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ScienceDirect
International Journal of Food Microbiology 112 (2006) 291–296
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Fluorescence in situ hybridisation detection of *Lactobacillus plantarum* group on olives to be used in natural fermentations ☆

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Abstract

At present there are very few studies on the bacterial diversity of olives and on the importance of the microbial species for the fermentation of olives aimed to table olives production. Most of the authors report on the occurrence of *Lactobacillus plantarum* as principal member of these communities or at least as the species responsible for the fermentation. In this study, fluorescence in situ hybridisation (FISH) with 16S rRNA probes was used to evaluate the occurrence of *L. plantarum* in olives. A 18-bp oligonucleotide probe was used in FISH experiments to evaluate the specificity of detection among *Lactobacillus* species. The probe was tested against 30 *Lactobacillus* species and appeared to be specific for *L. plantarum*, *L. paraplantarum* and *L. pentosus*. The probe was then used to investigate the occurrence of these species in 25 samples of olives (cultivar "Leccino") collected in Campania region (Southern Italy). The olives were washed in a saline solution and the suspensions were then analysed by FISH and observed by fluorescence microscopy. No hybridisation signal was detected in at least 30 fields of observation when the *L. plantarum*-specific probe was used, probably due to the low sensitivity of the FISH method. Olive samples were plated on Rogosa agar and about 40% of the samples did not give growth after 5 days. When colony growth was observed, bulk cells from Rogosa agar plates were collected and analysed by DNA extraction followed by 16S rDNA Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE). The different microbial species were identified by direct sequencing of DGGE bands. *Leuconostoc pseudomesenteroides* was the most frequently found species, occurring in more than 50% of the samples that had shown growth on Rogosa agar. The closest relatives of the species of the genera: *Leuconostoc*, *Pediococcus*, *Pseudomonas* and *Raoultella* were also identified suggesting that guided fermentation by using selected LAB starters is advisable for a safe and desired table olives production.

Keywords: Table olives; *Lactobacillus plantarum* group; FISH; PCR–DGGE

1. Introduction

The species of the genus *Lactobacillus* are widely occurring in many natural environments often playing important roles in fermentation processes and in the regulation of the relationships between species of complex ecosystems. Among them, the species belonging to the *Lactobacillus plantarum* group are of interest for food fermentations. *L. plantarum* and *L. pentosus* are associated with the fermentation of vegetables, dairy (Carr et al., 2002) and bakery products (Pepe et al., 2004). *L. paraplantarum* is the most recently introduced species (Curk et al., 1996); it has been isolated mainly from dairy products and it is believed to play a very similar role as the above species in food fermentations.

The identification of lactobacilli is one of the main issues of applied microbiology and rapid and reliable methods for their detection in natural ecosystems are of invaluable help.

Olives are the major fermented vegetables in western countries (Garrido Fernandez et al., 1997). Homemade production of naturally fermented table olives is very common in Mediterranean countries and the production methods vary according to local tradition. Most of the times the process is guided by the indigenous microbial flora whose composition is still to be satisfactorily assessed as very few studies have been carried out on the microflora of olives and on the microorganisms involved in the fermentation leading to table olives. Particularly, the composition of the microbial flora of the olives before brine making is one of the factors that could affect the dynamics of the fermentation and the quality of the product.

☆ This paper was presented at the 19th International ICFMH Symposium, Food Micro 2004, Potorož Slovenia, 12-16 September 2004.

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0168-1605/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2006.09.003

Lactic acid bacteria are recognised to play an important role in olive fermentation and *L. plantarum* and *L. pentosus* are, in fact, regarded as the main species leading this process being often used as starter in guided olives fermentation (Ruiz-Barba et al., 1994; Durán Quintana et al., 1999; Sánchez et al., 2001; Leal-Sánchez et al., 2003).

