Cheese surface microbiota complexity: RT-PCR-DGGE, a tool for a detailed picture?

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Running title: RT-PCR-DGGE: a tool for complex microbiota investigations

Cheese surface microbiota complexity: RT-PCR-DGGE, a tool for a detailed picture?

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ABSTRACT

In this work, a culture-independent approach, based on PCR-DGGE and RT-PCR-DGGE, has been used to study the succession of bacterial communities that are encountered in Fontina PDO cheese. As already found for other smear ripened cheeses, it appeared that coryneform bacteria were actively present and could therefore be considered determinant in rind formation. DGGE profiles, especially at the RNA level, have shown the presence of *Brevibacterium*, *Corynebacterium* and *Arthrobacter* genera. RT-PCR-DGGE gels have lead to a richer band profile than the one obtained on the basis of DNA analysis, thus indicating that RNA analysis can highlight bacterial species that DNA analysis is not able to show. Thus, the biodiversity of the Fontina PDO surface has been described better by means of RT-PCR-DGGE, and RNA molecules should be considered a more informative target than DNA.

Keywords: DGGE; cheese surface; RNA; coryneform bacteria

1. Introduction

Culture-independent methods have been developed to determine the diversity of microorganisms in natural ecosystems and to monitor the evolution of microbial population over time. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and PCR-temperature gradient gel electrophoresis (PCR-

TGGE) were first introduced some years ago in the environmental microbiology field and are now routinely used in many laboratories throughout the world as molecular methods to study population composition and dynamics in food-associated microbial communities. PCR-DGGE has been widely employed in dairy microbiology (Coppola et al., 2008; Ercolini, 2004; Randazzo et al., 2009). The diversity and dynamics of microbial populations in cheese, and during cheese manufacturing, have been profiled by means of DNA-based experiments by different authors (Alegrìa et al., 2009; Belén Flòrez and Mayo, 2006; Bonetta et al., 2008; Casalta et al., 2009; Coppola et al., 2001; Dolci et al., 2008, Ercolini et al., 2003; Gala et al., 2008; Randazzo et al., 2006). Reverse transcription (RT)-PCR-DGGE has been also performed, but to a lesser extent, to obtain a picture of the species metabolically active at a particular sampling instant (Alessandria et al., 2010; Dolci et al., 2010; Masoud et al., 2011; Randazzo et al., 2002; Rantsiou et al., 2008). Since the half-life of DNA may vary to a great extent in dead bacterial cells, and is highly dependent on environmental conditions (Cenciarini-Borde, 2009; Keer and Birch, 2003), the use of retrotranscribed RNA as a template is suggested by many authors to overcome this critical issue. DNA from lysed cells can persist for a long time in the environment, while RNA is a better indicator of viable microorganisms as it is degraded rapidly upon cell death (Santarelli et al., 2008). Thus, since the numbers of intact ribosomes approximately reflects the rates of protein synthesis, ribosomal RNA (rRNA) can be used as a marker of the general metabolic activity. Although it should be considered that rRNA molecules are characterized by a much higher level of protection than messenger RNA (mRNA), which has an average half-life that can be measured in minutes, the use of rRNA molecules as the target allows to avoid practical problems of extracting detectable levels of intact mRNA and of distinguishing mRNA from the corresponding homologous DNA sequences.

In this work, a culture-independent approach, based on PCR-DGGE and RT-PCR-DGGE, has been used to study the succession of microbial communities in Fontina PDO cheese, a traditional ripened Italian cheese, characterized by alternating dry salted and brine washed treatments, which allow the characteristic red-brown rind to develop. Fontina PDO is a semi-cooked cheese produced from full

