Metabolism of phenolic compounds by *Lactobacillus* spp. during fermentation of cherry juice and broccoli puree

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ABSTRACT

This study aimed to investigate the metabolism of phenolic acids and flavonoids during lactic acid fermentation of cherry juice and broccoli puree for potential food and pharmaceutical purposes. When fermenting cherry juice and broccoli puree, *Lactobacillus* spp. exhibited strain-specific metabolism of phenolic acid derivatives. The metabolism of protocatechuic, caffeic and *p*-coumaric acids through phenolic acid decarboxylases and reductases differed between mMRS and cherry juice and broccoli puree. The synthesis of reduced compounds was the highest during food fermentations and the substrate seemed to modulate the metabolism of phenolic compounds. The reduction of phenolic acids involves a hydrogen donor and the re-oxidation of the reduced co-factor NADH, which may provide a metabolic advantage through NAD^+ regeneration. Quinic acid reduction may replace fructose and pyruvate as hydrogen acceptors, and it may provide an energetic advantage to heterofermentative bacteria when growing in broccoli puree lacking of fructose. This study demonstrated that phenolics metabolism may confer a selective advantage for lactobacilli in vegetable and fruit fermentation, and the metabolic routes are strongly dependent on the intrinsic factors of substrate. Fermented cherry juice and broccoli puree, due to the selected bacterial bioconversion pathways, are enriched in phenolic derivative with high human bioavailability and biological activity.

Keywords: phenolics metabolism, lactic acid bacteria, NAD^+/ NADH ratio, functional foods, fruits, vegetables.

1. Introduction

Phenolic compounds are secondary plant metabolites whose structure includes one or more aromatic rings substituted by one or more hydroxyl groups. On the basis of their structure, phenolics are classified as phenolic acids (C_6-C_1 and C_6-C_3), flavonoids (C_6-C_3-C_6), xanthones (C_6-C_1-C_6), stilbenes (C_6-C_1-C_6), phenolic lipids, and tannins, which include polymeric condensed tannins (Rodrìguez et al., 2009). Phenolic compounds in food have attracted interest because of their health benefits, but also owing to their antimicrobial features and their impact on multiple sensory attributes of food, including flavor, astringency, and color of plant-based foods (Rodrìguez et al., 2009; Shahidi and Naczk, 2003).

It is increasingly recognized that health benefits of phenolic compounds are partially dependent on their microbial conversion (Selma et al., 2009). The microbial conversion of phenolics may occur *in situ* upon injury of the plant tissue, during food processing, particularly during food fermentations, and by human intestinal microbiota upon ingestion (Manach et al., 2004; Requena et al., 2010; Selma et al., 2009). Lactic acid bacteria are a major part of the fermentation microbiota of fermented plant foods (Di Cagno et al., 2013); their capacity to metabolize phenolic compounds thus impacts the profile of phenolic compounds in the human diet (Rodrìguez et al., 2009).

Phenolic acids and flavonoids are the most abundant classes of phenolics in vegetables and fruits (Lee et al., 2006; Naczk and Shahidi, 2006). Compared to other bacteria, including *Enterobacteriaceae*, *Clostridium* spp. and *Bacteroides* spp., *Lactobacillus* spp. are more resistant to phenolic compounds (Bel-Rhlid et al., 2013; Engels et al., 2011; Sanchez-Maldonado et al., 2011). Lactobacilli showing the capacity to metabolize phenolic compounds are usually isolated from fermented foods with high content of phenolic compounds (Campos et al., 2009; Rozes and Peres, 1998; Svensson et al., 2010; Van Beek and Priest, 2000). The ability to tolerate and metabolize these compounds is strain- or species-dependent (Cueva et al., 2010; Curiel et al., 2010; Svensson et al., 2010; Van Beek and Priest, 2000). *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus plantarum* are some of the lactobacilli, which metabolize phenolic acids through

66 decarboxylation and/or reduction activities. Further metabolic pathways identified in lactobacilli are
67 based on: glycosyl hydrolases, which convert flavonoid glycosides to the corresponding aglycones,
68 and esterase, degrading methyl gallate, tannins, or phenolic acid esters (Rodrìguez et al., 2009).
69 Only few studies describe the conversion of phenolics in food fermentations, i.e. sorghum and
70 pomegranate fermentations (Filannino et al., 2013; Svensson et al., 2010). The metabolism of
71 phenolics in food may confer a selective advantage for microorganisms and thus impacts the
72 selection of competitive starter cultures for vegetable and fruit fermentations (Clausen et al., 1994;
73 Goodey and Tubb, 1982). Moreover, phenol derivatives may be sources of flavor or off-flavors in
74 plant-based foods and are valuable intermediates in the biotechnological production of new flavor
75 and fragrance chemicals (Etiévant et al., 1989; Huang et al., 1993; Thurston and Tubb, 1981). As
76 indicated above, metabolites of phenolic compounds also exert biological activities at the human
77 cellular level (Manach et al., 2004).
78 This study aimed to investigate the metabolism of phenolic acids and flavonoids during lactic
79 acid fermentation of cherry juice and broccoli puree for potential food and pharmaceutical purposes
80 (Manach et al., 2004). Based on their high level, hydroxycinnamic acids and flavonols characterize
81 the phenolic profile of cherries. Low levels of hydroxybenzoic acids were also found (Jakobek et
82 al., 2009). Broccoli is one of the main dietary sources of flavonoids, mainly acylated derivatives
83 where sinapic, ferulic, caffeic and *p*-coumaric acids were linked to the flavonoid-glycoside
84 molecules (Moreno et al., 2006; Vallejo et al., 2004a). Phenolics from cherries and broccoli have
85 been reported to have several human health effects such as scavenging free radicals and antioxidant
86 activities, and the inhibition of human low-density lipoprotein oxidation (Moreno et al., 2006; Serra
87 et al., 2011). In addition to phenolic compounds, cherry and broccoli are good sources of other
88 bioactive compounds, such as ascorbic acid, glucosinolates, essential minerals, β-carotene, α-
89 tocopherol, vitamins, and dietary fibers (Di Cagno et al., 2011; Vallejo et al., 2004b).
90 Unquestionably, they represent optimal substrates exploitable for the production of functional
91 foods, and lactic acid fermentation may be a valuable biotechnology to enhance the concentration of

health-promoting compounds in these vegetable substrates. The interpretation of the bioconversion pathways under the environmental conditions of this study should highlight the physiological significance of the phenolics metabolism in lactic acid bacteria, and facilitates the selection of microbial starters for industrial scale fermentations.

2. Materials and methods

2.1 Preparation of media

The metabolism of phenolic compounds by *Lactobacillus* spp. was studied in cherry juice and broccoli puree. Cherry juice was produced as described by Di Cagno et al. (2009), with some modifications. In brief, frozen fruits were thawed, homogenized, treated at 121°C for 10 min, and centrifuged at 10,000 x g for 20 min. Frozen inflorescences from broccoli were thawed and homogenized to prepare broccoli puree. To avoid the inactivation of the endogenous myrosinase, which hydrolyses glucosinolates into numerous biologically active products, broccoli puree was not sterilized (Aires et al., 2009). Both media were stored at -20 °C before use.

