1 **Characterization of isogenic mutants with single or double deletions of four phenolic acid**

2 **esterases in *Lactiplantibacillus plantarum* TMW1.460**

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8 **Running title:** Phenolic acid esterases in *Lp. plantarum*

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**Abstract**

In plants, hydroxycinnamic and hydroxybenzoic acids occur mainly as esters. This study aimed to determine the contribution of individual phenolic acid esterases in *Lp. plantarum* TMW1.460, which encodes for four esterases: TanA, Lp_0796, Est_1092 and a homologue of Lj0536 and Lj1228 that was termed HceP. To determine which of the phenolic acid esterases present in *Lp plantarum* TMW1.460 are responsible for esterase activity, mutants with deletions in *lp_0796, est_1092, tanB, hceP*, or *hceP* and *est_1092* were constructed. The phenotype of wild type strain and mutants was determined with esters of hydroxycinnamic acids (chlorogenic acid and ethyl ferulate) and of hydroxybenzoic acids (methyl gallate, tannic acid and epigallocatechin-3-gallate). *Lp. plantarum* TMW1.460 hydrolysed chlorogenic acid, methyl ferulate and methyl gallate but not tannic acid or epigallocatechin gallate. The phenotype of mutant strains during growth in mMRS differed from the wild type as follows: *Lp. plantarum* TMW1.460Δ*hceP* did not hydrolyse esters of hydroxycinnamic acids; *Lp. plantarum* TMW1.460Δ*tanB* did not hydrolyse esters of hydroxybenzoic acids; disruption of *est_1092* or *Lp_0796* did not alter the phenotype. The phenotype of *Lp. plantarum* TMW1.460ΔΔ*hceP/est_1092* was identical to *Lp. plantarum* TMW1.460Δ*hceP*. The metabolism of phenolic acids during growth of the mutant strains in broccoli puree and wheat sourdough did not differ from metabolism of the wild type strain. In conclusion, esters of hydroxycinnamic and hydroxybenzoic acids each are hydrolysed by dedicated enzymes. The hydroxycinnamic acid esterase HceP is not expressed, or not active during growth of *Lp. plantarum* TMW1.460 in all food substrates.

**Keywords**

Phenolic acid esterase, tannase, ferulic acid esterase, chlorogenic acid, sourdough, *Lactiplantibacillus plantarum, Lactobacillus.*

44 **1. Introduction**

45 Phenolic compounds contribute to the sensory and nutritional properties of plant foods. Phenolic
46 acids are among the major phenolic compounds in fruits, vegetables, and cereals (Acosta-Estrada
47 et al., 2014). Phenolic acids have antimicrobial activity, and their metabolism can alter the sensory
48 properties of a fermented foods (Kobue-Lekalake et al., 2007; Sánchez-Maldonado et al., 2011).
49 Phenolic acids are bound to cell wall polysaccharides in various cereals and plants such as wheat,
50 rye, kidney beans and cruciferous vegetables (Shahidi and Yeo, 2018; Zhang et al., 2020). Esters
51 of phenolic acids with monosaccharides and alcohols, and ether linkages are also common (Shahidi
52 and Yeo, 2018). Dietary phenolic acids have been linked with beneficial effects on gut health and
53 free phenolic acids have been shown to have anti-inflammatory and anti-obesity effects in animal
54 models (Leonard et al., 2021).

55 Fermentation of plant foods with lactic acid bacteria increases the content of free phenolic acids
56 through activity of hydroxycinnamic acid esterases (EC 3.1.1.73), hydroxybenzoic acid esterases
57 (EC 3.1.1.20) and glycosyl hydrolases (Gänzle, 2019; Ripari et al., 2019; Svensson et al., 2010).
58 *Lactiplantibacillus plantarum* is one of the major organisms involved in fermentation of plant
59 foods including vegetables, cereal fermentation, but this organism also occurs in the human
60 intestinal tract and in insect associated persistence niches (Gänzle, 2022, 2019; Martino et al.,
61 2016). This nomadic lifestyle is thought to contribute towards the metabolic flexibility of the
62 species making it a suitable candidate for use as starter cultures and probiotics (Duar et al., 2017;
63 Pswarayi et al., 2022; Zheng et al., 2020).

64 The phenolic acid metabolism of *Lp. plantarum* has been studied extensively. Enzymes that are
65 biochemically characterized from this species include phenolic acid decarboxylases, reductases,
66 tannases and esterases (Gaur et al., 2020; Muñoz et al., 2017; Santamaría et al., 2018a, 2018b).