cream from raw cow milk from a single milking, which is fermented with selected autochthonous *Streptococcus thermophilus* and/or *Lactococcus lactis* strains. The milk is coagulated at 36°C and the curd is cut finely while the temperature is gradually raised to 46-48°C. The curd, after being left briefly to rest in whey, is collected in moulds and pressed to eliminate any residual whey. Cheese ripening, which takes place in natural caves under a temperature that varies from 5 to 12 °C, usually lasts three months. In a previous research, culture-dependent and –independent approaches were applied to study the evolution of microbial communities in Fontina PDO rind in order to assess the influence of the maturing environment on the surface ecology (Dolci et al., 2009). The complexity of the cheese surface microbiota is due to the richness of the microorganisms involved in smear rind formation. The difficulty of detecting the presence of these microorganisms, through standard culture-based methods, can be considered a challenge to understand this complex microbial ecology. Consequently, culture –independent methods offer a better way of investigating cheese surface microbiology. In this study, both PCR-DGGE and RT-PCR-DGGE were applied to surface and subsurface layers as well as to core samples of cheese in order to obtain a more complete "picture" of the succession and localization of bacterial communities in Fontina PDO cheese. Both DNA and RNA were used as the targets for DGGE analysis. The results suggest that RT-PCR-DGGE based on RNA targets gives more detailed information on the microbiota of the cheese.

2. Materials and methods

2.1 Sample collection

Fifteen Fontina PDO cheeses were produced from the same milk batch, with the addition of selected autochthonous *Streptococcus thermophilus*, *Lactococcus lactis* and *Lactobacillus delbrueckii* strain cultures. These cheeses were left to ripen in three different caves in the Aosta Valley region (northwest Italy): Prè-Saint-Didier (PS), Issogne (IS) and Ollomont (OL). In each cave, five cheeses were placed on the same shelf, under controlled temperature (ranging from 5 to 12 °C) and humidity (above 90%) conditions.

Samples of the cheese surfaces were collected and microbiologically analysed at 7, 28, 56 and 84 days from the production. In addition, two layers per sample were cut up and analysed: a 4-cm-thick section from the core of the product and a 3-cm-thick section from the subsurface, taken at precisely 2 cm under the rind.

2.2 Microbial analysis

Ten grams of the cheese samples was homogenized in a Ringer quarter strength solution (Oxoid, Basingstoke, UK), using a Stomacher machine, serially diluted in the Ringer solution and plated onto specific media for viable counts. Presumptive lactococci and lactobacilli were grown, respectively, on M17 agar, aerobically, and on MRS agar pH 5.8 (Fluka, Buchs SG, Switzerland), in anaerobic jars, and incubated at 37 °C for 48 h. Coryneforms were selected on Plate Count Agar (Oxoid) supplemented with 5% NaCl and 0.002% pimaricin (PCAC) at 30°C for 72 h, plus a further 96 h of daylight at room temperature. Yellow-to-orange colonies were assumed to belong to coryneform bacteria (Dolci et al., 2009).

A surface area of 100 cm^2 of each sample was wiped clean with a sterile swab, transferred to 10 mL of physiological solution, homogenized, decimally diluted in a Ringer solution and plated onto the previously mentioned selective media. Sampling was performed in triplicate. In order to avoid repeatedly scraping of the same area of the cheese surfaces, five cheeses per cave were considered for each sampling time.

2.3 Nucleic acid extraction

The total DNA and RNA were extracted directly from the samples, according to the protocol reported in Rantsiou et al. (2008). Briefly, 1 mL of homogenized cheese and surface samples were submitted to centrifugation for 10 minutes at maximum speed. The pellet was re-suspended in 120 µL of proteinase K buffer (50 mM Tris-HCl, 10 mM EDTA, pH 7.5, 0.5 % [wt/vol] sodium dodecyl sulphate), 20 µL of proteinase K (25 mg/mL, Sigma, Milan, Italy) and 20 µL of lysozyme (50 mg/mL, Sigma) and treated for 1 h at 50 °C. The mixture was transferred into 1.5 mL screw cup tubes, containing glass beads, and 150 µL of 2X breaking buffer (4 % Triton X-100 [vol/vol], 2 %

[wt/vol] SDS, 200 mM NaCl, 20 mM Tris, pH 8, 2 mM EDTA, pH 8), 300 µL phenol-chlorophorm-isoamyl alcohol (25:24:1, pH 6.7; Sigma) were added for DNA extraction, or 300 µl of phenol-chlorophorm (5:1, pH 4.7; Sigma) was added for the extraction of the RNA. Three cycles (30 sec at 4.5 motion/sec) were performed in a bead beater (Fast Prep-24, MP Biomedicals, Solon, OH). After the treatment, 300 µl of TE (10 mM Tris, 1 mM EDTA) was added to the tubes and centrifugation was performed at 14000 rpm for 5 min. The aqueous phase was moved to a new tube and precipitated with ice-cold absolute ethanol. The nucleic acids were collected by means of centrifugation at 14000 rpm for 10 min, washed briefly in 70% ethanol and re-suspended in 50 µl of sterile water. Three microliters of TURBO - DNase (Ambion, Milan, Italy) was added to digest the DNA in the RNA samples, with an incubation of 3 h at 37ºC. The presence of residual DNA in the RNA samples was checked by PCR (Cocolin et al., 2001).

