Università degli Studi di Parma

Faculty of Agriculture
Ph.D. in Food Science and Technology
XX° cicle 2005-2007

Ph.D. dissertation

Traditional and innovative approaches to evaluate microbial contribution in long ripened fermented foods: the case of Parmigiano Reggiano cheese

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Parma 2008

to my family, friends and professors

Scienza e Civilità

“Al di sopra e oltre qualsiasi giudizio sul nostro tempo, noi abbiamo un altro compito, la preoccupazione per ciò che costituisce il nostro eterno e più alto possesso, ciò che dà alla vita la sua importanza e che noi desideriamo lasciare ai nostri figli, più puro e più ricco di quanto non l’abbiamo ricevuto dai nostri padri.”

Albert Einstein, 1933

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1. INTRODUCTION

1.1 FERMENTED DAIRY FOODS AND LACTIC ACID BACTERIA

Humans place great value on technologies to improve the keeping qualities of foods, and one of the most ancient of these practices depends on lactic acid bacteria (LAB) to ferment milk. Because these bacteria are constituents of raw milk, cheese and other fermented milk foods have likely been part of the diet since humans first collected milk and held it in crude containers. Over the centuries, these "accidental" fermentations were controlled and moulded into the more than hundreds unique cheeses, yogurts, and fermented milks that are available today (Broadbent and Steele, 2005).

Because fermented dairy foods developed before the emergence of microbiology as a science, manufacturing processes for all varieties long relied upon naturally occurring LAB to acidify milk. It was not until discovery of the lactic acid fermentation by Pasteur in 1857, and development of pure LAB starter cultures later that century, that the door to industrialized cheese and milk fermentations opened. Since then, production of fermented milk and specially cheese have undergo dramatic, sustained growth (Broadbent and Steele, 2005).

To sustain such growth and productivity, the dairy industry has evolved into a leader in starter microbiology and fermentation technology. Decades of experience have proved that large-scale production of uniform, high-quality cheese is facilitated by the use of thoroughly characterized starter bacteria. Thus, even thought some traditional cheese fermentations rely on the natural souring of raw milk, the great majority of industrialized processes use starter cultures. Since future growth and economic vitality of dairy industry depends on starter cultures with known, predictable, and stable characteristics, fundamental understanding of LAB behaviour holds enormous value globally (Broadbent and Steele, 2005). In part, this doctoral thesis would like to cooperate to improve the knowledge in this field.

The term LAB mainly refers to the defining feature of the basal metabolism of these bacteria, the fermentation of hexose sugars yielding, primarily, lactic acid. It is thought that LAB emerged 1.5 to 2.0 billion years ago, whereas the milk environment is a relatively recent environment. The milk environment certainly arose after the emergence of mammals (approximately 60 million years ago) and more likely

became a viable environment for nonpathogenic microorganisms around the time that man domesticated dairy animals (approximately 10,000 yr ago (Fox and McSweeney, 2004). The LAB might be the most numerous group of bacteria linked to humans. They are naturally associated with mucosal surfaces, particularly the gastrointestinal tract, and are also indigenous to food-related habitats, including plant (fruits, vegetables, and cereal grains), wine, milk, and meat environments (Wood and Holzapfel, 1995, Wood and Warner, 2003). The LAB include both important pathogens, e.g., several *Streptococcus* species, and extremely valuable nonpathogenic species that are used for industrial fermentation of dairy products, meats, and vegetables, and they are also critical for the production of wine, coffee, silage, cocoa, and sourdough (Dunny and Cleary, 1991; Wood and Holzapfel, 1995; Wood and Warner, 2003). In addition, the LAB are a priceless source of antimicrobial agents, the bacteriocins (Cotter et al., 2005).

LAB are a relatively heterogeneous group that share several defining characteristics, including: low G+C content (<55 mol%); high acid tolerance; unable to synthesize porphyrins; and strictly fermentative metabolism with lactic acid as the major metabolic end product (Broadbent and Steele, 2005).

Being linked to the lactic acid production, the definition of LAB is biological rather than taxonomical, i.e., the LAB do not comprise a monophyletic group of bacteria. Most of the LAB belong to the order *Lactobacillales*, a group of nonsporulating, gram-positive bacteria, but a few LAB species belong to the *Actinobacteria* (Wood and Holzapfel, 1995). LAB, including members of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Tetragenococcus*, *Weissella*, and *Vagococcus* are defined as gram-positive, nonsporulating, catalase-negative, and facultative anaerobic bacteria (Axelsson, 1998). Certain LAB strains, most notably the strains from de genera *Lactobacillus*, are increasingly marketed as health-promoting, i.e., probiotic bacteria (Saxelin et al., 2005), while certain *Lactobacillus* strains are believed to produce bioactive health-beneficial peptides from milk proteins (Korhonen and Pihlanto, 2003). *Lactococcus lactis* is the most extensively studied LAB organism and the second most studied gram-positive bacterium with respect to its genetics, physiology, and molecular biology (Savijoki et al., 2006).

Undoubtedly, the most important application of LAB as aforementioned is their use as starter strains in dairy products. In particular, *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus*

helveticus, and *Lactobacillus delbrueckii* subsp. *bulgaricus* are widely used dairy starters and are of major economic importance. In the milk fermentation processes, the proteolytic system of LAB plays the key role because it enables these bacteria to grow in milk, thereby ensuring successful fermentation. LAB are fastidious microorganisms that require an exogenous source of amino acids, or peptides, which are provided by the proteolysis of casein, the most abundant protein in milk and the main source of amino acids (Savijoki et al., 2006).

Starter lactic acid bacteria (SLAB) are added to milk at the beginning of cheese manufacture. Nonstarter lactic acid bacteria (NSLAB) are present in the cheese matrix as a result of contamination during the manufacturing procedure or come from milk. In general, number of NSLAB begin to increase in the cheese matrix after the first two weeks postmanufacture. Therefore, when NSLAB cell numbers increase, most of the residual lactose in cheese has been utilized by SLAB (Diaz-Muñiz et al., 2006). Alternate potential energy sources for NSLAB in cheese are nucleic acids derived from the autolysis of SLAB (Thomas, 1986; Williams et al., 2000), amino acids (Kieronczyk et al. 2001), trace of carbohydrates, such as *N*-acetylglucosamine and ribose, liberated from glycoproteins and glycolipids present in milk (Williams and Banks 1997; Fox et al., 1998; Williams et al., 2000), and citrate (Hugenholtz, 1993; Fox and Wallace, 1997), up regulated when in presence of galactose and glucose (Dìaz-Muñiz et al., 2006). Consequently, the quantitative and qualitative microbial content of SLAB and NSLAB in cheese is the result of microbial growth capacity in milk and curd linked with the suffered technological pressure and bacterial cell autolysis (O'Cuinn et al., 1995; Fox et al., 1996; Addeo et al., 1997).

1.2 LACTIC ACID BACTERIA METABOLISMS INVOLVED IN CHEESE MANUFACTURE

1.2.1 Glycolysis

Fermenting lactose into L-lactic acid is a primary function of any starter culture in cheese manufacture. See an outline of potential routes of lactate formation in figure 1. Acid productivity is critical for controlling cheese quality because the culture determines the final pH and mineral content of the curd, which affects the protein structure and amount of residual coagulant in the curd, and, thus, texture and flavour properties. Lactate itself is also a component of cheese flavour and for example in Swiss-type cheeses serves as a key nutrient for propionibacteria. They convert it into propionic acid, which is another important flavour component, and carbon dioxide, which gives the cheese its "eyes". If starter bacteria rapidly deplete residual milk sugar in the curd, they can help to prevent its use as a substrate for undesiderable adventitious bacteria, such as, for example, heterofermentative bacteria, that can produce serious flavour and texture defects (Broadbent and Steele, 2005).

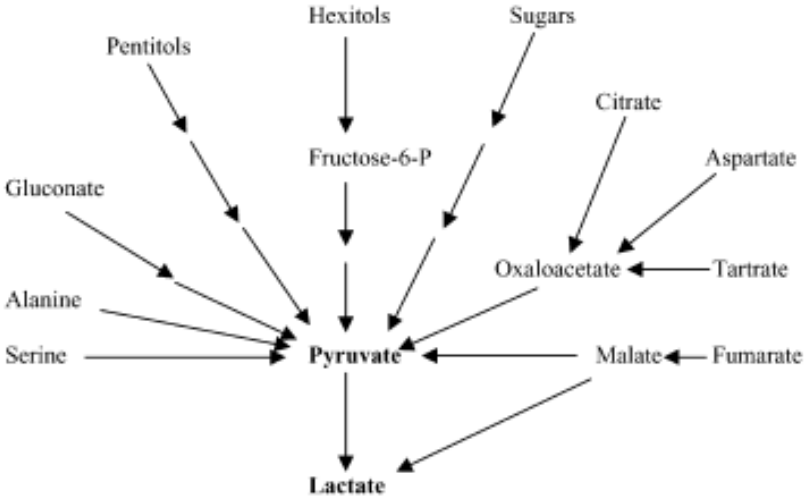

Figure 1. An outline of potential routes of lactate formation from sugars, polyols, organic acids and amino acids in lactic acid bacteria.

Depending on starter type used in cheese production, lactose is metabolized by the glycolytic or phosphoketalase (*Leuconostoc* sp.). The

principal products of lactose metabolism are L- or D-lactate or a racemic mixture of both, although some strains produce other products, e.g., ethanol (Vedamuthu, 1994). See a simplified scheme of major pathways of hexose (glucose) fermentation in LAB in figure 2.

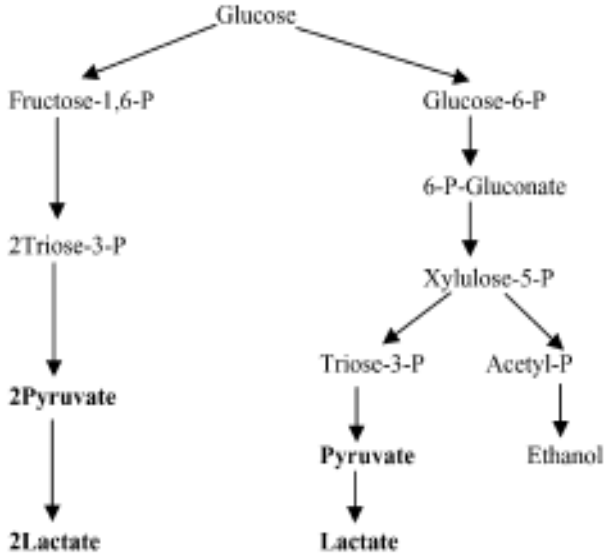

Figure 2. A simplified scheme of major pathways of hexose (glucose) fermentation in lactic acid bacteria.

St. thermophilus are unable to metabolize the galactose moiety of lactose and must grow with galactose-positive (Gal^+) microorganisms (e.g., Gal^+ Lactobacilli), or galactose will accumulate in the curd (McSweeny and Sousa, 2000). The production of D-lactate during ripening is probably greater in cheeses made from raw milk but, as far as we are aware, only few studies have investigated this until now (Steffen, 1971; Steffen, 1980). Racemization of lactate has little impact on flavour but may have undesirable nutritional consequences, particularly for infants. The solubility of Ca-D-lactate is less that of Ca-L-lactate, and Ca-D-lactate may crystallize in cheese forming white specks, particularly on cut surfaces (Fox et al., 1990).

The end product of lactose fermentation, lactate, can be catabolized further by some NSLAB. For example, it has been found that some lactobacilli and pediococci isolated from Cheddar cheese can oxidise lactate to acetate and CO_2 under aerobic conditions, and this lactate oxidation system in cheese lactobacilli and pediococci is operative under

the ripening conditions of Cheddar cheese (Thomas, 1986; Thomas, 1987). Furthermore, in the presence of O_2, some members of the NSLAB, particularly pediococci, can oxidize lacate to formate and acetate.

Negative products from glycolysis can be observed in certain hard cheeses. Late gas blowing and off-flavours result from the metabolism of lactate (or glucose) by *Clostridium* sp. To butyric acid and H_2 (Fox et al., 1993). These defects may be avoided by good hygiene, addition of NO_3^- or lysozyme, or by the physical removal of spores by bactofugation or microfiltration.

1.2.2 Citrate catabolism

LAB can use citrate to produce succinate or diacetyl (Fig. 3). Succinate, a compound with monosodium glutamate-like flavour-enhancing properties, has been isolated from several cheese varieties, and sensory studies suggest it contributes savoury flavour to Swiss-type cheese and to a full, aged flavour in Cheddar cheese. In Swiss and other cheeses where *Propionibacterium freudenreichii* subsp. *shermanii* attain high numbers, succinate production is attributed to aspartic acid catabolism by the propionibacteria. In Cheddar cheese and other varieties, however, NSLAB produce succinate from citrate via the reductive tricarboxylic acid pathway. The other important citrate-derived flavour component, diacetyl, imparts a “buttery” note whose importance in butter, buttermilk, and some cheese types has been recognized for decades. Diacetyl is formed by oxidative decomposition of α-acetolactate, an intermediate in the pathways for pyruvate metabolism and amino acid biosynthesis. In recent years, detailed knowledge of citrate metabolism and diacetyl production has yielded effective strategies for engineering *L. lactis* strains to enhance diacetyl production (Broadbent and Steele, 2005).

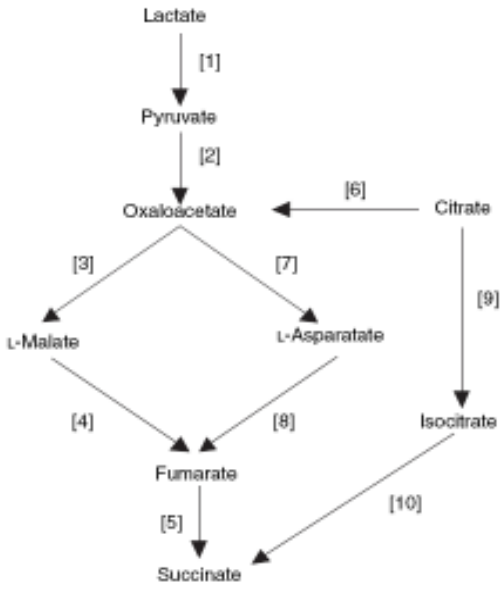

Figure 3. Potential succinate producing pathways of *Lactobacillus* strains. The enzymes designated are: 1- lactate dehydrogenase; 2- pyruvate casrboxylase; 3- malate dehydrogenase; 4- fumarase; 5- fumarate reductase; 6- citrate lyase; 7- aspartate aminotransferase; 8- aspartase; 9- aconitase; 10- isocitrate lyase.

Milk contains about 1,5 g/l of citrate, most of which is lost in the whey during cheesemaking, since ca. 94% of the citrate is in the soluble phase of the milk. Nevertheless, the low concentration of citrate in cheese curd (about 2 g/kg) is of great important since it may be metabolized to a number of volatile flavour compounds by mesophilic starters (citrate-positive, Cit^+, lactococci and *Leuconostoc* sp.). Citrate is not metabolized by *St. thermophilus* nor by thermophilic lactobacilli, but is metabolized by certain mesophilic lactobacilli in the NSLAB microflora (McSweeney and Sousa, 2000).

Cit^+ microorganisms may utilize citrate as an energy source. Conditions required for citrate utilization by *Lactobacillus casei* ATCC334 were recently studied by Dìaz-Muñiz and Steele (2006). Citrate was utilized by this microorganism in a modified chemically defined media as an energy source, solely in the presence of limiting concentrations of galactose. The presence of glucose inhibited citrate utilization by this microorganism even when added in limiting concentration. This results lead to presuppose that *Lb. casei* uses citrate as an energy source in ripening cheese only when the residual levels of

carbohydrate post-fermentation are limiting (<2.5 mM), and lactose or glucose are absent.

1.2.3 Lipolysis

Free fatty acids (FFA) formed by lipase or esterase activity on milk fat directly affect cheese flavour, and can have further effects by serving as precursors for esters and other flavour compounds. Moreover, esterases and lipases catalyse the hydrolysis or synthesis of esters, depending on cheese water activity and levels of other available fatty acids and alcohols. Enzymes involved in these reactions may come from rennet, from milk itself, and from SLAB or NSLAB. It is well established, for example, that pregastric lipases and esterases from ruminants are responsible for the sharp, fatty acid-based flavours that characterize some Italian cheese. In cheeses such as Parmigiano Reggiano that do not use pregastric lipases and esterases, however, flavour notes associated with lipolysis are probably due to indigenous milk enzymes and microbial enzymes. Most LAB lack lipolytic activity and have very low esterolytic activity, but in cheese with long ripening times these cells can generate enough free fatty acids and esters to impact flavour (Broadbent and Steele, 2005).

The lipase/esterase systems of starter bacteria have received much less attention then their proteolytic systems. *Lactococcus* sp. are only weakly lipolytic, but lactococci may be responsible for the liberation of quite high levels of FFA when present in high cell number or over extended ripening periods. Lipases/esterases of *Lactococcus* strains, which appear to be intracellular, have been studied (Fox et al., 1993; Cjick et al., 1997; Fox and Wallace, 1997). Obligately homofermentative lactobacilli used as starter (*Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbrueckii* subsp. *lactis*) also produce esterases, some of which have been studied (Khalid and Marth, 1990). Facultatively heterofermentative lactobacilli (e.g., *Lb. casei*, *Lactobacillus paracasei* and *Lactobacillus plantarum*) which dominate the NSLAB flora of many cheese varieties, are weakly lipolytic. *Micrococcus* and *Pediococcus* are also weakly lipolytic (Bhownik and Marth, 1990).

Fourteen different esters have been found in Emmental cheese (Rychlik et al., 1997), and esters have also been claimed to be important contributors to the flavour of PR cheese (Meinhart and Schreier, 1986). The most abundant of the 38 esters identified in PR cheese are ethyl

butanoate, ethyl hexanoate, ethyl acetate and methyl hexanoate (McSweeney et al., 1997).

LAB, in general, contribute relatively little to lipolysis, but additional cultures, e.g., moulds in the case of surface-ripened cheeses often have high activities in fat conversion (Molimard and Spinnler, 1996).

1.2.4 Proteolysis

Proteolysis and its secondary reactions also play a major role in bacterially ripened cheeses, making casein hydrolysis and its relationship to flavour development an area of intense research interest from decades. The hydrolysis of intact casein is almost exclusively catalysed by the coagulant (e.g., chymosin) and endogenous milk proteinases (e.g., plasmin), while LAB proteinases and peptidases are responsible for producing water-soluble peptides and free amino acids. Together, primary and secondary proteolysis of casein influences cheese flavour in at least three significant ways. First, casein network breakdown softens cheese texture, which facilitates the release of flavour compounds when the cheese is consumed. Second, some of the low-molecular-weight peptides produced in these reactions directly affect flavour, but this consequences is generally negative since these peptides impart bitterness. Third, the free amino acids that are liberated can also directly affect flavour. For instance, glutamate and aspartate residues enhance flavour. More commonly, released amino acids are precursors for a broad range of potent aroma compounds. These reactions are of particular interest because a growing body of evidence indicates that LAB's converting of free amino acids into aroma compounds is the rate-limiting step in the development of mature cheese aromas. The products of amino acid catabolism, which may arise via decarboxylation, deamination transamination, desulfuration, or side chain removal, can impart desirable or undesirable flavour attributes. Much of research on amino acid catabolism by LAB has been directed toward the fates of aromatic, sulphur-containing, and branched-chain of amino acids because of their key role in aroma. For example, converting methionine into volatile sulphur compounds such as methanethiol, hydrogen sulfide, dimethyl sulfide, and dimethyl trisulfide is thought to contribute desirable "sulphur" flavours to many cheese types, whereas breaking down leucine is the likely source of a desiderable nutty flavour note in Cheddar cheese. In contrast, breaking down aromatic amino acids contributes several undesirable "off-flavours" to cheese, including derivatives such as

indole, skatole, [para]-cresol, and phenyl acetaldehyde (Broadbent and Steele, 2005).

Amino acids are primarily needed for protein synthesis, but for example, *L. lactis* is not able to produce all amino acids from the central metabolism (Reiter and Oram, 1962) (Fig. 4). Certain genes coding for enzymes involved in the amino acid biosynthesis seem to be disrupted (Deguchi and Morishita, 1992; Van Kranenburg et al., 2002). To obtain all essential amino acids, *L. lactis* is able to take up small peptides and to a lesser extend amino acids from the environment. This uptake can be preceded by extracellular degradation of proteins (proteolysis and peptidolysis (Konings et al., 1989; Smid, 1991) (Fig. 4 (A)–(D)).

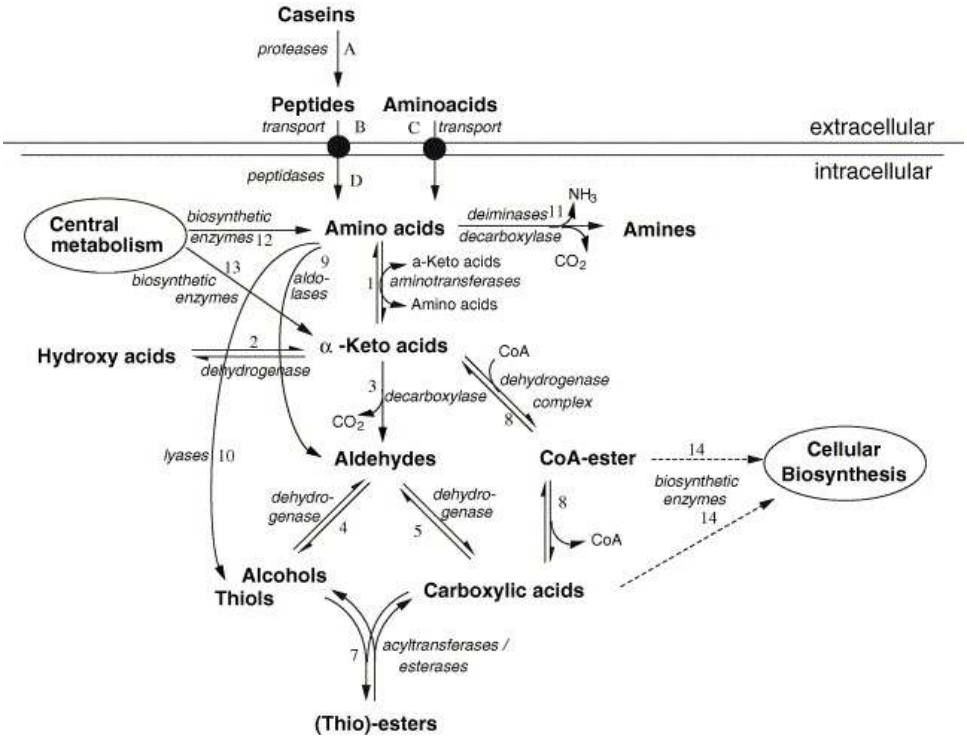

Figure 4. Overview of general protein conversion pathways relevant for flavour formation in dairy fermentations.

Peptide uptake occurs via oligopeptide transport systems (Opp system), and di-/tri-peptide transporters. In addition, various amino acid transport systems have been identified with a high specificity for structurally similar amino acids (Peltoniemi et al., 2002; Charbonnel et al., 2003). The Opp proteins belong to a superfamily of highly conserved

ATP-binding cassette transporters that mediate the uptake of casein-derived peptides (Higgins, 1992). The Opp system of *L. lactis* transports peptides up to at least 18 residues an the nature of these peptides significantly affects the transport kinetics involved (Detmers et al., 1997; Juillard et al., 1998). Described Opp systems for other LAB demonstrate to be similar to that described for *Lactococcus* (Garault et al., 2002; Peltoniemi et al., 2002). Other peptide transporters identified in *L. lactis* MG1363 and IL1403 strains include a proton motive force (PMF)-driven dipeptide/tripeptide DtpT and ATP-driven Dpp system (Hagting et al., 1994; Foucaud et al., 1995).

After the casein-derived peptides are taken up by the cells, they are degraded by a concerted action of peptidases with differing and partly overlapping specificities (Kunji et al., 1996). The intracellular endopeptidases, general aminopeptidases (PepN and PepC), and the X-prolyl dipeptidyl aminopeptidase (PepX) are the first enzymes to act on oligopeptides. Several endopeptidases were characterized from LAB and assigned a variety of names. All are metallopeptidases with the exception of the *Lb. helveticus* PepE, which was shown to exhibit a thiol-dependent activity (Fenster et al., 1997). A common feature of endopeptidases is their inability to hydrolyze intact casein but has the ability to hydrolyze internal peptide bonds of casein-derived peptides. For example, the α_{S1}-casein f1–23 and/or β-casein f193–209 are the most preferred substrates for the endopeptidases of starter LAB origin (Caira et al., 2003). A unique cleavage specificity on α_{S1}-casein f1–23 and on post-proline residues of β-casein f203–209 was recently demonstrated for a PepO from a nonstarter strain, *Lactobacillus rhamnosus* HN001 and for PepO2 from the starter strain *Lb. helveticus* CNRZ32 (Christensson et al., 2002; Chen et al., 2003). Besides cleaving oligopeptides from 7- to 17-residue-long, PepF is also important for protein turnover under conditions of nitrogen starvation in *L. lactis* (Monnet et al., 1994; Nardi et al., 1997).

