Inhibition of *Lactobacillus plantarum* by polyphenols extracted from two different kinds of olive brine

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J.L. RUIZ-BARBA, M. BRENES-BALBUENA, R. JIMENEZ-DIAZ, P. GARCIA-GARCIA AND A. GARRIDO-FERNANDEZ. 1993. The characteristic phenolic content of NaOH-treated and untreated olive brines is reported. Detection, quantification and isolation of the phenolic compounds were carried out by HPLC. Viability of *Lactobacillus plantarum* in the presence of single or combined fractions of these isolated compounds was tested. When assayed at the concentrations found in brines, only the single phenolic fraction containing hydroxytyrosol strongly inhibited *Lact. plantarum*. However, inhibition of *Lact. plantarum* was also observed when double phenolic fractions (e.g. the glucosides, oleuropein and verbascoside) were used; this showed a combined effect in the inhibition. The different permeability of fruit for polyphenols and organic compounds is discussed as the primary cause of the rapid fermentation of alkaline-treated, but not of untreated, olives.

INTRODUCTION

Processing of fermented vegetable products is increasingly important, mainly because of the special characteristics that it confers, such as flavour, qualities for preservation and health, but also because it is a field in which biotechnology promises substantial improvements in the next few years. Fermented green olives are of great interest in the Mediterranean countries. The usual process includes treatment of the fruits with NaOH (2%, w/v) before brining. It is in these brines that a lactic acid fermentation takes place, conducted mainly by *Lactobacillus plantarum*. The alkaline treatment is necessary to eliminate the bitterness of the fruits and to ensure a suitable growth of lactic acid bacteria that are otherwise generally accepted as being inhibited by the polyphenols contained in the untreated fruits (Juven and Henis 1970; Fleming *et al.* 1973; Ruiz-Barba *et al.* 1990). The antimicrobial activities of the phenolic compounds have also been shown to affect other micro-organisms (Juven and Henis 1970; Nychas *et al.* 1990) and this is a subject of current interest in view of their use as antimicrobial agents in food and cosmetics (Himejima and Kubo 1991).

Several authors have studied the bactericidal effect of both brines on untreated olives (Ruiz-Barba and Jiménez-Díaz 1989) and have isolated phenolic compounds extracted

from these olives or their brines (Federici and Bongi 1983; Ruiz-Barba *et al.* 1991). Nevertheless, different results have been reported for various phenolic compounds, probably because different antimicrobial assay methods were used. On the other hand, not all the significant polyphenols present in untreated olive brines have been tested, nor have their concentrations for the antimicrobial tests been adjusted to those in which they appear in the brines. In addition, we do not really know at present if there are any quantitative and/or qualitative differences in phenolic compounds between alkali-treated and untreated olive brines. If there are it could explain why *Lact. plantarum* can grow in the alkali-treated olive brines but is strongly inhibited in untreated olive brines.

The aim of this work was to establish whether the presence of any particular phenolic compound is the sole explanation of the inhibitory effect observed in untreated but not alkali-treated olive brines against *Lact. plantarum* at their usual concentration and/or if there is any additive inhibitory effect. As a very accurate method to show both the composition and changes of phenolic compounds in olive brines has recently been developed (Brenes *et al.* 1992) we have used this for our purposes.

MATERIALS AND METHODS

Bacteria

Lactobacillus plantarum ATCC 8014 was used as the test micro-organism. It was supplied by the Spanish Type

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Fig. 1 HPLC chromatograms of the total phenolics extracted with ethylacetate from non alkali-treated (a) and alkali-treated olive brines (b). Concentrations of phenolic compounds are given in brackets

Culture Collection and was grown in MRS (De Man *et al.* 1960) solid or liquid medium (Oxoid) at 30°C. Stock cultures were maintained in 20% glycerol (w/v) at -20°C, as static cultures.

