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Role of *Lactobacillus rhamnosus* in Parmigiano Reggiano cheese ripening: a genotypic and post-genomic study

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To my family, frieds and professors

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1. INTRODUCTION

1.1 Parmigiano Reggiano cheese

Parmigiano Reggiano (PR) is an important Italian cheese produced in a limited geographic area in Northern Italy. It is appreciated for its nutritional and sensorial properties and has a Protected Designation of Origin (PDO) in compliance with the EC Regulation 2081/9 (Gala et al., 2008; Malacarne et al., 2008; Gatti et al 2003). For different reasons, PR production system represents a unique dairy system. Nowhere else in Europe 1.7 million tons of milk (representing 15% of the Italian milk market) into a high quality product in 450 cheese dairies are processed, using predominantly artisan production techniques (http://www.parmigiano-reggiano.it). The high labor input required either in dairy farms and in the cheese dairies creates much more employment than any other dairy system: about 20.000 men and women are involved. The numbers of this artisan system highlight its relevant importance for the Italian economy: the PR cheese production is able to sustain economic development in less favored areas and has a significantly better environmental impact than industrial dairy farms. The final quality of PR is heavily dependent on the ability of the cheese-maker to process the different qualities of raw milk (which varies from season to season and from farm to farm) only using the dairy-based whey starter and rennet (http://www.parmigiano-reggiano.it).

PR is a semi-fat hard cheese characterized by a cooked, slowly and long matured paste. It is made up with raw and partly skimmed cow's milk (Gala et al., 2008; Gatti et al., 2003) from animals principally fed with forage from the area of origin. Only raw, unheated milk can be used and any additive is strictly forbidden (Malacarne et al., 2008). The evening collected milk and the morning one are delivered to the dairy within two hours from the end of each milking and the milking time of each one of the two milkings allowed daily must be limited within 4 hours of time. Milk should be cooled immediately after milking and kept at a temperature not below 18°C. The evening milk is partly skimmed by removing the cream naturally arisen to the surface in open-top stainless steel basins. The morning milk, just after arriving at the dairy, is mixed with the partly skimmed milk from the previous evening. It may also be partially skimmed by removing the naturally risen cream.

A maximum of 15% of the morning milk may be kept for processing the following day. In this case, the milk must be kept in the dairy in suitable refrigerated containers, equipped with special agitators and at a minimum temperature of 10°C, and poured into the resting basins in the evening of the same day.

Milk is then added with natural whey starter consisting of a natural culture of thermophilic lactic acid bacteria (SLAB) most of them acid-producing. They are obtained from the spontaneous

acidification of the whey left after the previous day's cheese processing and recovered overnight at a natural decreasing temperature gradient (Mucchetti and Neviani 2006). The inoculated milk is heated at 32-34°C, added with calf rennet powder (chymosyn preparation that contains less than 3-4% of pepsin) and coagulated within 8-12 min. The milk curdling takes place inside copper vats shaped like truncated cones, exclusively using the calf rennet. After curdling, the curd is broken up into grains and cooked. When the curd reaches the proper firmness it is broken down into minuscule granules and heated up to 53-56°C, depending from specific different dairy technology, under stirring in about 5-15 min. During cooking the acidifying activity of natural whey starter lactic acid bacteria, together with heating effects, favors the formation of the proper texture of curd granules and whey drainage. When the heating is turned off, the curd granules deposit at the bottom of the vat where they will aggregate together in about 30-50 min under the whey, at a temperature no higher the one reached at the end of the cooking process (Mucchetti and Neviani 2006). The high temperatures (53-55°C) used during curd cooking select the thermophilic LAB that so becomes the dominant microflora: thermophilic lactobacilli reach a concentration higher than 10^8 cfu ml^{-1} (Neviani et al. 1997) and *Lb. helveticus* is usually the main species encountered (Gatti et al., 2003).
These curd grains are then left to settle to the bottom of the vat in order to form a compact mass. The cheese mass is subsequently placed into special moulds for the moulding process and, after a few days, cheeses are immersed in a water and salt saturated solution. Maturation must last at least 12 months, starting from the cheese moulding. In summer the temperature of maturation rooms must not be lower than 16°C (http://www.parmigiano-reggiano.it).
PR cheese has the following features:

- cylinder shape with slightly convex to straight sides, upper and lower faces slightly chamfered;
- dimensions: diameter of upper and lower faces from 35 to 45 cm; side height from 20 to 26 cm;
- minimum wheel weight: 30 kg;
- external appearance: natural gold-colored rind;
- paste color: from pale straw-yellow to straw-yellow;
- typical aroma and taste of the mass: fragrant, delicate, tasty yet not sharp;
- paste texture: fine granules, breaks in brittles;
- rind thickness: approximately 6 mm;
- fat content: minimum 32 % of dry matter.

The area of production includes the territory of the provinces of Parma, Reggio Emilia, Modena and Mantova on the right bank of the Po river and Bologna on the left bank of the Reno river.

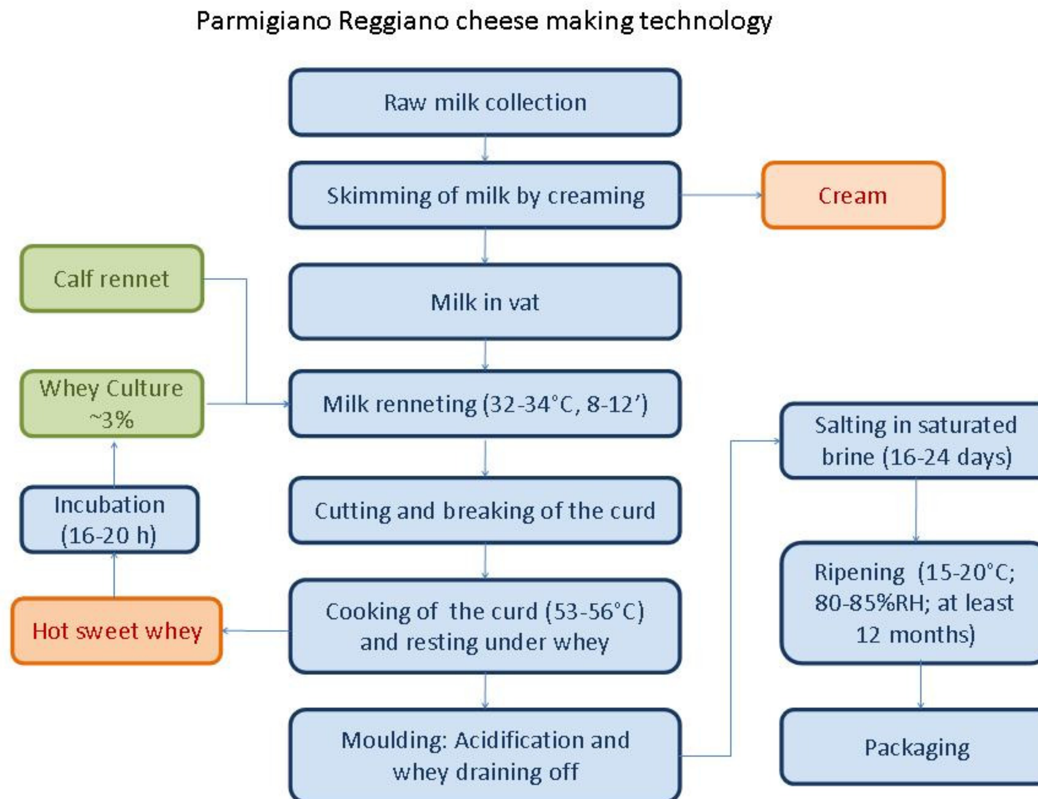

Figure 1. **Flowsheet of Parmigiano Reggiano cheese-making** (Neviani E., unpublished)

1.1.1 Microbiology of Parmigiano Reggiano cheese-making

Parmigiano Reggiano (PR) microflora arises from milk and from natural whey starter.

Milk is a nutritionally rich medium where all microbial groups, generally associated with food matrices, may be found: pathogenic, spoilage and useful microorganisms. Within the last group, lactic acid bacteria (LAB) are commonly isolated at significant concentrations (Franciosi et al., 2009). They are naturally present in milk as contaminants by the udder surface, milking equipment, stable environment and/or during transport and filling operations, storage surfaces, and dairy factory environment (Eneroth et al., 1998; Mc Phee and Griffiths, 2002).

Several species belonging to *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* genera are often recognized from raw milk (Franciosi et al., 2009; Wouters et al., 2002). LAB may play different roles in cheese-making: some species participate in the fermentation process, whereas some others are implicated in the maturation of cheese. In the first case, LAB rapidly ferment lactose producing high concentrations of lactic acid and are designated as starter LAB (SLAB), while LAB, which are not linked to acid production during the first hours of cheese-

making but involved in ripening process, are indicated as non-starter LAB (NSLAB). Table 1 reports the main SLAB and NSLAB isolated during cheese manufacturing and ripening.

Microflora	Genera/specie	Characteristics	Reference
SLAB	*Lactococcus lactis* *Leuconostoc spp.*	Mesophilic	Fox et al., 2004
	Streptococcus thermophilus *Lactobacillus delbrueckii* *Lactobacillus helveticus*	Thermophilic	
	Leuconostocs	Non lactobacillus	Chamba and Irlinger, 2004
NSLAB	*Lactobacillus farciminis*	Obligate homofermentative	Gobbetti et al., 2002; Coeuret et al., 2004; Svec et al., 2005
	Lactobacillus casei *Lactobacillus paracasei* *Lactobacillus plantarum* *Lactobacillus pentosus* *Lactobacillus curvatus* *Lactobacillus rhamnosus*	Facultative heterofermentative	
	Lactobacillus fermentum *Lactobacillus buchneri* *Lactobacillus parabuchneri* *Lactobacillus brevis*	Obligate heterofermentative	
	Pediococcus acidilactici, *Pediococcus pentosaceus* *Enterococcus durans* *Enterococcus faecalis* *Enterococcus faecium*	Non Lactobacillus	Chamba and Irlinger, 2004

Table 1. **SLAB and NSLAB isolated during cheese manufacturing and ripening** (Settanni et al., 2010).

Evolution of SLAB and NSLAB in raw milk cheese follows a general dynamic. SLAB are highly represented at the beginning of ripening and decrease regularly during ageing (Beuvier and Buchin,

2004; Franciosi et al., 2008). On the contrary, NSLAB are present at low concentrations after pressing but may increase within a few months (Fox et al., 2004).

In general, LAB are the dominant microflora in dairy products. Particularly, in the case of long ripened cheese, such as PR, it is well known that different LAB species and biotypes, arising from raw milk and starter, contribute, both as entire and lysed cells, to the biochemical events involved in cheese ripening. PR SLAB originating from natural whey starter are characteristic of the first hours of cheese production: they produce the lactic acid that influences important qualitative characteristics such as texture, moisture content and inhibition of pathogenic microorganisms. Subsequently, the mesophilic NSLAB microflora, originating from raw milk and the environment, start to increase after SLAB autolysis in a successive phase of cheese aging and becomes characteristic of cheese ripening (Addeo et al., 1997; Neviani et al., 1997; Gatti et al., 2008; De Dea Lindner et al., 2008). These adventurous NSLAB species, mainly involved in flavor formation, begin to increase when nearly all residual lactose in cheese has already been utilized by SLAB (Dìaz-Muñiz et al., 2006) and their composition varies with the processing applied to milk and with the age of cheese (Randazzo et al., 2009).

Consequently, the quantitative and qualitative microbial composition of a ripened cheese represents a dynamic environment resulting from the different microbial growth capacities in milk and curd and linked to the effects of the technological pressure and bacterial cell autolysis (O'Cuinn et al., 1995; Fox et al., 1996; Addeo et al., 1997). Microbiological characteristics of PR have been traditionally studied by Coppola and colleagues (1997; 2000), which isolated many strains from a great number of samples representative of each step of PR cheese production and ripening, by using traditional growth media. These studies demonstrate that a variegated lactic acid microflora participates in the ripening process. The LAB isolated from milk was essentially made up of facultative heterofermentative mesophilic strains, with a predominance of *Lb. paracasei* ssp. *paracasei* and of some strains ascribable to *Lb. paracasei* ssp. *tolerans*. The microflora of the starter was mainly composed by *Lb. helveticus* and, to a less extent, by some strains of *Lb. delbrueckii* ssp. *bulgaricus* and of *Lb. delbrueckii* ssp. *lactis*, *Lb. rhamnosus* and *Lb. brevis*. In both curd and whey after cooking, mainly *Lb. helveticus* and *Lb. paracasei* ssp. *paracasei* were found. Among lactococci, *Lc. lactis* ssp. *lactis* was predominant together with *Lc. raffinolactis*, *Lc. plantarum* and *Lc. lactis* ssp. *cremoris*. Among enterococci, *E. faecalis* was the predominant species accompanied by *E. faecium*. Among Micrococcaceae, *Kocuria kristinae*, *K. rosea*, *Kytococcus sedentarius*, *Arthrobacter agilis* and certain strains ascribable to *Staphylococcus* spp. have been isolated (Coppola et al., 2000). Other studies have focused on the biodiversity of different strains of *Lb.*

helveticus isolated from natural whey cultures (Gatti et al., 2003; Gatti et al., 2004). Investigations on microbial population through traditional agar-based methods typically reveal the most commonly occurring microorganisms and, among them, only those able to grow to a detectable level by forming colonies in the specific cultural conditions adopted. On the other hand, traditional cultural media aimed to recover the majority of microorganisms could be too generic and not selective enough to differentiate species, which are differently represented. In fact, some LAB strains, overall all NSLAB, could be present in little amounts in raw milk, in curd or in cheese. For these reasons, nutritionally complete media may underestimate the less abundant components of microflora that could be equally important for cheese ripening and flavor development (Neviani et al., 2009). It has already been underlined that the characterization of naturally occurring bacterial species present in cheese made from raw milk and the study of the dynamics of the floras present during the ripening process necessitate the use of reliable culture media (Denis et al., 2001). At this regard, in the case of a long ripened cheese, as an alternative to commercial synthetic agar-media, Gatti et al. (2003) and Fornasari et al. (2006) used sterilized sweet whey as a base constituent to produce two cultural media. These media resulted to better recovery the injured or strictly adapted to the whey environment SLAB microbial population, which in contrast was completely underestimated in MRS and M17 agar.

By the means of these culture dependent studies, the microbial ecology of ripened cheeses was not completely understood (Gatti et al., 2006).

Microbiological ecology and dynamics of PR have recently been studied using a culture-independent method, named length heterogeneity PCR (LH-PCR), to monitor the microbial dynamics during 24 months of PR ripening for both the whole and lysed cells. The availability of PR twin wheels allowed to have samples representative of the subsequent stages of the same cheesemaking process. This study showed that the thermophilic microflora of natural whey starter for PR is mainly composed by *Lb. helveticus* and *Lb. delbrueckii* ssp. *lactis* or ssp. *bulgaricus*. Authors observed that, after two months of ripening, whole cells of *Lb. helveticus* and *Lb. delbrueckii* ssp. *lactis* or ssp. *bulgaricus* were found in great amounts but none of these species was isolated from agar plates. These cells could be quiescent, might be viable but not cultivable, and might still not be lysed. In addition to these species, also *Lb. rhamnosus*, *Lb. casei*, or *Lb. plantarum* and *Lb. parabuchneri* or *P. acidilactici* were able to grow after 2 months of ripening. After 6 months of ripening, the same species were found even if no one of them seems to be dominant and from the sixth to the twentieth month of ripening any microbial evolutionary change

were observed. In the 24-month cheese whole-cell electropherogram, the major peak was attributable to *Lb. rhamnosus*, *Lb. casei*, or *Lb. plantarum* (Gatti et al., 2008).

The same samples have been studied using a culture dependent approach: Neviani and colleagues (2009) gave a partial picture of the typical microflora of PR cheese by studying the cultivability of LAB associated with its manufacturing and ripening. The highest number of LAB species was recovered from MRS medium. In particular, 7 different species, 1 SLAB (*Lb. helveticus*) and 6 NSLAB (*Lb. kefiri*, *Lb. fermentum*, *Lb. reuteri*, *Lb. plantarum*, *P. acidilactici* and *Lb. rhamnosus*) were cultivated in this medium. The first 6 species were found in the first 48 h of cheese production while *Lb. rhamnosus* was not cultivated in these samples using this medium. Whey agar medium (WAM) allowed to cultivate six different species, two SLAB (*Lb. helveticus*, *Lb. delbrueckii* ssp. *lactis*) and four NSLAB (*Lb. fermentum*, *Lb. casei*, *P. acidilactici* and *Lb. rhamnosus*). 5 of these species were found in the first 48 h of cheese production and *Lb. rhamnosus* was not cultivated using this medium in these samples. An innovative ripened cheese-based medium (CAM) recovered the lowest number of species (*Lb. fermentum*, *Lb. plantarum*, *P. acidilactici* and *Lb. rhamnosus).* All four species were isolated in the first 48 h of cheese production and, in particular, *Lb. rhamnosus* resulted to be the species more isolated (65% of the isolates), independently by the media used. However, it was possible to isolate this species on MRS and WAM only in the samples after brining, while it was isolated using CAM also from whey starter, milk and curd during the first 48 h of cheese production. Differently from MRS-vancomicin agar, which is a selective medium suitable to enumerate *Lb. rhamnosus* (Tharmaraj and Shah, 2003), the use of a cheese-based medium may promote the study of the microflora that better adapt to the changes in nutritional availability and technological parameters during production and ripening (Neviani et al., 2009).

1.1.2 *Lactobacillus rhamnosus*

Lb. rhamnosus is a facultative hetero-fermentative LAB frequently encountered in many fermented foods and beverages, including cheeses, fermented milks, baked goods, sausages and various vegetable juices. It is also one of the most intensively studied probiotic organisms (Saxelin et al., 2005; Bernardeau et al., 2008) and the discovered health-promoting effects associated with its consumption include a reduction in the risk for acute diarrhea in children (Szajewska et al., 2001) and atopic diseases (Kalliomaki et al., 2003) as well as relief for milk allergy/atopic dermatitis in infants (Isolauri et al., 2000; Majamaa et al., 1997; Viljanen et al., 2005), reduction of the risk of respiratory infections (Gluck and Gebbers, 2003; Hatakka et al., 2001) and the occurrence of dental caries (Nase et al., 2001). However, the molecular mechanisms underlying the positive effects of *Lb.*

rhamnosus GG (LGG) and other probiotic organisms on human health are poorly understood (Koskenniemi et al., 2009).

In Parmigiano Reggiano, *Lb. rhamnosus* has been isolated, and its DNA detected, from the beginning of ripening and becomes dominant from the 2th month to the 20th month of cheese ripening (Figure. 2) when most of the residual lactose in cheese has already been consumed by SLAB (De Dea Lindner et al., 2008; Gatti et al 2008; Neviani et al., 2009).

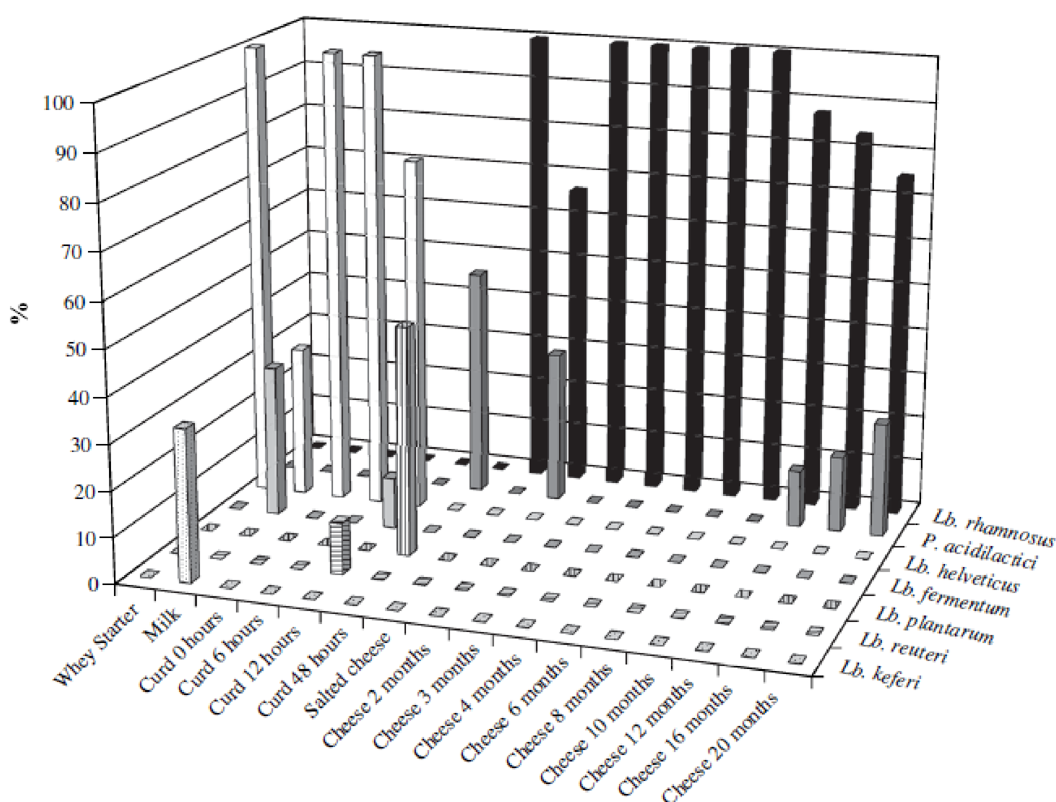

Figure 2. **Percentage of each LAB species on the total of strains isolated on MRS medium for samples at different cheese manufacturing and ripening times.** Bars – Dotted: *Lb. kefiri*; Horizontal lines: *Lb. reuteri*; Vertical lines: *Lb. plantarum*; Pale grey: *Lb. fermentum*; White: *Lb. helveticus*; Dark grey: *P. acidilactici*; Black: *Lb. rhamnosus* (Neviani et al. 2009).

In this environment *Lb. rhamnosus* has to use non conventional energy sources (Succi et al., 2005) but which kind of energy sources is used by NSLAB during cheese ripening are still debating. Potential sources could be amino acids, organic and fatty acids, glycerol or carbohydrates released from glycomacropeptide (GMP) (galactose, N-acetylgalactosamine and N-acetylneuraminic acid), glycoproteins, glycolipids of the milk-fat globule membrane (MFGM) (galactose, mannose, fucose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid) or from lysed cells of SLAB (ribose, deoxyribose, N-acetylglucosamine and N-acetylmuramic acid) (De Dea Lindner et

al., 2008; Neviani et al., 2009; Mather, 2000; Williams et al., 2000; Laht et al., 2002). The versatile adaptability of *Lb. rhamnosus* to different ecosystems (e.g., fermented foods, and human and animal gastrointestinal tracts) is probably related to its ability to regulate metabolic pathways and/or to constitutive genetic intra-specie variability. Comparative genome analysis of the *Lactobacillus* species indicates the combination of gene gain and gene loss occurs during environmental adaptation (Cai et al. 2009; Goh and Klaenhammer, 2009). *Lb. rhamnosus* and *Lb. plantarum* have the largest size of chromosome (3.0 and 3.3 Mb, respectively) which has recently been sequenced and annotated and contain a large number of regulatory and transport functions (Morita et al., 2009;). Anyway, there is a lack of information about *Lb. rhamnosus* metabolism under particular nutritional conditions that is necessary to improve with further investigations.

It is known that LAB typically have multiple amino acid auxotrophies and, therefore, are dependent on the transport of amino acids and/or transport and hydrolysis of exogenous peptides for growth. Growth to high cell-densities in milk relies on casein-derived peptides and amino acids provided by the proteolytic system (Savionky et al., 2006; Koskenniemi et al., 2009). However, environmental conditions of ripened cheese are only partially comparable with milk. Recently, a proteomics approach revealed that *Lb. rhamnosus* was able to produce several components of the proteolytic system. This study showed that two aminopeptidases, PepN and PepC, as well as an oligopeptidase PepF1, were more abundant in cells during the growth in whey, whereas a dipeptidase PepD, a dipeptidyl aminopeptidase PepX, and an oligopeptidase PepF2, as well as the oligopeptide transport protein OppD were more abundant in *Lb. rhamnosus* cells during growth in MRS (Koskenniemi et al., 2009). Even if whey is only partially comparable with cheese environment, these results evidence that potential sources could be also obtained by the hydrolysis of exogenous peptides like casein-derived peptides.

1.2 Molecular approaches for identification and characterization of microbial communities and LAB in cheese

The denomination of Protected Designation of Origin cheese (PDO), such as PR, assumes that a link exists among the area of origin, the traditional cheese-making procedures and the specific characteristics of the final product. Among their denomination, it is interesting to highlight the differences which exist among cheeses varieties, whose sensorial and texture characteristics are greatly influenced by microbial population, mainly composed by LAB, which are naturally present in raw milk, selected during manufacture, or added as natural cultures following the back-slopping practice (Randazzo et al., 2009). Hence, characterizing the cheese microbial population, with

special attention to LAB community, may contribute to understand the ecological processes that drive microbial interaction in cheese.

Further, the main interest of cheese microbiologists is to study the diversity and the dynamics of microorganisms during cheese manufacture and ripening, and try to correlate the occurrence of certain bacterial species and/or strains with specific flavor and sensorial traits of the final product. Traditionally, most knowledge of bacterial diversity in cheese is derived from culturing studies, based on microorganisms' growth on selective media and their subsequent identification at genus/ species level using phenotypic characterization. Although these methods may be reasonably sensitive, they do not always allow the discrimination of species or strains, nor the detection of the phylogenetic relationships among certain groups of bacteria. Hence, the application of molecular methods, in particular, the polymerase chain reaction (PCR)-based fingerprinting techniques, is useful in answering ecological questions (Justé et al., 2008). Although the use of these techniques is still in development, their application has already been shown to be a powerful tool for determining the structure of microbial communities in different environments and monitoring changes in microbial communities. At present a wide range of molecular approaches are available to study cheese communities. Molecular techniques, especially PCR-based methods, such as Repetitive Element Sequence-based (REP)-PCR fingerprinting and restriction fragment length polymorphism (RFLP) as well as pulse-field gel electrophoresis (PFGE) are regarded important for the specific characterization and detection of LAB strains (Holzapfel et al., 2001). Denaturing gradient gel electrophoresis (DGGE) and the temperature gradient gel electrophoresis (TGGE) analysis of the fecal 16S ribosomal DNA (rDNA) gene and its rRNA amplicons have shown to be powerful approaches in determining and monitoring the bacterial community in feces (Zoetendal et al., 1998). These techniques can be divided into: (i) cultivation-dependent techniques based on cultivation followed by phenotypic and molecular identification; and (ii) cultivation-independent molecular methods. In Figure 3, a schematic outline of most frequently used molecular approaches, used to study cheese microbial population, is illustrated.

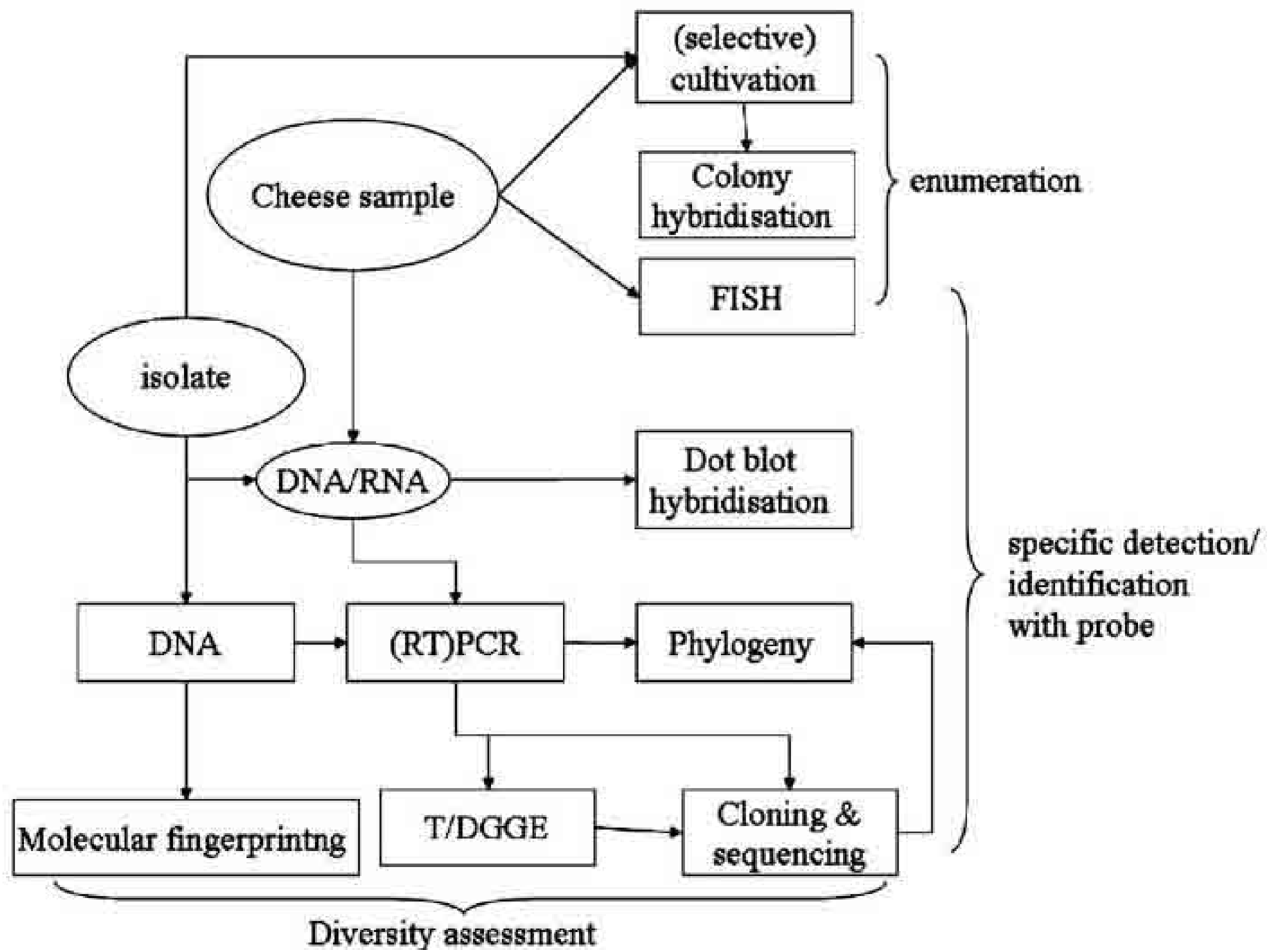

Figure 3. Flow chart of current molecular approaches used singularly or in combination to analyze cheese microbial communities (Randazzo et al., 2009).

1.2.1 Application of molecular methods in microbial communities

1.2.1.1 Denaturing Gradient Gel Electrophoresis (DGGE) and Temporal Temperature Gradient Electrophoresis (TGGE)

The general principle of Denaturing Gradient Gel Electrophoresis (DGGE) and Temporal Temperature Gradient Electrophoresis (TTGE) is the separation of individual *rRNA* genes based on differences in their chemical stability or melting temperature. Polyacrylamide gels consisting of a linear denaturing gradient formed by urea and formamide are employed for DGGE, whereas a linear temperature gradient is used during TGGE. The rRNA heterogeneity among bacterial species and even strains can be utilized in the fingerprinting of bacterial communities. Ribosomal RNA (rRNA) or total DNA is extracted from cheese samples, and fragments of this rRNA or DNA are amplified using universal primers. The separation of PCR amplicons, which are similar in size but different in

sequence and that are specific to a given species, is performed by DGGE or TTGE, offering a unique and comprehensive tool for the characterization of bacterial communities and their dynamics in a culture-independent way (Jany and Barbier, 2008). DGGE/TTGE of PCR-amplified *rRNA* gene amplicons offers several potential advantages over culture techniques as a method to monitor mixed bacterial populations. The main advantages of DGGE/TTGE are that it enables the monitoring of the spatial/temporal changes in microbial community structure and provides a simple view of the microbial species within a sample. However, it is noteworthy to state that there are some limitations associated with the use of PCR-DGGE/TTGE analysis of microbial community structure. Firstly, the 16S rDNA fragments with different sequences may have similar melting behavior and co-migrate, and resulting bands at the same position in the gel are not necessarily phylogenetically related (Muyzer et al., 1993). Band intensity may not truly reflect the abundance of microbial population, and perceived community diversity may be underestimated. Moreover, the amplified fragment of *16S rRNA* gene could generate several bands on a DGGE gel, instead of a single band that is representative of that particular species. In this case, the heteroduplexes can be eliminated by the analysis of the *rpoB* gene, or of other genes, which appear to exist in one copy only in bacteria (Dahllof et al., 2000) and the resolution may be improved by narrowing the gradient.

DGGE analysis has been successfully applied to study the diversity, the dynamics and the activity of bacterial community of several artisanal cheese types. In the Pecorino Siciliano cheese the technique allowed to reveal dramatic shifts in the bacterial community structure throughout the whole production and the dominance in the final products of *S. bovis* and *Lc. lactis* species (Randazzo et al., 2006). The development of molecular techniques based on sequence variability in *16S* and *23S rRNA* genes has led to an improved understanding of the microbial communities present in a variety of ecosystems, including different dairy products (Coppola et al., 2001; Ercolini et al., 2003; Randazzo et al., 2002).

