Manuscript Number: FOOD-D-14-00415R2

Title: Genetic diversity and dynamics of bacterial and yeast strains associated to Spanish-style green table-olive fermentations in large manufacturing companies

Article Type: Full Length Article

Keywords: olive fermentation; biodiversity; microbiota; genotyping; RAPD-PCR; *Lactobacillus pentosus*

Corresponding Author: Dr. José Luis Ruiz-Barba, Ph.D.

Corresponding Author's Institution: Instituto de la Grasa-C.S.I.C.

First Author: Helena Lucena-Adrós, MSc

Order of Authors: Helena Lucena-Adrós, MSc; Belén Caballero-Guerrero, MSc; Antonio Maldonado-Barragán, Ph.D.; José Luis Ruiz-Barba, Ph.D.

Abstract: We have genotyped a total of 1045 microbial isolates obtained along the fermentation time of Spanish-style green table olives from the fermentation yards (patios) of two large manufacturing companies in the province of Sevilla, south of Spain. Genotyping was carried out using RAPD-PCR fingerprinting. In general, isolates clustered well into the relevant phylogenetic dendrograms, forming separate groups in accordance to their species adscription. We could identify which bacterial and yeast genotypes (strains) persisted throughout the fermentation at each patio. Also, which of them were more adapted to any of the three stages, i.e. initial, middle and final, described for this food fermentation. A number of genotypes were found to be shared by both patios. Fifty seven of these belonged to five different bacterial species, i.e. *Lactobacillus pentosus*, *Lactobacillus paracollinoides/collinoides*, *Lactobacillus rari*, *Pediococcus ethanolidurans* and *Staphylococcus* sp., although most of them (51) belonged to *L. pentosus*. Four yeast genotypes were also shared, belonging to the species *Candida thaimueangensis*, *Saccharomyces cerevisiae* and *Hanseniaspora* sp. Two genotypes of *L. pentosus* were found to be grouped with those of two strains used in commercially available starter cultures, one of them bacteriocinogenic, which were used up to three years before this study in these patios, demonstrating the persistence of selected strains in this environment. Biodiversity was assessed though different indexes, including richness, diversity and dominance. It was found a statistically significant decrease in biodiversity between the initial and final stages of the fermentation in both patios. However, values of biodiversity indexes in the fermenters were very similar, and no significant differences were found in the total biodiversity between both patios. This study allowed us to identify a range of well adapted strains (genotypes), especially those belonging to the lactic acid bacteria, which could be useful to improve safety and quality of table olive fermentations.

Highlights

Genetic diversity and dynamics of bacteria and yeast associated to Spanish-style green table-olive fermentations in large manufacturing companies.

Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

Highlights

- We have genotyped 1045 microbial isolates from Spanish-style olive fermentations
- Isolates clustered well into phylogenetic dendrograms in accordance to their species
- Fifty seven bacterial genotypes were shared by the two fermentation yards studied
- Four yeast genotypes were shared by the two fermentation yards studied
- Two *L. pentosus* genotypes persisted years after use of commercial starter cultures

**Genetic diversity and dynamics of bacterial and yeast strains associated to
Spanish-style green table-olive fermentations in large manufacturing companies.**

Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and
José Luis Ruiz-Barba*

*Departamento de Biotecnología de Alimentos, Instituto de la Grasa, Consejo Superior
de Investigaciones Científicas (CSIC), 41012 Sevilla, Spain*

*For correspondence:
Departamento de Biotecnología de Alimentos, Instituto de la Grasa (CSIC), Avda.
Padre García Tejero, 4, Apto. 1078, 41012 Sevilla, Spain.
E-mail: jlrui@cica.es
Tel.: +34 54 69 08 50. Fax: +34 54 69 12 62.

Abstract

We have genotyped a total of 1045 microbial isolates obtained along the fermentation time of Spanish-style green table olives from the fermentation yards (*patios*) of two large manufacturing companies in the province of Sevilla, south of Spain. Genotyping was carried out using RAPD-PCR fingerprinting. In general, isolates clustered well into the relevant phylogenetic dendrograms, forming separate groups in accordance to their species adscription. We could identify which bacterial and yeast genotypes (strains) persisted throughout the fermentation at each *patio*. Also, which of them were more adapted to any of the three stages, i.e. initial, middle and final, described for this food fermentation. A number of genotypes were found to be shared by both *patios*. Fifty seven of these belonged to five different bacterial species, i.e. *Lactobacillus pentosus*, *Lactobacillus paracollinoides/collinoides*, *Lactobacillus rami*, *Pediococcus ethanolidurans* and *Staphylococcus* sp., although most of them (51) belonged to *L. pentosus*. Four yeast genotypes were also shared, belonging to the species *Candida thaimueangensis*, *Saccharomyces cerevisiae* and *Hanseniaspora* sp. Two genotypes of *L. pentosus* were found to be grouped with those of two strains used in commercially available starter cultures, one of them bacteriocinogenic, which were used up to three years before this study in these *patios*, demonstrating the persistence of selected strains in this environment. Biodiversity was assessed through different indexes, including richness, diversity and dominance. It was found a statistically significant decrease in biodiversity between the initial and final stages of the fermentation in both *patios*. However, values of biodiversity indexes in the fermenters were very similar, and no significant differences were found in the total biodiversity between both *patios*. This study allowed us to identify a range of well adapted strains (genotypes), especially those belonging to the lactic acid bacteria, which could be useful to improve safety and quality of table olive fermentations.

Keywords: olive fermentation, biodiversity, microbiota, genotyping, RAPD-PCR, *Lactobacillus pentosus*.

1. Introduction

Production of table olives is of major economical and social importance in Mediterranean countries (Garrido-Fernández et al., 1997). This vegetable fermentation can be produced through a wide variety of methods, although most of the world production is carried out by one of the three most common industrial preparations, i.e. Spanish-style, natural black olives and oxidised black olives (Rejano et al., 2010). Spanish-style fermentation of green olives represent up to 60% of the world table olive production (Botta and Cocolin, 2012), most of it being concentrated in Spain. This table olive preparation is characterised by an initial alkali (NaOH) treatment of the green fruits to remove most of their natural bitterness as well as bacterial-growth inhibitory compounds, mostly phenolic compounds (Rejano et al., 2010). A subsequent spontaneous fermentation takes usually place in the brine which finally covers the alkali-treated olives. This fermentation is mainly carried out by lactic acid bacteria (LAB) and, more precisely, strains of the species *Lactobacillus pentosus* (de Castro et al., 2002; Rejano et al., 2010; Ruiz-Barba and Jiménez-Díaz, 2012).

In a recent publication (Lucena-Padrós et al., 2014a), we reported a comprehensive study on the microbiota, bacterial and yeast species, that is associated to the Spanish-style fermentation of green olives in large-scale table olive manufacturing companies located in the province of Sevilla, southern Spain, where actually up to 63% of the national production is manufactured (season 2012/2013; AAO, 2013). In that study, more than one thousand isolates were obtained and identified at the species level using molecular criteria. In particular, 37 different bacterial species were isolated, belonging to 18 different genera, while 12 yeast species were isolated, belonging to 7 distinct genera. Moreover, a substantial number of bacterial and yeast species had not been described before either from Spanish-style olive fermentations or even from table olives, including a novel bacterial species, i.e. *Enterococcus olivae* (Lucena-Padrós et al., 2014b), previously identified as *Enterococcus saccharolyticus* (Lucena-Padrós et al., 2014a). Although we concluded that Spanish-style green olive fermentation is actually dominated by the species *L. pentosus*, it was also obvious the great biodiversity harboured by this food fermentation. In this study we aimed to further uncover such biodiversity by stepping down to the strain level. For this, and once known their species adscription, we fingerprinted all of the isolates through RAPD and grouped them into phylogenetic dendrograms so that we could examine the microbial population dynamics

now at the strain level. It is known that intra-species strain variability is a fact to take into account when examining the actual functionality of a microbial species in a food environment (Ravyts et al., 2012). Such variability dictates the outcome of analyses such as the microbial risk assessment in food (Lianou and Koutsoumanis, 2013) or the evaluation of the biotechnological properties of selected starter cultures (Leroy and De Vuyst, 2004). In particular, in this study we wanted to answer questions such as which strains are ubiquitous in the different fermenters of a particular fermentation yard ("patio"), which of them are dominant along the fermentation time, and which of them are shared by different *patios* located far away from each other in the province of Sevilla. The answers to these questions are of particular importance regarding the dominant species *L. pentosus* as well as those belonging to the LAB group, for it would reveal specific strains of this species with remarkable biotechnological properties useful to improve the output and safety of this food fermentation.

