Factors influencing the production of volatile phenols by wine lactic acid bacteria

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A B S T R A C T

This work aimed to evaluate the effect of certain factors on the production of volatile phenols from the metabolism of *p*-coumaric acid by lactic acid bacteria (LAB) (*Lactobacillus plantarum*, *L. collinoides* and *Pediococcus pentosaceus*). The studied factors were: pH, L-malic acid concentration, glucose and fructose concentrations and aerobic/anaerobic conditions. It was found that, in the pH range of 3.5 to 4.5, the higher the pH the greater the production of volatile phenols. This behaviour is correlated with the effect of pH on bacterial growth. Increasing levels of L-malic acid in the medium diminished the production of 4-vinylphenol (4VP) and stimulated the production of 4-ethylphenol (4EP) by *L. plantarum* NCFB 1752 and *L. collinoides* ESB 99. The conversion of 4VP into 4EP by the activity of the vinylphenol reductase may be advantageous to the cells in the presence of L-malic acid, presumably due to the generation of NAD^+, a cofactor required by the malolactic enzyme. Relatively high levels of glucose (20 g/L) led to an almost exclusive production of 4VP by *L. plantarum* NCFB 1752, while at low concentrations (≤5 g/L), 4EP is mainly or solely produced. Part of the glucose may be diverted to the production of mannitol as an alternative pathway to regenerate NAD^+. This is corroborated by the experiments done with fructose, a compound that can be used as an electron acceptor by some bacteria becoming reduced to mannitol. In anaerobiosis, the reduction of 4VP into 4EP is clearly favoured, which is consistent with the need to increase the availability of NAD^+ in these conditions. This study shows that the amount and the ratio 4VP/4EP produced by LAB are greatly affected by certain environmental and medium composition factors. The behaviour of the bacteria seems to be driven by the intracellular NAD^+/NADH balance.

1. Introduction

Some wine microorganisms can produce volatile phenols (vinylphenols and ethylphenols) from grape-derived hydroxycinnamic acids (*p*-coumaric and ferulic acids). Volatile phenols have characteristic aromas which, above a certain concentration threshold, have a negative effect on the overall aroma of a wine, imparting off-flavours described as "animal", "horse sweat", "leather" or "medicinal"; but at low concentrations (<420 μg/L of a mixture of 4-ethylphenol and 4-ethylguaiacol) have been cited as contributing positively to aroma complexity (Chatonnet et al., 1995; Fugelsang and Edwards, 2007; Ribéreu-Gayon et al., 2000).

The precursors of the volatile phenols are natural constituents of grape juice and wine — the hydroxycinnamic acids *p*-coumaric and ferulic acids (Chatonnet et al., 1995; Heresztyn, 1986). 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 (4-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 (4-ethylphenol [4EP] from 4VP and 4-ethylguaicol from 4-vinylguaiacol) (Chatonnet et al., 1992; Heresztyn, 1986).

White wines contaminated with volatile phenols normally contain variable amounts of vinylphenols, at a 4VP/4-vinylguaiacol ratio of 1:1, but generally contain no ethylphenols. Contaminated red wines, on the other hand, contain small amounts of vinylphenols but they possess large quantities of ethylphenols, in a typical 4EP/4-ethylguaiacol ratio of 8:1 (Chatonnet et al., 1992; Ribéreu-Gayon et al., 2000).

The contaminant yeasts *Dekkera*/*Brettanomyces* are recognised as the main organisms responsible for the production of volatile phenols (Heresztyn, 1986), although previous works have shown that some strains of lactic acid bacteria (LAB) are also capable of producing volatile phenols. Cavin et al. (1993) found that *Lactobacillus* and *Pediococcus* were able to metabolise phenolic acids. Chatonnet et al. (1992, 1995) compared the ability of LAB to synthesise volatile phenols with *Dekkera*/*Brettanomyces*. Some of the LAB strains studied were capable of producing large quantities of 4VP, but only traces of ethylphenols. Only *L. plantarum* was capable of producing significant quantities of 4EP, however in much lower concentrations than those produced by *Dekkera*/*Brettanomyces*. Working with lactobacilli isolated from whisky distilleries, van Beek and Priest (2000) have shown

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that several strains were also able to produce volatile phenols from hydroxycinnamic (*p*-coumaric and ferulic) acids. The authors also found that while most strains produced vinylphenols only, *L. plantarum* and *L. crispatus* were capable of producing ethylphenols from these phenolic acids. More recently, Couto et al. (2006) have shown that 37% of the strains studied (thirty five strains, twenty species) were capable of producing volatile phenols from *p*-coumaric acid in culture medium, although only 9% could produce 4EP.