1.2.1.2 Length Heterogeneity-PCR (LH-PCR)

Strain fingerprinting methods can characterize environmental LAB populations after strain isolation, allowing the construction of computerized databases of strains fingerprinting (Chan et al., 2003). The recently development of culture-independent methods offers a further technique to monitor LAB populations during the ensiling process (Brusetti et al., 2006). Lenght heterogeneity-PCR (LH-PCR) analysis is a culture-independent method and distinguishes different organisms based on natural variations in the length of the *16S rRNA* gene sequences. The technique combines selective PCR amplification of target gene, high-resolution electrophoresis and fluorescent detection. The

application of LH-PCR technique in ecological studies is still limited; it has been applied to depict the population structure within the LAB community present in controlled dairy ecosystems such as whey starters from PR and Grana Padano cheeses in order to identify different organisms (Lazzi et al., 2004).

1.2.1.3 Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and Single Strand Conformation Polymorphism (SSCP) analyses

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and Single Strand Conformation Polymorphism (SSCP) analyses are based on specific target sites for restriction enzymes, and on the secondary structure of single stranded DNA, respectively. T-RFLP analysis is a rapid and sensitive molecular approach that may assess subtle genetic differences between strains and may provide an insight into the structure of microbial communities. The technique combines selective PCR amplification of target genes with restriction enzyme digestion, high-resolution electrophoresis and fluorescent detection. The use of one fluorescently labelled primer restricts the analysis to the terminal fragment only. The absolute quantification is difficult to carry out since the obtained signal depends both on the absolute number of existing cells, and on the relative number of group cells to be quantified compared with other existing cells (Rademaker et al., 2006). The main advantages of T-RFLP over other community analysis techniques, such as DGGE/TGGE, are the improved resolution afforded by nucleic acid sequencing, compared to electrophoresis system ones, and the digital output. The use of this profiling technique has gained great popularity in cheese ecology (Rademaker et al., 2005).

SSCP is an electrophoretic technique which has been developed for the detection of mutations in genes and has been used to follow the dynamics of the global microbial population of a complex ecosystem. The rapidity and automation of SSCP method by capillary electrophoresis, compared to other molecular techniques, such as TGGE or DGGE, allows to analyze the microbial dynamics of different samples without the requirement of several gel conditions to separate sequences. However, similarly to DGGE/TGGE analyses, SSCP provides community fingerprints, which cannot be phylogenetically assigned. The principle of SSCP is that the mobility of a single-stranded DNA fragment is dependent on the secondary structure of the fragment. The secondary structure is determined by nucleotide sequence and the physiological environment (e.g., temperature, pH and ionic strength). A typical SSCP profile consists of two single-stranded DNA fragments and one double-stranded DNA fragment, although different conformations from one strand are also possible.

The main drawback of the technique is that the unknown bands on the gel cannot be sequenced directly, as in DGGE/TGGE, because volumes used during the analysis are too small and the DNA fragments cannot be recuperated.

SSCP has been applied in several ecosystems and, recently, it was used to analyze cheese microbial community. For example, the V2 or V3 variable region of the *16S rRNA* gene was used for SSCP fingerprinting of microbial community dynamics during the production and ripening of the RDO Salers cheese, a French farmhouse cheese produced exclusively with raw milk (Duthoit et al., 2005a,b).

1.2.2 Genotypic identification of LAB

Several molecular typing techniques have been developed during the past decade for the identification and classification of bacteria at or near the strain level. The major advantages of these DNA-based typing methods lie in their discriminatory power (Farber, 1996) and in their universal applicability. Among these, the most powerful are the genetic based molecular methods, known as DNA fingerprinting techniques, e.g., pulsed-field gel electrophoresis (PFGE) of rare-cutting restriction enzyme fragments, ribotyping, randomly amplified polymorphic DNA (RAPD), RFLP, DGGE, TGGE and amplification rDNA restriction analysis (ARDRA) and amplified fragment length polymorphism (AFLP) (Mohania et al., 2008). These methods have been extensively applied for the intraspecific identification and for genotyping of LAB isolated from several fermented foods as well as from human gastrointestinal tract (McCartney, 2002). These techniques have completely revolutionized the detection of DNA/RNA in microbial ecological studies, since they are not influenced by cells' physiological state and remain constant during the cells' growth. They also allow identification below the species level, and have been used to track specific bacteria and to reveal the diversity of bacterial populations, including dairy microbiota (Giraffa and Neviani, 2000). Nevertheless, fingerprinting techniques require prior cultivation, just like any conventional method. Moreover, the greatest hazards in studying the composition of mixed population include insufficient or preferential cell lysis, PCR inhibition, differential amplification and formation of chimeric or artefactual PCR products. A number of strategies are available to reduce and/or alleviate such biasing, e.g. incorporating PCR facilitators in the reaction, performing multiple reactions with different “universal” primer sets, or controlling the PCR conditions, such as number of cycles and elongation time (McCartney, 2002). The selection of the most appropriate typing method depends on cost, high throughput capacity and fingerprints reproducibility. Genetic fingerprinting techniques that are currently used for typing dairy LAB are described below.

1.2.2.1 Gene sequencing of the *16S RNA* gene

Macromolecules have been described as documents of evolutionary history and for decades they have been used to explore the phylogenetic diversity and evolutionary relatedness of organisms. The *16S rRNA* gene is the most common gene targeted in bacterial diversity studies. It is a well-conserved universal marker with constant and highly constrained functions that were established at early stages in its evolution and it is relatively unaffected by environmental pressures. These facts, along with the size of the gene, make it a good evolutionary clock (Kimura et al., 1997).
Sequence analysis is used to provide information about the nucleotide sequence of the *16S RNA* gene. Several programs to determine the closest relative of the DNA sequence are available on internet sites. Mostly these programs use homology searches provided by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) or FASTA (http://biogate.mlg.co.jp/tssfree/Fasta.html). The information on rRNA sequences has proved to be effective for comparative identification of microorganisms, leading to recognition of thousands of microbial species, including LAB species. However, DNA sequencing is generally expensive and requires a high degree of technical competence to perform. Moreover the *16S rRNA* gene shows discrimination pitfalls in the identification of closely related species. Firstly the *16S rRNA* genes are so well conserved that it results in a limited resolving power (Achenbach et al., 2001). Secondly even though the *16S rRNA* gene is a universal marker different bacterial species have different copy numbers of the gene. This leads to an over- and under-representation of some bacterial species when using *16S rRNA* genes as targets. Many genes other than *16S RNA*, present only in a single copy, have also been exploited for the differentiation of LAB species. Some of these genes are: the elongation factor Tu (*tuf*) gene (Ventura et al., 2003), the DNA repair recombinase (*recA*) gene (Felis and Dellaglio, 2005), the RNA polymerase B subunit (*rpoB*) gene (Rantsiou et al., 2004), and the chaperonin HSP60 (*cpn60*) gene (Dobson et al., 2004).

1.2.2.2 Restriction Fragment Length Polymorphism (RFLP)

Most genetic fingerprinting techniques are based on Restriction Fragment Length Polymorphism (RFLP) analysis, a profiling tool based on the banding patterns obtained from genomic or rRNA restriction digests. Because of the high specificity of restriction enzymes and the stability of chromosomal DNA, a reproducible pattern of fragments is obtained after the complete digestion of the chromosomal DNA by a particular enzyme. These variations in the banding patterns between strains are ascribed to basic differences in the DNA base composition of the organism examined. One general criticism about this method is the complexity of banding pattern. Nevertheless, using

the right enzyme and specified conditions RFLP could still be a relatively rapid and reliable technique. 16S rDNA-RFLP offers an effective and rapid method to isolate and distinguish the LAB coccus genera, such as the *Enterococcus*, the *Lactococcus* and the *Leuconostoc* genera, offering more correct results (Yanagida et al., 2005). Furthermore, PCR-RFLP has been successfully used to identify LAB species commonly isolated from wine (Claisse et al., 2007). Mainville and colleagues (2005) also isolated and characterized the LAB of kefir using phenotypical, biochemical, and genotypical methods. Polyphasic analyses of the results permitted the identification of the microflora to the strain level, indicating that a RFLP-based polyphasic analysis approach increased confidence in strain determination by helping to confirm strain groupings, and hence could have an impact on the phylogeny of the strains (Mainville et al., 2005). Deveau and Moineau (2003) also used RFLP for the differentiation and rapid characterization of *Lc. lactis* strains producing exopolysaccharides by analysis of their DNA restriction patterns, and concluded that the availability of such an effective RFLP-based cataloging system could benefit research aimed at identifying lactococcal strains. Further, RFLP analysis has been applied for the identification of SLAB isolated from different natural whey starter for PR cheese to demonstrate the presence of different *Lb. helveticus* biotypes related to a specific cheese ecosystem (Giraffa et al 2000; Gatti et al., 2003).

1.2.2.3 Pulsed Field Gel Electrophoresis (PFGE)

The crucial element of this technique is the selection of the restriction enzymes. Use of rare-cutting enzymes reduces the number of DNA fragments, but usually requires more sophisticated techniques, such as Pulsed-Field Gel Electrophoresis (PFGE), in order to separate the large DNA fragments in a continuously reorienting electric field (McCartney, 2002). The generated DNA fingerprint obtained depends on the specificity of the restriction enzyme used and on the sequence of the bacterial genome and is therefore characteristic of a particular bacterial species or strain. This fingerprint represents the complete genome and thus can detect specific changes (DNA deletion, insertions, or rearrangements) within a particular strain over time. Its high discriminatory power has been useful for the differentiation of strain belonging to the same LAB species, group-strain within a species, and to distinguish between strains of different LAB species, and even to place isolates in specific *Lactobacillus* species. Blaiotta et al. (2001) used Restriction Endonuclease Analysis by Pulsed-Field Gel Electrophoresis (REA-PFGE) to monitor the addition of LAB as starters to the "Cacioricotta" cheese by analysing isolates taken during the different phases of the fermentation. PFGE has been shown to be discriminatory in typing the closely related *Lb. delbrueckii* and *Lb. helveticus* strains (Bouton et al., 2002). The choice of the endonuclease is important to obtain

reliable isolate differentiation. However, it has been suggested that analysis of two or three restriction enzymes should be used to differentiate *Lactobacillus* strains (Vancanneyt et al., 2006). Nevertheless, due to the labor intensity, PFGE is not a feasible technique for large scale typing of isolates. Thus, only using a combination of several molecular techniques, a more effective strain differentiation is possible.

1.2.2.4 Ribotyping

Ribotyping, a variation of conventional RFLP, allows less complex patterns to be generated since the genomic DNA is digested by restriction enzymes and the resulting fragments are transferred to a membrane where they are hybridized with an rDNA probe. The probes used in ribotyping vary from partial sequences of the DNA genes or their spacer regions to the whole rDNA operon, which could be used as a probe in a classical multi-step southern blotting, or in automated ribotyping. If the probe contains conserved regions of rDNA, it can be used for the ribotyping of a wide range of bacteria, even those that are phylogenetically distant. Evidently, more fragments hybridize with probes that encompass a large region of the rDNA operon than with shorter probe. Thus, the discriminatory power of this technique depends on the size of the probe, but also on the restriction enzyme(s) used. Ribotyping allows to relate the presence of specific strain genotypes with the dairy ecosystem source, emphasizing the importance of raw milk as a source of strain genetic diversity in the PDO cheeses (Corroler et al.,1998; Bouton et al., 2002). Ribotyping has been shown to be a useful tool in differentiating *Lb. helveticus* strains in natural whey starters of Grana and Provolone cheeses (Giraffa and Neviani, 2000) and, recently, to differentiate *Enterococcus* and *Lactobacillus* strains isolated from a São Jorge cheese, an artisanal Portuguese cheese (Kongo et al., 2007).

1.2.2.5 Randomly Amplified Polymorphic DNA (RAPD)

Arbitrary amplification, also known as RAPD, has been widely reported as a rapid, sensitive, and inexpensive method for genetic typing of different strains of LAB (Ben Amor et al., 2007). The RAPD technique is a PCR-based discrimination method in which a short arbitrary primer anneals to multiple random target sequences, resulting in patterns of diagnostic value. In RAPD analysis, the target sequence(s) to be amplified is unknown and a primer with an arbitrary sequence (a 10-base pair sequence or a 10-bp sequence randomly generated by computer) is designed and synthesized. After these sequences have been synthesized they are used in PCR reactions with low-stringency annealing conditions, which results in the amplification of randomly sized DNA fragments that are separated by electrophoretic gel to give a fingerprint.

RAPD is currently being explored for the identification of LAB, including probiotic strains, and it is the most widely used technique in cheese environment, allowing the differentiation between species and, to some extent, also between strains within the same species. Thus, RAPD analysis is considered a reliable method to discern between starter and non-starter species in cheese or to monitor shifts in LAB community during cheese fermentation.

Thermophilic streptococci and lactobacilli were detected by RAPD analysis in several Italian, France and Spanish cheese types, such as Mozzarella cheese, made both from raw buffalo and bovine milk (Moschetti et al., 1998; Morea et al., 1998; De Candia et al., 2007), PR (Gatti et al., 2008) and Grana Padano cheeses (Cocconcelli et al., 1997), Beamfort, Comte and Manchego cheese types (Bouton et al., 2002; Sánchez et al., 2006). The presence of several strains belonging to *S. thermophilus* and *S. macedonicus* in Italian PDO cheeses (Asiago d'Allevo, Fontina, Montasio and Monte Veronese cheeses) was confirmed by using the RAPD (Andrighetto et al., 2002). In the Alturamura Scamorza cheese, a traditional stretched-curd cheese in Southern Italy, manufactured with the same method of Mozzarella cheese, RAPD technique, in combination to plating count, allowed the detection of *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* throughout the fermentation process (Baruzzi et al., 2002). RAPD analysis has been successfully performed to reveal (1) the heterogeneity of *Lb. kefir*, and *Lb. paracasei* in the Ricotta forte cheese (Baruzzi et al., 2000); (2) to show the diversity of strains within the *Lb. plantarum* species in Canestrato Pugliese cheese (Albenzio et al., 2001) and Roncal cheese type (Oneca et al., 2003); and (3) to evaluate the genetic diversity of lactococcal strains isolated from raw milk in RDO Camembert cheese (Corroler et al., 1998; Desmasures et al., 1998), Mozzarella cheese (Morea et al., 1999), Toma Piemontese cheese (Fortina et al., 2003), Raschera, and Castelmagno PDO cheeses (Dolci et al., 2008a,b).

The RAPD technique was used to type LAB strains isolated from Pecorino Toscano cheese where it was even possible to correlate the specificity of the microflora, which included mainly *S. thermophilus*, with the origin of the milk (Bizzarro et al., 2000). Nevertheless, De Angelis and colleagues (2001) demonstrated that the combination of 3 primers, which allowed the differentiation of 10 NSLAB type strains, did not find a species-specific DNA band which permitted complete separation of all the species considered. Recently, Coppola and colleagues (2006) analyzed RAPD-PCR patterns of *Lc. lactis* strains isolated from raw milk, curd and “Fior di latte” cheese, a traditional raw milk pasta filata cheese of the Naples area, and showed that 5 of 8 biotypes isolated from raw milk also persist during curd ripening. Statistical analysis of RAPD profiles can allow grouping of strains on the basis of their geographical and dairy origins (Moschetti et al., 1998). Some reports included the use of RAPD analysis, combined with other molecular

techniques, to determine strain diversity of *Leuconostoc* spp. isolated from Spanish and French cheeses (Cibik et al., 2000; Perez et al., 2002) and to provide a complete picture of the high heterogeneity between *E. faecium* and *E. faecalis* strains present in Pecorino Sardo and Caprino cheeses (Mannu et al., 1999; Suzzi et al., 2000).

Although RAPD is a simple and rapid technique a careful optimization and standardization are needed to improve its reproducibility. Differences in thermal cycles, DNA polymerases and their concentrations, DNA preparation methods, primer to template ratios and magnesium concentrations can cause variations in the RAPD patterns and, consequently, patterns obtained in different laboratories are not always comparable (Randazzo et al., 2009).

1.2.2.6 Repetitive Element Sequence-based PCR (REP-PCR)

Repetitive Element Sequence-based PCR (REP-PCR), unlike RAPD protocols in which patterns are obtained using a short non-specific primer, is based on the amplification of short repetitive sequence elements dispersed through the chromosome of diverse species (Versalovic et al., 1991).

This technique was applied to monitor the addition of starter cultures in Cheddar cheese (Dasen et al., 2003) and, most recently, to characterize NSLAB during the ripening of Zlatar cheese, an artisanal home-made cheese manufactured from raw cow's milk without the addition of starter culture (Terzic-Vidojevic et al., 2007). Although this genotypic fingerprinting method has been successfully applied to the identification and taxonomic classification of a high number of LAB, providing new insights in the distribution of bacterial species and/or strains in cheese ecology, it is known to be laborious and time-consuming and the outcome can be highly variable between laboratories.

1.2.2.7 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Amplified Ribosomal DNA Restriction Analysis (ARDRA) is essentially the reverse of ribotyping, i.e., the RFLP of 16S rRNA PCR amplicons.

It is a rapid technique based on the PCR amplification of the gene encoding 16S rRNA with universal primers and restriction of the amplicon, commonly using tetrameric endonucleases to generate multiple restriction fragments (Marilley and Casey, 2004). However, ribotyping generally affords greater discriminatory power than ARDRA due to the inclusion of the flanking regions of the *16S rRNA* genes in the fingerprint (Mohania et al., 2008). ARDRA analysis has successfully differentiated various species or strains within the *Lb. acidophilus* complex, *Lb. casei*, *Lb. delbrueckii*, *Lb. fermentum*, *Lb. helveticus*, *Lb. plantarum*, *Lb. reuteri*, *Lb. rhamnosus* and *Lb. sakei*

(Holzapfel et al., 2001; Giraffa and Neviani, 2000). Furthermore, this approach has been used to differentiate a variety of lactobacilli at species level, including *Lb. delbrueckii* and its three subspecies (*bulgaricus*, *delbrueckii* and *lactis*), *Lb. acidophilus* and *Lb. helveticus* (Roy et al., 2001).

1.2.2.8 Intergenic Spacer Region (ISR)

The use of the 16S–23S rRNA Intergenic Spacer Region (ISR) (Gurtler and Stanisich, 1996) is an alternative to ARDRA for detecting heterogeneity among and within species. Heterogeneity has been found in terms of both the number and the length of the spacers. The spacer is amplified by PCR with primers designed to anneal conserved regions flanking the 16S–23S rRNA ISR, usually located at the 3V-end and 5V-end of 16S and 23S rDNA, respectively. The discriminative power of this method was shown to be at the species level (Jensen et al., 1993). This approach has therefore broad applicability as a rapid, automatable method for bacterial identification and typing.

1.2.2.9 Amplification Fragment Length Polymorphism (AFLP)

Amplification Fragment Length Polymorphism (AFLP) combines the power of RFLP with the flexibility of PCR-based methods by ligating primer-recognition sequences (adaptors) to the digested DNA. Total genomic DNA is digested using two restriction enzymes, one with an average cutting frequency and a second with higher cutting frequency. Double-stranded nucleotide adapters are usually bound to the DNA fragments serving as primer binding sites for PCR amplification. The use of PCR primers, complementary to the adapter and the restriction site sequence, yields strain-specific amplification patterns (Vos et al., 1995). AFLP methods rapidly generate hundreds of highly replicable markers from the DNA of the organism. Thus, AFLP provides time and cost efficiency, allowing genotyping and fingerprinting with high reproducibility and high resolution. Originally developed for plant systematics, AFLP has been found to be a very useful fingerprinting technique for bacteria, applicable for species resolution as well as for strain differentiation. AFLP has been employed mostly in epidemiological studies and in investigations aiming to distinguish virulence markers in food-borne pathogens (such as *L. monocytogenes* and *Salmonella* ssp.). Regarding LAB, species-level discrimination has been shown for the phylogenetically closely related species *Lb. pentosus*, *Lb. plantarum* and *Lb. pseudoplantarum* using this method (Giraffa and Neviani, 2000). Further, AFLP analysis has been successfully applied to study the genetic diversity of *S. thermophilus* (Lazzi et al., 2009) and *Lb. plantarum* (Di Cagno et al., 2010).

1.3 Genomics and Post-Genomics studies in LAB

With the advances and cost reducing of DNA-sequencing, the genome determination becomes available for many microorganisms. Genomics is a recent conceptual approach for the biological study of microorganisms, which relies on the analysis of the complete genetic information they contain. This scientific discipline really emerged with the characterization of the first complete genome of the autonomous organisms *Haemophilus influenzae* (Fleischmann et al., 1995) and *Methanococcus jannaschii* (Bult et al., 1996). These studies were almost immediately followed by others describing laboratory model eukaryotes, i.e. *Saccharomyces cerevisiae* (Goffeau et al., 1996), and bacteria, i.e. *Escherichia coli* (Blattner et al., 1997) and *Bacillus subtilis* (Kunst et al., 1997). Such projects which initially required many years and a huge amount of work were markedly facilitated by numerous technological developments that came of age at the end of the 1990s. (Bertin et al., 2008). Moreover, the DNA sequence data represents only the first critical step in mining the bacteria genome. From 1995 the availability of the entire gene set of any organism facilitates the investigation of that organism at a global level. In tandem with the large-scale genome sequencing projects, new technologies, that allow the analysis of gene and protein expression profiles across the entire genome, are being developed. Functional genomics refers to the technologies that incorporate the functional analysis of the DNA sequence information. This new holistic approach to cell biology has produced new terminology. Biologists now refer to the transcriptome, the complement of mRNAs transcribed from a cell's genome and their relative levels of expression in a particular cell under a defined set of conditions; and the proteome, the complete complement of proteins encoded by the genome. The derived technologies, such as transcriptomic and proteomic, exploit the sequence data to produce genome-wide transcription and protein expression profiles (Callanan, 2005).

1.3.1 Genomics studies in LAB

Genetics research in "food-grade" LAB began in the early 1970s, during which period four basic types of genetic elements were characterized in dairy LAB: plasmid DNA, transposable elements, bacteriophages, and complete chromosomes. Genome sequence information for the first of several industrially important LAB starter species appeared in 2001, when Sorokin and coworkers released the genomic DNA sequence for *Lc. lactis* IL1403. Genome sequence information for several other important dairy LAB is also now available. To date, 25 LAB genomes (15 *Lactobacillus*, three *Lactococcus*, three *Streptococcus*, two *Leuconostoc*, one *Pediococcus*, and one *Oenococcus*) have been sequenced and published (Table 2) while 67 projects are in progress (59 *Lactobacillus*, three

Lactococcus, three *Leuconostoc*, one *Oenococcus*, and one *Streptococcus*) (Zhu et al., 2009). Recently The genome of *L. rhamnosus* ATCC 53103 has also been sequenced and annotated (Morita et al., 2009). The analysis of genomic sequences has shown that LAB have relatively small genomes (1.8–3.3 Mb) and the numbers of protein encoding genes differ from 1700 to 3200.

The availability of complete genomes for all major branches of *Lactobacillales* enables a more definitive analysis of their evolutionary relationships. Makarova et al. (2006) constructed phylogenetic trees from concatenated protein sequences, an approach shown to improve the resolution and increase robustness of phylogenetic analyses (Figure. 4).

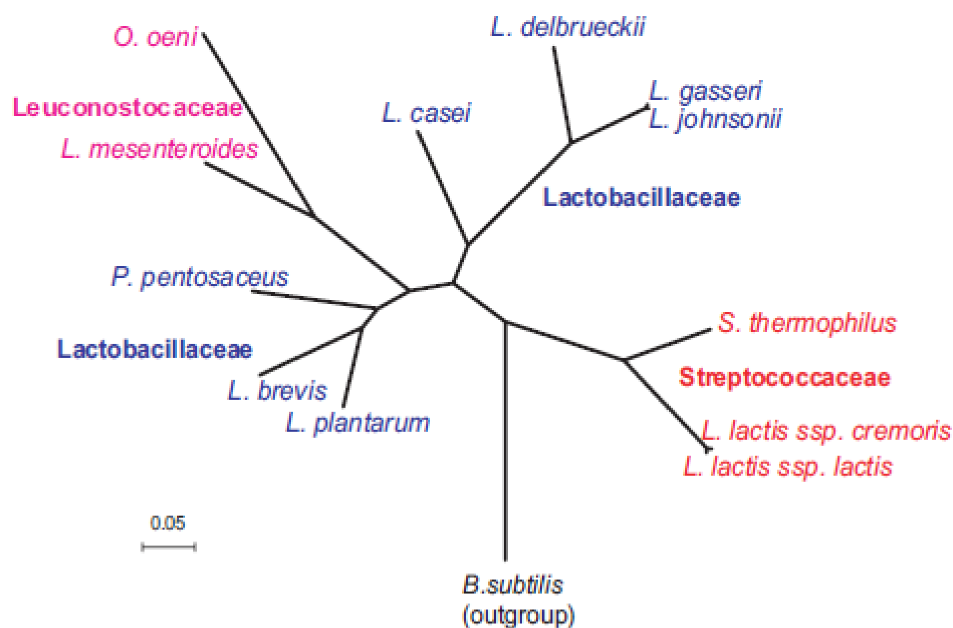

Figure 4. **Phylogenetic trees of *Lactobacillales* constructed on the basis of concatenated alignments of ribosomal proteins** (Makarova et al., 2006).

Strain names	Origin/usage	NCBI access no.	GC content (%)	Length (Kb)	Genome size (Mb)	No. of genes	No. of proteins	No. of pseudogenes	References
Lactobacillus acidophilus NCFM[a]	Probiotics	NC_006814	34.7	1,993	1.99	1,936	1,862	0	Altermann et al. (2005)
Lactobacillus brevis ATCC 367[b]	Beer fermentation, sourdough starter culture	NC_008497	46.2	2,291	2.34	2,314	2,218	52	Makarova et al. (2006)
		NC_008498 (pLVIS1)	38.6	13		12	11	1	
		NC_008499 (pLVIS2)	38.5	36		25	22	3	
Lactobacillus casei ATCC 334[a]	Swiss cheese, probiotics	NC_008526	46.6	2,895	2.92	2,909	2,751	82	Makarova et al. (2006)
		NC_008502 (pLSEI1)	42.2	29		20	20	0	
Lactobacillus casei BL23[b]	Starter culture, probiotics	NC_010999	46.3	3,079	3.08	3,044	3,044	0	
Lactobacillus delbrueckii ssp. *bulgaricus* ATCC 11842[c]	Yogurt fermentation	NC_008529	49.7	1,864	1.86	2,217	1,562	533	van de Guchte et al. (2006)
Lactobacillus delbrueckii ssp. *bulgaricus* ATCC BAA-365[c]	Yogurt fermentation	NC_008529	49.7	1,857	1.86	2,040	1,721	192	Makarova et al. (2006)
Lactobacillus fermentum IFO 3956[b]	Probiotics, animal & plant material	NC_010610	51.5	2,098	2.10	1,912	1,843	0	Morita et al. (2008)
Lactobacillus gasseri ATCC 33323[c]	Probiotics	NC_008530	35.3	1,894	1.89	1,898	1,755	43	Azcarate-Peril et al. (2008); Makarova et al. (2006)
Lactobacillus helveticus DPC 4571[c]	Cheese flavor development	NC_010080	37.1	2,080	2.08	1,838	1,610	155	Callanan et al. (2008)
Lactobacillus johnsonii NCC 533[c]	Probiotics	NC_005362	34.6	1,992	1.99	1,918	1,821	0	Pridmore et al. (2004)
Lactobacillus plantarum WCFS1[b]	Vegetable fermentation, probiotics	NC_004567	44.5	3,308	3.35	3,135	3,007	42	Kleerebezem et al. (2003); van Kranenburg et al. (2005)
		NC_006375 (pWCFS101)	39.5	2		3	3	0	
		NC_006376 (pWCFS102)	34.3	2		4	4	0	
		NC_006377 (pWCFS103)	40.8	36		43	43	0	
Lactobacillus reuteri F275[b]	Probiotics	NC_009513	38.9	2,000	2.00	2,027	1,900	39	
Lactobacillus reuteri JCM 1112[b]	Probiotics	NC_010609	38.9	2,039	2.04	1,901	1,820	0	Morita et al. (2008)
Lactobacillus sakei ssp. *sakei* 23K[b]	Meat fermentation	NC_007576	41.3	1,884	1.88	1,963	1,879	0	Chaillou et al. (2005)
Lactobacillus salivarius ssp. *salivarius* UCC118[b]	Probiotics	NC_007929	32.9	1,827	2.13	1,864	1,717	49	Claesson et al. (2006)
		NC_006529 (pSF118-20)	39.1	20		27	27	0	
		NC_006530 (pSF118-44)	39.6	44		49	47	2	

Strain names	Origin/usage	NCBI access no.	GC content (%)	Length (Kb)	Genome size (Mb)	No. of genes	No. of proteins	No. of pseudogenes	References
		NC_007930 (pMP118)	32.1	242		242	222	21	
Lactococcus lactis ssp. *cremoris* MG1363[c]	dairy/model strain	NC_009004	35.7	2,529	2.53	2,597	2,434	82	Wegmann et al. (2007)
Lactococcus lactis ssp. *cremoris* SK11[c]	Cheese production, flavor formation	NC_008527,	35.9	2,438	2.59	2,610	2,384	144	Makarova et al. (2006)
		NC_008503 (pLACR1)	34.4	14		11	10	1	
		NC_008504 (pLACR2)	30.4	9		8	6	2	
		NC_008505 (pLACR3)	35.4	74		66	61	3	
		NC_008506 (pLACR4)	34.8	47		39	35	4	
		NC_008507 (pLACR5)	33.5	14		8	8	0	
Lactococcus lactis ssp. *lactis* IL1403[c]	Milk fermentation soft cheese	NC_002662	35.3	2,365	2.37	2,425	2,321	1	Bolotin et al. (2001)
Leuconostoc citreum KM20[b]	Kimchi fermentation	NC_010471	39	1,796	1.90	1,785	1,702	1	Kim et al. (2008)
		NC_010470 (pLCK1)	37.4	39		49	49	0	
		NC_010466 (pLCK2)	38	31		36	36	0	
		NC_010467 (pLCK3)	33	18		20	20	0	
		NC_010479 (pLCK4)	36.9	12		13	13	0	
Leuconostoc mesenteroides ssp. *mesenteroides* ATCC 8293[b]	Vegetable fermentation, polymer production	NC_008531	37.7	2,038	2.08	2,073	1,970	19	Makarova et al. (2006)
		NC_008496 (pLEUM1)	35.4	37		35	35	0	
Oenococcus oeni PSU-1[b]	Wine making	NC_008528	37.9	1,780	1.78	1,864	1,691	122	Mills et al. (2005)
Pediococcus pentosaceus ATCC 25745[b]	Fermentation starter culture, preservation	NC_008525	37.4	1,832	1.83	1,847	1,755	20	Makarova et al. (2006)
Streptococcus thermophilus CNRZ1066[b]	Yogurt	NC_006449	39.1	1,796	1.80	2,000	1,915	0	Bolotin et al. (2004)
Streptococcus thermophilus LMD-9[b]	Yogurt	NC_008532	39.1	1,856	1.86	2,003	1,710	206	Makarova et al. (2006)
		NC_008500 (pSTER1)	37	4		4	4	0	
		NC_008501 (pSTER2)	35.1	3		2	2	0	
Streptococcus thermophilus LMG 18311[b]	Yogurt	NC_006448	39.1	1,796	1.80	1,974	1,889	0	Bolotin et al. (2004)

[a] Facultative heterolactic fermentative strain

[b] Heterolactic fermentative strain

[c] Homolactic fermentative strain

Table 2. Genome sequencing projects for lactic acid bacteria (Zhu et al., 2009).

Comparative genomic analysis of different LAB has provided a first view of the molecular basis of their adaptation at different environments and has revealed well equipped organisms for a wide range of metabolic activities, defense and stress responses, specifically needed to live, reproduce

and survive in plant-derived materials, diary and meat products, and animal and human mucosa (Mayo et al., 2008).

Given the close phylogenetic relationships of these organisms, comparison of gene content across the species and reconstruction of ancestral gene sets indicate that both the bulk of the genes lost, as well as the acquisition of key genes through horizontal gene transfer (HGT) and gene duplication (Altermann et al 2005, Bolotin et al., 2004) were due to the adaptation to nutrient-rich food environments (Mayo et al., 2008).