2. Materials and Methods

2.1. Origin of the microbial strains and growth conditions.

Microbial isolates were obtained from Spanish-style green-olive fermenting brines from two large table-olive manufacturing companies and identified to the species level in a previous work (Lucena-Padrós et al., 2014a). These isolates were propagated in their optimal culture media and conditions, as follows. Yeast species were grown aerobically in Glucose-Yeast Extract Agar (OGYE; Mossel et al., 1962) at 30 °C. Bacterial species belonging to the family Enterobacteriaceae were grown in MacConkey Broth Purple (Biokar Diagnostics, Beauvais, France). Bacterial species belonging to the genera *Bacillus*, *Clostridium*, *Paenibacillus*, *Propionibacterium*, *Sporolactobacillus* and *Paracoccus* were grown in Reinforced Clostridial Medium (RCM; Biokar Diagnostics), while those belonging to the genera *Aerococcus*, *Staphylococcus*, *Enterococcus* and *Vibrio* were cultivated in Brain Heart Infusion (BHI; Biokar Diagnostics) supplemented with 0.05% L-cysteine (AppliChem, Darmstadt, Germany). The rest of bacterial species were propagated in de Man-Rogosa-Sharpe (MRS; Biokar Diagnostics) supplemented with 0.05% L-cysteine. Except for *Paracoccus carotinifaciens*, which was grown aerobically at 30 °C, bacteria were cultivated anaerobically at 30 °C using a DG250 Anaerobic Workstation (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK), with a gas mixture consisting of 10% H₂-10%

CO₂-80% N₂. Bacteria used as reference strains (Table 1) for the RAPD-based clustering of strains belonging to the *L. plantarum* group were grown in MRS medium, anaerobically at 30 °C.

2.2. Genotyping by Randomly Amplified Polymorphic DNA (RAPD).

Total DNA was extracted directly from colonies by the rapid chloroform method described by Ruiz-Barba et al. (2005). Genotyping was carried out by RAPD using the primer OPL5 (Table 1) as described by Maldonado-Barragán et al. (2013). In the case of cocci, primer ISS1rev (Table 1) was used instead. Amplification products were resolved by electrophoresis through 2% (w/v) agarose gels (SeaKem, Biowhittaker Molecular Applications, USA) in 1x TAE buffer, stained with ethidium bromide (0.5 µg/ml), visualized under UV light and digitally recorded. DNA molecular weight marker 1 kb Plus DNA Ladder (Invitrogen) was used as size standard and as a normalization reference. The resulting RAPD profiles were normalized and analyzed for clustering with the Bionumerics 7.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). Only bands representing amplicons between 150 and 5000 bp in size were included in the analysis. Similarity dendrograms were constructed by the UPGMA clustering method, using the band-based Dice similarity coefficient. The quality of the cluster analysis was verified by calculating the cophenetic correlation value (in percentage) for each dendrogram, using the BioNumerics 7.0 software. Interpretation of values obtained for the similarity coefficients was as follows: 1.0, genetically indistinguishable isolated; 0.99 to 0.80, closely related isolates that are highly similar but not identical, which could be considered the same strain; 0.79 to 0.50, related isolates; <0.50, unrelated isolates (Tenover et al., 1995; Soll, 2000).

Reference strains of species belonging to the *L. plantarum* group listed in Table 1 were included in the cluster analysis of the RAPD profiles of bacilli in order to produce an improved distinction among species. As a result, the phylogenetic dendrograms of the bacilli strains were built separately for the *L. plantarum*-group isolates and the non-*L. plantarum*-group ones.

As a control, reproducibility of our PCR fingerprinting experiments was verified with a reduced number of strains. Reproducibility of different RAPD-PCR patterns for the same isolate was always above 80%, which correspond to the minimum level of repeatability for the RAPD fingerprinting technique (Tailliez et al., 1996).

2.3. Biodiversity analyses.

Biodiversity of the overall microbial load was evaluated with Margalef's index of genotypes richness (R), Shannon-Weaver's index of diversity (H') and Simpson's index of dominance (D), calculated as proposed by Ventorino et al. (2007). For strains belonging to the *L. pentosus* species, a strain diversity index (H_1) was calculated for each fermenter by the formula $H_1 = \sum P_i \ln P_i$, in which P_i is the relative abundance of each genotype calculated according to the following equation: $P_i = n_i/N$, where n_i was the number of isolates of each genotype and N is the total number of isolates of the same species. Mean values of biodiversity indexes considering time periods were compared through the ANOVA of repeated measures in each *patio*. Comparisons of mean values of biodiversity indexes between *patios* were done by t-Student's test at each fermentation stage. Bartlett and Levene tests were used to check for homogeneity of the variance, while Kolmogorov-Smirnov test was used to check for normality. A probability value of $P < 0.05$ was regarded to be statistically significant. When it was necessary, values were transformed before the parametric test was carried out. These analyses were performed using the SPSS 21.0 statistical software (SPSS Inc., Chicago, USA). Venn diagrams were drawn using the Venn Diagram Plotter (Pacific Northwest National Laboratory, Richland, WA, U.S.A.).

3. Results

3.1. Bacterial strains diversity.

Clustering of the 927 bacterial isolates of this study according to their RAPD profile was carried out separately for bacilli belonging to the *L. plantarum* group, for bacilli not belonging to the *L. plantarum* group, and for cocci.

3.1.1. Strain diversity in the *L. plantarum* group.

The isolates of bacteria belonging to the *L. plantarum* group, a total of 638, could be clustered according to their RAPD profile, using the primer OPL5 and a similarity level of at least 80%, into the 144 different genotypes summarized in Table S1. The isolates belonging to the three species detected, e.g. *L. pentosus*, *L. plantarum* and *L. paraplantarum*, could be grouped separately (not shown) in good accordance to the reference strains used (Table 1). Fingerprinting similarity of *L. pentosus* profiles

varied from 47% to 100%. With 137 distinct genotypes (Table S1), this species displayed the greatest genotypic variability. This could be the result of the higher number of isolates belonging to this species used for comparison (632). The number of different genotypes identified (91 and 97 from *patio* 1 and 2, respectively) as well as their diversity, measured as the number of isolates per RAPD profile (*ca.* 3.35), was similar in both *patios*. In spite of the large number of different genotypes of *L. pentosus* involved in olive fermentation at each *patio*, 56% of them (51 genotypes) were actually present in both *patios* (Table S1). Fig. 1 shows the population dynamics, expressed as frequency of isolation, of those *L. pentosus* genotypes present in five or more fermenters along the three fermentation stages in both *patios*. All of these 28 genotypes could be isolated from more than one fermentation stage, and 57 % of them were present all along the fermentation (Table S1). Remarkably, we found RAPD profiles of natural isolates similar ($\geq 80\%$) to the ones exhibited by two of the reference strains used to build the dendrogram, i.e. *L. pentosus* LPCO10 and *L. pentosus* 128/2, corresponding to genotypes nos. 7 and 104 in Table S1, respectively. Actually, isolates highly similar to strain LPCO10 were found in both *patios*, in a total of 9 fermenters (Table S1).