FISH with 16S rRNA probes is a valid tool in environmental microbiology and has found many applications for the in situ detection and identification of microorganisms in several habitats (Moter and Göbel, 2000; Amann et al., 2001). Recently, this technique was also introduced into food microbiology for the location of specific bacterial colonies in cheese (Ercolini et al., 2003a,b). The main advantage of FISH is its possibility to detect the microorganisms directly in their habitats without culture dependent isolation of cells or culture independent extraction of nucleic acids prior to the identification. The most commonly employed target for the identification of Bacteria by FISH is the 16S rRNA (Moter and Göbel, 2000), due to the presence of conserved and variable regions and to the abundance of 16S rRNA copies in ribosomes within the cells, which makes it possible to many probes to hybridise with a consequent significant yield in fluorescence. In this study we evaluated the specificity of a 16S rRNA probe for the selective detection of *L. plantarum* group by FISH among 30 species of *Lactobacillus* usually occurring in food. FISH was thereafter used to screen olives after the harvest for the presence of high loads of members of *L. plantarum* group. The occurrence of other lactic acid bacteria on olives to be used in natural fermentations before brine making was also evaluated.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Twenty-eight strains of *Lactobacillus* from the DSM collection were used in this study (Table 1). The strains were maintained in MRS broth (Oxoid, Milano, Italy) with 25% glycerol at −20 °C. They were cultivated in MRS broth at their optimal growth temperature for 16 h prior to FISH experiments.

2.2. Olive samples and analysis

Twenty-six black olive samples (cultivar "Leccino", numbered from 1 to 26) were collected during the harvesting period in different zones of the Campania region, Southern Italy, before brine making and fermentation. The olives were all of the same variety. 100 g samples of olives were washed by shaking in 100 ml of quarter-strength Ringer's solution (Oxoid) overnight at room temperature. 20 ml of the suspensions was centrifuged at 8000 rpm for 5 min at room temperature; the pellet was resuspended in 2 ml of quarter-strength Ringer's solution and divided into two aliquots of 1 ml. The first 1 ml aliquot was directly used in FISH experiments as described below. The other 1 ml aliquot was plated on Rogosa agar (Oxoid). Rogosa agar plates were incubated in anaerobiosis by using the Anaerogen kit (Oxoid) at 30 °C for five days. The microbial mass grown on the plates after the incubation was collected in cell bulks as previously described (Ercolini et al., 2003b) by using quarter-strength Ringer's solution (Oxoid).

Table 1
Lactobacillus strains used in this study and the results of FISH experiments with the LpbV3 and Eub338 16S rRNA probes

Strain	Eub338-FISH	LbpV3-FISH
Lactobacillus plantarum [1]DSM20174 [T]	+	+
Lactobacillus paraplantarum DSM10667[T]	+	+
Lactobacillus pentosus DSM20314 [T]	+	+
Lactobacillus acidophylus DSM20079 [T]	+	−
Lactobacillus alimentarius DSM20249 [T]	+	−
Lactobacillus amylophylus DSM20533 [T]	+	−
Lactobacillus amylovorus DSM20531 [T]	+	−
Lactobacillus arizonensis DSM13273	+	−
Lactobacillus bifermentans DSM20003 [T]	+	−
Lactobacillus brevis DSM20556	+	−
Lactobacillus buchneri DSM20057	+	−
Lactobacillus casei DSM20011	+	−
Lactobacillus coryniformis subsp. *coryn.* DSM20001[T]	+	−
Lactobacillus curvatus subsp. *curvatus*DSM20019 [T]	+	−
Lactobacillus delbrueckii subsp. *lactis* DSM20072 [T]	+	−
Lactobacillus farciminis DSM20184 [T]	+	−
Lactobacillus fermentum DSM20052 [T]	+	−
Lactobacillus gasseri DSM20243[T]	+	−
Lactobacillus helveticus DSM20075 [T]	+	−
Lactobacillus johnsonii DSM20553 [T]	+	−
Lactobacillus kimchii DSM13961	+	−
Lactobacillus paracasei subsp. *paracasei* DSM5622 [T]	+	−
Lactobacillus reuteri DSM20016 [T]	+	−
Lactobacillus rhamnosus DSM20021[T]	+	−
Lactobacillus sakei subsp. *sakei* DSM20017 [T]	+	−
Lactobacillus salivarius subsp. *salivarius* DSM20555 [T]	+	−
Lactobacillus salivarius subsp. *salicinius* DSM20554 [T]	+	−
Lactobacillus zeae DSM20178 [T]	+	−

[1]DSM: Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany.