Brining of olives

One hundred kg of whole green olives, Hojiblanca variety, were washed in water and divided into two batches of 50 kg. The fruits were placed in PVC jars and one batch was covered with 50 l of NaOH (2%, w/v) and left for 6 h. The NaOH solution was then poured away and the olives were washed in water for 15 h. The water was removed and the fruits were covered by a brine (6% NaCl at equilibrium, w/v). The other 50 kg batch was not treated with alkali and the untreated olives were covered by the same brine.

At 1 and 6 months after brining, samples of brines were removed from batch alkali-treated and untreated olives and analysed by HPLC for phenolic compounds.

Detection, quantification and isolation of phenolic compounds

Polyphenols from brines were extracted with ethylacetate, evaporated under vacuum and the residues dissolved in a

1:1 water/methanol mixture. Detection and quantification of phenols were carried out by HPLC in a Waters apparatus (Millipore, Mildford, MA) equipped with an array diode detector (Waters 994). A 25 cm × 4 mm i.d. column, filled with Spherisorb ODS-2, 5 µm (Supelco, Barcelona, Spain) was used. The wavelength selected was 280 nm. Separation was achieved by elution gradient with an initial composition of 95% water, with pH adjusted to 4.0 units with acetic acid and 5% of acetonitrile. Concentration of the latter solvent was increased to 25% in 20 min and up to 50% in another 20 min. A flow of 1 ml min⁻¹ was maintained. Phenolic compounds were identified by their retention times and absorption spectra in the 200–380 nm range, compared with reference compounds.

Purification was carried out by HPLC with a semi-preparative column and under the same conditions as those used for detection. The purified fractions were desiccated under vacuum, the residues dissolved in acetate buffer 0.05 N, pH 4.0 and then maintained frozen at -20°C under nitrogen.

Preparation of purified phenolic compounds for microbiological assays

HPLC-purified hydroxytyrosol (β -3-4 dihydroxyphenylethyl alcohol) and verbascoside (heterosidic ester of caffeic acid and hydroxytyrosol) were defrozen and then filter-sterilized (Millipore, 0.2 µm pore size). Oleuropein and luteolin-7-glucoside were purchased from Extrasynthese (Genay, France), and tyrosol (β -4-hydroxyphenylethyl-alcohol) was supplied by Sigma. They were prepared at the same concentrations as detected in brines, dissolved in acetate buffer 0.05 N, pH 4.0 and then filter-sterilized (Millipore, 0.2 µm pore size). Two-by-two combinations of single phenolic compounds were made by mixing them at the concentrations found in brines. In addition, hydroxytyrosol was prepared at the maximum, medium and minimum concentrations in which it appeared in brines.

Bactericidal effect

An overnight culture of *Lact. plantarum* ATCC 8014 was used to inoculate fresh MRS broth (10% inoculum). This was incubated at 30°C until it reached log-phase (O.D. = 1.2 at 590 nm, approx. 2×10^8 cfu ml⁻¹), washed three times in saline and then added (10%, v/v) to the different single or double polyphenol fractions. At different time intervals samples were removed and transferred to tubes containing a neutralizing fluid (Corre *et al.* 1990) consisting of (g l⁻¹): lecithin (Merck), 30.0; Tween-80 (Sigma), 20.0; yeast extract (Oxoid), 10.0. After 10 min, samples were plated on MRS agar with a Spiral apparatus (Model DS, Interscience, Saint-Nom-La-Breche,

Fig. 2 Effect of different polyphenols on the survival of *Lactobacillus plantarum* ATCC 8014. The total of viable cells was determined at each time. Values are represented as percentage of the control consisting of acetate buffer 0.05 N, pH 4.0. ○, Tyrosol (1.5 mmol/l), oleuropein (0.45 mmol/l), verbascoside (0.14 mmol/l) or vanillic acid (0.01 mmol/l); △, hydroxytyrosol (7.5 mmol/l); ●, hydroxytyrosol (7.5 mmol/l) and oleuropein (0.45 mmol/l); □, hydroxytyrosol (7.5 mmol/l) and verbascoside (0.14 mmol/l); ×, verbascoside (0.14 mmol/l) and oleuropein (0.45 mmol/l)

Fig. 3 Effect of hydroxytyrosol on the survival of *Lactobacillus plantarum* ATCC 8014. The total of viable cells was determined at each time. Values are represented as percentage of the control consisting of acetate buffer 0.05 N, pH 4.0

France). Survivor cell counts were determined after incubation at 30°C for 48 h. As a control, tubes containing acetate buffer (0.05 N, pH 4.0) were also inoculated and assayed as for the phenolic fractions.

