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L'activité protéolytique et antimicrobienne des bactéries lactiques isolées de produits laitiers fermentés traditionnels d'Azerbaidjan

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Présentée
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Discipline: Biochemistry

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Presented by

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Nantes, June 2012

Aynur Ahmadova

Title:

Proteolytic and antimicrobial activities of lactic acid bacteria isolated from traditional Azerbaijani fermented dairy products

Key-words:

proteolysis, caseins, lactic acid bacteria, bacteriocins, probiotics

Abstract:

This thesis was devoted for the study of proteolytic and antimicrobial activities of LAB isolated from traditional Azerbaijani fermented dairy products. LAB isolation was done from 46 samples of traditional fermented dairy products obtained from different regions of Azerbaijan. As a result of the screening 10 new LAB strains were isolated, from which 8 were proteolytic and belong to the species *Lactobacillus helveticus* (1 strain), *Lactobacillus paracasei* subsp. *paracasei* (1 strain) and *Enterococcus faecalis* (6 strains). Two strains were producers of bacteriocins and belong to species *Enterococcus faecium* and *Lactobacillus curvatus*. Specificity of proteolytic activities of studied LAB strains depended on the substrate cleaved and on bacterial growth phase. Found proteolytic activities were mainly directed against caseins. The optimal hydrolysis of substrates by studied strains was observed at 37-45 ºC and at neutral pH range. The studied strains produced several proteases, mainly metallo- and serine- proteases.

The proteolysis of caseins by strains *Lactobacillus helveticus* A75 and *Enterococcus faecalis* AN1 decreased their immunoreactivity as a result of lytic destruction of IgE binding linear epitopes.

The studied bacteriocinogenic strains inhibited the growth of closely related LAB strains and also pathogens such as *Listeria monocytogenes, Bacillus cereus. Lactobacillus curvatus* A61 also displayed antifungal activity. The mode of action of found bacteriocins was dependent on the test-organism used and was bacteriostatic or bactericidal. *Enterococcus faecium* AQ71 possesses genes coding enterocins P, L50A/B and A. *Lactobacillus curvatus* A61 possesses the structural gene of curvacin A. Antimicrobial substances produced by the studied LAB strains are heat stable and active in broad pH range.

The studied Azerbaijani LAB do not possess the virulence factors and multi-resistance to antibiotics. Thus they are safe for industrial application. Moreover, the studied Azerbaijani LAB strains are resistant to physiological concentrations of bile salts, what is one of the main characteristics of probiotic strains. All these results suggest that the studied bacteriocinogenic and proteolytic strains could be used safely as starter and co-cultures for the food industry.

Titre:

Activités protéolytiques et antimicrobiennes des bactéries lactiques isolées de produits laitiers fermentés traditionnels d'Azerbaïdjan

Mots-clés:

la protéolyse, les caséines, les bactéries lactiques, des bactériocines, probiotiques

Résumé :

Ce travail a été consacrée à la recherche des activités protéolytiques et antibactériennes de bactéries lactiques isolées de produits laitiers fermentés traditionnels d'Azerbaïdjan. Les souches étudiées ont été isolées de 46 échantillons de produits laitiers fermentés, obtenus dans différentes régions d'Azerbaïdjan. Huit souches ayant une activité protéolytique notable ont été purifiées et identifiées par amplification et par séquençage de l'ADN 16S comme étant *Lactobacillus helveticus* (1 souche), *Lactobacillus paracasei* subsp. *paracasei* (1 souche), *Enterococcus faecalis* (6 souches). Deux souches (1 *Lactobacillus curvatus* et 1 *Enterococcus faecium*) ayant une activité antibactérienne ont été purifiées et caractérisées. Les activités protéolytiques de ces souches ont été étudiées dans 2 systèmes: croissance dans du lait UHT et système cellulaire non prolifératif. Ces souches hydrolysent toutes les fractions de caséines ce qui démontre leur similarité avec les protéases de type PIII de *Lactococcus*. Les optima de température et de pH d'activité protéolytique sont de 37-45 °C et de pH neutre. Les effets de différents inhibiteurs sur les activités protéolytiques indiquent que les souches étudiées produisent des métallo-, sérine- et cystéine-protéases. Ces résultats ont été confirmés par l'analyse des souches par PCR gène spécifique avec des amorces de protéases de bactéries lactiques. L'immuno réactivité des caséines β et α_{S1} mesurée par interaction avec les IgE peut être considérablement réduite par hydrolyse avec *Lactobacillus helveticus* A75 et *Enterococcus faecalis* AN1 à la suite de la destruction des épitopes linéaires des IgE.

Les souches productrices de bactériocines étudiées dans ce travail inhibent la croissance des souches de bactéries lactiques étroitement liées et aussi des souches pathogènes comme *Listeria monocytogenes* et *Bacillus cereus*. La souche *Lactobacillus curvatus* A61 a également une activité antifongique. Le mode d'action de leurs bactériocines dépendant de l'organisme test utilisé était bactériostatique ou bactéricide. *Enterococcus faecium* AQ71 possède les gènes codant pour les enterocines P, L50A/B et A ; la souche *Lactobacillus curvatus* A61 présente un gène de curvacine. Les substances produites par les souches de LAB azerbaïdjanaises sont stables à la chaleur et actives dans une large gamme de pH.

L'analyse des souches par des méthodes de biologie moléculaire a révélé l'absence de gènes de facteurs de virulence. Les souches étudiées ne présentaient pas de multi-résistance aux antibiotiques. Par ailleurs, les souches de LAB azerbaïdjanaises étudiées dans ce travail sont résistantes aux concentrations physiologiques de sels biliaires, ce qui est l'une des caractéristiques principales des probiotiques. L'ensemble de ces résultats montre que les souches de LAB azerbaïdjanaises sélectionnées, productrices de bactériocines et de protéases, peuvent être utilisées sans risque majeur comme levains ou en co-culture dans les processus de fabrication alimentaire.

CONTENT

PUBLICATIONS – SCIENTIFIC COMMUNICATIONS

PUBLICATIONS

1. **Ahmadova, A.,** Dimov, S., Ivanova, I., Choiset, Y., Chobert, J.-M., Kuliev, A., & Haertlé, T. (2011). Proteolytic activities and safety of use of *Enterococci* strains isolated from traditional Azerbaijani dairy products. Eur Food Res Technol, 233:131–140.
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ABBREVIATIONS

ALA	alpha-Lactalbumin
ATCC	American type culture collection
AU	Arbitrary units
BLG	beta-Lactoglobulin
bp	bases pairs
CEP	cell envelope proteinase
CFU	Colony forming units
CMA	Cow Milk Allergy
DNA	Desoxy Ribonucleic Acid
dNTP	dideoxynucleotide
ELISA	Enzyme-Linked Immunosorbent Assay
F-ELISA	Fluorescent ELISA
GIT	Gastrointestinal tract
HPLC	High Performance Liquid Chromatography
IgE	Immunoglobulin E
LAB	Lactic Acid Bacteria
LB	Luria-Broth
β-ME	2-mercaptoethanol
4-MUP	4-methylumbelliferyl phosphate
MRS	Mann Rogosa Sharpe broth
MW	Molecular Weight
ONIRIS	L'école nationale vétérinaire, agroalimentaire et de l'alimentation, Nantes-Atlantique
PCR	Polymerase chain reaction
TEMED	N,N,N,′N′-tetramethylethylenediamine
PAGE	Poly-acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PVA/T	Polyvinyl Alcohol/Tween
RAPD	Random Amplified Polymorphic DNA
RP	Reverse Phase
SDS	Sodium Dodecyl Sulfate
UHT	Ultra-high-temperature processing
UPGMA	Unweighted Pair Group Method with Arithmetic averages
WHO	World Health Organization

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Proteolytic and antimicrobial activities of lactic acid bacteria isolated from traditional Azerbaijani fermented dairy products

INTRODUCTION

The interest of consumers for diverse fermented foods has increased in recent years due to their beneficial impact on consumer health. Consumption of fermented foods has many advantages including enhanced nutritional value, digestibility, therapeutic benefits and safety against spoilage and pathogenic bacteria. The successful fermentation of food depends on the production of different metabolites by starter cultures. Because lactic acid bacteria (LAB) and their metabolites have been consumed in high quantities by generations of people in fermented foods with no adverse effects, the LAB continue to be used as the preferred starters and adjunct cultures in the production of different fermented foods, including functional dairy products. They can contribute to microbiological safety and provide technological, nutritional and organoleptic advantages to a fermented product, through production of ethanol, acetic and lactic acid, aroma compounds, exopolysaccharides, bacteriocins and several enzymes.

LAB play an important role in the biochemical reactions taking place during cheese ripening due to their milk acidification, flavour development and proteolysis. Proteolysis is considered to be one of the most important biochemical processes involved in the manufacture of many fermented dairy products, independently of the contribution of the proteolytic/peptidolytic enzymes of LAB to organoleptic properties of the final milk products. The ability to secrete extracellular proteinases is a very important feature of LAB. They hydrolyze milk proteins, providing the amino acids essential for growth of LAB (Fira et al., 2001). Proteolysis is a desirable process improving milk digestibility and enhancing nutritional quality of final dairy products. It is well known that proteolytic system of LAB degrades proteins and hence, changes the texture, the taste and the aromas of fermented products.

It should be highlighted also that food allergies are rising significantly amongst the world populations. Cow's milk allergy (CMA) concerns ~2.5% of children below 3 years of age, hence the feeding of an infant allergic to cow milk casein may create serious trouble. Numerous milk proteins induce allergic responses and most of these have been shown to contain multiple allergenic epitopes (Chatchatee et al., 2001b; Ross et al., 2005). Most studies revealed that caseins and BLG are the main allergens in cow milk. Well described and well studied influence of the fermentative processing of foods and in particular the dairy food systems, could help to circumvent or limit the severity of some of the adverse allergenic reactions. Different attempts have been made to reduce the antigenicity of dairy proteins and various technological processes have been used for this purpose. One of the potential approaches to decrease antigenicity of milk proteins could be bacterial fermentation. Proteolysis with well selected strains occurring in fermented dairy (and other foods) systems could either change the allergen presentation or cleave

the allergenic protein epitopes. Hence, it could be expected that well managed fermentative transformations of dairy (and other food) products with appropriate LAB strains, would produce hypoallergenic products at least for some part of allergic patients. It was already reported that some LAB strains reduce the antigenic response of milk proteins (Bu et al., 2010; Kleber et al., 2006; Wroblewska et al., 1995). For these reasons, the well composed starters containing LAB strains with robust proteolytic activities able to reduce the allergenicity of fermented milk products are of great interest for the dairy industry.

Some but not all LAB strains may also increase the safety and quality of fermented products due to production of different antimicrobial compounds, which can prevent the growth of pathogenic and spoilage bacteria. Antimicrobial metabolites of LAB include organic acids, hydrogen peroxide and diacetyl and also additional metabolites such as small proteins or peptides named bacteriocins.

Bacteriocins are ribosomally synthesized antimicrobial proteinaceous compounds doted of general bactericidal activities, often towards bacteria that are closely related to the bacteriocin-producing strain. Because LAB strains used currently in food production have a 'generally recognized as safe' (GRAS) status, the detection and identification of bacteriocins produced by such bacteria have attracted lot of attention, since these substances can be used as 'natural' food preservatives (Cleveland et al., 2001).

The microbial populations present in artisanal dairy products may be very diverse in function of the local production technology and the geographic origins of the product. In the traditional dairy products manufactured on the farm, unique sensory characteristics, specific flavours and textures are often due to the activities of autochthonous and non-starter lactic acid bacteria (NSLAB), originating from milk and the surrounding microflora. Thus, the microflora of such artisanal fermented products, especially NSLAB microflora, could be the rich source for the screening of LAB starters with desired properties.

Research objectives. The aim of the present study was the isolation and the characterisation of proteolytic and bacteriocinogenic LAB strains from traditional fermented milk products of Azerbaijan and the determination of the potential applications of obtained strains for production of functional hypoallergenic dairy products and also their potential use as natural preservatives.

For this purpose, the following objectives were defined:

- Screening of proteolytic and bacteriocinogenic LAB strains from fermented dairy products, obtained from different regions of Azerbaijan;

- Study of the proteolytic activities of obtained LAB strains at different conditions and the determination of the specificity of proteolytic enzymes and the effects of different factors on proteolytic activities;
- Study of the effect of proteolytic activities of obtained LAB strains on the immunoreactivity of milk proteins;
- Biochemical characterisation of bacteriocins, produced by obtained LAB strains, also to define the optimal conditions of bacteriocin production and the mode of action of active compounds;
- Determination of the inhibition spectra of bacteriocinogenic LAB strains;
- Study the safety and probiotic properties of obtained proteolytic and bacteriocinogenic LAB strains.

The scientific novelty of research. The present research is the first report describing the screening of Azerbaijani dairy products for the presence of strongly proteolytic LAB strains. At the same time, the screening of Azerbaijani dairy products for the presence of bacteriocinogenic LAB was continued. As a result of screening, 10 new strains of LAB, from which 8 were protease-producers and 2 bacteriocin-producers, were obtained and the characterisation of their activity, their potential role in dairy industry, as well as, their safety for consumers and their probiotic properties were investigated. Till now, the isolation and characterisation of proteolytic LAB from Azerbaijani dairy products was not performed by any research group and the information in this area does not exist in the literature. Thus, the obtained results are of scientific significance regarding the metabolic activity of LAB isolated from geographic region where Azerbaijan is situated. Obtained results could yield new information about LAB metabolism during the manufacture of artisanal dairy products. Moreover, the results, obtained in the present work, give new information about proteolytic activity of dairy *Enterococci* strains, as the information in this domain is limited in the literature. It was shown that *Enterococci* harbour proteolytic systems with strong activities towards hydrolysis of caseins and the proteases involved in this activity are similar to the proteases of *Lactobacilli*.

In contrast bacteriocin activity of LAB from Azerbaijani dairy products was already studied. However, the identification of bacteriocin types and producer strains by modern molecular methods, which could be more informative regarding the diversity of bacteriocins among LAB isolated from different geographical areas, was performed for the first time in the present thesis.

The potential practical significance of obtained results. From dairy products, manufactured in traditional way at different regions of Azerbaijan, 8 proteolytic and 2

bacteriocinogenic LAB strains were obtained and well characterised. These new strains could be used as starter and adjunct cultures in dairy industry. Obtained proteolytic strains decrease the antigenicity of milk proteins and could be used for manufacture of hypoallergenic functional products. Isolated bacteriocinogenic strains could be used as natural bio preservatives increasing the shelf-life of containing them food products due to their strong anti-listerial and other antibacterial activities.

1. LITERATURE REVIEW

1. LITERATURE REVIEW

1.1. LAB in dairy fermentation

1.1.1. Properties and classification of LAB

The LAB might be the most numerous group of bacteria linked to humans. They are naturally associated with mucosal surfaces, particularly the gastrointestinal tract (GIT) and are also indigenous to food-related habitats, including plant (fruits, vegetables and cereal grains), milk and meat environments (Wood and Holzapfel, 1995; Wood and Warner, 2003).

The LAB form metabolically and physiologically related group of Gram-positive, catalase-negative, strictly fermentative bacteria, producing lactic acid as their major final product of sugar fermentation. Typical LAB are non-sporulating and non-respiring, devoid of cytochromes, aero- and acid-tolerant and fastidious. Group of LAB consist of both cocci and bacilli. LAB DNA is composed of less than 50 mol% G+C. Phylogenetically, they are a very diverse group of organisms and belong to the clostridial branch of Gram-positive bacteria.

The LAB include both non-pathogenic species that are used in industrial fermentation of dairy products, meats and vegetables. Some of them may be also pathogenic, e.g., some *Enterococcus* species. Certain LAB strains, mostly the strains from the genera *Lactobacillus*, are increasingly marketed as healthpromoting, i.e., probiotic bacteria (Saxelin et al., 2005), while certain *Lactobacillus* strains are believed to produce bioactive health-beneficial peptides from milk proteins (Korhonen and Pihlanto, 2003).

It is admitted at this stage of knowledge that LAB group embraces about 20 genera, of which *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* are considered as the major LAB genera associated with foods (Axelsson, 2004).

Two major pathways of hexose (e.g., glucose) fermentation can be distinguished among LAB. This includes glycolysis (Embden-Meyer pathway), which results in almost exclusively lactic acid as the end product (homofermentative) and the 6-phosphogluconate/phosphoketolase pathway, which results in significant amounts of other end products, such as ethanol, acetic acid and CO_2 in addition to lactic acid (heterofermentative). A simplified scheme of major pathways of hexose (glucose) fermentation in LAB is presented in Fig.1.1.

LAB can be divided into three groups according to fermentative characteristics: obligatory homofermentative, obligatory heterofermentative and facultative heterofermentative. Obligatory homofermentative *Lactobacilli* (*Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, etc.) degrade hexoses exclusively to lactic acid and do not ferment pentoses or gluconate. Obligatory heterofermentative *Lactobacilli* (*Lb. fermentum*, *Lb. brevis*, *Lb. reuteri*, *Lb.*

sanfranciscensis etc.) degrade hexoses to lactic acid and additional products such as acetic acid, ethanol and CO_2 and pentoses to lactic and acetic acids. Facultative heterofermentative *Lactobacilli* (*Lb. casei, Lb. plantarum, Lb. sakei*; etc.) ferment hexoses to lactic acid and may produce CO_2 from gluconate but not from glucose. They also ferment pentoses to produce lactic and acetic acid. Depending on species, LAB synthesize either L (+) or D (−) isomer of lactic acid or both.

The *Lactobacilli* can decrease pH in foods that contain fermentable carbohydrates till about pH 4 and they can grow up to a pH of about 7.0. In terms of their growth requirements, the LAB require preformed amino acids, vitamins B, purine and pyrimidine bases — hence their use in microbiological assays for these compounds. Although they are mesophilic, some can grow below 5 °C and some as high as 45 °C. With respect to pH range in which they can grow - some can grow at pH as low as 3.2, some at pH as high as 9.6 and most grow in the pH range 4.0–4.5.

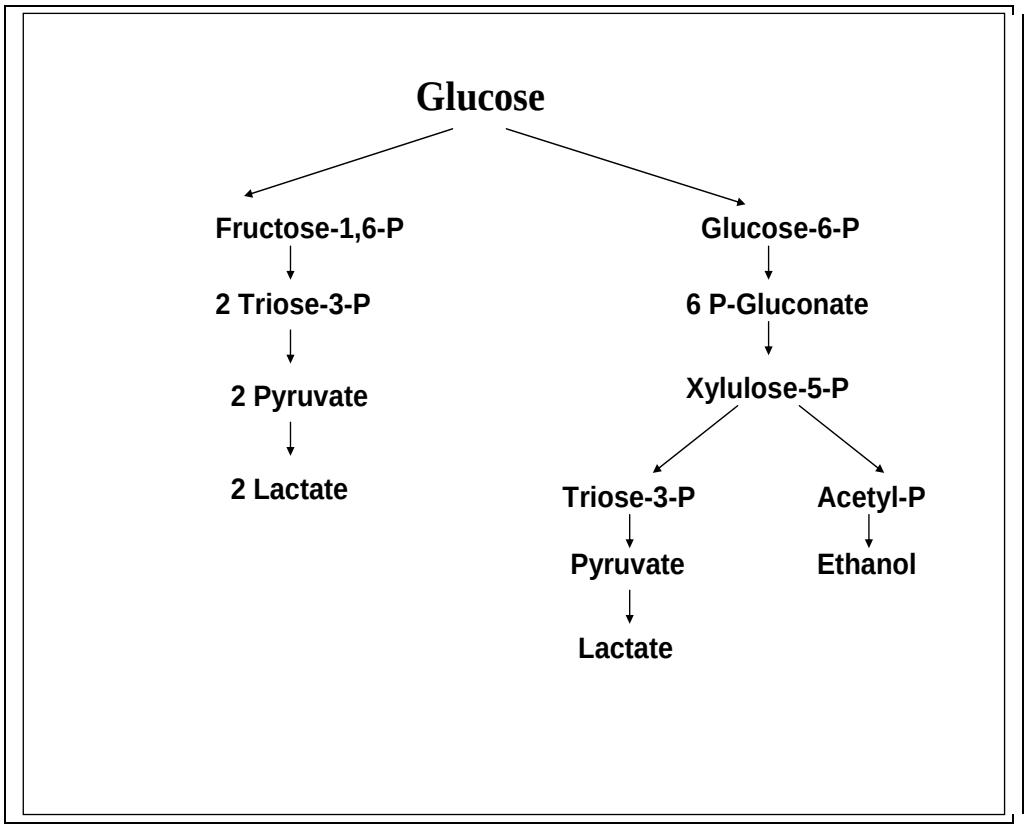


Figure 1.1. A simplified scheme of major pathways of hexose (glucose) fermentation in LAB

1.1.2. LAB in fermented dairy products

LAB are used in the production of wide range of fermented dairy products such as cheeses and yoghurts. They can contribute to microbiological safety and provide technological, nutritional and organoleptic advantages to a fermented product, through production of ethanol, acetic acid, aroma compounds, exopolysaccharides, bacteriocins and several enzymes.

1.1.2.1. Definition of fermentation

Fermentation is a very old traditional food processing technology with earliest records dating back to 6000 BC. The methodologies and knowledge associated with the manufacturing of fermented products were transmitted from generation to generation within local communities. By definition, fermentation is the process in which a substrate is subjected to biochemical modifications resulting from the metabolic activities of microorganisms and of their enzymes (Gotcheva et al., 2000). During fermentation energy (ATP) is derived from the partial oxidation of an organic compounds, such as carbohydrates . The simple organic end products formed from this incomplete biologic oxidation process also serve as final electron and hydrogen acceptors and all ATP is produced by substrate level phosphorylation. Fermentation is important in conditions when there is no oxidative phosphorylation to maintain the production of ATP (adenosine triphosphate) by glycolysis. In the late 1850-s, Pasteur demonstrated that fermentation is a vital process associated with the growth of specific microorganisms, and that each type of fermentation can be defined by the principal organic end product formed (lactic acid, ethanol, acetic acid, or butyric acid). During fermentation, pyruvate is metabolised to various compounds. Homolactic fermentation is the production of lactic acid from pyruvate; heterolactic fermentation is the production of lactic acid as well as other acids and alcohols.

