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Composition and dynamics of microbiota in different dairy ecosystems

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ABSTRACT

Cheese is a biologically and biochemically dynamic food containing microorganisms both deliberately added as starters and non-starter adventitious contaminants.

The composition of microbial population changes under the influence of continuous shifts in environmental conditions and interactions occurring among microorganisms during manufacturing and ripening. In cheese manufacturing, the selection of technological parameters can influence and even induce several biochemical processes needed for this product. The microbiota present in cheese is complex and its growth and activity represent the most important, but the least controllable steps.

The aim of the present thesis is the study of microbial diversity and dynamics of starter, non-starter and adventitious microorganisms involved in the cheese manufacturing and ripening processes. Thus, different ecosystems from raw milk to cheese and rind were investigated in order to comprehend the specific role played by microorganisms in each cheese making phase and to correlate the occurrence of certain microbial species with desired flavors and textures.

In particular, the microbial ecosystems in natural whey starters, hard Italian cheeses (i.e. Grana Padano and Parmigiano Reggiano), and natural rind of mold-ripened cheese (i.e. blue cheese) were investigated throughout cheese manufacturing and ripening by culture-dependent and culture-independent techniques.

By studying the microbiota in natural whey starters and cheese starting from the first days of manufacture, it was possible to describe the fermentative activity of starter lactic acid bacteria (SLAB) determining the ripening progress of the cheese. Whey starter titratable acidity did not seem to be related to the cell amount (total and cultivable cells) nor to the different species contribution. High concentrations of lactic acid and free aminoacids were found in cheeses with higher levels of *Lactobacillus helveticus* species and cultivable thermophilic bacterial densities. The presence of residual galactose was associated with higher contents of *Streptococcus thermophilus* species. In addition, the biotype composition of whey starters seemed to be far more important than the species composition in ensuring their good performances. A direct correlation between the composition of SLAB species and the acidifying efficacy in whey starters was not found. Contrarily, in the cheese matrix, the SLAB fermentative activity seemed to be species-dependent.

In hard cheese ecosystems, both SLAB and non-starter LAB (NSLAB) seemed to contribute to acidification and ripening. However, SLAB, and in particular *L. helveticus*,

resulted to be the species mainly subjected to the lysis occurring at 2 months of ripening. NSLAB were able to grow after brining, and became more relevant during ripening. NSLAB could arise both from the raw milk and the natural whey starter but their contribution to the development of cheese characteristics is still unknown. Furthermore, the presence of *L. helveticus* and *Lactobacillus delbrueckii* subsp. *lactis* in a non-cultivable state, up to 13 months of ripening, suggests that these species could play a different but still unknown role in cheese ripening.

The microbiota on the surface of a blue cheese during the natural rind development showed microbial diversity comprising fourteen genera of bacteria (*Enterococcus; Lactococcus; Leuconostoc; Macrococcus; Staphylococcus; Klebsiella; Brevibacterium; Corynebacterium; Brachybacterium;, Nocardiopsis;, Cobetia; Psychrobacter; Halomonas*; *Haererehalobacter*), two yeasts genera (*Candida; Debaryomyces*) and one filamentous fungal genus (*Penicillium*). High and comparable densities of viable bacteria and yeasts were observed. Bacterial succession was observed during rind formation and no genus remained constant throughout ripening. The *Staphylococcus* genus dominated the early stages and then *Brevibacterium* the later stages. By using interaction experiments, inhibition and stimulation were observed among several species; these interactions could explain how some microorganisms contribute to community formation. *Candida catenulata* and *Debaryomyces hansenii* enhanced the growth of *Staphylococcus equorum* while *C. catenulata* inhibited *D. hansenii* growth. However, thorough studies need to be performed in order to evaluate whether the above mentioned bacteria, yeasts and molds can be beneficial and play a role in flavor and texture development of other cheese varieties with similar natural rinds.

Overall, thanks to culture-dependent and culture-independent complementary approaches it was possible to identify which microorganisms were mainly involved in each dairy matrix and to address the importance of their presence that, if balanced, can help obtaining the distinctive features of each product.

RIASSUNTO

Il formaggio è un cibo biologicamente e biochimicamente dinamico contenente microorganismi sia intenzionalmente aggiunti come colture starter sia presenti come contaminanti avventizi di natura non-starter.

La composizione della popolazione microbica si modifica sotto l'influenza di continui cambiamenti delle condizioni ambientali e delle interazioni tra i microorganismi durante la produzione e la stagionatura. Nel processo produttivo del formaggio, la selezione di parametri tecnologici può influenzare o anche indurre vari processi biochimici necessari per l'ottenimento di questo alimento. Le popolazioni microbiche presenti nel formaggio sono complesse e la loro crescita e attività rappresentano le fasi più importanti ma meno controllabili del processo di caseificazione.

Lo scopo della presente tesi di dottorato è lo studio della diversità microbica e delle dinamiche delle colture starter, non-starter e dei microrganismi contaminanti coinvolti nei processi di produzione e stagionatura del formaggio. A tale scopo, diversi ecosistemi, dal latte crudo al formaggio, e la crosta sono stati studiati al fine di comprendere il ruolo specifico svolto dai vari microorganismi in ciascuna fase del processo di produzione e correlare la presenza di alcune specie microbiche con lo sviluppo di aromi e strutture desiderate del prodotto.

Nello specifico, gli ecosistemi microbici di sieroinnesti naturali, formaggi duri italiani (Grana Padano e Parmigiano Reggiano), e della crosta naturale di un formaggio erborinato stagionato, sono stati studiati per tutto il processo di produzione e stagionatura mediante tecniche culture-dependent e culture-independent.

Attraverso lo studio della microflora di sieroinnesti naturali e formaggi a partire dai primi giorni di produzione, è stato possibile descrivere l'attività fermentativa dei batteri lattici starter (SLAB) che determinano il processo di stagionatura del formaggio. L'acidità titolabile del sieroinnesto non è risultata essere in relazione né con la quantità di cellule (totali e coltivabili) né con il contributo specifico delle differenti specie. Elevate concentrazioni di acido lattico e aminoacidi liberi sono state trovate in formaggi con maggiori quantità della specie *Lactobacillus helveticus* e con alte densità di batteri termofili coltivabili. La presenza di galattosio residuo è risultata associata ad alti contenuti della specie *Streptococcus thermophilus*. Inoltre, la composizione in termini di biotipi dei sieroinnesti è apparsa molto più importante che la composizione in termini di specie nell'assicurare le buone performance dei sieroinnesti stessi. Una correlazione diretta tra la composizione delle specie SLAB e l'efficacia

di acidificazione nei sieroinnesti non è stata trovata. Al contrario, nella matrice formaggio, l'attività fermentativa degli SLAB è sembrata essere specie-dipendente.

Negli ecosistemi dei formaggi duri, sia gli SLAB che i LAB non-starter (NSLAB) sono apparsi contribuire all'acidificazione e alla maturazione. Tuttavia, gli SLAB, ed in particolare *L. helveticus*, sono risultati essere principalmente soggetti alla lisi che si verifica a 2 mesi di stagionatura. I NSLAB sono stati in grado di crescere dopo la salatura, diventando la maggioranza dei microorganismi durante la stagionatura. I NSLAB potrebbero provenire sia dal latte crudo che dal sieroinnesto naturale ed il loro contributo allo sviluppo delle caratteristiche del formaggio è ancora sconosciuto. Inoltre, la presenza di *L. helveticus* e *Lactobacillus delbrueckii* subsp. *lactis* in stato non coltivabile, fino a 13 mesi di stagionatura, suggerisce che queste specie potrebbero svolgere un ruolo diverso ma ancora sconosciuto nella stagionatura del formaggio.

La microflora della superficie di un formaggio erborinato durante lo sviluppo naturale della crosta ha mostrato una diversità microbica composta da 14 generi di batteri (*Enterococcus; Lactococcus; Leuconostoc; Macrococcus; Staphylococcus; Klebsiella; Brevibacterium; Corynebacterium; Brachybacterium;, Nocardiopsis;, Cobetia; Psychrobacter; Halomonas*; *Haererehalobacter*), due generi di lieviti (*Candida; Debaryomyces*) e un genere di fungo filamentoso (*Penicillium*). Sono state osservate elevate e comparabili densità di batteri e lieviti vitali. E' stata osservata inoltre, un'evoluzione batterica durante la formazione della crosta e nessun genere è rimasto costantemente presente per tutta la durata della stagionatura. *Staphylococcus* si è dimostrato il genere dominante durante le fasi precoci e successivamente è stato sostituito da *Brevibacterium* alla fine della stagionatura.

Sfruttando esperimenti di interazione, sono stati osservati effetti di inibizione e stimolazione tra le specie; queste interazioni potrebbero spiegare come alcuni microorganismi contribuiscano alla formazione della comunità microbica. *Candida catenulata* e *Debaryomyces hansenii* hanno incrementato la crescita di *Staphylococcus equorum* mentre *C. catenulata* ha inibito la crescita di *D. hansenii*. Tuttavia, studi approfonditi dovranno essere svolti per valutare se le specie batteriche, di lievito e muffe sopracitate possano essere utili e svolgere un ruolo nello sviluppo dell'aroma e della struttura di altre varietà di formaggi che presentino simile crosta naturale.

In conclusione, grazie ad approcci complementari culture-dependent e culture-independent, è stato possibile identificare quali microrganismi fossero principalmente coinvolti in ciascuna delle matrici casearie studiate, e definire l'importanza della loro presenza

che, se in giusto equilibrio, può favorire l'ottenimento delle diverse caratteristiche peculiari di ciascun prodotto.

A Luca

A mi Familia

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1. INTRODUCTION

1.1 Cheese

1.1.1 General aspects

The primary objective of cheesemaking originally was to convert milk to a less perishable product. The manufacture of most cheese varieties involves four basic ingredients: milk, rennet, microorganisms and salt. Cheese is the product of the coagulation of caseins in the milk forming a gel which occludes the fat and aqueous phase of milk. When sufficient acid is produced by lactic acid bacteria catabolizing lactose to lactic acid, the coagulation of caseins can be addressed (Fox, 2011b) by addition of proteinases from bacteria, molds, plants or animal tissues (referred as rennets) (Fox, 2011b). Successively, by cutting or breaking, the gel separates into curds and whey (Mucchetti and Neviani, 2006; Fox, 2011b).

During the storage of curds, complex processes involving a range of microbiological and biochemical reactions occur, that is ripening, resulting in changes in flavor, aroma and texture which will define the unique characteristic of the cheese (Cogan, 2002). Figure 1 summarizes the steps of cheese manufacture. From 400 to 1000 varieties of cheese are produced throughout the world (Fox *et al*., 2004). Differences between varieties are the result of modifications made in one or more basic steps of cheesemaking. That is, the quality of the milk, starter culture, technology, and ripening will combine to bring about biodiversity in any cheese.

1.1.2 Milk: the main ingredient

Milk is an excellent substrate for the growth of many microorganisms, including lactic acid bacteria, pathogens and spoilage organisms, because of its complex biochemical composition, near-neutral pH and high water content (Mucchetti and Neviani, 2006; Hassan and Frank, 2011). On average, cow milk is composed of approximately 87.4% water, 3.7% fat, 4.8% lactose, 3.4% protein and 0.7% mineral substances (Fox, 2011a). Differences in the principal constituents are found among milk from different animals (sheep, goat, etc.).

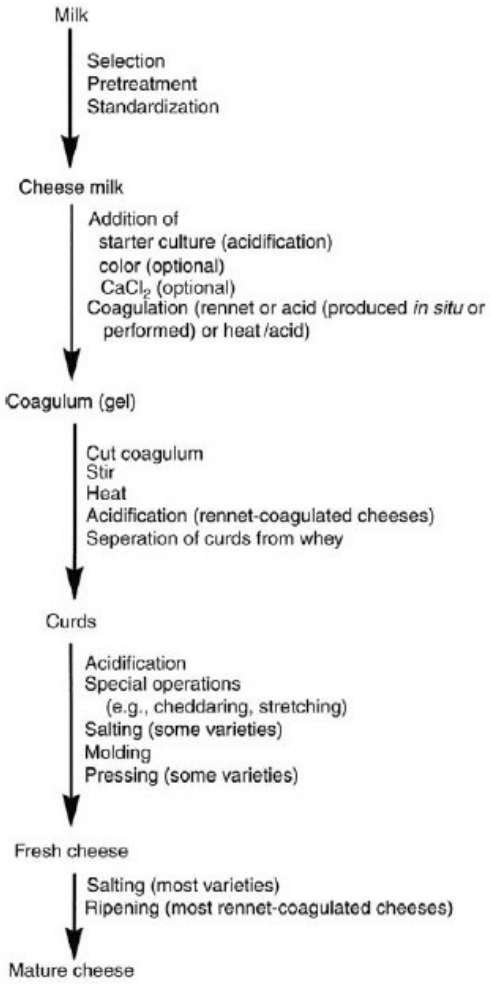

Figure 1. Outline of cheese manufacture (Fox, 2011b).

In healthy animals the secretory tissue of the udder is free of microorganisms. However, the mucosal membrane of the streak canal has a microflora that includes streptococci, staphylococci, micrococci (normally >50%), *Corynebacterium* spp., coliforms, lactic acid bacteria, and other bacteria. The level of milk contamination through the streak canal may vary between 10^2 and 10^4 cfu/ml (Roberts *et al.,* 2005). Moreover, milk becomes "contamined" by microorganisms from the farm or milking barn environment and from persons and equipment in contact with the milk (Hassan and Frank, 2011). Figure 2 shows the sources of contamination at a dairy farm. Microorganisms commonly found in air include micrococci, yeasts, molds and spores of *Bacillus*, all of which may survive heat processes and cause flavor or physical defects in processed products (Roberts *et al.,* 2005). Water supplies in farms often contain coliforms and psychrotrophic organisms (i.e. *Pseudomonas* spp.) and when used to rinse dairy equipment may be a source of contamination. It is well-recognized that the milking machines may contribute substantially to the raw-milk microflora, microorganisms such as micrococci, enterococci, aerobic spore-forming bacteria, and certain

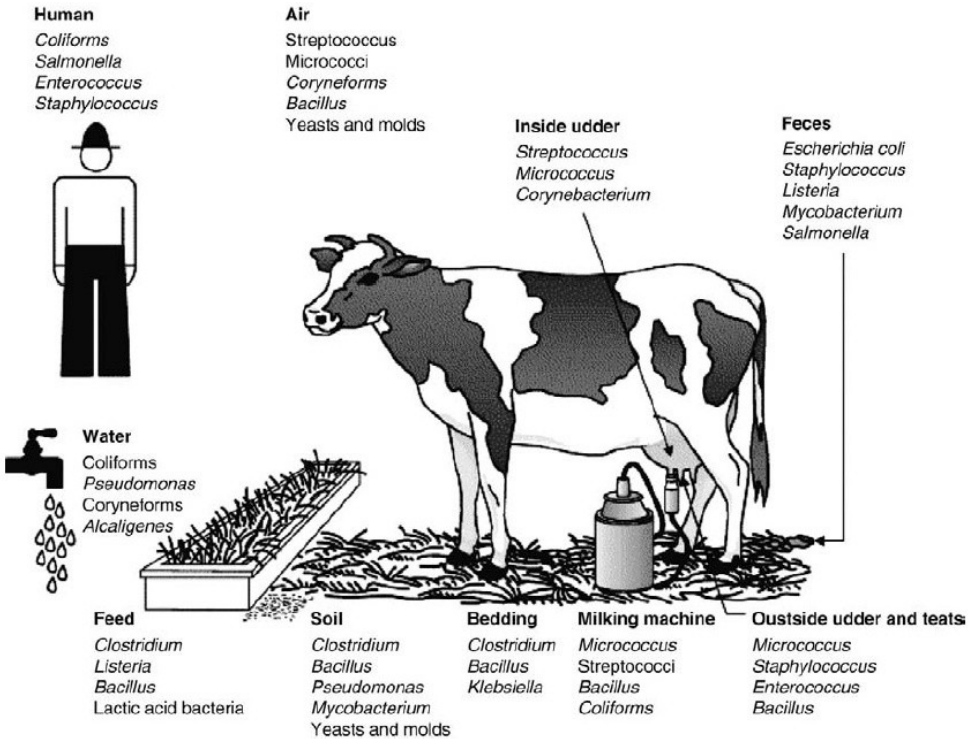

Figure 2. Diagram of the sources of contamination at dairy farm (Hassan and Frank, 2011).

lactobacilli may form biofilms. Spore-forming bacteria such as *Bacillus* spp., and *Clostridium* from the soil and feed, readily find their way into milk (Roberts *et al.,* 2005). Some spoilage microorganisms are particularly important to further processing of milk. These include thermoduric bacteria such as *Enterococcus* spp., which are important for products made from pasteurized milk, *Clostridium tyrobutyricum*, and *Cl. butyricum* from silage for hard-cheese production (Roberts *et al.,* 2005).

Thus, the indigenous bacterial microbiota of raw milk include several species of different affiliation including Firmicutes: *Aerococcus* spp., *Bacillus* spp., *Enterococcus* spp., *Lactococcus* spp., *Lactobacillus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Leuconostoc* spp., Actinobacteria: *Micrococcus* spp., *Corynebacterium* spp., *Brachybacterium* spp., *Dermacoccus* spp., *Kocuria* spp., *Leucobacter* spp., *Microbacterium* spp., *Arthrobacter* spp., *Brevibacterium* spp., *Propionibacterium* spp., Proteobacteria: *Acinetobacter* spp, *Enterobacter* spp., *Escherichia* spp., *Ochrobactrum* spp., *Pantoea* spp., *Paracoccus* spp., *Pseudomonas* spp., *Psychrobacter* spp., (Delbès *et al.*, 2007; Ercolini *et al*., 2009, Giannino et al., 2009, Rasolofo *et al*., 2010; Vacheyrou *et al*., 2011).

1.1.3 Classification of cheeses

Historically the names of cheeses were according to their characteristic such as structure (i.e. Grana, because of the grainy texture of the ripened cheese); the color of the mold that grow on it (i.e. Bleu d'Auvergne); the external aspect of the rind, i.e. Canestrato, derives from "canestri" (reed baskets) that were used to shape the cheeses; the milk source (i.e. Pecorino from sheep's milk); a particular production process, i.e. Mozzarella, derives from the verb "mozzare" (to cut off); the particular place where it ripens (i.e. Fossa, that means pit) and the place of production (i.e. Parmigiano Reggiano, Emmental, Gloucester) (Mucchetti and Neviani, 2006).

Actually, classification of cheeses can be based on different criteria such as moisture content, concentration of calcium, rheological properties, cooking temperature, secondary microflora or type of ripening (Fox *et al*., 2004). Traditional classification schemes have been based principally on moisture content, that is, extra-hard, hard, semihard/semisoft, or soft (Fox, 2011b). This classification is utilized by Codex Alimentarius (General Standard A6-1993). Although used widely, this scheme suffers from serious limitations since it groups cheeses with widely different characteristics. Figure 3 shows a summary of the classification of cheese (Fox, 2011b).

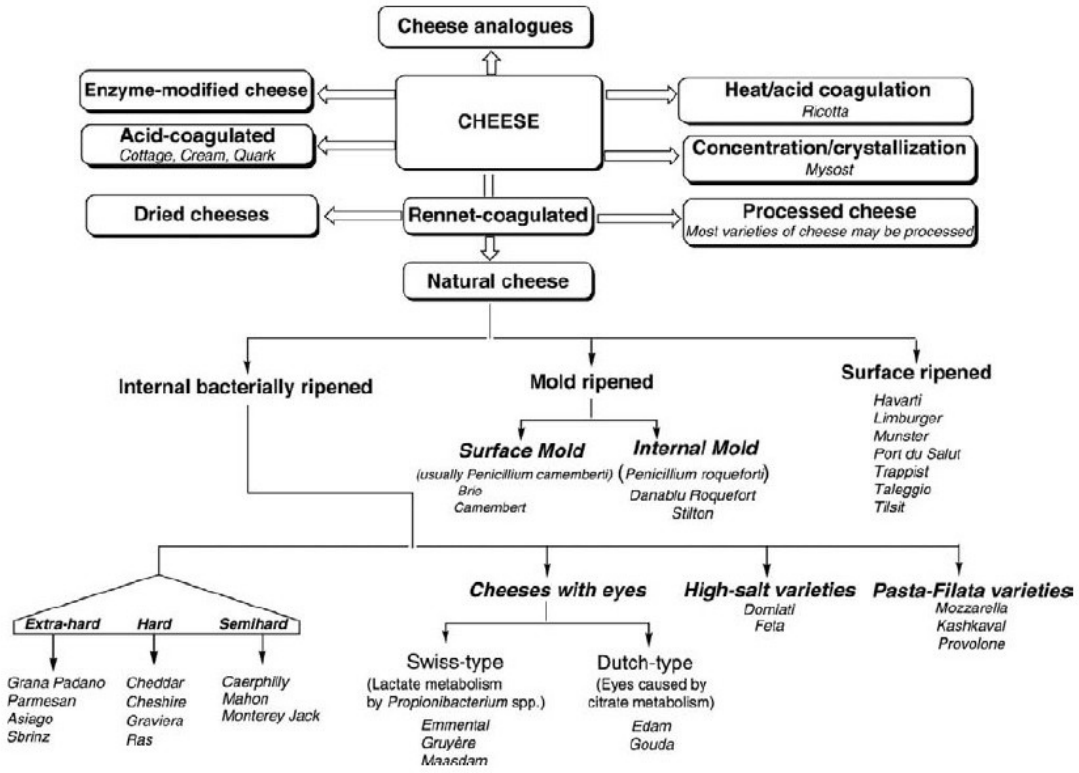

Figure 3. A scheme for the classification of cheese (Fox , 2011b).

1.2 Cheese microbiology

1.2.1 General aspects

Cheese microbiota is composed of a) starter lactic acid bacteria (SLAB), b) secondary microorganisms and c) adventitious organisms (Powell *et al*., 2011). None of the classifications or categories of cheeses have considered the microbial diversity characterizing different types of cheese. It is notoriously shown that cheese is a microbiologically dynamic food, hosting diverse metabolically active bacteria, yeasts and molds (Ndoye *et al.,* 2011). In many cases, despite being made under standard manufacturing conditions, cheese from different days at the same dairy or in different dairies exhibit variations in the final characteristics. The composition and activity of the microbiota is the least controllable of all the parameters involved in cheese production (Fox *et al*., 2000).

1.2.2 Starter cultures

Modern cheese manufacture usually involves deliberate addition of one or more lactic acid bacteria (LAB) species to ensure a proper fermentation. These deliberately added species include the so-called 'starter lactic acid bacteria' (SLAB), with the primary role of acidifying milk via the conversion of lactose into lactic acid at a predictable and controlled rate. Because they are present at very high cell densities in the matrix of young cheese, LAB starter cultures are generally thought to make the strongest contributions to cheese flavor development. SLAB possess an array of predominantly intracellular peptidases that degrade peptides formed by proteolytic agents to amino acids, which then act as precursors for a range of volatile flavor compounds. When starter culture cells lyse in cheese, the intracellular peptidases are also available to act upon peptides in the cheese matrix itself. LAB starter culture metabolism can also directly affect cheese flavor development by forming various compounds from lactose and citrate (Powell *et al*., 2011).

Starter bacteria are either added deliberately at the beginning of manufacture or may be natural contaminants of the milk, as is the case of many artisanal cheese varieties made from raw milk (Beresford *et al.,* 2001). Either mesophilic or thermophilic starter cultures are used, depending on the cheese being manufactured. Starter bacteria encountered most often are members of the genera *Lactococcus, Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus* (Beresford *et al.,* 2001).

Both mesophilic and thermophilic cultures can be subdivided into natural cultures also referred as mixed (undefined) cultures, which are produced every day at the dairy and which the number of strains is unknown, and defined cultures, which are composed of a known number of strains (Beresford *et al.,* 2001; Mucchetti and Neviani, 2006).

Natural mesophilic cultures are mainly composed of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*. Thermophilic natural whey starters are composed of undefined strains of lactobacilli such *as L. delbrueckii* subsp. *lactis* or *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus, L. fermentum* and *S. thermophilus* (Gatti *et al*., 2003; Lazzi *et al*., 2004; Fornasari *et al.,* 2006; Rossetti *et al.,* 2008; Santarelli *et al.,* 2008). These thermophilic starter cultures are those used in some traditional Italian hard cheese productions such as Grana Padano and Parmigiano Reggiano. They are produced by incubating cheese whey under conditions that favor the growth of thermophilic lactic acid bacteria (Mucchetti and Neviani, 2006).

The natural starter cultures are subjected to a great variability in terms of either microbial composition and performance. Industrial scale cheese production requires starters that give reproducible performance and are free of undesirable organisms. These goals are difficult to achieve using traditional methods. This has lead to the preparation of defined starters for industrial cheese production (Powell *et al*., 2011).

1.2.3 Secondary and adjuncts cultures

The secondary and adjunct cultures are added mainly for their effect on flavor, color, texture and eye formation in cheese. They are called secondary cultures to distinguish them from the primary acid-producing starters. Their contribution to acid production is limited or absent, moreover they are usually unique to the specific cheese variety. The principal secondary cultures used for the ripening include: *Propionibacterium freudenreichii* that are involved in flavor and eye formation in Swiss-type cheese, *Penicillium camemberti,* that are mainly involved in proteolysis in mold surface-ripened cheese as Camembert and Brie cheeses, *Penicillium roqueforti* involved in flavor, color, lipolysis and some proteolysis in blue-veined cheese (e.g. Stilton, Roquefort, Gorgonzola), and *Brevibacterium linens* that are involved in flavor and color in bacterial surface-ripened cheeses (e.g. Müster, Limburger, Tilster) (Ndoye *et al*., 2011; Rattray and Eppert, 2011).

1.2.4 Non-starter LAB and adventitious organisms

Adventitious ('contaminant') organisms are likely originated from the milk or processing plant environment (brine, wooden shelves, the cheese maker's hands) (Beresford *et al*., 2001). As adventitious organisms, they gain access to cheese and can contribute to flavor attributes or defects or have no impact on cheese ripening depending on the strain, the cell densities, and the cheese conditions (Beresford *et al*., 2001).

They consist of either non-starter lactic acid bacteria (NSLAB), other bacteria, yeasts and molds, which grow internally or externally on cheese (Robinson, 2002).

NSLAB are usually facultatively heterofermentative lactobacilli and pediococci which form a significant portion of the microbiota of most cheese varieties during ripening. Many species of mesophilic lactobacilli have been isolated from cheese, but those most frequently encountered belongs to the "*L. casei* group" (*L. casei/L. paracasei*, *L. rhamnosus*), *L. plantarum*, and *L. curvatus*. *Pediococcus acidilactici* and *P. pentosaceus* are the most frequently encountered pediococci in cheese (Beresford *et al*., 2001). In hard cheese varieties, NSLAB are the main population encountered in advanced stages of ripening (Crow *et al.*, 2001; Sheenan *et al*., 2007; Neviani *et al*., 2009), and they are considered to be involved in flavor formation during ripening. To date, it is still unknown which specific substrates they use for growth. At the time of ripening when they have been found, lactose has usually been exhausted. Thus, NSLAB may derive energy from a wide variety of compounds present in cheese, including lactic acid, citric acid, fatty acids, glycoproteins, glycolipids, amino acids, and even nucleotides that are released into the cheese matrix by dying starter bacteria. (Adamberg *et al*., 2005; Broadbent *et al.,* 2011). Culture media prepared from ripened cheese (i.e. Parmigiano Reggiano) are useful to recover this population, since they reproduce the natural composition in terms of nutrient availability found in the ripened cheese (Neviani *et al*., 2009). Recently, the growth of a *L. paracasei* strain was revealed in a medium made with a hard cheese (i.e. Cheddar), until 8 months of ripening. The growth substrates utilized by this strain was neither lactose, galactose nor citrate, because after 2 months they are completely exhausted. Authors supposed that NSLAB use for growth milk-derived complex carbohydrates and starter-derived components, however they are still unknown (Budinich *et al.,* 2011).

The presence of adventitious NSLAB introduces variability into the ripening process that cannot be easily controlled by the cheesemakers. As presented above, they may intensify or accelerate typical flavor development; they may impart atypical but nonetheless desirable flavor notes; or they may promote the development of undesirable off-flavors. Thus, several cheese productions may be subjected to fluctuations in the final characteristics (Franciosi *et*

al., 2008) between factories and among cheeses from the same factory (Williams *et al.,* 2002; Antonsson *et al*., 2003). For all these reasons, the dominance of desired NSLAB strains, is crucial to minimize microbial variability during the ripening process (Settanni and Moschetti, 2010). Of course, strains that consistently impart desirable flavor changes have value as adjunct cultures. At this regard, in some hard cheese manufactures (i.e. Cheddar), NSLAB are deliberately added to control and direct the ripening process, and they are referred as 'adjunct NSLAB' (Crow *et al.,* 2001; Broadbent *et al.,* 2003).

1.2.5 Cheese surface

One of the most noticeable events during cheese ripening is the gradual differentiation between the cortical layer and the inner mass. The outer layer tends to change developing a protective covering which gives the name of rind through which moisture evaporated. This is an essential component of all types of cheese, although it is more evident for some products. In some cases (fresh cheese, melted, etc.) an external protection are used to prevent the formation of the rind. In these cases, the protective function is exerted by a wrap, which assumes the function of artificial rind. Therefore, it can be distinguished two basic types of rind, which correspond to two different processes of maturation: the active and passive rinds (Salavadori del Prato, 1998).

The active rinds are formed after a rich and dense active microbial consortia develops on the cheese surface. These microorganisms coexist, interact and are essential for the ripening process and the final characteristic of the product. These type of cheese are characterized by a maturation process that proceeds from the surface to the core of the cheese. This is the case of the surface mold-surface ripened cheese, and bacterial surface-ripened cheese.

Active rinds are formed by natural or artificial inoculation, during and after salting and during ripening. The natural colonization is usually performed by contact with the environment, e.g., wooden shelves, that are naturally rich in the microflora involved (i.e. source of adventitious microflora) (Mounier *et al.,* 2006) or by technological practices, e.g., wiping down periodically with salt and water, or brines to keep the rind moist causing the spread of microorganisms naturally present in brines throughout the rind. Some cheese varieties that present natural rinds are the Stilton and Cabrales blue cheeses, in UK and Spain, respectively, that belong to the class of internally mold-ripened cheese and St. Nectaire cheese in France, that belongs to the class of surface mold-ripened.

However, the artificial inoculation can be done by spraying the surface with secondary cultures composed by suspensions of spores or other desired microorganisms (i.e. *Penicillium camemberti)* in Camembert cheese (Addis *et al.,* 2001), or by immersion in water and salt solutions containing desired microorganisms (i.e. secondary cultures of *Brevibacterium linens* in Gubbeen cheese) (Brennan *et al.* 2002). These latter cheeses that are periodically wipe down are therefore often identified with the name of washed-rind cheeses and are also called smear or red smear cheeses because of the development of viscous, red-orange smears on their surfaces during ripening composed of bacteria and yeast (Mounier *et al.,* 2005).

On the other hand, passive rinds do not take an active role in maturation of cheese, but still participate with their protective action and regulation of gas exchange and water with the inside. Furthermore, the cheeses with passive rinds are subject to a widespread ripening as is the case of Italian hard cheese (Parmigiano Reggiano and Grana Padano) (Salavadori del Prato, 1998).

The technological conditions during ripening process, humidity, temperature and the ecology of the microbiota in the brines and in the dairy room are factors that influence the microbiota on the surface (Mucchetti and Neviani, 2006). However, the identity and origin of the microbes present on the cheese surface has not been well-defined. Yet, the knowledge of the microbial composition on the cheese surface is a prerequisite for the development of secondary adjunct cultures and for the control of surface ripening with good hygienic practices avoiding the development of undesirable flora.

While the microbiota of the paste of the cheese is mainly composed by strictly or facultative anaerobic microorganisms, the cheese surface microbiota is related to aerobic metabolism. The most frequent species found in active rinds belong to different genera and families: among filamentous fungi *Penicillium* spp., *Geotrichum* spp. and *Mucor* spp., among yeasts: *Oospora* spp., *Candida* spp., *Mycoderma* spp., *Rhodotorula* spp., *Debaryomyces* spp., *Torulopsis* spp. and *Saccharomyces* spp., among bacteria: *Micrococcus* spp., *Staphylococcus* spp. and *Brevibacterium* spp.. (Marcellino and Benson, 1992; Roostita and Fleet, 1996; Salavadori del Prato, 1998; Rea *et al.,* 2007; Dolci *et al.,* 2009).

The cheese rinds vary in thickness according to the cheese and age of the cheese. An example of sequential appearance of microorganisms naturally on the cheese rind of semisoft French cheese (St. Nectaire, belonging to the class of surface mold-ripened cheese) during ripening, is shown in Figure 4. A succession of microorganisms can be seen, yeast and lactic acid bacteria dominated at earliest stages of ripening, and no microbial growth can be seen in the cheese curd. Then the molds begin to grow and their hyphae form channels where yeast

and bacteria can grow. At the end of ripening the rind is formed by molds, yeast and bacteria (coryneform bacteria) differently localized (Marcellino and Benson, 1992).

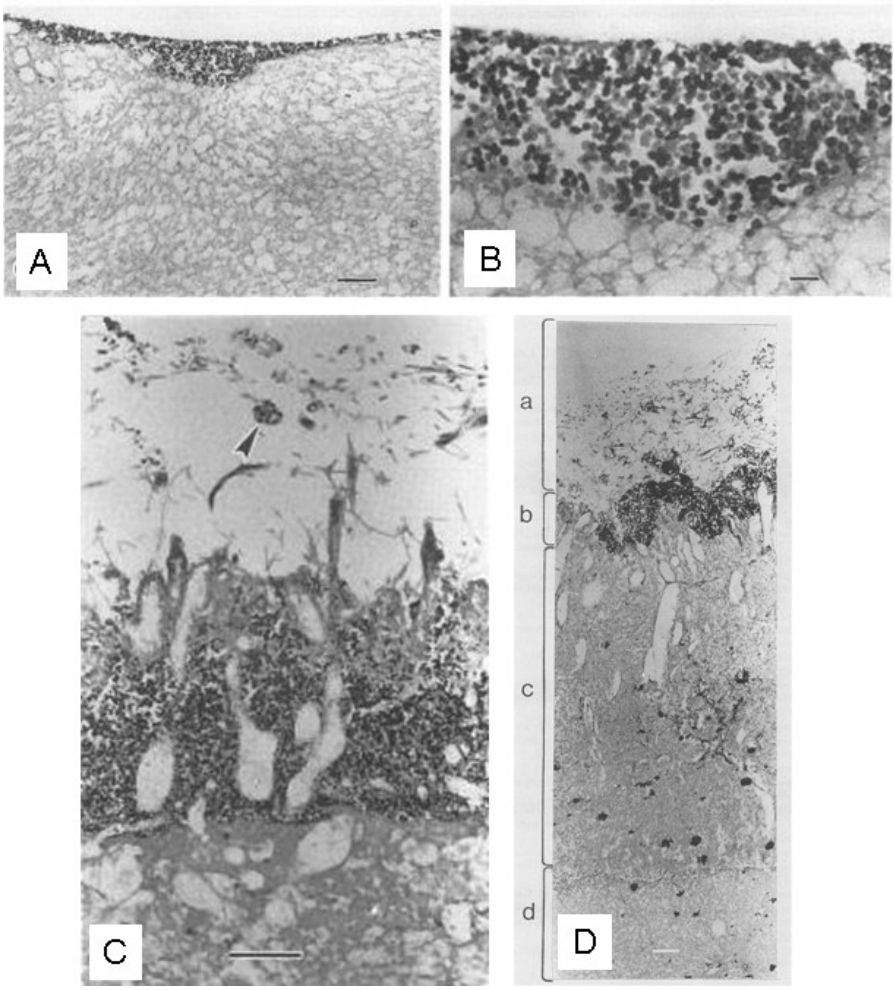

Figure 4. Light microscopy micrographs of a stained paraffin sections of semisoft cheese (St. Nectaire), belonging to the category mold-ripened (along with brie, camembert), showing nascent cheese rind during ripening. A) and B) after 1 to 2 days of ripening, Bar, 50 µm. C) at day 7 Aerial mycelia of (arrowhead) molds can be seen. The rind thickness measures 400 to 500 µm. Bar, 50 µm. D) at 37-day-old ripened cheese. (a) Fungal spores and collapsed hyphae, (b) layer of yeast and bacterial colonies, (c) dense fungal mycelia. (d) A clear demarcation between the rind and the curd can be distinguished. The rind measures 1.5 mm thick. Bar, 100 µm (Marcellino and Benson, 1992).