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. Therefore, the objective of this work was to study the influence of certain parameters (pH, L-malic acid concentration, glucose and fructose concentrations and aerobic/anaerobic conditions) on the production of volatile phenols by *L. plantarum*, *L. collinoides* and *P. pentosaceus*.

2. Materials and methods

2.1. Bacteria and growth conditions

The LAB strains *L. plantarum* NCFB 1752, *P. pentosaceus* NCFB 990 (National Culture of Food Bacteria, Reading, England) and *L. collinoides* ESB 99 (Escola Superior de Biotecnologia — UCP, Porto, Portugal (Couto and Hogg, 1994)) were used in this work. These strains were chosen due to their ability to convert *p*-coumaric acid into 4VP and/or 4EP as demonstrated by Couto et al. (2006).

Cultures were grown to late exponential phase in an MRS medium (Lab M, Lancashire, United Kingdom) at 25 °C. The initial pH was adjusted to 4.5 with a concentrated (6 mol/L) HCl solution before sterilisation (121 °C, 15 min). After sterilisation, 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.

2.2. Production of volatile phenols under different growth conditions

To study the influence of pH on the production of volatile phenols by LAB, cultures in late exponential phase were transferred to an MRS medium with 5% v/v ethanol supplemented with 50 mg/L of *p*-coumaric acid (trans-4-hydroxycinnamic acid; 98% purity, Sigma-Aldrich, Steinheim, Germany), with pH values ranging from 3.5 to 4.5. The different initial pH values were adjusted with a concentrated (6 mol/L) HCl solution before sterilisation (121 °C, 15 min). A concentrated solution of *p*-coumaric acid was prepared in ethanol (99.5% v/v) and added to the growth medium, after sterilisation. Cultures were incubated at 25 °C, without agitation, until stationary growth phase.

The effect of the presence of malic acid was studied in MRS medium with 5% v/v ethanol and 50 mg/L of *p*-coumaric acid (pH 4.5) to which L-malic acid from Merck (Darmstadt, Germany) was added to final concentrations of 0, 0.1, 2 and 4 g/L. Cultures were incubated at 25 °C, without agitation, until stationary growth phase.

The influence of glucose and fructose on the production of volatile phenols by LAB was studied in modified MRS medium with 5% v/v ethanol and final concentrations of glucose adjusted to 0, 1, 3, 5 and 20 g/L, and fructose added to 5 and 20 g/L (pH 4.5). In all the experiments the medium was supplemented with 50 mg/L of *p*-coumaric acid. Cultures were incubated at 25 °C, without agitation, until stationary growth phase.

The influence of oxygen on the production of volatile phenols by LAB was studied comparing aerobic versus anaerobic conditions. Cultures were grown in aerobic conditions in 100 ml of modified MRS medium with 5% v/v ethanol, either with 3 or 20 g/L of glucose (pH 4.5), in 250 mL loosely capped Schott flasks with agitation (150 rpm) at 25 °C. Anaerobic conditions were achieved by boiling the medium for 5 min and sparging the broth and the headspace with nitrogen gas for 15 and 5 min, respectively, to remove air and traces of oxygen. Resazurin (1 mg/L) (Sigma) was added as an indicator of redox potential. The medium was then autoclaved (121 °C, 15 min). Cultures in 250 mL tightly capped Schott flasks were incubated statically at 25 °C.

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. Assays were made in duplicate or triplicate.

2.3. Analysis of volatile phenols

The samples were centrifuged at 3000 *g* for 10 min and the supernatants were treated and analysed according to the method described by Bertrand (1981). 50.0 μL of 4-decanol (internal standard) was added to 50 mL of culture medium. 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 microliter 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, Texas). The injector (split/splitless) was heated to 220 °C with a split flow of 30 mL/min and a splitless time of 0.5 min. The carrier gas flow was adjusted to 1 mL/min. The temperature of the oven was maintained at 4 °C for 1 min and was then increased at a rate of 2 °C/min up to 220 °C. This temperature was then maintained for 30 min. The detection limit of the method was 0.05 mg/L for 4VP and 0.01 mg/L for 4EP.

