Chapter 2
Dairy Products

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Abstract Approaches for studying microorganisms in food have undoubtedly changed. Advances in molecular biology have provided more information on food-associated bacteria, and have also provided the scientific community with sound, reliable and effective methods for detection, identification and typing of microorganisms from food. The main interest of dairy microbiologists is to study the diversity and dynamics of microorganisms in dairy productions and, possibly, to correlate the occurrence of certain microbial species and strains with desired flavor and sensorial traits of the products. Various molecular methods can be used depending on the level of information required by research. Microbiologists can be interested in identification, detection or typing of bacteria from a certain environment. Identification and detection can benefit from the availability of both culture-dependent and culture-independent techniques, whereas typing is an analysis performed on isolates and is, thus, strictly related to culture-dependent methods. The aim of this chapter is to describe how dairy microbiologists have made use of such advanced techniques to provide new insights in the study of the microbial ecology associated to dairy fermentation.

1 Diversity and Microbiological Aspects of Dairy Products

1.1 Introduction

Fermented dairy products are an important part of traditional diet, although their production/consumption is more common in some countries than others. This is clear from cheese databases (*http://www.indexmundi.com*) or from consumption data of fermented milks reported by Tamine and Robinson (1999).

Notoriously, they include a very wide variety of products obtained from milk by means of different combinations of fermentation and other biochemical activities with different technological interventions. Product diversity may be due to chemical

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composition (mainly as moisture, fat and protein contents), texture, taste and aroma, as well as, quite typically, shape and size no less than final appearance. In the case of fermented milks, their diversity allows three great categories to be discriminated on the basis of the microbial activities typically occurring during their preparation: i) "Acidic," such as Yogurt and Yakult; ii) "Acid-alcoholic," such as Kefir and Koumys, and iii) "Viscous acid-alcoholic," such as Scandinavian fermented milks.

Oberman and Libudzisz (1998) report an interesting classification of fermented milks into four types characterized by the microbial cultures used for their preparation. The first type gathers those produced using lactococci and leuconostocs, as is the case of Scandinavian fermented milks. The second, represented by Yakult, is produced using *Lactobacillus* strains. The third type is produced using cultures of thermophilic streptococci and lactobacilli – typically Yogurt. Finally the fourth type, comprising Kefir and Koumys, characterized by mixed microbial populations of lactic acid bacteria, yeasts, micrococci and acetic acid bacteria.

In contrast cheeses are usually classified according to criteria that rarely take account of microbial content and/or activities in each product: the milk species (goat, sheep, buffalo) is mentioned when milk other than from cow is used; according to their texture, cheeses are qualified as hard, semi-hard or soft. In some cases the time required for cheese making up to suitability for best consumption is taken into consideration, speaking of "fresh" or "unripened," and "ripened" cheese; special categories are commonly recognized, such as "pasta filata cheese," "blue-veined," and "smeared." Fox and McSweeney (2004) reported a long list of voluminous scientific literature, encyclopedias, pictorial books, country-specific or variety-specific books on cheese. Here it may be useful to recall that Ottogalli (2001) proposed an intriguing cheese classification by first distinguishing "*lacticinia*" (obtained from milk, buttermilk, cream or whey promoting protein clotting without the use of enzymes, but through biological acidification, addition of lactic or citric acid, or by means of combined action of acid with heat; and represented, as main products, by Ricotta cheese, Queso blanco, Mascarpone) from "*formatica*" (true cheeses, obtained after milk clotting with animal, plant or microbial rennet, followed by whey draining). The latter are further split into six classes. Different classification schemes for cheese were reported by McSweeney, et al. (2004), none of them considering the microbial diversity characterizing different types of cheese. Mucchetti and Neviani (2006) have recently listed cheeses according to the following categories: i) cheeses produced with pasteurized milk and selected starter; ii) cheeses produced with pasteurized milk and natural starter; iii) cheeses produced with thermal treated milk and natural starter; iv) cheeses produced with raw milk and selected starter; v) cheeses produced with raw milk and natural starter, and vi) cheeses produced with raw milk without starter addition.

Actually, as well pointed out by Johnson (1998), the diversity of cheese making processes makes cheese a complex subject microbiologically; according to Johnson (1998) it is a misconception to think of cheese microflora in terms of the type of cheese, for example, all cheddars, blue cheeses, and so on. He emphasizes the occurrence of adventitious, non-starter, non-deliberately added contaminants that

can really cause each individual cheese (not type) to have its own unique microflora. He also considers conventional methods (both those currently used to isolate microorganisms and those to differentiate the isolates by biochemical tests) as unsatisfactory for studying a dynamic ecological system such as cheese microflora, and applications of molecular techniques like PCR (with reference, of course, to the time he was writing), just useful to determine the presence of individual species or strain, offering the possibility to identify the proverbial "needle-in-a-haystack."

Today we can assume that modern molecular methods of analysis within polyphasic approaches are available to obtain information about microorganisms occurring in the various dairy products; also with a view to discriminating species whose cells are viable at the moment of the analysis from those formerly present, but no longer active, due to one or more factors: technological stress, depletion of specific nutrients, microbial antagonism, modified adverse environmental conditions occurring during cheese manufacture and ripening. Beresford, et al. (2001) cited an interesting review by O'Sullivan (1999) to maintain that the development of culture-independent methods for microbial analysis has revolutionized microbial ecology. Advanced procedures have also been implemented to establish the location of specific microorganisms in particular parts of the product sample (Ercolini, et al. 2003a,b). A recent review by Spiegelman, et al. (2005) discussed the most powerful methods exploitable in environmental microbiology for the characterization of microbial consortia and communities. Zhang and Fang (2006) critically reviewed emerging techniques involving real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Friedrich and Lenke (2006) applied multiplex quantitative Real-Time PCR (q-PCR) and flow cytometry-FISH to enumeration of lactic acid bacteria (LAB) in a mesophilic dairy starter culture. Various approaches to studying gene expression in complex environments are also available (Saleh-Lakha, et al. 2005). Significant applications showing technical aspects, potentials and limits of these methods will be discussed in this chapter to draw special attention to those that can be particularly useful in studying the microbial ecology of cheese.

The study of microbial ecology associated with dairy fermentation is fundamental to understand the bases of important traits of dairy products. Interestingly, microbiological aspects are not usually taken into account in cheese classification systems.

The microbiota of each dairy product (as well as, of course, each fermented food) has its own history, during which the microbial population structure changes under the influence of continuous shifts in environmental factors occurring during its preparation. Therefore, microorganisms, at species and strain level, must be monitored at least during the most effective technological phases, where it is important to have certain microbial activities in order to achieve the expected quality of the final product. Changes in the microbial community during the various phases of dairy production are particularly important for achieving a satisfactory description of the microflora occurring, especially in typical products obtained by traditional procedures. This could help in understanding the basis for specific sensorial traits and/or their seasonal variations. In the case of PDO (Protected Denomination of Origin) cheeses, it would be important to recognize an association between microbial

diversity and the area of production that may enhance the link between microbiota, the environment and sensorial quality of these traditional productions.

A satisfactory study of the microbiology of dairy fermentations must also examine all the important technological phases of production. Therefore, the significant steps of cheese production will be described below with particular attention to their possible influence on the microbiota of cheese.

1.2 Technological Production Phases of Dairy Products

Milk Pre-treatment and Standardization

Although most industrial dairy products are produced from pasteurized milk, a large number of raw milk cheeses are increasingly described as celebrated traditional on-farm-made cheeses and commercially proposed as gastronomic specialities, emphasizing their distinctive flavor and suggesting the best way to consume them. In the microbiological literature these types of cheese are attracting increasing coverage, showing once more the importance of the biodiversity of raw milk native microflora to achieve the roundest, most pleasant and palatable traits. Studies are needed on molecular methods for monitoring both useful and dangerous microorganisms during practices performed in raw milk cheese making, such as milk storage/ripening (usually at a low temperature) or milk skimming for reducing the fat content. Indeed, cold storage is known to cause physical and chemical changes to milk, and to be selective for the development of psychrotrophic microflora. Great interest is merited by the case of partial milk-skimming by natural creaming typically performed within the manufacture of famous Italian semi-fat hard and long-ripened cheeses (Parmigiano-Reggiano and Grana Padano), promoting fat floating to the surface of the raw milk contained in wide trays for as long as six to 12 hours. Within this singular and uncommon practice, fat droplets appearing on the surface lead to microbial enrichment of the cream and a reduction in the microbial content of the skimmed milk lying below (Mucchetti and Neviani 2006).

Starter Addition

For starter cultures, a particularly valid nomenclature is proposed by Limosowtin, et al. (1996), who define “Mixed Strain Starters” (MSS) as the cultures that include many species and strains in unknown proportions, and “Defined Strain Starters” (DSS) as those containing known quantities of known strains. Within the first category, the authors list “Artisanal or Natural Mixed Starter Cultures” (NMSS), “Thermophilic Mixed Starter Cultures” (TMSS) and “Mesophilic Mixed Starter Cultures” (MMSS); within the second (DSS) “Thermophilic Defined Starter Cultures” (TDSS) and “Mesophilic Defined Starter Cultures” (MDSS). It can be assumed that most DSS, as

well as many single strain starters, are commercial starters produced by specialized laboratories; MSS – mainly NMSS – are prepared and managed by the dairies, often following traditional procedures that, for the same type of cheese, can differ somewhat among the various manufacturers. Many cheeses are traditionally produced without starter addition nor by using back-slopping practices. Natural whey cultures are microbial cultures naturally occurring in the whey extracted in previous cheese making, stored at room temperature or variously handled, and then used in the manufacture of the following day with a back-slopping practice. Instead, natural artisanal milk cultures are generally obtained by incubating, at appropriate temperatures, a large amount of raw milk after mild heating to destroy undesirable microflora. Indeed, they commonly contain strains of thermoduric microorganisms, such as thermobacteria, thermophilic streptococci and enterococci. Moreover, the dairy industry can benefit from the use of other microbial cultures called "adjuncts;" they are not essential for the technological process in itself, but are selected and used with specific additional purposes. They can be "reinforcing cultures" to be used to accelerate or standardize acidification; "flavoring cultures" to enhance aroma production; "protective cultures" to inhibit pathogens or spoilage agents, or "health cultures" to enrich the product with probiotic strains.

The addition of starters and/or adjuncts to cheese milk causes an immediate change in the microbiota of the technological ecosystem concerned, as loads of at least 10^6 cells per ml of milk of each important microbial type are applied. Thus, the structure of the population under investigation is strongly – and often permanently – influenced after the inoculum.

Milk Clotting

Rennet-coagulated cheeses represent, by far, the greater part of solid dairy products. Liquid, powder or tablet rennets are usually special preparations with little to no significant microbial content. Among traditional cheeses a number of more or less long ripened products are manufactured by the use of a rough type of rennet prepared in the form of a paste from the fourth stomach (abomasum) of suckling goat kid or lambkin. These rennets, in addition to chymosin and other proteases, contain some lipases that, during cheese ripening, are responsible for reactions that produce a distinct piquant taste. Several studies have shown that these rennet pastes might have a microbial content, representing a special additional source of microorganisms for the cheese (P. Deiana, personal communication). Modern research on the microbial content of raw rennet is, to our knowledge, nonexistent and no information is available about the possible presence of stressed or unculturable microorganisms.

In any case, depending on the type of cheese, coagulation time is variable, generally predefined through the quantity and quality (clotting strength) of the rennet. Milk fat droplets, whey with water-soluble components and microbial cells are entrapped within the casein network, e.g. inside the pores among aggregates of micelles. Microbial growth may also occur, of course, in a good nutritional environment and with favorable temperatures, no longer as planktonic cells but growing as colonies

in a solid matrix. Sampling at this stage of cheese making may be of interest for chemical investigation. However, microbial populations at the end of the clotting process cannot yet be referred to cheese, due to the fact that part of the microorganisms entrapped in the coagulum will be eliminated from the system by subsequent technological phases.

Curd Cutting, Cooking and Draining

Once the coagulum firmness required for the specific cheese variety has been reached, the curd is cut with knives or wire-tools into small pieces. Cut size also strongly depends on the cheese type, as the firmer and larger the curd pieces, the higher the moisture content of the cheese. According to the Italian dairy tradition, curd piece size is typically named after fruits or seeds of similar size: walnut, little walnut, almond, hazelnut, pea, small pea and grain. Long ripening cheeses require curd cutting at the smallest size (grain); fresh or brief-ripening cheeses at the largest one (walnut). With cutting, caseins continue to interact and squeeze out the whey entrapped (with all its water-soluble components, lactose included) and some microbial cells as well (more cocci than rods). Curd pieces shrink, become firmer and, depending again on the cheese variety, they can be differently processed. Syneresis may be enhanced by lowering the pH (hence, counting on starter effectiveness), increasing the temperature and stirring the curd (performing, in this case, the process of curd cooking). Alternatively, the curd pieces can be promptly separated from the whey, drained and subjected to the subsequent technological phase of molding. However, curd treatments are always ecologically important, involving selective pressures with major effects upon microorganisms and their activities.

Molding

Molding can be ecologically important due to the possibility of contamination occurring during curd handling. It is generally recognized that chemical cleaning and sanitation of tanks, vats and other tools used within cheese making – made of proper modern materials – can reduce contamination considerably, whereas it is more difficult to achieve satisfactory results when the cheese is exposed to the work environment. This is considered the main source of adventitious cheese microflora, commonly including non-starter LAB, and is responsible for important activities during cheese ripening. Molding is, therefore, another stage of cheese making during which the cheese microflora can change.

Salt Addition

Salt is added in cheese making to improve its taste and to lower the water activity. In some cases it is added as a solid to milled curd; in others, cheeses removed from

the forms are brined, and in further cases the cheese molds are dry salted by rubbing or sprinkling salt on their surfaces.

Salting causes selective pressure upon microorganisms. Brines are often a source of cheese contamination by salt-tolerant microorganisms: yeasts such as *Debaryomyces hansenii* and its imperfect form *Candida famata*; bacteria such as staphylococci, micrococci, enterococci, corynebacteria and some LAB.

Ripening

It is well-known that cheese ripening occurs in a variety of environmental conditions, depending on cheese type, and often in natural or cellar conditions, where it is uncontrolled and difficult to reproduce. Moreover, this final process of cheese making is difficult to describe because it consists of a complex succession of events conditioned by the previous technological stages, with the contribution of secondary adventitious microflora, under the influence of the cheese storage environment and, in some cases, caused by curing practices. Then, further complication may be encountered in some cases, where ripening proceeds without heterogeneity within the same cheese mold: blue-veined cheeses include portions strongly affected by both growth and activity of *Penicillium roqueforti*; surface-ripened cheeses are characterized by centripetal ripening due to diffusion of enzymes produced by the surface microflora. Such complexity requires polyphasic analytical approaches including physical, chemiometric, molecular and cultural procedures to be performed on several samples from various parts of the same cheese mold.

1.3 Microbial Diversity in Dairy Products

For about one decade, studies of the microbial ecology of cheese have focused on explaining the relationship between microbial population succession (in terms of implantation/growth/colonization), enzyme production/activity (with reference to milk and rennet enzymes too, in addition to those of a microbial origin) and cheese texture, taste and flavor development. Great emphasis has been given to the importance of microbial diversity and the role of non-starter microorganisms. Nevertheless, there is scant information, both in terms of quality and quantity, to be used for total technological control/management of cheese quality, or for producing pasteurized milk cheese with flavor resembling raw milk cheese.