2.4 PCR-DGGE and RT-PCR-DGGE analysis.

The extracted DNA and RNA were subjected to PCR and RT-PCR, with universal primers 338f (5'-ACT CCT ACG GGA GGC AGC AGC AG-3') and 518r (5'- ATT ACC GCG GCT GCT GG-3') (Ampe et al., 1999) annealing to the bacterial V3 region of the 16S rRNA gene (Alessandria et al., 2010). The amplicons were then subjected to DGGE analysis. A GC clamp (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') was attached to the end of 5' of primer 338f for the DGGE analysis. A denaturant gradient, ranging from 30 to 60%, was created in a 1X TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 M EDTA [pH 8]) in an 8% (wt/vol) polyacrylamide gel (acylamide-bis acrylamide, 37.5:1) with a Dcode universal mutation detection system (BioRad, Milan, Italy) (Dolci et al., 2008).

The gels were subjected to a constant voltage of 120 V for 4 h at 60°C, stained in 1X TAE containing 1X SYBR Green I (Sigma), and analysed under UV ~~by~~ using UVIpro Platinum 1.1 Gel Software (Eppendorf). Selected DGGE bands were extracted from the gels, checked by means of DGGE and sent for sequencing to MWG Biotech in order to identify the species (Dolci et al., 2008).

Partial 16S rRNA gene sequences were aligned with those in GenBank with the Blast program to determine the closest known relatives of the bands (Altschul et al., 1997).

2.5 Statistical analysis

The BioNumerics software (Applied Maths, Kotrijk, Belgium) was applied to normalize and submit the obtained DGGE profiles to Cluster Analysis. The Pearson product moment correlation coefficient was used to calculate the similarities in the DGGE patterns, and dendrograms were obtained by means of the unweighted pair group method with arithmetic averages.

3. Results and discussion

Lactic acid bacteria (LAB) were found with comparable values of 10^7-10^8 colony forming units/ cm^2 or g, in the surface, core and subsurface layer samples. Coryneforms were instead detected at 10^4-10^5 cfu/g in the core and subsurface layer samples, while they were initially present on the surface with values of 10^4-10^5 cfu/cm^2 and then reached 10^8-10^9 cfu/cm^2 at the end of the ripening.

As found in a previous study (Dolci et al., 2009), *S. thermophilus* and *L. lactis* were found to be present on the surface (Fig. 1a and 1b). Interestingly, direct analysis of rRNA revealed their active role in only a few samples (Fig. 1b). Moreover, RT-PCR-DGGE occasionally showed LAB belonging to *Lactobacillus* genera, that is, *Lb. delbrueckii*, *Lb. amylovorus* and *Lb. helveticus* (Fig. 1b), which are not commonly found in cheese surface niches. It can therefore be stated that the metabolically active role of the LAB population on the Fontina PDO surface requires further investigation.

It appeared, as for other smear-ripened cheeses, that coryneform bacteria were actively present and can therefore be considered determinant in rind formation. The DGGE profiles (Fig.s 1a and 1b), especially at an RNA level (Fig. 1b), showed the presence of *Brevibacterium*, *Corynebacterium* and *Arthrobacter* genera. The RT-PCR-DGGE gels in fact gave more detailed and richer band profiles than those obtained from the DNA analysis. The species obtained from the sequence information

bands detected on the DGGE gels were *Brevibacterium stationis* and *Corynebacterium variabile* (Tab. 1).

From the analysis of the core and subsurface layer samples, it emerges that *S. thermophilus* and *Lb. delbrueckii* were present during cheese manufacturing where, as known, they act as starter cultures (data not shown). As revealed by RT-PCR-DGGE, they also appeared to be metabolically active during the ripening stages, together with *L. lactis*, which was present with stronger bands than in the PCR-DGGE profiles (data not shown).

