UNIVERSITÀ DEGLI STUDI DI TORINO

Diversity and functional characterization of *Lactobacillus* spp. isolated throughout the ripening of a hard cheese

J. Bautista-Gallego[1*], V. Alessandria[1], M. Fontana[2], S. Bisotti[2], S. Taricco[2], P. Dolci[1], L. Cocolin[1], K. Rantsiou[1]

[1]University of Torino, DISAFA, Agricultural Microbiology and Food Technology Sector. Via Leonardo da Vinci 44, 10095 Grugliasco, Torino, Italy.

[2]Biraghi S.p.A., Via Cuneo 1, Cavallermaggiore 12030, Cuneo, Italy.

Running title: Lactobacilli ecology from a hard cheese

*Corresponding author

Joaquín Bautista-Gallego, Ph.D.

University of Turin, DISAFA

Via Leonardo da Vinci 44, 10095 Grugliasco, Italy.

Tel.: +39 011 6708553; fax: +39 011 6708549

e-mail address: joaquin.bautistagallego@unito.it

Abstract

The aim of this work was to study the *Lactobacillus* spp. intra- and inter- species diversity in a Piedmont hard cheese made of raw milk without thermal treatment and without addition of industrial starter, and to perform a first screening for potential functional properties. A total of 586 isolates were collected during the cheese production and identified by means of molecular methods: three hundred and four were identified as *Lactobacillus rhamnosus*, two hundred and forty as *Lactobacillus helveticus*, twenty six as *Lactobacillus fermentum*, eleven as *Lactobacillus delbrueckii*, three as *Lactobacillus pontis*, and two as *Lactobacillus gasseri* and *Lactobacillus reuteri*, respectively. A high genetic heterogeneity was detected by using the repetitive bacterial DNA element fingerprinting (rep-PCR) with the use of (GTG)$_5$ primer resulting in eight clusters of *L. helveticus* and sixteen clusters in the case of *L. rhamnosus*. Most of isolates showed a high auto-aggregation property, low hydrophobicity values, and a general low survival to simulated digestion process. However, sixteen isolates showed promising functional characteristics.

1. Introduction

In recent years, the consumption of cheese has increased rapidly owing to the fact that this dairy product fulfils many of the current dietary needs. In addition, cheeses have been described as potential probiotic food; it is hypothesized that they offer an excellent food based delivery vehicle by providing increased cell protection (Boylston et al., 2004). During recent years, numerous studies have been undertaken to obtain scientific evidences for the beneficial effects of fermented foods containing probiotic bacteria (Renault, 2002; Rafter, 2002). Semi-hard and hard cheeses, such as Cheddar (Darukaradhya et al., 2006; Phillips et al., 2006; Ong et al., 2006, 2007), Canestrato Pugliese (Corbo et al., 2001) and Gouda (Gomes et al., 1995), among others, have been tested as probiotic delivery vehicles.

The lactic acid bacteria (LAB) play an important role in the production of fermented foods, above all in cheese production. The isolation and characterisation of LAB from traditional dairy products have been carried out to obtain new industrially important cultures (Zhang et al., 2008). Pogačić et al. (2013) studied the microbiota during ripening of a long ripened hard cheese showing high biodiversity. Dolci et al. (2013) used RT-PCR-DGGE, as culture-independent method, to understand the microbial communities in Fontina PDO cheeses.

It is known that starter LAB (SLAB) and non starter LAB (NSLAB) dynamically evolve according to modification of environmental conditions and thus during cheese ageing (Gala et al., 2008). They play a fundamental role in the acidification of the curd as well as in physical and chemical transformation with the development of flavour in the final cheese. The evaluation of LAB microbiota is important in the understanding of their role in sensorial traits of the final cheese. For example, Piras et al. (2013) studied role of autochthonous microbiota on sensorial differences of the traditional Fiore Sardo cheese. As determined in previous studies, in Parmigiano Reggiano (Coppola et al., 1997; Gala et al., 2008; Neviani et

al., 2013) and Grana Padano (Giraffa et al., 1997; Zambonelli et al., 2002; Rossetti et al., 2008; Monfredini et al., 2012; Pogačić et al., 2013;) cheeses, *Lactobacillus* is the dominant genus in the natural whey employed and during ripening.

Apart from their role in cheese production and ripening, LAB with probiotic properties could confer an added value to the final product that is of interest to consumers (Stanton et al., 2001). Strains intended to be used for production of cheeses with probiotic characteristics have previously to be tested for some basic properties. Viability and survival of probiotic bacteria are ones of the most important prerequisites in order to provide therapeutic functions to the consumer (Godward et al., 2000; Godward and Kailasapathy, 2003), but also antibiotic resistance and the interaction with epithelial human cells. A number of factors are known to affect the viability of probiotic bacteria in dairy foods such as yoghurt and fermented milk, including low pH and refrigerated storage (Shah, 2000). Moreover, the resistance to human gastric transit constitutes an important selection criterion for probiotic bacteria (Bautista-Gallego et al, 2013; Saxelin et al., 2010). Bearing in mind this fact, some probiotic cheeses have been produced (Abadía-García et al., 2013; Albenzio et al., 2013a, 2013b; Bergamini et al., 2010) but limited information about probiotic bacteria in hard ripened cheeses is available. Grana type cheese could be considered an interesting candidate as functional food due to the presence of viable lactic acid bacteria at the end of the ripening (Coppola et al., 1997).

The main goals of this work were i) the isolation, identification and molecular characterization of *Lactobacillus* spp. isolated from a Piedmont hard cheese; and ii) a first *in vitro* study of certain characteristics related to the functional properties of these microorganisms.

2. Materials and methods

2.1. Sample collection

Three different cheese productions (D, E and F) of the same dairy plant, located in the Piedmont region (North West of Italy), were studied from whey (starter) and raw milk (Frisona cows) until the twelfth month of cheese ripening. The different samplings were carried out for (Figure 1): whey (1º), raw milk (2º), milk plus whey (3º), cutting curd (4º), curd after heating (5º), after pressing (6º), after storage room at 46 ºC (7º), after salting (8º), after thermostatic room (9º), and from the first to the twelfth ripening month (10-21º). At the end of this process, the same in the last thirty years, the final product has the following characteristics: an average weight of 32 kg, a diameter of 410 mm and a height of 215 mm. Each sample was subjected to microbiological analysis not more than 2 h after collection. Sampling on cheese loafs was carried out using a cheese trier, which was inserted perpendicular to the centre of the cheese and then rotated 360º.

2.2. Microbiological analysis

Ten millilitre or gram of samples were homogenized with 90 ml of Ringer solution (Oxoid, Milan, Italy) in a Stomacher machine (Savatec, Milan, Italy). Serial dilutions were plated in duplicate onto Rogosa Bios agar (Biolife, Milano, Italy), incubated anaerobically in jar, at 42 °C for 48 h. Rogosa medium was used as being selective for lactobacilli population. Ten randomly selected colonies were collected from each sample, purified by a streak on a new Rogosa agar plate, and finally stored at –80 °C with 20% glycerol.

After counting, data were subjected to one-way ANOVA and Duncan Test was used to determine differences at $P < 0.05$, using the statistical software, Statistica 7.0 for Windows (Statsoft, Tulsa, USA).