Numerous food products owe their production and characteristics to the fermentative activities of microorganisms. The digestibility, nutritional value, organoleptic qualities and shelf-life of foods are increased by fermentation. Many foods such as ripened cheeses, pickles, sauerkraut and fermented sausages are preserved products in that their shelf life is extended considerably over that of the raw materials from which they are made. Characterization of microorganisms responsible for the fermentation of various fermented products led to the isolation of starter cultures, which could be produced on a large-scale to supply industries involved in the manufacture of these products. In such fermented products LAB are the starter cultures with widespread use and important beneficial effects for consumers.

1.1.2.2. LAB metabolism involved in dairy fermentation

Glycolysis

The primary function of any starter LAB in cheese manufacture is fermenting lactose into lactic acid. Acid production is very important for controlling the cheese quality, the development of sensory characteristics in certain types of cheese. Raw milk has an initial pH of ~6.6 and for cheese manufacture this pH has to be reduced at the end of ripening to values < 6.0 but the exact final pH is depending on the type of cheese. A rapid decrease in pH during the initial steps of cheese preparation is crucial in cheese manufacture since it is essential for coagulation of dairy

proteins. Acidity determines the final pH and mineral content of the curd and also affects the protein structure and amount of residual coagulant in the curd and, thus, texture and flavour of final product. At the same time lactate contributes to cheese flavour and is substrate/nutrient for other micro flora in cheeses, like propionibacteria. Conversion of lactate into another important flavour component – propionic acid, as a result of bacterial metabolism, is also remarkable. Rapid metabolism of milk lactose in the curd can prevent the growth of undesiderable adventitious bacteria, such as, for example, some heterofermentative bacteria, which can produce serious flavour and texture defects (Broadbent and Steele, 2005). The principal products of lactose metabolism depend on starter type used and could be L- or D-lactate or a racemic mixture of both (Vedamuthu, 1994). It was found that the solubility of Ca-D-lactate is less than that of Ca-L-lactate and it can be crystallized in cheese forming white specks, particularly on exposed surfaces (Fox et al., 1990).

Although the main product of LAB metabolism is lactate, they can also produce significant amounts of acetate, formate and ethanol, depending on the growth conditions (Rea and Coagen, 2003). Like lactate, acetate and the others are recognised as 'flavour compounds' since they are important in determination of the taste of fermented dairy products (Giraffa, 2003).

Citrate catabolism

Milk contains about 1.5 g/L of citrate, most of which is lost in the whey during cheese making, since 94% of the citrate is in the soluble phase of the milk. Nevertheless, the low concentration of citrate in cheese curd is of great important since it may be metabolized to a number of volatile flavour compounds by mesophilic starters (citrate-positive lactococci, enterococci and *Leuconostoc* sp.) (McSweeney and Sousa, 2000; Sarantinopoulos et al., 2001). These compounds could be succinate or diacetyl. Succinate is a flavour enhancing compound specific for several cheese varieties, like Swiss-type Cheddar cheeses. The other important citrate-derived flavour component, diacetyl, is formed by oxidative decomposition of α-acetolactate, an intermediate in the pathways of pyruvate metabolism and amino acid biosynthesis. Diacetyl is also known to have inhibitory activity against some food spoilage micro-organisms (Charumati and Lambert, 1996).

Several studies of enterococcal citrate metabolism (Rea and Cogan, 2003; Sarantinopoulos et al., 2001) revealed the high ability of enterococci to metabolise citrate and this was one of the reasons of their food application. Detailed knowledge of citrate metabolism and diacetyl production obtained during recent years has yielded effective strategies for engineering *L. lactis* strains to enhance diacetyl production (Broadbent and Steele, 2005).

Lipolysis

The formation of free fatty acids (FFA) through lipolysis of milk and further conversion of produced free fatty acids to methylketones and thioesters by lipase and esterase activities can directly affect the cheese ripening process, flavour development and texture. Enzymes involved in these reactions may come from rennet, milk itself and starter lactic acid bacteria (SLAB) or NSLAB. According to Giraffa (2003), *esterases* can be arbitrarily classified as enzymes hydrolysing substrates in solutions whereas *lipases* hydrolyse substrates in emulsions.

Most of LAB have weak lipolytic activities and have very low esterolytic activities, but in cheeses with long ripening times they can generate enough free fatty acids and esters to modify flavours (Broadbent and Steele, 2005). Lipases/esterases of *Lactococcus* strains, which are intracellular, have been studied by several authors (Fox et al., 1993; Fox and Wallace, 1997). Obligatory homofermentative *Lactobacilli* used as starter (*Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis*) produce also esterases, some of which have been studied (Khalid and Marth, 1990). *Enterococci* of food origin are the most lipolytic and esterolytic, especially *E. faecalis* species, followed by either *E. durans* or *E. faecium* (Sarantinopoulos et al., 2001; Tsakalidou et al., 1993). However, their lipolytic activity depends on a number of factors and conditions, as for example the substrate where enterococci are present. The esterolytic activity of *Enterococci* is more efficient than the lipolytic activity and has the broader substrate specificity (Giraffa, 2003).

Proteolysis

Proteolysis is considered as one of the most important biochemical processes involved in manufacturing of many fermented dairy products. The proteolysis during ripening of cheese occurs due to coagulant (e.g., chymosin), endogenous milk proteinases (e.g., plasmin) and enzymes produced by SLAB and NSLAB. This proteolysis and its secondary reactions play major role in bacterially ripened cheeses (Giraffa, 2003). Hydrolysis of major milk proteins – caseins is started initially by the coagulant chymosine and endogenous proteinases and is followed by proteolysis by LAB proteinases and peptidases. Together, primary and secondary proteolysis of casein directly or indirectly influences cheese flavour formation. First of all, casein breakdown softens cheese texture, what facilitates the release of flavour compounds when the cheese is consumed. At the same time, some of the low-molecular-weight peptides released from caseins directly affect flavour but some of them could also give bitterness and can lead to off-flavour formation (Sarantinopoulos et al., 2001). The liberation of free amino acids can also directly affect flavours. For instance, glutamate and aspartate residues enhance flavour and are the taste stimulants. More commonly, released amino acids are precursors of many aroma

compounds. The products of amino acid catabolism, which may arise via decarboxylation, deamination, transamination, desulfuration or side chain removal, can convey desirable or undesirable flavour attributes to the treated product. For example, converting methionine into volatile sulfur compounds such as methanethiol, hydrogen sulfide, dimethyl sulfide and dimethyl trisulfide is giving desirable "sulfur" flavours to many cheese types, whereas breaking down leucine is the likely source of a desirable nutty flavour note in Cheddar cheeses.

In addition to the proteolysis by proteases and peptidases, the autolysis of LAB starters is considered to be another important element of cheese manufacture because this releases cytoplasmic peptidases into the curd and influences the flavour formation to proceed (Crow et al., 1995).

1.1.2.3. Role of starter and non-starter LAB in dairy fermentation

The earliest fermented food products relied on natural fermentation by micro flora present in the raw material. During such a natural fermentation the quality of the end product depends on the load and the spectrum of microorganisms populating raw material and on fermentation conditions.

Use of starter cultures has been developed to exploit previously performed successful fermentation in order to obtain good quality repetitive end-products. This technique named backslopping, i.e., inoculation of the raw material with a small quantity of inoculum from a past successful fermentation was used to optimize spontaneous fermentations of different products. During such fermentations the dominant strains can be seen as starter cultures that shortening the fermentation process and reducing the risks of fermentation failure.

LAB have been used for many years in the manufacture of fermented dairy products, such as cheeses, yogurts, etc. At present LAB play an important role in the dairy industry due to their unique metabolism and due to their products. These include acid production as a result of sugar metabolism, proteolytic activities of different proteases and peptidases and also the production of antimicrobial compounds, which can prevent or inhibit the growth of food-borne pathogens.

SLAB are added to milk at the beginning of manufacture process. NSLAB are those bacteria which were not added as part of the starter cultures. They are present in the cheese and other fermented dairy products as a result of contaminations during the manufacture or were already present in milk. In cheese, the quantitative and qualitative microbial content of SLAB and NSLAB is the result of microbial growth capacity in milk and curd linked with the bacterial cell autolysis. NSLAB use as a main energy source nucleic acids derived from the autolysis of SLAB, amino acids, trace of carbohydrates such as N-acetylglucosamine and ribose liberated

from glycoproteins and glycolipids present in milk and citrate, because most of lactose are utilized by SLAB (Williams et al., 2000; Kieronczyk et al. 2001; Diaz-Muniz et al., 2006).

The main role of SLAB in dairy products manufacturing is to acidify raw materials by producing large amounts of lactic acid (homofermentative bacteria) or lactic acid along with acetic acid, carbon dioxide and ethanol (heterofermentative bacteria). They are responsible for lactic acid generation, for milk acidification and curd formation. It is important to highlight the role of adjunct cultures. Adjunct cultures are defined as selected non-starters added to cheeses for other purposes than acid formation, which is exclusively devoted to the added starter. These adjunct cultures can be added to accelerate ripening, to produce desirable flavour or to act as probiotics (Giraffa, 2003). NSLAB play important role in dairy manufacture and their presence can affect the flavour, texture, aroma and appearance of dairy products positively or negatively or have no effect. They are also important part of artisanal fermented dairy products.

Among SLAB there are both mesophilic and thermophilic starters classified by their optimum growth temperatures 30-35 °C and 40-50 °C respectively. The species of mesophilic starters including *Lactococcus lactic* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Leuconostoc lactis* and *Leuconostoc cremoris* are used in the production of different cheeses, fermented milk products and ripened cream butter. Thermophilic lactic starter cultures, usually *Lb. bulgaricus*, *Lb. helveticus* and *S. thermophilus*, are used in the manufacture of yogurts and related fermented milks, and Swiss- and Italian-type cheeses (Robinson, 2002). A number of LAB used as starter cultures in fermented food have probiotic properties and may confer potential health benefits to the consumer. Probiotics can be defined as “live microorganisms of benefit to the host by improving its intestinal microbial balance when administered in adequate amounts” (FAO/WHO, 2001). Starter cultures have to meet certain selection criteria to be considered probiotic, i.e. they have to survive at low pH and in the presence of high bile salt concentrations and need to adhere to the mucosa or epithelial cells (Salminen et al., 1996; Reid and Burton, 2002). A variety of LAB strains are presently marketed as probiotics and the best-studied probiotic strains belong to species *Lb. acidophilus*, *Lb. fermentum*, *Lb. plantarum*, *Lb. brevis*, *Lb. jensenii*, *Lb. casei*, *Lb. delbrueckii*, *Lb. vaginalis* and *Lb. salivarius* (De Vries et al. 2006; Galdeano et al. 2007; Ranadheera et al. 2010). *Enterococci* also belong to the group of LAB but their application as probiotics remains controversial. However, *Enterococci* have a long history of application in food production because of their favourable metabolic activities such as lipolysis, esterolysis, citrate utilization, etc., contributing to the typical taste and flavour of fermented foods (Centeno et al. 1999; Manolopoulou et al. 2003).

Many publications have been published on bacteriocinogenic LAB isolated from fermented food products. Some of these bacteriocins were active against several food spoilage

and pathogenic bacteria, including Gram-negative bacteria (Todorov and Dicks, 2004, 2005; Todorov et al., 2006; Von Mollendorff et al., 2006). Bacteriocinogenic probiotic bacteria could be beneficial when used as starter cultures, since their use may extend the shelf-life of the products and provide the consumer with healthy dietary components at considerably low cost. To be qualified as starter cultures LAB have to be present in sufficient numbers in fermented products. Furthermore, starter cultures should not enhance acidification during storage and should not have adverse effects on the taste and aroma profiles (Heller, 2001).

NSLAB are presented by mixture of different *Lactobacilli* species predominantly constituted of *Lb. casei*, *Lb. plantarum* and *Lb. brevis* and also enterococci species, with *E. faecium* and *E. faecalis* being the most frequent species. NSLAB and particularly facultative heterofermentative *Lactobacilli* have higher proteolytic activities. Citrate metabolism of NSLAB also may have a significant role during cheese ripening.

A number of research works (Centeno et al., 1996, 1999; Oumer et al., 2001) has been carried out to evaluate the technological quality of selected enterococcal species and strains in cheese production. Most of these researches concluded clearly that *Enterococci* a role as adjunct cultures in food fermentation. In all of these studies, *Enterococci* showed the highest efficiency when added as adjuncts and positively affected tastes, aromas, colours and structures of the full-ripened cheeses, as well as the overall sensory profiles.

1.1.3. Safety of LAB

LAB present in fermented milk products enter into human intestines in large numbers where they interact with the local microflora. Because of the risks of transmission of potentially harmful strains by the food chain and the risk of contribution of some of LAB in spread of antimicrobial resistance, it is necessary to evaluate not only their functional and technological characteristics but also the safety of LAB strains before their application as starters or probiotics. Since the pathogenicity is not linked to particular species, the security and functional properties of each particular strain should be thoroughly studied and documented.

Special concern was expressed on the potential risk posed by resistance to antibiotics and existence of transferable genes among LAB. Genes encoding antibiotic resistance may be transferred into a susceptible strain via mobile genetic elements, such as plasmids and transposons resulting in new resistant bacterial strains (Noble et al., 1992; Danielsen and Wind, 2003).

Resistance to antibiotics, such as ciprofloxacin, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin and vancomycin was found in some *Lactobacilli* (Danielsen and Wind, 2003). Many strains of *Lb. plantarum*, *Lb. casei*, *Lb.*

salivarius, Lb. acidophilus carry intrinsic resistance towards vancomycin, what is due to the presence of D-alanine ligase-related enzymes (Elisha and Courvalin, 1995). In a study undertaken by Temmerman et al. (2002) 68.4% of the isolates from a total of 55 European probiotic products showed resistance against multiple antibiotics.

It is important also to evaluate the presence of mobile genetic elements for transfer of antibiotic resistance. Genes encoding antibiotic resistance and situated on plasmids were detected in *Lactobacillus* isolates from fermented food (Gevers et al., 2002; Danielsen, 2002) and in some cases *Lactobacillus* isolates were able to transfer *in vitro* these genes to another Gram-positive bacterium (Gevers et al., 2003). *S. thermophilus* strains isolated from Turkish yoghurts were found to be resistant to gentamicin and penicillin, however, no correlation was observed between the resistance to antibiotics and the occurrence of plasmids (Aslim and Beyatli 2004). However it is difficult to determine the safety of strain, which has resistance to antibiotic but does not carry mobile genetic elements.

In spite of the beneficial activities of *Enterococci*, they may present a danger as pathogens associated with nosocomial infections (Mundy et al., 2000). Their resistance to a wide range of antibiotics together with the presence of virulence factors has raised questions about their safe use as starter cultures (Klein, 2003). Besides that, the differentiation between safe and non-safe enterococcal strains is difficult because of the possibility of horizontal gene transfer to other strains. Huys et al. (2004) made phenotypic and genotypic assessment of resistance against tetracycline in *Enterococcus* isolates from European cheeses. They found that resistance against tetracycline could be linked to the presence of *tet* (M) genes in enterococcal isolates, all of which harboured a member of the Tn916–Tn1545 conjugative transposon family.

Vancomycin-resistant *Enterococci* have emerged in the last decade as a frequent cause of nosocomial infections. The number of vancomycin-resistant enterococci continues to increase and is not restricted only to clinical cases (Messi et al., 2006). The resistance to this antibiotic was found with high frequency among cheese derived isolates of *E. faecalis* and *E. faecium* (Citak et al., 2004). Resistance to another antibiotic - gentamicin in *Enterococci* is no longer limited to clinical isolates but can be found in many food products of animal origin (Franz et al., 2001). The high level of resistance of *Enterococci* and most of the Gram-positive bacteria to antibiotics is due to enzymes such as phosphorylases modifying the antibiotics (Lopes et al., 2003). The bi functional phosphorylase gene aac (60)-Ie-aph (20) Ia is the most detected in *E. faecalis* and *E. faecium* isolates from food.

Virulence factors (also known as virulence traits or virulence determinants) are factors, that are genetically encoded in some strains of some bacteria and that confer pathogenic effect on mammalian tissues and/or resistance against specific and non-specific defence mechanisms. As a result, virulence factors enable the bacteria to act as ‘opportunistic pathogens’. Several virulence

factors, such as aggregation substance, enterococcal surface protein, cytolysin, adhesion, have been studied in *Enterococci* (Ben Omar et al., 2004; Eaton and Gasson, 2001; Mundy et al., 2000; Nakayama et al., 2002). Aggregation substance (AS) is an adhesin that promotes adhesion to a variety of eukaryotic cells, including macrophages and neutrophils and different intestinal cells. Another adhesion protein - enterococcal surface protein (Esp) was suggested to contribute to the binding of enterococcal strains to the host extracellular matrix proteins. It may confer also colonisation and persistence properties to enterococci. Esp could even confer adherence to abiotic surfaces and biofilms, what may be dangerous in patients with medical implants. Adhesin-like *E. faecalis* and *E. faecium* endocarditis antigens *($EfaA_{fs}$, $EfaA_{fm}$)* are considered as potential virulence factors involved in human endocarditis. ß-haemolysin or Cytolysin (Cyl) is a cellular toxin that mostly shows a haemolytic phenotype. Therefore it is thought to be the most frequently involved virulence trait in haemolytic infectious activities, although not in all cases since other non-haemolytic strains of enterococci may also induce this type of infections. It has been shown experimentally that *cyl* could induce tissue damage. Among described virulence factors *Ace* and $EfaA_{fs}$ are the most frequently present in *Enterococci* of food origin (Martín-Platero et al., 2009; Veljovic et al., 2009).

Some LAB have been involved in local systemic infections linked to the consumption of probiotics (Salminen et al., 2002; Salminen et al., 2004). Except for enterococci and streptococci, the clinical significance of LAB is low, *Lb. rhamnosus* being the most frequently isolated LAB from clinical samples (Felten et al., 1999).

1.1.4. Azerbaijani fermented dairy products

Azerbaijan represents a geographical and natural interface between Europe and Asia. It is situated in the Southern Caucasus. Over half of its territory is covered with mountains while the rest of the country is made of plains and lowlands. 27% of the territory is above 1,000 m above sea level, but 18% is below the mean level of the World Oceans. The Major Caucasus, Minor Caucasus and Talish mountain ranges are rich in natural resources, covered with forests and meadows. The Kur-Araz lowland surrounded by these mountain ranges is the main area that provides the population with agricultural products. There are 9 climate zones ranging from arid to humid “subtropical” in Azerbaijan, which are contributing to a development of important biodiversity.

Immemorial pastoral traditions of multiple ethnic groups, which were transiting or dwelling in this region, are still maintained by present populations. Large quantities of different fermented dairy products such as cheeses, yogurts, ayrans etc., have been produced for centuries in Azerbaijan. The traditional ways of preparing various dairy products, are persisting and are

even further developed. In this part we will describe some of traditional fermented dairy products specific for Azerbaijan which were used in our study.

Motal cheese

"Motal" cheese - Azerbaijani original cheese, which is made from goat or sometimes from sheep's milk. This cheese is produced in small quantities by the local households in a very traditional and specific way. It has greasy, crumbly (friable) texture and has no definite form and external cover with artificial turf. It is shapeless lump of 20-25 kg. The fat content of this cheese is 35-40%.

Motal cheese is very different from regular goat cheese. It is produced in a skin of sheep or goat and has a strong taste somewhat reminding French Roquefort most probably because of it's long and difficult preparation process. Goat/sheep's milk is heated and fermented. Milk coagulates and the curd is put in a cloth bag for 3 days so it looses most of its water. After that, the curd is mixed with a 3% proportion of salt and is left in open air for about 18 h. This process is repeated a few times to get the right aroma. Finally, the cheese is placed in a goat or sheep skin and left to ripe for 120 days. The goat or sheep skins pass before use a long preparation process. They are salted and dried during half a year before the cheese is placed in them.

In Azerbaijan there are two kinds of motal cheeses. One is named *"Qarabagh motal"* and the other *"Qazakh motal"*. *"Qarabagh motal"* cheese is specific for Qarabakh region of Azerbaijan and *"Qazakh motal"* cheese is specific for eastern part of Azerbaijan. There are not big differences in their preparation processes.

White cheese

White cheese is the most popular kind of cheese in Azerbaijan. It is made of pasteurized cow, sheep and buffalo's milk by natural fermentative coagulation. This kind of cheeses could have hard or semi-hard consistence and white or slightly yellow colour. Some kind of white cheese, for example made of bufallo's milk, could contain very low amount of salt. In most of the cases during the manufacture of these cheeses they are ripened in the presence of 1.5 – 2% of salt. The ripening periods range from 20 days till 1.5 month. White cheese doesn't have the specific strong taste of motal cheese, but it is very tasty and is commonly consumed by most of the Azerbaijani population.

Kesmik and Shor

"Kesmik" is a soft cheese made from curdled milk. The name *"kesmik"* comes from *"kesmek"* or *"kesilmek"* what means *"to curdle"*. The common technique in making of these cheeses involves combining milk with a small amount of acid and heating it up until the curd

forms. The acidity may be provided by vinegar, lemon juice, a buttermilk, yogurt, sour cream or even citric acid. *"Shor"* is similar to *"kesmik"*, but it is drier, has a crumbly texture and can be sliced. It also could contain some amount of salt.

