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Degradation of tannic acid by cell-free extracts of
Lactobacillus plantarum

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Abstract

The ability of *Lactobacillus plantarum* CECT 748^T to degrade hydrolyzable tannins was evaluated. Three commercial tannic acids were incubated in presence of cell-free extracts containing soluble proteins from *L. plantarum*. By HPLC analyses, almost a complete tannic acid degradation was observed in the three samples assayed. By using HPLC-DAD/ESI-MS we partially determined the composition of tannic acid from *Quercus infectoria* galls. This tannic acid is a gallotannin mainly composed of monomers to tetramers of gallic acid. We studied the mechanism of its degradation by *L. plantarum*. The results obtained in this work indicated that *L. plantarum* degrade gallotannins by depolymerization of high molecular weight tannins and a reduction of low molecular weight tannins. Gallic acid and pyrogallol were detected as final metabolic intermediates. Due to the potential health beneficial effects, the ability to degrade tannic acid is an interesting property in this food lactic acid bacteria.

Keywords: Tannic acid; *Lactobacillus*; hydrolyzable tannins; phenolic compounds; gallic acid; pyrogallol.

46 **1. Introduction**

47

48 Vegetable tannins are water-soluble polyphenols that are present in many plant
49 foods. Tannins are present in a variety of plants utilized as food including food grains
50 like dry beans, faba beans, peas, and fruits such as apples, bananas, blackberries,
51 peaches, and plums.

52 Tannins are considered nutritionally undesirable because inhibit digestive
53 enzymes and affect the utilization of vitamins and minerals. Ingestion of large quantities
54 of tannins may result in adverse health effects. However, the intake of a small quantity
55 of the right kind of tannins may be beneficial to human health (Gu et al., 2003). Tannins
56 seem to be a double-edged sword. On the one hand, they are beneficial to health due to
57 their chemopreventive activities against carcinogenesis and mutagenesis (Horikawa,
58 Mohri, Tanaka, & Tokiwa, 1994), but on the other hand, they may be involved in cancer
59 formation, hepatotoxicity, or antinutritional activity (Ramanathan, Tan, & Das, 1992). It
60 is not advisable to ingest large quantities of tannins, since they may possess carcinogenic
61 and antinutritional activities, thereby possessing a risk of adverse health effects.
62 However, the intake of a small quantity of some tannins promotes beneficial health
63 effects. Thus, it is important to determine the right dose of the right kind of tannins to
64 promote optimal health.

65 The molar mass of tannin molecules affects tannin's characteristics directly. It
66 has been found that the higher the molar mass of tannin molecules is, the stronger the
67 anti-nutritional effects and the lower the biological activities are (Chung, Wei, &
68 Johnson, 1998). Small molecule tannins such as monomeric, dimeric and trimeric
69 tannins are suggested to have less anti-nutritional effects and can be more readily
70 absorbed. Marker biological and pharmacological activities such as anticarcinogenic

71 activity, host-mediated antitumor activity, antiviral activity, inhibition of lipid
72 peroxidation and some enzymes such as lipoxygenase, xanthine oxidase and
73 monoamine oxidase have been shown for medical herbs to contain small molecule
74 tannins (Okuda, Yoshida, & Hatano, 1992).

75 Tannins could be divided into hydrolyzable and condensed tannins, and these are
76 classified by structure and susceptibility to acid hydrolysis. Hydrolyzable tannins are
77 comprised of a polyol carbohydrate core (usually D-glucose) esterified to phenolic acids
78 such as gallic acid or ellagic acid, forming gallotannins and ellagitannins, respectively.
79 Mild acid hydrolysis of these tannins yields carbohydrates and phenolics. Tannic acid,
80 one of the most abundant reserve material of plants, is a gallotannin consisting of esters
81 of gallic acid and glucose, containing galloyl groups esterified directly to the glucose
82 molecule.

