A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation

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ABSTRACT

A novel starter culture consisting of two *Lactobacillus pentosus* strains was successfully used for Spanish-style green olive fermentations in an industrial-scale study. Both strains were previously isolated from olive fermentations at two different olive-processing industries in Southern Spain and were characterized attending to their carbohydrate utilization profiles, plasmid content, vitamin and amino acid utilization, and bacteriocin and lactic acid production, among other characteristics. The starter culture, consisting of strains *L. pentosus* LP RJL2 plus *L. pentosus* LP RJL3, was inoculated into 10,000-Kg glass fibre fermentors at 10^6 CFU/ml and 10^5 CFU/ml final concentrations, respectively, in five different olive-processing industries in Southern Spain. As controls, commercial starter-inoculated or traditional uninoculated fermentations under the same conditions were used. In all five experiments, the two strains of the paired starter rapidly colonized the brines to dominate the epiphytic micro flora and persisted throughout the fermentations. Decline in pH to reach values ca. 5.0 was achieved in the first 15-20 days, reaching values ca. 4.0 at the end of the process. Similarly, the lactic acid concentration in brines increased rapidly in the first 20 days of fermentation (0.3-0.4 g/100 ml) to give values higher than 0.8 at the end of the fermentation. In contrast, in uninoculated or commercial starter-inoculated brines increase in lactic acid concentration was slower and the final concentrations reached were also lower than in the *L. pentosus*-paired starter inoculated brines. Also, the decline in pH in uninoculated or commercial starter-inoculated brines was slower although reaching similar values at the end of the process. These results clearly show the performance and the usefulness of the new starter culture to control the olive fermentations.

1. Introduction

Among lactic acid bacteria (LAB), different species of the genus *Lactobacillus* have been established as important micro organisms in the production of fermented vegetables (Daeschel et al., 1987; Fleming et al., 1985; Buckenhüskes, 1993). Among these, only table olives, sauerkraut and cucumbers are of real economical significance, being the production of table olives the most important one (Anonymous, 2008). Thus, the world production of table olives was estimated in about 1.8 million tons in 2007-2008 from which Spain, the main world producer, contributed with about 31% of the total (Anonymous, 2008). From that amount of olives, nearly 800,000 tons were processed as Spanish-style green olives.

In the spontaneous, traditional Spanish-style green olive fermentation, both the fruits and the environmental conditions are handled in order to favour the development of a population of LAB in the fermentation brines which are responsible for the subsequent lactic acid fermentation. This LAB population is present as a contaminant at very low initial numbers, being composed mainly by strains of *L. plantarum* (Garrido Fernández et al., 1995), although more recent studies carried out in our laboratory show that most of these strains can be now classified as *L. pentosus* according to the molecular criteria described by Torriani et al. (2001). Thus, the olives are treated with a NaOH solution in order to hydrolyze the phenolic compounds which are bactericidal for *L. plantarum/pentosus* (Ruiz-Barba et al., 1990; Ruiz-Barba, 1991; Ruiz-Barba et al., 1993), washed to eliminate the excess of NaOH, and finally placed into 10,000-15,000 kg fibre glass containers and covered with brine. In these conditions, typically *L. plantarum/pentosus* become dominant over Gram-negative bacteria and other LAB within 2-3 weeks after brining the olives, and coexists until the end of the fermentation process (up to 3 months) with a yeast population (Fernández Díez et al., 1983; Ruiz-Barba, 1991; Ruiz-Barba et al., 1994; Garrido Fernández et al., 1995; Leal-Sánchez et al., 2003). By fermenting the sugars contained in the fruits, *L. plantarum/pentosus* contributes not only to the appropriated organoleptic characteristics of the final product but also to the preservation of the fermented olives because of production of large amounts of lactic acid. However, as it happens in much

other spontaneous vegetable fermentation, production of Spanish-style green olives relies upon micro organisms present in the natural micro flora of the fruits or in the processing plant in which the olives are fermented. This practice often leads to an abnormal sequence of micro organisms being the competing micro flora present in the raw material the predominating population over *L. plantarum/pentosus*. In these cases, lactic acid is not produced in the adequate amounts needed for olive preservation, thus leading to wide variations in the flavour and quality of the olives and often to spoilage of the fruits. To avoid these problems and to improve the quality of the fermented olives, technological control procedures such as the systematic use of suitable *L. plantarum/pentosus* starter cultures are necessary.

In spite of some available commercial inocula, the use of starter cultures is not a habitual practice in the olive processing industry (Daeschel et al., 1987; Fleming et al., 1985; Buckenhüskes, 1993). A reason for this is the lack of success in the microbiological control of the process because the strains used have not been optimized for this particular fermentation. However, it has been shown that with a very detailed selection of wild-type *L. plantarum/pentosus* strains carrying technologically relevant characteristics it is possible to obtain more effective starter cultures. Thus, the plantaricin S (PLS)-producing *L. plantarum* LPCO10 strain (now classified as *L. pentosus*) has extensively shown its ability to contribute to the preservation of the olives from spoilage when it was used as a starter culture in fermentors located in our pilot plant (Ruiz-Barba et al., 1994; Leal-Sánchez et al., 2003). Bacteriocin production by the LPCO10 strain proved to be essential to control the lactic acid fermentation of Spanish-style green olives (Ruiz-Barba et al., 1994). However, when it was inoculated in fermentors in olive processing plants placed in Southern Spain the results obtained were no as satisfactory as in the pilot plant experiments (unpublished results). This lesser effectiveness at the industrial level was probably due to an incomplete control of the natural micro flora which is often contaminating in excess the raw material. This indicated that, in order to be successfully used at the industrial level, a starter culture has to show other relevant characteristics apart from its ability to produce bacteriocin.