2.2 Microorganisms and growth conditions

Lactobacillus plantarum C2 and C5 isolated from carrot (Di Cagno et al., 2008), CIL6 from cherry (Di Cagno et al., 2011), POM1 from tomato (Di Cagno et al., 2009), 1MR20 from pineapple (Di Cagno et al., 2010), TMW 1.460 from spoiled beer (Ulmer et al., 2000), and *Lactobacillus spicheri* Lp38 from French wheat sourdough (Valcheva et al., 2006) were cultivated at 30°C in modified MRS (mMRS) medium, containing 10 g of maltose, 5 g of glucose and 5 g of fructose per liter (Tieking et al., 2005). *Lactobacillus fermentum* FUA3165 and *Lactobacillus reuteri* FUA3168, both isolated from “ting”, a fermented sorghum porridge from Botswana (Sekwati-Monang and Gänzle, 2011), were cultivated in mMRS medium at 37°C. Inocula for food fermentations were prepared by harvesting cells from 24 h cultures in mMRS and washing twice in physiological saline (0.9% NaCl, wt/vol). Cherry juice or broccoli puree were inoculated with 10% (vol/vol) of the cell suspensions, corresponding to ca. 10^8 cfu / g, and incubated at 34°C for 24 h. Samples were taken

before inoculation, and before and after fermentation. Viable cell counts were enumerated by surface plating on mMRS agar; the pH was measured by a glass electrode (Hamilton, Bonaduz, Switzerland). Cherry juice without bacterial inoculum was incubated under the same conditions and used as the control. The value of pH was not adjusted since it corresponds to that usually found after cherry juice fermentation (Di Cagno et al., 2011; Filannino et al., 2014). Broccoli puree without a bacterial inoculum was chemically acidified with lactic acid (final pH 3.5) to get the same value of pH as that found after broccoli puree fermentation, and used as the control.

2.3 Determination of organic acids

Bacterial cells were removed by centrifugation and equal volumes of perchloric acid (5%, vol/vol) were added to samples. The suspension was kept at 4°C overnight, centrifuged at 10,000 x g, 10 min, and filtered through a Millex-HA 0.22-μm pore size filter (Millipore Co.). Organic acids were determined using an Agilent 1200 HPLC system, which was equipped with an Aminex HPX-87 column (Bio-Rad, Mississauga, Canada) and a refractive index detector. Samples were eluted with 5 mM H_2SO_4 at a temperature of 70°C and a flow rate of 0.4 ml/min and analytes were detected (Galle et al., 2010). The concentrations of lactate, acetate, and malate were determined using external standards (Sigma, Oakville, Canada).

2.4 Extraction of free phenolic acids and flavonoids

The liquid-liquid extraction of free phenolic acids and flavonoids from cherry juice and broccoli puree was carried out as described by Svensson et al. (2010), with some modification. Thirty milliliters of cherry juice were acidified to pH 1.5 with hydrochloric acid and extracted with 120 ml of ethyl acetate (Fisher Scientific, Ottawa, ON, Canada). The mixture was shaken every 10 min for 30 min. The liquid-liquid extraction was repeated, and the extract was evaporated under vacuum at 30°C. Solids were redissolved in 1 ml of methanol. To extract phenolic compounds from broccoli puree, samples were mixed with 120 ml of aqueous methanol (70%, vol/vol). The mixture was shaken for 1 h and centrifuged at 4,225 x g for 10 min. The supernatant was removed, and the

142 residue was extracted again as described above. Methanol was evaporated under vacuum at 30°C,
143 solids were redissolved with 30 ml of Milli-Q water and acidified to pH 1.5 with hydrochloric acid.
144 The liquid-liquid extraction was carried out as described for cherry juice.

145 *2.5 Identification of free phenolic acids and flavonoids*

146 Identification of free phenolic compounds was carried out through LC-DAD-MS (Liquid
147 Chromatography-Diode Array Detector-Mass Spectrometry) analysis, using the Shimadzu UPLC
148 system, which was equipped with a degasser, binary pump, auto-sampler and a thermostated
149 column compartment. An SPD-M20A Prominence diode array detector (DAD) was connected to a
150 4000 Q TRAP LC-MS/MS System (MDS SCIEX, Applied Biosystems, Streetsville, ON, Canada).
151 A Kinetex PFP column (100 x 3.0 mm, 2.6 μm) was used for the separation of phenolics. DAD
152 detection was carried out between 100 and 400 nm. Eluent A consisted of 0.1% (vol/vol) formic
153 acid in water HPLC grade, and eluent B consisted of 0.1% (vol/vol) formic acid in acetonitrile
154 (90%, vol/vol) and water HPLC grade (10%, vol/vol). Samples were eluted with the following
155 gradient: 0% B (2 min), 0-2% B (2 min), 2-5% B (4 min), 5-10% B (2 min), 10-18% B (4 min), 18-
156 20% B (1 min), 20-28% B (5 min), 28-32% B (2.5 min), 32-90% B (6.4 min), 90-0% B (0.1 min).
157 Mass spectra were recorded in the negative mode; the flow rate was maintained at 0.9 mL min^{-1}
158 with the pneumatically assisted electrospray probe using high-purity nitrogen gas (99.995%) as the
159 nebulizing (GS1) and heating gas (GS2). The values for optimum spray voltage, source
160 temperature, GS1, GS2, and curtain gases were -4500 V, 600°C, and 55, 45, and 18 psi,
161 respectively. An information-dependent acquisition (IDA) method, EMS→EPI, was used to identify
162 phenolic compounds. Both Q1 and Q3 were operated at low and unit mass resolution. The spectra
163 were obtained over a range from m/z 100 to 2000 in 2 s. LIT fill time was set at 30 ms. The IDA
164 threshold was set at 100 cps, above which enhanced product ion (EPI) spectra were collected from
165 the three most intense peaks. The EPI scan rate was 4000 Da s^{-1}. Collision-induced dissociation
166 (CID) spectra were acquired using nitrogen as the collision gas under two different collision
167 energies. The collision energy (CE) and collision energy spread (CES) were 10 and 0 V. The

declustering potential (DP) was -20 V. Data acquisition was interfaced to a computer workstation running Analyst 1.5 (Applied Biosystems). External standards were analyzed under the same conditions and used for identification by comparison of elution volume, mass spectrum, and UV absorbance. Caffeic, *p*-coumaric, protocatechuic, and sinapic acids, rutin and quercetin were obtained from Extrasynthese (Genay, France). Chlorogenic, dihydrocaffeic and phloretic acids and catechol were purchased from Sigma (St Louis, MO, USA). Literature data for mass spectra and UV absorbance were used when external standards were not available.

2.6 Quantification of free phenolic acids and flavonoids

Quantification of free phenolic compounds was carried out through LC-DAD, using external standards dissolved in methanol under the UPLC conditions described above. Phenolic acids and flavonoids were detected at 280 and 320 nm, respectively. The calibration curves were linear with a correlation coefficient ≥ 0.99. For compounds, whose external standards could not be obtained, the concentration was calculated as equivalent of chlorogenic, coumaric, or sinapic acid. Compounds co-eluted with other compounds were quantified by LC-MS/MS and results were expressed as cps per min.

2.7 Metabolism of phenolic acids by Lactobacillus spp. strains in mMRS

mMRS medium was supplemented with protocatechuic, caffeic or *p*-coumaric acids at a concentration of 1 mmol / L (Sanchez-Maldonado et al., 2011). Overnight cells were single inoculated (5%, vol/vol) into mMRS supplemented media and incubated for 24 h at 34°C. Sterile media containing the corresponding phenolic compounds, without bacterial inoculum, were used as the control. After incubation, cells were removed by centrifugation (10,000 x g for 10 min), and the supernatant was acidified to pH 1.5 with hydrochloric acid. Ethyl acetate (3 ml) was used for liquid-liquid extraction. The extracts (5 µl) were analyzed through LC-DAD-MS using a Shimadzu UPLC system equipped with a Kinetex PFP column (100 x 3.0 mm, 2.6 µm) and a SPD-M20A Prominence diode array detector. The UPLC conditions, and the metabolite identification (at 280

nm) and quantification were as described elsewhere. Changes of substrates were calculated as percentage of the initial concentration, while products were expressed as percentage of total products.

