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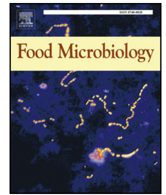

Metabolism of phenolic acids in whole wheat and rye malt sourdoughs

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ABSTRACT

This work aimed to study the phenolic acid metabolism of sourdough lactic acid bacteria (LAB) in laboratory media, and in sourdough fermentation with single cultures and in co-fermentations. Lactobacilli were selected from isolates obtained from 35 sourdough samples. Isolates (114 strains) were screened for phenolic acid decarboxylase gene *pdc* and EPS production. Ferulic acid metabolism of the 18 *pdc* positive strains was evaluated in mMRS; all *pcd* positive strains converted ferulic acid by decarboxylation and/or reduction. Single whole wheat and rye malt dough fermentation fermented with lactobacilli or yeasts were characterized with respect to free, conjugated, or bound phenolic acids. Concentrations of free, conjugated, or bound phenolic acids were not altered substantially in chemically acidified sourdoughs, or in yeast fermented doughs. *L. plantarum* metabolized free ferulic acid in wheat and rye malt sourdoughs; *L. hammesii* DSM 16381 metabolized syringic and vanillic acids and reduced levels of bound ferulic acid in wheat sourdoughs. Co-fermentation of *L. hammesii* and *L. plantarum* achieved release of bound ferulic acid and conversion of the resultant free ferulic acid to dihydroferulic acid and volatile metabolites. Phenolic acid metabolism in sourdoughs was enhanced by co-fermentation with strains exhibiting complementary metabolic activities. Results may enable improvement of bread quality by targeted conversion of phenolic acids during sourdough fermentation.

1. Introduction

Phenolic compounds are secondary metabolites in plants that provide protection against pathogens and ultraviolet radiation (Beckmann, 2000). Phenolic compounds have been considered nutritionally undesirable because some phenolic compounds precipitate proteins, inhibit digestive enzymes and thus inhibit nutrient absorption (McSweeney et al., 2001). A reduced rate of nutrient absorption, however, also reduces the glycemic index of foods and can be considered health-beneficial (Ding et al., 2013; Chung et al., 1998). In particular, dietary phenolic acids have antidiabetic effects (Vinayagam et al., 2015). The beneficial effects of phenolic compounds thus depend on their quantity and bioavailability (Chung et al., 1998; Lodovici et al., 2001), and on the nutritional status of the consumer.

Phenolic acids are the major class of phenolic compounds in cereals (Shahidi and Naczk, 2000; Shewry et al., 2010). Wheat and rye contain 0.5–1 g/kg phenolic acids; these predominantly occur in conjugated form (0.1–0.2 g/kg), or bound to cell wall polysaccharides 0.4–0.9 g/kg) with ferulic acid typically accounting for more than 50% of total phenolic acids (Li et al., 2008; Shewry et al., 2010). Cross-linking of cell wall polysaccharides and proteins by phenolic acids influences the bread-making quality of wheat and rye flours. The solubilization of arabinoxylans during fermentation improves the water binding capacity and the baking quality of rye flour and, to a lesser extent, of wheat flour (Gänzle, 2014). Moreover, release of bound phenolic acids increases their bioavailability (Gänzle, 2014; Katina et al., 2012). Moreover, microbial conversion of phenolic acids generates volatile phenolic compounds (Rodríguez et al., 2009) which impact bread flavor (Czerny and Schieberle, 2002).

Phenolic acids in wheat and rye include hydroxycinnamic acids (C6−C3 compounds) and hydroxybenzoic acids (C6−C1 compounds). Both classes of compounds have antibacterial activity (Sànchez-Maldonado et al., 2011). Lactic acid bacteria have a high tolerance to antimicrobial phenolic acids; their resistance is partially dependent on their capacity to convert phenolic acids to metabolites with reduced metabolic activity (Sànchez-Maldonado et al., 2011). Hydroxy-benzoic acids are metabolized by decarboxylation to volatile phenolic compounds (for review, see Rodríguez et al., 2009; Gänzle, 2014). Hydroxybenzoic acids are metabolized by decarboxylation to the corresponding vinyl-derivatives, by reduction of the double bond in the C3 side chain, or by sequential activity of both enzymes (Rodríguez et al., 2009). An example, ferulic acid is reduced to dihydroferulic acid, decarboxylated

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to 4-vinyl-2-methoxyphenol (vinyl-guaiacol), or decarboxylated and reduced to 4-ethyl-2-methoxyphenol (ethyl-guaiacol, Beek and Priest, 2000). Specific lactobacilli also hydrolyse esters of phenolic acids by ferulic acid esterase activity (Hole et al., 2012).