In this paper we report the use at the industrial level of a novel starter culture consisting

of two *L. pentosus* strains, i.e. the LP RJL2 strain, a PLS producer able to grow in the absence of two of the essential B-group vitamins, pyridoxal and PABA, and the LP RJL3 strain, characterized by a fast and predominant growth in the fermentation brines and by producing high amounts of exopolysaccharides (EPSs), among other relevant technological characteristics (Sánchez et al., 2006; Rodríguez-Carvajal et al., 2008).

2. Materials and methods

2.1 Bacteria and culture medium

The two *L. pentosus* strains used as inoculants in the present study belong to our culture collection. They were isolated in MRS medium (De Man et al, 1960; Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) originally from different Spanish-style green olive fermentations in two different factories in Southern Spain (Ruiz-Barba, 1991 and Ruiz-Barba et al. 1991). They were named LP RJL2 and LP RJL3, respectively, and they are registered in the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT) as strains *L. plantarum* CECT 5358 (LP RJL2) and *L. plantarum* CECT 5359 (LP RJL3). More recently, they were classified as *L. pentosus* according to the molecular criteria described by Torriani et al. (2001) (Figure 1b). Both strains were grown at 30 ºC in MRS medium as static cultures and they were maintained as frozen stocks at -20 ºC in distilled water plus glycerol (20%, v/v). Spontaneous streptomycin-resistant (Str^r, 500 µg/ml) or rifampin-resistant (Rif^r, 10 µg/ml) derivatives of strains LP RJL2 and LP RJL3, respectively, were isolated by sequential selection on MRS agar containing increasing concentrations of each antibiotic (Sigma Chemical Co., St. Louis, Mo.). *L. pentosus* 128/2, used as indicator strain in the bacteriocin assays, was grown in MRS medium at 30 ºC.

2.2 Strain characterization

The strains were phenotipically characterized according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). Test comprises Gram-staining, determination of the morphology by contrast-phase microscopy, catalase and oxidase activities, reduction of NO_3 to NO_2, gas production from glucose and gluconate, carbohydrate fermentation profile, and

enzymatic activity.

To determine their nutritional requirements in amino acids and vitamins, the strains were grown in the defined medium described by Morishita et al. (1981) in which each of the essential amino acid (L-soleucine, L-Glutamic acid, L-Leucine, L-Methionine, L-Phenylalanine, L-Tryptophan or L-Valine) or B-group vitamin (p-Amino Benzoic acid (PABA), D-Biotin, Nicotinic and Panthotenic acids or Piridoxal) was omitted in turn.

Tests for rapid acid production were conducted as described previously (de la Borbolla y Alcalá et al., 1958; Kostinek et al., 2007). Briefly, overnight cultures of each strain were inoculated (1%, v/v) into MRS broth at pH 6.2 and incubated at 30 ºC for 6, 24 and 96 h. At those times, pH and lactic acid production of the cultures were determined as previously described (Leal-Sánchez et al., 2003).

Genotypic characterization of the strains was achieved through PCR studies. Genomic DNA was extracted according to Ruiz-Barba et al. (2005). Multiplex PCR amplifications were done as described by Torriani et al. (2001) using the species-specific *recA*-based primers paraF (5'-GTCACAGGCATTACGAAAAC-3'), pentF (5'-CAGTGGCGCGGTTGATATC-3'), planF (5'-CCGTTTATGCGGAACACCTA-3'), and pREV (5'-TCGGGATTACCAAACATCAC-3'). PCR amplification of known *plS* cluster was carried out according to Maldonado et al. (2002), with specific primers BRI1 (5'-TTCTCATGCAAGGAGTGCCCATGC-3'), ARI2 (5'-TTCTCATGCAAGGAGTGCCCATGC-3'), 2B (5'-GTCATTATGATGTTGACAGCG), and 3A (5'-GCTTAGATTTCACAGCTTCGA-3'). For Randomly Amplified Polymorphic DNA (RAPD), the method of Rodas et al. (2005) was followed using the primer OPL5 (5'-ACGCAGGCAC-3´).

For plasmid extraction, the protocol of Anderson and McKay (1983) for isolating large plasmid DNA was followed.

To determine bacteriocin production, the method described by Jiménez-Díaz et al. (1993) was followed. Antimicrobial activity was assayed by the agar drop diffusion test using *L. pentosus* 128/2 as the indicator strain.

2.3 Brining procedure of the olives

The traditional Spanish-style green olive brining procedure was followed (Garrido Fernández et al., 1995). Fifteen industrial glass fiber-fermentors, located at five different olive processing industries in Southern Spain, were used. Each fermentor containing 10,000 kg of whole Hojiblanca or Manzanillo green olives was treated with a diluted NaOH solution (2.1%) for 6 h, followed by two washes with water to remove the excess of alkali, and then brined in about 5,000 l of 11% (w/v) NaCl solution (Garrido Fernández et al., 1997).

2.4 Preparation of bacterial inocula for olive fermentation

L. pentosus LP RJL2 and LP RJL3 were subcultured overnight at 30 ºC in 50 ml MRS broth containing 500 µg/ml streptomycin (MRS-Str) or 10 µg/ml rifampin (MRS-Rif), respectively. The next day, 2 l of fresh MRS were inoculated with the respective strain and then incubated at 30ºC for 16-18 h. These cultures at the early stationary phase of growth (ca. 5×10^9 CFU/ml) were used as inocula. Both strains were inoculated at a time in each fermentor at final concentrations of 10^6 CFU/ml and 10^5 CFU/ml of LP RJL2 and LP RJL3, respectively.

