Food Microbiology 77 (2019) 43–51

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Metabolism of phenolic acids in whole wheat and rye malt sourdoughs

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ARTICLE INFO

Keywords:
Phenolic acids
Ferulic acid
Phenolic acid decarboxylase
Sourdough
Pdc gene
Lactobacillus

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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https://doi.org/10.1016/j.fm.2018.08.009
Received 14 April 2018; Received in revised form 31 July 2018; Accepted 21 August 2018
Available online 21 August 2018

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′- GANAAYGGNT-GGGARTAYGA) 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.

Doughs fermented with yeast only were acidified to a pH of 4 or less by addition of a solution of lactic acid and acetic acid in a molar ratio of 4:1; acidification was carried out to prevent bacterial growth. For both types of flour, acidified controls acidified with lactic and acetic acid were incubated at 24 °C under the same conditions as sourdoughs to account for the activity of flour enzymes. Sourdoughs were freeze dried for analysis of phenolic acids; the pH, viable cell counts, and organic acids and monosaccharides were analysed with fresh sourdoughs.

2.7. Determination of pH and cell counts

After 24 h 1 g of sourdough was added to 9 ml of 18 MΩ water for pH analysis; subsequent tenfold dilutions of this suspension were prepared with 0.1% peptone to assess viable cell counts by surface plating of appropriate dilutions on mMRS agar, followed by incubation at 30 °C for 3 days. Plates were visually examined for a uniform colony morphology matching the inoculum to exclude bacterial contamination.

2.8. Organic acid and monosaccharides extraction and determination using HPLC analysis

After 24 h of fermentation at 30 °C, 200–500 mg of dough were mixed with an equivalent volume (0.2–0.5 mL) of 7% percloric acid, and incubated at 4 °C overnight to precipitate proteins. After centrifugation at 10,000 × g for 5 min, the supernatant was collected for analysis on a Aminex HP87X column linked to RI and UV–Vis detectors. Mobile phase was 5 mM sulfuric acid (5%) in HPLC water, using 70 °C as temperature and 0.4 ml/min as flow rate (Galle et al., 2010). The organic acids, monosaccharides and ethanol were quantified by comparison with calibration curves made with the respective standard having a coefficient of correlation ≥0.98. Results are shown as average of triplicate ± standard deviation.

2.9. Extraction of free, conjugated + free and bound phenolic acids from sourdoughs

Extraction of free, conjugated + free and bound phenolic acids was based on the protocol established by Li et al. (2008) with some modifications (Fig. 1). After addition of 1 mL 80% ethanol to 0.25 g of sample, samples were sonicated in a sonicator bath for 10 min and solids were removed by centrifugation at 8000 × g for 5 min at 4 °C. The extraction was performed for three independent fermentations for each condition.

To quantify free phenolic acids, phenolic acids were obtained by two consecutive extractions of 250 mg freeze dried sourdough with ethanol/water (1 ml of an 80:20 v/v mixture), followed by evaporation of the solvent under N_2 at 40 °C. Samples were re-dissolved in 500 μL of 2% acetic acid and 2 μL of 12 M HCl and extracted twice with 500 μL of ethyl acetate. The organic phase was recovered after and solvent was evaporated under N_2 at 40 °C. The residue was suspended in 0.1% (v/v) formic acid in methanol (100 μL) for UPLC analysis.

Conjugated phenolic acids from sourdoughs were extracted in in the same way; prior to extraction with ethyl acetate, conjugated phenolic acids were hydrolysed with 2 M NaOH for 4 h to convert conjugated phenolic acids to free phenolic acids (Fig. 1).

Bound phenolic acids were extracted from the pellet obtained after extraction of free and conjugated phenolic acids with ethanol/water. Bound phenolic acids in the pellet were hydrolysed with 400 μl 2 M NaOH for 4 h. The supernatant was collected by centrifugation, acidified with 120 μL of 12 M HCl to achieve pH 2, and extracted with ethyl acetate (Fig. 1). The organic phase was collected by centrifugation, solvent was evaporated under N_2 at 40 °C, and samples were re-dissolved in 100 μL 0.1% (v/v) formic acid in methanol **LC-DAAD-MS/MS analysis of phenolic acids and metabolites of phenolic acids.**

Phenolic acids and metabolites extracted from mMRS were analysed by UHPLC-DAAD as previously described (Sànchez-Maldonado et al., 2011). Extracts were separated on a Kinetex PFP column (100·x 3.0 mm, 2.6 μm) and quantified on a SPD-M20A Prominence diode array detector. The mobile-phase consisted of (A) 0.1% (v/v) formic acid in water and (B) 0.1% formic acid in water:acetonitrile (10:90, v/v). Samples were eluted with the following gradient: 0–20% B (1.5min), 20% B (4.5min), 20–90% B (7.5min), 90% B (8min). The assay was calibrated with standard compounds dissolved in o.1% formic in methanol.

Phenolic acids and metabolites extracted from sourdough were quantified by LC-DAAD-MS/MS as described by Filannino et al. (2015). MS/MS analysis was performed on a 4000 Q TRAP LC-MS/MS System (MDS SCIEX, Applied Biosystems, Streetsville, ON, Canada) and phenolic acids were identified with an information-dependent acquisition method with parameters identical to parameters described by Filannino et al. (2015). Phenolic acids were quantified with the UV280 nm signal after calibration with standard compounds dissolved in 0.1% formic in methanol.

