Proteolysis in Model Sourdough Fermentations

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Model wheat doughs started with six different lactic acid bacteria (LAB), with or without a commercial baker's yeast culture, were used to study proteolysis in sourdough fermentations. Cell counts, pH, and free amino acid concentration were measured. Sequential extraction of dough samples was performed to separate wheat proteins. The salt-soluble protein fraction (albumins and globulins) was analyzed by RP-HPLC and SDS-PAGE, whereas propanol-soluble (gliadins) and insoluble (glutenins) protein fractions were analyzed by SDS-PAGE only. Multivariate statistical methods were used for the analysis of results. The presence of yeasts and LAB affected RP-HPLC and SDS-PAGE patterns of the salt-soluble fraction in a complex way. The only changes in the gluten proteins that could be related to the presence of LAB were the appearance of new protein fragments (20 and 27 kDa) from gliadins and the degradation of high molecular weight glutenin subunits.

KEYWORDS: Wheat sourdoughs; proteolysis; lactic acid bacteria; yeasts

INTRODUCTION

Sourdough fermentation is a traditional process used in the baking industry to improve the quality and flavor of baked goods. The addition of sourdoughs in breadmaking affects in several different ways the final properties of the bread. During the fermentation process several metabolic activities take place, and each is responsible for the changes that occur in the characteristics of wheat flour as well as in aroma, taste, nutritive value, and shelf life of the bread.

Lactic acid bacteria (LAB) and yeasts have been reported to be the predominant microorganisms in sourdoughs (*1*) and, consequently, the organoleptic and nutritional properties of sourdough breads depend on their metabolism and their cooperative activities. Acidification and antimicrobial compounds produced by the sourdough microflora contribute to the stability of products and play an important role in the regulation of the complex interactions within the starter microflora and between the starter and contaminant microflora (*2*). Exopolysaccharides (EPS) can replace hydrocolloids used as texturizing, antistaling, or prebiotic additives in bread production, improving the textural and nutritional properties of the product (*3*). Production of CO_2 from heterofermentative LAB and yeasts influences the leavening process of the final dough, improving bread softening. Moreover, the interaction between LAB and yeasts may affect the synthesis of volatile compounds that contribute to the flavor and aroma of sourdough products (*1*). The hydrolysis of chelating agents such as phytic acid during

sourdough fermentation can have positive nutritional implications by increasing mineral bioavailability (*4*).

Dough properties and the nutritional value of bread are strongly influenced by protein content as well as the protein composition of the flour. The quality of cereal proteins may be improved by the fermentation process. The degradation of gluten proteins influences the rheology of wheat sourdoughs and, consequently, the texture of bread (*5*); additionally, modification of the gluten network improves the dough's machinability (*6*). Free amino acids and small peptides released during fermentation are important for rapid microbial growth as well as fermentative activity of yeasts and lactate production by LAB and as precursors for flavor development of baked goods. The proteolytic/peptidolytic activity of LAB can contribute to hydrolysis of bitter peptides and liberation of bioactive peptides (*7*). Several proteins of wheat flour and products of their hydrolysis have biological activities that can affect human health when wheat or its byproducts are consumed as foods. Peptides generated from the digestion of the wheat prolamins, which affect the human intestinal mucosa, resulting in celiac disease or gluten-sensitive enteropathy (*8*), may be degraded during proteolysis. Besides, there are also indications that some mental disorders, such as schizophrenia and autism, could be related to a diet rich in wheat proteins containing peptides with opioid-like activities (*9*).

Several theories about whether the proteolytic activity during sourdough fermentation originates from LAB and/or yeasts or from indigenous cereal enzymes have been proposed (*10*). Cereal flour, yeasts, and LAB contain proteases and peptidases that can contribute in different ways to the proteolytic events. Thiele et al. (*5*) concluded that proteolysis during sourdough

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Table 1. Dough Formulas for Preparation of Control Doughs and Sourdoughs

	neutral control	acid control	neutral yeast	acid yeast	LAB	LAB, buffer	LAB, yeast
flour (g)	20	20	20	20	20	20	20
water (mL)	20	20	18	18	18	16	16
NaCl (g)	0.4	0.4	0.4	0.4	0.4	0.4	0.4
acid solution[b] (µL)	0	600	0	600	0	0	0
penicillin G[c] (µL)	20	20	20	20	0	0	0
cycloheximide[c] (µL)	200	200	0	0	200	200	0
cloramphenicol[c] (µL)	200	200	200	200	0	0	0
LAB starter[a] (mL)	0	0	0	0	2	2	2
yeast starter (mL)	0	0	2	2	0	0	0
phosphate buffer[d] (mL)	0	0	0	0	0	2	2

[a] *Lb. plantarum* DBPZ1015 or *Lb. plantarum* DBPZ1025 or *Lb. pentosus* DBPZ0984 or *Lb. curvatus* DBPZ1020 or *W. cibaria* DBPZ1006 or *Leuc. mesenteroides* DBPZ1005. [b] Mixture of 4 volumes of lactic acid (90%) and 1 volume of acetic acid (98%). [c] 1 g/L cycloheximide and cloramphenicol; 50000 IU/mL of penicillin G. [d] 1 M sodium phosphate buffer, pH 6.8.

fermentation and the rheological consequences of gluten degradation are mainly related to the pH-mediated activation of cereal enzymes; the indigenous proteases of flour, in fact, are able to degrade cereal prolamins under acid conditions (*10*). On the contrary, other studies have shown that LAB had a substantial role in the proteolysis during sourdough fermentation (*6*, *8*).

The aim of the present study was to investigate the changes that occur during sourdough fermentation in wheat flour proteins, the sources of proteolytic activities, and the factors that may be responsible for these events such as activity of indigenous cereal enzymes, pH conditions, and microbial metabolism.

