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NON STARTER LACTIC ACID BACTERIA DURING CHEESE RIPENING: SURVIVAL, GROWTH AND PRODUCTION OF MOLECULES POTENTIALLY INVOLVED IN AROMA FORMATION

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“ "Say cheese",
calls the photographer in any part of the world
when photograph is to be taken.
This is the extent to which the taste of cheese
has influenced people such it brings a smile upon a mention”

Weimer, “Improving the flavour of cheese” 2007

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I. INTRODUCTION

I.I CHEESE: A BRIEF OVERVIEW

Cheese is the generic name of a group of fermented dairy products, produced throughout the world in a great diversity of flavours, textures, and forms; there are more than 1000 varieties of cheese. Cheese is an ancient food whose origins predate recorded history. It is commonly believed that cheese evolved in the "Fertile Crescent" between the Tigris and Euphrates rivers, in what is now Iraq, about 8000 years ago, during the so-called "Agricultural Revolution", when certain plants and animals were domesticated as sources of food. There is no conclusive evidence indicating where cheese-making originated. The first fermented dairy foods were produced by a fortuitous combination of events, the ability of a group of bacteria, the lactic acid bacteria (LAB), to grow in milk and to produce enough acid to reduce the pH of milk to the isoelectric point of the caseins, at which these proteins coagulate. Neither the LAB nor the caseins were designed for this outcome.

The ability of LAB to ferment lactose, a specific milk sugar, suggests that this characteristic was acquired relatively recently in the evolution of these bacteria. Their natural habitats are environment and/or the intestine, from which they presumably colonized the teats of dairy animals, contaminated with lactose; it is likely that through evolutionary pressure, these bacteria acquired the ability to ferment lactose.

Like mainly fermentation-derived food products, cheese cannot be easily and succinctly defined. Cheese is essentially a microbial fermentation of "curdled" milk. Milk is a rich source of nutrients for bacteria, which contaminate milk and grow well in the ambient conditions. Cheese manufacture accompanied the spread of civilization through Egypt, Greece, and Rome. Fermentation and/or salting, two of the classical principles for food preservation, were used to preserve meat, fish, vegetables and milk, and to produce beer, wine, fermented milks, butter, and cheese. Within large estates, individuals acquired special skills, which were passed on to succeeding generations. Traditionally, many cheese varieties were produced in limited geographical regions, especially in Italy. The localized production of certain varieties is now protected and encouraged through the European definition of Protected Denomination Origin (PDO), which legally defines the region and manufacturing technology for certain cheese varieties.

In the past there were thousands of farm-scale cheese-makers and there must have been great variation within any one general type; even today, there is very considerable inter and intra-factory variation in the quality and characteristics of well-defined varieties, in spite of the very considerable scientific and technological advances. The curds for many famous varieties of cheese, for example, Parmigiano Reggiano, Grana Padano, Emmental, and Roquefort, are produced in many farm-level dairies under the supervision of a producer consortium and the ripening of cheese and the marketing are organized by central facilities. Research on microbiology, chemistry, and technology of cheese started toward the end of the nineteenth century and continues today, as a result of which cheese science and technology are quite well understood. However, there are still large gaps in our knowledge, for example, the complete description of all cheeses flavour, how the flavour compounds are produced, the structure of some cheese, etc. With the gradual acquisition of knowledge on the chemistry and microbiology of milk and cheese, it becomes possible to study and control the changes involved in cheese-making.

The production of all varieties of cheese involves a generally similar protocol (Figure 1); various steps can be modified to give a product with the desired characteristics.

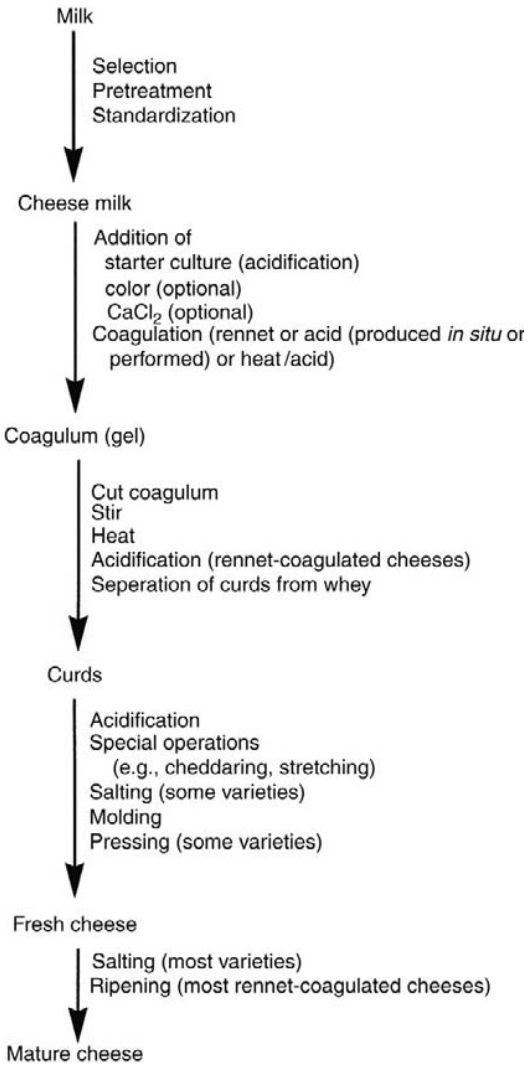

Figure 1: General cheese manufacture. Reproduced from Fox P.F., Guinee T.P., Cogan T.M., & McSweeney P.L.H. (2000), Fundamentals of Cheese Science.

Cheese manufacture is essentially a dehydration process in which the fat and casein in milk are concentrated between 6 and 12 fold, depending on the kind of cheese (*Cheese Chemistry, Physic and Microbiology, Fox, P.F., 2004*). The degree of dehydration is regulated by the combination of different operations, in addition to the chemical composition of the milk. In turn, the levels of moisture and salt, the pH and the cheese microflora regulate and control the biochemical changes that occur during ripening and hence determine the flavour, aroma and texture of the finished product. Thus, the nature quality of the finished cheese is determined largely by the manufacturing steps (*Cheese Chemistry, Physic and Microbiology, Fox, P.F., 2004*). However, it is during the ripening phase that the characteristic flavour and texture of the individual cheese varieties develop.

Cheese manufacture begins with the selection of milk. The adventitious microflora of milk is normally heterogeneous. Some of these microorganisms, especially the LAB, may be beneficial. In the olden time and still for some minor artisanal cheeses, the indigenous LAB were responsible for acid production but selected starter LAB cultures are used for acidification in most cases. One of the basic operations in the manufacture of most, if not all, cheese varieties is a progressive acidification throughout the manufacturing stage.

Another essential characteristic step in the manufacture of all cheese varieties is coagulation of the casein component of the milk protein system to form a gel which entraps the fat, if present. The majority of cheeses are produced by enzymatic (rennet) coagulation. A rennet-coagulated milk gel is quite stable if maintained under quiescent conditions but if it is cut or broken, it syneresis rapidly, expelling whey. The rate and extent of syneresis are influenced by how finely the coagulum is cut (small pieces promote syneresis), milk composition, especially Ca_2 and casein, pH, cooking temperature, rate of stirring of the curd-whey mixture and time. The composition of the finished cheese is to a very large degree determined by the extent of syneresis and since this is under the control of the cheese-maker, the differentiation of the individual cheese varieties really begins at this stage, although the composition of cheese-milk, the amount and type of starter and the amount and type of rennet are also significant in this regard. The temperature to which the curds can be cooked varies from 30°C (no cooking) for high-moisture cheeses (e.g. Camembert) to 55°C for low-moisture cheese (e.g. Parmigiano Reggiano). After cooking, the curds and whey are separated by various, variety-specific techniques. The curds for most varieties are transferred to moulds where further drainage and microbial acidification occur. Some cheeses, mainly acid-coagulated varieties, are consumed fresh. However, most cheese varieties undergo a period of ripening which varies from 2 weeks to more than 2 years (e.g. Parmigiano Reggiano). Many varieties may be consumed at any of the several stages of maturity, depending on the flavour preferences of consumers.

Throughout manufacture and ripening, the cheese production represents a finely orchestrated series of consecutive and concomitant biochemical events which, if balanced, lead to products with highly desirable aromas and flavours. Considering that, in general terms, a basically similar raw material (milk of different species) is subjected to a manufacturing protocol, the general principles of which are common to most cheese varieties. It is fascinating that such a diverse range of products can be produced.

For various reasons, a number of attempts have been made to classify cheeses into meaningful groups. Criteria to classify the cheeses are many and the variables by which it is possible to associate the cheese with a class, category or group can be several (Figure 2).

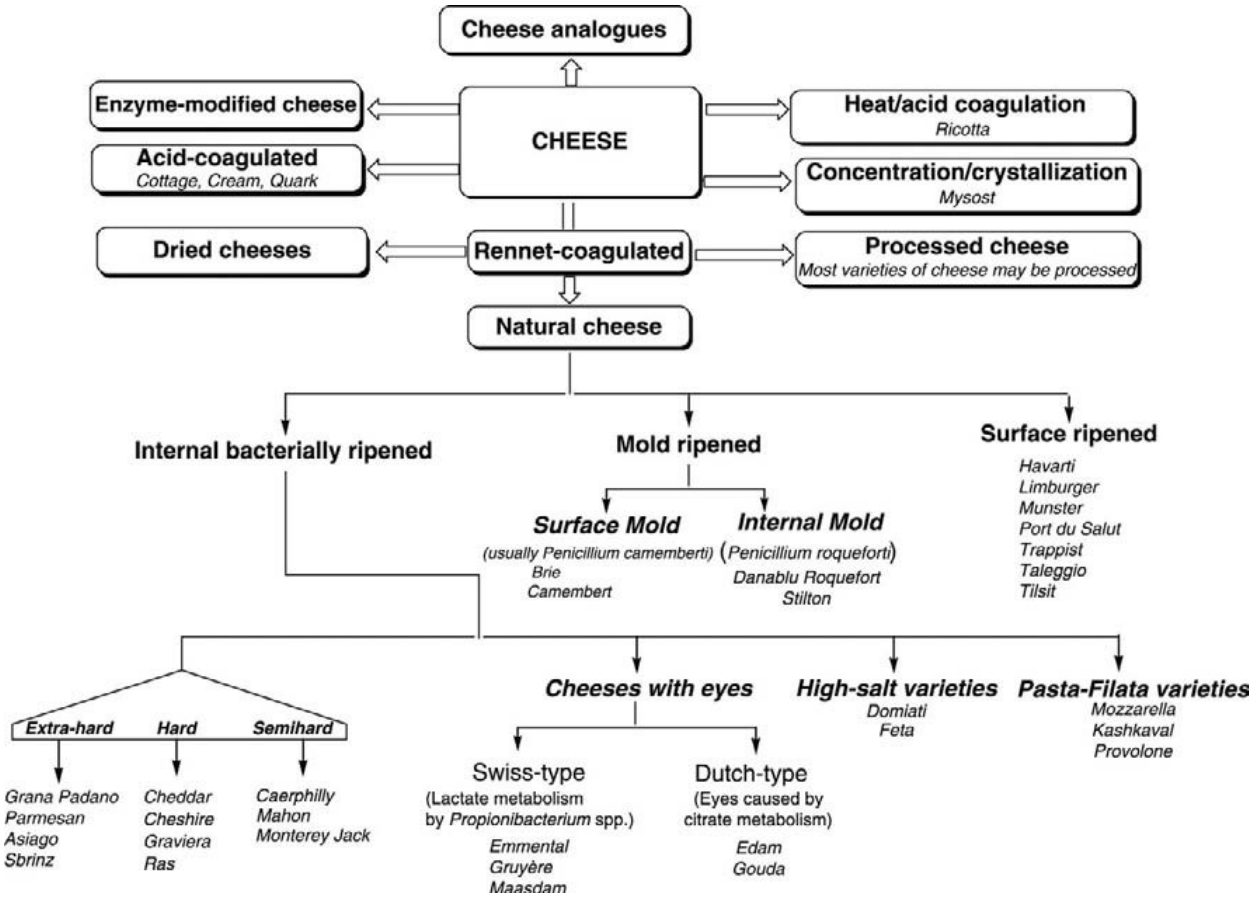

Figure 2: Classification of cheese. Reproduced from Fox P.F., McSweeney P.L.H., Cogan T.M., and Guinee T.P. (2004), Cheese: Chemistry, Physics and Microbiology.

A cheese can be considered unique, or indeed be the same product, simply defined with a name different from place to place.

Traditional classification schemes have been based principally on moisture content, that is, extra-hard, hard, semi-hard/semisoft, or soft. Although used widely, this scheme suffers from serious limitations since it groups cheeses with widely different characteristics, for example, Cheddar and Emmental are classified as hard cheeses although they have quite different textures and flavors, are manufactured by very different technologies, and the microbiology and biochemistry of their ripening are very different.

Therefore, the features that identify the product (consistency, production techniques, fat content, time of ripening) are many and different, so all can be considered the reference parameters. Based on these ones, the historical classifications were write (*Mucchetti & Neviani, 2006*).

Among the most important classifications there are those based on:

- Consistency of the paste (soft, semi-hard, hard),
- Time of aging (fresh, ripened, long ripened),
- Process temperature (cooked pasta, uncooked pasta),

but the criteria of differentiation may grow exponentially.

I.II CHEESE AND LACTIC ACID BACTERIA

Cheese cannot be made without lactic acid bacteria (LAB). They can be deliberately added and/or naturally present in milk.

Lactic acid bacteria (LAB) are industrially important microbes that are used worldwide in a variety of industrial food fermentations. 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 about 2 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 non-pathogenic microorganisms around the time that man domesticated dairy animals (approximately 10000 years ago) (*Fox & 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 & Holzapfel, 1995*). Due to the limited biosynthetic abilities (*Klaenhammer et al., 2005*) and their requirements in terms of carbon and nitrogen sources (*Salminen & Von Wright, 1998*), the natural habitat of LAB is represented by nutritionally rich environments.

Being linked to the lactic acid production, the definition of LAB is biological rather than taxonomical. LAB are a relatively heterogeneous group that share several defining

characteristics, including: low G+C content (<55 mol%) (*Kanlder & Weiss, 1986*); high acid tolerance; unable to synthesize porphyrins; and strictly fermentative metabolism with lactic acid as the major metabolic end product (*Broadbent & Steele, 2005*). LAB encompass a group of microorganisms Gram+, non-sporulating, catalase-negative. Except for a few species, LAB members are non-pathogenic organisms reputed Generally Recognized as Safe (GRAS) status. At present, nearly 400 LAB species have been recognized. They are generally classified into four families and seven genera as follow: family Lactobacillaceae (genera *Lactobacillus* and *Pediococcus*), family Leuconostocaceae (genera *Oenococcus* and *Leuconostoc*), family Enterococcoceae (genus *Enterococcus*) and family Streptococcaceae (genus *Streptococcus*) (*Zhang et al., 2011*).

In terms of their growth requirements, the lactic acid bacteria generally require sugars, preformed amino acids, B vitamins, and purine and pyrimidine bases. Although they are mesophilic, but some can grow below 5°C and some as high as 45°C. With respect to growth pH, some can grow as low as 3.2, some as high as 9.6, and most grow in the pH range 4.0–4.5. (*Modern Food Microbiology, Jay J.M., Loessner M.J., Golden D.A., 2005*).

Their contribution primarily consists of the rapid formation of lactic acid from the available carbon source resulting in acidification of the food raw material, which is a critical parameter for dairy products, especially for cheese manufacturing. Besides their lactic acid forming capacity, however, LAB also have the ability to contribute to other product characteristics like flavour, texture and nutrition. The most important application of LAB is therefore undoubtedly in the dairy production, mainly cheese (*Kleerebezem & Hugenholtz, 2003*).

The lactic acid bacteria used in the dairy fermentations can roughly be divided into two groups on the basis of their growth optimum. Mesophilic lactic acid bacteria have an optimum growth temperature between 20°C and 30°C and the thermophilic have their optimum between 30°C and 45°C.

Another possible division can be done considering their role in cheese production: starter lactic acid bacteria (SLAB) and non starter lactic acid bacteria (NSLAB).

STARTER LACTIC ACID BACTERIA

Cheese cannot be made without the use of some species of lactic acid bacteria (LAB), the major functions of which are to produce lactic acid from lactose during dairy products manufacture, mainly during curd acidification. These microorganisms are called starter bacteria because they "start" (initiate) the production of lactic acid. Generally, the starter bacteria are carefully selected and deliberately added to the milk before cheese making but, for some cheeses, no starter is added, and the cheese maker relies on adventitious contaminants present in the milk used to make the cheese.

LAB occur naturally in raw milk, and in the past, cheese making relied on this indigenous bacteria to promote acid production. However, this must have resulted in very variable fermentations. Cheese makers soon developed the technique of inoculating fresh milk with whey from the previous day's cheese making. This is a crude form of starter inoculation. In most cheeses, selected strains of LAB, are previously grown in milk or another medium and added deliberately to the milk at the beginning of cheese manufacture. In some countries with a long tradition of cheese making, for example in Italy, whey starters are used. In this case, fresh whey is incubated under controlled temperatures for use in the next day's production (Parmigiano Reggiano).

Two subtypes of artisanal (natural) starters are recognized, whey starters and milk starters, depending on the substrate and the technique used for their reproduction. Natural milk starter are still used in small cheese making plants in both Southern and Northern Italy for the production of traditional cheeses. The methodology used to prepare them and the microbiological composition of the raw milk used determine their microflora. This is usually dominated by *St. thermophilus* but other LAB such as enterococci (*Enterococcus faecium* and *Enterococcus faecalis*, mainly), lactobacilli (*L. helveticus* and *L. delbrueckii* subsp *bulgaricus/lactis*) may be present (*De Angelis et al., 2008; Mucchetti & Neviani, 2006*).

Natural whey cultures are prepared by incubating some of the whey drained from the cheese vat overnight under more or less selective conditions. In the manufacture of Parmigiano Reggiano and Grana Padano cheeses, whey is removed from the cheese vat at the end of cheese making at 50–54°C and it is incubated overnight at a controlled temperature (45°C), or in large containers in which the temperature decreases to 37–40°C, to a final pH as of low as 3.3.

The resulting whey culture is dominated by thermophilic LAB species belonging to *L. helveticus*, *L. delbrueckii*, and *St. thermophilus*. Similar whey cultures are used in the production of "pasta filata" cheese varieties in Italy and Comte cheese in France. Other types of natural whey cultures include deproteinized whey starters, are used for the manufacture of Pecorino cheese, and deproteinized whey starters with rennet are used for the manufacture of Swiss-type cheeses (Emmental, Sbrinz, Gruyere) in small cheese factories in the Alps (*Mucchetti & Neviani, 2006*).

SLAB can be classified as mesophilic or thermophilic. The latter are characteristics of Italian (Parmigiano Reggiano, Grana Padano) and Swiss (Emmental, Gruyere) cheese varieties, in which a high temperature (48-52°C generally) prevails during the cooking phases of cheese making. Mesophilic starters are used in all cheese varieties in which the temperature of the curd during the early stage of acid production does not exceed 40°C (*Cheese Chemistry, Physic and Microbiology, Fox P.F., McSweeney P.L.H., Cogan T.M. & Guinee T.P., 2004*). However, this distinction is losing some of its meaning, since mesophilic and thermophilic species are often found (or used) together in both mixed and defined starts for the manufacture of cheese like Mozzarella (*Parente et al., 1997*) and Cheddar (*Beresford et al., 2001*).

All starter lactic acid bacteria (SLAB) cultures available today are derived from artisanal starters of undefined composition (containing an undefined mixture of different strains and/or species). No special precautions are used to prevent contamination from raw milk or from the cheese making environment, and control of media and culture conditions during starter reproduction is very limited. As a result, even in any given cheese plant, natural starters are continuously evolving as undefined mixtures composed of several strains and/or species of LAB. The production of many traditional cheeses in Europe still relies on the use of natural mixed strain starters, which are an extremely valuable source of strains with desirable technological properties (phage resistance, antimicrobials, and aroma production) (*Parente & Cogan, 2004*).

Therefore, dairy starter cultures are actively growing cultures of LAB, used to drive the fermentation process.

Three main technological functions can be attributed to SLAB: acidification, texture enhancement and contribute in flavour development.

The process of acidification enhances the expulsion of whey from the curd during the cheese making process, and promotes the development of the texture of the cheese.

As mentioned above, fermenting lactose into L-lactic acid is a primary function of any starter culture in cheese manufacture. This fermentation is their energy- producing mechanism. The method of this metabolism differs in the various LAB species. Lactococci actively transport the lactose across the cell wall membrane as lactose-phosphate, which is hydrolysed to glucose and galactose-6-phosphate; the glucose molecule is then metabolised to L-lactate by the glycolytic pathway, and the galactose is metabolized *via* the tagatose-6- phosphate pathway.

The thermophilic LAB also use the glycolytic pathway, but they differ from lactococci in several aspects, namely the lactose uptake mechanism, and that *St. thermophilus* and *L. delbruekii* subsp *bulgaricus* are not able to metabolize galactose, whereas *L. helveticus* metabolize in *via* the Leloir pathway.

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 (*Broadbent & Steele, 2005*). The principal products of lactose metabolism are L- or D-lactate or a racemic mixture of both. The production of D-lactate is greater during ripening in cheeses made from raw milk but, as far as we are aware, only few studies have investigated this, until now (*Steffen, 1971; Steffen et al., 1980*). Racemisation 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 catabolised further by some NSLAB (see "*NON STARTER LACTIC ACID BACTERIA*").

SLAB are important in flavour development due to both the end products of fermentation (in particular diacetyl from citrate metabolism) and the slow proteolysis of the milk protein to peptides and amino acids by proteinase and peptidase of SLAB.

Generally SLAB can use citrate to produce succinate or diacetyl. Succinate production is attributed to aspartic acid catabolism by the propionibacteria in Swiss and other cheeses where *Propionibacterium sp*. attain high number. In Cheddar cheese and other varieties, SLAB are not the responsible of the production of succinate.

The other important citrate-derived flavour component, diacetyl, imparts a "buttery" note whose importance in butter. 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 & Steele, 2005*).

Milk contains about 1,5 g/l of citrate, most of which is lost in the whey during cheese making, since about 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 the microflora non starter (*McSweeney & Sousa, 2000*). Citrate is not metabolized by *St. thermophilus* nor by some species of thermophilic lactobacilli.

An important biochemical phenomenon leading to the formation of aromatic molecules is the proteolysis. Proteolysis and its secondary reactions play a major role in ripened cheeses, making casein hydrolysis and its relationship to flavour development an area of intense research interest. The hydrolysis of intact casein is almost exclusively catalysed by the coagulant (chymosin) and endogenous milk proteinases (plasmin), while LAB proteinases and peptidases are responsible for producing water-soluble peptides and free amino acids.

Proteolysis is a major event in cheese ripening; the proteolytic system of the microflora starter contributes, together with the activity of non starter microflora enzymes, to the production of hundreds of flavour compounds (*Cheese Chemistry, Physic and Microbiology, Fox P.F., McSweeney P.L.H., Cogan T.M. & Guinee T.P., 2004)*.

The proteolytic system of LAB is composed of a cell-wall bound proteinase, transport systems for amino acids, di- and tri-peptides and oligopeptides, a number of intracellular peptidases and some intracellular proteinases. Several reviews have been published on this topic (*Liu et al., 2010; Christensen et al., 1999*; *Siezen, 1999; Kunji et al., 1996*).

The role of proteolysis and amino acid catabolism has been addressed by several reviews *(Sousa et al., 2001*; *Yvon &Rejien, 2001*) and it is described in more detail in section "*THE CHEESE RIPENING*".

As regards lipolytic activity, obligate homofermentative lactobacilli used as starter (*L. helveticus*, *L. delbrueckii* subsp *bulgaricus* and *L. delbrueckii* subsp *lactis*) produce esterases, some of which have been studied (*Khalid & Marth, 1990*).

Free fatty acids, formed by lipase and esterase activity on milk fat, affect directly cheese flavour. Most SLAB lack lipolytic activity and they have very low esterase activity, but in cheese with long ripening times the LAB cells, (SLAB and non starter microflora) generate enough free fatty acids and esters to impact flavour (*Broadbent & Steele, 2005*). Therefore, SLAB contribute through these different ways, to the final characteristics of the products. These activities are performed by SLAB while they are viable in the cheese at the beginning of ripening when they reach numbers of 10^9 cfu/g. During ripening most of the starter bacteria lyse relatively rapidly, releasing their intracellular enzymes, which help the development of cheese flavour too. The ability to lyse is variable; some strains lyse relatively quickly, whereas others lyse slowly. Faster lysing strains generally produce cheese that develop flavour more rapidly than slower lysing strains. Lysis is caused by an intracellular muraminidase that hydrolyzes the bacterial cell wall. Lysis is influenced by several factors, including the level of salt in cheese.

NON STARTER LACTIC ACID BACTERIA

NSLAB are the lactic microflora not involved in curd acidification and generally naturally present in cheese matrix as a result of contamination during the manufacturing procedure or come from milk. This adventitious LAB microflora, result of contamination during the manufacturing procedure or come from milk, is very complex. If milk is considered the major source of NSLAB in production of the raw milk cheese (*Berthier et al., 2001*), NSLAB have also been isolated from drains, new vats, counter tops, and molds (*Somers et al., 2001*). NSLAB do not contribute to acid production in cheese vat (*Cogan et al., 1997*). This microbial population grows in long ripened cheese (Parmigiano Reggiano, Cheddar, etc) as it ripens over a period of weeks and months and comprises microorganisms that are able to grow under the conditions of the cheese environment, using the available substrates.

The NSLAB are a significant portion of microflora of most cheese varieties during ripening and have also been suggested as microbial fingerprint for traditional cheeses. *Beresford & Williams (2004)* have summarized the NSLAB that have been identified in more than 50 varieties of cheese. The role of NSLAB in cheese is not relatively clear. In many varieties the contribution of NSLAB to the development of characteristic of cheese remains a contentious topic.

It is known that the proteinase activity of NSLAB seems to be lower than that of SLAB and their contribution to casein hydrolysis appears to be relatively small (*Lynch et al., 1997*). In contrast the peptidase activity, at least in certain strains, contribute to the hydrolysis of bitter peptides to non-bitter peptides with the release of free amino acids (*Gagnaire et al., 2001*). Amino acid catabolism and the production of aroma compounds by NSLAB, appear to be one of the determinant properties of these microorganisms during cheese ripening (see "*THE CHEESE RIPENING*"). NSLAB exhibit a large diversity properties, but not all are studied in detail, and their effects on cheese characteristic vary from negative, to no effect, to positive effects. These properties are strongly strain dependent.

The NSLAB consist of various strains of pediococci and mostly of lactobacilli. Lactobacilli are divided into three groups on the basis of being either (I) obligatory homofermentative, (II) facultative heterofermentative, (III) obligatory heterofermentative (*Kandler & Weiss, 1986*).

The NSLAB regularly encountered in cheese include (II) facultative heterofermentative (FHL) lactobacilli (*Beresford et al., 2001*). The most frequently mesophilic lactobacilli isolated from cheese are: *L. casei*, *L. plantarum* and *L. rhamnosus* (*Fitzsimons et al., 1999; Coppola et al., 1997; Martley & Crow, 1993*).

Prolonged ripening times are a specific feature of Parmigiano Reggiano cheese ("*LONG RIPENED ITALIAN CHEESE: PARMIGIANO REGGIANO*"), therefore NSLAB have time to grow and become the dominant microflora. The evolution of the microflora of PR was studied up to 20 months of ripening (*De Dea Lindner et al., 2008; Coppola et al., 1997*). *L. casei*, *L. rhamnosus*, *P. acidilactici* were the dominant NSLAB microflora in PR. It is not yet clarified the nutrient source used by this microflora to grow and what energy source allows NSLAB to became the dominant microflora during cheese ripening. NSLAB, as all LAB, require sugar for growth and energy production. As all the lactose is fermented to lactic acid by the starter bacteria within the first few days of ripening in most cheeses, the energy source used by the NSLAB in cheese is not clear (*Beresford et al., 2001*). NSLAB can transform the L-isomer of lactate to the D-isomer, but this will not result in energy production (*Thomas & Crow, 1983*). The end product of lactose fermentation, lactate, can be catabolised further by some NSLAB.

