



CATÓLICA PORTO
BIOTECNOLOGIA

**CHARACTERIZATION OF PHENOLIC ACID
REDUCTASE AND DECARBOXYLASE ACTIVITIES OF
LACTIC ACID BACTERIA**

Master thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* as part of the European Master Degree in Food Science, Technology and Nutrition.



By Ana de Seabra Leão Ferreira Soares

Under the supervision of Francisco Campos and José António Couto

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Abstract

Hydroxycinnamic acids are natural constituents of grape juice and wine, and are precursors of volatile phenols produced by yeasts and lactic acid bacteria (LAB). The organoleptic defects due to the presence of this volatile phenols are usually associated with “animal”, “horsey”, “leather”, “phenolic” or “spicy” aromatic notes. The most common pathway for the degradation of hydroxycinnamic acids involves two enzymes. In first place, it occurs a decarboxylation by the phenolic acid decarboxylase (PAD), and secondly a reduction of the intermediate metabolite carried out by the vinylphenol reductase (VPR). Some LAB strains seem to be able to metabolise hydroxycinnamic acids through an alternative route, involving a reduction step by a phenolic acid reductase (PAR) followed by a decarboxylation reaction.

In order to characterize PAR and PAD/VPR activities, six different LAB strains were tested for the production of volatile phenols and non-volatile metabolites. The main objective of this work was to study the influence of certain factors/growth conditions on the extent of these activities. In the first part of this study, we evaluated the influence of the concentration of *p*-coumaric acid (1.0, 5.0 and 50 mg/L) as precursor for the production of volatile phenols. In the second part, the effect of the presence of L-malic acid and fructose on PAR and PAD/VPR activities with regard to the metabolism of *p*-coumaric acid was studied.

The results show that all of the LAB strains tested have the ability to produce volatile phenols, however, strain-dependent patterns were observed. Four of the strains were also found to produce phloretic acid from 50 mg/l of *p*-coumaric acid using the alternative metabolic route mentioned above, thus indicating PAR activity. L-malic acid and fructose seemed to stimulate VPR activity, while no alteration was observed on the PAR activity. Moreover, the initial concentration of *p*-coumaric acid used seems to play an important role on volatile phenols production.

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Abbreviations

4-EC	4-ethylcatechol
4-EG	4-ethylguaiacol
4-EP	4-ethylphenol
4-VC	4-vinylcatechol
4-VG	4-vinylguaiacol
4-VP	4-vinylphenol
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ESB	Escola Superior de Biotecnologia
FID	Flame Ionisation Detector
GC	Gas-Liquid Chromatography
HPD	Hydroxyphenylpropionic Decarboxylase
HPLC	High Performance Liquid Chromatography
<i>L.</i>	<i>Lactobacillus</i>
LAB	Lactic Acid Bacteria
LB	Bacteriocin producer strain
MLF	Malolactic Fermentation
MRS	de Man, Rogosa and Sharpe
NAD	Nicotinamide Adenine Dinucleotide
NCFB	National Collection of Food Bacteria, Reading, UK
<i>O.</i>	<i>Oenococcus</i>
<i>P.</i>	<i>Pediococcus</i>
PA	Phloretic acid
PAD	Phenolic Acid Decarboxylase
PAR	Phenolic Acid Reductase

UCP	Universidade Católica Portuguesa
v/v	Volume by volume
VP	Volatile Phenols
VPR	Vinylphenol Reductase

1. Introduction

Grapes are one of the most antique crops in history. Indeed, wine and man have a long history together, and Palaeolithic man was probably the first to become familiar with wine (Hornsey, 2007). Since Louis Pasteur clearly demonstrated that the alcoholic fermentation required the presence of yeast, unprecedented improvements in the winemaking processes have gone hand in hand with the development of modern microbiology

1.1. Wine Aroma and Phenolic Compounds

Several chemical compounds - mainly aldehydes, esters, alcohols, acids and phenolic compounds - have been described as normal components of wine aroma. Some of these compounds have a relatively low boiling point and are detectable by the human nose when present above the olfactory threshold. Some of them contribute positively to the wine aroma, although depending on the concentration levels, but others are responsible for off-flavours, decreasing the quality of the wine.

The composition of the volatile fraction is influenced by many factors, including grape must composition, pH, prevailing temperature of fermentation, which can affect the biosynthetic pathways in wine by yeast and bacteria (Swiegers *et al.*, 2005). In addition, viticulture factors, such as the quality of the grapes and soil, water management, climate, technological aspects, vinification methods (grape de-stemming, crushing and pressing technology, must treatment and skin contact time) and the ageing conditions (oak-ageing, bottle ageing), affect the amount and type of volatiles in wine (Gómez-Míguez *et al.*, 2007; Ivanova *et al.*, 2011; Ginjom *et al.*, 2011)

. The major phenolics found in wine are either members of the diphenylpropanoids (flavonoids) or phenyl-propanoids (nonflavonoids) (Jackson, 2008). They play an important role in the visual and gustative quality of wines, in particular, anthocyanins that are responsible for the colour of red wines, and tannins which confer the astringency and structure to the wine (Ivanova *et al.*, 2011). Non-flavonoids, especially resveratrol, are thought to have anti-carcinogenic effects due to their antioxidant properties, limiting the action of free radicals in the body. Epidemiological studies have suggested that a moderate consumption of alcoholic beverages, particularly

red wine, is associated with a reduction in overall mortality, attributed mostly to a reduced risk of coronary heart disease (Bakker and Clarke, 2012). These phenolic compounds are abundant in grape material (skins, seeds and stalks) as well as in wood used for storage (Campos *et al.*, 2003), therefore, the wine-making procedures determine the transference of these compounds to musts and wines.

1.2. Volatile phenols

The phenolic composition of wines include phenolic acids, such as hydroxycinnamic (*p*-coumaric, caffeic and ferulic) acids and hydroxybenzoic (gallic, protocatechuic, vanillic and syringic) acids, ranging from 100 to 200 mg/L in red wine and 10-20 mg/L in white wine (Ribéreau-Gayon *et al.*, 2006b).

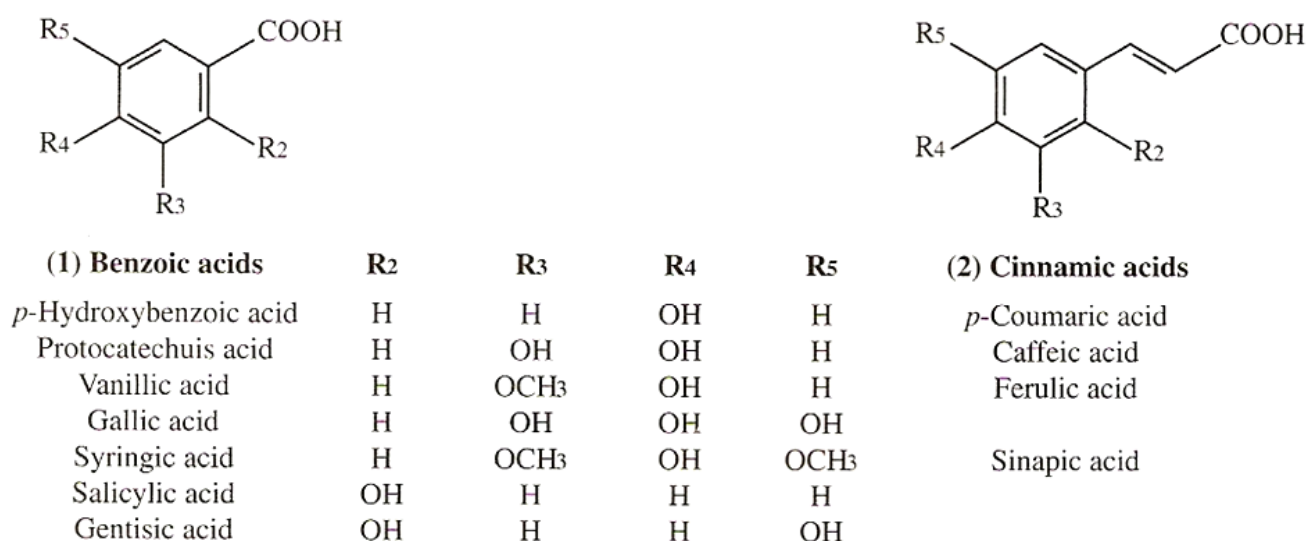


Figure 1. Phenolic acids in grapes and wine (Ribéreau-Gayon *et al.*, 2006b)

These phenolic acids are natural constituents of grape juice and wine, being differentiated by substitution of their benzene ring (Figure 1). They are mainly esterified, in particular with tartaric acid, however, the free form can be released by cinnamoyl-esterase activities. This enzyme is present in wine due to enzyme preparations added, and also due to lactic acid bacteria (LAB), that can exhibit cinnamoyl-esterase activities during malolactic fermentation. Although these phenolic

acids have no flavour or odour, they are precursors of volatile phenols produced by yeasts and lactic acid bacteria (Buron *et al.*, 2011).

Volatile phenols are found in many fermented beverages, such as beers (Lyumugabe *et al.*, 2013) and cider (Buron *et al.*, 2012), and have characteristic aromas which, above a certain concentration threshold, have a negative effect on the overall aroma of a wine (Silva *et al.*, 2011b). The most important molecules in this class are 4-vinylphenol (4-VP), 4-vinylguaiacol (4-VG), 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG). The organoleptic defects due to their presence are usually associated with “animal”, “horsey”, “leather”, “phenolic” or “spicy” aromatic notes, which also leads to a loss of freshness and fruitiness character (Couto *et al.*, 2006; Tamasi *et al.*, 2013) (Figure 2). The sensory threshold in wines for 4-EP is low, about 230 µg/L (Suarez *et al.*, 2007), but low concentrations (420 µg/L of a mixture of 4-EP and 4-EG) have been cited as contributing positively to aroma complexity (Fugelsang and Edwards, 2007; Ribéreu-Gayon *et al.*, 2000a).

