Wine phenolic compounds influence the production of volatile phenols by wine-related lactic acid bacteria

I. Silva, F.M. Campos, T. Hogg and J.A. Couto

CBQF/Escola Superior de Biotecnologia – Universidade Católica Portuguesa, Porto, Portugal

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Correspondence
José A. Couto, Escola Superior de Biotecnologia – Universidade Católica Portuguesa, Rua Dr António Bernardino de Almeida, 4200-072 Porto, Portugal.
E-mail: jacouto@esb.ucp.pt

Abstract

Aims: To evaluate the effect of wine phenolic compounds on the production of volatile phenols (4-vinylphenol [4VP] and 4-ethylphenol [4EP]) from the metabolism of *p*-coumaric acid by lactic acid bacteria (LAB).

Methods and Results: *Lactobacillus plantarum*, *Lactobacillus collinoides* and *Pediococcus pentosaceus* were grown in MRS medium supplemented with *p*-coumaric acid, in the presence of different phenolic compounds: nonflavonoids (hydroxycinnamic and benzoic acids) and flavonoids (flavonols and flavanols). The inducibility of the enzymes involved in the *p*-coumaric acid metabolism was studied in resting cells. The hydroxycinnamic acids tested stimulated the capacity of LAB to synthesize volatile phenols. Growth in the presence of hydroxycinnamic acids, especially caffeic acid, induced the production of 4VP by resting cells. The hydroxybenzoic acids did not significantly affect the behaviour of the studied strains. Some of the flavonoids showed an effect on the production of volatile phenols, although strongly dependent on the bacterial species. Relatively high concentrations (1 g l^{-1}) of tannins inhibited the synthesis of 4VP by *Lact. plantarum*.

Conclusions: Hydroxycinnamic acids were the main compounds stimulating the production of volatile phenols by LAB. The results suggest that caffeic and ferulic acids induce the synthesis of the cinnamate decarboxylase involved in the metabolism of *p*-coumaric acid. On the other hand, tannins exert an inhibitory effect.

Significance and Impact of the Study: This study highlights the capacity of LAB to produce volatile phenols and that this activity is markedly influenced by the phenolic composition of the medium.

Introduction

Volatile phenols may appear in wine at different stages of the winemaking process, mostly occurring during ageing prior to bottling, especially when wines are stored in oak barrels (Chatonnet *et al.* 1992). Although they are often regarded as negative, imparting off-flavours to red wines described as 'animal', 'horse sweat' or 'medicinal', some winemakers consider that at low concentrations (<420 μg l^{-1} of a mixture of 4-ethylphenol and 4-ethylguaiacol), volatile phenols can contribute positively to the complexity of the bouquet of wines (Chatonnet *et al.* 1995; Ribéreau-Gayon *et al.* 2000; Fugelsang and Edwards 2007). The precursors of the volatile phenols are natural constituents of grape juice and wine – the hydroxycinnamic acids *p*-coumaric and ferulic acids (Heresztyn 1986; Chatonnet *et al.* 1995). The conversion of these phenolic acids involves the sequential activity of two enzymes: the first is the cinnamate decarboxylase, which decarboxylates the hydroxycinnamic acid into the corresponding vinylphenol (4VP from *p*-coumaric acid or 4-vinylguaiacol from ferulic acid), and the second is the vinylphenol reductase, which reduces the vinylphenol into the corresponding ethylphenol (4EP from 4VP and 4-ethylguaicol from 4-vinylguaiacol) (Heresztyn 1986; Chatonnet *et al.* 1992; Boulton *et al.* 1996).

Brettanomyces, specifically the species *B. bruxellensis* and *B. anomalus*, and its ascosporogenous form *Dekkera* are considered the main organisms responsible for the production of volatile phenols (Heresztyn 1986). Chatonnet *et al.* (1992) have shown that although numerous micro-organisms could decarboxylate *p*-coumaric acid, only the yeasts from the genus *Brettanomyces* were capable of forming significant quantities of ethylphenols. Cavin *et al.* (1993) found that *Lactobacillus* and *Pediococcus* were able to metabolize phenolic acids. Chatonnet *et al.* (1995) compared the ability of lactic acid bacteria (LAB) to synthesize volatile phenols with *Dekkera/Brettanomyces*. Some of the LAB strains studied, namely *Lactobacillus brevis* and *Pediococcus pentosaceus*, were capable of producing large quantities of 4VP, but only traces of ethylphenols. Only *Lactobacillus plantarum* was capable of producing significant quantities of 4EP, however, in much lower concentrations than those produced by *Dekkera/Brettanomyces*. More recently, Couto *et al.* (2006) tested the ability of 20 different species of LAB (35 strains) to produce volatile phenols, in culture medium. They have shown that 37% of the strains studied were capable of producing volatile phenols from *p*-coumaric acid, and only 9% could produce 4EP. Chatonnet *et al.* (1997) studied the influence of polyphenolic compounds on the production of volatile phenols by LAB and found that the growth and the production capacity of *Lact. plantarum* were strongly affected by tannins. On the contrary, no effect was found on the synthesis of volatile phenols by *Dekkera/Brettanomyces*. The factors that may influence the capacity of LAB to produce volatile phenols and the ratio vinylphenols/ethylphenols obtained from this activity are not well known. Wine phenolic compounds have been shown to affect growth, and sugar, organic acid and amino acid metabolisms of LAB (Stead 1993, 1994; Vivas *et al.* 1997; Rozès and Peres 1998; Alberto *et al.* 2001; Campos *et al.* 2003; Rozès *et al.* 2003; Alberto *et al.* 2007; García-Ruiz *et al.* 2008, 2009; Campos *et al.* 2009), but its influence on the production of volatile phenols is not yet well known.

