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PCR-DGGE assessment of the bacterial diversity in Spanish-style green table-olive fermentations.

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Abstract

The bacterial ecology associated to Spanish-style green olive fermentations has been studied, attending to its dynamics along the time and its distribution, by a culture-independent approach based on PCR-DGGE. Forty-three 10-tonne fermenters were selected from the fermentation yards (*patios*) of two large table-olive manufacturing companies in southern Spain. The fermenting brines of 20 of these fermenters were previously analysed through culture-dependent methods, allowing comparisons of both methodologies. A statistical analysis of DGGE banding profiles obtained using Bacteria universal primers demonstrated significant evidences of discrimination of bacterial communities by location (*patio*) and fermentation stage. Specific microbial "fingerprints" could be established for these variables. At least 17 bacterial species were detected, most of them previously isolated from the same fermenters. Most of these species belonged to the lactic acid bacteria (LAB) group. Dominance of species within the *Lactobacillus plantarum* group was confirmed. *Marinilactobacillus* sp. and *Propionibacterium olivae*, which were not isolated in the previous culture-dependent study, were detected. *Alkalibacterium* sp. and *Halolactobacillus* sp. were detected for the first time in table olive fermentations. Using *Lactobacillus*-group specific primers, significant clustering within the DGGE banding profiles was observed, allowing discrimination regarding the actual fermentation stage. These results corroborated the previous culture-dependent study, and added the detection of *Alkalibacterium* sp. and *Pediococcus acidilactici*. The species *Alkalibacterium* sp., *Marinilactobacillus* sp. and *Halolactobacillus* sp. are characterized by their ability to carry out lactic acid fermentation under alkaline conditions and thus ascribed within the halophilic and alkalophilic lactic acid bacteria (HALAB). Their ubiquitous presence suggests that they could play an important role in Spanish-style olive fermentations, especially at the initial fermentation stage. Thus, they could contribute to brine conditioning before *Lactobacillus plantarum* group-driven lactic acid fermentation takes place.

Keywords: olive fermentation, biodiversity, PCR-DGGE, *Lactobacillus plantarum*-group, HALAB.

1. Introduction

Spanish-style green table olives, a fermented food of major economic importance in Mediterranean countries, are elaborated following traditional methods where spontaneous lactic acid fermentation takes place. This fermentation is usually driven by the microbiota acquired during the processing of the fruits at the factory's facilities and fermentation yards ("*patios*"). Therefore, like for other vegetable fermentations, organoleptic characteristics as well as preservation properties are very dependant on the autochthonous microbiota which is present in its processing environment (Giraffa, 2004; Ruiz-Barba and Jiménez-Díaz, 2012). Natural fermentations are usually characterized by a diverse and complex microbiota which may be difficult to examine using conventional microbiological, i.e. culture-dependent, methods (Ampe et al., 1999; Giraffa, 2004; Justé et al., 2008). The need for microorganism cultivation prior to identification through biochemical or molecular methodology has limited the knowledge of the actual microbial diversity. Thus, it has been postulated that above 90 % of the microorganisms present in natural environments can not be cultivated through conventional microbiological techniques (Amann and Kühn, 1998). Hugenholtz et al. (1998) raised this figure to 99%. Actually, it has been estimated that at least 25-50% of the active microbial community of fermented foods can not be cultivated in the laboratories (Ampe et al., 1999). To surpass these limitations, culture-independent techniques appeared in the 90s as different applications of the recently discovered PCR (Mullis et al., 1986). The technique that has most often been used as a culture-independent method to study microbial communities in food microbiology is PCR combined with denaturing gradient gel electrophoresis (PCR-DGGE) (Cocolin et al., 2013a). Recently, PCR-DGGE has been successfully applied by several authors to study table olive fermentations (Abriouel et al., 2011; Cocolin et al., 2013b; Muccilli et al., 2011; Randazzo et al., 2012). Typically, this technique involves the extraction of nucleic acids from the fermenting brines, followed by PCR amplification of 16S rDNA gene fragments, using universal or group-specific primers, and separation of the resulting PCR products by DGGE. The banding pattern of a sample can then be compared to that of known microbial species (included in *ad hoc* DGGE markers) or other microbial community profiles. In addition, fragments of interest (usually those with no matching to the markers) may also be

excised from the gels, reamplified through PCR, sequenced, and compared to 16S rDNA databases for identification.

