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CHANGES IN LARGE-DEFORMATION PROPERTIES DURING DOUGH FERMENTATION BY *Lactobacillus* STRAINS AND THEIR RELATIONSHIP WITH MICROSTRUCTURE

CAMBIOS EN LAS PROPIEDADES DE DEFORMACIÓN DE LA MASA DURANTE LA FERMENTACIÓN POR *Lactobacillus* Y SU RELACIÓN CON LA MICROESTRUCTURA

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Abstract

Assessment of the microstructural changes that occur in wheat doughs during fermentation due to the Lactobacillus type (*Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus sanfranciscensis*), and the changes associated to mechanical properties. To dough with a dough yield (DY) of 150 were added 50 mL of inoculum (10^9 CFUmL^{-1}) and fermented at 35°C for 24 h (76% RH). The uniaxial extensibility of the dough was determined at 0, 6, 12, 18 and 24 h of fermentation using a texture analyzer equipped with a Kieffer rig. The microstructure was observed by means of a Scanning Electron Microscope (SEM), and the images were analyzed to determine the fractal dimension (FD_{SDBC}) and Entropy (Ent). In all cases, the maximum extensibility (E_m) and the maximum resistance (R_m) of the doughs decreased with the progress of fermentation. *Lb. plantarum* and *Lb. sanfranciscensis* reduced E_m by 40 and 42%, respectively, while DY decreased by more than 85%. Fermentation increased the structural complexity of the dough by raising its FD_{SDBC} and Ent values, with differences depending on the type of bacteria. These results provide adequate criteria for the selection of bacteria for the development of sourdoughs and for understanding the structural and mechanical changes that occur during fermentation.

Keywords: lactobacilli, sourdough, mechanical properties, fractal dimension, microstructure.

Resumen

Se evaluaron los cambios microestructurales que ocurren en la masa de trigo durante la fermentación por efecto del tipo de lactobacilo (*Lactobacillus plantarum, Lactobacillus brevis* y *Lactobacillus sanfranciscensis*), así como los asociados a las propiedades mecánicas. Las masas preparadas con un RM (rendimiento de masa) de 150, fueron adicionadas con 50 mL de inóculo (10^9 UFC mL^{-1}) y se fermentaron a 35°C/24 h (HR 76%). Se determinó la extensibilidad uniaxial de las masas a las 0, 6, 12, 18 y 24 h de fermentación usando un texturómetro equipado con el gancho de Kieffer. Las imágenes de microscopía electrónica de barrido fueron analizadas para determinar la dimensión fractal. En todos los casos, la extensibilidad máxima (E_m) y la resistencia máxima de las masas (R_m), disminuyó por la fermentación. *Lb. plantarum* y *Lb. sanfranciscensis* redujeron la E_m en 40 y 42% respectivamente, mientras que la R_m en todos los casos se redujo en más de 85%. La fermentación incrementó la complejidad estructural aumentando los valores de FD_{SDBC} y Ent, diferenciado por el tipo de bacteria. Estos resultados proveen de criterios adecuados para la selección de bacterias en la elaboración de masas agrias y para el entendimiento de los cambios estructurales y mecánicos.

Palabras clave: lactobacilos, masas agrias, propiedades mecánicas, dimensión fractal, microestructura.

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1 Introduction

It is well known that the properties of wheat dough depend mainly on gluten proteins. In recent years, various treatments have been applied to improve the quality of these proteins or improve the gas retention process and produce bread with higher volume and softer crumb (Arendt *et al*., 2007; Gänzle, 2014). Thus, the production of bread by adding sourdoughs has regained prominence; this is an ancient biotechnological process that consists in fermenting a mixture of flour (wheat, oats, rice, among others) and water with yeast and lactic acid bacteria (LAB) belonging generally to the genus Lactobacillus (Neysens and De Vuyst, 2005; Rehman *et al*., 2006; De Vuyst and Vancanneyt, 2007; Rodríguez-Huezo *et al*., 2011), mainly *Lb. sanfranciscensis, Lb. plantarum, Lb. brevis, Lb. paracasei, Lb. acidophilus, Lb. debrueckii* subsp. *bulgaricus* and other bacteria from the genus Bifidobacterium (Gänzle *et al*., 2007; Lattanzi *et al*., 2013; Gänzle, 2014). The use of sourdoughs offers a number of advantages in baked goods technology, among which are a decrease of pH during fermentation, a better retention of gas, increased strength of the gluten network, inhibition of flour amylases, water binding of gluten and starch granules, swelling of pentosans, solubilization of the phytate complex and prevention of mal-fermentation and spoilage (Di Cagno *et al*., 2002; Decock and Capelle, 2005; Katina, 2005).

During fermentation, the proteolytic system of LAB releases peptides and amino acids of low molecular weight, which promotes the development of metabolic activity in the microorganisms, helping to obtain a better taste and lower content of allergenic peptides (De Angelis *et al*., 2005; Di Cagno *et al*., 2005, Gobbetti *et al*., 2007; Rizzello *et al*., 2007; this holds great promise for celiac patients, who are sensitive to the gliadin fraction of gluten and who cannot consume products containing even small amounts of gluten (Wieser, 1996; Di Cagno *et al*., 2002; Gerez *et al*., 2006; Holtmeier and Caspary, 2006; Loponen, *et al*., 2007; Curiel *et al*., 2014). The problem remains of how to make bread with sourdough without mixing it with unprocessed dough.

During the fermentation progresses, changes occur in the nature of the elements that constituting the structure of the dough, causing a decrease in viscosity (Arendt *et al*, 2007; Tlapale-Valdivia *et al*., 2010). When fermentation becomes extensive, proteolysis involves a structural change, which must be characterized in order to propose the addition of other re-structuring ingredients which will serve to obtain bread with the typical characteristics of the product. Although the beneficial effects of the partial use of sourdough for bread making have been well-documented (Takeda *et al*, 2001; Schober *et al*., 2003; Arendt *et al*., 2007; Gänzle *et al*., 2008), several aspects of this technology have not been fully understood. This relates not only to the microbial ecology of this type of dough and how the type of fermenting microorganism affects the viscoelastic properties of the dough during fermentation when it modifies the gluten, but also to the influence of sour fermentation, especially on dough structure.