MATERIALS AND METHODS

Strains and Culture Conditions. Two strains of *Lactobacillus plantarum* and one strain each of *Lactobacillus pentosus*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, and *Weissella cibaria* were used in this study. All strains were isolated from wheat sourdoughs used to produce Cornetto di Matera bread (*11*) and were maintained as freeze-dried stocks in the Culture Collection of the Department of Biologia, Difesa e Biotecnologie Agro-forestali, Università della Basilicata, Potenza, Italy. Commercial active dry yeast was obtained from Lesaffre Yeast Corp., North America. LAB strains were propagated overnight in modified MRS medium (mMRS) containing 10 g/L maltose and 5 g/L each of glucose and fructose (*5*). Cells were harvested by centrifugation (12000*g*, 10 min, 4 °C), washed twice, and resuspended in 0.85% (w/v) NaCl to obtain a final concentration of 10^7 colony-forming units (cfu)/g of dough. The yeast was revitalized with 0.85% (w/v) NaCl and added immediately to the dough to obtain a final concentration of 10^6 cfu/g of dough.

Sourdough Fermentations. Commercial breadmaking wheat flour with 12% protein content (w/w dry basis), 0.5% ash content (w/w dry basis), 13% moisture (w/w), and a Hagberg falling number value of 250 (Odlum Group, Dublin, Ireland) was used in all tests. Flour was not treated or fortified with additives. Doughs were prepared as described in **Table 1**, mixing water and flour with a sterile spatula for 3 min to obtain a dough yield (DY) of 200. The fermentations were carried out for 24 h at 30 °C. Two control fermentations, obtained by adding antibiotics to the dough mixtures, were carried out aseptically at pH 6.0 and 3.5, respectively. Samples were taken at 0, 6, and 24 h for the subsequent analysis.

Cell Counts and pH Measurement. LAB and yeasts were enumerated at the end of fermentation by plate counts on mMRS and YPD (yeast extract, 10 g/L; peptone, 20 g/L; glucose, 20 g/L) agar media, respectively. The pH values were measured on each dough suspension (1 g of dough and 9 mL of distilled water) after 0, 6, and 24 h of

fermentation using a glass electrode (pH-meter 210, Radiometer, Copenhagen, Denmark).

Extraction of Dough Samples. Wheat proteins and their fractions were extracted sequentially from dough samples using the following solvents: (1) 0.5 mol/L NaCl and 150 mmol/L sodium phosphate, pH 6.8 (*albumins* and *globulins*) (*5*); (2) 50% (v/v) 2-propanol in H_2O (*gliadins*); (3) 1.5% (w/w) SDS, 1% (w/w) DTT, and 0.063 mol/L Tris-HCl, pH 7.5 (*glutenins*) (*12*).

Solvent (6 mL) was added to 2 g of dough, and each extraction step was carried out for 30 min at room temperature (albumins, globulins, and gliadins) or at 70 °C (glutenins), vortexing the suspension every 10 min. Supernatants were recovered after centrifugation for 10 min at 25000*g* and 4 °C (albumins, globulins, and gliadins) or 20 °C (glutenins). A washing step with deionized water of the pellet following the saline extraction was included to remove salt and residual organic acids. The extracts were not combined. The dialyzed (Medicell International Ltd., London, U.K.; dialysis tubing cutoff 3500 Da, against deionized water at 4 °C for 24 h) albumin—globulin fraction was freeze-dried and stored at room temperature until analysis by SDS-PAGE and RP-HPLC. The gliadin and glutenin extracts were stored at −80 °C until analysis by SDS-PAGE.

Determination of Free Amino Acids. The concentration of free amino acids in the salt-soluble extracts of control doughs and sourdoughs was determined by using the trinitrobenzenesulfonic acid (TNBS) method (*13*). A calibration curve was prepared using leucine (Leu, Sigma) as standard (range = 0.0−1.0 mmol/L of Leu), and results were expressed as milligrams of Leu per kilogram of dough.

RP-HPLC Analysis of Dough Extracts. Salt-soluble extracts were analyzed by RP-HPLC. The freeze-dried samples were dissolved (10 mg/mL) in 0.1% (v/v) trifluoroacetic acid (TFA, sequential grade, Sigma) in deionized HPLC grade water (Milli-Q system, Waters Corp.) and centrifuged at 12000*g* for 5 min at room temperature. The supernatants were filtered through 0.45 µm cellulose acetate filters (Sartorius GmbH, Goettingen, Germany) and injected (150 µL) into a Varian System HPLC (Varian Associates Inc., Walnut Creek, CA) comprising an autosampler, a ProStar solvent delivery system with three pumps, and a ProStar programmable multiwavelength spectrophotometric detector, interfaced with a PC with a software package for system control and data acquisition (Varian Star Workstation 5). The column was an analytical 250 × 4 mm Nucleosil C8 (5 µm particle size, 300 Å pore size, operated at room temperature) with a 4.6 × 10 mm guard column (Capital HPLC Ltd., Broxburn, West Lothian, U.K.). Eluents used were A, 0.1% (v/v) TFA in deionized HPLC grade water, and B, 0.1% (v/v) TFA in acetonitrile (HPLC grade, Lab-scan Ltd., Dublin, Ireland). The separation was achieved using the following gradient: 100% A for 5 min, 50% B (v/v) from 6 to 55 min, 50% B (v/v) for 6 min, 60% B (v/v) from 62 to 66 min, and 60% B (v/v) for 3 min; the flow rate was 0.75 mL/min. The column was washed with 95% B (v/v) for 5 min, followed by equilibration with 100% A for 5 min before the next injection. The proteins were detected by measuring the absorbance at 214 nm. Some samples were run three times to measure the reproducibility of the patterns in the above conditions.