67 Two hydroxycinnamic acid esterases and two hydroxybenzoic acid esterases have been identified
68 in *Lp. plantarum* (Table 1) (Curiel et al., 2009; Esteban-Torres et al., 2015, 2013; Iwamoto et al.,
69 2008; Jiménez et al., 2014). In addition, two distinct cinnamoyl esterases (Lj0536 and Lj1228)
70 were identified in *Lactobacillus johnsonii* and other lactobacilli including *L. helveticus, L.*
71 *acidophilus* and *Limosilactobacillus fermentum* (Table1) (Kim and Baik, 2015; Lai et al., 2009;
72 Liu et al., 2016; Song and Baik, 2017) and biochemically characterized. These biochemically
73 characterized enzymes display overlapping substrate specificities and many genomes of *Lp.*
74 *plantarum* encode for multiple esterases, making it difficult to ascertain the contribution of specific
75 genes or enzymes to *in situ* activity in food fermentations. In addition, the substrates that were
76 used for biochemical characterization of phenolic acid esterases of lactobacilli (Table1) represent
77 only a small portion of the diverse esters of hydroxycinnamic and hydroxybenzoic acids that are
78 present in plants (Shahidi and Yeo, 2018). Experiments with isogenic pairs of strains with deletions
79 in one or multiple phenolic acid esterases are suitable to address this knowledge gap, however,
80 such experiments have not been reported in the scientific literature. This work therefore aimed to
81 identify putative phenolic acid esterases in *Lp. plantarum* TMW1.460 and study their role in
82 release of phenolic acids. Deletion mutants lacking genes coding for phenolic acid esterases were
83 constructed and tested for their esterase activity on different phenolic acid esters. Selected mutants
84 were further used in model food fermentations to characterize their esterase activity and its
85 influence on phenolic acid metabolite production.

86 **2. Materials and Method**

87 **2.1 Bacterial strains and growth conditions**

88 *Lactiplantibacillus plantarum* TMW1.460 and *Escherichia coli* EC1000 were streaked onto
89 modified De Man, Rogosa and Sharpe (mMRS) (Zhao and Gänzle, 2018) and Luria–Bertani (LB)

90 agar plates respectively from -80°C glycerol stocks, followed by subculturing in their respective
91 liquid media. Strains of *Lp. plantarum* were grown at 30°C under microaerophilic conditions,
92 while *E. coli* strains were grown at 37°C under aerobic conditions. MRS and LB media containing
93 5 mg/L and 300 mg/L erythromycin respectively were used where applicable. Strains and plasmids
94 used in this study are listed in Table 2.

95 **2.2 Chemicals**

96 Chlorogenic acid, methyl ferulate, methyl gallate, tannic acid, epigallocatechin gallate (EGCG),
97 pyrogallol, gallic acid and erythromycin (Em) were purchased from Millipore Sigma (St. Louis,
98 MO, USA). Ferulic acid and caffeic acid were purchased from Extrasynthèse (Genay, France);
99 dihydroferulic acid and dihydrocaffeic acid were obtained from MP Biomedicals (Illkrich, France).
100 Polyethylene glycol (PEG) 1500 was obtained from Thermo Scientific (Mississauga, ON,
101 Canada). MRS, LB media and agar were purchased from BD (Sparks, MD, USA). Ingredients
102 required for making mMRS media were obtained from BD (Sparks, MD, USA) or Millipore Sigma
103 (St. Louis, MO, USA).

104 **2.3 *In silico* identification of phenolic acid esterases in strains of *Lp. plantarum***

105 Genome sequence and protein fasta files of *Lp. plantarum* TMW1.460 (GCA_009864015.1) and
106 query sequences listed in Table 1 were downloaded from NCBI. Protein BLAST was performed
107 using query sequences with cut-off values of 80% query cover and 40% amino acid (aa) identity.
108 Nucleotide sequences of best match hit for each query were then used for primer design and genetic
109 manipulations (Table 3).

110 Whole genome nucleotide sequences of all *Lp. plantarum* strains (328) assembled at chromosomal
111 level (as of January 2022) were downloaded from NCBI database and reannotated using Prokka 1.13.7

112 (Seemann, 2014). In addition, genome sequences of *Lp. plantarum* TMW1.460 (Gaur et al., 2020),
113 *Lp. plantarum* FUA3584 and FUA3590 (Pswarayi and Gänzle, 2019), and 16 previously sequenced
114 lab strains were also used in the analysis (Table S1). Because the query sequence originates from the
115 bacterial species that was analysed, the presence or absence of genes were determined by performing
116 protein BLAST on each genome with cut-off values of 75% query cover and 70% amino acid (aa)
117 identity. In addition to the query sequences listed in Table 1, the sequence of Lp_2953 from *Lp.*
118 *plantarum* WCFS1 (YP_004890534.1) was used as a query sequence.

119 **2.4 Construction of phenolic acid esterase knockout mutants**

120 Upstream and downstream flanking regions (800-1000 bp) of identified esterases were PCR
121 amplified along with the plasmid backbone of pVPL3002 (Zhang et al., 2018). Ligation and
122 plasmid assembly was performed using Gibson assembly (Gibson et al., 2009). Plasmid constructs
123 were then transformed into electrocompetent *E. coli* EC1000 host cells (Leenhouts et al., 1996)
124 and plated on LB plates containing erythromycin (300 mg/L) after recovery. Transformants were
125 confirmed using colony PCR and subcultured overnight. pVPL3002 plasmid constructs were
126 isolated and purified using GeneJET Plasmid Miniprep Kit (Thermo Scientific) following
127 manufacturers instructions.