1.2.6 Cheese ripening and flavor development

The cheese ripening (maturation) is a complex process involving a range of microbiological and biochemical reactions. Microorganisms are present in cheese throughout ripening and contribute to the maturation process either directly through their metabolic activity or indirectly through the release of enzymes into the cheese matrix through autolysis (Fox *et al.,* 2004). During the ripening of cheese, three major biochemical events - glycolysis,

lipolysis, and proteolysis - occur, each of which is involved in flavor formation. The latter is probably the most important and also the most complex (Robinson, 2002). Metabolism of lactose, lactate, and citrate and related events are caused by living microorganisms (starter and/or non-starter), while lipolysis and proteolysis are catalyzed mainly by enzymes from the coagulant, milk, starter bacteria, adventitious non-starter bacteria, and, usually, secondary (adjunct) cultures (Mc Sweeney, 2011).

Lipolysis results in hydrolysis of the milk fat and the production of glycerol and free fatty acids, many of which, particularly the short-chain ones, have strong characteristic flavors (Robinson, 2002). Lactic acid bacteria (LAB) are weakly lipolytic. Levels of lipolysis may also be high in surface bacterial (smear) and mold-ripened cheeses. Very extensive lipolysis occurs in blue-mold cheeses, in which *Penicillium roqueforti* secretes potent lipases (Mc Sweeney, 2011).

Glycolysis is the conversion of lactose to lactic acid and is almost exclusively due to the growth of the starter bacteria and the lactate produced gives the freshly made cheese its overall acidic taste. They can also produce other compounds, e.g., diacetyl, acetate and acetaldehyde, which are important compounds in flavor formation in fresh cheeses; diacetyl is also an important flavor compound in hard cheeses (Weimer, 2007).

Cheese contains a broad range of proteinases and peptidases, which originate from the coagulant, milk, starter LAB, adventitious NSLAB, and secondary cultures (e.g., *Propionibacterium, Brevibacterium, Arthrobacter, Penicillium*) (Mc Sweeney, 2011). Native milk proteinase is called plasmin and is only significant in cheeses which are cooked to high temperatures because the cooking process inactivates chymosin. Chymosin is responsible for the initial hydrolysis of the casein, which the proteinases and peptidases released from the starter by autolysis, act on to produce smaller peptides and free amino acids. Many of the small peptides and amino acids contribute directly to flavor, but the starter bacteria also have enzymes that degrade the amino acids to amines, acids, alcohols, carbonyls, sulfur-containing compounds, which are also involved in flavor formation (Robinson, 2002).

During cheese ripening, texture will be modified by the combined actions of the LAB, secondary flora, the enzymes released, particularly proteolytic enzymes, and the cheese storage conditions (Wood, 1998; Gatti *et al*., 2008). Processing and ripening parameters such as pH, salt levels, water activity, and temperature influence the growth, metabolic state, viability, and rate of lysis of the LAB, and the activity and half-life of enzymes released upon lysis (Coolbear *et al.,* 2011).

1.2.7 Study of microbial ecology in dairy producs

Microorganism are not living as individuals instead they are part of a complex system where interact. Over the last decades, microbial studies in cheese and other matrices were based on cultivation of microorganism then identification by fenotypic studies. The methods that use a primarily cultivation step in *vitro* are known as culture-dependent techniques. These methods allow to partially identify the microbiota since not all microorganisms are able to cultivate in synthetic media (Jany and Barbier, 2008). Microbial ecology studies have gained more information with the development of molecular methods based on direct analysis of microbial DNA or RNA without any traditional cultivation step, the 'culture-independent techniques'.

Both approaches have advantages and disadvantages. Therefore, the combination of both approaches should be used to describe the contribution of individual micoorganisms and control microbiota (culture-based methods) and to determine the diversity, abundance and microbial activity (culture-independent) (Ndoye *et al.,* 2011). Culture-dependent methods are time-consuming and do not necessarily provide a comprehensive information on the composition of microbial communities. Minority microbial populations are often out-competed by more abundant species, and some species may be unable to cultivate (Jany and Barbier, 2008). Despite its disadvantages, culture-based approaches are extremely useful for understanding the physiological potential of isolated organisms. On the other hand, culture-independent techniques are fast and potentially more exhaustive.

Culture-independent techniques have been used to characterize microbial communities, to evaluate the *in situ* gene expression as well as to determinate the metabolic activities of cheese microbiota (Ndoye *et al.,* 2011). Among the molecular approaches that enable characterization of microorganisms, fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes (DeLong *et al.,* 1989; Amann *et al*., 1990) has been one of the most powerful and widely used techniques (Amann *et al*., 2001) in microbial ecology. FISH provides microbial identification, physical detection of uncultivable microorganisms, and distribution of microbial populations in several environments, including food products. FISH has been used to evaluate bacterial community structure and location in Stilton cheese (Ercolini *et al*., 2003a,b) and to detect Brevibacteria on the surface of Gruyère cheese (Kolloffel *et al*., 1999), to detect *Lactobacillus plantarum* on natural fermented olives (Ercolini *et al.,* 2006), and to quantify Leuconostoc populations in mixed dairy starter cultures (Olsen *et al.,* 2007).

Culture-independent methods are also suitable for determining the diversity of different food-associated microorganisms and to monitor dynamics over time. Most of them are based on polymerase chain reaction (PCR) amplification of total microbial DNA. The PCR amplicons from different species are discriminated by using gel or capillary separation and identified by comparison with databases or by sequencing. These techniques are known as fingerptinting methods, some of the most commonly used are polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and temporal temperature gel electrophoresis (TTGE). Length heterogeneity-PCR (LH-PCR) is another PCR-based fingerprinting technique. It can differentiate organisms based on species-specific variations in the length of 16S rRNA gene sequences. A fluorescently labelled oligonucleotide is used as a forward primer coupled to a reverse primer to amplify variable regions of the 16S rRNA gene, and labelled fragments are separated by capillary electrophoresis and detected by laser induced fluorescence with an automated gene sequencer (Randazzo *et al*., 2002; Ogier *et al*., 2002; Rademaker *et al*., 2005; Ercolini and Coppola, 2011).

Recently, high-throughput sequencing technologies have been developed that do not rely on the traditional Sanger chain termination method (Margulies *et al.,* 2005). These powerful sequencing platforms and processes can produce massive amounts of data in less time and at a lower cost. The sequence reads are shorter than conventional automated Sanger sequencing, each base position is sequenced many times on average to achieve deeper coverage (Haridas *et al.,* 2011). More than 300,000 sequences per run can be determined simultaneously, and eliminates the need for cloning and cultivation. A highly variable region of 16S rRNA gene is amplified using primers that target adjacent conserved regions, followed by direct sequencing of individual PCR products (Lopez-Velasco *et al.,* 2011). Because of this enormous information new potent softwares tools has been developed for data acquisition and analysis.

These sequencing platforms include the Illumina technology which sequence by chemical (www.illumina.com/technology/sequencing_technology.ilmn) synthesis by using a “reversible terminator-based method”, 454 sequencing which involves DNA capture beads (http://my454.com/products/technology.asp) and is based on the detection of pyrophosphate released during nucleotide incorporation, and SOLiD™ System (www.appliedbiosystems.com/absite/us/en/home/applications-technologies/solid-next-generation-sequencing.html) which uses “microfluidic FlowChips” (Haridas *et al.,* 2011).

Figure 5 summarizes the culture-dependent and culture-independent methods used to study the community structure and activity in cheese microbiota.

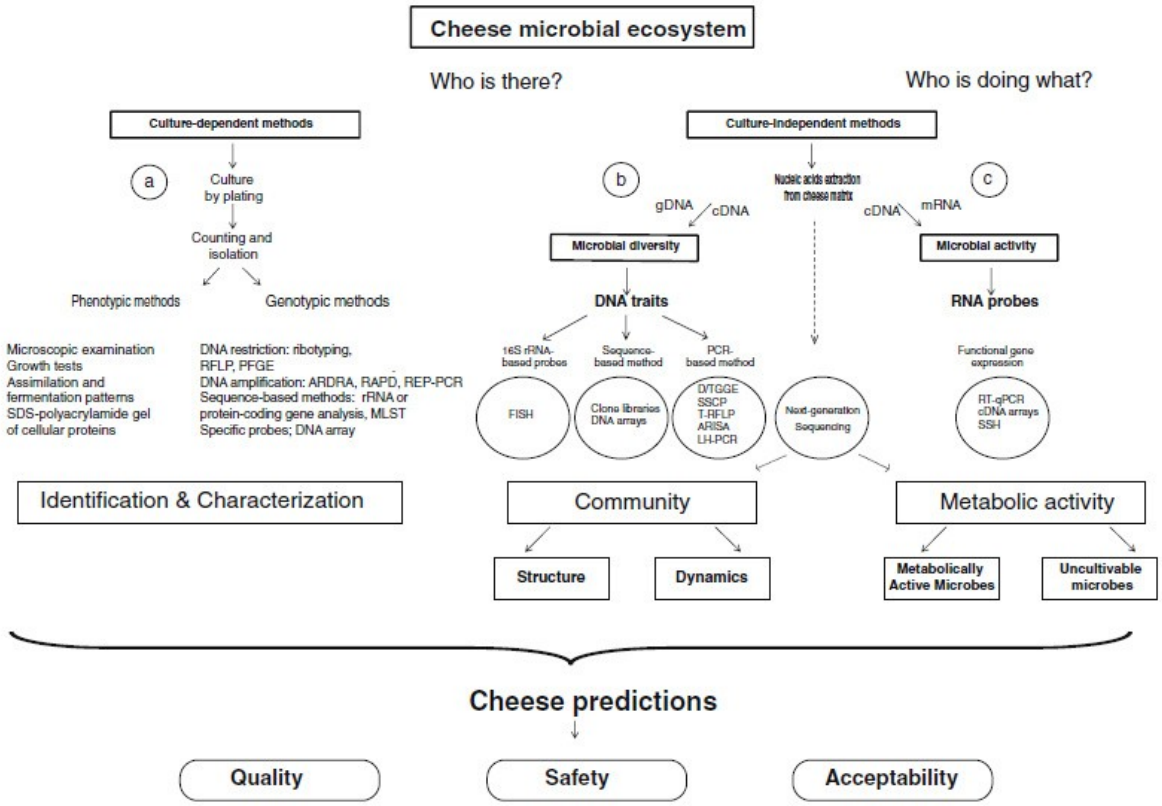

Figure 5. Flow diagram of culture-dependent and culture-independent methods to study the community structure and activity in cheese microbiota (Ndoye *et al*., 2011).

Briefly, Illumina sequencing requires that a DNA sample is converted into special sequencing libraries. This can be achieve by shearing DNA to a designated size and adding specific adapter sequences on both ends of the DNA molecules. This adapters allow molecules to be immobilized in one or more channels of a flow cell and amplified to form local clonal colonies. Sequencing primers, and four differently labelled nucleotides (also 3'-blocked) are provided and used for extension by DNA polymerases and non-incorporated nucleotides are washed away. The DNA is extended one nucleotide at a time and a camera takes images of the fluorescently labelled nucleotides. The dye along with the terminal 3′ blocker is chemically removed from the DNA, allowing a next cycle (Haridas *et al.,* 2011; Kirchner *et al*., 2011). Figure 6 shows esquematically the Illumina sample preparation and sequencing.

A reliable estimation of the relative abundance of microbial species can be obtained. To date, they have been used for culture-independent analysis in order to monitor microbial communities in various ecosystems such as soil (Acosta-Martinez *et al.,* 2008), gut (Andersson *et al.,* 2008), food (Lopez-Velasco *et al.,* 2011), fermented food (Humblot and Guyot 2009; Roh *et al.,* 2010; Park *et al.,* 2011) and cheese (Masoud *et al.,* 2011). The high throughput sequencing technologies offers a more global view of the community structure.

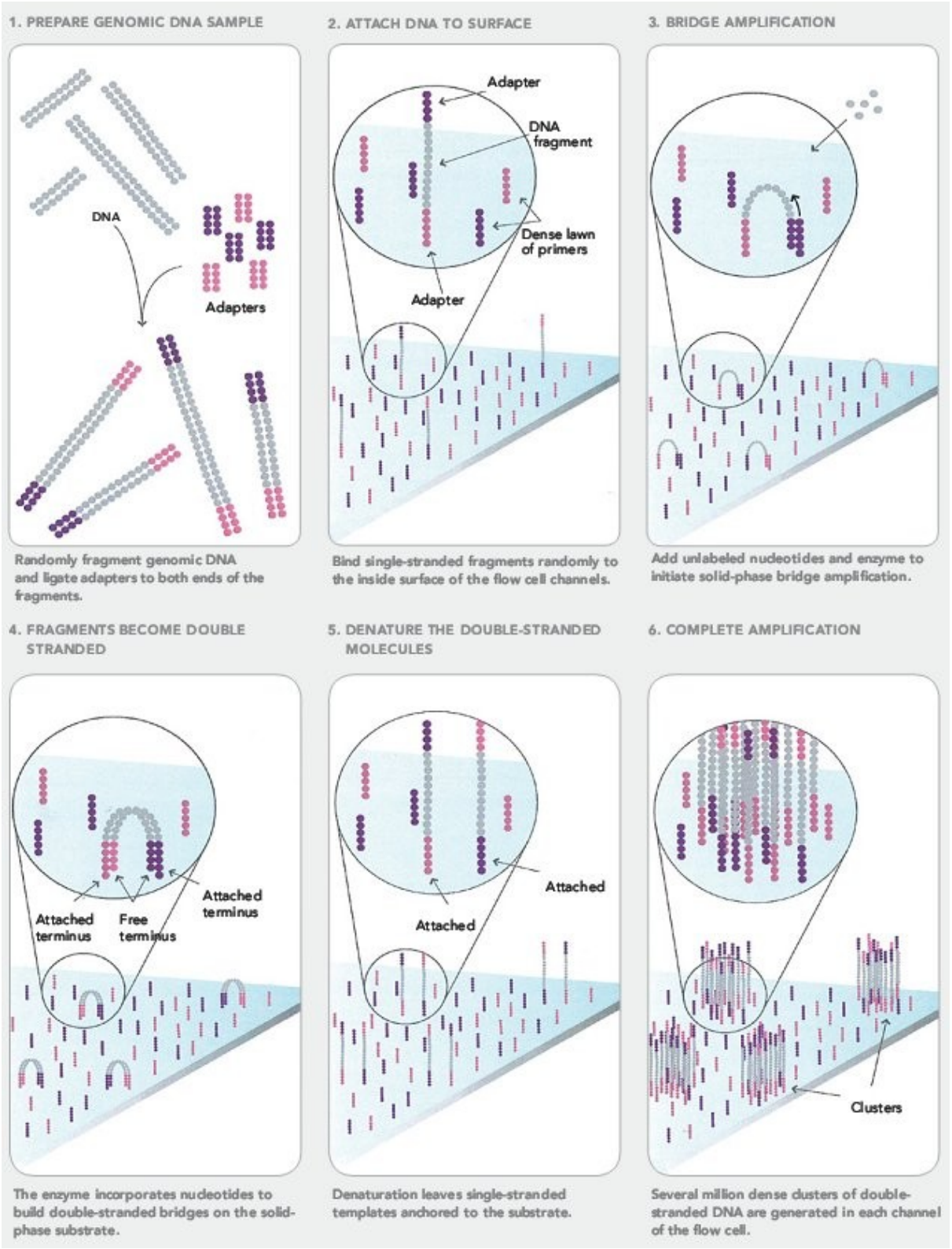

Figure 6. Illumina sample preparation and sequencing.

(http://seqanswers.com/forums/showthread.php?t=21)

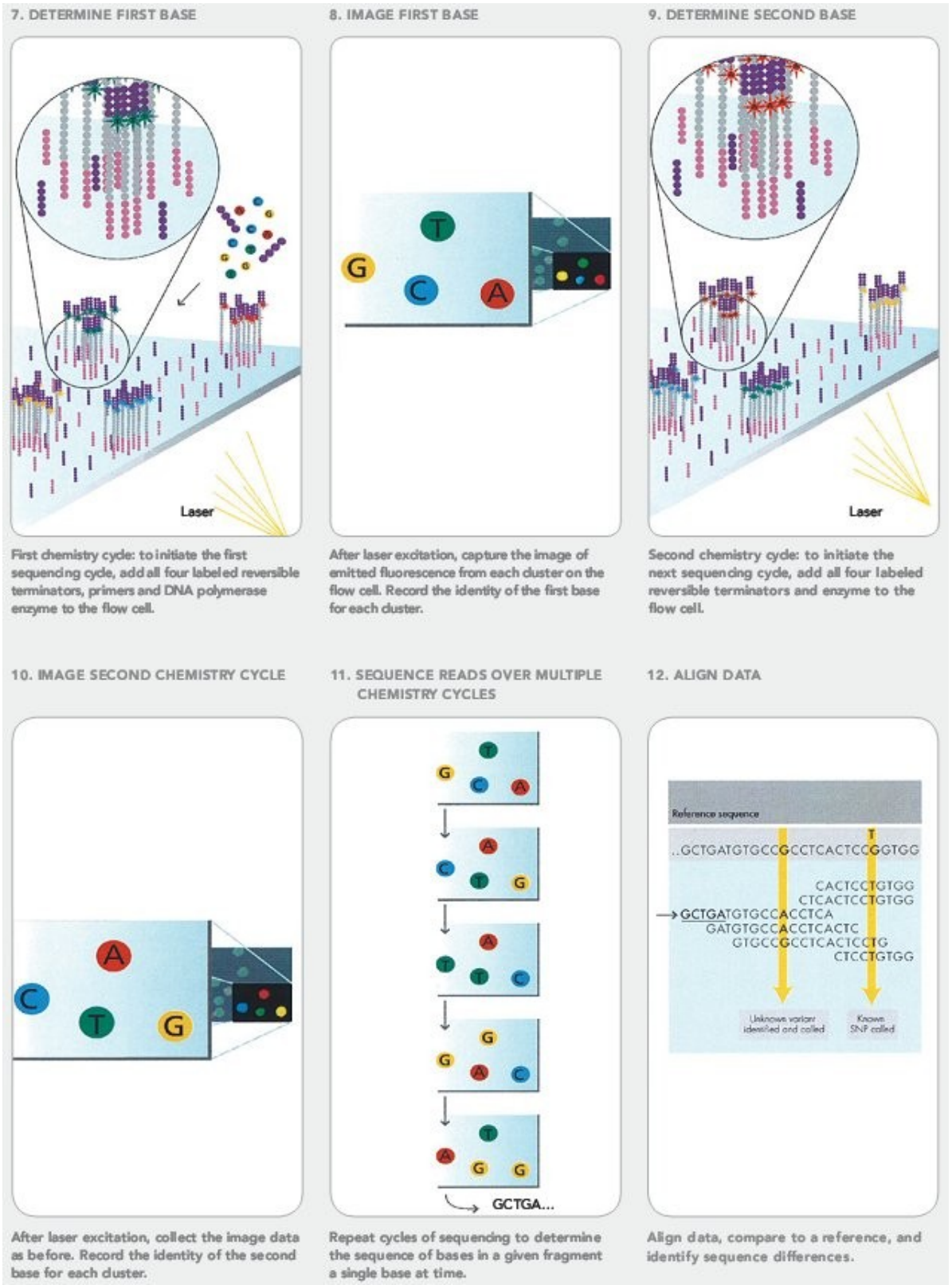

Figure 6 cont. Illumina sample preparation and sequencing (http://seqanswers.com/forums/showthread.php?t=21)

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2. AIMS OF THE THESIS

Cheese is a biologically and biochemically dynamic food. Microorganisms arising from starter, milk and the dairy environment contribute to all the steps throughout the manufacturing and ripening processes. Together with technological parameters are responsible to the great diversity of cheese varieties. Since it is as dynamic matrix, biochemical events must proceed under conditions controlled by technology to lead to products with desired peculiar aromas and textures. Microorganisms either deliberately added or adventitious, do not live as individuals, instead, they are part of an ecosystem in which they interact. These interactions lead to define the population that successively will determine the organoleptic characteristic of each cheese variety.

The general aim of this thesis was to study microbial diversity and dynamics of both starter and non-starter microorganisms involved in the cheese manufacturing and ripening processes. Thus, different ecosystems from raw milk to cheese and rind have been investigated in order to comprehend the specific role played by microorganisms in each cheesemaking phase and to correlate the occurrence of certain microbial species with desired flavor and texture.

For this reason, natural whey starters, hard Italian cheeses (i.e., Grana Padano and Parmigiano Reggiano), and natural rind of mold-ripened cheese (i.e., blue cheese) have been studied.

Through culture-based and culture-independent approaches, the microbiota of these different dairy matrices have been described with the purpose of trying to understand how starter, non-starter and adventitious microorganisms contribute or simply take part in the development of the distinctive features of each considered product.

3. RESULTS

3.1 Natural whey starter for Parmigiano Reggiano: culture-independent approach

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3.1.1 Abstract

The aim of this work was to obtain a deeper insight into the knowledge of microbial composition of Parmigiano Reggiano natural whey starters through different culture-independent methods. Eighteen different Parmigiano Reggiano natural whey starters sampled from three different provinces of this cheese production area and the non-acidified wheys from which they arose, have been studied by length heterogeneity PCR (LH-PCR) and fluorescent *in situ* hybridization (FISH). A high microbial composition variability between different samples has been observed. Revealing different images of the same community, LH-PCR and FISH have given a more accurate view of the not well-known Parmigiano Reggiano whey starter ecosystem. New lights have been shed on Parmigiano Reggiano natural whey starters microbial composition, highlighting how culture-independent approach could be used and improved to study this and other food ecosystems.

3.1.2 Introduction

Parmigiano Reggiano is an Italian protected designation of origin (PDO) cheese. It is a hard, cooked cheese made from raw partly skimmed cow's milk supplemented with natural whey starter (Neviani *et al*., 1998; Coppola *et al*., 2000). Natural whey starter is obtained from the previous day residual whey which is then incubated at a gradually decreasing temperature (Neviani and Carini 1994, Coppola *et al*., 1997). During cheese production, the composition of LAB (lactic acid bacteria) microbiota undergoes several changes, because of modifications of environmental conditions. This may lead to cellular stress, such as heat shock, adverse pH, reduction of redox potential, water activity, and nutrient content (Di Cagno *et al*., 2006). Thermophilic acidifying starters grow during the first few hours of hard cooked cheese

making, determing the key acidification step, drawing enzymatic potential and the environment that will prevail for microbial growth and activity throughout the ripening period (Charlet *et al*., 2008). These complex consortia of microorganisms have an important technological role in defining the quality of such an appreciated cheese as Parmigiano Reggiano. The study of the dynamics within the microbial populations has often been hampered by culturing techniques limitations, such as failure in detecting viable non-cultivable bacterial species and selection given by chosen growing parameters (Fleet 1999; Giraffa and Neviani, 2001). To overcome these drawbacks, different non-cultivable methods, including length heterogeneity polymerase chain reaction (LH-PCR) (Lazzi *et al.,* 2004, Gatti *et al*., 2008; Santarelli *et al*., 2008) and fluorescent *in situ* hybridization (FISH) (Ercolini *et al*., 2003 a,b; Fornasari *et al*., 2008) have been developed and applied to whey starter or other dairy food matrices. So far, few studies have been performed on natural whey starters for Parmigiano Reggiano (Cocconcelli *et al*., 1997; Coppola *et al.,* 2000; Gatti *et al.,* 2003; Gatti *et al.,* 2008). Through both culture-dependent and culture-independent techniques, these authors have found *Lactobacillus helveticus* to be the dominant species within the natural whey starters for Parmigiano Reggiano cheese. With the awareness of culture-based methods limits in detecting microorganisms in complex ecosystems such as fermented foods (Jany and Barbier*,* 2008), a deeper insight into the microbial composition of natural whey starters for Parmigiano Reggiano was aimed in this study. Using a polyphasic approach, eighteen different Parmigiano Reggiano natural whey starters, sampled from three different provinces of this cheese production area, were investigated, combining the culture-independent methods FISH and LH-PCR. Further, the microbial composition of the non-acidified wheys from which the whey starters arose, was considered.

3.1.3 Materials and methods

Whey starter samples

Eighteen whey starters for Parmigiano Reggiano were sampled from nine different dairies located in the provinces of Parma (A, B, C), Modena (D, E, F) and Reggio Emilia (G, H, I), belonging to the Parmigiano Reggiano PDO cheese area of production. Whey cultures (named "a" as "acidified") were collected right before addition to the vat milk. The 18 non-acidified wheys (named "na" as "non-acidified") from which whey starters arose, were collected from the vat 10 min after the end of the cooking process. From each dairy, samples were collected during two consecutive days of cheesemaking (e.g.: A_{na1}, A_{a1}, A_{na2}, A_{a2}). Samples were cooled

at 4-6°C at the dairy plant, shipped to the laboratory under refrigerated conditions and immediately analysed.

Acidity determination

Titratable acidity was obtained by titrating 50 ml of sample with NaOH 0.25 M, using phenolphthalein as indicator and the results have been expressed with Soxhlet Henkel degrees (°SH). The analysis was carried out in duplicate and the average values calculated to give an estimated error lower than 1%.

Microbial count

A ten-fold serial dilutions of natural whey starters and non-acidified whey samples were done in 0.05 mM sodium citrate (Sigma, Italy) buffer pH 7.5. To recover the cultivable bacterial population arising from natural whey starter, the non-selective Whey agar medium (WAM) was used with an incubation period of 48h at 42°C under anaerobic conditions. This rich medium is known to be the better substrate of growth for recovering whey starter LAB thank to its composition similar to the matrix of origin (Gatti *et al*., 2003). Plate counts were carried out in duplicate. The enumeration of the total bacterial cell numbers was performed according to the protocol by Mesa *et al.* (2003) using the DNA intercalating agents 4',6-diamidine-2-phenylindole, dihydrochloride (DAPI) (Sigma-Aldrich, Italy). A stock solution was prepared by dissolving 1 mg of DAPI in 10 mL of ultrapure water. The stain at a final concentration of 10 µg/ml was added to 1 ml of second-diluted natural whey starter, previously washed twice with sterilized distilled water and then incubated for 30 min at room temperature in the dark. After incubation, the samples were filtered on a black polycarbonate membrane (Millipore corp., MA, USA); the membrane was air-dried and mounted on a glass slide in Citifluor solution (Citifluor Ltd, London, UK). The bacteria on DAPI-stained membranes were enumerated by counting the total number of blue fluorescing bacteria. The number of bacteria was estimated from counts of 20 microscopic fields (at × 1000) and calculated as follows:

$$N = \frac{C \times A}{a \times V} \times D$$

where N is the number of cells per mL; C is the number of cells per observation field; A is the filtration area (mm^2); a is the observation field area (mm^2); V is the volume of filtered sample (ml); and D is the dilution factor.

Fluorescent *in situ* hybridization

Whey samples were washed twice in PBS and pellets were resuspended in 300 µl of PBS. Cells were fixed adding freshly prepared cold paraformaldehyde 4% (Sigma-Aldrich, Milano, Italy), 1:3 (v:v), and stored for 1h at 4°C according to Amann *et al.* (1990). After washing, pellets were resuspended in 50% (v/v) ethanol/PBS and stored at -20°C until further FISH analysis. About 20 µl of the fixed cell suspension were spotted on poly-L-lysine coated slides and let to dry at 46°C for 10 min in a oven. Spots were dehydrated in ethanol series by covering them with about 50 µl of 50%, 80% and 100% ethanol solutions for 3 min each and air dried. Specimens were treated by covering the spots with 10 µl of proteinase K (10 mg/ml) at 37°C for 10 min, to allow permeabilization of Gram-positive cells. Lbh1-fluorescein isothiocyanate (FITC) labelled probe (Bidnenko *et al*., 1998), specific for *L. helveticus* and St4-Cy3 labelled probe specific for *S. thermophilus* (Mercier *et al*., 2000), have been used. Both probes were synthesized and labelled by MWG (Ebersberg, Germany). After addition of 10 µl of the hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl pH 7.2, 45% formamide) containing 10 ng of each probe, slides were incubated in a dark humid chamber at 45°C for 4 hours. Unbound oligonucleotides were then removed by incubating slides in pre-warmed washing buffer (20 mM Tris-HCl pH 7.2, 0.01% SDS, 40 mM NaCl, 5 mM EDTA) at 46°C for 15 min and by rinsing with water.

Slides embedded in mounting oil were evaluated with a Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a C-SHG1 100 W mercury lamp. Nikon filter set B2A FITC was used for Lbh1 FITC-labelled probe (excitation wavelength, 450-490 nm; emission wavelength, 500–520 nm). Nikon filter set G-2E/C was used for St4 Cy3 labelled probe (excitation wavelength, 540/25 nm; emission wavelength, 605/55 nm). Pictures of each field were taken and then superimposed through the Nis Elements software (version 2.10 Nikon).

DNA extraction

Genomic DNA was extracted from each sample using an InCura DNA extraction kit (InCura srl, Cremona, Italy) according to the manufacturer's instruction. DNA was spectrophotometrically (Jasco V-530, Japan) quantified by measuring absorbance at 260 nm, diluted up to 20 ng/µl and stored at -20°C until use.

Length heterogeneity PCR

LH-PCR to analyse the V1 and V2 variable regions of the 16S rRNA gene was performed as previously described by Lazzi *et al.* (2004). The primer pair 63F (5'-AGGCCTAACACATGCAAGTC-3') 5'-end labelled with 6-carboxy-fluorescein dye (6-FAM) and 355R (5'-GCTGCCTCCCGTAGGAGT-3') was used for the analysis. Reaction and amplification conditions proposed by Lazzi *et al.* (2004) were slightly modified according to Gatti *et al.* (2008). 0.5 U of Platinum Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA) were used in 20 µL of reaction mixture. Initial denaturation at 94°C for 2 min was followed by 25 cycles consisting of denaturation at 95°C for 45 s, annealing at 49°C for 45 s, and extension at 72°C for 2 min. A final extension step of 72 °C for 7 min was carried out. LH-PCR products were stored at -20°C in the dark until use (usually < 1 week). For fragment analysis, 1 µl volumes of LH-PCR amplicons were mixed with 12 µl of deionized formamide (Applied Biosystems) plus 1 µl of internal size standard (GS500 LIZ®, Applied Biosystems, Foster City, CA, USA) and then denatured at 90°C for 2 min, followed by immediate chilling on ice. Capillary electrophoresis was performed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) under the following conditions: 47 cm capillary, polymer 4 (POP-4™), 15 s injection time, 15 kV injection voltage, 35 min electrophoresis at 15 kV and 60°C capillary temperature. The peaks of the electropherogram profiles, corresponding to amplicons of different length, were attributed to bacterial species according to an LH-PCR database obtained in a previous study (Gatti *et al*., 2008). Amplicons sizes were determed with GeneMapper v4.0 software (Applied Biosystems, Foster City, CA, USA). LH-PCR profiles were analysed by reference to the internal size standard using the local Southern size calling method, no-smoothing option and a threshold of 50 fluorescent units.

3.1.4 Results

Eighteen natural whey starters for Parmigiano Reggiano cheese and the eighteen non-acidified wheys from which they arose were investigated. Samples were collected from nine different dairies located in three different zones of Parmigiano Reggiano production, during two consecutive days of cheesemaking. Plate counts on WAM showed a very similar trend of cultivability among all whey starter samples. Lactic acid bacteria (LAB) able to grow in WAM ranged between 8.03 and 8.79 log cfu/ml, with a variability lower than 3% (Table 1).

Titratable acidity of each sample was also measured and results ranged between 26.73 and 33.8 °SH.

