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

Héctor Rodríguez, Blanca de las Rivas, Carmen Gómez-Cordovés *, Rosario Muñoz

Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain

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Abstract

The ability of *Lactobacillus plantarum* CECT 748^T to degrade hydrolysable 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* degrades gallotannins by depolymerisation 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.
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Keywords: Tannic acid; *Lactobacillus*; Hydrolyzable tannins; Phenolic compounds; Gallic acid; Pyrogallol

1. Introduction

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

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

The molar mass of tannin molecules affects the tannin's characteristics directly. It has been found that the higher the molar mass of tannin molecules, the stronger the anti-nutritional effects and the lower the biological activities (Chung, Wei, & Johnson, 1998). Small molecule tannins such as monomeric, dimeric and trimeric tannins are suggested to have less anti-nutritional effects and can be more readily absorbed. Marker biological and pharmacological activities such as anticarcinogenic activity, host-mediated antitumor activity, antiviral activity, inhibition of lipid peroxidation and some enzymes such as lipoxygenase, xanthine

* Corresponding author. Tel.: +34 91 5622900; fax: +34 91 564 4853.
E-mail address: cgcordoves@ifi.csic.es (C. Gómez-Cordovés).

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oxidase and monoamine oxidase have been shown for medical herbs to contain small molecule tannins (Okuda, Yoshida, & Hatano, 1992).

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

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

As far as we known, there is no information about the degradation mechanism of complex hydrolysable tannins, such as tannic acid, by *L. plantarum*. In addition, microbial degradation is one of the efficient ways to degrade large molecule tannins into smaller molecule tannins with valuable bioactivities. Therefore, in this paper, we demonstrated the degradation of several commercial tannic acids by *L. plantarum* and reported the mechanism of degradation followed by this food lactic acid bacteria.

2. Material and methods

2.1. Bacterial strain

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

2.2. Culture media and growth conditions

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

2.3. Preparation of cell-free extracts

To determine if *L. plantarum* possess intracellular enzymes able to degrade tannic acid, cell-free extracts containing all soluble proteins were prepared. *L. plantarum* CECT 748^{T} was grown in modified basal media under microaerobic conditions at 30 °C, until a late exponential phase. The cells were harvested by centrifugation and washed three times with phosphate buffer (50 mM, pH 6.5) and subsequently resuspended in the same buffer for cell rupture. Bacterial cells were disintegrated twice by using the French Press at 1100 psi pressure (Amicon French pressure cell, SLM Instruments). The cell disruption steps were carried out on ice to ensure the low temperature conditions required for most enzymes. The disintegrated cell suspension was centrifuged at 12,000*g* for 20 min at 4 °C, in order to sediment cell debris. The supernatant containing the soluble proteins was filtered aseptically using sterile filters of 0.2 μm pore size (Sarstedt, Germany). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Germany).

2.4. Enzymatic hydrolysis of tannic acid

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

2.5. HPLC-DAD analysis of tannic acid

The equipment used for the HPLC analysis consisted of a Waters (Milford, MA) chromatograph equipped with a 600-MS system controller, a 717 plus autosampler and a 996 photodiode array detector. Separation was performed on a reverse-phase Nova-pack C_{18} (250 mm × 4.0 mm i.d., 4.6 μm) cartridge at room temperature as described by Bartolomé, Peña-Neira, and Gómez-Cordovés (2000). A gradient consisting of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied as follows: 0–80% B linear from

0 to 55 min at a flow rate of 1.1 ml/min; 80–90% B linear from 55 to 57 min, 1.2 ml/min; 90% B isocratic from 57 to 70 min, 1.2 ml/min; 90–95% B linear from 70 to 80 min, 1.2 ml/min; 95–100% B from 80 to 90 min, 1.2 ml/min; followed by washing (methanol) and re-equilibration of the cartridge from 90 to 120 min, 1.1 ml/min. Detection was performed by scanning from 220 to 380 nm. Samples were injected in duplicate onto the cartridge after being filtered through a 0.45 μm PVDF filter (Teknokroma, Spain).