Ethylphenols are present in largest quantities in red wines, whereas vinylphenols are more common in white wines (Torrens *et al.*, 2004). Concentrations of more than 770 µg/L of a mixture of 4-VG/4-VP (1:1) in white wines can be responsible for heavy "pharmaceutical" odours reminiscent of sticking plaster (Chatonnet *et al.*, 1995).

	Compound	Concentration in wine (mg/L)	Aroma
Volatile phenols	4-Vinylphenol	0-1.15	Medicinal
	4-Vinylguaiacol	0-0.496	Smoky, vanilla
	4-Ethylphenol	0-6.047	Horse sweat
	4-Ethylguaiacol	0-1.561	Smoky, vanilla

Figure 2. Principal volatile phenols in wine; Adapted from Carrascosa *et al.*, 2011.

The formation of volatile phenols in wine is known to depend on the presence of precursors (hydroxycinnamic acids) and is proportional to the size of the

Brettanomyces/Dekkera population (Suarez *et al.*, 2007), which are capable of forming significant quantities of ethylphenols.

Although the yeasts *Dekkera/Brettanomyces* are recognized as the main organisms responsible for the production of volatile phenols (Suarez *et al.*, 2007), previous works have shown that some strains of LAB are also capable of producing volatile phenols in growth media, but at lower concentrations (Couto *et al.*, 2006; Chatonnet *et al.*, 1995; Cavin *et al.*, 1997). In 1997, Chatonnet *et al.* (1997) showed that strains of *Lactobacillus* and *Pediococcus* were capable of producing quantities of 4-VP similar to those formed by *Saccharomyces cerevisiae*. However, measurable quantities of 4-EP were always relatively low and, in all cases, much lower than those produced by *Dekkera/Brettanomyces*.

1.3.Lactic Acid Bacteria and Malolactic Fermentation

LAB are naturally present in wine as well as in winery equipment that is not well sanitized. A general description of them would be that they are Gram positive, non-spore forming, non-respiring cocci or rods, which produce lactic acid as the major end-product during the fermentation of carbohydrates (Hornsey, 2007). They are doubly important, whereas growth of some bacteria in certain wines is desirable (i.e., malolactic fermentation), while the growth of other species can lead to spoilage (Fugelsang, K.C. and Edwards, 2007).

Lactic acid is the primary metabolite of sugar metabolism that can be carried out by homo or heterofermentative pathways (Figure 3). Homofermentative bacteria transform nearly all of the hexoses that they use, especially glucose, into lactic acid; the oxidation reaction takes place generating the reduced coenzyme NADH and H⁺, which is at the end oxidized into NAD⁺ during the reduction of pyruvate into lactate. Bacteria using the heterofermentative pathway transform hexoses principally but not exclusively into lactate. The other molecules produced by this metabolism are essentially CO₂, acetate and ethanol (Ribéreau-Gayon *et al.*, 2006). The study of the homofermentative and heterofermentative metabolic pathways of sugars therefore permits the prediction of the nature of the products formed.

Yeasts are better adapted than LAB to growth in grape musts, which contain very high sugar concentrations (210 g/L) and have a low pH, about 3.0–3.8 (Lonvaud-funel, 1999). Therefore, alcoholic fermentation starts very quickly, and when all reducing sugars are fermented to ethanol, yeast levels decline and LAB growth occurs, due to their adaptation to the wine environment, namely low pH, high ethanol and low concentrations of nutrients (Campos *et al.*, 2003).

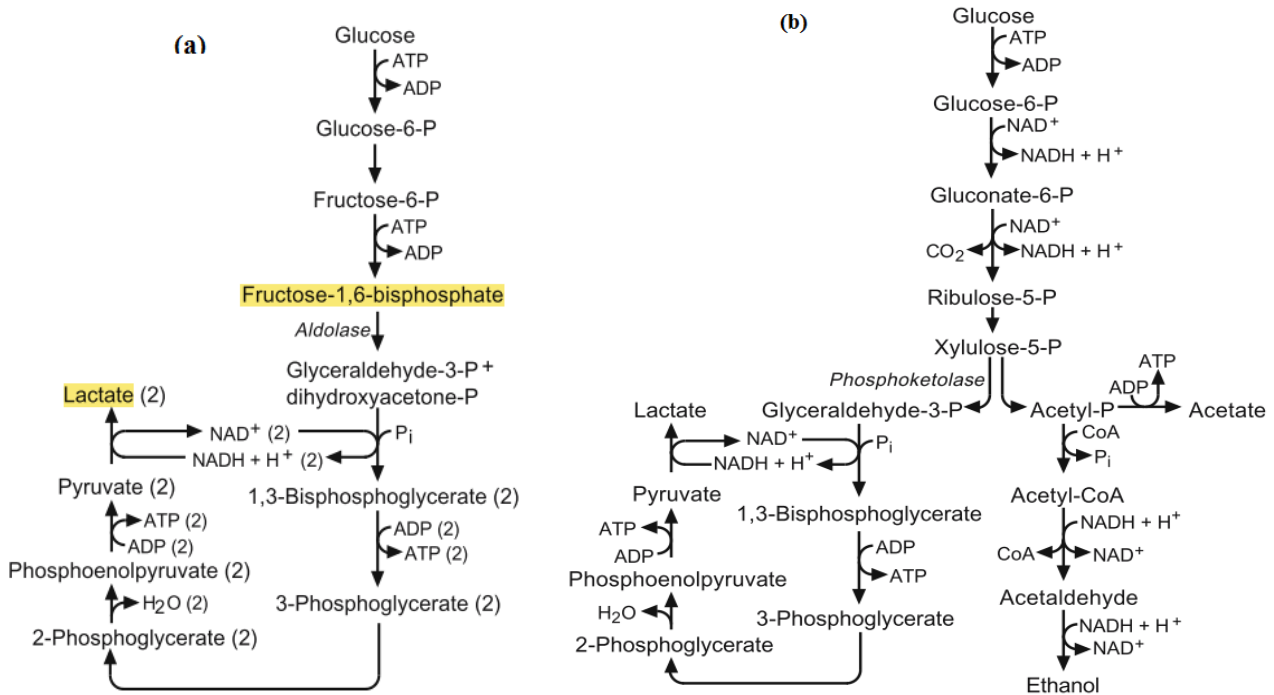


Figure 3. Homofermentative (a) and heterofermentative (b) pathways of sugar metabolism (Fugelsang and Edwards, 2007)

The most important bacterial genera in winemaking are *Pediococcus*, *Lactobacillus*, *Leuconostoc* and *Oenococcus*. *Lactobacillus*, mainly *Lactobacillus hilgardii*, use the residual sugar of the wine in slow alcoholic fermentations generating an increase of the volatile acidity (D-lactic acid and acetic acid) which results in a modification of the sensorial properties of the wine (Chatonnet *et al.*, 1997; Lonvaud-Funel, 1999). *Pediococcus* are known to cause ropiness in wine by the production of polysaccharides (König, Uden and Fröhlich, 2009), whereas *Oenococcus* is the species of choice to carry out the malolactic fermentation.

Malolactic fermentation (MLF) follows the alcoholic fermentation, and although the main reason it's carried out is because it leads to a reduction of wine acidity, due to the conversions of L-malic acid (dicarboxylic acid) into L-lactic acid (monocarboxylic

acid), many winemakers are increasingly viewing it as a means of adjusting wine flavour. Although more commonly promoted in red wines, it is now being applied to white wines (Jackson, 2008).

MLF may occur spontaneously, but also can be induced by inoculating wine with malolactic starter cultures, which are preparations usually containing cultures of one strain, used to inoculate a substrate in order to initiate fermentation (Comitini, *et al.*, 2005; Carrascosa *et al.*, 2011). The induction of MLF by inoculation offers some advantages, including greater control over the timing and length of this fermentation and also over the strain of lactic acid bacteria that carries out the process. The longer it takes for it to start, the higher the risk for the growth of *Dekkera/Brettanomyces*, which compromises the quality of the wine due to the formation of various spoilage compounds, especially volatile phenols. The selection of the starter culture is based on some specific criteria, such as resistance to low pH, resistance to ethanol, tolerance of low temperatures, and reduced metabolism of hexose/pentose sugars (Carrascosa *et al.*, 2011).

1.4.Biochemical Pathways of volatile phenols production

The precursors of volatile phenol biosynthesis are hydroxycinnamic (caffeic, *p*-coumaric and ferulic) acids (Figure 4), that are generally esterified with tartaric acid in grape must and wine, and can be released as free acids during wine making by some cinnamoyl-esterase activities (Buron *et al.*, 2012; Cavin *et al.*, 1997).

After this first step, there are two possible metabolic pathways for the conversion of hydroxycinnamic acids into volatile phenols. Most often, they are first decarboxylated into 4-vinyl derivatives and then reduced to 4-ethyl derivatives, involving the sequential activity of two enzymes: the first is the cinnamate decarboxylase or phenolic acid decarboxylase (PAD) which decarboxylates the hydroxycinnamic acid into the corresponding vinylphenol; the second enzyme involved is the vinylphenol reductase (VPR) which reduces the vinylphenol into the corresponding ethylphenol (Ribéreau-Gayon *et al.*, 2000). Some LAB like *L. plantarum*, *L. brevis* and *P. pentosaceus* are able to synthesize PAD, thus being able to decarboxylate the hydroxycinnamic acid, while others don't possess the gene encoding

a PAD, namely *O. oeni* and *L. hilgardii* (de la Rivas *et al.*, 2009). Given this, *Dekkera/Brettanomyces* could theoretically reduce vinyl phenols synthesized by other wine microorganisms and thereby benefit from the growth of other microorganisms (e.g., LAB). The reduction step seems to be mediated by the intracellular NAD⁺/NADH balance (Silva *et al.*, 2011a).