2.3. DNA extraction and PCR–DGGE analysis

DNA was extracted from 1 ml aliquots of bulk cells in Ringer's solution by using the Wizard DNA purification kit (Promega, Madison, WI, USA) as previously reported (Ercolini et al., 2004). The extracted DNA was used for the PCR amplification of the variable V3 region of the 16S rDNA by primers V3F and V3R followed by sequence-specific separation of the amplicons by denaturing gradient gel electrophoresis (DGGE). The materials and conditions for the PCR and DGGE analyses were previously reported (Ercolini et al., 2003b). DGGE bands to be sequenced were purified in water as described by Ampe et al. (1999). Eluted DNA (1 μl) of each DGGE band was re-amplified by PCR and the products that gave a single band co-migrating with the original band were then purified with a Qiaex PCR purification kit (Qiagen, Milano, Italy) and sequenced. Sequencing was performed by Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems) and using the primer V3R. To determine the closest known relatives of the partial 16S rDNA sequences obtained, searches were performed in public data

libraries (GenBank) with the Blast search program. The GenBank accession numbers for 16S rDNA partial sequences retrieved from DGGE bands are from AY660888–AY660892.

2.4. Fluorescence in situ hybridisation

FISH experiments were carried out by using the 16S rRNA probe S-S-Lb.pl-0468-a-A-18 LbpV3 previously designed by Ercolini et al. (2003b). The sequence of the LbpV3 probe is 5'-CCGTCAATACCTGAACAG-3'. In each experiment, a FISH reaction with the universal Eub338 16S rRNA probe (Amann et al., 1990) targeting all Bacteria was also carried out, both probes were labeled in 5′ position with Fluorescein (Invitrogen). The specificity of the probe LbpV3 was checked in FISH experiments involving the *Lactobacillus* species listed in Table 1. Moreover, FISH was used for the detection of *L. plantarum* group in the olive suspensions in quarter-strength Ringer's solution (Oxoid) prepared as above described. The fixation was performed by using 4% paraformaldehyde (Amann et al., 1990) in PBS (Oxoid) and applied to: (i) 0.3 of broth culture and (ii) 1 ml of concentrated olive suspension. About 20 μl of the fixed suspensions in PBS was spotted on a poly-L-lysine-coated slide, dried in an oven at 46 °C for 10 min, and dehydrated successively in 50, 80, and 100% ethanol solutions for 3 min each. Once the ethanol had evaporated, the specimens were used for the hybridisation. Pretreatment of all specimens with proteinase K (10 mg^{-1}; Sigma, Milano, Italy) was performed at 37 °C for 15 min in cultures and for 10 min for olive samples, followed by a washing step with ice-cold 1× PBS (Oxoid). The hybridisation buffer (25% formamide, 0.9 M NaCl, 0.01% SDS, 20 mM Tris–HCl [pH 7.2]) (30 μl) containing 10 ng of 16S rRNA probe (Invitrogen) was spotted onto the dry specimen, and the slides were incubated in a dark humid chamber at 42 °C for 3 h. A washing step in a pre-warmed washing buffer (20 mM Tris–HCl [pH 7.2], 0.01% SDS, 40 mM NaCl, 5 mM EDTA) was then performed at 42 °C for 15 min. Microscopic observations were carried out with the epifluorescence microscope Nikon E400 (Nikon Co. Ltd, Tokio, Japan). Images were captured by using the digital camera Nikon E4500 (Nikon Co. Ltd, Tokio, Japan). All FISH experiments were performed in triplicate to confirm the results; at least 30 fields were observed for each specimen at a magnification of ×100.