	Microbial log counts and standard deviation (SD)			
	Total expressed as cells number/ml		cultivable in WAM expressed as mean cfu/ml	
Samples	Mean	SD	Mean	SD
A_{a1}	9.15	0.12	8.03	0.51
A_{a2}	9.20	0.15	8.09	0.71
B_{a1}	9.26	0.18	8.36	0.78
B_{a2}	9.58	0.32	8.40	0.55
C_{a1}	9.89	0.16	8.64	0.88
C_{a2}	9.65	0.28	8.56	0.81
D_{a1}	9.51	0.43	8.34	0.66
D_{a2}	9.16	0.11	8.26	0.45
E_{a1}	9.46	0.15	8.43	0.78
E_{a2}	9.63	0.33	8.48	0.74
F_{a1}	9.94	0.14	8.79	0.89
F_{a2}	9.80	0.10	8.77	0.81
G_{a1}	9.52	0.28	8.11	0.63
G_{a2}	9.48	0.22	8.39	0.52
H_{a1}	9.47	0.17	8.73	0.59
H_{a2}	9.64	0.18	8.42	0.63
I_{a1}	9.61	0.25	8.29	0.75
I_{a2}	9.65	0.25	8.31	0.46

Table 1. Mean log count of total microbial population and cultivable in WAM microbial population of 18 natural whey starters

Moreover, in order to find confirmatory, complementary or alternative results, a culture-independent approach was used. Total cell count in natural whey starter samples ranged between 9.15 and 9.94 log cells/ml with a variability lower than 3% (Table 1). The species composition of whey starters and non-acidified wheys was investigated by LH-PCR. Three representative LH-PCR profiles referred to the whey starters are shown in Figure 1. The different fragment sizes in the LH-PCR profiles were attributed to bacterial species on the basis of a published LH-PCR database (Lazzi *et al*., 2004, Fornasari *et al*., 2006, Gatti *et al*., 2008). Because the areas under the peaks shown in the electropherograms are rough measure of the proportions among the species, their relative estimation was also possible (Suzuki *et al*.,

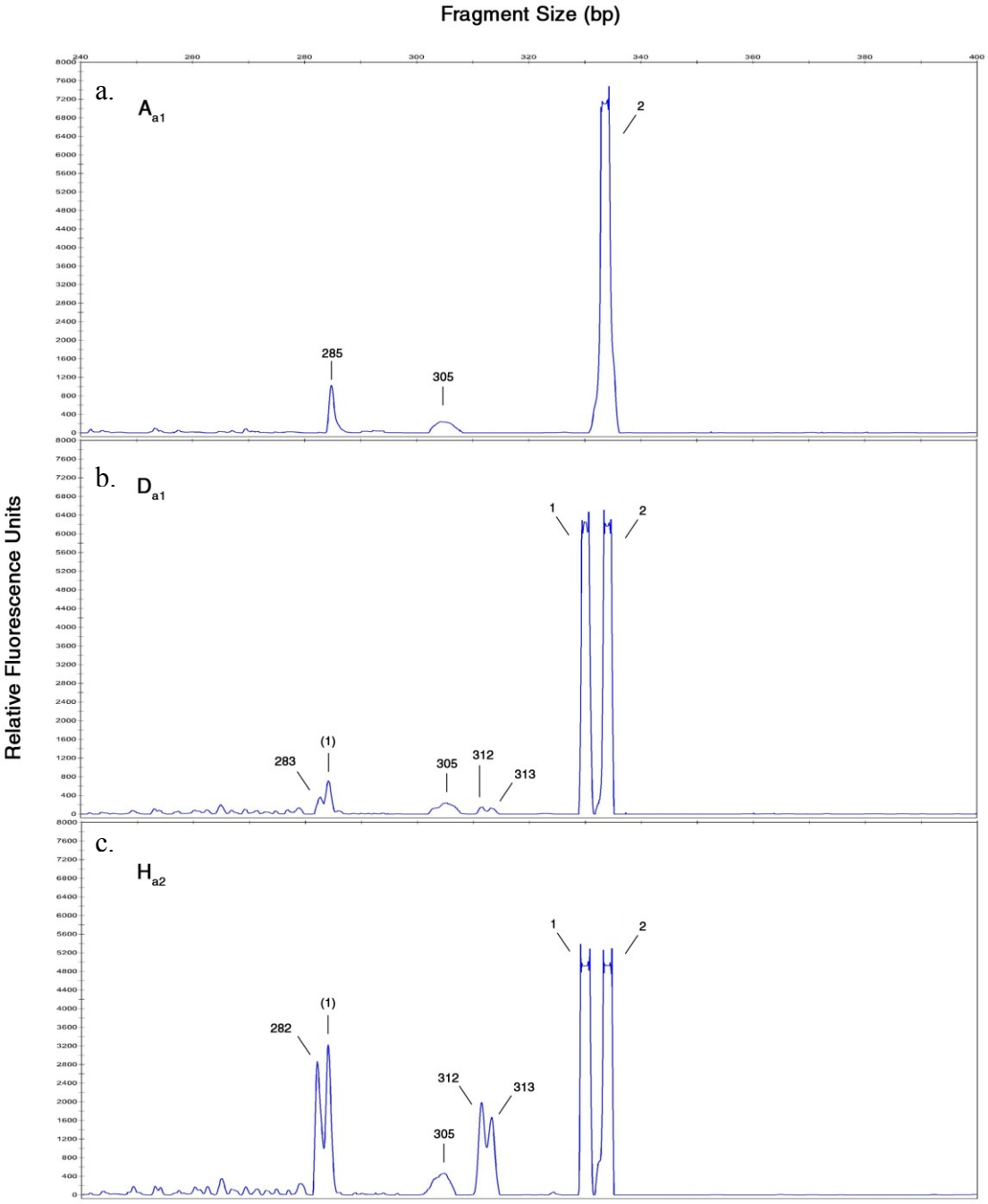

Figure 1. Length heterogeneity LH-PCR electropherograms of three whey starter samples representative (A_{a1}; D_{a1}; H_{a2}) of different cases occurring: *L. helveticus* as dominant species (a), comparable percentages of *L. helveticus* and *L. delbrueckii* (b), *L. helveticus* and *L. delbrueckii* percentages comparable also to the percentage of other species (c). The *x* axis shows peaks size in base pairs, and the *y* axis shows peak intensity in relative fluorescence units. The peak sizes were attributed to bacterial species according to LH-PCR published database as follows: **1** *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus*; **(1)** Secondary peak of 1; **2**, *L. helveticus*. Unattributed peaks are shown by the fragment lengths as base pairs.

1998). For the majority of the samples, the same peaks were frequently detected: 330 ± 1 bp (attributed to *Lactobacillus delbrueckii* subsp. *lactis* or subsp. *bulgaricus*), 334 ± 1 bp (*L. helveticus*), 319 ± 1 bp (*S. thermophilus*), 342 ± 1 bp and 345 ± 1 bp (attributed to *Lactobacillus fermentum*) and 305 ± 1 bp (non-attributed).

However no peak at 330 ± 1 bp (attributed to *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus*) was detected in the samples A_{na1}, A_{a1}, A_{na2} and A_{a2}. A peak attributable to *S. thermophilus* was revealed in the whey starters B_{a1}, C_{a1}, $G_{a1,}$ D_{a2}, E_{a2}, electropherograms. Two fragments of 342 ± 1 bp and 345 ± 1 bp revealed the presence of *L. fermentum* in the whey starters samples B_{a1} and B_{a2}, F_{a1}, F_{na2}, F_{a2} , G_{a1} G_{a2}, and I_{a2}. We noticed that for the samples G_{na1}, $G_{na2,}$ H_{na1}, I_{na1} and I_{na2} the 330 ± 1 bp peak (attributed to *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus*) fluorescence intensity was lower than in the deriving whey starters. H_{a1} and H_{a2} whey starters and H_{na1} and H_{na2} non-acidified wheys' profiles were characterized respectively by the presence of two non-attributed peaks at 312 and 313 ± 1 bp and at 305 ± 1 bp (data not shown). The composition of natural whey starters and non-acidified wheys sampled in this study, was investigated also by FISH. 23S rRNA Lbh1 probe specific for *L. helveticus* and 16S rRNA St4 probe specific for *S. thermophilus* have been used. Being respectively labelled with FITC (green) and Cy3 (red), the simultaneous visualization of both groups of hybridized cells was possible. For each sample, green hybridized rod-shaped cells (*L. helveticus*), red hybridized round-shaped cells (*S. thermophilus*) and non-hybridized rod-shaped cells have been observed (Figure 2). Non-hybridized cells could be either lactobacilli non-*L. helveticus* or non-viable (low RNA content) *L. helveticus*. The signal intensity of cells hybridized with oligonucleotide probes is in fact directly related to the cellular rRNA content (Bottari *et al*., 2006) which is a useful indicator of viability (Bentsink *et al*., 2002). Positivity to one or more of these conditions was indicated with "+" or "++" if representing the majority. A majority of *L. helveticus* hybridized cells has been observed for A_{na1}, A_{a1}, $A_{a2,}$ H_{a2} and $I_{a1.}$ Non-hybridized rod-shaped cells were found to be the major component for B_{na1}, B_{a1}, C_{na1}, C_{na2}, E_{a2}, G_{a1}, G_{na2}, G_{a2}, H_{na1}, H_{na2}, I_{na2} and I_{a2}. Hybridized cells of *S. thermophilus* have been observed for all the natural whey starter samples, and for almost all the non-acidified whey samples, except for A_{na1}, B_{na2}, G_{na2} and I_{na1} (Figure 3).

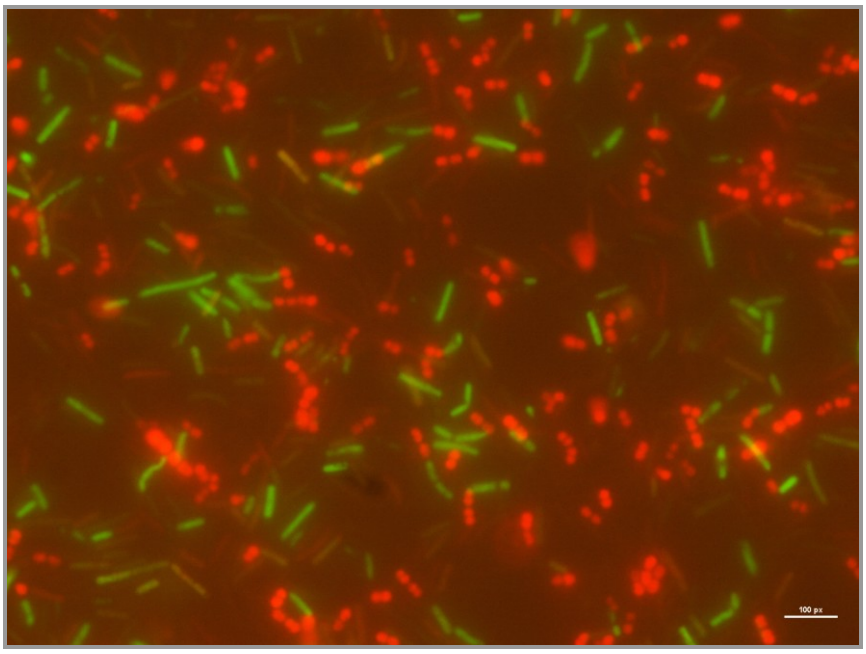

Figure 2. FISH on a sample of Parmigiano Reggiano natural whey starter. Simultaneous use of probes St4 (red label) and Lbh1 (green label). Hybridized *L. helveticus* cells appear green, Hybridized *S. thermophilus* cells appear red. Non-hybridized cells on the background could be either non-*L. helveticus Lactobacillaceae* or non-viable *L. helveticus*

3.1.5 Discussion

A polyphasic approach, combining different culture-independent methods such as FISH and LH-PCR, was used to investigate the microbial population in 18 Parmigiano Reggiano natural whey starters sampled from three different provinces of this cheese production area. In previous works, plate isolation revealed a simple composition of Parmigiano Reggiano whey starters microbiota, highlighting the prevalence of *L. helveticus* species and the presence of *L. delbrueckii* (Cocconcelli *et al*., 1997; Coppola *et al*., 2000).

The always more used culture-independent methods for the analysis of microorganisms in food have already shed light on the structure of microbial population of dairy environments (Andrighetto *et al*., 1998; Fitzsimmons *et al*., 1999; Mannu *et al*., 2000; Berthier *et al*., 2001; Dasen *et al*., 2003). Together with traditional counting methods, LH-PCR and FISH have been therefore used for a better investigate the complex ecosystem of Parmigiano Reggiano whey starter.

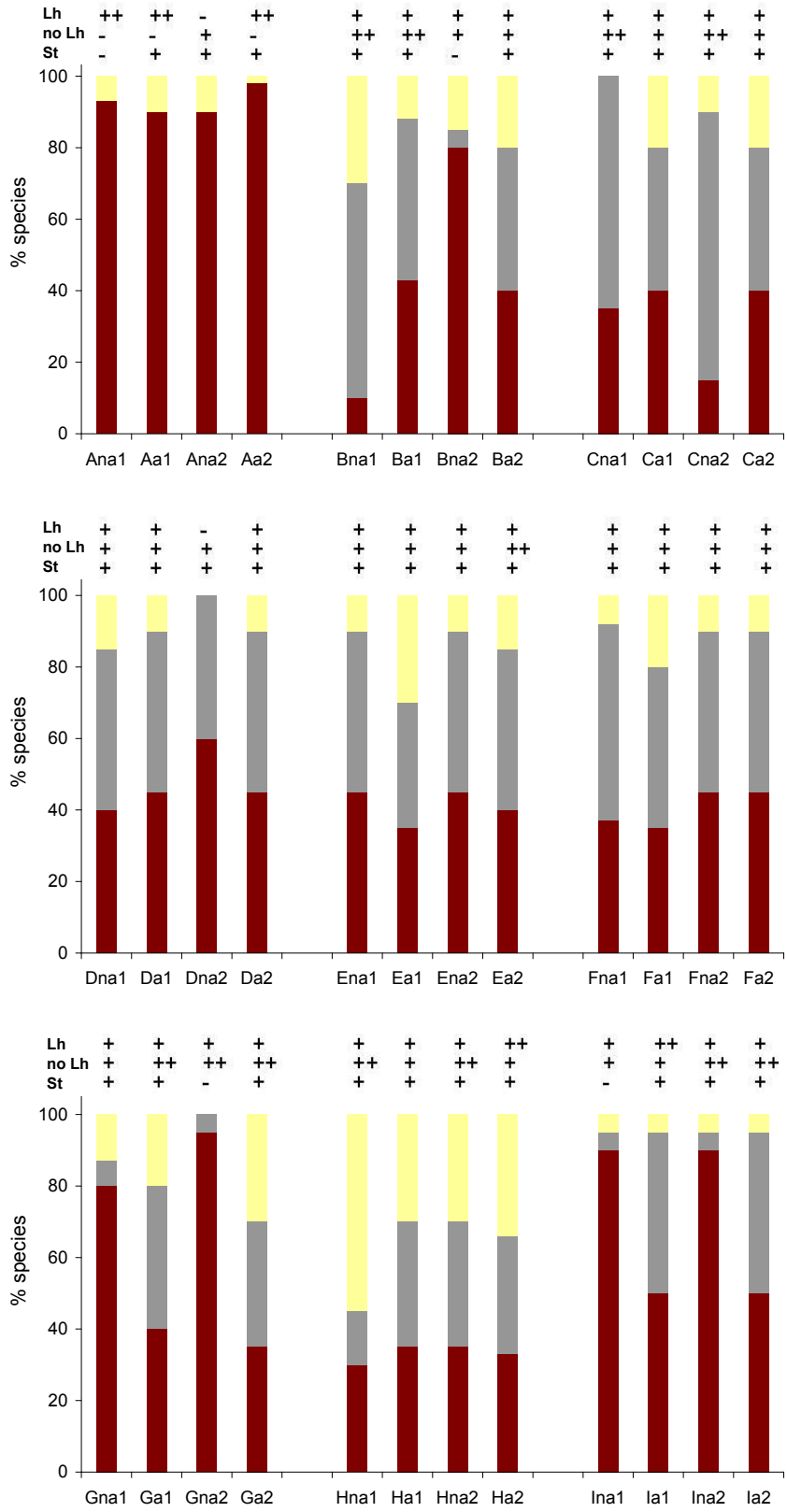

Figure 3. LH-PCR (bars) and FISH (grid) results for whey starter samples ($samples_a$) and non-acidified whey samples ($samples_{na}$). Bars- Percentage of *L. helveticus* species (red), *L. delbrueckii* species (grey) and other species (yellow) calculated on peaks relative abundances in LH-PCR profiles. Grid- Presence (+) or absence (-) of hybridized *L. helveticus* cells (Lh), hybridized *S. thermophilus* cells (St) and non-hybridized cells (no Lh) revealed by FISH. (++) indicates majority.

Plate counting was performed on WAM which gives better results in counting natural whey starter LAB, thank to its capability of reproducing the natural system of the whey, as proven by Gatti *et al*. (2003). Despite the use of WAM, the number of cfu/ml was up to 1 unit log lower than direct total count measured with DAPI (Table 1). Although the highest amount of cells measured with DAPI could be because of the presence of both nonviable cells and viable but non-cultivable cells, this result can support previous observations made by Gatti *et al*. (2006), who reported lower cultivability with respect to the total number of cells in a whey starter sample. This difference could be related to the presence of viable cells unable to duplicate in the agar medium. Data obtained for both natural whey starters for Grana Padano and Parmigiano Reggiano, are in agreement with Fleet *et al*. (1999) that calculated that plate culturing techniques reveal only 1-10% of the real microbial population present in natural environment.

The microbial loads of all the whey starter samples considered were very similar when analyzed with culture-dependent analysis. However, no information on microbial composition can be given by plate counting and possible biases can be introduced such as failure in culturing species that cannot grow under selected experimental conditions. LH-PCR and FISH have been therefore applyied to gain deeper knowledge of whey starter microbial composition. LH-PCR electropherograms of almost all samples revealed the presence of most frequently detected peaks, attributable to *L. helveticus, L. delbrueckii* subsp. *lactis* or subsp. *delbrueckii*, *S. thermophilus* and *L. fermentum.* According to a published database (Lazzi *et al*., 2004, Fornasari *et al*., 2006, Gatti *et al*., 2008), 330 ± 1 bp peak could be attributable to *L. delbrueckii* species as well as to *Enterococcus faecium* and *Enterococcus faecalis*. However, in whey samples microscopically observed after FISH analysis, all present round-shaped cells were hybridized by *S. thermophilus*-specific probe St4 excluding the presence of Enterococci. Our results are in agreement with Lazzi *et al*. (2004), Fornasari *et al*. (2006), Rossetti *et al*. (2008) and Santarelli *et al*. (2008), that found that the microbial composition of the natural whey starters for another Italian hard cooked cheese, such as Grana Padano, shows a constant presence of dominant species corresponding to *L. helveticus* and *L. delbrueckii* subsp. *lactis* and minor species corresponding to *S. thermophilus* and *L. fermentum.* Nevertheless, our results revealed a higher variability in the composition of different whey starters for Parmigiano Reggiano in comparison with the only natural whey starter for Parmigiano Reggiano so far analysed by LH-PCR (Gatti *et al*., 2008). Through culture-dependent approach, previous works on Parmigiano Reggiano natural whey cultures ascribed predominant whey microflora to *L. helveticus* (Cocconcelli *et al*., 1997; Coppola *et al*., 2000

Gatti *et al*., 2003). Differently from these authors, through LH-PCR results, we observed a variable microbial composition among whey starters. In fact, only two samples (A_{a1}, A_{a2}) were characterized by *L. helveticus* as dominant species (Figure 3), while the majority of whey starter samples have been found to have comparable percentages of *L. helveticus* and *L. delbrueckii* (B_{a1}, B_{a2}, C_{a1}, C_{a2}, D_{a1}, D_{a2}, E_{a2}, F_{a1}, F_{a2}, G_{a1}, I_{a1}, I_{a2}). In few whey starters (E_{a1}, G_{a2}, H_{a1}, H_{a2}), *L. helveticus* and *L. delbrueckii* percentages were also comparable to other species represented by non-attributed and attributed (i.e., *S. thermophilus*) peaks (Figure 3). We noticed that the whey starter samples with *L. helveticus* as dominant species (A_{a1}, A_{a2}), arose from non-acidified wheys where *L. helveticus* was the dominant species (A_{na1}, A_{na2}). Otherwise, other non-acidified wheys with a higher percentage of *L. helveticus* (B_{na2}, D_{na2}, G_{na1}, G_{na2}) gave rise to whey starters where *L. helveticus* was not the dominant species (B_{a2}, D_{a2}, G_{a1}, G_{a2}) (Figure 3). This variability could be related to different incubation conditions used by each dairy for the production of natural whey culture. The microbial composition and diversity of whey starters are, in fact, modulated by several factors, among which high thermophilic condition and deep acidification rate, responsible for a technological selection of microorganisms (Neviani *et al*., 1995; Fortina *et al*., 1998; Giraffa *et al*., 1998; Giraffa *et al* 2004). However, both whey starter samples with *L. helveticus* as dominant species (A_{a1}, A_{a2}) and the ones with comparable percentages of *L. helveticus* and *L. delbrueckii* (B_{a2}, D_{a2}) showed a high titratable acidity. On the other hand, both samples with comparable percentages of *L. helveticus* and *L. delbrueckii* (G_{a1}, $I_{a1,}$ I_{a2}) and samples with comparable percentages of *L. helveticus, L. delbrueckii* and of other species (G_{a2}), were characterized by a low titratable acidity. Therefore, whey starter titratable acidity, that plays a key role at the beginning of cheesemaking, did not seem to be related neither to cell amount (total and cultivable) nor to different species contribution. Even if no correlation between wheys acidity and species variability has been found, a correlation between wheys acidity and biotypes composition cannot be excluded. Whey starters and non-acidified wheys have been then analysed by FISH. Being respectively the dominant species and one of the most frequently detected minority species in whey starters for hard cooked cheeses (Cocconcelli *et al*., 1997; Coppola *et al*., 2000; Gatti *et al*., 2003; Rossetti *et al*., 2008), *L. helveticus* and *S. thermophilus* have been chosen as targets for FISH experiments. Considering natural whey starter samples, FISH results were in good agreement with the LH-PCR. However, a higher number of samples containing *S. thermophilus* was revealed by FISH. Both techniques have a limit of detection around 10^4 - 10^5 cells/ml (Lazzi *et al*., 2004, Fornasari *et al*., 2008), but with a very high percentage of *L. helveticus* or other species in the samples, S. *thermophilus* could have gone

undetected by LH-PCR. This could explain the better effectiveness of FISH in detecting *S. thermophilus*. Discordance between FISH and LH-PCR results was observed for some non-acidified whey samples ($A_{na1,}$ $A_{na2,}$ D_{na2}). Targeting respectively RNA and DNA, FISH is able to detect cells in a good physiological state, while LH-PCR to detect both living and dead cells. Cooking of the curd could affect integrity and thus lower RNA content of non-acidified whey microbial cells, making some of them undetectable by FISH. Otherwise, DNA of both live and dead or damaged cells can be estimated by LH-PCR (Gatti *et al*., 2008); slight differences observed with these two methods in non-acidified whey samples could be therefore explained.

3.1.6 Conclusions

An overall picture of the microflora of 18 Parmigiano Reggiano cheese whey starters was determined by LH-PCR analysis. This culture-independent approach highlighted on a great variability among the whey starter samples considered and deepened the information given by traditional culture-based techniques. A further knowledge on microbial composition of whey starter samples was provided by FISH analysis. These methods revealed different images of the same community, therefore a polyphasic approach, combining LH-PCR and FISH was worthwhile to obtain a more accurate view of the structure of Parmigiano Reggiano whey starters microbial community. Nevertheless, the frequent presence of several non-attributed peaks shown by LH-PCR in whey starter and non-acidified whey electropherograms draws attention on the fact that culture-independent methods need to be improved to reveal as accurately as possible the actual microbial composition of wheys and other food ecosystems. Further efforts might be therefore devoted in the future to address LH-PCR, as well as other culture-independent techniques, to a deeper knowledge of what these methods still do not exhaustively describe.

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3.2 Comparison of natural whey starters for Grana Padano cheese using sunray plots

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3.2.1 Abstract

Twenty one natural whey starters, collected from dairy factories located in six provinces of the Grana Padano production area, were characterized. Basic techniques such as acidity evaluation and microbial plate count together with more complex methods such as the Live/Dead® BacLight™ bacterial viability kit, have been used. Seven parameters including pH, Soxhlet Henkel degrees, microbial plate count in Man Rogosa Sharpe medium and Whey Agar medium together with count of total, viable and non-viable cells, were chosen to produce sunray plots. One plot for each natural whey starter sample was obtained by characterizing the status of the microbial culture and compared with three natural whey starter prepared in laboratory. In this way, a sunray trace was suggested to define the traits of a good natural whey starter. Another multivariate technique, principal component analysis (PCA), was applied and it was concluded that, for this particular data set composed by 24 object and 7 variables, PCA allowed to highlight the good and the bad samples, while sunrays plots, even if remaining only a descriptive and explorative analysis, allowed to better visualize the differences among all the samples.

3.2.2 Introduction

The Grana Padano Production disciplinary states that this Protected Designation of Origin (PDO) cheese must be produced with raw milk and natural whey starter. The starter is a natural culture of thermophilic lactic acid bacteria that grow in the whey produced at the beginning of dairy process (http://www.granapadano.com). The whey starter is produced by culturing the non-acidified whey resulting from daily cheesemaking, called sweet whey. Whey is fermented at a naturally decreasing temperature, that in approximately 20 hours decrease from about 54°C to about 35°C, performing a thermophilic selection. Whey starter is added in

the milk vat and sweet whey is recovered after curd cooking from each cheese production (Rossetti *et al*., 2008; Santarelli *et al*., 2008). The modality of preparation of the whey starter cultures warrants the survival of different biotypes useful to the development of the ecosystem itself, and a mixture of strains of the same species is necessary to the natural starter evolution (Gatti *et al*., 2004). The primary function of starter bacteria is to produce acid during the fermentation process; however, they also contribute to cheese ripening thanks to their enzymes involved in proteolysis and conversion of amino acids into flavor compounds (Fox and Wallace, 1997). Moreover, one important role of starter bacteria is to provide a suitable environment, with respect to redox potential, pH and moisture content in the cheese, allowing enzyme activity of rennet and starter, and making the growth of secondary flora to proceed favourably (Beredsford *et al*., 2001). For traditional Grana Padano production, it is the experience of the cheese maker's that defines the correct characteristics of a good natural whey starter. To date, the analytical techniques traditionally used for its characterization are very basic (pH, titratable acidity, plate count agar) and do not provide exhaustive information on the performance of the starter. These data are usually regularly recorded. The aim of this study was to apply a different approach to record these data set, together with the number of live/dead microbes, using a figurative method which could be useful when comparing different samples.

Sunray plots are basically a radial plot showing the importance of each variable in a sample, and that are used to compare more than two endpoints, or data sets, simultaneously. Sunray plots, or star plots, traditionally used for sensory analyses, have not been frequently reported in the literature for visualization of other data. As well as for sensory evaluation, this method has been used, for example, to show the relative importance of different descriptors chosen to predict the protein retention time in anion-exchange chromatography (Song *et al*., 2002; Tugcu *et al*., 2003) and to visualize plasma fatty acids chosen to screen and monitor the effects of infection following the use of adenoviral vectors in gene therapy (Paik *et al*., 2007). Sunray plots have also been useful for showing the differences between wine samples and coffee samples (Haswell and Walmsley 1998), in sensor responses of five typical malodours in fields (Romain *et al*., 2000) and in different types of propagation materials of banana 'Nanicão' (Scarpare Filho *et al*., 1998). However the use of sunray plots for microbial parameters has rarely been reported. In particular Hofman and collaborators characterized soil biological quality by means of microbial biomass determination and others seven chemicals parameters, describing an comparing their status by the sunray plots (Hofman *et al*., 2003).

Recent studies have been carried out to evaluate the microbial diversity of natural whey starter for Grana Padano cheese. Applying advanced microbiological techniques, such as random amplified polimorphic DNA PCR, temporal gradient gel electrophoresis (Andrighetto *et al*., 2004) and length heterogeneity PCR (Rossetti *et al*., 2008), the thermophilic dominant microflora characteristic of Grana Padano whey culture have been described. This study suggested a new approach for easily comparison and evaluation the quality of natural whey starter for Grana Padano cheese. To reach these goals, an alternative and easy way to visualize each sample in order to recognize the best or the worst characteristics, were considered. To implement this experimental study, 21 natural whey starters were characterized by three traditional parameters, such as count on Man Rogosa Sharpe (MRS), titratable acidity and pH, as well as four new parameters including Whey Agar (WAM) plate count agar and direct assessment of the total microbial population discriminating viable and non-viable cells.

3.2.3 Materials and Methods

Determination of the parameters

Twenty-one natural whey starter collected from 21 dairy factories involved in Grana Padano cheese production and located in the six main provinces of the Grana Padano production area (Brescia, Mantova, Piacenza, Padova, Vicenza and Verona) were obtained. Just before being added in the vat milk, samples were collected, cooled to 4°C, quickly transported to the laboratory and analyzed. Measurements of pH were performed in duplicate by using the pH212 pH meter (Hanna Instruments, Padova, Italy). Titratable acidity was obtained by titrating 100 ml of sample with NaOH 0.25 M, using phenolphtalein as indicator and the results were expressed as Soxhlet Henkel degrees (°SH). The analysis was carried out in duplicate.

Agar plate counts were performed using MRS agar pH 5.4 (Biolife, Milano, Italy) and whey agar medium (WAM; Gatti *et al*., 2003). Plates of MRS and WAM were incubated under anaerobic conditions (Anaerogen TM, Oxoid, Basingstoke, UK) at 42°C for 48 h. The counts were carried out in duplicate.

Fluorescence microscopy counts to assess the total (T), viable (V), and non-viable (NV) bacterial population were carried out using a Leica DMSL (Leica Microsystems, Wetzlar Germany) and LIVE/DEAD® BacLight™ bacterial viability kit, based upon SYTO®9 and Propidium iodide as previously described (Gatti *et al*., 2006). Each sample was prepared in duplicate and average values were calculated.

A variable usually employed in dairy factories is °SH which is used to calculate the amount of natural whey starter to add to the milk in the vat. Depending on milk acidity, the higher the acidity of the natural culture, the lower the amount to add. Moreover, positive traits of natural whey starters are a high number of T together with a high number of V.

With the aim to obtain samples to be considered positive and negative, three whey starters were prepared in the laboratory (samples G, B1 and B2) and were referred as control samples. The production started from the incubation at 45°C of one sweet whey obtained from the nearest dairy factory at less than 30 km. Values of pH and titratable acidity were monitored and microbial growth was evaluated by optical density at 650 nm (OD_{650}). Sample G was collected, and refrigerated (4°C), at the end of the exponential phase, after 20 h of incubation (high acidity and high OD_{650}), sample B1 was collected after 15 h (low acidity and low OD_{650}) and sample B2 after 25 h (high acidity and high OD_{650} in stationary phase) (data not shown).

Sunray plots

All the seven parameters obtained, count on MRS (MRS), on WAM (WAM), number of total cells (T), number of viable cells (V), number of non-viable cells (NV), tritrable acidity (°SH) and pH, (pH) were centered by average subtraction and normalized by dividing by standard deviation in the framework of the natural whey starters set evaluated. The standardized values were plotted into sunray plots with seven axes using Statistica 6.1 (StatSoft Italia Srl, Padova, Italy). Sunray plots are a subclass of circular icon plots in which the rays tend to form a circle. Each variable is represented by one ray or direction and all rays start in the center. The value of each variable is reflected by the distance from the center. These plots are basically a radial plot showing the importance of each variable in the sample. In this way, a unique plot for each whey starter sample was obtained, characterizing the status of the microbial culture. Samples G, B1 and B2 were used to represent one good and two bad natural whey starters, respectively.

Principal Component Analysis

The PCA analysis was performed using Statistica 6.1 (StatSoft Italia Srl, Padova, Italy).

3.2.4 Results and Discussion

Determination of the parameters

As expected, total cell counts (T) of the 21 natural whey starters for Grana Padano cheese were high and very similar to each other (CV 18%) (Table 1). As for the total count, the viable populations (V) were high and similar to each other, representing from 98% to 73% of the T population. The G sample, as expected, was characterized by a rather high percentage of viable cells (98%), showing that 20 h of incubation at 45°C were an optimal condition for producing the whey starter beginning from the selected sweet whey. Sample B1 showed the highest percentage of viable cells (99%) but a lower number of total cells because the culture was in stationary phase. The B2 sample exhibited the lowest percentage of viable cells (58%) as well as a low level of total cells, suggesting that the longer incubation, i.e., 10 h more than the B1 sample, had affected the microbial cells. The non-viable population (NV) gave a more variable result (CV 56%), representing on PC2 almost one-third of T population (Table 1).

Plate counts in MRS at pH 5.4 were more variable (CV of 68%) than those in WAM, ranging from 4% in PC2 to 46% in MN4. With respect to T, cultivable population in WAM ranged from 21% in PC2 to 100% in MN6, VI1 and VR1 and in MRS at pH 5.4 from 1% in PC2 to 44% in MN4. Cultivability of samples G and B1 in MRS at pH 5.4 and in WAM were similar, showing that this parameter depends upon the microbial biodiversity of the sample. In sample B2, cultivability was lower than for samples B1 and G, suggesting that acid stress can modify the capability of cells to duplicate in the agar medium.

The measurements of pH and titratable acidity are two different methods for evaluating the whey starter acidity. As expected the two parameters were not strongly correlated (correlation coefficient, -0.60). pH varied between 3.15 for BS4 to 3.49 for VI2. The highest value of pH in sample B1 was linked with low numbers of T and V values and high value of °SH. Differently form this control sample, for the experimental samples the same correlation was not found. It was observed that in samples characterized by a low percentage of viable bacteria (BS3, VI1, MN5 and MN2), the °SH were higher than 31.0, but in other samples in which the viable population was less than 86% (PC1, VI2 and PD1) the °SH was 29.0. In contrast, MN4 had 96% of viable cells and 32°SH. In the control samples similar percentage of viable cells were observed when acidity was 26.0 and 31.5 °SH (in B1 and G samples, respectively 99% and 98%), whereas the percentage of viable cells decreased in sample B2, when the acidity was 34.0 °SH. The relationship between acidity and cell viability could depend on the acid resistance of the dominant microbial population.