At each factory, two fermentors were inoculated with the double-inoculum starter culture and a third one, used as a control, was inoculated with the commercial starter Vegestart60 (Chr. Hansen A/S, Horsholm, Denmark) as recommended by the manufacturer. One fermentor remained uninoculated thus following the traditional process and serving as a control for spontaneous fermentation. As a common practice in the traditional method of elaboration, the pH of the brines was lowered by adding HCl until a value of about 6.0 was reached before adding the commercial starter culture Vegestar60 or in the uninoculated fermentors (Fernández Díez et al., 1983; Garrido Fernández et al., 1995; Leal-Sánchez et al., 2003). However, in those fermentors where the mixed-culture starter was applied the pH was not lowered before addition of the inoculum.

As most factories place the fermentors outside, all fermentations were carried out at the outer temperature.

2.5 Microbial counts

At specific time intervals during the fermentation, which was continued for 120 days, samples (100 ml) were taken from each fermentor. The number of CFU per ml of the different microbial populations was determined by spreading appropriate serial dilutions on solid culture media with a Spiral Plater (model Wasp 2, Don Whitley Sci. Ltd., Shirpley, U.K.). Yeasts were enumerated at 25 ºC in oxytetracycline-glucose-yeast extract (OGYE) agar (Oxoid) and lactic acid bacteria at 30 ºC on MRS agar (Oxoid) and MRS agar plus 0.02% (w/v) sodium azide (Sigma). *L. pentosus* LP RJL2 and LP RJL3 were differentially enumerated at 30 ºC on MRS agar containing 500 µg/ml streptomycin or 10 µg/ml rifampin, respectively. All samples were analysed in triplicate and for each dilution three plates were spread.

For a molecular identification of strains LP RJL2 and LP RJL3, ten colonies growing in MRS-Str or MRS-Rif at each sampling time were selected. PCR using PLS-*locus* specific primers and RAPD were used to identify *L. pentosus* LP RJL2, and *L. pentosus* LP RJL3 was identified by RAPD.

2.6 Analysis of olive brines

At the same sampling times as for the microbiological analysis, a 20-ml brine sample was obtained from each fermentation and then the pH, titratable acidity (expressed as percentage of lactic acid), and NaCl concentration were determined as described previously (Fernández Díez et al., 1985).

2.7 Statistical analysis

The data were statistically analyzed using the software package Statistica (data analysis software system) version 7.0 (StatSoft,Inc., Tulsa, Oklahoma, USA) by applying a one-way analysis of variance (ANOVA). Means of the values were considered significantly different when $P < 0.05$.

3. Results

3.1 *Characterization of L. pentosus LP RJL2 and LP RJL3 strains*

By using the species-specific *recA*-based PCR method to differentiate *L. plantarum*, *L. pentosus*, and *L. paraplantarum* described by Torriani et al. (2001), the two lactobacilli strains used in this work were typed as belonging to the species *L. pentosus*, and named LP RJL2 and LP RJL3, respectively (Figure 1B). However, they show many differences in their biochemical and physiological characteristics, their plasmid and RAPD profiles, their vitamin requirements and other properties that clearly distinguish each strain from the other, as well as both of them from other *L. pentosus* strains.

The biochemical and physiological characteristics of *L. pentosus* strains LP RJL2 and LP RJL3 are shown in Table 1. Although the carbohydrate utilization profile is very similar in both strains, they show some differences in the fermentation of certain sugars such as L-arabinose, D-arabinose, melizitose, β-gentibiose, arabitol, rhamnose or gluconate. Note that among other interesting physiological characteristics, *L. pentosus* LP RJL2 and LP RJL3 strains were able to grow in tauroglycolate but they did not hydrolyzed gelatinase, two properties exhibited by probiotic micro organisms (Lebeer et al., 2008).

Both the LP RJL2 and LP RJL3 strains were considered as fast acid producers as they were capable of lowering the pH in MRS broth to below 5.3 and 3.9 after 6 and 24 h, respectively. Acid productions (as % of lactic acid) at these time intervals were 0.36 and 1.35, respectively, for strain LP RJL2, and 0.54 and 1.44, respectively, for strain LP RJL3. The pH and acidity values for both strains after 96 h incubation in MRS were of 3.8 and 1.89, respectively.

As described previously for most of the *Lactobacillus* strains, *L. pentosus* LP RJL2 and LP RJL3 strains failed to grow in the absence of any of the amino acids tested (data not shown). However, differences in the patterns of vitamin requirement were observed. Thus, whereas *L.*

pentosus LP RJL2 was able to grow in the absence of PABA and piridoxal, *L. pentosus* LP RJL3 was not able to grow in the absence of any of the vitamins tested (data not shown).

RAPD and plasmid profiles, and the presence or absence of the *pIS locus* by PCR allowed a rapid identification and differentiation of both strains. In Figure 1A, the characteristic RAPD profile of both strains is shown, as well as the *pIS*α and *pIS*β structural genes for PLS production in *L. pentosus* LP RJL2. On the other hand, the number and molecular weight of plasmids harboured by both strains are quite different. Whereas the strain LP RJL2 exhibited nine plasmids ranging from 49 to 2.4 kb, *L. pentosus* LP RJL3 showed seven plasmids in the range of 45 to 3.5 kb (Figure 1C).

Other characteristics that clearly distinguish both strains were the ability of *L. pentosus* LP RJL2 to produce PLS whereas *L. pentosus* LP RJL3 does not (it does not carry the *pIS locus*). However, *L. pentosus* LP RJL3 produces EPSs in large amounts when it grows in MRS, showing characteristic ropy colonies on solid medium which greatly facilitates its identification.