Other peptidases capable of acting on oligopeptides are the broad-specificity metallopeptidase PepN and cysteine peptidase PepC proteins that were characterized from diverse LAB strains. Collectively, these enzymes can remove the N-terminal amino acids from a peptide, the specificity depending on the peptide length and the nature of the N-terminal amino acid residue (Kunji et al., 1996; Christensen et al.,1999). Di/tripeptides generated by endopeptidases, general aminopeptidases, and PepX are next subjected to additional cleavage by the tripeptidase, PepT, and dipeptidases, PepV and PepD. These enzymes prefer peptides containing hydrophobic amino acids including leucine, methionine, phenylalanine, or glycine. An enzyme possessing specificity toward

di/tripeptides with N-terminal leucine residues and dipeptides containing proline was biochemically characterized from *Lb*. *delbrueckii* subsp. *bulgaricus* (Klein et al., 1995). Other peptidases with more specific substrate specificities include: PepA, which liberates N-terminal acidic residues (glutammic acid and aspartic acid) from peptides that are three- to nine-residue-long; PepP, which prefers tripeptides carrying proline in the middle position; PepR and PepI, which act on dipeptides containing proline in the penultimate position; PepQ, which cleaves dipeptides carrying proline in the second position; and PepS, which shows preference for peptides containing two to five residues with Arg or aromatic amino acid residues in the N-terminal position (Kunji et al., 1996; Christensen et al.,1999; Fernandez-Espla and Rul,1999) (Table 1).

Peptidases	Type[#]	Substrate specificity[*]
Endopeptidases		$NH_2–X_n↓X_n–COOH$
PepO	M	
PepO2	M	
PepO3	M	
PepF	M	
PepF1	M	
PepF2	M	
PepE	C	
PepE2	C	
PepG	C	
Aminopeptidases		
PepN	M	$NH_2–X↓X_n–COOH$
PepC	C	$NH_2–X↓X_n–COOH$
PepS	M	
PepA	M	$NH_2–Glu/Asp↓X_n–COOH$
PepL	S	NH_2-Leu↓X_n–COOH
Tripeptidases		$NH_2–X↓X–X–COOH$
PepT	M	
Dipetidases		$NH_2–X↓X–COOH$
PepD	C	
PepV	M	
Proline-specific		
PepQ	M	$NH_2–X↓Pro–COOH$
PepI	S	$NH_2–Pro↓X_n–COOH$
PepR	S	$NH_2–Pro↓X–COOH$
PepX	S	$NH_2–X–Pro↓X_n–COOH$
PepP	M	$NH_2–X↓Pro–X_n–COOH$

Table 1. Peptidases from LAB. # Catalytic class of peptidases according to sequence analysis or biochemical characterization. * The arrow indicates the cleavage site. M: Metallopeptidase; C: Cysteine-peptidase; S: Serine peptidase

Intracellularly, most amino acids can be converted at first by aminotransferases (Fig. 4 (1)) to their corresponding α-keto acids. Other types of deaminating enzymes have not been found in LAB. α-Keto acids are central intermediates, and can be converted to hydroxy acids (Fig. 4 (2)), aldehydes (Fig. 4 (3)) and CoA-esters (Fig. 4 (8)). These reactions are mostly enzymatic, but some chemical conversion steps have also been described, like the formation of benzaldehyde from phenylpyruvic acid (Groot and De Bont, 1998; Smit, 2004). The aldehydes formed can

generally be dehydrogenated (Fig. 4 (4)), or hydrogenated (Fig. 4 (5)) to their corresponding alcohols or organic acids, which are in their turn substrates for esterases and acyltransferases (Fig. 4 (7)), leading to (thio)esters. One of the biological roles of these amino acid degrading pathways is the generation of precursors, which are needed for example in the sterol and branched-chain fatty acids synthesis (Oku and Kaneda, 1988). On the other hand, the hydrogenation of the α-keto acids may act as sink for excessive redox potential (NADH). The conversion of amino acids to alcohols via α-keto acids was first identified for the formation of so-called fusel alcohols (short-[branched-]chain alcohols) in yeast, where it is called the Ehrlich's pathway (Ehrlich, 1907).

Another important conversion route of amino acids is initiated by lyases (Fig. 4 (10)), like cystathionine β-lyase (EC 4.4.1.8), which is able to convert methionine to methanethiol (Alting et al., 1995; Dias and Weimer, 1998; McSweeney and Sousa, 2000). Threonine aldolase (EC 4.1.2.5) (Fig. 4 (9)) belongs to another class of lyases, and is able to convert threonine directly to acetaldehyde (Raya et al., 1986; Hugenholtz et al., 2000; Ott et al., 2000).

Another conversion pathway for amino acids is the deimination/decarboxylation to amines. These reactions have been studied, in regard to the health risk of biogenic amines (Leuschner et al., 1998). The direct decarboxylation of amino acids explains the presence of most of the amines found, but not the formation of secondary and tertiary amines (Adda et al., 1982).

The most potent flavour compounds in figure 4 are the aldehydes, alcohols, carboxylic acids and esters. Especially important are the aldehydes, alcohols, carboxylic acids and esters derived from the amino acids methionine, phenylalanine, threonine and the branched-chain amino acids. The importance of these amino acids for the cheese flavour is a combination of their abundance, their conversion rates, and the odour threshold of the compounds derived from them.

1.2.5 Application of strains with selected metabolism for improving dairy products

LAB strains differ in amino acid converting abilities and that these activities are in fact linked to the ability to synthesise amino acids. Sometimes, strains that have highest activity are strains isolated from natural sources and non-dairy environments, the so-called 'wild lactococci' (Smit et al., 2004). Ayad et al. (2000) focused on the ability of these strains and they were found to have unique and diverse

properties, when compared to commercially available starter strains. For instance, many of these strains do not degrade caseins, produce antimicrobial compounds and/or have low acidifying activity. However, when the dependency of these strains for amino acids in the growth medium was determined using the single omission technique (Cocaign-Bousquet et al., 1995), it was found that these strains had a much larger potential to synthesise their own amino acids as compared to industrial strains. Lactococci used in dairy fermentations are known for their limited capacity for biosynthesis of amino acids, which explains their complex nutritional requirements. Most of these strains require at least glutamate, valine, methionine, histidine, serine, leucine and isoleucine for growth, and the number of essential amino acids is strain-dependent (Chopin, 1993; Ayad et al., 1999). Industrial *Lactococcus lactis* subsp. *cremoris* strains require even more different amino acids for growth (Ayad et al., 1999). Wild *L. lactis* subsp. *cremoris* strains generally require two to three amino acids while some *Lactococcus lactis* subsp. *lactis* strains only need one or two amino acids. The absence of some amino acid biosynthetic pathways in dairy lactococci might be a consequence of their adaptation to dairy products, since in milk, the amino acids are readily available from the proteolytic degradation of caseins. Wild strains are not naturally associated with a rich environment such as milk, which makes them more dependent on their own biosynthesis of amino acids compared to industrial strains (Smit et al., 2005). Interestingly, lactococci isolated from natural niches were not only found to have a larger potential in amino acid production, but concomitantly, also found to be able to produce rather unusual flavour components and/or flavour profiles (Ayad et al., 1999). This natural biodiversity could offer new possibilities when explored and applied in industrial field.

Since the impact of NSLAB on cheese flavour will ultimately be determined by the metabolic activities of strains and species that dominate during ripening, the complex and dynamics nature of wild NSLAB communities is a major source of cheese flavour inconsistencies and random defects (Crow et al., 2001). As a result, an industrial effort to consistently produce uniform, high quality cheese requires technology to control the composition and growth of NSLAB communities throughout ripening. One strategy to control NSLAB composition and diversity is to use well-characterized adjunct cultures that can grow in ripening cheese, suppress the emergence of wild NSLAB and have no deleterious impact on flavour. The general principle of this strategy is supported by some academic trial that showed adjunct use may help to reduce the

susceptibility of cheese to culture-related quality defects (Frohlich-Wyder et al., 2002). The real key to this strategy, however, rests on whether or not adjuncts can effectively suppress the development of these wild NSLAB populations (Cogan et al., 2006).

1.3 IDENTIFICATION AND ANALYSIS OF LACTIC ACID BACTERIA

The microbial ecology of long ripened fermented foods appears complex and still not completely understood because the traditional agar-based and culture dependent methods have many limitations (Gatti et al., 2006). Investigations on microbial population, through traditional microbial methods, typically reveal the most prevalent organisms that can grow to a detectable level by forming colonies on selective media but not the minor components of microflora, that are equally important for cheese ripening and flavour development (Steele et al., 2006). A potential problem in counting NSLAB is that the low numbers in the first phase of, for example, cheese-making are occluded in a solid matrix to form microcolonies. Other possible problem is the inability to distinguish between the adventitious "wild" NSLAB and those LAB deliberately added (SLAB) (Cogan et al., 2007).

Traditional commercial cultural media, prepared with the aim to recover the majority of microorganisms, could be too generic and not always enough selective to differentiate species or biotypes present at different concentrations. LAB strains and biotypes which play an important role during ripening processes could be present in little amount in raw milk or in natural starter. For these reasons, commercial rich agar media may underestimate these minority microbial population however present in a food matrix. This information is really important for studying the microbial evolution in a fermented food with the aim to understand who are the bacteria really involved in the process (Gatti M., submitted for publication).

Identification of microorganisms, conducted in typical microbiological laboratories, is almost exclusively based on classical or traditional phenotypic tests. The results of these tests form the basis for formalized description of taxa, from family and genus to species and subspecies. The classical phenotypic characteristics of bacteria include morphological, physiological and biochemical characters (Goodfellow et al., 1985). The morphology of bacteria include both cell morphology (cell shape, the presence of endospores, flagella, organelles, inclusions, Gram staining) and colony morphology (color, size, shape, and texture). Description of physiological and biochemical characters imply information on the following properties: growth at different temperatures, pH values, salt concentrations, in different atmospheric and other conditions; growth in the presence of various substances, including antimicrobial ones; the presence and activity of diverse enzymes; and

utilization of organic compounds (Bergey's Manual of Systematic Bacteriology, 1984). Classical phenotypic analyses of LAB can be successfully replaced by commercial test systems, consisting of large sets of dehydrated reagents in sterile kits. An addition of inoculate initiates a response (growth, enzyme activity, etc.). The results are interpreted both visually and spectrophotometrically.

More precise identification of the microorganisms could be achieved in comprehensive studies, including analysis of their genomic characteristics. The situation has drastically changed after the advent of methods of molecular biology, based on investigation of the prokaryotic genome, in microbiology. The modern trends in systematics of microorganisms, including LAB, are directed at simplifying and formalizing taxonomical studies on the basis of using quantitative characters in analysis of genome similarity (Botina et al., 2006).

Lactic acid bacteria, isolated from different sources, including high-quality food products, whose microflora is naturally developed, generally belong to the following genera: *Lactobacillus, Lactococcus, Micrococcus, Pediococcus, Staphylococcus, Streptococcus,* and *Enterococcus*. Many authors have noted that identifying various genera of catalase-negative facultative anaerobic bacteria is problematic. Identifying species within the genera *Enterococcus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus*, and *Lactobacillus* by classical phenotypic methods presents particular difficulties for microbiologists (Facklam et al., 1989).

Bacteria of the species *St. thermophilus* and *Enterococcus durans, Enterococcus faecium, Enterococcus faecalis* are hard to distinguish using phenotypic methods because of their similar phenotypes. Strains with characteristics similar to enterococci and streptococci, which make their identification problematic, are also rather common. These strains, poorly identified and having a number of morphological and biochemical traits, similar to those of enterococci (Stepanenko, 1999).

The identification of lactic acid lactobacilli using only standard microbiological tests may also involve difficulties. For instance, identifying closely related *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *delbrueckii, Lb. delbrueckii* subsp. *lactis, Lb. rhamnosus, Lb. plantarum, Lb. casei*, and *Lb. paracasei* on the basis of their physiological and biochemical properties is problematic, because these bacteria share many characteristics. For their precise species attribution, modern methods based on polymerase chain reaction and gene sequencing are required (Andrighetto et al., 1998; Giraffa et al., 2004; Dellaglio et al., 2005).

The modern tools that have been developed for identifying microbes and analyzing their activity can be divided into those based on nucleic acids and other macromolecules and approaches directed at analyzing the activity of complete cells. The nucleic acid–based tools are more frequently used because of the high throughput potential provided by using PCR amplification or ex situ or in situ hybridization with DNA, RNA, or even peptide nucleic acid probes. Notably, these include 16S rDNA sequences that can be used to place diagnostics into a phylogenetic framework and can be linked to databases providing up to 160,000 sequences (Botina et al., 2006). These 16S rDNA–based methodologies are robust and superior to traditional methods based on phenotypic approaches, which are often unreliable and lack the resolving power to analyze the microbial composition and activity of bacterial populations. A panoply of approaches that are based on DNA sequences other than rDNAs have been applied frequently to different groups of bacteria (Amor et al., 2007).

In addition to analysis of individual macromolecules or their collective set in a LAB strain, whole cells can also be targeted. This offers the possibility of analyzing the physiological properties of intact cells in situ using fluorescently labeled probes or substrates in combination with high-throughput approaches such as flow cytometry. These systems are notably useful to provide information on the viability and stresses in lactic cultures (Amor et al., 2007).

1.3.1 16S ribosomal RNA probing

Over the last decade, hybridizations with ribosomal RNA (rRNA)-targeted probes have provided a unique insight into the structure and spatiotemporal dynamics of complex microbial communities (Amann et al., 2001). Nucleic acid probes can be designed to specifically target taxonomic groups at different levels of specificity (from species to domain) by virtue of variable evolutionary conservation of the rRNA molecules. Appropriate software environments such as the ARB package, a software environment for sequence data (http://www.arb-home.de/) and availability of large databases (http://rdp.cme.msu.edu/html/), or the online resource for oligonucleotide probes probeBase (http://www.microbial-ecology.de/probebase/index.html) offer powerful platforms for a rapid probe design and in silico specificity profiling. Oligonucleotide probes that are complementary to regions of 16S or 23S rRNA have been successfully used for the identification of LAB, and

hence, they offer the potential to be used as reliable and rapid diagnostic tools (Amor et al., 2007).

Nucleic acid probing is based on 2 major techniques: dot-blot hybridization and whole-cell in situ hybridization. Dot-blot hybridization is an ex situ technique in which total RNA is extracted from the sample and is immobilized on a membrane together with a series of RNAs of reference strains. Subsequently, the membrane is hybridized with a radioactively labeled probe, and after stringent washing, the amount of target rRNA is quantified. Because cellular rRNA content is dependent on the physiological activity of the cells, no direct measure of the cell counts can be obtained. In contrast to dot-blot hybridization, fluorescent in situ hybridization (FISH) is applied to morphologically intact cells and thus provides a quantitative measure of the target organism. The listed probes can all be used for dot-blot hybridizations, but for application in FISH, specific validation is required because some regions of the rRNA are not accessible because of their secondary structure and protection in the ribosome. Hence, the number of validated FISH probes is much smaller than that of the probes suitable for ex situ analysis (Amor et al., 2007).

1.3.2 Genotypic typing

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 most powerful of these are genetic-based molecular methods known as DNA fingerprinting techniques, e.g., pulsed-field gel electrophoresis (PFGE) of rare-cutting restriction fragments, ribotyping, randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP), which have been applied extensively for the intraspecific identification and genotyping of LAB and bifidobacteria isolated from fermented food products as well as from the human gastrointestinal tract (McCartney, 2002). Basically, these methods rely on the detection of DNA polymorphisms between species or strains and differ in their dynamic range of taxonomic discriminatory power, reproducibility, ease of interpretation, and standardization. Genetic fingerprinting techniques that are currently being used for typing dairy LAB are described below.

1.3.2.1 Pulse field gel electrophoresis

Restriction fragment length polymorphism (RFLP) analysis of bacterial DNA involves the digestion of genomic DNA with rare-cutting restriction enzymes to yield a few relatively large fragments. The restriction fragments are then size-fractionated using PFGE that allows separation of large genomic fragments. The generated DNA fingerprint obtained depends on the specificity of the restriction enzyme used and the sequence of the bacterial genome and is therefore characteristic of a particular species or strain of bacteria. 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 reported for the differentiation between strains of important bacteria, *Lb. casei* and *Lb. rhamnosus* (Tynkkynen et al., 1999), *Lb. helveticus* (Lortal et al., 1997), and others. More recently, a new approach combining RFLP with DNA fragment sizing by flow cytometry has been reported for bacterial strain identification (Larson et al., 2000). DNA fragment sizing by flow cytometry was found to be faster and more sensitive than PFGE, and this technique is also amenable to automation (Amor et al., 2007).

1.3.2.2 Ribotyping

Ribotyping is a variation of the conventional RFLP analysis. It combines Southern hybridization of the DNA fingerprints, generated from the electrophoretic analysis of genomic DNA digests, with rDNA-targeted probing. The probes used in ribotyping vary from partial sequences of the rDNA genes or the intergenic spacer regions to the whole rDNA operon (O'Sullivan, 1999). Ribotyping has been used to characterize strains of *Lactobacillus* from commercial products (Giraffa et al., 2000). However, ribotyping provides high discriminatory power at the species and subspecies level rather than on the strain level. PFGE was shown to be more discriminatory in typing closely related *Lb. casei* and *Lb. rhamnosus* as well as *Lactobacillus johnsonii* strains than either ribotyping or RAPD analysis (Tynkkynen et al., 1999; Ventura et al., 2002).

1.3.2.3 Randomly amplified polymorphic DNA

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 and bifidobacteria. This PCR-based technique makes use of arbitrary primers that are able to bind under low stringency to a number of partially or perfectly complementary sequences of unknown location in the genome of an organism. If binding sites occur in a spacing and orientation that allow amplification of DNA fragments, fingerprint patterns are generated that are specific to each strain (O'Sullivan, 1999). RAPD profiling has been applied to distinguish between strains of the *Lactobacillus acidophilus* group and related strains (Gancheva et al., 1999; Torriani et al., 1999; Tynkkynen et al., 1999). Several factors have been reported to influence the reproducibility and discriminatory power of the RAPD fingerprints, i.e., annealing temperature, DNA template purity and concentration, and primer combinations (Amor et al., 2007).

1.3.2.4 Amplified restriction 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 ligated 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).

1.3.3 Other PCR approaches

PCR-based approaches other than RAPD and AFLP have been used for molecular typing, such as amplified ribosomal DNA restriction analysis (ARDRA) (Delley and Germond, 2002), repetitive extragenic palindromic PCR (Rep-PCR) (Ventura et al., 2003), and triplicate arbitrary primed PCR (TAP-PCR) (Cusick and O'Sullivan, 2000), and have been shown to offer a high discriminatory power for the identification and differentiation of LAB.

Although these genotypic fingerprinting methods have been successfully applied to the identification and taxonomic classification of a number LAB and bifidobacteria, the outcome can be highly variable between laboratories. Furthermore, a basic limitation in genotypic typing procedures is that the organism to be typed must be isolated because DNA from other sources disturbs the DNA fingerprints. Considering the

cost/time-effectiveness and ambiguities that are still inherent to some of these techniques, 16S rRNA sequence–based methods (PCR amplification or nucleic acid probing) offer a viable option for the rapid and reliable identification of LAB and probiotic strains in mixed populations. Yet, DNA fingerprinting is a very powerful tool for the intraspecific classification of LAB and bifidobacteria provided that its methods are used in combination with other approaches. Subsequently, these methods may offer a useful means for the quality assurance of LAB starters and probiotic strains used in food products by monitoring their genetic stability and integrity over time.

1.3.4 Denaturing gradient gel electrophoresis

The separation of PCR-amplified segments of 16S rRNA genes different in sequence by denaturing gradient gel electrophoresis (DGGE) offers a unique and comprehensive tool for the characterization of bacterial communities. With DGGE, double-stranded DNA is denatured in a linearly increasing denaturing gradient of urea and formamide at elevated temperatures. As a result a mixture of amplified PCR products will form a banding pattern after staining that reflects the different melting behaviour of the various sequences. The DGGE-generated patterns make it possible to monitor shifts in the structure of microbial communities over time and/or following different treatments. Subsequent identification of specific bacterial groups or species present in the sample can be achieved either by cloning and sequencing of the excised bands or by hybridization of the profile using phylogenetic probes (Muyzer et al., 1993).

PCR-DGGE was successfully used to monitor the development of the microbial community and specifically the LAB population during the production and ripening of artisanal Sicilian cheese from milk to the ripened cheese (Randazzo et al., 2002). Thus, PCR-DGGE can offer an alternative tool for rapid detection and identification of LAB in food products.

1.3.5 Length heterogeneity-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 develop culture-independent methods offer a further technique to monitor LAB populations during the ensiling process (Brusetti et al.,

2006). Lazzi et al., (2004) and Santarelli et al., (2008) demonstrate that length heterogeneity (LH)-PCR (Suzuki et al., 1998; Ritchie et al., 2000) is helpful for characterizing the LAB of milk whey starters for Grana Padano cheese. This method allowed the detection of both dominant and nondominant bacterial species without cultivation and it was demonstrate to be easy, fast, reliable and highly reproducible.

1.3.6 Analyzing the viability of lactic acid bacteria

It is crucial that the viability of the LAB strain and stability of the desirable characteristics be maintained during processing, storage, and delivery of the final product (Tuomola et al., 2001). The plate count approach is often employed as the gold standard method to measure bacterial viability, it actually only indicates how many of the cells can replicate under the conditions provided for growth. The ability to reproduce might be repressed or blocked in a certain cell type, or reproduction might be limited to a certain set of conditions. Furthermore, cell populations that have been exposed to stress (e.g., oxidative, heat, freezing, osmotic stress, or starvation) can enter an unculturable state in which they still can maintain activity. Alternatively, fluorescent techniques in combination with flow cytometry (FCM) offer a powerful tool to analyze a cell population at the single-cell level because they can be used to measure different physical and biochemical parameters simultaneously and hence offer substantial information on the dynamics and physiological heterogeneity of a bacterial population (Davey and Kell, 1996). FCM has been used to detect and enumerate a number of lactobacilli and other LAB in milk and commercial probiotic products after chemical or enzymatic clearing of milk and staining of bacteria with fluorescent probes (Holm and Jespersen, 2003). The FCM assay was rapid (<1 h) and very sensitive ($<10^4$ bacteria/mL milk). Undoubtedly, FCM technique will provide a novel tool for the assessment of viability and stability of various lactic acid bacteria and probiotic-containing products (Amor et al., 2007).

1.4 ADVANCES IN THE STUDY OF LACTIC ACID BACTERIA

Though great progress has been made toward understanding LAB physiology and the processes that drive cheese flavour development, much remains to be learned about these reactions. Currently, significant research advances depend on recombinant DNA technology. The complexity of the peptidase enzyme system in LAB, for example, confounded earlier efforts to establish the role of individual enzymes in casein hydrolysis and cheese ripening. However, tools for constructing isogenic strains that differ in the activity of only single peptidases now are providing researchers with an effective approach to determine how individual enzymes contribute to cell growth and cheese properties (Broadbent and Steele, 2005).

By combining molecular tools with genomics, researchers in industry and academia are creating even greater opportunities to investigate the means by which LAB act within and respond to cheese and milk microenvironments.

Functional genomics is being used to investigate pathways for amino acid biosynthesis and catabolism in Lactobacillus helveticus CNRZ32. In cheese, converting amino acids into volatile cheese flavour compounds may occur directly or through interactions among starter, adjunct, and NSLAB components. Some strains can independently convert amino acids into aroma compounds, while others may produce or degrade only one or more metabolic intermediates (Broadbent and Steele, 2005).

The primary sequences of most enzymes involved in the conversion of amino acids reactions are relatively well-conserved, access to genome sequence information should dramatically enhance our ability to predict and test how individual organisms contribute to amino acid catabolism in cheese.

There is still a great deal to be learned in LAB metabolism. The combined strengths of genomics and molecular biology tools are certain to play a leading role in research to define the molecular dynamics of LAB in producing fine cheeses.

1.4.1 Genomic studies on lactic acid bacteria

Genomes of LAB contain both plasmid and chromosomal DNA (Broadbent, 2001). The characterization of plasmids in LAB has been an ongoing area of study for the past 30 years (Broadbent, 2001). Characterization of LAB chromosomes began in the early 1970s;

however, the most exciting developments in LAB genomics are now being fueled by nucleotide sequence information for complete genomes. Currently, the genome sequence is known or is being determined for more than 20 LAB (Table 2). The value of genome sequence information for dairy-related LAB cannot be overstated.

Because of their economic relevance, many of these sequences are being mined for intellectual property and are not yet available to the general scientific community. Nonetheless, nucleotide sequence data is publicly available for more than half of the sequenced LAB strains (Makarova et al., 2006).

Species	Strain	Genome size (Mb)	Project sponsor[1]	Public access?[1]
Lactobacillus acidophilus	ATCC700396	2.0	Dairy Management, Inc. and Rhodia, Inc. (US)	No
Lb. brevis	ATCC 367	2.0	JGI-LABGC (US)	Yes
Lb. casei	ATCC 334	2.9	JGI-LABGC (US)	Yes
Lb. casei	BL23	2.6	INRA (FRA)	No
Lb. delbrueckii ssp. *bulgaricus*	ATCCBAA-365	2.3	JGI-LABGC (US)	Yes
Lb. delbrueckii ssp. *bulgaricus*	ATCC11842	2.3	INRA and Genoscope (FRA)	No
Lb. delbrueckii ssp. *bulgaricus*	DN-100107	2.1	Danone Vitapole (FRA)	No
Lb. gasseri	ATCC 33323	2.0	JGI-LABGC (US)	Yes
Lb. helveticus	CNRZ32	2.4	Dairy Management, Inc. and Chr. Hansen, Inc. (US)	No
Lb. helveticus	DPC 4571	Not reported	Teagasc and University College, Cork (IRL)	No
Lb. johnsonii	NCC533	2.0	Nestlé (CHE)	Yes
Lb. plantarum	WCFS1	3.3	Wageningen Center for Food Sciences (NLD)	Yes
Lb. rhamnosus	HN001	2.4	Fonterra Research Center, NZ	No
Lactococcus lactis ssp. *cremoris*	SK11	2.3	JGI-LABGC (US)	Yes
Lc. lactis ssp. *cremoris*	MG1363	2.6	Univ. Groningen (NLD); INRA (FRA)	No
Lc. lactis ssp. *lactis*	IL1403	2.3	INRA and Genoscope (FRA)	Yes
Leuconostoc mesenteroides	ATCC 8293	2.0	JGI-LABGC (US)	Yes
Pediococcus pentosaceus	ATCC 25745	2.0	JGI-LABGC (US)	Yes
Streptococcus thermophilus	LMD-9	1.8	JGI-LABGC (US)	Yes
S. thermophilus	LMG18311	1.9	Univ. Catholique de Louvain (BEL)	No
S. thermophilus	CNRZ1066	1.8	INRA (FRA)	No
Other dairy-related bacteria:				
Bifidobacterium longum	NCC2705	2.3	Nestlé (CHE)	Yes
B. longum	DJ010A	2.1	JGI-LABGC (US)	Yes
Bifidobacterium breve	NCIMB8807	2.4	University College, Cork (IRL)	No
Brevibacterium linens	ATCC9174	3.0	JGI-LABGC (US)	Yes
Propionibacterium freundenreichii	ATCC6207	2.6	DSM Food Specialties (NLD)	No

Table 2. Genome sequencing projects for dairy-related lactic acid bacteria and other species.

