Study of the bacterial diversity of foods: PCR-DGGE versus LH-PCR

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Abstract

The present study compared the two culture-independent methods, polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE) and length-heterogeneity polymerase chain reaction (LH-PCR) for revealing food bacterial microbiota. Total microbial DNA and RNA were extracted directly from fourteen fermented and unfermented foods, and domain A of the variable regions V1 and V2 of the 16S rRNA gene was analyzed through LH-PCR and PCR-DGGE. Finally, the outline of these analyses was compared with bacterial viable counts obtained after bacterial growth on suitable selective media.

For the majority of the samples, RNA-based PCR-DGGE revealed species that the PCR-DGGE based on DNA analysis was not able to highlight. Either by analyzing DNA and RNA, LH-PCR identified several lactic acid bacteria (LAB) and coagulase negative cocci (CCN) species that were not identified by PCR-DGGE. This phenomenon was particularly evident in food samples with viable loads < 5.0 Log cfu g^{-1}. Furthermore, LH-PCR was able to detect an higher number of peaks in the analyzed food matrices respect to the PCR-DGGE signals. From these considerations an higher sensitivity of LH-PCR respect to PCR-DGGE may be suggested. However, PCR-DGGE, allowed the identification of some other species (LAB included) not identified by LH-PCR. By consequence, certain LH-PCR peaks not attributed to known species within the LH-PCR database could be solved by merging PCR-DGGE identification results. Overall, this study also showed that LH-PCR is a promising method for food microbiology, indicating the necessity to expand the LH-PCR database, which is based, up to now, only on LAB isolates from dairy products. This study also represents a contribution to the knowledge about the bacterial microbiota occurring in some foods that have been poorly investigated, such as seaweeds and soy-based products (tofu, soy “milk”, soy “yogurt”).

Highlights

- The foods’ bacterial microbiota was explored by LH-PCR, PCR-DGGE and viable counts,
- The total microbial DNA and RNA were extracted directly from foods
- Only partial overlapping of bacteria was found by using LH-PCR and PCR-DGGE
- A higher sensitivity of LH-PCR respect to PCR-DGGE may be suggested
- The LH-PCR database needs to be expanded

Keywords: bacterial microbiota; PCR-DGGE; LH-PCR; fermented and unfermented foods; DNA; RNA

1. Introduction

In the food microbiology field, there is a continuous and increasing interest in profiling microbial food ecosystems in order to characterize food fermentation, to preserve foods from spoilage and to investigate the ecology of food-borne pathogens. Indeed, the food microbiota can be distinguished as pathogens, spoilage and pro-technological depending on their role in the food ecosystem. The presence and the relative abundance of one or all these groups can vary based on the type of foods and the possible contamination (generally classified in primary or secondary) or after the deliberate adjunct of starter cultures, as are often applied for fermented food production.

Until 30 years ago, the growth of microorganisms on synthetic media was the only way to perform microbiological investigation of foods. After the development of PCR, it has become possible to develop several molecular techniques aimed to identify the food-borne microorganisms by avoiding cultivation. These "culture-independent techniques" analyze nucleic acids (DNA and/or RNA) extracted directly from food microbial cells in order to study the microbial ecology and dynamics of food ecosystems (Cocolin et al., 2013). Several advantages of the culture-independent methods over culture-dependent methods may be underlined: i) the chance to investigate food microbial populations independently from the capacity of the microorganisms to grow on synthetic media, which is often linked to the difficulty of a such media to reproduce the microbial natural habitat conditions; ii) the microbiota of a specific food is examined irrespective of the physiological status of the microbial cells [i.e., Viable But Not Cultivable (VBNC), stressed and/or injured cells]; iii) the less represented microbial cells may not be revealed through traditional microbiological methods; iv) the rapidity and reliability of PCR-based methods (Cocolin et al., 2013; Ercolini et al., 2004).

Furthermore, it is important to note that it is possible to analyze either DNA or RNA using culture-independent methods. By analyzing the total microbial DNA extracted directly from a food ecosystem, it is possible to gain information about the microbial diversity. RNA analysis [and in particular analyses of the ribosomal RNA (rRNA)] is useful to define the microbial species that are either metabolically active and consequently participate in food transformation/fermentation (Cocolin et al., 2013; Dolci et al., 2013), or dormant or dead non-lysed bacteria that can contain high numbers of ribosomes (Blazewicz et al., 2013).

Among the several culture-independent methods based on PCR, the polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE) and length-heterogeneity polymerase chain reaction (LH-PCR) techniques show great potential for outlining the microbial diversity. PCR-DGGE has been widely used for profiling microbiota of foods and environmental samples since the late 1990s (Aquilanti et al., 2016a; Cocolin et al., 2013). LH-PCR is most commonly used to monitor microbial community changes in soils or other environments different from foods (Brusetti et al., 2006;

Moreno et al., 2011; Suzuky, 1998). LH-PCR has been applied from 2008 to study the LAB composition of different dairy foods, such as milk, curd and cheeses during the ripening period and also in natural whey starters for cheese production (Gatti et al., 2014; Neviani et al., 2013). To our knowledge, this community-level molecular technique based on 16S rRNA gene analysis has never been used to investigate the microbiota of other foods. For this reason, the only bacterial food database available is that built with bacterial strains of dairy origin (Gatti et al., 2008; Lazzi et al., 2004). The aim of this study was to compare the efficiency of the two culture-independent methods PCR-DGGE and LH-PCR in studying the bacterial diversity of several fermented and unfermented foods in comparison with viable counts obtained after bacterial growth on different selective media. To the best of our knowledge these two techniques have never been compared before by analyzing either the total microbial DNA or RNA extracted directly from the food samples.

