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Bacteriocin production by lactic acid bacteria isolated from Rioja red wines

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L. NAVARRO, M. ZARAZAGA, J. SÁENZ, F. RUIZ-LARREA AND C. TORRES. 2000. Forty-two lactic acid bacteria (LAB) of the genera *Lactobacillus* (32), *Leuconostoc* (6), *Pediococcus* (3) and *Lactococcus* (1), isolated from Rioja red wines, were tested for antimicrobial activity. All these strains, as well as 18 *Leuconostoc oenos* and 19 yeast strains were used as indicators. Only nine strains showed antimicrobial activity, and all were of the species *Lactobacillus plantarum,* which constitutes the predominant microflora in Rioja red wines after alcoholic fermentation. *Lact. plantarum* strain J-51 showed the widest range of action, inhibiting the growth of 31 strains of the four studied LAB genera. *Lact. plantarum* J-51 antimicrobial activity was lost after treatment with proteases, suggesting a proteinaceous nature for this activity. It was found to be stable between pH 3 and 9 and under strong heating conditions (100 °C for 60 min). Polymerase chain reaction (PCR) analysis of *Lact. plantarum* J-51 genome revealed the presence of the *plnA* gene that encodes the plantaricin precursor PlnA. A 366-bp fragment was sequenced and showed 95% identity with *pln* locus of *Lact. plantarum* C-11. The deduced precursor peptide sequence showed one mutation (Gly7 to Ser7) at the double glycine leader peptide, and the three putative 26-, 23- and 22-residue active peptides remain identical to those of *Lact. plantarum* C-11. Therefore, antimicrobial peptides constitute a potent adaptation advantage for those strains that dominate in a medium such as wine, and can play an important role in the ecology of wine microflora.

INTRODUCTION

Bacteriocins are antimicrobial proteinaceous substances with a narrower spectrum of activity than antibiotics. They are secreted by some bacteria, which are thus adapted for competition against other micro-organisms growing in the same medium. Bacteriocins constitute only part of the inhibitors produced by lactic acid bacteria (LAB).

Bacteriocins produced by a variety of LAB have been reported (reviewed by De Vuyst and Vandamme 1994), and some have been intensively studied for application in food preservation (Gibbs 1987). However, few reports can be found either on bacteriocins produced by LAB of oenological origin (Lonvaud-Funel and Joyeux 1993; Strasser de Saad and Manca de Nadra 1993), or on bacteriocins present in a finished wine.

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During alcoholic and malolactic fermentation of wine, microbial interactions may happen between yeasts, LAB and yeast-LAB. A variety of factors may be involved in these interactions (organic acids, pH change, bacteriophages, etc.), and bacteriocins are important inhibitors of bacterial growth that should be taken into account. A few bacteriocinegenic LAB strains from wines have so far been described. Inhibitory effects between *Pediococcus pentosaceus*, *Lactococcus plantarum* and *Leuconostoc oenos* (also called *Oenococcus oeni*, Dicks, Dellaglio and Collins 1995), isolates from wines were described by Lonvaud-Funel and Joyeux (1993). A *Pediococcus pentosaceus* strain isolated from Argentinean wines was reported to produce pediocin N5p (Strasser de Saad and Manca de Nadra 1993). This bacteriocin showed high heat stability, optimum pH for activity in the acid range and resistance to ethanol and SO_2. Nisin is a bacteriocin isolated from *Lactococcus lactis* of non-oenological origin. Nevertheless, this bacteriocin has interest in wine-making, since it has been used to control spoilage by undesired LAB in wine and beer

(Delves-Broughton *et al*. 1996). Currently, there is increasing interest in bacteriocins as natural food preservatives; moreover, there are strong evidences that these antimicrobials may play an essential role in wine fermentation. This paper seeks to obtain further knowledge of these antimicrobials secreted by LAB during fermentation. The objectives of this study were to identify bacteriocin-producing LAB isolated from musts and red wines, to determine their spectrum of action and to characterize their inhibitory activities.