83 Some bacteria, yeast, and filamentous fungi have developed the ability to
84 degrade tannins into innocuous compounds (Bath, Singh, & Sharma, 1998). Lactobacilli
85 able to degrade methyl gallate, a simple galloylester of methanol, and a component of
86 tannic acid were isolated (Osawa, Kuroiso, Goto, & Shimizu, 2000). Later, this property
87 was confirmed in *L. plantarum* strains isolated from various food substrates (Nishitani
88 & Osawa, 2003; Nishitani, Sasaki, Fujisawa, & Osawa, 2004; Vaquero, Marcobal, &
89 Muñoz, 2004). It has been postulated that this enzymatic property have an ecological
90 advantage for this species, as it is often associated with fermentations of plants
91 materials.

92 As far as we known, there is no information about the degradation mechanism of
93 complex hydrolyzable tannins, such as tannic acid, by *L. plantarum*. In addition,
94 microbial degradation is one of the efficient ways to degrade large molecule tannins into
95 smaller molecule tannins with valuable bioactivities. Therefore, in this paper, we

96 demonstrated the degradation of several commercial tannic acids by *L. plantarum* and
97 reported the mechanism of degradation followed by this food lactic acid bacteria.

98

99 **2. Material and methods**

100

101 *2.1. Bacterial strain*

102

103 *L. plantarum* CECT 748^T (ATCC 14917, DSMZ 20174) isolated from pickled
104 cabbage was purchased from the Spanish Type Culture Collection. High tannase
105 activity was reported previously in this strain (Nishitani & Osawa, 2003; Nishitani et
106 al., 2004; Vaquero et al., 2004).

107

108 *2.2. Culture media and growth conditions*

109

110 The bacterium was cultivated in a modified basal medium described previously
111 for *L. plantarum* (Rozès & Peres, 1998) in order to study the degradation of tannic acid.
112 The basal medium has the following composition in g L⁻¹: glucose, 2.0; trisodium citrate
113 dihydrate (SO 0200, Scharlau), 0.5; D-, L-malic acid (AC 1420, Scharlau), 5.0;
114 casamino acids (223050, BD), 1.0; yeast nitrogen base without amino acids (239210,
115 BD), 6.7; pH adjusted to 5.5. The basal media was modified by the replacement of
116 glucose by galactose (216310, Difco) in order to avoid a possible glucose carbon
117 catabolite repression.

118

119 *2.3. Preparation of cell-free extracts*

120

121 To determine if *L. plantarum* possess intracellular enzymes able to degrade
122 tannic acid, cell-free extracts containing all soluble proteins were prepared. *L.*
123 *plantarum* CECT 748^T was grown in modified basal media under microaerobic
124 conditions at 30 °C until a late exponential phase. The cells were harvested by
125 centrifugation and washed three times with phosphate buffer (50 mM, pH 6.5), and
126 subsequently resuspended in the same buffer for cell rupture. Bacterial cells were
127 disintegrated twice by using the French Press at 1100 psi pressure (Amicon French
128 pressure cell, SLM Instruments). The cell disruption steps were carried out on ice to
129 ensure low temperature conditions required for most enzymes. The disintegrated cell
130 suspension was centrifuged at 12000g for 20 min at 4 °C in order to sediment cell
131 debris. The supernatant containing the soluble proteins was filtered aseptically using
132 sterile filters of 0.2 µm pore size (Sarstedt, Germany). Protein concentration was
133 determined using the Bio-Rad protein assay (Bio-Rad, Germany).

134

135 2.4. Enzymatic hydrolysis of tannic acid

136

137 The enzymatic hydrolysis of tannic acid by cell-free extracts of *L. plantarum*
138 CECT748^T was determined by using three commercial tannic acids: tannic acid 1 (TA1)
139 (T0125, Sigma), tannic acid 2 (TA2) (411074, Carlo Erba) and tannic acid 3 (TA3)
140 (48811, Fluka). Only the source of TA1 is known; TA1 is a hydrolysable tannin
141 obtained from gall nuts from *Quercus infectoria*. The standard assay to determine tannic
142 acid degradation was performed containing 1 mM tannic acid (final concentration) in
143 the reaction. *L. plantarum* cell-free extracts in phosphate buffer (50 mM, pH 6.5)
144 containing 900 µg of protein were incubated for different times at 37 °C in the presence
145 of each tannic acid (1 mM). As control, phosphate buffers containing 1 mM of each

146 different tannic acid were incubated in the same conditions. The reaction products were
147 extracted twice with ethyl acetate (Lab-Scan, Ireland).