3.2 *Monitoring of mixed L. pentosus LP RJL2 and LP RJL3 starter cultures throughout the olive fermentations*

Growth of micro organisms in the fermentation brines is shown in Figure 2. In all ten fermentors that were inoculated with the *L. pentosus* LP RJL2 and LP RJL3 mixed starter culture in the five different factories the progress of the micro organism populations was very similar, both becoming dominant and persisting throughout the process (up to 120 days) (Figure 2A and 2B). In all cases (Figure 2C), a yeast population rapidly colonized the brines and persisted throughout the fermentations, as it has been previously described (Fernández Díez et al., 1983; Ruiz-Barba et al., 1994; Garrido Fernández et al., 1995). In those fermentors which were inoculated with the mixed culture the epiphytic micro flora of LAB rapidly decreased in number and disappeared from the fermentation brines after a few days (15-20 days after brining of the olives) (Figure 2D).

It was noticeable that although the strain LP RJL3 was always inoculated at 10^5 CFU/ml it rapidly grew and outnumbered the strain LP RJL2, which was inoculated at 10^6 CFU/ml, perhaps due to its faster rate of growth or to a better adaptative response to the environmental conditions. On the other hand, although it could not be detected bacteriocin activity in any of the inoculated brines, all the tested colonies growing in MRS-Str were able to produce PLS, thus proving that they did not loss the bacteriocin-producer character during the process.

The uninoculated fermentors, which were used as controls, reflect the micro organisms that usually occur during the traditional Spanish-style green olive fermentation (Figure 3). In a few days after brining the olives, the epiphytic lactic cocci and yeasts rapidly colonized the brines (Figure 3B and 3C, respectively) but only the yeast population persisted together with the natural lactobacilli throughout the process (Figure 3A and 3C, respectively). As reported previously for the traditional fermentation, the epiphytic lactobacilli micro flora, which began to grow when the pH in the brines dropped below 6.0, reached high count levels after 20 days of fermentation and then became dominant (Fernández Díez et al., 1983; Ruiz-Barba et al., 1994; Garrido Fernández et al., 1995).

Finally, in the fermentors inoculated with the Vegestar60 starter culture, the lactobacilli population reached reasonably high count levels until the end of the fermentation (about 10^6 CFU/ml) (Figure 4A). However, as the starter culture does not carry any specific genetic or phenotypic marker, it was not possible to distinguish that strain from those naturally occurring. On the other hand, the epiphytic micro flora mainly consisting of lactic cocci appeared at the beginning of the fermentation (Figure 4C). They reached more than 10^4 CFU/ml and persisted until the day 30-40 of the process, when they did not appear at detectable levels. However, they were detected again at the end of the fermentation (by day 120). Finally, growth and levels of the yeast population were as expected for a normal development in the traditional fermentative process (Figure 4B).

3.3 *Physicochemical parameters of olive brines*

In spite of an initial pH of the brines of 9-10 (Figure 2E), inoculation with the *L. pentosus* LP RJL2 and LP RJL3 mixed starter culture produced a faster decrease of pH and acidification than those of uninoculated (Figure 3D) or commercial starter-inoculated (Figure 4D) brines, whose initial alkaline pHs were lowered to 6.0 with HCl, as a common practice in the olive processing industry. Whereas in the mixed starter culture inoculated fermentors a pH value of about 5.0 was achieved in the first 15-20 days, reaching about 4.0 at the end of the process, in uninoculated or commercial starter-inoculated brines the drop was slower although reaching similar values at the end of the process. Also, the lactic acid concentration in brines increased rapidly in the first 20 days of fermentation (0.3-0.4 g/100 ml) to give values higher than 0.8 at the end of the fermentation in those fermentors that had been inoculated with the double-strain starter culture (Figure 2F). In contrast, in uninoculated or commercial starter-inoculated brines the increase in the lactic acid concentration was slower and the final concentrations reached were also lower than in the *L. pentosus*-paired starter inoculated brines (Figures 3E and 4E, respectively).

4. Discussion

As many other fermentative processes, the spontaneous, traditional fermentation of Spanish-style green olives historically relies on LAB which are naturally present in the raw material (Daeschel et al., 1987; Fleming et al., 1985). Among them, the species *L. pentosus* (previously described as *L. plantarum*) plays a major role in the preservation of olives (Fernández Díez et al., 1983; Ruiz-Barba, 1991a; Ruiz-Barba et al., 1994; Garrido Fernández et al., 1995). In the last years, some *L. pentosus* strains have been used as starter cultures and they have shown to be useful to control the lactic acid fermentation of olives at small-scale olive fermentations (Ruiz-Barba et al., 1994; Sánchez et al., 2001; de Castro et al., 2002; Leal-Sánchez et al., 2003; Leal-Sánchez et al., 1998). However, as most of them have not been used at the industrial scale its performance in those natural conditions has not been tested yet.