Yogurt

Yogurt, named *"qatiq"* in Azerbaijan, is another fermented dairy product consumed in large quantities. It is prepared from cow or buffalo's milk. Preparation of yogurts is very simple. Normally it is prepared from pasteurised milk chilled to 37-40 °C by adding some amount of previously produced yogurt. After the inoculation, the milk stored at this temperature in glass containers or saucepan vessels for 4 - 8 h until the curd formed.

1.2. Proteolytic activity of LAB

LAB are fastidious in nature and require numerous essential growth factors. Even milk which is a rich growth medium contains a bit too low concentrations of free amino acids and peptides to efficiently support growth of LAB. However, milk is abundant with proteins, 80% of them are represented by caseins. Caseins contain all amino acids necessary for the growth of LAB in milk to high cell density. LAB have complex proteolytic systems capable of degrading caseins into peptides and free amino acids, realising this way their nutritional requirements when growing in milk. Their proteolytic systems consist of cell-envelope associated proteases (CEPs), transport systems to take up the resulting peptides and intracellular peptidases that release freeamino acids from the peptides (Fig. 1.2) (Kunji et al., 1996).

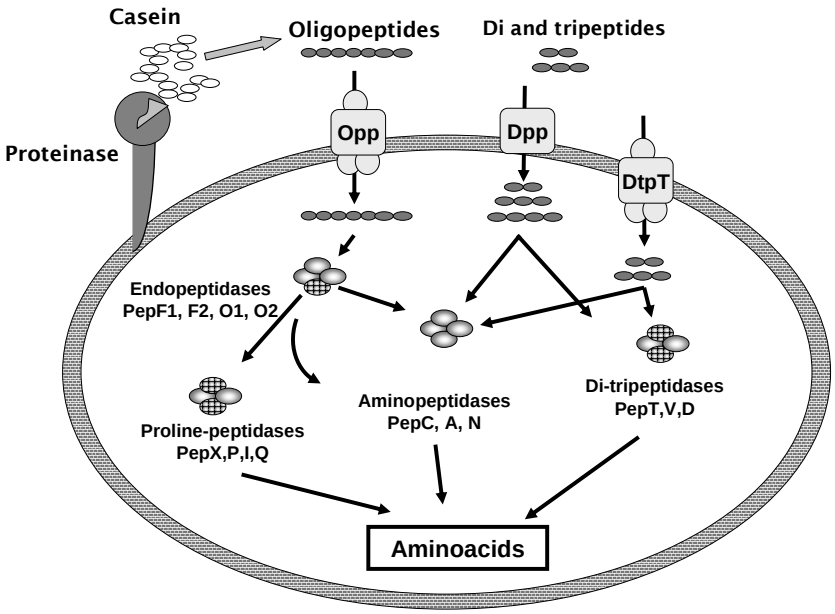


Figure 1.2. Schematic presentation of proteolytic system in LAB

1.2.1. Proteinases of LAB

General properties and function

Proteinases perform the first step of caseins hydrolysis yielding a large number of different oligopeptides. The proteinases of many different LAB have been identified and their biochemical and genetic properties, localization and specificities have been studied (Ong et al., 2006; Tzvetkova et al., 2007 ; Hébert et al., 2008 ; Pescuma et al., 2008 ; Piraino et al., 2008 ; Strahinic et al., 2010 ; Sadat-Mekmene et al., 2011; Hadji-Sfaxi et al., article in press). It was determined, that LAB proteinases have extracellular localisation and they are attached to cell-wall of producer. This is why they are called cell envelope proteinases (CEP). These data are supported by various results, such as liberation of proteinases from the cell-wall by lysis with lysozyme and the treatment with Ca^{2+}-free buffers, electron microscopy of immuno-gold labelled PrtP and the presence of typical N terminal signal sequences, which target the protease to the outside of the cell (Kunji, 1996; Kok and De Vos, 1994).

Most of LAB possess only one CEP, but the presence of two and more CEPs was reported in strains of *Lb. helveticus* and *Lb. bulgaricus* (Stefanitsi et al. 1995; Pederson et al. 1999; Sadat-Mekmene et al., 2011).

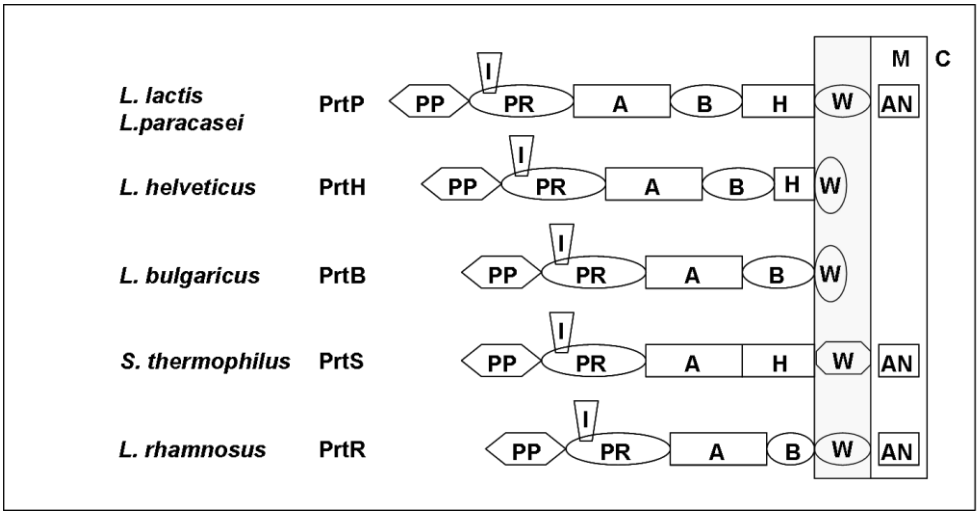
Five different types of CEPs were cloned and characterized from LAB, including PrtP from *L. lactis* and *Lb. paracasei*, PrtH from *Lb. helveticus*, PrtR from *Lb. rhamnosus*, PrtS from *S. thermophilus* and PrtB from *Lb. bulgaricus* (Kok et al., 1988; Holck and Naes, 1992; Gilbert et al., 1996; Pederson et al., 1999; Siezen, 1999; Pastar et al., 2003).

Biochemical properties and genetic organisation

The CEPs of dairy LAB as most of subtilisin-like serine proteases are synthesized as preproenzymes of approximately 2000 residues. They translocate through the cell membrane via signal peptide-dependent pathway and their activation occurs after cleavage of their propeptides. These CEPs contain several functional domains (Siezen, 1999) (Fig. 1.3).

The N terminus of the CEPs starts with the preprodomain (PP) corresponding to a signal sequence (~40 residues) required for secretion followed by a prosequence (~150 residues) that is removed by autocatalytic processing. The catalytic serine protease domain (PR) contains about ~500 residues and show higher degree of amino acids sequence similarity compared to other domains in different types of LAB proteases. The highest homology of this domain was found to be in PrtH and PrtP (65%) (Siezen, 1999). Insert domain (I) (~150 residues) possibly modulates the substrate specificity of CEPs. The A domain (~400 residues) is located directly after PR domain. Conserved sequences of this domain possibly represent the β-sheeted hydrophobic core of globular domain. The function of this A-domain is unknown, but there are speculations that it

is essential for regulation of proteolytic activity and/or specificity of the PR-domain (Siezen, 1999). The B domain (~500 residues) is located directly after the putative A domain. It is probably involved in stabilisation of the CEP activity/specificity since autoproteolysis most likely occurs within this domain. However, it seems that not in the case of all LAB it is required for proteolytic activity since it is absent in the streptococcal CEPs. Like the A-domain, it has a high predicted content of β-sheet secondary structure. It is possible that the conserved fragments form the hydrophobic core of the domain.



According to Siezen, 1999

Figure 1.3. Schematic representation of domain structure of CEPs from different LAB

The helix domain (H) (~200 residues) of α-helix secondary structure plays the role of a long single-helix spacer in positioning of the A and B domains outside the bacterial cell. This domain is present only in PrtP (210 aa), PrtS (367 aa) and PrtH (72 aa). Hydrophilic W domain (~100 residues) contains the typical amino acid composition of the cell wall domain of Gram-positive bacteria (rich in Pro-Gly and Ser-Thr) and plays the role of a cell wall spacer. In PrtH and PrtB this domain is responsible for binding to the cell wall (Pederson et al., 1999; Siezen, 1999). In *Lb. helveticus* W domain of PrtH is homologous to the family of S-layer proteins (Pederson et al., 1999; Leenhouts et al., 1999). Binding of PrtP and PrtS to the cell wall occurs by a different mechanism. Their C-terminal part contains the cell wall anchor domain (AN) situated directly after the W domain and carrying a sorting signal typical for many surface proteins of Gram-positive bacteria (Navarre and Schneewind, 1994).

Prt genes in *Lactobacilli* are genome-encoded, whereas in *Lactococci* can either be plasmid- or genome encoded. The gene sequences analysis showed that for activation of some proteases maturation protein is required. In *L. lactis* and *Lb. paracasei*, the *Prt*P gene is preceded by a divergently transcribed gene (*Prt*M) encoding a membrane-bound lipoprotein that was

shown to be essential for autocatalytic maturation of PrtP (Holck and Naes, 1992). In *Lb. helveticus, Lb. bulgaricus* and *S. thermophilus*, no prtM gene was identified in the regions flanking the genes encoding CEPs and the maturation process of their proteases does not require a PrtM-like chaperone (Siezen, 1999; Gilbert et al., 1996; Germond et al., 2003).

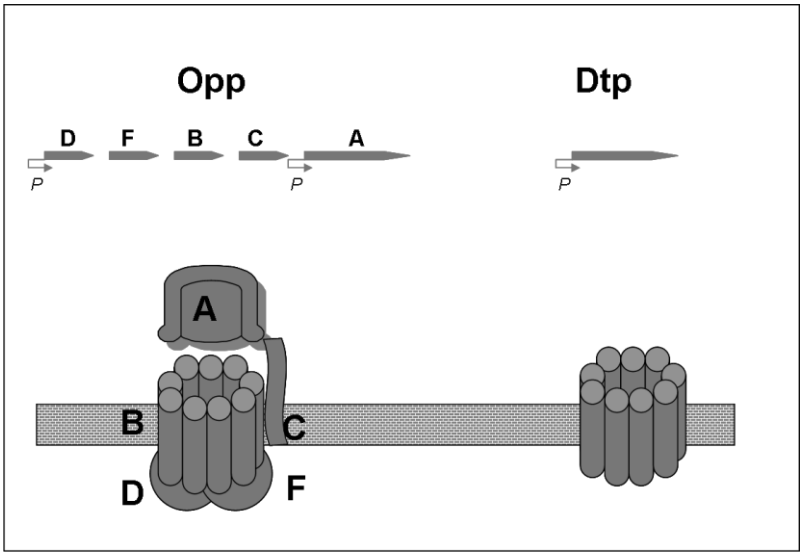
Specificity classes

Milk caseins are divided into the α_{s1}-, α_{s2}-, β- and κ-caseins. On the basis of degradation patterns of these types of caseins, two proteinase specificity-classes have been described in *Lactococci*, which are named as PI and PIII (Kunji et al. 1996). The PI-type proteases degrade primarily β-casein, which is cleaved into more than 100 different oligopeptides ranging from 4 to 30 amino acid residues and also κ-casein cleaved to a lesser extent (Juillard et al., 1995). PIII-type proteases are able to cleave α_{S1}-, β- and κ-caseins (Pritchard and Coolbear 1993). Further classification of proteinases was done according to their specificity toward the α_{S1}-casein fragment 1 to 23 (f1–23) and seven groups were distinguished (a, b, c, d, e, f and g) (Kunji et al., 1996).

For *Lactobacilli*, CEPs PI-, PIII-, the intermediate PI/PIII-type and some of novel type substrate specificities were reported, whereas a CEP (PrtS) exhibiting the intermediate PI/PIII-type specificity was purified from *S. thermophilus* (Fernandez-Espla et al., 2000).

1.2.2. Peptide transport system

The peptides formed after action of proteinases on casein are transported into the cell *via* peptide transport system (Fig.1.2). The peptide transporters identified in LAB include systems termed Opp, DtpT and Dpp. The oligopeptide transport system - Opp transporter of *Lactococcus lactis* was studied in detail. It is specific for oligopeptides and belongs to the superfamily of highly conserved ATP-binding cassette transporters (Higgins, 1992; Kunji et al., 1995). The genes encoding the different proteins composing this oligopeptide transport system - Opp, were cloned and characterized (Tynkkynen et al., 1993). Sequence analysis indicates that the Opp transporter consists of five subunits: two ATP-binding proteins OppD and OppF, two integral membrane proteins OppB and OppC and a substrate binding protein OppA (Fig.1.4). The *oppDFBCA* genes are probably organized in one operon, together with the endopeptidase *PepO* gene. The Opp system transports peptides up to at least 18 residues. Described Opp systems for other LAB demonstrate to be similar to that described for *Lactococcus* (Garault et al., 2002; Peltoniemi et al., 2002).



According to Kunji et al., 1996

Figure 1.4. Model of gene organisation of the Opp and Dtp system of *Lactococcus lactis*

Another peptide transporter is a proton motive force (PMF)-driven dipeptide/tripeptide DtpT system. It has a preference for hydrophilic and charged di- and tripeptides and was identified in both *Lactococci* and *Lactobacilli* (Hagting et al., 1994; Nakajima et al., 1997).

Dpp system has specificity to di-, tri- and tetrapeptides containing relatively hydrophobic branched-chain amino acids (BCAAs) and displays the highest affinity for tripeptides (Sanz et al., 2003).

1.2.3. Peptidases

After the casein-derived peptides are taken up by the LAB cells, they are degraded by a concerted action of peptidases (Kunji et al., 1996) (Fig.1.2).The first enzymes acting on oligopeptides are intracellular endopeptidases (PepO and PepF), general aminopeptidases (PepN and PepC) and the X-prolyl dipeptidyl aminopeptidase (PepX). Endopeptidases of LAB (PepO, PepF) were shown to be metallopeptidases with the exception of the *Lb. helveticus* PepE, which was shown to exhibit a thiol-dependent activity (Fenster et al., 1997). They can hydrolyze internal peptide bonds of casein derived peptides but are unable to cleave intact caseins. The α_{S1}-casein f1–23 and/or β-casein f193–209 are the most preferred substrates for the endopeptidases of starter LAB origin and for some nonstarter strains (Christensson et al., 2002; Chen et al., 2003).

Other peptidases able of cleaving oligopeptides are the broad-specificity metallopeptidase PepN and cysteine peptidase PepC. These aminopeptidases are highly conserved among dairy LAB. These enzymes can remove the N-terminal amino acids from a peptide and this specificity depends on the peptide length and on the nature of the N-terminal amino acid residue (Kunji et

al., 1996). PepN does not cleave dipeptides containing Pro in either two positions, while tripeptides, which contain Pro in either first or second position are hydrolysed by this peptidase. In *L. lactis* PepN shows a preference for dipeptides containing Arg as the N terminal residue, but it is also capable of cleaving other residues such as Lys and Leu. Inability to cleave dipeptides containing Pro was also observed for another aminopeptidase - PepC, but Pro-containing tripeptides were observed to be cleaved to some extent. Some preference for dipeptides with Ala, Leu or Lys in N-terminal position has been observed (Kunji et al., 1996).

Di/tripeptides generated by endopeptidases, general aminopeptidases and PepX are thereafter subjected to additional cleavage by the tripeptidase, PepT and dipeptidases, PepV and PepD. These enzymes prefer peptides containing hydrophobic amino acids including leucine, methionine, phenylalanine, or glycine. Other peptidases with more specific substrate specificities include: PepA, which liberates N-terminal acidic residues from peptides that are three- to nine-residue-long; PepP, which prefers tripeptides carrying proline in the middle position; PepR and PepI, which act on dipeptides containing proline in the penultimate position; PepQ, which cleaves dipeptides carrying proline in the second position and PepS, which shows preference for peptides containing two to five residues with Arg or aromatic amino acid residues in the N terminal position (Kunji et al., 1996; Christensen et al., 1999).

1.2.4. Regulation of proteolytic system

Regulation of the expression of proteolytic system components is a response of LAB to nitrogen balance in the cell. It is strongly depending on the peptide-protein content of the growth medium. Its regulation mechanisms were mostly studied in *L. lactis* with lesser extent in *Lactobacilli*. In *L. lactis* the synthesis of proteinase is repressed in the presence of casein and casitone (Laan et al., 1993). It was shown, that he expression of six transcriptional units, including prtP, prtM, opp-pepO1, pepD, pepN, pepC and pepX, was repressed upon the addition of casein hydrolysate to the growth medium and the expression was relieved only when the cells encountered nitrogen-limiting conditions (Guédon et al., 2001a). The expression of the peptide transport system is highly dependent on peptide composition of the media. Di/tripeptides with hydrophobic residues were suggested to act as effector molecules in the transcriptional regulation of the Opp system and thereby influence the whole proteolytic system (Kunji et al., 1996).

The regulation of components of proteolytic system was shown to be controlled by specific transcriptional regulator CodY. The regulator senses the internal pool of branched-chain amino acids (BCAAs) such as isoleucine, leucine and valine and use them as cofactors to represses the expression of genes comprising the proteolytic system in *L. lactis* (Guedon et al.,

2001b; Petranovic et al., 2004). *In vitro* assays revealed that CodY binds to the upstream sequences preceding the Opp-operon and that the BCAAs stimulate this binding. In summary, in nitrogen-rich medium the proteolytic system is repressed by CodY and the expression is relieved when cells encounter limiting amounts of BCAAs (Guedon et al., 2001b).

Regarding *Lactobacilli*, the peptide concentration in the growth medium was shown to control the PrtH and PrtR biosynthesis in *Lb. helveticus* and in *Lb. rhamnosus*, respectively (Hebert et al., 2000; Pastar et al., 2003). The PrtH activity of *Lb. helveticus* CRL 1062 was shown to be repressed in a peptide-rich medium, mostly in the presence of dipeptides leucine and proline (Hebert et al., 2000).

Environmental factors, such as the presence or absence of oxygen during the growth, are also known to affect the expression of certain peptidase genes. The carbohydrate composition of the media can also affect the expression of some peptidases. The biosynthesis of regulatory protein PepR1 depends on the glucose concentration in the culture medium. This regulatory protein directly controls the expression of some genes of proteolytic system in *Lb. delbrueckii* subsp. *lactis* and *Lb. bulgaricus*, particularly pepQ expression (Morel et al., 1999).

1.3. LAB and dairy allergens

1.3.1. Milk allergens

Food allergy is an abnormal immunological response due to a sensitisation to a food or food components. Clinical manifestations of food allergy consist of disorders in the digestive tract or in digestive organs as a result of an immunologic reaction. Among food allergens cow's milk is a member of the so-called "Big-8" food allergens, ranking alongside egg, soy, wheat, peanuts, tree nuts, fish and shellfish in terms of prevalence.

Milk allergy is an adverse reaction to proteins that are present in milk. Milk contains approximately 20 different proteins that can potentially be responsible of allergic reactions. Some of these proteins are considered as major allergens, some are known as minor allergens, while a small number has rarely been described as being responsible for clinical reactions. The simple question, which can be raised is - why the milk proteins are recognised by human immune system as harmful and cause allergic reactions at all?

As we know, for a variety of reasons, some babies cannot be breast-fed and require infant milk formulas, which can contain intact or hydrolyzed milk proteins, or simply mammalian milk. In Table 1.1 we can see the difference between protein composition of human and cow's milk. Some of milk proteins, considered as the major allergens, like α_{S1}-casein and BLG are absent in human milk, so it is not surprising that immune system of newborn children recognise them as foreign and potentially harmful. From another point, breast milk from mothers who have

consumed products containing cow milk might be another cause for the development of CMA due to the absorption of cow's milk proteins, their passage through the gut mucosa and their release in human milk.

Milk of all ruminant species (e.g. cow, goat and sheep) contains proteins with high degree of homology sharing similar structural, functional and biological properties. Cow's milk contains about 3–3.5% protein (w/w), which can be divided into two main classes: caseins and whey proteins. During last years a lot of studies on antigenicity/immune reactivity of milk proteins were performed and epitope mapping of a number of milk proteins was done. The mapping has revealed multiple allergenic epitopes, both for B cells that produce antibodies and for T cells that direct both antibody and cell mediated immune responses (Jarvinen et al., 2001; Chatchatee et al., 2001 a and b; Cerecedo et al., 2008; Lin et al., 2009).

Table 1.1. Main characteristics of major milk protein-allergens

Protein	mg/mL		Allergen name	Chemical characteristics			Function
	Human milk	Cow milk		MW (kDa)	aa	pI	
Caseins							
α_{S1}-casein	0	11.6	*Bos d 8*	23.6	199	4.9-5.0	Calcium binding
α_{S2}-casein	0	3.0		25.2	207	5.2-5.4	Calcium binding
β-casein	2.2	9.6		24.0	209	5.1-5.4	Calcium binding
γ-caseins γ1- γ2- γ3-	0	1.6		 20.6 11.8 11.6	 180 104 102	 5.5 6.4 5.8	
κ-casein	0.4	3.6		19.0	169	5.4-5.6	Stabilisation and coagulation of milk
Whey proteins							
ALA	2.2	1.2	*Bos d 4*	14.2	123	4.8	Lactose synthesis
BLG	0	3.0	*Bos d 5*	18.3	162	5.3	Lipid binding/albeit unknown
Ig	0.8	0.6	*Bos d 7*	160.0	-	-	Defence
BSA	0.4	0.4	*Bos d 6*	67.0	583	4.9-5.1	Transport of ligands and protection against free radicals
LF	1.4	0.3		800.0	703	8.7	Antimicrobial activity

According to Ross et al., 2005 and Monaci et al., 2006

Caseins

Caseins are major milk allergens and account for 80% of the total protein in cow's milk. Casein fraction is composed of α_{S1}-, α_{S2}-, β-, κ-, γ-caseins constituting 32%, 10%, 28% and 10% of the total protein content, respectively. These proteins have little primary structure homology

between each other but have high primary structure homologies with caseins from other mammalian species.