2. Materials and Methods

2.1. Reference strains and culture conditions

Two bacterial reference strains (*Lactobacillus brevis* DSMZ 20556 and *Lactobacillus plantarum* DSMZ 2601) were used as controls in the PCR-DGGE analyses. These cultures were purchased from the *Deutsche Sammlung von Mikrorganismen und Zellkulturen* (DSMZ Collection, Braunschweig, Germany, http://www.dsmz.de/) and grown on MRS agar (Oxoid, Basingstoke, UK) at 30°C for 48 h under anaerobiosis.

2.2. Sampling

The fourteen food samples were arbitrarily chosen among fermented and not fermented foods both of animal and vegetal origin. Particularly, *burrata*, butter, cream cheese, feta cheese, kefir, pasteurized milk, salami, seaweed, soy "milk", soy sprouts, soy "yogurt", table olives, tofu and tomatoes were analyzed. They were purchased in local groceries, and for each food sample we prepared a bulk made from 5 to 10 subsamples, taking into account different product brands and expiry dates.

2.3. Sample preparation

For solid foods, 10 g of each subsample was homogenized in 90 mL of sterile peptone water (0.1% peptone), by using a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy) at 260 rpm for 3 min. The bulk of each solid food sample was prepared by combining all subsample homogenates in a sterile container and mixing with a magnetic stirrer

for 15 min. The bulks of liquid food samples were prepared by pouring 10 mL of each subsample into a sterile container and mixing as described above.

2.4. Bacterial viable counts

Serial dilutions of the bulks were performed in sterile peptone water, and aliquots (100 µL for the spread-plate method or 1 mL for the pour-plate method) were streaked in triplicate onto opportune agar plates. Media and growth conditions used for enumeration of the main groups of the culturable bacteria (aerobic mesophilic bacteria, mesophilic and thermophilic streptococci, mesophilic and thermophilic lactobacilli, micrococci and staphylococci, *Pseudomonodaceae*, enterococi, total coliforms and *Enterobacteriaceae*) present in food samples are shown in Table 1. The results of the viable counts are expressed as mean ± standard deviation of the Log of colony-forming units (cfu) per gram or milliliter of sample.

2.5. DNA extraction from the food samples

An aliquot (1 mL) of bulk from each food sample was centrifuged at 16,000 g for 3 min, and the microbial DNA was extracted from the pellets using a DNeasy Blood & Tissue Kit (Qiagen, Venlo, The Netherlands) according to the kit manufacturer's instructions. The DNA quantity and purity were assessed using a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

2.6. RNA extraction from the food samples and cDNA synthesis

For the extraction of microbial RNA, a 2 mL aliquot of bulk from each food sample was centrifuged for 5 min at 16,000 g, the supernatants were discarded, the pellets were covered with RNA later Stabilization Solution (Ambion, Foster City, CA, USA), and they were stored at -80°C until the extraction. The total RNA was then extracted from the pellets with the RNeasy Mini Kit (Qiagen). The mechanical lyses of the cells, following the kit manufacturer's instructions, was performed using glass beads of 425-600 µm diameter (Sigma Aldrich, St. Louis, MO, USA) and a Mixer Mill MM 300 (Qiagen) at 30 Hz for 5 min. The RNA extraction proceeded according to the kit manufacturer's instructions.

One microliter of RNase-free DNase (DNase I Amplification Grade, Sigma Aldrich) was added to 8 µL of total extracted RNA, and the mixture was incubated at 37°C for 30 min to digest all residual DNA. The RNA samples were checked for the presence of residual DNA by PCR amplification, and if PCR products were obtained, the DNase

157 treatment was repeated to eliminate DNA. The quantity and purity of the extracted RNA was determined using a
158 Nanodrop ND 1000 (Thermo Fisher Scientific); RNA quality was further checked by agarose gel (1%) electrophoresis.
159 Prior to cDNA synthesis, the concentration of isolated RNA was normalized to 50 ng μL^{-1}; 10 µL were then reverse
160 transcribed in cDNA using SensiFAST cDNA Synthesis Kit for RT-qPCR (Bioline, London, UK) as recommended by
161 the manufacturer. Oligo (dT) and random hexamer primers were used to prime the synthesis of first-strand cDNA.
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163 *2.7. PCR-DGGE and reverse transcription (RT)-PCR-DGGE analysis*
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165 *2.7.1 DNA extraction from references strains*
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167 Some colonies of the pure reference cultures were suspended in 300 µL of TE buffer (10 mMTris–HCl pH 8.0, 1 mM
168 EDTA pH 8.0), and the suspension underwent DNA extraction using the method proposed by Hynes et al. (1992) with
169 some modifications as described by Osimani et al. (2015). The DNA quantity and purity were assessed as described in
170 paragraph 2.5.
171
172 *2.7.2. PCR-DGGE protocol*
173
174 Bacterial DNA and synthesized cDNA were amplified with primers 63F (5'- CAGGCCTAACACATGCAAGTC -3')
175 (Lane, 1991) and 355R (5'- GCTGCCTCCCGTAGGAGT -3') (Amann et al., 1990) which amplify domain A of the
176 variable regions V1 and V2 of the 16S rRNA gene; a theoretical amplicon length of approximately 276–327 bp was
177 expected (Castillo et al., 2006; Garcia-Garcerà et al., 2012; Grice et al., 2008; Suzuki et al., 1998). A GC clamp (5'-
178 CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') was attached to the 5' end of the
179 forward primer (63F). Approximately 50 ng of template DNA and 2 µL (about 50 ng) of cDNA were amplified in a 50
180 µL reaction volume containing 1.25 U of *Taq* DNA polymerase (AmpliTaq Gold, Life Technologies, Milan, Italy), 1X
181 reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.5 µM of each primer. The amplification reactions were
182 performed in a thermal cycler (My cycler, Bio-Rad Laboratories, Milan, Italy) using the following cycling program:
183 initial denaturation at 95°C for 10 min, followed by 25 cycles of denaturation at 95°C for 45 sec, annealing at 49°C for
184 45 sec and extension at 72°C for 2 min. The final extension was at 72°C for 7 min (Lazzi et al., 2004).
185 Five microliters of each PCR product was checked by electrophoresis in 1.5% (w/v) agarose gel in 0.5X TBE (45
186 mMTris-borate, 1 mM EDTA) containing 0.5 µg mL^{-1}ethidium bromide at 100 V for 45 min, using the Hyper Ladder