The five isolates identified as *L. plantarum*, along with the two reference strains used, *L. plantarum* NC8 and LPT70/3 (Table 1), were clustered together (not shown) with a similarity level of 59%. Three distinct genotypes could be distinguished among these isolates, all of them from the initial stage of fermentation (Table S1). No strain produced RAPD profiles with a similarity $\geq 80\%$ to any of the reference strains used, i.e. *L. plantarum* NC8 and 70/3. Finally, the only *L. paraplantarum* isolate clustered (not shown) with the reference strain used, i.e. *L. paraplantarum* CNRZ1885^T, at a similarity of 57%.

3.1.2. Strain diversity in the bacilli not-belonging to the *L. plantarum* group.

The isolates of bacilli which did not belong to the *L. plantarum* group, a total of 134, were clustered in a dendrogram using RAPD fingerprinting with primer OPL5 (Fig. S1). Clusters were arbitrarily identified at a similarity level of 50% and resulted in a coherent classification at the species level. These clusters were monospecific, with the exception of that grouping together the species *Clostridium jejuense* and *Clostridium xylanovorans*. On the other hand, the majority of species formed a single cluster, with the exceptions of *Lactobacillus paracollinoides/collinoides*, *Paenibacillus illinoisensis/xylanilyticus* and *Lactobacillus parafarraginis*. In the two first cases, the

two observed clusters could be in accordance with isolates belonging to each of the two possible species forming these groups, although such hypothesis has not been investigated further in our laboratory yet.

Finally, only genotypes nos. 5, 10 and 13 of *L. paracollinoides/collinoides*, and genotype no. 1 of *Lactobacillus rapi* were isolated from both *patios* (Fig. S1).

3.1.3. Strain diversity in the isolated cocci.

Cocci isolates, a total of 155, were clustered in a dendrogram using RAPD fingerprinting with primer ISS1rev (Fig. S2). Preliminary assays of RAPD patterns obtained for this group of isolates showed that primer ISS1rev performed better than OPL5 for identification purposes. The cophenetic correlation value calculated for the dendrogram shown in Fig. S2 was 0.81, indicating a good level of reliability. Cluster analysis of RAPD patterns revealed a high degree of diversity among cocci, which could be separated into nine different groups at an arbitrarily chosen similarity level of 50%. The patterns of genotypes assigned to the same species or that fell into the same major clusters exhibited certain common bands that can be considered representative for the species concerned, except for *Enterococcus olivae* (Fig. S2). Isolates of this species grouped in three different clusters, one of them as a subgroup into the cluster containing the *Pediococcus parvulus* isolates, the predominant coccus species in the middle and final stages in *patio* 2, with a similarity coefficient of 62.9%. Isolates identified as *P. parvulus* were genetically distant among them, showing a similarity coefficient of 44.8%, i.e. lower than the similarity level of our arbitrary cluster definition, but they were clearly distinguished from *Pediococcus ethanolidurans* isolates, which was the predominant species of cocci during the middle and final fermentation stages in *patio* 1. Only genotypes nos.1 of *P. ethanolidurans* and *Staphylococcus* sp. were isolated from both *patios* (Fig. S2).

3.2 Yeast strains diversity.

Clustering in a RAPD-based dendrogram of the 117 yeast strains of this study is shown in Fig. S3. The cophenetic correlation value calculated for this dendrogram was 0.79, indicating a good level of reliability. The twelve yeast species identified in this study grouped well into distinct individual clusters by using the primer OPL5. Nevertheless, discrimination between *Issatchenkia orientalis* and *Kluyveromyces lactis/marxianus* isolates was difficult at the similarity degree arbitrarily fixed in this

study (50%), most probably due to the similar range of amplified bands obtained for both species (Fig. S3). A similar situation occurred between *Candida parapsilopsis* and *Candida glabrata*, which are actually very related species (Silva et al., 2012). According to their frequency, two species appeared to dominate the Spanish-style olive fermentation: *Saccharomyces cerevisiae* and *Candida thaimueangensis*, which, taken together, represented up to 50% of the total yeast isolates. These species were isolated from virtually all 20 fermenters during the three stages of the fermentation. Four different genotypes of *S. cerevisiae* were isolated, each of them showing to be associated to a precise fermentation stage/s: genotypes nos. 1 and 2 were isolated from the initial and middle stages, while genotypes 3 and 4 were from the middle and final stages, respectively (Fig. S3). Moreover, genotype no. 2 could be isolated from both *patios*, being the only strain isolated from *patio* 2. On the other hand, isolates of the species *C. thaimueangensis* presented less complex (in terms of bands) but more varied genotypes, i.e. up to 7 ones (Fig. S3). Genotype no. 1 represented up to 50% of the isolates and was isolated from 10 different fermenters and, along with genotype no. 6, was isolated from both *patios*. Apart from these, only *Hanseniaspora* sp. showed a genotype which was shared by both *patios*. The rest of species and genotypes were limited to the fermenters of just one of the *patios* examined in this study.

3.3. Biodiversity analyses.

The richness, diversity, and dominance indexes of the overall genotypes in both *patios* at the three fermentation stages considered are shown in Fig. 2. No statistical difference in any of these biodiversity indexes was found between both *patios* at any fermentation stage. Fig. 2A shows the evolution of genotype richness, which was stable in *patio* 1 but not in *patio* 2, where a significant difference was observed between the initial and final fermentation stages. Moreover, evolution of diversity (H') and concentration of dominance (D) indexes were similar in both *patios*, although statistical significance of time effect was detected between the initial and final fermentation stages in both cases (Fig. 2B and 2C). The highest microbial diversity (1.61 and 1.24 for *patio* 1 and 2, respectively) was found to be associated to the lowest concentration of dominance (0.3 and 0.35 for *patio* 1 and 2, respectively) and characterized the initial fermentation stage (Fig. 2B and 2C). Conversely, during the middle and final stages, the lowest diversity index values (ca. 0.98) were associated to the highest dominance index values (ca. 0.52) in both *patios* (Fig. 2B and 2C). Finally, when specifically examining

the strain diversity index in the dominant species *L. pentosus* (H_1) in every individual fermenter at both *patios* through fermentation we did not appreciate significant differences in any case (Fig. 1).

3.4. Genotype population dynamics.

Dynamics of microbial genotypes isolated along the three fermentation stages of Spanish-style green olive fermentation is represented by proportional Venn diagrams in Fig. 3 for *patio* 1 (panel A) and 2 (panel B). Remarkably, 11 genotypes of the species *L. pentosus* in *patio* 1 and 6 genotypes in *patio* 2 were isolated at the three fermentation stages. In addition, one particular genotype of *Staphylococcus* sp. was also isolated all along the fermentation in *patio* 1. In a previous study (Lucena-Adrós et al., 2014a), these isolates could be assigned to the *Staphylococcus epidermidis* group, which is composed by the species *Staphylococcus epidermidis*, *Staphylococcus saccharoliticus*, *Staphylococcus capitis* and *Staphylococcus caprae* among others, which can not be easily distinguished just by their 16S rDNA sequence (Kwok and Chow, 2003). Finally, a total of 61 distinct genotypes could be isolated from both *patios*, belonging to 8 different microbial species, 5 bacterial and 3 yeast species (Fig. 4). It is noteworthy the high number of genotypes, a total of 51, of the dominant species *L. pentosus* which were shared by both *patios* (Fig. 4).

4. Discussion

This work extends, to the strain level, results shown in a previous publication (Lucena-Adrós et al., 2014a) on the characteristic species composition of the microbiota related to green olive fermentations at large-scale manufacturing companies. On that occasion, RAPD analysis was used to evaluate the selectivity and discrimination power of culture media used, as well as to control the clonal relationships between isolates from the same samples to avoid repetitive sequencing of the 16S rDNA from the same strains. In this work we have used the same fingerprinting technique to elucidate the intraspecific microbial diversity associated to olive fermentations. This allowed us to investigate also the possible role of specific strains during the fermentations, their distribution among the different fermenters of a given *patio* and assess their "cosmopolitan" character when comparing different *patios*. To do this, RAPD analysis was carried out independently on the different microbial groups

previously detected, i.e. yeast, cocci and bacilli belonging and not belonging to the *L. plantarum* group, establishing specific conditions for each group and including, in the case of the dominant *L. plantarum* group, reference strains. Proceeding in this manner, we obtained fairly good grouping of the different strains into the species clusters, allowing further analysis of strain dynamics.