Phenolic compounds are very important to grape and wine quality, because of its contribution to colour and flavour, and are responsible for important differences between red and white wines (Macheix *et al.* 1990; Jackson 2000). The wine phenolic composition depends on the raw material (grape variety, degree of maturity of the grapes, cultivation and local environmental conditions, nature of the soil and the state of health of the grapes), vinification techniques and wine ageing (Macheix *et al.* 1990). These compounds can be divided in two groups: nonflavonoids (which include phenolic acids, aldehydes and alcohols) and flavonoids (comprising anthocyanins, flavonols and flavanols). The two major subgroups of phenolic acids are hydroxybenzoic (vanillic, gallic, syringic, salicylic and gentisic) and hydroxycinnamic (*p*-coumaric, caffeic, ferulic and sinapic) acids, which are present in concentrations of 100–200 mg l^{-1} in red wines and 10–20 mg l^{-1} in white wines (Reguant *et al.* 2000; Ribéreau-Gayon *et al.* 2000). Flavonoids are molecules that possess two phenols joined by an oxygen-containing carbon ring structure. Among them, the flavonols are differentiated by the substitution of the lateral nucleus, producing kaempferol, quercetin and myricetin. The most important flavanols in wine are [+]-catechin and [-]-epicatechin, which are the monomers of tannins (Macheix *et al.* 1990). Tannins are polyphenols capable of precipitating proteins or other macromolecules such as polysaccharides. They can be separated into two classes: the hydrolysable tannins (which include gallotannins or ellagitannins that release gallic or ellagic acids, respectively, under hydrolytic conditions) and the condensed tannins (with catechin and epicatechin as basic structural units) (Macheix *et al.* 1990; Ribéreau-Gayon *et al.* 2000).

The objective of this work was to study the influence of phenolic acids (caffeic, ferulic, vanillic and gallic), flavonols (kaempferol, quercetin and myricetin), flavanols (catechin and epicatechin) and tannins on the production of volatile phenols by *Lact. plantarum*, *Lactobacillus collinoides* and *Ped. pentosaceus*.

Materials and methods

Bacteria and growth conditions

Three strains of LAB, *Lact. plantarum* NCFB 1752, *Ped. pentosaceus* NCFB 990 (National Culture of Food Bacteria, Reading, UK) and *Lact. collinoides* ESB 99 (Escola Superior de Biotecnologia, UCP, Porto, Portugal), were used. These strains were chosen because of their ability to convert *p*-coumaric acid into 4VP and/or 4EP as demonstrated by Couto *et al.* (2006).

Cultures were grown aerobically, without agitation, to late exponential phase in MRS medium (Lab M, Lancashire, UK). The initial pH was adjusted to 4·5 with a concentrated (6 mol l^{-1}) HCl solution before sterilization (121°C, 15 min). After sterilization, ethanol (99·5% v/v) was added to the medium to obtain a final concentration of 5% v/v. Cultures were maintained on slants prepared with MRS with 2% agar (Pronadisa, Madrid, Spain), pH 4·5, 5% v/v ethanol, at 4°C, with monthly transfers to maintain strain viability.

Influence of phenolic compounds on growth and volatile phenols production by LAB

Cultures in late exponential phase were transferred to MRS medium with 50 mg l^{-1} of *p*-coumaric acid (pH 4·5),

supplemented with different phenolic compounds at 0 (control), 50 and 100 mg l^{-1}. *p*-coumaric acid (trans-4-hydroxycinnamic acid; 98% purity), caffeic acid (dihydroxycinnamic acid; 99% purity), ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid; 99% purity), gallic acid (3,4,5-trihydroxybenzoic acid), vanillic acid (p-hydroxymethoxybenzoic; 97% purity), quercetin (3,3′,4′,5,7-pentahydroxyflavone, 98% purity) and kaempferol (3,4′,5,7-tetrahydroxyflavone, 90%) were obtained from Sigma-Aldrich (Steinheim, Germany). Catechin (trans-3,3′,4′,5,7-pentahydroxyflavane, 96% purity) and epicatechin [(-)-cis-3,3′,4′,5,7-pentahydroxyflavane, 90% purity] were obtained from Fluka (Buchs, Switzerland). Myricetin was obtained from Extrasynthese (Genay, France). Fresh concentrated solutions of phenolic acids (10 g l^{-1}) and flavonoids (5 g l^{-1}) were prepared in pure (99·5% v/v) ethanol, filter-sterilized and added to the growth media. The final ethanol concentration was adjusted to 5% v/v.

Cellular growth was monitored daily by measuring the absorbance at 660 nm, using a UV-VIS Nicolet evolution 300 spectrophotometer (Thermo Electron Corporation, Cambridge, UK) and optical cells of 1 cm path length. Dilutions with distilled water were made when the optical density value exceeded the linearity limit of Beer–Lambert's law.

When cultures reached the stationary phase, samples were taken for the quantification of volatile phenols (4EP and 4VP). All assays were made in triplicate.

Production of volatile phenols by resting cells and the influence of cultivation conditions

Bacteria were grown at 30°C, to late exponential phase, in MRS medium (pH 4·5, 5% v/v ethanol) supplemented with 50 mg l^{-1} of *p*-coumaric acid, or 50 mg l^{-1} of caffeic acid, or 50 mg l^{-1} of ferulic acid, or a mixture of 50 mg l^{-1} *p*-coumaric acid and 50 mg l^{-1} caffeic or ferulic acids. The cells were harvested by centrifugation (3000 ***g***, 10 min) and washed twice with phosphate buffer solution (KH_2PO_4, 50 mmol l^{-1}, pH 5·5) (Vivas *et al.* 1997). Cells were suspended in 70 ml of this buffer supplemented with 50 mg l^{-1} of *p*-coumaric acid. Samples were collected, and volatile phenols were quantified after 30 min, 4 and 8 h of incubation at 30°C. All assays were made in triplicate.