Recently, our group has reported comprehensive studies on the microbiota which is associated to Spanish-style green table-olive fermentations, both at the species and strain levels (Lucena-Padrós *et al.*, 2014b, 2014d). In these studies, more than one thousand microbial isolates were identified, which belonged to 49 bacterial and yeast species, included in 25 different genera. In addition, a novel bacterial species was isolated and described, i.e. *Enterococcus olivae* sp. nov. (Lucena-Padrós *et al.*, 2014a). Therefore, a high biodiversity could be observed in this food fermentation through culture-dependent techniques. However, according to the above mentioned observations of environmental microbiologists, a large proportion of microorganisms that are difficult or virtually impossible to detect with these techniques may have been excluded for different reasons (Giraffa and Neviani, 2001; Hugenholtz *et al.*, 1998). Therefore, these previous studies may not reflect the complete, real microbial communities involved in this fermentation. Consequently, a polyphasic approach involving culture-independent techniques should be adopted to attempt a more realistic view of the composition of this microbial community and its dynamics over fermentation time.

The aim of the present study is to assess the bacterial diversity of Spanish-style green olive fermentations through a culture-independent technique such as PCR-DGGE.

2. Materials and Methods

2.1. Origin of the samples and sampling strategy

Samples of Spanish-style green-olive fermenting brines were obtained from 43 10-tonne fermenters at two large manufacturing companies in the province of Seville, southern Spain. Samples were taken along the fermentation time (*ca.* 3 months) in coincidence with the initial, middle and final characteristic stages of green olives fermentations during the season 2010-2011. More specifically, fermentation had taken place for 1 to 14 (first two weeks), 35 to 48 (5th to 7th week), and 69 to 72 (10th to 12th week) days after brining, for the initial, middle and final sampling points, respectively. Twenty of these fermenting brines, 10 of them at each company (numbered consecutively 1-10 in this work to be

consistent with previous publications), have been analysed previously through culture-dependent techniques (Lucena-Padrós *et al.*, 2014b, 2014d). Fermentation set up as well as sampling strategy and sampling times have been previously described for these fermenters (Lucena-Padrós *et al.*, 2014b) and are valid also for the rest of fermenters analysed in this study. Fifty ml samples were taken from approximately the geometric centres of the fermenters as previously described (Lucena Padrós *et al.*, 2014b). Within *patio* 1, two different areas (1a and 1b, including 15 and 5 fermenters, respectively) were considered, while three different areas (2a, 2b and 2c, including 12, 8 and 3 fermenters, respectively) were considered in *patio* 2. Areas were defined considering their construction date and location within the *patio*, as well as the fact of sharing common facilities and equipment. Samples were stored at -80 °C in 20% (v/v) glycerol until analysed.

2.2. DNA extraction from brine samples

Extraction of genomic DNA from brine samples was performed according to a modification of the protocol described by Martín-Platero *et al.* (2007) as follows: 3 ml of brine per sample were subjected to centrifugation at 13,000 rpm at 4 °C for 60 s and the resulting pellet was washed with 1ml of TESAC buffer (10% sucrose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA). After a new centrifugation, the supernatant was discarded while the pellet, containing microbial cells, was resuspended in 100 µl of TESAC buffer supplemented with 10 mg/ml of freshly-made lysozyme (Sigma, St. Louis, MO) plus 40 µg/ml RNase (Sigma) and incubated at 37 °C for 30 min. The resulting protoplast cells were immediately lysed by adding 600 µl of lysis buffer (100 mM Tris-HCl [pH 8.0], 100 mM EDTA, 10 mM NaCl, 1% SDS) with the tube being gently inverted to mix thoroughly. The mixture was then incubated at room temperature for 15 min. To increase the purity of the DNA, the lysate was treated with 10 µl of proteinase K (10 mg/ml, Sigma) and incubated at 37 °C for 15 min. In addition, incubation at 55°C for 15 min followed by cooling to room temperature for 10 min was included to improve cell lysis and DNA yields. Subsequently, 200 µl of cold 7.5 M ammonium acetate were added, the solution was vortex-mixed for 10 s, chilled on ice for 10 min and then centrifuged at 13,000 rpm for 10 min to precipitate the proteins. Finally, the nucleic acids present in the supernatant were precipitated with an equal volume of isopropanol (ca. 950 µl) in a clean microfuge tube,

which was gently inverted several times and chilled on ice for 10 min. The genomic DNA was pelleted by centrifugation at 13,000 rpm for 10 min., washed once with 1 ml of 70% (v/v) ethanol and dried at room temperature. The washed DNA pellet was finally resuspended in 200 µl of molecular grade deionized water (Sigma). Samples (5 µl) were then analyzed by agarose gel electrophoresis to probe their integrity and estimate their concentration.