3. Results and discussion

3.1. Effect of pH on the production of volatile phenols

In the pH range investigated in this work (3.5 to 4.5), it was possible to observe that the higher the pH the greater the production of volatile phenols by the three bacterial strains studied (Table 1). This behaviour is correlated with the effect of pH on bacterial growth. Final OD_{660} values of the liquid cultures (recorded at the stationary growth phase) were considerably lower at pH 3.5 than at 4 and 4.5. The results show that the production of volatile phenols is more affected by pH variations in LAB than in *Dekkera bruxelensis* (recognised as the main organism responsible for the production of volatile phenols in

Table 1
Influence of pH on the production of 4-vinylphenol (4VP) and 4-ethylphenol (4EP) by *L. collinoides* ESB 99, *L. plantarum* NCFB 1752 and *P. pentosaceus* NCFB 990 (nd — not detected).

		L. collinoides ESB 99			*L. plantarum* NCFB 1752			*P. pentosaceus* NCFB 990		
		[4VP] (mg/L)	[4EP] (mg/L)	Final OD_{660}	[4VP] (mg/L)	[4EP] (mg/L)	Final OD_{660}	[4VP] (mg/L)	[4EP] (mg/L)	Final OD_{660}
pH	3.5	0.91 ± 0.01	nd	0.17 ± 0.01	1.21 ± 1.04	0.04 ± 0.00	0.48 ± 0.54	0.56 ± 0.39	0.04 ± 0.00	0.07 ± 0.01
	4.0	1.08 ± 0.08	6.71 ± 0.05	3.35 ± 0.04	3.27 ± 1.61	0.90 ± 0.50	1.27 ± 0.09	26.69 ± 0.37	0.05 ± 0.00	0.87 ± 0.27
	4.5	2.35 ± 0.21	11.80 ± 2.12	4.36 ± 0.06	20.68 ± 2.94	0.62 ± 0.44	2.89 ± 0.25	30.32 ± 1.31	0.04 ± 0.00	1.99 ± 0.05

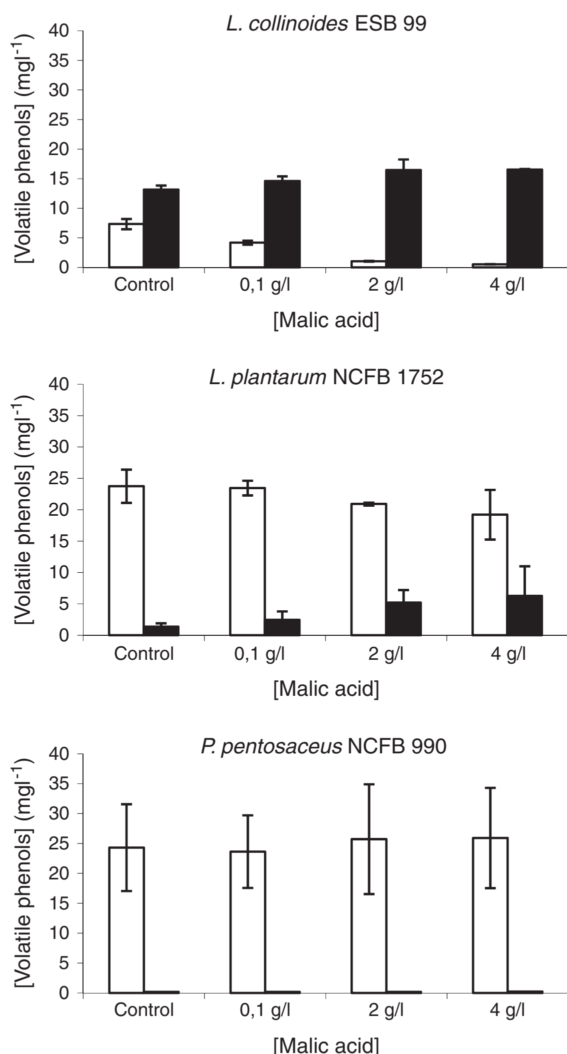

Fig. 1. Effect of L-malic acid on the production of 4-vinylphenol (white bars) and 4-ethylphenol (black bars) by *L. collinoides* ESB 99, *L. plantarum* NCFB 1752 and *P. pentosaceus* NCFB 990. Results are the average values of two experiments (with standard deviation).