There have been studies on the microstructure of bread dough using various microscopy techniques (Calderon-Dominguez *et al*., 2003; Prabhasankar *et al*., 2002; López-Guel *et al*., 2012; Peighambardoust *et al*., 2010). Image analysis has been proposed as a useful tool to quantify the changes on the dough and crumb structure of bread. The image parameters extracted from microscopy images provide numerical values that allow evaluating the size, shape, colour, roughness and textural features of the food materials during their processing (Arzate-Vázquez *et al*., 2012; Kerdpiboon and Devahastin, 2007; Chanona *et al*., 2003; Barletta and Barbosa, 1993; Peleg and Normand, 1985). The statistical methods approach tries to characterize the texture of an image region; the most popular one is based on the estimation of the second-order statistics (Haralick *et al*., 1973). Each textural feature is computed from a set of co-occurrence matrices (COM), which shows the statistical relationship of a pixel's intensity to the intensity of its neighboring pixels. A co-occurrence matrix is a square matrix whose elements correspond to the relative frequency of occurrence p (i, j) of two pixel values (one with intensity i and the other with intensity j) separated by a certain distance d in a given direction (Latif-Amet *et al*., 2000; Unay and Gosselin, 2002). The co-occurrence matrix is, therefore, a square matrix that has the size of the largest pixel value in the image. Another method for determining the textural features of an image is the analysis of the surface intensity of the image, which is obtained by plotting the pixel coordinates (x, y) versus the grey level corresponding to each pixel (z axis). When the surface intensity of an image is characterized using fractal dimension (FD), the particular textural feature evaluated is then called the fractal texture of the image. When FD is evaluated using the Shifting Differential Box Counting algorithm (SDBC), it is denoted as FD_{SDBC}. Wen-Shiung *et al*., (2003) reported that this

method improved the evaluation of the FD of texture as compared with the traditional Box Counting Method.

Thus, assessing the changes in the relationship between structure and function of dough is justified by the need to lay the technological foundations that allow making baked goods from sourdough with good sensory characteristics, without mixing the sourdough with traditionally fermented flour. The aim of this research was evaluate the changes associated with mechanical properties during fermentation with lactic acid bacteria (LAB) and the relationship with their microstructure of the protein network (gluten) when interacting with dough components.

2 Methodology

2.1 Materials

We used commercial white flour, suitable for manual baking (Hoja de Plata, Flour mill Elizondo SA de CV, Mexico), with 12% protein and 10% moisture; three types of lactobacilli: *Lb. brevis* CDBB-B380, *Lb. plantarum* CDBB-B-1091 were used, belonging to the Culture Collection of the CINVESTAV-IPN in Mexico City, and *Lb. sanfranciscencis*, isolated from pulque and donated by the Iberoamerican University. Each strain was suspended in Man Rogosa Sharpe broth (MRS) (Difco TM Lactobacilli MRS broth, USA) and incubated for 12 to 24 h under anaerobic conditions at 37 ± 1 ºC.

2.2 Determination of the maximum rate of growth of lactobacilli

Fresh, active 24 h cultures of each strain were streaked on MRS agar plates to which 10% sterile skim milk was added. The plates were incubated in anaerobiosis for 24 h at 37 ± 1°C. Ten colonies were picked from a culture and inoculated into the fermentation jar of a Multigen chemostat (New Brunswick Sci. Co. Inc., Edson NJ, USA) containing MRS broth. The temperature of the medium was maintained at 37 ± 2 °C, with stirring at 50 rpm.

It is sampled every 2 h for 24 h to determine the optical density at 600 nm (Perni *et al*., 2005) with a UV-visible spectrophotometer Varian Cary 50 Conc (Palo Alto, CA. U.S.A.) and to seed plates using the most probable number technique with five replicates (Woomer, 1994). The population was estimated according to standardized tables for five replicates, with a confidence level of 95%. The results of bacterial growth were fitted to mathematical models using Origin 7.5 (OriginLab Northampton, MA, USA). It calculated the time required for reaching maximum growth rate from predictive models and used it to produce the biomass.

2.3 Biomass production

Ten colonies of lactobacilli were taken and dispersed in 600 ml of MRS broth in the fermentation jar and each organism was grown for the time required to reach the maximum growth rate. The cells were collected by centrifugation (9000 g, 15 min, 4 °C) and washed twice with a sterile buffer solution of 10 mmol L^{-1} of potassium phosphate (pH 7) and suspended in sterile saline water. The inoculum was prepared from these cells at a concentration of 10^9 CFU mL^{-1}. An aliquot was plated on MRS agar for colony counting, incubating it anaerobically at 37±1 °C for 48 h (Gerez *et al*., 2006). This was the procedure followed for each of the strains.

2.4 Preparation of sourdoughs

The consistency of the sourdough is determined by a parameter called dough yield (DY) (Eq. 1) (Decokk and Cappelle, 2005):

$$DY = \frac{\text{amount of flour} + \text{amount of water}}{\text{amount of flour}} \times 100 \quad (1)$$

The doughs were prepared with 400 g of wheat flour and 150 ml of water, adding 50 ml of LAB inoculum (10^9 CFUmL^{-1}) in logarithmic growth phase and mixing for 8 min. Doughs were incubated at 35 °C for 24 h, with a relative humidity of 76% (De Angelis *et al*., 2005; Paraminthiotis *et al*., 2005). The changes in pH and titratable acidity during fermentation were determined.

2.5 Uniaxial extension tests

The uniaxial extensibility of the doughs was determined in a TA.XT2 texture analyzer (Texture Analyser, Stable Microsystems, UK), equipped with a Kieffer extensibility rig (Stable Micro Systems, UK). Samples were taken at different fermentation times, 0, 6, 12, 18 and 24 h, and pressed in the standard mold for 40 min according to the description by Suchy *et al*., (2000). The maximum resistance to extension (R_m) and the maximum extensibility (E_m) were obtained.

To compare the changing values of strength (E_m) and distance obtained with the texture analyzer, the curves were expressed as normalized values of E_m and

distance; for instance, strength is expressed as the Eq. (2):

$$\frac{\Delta E_m}{E_{m0}}, \quad \Delta E_m = E_{mt} - E_{m0} \tag{2}$$

Where E_{m0} and E_{mt} are force at the initial time and at any instant respectively.

2.6 *Evaluation of dough microstructure by means of Image Analysis*

Samples were taken every 6 h during fermentation. The sample sizes were approximately 2 cm x 2 cm x 0.5 cm, and dried under vacuum at room temperature for 24 h (Varriano-Marston, 1977). When dehydrated, they were immersed in liquid nitrogen to facilitate cracking. A portion of the center of each sample was taken and coated with gold palladium (2 min, 2 bar) using a Desk IV CTC Parker evaporator, Standard Model (Denton Vacuum LLC. NJ, USA); these portions were observed with a Scanning Electron Microscope (SEM) Jeol JSM-6390LV at 20 kV (JEOL. Akishima, Japan). The digital images were acquired using the Imix software (Princeton Gamma Tech, Princeton, NJ).