SDS-PAGE Analysis of Dough Extracts. All protein fractions were analyzed by SDS-PAGE using a Bio-Rad MiniProtean III apparatus (Bio-Rad Laboratories). Freeze-dried albumin—globulin extracts (∼3.0 mg) were dissolved in 0.5 mL of sample buffer (sample buffer 2×, Sigma), whereas 1 volume of gliadin and glutenin extracts were mixed with 3 or 4 volumes of sample buffer, respectively. Samples were heated at 95 °C for 5 min before the run. Electrophoresis was performed using separating (12% w/v T, 2.67% w/v C) and stacking (4% T, 2.67% C) gels (*14*). Gels were run at a constant current of 20 mA for 3 h using a Power Pack 3000 unit (Bio-Rad Laboratories). Staining was carried out according to the method of Pot et al. (*15*).

Data Processing and Statistical Analysis. RP-HPLC chromatograms (height and retention time of peaks) were processed by a logistic weighting function as described by Piraino et al. (*16*). Peak height was expressed as percent of the total, and the peaks with heights of <0.5% were discarded. Classes (45; class width = 1.48 min) were defined over the retention time (RT) axis of the chromatogram in the range from 5 min (starting class RT) to 70 min (last class RT). Flat range (FR) around the class center and the membership in the flat range

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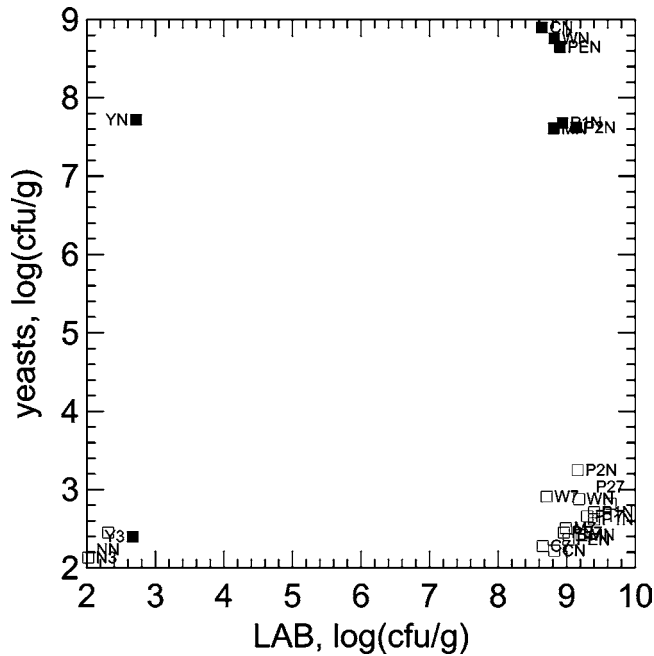
Figure 1. Microbial populations after 24 h of fermentation. P1, *Lb. plantarum* DBPZ1025; P2, *Lb. plantarum* DBPZ1015; PE, *Lb. pentosus* DBPZ0984; C, *Lb. curvatus* DBPZ1020; W, *W. cibaria* DBPZ1006; M, *Leuc. mesenteroides* DBPZ1005; N3, acidified control; NN, neutral control; 7, buffering condition; (○, △, □) 0, 6, and 24 h of fermentation, respectively; solid symbols, presence of yeasts.

Figure 2. Total free amino acid content during sourdough fermentation. P1, *Lb. plantarum* DBPZ1025; P2, *Lb. plantarum* DBPZ1015; PE, *Lb. pentosus* DBPZ0984; C, *Lb. curvatus* DBPZ1020; W, *W. cibaria* DBPZ1006; M, *Leuc. mesenteroides* DBPZ1005; N3, acidified control; NN, neutral control; 7, buffering condition; (○, △, □) 0, 6, and 24 h of fermentation, respectively; solid symbols, presence of yeasts.

(MFR) were 50 and 99%, respectively, in all cases. The 45 classes were used as variables for principal component analysis (PCA). SDS-PAGE patterns of the salt-soluble fraction were processed according to the method of Piraino et al. (*17*), using a logarithmic transformation of molecular weight (log kDa). Classes (16; class width = 0.040 log kDa), used as input for PCA, were obtained in the range from 20 kDa (starting class) to 80 kDa (last class); FR and MFR were 30 and 99%, respectively. SDS-PAGE patterns of gliadin and glutenin fractions were analyzed by visual matching. All statistical and graphic analyses were performed using Systat 10.0 for Windows (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Microbial Growth, pH Decrease, and Total Free Amino Acids. Sourdough fermentations were carried out using pure cultures of LAB and combinations of LAB and a commercial yeast. Additionally, doughs started with LAB strains in the presence of phosphate buffer to prevent the drop in pH caused by lactic acid production were used in an attempt to discriminate between effects of microbial metabolism and pH conditions. Two control fermentations, obtained by adding antibiotics to the dough mixtures, were carried out at neutral and acid condition (pH 6.0 and 3.5, respectively) to evaluate the proteolysis in the absence of microbial metabolism.

Addition of antibiotics allowed a good control of growth of LAB and/or yeasts. In fact, cell counts of yeasts and LAB in control doughs and in doughs that had not been inoculated with either group were <10³ cfu/g of dough.

