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High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol

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Abstract

A total of 76 *Lactobacillus plantarum* and *Oenococcus oeni* wild strains were recovered from traditionally elaborated Spanish red wines and were investigated with respect to their response to acid pH, lyophilisation, temperature and ethanol concentrations which are normally lethal to lactic acid bacteria. Both *L. plantarum* and *O. oeni* strains were able to grow at pH 3.2, were highly resistant to lyophilisation treatment and proliferated in the presence of up to 13% ethanol at 18°C. Therefore, it is shown that both species are highly tolerant to stress conditions and that similarly to *O. oeni* strains, *L. plantarum* strains are of interest in beverage biotechnology.

Keywords: *Lactobacillus plantarum*; *Oenococcus oeni*; Wine; Stress

1. Introduction

Lactic acid bacteria (LAB) are widely used in food biotechnology and efficient control of these microbiological processes requires an increase in our knowledge of bacterial behaviour under stress conditions. Various physical-chemical factors, such as ethanol, pH and temperature, are known to affect the growth of the LAB responsible for the malolactic fermentation (MLF) of wine [1]. Ethanol is generally regarded as one of the principal inhibitors of bacterial growth. Ethanol resistance varies with a number of conditions in the medium (i.e. pH and temperature) and *Oenococcus oeni* has long been reported as the LAB species most resistant to the presence of ethanol in wine (for a review, see [2]). *O. oeni* is used as a starter culture for MLF in wine and cider. The conversion of L-malic acid to L-lactic acid and CO_2 deacidifies wine, which leads to a significant influence on its quality and stability. MLF gives wines additional flavours and stability for ageing and is, therefore, sought for producing old red wines and some old white wines [1]. Many factors appear to affect MLF, which at the present cannot be fully controlled. This may lead to a number of processing problems, time consumption and risk of alteration of wine. When MLF is desired, the addition of bacteria is a general practice [3], but cells undergo rapid death due to the harsh environment (pH between 3.0 and 3.6, presence of ethanol and SO_2). The resistance to ethanol varies from strain to strain and it is generally accepted that all *O. oeni* strains grow in a medium containing 10% ethanol at pH 4.7 [4] and that small quantities of ethanol (3–4%) can stimulate their growth [3]. The optimum pH reported for the growth of *O. oeni* is between 4.3 and 4.8 and a pH of 3 or lower prevents almost all growth [4]. The optimum growth temperature in wine seems to be between 20 and 25°C [4] and at 15 °C or lower temperatures the possibility of bacterial growth in wine is slight [5]. When MLF is to be induced, it is important to know which organism is best suited, as the strain or strains must have the ability to grow under rather adverse conditions.

It has long been reported that exposure to stress conditions such as heat, ethanol or acid pH can provide protection against further hostile environmental conditions

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[6,7]. This adaptive response requires the expression of some defence mechanisms, so that bacteria become more tolerant to adverse conditions following exposure to mild stress conditions. Thus, acclimatisation to cold temperatures can render LAB cryotolerant [8–11] and accumulation of certain osmoprotectant organic compounds is a response to osmotic stress in certain LAB [12,13] and a method of survival of LAB subjected to drying [14]. *O. oeni* resistance mechanisms to wine stress conditions have been studied [15–20]. *Lactobacillus plantarum* responses to osmotic stress, which includes salt and non-electrolyte stress, as well as to cold shock [6] and oxidoreduction potential [21], have also been studied. This species is widely used in food biotechnology of fermented products of animal origin and its behaviour after freezing and drying has been the object of great interest [22–24]. Nevertheless, to date no study has been reported on the ability of *L. plantarum* strains to grow and proliferate under stress conditions of ethanol presence in the medium and acid pH.

The purpose of this study was to determine which *O. oeni* strains were best adapted to the adverse growth conditions of wine, and our results demonstrated that *L. plantarum* strains were as well adapted to those conditions of acid pH and ethanol presence, revealing a high tolerance to lyophilisation, to ethanol concentration up to 13% (v/v) in the medium, and to low pHs in the range 3.2–4. A comparison is established between the behaviour of *O. oeni* and *L. plantarum*. Results indicate that *L. plantarum* strains could constitute starter cultures for induction of MLF in wines, and that strains possess resistance mechanisms to survive and proliferate in hostile wine media.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 259 *L. plantarum* and *O. oeni* isolates were recovered from Spanish Rioja red wines produced during the period 1994–2000. Alcoholic fermentations were conducted in the presence of grape skins, seeds and stalks, after the addition of SO_2, and until the residual reducing sugar content was below 2.20 g l^{-1}. At this end point of alcoholic fermentation, wines (alcohol 12% by volume; pH $\geq$ 3.4) were drawn off from the yeast lees and allowed to undergo spontaneous MLF with the endogenous microflora (no starter inoculum was used). Samples were collected during this spontaneous MLF. Wine samples of 10 ml were spun at 100 $\times g$ for 3 min at 4°C (Sorwall RC-5 B refrigerated superspeed centrifuge). Pellets containing fermentation debris were discarded and supernatants were spun at 1000 $\times g$ for 10 min under the same conditions. Pellets were collected and after appropriate dilutions in sterile saline solution (0.9% NaCl) they were seeded onto MRS agar (Scharlau Chemie, Barcelona, Spain) plates with 200 μg of nystatin per ml (Acofarma, Terrassa, Spain). Samples were incubated at 30°C under strict anaerobic conditions (GasPak, Oxoid, Basingstoke, UK). These anaerobic conditions were used to prevent the growth of acetic acid bacteria and to accelerate LAB growth. Colony reisolation was carried out and a total of 259 isolates were used for this study. It should be pointed out that all the isolates were recovered from wines that after MLF followed the normal elaboration processes at their corresponding wineries, were bottled and underwent quality and sensorial analysis before commercialisation.