3. Results

3.1. LAB population of traditional sourdoughs: EPS and pdc screenings

Sourdough fermentations were carried out with reference strains and with isolates from 35 sourdoughs. Microbiota of 19 of the 35 sourdoughs was described previously (Ripari et al., 2016). The 35 sourdoughs harboured diverse microbiota that were composed of species of the genera *Lactobacillus, Pediococcus, Weissella, Leuconostoc,* and *Acetobacter* (Ripari et al., 2016 and Table S1 of the online supplementary material). An overview on the microbiota of the 35 sourdoughs is shown in Fig. S1A of the online supplementary material; the most common species were *Pediococcus pentosaceus* (23 isolates), isolates related to *Lactobacillus plantarum* (*L. plantarum, L. paraplantarum,* or *L. pentosus*, 21 isolates), *Leuconostoc* spp. (18 isolates), *Lactobacillus brevis* (9 isolates), and *Lactobacillus sanfranciscensis* (7 isolates). Isolates were characterized with respect to production of exopolysaccharides from sucrose and the presence of *pdc* coding for phenolic acid decarboxylase (Fig. S2). All *Leuconostoc* spp., most *Weissella* and *Acetobacter* spp., and some *P. pentosaceus* and *L. plantarum* produced EPS from sucrose. All strains of *Lactobacillus rossiae*, and most strains of *L. brevis* and the *L. plantarum* groups harboured the gene coding for phenolic acid decarboxylase (Table S1). Most of the sourdoughs contained at least one strain that was capable of EPS production from sucrose but only 21 of the 35 sourdoughs contained a *pdc* positive strain (Fig. S2B). Because EPS production by lactic acid bacteria in sourdough is well characterized (Galle et al., 2010; Ua-Arak et al., 2016), subsequent analyses focused on conversion of phenolic acids.

3.2. Metabolism of ferulic acid in mMRS

To confirm that the presence of pdc gene relates to the capacity to metabolise phenolic acids, fermentations of 18 *pdc*-positive strains in mMRS with ferulic acid were be performed. Metabolites of ferulic acid were detected in all culture supernatants (Fig. 2). Most strains and particularly strains of *L. plantarum* reduced ferulic acid concentrations by more than 75%. Vinyl guaiacol was detected in culture supernatants of *pdc* positive strains. Strains that reduced ferulic acid concentrations to less than 0.25 mmol/L also produced dihydroferulic acid, the product of ferulic acid reductase activity. In strains exhibiting both decarboxylase and reductase activities, the concentration of dihydroferulic acid was higher than the concentration of vinyl-guaiacol (Fig. 3). Only L. *plantarum* LA1 produced ethyl guaiacol, the product of decarboxylation and reduction of ferulic acid, in low concentrations (< 0.1 mmol/L).

3.3. Phenolic acid metabolism in whole wheat sourdoughs

To determine phenolic acid metabolism in sourdoughs, whole wheat

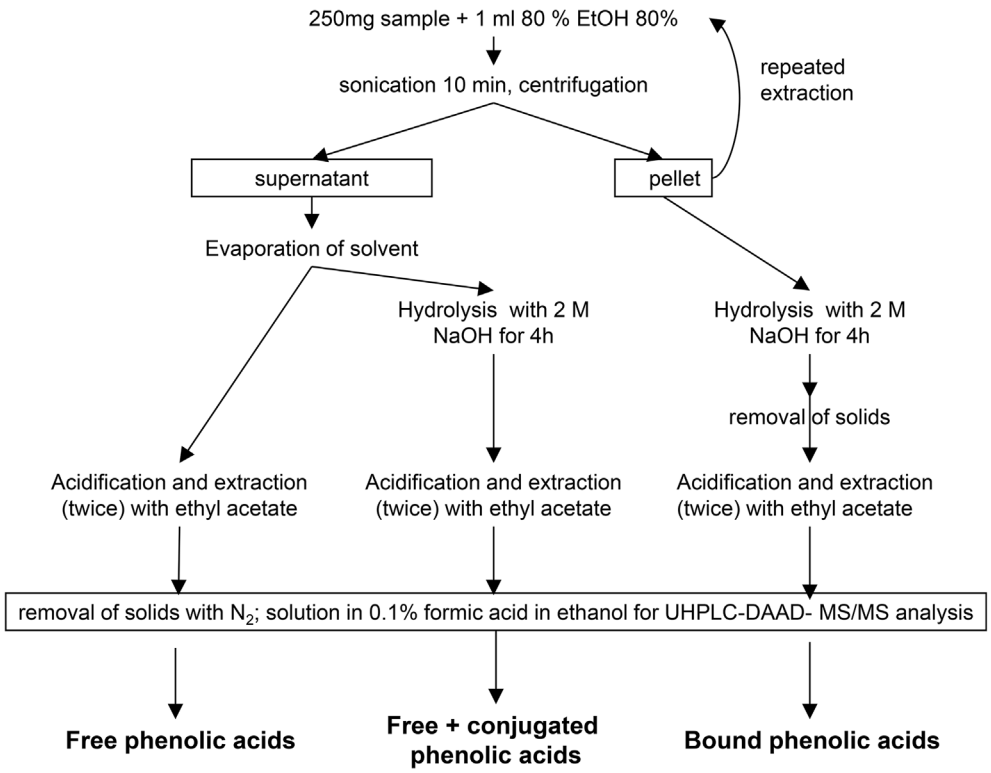

Fig. 1. Schematic overview of phenolic acid extractions from whole wheat dough and rye malt dough.