Therefore, given that molecular techniques can quantify both microbial species that can be encountered, targeting rRNA genes and their activity, and evaluating the expression of functional genes according to methods already applied to the study of natural environments (Saleh-Lakha, et al. 2005), it may be worth briefly recalling microorganisms and metabolic activities that are expected during the most important above-mentioned phases of cheese making.

In evaluating cheese milk quality, species- or biotype-specific DNA sequences can be targeted for detection of pathogenic microorganisms. Therefore, sequences of genes encoding for toxins can be used both to detect the producer's occurrence and ascertain the specific gene expression during milk cold-storage, ripening or partial skimming. During these pre-treatments, microbiological investigations should also deal with monitoring the effective growth of useful microflora in comparison with that of psychrotrophic flora regarded as spoilage agents. Of the latter, *Pseudomonas* spp., *Alteromonas putrefaciens, Alcaligenes faecalis, Arthrobacter globiformis, Serratia* spp., *Enterobacter* spp. and *Flavobacterium* spp. may be responsible for anti-technological activities: undesirable proteolytic and lipolytic activities, ammonia production, diacetyl mineralization and production of many off-flavors, like fruity, sweetish, fecal or putrid.

Within production of long-ripened cheese, the presence of the anaerobic spore-former *Clostridium tyrobutyricum* – considered the causative agent of late blowing spoilage – can be verified in cheese-milk by species-specific PCR according to Klijn, et al. (1995a) or by PCR-DGGE (Cocolin, et al. 2004).

In studying microbial ecology during cheese manufacture, microorganisms need to be monitored with reference to their taxonomic and metabolic diversity to be consistent with technological protocol. Of course, mixed and more complex microflora must be expected in raw milk cheeses and when natural starter cultures are used. In these cases, in fact, the acidification process relies on the indigenous microflora, usually taxonomically complex. Considering the temperature ranges suitable for the various LAB groups, both thermophilic and mesophilic species can occur. The most important species of the microflora occurring during the manufacture of the main types of cheese are reported in Table 2.1.

Only residual amounts of lactose are generally available at the end of cheese manufacture. Thus, the initially dominant starter microorganisms responsible for acidification and flavor production from lactose and citrate during the first phase of cheese making are destined to progressively decrease from the phase of cheese ripening. Thereafter, depending on the cheese variety, secondary and/or adventitious microflora begin their growth, carrying out their activities. The latter involve both metabolites formerly produced by starter bacteria and the other constituents of cheese curd, like proteins and fat, generally transformed to a lesser extent within the first stage of cheese making. Moreover, they complement biochemical activities arising from residual milk plasmin, rennet enzymes and starter cell autolysis. However, secondary and adventitious microflora should not be regarded as agents of single specific processes, but as contributors to the overall complex transformations influencing the final cheese quality.

Milk proteins and lipid metabolism by dairy bacteria, yeast and molds will release key flavor molecules that will characterize each kind of cheese. Such compounds are the result of the diversity of the microrganisms and their activities during cheese ripening.

Microbial ecology of cheese, as well as of other fermented dairy products, can greatly benefit from molecular tools supporting identification of microbial species and strains occurring during the production processes, discriminating and quantifying

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type

Taxon	Products
Starter Microorganisms (mainly responsible for production of lactic acid, ethanol, acetaldehyde and diacetyl)*	
Lactococcus lactis subsp. *lactis*	Viili **(1); Kefir (1); Cheddar (3); Edam-Gouda (4); Cottage cheese (6); Camembert (3); Brie (4);Blue-veined cheeses (3); Limburger and other surface-ripened cheeses (6); Artisanal Mozzarella (16); Water buffalo Mozzarella (8); Artisanal Emmenthal (9); Pecorino Sardo (9); Majorero (9); Fior di Latte di Agerola (10); Caciocavallo Silano (18); Canestrato Pugliese (22); Salers (25); Toma Piemontese (29); Fiore Sardo (30); Stilton (31); Ragusano (32)
Lactococcus lactis subsp. *lactis* biovar *diacetylactis*	Viili (1); Kefir (1); Blue-veined cheeses (3); Limburger and other surface-ripened cheeses (6); Water buffalo Mozzarella (8)
Lactococcus lactis subsp. *cremoris*	Viili** (1); Kefir (1); Cheddar (3); Edam-Gouda (4); Cottage cheese (6); Camembert (3); Brie (4); Blue-veined cheeses (3); Limburger and other surface-ripened cheeses (6); Water buffalo Mozzarella (8); Artisanal Emmenthal (9); Pecorino Sardo (9); Majorero (9); Canestrato Pugliese (22); Toma Piemontese (29)
Streptococcus salivarius	Salers (25)
Streptococcus thermophilus	Kefir (1); Yogurt (2); Gorgonzola (4); Brie (4); Emmenthal (4); Artisanal Emmenthal (9); Mozzarella (4); Artisanal Mozzarella (16); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Grana Padano (12); Parmigiano Reggiano (13); Pecorino Romano (14); Montasio (14); Provolone (14); Asiago (21); Scamorza Altamurana(23); Toma Piemontese (29); Ragusano (32)
Streotococcus macedonicus	Kasseri (19); Asiago (20); Montasio (20); Scamorza Altamurana(23); Salers (25); Toma Piemontese (29)
Enterococcus faecium	Artisanal Emmenthal (9); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Pecorino Sardo (9); Majorero (9); Caciocavallo Silano (18); Canestrato Pugliese (22); Salers (25); Fiore Sardo (27); Toma Piemontese (29)
Enterococcus faecalis	Artisanal Emmenthal (9); Artisanal Mozzarella (16); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Pecorino Sardo (9); Majorero (9); Caciocavallo Silano (18); Canestrato Pugliese (22); Salers (25); Fiore Sardo (27); Toma Piemontese (29); Ragusano (32)
Enterococcus durans	Scamorza Altamurana(23); Fiore Sardo (27); Toma Piemontese (29)
Leuconostoc lactis	Water buffalo Mozzarella (8); Ragusano (32)
Leuconostoc mesenteroides subsp. *mesenteroides*	Artisanal Emmenthal (9); Artisanal Mozzarella (16); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10);Pecorino Sardo (9); Majorero (9); Salers (25); Ragusano (32)

(continued)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
Leuconostoc mesenteroides subsp. *dextranicum*	Kefir (1); Water buffalo Mozzarella (8)
Leuconostoc mesenteroides subsp. *cremoris*	Viili (1); Kefir (1); Water buffalo Mozzarella (8)
Leuconostoc pseudomesenteroides	Salers (25);
Leuconostoc argentinum	Canestrato Pugliese (22); Ragusano (32)
Leuconostoc gelidum	Fior di Latte di Agerola (10)
Weissella hellenica	Artisanal Mozzarella (17)
Weissella paramesenteroides	Fior di Latte di Agerola (10)
Weissella viridescens	Scamorza Altamurana (23)
Lactobacillus delbrueckii subsp. *bulgaricus*	Yogurt (2); Gorgonzola (4); Emmenthal (4); Artisanal Emmenthal (9); Mozzarella (4); Fior di Latte di Agerola (10); Grana Padano (12); Parmigiano Reggiano (13); Pecorino Romano (14); Pecorino Sardo (9); Majorero (9); Caciocavallo Silano (18); Asiago (21); Ragusano (32)
Lactobacillus delbrueckii subsp. *lactis*	Kefir (1); Emmenthal (4); Artisanal Emmenthal (9); Water buffalo Mozzarella (8); Grana Padano (12); Pecorino Romano (14); Pecorino Sardo (9); Majorero (9); Parmigiano Reggiano (13); Caciocavallo Silano (18); Scamorza Altamurana(23); Ragusano (32)
Lactobacillus helveticus	Kefir (1); Emmenthal (4); Artisanal Emmenthal (9); Mozzarella (4); Artisanal Mozzarella (17); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Grana Padano (12); Parmigiano Reggiano (13); Pecorino Romano (14); Pecorino Sardo (9); Majorero (9); Montasio (14); Asiago (14); Provolone (14); Scamorza Altamurana(23)
Lactobacillus casei	Caciocavallo Silano (18); Canestrato Pugliese (22); Fiore Sardo (30); Ragusano (32)
Lactobacillus casei subsp. *casei*	Artisanal Mozzarella (17); Fior di Latte di Agerola (10)
Lactobacillus casei subsp. *pseudoplantarum*	Water buffalo Mozzarella (8)
Lactobacillus casei subsp. *alactosus*	Kefir (1)
Lactobacillus casei subsp. *rhamnosus*	Kefir (1)
Lactobacillus casei subsp. *paracasei*	Scamorza Altamurana(23)
Lactobacillus paracasei	Caciocavallo Silano (18); Salers (25); Fiore Sardo (30)
Lactobacillus paracasei biovar *Shirota*	Yakult (2)
Lactobacillus paracasei subsp. *paracasei*	Fior di Latte di Agerola (10)
Lactobacillus plantarum	Artisanal Mozzarella (17); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Caciocavallo Silano (18); Canestrato Pugliese (22); Salers (25); Fiore Sardo (30); Ragusano (32)
Lactobacillus kefir	Kefir (1)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
Lactobacillus brevis	Kefir (1)
Lactobacillus cellobiosus	Kefir (1)
Lactobacillus fermentum	Artisanal Emmenthal (9); Grana Padano (12); Artisanal Mozzarella (17); Caciocavallo Silano (18); Scamorza Altamurana(23); Ragusano (32)
Kluyveromyces lactis	Kefir (1)
Kluyveromyces marxianus subsp. *bulgaricus*	Kefir (1)
Kluyveromyces marxianus subsp. *marxianus*	Kefir (1)
Saccharomyces florentinus	Kefir (1)
Saccharomyces globosus	Kefir (1)
Saccharomyces unisporus	Kefir (1)
Co-starter or adjuncts (typical components of the secondary microflora of some products, often intentionally introduced)	
Lactobacillus acidophilus	Kefir (1); Bio-Yogurt (2)
Lactobacillus paracasei subsp. *paracasei*	Bio-Yogurt (2)
Lactobacillus paracasei biovar *Shirota*	Bio-Yogurt (2)
Lactobacillus rhamnosus	Bio-Yogurt (2)
Lactobacillus reuteri	Bio-Yogurt (2)
Bifidobacterium spp.	Bio-Yogurt (2)
Enterococcus faecium	Bio-Yogurt (2)
Enterococcus faecalis	Bio-Yogurt (2)
Pediococcus acidilactici	Bio-Yogurt (2)
Penicillium camemberti	Camembert (3); Brie (4)
Penicillium roqueforti	Roquefort and other blue-veined cheeses (3); Gorgonzola (4); Stilton (9); Danish blue (9); Stilton (31)
Geotrichum candidum	Viili (1); Brie (4); Limburger and other surface-ripened cheeses (6)
Propionibacterium fruedenreichii subsp. *shermanii*	Emmenthal and other Swiss type cheeses (4)
Brevibacterium linens	Camembert (3); Limburger and other surface-ripened cheeses (6); Smear ripened cheeses (9); Gubbeen (11)

(continued)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
Streptococcus thermophilus	Cheddar (9)
Lactobacillus helveticus	Cheddar (9)
Lactobacillus subsp. casei	Cheddar (9)
Lactobacillus casei subsp. *pseudoplantarum*	Cheddar (9)
Non-starter Microorganisms (generally adventitious: poorly acidifying lactic acid bacteria, micrococci, corynebacteria, yeasts and molds)	
Lactococcus raffinolactis	Fior di Latte di Agerola (10)
Lactococcus garvieae	Artisanal Mozzarella (16); Fior di Latte di Agerola (10); Salers (25); Toma Piemontese (29)
Streptococcus durans	Kefir (1)
Streptococcus filant	Kefir (1)
Streptococcus bovis	Artisanal Mozzarella (16); Scamorza Altamurana(23); Ragusano (32)
Streptococcus uberis	Artisanal Mozzarella (16)
Streptococcus parauberis	Fior di Latte di Agerola (10)
Streptococcus suis	Fior di Latte di Agerola (10); Toma Piemontese (29)
Streptococcus millieri	Salers (25)
Aerococcus viridans	Artisanal Mozzarella (16)
Enterococcus suphurans	Artisanal Mozzarella (16); Ragusano (32)
Enterococcus hirae	Ragusano (32)
Enterococcus faecalis	Stilton (31)
Pediococcus acidilactici	Parmigiano Reggiano (13); Ragusano (32)
Pediococcus pentosaceus	Cheddar (9); Salers (25)
Lactobacillus casei	Cheddar (6); Jarlberg (9); Norvegia (9); Herrgard (9); Greve (9); Gouda (9); Irish Cheddar (9); Parmigiano Reggiano (13)
Lactobacillus casei subsp. *casei*	Italian ewe's cheeses (33)
Lactobacillus casei subsp. *pseudoplantarum*	Italian ewe's cheeses (33)
Lactobacillus paracasei	Jarlberg (9); Norvegia (9); Herrgard (9); Greve (9); Gouda (9); Kefalotyri (9); Tenerife goat's milk cheese (9); Majorero (9); Arzua (9); Armada (9); Serra da Estrela (9); (9); Casu Axedu (9); Fontina (9); Swiss type (9); British Cheddar (9); Irish Cheddar (9); Pecorino (15); Comtè (24)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
Lactobacillus paracasei subsp. *paracasei*	Parmigiano Reggiano (13) Italian ewe's cheeses (33)
Lactobacillus paracasei subsp. *tolerans*	Parmigiano Reggiano (13)
Lactobacillus rhamnosus	Jarlberg (9); Norvegia (9); Herrgard (9); Greve (9); Gouda (9); Cheddar (9); Grana Padano (12); Parmigiano Reggiano (13); Caciocavallo Silano (18); Comtè (24); Fiore Sardo (30); Italian ewe's cheeses (33)
Lactobacillus plantarum	Cheddar (6); Feta (9); Teleme (9); Kefalotyri (9); Tenerife goat's milk cheese (9); Cabrales (9); Afuega'l Pitu (9); Majorero (9); Mahon (9); Arzua (9); Armada (9); Serra da Estrela (9); Casu Axedu (9); Fontina (9); Toma (9); Swiss type (9); Irish Cheddar (9); Pecorino (15); Stilton (31); Italian ewe's cheeses (33)
Lactobacillus paraplantarum	Fiore Sardo (30)
Lactobacillus brevis	Afuega'l Pitu (9); Majorero (9); Swiss type (9); British Cheddar (9); Irish Cheddar (9); Caciocavallo Silano (18); Canestrato Pugliese (22); Italian ewe's cheeses (33)
Lactobacillus fermentum	Majorero (9); Toma (9); British Cheddar (9); Italian ewe's cheeses (33)
Lactobacillus curvatus	British Cheddar (9); Irish Cheddar (9); Fior di Latte di Agerola (10); Caciocavallo Silano (18); Fiore Sardo (30); Italian ewe's cheeses (33)
Lactobacillus helveticus	British Cheddar (9)
Lactobacillus bifermentans	British Cheddar (9)
Lactobacillus buchneri	British Cheddar (9)
Lactobacillus parabuchneri	British Cheddar (9); Comtè (24)
Lactobacillus farciminis	British Cheddar (9)
Lactobacillus kefir	British Cheddar (9)
Lactobacillus graminis	Fior di Latte di Agerola (10); Fiore Sardo (30)
Lactobacillus sakei	Fior di Latte di Agerola (10); Caciocavallo Silano (18); Fiore Sardo (30)
Lactobacillus homoiochii	Fior di Latte di Agerola (10)
Lactobacillus maltaromicus	Fior di Latte di Agerola (10)
Lactobacillus pentosus	Fior di Latte di Agerola (10); Canestrato Pugliese (22); Fiore Sardo (30); Italian ewe's cheeses (33)
Lactobacillus gasseri	Scamorza Altamurana (23)
Carnobacterium divergens	Artisanal Mozzarella (16)