A second pathway, first described by Whiting *et al.* (1959) for *Lactobacillus pastorianus*, consists of a two-step reaction. First, a reduction step takes place carried out by a phenolic acid reductase (PAR), followed by a decarboxylation reaction through a putative hydroxyphenylpropionic decarboxylase (HPD) (Figure 4). Barthelmebs *et al.* (2000) showed that knocking out the *p*-coumaric acid decarboxylase gene, the bacteria was still able to weakly metabolize *p*-coumaric and ferulic acids into vinyl derivatives or into substituted phenyl propionic acids (e.g. phloretic acid), showing that both routes, can co-exist and compete for the hydroxycinnamic acid degradation. In sum, the main difference between the two routes is the intermediary compound, either vinylphenols or phloretic acid.

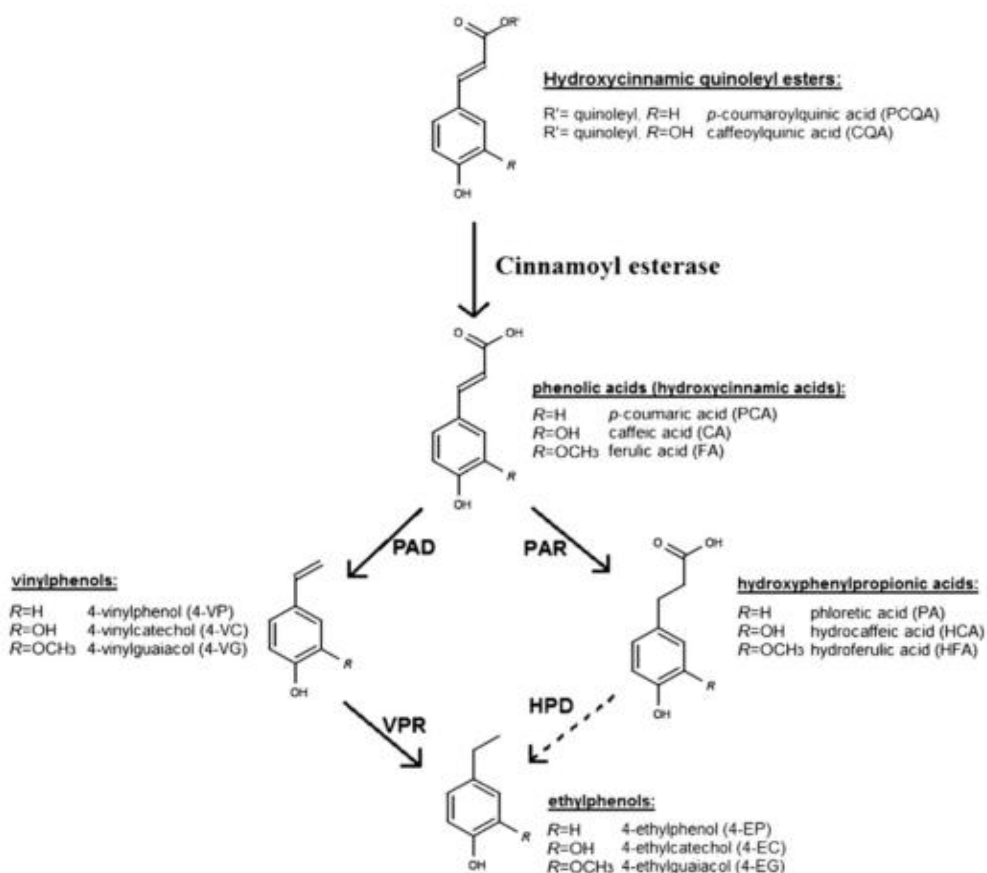


Figure 4. Biosynthetic pathways of volatile phenols from hydroxycinnamic esters. PAD, phenolic acid decarboxylase; VPR, vinylphenol reductase; PAR, phenolic acid reductase; HPD, putative hydroxyphenylpropionic decarboxylase (Buron *et al.*, 2012)

1.5. Factors influencing volatile phenols production by LAB

The contaminant yeasts *Dekkera/Brettanomyces* are recognized as the main organisms responsible for the production of volatile phenols, although previous works have shown that some strains of LAB are also capable of producing volatile phenols. Couto *et al.* (2006) showed that thirteen LAB strains (37%) produced volatile phenols from *p*-coumaric acid, although only three (9%) produced 4-EP. The reduction step was only found in the *Lactobacillus* genus: *L. brevis*, *L. collinoides*, and *L. plantarum*.

1.5.1. Influence of hydroxycinnamic acids, pH, L-malic acid and oxygen

Hydroxycinnamic acids, especially *p*-coumaric, were proven to be inhibitory for the growth of *O. oeni* and *Lactobacillus hilgardii*, inhibiting cell growth (Campos *et al.*, 2003; Campos *et al.*, 2009), but the tested strains didn't have the ability to produce volatile phenols (Couto *et al.*, 2006).

Regarding the bacterial strains that are capable of producing volatile phenols, Silva *et al.* (2011b) evaluated the effect of wine phenolic compounds on the production of volatile phenols by LAB, where the results suggest that caffeic and ferulic acids induce the synthesis of the PAD involved in the metabolism of *p*-coumaric acid, supporting 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 (Figure 1). Also, Couto *et al.* (2006) studied the influence of hydroxycinnamic acids concentration. The higher the *p*-coumaric acid content in the culture medium, the more volatile phenols were produced, both for 4-vinylphenol and 4-ethylphenol, except for *Lactobacillus plantarum* where it appeared that the production of 4-ethylphenol, in relation to 4-vinylphenol, was favoured at low concentrations of *p*-coumaric acid.

Other factors affect the production of volatile phenols by LAB, namely the presence of L-malic acid, which can stimulate the production of volatile phenols (Silva *et al.*, 2011a). During malolactic fermentation, LAB converts a dicarboxylic acid (L-malic acid) into a monocarboxylic acid (L-lactic acid) increasing pH (Carrascosa *et al.*, 2011), factor which Silva *et al.* (2011a) showed to influence volatile phenols

production, increasing its yield. This study also showed that L-malic acid stimulates the production of 4-EP while diminish the amount of 4-VP released to the culture medium by *L. collinoides* and *L. plantarum*. A possible explanation for this behaviour is that the the enzyme involved in the malolactic fermentation by LAB, requires the presence of NAD^+ cofactor for the decarboxylation of L-malic acid into L-lactic acid and CO_2 (Jackson *et al.*, 2008), which is generated when reducing 4-VP to 4-EP. This may also explain why in anaerobiosis, the production of 4-EP is also favoured (Silva *et al.*, 2011a). Under low oxygen concentrations the availability of NAD^+ can be limited, thus the reduction of 4-VP into 4-EP would allow the cells to regenerate NAD^+ .

1.5.2. Effect of glucose and fructose on the production of volatile phenols

Fermentation can result in the generation of excess oxidised NAD^+ (NADH). To maintain an acceptable redox balance, the bacteria must regenerate NAD^+ . (Jackson *et al.*, 2008). Silva *et al.* (2011a) investigated the influence of glucose and fructose concentration in some homo and/or heterofermentative LAB strains. At high concentrations of glucose (20 g/L) production of 4-VP by homofermentative or facultatively heterofermentative seemed to be favoured, although it was strain dependent, since *Pediococcus pentosaceus* (homofermentative) was not affected. This influence on the production of 4-VP may be because at relatively high levels of glucose, part of the carbohydrate might be diverted to the production of mannitol in homofermentative strains. Mannitol biosynthesis in homofermentative lactic acid bacteria starts with the glycolysis intermediate fructose 6-phosphate, which is an alternative pathway, instead of lactate formation, to regenerate NAD^+ , so the reduction step of *p*-coumaric acid metabolism would not be necessary under these circumstances. Since in homofermentative lactic acid bacteria the carbon flow from carbohydrates is directed mainly to lactate production, the formation of other fermentation products like mannitol is often only possible when strains are more or less hampered in the lactate production pathway, thus homofermentative LAB are found to produce small amounts of mannitol (Wisselink *et al.* 2002). A similar process occurs with the addition of fructose and the subsequent production of mannitol (via reduction of fructose).

In the case of heterolactic bacteria, they are unable to synthesize mannitol from glucose, therefore this via cannot be used as an alternative pathway to regenerate NAD^+ , thus reducing 4-VP to 4-EP for regeneration of NAD^+ . (Wisselink *et al.*, 2002)

The main objective of this work was to investigate the influence of certain factors/growth conditions on the bacteria metabolic pathway by studying PAR and/or PAD / VPR activities. To this end, LAB strains from previous works were selected, regarding their ability to produce volatile phenols as well as phloretic acid.

2. Material and Methods

2.1. Bacterial strains and growth conditions

The selection of the lactic acid bacterial strains was based on previous experiments (Couto *et al.*, 2006), regarding their capacity to produce volatile phenols as well as phloretic acid. The bacteria used in this study (Table 1) belong to the culture collection of Escola Superior de Biotecnologia da Universidade Católica Portuguesa (ESB).

Table1. List of species and strains surveyed

Species	Strains	Source
<i>Lactobacillus collinoides</i>	ESB 99	Wine
<i>Lactobacillus kefir</i>	NCFB 2737	Kefir Grains
<i>Lactobacillus mali</i>	NCFB 2168	Cider apple juice
<i>Lactobacillus sakei</i>	Lb 706	Meat
<i>Lactobacillus viridescens</i>	NCFB 8965	Cured meat products
<i>Pediococcus pentosaceus</i>	NCFB 990	Dried American beer yeast

ESB: Escola Superior de Biotecnologia, Porto, Portugal

Lb: Bacteriocin producer strain

NCFB: National Collection of Food Bacteria, Reading, UK

This work was divided in two parts, where in the first different amounts of *p*-coumaric acid were added to the medium (table 2).