MATERIALS AND METHODS

Strains and culture conditions

Forty-two LAB, all of them isolated from Rioja red wines (Northern Spain) during malolactic fermentation, were tested for antimicrobial production. They included *Lact. plantarum* (22), *Lactobacillus brevis* (8), *Lactobacillus paracasei* (2), *Leuconostoc mesenteroides* (6), *Ped. pentosaceus* (1), *Pediococcus damnosus* (1), *Pediococcus acidilactici* (1) and *L. lactis* (1). All these strains and 18 *Leuc. oenos* (isolated from wine), were used as indicators for bacteriocin production. Yeasts of different genera (some of them obtained from the Spanish Type Culture Collection) were also included as indicators: *Saccharomyces cerevisiae* (8), *Schizosaccharomyces pombe* (1), *Pichia* spp. (1), *Candida albicans* (1), *Rodothorula rubra* (1), *Torulaspora delbrueckii* (1), *Hanseniaspora uvarum* (1), *Zygosaccharomyces veronae* (1), *Saccharomyces bayanus* (1) and *Kloeckera apiculata* (1).

The stock culture collection was maintained at −20 °C in skim milk (Difco, Detroit, MI, USA) and cultures were propagated twice in Man-Rogose and Sharpe (MRS) agar plates (Oxoid, Hampshire, UK) at 30 °C in anaerobic conditions for 2–3 days.

Detection of antimicrobial activity

Bacteriocin production was evaluated by a modification of the 'spot on the lawn' method (Lewus and Montville 1991). Plates containing Trypticase Soy broth, plus 0·5% yeast extract and 1·5% agar (TSAYE, Difco), were spotted with putative bacteriocin-producing strains and incubated anaerobically overnight at 30 °C. Five-millilitre aliquots of Brain Heart Infusion (BHI, Difco) containing 0·8% agar were tempered at 45 °C and seeded with 40 μl of overnight cultures of a number of bacteriocin-sensitive strains which were used as indicators. The spotted TSAYE agar plates were carpeted with indicator strains and incubated anaerobically for 48 h at 30 °C. Growth inhibition was detected by a zone of clearing around the producer strain. Screening was performed under anaerobic conditions as well as in the presence of 5% of CO_2. Sabouraud soft broth (Difco) was used when yeasts were tested as indicators.

Bacteriocin extract preparation

The bacteriocin-producing *Lact. plantarum* strain J-51 was grown in MRS broth at 30 °C for 48 h. The final pH of the culture was 4·05. Crude extracts were prepared by centrifugation at 30 000 *g* for 30 min. Cell-free supernatants were 10-fold concentrated by speed vacuum and stored at −20 °C.

Assay for bacteriocin activity of *Lact. plantarum* strain J-51

Concentrated extract samples were assayed for antimicrobial activity by a modified disk-diffusion assay (Ferreira and Gilliand 1988). A TSAYE agar plate was overlaid with 5 ml of soft BHI agar seeded with 40 μl of an overnight culture of the indicator micro-organism (*Lact. plantarum* J-81). Sterile paper disks (Difco) were saturated with the concentrated extract and placed upon the surface of freshly seeded and solidified agar media. Plates were incubated for 24 h at 30 °C, and thereafter they were observed for the presence of clear zone surrounded disks.

Growth and bacteriocin production curves of *Lact. plantarum* strain J-51

Lact. plantarum strain J-51 was grown in MRS broth at 25 °C and 30 °C (initial inoculum 5×10^5 c.f.u. ml^{-1}) and growth curves were followed optically by OD at 625 nm. Culture samples (10 ml) were removed every hour, concentrated and assayed for activity as indicated above.

Characterization of the antimicrobial activity produced by Lact. plantarum strain J-51

Characterization of the antimicrobial activity of *Lact. plantarum* strain J-51 was carried out according to the following criteria:

(i) Sensitivity to proteolytic enzymes. Concentrated extracts of the culture medium of the producer *Lact. plantarum* strain J-51 were prepared as described above. They were adjusted to the optimum pH for each of the following proteases: trypsin, α-chymotrypsin, proteinase K, papaine, protease, lysozyme (all of them purchased from Sigma, St Louis, MO, USA), and were independently incubated with 1 g l^{-1} of each protease.