Influence of tannins on growth and production of volatile phenols by LAB

The condensed tannins used were extracted from *Vitis vinifera* grape seed tissues, according to the method described by de Freitas *et al.* (2003). Polyphenols were extracted from grape seed tissues (5 g) with 50 ml of ethanol/water/chloroform solution (1 : 1 : 2 v/v/v) using a blender. The upper aqueous layer containing the polyphenols was separated from the chloroform layer containing chlorophylls, lipids and other unwanted components. Ethanol was removed from the hydroalcoholic layer on a rotary evaporator at 30°C, and polyphenol compounds were extracted from the resulting aqueous solution with ethyl acetate (3 × 20 ml). The volume of ethyl acetate was reduced to 10 ml on a rotary evaporator at 30°C, and the condensed tannins were obtained by precipitation with 15 ml of hexane. The resulting solid was purified by column chromatography (TSK Toyopearl HW-40(s) gel) to yield catechin monomers and polymeric procyanidin fractions. These fractions were directly analysed by LSI/MS. The average molecular weight of the different fractions is as follows: fraction I – 291, fraction V – 1513, fraction VI – 2052. The commercial tannin used (Tanipepin, Proenol, Portugal) is a proanthocyanidic tannin extracted from grape seeds.

To test the effect of tannins, cultures in late exponential phase were transferred to MRS medium (pH 4·5) with 50 mg l^{-1} *p*-coumaric acid supplemented with different tannin fractions at 0, 50 and 100 mg l^{-1}. Fresh concentrated solutions of tannins (5 g l^{-1}) were prepared in pure (99·5% v/v) ethanol, filter-sterilized and added to the growth media. The final ethanol concentration was adjusted to 5% v/v. Cellular growth was monitored daily, and volatile phenols (4EP and 4VP) were quantified when cultures reached the stationary phase.

High concentrations of tannins (normally present in red wines in the range 1–4 g l^{-1}) could not be tested in culture medium because of precipitation. To avoid this, 1 g l^{-1} of commercial tannins was tested in phosphate buffer solution (KH_2PO_4, 50 mol l^{-1}, pH 5·5) supplemented with 50 mg l^{-1} of *p*-coumaric acid, to study its influence on the production volatile phenols by resting cells of *Lact. plantarum* NCFB 1752. After growing at 30°C to late exponential phase in MRS medium (pH 4·5, 5% v/v ethanol) supplemented with 50 mg l^{-1} of *p*-coumaric acid, cells were harvested by centrifugation (3000 ***g***, 10 min), washed twice and transferred to the buffer solution. Samples were collected, plated in duplicate in MRS medium containing 20 g l^{-1} agar, and volatile phenols were quantified after 4 and 8 h of incubation at 30°C. All assays were made in triplicate.

Analysis of volatile phenols

The samples were centrifuged (3000 ***g***, 10 min), and the supernatants were analysed according to the method described by Bertrand (1981). Fifty microlitres of 4-decanol

(internal standard) was added to 50 ml of culture medium (0·819 g l^{-1}). This mixture was successively extracted with 4, 2 and 2 ml of ether/hexane (1 : 1) by stirring for 5 min. The organic phases were collected, mixed and concentrated under a stream of nitrogen to approximately half of the initial volume. One microlitre of the extract was injected into a Perkin Elmer GC-FID (Shelton, CT, USA). The column employed was a FFAP type (BP 21·50 m × 0·25 mm × 0·2 μm), from SGE (Austin, TX, USA). The injector (split/splitless) was heated to 220°C with a split flow of 30 ml min^{-1} and a splitless time of 0·5 min. The carrier gas flow was adjusted to 1 ml min^{-1}. The temperature of the oven was maintained at 4°C for 1 min and was then increased at a rate of 2°C min^{-1} up to 220°C. This temperature was then maintained for 30 min. The detection limit of the method was 0·05 mg l^{-1} for 4VP and 0·01 mg l^{-1} for 4EP.

Statistical analysis

One-way analysis of variance (ANOVA) was used to test the effect of phenolic compound's concentration on the production of volatile phenols, and *post hoc* Tukey test was used for multiple mean comparisons. All statistical analyses were preformed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL).

Results

Influence of phenolic acids on growth and production of volatile phenols by LAB

Different phenolic acids at concentrations of 50 and 100 mg l^{-1} were tested for their influence on the production of volatile phenols by LAB. The growth rate and the final biomass of the tested strains were not significantly affected by the hydroxybenzoic (gallic and vanillic) and hydroxycinnamic (ferulic and caffeic) acids tested (data not shown). Gallic acid had no effect on the production of 4EP by *Lact. collinoides* ESB 99; however, a slight decrease in the amount of 4VP is noted (Fig. 1). In *Lact. plantarum* NCFB 1752, there was a slight increase in the amount of 4EP, while no differences were noticed concerning 4VP. Regarding *Ped. pentosaceus* NCFB 990, no influence was noticed on the production of 4VP (the only volatile phenol produced by this strain in the conditions studied). Vanillic acid did not affect the production of volatile phenols (Fig. 1).

Within the hydroxycinnamic acids, caffeic acid clearly stimulated the production of 4VP by *Lact. plantarum* NCFB 1752 and *Ped. pentosaceus* NCFB 990, while the reduction of 4VP into 4EP was not affected (Fig. 2). *Lactobacillus collinoides* ESB 99 was found to be less affected than the other bacteria. The presence of ferulic acid stimulated the metabolism of *p*-coumaric acid by *Lact. collinoides* ESB 99, increasing the production of 4EP but not affecting the amount of 4VP. The concentration of both volatile phenols was enhanced in *Lact. plantarum* NCFB 1752, while the production of 4VP by *Ped. pentosaceus* NCFB 990 was not affected. Caffeic and ferulic acids were added separately to MRS medium to check the possible use of these compounds as substrates for the production of 4VP and 4EP by LAB. No peaks, corresponding to these compounds, were detected on the GC-FID analyses (data not shown).

Following these results, the influence of the addition of hydroxycinnamic acids to the growth medium on the production of volatile phenols by resting cells was studied. It was found that, in nongrowing conditions, cells were only capable of producing 4VP (and not 4EP) from the metabolism of *p*-coumaric acid (Fig. 3). The three strains tested clearly produced more 4VP when the cells were grown in the presence of 50 mg l^{-1} of caffeic acid and 50 mg l^{-1} of caffeic acid plus 50 mg l^{-1} of *p*-coumaric acid. No statistical differences were found between the control and the cells grown in MRS supplemented only with *p*-coumaric acid, except for *Lact. plantarum* NCFB 1752. In the case of the cells grown in MRS supplemented with ferulic acid, the synthesis of volatile phenols by resting cells was not affected in any of the bacteria studied; however, an increase in the concentration of 4VP was found in the cultures grown in the presence of *p*-coumaric acid.