This study has highlighted the microbial diversity at the strain level harboured by the Spanish-style green olive fermentation ecosystem. The fingerprint technique used here allowed fair clustering of the isolates into discrete bacterial or yeast species in the vast majority of the cases. Primer OPL5 was useful in all cases but the cocci isolates, where primer ISS1rev was used instead. In the last case, complexity of the RAPD-PCR band patterns was not uniform for all species identified, and the number of average bands was lower than that obtained for other microbial groups with primer OPL5. In particular, in the case of *P. parvulus* and *E. olivae* the number of bands is too low to make a correct assessment of the actual similarity between isolates. Therefore a polyphasic approach would be recommended in this case. Although it can not be said for sure that two genotypes sharing $\geq 80\%$ similarity are the same strain, at least we can say they are closely related by common ancestors (Tailliez et al., 1996).

Most probably influenced by the fact that the isolates in this work were not randomly selected, as we pursued maximal diversity in the morphotypes isolated, high values of genotype richness were achieved. The low number of repeated genotypes per sample indicated that results of species diversity showed in our previous study (Lucena-Padrós et al., 2014a) were consistent at the strain (genotype) level too. In this occasion we carried out a global analysis of genetic microbial load in Spanish-style table olive fermentation, including genotypes of both yeast and bacterial species that were previously described as singletons and not taken into consideration for the diversity analyses (Lucena-Padrós et al., 2014a). Statistically significant changes in diversity (H') and concentration of dominance (D) observed in *patio* 1 were mainly due to a loss of genetic diversity associated to the yeast species along the fermentation, which was replaced by an increase of genotype diversity in the predominant bacterial species, i.e. *L. pentosus*. In contrast, in *patio* 2, the observed significant changes in diversity (H') and dominance (D) values was a consequence of an increase in genotype diversity of the dominant species *L. pentosus* that replaced the loss of diversity associated to *A. urenaequii/viridans*, the co-dominant species at the initial fermentation stage in this

patio. This fact was reinforced by the lost of genotype diversity associated to sporadic species, i.e. those isolated only at this stage.

In a previous work (Lucena-Padrós et al., 2014a) it was shown that some microbial species were quite ubiquitous, being present in most of the fermenters examined at each *patio*. Furthermore, a total of 8 bacterial and 3 yeast species were shared by both *patios* studied (Lucena-Padrós et al., 2014a). Now we have shown how some precise genotypes persist along the fermentation and even can be isolated from different *patios*, 35 km apart from each other. In particular, Fig. 4 shows that some genotypes belonging to 5 bacterial and 3 yeast species are shared by both *patios*. It is remarkable the number (51) of *L. pentosus* genotypes which could be isolated from both *patios*. Actually, with 137 different ($\geq 80\%$ similarity) RAPD-PCR profiles, the species *L. pentosus* displayed the greatest genotype diversity, although differences in diversity index values (H_1) in the fermenters throughout the fermentation were not significant (Fig. 1). Nevertheless, these facts were no doubt influenced by the large number of isolates obtained of this species (632) in comparison to those obtained from other bacterial or yeasts species. Of special interest are those genotypes which persist all along the fermentation and/or are present in both *patios* (Figs. 3 and 4), for they could display advantageous biotechnological properties of use in olive fermentations. In this sense, up to 16 different *L. pentosus* genotypes were found to persist all along the fermentation (Fig. 3). Also of interest are those genotypes of other 6 LAB species, i.e. *L. paracollinoides/collinoides*, *L. parafarraginis*, *P. ethanolidurans*, *Lactobacillus paracasei*, *L. rafi* and *P. parvulus*, which persist during the middle and final stages (Fig. 3), for they could play an important role in this fermentation which has not been investigated so far.

The fact that the reference strain *L. pentosus* LPCO10 was clustered along with other 13 *L. pentosus* isolates from a total of 9 fermenters at both *patios* (Table S1) could be very significative. This strain has been used as part of a commercial starter culture in the *patios* of many companies, although none of the companies studied here used any starter during the season examined here. Nevertheless, personnel at these two companies declared to have used commercial starters based on a mixed culture of *L. pentosus* LPCO10 and *L. pentosus* 128/2 in previous seasons, more specifically during seasons 2007-2008 and 2008-2009 in *patio* 1 and 2, respectively. Also, the strain *L. pentosus* 128/2 could also be clustered along with a single isolate from *patio* 2 (Table S1). As table olive season studied here was 2010-2011 in both *patios*, it suggests that

these strains were capable to remain in these *patios* for at least two or three years after deliberate inoculation took place. In the past, we demonstrated that bacteriocin production enhanced dominance and persistence of strains of *L. pentosus* and *L. plantarum* in Spanish-style olive fermentations (Ruiz-Barba et al., 1994; Ruiz-Barba et al., 2010; Ruiz-Barba and Jiménez-Díaz, 2012). Moreover, recent studies showed the ability to form biofilms of the *L. pentosus* strains LPCO10 and 128/2, both on abiotic and biotic surfaces, during Spanish-style olive fermentations (Domínguez-Manzano et al., 2012), including surfaces made of the same material as the fermenter inner walls (Dr. Rufino Jiménez-Díaz, Instituto de la Grasa-CSIC, personal communication). Therefore, although fermenters are emptied and thoroughly cleaned at the end of each season, the inherent porosity of the fermenters (made of glass fibre) along with the ability to form biofilms of some bacterial strains which have grown during the fermentation season, makes it very probable that a characteristic and well adapted microbiota persist season after season in a particular *patio*. Such characteristic microbiota, both at the species as well as the strain levels, could constitute a sort of "fingerprint" of a specific *patio* and therefore could help in the future to early detect problems in the fermentation or to establish the traceability of a table olive batch down to a specific *patio*. This matter is currently being investigated in our laboratory.

It is noteworthy the isolation of one genotype of *Staphylococcus* sp., previously assigned to the *S. epidermidis* group (Lucena-Padrós et al., 2014a), which was able to persist all along the olive fermentation and even was shared by both *patios* (Figs.3 and 4). As some of the species of this bacterial group have been defined as opportunistic pathogens (Ghebremedhin et al., 2008), this fact poses a note of worry on the existence of unwanted bacteria that appear to be well adapted to this environment. Therefore, it should encourage the necessity of carrying out thorough cleansing of the fermenters before the start of the new season to avoid persistence of some problematic microbial species. On the other hand, such more hygienic practises could prevent from establishing a favourable autochthonous and well adapted microbiota in the fermenters. This could be also the context with newly set up fermenters, especially those located in brand-new *patios*. In both situations, it is therefore advisable the use of appropriate starter cultures based on well adapted strains of LAB and, more specifically, strains of the species *L. pentosus* that contribute to adequate table olive fermentations season after season. In this work we have isolated a wide set of LAB genotypes for this purpose. The strains (genotypes) selected for these starter cultures should persist throughout the

fermentation and even after the season finishes. Therefore, they should display good competitive characteristics such as, for instance, bacteriocin production, as it has been demonstrated in the past (Ruiz-Barba et al., 1994; Ruiz-Barba and Jiménez-Díaz, 2012). Finally, it has been stressed the convenience of performing the assessment on the suitability of selected strains to be used for different biotechnological uses under the actual environmental conditions they are intended to be applied (Leroy and De Vuyst, 2004). Therefore, isolation and preservation of wild-type strains from traditional products should be encouraged. This study was aimed to contribute to this goal.