2.2. Bacterial species identification

Strain species were identified according to previously recommended methods, which included bacteria morphology, Gram staining, and carbohydrate fermentation patterns [25]. The API 50 CHL system (BioMérieux, La Balme, France) was also used. *O. oeni* and *L. plantarum* species were confirmed in all the studied isolates by the species-specific polymerase chain reaction (PCR) analyses described by Zapparoli et al. [26] and Quere et al. [27], respectively. *L. plantarum* strains were grown onto MRS agar (Scharlau) plates, at 30°C either under 98% humidity and 5% CO_2 atmosphere, or under strict anaerobic conditions (GasPak, Oxoid). MRS medium pH was 6.2. *O. oeni* strains were grown onto MLO (Scharlau) agar and under the same conditions. MLO medium pH was 4.7. Strains were stored in 20% w/v sterile skim milk (Difco, Madrid, Spain) at −20°C.

2.3. Clonal characterisation of strains by PFGE

Genomic DNAs were immobilised into agarose blocks, subjected to restriction enzyme digestion, and separated by pulsed field gel electrophoresis (PFGE) according to the following method. Bacterial cells from fresh cultures were recovered by centrifugation and immobilised in 1% agarose (pulsed field certified agarose, Bio-Rad, Hercules, CA, USA) in 0.5 $\times$ TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). Agarose blocks were incubated in bacterial lysis buffer [28] containing 2 mg ml^{-1} of lysozyme (Sigma-Aldrich, Madrid, Spain), and then incubated with proteinase K (1 mg ml^{-1}) (Sigma) in digestion buffer [28]. After these enzyme treatments, agarose blocks were cut (slices 1–2 mm) and digested with *Sfi*I restriction enzyme (Biolabs, Beverly, MA, USA) following the manufacturer's instructions. Gel blocks were loaded onto 1% (w/v) agarose D-5 (Pronadisa, Madrid, Spain) gels. DNA fragments were separated in 0.5 $\times$ TBE buffer in a CHEF DR II system (Bio-Rad Laboratories, Hercules, CA, USA). PFGE was performed at 14°C at a constant voltage of 4.5 V cm^{-1} with a switch time ramped from 5 to 45 s over a 24-h period. The CHEF DNA size standard lambda ladder (Bio-Rad) was used as the molecular size stan-

dard. The GelCompar 2.5 software (Applied Maths, Kortrijk, Belgium) was used for conversion, normalisation, and further processing of images. Comparison of the obtained PFGE patterns was performed with Pearson's product–moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA)

2.4. Lyophilisation

Twenty-five *L. plantarum* strains and 43 *O. oeni* strains were subjected to lyophilisation. Inocula with turbidity equivalents of 1 and 3 McFarland were prepared from fresh cultures of *L. plantarum* and *O. oeni* strains respectively (3×10^8 cfu ml^{-1} and 9×10^8 cfu ml^{-1} respectively). 25 μl of *L. plantarum* inoculum was added to 3 ml of MLO broth, and 100 μl of *O. oeni* inoculum was added to 6 ml of MLO broth. Cells were grown at 30°C with continuous shaking (Innova 4000 incubator shaker) and incubations were maintained until cells reached stationary phase. At this stationary phase colony enumeration was in the 10^8 cfu ml^{-1} range. Bacteria were collected by centrifugation at $2000\times g$ (Megafuge 1.0, Heraeus Instruments) for 15 min. Pellets were resuspended in 0.5 ml sterile skimmed milk and stored at −80°C. Lyophilisation was performed at −55°C for 16 h under vacuum (Freeze mobile 3.3 lyophiliser, Virtis Company).