100 bp (Bioline, London, UK) as a molecular weight standard. Gels were visualized under UV light and photographed with the Complete Photo XT101 system (Explera, Jesi, Italy).

A vertical electrophoresis system DCode (Bio-Rad Laboratories) was used for the DGGE analysis. PCR products (20 µL) obtained with primers $63F_{GC}$/355R were applied to 0.8 mm polyacrylamide gel [8% (w/v) acrylamide/bisacrylamide gel 37.5:1], containing a 30-60% urea-formamide denaturing gradient that increased in the direction of the electrophoresis (100% corresponded to 7 M urea and 40% (w/v) formamide), and run with 1X TAE buffer (0.04 mol L^{-1}Tris-acetate, 0.001 mol L^{-1} EDTA). The gels were run at a constant voltage of 130 V for 4.5 h at 60°C. After electrophoresis, the gels were stained for 20 min in TAE 1X containing SYBR Green I Stain 1X (Lonza, Walkersville, MD, USA), visualized under UV light and photographed with the Complete Photo XT101 system (Explera). To allow the standardization of band migration and gel curvature between different gels, a reference ladder made with 5 µL of the PCR products obtained from the DNA extracted from pure cultures of each of two reference strains was loaded in the gel. Amplification reactions and DGGE runs were performed in duplicate on the same extracts.

2.7.3. Sequencing of the DGGE bands and sequence analysis

All the DGGE bands were excised from the gels using sterile pipette tips, and the DNA from each band was eluted in 50 µL sterile deionized water overnight at +4°C, as performed by Garofalo et al. (2008). Five microliters of the eluted DNA was re-amplified under the same conditions as described in paragraph 2.7.2., but using forward primer 63F without the GC clamp. These PCR amplicons were then sent to Beckman Coulter Genomics (London, UK) for purification and sequencing. The electropherograms were also checked and edited to remove unreadable portions (mainly at 5' and 3' ends) or to detect double peaks. Finally, the sequences in FASTA format were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the basic BLAST search tools (Altschul et al., 1990).

2.8. LH-PCR analysis

The V1 and V2 16S rRNA gene regions of bacterial DNA and synthesized cDNA were amplified by using primers 63F and 355R. The 63F primer was 5′ end-labelled with 6-carboxyfluorescein (FAM). Length heterogeneity in the PCR amplicons was detected by capillary electrophoresis (ABI Prism 310, Applied Biosystems, Foster City, USA). The PCR and capillary electrophoresis conditions were those described by Bottari et al. (2010). The fragment sizes (base pairs) were determined using GeneMapper software version 4.0 (Applied Biosystems, Foster City, USA) and the local

Southern method to generate a sizing curve from the fragment migration of the internal size standard (GS500 LIZ®; Applied Biosystems Foster City, USA). The minimum noise threshold was set at 150 fluorescence units. The peaks, corresponding to amplicons of specific length on the electropherogram profiles, represent fragments of different sizes, and the areas under the peaks depend on the amounts of the fragments (Lazzi et al., 2004). Each peak, corresponding to amplicons of specific length on the electropherogram profiles, was putatively attributed to a bacterial species according to a published database (Lazzi et al., 2004; Gatti et al., 2008). LH-PCR assays were performed in duplicate on the same DNA extracts and cDNAs used for PCR-DGGE analyses.

3. Results

3.1. Bacterial viable counts

To support the culture-independent analyses, eight different selective media were used in order to enumerate and detail as much as possible the cultivable fraction of the main bacterial groups presumptively present in 14 food samples: mesophilic and thermophilic streptococci, mesophilic and thermophilic lactobacilli, micrococci and staphylococci, aerobic mesophilic bacteria, *Pseudomonodaceae*, enterococi, total coliforms and *Enterobacteriaceae*. The results of bacterial viable counts are shown in Tables 3 and 4. On the basis of the results of bacterial viable counts, the fourteen selected food samples were divided in two categories. The first category included samples with viable counts on PCA higher than 5.0 Log cfu g^{-1} or Log cfu mL^{-1} (*burrata*, feta cheese, kefir, salami, soy sprouts, soy "yogurt" and tofu) (Table 3), while the second category included samples with viable counts on PCA lower than 5.0 Log cfu g^{-1} or Log cfu mL^{-1} (butter, cream cheese, pasteurized milk, seaweed, soy "milk", table olives, and tomatoes) (Table 4).