Sample	Plate count on		Direct count			Acidity	
	WAM	MRS pH5.4	Total	Viable	Non viable	Titratable	
	(cfu/ml) *SD*	(cfu/ml) *SD*	(cells/ml) *SD*	(cells/ml) *SD*	(cells/ml) *SD*	°SH *SD*	pH *SD*
BS[a] 1	9.50×10^8 *9.90×10^7*	2.60×10^8 *2.26×10^7*	1.58×10^9 *3.54×10^7*	1.45×10^9 *1.41×10^7*	1.25×10^8 *2.83×10^6*	28.0 *0.14*	3.31 *0.01*
BS 2	1.40×10^9 *1.06×10^8*	2.21×10^8 *8.49×10^6*	1.55×10^9 *3.89×10^7*	1.47×10^9 *2.83×10^7*	7.50×10^7 *1.06×10^6*	31.0 *0.35*	3.48 *0.02*
BS 3	7.80×10^8 *5.66×10^7*	1.18×10^8 *5.66×10^6*	1.59×10^9 *6.36×10^7*	1.37×10^9 *2.12×10^7*	2.20×10^8 *7.07×10^6*	31.5 *0.35*	3.35 *0.02*
BS 4	9.40×10^8 *3.54×10^7*	2.14×10^8 *1.20×10^7*	1.35×10^9 *3.54×10^7*	1.25×10^9 *4.24×10^7*	1.00×10^8 *1.41×10^6*	33.0 *0.14*	3.51 *0.02*
MN[b] 1	6.40×10^8 *6.36×10^7*	7.20×10^7 *5.66×10^6*	1.50×10^9 *1.41×10^7*	1.35×10^9 *4.24×10^7*	1.50×10^8 *7.07×10^5*	30.0 *0.14*	3.42 *0.02*
MN 2	9.80×10^8 *4.95×10^7*	8.30×10^7 *5.66×10^6*	1.90×10^9 *4.24×10^7*	1.70×10^9 *2.83×10^7*	2.00×10^8 *5.66×10^6*	31.5 *0.07*	3.38 *0.01*
MN 3	4.82×10^8 *2.90×10^7*	1.60×10^8 *1.27×10^7*	1.40×10^9 *2.12×10^7*	1.35×10^9 *3.54×10^7*	5.00×10^7 *1.34×10^6*	32.0 *0.35*	3.48 *0.01*
MN 4	1.32×10^9 *8.49×10^7*	6.04×10^8 *4.67×10^7*	1.37×10^9 *1.77×10^7*	1.27×10^9 *2.12×10^7*	1.00×10^8 *1.41 x 1 0^6*	27.0 *0.28*	3.49 *0.01*
MN 5	4.05×10^8 *1.77×10^7*	9.86×10^7 *7.35×10^6*	1.00×10^9 *3.54×10^7*	8.75×10^8 *1.27×10^7*	1.25×10^8 *3.54×10^6*	33.0 *0.35*	3.38 *0.01*
MN 6	1.61×10^9 *1.84×10^8*	2.11×10^8 *1.56×10^7*	1.61×10^9 *3.18×10^7*	1.53×10^9 *4.24×10^7*	7.50×10^7 *1.06×10^6*	33.0 *0.35*	3.39 *0.01*
PC[c] 1	9.28×10^8 *4.45×10^7*	1.27×10^8 *5.66×10^6*	1.00×10^9 *7.07×10^6*	8.25×10^8 *6.36×10^7*	1.75×10^8 *1.70×10^7*	29.0 *0.28*	3.49 *0.02*
PC 2	2.45×10^8 *2.40×10^7*	1.00×10^7 *7.78×10^5*	1.16×10^9 *3.18×10^7*	1.13×10^9 *3.54×10^7*	2.50×10^7 *8.49×10^5*	30.0 *0.35*	3.53 *0.01*
PC 3	1.64×10^9 *9.19×10^7*	4.32×10^8 *2.33×10^7*	1.65×10^9 *4.60×10^7*	1.52×10^9 *2.83×10^7*	1.25×10^8 *5.66×10^6*	30.0 *0.14*	3.47 *0.01*
PC 4	9.19×10^8 *6.43×10^7*	1.69×10^8 *1.34×10^7*	1.16×10^9 *2.62×10^7*	1.08×10^9 *3.25×10^7*	7.50×10^7 *7.78×10^5*	32.0 *0.07*	3.39 *0.02*
PC 5	9.55×10^8 *6.72×10^7*	2.65×10^8 *2.05×10^7*	1.18×10^9 *2.47×10^7*	9.50×10^8 *2.12×10^7*	2.25×10^8 *4.24×10^6*	30.5 *0.07*	3.50 *0.04*
PD[d] 1	9.46×10^8 *6.65×10^7*	2.16×10^8 *1.27×10^7*	1.40×10^9 *1.20×10^8*	1.20×10^9 *1.41×10^7*	2.00×10^8 *2.26×10^7*	29.0 *0.28*	3.45 *0.03*
PD 2	1.30×10^9 *1.27×10^8*	1.59×10^8 *5.66×10^6*	1.48×10^9 *4.24×10^7*	1.08×10^9 *2.12×10^7*	4.00×10^8 *1.13×10^7*	30.0 *0.35*	3.57 *0.02*
VI[e] 1	1.93×10^9 *1.20×10^8*	9.80×10^7 *3.89×10^6*	1.93×10^9 *3.54×10^7*	1.68×10^9 *1.06×10^8*	2.50×10^8 *7.78×10^6*	31.0 *0.14*	3.49 *0.03*
VI 2	1.11×10^9 *8.49×10^7*	1.20×10^8 *9.90×10^6*	1.73×10^9 *4.95×10^7*	1.48×10^9 *3.54×10^7*	2.50×10^8 *8.49×10^6*	29.0 *0.11*	3.58 *0.01*
VR[f] 1	1.62×10^9 *1.27×10^8*	1.89×10^8 *1.91×10^7*	1.62×10^9 *1.13×10^8*	1.47×10^9 *3.54×10^7*	1.50×10^8 *1.41×10^6*	30.0 *0.18*	3.50 *0.01*
VR 2	1.43×10^9 *1.20×10^8*	1.74×10^8 *9.90×10^6*	1.45×10^9 *1.70×10^8*	1.30×10^9 *1.27×10^8*	1.50×10^8 *2.83×10^6*	30.0 *0.11*	3.36 *0.02*
Sample G	1.45×10^9 *7.78×10^7*	9.80×10^8 *8.77×10^7*	1.90×10^9 *5.66×10^7*	1.87×10^9 *2.83×10^7*	5.00×10^7 *1.70×10^6*	31.5 *0.04*	3.30 *0.01*
Sample B1	6.20×10^8 *3.54×10^7*	3.78×10^8 *2.97×10^7*	8.00×10^8 *1.56×10^7*	7.95×10^8 *5.66×10^6*	5.00×10^6 *9.19×10^4*	26.0 *0.14*	3.82 *0.01*
Sample B2	5.14×10^8 *1.72×10^7*	3.13×10^8 *1.92×10^7*	9.12×10^8 *2.05×10^7*	5.24×10^8 *9.90×10^6*	3.88×10^8 *5.66×10^6*	34.0 *0.35*	3.15 *0.01*

Table 1. Microbiological and chemical determinations of 21 natural whey starters for Grana Padano cheese collected in six different provinces and three control samples (positive control G, negative controls B1 and B2). SD standard deviation.

[a]BS. Brescia province, [b]MN. Mantova province, [c]PC. Piacenza province, [d]PD. Padova province, [e]VI. Vicenza province, [f]VR. Verona province

Sunray plots

Each sample plots was constructed by averaging the sample replicates first and normalizing the data to scale the plots correctly later. In this way it is possible to ensure that no distortion in the plots results due to relative magnitude. The sunray plots demonstrated the differences between samples fairly well.

To evaluate the quality of a natural whey starter, it is necessary to consider that its role during cheese manufacture is to produce the lactic acid that influences important quality characteristics such as texture, moisture content, absence of pathogenic microorganisms, and taste (Fox *et al*., 1997).

The rate of acid production, generally measured as °SH and/or pH, is critical for the production of cheese. Starters may also be required to produce acid at a consistently fast rate every day through the manufacturing period (Hugenholtz, 2008). To reach this goal the cells should be numerous, viable, and able to replicate in the milk in the vat and curd. These are the reasons why high values of T and V are considered positive traits. Correspondingly, a high value of NV is a negative trait. High °SH and low pH values can be considered as positive features only if they are related with a high percentage of V. In a sunray plot, this positive aspect is easily visualized in the two triangles: T-centre-V and V-centre-SH: the bigger they are, the better is the starter quality, as exemplified by sample G (Figure1). At the same time, the triangle pH-centre-VN must be smaller. According to the present approach, MN2, MN6, PC3 and VI1 can be considered good-quality samples, MN5, PC1 and PC2 can be considered low-quality samples, with the latter one appearing to be particularly poor.

The capacity of the cells to grow in the synthetic commercial medium MRS, even when it is acidified, is generally lower than their capacity to grow in WAM. As previously discussed (Lazzi *et al*., 2004), the reason for this difference is related to the cells' nutritional requirement and to the greater complexity of the WAM which attempts to reproduce the natural system of the whey. The capability of the cells to grow in MRS and WAM could be interpreted as a different possibility of adaptation to the curd and cheese environment. Even high count values in WAM and in MRS at pH 5.4 can be considered as positive traits, while low levels of these parameters may be considered as biodiversity, rather than as a negative factor.

PCA analysis

To confirm the efficiency of this unusual approach, the same pool of data was analyzed by means of a more common statistical method such as principal components analysis (Figure 2). It was possible to evaluate the difference of the sample G from samples B1 and B2 along

factor 1, mainly composed by T, V and WAM variables. On the other hand, samples B1 and B2 laid differently on the biblot along Factor 2 composed by variable °SH, NV (towards sample B2) and pH (towards sample B1). Sample G and samples B1 and B2 were at the extremities of the biblot and, as expected, variables T, V and WAM discriminated between the good and the bad samples. Bad samples differed from each other for their different acidity. According to the sunray plot, samples VI1, MN2, MN6 and PC3 were closest to G whereas samples MN5, PC1 and PC2 laid in the bad sample zone. Samples PD2, MN1 and BS4 overlapped in the middle of the biblot but their sunray plots were different. Similarly, in the "bad zone", PC1 and PC2 were very close and, in the "good zone", MN2 was very close to MN6, but their sunray plots were different. The not very high value of variance of the PCA analysis (62.56%) could be the reason of this discrepancy (Massart *et al.,* 1988). During PCA

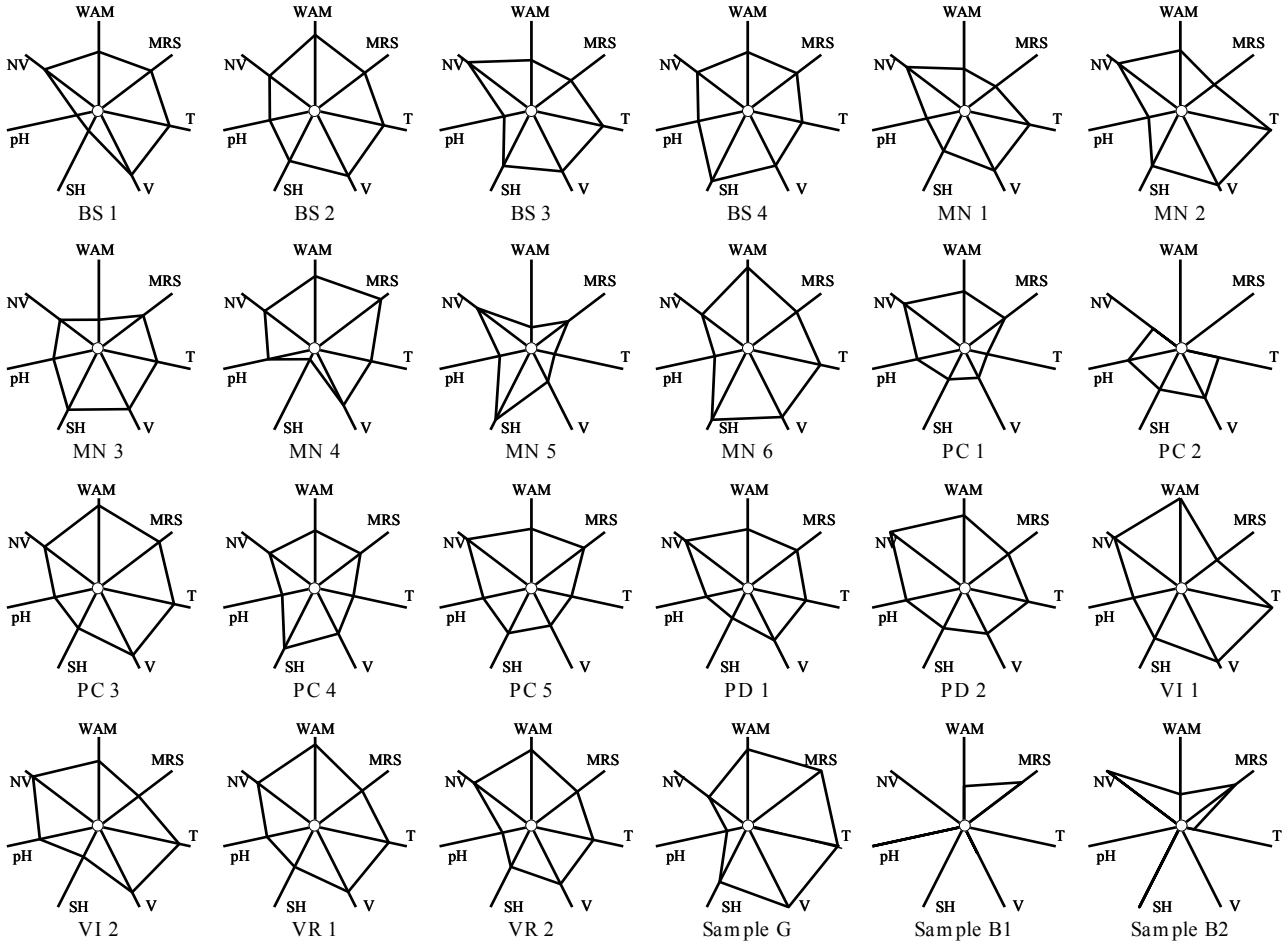
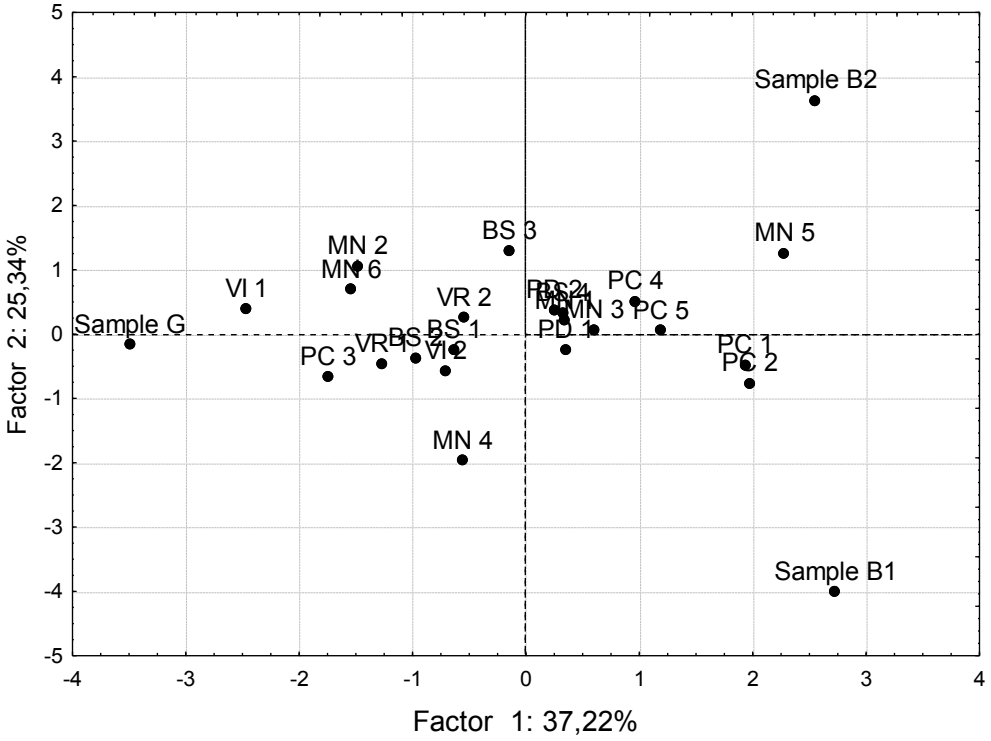


Figure 1. Sunray plots of the standardized chemical and microbial parameters for 21 natural whey starters for Grana Padano cheese and three control samples (G, B1 and B2). Each axis represent one parameter: cfu/ml in WAM (WAM), cfu/ml in MRS at pH 5.4 (MRS), total cells/ml (T), viable cells/ml (V), titrable acidity expressed as °SH (SH), pH and non-viable cells/ml (NV).

a)



b)

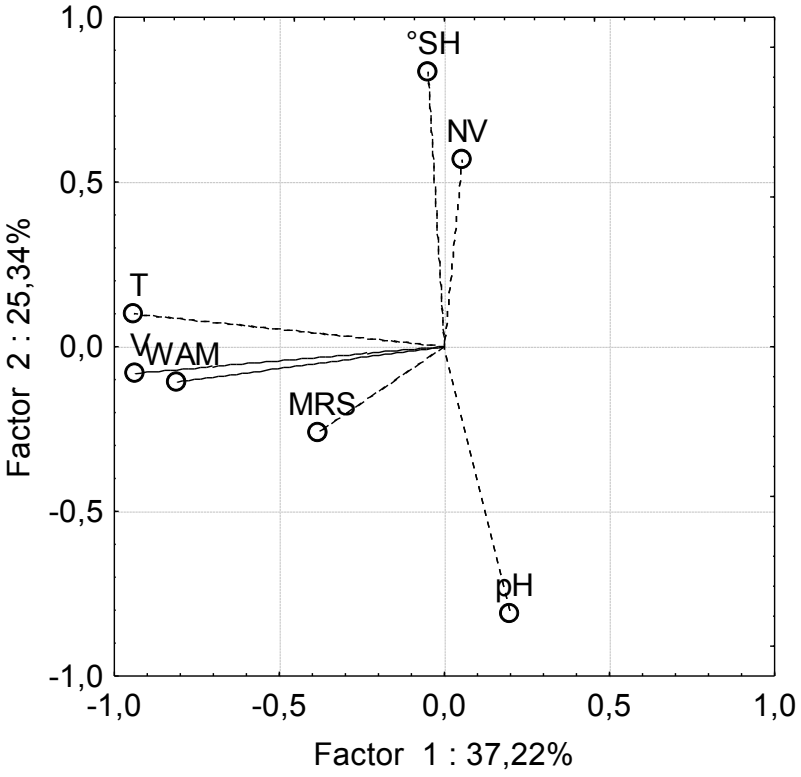


Figure 2. Scores (a) and loadings (b) plots for the first and second factors of principal component analysis carried out on 21 natural whey starters for Grana Padano cheese and three control samples (G, B1 and B2) and 7 variables: cfu/ml in WAM (WAM), cfu/ml in MRS at pH 5.4 (MRS), total cells/ ml (T), viable cells/ml (V), titrable acidity expressed as °SH (SH), pH and non-viable cells/ml (NV)

transformation from 7 original variables to 2 new ones, some information can be lost, information fully considered by sunray plots. Comparing the two explorative techniques, it could be concluded that, for this particular data set composed by 24 objects and seven variables, PCA allowed to highlight the good and the bad samples, while sunrays plots, even if remaining only a descriptive and explorative analysis, allowed to better visualize the differences among all the samples.

3.2.5 Conclusions

The interpretation of the chemical and microbiological data in natural whey starter for Grana Padano cheese, has been simplified. Considering their significance for natural whey starter quality, a new way for evaluating these parameters was proposed. This approach could be useful to summarize and outline the microbiological and chemical data and to readily compare different samples, as well as compiling and monitoring a data archive, for example to control their time stability.

3.2.6 Acknowledgements

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3.3 Variability of lactic acid production, chemical and microbiological characteristics in 24-hour Parmigiano Reggiano cheese

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3.3.1 Abstract

A proper level of acidification at early stages of manufacture is essential for a good quality hard cheese. In this work, chemical parameters and microbial characteristics of 18 Parmigiano Reggiano cheese during molding were analyzed in order to comprehend how the microbial growth takes place conditioning acidification performances. Based on lactic acid content, two groups of cheeses were observed. High numbers of *Lactobacillus helveticus* able to metabolize galactose but also growing faster in the cheese, appeared to be essential conditions to get adecuate lactic acid content. Low acidification and residual galactose were associated to higher levels of *Streptococcus thermophilus* in the cheese. While culturable thermophilic bacteria were fundamental for higher lactic acid production, non-culturable cells did not seem to contribute to acidification. This new findings could be extended to all hard-cooked cheeses produced with thermophilic natural cultures.

3.3.2 Introduction

Parmigiano Reggiano (PR), that was granted the protected designation of origin (PDO) by European laws, is an Italian hard-cooked cheese, ripened for a period of at least 1 year, during which major microbiological and biochemical changes occur. Raw and partially skimmed cow's milk supplemented with natural thermophilic starter culture are the main sources of lactic acid bacteria (LAB) in this cheese. The latter, consisting of a naturally acidified cheese

whey, is composed mainly by different strains of *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis* (Neviani and Carini, 1994; Cocconcelli *et al*., 1997; Mucchetti and Neviani, 2006) and by a minority presence of *Streptococcus thermophilus* and *Lactobacillus fermentum* (Bottari *et al*., 2010). During the traditional cheese manufacture, the high cooking temperature selects a thermophilic flora that is crucial for the cheese ripening, directly through their enzymes and indirectly through acidification (Bottazzi *et al.,* 1993a; Weimer, 2007; De Dea Lindner *et al*., 2008; Gatti *et al*., 2008). This thermophilic microflora that also remains in the whey, will originate the natural whey starter culture that will be used in the next day's cheesemaking, maintaining a microbiological linkage among productions. After vat extraction of the curd its transformation into cheese proceeds during a period in molds that can last 2 days (http://www.parmigiano-reggiano.it). It is during this phase, and particularly, during the first 24 hours that the acidification occurs and the main biochemical processes take place in the cheese together with the highest starter thermophilic bacterial growth (Fox *et al*., 1993; Pecorari *et al*., 2003). Highly acidifying thermophilic LAB added within the natural whey starter are the main responsible for this process, in which suitable fermentation of lactose leads to lactic acid production. In PR, such as in other Grana types, LAB from natural whey starters need from about 5-20 hours, depending on the zones of the cheese, in order to acidify the curd (Mucchetti and Neviani, 2006). Thus, lactic acid production, and correspondingly drop in pH, at the appropriate rate and time are key steps in the manufacture of a good quality cheese. Acidification inhibits the growth of pathogens and food-spoilage bacteria and enhances also the expulsion of whey from the curd during the cheesemaking process so producing curd with lower moisture levels (Mucchetti and Neviani, 2006; Powell *et al*., 2011).

In the first hours of PR cheese manufacture, slow acidification and the presence of residual fermentable sugar, as galactose, may occur. Moreover, large cheese wheel size (i.e. 50 kg) leads to a slow cooling, creating a gradient between internal and external zones during overall molding phase (Zapparoli and Neviani, 2005). In particular, at 24 hours after vat extraction, temperatures can reach 35°C and 27°C at internal and external zones, respectively (Giraffa *et al*., 1998; Pecorari *et al.,* 2003). In the absence of antagonist microflora, those are favorable conditions to the growth of undesirable bacteria, if present in the raw milk, that can lead to fermentations with gas formation with structural defects consequences on the cheese (Pellegrino *et al*., 1996; Pecorari *et al*., 2003). Spoilage bacteria such as *Clostridium butyricum, Clostridium sporogenes,* Coliforms and yeasts can find the optimal condition to grow causing early and late blowing in PR cheese (Tosi *et al*., 2006). Bottazzi *et al*. (1993b) stated that germination of *Cl. tyrobutyricum* spores takes place in the first 20-30 hours after

renneting in Grana cheese, a similar hard cheese. Moreover, early gas, mainly as CO_2, can be also due to the growth of heterofermentative non-starter LAB (NSLAB) able to metabolize residual galactose (Mucchetti and Neviani, 2006).

As stated above, acidification rate by starters in a proper level and time, is essential for a good quality PR cheese. Thus, the aim of this work was to evaluate the rate of acidification of PR cheese at 24 hours after vat extraction, during molding phase in order to investigate acidification performances together with microbial development. Thus, PR cheese were subjected to chemical and microbiological analyses. For microbial growth evaluation, different methodological approaches were used.

3.3.3 Materials and Methods

Cheese manufacture

Eighteen PR cheeses were sampled from 7 different dairies belonging to the PDO cheese production area. Five dairies supplied two cheeses, and 2 dairies 4 cheeses. PR cheeses (33-35 kg) were made according to the method approved by the Consortium (Council Regulation, 1992; http://www.parmigiano-reggiano.it). The vats (copper tanks) of 1,200 L of capacity were filled with a mixture of raw cow's milk from two consecutive milking. In which the milk from the evening milking was partially skimmed after overnight creaming (8-10 h at 12-18°C). The natural whey cultures were used as starters (2.5 - 3.2% v/v), in which were obtained by incubating under a gradient of temperature from about 50°C to 35–20°C for 18-24 h, the whey of the previous day's cheesemaking. Calf rennet powder were added and coagulation were obtained at 32-35°C. After the coagulation, the curd was cut then stirred and cooked for 5-15 min at 54-56°C. After 40-80 min to let deposit the curd, it was extracted from the vat and cut in two portions and then the two curd were molded for 48 h before being salted in saturated brine for 20-23 days. Cheese is ripened for 18-24 months at 18-22°C and 80-85% relative humidity.

Samples of cheese were collected aseptically at 24 hours after vat extraction during the molding phase and were kept at 4°C until arrival at the laboratory. As soon as they arrived they were subjected to microbiological analysis, moisture and pH determinations and stored at -20°C for further analysis within 2 days.

Microbial counts

Ten grams of grated cheese samples were suspended in 90 ml of 20 g/L of trisodium citrate solution (pH 7.5) (Sigma-Aldrich, St. Louis, USA) and homogenized for 2 min in a Blender (Seward, London, United Kingdom) at 230 rpm. Serial decimal dilutions in sterile quarter-strength Ringer solution (Oxoid, Basingstoke, United Kingdom) were made and spread plated on the following media and incubation conditions: MRS agar pH 5.4 (Oxoid, Basingstoke, UK) at 42°C and 25°C for 72 h (for the recovery of thermophilic and mesophilic lactobacilli, respectively), under anaerobic conditions (AnaerogenTM, Oxoid, Basingstoke, UK); M17 agar (for the recovery of streptococci) (Oxoid, Basingstoke, UK) at 42°C for 72 h under aerobic conditions and whey agar medium (WAM) (Gatti *et al*., 2003) at 42°C for 72 h, under anaerobic conditions (AnaerogenTM, Oxoid, Basingstoke, UK), (for enumeration of lactic acid bacteria arising from natural whey starter). Plates were made in triplicate and results were expressed as cfu/g of cheese.

Additionally, an aliquot (15 ml) of the cheese homogenates in trisodium citrate solution was used for viability counts. LIVE/DEAD *Bac*light Bacterial Viability kit (Molecular Probes, Oregon, USA) was used according to manufacturer's instructions. Cheese homogenates (15 ml) were centrifuged (10,000 rpm, 10 min, 4°C). The resulting pellet was washed twice in 15 ml of 20 g/L sodium citrate solution (pH 7.5) (Sigma-Aldrich, St. Louis, USA), then resuspended in 15 ml of sterile water and ten-fold diluted. Hence, 1 ml was used for viability counts as directed by the supplier. Samples stained with LIVE/DEAD were then filtered on black polycarbonate filters (0.2 μm pore size) (Millipore Corp., Billerica, MA, USA), visualized by an epifluorescence microscope (Nikon 80i, Tokyo, Japan) and counted as described by Bottari *et al.* (2010). Three separate experiments were made for each sample.

Fluorescence *in situ* hybridization (FISH)

FISH was performed on the ten-fold dilution of cheese samples as previously described by Bottari *et al.* (2010). Lbh1-FITC labelled probe (Bidnenko *et al*., 1998), specific for *L. helveticus* and St4-Cy3 labelled probe specific for *S. thermophilus* (Mercier *et al*., 2000), were used. Both probes were synthesized and labelled by eurofins MWG (Ebersberg, Germany). After addition of 10 μl of the hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl pH 7.2, 45% formamide) containing 10 ng of each probe, slides were incubated in a dark humid chamber at 45°C for 4 h. Slides embedded in mounting oil were evaluated with a Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a C-SHG1 100 W mercury lamp.

DNA extraction and LH-PCR

In order to obtain information about the community members in the cheese samples, genomic DNA was extracted directly from cheese samples and was subjected to LH-PCR fingerprinting analysis. Five grams of grated cheese samples were mortared and 450 mg were used for DNA extraction by using the General Rapid Easy Extraction System (GREES) DNA kit (InCura S.r.l., Cremona, Italy) according to the manufacturer's instructions.

V1 and V2 16S rDNA gene regions were amplified with primers 63F and 355R (Lazzi *et al*., 2004). The forward primer was 5′-end labelled with a 6-carboxyfluorescein (6-FAM) dye. Amplicons were then separated by capillary electrophoresis in an automated sequencer (Applied Biosystems, Foster City, USA). PCR and capillary electrophoresis conditions were performed as described by Bottari *et al.* (2010). The fragment sizes (base pairs) were determined with GeneMapper software version 4.0 (Applied Biosystems, Foster City, USA), local Southern method to generate a sizing curve from the fragment migration of the internal size standard (GS500 LIZ®; Applied Biosystems Foster City, USA) and a threshold of 150 fluorescence units. Amplicon lengths were attributed to bacterial species according to LH-PCR published databases (Lazzi *et al.,* 2004; Gatti *et al.,* 2008).

Compositional analyses

pH measurements were performed by using a potentiometer (Crison Instruments, Barcelona, Spain) and measuring directly in the cheese paste. The moisture content was determined by drying the sample at 102°C (IDF, 1982) and fat was measured by the Gerber's method as modified by Siegfeld (Savini, 1946). Ash content was determined on cheese and on water soluble extract of cheese by the gravimetric method according to Savini (1946) after calcination of the cheese samples in a muffle at 530°C. The water soluble extract of cheese was obtained as reported by Metzger *et al*. (2001). Lactose, lactic acid and galactose contents were determined using an enzymatic test kit according to Giudici *et al*. (1996).

Total nitrogen, pH 4.6-soluble nitrogen (SN), 12% trichloroacetic acid-soluble nitrogen (TCASN) and 5 % phosphotungstic acid-soluble nitrogen (PTASN) were separated using the procedure proposed by Gripon *et al*. (1975) and their values were assessed by the Kjeldahl method. Ammonia nitrogen (N-NH3) was determined according to Savini (1946). From nitrogen fractions, the values relative to the components of high molecular weight (Peptones N = NS - TCASN), peptides of low molecular weight (Peptides N = TCASN - PTASN - NNH3) and free amino acids (amino acids N = PTASN) were obtained.

Amino acids determination

A sample of grated cheese (10 g) was suspended in 45 ml of 0.1N HCl. D,L-Nor-Leu (0.5 ml of a 5 mM solution) was added as internal standard. The mixture was homogenized for 2 min with an Ultraturrax T 50 basic (IKA, Staufen, Germany) and centrifuged for 60 min at 4°C and 3,220 xg. The solution was filtered on a 45 µm filter and 3 ml of the filtrate were dried. The residue was dissolved in 2 ml of a 0.1% solution of formic acid and ultrafiltered for 30 min a 7,370 xg (molecular cut off of 10 kDa). The filtrate was dried and suspended in 300 µl of bidistilled water. The derivatization reaction was carried out on 10 µl of the solution using the AccQ*FluorTM reagent kit (Waters, Milford, MA, USA) following the manufacturer's instructions. The mixture was analyzed by HPLC on an Alliance 2695 separation module, at flow of 1 ml/min, by using an AccQ-Tag C18 column (3.9 x 150 mm) thermostated at 37°C. Eluent A: phosphate buffer AccQ*TagTM added of 1 L of bidistilled water; eluent B: CH3CN:H2O 60:40; gradient: 0-1 min linear gradient from 100% to 97% A, 1-13 min linear gradient from 97% to 93% A, 13-18 min linear gradient from 93% to 90% A, 18-38 min linear gradient from 90% to 67% A, 38-51 min isocratic 67% A, 51-52 min linear gradient from 67% to 0% A, 52-61 min isocratic 0% A, plus reconditioning (25 min). Detection was performed by a Waters 2475 fluorimetric detector, λ_{abs} = 250 nm, λ_{em} = 395 nm. Quantification was performed against a calibration curve obtained by the internal standard method

Statistical analysis

Data were analyzed by ANOVA multivariate (PASW statistics 18.0.0, Armonk, New York, USA) according to the following general linear model: $y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$. Where: y_{ij} = dependent variable; μ = common mean; α_i = effect of lactic acid content i = 1, 2; ε_{ij} = residual error. According to lactic acid content, cheese samples were divided in two groups: ≤ 1.8 and >1.8 g lactic acid/100 g cheese dry matter (DM). Significance of differences were tested by the Fisher's LSD (least significant difference) method.

3.3.4 Results

Among the 18 cheese samples analyzed 24 h after vat extraction, two distinctive groups based on lactic acid content were observed, named as "low lactic acid" (LLA) (< 1.8) , and ii) "high lactic acid" (HLA) (> 1.8), expressed as g on 100 g of cheese dry matter (DM). Among the cheese samples examined, seven (39%) belonged to the LLA group and eleven (61%) to

the HLA group. In particular, two dairies made cheeses that were included in LLA group, three dairies in HLA group and two dairies produced cheeses that belonged to both cheese groups.

Cheese composition

The basic composition of cheese samples is reported in Table 1. No differences between LLA and HLA cheese were observed for moisture, protein and fat content (g/100g cheese DM). Differently, LLA group was characterized by contents of ash lower ($P < 0.05$) than those of HLA group: 4.77 and 4.94 (g/100g cheese DM), respectively. Even the quota of water soluble ash of cheese (expressed as % of cheese ash) was lower ($P < 0.05$) in LLA group than in HLA group, being 30.79 % and 44.79 %, respectively. Lactose, galactose, and lactic acid

Composition	LLA		HLA		
	Mean	SD[a]	Mean	SD[a]	*P*[b]
Moisture (g/100g)	38.70	0.98	38.55	0.83	NS[c]
Protein (g/100g DM*)	48.75	1.06	48.18	2.79	NS
Fat (g/100g DM)	44.73	1.12	44.61	5.07	NS
Ash (g/100g DM)	4.77	0.15	4.94	0.16	≤0.05
Lactose (g/100g DM)	0.030	0.050	0.014	0.021	NS
Galactose (g/100g DM)	0.854	0.190	0.312	0.201	≤0.05
Lactic acid (g/100g DM)	1.400	0.478	2.154	0.162	≤0.05
Ripening index [d]	3.90	1.09	4.88	0.89	≤0.05
Water soluble Ash/Ash (%)	30.79	2.22	44.79	1.77	≤0.05
pH	5.401	0.154	5.220	0.196	≤0.05

Table 1. Composition and pH values of Parmigiano Reggiano cheeses 24 h after vat extraction, according to lactic acid content (LLA: ≤1.8; HLA: >1.8 g/100g cheese DM)

*DM: cheese dry matter
[a]Standard deviation
[b]p value
[c]not significant, p>0.05
[d]soluble nitrogen at pH 4.6/Total nitrogen (%)

contents (g/100 g cheese DM) and pH values of 24-h cheeses are shown in Table 1. Comparing to HLA cheeses, LLA cheeses were characterized by higher ($P < 0.05$) values of galactose (0.85 vs 0.31 g/100g cheese DM for HLA) and, as expected, lower ($P < 0.05$) values of lactic acid (1.40 vs 2.15 for HLA). Accordingly, pH values resulted lower ($P < 0.05$) in HLA than LLA in which the difference was on average 0.18 units. Nitrogen fractions of 24-h

cheeses reported no differences between LLA and HLA cheeses (data not shown), except for the ripening index (soluble nitrogen at pH 4.6, expressed as a percentage of total nitrogen) which its value resulted to be higher in HLA than in LLA cheese (4.88 vs 3.90 %, $P < 0.05$).

Amino acid composition

The contents of individual amino acids and their sum (expressed as mg/100 g of cheese protein) are reported in Table 2. Total free amino acids concentrations were significantly higher in 24-h cheese samples of HLA group than those in LLA. Moreover, significant differences could be detected in some amino acid concentrations such as Gly, His, Arg, Ala, Pro and Lys, that were higher in HLA cheese samples.