2.5. Measurement of acid pH tolerance

Twenty *L. plantarum* strains and 22 *O. oeni* strains were included in the study of acid pH tolerance. HCl was added to MLO broth to final pHs of 4.7, 3.6, 3.3 and 3.2. Inocula with turbidity equivalents of 1 McFarland were prepared from fresh cultures of *L. plantarum* strains (3×10^8 cfu ml^{-1}), and with turbidity equivalents of 3 McFarland for *O. oeni* strains (9×10^8 cfu ml^{-1}). 100 μl and 200 μl of these inocula were added to 6 ml of growth medium in the case of *L. plantarum* and *O. oeni*, respectively. Cells were incubated at 30°C without shaking. Bacterial growth was followed by OD at 600 nm until a plateau was reached, this took an average of 3–4 days in the case of *L. plantarum*, and 4–5 days for *O. oeni*.

2.6. Measurement of ethanol tolerance

Twenty-five *L. plantarum* strains and 51 *O. oeni* strains were included in the study of ethanol tolerance, for which MLO broth supplemented with ethanol to final concentrations of 7%, 12% and 13% (v/v) was used. Inocula with turbidity equivalents of 1 McFarland were prepared from fresh cultures of *L. plantarum* strains (3×10^8 cfu ml^{-1}), and with turbidity equivalents of 3 McFarland for *O. oeni* strains (9×10^8 cfu ml^{-1}). 25 μl of *L. plantarum* inoculum was added to 3 ml of growth medium, and 200 μl of *O. oeni* inoculum was added to 6 ml of growth medium. Samples were grown at 18°C and 10°C without shaking. Control samples were incubated without ethanol at 30°C, 18°C and 10°C. Bacterial growth was followed by OD at 600 nm until a plateau was reached. This took an average of 6–9 days.

2.7. Growth parameters

OD at 600 nm was followed for determination of bacterial growth. Absorbance data were plotted versus time for each strain and for each tested condition. Absorbance slopes and absorbance values reached at the plateau at stationary phase were used respectively for growth rate and maximal bacterial population determinations. Relative maximal bacterial populations and growth rates were calculated as percentages relative to respectively maximal bacterial population and growth rate reached by each strain under defined standard conditions for each type of experiment. In lyophilisation experiments, growth parameters were calculated relative to the corresponding values before subjecting cells to the process of lyophilisation. In the case of acid pH tolerance, growth parameters were calculated relative to strain growth parameters in standard MLO broth pH 4.7. In the case of ethanol tolerance, growth parameters were calculated relative to the growth parameters reached by each strain in standard MLO broth without ethanol.

3. Results and discussion

3.1. Strain identification

From the total 259 initial LAB isolates of this study, 211 isolates were identified as *O. oeni* and 48 as *L. plantarum*, as described in Section 2. The species-specific PCR analyses described by Zapparoli et al. [26] and Quere et al. [27] have been shown to be of high reliability and specificity for species identification, and the specific *O. oeni* 1025-bp and *L. plantarum* 280-bp amplicons were generated from all the investigated isolates (results not shown). After strain identification by PFGE, as described in Section 2, the total number of *O. oeni* strains was 56, and that of *L. plantarum* was 35. Fig. 1A shows the PFGE patterns obtained after *Sfi*I digestion of total bacterial DNAs of 11 *L. plantarum* isolates belonging to nine unrelated and one closely related patterns. Fig. 1B shows the PFGE patterns of total bacterial DNA of 10 *O. oeni* strains with 10 unrelated patterns.

3.2. Lyophilisation

A total of 43 *O. oeni* strains and 25 *L. plantarum* strains were subjected to lyophilisation and successfully survived after the process. This process is regarded as a gentle drying procedure when compared with other methods, such as

spray drying, and in fact it is the preferred method for wine starter preparation. *L. plantarum* strains maintained the same growth rate and the same maximal bacterial population as before being subjected to lyophilisation, as shown in Table 1, which indicates that 90.0% (±9.0%) of maximal population values and 97.2% (±24.4%) of growth rates were recovered after the process in a total of 25 studied strains. These results are in agreement with those reported by Linders et al. [29,30] who observed that one *L. plantarum* strain was able to survive successfully (61% residual activity) after a process of drying, when a high initial cell concentration was used for drying and without addition of solutes, such as betaine or carnitine, which were supposed to be beneficial for LAB during drying [31]. In our experiments, bacterial concentrations were kept high before subjecting them to lyophilisation (around 6×10^9 cfu ml^{-1}) and this could account for the high rate of recovery obtained.