2.3. PCR amplification and DGGE analysis.

To investigate the dominant bacterial communities by DGGE analysis, PCR products were generated using universal primers U968-GCf and L1401r that amplify the V6 to V8 region of Bacterial 16S rDNA (Nubel et al., 1996). The 40-nucleotide GC rich sequence at the 5' end of primer U968-GCf improves the detection of sequence variations of amplified DNA fragments by subsequent DGGE running (Muyzer et al., 1993). PCR amplification was performed as previously described (Martín et al., 2007a). *Lactobacillus* group-specific PCR was performed using primers targeting the V2–V3 region of the Bacterial 16S rDNA gene. To prevent a low amplicon yield, a nested PCR approach was used as described earlier (Heilig et al., 2002). This involved a first PCR reaction in which primers Bact27f (Lane, 1991) and Lab-677r (Heilig et al., 2002) were used, followed by a second PCR with primers Lab159f (Heilig et al., 2002) and Univ515-GCr (Lane, 1991). A 40-bp GC-clamp was attached to the 5' end of the Uni515r primer in order to facilitate the analysis of the PCR products by DGGE. PCR was performed as previously described (Martín et al., 2007b). PCR products that were used as templates in nested PCR were purified with the NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). PCR products were stored at -20°C until further use.

DGGE analysis of PCR amplicons was performed on the Dcode System apparatus (BioRad), as previously described (Muyzer et al., 1993). Samples were loaded into an 8% (w/v) polyacrylamide gel (acrylamide:bisacrylamide 37.5 : 1) in 0.5 TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 M EDTA pH 8.0). Optimal separation of the PCR products obtained with Bacterial 16S rDNA primers was achieved with 30-60% urea formamide denaturant gradient, increasing in the direction of electrophoresis, while optimal separation of the *Lactobacillus* group-specific PCR products was achieved with 35-55% urea

formamide denaturant gradient, increasing in the direction of electrophoresis. A 100% denaturant corresponds to 7 M urea and 40% (v/v) formamide. Electrophoresis was performed at a constant voltage of 75 V and at temperature of 60 °C for 16 h. The DNA bands were visualized by silver staining as previously described (Sanguinetti et al., 1994). It was possible to digitally normalize the DGGE profiles by comparison with a standard pattern (DGGE marker) using BioNumerics 7.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). This normalization enabled comparison between DGGE profiles from different gels that were run under comparable denaturing and eletrophoretic conditions. Cluster analysis of DGGE pattern profiles was performed using the UPGMA method based on the Dice similarity coefficient (band based).

2.4 Statistical analysis of Bacterial community structure

Similarities between microbial community profiles generated by DGGE analysis were assessed by Dice similarity index and the UPGMA clustering algorithm using the BioNumerics 7.0 software package. The same software package was employed in the discriminant analysis of Bacterial community banding patterns that was performed by the jackknife method to evaluate the stability of the resulting clustering and the integrity of the assignments of banding patterns to defined groups based on fermentation stage, *patio* or area within each *patio*. The percentage of correctly assigned observations for all fingerprints was computed and reported as the estimated rate of correct classification (ERCC) calculated as proposed by Ringbauer et al. (2006). The random ERCC was conservatively calculated as described in James et al. (2006). Expected values for a null hypothesis of random association were computed by comparing the percentages expected under the null hypothesis with the confidence limits for the percentage correctly classified. Confidence limits for the percentage correctly classified were obtained from statistical tables (Sokal and Rohlf, 1987). Moreover, the similarity matrix calculated from the presence-absence data sets using the Dice coefficient was used as data for the multidimensional scaling map (MDS) method, in which data were represented in a Euclidean plane (van Hanne et al., 1999; Bernhard et al., 2005). Every band pattern was shown as one plot, and highly similar band patterns were plotted close together. MDS analysis was performed using SPSS 21.0 software package (SPSS Limited-IBM, Woking,

UK). DGGE fingerprintings obtained with Bacteria universal primers were further analysed in terms of the phylotype richness (S; number of bands) (Sigler et al. 2004). U Mann-Whitney tests were applied to determine statistically significant differences between the S-values in both *patios* at each fermentation stage whereas the fermentation-time effect on phylotype richness in each *patio* was tested using Friedman tests. Significances were accepted at a level of probability (P) of 0.05.

