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Influence of oak wood and grape tannins on the lactic acid bacterium *Œnococcus œni* (*Leuconostoc oenos*, 8413)

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Abstract: Grape tannins, procyanidins, and oak wood tannins, ellagitannins, do not have the same effect on *Œnococcus œni*. Oligomer procyanidins which are extracted from grape seeds are powerful inhibitors, affecting bacterial viability in non-growing conditions, bacterial growth and malolactic activity. Pure ellagitannins on the contrary appear to be propitious to the viability of *Œ œni*, while the total oak extract is also a powerful inhibitor. We demonstrate that ellagitannins when oxidised have a strong inhibiting effect, as opposed to procyanidins which lose all effect when oxidised. One of the mechanisms involved may be compared to the one that occurs for medium-chain fatty acids, through adsorption on the bacterial walls.

Keywords: grapes; wood; proanthocyanidins; ellagitannins; lactic acid bacterium; metabolism; growth

INTRODUCTION

Alcoholic fermentation is followed by malolactic fermentation which brings about major changes in the composition and quality of wine.[1–4] However, how to trigger and control malolactic fermentation poses a number of problems.[5–7] The bacteria that change malic acid into lactic acid in wine mainly belong to the *Œnococcus œni* (*Leuconostoc oenos*) species.[8–10]

A number of studies have looked into the various factors that hinder the development of *Œ œni* in wine, most of them concentrating on pH, temperature, alcohol and SO_2. The special conditions existing in red wine, notably the great number of phenolic compounds, could be one of the main causes of inhibition. These compounds are found partly in the solid elements of the grape (skin and seeds) which are extracted during winemaking, and partly in the wood from the barrels during aging. Some phenolic compounds such as gallic acid and monoglucoside anthocyanins seem to have a positive effect.[11] On the other hand, the great quantity of tannins which are present seem to have a strong inhibiting effect.[12] Tannins involved are procyanidins in grapes[13] and ellagitannins in oak wood.[14] They have several forms of action: they can inhibit enzyme activity,[15] adhere to the cell walls[16] or form complexes with copper and iron.[17]

The present work studies the incidence of tannins on *Œ œni*, in particular the effect of these molecules on bacterial growth and metabolism.

MATERIALS AND METHODS

Source of tannins

Procyanidins were obtained from a *Vitis vinifera* L cv Cabernet Sauvignon seed hydroalcoholic extract (12 vol% ethanol, 5 g tartaric acid, NaOH to pH 3.5, distilled water to 1000 ml). Lipids were extracted from the hydroalcoholic extract using hexane, and the phenol acids using diethyl ether. The oligomer procyanidin (Pol) fraction was then isolated using ethyl acetate extraction. The organic phase was then evaporated and the extract freeze dried. Hemisynthesis was used to produce the B3 dimer (97% purity, by ^{1}H NMR Bruker DPX400) procyanidin which was then purified as described by Freitas.[18]

The oak wood extract which contains a major fraction of ellagitannins (68% w/w of total extract) was obtained by macerating *Quercus robur* L heartwood sawdust (60 mesh) in an acetone/water mixture (8:3 v/v) for 48 h at room temperature on a rotating table. The acetone was evaporated; the aqueous phase was then extracted with diethyl ether followed by ethyl acetate before freeze drying. Vescalagin (93% purity, by ^{1}H NMR Bruker DPX400), monomeric ellagitannins, was purified as described by Vivas *et al.*[12]

Culture conditions and media

Bacteria were grown in the following medium: casamino acid, 5 g; yeast extract, 4 g; KH_2PO_4, 0.55 g; KCl, 0.425 g; $CaCl_2$, 0.125 g; $MnSO_4$, 0.025 g; $MgSO_4$, 0.125 g; glucose, 5 g; malic DL acid,

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10g; Tween 80, 1ml; pH 4.5–4.8; H_2O to 1000ml. They were enumerated by plate counts on a tomato juice-enriched medium: yeast extract, 5g; neopeptone, 5g; malic DL acid, 10g; $MgSO_4$, 0.05g; $MnSO_4$, 0.02g; tomato juice, 250ml; pH 4.5–4.8; agar, 20g; H_2O to 1000ml. Plates were incubated for 5 days at 25°C under a CO_2+N_2 atmosphere (Queue incubator). In some experiments bacteria were grown in red Cabernet Sauvignon containing 2.85gl^{-1} malic acid and processed with 8gl^{-1} charcoal to eliminate phenolic compounds. Culture media were sterilised by heating at 120°C for 15min, and the wine by membrane filtration (Millipore HAWP, 0.45μm).