(continued)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
Carnobacterium piscicola	Artisanal Mozzarella (16)
Brevibacterium imperiale	Smear ripened cheeses (9)
Brevibacterium fuscum	Smear ripened cheeses (9)
Brevibacterium oxydans	Smear ripened cheeses (9)
Brevibacterium helvolum	Smear ripened cheeses (9)
Corynebacterium casei	Milleens (11); Gubbeen (11); Durrus (11); Adrahan (11)
Corynebacterium ammoniagenes	Smear ripened cheeses *** (9)
Corynebacterium betae	Smear ripened cheeses *** (9)
Corynebacterium insidiosum	Smear ripened cheeses *** (9)
Corynebacterium variabile	Smear ripened cheeses *** (9); Milleens (11); Gubbeen (11)
Arthrobacter arilaitensis	Milleens (11); Gubbeen (11); Durrus (11); Adrahan (11)
Arthrobacter mysorens	Durrus (11)
Arthrobacter citreus	Smear ripened cheeses *** (9)
Arthrobacter globiformis	Smear ripened cheeses *** (9)
Arthrobacter nicotianae	Smear ripened cheeses *** (9)
Microbacterium gubbeenense	Gubbeen (11)
Agrococcus sp. nov	Gubbeen (11)
Curtobacterium poinsettiae	Smear ripened cheeses *** (9)
Microbacterium imperiale	Smear ripened cheeses *** (9)
Rhodococcus fascians	Smear ripened cheeses *** (9)
Caseobacter variabilis	Smear ripened cheeses *** (9)
Macrococcus caseoliticus	Ragusano (32)
Micrococcus spp.	Limburger and other surface-ripened cheeses *** (6)
Micrococcus luteus	Smear ripened cheeses *** (9); Adrahan (11)
Micrococcus lylae	Smear ripened cheeses *** (9)
Kocuria kristinae	Smear ripened cheeses *** (9)
Kokuria roseus	Smear ripened cheeses *** (9)
Staphylococcus epidermidis	Durrus (11); Artisanal Mozzarella (16)
Staphylococcus equorum	Smear ripened cheeses *** (9); Milleens (11); Adrahan (11); Stilton (31)

(continued)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
Staphylococcus vitulus	Smear ripened cheeses *** (9)
Staphylococcus xylosus	Smear ripened cheeses *** (9)
Staphylococcus saprophyticus	Smear ripened cheeses *** (9); Gubbeen (11)
Staphylococcus carnosus	Artisanal Mozzarella (16)
Staphylococcus lentus	Smear ripened cheeses *** (9)
Staphylococcus sciuri	Smear ripened cheeses *** (9)
Halomonas venusta	Milleens (11); Gubbeen (11); Adrahan (11)
Saccharomyces cerevisiae	Salers (26)
Saccharomyces unisporus	Salers (26)
Candida utilis	Smear ripened cheeses *** (9)
Debaryomyces hansenii	Roquefort (5); Camembert (7); Limburger and other surface-ripened cheeses *** (6); Smear ripened cheeses *** (9); Danish blue (9); Milleens (11); Gubbeen (11); Durrus (11); Adrahan (11); Water buffalo Mozzarella (8); Salers (26); Sardinian ewe's cheeses (28)
Kluyveromyces lactis	Roquefort (5); Camembert (7); Water buffalo Mozzarella (8); Smear ripened cheeses *** (9); Salers (26); Sardinian ewe's cheeses (28)
Kluyveromyces marxianus	Salers (26); Sardinian ewe's cheeses (28)
Geotrichum candidum	Smear ripened cheeses *** (9); Sardinian ewe's cheeses (28)
Yarrowia lipolytica	Camembert (7) Limburger and other surface-ripened cheeses *** (6); Danish blue (9); Milleens (11); Sardinian ewe's cheeses (28)
Brettanomyces spp.	Water buffalo Mozzarella (8)
Dekkera anomala	Sardinian ewe's cheeses (28)
Candida spp.	Roquefort (5); Camembert (7); Limburger and other surface-ripened cheeses *** (6)
Candida zeylanoides	Salers (26)
Candida parapsilosis	Salers (26)
Candida silvae	Salers (26)
Candida intermedia	Salers (26)
Candida kefir	Kefir (1)
Candida catanulata	Sardinian ewe's cheeses (28)

(continued)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
Candida rugosa	Danish blue (9); Salers (26)
Candida sake	Sardinian ewe's cheeses (28)
Candida glutosa	Danish blue (9)
Zygosaccharomyces spp.	Danish blue (9)
Pichia guilliermondii	Salers (26)
Pichia fermentans	Sardinian ewe's cheeses (28)
Pichia membranefaciens	Sardinian ewe's cheeses (28)
Rhodotorula rubra	Sardinian ewe's cheeses (28)
Penicillium spp.	St. Nectair *** (9); Tome de Savoie *** (9); Taleggio *** (9)
Mucor spp.	St. Nectair *** (9); Tome de Savoie *** (9); Taleggio *** (9)
Cladosporium spp.	St. Nectair *** (9); Tome de Savoie *** (9)
Geotrichum spp.	St. Nectair *** (9); Tome de Savoie *** (9); Taleggio *** (9)
Cryprococcus laurentii	Sardinian ewe's cheeses (28)
Issatchenkia orientalis	Sardinian ewe's cheeses (28)
Epicoccum spp.	St. Nectair *** (9); Tome de Savoie *** (9)
Sporotrichum spp.	St. Nectair *** (9); Tome de Savoie *** (9)

*For cheeses produced without starter addition or back-slopping practice, we intended as "starter" those microorganisms well-known as acidifying or flavoring agents

** "Ropy" or "slime" variants

*** The surface microflora is fundamental for the typical quality of this cheese variety. In some cases it is exclusively adventitious; in some other "smears" of old cheeses are utilized as adjunct for promoting ripening of young cheeses. Therefore in these last cases the microbial species could be included among co-starters or adjuncts

(1) Oberman and Libudzisz, 1998; (2) Tamine and Robinson, 1999; (3) Johnson and Steele, 1997; (4) Stanley, 1998; (5) Besancon, et al. 1992; (6) Johnson 1998; (7) Nooitgedagt and Hartog 1988; (8) Coppola, et al. 1988; (9) Beresford, et al. 2001; (10) Coppola, et al. 2006; (11) Mounier, et al. 2005; (12) Lazzi, et al. 2004; (13) Coppola R., et al. 2000; (14) Bottazzi 1993; (15) Coda, et al. 2006; (16) Morea, et al. 1999; (17) Morea, et al. 1998; (18) Piraino, et al. 2005; (19) Tsakalidou, et al. 1998; (20) Pacini, et al. 2006; (21) Mucchetti and Neviani 2006; (22) Aquilanti, et al. 2006; (23) Baruzzi, et al. 2002; (24) Berthier, et al. 2001; (25) Callon, et al. 2004; (26) Callon, et al. 2006; (27) Cosentino, et al. 2004; (28) Cosentino, et al. 2001; (29) Fortina, et al. 2003; (30) Mannu, et al. 2000; (31) Ercolini, et al. 2003a; (32) Randazzo, et al. 2002; (33) De Angelis, et al. 2001

viable and active microorganisms. Extensive genomic sequencing of dairy microorganisms will be able to detect increasing numbers of new targets to monitor, and will allow considerable progress in describing microbial genetic diversity and its potential functional activity in fermented dairy products. It must, however, be recognized that only combined efforts of these approaches with proteomics, chemiometric measurements and sensorial evaluation can elucidate to an exploitable extent the complex and dynamic processes briefly discussed herein.

2 The Use of Molecular Methods in Dairy Microbiology

Approaches to studying microorganisms in food have undoubtedly changed. Advances in molecular biology have provided more information on food-associated bacteria and have also provided the scientific community with sound, reliable and effective methods for detection, identification and typing of microorganisms from food. The availability of such methods has made food scientists shift from a more traditional isolation and biochemical characterization of microbes from food, to a direct detection of microbes – not as microbes, but rather as "D/RNA from microbes" themselves. How have dairy microbiologists made use of these novel approaches so far? The main interest of dairy microbiologists is to study the diversity and dynamics of microorganisms in dairy produce and possibly to correlate the occurrence of certain microbial species and strains with desired flavor and sensorial traits of the products.

Various molecular methods can be used depending on the level of information required by research. Dairy microbiologists can be interested in identification, detection or typing. Identification and detection can benefit from the availability of both culture-dependent and culture-independent techniques, whereas typing is an analysis performed on isolates and is, thus, strictly related to culture-dependent methods.

Identification can be carried out at different levels. The dairy microbiologist can be interested in classifying his microbiota of interest at genus, species and sometimes strain level. Of course the methods to be employed can vary each time.

2.1 Culture-independent Approaches

Identification at genus/species level can be achieved by using culture-independent techniques such as PCR-DGGE/TGGE/SSCP. These methods have the advantage of providing identification and monitoring of a microbiota at species level without isolating the microorganisms on culture media. Instead of isolating bacteria of each sample from milk, natural starter, intermediate of production or the final cheese, direct DNA extraction can be achieved to provide a mixture of nucleic acids from most of the microorganisms present in the original dairy matrix. PCR amplification

is subsequently required, and the most commonly employed target for identification at species level is the DNA encoding for ribosomal RNA. In most cases, for the identification of *Bacteria*, portions of the 16S rRNA gene are used. The 16S rRNA gene is conserved and allows the development of PCR primers that can be used for all *Bacteria*. However, it also contains variable regions, whose variability of sequence is species-specific in most cases. Therefore, the result of PCR amplification will be a portion of the 16S rRNA gene from all the microbial species whose DNA was extracted *in situ*, and the sequence of the amplicon is likely to vary from species to species. This sequence variation will allow separation of the fragments according to formation of discrete regions of thermal (TGGE) or chemical (DGGE) denaturation or the formation of different single strand conformations (SCCP), but the final output of the analysis will always be a fingerprint. The fingerprint will be made of a number of bands corresponding (in most but not all cases) to as many microbial species and will represent the microbiological identity of the milk, starter, intermediate of production or cheese analyzed. The final identification of each species can then be obtained by the purification and sequencing of each band and by comparison with the available data bases (Gene Bank, *www.ncbi.nlm.nih.gov/blast/*).

2.1.1 Diversity and Dynamics of Natural and Selected Starter Cultures

The microbes occurring in the cheese may arise from the raw milk, from the environment and tools of production, or can be added as selected starter cultures under controlled conditions. Another interesting possible source of bacteria exists in natural starter cultures, currently employed in much traditional cheese making where fermentation is assured by the back-slopping of milk or whey cultures from previous preparations.

The studies of selected and natural starter cultures share common interests such as the knowledge of the fate of microorganisms present in the culture at the beginning of fermentation, and their interaction with background microbiota. These interactions are recognized to be fundamental in selecting the microorganisms actually dominating the process and contributing to the principal rheological and sensorial attributes of the cheese. In addition, the study of the accessory microbiota is also important since the bacteria occurring at lower loads, along with the dominant bacteria, can potentially contribute to the development of product flavor and taste, thanks to specific metabolic pathways (Beresford, et al. 2001).

Of the above-cited fingerprinting techniques the most commonly employed in dairy microbiology is PCR-DGGE. We will be giving some examples of how this has been used to study the diversity and dynamics of microbial communities in cheese production.

The PCR-DGGE approach has been exploited to directly identify microbial species occurring in natural whey cultures (NWCs) used as starter for water buffalo Mozzarella cheese manufacture (Ercolini, et al. 2001). Both thermophilic and mesophilic LAB were identified by sequencing of the V3 region of the 16S rRNA

gene from DGGE fragments of NWCs profiles, namely *Lb. delbrueckii, Lb. crispatus, Lactococcus* (*L.*) *lactis* and *Streptococcus* (*St.*) *thermophilus*. Moreover, the occurrence of contaminants such as *Alishewanella fetalis* could also be highlighted. In the same study, a novel PCR-DGGE approach was developed to rapidly check the diversity of the bacterial community after cultivation on specific or non-specific culture media. Briefly, after colony counting has been performed, the colonies from the plates can be collected in "bulks" and subjected to DNA extraction and PCR-DGGE analysis (Ercolini, et al. 2001). Consequently, a DGGE fingerprint can be obtained for each plate, dilution, and culture medium. This method to investigate the cultivable microbial community has been shown to have good potential in food microbiology (Ercolini, et al. 2001; Ercolini, et al. 2003a; Ercolini, et al. 2004; Ercolini 2004). Firstly, it provides an alternative to traditional tools for identification. Qualification of the dominant species could be achieved by sequencing of the DGGE bands arising from the patterns corresponding to the highest dilutions, in spite of the isolation of single colonies followed by purification and biochemical identification (Ercolini 2004). Analysis of DGGE profiles obtained from bulk cells provides an image of the cultivable community, while simultaneously allowing ecological information to be obtained. Ercolini, et al. (2001) counted a population of mesophilic streptococci of 10^8 cfu/ml in NWCs for Mozzarella cheese production, but realized, after bulk PCR-DGGE analysis of all the dilutions, that the only species reaching the value of 10^8 was the thermophilic *St. thermophilus* and that mesophilic cocci were only present at levels of 10^4 cfu ml^{-1}.

PCR-DGGE fingerprinting can also be useful to trace process dynamics during cheese making. The approach can be used to track the starter during production by examining the fingerprints of samples from raw material, through intermediate of production until the final product. This is important in both traditional and industrial dairy production. In the latter case the use of selected starter cultures ensures controlled fermentation and a standard quality of the final product. Analysis of DGGE fingerprints of the samples during manufacture can be important to ascertain that the starter culture is actually dominating the fermentation and can be of help in highlighting the occurrence of contaminating bacteria. On the other hand, in the case of traditional cheese production, one can trace the evolution of the contributing microbiota during the whole production and assess whether the raw milk or the natural whey/milk culture microflora actually contributes to cheese production. It can also show which microbial species of the natural starter survives fermentation, processing and the possible stresses imposed by the technology of production (pH, thermal stress, etc.). In a recent study the fate of the natural whey culture for the manufacture of traditional water buffalo Mozzarella cheese was investigated by PCR-DGGE (Ercolini, et al. 2004). The analysis of DGGE fingerprints from the intermediate samples during cheese production was shown to be useful to check the natural starter effectiveness and to determine the contribution of different groups of LAB during fermentation leading to the final Mozzarella cheese. All the DGGE profiles of dairy samples taken during manufacture were analyzed: raw milk, NWC, raw milk after NWC addition, curd before and after ripening, drained whey, stretched curd and final product (Ercolini, et al. 2004). A single

glance at the succession of the fingerprints (Fig. 2.1) explains all that occurred in the process: the raw milk had a complex profile, but none of the species occurring in the milk were present in the profiles of the other samples. As soon as the NWC was added to the milk, the profile changed into the NWC fingerprint, which was displayed by all further samples until the final water buffalo Mozzarella cheese (Fig. 2.1). In other words, in this specific manufacture, the NWC was the main performer in the fermentation, giving high loads of bacteria to the raw milk, concealing the raw milk microbiota in the fingerprints, but probably giving strength to the fermentation and allowing the process to be properly carried out in respect of tradition. In this case, the microbial succession could be registered as "pictures" of microbial groups involved in premium quality production. This procedure may find useful applications for the monitoring of non-premium quality products where poor quality arises from the lack of development of the NWC. This procedure can be easily applied to dairy plants, allowing process development and starter effectiveness to be checked by analyzing dairy samples by PCR-DGGE. In comparison with traditional culture-dependent microbiological analyses, molecular approaches can be considered a step forward for the innovation of tracing systems in food technology, and may play an important role in the quality control of traditional

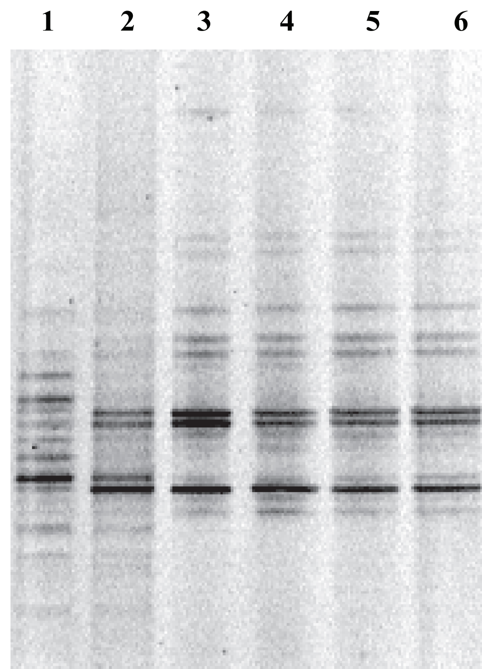

Fig. 2.1 PCR-DGGE profiles of dairy samples during water buffalo Mozzarella cheese manufacture Lanes: 1, raw milk; 2, milk after NWC addition; 3, NWC; 4, curd after ripening; 5, stretched curd; 6, final product. (From Ercolini, et al. (2004), permission granted)

products, allowing preservation of their typical identity and consumer protection when territory claims are involved.