2.5. Marker lanes

In order to enable the identification of bands in DGGE fingerprints, specific markers were built. Total DNA from pure cultures of a variety of selected bacterial species was obtained from isolated colonies using the rapid chloroform method described by Ruiz-Barba et al. (2005). Subsequent PCR was carried out applying the same procedure described above for brine samples. The resulting amplicons were purified using the NucleoSpin Extract II kit and equimolar amounts were mixed to obtain the DGGE markers. Three markers were used in DGGE analyses with universal primers U968-GCf and L1401r. Marker I comprised amplicons from (in increasing electrophoretic migration order) *Staphylococcus* sp. (epidermidis group) J1.12, *Clostridium xylanovorans* J6.31, *Clostridium jejuense* J14.32, *Clostridium schirmacherense/argentinense* J8.31, *Clostridium sporogenes* C22/10, and *Clostridium tyrobutyricum* 3.5 (Figure S1). Marker II comprised amplicons from *Lactobacillus pentosus* G11.2, *Lactobacillus parafarraginis* J31.6, *Lactobacillus collinoides/paracollinoides* J30.1, *Pediococcus parvulus* G72.8, *Lactobacillus paracasei* G31.12, *Pediococcus ethanolidurans* G73.4, and *Lactobacillus coryniformis* G38.8 (Figure S1). Marker III comprised amplicons from *Aerococcus viridans* G18.4, *Enterococcus casseliflavus* J1.9, *Weissella paramesenteroides/hellenica* J11.1, *Enterococcus faecium* J19.2, *Enterococcus faecalis* J20.4, *E. olivae* G16.11^T, *Propionibacterium olivae* IGBL1^T and *Propionibacterium acnes* J41.31. Marker IV was used in DGGE analyses of lactobacilli and related lactic acid bacteria (*Lactobacillus* group) with specific PCR primers, and comprised amplicons from *Lactococcus lactis* ssp. *lactis* IL1403, *Leuconostoc mesenteroides* L32, *P. parvulus* G72.8, *Pediococcus acidilactici* 347, *Lactobacillus rapi* G51.2B, *L. collinoides/paracollinoides* J30.1, *L. parafarraginis* J31.6, *Lactobacillus rhamnosus* G32.8, and *L. paracasei* G31.12 (Figure S1). Strains used in these

reference markers are natural isolates from Spanish-style green olive fermenting brines that were obtained and identified in a previous biodiversity analysis by culture-dependent methods in the same *patios* (Lucena-Padrós et al., 2014d) or belong to our collection and are representative of some of the bacterial species that might be associated to table olive fermentations.

2.6. Identification of DGGE electrophoretic bands

The bands observed in the DGGE analyses were identified using two different approaches. The first approach consisted on the comparison of the DGGE profiles of the samples with DGGE markers constructed previously (Figure S1). In addition, notorious DGGE bands that could not be identified by comparison with the DGGE markers were excised from the gels with sterile surgical blades and DNA was extracted using the protocol of Sanguinetti et al. 1994. Extracted DNA was used as a template for PCR reamplification using the primer pairs U968f (devoid of the GCclamp)/L1401r or Lab159f/Univ515r (devoid of the GCclamp) for DNA products which were primarily obtained from PCRs with universal or *Lactobacillus*-group specific primers, respectively. The resulting PCR products were purified using the Nucleospin Extract II kit and sequenced. The sequences obtained were compared to those of type strains present in the NCBI database using the BLAST algorithm and their identity was determined based on the highest scores.

3. Results

3.1. Distribution of the Bacteria community through PCR-DGGE analysis.

PCR-DGGE banding patterns obtained with Bacteria universal primers showed that amplicons were concentrated along the middle-bottom side of the gel gradient, i.e. in the range 45-60% urea. These banding patterns, organized in a dendrogram as a result of a UPGMA clustering analysis, are shown in Figure S2. An example of the evolution of banding patterns in 8 fermenters along the fermentation time is also shown in Figure 1. Overall, profiles from *patio* 1 were significantly less complex ($p < 0.05$) in terms of band number (phylogroup richness) than *patio* 2 ones (Figure 2). On the other hand, considering the dynamics of bacterial populations along the time, significant differences in phylogroup

richness could be found only in *patio* 2 (Figure 2). Cluster analysis of DGGE profiles through the UPGMA method is shown in Figure S2. A discriminant analysis of this clustering was carried out by the jackknife method. This analysis revealed that assignments of samples to specific groups based on location (*patio*) provided a consistent effect in the bacterial community composition ($p < 0.05$). This is shown in Table 1, where an ERCC value of 72.22 % was obtained whereas the random ERCC was 52.38%. It was remarkable that incorrect assignment of samples was observed only in samples from *patio* 1 (Table S1). This result is graphically visualized in the MDS plot shown in Figure 3, where samples from *patio* 1 showed a dispersed distribution while configuration of samples from *patio* 2 indicated the presence of a central core data group. Dimensions (axes x and y) of the spatial configuration map, generated by MDS scores, are simply for plotting purposes, although distances between data points indeed reflect the relationships between samples in the underlying data set. Furthermore, samples also grouped regarding the fermentation stage significantly ($p < 0.05$) (Table 1 and Table S2). As it was expected, analysis of the frequency of incorrect assignments at each fermentation stage revealed that changes in bacterial community structure were gradual, being this composition more similar at the middle and final fermentation stages. Dynamics of bacterial communities in each fermenter along the fermentation time is also reflected in the MDS plot shown in Figure 3, where mean changes in bacterial community structure (distance between data points) were higher between samples from the initial to the middle fermentation stages than from the middle to the final stages in most cases. When samples were grouped combining multiple significant factors such as location (*patio*) and fermentation stage, this resulted in a statistically significant ($p < 0.05$) partitioning of the samples, with an ERCC value of 56.35 % while the random ERCC was 18.25 (Table 1). Only 4.76 % of the samples (6 cases out of 126) sorted incorrectly when considering both mentioned factors (Table S3). Finally, when a discriminant analysis considering the different areas within each location (*patio*) was performed, significant differences were found only in the area sub-set from *patio* 2 (Table 1).