128 Electrocompetent cells of *Lp. plantarum* TMW1.460 and derivatives were prepared by washing
129 overnight cultures 2-3 times with ice cold 10% PEG 1500, followed by transformation with 2 µg
130 of plasmid DNA (2.5 kV, 400 Ω, 25 µF). Cells were recovered for 2-3 h in MRS medium, plated
131 on MRS containing erythromycin (5 mg/L) and incubated for 48-72 h. Erythromycin resistant
132 colonies were then cultured for a minimum of 2 passages at 42°C, followed by plating on
133 erythromycin-MRS plates to obtain single crossover colonies (SCO). To obtain double cross over
134 (DCO), washed cells were cultured in MRS medium containing no antibiotics for 10-20 passages.

135 Mutants were screened by replica plating on MRS and erythromycin-MRS plates. Erythromycin
136 sensitive colonies were then screened by colony PCR and mutants were confirmed by Sanger
137 sequencing.

138 **2.5 Characterization of phenolic acid esterase activity using HPLC**

139 Samples for HPLC were prepared with triplicate independent cultures using the protocol by
140 Svensson et al., 2010. Briefly, mMRS media was supplemented with 1mM of chlorogenic acid,
141 methyl ferulate, methyl gallate, tannic acid and EGCG. Overnight cultures of wild type and mutant
142 strains (10%) were inoculated into mMRS broth supplemented with phenolic acid esters and
143 incubated at 30°C for 1 or 10 d. Samples were then centrifuged, and supernatant was acidified
144 using hydrochloric acid to pH 1.5. Solvent extraction (2X) was performed using half volume ethyl
145 acetate followed by filtration.

146 Esters of hydroxycinnamic and hydroxybenzoic acids were quantified using an Agilent 1200 series
147 HPLC system equipped with a multi-wavelength UV detector and Eclipse XDB C-18 column
148 (4.6 by 150 mm; 5 μm). The column was eluted by gradient elution with 0.1% formic acid in water
149 (phase A) and 0.1% formic acid in 90% acetonitrile-water (v/v) (phase B) as described previously
150 by Gaur et al., 2020.

151 **2.6 Fermentation of whole wheat sourdough and broccoli purée**

152 Whole wheat flour and fresh broccoli were purchased from a local supermarket. Overnight cultures
153 of *Lp. plantarum* TMW1.460 wild type, TMW1.460 Δ*tanB* and TMW1.460 ΔΔ*hceP/est_1092*
154 were washed and resuspended in sterile tap water to a cell counts of about $10^8$ CFU/mL. Blended
155 broccoli and whole wheat flour (10g) were mixed with 10 mL of inoculum and incubated at 30°C
156 for 24 h along with chemically acidified controls. They were prepared by adding sterile tap water

157 and adjusting the pH using lactic acid for final volume of 10 mL. Experiments were performed
158 with three independent replicates.

159 After 24 h of fermentation, 1g of fermented samples were mixed with 9 mL of sterile DI water for
160 pH measurements along with unfermented controls. mMRS plates were plated with 10-fold serial
161 dilutions of fermented and unfermented samples prepared in 0.1% peptone water and incubated
162 for 24-48 h for cell counts. Remaining samples were freeze dried and stored at -20°C for further
163 analysis.

164 **2.7 Quantification of free phenolic metabolites in fermentation samples**

165 Extraction of free phenolics was performed using the protocol described by Ripari et al., 2019.
166 Briefly, solvent extraction was performed twice on freeze dried fermented samples (250 mg) using
167 1 mL of 80% ethanol. Collected supernatants were mixed and evaporated under nitrogen.
168 Precipitate obtained was dissolved in acetic acid (2%) with pH of the solution adjusted to 2 for a
169 final volume of 500 µL, followed by solvent extraction using ethyl acetate. Extract was once again
170 evaporated under nitrogen followed by resuspension of solids in 200-400 µL of 100% methanol
171 (0.1% formic acid).

172 Extracts containing free phenolic compounds were separated using the same HPLC, column and
173 solvent system as described in section 2.5. The gradient used was as follows- 10% to 42% B (35
174 min), 42%-48% B (15 min), 48% to 10% B (1 min) and isocratic with 10% B (14 min).
175 Quantification was performed using external standards at 280 and 330 nm. Injection volume was
176 10 µL with a flow rate of 0.3 mL/min.

177 **3. Results**

178 **3.1 Phenolic acid esterases in *Lp. plantarum* TMW1.460**

179 Four phenolic esterases have been characterized in *Lp. plantarum* including 3 intracellular
180 esterases and one extracellular tannase. Protein BLAST results revealed the presence of only
181 intracellular esterases in *Lp. plantarum* TMW1.460 while extracellular TanA was absent (Table 1).
182 Lp_0796 and Est_1092 are characterized as a hydroxycinnamic acid esterases but Est_1092 also
183 displayed activity on esters of hydroxybenzoic acids. TanB is characterized as an intracellular
184 tannase. All the three enzymes showed almost 100% amino acid identity to the query sequences
185 from other strains of *Lp. plantarum*. The genome of *Lp. plantarum* TMW1.460 also encoded an
186 additional uncharacterized phenolic acid esterase which shares 53% amino acid identity with 2
187 homologous phenolic acid esterases (Lj0536 and Lj1228) in *L. johnsonii,* hereafter referred as
188 HceP (Lai et al., 2009).

