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Metabolism of Gallic Acid and Catechin by *Lactobacillus hilgardii* from Wine

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The ability of *Lactobacillus hilgardii* 5w to metabolize gallic acid and catechin was evaluated. It was grown in a complex medium containing gallic acid or catechin. The metabolites were analyzed by high-performance liquid chromatography and identified by comparing the retention times and spectral data with the standards of a database. In gallic acid-grown cultures, gallic acid, pyrogallol, catechol, protocatechuic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzyl alcohol were detected. In catechin-grown cultures, catechin, gallic acid, pyrogallol, catechol, *p*-hydroxybenzoic acid, acetovanillone, and homovanillic acid were detected. This work presents evidence of gallic acid and catechin degradation by *L. hilgardii* from wine.

KEYWORDS: ***Lactobacillus hilgardii*; gallic acid; catechin**

INTRODUCTION

Grapes and wine contain a large array of phenolic compounds. There are three classes: One is composed of the nonflavonoids, which are derived from cinnamic and benzoic acids; the second class includes the flavonoids; and the third group involves tannins, composed of gallic or ellagic acids or polymeric molecules of flavonoids such as catechin or leucoanthocyanidins (*1*). In grapes, the flavonoids constitute 80% of the phenolic compounds and gallic acid is the major phenolic acid (*1*, *2*). The specific amounts and types of phenolics present in grapes and wine depend on a number of factors, including variety, maturity, seasonal conditions, storage, and processing practices (*3*). Phenols are responsible for red wine color, astringency, and bitterness, in addition to contributing somewhat to the olfactory profile of the wine (*1*).

Flavonoids and other phenolics have been reported to have multiple biological effects such as antioxidant activity, anti-inflammatory action, inhibition of platelet aggregation, and antimicrobial activities (*4*). It has been suggested that the moderate intake of wine provides protection against coronary heart disease (CHD) because of the antioxidant properties of the phenolic compounds. The "French paradox", i.e., the apparent compatibility of a high fat diet with a low incidence of CHD, has been attributed to the regular consumption of red wine (*5*).

It has been demonstrated that gallic acid could have a stimulatory (*6*−*8*) or inhibitory (*9*) effect on the growth of lactic acid bacteria. In a previous work, Alberto et al. (*10*) reported the effects of different concentrations of gallic acid and (+)-catechin in laboratory media on the growth and metabolism of *Lactobacillus hilgardii* 5w, a wine spoilage lactic acid bacterium. At concentrations normally present in wine, these pure phenolic compounds not only stimulated the growth rate but also resulted in greater cell densities during the stationary growth phase, and at high concentrations, they inhibited the bacterial growth. It was also reported (*11*) that there is an inverse relationship between total phenolic concentration in wine media and cell viability and a direct relationship between *L. hilgardii* tannin binding and its viability loss.

Much scientific interest has focused on the ability of some microorganisms to metabolize phenolic compounds. *Lactobacillus* and *Pediococcus* strains were found to be able to metabolize phenol carboxylic acids (ferulic and *p*-coumaric) (*12*). The inhibitory effect of hydroxycinnamic acids (caffeic, coumaric, and ferulic acids) on the growth of the wine spoilage lactic acid bacteria *Lactobacillus collinoides* and *Lactobacillus brevis* was studied (*13*). *L. collinoides* has the capacity to metabolize hydroxycinnamic acids by reduction of their side chain (*14*). Phenol carboxylic acids can be decarboxylated by yeasts and lactic acid bacteria to produce off-flavors with low taste thresholds (*15*, *16*). *Saccharomyces cerevisiae* is able to decarboxylate the side chain of hydroxycinnamic acids to produce vinyl derivates (*17*).

To our knowledge, there is no information about the degradation of gallic acid and catechin by lactic acid bacteria from wine.

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Table 1. Modification in the Phenolic Compound Concentrations of Red Wine after Incubation of *L. hilgardii* 5w

phenolic compound	initial concentration	incubation time 10 days			
		noninoculated wine		inoculated wine	
		4 °C	20 °C	4 °C	20 °C
anthocyanins	91.6 ± 4.6[a]	91.0 ± 2.2	89.0 ± 2.5	80.3 ± 4.0	40.8 ± 2.0
catechins	1098.0 ± 50.2[b]	1092.0 ± 47.3	1084.3 ± 50.4	1090.0 ± 58.4	565.7 ± 10.3
procyanidins	1086.0 ± 42.5[c]	1084.0 ± 51.2	1086.0 ± 43.7	645.0 ± 38.5	304.0 ± 27.2
total phenolics	2600.0 ± 87.0[d]	2598.0 ± 53.0	2596.3 ± 72.3	2050.0 ± 64.3	1500 ± 72.1

[a] mg L^{-1} malvidin 3-glucoside equivalent. [b] mg L^{-1} (+)-catechin equivalent. [c] mg L^{-1} cianidin clorhidre equivalent. [d] mg L^{-1} gallic acid equivalent. Each value is the mean of three replicates ± SD.