All samples were examined at 500X magnification and five different fields were captured at random. An image processing methodology was used to characterize the fermented sourdough surface using the Image J 1.37 software; it is an image analysis program in the public domain (National Institute of Health, USA. Available in: http://rsb.info.nih.gov/ij/). The procedure consisted of several steps used to carry out the image analysis, which included the following operations: a) the images acquisition through of SEM; b) the Pre-processing, this consisted in transforming the images from original images (24 bits) to grey level images (8 bits); c) Image segmentation included cropping of the images and d) The FD (Fractal Texture) and Entropy (Ent) were obtained from grey level images.

Image texture was analyzed studying the spatial dependence of the pixel values represented by a co-occurrence matrix $P_{d,\theta}$, with the entry $P_{d,\theta}(i, j)$ being the relative frequency or distance between two pixels d-pixels apart in direction θ to have values i and j, respectively. For a given directional orientation and distance between the patterns, 14 textural features can be extracted from a gray scale image using this matrix (Haralick *et al*., 1973). Entropy measures disorder or randomness in the image and is an indication of the complexity within an image; thus, more complex images have higher entropy values. The entropy of the images was evaluated using the GLCM_ Texture plug-in (v 1.0, available on line) as proposed by Haralick *et al*., (1973), and analyzed using the texture average of four directions, θ = 0, 45, 90,180º and distance, d = 1. Entropy was computed as the Eq. 3:

$$\text{Entropy} = -\sum_i \sum_j p(i, j) \log[p(i, j)] \tag{3}$$

Where $p(i, j)$ is the relative frequency of occurrence of two pixel values (one with intensity i and the other with intensity j).

The fractal texture of images was evaluated by power-law scaling to obtain their fractal dimension, using the Shifting Differential Box Counting method (FD_{SDBC}). FD_{SDBC} was calculated using the ImageJ software (1.37v) and the Mapfractalcount plug-in, v 1.0. FD_{SDBC} was estimated from the slope in the log (box count) vs. log (box size) plot and by Equation (4), where "N" is the number of boxes and "r" is the length of the side of box (Kerdpiboon *et al*., 2007).

$$FD_{SDBC} = \frac{\log(N)}{\log\left(r^{-1}\right)} \tag{4}$$

High values of FD_{SDBC} obtained by Equation (4) mean more complex or rougher gray level images of fermented sourdough, while low values of FD_{SDBC} can be associated to simpler or smoother images. All measured parameters were analyzed using electronic spreadsheet software (Excel 2000, Microsoft Office Corporation, USA). In addition, to compare the changing values of FD_{SDBC} and Entropy, they were expressed as normalized values.

3 Results and discussion

3.1 *Growth parameters of lactobacilli*

The growth curves of the test microorganisms were adjusted to the Gompertz model, from which the growth parameters presented in Table 1 were estimated (Zwietering *et al*., 1990). That table shows that *Lb. brevis* needs a time of 11.47 h to reach its maximum growth rate, followed by *Lb. plantarum* with a time of 9.47 h and *Lb. sanfranciscensis* with 6.24 h. Lb. brevis had a slower growth, probably because of a slower adaptation to the culture medium or greater nutritional requirements; a higher growth rate, as in the case of *Lb. sanfranciscensis*, may indicate greater adaptability to the environment.

Table 1. Growth parameters of the tested microorganisms

Microorganism	Time (h) for maximum growth rate	Maximum growth rate	Delay time (h)	Time for reaching stationary phase (h)	Adjustment to Gompertz model (R^2)
Lb. brevis	11.47	0.13	2.78	nd	0.997
Lb. plantarum	9.34	0.34	9.34	12.53	0.976
Lb. sanfranciscensis	6.24	0.44	3.78	9.81	0.982

Table 2. pH values of the doughs during fermentation with lactobacilli

Time of fermentation (h)	*Lb. brevis*		*Lb. plantarum*		*Lb. sanfranciscensis*	
	pH	Lactic acid (%)	pH	Lactic acid (%)	pH	Lactic acid (%)
0	6.21±0.04	0.027	6.21±0.03	0.027	6.24±0.04	0.027
6	5.70±0.02	0.036	4.91±0.05	0.090	5.17±0.03	0.045
12	4.82±0.03	0.135	3.97±0.04	0.405	4.20±0.05	0.252
18	4.26±0.03	0.180	3.83±0.03	0.450	4.02±0.02	0.423
24	4.07±0.02	0.477	3.75±0.04	0.765	3.95±0.03	0.612

3.2 Sourdough characteristics

A sourdough with a DY of 160 corresponds to firm dough, while one with a DY value of 200 corresponds to liquid dough. The experimental dough used in this study had a DY value of 150, meaning it was firm dough. It has been reported that more acetic acid than lactic acid is produced with smaller DY values, affecting the taste of the final product, while high DY values increase the acidification rate (Decokk and Cappelle, 2005). The changes observed in the pH values of the doughs during fermentation were dependent on the type of microorganism used, as shown in Table 2.

After 12 h of fermentation, the drop in pH was lower, ending with similar pH values at 24 h, although dough treated with *Lb. brevis* finished with a slightly higher pH value compared with doughs fermented with the other microorganisms (Table 2). *Lb. plantarum* is a facultative homofermentative bacteria that produces mainly lactic acid during fermentation (Wick *et al*., 2001; Gänzle *et al*., 2007), while *Lb. brevis* and *Lb. sanfranciscensis* are heterofermentative, mainly producing acetic acid and ethanol as their main metabolites. This could explain that the pH of dough fermented with *Lb. plantarum* decreased faster and further, as lactic acid is stronger than acetic acid (pKa = 3.5 and pKa = 4.76, respectively).

The pH value reached at the end of fermentation was slightly higher than that reported by Thiele *et al*. (2004) for doughs fermented with *Lb. sanfranciscensis*. Wick *et al*. (2001) reported a pH of about 3.3 in studies of the growth of *Lb. brevis* in MRS broth for 35 h, where the value remained constant after 25 h of reaction and an initial pH of 6.0. In the present study, the dough with *Lb. brevis* reached a higher pH value (4.07). It has been noted that when microorganisms are in complex, highly viscous systems like sourdoughs, the bacteria are trapped within the matrix that makes up the system. It can be hypothesized that the diffusion of nutrients and metabolites is slow, which affects the development and metabolism of bacteria (Vernocchi *et al*., 2008). It should also be considered that *Lb. brevis* was the microorganism with lower growth rate (Table 1).

3.3 Uniaxial extensibility of sourdoughs

In uniaxial extensibility tests for bread dough, it is generally accepted that a good dough should have a maximum force required for extension (R_m) of about 60 g, and that the distance to which the sample can be extended before rupture should be 14-15 cm (E_m), as both parameters are related to bread volume. Figures 1a, 1b and 1c show the extensograms of sourdoughs inoculated with *Lb. brevis, Lb. plantarum* and *Lb. sanfranciscensis* at different fermentation times; it can

be seen that in all cases E_m and R_m decreased as the time of fermentation increased. The fermented dough with *Lb. brevis* showed an E_m of 71.69 mm and an R_m of 57.00 g at time zero; after 6 h and up to 18 h, the E_m of the dough was 75.00 mm, while the R_m decreased, ending at 24 h with an E_m of 70.65 mm and an R_m of 8.00 g (1.45% and 85% decrease, respectively) (Fig. 1a).