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 (Fig. 5).

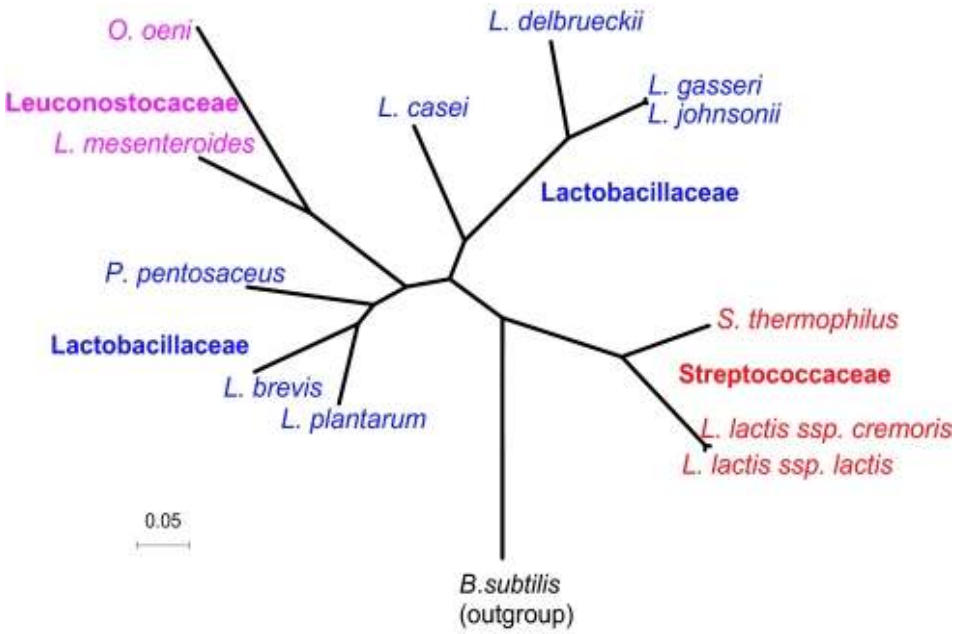

Figure 5. Phylogenetic trees of *Lactobacillales* constructed on the basis of concatenated alignments of ribosomal proteins. All branches are supported at >75% bootstrap values. Species are coloured according to the current taxonomy: *Lactobacillaceae*, blue; *Leuconostocaceae,* magenta; *Streptococcaceae,* red.

The availability 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 most vitamins and minerals. Examination of dairy LAB genomes shows that gene loss or inactivation as well as metabolic simplification is a central component of the evolution of these organisms to milk (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 favoured 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 horizontal gene transfer, 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 horizontal gene transfer. The availability of dairy LAB genomic sequences supports the view that these organisms have evolved from other LAB via gene loss or inactivation, as well as via horizontal gene transfer, 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).

Genomic sequences has also allowed researchers to rapidly discern the metabolic potential of the sequenced strains. For example, because proteolysis plays such a critical part in cheese ripening, the Steele research group and collaborators spent more than a decade examining the proteolytic system of *Lb. helveticus* CNRZ32 (Christensen et al., 1999; Broadbent and Steele, 2007). The outcome of these efforts was the characterization of 12 CNRZ32 genes that encode proteolytic enzymes. Despite these concerted efforts, initial annotation of the CNRZ32 genome sequence revealed a large number of additional genes in CNRZ32 whose products are predicted to contribute to the proteolytic enzyme system of this bacterium.

From this perspective, such data underscore both the power of genome sequence information for applied bacteriology, and the challenges one must face in interpreting and applying that information. Although sequencing efforts expanded the genetic database for the CNRZ32 proteolytic enzyme system by about 5-fold, efforts to confirm and characterize all the new gene assignments will require more time and resources. Nonetheless, functional analysis of the newly discovered endopeptidase genes has already identified enzymes with important roles in the hydrolysis of bitter peptides in cheese (Sridhar et al., 2005).

The availability of multiple genome sequences within a species allows for the study of strain-specific traits. For example, a comparison of the complete genome sequences of 2 strains of *Lb. delbrueckii* subsp. *bulgaricus* identified regions involved in bacteriophage resistance, a trait known to vary from strain to strain. Genome sequences, also allows studies to follow global gene regulation via DNA microarrays. A major strength of this technology is that it provides a nonbiased global view of an organism's transcriptional response to an environment of interest. This unbiased holistic view consistently yields unexpected observations that ultimately lead to the identification of genes with critical functions in the physiological system of interest.

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. However, testing these hypotheses is likely to take several years. Then, of course, perhaps the greatest challenge remains—taking this new knowledge and converting it into new or improved products for the consumer.

1.4.2 Proteomic studies on lactic acid bacteria

The availability of whole genome sequences is paving the way for global analyses of gene expression in LAB in vivo. Recent proteomic approaches on LAB exploit the available sequence data for protein identification by mass spectrometry (e.g., peptide mass mapping by MALDI-TOF, i.e., matrix-assisted laser desorption/ionization time-of-flight mass spectrometry). Recently, the protein expression profile of *L. lactis* growing in milk was published by Gitton et al. (2005). Using two-dimensional electrophoresis, Gitton et al. (2005), quantitatively detected about 900 protein spots and identified more than 330 distinct proteins. While 12 of the identified proteins were peptidases (PepA, PepC, PepDB, PepF, PepM, PepN, PepP, PepQ, PepO, PepO2, PepT, and PepV), only four of them (PepC, PepN, PepO, and PepF) were found to be upregulated in milk. Of the genes in the proteolytic machinery, the Opp system was found to be strongly upregulated. Gagnaire et al. (2004) utilized proteomic tools to characterize the Emmental cheese ripening process. This interesting approach provided information on the peptidases released into the cheese by the starter bacteria *Lb. helveticus* and *St. thermophilus* during ripening. Different peptidases also arose from *Lb. helveticus* (PepN, PepO, PepE, and PepQ) and *St. thermophilus* (PepN, PepX, and PepS). Remarkably, this work suggested the participation of specific peptidases of *Lb. helveticus* and *St. thermophilus* in the degradation of casein-derived peptides during the ripening process of Emmental cheese.

1.5 THE PARMIGIANO REGGIANO CHEESE

Parmigiano Reggiano is a Protected Designation of Origin (PDO) produced in specific areas of Northern Italy. In many respects this cheese production system is a unique dairy system. The processing of 1.7 million tons of milk (represent 15 percent of the Italian milk market) into a high quality product in 450 cheese dairies using predominantly artisan production techniques is not found anywhere in Europe (http://www.parmigiano-reggiano.it). The high labour input required both on dairy farms and in the cheeses dairies creates considerably more employment than any other dairy system. The numbers of this system highlight it relevant importance for the Italian economy. About 20.000 men and women work in this very special system. This artisan system is able to sustain economic development in less favoured areas and has a significantly better environmental impact than industrial dairy farms. The final quality of the cheese is heavily dependant 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) without using any additive excepted for the dairy-based whey starter and rennet (http://www.parmigiano-reggiano.it).

Parmigiano Reggiano is a semi-fat hard cheese obtained from cooked and slowly matured paste. It is made with cow's milk from animals whose feeding mainly consists of forage from the area of origin. The milk used is raw and cannot undergo any thermal treatments. The use of additives is strictly forbidden. The milk from the evening milking and that from the morning milking is delivered to the dairy within two hours from the end of each milking. Milk can be cooled immediately after milking and kept at a temperature not below 18°C. The milking time of each one of the two milkings allowed daily must be limited within 4 hours of time. The evening milk is partly skimmed by removing the cream naturally risen to the surface in open-top stainless steel basins. The morning milk, immediately 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 on 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. Starter whey is then added to the milk. This is a natural starter culture of lactic bacteria, most of them acid-producing,

obtained from the spontaneous acidification of the whey remaining after the previous day's cheese processing.

Thermophilic lactic acid bacteria selected by the process of curd cooking are the dominant microflora of natural whey starter. In particular natural whey starters contain thermophilic lactobacilli higher than 10^8 cfu ml^{-1} and *Lb. helveticus* is usually the dominant species (Gatti et al., 2003).

The milk curdling takes place inside copper vats shaped like truncated cones with the exclusive use of calf rennet. After curdling, the curd is broken up into grains and cooked. 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. After a few days, cheese are salted in a bath of salt 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) In figure 5., time/temperature conditions used in Parmigiano Reggiano standard production can be visualized.

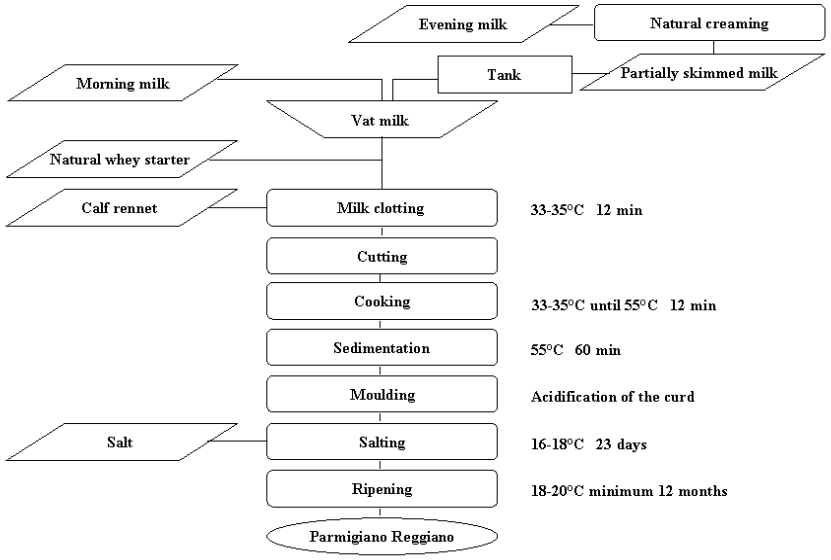

Figure 5. Parmigiano Reggiano cheese production protocol.

Parmigiano Reggiano 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-coloured rind;
- paste colour: 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 production region is the territory of the provinces of Parma, Reggio Emilia, Modena and Mantua on the right bank of the river Po and Bologna on the left bank of the river Reno.

Microbiological characteristics of Parmigiano Reggiano have been studied by Coppola et al., (1997; 2000) which isolated from traditional grown media, a great number of strains coming from a great number of samples representative of the production and of the earlier and advanced stage of ripening. Moreover other studies focused into the biodiversity of different strains of *Lb. helveticus* isolated from natural whey cultures for Parmigiano Reggiano production (Gatti et al., 2003; Gatti et al., 2004). However the microbial ecology of Parmigiano Reggiano appears still not completely understood because all theses studies were carried out using traditional agar-based and culture dependent methods which have many limitations (Gatti et al., 2006).

1.6 REFERENCES

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2. AIM OF THE THESIS

Future development and economic growth of dairy industry largely depends on microbial starter used to produce the great majority of industrial fermented products. Moreover, natural cultures implied in traditional cheeses manufacturing, are responsible for many good recognizable traits which make them appreciable all over the word. Even if, great progress has been made toward understanding lactic acid bacteria physiology, and processes that drive quality fermented food development, much remains to be learned.

For this reason, a Protected Designation of Origin, appreciated and economically important Italian cheese like Parmigiano Reggiano, has been chosen as subject for this study, to try a better understanding of how and when lactic acid bacteria are involved in these processes.

To reach this issue, both traditional and innovative approaches have been used. Troughout this Ph.D. thesis, an accurate and laborious sampling allowed to analyse Parmigiano Reggiano cheese in all stages of manufacturing and through two years of ripening.

Traditional microbial counts have been attended and compared with data obtained by means of innovative counting media and direct enumeration considering even cell viability. Cell autolysis has been also evaluated by direct microbial count and by the quantification of both cell lysis-released DNA and cell lysis-released enzymes. Moreover, length heterogeneity-PCR has been held to monitor microbial species evolution during a complete cheesemaking process. Furthermore, a mathematical model able to assess the effect of NaCl concentration, as well as pH and temperature on aminopeptidases activities has been performed.

2. SCOPO DELLA TESI

La crescita economica e lo sviluppo delle industrie casearie si basano ampiamente sugli starter microbici utilizzati per produrre la gran parte dei prodotti fermentati industriali. D'altro canto, le colture naturali impiegate nella produzione di formaggi tradizionali, sono responsabili di diversi tratti distintivi di qualità, che rendono questi prodotti apprezzabili in tutto il mondo. Nonostante siano stati fatti molti progressi nella comprensione della fisiologia dei batteri lattici e nei processi che indirizzano lo sviluppo di prodotti fermentati di qualità, molto rimane ancora da comprendere.

Per questo motivo, è stato scelto come oggetto di questo studio, un formaggio italiano, a Denominazione di Origine Protetta, apprezzato e di rilevanza economica come il Parmigiano Reggiano, allo scopo di ottenere una miglior comprensione di come e quando i batteri lattici siano coinvolti in questi processi.

A tale fine, sono stati utilizzati sia approcci tradizionali che innovativi. Nel corso di questa tesi di dottorato, un accurato e laborioso campionamento ha permesso di analizzare un formaggio Parmigiano Reggiano in tutti gli stadi della produzione e nei due anni di stagionatura.

Sono state effettuate conte microbiche tradizionali poi confrontate con i dati ottenuti da terreni di conta innovativi e attraverso conte dirette in grado di stimare anche la vitalità cellulare. E' stato anche valutato il grado di autolisi cellulare tramite conta microbica diretta e grazie alla quantificazione sia del DNA che degli enzimi rilasciati in seguito a lisi cellulare. Inoltre, è stata utilizzata la tecnica Length heterogeneity-PCR per monitorare l'evoluzione delle specie microbiche nel corso di un completo processo di caseificazione. Infine, è stato sviluppato un modello matematico in grado di stabilire l'effetto di NaCl, così come di pH e temperatura sull'attività aminopeptidasica.

3. RESULTS

3.1 Cheese Agar Medium: recovery and differentiation of long ripened cheese microflora through a new cheese based cultural medium

3.1.1 ABSTRACT

Aims: To recover and discriminate the long ripened cheese bacterial population during aging through a ripened cheese based medium.
Methods and Results: A twenty four months ripened Parmigiano Reggiano cheese was used to prepare the cheese agar medium (CAM). Sixteen wholes of Parmigiano Reggiano cheese were sampled, all produced at the same time, in the same dairy using the same milk and the same natural whey starter. During cheese manufacturing and ripening different samples were prepared and analysed using CAM in comparison with other traditional media. Randomly selected CFU from the different agar media plates were picked and isolated. Phylogenetic positions of 104 isolated strains at level of species and genus by subunit ribosomal 16S rRNA gene sequencing were studied by comparison with the corresponding sequences of LAB from the NCBI database. CAM distinguished SLAB and NSLAB population during the ripening and was able to recover the minority population originated from milk not always estimable using traditional media.
Conclusions: The use of the new cheese based agar medium (CAM) promoted the study of the microflora mainly linked to the changes in nutritional availability involved in Parmigiano Reggiano ripening.
Significance and Impact of the study: The use of new cheese based agar media may allow the study of long ripened cheese microflora that is hardly recovered on traditional media.

Keywords: Long ripened cheese, Parmigiano Reggiano cheese, cultural media, microbial ecosystem, lactic acid bacteria, ripening

3.1.2 INTRODUCTION

Lactic acid bacteria (LAB) are the dominant microflora in dairy products. Particularly in the case of long ripened cheese, like Parmigiano Reggiano (PR), it is well-know that, different LAB species and biotypes, arising from raw milk and starter, contribute to the biochemical events involved in cheese ripening. The natural whey starter, used during PR cheesemaking, is a natural culture of thermophilic LAB (SLAB) that have been selected during the previous day cheesemaking process by the high temperatures (53-55 °C) used during curd cooking (Neviani et al. 1997). After this process, whey is maintained and recovered overnight at a natural decreasing temperature gradient (Mucchetti and Neviani 2006). Thus, thermophilic LAB, such as *Lactobacillus helveticus* and *Lactobacillus delbrueckiii* subsp. *lactis*, become the dominant bacterial population in this kind of natural whey starter (Gatti et al. 2004).

SLAB of PR cheese belong to *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Streptococcus thermophilus* species (Gatti et al. 2003; Mucchetti and Neviani, 2006). Thermophilic microflora from the starter dominate in the first period of ripening while the mesophilic one, originated from raw milk and environment, increase after autolysis of the thermophilic microflora in a successive phase of the cheese aging (Addeo et al. 1997; Neviani et al. 1997). These adventurous species, non-starter LAB (NSLAB), begin to increase when most of the residual lactose in cheese has already been utilized by SLAB (Dìaz-Muñiz et al. 2006). Potential sources of energy for NSLAB growth in curd and cheese are nucleic acids derived from the autolysis of SLAB (Thomas 1986), amino acids (Kieronczyk et al. 2001), trace of carbohydrates, such as *N*-acetylglucosamine and ribose, liberated from glycoproteins and glycolipids present in milk (Williams and Banks 1997; Fox et al. 1998; Williams et al. 2000), and citrate (Hugenholtz 1993; Fox and Wallace, 1997; Dìaz-Muñiz et al. 2006).

Consequently, the quantitative and qualitative microbial content of SLAB and NSLAB in cheese is the result of microbial growth capacity in milk and curd linked with the suffered technological pressure and bacterial cell autolysis (O'Cuinn et al. 1995; Fox et al. 1996; Addeo et al. 1997).

The microbial ecology of ripened cheeses appears complex and still not completely understood because the traditional agar-based and culture dependent methods have many limitations (Gatti et al. 2006). Investigations on microbial population, through traditional microbial methods, typically reveal the most prevalent organisms that can grow to a

detectable level by forming colonies on selective media but not the minor components of microflora, that are equally important for cheese ripening and flavour development (Steele et al. 2006). A potential problem in counting NSLAB is that the low numbers in the first phase of cheese-making are occluded in a solid matrix to form microcolonies. Other possible problem is the inability to distinguish between the adventitious NSLAB and those LAB deliberately added (Cogan et al. 2007).

On the other hand, traditional commercial cultural media, prepared with the aim to recover the majority of microorganisms, could be too generic and not always enough selective to differentiate species or biotypes present at different concentrations. LAB strains and biotypes which play an important role during ripening processes could be present in little amount in raw milk or in natural starter. For these reasons, commercial rich agar media may underestimate these minority microbial population however present in a food matrix. This information is really important for studying the microbial evolution in a fermented food with the aim to understand who are the bacteria really involved in the process.

Several media have been developed with the aim to distinguish *Lact. acidophilus*, *Bifidobacterium* spp, *Lact*. *casei*, *Lact*. *rhamnosus* and *Propionibacterium* in yogurt and fermented milk (Cogan et al. 2007). 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 SLAB microbial population fully underestimated in MRS and M17 agar.

Technological parameters and food composition are responsible for the selection of the minority part of initial microbiological ecosystem present in vat milk. To better understand which part of this ecosystem has a specific role in cheese making and to select the most interesting LAB strains from the technological point of view, it could be useful to elaborate new agar media that can reproduce the real cheese composition during the fermentation process.

The aim of this work was to develop a ripened cheese based medium eligible to recovery the PR characteristic population. This innovative food base medium, together with the traditional ones as MRS and whey agar (WAM), was used to isolate more than 100 strains from the different count agar plates collected during the production and ripening of PR. Furthermore, we identify the SLAB and NSLAB isolated strains at level of genus and species by 16S rRNA gene sequencing to better understand which are the microorganisms able to growth in these three different media.

3.1.3 MATERIALS AND METHODS

Preparation and composition of Cheese Agar Medium (CAM)

An innovative cultural medium using as base component grated PR cheese was developed. Twenty four months ripened cheese was used to obtain the cheese agar medium (CAM). CAM was prepared by fine-grain grating and dissolving of the cheese in sodium citrate 0.07 mol L^{-1} pH 7.5 in proportion of 120 g L^{-1}. The solution was heated and homogenized for 40 min at 42 °C, centrifuged (8000 rpm for 15 min at 4 °C) and filtered through sterile gauze to retain the surfaced fat layer. Then, agar 1% (wt/vol) was added and the solution was sterilized for 15 min at 121 °C.

Composition	CAM
Proteins*	80.92
Lactose*	0.00
Galactose*	0.00
Lactic acid*	3.82
NaCl*	3.40
Ph	7.50
Sodium citrate*	20.64

Table 1 Cheese agar medium (CAM) composition. * g L^{-1}

Cheese sampling, microbial counts and isolation conditions

With the aim to obtain a representative sampling times during PR ripening period, an accurate cheesemaking was done. Sixteen cheese wholes were produced at the same time, in the same dairy using the same milk and the same natural whey starter. During cheese manufacturing, an aliquot of the total mass of whey starter, raw milk and curd at the vat extraction was sampled. For each of the following samples, curd after 6, 12 and 48 h, salted cheese (1 month) and 2, 3, 4, 6, 8, 10, 12, 16 and 20 months cheeses, the wholes were lengthwise cut in the vertical axis. A portion of each cheese section obtained was grated before the analysis.

Whey starter and milk were 10 fold serial diluted in 0.05 mol L^{-1} sodium phosphate buffer pH 7.0, while curd and cheese samples were first homogenized at 10 g for 3 min in a blender (Stomacher 400, Seward, UK) with sodium phosphate buffer and then serial diluted.

In order to recover the cultivable bacterial population, the following media and time/temperature conditions of incubation were used: MRS agar pH 5.4 (Oxoid Spa, Italy) at 42 °C for 48 h; whey agar medium (WAM) (Gatti et al. 2003) at 42 °C for 48 h and CAM at 42 °C for 72 h.

The media were all incubated under anaerobic conditions. The microbial counts were carried out in duplicate and the standard deviation of mean values was calculated.

Randomly selected CFU from the MRS, WAM and CAM plates were picked and isolated (Table 2), purified in MRS or M17 broth, depending on the cellular morphologic observed after microscopic examination, and anaerobically incubated overnight at 42 °C.

Identification of LAB

Genomic DNA of 104 isolated strains was extracted from overnight cultures by 5% Chelex 100 (Sigma-Aldrich Co., St. Louis, MO, USA) according to Giraffa et al. (2003). DNA quantity and purity were assessed by optical reading at 260 and 280 nm, as described by Sambrook et al. (1998).

Phylogenetic positions of the 104 strains at level of species and genus by subunit ribosomal 16S rRNA gene sequencing were studied. DNA sequencing was performed as described previously (Giraffa et al. 2003) and identification of LAB was deduced through BlastN alignment (www.ncbi.nlm.nih.gov/BLAST).

3.1.4 RESULTS

Preparation and composition of Cheese Agar Medium

In order to recovery minor bacterial populations that could be underestimated by the use of traditional media, an innovative cultural medium containing as base component PR cheese was developed. Different compositions in ripened cheese and dissolving solution concentration were tested during preparation together with variations in time and temperature parameters (data not shown). 120 g of ripened cheese and 0.07 mol L^{-1} pH 7.5 sodium citrate were used for the best formulation.

The chemical composition of this new medium (Table 1) was kindly determined by the Technology Laboratory of Parmigiano Reggiano Cheese Consortium (Reggio-Emilia, Italy).

Microbiological analysis of cheese

LAB cultivability was evaluated during PR manufacturing and ripening. Figure 1 shows the growth trends of cultivable population in MRS, WAM and CAM. The microbiological count of the whey starter used in cheese-making was estimated in $1.0x10^{7}$ CFU mL^{-1} in MRS, $5.0x10^{8}$ CFU mL^{-1} in WAM and 10 CFU mL^{-1} in CAM. The count

concerning caseification milk was: $1.0x10^2$ CFU mL^{-1} in MRS, $4.3x10^2$ CFU mL^{-1} in WAM and $2.2x10^3$ CFU mL^{-1} in CAM.

Microbial counts on the three media were different for the first 48 hours, and became similar only after cheese brining. When three months of ripening were reached, the values of microbial counts on MRS, WAM and CAM resulted overlapped and remained comparable until the end of ripening. After 9-10 months of ripening a significative decrease of the number of cultivable bacteria was observed. Bacterial counts in CAM resulted the lowest until the first month of ripening, corresponding to the end of brining. After this moment a significant increase of the cultivable population, equal to about three logarithmic unities, was observed in this medium (Figure 1).