The analysis of dairy LAB genomic sequences has shed new light on the evolution of these organisms to the milk new environment. Milk is a nutritionally rich, stable environment containing lactose as the primary carbohydrate and caseins as a rich potential source of amino acids; milk also contains vitamins and minerals. Examination of dairy LAB genomes shows that gene loss or inactivation, as well as metabolic simplification, are a central component of the evolution of these organisms to milk (Bolotin et al., 2001, 2004; Makarova et al., 2006; van de Guchte et al., 2006). This is particularly true in genes involved in amino acid biosynthesis. Selective pressure during growth in milk has favored microorganisms that are capable of obtaining amino acids from caseins via their relatively complex proteolytic enzyme system and loss or inactivation of genes for *de novo* biosynthesis of amino acids. Additionally, it is clear that HGT, a process by which an organism transfers genetic material to different organisms, has played a significant role in the evolution of these organisms. For example, the ability to utilize lactose as an energy source has evolved independently in different dairy LAB via HGT (Cogan et al., 2007). The availability of dairy LAB genomic sequences supports the view that these organisms have evolved from other LAB, rapidly resulting in organisms that are specialists for growth in milk (Bolotin et al., 2004; Makarova et al., 2006; van de Guchte et al., 2006). In fact, in the genomes of dairy LAB, such as *S. thermophilus* (Bolotin et al., 2004), *Lb. delbrueckii* ssp. *bulgaricus* (van de Guchte et al., 2006), and *Lb. helveticus* (Callanan et al., 2008), more than 10% coding genes lost their functions and are present as pseudogenes (Zhu et al., 2009).

The specialized adaptation to milk is particularly interesting because this fermentation environment would not exist without human intervention. The selective pressure came not only from the natural environment, but also from anthropogenic environments created by humans, which essentially domesticated these organisms over the last 5000 years through repeated transfer of LAB cultures for production of fermented dairy products such as cheeses (Schroeter and Klaenhammer, 2009).

The availability of genomic sequences has also allowed researchers to rapidly discern the metabolic potential of the sequenced strains. For example the complete genome sequence of the probiotic *Lb.*

rhamnosus ATCC 53103 strain revealed a relatively high number of putative genes involved in carbohydrate and amino acid metabolism and transport and defense mechanisms, compared with other sequenced lactobacilli. The availability of multiple genome sequences within a species allows the study of strain-specific traits. In a recent work, Kankainen and colleagues (2009), performed a comparative genomic analysis of *Lb. rhamnosus* GG (LGG), a commonly used probiotic bacterium, and *Lb. rhamnosus* Lc705, an industrial strain used as an adjunct starter culture in dairy products. The genome analysis revealed that strain LGG had lost the ability to use lactose, an important metabolic feature commonly exploited in dairy industrial applications, because of frameshifts in the antiterminator (*lacT*) and 6-phospho-B-galactosidase (*lacG*) genes. The authors also identified 40 and 49 genes predicted to encode potential glycosidases in the genomes of strains LGG and Lc705, respectively. Because of their annotation and predicted cellular location, several of these (10 in LGG and 9 in Lc705) may have participated in peptidoglycan hydrolysis and conversion of complex polysaccharides and prebiotics to simple carbohydrates. Furthermore, both strains encoded a cell envelope serine protease (PrtP), maturation protein (PrtM), and proteinase (PrtR), in addition to a similar set of 25 peptidases. Only within the Lc705 genome, a gene for an additional secreted subtilisin-like serine protease was predicted, which may have been involved in casein degradation (Kankainen et al., 2009).

Access to genomic information has provided researchers with an unprecedented opportunity to refine old and develop new hypotheses concerning how LAB effect the conversion of milk into a variety of fermented dairy products. The addition of the other ongoing sequence projects to the currently available set will not only facilitate comparative genomic analysis, but will also form the basis for extensive functional genomics approaches exploring both the transcriptome and proteome. The outcome of these challenging efforts will undoubtedly revolutionize one's understanding and knowledge of LAB.

1.3.2 Transcriptomics study in LAB

Transcriptomics refers to analyze the information of a whole transcriptome of an organism.

The transcriptome constitutes a snapshot of all actively expressed genes at any given time and represents the set of all RNA molecules, including messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), and non-coding RNA produced in one or group of cells. A major type of RNA is mRNA, which includes the protein-coding part of the genome and is translated into proteins that fulfill specific biological functions. Generally, transcriptomics indicates the global analysis of gene expression profile at mRNA level (Deng et al., 2010; Schirmer et al., 2010).

Genome-wide gene expression technologies mainly include DNA microarray (or gene chip), oligo-microarray, copy DNA (cDNA)-AFLP-Amplified fragment length polymorphism(cDNA-AFLP) and Serial Analysis of Gene Expression (SAGE) (Deng et al., 2010).

Currently the most popular transcriptomics methods are cDNA and oligo-microarrays (Deng et al., 2010). These methods proved to be powerful and are now widely used for genome-wide expression analysis in a wide range of organisms, including LAB (Klaenhammer et al., 2007; Xie et al., 2004, Raynaud 2005). Although fundamental differences exist between the two methods, their strength lies in the massive parallel nature of the analysis, which allows up to tens of thousands of genes to be analyzed simultaneously. Microarrays comprising complete gene sets are available for a number of organisms, such as yeasts (Wodicka et al., 1997), bacteria (Laub et al., 2000; Selinger et al., 2000) and *Caenorhabditis elegans* (Jiang et al., 2001), for which the entire genome sequence was determined. In LAB, cDNA microarrays were developed to study expression of the metabolic genes in *Lc. lactis* ssp. *lactis* IL1403 during stress condition associated with dairy fermentations (Xie et al., 2004) and to study the transport and catalytic machinery involved in carbohydrate utilization by *Lb. acidophilus* (Barrangou et al., 2006). cDNA microarray were also utilized to investigate features of the acid tolerance response in *Lb. casei* (Broadbent et al., 2010) and to obtain a genome-wide view of the transcriptional response towards lactic acid stress in *Lb. plantarum*. However, although cDNA microarrays are rapidly becoming the standard tool for genome-wide expression analysis, their use is limited to organisms for which the complete genome sequence or a large cDNA collection is available and furthermore, compared with other methods, it remains more expensive (Li et al., 2009; Breyne and Zabeau, 2001).

Alternative technologies used extensively in the analysis of transcription profile in plants (Bachem et al., 1996; Durrant et al., 2000; Milioni et al., 2001; He et al., 2003) but seldom in bacteria (Decorosi et al., 2005; Dellagi et al., 2000; Noel et al., 2001), are represented from rDNA fragment analysis based methods, such as cDNA-AFLP. This technology does not require any prior knowledge of gene sequences (Bachem et al., 1996), and its sensitivity and specificity have been compared to those of a microarray approach (Reijans et al., 2003). Moreover, the cDNA-AFLP approach allows the detection of rarely expressed genes and possibly the discrimination between homologous genes. Nevertheless, the cDNA-AFLP, since it needs radioactively labelled PCR primers, is potentially hazardous, time consuming, and also requires laboratories designated for radioisotope research (Decorosi et al., 2005). cDNA-AFLP is carried out according to the principle of AFLP. The AFLP technique is based on the digestion of DNA templates followed by the ligation of adapters to restriction fragments and the selective PCR amplification of subsets of these

fragments using selective AFLP primers. Similarly, the original cDNA-AFLP method (Bachem et al., 1996) involves (1) the reverse transcription of mRNA into double-stranded cDNA, followed by (2) the generation of a complex mixture of transcript-derived fragments (TDFs) by restriction enzyme digestion and ligation of specific adapters, (3) selective PCR amplification and finally (4) the visualization of the TDFs on high-resolution (sequence) gels (Vuylsteke et al., 2007). In the last years, different researches were published to improve the original cDNA-AFLP method (Weiberg et al., 2008; Decorosi et al., 2005). For example, Decorosi and colleagues (2005) developed a safe, easy, and labour-saving cDNA-AFLP technique based on non-radioactive fluorescent primers (cDNA-fluorescent amplified length polymorphism, cDNA-FAFLP) avoiding the problems linked with the use of radioisotopes.

However, the most important variant is the modification of the original protocol based on "one-gene-multiple-tag" into "one-gene-one-tag" as previously reported (Vuylsteke et al., 2007; Breyne and Zabeau, 2001). The "one-gene-one-tag" cDNA-AFLP protocol involves a reduction of TDFs to a single fragment for each cDNA by selecting the 3'-terminal restriction fragment of each transcript before selective amplification. To date, cDNA-AFLP was applied to study the gene expression in mammalian cells (Ekkapongpisit et al., 2007, Majima et al., 2000, Rizos et al., 2002), yeasts (Reijans et al., 2003), nematodes (Neveu et al., 2007), and some bacteria, mainly plant pathogens (Dellagi et al., 2000; Noel et al., 2001). That studies demonstrated that cDNA-AFLP is a powerful tool to study prokaryotic transcriptomes and to identify genes involved in the adaptation of bacteria at different environments. Thus, this technology could be useful to study the gene expression profile of the LAB during their growth in dairy environments.

1.3.3 Proteomics studies in LAB

The term proteome refers to the proteins expressed by a genome of a living cell at a particular point in time (Manso et al., 2005). The genome provides only static information, while the proteome provides an overall view of the cell machinery, which can be studied under various conditions and could provide information regarding dynamic processes. The elucidation of the expressed part of the genome is required to link genomic data to biological functions. Mainly based on high resolution two-dimensional electrophoresis (2-DE) (Klose, 1975; O'Farrell, 1975; Patterson and Aebersold, 1995) coupled with mass spectrometry (MS), proteomics is a powerful tool for analyzing several hundred proteins in complex mixtures (Mann et al., 2001), as different as samples of human body fluids (Lafitte et al., 2002), vegetable cells, yeast cells such as *Saccharomyces cerevisiae* (Boucherie et al., 1995) and several microorganisms used in dairy fermented products, as

reviewed by Champomier-Verges and colleagues (2002) and Manso and colleagues (2005). 2-DE proved to be a valuable tool for providing a proteome-wide view of the changes in the protein profile triggered by environmental stimuli such as stress or starvation. Nevertheless, even if the techniques were constantly improved they remained demanding and suffered from severe limitations in the ability of identification of the proteins of interest. Thus, this promising technique was not commonly used but rather confined to a limited number of highly specialized laboratories. In the mid-1990s several concurrent technical developments not only dramatically increased the popularity of proteomic expression profiling but also launched a new era of cellular physiology. The advent of genome sequencing enabled the highly sensitive and large-scale identification of proteins by Mass Spectrometry (MS), mainly Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) and Liquid Chromatography-Electrospray Ionization (LC-ESI) Tandem Mass Spectrometry (MS/MS). MS, in fact, is a versatile and indispensable tool in protein chemistry and proteomics. The mass spectrometer determines the mass-to-charge ratio (m/z) of peptide and protein ions that are generated by ESI or MALDI sources. Another proteomic technology such as tandem MS (MS/MS) enables the peptide to be sequenced allowing the identification of eventually post-translational modifications. The implementation of bioinformatics tools such as MASCOT or SEQUEST allowed the effective cross-examination of databases with the MS data and improvements in the software for the analysis allowed rapid recognition and visualization of the changes in the protein profile (Volker and Hecker, 2005). However, the coupling of 2DE with MALDI-TOF-MS/MS methodologies is a powerful tool in high-throughput proteomics (Renzone et al., 2005).

Proteomic studies now includes (1) protein expression profiling of biological samples in a given physiological state to afford a large-scale characterization of all detectable proteins including their post-translational modifications (structural proteomics) (Wasinger et al., 1995), (2) comparison of protein expression level in two or more physiological states (i.e. normal versus altered/pathological conditions), commonly referred as “comparative proteomics or quantitative proteomics” (Lasonder et al., 2002; Florens et al., 2002), (3) protein-protein interaction analysis (Uetz et al., 2000; Gavin et al., 2002), (4) subcellular protein localization analysis (Huh et al., 2003) and (5) definition of a biological role for open reading frames with poorly known function, also known as “functional proteomics” (Minshull et al., 2005).

In comparative proteomic studies, proteins from different biological states are quantitatively compared to obtain a thorough understanding of the biological processes affecting their expression and/or in which they are involved (Renzone et al., 2005). Although proteomic studies are essentially

based on a comparative approach, the preparation of a complete and reproducible reference gel is a prerequisite. Over the last 15 years, *in vitro* reference maps, as well as proteins overexpressed following stress adaptation, have been established dairy processing for most bacteria grown in either synthetic medium, in milk or in whey based medium. For lactic acid bacteria, these have been established for *Lc. lactis* (Anglade et al., 2000; Drews et al., 2004; Gitton et al., 2005), *Lb. delbrueckii* ssp. *bulgaricus* (Gouesbet et al., 2002), *Lb. casei* (Maze et al., 2004), *Lb. acidophilus* (Wang et al., 2005), *Lb. salivarius* (Kelly et al., 2005), *Lb. plantarum* (Cohen et al., 2006), *S. thermophilus* (Perrin et al., 2000; Guimont et al., 2002; Arena et al., 2006), *Lb. casei* (Hussain et al., 2007) and *Lb. rhamnosus* (Koskenniemi et al., 2009).

The effect of milk and cheese processing on bacteria has been investigated using proteomic approaches. Cheese processing was shown to impose harsh stress conditions on bacteria. The heat stress response of *Lb. helveticus* during propagation in cheese whey was found to involve expression of 48 proteins related to heat adaptation. Stress proteins (e.g., DnaK and GroEL), glycolysis-related machinery (e.g., enolase and GAPDH), and other regulatory proteins or factors (e.g., DNA-binding protein II and ATP-dependent protease; Di Cagno et al., 2006) were among them. Stress proteins from *P. freudenreichii*, *Lb. helveticus*, and *S. thermophilus* were found to be highly expressed in Emmental cheese showing adaptation of bacterial species to various stresses that occur during cheese manufacturing and ripening, such as acid, thermal, and osmotic stresses (Gagnaire et al., 2004).

For *Lb. rhamnosus* a proteomic approach was used to compare the adaptation of a probiotic strain, *Lb. rhamnosus* GG (LGG), to an hydrolyzed whey-based medium and a rich MRS laboratory medium. Collectively, the data showed that growth of LGG in whey medium increased the relative abundance of proteins involved in purine biosynthesis, galactose metabolism, and fatty acid biosynthesis. By contrast, growth of LGG in laboratory medium resulted in an increase amount of proteins involved in translation and the general stress response, as well as pyrimidine and exopolysaccharide biosynthesis. Moreover, several enzymes of the proteolytic system of LGG demonstrated growth medium-dependent production. This study demonstrated the fundamental effects of culture conditions on the proteome of LGG, which are likely to affect the functionality and characteristics of its use as a probiotic (Koskenniemi et al., 2009).

Using both metabolomic and proteomic approaches, the differential behavior of 2 strains of *Lc. lactis* was investigated in a model cheese made from milk concentrated by ultrafiltration (Gitton et al., 2008). The main differences concerned enzymes involved in metabolism of purines/pyrimidines,

lactose, and citrate. This work represented the first proteomic map of bacteria entrapped in a model of solid dairy matrix and opened the way to explore the bacterial proteome in all cheese varieties.
In conclusion, the availability of genomic sequences and the spectacular development in MS methodology enables large-scale proteomic studies that will be a decisive turning point in our knowledge on proteins from milk and bacteria involved in fermented dairy products.

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2. AIM OF THE THESIS

Lactic Acid Bacteria (LAB) constitute an heterogeneous group of bacteria that are traditionally used to produce fermented foods. They may play different roles in cheese-making where starter LAB (SLAB) participate in the fermentation process, whereas non starter LAB (NSLAB) are implicated in the maturation of cheese. Although the role of NSLAB in ripening has not yet been clarified, different authors have suggested their importance in the cheese ageing. In Parmigiano Reggiano (PR) cheese, NSLAB, autochthonic of raw milk and arising from the environment, are the protagonists of the different biochemical processes during the production and ripening stages.

Recent studies in microbiological ecology of PR cheese allowed a deeper insight of the microbial composition during the manufacturing and ripening stages of the same cheese-making process. Further, the study of samples representative of the subsequent stages of the same cheese-making by culture-independent methods led to determine the microbial succession during 24 months of PR ripening. It was demonstrated that SLAB are dominant until the 2nd month of ripening. Differently, after cheese brining, the species NSLAB, especially *Lb. rhamnosus*, are able to grow and increase in number, while SLAB cells undergo to autolysis. *Lb. rhamnosus* was shown to be the dominant species present after a lack of essential nutrients, such as sugars. Therefore, this species seems to well adapt to the absence of lactose in cheese, confirming an optimal adaptability to unfavorable growth conditions. Presumably, this characteristic was due to the ability of *Lb. rhamnosus* to use nitrogen fraction as an alternative energy source.

So far, only few studies are available about NSLAB, and in particular about *Lb. rhamnosus*, in PR and the exact role of these bacteria has not been investigated in greater depth. In particular, the biotypes of the dominant species during the maturation stage of cheese processing have never been studied. Indeed, it can only be hypothesized that the technological pressure determines, at different stages, the potential development of the biotype that may have a specific biochemical role leading to certain flavors and sensorial traits of the PR cheese.

For this reason, *Lb. rhamnosus*, the dominant species among NSLAB in PR, has been chosen as subject for the present study. This Ph.D thesis aims to better understand the role played by this species in cheese-making and ripening process, and to hypothesize the strategies used to adapt its energy metabolism to the environmental conditions met in PR cheese.

To reach this issue, different strategies has been pursued.

First, an intraspecific genotypic characterization has been used to investigate the diversity of *Lb. rhamnosus* strains isolated at different steps during the same manufacturing cycle of PR cheese. Secondly, an integrative approach, coupling metabolic and proteomic techniques with a novel

transcriptomic approach, was used to unravel the rules governing their adaptation to the PR cheese environment.

3. RESULTS

3.1 Evaluation of genetic polymorphism among *Lactobacillus rhamnosus* non-starter Parmigiano Reggiano cheese strains

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3.1.1 Abstract

Parmigiano Reggiano (PR) is an Italian cooked, long-ripened cheese made with unheated cow's milk and natural whey starter. The microflora is involved in the manufacturing of this cheese, arising from the natural whey starter, the raw milk and the environment. Molecular studies have shown that mesophilic non-starter lactic acid bacteria (NSLAB) are the dominant microflora present during the ripening of PR. In this study, a characterisation of *Lactobacillus rhamnosus* isolated from a single PR manufacturing and ripening process is reported, using a combination of genotypic fingerprinting techniques (RAPD-PCR and REP-PCR). The intraspecies heterogeneity evidenced for 66 strains is correlated to their abilities to adapt to specific environmental and technological conditions. The detection of biotypes that correlate with specific moments in cheese ripening or differential development throughout this process suggests that these strains may have specific roles closely linked to their peculiar technological properties.

3.1.2 Introduction

Parmigiano Reggiano (PR) is a hard-textured, cooked and longripened cheese whose denomination "Protected Designation of Origin" is linked to a strictly artisanal manufacturing process in specific areas of Northern Italy (http://www.parmigiano-reggiano.it), which involves the use of unheated, partially skimmed raw cow's milk supplemented with a natural whey starter (Mucchetti and Neviani, 2006). Non-starter lactic acid bacteria (NSLAB), autochthonic of raw milk and arising from the environment, as well the starter lactic acid bacteria (SLAB), added with the natural whey starter, are the protagonists of the different biochemical processes during the manufacturing and ripening stages involved in the development of PR cheese (Neviani et al., 2009). Although the role of NSLAB in ripening has not yet been clarified, different authors have suggested

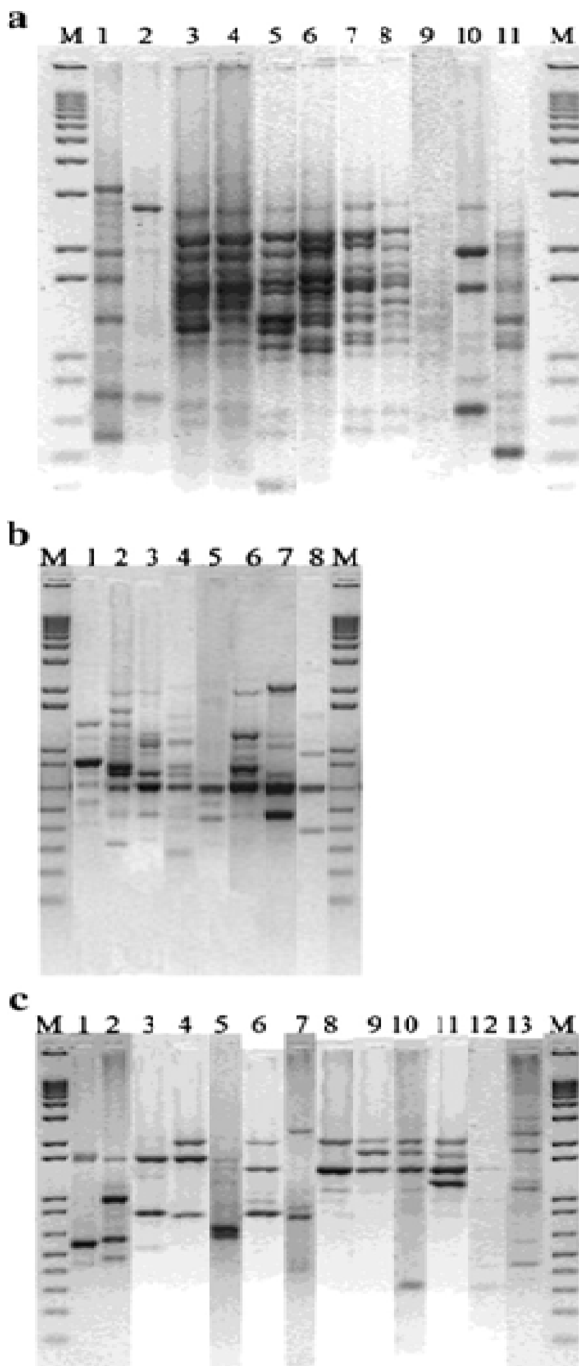
the importance in this process mainly in Cheddar cheese (Beresford et al., 1999; Fox et al., 1998; Peterson and Marshall, 1990) and also in PR cheese (Succi et al., 2005).

In recent years, some studies have demonstrated that *Lactobacillus rhamnosus*, with some other NSLAB microflora, was the dominant species present after a lack of essential nutrients, such as sugars, in PR ripening (De Dea Lindner et al., 2008; Gatti et al., 2008; Lazzi et al., 2007). This microflora originates from raw milk and the environment as a result of contamination during the manufacturing procedure, where it is present at low cell densities. Different from SLAB, after cheese brining, *Lb. rhamnosus* are able to grow and increase in number, while SLAB cells undergo autolysis (Gatti et al., 2008). When this occurs, most of the residual lactose in the cheese has already been utilised by the SLAB; therefore, this species seems to adjust well to the absence of lactose in cheese, confirming an optimal adaptability to unfavourable growth conditions (Succi et al., 2005). Presumably, this adaptability was due to the ability of this strain to use nitrogen fraction (amino acids and peptides) as an alternative energy source. This finding is confirmed by the optimal growth rate of microflora NSLAB, in particular *Lb. rhamnosus*, in a ripened cheese medium used instead of traditional cultural media (Lazzi et al., 2007; Neviani et al., 2009). This medium, in fact, allowed for the recovery of the NSLAB population in raw milk and fresh curd when they are present in little amounts (Lazzi et al., 2007; Neviani et al., 2009).

Although different studies have highlighted the importance of NSLAB in the ripening process of PR (Coppola et al., 2000; De Dea Lindner et al., 2008; Gala et al., 2008; Gatti et al., 2008; Neviani et al., 2009), the exact role of these bacteria has not been investigated in greater depth. In particular, the biotypes of the dominant species during the maturation stage of cheese processing have never been studied. Indeed, it can only be hypothesised that the technological pressure determines, at different stages, the potential development of the biotype that may have a specific biochemical role leading to certain flavours and sensorial traits of the PR. Molecular typing may be helpful to understand the adaptation of bacterial strains subjected to selective pressure while growing in milk. Therefore, this study aimed to characterise the diversity of *Lb. rhamnosus* strains isolated at different steps during the same manufacturing cycle of PR.

Intraspecific genotypic characterisation of *Lb. rhamnosus* has been already studied by Vancanneyt et al. (2006) using high-resolution DNA fingerprinting techniques (Fluorescent Amplified Fragment Length Polymorphism [FAFLP] and Pulse Field Gel Electrophoresis of macrorestriction fragments). In this work, we determined genotypic relatedness at the intraspecies level for 66 *Lb. rhamnosus* strains by means of two DNA fingerprinting techniques, i.e., randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and repetitive extragenic

palindromic-polymerase chain reaction REP-PCR analysis. The interesting aspect of this study is that the *Lb. rhamnosus* strains were isolated from samples collected during the same cheesemaking process. To our knowledge, no previous studies have investigated the biodiversity of microflora NSLAB present in the ripening process. Therefore, through this study, we aimed to understand if different biotypes are present at different stages of the ripening process.



3.1.3 Materials and methods

Bacterial strains

Sixty-six strains of *Lb. rhamnosus* isolated from different stages of PR manufacturing and ripening were used in this study. The strains were isolated from twelve samples (curd after 6 and 48 h, cheese at 1, 2, 3, 4, 6, 8, 10, 12, 16, and 20 months of ripening) collected during the same cheese-making period from a cheese factory able to guarantee the production of twin wheels (Gatti et al., 2008). All the strains were isolated, identified and maintained as described in previous work (Neviani et al., 2009).

Characterisation by RAPD-PCR

RAPD-PCR analysis was performed with the aim of differentiating the strains using the primers M13 and P1 according to Rossetti and Giraffa (2005) and De Angelis et al. (2001), respectively. Primers were used singly in two series of amplification. Amplification products were separated on a 1, 2% agarose gel in 1X TAE buffer. The repeatability of RAPD-PCR fingerprints was determined by triplicate loading of independent triplicate reaction mixtures prepared with the same strains.

Fig. 1. *Lb. rhamnosus* REP-PCR patterns obtained with primer GTG5 (a), and *Lb. rhamnosus* RAPD-PCR patterns obtained with primers M13 (b) and P1 (c). Each pattern shows the

representative fingerprints of the clusters with each primer and was obtained considering 80% similarity level for each single genetic characterisation (data not shown). (a), (b) and (c) M, DNA molecular size standards (1 kb plus DNA ladder; Invitrogen, Carlsbad, CA, USA).

Strain typing by REP-PCR.

The single oligonucleotide primer (GTG)5 (5′-GTGGTGGTGGTGGTG-3′) was applied for REP-PCR. Amplifications were performed as reported by Coudeyras et al. (2008). The repeatability of REP-PCR fingerprints was determined as above for RAPD-PCR.

Cluster analysis

Conversion, normalisation, and further analysis of the RAPD-PCR and REP-PCR patterns were carried outwith Bionumerics software (Version 3.0; Applied Maths, BVBA, Sint-Martens-Latem, Belgium). The two series of RAPD-PCR profiles and the profiles of REP-PCRwere combined to obtain a unique dendrogram. Cluster analysiswas carried out utilizing the unweighted pair group method with arithmetic average (UPGMA).

3.1.4 Results and discussion

The sixty-six *Lb. rhamnosus* isolated strains were differentiated at the strain level by the combined use of RAPD-PCR, using primers P1 and M13, and REP-PCR analysis using primer GTG5. RAPD-PCR analysis was used because it provides a good level of discrimination, mainly when the number of primers used to randomly amplify the bacterial genomeis high (De Angelis et al., 2001; Tailliez et al., 1998). These techniques were successfully applied to study NSLAB from cheddar cheeses (Fitzsimons et al., 1999) and NSLAB in Italian ewe cheese (DeAngelis et al., 2001). REPPCR has been successfully applied to strain typing for both Gram-positive and Gram-negative bacteria. This technique employs oligonucleotides designed to match consensus sequences of repetitive extragenic palindromic sequences and it seems more specific and reproducible than RAPD-PCR analysis (De Urraza et al., 2000). In this study, the repeatability of the RAPD-PCR and REP-PCR fingerprints was assessed by comparing the profiles obtained fromthree separate cultures of the same strain (data not shown). The similarity of the electrophoresis pattern results was greater than 80% using all the three primers, indicating a high level of repeatability for both techniques.

Due to the combined use of RAPD-PCR and REP-PCR techniques, a wide genotypic variability was found among *Lb. rhamnosus* strains. In the dendrograms obtained from primers GTG5, M13, and P1, considering an 80% of similarity level, we were able to group the isolates into 11, 8 and 13

clusters, respectively (data not shown). In Fig. 1 is shown the representative fingerprint for each of the three primers: 11 different patterns generated by primer GTG5, 8 different patterns obtained using primer M13, and 13 different patterns obtained using primer P1. Overall, the primer GTG5 determined a greater number of amplicons than did the primers M13 and P1 (Fig. 1). Notwithstanding, the P1 primer generated the greatest pattern diversity resulting in a more suitable way to highlight the intraspecies differences among the strains. To obtain a good discrimination at the strain level, the RAPD-PCR and REP-PCR profiles were combined to obtain the UPGMA dendrogram shown in Fig. 2.

At 80% similarity, the *Lb. rhamnosus* strains from curd and cheeses sampled at different times were grouped into 20 clusters (Fig. 2), which can be reasonably considered representative of 20 biotypes. Each cluster included 1–20 strains. The most different biotypes (less then 40% similarity) were formed by seven single strains that showed unique RAPD-PCR and REP-PCR patterns (clusters 14, 15, 16, 17, 18, 19, and 20). These biotypes, with the exception of 1019, were isolated in the first period of cheese maturation, in particular in the curd after 6 h and in the cheese after 48 h of brining (1 month cheese). After this time, these biotypes disappear, perhaps because of increasingly unfavourable conditions. On the contrary, the biotypes 2, 4, and 5 include strains isolated only at the end of ripening. Unfortunately, this finding might be due to the lower number of isolates obtained in the first samples (from curd 6 h to cheese 1 month) or to the difficulty in isolating these biotypes. The results of clusters 1, 3, 6, 12 and 13were the most heterogeneous as they included strains able to grow during all periods of ripening. Indeed, these five clusters (biotypes) are present in cheese from the first or second month of ripening until after ten months. Among them, biotype 3 is most frequently isolated and therefore might be considered dominant in the whole ripening process.

In consideration of its remarkable presence during the whole ripening period, the microbial population NSLAB can be considered as one of the main factors in determining typical cheese features. The intraspecies heterogeneity evidenced in *Lb. rhamnosus* strains during a cycle of PR manufacturing is certainly correlated to the adaptation of the strains to the specific environmental conditions and production manufacturing. In this regard, the detection of biotypes that mark specific moments in cheese ripening, or of those that can develop differently throughout the ripening process, suggests that they may have specific roles closely linked to their peculiar technological properties. The impact on typical cheese features by the different biotypes of *Lb. rhamnosus* isolated strains during ripening will be the object of further studies.

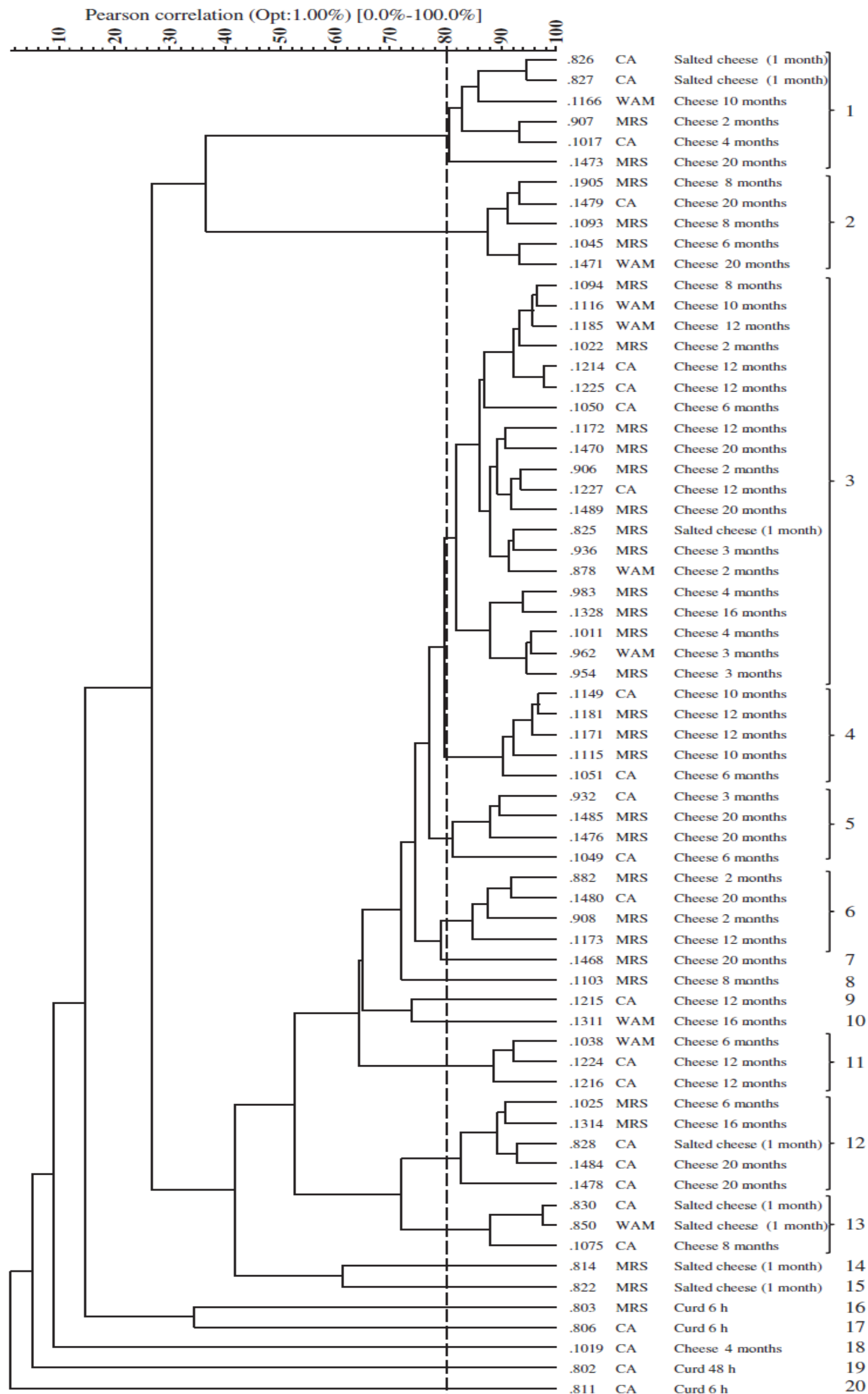


Fig. 2. UPGMA dendrogram of *Lb. rhamnosus* strains based on the combined analysis of RAPD-PCR patterns of the two primer P1 and M13 and REP-PCR patterns using the primer GTG5.