Table 2. Part I; Addition of different initial concentrations of substrate (*p*-coumaric acid)

Part I		
Parameter		Strains
Initial concentration of <i>p</i> -coumaric acid	1.0 mg/L	<i>L. collinoides</i> ; <i>L. kefir</i> ; <i>L. mali</i> ; <i>L. sakei</i> ; <i>L. viridiscens</i> ; <i>P. pentosaceus</i>
	5.0 mg/L	
	50 mg/L	

In the second part of the work, three strains were selected, regarding their type of hexose metabolism. *L. collinoides* is strictly heterofermentative, *L. sakei* is facultatively heterofermentative and *P. pentosaceus* is strictly homofermentative. L-malic acid or fructose was added to the medium at concentrations of 4.0 and 3.0 g/L, respectively.

Table 3. Part II; Addition of L-malic acid or fructose do the growth medium.

Part II		
Parameter		Strains
Addition of L-malic acid (4.0 g/L)	<i>Note:</i> Initial Concentration of <i>p</i> -coumaric acid: 5 mg/L	<i>L. collinoides; L sakei; P. pentosaceus</i>
Addition of fructose (3.0g/L)		

Bacterial growth was carried out in MRS (de Man, Rogosa & Sharpe) medium from Biokar Diagnostics (Beauvais, France), either liquid or solid. The latter was prepared by adding 20 g/L of agar (Laboratorios Conda, Madrid, Spain) to the liquid medium. The pH was adjusted to 4.5 in the liquid medium and 5.0 in the solid medium, using a hydrochloric acid 6.0 M solution. Afterwards, they were sterilized in Schott glass bottles (one cycle at 121 °C during 15 minutes) as well as all the material used, including glass pipettes and micropipette tips. Finally, sterilized medium were transferred to plastic tubes under aseptic conditions.

Pre-cultures were obtained from stock cultures stored at -80°C in MRS with 20% glycerol which were transferred to sterile liquid MRS broth and grown to late exponential phase for 2-3 days at 25 C°, without agitation.

The purity of the cultures was checked by the observation of liquid cultures under the microscope and by inoculating in solid medium. Cultures were stored in MRS Agar slopes at 4°C. For each experiment, fresh cultures where prepared from these stock cultures, and grown in MRS broth in plastic tubes for 24-48h at 25°C.

2.2. Inoculation of media

All samples were prepared in duplicate, under aseptic conditions.

With the intent to evaluate the production of volatile phenols and phloretic acid at different concentrations of *p*-coumaric acid, an experiment was conducted during the first part of this work, supplementing MRS broth with 1.0 mg/L, 5.0 mg/L and 50 mg/L of *p*-coumaric acid (trans-4-hydroxycinnamic acid, 98% purity), obtained from Sigma Aldrich (Steinheim, Germany).

The second part of this work consisted in separately evaluate the effects of malic acid and fructose in the production of volatile phenols and phloretic acid. L-malic acid (Sigma Aldrich Chemie GmbH, Steinheim, Germany) was added, at a concentration of 4.0 g/L, and fructose (Sigma Aldrich Chemie GmbH, Steinheim, Germany) was added at 3.0 g/L to liquid MRS medium, subsequently sterilized. In both experiments *p*-coumaric acid was added, at a concentration of 5.0 mg/L.

The different amounts of *p*-coumaric acid were weighted and dissolved in 10.0 ml of a deionised water/ethanol solutions (1:1, v/v), which were then filtered with a cellulose acetate syringe filter with 0.45 µm pore size (VWR International, USA). Afterwards, 250 µl of these solutions were added to Schott bottles (Schott AG, Mainz, Germany) containing 50 ml of MRS liquid medium, and finally, 500 µl of bacterial culture were inoculated, using fresh cultures.

All samples were incubated for 7 days, at 25 °C without agitation. After 7 days in the incubator, the culture media was centrifuged at 3000 x g for 10 minutes to remove the supernatant for later analysis.

2.3. Volatile fraction extraction

The following procedure was executed for each sample of each experience.

The supernatant was analyzed by a modified version of the method developed originally by Bertrand (1981) for analysis of volatile phenols, described as follows.

Samples were diluted (1:1, v/v) by mixing 25.00 ml of culture media, obtained from the centrifugation, with 25.00 ml of deionised water in a 100.0 ml volumetric flask, followed by a liquid-liquid extraction to analyze the volatile phenols.

The first step consisted in the addition of 50.0 μL of 3-octanol (447 mg/L in methanol) as internal standard. Three consecutive extractions were performed by stirring the solution for 5 minutes with 4.0 ml, 2.0 ml and 2.0 ml, respectively, with a solvent solution of diethyl-ether/hexane (1:1, v/v) (both reagents were obtained from Merck, Darmstadt, Germany). After mixing for 5 minutes, the solution was transferred to a separatory funnel where the organic phase was separated from the inorganic phase and collected to a headspace flask while the inorganic phase was recovered to repeat the extraction.

The organic phase from the consecutive extractions (transparent layer) was collected with a Pasteur pipette to a 1.50 ml vial and concentrated under a stream of nitrogen to approximately 0.50 ml for further analysis in the gas chromatograph (GC-FID)

2.4. Gas-liquid chromatography analysis

The analyses were performed with a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector (FID). One microlitres (1.0 μL) of sample was injected from the vials. Compounds were separated on a FFAP-type (BP 1,50 m x 0,22 mm x 0.25 μm) obtained from SGE (Austin, TX, USA). The injector (split/splitless) was heated to 220 $^{\circ}\text{C}$ with a split flow of 30 ml/min and a splitless time of 0.3 min. Hydrogen was used as carrier gas. The temperature program was as follows: 40 $^{\circ}\text{C}$ for 5 minutes after injection and then increased at a rate of 2 $^{\circ}\text{C}/\text{min}$ up to 220 $^{\circ}\text{C}$. This temperature was maintained for 30 min.

In order to obtain a calibration curve (Figure 5), five standard solutions were prepared in 50 mL volumetric flasks from a solution of 4-vinylphenol (10% purity) and a solution of 4-ethylphenol (99% purity). The final concentrations ranged from 2.03 up to 25.40 mg/L of 4-vinylphenol and 2.12 up to 26.5 mg/L of 4-ethylphenol. The internal standard was added to each standard solution and the extraction proceeded as described

in section 2.3. The average retention times for the analysed compounds are shown in table 4.

Table 4. Average retention times for volatile phenols and internal standard.

Compound	Average Retention Times (minutes)
3-octanol (Internal Standard)	29.56
4-ethylphenol	75.03
4-vinylphenol	85.31

Statistical analysis where performed for the calibration curves (IUPAC, 2007), the detection and quantification limits for each compound are presented in table 5.

Table 5: Detection limits for 4-vinylphenol and 4-ethylphenol.

Compound	Detection Limit mg/L	Quantification Limit mg/L
4-vinylphenol	1.5	4.8
4-ethylphenol	0.76	2.5

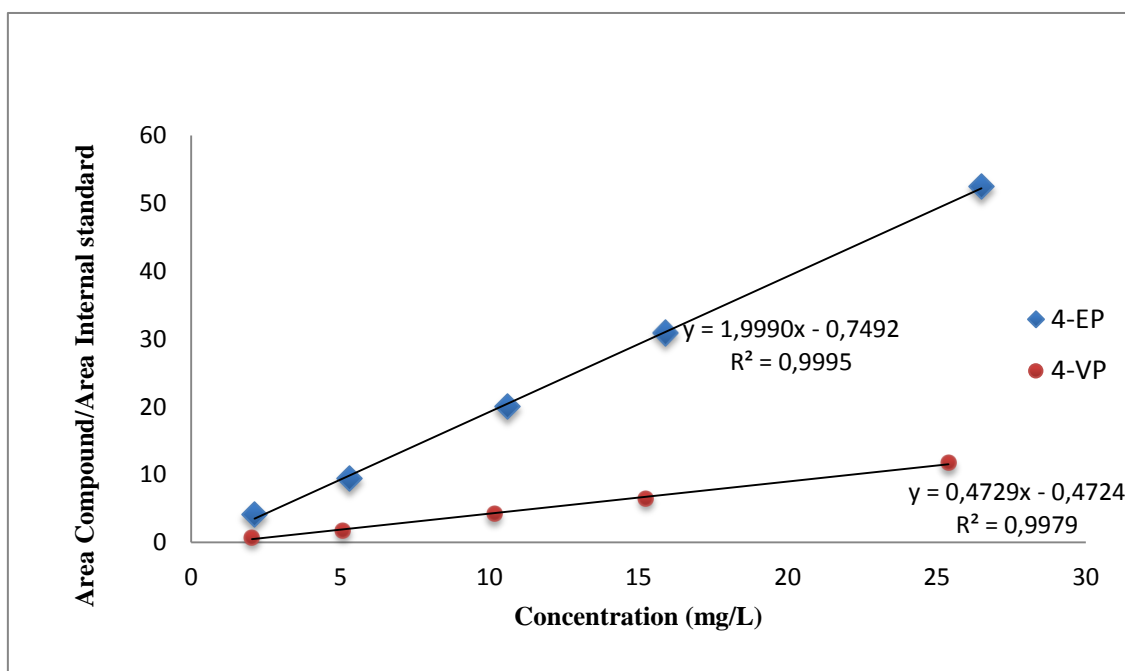


Figure 5. Calibration curve for 4-ethylphenol (4-EP) and 4-vinylphenol (4-VP).