3. Results and discussion

In Fig. 1 a sequence alignment of the LbpV3 probe targeted 16S rRNA fragment of the 28 species of *Lactobacillus* considered in this study is reported. The reported alignment considers a fragment from position 463 to position 500 of *E. coli* 16S rDNA. As shown in Fig. 1 the region targeted by the LbpV3 probe is exactly identical for *L. plantarum*, *L. paraplantarum* and *L. pentosus* while 1 to 7 nucleotide mismatches can be found in the same fragment of the other *Lactobacillus* species. Particularly, *L. arizonensis* (Swezey et al., 2000) is shown to be very related to the other members of the *L. plantarum* group displaying 1 mismatch only within the region targeted by the LbpV3 probe. The regions flanking the LbpV3 probe target also displayed from 1 to 4 nucleotide mismatches between the species of the *L. plantarum* group and all the other considered species. The suitability of 16S rRNA probes for FISH is related to the probe accessibility to 16S rRNA in its natural conformation (Fuchs et al., 1998; Fuchs et al., 2000). Therefore, nucleotide differences in 16S rRNA fragments of different species, which are lying outside the specific target of the FISH

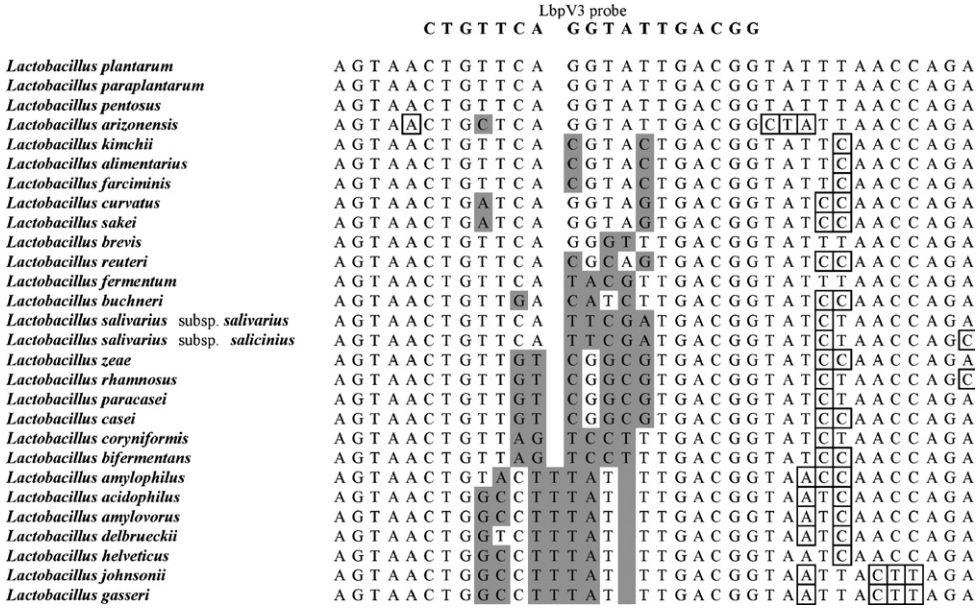

Fig. 1. Sequence alignment of the 16S rDNA (position 463 to 500 in *E. coli* numbering) of the 28 species of *Lactobacillus* used in this study. The sequences are aligned to the LbpV3 probe: nucleotide differences from the species of the *Lactobacillus plantarum* group in the probe region are highlighted in grey; differences falling outside the probe region are boxed.

probe, may also influence the specificity of the FISH detection because they can influence the conformation of the 16S rRNA in the ribosome and thus the availability of the target sequence for the specific probe.

The probe LbpV3 hybridised only the three species of the *L. plantarum* group in FISH experiments carried out at the established conditions of stringency. On the other hand, in each experiment the universal probe Eub338 gave positive results for all the other species used (Table 1). Cell permeabilization was shown to be fundamental for a positive response to FISH reaction, in fact, even using the universal probe Eub338, strains of *L. brevis* and *L. arizonensis* had to be treated up to 25 min at 37 °C with proteinase K in order to obtain probe access and hybridisation signal. The probe LbpV3 had been already shown to be capable of detecting *L. plantarum* in cheese but its specificity had been proven only against *L. curvatus*, *Lactococcus lactis*, *Enterococcus faecalis*, *Leuconostoc* spp. and *Staphylococcus equorum* (Ercolini et al., 2003b). On the basis of these results this probe can be considered valid to detect by FISH the species of the *L. plantarum* group. The FISH detection may be used to identify *Lactobacillus* isolates from food products or to rapidly screen food matrices for the presence of the species of the group. The probe does not provide a species-specific identification, as it does not discriminate between the three species of the *L. plantarum* group. However, other methods have been exploited to distinguish *L. plantarum*, *L. paraplantarum* and *L. pentosus* (Berthier and Ehrlich, 1998; Quere et al., 1997; Torriani et al., 2001b) and a rapid multiplex PCR targeting the *rec*A gene has been shown to be effective in the discrimination of the three species in a single assay (Torriani et al., 2001a). Therefore, FISH can rapidly assess the presence of the members of the *L. plantarum* group in food and environmental samples and, only when it is required, a further discriminative assay will be necessary to identify the species within the group.