After 24 h of fermentation, sourdoughs had microbial populations ranging from 8.6 to 9.6 log(cfu/g) for LAB and from 7.6 to 8.9 log(cfu/g) for yeasts (**Figure 1**). Addition of yeasts significantly decreased the final population of LAB (paired *t* test, *p* = 0.015). As a consequence, pH values were slightly higher when yeasts were used. This could be a consequence of microbial competition for carbon resources during sourdough fermentation. In fact, bacterial growth and production of lactic and acetic acids decrease when *Saccharo-*
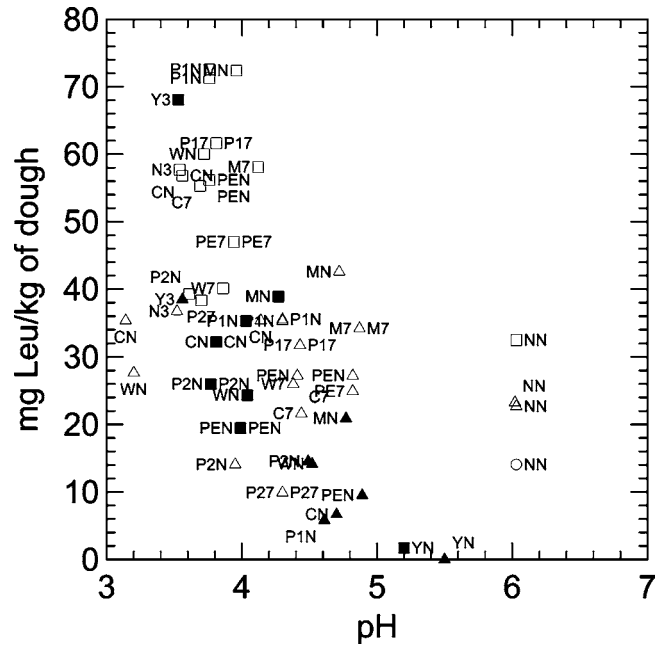
myces cerevisiae was associated with LAB strains because of the faster consumption of maltose and glucose by yeast. Therefore, the imbalance between yeast consumption and starch hydrolysis by flour enzymes led to the rapid depletion of soluble carbohydrates during sourdough fermentation which, in turn, decreases LAB acidification (*18*). In particular, in this study, cell counts of homofermentative LAB were affected by the presence or absence of yeasts to a lesser extent than cell counts of heterofermentative LAB. Probably, decrease of growth for *W. cibaria* and *Leuc. mesenteroides* could be due to the high consumption of aspartic acid and asparagine in sourdoughs started with yeasts (*19*); most heterofermentative sourdough bacteria, in fact, are auxotrophic for arginine, leucine, phenylalanine, tryptophan, tyrosine, and valine (*20*). On the other hand, the addition of LAB apparently stimulated the growth of yeast, which increased from 7.7 log(cfu/g) when no LAB was added to 8.6−8.9 log(cfu/g) when used in association with strains *Lb. curvatus*, *W. cibaria*, and *Lb. pentosus*. This suggests that the growth of yeasts could be enhanced by release of amino acids and/or peptides due to either LAB or native enzyme activities during sourdough fermentation. Proteolysis due to LAB, in fact, increases the total concentration of free amino acids, especially aliphatic, dicarboxylic, and hydroxy amino acids which, for the most part, are stimulatory for bacterial growth and are used by yeasts (*18*). This was confirmed in the present study by evaluating the free amino acid content of dough samples. Generally, the concentration of amino acids increased during fermentation and roughly doubled within 24 h (**Figure 2**). Sourdoughs and acidified control doughs showed an increase in total free amino acids compared to neutral control doughs. In the doughs fermented with yeasts and LAB, the amino acid concentration was lower (paired *t* test, *p* = 0.001) than that of doughs fermented with LAB only, indicating that the amino acid consumption is greater when yeasts are present. However, these results may be partly due to the higher pH values (on average, 0.25 unit, *p* = 0.001) due to inhibition of LAB growth,
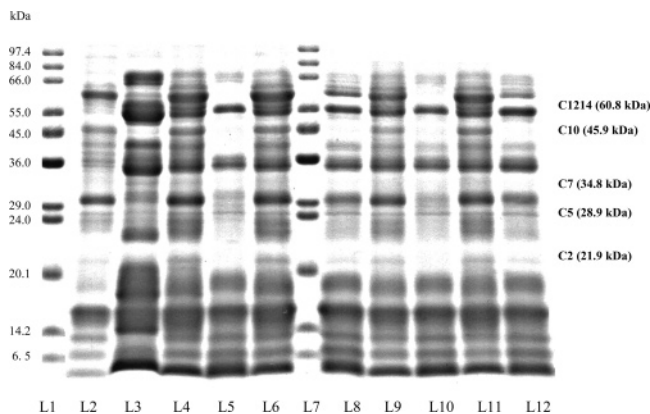
Figure 3. Example of SDS-PAGE of albumin and globulin fraction. C1214 (60.8 kDa), C10 (45.9 kDa), C7 (34.8 kDa), C5 (28.9 kDa), and C2 (21.9 kDa) are the main protein bands hydrolyzed during fermentation. L1 and L7, molecular weight markers (Sigmamarker Wide Range, Sigma); L2, albumins; L3, globulins; L4, neutral control at 6 h; L5, acidified control at 6 h; L6, *Leuc. mesenteroides* DBPZ1005 at 6 h; L8, *Leuc. mesenteroides* DBPZ1005 at 24 h; L9, *Lb. plantarum* DBPZ1025 with yeast at 6 h; L10, *Lb. plantarum* DBPZ1025 with yeast at 24 h; L11, *Lb. pentosus* DBPZ0984 with buffer at 6 h; L12, *Lb. pentosus* DBPZ0984 with buffer at 24 h.

which, in turn, may have limited proteolysis by indigenous enzymes. The lowest amino acid concentration was found in doughs fermented with yeasts only. Thiele et al. (*21*) found that the sourdough fermentations with LAB resulted in an increase of amino acid concentrations during fermentation, whereas the doughs started with mixed culture or with yeasts only contained a lower level of total amino acids, confirming that the presence of yeasts leads to a depletion of amino acids during sourdough fermentation.

Amino acid concentration did not increase linearly throughout the fermentation. At the beginning of incubation time (6 h) the level of amino acids was lower in the dough started with LAB compared to the acidified control dough, suggesting a period of adaptation of bacteria to the sourdough environment followed by a strong demand for assimilable nitrogen. Our results indicate that the amino acid levels in wheat doughs depend on several factors, principally, the pH value, the fermentation time, the proteolytic activity by the microflora and/or flour enzymes, and the consumption of amino acids by LAB and/or yeasts.