2.5. High-Performance Liquid Chromatography analysis

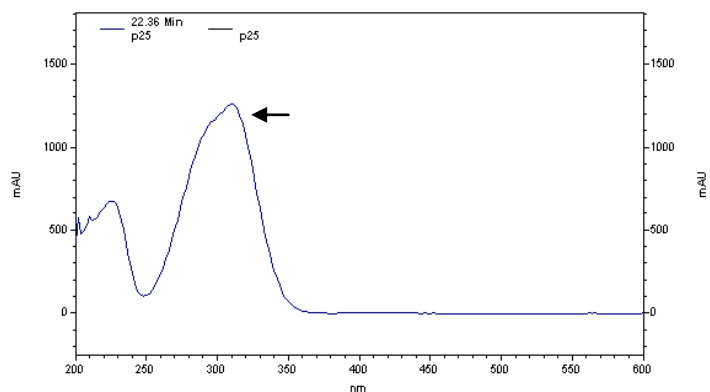
The analysis of residual *p*-coumaric left in the medium and phloretic acid produced was carried out by a Beckman Gold HPLC system (Beckman Coulter, USA) equipped with a Diode Array Detector and a Zorbax Eclipse XDB-C18 analytical column, 4.6 x 150 nm 5 micron from Agilent Technologies (Santa Clara, CA, USA). The automatic injector was used in a full loop of 20 μ l, with a mobile phase A composed of 100% acetonitrile (Fisher Scientific, Loughborough, UK), and a mobile phase B composed of 95% ultrapure water; 5% acetonitrile; 2.0 ml/L trifluoroacetic acid (98%) obtained from Sigma-Aldrich, Steinheim, Germany. The elution program was as follows: 0min: 100% B; 2min: 60%B; 28min: 100%B (during 3minutes), with flow rate fixed at 1.0 ml/min.

Both mobile phases were filtered and placed in an ultrasound bath for ten minutes to eliminate remaining air bubbles. Samples were filtered into the vials, using 0.45 μ m-pore syringe filters (VWR International, USA) prior to injection.

In order to identify and quantify the peaks, a calibration was performed for *p*-coumaric acid and phloretic acid. The UV absorption spectrum (Figure 6) and maximum wavelength absorbance (Table 6) for each acid were also used for identification of the compounds. Five standard solutions were prepared in 50.00 mL volumetric flasks containing different concentrations of phloretic acid, and *p*-coumaric acid.

All dilutions were microfiltered with syringe filters of 0.45 μ m pore size into vials that were then injected in the HPLC-DAD system. The average retention times and calibration curves are represented in table 6 and Figure 6 respectively.

(a)



(b)

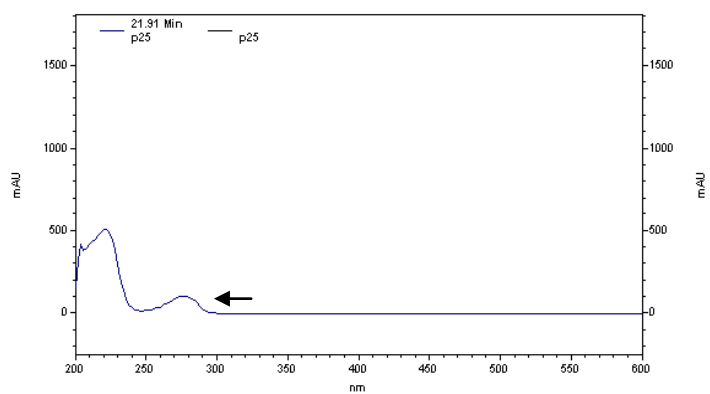


Figure 6. UV absorption spectrum of *p*-coumaric acid (a) and phloretic acid (b).

Table 6. Average retention times in the HPLC-DAD system and wavelength of maximum absorbance obtained for phloretic acid and *p*-coumaric acid.

Compound	Average Retention Time (minutes)	Wavelength of maximum absorbance (nm)
Phloretic acid	19.38	275-281
<i>p</i> -Coumaric acid	20.08	310-312

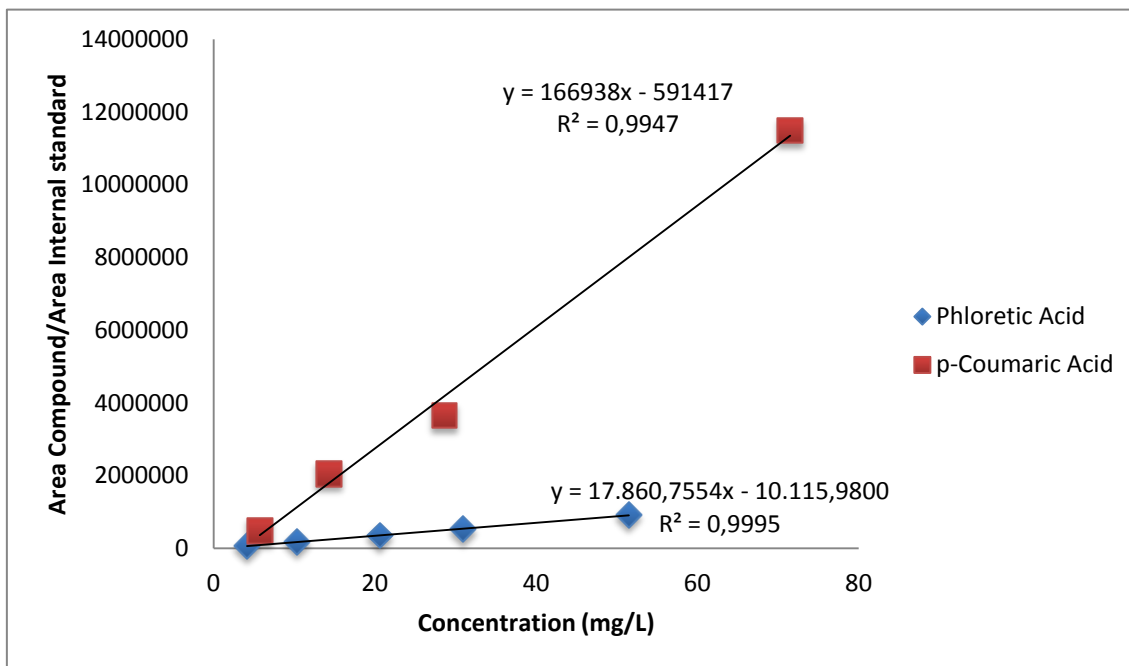


Figure 6: Calibration curves for phloretic acid and *p*-coumaric acid

Statistical analysis was performed for the calibration curves (IUPAC, 2007), the detection and quantification limits for each compound are presented in table 7.

Table 7. Detection limits for *p*-coumaric acid and phloretic acid.

Compound	Detection Limit mg/L	Quantification Limit mg/L
<i>p</i> -coumaric acid	7.9	26.0
Phloretic acid	3.1	10.2

3. Results

3.1 Volatile phenols production

In order to characterize PAR and PAD/VPR activities in the LAB surveyed, gas-liquid chromatography was used to quantify the production of volatile phenols by these bacteria (4-VP and 4-EP) from the precursor *p*-coumaric acid. The molar conversion rates of *p*-coumaric acid into volatile phenols were calculated by dividing the molar concentration of 4-VP and 4-EP by the initial molar concentrations of this precursor added to the medium. Some of the results are below the limit of detection calculated for the calibration curve and are marked in the tables. Results are the average values of two experiments.

3.1.1. Volatile phenols production from different concentrations of *p*-coumaric acid

In order to evaluate the bacterial behaviour, three different concentrations of *p*-coumaric acid were used (1.0, 5.0 and 50 mg/L) and the obtained results are presented in tables 8, 9, and 10, respectively. In some cases conversion rates higher than 100% were obtained. This may have happened due to a possible experimental error. Moreover, some of the results are below the detection limit.

Table 8. Production of 4-VP and 4-EP from *p*-coumaric acid at 1.0 mg/L in MRS Broth.

Bacterial Strains	4-VP (mg/L)	Molar conversion (%)	4-EP (mg/L)	Molar conversion (%)
<i>Lactobacillus collinoides</i> ESB 99	0,54*	74	0,03*	4
<i>Lactobacillus kefir</i> NCFB 2737	1,44	196	0,29*	39
<i>Lactobacillus mali</i> NCFB 2168	0,05*	7	0,49*	66
<i>Lactobacillus sakei</i> LB 706	1,57	215	0,04*	5
<i>Lactobacillus viridiscens</i> NCFB 8965	0,14*	19	nd	0
<i>Pediococcus pentosaceus</i> NCFB 990	0,33*	45	nd	0

nd – not detected; *Values below the limit of detection

When using 1.0 mg/L of *p*-coumaric acid, *L. collinoides*, *L. kefir* and *L. sakei* converted this acid into mostly 4-VP, while *L. mali* showed higher amounts of 4-EP (molar conversion rate 66%) than 4-VP. *L. viridescens* and *P. pentosaceus* did not produce 4-EP, presenting molar conversion rates for 4-VP of 19 and 45%, respectively. The decarboxylation of *p*-coumaric acid into 4-VP and consequent reduction to 4-EP suggests PAD and VPR activity

Table 9. Production of 4-VP and 4-EP from *p*-coumaric acid at 5.0 mg/L in MRS Broth.

Bacterial Strains	4-VP (mg/L)	Molar conversion (%)	4-EP (mg/L)	Molar conversion (%)
<i>Lactobacillus collinoides</i> ESB 99	0,45*	12	0,15*	4
<i>Lactobacillus kefir</i> NCFB 2737	0,59*	16	0,13*	3
<i>Lactobacillus mali</i> NCFB 2168	0,70*	19	0,16*	4
<i>Lactobacillus sakei</i> LB 706	0,44*	12	0,13*	3
<i>Lactobacillus viridiscens</i> NCFB 8965	0,52*	14	0,02*	0
<i>Pediococcus pentosaceus</i> NCFB 990	0,37*	10	0,30*	8

*Values below the limit of detection

In the experiments done with an initial concentration of 5.0 mg/L of *p*-coumaric acid, all of the strains produced higher quantities of 4-VP than 4-EP. *Lactobacillus mali* was the strain with higher molar conversion rate into 4-VP (19%), and *Pediococcus pentosaceus* showed the higher molar conversion rate into 4-EP (8%). It is noteworthy that some of the strains show lower concentrations of 4-VP and/or 4-EP at 5.0 mg/L than at 1.0 mg/L, namely *L. mali*, *L. kefir* and *L. sakei*.