Acknowledgements

This research was funded by the Spanish Ministry of Science and Innovation (MICINN), which included FEDER funds, through Projects AGL2009-07861 and AGL2012-33400, and by the Junta de Andalucía Excellence Projects AGR-04621 and AGR-07345. All these projects included FEDER funds. AMB and HLP were the recipients of grants awarded by the Spanish National Research Council (CSIC) through the JAE Doc (ref. JAEDoc 07-00163) and JAE pre (ref. JAEPRe 09-01649) Programmes, respectively. We want to express our most sincere gratitude to Juan Carlos Roldán, from JOLCA S.A., and Antonio Martín and Marta Sánchez, from GOYA en España S.A.U., for their invaluable collaboration in this study.

References

- AAO, 2013. Agencia para el Aceite de Oliva [Olive Oil Agency], Market Information. Available at: http://aplicaciones.magrama.es/pwAgenciaAO/InfGeneral.aao?opcion_seleccionada=1000&control_acceso=S&idioma=ING (accessed the 6th of february, 2013)
- Aukrust, T., Blom, H., 1992. Transformation of *Lactobacillus* strains used in meat and vegetable fermentations. Food Research International 25, 253-261.

Botta, C., Cocolin, L., 2012. Microbial dynamics and biodiversity in table olive fermentation: culture-dependent and -independent approaches. *Frontiers in Microbiology* 3, 245.

Botta, C., Cocolin, L., 2012. Microbial dynamics and biodiversity in table olive fermentation: culture-dependent and -independent approaches. *Frontiers in Microbiology* 3, 245.

Curk, M.-C., Hubert, J.-C., Bringel, F., 1996. *Lactobacillus paraplantarum* sp. nov., a new species related to *Lactobacillus plantarum*. *International Journal of Systematic Bacteriology* 46,595–598.

De Castro, A., Montaña, A., Casado, F.-J., Sánchez, A.-H., Rejano, L., 2002. Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter culture for Spanish-style green olive fermentation. *Food Microbiology* 19, 637–644.

Domínguez-Manzano, J., Olmo-Ruiz, C., Bautista-Gallego, J., Arroyo-López, F.N., Garrido-Fernández, A., Jiménez-Díaz, R., 2012. Biofilm formation on abiotic and biotic surfaces during Spanish style green table olive fermentation. *International Journal of Food Microbiology* 157, 230–238.

Garrido Fernández, A., Fernández Díez, M.J., Adams, R.M., 1997. In: *Table Olives: Production and Processing*. Chapman & Hall, London, pp. 134-197

Ghebremedhin, B., Layer, F., König, W., König, B., 2008. Genetic classification and distinguishing of *Staphylococcus* species based on different partial gap, 16S rRNA, hsp60, rpoB, sodA, and tuf gene sequences. *Journal of Clinical Microbiology* 46,1019–1025.

Jiménez-Díaz, R., Rios-Sánchez, R.M., Desmazeaud, M., Ruiz-Barba, J.L., Piard, J.C., 1993. Plantaricin S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. *Applied and Environmental Microbiology* 59, 1416-1424.

Kwok, A. Y., Chow, A.W., 2003. Phylogenetic study of *Staphylococcus* and *Micrococcus* species based on partial hsp60 gene sequences. *International Journal of Systematic and Evolutionary Microbiology* 53, 87-92

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology* 15, 67-78.

Lianou, A., Koutsoumanis, K.P., 2013. Strain variability of the behavior of foodborne bacterial pathogens: A review. *International Journal of Food Microbiology* 167, 310-321.

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A., Ruiz-Barba, J.L., 2014a. Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques. *Food Microbiology* 42, 154-165.

Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L., Maldonado-Barragán, A., 2014b. *Enterococcus olivae* sp. nov., isolated from Spanish-style green-olive fermentations. *International Journal of Systematic and Evolutionary Microbiology*, manuscript IJS/2014/062208, accepted for publication on 4th May 2014.

Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H., Ruiz-Barba, J.L., 2013. Induction of bacteriocin production by coculture is widespread among plantaricin-producing *Lactobacillus plantarum* strains with different regulatory operons. *Food Microbiology* 33, 40–47.

Mossel, D.A.A., Visser, M., Mengerink, W.H.J., 1962. A comparison of media for the enumeration of moulds and yeasts in foods and beverages. *Laboratory Practice* 11, 109-112.

Ravyts, F., Vuyst, L.D., Leroy, F., 2012. Bacterial diversity and functionalities in food fermentations. *Engineering in Life Sciences* 12, 356-367.

Rejano, L., Montañó, A., Casado, F.J., Sánchez, A.H., de Castro, A., 2010. Table olives: varieties and variations. In: Preedy, V.R., Watson, R.R. (Eds.), *Olives and Olive Oil in Health and Disease Prevention*, Academic Press, Oxford, pp. 5-15.

Ruiz-Barba, J.L., Cathcart, D.P., Warner, P.J., Jiménez-Díaz, R., 1994. Use of *Lactobacillus plantarum* LPCO10, a bacteriocin producer, as a starter culture in Spanish-style green olive fermentations. *Applied and Environmental Microbiology* 60, 2059- 2064.

Ruiz-Barba, J.L., Maldonado, A., Jiménez-Díaz, R., 2005. Small-scale total DNA extraction from bacteria and yeast for PCR applications. *Analytical Biochemistry* 347, 333-335.

Ruiz-Barba J.L., Caballero-Guerrero, B., Maldonado-Barragán, A., Jiménez-Díaz, R., 2010. Coculture with specific inducing bacteria notably enhances survival of the autoinducer-regulated bacteriocin producer *Lactobacillus plantarum* NC8 in olive fermentations. *Food Microbiology* 27, 413-417.

Ruiz-Barba, J.L., Jiménez-Díaz, R., 2012. A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. *Food Microbiology* 30, 253-259.

Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D.W., Azeredo, J., 2012. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiology Reviews* 36, 288–305.

Soll, D. R., 2000. The ins and outs of DNA fingerprinting the infectious fungi. *Clinical Microbiology Reviews* 13, 332–370.

Tailliez, P., Quénée, P., Chopin, A., 1996. Estimation de la diversité parmi les souches de la collection CNRZ: application de la RAPD à un groupe de lactobacilles. *Lait* 76, 147–158.

Tenover, F. C., Arbeit, R. D., Goering, R.V., Mickelsen, P. A., Murray, B. E., Persing, D. H., Swaminathan, B., 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial typing. *Journal of Clinical Microbiology* 33, 2233–2239.

Torriani, S., Felis, G.E., Dellaglio, F., 2001. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. *Applied and Environmental Microbiology* 67, 3450-3454.

Ventorino, V., Chiurazzi, M., Aponte, M., Pepe, O., Moschetti, G., 2007. Genetic diversity of a natural population of *Rhizobium leguminosarum* bv. *viciae* nodulating plants of *Vicia faba* in the vesuvian area. *Current Microbiology* 55, 512-517.

Veyrat, A., Miralles, M.C., Pérez-Martínez, G., 1999. A fast method for monitoring the colonization rate of lactobacilli in a meat model system. *Journal of Applied Microbiology* 87, 49-61.

Legends to Figures

Figure 1. Population dynamics, expressed as frequency of isolation (%), of the genotypes of *Lactobacillus pentosus* in the ten fermenters of the fermentation yard (*patio*) # 1 (panel A) and #2 (panel B). For each fermenter, from left to right, the three bars represent the genotype frequency at the initial, middle and final stages of fermentation, respectively. Genotype labelled as "Others" represents the grouping of those genotypes which were isolated from less than 5 fermenters, considering both *patios*. H_1 : Shannon strain diversity index for each fermenter throughout the fermentation.

Figure 2. Richness, diversity and dominance indexes of microbial genotypes at each fermentation stage in two Spanish-style table olive fermentation yards (*patios*). Panel A: Margalef's index of genotype richness (R); Panel B: Shannon-Weaver's index of diversity (H'); Panel C: Simpson's index of dominance (D). Data are shown as mean values with SEM; * statistically significant difference ($p < 0.05$).