3.2. PCR-DGGE and *RT-PCR-DGGE analyses*

The DGGE profiles obtained from the analysis of the bacterial DNA extracted directly from the 14 food samples are shown in Figure 1 (panel A). DGGE fingerprints of the bacterial communities of most of the food samples analyzed, such as tofu, soy "yogurt", kefir, feta cheese, salami, seaweeds, butter, soy "milk", and tomatoes, were rather simple, containing 1 to 3 different bands (Fig. 1-panel A). By contrast, soy sprouts, *burrata*, pasteurized milk, cream cheese, and table olives showed more complex DGGE profiles (from 4 to 6 bands).

As shown in Figure 1 (panel B), the gel obtained after RT-PCR-DGGE analysis was characterized by a profile richer in bands than that in the PCR-DGGE gel achieved by analyzing DNA. The closest relatives, the percent identities, and the

accession numbers of sequences obtained from the PCR-DGGE and RT-PCR-DGGE bands are reported in Table 2. For the majority of the samples, the sequencing results of the bands excised from the RT-PCR-DGGE gel highlighted species not previously detected by DNA analysis (Table 2, Table 3 and Table 4). The DNA-based PCR-DGGE technique allowed the detection of several bacterial species ascribed to *Lactobacillus*, *Leuconostoc*, *Staphylococcus*, *Pseudomonas*, *Anoxybacillus*, *Acinetobacter*, *Pantoea* and *Allomonas*, while some more species ascribed to *Bacillus*, *Bradyrhizobium*, *Sphingomonas*, *Sphingobium*, *Caldilinea*, *Sulfobacillus*, *Curvibacter*, *Lysinibacillus*, *Enterobacter*, *Aeromonas* and *Serratia* were identified through the RNA-based PCR-DGGE analysis.

Unexpectedly, for foods of plant origin (except soy sprouts), the sequencing results of some or all excised DGGE bands revealed the presence of eukaryotic chloroplast DNA related to the corresponding plants. Specifically, the DNA of *Glycine soja* chloroplast was detected in soy-based foods (tofu, soy "yogurt" and soy "milk"), while the chloroplast DNA of *Solanum lycopersicum* and *Olea europea* was found in tomatoes and table olives, respectively.

3.3. LH-PCR analysis

Results of LH-PCR analysis consist of electropherogram profiles as shown in Figure 2. Each peak of the LH-PCR electropherogram corresponds to a DNA (or cDNA) amplicon. The different amplicon sizes of the peaks were used to identify bacterial species by comparison with LH-PCR database. LH-PCR analysis performed on the 14 food samples did not generally show differences in terms of species identification within samples by analyzing microbial DNA or RNA (Tables 3 and 4). In detail, 42 DNA and 39 cDNA amplicons were found, but among these, only 11 DNA amplicons and 10 cDNA amplicons could be attributed to known bacterial species of the LAB and cocci coagulase-negative (CCN) categories. The remaining 31 DNA amplicons and 29 cDNA amplicons did not match any known species in the LH-PCR references database (unattributed amplicons). Among these unattributed amplicons, only 14 were in common between LH-PCR and RT-LH-PCR, while the other 33 (18 DNA amplicons and 15 cDNA amplicons) were different. In particular, soy "milk", butter and tomatoes showed only unattributed DNA and cDNA amplicons (Table 4).

3.4 PCR-DGGE vs LH-PCR

Considering the number of detected peaks by DNA and RNA-based LH-PCR respect the number of species detected by DNA and RNA-based PCR-DGGE method, LH-PCR seemed to be a generally more sensitive technique for study the bacterial diversity of foods (Tables 3 and 4). In general, LH-PCR was able to identify more LAB and CCN species respect PCR-DGGE, but this latter method was able to identify even other different species. As expected, a high

biodiversity was observed for the first group of food samples characterized by viable load on PCA higher than 5.0 Log cfu g^{-1} or mL^{-1} (Table 3). In detail, *Lb. delbrueckii* (feta cheese and kefir) and *Lb. helveticus* (kefir) were the only species detected by both methods. Other LAB species such as *Str. thermophilus* (burrata, feta cheese, kefir and salami), *Lb. helveticus* (burrata, feta cheese), *Lc. lactis* (feta cheese, kefir, salami, soy sprouts), *E. faecalis* (feta cheese, kefir, tofu), *Lb. rhamnosus* (feta cheese), *P. acidilactici* (feta cheese, salami) and *Lb. plantarum* (salami) the same as CCN species *K. kristinae* (soy sprouts, tofu) were detected only by LH-PCR. Some other LAB species as *Lb. crispatus* (burrata, kefir), *Lb. ruminis* (feta cheese), *Lb. sakei* (salami), and *Leuc. mesenteroides* (feta cheese) were identified exclusively by PCR-DGGE. This method also allowed the detection of other species ascribed to *Pseudomonas*, *Staphylococcus*, *Bacillus*, *Pantoea*, *Rhizobium*, *Enterobacter*, *Lysinibacillus*, *Aeromonas* and *Serratia* throughout different food samples. PCR-DGGE detected only chloroplast DNA in soy "yogurt" while LH-PCR method was able to identify two LAB species (*Lb. delbrueckii* and *Lc. lactis*) for this food sample. Moreover, LH-PCR showed 1 (salami) to 8 (tofu) unattributed peaks for this group of analyzed samples.