Moreover, the E_m of the dough inoculated with *Lb. plantarum* was 75.00 mm and the R_m 47.20 g at time zero; at 24 hours, the E_m and R_m decreased to 44.85 mm and 7.80 g, a decrease of 40.20 and 86.66%, respectively (Fig. 1b). Finally, in the dough fermented with *Lb. sanfranciscensis* (Fig. 1c), the E_m decreased 42.42% and the R_m decreased 86.54%.The fermented doughs that showed greater loss of extensibility were those inoculated with *Lb. plantarum* and *Lb. sanfranciscensis*. The smaller loss of extensibility was that of the dough fermented with *Lb. brevis* (1.45%). As for the maximum resistance to extension, the three types of dough showed a loss of approximately 80%.

Fig. 2a shows the normalized pH values (pH_n) for sourdoughs inoculated with *Lb. brevis, Lb. plantarum* and *Lb. sanfraciscencis*; it can be observed that in all cases the pH_n increases with time, which is typically associated with the fermentation process caused by the inoculation of lactobacilli. A greater acidification of the doughs is observed with the *Lb. plantarum* strain, intermediate values with *Lb. sanfraciscencis* and lower values with *Lb. brevis*.

Furthermore, the maximum strength values required for extension (R_m) and extensibility (E_m) were obtained from the data of the extension tests of sourdoughs (Fig. 1). For comparison purposes, these values were normalized as described in the materials and methods section. Fig. 2b shows the values of $\Delta R_m/R_{m0}$, where it can be observed that the maximum normalized strength decreases with time of fermentation in all cases, which may be associated to the growth of microorganisms which, in turn, might modify the structure of the dough and increase pH_n. A more drastic decrease is observed in the doughs with *Lb. brevis* and *Lb. sanfranciscencis*, while the dough containing *Lb. plantarum* shows a slower decrease in comparison with the doughs containing the other two strains. Likewise, the values of $\Delta R_m/R_{m0}$ were similar and remained approximately constant at 18 h of fermentation, a behavior that might be associated with the fermentation process, which affects the protein structure of the system and causes a consequent fall in dough strength, which decreases to minimum values after this type of prolonged fermentation, as shown in Fig. 1.

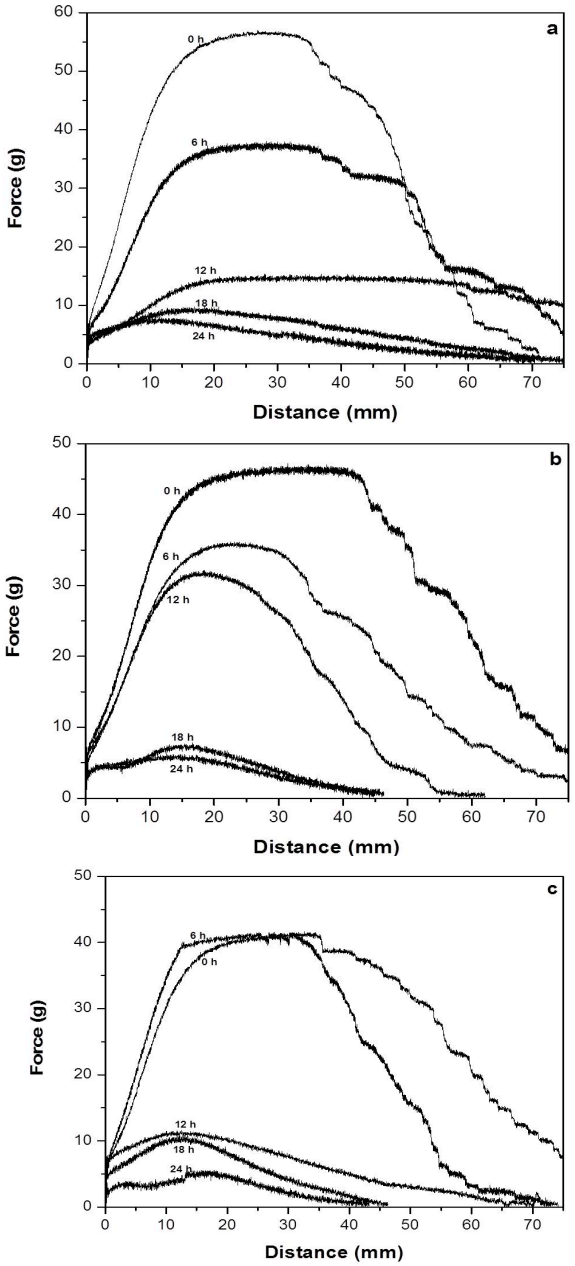

Figure 1. Extensibility of sourdoughs inoculated with a) *Lb. bevis*; b) *Lb. plantarum* and c) *Lb. sanfranciscensis* at different fermentation times.

On the other hand, the values of $\Delta E/E_0$ for the three strains (Fig. 2c) show a similar trend to that of $\Delta R_m/R_{m0}$, which was expected, as extensibility is directly related to the values of maximum strength because the fermentation process modifies the structure of the dough. The minimum