2.8 Statistical analyses

Data (at least three replicates) were subjected to one-way ANOVA, and pair-comparison of treatment means was achieved by Tukey's procedure at $P<0.05$, using the statistical software Statistica for Windows (Statistica 7.0 per Windows).

3. Results

3.1 Fermentation parameters of cherry juice and broccoli puree

Cherry juice and broccoli puree were used as model systems for fruit and vegetable fermentations. The strains of *Lactobacillus* spp. selected as starter cultures were previously isolated from foods with a relatively high content of phenolic compounds. Viable cell counts, pH and concentration of organic acids were determined during cherry juice and broccoli puree fermentation (Table S1). The bacterial inoculum was ca. 8.30 Log CFU/ml. After 24 h, the cell density of all strains increased of ca. 1.0 and 1.5 Log CFU/ml in cherry and broccoli, respectively.

Cherry juice had an initial value of pH of 4.01 ± 0.28. After fermentation, the value of pH remained almost constant or slightly decreased. The only exception was with *L. spicheri* Lp38, showing a slight increase (4.28 ± 0.24). The pH of broccoli puree decreased from 6.51 ± 0.03 to ca. 4.28 – 3.46. *L. spicheri* Lp38, *L. fermentum* FUA3165 and *L. reuteri* FUA3168 showed the lowest decreases (Table S1).

Lactic acid was the major fermentation end-product. In cherry fermentations, *L. plantarum* strains CIL6, POM1, 1MR20 and TMW1.460 showed the highest synthesis and accumulated about 130 mM lactate. *L. spicheri* Lp38 and *L. fermentum* FUA3165 synthesized 87 and 65 mol lactate / L, respectively and the lowest lactate concentrations, 27 mmol / L, was found in cherry juice fermented with *L. reuteri* FUA3168. Acetic acid was found in traces. A significant decrease of

malic acid was found only for *L. plantarum* POM1, *L. spicheri* Lp38, and *L. fermentum* FUA3165. *L. plantarum* strains also synthesized the highest level of lactic acid in broccoli fermentations (Table S1). The obligate heterofermentative *L. spicheri* Lp38, *L. fermentum* FUA3165 and *L. reuteri* FUA3168 synthesized 53 – 74 mmol / L lactate and 9 – 22 mmol / L actetate. The concentration of malic acid decreased significantly ($P<0.05$) in almost all broccoli fermentations.

3.2 Identification and quantification of free phenolic compounds in unfermented cherry juice and broccoli puree

Separation of free phenolic compounds was carried out through LC-DAD-MS. The highest peaks of free phenolic compounds in the extracts from unfermented cherry juice and broccoli puree were identified. External standards were analyzed under the same conditions and used for identification by comparison of retention time, mass spectrum, and UV absorbance. Literature data for mass spectra and UV absorbance were used when external standards were not available. The mass spectra, retention time, and UV absorbance of protocatechuic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, sinapic acid, rutin and quercetin matched those of the external standards (Table 1 and 2).

Unfermented cherry juice contained protocatechuic and *p*-coumaric acids as the most abundant phenolic acids, which were present at 34.3 ± 9.1 and 31.8 ± 7.4 mg / L, respectively. Intermediate concentrations were found for chlorogenic and caffeic acids (16.4 ± 3.6 and 14.7 ± 1.9 mg / L, respectively). Neochlorogenic acid and *p*-coumarolquinic acid were found at lowest level (2.75 ± 0.07 and 2.70 ± 0.02 mg / L, respectively). Flavonoids such as rutin (93.96 ± 16.89 mg / L) and quercetin (5.29 ± 0.88 mg / L) were also identified.

In unfermented broccoli puree, the highest peaks were attributed to sinapic acid esters (peaks 7 – 13). Chlorogenic and neochlorogenic acids were found at high concentrations (149 ± 33 and 249 ± 91 mg / L, respectively). Caffeic acid was found only in traces.

3.3 Identification and quantification of free phenolic compounds in cherry juice and broccoli puree

The profile of phenolic compounds of cherry juice and their metabolites was analyzed before and after fermentation (Fig. 1 and 2, and Table 1 and 3). The concentration of rutin, quercetin, chlorogenic acid, neochlorogenic acid, and *p*-coumarolquinic acid, did not change during aseptic incubation or during lactic fermentation (data now shown). However, most strains changed the concentration of phenolic acids. With exception of *L. plantarum* C2 and C5, all strains of *L. plantarum* decreased the concentration of protocatechuic acid by about 70% (Fig. 1). The degradation of protocatechuic acid corresponded to the accumulation of catechol in approximately equivalent concentrations (Fig. 1). Catechol synthesis was also found by *L. spicheri* LP38 and *L. fermentum* FUA3165 (Fig. 1). Caffeic acid was completely consumed by most *L. plantarum* strains and *L. spicheri* Lp38 (Fig. 2A). A slight decrease was also found in cherry juice fermented by *L. fermentum* FUA3165. Metabolism of caffeic acid by *L. plantarum* corresponded to the accumulation of the reduced metabolite, dihydrocaffeic acid (Fig. 2A). *L. plantarum* strains also consumed *p*-coumaric acid, strains of *L. plantarum* accumulated phloretic acid (Fig. 2B). *L. plantarum* C5 showed the weakest metabolism of caffeic and *p*-coumaric acids (Fig. 2); the conversion of caffeic and coumaric acids by *L. spicheri* LP38 did not correspond to the accumulation of reduced metabolites (Fig. 2).

Fermentation of broccoli puree did not change the concentrations of neochlorogenic acid, *p*-coumarolquinic acid, sinapoylhexose and the sinapic- and ferulic acid esters of gentiobiose (Table 2 and data not shown). Sinapic acid concentrations generally increased during fermentation of broccoli puree; this more than twofold increase was not significant but consistent in all fermentations with strains of *L. plantarum* (data not shown). The concentration of chlorogenic acid decreased after fermentation of broccoli puree with *L. reuteri* FUA3168 (Fig. 3); quinic acid concentrations decreased after fermentation with *L. plantarum* C2 and C5, *L. fermentum* FUA3165 and *L. reuteri* FUA3168. *L. fermentum* FUA3165 and *L. reuteri* FUA3168 produced dihydrocaffeic acid (Fig. 3), all lactobacilli with exception of *L. spicheri* Lp38 also produced phloretic acid (Table 3 and data not shown).

3.4 Metabolism of phenolic acids in mMRS

To confirm the strain-specific metabolism of protocatechuic, caffeic and *p*-coumaric acids in cherry juice and broccoli puree by phenolic acid decarboxylases and reductases (Rodrìguez et al., 2009), the metabolism of these compounds was further investigated in mMRS (Table 4). Differently from the protocol for extraction of phenolic compounds from cherry juice and broccoli puree, the extraction of metabolites from mMRS also allowed the detection of volatile metabolites of phenolic acids. With exception of *L. plantarum* C2, strains of *L. plantarum* decarboxylated protocatechuic acid to catechol (Table 4). *L. spicheri* Lp38, *L. fermentum* FUA3165 and *L. reuteri* FUA3168 partially metabolized protocatechuic acid but related metabolites were not identified. Caffeic acid was completely consumed by all strains of *L. plantarum*, *L. spicheri* Lp38 and *L. fermentum* FUA3165. Strains of *L. plantarum* and *L. spicheri* Lp38 decarboxylated caffeic acid to vinyl catechol ($[M - H]^-$ ion at m/z 135) (Table 4). *L. plantarum* CIL6 also produced ethylcatechol ($[M - H]^-$ ion at m/z 137). On the contrary, *L. fermentum* FUA3165 mainly reduced caffeic acid into dihydrocaffeic acid (Table 3 and 4). Dihydrocaffeic was also found at low levels in culture supernatants of *L. plantarum* C2 and TMW 1.460, and *L. reuteri* FUA3168. An unidentified metabolite of caffeic acid by *L. reuteri* FUA3168 was found, this compounds showed a mass spectrum with $[M - H]^-$ ion at 193 and fragment at m/z 161 and 134. *p*-Coumaric acid was metabolized to *p*-vinylphenol ($[M - H]^-$ ion at m/z 119) and phloretic acid by strains of *L. plantarum* except strain 1MR20. *L. spicheri* Lp38 and *L. fermentum* FUA3165 partially converted *p*-coumaric acid to *p*-vinylphenol and phloretic acids, respectively.

