.

LAB and yeasts were cultured on sourdough bacteria (SDB) (Kline and Sugihara, 1971) and Sabouraud (Difco Laboratories, Detroit, MI) media, respectively, at 28 °C for 24 h. Cells were harvested by centrifugation at 10000*g* for 10 min, washed twice with sterile, distilled water, and then resuspended in sterile water; the microbial suspension, diluted 1:10, gave an optical density (620 nm) of 1.25, yielding about 10^9 and 10^7 colony-forming units (CFU)/mL of LAB and yeasts, respectively.

Dough Kneading, Fermentation, and Baking. The wheat flour contained 12.8% moisture; protein (N × 5.70), 10.6% of dry matter (dm); fat, 1.79% of dm; and ash, 0.60% of dm. Wheat flour (250 g), 110 mL of tap water (or 125 mL for pentosan-containing doughs), and 40 mL of cellular suspension, containing one or more microorganisms at the described cellular concentrations, were used to produce dough with a continuous high-speed mixer (60*g*; optimal dough mixing time = 5 min) (Chopin & Co., Boulogne, Seine, France). Doughs (400 g) were individually placed in aluminum pans (25 cm × 10 cm × 8 cm height) and incubated at 28 °C for 150 (yeast and LAB starters) or 190 min (yeasts alone). Fermentation times were selected on the basis of assays conducted by a Chopin Rheofermentometer F_2 (Groupe Tripette et Renaud, Villeneuve-La-Garenne, Cedex, France), which showed a maximum volume of CO_2 produced by baker's yeast and *S. cerevisiae* 141 in 190 min and an initial activation of yeast metabolism by homo- and heterofermentative LAB (Gobbetti et al., 1995a). After fermentation, the sourdoughs were baked in a batch oven (Mondial Forni, Verona, Italy) at 220 °C for 30 min. Sourdough breads were then cooled at room temperature (~20 °C) for 90 min, vacuum packed in polyethylene bags of 95 μm thickness (Tillmanns S.p.a., Milan, Italy), and stored at 20 °C for 144 h. The presence of molds in a few stored loaves marked the end of the sourdough bread shelf life.

Addition of Organic Acids, Pentosans, and Enzymes. Chemical acidification of the dough (pH 4.0) started with *S. cerevisiae* 141 was obtained by adding lactic and acetic acids in the same proportion as determined in the sourdough fermented with homo- and heterofermentative LAB.

Pentosans and microbial enzymes with the exception of protease from *B. subtilis* (Prodotti Gianni, Milan, Italy) were furnished by Puratos n.v., Groot-Bijgaarden, Belgium. Pentosans were extracted from a wheat flour slurry by physical treatment and mainly consisted of the fraction of water-insoluble pentosans. The powder preparation of pentosans contained ~14.5% protein (N × 5.70), arabinose and xylose in a molar ratio of approximately 0.5:1.0, and only traces of galactose and glucose. After hydradation in tap water for 30 min at room temperature, with magnetic stirring, pentosans were added to the dough up to a final concentration of 1% (w/w). To permit comparison of loaves with the same moisture contents after baking, preliminary assays were performed to establish the quantity of water to add to the pentosan-containing breads. The selected quantity of water (125 mL) was probably lower than the optimum, as it is known that pentosans retain water up to 10 times their own weight, but permitted us to obtain breads with a satisfactory structure.

Endoxylanase from *B. subtilis* has an optimum temperature of 40 °C and a pH range of 4.5−7.0. The UI of the enzyme (0.21 UI/mg) was determined by using a Megazyme kit (Megazyme International Ireland Ltd., Co., Wicklow, Ireland) with azoxylan (birchwood) as substrate.

The powder preparation of endoxylanase was resuspended (0.5 mg/mL) in 0.05 M sodium acetate buffer, pH 4.5, containing 0.5 mg/mL bovine serum albumin. Forty milliliters of the endoxylanase solution was then added to the dough containing 1% (w/w) water-insoluble pentosans. The amount of water was proportionally reduced so as not to change the final water content of the dough.

Protease from *B. subtilis*, having both endo- and exopeptidase activities, has a range of pH of 5.0−9.0 (optimum pH 7.0) and was used at the concentration of 4 mg/400 g of dough.

α-Amylase from *Aspergillus oryzae* (75000 SKB activity) has an optimum temperature of 45 °C and a pH range of 4.5−6.0 and was used at the concentration of 50 mg/400 g of dough (0.012% w/w).