The strains and the sources of isolation are indicated on the right side of the dendrogram. The numbers reported in the figure represent the clusters formed at the cut-off point (vertical dotted line).

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3.2 Proteomic and metabolic diversity of *Lactobacillus rhamnosus* strains during adaptation to cheese-like environmental conditions

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3.2.1 Abstract

This work aimed at showing the metabolic and proteomic adaptation of *Lactobacillus rhamnosus* strains isolated at different times of Parmigiano Reggiano cheese ripening. Cultivation was carried out under cheese-like conditions (Cheese broth) and on pasteurized and micro-filtrated milk and MRS broth. As shown by Biolog system analysis, the large part of the *L. rhamnosus* strains had the capacity to use numerous chemical compounds as energy sources. Growth and acidification differed depending on the culture media. Compared to growth on MRS or pasteurized and micro-filtrated milk, all strains cultivated in Cheese broth showed a decrease of the synthesis of D,L-lactic acid and synthesized higher levels of acetic acid. Except for one strain, the others caused an increase of the concentration of free amino acids during cultivation on Cheese broth. The proteomic maps of five strains showing different metabolic traits were comparatively determined after growth on MRS and Cheese broth. The synthesis of proteins was probably affected by the time of isolation during cheese ripening and certainly by the growth media. A total of 93 protein spots which showed an increased level of synthesis during growth in MRS (46 spots) or Cheese broth (47 spot) were identified by MALDI-TOF-MS/MS or LC-nano-ESI-MS/MS. Compared to cells grown in MRS, *L. rhamnosus* strains cultivated under cheese-like conditions modified the synthesis of proteins related to protein biosynthesis, nucleotide and carbohydrate metabolisms, glycolysis pathway, proteolytic activity, cell wall and exopolysaccharide biosyntheses, cellular regulation, amino acid and citrate metabolisms, oxidation/reduction processes and stress response.

3.2.2 Introduction

Lactobacillus rhamnosus is a facultative hetero-fermentative lactic acid bacterium that is frequently encountered in many fermented foods and beverages, including cheeses, fermented milks, baked goods, sausages and vegetable juices. *L. rhamnosus* is also used as probiotic bacterium (4). During ripening of several cheese varieties, *L. rhamnosus* often becomes dominant within the population of mesophilic lactobacilli, referred to as the nonstarter lactic acid bacteria (NSLAB) (19, 20). NSLAB originate from raw milk and environment, as the consequence of contamination during cheese manufacture. After cheese brining, strains of *L. rhamnosus* possess the capacity of growing while starter lactic acid bacteria (SLAB) cells very often undergo to autolysis (20). During ripening, most of the residual lactose of cheese is rapidly consumed by SLAB. Therefore, *L. rhamnosus* has to use non conventional energy sources (43). Overall, the energy sources used by NSLAB during cheese ripening are still debating. Potential sources could be amino acids, organic and fatty acids, glycerol or carbohydrates released from glycomacropeptide (GMP) (galactose, N-acetylgalactosamine and N-acetylneuraminic acid), glycoproteins, glycolipids either from milk-fat globule membrane (MFGM) (galactose, mannose, fucose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid) or from lysed cells of SLAB (ribose, deoxyribose, N-acetylglucosamine and N-acetylmuramic acid) (19, 35, 36, 38, 48).

The versatile adaptation of *L. rhamnosus* to different ecosystems (e.g., fermented foods, and human and animal gastrointestinal tracts) is probably related to its capacity to regulate metabolic pathways, notwithstanding the constitutive genetic intra-species variability. Comparative genome analysis of *Lactobacillus* species indicated that combination of gene gain and gene loss frequently occurs during environmental adaptation (9, 24). Together with *Lactobacillus plantarum*, *L. rhamnosus* has the largest size (3.3 Mb) of chromosome which contains a large number of genes having regulatory and transport functions (31, 37). Previously, only a few reports described the diversity and the environmental adaptation of *L. rhamnosus* strains (34, 47). In spite of the extensive use and the frequent isolation from various food matrices, a very few information are reported regarding the global gene expression of *L. rhamnosus* under different environmental conditions. More detailed information are needed regarding the dynamic of global protein synthesis during growth and, especially, studies are needed which highlight the diversity between strains (8, 32, 34). Knowledge about the global protein synthesis patterns of *L. rhamnosus* may permit: (i) tools to be developed for screening tolerant or sensitive strains for biotechnology uses; (ii) an enhanced use in food processes and for medical purposes through the optimisation of growth, acidification, proteolysis and

probiotic effects; (iii) an enhanced growth and/or survival by appropriate preservation methods; and iv) the fitness and level of adaptation of a culture to be evaluated (14).
This work aimed at showing the metabolic and proteomic adaptation of *Lactobacillus rhamnosus* strains isolated at different times of Parmigiano Reggiano cheese ripening. Proteome maps of cells grown on Cheese broth or MRS and protein identification by MALDI-time of flight mass spectrometry (MALDI-TOF-MS/MS) and nano-electrospray ionization-ion trap mass spectrometry (ESI-MS/MS) were performed to achieve a global picture of the environmental adaptation within *L. rhamnosus* strains.

3.2.3 Materials and methods

Bacterial strains and culture condition

L. rhamnosus PR825, PR826, PR830, PR1019, PR1215, PR1224, PR1473, PR1479, PR1484 and PR1489 were identified by 16S rRNA gene sequence analysis (Culture Collection of the Department of Genetics, Biology of Microorganisms, Anthropology, Evolution, University of Parma, Italy) and used in this study. All strains were isolated during Parmigiano Reggiano (PR) cheese making (19). Isolation was carried out after one (PR825, PR826 and PR830), four (PR1019), twelve (PR1215 and PR1224) and twenty months (PR1473, PR1479, PR1484 and PR1489) of ripening. Strains were isolated using MRS broth (Oxoid Ltd., Basingstoke, United Kingdom) or Cheese agar medium (38), and routinely propagated at 37°C in MRS medium (Oxoid) under anaerobic conditions. Strains were also cultivated in pasteurized and micro-filtrated milk (Premium blu, Parmalat, Parma, Italy), and Cheese broth medium. Cheese broth was made as described by Neviani et al. (39), with some modifications. Fine-grain grated PR cheese ripened for 18-20 months was dissolved (120 g/L) in sodium citrate (Sigma-Aldrich Co., St. Louis, MO) 0.07 mol/L pH 7.5. The suspension was incubated at 42°C for 50 min under stirring condition (150 x *g*). After incubation, the suspension was centrifuged (8000 x *g* for 15 min at 4°C) and filtered through sterile gauze to retain the surfaced fat layer. Filtered liquid was acidified to pH 4.6 by adding HCl 1.0 M, sterilized for 15 min at 121°C and centrifuged at 8000 x *g* for 15 min to remove un-hydrolyzed proteins (casein and heat-denatured serum proteins). The supernatant was added of NaOH 1 M until pH 6.1 was reached. Freeze-dried cell lysate of *Lactobacillus helveticus* PR775 (isolated from PR cheese) was added at the final concentration which corresponded to ca. 8 log CFU/mL. The resulting medium was sterilized by filtration on 0.22 µm membrane filters (Millipore Corporation, Bedford, MA 01730).

Fermentative profile by Biolog system

Three days before the inoculation of Biolog AN plates (Biolog, Inc., Hayward, CA, USA), the strains were streaked twice on MRS agar plates. The plates were incubated at 37°C for 24 h and after the cells were used for Biolog assays. The wells of the Biolog AN plates were inoculated with 150 μL of bacterial suspensions adjusted to 65% transmittance as recommended by the manufacturer. Positive reactions were automatically recorded using a microplate reader with 590 nm wavelength filter. The characterization of the fermentative profile by Biolog system was integrated with three pentose carbohydrates: D-xylose, ribose and L-arabinose. Similarities between the fermentation profiles were calculated for all positive and negative characters using the Jaccard coefficient and UPMGA analysis as previously described (15).

Kinetics of growth and acidification

Growth and acidification by *L. rhamnosus* strains were assayed using three different media: MRS broth, pasteurized and micro-filtrated milk and Cheese broth. Harvested cells were washed in 50 mM phosphate buffer pH 7.0, centrifuged at 10,000 x *g* for 10 min at 4°C, and re-suspended in the different media at the cell density of ca. 7.2 log CFU/mL. The media were incubated at 37°C for 48 h. Cell numbers were determined by plating on MRS agar at 37°C for 48 h. The kinetic of acidification was monitored on-line by a pH-meter (Model 507, Crison, Milan, Italy). Concentrations of organic acids were determined by HPLC (High Performance Liquid Chromatography) using ÄKTApurifier™ systems (GE Healthcare Life Sciences). The analyses were carried out isocratically at 0.8 mL/min and 65°C with a 300 x 7.8 mm i.d. cation exange column (Aminex HPX-87H) (Bio-Rad Laboratories). Mobile phase was 0.013 N H_2SO_4 prepared by diluting reagent grade sulfuric acid with distilled water, filtering through a 0.45 μm membrane filter (Sartorius, AG, Göttingen, Germany), and degassing under vacuum. Analytical grade reagents were used as standards (Sigma Chemical).

Concentration of free amino acids

To determine free amino acids (FAA), MRS, pasteurized and micro-filtrated milk or Cheese broth cultures were centrifuged at 10,000 x g for 10 min. Proteins and peptides were precipitated by addition of 5% (vol/vol) cold solid sulfosalicylic acid, holding at 4°C for 1 h and centrifuging at 15,000 × *g* for 15 min. Total and individual FAA were analyzed by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, England) (42).

Protein extraction, two-dimensional electrophoresis (2-DE) and multivariate analysis of 2-DE protein patterns

For 2-DE analyses, harvested cells from MRS or Cheese broth were washed in 0.05 M Tris/HCl pH 7.5, centrifuged (15,000 × *g* for 15 min at 4°C) and frozen or directly resuspended in denaturing buffer containing 8 M urea, 4% CHAPS, 40 mM Tris base and 65 mM dithiothreitol (DTT) (GE Healthcare, Uppsala, Sweden). To extract total proteins, cells were disrupted with a Branson model B15 Sonifier by 3 cycles of sonication (1 min each). After pelleting of unbroken cells (15,000 × *g* for 15 min at 4°C), the protein content of the supernatant was measured by the method of Bradford (7).

Two-DE was carried out using the immobiline/polyacrylamide system, essentially as described by Görg et al. (25) and Hochstrasser et al. (27), using a Pharmacia 2-D-EF system (GE Healthcare). The same amount of total protein (45 μg) was used for each electrophoretic run. Isoelectric focusing was carried out on 18 cm immobiline strips providing a non-linear pH 3-10 gradient (IPG strips, GE Healthcare) by IPG-phore, at 15°C. Voltage was increased from 300 to 5000 V during the first 5 h, then stabilized at 8000 V for 8 h. After electrophoresis, IPG strips were equilibrated as described by De Angelis et al. (13). Following electrophoresis, IPG strips were equilibrated for 12 min against buffer A (6 M urea, 30% [vol/vol] glycerol, 2% [wt/vol] SDS, 0.05 M Tris-HCl [pH 6.8], 2% [wt/vol] DTT) and for 5 min against buffer B (6 M urea, 30% [vol/vol] glycerol, 2% [wt/vol] SDS, 0.05 M Tris-HCl [pH 6.8], 2.5% [wt/vol] iodoacetamide, 0.5% bromophenol blue). For the second dimension, 12.5% homogeneous SDS-PAGE gels were used. Voltage was 15 mA for gel by using a Ettan DALTsix electrophoresis unit (GE Healthcare). Gels calibration and spot detection were performed as described by De Angelis et al. (13). Gels were stained by using Silver and colloidal Coomassie Blue methods. The protein maps were scanned with a laser densitometer (Molecular Dynamics 300s) and analyzed with the ImageMaster 2D Platinum v6.0 computer software (GE Healthcare). Three gels from three independent experiments were analyzed and spot intensities were normalized as reported by Bini et al. (5). In particular, the spot quantification for each gel was calculated as relative volume (% VOL); the relative VOL was the VOL of each spot divided by the total VOL over the whole image. In this way, differences in colour intensities among the gels were eliminated (1, 13).. The induction factor is defined as the ratio between the spot intensity of a protein from cells cultivated in Cheese broth and the spot intensity of the same protein from cells grown in MRS. The reduction factor is defined as the ratio between the spot intensity of a protein from cells grown in MRS and the spot intensity of the same protein from cells cultivated in Cheese broth. All the induction or reduction factors were calculated based on the average of the spot

intensities of each of the three gels and standard deviation was calculated. The comparison between the level of synthesis for the same spot between strains and media was performed as percentage of relative volume, attributing the value of 100 to the condition wherein the protein had been synthesized at the highest level.

Principal component analysis (PCA) was carried out as described by Jacobsen et al. (29). Protein profiles and volume of each spots were exported from 2-DE gels and used as variables for PCA analysis. The variable selection was carried out by the cross-model validation (CMV) method, using the indicator variables as the y matrix and the protein spot volumes as the x matrix. CMV is an extended PLS/jack knife cross-validation method, but with an additional validation step. Each subset of samples is left out and validated against a model generated on the basis of cross-validation of the rest of the samples. Significance was reported as the percentage where each variable was marked as significant when tested on the external samples not included in the variable selection process. A cut off of 75% was chosen for the preliminary results to decrease the risk of missing some potential important proteins (29).

Protein identification by MALDI-time of flight mass spectrometry (MALDI-TOF-MS/MS) and multidimensional liquid chromatography (MDLC) coupled by nano-electrospray ionization-ion trap mass spectrometry (ESI-MS/MS).

Protein spots were excised from the gels manually and transferred to pierced V-bottom 96-well polypropylene microplates (Bruker Daltonik, Bremen, Germany) loaded with ultrapure water. Samples were digested automatically using a Proteineer DP robot (Bruker Daltonik) under the control of Control 1.2 software (Bruker Daltonik) according to the protocol of Shevchenko et al. (41), with minor variations. Gel plugs were submitted to reduction with 10 mM DTT in 50 mM ammonium bicarbonate (99.5% purity; Sigma Chemical, St. Louis, MO, USA) and alkylation with 55 mM iodoacetamide (Sigma Chemical) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) at a final concentration of 8 ng/µL in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion proceeded at 37ºC for 8 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Sigma Chemical) was added for peptide extraction, and the resulting digestion solutions transferred by centrifugation to V-bottom 96-well polypropylene microplates (Greiner Bio-One, Frickenhausen, Germany). MALDI samples were prepared by mixing equal volumes of the above digestion solution and a matrix solution composed of α-cyano-

4-hydroxycinnamic acid (Bruker Daltonik) in 50% aqueous acetonitrile and 0.25% trifluoroacetic acid. This mixture was deposited onto a 600 µm AnchorChip prestructured MALDI probe (Bruker Daltonik) (40) and allowed to dry at room temperature. Samples were automatically analyzed in an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik) (44) with an automated analysis loop using internal mass calibration, under the control of flexControl 2.2 software (Bruker Daltonik). In a first step, the MALDI-MS spectra were acquired by averaging 300 individual spectra in the positive ion reflector mode at 50 Hz laser frequency in a mass range from 800 to 4000 Da. Internal calibration of MALDI-MS mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2211.105. In a second step, precursor ions exceeding a threshold signal-to-noise ratio in the MALDI-MS mass spectrum were subject to fragment ion analysis in the tandem (MS/MS) mode. Precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector to average 1000 spectra. For MALDI-TOF-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region. Automated analysis of mass data was performed using the flex Analysis 2.2 software (Bruker Daltonik). MALDI-TOF-MS and MS/MS spectra were manually inspected in detail and reacquired, recalibrated and/or relabelled when necessary using the above programs as well as home-made software.

MALDI-TOF-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker Daltonik) to search a non-redundant protein database (NCBInr; ~10^7 entries; National Center for Biotechnology Information, Bethesda, US; or SwissProt; ~5 x 10^5 entries; Swiss Institute for Bioinformatics, Switzerland) using the Mascot software (Matrix Science, London, UK) (39). Other relevant search parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl; allow up to one missed cleavages; peptide tolerance ± 20 ppm; MS/MS tolerance ± 0.5 Da.

Protein identification was carried out also using a multidimensional liquid chromatography (MDLC) coupled with a nano-electrospray ionization (ESI)-ion trap mass spectrometry (MS). The HPLC apparatus consisted of an Ettan MDLC (GE Healthcare, Milan, Italy) equipped with a Zorbax 300 SD C18 precolumn (5 by 0.3 mm) and a Thermo Electron BioBasic-8 column (150 by 0.18 mm). The MDLC was connected to a Finningan LCQ Deca XP Max ion trap mass spectrometer (ThermoElectron Co., San Jose, CA) through the nano-ESI interface. Aliquots of 10 µL of each spot were injected. HPLC separation was carried out at a flow rate of 75 µL/min by using a gradient

elution with water (eluent A) and 84% acetonitrile (eluent B), both containing 0.1% (vol/vol) formic acid. The following program was used: 0% eluent B for 30 min; 0 to 100% (vol/vol) eluent B in 100 min, isocratic elution with 100% eluent B for 100 min, return to 0% eluent B in 5 min, and column reconditioning for 30 min. The flow rate at the nano-ESI source was 2.5 μL/min. The LCQ spectrometer, completely controlled by the Xcalibur software (ThermoElectron), operated in the positive ion mode (17).

Statistical analysis

Data (three replicates) from microbial growth, acidification, organic acids and FAA were subjected to one-way ANOVA (SAS, 1985), and pair-comparison of treatment means was achieved by Tukey's procedure at $p<0.05$, using the statistical software Statistica for Windows (Statistica 6.0 per Windows 1998). Multivariate statistical analysis (MSA) and PCA were carried out using Statistica 6.0 per Windows 1998 (StatSoft).

3.2.4 Results

Fermentative profiles of *L. rhamnosus* strains

The fermentative profiles of the *L. rhamnosus* strains used in this study were determined according to the Biolog system (see supplementary material, Table 1S). All strains fermented N-acetyl-d-glucosammine, D-fructose, dextrin, lactose, maltose, D-mannitol, D-mannose, turanose, α-hydroxy-butyric acid, α-ketobutyric acid, D,L-lactic acid, D,L-lactic acid methyl ester, pyruvic acid, pyruvic acid methyl ester, thymidine and uridine. N-Acetil-D-galactosamine, arbutin, D-galactose, glucose-6-phosphate, palatinose, salicin, D-sorbitol, D-trehalose, turanose and 2-deoxy-adenosine were fermented by ca. 80% of the strains. In spite of the above results, a rather large variability was found. Based on the fermentative profiles, strains were grouped at the similarity level of ca. 73% (Fig.1).

Kinetics of growth and acidification

Strains were inoculated into the different culture media at the cell density of ca. 6.8 log CFU/mL. The stationary phase was reached after ca. 24, 36 or 48 h of incubation at 37°C depending on the growth on MRS broth, pasteurized and micro-filtrated milk or Cheese broth, respectively. When cultivated in MRS broth, the final cell density varied from 8.79 ± 0.09 (PR1484) to 9.45 ± 0.11 (PR1224) CFU/mL (Fig. 2A). The median value was 9.25 CFU/mL. Compared to MRS broth, cell growth decreased in pasteurized and micro-filtrated milk (median value of 8.70 CFU/mL) and,

especially, in Cheese broth (median value of 8.49 CFU/mL). Compared to growth on pasteurized and micro-filtrated milk, only a few strains had higher (PR825, PR1019 and PR1479) cell density during cultivation in Cheese broth. *L. rhamnosus* PR1019 and PR1473 were, respectively, the strains showing the highest and lowest cell density in Cheese broth.

Acidification by *L. rhamnosus* strains also varied depending on the culture media. The values of ΔpH during growth in MRS broth varied from 1.67 ± 0.05 to 1.95 ± 0.1 (Fig. 2B). The median value was 1.88. The lowest value of ΔpH (1.67 ± 0.04) was found for strain PR1215. During growth in pasteurized and micro-filtrated milk, the values of ΔpH ranged from 1.03 ± 0.02 to 2.13 ± 0.07. The median value was 1.20. Acidification markedly decreased when strains were cultivated in Cheese broth. The median value of ΔpH was 0.13. PR1215, PR1479 and PR1484 were the strains showing the highest acidification in Cheese broth.

The concentration of D,L-lactic acid in MRS broth ranged from 43.3 ± 0.19 to 151,7 ± 4.8 mM (median value of 103.5 mM) (Fig. 3A). Almost similar results were found for strains grown in pasteurized and micro-filtrated milk (median value of 107.9 mM). As expected, the concentration of D,L-lactic acid markedly decreased when strains were cultivated in Cheese broth (median value of 4.5 mM). No synthesis of acetic acid was found when strains were cultivated in MRS broth (Fig. 3B). Only strains PR825, PR826 and PR1489 synthesized acetic acid during growth in pasteurized and micro-filtrated milk (27.5 ± 0.06 - 29.4 ± 1.04 mM). All strains synthesized acetic acid during cultivation in Cheese broth. The concentration varied from 39.7 ± 1.5 to 71.6 ± 2.1 mM. The median value was 39.7 mM. *L. rhamnosus* PR830, PR1019 and PR1215 showed the highest synthesis of acetic acid.

Concentration of free amino acids during growth in Cheese broth

Cheese broth without bacterial inoculum was incubated for 48 h at 37°C and used as the control. After incubation, the concentration of free amino acids (FAA) was 3614.6 ± 29.8 mg/kg. Fermentation by *L. rhamnosus* strains caused a variation of the concentration of FAA (Table 1)., Compared to control, all the other strains significantly (p<0.05) increased the concentration of FAA (the only exception was strain PR1473). The increase of FAA varied from ca. 38.4 ± 1.2 (PR1489) to 2658.0 ± 17.8 mg/kg (PR825). Overall, Cheese broth fermented with strains PR825 and PR826, and with PR1019 and PR1484 showed similar profiles of FAA. Compared to control, all FAA decreased during growth of *L. rhamnosus* PR1473. The only exception was Orn. With a few exceptions, Arg and Pro were the FAA found at lowest and highest concentration, respectively.

Compared to control, Cheese broth fermented with PR830 and PR1215 showed a marked decrease of the concentration of Ser and Arg .

L. rhamnosus PR825 (highest liberation of FAA - isolated after one month of cheese ripening), PR830 (highest synthesis of acetic acid - isolated after one month of cheese ripening), PR1019 (highest cell density - isolated after four month of cheese ripening), PR1215 (highest acidification - isolated after twelve month of cheese ripening) and PR1473 (lowest growth and FAA - isolated after twenty month of cheese ripening) were considered as the representative strains which showed distinguishing features during growth in Cheese broth. They were selected for further characterization.

Two-DE analysis and protein identification by MALDI-TOF-MS/MS and LC-nano-ESI-MS/MS.

After the stationary phase was reached, the cytosolic proteins of *L. rhamnosus* PR825, PR830, PR1019, PR1215 and PR1473 grown in MRS broth were used to construct the 2-DE reference maps. As shown using 3-10 IPG strips, most of the extracted proteins had values of pI ranging from *ca.* 4.0 to 9.2 and molecular weight (mol wt) which varied from *ca.* 10 to 90 kDa. Overall, strains PR825 and PR830, and strains PR1019 and PR1215 gave 2-DE maps almost similar, and further Figures 4A-F only referred to *L. rhamnosus* PR825, PR1019 and PR1473. Strains of *L. rhamnosus* commonly synthesized ($p<0.05$) 186 protein spots (data not shown). Other 117 protein spots were differentially synthesized (Figs. 4A-C and Table 2S). Within these latter protein spots, the maximum level of synthesis (100%) was found for 55 and 52 proteins in *L. rhamnosus* PR825 and PR830, respectively, and for 35 and 32 proteins in *L. rhamnosus* PR1019 and PR1215, respectively. The lowest number of protein (10 spots) which showed the maximum level of synthesis was found in strain PR1473 (Figs. 4A-C, and Table 2S).

The 2-DE maps of strains cultivated in Cheese broth were compared with those of strains grown on MRS broth. Among the 186 protein spots commonly synthesized during growth in MRS broth, 182 also showed the same level of synthesis during cultivation in Cheese broth. Other 121 protein spots, including the above 117 which differentiated the strains cultivated in MRS broth, were differentially ($p<0.05$) synthesized (Figs. 4A-F, and Table 2S). Although protein spots were almost the same, their synthesis varied depending on the culture media. Indeed, 55 and 43 (strain PR825), 54 and 43 (PR830), 53 and 42 (PR1019), 52 and 35 (PR1215), and 36 and 45 protein spots (PR1473) showed decreases or increases, respectively, when the growth in Cheese broth was compared to that in MRS

broth (Figs. 4A-F and Table 2S). The synthesis of 27 and 24 of the above decreasing or increasing proteins was common between *L. rhamnosus* strains.

PCA, based on the data obtained from 2-DE maps of *L. rhamnosus* strains grown in MRS or Cheese broth, was used to reduce the dimension data set and to visualize the related structure. Using the first and second Principal Component (PCs) only, a large part of the information data is shown in a simple 2D plot (Fig. 5). Indeed, PC1 and PC2 explained ca. 70% of the total variance of the data. PC1 showed the level of synthesis of each proteins, and PC2 showed the total number of proteins. Compared to other strains, PR1473 markedly differentiated from the other strains either during cultivation in MRS or in Cheese broth. The other strains grouped together, especially during cultivation in Cheese broth. The highest synthesis of proteins characterized the growth in MRS, especially for *L. rhamnosus* PR825 and PR830.

A total of 93 well-separated and prominent protein spots which showed decreased (46 spots, including the 27 protein spots commonly synthesized by all *L. rhamnosus* strains) or increased (47 spots, including the 24 protein spots commonly synthesized by all *L. rhamnosus* strains) levels of synthesis during growth in Cheese broth were identified by MALDI-TOF-MS/MS or LC-nano-ESI-MS/MS (Tables 2, 3 and 3S). Compared to cells grown in MRS, when cultivated in Cheese broth *L. rhamnosus* strains decreased the synthesis of proteins involved on: (i) synthesis of proteins (PS, 2 spots); (ii) nucleotide metabolism (NM, 7 spots); (iii) carbohydrate metabolism and glycolysis, (CMG, 11 spots); (iv) proteolytic enzyme system (PES, 2 spots); (v) cell wall biosynthesis (CWB, 3 spots); (vi) exopolysaccharide biosynthesis (EPSB, 4 spots); (vii) cellular regulation (R, 2 spots); (viii) stress response (SR, 2 spots); (ix) miscellaneous/hypothetical function (M, 3 spots); (x) metabolic process (MP, 2 spots) and (xi) hypothetical protein (HP, 8 spots). Compared to cells grown in MRS, when cultivated in Cheese broth *L. rhamnosus* strains increased the synthesis of other proteins involved on: (i) PES (7 spots); (ii) citrate metabolic process (CMP, 3 spots); (iii) amino acid metabolism (AM, 8 spots); (iv) oxidation/reduction processes (OR, 5 spots); (v) CM (8 spots); (vi) CWB (1 spot); (vii) R (1 spot); (viii) bifunctional ribosomal proteins (synthesis of proteins and stress response PS-SR, 4 spots); (ix) NM (2 spots); (x) SR (4 spots); (xi) M, (3 spots); and (xii) PS, (1 spot).

3.1.4 Discussion

This study reports an alternative (3, 38, 47) and integrated metabolic and proteomic approach to investigate the diversity and environmental adaptation of cheese-related (Parmigiano Reggiano) strains of *L. rhamnosus*. Usually, fermentative profiles of lactobacilli are characterized by the API

50 CH System. This study used the semi-automated, computer-linked technology of the Biolog system to get information about 95 different carbon sources. The major part of the *L. rhamnosus* strains had the capacity to use a large number of chemical compounds also found in cheese during ripening (19, 35, 38). A few free amino acids (FAA) were also used (L-alanyl-L-histidine, glycyl-L-methionine, L-methionine and L-threonine) as energy sources. Similar results were found for *Weissella* and *Lactobacillus* strains which used peptides and FAA only when an exogenous source of α-ketoglutaric acid was available (48). As expected, all strains grew and acidified better in MRS broth compared to pasteurized and micro-filtrated milk (16). Overall, cell growth was the lowest in Cheese broth. No synthesis of acetic acid was found during growth on MRS broth but high levels of this acid were synthesized by all strains during growth in Cheese broth. It seemed that *L. rhamnosus* strains had the capacity to activate different metabolic pathways depending on the culture media. Under stress condition (e.g., starvation and acid stress), lactobacilli may activate catabolic pathways of FAA to synthesize ATP directly or by proton translocation, thereby decreasing the level of ATP needed for proton balancing (33, 45). It was previously shown (45) that transamination and degradation of aromatic and branched-chain amino acids may generate ATP by proton-motive force-driven transhydrogenase reaction. The transhydrogenase is a highly favorable reaction, as it regenerates catabolic NAD and produces NADPH for biosynthesis. Anaerobic oxidation of NADH allows less ethanol formation and hence more acetate with concomitant synthesis of ATP (45). Other authors (26) demonstrated that *Lactobacillus* sp. incubated in the presence of glutamate were capable of synthesizing ATP, which suggested an important role of glutamate for energy metabolism. During cheese ripening, high levels of FAA are liberated through the activity of peptidases from primary starters and NSLAB (23). As expected, the concentration of FAA during cultivation on Cheese broth resulted as the balance between peptidase activities and catabolism of FAA. Except for *L. rhamnosus* PR1473, all strains caused an increase of the concentration of FAA with differences among individual FAA. The peptidase activities and the catabolism of FAA markedly varied between cheese-related lactic acid bacteria (12, 18, 21, 22, 23).

The proteomic approach aimed at elucidating the (i) intra-specific diversity of *L. rhamnosus* and (ii) the metabolic mechanisms involved during growth under culture conditions (e.g., lactose deficiency) which mimicked the cheese during ripening. Proteomic maps of five strains which showed different metabolic and technology features were obtained. Overall, the synthesis of ca. 60 - 70% of the cytosolic proteins of *L. rhamnosus* strains was not affected by the culture media. The remaining part (ca. 30-40%) of the total proteome seemed to vary depending on strains and environmental conditions. The effect of the growth medium on the proteome of *L. rhamnosus* was previously

studied (34). Recently, comparative studies on bacterial genomes showed that adaptation to dairy niches is associated either to metabolic simplification (loss of genes involved in carbohydrate, amino acid and cofactor metabolisms) or to increasing expression of genes related to peptide hydrolysis and amino acids catabolism (6, 10, 28, 46). By comparing the cultivation on Cheese broth and MRS, the proteomic maps of strains PR825 and PR830 showed the largest differences (Table 2S, Fig. 4A-F and Fig. 5). Both these strains were isolated only after one month of Parmigiano Reggiano cheese ripening which would suggest a relatively weak adaptation to this environment. On the contrary, strains isolated during late ripening (e.g., twelve and twenty months for PR1215 and PR1473, respectively) did not show noticeable large differences between the protein maps obtained after cultivation in MRS and Cheese broth. This would hypothesize a more pronounced adaptation to cheese-like conditions. The genomes of versatile microorganisms such as *L. plantarum, L. casei* and *L. rhamnosus* contain high numbers of coding sequences which are involved in carbohydrate utilization and transcriptional regulation. This reflects their requirement for dealing with diverse environmental conditions (9, 31). In agreement with the genome simplification found for dairy starters such as *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* (10, 46), the proteome simplification of *L. rhamnosus* strains under cheese-like conditions could also be hypothesized. Except for a few exceptions, the synthesis of proteins during cultivation on Cheese broth seemed to have distinguishing features which are common to almost all strains. According to the synthesis of acetic acid, all strains over-synthesized the acetate kinase. The synthesis of proteins involved on biosynthetic processes (protein and nucleotide metabolism; cell-wall biosynthesis and EPS production) decreased. On the contrary, peptidases (dipeptidyl aminopeptidase, aminopeptidase S, proline dipeptidase, bifunctional S2, S16 and S24 family peptidases and methionine aminopeptidase) and enzymes responsible for the catabolism of FAA (arginine deiminase, cystathionine β-liase, cysteine synthase and L-serine dehydratase β-subunit) were over-synthesized. Enzymes responsible for the citrate catabolism also increased during cultivation on Cheese broth. The growth of *L. rhamnosus* on citrate, and the concomitant synthesis of lactate, acetoin, acetate and diacetyl was previously reported (30). Enzymes involved in oxidation/reduction processes were also over-synthesized . This feature was probably related to the need of maintaining the balance between $NAD(P)^+$ and $NAD(P)H + H^+$, which is indispensable for the catabolic reactions involved in the synthesis of ATP (e.g., catabolism of glutamate) (2). Proteins related to stress response adaptation were also over-synthesized during growth on Cheese broth. Indeed, such environment could be considered as sub-optimal for growing *L. rhamnosus* strains (14).