RESULTS

Chromatograms of polyphenols extracted from both NaOH-treated and untreated olive brines are shown in Fig. 1. As can be seen, the main differences in the phenolic content of these brines were the glucoside compounds verbascoside, oleuropein and luteolin-7-glucoside, found to be present only in the latter. The concentration of each phenolic found in brines is given in Fig. 1.

None of the three phenolic compounds showed inhibition when tested against *Lact. plantarum* ATCC 8014 even at the maximum concentrations found in brines (Fig. 2). These concentrations were 0.15 mmol l⁻¹, 0.5 mmol l⁻¹ and 0.15 mmol l⁻¹ for verbascoside, oleuropein and luteolin-7-glucoside respectively. The same result was obtained when tyrosol or vanillic acid, which are in both alkali-treated and untreated olive brines, were assayed (Fig. 2). The concentrations used were 1.5 mmol l⁻¹ and 0.01 mmol l⁻¹ for tyrosol and vanillic acid, respectively. In contrast, hydroxytyrosol showed a strongly bactericidal effect against *Lact. plantarum* ATCC 8014 (Figs 2 and 3).

At the maximum concentration found in both alkali-treated and untreated olive brines (approx. 7.5 mmol l⁻¹) hydroxytyrosol killed up to 55% of the initial *Lact. plantarum* ATCC 8014 population within the first hour of incubation (Figs 2 and 3) and no survivors were detected after 2 h (Fig. 3). Hydroxytyrosol was also found to be bactericidal at low concentration (Fig. 3). Some 70% and 30% of surviving lactobacilli were detected after 2 h of incubation with 3.2 and 4.5 mmol l⁻¹ of hydroxytyrosol, respectively. After 4 h no survivors were detected in 4.5 mmol l⁻¹ hydroxytyrosol but some 20% of surviving cells were found at the same incubation time for 2.3 mmol l⁻¹. Finally, 100% of *Lact. plantarum* ATCC 8014 were killed after incubation for 24 h in 2.3 mmol l⁻¹ hydroxytyrosol.

When *Lact. plantarum* ATCC 8014 was added to different two-by-two phenolic compound fractions, a decrease in cell viability was found (Fig. 2). Thus, the verbascoside (0.14 mmol l⁻¹) plus oleuropein (0.45 mmol l⁻¹) fraction killed up to 95% of the lactobacilli after incubation for 1 h and 100% after 2 h. A similar strongly bactericidal effect was found when hydroxytyrosol (7.5 mmol l⁻¹) and verbascoside (0.14 mmol l⁻¹) were tested together (Fig. 2). For the pair hydroxytyrosol (7.5 mmol l⁻¹) and oleuropein (0.45 mmol l⁻¹), a decrease of some 70% in the initial *Lact. plantarum* population was observed after incubation for 1 h and 100% of the lactobacilli were killed after 2 h (Fig. 2).

DISCUSSION

This study has demonstrated the bactericidal effect of single phenolic compounds isolated from green olive brines against *Lact. plantarum* ATCC 8014. Furthermore, the additive antimicrobial effect of some of these phenolics has been shown, whereas when assayed as single fractions they had no bactericidal effect against the lactobacilli.

Ruiz-Barba *et al.* (1991) found that oleuropein has a marked bactericidal effect against *Lact. plantarum*. However, the concentration of oleuropein they used (about 7.7 mmol l^{-1}) was considerably higher than that which we found in olive brines (no more than 0.5 mmol l^{-1}). They used that concentration of oleuropein because this is the level of this glucoside in olive fruits. Fleming *et al.* (1973), however, used 0.4 mmol l^{-1} of oleuropein in 5% NaCl and reported no bactericidal effect against *Lact. plantarum* (cf. Fig. 2). To our knowledge the antimicrobial activity of vanillic acid, verbascoside and luteolin-7-glucoside had not been assayed before for antimicrobial activity, and our results are the first to be published. We have shown that none of them, when tested as single phenolic fractions, had bactericidal effect against the lactobacilli (Fig. 2).

