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

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ABSTRACT

A new starter culture consisting of two *Lactobacillus pentosus* strains was developed and successfully used for Spanish-style green olive fermentations in an industrial study. The inoculum, consisting of *L. pentosus* LP RJL2 and LP RJL3 strains, was inoculated in 10,000 kg glass fiber containers at 10^6 CFU/ml and 10^5 CFU/ml, final concentration respectively, in five different olive processing plants in the south of Spain. As a control, uninoculated fermentors were also used. In all inoculated fermentors, the paired starter rapidly colonized the brines to dominate the natural microbiota and persisted throughout fermentation. A decline in pH to reach about 5.0 was achieved in the first 15–20 days, reaching about 4.0 at the end of the process. 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 g/100 ml at the end of the process. In contrast, increasing lactic acid concentration was slower in uninoculated than in the inoculated brines, and the final concentrations were lower. Although reaching similar values at the end of the process, the decline in pH in uninoculated fermentors was slower than in the inoculated ones. These results show the efficacy of the new starter culture to control the lactic acid fermentation of Spanish-style green olives.

1. Introduction

Among lactic acid bacteria (LAB), different species of the genus *Lactobacillus* have been established as important microorganisms in the production of fermented vegetables (Daeschel et al., 1987; Fleming et al., 1985; Buckenhüskes, 1993; Panagou and Katsaboxakis, 2006; Panagou et al., 2008; Hurtado et al., 2008, 2010). Among these, only table olives, sauerkraut and cucumbers are of real economical significance in the western world, with the production of table olives being the most important in the Mediterranean region. The world production of table olives was estimated by the International Olive Oil Council (IOOC) to be about 2.2 million tons in 2007–2008, to which Spain, the main producer, contributed about 31% of the total (IOOC, 2008). Nearly 800,000 tons were processed as Spanish-style green olives.

In spontaneous, traditional Spanish-style green olive fermentation, both the fruits and the environmental conditions are handled in such a way as to favor the development of a population of LAB in the fermentation brines which is responsible for the subsequent lactic acid fermentation. This LAB population is present as a contaminant at very low initial numbers and is composed mainly of strains of *Lactobacillus plantarum* (Garrido Fernández et al., 1995), although more recent studies carried out in our laboratory show that most of these strains can now be classified as *Lactobacillus pentosus* according to the molecular criteria described by Torriani et al. (2001). The olives are treated with an NaOH solution in order to hydrolyze the phenolic compounds which are bactericidal for LAB (Ruiz-Barba et al., 1990; Ruiz-Barba, 1991; Ruiz-Barba et al., 1993), washed to eliminate the excess NaOH and finally placed in 10,000–15,000 kg glass fiber containers and covered with brine (8–10% NaCl). In these conditions, typically *L. pentosus* becomes 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, 1983; Fernández Díez et al., 1985; Ruiz-Barba, 1991; Ruiz-Barba et al., 1994; Garrido Fernández et al., 1995; Leal-Sánchez et al., 2003; Panagou and Katsaboxakis, 2006; Panagou and Tassou, 2006; Panagou et al., 2008; Hurtado et al., 2010). By fermenting the sugars contained in the fruits, *L. pentosus* contributes not only to the appropriate organoleptic characteristics of the final product but also to the preservation of the fermented olives as a result of the production of large amounts of lactic acid. However, as is true for many other spontaneous vegetable fermentations, the production of Spanish-style green olives relies on microorganisms present in the natural microbiota of the fruits or in the processing

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plant where the olives are fermented. As the use of starter cultures is not a common practice in olive fermentations, this often leads to an abnormal succession of microorganisms with the competing microbiota present in the raw material predominating over the indigenous *L. pentosus* population. In these cases, lactic acid is not produced in the adequate amount needed for olive preservation, thus leading to wide variations in the flavor and quality of the olives and often to spoilage of the fruits. To avoid these problems, technological control procedures such as the systematic use of suitable *L. 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 detailed selection of the wild-type *L. 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 located in southern Spain the results obtained were not as satisfactory as in the pilot plant experiments (unpublished results). This lower effectiveness at the industrial level was probably due to an incomplete control of the natural microbiota which often contaminates the raw material in excess. 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 on the successful use at the industrial level of a new 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 *p*-Amino Benzoic acid (PABA) and the LP RJL3 strain, characterized by rapid and predominant growth in the fermentation brines and by producing high amounts of exopolysaccharide (EPS), among other relevant technological characteristics (Sánchez et al., 2006).

2. Materials and methods

2.1. Bacteria and culture medium

The two *L. pentosus* strains were originally isolated from different Spanish-style green olive fermentations in two different factories in southern Spain (Ruiz-Barba, 1991; 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), as part of the Patent Application PCT/ES2002/000013 (Jiménez-Díaz and Ruiz-Barba, 2004). More recently, they were classified as *L. pentosus* according to the multiplex PCR amplifications using the species-specific *recA*-based primers described by Torriani et al. (2001).

The strains were grown at 30 °C in an MRS (de Man et al., 1960; Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) medium as static cultures and they were maintained as frozen stocks at −20 °C 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 the MRS medium at 30 °C.

2.2. Strain characterization

The strains were phenotypically characterized according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The test comprises Gram-staining, determination of the morphology by phase contrast 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, the strains were grown in a defined medium described by Morishita et al. (1981) in which each of the essential amino acids (L-Isoleucine, L-Glutamic acid, L-Leucine, L-Methionine, L-Phenylalanine, L-Tryptophan or L-Valine) or B-group vitamins (PABA, D-Biotin, Nicotinic and Panthotenic acids or Pyridoxal) was omitted in turn. The defined medium consisted of (per liter): D-glucose, 10 g; sodium acetate, 6 g; ammonium citrate, 1 g; KH_2PO_4, 3 g; K_2HPO_4, 3 g; $MgSO_4 \times 7H_2O$, 0.5 g; $MnSO_4 \times 7H_2O$, 0.05; $FeSO_4 \times 7H_2O$, 0.02; Tween 80, 1 ml; and agar, 15 g. Except for agar (Bacto-agar, Difco Laboratoires, Detroit MI, USA), all the components were purchased from Fluka (Fluka Chemie GmbH, Steinheim, Switzerland).

Tests for rapid acid production were conducted as described previously (de la Borbolla y Alcalá et al., 1958; Kostinek et al., 2007). Briefly, each strain was inoculated (1% of an overnight culture) in MRS broth (pH 6.5) and grown at 30 °C. The pH of the culture was determined after 6, 24 and 48 h of incubation. According to Kostinek et al. (2007), the strain capable of lowering the pH in MRS broth to below 5.3 and 3.9, after 6 and 48 respectively, was considered a fast acid producer.

A 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′-CAG TGGCGCGGTTGATATC-3′), planF (5′-CCGTTTATGCGGAACACCTA-3′), and pREV (5′-TCGGGATTACCAAACATCAC-3′). A PCR amplification of a known *plS* cluster was carried out according to Maldonado et al. (2002), with specific primers BRI1 (5′-TTCTCATGCAAGGAGTGCC-CATGC-3′), ARI2 (5′-TTCTCATGCAAGGAGTGCCCATGC-3′), 2B (5′-GTCATTATGATGTTGACAGCG), and 3A (5′-GCTTAGATTTCACAGCTTC GA-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-variety green olives, was treated with a diluted NaOH solution (2.1%) for 6 h, followed by two washes with water to remove the excess alkali and then brined in about 5000 l of 11% (w/v) NaCl solution (Garrido Fernández et al., 1997).