In this paper, we report the products obtained from gallic acid and catechin utilization by a lactic acid bacterium.

MATERIALS AND METHODS

Bacterial Strain. *L. hilgardii* 5w was isolated from Argentinean wine (*18*).

Culture Media and Growth Conditions. The bacterium was cultivated in a complex medium in order to study the metabolism of gallic acid and catechin. The complex FT80 medium (*19*) was used as basal medium with the following composition in g L^{-1}: casein acid hydrolysate (C 9386, Sigma, St. Louis, MO), 5.0; yeast extract (103753 Merck, Darmstadt, Germany), 4.0; glucose (108342, Merck), 5.0; fructose (105323, Merck), 3.5; D-L malic acid (100382, Merck), 10.0; KH_2PO_4 (105108, Merck), 0.6; KCl (104934, Merck), 0.45; $CaCl_2$ (C 4901, Sigma), 0.13; $MgSO_4$ (106067, Merck), 0.13; $MnSO_4$ (159265, Merck), 0.003; and 1 mL of tween 80 (P 1754, Sigma), pH 5.0, with 100 mg L^{-1} gallic acid (159630, Merck) or 200 mg L^{-1} catechin (C 1251, Sigma). When gallic acid or catechin was added to the autoclaved medium, they were filter sterilized. Inoculation (5% v/v) with bacteria previously grown in FT80 medium supplemented with phenolic compounds was followed by incubation in darkness without shaking, at 30 °C for 48 h under microaerophilic conditions. Microaerophilic growth was conducted in capped tubes two-thirds full. The cultures were carried out in duplicate.

Cell Suspensions in Wine Media. To determine if resting cells of *L. hilgardii* are able to modify the phenolic composition of the wine, we determined the concentration changes of the main families of wine phenolic compounds during culture incubation. The cells harvested by centrifugation from FT80 medium incubated overnight at 30 °C were washed twice with sterile water and subsequently resuspended (approximately 10^7 cells mL^{-1}) in Argentinean red table wine. The cell suspensions in wine were incubated at 4 and 20 °C for 10 days before analyses. Incubated wines without cells were used as controls to consider the potential occurrence of chemical reactions between anthocyanins and anthocyanins and phenols.

Colorimetric Determinations in Wine Suspensions. Anthocyanins were measured by changes in the color caused by pH modification (*20*). Catechin was determined using vanillin−hydrochloric acid reagent (*21*). Procyanidins (tannins) were analyzed by a reaction based upon their transformation into anthocyanins when heated in an acidic medium (*22*). Total phenolics were estimated by Folin−Ciocalteu reagent (*23*).

Analysis of Culture Supernatants. To determine the fate of gallic acid or catechin during *L. hilgardii* growth, culture supernatants from the bacterium grown at 30 °C in FT80 media supplemented with 100 mg L^{-1} gallic acid or 200 mg L^{-1} catechin were analyzed by high-performance liquid chromatography (HPLC). Cultures grown in the absence of phenolic compounds and cultures with phenolic compounds and without cells acted as controls. Culture samples taken at times 0, 12, 24, and 48 h were centrifuged (4000*g* for 10 min), and the cell-free supernatants were stored at −20 °C prior to HPLC analysis.

Phenolic Extraction of Culture Supernatants. Cell-free supernatants (25 mL) from FT80 media at different incubation times were concentrated to dryness at 30 °C under vacuum. The concentrates were extracted twice with 1 mL of acetone/water (90:10, v/v) and then twice with 1 mL of methanol/HCl (1000:1, v/v). The liquid fractions were filtered through a 0.45 μm filter, combined, and dried in a rotary evaporator at 30 °C. The residue was redissolved in 1 mL of methanol/water (50:50, v/v), and 20 μL was analyzed by HPLC as described below.