wines (Chatonnet et al., 1995; Heresztyn, 1986)). Benito et al. (2009) reported that the conversion of *p*-coumaric acid into 4EP by this organism is complete in the pH range of 2.6 to 4.2. Harris et al. (2010) did not observe any significant impact of pH on growth of most of the *Dekkera* species studied in the pH range of 3.0 to 5.0.

3.2. Effect of L-malic acid on the production of volatile phenols

The presence of L-malic acid in the culture medium, especially in the concentrations of 2 and 4 g/L, slightly stimulated the growth of the three strains as expressed by the final biomasses obtained (data not shown). The stimulatory effect of malic acid on the growth of wine LAB, such as *O. oeni*, is well documented (Firme et al., 1994; Kunkee, 1991; Loubiere et al., 1992; Saguir and Manca de Nadra, 1996). Cox and Henick-Kling (1989, 1995) were able to demonstrate, based on the chemiosmotic theory, the biochemical benefit of malolactic fermentation, as an ATP yielding process. The results of the present work show that L-malic acid markedly influences the production of volatile phenols, stimulating the production of 4EP while diminishing the amount of 4VP released to the culture medium by *L. collinoides* ESB 99 and *L. plantarum* NCFB 1752 (Fig. 1). A possible explanation for these results is that the conversion of 4VP into 4EP, by the activity of the vinylphenol reductase, may be advantageous to the cells in the presence of L-malic acid, presumably due to the production of NAD^+ by the reduction step of the hydroxycinnamic acids metabolic pathway. Indeed, the enzyme involved in the malolactic fermentation by LAB, the malolactic enzyme, requires the presence of NAD^+ cofactor and bivalent ions, particularly Mn^{2+}, for the decarboxylation of L-malic acid into L-lactic acid and CO_2 (Jackson, 2008). The production of volatile phenols by *P. pentosaceus* NCFB 990 was not affected by the different levels of malic acid studied. In fact, this species was unable to synthesise 4EP (only trace amounts) in any of the scenarios investigated.

3.3. Effect of glucose and fructose concentrations on the production of volatile phenols

The concentration of glucose in the culture medium strongly influenced the behaviour of *L. plantarum* NCFB 1752 (Fig. 2). This organism produced mainly 4VP in the presence of 20 g/L of glucose, while at 5 g/L and lower concentrations, 4EP is mainly or solely produced. When homofermentative or facultatively heterofermentative LAB (such as *L. plantarum*) grow on hexose substrates such as glucose, a typical homolactic fermentation is carried out. Pyruvate is reduced to lactic acid by the lactate dehydrogenase, thereby reoxidizing