Influence of flavonoids on growth and production of volatile phenols by LAB

Flavonoids (kaempferol, myricetin, quercetin, catechin and epicatechin), at the concentrations tested, did not significantly influence the growth rate and the final cell density of the bacteria (data not shown). Among the flavanols, catechin did not affect the production of volatile phenols by any of the bacteria tested (Table 1). Although the total amount of volatile phenols produced by *Lact. plantarum* NCFB 1752 (4VP + 4EP) was not considerably affected by the presence of epicatechin (100 mg l^{-1}), less 4VP was converted into 4EP. Among the flavonols, kaempferol and myricetin did not affect *Lact. plantarum* NCFB 1752 and *Ped. pentosaceus* NCFB 990 (Table 1) but stimulated the metabolic activity of *Lact. collinoides* ESB 99, leading to an increase in the production of 4VP. The higher the myricetin concentration used the greater the *p*-coumaric acid conversion. Quercetin exhibited a remarkable effect on the behaviour of *Lact. plantarum* NCFB 1752 (Table 1). Although less *p*-coumaric acid was converted into volatile phenols (lower total concentration of 4VP + 4EP), the amount of

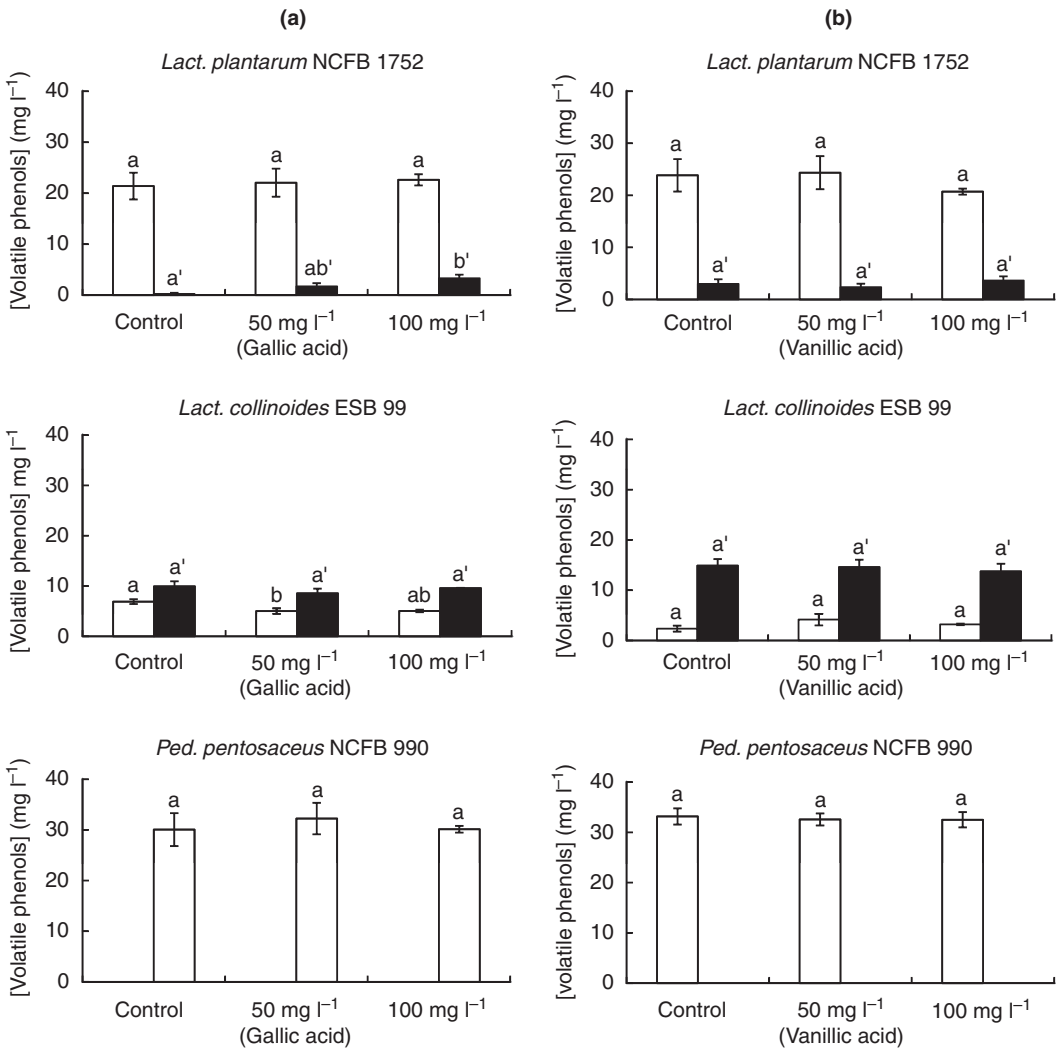

Figure 1 Effect of gallic (a) and vanillic (b) acids on the production of 4VP (white) and 4EP (black) by *Lactobacillus plantarum* NCFB 1752, *Lactobacillus collinoides* ESB 99 and *Pediococcus pentosaceus* NCFB 990. Results are the average values (with standard deviation) of experiments performed in triplicate. Bars with the same letters are not significantly different ($P < 0{\cdot}05$).

4EP produced was higher while the amount of 4VP decreased.

Influence of tannins on growth and production of volatile phenols by LAB

Tannins at 50 and 100 mg l^{-1} in MRS medium affected neither the growth nor the production of volatile phenols by the bacteria studied (Table 2). Tannins are present in wine in the range 1–4 g l^{-1}, so the effect of 1 g l^{-1} of commercial tannins on the production of volatile phenols by resting cells of *Lact. plantarum* NCFB 1752 was tested in phosphate buffer supplemented with 50 mg l^{-1} of *p*-coumaric acid. The results obtained show an inhibitory effect on the production of 4VP (Fig. 4).

