1 *RUNNING HEAD:*

2 WHOLE WHEAT SOURDOUGH BREAD WITH BIFIDOBACTERIA

3
4 **APPLICATION OF BIFIDOBACTERIA AS STARTER CULTURE IN WHOLE**
5 **WHEAT SOURDOUGH BREADMAKING**

6 **Juan Mario Sanz-Penella, Juan Antonio Tamayo-Ramos, Monika Haros^{*}**

7
8 Institute of Agrochemistry and Food Technology (IATA-CSIC), Av. Agustín Escardino 7

9 Parque Científico, 46980 Paterna-Valencia, Spain

10
11
12
13
14
15
16
17 *Corresponding author. Mailing address: Institute of Agrochemistry and Food
18 Technology (IATA-CSIC), Av. Agustín Escardino 7, Parque Científico, 46980 Paterna-
19 Valencia, Spain. Phone: +34 96 390 00 22, Fax: +34 96 363 63 01, E-mail:
20 mharos@iata.csic.es

21
22 This work was financially supported by grants AGL2006-09613/ALI, CSIC-200870I229
23 and Consolider Fun-C-Food CSD2007-00063 from the Ministry of Science and
24 Innovation, Spain (MICINN). The scholarship of J.M. Sanz Penella and the contract of
25 J.A. Tamayo Ramos from MICINN are greatly acknowledged.

ABSTRACT

This investigation is aimed at developing a new cereal-based product, with increased nutritional quality, by using *Bifidobacterium pseudocatenulatum* ATCC 27919 as starter in whole wheat sourdough fermentation, and evaluating its performance. Four different sourdough levels (5, 10, 15 and 20% on flour basis) in bread dough formulation were analysed. The effects of the use of bifidobacteria in sourdough bread were comparatively evaluated with controls (yeast and/or chemically acidified sourdough with antibiotics). The sourdough and dough fermentative parameters analysed were pH, total titratable acidity, D/L-lactic and acetic acids. Bread performance was evaluated by specific volume, slice shape, crumb structure and firmness, crust and crumb colour, pH, total titratable acidity, and D/L-lactic and acetic acids, phytate and lower *myo*-inositol phosphate contents. The sourdough breads showed similar technological quality to the control sample, with the exception of specific bread volume (decreased from 2.46 to 2.22 mL/g) and crumb firmness (increased from 2.61 to 3.18 N). Sourdough inoculated with bifidobacteria significantly increased the levels of organic acids in fermented dough and bread. The *Bifidobacterium* strain contributed to the fermentation process, increasing phytate hydrolysis during fermentation owing to the activation of endogenous cereal phytase and its own phytase, resulting in bread with significantly lower phytate levels (from 7.62 to 1.45 $\mu\text{mol/g}$ of bread in dry matter). The inclusion of sourdough inoculated with bifidobacteria made possible the formulation of whole wheat bread with positive changes in starch thermal properties and a delay and decrease in amylopectin retrogradation.

KEY WORDS: sourdough; *Bifidobacterium*; phytate-degrading enzyme; phytate; whole wheat bread

INTRODUCTION

Cereal grains are grown in greater quantities and provide more food energy worldwide than any other type of crop. Cereal foods produced and consumed in different ways are an essential component of daily diet. Health experts advise that whole grains are a healthy necessity in every diet, the consumption of at least half of the cereal servings as whole grains being the recommendation for adults (Whole Grains Council, USA). Epidemiological findings have indicated a protective role of whole grain foods against several diseases. Medical evidence clearly shows that whole grains reduce risks of certain diseases such as colorectal cancer, type 2 diabetes, coronary heart disease and obesity (Pereira et al., 2002; Mellen et al., 2008). Cereal goods, especially whole grain products, are source of fibre, vitamins, minerals and other biologically active compounds as phenolic compounds, lignans, phytosterols, tocopherols, tocotrienols and phytic acid, and processing may modify the amount and bioavailability of some of them (Slavin, 2004; Katina et al., 2005). In fact, the whole grain or fractions of cereal grain could be modified by sourdough fermentation to improve nutritional value or promote healthiness of cereal by-products (Katina et al., 2005). The use of sourdough is a common practice in many countries around the world. Sourdough fermentation can modify the flavour of products, stabilize or increase levels of various bioactive compounds, retard starch bioavailability, extend the shelf life of bread and improve mineral bioavailability (Katina et al., 2005). Texture, taste and smell of bread are the main characteristics taken into account by consumers to determine its quality. In this sense, there are numerous examples of improved texture and palatability in sourdough fermentation processes due to peptide, lipid and carbohydrate metabolism (Thiele et al., 2002; Gänzle et al., 2007). Although sensory quality is the basis for any successful bakery product, consumers are aware of nutrition/health interactions and consequently society demands healthier and more

76 nutritious foods. The effect of sourdough and cereal fermentation could enhance delivery
77 of nutrients to the bloodstream (Poutanen et al., 2009). As was mentioned above,
78 sourdough has great potential to modify the digestibility of starch, lowering the glycemic
79 index of the products mainly due to increased lactic and acetic acid levels (Katina et al.,
80 2005; De Angelis et al., 2009). Whereas lactic acid lowers the rate of starch digestion in
81 bread, acetic acid would delay the gastric emptying rate (Liljeberg et al., 1995; Liljeberg
82 & Björck, 1998).