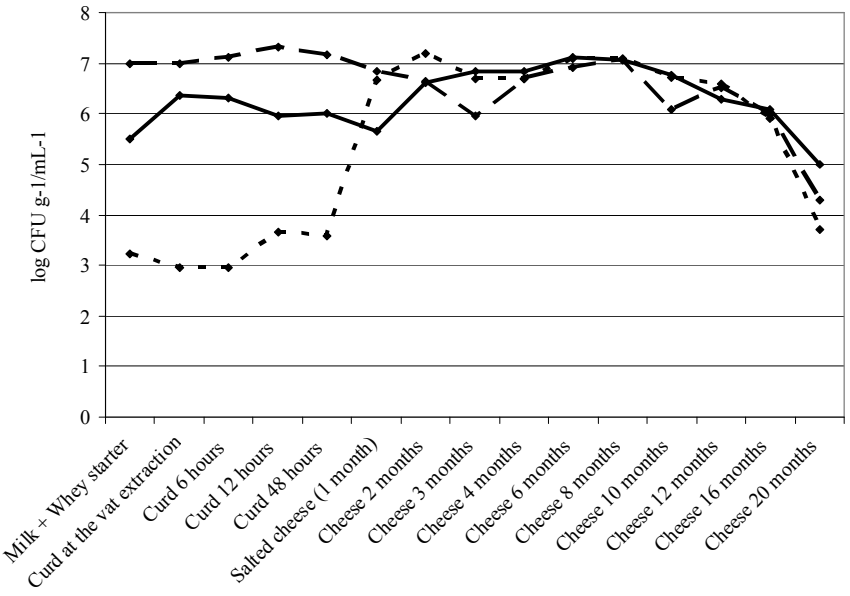

Figure 1 Evolution of cultivable population, expressed as $\log_{10}$ CFU g^{-1} or mL^{-1}, during different steps of Parmigiano Reggiano cheese manufacturing and ripening. Solid line: MRS; Dashed line: WAM; Dotted line: CAM.

Identification of LAB

A minimum of one to a maximum of eight colonies were randomly isolated for each medium and for each temporal sample (Table 2), for a total of 104 strains (42 from MRS, 26 from WAM and 36 from CAM).

After 16S rRNA gene sequencing of the strains, eight different species were identified and classified as SLAB and NSLAB.

Figure 2, 3 and 4 report the bar charts of percentage of total randomly identified LAB from tested media in the different temporal cheese samples. The percentage of each species with respect to the total number of the isolated strains in each sample was calculated.

The highest number of LAB species was recovered from MRS medium. In fact, seven different species, one SLAB (*Lact. helveticus*) and six NSLAB (*Lactobacillus fermentum, Lactobacillus kefiri*, *Lactobacillus reuteri*, *Lactobacillus plantarum, Lactobacillus rhamnosus* and *Pediococcus acidilactici*) resulted cultivable in this medium (Figure 2). Five of these species were found in the first 48 hours of cheese production. In the WAM medium case, five different species, two SLAB (*Lact. helveticus and Lact. delbrueckii* subsp. *lactis*,) and three NSLAB (*Lact*. *fermetum, Lact*. *rhamnosus* and *Ped*. *acidilactici*) were detected. Besides a high percentage of *Lact. rhamnosus* was found after cheese brining (Figure 3). CAM was useful to recover only three species: one SLAB (*Lact. helveticus*) and two NSLAB (*Lact. fermentum* and *Lact. rhamnosus*) (Figure 4). More than 80% of the strains cultivable in this medium belonged to *Lact. rhamnosus* species.

	MRS	WAM	CAM
Whey Starter	3	3	3
Caseification Milk	3	2	0
Curd at the vat extraction	4	2	0
Curd 6 hours	2	1	2
Curd 12 hours	8	2	4
Curd 48 hours	1	3	2
Salted cheese (1 month)	2	1	3
Cheese 2 months	2	1	6
Cheese 3 months	1	1	1
Cheese 4 months	1	1	2
Cheese 6 months	2	1	3
Cheese 8 months	4	1	1
Cheese 10 months	1	3	1
Cheese 12 months	3	1	4
Cheese 16 months	2	2	2
Cheese 20 Months	3	1	2
Total	42	26	36

Table 2 Number of randomly isolated CFU from MRS, Whey agar medium (WAM) and Cheese agar medium (CAM) plates.

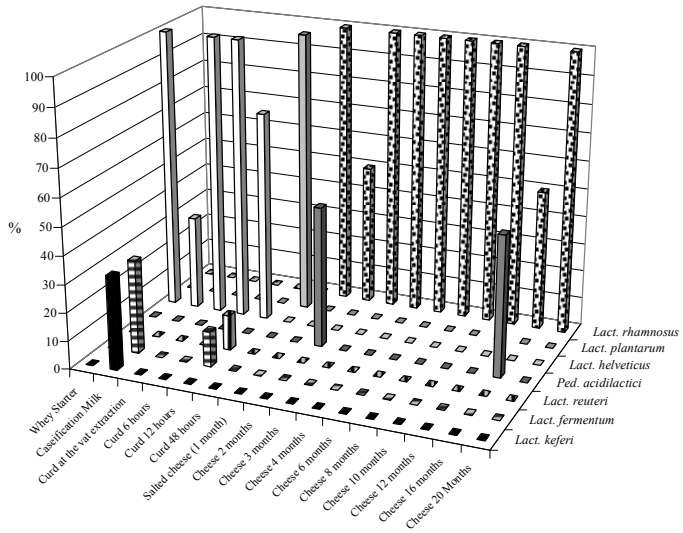

Figure 2 Percentage of each LAB species on the total of strains isolated on MRS medium for samples at different cheese manufacturing and ripening times. Bars – Black: *Lactobacillus kefiri*; Horizontal lines: *Lactobacillus fermentum*; Vertical lines: *Lactobacillus reuteri*; Dark grey: *Pediococcus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

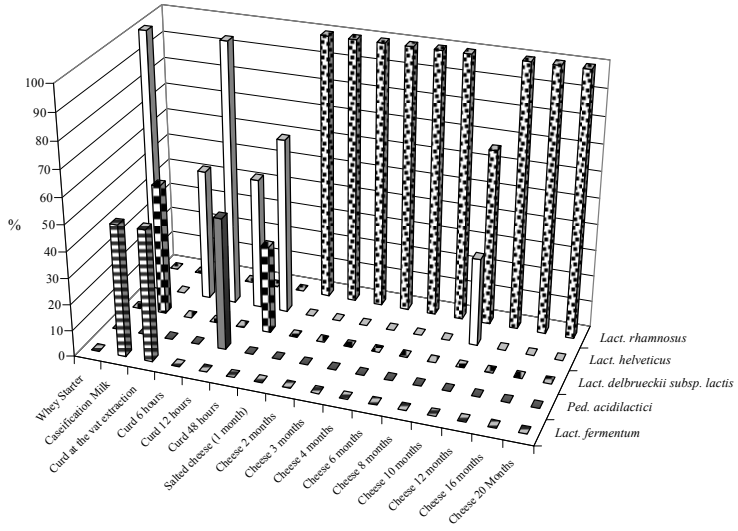

Figure 3 Percentage of each LAB species on the total of strains isolated on WAM medium for samples at different cheese manufacturing and

ripening times. Bars – Horizontal lines: *Lactobacillus fermentum*; Dark grey: *Pediococcus acidilactici*; Squared: *Lactobacillus delbruecki* subsp. Lactis; White: *Lactobacillus helveticus*; Dotted: *Lactobacillus rhamnosus*.

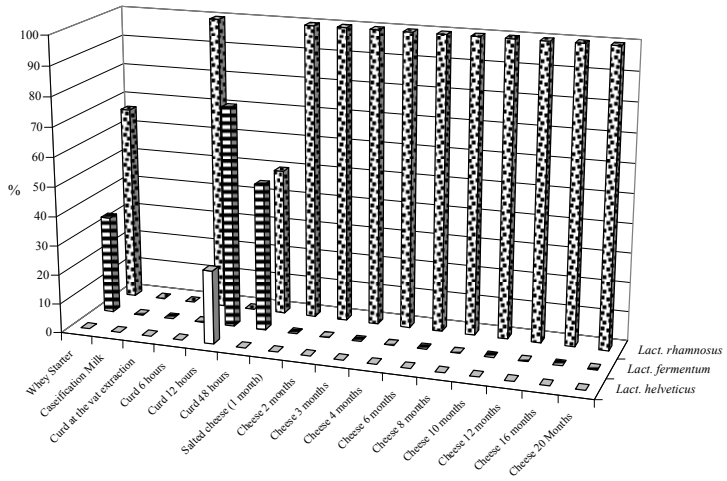

Figure 4 Percentage of each LAB species on the total of strains isolated on CAM medium for samples at different cheese manufacturing and ripening times. Bars – White: *Lactobacillus helveticus*; Horizontal lines: *Lactobacillus fermentum*; Dotted: *Lactobacillus rhamnosus*.

3.1.5 DISCUSSION

Regarding a generic ripened cheese, Broadbent and Steel (2005) wrote that the number of SLAB commonly exceeds 10^9 CFU g^{-1} when ripening begins. At the same time, NSLAB occur with initial numbers of about 10^2 CFU g^{-1}; this population grows and eventually goes to plateau at a cell density of 10^6-10^8 CFU g^{-1} after several months of aging. Our data demonstrate that the ripened cheese based medium CAM can distinguish the two different populations. This innovative medium seems able to recover minority populations coming from milk and hardly estimable on traditional media.

A partial picture of the microflora typical of PR cheese was achieved by studying the cultivable LAB associated with its manufacturing and ripening. Microorganisms with high nutritional requirement, principally originated from whey starter, find the best conditions to develop in rich substrates like MRS and WAM and they are

unable to grow in CAM. Moreover, SLAB counts were higher in WAM instead of MRS, in agreement with previous studies (Gatti et al. 2003)

Microorganisms with less nutritional demand, mainly originated from milk (NSLAB), are not estimable in MRS or WAM when they are the minority of a microbial population dominated by SLAB. However, thanks to their ability to metabolize carbon sources different from lactose, they can grow in CAM. When they become the dominant population, in absence of nutritional demanding microorganisms competitors, we can detect them even in MRS or WAM.

When SLAB and NSLAB are both present we are able to distinguish and isolate them only using all three different media (MRS, WAM and CAM).

We found that in the first 48 hours of production it was possible to isolate strains belonging to 8 different species, but only some of them (*Lact. delbrueckii* subsp. *lactis*, *Lact*. *keferi*, *Lact*. *fermentum*, *Lact*. *reuteri* and *Lact*. *plantarum*) during ripening too. After cheese brining, mesophilic lactobacilli as *Lact. rhamnosus* and mesophilic lactococci as *Ped. acidilactici* (minority species in the first 48 hours of production) resulted to be the microflora mainly isolated. Differently from Grana Padano cheese (Fornasari et al. 2006; Zago et al. 2007) NSLAB as *Lactobacillus casei*/*paracasei* and SLAB as *Streptococcus thermophilus* were not found. *Lact. fermentum* and *Lact. rhamnosus* were isolated from all three cultural media. *Lact. helveticus,* initially isolated from all three media, was found in 12 months cheese only on MRS medium. *Lact. delbrueckii* subsp. *lactis* was isolated only from WAM and *Lact. plantarum* and *Lact. reuteri*, very interesting for their lipolytic characteristics (Tungjaroenchai et al. 2003) were isolated only from MRS. The only not lactobacillus species, *Ped. acidilactici*, was isolated from 3 and 16 months cheese but we did not isolated it from CAM. To our knowledge this species, very common in sausage environment (Benito et al. 2007), is not so commonly found in long ripened cheese. The comparison among the strains belonging to the same species but isolated from different samples through different media, will be the object of a future work.

Differently from MRS-vancomicin agar, which is a selective medium suitable to enumerate *Lact. rhamnosus* (Tharmaraj et al. 2006), the use of a new cheese based medium (CAM) may promote the study of the microflora that better adapt to the changes in nutritional availability during ripening. We consider the data obtained in this study as the groundwork and the beginning of a more detailed investigation about PR ripening that wasn't the aim of this work.

Furthermore, since fermented foods are really complex microbial ecosystems they may be better investigated using alternative cultural approaches: the results obtained for ripened cheese could open a new field for other food based media.

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3.2 Parmigiano Reggiano cheese: evolution of lactic microflora and peptidase activities during production and ripening

3.2.1 ABSTRACT

The aim of this work was monitoring the microbial ecosystem evolution, cells viability and peptidase activities during production and ripening of Parmigiano Reggiano cheese. The microbial population and enzymatic activities in internal and external portions of cheese during 20 months of ripening were examined. The quantitative evolution of different microbial population was followed with two different approaches: directly, by cell viability counts by means of the BacLight® Bacterial Viability Kit and indirectly, by classical plate counts on traditional and innovative media. Furthermore, in order to highlight the role of the intracellular enzymes released after cellular lysis, peptidase activities in curd and cheese samples free from cells were evaluated.

Technological parameters select different SLAB and NSLAB in the different zones of cheese. During ripening a strong decrease of total microbial population and an important increase of peptidase activities were observed, probably due to the cellular autolysis occurred as an effect of cheesemaking technology on bacterial cells. In particular, curd-cooking temperature, brining and lack of available nutrients seem to regulate this phenomenon. A decrease of viable SLAB was observed after 48 hours of production. At the same time, NSLAB population initially present at low numbers, begin to grow and remain at high level for at least ten months. In the external and internal zones of PR cheese different microbial growth, cell autolysis and peptidases activities trends were observed. The higher aminopeptidases activities detected in the external zone might lead to a premature ripening respect to the internal one.

Keywords: Parmigiano Reggiano, cheese ripening, lactic acid microflora, bacterial cell autolysis, cell viability, peptidase activity

3.2.2 INTRODUCTION

Parmigiano Reggiano (PR) is a hard-textured, cooked, and long ripened cheese made from cow's raw milk supplemented with natural whey starter. It is made from the milk of cows fed with fodder grown in the region of origin and following strict manufacturing procedures (Council Regulation, 1992; www.parmigiano-reggiano.it). Only raw milk, unheated, obtained by mixing evening milk (partially skimmed by natural creaming) with full-cream milk of the following morning can be used. During the natural creaming two different phenomena occur: the removal of the bacteria ascending with the fat globules and the growth of the residual bacteria which depends on the environmental conditions (Panari et al., 2007). PR must be manufactured using only natural whey starter. It is a culture of thermophilic lactic acid bacteria (SLAB) selected by the process of curd cooking and obtained by the incubation at a naturally decreasing temperature of the sweet whey resulting from every day cheesemaking.

Other important technological characteristics regarding this DOP cheese are the use of calf rennet, the cooking temperature of the curd (54-55°C), the slow heat dissipation during lactic acid fermentation, the whey drainage from the curd, the brining with NaCl slow diffusion into the cheese, and the long ripening time of at least 12 months (Mucchetti & Neviani 2006).

Lactic acid bacteria are the dominant microflora in dairy products and it is well-known that cheeses are characterized by ripening kinetic and numerically different LAB species and biotypes coming both from raw milk and from the starter. Consequently, the presence of different LAB species in cheese is the result of microbial growth capacity in milk and curd, and of bacterial cell autolysis (Fox & Sweeney, 1996, O'Cuinn et al., 1995, Addeo, Mucchetti & Neviani, 1997). SLAB are added to the milk for cheese and fermented milks production due to their strictly fermentative metabolism with lactic acid as the major metabolic product. Adventurous species from the environment, called non starter lactic acid bacteria (NSLAB), are also present in the cheese matrix and begin to increase in numbers after the first two weeks postmanufacture, when most of the residual lactose in cheese has been utilized by SLAB (Diaz-Muniz, Banavara, Budinich, Rankin, Dudley & Steele, 2006). Alternative potential energy sources for NSLAB in cheese could be nucleic acids, derived from the autolysis of SLAB (Thomas, 1986), amino acids (Kieronczyk, Skeie, Olsem & Langsrud, 2001), sugars from glycoproteins and glycolipids present in milk (Williams & Banks, 1997,

Fox, McSweeney & Lynch, 1998, Williams, Withers & Banks, 2000), and citrate (Diaz-Muniz, Banavara, Budinich, Rankin, Dudley & Steele, 2006) (Liu, 2003).

Some studies (Neviani, Rossetti, Mucchetti, Addeo & Giraffa, 1997, Coppola, Nanni, Iorizzo, Sorrentino, Grazia, 2000) reported that in long ripened cheeses, the thermophilic microflora originated from natural whey starter dominates in the first period of ripening, while the mesophilic one, coming from raw milk, became prevalent in a successive phase of the cheese aging after the premature autolysis of the thermophilic microflora.

A very interesting research highlighted the effect of the curd cooking temperature on SLAB and NSLAB viability, cheese composition and ripening indices of an experimental semi-hard cheese (Sheehan, Fenelon, Wilkinson, McSweeney, 2007). Recent works showed the key role played by the cooking temperature on the acidification process in the first hours after the cheesemaking in PR cheese. Besides, the temperature at the end of cooking influenced the moisture content of cheese and affected the acidification curves of the cheese mass, particularly in the inner zone, where slight variations of temperature could advance or delay the beginning of the lactic fermentation (Sandri et al., 2007).

During the ripening period, LAB contribute to cheese flavour development through several basic mechanisms that include carbohydrate fermentation, conversion of milk proteins into peptides and free amino acids, catabolism of amino acids into aroma compounds, hydrolysis of milk lipids into free fatty acids, followed by their conversion in esters, and citrate catabolism (Fox & Wallace, 1997). In particular, for long ripened cheeses like PR, the proteolysis may be considered an essential multi-step biochemical event (Fox, Wallace, Morgan, Lynch, Niland & Tobin, 1996, Pereira, Gomes, Gomes & Malcata, 2007).

LAB enzymatic activity largely contributes to the definition of cheese organolectic properties and their proteolytic system can differ from specie to specie and from biotype to biotype. In the last years, some complex peptidase systems different in nature, specificity and localization were characterized (O'Cuinn et al., 1995, Takafuji, Iwasaki, Sasaki & Tan, 1995, Kunji, Mierau, Hagting, Poolman & Konings, 1996, Christensen, Dudley, Pederson & Steel, 1999, Savioky, Ingmer & Varmanen 2006). Residual enzymatic activity in cheese can be considered as the trace of the microbial ecosystem evolution during aging (Wilkinson, Guinee, O'Callaghan & Fox, 1994, Gatti et al 1999). In a comprehensive study of the relationship between starter autolysis and the proteolytic enzyme complement released in Cheddar cheese, Sheehan et

al. (2006) found that differences in lactococcal starter autolysis influenced the proteolytic activities of the released enzymes during ripening. Moreover, in a recent study, it was demonstrated the positive impact of *L. helveticus* autolysis on flavour precursor development in ripened Cheddar cheese (Hannon, Kilcawely, Wilkinson, Delahunty, Beresford 2007)

For all these reasons, the study of lactic microflora and peptidase activities should be very interesting to comprehend the essential biochemistry events during PR production and ripening. In this work, an accurate and laborious sampling allowed to analyse this PDO cheese during its production and through 20 months of ripening. This kind of sampling allowed us to study in an innovative way the complex ripening process. We evaluated the SLAB and NSLAB cultivability using traditional an innovative cultural media containing whey, curd and ripened cheese. Furthermore, cell viability was checked. In order to stress the role of microbial enzymes released after cellular lyses, we evaluated microbial aminopeptidases and dipeptidylaminopeptidase activities in the extract of cheese, free from cells, using β naphthylamide-aminoacids.

3.2.3 MATERIALS AND METHODS

Cheesemaking

With the aim to obtain a suitable number of PR wholes with high similarity, a cheese factory was selected for its equipments and technological conditions. To guarantee the production of twin wholes, the milk and whey starter were held and processed in the same tanks. Using volume counter pumps, the milk was distributed in equal volume in the vats and was worked according to PR production protocol (http://www.parmigiano-reggiano.it) illustrated in figure 1.

Natural whey starter was added in eight vats containing 1040 Kg of milk each one (510 Kg of partially skimmed evening milk and 530 Kg of whole morning milk) at 33 $g \cdot L^{-1}$ bringing the pH of the mixture to 6.20. After the cheesemaking and moulding, the wholes were held for 3 days and frequently turned to enable the complete whey drainage. Sixteen wholes were then salted by immersion in brine at 300 g $NaCl \cdot L^{-1}$ for 23 days. Ripening was held in aging rooms with 85% relative humidity for 20 months.

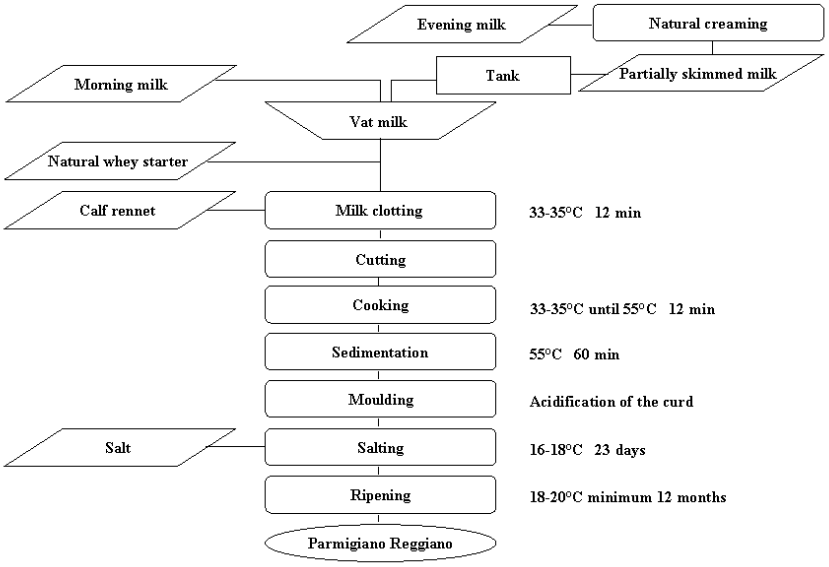

Figure 1. Parmigiano Reggiano cheese production protocol.

Dairy samples

Samples were collected from milk to 20-months ripened cheese. First of all, aliquots of the total mass of whey starter, raw milk and curd at the vat extraction were sampled. For each of the following samples, curd after 6, 12 and 48 h from extraction, salted cheese (1 month) and cheese at different stages of ripening (2, 3, 4, 6, 8, 10, 12, 16, 20 months), the wholes were lengthwise cut along vertical axis and a central 10 cm thickness cheese section was obtained. Internal and external (far 0.5 cm from cheese crust) portions were obtained from these cheese sections, mixed in equal weight and grated before the analysis.

Whey starter and milk were 10-fold serial diluted in 0.05 mol L^{-1} sodium citrate buffer pH 7.0, while 10 g of curd and cheese samples were first homogenized with sodium citrate buffer in a blender (Stomacher 400, Seward, UK) for 3 min and then serially diluted.

Physical-chemical and chemical analyses

The pH of samples was directly measured with a pH meter (350 pH, Beckman Coulter, Fullerton, CA, USA).

Lactose, galactose, L-lactate and D-lactate were determined according to Careri et al. 1996, by enzymatic assays in $g \cdot L^{-1}$ with Boehringer-Mannheim kits (Boehringer-Mannheim, Germany). Total lactate was obtained through the sum of L-lactate plus D-lactate.

Citrate, succinate, acetic acid and pyroglutamic acid were determined according to Careri et al. 1996. The analysis of these organic acid were performed in a liquid chromatography system with a variable wavelength UV/visible DAD 540 (Kontron Instruments, Italy) and refractometric REFRACTIVE INDEX 475 (Kontron Instruments, Italy) detectors. Analysis were performed using a cation exchange Aminex HPX 87H (Bio-Rad Laboratories, CA, USA) column and filtered and degassed 0.009 N reagent grade H_2SO_4 (Carlo Erba, Italy) as mobile phase at a flowrate of 0.6 $mL \cdot min^{-1}$. Eluates were monitored at 215 nm. The calibration curves were obtained preparing a standard mix of the organic acids (Sigma, USA). The resulting peaks areas were calculated for duplicate 25 µL injections and plotted against concentration using the software Kromasystem 2000 (Kontron Instruments, Italy).

Each assay was carried out in duplicate and average values expressed in $g \cdot 100\ g^{-1}$ for the lactose, galactose, L-lactate, D-lactate, citrate, succinate, acetic and pyroglutamic acid concentration during PR manufacturing and ripening were calculated.

Microbiological analysis

Whey starter and milk were 10 folds serial diluted in sodium citrate (Sigma, Italy) buffer 0.07 $mol \cdot L^{-1}$ pH 7.5 while curd and internal/external cheese samples were first homogenized at 10 g for 3 min in a blender (Stomacher 400, Seward, UK) with sodium citrate and then serial diluted. In order to recover the cultivable bacterial population, the following media and time/temperature conditions of incubation were used: MRS agar pH 5.4 (Oxoid Spa, Italy) at 42°C for 48h and 25°C for 72h; M17-SSW (Fornasari, Rossetti, Carminati & Giraffa, 2006) at 42°C for 42h and 25°C for 72h; Whey agar medium (WAM) (Gatti et al., 2003) at 42°C for 48h; Curd agar medium (CURDAM) (Lazzi et al 2007) and Cheese agar medium (CAM) (Lazzi et al. 2007, Gatti et al., submitted for publication) at 42°C for 72h. The media were all incubated under anaerobic conditions. The microbial counts were carried out in duplicate and the standard deviation of mean values was calculated.

Cell viability was directly checked using LIVE/DEAD BacLight® Bacterial Viability Kit (Molecular Probes Inc., Eugene, USA) and an epifluorescence microscope (Leica DSML, Leica Microsystems, Wetzlar, Germany) as reported in Gatti, Bernini, Lazzi and Neviani (2006). Curd

and cheese samples were diluted as previously described, centrifuged (8000 *g*, 10 min, 4°C) and pelleted. The resulting pellet was diluted in 1 mL of sterile water and treated with 0.7 μL of Syto9™ and 1 μL of PI. Direct counts of total, viable and not viable bacterial population were evaluated using a Thoma's chamber (Brand, Germany). Each sample was prepared in duplicate and average values were calculated.