Addition of phosphate buffer did not provide a good control of pH because of the high acidifying capability of LAB strains. pH values were on average 0.12 unit higher ($p = 0.001$) when

buffering was used, but this had no significant effect on LAB growth. The free amino acid content was slightly lower when the buffer was used, suggesting that proteolysis was mainly associated with the drop in pH that promotes the activation of cereal enzymes. However, pH is not the only factor determining the level of free amino acids. In fact, significant differences in free amino acid content were evident at similar pH values and time of incubation. Particularly, after 24 h of fermentation, sourdoughs started with *Lb. plantarum* DBPZ1025 had a concentration of free amino acids higher than that of sourdoughs inoculated with *Lb. pentosus* even if the final dough pH was the same (pH 3.75). Additionally, at the same fermentation time, the amount of free amino acids when *Lb. plantarum* DBPZ1025 was used as starter was higher than that of acidified control dough (pH 3.5), suggesting the substantial contribution of LAB to the proteolytic events, as previously demonstrated by Di Cagno et al. (*8*). Our results show the important role of LAB in sourdough proteolysis, although their proteolytic activity is strain specific. In contrast with our data, Thiele et al. (*21*) found that the release of amino acids did not exceed the amino acid concentrations of acidified doughs, indicating that the proteolytic activity of LAB is negligible compared with the proteolytic activity of wheat flour enzymes.

Hydrolysis of Salt-Soluble Proteins during Sourdough Fermentation. The changes that occurred in the salt-soluble wheat proteins during sourdough fermentation were analyzed qualitatively by SDS-PAGE. Albumin−globulin extracts contained polypeptides with molecular masses within the range of 10−80 kDa, with high concentrations of bands with molecular masses in the range of 66−50, 45−30, and 23−22 kDa. The banding patterns of dough samples indicated that sourdough fermentation resulted in the breakdown of high molecular mass (MM) proteins (**Figure 3**). The intensity of these bands, in fact, decreased with increasing fermentation time, and some bands completely disappeared because of the proteolytic activity. The differences between SDS-PAGE electropherograms of neutral and acidified control doughs suggested that the major effects in protein hydrolysis were attributable to the pH conditions and proteolytic activity of wheat flour enzymes. However, the presence of yeasts and LAB significantly affected the electrophoretic patterns of salt-soluble fractions at both 6 and 24 h of fermentation. A considerable degree of protein hydrolysis was detectable for doughs started with *Lb. plantarum* DBPZ1015 and *Lb. curvatus* after 6 h of fermentation. At the end of incubation time (24 h), the two *Lb. plantarum* strains, *Lb.*
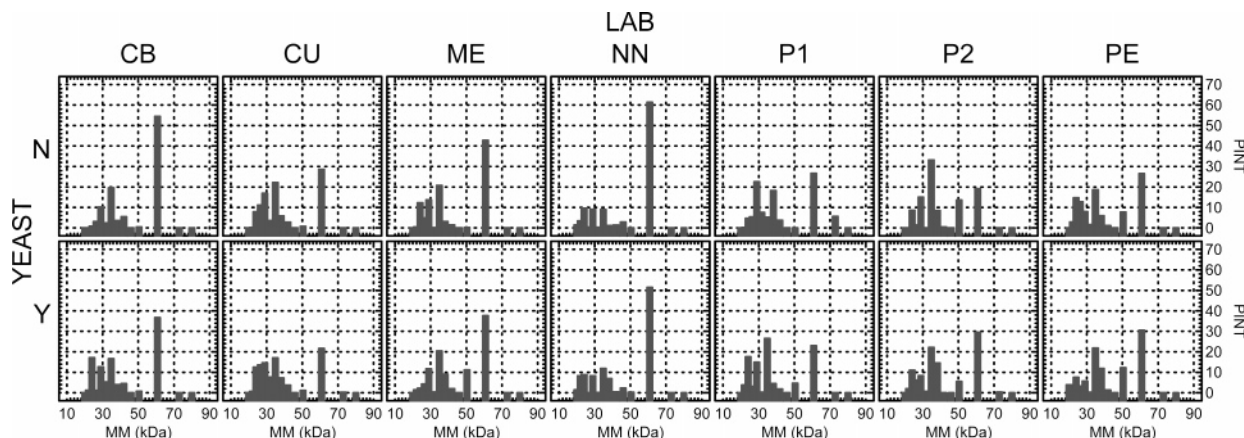
Figure 4. Multiplot of salt-soluble fraction SDS-PAGE patterns showing the interaction between LAB and yeasts after 24 h of fermentation. CB, *W. cibaria* DBPZ1006; CU, *Lb. curvatus* DBPZ1020; ME, *Leuc. mesenteroides* DBPZ1005; NN, neutral control dough; P1, *Lb. plantarum* DBPZ1025; P2, *Lb. plantarum* DBPZ1015; PE, *Lb. pentosus* DBPZ0984; PINT, band intensity as percentage of the total intensity.

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curvatus, and *Lb. pentosus* produced patterns which were different from that of the neutral control dough, indicating a higher degradation of salt-soluble proteins. Additionally, the association with yeasts resulted in an increase of low MM fractions compared to patterns of doughs in which LAB only were used (**Figure 4**). When combined with *Lb. curvatus* or with two *Lb. plantarum* strains, the presence of yeasts enhanced the degradation of the 60.8, 38.2−34.8, and 28.9 kDa proteins, increasing the fractions in the range of 26.4−24.1 kDa. Interaction between *Lb. pentosus* and yeasts increased the protein fragments ranging from 21.9 to 20.0 kDa, possibly as a consequence of the hydrolysis of 28.9−24.1 kDa proteins.