(ii) Stability under heating, extreme pH and organic solvents. Concentrated supernatant aliquots were heated at 100 °C for 10, 20, 30, 45 and 60 min. They were adjusted to different pHs, between 1 and 12, and treated with 10 and 25% chloroform and 5, 10, and 15% ethanol. After all these treatments, antimicrobial activity was assayed. Negative controls were always included.

(iii) Storage and filtration stability. Antimicrobial activities

of extracts were assayed after storage at – 20 °C, + 4 °C and room temperature, and after filtration with different filters: filter membrane 0·22 μm Millex-GV and Millex-GP 50 (Millipore, Beford, MA, USA), and acetate cellulose filter membrane 0·45 μm (Corning, Badhoevedorp, The Netherlands).

PCR studies were performed to determine the presence of the *plnA* gene described for *Lact. plantarum* C11, a strain wich was isolated from cucumbers in fermentation (Diep *et al*. 1994). PCR reactions were carried out using primers and conditions previously reported (Remiger, Ehrmann and Vogel 1996):

PlnA5p: 5′-GTA CAG TAC TAA TGG GAG-3′
S7: 5′-CTT ACG CCA TCT ATA CG-3′

The amplified PCR fragment (expected size 450 bp) was visualized after electrophoresis in 1·8% (w/v) agarose gels (80 V for 1 h). A 100-bp ladder was used as molecular weight marker. The double stranded polymerase chain reaction (PCR) amplicon was sequenced in forward and reverse senses using the ABI 373 DNA Sequencer.

RESULTS

Forty-two LAB strains of oenological origin of the genera *Lactobacillus, Leuconostoc*, *Pediococcus* and *Lactococcus* were screened for producers of growth inhibitory substances. The same 42 LAB strains and 18 additional *Leuc. oenos* strains were used as indicators for bacteriocin production. No antimicrobial activity was detected in six *Leuc. mesenteroides,* three *Pediococcus* spp. and one *L. lactis* strain. Nevertheless, antimicrobial activity was shown in nine out of 32 *Lactobacillus* spp. strains (28%), all of them of the species *Lact. plantarum* (nine out of 22 *Lact. plantarum*, 41%) (Table 1).

When bacteriocin detection was performed in the presence of 5% of CO_2, 16 positive strains were obtained. Nevertheless, when the assay was performed under anaerobic conditions, only nine of these strains maintained the antimicrobial activity and were therefore considered as producers. None of these nine strains showed activity against themselves when used as producers and indicators simultaneously.

In four of these nine *Lact. plantarum* producer strains (J-51, J-53, J-65 and J-75), the antimicrobial activity was shown against at least 13 out of the 42 LAB strains used as indicators, and they were considered high producers. One of these strains, *Lact. plantarum* J-51, showed inhibitory activity against 31 strains of the four studied genera of LAB: *Lactobacillus* (*Lact. plantarum, Lact. brevis and Lact. paracasei*), *Leuconostoc (Leuc. mesenteroides), Pediococcus* (*Ped. pentosaceus and Ped. acidilactici*), and *Lactococcus (L. lactis)*. The other three high producer *Lact. plantarum* strains did not show activity against *Leuc. mesenteroides*, but against *Lactobacillus* spp., *Pediococcus* spp. and *L. lactis*. The other five *Lact. plantarum* producer strains showed intermediate activity, inhibiting the growth of 1–3 indicators of the genera *Lactobacillus (Lact. plantarum, Lact. paracasei and Lact. brevis) and Ped. pentosaceus*. None of the nine producer strains showed inhibitory activity against either *Leuc. oenos*, or 19 yeasts of different species of oenological interest (*Saccharomyces cerevisiae, Schizosaccharomyces pombe, Pichia* spp., *Candida albicans*, *Rodothorula rubra*, *Torulaspora delbrueckii*, *Hanseniaspora uvarum*, *Zygosaccharomyces veronae*, *Saccharomyces bayanus* and *Kloeckera apiculata*).