Determination of peptidases activities

With the aim to investigate the phases of PR ripening in which LAB aminopeptidases could be involved in release of free amino acids, we studied six different aminopeptidases activities that release different N-terminal amino acids.

The aminopeptidase activities tested were those able to hydrolyze six different substrates: broad specificity aminopeptidase N (PepN) activity was determined using Lys-βNA, broad specificity aminopeptidase C (PepC) using Arg-βNA, proline iminopeptidase (PepI) activity using Pro-βNA, glutamyl aminopeptidase A (PepA) activity using Glu-βNA, peptidase with high specificity for leucine and alanine (PepL) using Leu-βNA and activity of the X-prolyl dipeptidyl aminopeptidase (PepX) was determined using Phe-Pro-βNA (Christensen et al. 1999, Savijoki, Ingmer & Varmamen, 2006).

Sample extract preparation prior to the dialysis was carried out according to Gatti, Fornasari, Mucchetti, Addeo and Neviani (1999). The sample suspension obtained after 24 h of dialysis in cellulose tube-shaped (Spectra/por, Spectrum Laboratories Inc, USA) with cut off of 3000 $g \cdot mol^{-1}$, was centrifuged (10000 *g*, 10 min, 4°C), filtered through cellulose acetate membrane with 0.22 μm pore size (Sartorius, Italy) and incubated with 0.650 $mmol \cdot L^{-1}$ solutions of β-naphthylamide derivates (Bachem Feinchemikalien AG, Switzerland) and 0.05 $mol \cdot L^{-1}$ phosphate buffer pH 7.0 at 40°C for the specific times, showed for each aminoacid in table I. The reaction was stopped adding 250 μL of 2.0 $mol \cdot L^{-1}$ HCl. The degree of hydrolysis was determined by measuring the coloured product of an azocopulation reaction by reading spectrophotometrically the optical density at 580 nm (A_{580nm}) according to Bouquien, Corrieu and Desmazeaud (1988). Each assay was carried out in duplicate and average values were calculated. The arbitrary unit of enzyme activity (EA) was defined as the number of micromoles of β-naphthylamide released in one hour in one mL of sample.

Substrates	Cheese sample*	Substrates βNA*	Buffer*	Water*	Time of analysis
Lys-βNA	50	125	125	200	1 hour
Arg-βNA	50	125	125	200	1 hour
Leu-βNA	50	125	125	200	3 hours
Pro-βNA	300	100	100	\	24 hours
Glu-βNA	350	75	75	\	24 hours
Phe-Pro-βNA	50	125	\	325	1 hour

Table I. Parameters of reaction to determinate peptidase activities with different substrates. ***** μL

3.2.4 RESULTS AND DISCUSSION

Chemical analysis

Organic acids are important indicators of biochemical metabolic processes occurring during the ripening of cheese. The results obtained from the organic acids evaluation reflect a kind of bacterial metabolism and fermentation which characterize PR cheese.

Table II shows that at the beginning of the process, lactose and galactose were totally consumed by thermophilic starter LAB. At the same time an expected increase of lactate was observed. Lactate produced from lactose is the most abundant organic acid in Parmigiano Reggiano cheese, ranging from 1.30 to 1.70 % (wt/wt) (Panari 1985, Careri et al. 1996). The concentrations of D- and L-lactic acid were approximately the same (L/D ratio approx. 1.1); in fact, both optical isomers are produced from lactose by the thermophilic LAB of the natural starter. The values for citrate and succinate are in agreement with the result described by Careri et al. 1996 in PR, but disagree with Coppola et al. 2000 that observed a gradual decrease in citrate concentration during aging. Citrate has not been used as an energy source by facultatively heterofermentative LAB even if many of them can metabolize it also when fermentable sugars are present (Cogan et al. 2007). Our findings demonstrate that probably the growth of the dominant organisms after the start of the ripening was not connected to their ability to utilize citrate as a source of energy. Acetic acid values found are in agreement with the results observed by Careri et al. 1996 in a normal PR fermentation. Pyroglutamic acid is related to the age of PR (Panari, 1985). The association of glutamic acid with pyroglutamic acid cyclase activity was demonstrated for thermophilic lactic species. The

cyclization of glutamic acid in pyroglutamic acid was proposed by Mucchetti et al. 2000 as a ripening marker in long ripened cooked cheese. Our results for this organic acid support these hypothesis.

	pH	Lactose	Galactose	L-Lactate	D-Lactate	Lactate	Citrate	Succinate	Acetic Acid	Pyroglutamic Acid
Milk	6.73	4.81	-	-	-	-	0.16	-	-	-
Whey starter	3.25	-	-	-	-	-	-	-	-	-
Curd at vat extraction	6.00	1.36	0.09	0.10	0.09	0.19	-	-	-	-
Curd 6 hours	5.75	0.40	0.39	0.22	0.20	0.43	-	-	-	-
Curd 12 hours	5.57	0.15	0.56	0.24	0.24	0.48	-	-	-	-
Curd 48 hours	4.36	0	0	0.47	0.58	1.05	0.05	0.03	0.04	-
Salted cheese (1 month)	5.35	0	0	0.77	0.61	1.38	-	-	-	-
Cheese 2 months	5.36	0	0	0.73	0.57	1.30	-	-	-	-
Cheese 3 months	5.37	0	0	0.72	0.67	1.39	-	-	-	-
Cheese 4 months	5.36	0	0	0.85	0.60	1.45	-	-	-	-
Cheese 6 months	5.33	0	0	0.73	0.69	1.42	0.05	0.04	0.09	0.18
Cheese 8 months	5.41	0	0	0.72	0.62	1.34	-	-	-	-
Cheese 10 months	5.37	0	0	0.74	0.63	1.37	-	-	-	-
Cheese 12 months	5.42	0	0	0.73	0.63	1.36	-	-	-	-
Cheese 16 months	5.41	0	0	0.69	0.63	1.32	-	-	-	-
Cheese 20 months	5.41	0	0	0.75	0.68	1.43	0.05	0.05	0.12	0.51

Table II. pH and mean values in g 100 g^{-1} for the concentration of lactose, galactose, L-lactate, D-lactate, lactate, citrate, succinate, acetic and pyroglutamic acid during Parmigiano Reggiano manufacturing and ripening. Total lactate was obtained through the sum of L-lactate plus D-lactate. - : Not determined.

Microbial counts and growth trends

LAB cultivability was evaluated during PR manufacturing and ripening. Figures 2 and 3 show the growth trends of cultivable population in MRS 5.4, M17-SSW, WAM, CURDAM and CAM for both internal and external zones of cheese. The microbiological count of the whey starter was estimated in $7.75x10^4$ colony forming units (CFU)·mL^{-1} in M17-SSW incubated at 42°C, $1.00x10^7$ CFU·mL^{-1} in MRS 5.4 42°C, $3.36x10^8$ CFU·mL^{-1} in WAM, $1.00x10^7$ CFU·mL^{-1} in CURDAM and 10

$CFU \cdot mL^{-1}$ in CA. The microbiological count for caseification milk was estimated in $4.10x10^3$ $CFU \cdot mL^{-1}$ in M17-SSW incubated at 25°C, $7.75x10^3$ $CFU \cdot mL^{-1}$ in M17-SSW 25°C, $1.35x10^2$ $CFU \cdot mL^{-1}$ in MRS 5.4 25°C, 90 $CFU \cdot mL^{-1}$ in MRS 5.4 42°C, $2.70x10^2$ $CFU \cdot mL^{-1}$ in WAM, $1.49x10^3$ $CFU \cdot mL^{-1}$ in CURDAM and $1.69x10^{-3}$ $CFU \cdot mL^{-1}$ in CA.

The figure 2 and 3 show as total microbial cultivable population was high and not significantly different for the first six months. After six months, a progressive decrease can be observed. The evolution of the microbial counts in the five growth media was different for the first 48 hours, and split in three groups after 4 months: highest counts in WAM, MRS 5.4 42°C and CAM; lowest in M17-SSW 25°C and 42°C. In particular, when three months of ripening were reached in the internal and external zone, the values of microbial counts on MRS 5.4, WAM and CAM resulted overlapped and remained comparable until the end of ripening. After 9/10 months of ripening a significant decrease of the number of cultivable bacteria was observed. Counts in CURDAM resulted low in 1-2 logarithmical unities, and the lowest count resulted for the streptococci and lactococci populations, evaluated in M17-SSW agar respectively at 42 and 25°C. It is possible to hypothesize that the majority of microbial population found in cheese after four months of ripening wasn't able to metabolize lactose, present in M17 and CURDAM (less than in M17), as energetic source.

Microbial counts in WAM confirmed that after the addition of whey starter, owing to dairy thermal parameters, in the first 48 hours, the thermophilic microflora become dominant respect to the mesophilic one (Fig. 2 and 3). In this phase, nutritional demanding microorganisms found in WAM the best conditions to grow (WAM attempts to reproduce the natural composition of the whey), supporting as the original population from whey starter is probably the one that better develop in this substrate. Different microbial counts in WAM for internal and external 6 hours curd demonstrate that anticipate refresh in the external curd may leader for better thermophilic (growth *optimum* 42-45°C) environmental conditions.

Microorganisms cultivated at 25°C in MRS 5.4 and at 42°C in CAM (probably NSLAB originate from milk) were unable to firmly grow in the first 48 hours, while in the advanced phase of ripening (Fig. 2 and 3) demonstrated ability to use alternative potential energy sources and metabolize also different carbon sources from lactose and citrate.

Regarding a general ripened cheese, Broadbent & Steel (2005) wrote that the number of SLAB commonly exceeds 10^9 $CFU \cdot g^{-1}$ of cheese when ripening begins. At the same time, NSLAB populations

occur with initial numbers of about 10^2 CFU·g^{-1} and begin to grow and eventually plateau at cell densities of 10^6-10^8 CFU·g^{-1} after several months of aging. Our results are in agreement with this general consideration. Moreover, we found that in this advanced phase of PR ripening (over 12 months), mesophilic lactobacilli counts result higher than mesophilic lactococci (mesophilic microrganisms able to use lactose) in both cheese portions.

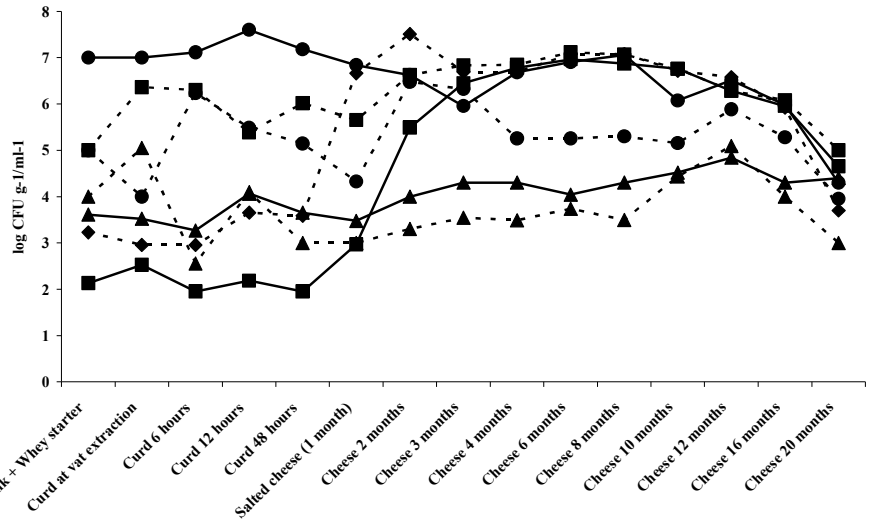

Figure 2. Evolution of cultivable population, expressed as log CFU·g-1 or mL-1, in internal zone, during different steps of Parmigiano Reggiano cheese manufacturing and ripening. Solid line ▲: M17-SSW 25°C; Dotted line ▲: M17-SSW 42°C; Solid line ■: MRS 5.4 25°C; Dotted line ■: MRS 5.4 42°C; Solid line ●: WAM 42°C; Dotted line ●: CURDAM 42°C; Dotted line ♦: CAM 42°C.

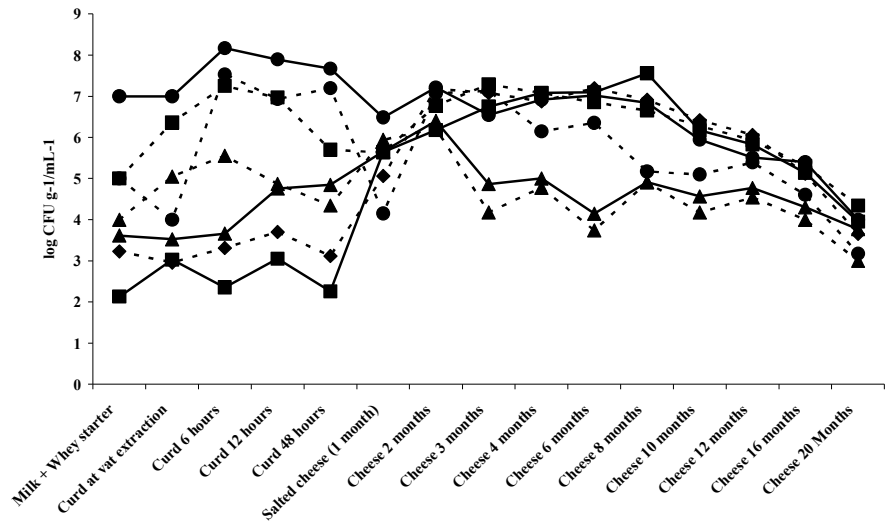

Figure 3. Evolution of cultivable population, expressed as log CFU·g-1 or mL-1, in external zone, during different steps of Parmigiano Reggiano cheese manufacturing and ripening. Solid line ▲: M17-SSW 25°C; Dotted line ▲: M17-SSW 42°C; Solid line ■: MRS 5.4 25°C; Dotted line ■: MRS 5.4 42°C; Solid line ●: WAM 42°C; Dotted line ●: CURDAM 42°C; Dotted line ♦: CAM 42°C.

Cell viability counts

Cell viability counts using LIVE/DEAD BacLight® Bacterial Viability Kit and an epifluorescence microscope (Nikon 80i, Tokyo, Japan) allowed to determinate viable (undamaged cell wall) and not viable (damaged cell wall) microorganisms. Viable plus not viable counts supply a direct total population. Total microbial direct count using cell viability methods gives additional information compared with classical plate counts, where there is the influence of adaptability condition, nutritional substrate and incubation parameters. Figures 2, 3 and 4, show that cultivable population was always lower than total direct counted population, especially at the beginning of cheese production. During ripening, the cultural medium CAM supported the recovery of total cultivable population, like demonstrated by an overlap of both plate and direct count results. In the first 12 hours of production, viable, not viable and total population, in the external portion of cheese, resulted to be higher than in the internal one (Fig. 4). In particular, viable cells in the inner of the curd are one logarithm lower than those in the external ring. This demonstrates the influence of technological thermal gradient applied

during curd cooking on the development of the microflora. This different thermal condition for internal and external zone will affect the peptidase activity in the first hours of production.

The assessment of cell viability allowed to highlight the bacterial lysis moment that can be correlated with the release of intracellular peptidase and aminopeptidase activity. As observed by Broadbent and Steele (2005), during aging, limiting condition for cell viability occurs in the cheese matrix. Absence of residual lactose, high levels of NaCl, low pH and drop in temperature contribute to cells autolysis, which releases intracellular enzymes and other components into the cheese matrix. In salted cheese (sample of first month), this lysis moment and a reduction of the number of viable cells, can be clearly observed both in the external and internal part. Between the first and the second month an increase of total population, above all in the external zone, has been recorded. After the second month of ripening, it is possible to observe a continuous and gradual decrease of population until the end of ripening (Fig. 4).

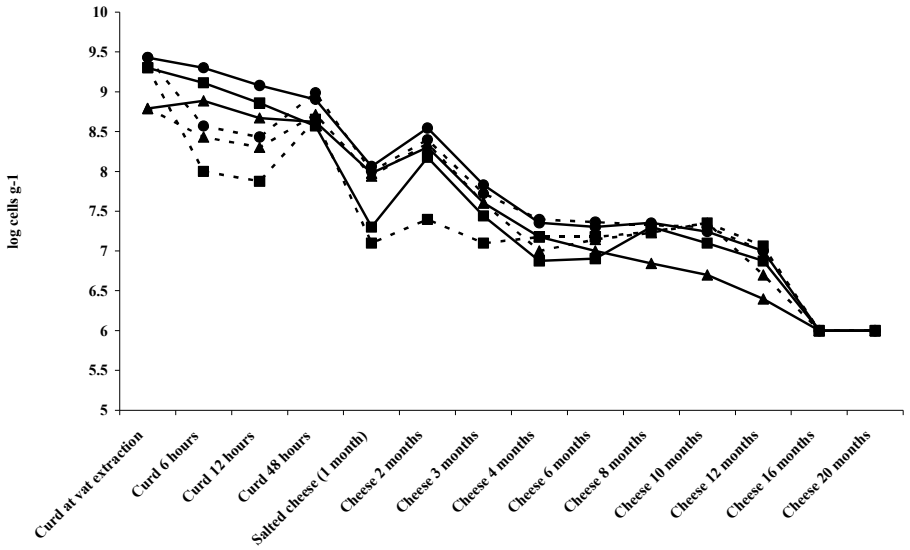

Figure 4. Evolution of viable ■, not viable ▲ and total population ●, expressed as log cells·g^{-1}, in internal (dotted line) and external zones (solid line), during different steps of Parmigiano Reggiano cheese manufacturing and ripening.

Enzymatic activity

Biochemical transformation of the curd proteins and peptides in the last months of ripening could be in part due to the citoplasmatic enzymes

released after cellular lysis. SLAB autolysis represents an important technological condition (Valence, Deutsch, Richoux, Gagnaire & Lortal, 2000, Lortal & Chapot-Chartier, 2005) as the peptidases released are responsible for water-soluble peptides and free amino acids production (Broadbent & Steel, 2005, Savijoki et al. 2006). The presence of free aminoacids into the cheese is the result of their initial presence in milk and of the chemical and biochemical events occurring during cheesemaking. LAB, in particular NSLAB, can utilize free aminoacids for protein synthesis, generation of metabolic energy, recycling of reduced cofactors, which are connected with cheese flavour formation (Christensen et al. 1999, Yvon and Rijnen 2001, Smit et al. 2005). The aminoacid catabolism involves a wide set of both general and specific regulators and shows significant differences among LAB (Fernandez e Zuniga 2006).

EA values reported in figures 5 and 6 for the internal and external zones of cheese are relative to aminopeptidases and dipeptidylaminopeptidase activities found in the cheese extract free from cells. These results reflect an enzymatic activity due only to cytoplasmatic enzymes released after bacterial lysis. In the external zone of cheese (Fig. 6), PepC, PepN, PepX and PepL activities result higher than in the internal one (Fig. 5) especially during the first six months of aging. In both zones, PepA and PepI activities result to be limited in contrast with the high quantity of proline and glutamic acid found in free form at the end of ripening by Resmini, Pellegrino, Hogenboom and Bertuccioli (1988) and Careri et al. (1996). In general, glutamic acid is the aminoacid mainly detected in aged cheese produced with a thermophilic starter (Careri et al. 1996). This apparent contradiction might be explained in different ways: i) the hydrolytic activity towards these amino acids is low but always attends during ripening; ii) these amino acids are highly present in the milk proteins (Swaisgood 1982); iii) proline does not undergo to a further conversion into degraded molecules and gradually increases in cheese (Fernandez and Zuniga 2006).

A significant increase of EA was detected in the first 48 hours of ripening. These results agree with the cellular exponential growth and the consequent cellular autolysis. A second EA increase can be observed for the internal zone during the 6^{th} and 8^{th} months (Fig. 5) and for the external zone during the 4^{th} and 6^{th} months (Fig. 6). Over the 20 months of ripening, similar quantitative ascending trends in the hydrolysis of specific substrates were noted for internal and external zones of cheese.

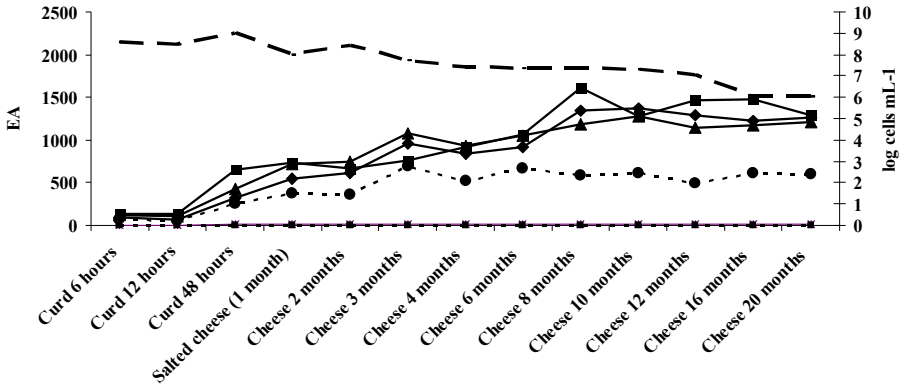

Figure 5. Evolution of aminopeptidases activities expressed as EA and total population expressed as log cells·g-1 (secondary axis – dashed line) in internal zone, during different steps of Parmigiano Reggiano cheese manufacturing and ripening. Solid line ■: PepX; Solid line ▲: PepN; Solid line ♦: PepC; Solid line ●: PepI; Dotted line ●: PepL; Dotted line ■: PepA.

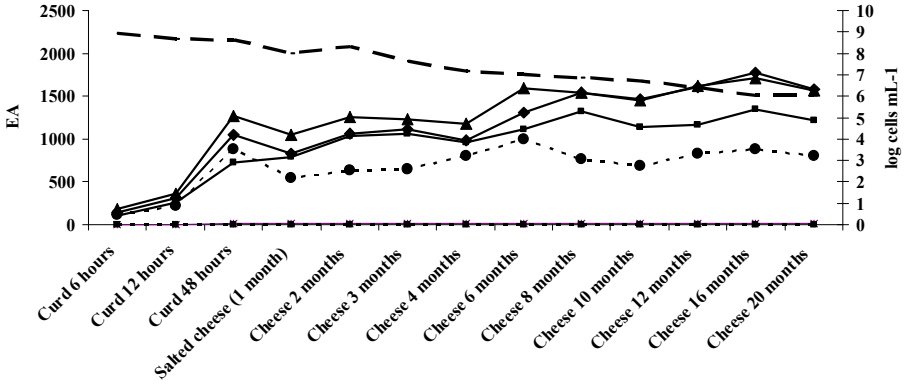

Figure 6. Evolution of aminopeptidases activities expressed as EA and total population expressed as log cells·g-1 (secondary axis – dashed line) in external zone, during different steps of Parmigiano Reggiano cheese manufacturing and ripening. Solid line ■: PepX; Solid line ▲: PepN; Solid line ♦: PepC; Solid line ●: PepI; Dotted line ●: PepL; Dotted line ■: PepA.

3.2.5 CONCLUSIONS

Parmigiano Reggiano cheese ripening process is a very complex biochemical event. Technological parameters determine a specific microbial selection in the different moment of cheese-making and ripening and even in the different cheese zones.

It could be summarized that the natural whey starter thermophilic microflora is involved not only in lactose depletion and curd acidification, but also in the production of a considerable amount of intracellular aminopeptidases released consequently to bacterial lysis. The different microbial increase in the various parts of cheese whole and the specific autolytic properties of cells induce the release of different amount of active peptidases. These enzymes could be involved in some stages of caseins hydrolysis and in the production of new free aminoacids contributing to cheese ripening.

The availability of such free amino acids and their further transformation in more simple compounds are of crucial importance for cheese flavour development and overall peculiar PR cheese characteristics. Therefore, we can speculate that the role of SLAB does not finish with the bacterial cell death as their enzymatic pattern strongly contributes to the biochemical events involved in cheese aging.

As regards NSLAB coming from raw milk, they remain viable for a long time during cheese ripening. These microrganisms, if compared to starter microflora, better adapt in cheese and are able to survive and grow even in lack of lactose. Moreover, our findings demonstrate that probably, the NSLAB growth is not connected to their ability to utilize citrate as a source of energy. It is presumable that these bacteria gain energy and regenerate NADH from the degradative pathways of amino acids (Fernàndez and Zuniga, 2006). These transformation could be essential for survival and multiplication of ripening bacteria in an environment characterized by stringent conditions. In addition, also this metabolic activity could be strictly linked to cheese flavour and aroma compounds formation. The real technological role of this secondary microflora could be deeply investigated.

Our results show the microbial population present in curd and cheese could play a particular role in the subsequent phases of ripening and could interact overlapping their metabolic pathways. Anyway, the growth dynamics of the different LAB species and biotypes and their interactions during PR cheesemaking and ripening are very complex and not still completely clarified. Hence, the knowledge of microbial dynamics and the correct identification of each microbial species

involved in PR cheese production could help to understand and protect its traditional quality.

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3.3 Microbial Dynamics During Parmigiano Reggiano Cheese Production and Ripening

3.3.1 ABSTRACT

The use of length heterogeneity PCR (LH-PCR) was exploited to monitor the evolution of Parmigiano Reggiano cheese microflora during production and ripening. With the aim to randomly isolate representative Parmigiano Reggiano microflora we used different nutritive media. Moreover, random amplification of polymorphic DNA PCR of the isolates was applied to choose biodiverse strains to create a LH-PCR database. This database was developed employing the peak profile obtained for isolated, molecular typed and identified by 16S rRNA gene sequencing strains.