This study describes the metabolic potential of *L. rhamnosus* strains and the strategies used to adapt the energy metabolism to the growth conditions. Adaptation of *L. rhamnosus* to cheese-like conditions is a complex process, which is related to proteome modification according to over- and under-synthesized proteins responsible for the global metabolism. First, this study combined metabolic data (fermentative profiles, synthesis of organic acids and FAA) and 2-DE analyses followed by the identification of a large number of proteins to highlight the physiological mechanisms of adaptation of *L. rhamnosus* which are responsible for survival in cheese during ripening.

3.2.6 References

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3.2.7 Legends to figures

Figure 1. Dendrogram of combined fermentative profiles of *Lactobacillus rhamnosus* strains as determined using the Biolog system. Cluster analysis was carried out on the Jaccard coefficient matrix by UPGMA cluster analysis.

Figure 2. Cell densities (CFU/mL) (A) and acidification (ΔpH) (B) of *Lactobacillus rhamnosus* PR825, PR826, PR830, PR1019, PR1215, PR1224, PR1473, PR1479, PR1484 and PR1489 during growth in MRS broth ( ), pasteurized and micro-filtered milk ( ) or Cheese broth ( ) for 24, 36 and 48 h, respectively, at 37°C. ΔpH was the differences between the pH of the medium at the inoculum (pH_{To}) and the pH at the end of incubation (pH_{Tf}). Data are the means ± SD of three separate experiments performed in triplicate. Box plots are shown also. The center line of each box represents the median (□), the top and bottom of the box represent the 75th and 25th percentile of the data, respectively. The top and bottom of the error bars represent the 5th and 95th percentile of the data, respectively. The circles in each box plot extend to the outliers (*).

Figure 3. Synthesis (mM) of lactic (A) and acetic (B) acids by *Lactobacillus rhamnosus* PR825, PR826, PR830, PR1019, PR1215, PR1224, PR1473, PR1479, PR1484 and PR1489 during growth in MRS broth ( ), pasteurized and micro-filtered milk ( ) or Cheese broth ( ) for 24, 36 and 48 h, respectively, at 37°C. Data are the means ± SD of three separate experiments performed in triplicate. Box plots are shown also. The center line of each box represents the median (□), the top and bottom of the box represent the 75th and 25th percentile of the data, respectively. The top and bottom of the error bars represent the 5th and 95th percentile of the data, respectively.

Figure 4. Two-dimensional electrophoresis (2-DE) analysis of protein synthesized by *Lactobacillus rhamnosus* PR825 (A,D), PR1019 (B,E) and PR1473 (C,F) cultivated in MRS (A,B,C) and Cheese broth (D,E,F) until the early-stationary phase of growth (37°C for 24 and 48 h, respectively) was reached. Numbered triangles and circles refer to proteins which are present in decreased and increased amounts, respectively, compared to cells grown in MRS.

Figure 5. Score plot of the first and second principal components (PC1 and PC2) after principal component analysis (PCA) based on the data obtained from two-dimensional electrophoresis (2-DE)

analysis of protein synthesized by *Lactobacillus rhamnosus* strains grown in MRS (PR825M, PR830M, PR1019M, PR1215M and PR1473M) and Cheese broth (PR825C, PR830C, PR1019C, PR1215C and PR1473C) until the early-stationary phase of growth was reached.

Table 1. Variation of the concentration (mg/kg) of free amino acids (FAA) found in Cheese broth after fermentation by *Lactobacillus rhamnosus* strains for 48 h at 37°C. Data are calculated as the differences between fermented and non inoculated Cheese broth (control) incubated at 37°C for 48 h.

Amino acids	Control	PR825	PR826	PR830	PR1019	PR1215	PR1224	PR1473	PR1479	PR1484	PR1489
Threonine	96.4 ± 3.2	138.6a	129.9°	123.2ab	119.4ab	58.4c	35.5d	-19.0f	110.7b	121.8ab	-2.3e
Serine	370.3 ± 12.6	318.6a	300.0a	-202.1e	278.2b	-337.8f	117.5c	-72.9d	263.2b	285.4b	-2.1de
Glutamic Acid	481.2 ± 24.4	214.4a	198.0a	213.1a	191.2a	177.4b	70.8c	-41.5e	183.4b	197.6a	1.3d
Glycine	129.9 ± 1.5	95.3a	89.8b	90.7b	86.6b	80.5c	36.9d	-24.2f	84.3c	89.2b	-1.1e
Alanine	116.6 ± 4.4	143.0a	135.9b	134.3b	131.4b	123.2c	40.2d	-19.8f	123.7c	131.8b	1.3e
Valine	247.3 ± 6.3	87.7a	84.8°	88.9a	85.1a	82.4a	36.3b	-26.7d	81.5a	89.5a	0.8c
Methionine	126.0 ± 1.5	159.0a	147.5ab	152.5a	142.7b	137.4b	47.8c	-22.7e	134.5b	144.6ab	1.9d
Isoleucine	293.9 ± 8.8	208.6a	198.5°	204.4a	196.7a	190.7a	88.0b	-53.4d	188.7a	201.2a	-0.8c
Leucine	396.2 ± 6.5	175.2a	167.8b	175.6a	169.7b	160.6b	77.1c	-58.2e	162.8b	175.0a	-0.3d
Tyrosine	80.9 ± 1.4	131.0a	112.9b	-65.2f	131.8a	12.2d	42.9c	-15.1e	114.5b	122.2ab	7.2d
Phenylalanine	236.2 ± 2.41	262.0a	240.7b	252.6a	240.3b	223.4c	91.3d	-47.1f	221.7c	237.6b	-0.2e
Histidine	125.2 ± 1.7	128.3a	121.5°	124.3a	118.8a	106.2b	46.0c	-22.8e	114.1ab	120.4a	2.2d
Trypthophan	28.3 ± 1.0	52.6a	46.8ab	50.3a	43.5b	39.3b	14.7c	-4.6e	39.9b	43.2b	2.5d

Table 1. continued

Amino acids	Cheese broth	PR825	PR826	PR830	PR1019	PR1215	PR1224	PR1473	PR1479	PR1484	PR1489
Ornitine	39.7 ± 2.3	115.4^{b}	118.8^{b}	194.1^{a}	48.8^{c}	-37.1^{f}	16.2^{d}	17.4^{d}	46.7^{c}	50.9^{c}	0.4^{e}
Lysine	403.6 ± 4.8	156.3^{a}	143.0^{c}	149.9^{b}	143.4^{c}	137.5^{c}	59.3^{d}	-50.0^{f}	137.1^{c}	149.4^{b}	-3.3^{e}
Arginine	54.1 ± 2.4	-51.4^{d}	-54.1^{d}	-52.6^{d}	79.1^{a}	-54.1^{d}	21.6^{b}	-54.1^{d}	67.2^{a}	71.7^{a}	0.1^{c}
Proline	388.8 ± 7.2	323.2^{a}	298.0^{ab}	273.7^{b}	287.6^{ba}	277.4^{b}	112.0^{c}	-56.4^{e}	273.7^{b}	291.3^{ab}	9.5^{d}
Total	3614.6 ± 29.8	2658.0^{a}	2480.3^{b}	1907.9^{d}	2494.4^{b}	1377.7^{e}	954.4^{f}	-571.3^{h}	2346.9^{c}	2523.4^{ab}	38.4^{g}

Data are means of three independent experiments twice analyzed. $^{a-f}$The values in the same raw with different superscript letters differ significantly ($p < 0.05$).

Table 2. Putative function of proteins over-synthesized in *Lactobacillus rhamnosus* strains when cultured in MRS broth compared to Cheese broth (Fold change ≥ 2.0. $p \leq 0.05$).

Spot[a]	Homologous protein	Function[b]	A.N.[c]
2	Protein translation elongation factor (FusA)	PS	YP_003172239.1.
4	Dipeptidyl aminopeptidase/acylaminoacyl-peptidase (YuxL)	PES	C7TDR6-1
5	Phosphoribosylformylglycinamidine synthase II (FGAM II)	NM	YP_003171555
6	Fructose-1.6-bisphosphatase (FBPase)	CMG	LRH_04694
7	Chaperone protein dnaK (DnaK)	SR	C2JXM9
8	Beta-glucoside-specific PTS system IIABC component (PTS II glc)	CMG	LRH_02452
9	Dihydrolipoamide acetyltransferase (DlaT)	CMG	B5QMX1
10	GMP synthase (GuaA)	NM	O85192
12	H(+)transorting two-sector ATPase, alpha subunit (atpA)	M	C7TBT3
13	H(+)transorting two-sector ATPase, alpha subunit (atpB)	M	C7TBS9
15	Hypothetical protein	HP	C7T8A2
16	CTP synthetase (Ctps)	NM	ZP_03212229
21	Hypothetical protein	HP	B2KU23-1
23	Transcription elongation factor NusA (NusA)	R	ZP_03210532
25	Dipeptidase (PepV)	PES	ZP_04441957
28	Glucose-6-phosphate isomerase (Pgi)	CMG	C7TBI7
30	Serine--tRNA ligase (SerRS)	NM	ZP_04442045
31	UDP-N-acetylmuramoylalanine-D-glutamate ligase (MurD)	CWB	C7TC34
32	Phosphomannomutase (Pmm)	CMG	ZP_03210749
34	Hypothetical protein	HP	Q1WVU0
35	Hypothetical protein	HP	Q38Z86
43	Inorganic pyrophosphatase/exopolyphosphatase (PPase/ExopolyPase)	M	ZP_03212660

Table 2. continued.

Spot[a]	Homologous protein	Function[b]	A.N.[c]
45	UDP-N-acetylmuramate-L-alanine ligase (MurC)	CWB	C7TK53
46	UDP-N-acetylglucosamine 2-epimerase (UDP-GlcNAc-2-epimerase) (MnaA)	CWB	YP_003174688
48	DNA-directed DNA polymerase III, beta chain (DnaN)	NM	C7TF57
49	Glucose-1-phosphate adenylyltransferase (ADPGlc PPase)	CMG	ZP_03212472
50	dTDP-glucose 4,6-dehydratase (RmlB)	EPSB	C7TE90
55	Phosphomannose isomerase (Pmi)	CMG	ZP_03212702
56	dTDP-glucose 4,6-dehydratase (RmlB)	EPSB	C7TE49
57	Aryl-alcohol dehydrogenase related enzyme (Badh)	CMG	ZP_03211231
66	Putative galactofuranosyltransferase (WelG)	EPSB	C7TE97
68	Phosphoribosylpyrophosphate synthetase (Prs-I)	NM	YP_807748
72	Hypothetical protein LRH_05549	HP	ZP_03210753
82	Hypothetical protein	HP	B5QJH1
85	Phosphoglycerate mutase 1 (Pgam 1)	CMG	ZP_03210875
86	Glucosamine-6-phosphate isomerase (Gnpda)	CMG	ZP_03211688
92	dTDP-4-dehydrorhamnose 3.5-epimerase (RmlC)	EPSB	YP_003174729
94	Pyrimidine regulatory protein PyrR (UPRTase)	NM	ZP_03211791
104	Hypothetical protein	HP	C7TLJ7
107	Small heat shock protein (sHSP)	SR	YP_002221594
109	Arginine repressor 1 (ArgR1)	R	B5QJX6
110	PTS system transporter subunit IIB (PTS IIB)	CMG	YP_003173174
115	Hypothetical protein LRH_11899	MP	ZP_03211087
116	Pyridoxine 5'-phosphate oxidase V (Pnpo)	MP	ZP_03211323
117	Hypothetical protein	HP	B5RSF8

[a]Spot designation correspond to those of the gels in Figure 4 A-F. [b]Protein synthesis, PS; Nucleotide metabolism (Purine biosynthesis), NM; Carbohydrate metabolism and Glycolysis, CMG; Proteolytic enzyme system, PES; Cell Wall biosynthesis, CWB; Exopolysaccharide biosynthesis, EPSB; Cellular regulation. R; Stress response, SR; Metabolic process, MP; Miscellaneous, M. [c]Accession number, A.N.

Table 3. Putative function of proteins over-synthesized in *Lactobacillus rhamnosus* strains when cultured in Cheese broth compared to MRS Broth (Fold change ≥ 2.0. $p \leq 0.05$).

Spot[a]	Homologous protein	Function[b]	A.N.[c]
3	Dipeptidyl aminopeptidase/acylaminoacyl-peptidase (DPP/:…)	PES	B5QMB6-1
18	Citrate (pro-3S)-lyase (CitC)	CMP	ZP_04442020
19	Citrate lyase. alpha subunit (CitF)	CMP	ZP_03211892
26	Oxaloacetate decarboxylase (Oxaloacetate decarboxylase. alpha chain) (OadA)	AM	C7TDW4
27	Dihydrolipoyl dehydrogenase (Dld)	OR	C7TC75
29	Pyridine nucleotide-disulphide oxidoreductase family protein	OR	YP_002221633
36	Ribose-5-phosphate isomerase A (Pri)	CM	CAR91534
39	Arginine deiminase (Adi)	AM	ZP_00604119
40	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase (MurF)	CWB	YP_003175234
41	Aminopeptidase PepS (PepS)	PES	YP_003170796.1.
44	ADP-glucose pyrophosphorylase (ADPGlc PPase)	CM	ZP_03212473
52	Glutamyl aminopeptidase (YsdC)	AM	C2JV86
53	Proline dipeptidase (Xaa-Pro dipeptidase)	PES	YP_003170538.1.
58	Transcriptional regulator	R	ZP_03212661
60	Acetate kinase (Acka)	CM	B5QKR4
61	S16 family peptidase (RP S2)	PES	C2JWU6
62	NADH dehydrogenase, FAD-containing subunit (Ndh)	OR	B5QM27
63	Cystathionine beta-lyase/cystathionine gamma-synthase	AM	LRH_02562
64	Phosphotransacetylase (Pta)	AM	C7TB61
70	Prolyl aminopeptidase (pepR)	PES	C7TAI8
71	Bifunctional S24 family peptidase/transcriptional regulator	PES	C2JVF6

Table 3. continued.

Spot[a]	Homologous protein	Function[b]	A.N.[c]
73	Glutamine amidotransferase (GATase)	CM	YP_003170280.1.
75	Cysteine synthase (CSase)	AM	C7TA20
76	Aldose 1-epimerase (GalM)	CM	ZP_04440939
77	Methionine aminopeptidase (Map)	PES	C7TBW8
79	L-serine dehydratase beta subunit (SdhB)	AM	C7TBW8
81	Ribosomal protein L1 (RP L1)	PS- SR	ZP_03212951
89	Hypoxanthine phosphoribosyltransferase (Hprt)	NM	ZP_04440348
90	Translation elongation factor P (EF-P)	PS- SR	ZP_03210599
91	Ribose-5-phosphate isomerase A	CM	C2JUN7
93	Glucosamine-6-phosphate deaminase/isomerase (NagB)	CM	YP_003172659.1.
95	Uracil phosphoribosyltransferase (Uprt)	NM	ZP_03211012
96	Glycine betaine/carnitine/choline ABC transporter. permease	SR	B5QQQ9
97	Glycerone kinase (Dha Kinase)	CM	ZP_04826040
98	Heat shock protein Hsp20 (HSP20)	SR	C2JUC9
99	50S ribosomal protein L5 (RP 50S-L5)	PS-SR	ZP_03212255
100	Thiol peroxidase (Tpx)	OR	ZP_04442480
102	4-Methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme. amidase family protein	M	ZP_03211639
103	Peptide methionine sulfoxide reductase msrA 2 (MsrA)	OR	C7TJ74
105	Phospholipid-binding protein	M	ZP_03211261
106	Hydrolase (YqeK)	AM	C7TDD7
108	D-tyrosyl-tRNA(Tyr) deacylase (Dtd)	M	C2JXJ5
112	Universal stress protein UspA nucleotide-binding protein (UspA)	SR	B5QKT5
113	Universal stress protein (Usp)	SR	ZP_04441763

Table 3. continued

Spot[a]	Homologous protein	Function[b]	A.N.[c]
118	30S ribosomal protein S10 (RpsJ)	PS-SR	B5QPR4
119	Citrate lyase acyl carrier protein (CitD)	CMP	B5QNR2
120	Asp-tRNA-Asn/Glu-tRNA-Gln amidotransferase C subunit (GatC)	PS	B5QKK8

[a]Spot designation correspond to those of the gels in Figure 4 A-F. [b]Cellular regulation, R; Citrate metabolic process, CMP; Aminoacid metabolism, AM; Oxidation reduction, OR; Carbohydrate metabolism, CM; Cell Wall biosynthesis, CWB; Proteolytic enzyme system, PES; Nucleotide metabolism (Purine biosynthesis), NM; Protein synthesis, PS; Stress response, SR; Miscellaneous. M; [c]Accession number, A.N.

Table 1S. Fermentative profiles of *Lactobacillus rhamnosus* strains as determined by Biolog system.

Source	PR 825	PR 826	PR 830	PR 1019	PR 1215	PR 1224	PR 1473	PR 1479	PR 1484	PR 1489
N-Acetil-D-Galactosamine	+	+	+	+	+	+	+	+	+	-
N-Acetil-D-Mannosamine	+	+	+	+	+	+	+	+	+	+
Adonitol	-	-	-	-	+	+	-	-	-	-
Amygdalin	-	+	+	-	-	-	+	-	-	-
D-arabitol	-	-	-	-	+	+	-	-	-	-
Arbutin	-	+	+	+	+	+	+	+	+	-
D-Cellobiose	-	+	-	-	+	+	+	-	+	-
Dextrin	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+
D-Galactose	-	+	+	+	+	+	+	+	+	-
D-Galacturonic Acid	-	+	-	-	+	+	+	-	+	-
Gentiobiose	-	+	-	-	-	+	-	-	-	-
D-Gluconic Acid	-	+	+	+	+	+	+	-	+	-
D-glucosaminic Acid	-	-	-	-	-	+	-	-	-	-
a-D-Glucose	+	+	+	+	+	+	+	+	+	+
Glucose-+-Phosphate	-	-	-	-	+	+	-	-	+	-
Glucose-6-Phosphate	+	+	+	-	+	+	+	+	+	-
Glycerol	-	-	-	-	+	-	+	-	-	-
D.L-a-Glycerol Phosphate	-	-	-	-	+	-	-	-	-	-
a-D-Lactose	+	+	+	+	+	+	+	+	+	+
Lactulose	-	+	+	+	-	+	-	-	+	-
Maltose	+	+	+	+	+	+	+	+	+	+
Maltotriose	+	+	-	-	+	+	-	-	+	-
D-Mannitol	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+
D-Melezitose	+	+	+	+	-	+	+	+	+	-
D-Melibiose	-	-	-	-	+	-	-	-	-	-
3-Methyl-D-Glucose	+	+	-	+	-	+	+	-	+	-
β-Methil-D-Galactoside	-	-	-	+	-	+	-	+	+	-
β-Methyl-D-Glucoside	-	+	-	-	+	+	-	+	-	-

Table 2S. Relative intensity (%) of proteins showing an increased and decreased level of synthesis in *Lactobacillus rhamnosus* PR825, PR830, PR1019, PR1215 and PR1473 when cultured in MRS or Cheese broth medium[*].

Spot	PR825		PR830		PR1019		PR1215		PR1473	
	MRS	Cheese broth	MRS	Cheese broth	MRS	Cheese broth	MRS	Cheese broth	MRS	Cheese broth
1	100.0[a]	0.1[d]	96.8[a]	3.1[d]	32.7[c]	0.5[d]	50.0[b]	0.9[d]	47.2[b]	0.6[d]
2	98.3[a]	61.0[c]	100.0[a]	50.5[c]	98.4[a]	50.0[c]	75.2[b]	48.3[c]	45.8[c]	25.2[d]
3	47.9[c]	75.5[b]	39.5[cd]	70.6[b]	50.5[c]	75.0[b]	21.5[d]	50.0[c]	12.5[de]	100.0[a]
4	73.7[b]	1.4[d]	100.0[a]	0.8[d]	96.1[a]	0.6[d]	96.4[a]	0.5[d]	50.7[c]	0.8[d]
5	100.0[a]	1.5[e]	78.4[b]	0.9[e]	75.0[b]	0.5[e]	50.0c	1.0[e]	15.5d	1.1[e]
6	100.0[a]	0.5[d]	75.0[b]	0.2[d]	70.0[b]	0.6[d]	70.5[b]	0.7[d]	10.0[c]	0.5[d]
7	99.5[a]	50.0[b]	100.0[a]	54.2[b]	97.5[a]	52.0[b]	94.8[a]	51.2[b]	99.8[a]	45.5[b]
8	100.0[a]	1.5[d]	99.6[a]	0.8[d]	98.5[a]	1.3[d]	75.6[b]	1.9[d]	10.2[c]	1.46[d]
9	97.6[a]	23.5[c]	100.0[a]	22.4[c]	27.4[c]	8.9[d]	48.7[b]	12.6[d]	49.6[b]	23.7[c]
10	100.0[a]	8.7[e]	98.5[a]	9.3[e]	21.5[c]	14.6[d]	21.7[c]	13.6[d]	27.8[b]	8.7[e]
11	96.5[a]	48.9[b]	100.0[a]	47.8[b]	98.4[a]	55.3[b]	97.4[a]	51.6[b]	52.4[b]	48.6[b]
12	98.7[a]	45.9[c]	99.2[a]	76.3[b]	100.0[a]	72.1[b]	97.3[a]	43.6[c]	95.5[a]	49.5[c]
13	95.3[a]	43.6[b]	95.6[a]	43.b	98.5[a]	53.1[b]	100.0[a]	52.7[b]	97.6[a]	47.2[b]
14	78.4[b]	27.2[d]	8.4[e]	30.8[d]	51.1[c]	0.0[f]	100.0[a]	0.0[f]	45.6[c]	0.0[f]
15	100.0[a]	54.3[c]	78.3[b]	47.4[c]	72.8[b]	48.9[c]	52.4[c]	27.9[d]	8.7[e]	0.0[f]
16	97.7[a]	79.2[b]	100.0[a]	73.2[b]	74.5[b]	78.4[b]	45.9[c]	49.8[c]	77.9[b]	78.4[b]
17	100.0[a]	47.3[c]	99.2[a]	48.7[c]	97.8[a]	77.4[b]	98.6[a]	45.6[c]	43.5[c]	42.1[c]
18	0.0[d]	27.4[b]	0.0[d]	10.6[c]	0.0[d]	0.0[d]	0.0[d]	12.5[c]	0.0[d]	100.0[a]
19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0[a]
20	100.0[a]	22.5[d]	77.8[b]	1.0[e]	48.9[c]	12.4[e]	76.4[b]	9.8[e]	11.9[e]	14.2[e]
21	98.7[a]	24.3[c]	100.0[a]	27.6[c]	97.6[a]	22.3[c]	97.6[a]	22.5[c]	78.9[b]	12.5[d]
22	100.0[a]	23.1[d]	77.8[b]	21.4[d]	76.4[b]	9.7[e]	47.8[c]	24.7[d]	28.6[d]	20.0[d]
23	98.7[b]	45.2[d]	96.9[b]	44.9[d]	100.0[a]	48.7[d]	77.4[c]	47.9[d]	76.2[c]	24.9[e]
24	98.5[a]	50.1[c]	100.0[a]	49.5[c]	97.5[a]	44.6[c]	72.6[b]	48.9[c]	23.1[d]	26.3[d]
25	100.0[a]	23.2[d]	98.7[a]	23.2[d]	97.1[a]	43.2[c]	74.2[b]	23.1[d]	23.6[d]	0.0[e]
26	0.0[e]	47.8[c]	10.7[d]	77.8[b]	0.0[e]	100.0[a]	12.3[d]	77.5[b]	0.0[e]	97.5[a]
27	50.6[c]	100.0[a]	27.8[d]	43.8[c]	26.7[d]	75.0[b]	0.0[e]	24.3[d]	27.5[d]	48.9[c]
28	99.6[a]	26.7[c]	96.6[a]	52.7[b]	99.1[a]	47.8[b]	100.0[a]	47.9[b]	52.3[b]	24.3[c]
29	77.8[b]	100.0[a]	9.5[c]	12.4[c]	11.7[c]	9.6[c]	9.1[c]	11.4[c]	10.8[c]	11.9[c]
30	98.7[a]	9.8[d]	99.5[a]	8.4[d]	77.4[b]	23.1[c]	27.1[c]	11.9[d]	100.0[a]	26.3[c]
31	100.0[a]	9.3[e]	98.7[a]	11.2[e]	78.9[b]	24.1[d]	49.5[c]	11.2[e]	27.1[d]	24.9[d]
32	98.5[a]	23.7[d]	99.1[a]	22.6[d]	100.0[a]	23.4[d]	77.4[b]	22.1[d]	49.7[c]	12.4[e]
33	0.0[c]	0.0[c]	0.0[c]	0.0[c]	100.0[a]	49.9[b]	0.0[c]	0.0[c]	0.0[c]	0.0[c]
34	100.0[a]	11.2[d]	98.6[a]	9.8[d]	77.6[b]	9.6[d]	79.6[b]	11.2[d]	47.2[c]	12.3[d]
35	98.6[a]	43.6[c]	100.0[a]	52.5[c]	99.7[a]	45.6[c]	78.4[b]	23.1[d]	45.8[c]	27.4[d]
36	9.8[c]	98.7[a]	9.5[c]	99.5[a]	12.9[c]	100.0[a]	11.9[c]	49.3[b]	9.5[c]	12.1[c]
37	0.0[d]	11.5[c]	0.0[d]	9.6[c]	0.0[d]	0.0[d]	0.0[d]	8.9[c]	75.7[b]	100.0[a]
38	0.0[d]	9.8[c]	0.0[d]	11.2[c]	0.0[d]	0.0[d]	0.0[d]	9.5[c]	22.8[b]	100.0[a]
39	9.8[c]	22.4[b]	11.7[c]	23.7[b]	9.7[c]	22.9[b]	9.8[c]	24.3[b]	9.3[c]	100.0[a]
40	73.4[b]	75.7[b]	78.6[b]	76.4[b]	49.8[c]	52.5[c]	49.2[c]	51.2[c]	9.7[d]	100.0[a]
41	46.9[c]	52.8[c]	47.9[c]	73.2[b]	50.4[c]	98.1[a]	47.6[c]	73.8[b]	53.1[c]	100.0[a]

Table 2S. continued.

Spot	PR825 MRS	PR825 Cheese broth	PR830 MRS	PR830 Cheese broth	PR1019 MRS	PR1019 Cheese broth	PR1215 MRS	PR1215 Cheese broth	PR1473 MRS	PR1473 Cheese broth
42	0.0^{c}	0.0^{c}	0.0^{c}	47.9^{b}	0.0^{c}	49.6^{b}	0.0^{c}	47.9^{b}	0.0^{c}	100.0^{a}
43	48.8^{c}	12.4^{d}	100.0^{a}	9.8^{d}	99.6^{a}	12.3^{d}	76.3^{b}	12.5^{d}	48.9^{c}	9.9^{d}
44	0.0^{e}	10.2^{d}	0.0^{e}	76.9^{b}	0.0^{e}	49.9^{c}	0.0^{e}	50.7^{c}	11.8^{d}	100.0^{a}
45	100.0^{a}	0.0^{c}	98.6^{a}	0.0^{c}	99.5^{a}	0.0^{c}	97.9^{a}	0.0^{c}	49.0^{b}	0.0^{c}
46	100.0^{a}	10.7^{e}	75.6^{b}	9.8^{e}	48.9^{c}	24.7^{d}	99.1^{a}	12.3^{e}	27.6^{d}	26.1^{d}
47	100.0^{a}	51.2^{b}	98.6^{a}	23.4^{c}	97.7^{a}	47.8^{b}	98.9^{a}	25.6^{c}	51.2^{b}	49.8^{b}
48	100.0^{a}	9.8^{e}	77.8^{b}	49.0^{c}	51.3^{c}	52.5^{c}	76.2^{b}	26.2^{d}	49.4^{c}	12.0^{e}
49	100.0^{a}	98.6^{a}	99.7^{a}	78.2^{b}	97.6^{a}	43.9^{c}	47.6^{c}	49.0^{c}	24.1^{d}	23.8^{d}
50	100.0^{a}	52.5^{b}	98.7^{a}	52.1^{b}	99.1^{a}	51.1^{b}	49.9^{b}	26.8^{c}	52.7^{b}	27.9^{c}
51	100.0^{a}	9.8^{d}	76.1^{b}	11.2^{d}	50.9^{c}	10.6^{d}	12.3^{d}	11.8^{d}	12.6^{d}	8.9^{d}
52	76.9^{b}	100.0^{a}	50.6^{c}	98.7^{a}	54.2^{c}	51.8^{c}	49.8^{c}	98.9^{a}	49.8^{c}	99.8^{a}
53	77.8^{a}	76.9^{a}	75.8^{a}	78.1^{a}	49.2^{b}	76.2^{a}	50.3^{b}	50.9^{b}	9.9^{c}	11.3^{c}
54	100.0^{a}	10.6^{c}	24.9^{b}	25.6^{b}	23.9^{b}	24.8^{b}	25.8^{b}	23.1^{b}	27.4^{b}	26.4^{b}
55	26.6^{c}	24.9^{c}	26.9^{c}	24.0^{c}	100.0^{a}	77.1^{b}	74.9^{b}	75.7^{b}	99.1^{a}	97.5^{a}
56	100.0^{a}	1.5^{e}	49.8^{b}	9.8^{d}	24.7^{c}	0.9^{e}	9.4^{d}	1.5^{e}	46.9^{b}	9.8^{d}
57	98.7^{a}	100.0^{a}	99.8^{a}	97.6^{a}	98.9^{a}	98.1^{a}	48.7^{b}	9.7^{c}	1.4^{c}	1.0^{c}
58	75.9^{b}	98.7^{a}	49.1^{c}	100.0^{a}	53.9^{c}	71.9^{b}	49.8^{c}	73.8^{b}	50.5^{c}	75.9^{b}
59	78.3^{b}	74.8^{b}	76.2^{b}	77.4^{b}	76.6^{b}	100.0^{a}	73.5^{b}	74.6^{b}	49.6^{c}	77.1^{b}
60	11.9^{d}	49.1^{c}	9.8^{d}	100.0^{a}	10.6^{d}	98.6^{a}	9.8^{d}	77.6^{b}	9.8^{d}	48.7^{c}
61	51.2^{c}	100.0^{a}	49.8^{c}	98.3^{a}	76.1^{b}	97.8^{a}	49.7^{c}	48.7^{c}	98.3^{a}	99.1^{a}
62	48.9^{b}	97.8^{a}	48.5^{b}	99.0^{a}	48.4^{b}	100.0^{a}	50.6^{b}	49.5^{b}	11.8^{c}	49.8^{b}
63	77.8^{b}	99.2^{a}	49.3^{c}	97.3^{a}	49.1^{c}	98.3^{a}	47.9^{c}	97.8^{a}	26.3^{d}	100.0^{a}
64	47.9^{c}	74.1^{b}	47.9^{c}	73.4^{b}	23.1^{d}	77.5^{b}	50.6^{c}	49.5^{c}	23.4^{d}	100.0^{a}
65	48.5^{b}	98.3^{a}	54.9^{b}	100.0^{a}	51.2^{b}	97.8^{a}	48.1^{b}	48.4^{b}	11.2^{c}	9.5^{c}
66	100.0^{a}	11.4^{d}	52.9^{b}	9.2^{d}	26.3^{c}	9.8^{d}	48.7^{b}	9.1^{d}	47.9^{b}	11.9^{d}
67	76.2^{b}	100.0^{a}	76.7^{b}	77.1^{b}	49.3^{c}	98.9^{a}	49.3^{c}	51.9^{c}	76.9^{b}	75.4^{b}
68	100.0^{a}	39.8^{c}	48.3^{b}	51.5^{b}	52.0^{b}	38.9^{c}	41.4^{c}	12.3^{d}	40.9^{c}	38.9^{c}
69	1.2^{c}	1.7^{c}	0.9^{c}	0.8^{c}	1.3^{c}	1.8^{c}	0.7^{c}	1.4^{c}	100.0^{a}	50.9^{b}
70	50.3^{b}	100.0^{a}	24.8^{c}	98.6^{a}	9.8^{d}	99.1^{a}	11.9^{d}	49.8^{b}	9.7^{d}	49.1^{b}
71	9.8^{e}	97.6^{a}	29.4^{d}	100.0^{a}	9.7^{e}	97.1^{a}	11.7^{e}	69.9^{c}	30.4^{d}	80.1^{b}
72	100.0^{a}	51.2^{b}	98.7^{a}	48.7^{b}	98.3^{a}	45.9^{b}	97.8^{a}	49.9^{b}	41.2^{c}	39.9^{c}
73	51.5^{c}	76.1^{b}	51.2^{c}	75.4^{b}	81.2^{b}	100.0^{a}	51.8^{c}	77.5^{b}	99.1^{a}	97.8^{a}
74	100.0^{a}	54.1^{b}	99.5^{a}	48.9	97.8^{a}	47.8^{b}	98.1^{a}	1.8^{c}	0.9^{c}	1.2^{c}
75	77.1^{b}	100.0^{a}	76.1^{b}	98.7^{a}	51.2^{c}	97.8^{a}	74.8^{b}	96.9^{a}	51.2^{c}	46.9^{c}
76	30.0^{b}	30.4^{b}	9.8^{d}	32.1^{b}	1.9^{e}	23.1^{c}	1.8^{e}	24.3^{c}	33.1^{b}	100.0^{a}
77	10.4^{b}	100.0^{a}	9.8^{b}	97.8^{a}	11.2^{b}	99.0^{a}	11.1^{b}	9.5^{b}	1.5^{c}	9.6^{b}
78	23.4^{b}	24.3^{b}	27.6^{b}	26.9^{b}	9.8^{c}	100.0^{a}	25.0^{b}	24.3^{b}	13.8^{c}	17.5^{bc}
79	1.0^{d}	81.3^{b}	0.9^{d}	79.6^{b}	0.8^{d}	100.0^{a}	1.1^{d}	76.7^{b}	0.9^{d}	49.9^{c}
80	100.0^{a}	48.9^{c}	77.3^{b}	51.2^{c}	76.1^{b}	50.9^{c}	76.7^{b}	24.3^{d}	48.9^{c}	51.2^{c}
81	1.2^{d}	100.0^{a}	0.8^{d}	76.5^{b}	10.4^{c}	97.8^{a}	11.1^{c}	77.2^{b}	1.1^{d}	99.4^{a}
82	98.8^{a}	77.3^{b}	100.0^{a}	75.7^{b}	98.6^{a}	77.1^{b}	98.7^{a}	77.1^{b}	99.0^{a}	51.3^{c}
83	76.4^{b}	51.5^{c}	77.7^{b}	52.1^{c}	76.2^{b}	51.3^{c}	77.9^{b}	49.1^{c}	1.9^{d}	100.0^{a}
84	50.3^{c}	77.4^{b}	48.9^{c}	76.7^{b}	23.9^{d}	100.0^{a}	49.8^{c}	27.1^{d}	9.5^{e}	9.8^{e}
85	98.9^{a}	77.8^{b}	98.9^{a}	100.0^{a}	95.1^{a}	76.4^{b}	99.0^{a}	97.2^{a}	23.8^{c}	11.5^{d}
86	27.8^{c}	10.0^{d}	26.1^{c}	9.8^{d}	27.6^{c}	1.9^{e}	48.2^{b}	0.9^{e}	100.0^{a}	24.7^{c}
87	48.7^{c}	75.2^{b}	51.2^{c}	77.3^{b}	54.3^{c}	99.1^{a}	49.8^{c}	100.0^{a}	4.9^{d}	5.7^{d}

Table 2S. continued.