With 50 mg/L of *p*-coumaric acid, *L. sakei* and *P. pentosaceus* almost fully decarboxylate this substrate into 4-VP. It can also be observed that *L. collinoides*, *L. kefir* and *L. mali*, reduced a high amount of 4-VP into 4-EP with molar conversion rates of 50%, 72% and 59% respectively, regardless of some 4-VP still being left in the medium. *L. viridescens* produced almost none of both volatile phenols. Figure 7 shows the comparison between the results with 5.0 mg/L and 50 mg/L of *p*-coumaric acid.

Table 10. Production of 4-VP and 4-EP from *p*-coumaric acid at 50 mg/L in MRS Broth.

Bacterial Strains	4-VP (mg/L)	Molar conversion (%)	4-EP (mg/L)	Molar conversion (%)
<i>Lactobacillus collinoides</i> ESB 99	6,41	18	18,70	50
<i>Lactobacillus kefir</i> NCFB 2737	0,91	2	26,84	72
<i>Lactobacillus mali</i> NCFB 2168	0,29*	1	22,02	59
<i>Lactobacillus sakei</i> LB 706	53,36	146	2,12	6
<i>Lactobacillus viridiscens</i> NCFB 8965	0,39*	1	0,24*	1
<i>Pediococcus pentosaceus</i> NCFB 990	61,26	167	0,10*	0

*Values below the limit of detection

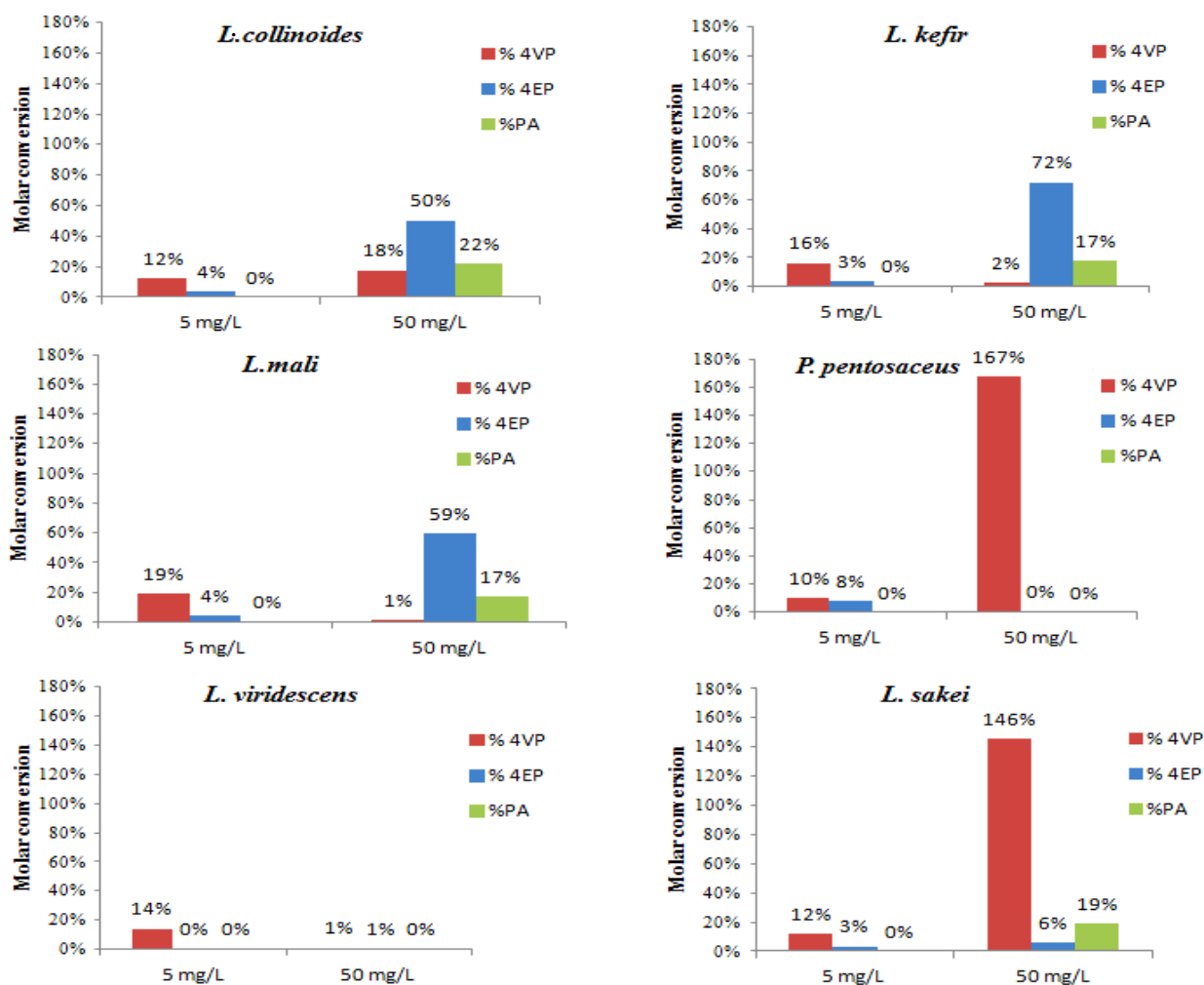


Figure 7. Production of 4-VP, 4-EP and phloretic acid (PA) with different concentrations of *p*-coumaric acid.

3.1.2. Volatile phenols production from *p*-coumaric acid with addition of L-malic acid to the medium

L-malic acid was added to the medium to a final concentration of 4.0 g/L, using an initial concentration of 5.0 mg/L of *p*-coumaric acid for the three strains selected. The results are shown in table 11.

To better understand the difference caused by the addition of L-malic acid, the results were compared with the ones obtained with 5.0 mg/L of *p*-coumaric acid in the first part of this work, which, in this section, will be referred to as controls. Figure 8 shows the comparison between these two results.

Table 11. Production of 4-VP and 4-EP from *p*-coumaric acid at 5.0 mg/L in MRS Broth with L-malic acid added to a concentration of 4.0 g/L.

Bacterial Strains	4-VP (mg/L)	Molar conversion (%)	4-EP (ml/L)	Molar conversion (%)
<i>Lactobacillus collinoides</i> ESB 99	1,71	47	0,13*	3
<i>Lactobacillus sakei</i> LB 706	1,60	44	0,19*	5
<i>Pediococcus pentosaceus</i> NCFB 990	0,79	22	0,05*	1

*Values below the limit of detection

In the presence of L-malic acid, *L. collinoides* produced higher quantities of 4-VP when comparing with the control, while production of 4-EP was approximately the same. *P. pentosaceus* showed decrease in 4-EP production (8% to 1% molar conversion rate), while 4-VP production was stimulated, doubling the conversion rate when comparing with respective control. *L. sakei*, on the other hand, showed an increase in both volatile phenols, more noticeable in 4-VP production.

Figure 8 presents the comparison of results for the production of volatile phenols with the addition of L-malic acid to the medium.

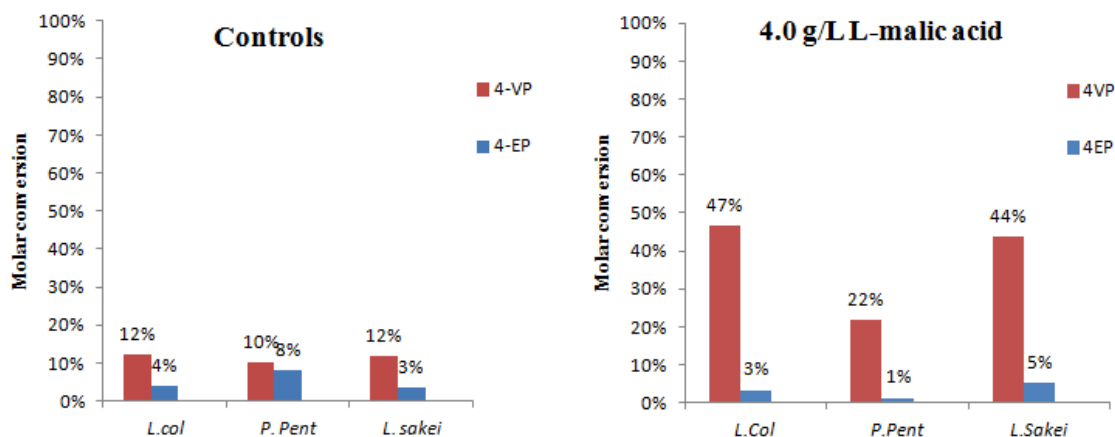


Figure 8. Production of 4-VP, 4-EP and phloretic acid (PA) with L-malic acid added to a concentration of 4.0 g/L, and respective controls.

3.1.3. Production of volatile phenols from *p*-coumaric acid with addition of fructose to the medium.

To evaluate the influence of fructose in the production of volatile phenols, 3.0 g/L of this hexose was added to the medium, along with a 5 mg/L concentration of *p*-coumaric acid. The controls used where the same as in 3.1.2. The results are presented in table 12.

Table 12. Production of 4-VP and 4-EP from *p*-coumaric acid at 5.0 mg/L in MRS Broth with fructose added to a concentration of 3.0 g/L.