148

149 *2.5. HPLC-DAD analysis of tannic acid*

150

151 The equipment used for the HPLC analysis consisted of a Waters (Milford, MA)
152 chromatograph equipped with a 600-MS system controller, a 717 plus autosampler, and
153 a 996 photodiode array detector. Separation was performed on a reverse-phase Nova-
154 pack C₁₈ (250 mm x 4.0 mm i.d., 4.6µm) cartridge at room temperature as described by
155 Bartolomé, Peña-Neira, & Gómez-Cordovés, (2000). A gradient consisting of solvent A
156 (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2,
157 v/v/v) was applied to follows: 0-80% B linear from 0-55 min at a flow rate of 1,1
158 ml/min; 80-90% B linear from 55-57 min, 1.2 ml/min; 90% B isocratic from 57-70 min,
159 1.2 ml/min; 90- 95% B linear from 70-80 min, 1.2 ml/min; 95-100%B from 80-90 min,
160 1.2 ml/min; followed by washing (methanol) and re-equilibration of the cartridge from
161 90-120 min, 1,1 ml/min. Detection was performed by scanning from 220 to 380 nm.
162 Samples were injected in duplicate onto the cartridge after being filtered through a 0.45
163 µm PVDF filter (Teknokroma, Spain).

164 The identification of degradation intermediates was carried out by comparing the
165 retention times and spectral data of each peak (Bartolomé et al., 1993) with those of
166 standards from commercial suppliers Extrasynthese or with previously purified
167 compounds.

168

169 *2.6. High-Performance Liquid Chromatography-Diode Array Detector-Electrospray*

170 *Mass Spectrometry (HPLC-DAD/ESI-MS)*

171
172 A Hewlett-Packard series 1100 (Palo Alto, CA) chromatography system
173 equipped with a diode array detector (DAD) and a quadrupole mass spectrometer
174 (Hewlett-Packard series 1100 MSD) with an electrospray interface was used. Separation
175 was performed on a reversed-phase Waters Nova-Pak C18 column at room temperature.
176 The solvent gradient described above was applied. DAD detection was performed from
177 220 to 380 nm, with 0.7 ml/min. The ESI parameters were as follows: drying gas (N₂)
178 flow and temperature, 10 L/min at 340 °C; nebulizer pressure, 40 psi; capillary voltage,
179 4000 V. The ESI was operated in negative mode, scanning from m/z 100 to 3000 using
180 the following fragmentator voltage gradient: 100 V from 0 to 200 *m/z* and 200 V from
181 200 to 3000 *m/z*.

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183

184 **3. Results and discussion**

185

186 **3.1. Tannic acid degradation by *L. plantarum***

187

188 Because food extracts contained a range of condensed and hydrolyzable
189 phenolic residues, we limited our studies to tannic acid as a relatively well-defined
190 commercially available hydrolyzable tannin preparation. We used three different
191 commercial preparations of tannic acid. Only the source of TA1 is available. TA1 is a
192 hydrolyzable tannin obtained from oak gall nuts from *Quercus infectoria*. As Ayed and
193 Hamdi (2002) described that *L. plantarum* produces an extracellular tannase after 24 h
194 growth on minimal medium of amino acids containing tannic acid, firstly we grown *L.*
195 *plantarum* cells in presence of tannic acid. However, no tannic acid degradation was

196 observed (submitted for publication). Therefore, in order to know if *L. plantarum*
197 posses intracellular enzymes to degrade tannic acid, cell-free extracts containing all the
198 soluble proteins were incubated at 37 °C during 20 h in presence of 1 mM of each
199 commercial tannic acid. Since the soluble proteins were present in phosphate buffer (50
200 mM, pH 6.5), control samples were prepared in this buffer and incubated in the same
201 conditions. The tannins were extracted and analysed by HPLC. Fig. 1 showed the HPLC
202 chromatograms obtained. Commercial tannic acids TA1, TA2, and TA3 are represented
203 in Fig. 1a, d, and g, respectively. It could be concluded that TA2 is more rich in
204 extracted hydrolysable tannins than TA1 and TA3. A total of 50 hydrolysable tannins
205 were detected in TA2, 20 in TA1, and only 8 in TA3. Spectral data from UV/vis
206 photodiode array detector were used to identify each compound as gallic acid type
207 based on the spectrum of gallic acid standards from 220 to 380 nm.