For example, it has been found that some lactobacilli isolated from Cheddar cheese can oxidise lactate to acetate and CO_2 under aerobic conditions, and this lactate oxidation system in cheese lactobacilli is operative under the ripening conditions of Cheddar cheese (*Thomas, 1986*; *Thomas, 1987*). Several substrates have been suggested as nutrient source used by NSLAB, including citrate, ribose, and amino acids (*Williams et al., 2000*).

Following research data indicated that citrate is not used as an energy source by NSLAB (*Palles et al., 1998*). Ribose released from RNA after starter autolysis could be used as a energy source (*Rapposch et al.,1999*). However, many strains of NSLAB isolated from mature Cheddar cheese are unable to ferment ribose. The energy source potentially used by NSLAB in physiological and nutritional conditions prevailing during cheese ripening is an open topic and it is discussed more depth in the following section.

I.III THE CHEESE RIPENING

Acid-coagulated cheeses, which constitute a major part of cheeses consumed in some countries, are ready for consumption at the end of curd manufacture. Although rennet-coagulated cheese may be consumed as fresh curd, most of these kind of cheese are ripened for a period ranging from about 3 weeks to more than 2 years. Many varieties may be consumed at any of several stages of maturity, depending on the flavour preferences of consumers. For PDO cheeses the disciplinary imposes that they can be sold after a specific time of ripening (e.g. Parmigiano Reggiano can be sold after at least 12 month) (*Mucchetti & Neviani, 2006*).

Although curds for different cheese varieties are recognizably different at the end of manufacture (mainly due to compositional and textural differences), the unique characteristics of each variety develop during ripening as a result of a complex set of biochemical reactions (*Cheese Chemistry, Physic and Microbiology, Fox P.F., McSweeney P.L.H., Cogan T.M. & Guinee T.P., 2004*).

The ripening process of cheese is very complex and involves microbiological and biochemical changes to the curd resulting in the flavour and texture characteristic of the particularly variety (*McSweeney, 2004*).

MICROBIOLOGICAL CHANGES DURING CHEESE RIPENING

Microbiological changes in cheese during ripening include the death and lysis of starter cells (SLAB) and the growth of secondary microflora (particularly NSLAB). Microbial changes during ripening have been discussed extensively by *Beresford & Williams (2004)*. The microflora associated with cheese ripening is extremely different; however, as mentioned above, it may be conveniently divided into two groups: the SLAB and the NSLAB. During cheese making, SLAB and NSLAB dynamically evolve. In Parmigiano Reggiano cheese and other long ripened cheese, SLAB are the dominant microflora at the culmination of curd acidification. When ripening start, they begin to undergo autolysis (*Gala et al., 2008; Gatti et al., 2008*). One definition of autolysis, proposed by not recent literature, is "the lytic event which is caused by action of the cell's own intracellular mureinases" (*Stolph & Starr, 1965*). However, this definition is not suitable and sufficient in the case of cheese. A proper definition must incorporate not only the starter related mechanism of autolysis but also reflected how autolysis can be influenced by cheese manufacturing conditions and cheese environment to obtain the desirable ripening events in cheese (*Crow et al., 1995*). Then, give a precise definition of autolysis in cheese matrix is not easy. Indeed, the mechanism of bacterial lysis is not the most important aspect, but consequences that the SLAB lysis had in cheese is fundamental. The mainly consequence of starter autolysis in cheese is changes in proteolysis during ripening. This aspect has been studied in several research work.

On the other hand, in literature, little is known about the influence and the eventually aftermaths of the SLAB autolysis products on NSLAB growth during ripening. While the SLAB autolyse, NSLAB grow in cheese (*Banks & Williams, 2004*). Several studies reported that, in Parmigiano Reggiano the number of SLAB commonly exceeds 10^9 cfu/g of cheese when ripening begins. After Parmigiano Reggiano brining, SLAB cells undergo autolysis (*Gatti et al., 2008*); NSLAB species, the initial population of which is typically less than 10^2 cfu/g, begin to grow and eventually plateau at cell densities of 10^6-10^7 cfu/g after 3-9 months of aging (*De Dea Lindner et al., 2008; Gatti et al., 2008; Coppola et al., 1997*), becoming the dominant cheese microbiota during ripening.

Cheese during ripening is a hostile environment, typically characterized by the presence of salt, low moisture, 4.9-5.3 pH value, low temperatures, and deficiency of nutrients (*Turner et al., 1986*).

The difficult conditions inhibit towards many microbial groups, except the NSLAB which are able to tolerate the environment of cheese (*Settani & Moschetti, 2010; Peterson & Marshall, 1990*).

Therefore, the cheese maturation process imposes several types of stresses (acid, oxidative, osmotic and nutritional) to NSLAB. Considering the nutritional stress, it is well documented that the cheese matrix lacks the availability of lactose as a major carbohydrate energy source for the growth of NLSAB (*Shakeel-Ur-Rehman et al., 2004; Waldron, 1997*) because of the lactose is completely use by SLAB in the first step of cheese making.

It was demonstrated that bacteria can adapt to nutritional stress by adopting a physiological state characterized by the down-regulation of nucleic acid and protein synthesis and the simultaneous up regulation of protein degradation and amino acids synthesis. This basal metabolism is understandably the result of a complicated global network that operates in response to nutrient limitation (*Chatterji & Ojha, 2001*).

The energy source that NSLAB can use in cheese are still unknown, but many hypotheses have been proposed in the literature. Potential energy source could be amino acids, organic and fatty acids, glycerol or carbohydrates released from glycocmacropeptide of k-casein (galactose, N-acetylgalactosamine, N-acetylneuraminic acid), from glycoproteins and glycolipids in the milk fat globule membrane (*Peterson & Marshall, 1990; Fox et al., 1998; Williams et al., 2000*).

The glycomacropeptide from k-casein carriers several carbohydrates group: galactose, N-acetylgalactosamine and N-acetylneuraminic acid, but it is mainly lost in whey during cheese manufacturing (*Walstra & Jenness, 1984*), as well as part of nucleotides (containing ribose, phophoribose and deoxyribose) (*Adamberg et al., 2005*). Therefore small amount of glycomacropeptide as well as other component are present in cheese and could be used by some NSLAB (*Adamberg et al., 2005*).

Carbohydrate released by enzymatic hydrolysis of glycoproteins in the milk fat globule membrane can be possible energy source for NSLAB (*Fox et al., 1998*).

Several proteins in milk fat globule membrane are glycosylated, some containing up to 50% (w/w) of carbohydrate residues (*Mather, 2000*). The glycosylated part of milk fat globule membrane proteins consists mainly of N-acetylglucosamine, N-acetylgalactosamine, mannose, galactose and N-acetylneuraminic acid (*Mather, 2000*).

There are no data available on concentrations of bound carbohydrates in cheese; theoretically, the total amount should be sufficient to support the growth of NSLAB to 10^7 cfu/g (*Adamberg et al., 2005*).

NSLAB have proteolytic and glycolytic enzymes that hydrolyse glycoproteins (*Williams & Banks, 1997*). *Adamberg et al., (2005)* reported that NSLAB, isolated from Danish cheeses, use carbohydrates from the glycomacropeptide of k-casein and glycosylated proteins of the milk fat globule membrane, the bacterial cell wall peptidoglycan. Those carbohydrates need to be released by hydrolytic enzymes during cheese ripening. The information about glycosidases in NSLAB is limited, but galactosidase activity and N-acetylgalactosaminidase activity both occur in *L. paracasei* strains (*Williams & Banks, 1997*).

Degradation of the amino acids arginine has been shown to be sufficient to support NSLAB develop, with enough ATP to permit growth (*Lath et al., 2002*). In the semi-hard cheeses the starter rapidly consume most of the arginine (*Ardo et al., 2002*).

Also citrate is not available for NSLAB in cheese containing citrate fermenting starter bacteria. Furthermore, NSLAB usually cannot produce energy from citrate in the absence of other fermentable carbohydrates. There is evidence that NSLAB can take up citrate into the cell but are not able to metabolise it (*Williams et al., 2000*). However, *Diaz-Muniz et al., (2006)* observed that *L. casei* can be able to use citrate in defined condition, when galactose is present in limiting concentrations; namely this NSLAB is able to use citrate as energy source during cheese ripening only when the residual levels of carbohydrate post fermentation are limiting (lower than 2.5 mM).

Interesting is the study of *Thomas (1987)* that showed that nutrients for growth of cheese NSLAB could be provided by autolysed starters.

Therefore, an indirect effect on cheese ripening from starter autolysis could be to influence the growth rates and types of NSLAB in cheese which, in turn, could influence cheese flavour.

Carbohydrates from the lysed starter cells can be used for bacterial growth. NSLAB have been shown to grow on suspensions of lysed starter bacteria (*Thomas, 1987*; *Rapposch et al., 1999*). The peptidoglycan from the lysed cells of starter Lactococci contains residues of N-acetylglucosamine and N-acetylmuramic acid (*Delcour et al., 1999*), while N-acetylglucosamine may be more easily released than N-acetylmuramic acid due to better affinity to autolysins in the peptidoglycan structure.

Furthermore, pentoses from the nucleic acids such as ribose from RNA and deoxyribose from DNA may be released from lysed starter cells.

Ribose has been observed as the main carbohydrate released in a suspension of lysing *L. helveticus* cells (*Rapposch et al., 1999*).

Concluding, it can be supposed that NSLAB isolated from ripened Parmigiano Reggiano cheese can survive and grow, using either nitrogenous compounds that result from casein hydrolysis (*Neviani et al., 2009*) or products of SLAB lysis (*Thomas, 1987; Williams et al. 2000*) as nutrient sources. About this latter there are not many experimental research work that demonstrate and support the NSLAB growth using the SLAB cells lysis product as nutrient source. Firstly aim of this PhD thesis was fill this gap.

BIOCHEMICAL EVENTS DURING CHEESE RIPENING

The biochemical reactions that occur during ripening are caused by one or more of the following agents:

- indigenous milk enzymes, especially proteinase, such as plasmin, and perhaps lipase (rennet paste) (*Sousa et al., 2001*),
- secondary activity of chimosin (*Reida et al.,1997*)
- SLAB or their enzymes,
- NSLAB and their enzymes.

The biochemical changes may be grouped into primary events that include the metabolism of residual lactose and of lactate and citrate, lipolysis and proteolysis. Following these primary events secondary biochemical events are very important for the development of many volatile flavour compounds and include the metabolism of fatty acids and the catabolism of amino acids (*McSweeney, 2004*).

It is possible to divided these biochemical reactions into three principal groups:

1) Catabolism of residues of lactose, lactic acid, and, in some varieties, citric acid; this results in changes in flavour and texture.
2) Lipolysis and the catabolism of fatty acids; in some varieties, for example, blue cheeses, these reactions dominate ripening; while in Parmigiano Reggiano the catabolism of fatty acids can be considered important for flavour production, but the lipolysis is very low because of low lipolytic activities of LAB.

3) Proteolysis and modification of amino acids, which are the most complex, and perhaps the most important, reactions in cheese ripening, especially in internal bacterially ripened varieties; they affect flavour and texture. (*Encyclopedia of Dairy Science, Fuquay J.W., Fox P.F., McSweeney P.L.H., 2010).*

These biochemical reactions during cheese ripening is an active area of research and has been studied by several authors (among them: *Smit et al., 2005; Curtin & McSweeney, 2004; Fox & McSweeney, 2004; Yvon & Rejien, 2001; Sousa et al., 2001; McSweeney & Sousa, 2000; Fox & Wallace, 1997; Fox & McSweeney, 1996, 1997; Fox & Stepaniak, 1993; Fox & Law, 1991; Fox et al., 1990, 1998; Fox, 1989; Grappin et al., 1985).*

In this session it briefly considers each of the three groups of primary events listed above.

1) As cheese is a fermented dairy product, a key feature of its manufacture is the metabolism of lactose to lactate by selected cultures of SLAB. Starter metabolize majority of lactose; in some cheese a low amount of lactose residue can remain and it can be used by NSLAB (*Fox & McSweeney, 2004*). Lactate produced from lactose is an important substrate for a range of reactions that occur in cheese during ripening. D-Lactate may be formed directly from lactose by NSLAB *in vitro* (*Fox et al., 2000*) or by racemisation of L -lactate. The rate at which L-lactate is racemised depends on the composition of the NSLAB flora (*Thomas & Crow, 1983*). The pathway for lactate racemisation probably involves oxidation of L-lactate by L-lactate dehydrogenase to form pyruvate, which is then reduced to d-lactate by the action of D-lactate dehydrogenase. Racemisation of lactate is significant because the solubility of Ca-DL-lactate is lower than that of Ca-L-lactate (*Dybing et al., 1988; Thomas & Crow, 1983*) and thus racemisation favours the formation of Ca-DL-lactate crystals, which are manifested in cheese as white specks (*McSweeney, 2004*).

   Lactate can be oxidized by LAB in cheese to products including acetate, ethanol, formate and CO_2 (*Fox et al., 2000*). However, the extent to which this pathway occurs in cheese depends on the NSLAB population and the availability of O_2 (*Fox & McSweeney, 2004; Thomas, 1987*).

   Citrate is an important precursor for flavour compounds in some kind of cheese made using mesophilic starter cultures (*Parente & Cogan, 2004; Fox et., 1990*). In some long ripened cheese citrate can be used by NSLAB when the residual level of carbohydrates

are limiting and lactose or glucose are absent (condition during cheese ripening) (*Diaz-Muniz et al., 2006*).

The products of NSLAB metabolism of citrate are acetoin, acetate and diacetyl (*Palles et al., 1998*).

2) In cheese, oxidative changes are very limited due to the low oxidation/reduction potential (about 250 mV) (*McSweeney & Sousa, 2000; Fox & Wallace, 1997*) However, triglycerides in all cheese varieties undergo hydrolysis by the action of indigenous, endogenous and/or exogenous lipases, which result in the liberation of fatty acids in cheese during ripening. The triglycerides of ruminant milk fat are rich in short-chain fatty acids that, when liberated, have low flavour thresholds that contribute significantly to the flavour of many cheese varieties. Although some lipolysis occurs in most or all cheeses, it is most extensive in some hard Italian varieties and in blue cheese (*McSweeney, 2004*).

   Lipolytic agents in cheese generally originate from the milk, the coagulant (in the case of rennet paste) and the cheese microflora (SLAB and NSLAB).

   LAB possess intracellular esterolytic lipolytic enzymes. As starter and nonstarter LAB are present in cheese in high numbers, enzymes from these organisms are responsible for the liberation of significant levels of fatty acids during the long ripening period of many internal bacterially ripened cheeses (*Collins et al., 2004*). Lipolytic enzymes from SLAB are intracellular (*Fernandez et al., 2000*), and hence are released into the cheese matrix on lysis.

3) Proteolysis is the most complex and, in most cheese varieties, is the most important of the primary biochemical events that occur in most cheeses during ripening.

   The pattern of proteolysis in many cheese varieties may be summarised as follows: the caseins are hydrolysed initially by residual coagulant activity retained in the curd and by plasmin (and perhaps other indigenous proteolytic enzymes) to a range of large and intermediate sized peptides that are hydrolysed by proteinases and peptidases from the starter LAB and NSLAB, to shorter peptides and amino acids (*McSweeney, 2004*).

   These biochemical reactions have a direct influence on flavour through the production of short peptides and amino acids, some of which are flavoured (often bitter), by facilitating the release of sapid compounds from the cheese matrix and by providing free amino acids that are substrates for a series of catabolic reactions (an important

secondary biochemical event during cheese ripening) that generate many important flavour compounds.

The proteinases and peptidases that catalyse proteolysis in cheese during ripening originate from four sources, namely, the coagulant, the milk, the SLAB and the NSLAB (*Sousa et al., 2001*).

LAB require many amino acids and thus have complex proteolytic systems to liberate the amino acids necessary for growth from the proteins in their environment. The proteinases and peptidases of LAB have been the subject of several studies (*Liu et al., 2010; Upadhyay et al., 2004; Christensen et al., 1999; Law & Haandrikman 1997; Kunji et al., 1996*). LAB also contain intracellular peptidases that are very important for the final stages of proteolysis in cheese during ripening and the ultimate liberation of free amino acids as substrates for catabolic reactions.

During cheese ripening also other biochemical reactions occur, these are known as secondary events, but not least. Among these the metabolism of free fatty acids (FFA) contribute to formation of cheese flavour. The pathways for the metabolism of FFA is reported in Figure 3.

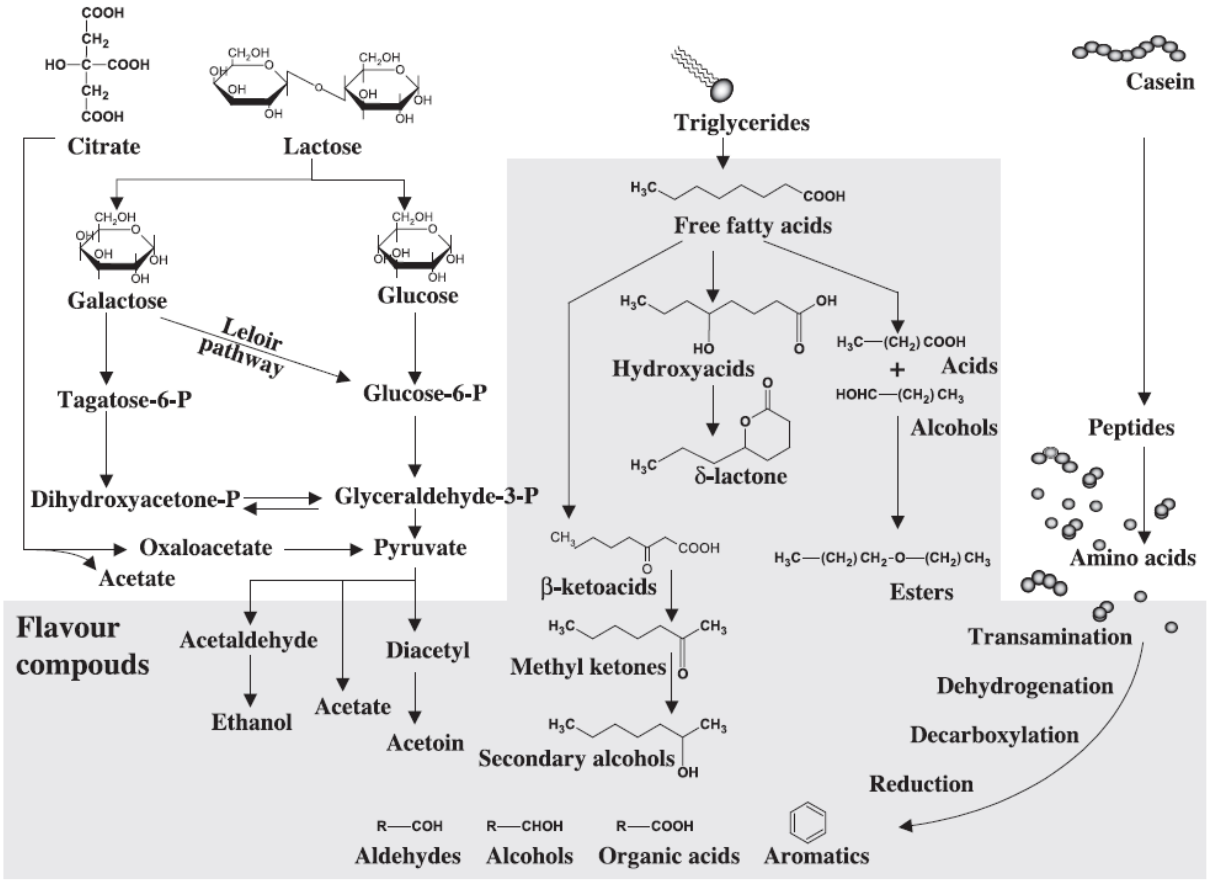

Figure 3: Biochemical pathways leading to the formation of flavour compounds. The gray surface indicated compounds with a flavor note (From Marilley & Casey, 2004)

Esters are found commonly in many cheese varieties and are produced by the reaction of an FFA with an alcohol. Ethanol is the limiting reactant in the production of esters; this alcohol is derived from the fermentation of lactose or from amino acid catabolism. *Holland et al., (2002)* suggested that esters are formed during cheese ripening by transesterification of an FFA from partial glycerides to ethanol. Thioesters are compounds formed by the reaction of FFAs with sulphydryl compounds, usually methanethiol (*McSweeney & Sousa, 2000*).

Also lactones were found in cheese, but during ripening their production is limited by the amount of precursors (hydroxyacids) (*McSweenwy & Sousa, 2000*).

The catabolism of amino acids during ripening is another main secondary events that produce flavour compounds (Figure 3). The amino acids catabolic pathways were reported in detail in a review of *Fernandez & Zuniga (2006)*. For each amino acid there is a catabolic pathway that leads to the formation of specific compounds. Amino acids

catabolism produces, in turn, a number of compounds, including ammonia, amines, aldehydes, phenols, indole and alcohols, which contribute as a whole to cheese flavour (*Urbach, 1995*). There are usually three recognisable steps in this complex process (*Tavaria et al., 2002*):

- reactions of decarboxylation, deamination, transamination, desulfuration and hydrolysis of side chain;
- conversion of the resulting compounds (amines and α-ketoacids);
- reduction of aldehydes to alcohols or their oxidation to carboxylic acids.

Several authors studies the flavour compounds formation by amino acids catabolism pathways (*Smit et al.,2005; Yvon & Rejien 2001; McSweeney & Sousa 2000*).

The pathways previously reported have been described in detail in literature.

The flavour, produced through these metabolisms, characterizing the aroma of different kind cheeses, have been studied in many research works (*Randazzo et al., 2010; Di Cagno et al., 2003; Qian & Reineccius, 2002; Moio et al.,2000; Moio & Addeo, 1998; Bosset & Gauch, 1993*).

As mentioned at the beginning, though assuming that the NSLAB have an important role during cheese ripening (*Urbach, 1995*), until now few studies has reported the specific contribute of NSLAB species in the flavour formation.

Therefore, this PhD thesis wants to relate the flavour compounds found in cheeses with the metabolic activities of NSLAB that grow during cheese ripening using as energy source an alternative food source as reported in the section "*MICROBIOLOGICAL CHANGES DURING CHEESE RIPENING*".

The investigation considered can open many perspectives on the metabolic capabilities of NSLAB. The role of starter microflora in Parmigiano Reggiano and other ripened cheeses is well known, however, little is known about NSLAB role and contribution during cheese ripening. To enhance cheese quality and the consistency of cheese flavour, also the dairy industry is interested in methods to control the NSLAB microbiota and metabolism of this microorganism.

II. LONG RIPENED ITALIAN CHEESE: PARMIGIANO REGGIANO

A brief overview on Parmigiano Reggiano cheese is reported below.

This brief paragraph aims to present the Parmigiano Reggiano cheese. This is the "reference" cheese of the studies reported in this PhD thesis. The LAB strains used in experiments were isolated from Parmigiano Reggiano cheese during a previously PhD thesis research work (*De Dea Lindner, 2008*). Therefore, it was considered convenient to present this cheese and its characteristics and to report the state of the art of the microbiological studies regarding this appreciated product, famous all over the world.

The classification of Italian cheeses is complicated, therefore it is used different names for the same or very similar cheeses in different regions, and especially because many varieties are consumed after different degree of ripening.

In this section it deals with one of the most famous Italian hard cheese: Parmigiano Reggiano (PR). This cheese is among the most famous international cheeses and have maintained its traditional features over time in spite of marked changes in cheese-making technology. PR is produced in a limited geographic area in Northern Italy. The area of production includes the territory of the provinces of Parma, Reggio Emilia, Modena and Mantova on the right bank of the Po river and Bologna on the left bank of the Reno river. It is appreciated for its nutritional and sensorial properties and received the Protected Designation of Origin (PDO) in compliance with the EC Regulation 2081/9 (*Gala et al., 2008; Malacarne et al., 2008; Gatti et al 2003*).

PR cheese derives its name from the grainy texture of the ripened cheese. PR is a semi-fat hard cheese characterized by a cooked, slowly and long matured paste.

It is made up with raw (milk cannot undergo any thermal treatments) and partly skimmed cow's milk (*Gala et al., 2008; Gatti et al., 2003*) from animals whose feeding mainly consist of forage from the area of origin. Only raw, unheated milk can be used and any additive is strictly forbidden (*Malacarne et al., 2008*). A mixture of milk from two consecutive milkings is used for PR and the milk from the evening milking is partially skimmed after overnight creaming. Milk is then added with natural whey starter. The natural whey cultures used as starters are obtained from the spontaneous acidification of the whey left after the

previous day's cheese processing and recovered overnight at a natural or controlled decreasing temperature gradient (*Mucchetti & Neviani, 2006*). The microbial composition of the natural starter is very complex. 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 and *L. helveticus* is usually the dominant species (*Gatti et al., 2003*). The inoculated milk is heated at 32-34°C, added with calf rennet powder and coagulated within 8-12 min. After curdling, the curd is broken up into grains and cooked at 54-56°C. During cooking the acidifying activity of natural whey starter lactic acid bacteria, together with heating effects, favours the formation of the proper texture of curd granules and whey drainage. When the heating is turned off, the curd granules deposit at the bottom of the vat where they will aggregate together in about 30-50 min under the whey, at a temperature no higher the one reached at the end of the cooking process (*Mucchetti & Neviani, 2006*). These curd grains settled to the bottom of the vat form a compact mass. The cheese mass is subsequently placed into special moulds for the moulding process where the most important acidifying activity of natural whey starter lactic acid bacteria occurs. After a few days, cheeses are immersed in a water and saturated salt solution. After 20 or 30 days PR is drained off brine and it is ripened for 20–24 months (must last at least 12 months). In summer the temperature of maturation rooms must not be lower than 16°C (*http://www.parmigiano-reggiano.it*). This cheese has a typical compact texture and melt in the mouth with a sweet and pronounced flavour, which is the result of a very slow ripening.

Microbiological characteristics of PR have been studied by several authors (*De Dea Lindner et al. 2008; Coppola et al., 2000; Coppola et al., 1997*) which isolated a high 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 *L. helveticus* isolated from natural whey cultures for PR production (*Gatti et al., 2003; Gatti et al., 2004*).

REFERENCES

Adamberg K., Antonsso M., Vogensen F. K., Nielsen E.W., Kask S., Moller P. L. & Ardo Y. 2005 Fermentation of carbohydrates from cheese sources by non-starter lactic acid bacteria isolated from semihard Danish cheese. Int Dairy J 15, 873–882.

ArdoY., Thage B.V. & Madsen J.S. 2002 Dynamics of free amino acid composition in cheese ripening. Aust J Dairy Technol 57, 109–15.

Banks J.M. & Williams G., 2004 The role of nonstarter lactic acid bacteria in Cheddar cheese ripening. Int J Dairy Tech 57, 145-152.

Beresford T. & Williams A. 2004 The microbiology of cheese ripening. In Cheese: Chemistry, Physics and Microbiology, Vol 1 General Aspects, 3rd edn Fox P. F., McSweeney P. L.H., Cogan T. M. & Guinee T. P., eds Elsevier, London.

Beresford T. P., Fitzsimons N. A., Brennan N. L., & Cogan T. M. 2001 Recent advances in cheese microbiology. Int Dairy J 11, 259–274.

Berthier E., Beuvier E., Dasen A. & Grappin R. 2001 Origin and diversity of rnesophilic lactobacilli in Comte cheese, as revealed by PCR with repetitive and species- specific primers. Int Dairy J 11, 293-305.

Bosset J.O. & Gauch R. 1993 Comparison of the volatile flavour compounds of six European 'AOC' cheeses by using a new dynamic headspace GC-MS method. Int Dairy J 33, 359-377.

Broadbent J.R. & Steele J.L., 2005 Cheese Flavor and the Genomics of Lactic Acid Bacteria. ASM News 71, 121-128.