Bacterial Strains	4-VP (mg/L)	Molar conversion (%)	4-EP (ml/L)	Molar conversion (%)
<i>Lactobacillus collinoides</i> ESB 99	1,55	42	0,16*	4
<i>Lactobacillus sakei</i> LB 706	1,22	33	0,07*	2
<i>Pediococcus pentosaceus</i> NCFB 990	1,19	33	nd	0

nd – not detected; *Values below the limit of detection

With fructose added to the medium, the three strains showed higher production of 4-VP. *L. collinoides* was the strain with higher stimulation of PAD, with a 42% of molar conversion rate when comparing with 12% of the respective control. As for 4-EP production, *P. pentosaceus*, production was null, showing no VPR activity, while *L. sakei* showed a very low decrease and *L. collinoides* maintained the amount produced. Figure 9 show the comparison between these results.

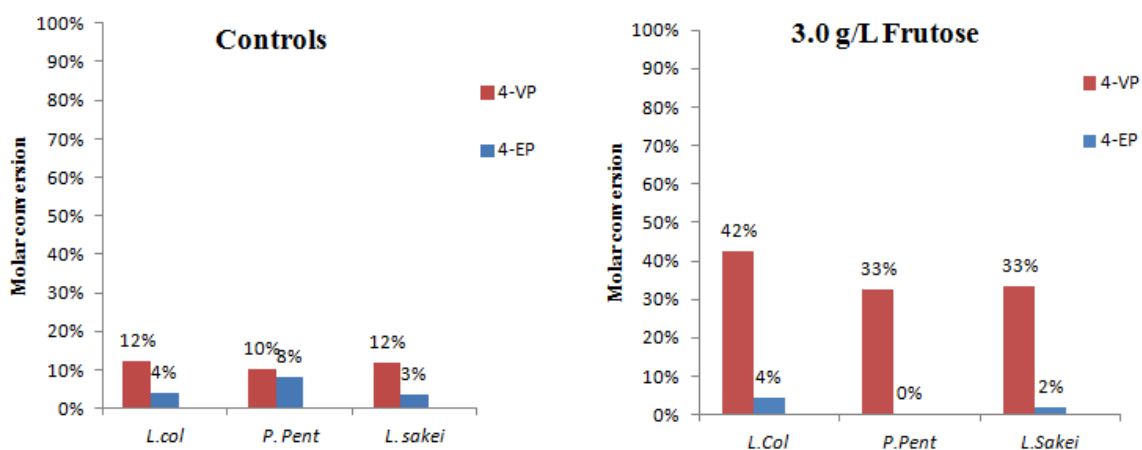


Figure 9. Production of 4-VP, 4-EP and phloretic acid (PA) with fructose added to a concentration of 3.0 g/L, and respective controls.

3.2 Non-volatile phenolic compounds production

After one week of incubation, the samples were analysed through HPLC-DAD in order to measure the concentrations of non-volatile phenolic metabolites, namely *p*-coumaric acid remaining in the growth medium (after fermentation) and the presence of phloretic acid, as a result of the reduction of *p*-coumaric acid. Molar concentration of phloretic acid was divided by the initial molar concentration of *p*-coumaric acid in order to calculate the molar conversion rates. Results are the average values of two experiments.

3.2.1. Non-volatile phenolic compounds production from different concentrations of *p*-coumaric acid

For the initial concentration of *p*-coumaric acid at 1.0 mg/L, phloretic acid was not found, and also no *p*-coumaric acid remained in the medium (data not shown). The results for the production of phloretic acid as well as the remaining amount of *p*-coumaric acid with 5.0 mg/L and 50 mg/L of initial *p*-coumaric acid are shown in table 13 and table 14 respectively. Some of the results are below the limit of detection calculated for the calibration curve and are pointed in the tables. Results are the average values of two experiments.

With 5.0 mg/L of *p*-coumaric acid, phloretic acid wasn't detected in any of the strains tested. As for residual *p*-coumaric acid, all of the strains appear not to fully consume this substrate.

Table 13. Production of phloretic acid from *p*-coumaric acid at 5.0 mg/L and residual *p*-coumaric acid in the medium.

Bacterial Strains	Phloretic acid (mg/L)	Molar conversion (%)	Residual <i>p</i> -coumaric acid (mg/L)	Residual <i>p</i> -coumaric acid (%)
<i>Lactobacillus collinoides</i> ESB 99	nd	0	3.07	61
<i>Lactobacillus kefir</i> NCFB 2737	nd	0	1.28	26
<i>Lactobacillus mali</i> NCFB 2168	nd	0	2.13	43
<i>Lactobacillus sakei</i> LB 706	nd	0	1.84	37
<i>Lactobacillus viridiscens</i> NCFB 8965	nd	0	1.13	23
<i>Pediococcus pentosaceus</i> NCFB 990	nd	0	1.91	38

nd – not detected; *Values below the limit of detection

Table 14. Production of phloretic acid from *p*-coumaric acid at 50 mg/L and residual *p*-coumaric acid in the medium.

Bacterial Strains	Phloretic acid (mg/L)	Molar conversion (%)	Residual <i>p</i> -coumaric acid (mg/L)	Residual <i>p</i> -coumaric acid (%)
<i>Lactobacillus collinoides</i> ESB 99	11,02	22	nd	0
<i>Lactobacillus kefir</i> NCFB 2737	8,81	17	nd	0
<i>Lactobacillus mali</i> NCFB 2168	8,62	17	nd	0
<i>Lactobacillus sakei</i> LB 706	9,51	19	nd	0
<i>Lactobacillus viridiscens</i> NCFB 8965	nd	0	13,65	27
<i>Pediococcus pentosaceus</i> NCFB 990	nd	0	5,73	11

nd – not detected *Values below the limit of detection

Of the six strains analysed with 50 mg/L of *p*-coumaric acid, four produced phloretic acid, being *L. collinoides* the strain with higher rate, presenting a molar

conversion of 22%. *L. kefir* and *L. mali* also transformed 17% of *p*-coumaric acid into phloretic acid, while *L. sakei* showed a conversion rate of 19%.

Only *L. viridescens* and *P. pentosaceus* presented residual *p*-coumaric in the medium. As for *L. kefir* and *L. sakei*, results suggest that *p*-coumaric acid was fully degraded since the total molar conversion rates were near 100%. On the other hand, *L. collinoides* and *L. mali* molar conversion rates were 90 and 77% respectively, and no residual *p*-coumaric acid was found in the medium, suggesting the possibility that some substrate was lost during filtration or that maybe it combined with some other component unknown at the time.

3.2.2. Non-volatile phenolic compounds production from *p*-coumaric acid with addition of L-malic acid to the medium.

L-malic acid was added to the medium to a final concentration of 4.0 g/L, using an initial concentration of 5.0 mg/L of *p*-coumaric acid for the three stains selected. The results were compared with the ones obtained with 5 mg/L of *p*-coumaric acid in the first part of this work, which, in this section, will be referred to as controls.

The presence of L-malic acid in the medium apparently didn't induce the production of phloretic acid in any of the strains. Residual *p*-coumaric acid found after the 7 days of incubation was only detected in *P. pentosaceus*, with lower amount left in the medium (21%) when comparing with the control (38%). Moreover, *L. collinoides* and *L. sakei* showed no residual *p*-coumaric acid in the medium, while residual amounts were found in the controls, 61% and 37%, respectively. Once again, the total molar conversion rates (50 and 49%, respectively) suggest that some of the substrate was lost.

3.2.3. Non-volatile phenolic compounds production from *p*-coumaric acid with addition of fructose to the medium.

The influence of fructose in the production of non-volatile metabolites was evaluated, with 3.0 g/L of this hexose added to the medium, along with a 5.0 mg/L concentration of *p*-coumaric acid. The controls used were the same as in 3.1.2.

Phloretic acid wasn't detected in any of the strains, while residual *p*-coumaric acid was only found in *P. pentosaceus* with lower amount left (12% conversion rate) when comparing with control (38% conversion rate). Furthermore, *L. collinoides* and *L. sakei* didn't show any residual *p*-coumaric acid in the medium, while residual amounts were found in the controls, 61% and 37%, respectively, suggesting the possibility that some substrate was lost during filtration or that maybe it combined with some other component unknown at the time.

4. Discussion

4.1. Influence of *p*-coumaric acid concentration in the production of VP and Non-volatile phenolic compounds

All tested strains were able to produce volatile phenols (4-VP and/or 4-EP) from different initial concentrations of *p*-coumaric acid. Some patterns were observed, with strain dependent behaviours being perceived. Phloretic acid was only detected in four of the six strains, when using the highest amount of *p*-coumaric acid.

Literature indicates that *L. collinoides* ESB 99, isolated from wine, has both PAD and VPR activity and is a strong producer of 4-EP (Silva *et al.*, 2011a). Couto *et al.* (2006) showed that 4-VP and 4-EP production by *L. collinoides* increased when supplementing the medium with higher amounts of *p*-coumaric acid (5.0, 50 and 500 mg/L). In this work, the conversion rate of 4-VP by *L. collinoides* did in fact increase when *p*-coumaric acid was augmented (from 5.0 to 50 mg/L), suggesting that the initial concentrations of substrate plays an important role in the final amount of volatile phenols produced.

Del Hoyo, 2012, studied more than thirteen LAB strains for phenolic acid reductase activity, being *L. collinoides* ESB 99 one of the strains that produced phloretic acid from *p*-coumaric acid at 50 mg/L. It can be noticed that both 4-VP and 4-EP increased from 5.0 to 50 mg/L of *p*-coumaric acid, but at lower concentrations, the stimulation of VPR activity seemed to be minor.