The antimicrobial activity of the three high producer *Lact. plantarum* J-53, J-65 and J-75 strains was evaluated at different pHs and heating conditions. These activities were stable after treatment at 100 °C for 30 min. They were stable only at acid pH and were irreversibly inactivated at pH > 4. After treatment with proteolytic enzymes (protease, α-chymotrypsin, proteinase K, lysozyme and papaine) antimicrobial activities of these strains were completely lost, although this loss of activity could be due either to the neutral pH during incubation with proteases, or to the proteolytic activity of the enzymes.

Lact. plantarum strain J-51 was selected for further study as antimicrobial producer due to its wide and clear inhibitory spectrum, and *Lact. plantarum* J-81 was used as indicator strain. Concentrated supernatants of *Lact. plantarum* J-51 cultures in MRS broth were submitted to a variety of treatments. The inhibitory activity of these extracts was completely lost upon 2-h treatment with protease, trypsin, α-chymotrypsin, proteinase K and papaine, but it was not affected by lysozyme, even after 24-h treatment (Table 2). This inactivation by proteases suggests a proteinaceous nature, a general characteristic of bacteriocins. The antimicrobial activity of *Lact. plantarum* strain J-51 was found to be stable between pH 3 and 9, became gradually lost at lower pHs. This activity was lost at pH 10, and was partially restored upon reversion to acid pHs. It was very stable under different storage temperatures, such as 4 °C, – 20 °C and room temperature, and under strong heating conditions (100 °C for 60 min) (Table 2). Nevertheless, thermostability was lower after a previous storage at – 20 °C. *Lact. plantarum* J-51 bacteriocin activity was also maintained after treatment with organic solvents, such as chloroform (25%) and ethanol (15%). When direct supernatant of *Lact. plantarum* J-51 culture medium was submitted to sterile filtration, no antimicrobial activity was detected in direct or concentrated filtrates.

Growth and bacteriocin production curves of *Lact. plantarum* J-51 at 25 °C and 30 °C were performed (Fig. 1). Stationary phase was reached more rapidly at 30 °C than at 25 °C. Under both temperature conditions, bacteriocin activity of supernatants was detected (according to the method used) when OD at 625 nm reached the value 2·7, which corresponded with the beginning of the stationary

Table 1 Growth inhibition of lactic acid bacteria (indicator strains) by *Lactobacillus plantarum* strains producers isolated from wine

Growth inhibition producer strains	Indicator strains																											
	Lactobacillus plantarum														*Lactobacillus brevis*						*Lactobacillus paracasei*		*Leuconostoc mesenteroides*			*Lactococcus lactis*	*Pediococcus pentosaceus*	*Pediococcus acidilactici*
	J31	J40	J55 J56	J26 J71	J51	J69 J58	J36	J34	J39 J73	J53 J75	J67	J30	J62	J61	J77	J78	J80	J63	J81	J64	J60	J52	J48	J47	J57	J59	J27	J83
Lact. plantarum J51	+	+	+	–	+	+	+		+		+	+	+	+	+	+	+	+	+	+	+	–		+		+	+	+
Lact. plantarum J53	+/-	+	+	–	–	–			–		–	–	–	+	+	+	–	+	–	+/-	+	–		–		+	+	+
Lact. plantarum J65	+/-	+	+	–	+	+			–		–	–	–	+	+	+	+	+	+	–	+	–		–		+	+	+
Lact. plantarum J75	+/-	+	+	–	+	–			–		–	+	+/-	–	–	+	+	+	+	–	+	+		–		+	+	+
Lact. plantarum J36	–	–	–	–	–	–			–		–	–	–	–	–	–	–	–	–	–	–	–		–		–	+	–
Lact. plantarum J39	–	+/-	–	+	–	–			–		–	–	–	–	+	–	–	–	–	–	–	–		–		–	+	–
Lact. plantarum J62	–	–	–	–	–	–			–		–	–	–	–	+	–	+	–	–	–	–	–		–		–	–	–
Lact. plantarum J67	–	–	–	–	–	–			–		–	–	–	–	–	–	–	–	–	–	+	–		–		–	–	–
Lact. plantarum J71	–	–	–	–	–	–			–		–	–	–	+	–	–	–	–	–	–	–	–		–		–	–	–