2.3. Molecular identification and characterization

LAB isolates were subjected to DNA extraction as previously reported (Mora et al., 2000; Cocolin et al., 2001). The molecular identification of the isolates was performed by PCR 16S–23S rRNA gene spacer analysis (RSA), and 16S rRNA gene sequencing. The RSA was carried out with primers G1 (GAAGTCGTAACAAGG) and L1 (CAAGGCATCCACCGT) (Dolci et al., 2008). The variable V1 and V3 region of 16S rRNA gene of representative isolates was amplified with primers P1V1 (5′-GCG GCG TGC CTA ATA CAT GC-3′) and P4V3 (5′-ATC TAC GCA TTT CAC CGC TAC-3′) (Cocolin et al., 2001). PCR was performed in a final volume of 25 μl containing 10 mM Tris–HCl (pH 8.3), 25 mM MgCl$_2$, 10 mM deoxynucleoside triphosphates, 1.25 U of *Taq* polymerase (Eppendorf, Hamburg, Germany), and 10 μM of each primer. One hundred ng of template DNA was added to the mixture. A PCR was performed in a MyCyler (BioRad, Hercules, CA, USA). The annealing temperature was 45°C, and a denaturation of 95°C. was used. The extension for each cycle was carried out at 72 °C for 1 min and the final extension was at 72 °C for 7 min. PCR products were analysed by electrophoresis in 1.5% Tris-acetate-EDTA agarose gels.

The amplified and purified fragments were sequenced by Eurofins MWG Operon (Ebersberg, Germany) and the sequences obtained were aligned with those in GenBank using the Blast program (Altschul et al., 1997) to determine the closest known relatives of isolates.

Then, rep-PCR fingerprinting was performed for molecular strain characterization with the single oligonucleotide primer (GTG)$_5$, following the protocol described by Gevers et al. (2001). Products of rep-PCR were electrophoresed in a 2% agarose gel and the profiles obtained were visualized under ultraviolet light using UVI pro platinum 1.1 Gel Software (Eppendorf, Germany). The resulting fingerprints were analyzed with the BioNumerics 4.6 software package (Applied Maths, Belgium). The similarity among digitalized profiles was

calculated using the Pearson correlation and an average linkage (UPGMA) dendrogram was derived from the profiles.

2.4. In vitro *phenotypic tests related to probiotic potential*

Autoaggregation assays were performed according to the methodology described by Bautista-Gallego et al. (2013) with some modifications. Briefly, 4 ml of an overnight culture containing ~8-9 log cfu/ml were homogenized by vigorous vortexing for 10 s and then incubated at room temperature for 5 h. At the end, 0.1 ml of the upper suspension was carefully removed, transferred to a new tube containing 3.9 ml of phosphate buffered saline (PBS), and then the absorbance at 630 nm (A_{630}) was measured in a microplate reader (Biotek ELx808). The autoaggregation percentage was expressed as a function of time until it was constant, using the formula $1-(A_5/A_0)\times100$, where A_5 represents the absorbance at 5 h, and A_0 the absorbance at time t=0 h.

Bacterial cell surface hydrophobicity was assessed by measuring microbial adhesion to hydrocarbons using the procedure described by Crow et al. (1995) with the modifications of Bautista-Gallego et al. (2013).

Simulated digestion process was studied using the protocol described by Corcoran et al. (2007) and Bautista-Gallego et al. (2013) with slight modifications. Survival was evaluated by determining viable count on MRS agar.

3. Results and discussion

3.1. *Lactobacilli counts and ecology*

Milk transformation into cheese is a dynamic process in which microorganisms intervene in a complex and coordinated manner. In the three productions investigated in this study, a reduction trend in the lactobacilli counts was detected from the storage room until

the end of the monitoring period (Table 1). The mean count in milk upon addition of whey was 6.33 log cfu/g and no significant changes were detected for the first three days. Then, significant difference (P<0.05) between the storage room pause and the salting step was detected, as reported in Table 1. Then the counts went down slowly until the first month of ripening (3.78 ± 0.10 log cfu/g). Next, lactobacilli population increased until the fourth month of ripening to 5.06 ± 0.02 log cfu/g to then slowly decrease until the end of the study (1.21 ± 0.23 log cfu/g). It should be noted that a weak increase was detected at the beginning of ripening. This may be due to the moving of cheeses to a different room with better humidity and temperature conditions. Standard deviations were always at low levels, showing a very similar behaviour between the three different productions studied here.

LAB strains associated with traditional raw milk cheeses are one of the important elements influencing the cheese characteristics and quality (Steele et al., 2013). The isolates of this study were grouped on the basis of polymorphism of 16S–23S rRNA gene spacer region, and an indication of the hypothetical genus they could belong to was obtained (Coppola et al., 2001; Fortina et al., 2003). According to RSA results (Figure 2), 16S rRNA gene sequencings were performed in order to determine the taxonomic positions of the isolates. The evolution of the different lactobacilli species is presented in Figure 2. A total of 586 colonies were isolated, 200, 201 and 185 from productions D, E and F, respectively. Seven species were identified through the whole manufacture and ripening process: *L. rhamnosus* (51.88% of all isolates); *L. helveticus* (40.96%); *L. fermentum* (4.44%); *L. delbrueckii* (1.88%); *L. pontis* (0.51%); *L. gasseri* (0.17%) and *L. reuteri* (0.17%). *L. helveticus* was the only species isolated until the pressing step, and only in raw milk it was accompanied by isolates of *L. gasseri* and *L. rhamnosus*. The predominance of *L. helveticus* at the first stages of cheese production has been claimed already by others authors (Fortina et al., 1998; Gatti et al., 2003). *L. helveticus* is relatively adaptable to different growth

conditions as a consequence of its ability to ferment a wide variety of different carbohydrates and to metabolize a wide variety of proteins. The lactic acid and oxidative tolerances of *L. helveticus* are also relatively high compared with other lactobacilli (Dimitrov et al., 2005). This species has been identified in Grana Padano (Pogačić et al., 2013), Fontina (Dolci et al., 2013), Provolone (Giraffa et al., 1998) and Parmigiano Reggiano cheeses (Gatti et al., 2003).

From the storage room and the use of salt until the fourth month of ripening, the highest microbial diversity was detected. *L. helveticus*, *L. delbrueckii*, *L. fermentum*, *L. rhamnosus*, *L. reuteri* and *L. pontis* were identified. The effect of salt in lactic acid bacteria growth has been previously studied (Arroyo-López et al., 2009).

In the next eight months of ripening *L. rhamnosus* was the sole species isolated and identified. Its prevalence potentially offers very interesting possibilities by the point of view of producing a cheese with probiotic microorganisms. This species has been previously identified in Parmigiano Reggiano (Bove et al., 2011), Dambo (Antonsson et al., 2003), Edam (Ahola et al., 2002) and Grana Padano (Pogačić et al., 2013). *L. rhamnosus* has been studied as a possible probiotic microorganism showing potential positive characteristics (Tuo et al., 2013). Moreover *L. rhamnosus* sp. strain GG, originally isolated from healthy human intestine, has most of the characteristics generally proposed for a good probiotic strain, including excellent survival in the stomach and small intestine and transient colonization of the gastrointestinal tract, which is based on its adhesion capacity to intestinal cells (Saxelin, 2010).