Figure 3. Microbial genotypes and species distribution along Spanish-style green olive fermentations in *patio* 1 (panel A) and *patio* 2 (panel B). Data are represented by Venn diagrams with proportional areas. The three fermentation stages, initial, middle and final, are indicated along with the number of distinct genotypes which could be isolated at each stage. In brackets, the number of different species to which these genotypes belong. For the intersection areas, the actual bacterial and yeast species are indicated. Figures in the square at the left side of each panel indicate the total number of genotypes that were not isolated from that *patio* and the number of microbial species they belong to (in brackets).

Figure 4. Number of microbial genotypes, and the species they belong to, shared by both fermentation yards (*patios*) examined in this study during Spanish-style green olive fermentation. The Venn diagram at the left side of the figure indicates the number of genotypes which have only been isolated at each *patio*, along with the number (in brackets) of species they belong to. The intersection of this Venn diagram represents the number of genotypes which are shared by both *patios*, as well as the number (in brackets) of species they belong to.

Table 1. Bacterial reference strains and oligonucleotides used in this study.

Bacterial strain	Relevant features	Reference
<i>Lactobacillus paraplantarum</i> CNRZ1885 ¹	From beer contaminant; reference strain ¹	Curk et al., 1996
<i>Lactobacillus pentosus</i> 128/2 ²	From olive fermentation; reference strain; starter strain ³	Ruiz-Barba et al., 2010
<i>Lactobacillus pentosus</i> LPCO10 ²	From olive fermentation; reference strain; starter strain ³	Jiménez-Díaz et al., 1993
<i>Lactobacillus plantarum</i> NC8	From grass silage; reference strain	Aukrust and Blom, 1992
<i>Lactobacillus plantarum</i> LPT70/3	From olive fermentation; reference strain	Maldonado-Barragán et al., 2013
Oligonucleotide	Sequence (5'-3')	Reference
OPL5	ACGCAGGCAC	Maldonado-Barragán et al., 2013
ISS1rev	GGATCCAAGACAACGTTTCAAA	Veyrat et al., 1999

¹ Reference strains were used as internal controls to build the phylogenetic dendrogram of those strains of the *Lactobacillus plantarum* group.

² These strains had been previously described as belonging to the *L. plantarum* species (Jiménez-Díaz et al., 1993), but later identified as *L. pentosus* (Ruiz-Barba et al., 2010; Ruiz-Barba and Jiménez-Díaz, 2012) according to the molecular methods and criteria of Torriani et al. (2001).

³ Strains that are part of a commercially available starter culture for table olive fermentation.

A

B

Figure 2. Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

A

B

Figure 3. Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

Figure 4. Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

Table S1. Strain diversity in the species of the *Lactobacillus plantarum* group isolated from Spanish-style green olive fermentations.

Species	Genotype ¹	Isolates ²	<i>Patio</i> ³	Fermenters ⁴	Stage ⁵
<i>Lactobacillus paraplantarum</i>	1	1	2	1	I
<i>Lactobacillus plantarum</i>	1	2	1	1	I
<i>Lactobacillus plantarum</i>	2	1	1	1	I
<i>Lactobacillus plantarum</i>	3	2	1	1	I
<i>Lactobacillus pentosus</i>	1	22	1;2	13	I;M;F
<i>Lactobacillus pentosus</i>	2	32	1;2	12	I;M;F
<i>Lactobacillus pentosus</i>	3	19	1;2	12	I;M;F
<i>Lactobacillus pentosus</i>	4	40	1;2	11	I;M;F
<i>Lactobacillus pentosus</i>	5	17	1;2	11	I;M;F
<i>Lactobacillus pentosus</i>	6	19	1;2	11	M;F
<i>Lactobacillus pentosus</i>	7 ⁶	13	1;2	9	I;M;F
<i>Lactobacillus pentosus</i>	8	9	1;2	8	I;M;F
<i>Lactobacillus pentosus</i>	9	10	1;2	8	I;M;F
<i>Lactobacillus pentosus</i>	10	9	2	7	I;F
<i>Lactobacillus pentosus</i>	11	9	1;2	7	I;M;F
<i>Lactobacillus pentosus</i>	12	10	1;2	7	I;M;F
<i>Lactobacillus pentosus</i>	13	8	1;2	7	I;F
<i>Lactobacillus pentosus</i>	14	15	1;2	7	M;F
<i>Lactobacillus pentosus</i>	15	8	1;2	6	I;M;F
<i>Lactobacillus pentosus</i>	16	6	1;2	6	M;F
<i>Lactobacillus pentosus</i>	17	6	1;2	6	I;M;F
<i>Lactobacillus pentosus</i>	18	6	1;2	6	M;F
<i>Lactobacillus pentosus</i>	19	8	2	6	M;F
<i>Lactobacillus pentosus</i>	20	7	1	5	I;M;F
<i>Lactobacillus pentosus</i>	21	11	1;2	5	I;F
<i>Lactobacillus pentosus</i>	22	6	1	5	I;M;F
<i>Lactobacillus pentosus</i>	23	7	1;2	5	I;M;F
<i>Lactobacillus pentosus</i>	24	8	1;2	5	I;M;F
<i>Lactobacillus pentosus</i>	25	5	1;2	5	I;M
<i>Lactobacillus pentosus</i>	26	5	2	5	I;F
<i>Lactobacillus pentosus</i>	27	5	1;2	5	M;F
<i>Lactobacillus pentosus</i>	28	7	1;2	5	M;F
<i>Lactobacillus pentosus</i>	29	9	2	4	I;M;F
<i>Lactobacillus pentosus</i>	30	7	1;2	4	I;M;F
<i>Lactobacillus pentosus</i>	31	7	1;2	4	I;M;F
<i>Lactobacillus pentosus</i>	32	4	2	4	I
<i>Lactobacillus pentosus</i>	33	4	1;2	4	M;F
<i>Lactobacillus pentosus</i>	34	4	2	4	M;F
<i>Lactobacillus pentosus</i>	35	4	1;2	4	M;F
<i>Lactobacillus pentosus</i>	36	4	1;2	4	I;M;F
<i>Lactobacillus pentosus</i>	37	4	1;2	4	M;F
<i>Lactobacillus pentosus</i>	38	4	1;2	4	I;F
<i>Lactobacillus pentosus</i>	39	4	1;2	4	M;F
<i>Lactobacillus pentosus</i>	40	5	2	4	F
<i>Lactobacillus pentosus</i>	41	5	2	4	M;F
<i>Lactobacillus pentosus</i>	42	5	1;2	4	M;F
<i>Lactobacillus pentosus</i>	43	7	1;2	4	F
<i>Lactobacillus pentosus</i>	44	4	1;2	4	I;F
<i>Lactobacillus pentosus</i>	45	4	1	3	I;M;F