208 It have been described that hydrolyzable tannins underwent autodegradation
209 under certain incubation conditions (Kumar, Gunasekaran, & Lakshman 1999); Fig. 1b,
210 e, and h showed the chromatograms of TA1, TA2, and TA3, respectively, after
211 incubation in phosphate buffer during 20 h at 37 °C. It could be observed a remarkable
212 autodegradation as compared to the chromatograms obtained from the non-incubated
213 samples (Fig. 1a, d, and g). Finally, if the commercial tannic acids were incubated in
214 presence of soluble proteins from *L. plantarum*, almost a complete degradation was
215 observed in all the tannic acid samples assayed (Fig. 1c, f, and i). These results confirms
216 that *L. plantarum* possess intracellular enzymes able to degrade complex hydrolysable
217 tannins, such as tannic acids.

218 As showed by Fig. 1a, d, and g, corresponding to commercial tannic acids, three
219 common peaks could be identified in all the three tannic acid samples tested. The
220 common peaks were named peak 1 (peak 9 in the chromatogram corresponding to TA1,

peak 20 in TA2, and peak 2 in TA3), peak 2 (peak 20 in TA1, peak 32 in TA2, and peak 4 in TA3), and peak 3 (peak 25 in TA1, peak 38 in TA2, and peak 8 in TA3). Since only the source of TA1 is known, we decided to identify peak 1, 2 and 3 from TA1 using LC/ESI-MS. Peak 1, which presented at retention time 39.9 min, was identified as a tetragalloyl glucose: $[M-H]^-$ 787, m/z 617 (corresponding to dehydrated digalloyl glucose), m/z 393 (not identified) and m/z 169 (corresponding to a fragment ion of a gallic acid) (Fig. 2a). Peak 2, at retention time 55.8 min, was identified as a pentagalloyl glucose: $[M-H]^-$ 939.2, m/z 469 methyl-(digalloyl-glucoside), m/z 393 (not identified), m/z 169 (deprotonated gallic acid) (Fig. 2b). And finally, peak 3, which presented at retention time 69.2 min, was identified as a hexagalloylglucose: $[M-H]^-$ 1091, m/z 469 methyl-(digalloyl-glucoside), m/z 393 (not identified) (Fig. 2c).

From these results it could be concluded that *L. plantarum* degrades complex galloylated esters of glucose present in several tannic acid samples. Since these compounds could be also found in food samples, therefore *L. plantarum* could be used to degrade them.

3.2. Mechanism of tannic acid degradation by *L. plantarum*

Since *L. plantarum* is able to degrade several commercial tannic acids, we decided to study the mechanism of this degradation. Therefore we incubated cell-free extracts of *L. plantarum* in presence of 1 mM tannic acid (TA1), and aliquots were withdrawn at different incubation times. Similarly, phosphate buffer containing 1mM TA1 was incubated in the same conditions. Fig. 3 showed the chromatograms obtained from *L. plantarum* extracts at different incubation times, non-incubated (Fig. 3a), 30 min incubation (Fig. 3b), 6 h incubation (Fig. 3c), and 24 h incubation (Fig. 3d). A