Chatterji D. & Ojha A.K., 2001 Revisiting the stringent response ppGpp and starvation signaling. Curr Opin Micorbiol 4,160-165.

Christensen J.E., Dudley E.G., Pederson J.A., & Steele J.L. 1999 Peptidases and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhoek 76, 217–246.

Cogan T.M., Barbosa M., Beuvier E., BianchisSalvadori B., Cocconcelli P.S., Fernandes I., Gomez J., Kalantzopou-los G., Ledda A. & Medina M. 1997 Characterization of the lactic acid bacteria in artisanal dairy products. J Dairy Res 64, 409-421.

Collins Y.F., McSweeney P.L.H. & Wilkinson M.G. 2004 Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. Int Dairy J 13, 841–866.

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

Coppola R., Nanni M., Iorizzo M., Sorrentino A., Sorrentino E., Chiavari C. & Grazia L. 2000 Microbiological characteristics of Parmigiano Reggiano cheese during the cheesemaking and the first months of the ripening. Lait 80, 479-480.

Crow V.L., Coolbear T., Gopal P.K., Martley F.G., McKayb L.L. & Riepeb H. 1995 The Role of Autolysis of Lactic Acid Bacteria in the Ripening of Cheese. Int Dairy J 5, 855-875.

Curtin A.C. & McSweeney P.L.H. 2004 Catabolism of amino acids in cheese during ripening. In Cheese: Chemistry, Physics and Microbiology, Vol 1 General Aspects, 3rd edn Fox P.F., McSweeney P. L. H., Cogan T. M. & Guinee T. P., eds Elsevier, London.

De Angelis M., De Candia S., Calasso M. P., Faccia M., Guinee T. P., Simonetti M. C. & Gobbetti M. 2008 Selection and use of autochthonous mixed starters for the manufacture of traditional Mozzarella cheese. Int J Food Microbiol 125, 123-132.

De Dea Lindner J., Bernini V., De Lorentiis A., Pecorari A., Neviani E. & Gatti M. 2008 Parmigiano Reggiano cheese: evolution of cultivable and total lactic microflora and peptidase activities during manufacture and ripening. Dairy Sci Technol 88, 511-523.

De Dea Lindner, 2008, PhD thesis "Traditional and innovative approaches to evaluate microbial contribution in long ripened fermented foods: the case of Parmigiano Reggiano cheese".

Delcour J., Deghorain T.M., Palumbo E. & Hols P. 1999 The biosynthesis and functionality of the cell-wall of lactic acid bacteria. Antonie van Leeuwenhoek 76, 159-184.

Di Cagno R., Banks J., Sheehan L., Fox P.F., Brechany E.Y, Corsetti A. & Gobbetti M. 2003 Comparison of the microbiological, compositional, biochemical, volatile profile and sensory characteristics of three Italian PDO ewes' milk cheeses. Int Dairy J 13, 961-972.

Dìaz-Muñiz I., Banavara D.S., Budinich M.F., Rankin S.A., Dudley E.G. & Steele J.L. 2006. Lactobacillus casei metabolic potential to utilize citrate as an energy source in ripening cheese: a bioinformatics approach. J Appl Microbiol 101, 872-882.

Dybing S.T., Wiegand J.A., Brudvig S.A., Huang E.A. & Chandan R.C 1988 Effect of Processing Variables on the Formation of Calcium Lactate Crystals on Cheddar Cheese. J Dairy Sci 71, 1701-1710.

Fernandez L., Beerthuyzen M. M., Brown J., Siezen R. J., Coolbear T., Holland R., & Kuipers O. P. 2000 Cloning, characterization, controlled overexpression, and inactivation of the major tributyrin esterase gene of Lactococcus lactis. Appl Environ Microbiol 66, 1360–1368.

Fernandez M. & Zuniga M. 2006 Amino Acid Catabolic Pathways of Lactic Acid Bacteria. Crit Rev Microbiol 32, 155-183.

Fitzsimons N.A., Cogan T.M., Condon S. & Beresford T. 1999 Phenotypic and Genotypic Characterization of Non-Starter Lactic Acid Bacteria in Mature Cheddar Cheese. Appl Environ Microbiol 65, 3418-3426.

Fox P.E., McSweeney P.L.H. & Lynch C.M. 1998 Significance of non-starter lactic acid bacteria in Cheddar cheese. Aust Dairy Technol 53, 83-89.

Fox P.F. & Law J. 1991 Enzymology of cheese ripening. Food Biotechnol 5, 239–262.

Fox P.F. & McSweeney P.L.H. 1996 Proteolysis in cheese during ripening. Food Rev Int 12, 457-509.

Fox P.F. & McSweeney P.L.H. 1997 Rennets: their role in milk coagulation and cheese ripening. In Microviology and Biochemistry of cheese and fermented milk, 2nd edn, Law, B.A. eds Chapman and Hall,London.

Fox P.F. & McSweeney P.L.H. 2004 Cheese: an overview. In Cheese: Chemistry, Physics and Microbiology, Vol 1, General Aspects, 3rd edn Fox P.F., McSweeney P.L.H., Cogan T.M., & Guinee T.P. eds Elsevier, London.

Fox P.F. & Stepaniak L. 1993 Enzymes in cheese technology. Int Dairy J 3, 509-530.

Fox P.F. & Wallace J. M. 1997 Formation offlavor compounds in cheese. Advances in Applied Microbiology 45, 17–85.

Fox P.F. 1989 Proteolysis during cheese manufacture and ripening. Journal of Dairy Science 72, 1379–1400.

Fox P.F., Guinee T.P., Cogan T.M. & McSweeney P.L.H. 2000 Cheese flavor. In Fundamentals of cheese science Gaithersburg, MD: Aspen Publishers Inc 282–384.

Fox P.F., Lucey J.A., & Cogan T.M. 1990 Glycolysis and related reactions during cheese manufacture and ripening. Critical Reviews in Food Science and Nutrition 29, 237–253.

Fox P.F., McSweeney P.L.H., Cogan T.M. & Guinee T.P. 2004 Cheese: Chemistry, Physics and Microbiology, Vol 1 General Aspects, 3rd edn, eds Elsevier London.

Fuquay J.W., Fox P.F. & McSweeney P.L.H. 2010 Encyclopedia of Dairy Science, 2nd edn, eds Elsevier London.

Gagnaire V., Thierry A. & Lèonil J. 2001 Propionibacteria and facultatively heterofermentative lactobacilli weakly contribute to secondary proteolysis of Emmental cheese. Lait 81, 339–353.

Gala E., Landi S., Solieri L., Nocetti M., Pulvirenti A. & Giudici P. 2008 Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. Int J Food Microbiol 125, 347–351.

Gatti M., De Dea Lindner J., De Lorentiis A., Bottari B., Santarelli M., Bernini V. & Neviani E. 2008 Dynamics of whole and lysed bacterial cells during Parmigiano-Reggiano cheese production and ripening. Appl Environ Microbiol 74, 6161-7.

Gatti M., Lazzi C., Rossetti L., Mucchetti G. & Neviani E. 2003 Biodiversity in Lactobacillus helveticus strains present in natural whey starter used for Parmigiano Reggiano cheese. Appl Environ Microbiol 95, 463–470.

Gatti M., Trivisano C., Fabrizi E., Neviani E. & Gardini F. 2004 Biodiversity among Lactobacillus helveticus strains isolated from different natural whey starter cultures as revealed by classification trees. Appl Environ Microbiol 70, 182-190.

Grappin R., Rank T.C. & Olson N.F. 1985 Primary proteolysis of cheese proteins during ripening. J Dairy Sci 68, 531–540.

Holland R., Liu S.Q., Wang T., Bennett M., Norris G., Delabre M.L., Lubbers M.L., Dekker J.W. & Crow V.L. 2002 Esterases of lactic acid bacteria. Australian Journal of Dairy Technology 57, 116 (Abstract).

Jay J.M., Loessner M.J. & Golden D.A. 2005 Modern Food Microbiology, 7th edition, Springer.

Kandler, O. & Weiss N. 1986 Regular, non-sporing Gram-positive rods. In Bergey's manual of determinative bacteriology, 8th edn, Vol 2 P.H.A. Sneath, N.S. Mair, M.E. Sharpe & J.G. Holt eds Williams and Wilkins Co., Baltimore.

Khalid N. M. & Marth E.H. 1990 Lactobacilli-Their Enzymes and Role in Ripening and Spoilage of Cheese: A Review. J Dairy Sci 73, 2669-2684.

Klaenhammer T.R., Barrangou R., Buck B.L., Azcarate-Peril M.A. & Altermann E. 2005 Genomic features of lactic acid bacteria effecting bioprocessing and health. FEMS Microbiol Rev 29, 393-409.

Kleerebezem M. & Hugenholtz J. 2003 Metabolic pathway engineering in lactic acid bacteria. Curr Opin Biotech 14, 232–237.

Kunji E.R., Mierau I., Hagting A., Poolman B. & Konings W.N. 1996 The proteolytic systems of lactic acid bacteria. Antonie Van Leeuwenhoek 70, 187-221.

Lath T.M., Kask S., Elias P., Adamberg K. & Paalme T. 2002 Role of arginine in the development of secondary microflora in Swiss type cheese. Int Dairy J 12, 831-840.

Law J. & Haandrikman A. 1997 Proteolytic enzymes of lactic acid bacteria. Int Dairy J 7, 1–11.

Liu M., Bayjanov J.R., Renckens B., Nauta A. & Siezen R.J. 2010 The proteolytic system of lactic acid bacteria revisited: a genomic comparison. BMC Genomics 11, 36.

Lynch C.M., McSweeney P.L.H., Fox P.F., Cogan T.M. & Drinan E.D. 1997 Contribution of starter lactococci and non starter lactobacilli to proteolysis Cheddar cheese with a controlled microflora. Lait 77, 441-459.

Malacarne M., Summer A., Formaggioni P., Franceschi P., Sandri. S., Pecorari M., Vecchia P. & Mariani P. 2008 Dairy maturation of milk used in the manufacture of Parmigiano Reggiano cheese: effects on physico-chemical characteristics, rennet-coagulation aptitude and rheological properties. J Dairy Res 75, 218-224.

Marilley L. & Casey M. G. 2004 Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. International Dairy Journal 90, 139-159.

Martley F.G. & Crow V.L. 1993 Interactions between non-starter microorganisms during cheese manufacture and repening. International Dairy Journal 3, 461-483.

Mather I.H. 2000 A Review and Proposed Nomenclature for Major Proteins of the Milk-Fat Globule Membrane. J Dairy Sci 83, 203-247.

McSweeney P.L.H. & Sousa M.J. 2000 Biochemical pathways for the production of flavour compounds in cheese during ripening. Lait 80, 293-324.

McSweeney P.L.H., 2004 Biochemistry of cheese ripening. Int J Dairy Technol 57.

Moio L. & Addeo F. 1998 Grana Padano cheese aroma. J Dairy Res 65, 317–333.

Moio L., Piombino P. & Addeo F. 2000 Odour-impact compounds of Gorgonzola cheese. J Dairy Res 67, 273–285.

Mucchetti, G. & Neviani, E. 2006 Microbiologia e tecnologia lattiero casearia. Qualità e sicurezza. eds Tecniche Nuove, Milan.

Neviani E., De Dea Lindner J., Bernini V., Gatti M. 2009 Recovery and differentiation of long ripened cheese microflora through a new cheese-based cultural medium. Food Microbiol 26,240-5.

Palles T., Beresford S., Condon S. & Cogan T.M. 1998 Citrate metabolism in Lactobacillus casei and Lactobacillus plantarum. J Appl Microbiol 85, 147–154.

Parente E. & Cogan T.M. 2004 Starter Cultures: General Aspects. In Cheese: Chemistry, Physics and Microbiology, Vol 1 General Aspects, 3rd edn Fox P F, McSweeney P L H, Cogan T M and Guinee T P, eds Elsevier, London.

Parente E., Rota M.A., Ricciardi A. & Clementi E. 1997 Characterization of natural starter cultures used in the manufacture of Pasta Filata cheese in Basilicata (Southern Italy). Int Dairy J 7, 775-783.

Peterson S.D. & Marshall R.T. 1990 Non starter lactobacilli in Cheddar cheese: a review. J Dairy Sci 73, 1395-1410.

Qian M. & Reineccius G. 2002 Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry. J Dairy Sci 85, 1362–1369.

Randazzo C.L., Pitino I., Ribbera A. & Caggia C. 2010 Pecorino Crotonese cheese: study of bacterial population and flavour compounds. Food Microbiol 27, 363-374.

Rapposch S., Eliskases-Lechner F. & Ginzinger W. 1999 Growth of facultatively heterofermentative lactobacilli on starter cell suspensions. Appl Environ Microbiol 65, 5597–5599.

Reid J.R., Coolbear T., Ayersb J.S. & Coolbear K.P. 1997 The action of chymosin on K-casein and its macropeptide: effect of pH and analysis of products of secondary hydrolysis. Int Dairy J 7, 559- 569.

Salminen S. & Von Wright A. 1998 Lactic Acid Bacteria: Microbiology and Functional Aspects, 2nd edn, eds Marcel Dekker, New York.

Settani L. & Moschetti G. 2010 Non starter lactic acid bacteria used to improve cheese quality and provided health benefits. Food Microbiol 27, 691-697.

Shakeel-Ur-Rehman, Waldron D.& Fox P. F. 2004 Effect of modifying lactose concentration in cheese curd on proteolysis and in quality of Cheddar cheese. Int Dairy J 14, 591–597.

Siezen R.J. 1999 Multi-domain, cell-envelope proteinases of lactic acid bacteria. Antonie van Leeuwenhoek 76, 139-155.

Smit G., Smit B.A. & Engels W.J.M. 2005 Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products FEMS Microbiol Rev 29, 591–610.

Somers E.B., Johnson M.E. & Wong A.C.L. 2001 Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. J Dairy Sci 84, 1926-1936.

Sousa M.J., Ardo Y. & McSweeney P.L.H. 2001 Advances in the study of proteolysis in cheese during ripening. Int Dairy J 11, 327–345.

Steffen C. 1971 Konzentration und Konfiguration des milchsare im reifenden Emmentalerkase, Diss. 4630, Eidy Tech. Hochschule, Zurich.

Steffen C., Glättli H., Steiger G., Flückiger E., Bühlmann C. , Lavanchy P. & Nick B. 1980 Vergleichende Untersuchungen von Greyerzerkasen mit und ohne Nachgarung. I Bakteriologische, biochemische, chemische und rheologische Untersuchungen, Schweiz. Milchwirtsch. Forsch. 9,9–27.

Stolph H. & Starr M.P. 1965 Bacteriolysis. Ann. Rev. Microbiol. 19, 79-104.

Tavaria F.K., Dahl S., Carballo F.J. & Malcata F.X. 2002 Amino Acid Catabolism and Generation of Volatiles by Lactic Acid Bacteria. J Dairy Sci 85, 2462-2470.

Thomas T.D. & Crow V.L. 1983 Mechanism of D(–)-lactic acid formation in Cheddar cheese. New Zealand Journal of Dairy Science and Technology 18, 131–141.

Thomas T.D. 1986 Oxidative acitivity of bacteria from Cheddar cheese. New Zealand Journal of Dairy Science and Technology 21, 37-47.

Thomas T.D. 1987 Cannibalism among bacteria found in cheese. New Zealand Journal of Dairy Science and Technology 22, 215-219.

Turner K.W., Lawrence R.C. & LeLievre J. 1986 A microbiological specification for milk for aseptic cheesemaking. New Zealand Journal of Dairy Science and Technology 21, 249-254.

Upadhyay V.K., McSweeney P.L.H., Magboul A.A.A. & Fox P.F. 2004 Proteolysis in cheese during ripening. In Cheese: Chemistry, Physics and Microbiology, Vol 1: General Aspects, 3rd edn Fox P F, McSweeney P L H, Cogan T M and Guinee T P, eds Elsevier, London.

Urbach G. 1995 Contribution of lactic acid bacteria to flavour compound formation in dairy products. Int Dairy J 5, 877–903.

Waldron D. 1997 Effect of altering lactose content on Cheddar cheese quality. M.Sc. thesis. National University of Ireland, Cork.

Walstra P. & Jennes R. 1984 Dairy chemistry and physics. Wiley, New York.

Williams A.G. & Banks M. 1997 Proteolytic and Other Hydrolytic Enzyme Activities in Non-starter Lactic acid Bacteria (NSLAB) Isolated from Cheddar Cheese Manufactured in the United Kingdom. Int Dairy J 7, 763-774.

Williams A.G., Withers S.E. & Banks J.M. 2000 Energy sources of non starter lactic acid bacteria isolated from Cheddar cheese. Int Dairy J 10, 17–23.

Wood B.J.B. & Holzapfel W.H. 1995 Lactic acid bacteria in contemporary perspective. The lactic acid bacteria: the genera of lactic acid bacteria. Springer.

Yvon M. & Rejien L. 2001 Cheese flavour formation by amino acid catabolism. Int Dairy J 11, 185–201.

Zhang Z.G., Ye Z.Q., Yu L. & Shi P. 2011 Phylogenomic reconstruction of lactic acid bacteria: an update. BMC Evol Biol 11, 1.

III. AIM OF THE THESIS

Lactic Acid Bacteria (LAB) constitute a heterogeneous group of bacteria that are traditionally used in the manufacturing of fermented foods. They may play different roles in cheese-making where starter LAB (SLAB) participate in the fermentation process, whereas non starter LAB (NSLAB) are implicated in cheese ripening. During cheese making, SLAB and NSLAB dynamically evolve. It was demonstrated that SLAB are dominant until the 2nd month of ripening. Differently, after cheese brining, NSLAB, are able to grow and increase in number, while SLAB cells undergo to autolysis. NSLAB were shown to be the dominant species present after a lack of essential nutrients, such as sugars. When NSLAB growth begins, lactose has usually been exhausted, so the energy source used by NSLAB for grow has not yet been clearly defined. Therefore, these species seem to adapt to the absence of lactose in cheese, confirming an optimal adaptability to stressful growth conditions. Presumably, this ability was due to the particular capabilities of NSLAB to use alternative energy source, such as the SLAB cell lysis products, amino acids and other compounds (carbohydrates released from glycocmacropeptide of k-casein and from glycoproteins and glycolipids in the milk fat globule membrane).

Until now, the role of NSLAB during ripening, in this lacking sugar conditions, has not yet been clarified, even if different authors have suggested their importance in the cheese ageing. In fact, during cheese ripening different biochemical changes occur; flavour and texture characteristic of the cheese variety develop during time of aging. In long ripened cheese, such as Parmigiano Reggiano, NSLAB, arising from raw milk and from the environment, are the protagonists of the different biochemical processes during ripening that can lead to the formation of different compounds potentially involved in cheese flavour. Several authors hypothesized that NSLAB are able to use SLAB lysis products but experimentally this is not demonstrate, and the effects of the metabolic activity, during grow, using SLAB lysis product, involved on final cheese characteristics were not investigate.

This PhD thesis aims were: i) the study of NSLAB ability to survive and grow using as energy source only SLAB cell lysis product, ii) the study of their capabilities, in ripened cheese conditions, to produce compounds potentially involved in Parmigiano Reggiano cheese flavour.

To reach these issues, different researches have been assessed.

First, it was investigate the ability of two NLSAB strains, *L. rhamnosus* and *L. casei,* isolated from 20 month ripened Parmigiano Reggiano, to grow using SLAB cell lysis product as a food source. Secondly, as integrative approach, it was analyzed the NSLAB "volatilomes" during growth in this condition, to observe if NSLAB, using SLAB cell lysis product, can be able to produce cheese flavour compounds. Lastly, the ability of *L. rhamnosus* and *L. casei* to synthesize new molecules, γ-glutamil-amino acids and lactoyl-amino acids, potentially involved in flavour was evaluated.

IV. RESULTS

IV.I CANNIBALISM AMONG NON STARTER LACTIC ACID BACTERIA FOR SURVIVAL DURING CHEESE RIPENING

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ABSTRACT

During cheese making, starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) dynamically develop. In Parmigiano Reggiano cheese, SLAB are the dominant microflora at the culmination of curd acidification. They utilise lactose and, when ripening begins, they start to undergo autolysis. Simultaneously there is an increase of NSLAB, which dominate cheese microbiota during ripening. It has been hypothesised that NSLAB can utilise alternative carbon sources in this lacking sugar condition. NSLAB isolated from ripened Parmigiano Reggiano cheese can survive and grow, using products of SLAB lysis as nutrient sources. The aim of this study was to evaluate whether four different NSLAB strains, including two *Lactobacillus casei* and two *Lactobacillus rhamnosus* strains isolated from ripened Parmigiano Reggiano, were able to grow using only SLAB (*Lactobacillus helveticus* isolated from natural whey starter) cell lysate as a nutrient source. NSLAB growth on lysed SLAB cells was evaluated using a novel Cell Sonicated Substrate Membrane System. All NSLAB strains examined grew under these conditions, confirming

their ability to use the products released by SLAB cell lysis as their sole energy source. This conclusion was supported by changes observed in the composition of the substrate.

INTRODUCTION

Lactic acid bacteria (LAB) represent an important microflora for cheese manufacturing primarily due to their ability to metabolise lactose into lactic acid and to contribute to the development of cheese flavour during ripening. Considering curd cooked cheese, like grana cheeses, starter lactic acid bacteria (SLAB) are generally thermophilic microflora added to milk at the beginning of cheese manufacture. Non-starter lactic acid bacteria (NSLAB) are mesophilic microflora that originate from raw milk and from the environment as a result of contamination during the manufacturing process (*Settani et al., 2010*). During cheese making, SLAB and NSLAB dynamically evolve according to modifications of environmental conditions that frequently serve as cellular stresses, such as heat shock, adverse pH, water activity, NaCl concentration and nutrient composition (*Gala et al., 2008*). The presence of different amounts of SLAB and NSLAB, is the result of their ability to grow in milk and curd and of bacterial cell autolysis (*Fox et al., 1996*). In Parmigiano Reggiano (PR), a Protected Designation of Origin (PDO) long-ripened Italian cheese, the number of SLAB commonly exceeds 10^9 cfu/g of cheese when ripening begins. After PR brining, SLAB cells undergo autolysis (*Gatti et al., 2008*); NSLAB species, the initial population of which is typically less than 10^2 cfu/g, begin to grow and eventually plateau at cell densities of 10^6-10^7 cfu/g after 3-9 months of aging (*De Dea Lindner et al., 2008*; *Gatti et al., 2008; Coppola et al., 1997*), becoming the dominant cheese microbiota during ripening. This demonstrates the ability of NSLAB to grow in hostile, low-sugar environments (*Settani et al., 2010*). In fact, when NSLAB cell numbers increase, most of the residual lactose in cheese has been utilised by SLAB (*Dìaz-Muñiz et al., 2006*). It has been hypothesised that NSLAB utilise carbohydrates derived from glycomacropeptides of casein and glycoproteins derived from fat globule membranes (*Fox et al., 2004*), although this, to our knowledge, has not been demonstrated experimentally. It has also been proposed that fats and residual citrate, which are present at low levels, do not represent relevant nutritional sources (*Fox et al., 2004*). The same authors have hypothesised that peptides and amino acids, which are present at high concentrations, constitute the main nutrient for NSLAB growth. In a related study, *Thomas (1987)* considered the possibility

that NSLAB uses the products of starter-cell autolysis as a carbon source for growth. This author pointed out that upon cessation of growth, starter cells slowly autolyse.

The resulting products, which include sugars from cell walls, nucleic acids (*Rapposh et al., 1999*) and peptides, are not typically catabolised, thus representing potential carbon sources for the growth of NSLAB, which generally have more extensive metabolic capacities (*Budinich et al., 2011*; *Peterson et al., 1990*). Additionally, *Williams et al. (2000)* proposed that the increase in NSLAB cell numbers is dependent upon their ability to utilise potentially available substrates, including bacterial metabolites and products of cell lysis, such as DNA, ribose, hexosamine, citrate, lactate, fatty acids, peptides and amino acids. According to *Thomas (1987)* and *Williams et al. (2000)*, it may be assumed that the lysis of some organisms leads to the production of carbon, nitrogen, fatty acids and nucleic acids that could be used as energy sources for bacterial cells in lacking sugar environment, such as ripened cheese. Thus, it can be supposed that NSLAB isolated from ripened PR cheese can survive and grow, using either nitrogenous compounds that result from casein hydrolysis (*Neviani et al., 2009*) or products of SLAB lysis (*Thomas, 1987*; *Williams et al. 2000*) as nutrient sources. The aim of this study was to evaluate whether 4 different PR NSLAB strains, 2 *Lactobacillus casei* and 2 *Lactobacillus rhamnosus*, were able to grow using only SLAB (*Lactobacillus helveticus*) cell lysate as a nutrient source. Growth was studied using a novel Cell Sonicated Substrate Membrane System (CSSMS). To support the evidence of the growth, changes in substrate composition were evaluated. In fact, demonstrate that NSLAB grow on the SLAB lysis product would allow to confirm that they can be able to grow in lacking sugar conditions. Confirming this, many perspectives would be opened on the metabolic capabilities of these microorganisms. The role of starter microflora in Parmigiano Reggiano and other ripened cheeses is well known (*Pecorari et al., 2003*), however, little is known about NSLAB role and contribution during cheese ripening. Confirming the hypothesis that NSLAB can grow using SLAB as nutrient source, many new promising fields of study would be opened. A better knowledge of the role of this microflora in ripened cheese, as well as of the aromatic contribution of NLSAB on cheese flavour, would be possible.

MATERIALS AND METHODS

Strains

Four NSLAB strains previously isolated from ripened PR cheese, 2 *L. rhamnosus* (1216 and 1473) and 2 *L. casei* (1056 and 1247), and three SLAB strains previously isolated from PR natural whey starter (*Neviani et al., 2009*), *L. helveticus* (770, 772 and 780), were used. All strains were maintained as stock cultures at -80°C in MRS broth (Oxoid, Basingstoke, UK) supplemented with 15% glycerol (w/v).

Lysed SLAB Cell-Based Medium

To prepare the lysed SLAB cell-based medium (LCM), three *L. helveticus* strains were anaerobically (Gas Generating Kit, Oxoid, Basingstoke, UK) passaged twice with a 2% starting inoculum in 20 mL of MRS broth at 42°C for 24 h. Twenty mL of each culture were mixed in 1 L of MRS broth at 42°C for 24 h. After incubation, the cell concentration was determined by microscopic counting (*Gatti et al., 2006*). The mixed culture was harvested by centrifugation at 10000 rpm for 10 min at 4°C (Centrifuge, 5810R, Eppendorf, Hamburg, Germany), and the cells were resuspended in 60 mL of phosphate buffer (50 mM, pH 7) (Carlo Erba Reagents, Milan, Italy). The cell suspension was sonicated with a titanium probe operated at 70 W for 30 cycles (30 s pulse on and 1 min pulse off) (Sonoplus HD3100, Bandelin, Berlin Germany) and cooled on ice. Sonication was repeated twice. MRS agar plate count was performed in duplicate to verify the performance of sonication. The efficacy (E) of sonication was determined using the following formula: $E=100-(n_t/n_0)*100$, where n_0 = cfu/mL of *L. helveticus* culture and n_t = cfu/mL of *L. helveticus* culture after sonication. LCM was prepared by mixing this sonicate with agar (4 g/mL) (50:50, v/v).