<i>Lactobacillus pentosus</i>	46	5	1	3	I;M
<i>Lactobacillus pentosus</i>	47	3	1;2	3	F
<i>Lactobacillus pentosus</i>	48	3	1;2	3	I;F
<i>Lactobacillus pentosus</i>	49	3	1	3	I;M
<i>Lactobacillus pentosus</i>	50	3	1;2	3	M;F
<i>Lactobacillus pentosus</i>	51	3	1;2	3	F
<i>Lactobacillus pentosus</i>	52	3	2	3	M;F
<i>Lactobacillus pentosus</i>	53	3	1	3	I;M;F
<i>Lactobacillus pentosus</i>	54	4	2	3	I;F
<i>Lactobacillus pentosus</i>	55	4	1	3	F
<i>Lactobacillus pentosus</i>	56	4	1;2	3	I;M;F
<i>Lactobacillus pentosus</i>	57	4	1;2	3	I;F
<i>Lactobacillus pentosus</i>	58	4	1;2	3	F
<i>Lactobacillus pentosus</i>	59	4	1	3	M
<i>Lactobacillus pentosus</i>	60	5	1;2	3	M;F
<i>Lactobacillus pentosus</i>	61	5	1	3	F
<i>Lactobacillus pentosus</i>	62	6	1	3	F
<i>Lactobacillus pentosus</i>	63	3	1	2	I;M
<i>Lactobacillus pentosus</i>	64	3	1	2	M;F
<i>Lactobacillus pentosus</i>	65	3	1	2	M;F
<i>Lactobacillus pentosus</i>	66	2	2	2	M
<i>Lactobacillus pentosus</i>	67	2	2	2	M;F
<i>Lactobacillus pentosus</i>	68	2	1	2	M
<i>Lactobacillus pentosus</i>	69	2	1	2	I
<i>Lactobacillus pentosus</i>	70	2	2	2	F
<i>Lactobacillus pentosus</i>	71	2	2	2	I;M
<i>Lactobacillus pentosus</i>	72	2	2	2	I;M
<i>Lactobacillus pentosus</i>	73	2	1	2	M;F
<i>Lactobacillus pentosus</i>	74	2	2	2	F
<i>Lactobacillus pentosus</i>	75	2	1	2	I;F
<i>Lactobacillus pentosus</i>	76	2	1;2	2	F
<i>Lactobacillus pentosus</i>	77	2	2	2	I;M
<i>Lactobacillus pentosus</i>	78	2	1;2	2	I;M
<i>Lactobacillus pentosus</i>	79	2	1;2	2	M;F
<i>Lactobacillus pentosus</i>	80	2	2	2	I
<i>Lactobacillus pentosus</i>	81	2	1;2	2	I;F
<i>Lactobacillus pentosus</i>	82	2	1;2	2	M;F
<i>Lactobacillus pentosus</i>	83	2	1;2	2	M;F
<i>Lactobacillus pentosus</i>	84	2	2	2	F
<i>Lactobacillus pentosus</i>	85	2	1;2	2	F
<i>Lactobacillus pentosus</i>	86	2	1;2	2	M;F
<i>Lactobacillus pentosus</i>	87	2	1	2	I;M
<i>Lactobacillus pentosus</i>	88	2	1	2	M
<i>Lactobacillus pentosus</i>	89	2	1	2	F
<i>Lactobacillus pentosus</i>	90	3	2	2	M;F
<i>Lactobacillus pentosus</i>	91	3	1	2	I
<i>Lactobacillus pentosus</i>	92	3	1	2	F
<i>Lactobacillus pentosus</i>	93	3	1	2	F
<i>Lactobacillus pentosus</i>	94	4	2	2	M
<i>Lactobacillus pentosus</i>	95	4	1;2	2	M;F
<i>Lactobacillus pentosus</i>	96	6	2	2	M
<i>Lactobacillus pentosus</i>	97	6	1	2	I
<i>Lactobacillus pentosus</i>	98	7	2	2	F
<i>Lactobacillus pentosus</i>	99	1	1	1	M

<i>Lactobacillus pentosus</i>	100	1	2	1	F
<i>Lactobacillus pentosus</i>	101	1	2	1	F
<i>Lactobacillus pentosus</i>	102	1	2	1	M
<i>Lactobacillus pentosus</i>	103	1	2	1	F
<i>Lactobacillus pentosus</i>	104 ⁷	1	2	1	M
<i>Lactobacillus pentosus</i>	105	1	1	1	M
<i>Lactobacillus pentosus</i>	106	1	2	1	I
<i>Lactobacillus pentosus</i>	107	1	1	1	M
<i>Lactobacillus pentosus</i>	108	1	2	1	M
<i>Lactobacillus pentosus</i>	109	1	1	1	F
<i>Lactobacillus pentosus</i>	110	1	2	1	M
<i>Lactobacillus pentosus</i>	111	1	1	1	M
<i>Lactobacillus pentosus</i>	112	1	2	1	I
<i>Lactobacillus pentosus</i>	113	1	2	1	I
<i>Lactobacillus pentosus</i>	114	1	1	1	F
<i>Lactobacillus pentosus</i>	115	1	1	1	I
<i>Lactobacillus pentosus</i>	116	1	1	1	F
<i>Lactobacillus pentosus</i>	117	1	2	1	I
<i>Lactobacillus pentosus</i>	118	1	2	1	F
<i>Lactobacillus pentosus</i>	119	1	2	1	F
<i>Lactobacillus pentosus</i>	120	1	2	1	I
<i>Lactobacillus pentosus</i>	121	1	1	1	F
<i>Lactobacillus pentosus</i>	122	1	1	1	F
<i>Lactobacillus pentosus</i>	123	1	2	1	F
<i>Lactobacillus pentosus</i>	124	1	2	1	I
<i>Lactobacillus pentosus</i>	125	1	1	1	I
<i>Lactobacillus pentosus</i>	126	1	1	1	F
<i>Lactobacillus pentosus</i>	127	1	1	1	F
<i>Lactobacillus pentosus</i>	128	2	2	1	F
<i>Lactobacillus pentosus</i>	129	2	2	1	F
<i>Lactobacillus pentosus</i>	130	2	2	1	I
<i>Lactobacillus pentosus</i>	131	2	1	1	F
<i>Lactobacillus pentosus</i>	132	2	1	1	F
<i>Lactobacillus pentosus</i>	133	3	2	1	F
<i>Lactobacillus pentosus</i>	134	3	2	1	M
<i>Lactobacillus pentosus</i>	135	4	2	1	F
<i>Lactobacillus pentosus</i>	136	4	2	1	F
<i>Lactobacillus pentosus</i>	137	5	1	1	F

¹Genotyping is based on RAPD profiles, and it is the result of clustering isolates exhibiting more than 80% similarity.

²Number of isolates belonging to a distinct genotype.

³Indicates from which fermentation yard (*patio*) that genotype could be isolated.

⁴Number of fermenters, out of a total of 20, from which that genotype could be isolated.

⁵Fermentation stages at which that genotype could be isolated. I, initial; M, Middle; F, Final.

⁶The reference strain *L. pentosus* LPCO10 clustered in this RAPD profile with $\geq 80\%$ similarity.

⁷ The reference strain *L. pentosus* 128/2 clustered in this RAPD profile with $\geq 80\%$ similarity.

A phylogenetic tree showing the relationships between 100 taxa. The tree is rooted at the bottom left. Bootstrap values are indicated at the nodes. The scale bar at the top left ranges from 0 to 100. The taxa are labeled with numbers 1 to 100. The tree is divided into several major clades, with some clades highlighted in purple. The taxa are numbered as follows: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100.