Cluster analysis of the bacterial cheese surface (Fig. 2) revealed an important difference between the profiles obtained from the DNA and rRNA analyses. Two main clusters were detected with a very low percentage of similarity, and grouping rRNA samples (Fig. 2, cluster 1) and DNA samples (Fig. 2, cluster 2), as well as two rRNA samples clustering with DNA samples (Fig. 2, see the asterisk mark). Apparently the two approaches produce different information. While DNA based DGGE uses a pool of DNA from live VBNC (viable but non-culturable) and dead bacterial cells as a template, rRNA profiles instead highlight microorganisms that play an active role. In this sense, the "history" of the different microbial species followed in the analysed samples is recorded in electrophoretic profiles.

Moreover, dendrograms of rind samples, partially clustered on the basis of the different cave origins, (Fig. 2) and different species, have been highlighted by means of the RT-PCR-DGGE profiles (Fig. 1b), depending on the maturing environment. In particular, the surfaces of the cheeses ripened in the Prè-Saint-Didier cave have shown a large variety of microbial species, compared to the samples matured in the Ollomont and Issogne caves. This evidence supports the hypothesis that was made in a previous paper (Dolci et al., 2009) according to which the ripening environment seems to influence the microbiota that develops on Fontina PDO cheese and, consequently, the quality of the final product.

In this study, a detailed picture of the microbiota was observed when rRNA was targeted, and bacterial species that were not highlighted by means of the DNA analysis were detected (Tab. 1).

The biodiversity of the Fontina PDO surface was described better by RT-PCR-DGGE, and RNA molecules should be considered a more informative target than DNA. Bacterial rRNA genes are organized into a co-transcribed operon and from 1 to as many as 15 copies of the operon may be dispersed in a bacterial genome. For example, *Lb. delbrueckii* harbors at least six copies of the rRNA operon and these are heterogeneous in their sequences (Randazzo et al., 2002). In this sense, rRNA could be proportionally more abundant in some microbial cells than DNA copies, and this could lead to more detailed profiles in RT-PCR-DGGE and the evidence of "hidden" species in DGGE DNA-based profiles. It has not in fact been possible to highlight active microbial populations with low counts by means of DGGE analysis of the DNA molecules.

It has been estimated that populations that constitute less than 1% of the total community are generally not represented in DGGE profiles, and this therefore constitutes a detection threshold (Forney et al., 2004). As a result, the actual species richness of a community is not fully described and it may be impossible to determine it precisely. In fact, since PCR amplification of nucleic acid is a competitive enzymatic reaction, the small subunit rRNA templates in a sample are amplified according to their abundance. Nevertheless, RT-PCR-DGGE is able to assess the most active dominant populations at a single time in food fermentations more effectively than PCR-DGGE and thus gives a more significant image of the changes in the food environment (Bokulich and Mills, 2012).

Thus, in this paper, we suggest that RT-PCR-DGGE should be considered a better tool to profile microbial populations, and that the rRNA molecule is a worthwhile target for a better and more complete understanding of the microbial communities in food ecosystems.

References

Alegrìa, A., Alvarez-Martìn, P., Sacristàn, N., Fernàndez, E., Delgado, S., Mayo, B., 2009. Diversity and evolution of the microbial populations during manufacture and ripening of Casìn, a traditional Spanish, starter-free cheese made from cow's milk. International Journal of Food Microbiology 136, 44-51.

Alessandria, V., Dolci, P., Rantsiou, K., Pattono, D., Dalmasso, A., Civera, T., Cocolin, L., 2010. Microbiota of the Planalto de Bolona: an artisanal cheese produced in uncommon environmental conditions in the Cape Verde Islands. World Journal of Microbiology and Biotechnology 26, 2211-2221.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

Ampe, F., Ben Omar, N., Moizan, C., Wacher, C. Guyot, J.P., 1999. Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. Applied and Environmental Microbiology 65, 5464-5473.

Belén Flòrez, A., Mayo, B., 2006. Microbial diversity and succession during the manufacture and ripening of traditional, Spanish, blue-veined Cabrales cheese, as determined by PCR-DGGE. International Journal of Food Microbiology 110, 165-171.

Bokulich, N.A., Mills, D.A., 2012. Next-generation approaches to the microbial ecology of food fermentations. BMB reports 45, 377-389.