246 significant reduction in the total amount of hydrolyzable tannins was already observed
247 at 30 min incubation. In order to know how this reduction was produced, we divided
248 the chromatograms in three zones defined by the peaks 1, 2 , and 3, previously
249 identified. Zone A comprises from the beginning of the chromatogram to peak 1
250 included; zone B from peak 1 to peak 2 included; and zone C, from peak 2 to peak 3
251 included. Table 1 showed the relative area of these peaks and zones on each
252 chromatogram, corresponding to tannic acid incubated in phosphate buffer (control,
253 autodegradation or chemical degradation) or in presence of soluble proteins from *L.*
254 *plantarum* (biodegradation). In both incubation conditions, autodegradation and
255 biodegradation by *L. plantarum*, it could be observed that during the time course
256 incubation, peak 1 and zone A showed an increase, whereas peaks 2 and 3, and zones B
257 and C diminished. This indicated a selective hydrolysis of low polar and high polymeric
258 tannins. The hydrolysis observed in the sample incubated in phosphate buffer is not
259 surprising, since tannins are highly reactive and unstable compounds under several
260 incubation conditions.

261 In samples incubated in presence of *L. plantarum* proteins, at 30 min incubation
262 the area ratios between peak 1 and peak 3, and peak 1 and peak 2 increased twice in
263 both. At longer incubation times, peaks 1, 2 and 3 were undetected in the
264 chromatograms. Similarly in the defined zones, at 30 min incubation zone A showed a
265 63.5% increase, whereas, zones B and C diminished 38.3 and 25.2%, respectively. From
266 these results, it could be deduced that *L. plantarum* degrades tannic acid by degrading
267 low polar tannins, and presumably more polymerised, and converting them to tannins
268 possessing higher polarity and a lower polymerisation degree.

269 In order to confirm these results, we identified some of the peaks included in
270 zones A, B, and C from TA1 by LC/ESI-MS. Zone A includes from monomers ($[M-H]^-$

271 169, gallic acid) to tetramers of gallic acid and glucose ($[M-H]^-$ 787 and ions at m/z 617
272 dehydrated digalloyl-glucoside). Zone B comprises tetragalloyl-glucose and
273 pentagalloylglucose derivatives ($[M-H]^-$ 939, m/z 393, m/z 169, gallic acid). In zone 3
274 could be found hexagalloyl-glucosides ($[M-H]^-$ 1091, m/z 469 methyl-(digalloyl-
275 glucoside) and m/z 393, not identified, also found at pedunculata oak and myrabolans
276 tannins (Vivas, Bourgeois, Vitry, Glories, & de Freitas, 1996). The TA1 manufacturer
277 indicates that, by HPLC, the product is composed of monomers to pentamers of galloyl
278 glucose, but higher molecular weight isomers were detected by us. Also, in tannic acid
279 from *Quercus infectoria* galls was reported to contain only up to heptagalloyl-glucose
280 isomers (Vivas et al., 1996). The composition described for TA1 is also similar to that
281 reported in two other oak species (*Q. alba* and *Q. robur*) (Mämmela, Savolainen,
282 Lindroos, Kangas, & Vartiainen, 2000), in acorns of *Q. ilex* and *Q. rotundifolia* (Cantos,
283 Espin, López-Bote, de la Hoz, Ordoñez, & Tomás-Barberán, 2003), and in tannic acid
284 from tanoak (*Lithocarpus densiflorus*) acorn (Meyers, Swiecki, & Mitchell, 2006).
285 Cantos et al. (2003) distinguished 32 different phenolic compounds from the acorns of
286 *Quercus* species. All of them were gallic acid derivatives. The series of galloyl glucose
287 esters was also detected as different isomers of galloyl glucose, from monogalloyl to
288 pentagalloyl glucose. Mämmela et al. (2000) also identified castalagin/vescalagin in oak
289 extract by their molecular weight. The small differences encountered among these
290 tannic acids can be attributed to the fact that different plant varieties have been shown to
291 produce different types and quantities of phenolic compounds (Hakkinen & Torronen,
292 2000).

293 From the data of chromatograms analysed in Table 1, we calculated the loss of
294 total area detected. We obtained that during a prolonged incubation period (24 h) in the
295 conditions used in this assay, TA1 showed a 63 % autodegradation. However, tannic

296 acid degradation is highly increased by the presence of *L. plantarum* cell-free extracts,
297 which, only on a 6 h incubation period, degraded as much as 95% of the tannic acid
298 compounds.