189 **3.2 Phenotypic characterization of phenolic acid esterase activity and metabolism**

190 To confirm which of the phenolic acid esterases present in *Lp plantarum* TMW1.460 are
191 responsible for the esterase activity, mutants with deletions in *lp_0796, est_1092, tanB,* or *hceP*,
192 or *hceP* and *est_1092* were constructed. The wild type strain and the mutants were inoculated in
193 mMRS supplemented with hydroxycinnamic and hydroxybenzoic acid esters and incubated at
194 30°C for 1 or 10 d. The wild type strain showed esterase activity on esters of both hydroxycinnamic
195 and hydroxybenzoic acids (Table 4). Chlorogenic acid, methyl ferulate and methyl gallate were all
196 hydrolyzed to release caffeic acid, ferulic acid and gallic acid respectively.

197 *Lp. plantarum* TMW1.460 Δ*lp_0796* and TMW1.460 Δ*est_1092* had the same phenotype as the
198 wild type strain with respect to phenolic acid esterase activity. *Lp. plantarum* TMW1.460 Δ*tanB*
199 also hydrolysed hydroxycinnamic acid esters but did not hydrolyze methyl gallate to gallic acid.
200 Conversely, the strain *Lp. plantarum* TMW1.460 Δ*hceP* only hydrolyzed methyl gallate but did
201 not hydrolyse chlorogenic acid or methyl ferulate. *Lp. plantarum* TMW1.460 Δ*hceP*/Δ*est_1092*

202 showed the same phenotype as the TMW1.460 Δ*hceP* mutant strain under all conditions tested.
203 None of the strains hydrolyzed epigallocatechin gallate and tannic acid. Pyrogallol, the
204 decarboxylation metabolite of gallic acid was also detected for all samples in which methyl gallate
205 was hydrolyzed to gallic acid. However, no metabolites of released hydroxycinnamic acids were
206 detected for any of the strains after a 24 h incubation.

207 Since the phenolic acid esterases present in *Lp plantarum* TMW1.460 have been previously
208 characterized as intracellular enzymes, strains were also incubated for a period of 10 d to observe
209 any possible differences in metabolic activity. None of the strains showed any change in the
210 metabolism after extended incubation for any of the substrates except ferulic acid. Dihydroferulic
211 acid was detected in all strains which showed esterase activity on methyl ferulate after 10 d
212 incubation.

213 **3.3 Characterization of esterase activity and phenolic acid metabolism in food fermentations**

214 To determine the influence of phenolic acid esterases on metabolite formation during food
215 fermentations, the wild type strain, and the mutants *Lp. plantarum* TMW1.460 Δ*tanB* and
216 TMW1.460 Δ*hceP*/Δe*st_1092* were used for fermentation of broccoli and whole wheat flour. The
217 cell counts after 24 h fermentation were 9-9.5 log CFU/mL with pH value dropping to 3.75 and
218 3.45 for broccoli puree and whole wheat sourdough respectively (Table 5).

219 The major phenolic acid metabolites detected in broccoli fermentations were dihydrocaffeic acid
220 and dihydrosinapic acid. Dihydroferulic acid was the major metabolite detected in in whole wheat
221 sourdoughs (Figure 1). No significant differences were observed among the wild type and mutant
222 strains for both fermentation substrates used in this study.

223 **4. Discussion**

224 Phenolic acid esterase identified in lactobacilli include TanB of *Lp. plantarum* ATCC 14917, the
225 extracellular tannase TanA, and the hydroxycinnamic acid esterases Lp_0796 and Est_1092 that
226 were characterized in *Lp. plantarum* (Curiel et al., 2009; Esteban-Torres et al., 2015, 2013;
227 Iwamoto et al., 2008; Jiménez et al., 2014). Strains of *Lp. plantarum* also possess a homolog of a
228 cinnamoyl esterase (this study). The presence of multiple phenolic acid esterases with potentially
229 overlapping substrate specificity in a single strain makes the accurate estimation of genotype and
230 phenotype relationships challenging. In addition, the characterization of heterologously expressed
231 proteins may accurately predict the *in situ* activity of enzymes in food fermentations. This study
232 therefore characterized the role of phenolic acid esterases in *Lp. plantarum* TMW1.460 by
233 characterization of isogenic mutants.

234 The hydroxycinnamic acid esterase Lp_0796 was characterized in *Lp. plantarum* WCFS1. Cell
235 free extracts of this strain exhibited a very low activity on methyl esters of ferulic and *p*-coumaric
236 acid while whole cells of this strain did not hydrolyze these substrates (Esteban-Torres et al., 2013).
237 Est_1092 is also active on methyl esters of hydroxycinnamic acids (Esteban-Torres et al., 2015).
238 The genome of *Lp. plantarum* TMW1.460 encodes for both Lp_0796 and Est_1092. *Lp. plantarum*
239 TMW1.460 Δ*lp_0796* and TMW1.460 Δ*est_1092* mutants showed the same phenotype as the wild
240 type strain, indicating that another hydroxycinnamic acid esterase is present.