Bonetta, S., Bonetta, S., Carraro, E., Rantsiou, K., Cocolin, L., 2008. Microbiological characterisation of Robiola di Roccaverano cheese using PCR-DGGE. Food Microbiology 25, 786–792.

Casalta, E., Sorba, J.M., Aigle, M., Ogier, J.C., 2009. Diversity and dynamics of the microbial community during the manufacture of Calenzana, an artisanal Corsican cheese. International Journal of Food Microbiology 133, 243-251.

Cenciarini-Borde, C., Courtois, S., La Scola, B., 2009. Nucleic acids as viability markers for bacteria detection using molecular tools. Future Microbiology 4, 45-64.

Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Applied and Environmental Microbiology 67, 5113-5121.

Coppola, S., Blaiotta, G., Ercolini, D., Moschetti, G., 2001. Molecular evaluation of microbial diversity occurring in different types of mozzarella cheese. Journal of Applied Microbiology 90, 414-420.

Coppola, S., Blaiotta, G., Ercolini, D., 2008. Dairy Products. In: Cocolin L., Ercolini, D. (Eds.). Molecular techniques in the microbial ecology of fermented food. Springer, New York, pp. 31-90.

Dolci, P., Alessandria, V., Rantsiou, K., Rolle, L., Zeppa, G., Cocolin, L., 2008. Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. International Journal of Food Microbiology 122, 302-311.

Dolci, P., Alessandria, V., Rantsiou, K., Bertolino M., Cocolin L., 2010. Microbial diversity, dynamics and activity throughout manufacturing and ripening of Castelmagno PDO cheese. International Journal of Food Microbiology 143, 71-75.

Dolci, P., Barmaz, A., Zenato, S., Alessandria, V., Cocolin, L., Rantsiou, K., Ambrosoli, R., 2009. Maturing dynamics of surface microflora in Fontina PDO cheese studied by culture-dependent and -independent methods. Journal of Applied Microbiology 106, 278-287.

Ercolini, D, 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. Journal of Microbiological Methods 56, 297-314.

Ercolini, D., Hill, P.J., Dodd, C.E.R., 2003. Bacterial community structure and location in Stilton cheese. Applied and Environmental Microbiology 74, 3540-3548.

Forney, L.J., Zhou, X., Brown, C.J., 2004. Molecular microbial ecology: land of the one-eyed king. Current Opinion in Microbiology 7, 210-220.

Gala, E., Landi, S., Solieri, L., Nocetti, M., Pulvirenti, A., Giudici, P., 2008. Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. International Journal of Food Microbiology 125, 347-351.

Keer, J.T., Birch L., 2003. Molecular methods for the assessment of bacterial viability. Journal of Microbiological Methods 53, 175-183.

Masoud, W., Takamiya, M., Vogensen, F. K., Lillevang, S., Abu Al-Soud, W., Sørensen, S. J., Jakobsen, M., 2011. Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis and pyrosequencing. International Dairy Journal 21, 142-148.

Randazzo, C.L., Caggia, C., Neviani, E., 2009. Application of molecular approaches to study lactic acid bacteria in artisanal cheeses. Journal of Microbiological Methods 78, 1-9.

Randazzo, C.L., Torriani, S., Akkermans, A.D.L., De Vos, W.M., Vaughan, E.E., 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal sicilian cheese as evaluated by 16S rRNA analysis. Applied and Environmental Microbiology 68, 1882-1892.

Randazzo, C.L., Vaughan, E.E., Caggia, C., 2006. Artisanal and experimental Pecorino Siciliano cheese: Microbial dynamics during manufacture assessed by culturing and PCR-DGGE analyses. International Journal of Food Microbiology 109, 1-8.

Rantsiou, K., Urso, R., Dolci, P., Comi, G., Cocolin, L., 2008. Microbiota of Feta cheese from four Greek manufacturers. International Journal of Food Microbiology 126, 36–42.