L. delbrueckii has been identified as dominant species in Grana Padano whey and cheese by Massoni et al. (1982). Its presence has also been described in Akawi cheese (Ayyash et al., 2012). *L. fermentum* has been identified in Grana Padano (Pogačić et al., 2013), Feta (Rantsiou et al., 2008) and Parmigiano Reggiano cheeses (Solieri et al., 2012).

3.2. Molecular characterization

The 586 isolates were then subjected to molecular characterization to define their intra-species biodiversity, which was successfully accomplished by means of rep-PCR using the $(GTG)_5$ primer (Gevers et al., 2001; Svec et al., 2005). To improve the understanding of the diversity of lactobacilli in this cheese production, dendrograms for *L. helveticus* and *L. rhamnosus,* the dominant species, were constructed. *L. helveticus* showed eight clusters with a similarity coefficient of 60% (Figure 4) suggesting a high genetic variability already demonstrated by a previous study (Rossetti et al., 2008). Cluster II had the largest proportion of isolates (67.5%), but no special distribution can be emphasized. This cluster was formed by 54, 60 and 48 isolates of productions D, E and F, respectively. Cluster I was mainly characterized by isolates from "cutting curd" and "after heating" of all productions. Cluster VI was formed by only isolates from production D.

In the case of *L. rhamnosus*, 16 clusters were detected with the same similarity coefficient used before (Figure 5). Cluster XIII had 53.3% of all isolates and was formed by 39, 62 and 62 isolates of productions D, E and F, respectively. However, and as the main cluster of *L. helveticus*, no specific correlation can be deduced. Furthermore, in clusters I, II, III and IV there were isolates mainly from the eighth and ninth ripening months (all productions) whereas isolates of sixth and seventh ripening months formed cluster XIV.

Based on the characterization results, there is a succession of strains belonging to the two numerically more important species identified in this study according to their roles during the cheese ripening. First, cluster II of *L. helveticus* was isolated from whey and raw milk until the third ripening month. Then cluster XIII of *L. rhamnosus* was detected from the thermostatic room until the end of this study. Bove et al. (2011) also suggested that biotypes of *L. rhamnosus* related to specific moments of Parmeggiano Reggiano ripening may have

specific roles linked to their peculiar technological properties. Furthermore Solieri et al. (2012) also verified that in the case of *L. rhamnosus* and *L. paracasei*, ripening time appears to play a role in composition of cultivable NSLAB in cheese.

3.3. Functional characterization

Auto-aggregating and hydrophobicity properties have been described previously as very useful to select potentially probiotic bacteria (Meira et al., 2012; Xu et al., 2009). These two properties are likely to be correlated with adhesion ability to epithelial cells and mucosal surfaces (Xu et al., 2009). In this study, the results obtained from the sedimentation assays showed that lactobacilli isolates had a strong auto-aggregating phenotype (Figure 6A). Isolates from all productions had the same behaviour and no differences could be detected between them. The largest number of isolates (394, 67.24% of all isolates) exhibited maximum values, from 90 to 100% of auto-aggregation. Three isolates (16E7, 14F4 and 11F6) showed the lowest values, below 10%. In general, this behaviour agrees with the data presented by Bautista-Gallego et al. (2013), that determined the interval between 50% and 80% as the most abundant and their selected strains were between 27.00 and 76.51%.

On the contrary, the hydrophobicity showed an opposite pattern. Most of the isolates (89.42%) had values between 0% and 5% (Figure 6B). This result indicates that most of the lactobacilli isolated in this study were weakly hydrophobic, although some of them; namely 18E5, 18E10 and 21E8 (all of them *L. rhamnosus*), showed levels higher than 40%. Some studies have shown that the hydrophobicity values of some strains of lactobacilli (Lähteinen et al., 2010; Lee and Poung, 2002; Kaushik et al., 2009) and bifidobacteria (Del Re et al., 2000; González et al., 2013) varied from 23 to 95% and from 20 to 70%, respectively. However, Rehaiem et al. (2014) found that *Enterococcus faecium* MMRA had a high mean

value of hydrophobicity (74.06 ± 2.06%). They considered that this may contribute to adhesion of bacterial cells to host tissues and favouring the bacterial maintenance in the human gastrointestinal tract.

In this study and other studies (Bautista-Gallego et al., 2013), survival during transit through the gastrointestinal tract has been considered as one of the most critical factors. The sequential simulated gastrointestinal and pancreatic digestions have been applied to all isolates. The protocol applied in this study should be considered more realistic than those testing separately gastric and pancreatic digestion. Moreover, due to the characteristics of this screening only robust strains may pass. The majority of strains revealed total inhibition or very low survival, and the simulated gastric process resulted to be the most inhibitory step (data not shown). Nonetheless, sixteen strains belonging to *L. helveticus* (6), *L. rhamnosus* (9) and *L. fermentum* (1) exhibited the best survival behaviour (at least 3 $\log_{10}$ cfu/ml alive after the digestion process) as determined by plating (Table 2). Although these results (percentage, Table 2) are lower than reported by other studies, the residual populations are similar. In addition, these survival rates are close or higher than data obtained for *L. casei* Shirota and *L. rhamnosus* GG by Bautista-Gallego et al. (2013). These authors also reported a survival ratio of 0.008% and 0.007 % (a reduction of ≈4 log) for selected strains, that it's a residual population between 3 and 4.2 $\log_{10}$ cfu/ml. In another study carried out with a similar protocol, different strains of *L. plantarum* isolated from fermented food and from the intestine were found to show tolerance to overall digestion, with a survival rate of between 0.003-10% (Haller et al., 2001).

Lactobacilli have been investigated previously for their probiotic properties. *L. helveticus* species can display functional characteristics similar to those of *L. acidophilus*, *L. rhamnosus*, and *Bifidobacterium animalis* subsp. *lactis* (Taverniti and Guglielmetti, 2012). *L. rhamnosus* has been already isolated from Italian hard cheeses and studied for its

probiotic potentials, which were found to be analogous to *L. rhamnosus* GG (Succi et al. 2005). In this study, *L. rhamnosus* showed a good bile salt and acid tolerance as has been previously described for this species. Ahola et al. (2002) studied the effect of probiotic Edam cheese containing *L. rhamnosus* LC705 and *L. rhamnosus* GG ATCC53103 (LGG) on the risk of dental caries. The authors concluded that eating probiotic cheese could reduce the risk of dental caries in general, although no significant statistical difference was observed in salivary microbial populations. Mäkeläinen et al. (2009) showed that *L. rhamnosus* HN001 in cheese survives in the gastrointestinal tract and that the cheese matrix does not seem to affect the probiotic survival. Pitino et al. (2012) described that the use of cheese as a probiotic food carrier presents real advantages in the survival of probiotic bacteria especially during gastric digestion.