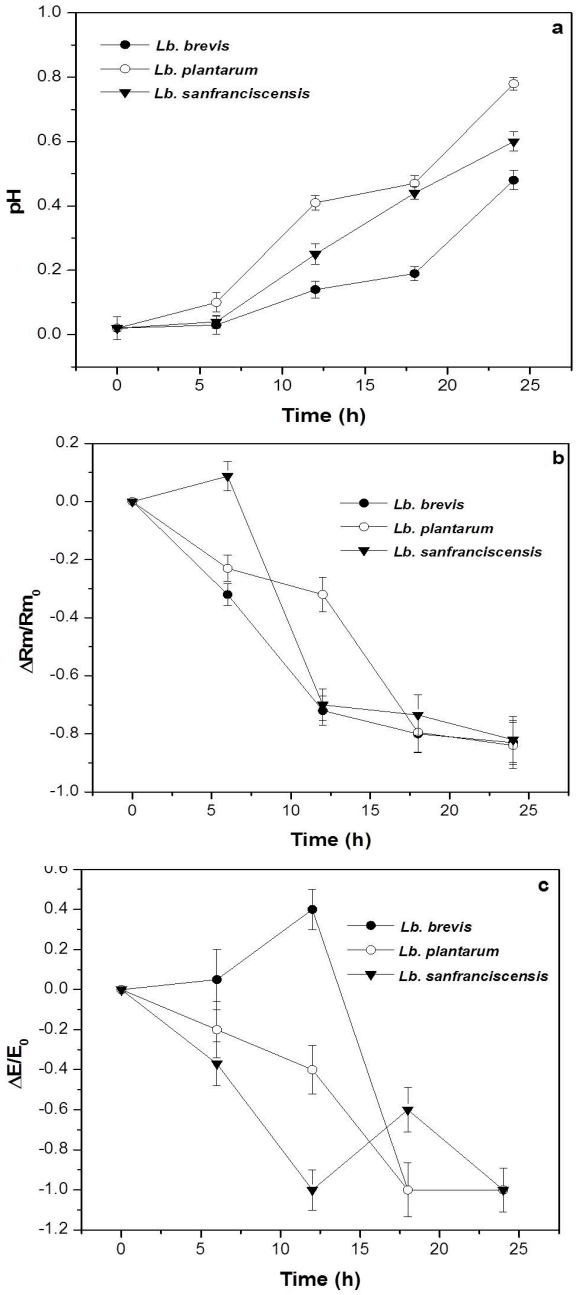

Figure 2. Normalized values of: a) pH, b) R_m, c) E_m of doughs fermented with *Lb. brevis*, *Lb. plantarum* and *Lb. sanfranciscensis* at different fermentation times.

values of $\Delta E/E_0$ were reached at 24 h of fermentation, with similar levels for the three strains studied in this work. Likewise, the dough with *Lb. plantarum* shows a slower decrease of E_m in comparison with the other two strains.

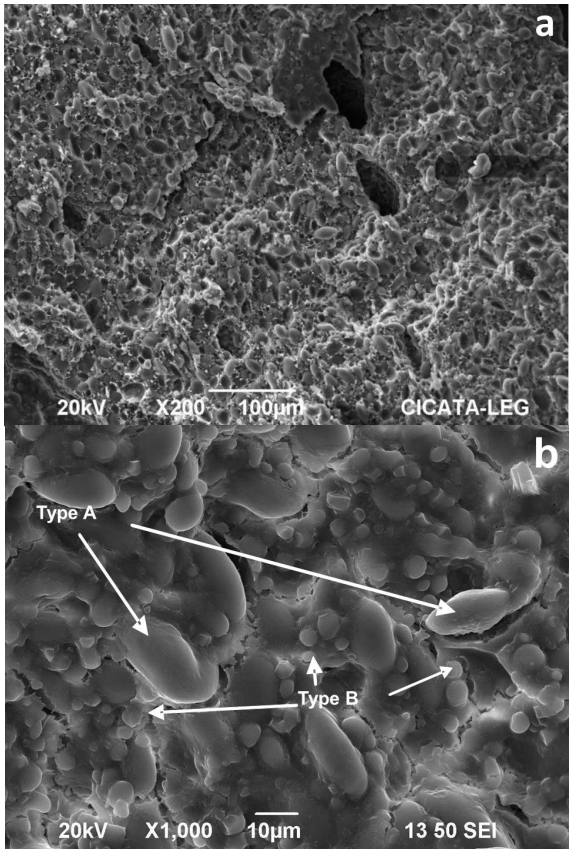

Figure 3. Representative structure of wheat doughs (a) with intercalated Type A and Type B starch granules (b).

3.4 Microstructure of fermented doughs

The analysis of the microstructure of the dough using SEM was adequate to reveal aspects of the formation and breakdown of the structure. Generally, it is possible to observe in the dough a continuous protein matrix (Fig. 3) with some gas cells of various sizes (Fig. 3a) and interleaved with starch granules (Fig. 3b).

The micrographs obtained for each fermentation time and for each type of microorganism used are presented in figs. (4)-(6). All correspond to a magnification of 1000x. It is possible to see a membrane-like gluten matrix, covering most of the starch granules (t_0). A dense distribution of the starch granules covered by the protein matrix can also be noted. Lenticulated granules of larger size (type-A) can be seen, and others smaller and spherical (type-B) (Fig. 3) resulting from the intimate contact of the components of the endosperm at the cellular level; these spherical granules are uniform size.

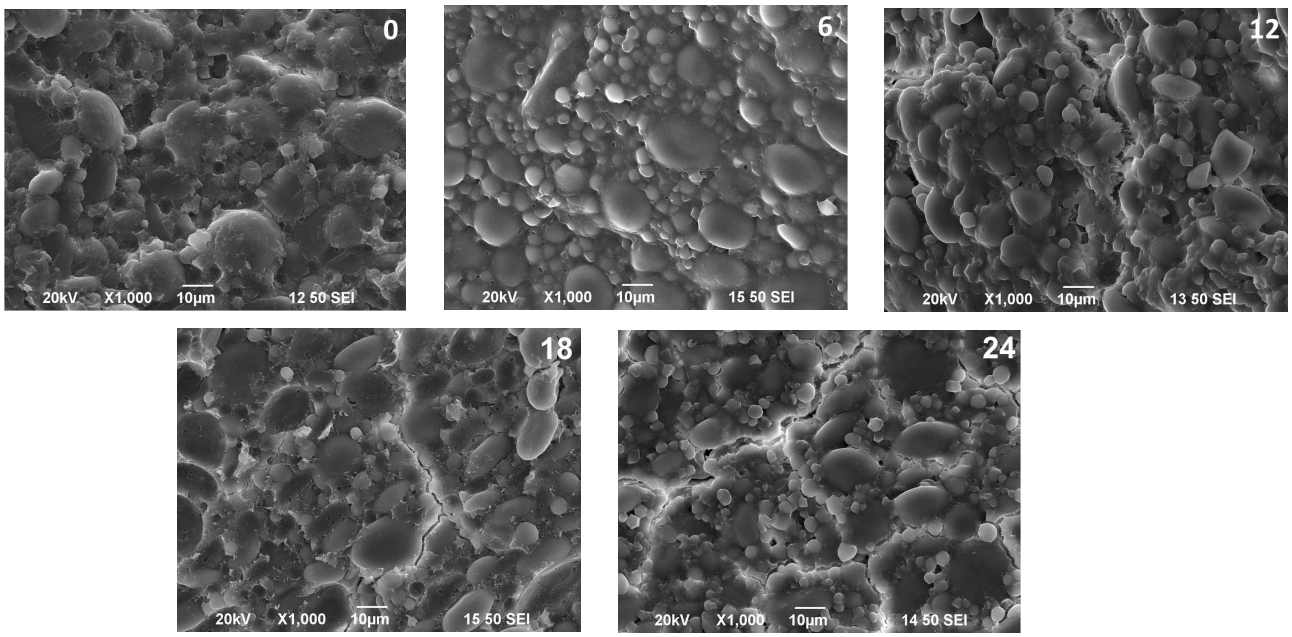

Figure 4. Micrographs of the structural modification of the dough fermented with *Lb. brevis*.