Multivariate Analysis of SDS-PAGE Patterns of the Salt-Soluble Fraction. To determine the main factors influencing protein hydrolysis during fermentation, PCA of the covariance matrix of electrophoretic data for the albumin and globulin fraction was carried out. After a preliminary run, all variables having loadings lower than 0.5 were discarded and, consequently, only variables C1214 (pooled from band classes C12, C13, and C14), C11, C10, C8, C7, C5, C4, and C3 were retained in the model. Two principal components were extracted explaining 79.8% of the total variance. **Figure 5** shows the score and loading plots (panels **a** and **b**, respectively).

Most of the variance (69.4%) was associated with the first principal axis, which, in turn, was strongly related with the sum of intensities of bands ranging from 55.3 to 66.5 kDa (C1214, class centered at 60.8 kDa); additionally, the first principal component (PC) was weakly related to changes in bands belonging to classes C4 (26.4 kDa) and C8 (38.2 kDa). Variation over the second principal axis (10.4% of the total variance) was associated with variables C7 (34.8 kDa), C11 (50.4 kDa) and C3 (24.1 kDa), and C5 (28.9 kDa). The latter two groups of bands were inversely correlated. Samples were divided into two groups on the factor 1 axis according to time of fermentation because of differences in the group of bands centered at 60.8 kDa (C1214), which loaded heavily on factor 1. Samples at 6 h were more tightly grouped together than samples at 24 h with the exception of the acidified control dough sample (N3), the pH of which was low from the starting time of fermentation. Dough samples started only with yeasts, at both 6 and 24 h, were located in the left section of the graph (6 h group), showing that yeasts had the lowest proteolytic activity. Replicates of the same samples were very close, showing that the analysis was repeatable.

Analysis of single bands as a function of time and pH showed that bands belonging to the class centered at 60.8 kDa decreased with time and pH. The presence of yeasts enhanced the degree of hydrolysis of these protein bands especially when associated with *Lb. curvatus*, *Lb. pentosus*, or *Lb. plantarum* DBPZ1015. Bands belonging to class C10 (45.9 kDa) completely disappeared after 24 h. On the other hand, the intensity of bands of class C4 (26.4 kDa) increased with time and, to a lesser extent, with decreasing pH. In fact, a high intensity of bands at 26.4 kDa was detected when *Lb. curvatus* and *Lb. pentosus*, alone or in combination with yeasts, were used for sourdough fermentation, suggesting that microbial activity, together with change in pH, was implicated in proteolysis.

Quantitative Analysis of the Salt-Soluble Fraction by RP-HPLC. The RP-HPLC chromatograms of the salt-soluble fraction indicated that fermentations caused detectable changes in protein profiles. Incubation of acidified control dough caused a strong decrease over time of peaks around RT 50 min and an increase of peaks around RT 40 min. The same trend was observed for RP-HPLC patterns of sourdoughs started with *W.*

Figure 5. (**a**) Score plot of the first two PCA factors. P1, *Lb. plantarum* DBPZ1025; P2, *Lb. plantarum* DBPZ1015; PE, *Lb. pentosus* DBPZ0984; C, *Lb. curvatus* DBPZ1020; W, *W. cibaria* DBPZ1006; M, *Leuc. mesenteroides* DBPZ1005; N3, acidified control; NN, neutral control; 7, buffering condition; (○, △, □) 0, 6, and 24 h of fermentation, respectively; solid symbols, presence of yeasts. (**b**) Loading plot of the first two PCA factors.

cibaria, *Lb. curvatus*, and *Lb. plantarum* DBPZ1015 after 24 h. No treatment resulted in significant changes in profile of the peaks eluting at the beginning or at the end of the gradient.

After 6 h of fermentation, the interaction with yeasts did not affect the RP-HPLC patterns of sourdough started with *W. cibaria* and *Leuc. mesenteroides*; some differences were evident for the two *Lb. plantarum* strains and, to a lesser extent, for *Lb. pentosus*. At the end of fermentation (24 h), changes in protein profiles were detectable for most strains when the yeast was present; generally, low RT fractions were higher when LAB were not combined with yeasts (**Figure 6**).

Figure 6. Multiplot of salt-soluble fraction RP-HPLC patterns showing the interaction between LAB and yeasts after 24 h of fermentation. CB, *W. cibaria* DBPZ1006; CU, *Lb. curvatus* DBPZ1020; ME, *Leuc. mesenteroides* DBPZ1005; NN, neutral control dough; P1, *Lb. plantarum* DBPZ1025; P2, *Lb. plantarum* DBPZ1015; PE, *Lb. pentosus* DBPZ0984; PEAKH, height of peaks as percentage of total height.

PCA carried out on the covariance matrix was applied on the data set. The variables with low loadings on all components were discarded, and only the classes between C24 and C34 (RT from 38.97 to 53.75 min, respectively) were included in the model. Two principal components explaining 78.8% of the total variance were extracted, and a varimax rotation was carried out to simplify the graphical interpretation of results. Score and loading plots are shown in panels **a** and **b**, respectively, of **Figure 7**.

The first PC (48.8% of the total variance) was negatively associated with C30 (peak with RT 47.8 min) and weakly associated with C29 (RT 46.36 min), whereas all of the other variables had low loading on this component. The second PC (30% of the total variance) was strongly and negatively related to the changes of peaks centered at RT 40.45 min (C25). C24 (RT 38.97 min), C26 (RT 41.93 min), and C28 (RT 44.88 min) also had smaller negative loadings on PC2, whereas all of the other variables had smaller positive loadings.

A small group of samples in the lower right section of the graph was very similar to the acidified control dough at 24 h, showing a decrease of peak at RT 47.8 min and an increase of smaller peaks at RT 40.45 min and RT 41.93 min. This trend was mainly detectable when *Lb. curvatus*, *W. cibaria*, and *Lb. plantarum* DBPZ1025 were used as starter and were not combined with yeasts. These results suggest once more that the pH is not the only factor in determining the changes in proteolysis during fermentation and that the microbial metabolism is also implicated. Another large group of treatments at both 6 and 24 h in the upper right section of the graph exhibited patterns similar to neutral control dough at 6 and 24 h and to acidified control dough at 6 h, indicating a moderate extent of protein degradation.