4. Conclusion

The isolation, identification and characterization of *Lactobacillus* species involved in this Piedmont hard cheese production provides the opportunity to study the functional characteristics and to select the most suitable strains for a potential native starter. In addition, the use of strains with functional properties can provide a higher value and acceptability by consumers. From this study some strains of *L. rhamnosus* and one of *L. fermentum* have been identified as the best candidates of this study to carry out more tests. 12D5 was the best candidate but also 10D9 and 11F9 (all of them *L. rhamnosus*), and 10F3 (*L. fermentum*) showed the best survival results in simulated transit through the gastrointestinal tract. These strains had good autoaggregation values (higher than 52.75%), hydrophobicity higher than 8% and at least a 3 log cfu/ml of residual population to the simulated digestion process. The use of cheese as probiotic food carrier presents potential advantages and it is a valuable alternative for the cheese industry. In the future, the survival

of the selected functional strains during the ripening process, additional probiotic properties and the effect in the sensorial characteristics of the cheese should be determined.

References

Abadía-García, L., Cardador, A., Martín del Campo, S.T., Arvízu, S.M., Castaño-Tostado, E., Regalado-González, C., García-Almendarez, B., Amaya-Llano, S.L., 2013. Influence of probiotic strains added to cottage cheese on generation of potentially antioxidant peptides, anti-listerial activity, and survival of probiotic microorganisms in simulated gastrointestinal conditions. International Dairy Journal 33, 191-197.

Ahola, A.J., Knnuttila-Yli, H., Suomalainen, T., Poussa, T., Ahlsrom, A., Meurman, J.H., Korpela, R., 2002. Short-term consumption of probiotic containing cheese and its effect on dental caries risk factors. Archives of Oral Biology 47(11), 799-804.

Albenzio, M., Santillo, A., Caroprese, M., Ruggieri, D., Napolitano, F., Sevi, A., 2013a. Physicochemical properties of Scamorza ewe milk cheese manufactured with different probiotic cultures. Journal of Dairy Science 96, 2781-2791.

Albenzio, M., Santillo, A., Caroprese, M., Braghieri, A., Sevi, A., Napolitano, F., 2013b. Composition and sensory profiling of probiotic Scamorza ewe milk cheese. Journal of Dairy Science 96, 2792-2800.

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.

Antonsson, M., Molin, G., Ardö, Y., 2003. *Lactobacillus* strains isolated from Danbo cheese as adjunct cultures in a cheese model system. International Journal of Food Microbiology 85, 159-169.

Arroyo-López, F.N., Bautista-Gallego, J., Chiesa, A., Durán-Quintana, M.C., Garrido-Fernández, A., 2009. Use of a D-optimal mixture design to estimate the effects of diverse chloride salts on the growth parameters of *Lactobacillus pentosus*. Food Microbiology 26, 396-403.

Ayyash, M.M., Sherkat, F., Shah, N.P., 2012. The effect of NaCl substitution with KCl on Akawi cheese: Chemical composition, proteolysis, angiotensin-converting enzyme-inhibitory activity, probiotic survival, texture profile, and sensory properties. Journal of Dairy Science 95, 4747-4759.

Bautista-Gallego, J., Arroyo-López, F.N., Rantsiou, K., Jiménez-Díaz R., Garrido-Fernández, A., Cocolin, L., 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Research International 50, 135-142.

Bergamini, C., Hynes, E., Meinardi, C., Suárez, V., Quiberoni, A., Zalazar, C., 2010. Pategrás cheese as a suitable carrier for six probiotic cultures. Journal of Dairy Research 77, 265-272.

Bove, C.G., De Dea Lindner, J., Lazzi, C., Gatti, M., Neviani, E., 2011. Evaluation of genetic polymorphism among *Lactobacillus rhamnosus* non-starter Parmigiano Reggiano cheese strains. International Journal of Food Microbiology 144, 569-572.

Boylston, T.D., Vinderola, C.G., Ghoddusi, H.B., Reinheimer, J.A., 2004. Incorporation of bifidobacteria into cheeses: challenges and rewards. International Dairy Journal 14, 375-387.

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, R., Nanni, M., Iorizzo, M., Sorrentino, A., Sorrentino, E., Grazia, L., 1997. Survey of lactic acid bacteria isolated during the advanced stages of the ripening of Parmigiano Reggiano cheese. Journal of Dairy Science 64, 305-310.

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.

Corbo, M.R., Albenzio, M., De Angelis, M., Sevi, A., Gobbetti, M., 2001. Microbiological and biochemical properties of Canestrato Pugliese hard cheese supplemented with bifidobacteria. Journal of Dairy Science 84, 551-561.

Corcoran, B.M., Stanton, C., Fitzgerald, G.F., Ross, R.P., 2007. Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice. Microbiology 153, 291–299.

Crow, V., Gopal, P., Wicken, A., 1995. Cell surface differences of lactococcal strains. International Dairy Journal 5, 45-68.

Darukaradhya, J., Phillips, M., Kailasapathy, K., 2006. Selective enumeration of *Lactobacillus acidophilus*, *Bifidobacterium* spp., starter lactic acid bacteria and non-starter lactic acid bacteria from Cheddar cheese. International Dairy Journal 16(5), 439-445.

Del Re, B., Sgorbati, B., Miglioli, M., Palenzona, D., 2000. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Letters in Applied Microbiology, 31, 438–442.

Dimitrov, Z., Michaylova, M., Mincova, S., 2005. Characterization of *Lactobacillus helveticus* strains isolated from Bulgarian yoghurt, cheese, plants and human faecal

samples by sodium dodecilsulfate polyacrylamide gel electrophoresis of cell-wall proteins, ribotyping and pulsed field gel fingerprinting. International Dairy Journal 15, 998-1005.

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., Zenato, S., Pramotton, R., Barmaz, A., Alessandria, V., Rantsiou, K., Cocolin, L., 2013. Cheese surface microbiota complexity: RT-PCR-DGGE, a tool for a detailed picture? International Journal of Food Microbiology 162, 8-12.

Fortina, M.G., Nicastro, G., Carminati, D., Neviani, E., Manachini, P.L., 1998. *Lactobacillus helveticus* heterogeneity in natural cheese starters: the diversity in phenotypic characteristics. Journal of Applied Microbiology 84, 72-80.

Fortina, M.G., Ricci, G., Acquati, A., Zeppa, G., Gandini, A., Manachini, P.L., 2003. Genetic characterization of some lactic acid bacteria occuring in an artisanal protected denomination origin (POD) Italian cheese, the Toma piemontese. Food Microbiology 20, 397-404.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.

Gatti, M., Lazzi, C., Rossetti, L., Mucchetti, G., Neviani, E., 2003. Biodiversity in *Lactobacillus helveticus* strains present in natural whey starter used for Parmigiano Reggiano cheese. Journal of Applied Microbiology 95, 463-470.

Gevers, D., Huys, G., Swings, J., 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiology Letters 205, 31-36.

Giraffa, G., Mucchetti, G., Addeo, F., Neviani, E., 1997. Evolution of lactic acid microflora during Grana cheesemaking and ripening. Microbiologie-Aliments-Nutrition 15, 115-122.

Giraffa, G., De Vecchi, P., Rossi, P., Nicastro, G., Fortina, M.G., 1998. Genotypic heterogeneity among *Lactobacillus helveticus* strains isolated from natural cheese starters. Journal of Applied Microbiology 85, 411-416.