On the other hand, most of the food fermentations rely on mixed cultures of micro organisms and it has been established that there are many important factors that positively influence the ecological success of a mixed starter culture (Sieuwerts et al., 2008). Thus, the use of mixed cultures *versus* the common, single-micro organism starter cultures has been proposed (Sieuwerts et al., 2008). In the case of olive fermentations, an ecological succession of different *L. plantarum/pentosus* strains throughout the fermentation process has been previously reported (Ruiz-Barba, 1991; Ruiz-Barba et al. 1991 and 1994). As these strains have different biochemical and physiological characteristics it would be assumed that they have evolved to optimize their metabolism to the particular conditions of olive brines. Thus, it would be assumed that many of them could be useful to take part of a starter culture for olive fermentation. For those reasons, and based in our previous experience, we carefully selected two *L. pentosus* strains among those belonging to our strain collection in basis to many useful technological properties for olive fermentations (e.g., such as bacteriocin production, less requirement of B-group vitamins, lactic acid and EPS production, tolerance to high pH values, etc.) and we used them as a mixed starter culture at industrial level in tanks placed in different olive processing plants in South Spain. Among other remarkable technological characteristics, *L. pentosus* LP RJL2 was selected in basis

to its ability to produce the bacteriocin PLS, for its rapid acidification properties, and for growing in the absence of two of the essential B-group vitamins for lactobacilli, pyridoxal and PABA. On the other hand, *L. pentosus* LP RJL3 was selected mainly in basis to its ability to produce EPSs which confers to the strain a ropy phenotype. At industrial level, production of EPS improves the viscosity of the brines during the Spanish-style green olive fermentation. This viscosity, named "filado", contributed to obtain a final product with the typical flavour and aroma of olives of consistently high quality (Fernández Díez, 1983; Garrido Fernández et al., 1995).

The *L. pentosus*-paired starter culture described here has proved to be useful to control the olive fermentations at industrial level. In contrast to the results obtained with *L. pentosus* LPCO10 at pilot plant level (Leal-Sánchez et al., 2003), the mixed culture was shown to be more powerful than the single *L. pentosus* LPCO10 starter culture as these two strains completely dominated the entire fermentations by eliminating any competing natural LAB micro flora present in the olive brines. However, the yeast population that plays a role in encouraging the growth of *L. pentosus* in Spanish-style green olive fermentation was present together with the two lactobacilli throughout the fermentation process (Ruiz-Barba and Jiménez-Díaz, 1995). The fact that the strain LP RJL3, which was inoculated in the fermentors at a final concentration 10-time lower than that of *L. pentosus* LP RJL2, rapidly grew and outnumbered the strain LP RJL2 can be attributed to its faster rate of growth or to a better adaptation to the brine environment. However, the persistence of the bacteriocin-producing LP RJL2 strain along the fermentative process was assumed to be essential to control the natural micro flora of the brines. That control would be mainly exerted by producing PLS into the brines, as it has been demonstrated before for the strain LPCO10 (Ruiz-Barba et al., 1994; Leal-Sánchez et al., 1998; Leal-Sánchez et al., 2003).

On the other hand, it has been stated that production of lactic acid by *L. plantarum/pentosus* contributes to the preservation of the fermented olives avoiding spoilage of the fruits (Fernández Díez, 1983; Fernández Díez et al., 1985; Garrido Fernández et al., 1995). In the case of the mixed culture used here, large amounts of lactic acid were produced in the early stages of the fermentation and the final levels reached were always higher than those obtained in commercial-inoculated and uninoculated fermentors thus proving the performance of that

1 double-strain starter for its industrial use.

2 An interesting fact was the ability of both strains to grow and proliferate in brines at
3 alkaline pH. As the olives are treated with NaOH, pH values of the brines at the beginning of the
4 fermentation are usually high (more than 10). For that, it is a recommended trait to decrease the
5 pH of the brines to 6.0 before inoculation by adding acids, by bubbling CO_2 or simply waiting until
6 the natural micro flora dropped it, although this last practice could be of risk because many
7 spoilage bacteria could develop before the natural lactobacilli have grown and produced large
8 amounts of lactic acid. Thus, the ability to grow at pH above 9-10 will provide to the double-
9 strain starter culture with an additional selective advantage over the natural micro flora: it will
10 rapidly colonize the brines and thus it will avoid the growth of any opportunistic bacteria that
11 can cause spoilage of the fruits or simply lead to erratic fermentations. The ability of *L. pentosus*
12 to grow in alkaline olive brines has been previously reported (Sánchez et al., 2001), thus pointing
13 to the possibility of an adaptive response of this species.

14 In conclusion, therefore, it would seem that dominance of the *L. pentosus*-paired starter
15 culture would be exerted by its rapid growth under fermentation conditions, e.g. initial alkaline
16 pH and limited amounts of B-group vitamins, and by their ability to produce bacteriocins and a
17 rapid acidification of the fermentation brines. The results presented here clearly show the
18 performance and the usefulness of this new starter culture to control the olive fermentations at
19 industrial level.

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Legends to the figures

Figure 1.- Genotypic characterization of *Lactobacillus pentosus* strains LP RJL2 and LP RJL3 isolated from natural olive fermentations. (A): RAPD-PCR patterns from total DNA of LPRJL3 (lane 1) and LP RJL2 (lane 3), and PCR with specific primers of the plantaricin S *locus* in LP RJL2 (see Material and Methods) showing the 397-bp fragment corresponding to *pISB* and *pISA* plus the intergenic region (lane 4). As a molecular weight marker, the 1Kb Plus DNA Ladder (Invitrogen) was used (lane 2). (B): PCR with species-specific primers of total DNA of LP RJL2 (lane 1) and LP RJL3 (lane 3) showing the 218-bp amplicon corresponding to the species *Lactobacillus pentosus*. The 1 Kb Plus DNA Ladder was used as molecular weight references (lane 2). (C) Plasmid profiles of strains LP RJL2 (lane 1) and LP RJL3 (lane 3). Plasmids from *Escherichia coli* V517 (Macrina et al., 1978) was used as a source of size reference for plasmid molecules (lane 2).