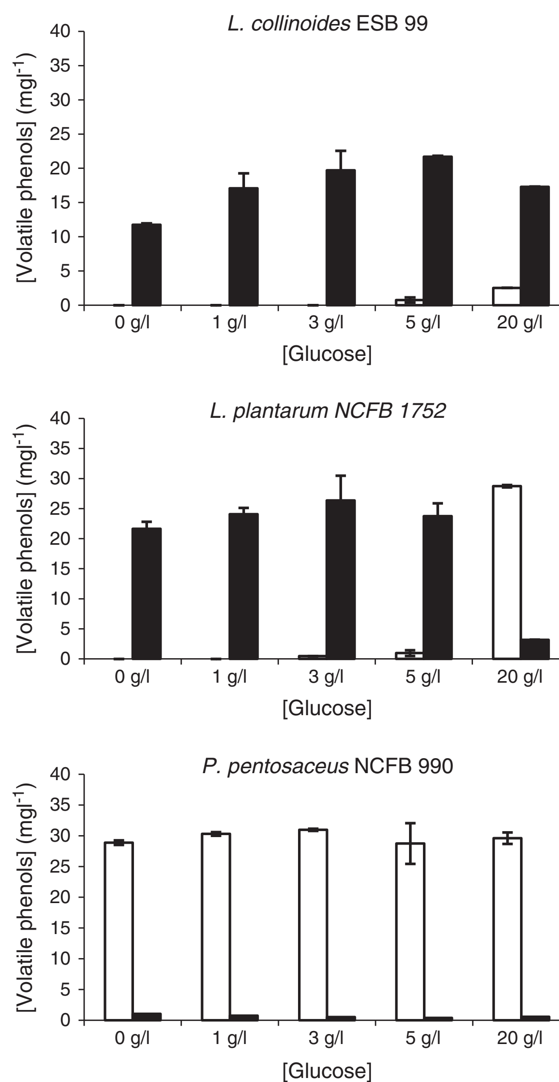

Fig. 2. Effect of glucose concentration on the production of 4-vinylphenol (white bars) and 4-ethylphenol (black bars) by *L. collinoides* ESB 99, *L. plantarum* NCFB 1752 and *P. pentosaceus* NCFB 990. Results are the average values of two experiments (with standard deviation).

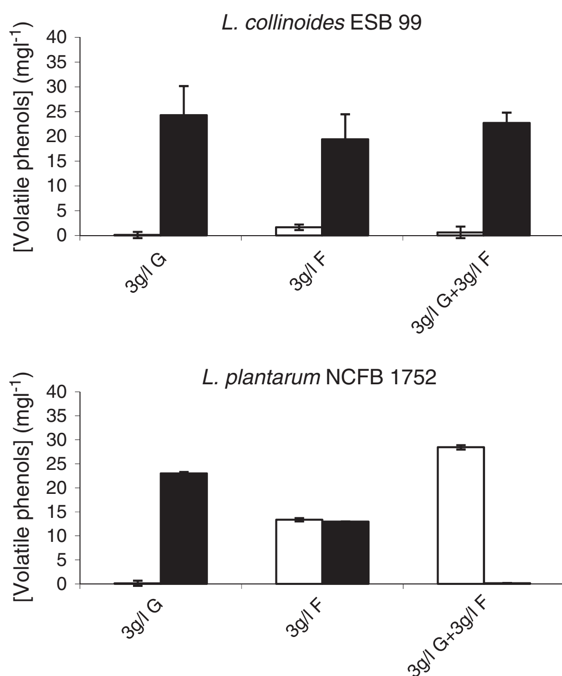

Fig. 3. Effect of fructose concentration on the production of 4-vinylphenol (white bars) and 4-ethylphenol (black bars) by *L. collinoides* ESB 99 and *L. plantarum* NCFB 1752. G — glucose; F — fructose. Results are the average values of two experiments (with standard deviation).

the NADH formed during the early glycolytic steps (Wisselink et al., 2002). The results of this work suggest that at relatively high levels of glucose (20 g/L), part of the carbohydrate might be diverted to the production of mannitol. The biosynthesis of this polyol can be performed by homofermentative/facultatively heterofermentative LAB from the glycolysis intermediate fructose 6-phosphate (Wisselink et al., 2002), functioning as an alternative pathway, instead of lactate formation, to regenerate NAD^+. In these conditions, cells might not need to synthesise NAD^+ (presumably the co-enzyme formed from the reduction of 4VP) by the reduction step of *p*-coumaric acid metabolism, which explains the accumulation of 4VP. A more comprehensive metabolic characterization of *L. plantarum*, concerning the effect of glucose concentration on the conversion of phenolic acids, will be taken into account in future work. The behaviour of *L. collinoides* ESB 99 was only slightly affected by the variation of the glucose concentration. Heterolactic bacteria (such as *L. collinoides*) are unable to synthesise mannitol from glucose, therefore this via cannot be used as an alternative pathway to regenerate NAD^+ (Wisselink et al., 2002). This explains the advantage of this strain in regenerating NAD^+ through the reduction of 4VP into 4EP. The production of volatile phenols by *P. pentosaceus* NCFB 990 was not affected by the glucose concentration. Low levels of glucose (0–5 g/L) affected the growth of all strains (results not shown), however they were still able to produce significant amounts of volatile phenols.