3.2. Identification of the Bacteria community through PCR-DGGE.

To identify the bands observed in the PCR-DGGEs, DGGE profiles of the samples (Figure 1 and Figure S2) were compared to those of the three different markers constructed *ad hoc* (markers I, II and III in Figure S1). Matching banding patterns were found for all of the species in marker II, except for *L. coryniformis*, and marker III, except for *E. faecalis* and *E. faecium* (Table 2). However, none of the species included in marker I were detected. When DGGE bands which did not match any of those species included in the markers were excised from the gels, purified and sequenced, the presence of *Alkalibacterium sp.*, *Halolactobacillus sp.*, *Marinilactobacillus sp.*, *Vibrio furnissi/fluvialis*, and *Vibrio sp.* was revealed (Table 2, Figure 1 and Figure S2). Also, extra bands corresponding to *L. collinoides/paracollinoides* were detected (Figure 1 and Figure S2).

3.3. Distribution of the *Lactobacillus*-group community through PCR-DGGE analysis.

PCR-DGGE profiles using *Lactobacillus*-group specific primers were obtained from samples of fermenting brines at *patio 2*. These DGGE profiles are shown in Figure S3, where they are organized in a dendrogram as a result of a UPGMA clustering analysis. Such clustering shows the dynamics of the *Lactobacillus*-group community across the fermentation time. This was corroborated by a discriminant analysis of banding-pattern assignment performed by the jackknife method, showing that the mean of ERCC regarding the fermentation stage was significantly higher than the calculated random ERCC (Table 3). However, the mean of ERCC regarding the different areas within *patio 2* was significantly lower than the random ERCC value (Table S6). Therefore, PCR-DGGE profiles of samples collected from fermenters placed in different areas within *patio 2* did not show a clear discrimination regarding the *Lactobacillus*-group community.

3.4. Identification of the *Lactobacillus*-group community through PCR-DGGE.

Comparison of *Lactobacillus* group-specific PCR-DGGE profiles to marker IV allowed the identification of all the species contained in this marker but *L. lactis* and *L. mesenteroides* (Table 4). These two species had not been isolated from these fermenters in a previous culture-dependent study either (Lucena-Adrós et al., 2014b). However, the presence of *P. acidilactici*, which had not been isolated previously, was observed and verified after band extraction and DNA sequencing (Table 4). When DGGE bands which

did not match any of the species included in marker IV were excised from the gels, purified and sequenced, the presence of *A. viridans*, *Alkalibacterium* sp., and species of the *L. plantarum* group was also revealed (Table 4). The relative presence of the bacterial species detected in this way throughout the fermentation time is also shown in Table 4.

4. Discussion

To our knowledge, this is the first study on bacterial community diversity and its dynamics made on industrial-scale traditional Spanish-style green table-olive fermentations carried out using a culture-independent approach such as PCR-DGGE. As near 50% of the fermenters had been analysed through culture-dependent techniques in a previous study (Lucena-Padrós et al., 2014b), these results allow a quite relevant comparison of both methodologies.

Clustering analysis of the PCR-DGGE profiles obtained with Bacteria universal primers showed a trend of grouping by location (*patio*), which was corroborated by jackknife analysis of similarity coefficients (Table 1). As expected, these results confirmed that the actual *patio* was a statistically significant factor influencing the bacterial community structure. Therefore, PCR-DGGE analysis could be a powerful tool for scientific purposes or even control authorities to characterize fermenting-olive brine samples and ascribe them to a specific location, contributing so to the traceability of the product. It could also allow the assessment of whether a normal fermentation is taking place in the fermenters within a specific *patio* or some alteration, which could end up in spoilage of the product, is taking place. Corrective steps could be done in time in the last case. Clustering and jackknife analyses performed on the PCR-DGGE profiles showed that the fermentation stage factor exerts also a quite significant effect on bacterial community structure (Table 1). Through this methodology it could be determined whether a brine sample is naturally evolving through the fermentation time. Early detection of spoilage microorganisms would be very helpful especially at the final fermentation stage. Actually, *P. olivae* and *P. acnes* could be detected, two microorganisms which can cause spoilage at the end of the usual lactic acid fermentation of table olives, in 10% of the fermenters at *patio* 1 (Table 2). In fact, *P. olivae* was first isolated, and described as a novel species, from

spoiled packaged Spanish-style green table olives (Lucena-Padrós et al., 2014c). On the other hand, the discriminant analysis by jackknife method revealed significant differences between PCR-DGGE profiles among the areas considered within *patio* 2. This makes sense, for the two areas defined within *patio* 1 are the same age, while those within *patio* 2 were built and utilized for the first time at quite different seasons, as declared by the respective manufacturing companies' personnel. The facilities and equipment used for the initial processing of the raw fruits, i.e. the characteristic alkali treatment and subsequent washing of the excess of lye, are unique in both *patios*. Therefore, differences in the microbiota should be due mainly to the fermenters themselves, especially to the number of seasons they have been used previously as well as their specific location within each *patio*. A microbial "fingerprint" which is characteristic of a specific *patio* could be determined, as it was pointed out in a recent publication (Lucena-Padrós et al., 2014d).