83 On the other hand, phytic acid (*myo*-inositol [1,2,3,4,5,6]-hexakisphosphate, InsP_6) or
84 phytates (its salts), which are considered to be the major factor causing negative effects
85 on mineral uptake in humans and animals, is a precursor of generation of bioactive
86 compound (Fretzdorff & Brümmer, 1992; Lopez et al., 2001; Nielsen et al., 2007; Haros
87 et al., 2009). The phytates are capable to form complexes that strongly reduce the
88 absorption of many minerals as iron, zinc, calcium, magnesium, manganese and copper
89 (Lopez et al., 2002; Konietzny & Greiner, 2003). However, the phytate hydrolysis
90 decreases the negative effects on mineral absorption and generates lower *myo*-inositol
91 phosphates that have been suggested to be compounds with specific biological activity
92 and may positively affect human health (Shi et al., 2006; Haros et al., 2009). The phytase
93 is the enzyme that catalyses the hydrolysis of InsP_6 to a mixture of *myo*-inositol pentakis-
94 , tetrakis-, tri-, di-, monophosphates (InsP_5 , InsP_4 , InsP_3 , InsP_2 , InsP_1 , respectively) and
95 orthophosphate. The reduction of InsP_6 content during the bread making process depends
96 on phytase action, which in turn depends on many factors including bran content, pH,
97 temperature, water content, particle size distribution, fermentation time, exogenous
98 phytase addition and process (Haros et al., 2001; Lopez et al., 2002; Sanz Penella et al.,
99 2008, 2009; Rosell et al., 2009). The cereal has an endogenous phytase, which its optimal
100 pH of action is around 4.5 in wheat and rye doughs, hence the use of sourdough or

acidified sponges increase the InsP_6 hydrolysis (Fretzdorff & Brummer, 1992; Lopez et al., 2001; Reale et al., 2004). Phytases could be produced by a wide range of plants, bacteria, and fungi; and some of them are commercially used for animal nutrition, although are not considered of food grade (Haros et al., 2009). It was reported that strains of *Bifidobacterium* show phytase activity, suggesting their possible utility in producing bakery products (Haros et al., 2005; 2007). Sanz Penella et al. (2009) investigated the use of bifidobacteria with high phytate-degrading activity as starter cultures in two formulations of bread (100% and 50% of whole wheat flour) resulting in breads with significantly lower levels of phytates. Palacios et al. (2008) investigated the use of *Bifidobacterium* strains as starter during long fermentation process of whole-wheat dough, which showed a good adaptation to the dough ecosystem and contributed to different acidification degrees promoting the phytate hydrolysis. Many new interesting applications for sourdough still remain to be explored, such as the use of *Bifidobacterium* starter cultures for improving phytate hydrolysis, or the production of organic acids and novel bioactive compounds. This research is aimed at developing new cereal-based products of increased nutritional quality and containing lower amounts of InsP_6 , by using bifidobacteria of human origin, *Bifidobacterium pseudocatenulatum* ATCC27919, as a starter in whole wheat sourdough fermentation.

MATERIALS AND METHODS

Materials

Commercial Spanish whole wheat flour was purchased from the local market. The characteristics of flour were (g kg^{-1} in dry matter): moisture 141.6 ± 0.3 , protein ($\text{N} \times 5.7$) 111.7 ± 0.6 , lipids 17.6 ± 0.2 , and ash 8.4 ± 0.1 . Compressed yeast (*Saccharomyces cerevisiae*, Levamax, Spain) was used as a starter for the bread making process, whereas *Bifidobacterium pseudocatenulatum* ATCC 27919, originally isolated from faeces of infants, was used as starter in sourdough fermentation.

Microbial growth conditions.

Bifidobacteria were grown in Garche broth in which inorganic phosphate (K_2HPO_4 and NaH_2PO_4) was replaced by 0.74 g/L phytic acid dipotassium salt (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 M 3-[N-Morpholino] propanesulphonic acid buffer (MOPS, Sigma-Aldrich, St. Louis, MO, USA) (Haros et al., 2007). The medium was inoculated at 5 % (v/v) with 18-hour old cultures, previously propagated under the same conditions. Cultures were incubated at 37 °C in anaerobic conditions (AnaeroGen™, Oxoid, England) until the beginning of the stationary phase of growth (~14-18 hours). Bacterial cells were harvested by centrifugation ($10,000 \times g$, 15 min., 4 °C, Sorvall RC-5B, DuPont Instruments), washed twice and suspended in 0.085 % NaCl solution (Sanz Penella et al., 2009). The obtained cell suspensions were used to inoculate the sourdough. Microbial counts in sourdough and dough samples were determined by plate count on selective media. Sourdough and dough samples from each formulation (1 g) were homogenised with 9 mL of peptone water (Scharlau Chemie, Barcelona, Spain), serially diluted and plated on agar. Bifidobacteria counts were determined after sourdough incubation and dough fermentation periods in Garche agar, using the double layer technique, after

anaerobic incubation at 37 °C for 48 h (Haros et al., 2005). Yeast counts were determined in Rose Bengal Agar (Scharlau Chemie, Barcelona, Spain) after aerobic incubation at 30 °C for 72 h (Sanz Penella et al., 2009).

Bread-making process

The control bread dough formula consisted of whole wheat flour (500 g), compressed yeast (2.5 % flour basis), sodium salt (1.8 % flour basis), tap water (up to optimum absorption, 500 Brabender Units, 65.0 %) and ascorbic acid (0.01 % flour basis). The ingredients were mixed for 4.5 min, rested for 10 min, divided (100 g), kneaded and then rested (15 min). Doughs were manually sheeted and rolled, proofed (up to optimum volume increase, at 28 °C, 85 % relative humidity) and baked (165 °C, 30 min) according to Haros et al. (2001).

Whole wheat sourdough without yeast were prepared and added in five levels to bread doughs formula: 0, 5, 10, 15 and 20 % in flour basis (Control, WDS-5, WDS-10, WDS-15 and WDS-20, respectively). The sourdough formulation consisted in a mixture of flour and water (1:2, v/v) with an inoculum $\sim 5.5 \times 10^8$ CFU of *B. pseudocatenulatum* per gram of flour, incubated for 18 hours at 37 °C in anaerobic conditions. The control acid sourdough consisted of the same formulation and conditions as described above without the addition of *Bifidobacterium* strain, including a mixture of antibiotics at 1 % v/v (Penicillin, 50 U/mL; Streptomycin, 0.05 mg/mL; Neomycin, 0.1 mg/mL; and Cycloheximide, 0.5 mg/mL from Sigma-Aldrich Steinheim, Germany). The control acid sourdough pH was adjusted at 4.17 with a mixture of lactic and acetic acids (1:2 v/v), to reach the same pH of sourdough biologically acidified with using bifidobacteria. Fermentation was monitored by measuring pH, temperature and volume increase of the dough at regular period times. After the fermentation step, doughs were baked in an

electric oven and cooled at room temperature for 75 min for their subsequent analysis (Sanz Penella et al., 2009).