Bacterial strain 8413 comes from the collection of the Faculty of Enology (University of Bordeaux). Bacterial growth was followed either by plate counting or by measurement of OD at 600nm (1unit of OD corresponds approximately to 1.3×10^9UFCml^{-1}). Malic acid was determined by an enzymatic method (Boehringer Manheim). Malolactic activity of resting cells was estimated with a pCO_2 electrode according to the method of Vivas *et al.* [11]

RESULTS

Influence of tannins on *Œ œni* viability in non-growing conditions

Non-growing bacteria were prepared by suspension of *Œ œni* in phosphate buffer. The required biomass (around 10^6UFCml^{-1}) was obtained by centrifuging a stationary phase culture at $8000\times g$ for 10min at 4°C. Cells were washed twice with phosphate buffer and finally suspended in 10ml of phosphate buffer supplemented with different phenolic substrates.

When placed in phosphate buffer, the *Œ œni* population behaved differently according to what type of tannin was present (Table 1). The B3 dimer procyanidin and Pol mixture adversely affected viability; this result was accentuated when the quantity was increased. On the other hand, vescalagin, a pure ellagitannin, markedly improved viability, while the oak total phenolic extract had an adverse effect like procyanidins.

Table 1. Influence of procyanidins and ellagitannins on *Œ œni* viability in phosphate buffer

	Concentration (mgl^{-1})	Log(N_t/N_0)[a]
Control	—	−4.0±0.3
Procyanidins		
B3 dimer	100	−4.6±0.5
Seed extract	50	−5.2±0.4
	100	−6.8±0.6
Ellagitannins		
Vescalagin	50	−3.2±0.2
Oak wood extract	50	−4.2±0.3
	100	−4.8±0.4

[a] N_0 initial population; N_t, population after 48h.

Table 2. Influence of seed extract and oak wood extract on growth of *Œ œni* and rate of malate decarboxylation in wine treated with charcoal

	Lactic acid bacteria (log UFCml^{-1})		Rate of malate decarboxylation (μmol$l^{-1}h^{-1}$)	
	2 days	20 days	2 days	20 days
Seed extract (mgl^{-1})				
0	7.6	6.2	43	36
50	6.3	6.3	35	47
100	6.2	6.5	29	42
Oak wood extract (mgl^{-1})				
0	6.3	6.3	38	25
50	5.3	5.1	22	0
100	5.0	4.6	19	0

Influence of seed and oak tannins on *Œ œni* population in a wine treated with charcoal

Bacterial growth and the rate of malic acid degradation during malolactic fermentation in a wine were studied. A control batch and a series of batches supplemented with either seed extract or oak extract at two concentrations (50 and 100mgl^{-1}) were prepared. The experimental results are given in Table 2. The seed extract lowered the population as well as the malate decarboxylation rate during the first few days of the experiment; the effect appeared to stop after 20 days. With the oak extract the inhibiting effect remained constant with time. Eventually, malolactic fermentation stopped in these media, leaving 2.5gl^{-1} residual malic acid. With both tannins the slowing down of the malate decarboxylation rate was proportional to their concentration.