241 The genome of *Lp. plantarum* TMW1.460 also encodes for a homolog of the *L. johnsonii*
242 cinnamoyl esterases Lj0536 and Lj1228 which we termed as HceP. Lj0536 was active only on
243 ethyl ferulate while Lj1228 was also hydrolysed esters of other hydroxycinnamic acids (Lai et al.,
244 2009). The genomes of *L. gasseri* and *Limosilactobacillus reuteri* also encode for homologs of
245 HceP and *L. gasseri* and *Lm. reuteri* displayed chlorogenic acid esterase activity in sunflower flour

246 and broccoli puree, respectively (Filannino et al., 2015; Fritsch et al., 2016). *Lp. plantarum*
247 TMW1.460 Δ*hceP* lost esterase activity with methyl ferulate and chlorogenic acid as substrates.

248 *Lp. plantarum* TMW1.460 thus possess three hydroxycinnamic acid esterases but only deletion of
249 *hceP* resulted in the loss of esterase activity in laboratory media. In *Lp. plantarum* DSM1055,
250 *est_1092* and *hceP* were over-expressed in response to methyl-ferulate while *lp_0796* was down-
251 regulated (Esteban-Torres et al., 2015). However, Lp_0796 was not over-expressed during growth
252 of *Lp. plantarum* in cereal substrates while HceF, the homologue to HceP in *Lm. fermentum*, was
253 over-expressed during growth in millet malt (Pswarayi et al., 2022). Current data on the expression
254 of phenolic acid esterases is too limited to differentiate whether these differences in gene
255 expression relate to differences between strains, or between different substrates.

256 The intracellular tannase TanB was characterized in *Lp. plantarum* ATCC 14917 (Curiel et al.,
257 2009; Iwamoto et al., 2008). The extracellular tannase TanA was required for esterase activity by
258 whole cells (Jiménez et al., 2014). *Lp. plantarum* TMW1.460 only encodes for TanB which
259 explains the absence of activity on tannic acid and EGCG in growing cultures. The wild type strain
260 but not its TanB-negative mutant released gallic acid from methyl gallate (this study), confirming
261 its role as a hydroxybenzoic acid esterase (Reverón et al., 2017).

262 To determine which of the esterases that were identified in the genome of *Lp. plantarum*
263 TMW1.460 are part of the core genome of the species, we analysed their presence in 345 strains
264 of *Lp. plantarum*. These analyses revealed that the hydroxycinnamic acid esterases Lp_0796 and
265 HceP, and the hydroxybenzoic acid esterase TanB are present in > 99% of the 345 strains analyzed.
266 The extracellular tannase TanA and the hydroxycinnamic acid esterase Est_1092 are accessory
267 genes present in only 14% and 16% of *Lp. plantarum* strains respectively (Table 6).

268 Fermentation of food substrates with strains of *Lp. plantarum* demonstrated phenolic acid
269 metabolism including esterase activities on chlorogenic acid and ferulic acid esters (Filannino et
270 al., 2015; Hole et al., 2012; Ripari et al., 2019). To confirm that phenolic acid esterases
271 characterized in this study are responsible for *in situ* activity, strains were fermented in broccoli
272 puree and whole wheat sourdough (this study), i.e. substrates for which prior data for strain *Lp.*
273 *plantarum* TMW1.460 is available (Filannino et al., 2015; Ripari et al., 2019). Fermentation with
274 wild type and mutant strains equally increased the concentration of metabolites from free phenolic
275 acid, suggesting that the disruption of the phenolic esterase genes did not alter the phenotype of
276 the strains. Unfermented broccoli is rich in sinapic acid esters and chlorogenic acid while whole
277 wheat flour is rich in bound ferulic acid (Filannino et al., 2015; Ripari et al., 2019). Our results
278 match the data of previous studies with high concentrations of dihydrocaffeic acid and
279 dihydrosinapic acid detected in broccoli fermentations and dihydroferulic acid in whole wheat
280 sourdoughs. Decarboxylated metabolites for *p*-coumaric acid and ferulic acid were below
281 detection limit, while vinyl catechol was not identified due to lack of the external standard.

282 Why does the *in situ* phenotype of esterase mutants not match the *in vitro* phenotype (this study)
283 or the activity of the respective enzymes (Table 1)? Plant substrates appear to impact gene
284 expression with stimuli that remain to be determined. for example, *Levilactobacillus hammesii*
285 DSM16341 metabolized hydroxybenzoic acids in wheat sourdoughs but not in rye malt
286 sourdoughs (Ripari et al., 2019). Similarly, fermentation of cherry juice with strains of *Lp.*
287 *plantarum* TMW1.460 resulted in higher concentrations of phenylpropionic acid, while vinyl- and
288 ethyl-derivatives were the major metabolites upon culture in mMRS (Filannino et al., 2015). This
289 indicates that phenolic acid metabolism enzymes are differentially expressed in different food

290 systems but the chemical cues that regulate expression of enzymes involved in metabolism of
291 phenolic compounds by lactobacilli are unknown.