NSLAB Growth Assay

NSLAB strains were anaerobically (Gas Generating Kit, Oxoid, Basingstoke, UK) passaged twice with a 2% starting inoculum in 6 mL of MRS broth at 30°C for 24 h. To obtain NSLAB cells for inocula, each viable *L. casei* (1056 and 1247) and *L. rhamnosus* culture (1473 and 1216) was washed in Ringer solution (Oxoid, Basingstoke, UK) and harvested at 10000 rpm for 10 min at 4°C (Centrifuge, 5810R, Eppendorf, Hamburg, Germany). Each washed culture was filtered onto a 0.2 µm white isopore PC membrane (Millipore, Billerica, MA) using a syringe filter holder. Before the addition of the PC membranes, Tissue Cell Culture Inserts (TCI) (Millicell, Millipore, Billerica, MA) were inverted and filled with 3 mL of

LCM. TCI were then placed upside down in a sterile six-well multi-dish plate (Nunc A/S, Roskilde, Denmark). The underside of the inoculated PC membrane was placed on the fixed anopore membranes of the TCI (*Ferrari et al., 2005*). One Sonicated Cell Substrate Membrane System (CSSMS) per strain was incubated at 30°C for 48 h. In the same way, a reference sample, CSSMS without NSLAB inoculum, was prepared and incubated at the same conditions. After incubation, each filter was stained with DAPI (4,6-diamidino-2-phenylindole) and observed with a fluorescence microscope (Nikon Eclipse 80i epifluorescence microscope equipped with a C-SHG1 100 W mercury lamp, Nikon, Tokyo, Japan). This assay was repeated four times. The CSSMS substrates from the 4 replicates for each strain were mixed and lyophilised. Finally, five samples were obtained: a reference sample (CSSMS substrate without inoculum) and CSSMS substrates after the growth of 2 strains of *L. casei* (Lc1056 and Lc1247) or 2 strains of *L. rhamnosus* (Lr1473 and Lr1216). Four negative controls were assembled in duplicate by inoculating agar with each NSLAB strain and incubation at 30°C for 48 h.

Changes in Fatty Acids, Sugars and Nitrogen

The CSSMS substrates of the reference sample and of the samples after *L. rhamnosus* growth (Lr1216 or Lr1473) or after *L. casei* growth (Lc1506 or Lc1247) were lyophilized. From the freeze-dried samples were then extracted the different fractions.

Fatty acids were extracted from CSSMS lyophilized substrates before NSLAB growth (reference sample), after *L. rhamnosus* growth (Lr1216 and Lr1473) and after *L. casei* growth (Lc1506 and Lc1247). Extraction was performed with 60 mL of diethyl ether using a Soxhlet apparatus (Velpscientifica, Italy) for 1 h. The extract was dissolved in 1 mL of hexane and injected into a gas chromatography-mass spectrometer (GC/MS). GC/MS analysis was performed using an Agilent Technologies 6890 gas chromatograph coupled to an Agilent Technologies 5973 mass spectrometer (Waters, Milford, MA).

A 30 m x 0.25 mm fused silica capillary column coated with 0.25 µm 95% polydimethylsiloxane/5% phenyl (SLB 5, Supelco) (Sigma Aldrich, St Louis, MO) was installed in the GC oven. The GC oven was held for 3 min at 40°C, ramped 20°C/min to 220°C, which was held for 8 min, and was then ramped 20°C/min to 270°C, which was held for 7 min and 30 s (the total run time was 30 min). The carrier gas helium (initial pressure of 8 psi) was transported with a constant flow rate of 1 mL/min through the column.

The injector temperature was 280°C. The full-scan mode was used.

For the analysis of nitrogenous compounds, 2 g of freeze-dried sample was added to 40 mL of phosphate buffer (pH 7, 50 mM). Samples were centrifuged (5°C for 30 min at 12000 rpm), filtered through a 0.22 µm filter and concentrated to obtain a final volume of 4 mL. Afterwards, the samples were ultra-filtered with a molecular cut-off of 100 kDa and 30 kDa to obtain 3 fractions: >100 kDa, 30-100 kDa and <30 kDa. All the fractions were dried under nitrogen flow. The 30-100 kDa fraction was analysed by Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE). The extracted proteins were quantified with a Q-bit spectrophotometer (Invitrogen, Carlsbad, CA, USA) (485-590 nm). Two concentrations (25 µg/mL and 50 µg/mL) were loaded onto a precast 12% Bis-Tris SDS-PAGE gel (BioRad, Richmond, California) with BioRad Range SDS-PAGE Molecular Weight Standards (BioRad) and separated in XT NG running buffer (BioRad) for 55 min at 150 V. Gels were stained with a solution of Coomassie blue (40% methanol, 10% acetic acid, 1 g/L Coomassie blue) for 2 h. Subsequently, the gel was de-stained with the same solution, without Coomassie blue, for 40 min, replacing the solution 2-3 times according to the BioRad precast gel instructions. The <30 kDa fraction was ultra-filtered using filters with a 5 kDa cut-off. The protein fraction between 30 kDa and 5 kDa was injected into LTQ-ORBITRAP. LTQ-ORBITRAP technology can be used for the identification of proteins and digestion products in biological samples. In the present work, we investigated whether the peptide fraction from 5 kD-30 kDa could be used as a food source by NSLAB. In particular, our aim was to determine whether the 5-30 kDa fraction was degraded by bacteria to recover amino acids or small peptides from these proteins. Briefly, using a computer-aided, data-dependent elaboration, it is possible to recognise the protein sequence from the peptides in the sample.

Analysis conditions were the following: Jupiter 4u Proteo 90A 300 µm X 15 cm column; eluent A: H_2O+0.2% formic acid; eluent B: acetonitrile (ACN)+0.2% formic acid; and a duration of 75 min.

The <5 kDa fraction was used for amino acid analysis using a procedure based on the AccQTag™ protocol (Waters). Briefly, after the derivatisation step, the amino acid fraction was analysed by HPLC using a C18 Spherisorb ODS-2 column (Waters, Milford, MA, USA) and a Waters 474 spectrofluorimetric detector according to the method of Cavatorta *et al.* (2007).

RESULTS AND DISCUSSION

NSLAB Growth Assay

To simulate the PR SLAB bacterial lysis that naturally occurs during ripening, *L. helveticus* cells were sonicated. The concentration of the *L. helveticus* mixed cell culture was determined to be 10^8 cells/mL by microscopic counting. The MRS plate count of the sonicated cells was below the limit of detection, demonstrating a sonication efficacy (E) of greater than 99.99999901%. To evaluate NSLAB growth, the CSSMS system was used. CCSMS separated the inoculated cells, NSLAB strains *L. casei* and *L. rhamnosus,* from the sonicated SLAB cells (*L. helveticus*), while allowing the diffusion of nutrients from the bottom to the top and of metabolites from the top to the bottom. NSLAB growth was detected by observing micro-colony formation by fluorescence microscopy (Figure 1, Figure 2). We have determined that fluorescence microscopy can accurately and objectively assess growth of these species. In fact, fluorescence microscopy allowed us to observe the formation of small micro-colonies on agar plates that could not be observed with the naked eye. All the NSLAB strains studied grew in this system. None of the negative controls, which consisted of agar inoculated with the four NSLAB strains, showed cell growth, confirming that agar alone cannot be a nutrient source. LMC growth medium was composed of agar and sonicated *L. helveticus* cells, which was the only available nutrient source. This result confirms that *L. casei* and *L. rhamnosus* can use the products released after SLAB cell lysis as an energy source, which *Thomas (1987)* had previously hypothesised.

Changes in Fatty Acids, Sugars and Nitrogenous Compounds

To understand which components were used as nutrients or produced as metabolites, chemical analysis were performed for all the samples on the substrate filling the TCI, after the growth of *L. casei* (Lc1056 or Lc1247) or *L. rhamnosus* (Lr1473 or Lr1216). Obtained results were compared with those coming from chemical analysis of the substrate of the reference sample (i.e. CSSMS without inoculum). Lipids were extracted from the five samples with a Soxhlet extractor and analysed by GC/MS chromatography. The chromatograms revealed several differences between the reference sample and the samples obtained after microbial growth. Peaks corresponding to myristic acid (peak 2, C14:0, retention time (rt) 11 min), palmitic acid (peak 3, C16:0, rt 14 min), oleic acid (peak 4, C18:1, rt 16 min), stearic acid (peak 5, C18:0, rt 16,5 min) and other long-chain fatty acids

were identified. Peak 1 was not identified. Relative abundance percentages were calculated for the 11 most significant peaks (Figure 3). The behaviour of the four strains was clearly different and not species-specific. The greatest differences were observed after the growth of *L. rhamnosus* 1473, which was able to produce long-chain fatty acids (peaks 6 to 11).

In contrast, the relative abundance of these compounds decreased after the growth of *L. rhamnosus* 1216, demonstrating long-chain fatty acid consumption. After *L. casei* 1056 growth, only a decrease in C18 (peak 4) and an increase in peak 1 were observed. *L. casei* 1247 growth did not significantly alter the relative abundance of fatty acids.

The modification of the fatty acid profile may be due to lipase and esterase activity that is known to be present in nonstarter lactobacilli (*Banks et al., 2004*). This is confirmed by the heterogeneity of the profiles observed after the growth of NSLAB. *Banks et al. (2004)* observed that greater activity of these enzymes increases the consumption of long-chain fatty acids. This suggests that *L. rhamnosus* 1216 could have a high lipase and esterase activity, while *L. rhamnosus* 1473 seems to lack these enzymatic activities. In particular, it was observed that the activities of these enzymes were strain-specific. In the majority of strains, the activity increased as the carbon chain length of the fatty acids decreased (*Banks et al., 2004*). Interestingly, it has been observed that *L. rhamnosus* was able to produce fatty acids, particularly long-chain fatty acids.

The 30-100 kDa proteic fraction was analysed by SDS-PAGE (Figure 4). Two concentrations (25 µg/mL and 50 µg/mL) of protein were loaded to highlight possible differences. The highest concentration revealed several differences between the reference sample and samples obtained after strain growth. Band 1 (approximate weight of 24 kDa) was consumed by all strains. In contrast, band 2 (approximately 36 kDa), which was absent in the reference sample, was produced after *L. casei* 1056 and *L. rhamnosus* 1216 growth, while band 3 (approximately 46 kDa) increased after the growth of all strains. This can be explained by the assumption that proteins greater than 100 kDa in weight are degraded by bacterial proteases (*Kunij et al., 1996*). Two fractions were obtained by the ultra-filtration of the <30 kDa fraction; fractions between 5kDa and 30 kDa were analysed by LTQ-ORBITRAP, and fractions <5 kDa were analysed for amino acid determination.

The data obtained by LTQ-ORBITRAP analysis are very complex, requiring advanced study and specific interpretations, which are not the objective of this work. In particular, the protocol allowed for the identification of 876 proteins in the reference sample as *Lactobacillus acidophilus* proteins, a species phylogenetically related to *L. helveticus* (*Zhang et al., 2011*). For each protein, fragments allowing for recognition by the instrument were observed. To understand whether this protein fraction could be used by NSLAB, the composition of peptides derived from proteins found in all samples after NSLAB growth was compared to the reference sample; an example of this comparison is shown in Figure 5. This comparison revealed the degradation of fragments of the reference sample into smaller peptides, which indicated that NSLAB used these proteins as a nutrient source. NSLAB growth led to a change in the peptide composition of the substrate. Accordingly, it is known that *L. casei* is equipped with a proteolytic enzyme system that allows it to acquire amino acids from proteins present in its environment (*Cai et al., 2009*). Peptides and amino acids that are liberated by the action of proteolytic enzymes may be translocated to the cytoplasm by a variety of peptide and amino acid transporters (*Cai et al., 2009*). No differences in the quantity of amino acids (fraction <5 kDa) were detected between the reference and experimental samples (data not shown). Amino acids are a high-quality substrate for bacteria, and peptides and amino acids are catabolised by lactobacilli when provided a keto acid acceptor that facilitates the aminotransferase-mediated degradative pathway (*Banks et al., 2004*; *Williams et al., 2000*). It is likely that no differences were found because bacterial metabolism also produces amino acids. Therefore, it can be assumed that *L. casei* and *L. rhamnosus* probably consume amino acids during growth but that, at the same time, some amino acids are produced by bacterial metabolism in quantities similar to what is consumed. Therefore, the results of our analysis do not show a significant change in amino acid quantities.

Despite the paucity of nutrients and, in particular, the absence of sugars that characterise cheese during ripening, NSLAB present in Parmigiano Reggiano can survive and even grow. It is known that these lactic acid bacteria can use nitrogenous compounds as nutrient sources. It has also been proposed that the cell degradation components of some microorganisms could serve as sources of carbon, nitrogen, phosphorous and nucleic acid precursors for less nutritionally demanding bacterial cells.

The experiments conducted to substantiate this hypothesis confirmed that *L. helveticus* lysate provides sufficient nutrients to allow the growth of *L. casei* and *L. rhamnosus in vitro*. The observation of changes in the lipid, and protein fractions let us to hypothesize that these compounds can be consumed or produced by bacterial metabolism during growth. However, the analysis performed in this study provides new perspectives on NSLAB cannibalism as a survival strategy, offering different clues that could support the initial hypothesis of NSLAB growth on SLAB lysate. Further work and a deeper analysis will be useful to better understand which compounds are consumed and which are produced by NSLAB during growth in ripened cheese.

REFERENCES

Banks J.M. & Williams G. 2004 The role of nonstarter lactic acid bacteria in Cheddar cheese ripening. Int J Dairy Tech 57, 145-152.

Budinich M.F., Perez-Díaz I., Cai H., Rankin S.A., Broadbent J.R. &Steele J.L. 2011 Growth of Lactobacillus paracasei ATCC 334 in a cheese model system: A biochemical approach. J Dairy Sci 94, 5263-5277.

Cai H., Thompson R., Budinich M.F., Broadbent J.R. & Steele J.L. 2009 Genome sequence and comparative genome analysis of Lactobacillus casei: insights into their niche-associated evolution. Genome Biol Evol 1, 239-257.

Cavatorta V., Sforza S., Lambertini F., Bellini M., Stefani F., Galaverna G., Dossena A., Panari G. & Nocetti M. 2007 Evaluation of the amino acid and peptide fraction of Parmigiano – Reggiano cheese up to the eight month of ripening. Sci Tec Latt Cas 58, 183-203.

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

De Dea Lindner J., Bernini V., De Lorentiis A., Pecorari A., Neviani E. & Gatti M. 2008 Parmigiano Reggiano cheese: evolution of cultivable and total lactic microflora and peptidase activities during manufacture and ripening. Dairy Sci Technol. 88, 511-523.

Dìaz-Muñiz I., Banavara D.S., Budinich M.F., Rankin S.A., Dudley E.G. & Steele J.L. 2006 Lactobacillus casei metabolic potential to utilize citrate as an energy source in ripening cheese: a bioinformatics approach. J Appl Microbiol 101, 872-882.

Ferrari B.C., Binnerup S.J. & Gillings M. 2005 Microcolony cultivation on a soil substrate membrane system selects for previously uncultured soil bacteria. Appl Environ Microbiol 71, 8714-8720.

Fox P.F., McSweeney P.L.H., Cogan T.M. & Guinee T.P. 2004 Cheese - Chemistry, Physics and Microbiology Elsevier, London UK, Springer.

Fox P.F. & McSweeney P.L.H. 1996 Proteolysis in cheese during ripening. Food Rev. Int 12, 457-509.

Gala E., Landi S., Solieri L., Nocetti M., Pulvirenti A. & Giudici P. 2008 Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. Int J Food Microbiol 125, 347–351.

Gatti M., Bernini V., Lazzi C. & Neviani E. 2006 Fluorescence microscopy for studying the viability of micro-organisms in natural whey starters. Lett Appl Microbiol 42, 338-43.

Gatti M., De Dea Lindner J., De Lorentiis A., Bottari B., Santarelli M., Bernini V. & Neviani E. 2008 Dynamics of whole and lysed bacterial cells during Parmigiano-Reggiano cheese production and ripening. Appl Environ Microbiol 74, 6161-7.

Kunji E.R., Mierau I., Hagting A., Poolman B. & Konings W.N. 1996 The proteolytic systems of lactic acid bacteria. Antonie Van Leeuwenhoek 70, 187-221.

Neviani E., De Dea Lindner J., Bernini V. & Gatti M. 2009 Recovery and differentiation of long ripened cheese microflora through a new cheese-based cultural medium. Food Microbiol 26, 240-5.

Peterson S.D. & Marshall R.T. 1990 Non starter lactobacilli in Cheddar cheese: a review. J Dairy Sci 73, 395-1410.

Pecorari, M., Gambini, G., Reverberi, P., Caroli, A., & Panari, G. 2003 Glycolysis in Parmigiano-Reggiano cheese during the first ripening phase. Sci Tec Latt Cas, 54, 149-162.

Rapposch S., Eliskases-Lechner F. & Ginzinger W. 1999 Growth of Facultatively Heterofermentative Lactobacilli on Starter Cell Suspensions. Appl Environ Microbiol 65, 5597–5599.

Settani L. & Moschetti G. 2010 Non starter lactic acid bacteria used to improve cheese quality and provided health benefits. Food Microbiol 27, 691-697.

Thomas T.D. 1987 Cannibalism among bacteria found in cheese. N. Z. J. Dairy Sci. Technol. 22, 215-219.

Williams A.G., Withers S.E. & Banks J.M. 2000 Energy sources of non starter lactic acid bacteria isolated from Cheddar cheese. Int Dairy J 10, 17–23.

Zhang Z.G., Ye Z.Q., Yu L. & Shi P. 2011 Phylogenomic reconstruction of lactic acid bacteria: an update. BMC Evol Biol 11, 1.

FIGURES

***Figure 1**: Fluorescence microscope images with DAPI staining of the L. rhamnosus CSSMS system (scale 100px). **a**: L. rhamnosus 1216 cells immediately after inoculation; **b**: L. rhamnosus 1473 cells immediately after inoculation; **c**: L. rhamnosus 1216 microcolonies after incubation at 30°C for 48 h; **d**: L. rhamnosus 1473 microcolonies after incubation at 30°C for 48 h.*

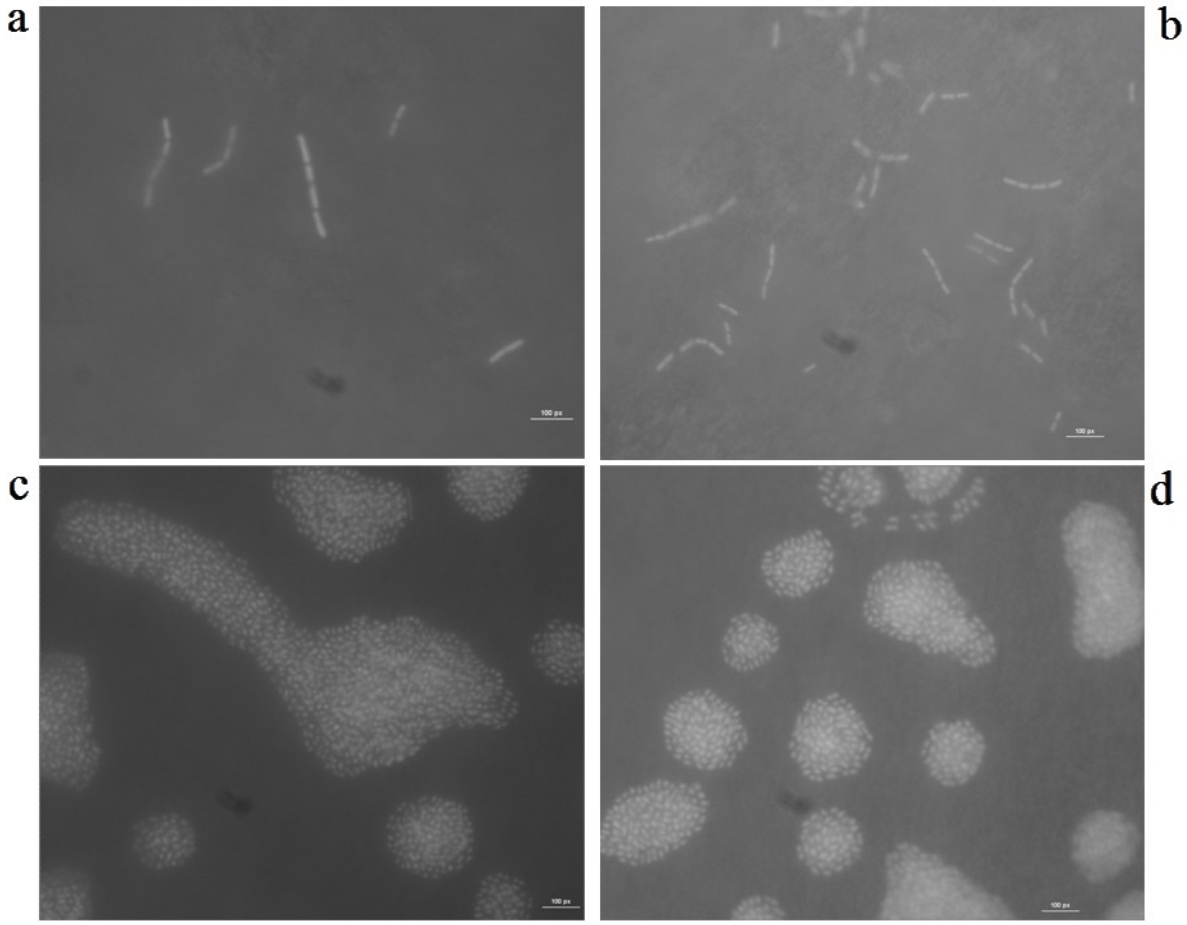

***Figure 2**: Fluorescence microscope images with DAPI staining of the L. casei CSSMS system (scale 100px). **a**: L. casei 1056 cells immediately after inoculation; **b**: L. casei 1247 cells immediately after inoculation; **c**: L. casei 1056 microcolonies after incubation at 30°C for 48 h; **d**: L. casei 1247 microcolonies after incubation at 30°C for 48 h.*

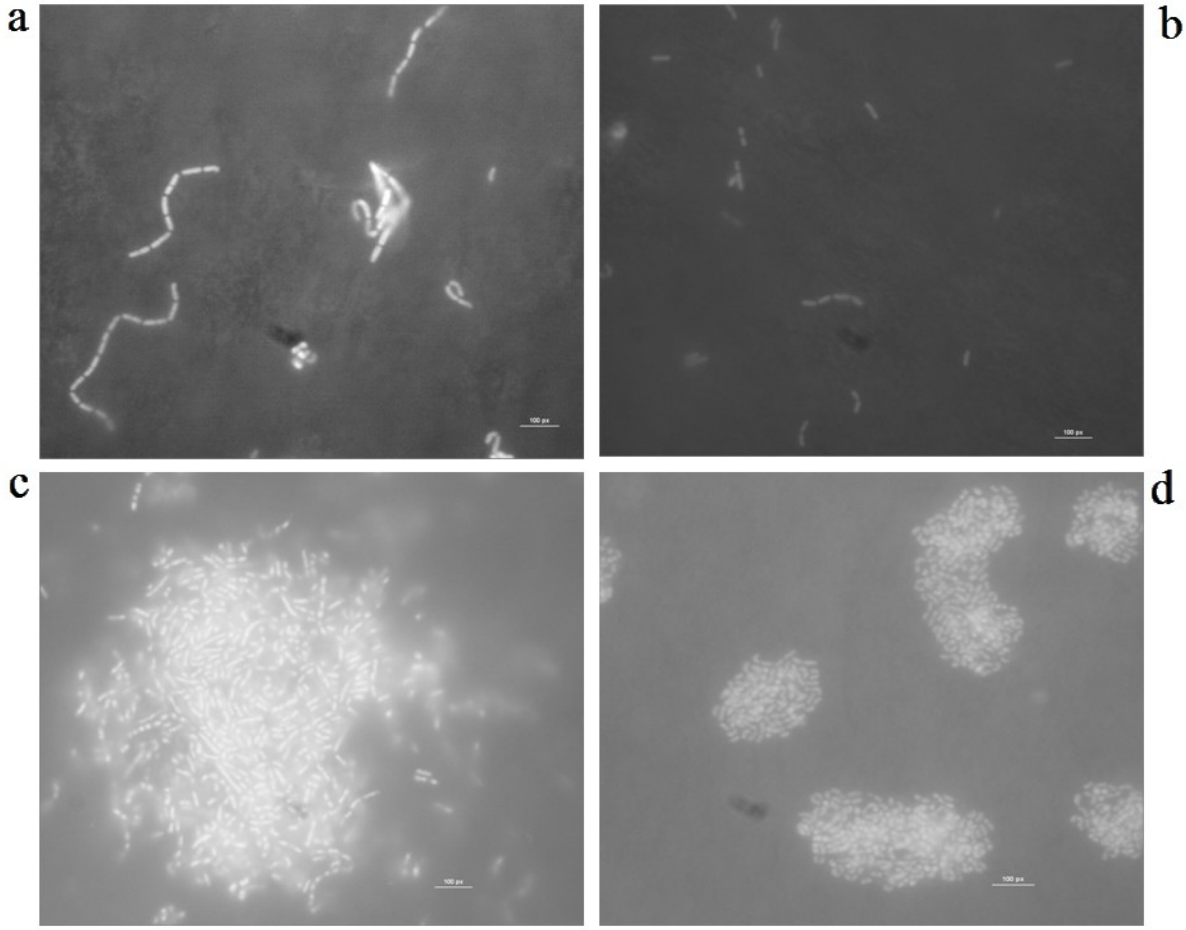

***Figure 3**: Relative abundance percentages of fatty acids determined on CSSMS substrates of the reference sample and after L. rhamnosus and L. casei growth. Numbers from 1 to 11 correspond to different fatty acids. Peaks identification: Peak 1: not identified, Peak 2: C14:0 myristic acid, Peak 3: C16:0 palmitic acid, Peak 4: C18:1 oleic acid, Peak 5: C18:0 stearic acid, Peaks from 6 to 11: long chain fatty acids.*

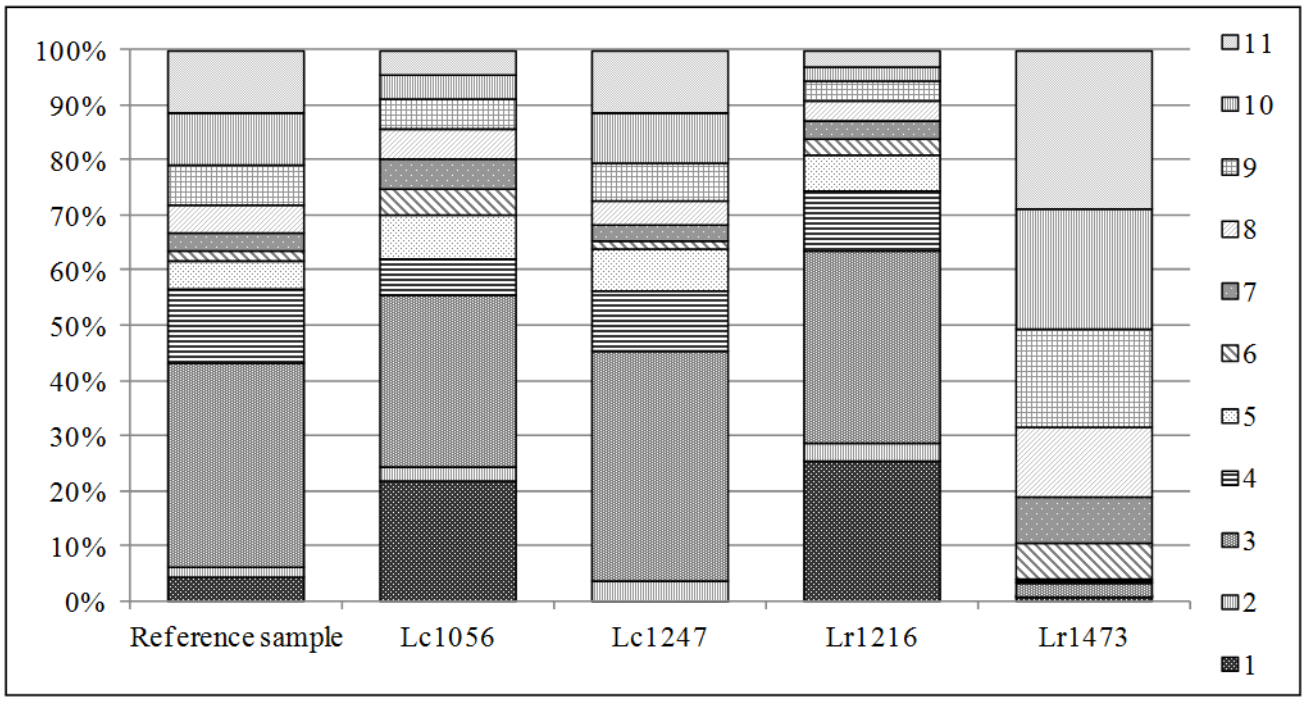

***Figure 4**: SDS-PAGE analysis of the 30-100 kDa proteic fraction. The standard BioRad Range SDS-PAGE Molecular Weight Standards (BioRad) was used. Two concentration of protein (25 µg/mL and 50 µg/mL) were loaded. Lane a: reference sample; lane b: Lr1473; lane c: Lc1056; lane d: Lr1216; lane e: Lc1247. Band 1 (~ 24 kDa), band 2 (~ 36 kDa), band 3 (~ 46 kDa).*