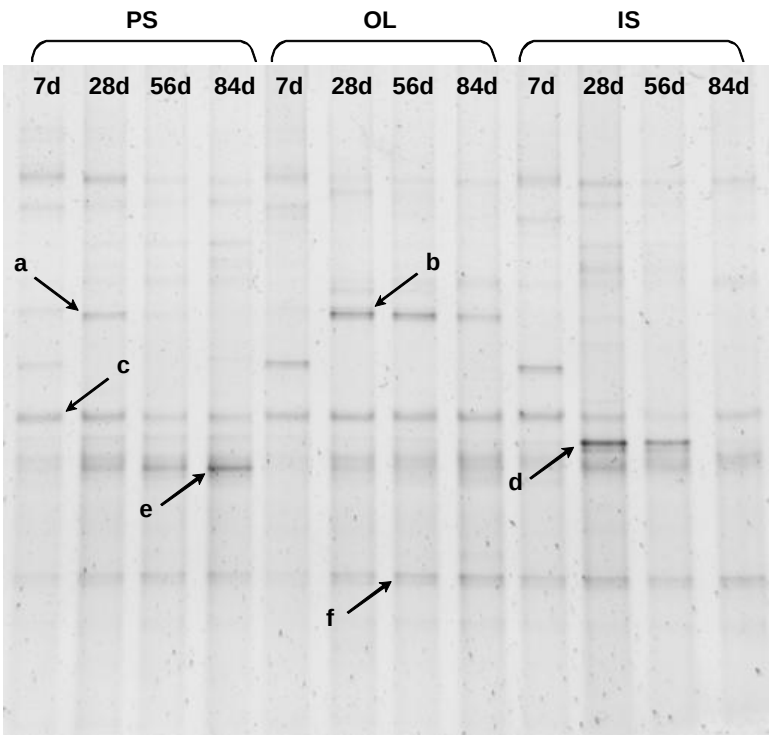
Santarelli, M., Gatti, M., Bernini, C. V., Zapparoli, G. A., Neviani, E., 2008. Whey starter for Grana Padano cheese: effect of technological parameters on viability and composition of the microbial community. Journal of Dairy Science 91, 883-891.

Figure legends:

Figure 1. DGGE profiles obtained from bacterial DNA (a) and RNA (b) extracted from surface samples of cheese analysed throughout Fontina PDO production (from day 7 to day 84) ripened in caves at Prè-Saint-Didier (PS), Ollomont (OL) and Issogne (IS). The bands were identified by sequencing and the obtained results are reported in Table 1.

Figure 2. Dendogram obtained from the cluster analysis of DGGE profiles of bacterial microbiota detected on the Fontina PDO surface. The surface samples obtained from the cheeses ripened in the Prè-Saint-Didier, Ollomont and Issogne caves are indicated with PSD, OLL and ISS, respectively.

Figure 1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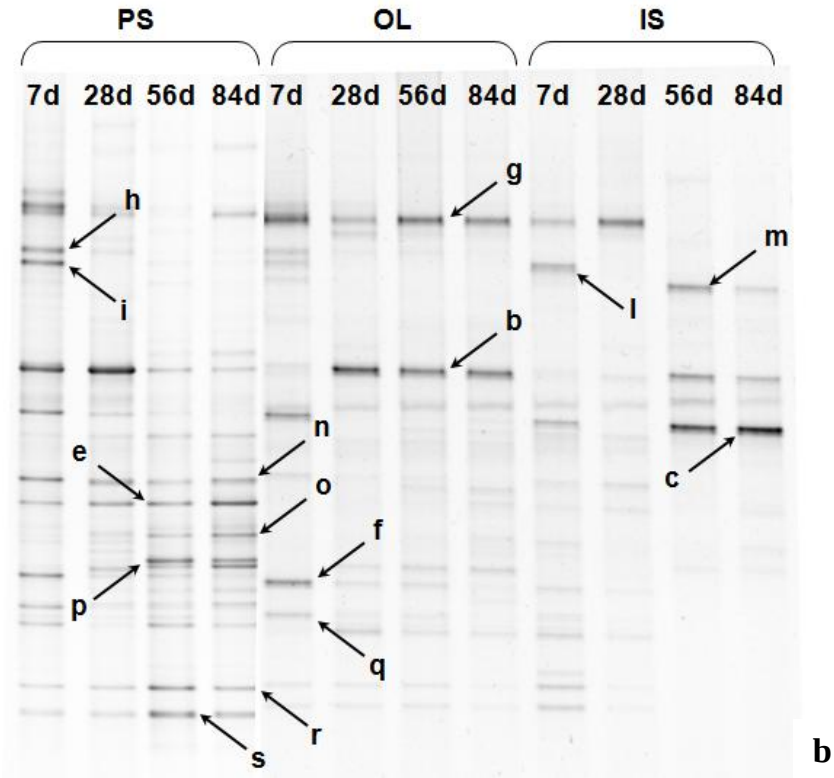
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Figure 2