Godward, G., Kailasapathy, K., 2003. Viability and survival of free and encapsulated probiotic bacteria in cheddar cheese. Milchwissenschaft 58, 624-627.

Godward, G., Sultana, K., Kailasapathy, K., Peiris, P., Arumugaswamy, R., Reynolds, N., 2000. The importance of strain selection on the viability and survival of probiotic bacteria in dairy foods. Milchwissenschaft 55, 441-445.

Gomes, A.M.P., Malcata, F.X., Klaver, F.A.M., 1995. Incorporation and survival of *Bifidobacterium* sp. strain Bo and *Lactobacillus acidophilus* strain Ki in a cheese product. Netherlands Milk and Dairy Journal 49, 71-95.González, I., Ruiz, L., Gueimonde, M., Margolles, A., Sánchez, B., 2013. Factors involved in the colonization and survival of bifidobacteria in the gastrointestinal tract. FEMS Microbiology Letters 340, 1-10.

Haller, D., Colbus, H., Ganzle, M.G., Scherenbacher, P., Bode, C., Hammes, W.P., 2001. Metabolic and functional properties of lactic acid bacteria in the gastro-intestinal ecosystem: A comparative in vitro study between bacteria of intestinal and fermented food origin. Systematic and Applied Microbiology 24: 218-226.

Kaushik, J.K., Kumar, A., Duary, R.K., Mohanty, A.K., Grover, S., Batish, V.K., 2009. Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. PLoS One 4, e8099.

Lähteinen, T., Malinen, E., Koort, J.M., Mertaniemi-Hannus, U., Hankimo, T., Karikoski, N., Pakkanen, S., Laine, H., Sillanpää, H., Söderholm, H., Palva, A., 2010. Probiotic properties of *Lactobacillus* isolates originating from porcine intestine and feces. Anaerobe 16, 293-300.

Lee, Y.K., Poung, K.Y., 2002. Competition for adhesin between probiotics and human gastrointestinal pathogens in the presence of carbohydrate. British Journal of Nutrition 88, S101–S108.

Mäkeläinen, H., Forssten, S., Olli, K., Granlund, L., Rautonen, N., Ouwehand, A.C., 2009. Probiotic lactobacilli in a semi-soft cheese survive in the simulated human gastrointestinal tract. International Dairy Journal 19, 675-683.

Massoni, F., Neviani, E., Vezzoni, A., Todesco, R., 1982. Tecnologia del Grana: II la microflora dei sieroinnesti. Attività caseinolitica. Industrie del Latte, 18, 25-28.

Meira, S.M.M., Helfer, V.E., Velho, R.V., Fernanda Cortez Lopes F.C., Brandelli, A., 2012. Probiotic potential of *Lactobacillus* spp. isolated from Brazilian regional ovine cheese. Journal of Dairy Research 79, 119-127.

Monfredini, L., Settanni, L., Poznanski, E., Cavazza, A., Franciosi, E., 2012. The spatial distribution of bacteria in Grana-cheese during ripening. Systematic and Applied Microbiology 35, 54-63.

Mora, D., Parini, C., Fortina, M.G., Manichini, P.L., 2000. Development of molecular RAPD marker for the identification of *Pediococcus acidilactici* strains. Systematic and Applied Microbiology 23, 400-408.

Neviani, E., Bottari, B., Lazzi, C., Gatti, M., 2013. New developments in the study of the microbiota of raw-milk, long-ripened cheeses by molecular methods: the case of Grana Padano and Parmigiano Reggiano. Frontiers in Microbiology 4, article 36.

Ong, L., Henriksson, A., Shah, N.P., 2006. Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus*, *Lb. casei*, *Lb. paracasei* and *Bifidobacterium* spp. and the influence of these bacteria on proteolytic patterns and production of organic acid. International Dairy Journal 16, 446-456.

Ong, L., Henriksson, A., Shah, N.P., 2007. Proteolytic pattern and organic acid profiles of probiotic Cheddar cheese as influenced by probiotic strains of *Lactobacillus acidophilus*, *Lb. paracasei*, *Lb. casei* or *Bifidobacterium* sp. International Dairy Journal 17(1), 67-78.

Phillips, M., Kailasapathy, K., Tran, L., 2006. Viability of commercial probiotic cultures (*L. acidophilus*, *Bifidobacterium* sp., *L. casei*, *L. paracasei* and *L. rhamnosus*) in cheddar cheese. International Journal of Food Microbiology 108, 276-280.

Pitino, I., Randazzo, C.L., Cross, K.L., Parker, M.L., Bisignano, C., Wickham, M.S.J., Mandalari, G., Caggia, C., 2012. Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. Food Microbiology 31, 57-63.

Pogačić, T., Mancini, A., Santarelli, M., Bottari, B., Lazzi, C., Neviani, E., Gatti, M., 2013. Diversity and dynamic of lactic acid bacteria strains during aging of a long ripened hard cheese produced from raw milk and undefined natural starter. Food Microbiology 36, 207-215.

Rafter, J.J., 2002. Scientific basis of biomarkers and benefits of functional foods for reduction of disease risk: cancer. British Journal of Nutrition 88, S219-224.

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

Rehaiem, A., Belgacem, Z.B., Edalatian, M.R., Martínez, B., Rodríguez, A., Manai, M., Pérez Guerra, N., 2014. Assessment of potential probiotic properties and multiple bacteriocin encoding-genes of the technological performing strain Enterococcus faecium MMRA. Food Control 37, 343-350.

Renault, P., 2002. Genetically modified lactic acid bacteria: applications to food or health and risk assessment. Biochimie 84, 1073-1087.

Rossetti, L., Fornasari, M.E., Gatti, M., Lazzi, C., Neviani, E., Giraffa, G., 2008. Grana Padano cheese whey starters: Microbial composition and strain distribution. International Journal of Food Microbiology 127, 168-171.

Saxelin, M., Lassig, A., Karjalainen, H., Tynkkynen, S., Surakka, A., Vapaatalo, H., Järvenpää, S., Korpela, R., Mutanen, M., Hatakka, K., 2010. Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. International Journal of Food Microbiology 144, 293-300.

Shah, N.P., 2000. Probiotic bacteria: selective enumeration and survival in dairy foods. Journal of Dairy Science 83, 894-907.

Solieri, L., Bianchi, A., Giudici, P., 2012. Inventory of non starter lactic acid bacteria from ripened Parmigiano Reggiano cheese as assessed by a culture dependent multiphasic approach. Systematic and Applied Microbiology 35, 270-277.

Stanton, C., Gardiner, G., Meehan, H., Collins, K., Fitzgerald, G., Lynch, P.B., Ross, R.P., 2001. Market potential for probiotics. American Journal of Clinical Nutrition 73, 476S-483S.

Steele, J., Broadbent, J., Kok, J., 2013. Perspectives on the contribution of lactic acid bacteria to cheese flavour development. Current Opinion in Biotechnology 24, 135-141.

Succi, M., Tremonte, P., Reale, A., Sorrentino, E., Grazia, L., Pacifico, S., Coppola, R., 2005. Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. FEMS Microbiology Letters 244, 129-137.