Free amino acids	LLA		HLA		p[b]
	Mean	SD[a]	Mean	SD[a]	
Asparagine	15.88	7.98	21.37	7.04	NS[c]
Serine	32.04	8.02	33.57	8.60	NS
Glutamine	96.43	20.19	102.25	19.04	NS
Glycine	13.32	1.80	18.37	2.98	≤0.05
Histidine	36.65	12.08	54.69	10.08	≤0.05
Arginine	49.83	9.43	64.43	11.09	≤0.05
Threonine	24.49	8.40	24.82	7.70	NS
Alanine	45.47	14.29	63.78	15.20	≤0.05
Proline	60.75	14.48	81.17	16.09	≤0.05
Tyrosine	34.09	5.68	35.49	10.70	NS
Valine	40.17	6.87	46.95	7.62	NS
Metionine	13.09	4.13	15.63	4.35	NS
Lysine	103.66	20.25	131.21	12.65	≤0.05
Isoleucine	25.12	5.56	26.56	5.43	NS
Leucine	56.99	9.65	59.58	16.43	NS
Phenylalanine	26.97	4.32	25.35	8.97	NS

Table 2. Free amino acids (mg/100g of cheese protein) of Parmigiano Reggiano cheeses after 24 h of vat extraction according to lactic acid content (LLA: ≤1.8. HLA: >1.8 g/100g cheese DM)

[a]Standard Deviation
[b]*p* value
[c]not significant, $p>0.05$

Microbial counts

Direct microscopic counts showed high total cell numbers (more than 8 log cells/g) and no significant differences between the two groups were observed (Table 3). Cultivable mesophilic lactobacilli counts (evaluated in MRS agar pH 5.4 at 25°C) were similar for both LLA and HLA samples, however thermophilic lactobacilli counts (MRS at 42°C) showed significant differences between LLA and HLA groups, being 6.95 cfu/g and 8.21 cfu/g, respectively (Table 3). Moreover, streptococci population also showed differences ($P < 0.05$) among the two groups, (4.46 and 5.47 cfu/g, respectively).

Composition	Units of measurement	LLA		HLA		p[b]
		Mean	SD[a]	Mean	SD[a]	
Total	Log cells/g	8.48	0.40	8.66	0.34	NS[c]
Mesophilic lactobacilli	Log cfu/g	1.44	1.02	1.52	0.77	NS
Thermophilic lactobacilli	Log cfu/g	6.95	0.50	8.21	0.38	<0.05
Streptococci	Log cfu/g	4.46	0.71	5.47	1.06	<0.05
Cultivable in WAM	Log cells/g	6.63	0.80	7.89	0.28	<0.05
Viable	Log cells/g	8.14	0.41	8.44	0.30	NS
Non-viable	Log cells/g	8.18	0.42	8.19	0.45	NS

Table 3. Microbial counts of 24-hour-cheese samples according to lactic acid content (LLA: ≤1.8, HLA: >1.8 g/100 g cheese DM).

Mesophilic lactobacilli: MRS (Man Rogosa Sharpe) at 25°C, in anaerobiosis; Thermophilic lactobacilli: MRS at 42°C in anaerobiosis, Streptococci population: M17 at 25°C, aerobiosis. WAM (whey agar medium) at 42°C under anaerobic conditions for enumeration of lactic acid bacteria arising from natural whey starter. Total, viable and non-viable were performed by direct microscopic counts.

[a]Standard Deviation

[b]*p* value

[c]not significant, $p>0.05$

Cultivable bacterial population in WAM, for enumeration of LAB arising from natural whey starter, showed differences ($P < 0.05$) between the two groups of cheese. That is, LLA cheeses highlighted density mean values of 6.63 log cfu/g, and HLA cheeses showed higher values of 7.89 cfu/g. Only microbial thermophilic counts in the three growth media (MRS, M17, WAM) evidenced higher cell densities in HLA cheese group. Viability counts were performed by using fluorescence microscopy. Differently from cultivable counts, no significant differences were observed between LLA and HLA groups of samples.

Determination of cheese community structure

Bacterial community structure of cheeses at 24 h after vat extraction was determined by LH-PCR and FISH. 16S rRNA gene of bacterial DNA extracted directly from cheese samples were amplified by PCR, then amplicons were separated in an automated gene sequencer and fragment lengths were assigned to different species according to published databases (Lazzi *et al.,* 2004; Gatti *et a*l., 2008). Among the 18 cheese samples, three different profiles based on the presence of peaks were observed (Figure 1). Profile A was characterized by peaks at 330 ±1 bp (attributed to *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus*); 334 ± 1 bp (*L. helveticus*), and double peaks at 342 ± 1 bp and 345 ± 1 bp (attributed to *L. fermentum*). A peak at 305 ± 1 bp was also detected (non-attributed).

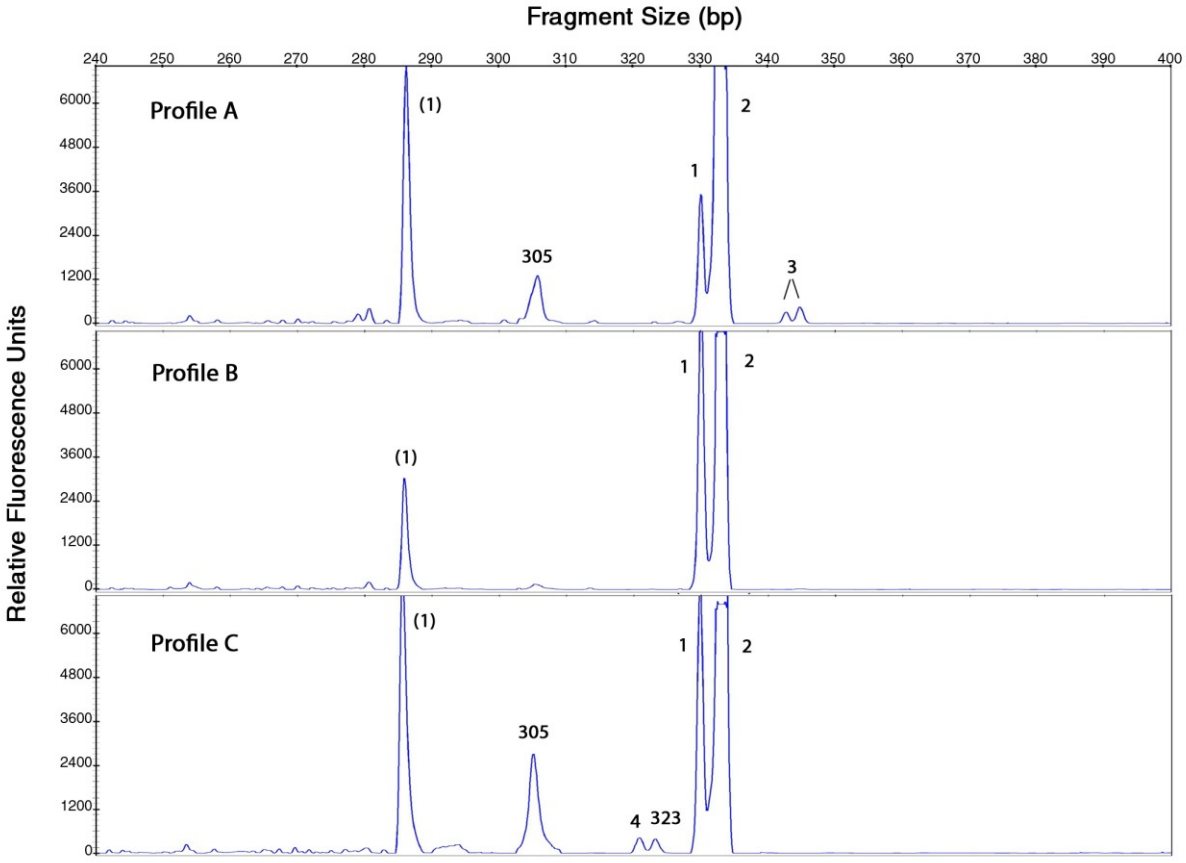

Figure 1. Three representative electropherogram profiles of DNA fragments amplified by PCR belonging to 24-h cheese samples. The *x* axis shows the peak size in base pairs, and the *y* axis shows the peak intensity in relative fluorescence units. Numbers represent the species, according to a published LH-PCR database (Lazzi *et al.,* 2004), as follows: **1**, *L. delbrueckii* subsp. *lactis*; **(1),** secondary peak of 1; **2**, *L. helveticus*; **3**, *L. fermentum*; **4**, *S. thermophilus*; 305 and 323 are non-attributed peaks.

Profile B was characterized by the presence of only two peaks at 330 ±1 bp and 334 ± 1 bp, according to the database belonged to *L. delbrueckii* subsp. *lactis* and *L. helveticus* species, respectively. No other peaks were detected. Finally, profile C presented peaks at 330 ±1 bp and 334 ± 1 bp, attributed to *L. delbrueckii* subsp. *lactis* and *L. helveticus*, respectively, and a peak at 320 ± 1 bp attributed to *S. thermophilus*. Other peaks at 305 bp and 323 bp were also detected and non-attributed to any species in the database.

Most cheese samples (45%) were characteristic of profile A, 33% displayed profile B, whereas profile C was revealed in 22% of cheese samples 24 h after vat extraction. However, no correlation between profile types and groups according to lactic acid content was observed.

Bacterial identification by FISH analysis was performed by specific probes for *L. helveticus* and *S. thermophilus* species. The signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content which is a useful indicator of viability (Bottari *et al*., 2006). An evaluation of relative abundance of the two targeted viable species was done, for each sample, according to Bottari *et al.,* (2010). Figure 2 shows two representatives results. It was observed that LLA cheese group was characterized by a majority of *S. thermophilus* hybridized cells respect to *L. helveticus* hybridized cells. On the contrary, HLA cheese samples showed a majority of *L. helveticus* hybridized cells.

(a) FISH LLA (b) FISH HLA

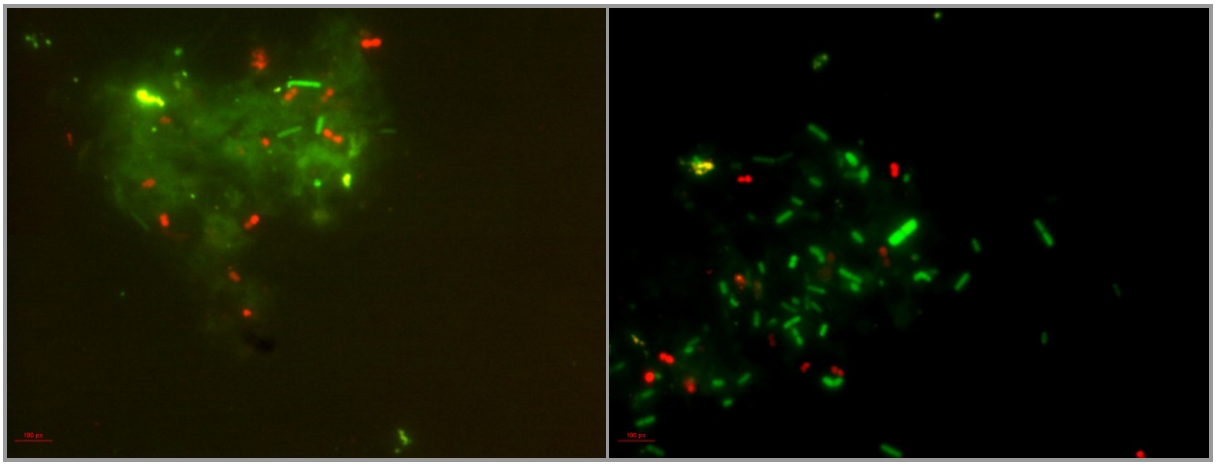

Figure 2. FISH of 24-h cheese samples classified as LLA (a) and HLA (b). The simultaneous use of probes St4 (red label) and Lbh1 (green label), revealed the presence of a majority of hybridized *S. thermophilus* cells (a). A lower amount of hybridized *L. helveticus* cells was also present. While majority of hybridized *L. helveticus* cells (b) was revealed in HLA. A lower amount of hybridized *S. thermophilus* cells was also present. Non-hybridized cells on the background could be either non-*L. helveticus Lactobacillaceae* or non-viable *L. helveticus.*

3.3.5 Discussion

During the first 24 hours of molding phase, acidification is the main biochemical process that takes place in the cheese. It is obtained by conversion of lactose into lactic acid by starter LAB. Acid production at the appropriate rate and time is a key step in the manufacture of a good quality cheese (Fox *et al.,* 1993; Powell *et al*., 2011). Moreover, during cheese manufacture, milk acidification cause a decrease in pH which in combination with cooking and stirring, promotes the syneresis of the curd and the expulsion of the whey (Walstra, 1993). To achieve this effect, fast acidification by starter LAB activity should occur. In Italian hard cheese (i.e. PR) acidification of the curds was reported during the first 48 h during molding phase (Pecorari *et al.,* 2003).

In this research, 18 different cheeses were sampled from 7 dairies at 24 hours after vat extraction during molding in order to gain knowledge and comprehend how the microbial growth takes place in different cheeses and to evaluate not only fermentative activity but also acidification rate performances by natural whey starter LAB. Chemical parameters and microbial characteristic of 18 cheeses were analyzed. It was found that cheeses could be divided into two groups according to the lactic acid content produced after 24 hours of vat extraction during molding, that is cheese with low and high lactic acid content, (referred as LLA and HLA, respectively).

Even though 5 of 7 dairies produced cheeses which belonged to one group, two dairies produced cheeses which belonged to either LLA and HLA groups. This result underline the variability of production among dairies but also within the same dairy.

Regarding basic cheese composition, all values were consistent with those reported by Panari *et al*. (2003) for Parmigiano Reggiano cheese at 24-48 hours after vat extraction. Besides higher lactic acid content, HLA cheese group was characterized by lower values of galactose, pH and higher contents of both ash and water soluble ash (as % of cheese ash) than LLA group. The lower pH values reported in HLA may be responsible for a greater solubilization of the calcium phosphate bound to the casein matrix (Lucey and Fox, 1993) leading to a higher proportion of water soluble ash in those cheeses.

The modification of calcium and phosphorus equilibrium that are encountered in cheese in the early stages of ripening are strongly correlated with changes in its rheological properties and influence the development of biochemical, chemical and physical processes during ripening (Lucey *et al*., 2005). As a consequence, the different extent of mineral solubilization observed in this work may lead to a different development of cheese ripening between LLA and HLA cheeses.

In a previous study, carried out on six PR cheeses at 23 hours after vat extraction, Pecorari *et al*. (2003) reported lactic acid content and pH values comparable with those observed for HLA cheeses but clearly different when compared to LLA cheese ones. Therefore, increasing the number of samples coming from different dairies, 24 h-cheeses with less acidification rates was observed. Furthermore, differently from what observed here, galactose was detectable in lower amounts (0.009 g/100 g 23-h cheese) compared to amounts in HLA group and, in particular, in LLA cheeses. The remaining of galactose and lactose in early cheese can be consequences of inefficient fermentative activities from the starter (Mucchetti and Neviani, 2006). The natural whey starters utilized for each cheese production were considered of good quality regarding its microbiological and chemical parameters (data not shown).

In this study, both cheese groups (LLA and HLA) showed similar lactose consumption, but differed in the galactose content, particularly higher in LLA group. This let hypothesize that starter bacteria, particularly in LLA group, were characterized by LAB species or strains that consume galactose with at slow rate or might not be able to consume it. The dominant species found in all 24 h cheeses by LH-PCR belonged to *L. helveticus* and *L. delbrueckii* subsp. *lactis* and *S. thermophilus*, these species can utilize galactose as a carbon source, however, it is known that *S. thermophilus* metabolize galactose strain dependent (Robinson, 2002). At this regard, although lactose is efficiently transported into the cell and subsequently hydrolyzed by an intracellular β-galactosidase, many strains of *S. thermophilus,* used in the dairy industry, ferment only the glucose moiety of lactose, while the galactose moiety is excreted into the medium in equimolar amounts with the lactose uptake (de Vin *et al*., 2005). De Vin *et al.* (2005) found that some *S. thermophilus* strains start to consume the galactose excreted into the medium only after lactose depletion whereas others were not able to consume it or they did it at different rates and to various extent, but never to completion. In this work, higher amounts of viable *S. thermophilus* hybridized cells revealed by FISH in LLA cheeses may confirm this hypothesis.

Similarly to our observations, Mucchetti *et al*. (2002) found that higher amounts of non-metabolized galactose remained after 24 h of vat extraction in experimental mini-cheese made with a defined *S. thermophilus* strain as starter than by using undefined natural cultures or *L. helveticus* or *L. delbrueckii* subsp. *lactis* strains alone. Furthermore, as expected, HLA cheeses characterized by higher proportions of starter bacteria able to consume galactose (i.e. *L. helveticus* revealed by FISH), showed higher lactic acid content than LLA. Thus, besides the evidenced differences in metabolizing galactose at species level, the accumulation of residual

galactose at 24 h could be also demonstrated by lower thermophilic cultivable bacterial densities (~1 log unit) found in LLA group.

In PR cheese, as other cheeses, special attention must be given to the availability of residual galactose that may lead to undesirable fermentation to CO_2 by increasing growth of *Clostridium* sp. and/or heterofermentative LAB, Coliforms and yeasts. This results in late and early blowing causing textural defects such as slits and fractures in cheese (Bottazzi *et al*., 1993b; Pellegrino *et al*., 1996).

By FISH it was found that HLA cheese group presented higher amounts of viable *L. helveticus* hybridized cells. As highly acidifying species (Tunuer and Martley, 1983; Kandler and Weiss, 1986), this result agrees with higher average of lactic acid content and lower pH values found in those cheeses. On the other hand, the same cheeses at vat extraction (0 h) were also analyzed in this work, and the lactic acid content was significantly higher in LLA cheeses than HLA cheese samples (data not shown). This reversal of lactic acid level was in good agreement with the presence of a majority of *S. thermophilus* cells in LLA cheese group. In fact, *S. thermophilus* is reported to acidify more rapidly than lactobacilli (Béal and Corrieu, 1994; Mucchetti and Neviani, 2006), but fast acidification does not mean high acidification, as demonstrated by Spinnler and Corrieu, (1989), that concluded that maximum acidification rate of *L. helveticus* was generally higher than that of *S. thermophilus*. Therefore, despite their presence is not necessarily related with their development in curd, the significance of *S. thermophilus* in the whey starter hast to be reconsidered. In particular, being whey starter a natural mix of LAB strains, the occurrence of S. *thermophilus* strains lacking in β-galactosidase is likely undesired.

Interestingly, differently from higher cultivable counts in HLA cheese group, no differences were found in the total, viable and non-viable cells between the two groups of samples. This could be ascribed to the presence of viable (detected microscopically) but not culturable, in agar media, bacteria. This fraction represents microorganisms that even viable on the cheese are not able to proliferate on agar media but could be capable to grow in cheese. However, the relevance of this population during ripening needs further studies, and being non-culturable, they still remain unknown.

Differently from FISH, thermophilic lactobacilli cultivable counts on MRS were higher than streptococci in M17 in both cheese groups. This could be explained by the presence of *L. delbrueckii* species, lactobacilli population cultivated on MRS could belong also to *L. delbrueckii* species that are not targeted by FISH.

Species composition determined by LH-PCR showed that profile B, characterized by dominant presence of *L. helveticus* and *L. delbrueckii*, was in agreement with those previously observed in Parmigiano Reggiano cheese at 48 h after vat extraction (Gatti *et al.,* 2008). Differently from these authors, that studied only one cheese throughout ripening, in this study 18 cheeses at 24 hours after vat extraction were analyzed. The variability in terms of species composition observed in the cheese samples could be explained by different operational conditions in each dairy, although the cheeses are produced under strict production rules.

In agreement with Beresford and Williams (2004), mesophilic lactobacilli cellular densities, (evaluated by cultivable method MRS at 25°C), were lower than 2 log unit, with no differences observed between the LLA and HLA cheese groups. This microbial population, mainly represented by non-starter lactic acid bacteria (NSLAB), do not contribute to acid production during the first hours of cheesemaking and proliferate after brining (2 months) reaching densities of 10^7 cfu/g cheese (De Dea Lindner *et al.,* 2008, Gatti *et al.,* 2008). NSLAB are considered to have potential impact on flavor formation during cheese ripening (Broadbent *et al.,* 2011).

The contents of nitrogen fractions and individual free amino acids allowed to highlight some issues on cheese proteolysis from a quantitative and qualitative point of view, respectively. The ripening index was the only parameter which resulted to be different between the two groups, being higher in HLA than in LLA cheese. However, even in correspondence of the extraction from the vat, the ripening index was higher in HLA cheese (data not shown). Hence, the different degree of casein solubilization reported here, should not be related to biochemical process that take place in cheese during the first 24 hours. From a microbiological point of view, it could only be speculated that a different microbial development occurred already in the vat milk. This could sustain the hypothesis of a better development of *L. helveticus* at the early phase being the most proteolytic species of the natural whey starters.

Independently of the classification of cheese, the most abundant amino acids were lysine and glutamic acid while the lowest values were observed for methionine and asparagine. Cavatorta *et al*. (2007) observed a similar pattern of free amino acids in PR cheese sampled 12 hour after vat extraction.

Regarding single amino acids concentrations, glycine, histidine, arginine, alanine, proline and lysine were significantly higher in HLA than in LLA cheese. Their values ranged from 27 % (Lys) to 49 % (His). The content of free amino acids was also assessed on the same cheese samples just after the vat extraction (0 h). At that point of the cheesemaking process no

differences in the contents of singles amino acids and their sum were observed between LLA and HLA cheeses (data not shown). According to these results, it seems that the acidification of the cheese by SLAB occurred in association with an increase of some specific pepdidase activities. This could be related to the metabolism of growing cells but also to an early SLAB autolysis which has already been observed in PR (De Dea Lindner *et al.,* 2008; Gatti *et al.,* 2008), Grana Padano (Zago *et al*., 2007) and Swiss cheese (Valence *et al*., 1998; Valence *et al*., 2000). Particularly, *L. helveticus* strains, characterized by rapid autolysis, are used as a flavor adjunct to accelerate ripening in other hard cheese varieties (Valence *et al*., 1998; Kiernan *et al*., 2000). However, the enzymes released after lysis were not determined in this study.

The higher peptidase activity observed in HLA cheese samples compared to LLA group were associated with majority of viable *L. helveticus* cells together with higher cell counts in this cheese group. In fact, *L. helveticus* has been reported to have a higher proteolytic activity compared to *S. thermophilus* and also to *L. delbrueckii* subsp. *lactis* (Sasaki *et al*., 1995; Deutsch *et al*., 2000; Gatti *et al.,* 2003). Since different *L. helveticus* strains manifest different proteolysis rates (Gatti *et al*., 1999), the great variability among *L. helveticus* strains found not only in natural whey starters but also in cheese during molding (Giraffa and Neviani, 1999; Gatti *et al.,* 2003) has to be also considered.

3.3.6 Conclusions

Lactic acid production, and correspondingly drop in pH, at the appropriate rate and time are key steps in the manufacture of a good quality cheese. In this research, chemical parameters and microbial characteristics of 18 different cheeses, sampled from 7 dairies producing Parmigiano Reggiano, at 24 hours after vat extraction during molding, were analyzed in order to comprehend how the microbial growth takes place conditioning acidification performances of natural whey starter.

While the most part of samples showed high lactic acid amount, about 40% of cheeses showed a low lactic acid content. Moreover, a variability among different dairies productions but also within the same dairy was observed. The different content of lactic acid was associated with a different degree of solubilization minerals with possible repercussion on cheese rheology and, consequently, on the development of chemical, biochemical and microbial processes that take place throughout ripening.

The presence of a high number of *L. helveticus* cells and in particular, of those strains that were able to metabolize galactose but also growing faster in the cheese, appear to be essential conditions to get adequate lactic acid content and thus the correct curd acidification. In the case of a scarce growth of these *L. helveticus*, galactose would remain non-metabolized. The presence of residual galactose was associated to higher contents of *S. thermophilus* species. In this sense, despite their presence is not necessarily related with their development in the curd, the significance of *S. thermophilus* in the whey starter has to be reconsidered. In particular, being whey starter a natural mix of LAB strains, the occurrence of *S. thermophilus* strains lacking in β-galactosidase is likely undesired

Total cell densities, which include the cultivable and non-culturable microbiota, did not appear to be fundamental for a correct curd acidification and, while cultivable thermophilic bacteria were essential for higher lactic acid production, non-culturable cells did not seem to contribute to acidification, or they are not metabolically active. Finally, the biotypes composition of whey starter LAB seemed to be far more important than the species composition in insuring their good performances.

The approach used in this study brought technologically very useful answers for the correct production of Parmigiano Reggiano cheese but could be extended to all hard-cooked cheeses produced with thermophilic natural cultures.

3.3.7 Acknowledgements

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3.4 Survey on community and dynamics of lactic acid bacteria in Grana Padano cheese

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3.4.1 Abstract

This study analyze diversity and dynamics of the microbial community in Grana Padano (GP) cheese during fermentation and ripening. Differently from other works, this research evaluated six GP cheesemaking processes of different dairies. Therefore, it was possible to evaluate not only the trend of microbial dynamics but also differences among productions. By means of length heterogeneity (LH)-PCR, profiles of the bacterial community were obtained and comparisons were carried out. Starter lactobacilli were the main species during the acidification steps of GP production and non-starter lactic acid bacteria (NSLAB) were able to grow after brining, becoming dominant during ripening. By pre-incubation of skimmed raw milk, it was possible to isolate lactobacilli strains: *Lactobacillus helveticus, Lactobacillus rhamnosus, Lactobacillus delbrueckii* subsp. *lactis/bulgaricus* and *Lactobacillus fermentum*, some of which of dairy interest in GP cheesemaking. It was demonstrated that the microorganisms able to grow under the specific cheese environmental conditions during ripening, could arise both from raw milk and natural whey starters. The presence of starter lactic acid bacteria (SLAB) such as *L. helveticus* and *L. delbrueckii* subsp. *lactis* in the first hours of production highlighted the well-known role of natural cultures in curd acidification while their presence, as a non-cultivable state, up to 13 months of ripening suggested a different unknown role in cheese ripening. Ecological indices throughout manufacturing and ripening were used. The vat skimmed raw milk ecosystem showed higher diversity, evenness and richness of bacterial community compared to the natural whey starter ecosystem. Among cheese ecosystems, diversity, evenness and richness showed changing trends. Differences in the qualitative and quantitative rate of cell lysis of SLAB and NSLAB population were found among 2-month cheeses with potential effects on ripening process and flavor development.

3.4.2 Introduction

Grana Padano (GP) is a Protected Designation of Origin (PDO) cheese produced from partially skimmed cow's raw milk and by the addition of natural cultures of lactic acid bacteria as starter (SLAB). Since spore-forming bacteria are usually found in silage and fermented feed, raw milk provide a ready medium for transmission of Clostridia spores. For this reason, and to inhibit late blowing caused by butyric fermentations in the cheese, the addition of lysozyme to the vat skimmed milk (20 ppm) is allowed (Carini *et al.,* 1985).

GP microbial ecosystem is due to a complex dynamic equilibrium between SLAB and secondary flora that develops during ripening, including non-starter lactic acid bacteria (NSLAB). The sources of NSLAB are likely milk and dairy environment (Cogan *et al.,* 2002). All these populations are responsible for the development of the characteristic Grana cheese flavor.

The natural whey starter is a complex microbial association of lactic acid bacteria (LAB), not only because of the presence of various species, but also because of the large number of biotypes (Giraffa *et al.,* 1998). This whey culture consists mainly of thermophilic undefined strains of *L. helveticus* and *L. delbrueckii* subsp. *lactis* and secondarily of *S. thermophilus* and *L. fermentum* (Fornasari *et al.,* 2006; Rossetti *et al.,* 2008; Santarelli *et al.,* 2008; Gatti *et al.,* 2011).

The skimmed raw milk used in GP cheesemaking, is obtained by a natural creaming process of whole raw milk for 8 to 12 hours at a temperature between 10°C and 20°C (http://www.granapadano.it). During this process two opposite phenomena occur: the removal of the bacteria ascending with the fat globules and their growth that depends on environmental conditions (Panari *et al.,* 2007).

Most raw milk microorganisms associated with hard cheese varieties are NSLAB mainly belonging to the *Lactobacillus casei-plantarum* group (Coppola *et al.,* 1997; Coppola *et al.,* 2000; Cogan *et al.,* 2002). By using culture-independent methods, different authors found that raw milk microbial composition was composed of lactobacilli and streptococci some of which of technological interest (Neviani *et al.,* 2009; Franciosi *et al.,* 2010, 2011).

The cheese ripening is a complex process involving a range of microbiological and biochemical reactions (Cogan *et al.,* 2002). In hard raw milk Italian cheeses such as Parmigiano Reggiano (PR) and GP cheeses, in which natural whey starter is used, high densities of viable microorganisms are present in cheese throughout ripening (Coppola *et al*., 1997; 2000; Zago *et al.,* 2007; De Dea Lindner *et al.,* 2008; Gala *et al.,* 2008). NSLAB are the dominant species at advanced stages, and are considered important microflora for the

development of cheese flavor because of their proteolytic and peptidolytic activities (Bottazzi, 1993; Gobbetti, 2004). Moreover, the rapid release of intracellular enzymes due to autolysis of LAB in the cheese matrix accelerates the ripening process, and in some cases improves the flavor (Kiernan *et al.,* 2000). In other cheese varieties, most studies were focused on autolysis of mesophilic starters (Crow *et al.,* 1995; Lortal *et al.,* 2005; Hannon *et al.,* 2007). Autolysis of thermophilic cultures also occurs, but it has not been studied to the same extent. Investigations reported autolysis of *L. helveticus* in Swiss-type cheeses (Valence *et al.,* 1998) and Cheddar (Kiernan *et al.,* 2000). Thermophilic SLAB autolysis has been observed in GP cheese (Zago *et al.,* 2007) and PR (De Dea Lindner *et al.,* 2008; Gatti *et al.,* 2008).

Microbial composition in PR cheese has been studied at a late stage of ripening (Gala *et al.,* 2008), and throughout manufacturing and ripening (De Dea Lindner *et al.,* 2008; Gatti *et al*., 2008; Neviani *et al*., 2009), whereas, to our knowledge, only one study was focused on population dynamics in GP cheese (Zago *et al*., 2007). These authors studied cheese microbiota of an experimental GP by culture-based techniques until 8 months of ripening. Authors reported that during ripening a decrease of total thermophilic lactobacilli and an increase of mesophilic lactobacilli (mostly belonging to *L. casei* or *L. paracasei* and *Lactobacillus rhamnosus*) occurred and a high heterogeneity in terms of strains was observed. Moreover, even if a decrease in *L. helveticus* densities was shown, a global estimation of autolysis was not considered.

The aim of this work was to describe cheese microbiota diversity and dynamics (SLAB and NSLAB) during fermentation and ripening of GP from six different dairies. Contrarily from Zago *et al.* (2007), who considered only one cheesemaking by using a culture-based approach, this work considered six cheesemaking processes of different dairies. Therefore, it was possible to evaluate not only the general trend of microbial dynamics, but also differences among productions. Culture-independent approaches were chosen in order to understand the contribute of viable, non-viable and lysed cells in ripening of the six different GP cheeses. A better knowledge of microbiota dynamics could be in fact useful to address technology favoring a suitable microbial evolution to be sought by each dairy.

3.4.3 Materials and Methods

Cheese manufacture and sampling

Six dairies of six provinces of the GP production area were considered for this study. In order to evaluate the cheese microbiota over time during each dairy cheesemaking, twin

wheels were produced from the same milk and whey starter in each dairy. Raw milk was treated according to the GP production protocol (http://www.granapadano.it; Gobbetti, 2004). A single milking was used for Grana Padano manufacture, and the milk was skimmed by creaming for about 8 h at 8-20°C. Partially skimmed milk was transferred to the vats (copper tanks) of 1,200 L of capacity. The vat skimmed raw milk was added with lysozyme (20 ppm) as anticlostridial agent. The natural whey cultures were used as starters (2.5 - 3.2% v/v), in which were obtained by incubating under a gradient of temperature from about 50°C to 35–20°C for 18-24 h, the whey of the previous day's cheesemaking. Calf rennet powder were added and coagulation were obtained at 31-33°C. After the coagulation, the curd was cut then stirred and cooked for 5-15 min at 53-54°C. After 40-80 min to let deposit the curd, it was extracted from the vat and cut in two portions and then the two curds were molded for 48 h. Then cheese were salted in saturated brine for 20-23 days and ripened for 12-16 months at 18-22°C and 80-85% relative humidity.

Samples were collected from vat skimmed raw milk up to cheese ripened for 13 months. Samples consisted of aliquots of the bulk skimmed raw milk (prior to the addition of lysozyme and natural whey starter culture), natural whey starter culture, cheese at 48 h after vat extraction and at different stages of ripening (2, 6, 9 and 13 months). According to the stage of ripening, cheeses were sampled from each twin wheels and were cut in slices. All samples were shipped to the laboratory as soon as they were collected and analyzed immediately upon arrival.

Microbial counts

Samples of skimmed raw milk (10 ml), natural whey starter (10 ml), and cheese (10 g) at 48 h after vat extraction, and at 2, 6, 9 and 13 months were used for the analysis. Ten grams of grated cheese samples were suspended in 90 ml of a 20 g/L trisodium citrate solution (pH 7.5) (Sigma-Aldrich, St. Louis, USA) and homogenized for 2 min in a Blender (Seward, London, United Kingdom). Decimal dilutions of milk and homogenates were made in quarter-strength Ringer solution (Oxoid, Basingstoke, United Kingdom) and plated in triplicate in the appropriate medium as described as follows.

Cultivable NSLAB, that can be considered potentially able to survive and grow in the ripened cheese were enumerated in cheese agar medium (CAM), constituted of GP grated cheese that is characterized by a high amount of digested proteins and NaCl and by the absence of milk sugars (Neviani *et al.,* 2009). CAM was incubated at 30°C for 3 days under anaerobic conditions. Total bacterial counts of vat skimmed raw milk was performed on Plate

Count Agar (Oxoid, Basingstoke, United Kingdom) supplemented with 10 g/L of powdered skimmed milk (MPCA) (Difco Skim milk, Sparks, MD, USA). Plates were incubated at 30°C for 3 days under anaerobic conditions. Thermophilic LAB were determined in whey starter samples by using whey agar medium (WAM) prepared as described by Gatti *et al.* (2003). Plates were incubated at 42°C for 2 days under anaerobic conditions. Total lactobacilli were enumerated on Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, United Kingdom), incubating plates at 30°C for 2 days under anaerobic conditions.

Culture-independent viable counts

Samples were subjected to viability counts by using the LIVE/DEAD *Bac*light Bacterial Viability kit (Molecular Probes, Oregon, USA) and fluorescence microscopy (Gatti *et al.,* 2006). One millilitre of 100-fold diluted natural whey starter, and 1 ml of skimmed raw milk were used. Grated cheese homogenates in trisodium citrate (15 ml) were centrifuged (10,000 rpm, 10 min., 4°C).