4. Discussion

Lactobacillus spp., particularly *L. plantarum*, are found in many vegetable and fruit fermentations. Nevertheless, the metabolism of phenolic compounds by lactobacilli in food fermentations is poorly characterized. This study aimed at giving new insights on the bioconversion pathways of several phenolics during fermentation of cherry juice and broccoli puree.

Lactobacilli metabolize phenolic acids by strain-specific decarboxylase and/or reductase activities (Rodrìguez et al., 2009). Caffeic and *p*-coumaric acids are decarboxylated to the corresponding vinyl derivatives through the activity of a phenolic acid decarboxylase (PAD) (Fig. 4) (Rodrìguez et al., 2009). The vinyl derivatives are subsequently reduced to their corresponding ethyl derivatives (Rodrìguez et al., 2009). However, the enzymes responsible for the decarboxylation of protocatechuic acid by lactobacilli are unknown; likewise, the phenolic acid reductase activity converting hydroxy-cinnamic acids to the corresponding dihydro-derivatives has not been characterized. In cherry juice and broccoli puree, *Lactobacillus* spp. exhibited strain-specific metabolism of phenolic acids including hydroxybenzoic acids (protocatechuic acid), hydroxycinnamic acids (caffeic and *p*-coumaric acids), and hydroxycinnamic acid derivatives (chlorogenic acid) (Fig. 4). *L. plantarum* consistently exhibited the most extensive conversion of protocatechuic, caffeic and *p*-coumaric acids during cherry juice fermentation. In contrast, *L. reuteri* FUA3168 did not affect the profile of phenolic acids but this organism was the only strain capable of hydrolysis of chlorogenic acid, confirming prior observations of chlorogenic acid conversion in laboratory culture (Sanchez-Maldonado et al., 2011). The metabolism of phenolic acids by *L. plantarum* was strain specific. In particular, strains *L. plantarum* C2 and C5 did not metabolize protocatechuic acid in cherry juice and caffeic and *p*-coumaric acids were only slightly used by *L. plantarum* C5. Remarkably, these two strains also produced a lower concentration of lactic acid when compared to other strains of *L. plantarum*. Taken together, these differences indicate that phenolic acid conversion corresponds to a lower overall metabolic activity and competitiveness in acidic fermentation substrates with high concentrations of phenolic acids. It was previously shown that phenolic acids degradation by lactic acid bacteria is an important mechanism for detoxification of these compounds (Sanchez-Maldonado et al., 2011). Other metabolic stress responses, e.g., the decarboxylation of malic acid and the catabolism of amino acids, also contribute to acid resistance of *L. plantarum* C2 (Filannino et al., 2014).

Hydroxycinnamic acids were almost exclusively decarboxylated during growth of *L. plantarum* in mMRS. In contrast, conversion of caffeic and *p*-coumaric acids by *L. plantarum* in cherry juice accumulated high concentrations of reduced derivatives (dihydrocaffeic acid and phloretic acid, respectively) (Fig. 4). An increase of phloretic acid was also found during fermentation of broccoli puree, probably derived from release and reduction of bound *p*-coumaric acid. Although the extraction of phenolic compounds from cherry juice and broccoli puree did not allow the quantification of vinyl derivatives, the considerable production of reduced compounds in food fermentations indicates that the fermentation substrate modulates the metabolism of phenolic compounds. The reductase activity is induced by hydroxycinnamic acids and it is mostly active in the presence of glucose (Barthelmebs et al., 2000). The high content of glucose in cherry juice (Filannino et al., 2014) may represent an inducing factor. The reduction of phenolic acids involves a hydrogen donor and may involve the reoxidation of the reduced co-factor NADH, thus providing a metabolic advantage through the NAD^+ regeneration. In keeping with this hypothesis, the obligate heterofermentative *L. fermentum* FUA3165 showed mainly reductase activity and the ration of decarboxylation to reduction of hydroxycinnamic acids was independent of the fermentation substrate. It is possible to state that the metabolism of phenolic acids is driven by the intracellular NAD^+/ NADH balance. These findings were further supported by Silva et al. (2011), which showed that during growth of *L. plantarum* and *Lactobacillus collinoides* under anaerobiosis or in absence of additional electron acceptors (e.g., fructose), the reduction of 4-vinylphenol to 4-ethylphenol is favored, according with the need to increase the availability of NAD^+.

L. reuteri FUA3168 was the only strain able to hydrolyze chlorogenic acid. This metabolism was not found during cherry juice fermentation, confirming the effect of the media and substrate concentration of the conversion of phenolic compounds. The hydrolysis of chlorogenic acid by *L. reuteri* FUA3168 leads to the synthesis of caffeic acid and quinic acid (Fig. 3 and 4). Although caffeic acid concentrations in broccoli puree did not increase, a considerable production of its metabolite dihydrocaffeic was found. Similarly, quinic acid was further metabolized. Quinic acid

metabolism was also observed in broccoli puree fermented by *L. fermentum* FUA3165 and *L. plantarum* C2 and C5. Only one prior report indicates the ability of *L. plantarum* and one obligately heterofermentative *Lactobacillus* spp. to metabolize quinic acid (Whiting and Coggins, 1971). In *L. plantarum*, the conversion of quinic acid to catechol increases the ambient pH. In contrast, the obligate heterofermentative *Lactobacillus* spp. reduced quinic acid to dihydroshikimic acid, thus replacing fructose and pyruvate as hydrogen acceptor. Therefore, the metabolism of quinic acid likely provided an energetic advantage to *L. fermentum* FUA3165 and *L. reuteri* FUA3168 during fermentation of broccoli puree, a substrate which does not provide fructose as electron acceptor. These findings were also supported by the substantial synthesis of the acetic acid.

Although, functional value of cherry and broccoli phenolics is well known (Manach et al., 2004), their biological properties depend on their absorption in the gastrointestinal tract. For instance, human tissues and biological fluids do not possess esterases capable of hydrolyzing chlorogenic acid to release caffeic acid, and only the colonic microbiota would be capable of carrying out this hydrolysis (Manach et al., 2004). Free caffeic acid is better absorbed in the stomach and in the small intestine than chlorogenic acid (Olthof et al., 2001; Rechner et al., 2001). In addition, dihydrocaffeic acid has a high human bioavailability and has a higher antioxidant property than its precursor caffeic acid (Silva et al., 2000, Huang et al., 2004). Thus, fermented cherry juice and broccoli puree, due to the selected bacterial bioconversion (Fig. 4), are enriched in phenolic derivatives with high human bioavailability, which may improve the inherent functional value of these fruit and vegetable matrices.