Studies on the hydrolysis of albumins and globulins during sourdough fermentation are rare compared to those on the hydrolysis of gliadins and glutenins and, consequently, a comparison of our results with data reported in the literature is not easy. Besides, the interpretation of results could be difficult because of the complexity of this protein fraction, which could contain soluble peptides deriving from hydrolysis of gliadins and/or glutenins. Di Cagno et al. (*8*) found a significant proteolysis of water- and salt-soluble fractions during fermentation of doughs started with selected lactobacilli. Additionally, degradation of albumins and globulins was observed in acidified doughs by Thiele et al. (*22*). The activity of a gluten aspartic proteinase (GlAP) had no visible effect on SDS-PAGE patterns of wheat albumins and globulins (*23*), whereas a small effect

on this fraction was observed only after 72 h of digestion with a second aspartic proteinase (GlAP 2) (*24*).

Analysis of SDS-PAGE Patterns of Gliadin and Glutenin Fractions. Gliadins and glutenins were degraded to a lesser extent than albumins and globulins during sourdough fermentation (data not shown). Hydrolysis of the gliadin fraction resulted in new protein fragments with MM of ca. 20 and 27 kDa, after 24 h of fermentation. In particular, a high intensity of the band corresponding to the protein fragment of 27 kDa was present in the patterns of acidified control dough and sourdoughs started with *Lb. plantarum* DBPZ1015 without yeasts. The intensity of the band at 20 kDa, instead, was higher in sourdoughs started with *Lb. curvatus* combined with yeasts and acidified control dough.

The electrophoretic patterns of glutenin fractions indicated that fermentation resulted in the breakdown of high MM proteins. The changes in glutenin proteins were mainly detectable in the banding pattern of acidified control dough, which were characterized by complete disappearance of bands with MM in the range of 110−85 kDa and by decreasing intensity of bands with MM ranging from 65 to 60 kDa and from 54 to 34 kDa. New protein fragments with MM of ∼57 kDa resulted from the proteolytic activity of wheat flour enzymes. A similar degree of protein hydrolysis was evident for the doughs started with two *Lb. plantarum* strains, alone or in combination with yeasts, and for doughs started with *Lb. curvatus* and *Leuc. mesenteroides* in association with yeasts.

However, attempts to perform a multivariate analysis of the results were unsuccessful, probably because of difficulties in resolving the intensity of the bands ranging from 36 to 45 kDa, of both gliadins and glutenins, which were most intense, making all samples very similar.

Proteolysis during fermentation has been studied by a number of authors but remains unclear because of difficulties in discriminating between the effects of pH, microbial metabolism, and indigenous enzyme activities. In the literature, in fact, are reported several data about the hydrolysis of gluten proteins, but many of these are divergent because of the complexity of the phenomenon. Gluten proteins contribute to dough strength, gas retention and rise, and improvements of texture and flavor during sourdough fermentation; their structural modifications by proteolytic enzymes could be used to change gluten functionality in bakery applications (*25*). However, the insolubility of gluten in water may limit the substrate concentration for proteolytic enzymes (*21*), making protein degradation difficult. Besides, during sourdough fermentation, glutenin subunits were hydrolyzed, and the resulting low molecular mass

Proteolysis in Model Sourdough Fermentations

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(a)

(b)
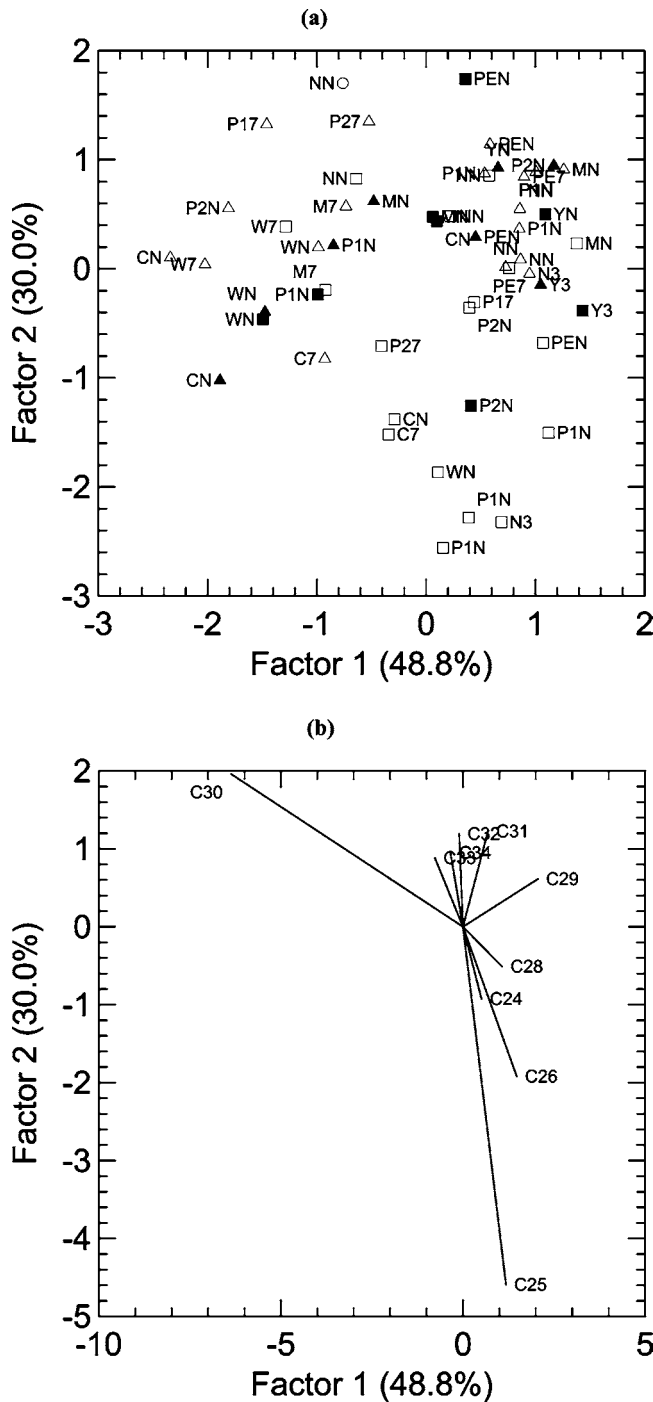
Figure 7. (**a**) Score plot of the first two PCA factors. P1, *Lb. plantarum* DBPZ1025; P2, *Lb. plantarum* DBPZ1015; PE, *Lb. pentosus* DBPZ0984; C, *Lb. curvatus* DBPZ1020; W, *W. cibaria* DBPZ1006; M, *Leuc. mesenteroides* DBPZ1005; N3, acidified control; NN, neutral control; 7, buffering condition; (○, △, □) 0, 6, and 24 h of fermentation, respectively; solid symbols, presence of yeasts. (**b**) Loading plot of the first two PCA factors.