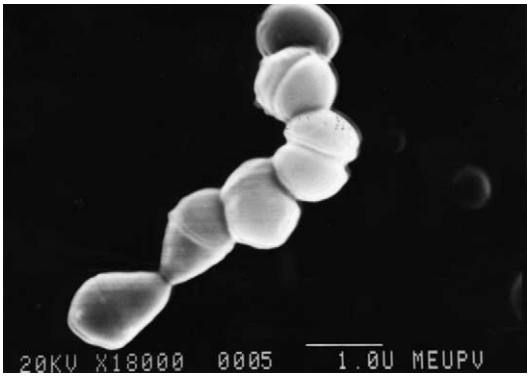

Fig. 2. Digitalised electron micrograph of *O. oeni* cells in growth phase after undergoing lyophilisation. Magnification: ×18000.

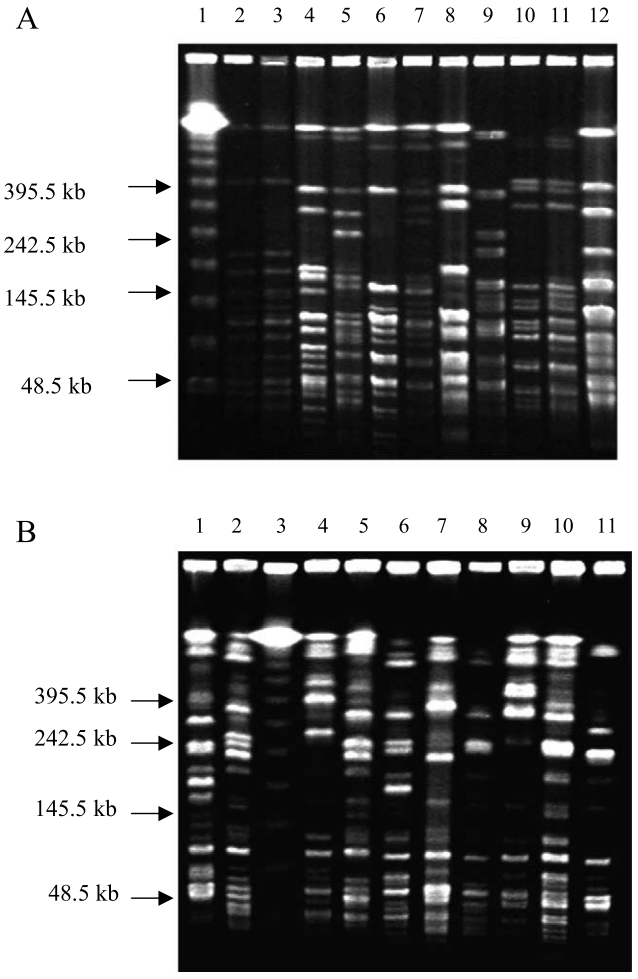

Fig. 1. A: PFGE patterns of *Sfi*I digests of genomic DNA from *L. plantarum* strains. Lanes: 1: Lambda DNA marker; 2: V-6; 3: V-8; 4: E-8; 5: E-14; 6: J-36; 7: J-36; 8: J-51; 9: J-66; 10: J-70; 11: J-34; 12: J-58. B: PFGE patterns of *Sfi*I digests of genomic DNA from *O. oeni* strains. Lanes: 1: IS 33; 2: IS 138; 3: Lambda DNA marker; 4: IS 141; 5: IS 142; 6: IS143; 7: IS144; 8: IS 146; 9: IS 147; 10: IS 148; 11: IS 149.

In the case of our *O. oeni* strains, they behaved similarly and maintained the same average growth rate and maximal population as before lyophilisation, 107.3% and 124.2% respectively in a total of 43 unrelated strains (Table 1). Nevertheless, in some cases *O. oeni* cells acquired an anomalous morphology reaching a bacterial width of 1 μm, as shown in Fig. 2, and they proliferated at a higher rate after lyophilisation. Forty-eight per cent of the studied *O. oeni* strains showed to some extent this larger morphology and the increased growth rate after lyophilisation. Probably this process, involving low temperatures (below −45°C) and high vacuum, would provoke cell death and most resistant bacteria, presumably those presenting larger morphology, survived successfully and rendered higher growth rates. To our knowledge, this is the first time that such an increase in cell growth rate and cell size is reported for LAB after lyophilisation. Maicas et al. [32] also reported high survival rates for *O. oeni* cells, up to 62.5%, after freeze-drying and 1 year of storage at 4°C.

Regarding lyophilisation, our results reveal that all the *O. oeni* and *L. plantarum* isolates from wines of this study did not decrease their growth rate after lyophilisation, which means a high degree of resistance to adverse conditions and, moreover, saves supplements of additional cryoprotectants to bacterial starters before lyophilisation.