The small starch granules, with the proteins and non-starch polysaccharides, form strung structures (Kim *et al*., 2003), which are more evident in the micrographs corresponding to 24 h of fermentation (Figs 4 and 5).

The microstructural changes that occurred during the fermentation progressed were evident. However, it is also worth noting that the microstructure of the dough is different depending on the type of microorganism used. Thus, it can be seen that the protein cover has practically disappeared in the dough fermented with *Lb. plantarum*, at 24 h (Fig. 5).

Rojas *et al*. (2000) have pointed out that during fermentation, the gluten and the soluble solid matrix become more dense, continuous and better distributed, which can be seen by comparing the micrographs of the doughs fermented with *Lb. brevis* at t_0 and t_6 (Fig. 4).

As fermentation progressed, it was possible to see the loss of continuity of the protein matrix, with clearly seen uncovered starch granules, especially in the dough fermented with *Lb. plantarum* (Fig. 5) (t_{18} and t_{24}), followed by those containing *Lb. brevis* (Fig. 4) (t_{18} and t_{24}) and to a lesser extent, in the dough fermented with *Lb. sanfranciscensis* (Fig. 6).

In the dough fermented with *Lb. brevis* it is worth noting the detachment of the starch granules from the protein matrix at 18 h of fermentation, which may be considered an indicator of hydrolysis degree of the protein phase (Fig. 4). Thus, it appears that an increased degradation of the protein matrix had occurred at 24 h of fermentation, and it was more intensive when using *Lb. plantarum*, followed by *Lb. brevis*, while *Lb. sanfranciscensis* caused the least intensity. This breaking of the protein matrix has been related to a decrease in bread volume and it has been proposed that the membrane-like structure of gluten is optimal for gas retention, so that the loss of this structure will result in poor gas retention ability (Kim *et al*., 2003).

Thiele *et al*. (2004) determined the hydrolysis and depolymerization of the gluten proteins during fermentation in sourdoughs. They reported that during this process, a substantial hydrolysis of gliadin and glutenin occurs, involving the participation of the enzymes that are naturally present in the flour. LABs may also exhibit proteolytic activity, but their activities are specific and it seems that they only play a minor role in the total proteolytic activity. However, it was observed in this study that each type of microorganism used for fermentation had a different effect on the protein structure of the dough.

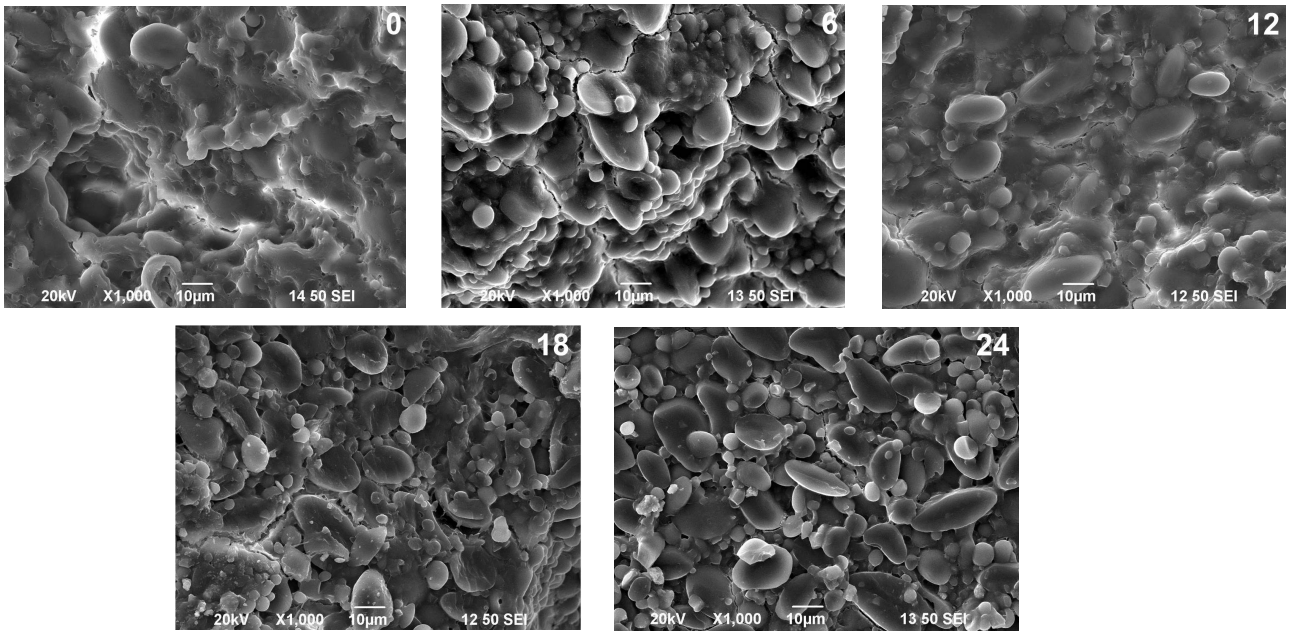

Figure 5. Micrographs of the structural modification of the dough fermented with *Lb. plantarum*. Al t_{18}, the damage to the starch granules (DS) is evident.