The α_{S1}-casein is a single-chain phosphoprotein composed from 199 amino acids, characterized by a high content of prolyl residues distributed throughout the molecule and a lack of disulfide bonds, resulting in a reduced or "rheomorphic" secondary and tertiary structure (Holt and Sawyer, 1993). The nearly 70% of its molecule has unordered structure, with only a small amount of secondary structure, such as α-helix or β-sheets. Thus, there is a high possibility that the major allergenic epitopes of this protein most of the time are linear rather than conformational. Spuergin et al. (1996) identified three IgE binding regions (AA 19-30, AA 93-98 and AA 141-150) on α_{S1}-casein by testing the sera from 15 patients with acute clinical reactions to cow's milk. Later more IgE-binding regions (AA 17-36, AA 39-48, AA 69-78, AA 83-102, AA 109-120, AA 123-132, AA 139-154, AA 159-174, AA 173-194, AA 28 – 50 and AA 49-62) of this protein were identified by other groups (Chatchatee et al., 2001a; Cerecedo et al., 2008; Lin et al., 2009).

The α_{S2}-casein is the most hydrophilic of all caseins due to the presence of anionic groups. The post-translational modification occurring in this protein is the formation of disulfide bonds, which do not participate in the interactions with other caseins (Farrell et al., 2004). α_{S2}-Casein also has some IgE specific regions (AA 1-20, AA 13-32, AA 67-86 and AA181-207), which have been identified and studied (Cerecedo et al., 2008; Lin et al., 2009).

The β-casein is the second most abundant protein in cow's milk, after a_{S1}-casein, constituting 28% of the total milk proteins. Although it has little homology to a_{S1}-casein, these two proteins bear some similarities in structure. As a_{S1}-casein it is a phosphoprotein with 209 AA and has high proline content and no disulphide bonds. It possesses no defined secondary structure and presents diminished tertiary interactions.

κ-Casein, is lower molecular weight casein composed of 169 amino acids. It differs significantly from other caseins by the presence of bound carbohydrate and disulphide bonds and also has been shown to fold in unspecified tertiary structures.

IgE binding epitopes of β- (AA 25-50, AA 52-74, AA 154-173 and AA 16-39) and κ- (AA 34-53) caseins were identified by some authors and are most probably involved in allergenicity of these caseins (Cerecedo et al., 2008; Lin et al., 2009).

Whey proteins

About 20% of the total proteins of cow's milk are constituted from whey (lactoserum) proteins. One of the main proteins of whey fraction is β-lactoglobulin (BLG). It accounts for 10 and 50% of the protein fraction of milk and of lactoserum, respectively and it is absent in milk of

primates and rodents. This protein belongs to the lipocalin superfamily and is capable of binding a wide range of molecules including retinol, β-carotene, saturated and unsaturated fatty acids and aliphatic hydrocarbons (Breiteneder and Mills, 2005). The quaternary structure of BLG fluctuates between monomers, dimers or oligomers depending on the pH, temperature and ionic strength. At physiological pH it has a dimeric structure, each monomer being an 18-kDa globular protein of 162 amino acids containing five cysteyl residues, four of which are forming intra-chain disulphide bridges (Sawyer and Kontopidis, 2000). Due to its rigid spatial conformation, BLG is highly resistant to gastric digestion, what partially causes its allergenicity.

There are ten known genetic variants of bovine BLG. The most abundant variants are termed BLG A and BLG B differing at two locations: Gly64 and Ala118 in BLG variant B are replaced by Asp and Val respectively in BLG variant A (Farell et al., 2004).

BLG is considered to be the major cow's milk allergen, that is, the protein most frequently and intensively recognized by human IgE (Wal, 2002). Its antigenicity has been extensively studied and its epitopes are now well identified. Some of them are short linear sequences, whereas others are large fragments constituting conformational epitopes. Selo et al. (1999) identified several epitopes on the molecule of BLG using trypsin digestion. The main epitopes recognised by 89-97% of studied human sera were fragments AA102-124, AA41-60 and AA149-162. Other fragments recognised were AA1-8, AA25-40, AA92-100, AA9-14, AA84-91 and AA92-100 (40-72% of sera). Similar results were obtained by Jarvinen (2001), who identified seven different IgE epitopes by using synthetic peptides covering the amino acid sequence of BLG. Another epitopes were identified by Cerecedo et al. (2008) (AA 58-77) and by Lin et al. (2009) (AA 106-119) using a peptide microarray–based immunoassay.

Another important whey protein is α-lactalbumin (ALA), which represents about 25% of whey proteins. It is a monomeric globular calcium binding protein composed of 123 amino acid residues and has four disulfide bonds. There are two variants of ALA. The aminoacid composition of ALA from cow's milk shows a 72% sequence identity with human ALA what makes it an ideal protein for the nutrition of human infants. Epitope studies of ALA showed the presence of large peptides and hence the importance of conformational epitopes in IgE-binding to native protein (Maynard et al., 1997). Four linear IgE-binding epitopes were identified on ALA: AA 1-16, AA 13-26, AA 47-58, AA 93-102 (Jarvinen et al., 2001).

Bovine serum albumin (BSA) is another whey protein constituting 5% of the total whey proteins. It is organised in three homologous domains and consists of nine loops connected by 17 disulfide bonds protected in the core of the protein. This is why it has quite stable tertiary structure even under denaturing conditions. Several studies have been performed in order to evaluate the antigenic potential of this protein and to identify major BSA epitopes, but the obtained results are very disparate with each other (Monaci et al., 2006).

Lactoferrin (LF) molecule is formed by a single polypeptide chain folded into two globular lobes, each of them having high-affinity iron binding sites, connected by a 3-turn helix. It contains five potential glycosylation sites and the molecular weight of this protein varies depending on the extent of its glycosylation. The highest concentration of this protein is found in human milk and it plays important role in the defence of the organism against infections and inflammations due to its ability to sequester iron from the environment and thereby removing this essential nutrient for bacterial growth (Ward et al., 2002) but also due to the capacity of producing free oxygen radicals by Fenton reaction. The potential allergenicity of LF is still discussed but some authors reported the presence of specific IgE to this protein in blood sera of some milk allergic patients (Taylor et al., 2004).

1.3.2. Mechanism of allergy to milk

Cow's milk allergy (CMA) is a complex disorder, which involves a spectrum of immunological mechanisms. The incidence of CMA varies with age. It is a dominant food allergy in early childhood (2 – 6% frequency) when the immune system is relatively immature and susceptible to sensitization by environmental antigens. It decreases with age (incidence of 0.1–0.5% in adulthood) due to naturally acquiring tolerance to cow's milk (Woods et al., 2002; Wood, 2003; Garcia-Ara et al., 2004). However, there is still a persistence of propensity for the development of atopic symptoms, such as asthma, hay fever or dermatitis in infants who recovered from CMA (Høst et al., 2002). Therefore, the attempts to prevent CMA are of high priority either by avoidance of the allergenic proteins (all milk products) or by induction of oral tolerance. CMA is classified both as IgE-mediated allergy and non-IgE-mediated allergy (Ross et al., 2005). IgE-mediated allergy is the best understood allergy mechanism and, in comparison with non-IgE-mediated reactions, it is relatively easily diagnosed. IgE-mediated allergy is often referred to as "immediate hypersensitivity" and development of this type of allergy occurs in two stages. During the first "sensitization" step the immune system is programmed to produce IgE antibodies against milk proteins. These serum allergen-specific IgE antibodies bind to high-affinity IgE receptors (FceRI) on the surface of mast cells and basophiles, forming there an allergen-specific trigger. Subsequent exposure to milk proteins leads to "activation" of mast cells and basophiles when these cell-associated milk specific IgE bind the allergenic epitopes on the milk proteins and triggers the rapid release of powerful inflammatory mediators leading to allergy symptoms. In children IgE-mediated mechanism of CMA occurs in half of the cases but is rare in adults (Heine et al., 2002).

Non-IgE-Mediated CMA known also as "delayed hypersensitivity occurs in significant proportion of infants and the majority of adults. In this case milk protein-specific IgE are absent

in the circulation and most of the diagnostic test based on IgE detection give negative results. The precise immunopathological mechanisms of non-IgE mediated CMA remain unclear. A number of mechanisms have been proposed, including type-1 T helper cell (Th1) mediated reactions, the formation of immune complexes leading to the activation of Complement or T-cell/mast cell/neuron interactions inducing functional changes in smooth muscle action and intestinal motility (Augustin et al., 2001; Osterlund et al., 2003).

1.3.3. Potential application of LAB to decrease the allergenicity of milk proteins

During the manufacture of food, their allergenicity may be altered by various processing parameters. The immune reactivity of food proteins may be unchanged, decreased or increased by food processing. The inactivation or destruction of epitope structures or the formation of new epitopes or better access of cryptic epitopes by denaturation of the native allergen constitute the molecular basis of such changes (Besler et al., 2001). Also, the antigenicity is decreasing with declining size of antigenic peptides.

Different attempts have been made in the past to reduce the antigenicity of milk proteins and various technological processes have been applied. Attempts to modify the protein components of cow milk in an effort to reduce their allergenic potential have included the application of heat treatment, enzymatic treatment with a variety of enzymes and combinations of these processes, such as heating and glycation (Taheri-Kafrani et al., 2009; El Mecherfi et al., 2011). Heating has only a small effect on the antigenic/allergenic properties of cow's milk proteins, even though some authors have shown a reduction in whey protein antigenicity. Furthermore, heating processes can only modify conformational epitopes, which might lose their binding capacity to specific IgEs, but linear epitopes remain unaffected by structural changes and maintain their antigenic potential after heating. Milk proteins contain both types of epitopes and, even though a slight reduction of antigenicity can be observed with whey proteins, only insignificant alterations in binding properties are reported with caseins after heating (Restani et al., 2009).

However, it should be taken into account that in spite of the alteration of antigenicity of some milk proteins by severe heat and enzymatic treatments, significant loss of the nutritional quality, as well as the development of bitterness and off-flavors of the product, has to be expected. Meanwhile, the proteolytic digestion might itself generate new antigenic substances (El-Agamy, 2007).

Several cow's milk protein hydrolysates produced by the action of various food-grade enzymes on the isolated caseins or whey proteins are present on the world market. These formulas are classified as partial or extensive hydrolysates according to the molecular weight of the peptides derived. Only extensively hydrolyzed products can be considered sufficiently safe

for allergic patients and their use is often recommended as a first alternative in children with a confirmed diagnosis of CMA.

Another approach to decrease antigenic/allergenic potential of milk proteins is bacterial fermentation. It has been demonstrated that fermentation can induce the degradation of some food allergens. Microbial fermentation can be sometime associated with proteolytic reactions, which can induce degrade milk protein allergens. Nakamura et al. (1993), applying proteases of plant or bacterial origin, demonstrated that extensive protein hydrolysis produced a significant reduction in the antigenicity of whey proteins and suggested that the antigenicity of proteins is decreased by the destruction of epitopes present on the native protein.

It is well known that LAB harbour a complex system of proteinases and peptidases, which enable them to produce essential amino acids during their growth in milk (Kunji et al., 1996). During fermentation processes, milk proteins are acidified by the production of lactic acid and are hydrolyzed by LAB proteases and peptidases. This proteolysis is followed by a reduction of the number of epitopes and consequently by the decrease in antigenicity of hydrolyzed proteins (Cross et al., 2001; Bertrand-Harb et al., 2003; Nentwich et al., 2004). For these reasons, such starters as *Lactobacillus* strains able to reduce the allergenicity of fermented milk products are of great interest for the dairy industry.

It was already reported that some LAB can reduce the antigenic response of milk proteins (Wroblewska et al., 1995). Besides, the reduction of milk protein antigenicity depends on the types of LAB and on the conditions of fermentation (Kleber et al., 2006, Guanhao Bu et al., 2010).

Lactic acid fermentation was applied to reduce the BLG antigenicity of sweet whey and skim milk, and the products have been reported to have beneficial effects on the host including the activation of the immune system (Shida et al., 1998). Kleber et al. (2006) studied the ability of some LAB strains to reduce the BLG antigenicity in skim milk and sweet whey by means of an indirect competitive ELISA and using polyclonal antibodies. They found reduction of more than 70% in sweet whey and more than 90% in skim milk compared to the initial values. In addition, some co-cultures of LAB reduced the antigenicity showing strong synergy. The enzymes produced by different LAB are more or less specific and by using co-cultures it is more possible to obtain higher degree of native allergen hydrolysis. Fermentation of sterilized cow's milk using a mixture of meso- and thermophilic LAB resulted in a 99% decrease of antigenicity of ALA and BLG (ELISA, rabbit antibodies) (Jedrychowski et al., 1999). Different studies revealed that the combination of *Lactobacilli* strains with *S. thermophilus* during fermentation of milk and whey resulted in higher degree of immuno reactivity loss of native allergens. In a recent study Bu et al. (2010) found that combined strains of *Lb. helveticus* and *S. thermophilus* were the most effective in reducing the antigenicity of ALA and BLG in skim milk compared with *Lb.*

helveticus alone and *S. thermophilus*. More precisely ALA antigenicity was reduced by 87% after fermentation with combined strains of *Lb. helveticus* and *S. thermophilus.* However, ALA antigenicity was inhibited by 71% and 49% with *Lb. helveticus* alone and *S. thermophilus* alone, respectively.

During fermentation, release of proteases and peptidases increases by the time, resulting thus in more effective hydrolysis of milk proteins with higher epitopes cleavage. However, it is also possible that further cleavage of the peptides into smaller peptides and amino acids by peptidases may unravel some hidden epitopes or linear epitopes. Bu et al. (2010) found that at the beginning of fermentation antigenicity of BLG was gradually decreased, but at longer fermentation time it was even slightly increased, what indicated that there is no linear correlation between the hydrolysis degree of milk protein and the antigenicity of whey proteins during the fermentation (Bu et al., 2010). Ehn et al. (2005) used different strains of *Lb. helveticus* to hydrolyse whey proteins of milk and observed more than 80% hydrolysis of BLG, but this did not affect the epitope recognition by IgE as judged by the unchanged inhibition pattern (ELISA, human serum) after this treatment. This suggests that the extracellular proteolytic activity in the fermentation process did not degrade the IgE epitopes extensively. It is also possible that the degradation was only partial, leaving peptides long enough to bind the antibodies or as we indicated above it is possible that there was a better access to some previously buried epitopes. It is also possible that the preheating temperature might have a significant impact on the changes in the antigenicity of whey proteins. In this study authors used preheating conditions for milk (72 °C for 30 s) to simulate the industrial process of pasteurization and for some milk samples (90 °C for 4 min), similar to the industrial process followed in making fermented milk products. Kleber et al. (2006) studied the effect of different LAB on the antigenicity of BLG in skim milk by a treatment at 90 °C for 40 min and found that the antigenicity of BLG decreased by 84–98% compared with unfermented skim milk. Jedrychowski and Wroblewska (1999) claimed that after the fermentation with LAB the antigenicity of sterilised milk (110 °C, 10 min) was reduced by over 99%.

In conclusion, we can say, that even if the *in vitro* analysis showed many promising results in decrease of antigenicity of milk proteins, it does not mean the decrease of allergenicity to the milk. Jedrychowski and Wroblewska (1999) studied the effect of lactic acid fermentation on antigenicity and found a reduced binding ability to rabbit polyclonal antibodies. When milk allergic patients were skin tested with whey samples from fermented whole milk, the allergic reaction was only slightly attenuated. Further *in vitro* and *in vivo* studies are necessary to understand better mechanism of allergenicity and ways to decrease it. Moreover, it is also interesting to study the combination of various methods, such as fermentation and hydrolysis by

proteases from animals or plants, heat treatment, microwave irradiation and high pressure. These will give some help in the development of hypoallergenic milk products.

1.4. Antimicrobial activity of LAB

1.4.1. Antimicrobial compounds produced by LAB

The antimicrobial effect of LAB resulting from their fermentative activities may be due to the production of a number of antimicrobial substances such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins. These antimicrobial compounds give to these organisms a competitive advantage over other microorganisms. In most cases, the acidity is probably the primary antimicrobial factor in the preservation of lactic acid-fermented foods, since the lactic acid is the main metabolic product of LAB fermentation. The reduction of pH due to lactic or acetic acid production by LAB influences the cellular metabolism and inhibits the growth of several contaminating microorganisms. The antimicrobial effects of hydrogen peroxide producing the oxidation of sulfhydryl groups cause the destruction of many pathogens. The hydrogen peroxide may also be a precursor for the production of free radicals, which can damage DNA of other microorganisms. Carbon dioxide may exert its antimicrobial effect in several ways such as by rendering the environment more anaerobic, inhibiting the enzymatic decarboxylation and disrupting cell membranes by the accumulation of the gaseous phase in the lipid bilayer. Diacetyl inhibits the growth of Gram-negative bacteria by reacting with the arginine-binding protein, thus affecting the arginine utilization.

Gradually it became clear that not only organic acids, hydrogen peroxide and diacetyl, but also additional metabolites often amplify the antimicrobial capacity of starter cultures. One of such metabolites is the bacteriocins. Bacteriocins are bacteria-produced proteinaceous antimicrobial compounds that have general bactericidal activity, often against bacteria that are closely related to the bacteriocin-producing strain. Bacteriocins exert their activities via the insertion in membranes and pore formation, the depolarization of the target membrane leading to the rapid efflux of low molecular weight compounds from the cell (Bruno and Montville, 1993; Mishra and Lambert, 1996). Because LAB strains are ‘generally recognized as safe’ (GRAS) in food production use, the detection and identification of bacteriocins produced by such bacteria have therefore received much attention, since these substances can be applied as ‘natural’ food preservatives (Cleveland et al., 2001).

1.4.2. Bacteriocins

Bacteriocins of LAB are ribosomally produced peptides (usually 30–60 amino acids) displaying potent antimicrobial activities against other often closely related to producer bacterial

strains. Most of the bacteriocins of LAB are usually thermostable cationic, hydrophobic, or amphiphilic molecules. They elicit their lethal effects by permeabilizing the cell membrane of target organisms, in certain cases by targeting intermediates of the cell wall biosynthesis, or possibly proteins of sugar phosphotransferase systems or formation of pores, by degradation of cellular DNA and by disruption through specific cleavage of 16S rRNA. They could be either chromosomally or plasmid encoded.

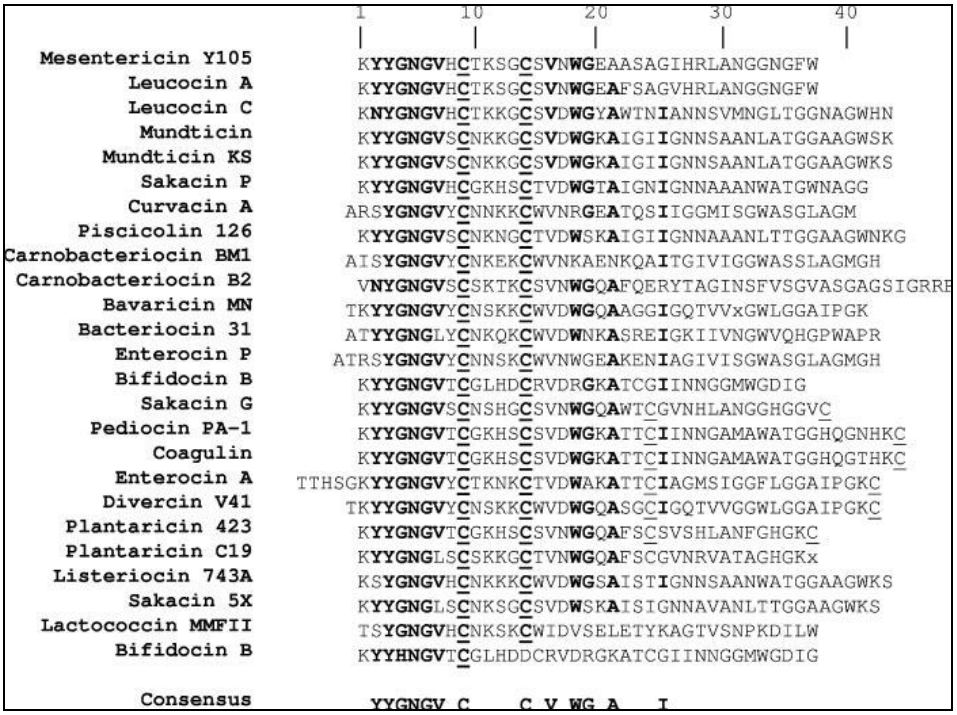
1.4.2.1. Classification and structure of bacteriocins

LAB bacteriocins are commonly classified into 4 groups that also include bacteriocins from other Gram-positive bacteria (Klaenhammer, 1993; Nes et al., 1996).

Class I bacteriocins are small (<5 kDa) peptides containing the unusual amino acids lanthionine (Lan), α-methyllanthionine (MeLan), dehydroalanine and dehydrobutyrine. This class of bacteriocins is called lantibiotics (from lanthionine-containing antibiotic). Lantibiotics are further subdivided into type A and type B lantibiotics according to chemical structures and antimicrobial activities. Type A lantibiotics are elongated, screw-shaped peptides with a positive charge. They exert their activity through the formation of pores in bacterial membranes. The well known bacteriocin nisin is a member of this group. Type B lantibiotics are more compact, small globular peptides with negative, positive or no net charge. The antimicrobial activity of these peptides is related to the inhibition of specific enzymes. Another classification scheme of lantibiotics was proposed by Cotter et al. (2005a), which was based on alignment of the amino acid sequences of the unmodified structural peptides.

Class II bacteriocins are small (<10 kDa), heat-stable, non-lanthionine-containing peptides. Their biosynthesis does not undergo extensive post-translational modification. The bacteriocins of this large group are classified in four subclasses (II a – d) (Cotter et al., 2005b).