Studies on metabolism of phenolic compounds were conducted mainly with *L. plantarum*. *L. plantarum* occurs in intestinal ecosystems and in insects, in association with plants, and in many food fermentations (Martino et al., 2016). The origin of strains of *L. plantarum* is unrelated to either the phylogenetic position or the metabolic potential, demonstrating that strains of *L. plantarum* frequently transition from one niche to another (Duar et al., 2017; Martino et al., 2016). This lifestyle has been termed "nomadic" and is associated with a relatively large genome size, corresponding to a broad metabolic diversity (Duar et al., 2017). *L. plantarum* frequently occurs in fermentation of plant foods rich in phenolic compounds including table olives, sauerkraut and cucumbers (Ruiz-Barba and Jimenez-Diaz, 1994; Plengvidhya et al., 2007; Costilow et al., 1956). The phenolic acid decarboxylase Pdc/PadA of *L. plantarum* decarboxylates hydroxycinnamic acids including p-coumaric and caffeic acids (Cavin et al., 1997). Mutational disruption of *pdc* in *L. plantarum* revealed the presence of a second, uncharacterized phenolic acid decarboxylase which is induced by ferulic acid (Barthelmebs et al., 2000). *L. plantarum* expresses phenolic acid and vinylphenol reductases as an alternative pathway for metabolism of hydroxycimmanic acids (Santamaría et al., 2018a, 2018b). In addition, hydroxybenzoic acid decarboxylases exist in *L. plantarum* and some other lactobacilli (De Las Rivas et al., 2009; Filannino et al., 2015).

The conversion of phenolic acids in sourdough affects nutritional and technological properties of bread (Gänzle, 2014), however, data on conversion of phenolic acids in rye is limited to simulated rye doughs without microbial activity (Boskov Hansen et al., 2002) or yeast-fermented rye dough with uncharacterized bacterial microbiota (Katina et al., 2012). This study therefore aimed to assess conversion of phenolic acids in wheat and rye sourdoughs. Lactic acid bacteria were screened for genes coding for phenolic acid decarboxylases (*pdc*) and phenolic acid conversion by *pdc*-positive isolates was verified by metabolite analysis. Selected isolates and two reference strains with well-characterized metabolism of phenolic acids (Sànchez-Maldonado et al., 2011) were studied with respect to their impact on phenolic acid compounds in whole wheat and rye malt sourdoughs. Free, conjugated, and bound phenolic acids were quantified by LC-MS/MS (Li et al., 2008).

2. Materials and methods

2.1. Strains and growth conditions

The strains analysed in this study were isolated from Italian sourdoughs; *Lactobacillus plantarum* TMW 1460 and *Lactobacillus hammesii* DSM 16381 were used as reference strains known to metabolise phenolic acids (Sànchez-Maldonado et al., 2011; Valcheva et al., 2005). *Candida humilis* FUA4001 and *S. cerevisiae* FA1 represent sourdoughs isolates obtained previously (Ripari et al., 2016). Yeasts and lactic acid bacteria were cultivated in modified de Man, Rogosa Sharpe medium (mMRS, Gänzle et al., 1998) at 30 °C.