Identification using universal primers revealed the presence of at least 17 different bacterial species (Table 2). Most of these species belonged to the LAB group. Although most unknown electrophoretic bands were extracted and prepared for DNA sequencing, not all of them rendered a result of enough quality as to be considered. This fact could be due to the use of silver staining of the DGGE gels that, although offering more sensitivity than other staining methods, it is known to cause trouble in subsequent PCR amplifications and sequencing (as well as more background problems). At least 13 of the species identified with this procedure had been already detected through culture-dependent techniques applied on 20 of the fermenters studied here, when up to 37 different species were isolated and identified (Lucena-Padrós et al., 2014b). On the other hand, some species were not isolated in the cited previous study. This is the case of *Marinilactobacillus* sp., *P. olivae*, *Alkalibacterium* sp. and *Halolactobacillus* sp.. Actually, the last two species had not been described before in table olives. However, some of the species included in the markers were not detected in any of the sample. This is the case of those included in marker I, which included species which could be involved in table olive spoilage, and also *L. coryniformis* and *E. faecium/faecalis*, in marker II and III, respectively. In total, up to 24 bacterial species which were isolated in the previous culture-dependent study were not detected now. Some authors have postulated that the detection of individual members from mixed microbial populations cannot be detected by PCR-DGGE when their concentration is lower

than 10^3 CFU/ml or g (Cocolin et al., 2001a, 2001b). Actually, increasing complexity of these populations can raise this detection limit according to these authors. In the present case, only *Staphylococcus* sp. and *L. coryniformis*, which were previously isolated from these samples in the range 10^1 - 10^5 and 10^4 - 10^6 CFU/ml, respectively, should have been detected according to the mentioned criterion. In the case of *L. rapi*, previously isolated in the range 10^3 - 10^6 CFU/ml, was actually detected through PCR-DGGE using *Lactobacillus* group-specific primers (Table 4). The elevated complexity, in terms of microbial species, determined through culture-dependent techniques in these table-olive fermenting brines could explain the failure in the detection of some species. This fact could be alleviated by the use of group-specific primers, which enhances the sensitivity of this technique by reducing the complexity of the population under study.

In *patio* 2, the distribution of the *Lactobacillus*-group community by using specific primers was studied. This appeared an obvious selection as it was previously determined, by culture-dependent techniques, that a LAB such as *L. pentosus* dominated these fermentations and more than 43% of the bacterial species isolated belonged to the LAB group (Lucena-Padrós et al., 2014b). Clustering of the corresponding PCR-DGGE profiles showed that they could be grouped based on the fermentation stage at which the samples were taken. This fact could be quite helpful to determine if a correct fermentation is taking place without the use of time-consuming traditional techniques. However, discrimination by areas was not possible, perhaps indicating that the *Lactobacillus*-group community is well installed and adapted in all of the fermenters in this *patio*, regardless their age. Although it was known that *L. pentosus* was the dominant species in these fermentations, it was not included in the corresponding marker because of the existence of a characteristic "fuzzy" band appearing when the primers used in this study were applied to the species constituting the *L. plantarum* group (Figure S3). Martín et al. (2007b), when studying the diversity of the *Lactobacillus* group in breast milk and vagina through PCR-DGGE, reported the same observation for *L. plantarum*. Electrophoretic bands corresponding to species within the *L. plantarum* group could be detected after band extraction and DNA sequencing, apart from the fact that this species was perfectly detectable through the Bacteria universal primers used here. Also after band extraction and sequencing, the presence of *Alkalibacterium* sp. was confirmed and *P. acidilactici* was detected. The last

species had not been isolated in the previous study nor was it detected using universal primers.