The enzymes were dissolved in tap water and added to the dough during mixing.

Determination of α-Amylase Activity. α-Amylase activity (U/L) of cell-free supernatant from *L. sanfranciscensis* CB1 *Amy* and *L. amylovorus* CNBL1008, after 24 h of growth on SDB medium, and of the watery solution (0.012% w/w) of the powder preparation from *A. oryzae* was determined with a kinetic−colorimetric method (G.D. S.r.l., Milan, Italy) using *p*-nitrophenylmaltoeptaoside (PNPG7) as substrate. The rate of increase of the color produced by the liberation of *p*-nitrophenol was followed spectrophotometrically at 405 nm. The extracellular α-amylase activity of the two LAB strains was expressed as a percentage of the activity of the *A. oryzae* preparation.

Sourdough and Bread Characteristics. LAB and yeasts were determined before and after dough fermentation by plating on SDB and Wallerstein Laboratory (WL) Nutrient (Difco) media at 28 °C for 72 h, respectively.

The pH, total titratable acidity (TTA), and lactic acid concentration (Boehringer Mannheim) were determined as described by Corsetti et al. (1994). The loaf volume was measured by rapeseed displacement (Barber et al., 1992). After differential scanning calorimetry (DSC) measurements, the dry matter of each individual bread was determined by puncturing and drying the pan at 105 °C for 12 h.

The water activity (a_w) was measured on the crumb center and top crust of each loaf after 2 and 144 h of storage. An Aqua Lab Decagon Devices (950 NE Nelson CT, Pullman, WA) instrument was used.

Crumb Firmness Measurements. The crumb firmness was determined using an Instron Universal Testing Machine (UTM) (Instron Corp., Canton, MA). Analyses were conducted according to AACC method 74-09 (AACC, 1986) on 25 mm thick slices, which were compressed to 40% of their original height. The force [newtons (N)] reading, measured at 25% compression, expressed the resistance of the crumb to the penetrating plunger and represents crumb firmness.

DSC. For DSC a Perkin-Elmer DSC-4 (Perkin-Elmer, Norwalk, CT) fitted with a Thermal Analysis Data Station and a Graphics Plotter HP7470A was used. Three loaves were analyzed for each sourdough bread production. Before analysis, one slice was cut from the middle of the loaf, and pieces were taken in triplicate from the center (3.5 cm diameter) of the slice and subsequently blended. Aliquots of crumb (~20 mg) were then placed in a preweighed sample pan, which was sealed and reweighed. The sample pan was placed in the calorimeter at 30 °C, allowed to equilibrate for 10 min, and heated to 85 °C at 10 °C/min. An aluminum pan, of about the same weight, was used as reference. Endotherms were analyzed (Thermal Analysis Data Station) (Perkin-Elmer) to calculate enthalpy values (ΔH_g). The temperature axis of the instrument was calibrated using indium.

The variations of enthalpy during bread storage were calculated as the percentage increase with respect to values determined after 2 h of storage. This was assumed to indicate fresh breads, which contained practically no retrograded amylopectin (Siljeström et al., 1988).

Statistical Analysis. Sourdough and bread characteristics and DSC values after 2 h of storage were analyzed by a one-way analysis of variance. Each analysis was based on three observations per starter. Data on UTM and DSC collected at 24, 48, 120, and 144 h were analyzed over time, as a factorial experiment, to evaluate the possible interactions starters−time of storage. Significant differences were defined at $P < 0.05$, and means were separated by least significant differences. Correlation coefficients between volume, firmness, and DSC values of bread were also calculated.

All statistical analyses were carried out using the SAS package (SAS Institute Inc., 1985), available at the Computer Centre of the University of Perugia.

RESULTS AND DISCUSSION

The LAB used to produce sourdough breads were previously selected on the basis of various characteristics. *L. sanfranciscensis* 57 and *L. plantarum* 13 were chosen because of their high acidification rate, moderate proteolytic activity, and marked contribution to the synthesis of volatile compounds during sourdough fermentation (Gobbetti, 1998). In contrast, *L. fructivorans* DA106 and *L. farciminis* CC1 are sourdough LAB with low acidification rates. *L. hilgardii* S32 was previously selected for its capacity to specifically use pentoses liberated from pentosans through the endoxylanase activity, which enhances bacterial cell yield, acidification rate, and, especially, production of acetic acid. Using *L. hilgardii* S32 together with pentosans and endoxylanase increases the sensory properties of the sourdough bread and, in particular, reduces the fermentation quotient (molar ratio between lactic and acetic acids) (Gobbetti et al., 1999). *L. sanfranciscensis* CB1 *Amy* and *L. amylovorus* CNBL1008 produced variable degrees of extracellular amylase activity.