3.3. Acid pH resistance

Twenty different *L. plantarum* strains were grown to study the effect of pH on bacterial growth. In these experiments bacterial populations at growth plateau were in the order of magnitude of 10^8–10^9 cfu ml^{-1}. Table 2 shows that when pH was decreased from 4.7, which is the standard pH of MLO broth, to 3.3, the average maximal population of *L. plantarum* strains only decreased to 80.8% and the average growth rate decreased to 56.9% with respect to optimal conditions at pH 4.7 in MLO broth. When pH was 3.2, the average maximal bacterial popula-

Table 1
Effect of lyophilisation on the growth of 68 *L. plantarum* and *O. oeni* wild strains

L. plantarum strain	Maximal population[a] after lyophilisation (%)	Growth rate[a] after lyophilisation (%)	*O. oeni* strain	Maximal population[a] after lyophilisation (%)	Growth rate[a] after lyophilisation (%)
J-21	99.9	105.5	IS 13	220.0	100.0
J-23	103.6	106.0	IS 17	76.4	78.7
J-30	106.5	109.1	IS 26	91.5	97.3
J-36	107.2	108.7	IS 31	68.6	71.4
J-39	109.7	52.6	IS 36	106.5	112.2
J-51	107.0	111.2	IS 42	149.9	63.2
J-53	107.3	51.1	IS 45	85.0	122.3
J-55	97.7	114.7	IS 47	68.9	73.8
J-58	102.5	105.4	IS 48	65.2	134.2
J-61	102.3	106.3	IS 53	295.0	148.6
J-62	98.2	99.7	IS 75	91.5	101.3
J-66	100.7	106.2	IS 94	131.4	73.7
J-67	93.3	44.0	IS 97	99.6	88.6
J-69	81.0	86.2	IS 122	112.0	152.5
J-70	101.0	105.1	IS 129	304.2	125.4
J-71	107.9	106.5	IS 135	81.3	189.5
J-73	103.8	118.6	IS 142	107.5	93.1
T-19	94.0	147.6	IS 143	181.8	175.6
T-20	89.1	118.1	IS 145	94.6	117.4
T-43	90.1	93.3	IS 151	110.7	121.8
T-53	88.6	101.0	IS 158	100.8	121.2
E-8	86.7	85.7	IS 159	125.4	50.7
E-14	114.7	48.5	IS 163	114.1	116.8
I-3	94.2	99.0	IS 164	85.6	161.1
V-8	87.7	99.5	IS 166	121.8	74.4
Mean ± S.D.	90.0 ± 9.0	97.2 ± 24.4	IS 167	49.4	64.9
			IS 174	166.3	101.2
			IS 183	174.8	91.6
			IS 186	152.8	78.6
			IS 189	151.3	137.0
			IS 202	173.5	75.7
			IS 209	207.1	90.9
			IS 210	195.4	274.0
			J-101	95.7	60.6
			J-102	84.8	109.5
			J-110	125.1	168.5
			J-113	81.4	107.8
			T-14	62.8	62.0
			T-27	135.1	84.7
			T-31	133.8	124.7
			T-38	102.2	75.4
			T-40	86.4	79.5
			T-56	72.0	64.4
			Mean ± S.D.	124.2 ± 57	107.3 ± 43

[a]Maximal populations and growth rates were calculated for each strain as percentages relative to the corresponding values before lyophilisation. Bacterial growth was followed by OD at 600 nm as described in Section 2. Absorbance values reached at the plateau at stationary phase were used for maximal bacterial population determinations.

tion dropped to 67.0% and the average growth rate decreased to 37.9% of the corresponding values under optimal conditions. These results all together showed that in the pH range from 3.2 to 3.6, which is the normal pH range for Rioja red wines, our *L. plantarum* strains proliferated successfully. This resistance may be a variable feature within the species and may be dependent on the strain and its source. Nevertheless, our whole collection of strains isolated from wines showed this resistance.

The 22 *O. oeni* strains, studied under the same pH conditions as for *L. plantarum* strains, behaved in a similar fashion, in that the average bacterial population decreased to 81.8% of the maximal value under standard conditions, and the average growth rate decreased to 60.0% when pH was lowered from 4.7 to 3.3, and average decreases to 72.3% and 53.6% in bacterial population and growth rates respectively were obtained at pH 3.2 (Table 2). Bacterial populations at stationary phase in the case of *O. oeni* strains were in the order of magnitude of 10^7–10^8 cfu ml^{-1}. These results indicate that our strains were able to

Table 2
Effect of acid pH on the growth of 44 wild *L. plantarum* and *O. oeni* strains