The utilisation of fructose as the sole carbon and energy source or in combination with glucose strongly influenced the production of volatile phenols by *L. plantarum* NCFB 1752, especially at low levels of sugar concentration (3 g/L) (Fig. 3). When fructose is used instead of glucose or in combination with glucose, the production of 4EP is diminished while the production of 4VP is favoured. This effect might be related with the reduction of fructose into mannitol, accompanied by the regeneration of NAD^+. The fermentative metabolism of hexoses can result in the generation of excess reduced NAD^+ (NADH). To maintain an acceptable redox balance, the bacteria must be able to regenerate NAD^+ (Jackson, 2008). It is known that some species reduce fructose to mannitol, presumably for this purpose (Nielsen and Richelieu, 1999; Salou et al., 1994). In these conditions cells wouldn't need to synthesise NAD^+ by the reduction step of *p*-coumaric acid metabolism, thus leading to the accumulation of 4VP. A similar effect of the addition of fructose was found in the heterofermentative strain *L. collinoides* ESB 99 but to a lesser extent than in *L. plantarum* NCFB 1752.

3.4. Effect of the oxygen level the production of volatile phenols

The level of oxygen present in the culture medium strongly influenced the production o volatile phenols by the bacterial strains studied. In aerobic conditions, the lactobacilli only produced 4VP (Fig. 4). In anaerobiosis, the total concentration of the volatile phenols (4VP + 4EP) produced was lower, however the reduction of 4VP into 4EP was clearly favoured. Since under reductive conditions cells may experience a shortage of NAD^+, the reduction of 4VP into 4EP would allow the cells to increase the availability of NAD^+. Under low oxygen concentrations the availability of NAD^+ can be limited so that carbohydrate metabolism is inhibited (Jackson, 2008). It is known that a number of flavoprotein oxidase enzymes are responsible for the direct interaction of LAB with oxygen (Condon, 1987). This makes possible the oxidation of $NADH_2$ with oxygen serving as the final electron acceptor.

As mentioned in the introduction, the conversion of phenolic acids into volatile phenols is a two step process comprising the decarboxylation of the precursor and the reduction of the vinylphenol intermediate. The results of this work suggest that the conversion of 4VP into 4EP, catalyzed by the vinylphenol reductase, may lead to the re-oxidation of NADH. An analogous statement was made by Fugelsang and Edwards (2007) for *Brettanomyces*/*Dekkera*: since 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 growth of this organism in red wines.

This study highlights the capacity of wine lactic acid bacteria to produce volatile phenols and that the amount and the ratio 4VP/4EP produced are considerably affected by environmental and medium composition factors. The behaviour of the bacteria seems to be driven by the intracellular NAD^+/NADH balance. Further research is required

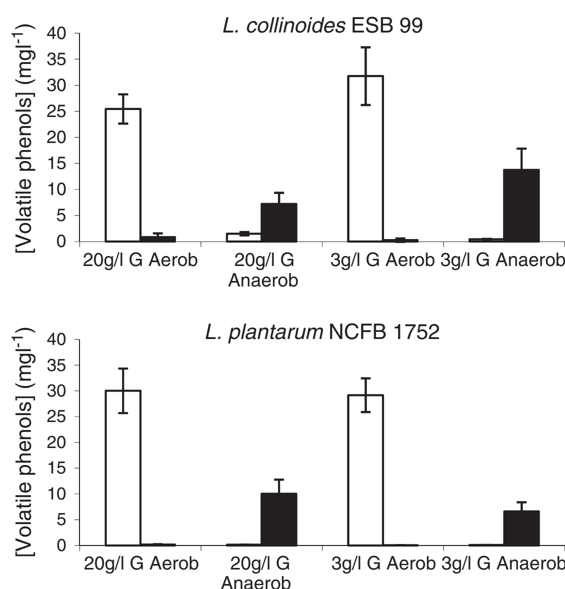

Fig. 4. Effect of oxygen on the production of 4-vinylphenol (white bars) and 4-ethylphenol (black bars) by *L. collinoides* ESB 99 and *L. plantarum* NCFB 1752. G — glucose. Results are the average values of three experiments (with standard deviation).

to elucidate the real impact of the metabolic activity of lactic acid bacteria on the levels of volatile phenols found in wines.

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