Subclass IIa includes the most studied bacteriocins - pediocin-like peptides. This subgroup has attracted much of the attention due to their anti-Listeria activity (Ennahar et al., 2000). They range in size from 37 to 48 amino acid residues and are cationic at neutral pH. Sequence alignment of class IIa bacteriocins reveals that they consist of a highly conserved hydrophilic and charged N-terminal part harboring the consensus sequence YGNGV(X)C(X)4C(X)V(X)4A (X denotes any amino acid) (Fig. 1.5) and a more variable hydrophobic and/or amphiphilic C-terminal part. Based on amino acid sequence alignments, the class IIa bacteriocins are further devided into three or four subgroups (Drider et al., 2006).



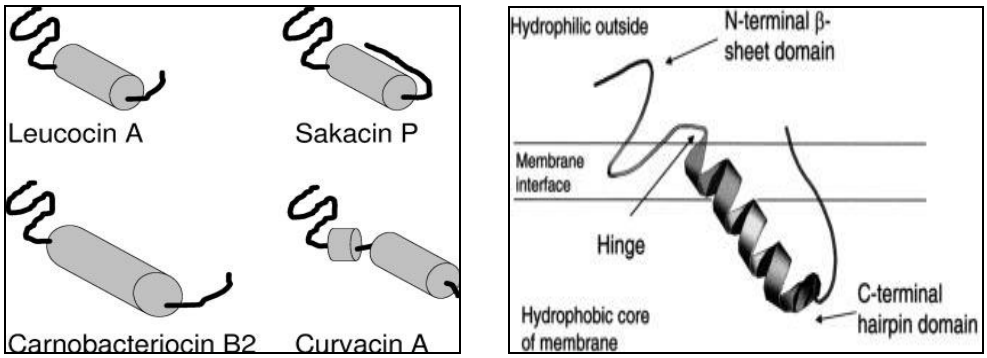
According to Drider et al., 2006

Fig. 1.5. Sequence alignment of class IIa bacteriocins

Today, the 3D structures of some class IIa bacteriocins have been determined by nuclear magnetic resonance (NMR) spectroscopy. They consist of an N-terminal β-sheet-like domain which is structurally stabilized by the conserved disulfide bridge and a C-terminal domain consisting of one or two α-helices, often ending with a structurally extended C-terminal tail (Fimland et al., 2005) (Fig. 1.6). In the C-terminal part, a few class IIa bacteriocins, such as sakacin G, plantaricin 423, pediocin PA-1/AcH, divercin V41, and enterocin A, contain an additional C-terminal disulfide bridge which plays an important role in stabilizing the 3D structure of the C-terminal domain. Often these structurally stabilized bacteriocins display higher antimicrobial potencies than those containing only one disulfide bridge, especially at higher temperatures (Fimland et al., 2000; Drider et al., 2006).

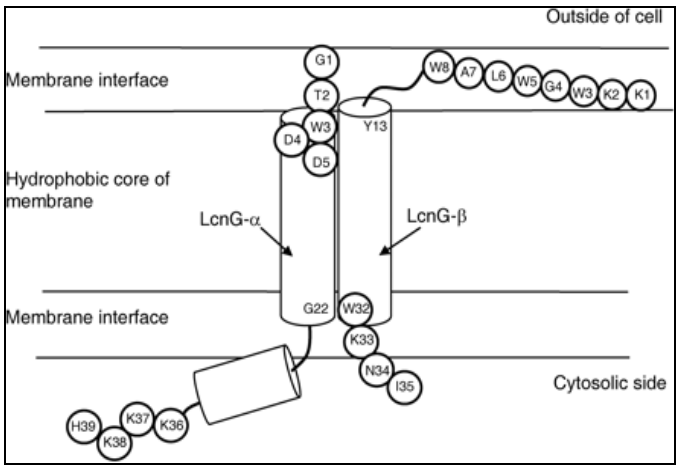
Subclass IIb contains two–peptide bacteriocins requiring 2 different peptides for activity. One of the members of this subclass bacteriocins is lactococcin G. It consists of two peptides, LcnG-a (39 amino acid residues) and LcnG-b (35 amino acid residues). Both peptides must be present in about equal molar amounts in order to obtain optimal antimicrobial activity. Circular dichroism (CD) and NMR spectroscopy show that the two peptides, LcnG-a and LcnG-b, are unstructured in aqueous environments, but adopt mainly α-helical structures when they are individually exposed to membranes. Moreover, when the two peptides together are exposed to membrane-like entities such as liposomes, additional α-helical structuring is forming, indicating the formation of an active structure (Hauge et al., 1998). According to NMR spectroscopy model

(Fig. 1.7) two peptides of lactococcin G form a parallel helix–helix structure consisting of the N-terminal half of LcnG-a (from about Trp-3 to Gly-22) and the C-terminal half of LcnG-b (from about Tyr-13 to Trp-32). The cationic C-terminal end (residues 35–39: R-K-K-K-H) of LcnG-a is unstructured and forced through the target-cell membrane by the membrane potential, thereby positioning the C-terminal ends of the two peptides inside the target cell. The tryptophanrich N-terminal end of LcnG-b is proposed to be relatively unstructured and to position itself in the outer membrane interface, thus forcing the N-terminal ends of the two peptides to remain on the outer side of the target-cell membrane and the helix–helix segment to transverse the membrane.



According to Drider et al., 2006 and Johnsen et al., 2005

Fig. 1.6. Schematic presentation of some class IIa bacteriocins



According to Oppegard et al., 2010

Fig. 1.7. NMR spectroscopy model of lactococcin G

Subclass IIc contains the cyclic unmodified peptides, including sec-dependently secreted bacteriocins. Subclass IId bacteriocins are non-pediocin linear peptides. The class III

bacteriocins are large (>30 kDa) heat-labile proteins, which still are not well characterized. The class IV consists of complex bacteriocins that require carbohydrate or lipid moieties for activity.

Recently, Zouhir et al. (2010) proposed new classification scheme of bacteriocins and grouped them in 12 classes. However the classification scheme described above, where bacteriocins of Gram-positive bacteria are mainly devided in 4 classes, is more common and widely used.

1.4.2.2. Genetic organisation and biosynthesis

The synthesis of bacteriocins occurs on ribosomes. The genes coding their production may be grouped as being the structural prebacteriocin gene, immunity gene, transporter gene and additional genes. All these genes are organized in operon clusters (Nes et al., 1996; McAuliffe et al., 2001). These operons can be located on conjugative transposable elements, chromosome or plasmid (Dufour et al., 2000; Banerjee and Hansen, 1988; Altena et al., 2000).

In case of linear unmodified bacteriocins, which include the plantaricins, carnobacteriocins and sakacins, it appears that specific inducing peptides or peptide pheromones stimulate synthesis of bacteriocins that are usually located on the same gene cluster (Quadri et al., 1997). The lantibiotic biosynthesis operons usually contain structural genes coding the prepeptide (LanA - the abbreviation lan refers to homologous genes of different lantibiotic gene clusters), enzymes responsible for modification reactions (LanB,C/LanM), processing proteases responsible for removal of the leader peptide (LanP), the ABC (ATP-binding cassette), superfamily transport proteins involved in peptide translocation (LanT), regulatory proteins (LanR, K) and proteins involved in producer self-protection (immunity) (LanI, FEG) (Bierbaum et al., 1996; Uguen et al., 2000).

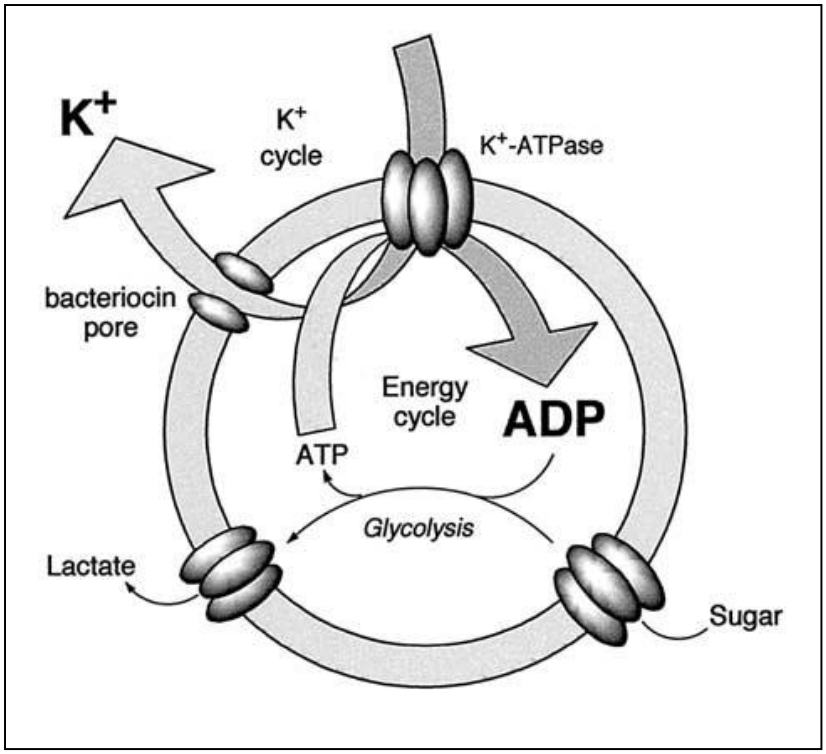
Class II bacteriocins require a minimum of four genes: gene coding prepeptide, immunity protein, ABC-type transporter and membrane-bound accessory protein, which is essential for export (Holo et al., 1991). In some cases (sakacin P, curvacin A) regulatory genes have been found to be necessary for production. The products of these regulatory genes include induction factor (IF), which could be dedicated induction factor or bacteriocin peptide itself and a two-component system composed of a membrane-bound histidine protein kinase (HPK) and a cytoplasmatic response regulator (RR). Induction factor acts as external signal that triggers transcription of bacteriocin genes (Ennahar et al., 2000).

Most bacteriocins are synthesized as biologically inactive propeptides carrying an N-terminal leader peptides that are attached to the C-terminal propeptides. During the maturation process, the leader peptide is removed and the C-terminal propeptide is modified to the active bacteriocin. In case of lantibiotics, the serine, threonine and cysteine residues in their propeptide

parts undergo extensive post-translational modification to form Lan/MeLan. The biosynthetic pathway of lantibiotics follows a general scheme: formation of prepeptide, modification reactions, proteolytic cleavage of the leader peptide and the translocation of the modified prepeptide or mature propeptide across the cytoplasmic membrane. Cleavage of the leader peptide may occur before, during, or after export from the cell. Leader peptides may serve as a recognition site, which directs the prepeptide towards maturation and transport proteins, protect the producer strain by keeping the lantibiotic in an inactive state while it is inside the producer and interacts with the propeptide domain to ensure a suitable conformation essential for enzyme-substrate interaction (Sablon et al., 2000; McAuliffe et al., 2001). Class II bacteriocins are synthesized as a prepeptide containing a conserved N-terminal leader and a characteristic double-glycine proteolytic processing site, with the exception of class IIc bacteriocins, which are produced with a typical N-terminal signal sequence of the sec-type and processed and secreted through the general secretory pathway (Leer et al., 1995; Worobo et al., 1995). Class II bacteriocins do not undergo extensive post-translational modification. After formation of prepeptide, it removes the leader peptide and is exported from the cell through a dedicated ABC-transporter and its accessory protein (Nes et al., 1996; Ennahar et al., 2000). The biosynthesis of lantibiotics and nonlantibiotics is usually regulated by well-known 2-component regulatory systems. These regulatory systems consist of 2 signal-producing proteins, a membrane-bound histidine protein kinase (HPK) and a cytoplasmic response regulator (RR) (Nes et al., 1996).

1.4.2.3. Mode of action

The anionic lipids of the cytoplasmic membranes are the primary receptors of LAB bacteriocins before initiation of pore formation. One of well known bacteriocins - nisin is often compared to a surface-active cationic detergent, which adsorption to the bacterial cell envelope is the necessary first step of membrane disruption. This is then followed by the inactivation of sulfhydryl groups. Lacticin 3147 is a two-peptide lantibiotic composed of peptides termed lac1 and lac2. Both components are required for full antagonistic activity that is a result of the formation of ion-specific pores in the membranes of Gram-positive target cells. Other known two-peptide bacteriocins are lacticin F, lactococcin G and thermophilin 13. In many of these two-peptide bacteriocins the bactericidal effect occurs as a result of permeabilization of the cell membranes (Nes and Holo, 2000). This pore-forming ability leads to cell leakage and the efflux of K+ ions leading to dissipation of membrane potential and inhibition of amino acid uptake. Cell death may be finally affected by a futile cycle of ATP-driven potassium uptake and bacteriocin mediated potassium release in combination with increased ATP hydrolysis by an ATPase (Fig. 1.5) (Jack et al., 1995).

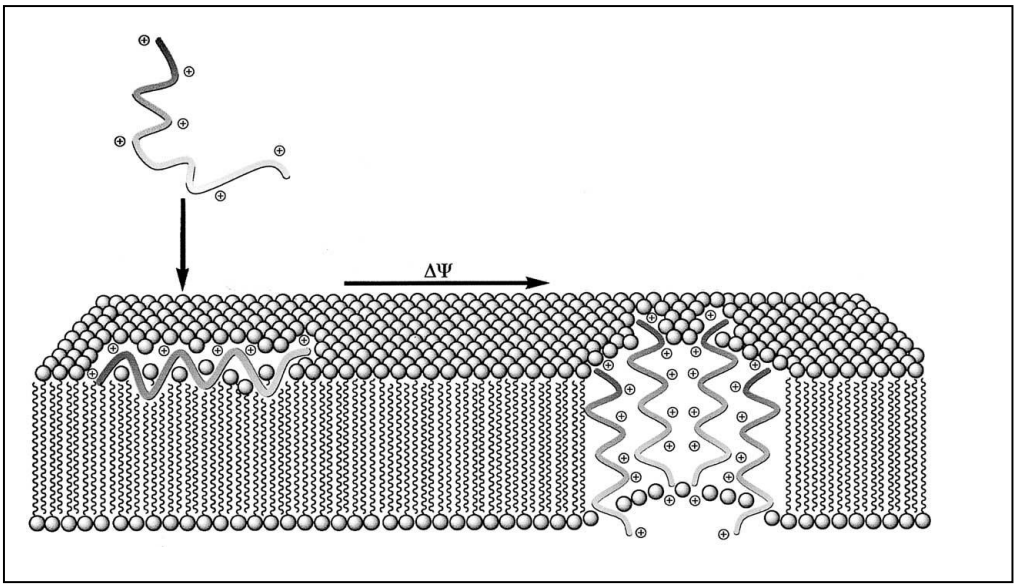


According to Garneau et al., 2002.

Figure 1.8. Proposed model of cell killing by pore-forming bacteriocins

In certain single-peptide bacteriocins it has been shown that after contact with membranes the otherwise random-coiled peptides adopt ordered helical structures (Demel et al., 1996). The formation of such amphipathic helices is the critical conformational change required for pore formation by the barrel-stave or wormhole mechanism (Moll et al., 1996; Ludtke et al., 1996; Abee, 1995). These mechanisms rely upon stabilizing interactions between membrane phospholipids and the cationic residues of the peptide allowing for the insertion of hydrophobic regions into the outer layer of the membrane. Once associated with the membrane surface a number of the ordered bacteriocins could potentially aggregate. The bacteriocin complex in principle can completely cross the membrane forming a transient pore (Fig.1.6).

It has been shown, that the single-peptide lantibiotic nisin has specificity for the peptidoglycan precursor lipid II (Wiedemann et al., 2001). Thus nisin displays a dual mode of action one of them is lipid II mediated pore formation. The N-terminal region of nisin is essential for lipid II recognition and the prevention of peptidoglycan biosynthesis. The C-terminal region of nisin and a flexible "hinge" region were then shown to be the essential pore-forming components (Sahl and Bierbaum, 1998).



According to Garneau et al., 2002.

Figure 1.9. Barrel-stave/wormhole mechanism of pore formation by cationic peptides, which do not require a receptor

1.4.3. Bacteriocinogenic LAB in biopreservation of fermented milk products

Biopreservation is the extension of the shelf-life and improvement of the safety of food using microorganisms and their metabolites. It is well known that LAB can produce a wide range of antimicrobial compounds, which can inhibit or reduce undesirable flora in food products. In the food preservation context, bacteriocins produced by LAB proved to have an interesting technological potential because they are strongly active against the food spoilers and food-borne pathogens such as *Listeria monocytogenes*, *Clostridium* spp. (including *C. botulinum* and *C. perfringens*), *Staphylococcus aureus, Bacillus* spp., *Brochothrix* spp., *Vibrio cholerae* and spoilage LAB. Because LAB strains have GRAS status in food production use, the detection and identification of bacteriocins produced by such bacteria has therefore received much attention, since these substances can be applied as 'natural' food preservatives. Alternatively, bacteriocinogenic LAB are also attractive to be used as co-cultures in food fermentation where they could contribute to the competitiveness of the producer strains and to the prevention of food spoilage and contamination.

Three approaches are commonly used in the application of bacteriocins for biopreservation of foods:

1. Inoculation of food with LAB, producing bacteriocins in the products. The ability of the LAB to grow and produce bacteriocins in the products is crucial for their successful use.
2. Addition of purified or semi-purified bacteriocins as food preservatives.

3. Use of a product previously fermented with a bacteriocin producing strain as an ingredient in food processing.

The use of bacteriocins, the producer strains or both is of particular interest for the food industry since it may be help to secure the microbial safety of the food products (Caplice and Fitzgerald, 1999). Until now nisin remains the most commercially important bacteriocin, although other bacteriocins have been characterized and developed for possible approvals and uses (Chikindas and Montville, 2002).

In recent years, various bacteriocins produced by *Lb. plantarum* species, *Lb. casei*, *Lb. lactis*, *Leuconostoc lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis and S. thermophilus* were isolated from fermented dairy products. These bacteriocins showed broad inhibition spectra including some pathogenic organisms in foods such as *Listeria monocytogenes*, *Escherichia coli*, *Shigella spp.*, *Staphylococcus aureus* and *Escherichia aerogenes* (Luo et al., 2011; Xie et al., 2011). Some *Enterococci* of dairy origin (*E. faecalis* and *E. faecium*) have also been reported to produce different types of bacteriocins (enterocins), with antimicrobial activities limiting food spoilage or inhibiting growth of pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus* spp. (Batdorj et al., 2006; Hadji-Sfaxi et al., 2011; El-Ghaish et al., 2011b). Bacteriocin-producing *Enterococci* strains may play an important role in the natural preservation of food products by controlling, competing and inhibiting the growth of undesirable bacteria, preventing thus the adulteration of foods caused particularly by *Listeria monocytogenes* and other food-borne pathogens what has led to severe disease outbreaks in the past. Hence, *Enterococci* show also a potential for dairy application as bio-preservatives or protective cultures

2. MATERIAL AND METHODS

2. MATERIAL AND METHODS

2.1. Samples of fermented dairy products

Forty six samples of traditionally fermented dairy products were obtained from individual households in different regions of Azerbaijan during 2009 – 2010 years. They were manufactured in traditional way in country households without the addition of any starter culture. Thus, LAB in these dairy products corresponds to the microflora in localities where dairy products are manufactured.

LAB isolation was done from final ready-to-eat dairy products (5 yogurts, 41 cheeses) (Table 2.1). These dairy products samples could be classified in 4 groups. First group includes samples of cheese named *"Motal"*. It is prepared from pasteurized sheep or goat's milk. This type of cheese has very specific and traditional preparation method. The ripening of *"Motal"* cheese is performed in sheep or goat skin with 3% NaCl for 3-5 months. It has hard consistence and white or yellow colour.

Second group includes samples of white cheese *("Brinza"*) manufactured from cow, sheep and buffalo's milk. All samples of this cheese are prepared in more or less similar way. The pasteurization of milk is done immediately after milking by warming the milk to 70 °C. Cheeses are made by addition of the 1 – 1.5% (v/v) homemade rennet to pasteurized milk, which is cooled subsequently to 37 °C. The curd formation takes around 2 h. Afterwards the curd is cut into small pieces and transferred in cotton bag and pressed for whey extraction for 6 - 8 h. Then, the curd without whey is put into a barrel with brine containing from 1.5 till 2% NaCl and ripening is taking from 20 till 45 days (depending on cheese sample). After ripening period, cheese is white with a semi-hard consistence.

Third group included samples of yogurt, which were prepared from cow or buffalo's milk. The production of yogurt was done as follows: 10 mL of old, previously produced yogurt was added as starter to 1 L of pasteurized milk chilled to 37 - 40 °C. After the inoculation, the milk was stored at this temperature in glass containers or saucepan vessels and incubation was carried out for 4 - 8 h until the curd was formed.

And the last group includes samples of "Shor" – soft cheese made from coagulated milk.

The samples of the above-mentioned cheeses and yogurts were collected from farmhouses in sterile plastic containers and transported to the laboratory.