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

2.6. High-performance liquid chromatography-diode array detector–electrospray mass spectrometry (HPLC-DAD/ESI-MS)

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

3. Results and discussion

3.1. Tannic acid degradation by L. plantarum

Because food extracts contained a range of condensed and hydrolysable phenolic residues, we limited our studies to tannic acid as a relatively well-defined commercially available hydrolysable tannin preparation. We used three different commercial preparations of tannic acid. Only the source of TA1 is available. TA1 is a hydrolysable tannin obtained from oak gall nuts from *Q. infectoria*. As Ayed and Hamdi (2002) described that *L. plantarum* produces an extracellular tannase after 24 h growth on minimal medium of amino acids containing tannic acid, we initially grew *L. plantarum* cells in the presence of tannic acid. However, no tannic acid degradation was observed (submitted for publication). Therefore, in order to find out if *L. plantarum* posses intracellular enzymes to degrade tannic acid, cell-free extracts containing all the soluble proteins were incubated at 37 °C during 20 h in presence of 1 mM of each commercial tannic acid. Since the soluble proteins were present in phosphate buffer (50 mM, pH 6.5), control samples were prepared in this buffer and incubated in the same conditions. The tannins were extracted and analysed by HPLC. Fig. 1 showed the HPLC chromatograms obtained. Commercial tannic acids TA1, TA2, and TA3 are represented in Fig. 1a, d, and g, respectively. It could be concluded that TA2 is more rich in extracted hydrolysable tannins than TA1 and TA3. A total of 50 hydrolysable tannins were detected in TA2, 20 in TA1, and only 8 in TA3. Spectral data from the UV/vis photodiode array detector were used to identify each compound as gallic acid type based on the spectrum of gallic acid standards from 220 to 380 nm.

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

As shown in Fig. 1a, d and g, corresponding to commercial tannic acids, three common peaks could be identified in all the three tannic acid samples tested. The 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 be 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