The availability of twin wholes, allowed us to have samples representative of the subsequent stages of the same PR dairy process. The use of a culture independent method as LH-PCR overcame traditional agar plate and culture dependent method limitations. Moreover, the modality of LH-PCR samples preparation allowed to evaluate both the entire and lysed cells evolution during cheesemaking and ripening.

L. helveticus and *L. delbrueckii* subsp. *lactis* were the dominant species until the second month of ripening, even if an increasing number of them underwent to autolysis process. One month after brining, at least two new species were able to grow in the cheese: *L. rhamnosus* or *L. casei* or *L. plantarum*, and *P. acidilactici* or *L. parabuchneri*. After six months of ripening, the same species were found even if no one of them seems to be dominant. Interestingly, in this stage of ripening, also *L. rhamnosus* or *L. casei* or *L. plantarum* which seems to increase, underwent to autolysis process. From the sixth to the twentieth month of ripening any microbial evolutionary change was observed. In the 24 months cheese electropherogram referred to entire cells, it was possible to observe a trend reversal, where the major peak correspond to *L. rhamnosus* or *L. casei* or *L. plantarum*. Notably, in the lysis-released DNA electropherogram of the same cheese sample, a significant decrease of fluorescence intensity was observed.

Monitoring both entire and lysed cells through LH-PCR allows to coming aware of the importance of this two fractions in PR cheese production and ripening. This approach opens perspectives for insight microbial evolution in fermented food environment.

Keywords: Length heterogeneity PCR (LH-PCR), lactic acid bacteria, microbial dynamics, long ripened cheese

3.3.2 INTRODUCTION

Parmigiano Reggiano (PR) is a hard-textured, cooked, and long ripened cheese made from cow's raw milk supplemented with natural whey starter. PR is a Protected Designation of Origin (PDO) cheese, produced in specific areas of Northern Italy that must be made using only unheated and partially skimmed by natural creaming raw milk, and natural whey starter. Other important technological characteristics of this PDO cheese are the use of calf rennet, the cooking temperature of the curd (54-55°C), the slow heat dissipation during lactic acid fermentation, the whey drainage from the curd, the brining and the uptake of NaCl, that slowly spread into the cheese, and a long ripening time of at least 12 months (17).

Microbiological features of PR have been studied by Coppola et al (3, 4) who isolated on traditional growth media a great number of strains from a large amount of samples representative of the production and of the earlier and advanced stages of ripening. Moreover other studies focused into the biodiversity of different strains of *Lactobacillus helveticus* isolated from natural whey cultures for PR production (7, 9). However the microbial ecology of PR appears still not completely understood as all these studies were carried out by the use of traditional agar-based and culture-dependent methods which imply many limitations (10).

Investigations on microbial population, through traditional microbial methods, typically reveal the most commonly occurring microorganisms and, among them, only those able to grow to a detectable level by forming colonies on selective media. Besides, these methods often underestimate the less abundant components of microflora, that are equally important for cheese ripening and flavour development (26). Moreover all previous studies have been performed by sampling condition able to show the microbial biodiversity of PR and not its real evolution during the production and ripening. From this perspective, a detailed knowledge of lactic acid bacteria (LAB) dynamics during manufacturing and ripening stages is necessary for a deeper insight into the complex process which bring to the outcome of this high quality cheese.

Strain fingerprinting methods can characterize dairy environmental LAB population allowing the construction of computerized database containing strain fingerprints (24). Recently developed culture-independent methods, like length heterogeneity PCR, can be used to monitor LAB population. In particular, this technique has already been

applied to identify LAB from natural whey starter for Grana Padano cheese (14, 25) and to monitor LAB population of ensiling process fermentation (1). To our knowledge, LH-PCR has never been used to follow a food fermentation process. During fermented food production, particularly in cheesemaking, the presence of different amount of LAB is the result of microbial ability to grow in milk and curd, and of bacterial cell autolysis (6, 21). This phenomenon occurs subsequently to cell death and leads to the release of cytoplasmatic contents, including DNA (22, 23). The actual amount of DNA deriving from autolytic bacterial cells is still unclear and largely dependent on the conditions preceding cell death such as bacterial starvation (18).

Recent studies suggest that extracellular DNA is an important component of bacterial biofilms (27, 30) and that DNA from lysed cells remain largely accessible to residual bacteria (19).

In the present study, LH-PCR was used to monitor microbial evolution during a complete PR cheesemaking process. LH-PCR profiles from intact cells DNA and DNA arising form lysed cells extracted at different stages of production and ripening, were compared with a database containing profiles of strains isolated from the same cheese samples and identified by 16S-rRNA gene sequencing. In this way we were able to evaluate the microbial dynamics during 24 months of PR ripening.

3.3.3 MATERIALS AND METHODS

Cheesemaking. With the aim to obtain a suitable number of PR wholes with high similarity, a cheese factory was selected for its equipments and technological conditions. To guarantee the production of twin wholes, milk and whey starter were held and processed in the same tanks. Using volume counter pumps, milk was distributed in equal volume in the vats and was worked according to PR production protocol (http://www.parmigiano-reggiano.it).

Natural whey starter was added in eight vats containing 1040 Kg of milk each one (510 Kg of partially skimmed evening milk and 530 Kg of whole morning milk) at 33 $g \cdot l^{-1}$ bringing the pH of the mixture to 6.20. After the cheesemaking and moulding, the wholes were held for 3 days and frequently turned to enable the complete whey drainage. Sixteen wholes were then salted by immersion in brine at 300 g $NaCl \cdot l^{-1}$ for 23 days. Ripening was held in aging rooms with 85% relative humidity and about 18°C temperature for 24 months.

Cheese sampling and bacterial recovering. Samples were collected from milk to 24 months ripened cheese. First of all, aliquots of the total mass of whey starter, raw milk and curd at the vat extraction were sampled. For each of the following samples, curd after 6, 12 and 48 h from extraction, salted cheese (1 month) and cheese at different stages of ripening (2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 months), the wholes were lengthwise cut along vertical axis and a central 10 cm thickness cheese section was obtained. Internal and external (far 0.5 cm from cheese crust) portions were obtained from these cheese sections, mixed in equal weight and grated before the analysis.

Whey starter and milk were 10-fold serial diluted in 50 mM sodium citrate buffer pH 7.0, while 10 g of curd and cheese samples were first homogenized with sodium citrate buffer in a blender (Stomacher 400, Seward, UK) for 3 min and then serially diluted.

In order to recover an heterogeneous bacterial population, different types of nutritional media and the following time/temperature conditions of incubation were used: MRS agar pH 5.4 (Oxoid Spa, Italy) at 42°C for 48 h and 25°C for 72 h; M17-SSW (5) at 42°C for 42 h and 25°C for 72 h; Whey agar medium (WAM) (7) at 42°C for 48 h; Curd agar medium (CURDAM) (15) and Cheese agar medium (CAM) (15, M. Gatti, submitted for publication) at 42°C for 72 h. All the media were incubated under anaerobic conditions.

Isolation, grouping and identification of LAB by RAPD-PCR and 16S-rRNA gene sequencing. A representative number of colonies forming unit (CFU) obtained in the five growth media from all samples were randomly selected, picked and isolated. One hundred and two strains were purified in MRS (Oxoid Spa, Italy) or M17 (Oxoid Spa, Italy) broth, depending on the cellular morphology observed after microscopic examination.

Genomic DNA of the isolated strains was extracted from overnight cultures (anaerobic incubation at 42°C) by a chelex-based procedure according to Rossetti and Giraffa (24). The presence of DNA was checked on 1,5 % (wt/vol) agarose ethidium bromide gel and DNA concentration and purity were determined spectrophotometrically at 260 and 280 nm (Jasco V-530, Japan).

Phylogenetic positions of the strains at level of species by subunit ribosomal 16S- rRNA gene sequencing were studied. DNA amplification and sequencing were performed as previously described by Giraffa et al. (11), and each sequence obtained was checked manually and searched for

sequence homology using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

The LAB strains identified by 16S-rRNA were genotypically related by random amplification of polymorphic DNA-PCR **(**RAPD-PCR) molecular typing. Genomic DNA from the strains was amplified with the universal primer M13 (5'-GAGGGTGGCGGTTCT-3') (12).

The PCR reaction was performed by using 25 µl (final volume) mixtures containing 20 mM Tris-HCl at pH 8.0, 200 µM of each deoxynucleoside triphosphate, 2 µM of primer (MWG Biotech, Germany), 3.0 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 2.5 ng μl^{-1} of total DNA. The cycling program consisted as follows: 40 cycles of denaturation (60 s at 94°C), annealing (20 s at 45°C) and extension (2 min at 72°C). RAPD profiles were visualized by electrophoresis at a constant voltage of 50 V for 6 h on a 1.5% (wt/vol) agarose ethidium bromide gel. Clustering of the patterns was achieved through the unweighted pair group method and employing arithmetic averages (UPGMA) using the BioNumerics™ software package version 3.0 (Applied Maths BVBA, Belgium). Calculation of similarity of the PCR fingerprinting profiles were based on the Pearson product-moment correlation coefficient. Strains with similarity coefficient higher than 70% in the dendrogram were considered belonging to the same biotype.

LH-PCR of isolated LAB and set up of an LH-PCR database. Two strains for each species identified by 16S-rRNA were selected from different RAPD-PCR clusters to set up the LH-PCR database. Type strains were also used to enrich the database when species usually found in PR (3, 4) were not isolated in the samples.

Domain A of the variable region of the 16S rRNA gene was analyzed by LH-PCR (14, 25). The primer pair 63F (5'-CAGGCCTAACACATGCAAGTC-3') 5' end labeled with 6-carboxy-fluorescein (6-FAM) and 355R (5'-GCTGCCTCCCGTAGGAGT-3') were used for the analysis (14). The reaction and amplification conditions were performed as previously described (14) with a slight modification in which 2.5 U of Platinum *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA) were used in 20 µl of reaction mixture. Initial denaturation at 94°C for 2 min was followed by 25 cycles consisting of denaturation at 95°C for 45 s, annealing at 49°C for 45 s, and extension at 72°C for 2 min. There was a final extension step that consisted of 72°C for 7 min. LH-PCR products were stored at -20°C in the dark until use (usually less than 1 week).

For fragment analysis, 1 µl volumes of LH-PCR amplicons were mixed with 12 µl of deionized formamide (Applied Biosystems, Foster City, CA, USA) and with 1 µl of internal size standard (GS500 LIZ®, Applied Biosystems, Foster City, CA, USA) and then denatured at 90°C for 2 min followed by immediate chilling on ice. Length heterogeneity of the PCR amplicons was detected by capillary electrophoresis on the ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Electrophoresis conditions were as follows: 47 cm capillary, performance optimized polymer 4 (POP-4™), 15 s injection time, 15 kV injection voltage, 35 min electrophoresis at 15 kV and 60°C capillary temperature.

The peaks of the electropherogram profiles correspond to amplicons of different length, and the areas under the peaks represent the relative abundance of each amplicon. Amplicons sizes were determined with GeneMapper v4.0 software (Applied Biosystems, Foster City, CA, USA). LH-PCR profiles were analyzed by reference to the internal size standard using the local Southern size calling method and no-smoothing option, and a threshold of 50 fluorescent units was used.

LH-PCR analysis of cheese samples. LH-PCR was performed to investigate the microbial community evolution in PR cheese. Grated cheese samples were diluted 1:10 in 50 mM sodium citrate buffer pH 7.0 and homogenized for 3 min, while whey starter and milk samples were directly submitted to the following step. In order to separate bacterial cells from the lysed cells free DNA, 1 ml of each sample was filtered on a 0.2-µm filter (Whatman, Germany) to obtain free-cell fraction and another milliliter was non-filtered and directly placed in a 1.5 ml microtube. The non-filtered fraction was treated with DNase for digestion of free DNA from lysed cells. Digestion was performed with 0.14 U μl^{-1} of Amplification Grade DNase I (Sigma-Aldrich, Co., St. Louis, MO, USA) under conditions given by the supplier. The samples were centrifuged at 7,500 rpm for 5 min and the pellets were suspended in 100 µl of pure water with the addition of 20 µl 10X Reaction buffer (Sigma-Aldrich, Co., St. Louis, MO, USA) and 20 µl Amplification Grade DNase. This mixture was incubated for 1 h at room temperature. DNase was inactivated adding 20 µl of Stop Solution (Sigma-Aldrich, Co., St. Louis, MO, USA) and heating at 70°C for 10 min.

Afterward, genomic DNA was extracted both from the filtered untreated fractions (lysed cells) and from the non filtered treated ones (entire cells) by means of Qiagen-DNeasy Blood & Tissue kit (QIAGEN GmbH, Hilden, Germany) as described by the manufacturer. DNA was spectrophotometrically (Jasco V-530, Japan) quantified by measuring

absorbance at 260 nm, diluted up to 20 ng μl^{-1} and stored at -20°C until use.

LH-PCR was performed as previously described for strains.

3.3.4 RESUTS AND DISCUSSION

Microbial cultivation. The data obtained in this first phase of the research are useful for a more accurate interpretation of the LH-PCR results. Microbial counts of 16 samples from as many again stages of PR manufacturing and ripening were performed on different media: MRS 5.4, M17-SSW, WAM, CURDAM and CAM (Table 1).

	M17-SSW		MRS pH 5.4		WAM	CURDAM	CAM
Samples	25°C	42°C	25°C	42°C	42°C	42°C	42°C
Whey Starter	n.d	4,89	n.d	7,00	8,53	7,00	1,00
Caseification Milk	3,61	3,89	2,13	1,95	2,43	3,17	3,23
Curd at vat extraction	3,52	5,05	3,03	6,36	7,00	4,00	2,96
Curd 6 h	3,66	5,55	2,35	7,25	8,17	7,53	3,31
Curd 12 h	4,76	4,87	3,05	6,97	7,89	6,93	3,70
Curd 48 h	4,85	4,34	2,26	5,70	7,67	7,19	3,11
Salted cheese	5,65	5,94	5,64	5,63	6,48	4,15	5,06
Cheese 2 months	6,40	6,21	6,18	6,77	7,21	7,07	7,18
Cheese 3 months	4,86	4,18	6,75	7,29	6,54	7,13	7,08
Cheese 4 months	5,00	4,78	7,08	7,08	6,91	6,15	6,88
Cheese 6 months	4,15	3,74	7,10	6,85	7,02	6,35	7,19
Cheese 8 months	4,91	4,90	7,56	6,65	6,84	5,17	6,91
Cheese 10 months	4,57	4,17	6,17	6,28	5,94	5,10	6,42
Cheese 12 months	4,77	4,54	5,83	5,93	5,51	5,39	6,06
Cheese 16 months	4,30	4,00	5,13	5,30	5,40	4,60	5,11
Cheese 20 months	3,77	3,00	3,95	4,34	4,00	3,18	3,65

TABLE 1. Logarithmic bacterial counts in different media (log CFU ml^{-1} for the first two samples and log CFU g^{-1} for the others) of cultivable lactic acid thermophilic and mesophilic bacteria during Parmigiano Reggiano cheese production and ripening. n.d. not determined

Non-starter LAB from caseification milk ranged between 1.95 log CFU ml^{-1} on MRS pH 5.4 plates incubated at 42°C and 3.89 log CFU ml^{-1} on M17-SSW incubated at 42°C, giving comparable growth on the other media. The natural whey starter, as expected, was characterized by

a high number of thermophilic lactobacilli recovered on MRS pH 5.4 and WAM, a low number of streptococci estimated on M17-SSW and a very low number of non nutritional demanding LAB cultivable on the new experimental medium CA (15, M. Gatti, submitted for publication) (Table 1).

Microbial counts in WAM confirmed that in the first 48 h after the addition of whey starter, owing to dairy thermal parameters, the thermophilic microflora overcame the mesophilic microflora. In this phase, nutritional demanding microorganisms were found on WAM, which attempts to reproduce the natural ecosystem of whey and for this reason, the best conditions for their development. The original population from whey starter is probably the best adapted to grow in this kind of substrate.

Total cultivable microbial population was high and not variable during the first six months. After that, a continuous decrease was observed. After 9/10 months of ripening a significant decrease of cultivable bacteria was observed. Counts in CURDAM fall about 1-2 orders of magnitude, and the lowest count resulted for the streptococci and lactococci populations, evaluated on M17-SSW agar at 42°C and 25°C respectively (Table 1). Similar growth trends were observed in previous studies (3, 4) even if results are not easily comparable because different media have been used.

Strains isolation, clustering and set-up of an LH-PCR database. To set-up an appropriate LH-PCR database, a total of 102 strains were randomly isolated from 5 media with different nutritional composition and aptitude for isolating diverse microflora. These strains were collected from all sixteen stages of PR manufacturing and ripening. With the aim of clustering strains with genotypic relatedness, all species identified by 16S-rRNA gene sequencing were fingerprinted by RAPD-PCR. Strains belonging to the same species but with a different RAPD-PCR profile and coming from different stages of ripening were chosen to generate the database. Moreover, six type strains of the species commonly isolated from PR cheese, were included.

Frequently, a combination of results obtained using several primers are necessary to increase the discriminatory RAPD-PCR resolution. In this study, just one primer was used, because the aim of RAPD-PCR was to show inter-species biodiversity. The profiles obtained generally showed low genotypic relatedness among the species (Fig. 1). The strains analyzed can be separated into different clusters based on polymorphisms, which consist of presence or absence of amplicons with

different sizes. The dendrogam revealed 11 genotypically related clusters of strains within 70% of similarity. These data support the biodiversity of the strains and species present in a complex system like PR cheese.

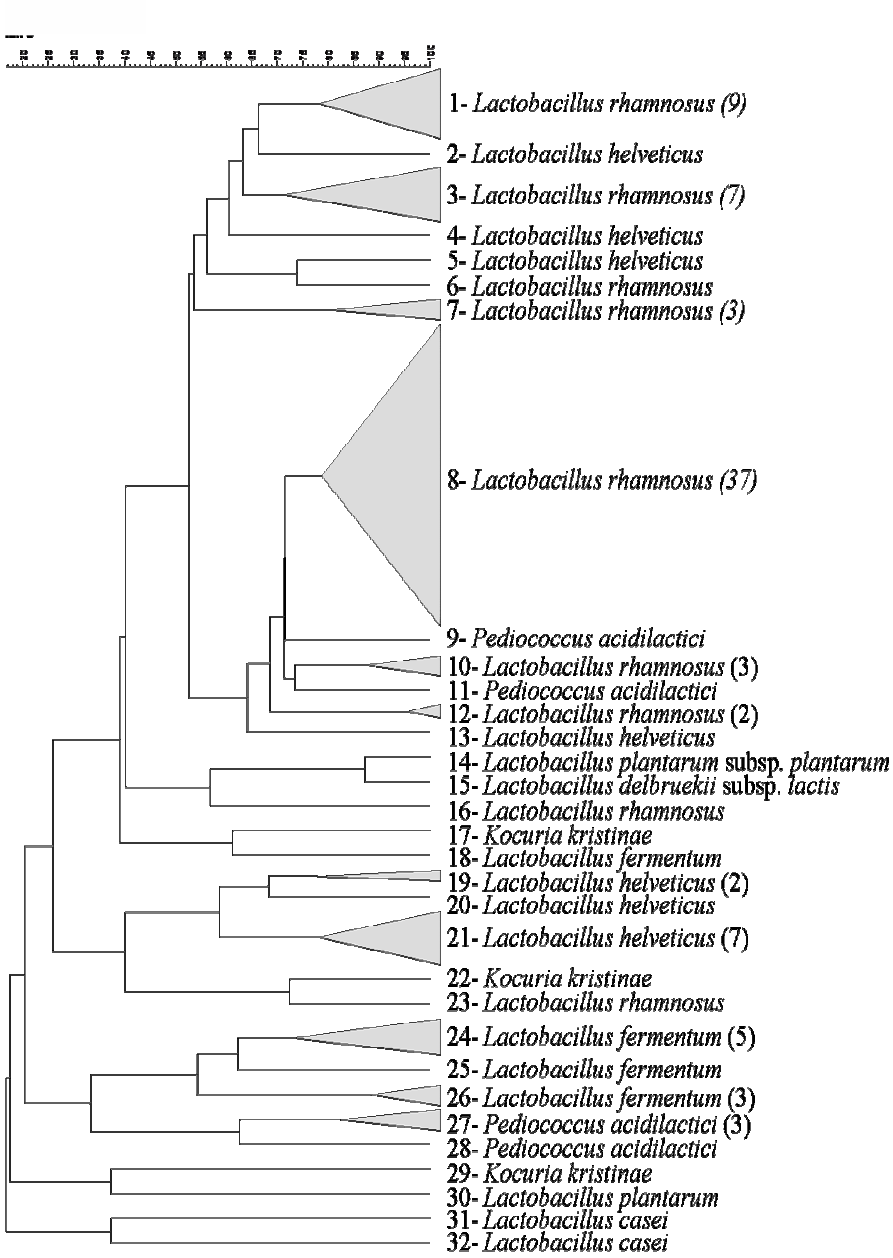

FIG. 1. Cluster analysis of RAPD-PCR patterns obtained with a primer M13 of strains isolated from samples. Clustering was performed by an UPGMA of Pearsons product moment correlation coefficient (expressed as a percentage). Numbers from 1 to 32 report the RAPD-PCR clusters or branches. The number of strains isolated for each cluster is indicated between brackets.

Fourteen isolated strains identified by 16S-rRNA gene sequences and six type strains were used to set-up the LH-PCR database (Table 2). The database reports fragment lengths from 310 to 346 bp. LH-PCR fragment sizes of the LAB agree with Lazzi et al. (14), which investigated the LH-PCR DNA profiles of the most representative LAB species usually occurring in natural whey starters for Grana Padano cheese. Differently from these authors, other secondary peaks where found. As expected, strains belonging to different clusters but to the same species gave the same fragment sizes in base pairs (Table 2).

Strain n°	RAPD-PCR cluster or branch	Number of isolates†	Origin	16S-rRNA Blast closest relative or Type strain	Acession number‡	% match	Fragment size* (bp)
750	29	1	Curd 12 h	*Kocuria kristinae*	AF375912	100	310
714	15	1	Milk	*Lactobacillus delbrueckii* subsp. *lactis*	AB289095	99	330 (284)
779	2	1	Curd 48 h	*Lactobacillus helveticus*	AB008210	100	334
776	21	7	Curd 6 h	*Lactobacillus helveticus*	AB008210	98	334
1056	31	1	Cheese 6 months	*Lactobacillus casei*	AJ558112	100	335
1247	32	1	Curd 12 h	*Lactobacillus casei*	AB008205	100	335
830	7	3	Salted cheese	*Lactobacillus rhamnosus*	EU184020	100	336 (290)
1489	8	37	Cheese 20 months	*Lactobacillus rhamnosus*	EF533991	99	336 (290)
710	14	1	Curd 48 h	*Lactobacillus plantarum* subsp. *plantarum*	EF577047	99	337 (290, 308)
1026	18	1	Cheese 6 months	*Lactobacillus fermentum*	AF477498	99	342, 344
730	26	3	Curd 6 h	*Lactobacillus fermentum*	EU221276	99	342, 344
1466	9	1	Cheese 20 months	*Pediococcus acidilactici*	EU147316	99	345
805	27	3	Whey starter	*Pediococcus acidilactici*	EU147316	100	345
Type LMG 6897	/	/	/	*Lactococcus lactis* subsp. *cremoris*	/	/	318 (272)
Type LMG 6896	/	/	/	*Streptococcus thermophilus*	/	/	319 (273)
Type LMG 6890	/	/	/	*Lactococcus lactis* subsp. l*actis*	/	/	318 (272, 291)
Type LMG 11423	/	/	/	*Enterococcus faecium*	/	/	328, 331 (284, 301, 303)
Type LMG 7937	/	/	/	*Enterococcus faecalis*	/	/	329 (283, 299)
Type LMG 6901	/	/	/	*Lactobacillus delbrueckii* subsp. *bulgaricus*	/	/	330
Type LMG 11457	/	/	/	*Lactobacillus parabuchneri*	/	/	345 (255, 299)

TABLE 2. LH-PCR fragment length database of 14 strains isolated from cheese samples belonging to different RAPD-PCR clusters and six type strains. † Refers to the quantity of strains isolated for this RAPD-PCR

cluster or branch; ‡ GenBank; * The fragment length is reported in base pairs with an approximation of ±1 bp. The lengths of the secondary peaks are reported in brackets.

LH-PCR analysis of microbial community. LH-PCR and RT-LH-PCR have already been successfully used for the analysis of fresh dairy products (14, 25), and to monitoring lactic acid bacteria dynamics during maize ensiling (1). To our knowledge this technique has never been used to study a ripened cheese where viable, not viable and lysed microbial cell are contemporaneously present. Analysis of intact DNA extracted from degraded specimens and tissue sample has become a useful tool for criminal and conservation forensics. In an intriguing review Nielsen and co-authors studied the release, breakdown and persistence of bacterial and plant DNA in soil, sediment and water (20). The recovery of DNA in processed food has already been used for detection and quantification of genetically modified ingredients (16). However, to our knowledge this approach has never been used to evaluate the presence of DNA from lysed cells in fermented food.

Fig. 2 and 3 show the LH-PCR profiles obtained from the entire and lysed cells of LAB in different stages of PR cheese manufacturing. Only the electropherograms referred to the most representative samples (Whey starter, Curd 48 h, Salted cheese, 2, 6 and 24 months cheese) are shown; the other analysed samples did not add any further information and thus are not reported.