The bacterial diversity of the second group of foods characterized by viable load on PCA lower than 5.0 Log cfu g^{-1} or mL^{-1} (Table 4) was poorly described by LH-PCR as some food samples like butter, soy "milk" and tomatoes had only unattributed peaks (up to 5), while other samples had only few identified peaks. *Str. thermophilus* was the only species identified in cream cheese and pasteurized milk by this method. This species was also detected in table olives together with *E. faecalis*, *Lb. helveticus*, *Lb. fermentum* and *Lb. plantarum*; the latter species was identified even by RT-PCR-DGGE. The only two species detected by LH-PCR in seaweeds were *E. faecium* and *K. kristinae*. The only LAB detected exclusively by PCR-DGGE was *Leuc. mesenteroides* in cream cheese and table olives. Using this method, other species ascribed to *Pseudomonas*, *Bradyrhizobium*, *Curvibacter*, *Acinetobacter*, *Bacillus*, *Anoxybacillus*, *Undibacterium*, *Lewinella*, *Sphingobium*, *Caldilinea*, *Sulfobacillus*, *Allomonas* and *Sphingomonas* were also detected throughout different food samples.

4. Discussion

In the present study, PCR-DGGE and LH-PCR analyses were carried out by using either DNA or RNA as template to study the bacterial diversity of different foods. Specifically, rRNA has been widely applied to assess the metabolically active populations of foods and environmental communities because it is has a much shorter half-life time than DNA after cell lyses (Blazewicz et al., 2013; Bleve et al., 2003; Cocolin et al., 2013; Dolci et al., 2013, 2015). Furthermore, the detection of rRNA is linked to the presence of ribosomes and therefore protein synthesis, indicating that it can be considered a suitable marker of viability (Cocolin et al., 2013; Dolci et al., 2013, 2015). However, most up-to-date

311 literature (not restricted to food-ecosystems) reveals that the general use of rRNA as a reliable indicator of metabolic
312 state in microbial assemblages has limitations. Blazewicz et al. (2013) highlights the complex and often contradictory
313 relationships between rRNA, growth and activity. For example, dormant cells can contain high numbers of ribosomes.
314 Therefore, while DGGE and LH-PCR, based on DNA analyses, highlights the presence of bacteria either dead (intact
315 and lysed) or viable or dormant, RT-PCR-DGGE and RT-LH-PCR analyses reveal the non-lysed dead cells, viable
316 bacteria as well as the dormant cells within the food ecosystems under study. Interestingly, in the present study, in some
317 cases RT-PCR-DGGE found bacterial species that PCR-DGGE did not (each food sample was analyzed twice and the
318 results were reproducible). This result is in agreement with a previous study performed by Dolci and colleagues (2013)
319 on Fontina PDO cheese. Indeed, they found that RNA was a more informative target than DNA in profiling the bacterial
320 dynamics of this smear-ripened cheese. This phenomenon was explained by the higher abundance of rRNA copy
321 numbers respect to 16S rRNA gene copy numbers for bacterial cells (Dolci et al., 2013; Prosser et al., 2010). This effect
322 is even more evident for species with several copies of rRNA operon (is possible to have from 1 to 15 copies)
323 distributed within bacterial chromosome. During PCR amplification the most abundant template are amplified and
324 therefore RNA target analyses may result by higher sensitivity respect to DNA target analyses. It has been proposed that
325 low represented bacterial community (less than 1% of the total microbiota) cannot be detect by using DNA-based PCR-
326 DGGE thus limiting the complete description of a bacterial ecosystem. On the opposite, RT-PCR-DGGE may overcome
327 this detection threshold and thus provides a more complete and reliable picture of the microbiota within a food
328 environment (Dolci et al., 2013; Prosser et al., 2010). A few other microbiological studies have used RT-PCR-DGGE to
329 profile the microbiota of foods and in particular cheeses, such as *Planalto de Bolona*, Castelmagno, Ragusano and feta
330 cheese (Alessandria et al., 2010; Dolci et al., 2010; Randazzo et al., 2002; Rantsiou et al., 2008). To our knowledge,
331 RT-LH-PCR has been applied only twice, and for studying natural whey starters used to produce Grana Padano cheese
332 (Rossetti et al., 2009; Santarelli et al., 2008).
333 Regarding PCR-DGGE, it is interesting to note that by using either DNA or RNA, in some food samples, despite the
334 complex DGGE profiles, after sequencing of all of the DGGE bands with different electrophoretic mobilities in the gel,
335 the same species were found. This PCR-DGGE drawback is mainly related to the presence of 16S rRNA gene
336 heterogeneous multi-copies in the bacterial genome of the same species (Cocolin et al., 2013; Dolci et al., 2015;
337 Ercolini, 2004; Garofalo et al., 2015 a, b).
338 The DNA from the three food samples based on soy (tofu, soy "yogurt" and soy "milk") showed the same DGGE
339 profile, probably related to soy as raw material. In these three soy-based foods, together with tomatoes and table olives,
340 the sequence alignments with sequences deposited in GenBank showed the presence of chloroplast DNA with high
341 identity percentage (99%). This result could be explained by the fact that when studying the composition of bacteria in