Spot	PR825		PR830		PR1019		PR1215		PR1473	
	MRS	Cheese broth	MRS	Cheese broth	MRS	Cheese broth	MRS	Cheese broth	MRS	Cheese broth
88	98.4^{a}	50.2^{b}	97.8^{a}	47.9^{b}	100.0^{a}	47.9^{b}	11.2^{c}	0.8^{d}	1.2^{d}	47.8^{b}
89	26.5^{c}	23.7^{c}	27.9^{c}	23.8^{c}	25.8^{c}	100.0^{a}	48.9^{b}	51.0^{b}	10.9^{d}	26.1^{c}
90	48.7^{c}	77.3^{b}	51.4^{c}	76.4^{b}	9.7^{d}	100.0^{a}	9.8^{d}	76.2^{b}	75.4^{b}	99.1^{a}
91	9.5^{d}	48.6^{b}	9.8^{d}	100.0^{a}	11.3^{d}	99.5^{a}	10.4^{d}	51.2^{b}	9.7^{d}	41.2^{c}
92	51.2^{b}	31.9^{c}	100.0^{a}	52.4^{b}	97.5^{a}	49.7^{b}	51.2^{b}	26.1^{d}	99.1^{a}	24.9^{d}
93	50.1^{c}	75.7^{b}	49.8^{c}	98.9^{a}	51.2^{c}	100.0^{a}	48.9^{c}	97.8^{a}	9.8^{d}	75.2^{b}
94	100.0^{a}	9.8^{e}	51.1^{c}	26.5^{d}	50.1^{c}	76.3^{b}	52.1^{c}	49.1^{c}	9.1^{e}	11.0^{e}
95	1.3^{c}	1.5^{c}	0.9^{c}	0.7^{c}	1.2^{c}	0.9^{c}	0.8^{c}	1.2^{c}	77.0^{b}	100.0^{a}
96	48.9^{c}	100.0^{a}	31.2^{d}	97.9^{a}	52.1^{c}	97.8^{a}	29.7^{d}	98.9^{a}	48.9^{c}	76.1^{b}
97	1.0^{b}	0.9^{b}	1.1^{b}	100.0^{a}	0.8^{b}	0.9^{b}	1.2^{b}	1.4^{b}	0.9^{b}	1.2^{b}
98	51.3^{b}	47.9^{b}	49.8^{b}	51.3^{b}	11.4^{d}	49.6^{b}	9.8^{d}	10.5^{d}	41.2^{c}	100.0^{a}
99	77.6^{b}	100.0^{a}	77.2^{b}	98.7^{a}	77.7^{b}	74.1^{b}	74.8^{b}	74.2^{b}	78.1^{b}	99.2^{a}
100	9.8^{c}	9.7^{c}	11.2^{c}	10.9^{c}	9.7^{c}	100.0^{a}	9.8^{c}	76.3^{b}	9.8^{c}	11.2^{c}
101	10.2^{e}	76.4^{b}	9.0^{e}	48.9^{c}	9.8^{e}	100.0^{a}	9.7^{e}	23.6^{d}	9.8^{e}	11.3^{e}
102	49.9^{c}	77.3^{b}	48.7^{c}	100.0^{a}	51.2^{c}	98.7^{a}	48.9^{c}	48.7^{c}	26.7^{d}	24.8^{d}
103	9.7^{c}	76.5^{b}	1.2^{d}	76.5^{b}	0.9^{d}	100.0^{a}	0.7^{d}	76.7^{b}	11.2^{c}	98.7^{a}
104	100.0^{a}	76.4^{b}	99.3^{a}	76.5^{b}	97.8^{a}	76.4^{b}	96.4^{a}	51.2^{c}	52.4^{c}	48.9^{c}
105	9.8^{e}	9.7^{e}	11.2^{e}	10.9^{e}	9.6^{e}	11.2^{e}	20.7^{d}	51.2^{c}	76.4^{b}	100.0^{a}
106	10.4^{d}	51.3^{b}	11.9^{d}	51.2^{b}	11.3^{d}	100.0^{a}	26.1^{c}	98.9^{a}	1.4^{e}	51.2^{b}
107	10.3^{b}	11.2^{b}	100.0^{a}	9.8^{b}	99.1^{a}	9.8^{b}	9.5^{b}	11.3^{b}	10.4^{b}	12.0^{b}
108	26.0^{c}	100.0^{a}	24.6^{c}	98.7^{a}	23.6^{c}	99.1^{a}	26.6^{c}	97.8^{a}	9.8^{d}	51.2^{b}
109	100.0^{a}	1.3^{e}	77.3^{b}	0.9^{e}	26.7^{d}	1.2^{e}	51.3^{c}	1.1^{e}	0.8^{e}	0.7^{e}
110	100.0^{a}	1.1^{c}	9.8^{b}	0.9^{c}	11.2^{b}	0.8^{c}	9.6^{b}	0.7^{c}	0.8^{c}	1.1^{c}
111	0.9^{c}	51.2^{b}	1.3^{c}	52.1^{b}	1.0^{c}	100.0^{a}	51.0^{b}	48.9^{b}	47.8^{b}	52.1^{b}
112	1.6^{e}	9.8^{d}	26.3^{c}	51.2^{b}	27.7^{c}	100.0^{a}	24.3^{c}	26.7^{c}	25.1^{c}	51.2^{b}
113	75.3^{b}	100.0^{a}	1.0^{c}	1.1^{c}	0.9^{c}	0.8^{c}	1.1^{c}	0.9^{c}	0.8^{c}	1.3^{c}
114	100.0^{a}	9.8^{d}	99.7^{a}	9.7^{d}	98.7^{a}	48.9^{b}	97.8^{a}	24.8^{c}	9.7^{d}	9.5^{d}
115	10.2^{c}	9.7^{c}	100.0^{a}	10.5^{c}	98.5^{a}	26.6^{b}	9.7^{c}	9.6^{c}	11.9^{c}	11.2^{c}
116	9.7^{b}	9.8^{b}	11.0^{b}	10.4^{b}	12.1^{b}	8.9^{b}	9.1^{b}	10.2^{b}	100.0^{a}	11.6^{b}
117	98.3^{a}	0.9^{c}	99.1^{a}	1.0^{c}	100.0^{a}	0.9^{c}	95.9^{a}	0.8^{c}	51.2^{b}	1.2^{c}
118	1.1^{b}	100.0^{a}	0.9^{b}	0.8^{b}	1.1^{b}	1.0^{b}	1.3^{b}	0.8^{b}	0.7^{b}	98.9^{a}
119	10.0^{d}	100.0^{a}	10.5^{d}	10.0^{d}	10.2^{d}	9.8^{d}	11.4^{d}	75.0^{b}	12.0^{d}	50.5^{c}
120	48.7^{c}	51.0^{c}	48.2^{c}	50.7^{c}	47.3^{c}	75.0^{b}	51.5^{c}	74.8^{b}	25.2^{d}	100.0^{a}
121	100.0^{a}	42.5^{c}	96.4^{a}	53.9^{c}	94.7^{a}	69.2^{b}	92.8^{a}	45.7^{c}	51.2^{c}	49.0^{c}

[*] Cultivation in MRS was at 37°C for 24 and 48 h, respectively. The spot quantification for each gel was calculated as relative volume (% VOL); the relative VOL was the VOL of each spot divided by the total VOL over the whole image. In this way, differences in colour intensities among the gels were eliminated. For each protein of different strains and medium. each single spot was quantified as percentage of relative volume. attributing the value of 100 to the condition wherein the protein had been synthesized at the highest level. [a-f] The values in the same raw with different superscript letters differ significantly ($p < 0.05$).

Table 3S. Identity score with homologous proteins of other microorganisms, number of peptides and sequence coverage of proteins showing an increased or decreased level of synthesis in *Lactobacillus rhamnosus* PR825, PR830, PR1019, PR1215 and PR1473 when cultured in MRS or Cheese broth medium.

Spot[a]	Identity score (%)[b]	Organism	Number of peptides	Sequenze coverage (%)
1	100	*L. rhamnosus* HN001	16	27
2	95	*L. rhamnosus* ATCC 53103	10	20
3	100	*L. rhamnosus* HN001	11	23
4	100	*L. rhamnosus* ATCC 53103	12	31
5	98	*L. rhamnosus* GG	10	19
6	100	*L. rhamnosus* HN001	8	20
7	100	*L. rhamnosus* LMS2-1	5	11
8	98	*L. rhamnosus* HN001	7	24
9	100	*L. rhamnosus* HN001	7	12
10	100	*L. rhamnosus* ATCC 7469	6	19
12	95	*L. rhamnosus* ATCC 53103	18	45
13	100	*L. rhamnosus* ATCC 53103	12	37
15	100	*L. rhamnosus* ATCC 53103	11	39
16	99	*L. rhamnosus* HN001	12	25
18	100	*L. rhamnosus* LMS2-1	12	29
19	100	*L. rhamnosus* HN001	12	30
21	100	*L. rhamnosus* HN001	10	26
23	95	*L. rhamnosus* HN001	18	47
25	100	*L. rhamnosus* LMS2-1	12	41
26	100	*L. rhamnosus* ATCC 53103	10	32
27	98	*L. rhamnosus* ATCC 53103	12	33
28	100	*L. rhamnosus* ATCC 53103	11	40
29	100	*L. rhamnosus* HN001	15	46
30	100	*L. rhamnosus* LMS2-1	24	51
31	95	*L. rhamnosus* ATCC 53103	7	14
32	100	*L. rhamnosus* HN001	14	38
34	94	*L. salivarius* UCC118	5	20
35	88	*L. sakei* 23K	4	10
36	100	*L. rhamnosus* Lc 705	12	60
39	85	*E. faecium*	9	39
40	100	*L. rhamnosus* Lc 705	12	33
41	100	*L. rhamnosus* ATCC 53103	7	25
42	100	*L. casei ATCC* 334	11	50
43	90	*L. rhamnosus* HN001	12	41
44	100	*L. rhamnosus* Lc 705	8	17
45	100	*L. rhamnosus* Lc 705	11	38

46	100	*L. rhamnosus* Lc 705	13	46
48	99	*L. rhamnosus* Lc 705	8	37
49	100	*L. rhamnosus* HN001	8	30
50	100	*L. rhamnosus* ATCC 53103	5	13
52	96	*L. rhamnosus* LMS2-1	6	24
53	100	*L. rhamnosus* ATCC 53103	10	30
55	99	*L. rhamnosus* HN001	13	61
56	100	*L. rhamnosus* ATCC 53103	6	20
57	100	*L. rhamnosus* HN001	19	64
58	100	*L. rhamnosus* HN001	8	28
60	100	*L. rhamnosus* HN001	4	14
61	98	*L. rhamnosus* LMS2-1	8	17
62	100	*L. rhamnosus* HN001	7	28
63	97	*L. rhamnosus* HN001	9	24
64	99	*L. rhamnosus* ATCC 53103	8	37
65	100	*L. rhamnosus* ATCC 53103	7	26
68	100	*L. casei* ATCC 334	7	26
69	100	*L. rhamnosus* GG	3	5
70	100	*L. rhamnosus* ATCC 53103	5	27
71	100	*L. rhamnosus* LMS2-1	2	10
72	98	*L. rhamnosus* HN001	2	12
73	100	*L. rhamnosus* ATCC 53103	7	30
75	100	*rhamnosus* ATCC 53103	13	64
76	100	*L. rhamnosus* LMS2-1	15	64
77	95	*L. rhamnosus* ATCC 53103	10	20
79	100	*L. rhamnosus* ATCC 53103	6	32
81	100	*L. rhamnosus* HN001	8	37
82	100	*L. rhamnosus* HN001	7	35
85	99	*L. rhamnosus* HN001	11	41
86	100	*L. rhamnosus* HN001	19	63
89	100	*L. rhamnosus* HN001	3	21
90	100	*L. rhamnosus* HN001	3	21
91	100	*L. rhamnosus* LMS2-1	4	29
92	95	*L. rhamnosus* Lc 705	10	57
93	100	*L. rhamnosus* ATCC 53103	6	34
94	100	*L. rhamnosus* HN001	6	44
95	100	*L. rhamnosus* HN001	9	35
96	100	*L. rhamnosus* HN001	5	27
97	93	*S. epidermidis* BCM-HMP0060	6	36
98	90	*L. rhamnosus* LMS2-1	2	12
99	100	*L. rhamnosus* HN001	1	8
100	100	*L. rhamnosus* LMS2-1	8	52
102	100	*L. rhamnosus* HN001	8	58
103	98	*L. rhamnosus* Lc 705	2	8
104	98	*L. rhamnosus* Lc 705	3	10
105	97	*L. rhamnosus* HN001	5	35

106	99	*L. rhamnosus* ATCC 53103	3	12
107	100	*L. rhamnosus* HN001	8	43
108	100	*L. rhamnosus* LMS2-1	7	34
109	100	*L. rhamnosus* HN001	5	27
110	98	*L. rhamnosus* Lc 705	3	20
112	100	*L. rhamnosus* HN001	7	25
113	100	*L. rhamnosus* LMS2-1	6	32
115	100	*L. rhamnosus* HN001	6	76
116	99	*L. rhamnosus* HN001	4	26
117	100	*L. rhamnosus* HN001	4	30
118	100	*L. rhamnosus* HN001	3	29
119	100	*L. rhamnosus* HN001	6	27
120	100	*L. rhamnosus* HN001	5	30

[a]Spot designations correspond to those of the gels in Figs. 4A, B, C, D, E and F and Tables 2, 3 and 2S.

[b]Identity score of the sequence retrieved in databases.

Figure 1. Bove et al.

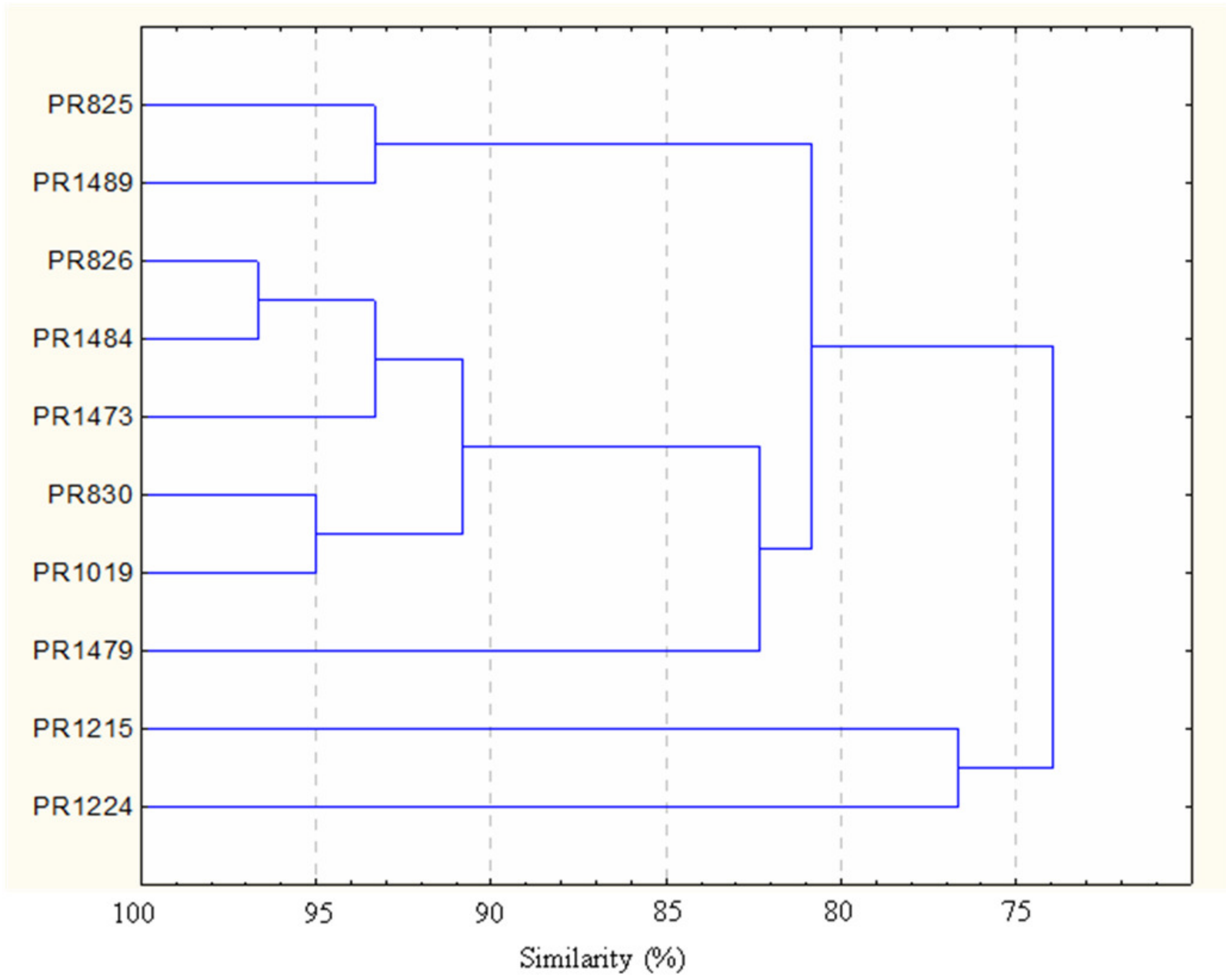
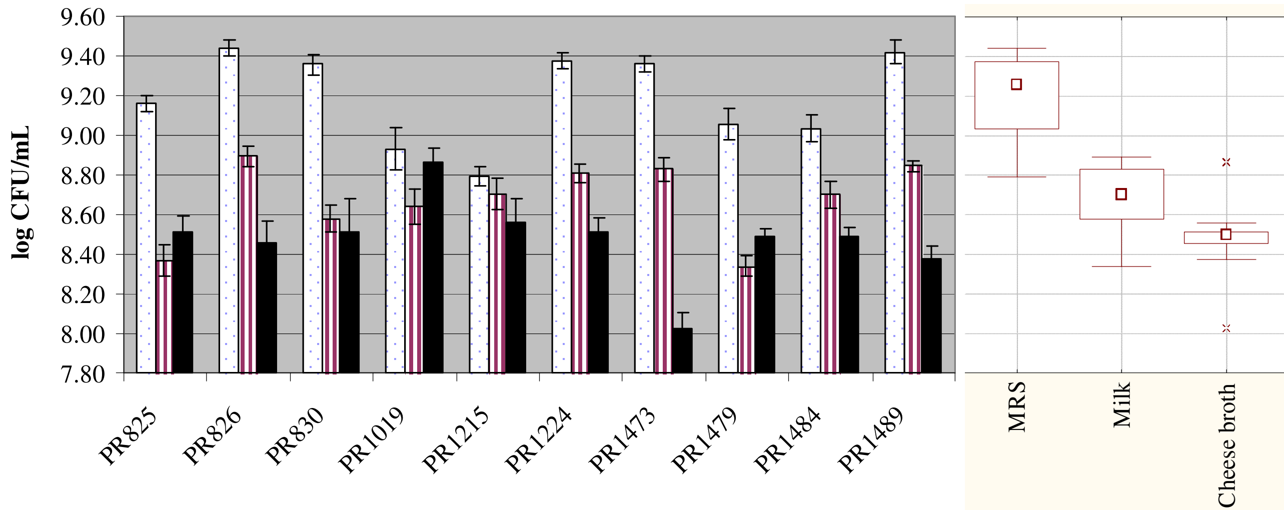


Figure 2. Bove et al.

A



B

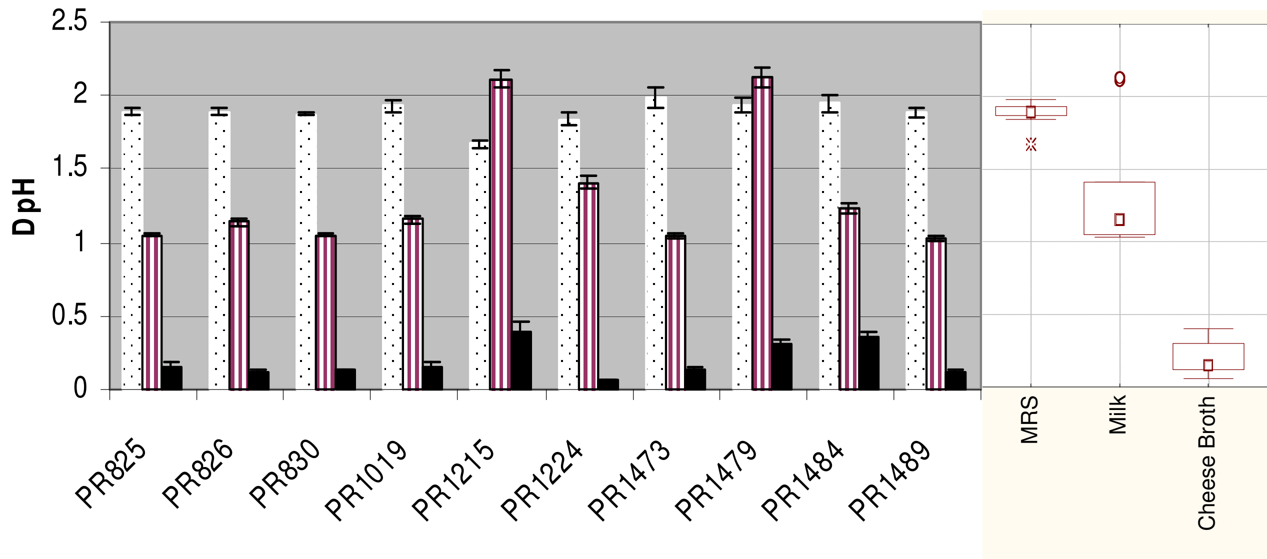
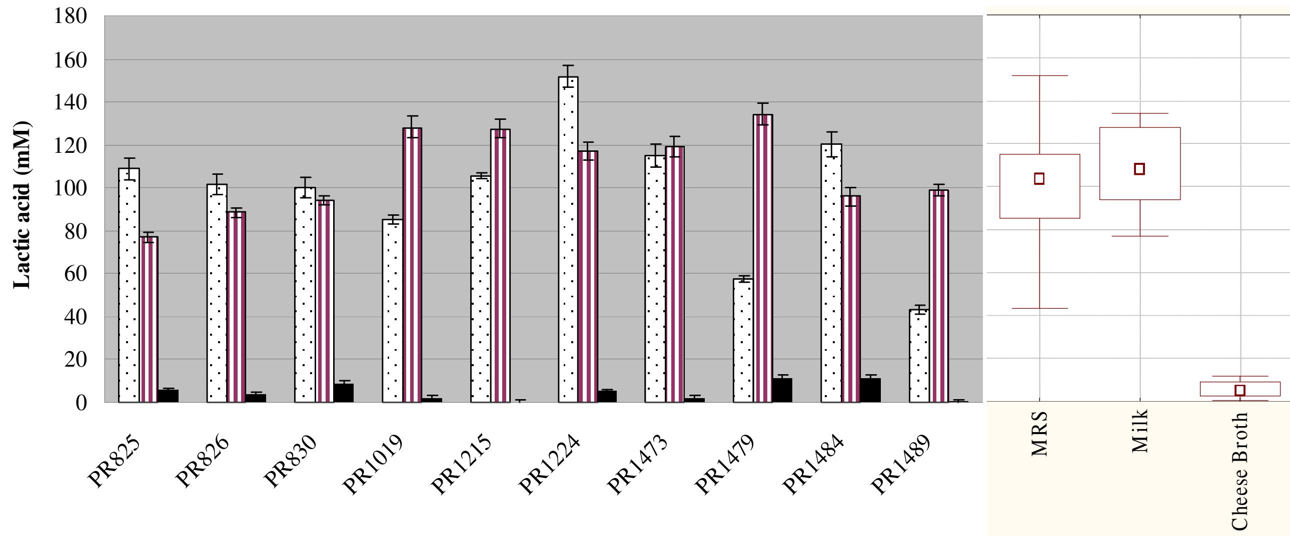
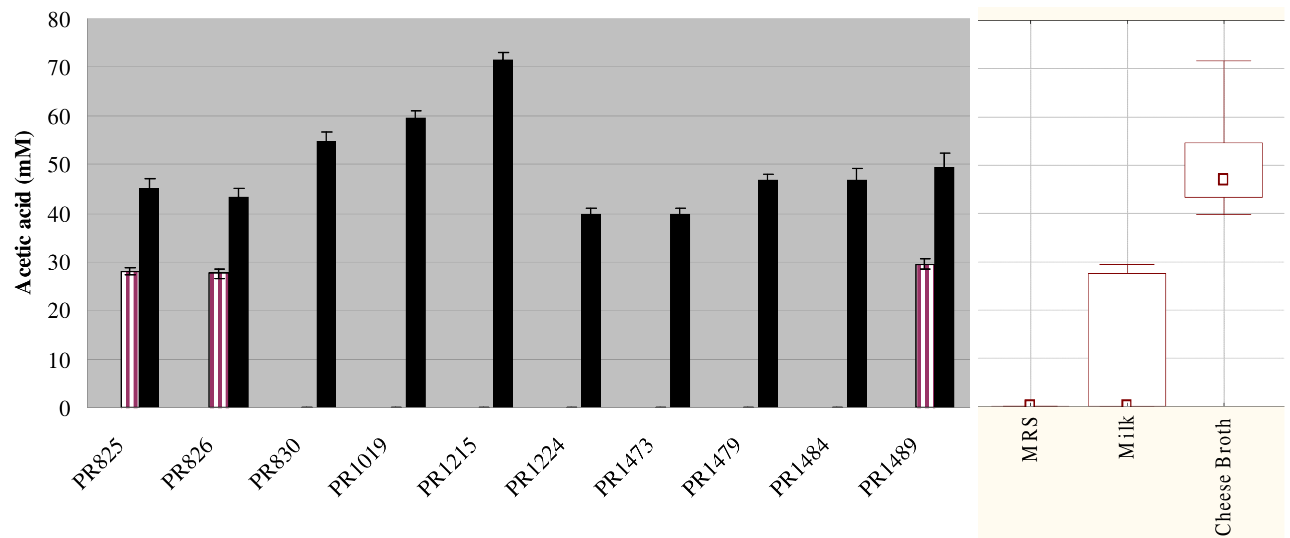


Figure 3. Bove et al.



A



B

Figure 4A. Bove et al.

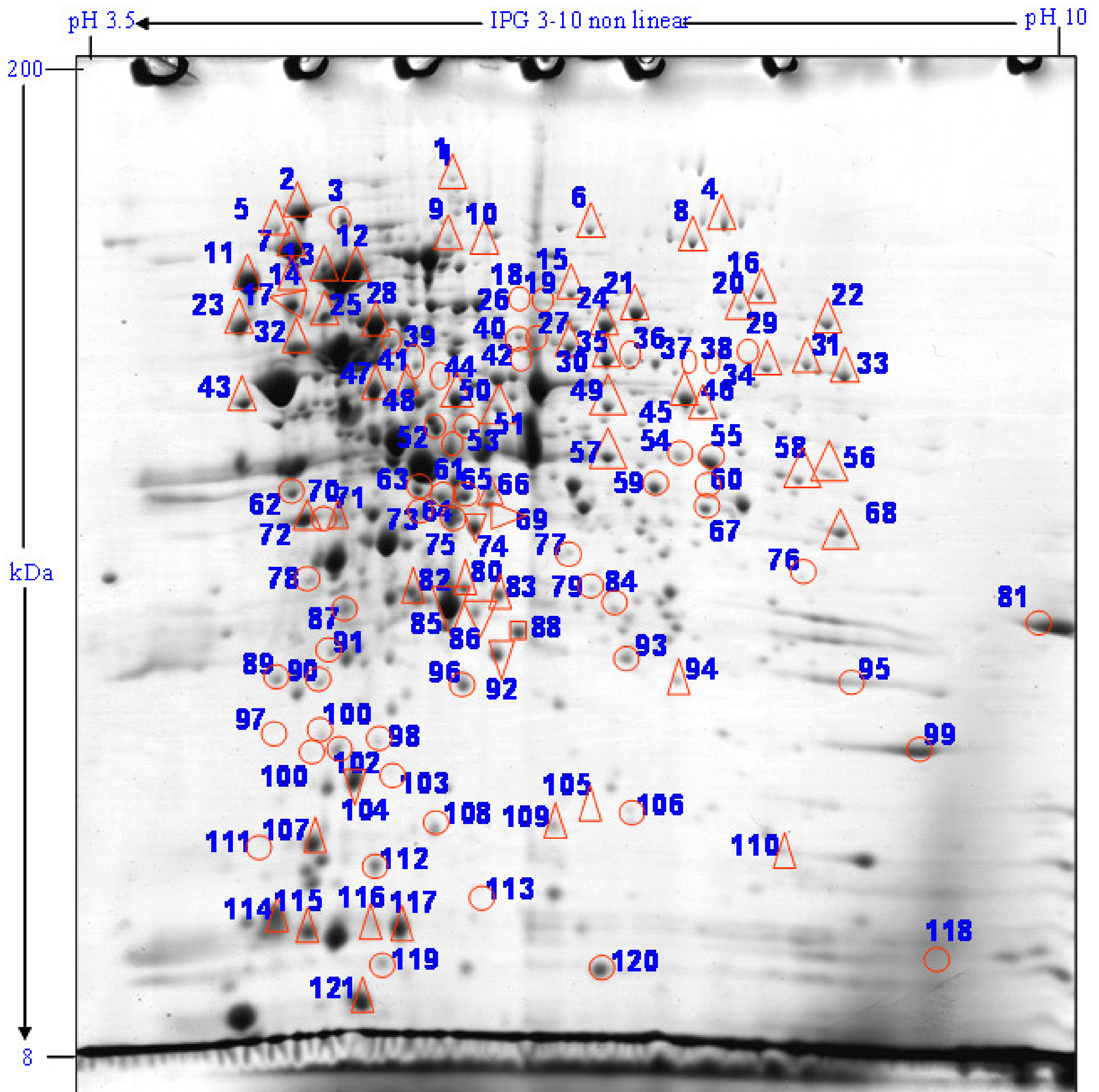


Figure 4B. Bove et al.