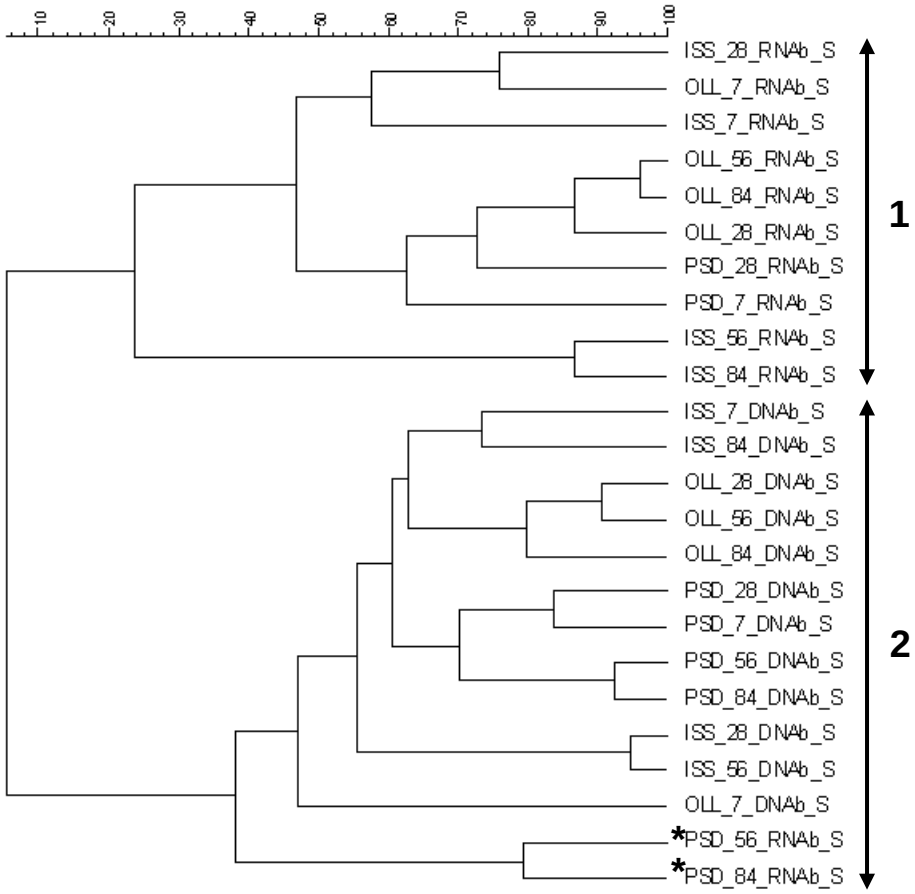
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Table 1. Identification of DGGE bands on the basis of a BLAST sequence comparison in GenBank.

Band	Closest relative	DNA analysis[a]	RNA analysis[a]	% Identity	Accession no.
a	*Psychrobacter* sp.	+	-	98%	EU237135.1
b	*Lactococcus lactis* subsp. *lactis*	+	+	98%	FJ378886.1
c	*Streptococcus thermophilus*	+	+	99%	FJ667772.1
d	Uncultured *Corynebacterium*	+	-	100%	EU029389.1
e	*Corynebacterium* sp.	+	+	99%	EF405602.1
f	*Brevibacterium stationis*	+	+	98%	FJ172667.1
g	Uncultured bacterium clone	-	+	99%	EU276170.1
h	*Staphylococcus* sp.	-	+	98%	EU278341.1
i	*Lactobacillus helveticus*	-	+	99%	FJ640998.1
l	*Lactobacillus amylovorus*	-	+	100%	EF439704.1
m	*Lactobacillus delbrueckii*	-	+	100%	EU886724.1
n	*Corynebacterium variabile*	-	+	100%	AM411116.1
o, q	*Arthrobacter* sp.	-	+	98%	FJ626618.1
p	Uncultured bacterium	-	+	99%	AM117141.1
r, s	*Brevibacterium* sp.	-	+	97%	EU931543.1

[a] The plus and minus signs mean the presence or absence on DGGE gels of the band referring to the indicated species.