With procyanidins the interruption of inhibition may be caused on the one hand by the ability of the bacteria to adapt to the medium, and on the other by a loss of the inhibiting effect of the tannins with time. The first assumption was dismissed after recultivating in the presence of procyanidins the bacteria harvested from a previous culture with procyanidins. No growth occurred, showing that the bacteria could not tolerate the inhibitor. To verify the second hypothesis, we prepared a control batch along with batches of media (wine treated with charcoal) containing tannin solutions exposed to air for 1, 2, 5, 20 and 30 days. These media had different degrees of oxidation, which were evaluated by measurement of OD at 420nm. The percentage of inhibition was calculated in relation to the growth recorded in the control batch after a 24h incubation period. The same experiment was carried out with the oak extract. Fig 1 shows that the more the procyanidin solution was oxidised, the more the inhibition decreased, and it eventually after 30 days. Under the same conditions the oak wood solution had a significantly more inhibiting effect after oxidation compared to the fresh state.

Interpreting one of the forms of action of a tannin

The suspected mode of action is related to the

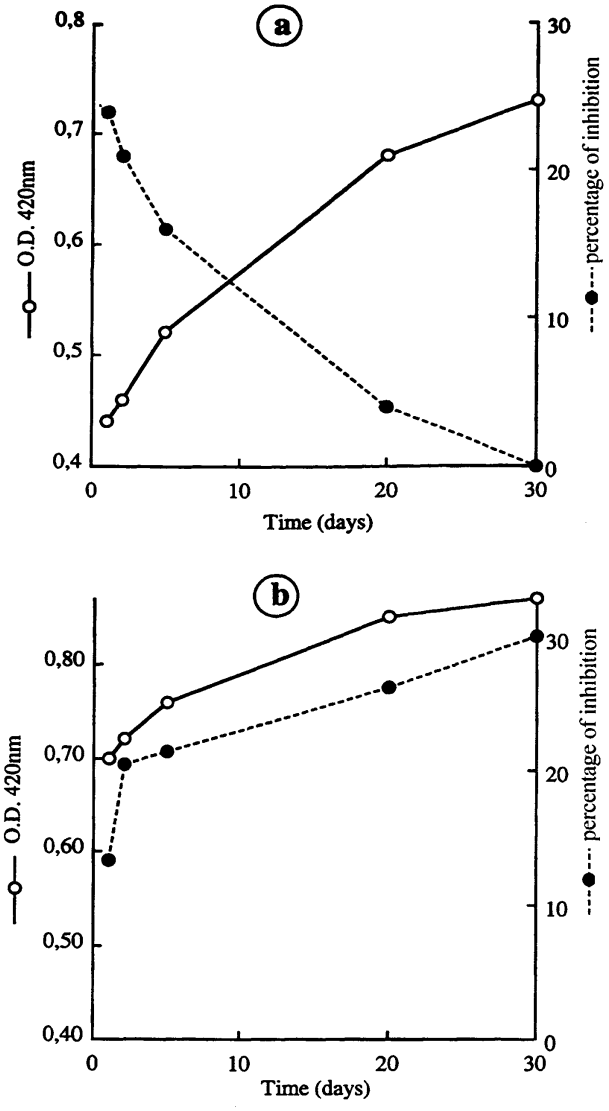

Figure 1. Influence of oxidation level of seed extract (a) and oak wood extract (b) on *Œ œni* growth inhibition.

adsorption capacity of tannins on the cell walls of bacteria. The loss of the inhibition effect of procyanidins could thus be caused by a partial loss of their capacity to be adsorbed. To evaluate the degree of loss, tannin solutions oxidised in the presence of the same quantities of viable bacteria and inactivated bacteria were incubated for 72h. The results given in Fig 2 show that the bacteria had a low adsorption capacity for the coloured oxidised forms from the seed extract. In contrast, the coloured oxidised forms from the oak extract were nearly entirely fixed or absorbed or taken up by the bacteria. On the whole, we observed that bacteria inactivated by heat adsorb the oxidised forms of tannins more than do viable bacteria.

DISCUSSION

Some of the main components of red wine consist of phenolic compounds, mostly in the form of tannins. The shielding effect of these polyphenols against microbial aggression in plant tissues is well known.[16] They act in fact as a passive defence. In wine, bacterial growth difficulties and the difficult onset of malolactic fermentation could be caused by interactions with these compounds. There are very few published studies however that link malolactic fermentation problems with tannin toxicity.[2,12,19] During the traditional winemaking process in vats, grape tannins can affect lactic acid bacteria, but oak tannins were also studied because they become soluble in wine during malolactic fermentation in barrels.