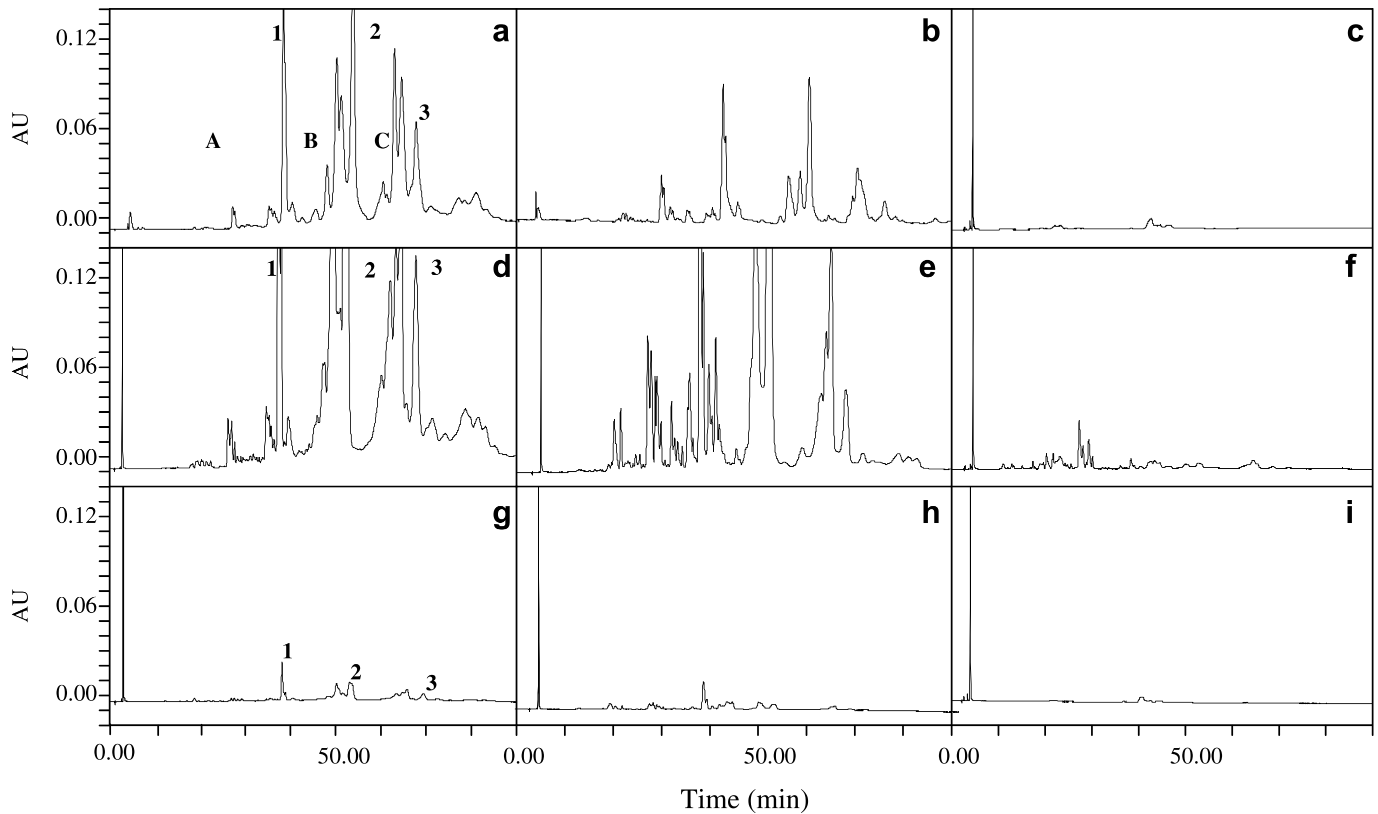

Fig. 1. HPLC chromatograms showing the composition of three different commercial tannic acids and their hydrolysis after 20 h incubation at 37 °C in phosphate buffer or in the presence of total soluble proteins from *L. plantarum* CECT 748[T]. The commercial tannic acids assayed were TA1 (a, b, and c),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

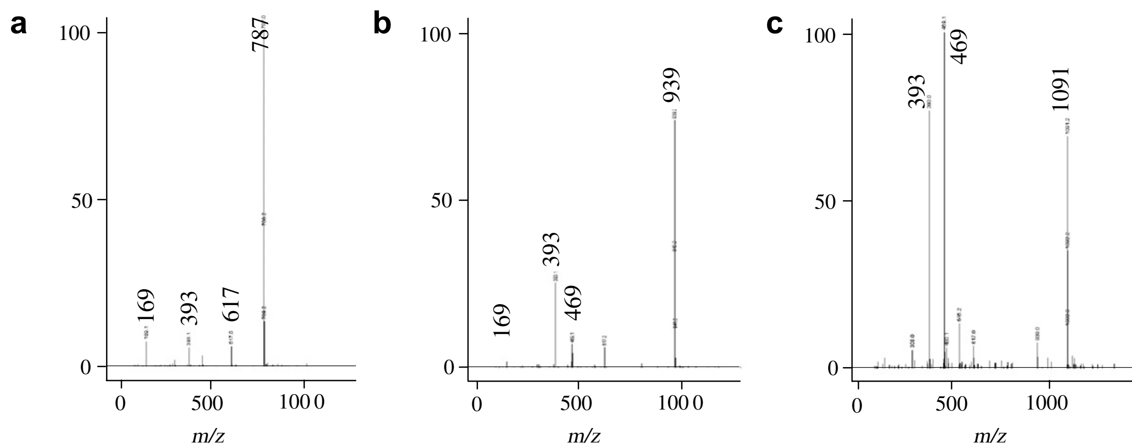

Fig. 2. Mass fragmentation pattern of peak 1 (a), peak 2 (b) and peak 3 (c) identified in commercial tannic acids. (a) Peak 1 (TR: 39.9 min) $[M-H]^-$ 787 tetragalloyl-glucoside, *m/z* 617 dehydrated digalloyl-glucoside, *m/z* 393 (not identified), *m/z* 169 (loss of the gallic acid). (b) Peak 2 (TR: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

degradation. Therefore we incubated cell-free extracts of *L. plantarum* in the presence of 1 mM tannic acid (TA1) and aliquots were withdrawn at different incubation times. Similarly, phosphate buffer containing 1 mM TA1 was incubated in the same conditions. Fig. 3 shows 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