eukaryotic organisms, a separation of nucleic acids from bacteria and eukaryotes may be impossible (Huys et al., 2008). Furthermore, the primers used in this study, targeting the V1 variable region of the bacterial 16S rRNA gene, did not present a complete specificity for bacterial DNA and amplified eukaryotic DNA as well. The presence of eukaryotic chloroplast DNA bands was more frequent in PCR-DGGE than in RT-PCR-DGGE. Large amounts of eukaryotic chloroplast/mitochondria DNA can interfere with the PCR amplification decreasing the amount of bands from the microorganisms. This negative effect can probably be reduced in RT-PCR-DGGE, because cDNA was a much purified starting material for PCR, thus further increasing the efficiency of this method with respect to DNA-based PCR-DGGE. In particular, with PCR-DGGE or LH-PCR it was not possible to define the bacterial ecology of soy "milk" except for the detection of *B. megaterium* through RT-PCR-DGGE. This result can be indeed explained by a very low bacterial contamination (low viable counts) probably resulting in a small amount of bacterial DNA extracted compared to eukaryotic DNA from soy. In contrast, despite the high viable counts obtained in soy "yogurt", *Lb. delbrueckii* and *Lc. lactis* were the bacterial species identified solely by LH-PCR. In tofu, RT-PCR-DGGE allowed the identification of various species and genera belonging to *Enterobacteriaceae*, such as *En. cloacae*, *Enterobacter* sp., *En. ludwigii*, *Serratia* sp., and *Aeromonas* sp. This result was also confirmed by the bacterial load obtained on VRBA (5.1 ± 0.02 Log cfu g^{-1}) and VRBGA (3.8 ± 0.13 Log cfu g^{-1}) for this type of soy-based food. By LH-PCR and RT-LH-PCR, we detected *E. faecalis* and *K. kristinae*. This finding is in agreement with previous studies conducted on traditional fermented soybean products (Feng et al., 2013) and with the viable counts on M17, MRS, SBA and MSA.

In soy sprouts, spoilage agents such as *Pseudomonas* sp. and *Ps. plecoglossicida* together with *Pantoea* sp., *P. anthophila* and *R. giardinii* were found by means of PCR-DGGE and RT-PCR-DGGE. The detection of *Pseudomonas* sp. was also confirmed by viable counts on PAB, highlighting a high level of this contaminant corresponding to 8.3 ± 0.08 Log cfu g^{-1}. *Pseudomonas* sp. includes Gram-negative rod-shaped bacteria considered food spoilage agents of proteinaceous foods such as meat, poultry, fish, shellfish, milk and some dairy products (Franzetti and Scarpellini, 2007; Liao, 2006). Soy natural contaminants *K. kristinae* (Feng et al., 2013) and *Lc. lactis* were also found by LH-PCR and RT-LH-PCR.

Among the complex and extremely variable microbiota of kefir (Prado et al., 2015), *Lb. helveticus* and *Lb. delbrueckii* (although this latter with sequence identity lower than 97%) were found by either PCR-DGGE or LH-PCR with both approaches (DNA and RNA). RT-PCR-DGGE also found *L. fusiformis*, a new species previously detected in soy-based fermented foods (Chettri and Tamang, 2015), while LH-PCR and RT-LH-PCR detected other LAB, such as *Lc. lactis*, *E. faecalis* and *Str. thermophilus*. This microbial pattern was also confirmed by the high viable counts on LAB media (MRS and M17) and on enterococci selective media (SBA) on the order of 7-8 Log cfu g^{-1}.

In feta cheese, *Lb. delbrueckii* was revealed by using DNA and RNA as target by both molecular techniques applied in this study. Six other LAB species, *Str. thermophilus*, *Lc. lactis*, *E. faecalis*, *Lb. helveticus*, *Lb. rhamnosus* and *P. acidilactici*, were also identified by LH-PCR and RT-LH-PCR and had high viable counts on selective media for LAB and specifically for enterococci (from 5.1 to 7.5 Log cfu g^{-1}).

The microbiota of salami had different profiles from the two techniques. PCR-DGGE and RT-PCR-DGGE confirmed the presence of *St. saprophyticus*, *Lb. sakei* and *St. xylosus*, as frequently reported in the literature (Aquilanti et al., 2016). Intriguingly, high loads on the micrococcus- and staphylococcus-selective medium MSA were observed (7.2 ± 0.04 Log cfu g^{-1}). LH-PCR and RT-LH-PCR showed the dominance of *Lb. plantarum*, *P. acidilactici*, *Lb. fermentum*, *Lc. lactis*, *Str. thermophilus* and *E. faecalis*. High viable counts on LAB- and enterococcus-selective media (MRS, M17 and SBA), ranging from 5.1 to 7.6 Log cfu g^{-1}, were found.

In *burrata*, *Pseudomonas* sp. and *Ps. fragi* were detected by PCR-DGGE either by analyzing DNA or RNA, which was also supported by their viable counts on PAB on the order of 4 Log cfu g^{-1}. *Lb. crispatus* was also identified by DNA-based PCR-DGGE. Thermophilic LAB, commonly used as starter cultures to produce high-moisture mozzarella cheese, such as *Str. thermophilus* and *Lb. helveticus* (De Angelis and Gobbetti, 2011), were detected only by LH-PCR and RT-LH-PCR. This finding is in agreement with high viable counts (> 5.0 Log cfu g^{-1}) on M17 and MRS agar plates incubated at 42°C.