Svec, P., Drab, V., Sedlacek, I., 2005. Ribotyping of *Lactobacillus casei* group strains isolated from dairy products. Folia Microbiologica 50, 223-228.

Taverniti, V., Guglielmetti, S., 2012. Health-promoting properties of *Lactobacillus helveticus*. Frontiers in Microbiology 3, 392.

Tuo, Y., Zhang, W., Zhang, L., Ai, L., Zhang, Y., Han, X., Yi, H., 2013. Study of probiotic potential of four wild *Lactobacillus rhamnosus* strains. Anaerobe 21, 22-27.

Xu, H., Jeong, H.S., Lee, H.Y., Ahn, J., 2009. Assessment of cell surface properties and adhesion potential of selected probiotic strains. Letters in Applied Microbiology 49, 434–442.

Zambonelli, C., Chiavari, C., Benevelli, M., Coloretti, F., 2002. Effects of lactic acid bacteria autolysis on characteristics of fermented foods. Food Technology and Biotechnology 40, 347-351.

Zhang, H.P., Xu, H., Wang, J.G., Bilige, M., Sun, T.S., Li, H.P., Guo, M.R., 2008. A survey on chemical and microbiological composition of kurut, naturally fermented yak milk from Qinghai in China. Food Control 19, 578-586.

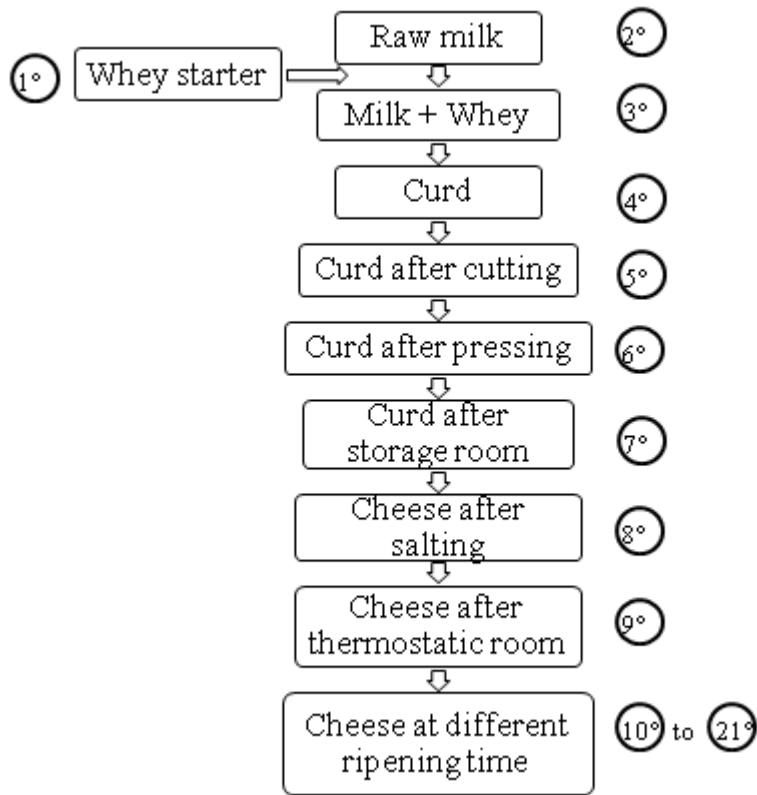**Figure 1.** Diagram of cheese production. Different samplings are shown in circle.