The association of *S. cerevisiae* 141−*L. sanfranciscensis* 57−*L. plantarum* 13 was chosen as standard sourdough to assay the effects of other combined LAB and several additives because it is the most frequently isolated and it is positively related to the sensory quality of sourdough breads (Gobbetti et al., 1995b).

S. cerevisiae 141 was also used alone to simulate bread-making processes that excluded the use of the sourdough starter.

Characteristics of Sourdoughs. The initial cell numbers of yeasts ($\sim 10^6$ CFU/g) and LAB ($\sim 10^8$ CFU/g) decreased at the end of sourdough fermentation, ranging from 1.1×10^5 to 5.6×10^5 CFU/g and from 3.1×10^7 to 6.2×10^7 CFU/g, respectively (Table 1). Only the chemically acidified and yeasted dough showed a slight increase in the yeast inoculum, with cells reaching 2.3×10^6 CFU/g at the end of fermentation.

Whereas the sourdough started with *S. cerevisiae* 141 had a final pH of 5.90, all other sourdoughs that also used LAB had lower pH values ranging from 4.60 to 3.96. In particular, when pentosans, endoxylanase, and *L. hilgardii* S32 were added, the standard sourdough (*S. cerevisiae* 141−*L. sanfranciscensis* 57−*L. plantarum* 13) had the lowest pH (3.96) and the highest TTA value (9.5 mL of 0.1 N NaOH/10 g of dough). In two recent papers (Gobbetti et al., 1999, 2000) we showed that by using a mix of water-insoluble pentosans, α-L-arabinofuranosidase from *Aspergillus niger*, or, especially, endoxylanase from *B. subtilis*, pentosans are a source of xylose and arabinose, which accelerate the acidification rate and positively interfere with the metabolism and acetic acid production of selected sourdough lactobacilli, such as *L. hilgardii* S32 and *L. plantarum* 20B. Acetic acid in sourdough is positive because it improves the sensory properties, also by affecting the fermentation quotient (molar ratio between lactic and acetic acids) (Gobbetti et al., 1995c) and has antiropeness (Rochen 1996) and antimold activities (Corsetti et al., 1998b).

Low acidity (pH 4.6 and TTA = 4.8 mL of 0.1 N NaOH/10 g of dough) was produced when *S. cerevisiae* 141 was associated with *L. fructivorans* DA106 and *L. farciminis* CC1.

Characteristics of the Breads. The main differences of acidity (pH and TTA values) that characterized the sourdoughs were maintained among the breads (Table 1).

Apart from the additives used, all of the sourdough breads produced with the LAB and *S. cerevisiae* 141 association had a significantly higher volume (805−910 mL) than that of bread produced with *S. cerevisiae* 141 alone (764 mL). The increase of the bread volume was not solely related to the LAB acidification, which may enhance the capacity of the gluten to retain CO_2 (Gobbetti et al., 1995a), because also the lowest acid sourdough (*L. fructivorans* DA106 and *L. farciminis* CC1 starters) had an intermediate volume of 841 mL. Compared to the standard sourdough bread (833 mL), the addition of fungal α-amylase markedly increased the volume to 910 mL, whereas the addition of the protease decreased the volume to 805 mL. A volume increase similar to that found with the fungal enzyme was not observed with the addition of *L. amylovorus* CNBL 1008 and *L. sanfranciscensis* CB1 *Amy*. The use of water-insoluble pentosans alone or in combination with endoxylanase enzyme and *L. hilgardii* S32 increased the bread volume when compared to the standard sourdough (869 and 854 mL, respectively, versus 833 mL). Michniewicz et al. (1992) reported the increase of the specific loaf volume only when water-soluble pentosans were used, whereas Jelaca and Hlynka (1972) observed either a beneficial or detrimental effect on loaf volume with the addition of water-insoluble pentosans, depending on the source of the material. In our conditions, due to the use of a pentosan fraction that was not entirely representative of the insoluble pentosans, a combined effect of different components could be supposed.