In table olives, chloroplast DNA of *Oleae europaea* was found together with bacterial species ascribed to *Leuc. mesenteroides* and *Ac. johnsonii* by DNA-based PCR-DGGE. *Lb. plantarum*, *Br. cytisi*, *Ac. baumannii*, *Ps. fluorescens*, and *Sphingomonas* sp. were also found at the RNA level. No viable counts on PAB were found thus indicating that the rRNA from *Pseudomonas* could derive from VBNC or dormant or dead non-lysed cells. Similarly, *E. faecalis* was identified by RT-LH-PCR despite no growth was observed on enterococci selective media (SBA). Moreover, *Lb. fermentum*, *Lb. helveticus*, *Str. thermophilus* and *Lb. plantarum* were found by combination of LH-PCR and RT-LH-PCR in accordance with the results of the viable counts on LAB selective media (MRS and M17), ranging from 3.8 to 3.9 Log cfu g^{-1}.

Members of the genus *Acinetobacter* were also detected by PCR-DGGE (DNA and RNA) in pasteurized milk together with *Pseudomonas* sp., *U. oligocarboniphilum* and thermal-resistant spore-forming bacilli such as *A. flavithermus* and *Bacillus* sp. Again, there was no visible bacterial growth on PAB as previously observed for table olives. The only species identified by RT-LH-PCR was *Str. thermophilus*, as previously reported by Delgado and colleagues (2013). Despite the low viable counts on M17 agar at 42°C (2.3 Log ufc mL^{-1}) RT-LH-PCR was able to detect *Str. thermophilus* showing a higher sensitivity of this method respect PCR-DGGE.

To our knowledge, a paucity of data are present in the literature concerning the microbiota of seaweeds. In the present study, although with a very low percentage of identity (78%), the closest relatives to *Lewinella* sp. were found in seaweeds through PCR-DGGE. Because species belonging to this genus are marine bacteria (Oh et al., 2009), it is possible to speculate that it is effectively present in this matrix. Furthermore, other species with environmental origin were found by using RT-PCR-DGGE. In detail, besides *B. smithii*, *C. aerophila* was isolated from a hot spring sulfur-turf in Japan (Sekiguchi et al., 2003), *S. acidophilus* was isolated from a hydrothermal vent in the Pacific Ocean (Li et al., 2011), and *Sphingobium* sp. comprises species generally isolated from soil (Singh and Lal, 2009). Despite low bacterial load found on MSA (2.0 Log cfu g^{-1}) and on SBA (1.4 Log cfu g^{-1}) *K. kristinae* was detected by DNA and RNA based LH-PCR, while *E. faecium* was identified only by DNA-based LH-PCR, thus confirming the high sensitivity of this technique. The presence of these species on seaweeds may be due to human contamination (*K. kristinae*) or could arise from aquatic habitats (*E. faecalis*) (Alexander et al., 2015).

Even if non bacterial growth was detected on *Pseudomonas* selective media (PAB) for cream cheese, this food sample was contaminated by *Pseudomonas* sp. and *Ps. fragi* at the DNA and RNA levels detected by using PCR-DGGE, together with *Leuc. mesenteroides* (DNA level), *Bradyrhizobium* sp. and *Cu. lanceolatus* (RNA level). *Str. thermophilus*, which is commonly used as starter to produce this type of cheese (Buriti et al., 2007), was the only species detected by DNA based LH-PCR. Despite the relatively high bacterial load of 3.8 Log cfu g^{-1}, enumerated after growth on M17 agar at 42°C, the latter species was not identified by RNA based LH-PCR. This result could indicate the growth of other thermophilic LAB which could be identified after database enlargement since two unattributed peaks were detected at RT-LH-PCR.

Like cream cheese, butter was contaminated with spoilage bacteria, specifically *Ps. putida* and *Ps. reinekei*, at the DNA and RNA levels by using PCR-DGGE. Intriguingly, no viable cells were found on PAB, probably because *Pseudomonadaceae* species were present in their VBNC form or dormant or dead non-lysed cells. In contrast, low viable counts on VRBA (2.4 ± 0.05 Log cfu g^{-1}) and VRBGA (2.3 ± 0.02 Log cfu g^{-1}) were found, although *Enterobacteriaceae* were not identified with PCR-DGGE and LH-PCR. These results could be explained by the low detection limit of these techniques or by the limited LH-PCR database as some unattributed peaks that were detected could be related to *Enterobacteriaceae*.

The presence of chloroplast DNA of *Solanum lycopersicum* in tomatoes was detected by PCR-DGGE together with the viable contaminant *Staphylococcus* sp. although with sequence identity lower than 97%, while LH-PCR was not able to identify any bacterial species by using DNA or RNA as the target.

Overall, by comparing PCR-DGGE and LH-PCR results, some interesting evidences emerged. In contrast to PCR-DGGE, which allows the identification of the species by comparing the obtained sequences with a huge number of

sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/), LH-PCR peaks identification is based, up to date, on a dairy database mainly composed by LAB species (Gatti et al., 2008; Lazzi et al., 2004). One of the intriguing results of this study is that the identification of unattributed LH-PCR peaks, could be solved by merging PCR-DGGE identification results. For instance, on the basis of the PCR-DGGE results, the unattributed 306-base-pair amplicon detected by LH-PCR in table olives, pasteurized milk, cream cheese, butter, burrata and soy sprouts could be hypothesized to be a member of the genus *Pseudomonas*. On the other hand, LH-PCR allowed the detection of some LAB and CCN species not identified by PCR-DGGE. This phenomenon was particularly evident in food samples with viable loads < 5.0 Log cfu g^{-1}, specifically in pasteurized milk and seaweeds, thus suggesting the higher sensitivity of LH-PCR respect PCR-DGGE. In samples with viable bacterial loads > 5.0 Log cfu g^{-1} (burrata, kefir, feta cheese and salami), instead, LAB species were generally also detected by PCR-DGGE. These findings were expected, as LAB are the dominant bacteria in these fermented foods. Discrepancies among LH-PCR and PCR-DGGE results in species identification could be due to different sensitivity of the techniques. For both these techniques, the available literature reports the ability to detect only the dominant species. In particular, a detection limit of 3 Log cfu per milliliter or per gram was found for PCR-DGGE (Cocolin et al., 2013) and approximately 4-5 Log cfu per milliliter or per gram was indicated for LH-PCR (Lazzi et al., 2004; Santarelli et al., 2013) although the detection limit for this latter method was defined only for milk and dairy products (Lazzi et al., 2004; Santarelli et al., 2013). However, this limit may vary depending on the complexity of the microbiota and of the nature of the food matrix (Cocolin et al., 2013; Ercolini et al., 2004). Furthermore, in the present study, LH-PCR was able to detect an higher number of fragments in the food matrices analyzed respect to the PCR-DGGE signals. This phenomenon could be explained by the fact that LH-PCR amplicons are separated through capillary electrophoresis, differently from PCR-DGGE that uses gel separation. Capillary electrophoresis has been reported to have higher sensitivity, resolution and discriminatory power compared to gel-based electrophoresis (Liljander et al., 2009; Beaubier et al., 2000). Moreover, DGGE bands from more than one species may comigrate which could result in underestimation of bacterial diversity (Heuer et al., 2001; Ercolini, 2004). Additionally, fair bands on the gel images may possibly be invisible to the eye and therefore remain undetected. Therefore, from all these considerations it is reasonable to suppose that in terms of sensitivity and resolution, the performance of LH-PCR could be greater than PCR-DDGE.

5. Conclusions

The efficiency of the two culture-independent methods PCR-DGGE and LH-PCR was compared by analyzing the bacterial ecology of 14 different foods (fermented and non fermented) using both DNA and RNA extracted directly

from food matrices as targets. Furthermore, the obtained results were compared with viable counts on different selective media chosen on the basis to cover the main bacterial groups presumptively present in tested food samples. Both culture-independent methods showed to be able to identify bacteria at species level fast and accurately without need of cultivation and isolation steps which are often laborious and time consuming. A generally good correlation was seen between the species identified by culture-independent methods and corresponding bacterial groups enumerated on eight different selective media (particularly evident for RNA-based PCR-DGGE and LH-PCR). For the majority of the samples, RT-PCR-DGGE revealed more species respect PCR-DGGE thus confirming the importance to use RNA as target instead of DNA to elucidate the food bacterial diversity. LH-PCR was able to identify several LAB and CCN species not detected by PCR-DGGE. This phenomenon was particularly evident in food samples with viable loads < 5.0 Log cfu g^{-1}, thus suggesting the higher sensitivity of LH-PCR respect to PCR-DGGE. Furthermore, if considering the number of detected peaks, LH-PCR showed again higher sensitivity when compared with PCR-DGGE method, but due to limited database based only on milk and dairy products, its main disadvantage remain the impossibility to identify those peaks. On the other hand, PCR-DGGE, a widely employed molecular technique in the food microbiology field allowed identification of some other species (LAB included) not identified by LH-PCR, hence some peaks corresponding to DNA or cDNA amplicons not identified by LH-PCR might probably be related to species identified by PCR-DGGE. As only partial overlapping of bacteria was found by using the two techniques, a combined use of LH-PCR and PCR-DGGE could be useful to describe accurately the food microbiota. The results of this study also showed that LH-PCR is a promising method for food microbiology, hence a further effort on LH-PCR database enlargement is recommended. This study also represents a contribution to the knowledge about the bacterial microbiota occurring in some foods that have been poorly investigated, such as seaweeds and soy-based products (tofu, soy “milk”, soy “yogurt”).

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Figure captions

Fig. 1. Bacterial DGGE profiles of the DNA (panel A) and RNA-cDNA (panel B) extracted directly from food samples and amplified with primers $63F_{GC}$ and 355R. The bands indicated by the letters were excised, re-amplified and subjected to sequencing. The identification of the bands is reported in Table 1; "L" indicates Ladder composed of *Lactobacillus plantarum* DSMZ 2601 (1) and *Lactobacillus brevis* DSMZ 20556 (2).

Fig. 2. Examples of bacterial LH-PCR electropherogram profiles of the DNA (panel A) and RNA-cDNA (panel B) extracted directly from food samples (soy "milk" and feta cheese) and amplified with primers 63F and 355R. The X-axis shows peak sizes as base pairs (bp) and the Y-axis shows the peak intensity as relative fluorescence units. Some peaks sizes were attributed to bacterial species according to the LH-PCR published database (Lazzi et al., 2004; Gatti et al., 2008), as follows: *Streptococcus thermophilus* (318), *Lactococcus lactis* (319), *Enterococcus faecalis* (329), *Lactobacillus delbrueckii* (330), *Lactobacillus helveticus* (334), *Lactobacillus rhamnosus* (336, 290) and *Pedicoccus acidilactici* (345). The other peaks are unattributed.