Figure 2.- Microbial growth and changes in pH and lactic acid in brines inoculated with the novel *L. pentosus*-paired starter culture during the fermentation of Spanish-style green olives at industrial factories. Panels A and B, growth of strains LP RJL2 (■) and LP RJL3 (●), respectively. Panels C and D, growth of yeasts (⧫) and lactic cocci (Δ). Panels E and F, changes in pH (✚) and in lactic acid concentration (✳), respectively. Values represent means ±.95 confidence interval.

Figure 3.- Microbial growth and changes in pH and lactic acid in uninoculated brines. Panel A, growth of the naturally occurring *Lactobacillus* strains (□). Panels B and C, growth of yeasts (◇) and lactic cocci (Δ), respectively. Panels E and F, changes in pH (✚) and in lactic acid concentration (✳), respectively. Values represent means ±.95 confidence interval.

Figure 4.- Microbial growth and changes in pH and lactic acid in fermentors inoculated with the

commercial starter culture Vegestar60. Panel A, growth of the *Lactobacillus* population (□). Panels B and C, growth of yeasts (◇) and lactic cocci (Δ), respectively. Panels E and F, changes in pH (+) and in lactic acid concentration (✳), respectively. Values represent means ±.95 confidence interval.

Table 1

Some morphological, biochemical, and enzymatic characteristics of *L. pentosus* LP RJL2 and LP RJL3 strains

Characteristic	*L. pentosus* LP RJL2	*L. pentosus* LP RJL3
Morphology		
Shape	rods in chains	rods in chains
Gram stain	+	+
Motility	-	-
Physiological characteristics		
Catalase	-	-
Oxydase	-	-
Gas from		
Glucose	-	-
Gluconate	+	-
Growth in sodium tauroglycolate	+	+
Fermentation of		
L-Arabinose	-	+
D-Arabinose	-	-
Ribose	+	+
L-Xylose	-	-
D-Xylose	+	+
Galactose	+	+
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	+	+
Sorbitol	+	+
Amygdalin	+	+
Arbutin	+	+
Esculin	+	+
Salicin	+	+
Cellobiose	+	+
Maltose	+	+
Lactose	+	+
Mellibiose	+	+
Sucrose	+	+
Threalose	+	+
Melizitose	-	+
D-Raffinose	+	+
β-Gentibiose	+	-
Rhamnose	-	+
Arabitol	+	-
Gluconate	+	-
Adonitol	-	-
Hydrolytic enzymatic actitivites		
α-metil-D-glucoside	-	+
N-acetyl-β-glucosamin	+	+
β-galactosidase	+	+
Gelatinase	-	-