HPLC Analysis. *Chromatographic Apparatus and Conditions.* A Waters (Milford, MA) chromatograph equipped with a 600-MS system controller, a 717 plus autosampler, and a 996 photodiode array detector was used. A gradient 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 to a reversed-phase Nova-pack C_{18} column (30 cm × 3.9 mm i.d.) as follows: 0−55 min, 80% B linear, 1.1 mL min^{-1}; 55−57 min, 90% B linear, 1.2 mL min^{-1}; 57−70 min, 90% B isocratic, 1.2 mL min^{-1}; 70−80 min, 95% B linear, 1.2 mL min^{-1}; 80−90 min, 100% B linear, 1.2 mL min^{-1}; 90−120 min, washing, and reequilibration of the column (*24*). Detection was performed by scanning from 200 to 380 nm.

Identification of Intermediate Products. Spectrophotometric and chromatographic methods such as gas chromatography and HPLC are largely involved with the identification of metabolic intermediates, which are often excreted into the growth medium. The identification of pathway intermediates was carried out by comparing the retention times and spectral data of each peak with those of standards (*25*) from Sigma (Deisenhofen, Germany), Fluka (Buchs, Switzerland), and Aldrich (Steinheim/Albuch, Germany) or with previously purified compounds (*26*).

Phenolic Compounds Adsorption by *L. hilgardii*. The adsorption of phenolic compounds on cell walls was carried out using heat-inactivated cells. Inactivation was conducted by heating the cells (approximately 2×10^8 cells mL^{-1}), in FT80 medium at 100 °C for 20 min. Inactivated cells of *L. hilgardii* were resuspended in FT80 media with added gallic acid (100 mg L^{-1}) or catechin (200 mg L^{-1}) for 48 h at 30 °C. After this time, the concentration of phenolic compounds was measured in the culture-free supernatant.

RESULTS

Modification in the Concentrations of Red Wine Phenolic Compounds. Table 1 shows the decrease of anthocyanins, catechins, procyanidins, and total phenolics caused by *L. hilgardii* 5w survival in wine. The decreases in anthocyanins concentration in the cell-free supernatants at 10 days of storage at 4 and 20 °C were 12.3 and 55.4%, respectively. There was no change in the catechin concentration at 4 °C, but at 20 °C, a decrease of 48.5% was observed. The reductions of procyanidins at 4 and 20 °C were 40.6 and 72.0%, respectively. No modification in the concentration of phenolic compounds was observed in the control noninoculated and incubated wine.

Gallic Acid and Catechin Consumption. Table 2 shows gallic acid and catechin consumption by growing cells of *L. hilgardii* 5w. The percentages of gallic acid and catechin consumed from FT80 medium at 48 h incubation at 30 °C were 26 and 37%, respectively. The higher consumption rate of gallic acid was observed between 12 and 24 h. It was 12.7-fold higher than in the first 12 h and 10.7-fold higher than the range from 24 to 48 h of incubation. Catechin utilization began immediately after the beginning of growth, and catechin was consumed more rapidly between 24 and 48 h of incubation. At this incubation