5. Conclusions

This study investigated the bioconversion of phenolic compounds in food fermentations. Phenolic acid metabolism contributes to the stress response of lactobacilli to adverse conditions; moreover, the catabolism of phenolic acids by obligately heterofermentative species is linked to the reoxidation of the reduced co-factors and thus provides an energetic advantage. Phenolics metabolism may thus confer a selective advantage for lactobacilli in vegetable and fruit

fermentation. This study also demonstrates that the metabolism of phenolic compounds is strongly dependent on the composition and intrinsic factors of fruits and vegetables; information derived from laboratory media is thus not fully transferable to food fermentations. Phenol derivatives may also exert biological activities. Therefore, bioconversion pathways highlighted in this study could be exploited for food and pharmaceutical purposes.

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503 **Legends to figures**

504 **Fig. 1.** Quantification protocatechuic acid (black bars) and catechol (white bars) in unfermented
505 cherry juice, in cherry juice incubated without bacterial inoculum (control), and in cherry juice
506 fermented with *Lactobacillus plantarum* C2, C5, CIL6, POM1, 1MR20, and TMW1.460,
507 *Lactobacillus spicheri* Lp38, *Lactobacillus fermentum* FUA3165 or *Lactobacillus reuteri*
508 FUA3168. Data are shown as means ± standard deviations of three independent experiments.
509 Significant differences ($P<0.05$) to unfermented cherry juice are indicated by an asterisk.

510 **Fig. 2.** Quantification caffeic and coumaric acids, and relative quantification of dihydrocaffeic and
511 phloretic acids in unfermented cherry juice, in cherry juice incubated without bacterial inoculum
512 (control), and in cherry juice fermented with *Lactobacillus plantarum* C2, C5, CIL6, POM1,
513 1MR20, and TMW1.460, *Lactobacillus spicheri* Lp38, *Lactobacillus fermentum* FUA3165 or
514 *Lactobacillus reuteri* FUA3168. **Panel A,** caffeic acid (black bars) and dihydrocaffeic acid (white
515 bars). **Panel B,** *p*-coumaric acid (black bars) and phloretic acid (white bars). Data are shown as
516 means ± standard deviations of three independent experiments. Significant differences ($P<0.05$) to
517 unfermented cherry juice are indicated by an asterisk.

518 **Fig. 3.** Quantification chlorogenic acid, and relative quantification of caffeic, quinic, and
519 dihydrocaffeic acids in unfermented broccoli purree, in chemically acidified broccoli purree
520 (control), and in broccoli puree fermented with *Lactobacillus plantarum* C2, C5, CIL6, POM1,
521 1MR20, and TMW1.460, *Lactobacillus spicheri* Lp38, *Lactobacillus fermentum* FUA3165 or
522 *Lactobacillus reuteri* FUA3168. Chlorogenic acid (black bars) was quantified by UHPLC-DAD,
523 relative quantification of caffeic acid (gray bars), quinic acid (gray hatched bars) and dihydrocaffeic
524 acid (white bars) was achieved by LC/MS. Data are shown as means ± standard deviations of three
525 independent experiments. Significant differences ($P<0.05$) to unfermented broccoli puree are
526 indicated by an asterisk.

527 **Fig. 4.** Schematic representation of the metabolic pathways of protocatechuic, *p*-coumaric,
528 chlorogenic and caffeic acids (for review see reference 1). Red arrows refer to presumptive

metabolic pathways adopted by *Lactobacillus* spp. strains during fermentation of of cherry juice (ChJ) and broccoli puree (BrP).

Figure 1 Quantification protocatechuic acid (black bars) and catechol (white bars) in unfermented cherry juice, in cherry juice incubated without bacterial inoculum (control), and in cherry juice fermented with *L. plantarum* C2, C5, CIL6, POM1, 1MR20, and TMW1.460, *L. spicheri* Lp38, *L. fermentum* FUA3165 or *L. reuteri* FUA3138. Data are shown as means ± standard deviations of three independent experiments. Significant differences (*P<0.05)* to unfermented cherry juice are indicated by an asterisk.

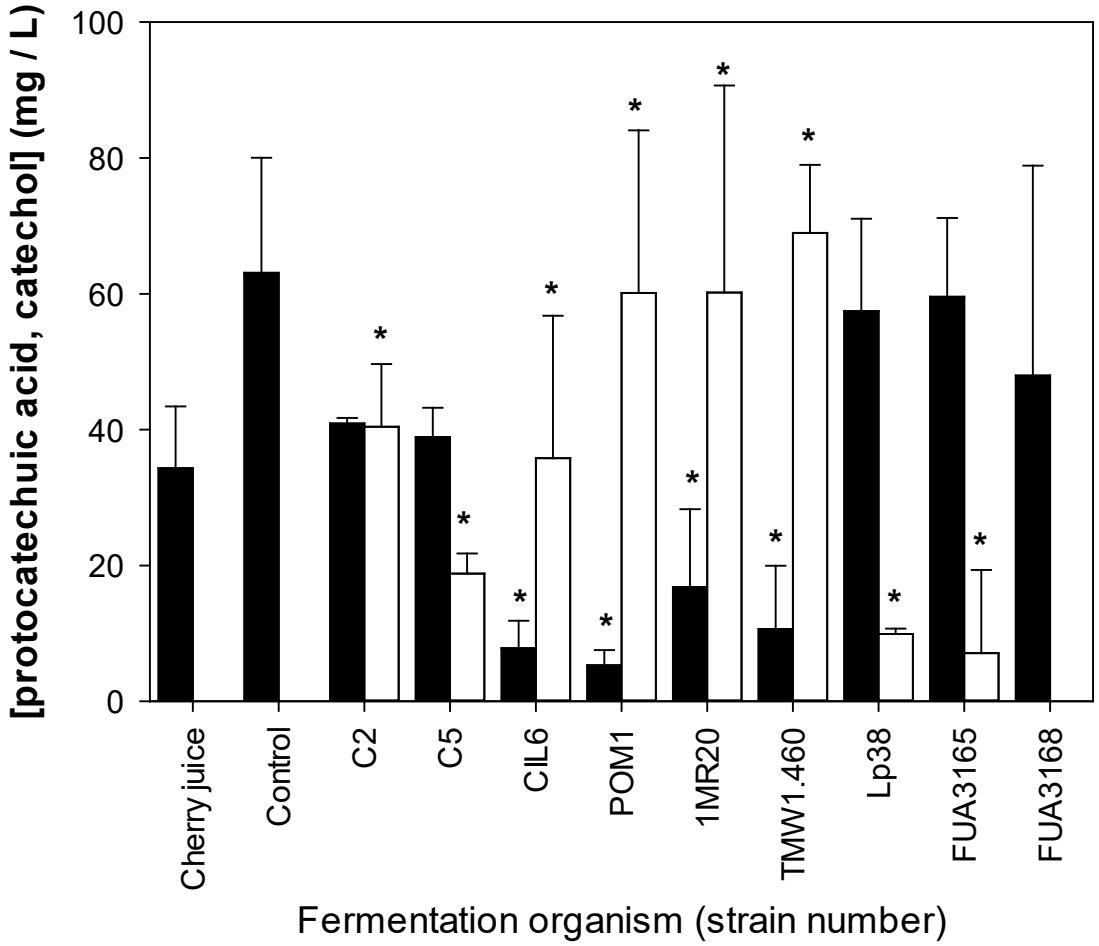

Figure 2. Quantification caffeic and coumaric acids, and relative quantification of dihydrocaffeic and phloretic acids in unfermented cherry juice, in cherry juice incubated without bacterial inoculum (control), and in cherry juice fermented with *L. plantarum* C2, C5, CIL6, POM1, 1MR20, and TMW1.460, *L. spicheri* Lp38, *L. fermentum* FUA3165 or *L. reuteri* FUA3138. **Panel A,** caffeic acid (black bars) and dihydrocaffeic acid (white bars). **Panel B,** *p*-coumaric acid (black bars) and phloretic acid (white bars). Data are shown as means ± standard deviations of three independent experiments. Significant differences (*P<0.05)* to unfermented cherry juice are indicated by an asterisk.