The highest value of the bread volume was observed in the chemically acidified and yeasted breads (931 mL). This was probably due to the highest yeast numbers and chemical acidification, which favor yeast metabolism at a constant pH 4.0.

Water Activity (a_w) in the Crumb and Crust. To eliminate the effect of different moistures on firming and staling kinetics, on the basis of preliminary assays, all of the breads were produced to have the same moisture content after baking. Because they were wrapped in polyethylene bags, all of the breads maintained a constant value of moisture of ~42% during storage.

The kinetics of a_w varied during storage and differed between the bread crumb and crust (Table 2). After 2 h of baking, the a_w values of the crumb varied from 0.992 in the chemically acidified and yeasted bread to 0.986 in standard sourdough bread with pentosans added. The a_w then decreased during the 144 h of storage in all of the breads, reaching similar values that ranged from 0.980 to 0.985. Apart from some differences after 2 h of baking, the a_w of the crust increased in all of the breads and ranged from 0.939 to 0.962 after 144 h.

Table 1. Characteristics of Sourdough and Corresponding Bread Samples

	sourdoughs				breads		
starter	CFU/g of yeasts	CFU/g of bacteria	pH	TTA, mL of NaOH	pH	TTA, mL of NaOH	vol (mL)
S. cerevisiae 141	1.1×10^5b (0.15)[a]		5.90a (0.021)	2.4f (0.10)	5.98a (0.031)	2.2f (0.15)	764h (5.6)
S. cerevisiae 141 (chemically acidified)	2.3×10^6a (0.55)		4.00cd (0.010)	7.5cd (0.25)	4.18cd (0.032)	5.6cd (0.13)	931a (3.0)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13	3.4×10^5b (0.30)	4.2×10^7a (0.50)	3.97cd (0.047)	7.8c (0.11)	4.15cd (0.025)	5.8cd (0.20)	833f (3.6)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + α-amylase	1.1×10^5b (0.17)	4.1×10^7a (0.36)	3.99cd (0.031)	7.4cd (0.32)	4.19cd (0.031)	5.8cd (0.21)	910b (6.1)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. amylovorus* CNBL 1008	2.2×10^5b (0.15)	4.1×10^7a (0.29)	4.03c (0.061)	7.4cd (0.25)	4.21c (0.045)	5.9c (0.06)	844e (3.0)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. sanfranciscensis* CB1 *Amy*	5.6×10^5b (0.51)	6.2×10^7a (0.31)	4.04c (0.068)	7.3d (0.25)	4.17cd (0.017)	5.4d (0.15)	838ef (9.7)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + protease	2.4×10^5b (0.55)	5.0×10^7a (0.49)	3.97cd (0.021)	7.6cd (0.30)	4.14cd (0.040)	5.8cd (0.32)	805g (5.0)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + pentosans	1.8×10^5b (0.60)	3.1×10^7a (0.33)	4.04c (0.026)	8.7b (0.32)	4.20c (0.051)	6.7b (0.26)	869c (4.0)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. hilgardii* S32 + pentosans + endoxylanase	3.3×10^5b (0.61)	4.2×10^7a (0.37)	3.96d (0.020)	9.5a (0.10)	4.13d (0.046)	7.5a (0.25)	854d (5.2)
S. cerevisiae 141−*L. fructivorans* DA106−*L. farciminis* CC1	1.5×10^5b (0.45)	4.5×10^7a (0.62)	4.60b (0.035)	4.8e (0.17)	4.92b (0.058)	3.8e (0.12)	841e (3.6)

[a] Mean values in the same column followed by the same letters are not different at $P > 0.05$. Values in parentheses are standard deviation of means.