Bread Performance

The technological parameters analysed were: loaf specific volume (cm^3/g), width/height ratio of the central slice or slice shape (cm/cm), moisture content (%) and crumb firmness, determined by a texture profile analysis using the Texture Analyser TA-XT Plus (Stable Micro Systems, Surrey, United Kingdom) (Sanz Penella et al., 2009). Each parameter was measured at least per triplicate.

Digital image analysis was used to measure the bread crumb structure. Images were previously squared at 240 pixels per cm with a flatbed scanner (HP ScanJet 4400C, Hewlett Packard, USA) supported by the HP PrecisionScan Pro 3.1 Software. Two 10 mm x 10 mm squares field of view of central slice (10 mm thick) of each of three loaves were used, thereby yielding 6 digital images per each baking. Data was processed using Sigma Scan Pro Image Analysis Software (version 5.0.0, SPSS Inc., USA). The crumb grain features chosen were: cell area/total area, cm^2/cm^2 ; wall area/total area, cm^2/cm^2 ; number of cells per cm^2 ; and mean cell area, mm^2 (Sanz Penella et al., 2009).

The tristimulus colour parameters L^* (lightness), a^* (redness to greenness), b^* (yellowness to blueness) of the baked loaves (crumb and crust) were determined using a digital colorimeter (Chroma Meter CR-400, Konika Minolta Sensing, Japan), previously calibrated with the white plate supplied by the manufacturer. The instrument settings were illuminant C, display L^* a^* b^* , and observer angle 10° . From the parameters determined hue angle (h^*), chroma (C^*) and total colour difference (ΔE^*) were calculated by the equations: $h^*_{ab} = \arctan(b^*/a^*)$; $C^*_{ab} = (a^{*2} + b^{*2})^{1/2}$; $\Delta E^* = [(\Delta L^*)^2 +$

$(\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Each sample was measured 18 times in different sample points to minimize the heterogeneity produced by the bran.

Initial $\text{Ins}P_6$ concentration in whole wheat flour, $\text{Ins}P_6$ residual amount and lower *myo*-inositol phosphates generated after fermentation and baking in bread were measured by using the high pressure liquid chromatographic method described by Türk and Sandberg (1992), later modified by Sanz Penella et al. (2008).

Preliminary sensory analysis of fresh breads was performed by a panel of 20 non-trained tasters, who usually consume whole wheat bread, using a simple scale of acceptance (dislike very much, dislike, like, like very much).

Total titratable acidity (TTA) determination, D/L- lactic and acetic acids

Ten grams of sourdough, dough or bread, blended with 100 mL of acetone:water (5:95, v/v) under constant agitation, were titrated against 0.1 N NaOH until a final pH of 8.5. The results were expressed as the volume (mL) of NaOH 0.1 N needed for titrating 10 g of sourdough, fermented dough or bread. Concentrations of D-lactic acid, L-lactic acid and acetic acid were analysed using the specific enzymatic methods of Boehringer Mannheim/R-Biopharm by UV method (Polar Star Omega BMG LABTECH, Germany). The results were expressed as μmoles of D/L lactic or acetic acid per gram of sourdough, fermented dough or bread.

Differential scanning calorimetry (DSC) analysis

The thermal properties of starch flour during the baking of fermented dough (gelatinization) and changes induced during the bread storage (amylopectin retrogradation) were carried out on a calorimeter (DSC-7, Perkin-Elmer). Indium (enthalpy of fusion 28.41 J/g, melting point 156.4 °C) was used to calibrate the

calorimeter. Fermented dough samples (30-40 mg) were weighted directly into DSC stainless steel pans (LVC 0319-0218, Perkin-Elmer) and hermetically sealed (Quick-Press, 0990-8467, Perkin-Elmer). Calorimeter scan conditions were used according to the methodology described by Leon et al. (1997), later modified by Sanz-Penella et al. (2010). Briefly, to simulate the temperature profile in the centre of the bread crumb during baking, the samples were kept at 30 °C for 1 min, were heated from 30 to 110 °C at 11.7 °C/min, were kept at this temperature until 5 min, and cooled to 30 °C at 50 °C/min. To analyse amylopectin retrogradation, heated-cooled pans were stored at 4 °C for 0, 1, 2, 4, 7, 10 and 15 days, and heated again in the calorimeter from 30 to 110 °C, at 10 °C/min (Sanz-Penella et al., 2010). An empty pan was used as a reference and three replicates of each sample were analysed.

The parameters recorded were onset temperature (T_o), peak temperature (T_p) and conclusion temperature (T_c) of gelatinization and retrogradation. Straight lines were drawn between T_o and T_c and the enthalpies associated with starch gelatinisation and retrogradation (ΔH_g and ΔH_r , respectively) were calculated as the area enclosed between the straight line and the endotherm curve. The enthalpies were expressed in Joules per grams of dry matter.

Statistical analysis

Multiple sample comparison of the means and Fisher's least significant differences (LSD) were applied to establish statistical significant differences between treatments. All statistical analyses were carried out with the software Statgraphics Plus 7.1 (Bitstream, Cambridge, MN) and differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Characteristics of sourdough