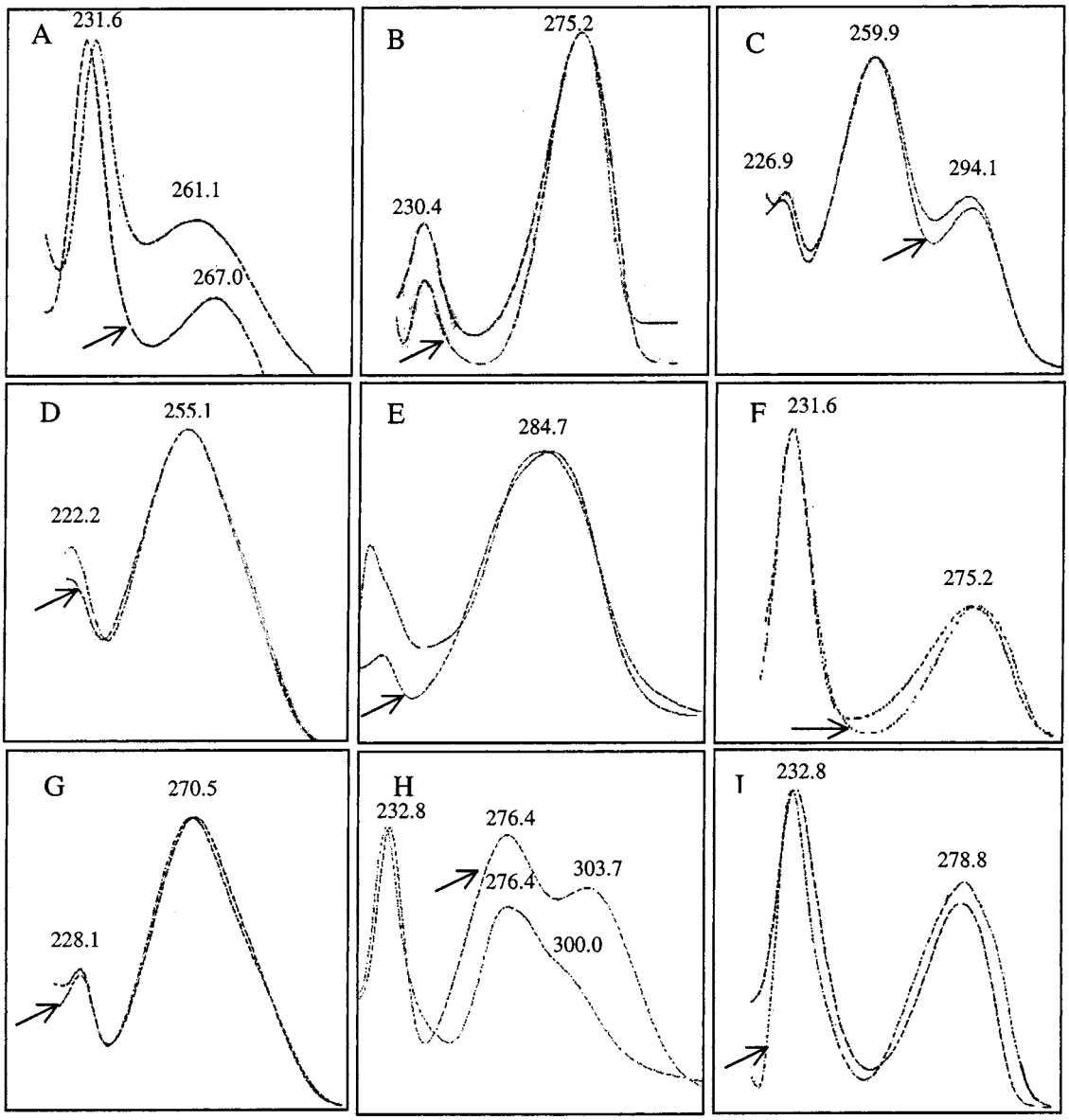

Figure 1. Comparison between spectra of the phenolic metabolites identified and the standards of the database. (**A**) Pyrogallol, (**B**) catechol, (**C**) protocatechuic acid, (**D**) *p*-hydroxybenzoic acid, (**E**) *p*-hydroxybenzaldehyde, (**F**) *p*-hydroxybenzyl alcohol, (**G**) gallic acid, (**H**) acetovanillone, and (**I**) homovanillic acid. The arrow indicates the line corresponding to the standard.

Table 2. Modification in Gallic Acid and Catechin Contents by Growing and Inactivated Cells of *L. hilgardii* 5w in FT80 Medium

	control		heat-inactivated cells		growing cells	
incubation time (h)	gallic acid	catechin	gallic acid	catechin	gallic acid	catechin
0	80.3[a]	158.2	81.5	159.1	80.9	156.3
12	79.7	157.1	81.0	158.1	79.6	148.2
24	79.1	155.4	80.4	156.7	63.0	139.4
48	78.5	153.2	79.8	154.8	59.9	98.98

[a] Results are expressed in mg L^{-1}. Relative standard deviation (RSD) $\leq$ 3%. Control: FT80 medium, pH 5.0, with phenolic compounds and without cells. The assay was carried out at 30 °C.

time, the consumption rate of catechin was 2.2-fold higher than in the first 12 h of incubation and 1.3-fold higher than the next 12 h.

Adsorption of Gallic Acid and Catechin. Heat-inactivated cells were used in order to study adsorption of phenolic compounds on cell walls. When inactivated cells of *L. hilgardii* 5w were incubated for 48 h in FT80 medium with gallic acid (100 mg L^{-1}) or catechin (200 mg L^{-1}), no adsorption of phenolic compounds was observed. **Table 2** shows that there were no changes in the phenolic concentrations during the incubation.

Identification of Intermediates from Gallic Acid and Catechin. The elucidation of the structures of unknown phenolic compounds was carried out according to Bartolomé et al. (*25*), who reported a detailed study of the possibilities and methodology for the use of a photodiode array detector. This chromatographic detector has led to considerable improvements in the HPLC analysis of phenolic compounds, as not only the retention time but also the UV spectrum can be used for identification purposes.