The dynamics of complex starter mixture can also be monitored to assess whether they are influenced by certain technological interventions in a cheese making process. Recently, PCR-DGGE was also applied to check the response of the smear microbiota of smear-ripened cheese to the use of a bacteriocin-producing culture used as protective adjunct to combat the occurrence of *Listeria monocytogenes* (O'Sullivan et al. 2006). The authors proved that there was apparently no effect on the smear microbial flora of the cheeses treated with the bacteriocin-producing culture in comparison with the untreated control (O'Sullivan et al. 2006). Even though PCR-DGGE profiling is not always very precise in describing all the species occurring in a certain environment (Ercolini, 2004), in cases such as this it can be useful to assess whether or not the microbiological state of a starter has changed, according to environmental and technological variations. Again, the approach to studying dairy cultures has changed since, in such cases, traditional analysis of the microbiota would have been performed by plate counts, isolation followed by biochemical identification, etc., to have information on the response of a certain flora to varied conditions.

Fingerprinting techniques can also be used to characterize wide numbers of natural starter cultures, especially when they are used by different dairies of a particular geographic region to produce the same type of cheese. Effective application of PCR-DGGE for grouping NWCs was reported by Mauriello, et al. (2003). In this study PCR-DGGE analysis was used for discriminating natural starters for traditional water buffalo Mozzarella cheese production that were sampled from different dairies in southern Italy. The profiles showed that the microbial composition of the starters was strongly dependent on their geographical origin, with starters from the same area displaying closely related DGGE profiles. It was also demonstrated that the flavors (detected by chromatographic methods) potentially provided by the development of each starter during curd ripening were linked to the complexity of the microbial flora shown by DGGE, and thus to the geographical origin of the products (Mauriello, et al. 2003). This constitutes evidence that the microbial diversity of natural starter cultures and its evolution during fermentation may represent important proof of authenticity for the traceability of origin and mode of production of traditional dairy products.

2.1.2 Diversity and Dynamics of Microbial Populations in Cheese and during Cheese Manufacture

The same approach used for monitoring the fate of starter cultures during cheese production can be used to obtain structure and identification of microbial communities in cheese and during cheese manufacture.

Randazzo, et al. (2002) examined the microbial succession in manufacturing of Ragusano, an artisanal Sicilian cheese. The variable region V6-V8 of the 16S rRNA gene was used in DGGE analysis to identify the total microflora, while specific

primers for *Lactobacillus* were employed spanning the V1-V3 region. Analysis of the active microflora was also performed by 16S rRNA RT-PCR followed by DGGE. DGGE profiles from samples taken during cheese production indicated dramatic shifts in the microbial community structure. Cloning and sequencing of rRNA gene amplicons revealed that mesophilic bacteria, including leuconostocs, *L. lactis* and *Macrococcus caseolyticus*, were dominant in the raw milk while *St. thermophilus* prevailed during fermentation. Other rod-shaped LAB, especially *Lactobacillus fermentum* and *Lb. delbrueckii*, were also found during ripening. Moreover, the authors found that *Lb. delbrueckii* was not cultivable, while some isolated species of enterococci and pediococci could be not found in the DGGE profiles.

The bacterial community occurring in Stilton cheese was structured by PCR-DGGE and sequencing of the 16S rRNA regions V3 and V4-V5 (Ercolini, et al. 2003a). The traditional British PDO cheese was shown to be colonized by a complex microbial flora including *Lb. plantarum, Lb. curvatus, L. lactis, Staphylococcus* (*S.*) *equorum, Enterococcus* (*E.*) *faecalis, Leuconostoc* (*Lc.*) *mesenteroides*. It was found that microbial diversity revealed from the same DNA templates amplifying two different regions of the 16S rRNA gene could be different. Indeed, the presence of *Leuconostoc* in Stilton cheese was revealed only by analyzing the V4-V5 region of the 16S rRNA gene while the species was not detected when the V3 region was targeted (Ercolini, et al. 2003a). Targeting more than one variable region may potentially widen the microbial diversity detected but could be more time-consuming. However, even if one region is targeted, other experiments should be done to ascertain whether other microbial species are present, but not detected.

The comparison of PCR-DGGE profiles of different cheeses of the same category, or of the same cheese manufactured by different procedures, can help assess the behavior of starter bacteria or adventitious microbial flora. For example, Randazzo, et al. (2006) found that the dominant bacteria in the manufacture of Pecorino Siciliano cheese were *St. bovis* and *L. lactis*, although various cheese making procedures were tested. In addition, Obodai and Dodd (2006) showed that *Lb. delbrueckii* subsp. *delbrueckii* and *St. thermophilus* were the principal bacterial species involved in the production of nyarmie, a Ghanaian fermented milk product obtained by natural fermentation. The authors concluded that these thermophilic bacteria, or alternatively mesophilic bacteria, could be selected as starter cultures in order to improve and/or standardize the quality of Nyarmie (Obodai and Dodd 2006).

Another culture-independent fingerprinting technique, very similar in principle to PCR-DGGE, is PCR-TTGE. This method has been used on several occasions to describe dairy ecosystems (Ogier, et al. 2002; Lafarge, et al. 2004; Ogier, et al. 2004; Parayre, et al. 2007). Initially, the technique was used to set up a species database in which each species or group of species was characterized by a specific TTGE fingerprint (Ogier, et al. 2002). The variable V3 region of the 16S rRNA gene of about 50 microbial species possibly occurring in dairy ecosystems and belonging to the genera *Lactobacillus, Enterococcus, Lactococcus, Leuconostoc,*

Streptococcus, Weissella and *Staphylococcus* was analyzed in TTGE to develop the database. However, TTGE fingerprints characterized by multiple bands for one species were highlighted. In addition, cases of TTGE co-migration of V3 amplicons from different species were also found within the *Lb. acidophilus* and *Lb. plantarum* groups, and within the species of *Leuconostoc* and *Enterococcus* (Ogier, et al. 2002). In fact, the latter evidence can represent a problem in obtaining reliable identification of microbial species by simple comparison of TTGE bands in a cheese fingerprint with the migration distances of the species of the database.

Use of the migration map to identify dairy bacteria was validated by analyzing dairy preparations from defined microbial content to increasing microbial complexity; in the latter samples bands that could not be recognized in the migration field of the species of the database were identified by cloning and sequencing, and were often shown to be Gram negative contaminants (Ogier, et al. 2002). In a further study, Ogier and co-workers (2004) extended the database to 150 microbial species, including possible contaminants and spoilage bacteria. The database of high G+C% bacteria was set up by DGGE analysis of V3 amplicons. The authors analyzed several dairy products such as Morbier, Munster, Epoisses and Leerdamer Swiss cheese, identifying a large number of bacteria by using their database and, in cases of unidentified bands, by cloning and sequencing the fragments and/or using species-specific PCR assays to sort out uncertainty in some identifications (Ogier, et al. 2004). As expected, it was found that raw milk cheeses such as Morbier were richer in microbial diversity than Leerdamer cheese obtained from pasteurized milk. It was shown that while LAB dominated in the core of the cheese, high G+C% coryneform bacteria such as *Corynebacterium variabile, C. mastitidis, C. casei, Arthrobacter* spp. and *Brevibacterium linens* could be identified from the surface of the cheeses (Ogier, et al. 2004). This is further confirmation that micro-environments characterized by different ecological factors can develop across a cheese matrix and, therefore, a heterogeneous spatial distribution of microbial species can occur at the end of ripening, as also shown by 16S rRNA FISH analysis of Stilton cheese by Ercolini, et al. (2003a). Recently, a PCR-TTGE approach was used for the optimization of a DNA extraction method from dairy products with a fingerprint reproducibility of 89 percent (Parayre, et al. 2007).

An original approach has also been developed to achieve a culture-independent microbial characterization of dairy samples, based on direct DNA/RNA extraction followed by PCR amplification and SSCP analysis by capillary electrophoresis. This approach was used on several occasions to study the microbial diversity of Salers, a Registered Designation Origin (RDO) semi-hard cheese from raw milk produced in France (Duthoit, et al. 2003, 2005a and 2005b; Callon, et al. 2006). Duthoit, et al. (2003) established their most suitable conditions for DNA extraction and employed different targets for 16S rRNA gene amplification to highlight the presence of LAB and high G+C% bacteria. An initial cloning strategy of the PCR products from curd, followed by sequencing and screening of the clone library by SSCP, was adopted to identify the different

SSCP peaks and to be able to recognize the corresponding microbial species in the analysis of cheese during ripening. LAB such as *L. lactis, St. thermophilus, E. faecium, Lc. paramesenteroides, Lc. mesenteroides, Lb. plantarum* and *Lb. pentosus* were identified; in addition, high G+C% corynebacteria such as *C. bovis, C. variabilis, C. afermentans* and *C. flavescens* were detected (Duthoit, et al. 2003). The authors showed that different results may be obtained by amplifying the V2 or V3 regions of the 16S rRNA gene, and that different microbial species may give the same migration properties in PCR-SSCP. Moreover, the identified species showed different trends according to cheese production (from different dairies) and time of ripening. *Enterobacteriaceae* occurred in raw milk, but disappeared during curd ripening, while the proportion of LAB species varied according to sample and time of ripening.

The diversity of the microbial populations in Salers cheese was also assessed by using RNA as a template in RT-PCR to obtain fingerprints of the active microbiota (Duthoit, et al. 2005a and 2005b). By comparing DNA and RNA SSCP profiles the authors realized that the revealed microbial species were different and that the active microbial species found in SSCP profiles did not always match those detected by DNA-based PCR-SSCP. The authors concluded that RNA-based SSCP was more pertinent than DNA-based SSCP to measure the diversity of the microbial community of Salers (Duthoit, et al. 2005b). The comparisons of microbial diversity were based on the calculation of diversity indexes, often taking into account the peak ratios of the different microbial species (Duthoit, et al. 2003, 2005a and 2005b). However, this strategy may be significantly biased by selective PCR amplification phenomena as often reported (Reysenbach, et al. 1992; Suzuki and Giovannoni 1996; Ercolini, et al. 2001b; Ercolini 2004), and also by the abundance of the number of rRNA gene copies (Farrelly, et al. 1995). Therefore, the dominance of a particular peak/species may be due to its selective amplification in PCR and not to its actual abundance in that particular dairy sample (Ercolini 2004).

The microbial diversity detected in Salers cheese by PCR-SSCP was tentatively compared to the sensorial properties of the cheese (Duthoit, et al. 2005a). The sensorial attributes of the cheese were shown not to be correlated to the producer, but to be influenced by ripening time. Changes in sensorial properties during ripening could be correlated to the variation in the RNA-based SSCP profiles. Moreover, bacteria not usually considered of technological interest – such as corynebacteria, *Enterobacteriaceae, Bacillus* spp., and some unidentified SSCP peaks – were shown to probably be involved in the development of texture, taste, flavor or aroma (Duthoit, et al. 2005a). However, the general profiling of the bacterial population at species level by SSCP fingerprinting cannot provide enough information on the sensorial quality of Salers cheese, which remains, as for most cheeses, fairly unpredictable. Diversity at strain level in metabolic activities (Giraffa, et al. 2001) and aroma production (Mauriello, et al. 2001) must be investigated to enhance our knowledge of how microbial succession affects the sensorial quality of cheeses.

The PCR-SSCP was also developed to identify staphylococcal populations in dairy products (Delbes and Montel 2005). A nested-PCR assay was developed by

using primers for selective amplification of the 16S rRNA gene of staphylococci, and SSCP analysis was applied to identify the staphylococci during the production of raw milk cheeses. *S. equorum, S. saprophyticus* and *S. aureus* were detected as dominant species in the dairy samples (Delbes and Montel 2005). Moreover, the amplicons from *S. aureus* were always found to be more abundant than the other species, even when equal amounts of DNA templates were used, probably due to preferential PCR amplification or 16S rRNA copy number heterogeneity (Coenye and Vandamme 2003).

Different yeast species can also occur in dairy products and can play a role in curd ripening and aroma development. The PCR-SSCP approach was also implemented to profile the yeast community in Salers cheese (Callon, et al. 2006). Yeast-specific primers were designed to amplify the V4 region of the 18S rRNA gene for SSCP analysis. The yeast species most frequently found in Salers were *Kluyveromyces lactis, K. marxianus, Candida zeylanoides, Debaryomyces hansenii* and *Saccharomyces cerevisiae* (Callon, et al. 2006).

Overall, the SSCP approach may be useful for profiling the microbial populations in dairy products and to observe the marked variation in microbial species composition during ripening. However, identification of the microbial species based on the co-migration of the PCR amplicons of reference species may be unsatisfactory since the occurrence of certain bacteria in the dairy sample is not always predictable, and also due to possible co-migration of amplicons from different species.

2.1.3 Microbial Profiles of Dairy Products for Quality Assessment

PCR-DGGE applied to template DNA directly extracted from a food matrix generates a specific profile of that product in that moment, given the conditions used. The fingerprint gives a "picture" of the microbiota of the product and can be taken into account as a specific trait of that food just like other biochemical, structural or sensorial properties. PCR-DGGE fingerprinting of food and drinks has been tested by several authors who discovered it can identify the microbiological traits of food products and may represent a tool for quality control.

Coppola, et al. (2001) discriminated between industrial and artisanal pasta filata cheeses by comparing the DGGE profiles of different commercial products. The authors found that PCR-DGGE was better than 16S-23S intergenic spacer region analysis at differentiating pasta filata cheeses. Cluster analysis of the fingerprints showed the dairy products grouped according to their microbial complexity; it was found that traditional pasta filata cheeses had profiles which were rich in bands, and that the degree of complexity of the microbial flora decreased when the product was of industrial manufacture. On the basis of their results, Coppola, et al. (2001) suggested that PCR-DGGE can be considered valid for discriminating traditional and industrial cheeses – also for legal purposes – when products obtained through prescribed manufacturing regulations are analyzed. The potential of PCR-DGGE in differentiating dairy products was further confirmed by Ercolini, et al. (2002) through profiling different kinds of

cheese, and showing them to be very different from each other. Unfortunately, it was also demonstrated that different samples of the same category of cheese could display different DGGE profiles (Ercolini, et al. 2002), thus compromising the use of the technique to develop class-specific profiles for cheese classification.