Sensitive strain inhibited+; not inhibited –; low inhibition+/-

Table 2 Antimicrobial activity of *Lact. plantarum strain* J-51

Producing organism	*Lact. plantarum* J-51
Origin	Wine
Spectrum of activity	*Lactobacillus* *Lactococcus* *Leuconostoc* *Pediococcus*
Degradation by proteases (2h)	Protease, trypsin, α-chymotrypsin, proteinase K and papaine
Heat stability	100° C, 60 min
pH stability	3–9
Filtration	Lost
Organic solvent stability	Chloroform, ethanol
Detection	Early stationary growth phase ($OD_{625\,nm} = 2{\cdot}7$)

phase of *Lact. plantarum* J-51 growth. Halo diameters did not increase, nor decrease during 10 h of stationary growth phase.

Genomic DNA of *Lact. plantarum* strain J-51 was analysed for the presence of *plnA* gene, formerly described for *Lact. plantarum* C11. PCR reaction with the primers described above, and under optimized conditions, resulted in amplification of a DNA fragment from *Lact. plantarum* J-51. This amplicon showed the expected 450 bp size.

The amplified PCR fragment was sequenced and showed 95% identity (351 out of 366 bp) with the sequence of *Lact. plantarum* C11 operon obtained from EMBL Gene Bank (accession code X75323). When the sequence was translated into amino acid sequence and compared with *Lact. plantarum* C11 plantaricin precursor sequence, an identity of 97% (47 of 48 amino acids) was obtained. One mutation of Gly-7 to Ser-7 was found in the leader peptide (Fig. 2), remaining fully identical the active peptide sequence.

DISCUSSION

Bacteriocin production was evaluated in 42 LAB of oenological origin, of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Lactococcus*. Antimicrobial activities secreted to the culture media were demonstrated for nine of these strains (21·4%), being all the producer strains of the species *Lact. plantarum* (nine out of 22 *Lact. plantarum* strains, 41%). No antimicrobial activity was detected in the culture media of the *Lactococcus*, *Leuconostoc* or *Pediococcus* strains of this study. Strasser de Saad and Manca de Nadra (1993) reported one *Ped. pentosaceus* strain, among 20 LAB isolates from Argentinean wines (5%), with inhibitory activity against the other LAB strains, which included: *Pediococcus* spp., *Leuc. oenos* and *Lactobacillus* spp. Lonvaud-Funel and Joyeux (1993) studied the antagonism between LAB isolates from grape