PCR-DGGE analysis is known to only provide monitoring of main microbial populations (Murray et al., 1996; Muyzer et al., 1993). However, elucidation of DGGE profiles provides more information about the genetic structure of the dominant populations than the total microbial richness which is calculated through conventional culture-dependent techniques (Muyzer and Smalla, 1998). This is of special interest in complex microbiological systems such as natural table olive fermentations. In general terms, the results obtained through PCR-DGGE corroborated previous conclusions obtained by culture-dependent techniques. Thus, prevalence of species belonging to the *L. plantarum* group was shown, what is in accordance with the ubiquitous isolation of *L. pentosus* in the previous study (Lucena-Padrós et al., 2014b). Other characteristic species were shown to be *patio* or stage specific (see Table 2). This is the case of *E. casseliflavus* and *Vibrio* sp. in *patio* 1, and *A. viridans*, *E. olivae* and *P. parvulus*, more characteristic of *patio* 2. These results confirmed previous culture-dependent observations (Lucena-Padrós et al., 2014b). However, this study revealed the existence of new characteristic species, such as the ubiquitous *Alkalibacterium* sp. and *Marinilactobacillus* sp.. Ntougias and Russell (2001) isolated *Alkalibacterium olivoapovliticus* from wash waters after alkali treatment of table olives, but not from the olive fermentation itself, while *Marinilactobacillus* sp. has been detected by culture-independent methods from table olive fermentations (Cocolin et al., 2013a). The fact that none of these species were detected in the cited previous study could be most probably due to the absence of specific culture media on that occasion. Although these halophilic/alkalophilic bacteria were detected all throughout the fermentation, it is probable that actual viable cells were not present at the three stages considered, for it is known that they do not withstand low pH values (Yumoto et al., 2014). The use of culture-independent techniques such as RT-PCR-DGGE could contribute to obtain more realistic data about which species are playing a major role at each stage in these complex as well as long lasting food fermentations. Along with *Halolactobacillus* sp., detected for the first time in table olive fermentations in *patio* 2, these bacteria are characterized by their ability to carry out lactic acid fermentation under alkaline conditions, and this is why they are ascribed to the halophilic alkalophilic lactic acid bacteria (HALAB) group (Yumoto et al.,

2014). Current efforts in our laboratory are actually aimed to isolate this class of bacteria using specific culture media to assess their role in this table olive fermentation, especially at the initial stage.

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Legends of the Figures

Figure 1. Normalized PCR-DGGE profiles of 16S rRNA gene V6-V8 variable regions, representing the biodiversity of Bacteria in the fermentation brines of eight fermenters from two different fermentation yards (*patios*) at each fermentation stage (I, initial; M, middle; F, final). M1, M2 and M3, PCR-DGGE identification markers used in this study. The classes of bands identified by gel excision and subsequent DNA sequencing are indicated at the right side of each band as follows: a, *Lactobacillus collinoides/paracollinoides*; b, *Alkalibacterium* sp. (the black bar indicates a group of four characteristic bands for species within this genus); c, *Marinilactobacillus* sp.; d, *Halolactobacillus* sp.; e, *Vibrio* sp.; f, *Vibrio furnisii/fluvialis*.

Figure 2. Phylotype richness of DGGE profiles at each fermentation stage in two Spanish-style table olive fermentation yards (*patios*). Richness data are shown as mean number of bands on the DGGE profiles with SEM. * Statistically significant difference ($p < 0.05$).

Figure 3. Multidimensional scaling ordinations of pairwise estimates of similarity (Dice' coefficient) calculated from PCR-DGGE profiles using Bacteria universal primers during Spanish-style green olive fermentations in two fermentation yards (*patios*). Data at each fermentation stage considered (triangles, initial; squares, middle; rhombus, final) of the same fermenter are connected by lines (dot lines and solid symbols, fermenters from *patio* 1; lines and open symbols, fermenters from *patio* 2). Core data group of *patio* 2 fermenters is circled. Value of normalized stress is indicated.

Figure 1. Helena Lucena-Padrós, Esther Jiménez, Antonio Maldonado-Barragán, Juan Miguel Rodríguez, and José Luis Ruiz-Barba

Figure 2. Helena Lucena-Padrós, Esther Jiménez, Antonio Maldonado-Barragán, Juan Miguel Rodríguez, and José Luis Ruiz-Barba

Figure 3. Helena Lucena-Padrós, Esther Jiménez, Antonio Maldonado-Barragán, Juan Miguel Rodríguez, and José Luis Ruiz-Barba

Table 1. Comparisons of ERCCs of different groupings made after PCR-DGGE analysis of the bacterial community in Spanish-style olive fermentations by jackknife analysis (Dice coefficient).

Group type	ERCC (%)	Random ERCC (%)	Statistical difference ($p < 0.05$)
Location (<i>patio</i>)	72.22	52.38	Yes
Fermentation stage	61.90	34.13	Yes
Location x Fermentation stage	56.35	18.25	Yes
Area			
Sub-set: <i>patio</i> 1	83.33	75	No
Sub-set: <i>patio</i> 2	68.20	54.55	Yes

Table 2. Prevalence of bacterial species identified through PCR-DGGE using universal primers at each fermentation stage (initial, middle and final) of Spanish-style green olive fermentations in two different fermentation yards (*patios*).