292 Strains of *Lp. plantarum* displayed strain specific activity in altering quinic acid concentrations in
293 broccoli fermentation, but chlorogenic acid concentrations remained unchanged (Filannino et al.,
294 2015; Ye et al., 2019). Concentrations of caffeic acid and its metabolites also remained unchanged
295 in broccoli samples fermented with *Lp. plantarum* TMW1.460 and its Δ*est_1092*/Δ*hceP* esterase
296 mutant strain (this study). *Lm. reuteri* FUA3168, which also encodes for an HceP homolog
297 significantly reduced chlorogenic acid concentrations in broccoli fermentation (Filannino et al.,
298 2015). This variation in metabolic activity may also be attributed to the effect of substrate
299 composition on the expression of phenolic acid esterases. Past studies that quantified the
300 expression of enzymes involved in metabolism of phenolic acids in lactobacilli in laboratory media
301 used either free hydroxycinnamic acids or their methyl esters which do not match the composition
302 and diversity of phenolic acid esters in plants.

303 The presence of multiple enzymes with overlapping substrate specificities in lactobacilli likely
304 reflects the diversity of phenolic compounds including esters of phenolic acids in plants. The
305 accumulation of phenolic compounds helps in survival and adaptation of plants to different
306 environments (Lattanzio et al., 2012; Wu et al., 2019). Chlorogenic acid is abundant in foods such
307 as coffee and broccoli while also being widespread across different edible plants (Santana-Gálvez
308 et al., 2017). Phenolic acid glycosides are less common compared to flavonoid glycoside esters
309 but are found in flaxseeds, mustard, and canola, and in red peppers (Alu'datt et al., 2017; Engels
310 et al., 2012; Materska et al., 2003). Cereals such sorghum and oats are rich in glycerol esters of
311 phenolic acids (Svensson et al., 2010; Varga et al., 2018).

312 Plant associated lactobacilli take advantage of the esters of phenolic acids by using
313 hydroxycinnamic acids for co-factor recycling (Filannino et al., 2019), and for release of
314 carbohydrate moieties for use as carbon source. The release of free phenolic acids from the diverse
315 phenolic acid esters is thus likely to be ecologically relevant for plant associated lactobacilli, which
316 is further supported by the presence of two esterases and one tannase in the core genome (Brochet
317 et al., 2021)(Table 6). Because the diverse esters of phenolic acids are likely hydrolyzed by
318 different esterases, the determination of the effect of individual enzymes in food substrates requires
319 identification and quantification of phenolic acid esters by LC-MS/MS in future studies.

320 In conclusion, this study expanded on the role of different phenolic acid esterases present in *Lp.*
321 *plantarum* TMW1.460 and characterized a novel hydroxycinnamic acid esterase HceP responsible
322 for hydrolysis of chlorogenic acid and methyl ferulate. While direct use of enzymes or
323 heterologous overexpression can be routes for targeted metabolite production as shown by Landete
324 et al., 2021 for Est_1092 and TanA, the biochemically characterized enzymes are not necessarily
325 active *in situ* in food fermentations. This may be due to the complementing substrate specificities
326 of enzymes and differential gene expression in varied phenolic substrate compositions. We also
327 provided evidence of differential metabolism between laboratory media and food fermentations
328 while confirming the role of different esterases by generation of isogenic mutants.

329 **Acknowledgements**

330 We would like to thank Nanzhen Qiao for providing scripts and assistance in bioinformatic
331 analyses. The Natural Sciences and Engineering Research Council of Canada, the Canada
332 Research Chairs Program and the China Scholarship Council are acknowledged for funding.

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508 **Figure legends**

509 **Figure 1.** Characterization of esterase activity via quantification of free phenylpropionic acid
510 metabolites in broccoli and whole wheat fermented samples. Both food matrices were fermented
511 with *Lp. plantarum* TMW1.460 strains for 24 h at 30°C. WT- Wild type, *tanB*- tannase, *hceP* and
512 e*st_1092*- phenolic acid esterases. Data are shown as means ± standard deviation of three
513 independent fermentations.

514

**Table 1.** *In silico* identification of phenolic acid esterases in *Lp. plantarum* TMW1.460.

| Esterase | Query Sequence ID | Known substrates | Locus tag in TMW1.460 | Amino acid identity (%) | Protein length (aa) | Reference |
|---|---|---|---|---|---|---|
| Lp_0796 | YP_004888771.1 | Hydroxycinnamic acid esters: Methyl ferulate, methyl caffeate, methyl *p*-coumarate, methyl sinapinate | GB998_RS03015 | 100 | 249 | (Esteban-Torres et al., 2013) |
| Est_1092 | WP_015825406.1 | Hydroxycinnamic acid esters: Methyl ferulate, methyl caffeate, methyl *p*-coumarate, methyl sinapinate, Hydroxybenzoic acid esters: Methyl gallate, methyl vanillate, ethyl gallate, ethyl protcatechuate, epigallocatechin gallate | GB998_RS05085 | 100 | 295 | (Esteban-Torres et al., 2015) |
| TanA | WP_003640628.1 | Hydroxybenzoic acid esters: Methyl gallate, ethyl gallate, propyl gallate, ethyl protcatechuate, gallocatechin gallate epigallocatechin gallate, tannic | - | - | - | (Jiménez et al., 2014) |
| TanB | YP_004890536.1 | Hydroxybenzoic acid esters: Methyl gallate, ethyl gallate, propyl gallate, lauryl gallate, ethyl protcatechuate, gallocatechin gallate epigallocatechin gallate, tannic acid | GB998_RS11910 | 99 | 469 | (Curiel et al., 2009; Iwamoto et al., 2008) |
| Lj0536, Lj1228 | WP_004898050.1, WP_011162057.1 | Chlorogenic acid, ethyl ferulate, rosmarinic acid | GB998_RS11900 | 53 | 249 | (Lai et al., 2009) |