The results obtained with hydroxytyrosol (Figs 2 and 3) corroborate the work of Ruiz-Barba *et al.* (1990) in which they stated that this phenolic compound had a bactericidal effect against strains of *Lact. plantarum* isolated from green olive fermentations. They used 3.8 mmol l^{-1} hydroxytyrosol in 4% NaCl as it appeared in olive brines. We have used acetate buffer to test hydroxytyrosol in order to minimize the additive effect of NaCl, described by Fleming *et al.* (1983). Tyrosol has no antimicrobial effect (Fig. 2) as Ruiz-Barba *et al.* (1990) have shown when they used 0.18 mmol l^{-1} of this phenolic compound.

Our results also demonstrated an additive antibacterial activity against *Lact. plantarum* of verbascoside, oleuropein and hydroxytyrosol when tested together two-by-two (Fig. 2). The bactericidal effect of hydroxytyrosol was even greater when combined with oleuropein or verbascoside (Fig. 2) than when isolated (Figs 2 and 3).

Although our results agree with the inhibition of bacterial growth observed in untreated green olive brines (Federici and Bongi 1983; Ruiz-Barba and Jiménez-Díaz 1989; Ruiz-Barba *et al.* 1990), they differ about the absence of inhibition of *Lact. plantarum* in alkali-treated green olive brines (Juven and Henis 1970; Rodríguez de la Borbolla y Alcalá 1979). The presence of the glucoside phenolics oleuropein, verbascoside and luteolin-7-glucoside in the untreated olive brines could explain this because, as is shown, in combination they are bactericidal against lactobacilli. However, hydroxytyrosol is also highly bactericidal and it appears in both alkali-treated and untreated olive brines.

Hydroxytyrosol is a major phenolic compound in NaOH-treated olive brines because of the hydrolysis of glucosides, especially oleuropein (Amiot *et al.* 1990). This is also a major compound in untreated olive brines as a result of acidic hydrolysis (Brenes *et al.* 1992) although it reaches a similar level much later in the brines of untreated olives. It was found that after 1 month the concentrations of hydroxytyrosol in NaOH-treated olive brines were between $6\text{--}8 \text{ mmol l}^{-1}$, while these levels were reported in untreated olive brines only after preservation for 8–9 months (Brenes *et al.* 1992). Thus the question is why *Lact. plantarum* is able to grow in the NaOH-treated olive brines even in the presence of such high levels of hydroxytyrosol.

In our opinion the answer must be closely related to the higher permeability of the fruits after NaOH treatment. Thus, diffusion of hydroxytyrosol into the brines is faster; but so also is that of many other organic compounds necessary for the growth of lactobacilli, e.g. sugars, proteins, vitamins, fatty acids, amino acids, etc. (Juven and Henis 1970; Rodríguez de la Borbolla y Alcalá 1979). Proteins and amino acids have been shown to bind polyphenols extensively (Pierpoint 1983) and, as a consequence, they neutralize their inhibitory effect (Ruiz-Barba *et al.* 1990). On the other hand, sugars, vitamins and certain fatty acids support the growth of lactobacilli (Ruiz-Barba *et al.* 1991). A rapid availability of all these beneficial compounds in the NaOH-treated olive brines could support such a heavy growth of lactobacilli that polyphenols remaining active (e.g. hydroxytyrosol) cannot inhibit the whole of the population at once. This is not the case with untreated fruits, where nutritional and neutralizing compounds take a long time to emerge from the fruits. In summary, we think that it could be the balance of inhibitory polyphenols/nutrients and neutralizing compounds that determine whether an olive brine is going to ferment properly. Experiments are being carried out to estimate the limits of this balance.

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