Discussion

It is known that certain strains of LAB are able to produce volatile phenols from the metabolism of phenolic acids (Cavin *et al.* 1993; Chatonnet *et al.* 1995, 1997; Couto *et al.* 2006). The results obtained in this work show that various phenolic compounds can affect the transformation of *p*-coumaric acid into volatile phenols by LAB in culture medium. The hydroxycinnamic acids tested stimulated the enzymatic activity involved in the metabolism of *p*-coumaric acid of the strains studied. Caffeic acid caused a notable increase in the consumption of *p*-coumaric acid by *Lact. plantarum* NCFB 1752, which can be explained by the enhancement of the cinnamate decarboxylase activity. The vinylphenol reductase activity

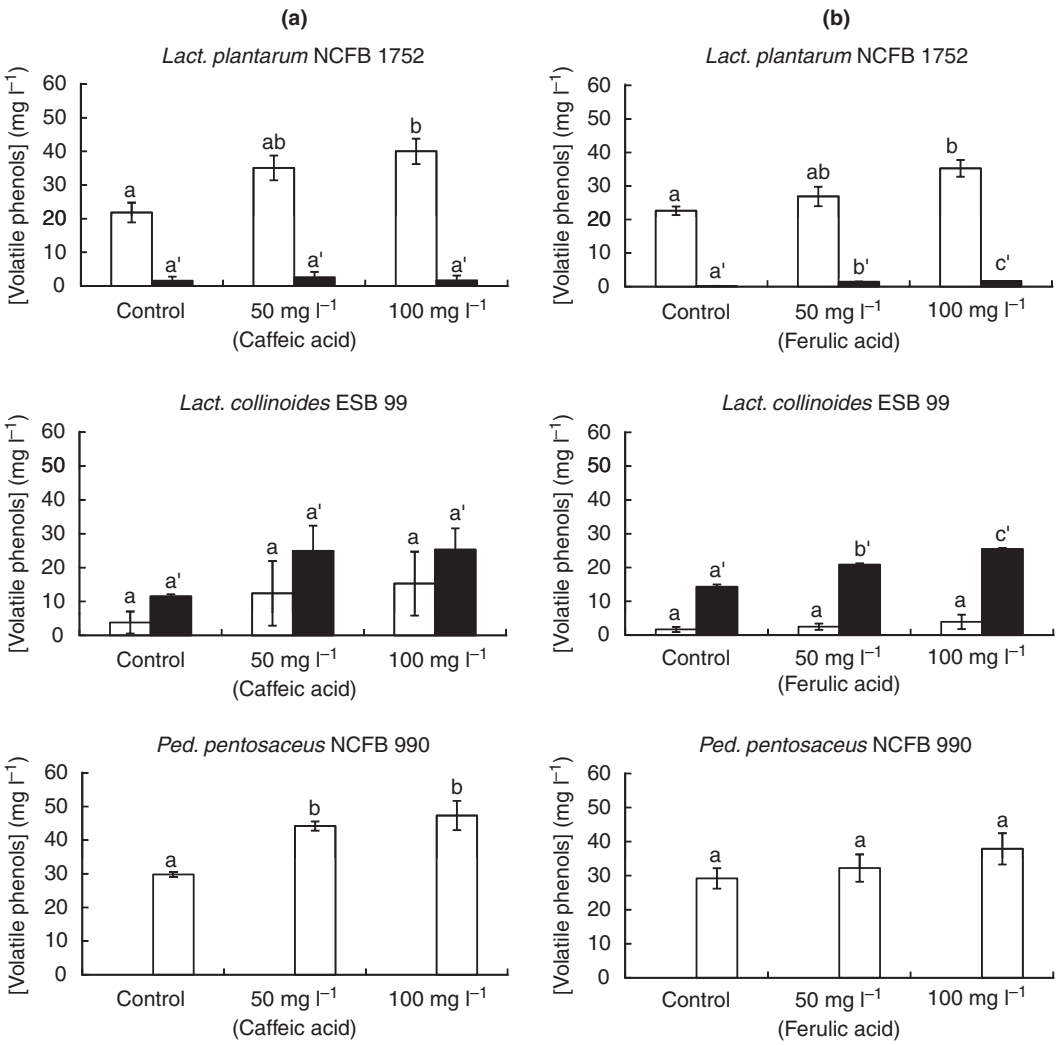

Figure 2 Effect of caffeic (a) and ferulic (b) acids on the production of 4VP (white) and 4EP (black) by *Lactobacillus plantarum* NCFB 1752, *Lactobacillus collinoides* ESB 99 and *Pediococcus pentosaceus* NCFB 990. Results are the average values (with standard deviation) of experiments performed in triplicate. Bars with the same letters are not significantly different ($P < 0{\cdot}05$).

was not, apparently, affected. A similar result was found in the experiments with ferulic acid except that the vinylphenol reductase was slightly stimulated. The *Ped. pentosaceus* NCFB 990 enzymatic activity was only affected by caffeic acid. In general, hydroxycinnamic acids affected more the production of volatile phenols than hydroxybenzoic acids. Campos *et al.* (2003) also found a higher effect of hydroxycinnamic acids as regards the inhibitory effect of phenolic acids on the growth of *Oenococcus oeni*. Hydroxycinnamic acids, because of their propenoic side chain, are much less polar than the corresponding hydroxybenzoic acids, which may facilitate the transport of these molecules across the cell membrane. Because the polarity of the molecules alone cannot explain the different effects of hydroxycinnamic acids on the growth of various micro-organisms (Baranowski *et al.* 1980; Herald and Davidson 1983; Stead 1993, 1995; Rozès and Peres 1998; Reguant *et al.* 2000; Campos *et al.* 2003) and on the production of volatile phenols, a possible explanation for the results presented in this work is the chemical similarity between the hydroxycinnamic acids. Phenolic acids are weak acids, with typical pKa values ranging from 4·2 to 4·5 for most of them (Ramos-Nino *et al.* 1996). Thus, the effect of ionization is expected to be similar among the phenolic acids studied. Generally, the resting cells that have grown in the presence of hydroxycinnamic acids were more efficient in the transformation of *p*-coumaric acid into 4VP. This induced stimulatory effect was higher for caffeic acid, which is in accordance with the results obtained with the growing cultures, where