Dairy products with a defined microbial flora, produced by using starter cultures and/or in controlled conditions, are the easiest matrices to control as they usually display a simple DGGE profile where each band corresponds to the species expected to be there. Among these are yogurts and probiotic beverages or preparations. Recently, PCR-DGGE was shown to be effective in corroborating the occurrence of certain microbial species in yogurt and lyophilized probiotic preparations (Fasoli, et al. 2003; Temmerman, et al. 2003). A general congruence between microorganisms declared on the label and those revealed by PCR-DGGE was found by Fasoli, et al. (2003) for probiotic yogurts. However, the authors also found some discrepancies for probiotic preparations such as incorrect identification of some *Bacillus* and *Bifidobacterium* species and the presence of microbial entities not declared in the label (Fasoli, et al. 2003). These results are consistent with those obtained by Temmerman, et al. (2003) in analyzing several probiotic preparations by PCR-DGGE; the authors found the technique very useful to ascertain the occurrence of probiotics in the analyzed matrices, especially if compared to lengthy and uncertain traditional procedures. The PCR-DGGE control of such products rapidly and reliably revealed a rather high percentage of incorrectly labeled probiotic products, and viable counts often showed low loads of the declared species, thus compromising the probiotic value of the products (Temmerman, et al. 2003).

2.1.4 Specific Location of Microbial Colonies in Cheese by FISH

A further possible goal in microbial ecology can be to examine the spatial distribution of the bacteria within a cheese matrix. This can be achieved by means of fluorescence *in situ* hybridization (FISH) using 16S rRNA probes (Bottari, et al. 2006).

First of all, the FISH experiments apply to thin cheese sections that can endure the hybridization procedure. This can be achieved by using a polymerizing glycol methylacrylate resin to embed the cheese specimen to be cut in thin sections. This method was developed by Ercolini, et al. (2003b) and proved to be a successful alternative to cryo-sectioning for the achievement of down to 5 µm dairy sections withstanding the hybridization buffers.

16S rRNA probes were developed for the specific detection of *L. lactis, Lc. mesenteroides* and *Lb. plantarum* to locate their colonies in Stilton cheese (Ercolini, et al. 2003a). A location and differential distribution of the microbial species was shown by FISH in the core, underneath the crust, and along the veins of Stilton cheese (Ercolini, et al. 2003a). The combined use of the universal Eub338 (Amann, et al. 1990) probe and the specific probes developed in FISH experiments

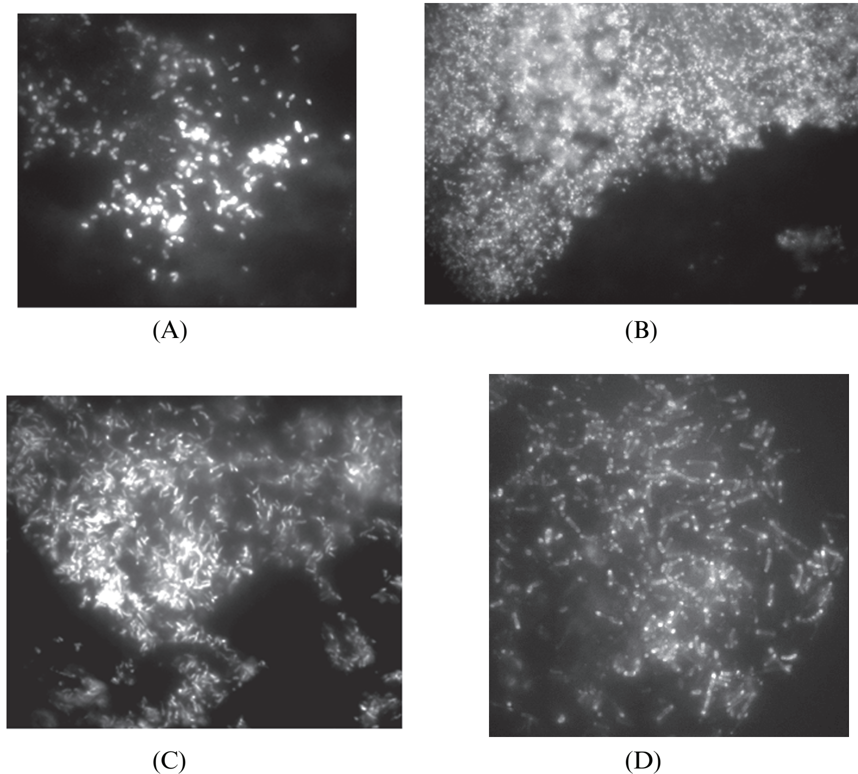

Fig. 2.2 Fluorescence *in situ* hybridisation of Stilton cheese sections (A) *L. lactis* microcolony from the core detected by using a *L. lactis* specific probe; (B) Microcolony of cocci along the vein detected by the universal probe Eub 338; (C) Colony of rods underneath the veins detected by probe Eub 338; (D) Microcolony of *Lb. plantarum* underneath the crust detected by using the *Lb. plantarum* specific probe. Adapted from Ercolini, et al. (2003a)

on Stilton cheese sections showed a differential spatial distribution of the bacterial flora within the dairy matrix (Fig. 2.2). A significant difference was detected between the core and the rest of the cheese; the former being much less rich in bacterial colonies. The colony density in the core was about 5-fold lower than the surface and the veins, although the micro-environment in the latter two was outcompeted by mold development. In the core most of the microcolonies were *L. lactis* even though a considerable amount of *Lc. mesenteroides* were also detected. No rod-shaped bacteria were found in the core; instead, conspicuous amounts of *Lb. plantarum* were found underneath the crust (Ercolini, et al. 2003a). Across the veins, moreover, a few micro-colonies of *Lb. plantarum* could be observed while

micro-colonies of shorter rods resembling *Lb. curvatus* were much more abundant. On the surface, and much more along the internal margin of the veins, very large colonies of non-*Lactococcus* cocci were detected and supposed to be staphylococci even though identification has not been achieved.

The differential spatial distribution of microbial species in cheese has a major impact on knowledge in dairy science. The location of the different species in different zones of the matrix implies differential utilization of the dairy nutrients, consequently affecting the impact of flavor to the cheese which might result in a pool of various compounds that are not released homogeneously from the cheese, but arise from different sites with different microbial activity. Moreover, the production of anti-microbial compounds is also localized, thus affecting flora development. When poor quality products arise it may be due to the lack of development of one or more of these micro-environments.

3 Molecular Identification and Characterization of Microbial Strains Isolated from the Dairy Environment

Cultivable microflora of milk and dairy products is mainly represented by LAB (*Lactobacillus, Streptococcus, Enterococcus, Lactococcus, Leuconostoc, Wiessella* and *Pediococcus*). However, strains of other genera such as *Propionibacterium, Staphylococcus, Corynebacterium, Brevibacterium*, yeasts and molds can also occur. Today (November 2006; see also *www.bacterio.cict.fr*), the genus *Lactobacillus* contains 119 species, the genus *Streptococcus* 67 species, the genus *Enterococcus* 34 species, the genus *Lactococcus* 5 species, the genus *Leuconostoc* 14 species, the genus *Wiessella* 11 species, the genus *Pediococcus* 11 species and the genus *Propionibacterium* 13 species. The main microbial species and their occurrence in dairy products are highlighted in Table 2.1.

The problems of traditional identification methods, even when based on miniaturized easy-to-handle kits or devices, make their use difficult for a reliable identification or biochemical typing of microbial taxa from food.

For these reasons, significant efforts have been made to develop alternative identification methods combining speed, reliability and low cost. These criteria are met by methods based on molecular rather than phenotypic traits. The greatest advantage of DNA-based identification techniques is that these methods focus on the unique nucleic acid sequence of the microorganisms rather than on the phenotypic expression of products that are encoded by the respective genes. Polyphasic taxonomy, however, combines pheno- and genotypic information and forms the basis for current systematic bacteriology.

Moreover, new microbial species are continually being classified, making further identification tools necessary. Therefore, molecular methods have been increasingly used in order to simplify characterization procedures, to provide rapid and reliable identification, or to validate phenotypically determined taxa. Indeed, thanks to the results of the application of molecular tools, in the course

of 2006 new species of LAB were described in the genus *Lactobacillus* (Aslam, et al. 2006; Konstantinov, et al. 2006; Osawa, et al. 2006; Rodas, et al. 2006; Vancanneyt, et al. 2006a), *Enterococcus* (Carvalho, et al. 2006; Svec, et al. 2006), *Streptococcus* (Glazunova, et al. 2006), *Leuconostoc* (Chambel, et al. 2006) and *Pediococcus* (Franz, et al. 2006). By contrast, in the same period, some species of *Lactobacillus* (Naser, et al. 2006a-b; Dellaglio, et al. 2006; Felis, et al. 2006), *Enterococcus* (Naser, et al. 2006c) and *Leuconostoc* genera (Vancanneyt, et al. 2006b) were re-classified.

The primary objectives of food microbiological analysis are the control of food quality, food preservation, evaluation of starter culture efficiency, and monitoring of particular species/strains during manufacturing. With reference to the development of starter cultures, with consistent and predictable performance, it is widely recognized that extensive characterization of the strains and more detailed knowledge of their physiology, metabolism and genetics are required. Moreover, the increasing number of available commercial strains used as starters requires reliable methods to accurately differentiate strains at both species and biotype levels in pure and mixed cultures in order to defend rights and eliminate risks of confusion during their use.

The taxonomic level of microbial discrimination depends on the sensitivity of the technique used and may range from genus (or species) to strain level (sub-typing or strain typing). The ability of a molecular typing system to discriminate among genetically unrelated isolates is a reflection of the genetic variation seen in the chromosomal DNA of the bacterial species. Usually this variation is high, and differentiation of unrelated isolates can be accomplished using any of a variety of techniques. However, often technologically important traits of dairy microorganisms are not uniformly distributed within a species. Thus, the most important biotypes are often a smaller subset of the many strains that constitute a species. As a consequence, this subset may exhibit relatively little genetic diversity, and it can be difficult to differentiate among strains even with molecular techniques.

Type-ability refers to the ability of a technique to assign an unambiguous result (type) to each isolate. Non-type-able isolates are more common with phenotypic methods, but can also occur with genotypic methods. The reproducibility of a method means the ability to yield the same result upon repeat testing of a bacterial strain. Poor reproducibility may reflect technical variation in the method or biological variation occurring during in vivo or in vitro passage of the organisms to be examined. The discriminatory power of a technique refers to its ability to differentiate among unrelated isolates, ideally assigning each to a different type. In general, phenotypic methods have less discriminatory power than genotypic methods. Most molecular methods require costly material and equipment, but are relatively easy to learn and are applicable to variety of species. On the other hand, phenotypic methods also involve costs in labor and material and are restricted to a few species (sero-typing, phage-typing). Characteristics of some typing systems are reported in Table 2.2.

Although the classical phenotype-based (biotyping) methods are still of importance for daily routine analyses, genotypic methods have increasingly contributed to the in-depth characterization of microorganisms and their differentiation. It may be assumed that the combination of different fundamental and advanced methods

Table 2.2 Characteristics of Some Systems for Typing of Dairy Bacteria

Typing systems	Proportion of strains typeable	Reproducibility	Discrimination Power	Ease of Interpretation[1]	Ease of Performance	Cost (Time and money)
Phenotypic biotyping	All	Poor	Poor	Moderate	Easy	High
Antimicrobial susceptibility patterns	All	Good	Poor	Easy	Easy	Moderate
Serotyping	Low	Good	Fair	Moderate	Moderate	Moderate
Plasmid profiling	Most	Good	Good	Moderate	Good	Moderate
REA-CE	All	Good	Good	Difficult	Good	Low
REA-PFGE	All	Excellent	Excellent	Good	Moderate	High
RLFP (including ribotyping)	All	Excellent	Moderate to Excellent	Moderate	Difficult	High
RAPD-PCR (including its variants)	All	Good	Good	Moderate	Good	Low
AFLP	All	Good	Good	Moderate	Good	Low to moderate
SDS-PAGE of WCPs	All	Good	Good	Moderate	Good	Moderate

[1] In all cases the ease of interpretation can be increased by applying objective reading and analysis systems.

in a polyphasic approach will provide a suitable solution for reliable identification and characterization of strains.

Indeed, for the identification and characterization of cultivable microflora of dairy products, in addition to phenotypic traits, molecular techniques with different specificity levels have been applied in the last decade. Tables 2.4 to 2.12 summarize the strategies applied for these purposes in research of micro-organisms isolated from the dairy environment. Table 2.3 groups the experiences of researchers who contemporaneously considered more than one LAB genus, while Tables 2.4 to 2.12 report research aiming to characterize strains of the genera *Lactobacillus, Streptococcus, Lactococcus, Enterococcus, Leuconostocs, Pediococcus, Propionibacterium* and yeasts.

3.1 Strain Typing

The most widely used typing techniques for the characterization of the microflora of dairy products are: RAPD-PCR (and similar techniques, such as REP-PCR, AP-PCR, BOX-PCR); REA-PFGE; AFLP and SDS-PAGE of WCPs. These techniques are used alone or in combination (Tables 2.3 to 2.11).