L. plantarum strain	Relative maximal population[a] at pH			Relative growth rate[a] at pH		
	3.6	3.3	3.2	3.6	3.3	3.2
J-26	79.7	78.2	50.9	90.1	98.2	26.5
J-34	76.2	79.0	64.9	40.6	46.4	64.0
J-39	61.6	62.4	54.4	62.7	46.7	37.3
J-40	74.3	76.5	9.5	27.8	23.4	8.3
J-51	81.7	74.0	63.3	58.1	44.7	31.7
J-61	88.1	89.8	81.3	72.4	71.9	52.6
J-62	70.5	72.0	76.5	56.6	44.6	35.7
J-66	87.4	85.9	74.1	49.5	49.9	54.4
J-67	80.6	74.0	63.3	41.6	66.7	58.7
J-69	86.5	83.3	75.5	68.3	68.8	43.6
J-70	70.2	73.8	67.6	55.3	47.3	35.2
J-71	86.1	89.7	76.4	58.3	59.9	42.9
J-72	70.0	75.3	28.7	65.8	71.5	14.1
J-73	89.7	92.2	83.2	65.0	67.7	44.1
T-19	80.8	81.6	79.3	61.6	44.2	37.3
T-20	94.7	90.3	93.5	68.2	68.8	41.8
T-43	77.0	79.8	65.1	40.0	47.7	22.9
T-53	89.1	84.0	80.1	58.9	58.6	34.4
E-8	87.0	88.8	76.5	53.6	49.4	32.8
E-14	84.2	84.8	75.9	50.7	62.0	39.1
Mean ± S.D.	80.8 ± 8.4	80.8 ± 7.7	67.0 ± 19.5	57.3 ± 13.7	56.9 ± 15.8	37.9 ± 13.8
O. oeni strain						
IS 18	100.2	94.9	102.9	93.2	82.3	67.1
IS 40	79.8	81.8	68.2	31.4	34.0	23.9
IS 59	156.4	79.5	59.4	173.0	39.4	62.5
IS 95	56.0	58.5	52.7	49.6	44.0	38.0
IS 111	68.4	60.1	66.2	43.9	40.9	31.0
IS 127	77.8	98.1	78.0	50.4	76.9	47.1
IS 155	55.5	58.3	54.3	50.6	58.7	59.9
IS 159	65.9	58.5	57.8	43.9	31.3	31.3
IS 167	98.3	107.7	84.1	115.8	108.3	47.4
IS 177	80.7	96.3	95.7	57.6	59.2	55.6
IS 180	72.3	78.3	70.1	62.1	60.1	60.3
IS 189	63.4	75.1	65.8	53.8	47.4	56.2
IS 209	33.5	32.9	39.0	29.6	23.6	27.1
IS 210	84.	95.6	93.6	72.1	105.7	97.1
J-101	52.1	131.1	63.7	43.1	46.1	60.0
J-102	79.6	86.0	66.8	66.4	74.5	48.9
J-113	91.6	87.9	79.8	58.2	55.2	56.6
J-116	97.6	87.0	89.8	79.8	63.5	76.0
T-14	78.2	78.8	87.6	64.0	57.6	100.3
T-27	109.9	76.2	68.5	58.9	60.9	58.5
T-38	86.1	133.3	111.0	48.4	111.5	53.8
T-56	45.7	43.3	36.1	36.5	37.7	19.6
Mean ± S.D.	78.8 ± 25.7	81.8 ± 24.7	72.3 ± 19.4	62.8 ± 31.6	60.0 ± 24.7	53.6 ± 20.8

[a]Relative maximal bacterial populations and growth rates were calculated as percentages relative to the corresponding parameters for each strain growing in standard MLO broth (pH 4.7). Bacterial growth was followed by OD at 600 nm, as described in Section 2. Absorbance values at stationary phase and absorbance slopes were used for maximal bacterial population and growth rate determinations respectively.

grow in the pH range 4–3.2 with high growth rates (relative values above 50%), and reveal a high adaptation of bacterial cells to wine acid pH. *O. oeni* resistance to wine low pH has been widely reported [4], in addition, our results show that also our collection of *L. plantarum* strains were able to growth successfully at wine pH 3.2, and that bacterial populations reached similar values as in the case of *O. oeni* strains, which traditionally had been considered as the most tolerant to acid pH among wine LAB species [1,3].

3.4. Resistance to ethanol

All the studied strains (25 *L. plantarum* strains and 51 *O. oeni* strains) showed maximal growth rates and maximal populations when grown in a medium free of ethanol