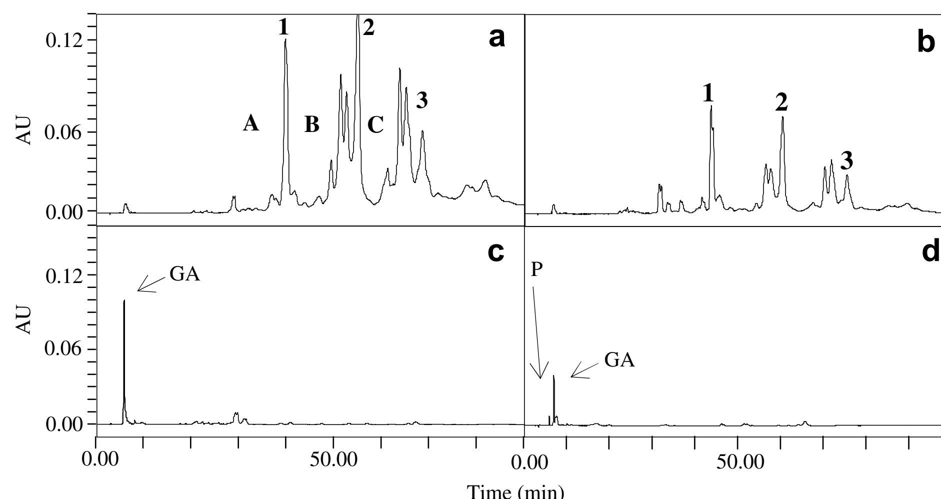

Fig. 3. HPLC chromatograms showing disappearance of tannic acid and appearance of hydrolysis products over time during the hydrolysis of TA1 by cell-free extracts of *L. plantarum* CECT 748^T. Incubation time: 0 h (a), 30 min (b), 6 h (c) and 24 h (d). Peaks 1, 2 and 3 are shown. Chromatogram regions,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

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

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

In order to confirm these results, we identified some of the peaks included in zones A, B and C from TA1 by LC/ESI-MS. Zone A includes from monomers ($[M-H]^-$ 169, gallic acid) to tetramers of gallic acid and glucose ($[M-H]^-$ 787 and ions at m/z 617 dehydrated digalloyl-glucoside). Zone

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	12.2	20.3	8.98	15.9	48.2	35.9
	30 min	16.8	19.6	7.76	23.2	45.8	31.0
	6 h	19.2	24.6	5.02	25.3	48.9	25.7
	24 h	21.8	21.6	3.04	37.3	40.4	22.3
L. plantarum	0	13.1	20.3	8.76	16.5	45.7	37.8
	30 min	23.3	18.5	6.72	37.6	35.4	26.1
	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.

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

From the data of chromatograms analysed in Table 1, we calculated the loss of total area detected. We observed that during a prolonged incubation period (24 h) in the conditions used in this assay, TA1 showed a 63% autodegradation. However, tannic acid degradation is highly increased by the presence of *L. plantarum* cell-free extracts, which only on a 6 h incubation period, degraded as much as 95% of the tannic acid compounds.