In this work, residual *p*-coumaric acid in the medium was quantified. Regarding *L. collinoides*, at 5.0 mg/L, 61% of this substrate was detected at the end of the experiment. A possible explanation for this fact is that, at lower *p*-coumaric acid concentration the bacteria doesn't fully metabolize this acid, but when increasing the concentration, the toxicity also increases, leading to its transformation in 4-VP, a less toxic compound for the microorganism (Couto *et al.*, 2006). PAR activity seemed to be stimulated as well (22% of molar conversion rate to phloretic acid), although it appears to be a less effective detoxification system according to Barthelmebs *et al.*, (2000) study with in *L. plantarum*. These authors demonstrated the existence of another inducible phenolic acid decarboxylase (PAD2) that, as well as PAR activity, showed slower

conversion of *p*-coumaric acid, thus presenting a disadvantage when detoxifying the cell. Toxicity of phenolic acids is thought to be due to the dissipation of the cell Δ pH (uptake of the protonated form and the consequent release of H⁺ in the interior of the cell) and to a specific mechanism of phenolic acids (Barthelmebs *et al.*, 2000).

Apart from some strains of *L. collinoides*, *L. mali* is also an important bacteria in cider production. The strain chosen for this work, *L. mali* NCFB 2168, was isolated from cider apple juice. Previous work (Couto *et al.*, 2006; Buron *et al.*, 2011) have shown that *L. mali* produce 4-VP at high conversion rates, thus exhibiting decarboxylation activity but not reduction activity. The results from the present work suggest the opposite, with high conversion rates of 4-EP when using 50 mg/L of *p*-coumaric acid. With 5.0 mg/L, it was detected a low VPR activity.

The same behaviour was observed with *L. kefir*, which presented high conversion rates of 4-EP, contrary to results obtained from Couto *et al.* (2006), where production was practically null. Although del Hoyo (2012) detected PAR activity in both *L. mali* and *L. kefir*, in agreement with this work, volatile phenols production is not consistent with previous works.

P. pentosaceus is well known for its PAD activity, Cavin *et al.* (1993) first demonstrated this ability, which later was confirmed by Couto *et al.* (2006) and de las Rivas *et al.* (2009). The results of the present work also indicate that *P. pentosaceus* NCFB 990 fully decarboxylates *p*-coumaric acid in to 4-VP at 50 mg/L of this substrate. At lower concentrations, results suggest some VPR activity, with 4-EP molar conversion of 8% and a much lower PAD activity when comparing with 50 mg/L results, with 4-VP molar conversion rate of 10% (Figure 7). Couto *et al.* (2006) also showed that higher conversion yields for 4-VP were obtained with 500 mg/L of *p*-coumaric acid than with 50 mg/L, reinforcing the idea that the initial concentration of *p*-coumaric acid seems to play an important role in the synthesis of volatile phenols. Despite the fact that in her work del Hoyo (2012) detected PAR activity by this strain, no production of phloretic acid was detected in this work. With 50.0 mg/L of *p*-coumaric acid this behaviour was expected since the 4-VP molar conversion rate demonstrates full decarboxylation of this substrate (167% of conversion rate). The incoherence regarding this experience, is the residual *p*-coumaric acid left in the medium. With the high conversion rate presented for 4-VP it shouldn't be any left.

There are not many studies regarding the production of volatile phenols by *L. sakei* and *L. viridescens*. Couto *et al.*, (2006), studied a different strain of *L. sakei*, showing that higher *p*-coumaric concentration resulted in high amounts of 4-VP, while VPR activity was basically null. The results were similar with the strain used in this work. *L. sakei* LB 706 showed a higher PAD activity at 50 mg/L than with 5.0 mg/L, while VPR activity was very low with both concentrations used. In accordance with del Hoyo (2012), this strain showed PAR activity, producing phloretic acid with 50 mg/L of *p*-coumaric acid.

On the other hand, for *L. viridescens* NCFB 8965, which Couto *et al.* (2006) identified as high producer of 4-VP, no significant volatile phenols were detected in this work at 50 mg/L. The only PAD activity appear to occur at 5.0 mg/L, with a molar conversion rate of 14% for 4-VP. However, it should be pointed out that these authors used a different growth medium which was supplemented with 5% v/v ethanol. Moreover, with 50 mg/L, around 75% of *p*-coumaric acid was used, suggesting that the strain consumed the substrate but didn't decarboxylate or reduced it to either 4-VP or phloretic acid, respectively. This suggests that either *p*-coumaric acid was lost during the experience, or that resulting metabolites are unknown at this stage.

In all strains, residual *p*-coumaric acid was detected in the medium supplemented with 5.0 mg/L of this phenolic acid. This may be explained by the toxicity that hydroxycinnamic acids presents to the bacteria, which increases when using higher amounts of *p*-coumaric acid, leading to a response from the bacteria.

4.2. Influence of L-malic acid in the production of VP and Non-volatile phenolic compounds

When adding L-malic acid to the medium, it is expected a stimulation of bacterial growth due to an increase of pH (Fugelsang *et al.*, 2007). Silva *et al.* (2011a) studied some factors that may influence the volatile phenols production, including the addition of L-malic acid to the medium, where *L. collinoides* ESB 99 produced high quantities of 4-EP while diminishing 4-VP. In the present work, the production of 4-EP was approximately the same when comparing to the control. On the other hand, 4-VP increased, meaning that decarboxylation step was favoured. One possible explanation

for this occurrence is that the bacteria didn't need to reduce 4-VP to 4-EP to regenerate NAD^+ . On the other hand, lactic acid bacteria are the only bacterial group that is both strictly fermentative and able to grow in the presence of oxygen (Jackson *et al.*, 2008). If trace amounts of oxygen were present during bacterial growth, it is possible that they regenerate NAD^+ from oxygen. However, there is no way of telling if the L-malic acid was consumed at all, since it wasn't measured at the beginning or at the end of the experience.

Regarding *P. pentosaceus* NCFB 990, in the presence of L-malic acid, Silva *et al.* (2011a) didn't register any change in the behaviour of this strain. In this study, however, this strain showed a decrease in VPR activity, while PAD activity was stimulated, but to a lesser extent than the other two strains tested. Moreover, this species showed a weak 4EP synthesis in any of the scenarios investigated, in accordance with previous studies (Couto *et al.*, 2006; Silva *et al.*, 2011a). Chatonnet *et al.* (1995) also shows that *P. pentosaceus* 4-EP production is almost null, and also that the consumption of L-malic acid in the presence of phenolic acids is not affected.

To our knowledge, there are not many studies regarding production of volatile phenols by *L. sakei*, and none about the influence of L-malic acid related to this event.. The present results show that L-malic acid stimulated this strain to produce volatile phenols, especially 4-VP. VPR activity, responsible for the reduction of 4-VP to 4-EP, was slightly stimulated, suggesting that the bacteria poorly resort to this step to obtain NAD^+ .

None of the three strains produced phloretic acid from the metabolism of 5 mg/L of *p*-coumaric acid. As for the residual *p*-coumaric acid found in the medium, it was only found in *P. pentosaceus*. The results suggest that *p*-coumaric was converted into metabolites that weren't detected in this study or lost during some procedures.

In summary, at lower concentration of *p*-coumaric acid (5.0 mg/L) L-malic acid seemed not to stimulate VPR activity, as it was observed at higher concentration of this substrate by Silva *et al.* (2011a). On the contrary, PAD activity appears to increase.

4.3. Influence of fructose in the production of VP and Non-volatile phenolic compounds

Silva *et al.* (2011a) suggested that when fructose is added in combination with glucose, production of 4-EP by some lactic acid bacteria was diminished while the production of 4-VP was favoured.

As mentioned before, since *L. collinoides* ESB 99 is strictly heterofermentative, it may reduce fructose to mannitol as an alternative pathway to the regeneration of NAD⁺. In accordance with Silva *et al.* (2011a), the results obtained in this work suggest that production of 4-VP by *L. collinoides* ESB 99 was indeed stimulated, while production of 4-EP was not affected and remained low, when comparing with controls.

In the experiments done with *P. pentosaceus* and *L. sakei*, which are, respectively, homofermentative and facultatively heterofermentative, the presence of fructose increased the production of 4-VP at the expense of the production of 4-EP.

Phloretic acid wasn't detected in any of the three strains studied, so the presence of fructose didn't change, in this respect, the metabolism of 5.0 mg/L of *p*-coumaric acid. Regarding the residual *p*-coumaric acid found in the medium, only *P. pentosaceus* showed remaining amounts. According to the conversion rates, the results suggests that either *p*-coumaric acid was lost during the experience, or that resulting metabolites are unknown at this stage.

Once again, this is an interesting finding, suggesting that unknown metabolites might have been synthesized.

5. Conclusion

This work focused on characterizing PAR and PAD/VPR activities of LAB, as an attempt to clarify the metabolic pathways of *p*-coumaric acid degradation.

The initial concentration of *p*-coumaric used seems to have high importance in the extension of volatile phenols production, and also in the effect that L-malic acid has in their production. When comparing to previous studies, at low concentration of *p*-coumaric acid, L-malic acid appears to stimulate PAD activity at a higher extent than VPR activity. In some of the strains studied, the production of volatile phenols was higher at lower concentrations, which is an important fact since phenolic compounds exist in wine at concentrations about 0.01–0.2% (Soleas *et al.*, 1997), much lower than the ones used in this study. Further studies need to be carried out trying to recreate wine environment.

The importance of characterizing PAD/VPR and PAR activities is that if the later one is to be induced and become the main pathway for *p*-coumaric degradation, this could be the basis for a strategy to diminish the concentration of volatile phenols precursors. In this work, PAR activity was only detected when using 50 mg/L of *p*-coumaric acid. The addition of L-malic acid or fructose didn't show any influence on this enzyme activity. However, further experiments need to be carried out in order to have more information about this subject.

During this work, some limitations might have affected the quantification of metabolites production when using the HPLC-DAD system, since very small amounts of initial *p*-coumaric acid were used. Moreover, the time of waiting when using GC could also have affected the volatile fractions, including the internal standard which is more volatile than the other compounds in the extracts.

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