Table 2. Water Activity (a_w) of Sourdough Bread Crumb and Crust during Storage

starter	crumb, 2 h	crumb, 144 h	crust, 2 h	crust, 144 h
S. cerevisiae 141	0.990bc (0.0015)[a]	0.984ab (0.0031)	0.902c (0.0021)	0.951bcd (0.0031)
S. cerevisiae 141 (chemically acidified)	0.992ab (0.0021)	0.982bcd (0.0029)	0.903c (0.0025)	0.948d (0.0060)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13	0.990bc (0.0031)	0.985a (0.0030)	0.922b (0.0031)	0.952bc (0.0040)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + α-amylase	0.991bc (0.0015)	0.980d (0.0015)	0.930a (0.0072)	0.962a (0.0038)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. amylovorus* CNBL 1008	0.988cd (0.0020)	0.983abcd (0.0021)	0.900c (0.0031)	0.943e (0.0025)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. sanfranciscensis* CB1 *Amy*	0.995a (0.0031)	0.983abc (0.0030)	0.892d (0.0038)	0.952bc (0.0020)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + protease	0.995a (0.0046)	0.982bcd (0.0029)	0.903c (0.0038)	0.953b (0.0015)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + pentosans	0.986d (0.0046)	0.982bcd (0.0010)	0.894d (0.0044)	0.953b (0.0038)
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. hilgardii* S32 + pentosans + endoxylanase	0.987cd (0.0026)	0.983abcd (0.0031)	0.881e (0.0012)	0.949cd (0.0035)
S. cerevisiae 141−*L. fructivorans* DA106−*L. farciminis* CC1	0.990bc (0.0015)	0.981cd (0.0040)	0.898cd (0.0075)	0.939f (0.0021)

[a] Mean values in the same column followed by the same letters are not different at $P > 0.05$. Values in parentheses are standard deviation of means.

Crumb Firmness. The firmness of all the breads increased markedly during 144 h of storage, with values ranging from 8.7 to 24.8 N (Table 3). Starters differed significantly and, as averages over time, the presence of *L. hilgardii* S32 together with pentosans and endoxylanase had the lowest firmness value ($P < 0.05$).

The firmness at 24 h and the firmness kinetics of the sourdough bread produced by *L. sanfranciscensis* 57−*L. plantarum* 13−*S. cerevisiae* 141 (standard sourdough) and of the bread produced with *S. cerevisiae* 141 alone did not differ significantly (firmness values of 11.0 and 9.7 N, respectively). A similar kinetics was also found in the bread produced with the *S. cerevisiae* 141−*L. fructivorans* DA106−*L. farciminis* CC1 association.

In a recent paper, Armero and Collar (1998) reported that the sourdough process led to harder breads than the straight process. Probably, as shown in a previous study (Corsetti et al., 1998a), the use of a strain of LAB with particular characteristics appears to be a fundamental requisite to retard firmness.

The addition of the fungal α-amylase to the standard sourdough gave one of the initial softest breads (6.3 N) and was effective in decreasing the firmness rate during the 120 h of storage. A softener effect, more pronounced in the presence of a soudough fermentation, was reported for fungal α-amylase (Armero and Collar, 1998). The positive effect of the amylase activity was confirmed, although to a lesser extent, by the addition of both *L. amylovorus* CNBL 1008 and *L. sanfranciscensis* CB1 *Amy* to the standard sourdough; the amylase activities of the two strains were 9 and 5.2% of the fungal α-amylase, respectively. The slightly lesser effect of the engineered LAB strain could depend on the lower extracellular amylase activity compared to that of *L. amylovorus* CNBL 1008. Bacterial amylases from *B. subtilis* are frequently used in bread-making as antistaling additives (Kulp and Ponte, 1981) and, in general, bacterial α-amylases are preferred to the fungal ones because of their higher thermostabilities. Nevertheless, the use of thermostable bacterial amylase may be associated with a persisting activity during baking, which produces a large amount of dextrins and a sticky bread (Dziezak, 1991).

The addition of protease to the standard sourdough increased the crumb firmness (final value of 32.1 N); this was probably due to extensive hydrolysis of wheat protein, which also caused a flattening of the bread. A coarser crumb was observed in the bread when protease was added. The elastic modulus of the crumb has been shown to depend on the size, shape, and distribution of the crumb alveoli, which are related to the behavior of the gluten network during the expansion of CO_2 in leavening and baking (Fessas and Schiraldi, 1998).

When pentosans or pentosans, endoxylanase enzyme, and *L. hilgardii* S32 were added to the standard sourdough, a more effective decrease of firmness kinetics was found. Kim and D'Appolonia (1977) showed that water-insoluble pentosans decreased firmness more than water-soluble pentosans. Michniewicz et al. (1992) reported that this effect was a direct consequence of the highest moisture content of the breads supplemented with pentosans. As previously reported, all of the breads produced had the same moisture content (~42%), which did not change during storage. In the latter case, a combined effect of pentosans and selected sourdough LAB may have been responsible for the decreased crumb firmness. Breads produced with the standard sourdough with pentosans and pentosans, endoxylanase enzyme, and *L. hilgardii* S32 added were from sourdoughs that had very low pH values (4.04 and 3.96) and especially high TTA values (8.7 and 9.5 mL of 0.1 N NaOH/10 g of dough).