**Table 2.** Strains and plasmids used in this study.

| Strains | Description | Reference |
|---|---|---|
| *Lactiplantibacillus plantarum* TMW1.460 | Isolated from spoiled beer | (Ulmer et al., 2000) |
| *Lp. plantarum* TMW1.460 Δ*lp_0796* | Single deletion mutant of *Lp. plantarum* TMW1.460 | This study |
| *Lp. plantarum* TMW1.460 Δ*est_1092* | Single deletion mutant of *Lp. plantarum* TMW1.460 | This study |
| *Lp. plantarum* TMW1.460 Δ*tanB* | Single deletion mutant of *Lp. plantarum* TMW1.460 | This study |
| *Lp. plantarum* TMW1.460 Δ*hceP* | Single deletion mutant of *Lp. plantarum* TMW1.460 | This study |
| *Lp. plantarum* TMW1.460 Δ*est_1092*/Δ*hceP* | Double deletion mutant of *Lp. plantarum* TMW1.460 | This study |
| *Escherichia coli* EC1000 | Cloning host for pVPL 3002 based plasmids, RepA+, $Km^{R}$ [a] | (Leenhouts et al., 1996) |
| **Plasmids** | | |
| pVPL 3002 | pORI19 derived suicide vector, $Em^{R}$ [a] | (Zhang et al., 2018) |
| pVPL3002/ΔLp_0796 | pVPL 3002 containing *lp_0796* flanking regions, $Em^{R}$ | This study |
| pVPL3002/ΔEst_1092 | pVPL 3002 containing *est_1092* flanking regions, $Em^{R}$ | This study |
| pVPL3002/ΔTanB | pVPL 3002 containing *tanB* flanking regions, $Em^{R}$ | This study |
| pVPL3002/ΔHceP | pVPL 3002 containing *hceP* flanking regions, $Em^{R}$ | This study |

[a] $Em^{R}$ and $Km^{R}$ indicate an erythromycin and kanamycin resistant phenotype, respectively.

**Table 3.** Primers used in this study for construction of mutants.

| Primer (forward, F; reverse, R) | Description | Primer Sequences (5'→3') |
|---|---|---|
| oVPL 188 F | amplifies pVPL3002 backbone | ATCCTCTAGAGTCGACCTGC |
| oVPL 187 R | (Zhang et al., 2018) | TACCGAGCTCGAATTCACTGG |
| oVPL97 F | insert check in pVPL3002 plasmids | CCCCCATTAAGTGCCGAGTGC |
| oVPL49 R | (Zhang et al., 2018) | ACAATTTCACACAGGAAACAGC |
| Lp_0796 U/S F | upstream flanking region of *lp_0796* in | CAGTGAATTCGAGCTCGGTAGGCATCTTCTTGCCAATC |
| Lp_0796 U/S R | *Lp. plantarum* TMW1.460 | CCAATTGGTGGGCATGTTGGCCATGTTC |
| Lp_0796 D/S F | downstream flanking region of | CCAACATGCCCACCAATTGGAACAAGATG |
| Lp_0796 D/S R | *lp_0796* in *Lp. plantarum* TMW1.460 | GCAGGTCGACTCTAGAGGATCGTGGCAACATTGGAATC |
| Lp_0796 DCO F | DCO screening for Δ*lp_0796* in *Lp.* | CGGCAATGCTGTTATTTTGAATTTG |
| Lp_0796 DCO R | *plantarum* TMW1.460 | TGTGACCGAATCACACTTTGGAAA |
| Est_1092 U/S F | upstream flanking region of *est_1092* | CAGTGAATTCGAGCTCGGTAGCATGTTAAACGGATGAAT |
| Est_1092 U/S R | in *Lp. plantarum* TMW1.460 | TTCCTCCAGACCTCCATTTATTGGCTCTATC |
| Est_1092 D/S F | downstream flanking region of | TAAATGGAGGTCTGGAGGAAAATATGATGAAG |
| Est_1092 D/S R | *est_1092* in *Lp. plantarum* TMW1.460 | GCAGGTCGACTCTAGAGGATGTCGTTGCCAAGTTCAAG |
| Est_1092 DCO F | DCO screening for Δ*est_1092* in *Lp.* | CCTTGCGATAATCACGGTTTTTATTTTACC |
| Est_1092 DCO R | *plantarum* TMW1.460 | CCAGCACATCCATAATGGTTGGTGA |
| TanB U/S F | upstream flanking region of *tanB* in | CAGTGAATTCGAGCTCGGTAATAACCGCAGCAACCATTG |
| TanB U/S R | *Lp. plantarum* TMW1.460 | AGGTCACAAGTGAATACGATGAGTGAAAGC |
| TanB D/S F | downstream flanking region of *tanB* in | ATCGTATTCACTTGTGACCTCCATTTCTATC |
| TanB D/S R | *Lp. plantarum* TMW1.460 | GCAGGTCGACTCTAGAGGATTTCCGACGATTCTAGTTC |
| TanB DCO F | DCO screening for Δ*tanB* in *Lp.* | AAAACTGTTAAAGTTCGTCGATGCT |
| TanB DCO R | *plantarum* TMW1.460 | AAATAATTCGAGTGACGTCGATTCC |
| HceP U/S F | upstream flanking region of *hceP* in | CAGTGAATTCGAGCTCGGTACGGCTTTACGACCTATATG |
| HceP U/S R | *Lp. plantarum* TMW1.460 | TGAGATGACATGACTTACGCCAGTTAATATAATG |
| HceP D/S F | downstream flanking region of *hceP* in | GCGTAAGTCATGTCATCTCACTTATTCATTATTCACAC |
| HceP D/S R | *Lp. plantarum* TMW1.460 | GCAGGTCGACTCTAGAGGATGTGTTATTGGGACCGGCATTTG |
| HceP DCO F | DCO screening for Δ*hceP* in *Lp.* | GCTTTTTCCACCGACTTAAAGATTTTC |
| HceP DCO R | *plantarum* TMW1.460 | TTTCGGCAGGTGTTTCTAATGCTAT |