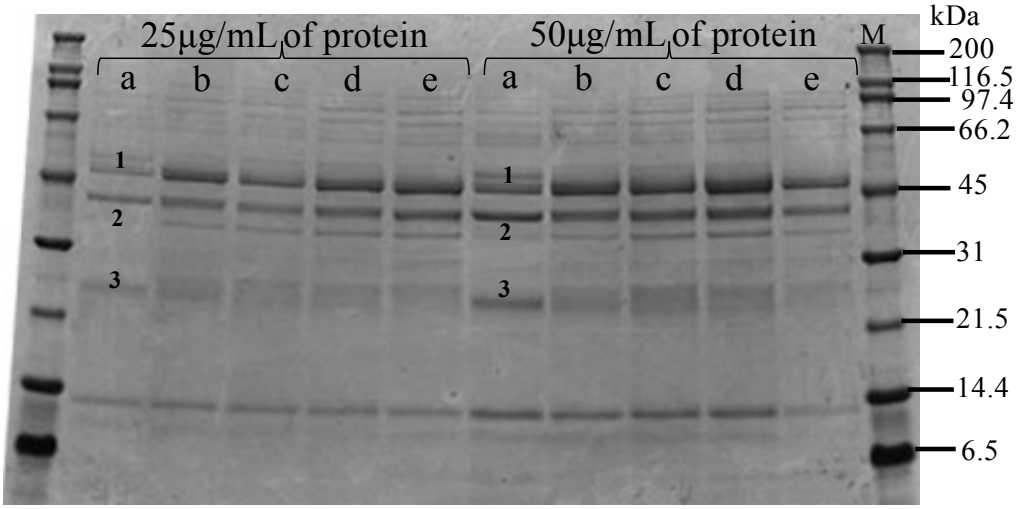

***Figure 5**: Example of a comparison of peptides extracted from the reference sample (highlighted in different shades of gray) and from sample Lr1473 (indicated by solid lines). Each different shade of gray corresponds to a different peptide coming from a protein of the reference sample. Each different length of solid line corresponds to different peptide coming from the same protein after NLAB growth. Peptides from protein Q5FKU9 are shown as an example.*

Protein name	Description	MW (Da)
Q5FKU9	Cell division protein OS	48119

MDFTFDSDDNKNAVIKVIGVGGAGGNAVNRMIDDGVQGVSFIAANTDVQALNSNKAENK
IQLGPKLTRGLGAGSHPEVGQKAAEESEQTIEDALKGADMIFITAGMGGGTGTGAAPVVA

KIARETGALTVGVVTRPFSFEGPKRSKNAAEGISQLKQYVDTLVIIANNRLLEMVDKKTPM
MDAFKEADNVLKQGVQGISDLITSTDYVNLDFADVKTVMENQGAALMGIGRASGENRTV
EATKLAISSPLLEVSIDGAKQVLLNITGGPDLTLFEAQDASEIVSKAAGDDVNIIFGTSINPNL

GDEVVVTVIATGIDSEAEEAASKQLPGRSHQIKAQPKKESEPAQNNDVVQPKVQTVDRPET
IQPENNVSEPEVKKPKQTMVDPTSVWDLNNNQDNQRRNTKPAEPEEDSEKFNAFSDQEQE
GISQIETSAQDTSDDNSDIPFFKHRGEN

IV.II NSLAB VOLATILOMES CONTRIBUTE TO CHEESE FLAVOUR

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ABSTRACT

The outstanding role of lactic acid bacteria (LAB) in cheese flavour formation is known. In long ripened cheese the flavour formation occurs during ripening. In several studies, it has been reported that, in long ripened cheese varieties, NSLAB population dominate during aging, when the SLAB are mainly autolysed. The LAB capabilities to contribute to cheese flavour formation have already been studied. The specific NSLAB metabolic reactions, that lead to flavour compounds formation and that occur in ripened cheese, are not clearly yet. In ripened cheese the nutrient source available can be the small peptides and/or amino acids, and the SLAB cell lysis products. Thus, the aim of this research was to evaluate the volatile flavour compounds producing capabilities of NSLAB during cheese ripening, using as energy source the only nutrients available in ripened cheese. Then, the potentially contribution of their volatilome on the total cheese flavour was discussed. For this purpose, the volatile components produced in two different growing media by 4 different NSLAB strains (2 *Lactobacillus casei* and 2 *Lactobacillus rhamnosus*), previously isolated from ripened PR, were investigated. The volatile compounds produced on Cheese based medium and on SLAB cell lyses based medium were analyzed through head-space

GC/MS to understand which volatile compounds can be produced from different ripened cheese components.

INTRODUCTION

The flavour perception is very important for the distinction of fermented dairy products and for determination of their quality (*Olson, 1990*). The combination of a large number of sapid volatile and non-volatile compounds, in the correct ratios and concentration, leads to cheese flavour formation (*McSweeney & Sousa, 2000*). In fact, there is a unique flavour for every cheese and it is produced by a large range of specific compounds that are present in varying quantities in each type of cheese and that allow to constitute the typical cheese flavour (*Weimer, 2007*).

The flavour of different cheeses has already been studied. The aromatic profile of several kind of cheese was characterized: Cheddar (*Zehentbauer & Reineccius, 2002*), Camembert (*Kubickovà & Grosh, 1997*), Gorgonzola (*Moio et al., 2000*), Grana Padano (*Moio & Addeo, 1998*), Parmigiano Reggiano (*Qian & Reineccius, 2002*), Pecorino Crotonese (*Randazzo et al., 2010*), Pecorino Romano and Fiore Sardo (*Di Cagno et al., 2003*). Up to 600 volatile compounds have been detected and identified (*Maarse & Visscher, 1989*).

Cheese is a biochemically dynamic product, in fact, during its ripening significant microbial and biochemical changes occur. The freshly-made curds of several kind of cheese have bland and similar flavours; during the ripening, which can be different among the cheese type and it differs for a period ranging from two weeks to two or more years, flavour compounds are produced (*McSweeney & Sousa, 2000*). Thus, the formation of flavours is a rather slow phenomena that involves several chemical and biochemical reactions that occur during aging (*Smit et al., 2005*). This complex process involved three major metabolic pathways: i) metabolism of lactate and citrate; ii) liberation of free fatty acids (FFA) and their subsequent metabolism; iii) degradation of casein matrix of the curd to a range of peptides, then to free amino acids (FAA) and ultimately the catabolism of FAA (*Yvon & Rijnen, 2001; McSweeney & Sousa, 2000*). Metabolic volatile compounds derived from amino acids were studied (*Fernandez & Zuniga, 2006*; *Tavaria et al., 2002*) and the lactic acid bacteria (LAB) ability to generate flavour compounds by amino acids catabolism was observed (*Yvon & Rijnen, 2001*). The lipolysis and the pathways of FFA catabolism that directly affect cheese flavour have been reported (*Collins et al., 2003*). Also

the capabilities of LAB to use lactate and citrate and to produce, from these, flavour compounds has been confirmed (*McSweeney, 2004*). Therefore LAB can be able to produce the most important aromatic compounds of several varieties of cheese (*Smit et al., 2005*).

It is commonly accepted that bacteria involved in cheese ripening and their catabolic abilities, leading to the production of different compounds, characterise the flavour profile of cheese (*Law et al.,1976; Fox & Wallace, 1997*). Although a link between the most important bacteria of cheese, lactic acid bacteria (LAB), and cheese flavour was first postulated more than 100 years ago, the complexities in microbiology, enzymology, and the cheese micro-environments made difficult to establish a definitive and specific role of these bacteria in flavours formation (*Broadbent & Steele, 2005*). Therefore, research in this area mainly focuses on some single species: *Lactococcus lactis*, which is added as a starter for Cheddar, Gouda, and other cheeses (*Broadbent & Steele, 2005*), *Lactobacillus delbrueckii* subsp. *bulgaricus* which is widespread used both as starter or adjunct cultures (*Yvon et al., 2001*). However, not only starter LAB (SLAB) compose microbiota of cheese but also adventitious species and nonstarter LAB (NSLAB) are present and may affect cheese flavour. While several studies have examined the metabolic capabilities of LAB leading to the production of flavour compounds (*Smit et al., 2005; Marilley & Casei, 2004; Helinck, 2004; Tavaria et al., 2002; Urbach, 1995; Olson 1990*) but the effect of metabolic products of NLSAB species were not deeply investigated yet. Considering that the majority of flavour compounds is generated during the long aging period, it would be interesting to investigate if NSLAB microflora, that dominate during maturation of cheese, can contribute to the aromatic compounds synthesis and how this contribute is achieved.

During long-ripened cheese making, SLAB and NSLAB dynamically evolve (*Gala et al., 2008*). In Parmigiano Reggiano (PR), a long-ripened Italian cheese, the number of SLAB commonly exceeds 10^9 cfu/g of cheese when ripening begins. After PR brining, SLAB cells undergo autolysis (*Gatti et al., 2008*) and NSLAB population (initially is less than 10^2 cfu/g) grows at cell densities of 10^6-10^7 cfu/g (*De Dea Lindner et al., 2008; Gatti et al., 2008; Coppola et al., 1997*). NSLAB became the dominant microflora of cheese until the end of ripening. Their development during ripening can be due to their capabilities to use the major compounds of ripened cheese as a nutrient sources for growth, such as small peptides and amino acids, which are present in at high concentrations (*Fox et al., 2004*). Moreover other compounds in ripened cheese are the products of starter cells autolysis

which include sugars and phospholipids from cell walls, nucleic acids and peptides, representing a carbon sources for the growth of NSLAB (*Budinich et al., 2011; Rapposh et al., 2009; Thomas, 1987*).

The aim of this research was to evaluate the volatile compounds produced by NSLAB during cheese ripening, using as energy source the only nutrients available in ripened cheese, and to discuss the potentially contribution of their volatilome on the total cheese flavour.

For this purpose the growth and the volatile compounds production of 2 *Lactobacillus casei* and 2 *Lactobacillus rhamnosus* strains in two different growing media, cheese based (*Neviani et al., 2009*) and SLAB cell lyses based media, were evaluated. The volatile compounds produced were analyzed through head-space GC/MS and the metabolic pathways involved in their production were discussed.

Several research focused their attention on the selection of LAB strains with interesting activities contributing in cheese flavour, to diversify flavour and to accelerate ripening of cheese whose production is not regulated by disciplinary. The accelerate ripening is of great economical interest in cheese manufacture, because ripening is a time consuming and expensive process that is still not well mastered and the final flavour of cheese partly determines consumer choice. This research can be considered a step in this direction.

MATERIALS AND METHODS

Strains

Four NSLAB strains belonging to University of Parma collection and previously isolated from ripened Parmigiano Reggiano cheese, 2 *L. rhamnosus* (Lr1216 and Lr1473) and 2 *L. casei* (Lc1056 and Lc1247) (*Neviani et al., 2009*), and three SLAB strains belonging to University of Parma collection and previously isolated from PR natural whey starter (*Neviani et al., 2009*), *L. helveticus* (Lh770, Lh772 and Lh780), were used. All strains were maintained as stock cultures at -80°C in MRS broth (Oxoid, Basingstoke, UK) supplemented with 15% glycerol (w/v).

Preparation of culture media

Two different culture media were used: cheese broth medium (CBM) (*Neviani et al., 2009*) and a medium containing only SLAB cells lysis product (LCM) (*Sgarbi et al., unpublished data*). Cheese broth medium was prepared according to the protocol proposed by *Neviani*

et al., 2009, formulated with 20 months ripened Parmigiano Reggiano cheese without agar addition. To prepare the lysed SLAB cell-based medium (LCM), the three *Lactobacillus helveticus* strains were anaerobically (Gas Generating Kit, Oxoid, Basingstoke, UK) grown twice with a 2% starting inoculum in 20 mL of MRS broth at 42°C for 24 h. Twenty mL of each culture were mixed in 1 L of MRS broth at 42°C for 24 h. After incubation, the 10^8 cell concentration was determined by microscopic counting (*Gatti et al., 2006*). The culture was harvested by centrifugation at 10000 rpm for 10 min at 4°C (Centrifuge, 5810R, Eppendorf, Hamburg, Germany), and the cells were resuspended in 60 mL of phosphate buffer (50m*M*, pH7) (Carlo Erba Reagents, Milan, Italy). The cell suspension was sonicated with a titanium probe operated at 70 W for 30 cycles (30 s pulse on and 1 min pulse off) (Sonoplus HD3100, Bandelin, Berlin Germany) and cooled on ice. Sonication was repeated twice. MRS agar plate count was performed in duplicate to verify the performance of sonication. The efficacy (E) of sonication (the ability of the method to kill the *L. helveticus* cells) was determined using the following formula: $E=100-(n_t/n_0)*100$, where n_0 = cfu/mL of *L. helveticus* culture and n_t = cfu/mL of *L. helveticus* culture after sonication.

NSLAB growth evaluation

Lc1056, Lc1247, Lr1216 and Lr1473 were anaerobically (Gas Generating Kit, Oxoid, Basingstoke, UK) grown twice with a 2% starting inoculum in 6 mL of MRS broth at 30°C for 24 h. To obtain NSLAB cells for inocula, each viable cultures were washed in Ringer solution (Oxoid, Basingstoke, UK) and harvested at 10000 rpm for 10 min at 4°C (Centrifuge, 5810R, Eppendorf, Hamburg, Germany). Each NSLAB cultures were inoculated in order to obtain a concentration of 10^6 cfu/mL in vials containing 5 ml of LCM and CBM. Cultures were incubated at 30°C for 4 and for 6 days respectively for CBM and LCM. Before and after incubation microbial counts were carried out in triplicate on MRS agar incubating at 30°C for 48 hours anaerobically. Data are expressed as mean and standard deviation.

Volatilome determination

After incubation, one vials for each media and for each strains were used to volatiloma evaluation. LCM and CBM without inoculums and incubated in the same condition was used as blank. Experiments were carried out in triplicate.

Analysis of volatile compounds in the vials headspace, for each media and for each strain, was performed by gas chromatography mass spectrometry (GC/MS) using an Agilent

Technologies 6890 gas chromatograph coupled to an Agilent Technologies 5970 mass spectrometer (Waters, Milford, MA). A SPME fiber assembly divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS) coating 50/30µm (Supelco Inc., Bellefonte, PA) were used after preconditioning according to the manufacturer's instruction manual. The samples were pre-heated 10 min at 45°C. The SPME fiber was exposed to each sample at 40°C for 40 min, and finally, the fiber was inserted into the injection port of the GC for 5 min of sample desorption. A Varian CB 7773 capillary column (50 m x 320 µm x 1,2 µm) was used. Volatile compounds were separated under the following conditions: helium carrier gas (1 mL/min), initial column temperature 50°C for 1 min, heated to 65°C at 4.5°C/min, followed by heating to 230°C at 10°C/min, maintained for 25 min. Injector, interface, and ion source temperatures were 250, 250, and 230°C, respectively. Compounds were identified by comparison of the mass spectral data with those of the Nist Mass Spectral databases library (NIST/EPA/NIH version 1998).

RESULTS AND DISCUSSION

NSLAB growth evaluation

In this research it was studied how the NSLAB can contribute to the cheese flavour formation, using the nutrient source available in ripened cheese, evaluating their growth and the production of volatile aroma compounds. To this purpose, four NSLAB strains, 2 *L. casei* and 2 *L.rhamnosus,* previously isolated from ripened Parmigiano Reggiano cheese (*Neviani et al., 2009*) were considered. To simulated the ripened cheese nutritional conditions, NSLAB strains were cultivated in two different media: CBM, a ripened PR cheese-based medium (*Neviani et al., 2009*), and LCM composed only by the SLAB (*L. helveticus)* cell lysis products.

After incubation (4 days for CBM and 6 days for LCM at 30°C) the *L. casei* and *L. rhamnosus* growth was observed. For all strains considered in both the media, there was an increase of about at least one logarithmic unit in MRS agar. Generally the NSLAB strains grew from about 10^6-10^7 cfu/mL to more than 10^8 cfu/mL (Table 1).

Volatilome determination

After establishing the growth, the capabilities of *L. casei* (Lc1056, Lc1247) and *L. rhamnosus* (Lr1216 and Lr1473) to produce volatile compound involved in cheese flavour were evaluated through GC/MS analysis of the head-space of sealed vials in which they were

grown. The experiments were carried out in triplicate. Each gas chromatograms obtained, contained a high numbers of peaks, the majority of which were identified by comparison of the mass spectral data with those of the Nist Mass Spectral databases library (NIST/EPA/NIH version 1998). All the volatile compounds identified in LCM and CBM media, grouped by chemical classes, are reported in Table 2. The relative abundance percentages were calculated for the identified peaks and the mean and the standard deviation of the three samples for each media and for each strains were reported (Table 2).
The chromatograms revealed several differences between the head-space of the blanks (LCM and CBM without inoculums, incubated at the same conditions) and the head-space of samples after microbial growth.

Volatilome production in LCM

The LCM medium was composed by only SLAB (*L. helveticus*) cell lysis products, obtained by mechanic break of SLAB culture. This medium simulates the product of autolysis of starter cells, therefore it contains mainly fatty acids, amino acids, N-acetyl-glucosamine and ribose that can be a source of nutrients available in ripened cheese for NSLAB growth (*Budinich et al., 2011; Adamberg et al., 2005; Thomas, 1987*).

Since the growth observed demonstrates the use of LCM components as nutrient source, the substances produced metabolizing these compounds were evaluated. It was verify, through GC/MS analysis of head-space of inoculated vials, if the metabolism, involved in consume of the cell lysis products, could lead to volatile flavour compounds formation.

Important differences in the composition of the head-space of LCM samples were observed. These differences were not only among the blank (not inoculated LCM) and LCM samples in which NSLAB were grown; but also the differences were observed among the samples on which the two different species, *L. casei* and *L. rhamnosus*, grew.

One of the compound more present in the head-space of the samples is an alkane, 6,6-dimethyl-undecane: about 12% in the blank decreased in head-space of samples after *L. casei* growth (Lc1056 about 7% and Lc1247 about 8%). On the contrary after *L. rhamnosus* growth, 6,6-dimethyl-undecane increased until about 17% and 15% for Lr1216 and Lr1473 respectively. Another alkane molecules found in LCM samples is the tetramethyl-cyclohexane. Its concentration between 3.63 % and 4.08% did not change during NSLAB growth.

Even if the metabolic pathway that lead to the production of these two molecules have not been previously described, their hydrocarbon structure (alkane) could indicate, as a possible source, the metabolism of fatty acids (*Collins et al., 2003*). In several works, molecules belonging to the alkanes classes, such as dodecane (*Curioni & Bosset, 2002; Moio & Addeo, 1998*), hexane, heptane and octane (*Randazzo et al., 2010*) were detected in different cheeses. Fatty acids act as precursor molecules for a series of catabolic reactions leading to the production of several aroma compounds, including alkanes (*Collins et al., 2003*). However the 6,6-dimethyl-undecane was never mentioned among the volatile compounds characteristic of the cheese flavour.

Even more difficult was the identification of the origin of the 3,3-dimethyl-butanamide The concentration of this molecule (between 7.99% and 11.03%) was not significantly different neither respect to the blank (8.40%), nor between the samples after the different NSLAB species growth. This compound, which has a baked aroma, was found in Parmigiano Reggiano aroma (*Qian & Reiniccius, 2002*). The 3,3-dimethyl-butanamide can be considered in the class a nitrogen containing compounds. Assuming this, it can be possible to state that this volatile molecule can be a degradation product of tryptophan as other nitrogen containing compounds (*Curioni et al., 2002*).

The other molecules detected in the head-space of LCM samples are attributable to metabolic pathways reported in the literature. These molecules are discussed below, arranged them by chemical compounds classes.

Total ketones are generally characterised by an increase after NSLAB growth, from about 2% in the blank to about 5-6% in the head-space samples after *L. casei* and *L. rhamnosus* growth, independently of inoculated strain. It was possible to observe the increase of 2-heptanone, 4-methyl-2-heptanone, 2-tridecanone and 2-pentadecanone. Ketones are common constituents of most dairy products; due to their typical odours and their low perception threshold, ketones are known for their contribution to cheese aroma (*Curioni & Bosset, 2002*). Among the ketones detected, 2-heptanone is an important flavour compound of Emmental and natural and creamy Gorgonzola (*Curioni & Bosset, 2002*; *Moio et al., 2000*). 2-nonanone, whose amount increases only in sample Lr1473, is another predominant methyl-ketone in natural Gorgonzola and ripened Ragusano cheeses (*Curioni & Bosset, 2002*). Furthermore 2-heptanone, 2-nonanone, 2-tridecanone were found in Grana Padano

(*Moio & Addeo, 1998*). Ketones were also the important class of flavour compounds in Fiore Sardo and Pecorino Romano (*Di Cagno et al., 2003*).

General pathways by which ketones can be produced is reported by *McSweeney & Sousa, 2000*. After the release of fatty acids (FFA) by lipolysis, the FFA were oxidated to β-ketoacids and decarboxylated to ketones or alkan-2-ones with one less C-atom. Therefore the ketones found in LCM medium were related to the NSLAB metabolism of fatty acids. For this reason, it is possible to hypothesize that the lipids/fatty acids, arising from cellular membrane of SLAB, were metabolized through this pathway.

Regarding the class of aldehydes, firstly, benzaldehyde deserves a specific consideration. Benzaldehyde was the only aldehyde found in the blank (about 2%). After *L. casei* growth significant change of its concentration was not observed, while after the growth of the two *L. rhamnosus* strains, its content increased up to about 6% and more. This aldehyde is produced as a result of the catabolic pathway of aromatic amino acids and particularly of phenylalanine or tyrosine. It seems that benzaldehyde arises from chemical conversion of α-keto-acids, such as phenyl pyruvic acid and p-hydroxy-phenyl pyruvic acid, which are produced by transamination of phenylalanine or tyrosine (*Fernandez & Zuniga, 2006; Marilley & Casey, 2003; Yvon & Rijen, 2001*). However, there is no evidence of a direct production of energy from these steps.

The amount of the other aldehydes, 2-decenal, nonanal and 8-octadecenal, changed in relation to the strains. These molecules were found in concentrations between about 2% and about 6% only in head-space after microbial growth. Especially nonanal was found only after Lr1473 growth, 2-decenal was present only after the 2 *L. rhamnosus* strains growth and 8-octadecenal was found in samples after the growth of 2 *L. rhamnosus* and Lc1247. After Lc1056 growth these aldehydes were not detected. 2-decenal, nonanal and 8-octadecenal are straight chain aldehydes, hence they can be produced by β-oxidation of unsaturated fatty acids (*Curioni & Bosset, 2002*). In fact the straight chain aldehydes are presumably produced by fatty acid metabolic pathways, differently from branched chain aldheydes, originated by catabolism of amino acids (*Belitz et al., 2004*). Some aldehydes found in the samples are quite common among the cheeses. For example, nonanal is the most important and is characterised by green grass and herbaceous aroma (*Curioni & Bosset, 2002*).

Regarding the class of alcohols, ethanol was found in blank sample (10.92%) and in similar concentration in head-space of samples after *L. rhamnosus* growth (Lr1216 11% and Lr1473 7%). After *L. casei* growth the concentration of ethanol increased at least two time respect to the blank, Lc1056 19.58% and Lc1247 22%. Ethanol can originated from acetaldehyde, by the metabolic pathway of lactate; it is known that NSLAB can metabolize lactate through different metabolic ways (*Marilley & Casei, 2004*; *McSweeney & Sousa, 2000*). In the LCM this metabolic pathways can be excluded because in the medium there is not lactate. However, ethanol can be originated from pentose sugars pathway (*Biotechnology of lactic acid bacteria, 2010*). Starting from a hexose sugars, pentose sugars can be obtained, concomitantly with the reduction of NAD^+. N-acetyl-glucosamine, arising from cell wall, can be present in LCM, therefore it is possible to state that it can be metabolised, leading to the production of ethanol with the concomitant oxidation of NADH.

An alternative metabolic way for ethanol production can be the catabolism of amino acids (*Ardo, 2006*). It was reported that, by threonina catabolism, a molecules of acetaldehyde can be produced, and with the concomitant oxidation of NADPH, ethanol can be obtained (*Ardo, 2006*).

Instead, other alcohols present in blank, decreased after NSLAB growth. For example, 3-methyl-pentanol, 6.12% in the blank, decrease after all strains growth to concentration of about 1-3%. A similar trend was observed for 1-octanol. 1-hexanol, present in concentration of about 8.65% in the blank, disappeared in the samples after NSLAB growth, with the exception of Lc1247 which was unable to consume it completely.

On the other hand, 2-heptanol was found after all NSLAB growth. 2-heptanol was found in flavour of Gorgonzola (*Moio et al., 2000*) and Grana Padano cheeses (*Moio & Addeo, 1998*). This molecules odour is herbaceous, green and oily (*Curioni & Bosset, 2002*). Secondary alcohols, like 2-heptanol, are formed by enzymatic reduction of the corresponding methyl ketones, which can arise from fatty acids by β-oxidation or from β-ketoacids (*Molimard & Spinnler, 1996*).

It was interesting to note that 3-methyl-1-butanol amount did not change after NSLAB growth, with the exception of Lc1247 able to triplicate the initial amount. This alcohol probably derived from the degradation of alanine, valine and leucine, and confers a pleasant aroma of fresh cheese (*Randazzo et al., 2006; Moio et al., 1993*).

Many metabolic pathways are involved in the biosynthesis of the alcohols: lactose/lactate metabolism, aldehydes reduction, amino acid catabolism as well as degradation of linoleic and linolenic acids (*Molimard & Spinnler, 1996*). In LCM, it is possible to suppose that the catabolism of amino acids is the most probably metabolic pathway for alcohols production by NSLAB. Through the amino acids catabolism it is possible to obtain aldehydes, which are then reduced to alcohols (*Urbach, 1995*). The fact that the aldehydes related to the alcohols found were not detected, leads to confirm this hypothesis.

Also aromatic alcohols show interesting differences. The concentration of 2,4-bis-dimethyl-ethyl-phenol was comparable in the blank and in the samples after *L. casei* growth, and disappeared after *L. rhamnosus* growth. In addition, the 1,1,3,3-tetramethyl-4-butyl-phenol, more than 6% in the blank, showed a strain dependent trend.

Phenolic compounds appear to give a positive contribution to cheese flavour when they are present in a threshold concentration, but they turn to be towards an unpleasant note as their concentration increases. The sensory quality ranges from sharp, medicinal, sweet, aromatic to smoky, charred, caramel, unpleasant and ''sheep-yard" (*Curioni & Bosset, 2002*). In literature only the origin of phenol molecules without methyl group has been reported (*Majcher et al., 2011; McSweeney, 2004; Curioni & Bosset, 2002*).

Finally, acetic acid, absent in the blank, was found in concentrations ranging about 1% after *L. casei* growth and about 3% after *L. rhamnosus* growth. The presence of acetic acid can be explained through multiple metabolic pathways. The metabolism of lactate (*McSweeney, 2004*), in lacking nutrient conditions, leads to formation of pyruvate, that was converted into acethyl-phosphate and then in acetic acid. In the metabolism of citrate (*McSweeney, 2004; Palles et al., 1998*) the citrate lyase can catalyzes the cleavage of citrate to oxaloacetate and acetyl-CoA, thus leads to the formation of acetic acid. Also the catabolism of amino acids can lead to acetic acid formation. *Liu et al., 2003* reported in fact that acetic acid can be produced by serine catabolism.

