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Effect of selected strains of lactobacilli on the antioxidant and anti-inflammatory properties of sourdough

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ABSTRACT

Sourdough fermentation of cereal foods is an excellent source of obtaining peptides due to the ability of lactic acid bacteria to activate cereal proteases and produce strain-specific peptidases. With the aim of identifying the lactic acid bacterial strains potentially most effective in producing bioactive peptides, 131 lactobacilli isolates from Italian sourdoughs, used in baking technology, have been screened for proteolytic and peptidase activity. Of these, 23 strains were selected and singly inoculated in liquid sourdoughs from which a Low Molecular Weight fraction containing peptides was obtained. Evaluation of the antioxidant and anti-inflammatory activities of the extracts was performed on cultured cells (RAW 264.7 murine macrophage, murine H-end endothelium cells and Human intestinal Caco-2 cells) by assaying Reactive Oxygen Species (ROS) content, NFkB/IkB expression level and Interleukin-1β production. As a result, three lactobacilli strains showed a high antioxidant and anti-inflammatory ability enabling the development of model sourdoughs that will potentially increase the nutritional benefits of bread.

1. Introduction

The sourdough process is one of the oldest biotechnological processes in the leavening of cereal products (De Vuyst and Vancanneyt, 2007). This technique is widespread in Italy, where several traditional sweet leavened baked goods (e.g. Panettone, Colomba, Lagaccio etc) and typical breads (e.g. Altamura, Tuscan and Dittaino breads) are manufactured according to this procedure (Giannone et al., 2018; Palla et al., 2017; Pasqualone et al., 2010; Raimondi et al., 2017; Venturi et al., 2012). Recently, surveys in new fermentation technologies and starter cultures with defined metabolic properties have been aimed at increasing the health benefits of breads (Gobbetti et al., 2005, 2007; Koistinen et al., 2018; Rizzello et al., 2017). The sourdough fermentation may not only influence the features of leavened baked goods but can also have positive effects on human health (Gänzle, 2014; Gobbetti et al., 2014). A recent study demonstrates the ability of sourdough lactic acid bacteria to release antioxidant peptides through the proteolysis of native cereal proteins (Coda et al., 2012). The degradation of native cereal proteins during sourdough fermentation is dependent on cereal enzymes (primary proteolysis) and bacterial metabolic activity (secondary proteolysis) (Meisel, 2005; Zhao et al., 2016). Acidification by lactic acid bacteria reduces the disulfide bonds of gluten increasing the solubility of gluten proteins and consequently their susceptibility to enzymatic degradation. This acidification capability of lactic acid bacteria shifts the ambient pH to the optimum pH of aspartic proteases, the major proteinase in dormant grains of wheat and rye (Brijs et al., 1999), promoting the primary activity of cereal proteases and thus the liberation of various sized peptides. Peptidases of sourdough lactic acid bacteria (secondary proteolysis) complete proteolysis liberating free amino acids, which, in turn, are subjected to various catabolic reactions by the same microorganisms (Gänzle et al., 2008; Gänzle, 2014). The proteolytic and peptidase activities of LAB sourdough are known to be strain dependent properties (Gerez et al., 2008), therefore a unique microbial strain frequently does not have all the necessary enzymes for the degradation of cereal proteins (Coda et al., 2012). Thus, the selection of specific lactic acid bacteria strains possessing different

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enzymatic activities could be a suitable tool to obtain sourdoughs that contain the various bioactive peptides responsible for different biological roles on human health (Leroy and De Vuyst, 2004). These peptides, which generally increase during food fermentation by lactic acid bacteria (Korhonen and Pihlanto, 2007), are fragments of native proteins with cryptic amino acid sequences that depending on their amino acid sequence may have a positive impact on human health (Kitts and Weiler, 2003; Zou et al., 2016), affecting the cardiovascular, digestive, immune and nervous systems. (Korhonen and Pihlanto, 2007; Li and Yu, 2015). Recent evidence suggests that bioactive peptides can have antimicrobial, antiproliferative and antioxidant properties (Elias et al., 2008; Malaguti et al., 2014; Meisel, 2005). Although numerous degenerative aging diseases can have different etiologies, they are characterized by abnormalities in inflammatory response and oxidative stress, thus enhancing interest in antioxidant peptides acting as inhibitors of lipid peroxidation, as direct scavengers of free radicals, and/or as agents to chelate transition metal ions that catalyze the generation of radical species (Sarmadi and Ismail, 2010). Antioxidant peptides are usually constituted of 2 to 20 amino acidic residues and have molecular masses of < 6.0 kDa. The antioxidant activity of peptides seems to be strongly correlated with amino acid composition, conformation and hydrophobicity (Zou et al., 2016). In addition to the antioxidant properties, bioactive peptides can also modulate inflammatory processes (Malaguti et al., 2014). Lunasin from soybean is the most widely studied peptide and can prevent the activation of the transcription factor NF-kB and inhibit expression of pro-inflammatory biomarkers such as interleukin-6, cyclooxygenase-2, and inducible nitric oxide synthase (Hernández-Ledesma et al., 2013; Wan et al., 2017; Hsieh et al., 2018). The anti-inflammatory properties of other bioactive peptides from fermented foods are currently under investigation and information about the role of peptides different from Lunasin in inflammation is still incomplete and mainly deals with the bioactive peptides from milk (Chakrabarti et al., 2014; Brown et al., 2017). Of particular interest are some identified Lunasin-like peptides that are recently recovered from soybean, amaranth, wheat flours and legumes (Rizzello et al., 2015). Their concentration increases after fermentation by lactic acid bacteria and, therefore, these bacteria are recognized as the most useful microorganisms in bioactive peptides enrichment in fermented foods (Rizzello et al., 2015, 2016). In the light of these studies, the interest for the selection of lactic acid bacteria strains to be used as starter cultures for the manufacture of healthier leavened baked goods is increasing (Rizzello et al., 2017). The interest is of particular relevance in Italy where several traditional and typical breads, three of which are awarded with Protected Designation of origin, are produced by using sourdoughs (Giannone et al., 2018; Palla et al., 2017; Pasqualone et al., 2010).