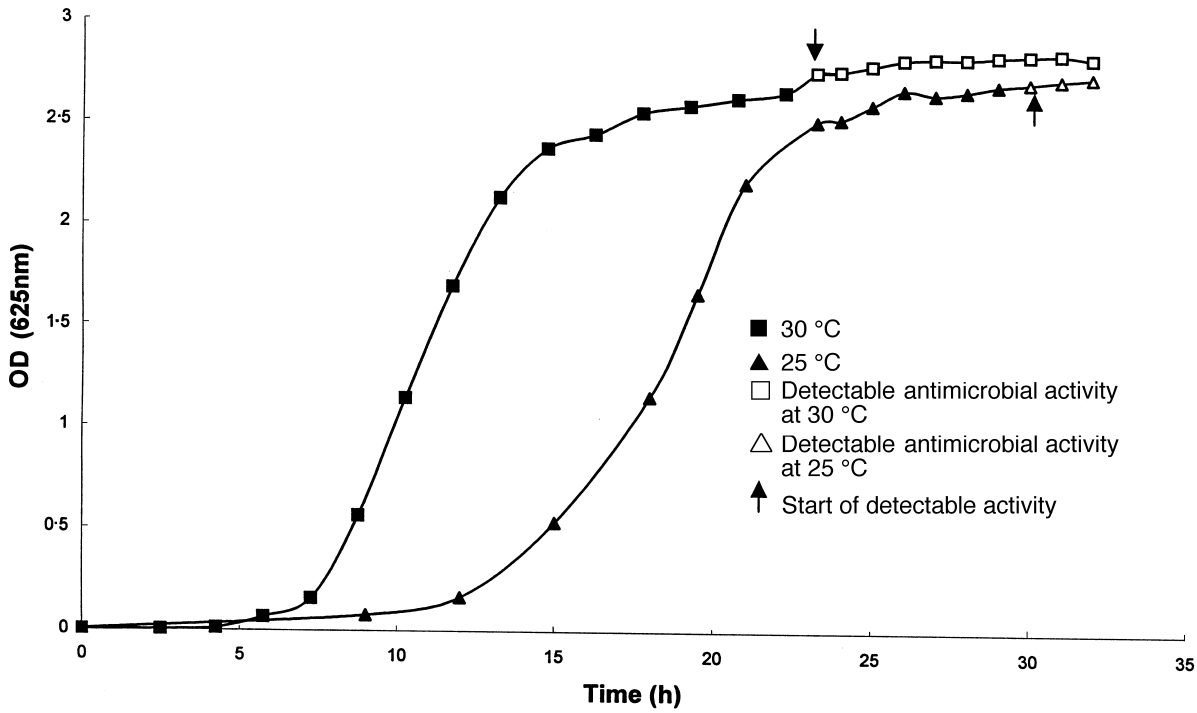

Fig. 1 Growth curves of *Lactobacillus plantarum* strain J-51 and antimicrobial activity at 30 °C and 25 °C. Hollow symbols show detectable antimicrobial activity and arrows show start of detectable antimicrobial activity

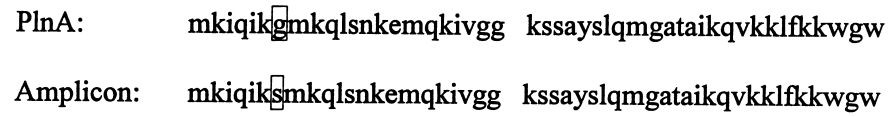

Fig. 2 Comparison of the peptide sequences of PlnA (X75323) and that encoded by the PCR amplicon of *Lact. plantarum* strain J-51

musts and wines, which included six *Lactobacillus* spp., one *Pediococcus* sp., and three *Leuconostoc* spp. strains. These authors found the highest inhibitory activities by one *Ped. pentosaceus* strain and one *Lact. plantarum* strain against *Leuc. oenos* and *Leuc. mesenteroides* strains. In our study all samples were taken from red musts and wines of the Spanish region of Rioja. In a former study of these red wines, Saenz *et al.* (1995) had reported that *Lact. plantarum* and *Leuc. oenos* were the most frequent species found in these wines, *Lact. plantarum* reaching up to 46% of total isolates. This could be the reason why in our study only *Lact. plantarum* strains were found to be producers of inhibitory substances, and therefore, the best adapted to growing in these wines.