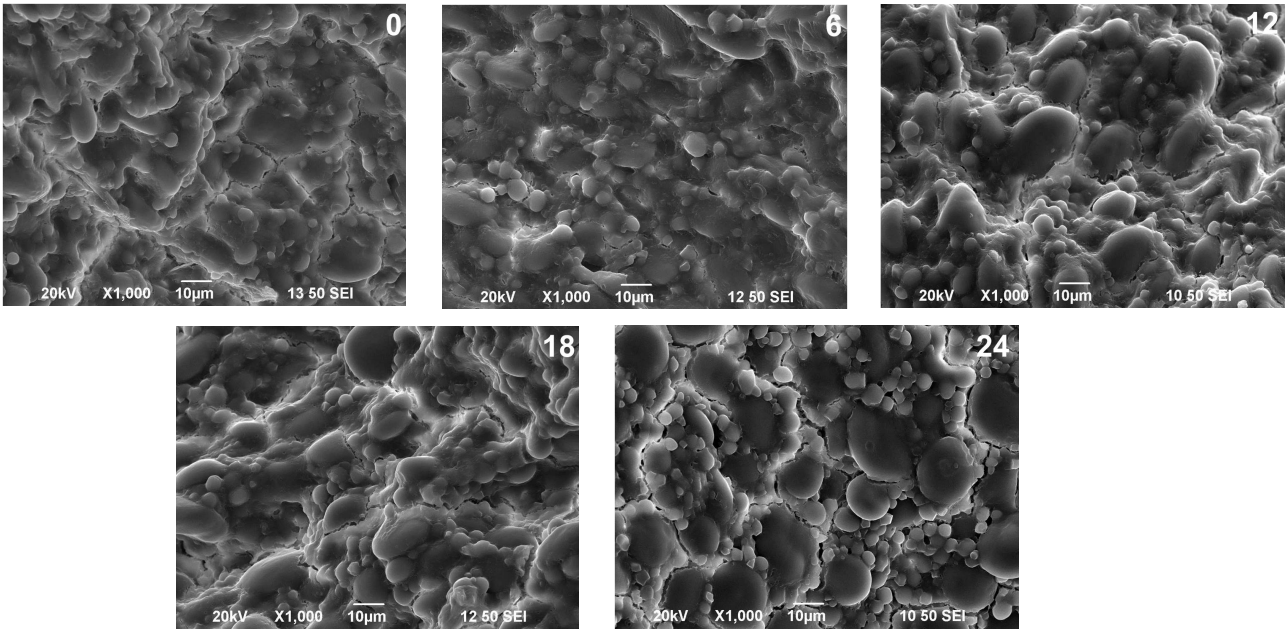

Figure 6. Micrographs of the structural modification of the dough fermented with *Lb. sanfranciscensis*.

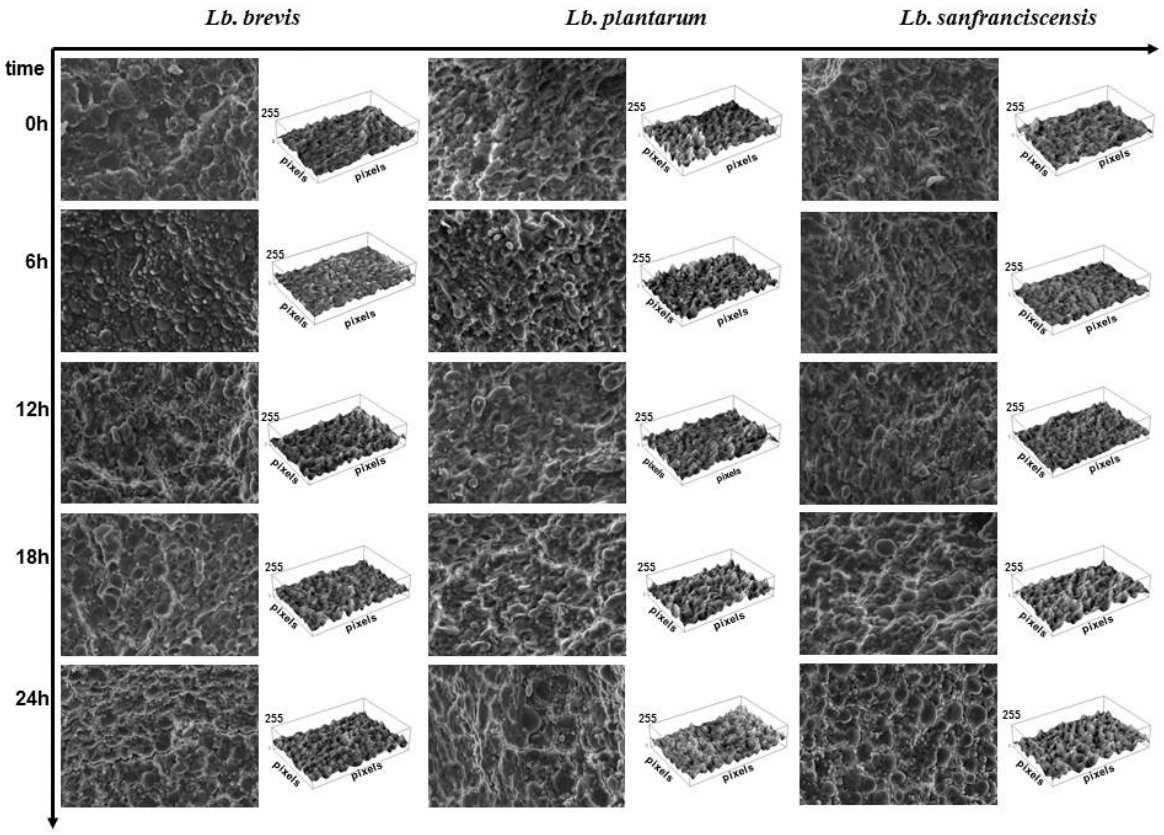

Figure 7. Gallery of SEM images at 500x (20kV), 50μm, and their corresponding Surface Intensity grayscale plots of the doughs fermented with different types of lactic acid bacteria and different fermentation times.

Lb. plantarum, which had the highest rate of acidification, significantly affected the microstructure of the dough, perhaps promoting greater enzymatic activity of proteases from the first hours of fermentation, since at 6 hours of the process, the dough had reached a pH of 4.91 (0.090 % lactic acid), close to the optimum activity value of the flour enzymes (Bleux *et al*., 1997). Lower enzymatic activities could be expected in the doughs containing other test microorganisms, as at 6 h of the process the pH value was 5.17 (0.045 % lactic acid) for *Lb. sanfranciscensis* and 5.70 (0.036 % lactic acid) for *Lb. brevis*. However, at 12 hours of the process, all doughs had reached pH values of about 4.0 (Table 2), which allows inferring a high enzymatic activity of the flour enzymes.

Thiele *et al*., (2004) indicated that the growth and metabolism of LABs ceased when the dough reached pH values of between 3.6 and 3.8. They also mentioned that the hydrolysis of glutenins is mainly dependent on the pH and is not related to the specific proteinases of LABs. If so, no changes in rheological properties should be found in the dough for similar pH values. That is, the doughs fermented with *Lb. brevis* and *Lb. sanfranciscensis*, which behave similarly in terms of the decrease of pH, would have a similar rheology. However, it has been reported that different strains of *Lb. plantarum* are acid tolerant, able to grow at pH 4.0 (Cebeci and Gürakan, 2003), which could be related to the microstructure presented by the fermented doughs at 24 h, where greater degradation of the protein matrix can be seen (Fig. 5) compared with the other doughs (figs (4)-(6). The ability to tolerate acidic conditions allows considering this microorganism as a promising probiotic.