It is possible to suppose that in LCM, the acetic acid formation can be due to the presence of the enzyme acetate kinase that leads to formation of acetic acid and ATP from acetyl-phosphate.

Considering all the results obtained for LCM, it is possible to summarize that: i) ketones increase during NSLAB growth and they can be produced by fatty acids catabolism, ii) aldehydes total amount increase after NSLAB growth with the exception of Lc1056. The

aldehydes produced were all straight chain aldehydes, hence they derived from fatty acids. iii) Benzaldehyde was produced only by *L. rhamnosus*, and originated by catabolic pathways of phenylalanine and tyrosine, iv) alcohols detected in the samples show a strain dependent trend, but they all probably derived from amino acids, v) ethanol was produced only by *L. casei*.

Therefore it can concluded that, in a LCM medium, containing only the lysis product of the SLAB, the catabolic pathways of amino acids and fatty acids were the most important for NSLAB. The reason for this could be that in this media fatty acids and amino acids are the more present compounds (arising from the break cells of *L. helevticus*).

Volatile compounds produced in CBM

CBM was a 20 months ripened cheese-based medium containing as nutrient source amino acids, small peptides, and product of SLAB autolysis (*De Dea Lindner et al., 2008*). For this reason it is possible to consider it a cheese model medium.

The trend of the volatile profile of head-space samples after NSLAB growth in CBM showed some similarities with that observed after the *L. casei* and *L. rhamnosus* growth in LCM.

6,6-dimethyl-undecane and 3,3-dimethyl-butanamide were found, either in the blank and after microbial growth, at lower concentrations (about 6% and 3% respectively).

Pentadecane, 4-cyclohexil-undecane were found in a concentration of about 2%-3% and did not significantly change after NSLAB growth. As previously discussed the presence of alkanes can be related to fatty acids metabolic pathway (*Collins et al., 2003*).

The most significant differences between head-space of blank and of CBM after NSLAB growth were observed for the concentration of aldehydes and ketones. In particular, as regards to ketones, after NSLAB growth it was found a significant amount of acetone, diacetyl (2,3-butanedione) and acetoin (3-hydroxy-butanone) with the exception of Lr1473 that show a specific behaviour. Acetone was produced in greater quantity by Lc1056 (4.33%), while the other strains produce it only in a lower quantity (about 1%-2%). High amount of diacetyl and acetoin were found after all NSLAB growth, with the exception of Lr1473 which was unable to produce them. Differently from CBM acetone, diacetyl and acetoin had not been produced in LCM.

Acetone was found in Cheedar cheese (*McSweeney & Sousa, 2000*) and other cheese. This is a volatile compound likely formed via acetyl-CoA metabolic pathways by *L. casei* (*Budinich et al., 2011*) and by *L. rhamnosus* (*Ramzan et al., 2010*).

Diacetyl (2,3-butanedione) can be obtained from pyruvate, product of lactose and citrate metabolism. This compound is appreciated for its buttery and nut-like notes. It is a key aroma component of Camembert, Cheddar and Emmental (*Curioni & Bosset, 2002*). It was reported that NSLAB can be able to metabolise citrate to acetoin and probably to diacetyl (*Palles et al., 1998; McSweeney, 2004*). It is possible to state that in CBM citrate and lactate were used and led to these compounds formation.

Among the others ketones, the presence of 2-heptanone, major constituent of the headspace of the blank sample, decreased after all NSLAB growth. It was interesting to observe for three strains the increase of 2-nonanone which was not produced in greater amount in LCM medium. Since it is a methyl-ketone, it was possible to assume, that like other methyl-ketones, 2-nonanone and 2-heptanone derived from the catabolism of fatty acids.

Among the aldehydes detected in the samples, 3-methyl-butanal, hexanal, octanal and nonanal decreased until disappearing after *L. casei* and *L. rhamnosus* growth. This trend is just opposite to that observed in LCM, where some aldehydes appeared after the growth. It was possible to hypothesized that aldheydes can be rapidly reduced to primary alcohols or even oxidised to the corresponding acids (*Curioni & Bosset, 2002*).

As observed in the LCM medium, benzaldehyde greater increased, with the exception of Lc1056.

In CBM alcohols were detected in similar concentration in the head-space after *L. rhamnosus* and *L. casei* growth and it decreased after Lc1056 growth. 1-hexadecanol was the alcohol present in highest percentage (about 2%-3%) but it did not change significantly after NSLAB incubation.

Finally, acetic acid absent in the blank, increased after NSLAB growth in particular for Lc1056 (5.29%). Higher quantity of acetic acid found after growth in CBM respect to LCM could be due also to the metabolism of lactate and citrate. In agreement with this state, it was reported that from lactate, NSLAB can be able to produce acetic acid though piruvate, in lacking sugar condition (*McSweeney & Sousa, 2000*).

Considering all the results obtained for CBM it is possible to summarize that the major compounds produced in this medium were acetoin, diacetyl and acetone, while in LCM the volatile compound produced in greater amount was the ethanol. These results lead to hypothesize that in cheese based medium there was a high amount of pyruvate that can be converted in acetone, acetoino and diacethyl. The pyruvate can be originated from lactate and citrate, that were present in CBM. In LCM these organic acids were not present, thus the NSLAB used mainly FFA, FAA and/or sugars linked on cellular wall.

REFERENCES

Adamberg K., Antonsso M., Vogensen F.K., Nielsen E.W., Kask S., Moller P.L. & Ardo Y. 2005 Fermentation of carbohydrates from cheese sources by non-starter lactic acid bacteria isolated from semihard Danish cheese. Int Dairy J 15, 873–882.

Ardo Y. 2006 Flavour formation by amino acids catabolism. Biotechol Adv, 24, 238-242.

Belitz H.D., Grosh W. & Schieberle P. 2004 Food chemistry. Springer, Berlin.

Broadbent J.R. & Steele J.L. 2005 Cheese Flavor and the Genomics of Lactic Acid Bacteria. ASM News 71, 121- 8.

Budinich M.F., Perez-Díaz I., Cai H., Rankin S.A., Broadbent J.R. & Steele J.L. 2011 Growth of Lactobacillus paracasei ATCC 334 in a cheese model system: A biochemical approach. J. Dairy Sci 94, 5263–5277.

Collins Y.F., McSweeney P.L.H. & Wilkinson M. G. 2003 Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. Int Dairy J 13, 841-866.

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

Curioni P.M. G. & Bosset J.O. 2002 Key odorants in various cheese types as determined by gas chromatography-olfactometry. Int Dairy J 12, 959-984.

De Dea Lindner J., Bernini V., De Lorentiis A., Pecorari A., Neviani E. & Gatti M. 2008 Parmigiano Reggiano cheese: evolution of cultivable and total lactic microflora and peptidase activities during manufacture and ripening. Dairy Sci Technol 88, 511-523.

Di Cagno R., Banks J., Sheehan L., Fox P.F.,. Brechany E.Y., Corsetti A. & Gobbetti M. 2003 Comparison of the microbiological, compositional, biochemical, volatile profile and sensory characteristics of three Italian PDO ewes' milk cheeses. Int Dairy J 13, 961-972.

Fernandez M. & Zuniga M. 2006 Amino Acid Catabolic Pathways of Lactic Acid Bacteria. Crit Rev Microbiol 32, 155-183.

Fox P.F. & Wallace J.M. 1997 Formation offlavor compounds in cheese. Adv Appl Microbiol 45, 17–85.

Fox P.F. & McSweeney P.L.H. 2004 Cheese: an overview. In Cheese: Chemistry, Physics and Microbiology. P.F. Fox, P.L.H. McSweeney, T.M. Cogan & T.P. Guinee, , Vol 1 General Aspects, 3rd edn, eds. Elsevier, London.

Gala E., Landi S., Solieri L., Nocetti M., Pulvirenti A. & Giudici P. 2008 Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. Int J Food Microbiol 125, 347–351.

Gatti M., De Dea Lindner J., De Lorentiis A., Bottari B., Santarelli M., Bernini V. & Neviani E. 2008 Dynamics of whole and lysed bacterial cells during Parmigiano-Reggiano cheese production and ripening. Appl Environ Microbiol 74, 6161-7.

Kubickovà J. & Grosh W. 1997 Evaluation of potent odorants of Camembert cheese by diluition and concentration techniques. Int dairy J 7, 65-70.

Helinck S., Le Bars D., Moreau D. & Yvon M. 2004 Ability of thermophilic lactic acid bacteria to produce aroma compounds from amino acids. Appl Environ Microbiol 70, 3855-3861.

Law B.A., Castanon M.J., Sharpe M.E. 1976 Effect of non-starter bacteria on the chemical composition and flavour of Cheddar cheese. J Dairy Res 43, 117–125.

Liu S.Q., Holland R. & Crow V.L. 2003 The potential of dairy lactic acid bacteria to metabolise amino acids via non transaminating reactions and endogenous tansamination. Int J Food Microbiol 86, 257-269.

Maarse H. & Visscher C.A. 1989 Volatile compounds in food: qualitative and quantitative data. (6th edn). TNO-CIVO Food Analysis Institute, Zeist, The Netherlands.

Majcher M. A., Goderska K., Pikul J. & Jelen H. H. 2011 Changes in volatile, sensory and microbial profiles during preparation of smoked ewe cheese. J Sci Food Agric 91, 1416-1423.

Marilley L. & Casey M.G. 2004 Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. Int Dairy J 90, 139-159.

McSweeney P.L.H. 2004 Biochemistry of cheese ripening. Int J Dairy Tech, 57, 127-144.

McSweeney P.L.H. & Sousa M.J. 2000 Biochemical pathways for the production of flavour compounds in cheese during ripening. Lait 80, 293-324.

Moio L., Dekimpe J., Etièvan P.X. & Addeo F. 1993 Volatile flavour compound of water buffalo Mozzarella cheese. Italian J Food Sci 5, 57-68.

Moio L. & Addeo F. 1998 Grana Padano cheese aroma. J Dairy Res 65, 317–333.

Moio L., Piombino P. & Addeo F. 2000 Odour-impact compounds of Gorgonzola cheese. J Dairy Res 67, 273–285.

Molimard, P., & Spinnler, H.E. 1996 Review: compounds involved in the flavor of surface mold-ripened cheeses: Origins and properties. J Dairy Sci 79, 169–184.

Mozzi F., Raya R.R. & Vignolo G.M. 2010 Biotechnology of lactic acid bacteria. Novel Application. Wiley-Blackwell, USA.

Neviani E., De Dea Lindner J., Bernini V. & Gatti M. 2009 Recovery and differentiation of long ripened cheese microflora through a new cheese-based cultural medium. Food Microbiol 26,240-5.

Olson N. F. 1990 The impact of lactic acid bacteria on cheese flavor. FEMS Microbiol Lett 87, 131-147.

Palles T., Beresford S., Condon S. & Cogan T.M. 1998 Citrate metabolism in Lactobacillus casei and Lactobacillus plantarum. J Appl Microbiol 85, 147–154.

Peterson S. D. & Marshall R. T. 1989 Non starter lactobacilli in Cheddar cheese: a review. J Dairy Sci 73, 1395-1410.

Qian M. & Reineccius G. 2002 Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry. J Dairy Sci 85, 1362–1369.

Ramzan M., Nuzhat H., Tita M. & Tita O. 2010 Evaluation of volatile flavouring compounds in Cheddar cheese, manufactured by using Lactobacillus rhamnosus as an adjunct culture. Journal of Agroalimentary Processes and Technologies 16, 188-195.

Randazzo C.L., Pitino I., Ribbera A. & Caggia C. 2010 Pecorino Crotonese cheese: study of bacterial population and flavour compounds. Food Microbiol 27, 363-374.

Randazzo C.L., De Luca S., Todaro A., Restuccia C., Lanza C. M., Spagna G. & Caggia C. 2006 Preliminary characterization of wild lactic acid bacteria and their abilities to produce flavour compounds in ripened model cheese system. J Appl Microbiol 103, 427-435.

Rapposch S., Eliskases-Lechner F. & Ginzinger W. 1999 Growth of facultatively heterofermentative lactobacilli on starter cell suspensions. Appl Environ Microbiol 65, 5597–5599.

Settani L.& Moschetti G. (2010) Non starter lactic acid bacteria used to improve cheese quality and provided health benefits. Food Microbiol 27, 691-697.

Smit G., Smit B.A. & Engels W.J.M. 2005 Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiol Rev 29, 591–610.

Tavaria F.K., Dahl S., Carballo F.J. & Malcata F.X. 2002 Amino Acid Catabolism and Generation of Volatiles by Lactic Acid Bacteria. J Dairy Sci 85, 2462-2470.

Thomas T.D. 1987 Cannibalism among bacteria found in cheese. New Zeal J Dairy Sci Tech 22, 215-219.

Urbach G. 1995 Contribution of lactic acid bacteria to flavour compound formation in dairy products. Int Dairy J 5, 877-903.

Weimer B. C. 2007 Improving the Flavour of Cheese. Woodhead Publishing.

Yvon M. & Rejien L. 2001 Cheese flavour formation by amino acid catabolism. Int Dairy J 11, 185–201.

Zehentbauer G. & Reineccius G.A. 2002 Determination of key aroma components of Cheddar cheese using dynamic headspace dilution assay. Flavour Frag J 17, 300–305.

TABLES

***Table 1**: Plate count in MRS (log cfu/mL) of L.casei 1056, L.casei 1247, L.rhamnosus 1216 and L.rhamnosus 1473, inoculated in LCM and CBM. Count was carried out in triplicate before (t_0) and after 4 and 6 days respectively of incubation at 30°C. Data are expressed as mean and standard deviation.*

	LCM		**CBM**	
Strain	**t_0**	**6 days**	**t_0**	**4 days**
Lc1056	$1.8x10^7 \pm 3.8x10^6$	$1.5x10^8 \pm 4.1x10^7$	$7.5x10^6 \pm 3.7x10^5$	$2.8x10^8 \pm 1.1x10^8$
Lc1247	$2.4x10^7 \pm 4.8x10^6$	$3.6x10^8 \pm 6.3x10^7$	$2.2x10^7 \pm 3.4x10^6$	$2.2x10^8 \pm 1.1x10^8$
Lr1216	$5.4x10^6 \pm 1.8x10^5$	$9.0x10^7 \pm 1.7x10^7$	$2.7x10^7 \pm 1.9x10^6$	$8.7x10^7 \pm 5.8x10^7$
Lr1473	$2.3x10^7 \pm 2.2x10^6$	$1.3x10^8 \pm 8.2x10^6$	$2.8x10^7 \pm 2.6x10^5$	$1.3x10^8 \pm 3.2x10^7$

***Table 2**: The chemical classes of volatiles compounds identified in LCM (2a) and CBM (2b), after growth of L.casei 1056, L. casei 1247, L. rhamnosus 1216 and L. rhamnosus 1473. Data, expressed relative abundance percentage, was reported as mean of triplicate experiments.*

2a

	Blank		Lc1056		Lc1247		Lr1216		Lr1473	
Compounds	**% mean**	**SD**	**% mean**	**SD**	**% mean**	**SD**	**% mean**	**SD**	**% mean**	**SD**
4,4-dimethyl-2-pentanone	0.87	0.15	0.72	0.62	0.64	0.41	1.20	0.59	1.09	0.60
2-tetradecanone			1.42	0.16						
2-heptanone					1.40	0.30			1.83	0.71
4-methyl-2-eptanone			2.27	0.20	2.35	0.16			0.80	0.43
2-nonanone	0.86	0.07	0.96	0.11	0.68	0.44	0.73	0.34	1.41	0.27
2-tridecanone			0.40	0.30			1.50	0.84		
2-penta-decadenone							1.51	1.39		
Ketones	**1.73**	**0.21**	**5.78**	**1.38**	**5.06**	**1.31**	**4.95**	**3.15**	**5.13**	**2.02**
8-octadecenal					2.00	0.50	1.78	0.88	1.57	0.84
nonanal									2.72	0.44
benzaldeide	1.91	0.45	1.36	0.56	1.58	0.13	5.76	1.36	7.29	1.15
2-decenal							1.28	0.54	0.82	0.45
Aldehydes	**1.91**	**0.45**	**1.36**	**0.56**	**3.58**	**0.63**	**8.82**	**2.78**	**12.39**	**2.89**
ethyl alcohol	10.92	1.21	19.58	1.15	22.00	0.57	11.29	0.39	7.23	0.46
3-methyl-1-butanol	1.16	0.05	1.52	0.49	3.07	0.12			1.51	0.82
3-methyl-pentanol	6.12	0.24	1.14	0.23	2.35	0.58	3.30	0.45	2.68	0.27
6,6-dimetil-1,3-heptadien-5-ol	1.34	0.83	1.60	0.27						
1-esanol	8.65	0.67			3.33	0.20				
2-heptanol			0.47	0.35	2.40	0.65	3.06	0.26	2.91	0.58
2-ethyl-1-esanolo	3.63	0.36	3.15	0.21			3.06	0.13	3.80	0.46
1-octanol	5.22	0.20	2.53	0.29	4.50	0.48	3.54	0.45	3.43	0.71
methylthio-4-phenol	0.73	0.51	1.10	0.48	0.93	0.60				
1-nonanol	1.30	0.07	1.79	0.06	2.77	0.52	1.93	0.27	1.62	0.38
1-undecanol	1.00	0.11	3.54	0.02	3.64	0.23	1.81	0.34	1.91	0.31
phenylmethanol	0.73	0.45								
dodecanethiol	1.50	0.02	2.68	0.21	2.92	0.45	1.51	0.90	2.08	0.22
2,4-bis-(1,1-dimethylethyl)-phenol	3.25	0.33	3.72	0.04	4.86	0.22				
phenol-4-(1,1,3,3-tetramethylbutyl)	6.07	1.03	4.97	1.10	1.38	1.02	3.52	2.20	8.65	2.46
Alcohols	**51.63**	**6.08**	**47.78**	**4.91**	**54.15**	**5.66**	**33.02**	**5.39**	**35.82**	**6.67**
benzoic acid										
2,2-dimethyl-propionic acid			1.46	0.06	1.28	0.18				
acetic acid			1.72	0.61	1.28	0.05	3.30	3.11	2.91	0.05
3-methylpentanoic acid	1.82	0.07								
hexadecanoic acid-methylester	1.37	1.03								
Acids	**3.19**	**1.10**	**3.19**	**0.67**	**2.55**	**0.23**	**3.30**	**3.11**	**2.91**	**0.05**
6,6-dimethyl-undecane	12.85	3.39	7.92	2.94	7.68	1.98	17.02	3.07	15.31	5.16
3,3-dimetil-butanamide	8.40	2.18	11.03	4.84	7.99	3.43	9.71	2.93	9.49	5.53
tetramethyl-cyclohexane	3.63	0.25	3.59	0.47	4.44	0.60	4.08	0.74	4.51	0.85
others	16.66	5.77	19.35	5.24	14.54	4.01	19.11	6.67	14.43	4.58
Other compound	**41.53**	**11.59**	**41.89**	**13.49**	**34.65**	**10.02**	**49.92**	**13.50**	**43.74**	**16.13**
Total	100.00	19.43	100.00	21.01	100.00	17.85	100.00	27.93	100.00	27.76

2b

	Blank		Lc1056		Lr1216		Lr1473		Lc1247	
Compounds	**%mean**	**SD**	**%mean**	**SD**	**%mean**	**SD**	**%mean**	**SD**	**%mean**	**SD**
acetone			4.33	0.16	1.25	0.12	1.60	0.57	1.56	0.12
2,3-butanedione			7.39	0.14	13.73	0.59			17.29	0.41
2-pentanone							1.81	0.57		
2-heptanone	17.56	0.26	11.68	0.73	8.34	0.26	12.25	1.44	9.01	0.19
3-hydroxybutanone			14.53	5.17	19.88	1.00			5.61	1.25
2-nonanone	2.03	0.08	3.40	0.05	4.34	0.91	4.37	1.03	1.50	0.11
2-undecanone	1.15	0.02	2.04	0.04	1.98	0.46	1.86	2.02	0.82	0.03
2-tridecanone	1.06	0.06	2.22	0.28	1.95	0.06	1.71	2.42	0.72	0.01
Ketones	**21.80**	**0.42**	**45.59**	**6.57**	**51.47**	**3.40**	**23.60**	**8.05**	**36.52**	**2.13**
3-methyl-butanal	2.49	0.19								
hexanal	6.69	0.02								
8-octadecenal	4.68	0.14	3.72	0.03	2.15	0.02	3.46	1.09	2.93	0.01
nonanal	7.66	0.08							1.47	0.04
2-nonenal	4.28	0.15							1.16	0.07
decanal	1.59	0.01			0.26	0.15			0.71	0.07
benzaldeide	6.58	0.10	5.55	0.75	23.42	3.76	32.06	6.53	26.95	1.34
Aldehydes	**33.95**	**0.69**	**9.27**	**0.78**	**25.82**	**3.93**	**35.52**	**7.62**	**33.22**	**1.53**
1-hexadecanol	3.45	0.05	3.10	0.09	1.46	0.24	3.28	0.97	2.25	0.11
1-octanol	1.59	0.03					1.09	2.06	1.44	0.03
methylthio-4-phenol	1.74	0.08	1.10	0.34	0.86	0.16	1.62	0.41	1.49	0.07
2-furan-methanol			0.73	0.23					0.40	0.15
phenylethanol			1.23	0.04						
Alcohols	**6.77**	**0.16**	**6.16**	**0.70**	**2.32**	**0.40**	**5.99**	**3.44**	**5.58**	**0.36**
acetic acid			5.29	0.65	1.43	0.15	3.47	1.34	1.71	0.21
n-decanoic acid							2.07	0.51		
Acid			**5.29**	**0.65**	**1.43**	**0.15**	**5.53**	**1.85**	**1.71**	**0.21**
6,6-dimethyl-undecane	8.25	0.84	9.01	1.09	5.10	0.87	7.54	0.83	5.44	0.97
3,3-dimetil-butanamide	4.65	0.85	4.94	0.89	2.27	0.96	3.90	0.64	2.36	0.84
4-cyclohexyl-undecano	3.22	0.06	3.15	0.03	1.98	0.14	2.68	1.33	2.54	0.01
pentadecane	4.08	0.01	3.42	0.18	2.02	0.13	2.65	1.59	3.19	0.02
others	17.26	0.01	13.15	0.01	7.60	1.20	12.60	3.51	9.43	0.44
Others compounds	**37.47**	**1.56**	**33.69**	**1.96**	**18.97**	**3.32**	**29.36**	**7.88**	**22.97**	**2.29**
Total	100.00	2.82	100.00	10.66	100.00	11.19	100.00	28.84	100.00	6.51

IV.III NON PROTEOLYTIC AMINOACYL DERIVATIVES IN PARMIGIANO REGGIANO AND OTHER CHEESES ARE PRODUCED BY LACTIC ACID BACTERIA

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ABSTRACT

Aminoacyl derivates of non-proteolytic origin with interesting sensory properties have been identified in cheeses. In the present work a correlation between the accumulation of γ-glutamyl-amino acids and lactoyl-amino acids, time of ripening and microflora has been observed in twelve different cheeses considered in a preliminary survey. Non proteolytic aminoacyl derivatives amounts were higher in cheeses characterized by long ripening time and lactobacilli microflora. An enzymatic activity producing γ-glutamyl-phenylalanine was also observed in Parmigiano Reggiano water soluble extracts. *Lactobacillus helveticus* and *Lactobacillus rhamnosus*, chosen as representative of starter and non starter microflora of Parmigiano Reggiano cheese, were used as model bacteria in order to verify their ability to produce γ-glutamyl-phenylalanine and lactoyl-phenylalanine during growth and after cell lyses. While the first was produced only by lysed cells, the latter was produced either by growing or lysed cells in different amount depending on the species, the cells condition and time of incubation.

INTRODUCTION

Cheese ripening involves a complex series of biochemical and chemical events, that leads to the characteristic taste, aroma and texture of each cheese variety (*Sousa et al., 2001*). During cheese ripening, proteolysis, one of the most important biochemical phenomena, releases free amino acids and small peptides in cheeses, as a consequence of casein degradation activity. Thus, cheeses contain low molecular weight nitrogenous compounds, that play a key role in a flavour development. Several researches reported the study of cheese flavour formation and investigated the amino acids catabolism and the degradation of small peptides (*Helinck et al., 2004; Yvon et al., 2001; Sousa et al., 2001; Urbach, 1995*). However, recently, small aminoacyl derivatives of non-proteolytic origin with interesting sensory properties have been identified in cheeses (*Sforza et al., 2009; Toelstede et al., 2009; Roudot-Algaron et al., 1994*) and other foods (*Sforza et al., 2006*). These non proteolytic aminoacyl derivatives (NPAD) are *de novo* formed during ageing starting from free amino acids and other organic acids present in high quantity, as starting compounds. The first compound to be identified and characterized are those in which glutamic acid is involved in a amide bond throuhg its glutamyl moiety in γ position.

In Comtè cheese γ-glutamyl-phenylalanine (γ-Glu-Phe), γ-glutamyl-tyrosine (γ-Glu-Tyr) and γ-glutamyl-leucine (γ-Glu-Leu) have been identified and characterized by the sensory point of view (*Roudot-Algaron et al., 1994*).

Very recently, a group of γ-glutamyl amino acids, responsible of "kokumi" flavour was also found in Gouda cheese (*Toelstede et al., 2009*). Simultaneously, in Parmigiano-Reggiano cheeses, the accumulation of γ-glutamyl amino acids was demonstrated to occur during the ageing time, and was also associated with the formation of other molecules of non proteolytic origin, namely lactoyl-amino acids and, to a lesser extent, pyroglutamyl-amino acids (*Sforza et al., 2009*), demonstrating for the first time the natural presence of lactoyl-amino acids in foods. Their presence was also observed in other aged cheese, namely Asiago and Grana Padano, at different time of ripening, with an increasing trend according to the ageing time. γ-Glutamyl amino acids in aged Parmigiano Reggiano cheese (24 months), were found to be present at a concentration around 50 mg in 100 g of cheese (*Sforza et al., 2009*). Common features observed for all NPADs hinted to an enzymatic origin: all of them were formed by glutamic acid, lactic acid or pyroglutamic acid, organic acids commonly found in cheese linked to an apolar amino acid (Phe, Val,

Ile, Leu, Met, Tyr), suggesting a common specificity in substrate choice. Moreover, the reaction appears to be highly stereo-selective, since only the L-configuration has been found in the acyl moieties, although the corresponding D-acids are known to be present in long ripened cheeses (*Careri et al., 1996*).

It was speculated that this enzymatic activity might derive from the lactic acid bacteria (LAB), either starter or non-starter (*Sforza et al., 2009*) because of the presence of high amount of these compounds in cheese where LAB are the dominant microflora. Anyway, no experimental evidence has yet demonstrated this hypothesis.

The aim of this work was to assess if these taste-active compounds, strongly contributing to the pleasant flavour of ripened cheeses, can be originated by LAB enzymatic activity. First, γ-glutamyl-amino acids and lactoyl-amino acids in different kinds of cheese were evaluated, in order to outline the correlation existing between cheese microflora, cheese ripening and NPAD content. Then, *Lactobacillus helveticus* and *Lactobacillus rhamnosus*, chosen as representative of starter and non starter LAB of Parmigiano Reggiano cheese, were used as model bacteria in order to verify their ability to produce γ-glutamyl-amino acids and lactoyl-amino acids during growth and after cell death and lysis.

MATERIALS AND METHODS

Cheese samples

Twelve different Italian cheeses were purchased from different retails and analyzed for a preliminary survey (Table 1). All samples were placed in plastic bags and stored immediately in a freezer at -20°C until analysis.