The sourdough inoculated with bifidobacteria used in this study became more acidic at the end of the incubation period (from initial pH 5.4 to final pH 4.2) owing to fermentative activity of the microbial metabolism. As observed in the literature, the values of pH in ripe sourdoughs using typical starters such as *Lactobacillus sanfranciscensis*, *Lactobacillus plantarum* and/or *Lactobacillus brevis* can vary between 3.5 and 4.3, depending on the type of flour, the process and the starter cultures used (Collar et al., 1994; De Angelis et al., 2009; Thiele et al., 2002; Robert et al., 2006). During the incubation period there was a considerable production of organic acids (mainly acetic and lactic acids) reaching TTA values around 17.0 mL, with the production of lactic acid exclusively in its levorotatory form (Table 1). Robert et al. (2006) reported lower values of TTA in sourdoughs inoculated with *L. plantarum* or *Leuconostoc* sp. In the current study the molar ratio between lactic and acetic acids was 0.37. It should be pointed out that acetic acid production is greater than lactic acid production in bifidobacteria. This value corresponds to typical molar ratios found as the result of sugar catabolism in bifidobacteria, 0.40-0.70, mainly depending on the strain and the sugar (Van der Meulen et al., 2006). Metabolism of carbohydrates varies depending on the species of *Bifidobacterium*, and even the strains, the type of soluble sugars and the processing conditions. The metabolite production for *B. pseudocatenulatum* after growing in synthetic medium containing different energy sources showed a molar ratio between 0.38 and 0.74 (results not shown). Although the optimum in industrial sourdough fermentation of wheat is considered around 2.5 (Röcken 1996; Hammes & Gänzle, 1998), this value can vary over wider ranges (Barber et al., 1991). Lactic and acetic acid production was considered the main reason for the decrease

in the value of pH after sourdough fermentation. Lactic acid bacteria (LAB) counts showed 4.0×10^9 CFU per gram of flour after the incubation period, which represented a considerable increase from the initial value. The LAB population was in the range of counts found in mature sourdoughs (Hammes et al. 2005; Robert et al., 2006). The pH value reached at the end of sourdough fermentation and the colony counts indicated that the inoculated bifidobacterial strain could adapt to the dough environment, increasing its viability.

Characteristics of fermented dough

The inclusion of sourdough in the bread formulation caused a significant decrease in the dough pH, from 5.38 to 4.57, as was expected (Table 1). However, the dough pH remained unchanged during the yeast fermentation process until the optimum volume increase was reached. Similar pH values were found by Collar et al. (1994) when sourdough was added in a proportion of between 10 and 25 % on flour basis. Dough volume showed a constant increase during the fermentation period, reaching a maximum after approximately 60 minutes at 28 °C. The presence of sourdough in the bread formulation did not significantly modify the optimum dough volume. However, the addition of sourdough to the formulation significantly increased the LAB counts (from 2.2×10^4 CFU/g to 1.1×10^7 CFU/g, control and WDS-20 samples, respectively), whereas the yeast counts remained almost constant (3.7 - 5.0×10^7 CFU/g). These LAB and yeast counts were consistent with previous reports shown by other authors (Palacios et al., 2006, 2008). TTA values in fermented dough ranged from 4.60 to 9.23, showing a constant and significant increase mainly due to the production of lactic and acetic acid during sourdough fermentation (Table 1). This highlights the considerable acidic production of the *Bifidobacterium* strain used in this study, which may be important for

enhancing flavour and delaying bread staling. The D-lactic acid content decreased with the rise in the sourdough percentage added to the formulation, whereas the L-lactic and acetic acids presented a significant increase. The molar ratio between the D/L-lactic and acetic acids remained between 0.45 and 0.47 in all formulations with sourdough inoculated with bifidobacteria (Table 1). *L. plantarum*, *L. brevis* and *Leuconostoc sp.* were reported to produce greater amounts of lactic acid (5.5-13.3 $\mu\text{mol/g}$) than the culture used in this study, and lower amounts of acetic acid (1.66-5.82 $\mu\text{mol/g}$), in fermented dough made with sourdough (Collar et al., 1994; Robert et al., 2006).

Acidic characteristics of bread

TTA values in the bread were recorded from 4.19 to 10.60 (Table 1). These results were in the range found by other researcher in bread with sourdough inoculated with lactobacilli (Katina et al., 2009), although this parameter could vary over a wider range. The D/L-lactic and acetic acids showed the same tendency as was found in the fermented dough: the amount of D-lactic acid decreased with the increase in sourdough in the formulation, whereas the L-lactic and acetic acids showed an opposite behaviour (Table 1). *L. plantarum* and *L. brevis* resulted in lower acetic acid production (1.2-2.3 $\mu\text{mol/g}$) than the levels found in this study, whereas the amount of lactic acid was significantly higher, reaching values up to 40.9 $\mu\text{mol/g}$ (Collar et al., 1994). The molar ratio between D/L-lactic and acetic acids registered an increase from 0.91 in the control sample to 1.29-1.45 with the addition of 15-20% of sourdough in the bread formula. During the breadmaking process there is a weight loss, 95% of which is due to water evaporation and 5% due to organic acid loss, mainly in crust and outside crumb of the bread, the loss of acetic acid during baking being greater than that of lactic acid (Spicher, 1983). This greater loss of acetic acid was responsible for the increase in the molar ratio between the

D/L-lactic and acetic acids of the bread compared to the values recorded in the fermented dough. It is important to note that the increase in the amount of these organic acids caused by the use of sourdough has been shown to lower the glycemic index of bread products (Liljeberg et al., 1995; Liljeberg & Björck, 1998).

Bread performance

The effect of the addition of sourdough on bread quality was analysed (Table 2). In general, technological parameters did not show significant differences between samples. The loaf moisture ranged between 34.74 and 36.04 without significant changes. The sample with 20 % sourdough content (WDS-20) showed a significantly lower loaf specific volume than the control, whereas the slice shape remained without significant differences, but tended to decrease (Table 2). There is considerable consensus with regard to the positive effects of the addition of sourdough on bread volume and crumb structure (Arendt et al., 2007). Despite this, Collar et al. (1994) developed lower volume breads when using a high percentage of sourdough with *L. plantarum* and *L. brevis* as starters. The acidification of the sourdough and partial acidification of the bread dough impact on structure-forming components like gluten and starch. During incubation of sourdough and dough fermentation, biochemical changes occur in the carbohydrate and protein components of flour owing to the action of microbial and endogenous enzymes. The possible proteolytic activity associated with the *Bifidobacterium* strain, which would take place during the incubation period of sourdough incubation and dough fermentation, would attack gluten-associated proteins and weaken the gluten network, leading to breads with a lower specific volume. This proteolytic activity has been observed in several lactobacilli strains found in different sourdoughs, which might contribute an improvement in bread flavour (Rollan et al., 2005).