Figure 2. RSA profiles obtained from the LAB isolated from the cheese productions. Lanes 1 and 10: 1 Kb DNA Ladder (Promega); Lanes 2 to 9: amplification profiles obtained by RSA analysis. Lane 2: *L. gasseri*; Lane 3: *L. helveticus*; Lane 4: *L. rhamnosus*; Lane 5: *L. pontis*; Lane 6: *L. delbrueckii*; Lane 7: *L. fermentum*; Lane 8: *L. reuteri*.
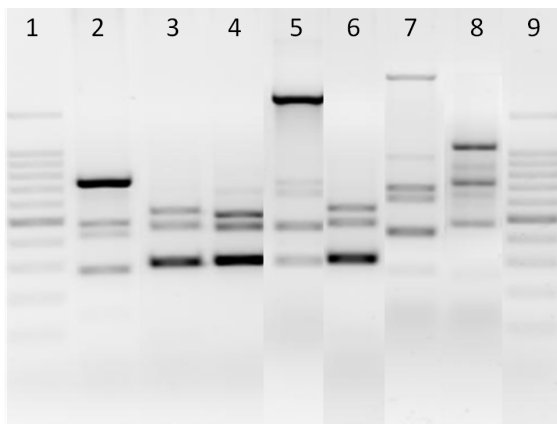
Figure 3. Diversity and evolution of species through the Piedmont hard cheese production, from whey and raw milk to the twelfth ripening month.
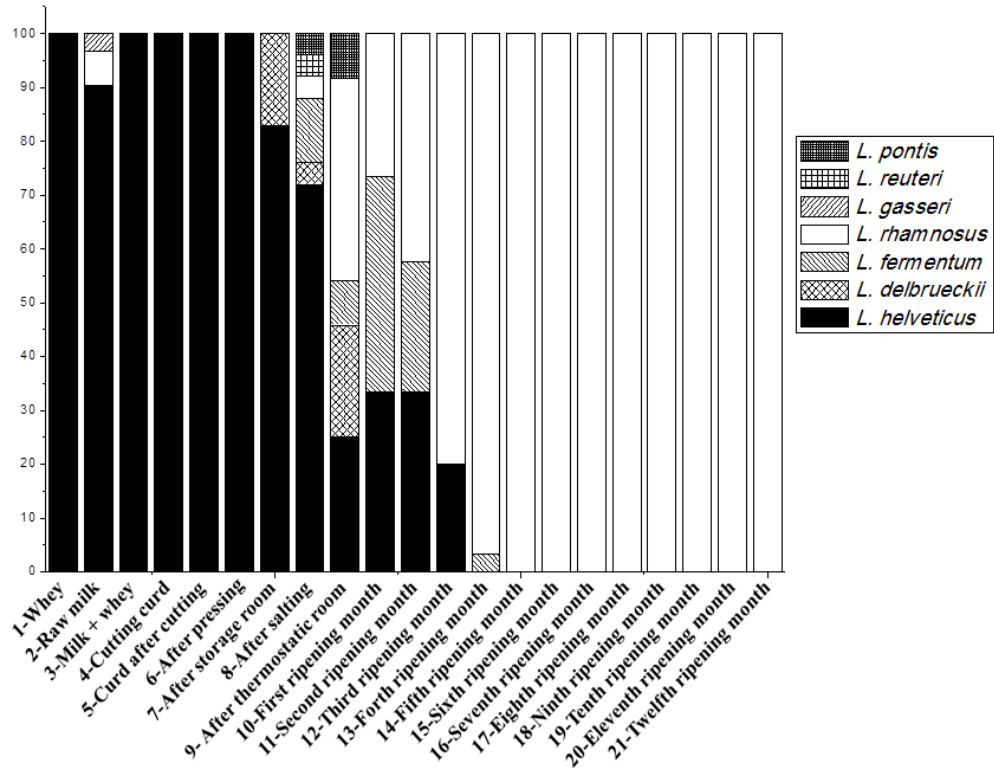
Figure 4. Dendrogram generated after cluster analysis of the digitized rep-PCR fingerprints of *L. helveticus* isolates (with a 60% coefficient of similarity). Clusters are indicated with roman numerals.
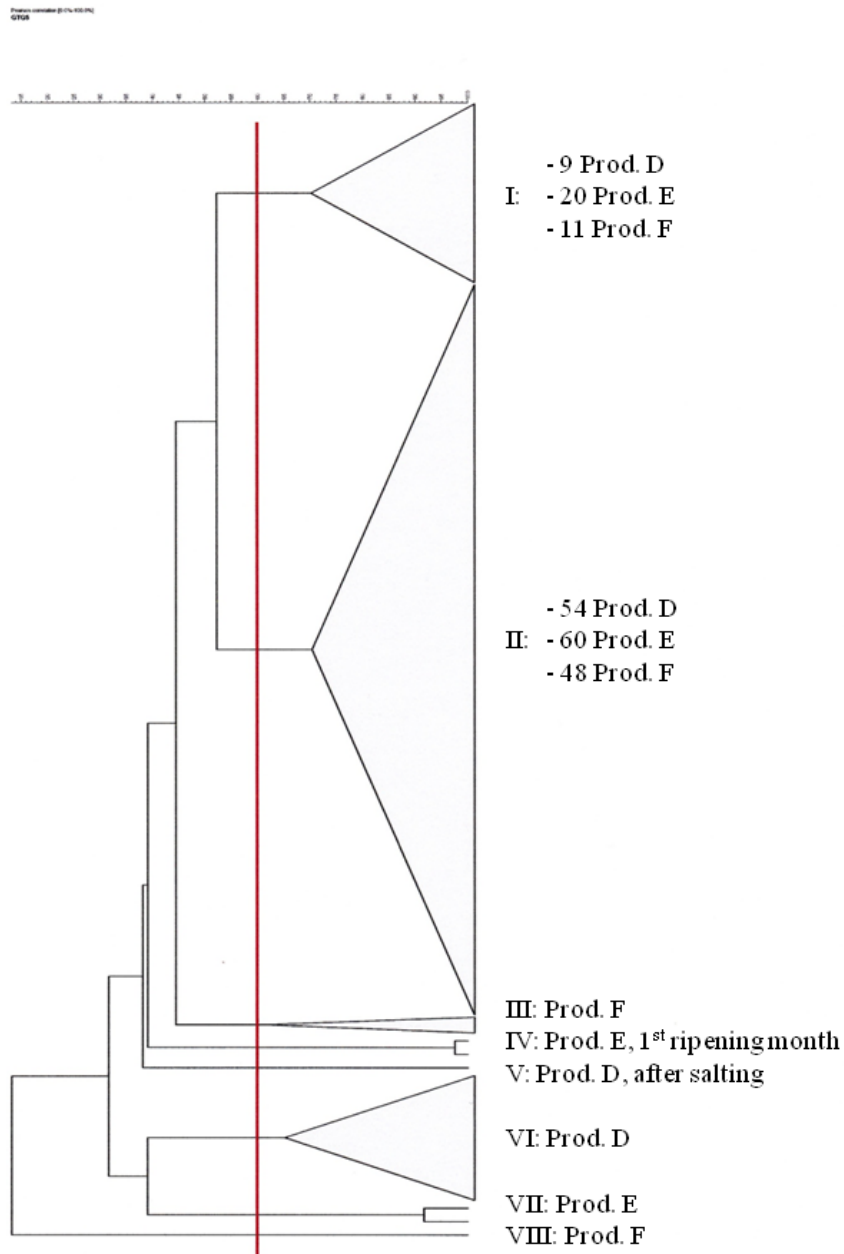