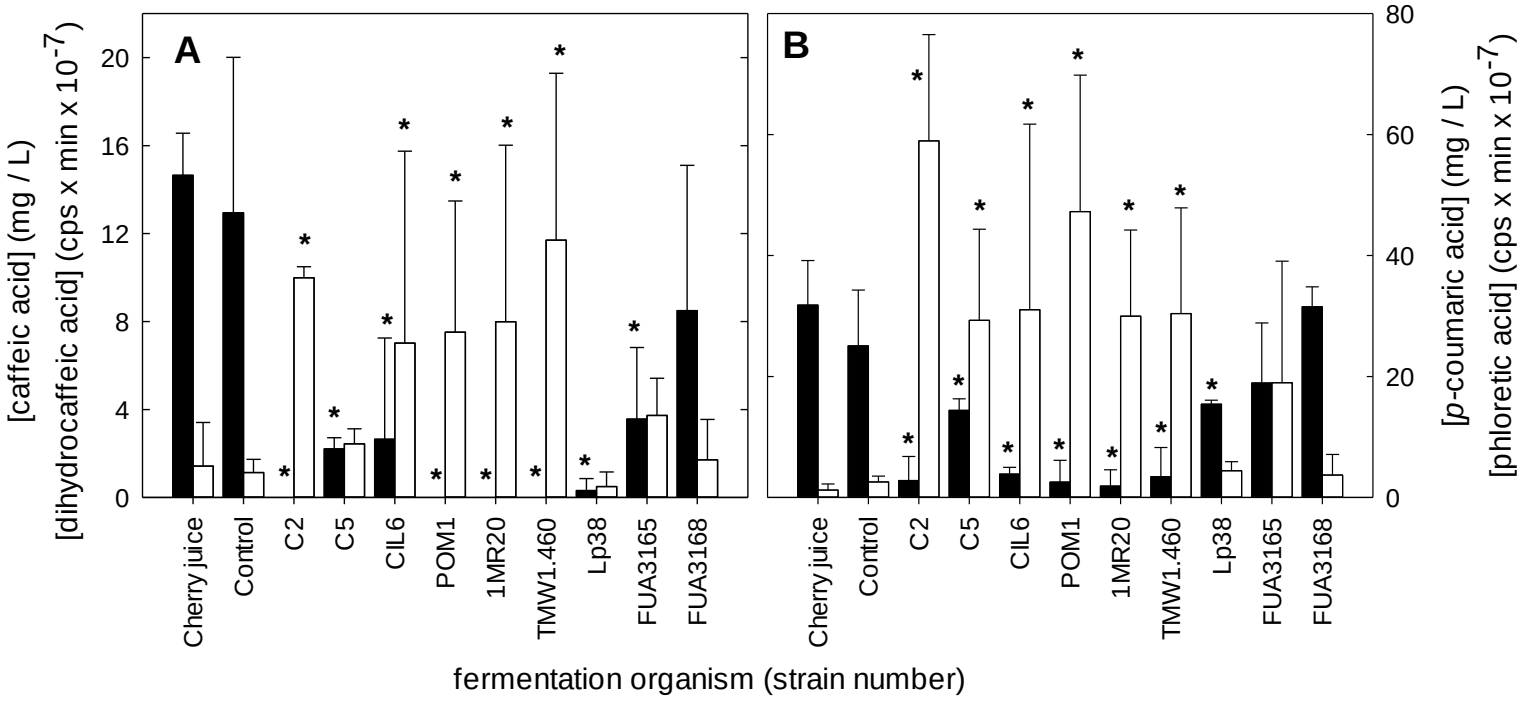

Fig. 3. Quantification chlorogenic acid, and relative quantification of caffeic, quinic, and dihydrocaffeic acids in unfermented broccoli purree, in chemically acidified broccoli purree (control), and in broccoli purree fermented with *L. plantarum* C2, C5, CIL6, POM1, 1MR20, and TMW1.460, *L. spicheri* Lp38, *L. fermentum* FUA3165 or *L. reuteri* FUA3138. Chlorogenic acid (black bars) was quantified by UHPLC-DAD, relative quantification of caffeic acid (gray bars), quinic acid (gray hatched bars) and dihydrocaffeic acid (white bars) was achieved by LC/MS. Data are shown as means ± standard deviations of three independent experiments. Significant differences (*P<0.05)* to unfermented broccoli purree are indicated by an asterisk.

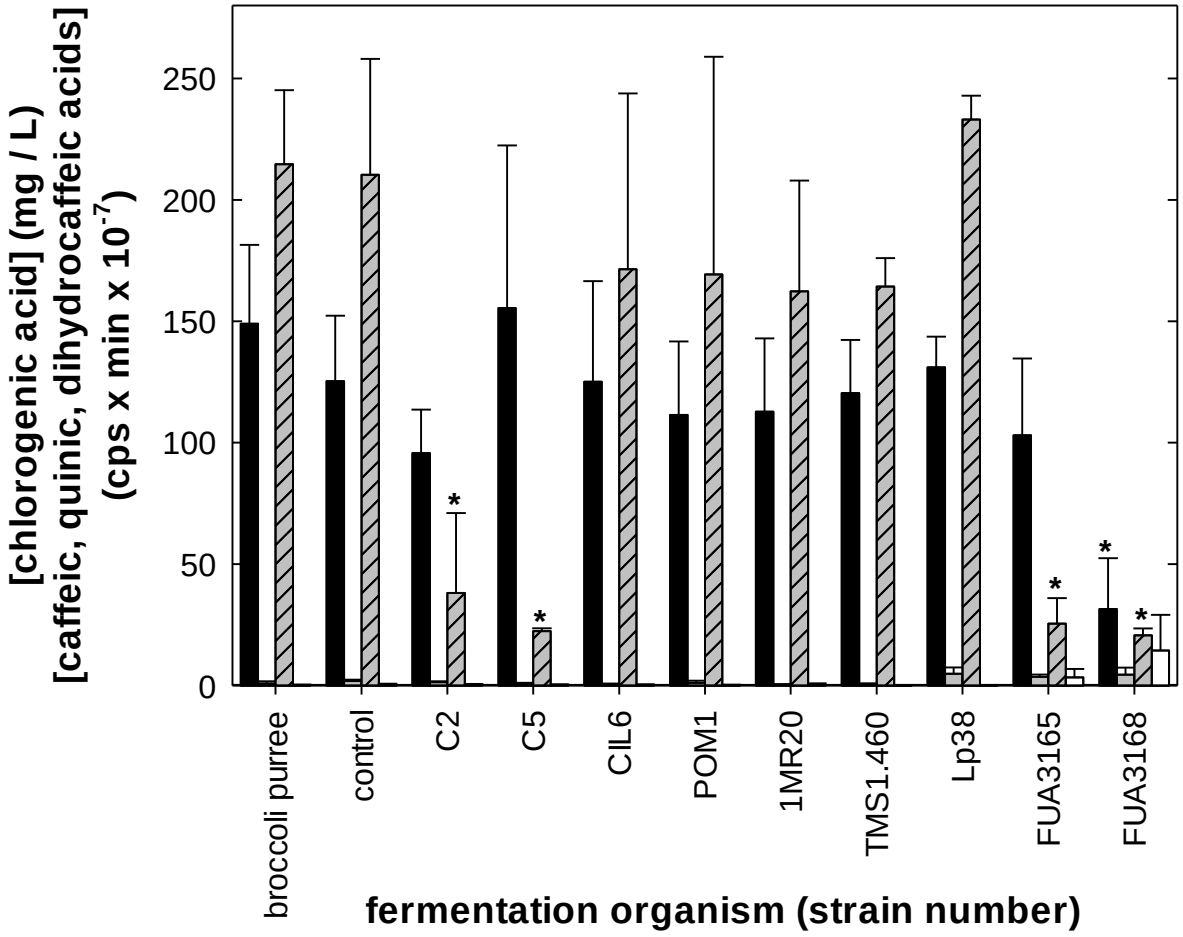

Figure 4 Schematic representation of the metabolic pathways of protocatechuic, *p*-coumaric, chlorogenic and caffeic acids (for review see Rodriguez et al., 2009). Red arrows refer to presumptive metabolic pathways adopted by *Lactobacillus* spp. strains during fermentation of cherry juice (ChJ) and broccoli puree (BrP).