299 The mechanism of tannic acid degradation followed by *L. plantarum* extracts
300 seems to be quite similar to that observed during autodegradation, tannins highly
301 polymerized are hydrolyzed to less polymerized and higher polar tannins. Tannic acid
302 degradation into monomeric products by *L. plantarum* is in accordance to the
303 depolymerization of phenolic compounds present in olive mill wastewater observed by
304 Kachouri and Hamdi (2004). They reported that olive mill wastewater fermented with
305 *L. plantarum* shows a depolymerization of high molecular weight of phenolic
306 compounds and a reduction of low molecular weight phenolics compounds. The
307 application of *L. plantarum* favours the increase of all phenolic compounds in olive oil,
308 especially by depolymerization, and inhibited the polymerization of phenolic
309 compounds during storage being the responsible for the darkening of the olive mill
310 wastewater (Ayed & Hamdi, 2003).

311 As observed in Fig. 3c and d, the degradation of TA1 by *L. plantarum* produces
312 two intermediates. At 6 h incubation, a peak which presented at retention time of 5.7
313 min and showed spectra with maximum similar to gallic acid (Fig. 4a) and [M-H]⁻ 169
314 was found. After 24 h incubation, this peak was markedly reduced, and appeared a new
315 peak showing pyrogallol [M-H]⁻ 125 and UV spectra (Fig. 4b). Therefore, the proposed
316 biochemical pathway for the degradation of tannic acid by *L. plantarum* implies that
317 tannic acid is hydrolyzed to gallic acid and glucose, and the gallic acid formed is
318 decarboxylated to pyrogallol. The presence of a tannase and a gallate decarboxylase in
319 *L. plantarum* has been previously reported (Osawa et al., 2000). These activities were
320 only detected by low precise colorimetric methods; therefore these enzymatic activities

321 remains to be precisely determined. Similarly, in an attempt to elucidate the metabolic
322 pathway of tannic acid degradation by *Citrobacter freundii* TB3, Kumar et al. (1999)
323 (Kumar et al., 1999) detected glucose, gallic acid and pyrogallol as metabolic
324 intermediates, and chromatographic analyses revealed that there was no other aromatic
325 compound formed from pyrogallol. Degradation of hydrolyzable tannins is best
326 understood in fungal systems. The oxidative degradation of hydrolyzable tannins has
327 been studied in detail in *Aspergillus* spp. and the pathways of gallic acid degradation
328 have been determined. In *A. flavus*, gallic acid is degraded to oxaloacetic acid and
329 finally pyruvic acid through tricarboxylic acid intermediate. In *A. niger*, pyrogallol, the
330 decarboxylated derivative of gallic acid is also oxidatively broken down into *cis*-
331 aconitic acid, which then enters the citric acid cycle (Bhat et al., 1998).

332

333 To improve our understanding of hydrolyzable tannins degradation by *L.*
334 *plantarum*, further works on the mechanism of action are required. The evidences
335 presented in this work suggests that *L. plantarum* possesses intracellular enzymes able to
336 degrade complex hydrolyzable gallotannins, such as tannic acid by depolymerization of
337 high molecular weight tannins and a reduction of low molecular weight tannins. Since
338 the molar mass of tannin molecules affects tannin characteristics, and small molecule
339 tannins are suggested to have less antinutritional effects and stronger biological
340 activities, *L. plantarum* might represent an efficient food lactic acid bacteria to obtain
341 health beneficial tannins.

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346 **Acknowledgments**

347

348 This work was supported by grants AGL2005-000470 and AGL2005-06640-
349 CO2-02 (CICYT), FUN-C-FOOD Consolider 25506 (MEC), VIN03-006-C2-01 and
350 RM03-002 (INIA) and S-0505/AGR-0153 (CAM). The technical assistance of M. I.
351 Izquierdo, M. V. Santamaría and A. Gómez is greatly appreciated. H. Rodríguez was a
352 recipient of a predoctoral fellowship from the I3P-CSIC.