Compared to breads produced with the standard sourdough or those to which fungal α-amylase, amylolytic LAB strains, or pentosans were added, the chemically acidified and yeasted bread, which had the highest volume, had a low value of crumb firmness at 24 h (7.2 N) but a greater increase of firmness during the storage. According to some authors (Maleki et al., 1980) who reported that breads with higher volume were softer during storage, we found, considering the volume and firmness values for all of the breads, a highly significant correlation ($r = -0.70$) between these two parameters; moreover, the use of selected strains and additives such as pentosans and α-amylase resulted in bread that, despite a smaller volume than the control bread, showed a lower firmness during storage.

DSC. A significant interaction was found for starters−time of storage. Staling increased during storage and, as an average over time, starters highly differed. Compared to the bread produced with *S. cerevisiae* 141 alone, chemical acidification was only effective for decreasing the starch retrogradation at 24 h (177 versus 211%), but later the two breads behaved similarly (550 versus 539% at 144 h) (Table 4).

The standard sourdough (*S. cerevisiae* 141−*L. sanfranciscensis* 57−*L. plantarum* 13) had a lower initial increase of enthalpy value and, particularly, a lower increase of enthalpy during storage with respect to breads produced with *S. cerevisiae* 141 or breads chemically acidified and yeasted. Piazza and Masi (1992) reported that a biological acidification may help maintain bread freshness because it influences the moisture redistribution throughout the loaf during storage. Nevertheless, if the acidification was rather low or if different strains were used, the delay of staling was not found; the sourdough started with the association of *S. cerevisiae* 141−*L. fructivorans* DA106−*L. farciminis* CC1 showed a kinetics similar to that of the bread produced with *S. cerevisiae* 141 alone.

As with bread firmness, when fungal α-amylase was added to the standard sourdough, the enthalpy values decreased further during 144 h of storage (final value of 313%). The same effect was found, although to a lesser extent, when *L. amylovorus* CNBL 1008 and *L. sanfranciscensis* CB1 *Amy* were added to the standard sourdough. Although some authors who used amylases of different sources did not report a significant effect on crumb softness of white bread (Maleki et al., 1972), the hydrolysis of α-D-(1−4) glucosidic linkages of starch by amylases converted amylose and amylopectin to soluble dextrin, which has less tendency to retrograde (Kulp and Ponte, 1981).

The addition of the protease increased the ΔH_g of the standard sourdough after 24 h as well as during storage. As reported for crumb firmness, the gluten hydrolysis probably had a negative influence also on starch retrogradation.

The greatest effect in delaying the staling was found when water-insoluble pentosans were added to the standard sourdough: the increased ΔH_g values during storage were limited to 49−188%. About the same kinetics was determined when water-insoluble pen-

Table 3. Firmness Values (Newtons) of Sourdough Bread Crumb during Storage

starter	24 h	48 h	120 h	144 h	av over time[a]
S. cerevisiae 141	9.7uv (0.26)[b]	18.7kl (0.38)	23.9fgh (0.68)	25.2def (1.28)	19.4D
S. cerevisiae 141 (chemically acidified)	7.2x (0.72)	16.9no (0.36)	22.7hi (0.68)	24.4efg (1.17)	17.8E
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13	11.0tu (0.45)	18.4klm (0.65)	24.9defg (1.40)	25.8de (0.06)	20.0CD
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + α-amylase	6.3x (0.20)	11.4st (0.85)	21.1j (1.37)	23.7gh (0.95)	15.6F
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. amylovorus* CNBL 1008	8.9vw (0.42)	13.8qr (0.64)	21.7ij (1.25)	25.9de (1.59)	17.6E
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. sanfranciscensis* CB1 *Amy*	9.3v (0.42)	14.9pq (1.15)	24.5defg (0.21)	27.8c (0.93)	21.1B
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + protease	11.3st (0.25)	21.6ij (0.50)	29.7b (2.50)	32.1a (2.93)	23.7A
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + pentosans	6.4x (0.20)	12.6rs (0.71)	15.8op (1.11)	17.5klmn (1.30)	14.2G
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. hilgardii* S32 + pentosans + endoxylanase	7.8wx (0.30)	13.2r (0.26)	16.3op (0.71)	19.5k (0.55)	13.1H
S. cerevisiae 141−*L. fructivorans* DA106−*L. farciminis* CC1	8.9vw (0.21)	18.5klm (0.58)	24.5defg (0.58)	26.0d (2.27)	19.5D
av over starter[c]	8.7D	16.0C	22.5B	24.8A	