Extraction of the oligopeptide fraction in cheeses

The peptide fraction was extracted according to *Sforza et al., 2004*. Ten g of grated cheese were suspended in 45 mL of 0.1 N HCl. (L,L)-phenylalanyl-phenylalanine (Phe-Phe) was added as internal standard (2.5 mL of a 1 mM solution). The suspension was homogenized for 2 min and then centrifuged at 4000 rpm for 60 min at 4°C with Centrifuge 5810R (Eppendorf, Hamburg, Germany). The solution was filtered through paper filters and then extracted three times with 100 mL of ethyl ether. The aqueous solution was filtered through 0.45 μm filter Millipore (Billerica, MA, USA). A total of 3 mL of the resulting solution was dried and redissolved in 2 mL of a 0.1% formic acid solution (pH 3). The

solution was ultra-filtered through filters with nominal molecular cut-off 10000 Da (Millipore) at 7000 rpm. The filtrate was dried, redissolved in 1.2 mL of a 0.1% HCOOH solution, transferred and again dried under nitrogen. Each sample was extracted at least twice. Samples were redissolved in 500 µL of 0.1% aqueous HCOOH solutions and analyzed by ultra-performance liquid chromatography/electrospray ionization mass spectrometry (UPLC/ESI-MS) (Waters, MA, USA).

Ultra performance liquid chromatography/ electrospray ionization mass spectrometry (UPLC/ESI-MS) analysis of oligopeptide fraction of cheeses

The oligopeptide fraction extracted from cheeses was analyzed through UPLC/ESI-MS.

UPLC/ESI-MS analysis were performed on Acquity UPLC (Waters) according to the following conditions. Eluent A was H_2O (0.2% CH_3CN and 0.1% HCOOH); eluent B was CH_3CN (0.2% H_2O and 0.1 % HCOOH). The elution program was as follow: isocratic 100% A for 7 min, a linear gradient from 100% A to 50% A between 7-50, isocratic 50% A for 2 min, reducing to 0% A between 53 and 58 min, followed by reconditioning. The chromatographic column used was a Aquity UPLC BEH C_{18} (1.7 µm, 2.1x150 mm) (Waters) and the flow rate was 0.2 mL/min. The detection was performed on Aquity SQD single quadrupole ESI mass spectrometer (Waters). The mass detector was used in the positive ion mode, by setting the capillary voltage 3.2 kV, the cone voltage at 30 V, the source temperature at 100°C, the desolvation temperature at 200°C, the cone gas (N_2) at 100 L/h and the desolvation gas (N_2) at 650 L/h. The acquisition was performed in the total ion current mode (TIC, 100-2000 m/z) with a scan time of 1 s. The data were collected and processed with MassLynx 4.0 software (Waters).

Quantitative determination of the γ-glutamyl- and lactoyl -amino acids in cheeses samples

For *N*-γ-Glutamyl amino acids (γ-Glu-Val, γ-Glu-Met, γ-Glu-Tyr, γ-Glu-Ile, γ-Glu-Leu, γ-Glu-Phe), and *N*-Lactoyl amino acids (Lac-Val, Lac-Met, Lac-Tyr, Lac-Ile, Lac-Leu, Lac-Phe) extract ion chromatograms (XICs) were obtained from the full-scan chromatograms by extracting the signals corresponding to the ions reported in precedent research work (*Sforza et al., 2009*). The XIC corresponding to the internal standard Phe-Phe was also obtained. An authentic standard of γ-Glu-Phe was synthesized as previously described (*Sforza et al., 2009*) and used to obtain a calibration curve against the internal standard Phe-Phe. All the NPADs were then quantified through the internal standard method assuming

for all of them a response factor equal to that of γ-Glu-Phe. Each sample was analysed in duplicate.

Determination of the presence of an enzymatic activity producing non-proteolytic aminoacyl derivatives in Parmigiano Reggiano extracts

In order to evaluate the presence of active enzyme(s) able to produce γ-Glu-Phe, Parmigiano Reggiano cheese sample aged 12 months was diluted in phospate buffer saline (PBS) 8 mM, pH 7. After homogenization, the soluble extract was filtered on paper filter and membrane with cut off 0.45 µm. The Parmigiano Reggiano cheese PBS soluble extract was incubated at 37°C for 24 h with $^2H_5$-heavy labeled phenylalanine ($^2H_5$-Phe), at increasing concentrations. The eventual presence of $^2H_5$-heavy labeled-γ-glutamyl-phenylalanine ($^2H_5$-γ-Glu-Phe) was assessed by UPLC/ESI-MS, and its amount was calculated as ratio to unlabelled γ-Glu-Phe. Experiment was carried out in duplicate.

Strains and culture preparation

Lactobacillus helveticus (Lh770) and *Lactobacillus rhamnosus* (Lr830), belonging to University of Parma collection and previously isolated respectively from natural whey starter and from ripened Parmigiano Reggiano cheese (*Neviani et al., 2009*) maintained as stock cultures at -80°C in MRS broth (Oxoid, Basingstoke, UK) supplemented with 15% glycerol (w/v) were used. *L. helveticus* and *L. rhamnosus* strains were anaerobically (Gas Generating Kit) (Oxoid) revitalized, 2% (v/v) inoculums, in MRS broth at 42°C and 30°C respectively for 24 h to obtain viable cells cultures.

To prepare lysed cell, 20 mL of each strains culture were inoculated in 1 L of MRS broth at 42°C and 30°C respectively, for 24 h. After incubation, the 10^8 cell/mL concentration was determined by microscopic counting (*Gatti et al., 2006*). The *L. helveticus* and *L. rhamnosus* cultures were harvested through centrifugation at 10000 rpm for 10 min at 4°C (Centrifuge 5810R) (Eppendorf), and the cells were resuspended in 60 mL of phosphate buffer (50 mM, pH 7) (Carlo Erba Reagents, Milan, Italy). The cell suspensions were sonicated with a titanium probe operated at 70 W for 30 cycles (30 sec pulse on and 1 min pulse off) (Sonoplus HD3100, Bandelin, Berlin Germany) and cooled on ice. Sonication was repeated twice. MRS agar plate count was performed in duplicate to verify the performance of sonication. The efficacy (E) of sonication was determined using the following formula: $E=100-(n_t/n_0)*100$, where n_0 = cfu/mL of L. helveticus 770 and *L. rhamnosus* 830 cultures and n_t = cfu/mL of cultures strains after sonication.

Determination of non-proteolytic aminoacyl derivatives produced by LAB strains

A solution containing 2.7 mL of MRS broth, 500 µL of 2 precursors, i) glutamic acid 11.62 mM and phenylalanine 3.25 mM, ii) lactic acid 21 mM and phenylalanine 3.25 mM, iii) glutamine 10 mM and phenylalanine 3.25 mM, and 300 µL of *L. helveticus* or *L. rhamnosus* (10^8 cfu/mL) were incubated for 24, 48, 72 h in anaerobic conditions at 42°C and 30°C respectively. After incubation the solutions were added with 200 µL of HCl 2N (in order to achieve pH 3) and filtered through 0.45 µm, 0.22 µm filters (Millipore) and ultra filtered through filters cut off 10000 Da (Millipore) at 7000 rpm. The filtrate was dried and re-dissolved in 1.2 mL of a 0.1% formic acid solution. The solutions were dried under nitrogen flow and store at -20°C. Before being analyzed by UPLC/ESI-MS, samples obtained from viable cells were dissolved in 200 µL of 0.1% aqueous HCOOH and analyzed for assessing the presence of γ-Glu-Phe and Lac-Phe through UPLC/ESI-MS at the same condition described for cheese samples, except for the acquisition mode, which was performed in the Single Ion Recording (SIR) mode, monitoring the following ions: γ-Glu-Phe: m/z 120, 166, 232, 278, 295; Lac-Phe: m/z 238, 192, 166, 120.

Determination of non-proteolytic aminoacyl derivatives produced by lysed LAB strains

In a falcon tube, 2.7 mL of phosphate buffer (50 mM, pH7), 500 µL of precursors, i) glutamic acid 11.62 mM and phenylalanine 3.25 mM, ii) lactic acid 21 mM and phenylalanine 3. 25 mM and iii) glutamine 10 mM and phenylalanine 3.25 mM and 300 µL of lysed cells were prepared. *L. helveticus* and *L. rhamnosus* lysed cells mixed with precursors were incubated for 24, 48, 72 hours in anaerobic conditions at 42°C and 30°C respectively. After incubation, 23 mL of HCl 0.1N (to achieve pH 3.00) were added to samples. Solutions were centrifuged at 4°C for 10 minutes at 10000 rpm and then filtered through 0.22 µm filters. The filtrate was extracted 2 times with 100 mL of ethyl ether, dried and re-dissolved in 2 mL of a 0.1% formic acid solution. Two mL of extract was ultra filtered through filters with cut off 10000 Da at 7000 rpm. The filtrate was dried and re-dissolved in 1.2 mL of a 0.1% formic acid solution. The solution was dried under nitrogen flow and store at -20°C. Before being analyzed by UPLC/ESI-MS, samples obtained from lysed cells were dissolved in 200 µL of 0.1% aqueous HCOOH analyzed for assessing the presence of γ-Glu-Phe and Lac-Phe through UPLC/ESI-MS at the same condition described for cheese samples, except for the acquisition mode, which was performed in the

Single Ion Recording (SIR) mode, monitoring the following ions: γ-Glu-Phe: m/z 120, 166, 232, 278, 295; Lac-Phe: m/z 238, 192, 166, 120.

RESULTS AND DISCUSSION

Preliminary survey of the presence of γ-glutamyl- and lactoyl-amino acids in different type of cheeses

NPADs with interesting sensory properties have been already identified in cheeses. These compounds have been previously found in ripened food, but in greater quantities in fermented and ripened food, in particular in cheeses such as Asiago, Parmigiano Reggiano and Grana Padano (*Sforza et al., 2009*). With the purpose to evaluate their presence in a wider variety of cheeses, different cheeses were considered in a preliminary survey in order to correlate their microbiological and technological characteristics with the presence of γ-glutamyl-amino acids and lactoyl-amino acids. Twelve different type of Italian cheeses with different known characteristics such as the use of raw or pasteurized milk, the use of starters and the time of ripening, were chosen for a preliminary survey (Table 1). The amount of each γ-glutamyl-amino acids and lactoyl-amino acids was determined through UPLC/ESI-MS analysis of the peptide fraction by the internal standard method, against the standard Phe-Phe, which was added to all samples before extraction, and using as authentic standard synthetic γ-Glu-Phe, assuming for all the other compounds the same response factor. This probably led to a slight underestimation of lactoyl-amino acids, which are known to have a lower response factor of γ-glutamyl-amino acids. Figure 1 show the sum (mg in 100g of cheese) of all γ-Glutamyl-amino acids quantity (γ-Glu-Val, γ-Glu-Met, γ-Glu-Tyr, γ-Glu-Ile, γ-Glu-Leu, γ-Glu-Phe) and all Lactoyl-amino acids quantity (Lac-Val, Lac-Met, Lac-Tyr, Lac-Ile, Lac-Leu, Lac-Phe) contained in the samples.

In Bagoss and Grana Padano cheese the highest amount of non proteolytic aminoacyl derivatives were found. In particular in Grana Padano cheese the highest quantities of these compounds were observed (Figure 1). This result is in agreement with the amount previously described by Sforza and colleagues (*Sforza et al., 2009*). Bagoss is a ripened Italian mountain cheese. Even if it is traditionally produced without the addition of natural whey starter it can be considered an ancient version of Grana Padano because technological parameters of production are very similar (*Mucchetti & Neviani, 2006;*

Mucchetti et al., 2000). The typical microflora of both cheeses is thermophilic lactobacilli, added with natural whey starter for Grana Padano (*Zago et al., 2007*) and naturally selected by the technological parameter for Bagoss (*Mucchetti & Neviani, 2006; Mucchetti et al., 2000*). Non starter lactic acid bacteria, typical of long ripened cheeses, is generally composed by mesophilic lactobacilli (*Settanni et al., 2010*).

Four different kind of Pecorino, (Fiore Sardo, Pecorino Romano, Pecorino Sardo and Pecorino Toscano) with different time of ripening have been considered. Among them, in Fiore Sardo, ripened over 24 months, γ-glutamyl-amino acids and lactoyl-amino acids were detected in great amount (Figure 1). The microflora of Fiore Sardo is mainly characterized, by mesophilic lactobacilli (*Mannu et al., 2000*). On the other hand, the quantities found in another type of Pecorino, Pecorino Romano and Pecorino Sardo, are quite low (Figure 1). In Pecorino Sardo only lactoyl-amino acids were detected. Differently from Fiore Sardo these samples were characterized by a shorter time of aging, respectively 8 months and 2-3 months and a different microbial population, mainly thermophilic lactobacilli and *Streptococcus thermophilus* for Pecorino Romano (*Gatti et al., 1999*) and mainly lactococci for Pecorino Sardo (*Mannu et al., 2000*). The non proteolytic aminoacyl derivatives have not been produced in Pecorino Toscano microbiologically characterized by the presence of lactococci and *Streptococus thermophilus* and ripened only 30 days (*De Angelis et al., 2001*) (Figure 1).

Differently from Grana types and Pecorino cheeses, only the γ-glutamyl-amino acids have been revealed in Gorgonzola cheese and Pannerone. Probably the presence of mold in Gorgonzola plays an important role in the final composition of the product. In fact the microflora of this blue veined cheese involves also *Penicillium roqueforti* which is responsible not only of proteolytic activities but contributed markedly to lipolysis (*Gobbetti et al., 1997*). Differently from the other cheeses, the high amounts of γ-glutamyl-amino acids in blue veined cheese might be due to the γ-glutamyl transferase (GGT) activity of the blue mold *Penicillium roqueforti* (*Toelstede et al., 2009*).

Pannerone is particular handcrafted from raw milk, produced without starter and salt. The microflora of Pannerone is typically characterized by coliforms which are considered important for cheese sensory properties, because of their ability to produce gas and aromatic compounds (*Mucchetti et al., 2009*). Interestingly GGT activity of *E. coli* have been

already described by Suzuki (*Suzuki et al., 1986*). Similarly to Gorgonzola, γ-glutamyl-amino acids found in this short ripened cheese should be due to this enzyme.

Considering the non ripened cheeses, such as Crescenza and short ripening cheeses such as Italico, Bitto and Caciocavallo Silano, γ-glutamyl-amino acids and lactoyl-amino acids were not found. These data support the hypothesis that the production of these compounds is slow and therefore needs long time for their accumulation (thus they are more evident in long aged cheeses) and happens as secondary activity during ripening.

Well conscious that these results are relative to cheeses bought on the market of which is not exactly known the manufacturing, however the aim of this preliminary survey is only an approach which would lay the foundations for a constructive microbiological experiment. For these reasons, and consequently oversimplifying the obtained results, it should be possible speculate that: i) the non-ripened cheeses did not contain NPADs, ii) long ripened cheese characterized by lactobacilli microflora contain the higher amount of NPADs. This appears consistent with the observations that there is a correlation between the long ripening time and the presence of dominant microflora lactobacilli, with the higher production of NPADs. All these data let us to speculate that these compounds can be originated by an enzyme activity of lactobacilli.

The differential amount ratio of lactoyl and γ-glutamyl-amino acids in the different cheeses also appear to support the hypothesis that different kind of enzymes might be involved in their production, also suggesting that some enzymes might be more specific for lacotyl derivatives and other for γ-glutamyl derivatives.

Determination of the presence of an enzymatic activity producing non-proteolytic aminoacyl derivatives in Parmigiano Reggiano extracts

The presence of a specific enzymatic activity in Parmigiano Reggiano water soluble extracts was evaluated incubating the Parmigiano Reggiano cheese (12 months of ageing) PBS extract at 37°C for 24 h, with $^{2}H_5$-Phe as precursor in different amounts, and then assessing the content of $^{2}H_5$- γ-Glu-Phe by UPLC/ESI-MS.

Figure 2 shows the ratio between the produced $^{2}H_5$-γ-Glu-Phe and naturally present unlabelled γ-Glu-Phe in the Parmigiano Reggiano cheese after incubation with different amounts of $^{2}H_5$-Phe.

Since the chemical formation of an amide bond between a free carboxylic acid and a free amino group in these conditions (water solution at slightly acidic pH) is very unlikely, this experiment suggested that γ-Glu-Phe can actually be produced by an enzymatic activity present in Parmigiano Reggiano water soluble extracts, starting from the precursors, with produced amounts related to the amount of the amino acidic precursors. It is known that during cheese aging, starter LAB (SLAB) and non starter LAB (NSLAB) dynamically evolve according to modifications of environmental conditions (*Gala et al., 2008*). A previous study reports that in Parmigiano Reggiano cheese SLAB are the dominant microflora at the maximum of curd acidification; when ripening begins, they begin to undergo autolysis, allowing the increase of NSLAB, which dominate cheese microbiota during ripening (*Gatti et al., 2008*). NSLAB, able to growth after brining, begin to autolysis after 8-10 months (*De Dea Lindner et al., 2008*). Thus microbial enzymatic activities of 12 months Parmigiano Reggiano arised from SLAB lysed cells, NSLAB whole and lysed cells. For this reason, our analytical approach was to evaluate the production of non-proteolytic aminoacyl derivatives by SLAB and NSLAB cells either in viable condition and after lysis.

Determination of non-proteolytic aminoacyl derivatives produced by LAB strains

It was evaluated whether lactobacilli are able to produce non-proteolytic aminoacyl derivatives during the ripening process. For this purpose two LAB strains, belonging to *Lactobacillus* genus, isolated from Parmigiano Reggiano cheese, were considered. In particular it has been studied one *L. helveticus* strain as typical SLAB and *L. rhamnosus* strain as typical NSLAB. The hypothetical enzymatic bacteria activity that lead to the production of γ-glutamyl-amino acids and lactoyl-amino acids was assessed by checking the production of γ-glutamyl-phenylalanine and lactoyl-phenylalanine in presence of the suitable precursors. Viable cells of *L. helveticus* and *L. rhamnosus* strains were cultivated in MRS broth with or without two different precursors combination: i) glutamic acid and phenylalanine and ii) lactic acid and phenylalanine. Precursor ratio were chosen in order to simulate the actual ratios of these compounds in aged Parmigiano-Reggiano cheese. In order to detect the target molecules (γ-glutamyl-phenylalanine and lactoyl-phenylalanine respectively) an efficient extraction methods was set up (details in the experimental section). No quantification of the two target compounds was attempted in this case, but their relative amount was estimated only according to the area of the target molecule in the corresponding XIC. The detection of non-proteolytic aminoacyl derivatives was

carried out after 24, 48 and 72 h of incubation at 42°C for *L. helveticus* and 30°C for *L. rhamnosus*. It was observed that both *L. helveticus* and *L. rhamnosus* viable cells were unable to produce non-proteolytic aminoacyl derivatives without the addition of precursors (data not shown).

None of the two species, in presence of the precursors glutamic acid and phenylalanine, was able to produce, after 72 hours, γ-glutamyl-phenylalanine (data not shown). On the other hand, in the presence of lactic acid and phenylalanine as precursors, both the species were able to produce lactoyl-phenylalanine (Figure 3a). In particular, increasing amounts of lactoyl-phenylalanine were produced by *L. helveticus* during the incubation time. Respect to *L. helveticus*, *L. rhamnosus* produced an higher quantity of lactoyl-phenylalanine reaching the maximum in 48 h (Figure 3a).

It was interesting to note that, even without lactic acid as a precursor, but adding phenylalanine only as precursor, both the strains were able to produce lactoyl-phenylalanine (Figure 3b). *L. helveticus* was able to produce about the same amount of lactoyl-phenylalanine with or without lactic acid addition. Differently, *L. rhamnosus,* without lactic acid addition, was able to produce only half of the amount of lactoyl-phenylalanine produced with the addition of the lactic acid as precursor (Figure 3b). This could due to the different ability of *Lactobacillus* strains to convert glucose in lactic acid (*Kandler et al., 1986*) which became available as precursor for non-proteolytic aminoacyl derivatives conversion. *L. helveticus* is an high acidifying species therefore the addition of lactic acid as a precursor is irrelevant for this species (*Kandler et al., 1986*). Instead, the lower capacity of *L. rhamnosus* to convert lactose in lactic acid leads to a minor amount of precursor which can be converted in lactoyl-phenylalanine.

Considering the results obtained with 12 months Parmigiano Reggiano sample and the important role of natural lysis of lactobacilli in cheese ripening (*Lortal et al., 2005*), even the ability of lysate cells to produce γ-glutamyl-phenylalanine and lactoyl-phenylalanine was evaluated. To mimic the bacterial autolysis that naturally occurs during ripening, *L. helveticus* and *L. rhamnosus* cells were sonicated. MRS plate count agar of the cells after sonication was below the limit of detection (none colonies in 1 mL), demonstrating a sonication efficacy (E) greater than 99.99%.

L. helveticus and *L. rhamnosus* lysed cells were incubated for 24, 48, 72 h at 42°C in presence and in absence of two different precursors combination: i) glutamic acid and phenylalanine and ii) lactic acid and phenylalanine. *L. helveticus* and *L. rhamnosus* lysed cells were not able to produce non proteolytic aminoacyl derivatives without the addition of precursors (data not shown).

Differently from viable cells, adding glutamic acid and phenylalanine, γ-glutamyl-phenylalanine was produced by both the strains (Figure 4). In particular *L. helveticus* was able to produce detectable amount only after 48 h and to redouble the production after 72 h (Figure 4). Lysed cells of *L. rhamnosus* was able to produce remarkable quantity of γ-glutamyl-phenylalanine just after 24 hour of incubation.

Both the species were able to produce lactoyl-phenylalanine in presence of lactic acid and phenylalanine as precursors (Figure 5). While *L. helveticus* lysed cells were able to produce amount of lactoyl-phenylalanine comparable to those produce by viable cells, using *L. rhamnosus* lysed cells the production increase almost tenfold respect to growing cells (Figure 5). Lactoyl- phenylalanine produced by lysed *L. rhamnosus* was the best result obtained among all the experiments. As expected, non-growing cells were not able to produce lactoyl-phenylalanine in absence of lactic acid as precursors, even if phenylalanine was added.

Anyway, even in the best cases, when the highest intensities for γ-glutamyl-phenylalanine and lactoyl-phenylalanine was observed, the efficiency of the process seems to be very low. An estimation of the conversion of precursors to products, done by comparing the intensities of γ-glutamyl-phenylalanine and lactoyl-phenylalanine to free phenylalanine, allowed to estimate a percentage of conversion not higher, in the best cases, of 0.01%.

Considering that γ-glutamyl-phenylalanine were produced only by lysed cells and lactoyl-phenylalanine was produced either by growing or lysed cells it should be possible suppose that: i) enzymes necessary to produce γ-glutamyl-phenylalanine are not expressed during growth in MRS of *L. helveticus* and *L. rhamnosus* since they are not involved in the main pathways, or they are in the cytoplasm and active only after cell lysis, ii) enzymes necessary to produce lactoyl-phenylalanine are active during cells growth and also active after breaking of the cells but their performance is different between the two species, iii) enzymes released after breaking of *L. rhamnosus* cells are the most effective for the reaction.

The accumulation during ripening of NPADs, and in particular γ-glutamyl amino acids previously observed in Parmigiano Reggiano (*Sforza et al., 2009*) should be thus justified by the activity of the enzymes which are gradually released in cheese after the natural autolysis of *L. helveticus* at the beginning and *L. rhamnosus* subsequently (*Gatti et al., 2008*).
To our knowledge lactoyl-amino acids production have not been studied yet and this is the first study dealing with their origin in foods. On the other hand, γ-glutamyl-amino acids were reported as a products of GGT activity (*Suzuki et al., 2007*). GGT catalyzes the lysis of γ-glutamil compounds, for example glutathione, and the transfer, through a transamidation reaction, of their γ-glutamil moieties to amino acids and peptide (*Suzuki et al., 2002*). This enzyme is known to use mainly glutamine as a substrate (*Toelstede et al., 2009*). It has been suggested that this reaction might be reponsible for the γ-glutamyl-amino acids presence in cheese (*Toelstede et al., 2009*).
In order to test this hypothesis, the same tests reported above on *L. helveticus* and *L. rhamnosus* activity, were done using glutamine and phenylalanine as precursors in order to evaluate the γ-glutamyl-phenylalanine production. No production was observed by viable cells, while a trace amount was produced by *L. rhamnosus* lysed cells (data not shown). Although GGT is known to be present in milk and thus in Parmigiano Reggiano cheese (*Belitz et al., 2009*), the high quantity of γ-glutamyl-phenylalanine produced by lactobacilli using glutamic acid as precursor, suggest that the consistent amount found in cheeses is rather to be due to a bacterial enzymatic activity different from GGT activity. The reaction responsible of non-proteolytic aminoacyl derivatives production seems to be an amidation starting from glutamic acid rather than a transamidation starting from glutamine or other similar precursor, the path usually followed by GGT. This is also consistent with the simultaneous presence of lactoyl- amino acids in the analyzed cheeses, whose presence cannot be due to the GGT activity.

CONCLUSION

The lactobacilli characterizing Parmigiano Reggiano cheese, *L. helveticus*, main species of natural whey starter and representative of SLAB, and *L. rhamnosus*, main species found in ripened cheese and representative on NSLAB, were able to produce both γ-glutamyl-phenylalanine and lactoyl-phenylalanine. In vitro, during growth *L. helveticus* and *L. rhamnosus* produced lactoyl- amino acids but did not produced γ-glutamyl- amino acids.

In lysed condition great amount of both non-proteolytic aminoacyl derivatives were produced.

It is well known that during cheese production and ripening lactose is converted in lactic acid by the action of SLAB and amino acids, such as glutamic acid, are released by casein degradation, due SLAB and NSLAB activity mainly occurring after lyses. For this reason the two precursors of the successive chemical transformation are fully available in cheeses, together with the aminoacyl acceptors of the acyl moieties. Thus after cells lyses, it is feasible to hypothesize that the enzyme responsible of these activities are released, allowing the production and the accumulation of these non-proteolytic aminoacyl derivatives.

The data contained in this study are consistent with the hypothesis that the non-proteolytic aminoacyl derivatives are produced by a bacterial enzymatic activity. In particular lactic acid bacteria, such as *L. helveticus* and *L. rhamnosus*, have been demonstrated to be able to produce γ-glutamyl- amino acids and lactoyl- amino acids *in vitro.*

Anyway, the accumulation of these compounds in cheeses appear to be very low and the final amount around 50 mg in100 g of cheese is a consequence of the long time of ageing and of the accumulation of the precursors. The enzymatic activity which leads to these compound production might be useful for the food industry, given their interesting sensory properties. Further studies will be helpful in order to isolate the responsible enzymes and to identify the optimal conditions to increase the yields.

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REFERENCES

Belitz H.D., Grosch W. & Schieberle P. 2009 Food Chemistry. 4th edn Berlin, Springer, Germany.

Careri M., Spagnoli S., Panari G., Zannoni M. & Barbieri G. 1996 Chemical parameters of the non-volatile fraction of ripened Parmigiano-Reggiano cheese. Int Dairy J 6, 147–155.

De Angelis M., Corsetti A., Tosti N., Rossi J., Corbo R. & Gobbetti M. 2001 Characterization of non-starter lactic acid bacteria from Italian ewe cheeses based on phenotypic, genotypic, and cell wall protein analyses. Appl Environ Microbiol 67, 2011-2020.

De Dea Lindner J., Bernini V., De Lorentiis A., Pecorari A., Neviani E. & Gatti M. 2008 Parmigiano Reggiano cheese: evolution of cultivable and total lactic microflora and peptidase activities during manufacture and ripening. Dairy Sci Tech 88 511–523.

Gala E., Landi S., Solieri L., Nocetti M., Pulvirenti A. & Giudici P. 2008 Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. Int J Food Microbiol 125, 347–351.

Gatti M., Fornasari M. E., Mucchetti G., Addeo F. & Neviani E. 1999 Presence of peptidase activities in different varieties of cheese. Lett Appl Microbiol 28, 368-372.

Gatti M., Bernini V., Lazzi C. & Neviani E. 2006 Fluorescence microscopy for studying the viability of micro-organisms in natural whey starters. Lett Appl Microbiol 42, 338-343.

Gatti M., De Dea Lindner J., De Lorentiis A., Bottari B., Santarelli M., Bernini V. & Neviani E. 2008 Dynamics of whole and lysed bacterial cells during Parmigiano-Reggiano cheese production and ripening. Appl EnvironMicrobiol 74, 6161-6167.