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422

423

424 **Figure captions**

425

426 Fig. 1. HPLC chromatograms showing the composition of three different commercial
427 tannic acids and their hydrolysis after 20 h incubation at 37 °C in phosphate buffer or in
428 presence of total soluble proteins from *L. plantarum* CECT 748^T. The commercial
429 tannic acids assayed were TA1 (a, b, and c), TA2 (d, e, and f), and TA3 (g, h, and i).
430 The hydrolysis products of TA1, TA2, and TA3 after incubation in phosphate buffer are
431 showed in chromatograms b, e, and h, respectively. Chromatograms c, f, and i showed
432 *L. plantarum* hydrolysis of TA1, TA2, and TA3, respectively. Common peaks 1, 2, and
433 3 are indicated in the three commercial tannic acids. Chromatogram regions, zone 1, 2,
434 and 3, are also indicated in TA1. The chromatograms were recorded at 280 nm.

435

436 Fig. 2. Mass fragmentation pattern of peak 1 (a), peak 2 (b), and peak 3 (c) identified in
437 commercial tannic acids. (a) Peak 1 (TR: 39.9 min) [M-H]⁻ 787 tetragalloyl-glucoside,
438 *m/z* 617 dehydrated digalloyl-glucoside, *m/z* 393 (not identified), *m/z* 169 (loss of the
439 gallic acid). (b) Peak 2 (TR: 55.8) [M-H]⁻ 939 pentagalloyl-glucoside, *m/z* 469 methyl
440 (digalloyl-glucoside), *m/z* 393 (not identified). *m/z* 169 (deprotonated gallic acid). (c)
441 Peak 3 (TR: 69.2 min)[M-H]⁻ 1091 hexagalloyl-glucoside, *m/z* 469 methyl-(digalloyl-
442 glucoside), *m/z* 393 (not identified).

443

444 Fig. 3. HPLC chromatograms showing disappearance of tannic acid and appearance of
445 hydrolysis products over time during the hydrolysis of TA1 by cell-free extracts of *L.*
446 *plantarum* CECT 748^T. Incubation time: 0h (a), 30 min (b), 6h (c), and 24h (d). Peaks 1,
447 2, and 3 are showed. Chromatogram regions, zone A, B, and C, are also indicated in
448 TA1. The gallic acid (GA) and pyrogallol (P) detected during the incubation are
449 indicated. Chromatograms were recorded at 280 nm.

450

451 Fig. 4. Comparison between spectra of gallic acid and pyrogallol identified as
452 intermediates during tannic acid hydrolysis and the standards of the database. (a) gallic
453 acid, and (b) pyrogallol. The uncontinuous line indicates the line corresponding to the
454 standard.

Table 1 (Rodríguez, de las Rivas, Gómez-Cordovés, & Muñoz)

Table 1.
Content of individual compounds in tannic acid incubated in presence of cell-free extracts of *L. plantarum*.

Sample	Time	Peak Relative area (%)			Zone Relative area (%)		
		1	2	3	A	B	C
Control ^a	0	12.22	20.31	8.98	15.89	48.18	35.92
	30 min	16.75	19.58	7.76	23.24	45.75	31.01
	6 h	19.16	24.56	5.02	25.34	48.91	25.74
	24 h	21.81	21.56	3.04	37.31	40.37	22.32
<i>L. plantarum</i>	0	13.14	20.34	8.76	16.53	45.70	37.76
	30 min	23.31	18.46	6.72	37.55	35.36	26.08
	6 h	ND ^b	ND	ND	100	ND	ND
	24 h	ND	ND	ND	100	ND	ND

^a Tannic acid in phosphate buffer (50 mM, pH 6.5).

^b ND, not detected.

Figure 1

Figure 1 (Rodríguez, de las Rivas, Gómez-Cordovés, & Muñoz)

Figure 2

Figure 2 (Rodríguez, de las Rivas, Gómez-Cordovés, & Muñoz)

Figure 3

Figure 3 (Rodríguez, de las Rivas, Gómez-Cordovés, & Muñoz)

Figure 4

Figure 4 (Rodríguez, de las Rivas, Gómez-Cordovés, & Muñoz)