The pellet obtained was washed twice in 15 ml of 20 g/L sodium citrate solution (pH 7.5) (Sigma-Aldrich, St. Louis, USA) then resuspended in 15 ml of sterile water and 10-fold diluted. Hence, 1 ml was used for viability counts according to manufacturer's instructions. Samples stained with LIVE/DEAD were then filtered on black polycarbonate filters (0.2 µm pore size) (Millipore Corp., Billerica, MA, USA), visualized by an epifluorescence microscope (Nikon 80i, Tokyo, Japan) and counted as described by Bottari *et al.* (2010). Three separate counts were performed for each sample. Results were expressed as total, viable and non-viable cells/ml or cells/g.

DNA extraction from milk, whey and cheese samples

Bacterial genomic DNA was extracted directly from samples by using the General Rapid Easy Extraction System (GREES) DNA kit (InCura S.r.l., Cremona, Italy) according to the manufacturer's instructions. Before DNA extraction, milk samples were clarified as described by Rasolofo *et al.* (2010), and 1 ml was used for the experiment. Grated 48-h cheeses were mortared and 450 mg were used for DNA extraction. Before DNA extraction, cheese samples at 2, 6, 9 and 13 months were divided in two fractions and treated differently in order to discriminate the DNA from whole and lysed cells as described by Gatti *et al.* (2008). The free-cell fraction was obtained by filtration and the whole-cell fraction was treated with DNase in order to digest free DNA arising from lysed cells. DNA was extracted from one millilitre of

the filtered untreated fraction (lysed cells) and from 1 ml of the treated fraction (whole cells). One millilitre of natural whey starter was used for DNA extraction without any treatment.

PCR and length heterogeneity (LH) analysis

Length heterogeneity-PCR (LH-PCR) (Applied Biosystems, Foster City, USA) was used in order to determine the community composition.

V1 and V2 16S rDNA gene regions were amplified with 63F and 355R primers previously described by Lazzi *et al.* (2004), 63F primer was labelled with 6-carboxyfluorescein (FAM). Length heterogeneity of the PCR amplicons was detected by capillary electrophoresis (ABI Prism 310, Applied Biosystems, Foster City, USA). PCR and capillary electrophoresis conditions were performed as described by Bottari *et al.* (2010). The fragment sizes (base pairs) were determined by using GeneMapper software, version 4.0 (Applied Biosystems, Foster City, USA), local Southern method to generate a sizing curve from the fragment migration of the internal size standard (GS500 LIZ®; Applied Biosystems Foster City, USA) and the minimum noise threshold was set at 150 fluorescence units. Amplicon lengths were attributed to bacterial species according to LH-PCR published databases (Lazzi *et al.,* 2004; Gatti *et al.,* 2008).

Isolation and identification of species

Bacterial isolation from skimmed raw milk, natural whey starter, cheese at 48 h after vat extraction and at different stages of ripening (2, 6, 9 and 13 months) were performed from countable plates. Moreover, in order to better recover microorganisms from skimmed milk which could be of dairy interest, aliquots of skimmed vat raw milk were incubated at 30°C or 42°C under anaerobic conditions for 12 h. Subsequently, pre-treated and control (non-treated) skimmed raw milk samples were plated on MPCA and incubated at 30°C or 42°C for 72 h under anaerobic conditions.

Different colony morphologies were selected and purified by restreaking in MRS (Oxoid, Basingstoke, United Kingdom) for rod-shaped microorganisms and in M17 (Oxoid, Basingstoke, United Kingdom) for cocci, and were incubated at the same temperature of each pre-treatment under anaerobic conditions. The isolates were stored in MRS and M17 broth containing 20% (v/v) glycerol at −80°C.

Genomic DNA of isolates was extracted from overnight cultures by using the DNeasy kit (Qiagen S.r.l., Milan, Italy) according to the manufacturer's instructions. For a rapid screening of the isolates based on the LH-PCR database, the LH-PCR fingerprinting method (Lazzi *et*

al., 2004; Gatti *et al.,* 2008) was performed as described above. Moreover, selected isolates were identified based on 16S rRNA gene sequencing. DNA amplification and sequencing were performed as previously described (Giraffa *et al.* 2003). Consensus 16S rRNA sequences were compared to known sequences in the NCBI ribosomal database using (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) the basic local alignment search option.

Statistical analysis

Data were analyzed by multivariate ANOVA (PASW statistics 18.0.0, Armonk, New York, USA) according to the following general linear model: $y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, in which y_{ij} = dependent variable; μ = common mean; α_i = type of sample i = 1, ...7; ε_{ij} = residual error. The factor "type of sample" was divided into seven levels: milk, natural whey, cheese at 48 h, cheese at 2 months, 6 months, 9 months and 13 months. Significance of differences was tested by the Fisher's LSD (least significant difference) method.

Diversity indices

Richness and diversity indices of bacterial population were estimated by using the height of each peak (measured as intensity of fluorescence) in the LH-PCR electropherogram profiles. Diversity indices (Shannon and Simpson) were calculated as follows (Magurran, 2004; Buckland *et al.,* 2005): Shannon index, $H = -\sum p_i \ln(p_i)$, and Simpson index, $D = \sum p_i^2$. The Simpson's index value is given as 1-D since this way of presenting means that a higher value reflects higher diversity. Richness (S = the number of species) and Evenness ($E = H/H_{max}$; $H_{max} = \ln S$) were also calculated. The relative frequencies (p_i) were obtained by dividing the height of each peak with the total height of all peaks in the electropherogram profile for each sample. Standard errors (SE) were calculated for milk, whey and cheese samples.

3.4.4 Results

SLAB and NSLAB enumeration

The culture-based and culture-independent enumeration of the microbial population are presented in Table 1. Cultivable NSLAB, evaluated by CAM, in raw milk were higher ($P < 0.05$) than those in natural whey starters. A significant increase with respect to cheese at 48 h (not significantly different from milk) was observed in all cheeses, except for 13 month-ripened cheese. No significant differences were observed between 2, 6, and 9 month-cheeses.

Total lactobacilli evaluated in MRS medium, showed a similar trend as in CAM. Natural whey starters showed significantly higher viable cell densities compared to raw milk. This corresponded to a higher cell viability (77%) of total cells (calculated as the number of viable cells respect to the number of total cells) compared to raw milk (58%). Total cultivable microbiota in natural whey starters was evaluated by WAM. As well as for raw milk samples, it was observed that the viable cell number in the whey starters was higher than the cultivable counts. Viable cell densities did not show significant differences between cheese at 48 h, 2, 6 and 9 months in which high cell densities were observed. Despite a significant decrease of viable cells was observed at 13 months, the viable cell number was still higher (log 7.11 ± 0.41) than cultivable lactobacilli in MRS (log 4.53 ± 0.88) and NSLAB in CAM (log 4.82 ± 0.57). A great number of viable but non-cultivable microorganisms was present in the ripened cheese. Non-viable cells were observed during the overall period with no significant differences ($P > 0.05$) among cheeses. Differences between viable and non-viable cells were, for almost all samples, lower than 1 log unit. Figure 1 shows the dynamics of cultivable NSLAB for the six cheesemaking processes, showing the dispersion of data between dairies.

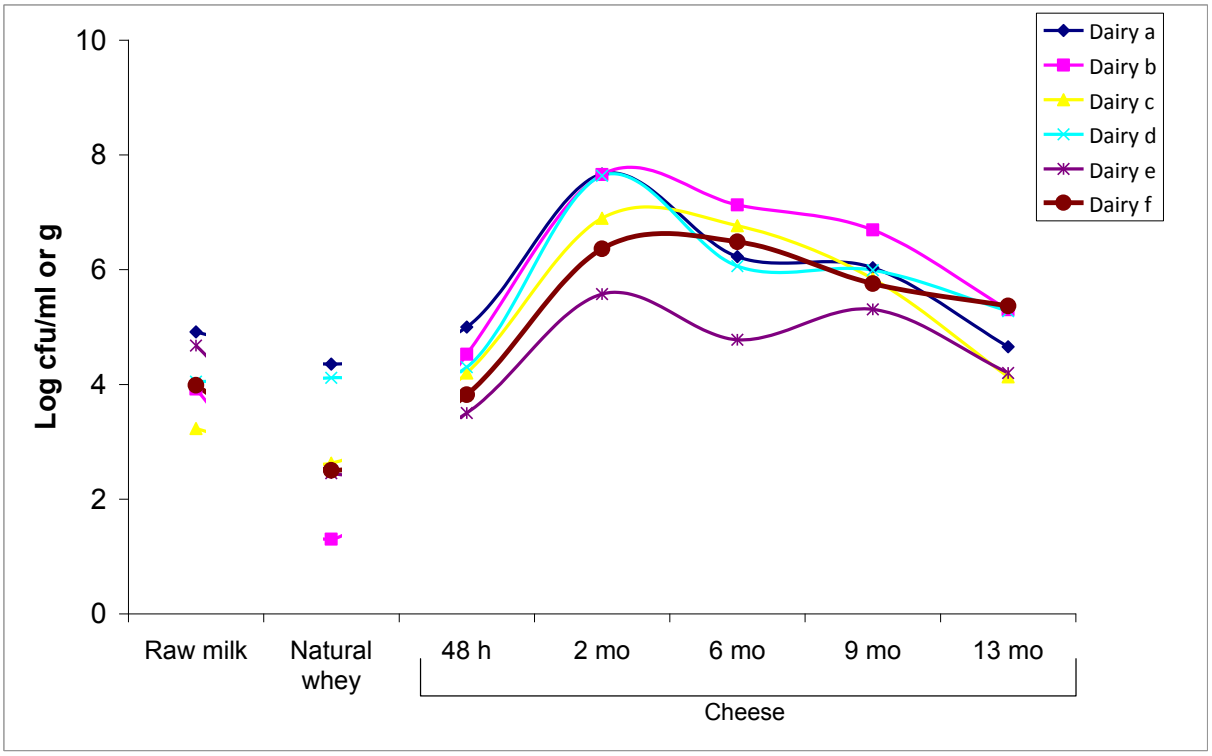

Figure 1. Cultivable NSLAB dynamics during the cheesemaking process.

	Bacterial count (mean ± standard deviation)						
	log cfu/ml or g		log cells/ml or g			log cfu/ml	
	CAM†	MRS	Viable	Non-viable	Total	MPCA	WAM
Milk	4.13 ± 0.60 b	4.29 ± 0.64 a	6.04 ± 0.45 a	5.09 ± 1.32 a	6.19 ± 0.55 a	4.80 ± 0.66	nd
Whey culture	2.89 ± 1.15 a	nd‡	9.07 ± 0.12 d	8.49 ± 0.24 c	9.19 ± 0.05 d	nd	8.10 ± 0.11
Cheese 48h	4.22 ± 0.53 b	4.37 ± 0.52 a	7.62 ± 0.42 c	7.01 ± 0.55 b	7.74 ± 0.43 c	nd	nd
Cheese 2 mo††	6.97 ± 0.86 c	6.89 ± 0.87 c	7.53 ± 0.45 c	6.81 ± 0.41 b	7.64 ± 0.39 bc	nd	nd
Cheese 6 mo	6.24 ± 0.81 c	6.41 ± 0.84 bc	7.40 ± 0.30 bc	7.00 ± 0.34 b	7.58 ± 0.25 bc	nd	nd
Cheese 9 mo	5.94 ± 0.45 c	5.76 ± 0.56 b	7.24 ± 0.15 bc	7.15 ± 0.22 b	7.52 ± 0.14 bc	nd	nd
Cheese 13 mo	4.82 ± 0.57 b	4.53 ± 0.88 a	7.11 ± 0.41 b	6.71 ± 0.48 b	7.30 ± 0.36 b	nd	nd
SE §	0.30	0.30	0.14	0.25	0.14	nd	nd

Table 1. Culture-based and culture-independent approaches for enumeration of the SLAB and NSLAB population. Each value is a mean of six samples for each matrix from different dairies throughout ripening. Different letters in the same column indicate that values were significantly different ($P < 0.05$), as determined by ANOVA and least significant difference.

†CAM (Cheese agar medium), MRS (Man Rogosa Sharpe), Milk Plate count agar (MPCA), Whey agar medium (WAM). All media were incubated at 30°C in anaerobiosis, except for WAM incubated at 42°C.

†† mo: months

§ SE: Standard Error

‡ nd: not determined

Isolation and identification of bacteria

A total of 334 colonies were isolated from the different counting media for all the samples. Bacterial isolation from plate count of skimmed raw milk was also performed after a milk pre-treatment in order to better recover bacteria, naturally occurring in the milk, that could be of dairy interest. Thus, regarding skimmed milk samples, a total of 56 colonies were isolated from control (without pre-treatment) and treated samples. Bacterial strains were analyzed by LH-PCR, and in order to confirm results, some strains were identified by 16S rRNA gene sequencing (Table 2).

The majority of strains from skimmed milk provided peaks at 319 bp. According to 16S rRNA gene sequencing, they corresponded to *Streptococcus uberis* and *Lactococcus* genus such as *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris*. Some of these strains were found only in control milk samples. The second most representative isolate was represented by a peak at 330 bp that belonged to *Lactobacillus delbrueckii* subsp. *lactis* or subsp. *bulgaricus.* This strain was isolated only from milk pre-treated at 42°C. Peaks at 317 bp were also frequently observed on isolates which corresponded to *Leuconostoc mesenteroides* subsp. *mesenteroides* and *L. lactis* subsp. *cremoris*. *L. mesenteroides* subsp. *mesenteroides* was only recovered in milk pre-treated at 30°C. Microorganisms belonging to the *Enterococcus* genus such as *E. faecalis* and *E. faecium* were also identified in milk pre-treated at 30°C and 42°C, respectively, giving peaks at 328 bp. *L. helveticus* (334 bp) and *L. hilgardii* (344 bp) were isolated from milk pre-treated at 30°C whereas *L. fermentum* (342 and 344 bp) and *L. gasseri* (340 bp) from milk pre-treated at 42°C. *L. rhamnosus* (336 bp) was isolated from both milk pre-treated at 30°C and 42°C.

Regarding bacteria isolated from natural whey starter and cheese at 48 h after vat extraction, all strains were identified as *L. helveticus*, *L. delbrueckii* subsp. *lactis/bulgaricus* and *L. fermentum.* All strains isolated from cheese at 2, 6, 9 and 13 months belonged instead to *L. rhamnosus*, *L. casei/paracasei* and *Pediococcus acidilactici* (data not shown).

Culture-independent analysis: LH-PCR

Figure 2 shows the superimposed LH-PCR electropherograms of the six different samples representing the microbial composition and dynamics during GP cheese production. Bacterial composition of vat skimmed raw milk showed that peaks of 280, 305, 309, 319 and 331 bp were mainly detected.

LH-PCR peak (bp)[a]	LH-PCR peak frequency (total of isolates = 56)	16S rRNA gene sequencing				
		Closest related sequence	GenBank	% identity	n° strains	milk treatment[b]
317	8	*Ln. mesenteroides* subsp. *mesenteroides*	HM058688.1	100	1	ON 30°C
		L. lactis subsp. *cremoris*	CP002094.1	99	2	control; ON 30°C
319	21	*L. lactis* subsp. *Lactis*	HM218818.1	99	8	control; ON 30°C
		S. uberis	AM946015.1	99	4	control
329	1	*Lactococcus sp.*	EF204374.1	99	1	control
328	4	*E. faecalis*	HQ293064.1	100	3	ON 30°C
		E.faecium	JN560911.1	99	1	ON 42°C
330	10	*L. delbrueckii lactis/bulgaricus*	AB680073.1 CP002341.1	100	10	ON 42°C
334	4	*L. helveticus*	HM218460.1	100	2	ON 30°C
336	3	*L. rhamnosus*	AF375898.1	99	3	ON 30°C; ON 42°C
340	1	*L. gasseri*	JN813104.1	99	1	ON 42°C
342, 344	3	*L. fermentum*	JN944698.1	99	3	ON 42°C
344	1	*L. hilgardii*	HM218530.1	99	1	ON 30°C

Table 2. Identification of species isolated from six milk samples subjected to pre-treatments and without treatment (control)

[a]The fragment length is reported with an approximation of ±1 bp.

[b]Treatment consisted in incubating milk at 30°C or 42°C under anaerobic conditions for 12 h (ON). Control were not subjected to any treatment.

According to the published database (Gatti *et al.,* 2008), the 330 bp peak could be attributable to *L. delbrueckii* species, while the peak of 319 bp could be attributable either to *S. thermophilus* or *L. lactis* subsp. *lactis*. The peaks of 280, 305 and 309 bp fragment lengths were not identified since they did not match with any species in the LH-PCR database.

In the majority of natural whey starter samples, the same LH-PCR peaks were detected and attributed to homofermentative thermophilic lactobacilli: the 330 bp peak was attributable to *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus* and the 334 bp peak to *L. helveticus*. Both SLAB species were dominant, while less abundant peaks at 319 bp were attributable to *S. thermophilus*. At 48 h after vat extraction, electropherograms of cheeses showed new less abundant peaks at 342 and 344 bp attributable to the heterofermentative NSLAB *L. fermentum*. These peaks were encountered until 9 months of ripening. Unidentified fragments at 323 and 325 bp were present in almost all 48-h cheese samples. The bacterial community structure of cheeses from 2 months was represented by the whole cells recovered from each stage of ripening. At two months, 336 and 345-bp peaks, belonging to *L. rhamnosus* or *L. casei/paracasei* and *P. acidilactici* respectively, were observed and persisted until 13 months of ripening. Many cheese samples showed *L. rhamnosus* or *L. casei/paracasei* as a dominant species. The peak attributable to *L. delbrueckii* subsp. *lactis/bulgaricus* was less abundant in the majority of 13-month ripened cheeses and none of them showed the presence of peaks belonging to *L. fermentum*.

Diversity indices

By using the LH-PCR technique it was possible to evaluate the diversity of the vat skimmed raw milk, natural whey and cheese ecosystems at different stages of ripening for six cheese manufacture processes. Table 3 presents the ecological indices during manufacture and ripening. The vat skimmed raw milk ecosystem presented higher diversity, evenness and richness compared to natural whey starter ecosystem. Among cheese ecosystems, diversity, evenness and richness demonstrated changing trends, with an increase during the first 2 months and then a gradual decrease during ripening. The highest number of species in the community (richness) was observed for cheese at 2 months of ripening.

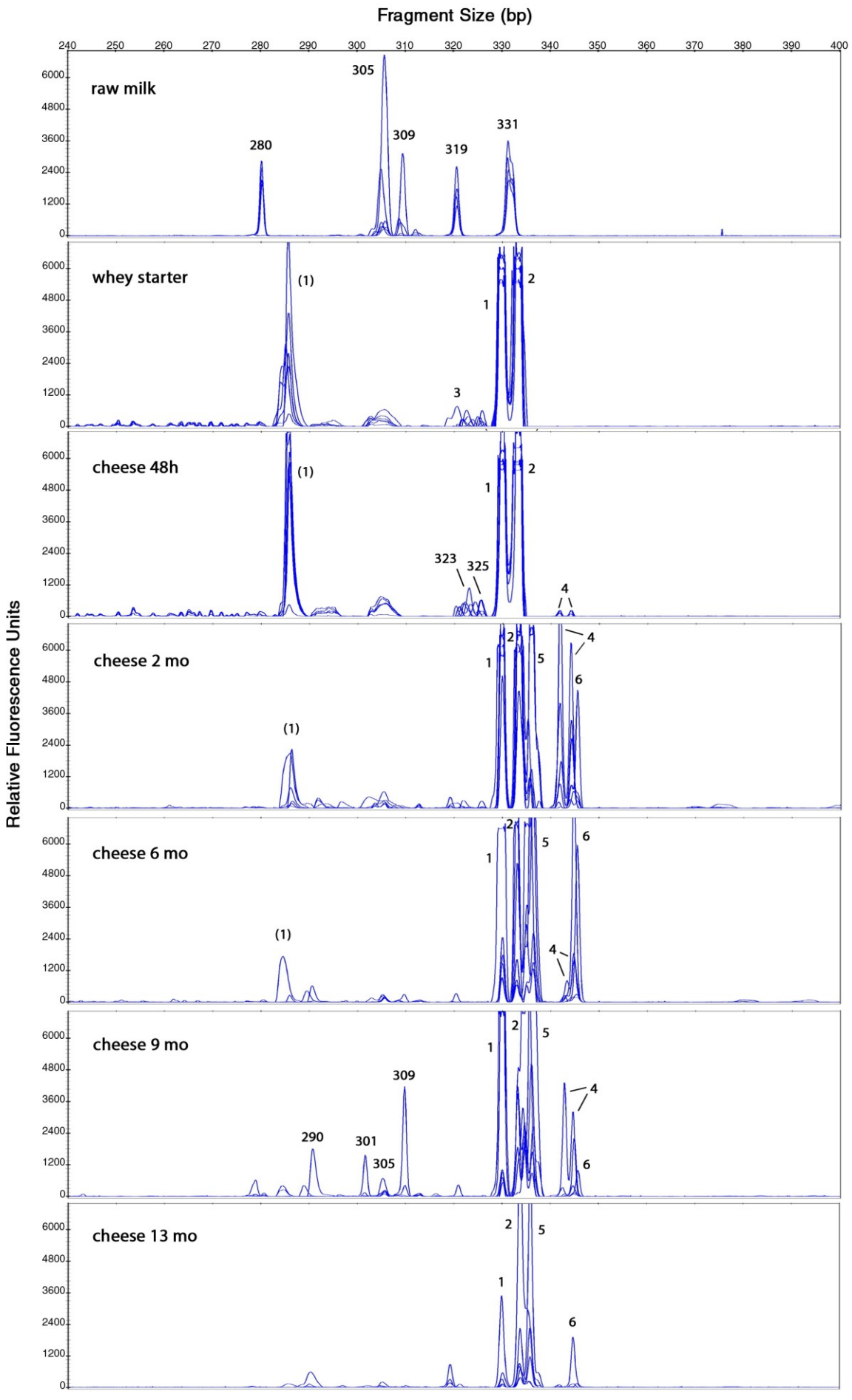
Fragment Size (bp)
240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400
Relative Fluorescence Units
0 1200 2400 3600 4800 6000
raw milk
305
309
280
319
331
whey starter
(1)
1
2
3
cheese 48h
(1)
1
2
323
325
4
cheese 2 mo
(1)
1
2
5
4
6
cheese 6 mo
(1)
1
2
5
6
4
cheese 9 mo
290
301
305
309
1
2
5
4
6
cheese 13 mo
1
2
5
6

Figure 2. Dynamics of SLAB and NSLAB throughout the Grana Padano cheesemaking process by LH-PCR. Six separate electropherograms from different dairies of each ecosystem (raw milk, natural whey starter, cheese 48h, cheese 2, 6, 9, 13 months) were superimposed (on the top of one another). For cheeses at 2, 6, 9 and 13 months, the whole cell fraction is represented. The X-axis shows peak sizes as base pairs (bp) and the Y-axis shows the peak intensity as relative fluorescence units. The peak sizes were attributed to bacterial species according to LH-PCR database as follows: **1**: *L. delbrueckii* subsp. *lactis/bulgaricus* (330 bp); **(1)**, secondary peak of 1; **2**: *L. helveticus* (334 bp); **3**: *S. thermophilus* or *L. lactis* subsp. *lactis* (319 bp); **4**: *L. fermentum* (342 and 344 bp*)*; **5**: *L. rhamnosus* or *L. casei/paracasei* (336 bp); **6**: *P. acidilactici* (345 bp*).* Unattributed peaks are shown by the fragment lengths as base pairs.

	Diversity indices			
	Simpson[a]	Shannon	Evenness	Richness
	D	*H*	*E*	*S*
Milk	0.63 (0.02)	1.08 (0.05)	0.91 (0.01)	3.67 (0.25)
Whey culture	0.55 (0.01)	0.85 (0.02)	0.72 (0.02)	3.50 (0.14)
Cheese 48 h	0.57 (0.01)	0.84 (0.04)	0.75 (0.04)	4.17 (0.19)
Cheese 2 mo[b]	0.72 (0.01)	1.43 (0.03)	0.93 (0.04)	5.00 (0.21)
Cheese 6 mo	0.67 (0.01)	1.25 (0.03)	0.85 (0.01)	4.33 (0.08)
Cheese 9 mo	0.63 (0.02)	1.20 (0.07)	0.80 (0.02)	4.50 (0.17)
Cheese 13 mo	0.50 (0.03)	0.88 (0.05)	0.75 (0.03)	3.50 (0.20)

Table 3. Ecological indices during the cheesemaking by LH-PCR. Mean of six samples for each matrix throughout ripening (standard errors) are shown.

[a]Simpson ($D = \sum p_i^2$); the Simpson's index value is given as 1-D. p_i is the relative abundance of a given LH-PCR peak; Shannon ($H = -\sum p_i \ln(p_i)$, where pi). Richness (S) is equal to the number of species. Evenness (E), the relative abundance with which each species is represented, ($E = H/H_{max}$; $H_{max} = \ln S$).

[b] mo: months

Two-month cheese cell lysis

The rates of bacterial lysis were monitored by determining the free DNA released from dead or damage cells in two-month cheeses. Lysis rates were calculated as the sum of the areas of all peaks in the LH-PCR profiles of free DNA fraction of each sample. The relative percentage of cell lysis was calculated by measuring the individual and total peak area (Table

4). The results showed that cheeses c, d and f had the highest lysis values and in particular, two-month cheese f showed the most abundant lysed cell fraction.

	Relative percentage of lysed cells[b] (%) Mean (SD)					
Peaks lengths (bp)[a]	Cheese a	Cheese b	Cheese c	Cheese d	Cheese e	Cheese f
330	43 (4)	38 (3)	36 (1)	9 (7)	8 (5)	54 (1)
334	25 (5)	51 (2)	64 (1)	45 (1)	92 (5)	46 (1)
336	13 (1)	11 (1)		43 (8)		
342-344	20 (5)			2 (0)		
Total peak area (lysis rate)	25,803	17,604	90,471	100,427	87,376	170,114

Table 4. Relative percentage of lysed cells in two-month cheese. Three separated LH-PCR experiments (n = 3) were performed for mean and standard deviation (SD) calculation.

[a]Peak lengths correspond to: *L. delbrueckii* (330), *L. helveticus* (334), *L. rhamnosus* or *L. casei/paracasei* (336) and *L. fermentum* (342 and 344).

[b]Percentage was calculated based on the ratio of each single peak area, corresponding to different species, and the total peaks area (lysis rate).

Furthermore, it was observed that lysed cells belonged to *L. helveticus* and *L. delbrueckii* subsp. *lactis* for all the two-month cheeses. The highest percentage of lysed cells corresponded to *L. helveticus,* except for cheese a and f in which *L. delbrueckii* gave the highest values. This result was consistent with the whole cell fraction of these cheeses, that evidenced *L. delbrueckii* as dominant species (data not shown). Lysed NSLAB *L. rhamnosus* or *L. casei/paracasei* were detected in cheese samples a, b and d as minor percentages. Interestingly, cheese d showed a comparable percentage of *L. helveticus* cell lysis (~ 43% of total cells). Lysed cells belonging to *L. fermentum* were detected in cheese d and in cheese a, which in particular, showed high and comparable percentages of *L. helveticus* (~ 20%).

3.4.5 Discussion

The overall process of GP cheesemaking was studied in six different dairies. Each dairy supplied samples of the complete cheese production, from vat skimmed raw milk until 13 months of ripening. The same cheese wheels were studied over time. Thus, skimmed raw milk, whey starter and cheese microbial ecosystems were studied during ripening by culture-

based and culture-independent approaches. Microbial population dynamics, ecological diversity and the lysis rate at two months of ripening were investigated.

As previously suggested, by using the cheese-based medium (CAM), it was possible to study the microbiota that better adapts to the nutritional characteristics of a sugar-free medium and that can be considered potentially able to survive and grow in the cheese environment and during ripening (Neviani *et al.,* 2009). These authors isolated NSLAB from milk, whey starter, and curd only using CAM. This medium is able to recover the secondary microflora, attributable to the NSLAB group, which do not contribute to acid formation throughout manufacturing but can play a significant role during ripening. Compared to natural whey cultures, results showed that the vat skimmed milk appeared to provide higher numbers of these microorganisms that could possibly develop later in cheese. Similar observations were reported for PR, a hard cheese similar to GP (De Dea Lindner *et al.,* 2008). Natural whey starters for GP showed high microbial counts in WAM, high cell viability and similar species composition as observed by other authors (Fornasari *et al*., 2006; Rossetti *et al.,* 2008; Santarelli *et al.,* 2008; Gatti *et al.,* 2011). Although as lower numbers, some LAB able to grow in CAM were also present in whey starters. Thus, it is possible to state that natural whey starter is mainly composed of LAB involved in acid production during manufacture but also that a very low percentage (less than 0.01%) of NSLAB is present.

Independently from their origin, cultivable NSLAB were present at low densities in cheeses at 48 h and significantly increased to high densities at 2, 6 and 9 months, then decreased at 13 months. In these latter samples, cultivable bacteria enumerated in CAM and MRS highlighted a similar trend over time. It is not surprising that bacterial enumeration in MRS was similar to that in CAM since low nutritional demanding NSLAB, adapted to grow in a low sugar medium as CAM, could better grow in MRS. The growth trend of the NSLAB cultivable population agreed with findings by De Dea Lindner *et al.* (2008) during PR cheesemaking.

Fluorescence microscopy is able to determine the densities of both SLAB and NSLAB without differentiating them. The number of viable cells was higher than total cultivable microbiota for milk, whey and cheese ecosystems. This suggests the presence, in all samples, of a population that is viable but noncultivable, as demonstrated by other authors (Ward *et al.,* 1990; Head *et al.,* 1998). Furthermore, these data highlight the limit of the plate counting technique that was able to reveal no more than 10% of bacteria. Similar observations were reported by other authors who compared culture-based and culture-independent approaches in dairy ecosystems (Valence *et al.,* 2000; Gatti *et al.,* 2006; Bottari *et al.,* 2010).

In agreement with Franciosi *et al.* (2011), the majority of isolates obtained from vat skimmed raw milk belonged to the *Streptococcaceae* LAB family: *L. lactis* subsp. l*actis,* and subsp. *cremoris, S. uberis*, and *L. mesenteroides* subsp. *mesenteroides*. These species were found only from control milk. *L. lactis* and *L. mesenteroides* are commonly used as dairy LAB starters in other cheeses and fermented milk (Cogan *et al.,* 2002); however, in ripened GP cheese they have never been reported. *S. uberis* is involved in clinical and subclinical intramammary infections in lactating and non-lactating dairy cows (Rambeaud *et al.,* 2003). Thus, it is not surprising to isolate this species in raw milk or in fresh cheeses such as artisanal mozzarella (Morea *et al.,* 1999).

Moreover, SLAB and NSLAB belonging to the genus *Lactobacillus* are of great technological interest for acidification and ripening (Cogan *et al.,* 2002); in the present study *L. helveticus, L. delbrueckii* subsp. l*actis;* subsp. *bulgaricus*, and *L. rhamnosus* were identified in cultured raw milks. The isolation of these LAB has already been reported in raw milk during PR cheesemaking (Neviani *et al.,* 2009). Furthermore, by using a culture-independent approach, Franciosi *et al.* (2010) detected the same species in raw milk for a type of GP, "Grana Trentino" cheese, however they also reported *S. thermophilus*. In the present study, *S. thermophilus* was not isolated either from control or pre-treated milk. However, by culture-independent LH-PCR analysis, a peak at 319 bp attributable to *S. thermophilus* according to the database (Lazzi *et al.,* 2004; Gatti *et al.,* 2008) could reveal its presence.

The finding of SLAB such as *L. helveticus*, *L. delbrueckii* and also the NSLAB *L. fermentum* in pre-treated raw milk highlight that the whey starter is not the unique source of these lactobacilli (Fornasari *et al.,* 2006; Mucchetti and Neviani, 2006). The fact that after incubation of milk, a considerable number of these strains was recovered suggests that they could grow during the first stages in the vat and successively they could have a role in acidification together with the SLAB added from the whey cultures. However, more studies have to be performed at strain level to confirm this hypothesis. *L. fermentum*, that is normally present in GP natural whey starters (Fornasari *et al.,* 2006), is involved in gas production responsible for the desired tiny holes in Grana cheese. However, this is considered a positive feature only if it is present at low amounts (Bottazzi, 1981).

In particular, the fact that some strains of *L. rhamnosus* and *L. fermentum* were isolated after a pre-treatment of milk at 42°C, induces to hypothesize that only the strains with a thermophilic characteristic will be able to resist to technological conditions and grow later in cheese as previously reported (De Dea Lindner *et al.,* 2008). It is not clear what alternative energy source NSLAB use to grow in cheese after the simple carbohydrates and citrate are

exhausted (Budinich *et al.,* 2011). These authors supposed that NSLAB use for growth milk-derived complex carbohydrates and starter-derived components, however these are still unknown. *L. gasseri* and *L. hilgardii* strains have not been identified before in raw milk of GP and PR (Coppola *et al.,* 1997; 2000; Neviani *et al.,* 2009; Franciosi *et al.,* 2010; 2011). Both species were isolated from soft cheese and they do not have any known role during acidification and flavor formation (Baruzzi *et al.,* 2000).

LH-PCR results in raw milk were in good agreement with bacterial isolation, revealing mainly the presence of *S. uberis* or *L. lactis* subsp. *lactis* and subsp. *cremoris* (319 bp) and *L. delbrueckii* subsp. *lactis*/*bulgaricus* (330 bp). Only the peak at 330 bp was detected according to Gatti *et al.* (2008). These authors studied raw milk for PR cheese by LH-PCR and also found peaks at 334 bp (*L. helveticus*), 336 bp (*L. rhamnosus, L. casei/paracasei* or *L. plantarum*), and 339 bp (not attributed). Since the LH-PCR technique allows to represent the dominant species in a community (Ndoye *et al.,* 2011), it is possible to consider these two milk as different. The different origins of the milk from specific geographic areas, delimited by two different official regulations, could justify this difference (http://www.parmigiano-reggiano.it; http://www.granapadano.it).

Peaks attributed to *L. helveticus* and *L. delbrueckii* subsp. *lactis* species were found in all natural whey starter samples according to previous works (Fornasari *et al.,* 2006; Rossetti *et al.,* 2008; Santarelli *et al.,* 2008; Gatti *et al.,* 2011). Differently from these authors, *S. thermophilus* and the heterofermentative *L. fermentum* were undetected. From cheese at 48 h after vat extraction until 13-month cheeses, DNA from whole cells of SLAB *L. helveticus* and *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus* were identified in all samples during ripening. This data agreed with microbial dynamics of whole SLAB cells in PR cheese (Gatti *et al*., 2008), differently from PR, GP cheese showed the presence of the NSLAB *L. fermentum* during ripening and in some cases with high abundance. However, no strains belonging to any of these species were isolated after brining (data not shown). It is not clear whether this population, composed of whole cells that are unable to be cultured, has a role in ripening and needs further studies. Differently from this observation, Zago *et al.* (2007), by using a culture-based approach, detected *L. helveticus* and *L. delbrueckii* cultivable cells in 2-month cheese that progressively decreased during ripening until 8 months.