A wide range of indicators (60 LAB strains and 19 yeast strains) was used for screening procedures. Some authors refer the importance of the number and species of indicator strains selected for a good screening of bacteriocin production (Rammelsberg and Radler 1990). It has been postulated that almost all strains would be able to produce some type of bacteriocin as a defence mechanism and to compete against some of the other micro-organisms present in a mixed population (Lewus and Montville 1991). Grape juice in fermentation is a culture medium where yeast rapidly dominates because it is well adapted to growing in this sugar-containing media under anaerobic conditions. LAB are able to compete with yeast on some occasions, and under standard fermentation conditions, LAB start to proliferate when sugar content is insufficient for yeast growth. We have included a wide range of LAB of oenological origin and yeasts as indicators to detect growth inhibitory activities of oenological importance. Four out of the nine producer *Lact. plantarum* strains of our study showed inhibitory activity against indicator strains of the genera: *Lactobacillus, Pediococcus, Leuconostoc* and *Lactococcus.* A similar wide inhibitory spectrum against LAB was reported for a bacteriocin from *Lact. plantarum* B33 isolated from grapevine leaves (Radler 1991). Rammelsberg and Radler (1990) reported that among 79 strains of the genus *Lactobacillus* isolated from plants or fermenting material, 12 strains (15%) inhibited at least one of the nine indicator strains of the species *Lact. brevis, Ped. damnosus* and *Leuc. oenos*. None of our nine producer *Lact. plantarum* strains showed inhibitory activity against 19 yeasts of different species of oenological interest, which reflects the excellent adaptation of yeast to the wine fermentation medium. LAB inhibitory activity against yeast was reported for a few *Lact. plantarum* strains, and all of them were isolated from grapevine leaves and not from musts in fermentation (Lonvaud-Funel *et al.* 1988; Radler 1991). None of our nine producer *Lact. plantarum* strains showed inhibitory activity against *Leuc. oenos*, which confirms the previous result that strains of the species *Lact. plantarum* and *Leuc. oenos* are the most abundant and therefore the best adapted to growing in Rioja red wines (Saenz *et al.* 1995).

Production of hydrogen peroxide or lactic acid from glucose is often reported to inhibit LAB (Dahiya and Speck 1968). This possibility was ruled out in our experiments by using TSAYE agar medium and anaerobic conditions (Lewus and Montville 1991), either strictly anaerobic conditions or an atmosphere of 5% CO_2.

The antimicrobial activities of the four high producer *Lact. plantarum* strains J-51, J-53, J-65 and J-75 were further studied. *Lact. plantarum* J-51 antimicrobial activity was stable in the pH range of 1–9, being inactive at higher pHs, as most bacteriocins and bacteriocin-like substances produced by LAB, such as nisin, pediocins, sakacin A (De Vuyst and Vandamme 1994), brevicin B37, caseicin 80 (Rammelsberg and Radler 1990) and plantaricin D (Franz *et al.* 1998), which show activity in a wide range of pHs. *Lact. plantarum* J-53, J-65 and J-75 strains produced antimicrobial activities which were stable only in the pH range of 1–4, becoming inactive at higher pHs. A similar behaviour has been reported for plantaricin A (Daeschel, McKenney and McDonald 1990), plantaricins S and T (Jimenez-Diaz *et al.* 1993), plantaricin C (Gonzalez *et al.* 1994), plantaricin F (Fricourt *et al.* 1994) and other bacteriocins (De Vuyst and Vandamme 1994), which are active only in the acid range (pH < 7).

The antimicrobial substances produced by *Lact. plantarum* J-51, J-53, J-65 and J-75, remained active after treatment at 100 °C for at least 30 min. Thermostability is a general characteristic of plantaricins (Daeschel *et al.* 1990; Jimenez-Díaz *et al.* 1993; Fricourt *et al.* 1994; Franz *et al.* 1998) and some other bacteriocins, such as nisin (Rogers 1928), pediocin PA-1 (González and Kunka 1987), brevicin 37 and lactacin F (Muriana and Klaenhammer 1987; Rammelsberg and Radler 1990). Bacteriocin thermoresistance can be due either to a small and low complexity structure (even lacking a tertiary structure), or to a compact globular structure stabilized by covalent bonds, as suggested by De Vuyst and Vandamme (1994).