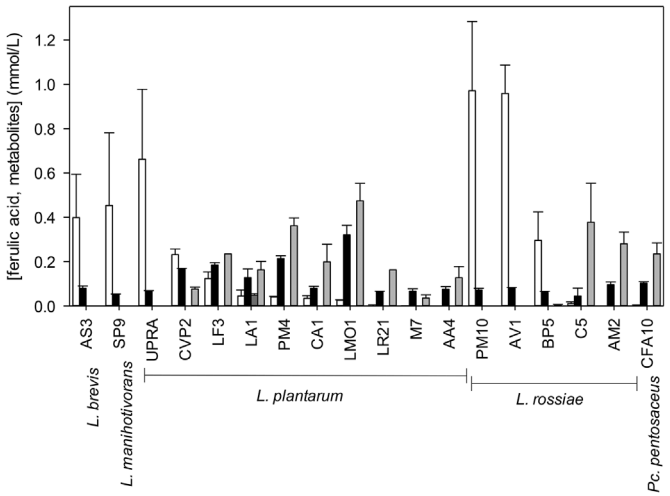

Fig. 2. Concentration of ferulic acid and its metabolites after fermentation with *pdc* positive strains. Ferulic acid was added to the medium at a concentration of 1 mmol/L. White bars, ferulic acid; black bars, vinylguaiacol; dark gray bars, ethyl guaiacol; light gray bars, dihydroferulic acid. Data are shown as means ± standard deviations of three independent fermentations.

sourdoughs were fermented with *L. brevis* AS3, a carboxylase positive but reductase negative strain, *L. plantarum* MAXIII and TMW1.460 with decarboxylase and reductase activities, and *L. hammesii* DSM 16381, a strain with esterase activity that is not capable of ferulic acid conversion (Sànchez-Maldonado et al., 2011, Fig. 3 and data not shown). Two doughs were fermented with *S. cerevisiae* and *C. milleri* for comparison. Viable cell counts, concentration of organic acids and pH are shown in Table 1. In all sourdoughs, the presence of a uniform colony morphology that matched the colony morphology of the strain used as inoculum demonstrated that the inoculum dominated the fermentation microbiota in all sourdoughs. Doughs fermented with yeasts remained free of bacterial contaminants (Table 1 and data not shown). Sourdough fermentation reduced the pH to pH 3.5 to 3.7; doughs fermented with yeasts had a pH of 4.10–4.16 and the chemically acidified dough has a pH of 3.96. The concentration of organic acids in sourdough matched the fermentation type of the respective cultures (Table 1).

Quantification of phenolic acids differentiated between free phenolic acids, conjugated phenolic acids, and bound phenolic acids (Fig. 3A, B, and 3C). Ferulic acid was the most abundant phenolic acid in all samples (Fig. 3); it occurred mainly in bound form (Fig. 3C). Syringic acid was the second most abundant phenolic acid, it occurred in free and conjugated form but was not bound to insoluble dough components (Fig. 3). Vanillic and dihydroxybenzoic acids were minor components in all fractions; 4-coumaric and syringic acids were minor components in free and conjugated phenolic acids.

The content and profile of phenolic acids did not change substantially in the chemically acidified control (Fig. 3), indicating that cereal enzymes are not major contributors to the conversion of phenolic acids during sourdough fermentation. Likewise, yeasts did not to degrade phenolic acids in whole wheat sourdough, or substantially change their distribution in free, conjugated, or bound fractions.

L. plantarum TMW1.460 and MAXXIII degraded most of the free ferulic acid but the concentration of bound ferulic acid remained unchanged (Fig. 3). Fermentation with L. *hammesii* DSM16381 reduced the concentration of bound ferulic acid in comparison to the chemically acidified control or sourdoughs fermented with other lactobacilli; however, a corresponding increase in free or conjugated phenolic acids was not observed. *L. hammesii* DSM 16381 also degraded free vanillic and syringic acids, indicating that the strain decarboxylates hydroxybenzoic acids but not hydroxycinnamic acids.