According to the present work, *L. rhamnosus* or *L. casei/paracasei* and *P. acidilactici* have been previously observed and in some cases isolated from PR (Gala *et al.,* 2008; Gatti *et al.,* 2008; Neviani *et al.,* 2009). Their presence in ripened cheese underlines their ability to use alternative potential energy sources other than lactose for growth (Williams *et al.,* 2000). In

GP, viable *L. rhamnosus* and *L. casei* were previously found (Zago *et al.,* 2007; Belletti *et al.,* 2009). However, by LH-PCR it was not possible to distinguish them since both generate the same 16S rRNA amplicon length (Lazzi *et al.,* 2004; Gatti *et al.,* 2008). The specific role of both *Pediococcus* spp. (Cogan *et al.,* 2002) and *L. rhamnosus* (Bove *et al.,* 2011) are not fully understood.

LH-PCR was able to show differences in the diversity, evenness and richness of bacterial community among ecosystems and during ripening. Higher diversity indices, species evenness and richness were observed for the vat skimmed raw milk ecosystem compared to natural whey starter. Diversity, evenness and richness of bacterial community demonstrated an increase during the first 2 months and then a gradual decrease during ripening. This seems to be correlated to the growth of NSLAB reported during the first months of ripening evaluated by culturing and LH-PCR.

Regarding bacterial enumeration, it was observed that between cheeses at 48 h and 2, 6 and 9 months, there was a transition period in which viable cell densities remained invariable among cheeses while an increase in cultivability has been highlighted. It seems that a balance of both the whole and lysed cells was created and unrevealed by fluorescence microscopy. It is known that a decrease in cultivability of SLAB occurs in early stages of hard cheese manufacture (Valence *et al.,* 2000; Deutsch *et al.,* 2002; Hannon *et al.,* 2007), in PR (Pecorari *et al*., 2003; De Dea Lindner *et al.,* 2008) and GP (Giraffa *et al.,* 1998; Zago *et al.,* 2007). Because of this, the lysis rate at 2 months was studied, expecting to find an early SLAB lysis. Moreover, since no correlation between the autolysis *in vitro* and in cheese trials was demonstrated (Valence *et al.,* 2000; Kenny *et al.,* 2006), in this work, a culture-independent approach was chosen to better discriminate which species undergo lysis. Hence, the study of the two-month ripened cheeses allowed to estimate the total lysis rate and how it affected each LAB species. Free DNA from lysed *L. helveticus* and *L. delbrueckii* subsp. *lactis* was found in all the two-month cheeses, confirming that after acidification, starter lysis occurs. Contrarily to Gatti *et al.* (2008) that did not reveal the presence of whole and lysed cells of *L. fermentum* during PR cheese ripening, *L. fermentum* lysed cells were observed and for many cheese samples high relative percentages were reported. The extent of cell lysis (lysis rate) in cheese affects the proteolysis rate and the length of ripening (Cogan *et al.,* 2002). Autolysis of different species was shown to induce the release of different enzymes that determine the degradation of casein derivatives during ripening (Kunji *et al.,* 1996). The effect of this process may highly affect the texture and flavor of cheese in each dairy.

3.4.6 Conclusions

By means of LH-PCR fingerprinting technique, only the most abundant members are represented, however bacterial communities profiles could be described and compared. Differently from other works, this research considered six cheesemakings of different dairies, allowing to evaluate both the trend of microbiota dynamics and the differences among different productions.

GP characteristic microbiota can arise from two main sources: milk and natural whey starters. By LH-PCR, it was observed that the microbial biodiversity was different among these ecosystems. Starter lactobacilli were the main species during the acidification steps of GP production while NSLAB were able to grow after brining, and became abundant during ripening. Their origins were both the raw milk and the natural whey starter. However thorough studies have to be performed on SLAB and NSLAB isolates, also at strain level, to better understand whether they have an aptitude to acidification and ripening.

The presence of *L. helveticus* and *L. delbrueckii* subsp. *lactis* during the first hours of production highlighted the well-known role of natural cultures in curd acidification while their presence, in a non-culturable state, up to 13 months of ripening may suggest a different unknown role in cheese ripening. There was a relevant presence of viable but non-culturable cells in cheese throughout ripening and during all the manufacturing process. Moreover, whole starter cells were found until advanced stages of ripening but they could not be cultured after brining. Because of the unculturable nature of this population, to date, their potential technological aptitudes are not clear. However, only metagenomic studies could provide some thorough information.

The pre-treatment of milk resulted to be useful and potentially applicable to other hard cooked cheese products in order to reveal the thermophilic population that has major chances to resist the cooking of the curd.

By monitoring SLAB and NSLAB cell autolysis in two-month cheeses, it was observed that SLAB represented the major component that underwent lysis at that time of ripening. The highest percentages of lysed cells corresponded to *L. helveticus* but also NSLAB cell lysis was observed. Despite being made under standard manufacturing conditions, 2-month cheese analyzed from different dairies exhibited variations in microbial cell lysis. Their lysis rate, which appeared to be variable among samples, could contribute to differentiate each cheese production. Bacteria from natural whey starter and from milk were encountered in all steps of

cheesemaking and ripening, and together with technological parameters contribute and are responsible for the peculiar organoleptic features of the cheese.

3.4.7 Acknowledgements

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3.5 Characterization and dynamics of surface microbiota during natural rind development

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3.5.1 Abstract

In some cheese varieties, a complex microbiota develops on the cheese surface during the ripening period. For instance, in smear cheese, this microbiota is involved in ripening proceeding from the surface to the interior of the cheese. In other cheese varieties in which ripening proceeds mainly from the interior, for example due to mold metabolism, little is known about the composition or role of the surface microbiota in flavor formation. The aim of this work was to describe the microbial diversity of a natural rind, understand how the development of a rind proceeds by natural microbial colonization, and examine interactions among microorganisms in order to predict possible contributions to community formation. Approaches that combine culture-based and next-generation sequencing were used. The microbiota on the surface of the blue cheese during natural rind development showed a microbial diversity comprising fourteen genera of bacteria (*Enterococcus; Lactococcus; Leuconostoc; Macrococcus; Staphylococcus; Klebsiella; Brevibacterium; Corynebacterium; Brachybacterium;, Nocardiopsis;, Cobetia; Psychrobacter; Halomonas*; *Haererehalobacter*), two yeast genera (*Candida; Debaryomyces*) and one filamentous fungal genus (*Penicillium*). High and comparable densities of viable bacteria and yeasts were observed. Bacterial succession was observed during rind formation in which *Staphylococcus* dominated the early stages and then *Brevibacterium* the later stages. Inhibition and stimulation have been observed among several species, which could explain some contributions to community formation. This natural rind can be considered an active rind formed by a rich and dense microbial consortium. However, thorough studies have to be performed in order to evaluate whether these bacteria, yeast and molds play a role in flavor and texture development that could be beneficial in other cheese varieties with diverse natural rind microbiota.

3.5.2 Introduction

Cheese microbiota is composed of starter lactic acid bacteria (SLAB), secondary microorganisms and adventitious organisms (Powell *et al*., 2011). Starter cultures are responsible for fast acidification and flavor formation during ripening, whereas the secondary microorganisms are added mainly for their effect on flavor, color and texture. Their contribution to acid formation is limited or absent and, moreover they are usually unique to the specific cheese variety (Ndoye *et al*., 2011; Rattray and Eppert, 2011). The adventitious organisms likely originate from the milk and/or dairy environment (brine, wooden shelves, the cheesemaker's hands); these include NSLAB, other bacteria, yeasts and molds that in many cases determine the final organoleptic characteristics of the cheese (Mounier *et al*., 2006; Settani and Moschetti, 2010).

Many aged cheese varieties develop a rich and dense microbial consortium on the surface, thus forming a rind. These microorganisms can be deliberately added as secondary cultures and/or be adventitious. They coexist, interact and are essential for the ripening process and the final characteristics of the product. The technological conditions during the ripening process, humidity, temperature and the ecology of the microbiota in the brines and in the dairy room are factors that influence the microbiota in the surface (Mucchetti and Neviani, 2006).

There are rinds that develop after being immersed or wiped down periodically with liquid (brines or beer) to keep them moist. During ripening, a viscous, red-orange smear composed of bacteria and yeast develops on their surfaces (Mounier *et al*., 2005, 2006). These cheeses are also called bacterial surface-ripened or smear-ripened cheeses since the microbiota and their enzymes promote the ripening process from the surface to the interior of the cheese. Other rinds develop after inoculation with defined strains of molds which are essential for the development of the texture and flavor; in some cases the growth of another molds is an undesirable event. In this case, these cheeses are also called mold surface-ripened cheeses. Moreover, there are cheeses to which no treatment is given during the overall period of ripening and the rind forms naturally, some of which include the blue-veined cheeses (e.g. Stilton in UK and Cabrales in Spain) and the surface mold-ripened cheese St. Nectaire in France.

Most studies have focused on the evaluation of microbial composition and dynamics in the smear (Brennan *et al*., 2002; Feurer *et al*., 2004; Ogier *et al*. 2004; Mounier *et al*., 2005, 2006; Rademaker *et al*., 2005; Rea *et al*., 2007; Cocolin *et al*., 2009; Dolci *et al*., 2009; Fontana *et al.* 2010). At the end of ripening, this microbial consortium on the surface of smear cheeses is

mainly composed of Gram-positive bacteria, including *Micrococcus*, *Staphylococcus*, various coryneform species, such as *Corynebacterium*, *Arthobacter* and *Brevibacterium linens* and yeasts such as *Debaryomyces*, *Kluyveromyces* and *Candida* (Brennan *et al*., 2002; Feurer *et al.*, 2004; Rademaker *et al*., 2005; Mounier *et al*., 2005, 2006, Rea *et al*., 2007; Cocolin *et al*., 2009; Dolci *et al*., 2009). Furthermore, the ecosystem on the surface results to be more diverse than that of the interior (Ogier *et al*., 2004).

In terms of progression of microbial populations, yeasts predominate initially and bacteria become dominant later. Authors reported that initially yeasts catabolize the lactate produced by the starter bacteria to CO_2 and H_2O which cause an increase of pH that favors the growth of salt-tolerant bacteria. Generally, staphylococci dominate the earliest stages (day 4), and coryneforms, particularly *Corynebacterium variabile* and *Corynebacterium casei*, dominate the later stages of ripening (Mounier *et al*., 2006), showing an important role in rind formation. The reason why staphylococci are generally replaced by coryneforms during ripening is not clear (Rea *et al.,* 2007). Reports suggest that yeasts produce vitamins and other compounds which stimulate the growth of *B. linens* (Rea *et al*., 2007).

Lactic acid bacteria are known to play a role in acidification and ripening in many cheeses (Powell *et al*., 2011), however, it is not clear yet whether they have a role in rind formation (Fontana *et al*., 2010). In a study focused on the dynamics of surface microbiota of an Italian smear cheese, the authors found high microbial counts of LAB in the rinds after 90 days of ripening (Dolci *et al*., 2009).

However, very few studies evaluated microbial composition and changes during ripening in natural rinds (Marcellino and Benson, 1992). The knowledge of the microbial composition on the cheese surface is a prerequisite for the development of secondary adjunct cultures and for the control of surface ripening through good hygienic practices avoiding the development of undesirable flora (Cocolin *et al.*, 2009; Monnet *et al*., 2010). This is not only true for surface ripening purposes; in blue-veined cheese in which ripening proceeds from the interior, spoilage fungi belonging to *Penicillium* spp. can colonize and grow on the surface. This causes the formation of off-flavors or discoloration of the cheese in the form of brown spots (Ardö, 2011). Performing a study of the microbial succession in smear cheeses may not provide real information on progression since the cheese surface is washed frequently, and thus disturbed, during ripening (Brennan *et al.,* 2002).

In many cases, strains are deliberately inoculated onto the surface of the cheese to promote ripening. However, a few studies showed that at the end of ripening the inoculated species

were not found, which may be a result of negative interactions among microorganisms (Brennan *et al*., 2002; Feurer *et al.,* 2004).

Thus, microbial succession studies in natural rind cheeses are needed. The fact that the surface of this type of cheese is not disturbed during ripening could allow the study of the natural microbial progression over time, with attention to possible interactions that may occur among microorganisms. These could be inhibitory through the production of antibiotics or competition for nutrients, or stimulatory through the production of growth factors. Moreover, the microbiota that is naturally able to grow in the surface of this type of cheeses could also have a role in flavor formation or protection of the surface from the growth of undesirable microorganisms.

The studies on cheese rind microbiota are mainly based on culture-based and culture-independent approaches in which fingerprinting methods are the most common (Feurer *et al*., 2004; Mounier *et al.*, 2005; Cocolin *et al*., 2009). Recently, high-throughput sequencing technologies, that do not rely on the traditional Sanger chain termination method, have been developed (Margulies *et al.,* 2005). Two of the three sequencing platforms include the Illumina technology (www.illumina.com/technology/sequencing_technology.ilmn) and 454 sequencing (http://my454.com/products/technology.asp). These platforms differ in that the Illumina technology uses a "reversible terminator-based method" whereas 454 sequencing involves DNA capture beads. These sequencing platforms and processes can produce massive amounts of data in less time and at a lower cost (Haridas, 2011). These technologies use specific barcodes (sequences) in order to analyze thousands of samples in a single run. To date, they have been used for culture-independent analysis in order to monitor the microbial community structure and abundance of community members in various ecosystems such as soil (Acosta-Martinez *et al.,* 2008), gut (Andersson *et al.,* 2008), food (Lopez-Velasco *et al.,* 2011), fermented food (Humblot and Guyot 2009; Park *et al*., 2011; Roh *et al.,* 2010) and cheese (Masoud *et al.,* 2011).

The aim of this work was to describe the microbial diversity of a natural rind, understand how the development of a rind proceeds by natural microbial colonization, and examine the interactions among microorganisms in order to predict possible contributions to community formation. Approaches that combine culture-based and next-generation sequencing were used.

3.5.3 Materials and Methods

Cheese manufacture

Cheese rind samples at different times of ripening were collected from a blue-veined cheese manufactured in a farm located in Vermont, USA. The cheeses were made using raw cow's milk derived from daily milking and a defined mesophilic starter culture composed of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Lactobacillus acidophilus*, *Bifidobacterium* sp. and *Leuconostoc mesenteroides*. *Kluyveromyces lactis* and conidia of *Penicillium roqueforti* were used as ripening cultures.

The raw milk was heated at 30°C, inoculated with mesophilic lactic acid starters and *P. roqueforti* conidia, and finally added with rennet. The curd was cut and stirred and then placed in molds, and whey was drained off during ~10–48 h without the application of external pressure, and the molds were turned frequently. The cheeses were salted manually by scrubbing the surface with dry salt. Subsequently, the cheeses were ripened at 9°C and 85% relative humidity. Piercing of the cheeses has been performed one or more times during ripening for 6 weeks. During the overall period of ripening (~65 days), a natural rind develops on the surface.

Rind sample collection for determination of community structure

Cheeses at different times of ripening were used for the study. Cheese surface samples were collected during rind formation at 10, 24, 40, 56, 71 and 86 days of ripening. Rind samples were taken scraping the surface with a sterile razor, then transferred into a sterile tube and immediately frozen at -20°C. All samples were shipped to the laboratory as soon as they were collected and immediately analyzed.

Rind sample collection for determination of chemical parameters

pH and sodium chloride (NaCl) in cheese surface were measured by using a pHmeter equipped with surface electrodes: pH microelectrode (MI-410 Combination pH electrode, Microelectrodes, Inc. Bedford, USA) and sodium microelectrode (MI-425 Sodium Electrode, Microelectrodes, Inc. Bedford, USA) with tips of 1.5 mm in diameter. Calibration of the sodium microelectrode was achieved by measuring millivolt (mv) potentials of pure standard sodium chloride solutions (0.001; 0.01; 0.1; 1 and 5 M). Sodium concentration was determined by a linear regression equation. Cheese surface samples were obtained as explained above and

collected at 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70 days of ripening from three different batches. Samples at 0 days (immediately after salting) were also considered for pH measurement. Aliquots of rinds were mixed with an equal volume of milliQ water and directly measured.

Microbial count and isolation

Total viable aerobic cell enumeration of bacteria and yeasts was performed using milk plate count agar (MPCA) (BD Difco, Sparks, USA) and yeast extract peptone and glucose agar (YPD) (BD Difco, Sparks, USA) supplemented with chloramphenicol to inhibit bacterial growth, respectively. One-hundred milligrams of rinds at different time of ripening were 10-fold diluted in 1× PBS, spread plated on MPCA and YPD media and incubated for 5 days at 20°C under aerobic conditions. Enumeration plates were performed in triplicate and results were expressed as cfu/g of cheese rind.

Different colony morphologies were selected, according to macroscopic and microscopic observations during rind formation, purified by restreaking in triptone soya agar medium (TSA) (BD Difco, Sparks, USA) for bacteria and in YPD medium for yeast, and then incubated for 5 days at 20°C under aerobic conditions. Bacterial and yeast isolates were stored in Luria-Bertani (LB) (Oxoid, USA) and in YPD (BD Difco, Sparks, USA) broth, respectively, containing 20% glycerol at -80°C.

DNA extraction from isolates and cheese rind

Genomic DNA of the isolates was extracted from overnight cultures by using Ultraclean Microbial DNA isolation kit (MO BIO Laboratories, Inc. Carlsbad, CA, USA) according to the manufacturer's instructions. One-hundred milligrams of each rind were used for genomic DNA extraction directly from samples. PowerSoil DNA isolation kit (MO BIO Laboratories, Inc. Carlsbad, CA, USA) was used according to the manufacturer's instructions. DNA quantification and purity measurements were performed by using a NanoDrop (ND-1000) spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Identification of bacterial and fungal isolates

16S rRNA gene and internal transcribed spacer (ITS) regions between the 18S and 28S rRNA genes were used for DNA sequencing in order to identify bacteria and fungi, respectively.

A pair of universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991), was used for PCR amplification of bacterial

16S rRNA gene. ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Brunes 1993) and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,*1990) were used for fungal rRNA gene amplification.

PCR was performed using a MJ Research Tetrad PTC-225 Thermo Cycler and 96-well polypropylene plates in 50 µl of reaction mixture containing 1 ng/µl of template DNA, 1× Phusion High-Fidelity PCR Master Mix (Biolabs, New England, USA), and 0.5 µM of each primer. The PCR conditions were performed as follows: 10 s at 98°C, followed by 30 cycles at 98°C for 10 s, 30 s at 55°C and 1 min at 72°. A final extension step at 72°C for 5 min was carried out. Sequences were analyzed for sequence homology by using the basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Barcoded Illumina sequencing of bacterial community

High-throughput sequencing with Illumina/Solexa technology was performed (www.illumina.com/technology/sequencing_technology.ilmn) in order to describe the diversity of rind bacterial community. Total bacterial genomic DNA extracted directly from cheese rinds at 10, 24, 40, 56, 71 and 86 days of ripening was subjected to conventional PCR amplification of the 16S rRNA gene. Then, PCR amplicons from multiple individuals of the rind community were sequenced by Illumina technology. Since Illumina can run of up to ≈1,500 samples per lane, specific barcodes were used, according to Caporaso *et al.* (2010a), in order to assign the sequence to its appropriate sample.

The V4 region (300 bp) of the 16S rRNA gene was amplified in each sample using the constructed primer pairs (F515/R806) described by Caporaso *et al.,* (2010a). The reverse PCR primer was barcoded with a 12-nucleotide sequence. Thus, 6 different barcodes were used for the 6 rind samples. Moreover, forward and reverse primers contained a linker sequence and an adaptor sequence (Illumina adaptor). These primer constructs yielded a library of amplification products that contained the barcode and Illumina adaptors.

PCR amplification was performed using a MJ Research Tetrad PTC-225 Thermo Cycler and 96-well polypropylene plates. Each sample of rind was amplified in triplicate. PCR reactions were performed in a final volume of 25 µl, containing 1× Phusion High-Fidelity PCR Master Mix (Biolabs, New England, USA), 0.5 µM of each primer constructs, and ~4 ng/µl of genomic DNA.

Amplification conditions reported by Carporaso *et al.,* (2010a) were slightly modified. PCR amplification cycling conditions consisted of an initial step of 98°C for 3 min, followed

by 25 cycles at 98°C for 45 s, 50°C for 30 s, and 72° for 45 s. A final elongation step of 5 min at 72°C was added to ensure complete amplification.

Amplicons were cleaned with Ampure XL (Agencourt, Beckman Coulter, USA). Amplicon DNA concentrations were determined using the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen, USA) according to the manufacturer's intructions.

A composite sample for sequencing was created by combining equimolar ratios of amplicons from the individual samples, followed by gel purification and ethanol precipitation as described by Costello *et al*. (2009) to remove any remaining contaminants and PCR artifacts. The samples, along with aliquots of the three sequencing primers, were subjected to sequencing by Illumina Genome Analyzer HiSeq 2000 (Illumina Inc., USA).

Sequencing runs were 100 nucleotide lengths and paired end reads. The criteria to select the reads for subsequent analysis were chosen as reported in the work by Caporaso *et al.* (2010a), in which a quality-filtering strategy was developed. Identification and relative abundance of reads were calculated from comparison of the reads to the Greengenes database (the greengenes reference collection) filtered at 99% identity using the Quantitative Insights Into Microbial Ecology (QIIME) software package (Caporaso *et al.,* 2010b).

Pyrosequencing of fungal community

PCR amplification of the ITS region of rDNA of genomic DNA extracted from samples was performed. The fungal-specific primer pairs ITS1F (Gardes and Bruns, 1993) and ITS4R (White *et al.*, 1990) with tag and adaptors sequences were use according to Amend *et al*. (2010). PCR reaction and 454 sequencing were performed according to these authors.

Microbial interaction test

To evaluate whether the presence of microorganisms is a consequence of interactions, the spot-on-lawn assay was employed for screening their ability to produce stimulatory or inhibitory substances to other microorganisms. Bacterial and yeast isolates were screened as described by Tong *et al*. (2010).

Bacteria and yeasts were grown overnight onto the appropriate media and according to optimal growth conditions. Once grown, test strains were resuspended in fresh medium and checked for optical density at 600 nm (OD_{600}). Each strain was diluted to an OD of 0.5. Trypticase soy agar (1.5%) was solidified in petri dishes of 120 mm in diameter, and the surface was spotted with 3 µl of each isolate to be tested, spaced approximately 3 cm apart.

The spots were allowed to diffuse into the agar and then incubated for 5-7 days at 20°C. Plates were prepared in duplicates.

Lawns of the isolates were prepared by inoculating about 10^7 cells in 6 ml of Trypticase soy broth soft-overlay (0.75% agar) medium at 50°C, and poured over the hard agar (1.5% agar) plates with the spots. Plates were incubated for 2 days at 20°C. After incubation, plates were checked for inhibition zones or stimulation of growth.

3.5.4 Results

Cheese rind chemical composition

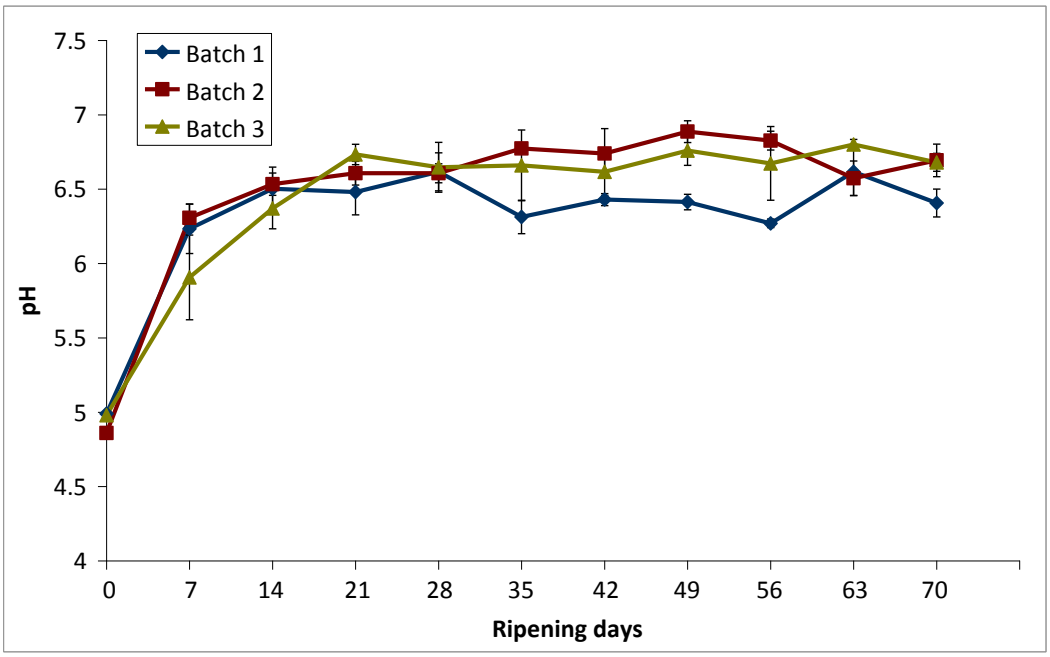
The pH and sodium chloride variations on the cheese surface during rind development are shown in Figures 1A and 1B. The surface deacidification occurred between 0 and 14 days. pH was about 4.9 at 0 days and at 14 days reached values of about 6.5, which remained almost constant throughout ripening. Sodium chloride decreased over time from 0.56-0.87 M at 7 days to 0.12 M at 63 days.

Bacterial and fungal community composition determined by high throughput sequencing (Illumina and 454 platforms) at the end of ripening (56 days).

16S rRNA gene amplicons for bacteria and ITS rDNA for fungi were evaluated by Illumina and 454 sequencing, respectively, in order to describe the bacterial and fungal communities on the cheese rind at 56 days of ripening. A total of ~10,000 and 8,246 reads was obtained for bacteria and fungi, respectively. The classification of sequences obtained from the 16S rDNA and ITS libraries are shown in Figure 2A and 2B, respectively.

The 16S rDNA Illumina library showed that the most abundant bacteria belonged to the genus *Staphylococcus* (57% of total reads) and the second most numerous corresponded to the *Brevibacterium* genus (25% of total reads). Sequences belonging to *Corynebacterium, Brachybacterium, Nocardiopsis*, *Cobetia*, *Psychrobacter*, *Halomonas* and *Haererehalobacter* genera were also observed at lower percentages (1-5% of total reads).

A.



B.

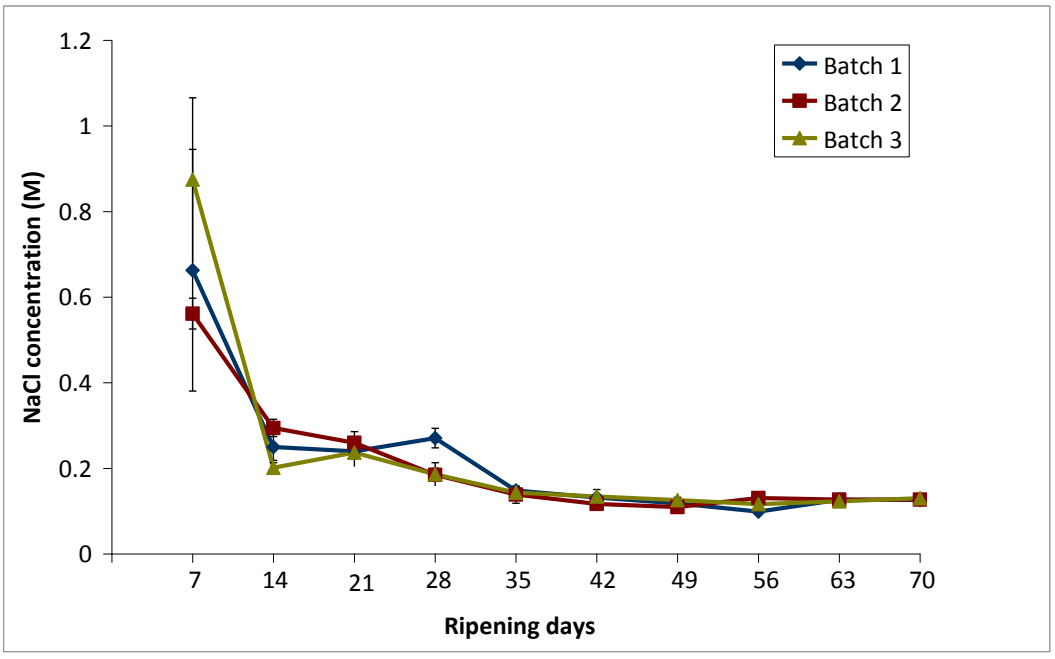


Figure 1. pH (A). and NaCl (B). variations on the surface of cheese during ripening in 3 batches. Three separated measurements (n = 3) were performed for mean and standard deviation (SD) calculation.

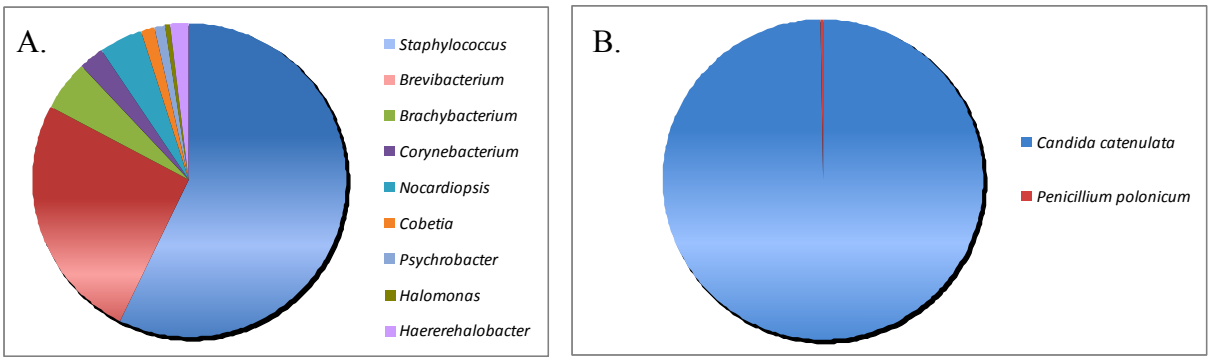


Figure 2. Phylogenetic classification of sequences obtained by high-throughput sequencing analysis in the cheese rind at 56 days of ripening. (A) Bacterial community evaluated by Illumina technology and (B) Fungal community evaluated by 454 sequencing. Each genus is expressed as relative abundance of the 16S rRNA gene sequences for bacteria and ITS region of rDNA for fungi.

The fungal library was obtained by 454 pyrosequencing analysis; almost all sequence reads (99%) belonged to the *Candida* genus and minor percentages (1% of total reads) corresponded to the *Penicillium* genus. All sequence reads of the genera *Candida* and *Penicillium* were found belonging to *Candida catenulata* and *Penicillium polonicum*, respectively.

Bacterial community composition and dynamics over time determined by Illumina sequencing

High-throughput 16S rRNA gene sequencing by Illumina technology was performed to describe the community structure during rind formation. Figure 3 shows representative pictures of a typical series of changes during rind formation. Pink color may be due to yeast colonies. Fuzzy white colonies of molds could be observed at 32 days.

An average of 10,000 sequences was analyzed per sample (i.e., at 10, 24, 40, 56, 71 and 86 days) and the average sequence length was 100 bp. Figure 4 and Table 1 show the temporal pattern of community development during rind formation. A succession of bacterial species was observed during ripening. The *Staphylococcus* genus was present during the overall ripening period comprehending 31.38-85.43% sequence reads of the 16S rDNA amplicons.

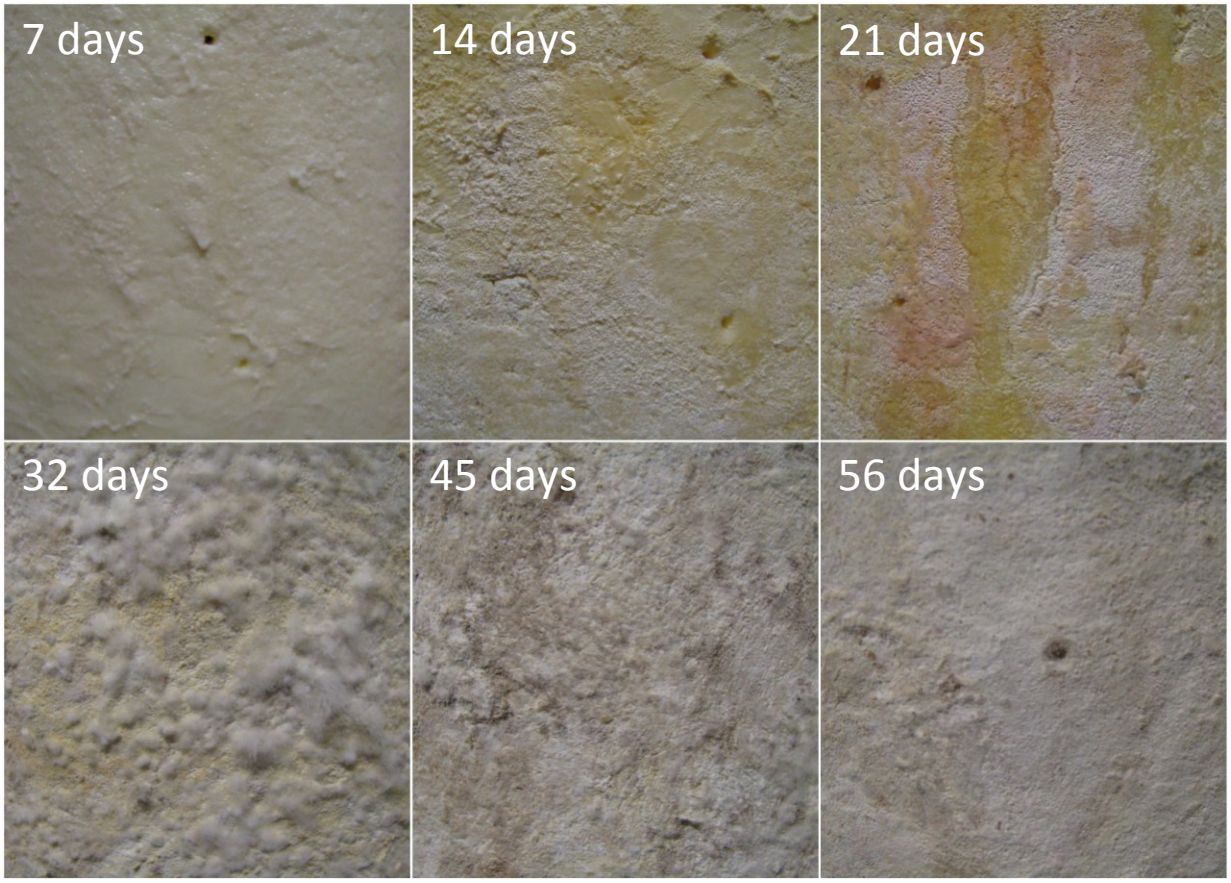


Figure 3. Representative pictures of a typical series of changes during rind formation

At the initial stages (10 days) and until 56 days of ripening, cheese rind was mainly dominated by the *Staphylococcus* genus. At the earliest stages of ripening (10 days), the occurrence of the S*taphylococcus* genus coexisted with a minor presence of *Lactococcus, Erwinia* and *Leuconostoc* genera. Less than 1% of sequence reads was identified as *Halomonas* and some Coryneform bacteria. At 24 days, the *Staphylococcus* genus showed a low decrease but subsequently, at 40 days, increased reaching maximum values of 85% of sequence reads. From 40 days until the end of ripening this genus showed a gradual decrease in abundance and *Brevibacterium* became the dominant member. Sequences of Coryneform bacteria such as *Brevibacterium*, *Corynebacterium* and *Brachybacterium* increased in abundance during ripening. In particular, *Brevibacterium* resulted the most numerous sequence at 86 days (52% of total reads of the 16S rDNA library). Sequences that belonged to the *Psychrobacter* genus were present from 40 days and increased until 71 days.