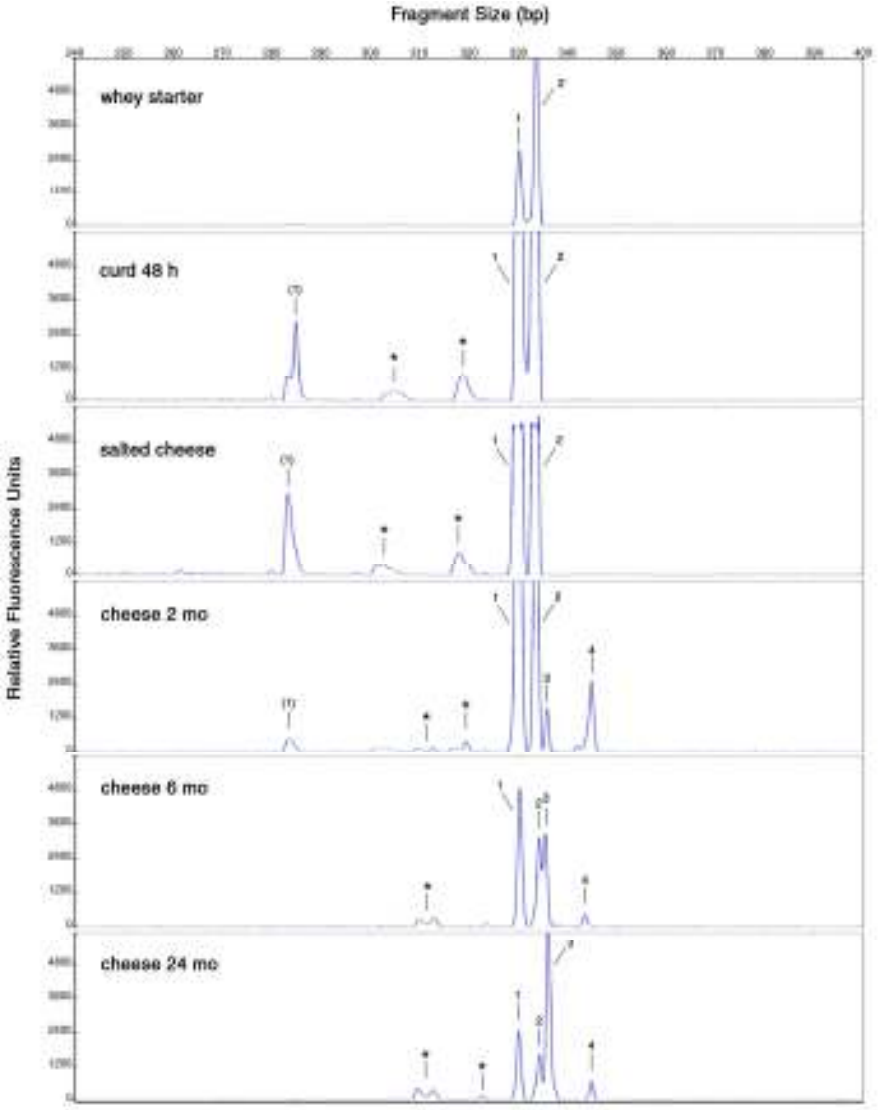

FIG. 2. Length heterogeneity LH-PCR electropherograms of entire cells of LAB community present in the samples studied at different stages of manufacturing and ripening of Parmigiano Reggiano cheese. The *x* axis shows peak size in base pairs, and the *y* axis shows peak intensity in relative fluorescence units. The peak sizes were attributed to bacterial species according to the LH-PCR database (Table 2) as follows: 1, *L. delbrueckii* subsp. lactis or subsp. *bulgaricus*; (1) Secondary peak of *L. delbrueckii* subsp. *lactis*; 2, *L. helveticus*; 3, *L. rhamnosus* or *L.* casei or *L. plantarum*; 4, *L. parabuchneri* or *P. acidilactici.* Not attribuited peaks are indicated by *.

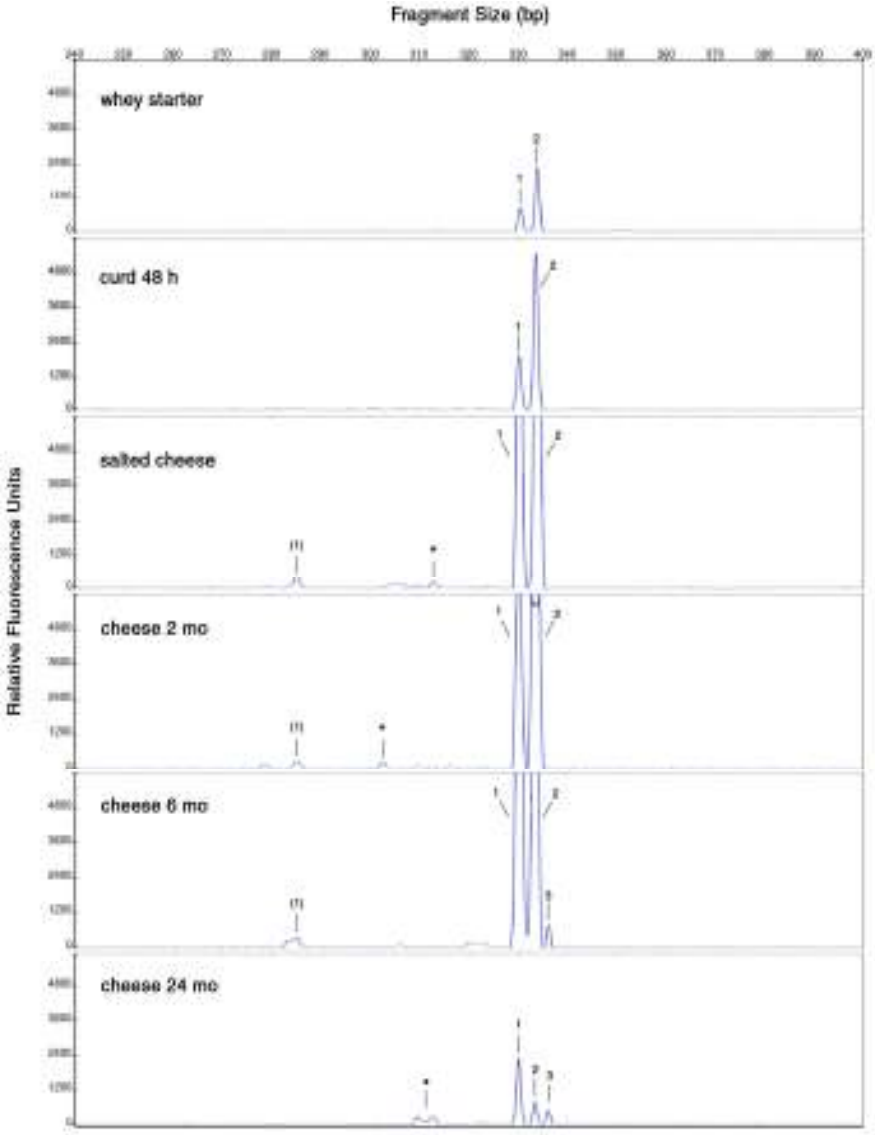

FIG. 3. Length heterogeneity LH-PCR electropherograms of lysed cells of LAB community present in the samples studied at different stages of manufacturing and ripening of Parmigiano Reggiano cheese. The *x* axis shows peak size in base pairs, and the *y* axis shows peak intensity in relative fluorescence units. The peak sizes were attributed to bacterial species according to the LH-PCR database (Table 2) as follows: 1, *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus*: (1) Secondary peak of *L. delbrueckii* subsp. *lactis*; 2, *L. helveticus*; 3, *L. rhamnosus* or *L. casei* or *L. plantarum*. Not attribuited peaks are indicated by *.

The different fragment sizes in the LH-PCR profiles were attributed to bacterial species according to the LH-PCR database.

The peaks detected in the raw milk entire cells electropherogram were: 330 bp (attributed to *Lactobacillus delbrueckii* subsp. *lactis* or subsp. *bulgaricus* or *Enterococcus faecium* or *Enterococcus faecalis*), 334 bp (*L. helveticus*), 336 bp (*Lactobacillus rhamnosus* or *Lactobacillus plantarum* or *Lactobacillus casei*), and 339 bp (not attributed). The fluorescence intensity was more than 10-fold lower than the average found in the other samples. As expected the amount of DNA from filtered raw milk, released from lysed cells, was too low to be amplified (data not shown). In fact the milk suitable for PR caseification has to be fresh, eventually refrigerated at a temperature not below 10°C for less than 12 h. During the natural creaming, non starter lactic acid bacteria grow depending on environmental condition (17).

The LH-PCR profile from entire LAB of natural whey starter showed 2 peaks (Fig. 2), corresponding to *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus* and *L. helveticus* species. Differently from previous studies on natural whey starter for a similar cheese as Grana Padano (5, 14, 25), *Streptococcus thermophilus* peak (320 ± 1 bp) was not found. LH-PCR profile from lysis-released DNA (Fig. 3) of natural whey starter was similar to the entire cells profile. Whey starter is expected to have a high percentage of metabolically active cells. Our results highlight an autolysis phenomenon which could have happened after the entering of cells in a non viable state, previously described in natural whey starter for Grana Padano cheese (10).

In the 48 h curd entire cells electropherogram (Fig. 2), two major peaks attributable to *L. helveticus* and *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus* were found. Two other not attributed peaks of minor fluorescence intensity were detected. In the LH-PCR profile from lysis-released DNA (Fig. 3) of 48 h curd only the two attributable peaks were found.

The data show that, after one month of brining, salted cheese sample is characterized by a peak pattern of entire cells similar to the previous one (Fig. 2), while in the lysis-released DNA profile (Fig. 3), the peaks are higher than that obtained with 48 h curd. This result could highlight an increase of cells autolysis after the brining.

At 2 months of ripening, in addition to *L. helveticus* and *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus,* two other peaks, attributable to *L. rhamnosus* or *L. casei* or *L. plantarum* and to *Lactobacillus parabuchneri* or *Pediococcus acidilactici,* appear in the

electropherogram referred to entire cells (Fig 2). They did not appear in the electropherogram referred to lysed cells (Fig. 3)

LAB microbial composition of 6 months cheese samples maintained the same electropherogram profile of previous samples in the case of entire cells (Fig. 2), only reducing in the peaks intensity. Instead, in lysis-released DNA electropherogram (Fig. 3), a peak attributable to *L. rhamnosus* or *L. casei* or *L. plantarum* appears. This trend persisted without appreciable variations, in the subsequent samples until 24 month of ripening.

In the 24 months cheese electropherogram referred to entire cells (Fig. 2), it is possible to observe a trend reversal, where the major peak becomes that attributable to *L. rhamnosus* or *L. casei* or *L. plantarum*. Notably, in the lysis-released DNA electropherogram of the same sample cheese, a significant decrease of fluorescence intensity was observed. The lack of this decrease in the 20 months lysed cells cheese sample (data not shown), seems to be related to some process occurring during these latest four months of cheese ripening, when a particularly hostile nutritional environment maybe establishes.

The DNA degradation can be due to many factors that affect its chemical and physical integrity in various environments. Nielsen et al. (20), describe the effect of DNase able to convert DNA to deoxyribose, inorganic orthopospate, and purine and pyrimidine bases in soil, water and sediment. Moreover they described numerous physical condition and chemical compounds that severely compromise the integrity of DNA molecules exposed to the extracellular environment (20). Another research evaluated the effect of meat sample composition on the persistence of chromosomal and plasmid DNA, concluding that the DNA originated from dead cells can cause false-positive quantitative real-time PCR results in food (32). On the other hand, a study about the natural transformation and environmental fate of DNA released by cell death, evidenced that the presence of cell debris, proteins released during lysis and the DNA complexing agents like DNA-binding proteins, did not dramatically influenced transformation efficiencies (13). Even if pure nucleic acids are generally not sufficient as a sole carbon source for bacteria, it has been demonstrated that *Serratia marcescens* and *Escherichia coli* are capable of utilizing DNA exclusively for carbon (20). In 1986, Thomas et al. (29) and in 2000, Williams et al. (31), hypothesized that non-starter LAB could use, as an alternative potential energy sources, nucleic acids derived from the autolysis of starter ones. In agreement with Thomas intuition and Nielsen et al. (20) studies, we can suppose that DNA degradation of some microorganisms could be a

source of carbon, nitrogen, phosphorus and nucleic acid precursor for less nutritional demanding bacterial cells in a particularly hostile nutritional environment. This hypothesis could be supported by sugar contents measured (M. Gatti, submitted for publication) in the cheese samples considered in this research. We observed, in fact, that at the beginning of the process, lactose and galactose were totally consumed. At the same time an expected increase of lactate was observed. The values found for lactate, citrate and succinate, in agreement with Careri et al. (2), but in disagreement with Coppola et al. (4), were the same in all the samples during aging. Thus, lactate and citrate have not been used as an energy source by survived microorganisms (data not shown). However, amino acid metabolism should be considered as an alternative energetic source.

3.3.5 CONCLUSIONS

Parmigiano Reggiano is a Protected Designation of Origin, appreciated and economically important Italian cheese. Microbiological features of this product have been already studied by means of traditional approaches.

In our opinion this paper leads to new findings that advance the understanding of microbial dynamics in a complex fermented ecosystem. Having twin wholes available, allowed us to have samples representative of the subsequent stages of the same PR dairy process. The use of a culture independent method as LH-PCR overcame traditional agar plate and culture dependent method limitations. Moreover, the modality of LH-PCR samples preparation allowed to evaluate both the entire and lysed cells evolution during cheesemaking and ripening.

To our knowledge this approach has never been used to study a ripened cheese where viable, not viable and lysed microbial cells coexist.

In this research we have observed that the thermophilic microflora of natural whey starter for PR is mainly composed by *L. helveticus* and *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus*. Being LH-PCR technique unable to distinguish *L. delbrueckii* subspecies, we can not attribute with certainty the former or the latter subspecies to the revealed peak. However, the presence of a secondary peak characteristic just for *L. delbrueckii* subsp. *lactis,* suggest that this is the species that better develop during the first 48 hours of production. Differently from Coppola et al (2000) we never isolated *L. delbrueckii* subsp. *bulgaricus* species which could have been viable but hardly cultivable. Identification of other species grown in the curd has not been possible. In fact, peaks in

the 300-325 bp range were not attributed due to the lack of database peaks in this range. *L. helveticus* and *L. delbrueckii* subsp. *lactis* were the dominant species until the second month of ripening, even if an increasing number of them underwent to autolysis process.

One month after brining, at least two new species were able to grow in the cheese: *L. rhamnosus* or *L. casei* or *L. plantarum*, and *P. acidilactici* or *L. parabuchneri*. Strains belonging to the first four species have been isolated during this research. In this stage of PR production, lysed cells of *L. helveticus* and *L. delbrueckii* species were revealed. Non attributable peaks were found in both lysed and entire cells LH-PCR profiles suggesting the presence of other unknown species. After six months of ripening, the same species were found even if no one of them seems to be dominant. Interestingly, in this stage of ripening, also *L. rhamnosus* or *L. casei* or *L. plantarum* which seems to increase, undergo to autolysis process. From the sixth to the twentieth month of ripening any microbial evolutionary change were observed. It is well known that during long PR cheese ripening, autolysis of bacterial cells results in liberation of the cytoplasm content, including intracellular enzymes. Intracellular enzymes released are considered to be highly important for cheese ripening as they play a key role in texture changes and flavour development (Broadbent, Steel 2005). Moreover the cytoplasmatic content includes even DNA. It could be converted in molecules available as energetic source when a particularly hostile nutritional environment establishes. This hypothesis could explain the DNA amount reduction we observed during the last four months of ripening.

Monitoring both entire and lysed cells through LH-PCR allows to coming aware of the importance of this two fractions in PR cheese production and ripening. This approach opens perspectives for insight microbial evolution in fermented food environment.

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3.4 A Model to Assess LAB Aminopeptidase Activities in Parmigiano Reggiano Cheese During Ripening

3.4.1 ABSTRACT

The aim of this work was to investigate in which phases of ripening of Parmigiano Reggiano cheese, lactic acid bacteria aminopeptidases present in cheese extract could be involved in release of free amino acids and better understand the behavior of these enzymes when suffer physical-chemical parameters changes that force an actuation far from their *optimum*. In particular we evaluated six different substrates to reproduce PepN, PepC, PepA, PepL, PepI and PepX activities releasing different N-terminal amino acids. The effects of pH, NaCl concentration and temperature on the enzymes activities of amino acid βNa-substrates were determined by modulating the variables in 19 different runs according to a three factor, five-level Central Composite Design. This experimental design allowed us to tune a mathematical model able to assess the effect on aminopeptidases activities over a range of values covering different environmental conditions in different zones of the wheel at different aging times. The aminopeptidases tested in this work were present in cell free Parmigiano Reggiano cheese extract after a 17 months ripening and were active when tested in model system. The modeling approach explicit that to highlight the individual and interactive effects of chemical-physical variables on enzyme activities, is helpful to determine the true potential of an aminopeptidase in cheese. Our results evidenced that the six different LAB peptidases participate to cheese proteolysis and are induced or inhibited by the cheese production parameters which, in turns, depend on the cheese dimension. The models elaborated varying pH, temperatures and salt concentration resulted to be a useful, low costly and time-consuming tool to understand the role of the main peptidases in the different phases of cheese ripening in relation to the major environmental factors influencing enzyme activity.

Keywords: aminopeptidase activity, lactic acid bacteria, Parmigiano Reggiano cheese, predictive model

3.4.2 INTRODUCTION

Parmigiano Reggiano (PR) is a typical Italian hard cooked cheese produced from raw partially skimmed milk with the aid of a natural whey starter culture mainly composed of thermophilic lactobacilli like *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis*, which largely dominate the bacterial population during the first part of ripening (Mucchetti et al., 2006).

During the ageing process, PR milk curd caseins undergo to an extensive degradation. This proteolysis occurs in two steps. In the first step, peptides are generated through the action of plasmin (Ferranti et al., 1997; Gaiaschi et al., 2000; Considine et al. 2002) and bacterial proteinases (Nielsen, 2002; Caira et al., 2003). In the second step, bacterial aminopeptidases liberate free amino acids (Resmini et al., 1985) and different sized peptides (Addeo et al., 1992; Fornasari et al. 2003) from the peptides generated in the first one (Gatti et al. 1999; Gatti et al. 2004).

It is well known that the total amount of free amino acids increases during ripening of PR (Resmini et al., 1985) and some aminopeptidase of lactic acid bacteria (LAB) are present and active throughout ripening in Grana Padano, PR (Gatti et al., 1999; Sforza et al., 2004), Emmental (Gagnaire et al. 1998), Cheddar or Saint Paulin cheeses (Chapot-Chartier et al. 1994; Wilkinson et al., 1994; Crow et al., 1995). However, in which phase of the cheese manufacture and ripening these LAB peptidases play their role, and how they act in relation to the physical-chemical parameters, it is not well known. In fact, from the initial steps of the cheese making up to the end of ripening, changes of the parameters pH, temperature and NaCl concentration modify the environment and force the enzymes to act under condition far from their *optimum*. Cooking temperature of the curd, slow heat dissipation during lactic acid fermentation, whey drainage from the curd, drop of pH within the first 24 hours and its slow climb (Pellegrino et al., 1997; Neviani et al., 1998; Mucchetti and Neviani, 2006), brining and uptake of NaCl and its slow diffusion into the cheese (Mucchetti and Neviani, 2006) are factors that, deeply modifying the matrix, regulate growth of microorganisms, induce autolysis and influence the activities of bacterial peptidases.

Lact. helveticus and *Lact. delbrueckii* subsp. *lactis* harbor aminopeptidases able to cleave different N-terminal amino acids from peptides (Kunji et al., 1996; Savijoki et al., 2006). Peptidase activities are high in cheese where the high number of microorganisms is associate with an extended autolysis (Gatti et al., 1999). The activity of these

peptidases is very important during ripening because they supply free amino acids which can be further metabolized with a relevant impact on cheese flavor or can be useful for not starter LAB (Fernandèz and Zuniga, 2006). The amino acid resulting from proteolysis are the major precursor of specific flavors compounds, such as various alcohols, aldehydes, acids, esters, and sulfur compounds (Smit et al., 2005). For example, the branched amino acid leucin is firstly converted to the correspondent α-ketoacid (α-ketoisocaproate) which can be transformed by oxidative decarboxylation (isovalerate), decarboxylated to aldehydes (3-methylbutanal) and related alcohols (via alcohol dehydrogenase) or reducted to hydroxyacid (2-hydroxyisocaproate).

A typical PR cheese wheel has a diameter of about 44 cm and is high about 20 cm. The total weight is around 35-38 kg. Given these proportions and the conditions of cheese-making and ripening, the values of pH, NaCl concentration and temperature can markedly vary in relation to the stages of ripening and the zone of the cheese shape. Each of these zones is characterized by variables which can remarkably differ from the other zone during the ripening (Mucchetti and Neviani, 2006).

The aim of this work was to investigate in which stages of ripening of Parmigiano Reggiano cheese, LAB aminopeptidases present in cheese extract could be involved in release of free amino acids. In particular we evaluated six different substrates to reproduce PepN, PepC, PepA, PepL, PepI and PepX activities releasing different N-terminal amino acids.

An experimental design allowed to tune a mathematical model able to assess the effect of NaCl concentration, as well as pH and temperature on aminopeptidases activities over a range of values covering different environmental conditions in different zones of the wheel at different aging times. This offers a prediction with minimum requirements on experimental data, which makes the approach innovative, less costly and time-consuming than direct analyses.

3.4.3 MATERIALS AND METHODS

Cells free cheese extract preparation

Seventeen months ripened PR cheese was kindly supplied by the Consorzio del formaggio Parmigiano-Reggiano (Reggio Emilia, Italy). Fifteen grams of cheese previously minced with sterilmixer (pbi International, Milano, Italia) (11,000 rpm for 10 sec) were homogenized with 50 mL of 0.05 mol l-1 sodium phosphate buffer pH 7.0 with Stomacher 400 circulator (Pbi International, Italy) (230 rpm for 5 min), centrifuged (12,000 x g for 10 min at 4°C), filtered 0.22 µm pore size

(Sartorius, Firenze, Italy), 24 h dialyzed in cellulose tube-shaped (Spectra/por, Spectrum Laboratories Inc, USA) with cut off 3,000 daltons and lyophilized.

Upon use the lyophilized sample was diluted in the same initial volume of citric buffer 0.05 mol l-1 at five different pH defined for the model system like demonstrated ahead.

Choice of parameters and peptidase activities

In order to describe the phases of ripening of PR cheese, five different values of three different parameters (pH, NaCl concentration on dry matter in percentage, and temperature) were chosen (Table 1). The choice of these values covered the range of these parameters characterizing the long ripening of PR, the different zone of the whole and the possible different environmental condition of aging rooms.

The aminopeptidase activities tested were those able to hydrolyze six different substrates: broad specificity aminopeptidase N (PepN) activity was determined using Lys-βNA, broad specificity aminopeptidase C (PepC) using Arg-βNA, proline iminopeptidase (PepI) activity using Pro-βNA, glutamyl aminopeptidase A (PepA) activity using Glu-βNA, peptidase with high specificity for leucine and alanine (PepL) using Leu-βNA and activity of the X-prolyl dipeptidyl aminopeptidase (PepX) was determined using Phe-Pro-βNA (Christensen et al. 1999).

Run	pH	NaCl (%)	Temperature (°C)	Peptidase					
				PepC	PepX	PepI	PepL	PepA	PepN
1	5.00	6.0	12	8.70	240.50	0.38	2.02	0.41	7.02
2	5.25	1.5	15	52.40	315.28	1.15	19.95	0.85	39.72
3	5.25	1.5	22	106.33	501.70	2.04	38.82	1.33	80.73
4	5.25	4.5	15	49.57	352.30	1.03	16.40	0.87	32.92
5	5.25	4.5	22	99.97	589.40	1.73	34.23	1.15	66.73
6	5.75	1.5	15	224.93	492.36	2.87	64.06	1.31	167.49
7	5.75	1.5	22	372.67	699.27	4.34	105.72	1.82	277.80
8	5.75	4.5	15	194.26	510.38	2.30	55.71	1.17	139.50
9	5.75	4.5	22	335.56	717.57	3.70	90.89	1.55	259.51
10	5.00	3.0	19	28.74	354.35	0.80	13.30	0.75	23.09
11	6.00	3.0	19	406.85	677.48	4.01	122.96	1.59	298.25
12	5.50	0.0	19	190.23	462.64	2.77	53.46	1.56	150.56
13	5.50	6.0	19	141.17	556.89	2.01	43.74	0.97	96.86
14	5.50	3.0	12	72.28	347.07	0.77	21.38	0.69	58.03
15	5.50	3.0	25	261.52	750.36	3.49	77.98	1.46	183.90
16	5.50	3.0	19	184.64	558.58	2.36	51.76	1.03	143.56
17	5.50	3.0	19	178.49	565.40	2.28	54.20	1.22	133.92
18	5.50	3.0	19	183.40	566.30	2.27	47.29	1.13	139.27
19	6.00	0.0	25	546.52	850.90	5.93	152.34	2.36	517.22

Table 1. Experimental design used to test the influence of pH, NaCl concentration and temperature on the selected peptidase activities. In the columns on the right are reported the values expressed as arbitrary unit enzymatic activity EA (μmol β-naphthylamide released h^{-1} mL^{-1} of sample) for every peptidase in each run of the experimental design.

Enzymes assay

Peptidase activities were assayed by using 0.656 mol l^{-1} solutions of each βNa-substrates, prepared as provided by the manufacturer (Bachem Feinchemikalien AG, Bubendorf, Switzerland) in different solution of sodium phosphate buffer 0.05 mol l^{-1} pH defined for the model system without or with NaCl presence. 250 μL of the sample and 250 μL of βNA-substrate were incubated for different periods, individualized for each amino acid-βNa (Lys-βNA 4 hours; Arg-βNA and Phe-Pro-βNA 6 hours; Leu-βNA 16 hours; Pro-βNA and Glu-βNA 30 hours), at the temperature defined for the model system. The reaction was stopped adding 250 μL of 2.0 mol l^{-1} HCl. The degree of hydrolysis was determined by measuring the colored product of an azocopulation reaction by reading spectrophotometrically the optical density at 580 nm (A_{580nm}) according to Bouquien et al. (1988).