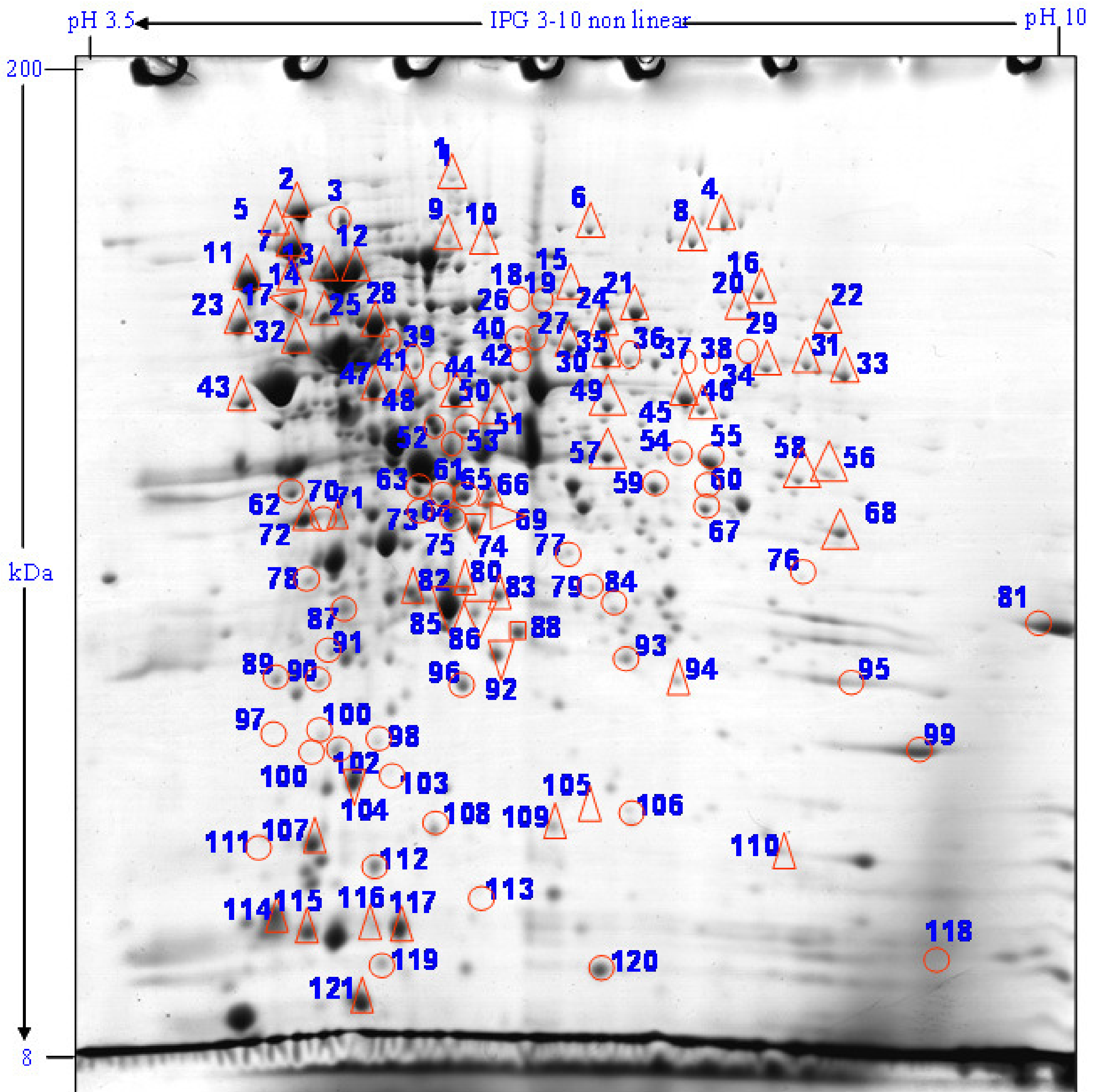


Figure 4C. Bove et al.

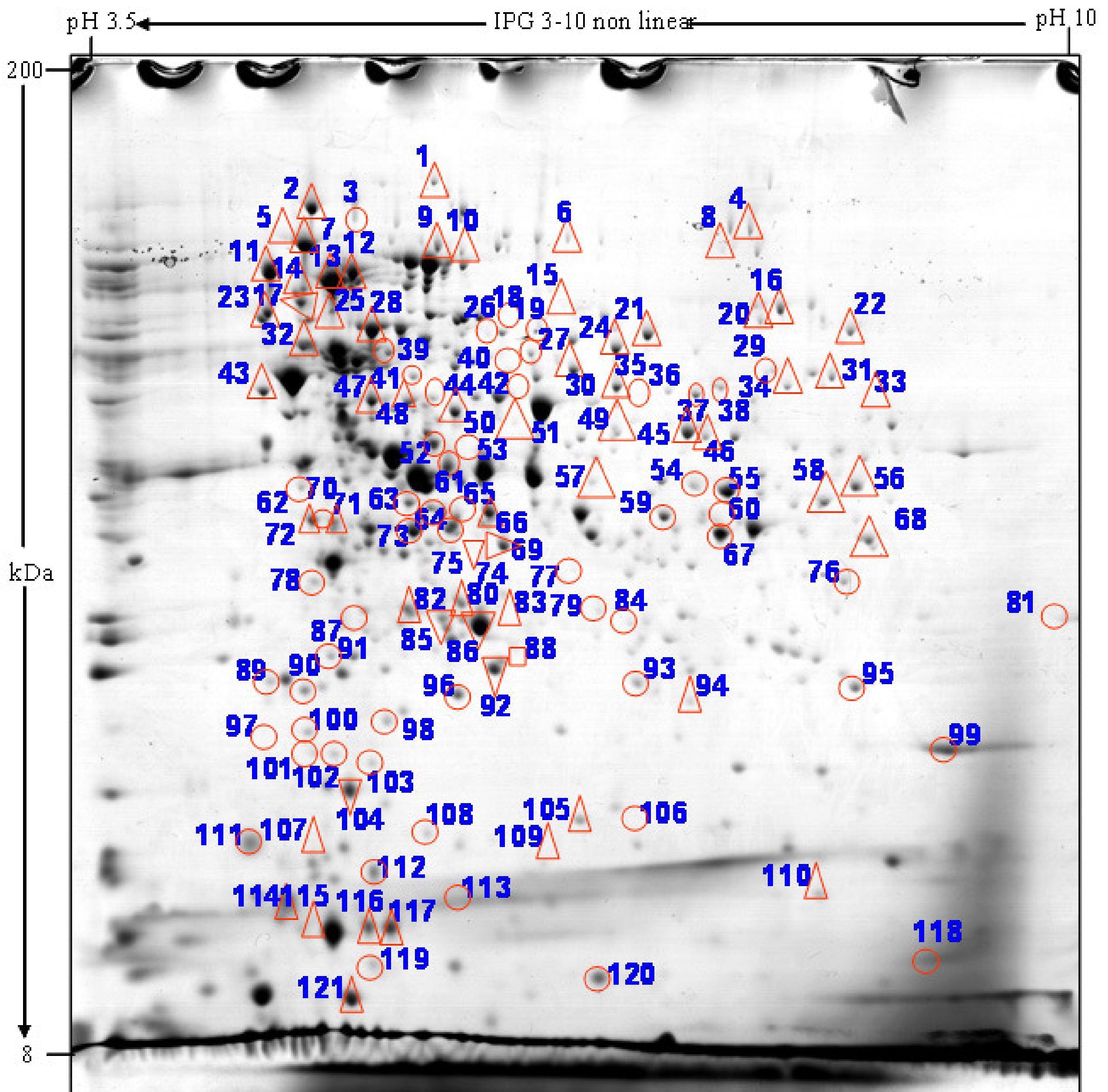


Figure 4D. Bove et al.

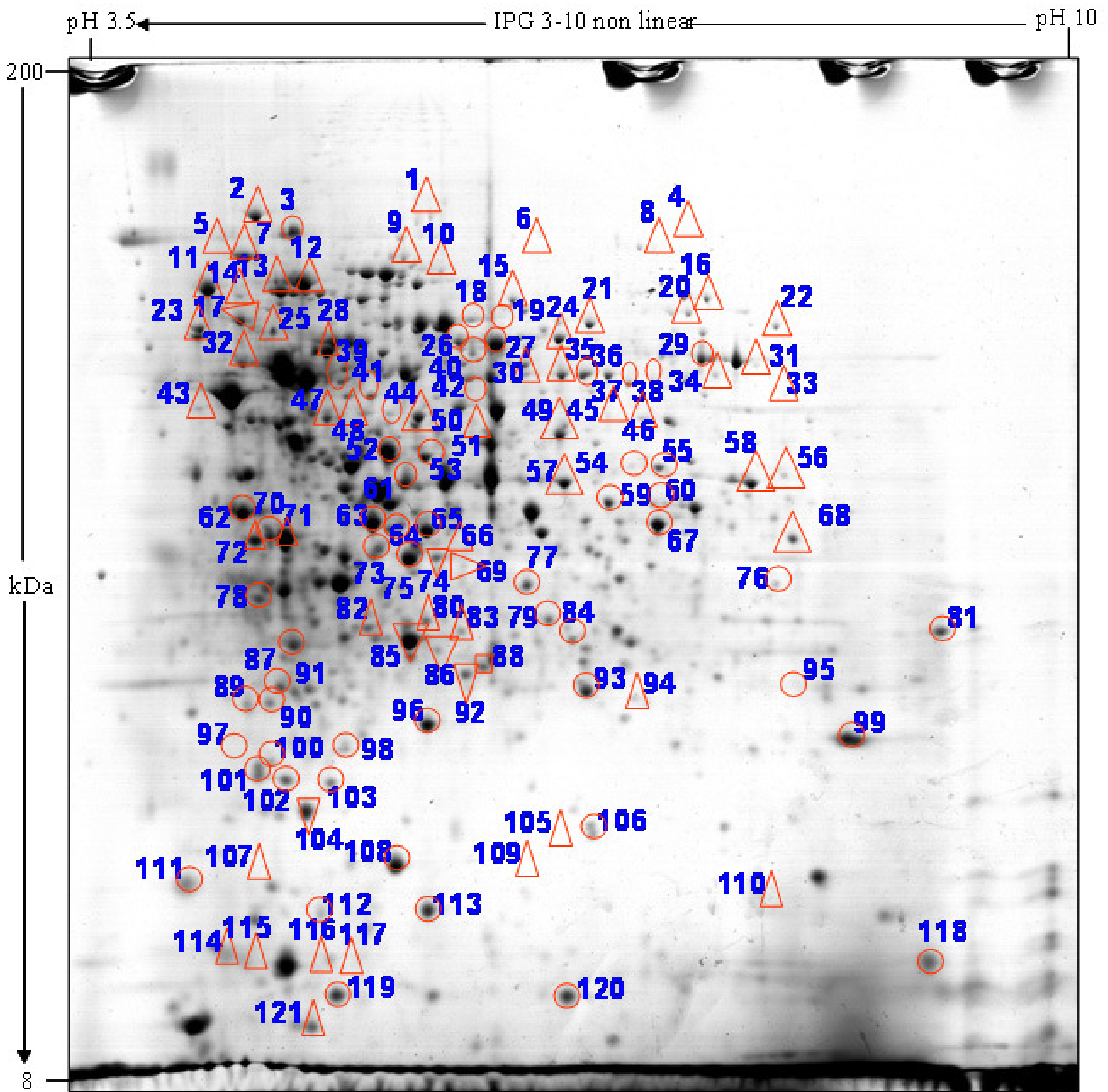


Figure 4E. Bove et al.

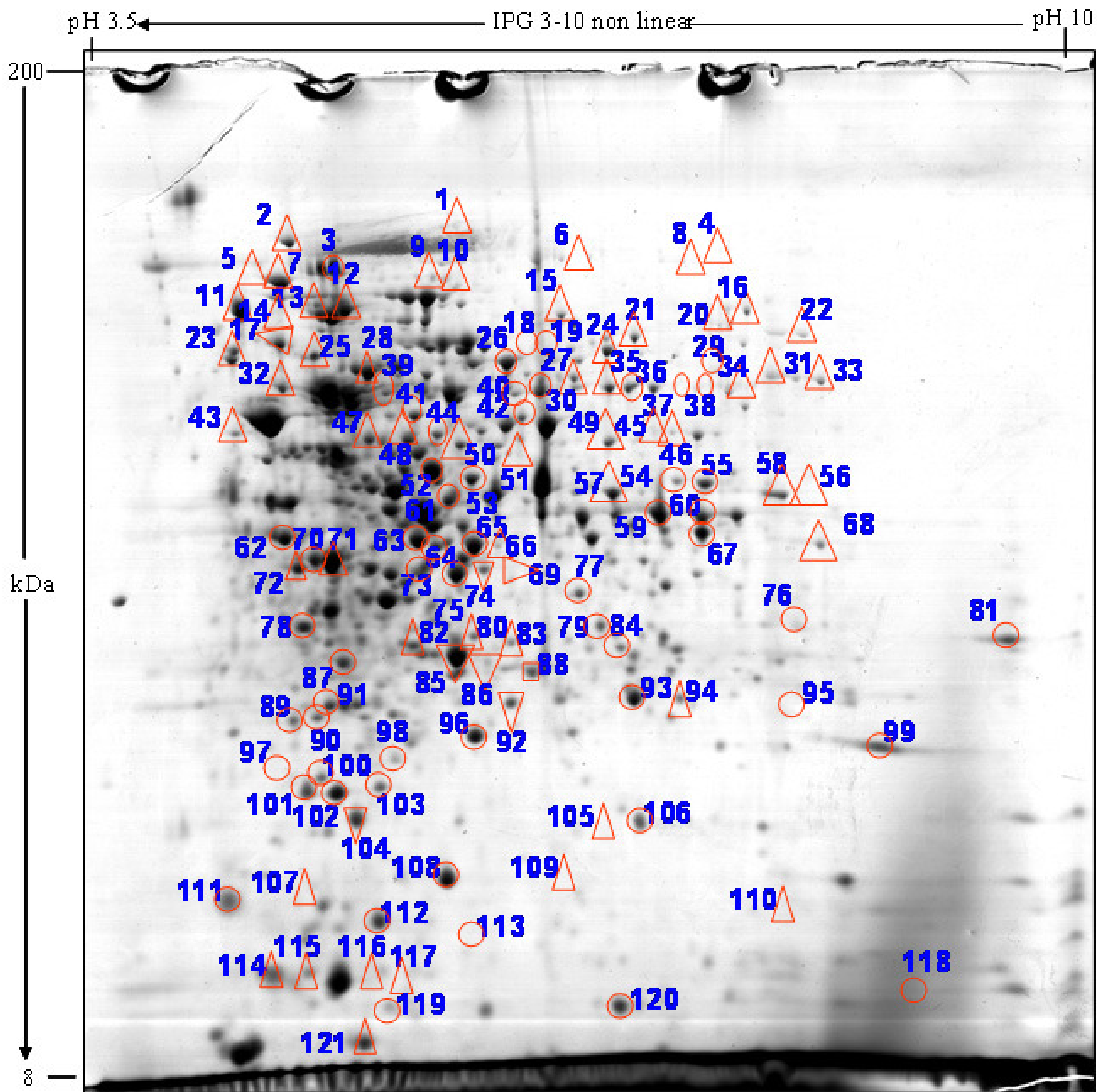


Figure 4F. Bove et al.

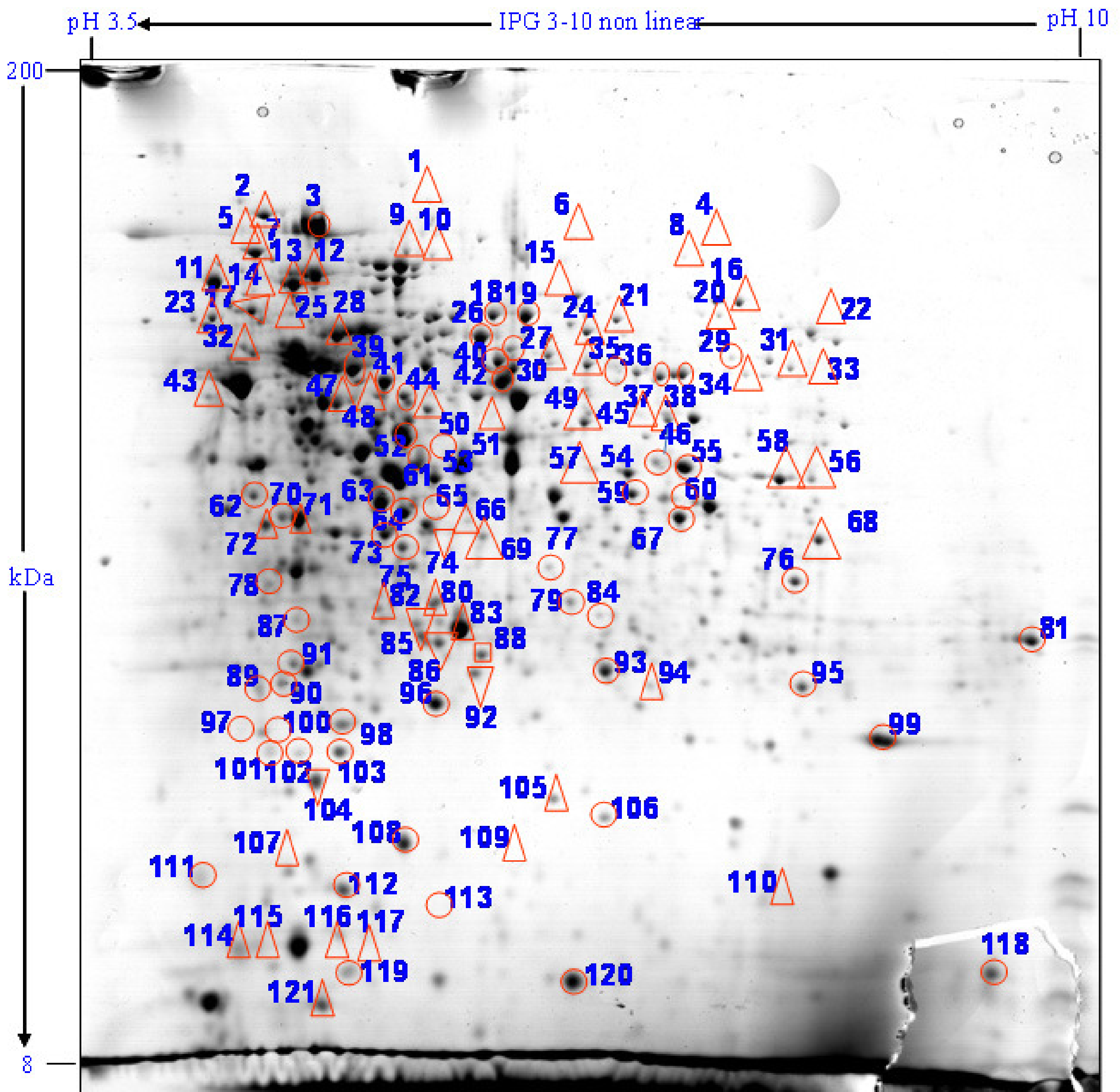
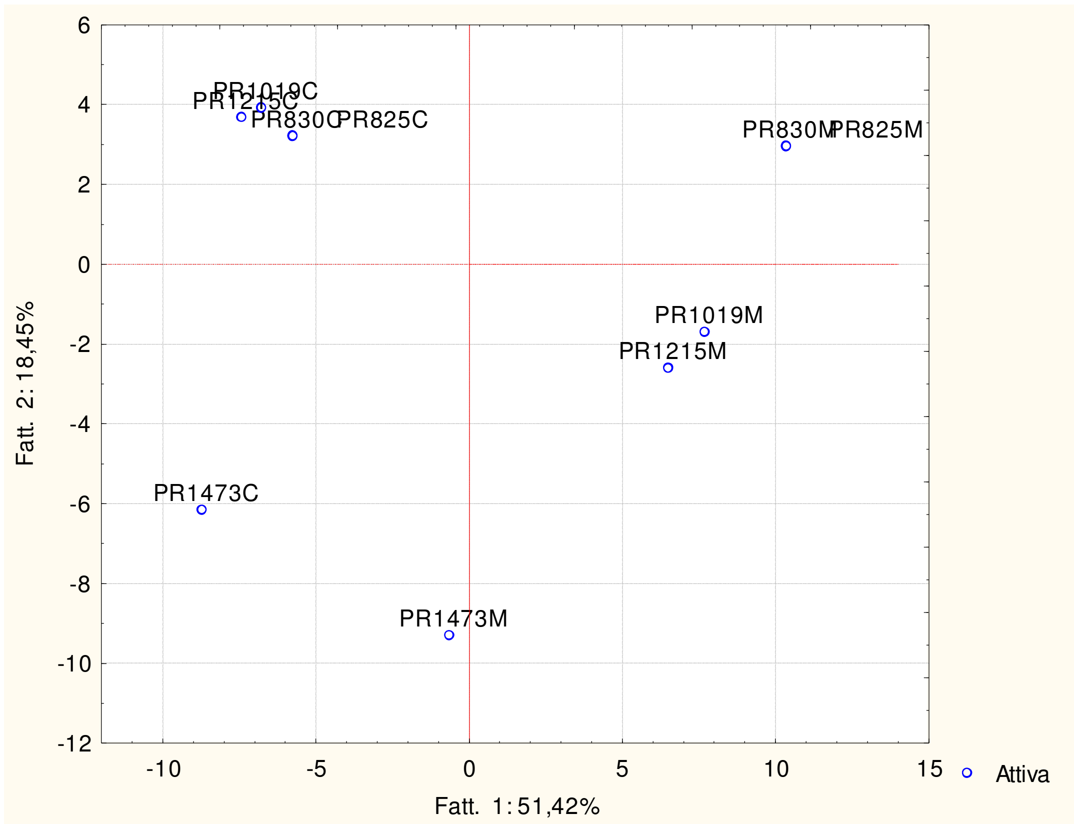


Figure 5. Bove et al.



3.3 Transcriptional responses in *Lactobacillus rhamnosus* during growth in cheese-like medium

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3.3.1 Abstract

Lactobacillus rhamnosus is a dominant species during cheese ripening and exhibits a great adaptability to unfavorable growth conditions.

In this work we compare the gene expression profile of a *L. rhamnosus* strain isolated from Parmigiano Reggiano cheese grown in a rich medium (MRS) versus cheese–like medium (CB) by a novel cDNA-AFLP protocol.

Two techniques, capillary electrophoresis cDNA-AFLP (ce-cDNA-AFLP) and gel electrophoresis cDNA-AFLP (ge-cDNA-AFLP) were applied to generate unique transcript tags from reverse-transcribed messenger RNA using restriction enzymes and selective PCR amplification.

Both methods highlight that *L. rhamnosus* modifies the expression of a large part of genes when cultivated in cheese-like conditions (CB) compared to growth under *in vitro* optimal conditions (MRS). In particular, the profiles of the strain grown in CB were more complex probably because the cell activate different metabolic pathways to generate energy and to respond to the environmental changes. This research is the first one done on *L. rhamnosus* isolated from cheese. Further analysis will have to be focused on gel extraction and identification of expressed genes to better understand *L. rhamnosus* physiological mechanisms of adaptation to cheese-like substrates that are fundamental for survival in cheese during manufacture.

Keywords: *Lactobacillus rhamnosus*; cDNA-AFLP; gene expression.

3.3.2 Introduction

Lactic acid bacteria (LAB) play different roles in cheese-making. Starter LAB (SLAB) participate in the fermentation process, whereas non starter LAB (NSLAB), probably present in all cheeses, are involved in the maturation of cheese, influencing cheese flavour development (Beresford et al.,

2001; Beresford and Williams, 2004). Although the role of NSLAB in cheese ripening has not yet been clarified, different authors have suggested their importance in the cheese ageing (Settani et al 2010). In Parmigiano Reggiano cheese it was demonstrated that SLAB are dominant until the 2nd month of ripening and, after cheese brining, the species NSLAB, especially *Lactobacillus rhamnosus*, are able to grow and increase in number, while SLAB cells undergo autolysis (Gatti et al 2008, De Dea Lindner et al 2008). *L. rhamnosus* was shown to be the dominant species present when essential nutrients, such as sugars, are lacking. Therefore, this species seems to well adapt to the absence of lactose in cheese, confirming an optimal adaptability to unfavorable growth conditions. Presumably, this characteristic is due to the ability of *L. rhamnosus* to use nitrogen fraction as an alternative energy source. So far, only few studies are available about *L. rhamnosus* in cheese ripening, and the exact role of these bacteria has not been clarified yet (Neviani et al 2009,De Dea Lindner et al., 2008; Gatti et al., 2008;). It was demonstrated that the intraspecies heterogeneity, found for 66 *L. rhamnosus* strains isolated during the same Parmigiano Reggiano cheesemaking, seems to be correlated to their abilities to adapt to the hostile environment of the cheese throughout the ripening (Bove et al., 2011). It was found that the great part of *L. rhamnosus* strains belonged to few biotypes which were present in cheese from the first or second month of ripening up to ten months and beyond (Bove et al., 2011). The detection of biotypes correlated with specific steps in cheese ripening suggested that these strains may have specific metabolic activities which allow an adaptation to the microenvironment of cheese ripening.

The high degree of adaptation of *L. rhamnosus* to different environments and conditions were studied towards several approaches (Succi et al., 2005; Koskenniemi et al., 2009) and probably involves many metabolic and physiological changes. To identify and analyse genes involved in biological processes, genome-wide expression analysis is considered one of the most efficient tool (Breyne and Zabeau, 2001). In this regard, cDNA-AFLP is a method for genome wide expression analysis often proposed as an alternative to microarrays methods. Above all its application is suggested to study organisms with complete genome sequence or cDNA collection unavailability (Breyne and Zabeau, 2001).

cDNA-AFLP is carried out according to the principle of AFLP (Amplified Fragment Length Polymorphism). The AFLP technique is based on the digestion of DNA templates followed by the ligation of adapters to restriction fragments and the selective PCR amplification of subsets of these fragments using selective AFLP primers. Similarly, the original cDNA-AFLP method (Bachem et al., 1996) involves the reverse transcription of mRNA into double-stranded cDNA, followed by the generation of a complex mixture of transcript-derived fragments (TDFs) by restriction enzyme

digestion and ligation of specific adapters, selective PCR amplification and finally the visualization of the TDFs on high-resolution (sequence) gels (Vuylsteke et al., 2007).
Recently different researches were published to improve the original cDNA-AFLP method (Weiberg et al., 2008; Decorosi et al., 2005; Kadota et al., 2007; Xiaohu et al., 2009). Nevertheless, the most important variant is the modification of the original protocol based on "one-gene-multiple-tag" into "one-gene-one-tag" as reported Vuylsteke et al. (2007) and Breyne and Zabeau (2001). The "one-gene-one-tag" cDNA-AFLP protocol involves a reduction of TDFs to a single fragment for each cDNA by selecting the 3'-terminal restriction fragment of each transcript before selective amplification. Recently, Levterova et al. (2010) have developed a cDNA-AFLP strategy to study the gene transcription in fungi based on the use of capillary electrophoresis.
Although cDNA-AFLP technique has been extensively applied to analyze transcription profiles of eukaryotic organisms (Bensch and Akesson, 2007; Reijans et al., 2003; Ekkapongpisit et al., 2007; Neveu et al., 2007) there are few data regarding cDNA-AFLP application to bacteria (Dellagi et al., 2000; Noel et al., 2001). This is probably due to the difficulties of working with bacterial mRNA. A major technical challenge for transcriptome sequencing is the low relative abundance of mRNAs in total cellular RNA (1-5%) (Neidhardt et al., 1996), mainly made of rRNAs and tRNAs (Karpinets et al., 2006). Furthermore, unlike eukaryotic mRNAs, bacterial mRNA generally lacks in poly(A)-tail which makes their isolation and analysis complicated (He et al., 2010).
In this study we developed, for the first time, a cDNA-AFLP protocol to study the profiling gene expression of *L. rhamnosus* strain isolated from Parmigiano Reggiano cheese. The novel transcriptomic approach was applied to evaluate changes in gene expression profile during the growth of *L. rhamnosus* in a rich medium (MRS) versus cheese–like medium, a medium based on grated Parmigiano Reggiano ripened cheese, characterized by the absence of milk sugars and rich on peptides, amino acids and NaCl (Neviani et al., 2009).

3.3.3 Materials and methods

Bacterial strains and media

L. rhamnosus 1473, previously isolated from 20 months ripened Parmigiano Reggiano (PR) cheese, belonging to biotype n°1 (Bove et al., 2011), has been used in this study. The strain, isolated on MRS agar (Oxoid Ltd., Basingstoke, United Kingdom), was maintained as stock culture at -80°C in MRS broth with 15% glycerol (w/v) added. For RNA extraction, it was first propagated twice, with a 2% inoculum, in MRS broth at 37°C for 24 h; then the culture was anaerobically (Gas Generating Kit, Oxoid, Basingstoke, UK) propagated twice at 37°C, until the stationary phase was reached, in

two different media: MRS broth and a cheese-like broth called cheese broth (CB). CB was prepared partially modifying the protocol described by Neviani et al. (2009). Fine-grain grated 20 months ripened PR cheese was dissolved (120 g/L) in sodium citrate (Sigma-Aldrich Co., St. Louis, MO, USA) 0.07 mol/L pH 7.5. The suspension was incubated at 42°C for 50 min under stirring condition (150 x *g*); then it was centrifuged (8000 x *g* for 15 min at 4°C) and filtered through sterile gauze to retain the surfaced fat layer. Filtered liquid was acidified to pH 4.6 by adding HCl 1.0 M, sterilized for 15 min at 121°C and centrifuged at 8000 x *g* for 15 min to remove un-hydrolyzed proteins (casein and heat-denatured serum proteins). The supernatant was added of NaOH 1 M until pH 6.1 was reached. Freeze-dried cell lysate of *Lactobacillus helveticus* PR775, previously isolated from PR cheese (University of Parma collection), was added at a final concentration corresponding to 8 log CFU/mL. The resulting medium was sterilized by filtration on 0.22 µm membrane filters (Millipore Corporation, Bedford, MA 01730, USA). For preparation of freeze-dried cell lysate, *L. helveticus* PR775, was maintained as stock culture at -80°C in MRS broth with 15% glycerol (w/v) added. It was first propagated twice, with a 2% inoculum, in MRS broth at 37°C for 24 h; then the culture was propagated in MRS at 37°C until the stationary phase was reached. Harvested cells were washed in 0.05 M Tris/HCl pH 7.5, centrifuged (15,000 × *g* for 15 min at 4°C) and frozen or directly resuspended in 0.05 M Tris/HCl pH 7.5. Cells were disrupted with a Branson model B15 Sonifier by 3 cycles of sonication (1 min each). After pelleting of unbroken cells (15,000 × *g* for 15 min at 4°C), the supernatant was maintained as stock at -20°C.

RNA extraction and cDNA synthesis

The growth of *L. rhamnosus* 1473 at 37°C both into MRS and CB was monitored by Optical Density (OD) measurement (Jasco, Tokio, Japan) until the stationary phase was reached. A total of 10^9 cells/ml at stationary phase was harvested, total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and stabilized using RNeasy Protect Bacteria Mini Kit (Qiagen, Valencia, CA, USA). The protocol included the removal of DNA from the samples by DNAseI using the RNase-Free DNase Set (Qiagen, Valencia, CA, USA). Total RNA was resuspended in 50 µl of RNAse Free water and checked on 0.8% agarose gel (Sigma-Aldrich, Milano, Italy). The quantity and purity of RNA were assessed by OD measurement at 260 and 280 nm (Jasco, Tokio Japan). Prior to cDNA-synthesis, total RNA was enriched in mRNA and then the mRNA was polyadenylated.

By using MicrobExpress bacterial mRNA enrichment kit (Ambion, Applied Biosystems, Monza, Italy), 10 µg of total RNA from three independent biological replicates were enriched in mRNA by

removing the 16S and 23S ribosomal RNAs (rRNA). The enriched mRNA was purified from 5S RNA using the MEGAclear Purification Kit (Ambion, Applied Biosystems, Monza, Italy) and quantified by spectrophotometer (Jasco, Tokio, Japan).

In order to add a poly(A) tail to RNA transcripts we used the Poly(A) Tailing Kit (Ambion, Applied Biosystems, Monza, Italy).The poly-A RNA (2 µg) was used in the synthesis of double-stranded cDNA according to the protocol reported by Vuylsteke et al. (2007) except for the following modifications.