Gobbetti M., Burzigotti R., Smacchi E., Corsetti A., & De Angelis M. 1997 Microbiology and biochemistry of gorgonzola cheese during ripening. Int Dairy J 7, 519-529.

Kandler O. & Weiss N. 1986 Regular, non sporing Gram-positive rods. In Bergey's Manual of Systematic Bacteriology (pp. 1209-1234). P.H.A. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt eds Baltimore, MD, USA.

Helinck S., Le Bars D., Moreau D. & Yvon M. 2004 Ability of thermophilic lactic acid bacteria to produce aroma compounds from amino acids. Appl Environ Microbiol 70, 3855-3861.

Lortal S. & Chapot-Chartier M.P. 2005 Role, mechanisms and control of lactic acid bacteria lysis in cheese. Int Dairy J 15, 857–871.

Mannu L., Comunian R. & Scintu F. 2000 Mesophilic lactobacilli in Fiore Sardo cheese: PCR-identication and evolution during cheese ripening. Int Dairy J 10, 383-389.

Mannu L. & Paba A. 2002 Genetic diversity of lactococci and enterococci isolated from home-made Pecorino Sardo ewes' milk cheese. J Appl Microbiol 92, 55-62.

Mucchetti G., Locci F., Gatti M., Neviani E., Addeo F., Dossena A. & Marchelli R. 2000 Pyroglutamic Acid in Cheese: Presence, Origin, and Correlation with Ripening Time of Grana Padano Cheese. J Dairy Sci 83, 659-665.

Mucchetti G., & Neviani E. 2006 Microbiologia e tecnologia lattiero casearia. Qualità e sicurezza. Tecniche Nuove, Milano, Italy.

Mucchetti G., Ghiglietti R., Locci F., Francolino S., Bonvini B., Remagni M.C., Zago M., Iezzi R. & Carminati D. 2009 Technological, microbiological and chemical characteristics of Pannerone, a traditional Italian raw milk cheese. Dairy Sci Tech 89, 419–436.

Neviani E., De Dea Lindner J., Bernini V. & Gatti M. 2009 Recovery and differentiation of long ripened cheese microflora through a new cheese-based cultural medium. Food Microbiol 26, 240-245.

Roudot-Algaron F., Kerhoas L., Le Bars D., Einhorn J. & Gripon J.C. 1994 Isolation of γ-Glutamyl Peptides from Comté Cheese. J Dairy Sci 77, 1161-1166.

Settanni L. & Moschetti G. 2010 Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiol 2, 691-697.

Sforza S., Galaverna G., Dossena A., Marchelli R., Neviani E. & Pinelli C. 2004 Study of the oligopeptide fraction in Grana Padano and Parmigiano-Reggiano cheeses by liquid chromatography electrospray ionization mass spectrometry. European Journal of Mass Spectrometry, 10, 421-427.

Sforza, S., Galaverna, G., Schivazappa C., Marchelli, R., Dossena, A. & Virgili R. 2006 Effect of extended aging of Parma dry-cured ham on the content of oligopeptides and free amino acids. Journal of Agricultural and Food Chemistry 54, 9422-9429.

Sforza S., Cavatorta V., Galaverna G., Dossena A., & Marchelli R. 2009 Accumulation of non-proteolytic aminoacyl derivatives in Parmigiano Reggiano cheese during ripening. Int Dairy J 19, 582-587.

Sousa M.J., Ardö Y. & McSweeney P.L.H. 2001 Advances in the study of proteolysis during cheese ripening. Int Dairy J 11, 327-345.

Suzuki H., Kajimoto Y. & Kumagai H. 2002 Improvement of the Bitter Taste of Amino Acids through the Transpeptidation Reaction of Bacterial γ-Glutamyltranspeptidase. Journal of Agricultural and Food Chemistry 50, 313-318.

Suzuki H. Kumagai H. & Tatsurokuro T. 1986 γ- Glutamyltranspetidase from Escherichia coli K-12: purification and proprierties. Journal of Bacteriology 168, 1325-1331.

Suzuki H., Yamada C. & Kato K. 2007 γ- Glutamyl compounds and their enzymatic production using bacterial γ- glutamyltranspetidase. Amino Acids 32, 333-340.

Toelstede S. & Hoffman T. 2009 Kokumi-active glutamyl peptides in cheeses and their biogeneration by Penicillium riquefortii. Journal of Agricultural and Food Chemistry 57, 1440-1448

Toelstede S., Dunkel A. & Hoffman T. 2009 A series of kokumi peptides impart the long-lasting mouthfulness of matured Gouda cheese. Journal of Agricultural and Food Chemistry 57, 1440-1448.

Urbach, G. 1995 Contribution of lactic acid bacteria to flavor compound formation in dairy products. Intl Dairy J 5, 877-903.

Yvon M. & Rijnen L. 2001 Cheese flavor formation by amino acids catabolism. Int Dairy J 11, 185-201.

Zago M., Fornasari M.E., Rossetti L., Bonvini B., Scano L. & Carminati D. 2007 Population dynamics of lactobacilli in Grana cheese. Annals of Microbiology 57, 349-353.

TABLES

Table 1: *General characteristics of the cheeses chosen for the preliminary survey.*

Samples	Milk	Starter	Ripening time (months)	Reference
Bagoss	Raw	Without	More than 12	*Gatti et al., 1999*
Grana Padano	Raw	Thermophilic lactobacilli	More than 12	*Gatti et al., 1999*
Fiore Sardo	Raw	Without	24	*Mannu et al., 2000*
Pecorino Romano	Raw	Thermophilic lactobacilli *Streptococcus thermophilus*	8	*Gatti et al., 1999*
Pecorino sardo	Raw	Lactococci	More than2	*Mannu et al., 2002*
Pecorino Toscano	Pastorized	Lactococci *Streptococcus thermophilus*	1	*De Angelis et al. 2001*
Gorgonzola	Pastorized	*Streptococcus thermophilus, Lactobacillus delbrueckii* subsp *bulgaricus, Penicillium roqueforti*	5	*Gatti et al., 1999*
Pannerone	Raw	Without	Less than 2	*Mucchetti et al., 2009*
Crescenza	Pastorized	*Streptococcus thermophilus*	Less than 1	*Gatti et al., 1999*
Italico	Pastorized	*Streptococcus thermophilus*	Less than 2	*Gatti et al., 1999*
Bitto	Raw	Without	2	*Colombo et al., 2009*
Caciocavallo Silano	Raw	Thermophilic lactobacilli *Streptococcus thermophilus*	2	*Ercolini et al., 2008*

FIGURES

Figure 1. *Sum (mg in 100 g of cheese) of all N-γ-Glutamyl-amino acids quantity (γ-Glu-Val, γ-Glu-Met, γ-Glu-Tyr, γ-Glu-Ile, γ-Glu-Leu, γ-Glu-Phe) (grey) and all N-Lactoyl-amino acids quantity (Lac-Val, Lac-Met, Lac-Tyr, Lac-Ile, Lac-Leu, Lac-Phe) (black) in different type of cheeses. Bars indicate the standard deviation of data from two experiments.*

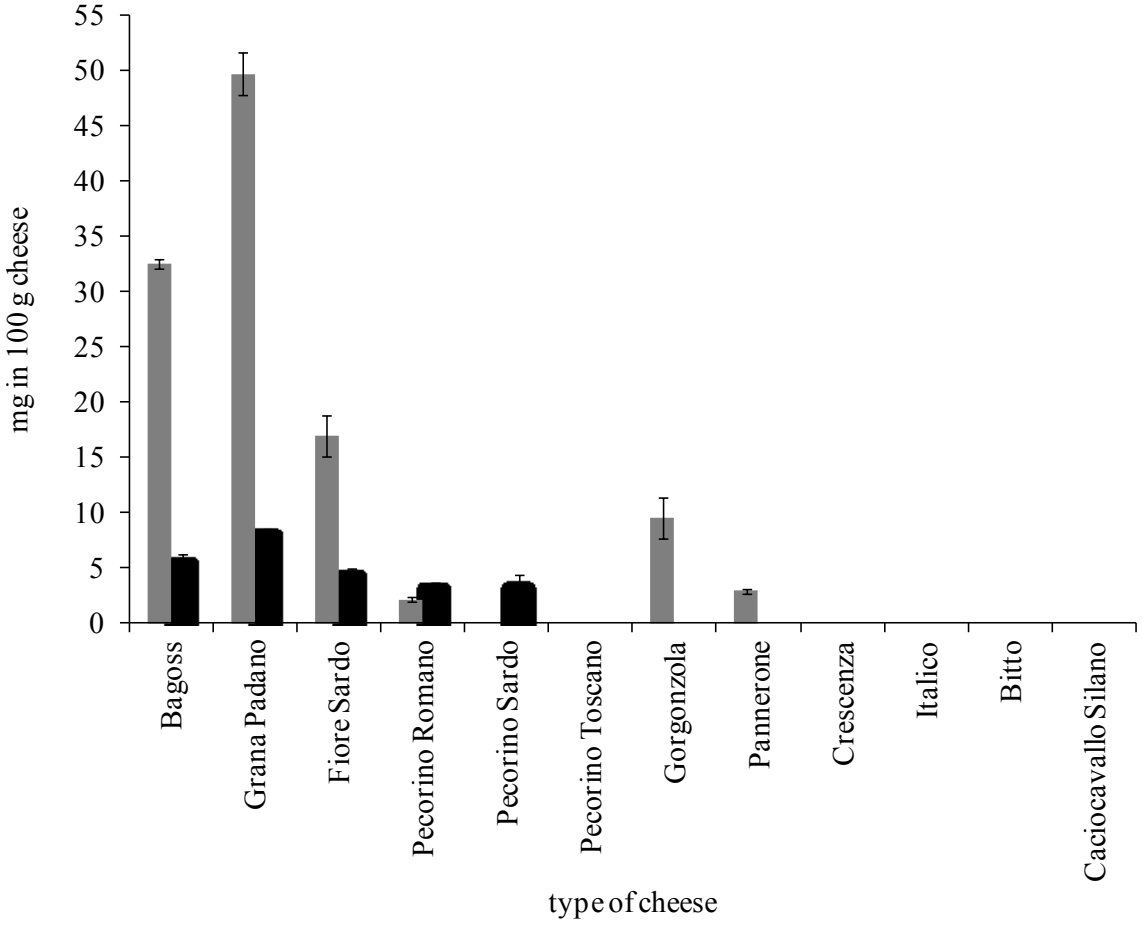


Figure 2. *Ratio of produced $^2H_5$-heavy labeled- γ-glutamyl-phenylalanine ($^2H_5$-γ-Glu-Phe) and naturally present unlabelled-γ-glutamyl-phenylalanine (γ-Glu-Phe) in the Parmigiano Reggiano cheese with different amount of $^2H_5$-heavy labeled phenylalanine ($^2H_5$-Phe) added as precursor.*

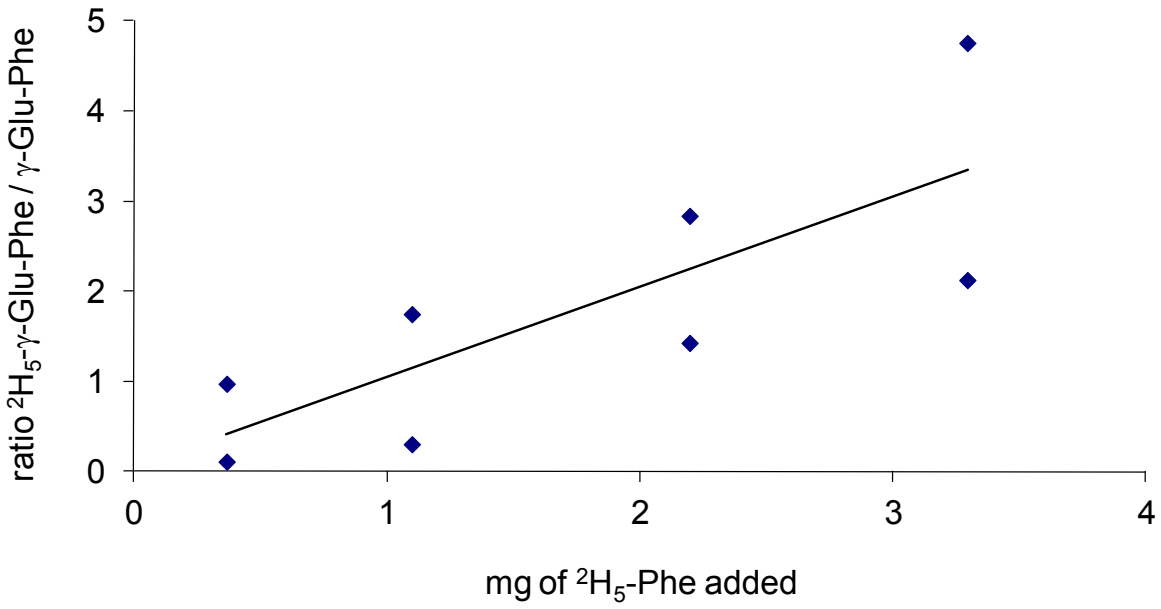
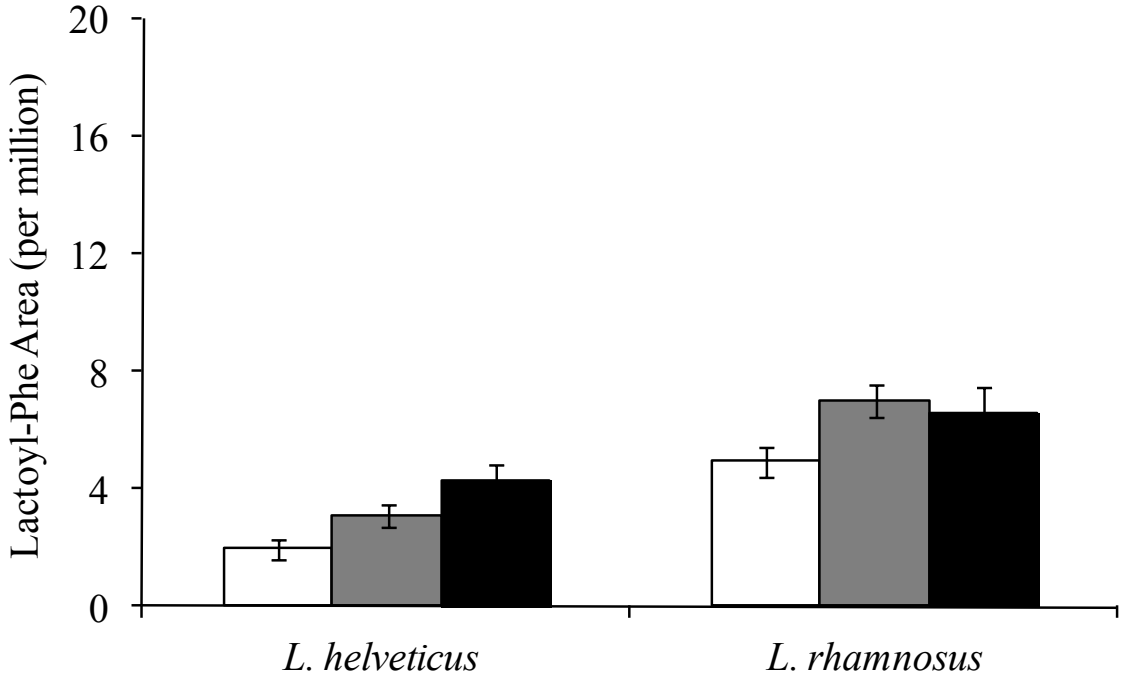


Figure 3. *Production of Lactoyl-Phe by viable L. helveticus and L. rhamnosus incubated for 24 (white), 48 (grey) and 72 (black) hours in presence of lactic acid and phenylalanine (**3a**) and glutamic acid and phenylalanine as precursor (**3b**). Bars indicate the standard deviation of data from two experiments.*

3a



3b

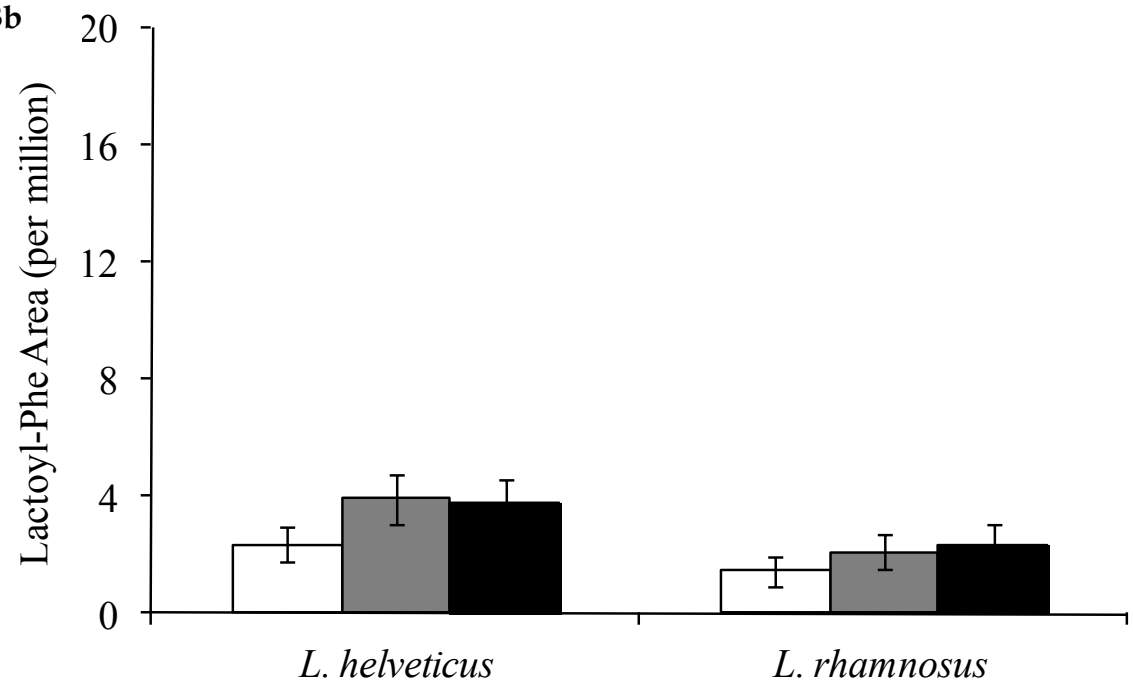


Figure 4. *Production of γ-Glutamyl-Phe by lysed L. helveticus and L. rhamnosus incubated for 24 (white), 48 (grey) and 72 (black) hours in presence of glutamic acid and phenylalanine as precursor. Bars indicate the standard deviation of data from two experiments.*

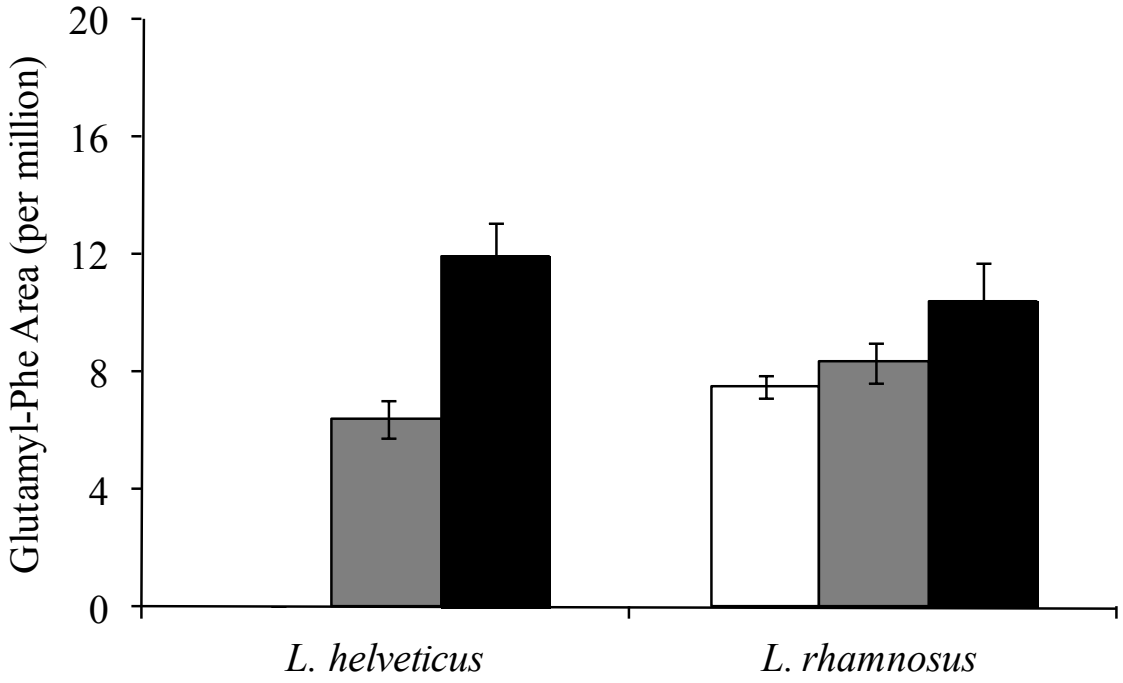
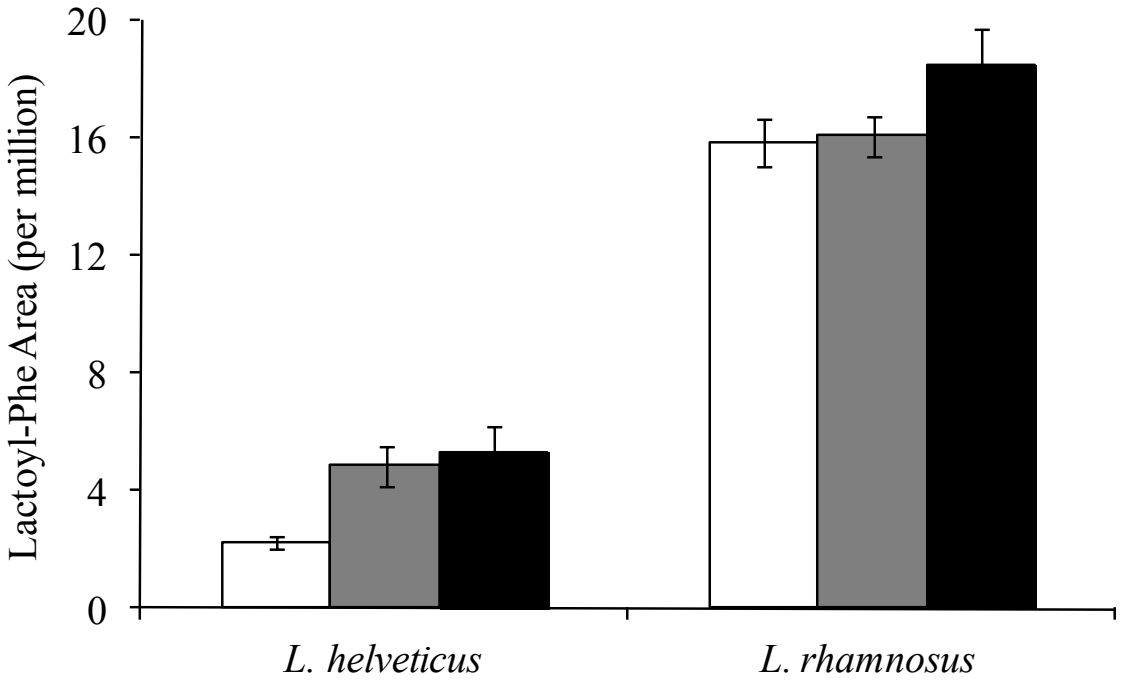


***Figure 5**. Production of Lactoyl-Phe by lysed L. helveticus and L. rhamnosus incubated for 24 (white), 48 (grey) and 72 (black) hours in presence of lactic acid and phenylalanine. Bars indicate the standard deviation of data from two experiments.*



V. GENERAL CONCLUSIONS

Prolonged aging times are a specific feature of long ripened cheese like Parmigiano Reggiano. Ripening usually involves several phenomena, such as evolution of the cheese dominant microflora and biochemical reactions that lead to the production of molecules involved in the cheese flavour. The LAB are the protagonists of these events.

The microbiological evolution during ripening include the death and lysis of SLAB (the dominant microflora at the culmination of curd acidification) and the growth of secondary microflora, particularly NSLAB. In Parmigiano Reggiano cheese NSLAB growth occurs in lacking sugars conditions, because the lactose has already been exhausted by SLAB during the initial step of cheese making.

The ability of NSLAB to survive in these conditions is due to their particular capabilities to use alternative energy sources, such as the SLAB cell lysis products.

The research work firstly reported in this Ph.D thesis, confirmed the NSLAB ability to survive and grow using as nutrient source only SLAB cell lysis products. In fact, the experiments conducted to sustain this hypothesis confirmed that *L. helveticus* (SLAB) lysate products provide sufficient nutrients to allow the growth of two NSLAB species, *L. casei* and *L. rhamnosus, in vitro*. Then, changes in the SLAB lysis product composition were observed after NSLAB grew on this. This suggests that some compounds can be either consumed or produced by bacterial metabolism during growth. The analysis performed in this study provides new perspectives on NSLAB survival strategy.

Secondly, this PhD research aimed to investigate if the NSLAB metabolism in these lacking sugar conditions, could contribute to the production of volatile molecules involved in cheese flavour formation. Therefore, the potential contribution to cheese aroma formation by NSLAB during cheese ripening, was studied. The volatilomes of *L. rhamnosus* and *L. casei,* grown using SLAB cell lysis products or a cheese model medium were studied. The results allowed to suppose that some important molecules involved in flavour formation can be produced in both conditions. The

metabolic products obtained by lactate/citrate metabolism, amino acids (FAA) catabolism, free fatty acids (FFA) metabolism and by the use of the few sugars contained in ripened cheese, such as sugars deriving from SLAB cellular wall (i.e. N-acetyl-glucosamine), were detected. The presence of these volatile compounds confirmed that the NSLAB can contribute to develop flavour of ripened cheese.

Finally, recent researches identified non proteolytic aminoacyl derivatives, (γ-glutamyl-amino acids and lactoyl-amino acids) with interesting sensory properties in cheese. These molecules seem to be involved in cheese flavour and it was observed that these compounds accumulate during cheese ripening. Therefore the ability of NSLAB and SLAB to produce these new molecules was evaluated. In this PhD thesis, it was observed that, *L. helveticus,* representative of SLAB, and *L. rhamnosus,* representative on NSLAB, were able to produce both γ-glutamyl-phenylalanine and lactoyl-phenylalanine by a bacterial enzymatic activity. While the first was produced only by lysed cells, the latter was produced either by growing or lysed cells in different amount depending on the species, the cell condition and time of incubation. Having regard to the low yield of the reactions, the greater amount of these compounds, found in cheese, is a consequence of the long time of ageing and of the accumulation of the amino acidic precursors.

Concluding, all the observed abilities of NSLAB and their activities during cheese ripening could be harnessed to several technological extents. In particular, the description of species specific features involved in flavour formation could be of great interest for the worldwide cheese producers

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Studies

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Publications list

Di Cagno R., Minervini G., Sgarbi E., Lazzi C., Bernini V., Neviani E., Gobbetti M. Comparison of phenotypic (Biolog System) and genotypic (Random Amplified Polymorphic DNA-polymerase chain reaction, RAPD-PCR, and Amplified Fragment Length Polymorphism, AFLP) methods for typing *Lactobacillus plantarum* isolates from raw vegetables and fruits. *International Journal of Food Microbiology* 143, 246-53 (2010).

Bernini V., Sgarbi E., Bove CG.,Gatti M., Neviani E. A polyphasic approach to detect enterotoxigenic *Staphylococcus aureus* and diarrheagenic *Escherichia coli* in raw milk Italian cheeses by multiplex PCR. *Journal of Food Protection* 73, 2281-2284 (2010).

Lazzi C., Bove CG., Sgarbi E., Gatti M., La Gioia F., Torriani S., Neviani E.. Application of AFLP fingerprint analysis for studying the biodiversity of *Streptococcus thermophilus*. *Journal of Microbiology Methods* 79, 48-54 (2009).

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