The crumb textural profile of samples to which sourdough had been added showed no significant difference compared with the control (data not shown). However, the crumb firmness showed a constant increase from 2.61 in the control sample to 3.18 N in the formulation with 20 % sourdough (Table 2). Increased firmness with addition of sourdough was at least partly due to the lower specific volume found in these samples. Softer breads were found after the inclusion of mature sourdough in the bread formulation, which might depend on the number of stages used in sourdough preparation (Barber et al., 1991).

The parameters that describe crumb grain features did not show any significant difference between samples (Table 2). Despite this, the cell area and number of cells showed a slight correlation, with the value decreasing when a greater percentage of sourdough was added to the formulation (15-20 %). The technological parameters (loaf specific volume, width/height ratio and firmness) could corroborate this tendency (Figure 1). However, although these differences were statistically significant, they were unimportant in the sensory analysis (results not shown). The values of the mean cell area ranged from 0.95 to 1.32, with no differences appearing between breads made with sourdough and the control (without sourdough).

The effect of the addition of sourdough inoculated with bifidobacteria on the crust and crumb colour was determined (Table 3). Generally, the sourdough did not present significant changes in the crust or crumb colour of the bread in comparison to the control.

The total colour difference in bread crust and crumb, which represents the total colour difference between the samples with sourdough and the control sample, was less than 5 units (from 0.67 to 3.16), indicating that no differences were detectable by visual observation. So, although some significant changes were recorded in a few colour parameters, they were not perceptible to consumers by visual observation (Figure 1).

In general, the breads made with sourdough (5-15 %) had high consumer acceptance (82-93 % of tasters), but with lower scores than the control breads. Bread made with 20 % sourdough showed the lowest degree of acceptance, mainly because of its higher acidity, being accepted by 40 % of the tasters.

Degradation of phytate and generation of lower myo-inositol phosphates

The phytate content in the control bread was reduced by 28 % over baseline in the flour (Table 4). Its reduction and the generation of lower *myo*-inositol phosphates were mainly due to endogenous cereal phytase, since it is known that phytates decrease during the breadmaking process as a consequence of the activity of this enzyme (Haros et al., 2001). The addition of sourdough to the bread formula produced a significant decrease in the amount of InsP_6 . This reduction was greater when the amount of sourdough increased in the formulation, from 7.62 $\mu\text{mol/g}$ (control sample) to 1.45 $\mu\text{mol/g}$ (WDS-20). Leenhardt et al. (2005) reported that slight acidification of dough (pH 5.5) with sourdough containing *L. brevis* allowed a significant phytate breakdown, up to 70 % of the initial flour content compared to 40 % in the control sample. Sourdough fermentation with a multi-species starter including *L. plantarum* and *L. mesenteroides* was more efficient than yeast fermentation in reducing phytate content in whole wheat bread, reaching values around 25 % hydrolysis after 1 hour of fermentation (Lopez et al. 2001).

The acidified control, which was supplemented by the amount of acids (lactic and acetic acids) required to mimic the pH reached by sourdough fermented by bifidobacteria, showed an intermediate concentration of InsP_6 (Table 4). This indicated that the endogenous phytase was also activated by the reduction of pH during the fermentation period. As mentioned above, the addition of sourdough produced a decrease in pH from 5.38 (control dough) to 4.57 (WDS-20). A similar observation was obtained in the dough

with 20 % acid control sourdough, which reached a pH of 4.58. Given that endogenous phytase acts during the breadmaking process and its optimum pH is around 4.1-4.5, acidification of dough due to microbial metabolism could activate this enzyme (Leenhardt et al., 2005). However, hydrolysis of InsP_6 of samples containing 20 % sourdough inoculated with bifidobacteria (WDS-20) was significantly higher than samples with chemically acidified sourdough in the same percentage of addition. This suggests that the additional hydrolysis was due to phytase activity of *B. pseudocatenulatum*, which has already been studied in previous investigations (Haros et al., 2005, 2009; Sanz Penella et al., 2009).

The amount of lower *myo*-inositol phosphates showed a significant increase with the addition of sourdough inoculated with the *Bifidobacterium* strain, mainly in the amounts of InsP_4 and InsP_3 . The intake of breads with a higher amount of lower *myo*-inositol phosphate could have positive effects on human health by increasing the bioavailability of minerals or as a result of their bioactive functions in the body, especially InsP_3 (Shi et al., 2006; Haros et al., 2009). Although the *Bifidobacterium* strain showed phytase activity, cereal activity was the predominant activity compared to the microbial enzyme during the breadmaking process. The additional InsP_6 hydrolysis by *Bifidobacterium* during sourdough incubation and dough fermentation might change the *myo*-inositol phosphate profile in the final product (results not shown).

Thermal parameters of wheat starch in bread

The differential scanning calorimeter was used as an oven to bake the bread dough inside the capsules. This procedure allows determination of the thermal behaviour of wheat starch during the baking process using hermetic capsules. When the temperature of the fermented dough increased from 30 to 110 °C, the thermograms obtained from all the

samples showed two different endotherms. The first peak of the thermogram corresponds to the gelatinization process of the amorphous phase of the starch. It was observed between 67.3 °C and 80.8 °C, reaching enthalpy values from 0.45 to 0.57 J/g (control and WDS-20, respectively). The addition of sourdough produced a slight but significant decrease in onset temperature (67.4 °C) compared to the control dough (68.1°C), although there were no significant differences between treatments adding from 5 to 20 % of sourdough. With regard to the peak temperature (T_p), all samples remained constant with no significant differences. A similar observation was recorded in the conclusion temperature (T_c). Regarding the enthalpy of gelatinization, the addition of sourdough provided a slight increase, from 0.46 J/g to 0.57 J/g (control and WDS-20, respectively). This increase was significant compared to the control sample when 15-20 % sourdough was added to the dough (WSD-15 and WSD-20). Both samples had higher ΔH_g values and similar gelatinization temperatures compared with other samples, suggesting better starch hydration during the period of fermentation (Leon et al., 1997).