The aim of this study was to investigate, by in vitro and ex-vivo assays, the potential antioxidant and anti-inflammatory properties of sourdoughs made with lactic acid bacteria belonging to different species and possessing different proteolytic and peptidase activities. These strains originated from sourdoughs commonly used in the manufacture of various Italian baked goods. Although synthetic antioxidants are more effective, natural antioxidants have a simpler structure, higher stability and a non-hazardous immune reaction (Sarmadi and Ismail, 2010). The selection of bacteria strains able to produce bioactive peptides represents an important step in creating a bread possessing enhanced health properties.

2. Materials and methods

2.1. Lactic acid bacteria and culture conditions

Several lactic acid bacteria (LAB) isolates (131) from the culture collection of the Department of Agricultural, Food and Forestry Systems of the University of Florence (Italy) were used in this study (Table 1). LAB belonging to five *Lactobacillus* species (*L. brevis*, *L. farciminis*, *L. plantarum*, *L. rossiae* and *L. sanfranciscensis*) were isolated from thirteen Italian wheat sourdoughs (A, B, C, D, E, F, G, H, I, L, M, N, O) used to produce various baked goods such as Colomba, Panettone, Lagaccio and specific breads. The isolates were routinely propagated for 24 h at 30 °C in MR3i, a medium containing (in g/L): maltose 20, glucose 6, fructose 6, polypeptone 10, meat extract 5, yeast extract 12, sodium gluconate 2, sodium acetate trihydrate 5, ammonium citrate trihydrate 2, di-potassium hydrogen phosphate 2, magnesium sulfate heptahydrate 0.2, manganese sulfate tetrahydrate 0.05, Cysteine-HCl 0.5, vitamins mix 1 mL, Tween 80 1 mL, Fresh Yeast Extract 15 mL, at a final pH of 5.6.

2.2. Proteolytic activity of lactic acid bacteria

LAB isolates were grown overnight at 30 °C in the MR3i medium before carrying out the assays. Bacterial cell counts were performed by the Thoma counting chamber to inoculate 10^9 cell/mL into Gluten Maltose Broth (GMB), a medium containing gluten as sole nitrogen source as described by Pepe et al. (2003). Initially, and again after 24 h, GMB cultures were centrifuged at 10,000 × *g* for 10 min and supernatants were used to determine hydrolyzed gluten proteins using Bradford spectrophotometric assay (SigmaAldrich, St. Louis, MO, USA). A calibration curve was prepared using BSA (Sigma Aldrich, St. Louis, MO, USA) as standard, at concentrations ranging from 0 to 200 mg/L. The absorbance increase was measured using uninoculated GMB as control and proteolytic activity of each LAB culture was expressed as BSA mg/L.

2.3. Peptidase activity of lactic acid bacteria

Stationary phase cells grown overnight in MR3i broth and counted by the Thoma chamber to obtain a cell suspension of 10^9 cell/mL concentration were harvested by centrifugation at 12,000 × *g* for 5 min, washed twice with sterile 50 mM potassium phosphate buffer, pH 7.0, and re-suspended in the same buffer. Two general aminopeptidase (PepNC), proline iminopeptidase (PepI), glutamyl aminopeptidase (PepA) and X-prolyl dipeptidyl aminopeptidase (PepX) activities were measured according to Macedo et al. (2000) using Lys-p-nitroanilide (pNA), Leu-p-NA, Pro-p-NA, Glu-p-NA and Gly-Pro-p-NA (Sigma Aldrich, St. Louis, MO, USA), respectively, as synthetic substrates. The assay mixture contained 30 µL of a 20 mM synthetic substrate methanol solution; 195 µL of 50 mM potassium phosphate buffer, pH 7.0; 95 µL of 0.05% (w/v) sodium azide solution; and 75 µL of cell suspension. After incubation at 30 °C for 4 h the reaction was stopped by addition of 900 µL of 10% (v/v) acetic acid. The release of p-nitroanilide (p-NA) was measured spectrophotometrically at 410 nm after centrifugation of the reaction mixture at 12,000 × *g* for 5 min. In order to determine the peptidase activity, the data obtained were compared to a calibration curve prepared using p-NA at concentrations in the range 0.1–20.0 mM.

2.4. Sourdough fermentation and water-soluble extracts

Cultures of selected LAB strains, grown overnight in MR3i broth, were inoculated at a concentration of 10^9 UFC/mL into model doughs. As reported by Coda et al. (2012), the liquid doughs were prepared by mixing water and wheat flour (type “00”) with a sterile spatula for 3 min to obtain a dough yield [DY = (mass of dough/mass of flour) × 100], of 333. Non-inoculated neutral control (C) and acid control (AC) doughs containing erythromycin 0.05 mg/g, and at pH 6.0 and 3.5, respectively, were obtained and incubated under the same conditions.

According to the commonly used backslopping conditions, sourdoughs were sampled after 6 h and 24 h of incubation at 30 °C under stirring conditions (approx. 100 rpm). The water-soluble extracts (WSE) of dough samples were obtained by extracting doughs with sterile water (1:3 w/v) and centrifugation at 14,000 × *g* for 20 min at 4 °C (Coda et al., 2012). Additionally, RAPD PCR analysis was performed