3.4. Phenolic acid metabolism in rye malt sourdoughs

Conversion of phenolic acids was also studied in rye malt flour to determine whether malt enzymes influence conversion of phenolic acids and their distribution in free and bound fractions. Viable cell

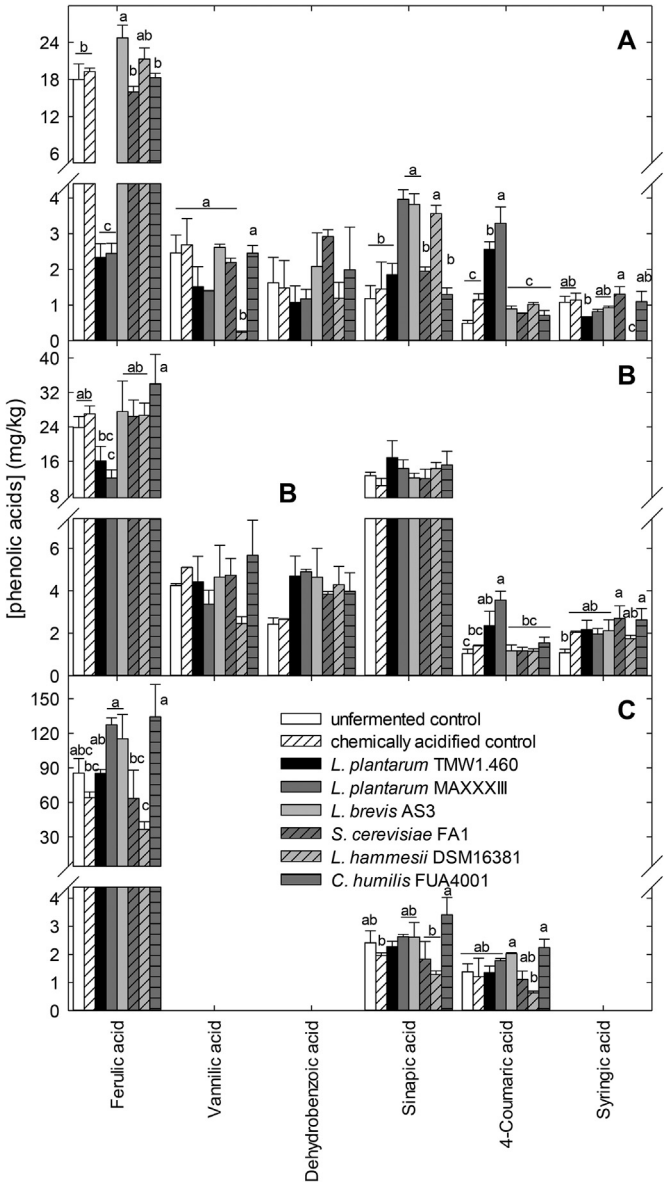

Fig. 3. Concentration of free (**Panel A),** conjugated plus free (**Panel B),** and bound **(Panel C)** phenolic acids in whole wheat sourdoughs. Strains were fermented with single strain as indicated. Data represent means ± standard deviation of three independent fermentations. Values for the same compound in the same fraction are different ($P < 0.005$) if they do not share a common superscript.

counts, concentration of organic acids, and the pH values after 24 h of fermentation are shown in Table 1. Rye malt sourdoughs supported formation of a higher concentration of organic acids when compared to whole wheat sourdoughs, reflecting the higher buffering capacity of rye malt when compared to whole wheat (Table 1).

The number of phenolic acids in rye malt and their concentrations were higher when compared to whole wheat. Ferulic acid, vanillic acid, chlorogenic acid, sinapic acid, syringic acid, 4-coumaric acid, and caffeic acid were detected (Fig. 4). Ferulic acid was the most abundant compound in all fractions; sinapic acid was abundant in conjugated phenolic acids. Bound phenolic acids included sinapic acid, 4-coumaric acid and chlorogenic acid as well as low amounts of caffeic and cinnamic acids in addition to ferulic acid (Fig. 4A, B and 4C). The concentration of free vannilic ($p = 0.032$) and syringic acids ($p < 0.001$) increased in chemically acidified rye malt doughs, likely reflecting enzyme activities of the rye malt. *L. plantarum* MAXXIII metabolized free ferulic acid (Fig. 4A). Fermentation with L. *hammesii* increased the concentration of free ferulic, sinapic and 4-coumaric acids but also increased recovery of ferulic acid and some other phenolic acids in the conjugated and bound phenolic acids relative to the unfermented and the chemically acidified sourdoughs (Fig. 4C). *S. cerevisiae* FA1 generally did not degrade phenolic acids of rye malt dough, or influence the distribution of phenolic acids in the three fractions.

3.5. Co-fermentation in whole wheat sourdoughs

L. plantarum and *L. hammesii* exhibited complementary activities with respect to their ability to release phenolic acids from the bound fraction (*L. hammesii*), and the ability to convert hydroxycinnamic acids (*L. plantarum*). To determine whether co-fermentation of *L. hammesii* and *L. plantarum* increases conversion of bound and free phenolic acids, whole wheat sourdoughs were inoculated with *L. hammesii* in combination with 6 different strains of *L. plantarum*. Cell counts and metabolite concentrations of the sourdoughs are shown in Table 2. Acetate and ethanol concentrations in these sourdoughs were higher than in sourdoughs fermented with *L. plantarum* (Table 1 vs. Table 2) but lower than in sourdoughs fermented with *L. hammesii* (Table 1 vs. Table 2), indicating growth and metabolic activity of both strains.