Table 2.1. Samples of fermented dairy products used in the study

City	Fermented milk product	Description	Number of samples
Barda	White cheese	semi-hard, cow's milk	2
	Yogurt	cow's milk	1
Dashkesen	White cheese	semi-hard, cow's milk	1
	Yogurt	bufallo's milk	1
	Shor (curd cheese)	white, soft, cow's milk	1
Hajikabul	Shor (curd cheese)	white, soft, cow's milk	2
	Yogurt	cow's milk	1
Ismailly	White cheese	semi-hard, cow's milk	2
	White cheese	semi-hard, sheep's milk	1
Imishly	Motal cheese	white, hard, goat's milk	2
	Shor (curd cheese)	white, soft, cow's milk	1
	White cheese	semi-hard, cow's milk	1
Lankaran	White cheese	semi-hard, cow's milk	2
Qazakh	Motal cheese	white, hard, sheep's milk	4
	Motal cheese	yellow, hard, sheep's milk	2
	Yogurt	bufallo's milk	1
	White cheese	semi-hard, buffalo's milk	1
Qanja	Motal cheese	yellow, hard, goat's milk	3
	White cheese	semi-hard, cow's milk	1
Quba	White cheese	semi-hard, cow's milk	2
	White cheese	semi-hard, sheep's milk	1
Sabirabad	Motal cheese	yellow, hard, sheep's milk	2
	White cheese	semi-hard, cow's milk	1
Siazan	White cheese	semi-hard, cow's milk	1
	White cheese	semi-hard, buffalo's milk	1
Shamakhi	White cheese	semi-hard, cow's milk	2
Shaki	Motal cheese	yellow, hard, goat's milk	2
	White cheese	semi-hard, cow's milk	1
	Yogurt	cow's milk	1
Zaqatala	White cheese	semi-hard, cow's milk	2

2.2. Media, chemicals and reagents

Skimmed milk powder, Triton X-100, 3% hydrogen peroxide were obtained from Merck (Darmstadt, Germany). The following products were purchased from Sigma-Aldrich (St Louis, MO, United States): Glycerol, sodium citrate, Tris, potassium chloride, Luria-Bertani (LB), ampicillin, vancomycin, ciprofloxacin, proteinase K, trypsin, α-chymotrypsin, catalase, α-amylase, lipase, sodium dodecyl sulfate (SDS), ethylene diamine tetra acetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), Pefabloc SC (4 - (2-aminoethyl) fluoride hydrochloride benzenesulfonyl (AEBSF), trifluoroacetic acid, sodium acetate, potassium chloride (KCl), sodium phosphate dibasic dodecahydrate (Na_2HPO_4 $12H_2O$), potassium dihydrogen pure (KH_2PO_4), Tween 80, polyvinyl alcohol and 4-methylumbelliferyl phosphate (4-MUP). The

culture media MRS (de Man-Rogosa-Sharpe), M17 and BHI (Brain Heart Infusion) were obtained from Biokar (Beauvais, France). Agar, yeast extract were purchased from Fisher Bioblock Scientific (Illkirch, France). Sodium chloride, sodium hydroxide and glycine were obtained from Panreac (Lyon, France). The culture medium YPD (yeast extract-peptone-glucose) was purchased from Serva (St Germain en Laye, France). The supplier of hydrochloric acid and acetonitrile was Carlo Erba (Val de Reuil, France) and the supplier of antibiotics (chloramphenicol, penicillin, tetracycline and gentamicin) was Euromedex (Souffelweyersheim, France). Acrylamide solution was obtained from Fisher Bioreagents (New Jersey, USA). Urea and glucose were from ACROS (New Jersey, USA); 2-mercaptoethanol and Coomassie blue R250 were from Fluka (Germany). Purified milk proteins used as substrates were of analytical grade.

2.3. Screening and isolation of LAB isolates

2.3.1. Screening and isolation of proteolytic LAB isolates

Two methods were used for the screening of proteolytic LAB isolates. First method was screening directly from cheese samples. For this, two grams of each sample was taken and homogenized with pestle in sterile conical flasks containing 2 mL of sterile 2% (w/v) trisodium citrate solution. Decimal serial dilutions of the homogenates were prepared in sterile 0.85% (w/v) sodium chloride. For preliminary screening of proteolytic enzymes producing strains, 1 mL from each dilution was plated on M17 or de MRS agar (1.5%, w/v) supplemented with skim milk (20%, v/v, Délisse, UHT skim milk, France). Inoculated plates were incubated at 37 °C and 30 °C for 48 h to detect proteinases giving rise to clear haloes surrounding colonies, which were taken as a positive indicator of proteolysis (Pailin et al., 2001). Colonies producing clear haloes were randomly taken from agar plates and sub-cultured in M17 and MRS broth.

At the same time the isolation of LAB by standard microbiological method was performed. For this, decimal serial dilutions of the homogenised cheese samples were plated on the surface of M17 or MRS agar (1.5%, w/v) without the addition of milk. After incubation at 37 °C and 30 °C for 48 h colonies were randomly choosed and transformed to liquid media. After 2 recultivations obtained isolates were screened for production of proteolytic enzymes by another method (El-Ghaish et al., 2010). Cultures were inoculated (5%, v/v) in UHT skim milk and incubated at 37 °C for 24 h. After second recultivation, samples of coagulated milk were analysed for protein hydrolysis by SDS-PAGE. Samples were mixed with solubilization buffer for electrophoresis (50 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 3% 2-mercaptoethanol, 0.07% bromophenol blue) at a 1:10 volume *ratio* and were heated at 100

°C for 3 min before loading to the gel. Controls were prepared by inoculation of equivalent volume of media (M17 or MRS) in UHT skim milk.

Purity of the isolates was checked by repetitive streaking on fresh suitable agar media, followed by microscopic examination. Purified isolates were reconstituted in sterile skim milk (12.5%, w/v) supplemented with 30% (w/v) glycerol and stored at –80 °C. Before using cultures were propagated twice in suitable media.

2.3.2. Screening and isolation of BLIS-producing LAB isolates

Preliminary screening for BLIS (bacteriocin-like inhibitory substances)-producing LAB strains were performed by modified deferred antagonism test originally described by Tagg et al. (1976). Samples were homogenized and serially diluted ten-fold with saline solution (0.9% NaCl). Aliquots (1 mL) were plated on MRS and M17 agar (1.5% w/v) medium, then incubated at 30 and 37 °C for 48 h. Multiple plates of serial dilutions (two or three plates providing a total of approximately 50 colonies from each sample) were overlaid with MRS agar (0.8% w/v) medium containing indicator strain and incubated at 37 °C for another 24h. The indicator strains used were *Lactobacillus bulgaricus* 340 and *Lactobacillus brevis* F145. Colonies producing zones of growth inhibition in the indicator lawn were randomly selected from agar plates and sub-cultured in M17 and MRS broth. Purity of the isolates was checked by repetitive streaking on fresh suitable agar media, followed by microscopic examination. Purified isolates were reconstituted in MRS or M17 media supplemented with 30% (w/v) glycerol and stored at –80 °C. Before using cultures were propagated twice in suitable media.

2.4. Identification of LAB strains

2.4.1. Phenotypic identification

Protease- and BLIS-producing LAB isolates were screened and isolated as it was described in the previous part of Materials and Methods. Phenotypic identification of isolates was performed by cell morphology examination, Gram staining and chemically for catalase production. Gram-positive, catalase-negative rods and cocci were presumptively identified as LAB.

2.4.2. Genotypic identification

Total DNA from bacterial strains was isolated by DNeasy purification kit (Qiagen, Hilden, Germany) from a 3 mL overnight culture inoculated with a single colony according to the manufacturer recommendations and stored at -20 °C until use. Genotypic identification of

obtained isolates was performed by genus / species – specific PCR 16 S rDNA fragment sequencing and RAPD-PCR fingerprinting.

16 S rDNA fragment sequencing

16S rDNA fragments were amplified with universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5' TAAGGAGGTGATCCAGGC3') according to Weisburg et al. (1991). DNA amplifications were performed in DNA thermal cycler model (Techno, Barloworld scientific, Cambridge, UK). Mix reaction contained: PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 U Taq DNA polymerase (Qiagen Gmbh, Hilden, Germany), 1 mM of each primer and 40 ng DNA in a final volume of 50 µL. PCR amplifications were performed under the following conditions: denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1.15 min and DNA extension at 72 °C for 1.15 min. A final extension was added at 72 °C for 5 min. Amplicons were analyzed on 1% (w/v) agarose gel with ethidium bromide (0.5 mg/mL) in 0.5 X TAE (Tris Acetate-EDTA) buffer for 30 min at 100 V and made visible by UV trans-illumination. DNA sequencing was carried out by MilleGen sequencing service (Labège, France). The blast algorithm (http://blast.ncbi. nlm.nih.gov/Blast) was used to determine the most related sequence relatives in the NCBI nucleotide sequence database.

Genus / species – specific PCR

Enterococci were identified using *Enterococcus* genus specific primers Ent 1 (5'-TACTGACAAACCATTCATGAT-3') and Ent 2 (5'-AACTTCGTCACCAACGCGAAC-3') as described by Ke et al. (1999).

In the case of *Lactobacilli* identification by species-specific PCR was applied after 16S rDNA sequencing in order to clarify if the strain belongs to *Lb. curvatus* or *Lb. sakei* species. PCR reaction was performed using primers and conditions described previously (Berthier and Ehrlich, 1998). For species-specific amplification, the primers coding 16S/23S spacer region of *Lb. sakei* (Ls: 5'-ATGAAACTATTAAATTGGTAC-3') and 16S/23S spacer region of *Lb. curvatus* (Lc: 5'-TTGGTACTATTTAATTCTTAG-3') were used. Each of them was paired with the primer coding conserved spacer region of 16S rDNA of *Lactobacilli* (16S: 5'-GCTGGATCACCTCCTTTC-3'). PCR amplifications were performed in 20 µL reaction mixtures containing 40 ng of bacterial DNA, 1X GoTaq reaction buffer, 1.0 mM $MgCl_2$, 1 U of GoTaq DNA polymerase, 250 µM of dNTPs mix (all from Promega) and 300 nmol of each primer. Amplifications were carried out using a DNA thermal cycler model (Techno, Barloworld scientific, Cambridge, UK) using a program consisting of the initial denaturation at 94 °C for 5

min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at an appropriate temperature depending on mT of primers for 1 min, elongation at 72 °C for 1 min and a final extension step of 5 min at 72 °C. For each PCR, a negative control (sample without template DNA) was included. Amplification products were separated on a 2% agarose gel in 0.5 x TAE buffer for 30 min at 100 V, stained with ethidium bromide (0.5 mg/mL) and visualised by UV trans-illumination.

RAPD-PCR

The isolated *Enterococci* strains were genomically typed by RAPD-PCR using primer E1 (5'-TCACGCTGCA-3') (Barbier et al., 1996). PCR amplification was performed in a total volume of 20 µL containing 40 ng of bacterial DNA, 5 µl 5 X GoTaq reaction buffer, 1.5 mM $MgCl_2$, 0.4 U of GoTaq DNA polymerase, 200 µM of dNTPs mix (all from Promega, Madison WI, USA) and 200 nmol of primer. Amplification was carried out in a "2720 thermal cycler" model (Applied Biosystems) using the following program: initial denaturation at 95 °C for 5 min, 4 cycles of 45 sec at 94 °C, 2 min at 30 °C and 30 sec at 72 °C ; 10 cycles of 5 sec at 94 °C, 30 sec at 36 °C and 30 sec at 72 °C; 10 cycles of 5 sec at 94 °C, 30 sec at 36 °C and 40 sec at 72 °C; 10 cycles of 5 sec at 94 °C, 30 sec at 36 °C and 50 sec at 72 °C; and 10 cycles of 5 sec at 94 °C, 30 sec at 36 °C and a final extension step of 10 min at 72 °C. In the negative control reaction, the DNA template was replaced by sterile deionized water. Amplicons were analyzed on a 1.5% agarose gel with ethidium bromide (0.5 mg/mL) in 0.5 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The RAPD patterns were documented and analyzed using the Gene Tools v.4.01 software (SynGene). Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm.

2.5. Proteolytic activity assays

Proteolytic activities of isolated strains were analyzed in two systems: in UHT skim milk and in non-proliferative cells system.

2.5.1. Proteolytic activity in UHT skim milk

To analyze the proteolytic activities in milk, overnight cultures of isolated strains were inoculated (5%, v/v) in UHT skim milk and incubated at 37 °C (El-Ghaish et al., 2010). Controls were prepared by inoculation of equivalent volume of media (M17 or MRS) in UHT skim milk. At different time intervals, samples were taken and mixed with solubilization buffer for electrophoresis (50 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 3% 2-mercaptoethanol, 0.07% bromophenol blue) at a 1:10 volume ratio. Samples were heated at

100 °C for 3 min and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Acidification ability was followed by measuring pH decrease after 3, 6, 9, 12 and 24 h incubation.

2.5.2. Proteolytic activities in non-proliferative cells system

Fresh pre-cultures were prepared by inoculation of strains in suitable media and overnight incubation at 37 °C. Induction of proteinase production was achieved by incubation of pre-cultures on milk-citrate-agar media (4.4% reconstituted skim milk, 0.8% Na-citrate, 0.1% yeast extract, 0.5% glucose, 1.5% agar (w/v)) (Fira et al., 2001). The plates were incubated for 48 h at 37 °C prior to cells collection. Collected fresh cells were washed twice with 0.8% (w/v) saline solution containing 5 mM $CaCl_2$ and re suspended to a final OD_{600} of 20 in 100 mM sodium phosphate (pH 7.2). The cell suspensions were mixed with substrate dissolved in the same buffer at a 1:1 volume *ratio* and incubated at 37 °C. Na-caseinate (12 mg/mL), β-casein (5 mg/mL), α_{S1}-casein or whey proteins (5 mg/mL, denaturated at 85 °C for 20 min) were used as substrates. Equivalent substrate solutions without cells, incubated for the same period, were used as controls. At different time intervals, samples were taken and cells were pelleted by centrifugation (10 min at 10 000 *g*). The clear supernatants were mixed with solubilization buffer at 1:1 volume ratio and heated at 100 °C for 3 min. Protein hydrolysis and peptide formation were analyzed by SDS-PAGE and Tricine SDS-PAGE. Supernatants were also analyzed by Reversed Phase High Performance Liquid Chromatography (RP-HPLC).

2.6. API ZYM

The cells suspensions of studied cultures were prepared according to instructions of manufacturer and dense were used to inoculate the API-ZYM kits (Biomerieux, France). Enzyme activity was graded from 0 to 5 by comparing the colour developed within 5 min to the API-ZYM colour reaction chart and was expressed on a scale from 0 (no activity) to 5 (maximum activity).

2.7. Influence of different factors on proteolytic activity

2.7.1. Effect of pH and temperature on proteolytic activity

To test the influence of pH on protease activities, the cells suspensions were prepared as described above in 100 mM Na-phosphate with different pH values (5.4, 5.7, 6.0, 6.5, 7.2 and 8.0) and mixed with substrate dissolved in the same buffer at a 1:1 volume *ratio*. Reaction mixtures were incubated at 37 °C. Similarly, to determine the effect of temperature on

proteolytic activity, the cells suspensions were prepared in 100 mM Na-phosphate (pH 7.2) and reaction mixtures were incubated at different temperatures (20, 30, 37, 45 and 55 °C). Na-caseinate (12 mg/mL) was used as substrate. Equivalent substrate solution without cells, incubated for the same period, was used as control. At different time intervals, samples were taken and the cells were pelleted by centrifugation (10 min at 10 000 *g*). The supernatants were mixed with solubilization buffer at a 1:1 volume ratio and heated at 100 °C for 3 min. Protein hydrolysis and peptides formation were analyzed by SDS-PAGE.

2.7.2. Effect of inhibitors

Cells suspensions were prepared as described before and incubated with different protease inhibitors (10 mM final concentration of EDTA, iodoacetic acid, phenylmethylsulfonyl fluoride (PMSF) or Pefabloc) for 2 h at 37 °C, prior to addition of 12 mg/mL Na-caseinate. Substrate solutions without cells and substrate-cells mixture without inhibitor were used as controls. At defined intervals, samples were taken and cells were pelleted by centrifugation (10 min at 10 000 *g*). The clear supernatants were mixed with solubilization buffer at a 1:1 volume ratio and heated at 100 °C for 3 min. Protein hydrolysis and formation of peptides was analyzed by SDS-PAGE.

2.8. Analysis of protein hydrolysis and peptide formation

2.8.1. SDS-PAGE

Gels were run on vertical slab electrophoresis cells (BIORAD Mini PROTEAN 3 System, Hercules, CA, USA). Analysis of caseins hydrolysis was carried out on SDS-PAGE by loading 12% polyacrylamide gel with prepared samples (Laemmli, 1970). After running at 10 mA on the stacking gel and 20 mA on the running gel, proteins and peptides were observed by staining gels with Coomassie Brilliant Blue R-250 (Sigma-Aldrich) followed by a convenient destaining in a solution made of ethanol (30%, v/v) and acetic acid (5%, v/v) in distilled water. Degradation of whey proteins in the studied samples was measured by Tricine SDS–PAGE electrophoresis on 17% (w/v) polyacrylamide gels (Schägger and von Jagow, 1987). After running at 10 mA on the stacking gel and 20 mA on the running gel, gels were stained with Coomassie Brilliant Blue G-250 (Sigma-Aldrich) according to Devouge et al. (2007). The gels were scanned with Image scanner III (GE Healthcare, USA). The degradation of proteins and the percentages of hydrolysis were quantified by densitometry analysis of gels with Fuji Film Image Gauge V3.0 software (Fuji Photo Film Co. Ltd. Japan). The obtained data were expressed as the ratio of the area and intensities of the bands. The reduction in the intensity of bands during

incubation with respect to the original intensities was expressed as percentage of hydrolysis (Ong et al., 2006).

2.8.2. RP-HPLC

Protein hydrolysis and peptides profiles arising from enzymatic activities of the studied strains were analyzed by RP-HPLC. Supernatants obtained after incubation of cells with different substrates in non-proliferative cells system were mixed (1/1, v/v) with 6 M urea and centrifuged (12 000 *g*, 5 min) before injection to the column. RP–HPLC was performed using Waters HPLC system (Alliance system, Milford, MA). Column (Symmetry C_{18}, 5 µm, 2.1 mm x 150 mm) was equilibrated with solvent A (H_2O, 0.11% trifluoroacetic acid (TFA), v/v) solution at a flow rate of 0.2 mL/min. After sample injection, column was washed 5 min with 100% of solvent A, then a linear gradient from 0 to 100% of solvent B (80% acetonitrile, 20% H_2O, 0.09% TFA, v/v/v) for 30 min was applied. Column was washed 2 min with 100% of solvent B before equilibration with solvent A. Detection was performed between 220 and 330 nm using a diode array spectrophotometer (model 996, Waters).

2.8.3. LC/MS/MS analyses

Mass spectrometry analyses were conducted on the platform "Biopolymers-Interaction-Structural Biology" located at the INRA centre of Nantes (INRA Research Unit 1268) (http://www.nantes.inra.fr/plateformes_et_plateaux_techniques/plateforme_bibs).

Casein peptides were separated by RP-HPLC with a Waters HPLC system as described above, coupled to a Finnigan LCQ ion trap spectrophotometer (Finnigan MAT, San Jose, CA). Spectra were acquired in automated MS/MS mode. The scan rate for MS mode was set between the masses of 400 to 2000 Da. Peptide identification was performed using the Mascot software (version 2.5, Matrix Science) on the MS/MS ion search mode with the following parameters: enzyme: none, peptide mass tolerance: 1.5 Da, fragment mass tolerance: 0.6 Da, fixed modification: phosphorylation. Identification was performed by comparing the obtained peptides with casein sequences.

2.9. Competitive F-ELISA

Sera. For competitive ELISA, a pool containing the series of sera was constituted. Two pools of sera collected at different times were used in this study. First pool contained series of 18 sera and second pool contained series of 8 sera from CMA patients. Sera were collected in the Laboratory of Immuno-Allergology of Academic Hospital (Angers, France) and their use was

approved by the internal Ethical Committee of the hospital. α_{S1}-Casein and β-casein -specific IgE concentrations were determined by direct-ELISA immunoassay.

Samples preparation. α_{S1}-Casein and β-casein (2mg/mL final concentration) were hydrolysed by strains in nonproliferative cells system. After 24 h incubation at 37 °C cells were pelleted by centrifugation and supernatants were taken for analyses.

Competitive F-ELISA. MaxiSorp bottom flat white 96 micro titration plates (Nunc, Roskillde, Denmark) were coated 3h at 30 °C with either 100µL per well of native α_{S1}- or β-casein diluted to 5 µg/mL in a 1M PBS pH 7.4 (0.136 M NaCl, 2.68 mM KCl, 1.76 mM KH_2PO_4 and 10.14 mM $Na_2HPO_4x12\ H_2O$), or with mouse monoclonal antihuman IgE antibody clone M604199 (Ab1, Fitzgerald, Concord, USA) diluted at 1:2500 (1.6 µg/mL) in PBS. Negative control (blank) was prepared by replacing α_{S1}-casein with equal volume (100 µL) of PBS. After coating, wells were washed three times with PBS containing 0.1% (v/v) Tween-20 (PBS/T) and saturated with 250 µL of the PBS/T solution containing 1% (w/v) polyvinyl alcohol (PBS/T/PVA) for 1 h at 37 °C. Standard curve was performed by adding 100 µL of serial dilutions from 160 to 0,15 ng/mL plus one dilution at 1 ng/mL of human serum IgE, prepared in PBS/T/PVA (WHO international reference preparation Code 75/502), to the wells coated with the antihuman IgE antibody only.

The pool of sera (diluted in PBS/T/PVA) was preincubated for 1 h at 37 °C in the presence of increasing concentrations of competitor (native and hydrolysed by strain α_{S1}- or β-caseins diluted in PBS/T/PVA) and added in the wells previously coated with allergen. The final dilution of the pool was 1:40 and the final concentration of the inhibitor ranged from 0 to 200 µg/mL (1:20 serum pool dilution and inhibitor solution were mixed v/v to a final volume of 100 µL). In the wells coated with PBS as a negative control, 100 µL of pool dilution mixed v/v with PBS was added. After an overnight incubation at 4 °C the plates where washed 3 times with PBS/T and incubated for 2 h at 37 °C with an alkaline phosphatase conjugated polyclonal antihuman IgE (Ab2: anti-human IgE (E-chain specific- Alkaline phosphatase conjugate produced in goat, Sigma A3525) diluted 1:1000 in PBS/PVA. The secondary antibody binding was revealed, after three washing steps with PBS/T, by the addition of 4-methylumbelliferyl phosphate (4-MUP Sigma M-3168) substrate diluted 1:5 in 1 M Tris-HCl, pH 9.8. The fluorescence emission was measured after 90 min incubation at room temperature with the FLx_{800} plate reader (Biotech Instruments, Inc., Winooski, VT) fitted with 360 nm excitation filter and a 440 nm emission filter. Controls included secondary antibody on capture antibody (no IgE), secondary antibody on IgE (no capture antibody), secondary antibody on allergens (no patient serum) and secondary antibody on serum (no allergen).