The effect of the addition of sourdough on the retrogradation kinetics during storage was analysed (Figure 2). During the first days of storage no significant differences between samples were found. After the seventh day there was a significant reduction in enthalpy with the increase in the amount of sourdough in the formulation (Figure 2). After 15 days of storage, retrogradation enthalpy achieved an asymptotic behaviour, reaching a value of 1.44 J/g (control sample), whereas the samples with sourdough showed significantly lower values (between 1.24 and 1.00 J/g). The phenomenon of retrogradation is closely related to the ageing of bread (Barcenas et al., 2003a), which depends on the formulation, among other factors. The inclusion of sourdough in the breadmaking process could delay ageing, which is related to the physical changes that take place in starch retrogradation (Barcenas et al., 2003b).

444

445 **CONCLUSIONS**

446 Sourdough inoculated with bifidobacteria could make possible the formulation of whole
447 wheat bread that allows an increase in phytate hydrolysis, enhancement of organic acid
448 levels that modify starch digestibility, and a delay/decrease in amylopectin
449 retrogradation, with high acceptance by consumers. *Bifidobacterium* strains are
450 GRAS/QPS microorganisms (Generally Regarded as Safe/Qualified Presumption of
451 Safety), do not significantly affect bread performance and increase its nutritional value,
452 and could therefore be used as starters in sourdough formulations, producing a quality
453 similar to the control sample.

454

455

456

REFERENCES

- Arendt EK, Ryan LAM & Dal Bello F (2007) Impact of sourdough on the texture of bread. *Food Microbiology*, 24, 165-174.
- Barber S, Báguena R, Benedito de Barber C & Martinez-Anaya MA (1991) Evolution of biochemical and rheological characteristics and breadmaking quality during a multistage wheat sour dough process. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*, 192, 46-52.
- Barcenas ME, Haros M, Benedito C & Rosell CM (2003a) Effect of freezing and frozen storage on the staling of part-baked bread. *Food Research International*, 36, 863-869.
- Barcenas ME, Haros M & Rosell CM (2003b) An approach to studying the effect of different bread improvers on the staling of pre-baked frozen bread. *European Food Research and Technology*, 218, 56-61.
- Collar C, Benedito de Barber C & Martinez-Anaya MA (1994) Microbial sourdoughs influence acidification properties and breadmaking potential of wheat dough. *Journal of Food Science*, 59, 629-633.
- De Angelis M, Damiano N, Rizzello CG, Cassone A, Di Cagno R & Gobbetti M (2009) Sourdough fermentation as a tool for the manufacture of low-glycemic index white wheat bread enriched in dietary fibre. *European Food Research and Technology*, 229, 593-601.
- Fretzdorff B & Brümmer JM (1992) Reduction of phytic acid during breadmaking of whole-meal breads. *Cereal Chemistry*, 69, 266-270.
- Gänzle MG, Vermeulen N & Vogel RF (2007) Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. *Food Microbiology*, 24, 128-138.

479 Hammes WP & Gänzle MG (1998) Sourdough breads and related products. In: Woods
 480 (ed) Microbiology of Fermented Foods, pp 199-216. Blackie Academic and Professional,
 481 London, UK.

482 Hammes WP, Brandt MJ, Francis KL, Rosenheim J, Seitter MFH & Vogelmann SA
 483 (2005) Microbial ecology of cereal fermentations. Trends in Food Science and
 484 Technology, 16, 4-11.

485 Haros M, Rosell CM & Benedito C (2001) Use of fungal phytase to improve
 486 breadmaking performance of whole wheat bread. Journal of Agricultural and Food
 487 Chemistry, 49, 5450-5454.

488 Haros M, Bielecka M & Sanz Y (2005) Phytase activity as a novel metabolic feature in
 489 Bifidobacterium. FEMS Microbiology Letters, 247, 231-239.

490 Haros M, Bielecka M, Honke J & Sanz Y (2007) *Myo*-inositol hexakisphosphate
 491 degradation by Bifidobacterium infantis. International Journal of Food Microbiology,
 492 117, 76-84.

493 Haros M, Carlsson N-G, Almgren A, Larsson Alminger M, Sandberg A-S & Andlid T
 494 (2009) Phytate degradation by human gut isolated Bifidobacterium pseudocatenulatum
 495 ATCC27919 and its probiotic potential. International Journal of Food Microbiology, 135,
 496 7-14.

497 Katina K, Arendt E, Liukkonen KH, Autio K, Flander L & Poutanen K (2005) Potential
 498 of sourdough for healthier cereal products. Trends in Food Science and Technology, 16,
 499 104-112.

500 Katina K, Maina NH, Juvonen R, Flander L, Johansson L, Virkki L, Tenkanen M &
 501 Laitila A (2009) *In situ* production and analysis of Weissella confusa dextran in wheat
 502 sourdough. Food Microbiology, 26, 734-743.

503 Konietzny U & Greiner R (2003) Encyclopedia of Food Science and Nutrition (2nd ed),
 504 pp 4555-4563. Elsevier, London, UK.

505 Leenhardt F, Levrat-Verny MA, Chanliaud E & Remesy C (2005) Moderate decrease of
 506 pH by sourdough fermentation is sufficient to reduce phytate content of whole wheat
 507 flour through endogenous phytase activity. Journal of Agricultural and Food Chemistry,
 508 53, 98-102.

509 Leon A, Duran E & Benedito de Barber C (1997) A new approach to study starch
 510 changes occurring in the dough-baking process and during bread storage. Zeitschrift für
 511 Lebensmittel-Untersuchung und -Forschung A - Food Research and Technology, 204,
 512 316-320.

513 Liljeberg H, Lönner C & Björck I (1995) Sourdough fermentation or addition of organic
 514 acids or corresponding salts to bread improves nutritional properties of starch in healthy
 515 humans. Journal of Nutrition, 125, 1503-1511.

516 Liljeberg H & Björck I (1998) Delayed gastric emptying rate may explain improved
 517 glycaemia in healthy subjects to a starchy meal with added vinegar. European Journal of
 518 Clinical Nutrition, 52, 368-371.