The impact of co-fermentation on the concentration of free, conjugated and bound phenolic acids was analysed by LC-DAAD-MS/MS (Fig. 5). The concentration of phenolic acids in unfermented and chemically acidified doughs was comparable to the controls that were prepared for sourdoughs fermented with single strains of yeasts or lactobacilli (Fig. 3). Irrespective of the presence of *L. hammesii*, strains of *L. plantarum* metabolized free ferulic acid in whole wheat sourdoughs (Figs. 3A and 5A). Co-fermentation of *L. hammesii* with *L. plantarum* LA1, CVP2, MAXXIII and M7 resulted in levels of conjugated and bound ferulic acid ranging between the concentration in the unfermented dough and the concentration in the chemically acidified control (Fig. 5C), indicating that ferulic acid esterase activity of *L. hammesii* has only a limited influence on the release of ferulic acid from ester linkages in these sourdoughs. However, co-fermentation of *L. hammesii* in combination with *L. plantarum* LM01, PM4, MAXXIII and M7 depleted conjugated (strain MAXXIII M7) or bound (strains LM01 and PM4) ferulic acid, indicating that co-fermentation of *L. plantarum* with a ferulic acid esterase positive strain increased conversion of this compound during fermentation (Fig. 5B and C).

To assess the impact of co-fermentation on metabolism of phenolic acids, we additionally analysed metabolites from ferulic acid. The analytical setup provides quantitative information on the concentration of dihydroferulic acid but only qualitative information is obtained on the volatile compounds vinyl guaiacol and ethyl guaiacol, because the method use for the extraction from dough samples involves one evaporation step. In keeping with the metabolite patterns observed in mMRS-ferulic acid (Fig. 2), all strains of *L. plantarum* produced dihyroferulic acid and vinyl-guaiacol as major metabolites from ferulic acid during growth in rye malt sourdough (Fig. 5A). The highest concentration of dihydroferulic acid was observed in rye malt sourdoughs fermented with *L. hammesii* and *L. plantarum* LM01 (Fig. 5A); a high capacity for formation of dihyroferulic acid was also observed in fermentation with *L. plantarum* LM01 in mMRS-ferulic acid (Fig. 2). All rye malt sourdoughs fermented with *L. plantarum* and *L. hammesii* also contained ethyl-guaiacol (Fig. 5A), a metabolite that was produced only by *L. plantarum* LA1 during growth in mMRS-ferulic acid (Fig. 2).

4. Discussion

The present study investigated metabolism of phenolic acids of lactobacilli in sourdoughs. Strains of lactobacilli for use in sourdough fermentation were selected from reference strains (Filannino et al., 2015; Sànchez-Maldonado et al., 2011), and by screening 114 isolates of lactic and acetic acid bacteria isolated from sourdoughs. In model

Table 1
Metabolite concentrations, pH, and viable cell counts in whole wheat and rye malt sourdoughs fermented with single strains of LAB or yeast. Samples were analysed in unfermented doughs (control) or after 24 h of fermentation (all other doughs). Data are shown as means ± of three independent fermentations.

Strain	[acetate] (mmol/kg)	[lactate] (mmol/kg)	[ethanol] (mmol/kg)	pH	cell count log(cfu/g)
whole wheat sourdoughs					
Unfermented control	0.0 ± 0.0	0.00 ± 0.00	0.0 ± 0.0	6.00 ± 0.04	< 4
Chemically acidified dough	15.8 ± 0.6	37.5 ± 2.2	0.0 ± 0.0	3.96 ± 0.06	< 4
L. brevis AS3	18.2 ± 1.0	102.4 ± 4.4	54.4 ± 3.0	3.72 ± 0.02	9.9
L. plantarum MAXXIII	4.0 ± 0.3	102.7 ± 4.6	0.0 ± 0.0	3.55 ± 0.02	10.1
L. plantarum TMW1460	5.1 ± 0.5	107.2 ± 3.4	0.0 ± 0.0	3.49 ± 0.03	10.0
*L. hammesii*DSM16381	20.8 ± 0.3	107.7 ± 1.6	51.9 ± 1.4	3.56 ± 0.06	10.0
S. cerevisiae FA1	13.1 ± 0.5	39.4 ± 2.8	215.8 ± 7.2	4.16 ± 0.10	8.0
C. humilis FUA4001	12.1 ± 0.7	49.1 ± 0.6	131.4 ± 13.3	4.10 ± 0.05	7.9
Rye malt sourdoughs					
Control	0.0 ± 0.00	0.00 ± 0.00	0.0 ± 0.0	5.54 ± 0.01	< 4
Chemically acidified	Nd	nd	Nd	3.86 ± 0.12	< 4
L. hammesii DSM16381	35.6 ± 2.0	118.7 ± 4.2	32.1 ± 1.4	3.92 ± 0.03	9.70
L. plantarum MAXXIII	3.4 ± 3.0	140.6 ± 16.8	35.2 ± 3.7	3.90 ± 0.02	9.80
S. cerevisiae FA1	20.5 ± 1.2	80.1 ± 5.9	278.7 ± 27.6	4.13 ± 0.03	7.90