Each assay was carried out in duplicate and average values were calculate. The arbitrary unit of enzyme activity (EA) was defined as the number of micromoles of β-naphthylamide released in one hour in one mL of sample.

Modelization of enzymatic activities

The effects of pH, NaCl concentration and temperature on the enzyme activity on six different βNa-substrates were determined by modulating the variables in 17 different runs according to a three factor, five-level Central Composite Design (CCD) (Box et al. 1978). In addition to these 17 combinations, other two runs were added in order to consider favorable (run 19, Table 1) and unfavorable (run 18, Table 1) extreme conditions for the enzyme activity and amplify the sampling space (Table 1). These two combinations are respectively the nearest and the farthest from the optimal growth condition of starter LAB, source of the enzymes object of this research.

Modeling was aimed at describing the enzyme activity as function of the independent variables of the experimental design. A software package (Statistica for Windows, Statsoft, Tulsa, OK) was used to estimate the coefficients of the quadratic polynomial equation regression analysis:

$$y = B_0 + \Sigma B_i x_i + \Sigma B_{ii} x_i^2 + \Sigma B_{ij} x_i x_j$$

were y is the dependent variable (peptidase activity), B_0 is the constant, B_i, B_{ii} and B_{ij} are regression coefficients of the model, and x_i and x_j are the independent variables (pH, NaCl concentration and temperature). In the final models, variables with significance lower than 95% ($P < 0.05$) were excluded through a backward stepwise regression.

3.4.4 RESULTS AND DISCUSSION

Peptidase activities

In Table 1 are reported the six enzymatic activities, expressed as the number of micromoles of β-naphthylamide released in one hour in one mL of sample, detected in each run of the experimental design. As expected, the conditions defined as worst and better case for the expression of the activities (run 18 and 19, respectively) were characterized by their lower and higher values. Quantitatively, in favorable condition the higher activity was PepX, followed by PepC and PepN which were very similar. PepL activity was more then five time lower than PepX, and PepA and PepI were very low. These data are in agreement with those previously found in PR cheese (Gatti et al 1999) and in thermophilic *Lactobacillus* of natural whey starter culture traditionally used for its production (Gatti et al 2004). In spite of the low

activity of PepI and PepA, proline and glutamic acid are free amino acids mainly detected in ripened Parmigiano Reggiano (Careri et al. 1996). Regarding proline, it is possible to suppose that specialized aminopeptidases, like PepR or PepQ, capable of hydrolyzing proline-containing sequences (Kunji et al., 1996), are important for the degradation of casein-derived peptides because of the high content of proline in these molecules (Mucchetti et al 2006). As starter LAB do not further metabolize glutamic acid, its accumulation in cheese reflect this phenomenon. Moreover, the association of glutamic acid with pyroglutamic acid (pGlu) cyclase activity was demonstrated for thermophilic lactic species. The cyclization of glutamic acid in pGlu was proposed by Muchetti et al. (2000) as a ripening marker in long ripened cooked cheese.

Modelling of the peptidase activities

The data obtained for each activity in the 19 runs of the experimental design were fitted with a quadratic polynomial equation using a backward stepwise procedure which allowed to simplify the models by keeping in the final equation only the terms with a significance higher than 95% (P<0.05). The final models obtained are showed in Table 2. All the three independent variables were included in at least one term of each equation. The models were all highly significant, as demonstrated by the diagnostics reported in Table 2 (R^2 and P-values derived from the F-test) and by the relationship between predicted and observed values (data not shown).

To evidence the effects exerted by the 3 variables on the behavior of the enzymatic activities, a first series of graphics were drawn with equations obtained by dividing the models by the maximum activity recorded (*i.e.* the value recorded in run 19). With such transformation, the predicted activities were normalized and varied between 0 (no activity) and 1 (maximum activity observed in run 19). In Figure 1a are reported the normalized influence due to the pH variation when the other 2 variables were kept constant at their mean values (*i.e*. temperature 19°C and NaCl 3%). pH has a similar influence on PepN, PepC, PepL and PepA, which increased their activities more than proportionally (due to the presence in the model of the quadratic term with a positive sign) with the diminution of acidity. This increase was approximately linear for PepI, while the positive effect of pH increase on PepX was gradually reduced at the higher values of pH. Increasing amount of NaCl (pH 5.5 and temperature 19°C) determined a general linear decrease of all the enzymatic activities considered with the exception of PepC, which was

scarcely affected by this variable, and PepX whose activity was higher at increasing NaCl percentage, at least until concentration of about 4% (Figure 1b).

The effect of temperature (showed in Figure1c for pH 5.5 and NaCl 3%) is similar and an almost linear increase of all the enzymatic activities considered was observed when the temperature augmented from 12 to 25°C. If in the winter season the temperature of aging rooms decreases, for example 12°C like predicted in the CCD, the hydrolysis would be reduced until 50%. The maintenance of temperature around 18°C could be an important compromise to safeguard a good level of enzymatic activity, to limit the development of undesirable microorganisms and to obtain a correct cheese exudation. This consideration is in agreement with the Parmigiano Reggiano disciplinary of production which state that the temperature has not to falls below 16°C (http://www.parmigiano-reggiano.it).

The normalization of the enzymatic activity responses indicated that generally they were negatively affected by pH and temperature decreases, while NaCl concentration increase, within the range of concentration considered here, was favorable only for the dipeptidyl aminopeptidase PepX and negative for all the other peptidases tested. However, the evaluation of the Pareto charts (data not shown) that illustrate the dominant terms in the models affecting the response variables and their statistical significance showed that the interaction pH×Temperature was the most important in 4 of the 6 models (*i.e.* PepX, PepN, PepC and PepI) and particularly in the three more active peptidases. Temperature and pH were the most important variables for PepA and PepL, respectively. To better evidence the effects of the interactions, in Figures 2 and 3 are reported the surface responses obtained for PepL and PepX.

The increase of NaCl from 0 to 6% caused a limited reduction of PepL independently on pH and temperature (Figure 2a and b). The importance of the interactive effect pH×temperature is evident in Figure 2c which shows that the increase caused by higher pH values is enhanced by increasing temperature. PepX was, on the contrary, strongly influenced by NaCl concentration (Figure 3a and b). In figure 3c, drawn keeping constant the temperature at the mean value 19°C, this effect was clear at the less favorable pH but was limited by pH values near to 6.0. On the contrary, the positive effect of the increase of NaCl concentration was not limited by the temperature increase, even if there was no interaction between these 2 variables. Finally, the PepX activity was higher with both temperature and pH increases.

It is interesting to note that the increase of NaCl percentage on dry matter characterizing PR in different zones of the cheese during the ripening had different effect on PepX with respect to other aminopeptidases tested. This information may induce to presuppose that the salt works as a co-factor in this hydrolytic reaction important in the cheese ripening.

Coefficient	PepC	PepX	PepI	PepL	PepA	PepN
Intercept	4994.126	-6946.460	-0.421	1817.157	-2.261	5572.290
pH	-1768.591	2138.964	-	-681.591	-	-1824.740
NaCl	-[a]	284.280	-	-	-	-
Temperature	-118.228	-	-0.812	-22.029	0.079	-143.868
pH^2	154.353	-162.696	-	63.203	0.075	145.857
$NaCl^2$	-0.833	-5.203	-	-0.244	0.011	-
$Temperature^2$	-	-	-	-	-	-
pH×NaCl	-	-43.223	-0.018	-	-	-1.623
pH×Temperature	23.879	5.467	0.178	4.707	-	28.155
NaCl×Temperature	-	-	-	-	-0.007	-
R^2	0.991	0.997	0.980	0.993	0.967	0.992
F-test (*P*-value)	382.653 (>0.000001)	600.153 (>0.000001)	254.831 (>0.000001)	369.517 (>0.000001)	101.800 (>0.000001)	333.234 (>0.000001)

Table 2. Models obtained for each peptidase activity by fitting the experimental data with a second order polynomial equation. In the final models only the terms with a significance higher than 95% ($P<0.05$) were considered, according to a backward stepwise procedure. The R^2 and the F-test (with the corresponding *P* value) are also reported.

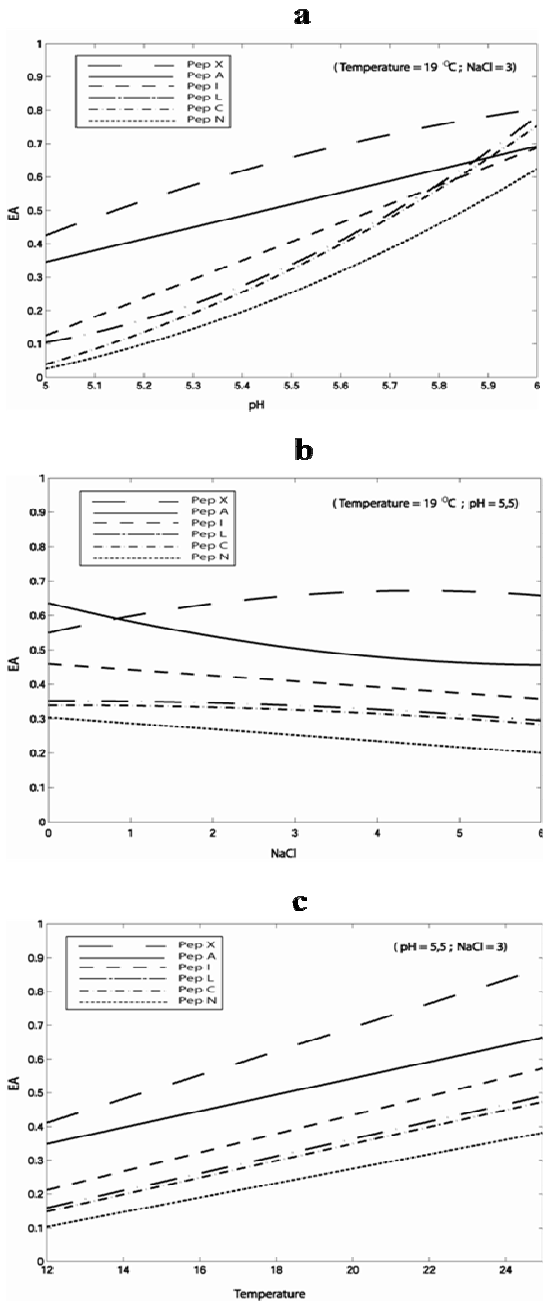

Figure 1. Effect of single environmental variables (a, pH; b, NaCl concentration; c, temperature) on enzyme activities (EA). Enzyme activities are reported as percentage of the values obtained under most favorable conditions (Run 19).

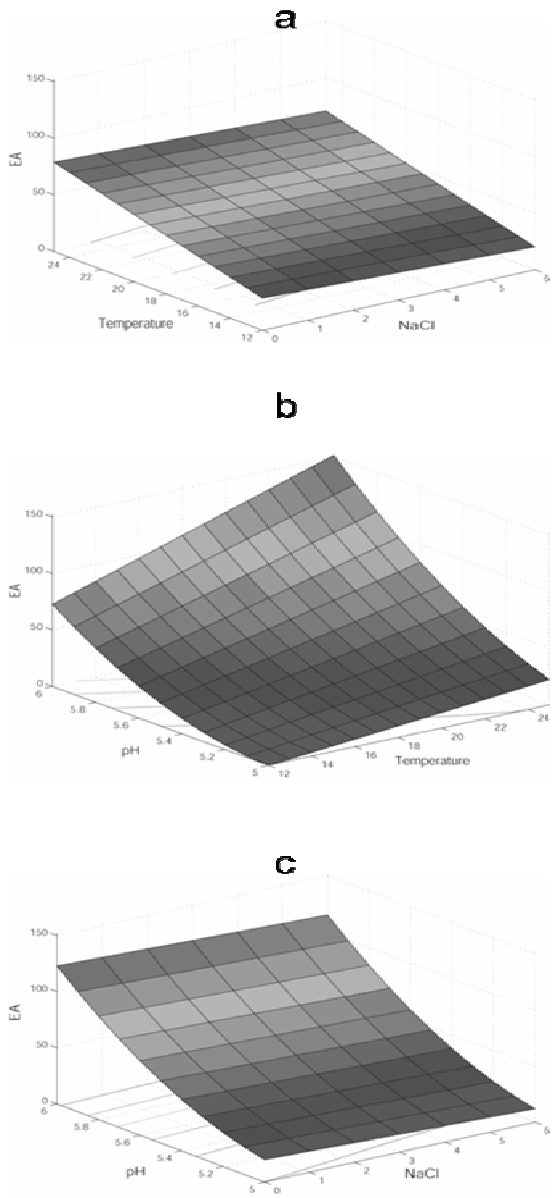

Figure 2. Combined effect of two environmental variables (a, temperature and NaCl concentration; b, pH and temperature; c, pH and NaCl concentration) on the PepL enzymatic activity (EA) (μmol β-naphthylamide released h^{-1} mL^{-1} of sample). The variable not present in each figure is kept constant to its central value (i.e. pH 5.5, NaCl 3.0%, temperature 19°C).

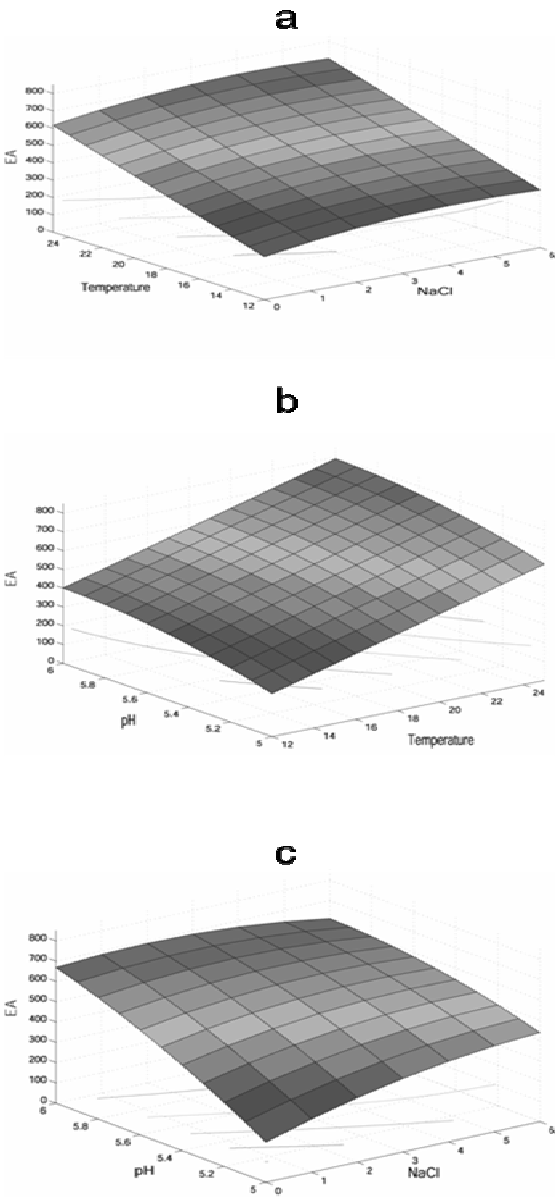

Figure 3. Combined effect of two environmental variables (a, temperature and NaCl concentration; b, pH and temperature; c, pH and NaCl concentration) on the PepX enzymatic activity (EA) (µmol β-naphthylamide released h^{-1} mL^{-1} of sample). The variable not present in each figure is kept constant to its central value (i.e. pH 5.5, NaCl 3.0%, temperature 19°C).

Enzymatic activities prediction

Enzymatic activities were predicted using the six models in two different zones of cheese with different environmental values that characterized the cheese during ripening (Table 3). The values reported in the table 3 simulate the trend of the independent variables during the ripening after brining period (about 1 month) until 12 months with optimal aging room temperature kept constant at 18°C. These values have been defined on the basis of literature data (Mucchetti and Neviani 2006) as well as experimental cheesemakings carried out at the Centro di Ricerca per le Produzioni Foraggere e Lattiero-casearie (CRA-FLC, Lodi, Italy) (Mucchetti and Fornasari, personal communication). In general, NaCl concentration decreases moving towards the inner part of the cheese even if the difference becomes less pronounced during ripening. pH is slightly lower in the inner zone, but the difference is reduced (few decimal units) and an increase can be observed during ripening.

Considering that the external part of cheese, corresponding to 3 cm surface layer under rind, and the internal part of the shape that is the innermost part of the cheese, enzymatic activity of all peptidases except for PepX, in external part is minor with respect to the inner part (Figure 4). The conditions which characterized NaCl concentration and pH during ripening determined a progressive increase of all the enzymatic activities considered here. However, for five peptidase activities (and namely, PepN, PepC, PepA, Pep I and PepL, the latter showed in Figure 4) the highest values were always predicted in the inner part of cheese and, in addition, the difference between the external and internal activities throughout all the ripening period remained quite constant. In contrast, PepX activity was predicted higher in the external cheese area (due to the NaCl content) but the difference between inner and external cheese was reduced during ripening because of a more homogeneous distribution of salt. Variation in temperature of 2°C determined changes in enzyme activities ranging between 10 and 20% (data not shown).

However, in these experiments, we did not considered the enzyme concentration that is strictly linked to the microbial development and autolysis that are higher in the external part of cheese. Therefore, in the external part, lower peptidases activity would be compensate by major enzyme concentration. A study is currently being performed to verify this hypothesis.

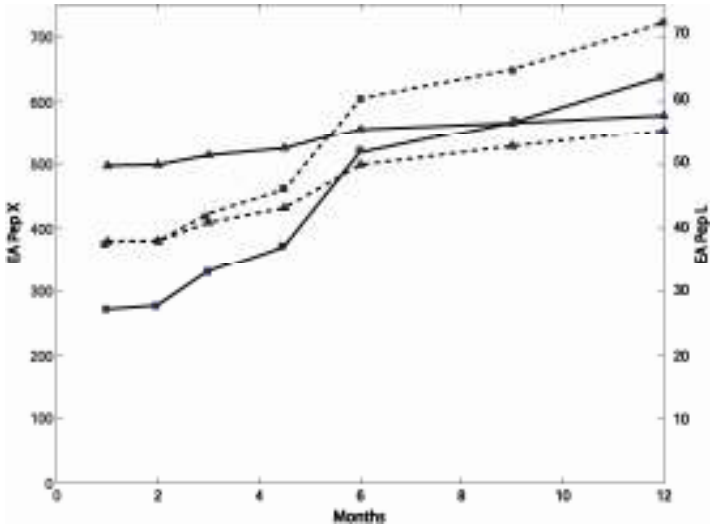

Figure 4. Predicted enzymatic activities of PepX (Δ) and PepL (■) in two different zones of Parmigiano Reggiano cheese wheel during different steps of the cheese ripening at 18°C. Dotted line = Internal zone. Solid line = External zone.

3.4.5 CONCLUSIONS

The conversion of peptides to free amino acids by peptidase system is a central metabolic activity in LAB and become the major phenomena during ripening of aged cheese above all when, after the decline of viability of the thermophilic starter, cellular autolysis occurs (Gagnaire et al., 1998) and the enzymes are free and active in the cheese even after a long ripening time (Gatti et al., 1999). The availability of such free amino acids is crucial for cheese flavour and overall characteristics because of the further transformations which they are subjected to. These transformation can also be fundamental for survival and multiplication of ripening bacteria in an environment characterized by stringent conditions. In fact, it is presumable that bacteria gain energy and regenerate NADH from the degradative pathways of amino acids (Fernàndez and Zuniga, 2006).

A variety of proteolytic enzymes released by lysed LAB cells are present in an active form in the cheese, which together with the indigenous milk proteinases, contributes to an extensive degradation of cheese casein. The peptidases tested in this work were present in cell free PR extract after a 17 months ripening and were active when tested in model system. The β-napthylamide derivatives has been chosen to

evaluate the activities of the free enzymes arising from starter lactic acid bacteria autolysis (Christensen et al., 1999). PepX was found in a lot of mesophilc and thermophilic LAB, while PepN (Savijoki et al., 2006) and PepC could represent *Lact. helveticus* and *Lact. delbrueckii* subsp. *lactis* activities (Dudley and Steel, 1994; Varmamen et al., 1994; Christensen et al., 1999). Interesting, Arg-ßNA, used to identify PepC, could evidence even PepS activity purified in *St. thermophilus* (Fernanzed-Espla and Rul, 1999). PepL (Savijoki et al., 2006) could be representative of *Lact. delbrueckii* subsp. *lactis* activities (Klein et al., 1995). Proline in N-terminal position is generally hydrolyzed by PepI (Savioky et al., 2006) and Glu-ßNA is the substrate preferably hydrolyzed by glutamil aminopeptidase PepA purified from *S. thermophilus* (Rul et al., 1995).

The modeling approach explicit that to highlight the individual and interactive effects of chemical-physical variables, such as pH, NaCl concentration and temperature on enzyme activities, is helpful to determine the true potential of an aminopeptidase in cheese. The pH had the greatest effect on enzyme activity although temperature plays a relevant role too. Salt concentration seems to be important to slow down PepC, PepA, PepI and PepL. Interestingly it did not affect PepN and positively affected PepX. Salt tolerance is an important feature for starter LAB enzymes and not starter LAB cheese microflora since high concentration of NaCl enter in the whole during the ripening after the brining period.

Our results evidenced that the six different LAB peptidases participate to cheese proteolysis and their activity is enhanced or inhibited by the cheese ripening parameters which, in turns, depend on the cheese dimension. The models elaborated varying pH, temperatures and salt concentration resulted to be a useful, low costly and time-consuming tool to understand the role of the main peptidases in the different phases of cheese ripening in relation to the major environmental factors influencing enzyme activity.

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4. GENERAL CONCLUSIONS

During this Ph.D. thesis, traditional and innovative approaches to evaluate the microbial contribution in a long ripened cheese were applied. Methodologies were studied and developed in the field of microbial culture and enzymology. An accurate and laborious sampling of Parmigiano Reggiano cheese in all stages of manufacturing and during two years of ripening allowed to reach the research aim. The lactic acid bacteria behaviour when acting into a fermentative process like cheese ripening, demonstrates to be the major subject of this thesis.

Since fermented foods are really complex microbial ecosystems, they may be better investigated by the use of alternative cultural approaches. A preliminary tune of a potential nutritional poor agar medium, based on a matrix similar to ripened cheese, was carried out for recovering less nutritional demanding microorganisms. This developed medium (Cheese Agar), allows to culture the Parmigiano Reggiano minor microflora, coming from milk, that better adapts to a decrease in nutritional availability during ripening and it is hardly estimated on traditional media. The results obtained open a new field for other food based media.

In the second step of the research, microbial ecosystem evolution, cells viability and peptidase activities were monitoring during production and ripening of Parmigiano Reggiano. The accurate way of sampling allowed to follow the specific microbial selection due to the technological parameters in the different moment of cheese-making and ripening and even in the different cheese zones. Moreover, the real technological role of the secondary microflora was deeply investigated and a better understanding of its autolysis moment and nutritional requirements was reached.

Length heterogeneity PCR (LH-PCR) technique was applied for the first time to monitor the microbial succession in a complex fermented ecosystem as Parmigiano Reggiano cheese. The twin wholes employed in this study were representative samples of the subsequent stages of the same dairy process. The use of a culture independent method as LH-PCR overcame traditional agar plate and culture dependent method limitations. The modality of LH-PCR samples preparation allowed to evaluate both the entire and lysed cells evolution during cheesemaking and ripening. Thermophilic microflora of natural whey starter, added to drive the cheese fermentative process, was the dominant population until the second months of ripening. In subsequent months, until the sixth, a trend

reversal was observed in LH-PCR data, confirming that environmental conditions in this phase, favour mesophilic microflora development since their overcoming. This approach has never been used to study a ripened cheese where viable, not viable and lysed microbial cells coexist.

The aim of the last study proposed was the investigation of the stages of Parmigiano Reggiano ripening in which lactic acid bacteria aminopeptidases present in cheese extract could be involved in the release of free amino acids. An experimental design allowed to tune a mathematical model able to assess the effect of NaCl concentration, as well as pH and temperature, on aminopeptidases activities over a range of values covering different environmental conditions, zones of the wheel and aging times. The modeling approach explicated individual and interactive effects of the three aforementioned chemical-physical variables. The pH had the greatest effect on enzyme activity although temperature played a relevant role too. Salt concentration seemed to be important to slow down PepC, PepA, PepI and PepL. Results evidenced that the six different LAB peptidases tested were involved in cheese proteolysis. Their activity was enhanced or inhibited by the ripening parameters which, in turns, depended on the cheese dimension. The models resulted to be useful and offered a prediction with minimum requirements on experimental data, which made the approach innovative, less costly and time consuming than direct analyses.

Results obtained during this doctorate thesis lead to new findings that advance the understanding of microbial dynamics in an appreciated and economically important Italian cheese like Parmigiano Reggiano.

The approach utilized, the methods and results obtained, open perspectives for insight into the microbial evolution in fermented food environment.

5. CURRICULUM VITAE

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Studies

1997-2000 Degree in Pharmacy Science. Pontifícia Universidade Católica do Paraná – Curitiba (PR) – Brazil.

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2003 Master in Tecnologia per la Sicurezza degli Alimenti (Food Safety Technology). Faculty of Agriculture. University of Parma – Italy. Conclusive stage in quality assurance department. Parmalat S.p.A. Collecchio, Parma – Italy.

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Experiences abroad

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