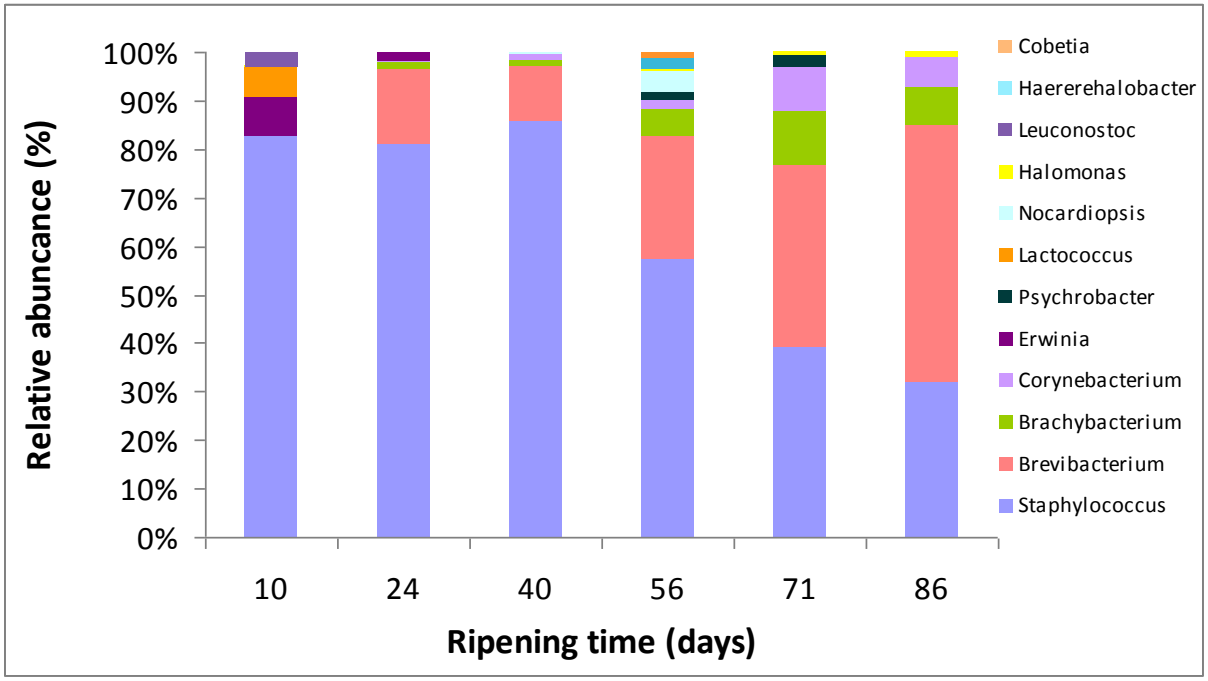


Figure 4. Comparison of the bacterial community structure of cheese rind during ripening determined by Illumina analysis.

		Relative abundance (%)					
		Ripening time (days)					
Phylum	Genus	10	24	40	56	71	86
Firmicutes	*Staphylococcus*	81.66	79.82	85.43	56.69	38.29	31.38
Firmicutes	*Lactococcus*	6.01	0.06	0.03	0.01	0.02	0.04
Firmicutes	*Leuconostoc*	3.01	0.05	0.01	0.01	0.02	0.01
Actinobacteria	*Brachybacterium*	0.04	1.54	1.02	5.14	10.69	7.78
Actinobacteria	*Corynebacterium*	0.02	0.30	1.61	2.35	9.07	5.97
Actinobacteria	*Brevibacterium*	0.23	15.01	11.38	25.36	37.23	52.26
Actinobacteria	*Nocardiopsis*			0.10	4.55	0.01	0.04
Proteobacteria	*Cobetia*				1.24		
Proteobacteria	*Psychrobacter*			0.03	1.15	1.99	
Proteobacteria	*Halomonas*	0.03	0.01	0.11	0.73	0.79	1.03
Proteobacteria	*Erwinia*	7.78	1.58	0.04		0.12	0.03
Proteobacteria	*Haererehalobacter*				1.79		

Table 1. Taxonomic affiliation of 16S rRNA gene amplicons during cheese rind formation. Percentages were calculated from comparison of the reads to the Greengenes database using the QIIME program.

Viable cheese rind community

Plate counting and identification of individual species by gene sequencing were performed to gain knowledge of the viable population contributing to cheese rind formation. During ripening at 10, 24, 40, 56, and 71 days, cultivable bacteria and yeasts were enumerated and recovered in generic media. The total aerobic bacterial counts in milk plate count agar (MPCA) at the earliest (10 days) and latest (71 days) stages of ripening were 7.68 and 10.62 log cfu/g of cheese rind, respectively (Table 2). For fungal population, yeast extract peptone and glucose (YPD) agar supplemented with chloramphenicol were used. Yeast counts ranged from 7.48 log cfu/g at 10 days to 10.11 log cfu/g of cheese rind at 56 days.

	total aerobic bacteria		yeasts	
time (days)	mean (log cfu/g)	SD (log cfu/g)	mean (log cfu/g)	SD (log cfu/g)
10	7.68	0.34	7.48	0.32
24	10.27	0.18	10.29	0.11
40	9.81	0.03	nd	nd
56	10.03	0.09	10.11	0.13
71	10.62	1.23	nd	nd

Table 2. Microbial enumeration by plate counting of bacteria and yeasts in cheese rind over time.

Three separated batches were enumerated (n = 3) for each time point for mean and standard deviation calculation. nd: not determined.

In order to describe the overall cultivable population, macroscopic and microscopic observations of colonies were performed. Thus, 59 different colony morphologies were distinguished in 138 colonies observed during rind formation. 46 colonies were bacterial cells, 7 colonies were yeast cells and 6 were filamentous fungi. Thus, the 59 colony morphotypes were isolated in the appropriate media and the bacterial and fungal species were identified by rDNA sequencing (Table 3). Species belonging to the *Staphylococcus* genus were the most frequently observed among isolates, and were identified as *Staphylococcus saprophyticus* (14% of total isolates), *Staphylococcus equorum* (14%), *Staphylococcus succinus* (7%) and *Staphylococcus xylosus* (7%). *Lactococcus lactis* was the most abundant lactic acid bacterium, with 12% of isolates, and *Leuconostoc mesenteroides* (7%) and species belonging to *Enterococcus* genus were also present. *Enterococcus* was identified as *Enterococcus faecalis* (8%) and *Enterococcus casseliflavus* (3%). The yeasts were also detected and comprised

Candida catenulata with 10% of the total isolates and a minority presence of *Debaryomyces hansenii* (2%). *Macrococcus caseolyticus* (3%) and *Klebsiella* sp. (3%) were observed less frequently among isolates. Among filamentous fungi, the most abundant species was *Penicillium commune* or *Penicillium camemberti* (5%), followed by *Penicillium aurantiogriseum/allii* (3%) and *Penicillium roqueforti* (2%).

Phyla	number of isolates (n = 59)	closest sequence relative (species)[a]	% identity	GenBank accession no.
Firmicutes	2	*Enterococcus casseliflavus*	99	AB547326.1
Firmicutes	5	*Enterococcus faecalis*	99	CP002621.1
Firmicutes	7	*Lactococcus lactis*	100	HQ721275.1
Firmicutes	4	*Leuconostoc mesenteroides*	99	JF261155.2
Firmicutes	2	*Macrococcus caseolyticus*	99	FJ263452.1
Firmicutes	8	*Staphylococcus equorum*	99	GU595329.1
Firmicutes	8	*Staphylococcus saprophyticus*	99	FJ210844.1
Firmicutes	4	*Staphylococcus succinus*	99	GU084442.1
Firmicutes	4	*Staphylococcus xylosus*	100	HM854231.1
Proteobacteria	2	*Klebsiella* sp.	99	AF129440.1
Ascomycota	6	*Candida catenulata*	99	GU246267.1
Ascomycota	1	*Debaryomyces hansenii*	99	GQ458041.1
Ascomycota	1	*Penicillium roqueforti*	99	AB479313.1
Ascomycota	2	*Penicillium aurantiogriseum/allii*	99	AY280957.1
Ascomycota	3	*Penicillium commune/camemberti*	99	GQ458026.1

Table 3. Identification by traditional 16S and ITS sequencing of bacteria, yeast and filamentous fungi isolated from cheese surface at different stages of ripening.

[a]Based on complete 16S rRNA and internal transcribed spacer (ITS) regions between the 18S and 28S rRNA genes.

Microbial interaction test

A spot-on-lawn assay was performed in order to determine whether interactions occurred among microorganisms. Thus, isolates from the natural rind throughout cheese ripening, particularly bacteria and yeasts, were used for the analysis. Table 4 summarizes interaction data. It was observed that *C. catenulata* spot inhibited *D. hansenii* highlighted by zones of inhibition (Figure 5). None of the bacteria was inhibitory to the yeasts *C. catenulata* and *D. hansenii*. Both *C. catenulata* and *D. hansenii* spots enhanced the growth of *S. equorum*. This was represented by both an increased number and largest colonies near the spot. One strain of

S. saprophyticus was inhibited by lactic acid bacteria, i.e., *E. casseliflavus, E. faecalis* and *L. lactis*. However, other two *S. saprophyticus* strains were not inhibited by LAB.

			Spot												
		Species	*C. catenulata*	*D. hansenii*	*E. casseliflavus*	*E. faecalis*	*L. lactis*	*L. mesenteroides*	*M. caseolyticus*	*S. equorum*	*S. saprophyticus 19*	*S. saprophyticus 34*	*S. succinus*	*S. saprophyticus 10*	*Klebsiella* sp.
Overlay	Ascomycota	*Candida catenulata*													
		Debaryomyces hansenii													
	Firmicutes	*Enterococcus casseliflavus*													
		Enterococcus faecalis													
		Lactococcus lactis													
		Leuconostoc mesenteroides													
		Macrococcus caseolyticus													
		Staphylococcus equorum													
		Staphylococcus saprophyticus 19													
		Staphylococcus saprophyticus 34													
		Staphylococcus succinus													
		Staphylococcus saprophyticus 10													
	Proteobacteria	*Klebsiella sp.*													

negative interaction
positive interaction
no interaction

Table 4. Interactions of bacteria and yeast isolates from the cheese rind.

To more appropriately screen yeast-yeast interactions, another spot-on-lawn experiment was performed evaluating the different strains isolated from the natural rind and from smear rind from another cheese (data not shown). Thus, for *C. catenulata*, the 6 different strains isolated from the natural rind and 3 strains isolated from the smear rind were used. For *D. hansenii*, 1 strain from the natural rind and 8 strains from the smear rind were used. For the experiment, the 9 strains of *C. catenulata* were spotted and were overlaid with each of the 9 strains of *D. hansenii*. The same approach was used for the 9 strains of *D. hansenii*. Figure 6 shows some representative pictures in which inhibition zones can be observed. On the *C. catenulata* spots there were zones of inhibition of *D. hansenii* growth that resulted to be of different intensities among strains. None of the *D. hansenii* strains inhibited *C. catenulata* (Figure 6 A).

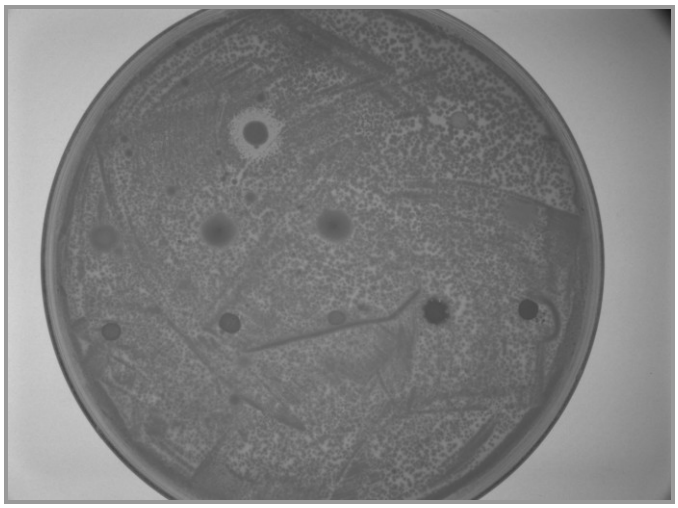


Figure 5. Image of a spot-on-lawn assay on a petri dish of 120 mm. Spots of different microorganisms (colonies) in an overlay of *D. hansenii* growth are shown. Inhibition halo of *D. hansenii* growth can be observed for the *C. catenulata* spot, whereas bacterial spots did not show any interaction.

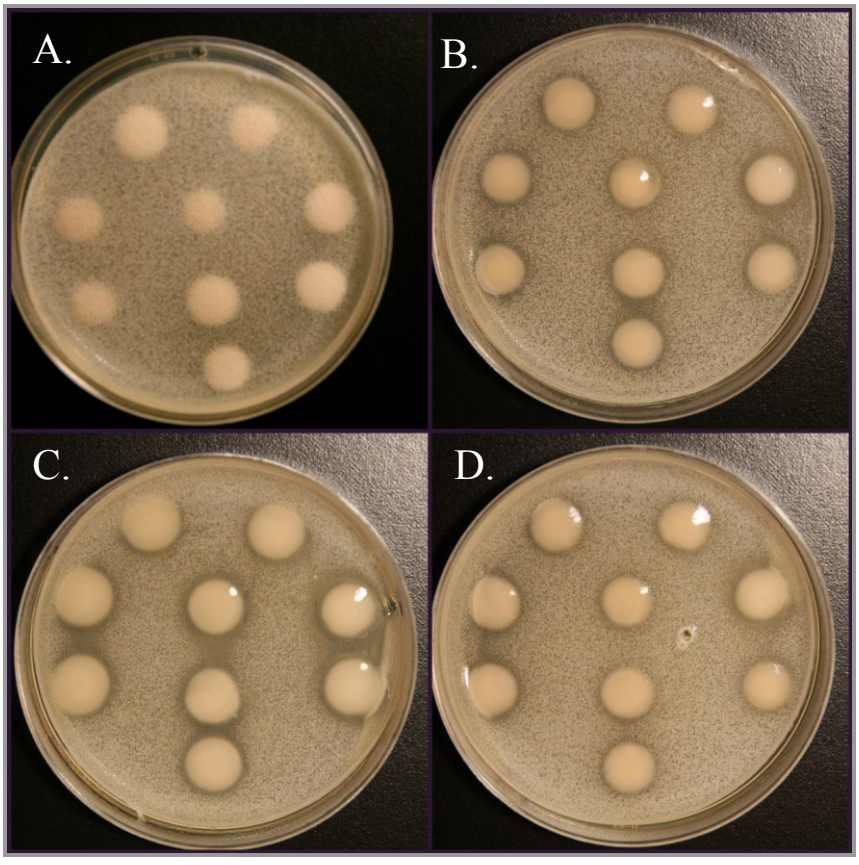


Figure 6. Image of a spot-on-lawn assay. (A). *D. hansenii* spots (9 strains) in an overlay of *C. catenulata*. (B)., (C). and (D): *C. catenulata* spots (9 strains) in an overlay of 3 *D. hansenii* strains. Inhibition halos of different intensities of *D. hansenii* growth can be observed among the different *Candida* strains.

3.5.5 Discussion

By means of high-throughput sequencing (Illumina and 454 sequencing), it was possible to examine the diversity of the microbial rind community at the end of ripening (56 days). PCR amplification of the 16S rRNA gene for bacteria and ITS region for fungi from bulk cheese rind DNA and subsequent sequencing by next-generation sequencing technology were performed. Bacterial and fungal populations were identified. In terms of diversity, it was observed that there was much greater diversity among bacteria than among fungi, particularly regarding *Staphylococcus* species. The most abundant component of the bacterial consortium was represented by the *Staphylococcus* genus while among yeasts and filamentous fungi *Candida catenulata* and *Penicillium commune*/*P. camemberti* were the most frequently observed, respectively. The contribution of each population (i.e. bacteria and fungi) could not be compared since separated sequencing experiments were performed. Only by plate counting it was possible to quantify and compare the contribution of each population. Further studies are currently focused on quantification of these two populations. Moreover, since bulk cheese rind DNA was analyzed, it was not possible to determine the viability of these organisms in the rind. Thus, in order to obtain more information regarding what species are actively present and to perform interaction studies that may explain the progression of species during rind formation, plate counting was performed and different representative colony morphologies were identified. In agreement with Illumina sequencing, by culturing and species identification, it was shown that the *Staphyloccus* genus dominates among isolates, in particular *S. saprophyticus* and *S. equorum* were detected as the dominant species and *S. succinus* and *S. xylosus* as minor components. Different origins could be attributed to these microorganisms. Staphylococci are found living naturally on the skin and mucous membranes of warm-blood animals and humans (Mounier *et al.,* 2006) but are also isolated from milk, cheese (Irlinger *et al*., 1997, Addis *et al*., 2001, Ercolini *et al.,* 2003; Rasolofo *et al*., 2010) and smear rinds (Brennan *et al.*, 2002; Mounier *et al.,* 2005, Rea *et al*., 2007). All strains recognized for their technological value and involved in desirable reactions (flavor and aroma formation) during cheese ripening are coagulase-negative staphylococci (Irlinger, 2008).

The fungal community evaluated by 454 sequencing of the cheese rind at the end of ripening was represented mainly by the yeast *C. catenulata* and, in minor abundance, by the filamentous fungi *P. polonicum. P. polonicum* is a common mold found on dry-cured meat products (Núñez *et al*., 2000). In agreement with 454 sequencing, a high number of isolates corresponding to viable cells of the yeast *C. catenulata* were found by culturing; moreover,

viable cells of *D. hansenii* were also isolated in minor abundance but were not detected by 454 sequencing at 56 days of ripening. This may suggest that viable *D. hansenii* was present at the beginning of the rind development and became undetectable during ripening. Since *D. hansenii* is well-adapted to grow under the rind environmental conditions (i.e. high NaCl concentration, high pH, presence of lactose and lactate) (Mounier *et al*., 2008; Dolci *et al*., 2009), we can hypothesize that this can be a consequence of some interactions among organisms causing inhibition. In fact, in this work, inhibition of the growth of *D. hansenii* by *C. catenulata* was observed by using a spot-on-lawn experiment and a different degree of inhibition was observed among strains. Yeasts are known to positively contribute to flavor and texture development in blue cheese (Roostita and Fleet, 1996). *Candida* spp. has been isolated in the core and surface of a Danish blue cheese (van den Tempel and Jakobsen, 1998) and *D. hansenii* was proved to have lipolytic activity and a weak proteolytic activity in blue cheese (van den Tempel and Jakobsen, 2000; Addis *et al*., 2001). Moreover, the yeasts are known to play a role in the deacidification of the cheese surface by oxidizing lactic acid to lactate, and that leads to the growth of acid-sensitive and salt-tolerant bacteria such as coagulase-negative staphylococci and coryneform bacteria. In smear cheese rinds, at the early stages of rind formation, the yeasts dominate, reaching high numbers (~6 log cfu/g) and then remained stable or decrease (Mounier *et al*., 2008; Cogan, 2011). The general trend is that the yeasts dominate during the early stages and are followed by bacterial domination at the end of ripening (reaching ~7-8 log cfu/g). The yeasts metabolize the lactic acid produced by starter LAB and together with NH_3 production from deamination of amino acids, cause the increase of pH on the surface where bacteria can grow (Brennan *et al.,* 2002). Differently from the trend in smear cheeses, in this work it was observed that bacterial and yeast populations developed at the same time from about 7 to 10 log cfu/g of cheese rind at the late stages of ripening. The pH variations on the surface showed an increase in the first 14 days. Particularly pronounced during the first 7 days, this result agrees with pH variations on the surface of smear cheese that registers a maximal increase during the first 6 days of ripening (Mounier *et al*., 2008). Thus, this deacidification of the cheese surface was highly correlated with the growth of bacteria and yeasts during the first 24 days. Since it was not possible to gain quantitative information about the contribution of bacterial and fungal species, real-time PCR experiments will be performed for quantification of bacterial and fungal communities during natural rind development.

Conidia of *P. roqueforti* were added to the cheese milk to confer the typical color and flavor to the cheese by lipolytic and proteolytic processes. This mold was isolated from the cheese surface, but it was not found by 454 sequencing at 56 days of ripening. This was not

surprising since the growth of *P. roqueforti* is inhibited by high salt concentration, being more concentrated near the surface of the cheese where the salting proceeds (Ardö, 2011). In this work, salt concentration on the rind was registered to be initially high, about 0.87 M, at 7 days and about 0.12 M at 56 days of ripening.

Lactic acid bacteria such as *Lactococcus lactis* and *Leuconostoc mesenteroides* were isolated from the rind. Since these species were used as starters cultures, they were probably recovered from the early stages of ripening when the rind is still developing and is basically curd, in which they are well-adapted to grow. The bacterial composition evaluated over time during natural rind development by Illumina sequencing confirmed this observation. *Lactococcus* and *Leuconostoc* were detected in rinds at 10 days and strongly decreased to very low abundances from 24 days of ripening. The high salt concentration (about 0.87 M), high pH (about pH 6.1), and the aerobic conditions on the rinds were not optimal for LAB growth. This result disagrees with what found by Dolci *et al.* (2009) who found high levels of LAB in smear rind cheese at 90 days of ripening. The analysis on Stilton cheese (a similar blue cheese) agrees with our observations; Ercolini *et al.* (2003) revealed the presence of different microenvironments based on the spatial distribution of bacteria and according to pH, oxygen and nutrient gradients in the core compared to the veins and the rind. These authors found *Leuconostoc* microcolonies distributed in the core, veins and rind; lactococci were found only in the internal part of the veins and core, whereas *Lactobacillus plantarum* was detected only underneath the surface. Gas production by *Leuconostoc* sp. is considered to be important for the subsequent development of *P. roqueforti* and hence good flavor (Robinson, 2002).

Subdominant species belonging to the non-starter LAB *Enterococcus* genus, such as *Enterococcus faecalis* and *Enterococcus casseliflavus*, were also isolated. These species are normally found in raw milk (Delbès *et al.,* 2007; Giannino *et al.,* 2009) and *Enterococcus* spp. was isolated at high levels from cheese cores in blue mold cheese made from raw milk (Ardö, 2011). Surprisingly, this genus was not revealed by Illumina sequencing during the overall rind development. Any important role of the non-starter LAB in blue cheese, however, was not demonstrated (Ardö, 2011).

In order to describe the temporal pattern of rind development during ripening, bacterial 16S rDNA was analyzed by Illumina sequencing. A microbial succession was observed and no genus remained constant throughout ripening. The progression of different bacterial genera on the cheese surface over time corresponded to variations in pH and NaCl concentrations during ripening. The increase of pH in the cheese surface during the first 2 weeks could have favored the growth of secondary bacterial species, such as staphylococci and coryneform bacteria.

Moreover, salt concentration was initially high and then drastically decreased at 14 and 21 days (to ~ 0.25 M); this could have consequences on the selection of salt-tolerant bacteria. In fact, between 10 and 24 days, the diversity changed considerably. LAB were greately reduced (as explained above) and *Brevibacterium* spp. and *Brachybacterium* spp. began to grow. Thus, *Staphylococcus* dominated the early stages and Coryneform bacteria, particularly the *Brevibacterium* genus, dominated later. Coryneform bacteria are known to play the most important role in surface-ripening, in particular, in smear cheeses (Dolci *et al.,* 2009). However, among all the bacteria found in the smear, only the role of *Brevibacterium linens* in ripening was studied in detail (Cogan, 2011). *B. linens* produces several proteinases, peptidases, and lipases, many of which have been purified and characterized, and are also involved in ripening (Cogan, 2011). In this work, *Corynebacterium* and *Brachybacterium* genera were also found and increased during ripening. Similarly, *Brachybacterium* spp. was detected on the surface of hard French cheeses (Schubert *et al*., 1996) and the *Corynebacterium* genus was detected in the core of Danish soft cheese at low percentages by the next-generation pyrosequencing technology at 14 and 56 days of ripening (Masoud *et al.,* 2011). Among coryneform bacteria, *Brevibacterium* dominated throughout ripening. Being the most abundant at the end of ripening, thorough studies has to be performed to understand whether these bacteria contribute to ripening in this cheese variety.

Some of the patterns observed in the succession may be a result of microbial interactions. Thus, in order to indentify some of these interactions, isolation of the individual species were subjected to interaction experiments (agar overlay technique). Similarly to the findings by Addis *et al*. (2001), in this work, yeast-yeast and yeast-bacterium interactions were observed. In the first case, *C. catenulata* inhibited *D. hansenii* and in the second case, *C. catenulata* and *D. hansenii* enhanced the growth of *S. equorum*. The negative interaction among yeasts could be responsible for absence of *D. hansenii* species detection during rind formation. Furthermore, growth factors produced by yeasts may explain the stimulation of *S. equorum* growth (Cogan, 2011). However, the mechanisms involved in these interactions remain unclear.

Understanding the nature and extent of these interactions within the cheese microbial community could lead to insights on how to manipulate the microorganisms involved, however, more studies have to be performed to discover the molecules involved in this interactions.

3.5.6 Conclusions

Combining the next-generation sequencing platforms and traditional culturing techniques, it was possible to describe in details the microbial community structure on the surface of a blue cheese made with raw cow milk and mesophilic LAB starters in which the rind naturally develops during ripening. These new sequencing platforms are powerful since allow not only to describe the community structure but also to evaluate the abundance of the community members, also considering the minor components.

The microbiota on the surface of the blue cheese during natural rind development showed microbial diversity comprising fourteen genera of bacteria (*Enterococcus; Lactococcus; Leuconostoc; Macrococcus; Staphylococcus; Klebsiella; Brevibacterium; Corynebacterium; Brachybacterium;, Nocardiopsis;, Cobetia; Psychrobacter; Halomonas*; *Haererehalobacter*), two yeast genera (*Candida; Debaryomyces*) and one mold genus (*Penicillium*). Bacterial succession was observed during rind formation and no genus remained constant throughout ripening. *Staphylococcus* dominated the early stages and then Coryneform bacteria the later stages. In particular, *Brevibacterium* was the most numerous genus at the end of ripening.

The species of the *Staphylococcus* genus were found to be *S. saprophyticus*, *S. equorum*, *S. succinus* and *S. xylosus*, while *C. catenulata* and *D. hansenii* were found among yeasts. *Penicillium* genus comprised *P. commune*/*P. camemberti, P. aurantiogriseum/allii, P. roqueforti* and *P. polonicum*. Furthermore, high and comparable densities of viable bacteria and yeasts were observed. Inhibition and stimulation have been observed among several species that could explain some contributions to community formation.

This natural rind was formed by natural microbial colonization through the contact with the environment during salting and ripening for 71 days. It can be considered an active rind formed by a rich and dense microbial consortium. Thorough studies have to be performed in order to evaluate whether these bacteria, yeasts and molds play a role in the cheese ripening process. In this blue cheese with natural rind, this contribution may hardly be observed because the ripening process proceeds mainly from the interior by the proteolytic and lipolytic activities of *P. roqueforti*. However, other cheese varieties could be benefited by this natural microbiota.

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4. GENERAL CONCLUSIONS

Cheese is a biologically and biochemically dynamic food in which the microbial population structure changes under the influence of continuous shifts in environmental conditions and interactions among microorganisms during manufacturing and ripening. In cheese manufacturing, the selection of technological parameters can influence and even induce several biochemical processes needed for this product. The microbiota present in cheese is complex and their growth and activity represent the most important, but the least controllable steps. Cheese microorganisms can be either deliberately added as starters or simply adventitious contaminants, that is non-starter organisms. In this PhD thesis different ecosystems such as natural whey starters, hard Italian cheeses (i.e., Grana Padano and Parmigiano Reggiano) and natural rind of mold-ripened cheese (i.e., blue cheese), were investigated following cheese manufacturing and ripening by culture-dependent and culture-independent techniques.

In this PhD thesis, by studying microbiota in natural whey and in the cheese since the first days of manufacture, it was possible to describe starter lactic acid bacteria (SLAB) fermentative activity determining the ripening progress of the cheese. Whey starter titratable acidity did not seem to be related to the cell amount (total and cultivable cells) nor to the different species contribution. It was found that the SLAB microbiota in the whey ecosystem did not necessary behave on the same way on the cheese ecosystem. In fact, a direct correlation between the SLAB species composition and acidifying efficacy in whey starters was not found. Contrarily, in the cheese matrix, the SLAB fermentative activity seemed to be species-dependent. High concentrations of lactic acid and free amino acids were found in cheeses with higher levels of *L. helveticus* species and cultivable thermophilic bacterial densities. The presence of residual galactose was associated to higher contents of *S. thermophilus* species. In this sense, despite their presence is not necessarily related with their development in the curd, the significance of *S. thermophilus* in the whey starter has to be reconsidered. In particular, being whey starter a natural mix of LAB strains, the occurrence of *S. thermophilus* strains lacking in β-galactosidase is likely undesired. Finally, the biotypes composition of whey starters seems to be far more important than the species composition in insuring their good performances.

Hard cheese resulted a useful system to study the LAB population dynamics in one environment characterized mainly by the absence of sugar since the first months of ripening. In these ecosystem, both SLAB and non-starter LAB (NSLAB) seemed to contribute to

acidification and ripening. However, SLAB, and in particular *L. helveticus*, resulted to be the species mainly subjected to the lysis occurring at 2 months of ripening. NSLAB are the lactic microflora not involved in curd acidification and, generally naturally present in cheese matrix. NSLAB were able to grow after brining, and became more relevant during ripening. They could arise both from the raw milk and the natural whey starter but their contribution to the development of cheese characteristics is still unknown. Furthermore, the presence of *L. helveticus* and *L. delbrueckii* subsp. *lactis* in a non-cultivable state, up to 13-month of ripening, suggested these species could play a different but still unknown role in cheese ripening.

Differently from PR and GP, in which ripening is mainly due to the microorganisms that are in the core, in other cheese varieties, a complex microbiota develops on the cheese surface naturally (i.e., Stilton blue cheese) and could contribute to ripening. The importance of this natural microbiota in defining cheese features, is still under study. Moreover, little is know about the composition and diversity of this wide microbial population that could also be involved in flavor formation. Thus, the cheese surface was elected as a suitable ecosystem to study what adventitious microorganisms, differently from the starters, bring to cheese ripening. However, the species contribution is difficult to determine. The microbiota on the surface of a blue cheese during natural rind development showed a microbial diversity comprising fourteen genera of bacteria (*Enterococcus; Lactococcus; Leuconostoc; Macrococcus; Staphylococcus; Klebsiella; Brevibacterium; Corynebacterium; Brachybacterium;, Nocardiopsis;, Cobetia; Psychrobacter; Halomonas*; *Haererehalobacter*), two yeasts genera (*Candida; Debaryomyces*) and one filamentous fungal genus (*Penicillium*). High and comparable densities of viable bacteria and yeasts were observed. Bacterial succession was observed during rind formation and no genus remained constant throughout ripening. The *Staphylococcus* genus dominated the early stages and then was replaced by *Brevibacterium* at the end of ripening. By using interaction experiments, inhibition and stimulation were observed among several species; these interactions could explain how some microorganisms contribute to community formation. *Candida catenulata* and *Debaryomyces hansenii* enhanced the growth of *Staphylococcus equorum* while *C. catenulata* inhibited *D. hansenii* growth. However, thorough studies need to be performed in order to evaluate whether these bacteria, yeast and molds can be beneficial and play a role in flavor and texture development of other cheese varieties with similar natural rinds.

By these complementary approaches it was possible to describe which microorganisms were mainly involved in each dairy matrix and to address the importance of their presence, that if balanced, can help obtaining the distinctive features of each product.

5. CURRICULUM VITAE

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Education

2009 – 2011 University of Parma, Parma, Italy.
Ph.D. in **Food Science and Technology**. Grant by the Italian Government.
1997 – 2003 National University of Litoral, Santa Fe, Argentina.
M.Sc. in **Biotechnology**

Experience abroad

March – September, 2011 **Research at Harvard University** - FAS Center for Systems Biology, Cambridge, MA, USA.
August – December, 2002 **Research at Federal University of Rio Grande do Sul** – Study grant at Laboratory of Virology, Center of Biotechnology, Porto Alegre, Brazil.

Conferences

- **II° National convention SIMTREA**. Role of microbiology in agriculture, agri-food and environmental fields. University of Sassari. 10-12 June 2009. Sassari, Italy.
  -Poster: Whey starter for Parmigiano Reggiano: culture-independent approach.
- **14th workshop on developments in the Italian PhD research on food science technology and biotechnology.** 16-18 September 2009. Oristano, Italy.
  - Poster: Detection and evaluation of viability and activity of microorganisms in food by using culture-independent approaches.
- **22nd International ICFMH Symposium Food Micro 2010**. 30 August – 3 September 2010. Copenhagen, Denmark.
  - Poster: Dynamics of microbial communities in Grana Padano cheese
- **15th workshop on the on developments in the Italian PhD research on food science technology and biotechnology**. 15 – 17 September 2010. Naples, Italy.
  - Poster: Survey on community and dynamics of lactic acid bacteria in Italian PDO hard cheeses.
- **AItel II Dairy Congress**. 21 September 2010. Turin, Italy.
  - Poster: Dynamics of microbial populations in Grana Padano cheese. Award of originality and scientific validity of research.
- **XII Argentinian Congress of Microbiology.** 17-20 October 2010. Buenos Aires, Argentina.
  - Oral presentation: Evaluation of microbial population dynamics during Grana Padano Italian cheese manufacturing and ripening.
- **American Cheese Society Conference**. 3-6 August 2011, Montreal, Canada.
- **SIMTREA.** Microbial diversity, environmental stress and adaptation, 26-28 October 2011. Milan, Italy.

Publications

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Gatti M., Lazzi C., Bernini V., De Dea Lindner J., Bottari B., **Santarelli M.**, Turroni F., Muchetti G. and Neviani E. (2006). Un sistema modello per prevedere l'attività amminopeptidasica dei batteri lattici durante la maturazione del Parmigiano-Reggiano. *Scienza e Tecnica Lattiero-Casearia,* 57: 407-419.

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