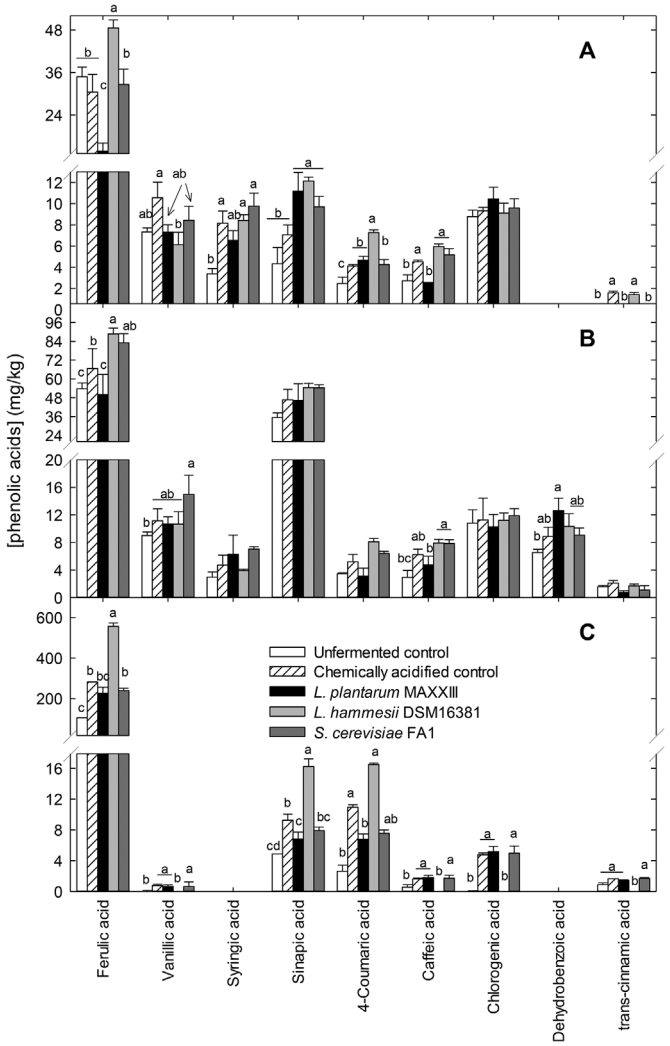

Fig. 4. Concentration of free (**Panel A),** conjugated plus free (**Panel B),** and bound **(Panel C)** phenolic acids in rye malt sourdoughs. Strains were fermented with single strains as indicated. Data represent means ± standard deviation of three independent fermentations. Values for the same compound in the same fraction are different ($P < 0.005$) if they do not share a common superscript.

sourdoughs that were inoculated with one or two defined strains, the strains were differentially enumerated by culture-based differential enumeration. If sourdoughs are inoculated defined strains that differ in their colony morphology, differential enumeration based on colony morphology is more reliable than qPCR or sequence based methodologies (Lin and Gänzle, 2014; Meroth et al., 2003; Scheirlinck et al., 2009; Sekwati-Monang et al., 2012; Zheng et al., 2015b). The selection of strains for sourdough fermentation also aimed to explore the potential for co-fermentation with strains exhibiting complementary enzyme activities to enhance the conversion of phenolic acids.

The characterization of 35 sourdoughs provided 117 isolates that represented the diversity of spontaneous sourdoughs and back-slopped type I sourdoughs (Gänzle and Ripari, 2016). In keeping with prior report on EPS production by sourdough microbiota, EPS production of sourdough isolates was mainly attributed to sucrose-dependent production of dextrans or fructans by *Weissella* spp. and *Leuconostoc* spp. (Van der Meulen et al., 2007; Galle et al., 2010). In addition, sourdoughs contained *Acetobacter* spp. (Ripari et al., 2016). *Acetobacter* spp. are rarely isolated from traditional sourdough (Gänzle and Ripari et al., 2016) but have been employed for production of high molecular weight EPS in sourdoughs (Ua-Arak et al., 2016).

Phenolic acid metabolism of cereal-associated lactobacilli has been investigated in isolates from whiskey and sorghum sourdoughs (Beek and Priest, 2000; Svensson et al., 2010), however, a screening of sourdough isolates has not been reported. Phenolic acid decarboxylase was not only identified in *L. plantarum*, the species for which phenolic acid metabolism is best described (Rodríguez et al., 2009; De Las Rivas et al., 2009; Cavin et al., 1997) but also in *L. brevis* and *L. rossiae*. The antibacterial activity of phenolic acids is higher when compared to the products of bacterial metabolism (Sànchez-Maldonado et al., 2011). Therefore, phenolic acid metabolism by lactobacilli increases the ecological fitness in substrates that contain high concentrations of phenolic acids, e.g. sorghum (Sekwati-Monang et al., 2012). Moreover, reduction of phenolic acids by heterofermentative lactobacilli regenerates reduced co-factors and thus increases the energy yield in the phosphoketolase pathway (Filannino et al., 2016; Gänzle, 2015). All strains that tested positive in the PCR screening for presence of *pdc* also metabolized ferulic acid in mMRS (this study). However, because multiple decarboxylases with differential substrate specificity are present in genomes of lactobacilli (Rodríguez et al., 2009; Barthelmebs et al., 2000), screening for presence of *pcd* only is unlikely to accurately predict phenolic acid metabolism.