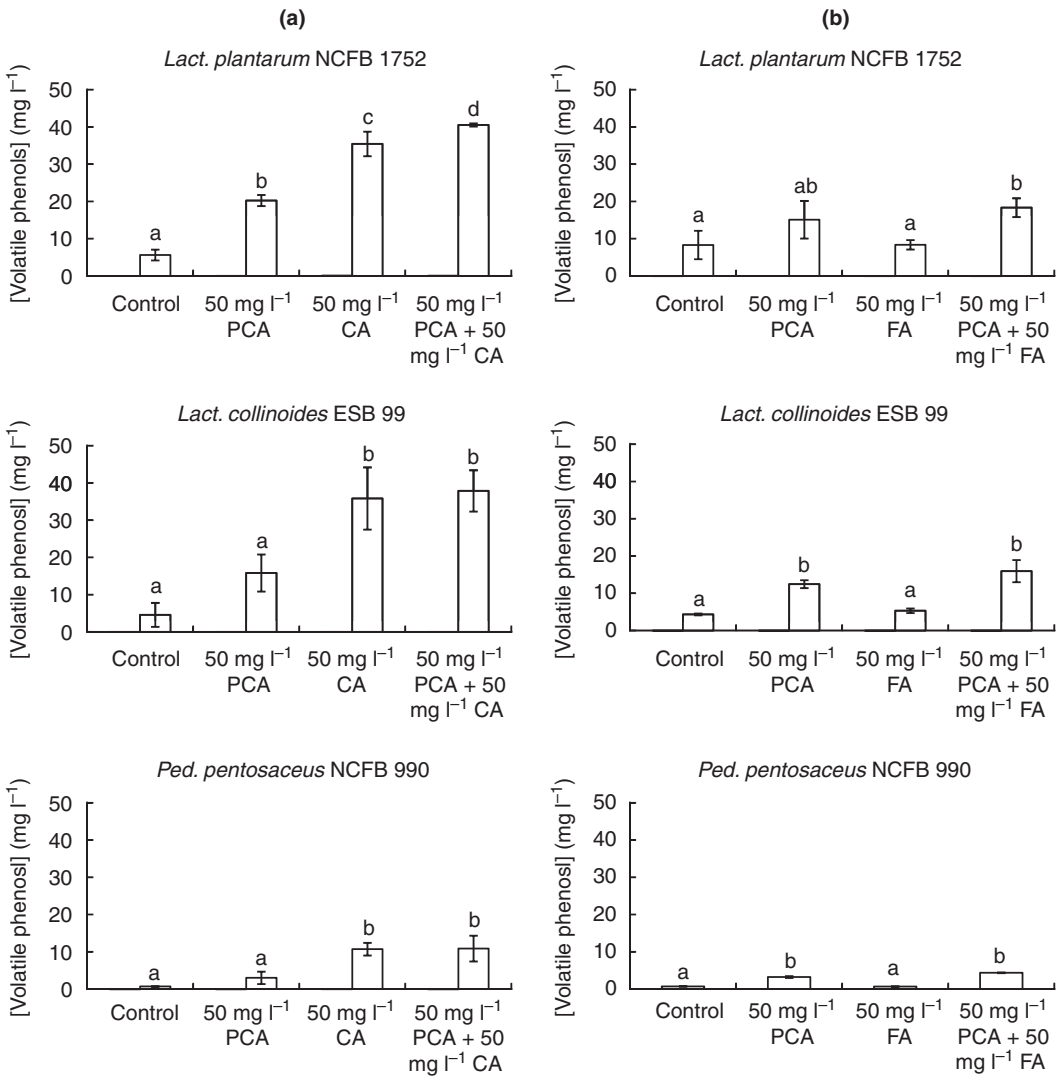

Figure 3 Influence of pre-cultivation conditions on the production of volatile phenols (4VP) by resting cells of *Lactobacillus plantarum* NCFB 1752, *Lactobacillus collinoides* ESB 99 and *Pediococcus pentosaceus* NCFB 990 grown in the presence of caffeic and/or *p*-coumaric acid (a) or ferulic and/or *p*-coumaric acid (b). Results are the average values (with standard deviation) of experiments performed in triplicate. Bars with the same letters are not significantly different ($P < 0{\cdot}05$). PCA: p-coumaric acid, FA: ferulic acid; CA: caffeic acid; control: non-induced cells (pre-cultivated in the absence of hydroxycinnamic acids).

a greater effect on the cinnamate decarboxylase activity was obtained in the presence of caffeic acid. The results support the hypothesis of enzyme induction by molecules structurally similar to the substrate. (The main difference between the hydroxycinnamic acids studied is the ring C3 substitution, which is −H for *p*-coumaric acid, −OH for caffeic acid and −OCH3 for ferulic acid.) Hashidoko *et al.* (2001) have shown the induction of the hydroxycinnamate decarboxylase in *Klebsiella oxytoca* by the substrates and by compounds structurally analogous to the substrates. The inducibility of the metabolism of *p*-coumaric acid was also suggested by Cavin *et al.* (1993, 1997a) for *Lact. plantarum* and Barthelmebs *et al.* (2000a) for *Ped. pentosaceus*. *Lactobacillus plantarum* displays substrate-inducible *p*-coumaric acid decarboxylase (PDC) encoded by the *pdc* gene, which converts *p*-coumaric acid into 4VP (Cavin *et al.* 1997b; Gury *et al.* 2004; Licandro-Seraut *et al.* 2008). van Beek and Priest (2000) demonstrated the wide distribution of similar *pdc* genes in various strains of *Lactobacillus* isolated from whisky fermentations. The induction of *Lactobacillus crispatus* with a mixture of ferulic and *p*-coumaric acids resulted in a high decarboxylation activity, suggesting that the ferulic acid decarboxylase could use *p*-coumaric acid as a