519 Lopez HW, Krespine V, Guy C, Messenger A, Demigne C & Remesy C (2001) Prolonged
 520 fermentation of whole wheat sourdough reduces phytate level and increases soluble
 521 magnesium. Journal of Agricultural and Food Chemistry, 49, 2657-2662.

522 Lopez HW, Leenhardt F, Coudray C & Remesy C (2002) Minerals and phytic acid
 523 interactions: is it a real problem for human nutrition? International Journal of Food
 524 Science and Technology, 37, 727-739.

525 Mellen P, Walsh T & Herrington D (2008) Whole grain intake and cardiovascular
526 disease: a meta-analysis. *Nutrition Metabolism and Cardiovascular Diseases*, 18, 283-
527 290.

528 Nielsen MM, Damstrup ML, Dal Thomsen A, Rasmussen SK & Hansen A (2007)
529 Phytase activity and degradation of phytic acid during rye bread making. *European Food*
530 *Research and Technology*, 225, 173-181.

531 Palacios MC, Sanz Y, Haros M & Rosell CM (2006) Application of *Bifidobacterium*
532 strains to the breadmaking process. *Process Biochemistry*, 41, 2434-2440.

533 Palacios MC, Haros M, Sanz Y & Rosell CM (2008) Selection of phytate-degrading
534 human bifidobacteria and application as starter cultures in whole-wheat dough
535 fermentation. *Food Microbiology*, 25, 169-176.

536 Pereira M, Jacobs D, Pins J, Raatz S, Gross M, Slavin J & Seaquist E (2002) Effect of
537 whole grains on insulin sensitivity in overweight hyperinsulinemic adults. *American*
538 *Journal of Clinical Nutrition*, 75, 848–855.

539 Poutanen K, Flander L & Katina K (2009) Sourdough and cereal fermentation in a
540 nutritional perspective. *Food Microbiology*, 26, 693-699.

541 Reale A, Mannina L, Tremonte P, Sobolev AP, Succi M, Sorrentino E & Coppola R
542 (2004) Phytate degradation by Lactic Acid Bacteria and Yeasts during the wholemeal
543 dough fermentation: A P-31 NMR study. *Journal of Agricultural and Food Chemistry*,
544 52, 6300-6305.

545 Robert H, Gabriel V, Lefebvre D, Rabier P, Vayssier Y & Fontagne-Faucher C (2006)
546 Study of the behaviour of *Lactobacillus plantarum* and *Leuconostoc* starters during a
547 complete wheat sourdough breadmaking process. *LWT-Food Science and Technology*,
548 39, 256-265.

549 Röcken W (1996) Applied aspects of sourdough fermentation. *Advances in Food*
550 *Science*, 18, 212-216.

551 Rollan G, De Angelis M, Gobbetti M & De Valdez GF (2005) Proteolytic activity and
552 reduction of gliadin-like fractions by sourdough lactobacilli. *Journal of Applied*
553 *Microbiology*, 99, 1495-1502.

554 Rosell CM, Santos E, Sanz Penella JM & Haros M (2009) Wholemeal wheat bread: A
555 comparison of different breadmaking processes and fungal phytase addition. *Journal of*
556 *Cereal Science*, 50, 272-277.

557 Sanz Penella JM, Collar C & Haros M (2008) Effect of wheat bran and enzyme addition
558 on dough functional performance and phytic acid levels in bread. *Journal of Cereal*
559 *Science*, 48, 715-721.

560 Sanz Penella JM, Tamayo Ramos JA, Sanz Y & Haros M (2009) Phytate reduction in
561 bran-enriched bread by phytase-producing *Bifidobacteria*. *Journal of Agricultural and*
562 *Food Chemistry*, 57, 10239-10244.

563 Sanz-Penella JM, Wronkowska M, Soral-Smietana M, Collar C & Haros M (2010)
564 Impact of the addition of resistant starch from modified pea starch on dough and bread
565 performance. *European Food Research and Technology*, 231, 499-508.

566 Shi Y, Azab AN, Thompson MN & Greenberg ML (2006) Inositol phosphates and
567 phosphoinositides in health and disease. In: Majumder & Biswas (ed) *Biology of*
568 *Inositols and Phosphoinositides*, pp 265-292. Springer Press, Subcellular Biochemistry.

569 Slavin J (2004) Whole grains and human health. *Nutrition Research Reviews*, 17, 99-
570 110.

571 Spicher G (1983) Baked goods. In: Rehm & Reed (ed) *Biotechnology. Food and feed*
572 *production with microorganisms*, pp 1-115. Verlag Chemie, Weinheim, Germany.

573 Thiele C, Gänzle MG & Vogel RF (2002) Contribution of sourdough lactobacilli, yeast
574 and cereal enzymes to the generation of amino acids in dough relevant for bread flavour.
575 Cereal Chemistry, 79, 45-51.

576 Türk M & Sandberg AS (1992) Phytate degradation during breadmaking - effect of
577 phytase addition. Journal of Cereal Science, 15, 281-294.

578 Van der Meulen R, Adriany T, Verbrugghe K & De Vuyst L (2006) Kinetic analysis of
579 bifidobacterial metabolism reveals a minor role for succinic acid in the regeneration of
580 NAD⁺ through its growth-associated production. Applied and Environmental
581 Microbiology, 72, 5204-5210.

582

LEGENDS

Figure 1. Aspect of the loaf, central slice and crumb image of bread. Bread formulation WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough inoculated with bifidobacteria, respectively.

Figure 2. Effect of sourdough addition on the amylopectin retrogradation during baked dough aging. Bread formulation WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough inoculated with bifidobacteria, respectively.