The validated probe was used for the screening of olive samples for the presence of members of the *L. plantarum* group. Twenty-six samples of olives (cultivar "Leccino") to be used in natural fermentations were collected from different zones of the Campania region, Southern Italy. All of them were subjected to FISH experiments as described in Materials and methods but none of them gave positive result to FISH experiments with the probe LbpV3. However, all the samples yielded positive results by using the universal probe Eub338 (Amann et al., 1990). As shown in Fig. 2 (Panels A and B), both rods and cocci could be detected. In order to prove that if a member of the *L. plantarum* group had been present, it would have been detected by LbpV3-FISH, experiments were carried out by artificially contaminating the Ringer's suspension of olive samples by 10^6 loads of *L. plantarum* DSM20174. As shown in Fig. 2 (Panel C) it was possible to detect *L. plantarum* DSM20174 when present in the samples. However, the conditions of fixation and permeabilization have been once again fundamental in analysing olive samples. In fact, the duration of fixation (2 h) and the time of treatment of the samples with proteinase K (8 min) were established as optimal for detecting lactobacilli in the presence of Gram negative bacteria. The presence of Gram negative bacteria on the surface of olives was demonstrated by several authors (Borcakli et al., 1993; Tassou et al., 2002). Longer fixation or exposition to proteinase K led to lysis of Gram negative bacteria during FISH reactions and caused a consistent background signal when the specimens were observed, due to the probe binding to cell lysates on the slides (results not