Table 1 Effect of flavonoids on the production of 4VP and 4EP by *Lactobacillus plantarum* NCFB 1752, *Lactobacillus collinoides* ESB 99 and *Pediococcus pentosaceus* NCFB 990. Results are the average values (with standard deviation) of experiments performed in triplicate

		Lact. plantarum NCFB 1752		*Lact. collinoides* ESB 99		*Ped. pentosaceus* NCFB 990	
Phenolic compound	Concentration (mg l^{-1})	4VP (mg l^{-1})	4EP (mg l^{-1})	4VP (mg l^{-1})	4EP (mg l^{-1})	4VP (mg l^{-1})	4EP (mg l^{-1})
Catechin	0	22·55 ± 3·56 a	4·50 ± 3·07 a′	6·19 ± 2·87 a	10·96 ± 3·06 a′	27·54 ± 4·26 a	nd
	50	23·70 ± 2·97 a	4·11 ± 1·29 a′	6·19 ± 2·63 a	12·90 ± 2·80 a′	21·76 ± 4·93 a	nd
	100	23·82 ± 2·89 a	5·85 ± 0·41 a′	6·28 ± 2·85 a	11·31 ± 2·89 a′	25·38 ± 3·63 a	nd
Epicatechin	0	14·98 ± 0·79 a	6·52 ± 0·00 a′	1·92 ± 0·44 a	16·07 ± 1·03 ab′	29·26 ± 2·08 a	nd
	50	17·76 ± 0·89 a	5·72 ± 0·02 a′	2·06 ± 0·50 a	14·86 ± 0·98 a′	28·91 ± 2·96 a	nd
	100	22·08 ± 0·27 b	3·47 ± 0·83 b′	2·15 ± 0·23 a	17·25 ± 0·67 b′	30·25 ± 2·18 a	nd
Kaempferol	0	19·57 ± 2·71 a	6·68 ± 2·27 a′	1·78 ± 0·20 a	13·13 ± 1·41 a′	30·60 ± 1·74 a	nd
	50	21·07 ± 6·88 a	4·65 ± 2·62 a′	5·81 ± 0·76 b	16·28 ± 0·35 a′	30·67 ± 2·31 a	nd
	100	18·08 ± 2·95 a	6·48 ± 3·46 a′	3·73 ± 0·03 c	15·04 ± 0·00 a′	30·99 ± 0·28 a	nd
Myricetin	0	15·77 ± 5·21 a	6·90 ± 4·63 a′	1·15 ± 0·63 a	15·65 ± 1·12 a′	21·08 ± 2·82 a	nd
	50	18·57 ± 7·02 a	6·19 ± 4·97 a′	3·28 ± 0·63 a	15·08 ± 1·40 a′	22·36 ± 9·28 a	nd
	100	19·14 ± 7·89 a	5·55 ± 4·67 a′	8·88 ± 0·31 c	14·25 ± 0·44 a′	29·42 ± 4·44 a	nd
Quercetin	0	17·07 ± 2·76 a	5·03 ± 1·57 a′	2·16 ± 1·12 a	9·11 ± 2·67 a′	26·17 ± 1·39 a	nd
	50	2·17 ± 1·56 b	13·77 ± 1·63 b′	1·93 ± 0·77 a	9·03 ± 3·38 a′	27·26 ± 2·62 a	nd
	100	1·64 ± 1·42 b	11·63 ± 1·97 b′	1·97 ± 0·87 a	10·30 ± 1·61 a′	24·42 ± 7·67 a	nd

nd, not detected.
Data with the same letters are not significantly different ($P < 0·05$).

Table 2 Effect of different fractions of tannins on the production of 4VP and 4EP by *Lactobacillus plantarum* NCFB 1752, *Lactobacillus collinoides* ESB 99 and *Pediococcus pentosaceus* NCFB 990. Results are the average values (with standard deviation) of experiments performed in triplicate

		Lact. plantarum NCFB 1752		*Lact. collinoides* ESB 99		*Ped. pentosaceus* NCFB 990	
Phenolic compound	Concentration (mg l^{-1})	4VP (mg l^{-1})	4EP (mg l^{-1})	4VP (mg l^{-1})	4EP (mg l^{-1})	4VP (mg l^{-1})	4EP (mg l^{-1})
Tannins fraction I	0	25·80 ± 0·53 a	2·51 ± 0·32 a′	6·66 ± 0·79 a	17·93 ± 0·25 a′	26·58 ± 0·74 a	nd
	50	26·73 ± 0·86 a	2·55 ± 0·62 a′	5·54 ± 0·51 a	19·43 ± 0·27 a′	26·85 ± 0·43 a	nd
	100	24·09 ± 2·47 a	3·60 ± 1·11 a′	5·89 ± 0·08 a	17·67 ± 1·01 a′	27·26 ± 0·61 a	nd
Tannins fraction V	0	13·83 ± 3·38 a	4·72 ± 0·62 a′	2·41 ± 0·85 a	14·96 ± 0·85 a′		
	50	16·41 ± 0·09 a	5·40 ± 6·08 a′	3·48 ± 0·06 a	16·43 ± 0·21 a′		
	100	15·66 ± 0·09 a	5·32 ± 0·82 a′	3·20 ± 0·22 a	17·04 ± 0·62 a′		
Tannins – fraction VI	0					26·62 ± 0·81 a	nd
	50					26·67 ± 0·89 a	nd
	100					27·46 ± 0·90 a	nd

nd, no detected.
Data with the same letters are not significantly different ($P < 0·05$). Blank cells mean assays not undertaken.

substrate or that a *p*-coumaric acid decarboxylase could be induced by ferulic acid. Further results obtained using RT-PCR contradicted the hypothesis of *p*-coumaric acid decarboxylase induction by ferulic acid and supports the presence of two enzymes: a *p*-coumaric acid decarboxylase induced by *p*-coumaric acid and a ferulic acid decarboxylase induced by ferulic acid with activity on *p*-coumaric acid (van Beek and Priest 2000). The results obtained in this work, likewise, suggest that caffeic acid may induce the synthesis of a decarboxylase with activity on *p*-coumaric acid.