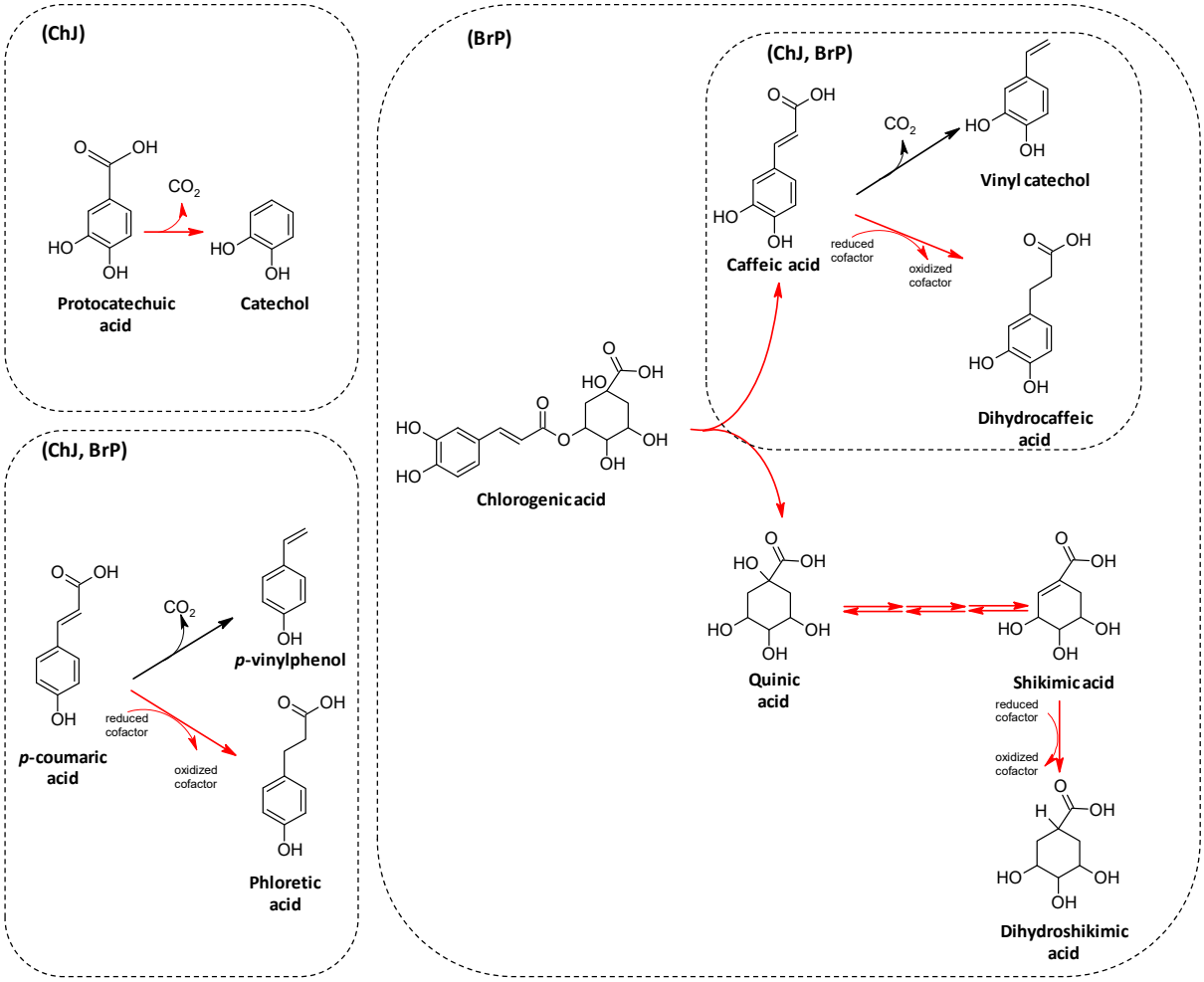

Table 1. LC-DAD-MS (Liquid Chromatography-Diode Array Detector-Mass Spectrometry) identification of phenolic compounds in extracts of unfermented cherry juice

Peak	Retention time (min)	Identity	Class of compound	*m/z* $[M-H]^-$	m/z MS^n	HPLC DAD (nm)
1	6.20	protocatechuic acid[a]	hydroxybenzoic acids	153	109	258, 293
2	7.87	neochlorogenic acid[b]	hydroxycinnamic acid derivatives	353	179, 191, 135	324
3	9.49	*p*-coumarolquinic acid[b]	hydroxycinnamic acid derivatives	337	163, 191	310
4	10.37	chlorogenic acid[a]	hydroxycinnamic acids derivatives	353	179, 191, 135	328, 325, 299
5	10.81	caffeic acid[a]	hydroxycinnamic acids	179	135	319
6	12.41	*p*-coumaric acid[a]	hydroxycinnamic acids	163	119	309
7	13.53	rutin[a]	flavonoids	609	301	352, 255
8	19.6	quercetin[a]	flavonoids	301	151	369

[a]Identification of compounds with external standards.

[b]Tentative identification of compounds on the basis of mass spectra and UV spectra reported in the literature.

Table 2. LC-DAD-MS (Liquid Chromatography-Diode Array Detector-Mass Spectrometry) identification of phenolic compounds in extracts of unfermented broccoli purree

Peak	Retention time (min)	Identity	Class of compound	*m/z* [M-H]$^-$	m/z MS^n	HPLC DAD (nm)
1	2.01	quinic acid[b]	Cyclohexane-carboxylic acids	191	190, 173	255
2	9.44	neochlorogenic acid[b]	hydroxycinnamic acid derivatives	353	179, 191, 135	324
3	11.12	p-coumarolquinic acid[b]	hydroxycinnamic acid derivatives	337	163, 191, 119	310
4	11.77	chlorogenic acid[a]	hydroxycinnamic acid derivatives	353	191, 179, 135	328, 325, 299
5	12.07	caffeic acid[a]	hydroxycinnamic acids	179	135	319
6	15.47	sinapic acid[a]	hydroxycinnamic acids	223		323, 327
7	12.21	sinapoylhexose[b]	hydroxycinnamic acid derivatives	367	161, 205, 193, 191, 179, 135	325, 328
8	13.25	sinapoylhexose[b]	hydroxycinnamic acid derivatives	367	161, 135, 179, 205	298, 313
9	18.13	1,2-disinapoyl gentiobiose[b]	hydroxycinnamic acids derivatives	753	223, 205, 193, 529, 175, 160	328
10	18.28	1-sinapoyl-2-feruloyl gentiobiose[b]	hydroxycinnamic acid derivatives	723	499, 205, 175, 223, 193, 208, 160	309
11	19.47	1,2,2'-trisinapoyl gentiobiose[b]	hydroxycinnamic acid derivatives	959	205, 223, 735, 529	352, 255
12	19.74	1,2' disinapyl-2-feruloyl gentiobiose[b]	hydroxycinnamic acid derivatives	929	205, 175, 223, 207, 160, 529, 705	369
13	20.01	1-sinapoyl-2, 2'-diferuloylgentiobiose[b]	hydroxycinnamic acid derivatives	899	175, 205, 223, 160, 193, 134	325, 328

[a]Identification of compounds with external standards.