2.2. Isolation and identification of LABs

Lactic acid bacteria and yeasts were isolated from Italian sourdoughs as described (Ripari et al., 2016). Sourdough samples were diluted in peptone water and appropriate dilutions were plated on mMRS. At least ten colonies with different morphologies were purified and maintained at −80 °C with glycerol as cryoprotectant. DNA was isolated from LAB using the DNeasy Blood & Tissue kit (Qiagen, Toronto, Canada) with the automated extractor QIAcube (Qiagen). Isolates from sourdough were analysed by RAPD–PCR using M13-5′- GAGGGTGGC GGTTCT-3′ (Huey and Hall, 1989) to eliminate clonal isolates. Bacterial isolates with different RAPD profile were identified by sequencing after PCR amplification of genes coding for 16S rRNA, using primers P0 (GAGAGTTTGATCCTGGCTCAG) and P6 (CTACGGCTACCTTGTTACGA) (Picard et al., 2000).

2.3. EPS production

Each strain was analysed for EPS production on agar plates. Strains are transferred on mMRS agar containing 5% of sucrose. Plates were incubated at 30 °C for 4–5 days. EPS formation was assessed visually and by assessing colonies with a sterile toothpick.

2.4. Molecular screening, amplification of pcd

The *pdc* gene encoding the p-coumaric acid decarboxylase was amplified by PCR using degenerative primers 49 (5′- GANAAYGGNTGGGARTAYGA) targeting the Pdc sequence (D/E)NGWEYE, and primer 50 (5′-GGRTANGTNGCRTAYTTYT) targeting EKY(A/E)TYP, [R = G or A; Y = G, C, or A; and N = G, A, C, or T]. These degenerate primers were based on well-conserved domains approximately 100 amino acids apart of the PDC proteins (De Las Rivas et al., 2009). PCR reactions were performed in a total volume of 25 µL containing 2 µL of template DNA (approximately 10 ng), 1x buffer, 2.5–2 mM MgCl2, 200 µM of each dNTP, 1 U of AmpliTaq DNA polymerase, and 1–0.8 µM of each primer. The reactions were performed using the following cycling parameters: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50–60 °C for 1min, and extension at 72 °C for 30s. For the final extension, 7 min at 72 °C. At the end of the amplification the result was observed through electrophoresis in 2% agarose gel. The size of the bands was estimated by comparison with a marker (1 Kb plus DNA ladder GeneRuler). The expected size of the amplicon was 321 bp.

2.5. Fermentation in synthetic medium and phenolic acids extraction

Metabolism of ferulic acid was investigated using the protocol described in Sànchez-Maldonado et al. (2011) with modifications. Each strain positive for the presence of *pdc*, was inoculated in 10 ml of mMRS broth. After incubation for 24 h at 30 °C, 1 ml of this suspension was then added to another 10 ml of mMRS and incubated for 18 h at 30 °C. Standards of all compounds that were quantified were obtained from SigmaAldrich (Mississauga, ON, Canada) and dissolved in a solution of 50% methanol and 50% buffer, sterilized by filtration. Ferulic acid concentrations are below the detection limit in standard mMRS; the final concentration of phenolic acids in mMRS was 1 mmol/L mMRS supplemented with ferulic acid was inoculated with each preculture and incubated for 24 h at 30 °C. Sterile media were used as control.

The extraction of ferulic acid and its metabolites was performed on the supernatant, obtained by centrifugation of tubes at 8000 x*g* for 10 min. To achieve pH 1.5, 80 µl of HCl 25% v/v in water were added. After addition of ethyl acetate 500 µl, the solution was mixed for one minute every 10 min for a total of 30 min. Following, centrifugation 8000 x*g* for 5 min. The extraction was repeated with another 500 µl of ethyl acetate. The supernatants collected were placed in screwcap vials for UPLC analysis after filtration with a 0.2 µm.

2.6. Model sourdoughs

Model sourdoughs were prepared in triplicate independent fermentations by mixing 10 g of whole white flour or rye malt flour, with 10 ml of sterile tap water in which the cultured cells were resuspended. The initial bacteria count was 1×10^8 CFU/g, for yeasts it was 1×10^6 CFU/g.

In case of co-fermentation with two strains, both strains were added to achieve approximately 1×10^8 CFU/g for each strain. The sourdoughs were placed in sterile tubes, and incubated at 30 °C for 24 h.

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