According to the studies performed with LPD1 (a *Lact. plantarum* mutant strain deficient in PDC activity constructed to investigate alternate pathways for phenolic acid degradation), *Lact. plantarum* reduces phenolic acids into substituted phenyl propionic acids and subsequently decarboxylates these acids into 4-ethyl derivatives (Barthelmebs *et al.* 2000b). Therefore, *Lact. plantarum* appears to have *p*-coumaric and ferulic acid reductase activities (PAR) induced by both substrates. The existence of this phenolic acid reductase acitivity is a possible explanation for the higher production of 4EP in the presence of ferulic acid.

Noninduced resting cells (grown in the absence of hydroxycinnamic acids – control samples) were able to produce a certain amount of 4VP. Despite some studies

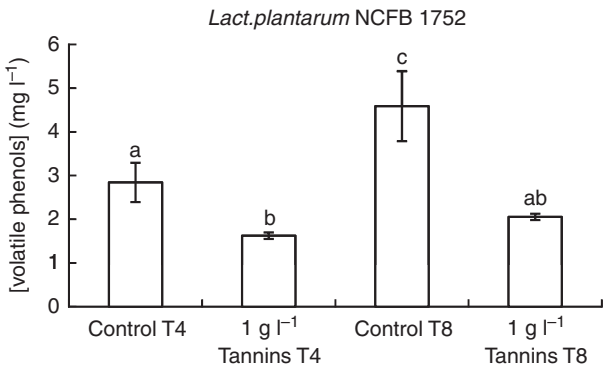

Figure 4 Effect of commercial tannins on the production of 4VP by resting cells of *Lactobacillus plantarum* NCFB 1752. Results are the average values (with standard deviation) of experiments performed in triplicate. Bars with the same letters are not significantly different ($P < 0{\cdot}05$). T4: results obtained after 4 h of incubation; T8: results obtained after 8 h of incubation.

refer that noninduced cells cannot decarboxylate phenolic acids (Cavin *et al.* 1993, 1997a; Barthelmebs *et al.* 2000b, 2001), our results are in accordance with a recent work that demonstrated that noninduced resting cells contain decarboxylases able to metabolize *p*-coumaric acid into 4VP (Rodríguez *et al.* 2008).

A noteworthy observation was the production of only 4VP, and not 4EP, in the experiments carried out with resting cells of *Lact. plantarum* NCFB 1752 and *Lact. collinoides* ESB 99. Because a reduced cofactor is generally required for the enzymatic reduction activity, it is likely that the production of volatile phenols, specifically the reduction of 4VP into 4EP, is a source of NAD^+ during the growth of *Brettanomyces/Dekkera* in red wines (Fugelsang and Edwards 2007). Because the resting cells are in nongrowing conditions, there is no need for NAD^+ regeneration; therefore, the reduction step and consequently the production of 4EP do not occur.

None of the flavonoids tested influenced the *Ped. pentosaceus* NCFB 990 metabolism, while different effects on the *Lactobacillus* species were observed. Kaempferol and myricetin only influenced the metabolic activity of *Lact. collinoides* ESB 99 by the stimulation of the cinnamate decarboxylase activity. Results obtained in the presence of epicatechin suggested the enhancement of the cinnamate decarboxylase and the inhibition of the vinylphenol reductase activities in *Lact. plantarum* NCFB 1752, while a stimulatory effect of both enzymes was observed for *Lact. collinoides* ESB 99. It is important to emphasize the strong effect of quercetin, which caused a notable shift on *Lact. plantarum* NCFB 1752 behaviour. The conversion rate of *p*-coumaric acid into volatile phenols decreased owing to the inhibition of the cinnamate decarboxylase; nevertheless, the vinylphenol reductase activity was stimulated, causing a significant increase in the 4EP concentration. In general, flavonoids had a higher effect on the cinnamate decarboxylase activity than in the vinylphenol reductase. Chatonnet *et al.* (1997) studied the capacity of *Saccharomyces cerevisiae*, *B. bruxellensis* and LAB such as *Lact. plantarum*, *Pediococcus damnosus* and *Leuconostoc oenos* to produce volatile phenols in the presence of flavanols. Generally, catechin (1 g l^{-1}) caused a decrease in the formation of volatile phenols, except for *Lact. plantarum*. The catechin concentration tested in our experiments was much lower, which probably explains the absence of influence on the production of volatile phenols. The polarity of phenolic compounds may be related to their ability to affect the LAB behaviour. Puupponen-Pimia *et al.* (2001) have shown that the number of hydroxyl groups in the B-ring in flavonols is associated with the antimicrobial activity of phenolic compounds against probiotic and other intestinal bacteria. Contrarily, Figueiredo *et al.* (2008) concluded that the flavonols' toxicity over *O. oeni* and *Lactobacillus hilgardii* could not be directly related to the degree of hydroxylation of the B-ring. Our results suggest that the effect of flavonoids on the metabolism of *p*-coumaric acid depends on the bacterial species and is not directly related to the polarity of the compounds.

Low concentrations (50 and 100 mg l^{-1}) of tannins did not affect the production of volatile phenols in culture medium, while 1 g l^{-1} strongly affected the metabolism of *p*-coumaric acid into 4VP by resting cells in phosphate buffer. (This concentration of tannins could not be tested in culture medium owing to precipitation.) This effect seems to be related to the ability of tannins to combine with enzymes (Scalbert 1991; Jackson 2000; Ribéreau-Gayon *et al.* 2000). Chatonnet *et al.* (1997) demonstrated a significant decrease in the quantity of volatile phenols synthesized by *Lact. plantarum* by concentrations of procyanidic tannins above 1 g l^{-1}, and almost a total inhibition at about 3 g l^{-1}. Figueiredo *et al.* (2008) described the strong effect of 500 mg l^{-1} of different tannin fractions extracted from grape seeds on the cell viability of *Lact. hilgardii* and especially of *O. oeni*.

This work clearly demonstrates that certain phenolic compounds influence the metabolism of *p*-coumaric acid by LAB. The ability of these bacteria to produce phenolic off-flavours in real wine conditions requires investigation.

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