Li et al. (2008) developed a protocol for quantification of free, conjugated, and bound phenolic acids. Alkaline hydrolysis before extraction releases bound phenolic acids; alkaline hydrolysis after extraction hydrolyses phenolic acids that are conjugated to other compounds including phenolic acid dimers, glycosides, or phenolic acid esters of polyols (Li et al., 2008). Composition and concentration of phenolic acids that were determined in the present study for whole

Table 2
Cell counts, pH, and metabolite concentrations in whole wheat sourdoughs fermented with a combination of *L. hammesii*DSM16381 and strains of the *L. plantarum* group as indicated. Data represent means ± standard deviations of triplicate independent fermentations.

Fermentation organisms (strain numbers)	[metabolites] (mmol/L)				cell count log(cfu/g)	pH
	ethanol	mannitol	acetate	lactate		
DSM16381 + PM4	18.6 ± 2.8	6.8 ± 0.4	11.4 ± 0.4	107.9 ± 1.4	10.1	3.16 ± 0.06
DSM16381 + LM01	7.6 ± 1.0	2.6 ± 2.1	5.9 ± 5.3	76.3 ± 1.9	9.70	3.32 ± 0.06
DSM16381 + CVP2	19.8 ± 2.1	5.7 ± 0.5	10.4 ± 0.7	111.0 ± 1.2	10.1	3.4 ± 0.04
DSM16381 + M7	12.8 ± 0.6	6.3 ± 0.6	10.7 ± 1.0	117.8 ± 4.4	10.3	3.23 ± 0.03
DSM16381 + MAXXIII	19.7 ± 2.5	6.8 ± 0.6	11.6 ± 0.4	112.3 ± 2.9	10.2	3.35 ± 0.04
DSM16381 + LA1	14.1 ± 3.6	7.0 ± 1.0	10.8 ± 0.3	111.5 ± 0.7	10.1	3.15 ± 0.03
Chemically acidified control	0.0 ± 0.0	0.4 ± 0.1	18.0 ± 2.5	55.74 ± 1.3	< 4	3.57 ± 0.20
Unfermented control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	< 4	5.91 ± 0.02

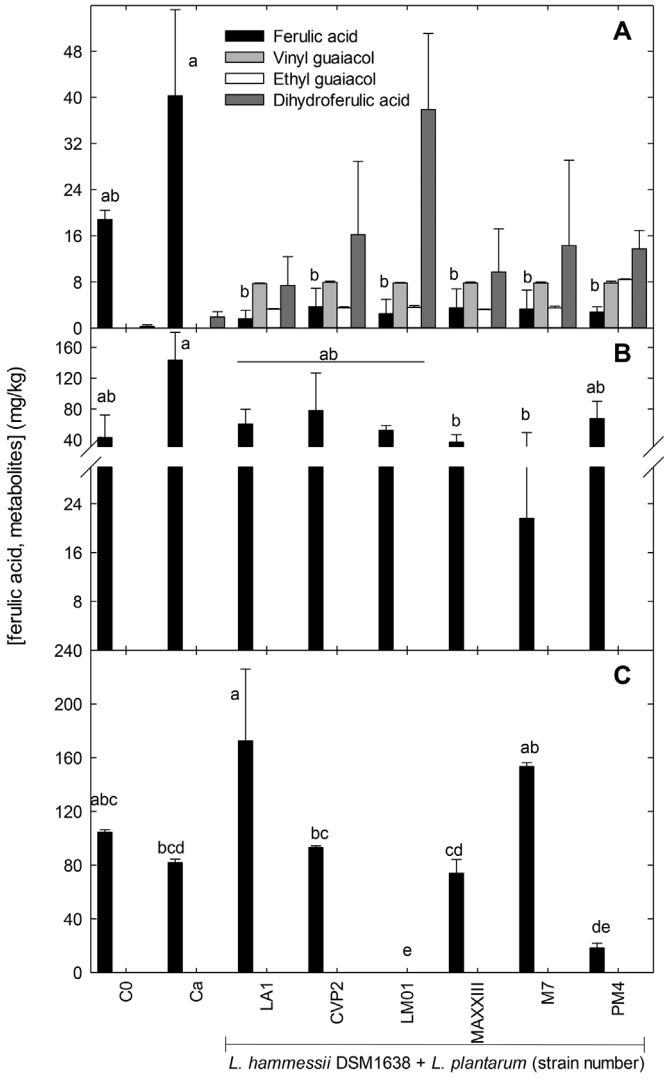

Fig. 5. Concentration of free (**Panel A),** conjugated plus free (**Panel B),** and bound **(Panel C)** ferulic acid and its metabolites in whole wheat sourdoughs. Black bar, ferulic acid, light gray bars, vinyl guaiacol, white bars, ethyl guaiacol, dark dray, dihydroferulic acid. C0, unfermented control (0 h); CA, chemically acidified controls; other samples were fermented with *L. hammesii* and one strain of *L. plantarum* as indicated. Data represent means ± standard deviation of three independent fermentations. Values for ferulic in the same fraction are different ($P < 0.005$) if they do not share a common superscript.