peptides remained associated with the gluten macropolymer (*5*). Solubilization of the gluten polymers was revealed by size exclusion chromatography (SEC) analysis of SDS-soluble proteins by Thiele et al. (*5*). The majority of gluten proteins were recovered as soluble or insoluble polymers in unfermented doughs and as SDS-soluble monomeric proteins after 24 h of fermentation at pH values of <4.0.

Thiele et al. (*5*) found that hydrolysis of glutenins was mainly related to the drop in pH during the fermentation process which,

in turn, promotes activation of indigenous flour enzymes, rather than to specific proteinases of LAB. In fact, compared to the degradation of gliadin and glutenin proteins in aseptic acidified doughs, the additional proteolytic activities of microbial enzymes were small. Microbial fermentation affects the size distribution of peptides resulting from proteolytic degradation of wheat proteins. In particular, during fermentation of doughs started with lactobacilli, the concentration of larger peptides decreases, resulting in increasing levels of smaller molecules such as dipeptides and amino acids, whereas the overall amount of low molecular weight glutenin subunits (LMW-GS) remain unaltered. On the contrary, when fermentation was carried out in the absence of any starter, the most LMW-GS resulted in a high concentration of peptides (*22*).

Autodigestion of gluten proteins is mainly associated with the activity of proteolytic enzymes of flour, such as aspartic and serine proteinases. A gluten aspartic proteinases (GlAP) has been shown to have maximal activity at pH 3.0 for the hydrolysis of gluten proteins (*23*). SDS-PAGE banding patterns of wheat proteins incubated with GlAP, reported in this study, showed a high degradation of high molecular weight glutenin subunits (HMW-GS), and several protein bands with molecular masses in the range of 9−19, 30−34, 57, and 72−96 kDa were formed; at the same time, protein fragments in the ranges of 9−17 and 23−26 kDa from LMW-GS and in the range of 30−32 kDa from gliadins appeared.

A second aspartic proteinases (GlAP 2), in contrast with the first GlAP, was unable to hydrolyze gliadins and LMW-GS; similar to GlAP, HMW-GS were hydrolyzed with the release of protein fragment with MM of 31−33, 42, and 72 kDa (*24*).

Proteolysis of gluten by sourdough LAB has been reported by Wehrle et al. (*6*).

Among LAB, *Lb. sanfranciscensis* CB1 has been shown to have a particular capability to degrade proteins or peptides during sourdough fermentation. The proteolytic system of *Lb. sanfranciscensis* CB1, which is characterized by a cell envelope-associated serine proteinase, a metal-dependent dipeptidase, and a general aminopeptidase, had a considerable capacity to produce TFA-soluble peptides from gliadins (*26*).

Di Cagno et al. (*8*) demonstrated a considerable degradation of gliadin fractions during sourdough fermentation; particularly, proteinases and peptidase enzymes of selected lactobacilli showed hydrolysis of the 31−43 fragment of A-gliadin, a peptide that affects the human intestinal mucosa of celiac patients. Hydrolysis of gliadins by LAB, in addition, seemed to affect positively the degree of softening of the dough and its stability during fermentation. The same work has shown that the glutenin fraction was not degraded by proteolytic enzymes.

During fermentation of wheat doughs started with *Lb. brevis* VTT E 95612, *Lb. plantarum* VTT E 78076, and *S. cerevisiae* VTT B81047, gluten proteins were highly degraded and a new protein fragment (MM 30 kDa) appeared in the alcohol-soluble fraction. Very similar changes are also evident in the fermentation process carried out under acid conditions (pH 3.7), in the absence of any starter microorganism and in the presence of antibiotics, because of the action of indigenous cereal proteinases (*10*).

Our results showed that proteolysis by yeasts was negligible compared to the activity of LAB and indigenous flour enzymes. Very few yeasts are, in fact, strongly proteolytic. However, some strains have caseinolytic activity; for example, *Yarrowia lipolytica* and a number of strains of *Candida* and *Saccharomyces* spp. have been reported to produce extracellular proteinases with

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various applications in the food industry, such as gelatin liquefaction (*7*).

In the present study, the SDS-PAGE banding pattern and RP-HPLC chromatograms of proteins and peptides extracted from wheat dough samples indicated the presence of proteolytic activity during fermentation. In particular, the present study confirms that proteolytic events during sourdough fermentation are largely related to the activity of indigenous cereal enzymes, which is promoted by decreasing pH during fermentation; at the same time, our results show a significant role for LAB in proteolysis, suggesting their usefulness in many applications in the bakery industry.

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