The experiment was realized in duplicate.

The calculation of the results was performed as described by Taheri-Kafrani et al. (2009). Inhibition percentage was calculated by using the following formula with Wt native caseins as reference proteins:

[(Signal $_{fluo}$ without inhibitor-Signal $_{fluo}$ with inhibitor) / (Signal $_{fluo}$ without inhibitor – signal $_{fluo}$ 100% inhibition)]x 100

The concentration of protein (inhibitor) needed to inhibit 50% of IgE-binding (IC50) was calculated from the inhibition curves by relating fluorescence intensity to inhibitor concentrations. To relate the fluorescence intensity to IgE concentrations, a sigmoid four parameter model [$y = d + (a-d)/1 + (x/c)^b$] was adjusted to standard curve data by nonlinear regression using the Solver option from Microsoft Excell 2000.

2.10. Amplification of proteinase genes by PCR

Genomic DNA was isolated as described previously (see section 2.4.2). For *Lactobacilli* the presence of genes encoding intergenic region of *prtP/prtM***,** catalytic domains of *prtB*, *prtH* and *prtR* was tested by polymerase chain reaction (PCR), using primers and conditions (Table 2.2) described previously (Strahinic *et al*., 2010). For *Enterococci* primers coding metallo- and serine proteases were constructed on the base of published genome sequences. Primers design was performed using Primer BLAST software. Several sets of primers covering different regions of both genes were created (Table 2.2). The oligonucleotide primers were purchased from Eurogentec (Belgium). The 50 µL reaction mixtures containing 40 ng of bacterial DNA, 1 x GoTaq reaction buffer containing 1.5 mM $MgCl_2$, 1 U of GoTaq DNA polymerase, 200 µM of dNTPs mix (all from Promega) and 200 nmol of each primer were prepared. PCR reactions were carried out in DNA thermal cycler model (Techno, Barloworld scientific, Cambridge, UK) using a following program: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at an appropriate temperature depending on mT of primers for 1 min, elongation at 72 °C for 1.5 min and a final extension step of 7 min at 72 °C. A negative control (sample without template DNA) was included for each reaction. PCR products were visualised (UV trans-illumination) after running of samples in 1.5% agarose gel (0.5 mg/mL ethidium bromide) in 0.5 x TAE buffer during 30 min at 50 V. The PCR products were purified by PureLink Quick Gel Extraction Kit (Invitrogen, France) and sequenced by MilleGen sequencing service (Labège, France). To determine the most related sequence relatives in the NCBI nucleotide sequence database the blast algorithm (http://blast.ncbi. nlm.nih.gov/Blast. cgi?CMD=web& PAGE_TYPE=BlastHome) was applied.

Table 2.2. Primers used for the detection of proteinase genes

Target gene	Primers (5' – 3')	Product size (bp)	Annealing temp (°C)
Lactobacilli			
prtP/prtM intergenic region	M70 : GCA-TGA-ATT-CAA-TGC-ACG-ATA-AAT-GAG P 70: GCT-TGA-ATT-CGT-TGT-CGC-TGC-GGT-TGT	685	56
prtB catalytic domain	B10: GGT-GTT-GCT-CCT-GAT-GCC-CAG-C B20: CCC-CGT-TTA-ACA-ACT-GCA-AGT-T	597	56
prtH catalytic domain	P23: GCT-TGG-ATA-GTA-GCG-TTA-GC P25: GGT-GAA-CAA-ACT-GAA-GAC-G	1034	56
prtR catalytic domain	TI2: CAA-CAC-CGG-GAC-CAC-GGT-G XbA: CTG-ATC-GTG-GAC-GGT-GTT-GC	1052	55
Enterococci			
*Ser*E1	F: GCG-GCC-AGC-TCT-CGG-GAT-TAC R: CAG-CAG-GGG-TCG-CTG-CTT-CA	134	56
*Ser*E7	F: AGC-TGG-CGG-CTT-ACT-TGG-TGG R: GTG-GAT-GTC-GTC-GTC-GCC-GC	102	55
*Met*E11	F: TGC-GAA-TGG-CTG-GGA-TGG-GC R: AGT-GGC-GCA-ATC-CGC-ACC-TC	280	56
*Met*E21	F: AGT-GGC-GCA-ATC-CGC-ACC-TC R: TGC-GAA-TGG-CTG-GGA-TGG-GC	280	56
*Met*E23	F: GCT-TCT-GCG-GCT-GGG-CCA-TT R: GGT-GCG-GAT-TGC-GCC-ACT-TG	201	55

2.11. Bacteriocin assay and spectra of inhibitory activity

Bacteriocin assay

The antimicrobial activity in cell-free supernatants (CFS) was determined by well diffusion method (Schillinger and Lucke 1989) with slight modification. CFSs of overnight (16-18 h) cultures were obtained by centrifugation (10000 x g, 15 min, 4 °C) and the pH was adjusted to 6.5 with 1 N NaOH. To avoid proteolytic degradation of the bacteriocins, CFSs were treated for 10 min at 80 °C. Soft nutrient agar (0.8%, w/v) was solidified in a sterile Petri dish after addition of indicator strains culture, grown to the early stationary growth phase in nutrient medium. Wells were made in the lawn of hardened soft agar in Petri dishes and aliquots (50 µL) of supernatants of overnight cultures were poured in the wells. The plates were left for 40 min at room temperature in sterile conditions before incubating them at the adequate temperature of growth of the test micro organism. A clear zone of inhibition of at least 2 mm in diameter around cutted wells was recorded as positive.

Antimicrobial activity spectra

The bacterial strains used as indicator micro-organisms for evaluation of inhibitory activity spectra are listed in Table 3.7. Before use, the strains were propagated twice in the

appropriate broth media overnight. Agar media were prepared by addition of 0.8% (w/v) agar to the medium. Antimicrobial activity against different indicator strains was detected by spot-on-lawn method (Yamamoto et al. 2003). CFSs of overnight (16-18 h) cultures were obtained as described above. Soft media agar was solidified in a sterile Petri dish after addition of indicator strains culture, grown to the early stationary growth phase. After 30 min of drying, aliquots (10 µL) of supernatants were spotted onto the agar layer. The plates were left for 40 min at room temperature in sterile conditions before incubating them at the adequate temperature of growth of the test microorganism. A clear zone of inhibition of at least 2 mm in diameter was considered as positive.

2.12. Antifungal activity

The antifungal activity assay was performed by a modified overlay method (Magnusson and Schnurer, 2001). The fresh cultures of tested strains were plated into 13 mL of MRS 0.8% agar modified as follows: pH was adjusted to 6.2 and sodium acetate was omitted. After incubation for 48 h at 30 °C, a fungi spore-suspension (104 spores/mL) was spot inoculated on the agar plate, followed by incubation for 72 h, at 25 °C, to promote fungal growth. The overall growth of fungi was compared to that in control plate and evaluated as: no inhibition; identical fungi growth; medium or strong inhibition, retarded growth of fungi; very strong inhibition, no fungal growth.

2.13. Effect of pH, temperature, enzymes and different chemicals on antimicrobial activity

2.13.1 Effect of pH and temperature

CFS was obtained as described before. The pH sensitivity of the active substances was estimated by adjusting the pH of CFSs between 3 and 10 by using 1 N NaOH or 1 N HCl. After 2 h incubation at 37 °C, the pH was adjusted to 6.5 and the residual activity was tested as described earlier. MRS media adjusted to pH between 3 and 10 and untreated CFSs were used as controls.

To evaluate the heat stability of the active substances, CFSs (1 mL) of the overnight cultures of tested strains were incubated at 50 °C for 30 min, at 100 °C for 5, 15 and 30 min and autoclaved at 121 °C for 15 min. The residual activity was then tested as described previously by the agar-well diffusion assay against indicator strain *Lb. brevis* F.145. Untreated CFSs were used as control.

2.13.2 Effect of enzymes

To test enzymatic inactivation of active substances, the CFSs were treated with the following enzymes (final concentration 0.1 mg/mL and 1 mg/mL): protease TypeX (from *Bacillus thermoproteolyticus* rokko, Sigma) and α-chymotrypsin Type II (from bovine pancreas, Sigma) in 0.05 M Tris HCl (pH 7); protease Type VIII (from *Bacillus licheniformis*, Sigma) in 0.05 M Tris HCl (pH 8); pronase E (from *Streptomyces griseus*, Merck) in 0.05 M Tris HCl (pH 8); lipase type VII (from *Candida rugosa*, Sigma) in 0.05 M Tris HCl (pH 8); catalase (from bovine liver, Sigma) in 0.01 M phosphate buffer (pH 7); amylase (Sigma) in 0.01 M Tris HCl (pH 7). After 2 h of incubation at 37 °C enzyme activity was stopped by heating at 100 °C for 10 min. Untreated CFSs and reaction mixtures, where supernatants were replaced by MRS media (also incubated at 37 °C for 2 h) were used as controls. The remaining activity was tested by the agar-well diffusion assay against indicator strain *Lb. brevis* F.145.

2.13.3 Effect of different chemicals on antimicrobial activity

The CFSs of the tested strains were obtained as described before. CFSs were treated with SDS (sodium dodecyl sulfate) at a final concentration of 1% (w/v), TritonX-20, Triton X-80, Triton X-100, β-mercaptoetanol at a final concentration of 1% (v/v), Na-EDTA (ethylene diamine tetra acetic acid) at a final concentration of 1mM and NaCl at a final concentration of 6.5% (w/v). After 2 h of incubation at 37 °C the residual activity of treated CFSs was tested as described before. Untreated CFSs of each producer strain and the same chemicals re-suspended in sterile MRS media served as controls. The remaining activity was tested by the agar-well diffusion assay against indicator strain *Lb. brevis* F.145.

2.14. Kinetics of bacteriocin production

The time course of bacteriocin production was determined in MRS media at 37 and 30 °C depending on the strain tested. MRS broth was inoculated with overnight cultures (2% v/v) and incubated under non regulated pH conditions. Antimicrobial activity (AU/mL) of the bacteriocin and modifications in pH and optical density (OD_{600nm}) of the cultures were determined at regular intervals (1h) for 24 h. Antimicrobial activity in AU/mL was calculated according to spot-on-lawn method (Yamamoto et al. 2003). CFSs were obtained as described previously. The resulting sample was serially diluted twofold with Na-phosphate (100mM, pH 6.5) and each dilution was spotted on the lawn of agar media containing sensitive strain. The plates were incubated at 37 °C overnight and the titer was defined as the reciprocal of the highest dilution (2^n) that resulted in inhibition of the indicator lawn. Thus, the AU of antimicrobial activity per millilitre was defined as $2^n \times 1000$ µl 10 $µl^{-1}$. *Lb. brevis* F145 was used as sensitive strain.

2.15. Partial purification of bacteriocins

The CFSs from 0.5 L of overnight cultures were obtained as described before. Ammonium sulfate was gently added to the supernatant maintained at 4 °C to obtain 60% saturation and the mixture was stirred for 4 h at 4 °C. After centrifugation for 1 h at 20000 x g at 4 °C, the resulting pellet was re-suspended in 30 mL of 25 mM ammonium acetate buffer (pH 6.5) and loaded on a SepPakC18 cartridge (Waters, Millipore, Milford, MA). The cartridge was activated by loading 80% iso-propanol / 25 mM ammonium acetate buffer (pH 6.5), sample loaded and elution was performed using 25 mM ammonium acetate buffer (pH 6.5) containing 20, 40, 60 and 80% isopropanol. Bacteriocins were eluted with 60% isopropanol in 25 mM ammonium acetate buffer (pH 6.5). After drying under reduced pressure (Speed-Vac), the 60% isopropanol fraction was dissolved in ultra purified water and used in the characterisation tests.

2.16. Effects of the bacteriocin-containing cell-free supernatants on the growth of indicator strains

The effects of CFSs of strains *Lb. curvatus* A61 and *E. faecium* AQ71, on the growth of indicator strains (*Lb. brevis* F145 and *Listeria monocytogenes* 506) were tested according to Todorov et al. (2010). Cell-free supernatants were prepared as described before. Aliquots (10 mL) of filter-sterilized (0.20 μm, Millipore) CFS were added to 100 mL culture of indicator strain in early exponential phase (3-h-old culture) and incubated at 37 °C. The optical density at 600 nm (Ultraspec 2000, Pharmacia Biotech, Piscataway, NJ) was monitored at 1 h intervals during 24 h.

2.17. Adsorption of bacteriocins on indicator strains

Adsorption of partially purified bacteriocins on the target pathogens was tested according Todorov (2008). Selected indicator strains were *Listeria monocytogenes* 506, 409, 302 (sensitive) and 211 (resistant) (Table 1). Cells of overnight cultures of target strains were pelleted by centrifugation (8000 x g, 15 min, 4 °C), washed twice with sterile Na-phosphate (5 mM, pH 6.5) and re-suspended to the original volume in the same buffer. The pH was adjusted to 6.5 with sterile 0.1 M NaOH and each cell suspension was mixed with equal volumes of partially purified bacteriocins. Reaction mixtures were incubated for 1h at 37 °C. After removal of cells (8000 x g, 15 min, 4 °C), the activity of unbound bacteriocin was determined by calculation of AU/mL and compared with original bacteriocin activity by following formula:

% of adsorption = 100 - [(bact activity after treatment/original bact activity) x 100].

2.18. Auto-aggregation and co-aggregation

Strains were grown up to 24 h in in MRS broth. The cells were harvested by centrifugation (7000 x g, 10 min, 20 °C), washed and re-suspended in sterile solution of 0.85% NaCl (w/v) to OD_{600nm} = 0.3 using spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Piscataway, NJ). After 1 h incubation at 37 °C the cells suspensions were centrifuged at 300 x g for 2 min at 20 °C and upper part of suspensions were taken for OD_{600nm} measurement. Auto-aggregation was determined using the following equation (Todorov et al. 2008): % auto-aggregation = $[(OD_0 - OD_{60})/OD_0] \times 100$, where OD_0 refers to the initial OD and OD_{60} refers to the OD determined after 1 h of incubation.

For evaluation of co-aggregation, the cell suspensions of studied strains *Lb. curvatus* A61 and *E. faecium* AQ71 were prepared as described above. The indicator strains *Listeria monocytogenes* 506, 409 and 302 (sensitive to the bacteriocins) and *Listeria monocytogenes* 211 (resistant to the bacteriocins) were cultivated in BHI, at 37 °C for 24 h and the cells suspensions were prepared in a similar way as for LAB strains. Cells suspensions of LAB strains were mixed with each other, as well with each indicator strain at the ratio 1/1 (v/v) and incubated for 1 h at 37 °C. Not paired cells suspensions of each strain (LAB and indicator) were also prepared in the similar way. Over the incubation time the cells suspensions were centrifuged at 300 g for 2 min at 20 °C and upper part of suspensions were taken for OD_{600nm} measurement. The degree of co-aggregation of paired cells suspension was calculated using the following equation (Todorov et al., 2008): % co-aggregation = $[(OD_0 - OD_{60})/OD_0] \times 100$, where OD_0 refers to the initial OD and OD_{60} refers to the OD determined after 1 h of incubation.

2.19. PCR amplification of bacteriocin genes

The presence of genes encoding different bacteriocin genes was tested by polymerase chain reaction (PCR), using primers and conditions described previously (Table 2.3). PCR reactions were performed in 100 µL volume of mixtures containing 30 ng of bacterial DNA, 1 x PCR reaction buffer, 1.5 mM $MgCl_2$, 0.5 U of Taq DNA polymerase, 200 µM of dNTPs mix and 1 µM of each primer. PCR amplifications were carried in Veriti® 96-Well Thermal Cycler (Applied Biosystems) using a program consisting of the initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at an appropriate temperature depending on Tm of primers for 1 min, elongation at 72 °C for 1.0 min and a final extension step of 7 min at 72 °C. As a negative control sample without template DNA was used. Amplicons were loaded to 2% agarose gel and runned in 0.5 x TAE buffer for 30 min at 100 V. Visualisation of amplifications products was made by UV trans-illumination after staining with ethidium bromide (0.5 mg/mL). The PCR products were purified by QIAquick PCR purification

kit (Qiagen) and sequenced by Human Genome Research Center of University of Sao Paulo (Sao Paulo, Brazil). The blast algorithm (http://blast.ncbi. nlm.nih.gov/Blast) was applied to determine the most related sequence relatives in the NCBI nucleotide sequence database.

Table 2.3. Primers used for detection of bacteriocin genes

Target gene	Primer (5' – 3')	Product size (bp)	Ann. Temp (ºC)	Reference
Plan NS8	F: GGT-CTG-CGT-ATA-AGC-ATC-GC R: AAA-TTG-AAC-ATA-TGG-GTG-CTT-TAA-ATT-CC	207	45	Maldonado et al., 2004
Plan S	F : GCC-TTA-CCA-GCG-TAA-TGC-CC R : CTG-GTG-ATG-CAA-TCG-TTA-GTT-T	450	45	Reminger et al., 1996
Plan W	F : TCA-CAC-GAA-ATA-TTC-CA R : GGC-AAG-CGT-AAG-AAA-TAA-ATG-AG	165	41	Gyu-Sung et al., 2010
Sak GA1	F: TTA-GAA-CTA-CAC-TGA-TCG-TG R: TGG-AAG-AAT-GAG-TAC-TTG-TT	186	38	Todorov et al., 2011
Sak GA2	F: CGT-TAC-AAC-AGA-ACT-TCA-AG R: TGG-AAG-AAT-GAG-TAC-TTG-TT	186	38	Todorov et al., 2011
Sak P	F: ATG-GAA-AAG-TTT-ATT-GAA-TTA R: TTA-TTT-ATT-CCA-GCC-AGC-GTT	186	40	Reminger et al., 1996
Ped PA1	PedrpoF: CAA-GAT-CGT-TAA-CCA-GTT-T PedC1041: CCG-TTG-TTC-CCA-TAG-TCT-AA	1238	44	Van Reenen et al., 1998
CurvA	CurAF: GTA-AAA-GAA-TTA-AGT-ATG-ACA CurvAR: TTA-CAT-TCC-AGC-TAA-ACC-ACT	171	40	Reminger et al., 1996
Ent A	F: GAG-ATT-TAT-CTC-CAT-AAT-CT R: GTA-CCA-CTC-ATA-GTG-GAA	542	45	Aymerich et al., 1996
Ent B	F: GAA-AAT-GAT-CAC-AGA-ATG-CCT-A R: GTT-GCA-TTT-AGA-GTA-TAC-ATT-TG	159	41	Du Toit et al., 2000
Ent P	F: ATG-AGA-AAA-AAA-TTA-TTT-AGT-TT R: TTA-ATG-TCC-CAT-ACC-TGC-CAA-ACC	216	41	Gutierrez et al., 2002
Ent L50A	F: CCA-TGG-GAG-CAA-TCG-CAA-AA R: AAG-CTT-AAT-GTT-TTT-TAA-TCC-ACT-CAA-T	135	50	Batdorj et al.,2006
Ent L50B	F: ATG-GGA-GCA-ATC-GCA-AAA-TTA R: TAG-CCA-TTT-TTC-AAT-TTG-ATC	252	49	Cintas et al., 1998
Ent 31	F: CCT-ACG-TAT-TAC-GGA-AAT-GGT R: GCC-ATG-TTG-TAC-CCA-ACC-ATT	130	58	de Vuyst et al., 2003
Ent Q	F: GGA-ATA-AGA-GTA-GTG-GAA-TAC-TGA-TAT-GAG-AC R: AAA-GAC-TGC-TCT-TCC-GAG-CAG-CC	653	60	de Vuyst et al., 2003
Ent AS48	F: GAG-GAG-TAT-CAT-GGT-TAA-AGA R: ATA-TTG-TTA-AAT-TAC-CAA	253	56	de Vuyst et al., 2003

2.20. Probiotic properties and safety of isolated LAB strains

2.20.1. Growth in the presence of bile salts and at different pH values

Studied strains were grown in MRS broth adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0, 9.0, 11.0 and 13.0 with 1 M HCl or 1 M NaOH before autoclaving. To check the bile resistance, strains were grown in MRS broth containing 0.2, 0.3, 0.5, 0.6, 1.0, 2.0 and 3.0% oxbile (Sigma). Cultures grown in MRS broth without bile served as control. All tests were conducted in sterile flat-bottom 96-well microtitre plates (TPP; Zellkultur testplatte, Trasadingen, Switzerland). Each well was filled with 100 µL of the medium and inoculated with 5 µL of the overnight cultures. Optical density readings were recorded at 600 nm every hour for 12 h, using a microtitre plate reader (TPP).

2.20.2. Assessment of resistance to antibiotics

Resistance to antibiotics was tested by disk diffusion method. Eight antibiotics were used: ampicillin, ciprofloxacin, vancomycin (powder, all from Sigma), tetracycline, kanamycin, chloramphenicol, gentamicin and penicillin and (powder, all from Euromedex). Applied concentrations of antibiotics ranged from 0.2 to 512 µg/mL. The level of susceptibility to antibiotics was reported as: resistant, intermediate or sensitive according to the observed breaking points recommended for *Enterococci* by the NCCLS antimicrobial susceptibility testing standards (NCCLS, 2004), and for *Lactobacilli* according to European Commission's Scientific Committee on Animal Nutrition and by Danielson and Wind (2003).