For randomly fingerprinting characterization (RAPD-PCR and similar techniques), analysis has been made of patterns obtained by using one primer (Morea, et al. 1999; Baruzzi, et al. 2002; Jenkins, et al. 2002; Gobbetti, et al. 2002, Poznanski, et al., 2004, Rossetti and Giraffa 2005; Coppola, et al. 2006, Zamfir, et al. 2006; for other references see Tables 2.3 to 2.11), two or more primers in separate reactions (Succi, et al. 2005, Sanchez, et al. 2006; Giraffa, et al. 2004; for other references see Tables 2.3 to 2.11) or two primers in the same reaction (Bouton, et al. 2002; Callon, et al. 2004). Generally, all the above research used thermal PCR conditions with a single annealing temperature. By contrast, others applied two (Gobbetti, et al. 2002; Baruzzi, et al. 2002; Baruzzi, et al. 2000) or three (Jenkins, et al. 2002; Cusick and O'Sullivan 2000) cycling conditions with different annealing temperatures. As summarized by Table 2.12, for each group of microorganisms a different primer(s) was used. The exceptions are represented by RAPD-PCR primers M13, Coc, P32, P1A and PC1 that were used to characterize strains of different LAB genera and yeasts. Finally, Rossetti and Giraffa (2005) established a large RAPD-PCR fingerprint database to identify dairy LAB (*Lb. casei, Lb, plantarum, Lb. rhamnosus, Lb. helveticus, Lb. delbrueckii, Lb. fermentum, Lb. brevis, E. faecium, E. faecalis, St. thermophilus and L. lactis*) on the basis of their M13 RAPD-PCR pattern. In particular, the RAPD technique is quite straightforward and quick, and analysis can generally be performed starting from a lysate of a bacterial colony without the need of extensive DNA purification. However, it is well known that RAPD profiles can be sensitive to even modest changes in the reaction conditions, and this can lead to problems of reproducibility, particularly regarding the minor faint bands, which are not always well conserved among replicates of the same sample. Moreover, although the use of RAPD-PCR protocols was efficiently applied

Table 2.3 Molecular Approaches Used for the Identification, Characterization and Monitoring of LAB Isolated from Dairy Products

Source	Methods Applied[a]	Aims[b]	Reference
Different sources	Statistical analysis of RAPD-PCR fingerprints (*M13*), sp-PCR (Berthier and Ehrlich 1998; Chagnaud, et al.. 2001; Corroler, et al.. 1998; Drake, et al.. 1996a; Dutka-Malen, et al. 1995; Guarneri, et al. 2001; Lick, et al. 1996; Tilsala-Timisjarvi and Alatossava 1997; Torriani, et al. 1999).	I, C	Rossetti and Giraffa 2005
Natural Whey Starters	LH-PCR, sp-PCR (Lick, et al. 1996; Torriani, et al. 1999; Tilsala-Timisjarvi and Alatossava 1997; Chagnaud, et al. 2001).	I	Lazzi, et al. 2004
Toma Piemontese Cheese	16S-23S rDNA ISR polymorphism (Jensen, et al. 1993), sp-PCR (Dutka-Malen, et al. 1995; Lick, et al. 1996; Cheng, et al. 1997; Berthier and Ehrlich 1998; Zlotkin, et al. 1998; Ward and Timmins 1999; Ke, et al. 1999; Corroler, et al. 1999), 16S rDNA sequencing.	I	Fortina, et al. 2003
Italian ewe's milk cheeses	Numerical analysis of SDS-PAGE of WCPs fingerprints	I, C	De Angelis, et al. 2001
Caciocavallo cheeses	Numerical analysis of SDS-PAGE of WCPs fingerprints	I, C	Piraino, et al. 2005
Different sources	Artificial neural networks analysis of SDS-PAGE of WCPs fingerprints	I, C	Piraino, et al. 2006
Romanian Dairy products	rep-PCR fingerprinting (*REP-PCR*), SDS–PAGE of WCPs, 16S rRNA gene sequencing	I, C	Zamfir, et al. 2006
Tenerife cheese	SDS-PAGE of WCPs fingerprints	I, C	Perez, et al. 2000
Different sources	Sau-PCR	I, C	Corich, et al. 2005
Caciocavallo Pugliese cheese	Two step RAPD-PCR (*Coc*), 16S rDNA partial sequencing (Cocconcelli, et al. 1997)	I, M	Gobbetti, et al. 2002
Canestrato Pugliese Cheese	16S rDNA-RFLP (Jayarao, et al. 1992), 16S rDNA partial sequencing, sp-PCR (Torriani, et al. 2001)	I, M	Aquilanti, et al. 2006

(continued)

Table 2.3 Molecular Approaches Used for the Identification, Characterization and Monitoring of LAB Isolated from Dairy Products (continued)

Source	Methods Applied[a]	Aims[b]	Reference
Fior di Latte Cheese	RAPD-PCR (*Primm 239*), *Sma* I REA-PFGE (Moschetti, et al. 1997), 16S-23S rDNA ISR polymorphism (Blaiotta, et al. 2002), sp-PCR (Fortina, et al. 2001; Moschetti, et al. 2000)	I, M	Coppola, et al. 2006
Mozzarella cheese	RAPD-PCR (*Coc*), 16S rDNA partial sequencing	I, M	Morea, et al. 1999
Nostrano di Primiero Cheese	RAPD-PCR (*PC1*), 16S rDNA partial sequencing	I, M	Poznanski, et al. 2004
Raw Milk Salers Cheeses	REP-PCR (*Rep-1R-Dt* plus *REP2-D*) ge-PCR (Deasy, et al. 2000), sp-PCR (Berthier and Ehrlich 1999; Berthier, et al. 2001; Ward and Timmins 1999)	I, M	Callon, et al. 2004
Scamorza Altamurana Cheese	Two step RAPD-PCR (*Coc*), 16S rDNA partial sequencing	I, M	Baruzzi, et al. 2002
Swiss cheese starter cultures	Triplex AP-PCR (*P32*), *Sma* I and *Apa* I REA-PFGE	C	Jenkins, et al. 2002

[a] The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12); sp-PCR, ge-PCR are species- and genus-specific PCR assays, respectively (Table 2.13).
[b] identification; C, characterization; M, monitoring.

Table 2.4 Molecular Approaches Used for the Identification, Characterization and Monitoring of Lactobacilli Isolated from Dairy Products

Source	Methods Applied[a]	Aims[b]	Reference
Different sources	multiplex sp-PCR (seven *Lactobacillus* species)	I, D	Kwon, et al. 2004
Different sources	Box-PCR (*BOXA1R*)	I, D (LD, ST)	De Urraza, et al. 2000
Parmigiano Reggiano cheese	RAPD-PCR (*M13* and *D8635*), sp-PCR (Ward and Timmins 1999)	I,C (LR)	Succi, et al. 2005
Spanish Goat Cheeses	RAPD-PCR (*OPL-05* and *P1*)	C	Sanchez, et al. 2005
Different sources	SDS-PAGE of WCPs fingerprints, *Not* I REA-PFGE, ARDRA-PCR, Ribotyping	C (LD)	Moschetti, et al. 1997
Natural Starter Cultures	RAPD-PCR (*D12554* and *M13*), *Not* I REA-PFGE	C (LDL)	Giraffa, et al. 2004
Different sources	SDS-PAGE of WCPs fingerprints, Ribotyping,*Sma* I REA-PFGE	C (LH)	Dimitrov, et al. 2005
Natural Whey Starter Cultures	SDS-PAGE of surface proteins fingerprints, IS1201 *Eco* RI-RFLP (Reinheimer, et al. 1996)	C (LH)	Gatti, el al. 2004
Natural Whey Starter Cultures and Cheeses	MLRT (eight housekeeping loci), and ARDRA-PCR	C (LH)	Borgo, et al. In Press
Different sources	Sequence analysis of S-layer-encoding genes	C (LH)	Gatti, el al. 2005
Different sources	MLST (six housekeeping loci), Ribotyping and 16S-23S rDNA ISR-RFLP	C (LP)	De Las Rivas, et al. 2006
Natural Whey Cultures	RAPD-PCR (Coc), 16S rDNA partial sequencing	C, M	Cocconcelli, et al. 1997
Ricotta Forte Cheese	Two step RAPD-PCR (*Coc*), 16S rDNA partial sequencing, 16S-23S rDNA sequencing (Berthier and Ehrlich 1998), presence of *prt*P gene (Klijn, et al. 1995)	C, M	Baruzzi, et al. 2000
Manchego cheese	RAPD-PCR (*OPL-05* and *P1*)	C, M	Sanchez, et al. 2006
Danbo Cheese	RAPD- PCR (*P1*) and TTGE (Vasques, et al. 2001)	C, M	Antonsson, et al. 2003
Different sources	RAPD-PCR (*Coc, M13* and *M14*) and AFLP	D (LP)	Torriani, et al. 2001a
Fiore Sardo Cheese	sp-PCR (Drake, et al. 1996b; Berthier and Ehrlich 1998; Ward and Timmins 1999)	I, M	Mannu, et al. 2000a
Comté Cheese	RAPD-PCR (*BO6, BO10*), REP-PCR (REP-1R-DT1 plus REP2-D), *Sgr* AI and *Xho* I REA-PFGE, sp-PCR (Drake, et al. 1996b)	I, M	Bouton, et al. 2002
Different sources	PCR detection of bacteriophages	C (LDL)	Zago, et al. 2006
Different sources	Identification and typing techniques	Review	Coeuret, et al. 2003

[a] The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12). sp-PCR, species-specific PCR assays (Table 2.13).

[b] I, identification; C, characterization; M, monitoring; D, differentiation. In parenthesis: LD, *Lb. delbrueckii*; LDL; *Lb. delbrueckii* subsp. *lactis*; LH, *Lb. helveticus*; LP, *Lb. plantarun*; LR, *Lb. rhamnosus* ST, *S. thermophilus.*

Table 2.5 Molecular Approaches Used for the Identification and Characterization of Streptococci Isolated from Dairy Sources

Methods Applied[a]	Aims[b]	Reference
Sequence analysis of *dna*J and *gyr*B genes	I, D	Itoh, et al. 2006
Sequence analysis of 16S-23S rDNA ITS, multiplex ITS-SSCP	I, D	Mora, et al. 2003
16S-23S rDNA ITS patterns, RAPD-PCR (*XD8* and *XD9*), *Sma* I REA-PFGE	I, D, C (EN also)	Moschetti, et al. 1998
RAPD-PCR (*D11344* and *M13*) and sp-PCR (this study)	I, D (SM)	Lombardi, et al. 2004
RAPD-PCR (*D11344* and *M13*) and sp-PCR (Lick, et al. 1996)	C (ST)	Andrighetto, et al. 2002
RAPD-PCR (*XD9*) and *galR*-*gal*K intergenic region sequence analysis	C (ST)	de Vin, et al. 2005
Sequences analysis of the *lac*SZ operon, group-specific PCR systems (strain-specific PCR, *lac*S-PCR-SSCP, *lac*S-PCR-DGGE)	C (ST)	Ercolini, et al. 2005
RAPD- PCR (*M13*) and sp-PCR (Lick, et al. 1996)	C (ST)	Giraffa, et al. 2001
sp-PCR (Lick, et al. 1996), RAPD- PCR (*OPI-02mod, XD9*), REA of epsC-D locus	C (ST)	Mora, et al. 2002a
Sma I, *Sfi* I, *Bss* HII *and Not* I REA-PFGE	C (ST)	O'Sullivan and Fitzgerald 1998

[a] The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12). sp-PCR, species-specific PCR assays (Table 2.13).

[b] I, identification; C, characterization; D, differentiation. In parenthesis: ST, *S. thermophilus*; SM, *S. macedonicus*; EN, enterococci.

Table 2.6 Molecular Approaches Used for the Identification, Characterization and Monitoring of Lactococci Isolated from Dairy Sources

Source	Methods Applied[a]	Aims[b]	Reference
Different sources	RAPD-PCR (*P1A, P2C* and *P3A*)	C (LL)	Tailliez, et al. 1998
Pecorino Sardo Cheese	sp-PCR (Garde, et al. 1999), Plasmid profile, *Sma* I REA-PFGE	I, C (LL)	Mannu, et al. 2000b
Different sources	sp-PCR (Nomura, et al. 2002), triplex RAPD-PCR (*P1A, P2A* and *P3A*), plasmid profile	I, C (LL)	Nomura, et al. 2006
Fior di Latte Cheese	16S-23S rDNA ITS patterns	I, D	Blaiotta, et al. 2002
Spain Starter-free Cheeses	RAPD-PCR (*RAP3*) *Mbo* II and *Hha* I ARDRA patterns, *Apa* I *or Sma* I REA-PFGE, SDS-PAGE of WCPs fingerprints	I, D (EN also)	Delgado and Mayo 2004
Fior di Latte Cheese	multiplex PCR (16S-23S rDNA ITS/*nis*A gene), *Sma* I REA-PFGE	M (LL *nis*+)	Moschetti, et al. 2001
Pecorino Sardo Cheese	sp-PCR (Ke, et al. 1999; Mannu, et al. 2000b), Plasmid profile, *Sma* I REA-PFGE	M (LL, EN)	Mannu and Paba 2002

[a] The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12). sp-PCR, species-specific PCR assays (Table 2.13).
[b] I, identification; C, characterization; D, differentiation; M, monitoring. In parenthesis: LL, L. lactis; EN, enterococci.

Table 2.7 Molecular Approaches Used for the Identification, Characterization and Monitoring of Enterococci Isolated from Dairy Sources

Source	Methods Applied[a]	Aims[b]	Reference
Portuguese cheese	RAPD-PCR (*M13* and *D8635*), proteins profile, fatty acids profile	C	Silva, et al. 2003
Different sources	Detection of virulence and vancomycin resistance gene markers by PCR	C	Khan, et al. 2005
Northwest Italian Dairy Products	RAPD-PCR (*M13* and *D8635*), sp-PCR (Dutka-Malen, et al. 1995)	I, C	Morandi, et al. 2006
Bryndza Cheese	sp-PCR (Dutka-Malen, et al. 1995; Knijff, et al. 2001), Detection of virulence genes by PCR (Dupre, et al. 2003; Jurkovic, et al. 2006)	I, C	Jurkovic, et al. 2006
Batzos Cheese	RAPD-PCR (*M13* and *D8635*), sp-PCR (Dutka-Malen, et al. 1995), *Sma* I REA-PFGE, plasmid profile	I, C	Psoni, et al. 2006
Fiore Sardo Cheese	RAPD- PCR (*XD9* and *M13*)	I, C.	Cosentino, et al. 2004
Artisanal Italian Cheeses	16S rDNA sequencing, DNA-DNA hybridization, 16S-23S rDNA ITS pattern	I, D	Fortina, et al. 2004
Different sources	MLSA (sequence analysis of *rpo*A, *phe*S and *atp*Agenes)	I, D	Naser, et al. 2005a,b
Semicotto Caprino Cheese	RAPD-PCR (*M13* and *D8635*)	I, M	Suzzi, et al. 2000
Cheddar-type Cheese	RAPD-PCR (*D11344*), *Sma* I REA-PFGE	I, M	Gelsomino, et al. 2002
Different sources	Identification and typing techniques	Review	Domig, et al. 2003

[a] The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12). sp-PCR, species-specific PCR assays (Table 2.13).

[b] I, identification; C, characterization; D, differentiation; M, monitoring.

Table 2.8 Molecular Approaches Used for the Identification and Characterization of Leuconostocs Isolated from Dairy Sources

Source	Methods Applied[a]	Aims[b]	Reference
Different sources	multiplex sp-PCR (5 species)	I, D	Lee, et al. 2000
Different sources	Protein profile, Ribotyping, ARDRA-PCR, *Apa* I REA-PFGE	I, D	Villani, et al. 1997
Different sources	RAPD-PCR (*Primm 239*), sp-PCR (this study)	I (LM)	Moschetti, et al. 2000
French Cheeses	RAPD-PCR (*P1A* and *P3A*), 16S rDNA sequencing, sp-PCR (this study)	C (LM, LCT)	Cibik, et al. 2000

[a] The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2-12). sp-PCR, species-specific PCR assays (Table 2-13).

[b] I, identification; C, characterization; D, differentiation. In parenthesis: LM, *Ln. mesenteroides*; LCT, *Leuconostoc citreum*.

Table 2.9 Molecular Approaches Used for the Identification and Characterization of Pediococci Isolated from Dairy Sources

Source	Methods Applied[a]	Aims[b]	Reference
No dairy origin	RAPD-PCR (*P1*)	I	Nigatu, et al. 1998
Different sources	Multilocus Hybridization Typing (16S rDNA, *rpo*C, *ldh*D, *ldh*L, and *met*S probes)	C (PAC)	Mora, et al. 2002b
Different sources	RAPD-PCR (*P1* and *P2*), *Apa* I, *Sma* I, *Asc* I, *Not* I, or *Sfi* I REA-PFGE	I, C	Simpson, et al. 2002

[a] The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12).

[b] I, identification; C, characterization. In parenthesis: PAC, P. acidilactici.

by Rossetti and Giraffa (2005), for LAB identification the 16S rRNA gene sequencing for some strains was needed.

RAPD-PCR and similar techniques may have the advantage of facilitating simultaneous strain typing, species affiliation determination and individual strain differentiation. Application of these techniques for analyzing isolates from the most important steps of cheese fermentation allowed monitoring of LAB species and biotypes within species during cheese manufacturing and ripening, and the detection of dominant biotypes in each fermentation phase (for references see Tables 2.3 to 2.11).