Grape seed extracts and oak extracts mainly contain tannins. The extract purification protocol enables the fatty acids and simple phenols to be eliminated. We completed our observations by comparing the results obtained with the extracts and with pure molecules on the viability of non-growing bacteria. For comparison with the grape seed extract we tested the effect of a dimer procyanidin (B3); the effect of the wood extract was compared with that of a monomeric ellagitannin (vescalagin). Procyanidins when mixed with the seed extract or used pure in the dimer B3 form acted as inhibitors. Pure ellagitannins in the form of vescalagin improved the overall viability of the bacterial population, unlike the total wood extract which proved to be toxic. This result suggested that certain forms of tannins in the extract acted as inhibitors, whereas they did not when in native form. The beneficial effect of vescalagin on bacterial viability may be caused by the β-glucosidase activity of *Œ œni* which allows the cells to capture the glucose in the ellagitannin (results not shown). A fresh solution of vescalagin has practically no effect on growing populations, at least with the amount used for the experiment (20 mg l^{-1}); however, if the same solution is oxidised (30 days), growth is inhibited by 18%. Procyanidins reacted in the reverse way: they were inhibitors only in their native form. Their inhibiting effect decreased with their degree of oxidation, evaluated by the increase in OD at 420nm

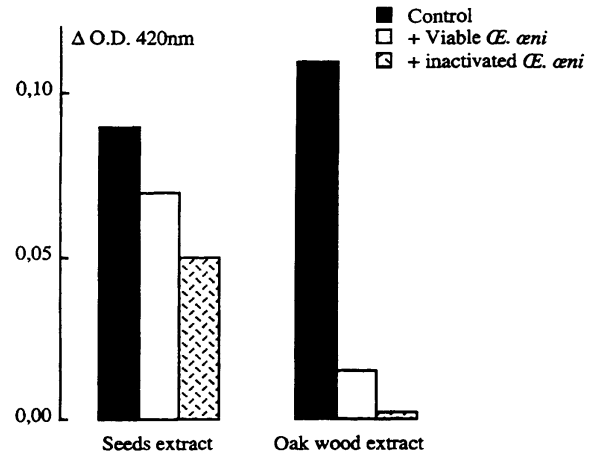

Figure 2. Influence of *Œ œni* biomass on coloration of oxidised seed extract and oak wood extract. Viable bacteria were collected from the stationary phase. Inactivated bacteria were obtained after incubation for 20min at 100°C in distilled water. Results correspond to the residual colour after removal of the bacteria.

of the solution. When oxidised, procyanidins were not adsorbed much on the surface of the bacteria. Recent work has shown that the oxidation of procyanidins decreased their ability to form stable bonds with proteins.[18] Bacterial cells which have been inactivated by heat adsorbed the coloured oxidised forms of tannins more easily. During the treatment, polysaccharides are released and the cell contact area is larger (scanning electron microscope; results not shown).

Indeed, $Gram^+$ bacteria polysaccharides, such as peptidoglycane in *Œ œni*, allow hydrogen links to form between the cell walls of bacteria and tannins.[20] In contrast, the cell wall structure of $Gram^-$ bacteria, in particular the presence of the external lipid layer, protects the cells from the inhibiting effects of tannins.[21]

However, despite the large amount of procyanidins present, malolactic fermentation in red wine only starts after a varying latency period. The simultaneous presence of activating substances and toxic molecules allows an equilibrium that becomes more propitious to the growth of lactic acid bacteria at a given moment. It should be noted also that wines contain few oligomeric procyanidins; most tannins are polymerised and are partly combined with other components, which makes them less toxic.[2,20] From the technological viewpoint, one can predict that the onset of malolactic fermentation will be problematic in wines which are rich in oligomeric procyanidins. This situation arises particularly when grapes are harvested before they are ripe enough, when the seeds are still too rich in tannin.

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