+, positive; -, negative

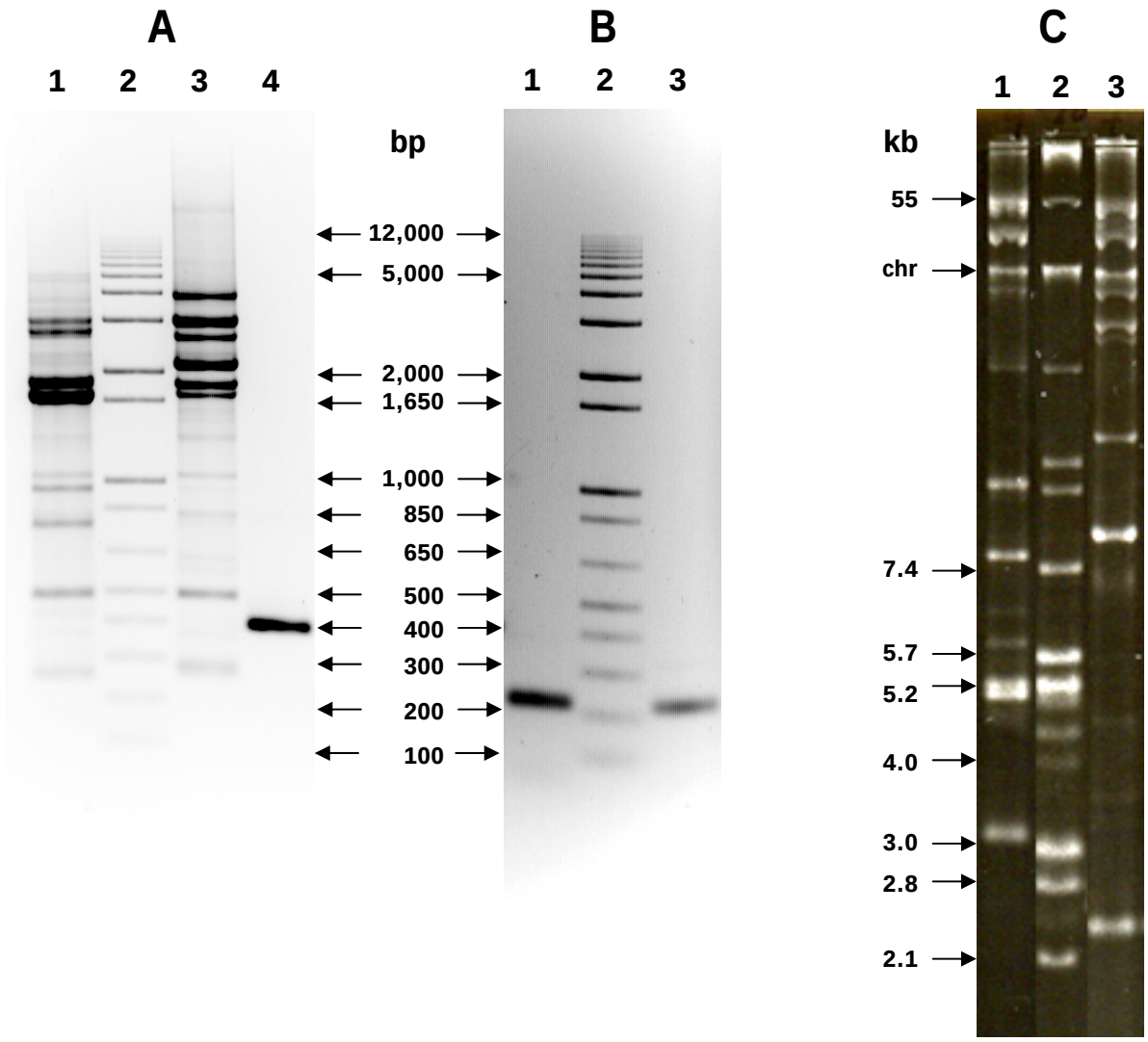


FIGURE 1. Ruiz-Barba, J.L., Jiménez-Díaz, R.

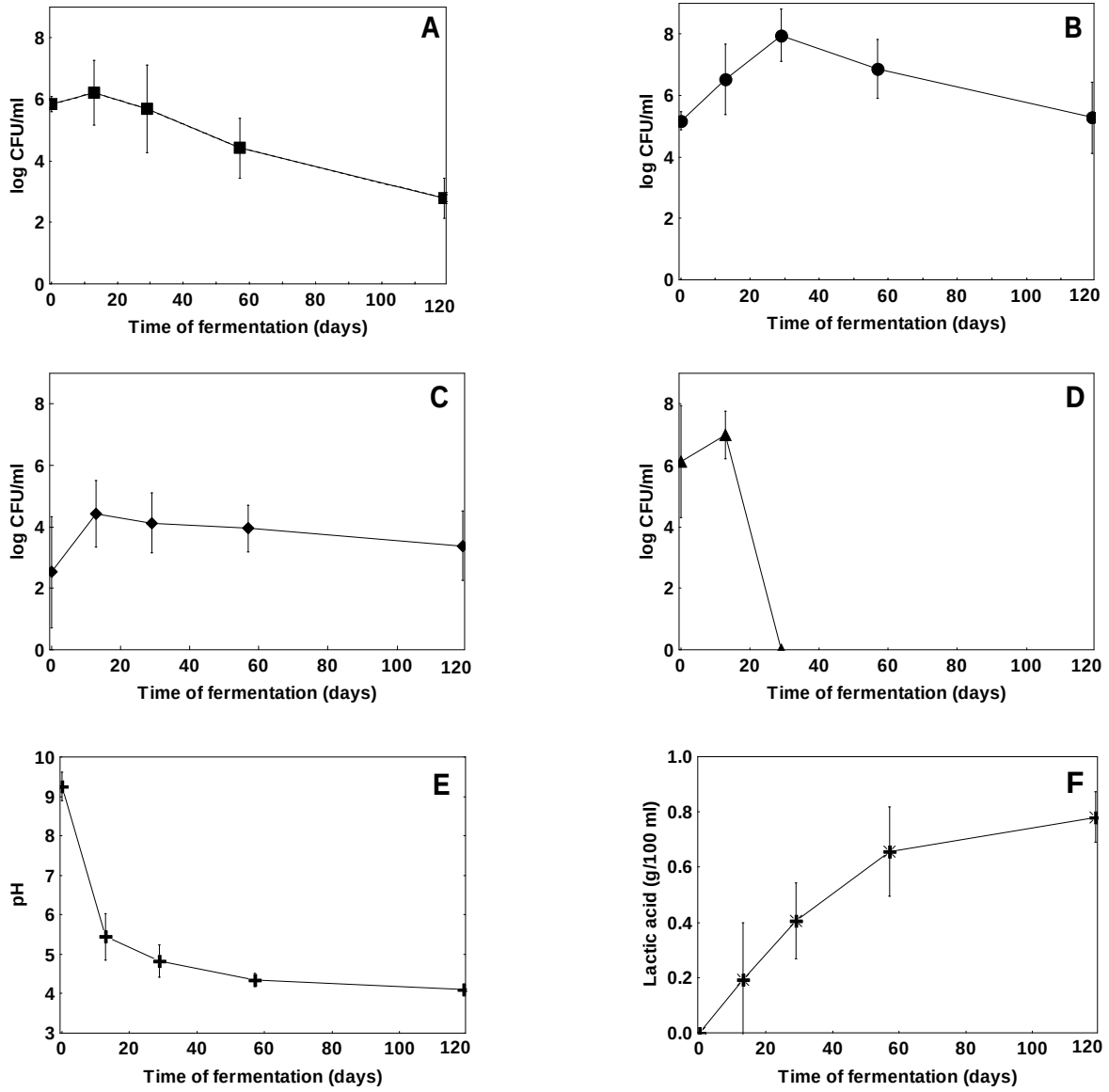
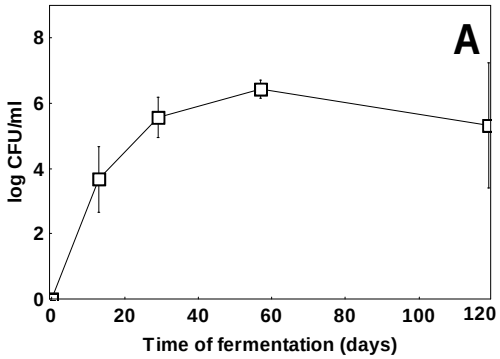
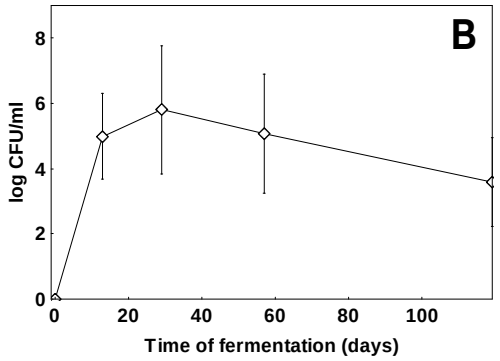
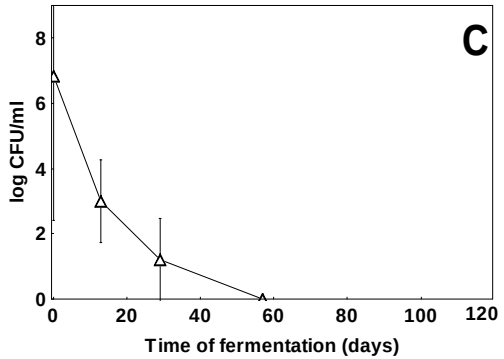
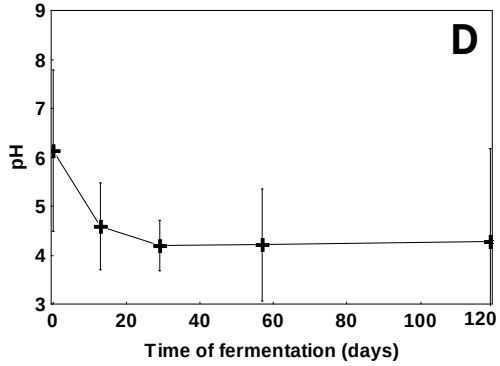