Morea, et al. (1999) monitored LAB during manufacture of Mozzarella cheese. Of the 25 RAPD-PCR biotypes found, only one (referable to *St. thermophilus* species) was detected in all samples analyzed (whey, curd, Mozzarella after shaping and after 24 hours of storage). Two other biotypes (referable *Enterococcus* spp. and *L. lactis* species, respectively) were found in curd and cheese samples. Coppola, et al. (2006) analyzed RAPD-PCR patterns of *L. lactis* strains from raw milk, curd and

Table 2.10 Molecular Approaches Used for the Identification and Characterization of Propionibacteria Isolated from Dairy Sources

Source	Methods Applied[a]	Aims[b]	Reference
Dairy	Partial Least Squares (PLS) regression analysis of RAPD-PCR patterns (*P1B* and *P2B*), 16S rDNA sequencing	I, C	Matte-Taillez, et al. 2002
Different sources	RAPD-PCR (*OPL-01, OPL-02* and *OPL-5*) and *Sma* I CGE-REA profiles analysis	I, C	Rossi, et al. 1998
Different sources	sp-PCR (this study)	I, D	Rossi, et al. 1999
Dairy	*rec*A gene sequence analysis	I, D	Rossi, et al. 2006
Swiss cheese starter cultures	Triplex AP-PCR (*P32*), *Spe*I and *Xba*I REA-PFGE	C	Jenkins, et al. 2002

[a] The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12). sp-PCR, species-specific PCR assays (Table 2.13).

[b] I, identification; C, characterization; D, differentiation.

Table 2.11 Molecular Approaches Used for the Identification and Characterization of Yeasts Isolated from Dairy Sources

Source	Methods Applied[a]	Aims[b]	Reference
Italian Dairy Products	RAPD-PCR (*M13* and *RF2*)	I, D	Andrighetto, et al. (2000)
Hungarian dairy products	18S rDNA/ITS1-RFLP, RAPD-PCR (*M13*)	I, D	Vasdinyei and Deak (2003)
Sardinian ewe's Dairy Products	no molecular techniques	OC	Cosentino, et al. (2001)
Raw Milk Salers Cheeses	26S rDNA gene sequencing, SSCP (of V4 region of 18S rDNA)	I	Callon, et al. (2006)
Smear-Ripened Cheeses	Mitochondrial DNA restriction fragment length polymorphism (mtDNA-RFLP), Fourier-transform infrared spectroscopy (FTIR)	I, C	Mounier, et al. (2005, 2006)

[a] The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12).

[b] I, identification; C, characterization; D, differentiation; OC, only the occurrence was evaluated.

Fior di latte cheese, and showed that five out of eight biotypes isolated from raw milk also persist during curd ripening. Moreover, isolates showing one unique RAPD-PCR pattern were detected in all the monitored phases. The results underlined the importance of raw milk as a source of important bacteria for fermentation. Moreover, statistical analysis of RAPD-PCR results can allow grouping of strains on the basis of their geographical and dairy origin (Moschetti, et al. 1998). RAPD-derived probes and primers have been described for identification at species level, and even at strain level (Quere, et al. 1997; Erlandson and Bat 1997).

Table 2.12 The Most Used RAPD-PCR Primers for the Characterization of Dairy Microorganisms

Name[a]	Sequence (5'—3')	Used for characterization of	Used by
BOXA1R	CTACGGCAAGGCGACGCTGACG	*Lactobacillus* *Streptococcus*	Versalovic, et al. 1994; De Urraza, et al. 2000
Coc	AGCAGCGTGG	LAB *Lactobacillus*	Cocconcelli, et al. 1995; Cocconcelli, et al. 1997; Morea, et al. 1999; Baruzzi, et al. 2000; Baruzzi, et al. 2002; Torriani, et al. 2001a; Gobbetti, et al. 2002
D11344	AGTGAATTCGCGGTGAGATGCCA	*Streptococcus* *Enterococcus*	Akopyanz, et al. 1992; Gelsomino, et al. 2001; Gelsomino, et al. 2002; Andrighetto, et al. 2002; Lombardi, et al. 2004
D1254	CCGCAGCCAA	Lactobacillus	Akopyanz, et al. 1992; Giraffa, et al. 2004
D8635	GAGCGGCCAAAGGGAGCAGAC	Lactobacillus Enterococcus	Akopyanz, et al. 1992; Suzzi, et al. 2000; Silva, et al. 2003; Succi, et al. 2005; Morandi, et al. 2006; Psoni, et al. 2006
M13	GAGGGTGGCGGTTCT	LAB Lactobacillus *Streptococcus* *Enterococcus* *Yeasts*	Huey and Hall 1989; Suzzi, et al. 2000; Andrighetto, et al. 2000; Giraffa, et al. 2001; Torriani, et al. 2001a; Andrighetto, et al. 2002; Silva, et al. 2003; Vasdinyei and Deak 2003; Cosentino, et al. 2004; Lombardi, et al. 2004; Giraffa, et al. 2004; Rossetti and Giraffa 2005; Succi, et al. 2005; Morandi, et al. 2006; Psoni, et al. 2006
M14	GAGGGTGGGGCCGTT	*Lactobacillus*	Torriani, et al. 2001a
OPI-02mod	GCTCGGAGGAGAGG	*Streptococcus*	Mora, et al. 2002a
OPL-01	GGCATGACCT	*Propionibacterium*	Rossi, et al. 1998
OPL-02	TGGGCGTCCAA	*Propionibacterium*	Rossi, et al. 1998
OPL-05	ACGCAGGCA	*Lactobacillus*	Sanchez, et al. 2005; Sanchez, et al. 2006
OPL-05A	ACGCAGGCAC	*Propionibacterium*	Rossi, et al. 1998
P1 (9mer, LPL)	ACGCGCCCT	*Lactobacillus* *Pediococcus*	Johansson, et al. 1995; Nigatu, et al. 1998; Fitzsimons, et al. 1999; Antonsson, et al. 2003; Simpson, et al. 2002; Sanchez, et al. 2005; Sanchez, et al. 2006
P1A (RAP3, BO6)	TGCTCTGCCC	*Lactobacillus* *Lactococcus* *Enterococcus*	Tailliez, et al. 1998; Mangin, et al. 1999; Cibik, et al. 2000; Bouton, et al. 2002; Delgado and Mayo 2004; Nomura, et al. 2006
P1B	CGGCCTGGAC	*Propionibacterium*	Matte-Tailliez, et al. 2002

(continued)

Table 2.12 The Most Used RAPD-PCR Primers for the Characterization of Dairy Microorganisms (continued)

Name[a]	Sequence (5'—3')	Used for characterization of	Used by
P2	ATGTAACGCC	*Pediococcus*	Fitzsimons, et al. 1999; Simpson, et al. 2002
P2A	GGTGACGCAG	*Lactococcus*	Mangin, et al. 1999; Nomura, et al. 2006
P2B	CGCCCTGCCC	*Propionibacterium*	Matte-Tailliez, et al. 2002
P2C	GTGACGCAG	*Lactococcus*	Tailliez, et al. 1998
P32	CAGCAGCCGCGGTAATWC	LAB	Cusick and O'Sullivan 2000; Jenkins, et al. 2002
P3A (BO10)	CTGCTGGGAC	*Lactobacillus* *Lactococcus* *Leuconostoc*	Tailliez, et al. 1998; Mangin, et al. 1999; Cibik, et al. 2000; Bouton, et al. 2002; Nomura, et al. 2006
PC1	AGCAGGGTCG	LAB	Poznanski, et al. 2004
Primm 239	CTGAAGCGGA	*Leuconostoc* *Lactococcus*	Moschetti, et. al. 2000; Coppola, et al. 2006
REP-1R-Dt	NCGNCGNCATCNGGC	LAB	Versalovic, et al. 1991; Callon, et al. 2004
REP-1R-DT1	IIINCGNCGNCATCNGGC	*Lactobacillus*	Bouton, et al. 2002
REP2-D	NCGNCTTATCNGGCCTAC	LAB *Lactobacillus*	Versalovic, et al. 1991; Callon, et al. 2004; Bouton, et al. 2002
REP-PCR	GTGGTGGTGGTGGTG	LAB	Zamfir, et al. 2006
RF2	CGGCCCCTGT	Yeast	Paffetti, et al. 1995; Andrighetto, et al. 2000
RP	CAGCACCCAC		Ward and Timmins 1999
		Lactobacillus	
XD8	CAAGGCATCC	*Streptococcus* *Enterococcus*	Moschetti, et al. 1998
XD9	GAAGTCGTCC	*Streptococcus* *Enterococcus*	Moschetti, et al. 1998; Mora, et al. 2002a; Cosentino, et al. 2004; de Vin, et al. 2005

[a] Some primers were grouped (different authors named primers showing the same sequence with different names) some others were renamed (primers with different sequences were named with same name).

REA-PFGE, performed by rare cutting endonucleases, has also been widely applied to type LAB isolates. The choice to use the endonuclease is of crucial importance to obtain reliable differentiation of the isolates. Endonuclease *Sma* I was used to type streptococci (Moschetti, et al. 1997; O'Sullivan and Fitzgerald 1998), enterococci (Gelsomino, et al. 2001; Psoni, et al. 2006), *L. lactis* (Moschetti, et al. 2001; Mannu, et al. 2000b; Delgado and Mayo 2004) and *Lb. helveticus* (Coppola, et al. 2006), *Apa* I for typing *Ln. mesenteroides* (Villani, et al. 1997), *Not* I for typing *Lb. delbrueckii* (Moschetti, et al. 1997; Giraffa, et al. 2004) and *Pediococcus* spp. (Simpson, et al. 2002). In some cases more than one endonuclease was used. Patterns obtained by *Sma* I and *Apa* I were analyzed by Jenkins, et al. (2002) to differentiate Swiss cheese starter culture strains of *Lb. helveticus, St. thermophilus* and *Prop. Freudenreichii*, and by Delgado and Myo (2004) to evaluate genetic diversities of *Lc. lactis* and *Enterococcus* spp. isolated from Spanish starter-free cheeses. *Sgr* AI and *Xho* I were applied by Bouton, et al. (2002) to monitor *Lb. helveticus* and *Lb. delbrueckii* subsp *lactis* strains isolated during Comtè cheese ripening. Simpson, et al. (2002) evaluated the discrimination power of different endonucleases (*Apa* I, *Sma* I, *Asc* I, *Not* I, *Sfi* I) for differentiation of *Pediococcus* spp. strains. REA-PFGE, albeit a laborious and expensive method, is highly reproducible and is, therefore, considered to offer the highest resolution for strain differentiation of LAB. Generally, analysis of RAE-PFGE patterns obtained by one well-chosen enzyme can provide fine, reliable differentiation. However, it has been suggested that analysis of two or three restriction enzymes should be used to differentiate *Lactobacillus* strains (Vancanneyt, et al. 2006c).

Blaiotta, et al. (2001) used REA-PFGE to monitor the addition of LAB, used as starter, to Cacioricotta cheese. By analyzing isolates from different phases of the fermentation the technique made it possible to evaluate the growth kinetics of each starter strain during the process.

Bouton, et al. (2002) used fingerprinting PCR-based methods and PFGE for typing and monitoring homofermentative lactobacilli during Comté cheese ripening. Isolates, which exhibited unique patterns by RAPD or REP-PCR, were distinguishable by PFGE. By contrast, some strains which were distinguishable by RAPD or REP-PCR were related by PFGE. These discrepancies were explained by the different exploration of DNA polymorphism (the whole DNA chromosome for PFGE, and region amplified by primers for RAPD and the REP-PCR). The use of second restriction enzymes would certainly be useful in this case.

Jenkins, et al. (2002), in analyzing genetic diversity in Swiss cheese starter cultures, found that strains with > 87 percent similarity by REA-PFGE consistently had the same acidification rate.

As it is a time-consuming technique, REA-PFGE was applied when fine strain typing was needed, when a small number of isolates have to be typed and when other strain typing techniques may be unreliable. Therefore, in many cases, it is applied as a supplementary technique to confirm or improve results obtained by other typing methods. Moschetti, et al. (1997) analyzed *Not* I-REA-PFGE patterns of *Lb. delbrueckii* subsp. *bulgaricus* isolated from commercial yogurt and showed that some strains isolated from products of different dairies displayed the same pattern,

suggesting that different dairies used the same starter. Similar results were obtained by Vancanneyt, et al. (2006c) who applied REA-PFGE to confirm and/or improve results obtained by AFLP analyzing *Lb. rhamnosus* strains isolated from different commercial probiotic preparation. Coppola, et al. (2006) analyzed *Lb. helveticus* strains isolated during manufacture of fior di latte cheese by RAPD-PCR and *Sma*I-REA-PFGE. Of 55 strains only four RAPD-PCR profiles were found by using primer Primm 239 (reliably used to differentiate *Lc. lactis* strains). Therefore, for a more appropriate biotyping, *Sma*I-REA-PFGE was applied. Using this last technique, a total of 13 different patterns were found. Also in this case, as already shown in *Lc. lactis* strains, strains showing the same profile were found in milk, in curd at the beginning of ripening and in curd at the end of ripening.

Overall, the most reliable method for strain differentiation is still REA-PFGE analysis and, therefore, its application is going to be fundamental for the monitoring of microorganisms in dairy processing.

The AFLP technique was used only to differentiate and characterize some species of the *Lb. plantarun* group (*Lb. plantarum, Lb. pentosus and Lb. paraplantarum*) by Torriani, et al. (2001a). Fluorescent AFLP (FAFLP) was also applied to type probiotic *Lb. rhamnosus* strains by Vancanneyt, et al. (2006c). The AFLP technique normally displays good levels of reproducibility and reliability – apart from some reported problems related to the initial DNA concentration or to the endonuclease or ligase treatment efficiency – but it is quite laborious and time-consuming, given that it requires two enzymatic reactions and large polyacrylamide gels to reach a good level of band separation. Although the observed strain-to-strain variations in the FAFLP patterns within a given cluster may reflect strain-specific differences, such variations are, in most cases, introduced during data processing. Therefore, for strain typing, FAFLP should be complemented by other fingerprinting techniques such as PFGE (Vancanneyt, et al. 2006c). However, FAFLP performed by multiple primer combination has proved to be a valid and powerful tool to reveal intraspecies diversities (Vancanneyt, et al. 2002). Recently, a simplified AFLP technique, called Sau-PCR, was applied to LAB fingerprinting (Corich, et al. 2005). Results suggest that Sau-PCR may be considered for DNA fingerprinting based on analyses as a possible alternative to the RAPD technique in cases where reproducibility or polymorphism levels are not satisfactory, and as an alternative to the AFLP technique, but with lower costs in terms of time and equipment, when a restriction-plus-amplification approach is preferred. However, AFLP, FAFLP and Sau-PCR were never used for typing large numbers of isolates from the dairy environment.

SDS-PAGE of WCPs was also applied to characterize cultivable dairy microflora. Villani, et al. (1997) evaluated diversities of *Ln. mesenteroides* strains isolated from dairy and non-dairy environments; Moschetti, et al. (1997) of *Lb. delbrueckii* isolated from yogurt, raw and pasteurized milks; Rossi, et al. (1998) propionibacteria from different dairy sources; Silva, et al. (2003) isolated enterococci from an artisanal Portuguese cheese, and Delgado and Mayo (2004) isolated lactococci and enterococci from Spanish starter-free cheeses. Piraino, et al. (2005) and Zamfir, et al. (2006) applied SDS-PAGE of WCPs to identify and characterize LAB occurring

in caciocavallo cheeses and Romanian dairy products, respectively. Finally, Piraino, et al. (2006) applied unsupervised and supervised artificial neural networks for the identification of LAB (*Lactobacillus, Leuconostoc, Enterococcus, Lactococcus* and *Streptococcus*) on the basis of their SDS-PAGE of the WCP pattern. SDS-PAGE of surface proteins was applied by Gatti, et al. (2004) to differentiate *Lb. helveticus* strains isolated from different natural whey starter cultures. However, there is some evidence of poor differentiation of some LAB species by this technique. De Angelis, et al. (2001), analyzing LAB of 12 Italian ewe's milk cheeses, showed that strains of *Lb. plantarum* and *Lb. pentosus* grouped in the same cluster. Gancheva, et al. (1999), analyzing a set of 98 strains belonging to nine species of the *Lb. acidiphilus* group had difficulty differentiating between *L. johnsonnii* and *Lb. gasseri* strains, and between those of *Lb. gallinarum* and *Lb. amylovorus*.