For first-strand complementary DNA synthesis, 1 µl of 100 µm oligo dT25bio primer (Eurofins MWG Operon, Ebersberg, Germany) was added to 20 µl of RNA sample, the mixture was heated to 65°C for 5 min and quick-chilled on ice. The sample was collected by brief centrifugation and 4 µl of 5X buffer, 2 µl of DTT 0.1 M and 1 µl of dNTPs mix 10 mM (Invitrogen Life Technologies, Carlsbad, CA, USA) were added. The sample was placed at 42°C for 2 min to equilibrate the temperature, then was added with 2 µl of SuperScript™ II RT (200 U/ µl) (Invitrogen Life Technologies, Carlsbad, CA, USA) and incubated at 42°C for 2 h. For second-strand cDNA synthesis 32 µl of 5X second-strand buffer, 3 µl of dNTPs mix (10 mM), 6 µl of DTT 0.1 M, 1.5 µl of *E. Coli* Ligase (10 U/µl), 0.2 µl of Ribonuclease H (10U/µl), 5 µl of DNA polymerase I (10U/µl) (Invitrogen Life Technologies, Carlsbad, CA, USA) were mixed and water was added up to 120 µl. After an incubation of 2 h at 16°C, the double-stranded cDNA was purified and concentrated in 30 µl of elution buffer NE by using the NucleoSpin Extract II Kits (Macherey-Nagel, Düren, Germany). The cDNA was checked on 0.8% agarose gel (Sigma-Aldrich, Milano, Italy) and quantified by spectrophotometer (Jasco, Tokio, Japan).

cDNA-AFLP analysis

Preparation of template

The template for cDNA-AFLP analysis was prepared making some modification to the procedure reported by Vuylsteke et al. (2007). The protocol of Vuylsteke et al. (2007) describes the generation of cDNA-AFLP fragments detected using infrared dye (IRD) detection technology and the Odyssey Infrared Imaging System. A first restriction mixture containing 1 µl of *Eco*RI (10U/ µl), 4 µl of NEBuffer 4, 0.2 µl of BSA 100X (New England Biolabs, Ipswich, MA, USA) and 14,8 µl of free-nuclease water was added to 20 µl of double-stranded cDNA (about 500 ng). This reaction mixture was incubated for 2 h at 37°C. Subsequently, the mixture was added to 40 µl of solution containing 10 µl of Dynabeads M-280 streptavidin (Invitrogen Life Technologies, Carlsbad, CA, USA) and 40

µl of 2X STEX buffer (40 ml NaCl 5M, 2 ml Tris-HCl 1 M pH 8.0, 400 µl EDTA 0.5 M pH 8.0, water up to 100 ml). This reaction mixture was incubated for 30 min at room temperature. This step allows the immobilization of biotinylated 3'-terminal cDNA fragments on streptavidin-coated Dynabeads. The Dynabeads were collected, washed and resuspended in 100 µl of 1X STEX buffer. Subsequently, a second restriction mixture containing 1 µl of *Mse*I (10U/ µl), 4 µl of NEBuffer 4, 0.1 µl of BSA 100X (New England Biolabs, Ipswich, MA, USA) and 4,9 µl of free-nuclease water was added. The mixture was incubated for 2 h at 37°C under stirring condition. The surnatant (40 µl) containing the cDNA fragment digested was added to 10 µl of ligation mixture. This consists in a total volume of 10 µl containing 1 µl EcoRI adapter (EcoRI-Forw: 5'-CTCGTAGACTGCGTACC-3'; EcoRI-rev: 5'-AATTGGTACGCAGTCTAC-3') (Vuylsteke et al., 2007) (5 pmol/ µl) (Eurofins MWG Operon, Ebersberg, Germany), 1 µl MseI adapter (50 pmol/ µl) (MseI-Forw: 5'-GACGATGAGTCCTGAG-3'; MseI-rev: 5'-TACTCAGGACTCAT-3') (Vuylsteke et al., 2007) (Eurofins MWG Operon, Ebersberg, Germany), 1 µl NEBuffer 4 (New England Biolabs, Ipswich, MA, USA), 1 µl T4 DNA ligase (1U/ µl), 1 µl ATP (10 mM), 0.1 µl of BSA 100X (Invitrogen Life Technologies, Carlsbad, CA, USA) and 4,9 µl of free-nuclease water. This reaction mixture was incubated for 3 h at 37°C. Subsequently, the mixture was diluted to 100 µl with TE buffer 0.1 M and stored at 20°C.

Non selective PCR

The "non-selective" primers EcoRI-0 (5'-GACTGCGTACCAATTC-3') and MseI-0 (5'-GATGAGTCCTGAGTAA-3') (Eurofins MWG Operon, Ebersberg, Germany) were used for pre-amplification of the diluted template. Each pre-amplification mixture contained 5 µl of the diluted template previously described, 1.5 µl of unlabelled MseI-0 primer (10 µM) and 1.5 µl of labelled IRD700EcoRI-0 primer (10 µM), 0,2 µl of Taq DNA polymerase (5U/ µl), 5 µl 10X PCR buffer, 5 µl $MgCl_2$ (25 mM), 2 µl dNTPs (5 mM), 29,8 µl of free-nuclease water. The reaction was subjected to the following PCR conditions: initial denaturation at 94°C for 2 min, 25 cycles consisted of 30 s denaturation at 94°C, 1 min annealing at 56°C, 1 min extension at 72°C and 1 cycle of final extension for 10 min at 72°C. All amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Hamburg, germany). The pre-amplification product was diluted 600 times with TE buffer 0.1 M and stored at -20°C.

Selective PCR and capillary electrophoresis (ce-cDNA-AFLP)

The "selective" primers 5'FAM-EcoRI-N, labeled with 5′-Carboxy fluorescein (FAM), and unlabelled MseI-N (Table 1 and Table 2) (Eurofins MWG Operon, Ebersberg, Germany) were used for selective amplification of the diluted pre-amplification product. PCR mixture contained 5 µl of diluted pre-amplification product, 0.8 µl of labelled IRD700EcoRI-N primer (1 µM), 3 µl of unlabelled MseI-N primer (2 µM), 0,2 µl of AmpliTaq (5U/ µl) (Ambion, Applied Biosystems, Monza, Italy), 2 µl 10X PCR buffer, 2.4 µl $MgCl_2$ (25mM), 0.8 µl dNTPs (5mM) (Invitrogen Life Technologies, Carlsbad, CA, USA), 5,8 µl of free-nuclease water.

The thermocycling conditions were designed as follows: initial denaturation at 94°C for 2 min, 13 cycles consisting of 10 s denaturation at 94°C, 30 s annealing at 65°C (reduced each cycle by 0,7 °C), 1 min extension at 72°C, 23 cycles consisting of 10 s denaturation at 94°C, 30 s annealing at 56°C, 1 min extension at 72°C (extended 1 s per cycle) and 1 cycle of final extension for 10 min at 72°C. All amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Hamburg, Germany). The cDNA-AFLP analysis of the amplified products was performed making some modification to the procedure reported by Lazzi et al. (2009). The amplified products from selective amplification were added to 1 µl of GeneScan-500 [ROX] size standard (Applied Biosystem-Pe Corporation) and 24 µl of deionized formamide. The mixture was heated 5 min and cooled 10 min on ice. Samples were loaded and run on the ABI Prism 310 (Applied Biosystem-Pe Corporation) according to the AFLP Microbial Fingerprinting protocol (Applied Biosystem-Pe Corporation) and analyzed using Genemapper Analysis Software (Applied Biosystem-Pe Corporation) according to the manufacturer's instructions. The data for each run were saved as an individual GeneScan file and displayed as an electropherogram. A threshold of 100 RFU (Relative Fluorescent Unit) was considered in scoring to consider only sharp and easily distinguishable peaks; all the signals under this value were treated as background and not scored.

Selective PCR and gel electrophoresis (ge-cDNA-AFLP)

The "selective" primers IRD700EcoRI-N, labeled with a infrared dye (IRDye™ 700 phosphoramidite), and unlabelled MseI-N (Table 1 and Table 2) (Eurofins MWG Operon, Ebersberg, Germany) were used for selective amplification of the diluted pre-amplification product. PCR mixture contained 5 µl of diluted pre-amplification product, 0.8 µl of labelled IRD700EcoRI-N primer (1 µM), 3 µl of unlabelled MseI-N primer (2 µM), 0,2 µl of AmpliTaq (5U/ µl) (Ambion, Applied Biosystems, Monza, Italy), 2 µl 10X PCR buffer, 2.4 µl $MgCl_2$ (25mM), 0.8 µl dNTPs (5mM) (Invitrogen Life Technologies, Carlsbad, CA, USA), 5,8 µl of free-nuclease water.

The thermocycling conditions were designed as follows: initial denaturation at 94°C for 2 min, 13 cycles consisting of 10 s denaturation at 94°C, 30 s annealing at 65°C (reduced each cycle by 0,7 °C), 1 min extension at 72°C, 23 cycles consisting of 10 s denaturation at 94°C, 30 s annealing at 56°C, 1 min extension at 72°C (extended 1 s per cycle) and 1 cycle of final extension for 10 min at 72°C. All amplifications were performed in a Mastercycler Ep Gradient S (Eppendorf, Hamburg, Germany). Electrophoresis was done through a 6% Long Range gel as reported by Vuylsteke et al. (2007). Amplification products (6 µl) obtained from selective amplification were mixed with 3 µl of IR2 Stop Solution Red (LI-COR Biosciences, NE, USA), denatured at 95°C for 3 min and then cooled on ice for 10 min. The 50-700 bp IRDye700 Sizing Standard (LI-COR Biosciences, NE, USA) was denatured at 95°C for 2 min and cooled on ice for 10 min. Samples were loaded onto the pre-running gel (30 min at 40 mA), and 50-700 bp IRDye700 Sizing Standard (LI-COR Biosciences, NE, USA) was loaded every 6 lane for normalization purposes. The gel was run for 4,5 h at 20 mA and, after electrophoresis, Odissey (LI-COR Biosciences, NE, USA) at 700 nm was used for gel scanning.

3.3.4 Results and discussion

In this study we report the application of cDNA-AFLP methodology for studying the adaptation of *L. rhamnosus* to cheese environment. We applied two techniques, capillary electrophoresis cDNA-AFLP (ce-cDNA-AFLP) and gel electrophoresis cDNA-AFLP (ge-cDNA-AFLP), to obtain the expression profile of the whole genome of *L. rhamnosus* grown in cheese-like medium CB respect to MRS.

In the first instance, ce-cDNA-AFLP was carry out to monitor transcriptional changes related to the growth in the two different media. Subsequently, ge-cDNA-AFLP allowed us to confirm the different expression profiles in MRS and CB and to obtain fragment that could be extracted and identified.

In both methods we used a novel protocol to analyze the expression of genes involving in the growth of *L. rhamnosus* strain in different nutritional condition.

The cDNA-AFLP method reported in our study allows to generate unique transcript tags from reverse-transcribed messenger RNA using restriction enzymes and selective PCR amplification. The protocol was developed on the basis of what Vuylsteke et al. (2007) used for studying gene expression in eukaryotic species. Principal changes at the original cDNA-AFLP procedure consist in the isolation of mRNA from total RNA and in the addition of a 3'poly(A) tail to mRNA. The subtractive hybridization by rRNA probe (MicrobExpress bacterial mRNA enrichment kit) was

found to be most efficient in enriching total RNA in mRNA and it has already been successfully applied in a recent study of microbiota metatranscriptomic (Booijink et al., 2010). Since the selective cDNA synthesis of mRNAs based on reverse transcription of the 3'-poly(A) tail is not applicable to prokaryotic messengers we added a poly(A) tails to mRNA prior to cDNA-synthesis. This allows to use an oligodT biotinilated in the retrotrascription reaction: in this way, it is possible to retrotranscript all mRNA by using a unique primer and, in the subsequently reactions of enzymatic digestion, only a cDNA fragment deriving from each mRNA species will be captured. RNA was prepared from *L. rhamnosus* grown to stationary phase in CB or MRS. The media CB and MRS represent different conditions for growth of *L. rhamnosus* and thus potentially stimulate differential gene expression. MRS is a standard laboratory complete medium containing a complex nitrogen source consisting of peptone (protein hydrolysate) and extracts (from meat and yeast) (De Man et al., 1960). CB is a medium based on grated PR ripened cheese, characterized by the absence of milk sugars and rich in peptides, amino acids and NaCl (Neviani et al., 2009).

Double stranded cDNA syntetized as previously described from *L. rhamnosus* grown in CB and MRS was used as template for AFLP analysis. We used three combinations of fluorescently labelled primers (Table 2) to obtain fragments 50-500 bp lenght. The selectivity of the primer pairs was determined by one or two additional nucleotides.

The different selective primer combinations generated profiles characterized both by a different number of fragments and by fragment with different molecular weight. In this way, when different selective nucleotides were added to the primers, it was possible to screen and visualize the majority of both abundantly and weakly expressed genes (Breyne et al. 2003).

In Table 2 are reported the number of fragments as results of capillary and gel electrophoresis. A significant number of fragments are obtained when performing PCR amplification with the use of the 5′-Carboxy fluorescein (FAM) fluorescently labeled primer in ce-cDNA-AFLP protocol (Fig.2). The fragments generated by ce-cDNA-AFLP, visualized as peaks in the electropherograms after selective amplification, ranged from 15 to 35 depending upon primer combinations and conditions of growth (Fig.2).

The highest number of peaks in ce-cDNA-AFLP and fragments in ge-cDNA-AFLP was produced either in MRS and in CB by *Eco*RI-AC/*Mse*I-T (Tab. 2 Figures 2c1, 2c2, 3). Nevertheless, the pairs primers *Eco*RI-A/*Mse*I-AC and *Eco*RI-T/*Mse*I-AC originated the higher number of different fragments between the strain grown in MRS and in CB, both for gel electrophoresis and for capillary electrophoresis (Table 2). Number of peaks detected in CB was always highest than in MRS, on the contrary number of fragments detected in CB was lowest than in MRS for *Eco*RI-

AC/*Mse*I-T (Table 2). This result is not completely unexpected and cannot be considered an incongruity. In fact the sensitivity of these two techniques is different and in particular the capacity of genes separation using the gel is lower than that obtained using capillary electrophoresis. The lower number of amplification products generated from cDNA templates in the ge-cDNA-AFLP methods may have been due to the presence of transcripts with the same or similar molecular weight that lead to a unique band in the gel. For this reason the total number of gel fragments could be lower than the total number of genes.

Remarkable differences were observed in the growth medium-dependent transcriptomes of *L. rhamnosus*. Overall, the use of 3 pairs primers allowed to detect 64 genes expressed in MRS and 96 in CB. 51 different transcripts, 9 of those expressed only during growth in MRS while 42 in CB, were found to be differentially expressed when transcriptomes of *L. rhamnosus* grown in different media were compared. As a whole, the profiles of the strain grown in CB were more complex (Fig. 3).

The larger number of amplification products generated from CB-derived template in respect to those generated from MRS-derived template (Table 2), is representative of a more complex gene expression of *L. rhamnosus* when grown in a cheese-like environment. The transcriptomic profiles resulted affected by the growth medium probably because it induces the activation of different metabolic pathways in the cell to generate energy and to respond to the environmental changes. Different studies demonstrated that the adaptation to dairy niches is associated either with a metabolic simplification as loss of genes involved in carbohydrate, amino acid and cofactor metabolisms, or with an increase of expression of genes involved in peptide hydrolysis and amino acids catabolism (Bolotin et al., 2004; Hols et al., 2005; van de Guchte et al., 2006; Callanan et al., 2008).

3.3.5 Conclusion

Adaptation of bacteria to their environment is considered a very interesting research field because involving many metabolic and physiological changes. *L. rhamnosus* species, among Lactobacilli, demonstrated to be able to adapt to very unfavourable environments such as the ripening of PR cheese and even to better grow in these condition becoming a dominant species. The study of *L. rhamnosus* growth in a medium reproducing these ripening conditions allowed us to better investigate these metabolic aspects.

This study represent one of the few concerning bacterial transcriptomic analysis toward cDNA-AFLP approaches. This technique resulted an useful tool to highlight that *L. rhamnosus* modify the

expression of a large part of genes when cultivated in cheese-like conditions (CB) compared to growth under *in vitro* optimal conditions (MRS).

In our research we used cDNA-AFLP approach to investigate changes in gene expression occurred in *L. rhamnosus* during growth in a commercial medium and in a cheese-like substrate. The two applied techniques, capillary electrophoresis cDNA-AFLP (ce-cDNA-AFLP) and gel electrophoresis cDNA-AFLP (ge-cDNA-AFLP), appear to have different performance: the first is more sensitive and able to evidence a greater number of genes, the latter is necessary to identify them.

This research is the first one done on *L. rhamnosus* isolated from cheese. However further analysis will have to be focused on gel extraction followed by the identification of expressed genes. This could be interesting in order to deepen the knowledge of the basal metabolism through the identification of constitutive genes, as well as to analyze how cells react to the dairy environment through the identification of differently expressed genes in the two culture media.

Moreover the expected results could lead to the discovery of genes putatively involved both in ripening processes and in the definition of organoleptic characteristics of the Parmigiano Reggiano cheese, as well as to understand *L. rhamnosus* physiological mechanisms of adaptation to cheese-like substrates that are fundamental for survival in cheese during manufacture.

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3.3.7 Legends to figures

Figure 1. Schematic overview of the cDNA-AFLP procedure with 3' end capture applied to RNA isolated from *L. rhamnosus* 1473. Total RNA isolated from *L. rhamnosus* 1473 is enriched for mRNA by subtractive hybridization as indicated by the manufacturer (MicrobExpress; Ambion, Huntington, United Kingdom). Poly(A) tail is added to RNA transcripts using the Poly(A) Tailing Kit (Ambion, Applied Biosystems, Monza, Italy). cDNA is synthesized by reverse transcription using a biotinilated oligo dT, followed by second-strand synthesis. Digestion with first restriction enzyme, capture with the aid of streptavidin coated magnetic beads followed by the digestion with the second restriction enzyme, the removal of the 3' fragments and ligation of adaptors. Selective amplification and separation of the fragments on a polyacrylamide gel and capillary electrophoresis and visualization.

Figure 2. Electropherograms of *L. rhamnosus* 1473 cDNA-AFLP profiles using different primer combinations. EcoRI-A/MseI-AC in MRS (a1) and CB (a2), EcoRI-T/MseI-AC in MRS (b1) and CB (b2), EcoRI-AC/MseI-T in MRS (c1) and CB (c2)

Figure 3. cDNA-AFLP fingerprint obtained with two different primer combinations.
M, 50-700 bp IRDye700 Sizing Standard; lane 1 and 2, cDNA-AFLP fingerprinting of *L. rhamnosus* cultured in MRS and Cheese Broth respectively using EcoRI-A/MseI-AC primer combination; lane 3 and 4, cDNA-AFLP fingerprinting of *L. rhamnosus* cultured in MRS and Cheese Broth respectively using EcoRI-T/MseI-AC primer combination; Lane 5 and 6, cDNA-AFLP fingerprinting of *L. rhamnosus* cultured in MRS and Cheese Broth respectively using EcoRI-T/MseI-AC primer combination.

Table 1. Sequences of the primers and adaptors used for cDNA-AFLP

Primers and adaptors	Initials	Sequences (5'-3')
EcoRI adaptor	EcoRI Forw	5'-CTCGTAGACTGCGTACC-3'
EcoRI adaptor	EcoRI Rev	5'-AATTGGTACGCAGTCTAC-3'
MseI adaptor	MseI Forw	5'-GACGATGAGTCCTGAG-3'
MseI adaptor	MseI Rev	5'-TACTCAGGACTCAT-3'
EcoRI-0	EcoRI-0	5'-GACTGCGTACCAATTC-3'
EcoRI-A	FAM-EcoRI-A IRD-EcoRI-A	5'- GACTGCGTACCAATTCA-3'
EcoRI-T	FAM-EcoRI-T IRD-EcoRI-T	5'- GACTGCGTACCAATTCT-3'
EcoRI-AC	FAM-EcoRI-AC IRD-EcoRI-AC	5'- GACTGCGTACCAATTCAC-3'
MseI-0	MseI-0	5'- GATGAGTCCTGAGTAA-3'
MseI-T	MseI-T	5'- GATGAGTCCTGAGTAAT-3'
MseI-AC	MseI-AC	5'- GATGAGTCCTGAGTAAAC-3'

Table 2. Primer combinations used in cDNA-AFLP and number of fragments obtained

Primer combinations	Media	ce-cDNA-AFLP peaks	ge-cDNA-AFLP fragments
EcoRI-A/MseI-AC	MRS	16 (Figure 2a1)	16 (Figure 3 lane 1)
	CB	32 (Figure 2a2)	24 (Figure 3 lane 2)
EcoRI-T/MseI-AC	MRS	15 (Figure 2b1)	11 (Figure 3 lane 3)
	CB	29 (Figure 2b2)	16 (Figure 3 lane 4)
EcoRI-AC/MseI-T	MRS	33 (Figure 2c1)	33 (Figure 3 lane 5)
	CB	35 (Figure 2c2)	27 (Figure 3 lane 6)

Figure 1. Bove et al.

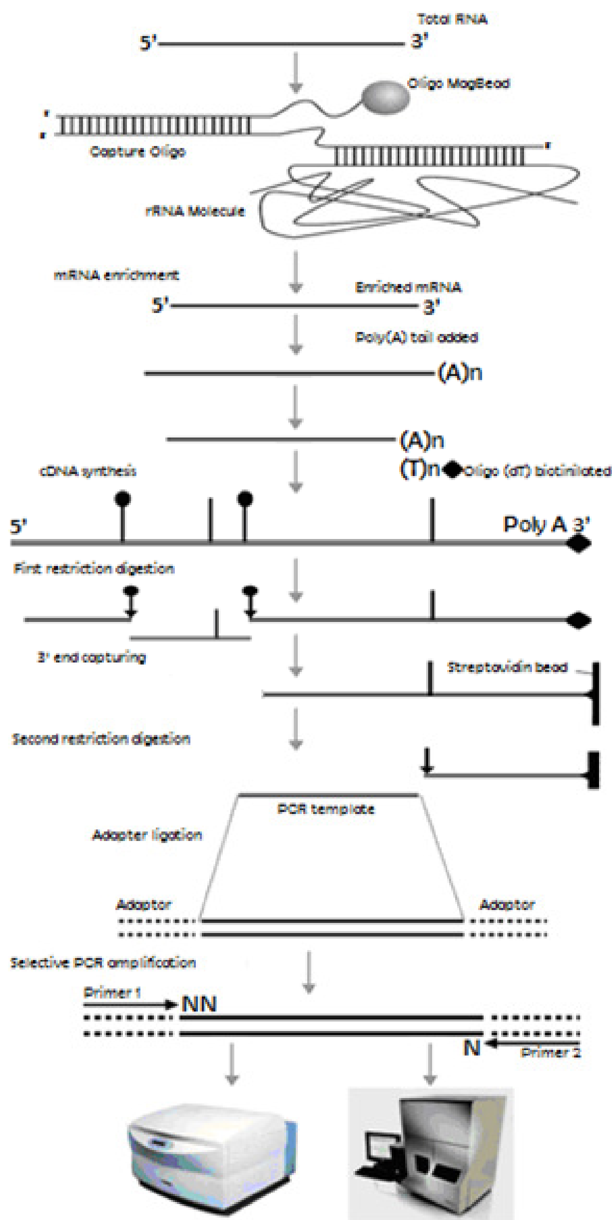


Figure 2. Bove et al.

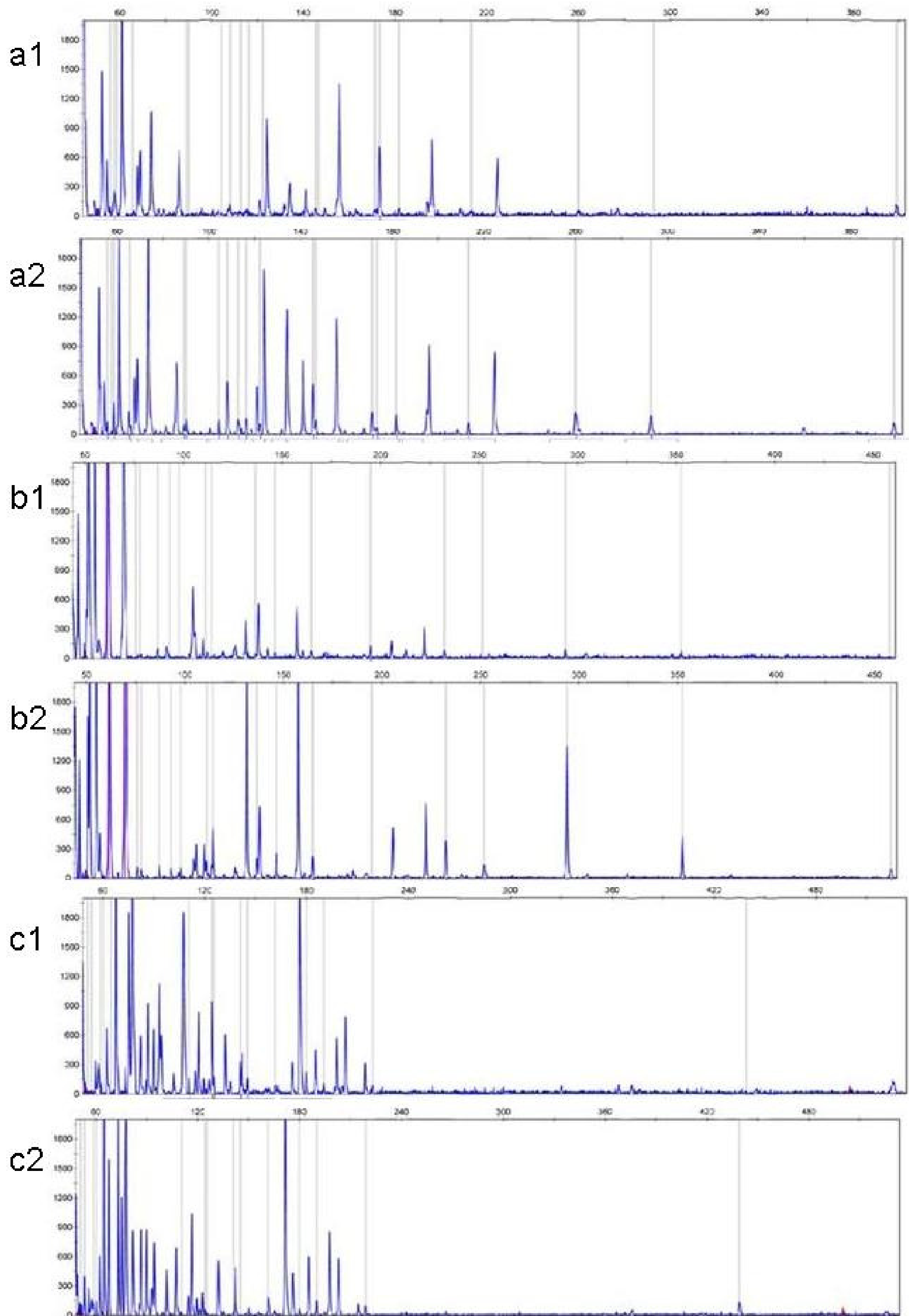
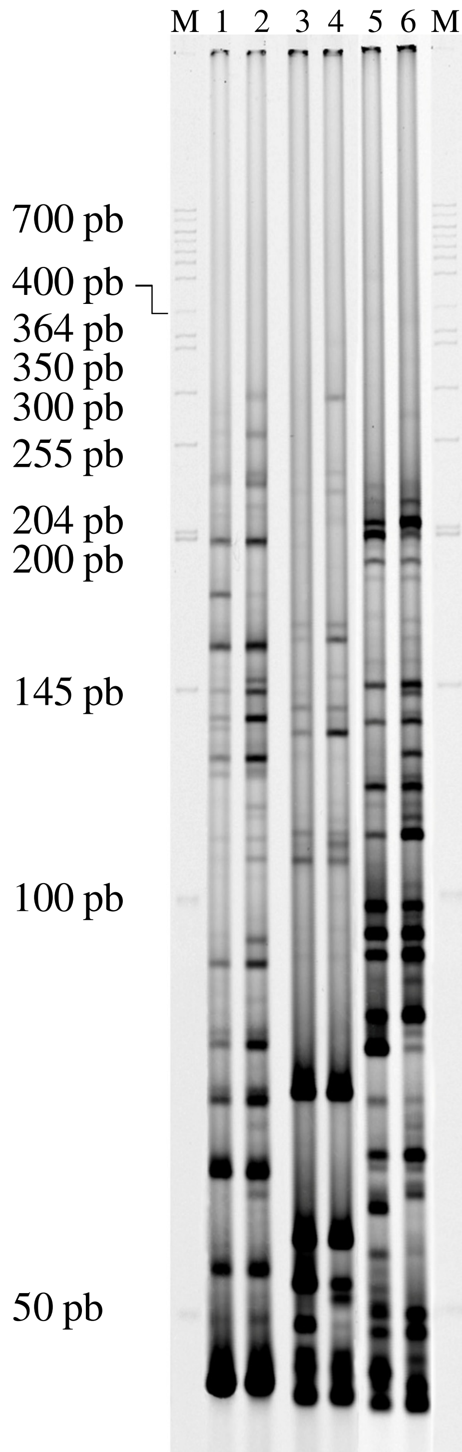


Figure 3. Bove et al.



4. GENERAL CONCLUSIONS

Non-starter lactic acid bacteria (NSLAB) are the protagonists of the different biochemical processes during the manufacturing and ripening of Parmigiano Reggiano (PR) cheese. In recent years, some studies demonstrated that, among NSLAB, *Lb rhamnosus* was the dominant species present in PR during ripening. Although the presence of NSLAB in the ripening process of PR is already known the exact role of these bacteria has not been clarified yet. In particular, the biotypes of the dominant species, such as *Lb. rhamnosus*, during the maturation stage of cheese processing have never been studied. Indeed, it can only be hypothesised that the technological pressure, during PR manufacturing, determines, at different stages, the potential development of the biotype that may have a specific biochemical role leading to certain flavours and sensorial traits of the PR.

During this Ph.D. thesis, genotypic, metabolic and post-genomics approaches were applied to evaluate the *Lb. rhamnosus* contribution in PR ripened cheese and advance in knowledge was reached.

In the first study, genotypic relatedness at the intraspecies level for 66 *Lb. rhamnosus* strains isolated during the same cycle of PR cheesemaking, was determined. Biodiversity has been estimated by means of two DNA fingerprinting techniques, i.e., randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and repetitive extragenic palindromic-polymerase chain reaction REP-PCR analysis. It was found an intraspecies heterogeneity in *Lb. rhamnosus* strains which is certainly correlated to their abilities to adapt to specific environmental and technological conditions. In this regard, the detection of biotypes that mark specific steps in cheese ripening, or of those that can develop differently throughout the ripening process, suggests that they may have also specific roles closely linked to their peculiar metabolical properties.

In the second step of the research the metabolic data (fermentative profiles, synthesis of organic acids and FAA) and 2-DE analyses followed by the identification of a large number of proteins were combined to highlight *Lb. rhamnosus* physiological mechanisms of adaptation that are responsible for survival in cheese during ripening.

As shown by fermentative profiles analysis, the major part of the *Lb. rhamnosus* strains had the capacity to use numerous chemical compounds as energy sources. Growth and acidification differed depending on the culture media. Compared to growth on MRS or pasteurized and micro-filtrated milk, all strains cultivated in cheese-like medium (cheese broth, CB) showed a decrease of the synthesis of D,L-lactic acid and synthesized higher levels of acetic acid. Except for one strain, the others caused an increase of the concentration of free amino acids during cultivation on CB.

A proteomic approach was applied to elucidate the (i) intra-specific diversity of *Lb. rhamnosus* and (ii) the metabolic mechanisms involved in growth in CB which mimicked the cheese during ripening. The proteomic maps of five strains showing different metabolic traits were comparatively determined after growth on MRS and CB. The result showed that synthesis of ca. 60 - 70% of the cytosolic proteins of *Lb. rhamnosus* strains was not affected by the culture media. The remaining part (ca. 30-40%) of the total proteome seemed to vary depending on strains and growth media. A total of 93 protein spots which showed an increased level of synthesis during growth in MRS (46 spots) or CB (47 spot) were identified by MALDI-time of flight mass spectrometry (MALDI-TOF-MS/MS) and nano-electrospray ionization-ion trap mass spectrometry (ESI-MS/MS). Compared to cells grown in MRS, *Lb. rhamnosus* strains cultivated under cheese-like conditions increased mainly the synthesis of proteins involved on proteolytic activity, oxidation/reduction processes, stress response, cellular regulation, citrate and amino acid metabolisms. It was confirmed that *Lb. rhamnosus* strains have the capacity to activate different metabolic pathways depending on the culture media.

In the last study an innovative cDNA-AFLP protocol was developed to investigate the changes in gene expression profile during the growth of one *Lb. rhamnosus* strain isolated in 20 month ripened PR and therefore able to growth and survive in this hostile environment. Changes in gene expression was evaluated in CB versus a rich medium (MRS). This study represents one of the few concerning bacterial transcriptomic analysis through cDNA-AFLP approaches. This technique allowed to generate unique transcript tags from reverse-transcribed messenger RNA using restriction enzymes and selective PCR amplification. Results evidenced that *Lb. rhamnosus* is able to modify the expression of a large part of genes when cultivated in cheese-like conditions (CB) compared to growth under in vitro optimal conditions (MRS).

However, these genes still have to be recognized thus it is not possible at the moment to correlate them with proteins found with the proteomic study. For this reason further analysis will have to be focused on gel extraction followed by the identification of expressed genes. This will allow to deepen the knowledge of the basal metabolism through the identification of constitutive genes, as well as to analyze the reactions of the cells to the dairy environment by the identification of differently expressed genes in the two cultured media. Moreover the expected results could lead to the discovery of *Lb. rhamnosus* genes putatively involved both in the physiological mechanisms of adaptation to cheese-like substrates, that are fundamental for survival during ripening, and in the definition of organoleptic characteristics of the PR cheese.

5. CURRICULUM VITAE

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Publications list

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