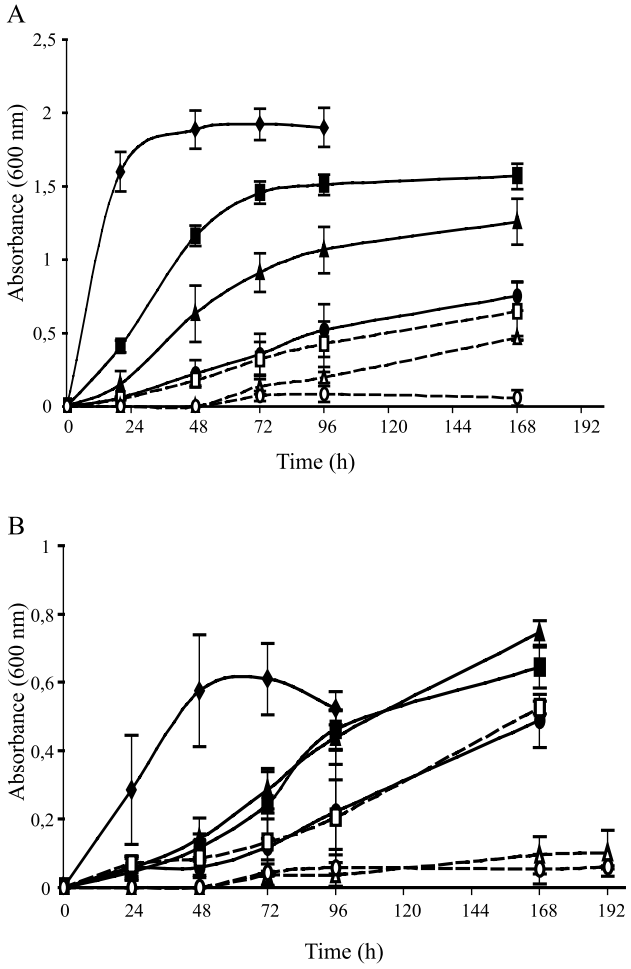

Fig. 3. Effect of ethanol and temperature on (A) *L. plantarum* growth and (B) *O. oeni* growth. Cells were incubated in MLO broth in the following conditions: ♦ 30°C without ethanol; ■ 18°C without ethanol; ▲ 18°C and 7% ethanol; ● 18°C and 12% ethanol; □ 18°C and 13% ethanol; △ 10°C without ethanol; ○ 10°C and 12% ethanol. Sampling point is the average of three independent experiments corresponding to the growth of three different strains representative of the behaviour of all the studied *L. plantarum* and *O. oeni* strains.

(MLO broth) and at 30°C, as expected. The combined effect of the presence of 12% ethanol in the medium and low incubation temperature (10°C) prevented bacterial growth of all strains of both species *L. plantarum* and *O. oeni* of this study, as shown in Fig. 3A,B, which represent growth curves of *L. plantarum* and *O. oeni* strains respectively. Sampling points were the average of three independent experiments corresponding to the growth of three different strains representative of the behaviour of all the studied *L. plantarum* and *O. oeni* strains. Similar results were obtained by Britz and Tracey [4] with a collection of 54 *O. oeni* strains. They showed that the combination of 15°C and 13% ethanol reduced to 15% the number of *O. oeni* strains able to grow under these conditions.

When we grew *L. plantarum* strains at 18°C in the absence of ethanol, their growth rate decreased 70%, a short lag phase reaching up to 24 h appeared in some cases, and bacterial population decreased 18% when compared with optimal conditions at 30°C (Fig. 3A). The presence of 12% ethanol in the medium affected *L. plantarum* growth rate, which decreased when compared with control samples incubated at 18°C without ethanol (Fig. 3A). Table 3 shows the results of relative maximal populations of the 25 *L. plantarum* strains that were studied. Bacterial population decreased in presence of 7%, 12% and 13% ethanol, to respectively 78.6%, 45.6% and 39.5% of the maximal population without ethanol at 18°C incubation temperature. Nevertheless, bacterial populations reached high values, around 10^8 cfu ml^{-1}, under conditions of 13% ethanol and 18°C, which are values in the same range as those reached by *O. oeni* strains under the same growth conditions. Some *L. plantarum* strains have been described as able to grow in wine [1] and, therefore, they should develop some mechanisms of ethanol resistance, such as changes in membrane lipid composition. Nevertheless, this is the first time, to our knowledge, that similar bacterial populations are reported for *L. plantarum* and *O. oeni* strains grown in 13% ethanol and 18°C.

When *O. oeni* strains were incubated at 18°C, growth rates decreased 38% when compared with growth rates under optimal conditions of 30°C and absence of ethanol (Fig. 3B), and a lag phase of 48–72 h appeared. After lag phase and cell proliferation, bacterial populations at 18°C reached values very similar to those obtained under optimal conditions (data not shown). Table 3 shows the relative maximal populations of the 51 *O. oeni* strains that were studied in these experiments and reveals that *O. oeni* strain growth was activated when 7% ethanol was present in the medium, giving higher bacterial populations than in control experiments at 18°C without ethanol. Small percentages of ethanol had been previously reported to activate bacterial growth, and thus 3–4% [3] or 5–6% [1] had been reported to activate *O. oeni* growth. In our *O. oeni* strains, 7% ethanol gave higher bacterial populations than in the absence of ethanol in the medium. An increase in ethanol content up to 12–13% (which are normal alcohol content values in the original red wines from which strains were isolated) produced no major decrease in bacterial populations with respect to populations reached at 18°C without ethanol, which revealed the high degree of adaptation of our *O. oeni* strains to growth in wine and, therefore, to relatively high amounts of ethanol in the medium. Bacterial populations under these conditions reached values around 10^8 cfu ml^{-1}, which was in the same range as the average bacterial population of *L. plantarum* strains grown under the same conditions.