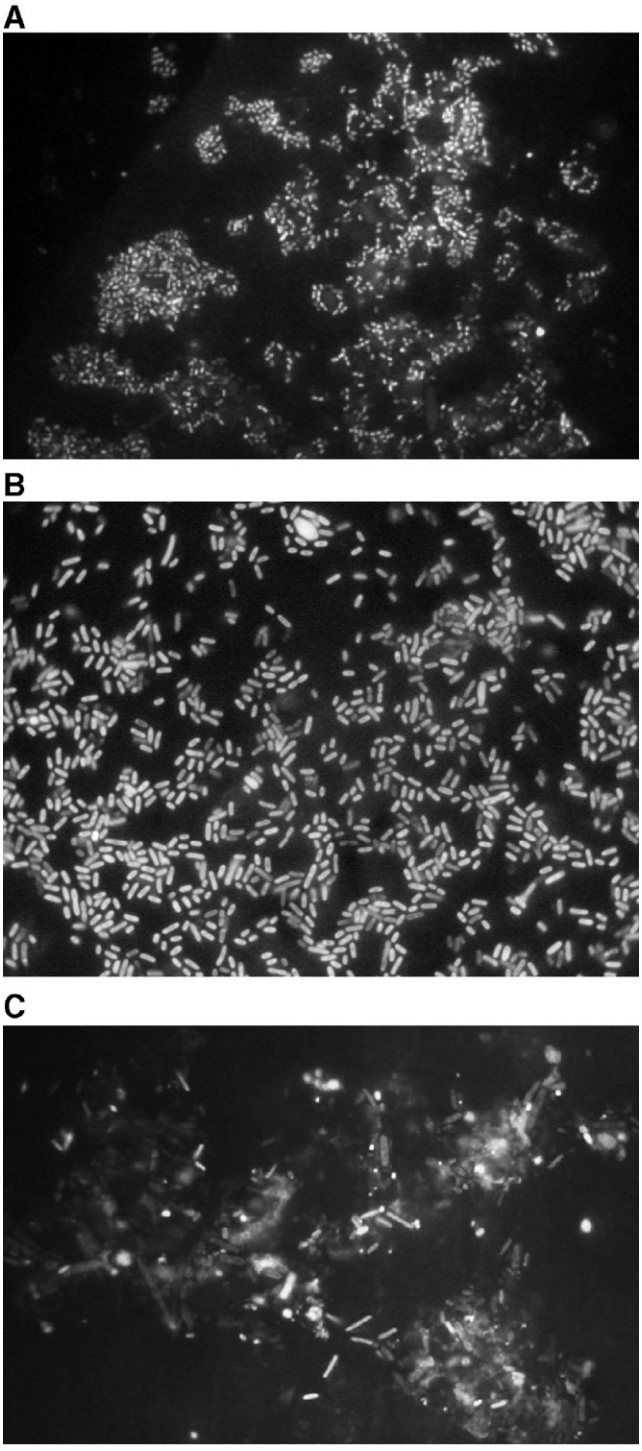

Fig. 2. FISH of olives suspension in Ringer's solution. Panels: A and B, olives suspension hybridised with the probe Eub338, cocci (A) and rods (B); C, olive suspension containing *L. plantarum* DSM 20174 hybridised with the probe LbpV3.

shown). Therefore, fixation and permeabilization conditions need to be previously optimised when Gram positives have to be detected by FISH in samples also containing Gram negative bacteria.

According to these results, members of the *L. plantarum* group were either absent in our olive samples or were present at loads beneath the detection limit of FISH that is recognised to be around 10^5 cfu^{-1} (Amann et al., 1995). In our case the minimal detectable potential load of *L. plantarum* could be under 10^5 also considering that 10 were concentrated and analysed. This aspect was further investigated by cultivating the lactic acid bacterial flora of the olive samples on Rogosa agar. Only 16 out of 26 olive samples showed microbial growth on Rogosa agar after 5 days of incubation at 30 °C in anaerobiosis. As a matter of fact, Rogosa is recognised as a selective medium for lactobacilli and was used in order to detect the lactic acid bacteria possibly responsible of olive fermentation.

Only 5 to 70 microbial colonies were found on the plates even though dilutions were not performed and 10 of olive suspensions were concentrated and plated on Rogosa. The microbial colonies were collected in bulks and analysed by PCR–DGGE after DNA extraction. The PCR–DGGE analysis of bulk cells from culture media has been proved to be a valid approach to rapidly assess bacterial species diversity after cultivation (Ercolini et al., 2003b; Ercolini, 2004). The PCR–DGGE profiles are shown in Fig. 3. A 16S rDNA V3 amplicon from *L. plantarum* DSM20174 was loaded in each DGGE gel as marker but none of the DGGE bands migrated the same distance in the gel suggesting that *L. plantarum* did not occur on Rogosa plates. All the DGGE fragments were sequenced; all of the sequences retrieved corresponded to portions of 16S rDNA of Bacteria. Fragments migrating the same distance in the gel gave exactly the same sequences. The closest relatives, the percent identities, and the accession numbers of the closest relatives are reported in Table 2. None of the sequenced fragments was the closest relative of a species of the genus *Lactobacillus*. *Leuconostoc pseudomesenteroides* was the most frequently found species, occurring in more than 50% of the samples that had shown growth on Rogosa agar. *Leuconostoc mesenteroides* was also found in 4 out of 16 samples while *Pediococcus pentosaceus* was present in one sample only. Noteworthy, Gram negative bacteria were also found such as *Pseudomonas* spp. and *Raoultella terrigena* even though a culture medium for lactobacilli was employed. This suggests that Gram negatives were abundant in the olives analysed.