Table 1. Values of the total titratable acidity (TTA) and concentration of organic acids of sourdough, fermented dough and whole wheat bread^a

Table 2. Technological parameters and crumb structure of whole wheat bread^a

Table 3. Effect of addition of sourdough inoculated with bifidobacteria in crust and crumb colour of whole wheat bread^{ab}

Table 4. Effect of sourdough addition on *myo*-inositol phosphates content in whole wheat bread^a

606 **Table 1.**

Sample	pH	TTA mL 0.1N NaOH	D-Lactic acid $\mu\text{mol/g}$	L-Lactic acid $\mu\text{mol/g}$	Acetic acid $\mu\text{mol/g}$
Sourdough^b					
Sourdough	4.17±0.01	17.24±1.77	n.d.	25.23±2.16	68.16±1.16
Dough^c					
Control	5.38±0.04a	4.60±0.05a	0.96±0.10a	1.83±0.87a	2.51±0.05a
WDS-5	5.15±0.16b	5.91±0.55b	0.73±0.03b	4.26±0.15b	10.66±1.07b
WDS-10	4.79±0.35c	7.62±0.99c	0.47±0.08c	6.12±0.43b	14.74±1.55c
WDS-15	4.76±0.16c	8.21±0.63cd	0.30±0.11c	8.84±0.86c	19.97±0.87cd
WDS-20	4,57±0.11c	9.23±0.32d	0.05±0.06d	10.55±0.54d	23.53±0.24d
Bread^b					
Control	5,72±0.06a	4.19±0.16a	0.57±0.10 a	1.46±0.19a	2.23±0.87a
WDS-5	5,50±0.07ab	5.74±0.14b	0.39±0.18 a	7.88±1.11b	8.61±0.43b
WDS-10	5,17±0.32bc	7.59±0.31c	0.18±0.08 a	9.78±1.60b	8.75±0.79b
WDS-15	5,12±0.12c	9.21±0.16d	n.d.	13.63±0.32c	9.39±1.60b
WDS-20	4,96±0.06c	10.60±0.41e	n.d.	15.35±1.22c	11.93±1.40b

607 ^an.d.: not detected, TTA: Total titratable acidity; Bread formulations WDS-5, WDS-10, WDS-15 and

608 WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough inoculated with bifidobacteria, respectively.

609 Mean, ^bn=8; ^cn=4; values followed by the same letter in the same column are not significantly different at

610 95% confidence level. Statistical analysis of the different categories was performed separately.

611 **Table 2.**

Sample	Specific volume ^b mL/g	Width/height ratio ^b cm/cm	Hardness ^b N	Cell area/ Total area ^c , cm ² /cm ²	Wall area/ Total area ^c , cm ² /cm ²	Cells ^b /cm ²	Mean cell area ^c , mm ²
Control	2.46±0.13a	1.80±0.10a	2.61±0.31a	0.397±0.099a	0.613±0.099a	299±101a	1.10±0.20ab
WDS-5	2.31±0.13ab	1.74±0.06a	2.51±0.29a	0.351±0.059a	0.649±0.059a	282±37a	1.03±0.18ab
WDS-10	2.38±0.09ab	1.89±0.12a	2.67±0.13ab	0.393±0.030a	0.607±0.030a	264±55a	1.32±0.26a
WDS-15	2.35±0.05ab	1.67±0.29a	3.09±0.30bc	0.343±0.050a	0.657±0.050a	302±52a	0.95±0.17b
WDS-20	2.22±0.12b	1.68±0.24a	3.18±0.25c	0.350±0.053a	0.650±0.053a	274±51a	1.20±0.24ab

612 ^aBread formulations WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough inoculated with bifidobacteria,
613 respectively.

614 Mean, ^an=6; ^bn=12; values followed by the same letter in the same column are not significantly different at 95% confidence level

615 **Table 3.**

Sample	Crust			Crumb		
	<i>L</i> *	<i>C</i> *	<i>h</i> *	<i>L</i> *	<i>C</i> *	<i>h</i> *
Control	49.2±4.0ab	32.0±2.4ab	64.3±2.9abc	55.5±1.9a	20.5±0.9a	77.7±2.0a
WDS-5	50.0±2.0a	33.0±1.4a	65.3±2.1a	58.4±1.4b	21.0±1.0bc	77.5±1.5a
WDS-10	48.2±3.8ab	33.1±2.9a	64.6±3.1ab	55.6±1.9a	20.7±0.6ab	76.5±1.4b
WDS-15	46.9±5.1ab	31.9±2.6ab	62.7±4.2bc	57.8±1.3b	21.2±0.7c	77.1±1.1ab
WDS-20	46.3±3.4b	31.4±2.1b	62.3±2.8c	57.8±1.7b	21.2±0.9c	77.6±1.2a

616 ^aBread formulations WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough
617 inoculated with bifidobacteria, respectively.

618 ^bMean, n=18; values followed by the same letter in the same column are not significantly different at 95%
619 confidence level

620

621**Table 4.**

Sample	%	<i>myo</i> -inositol phosphates ^{cd}				
		Hydrolysis				
		$\mu\text{mol/g}$ of bread d.m.				
		InsP ₆	InsP ₅	InsP ₄	InsP ₃	InsP ₆ + InsP ₅
Control	22.2±5.9a	7.62±0.58a	1.25±0.04a	0.80±0.08a	0.55±0.04a	8.86±0.61a
WDS-5	43.7±3.2b	5.51±0.31b	1.45±0.14ab	1.76±0.13b	1.05±0.13b	6.96±0.18b
WDS-10	63.4±3.8c	3.58±0.37c	1.32±0.05a	2.43±0.34c	1.21±0.14b	4.91±0.42c
WDS-15	75.0±2.9d	2.44±0.28d	1.75±0.13c	3.23±0.40de	1.25±0.23b	4.19±0.39d
WDS-20	85.2±3.7e	1.45±0.36e	1.73±0.31bc	3.07±0.48d	1.25±0.19b	3.17±0.67e
WDAcCS-20	79.8±0.7d	1.98±0.07d	2.02±0.18d	3.71±0.11e	1.54±0.04c	4.00±0.23d

622Bread formulations WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough
623inoculated with bifidobacteria, respectively. WDAcCS-20: wheat dough with 20% acid control sourdough with
624antibiotics.

625^bMean, n=4; values followed by the same letter in the same column are not significantly different at 95% confidence
626level

627^cInsP₃ to InsP₆: *myo*-inositol phosphate containing 3-6 phosphates per inositol residue

628^dd.m.: dry matter

629

630

Figure 1.

Figure 2.