Figure 2. Ruiz-Barba, J.L., and R. Jiménez-Díaz









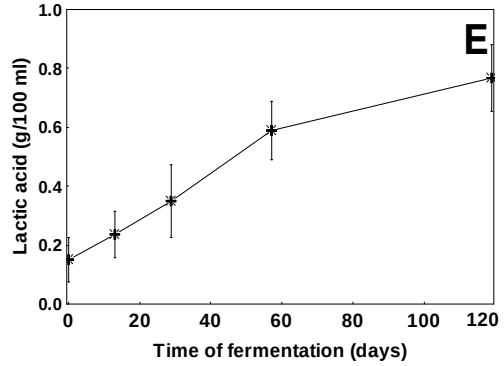
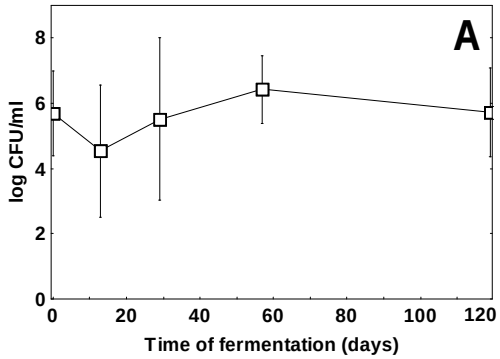
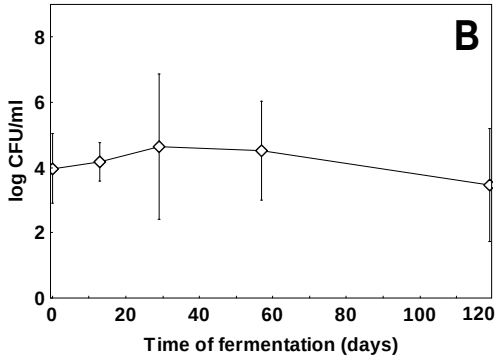
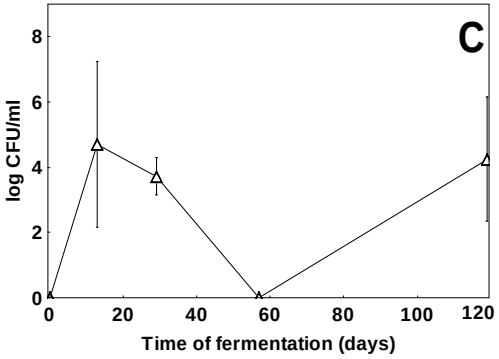
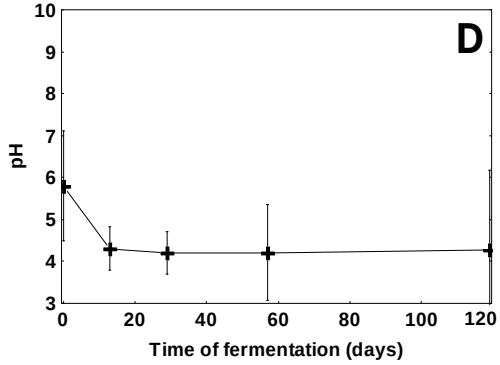


Figure 3. Ruiz-Barba, J.L., and R. Jiménez-Díaz









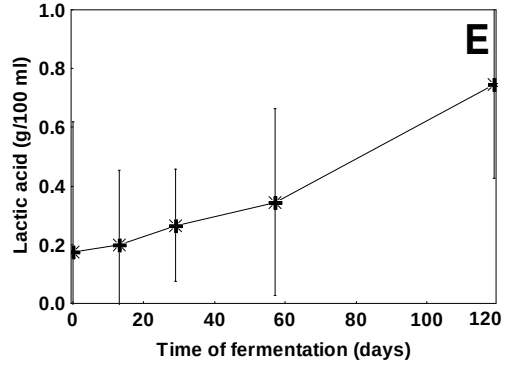


Figure 4. Ruiz-Barba, J.L., and R. Jiménez-Díaz