Species	Patio 1			Patio 2		
	Initial	Middle	Final	Initial	Middle	Final
<i>Aerococcus viridans</i>	10.0*	10.0	10.0	95.0	95.7	100
<i>Alkalibacterium</i> sp. ^{1,2,3}	100	100	95.0	100	100	100
<i>Enterococcus casseliflavus</i>	95.0	80.0	55.0	55.0	60.9	52.2
<i>Enterococcus olivae</i>	55.0	50.0	50.0	80.0	100	100
<i>Halolactobacillus</i> sp. ^{1,2,3}	0.0	0.0	0.0	50.0	4.4	4.4
<i>Lactobacillus paracasei/rhamnosus</i>	55.0	65.0	15.0	40.0	39.1	47.8
<i>Lactobacillus parafarraginis</i>	10.0	35.0	65.0	0.0	13.0	43.5
<i>Lactobacillus collinoides/paracollinoides</i>	15.0	55.0	85.0	100	78.3	87.0
<i>Lactobacillus plantarum</i> group*	100	100	100	100	100	100
<i>Marinolactobacillus</i> sp. ^{1,2}	50.0	60.0	60.0	100	100	100
<i>Pediococcus ethanolidurans</i>	10.0	20.0	25.0	0.0	0.0	0.0
<i>Pediococcus parvulus</i>	15.0	20.0	60.0	100	95.7	95.7
<i>Propionibacterium olivae</i> ²	10.0	0.0	10.0	0.0	0.0	0.0
<i>Propionibacterium acnes</i>	10.0	0.0	10.0	0.0	0.0	0.0
<i>Vibrio furnisii/fluvialis</i> ¹	85.0	0.0	0.0	0.0	0.0	0.0
<i>Vibrio</i> sp. ¹	85.0	5.0	0.0	30.0	4.4	0.0
<i>Weissella paramesenteroides</i>	30.0	35.0	30.0	65.0	52.2	56.5

*Results are expressed as percentage of fermenters where a specific bacterial species was detected through PCR-DGGE.

¹Bacterial species which were detected after extraction of the corresponding PCR-DGGE band and subsequent DNA sequencing. ²Bacterial species which were not detected previously in the fermenters under study through culture-dependent techniques (Lucena-Padrós et al., 2014b). ³Bacterial species which have not been previously isolated from table olive fermentations. *Includes the species *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*, which were indistinguishable using this technique.

Table 3. Assignment of banding patterns to fermentation stage groups of the *Lactobacillus*-group community in Spanish-style olive fermentations by jackknife analysis (Dice coefficient).

Fermentation stage	Banding patterns (%) assigned to groups		
	Initial (n=20)	Middle (n=23)	Final (n=23)
Initial	85.0	4.3	0.0
Middle	5.0	60.9	0.0
Final	10.0	34.8	100

ERCCs of patterns to host class are in boldface. The mean ERCC was 81.82%, while the random ERCC was 34,85%. Differences are statistically significant ($p < 0.05$).

Table 4. Prevalence of bacterial species identified through PCR-DGGE using *Lactobacillus*-group specific primers at each fermentation stage (initial, middle and final) of Spanish-style green olive fermentations in fermentation yard (*patio*) # 2.

Species	Fermentation stage		
	Initial	Middle	Final
<i>Aerococcus viridans</i> ¹	95.0*	26.1	39.1
<i>Alkalibacterium sp.</i> ^{1,2,3}	25.0	13.0	8.7
<i>Lactobacillus paracasei</i>	50.0	78.3	60.9
<i>Lactobacillus parafarraginis</i>	5.0	82.6	91.3
<i>Lactobacillus paracollinoides/collinoides</i>	20.0	73.9	95.7
<i>Lactobacillus plantarum</i> group ^{1,4}	100	95.7	95.7
<i>Lactobacillus rapi</i>	25.0	8.7	26.1
<i>Lactobacillus rhamnosus</i>	0.0	47.8	30.4
<i>Pediococcus acidilactici</i> ²	5.0	4.4	8.7
<i>Pediococcus parvulus</i>	0.0	26.1	65.2

* Results are expressed as percentage of fermenters where a specific bacterial species was detected through PCR-DGGE. ¹Bacterial species which were detected after extraction of the corresponding PCR-DGGE band and subsequent DNA sequencing. ²Bacterial species which were not detected previously in the fermenters under study through culture-dependent techniques (Lucena-Padrós et al., 2014b). ³Bacterial species which have not been previously isolated from table olive fermentations. ⁴Species within the *L. plantarum* group, including *L. plantarum*, *L. pentosus* and *L. paraplantarum*, are not distinguishable by 16S rDNA sequence analysis.

Highlights

- Bacterial ecology of Spanish-style olive fermentations studied by PCR-DGGE
- PCR-DGGE profiles discriminated by location of the fermenters and fermentation stage
- PCR-DGGE analyses confirmed most previous culture-dependent results
- *Alkalibacterium* and *Halolactobacillus* first detected in table olive fermentations