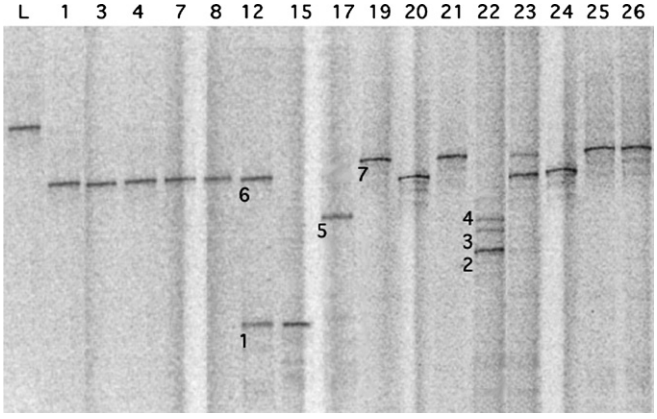

Fig. 3. PCR–DGGE profiles of bulk cells from Rogosa agar plates. The lane number also indicates the sample number. Lane L, PCR amplicon from *Lactobacillus plantarum* DSM 20174.

Table 2
Sequencing results of 16S-V3 rDNA fragments from DGGE gels

Band number [a]	Closest relative	% Identity	Source [b]
1	*Raoultella terrigena*	96%	AY292874
2	*Pseudomonas* spp.	99%	AY342005
3	*Pseudomonas* spp.	99%	AY342005
4	*Pseudomonas* spp.	99%	AY342005
5	*Pediococcus pentosaceus*	98%	AJ305321
6	*Leuconostoc pseudomesenteroides*	98%	AF515228
7	*Leuconostoc mesenteroides*	98%	AY453065

[a] Bands are numbered as indicated in Fig. 3.
[b] Accession number of the sequence of the closest relative found in GenBank with the Blast search.

Lactobacilli are often indicated as the principal bacteria involved in olive fermentation (Ciafardini et al., 1994; Ruiz-Barba et al., 1994; Durán Quintana et al., 1999; Sánchez et al., 2001; Randazzo et al., 2004); however, as result of this study, none of the species of this genus was found in the raw material analysed. In natural olive fermentations lactobacilli are usually detected by cultivation methods at significant loads after at least 10 days from the beginning of the technological process (Ruiz-Barba et al., 1994; Sánchez et al., 2001; Tassou et al., 2002). In this study lactobacilli were not found even though 10 of raw olive suspensions were analysed. Our results suggest that lactobacilli may also originate from the environment or tools of production and not exclusively from the olives. Very low percentages of lactobacilli were found on olive leaves by Ercolani (1991). Moreover, a significant occurrence of leuconostocs on olives was highlighted in this study. Indeed they might play a role in the fermentation as they usually do in other kinds of food fermentation (Carr et al., 2002). However, they are not usually recognised as halotolerant, which makes it difficult for them to contribute to olives production in brine. On the other hand, a high load population of lactic cocci was observed during olive fermentation in other studies (Durán Quintana et al., 1994; Leal-Sánchez et al., 2003).

At the best of our knowledge this is the first study that investigates on the LAB ecology of raw olives to be used in natural fermentations. A few other studies have dealt with the ecology of fermented olives as the final product (Nychas et al., 2002; Tassou et al., 2002; Randazzo et al., 2004) or have described conditions for guided olive fermentations (Ruiz-Barba et al., 1994; Durán Quintana et al., 1999; Sánchez et al., 2001; de Castro et al., 2002; Leal-Sánchez et al., 2003). The knowledge of the ecology of olives to be used in natural fermentation is important in order to define the appropriate conditions for fermentation. The fermentation is fundamental in order to have an appropriate decrease in pH and the development of a lactic acid microbial flora that can out compete and prevent the growth of spoilage microorganisms such as moulds and yeasts as well as potential pathogenic microbes. FISH may

be a useful tool for the detection of high loads of lactobacilli during the fermentation. The olive samples analysed in this study were shown to be mostly populated by leuconostocs among LAB microbial species. However, the lack of significant loads of lactobacilli and the occurrence of Gram negative bacteria suggest that the use of starter cultures for guided olive fermentations is advisable and highlights the need to deepen the studies on the ecology of olives as raw materials, in order to select the appropriate conditions of fermentation aimed to the improvement of the quality and safety of table olives.

Acknowledgements

We would like to thank Prof. Salvatore Coppola for encouragement and Massimo D'Agostino for technical support.

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