We conclude that our collection of *L. plantarum* strains can tolerate the combination of acid pH and ethanol concentration in the medium, and proliferate under conditions that are normally lethal to LAB. They survive and proliferate at pH 3.2, they grow in the presence of 13% ethanol at 18°C reaching bacterial populations of 10^8 cfu ml^{-1}, in the same range as *O. oeni* populations adapted to growing

Table 3
Effect of ethanol on the growth of 76 *L. plantarum* and *O. oeni* wild strains: relative maximal bacterial populations[a]

L. plantarum strain	7% ethanol	12% ethanol	13% ethanol	*O. oeni* strain	7% ethanol	12% ethanol	13% ethanol	*O. oeni* strain	7% ethanol	12% ethanol	13% ethanol
J-21	61.9	40.8	43.5	J-101	63.6	150.2	111.9	IS 53	116.0	130.4	58.0
J-23	73.2	44.0	53.7	J-102	353.9	262.3	172.5	IS 63	132.1	54.3	23.6
J-30	72.3	43.6	36.0	J-110	103.9	92.5	84.9	IS 75	81.9	55.5	57.1
J-36	86.0	37.8	38.8	J-113	100.6	80.7	76.1	IS 94	98.1	68.2	68.4
J-39	80.8	47.6	29.3	T-3	139.5	85.6	67.2	IS 97	97.2	107.6	15.8
J-51	68.8	38.8	38.2	T-14	247.4	134.4	121.5	IS 122	122.5	38.9	40.1
J-53	74.3	38.8	42.4	T-27	37.4	35.0	17.6	IS 135	162.9	77.0	30.7
J-55	78.9	51.4	51.8	T-31	113.9	66.3	45.3	IS 142	215.4	226.7	53.5
J-58	79.2	51.5	46.5	T-40	102.0	58.7	55.5	IS 143	157.2	137.9	211.8
J-61	82.1	45.8	36.6	T-56	46.6	19.9	16.8	IS 144	85.9	24.9	76.7
J-62	89.1	38.7	35.7	IS 11	88.2	97.8	97.8	IS 145	108.5	75.7	37.9
J-66	74.6	43.4	37.4	IS 13	129.0	68.2	–	IS 147	93.3	77.1	36.0
J-67	81.2	40.9	38.0	IS 17	125.2	105.1	–	IS 151	122.4	109.7	26.7
J-69	84.0	43.7	36.4	IS 24	178.3	47.9	–	IS 159	126.1	88.0	20.6
J-70	82.8	34.6	25.2	IS 26	103.1	73.1	77.0	IS 161	97.2	74.5	114.4
J-71	83.8	45.7	26.7	IS 31	72.4	67.4	23.4	IS 163	100.8	72.0	79.4
J-72	72.5	51.2	47.5	IS 33	221.1	173.4	–	IS 164	98.0	53.6	81.0
J-73	98.2	33.6	23.3	IS 36	88.2	78.4	–	IS 166	64.0	88.2	48.7
T-19	86.1	60.7	50.9	IS 41	84.8	42.8	21.3	IS 167	77.2	82.2	84.8
T-20	86.0	52.0	40.3	IS 42	152.7	91.6	97.4	IS 174	125.3	69.3	73.3
T-43	85.0	64.1	50.1	IS 43	86.7	39.2	34.4	IS 183	112.1	83.1	87.1
T-53	74.0	45.8	41.9	IS 44	115.7	82.4	63.9	IS 189	115.5	66.3	58.1
E-8	81.9	66.3	50.9	IS 45	88.5	26.2	25.8	IS 202	93.8	45.5	46.6
E-14	62.6	40.5	43.6	IS 47	80.6	75.0	62.9	IS 204	56.7	83.4	100.4
V-8	66.3	37.4	22.5	IS 48	145.6	122.3	34.4	IS 209	83.0	58.0	74.8
								IS 210	64.7	79.7	92.0
Mean ± S.D.	78.6 ± 8.6	45.6 ± 8.6	39.5 ± 9.1					Mean ± S.D.	115.2 ± 53.5	84.4 ± 45.5	65.3 ± 39.8

[a]Relative maximal bacterial populations were calculated as percentages relative to the corresponding value for each strain growing in MLO broth without ethanol. Strains were grown at 18°C in MLO broth in the presence on different concentrations of ethanol, as described in Section 2. Bacterial growth was followed by OD at 600 nm. Absorbance values reached at the plateau at stationary phase were used for maximal bacterial population determinations.

in wine. And, therefore, it is concluded that *L. plantarum* strains could constitute starters for inducing MLF and are of interest in wine production.

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