wheat flour and rye malt (Figs. 5 and 6) match the composition and concentration of phenolic acids previously observed in wheat and rye (Li et al., 2008; Shewry et al., 2010). In wheat, ferulic acid was the most abundant compound in all three fractions and particularly in the bound phenolic acids; sinapic acid was most abundant in the conjugated phenolic acids and other hydroxycinnamic or hydroxybenzoic acids were minor constituents (Li et al., 2008, Fig. 5). Also matching prior observations for rye varieties, the concentration and variety of phenolic acids in rye malt was greater when compared to wheat with ferulic acid as most abundant compound in all three fractions (Shewry et al., 2010, Fig. 6). Of note, the alkaline hydrolysis of phenolic acid esters including chlorogenic acid also results in degradation of some compounds, particularly phenolic acids with o-dihydroxy moieties (Sanchez-Maldonado et al., 2014). For some compounds, e.g. sinapic acid and 4-coumaric acid, the recovery from fermented samples was consistently higher than the recovery of unfermented samples. This may relate to the differential degradation of phenolic acids during alkaline hydrolysis or to hydrolysis of phenolic acid esters by rye enzymes (Boskov-Hansen et al., 2002).

Sourdough isolates of *C. humilis* and *S. cerevisiae* used in the present study did not metabolise phenolic acids or phenolic acid esters, although feruloyl esterase activity was previously shown for brewer's yeast (Coghe et al., 2004). The production of volatiles by *S. cerevisiae* is strain specific and aims at attraction of insects to overcome dispersal limitation (Davis et al., 2013). The metabolism of phenolic acids in sourdough by lactobacilli was strain specific, matching prior reports with respect to strain specific phenolic acid metabolism of lactobacilli in other substrates (Svensson et al., 2010; Filannino et al., 2015; Rodríguez et al., 2009). In the present study, fermentation with *L. brevis* had only a limited impact on the concentration and distribution of phenolic acids in sourdough, matching the limited conversion of ferulic acid *in vitro* (Figs. 4 and 5). Strains of L. *plantarum* consistently metabolized free ferulic acid in wheat and rye malt sourdoughs, matching the capacity of this species for ferulic acid metabolism *in vitro*. Remarkably, phenolic acids other than ferulic acid were not converted. Hydroxycinnamic acid reductases and decarboxylases have differential activity with ferulic acid and caffeic or 4-coumaric acid as substrates (Svensson et al., 2010; Sànchez-Maldonado et al., 2011; Barthelmebs et al., 2000; Santamaría et al., 2018a). The phenolic acid decarboxylase of *L. plantarum* recognizes caffeic and coumaric acids but not ferulic acid as substrate; however, the homologous enzyme from *B. subtilis* decarboxylates all three hydroxycinnamic acids (Cavin et al., 1997, 1998). *L. hammesii* metabolized hydroxybenzoic acids in wheat but not in rye malt sourdoughs, possibly reflecting that the fermentation substrate influences the expression of enzymes active on phenolic acids (Filannino et al., 2015). This strain also increased concentrations of free ferulic acid by esterase activity but did not hydrolyse chlorogenic acid. Because the genome of this strain does not encode for one of the feruloyl esterases that were characterized on the biochemical level (Zheng et al., 2015a; Hole et al., 2012), this activity is attributable to a previously uncharacterized enzyme. Hydrolysis of phenolic acid esters by esterase positive lactobacilli increases the bioavailability of dietary phenolic compounds in oats and barley (Hole et al., 2012) and reduced the chlorogenic acid content in sunflower seed flour (Fritsch et al.,

2016).

Strain-specific metabolism of phenolic acids allowed the unprecedented use of co-fermentation with strains that exhibit complementary pattern of metabolism. Esterase activity of *L. hammesii* increased the concentration of ferulic acid which was further converted to dihydroferulic acid and volatile phenolic compounds by *L. plantarum*. Vinyl-guaiacol with a smoky flavour note and ethyl-guaiacol with a clove like/spicy aroma are undesirable flavour compounds in beer or wine (Wackerbauer et al., 1982; Shinohara et al., 2000), but may contribute to the typical flavour of bread.

In conclusion, this study characterized phenolic acid metabolism of lactic acid bacteria isolated from sourdough. The phenolic acid decarboxylase pdc$^+$ was present mainly in *L. rossiae*, *L. brevis*, and *L. plantarum*. Pdc positive strains metabolized ferulic acid *in vitro* and in wheat and rye sourdoughs, however, Pdc positive L. *plantarum* did not metabolise other hydroxycinnamic or hydroxybenzoic acids in wheat and rye malt sourdoughs. This result indicates that phenolic acid metabolism by lactobacilli is dependent on multiple decaboxylases and reductases which are only partially characterized. Likewise, *L. hammesii* released bound ferulic acid by an uncharacterized esterase. Phenolic acid metabolism in sourdoughs was enhanced by co-fermentation with strains exhibiting complementary metabolic activities. The impact of phenolic acid metabolism on bread quality and on health-beneficial effects of phenolic compounds remains subject to future investigations.

Acknowledgements

Laihian Mallas (Laihian, Finland) is acknowledged for providing rye malt. The Natural Science and Engineering Research Council of Canada (NSERC) is acknowledged for funding under the Discovery Grant and the Canada Research Chairs Programs.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.08.009.

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