Table 3 shows the aromatic metabolites analyzed by HPLC after extraction from FT80 media. The identities of the metabolites were determined by comparing the retention times and spectral data with the standards of a database. In media

Table 3. Detection of Aromatic Products in Culture Media during 48 h of Growth of *L. hilgardii*[a]

products	aromatic growth substrate		retention time (min)
	gallic acid	catechin	
acetovanillone	−	+	28.4
catechol	+	+	8.1
gallic acid	+	+	5.7
homovanillic acid	−	+	22.0
p-hydroxybenzyl alcohol	+	+	9.1
p-hydroxybenzaldehyde	+	−	18.1
p-hydroxybenzoic acid	+	+	14.5
protocatechuic acid	+	−	9.5
pyrogallol	+	+	3.0

[a] The + symbol indicates that the compound was detected. The assay was carried out in FT80 medium, pH 5.0, at 30 °C.

from gallic acid-grown cultures, gallic acid, pyrogallol, catechol, protocatechuic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzyl alcohol were detected. In catechin-grown cultures, catechin, gallic acid, pyrogallol, catechol, *p*-hydroxybenzoic acid, acetovanillone, and homovanillic acid were detected.

Figure 1 shows the UV spectra of the phenolic compounds determined using the diode array detector. This spectral study shows the usefulness of the diode array detector for the identification of the phenolic compounds. Incubated media with cells and without phenolic compounds and incubated media without cells and with phenolic compounds were used as controls.

DISCUSSION

In wine media, *L. hilgardii* 5w was able to modify the concentrations of anthocyanins, catechins, and total phenolics with a greater decrease observed at 20 °C than at 4 °C. This fact could be related to the greater cell activity at 20 °C than at 4 °C. The procyanidins (tannins) decreased in wine during storage, with a greater decrease observed at 20 °C than at 4 °C. This fact could be related to the binding of tannins to bacteria reported previously (*11*). The inhibitory effects of tannins on bacteria have been associated with binding to extracellular polymers, inhibition of cell membrane and enzyme activity, and substrate and metal ion deprivation (*27*).

Gallic acid and catechin did not bind to *L. hilgardii* 5w cells in FT80 medium as we determined in the adsorption experiment with inactivated cells. These results are in agreement with Vivas et al. (*7*) who demonstrated in *Leuconostoc oenos* that there was not adsorption of gallic acid on cell walls.

Using viable cells, we observed a reduction of the phenolic compounds concentration in the media, but using inactivated cells, we determined that there was no modification in the concentration of phenolic compounds. So the diminution could be a consequence of phenolic compounds utilization and not due to adsorption on cell walls.

Pyrogallol is known to serve as an oxygen scavenger and to reduce the redox potential of the media. As lactic acid bacteria grow better in the absence of oxygen, the pyrogallol produced from gallic acid and catechin metabolism could be related to this property. Alberto et al. (*10*) reported a higher growth of *L. hilgardii* 5w in the media FT80 with added gallic acid or catechin. A pathway for the anaerobic catabolism of gallic acid by *Eubacterium oxidoreducens* was proposed, with pyrogallol as the only detectable product (*28*). We also found protocatechuic acid from gallic acid. In *Penicillium simplicissimun*, gallic acid is metabolized through a dihydrogallic acid, which subsequently loses water to produce protocatechuate derivatives (*29*).

Barz et al. demonstrated the ability of various fungi to disintegrate flavonoids in phenolic acids in a first step and then disintegrate carboxylic acid to primary metabolites. *L. hilgardii* 5w has the property to produce gallic acid (phenolic acid) from catechin (flavonoid compound).

From gallic acid and catechin, *L. hilgardii* 5w also produced catechol, protocatechuic, and trace amounts of *p*-hydroxybenzyl alcohol, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde. *Streptomyces setonii* catabolized benzoate, guaiacol, and vanillate through catechol and *p*-hydroxybenzoate through protocatechuate (*31*). *S. setonii* also catabolizes cinnamic acid via benzaldehyde, benzoic acid, and catechol; *p*-coumaric acid was catabolized via *p*-hydroxibenzaldehyde, *p*-hydroxybenzoic acid, and protocatechuic acid, and finally, ferulic acid was catabolized via vanillin, vanillic acid, and protocatechuic acid (*32*). A strain of *Pseudomonas* was capable to metabolize ferulic acid via vanillin, vanillic acid, and protocatechuic acid (*33*).

The results of the metabolism and possible binding of phenolics by *L. hilgardii* 5w provide useful information for the wine industry and contribute to new findings on the metabolism of important wine substances such as phenolic compounds. Further assays (using radioactive-labeled compounds) are needed to clarify the complete mechanism of gallic acid and catechin degradation.

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