Gänzle *et al*. (2008) mention that *Lb. sanfranciscensis* and many other LABs of sourdoughs have no extracellular proteolytic activity and that it is the enzymatic activity of the proteases present in wheat flour that supports the growth of non-proteolytic lactobacilli. This bacterium transports peptides and

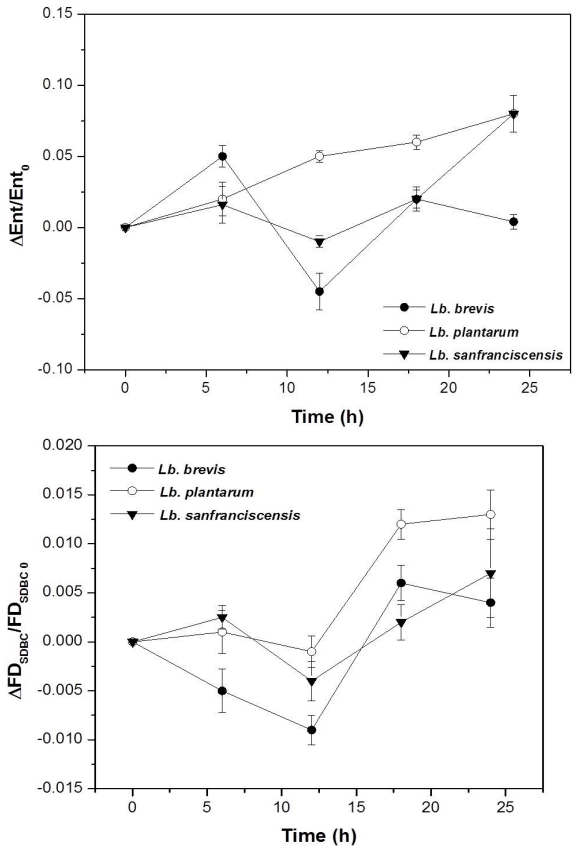

Figure 8. Ent and FD_{SDBC} values of the doughs fermented with different types of lactic acid bacteria and different fermentation times.

degrades them to amino acids through intracellular peptidases. They also note that the susceptibility to proteolysis increases by the acidification of LABs and the reduction of disulfide bonds in the gluten protein.

Peptide hydrolysis (secondary proteolysis) by LABs accumulates amino acids in the dough independently of the strain used for fermentation, while protein degradation weakens the dough. The fermentation with LAB affects the size distribution of the peptides resulting from proteolytic degradation, decreasing the presence of large peptides and increasing the levels of smaller molecules such as amino acids and dipeptides (Zotta *et al*., 2006). It has been noted that the only change in gluten proteins that can be attributed to the presence of LAB in sourdough is the emergence of new protein fragments (20 and 27 kDa) from gliadins and the degradation of glutenin subunits with high molecular weight (Zotta *et al*., 2006).

Starch granules also suffered changes; type-A granules, in particular, suffered degradation, which can be noted by the erosion on their surface. This phenomenon appears most intensely in the dough fermented with *Lb. plantarum* (Fig. 5, t_6 to t_{24}), while in the doughs fermented with *Lb. brevis* and *Lb. sanfranciscensis* the degradation occurs after 18 h of fermentation (Fig. 4 and 6). As fermentation progresses, type A granules seem to form a separate phase from that conformed by the protein matrix, whereas type B granules are directly embedded within the protein matrix. According to Blaszczak *et al*., (2004) the differences in the result of phases separation are due to the variations on the swelling capacity and the concentration of granules small and larger.

Finally, the Fig. 7 shows an image gallery of the microstructural changes on by the doughs inoculated with *Lb. brevis*, *Lb. plantarum* and *Lb. sanfranciscensis* at different fermentation times, and their corresponding intensity graphs in grayscale. In general, it can be seen that in the doughs fermented with the three types of lactobacilli, the complexity of the microstructure increases with the time of fermentation as a result of the microbial activity on the structural network of the system. The changes in the structural complexity were characterized by normalized entropy (Ent) and fractal texture (FD_{SDBC}) (Fig. 8) values. Both image texture descriptors showed a gradual increase with fermentation time, the FD_{SDBC} values showing a larger difference when compared with Ent values (figs. 8a and 8b). This tendency indicates greater image heterogeneity (Haralick *et al*., 1973; Quevedo *et al*., 2002, 2008) and greater apparent roughness (Quevedo *et al*, 2002, 2008; Gonzales-Barron and Butler, 2008; Perez-Nieto *et al*, 2010). However, the structural deformation was more marked in the dough fermented with *Lb. plantarum*, as these showed a faster increase in the texture parameters used, compared with the doughs fermented with the other strains under study. These correlates are relationship with the behavior of the normalized pH, which also showed a tendency to increase more rapidly compared to the other two treatments. Morphological and rheological changes that occurred in fermented doughs can be associated with the production of lactic and acetic acid by the LABs and the consequent, change in pH, which induces a higher activity of the enzyme system of wheat flour, adding to the proteolytic action of the bacterial species (Thiele *et al*., 2002, 2004; Zotta *et al*., 2006). It was observed that as fermentation progressed, the adhesive behavior observed when handling the sourdoughs increased, which may be attributable to the combination of the

activity of the LABs, the secreted exopolysaccharides and the pentosans present in the wheat flour (Di Cagno *et al*., 2002). Furthermore, it has been reported that low pH values modify the solubility of the proteins, and induce a net positive charge, which promotes electrostatic repulsions between the proteins, and makes their functional groups more reactive. However, intermolecular electrostatic repulsive forces prevent the formation of new bonds, increasing the smoothness of gluten and generating a weak structure (Arendt *et al*., 2007).

Conclusions

Physicochemical, rheological and textural parameters (FD_{SDBC} and Ent) provide relevant information to evaluate the changes occurred during the fermentation of the doughs as a function of the type of lactic acid bacteria. It was possible to differentiate the effect on the E_m and R_m of the dough; there was greater activity of *Lb. sanfranciscensis* for the first parameter and of *Lb. plantarum* for the second. Fermentation increased the structural complexity, which could be characterized by the fractal dimension and entropy values, allowing us to describe the heterogeneity of the doughs with respect to the type of bacteria tested. The results presented may provide useful criteria to select the types of microorganisms and fermentation times needed to modify the structure of these types of dough. Furthermore, this study provides relevant information for understanding the structural changes occurring during fermentation and their relationship with the functionality of the systems tested.

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Nomenclature

DY	dough yield
CFU	Colony Forming Units
Ent	entropy
FD	fractal dimension
$SDBC$	shifting Differencial Box Couting
E_m	maximum extensibility
R_m	maximum resistance
ΔE_m	changes in Strength values
E_{m0}	force at the initial time
E_{mt}	force at any instant
t	fermentation time (h)
d	distance
pH_n	normalized values of pH
$\Delta R_m/R_{m0}$	normalized values of maximum resistance
$\Delta E/E_0$	normalized values of extensibility
$\log(N)$	the number of boxes
$\log(1/r)$	the length of the side of box
$P(i,j)$	the relative frequency of occurrence of two pixel values
Greek symbols	
θ	direction to values i and j

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