Identification	Isolate	Genotype	Patio	Fermenter	Stage
<i>Lactobacillus paracollinoides/collinoides</i>	J50.8B	1	1	6	F
<i>Lactobacillus paracollinoides/collinoides</i>	J50.9B	1	1	6	F
<i>Lactobacillus paracollinoides/collinoides</i>	J41.3B	2	1	2	F
<i>Lactobacillus paracollinoides/collinoides</i>	J28.5	3	1	3	M
<i>Lactobacillus paracollinoides/collinoides</i>	J45.3	3	1	5	F
<i>Lactobacillus paracollinoides/collinoides</i>	J42.5	3	1	8	F
<i>Lactobacillus paracollinoides/collinoides</i>	J45.7B	3	1	5	F
<i>Lactobacillus parafarraginis</i>	J34.3	1	1	7	M
<i>Lactobacillus parafarraginis</i>	J31.6	1	1	4	M
<i>Lactobacillus parafarraginis</i>	J48.6B	2	1	3	F
<i>Lactobacillus parafarraginis</i>	J50.1	2	1	6	F
<i>Lactobacillus parafarraginis</i>	J35.4	3	1	1	M
<i>Lactobacillus parafarraginis</i>	J50.6B	3	1	6	F
<i>Lactobacillus parafarraginis</i>	J45.5	4	1	5	F
<i>Lactobacillus parafarraginis</i>	J33.3	5	1	10	M
<i>Lactobacillus parafarraginis</i>	J45.8B	5	1	5	F
<i>Lactobacillus rapi</i>	J54.2B	1	1	7	F
<i>Lactobacillus rapi</i>	G32.4	1	2	9	M
<i>Lactobacillus rapi</i>	G40.4	2	2	5	M
<i>Lactobacillus rapi</i>	G40.2	3	2	5	M
<i>Lactobacillus rapi</i>	G51.2B	3	2	10	F
<i>Lactobacillus rapi</i>	G58.3B	3	2	3	F
<i>Paenibacillus</i> sp.	G11.31	1	2	10	I
<i>Paenibacillus</i> sp.	G17.33	2	2	2	I
<i>Paenibacillus illinoisensis/xylanilyticus</i>	G16.31	1	2	1	I
<i>Lactobacillus paracasei</i>	G33.12	1	2	6	M
<i>Lactobacillus paracasei</i>	G34.12	1	2	7	M
<i>Lactobacillus paracasei</i>	G35.12	1	2	8	M
<i>Lactobacillus paracasei</i>	G33.13	1	2	6	M
<i>Lactobacillus paracasei</i>	G34.2	1	2	7	M
<i>Lactobacillus paracasei</i>	G36.14	1	2	1	M
<i>Lactobacillus paracasei</i>	G37.12	1	2	2	M
<i>Lactobacillus paracasei</i>	G60.3B	1	2	5	F
<i>Lactobacillus paracasei</i>	G35.13	1	2	8	M
<i>Lactobacillus paracasei</i>	G35.1B	1	2	8	M
<i>Lactobacillus paracasei</i>	G40.8	1	2	5	M
<i>Lactobacillus paracasei</i>	G60.4	2	2	5	F
<i>Lactobacillus paracasei</i>	G33.2B	3	2	6	M
<i>Lactobacillus paracasei</i>	G34.10	3	2	7	M
<i>Lactobacillus paracasei</i>	G35.2	3	2	8	M
<i>Lactobacillus paracasei</i>	G35.7	3	2	8	M
<i>Lactobacillus paracasei</i>	G35.8	3	2	8	M
<i>Lactobacillus paracasei</i>	G35.10	3	2	8	M
<i>Lactobacillus paracasei</i>	G33.5	3	2	6	M
<i>Lactobacillus paracasei</i>	G31.12	3	2	10	M
<i>Lactobacillus paracollinoides/collinoides</i>	J30.1	4	1	6	M
<i>Lactobacillus paracollinoides/collinoides</i>	J46.9B	4	1	9	F
<i>Lactobacillus paracollinoides/collinoides</i>	J30.14	4	1	6	M
<i>Lactobacillus paracollinoides/collinoides</i>	J28.4	5	1	3	M
<i>Lactobacillus paracollinoides/collinoides</i>	J35.3	5	1	1	M
<i>Lactobacillus paracollinoides/collinoides</i>	J26.6	5	1	9	M
<i>Lactobacillus paracollinoides/collinoides</i>	J30.2	5	1	6	M
<i>Lactobacillus paracollinoides/collinoides</i>	J31.2	5	1	4	M
<i>Lactobacillus paracollinoides/collinoides</i>	J46.8B	5	1	9	F
<i>Lactobacillus paracollinoides/collinoides</i>	G59.3B	5	2	4	F
<i>Lactobacillus paracollinoides/collinoides</i>	G52.1	5	2	9	F
<i>Lactobacillus paracollinoides/collinoides</i>	J21.2	6	1	2	M
<i>Lactobacillus paracollinoides/collinoides</i>	J25.3	6	1	5	M
<i>Lactobacillus paracollinoides/collinoides</i>	J28.6	6	1	3	M
<i>Lactobacillus paracollinoides/collinoides</i>	J41.4	7	1	2	F
<i>Lactobacillus paracollinoides/collinoides</i>	J41.6	7	1	2	F
<i>Lactobacillus paracollinoides/collinoides</i>	J50.4	7	1	6	F
<i>Lactobacillus paracollinoides/collinoides</i>	J21.5	8	1	2	M
<i>Lactobacillus paracollinoides/collinoides</i>	J30.3	8	1	6	M
<i>Lactobacillus paracollinoides/collinoides</i>	J46.2	9	1	9	F
<i>Lactobacillus paracollinoides/collinoides</i>	J31.4	10	1	4	M
<i>Lactobacillus paracollinoides/collinoides</i>	J31.9	10	1	4	M
<i>Lactobacillus paracollinoides/collinoides</i>	G55.4	10	2	8	F
<i>Lactobacillus paracollinoides/collinoides</i>	J25.4	11	1	5	M
<i>Lactobacillus paracollinoides/collinoides</i>	J41.4B	12	1	2	F
<i>Lactobacillus paracollinoides/collinoides</i>	J22.4	13	1	8	M
<i>Lactobacillus paracollinoides/collinoides</i>	G51.1	13	2	10	F
<i>Lactobacillus paracollinoides/collinoides</i>	J21.9	14	1	2	M
<i>Lactobacillus paracollinoides/collinoides</i>	J22.1	14	1	8	M
<i>Lactobacillus paracollinoides/collinoides</i>	J22.2	14	1	8	M
<i>Lactobacillus rhamnosus</i>	G32.8	1	2	9	M
<i>Lactobacillus rhamnosus</i>	G57.6	2	2	2	F
<i>Lactobacillus rhamnosus</i>	G57.2	3	2	2	F
<i>Lactobacillus rhamnosus</i>	G59.4	3	2	4	F
<i>Lactobacillus rhamnosus</i>	G37.1B	4	2	2	M
<i>Lactobacillus rhamnosus</i>	G57.4B	5	2	2	F
<i>Enterobacter hormaechei</i>	J11.12	1	1	4	I
<i>Escherichia coli</i>	G20.17	1	2	5	I
<i>Paracoccus carotinifaciens</i>	G19.31	1	2	4	I

Figure S1. Helena Lucena-Adrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

Figure S2. Helena Lucena-Adrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

Figure S3. Helena Lucena-Padrós, Belén Caballero-Guerrero, Antonio Maldonado-Barragán and José Luis Ruiz-Barba*

Supplementary material - Figure Legends

Figure S1. Phylogenetic dendrogram obtained from RAPD-PCR profiles with primer OPL5 of 134 isolates of bacilli which did not belong to the *L. plantarum* group during Spanish-style green olive fermentations from two different fermentation yards (*patios*). The number of fermenters, out of a total of 20, from which a particular genotype could be isolated is indicated in the column labelled "Fermenter". The fermentation stage at which a particular genotype could be isolated is indicated in the column labelled "Stage": I, initial; M, Middle; F, Final. Scale line at the top indicates the percentage of similarity. The 1 Kb Plus DNA ladder (Invitrogen) used to normalize banding patterns is represented at the top of the figure.

Figure S2. Phylogenetic dendrogram obtained from RAPD-PCR profiles with primer ISS1rev of 155 isolates of cocci during Spanish-style green olive fermentations from two different fermentation yards (*patios*). The number of fermenters, out of a total of 20, from which a particular genotype could be isolated is indicated in the column labelled "Fermenter". The fermentation stage at which a particular genotype could be isolated is indicated in the column labelled "Stage": I, initial; M, Middle; F, Final. Scale line at the top indicates the percentage of similarity. The 1 Kb Plus DNA ladder (Invitrogen) used to normalize banding patterns is represented at the top of the figure

Figure S3. Phylogenetic dendrogram obtained from RAPD-PCR profiles with primer OPL5 of 117 yeast isolates during Spanish-style green olive fermentations from two different fermentation yards (*patios*). The number of fermenters, out of a total of 20, from which a particular genotype could be isolated is indicated in the column labelled "Fermenter". The fermentation stage at which a particular genotype could be isolated is indicated in the column labelled "Stage": I, initial; M, Middle; F, Final. Scale line at the top indicates the percentage of similarity. The 1 Kb Plus DNA ladder (Invitrogen) used to normalize banding patterns is represented at the top of the figure.