[a] Mean values over time followed by the same capital letters are not different at $P > 0.05$. [b] Mean values followed by the same lower case letters are not different at $P > 0.05$. Values in parentheses are standard deviation of means. [c] Mean values over starter followed by the same capital letters are not different at $P > 0.05$.

Table 4. DSC Data for Melting Amylopectin Crystallites in the Crumb of Bread Stored for 2 h and Percentage Increases of Enthalpy (ΔH_g) during Storage

	DSC after 2 h of storage	% increase in ΔH_g during storage[a]				
starter	ΔH_g (J/g)	24 h	48 h	120 h	144 h	av over time[b]
S. cerevisiae 141	0.28bcd (0.04)[c]	211jk (7.2)	356fg (9.1)	550a (18.0)	539ab (26.5)	414A
S. cerevisiae 141 (chemically acidified)	0.28bcd (0.01)	177lm (12.5)	355fg (27.3)	538ab (24.0)	550a (7.0)	405AB
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13	0.32bc (0.01)	158mn (1.7)	254i (1.7)	386de (23.3)	394d (6.2)	298D
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + α-amylase	0.34b (0.01)	94pq (6.0)	163m (11.9)	305h (14.8)	313h (4.6)	219G
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. amylovorus* CNBL 1008	0.30bc (0.01)	120op (20.1)	181lm (13.6)	322h (18.2)	367ef (16.5)	248F
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. sanfranciscensis* CB1 *Amy*	0.26cd (0.01)	134no (10.6)	220j (40.7)	360ef (11.7)	402d (11.7)	279E
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + protease	0.24d (0.01)	186kl (19.5)	322h (19.0)	519b (31.6)	493c (20.9)	380C
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13 + pentosans	0.50a (0.01)	49r (3.0)	51r (10.5)	161m (9.0)	188kl (8.0)	112I
S. cerevisiae 141−*L. sanfranciscensis* 57−*L. plantarum* 13−*L. hilgardii* S32 + pentosans + endoxylanase	0.30bcd (0.01)	68qr (13.6)	87q (10.0)	210jk (1.7)	252i (22.0)	154H
S. cerevisiae 141−*L. fructivorans* DA106−*L. farciminis* CC1	0.28bcd (0.02)	215j (12.5)	340gh (16.0)	523ab (2.3)	527ab (23.8)	401AB
av over starter[d]		141D	233C	387B	402A	

[a] Percentage increases refer to the ΔH_g calculated after 2 h of storage. [b] Mean values over time followed by the same capital letters are not different at $P > 0.05$. [c] Mean values followed by the same small letters are not different at $P > 0.05$. Values in parentheses are standard deviation of means. [d] Mean values over starter followed by the same capital letters are not different at $P > 0.05$.

tosans were integrated with the endoxylanase enzyme and *L. hilgardii* S32. In these cases, DSC data showed a high correlation with the UTM determinations. When pentosans were used in bread-making, the effectiveness in delaying staling was mainly attributed to the decreased starch component available for crystallization (Kim and D'Appolonia, 1977).

Conclusion. The effects of bakers' yeast, chemical acidification, various sourdough LAB, and additives on bread firmness and staling were studied. The finding were as follows: (i) biological, and not chemical, acidification delays starch retrogradation with respect to the bread produced with bakers' yeast alone; (ii) the effect of biological acidification depends on the level of acidity produced and/or on the sourdough LAB strains; (iii) additives such as amylases or pentosans increase the effect of biological acidification on bread firmness and staling; and (iv) selected combinations of LAB strains and additives help delay the onset of physicochemical changes in the bread during storage.

ABBREVIATIONS USED

CFU, colony forming units; DSC, differential scanning calorimetry; LAB, lactic acid bacteria; SDB, sourdough bacteria; TTA, total titratable acidity; UTM, universal testing machine.

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