**Table 4.** Characterization of phenolic acid esterase activity and metabolite production of *Lp. plantarum* TMW1.460 and its knockout mutants after incubation with 1mM of different substrates. Shaded / + and unshaded / - boxes represent presence and absence of the phenotype for the metabolites as detected by the HPLC

| Strain Name | Incubation time (Days) | Chlorogenic Acid | Methyl Ferulate | | Methyl Gallate | | Tannic Acid | Epigallocatechin-3- gallate |
|---|---|---|---|---|---|---|---|---|
| | | Caffeic Acid | Ferulic Acid | Hydro-ferulic Acid | Gallic Acid | Pyrogallol | Gallic Acid | |
| *Lp. plantarum* TMW1.460 WT | 1 | + | + | - | + | + | - | - |
| | 10 | + | + | + | + | + | - | - |
| *Lp. plantarum* TMW1.460 Δ*lp_0796* | 1 | + | + | - | + | + | - | - |
| | 10 | + | + | + | + | + | - | - |
| *Lp. plantarum* TMW1.460 Δ*est_1092* | 1 | + | + | - | + | + | - | - |
| | 10 | + | + | + | + | + | - | - |
| *Lp. plantarum* TMW1.460 Δ*tanB* | 1 | + | + | - | - | - | - | - |
| | 10 | + | + | + | - | - | - | - |
| *Lp. plantarum* TMW1.460 Δ*hceP* | 1 | - | - | - | + | + | - | - |
| | 10 | - | - | - | + | + | - | - |
| *Lp. plantarum* TMW1.460 Δ*hceP*/ Δ*est_1092* | 1 | - | - | - | + | + | - | - |
| | 10 | - | - | - | + | + | - | - |

**Table 5.** Cell counts and pH of samples fermented with *Lp. plantarum* TMW1.460 and its mutant strains at 30°C for 24 h.

| Sample | Cell count (Log CFU/g) | | pH | |
|---|---|---|---|---|
| | Broccoli puree | Whole wheat sourdough | Broccoli puree | Whole wheat sourdough |
| Unfermented control | 5.50 ± 0.25 | 4.54± 0.11 | 6.75± 0.03 | 5.88± 0.02 |
| Chemically acidified | <4 | <4 | 3.50± 0.02 | 3.50± 0.02 |
| *Lp. plantarum* TMW1.460 WT | 8.94± 0.23 | 9.40 ± 0.28 | 3.75± 0.02 | 3.45± 0.04 |
| *Lp. plantarum* TMW1.460 Δ*tanB* | 9.04 ± 0.22 | 9.32 ± 0.13 | 3.73± 0.03 | 3.40± 0.03 |
| *Lp. plantarum* TMW1.460 Δ*hceP*/ Δe*st_1092* | 9.11 ± 0.20 | 9.25 ± 0.11 | 3.76± 0.02 | 3.43± 0.05 |

**Table 6.** Presence of genes coding for hydroxycinnamic and hydroxybenzoic acid esterases in 344 genomes of *Lactiplantibacillus plantarum*. Strain and genome accession numbers are shown in Table S1 of the online supplementary material. Data shown as positive hits for protein BLAST with >75% query cover and >70% amino acid identity.

| Esterase | Number (percentage) of positive strains |
|---|---|
| Lp_0796 | 344 (100 %) |
| HceP | 341 (99 %) |
| Est_1092 | 55 (16 %) |
| TanB | 344 (100 %) |
| TanA | 48 (14 %) |

**Figure 1.**