However, statistical analysis of SDS-PAGE of WCPs provides an effective tool for the classification and identification of LAB (Piraino, et al 2005 and 2006). By applying this technique Piraino, et al. (2005) demonstrated the possibility of discriminating PDO cheeses from non-PDO and showed that the microflora of PDO cheeses was less heterogeneous than that of non-PDO cheeses, and consisted mainly of non-starter LAB. Finally, in some cases the discrimination power of this technique was comparable to that of REA-PFGE (Delgado and Mayo 2004).

In addition to the above typing options, some authors have developed assays targeting genes encoding for key proteins or enzymes in food-borne bacteria. Gatti, et al. (2005) evaluated diversities of surface layer (S-layer) protein genes in *Lb. helveticus* strains and demonstrated that heterogeneity exists in genes of this species. However, cluster analysis of the sequences separated strains into only two main clusters. Ercolini, et al. (2005) evaluated sequence diversities of *lac*ZS operon of dairy *St. thermophilus* strains. Due to sequence polymorphism it was possible to design PCR-DGGE and PCR-SSCP systems allowing four and two groups, respectively, to be detected among strains analyzed. Moreover, a specific PCR system allowing detection of only one group of strains was designed. De Vin, et al. (2005), analyzing *gal*R-*gal*K (regulator and galactokinase genes, respectively) intergenic region of 49 *St. thermophilus* strains, found eight different genotypes. Of the latter, only four were related to the Gal-positive phenotype.

MLST (multi-locus sequence typing), which exploits the genetic variation present in six housekeeping loci, was recently applied to determine the genetic relationship among *Lb. plantarum* isolates (De Las Rivas, et al. 2006). Of the 16 strains analyzed, there were 14 different allelic combinations, with 12 of them represented by only one strain. MLHT (multi-locus hybridization typing) performed by five housekeeping gene probes was used by Mora, et al. (2002b) to subgroup *P. acidilactici* strains. MLRT (multi-locus restriction typing) analyzing restriction patterns from eight loci of housekeeping genes was applied by Borgo, et al. (2007) to characterize *Lb. helveticus* strains isolated from whey starter cultures and cheeses. High heterogeneity among strains was shown and an excellent association was observed between restriction profiles and origin of most of the isolates analyzed.

These last typing or sub-grouping approaches (Gatti, et al. 2005; Ercolini, et al. 2005; De Las Rivas, et al. 2006; Mora, et al. 2002b) have not yet been applied to

characterize or monitor wild strains isolated from dairy ecosystems. However, these techniques have reached a great level of automation and will surely have an important role in the rapid typing of bacteria of dairy origin.

Ribotyping was applied to evaluate genetic diversity of *Leuconostoc* spp. (Villani, et al. 1997), to differentiate *Lb. delbrueckii* subspecies (Moschetti, et al. 1997), to differentiate and characterize strains of *Lb. casei* group species (Svec, et al. 2005) and to subgroup *Lb. plantarum* strains (De Las Rivas, et al. 2006). Originally, ribotyping was intended for taxonomic use (Grimont and Grimont 1986), but it was also later applied for typing strains. However, due to its weak discriminatory power as a typing method, other techniques have replaced it. With a commercially available system, all stages of manual ribotyping can be performed and the basic protocol takes at least five days. However, the development of an automated ribotyping system, the RiboPrinter®, (Qualicon Inc., Wilmington, Del., U.S.) made it possible to shorten the procedure to eight hours. The process is highly standardized and data are stored electronically. In addition, data can be exchanged between different laboratories. Using more than one enzyme, the RiboPrinter® proved to be a valuable primary typing method for pathogens (Grif, et al. 2003). Research performed by Brunner, et al. (2000) provides evidence that PFGE and automated ribotyping are two reliable methods that can be useful for epidemiologic investigations on group A streptococci. Most strains belonging to the *Lb. casei* group and the *Lb. acidophilus* group were discriminated at the species level by automated ribotyping (Chun, et al. 2001). Massi, et al. (2004) compared automated ribopatterns of seven probiotic *Lactobacillus* strains (*Lb. acidophilus, Lb. delbrueckii* subsp. *bulgaricus, Lb. casei, Lb. plantarum, Lb. brevis, Lb. salivarius* subsp. *salicinius, Lb. gasseri*) with those reported in the RiboPrinter® database. All probiotic *Lactobacillus* strains gave specific new fingerprinting patterns, as none of them was included in the pre-existing ribogroups of the RiboPrinter® database. Due to the ribotyping specificity, the authors concluded that the method represents a powerful tool for strain-specific detection of these lactobacilli. However, Kitahara, et al. (2005), analyzing automated ribopatterns of 22 *Lb. sanfranciscensis* strains, obtained only four clusters at less than 80 percent similarity, while Basaran, et al. (2001) obtained only 10 different ribopatterns analyzing 20 lactococci. However, cluster analysis of data allowed differentiation of *Lc. lactis* subsp. *cremoris* strains from those of *Lc. lactis* subsp. *lactis*. Beaslay and Saris (2004) applied RiboPrinter® technology to differentiate nisin producing *Lc. lactis* strains isolated from human and cow's milk.

3.2 Identification at Genus, Species and Subspecies Level

Species identification can be achieved by statistical analysis of fingerprint data obtained from the above described approaches, even if they are commonly used for strain typing. Moreover, other techniques can be used to achieve identification at species level.

PCR-RFLP of the amplified 16S rRNA gene (ARDRA-PCR) or 16S-23S rRNA intergenic spacer region (ISR-RFLP-PCR) was also applied to identify or characterize dairy LAB. Villani, et al. (1997) analyzed *Eco RI* and *Hind III* ARDRA-PCR patterns of *Leuconostoc* spp. and showed that the technique is unreliable for species differentiation. By contrast, Aquilanti, et al. (2006) identified enterococci by analyzing *MboII, MspI* and *RsaI* PCR-ARDRA patterns. De Las Rivas, et al. (2006) found inconsistent differences on analyzing the ISR-RFLP-PCR of *Lb. plantarum* strains. Indeed, the latter approach was proposed to identify and differentiate *Lactobacillus* species (Moreira, et al. 2005). Baruzzi, et al. (2000) differentiated *Lb. planatrum* and *Lb. paraplantarum* after sequence analysis of the 16S-23S rDNA ISR. Flint and Angert (2006), on the basis of the 16S-23S rDNA ISR sequence, designed a strain-specific PCR primer to identify and monitor *Lactobacillus* spp. HOFG1 (closely related *L. animalis* or *L. murinus* species) in cattle feed.

16S-23S rDNA ISR pattern analysis allows differentiation of dairy streptococci, enterococci and lactococci (Moschetti, et al. 1998; Blaiotta, et al. 2002; Fortina, et al. 2003; Mora, et al. 2003) while it is unreliable for identification and differentiation of lactobacilli.

However, the continuously accumulating set of fingerprinting data and the construction of reliable databases require a high degree of standardization in experimental methodology. It is also important to have high-performing bioinformatic tools at one's disposal to get the best possible information from huge quantities of fingerprinting data. The development of bioinformatics has enabled improvement of the interpretation and elaboration of microbiological data. Many bioinformatic software programs or on-line tools, which are often commercially available, enable nucleic acid (or protein) sequences, fingerprinting profiles and phenotypic data to be analyzed and integrated. Some computerized databases of LAB fingerprints are also available, such as the RFLP database of total DNA patterns (Chan, et al. 2003), SDS-PAGE protein databases (Pot, et al. 1994; Leisner, et al. 1999) and the commercial RiboPrinter® system (Dawson 2001). Acquisition of specialized programs, which are expensive and demand a high level of technical skill for their efficient use, is necessary so that the most important international microbial collections can manage, compare and implement databases with information on genotypic and phenotypic data (Rossetti and Giraffa 2005).

For reliable identification of strains, partial or full 16S rRNA gene sequences have been extensively compared (see Tables 2.3 to 2.11). However, the 16S rRNA gene shows discrimination pitfalls in the identification of closely related LAB species. The genes present in only one copy, such as the Elongation factor Tu (*tuf*) gene (Chavagnat, et al. 2002; Ventura, et al. 2003), the DNA repair recombinase (*recA*) (Felis and Dellaglio 2005), the chaperonin Hsp60 (*Cpn*60) (Dobson, et al. 2004) and the RNA polymerase B subunit (*rpoB*) (Rantsiou, et al. 2004), have been exploited for the differentiation of *Lactobacillus* species. These genes have significant advantages over the 16S rRNA gene because of their species-discrimination power, indicated by published studies to be one order of magnitude higher than that of the 16S rRNA gene (Ventura, et al. 2003). Itoh, et al. (2006) performed sequence analysis of *dna*J (a member of the Hsp70 protein family) and *gyr*B (the B-subunit

of DNA gyrase, topoisomerase type II) genes of streptococci and concluded that they are efficient alternative targets for the classification of the genus *Streptococcus*, and that *dna*J is suitable for phylogenetic analysis of closely related *Streptococcus* strains. Goh, et al. (2000) sequenced a 552-bp region of the chaperonin 60 gene (*Cpn*60) and demonstrated that clustering of the analyzed species is similar to the published *Enterococcus* trees based on 16S rRNA gene sequences. Poyart, et al. (2000) partially sequenced the gene encoding manganese-dependent superoxide dismutase (*sod*A) in 19 enterococcal-type strains. Their results confirm that the *sod*A gene constitutes a more discriminative target sequence than the 16S rRNA gene and allows differentiation among closely related bacterial species. Rossi, et al. (2006) suggest that the *rec*A gene can be used as an alternative to the 16S rRNA gene as a target for detecting/identifying propionibacteria species, but it is less reliable as a molecular marker for their classification and intraspecies distinction.

Species-specific single or multiplex PCR assays were designed and used for rapid identification of LAB occurring in dairy products (Tables 2.3 to 2.11). As reported in Table 2.13, specific PCR systems are now available for the most important bacterial species occurring in dairy products. Moreover, PCR was also used to detect specific genes encoding for particular traits. Some of these systems are: detection of the *prt*P gene (coding for a cell envelope proteinase in LAB) (Klijn, et al. 1995); detection of virulence or resistance factors in enterococci (Khan, et al. 2005; Domig, et al. 2003); detection of genes involved in the production of biologically active amines such as histamine, tyramine and putrescine in LAB (Fernandez, et al. 2004; Marcobal, et al. 2005; Aymerich, et al. 2006).

Table 2.13 Some Available Genus- and Species-specific PCR Assays for the Rapid Identification and/or Differentiation Dairy Microorgamisms

Genera	Level of specificity	References
Lactobacillus (*Lb.*)	*Lb. plantarum* group species differentiation	Torriani, et al. 2001
	Lb. helveticus	Fortina, et al. 2001
	Differentiation of*Lb. casei* group species	Ward and Timmins 1999
	Lb. plantarum, Lb. curvatus and *Lb. sakei*	Berthier and Ehrlich 1998
	Lb. acidophilus, Lb. delbrueckii, Lb. casei, Lb. gasseri, Lb. plantarum, Lb. reuteri and *Lb. rhamnosus*	Kwon, et al. 2004
	Lb. delbrueckii subsp. *lactis* and *Lb. delbrueckii* subsp. *bulgaricus*	Torriani, et al. 1999
	Lb. helveticus, Lb. paracasei and *Lb. rhamnosus*	Tilsala-Timisjarvi and Alatossava 1997
	Lb. fermentum, Lb. casei/paracasei, Lb. plantarum, Lb. reuteri and *Lb. salivarius*	Chagnaud, et al. 2001
	Lb. acidophilus, Lb. casei, Lb. brevis	Massi, et al. 2004
	Lb. brevis	Guarneri, et al. 2001
	*Lb. casei, Lb. delbrueckii and Lb. helveticus/ acidophilus*groups	Drake, et al. 1996a-b

(continued)

Table 2.13 Some Available Genus- and Species-specific PCR Assays for the Rapid Identification and/or Differentiation Dairy Microorgamisms (continued)

Genera	Level of specificity	References
Streptococcus (*S.*)	*S. thermophilus*	Lick, et al. 1996
	S. macedonicus	Papadelli, et al. 2003
	S. macedonicus	Lombardi, et al. 2004
Enterococcus (*E.*)	*Enterococcus* spp.	Deasy, et al. 2000
	E. faecium and *E. faecalis*	Dutka-Malen, et al. 1995
	Enterococcus spp.	Ke, et al. 1999
	E. faecium	Cheng, et al. 1997
	E. durans and *E. hirae*	Knijff, et al. 2001
Lactococcus(*Lc.*)	*Lactococcus* spp.	Deasy, et al. 2000
	Lc. lactis subsp. *lactis* and *Lc. lactis* subsp. *cremoris*	Corroler, et al. 1998
	Lc. lactis (histidine biosynthesis operon)	Corroler, et al.1999
	Lc. garvieae	Zlotkin, et al. 1998
	Lc. lactis	Mannu, et al. 2000b
	Lc. lactis subsp. *lactis* and *Lc. lactis* subsp. *cremoris*	Garde, et al. 1999
	Lc. lactis subsp. *lactis* and *Lc. lactis* subsp. *cremoris*	Nomura, et al. 2002
	Species and subspecies of*Lactococcus*genus	Pu, et al. 2002
Leuconostocs(*Ln.*)	*Ln. mesenteroides* subsp. *mesenteroides*	Moschetti, et al. 2000
	Ln. carnosum, *Ln. citreum* and *Ln. mesenteroides*, *Ln. gelidum* and *Ln. lactis*.	Lee, et al. 2000
	Ln. mesenteroides, Ln. lactis, Ln. citreum and *Weissella paramesenteroide.s*	Cibik, et al. 2000
Propionibacterium	Identification and differentiation of dairy propionibacteria and *P. acnes*	Rossi, et al. 1999

4 Concluding Remarks and Perspectives

In conclusion, the use of molecular tools in dairy microbiology has improved the knowledge on the succession of microbial strain and species during cheese manufacture and ripening. The culture-independent techniques can provide a rapid assessment of the microbial diversity while del culture-dependent molecular methods are of invaluable help in defining and monitoring microbial biotypes during important phases of cheese making. The sequence-based identification is expected to increase its impact and potential owing to the availability of high throughput sequencing platforms. The major perspective in the nearest future is the possibility to monitor not only the microorganisms, but also their activities during dairy fermentations. Owing to the introduction of real-time PCR systems coupled with the appropriate procedure of RNA extraction from food, it will hopefully be possible to understand which of the important activities are being carried out in a certain phase of the production and which are the environmental stresses affecting such activities. Therefore, the future target of food microbiology will be not only the wondering of "who is there," but also

"who is doing what" with the final aim of improving technological processes and cheese quality and safety. Chemiometric and sensorial attributes should be associated to the evaluation of microbial diversity and activity in all the phases of interest to get a clear idea of the real association between microbiota, microbial metabolism and cheese quality. Therefore, only combined efforts of these approaches with proteomics, chemiometric measurements and sensorial evaluation can elucidate at an exploitable extent the complex and dynamic processes discussed here.

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