The widest inhibitory spectrum of *Lact. plantarum* J-51 (31 strains, out of the total 60 LAB indicator strains, were inhibited by J-51) indicated a strong and efficient antimicro-

bial activity. This activity was not affected by lysozymes but was completely lost after treatment with protease, trypsin, α-chymotrypsin, proteinase K and papaine. These experiments demonstrated that the inhibitory activity was due to a proteinaceous molecule, or several proteinaceous molecules, sensitive to proteases. A recent study by Niku-Paavola *et al.* (1999) reported a number of non-proteinaceous antimicrobial molecules secreted by *Lact. plantarum*, among which benzoic acid, mevalonolactone and lactic acid were included. In our case, the antimicrobial activity of *Lact. plantarum* J-51 was clearly due to one or several proteinaceous molecules. Daeschel, Green and Watson (1996) proposed the use of lysozyme as antimicrobial for controlling malolactic fermentation and bacterial spoilage in wine. The resistance of *Lact. plantarum* J-51 antimicrobial activity to lysozyme, may suggest a possible combination with this enzyme to control LAB growth in wines, and therefore their use to control malolactic fermentation in wineries.

Strasser and Manca de Nadra (1993) reported that pediocin N5p loosed its activity by filtration and by treatment with chloroform and ethanol, and they suggested a somewhat lipophilic nature for pediocin N5p. *Lact. plantarum* J-51 antimicrobial activity was lost after filtration through low adsorption hydrophilic membranes and was stable after treatment with chloroform and ethanol, which suggests the absence of lipophilic structures in the plantaricin molecule(s). The possible formation of aggregates could explain the loss of activity after filtration, as well as the small halos of growth inhibition observed in screening experiments (about 14 mm in diameter, data not shown).

Whereas most antibiotics (usually classified as secondary metabolites) are synthesized during the stationary growth phase, almost all bacteriocins produced by LAB were believed to display primary metabolite kinetics (De Vuyst and Vandamme 1994). Nevertheless, in recent years many studies have shown bacteriocin production during the stationary growth phase. Thus, plantaricin F (Poynter, Brown and Hayasaka 1997), pediocin N5p (Strasser and Manca de Nadra 1993) and pediocin PD-1 (Green *et al.* 1997) were synthesized during exponential growth phase and their production reached a maximum at the end of this phase. Plantaricin S and T, both produced by *Lact. plantarum* LPCO10 isolated from green olives (Jimenez-Díaz *et al.* 1993), were produced during exponential phase (plantaricin S) and stationary growth phase (plantaricin T). Similarly, in our study, antimicrobial activity of *Lact. plantarum* J-51 was detected at the end of the exponential phase and during early stationary growth phase (OD – 2·7).

The 450-bp amplicon obtained by PCR analysis of *Lact. plantarum* J-51 genomic DNA, using specific primers for *plnA* gene, demonstrated that *Lact. plantarum* J-51 possesses this gene. It is possible that this strain harbours all the other genes that constitute the full operon, or even the whole plantaricin locus that comprises several operons (Anderssen *et al.* 1998). Sequencing of the fragment revealed 95% identity with *Lact. plantarum* C11 *pln* locus (Diep, Havarstein and Nes 1996) over a 366-bp overlap, which is a high homology, as expected from strains of the same species. The deduced peptide sequence was identical in 47 residues over a 48-residue sequence (Gly7 mutated to Ser7). *PlnA* gene of *Lact. plantarum* C11 encodes a precursor of three active bacteriocins: 26-, 23- and 22-residue peptides (Anderssen *et al.* 1998). The mutation (Ser7) in our strain J-51 was located at the double glycine leader peptide, and therefore the putative active peptides of strain J-51 remain identical to those PlnA peptides of *Lact. plantarum* C11. All these peptides are characterized by their cationic character, their potential to form amphiphilic α-helices, and the double glycine leader peptide with hydrophobic character. More studies are being carried out in our lab to determine the full operon and whether other genes encoding for other plantaricin precursor peptides (PlnE, PlnF, PlnJ, PlnK, PlnN) are also present in *Lact. plantarum* strain J-51.

This study demonstrated production of bacteriocins by LAB of oenological origin, which indicates strong competition among LAB in a medium such as wine in fermentation. Those strains better adapted to competition against other bacteria will dominate and conduct fermentation. Further studies focused on LAB bacteriocin genes, their expression and bacteriocin influence on the ecology of wine, are in progress in our laboratory and will render more information about the complex process of vinification.

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