[b]Tentative identification of compounds on the basis of mass spectra and UV spectra reported in the literature.

Table 3. LC-DAD-MS (Liquid Chromatography-Diode Array Detector-Mass Spectrometry) identification of phenolic acid metabolites of lactobacilli.

Retention time (min)	Identity	Class of compound	*m/z* $[M-H]^-$	m/z MS^n	HPLC DAD (nm)
5.77	catechol[a]	hydroxybenzoic acid derivatives	109	108	275
8.87	dihydrocaffeic acid[b]	hydroxycinnamic acids derivatives	181	137	279
11.00	phloretic acid[b]	hydroxycinnamic acids derivatives	165	147	276

[a]Identification of compounds with external standards.

[b]Tentative identification of compounds on the basis of mass spectra and UV spectra reported in the literature.

Table 4. Metabolism of protocatechuic, caffeic and *p*-coumaric acids *Lactobacillus* spp. strains in mMRS.

	Protocatechuic acid		**Caffeic acid**		***p*-Coumaric acid**	
	% substrate degradated	**metabolites (% of products)**	**% substrate degradated**	**metabolites (% of products)**	**% substrate degradated**	**metabolites (% of products)**
L. plantarum C2	55 ± 29	n.d.	100 ± 0	vinyl catechol (97 ± 1) dihydrocaffeic acid (3 ± 0.1)	41 ± 2	*p*-vinylphenol (92 ± 4); phloretic acid (8 ± 0)
L. plantarum C5	100 ± 1	catechol (100 ± 23)	100 ± 0	vinyl catechol (100 ± 75)	34 ± 27	*p*-vinylphenol (86 ± 36); phloretic acid (14 ± 6)
L. plantarum CIL6	100 ± 0	catechol (100 ± 2)	100 ± 0	vinyl catechol (80 ± 8) ethylcatechol (20 ± 2)	94 ± 3	*p*-vinylphenol (95 ± 14); phloretic acid (5 ± 0)
L. plantarum POM1	100 ± 0	catechol (100 ± 10)	100 ± 0	vinyl catechol (100 ± 0)	98 ± 1	*p*-vinylphenol (93 ± 19); phloretic acid (7 ± 1)
L. plantarum 1MR20	100 ± 0	catechol (100 ± 71)	100 ± 0	vinyl catechol (100 ± 31)	0 ± 22	*p*-vinylphenol (tr.); phloretic acid (tr)
L. plantarum TMW 1.460	100 ± 0	catechol (100 ± 61)	100 ± 0	vinyl catechol (93 ± 54) dihydrocaffeic acid (7 ± 5.1)	95 ± 3	*p*-vinylphenol (92 ± 9); phloretic acid (8 ± 3)
L. spicheri Lp38	63 ± 5	n.d.	88 ± 1	vinyl catechol (100 ± 3)	22 ± 8	*p*-vinylphenol (100 ± 1)
L. fermentum FUA3165	56 ± 37	n.d.	100 ± 0	vinyl catechol (12 ± 6) dihydrocaffeic acid (88 ± 39)	22 ± 30	phloretic acid (tr.)
L. reuteri FUA3168	68 ± 12	n.d.	1 ± 13	n.i. (100 ± 17) dihydrocaffeic acid (tr.)	4 ± 14	*p*-vinylphenol (100 ± 2); phloretic acid (tr.)

Data are shown as means ± standard deviations of three independent experiments.

n.i., not identified; tr., traces.

Table S1. Cell density (Log cfu/ml), pH values and concentration (mM) of organic acids (lactic, acetic and malic acids) of cherry juice (ChJ) and broccoli puree (BrP) fermented for 24 h at 34°C with *Lactobacillus* spp.

Lactobacillus spp.	Cell count (Log CFU/ml)		pH		Lactic acid (mM)		Acetic acid (mM)		Malic acid (mM)	
	ChJ	BrP	ChJ	BrP	ChJ	BrP	ChJ	BrP	ChJ	BrP
unfermented	-	-	4.01 ± 0.28^{b}	6.51 ± 0.03^{a}	n.d.	4 ± 0^{c}	n.d.	4 ± 5^{c}	464 ± 27^{a}	21 ± 15^{a}
L. plantarum C2	9.13 ± 0.16^{a}	9.51 ± 0.08^{a}	3.72 ± 0.05^{cd}	3.47 ± 0.10^{d}	108 ± 14^{ab}	185 ± 30^{a}	tr.	5 ± 2^{c}	406 ± 14^{ab}	2 ± 0^{b}
L. plantarum C5	9.32 ± 0.24^{a}	9.51 ± 0.34^{a}	3.62 ± 0.11^{d}	3.51 ± 0.02^{d}	78 ± 14^{abc}	172 ± 21^{a}	tr.	6 ± 3^{c}	425 ± 19^{ab}	6 ± 2^{ab}
L. plantarum CIL6	9.16 ± 0.35^{a}	9.53 ± 0.07^{a}	3.83 ± 0.01^{bc}	3.45 ± 0.06^{d}	135 ± 33^{a}	185 ± 31^{a}	tr.	5 ± 3^{c}	407 ± 13^{ab}	1 ± 0^{b}
L. plantarum POM1	9.28 ± 0.29^{a}	9.44 ± 0.13^{a}	3.89 ± 0.05^{bc}	3.45 ± 0.10^{d}	132 ± 26^{a}	185 ± 34^{a}	tr.	5 ± 3^{c}	394 ± 20^{b}	2 ± 1^{b}
L. plantarum 1MR20	8.96 ± 0.27^{a}	9.22 ± 0.11^{a}	3.90 ± 0.02^{bc}	3.46 ± 0.05^{d}	133 ± 28^{a}	176 ± 28^{a}	tr.	5 ± 4^{c}	398 ± 14^{ab}	4 ± 1^{ab}
L. plantarum TMW1.460	9.20 ± 0.25^{a}	9.50 ± 0.20^{a}	3.91 ± 0.17^{bc}	3.43 ± 0.06^{d}	134 ± 19^{a}	186 ± 30^{a}	tr.	4 ± 3^{c}	402 ± 21^{ab}	1 ± 0^{b}
L. spicheri Lp38	9.27 ± 0.34^{a}	8.93 ± 0.13^{b}	4.28 ± 0.24^{a}	4.02 ± 0.06^{c}	87 ± 24^{abc}	74 ± 9^{b}	tr.	9 ± 2^{bc}	388 ± 22^{b}	3 ± 1^{ab}
L. fermentum FUA3165	8.81 ± 0.37^{a}	9.15 ± 0.03^{ab}	3.96 ± 0.15^{b}	4.28 ± 0.23^{b}	65 ± 26^{bc}	53 ± 12^{bc}	tr.	19 ± 7^{ab}	387 ± 41^{b}	1 ± 0^{b}
L. reuteri FUA3168	9.06 ± 0.28^{a}	9.16 ± 0.13^{ab}	3.83 ± 0.17^{bc}	4.24 ± 0.21^{b}	27 ± 8^{c}	60 ± 13^{bc}	tr.	22 ± 4^{a}	432 ± 20^{a}	0 ± 0^{b}

Data are shown as means ± standard deviations of three independent experiments.

n.d., not detected; tr., traces.

$^{a–d}$, means within the column with different superscript letters are significantly different ($P<0.05$).