The mechanism of tannic acid degradation followed by *L. plantarum* extracts seems to be quite similar to that observed during autodegradation, tannins which are highly polymerised are hydrolysed to less polymerised and higher polar tannins. Tannic acid degradation into monomeric products by *L. plantarum* is in accordance with the depolymerisation of phenolic compounds present in olive mill wastewater observed by Kachouri and Hamdi (2004). They reported that olive mill wastewater fermented with *L. plantarum* shows a depolymerisation of high molecular weight of phenolic compounds and a reduction of low molecular weight phenolics compounds. The application of *L. plantarum* favours the increase of all phenolic compounds in olive oil, especially by depolymerisation, and inhibited the polymerisation of phenolic compounds during storage hence being responsible for the darkening of the olive mill wastewater (Ayed & Hamdi, 2003).

As observed in Fig. 3c and d, the degradation of TA1 by *L. plantarum* produces two intermediates. At 6 h incubation, a peak which presented at retention time of 5.7 min and showed spectra with maximum similar to gallic acid (Fig. 4a) and $[M-H^-]$ 169 was found. After 24 h incubation, this peak was markedly reduced and appeared a new peak showing pyrogallol $[M-H^-]$ 125 and UV spectra (Fig. 4b). Therefore, the proposed biochemical pathway for the degradation of tannic acid by *L. plantarum* implies that tannic acid is hydrolysed to gallic acid and glucose, and the gallic acid formed is decarboxylated to pyrogallol. The presence of a tannase and a gallate decarboxylase in *L. plantarum* has been previously reported (Osawa et al., 2000). These activities were only detected by low precise colorimetric methods, therefore these enzymatic activities remains to be precisely determined. Similarly, in an attempt to elucidate the metabolic pathway of tannic acid degradation by *Citrobacter freundii* TB3, Kumar et al. (1999) detected glucose, gallic acid and pyrogallol as metabolic intermediates and chromatographic analyses revealed that there was no other aromatic compound formed from pyrogallol. Degradation of hydrolysable tannins is best understood in fungal systems. The oxidative degradation of hydrolysable tannins has been studied in detail in *Aspergillus* spp. and the pathways of gallic acid degradation have been determined. In *Aspergillus flavus*, gallic acid is degraded to oxaloacetic acid and finally pyruvic acid through a tricarboxylic acid intermediate. In *Aspergillus niger*, pyrogallol, the decarboxylated derivative of gallic acid is also oxidatively broken down into *cis*-aconitic acid, which then enters the citric acid cycle (Bhat et al., 1998).

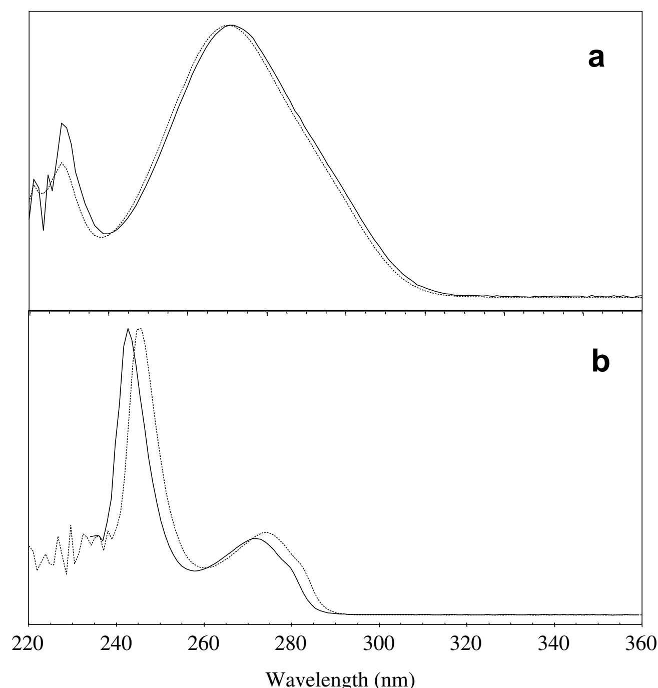

Fig. 4. Comparison between spectra of gallic acid and pyrogallol identified as intermediates during tannic acid hydrolysis and the standards of the database. (a) Gallic acid and (b) pyrogallol. The uncontinuous line indicates the line corresponding to the standard.

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

Acknowledgments

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