- 9 Prod. D
I: - 20 Prod. E
- 11 Prod. F

- 54 Prod. D
II: - 60 Prod. E
- 48 Prod. F

III: Prod. F
IV: Prod. E, 1ˢᵗ ripening month
V: Prod. D, after salting

VI: Prod. D

VII: Prod. E
VIII: Prod. F

Figure 5. Dendrogram generated after cluster analysis of the digitized rep-PCR fingerprints of *L. rhamnosus* isolates (with a 60% coefficient of similarity). Clusters are indicated with roman numerals.
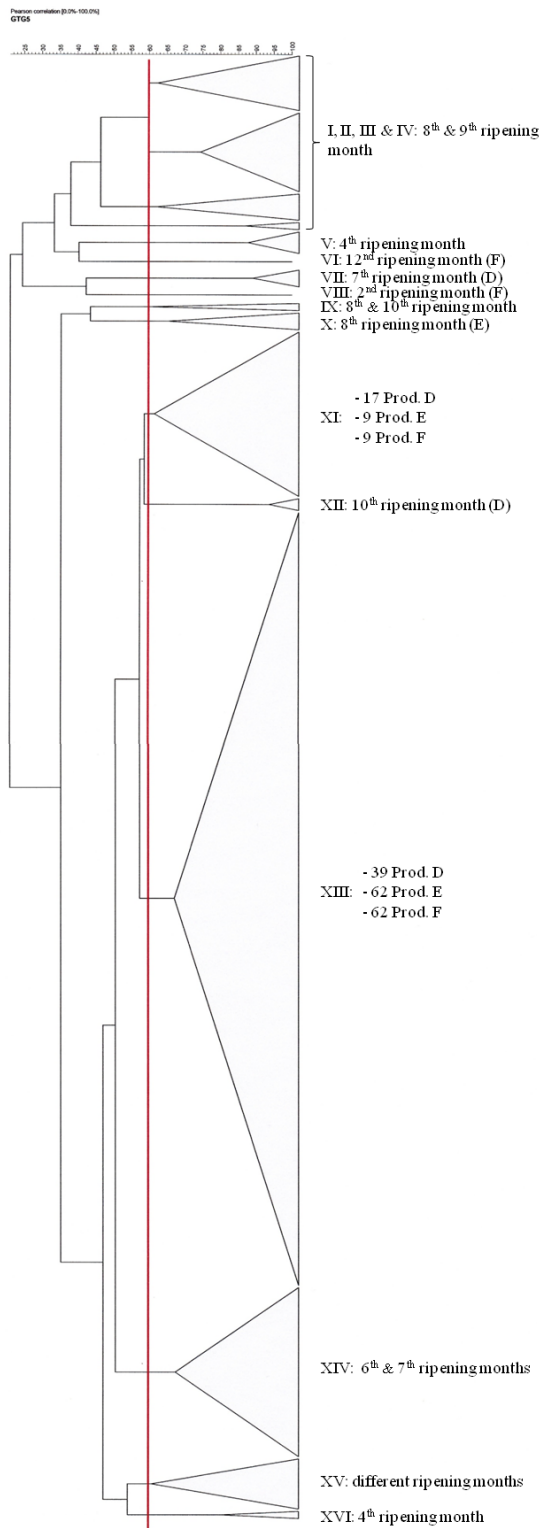
Figure 6. Histograms showing the distribution in each production of the auto-aggregation (a) and hydrophobicity (b) characteristics.
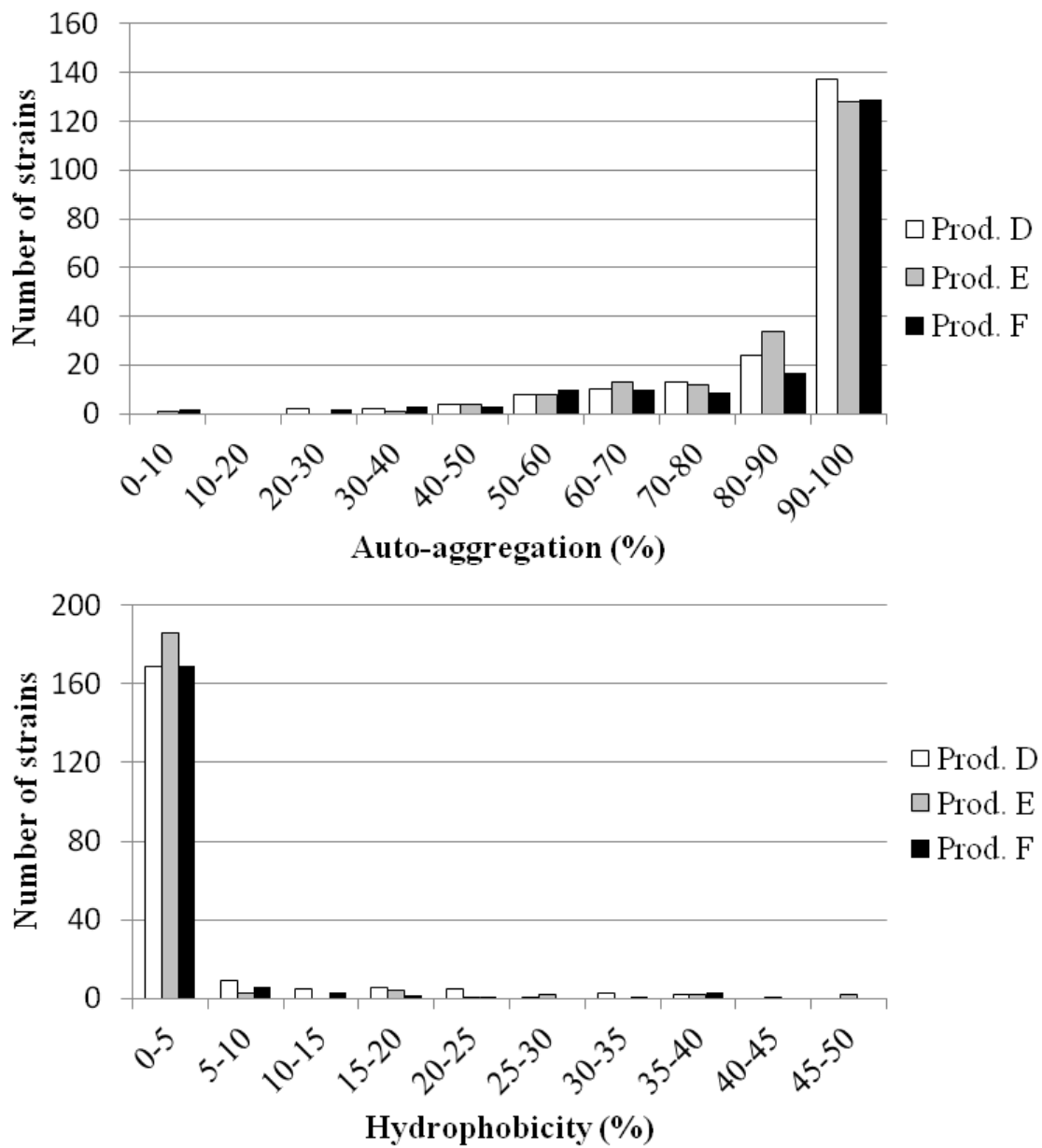
Table 1. Microbial sampling and counts during artisanal hard cheese production. Average and standard deviation (SD) of the counts determined for the three productions are presented.

Sampling	Average time of each phase	Average time in total (days)	Average Lactobacilli counts ($\log_{10}$ cfu/g)*	SD
1-Whey starter			7.54^a	0.31
2-Raw milk			3.70^{gh}	0.29
3-Milk + whey	12 min.		6.33^{bc}	0.17
4-Curd after cutting	15 min.	1	6.17^{bc}	0.20
5-Curd after heating	20 min.		6.20^c	0.06
6-After pressing	24 h	2	6.51^b	0.27
7-After storage room	4 d	5	5.79^c	0.09
8-After salting	21 d	26	4.23^{fg}	0.28
9-After thermostatic room	1 d	27	4.21^{fg}	0.14
10-First ripening month	30 d	57	3.78^{fgh}	0.10
11-Second ripening month	30 d	87	4.01^{fgh}	0.15
12-Third ripening month	30 d	117	4.42^{ef}	0.22
13-Forth ripening month	30 d	147	5.06^d	0.02
14-Fifth ripening month	30 d	177	3.50^h	0.37
15-Sixth ripening month	30 d	207	4.05^{fgh}	0.79
16-Seventh ripening month	30 d	237	4.93^{de}	0.57
17-Eighth ripening month	30 d	267	3.61^{gh}	0.09
18-Nineth ripening month	30 d	297	2.72^i	0.74
19-Tenth ripening month	30 d	327	2.66^i	0.62
20-Eleventh ripening month	30 d	357	1.24^l	0.05
21-Twelveth ripening month	30 d	387	1.21^l	0.23

* Results of variance analysis and Duncan test performed on parameters obtained by Lactobacilli counts during the different sampling points. Values with different superscript letters are significantly different, $P < 0.05$.

Table 2. Viable counts through simulated digestion process for 16 strains that exhibited good survival. Average and standard deviation (SD) of the counts determined in duplicate are presented.

Strains	Specie	Initial concentration		After digestion process		Overall survival rate (%)
		Average counts (log$_{10}$ cfu/ml)	SD	Average counts (log$_{10}$ cfu/ml)	SD	
2D5	*L. helveticus*	9.57	0.03	3.68	0.33	0.00013
4D5		9.54	0.02	3.71	0.15	0.00015
5D10		9.54	0.09	4.12	0.26	0.00038
6E8		9.38	0.10	3.61	0.29	0.00017
1F4		9.02	0.12	3.53	0.44	0.00032
1F8		9.30	0.08	3.45	0.31	0.00014
10D9	*L. rhamnosus*	9.49	0.11	3.56	0.16	0.00012
12D5		9.19	0.04	3.72	0.25	0.00034
17D10		9.15	0.16	3.48	0.30	0.00021
13E7		9.19	0.09	3.37	0.16	0.00015
14E4		9.56	0.12	3.49	0.34	0.00009
21E1		9.47	0.10	4.63	0.11	0.00145
11F9		9.69	0.07	3.58	0.42	0.00008
15F9		9.65	0.09	3.19	0.26	0.00003
15F10		9.53	0.03	3.13	0.19	0.00004
10F3	*L. fermentum*	9.55	0.07	3.32	0.27	0.00006