2.20.3 Screening for the presence of virulence genes

The presence of genes encoding cytolysin (*cylA, cylB, cylM, cylLL, cylLS*), gelatinase (*gelE*), enterococcal surface protein (*esp*), cell wall adhesins (*efaAfs*) aggregation substance *(asa1*), adhesin of collagen protein (*ace*) and fsr locus (*fsrA, fsrB and fsrC*) was tested by polymerase chain reaction (PCR), using primers and conditions described previously (Table 2.4). The oligonucleotide primers were purchased from Eurogentec (Belgium). PCR amplifications were performed in 20 µL volume. Reaction mixtures contained 40 ng of bacterial DNA, 5 µL 5 x GoTaq reaction buffer, 1.5 mM $MgCl_2$, 1 U of GoTaq DNA polymerase, 200 µM of dNTPs mix (all from Promega) and 200 nmol of each primer. Amplifications were carried out using a DNA thermal cycler model (Techno, Barloworld scientific, Cambridge, UK). The program consisting of the initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at an appropriate temperature for 1 min (Table 1), elongation at 72 °C for 1 min and a final extension step of 7 min at 72 °C was applied. For each PCR, a negative control

(sample without template DNA) and a positive control (sample with DNA from strain *E. faecalis* MMH594) were included. 1.5% agarose gel was used for analysis of PCR products as described previously.

Table 2.4. Primers and PCR conditions used for the screening of virulence genes

Primers	Specificity	Ann. temp (°C)	Prod. size (bp)	Reference
TE5 / TE6	Cell wall adhesins expressed in serum by *E. faecalis (efaAfs)*	54	705	Eaton et al.,2001
ASA11 / ASA12	Aggregation substance (*asa1*)	56	375	Vankerckhoven et al., 2004
ESP14F / ESP12R	Enterococcal surface protein (*esp*)	56	510	Vankerckhoven et al., 2004
ACE-F / ACE-R	Adhesin of collagen protein (*ace*)	56	1008	Ben Omar et al., 2004
CYT I / CYT IIb	Cytolysin (*cylA*)	58	688	Vankerckhoven et al., 2004
cylLL1 / cylLL2	*cylLL*	54	253	Semedo et al., 2003
cylLs1 / cylLs2	*cylLs*	54	240	Semedo et al., 2003
cylM1 / cylM2	*cylM*	58	2940	Semedo et al., 2003
cylB1 / cylB2	*cylB*	52	2020	Semedo et al., 2003
GelE1F / GelE1R	Gelatinase (*gel*)	45	213	Qin et al., 2001
fsrAF1 / fsrAR1	*fsrA*	43	740	Qin et al., 2001
fsrBF1 / fsrBR1	*fsrB*	43	710	Qin et al., 2001
fsrCF1 / fsrCR1	*fsrC*	43	1300	Qin et al., 2001

3. RESULTS AND DISCUSSION

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of proteolytic LAB

A collection of 431 isolates (Table 3.1) from traditional Azerbaijani dairy products was screened for the presence of proteolytic enzymes using skim milk agar technique and SDS-PAGE analysis of UHT skim milk inoculated with isolates.

Table 3.1. Presentation of LAB isolates obtained from dairy products from different regions of Azerbaijan

City	Fermented milk product	Number of samples	Number of LAB isolates	
			Bacilli	Cocci
Barda	White cheese	2	3	12
	Yogurt	1	0	4
Dashkesen	White cheese	1	2	7
	Yogurt	1	1	3
	Shor (curd cheese)	1	0	4
Hajikabul	Shor (curd cheese)	2	2	5
	Yogurt	1	0	2
Ismailly	White cheese	3	7	14
Imishly	Motal cheese	2	9	18
	Shor (curd cheese)	1	2	3
	White cheese	1	5	7
Lankaran	White cheese	2	7	13
Qazakh	Motal cheese	6	25	42
	Yogurt	1	1	6
	White cheese	1	7	9
Qanja	Motal cheese	3	7	25
	White cheese	1	1	18
Quba	White cheese	3	10	24
Sabirabad	Motal cheese	2	3	17
	White cheese	1	4	8
Siazan	White cheese	2	2	12
Shamakhi	White cheese	2	7	7
Shaki	Motal cheese	2	6	14
Sumqait	White cheese	1	3	8
	Yogurt	1	0	3
Zaqatala	White cheese	2	13	19
TOTAL				
15		46	127	304

As it was described in Materials and Methods chapter we performed the screening by two methods. First, we screened proteolytic isolates directly from the cheese samples using milk-agar technique. Using this method, 56 isolates were obtained, which gave clear zones on skim milk agar after 24 h incubation. Example of the results obtained with this technique is presented in Fig. 3.1.

Examination with optical microscope, test for production of catalase and Gram test resulted in total 32 isolates, which were Gram-positive and catalase-negative cocci (28) and bacilli (4). These isolates were preliminarily classified as proteinase-positive LAB and their proteolytic activities were confirmed by another method. Thus, isolates were inoculated into UHT skim milk and incubated for 24 h at 37 °C. After incubation samples of fermented milk were analyzed by SDS-PAGE. Among tested 22 isolates only 6 isolates (cocci) presented proteolytic activities (Fig. 3.2, Lanes 1-6), while 18 isolates did not show any measurable proteolytic activity (data not shown).

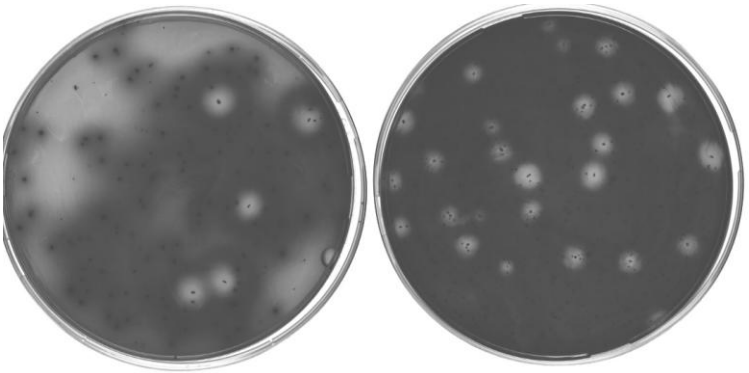


Figure 3.1. Screening of proteolytic LAB isolates by milk-agar technique

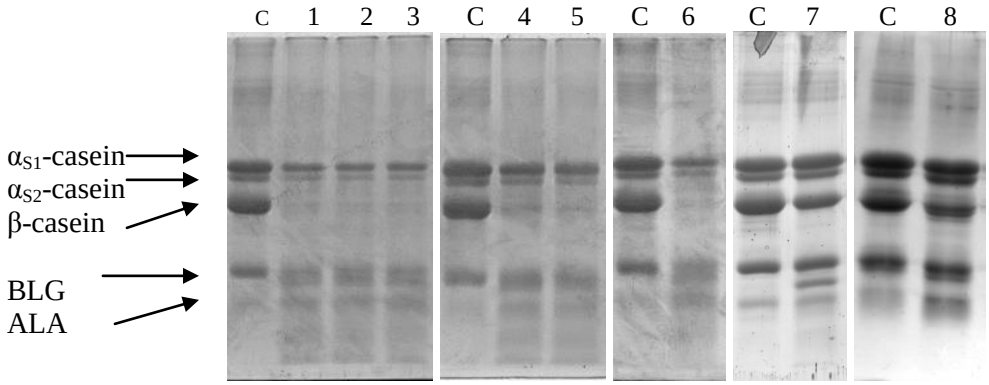


Figure 3.2. SDS-PAGE of UHT skim milk hydrolyzed by obtained LAB isolates after 24 h. Lanes from 1 to 8 isolates named AN1, A121, A1221, A124, A1232, A71, A75, A581

At the same time, randomly selected colonies were isolated. They did not give clear zones on skim milk agar. It is supposed that for some LAB this medium is not optimal for production of proteases. It is known that some LAB could grow and exhibit higher metabolic activities in liquid compared to solid media. Also, as it is known, proteolytic system of LAB has very sensitive and complex regulation. So, the expression of proteases could differ depending on temperature, media and other factors. The used conditions for the screening of proteolytic LAB

strains, could be optimal for proteases of some of them and at the same time could inhibit the proteases expression in the other strains.

SDS-PAGE analysis of fermented milk samples resulted in 2 more isolates (bacilli), however, their activity was very low (Fig. 3.2. Lanes 7 and 8).

Total results of screening and identification of proteolytic LAB are presented in Table 3.2. In total, the screening for proteolytic LAB resulted in 6 isolates (cocci) with high proteolytic activities and 2 isolates (bacilli) with lower proteolytic activities. In case of bacilli 16S rDNA fragment sequencing resulted in identification of one isolate as *Lb. helveticus* and another isolate as *Lb. paracasei* subsp. *paracasei* (more than 99% identity). It is known, that in some cases of lactobacilli 16S rDNA fragment sequencing is not enough for the species classification of the strain, thus, we can conclude that two obtained lactobacilli isolates are most probably belong to the above mentioned species, however additional identification tools are necessary for final taxonomic classification of these isolates. The source of the natural isolate *Lactobacillus helveticus* A75 was a semi-hard white cheese manufactured from cow's milk by addition of 1% (v/v) homemade rennet to pasteurized milk. Cheese ripened for 1.5 month in the presence of 3% NaCl. *Lb. paracasei* subsp. *paracasei* A581 were isolated from soft white cheese named *"Shor"*, manufactured from cow's milk.

Table 3.2. Identified proteolytic strains isolated from cheese samples manufactured in different regions of Azerbaijan

Fermented milk product	**City**	**Strain**
White cheese	Qazakh	*Lactobacillus helveticus* A75
Shor	Hajikabul	*Lactobacillus paracasei* subsp. *paracasei.* A581
Motal cheese	Ismailly	*Enterococcus faecalis* AN1
White cheese	Quba	*Enterococcus faecalis* A71
White cheese	Barda	*Enterococcus faecalis* A121
		Enterococcus faecalis A124
		Enterococcus faecalis A1221
		Enterococcus faecalis A1232

The source of these highly proteolytic cocci was found in 3 cheese samples manufactured from cow's milk by addition of 1% (v/v) homemade rennet to pasteurized milk. Two samples were semi-hard white cheeses ripened during 1.5 month in the presence of 3% NaCl. Third sample was yellow cheese with hard consistence and the ripening of this cheese was performed

inside the sheepskin in the presence of 3% NaCl for 4 months. These isolates showing high proteolytic activity in UHT skim milk were classified to *Enterococcus* genus by genus specific primers (Fig 3.3) and 16S rDNA fragment sequencing resulted in identification of all strains as *Enterococcus faecalis*. In order to identify if these isolates are different strains, or just the multiply copy of one, their genetic diversity was investigated by RAPD-PCR analysis. In RAPD-PCR, also referred to as arbitrary primed PCR, patterns are generated by the amplification of random DNA segments with single short (typically 10 bp) primers of arbitrary nucleotide sequence. The primer is not targeted to amplify any specific bacterial sequences and will hybridise at multiple random chromosome locations and initiate DNA synthesis. After separation of the amplified DNA fragments by agarose gel electrophoresis, a pattern of bands results, which is characteristic of the particular bacterial strain.

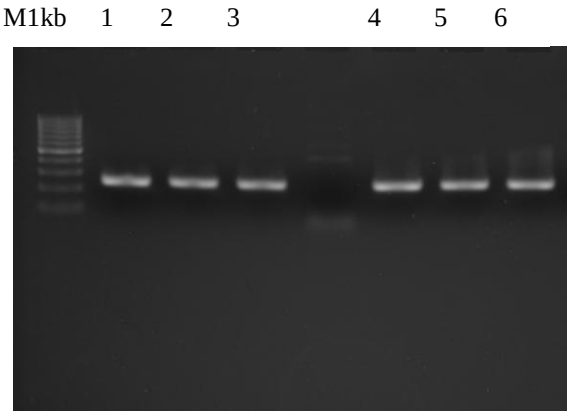


Figure 3.3. Identification of proteolytic cocci isolates with genus-specific primers. 1.2% agarose gel of amplified PCR products.
Lanes from 1 to 6 isolates named AN1, A121, A1221, A124, A1232, A71

Cluster analysis of the obtained patterns divided the studied strains into 3 clusters (Fig. 3.4) one of which consisted from 3 sub-clusters. This cluster was composed of 4 strains isolated from the same cheese sample. Strains A121 and A124 grouped in the first sub-cluster showed similar profiles (nearly 92%) and can therefore be considered as extremely close genotypically and perhaps identical. Strains A1221 and A 1232 also showed high level of similarities with these strains (77% and 68%, respectively). Other two strains (A71 and AN1) showed different RAPD patterns with similarity levels of 16% and 25%, respectively. These strains were isolated from two different cheese samples.

Although genotyping techniques may serve to reduce the number of isolates when screening for novel enzymatic activities, it has been reported that in the group of *L.lactis* even 100% identity in RAPD patterns showed large differences in phenotypical traits such as proteinase activity and acidifying capacity (De la Plaza et al., 2006). For this reason, we included

all strains, even that which gave identical pattern in RAPD fingerprinting for further investigations.

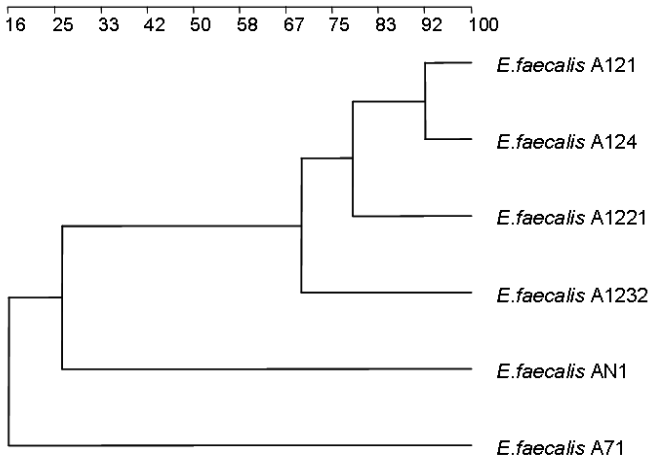


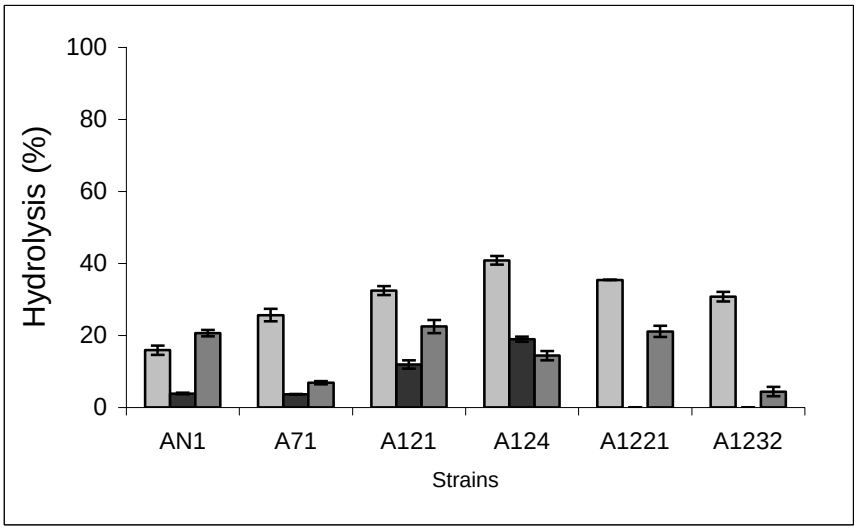
Figure 3.4. Cluster analysis of RAPD-PCR bands profiles. Dendrogram obtained by means of UPGMA (Unweighted Pair Group Method using Arithmetic Average) clustering algorithm

3.2. Proteolytic activities

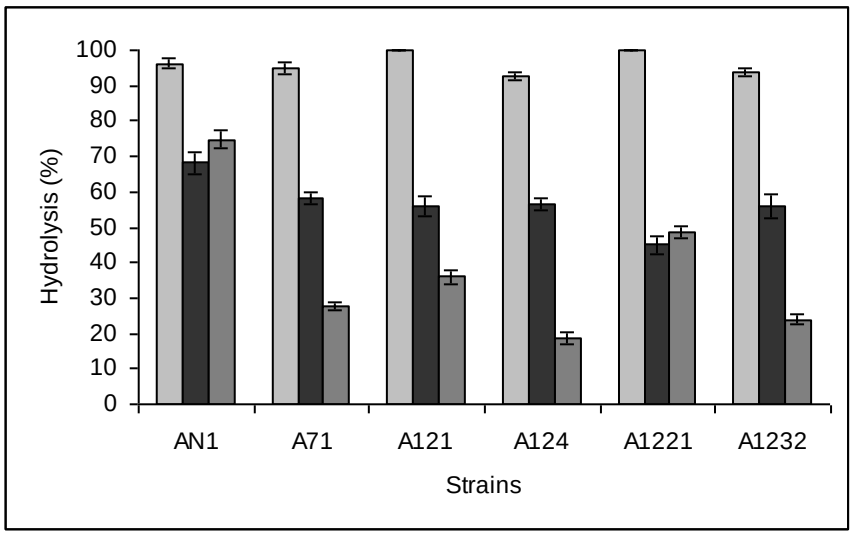
3.2.1. Proteolytic activities in UHT skim milk

Proteolytic activities of identified strains grown in pH uncontrolled conditions in UHT skim milk revealed that among studied microorganisms only *Enterococci* showed remarkable activity. *Lactobacilli* presented very low activity even after 24 h incubation with very little hydrolysis of only β-casein (Fig. 3.2. Lanes 7 and 8).

In case of *Enterococci* different extents of α_{S1}-, α_{S2}- and β-caseins, and β-lactoglobulin (BLG) hydrolysis was observed. Hydrolysis of α-lactalbumin (ALA) was not seen in any of studied strain. In order to facilitate interpretation and discussion of results we present the proteolysis *ratio* in percentage of hydrolysis, which was calculated as described in Materials and methods section. Proteolysis was observed after 3 h of cultivation in milk and increased with the time of incubation (Fig. 3.5 a, b). Highest degree of hydrolysis was observed for β-casein (more than 90% after 24 h incubation for all the studied strains). Degradation of α_{S1}- and α_{S2}-caseins was lower with different degrees of hydrolysis depending on the strain: from 45 to 70% for α_{S1}-casein and from 20 to 75% for α_{S2}-casein after 24 h incubation. Hydrolysis of BLG (Fig. 3.6) was also high (from 45 to 65% after 24 h incubation). The proteolytic activity of these *Enterococci* was detally described in our publication (Ahmadova et al., 2011). Similar to our results were observed in the study of El-Ghaish et al. (2010) where they observed efficient hydrolysis of milk proteins by dairy *Enterococci* isolates.



a



b

Figure 3.5. Hydrolysis of different caseins of skim milk during 3 h (**a**) and 24 h (**b**) fermentation with *E. faecalis* strains at 37 ºC: □ β-casein; ■ α_{S1}-casein; ■ α_{S2}-casein

Hydrolysis of β-casein was observed after 3 h incubation for all strains. However, hydrolysis of α_{S1}- and α_{S2}-caseins was not observed in all strains at this time point, when the pH of the medium was still close to neutral (pH 6.0–6.5). After 24 h incubation, when the pH of milk decreased below 4.8, hydrolysis yield of α_{S1}-, α_{S2}- and β-caseins increased. This is clearly observed in the case of strain AN1, which showed lower proteolytic activity towards caseins and BLG (Fig. 3.5 and Fig. 3.6) as compared with other strains at the beginning of the exponential growth phase (after 3 h incubation) when the pH of milk was near neutral value and highest

degree of hydrolysis (96% of β-, 68% of α_{S1}-, 74% of α_{S2}-caseins and 65% of BLG) at the end of incubation time when the pH decreased below 4.8.

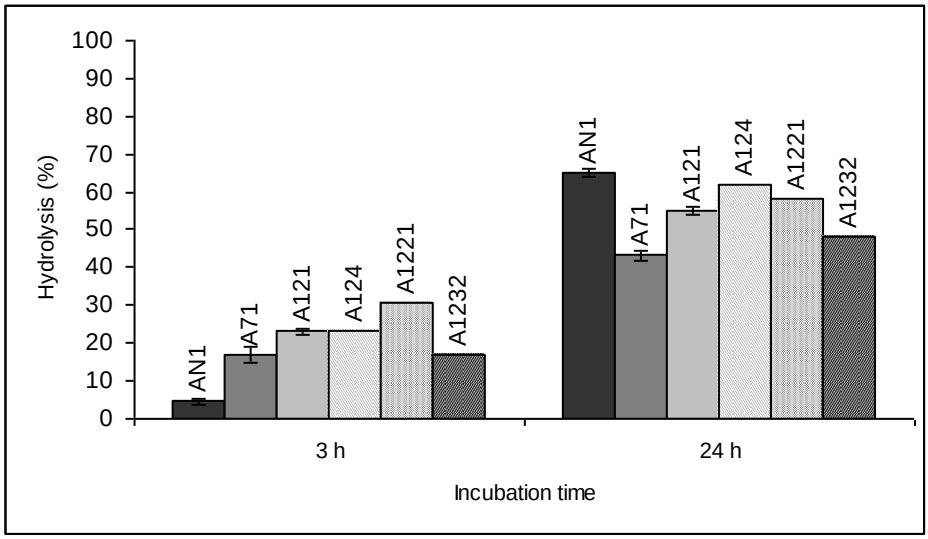


Figure 3.6. Hydrolysis of BLG of skim milk during fermentation with *E. faecalis* strains at 37 ºC. From left to right: AN1, A 71, A 121, A 124, A 1221 and A 1232

It seems that proteases produced by the studied strains are different in their substrate preferences since the start point of β- and α_S-caseins hydrolysis was not the same for all strains. In another study, Psoni et al. (2006) found that *Enterococci* isolates hydrolyzed faster β-casein than other caseins, but at the end of incubation α_S-caseins were more hydrolyzed. In the same study selected *Enterococci* strains hydrolyzed preferentially α_{S2}-casein.

3.2.2. Proteolytic activities in non-proliferative cells system

The ability of the strains to hydrolyze different fractions of milk proteins was also determined in non-proliferative cells system. In this case, after induction of proteinase activity on milk-citrate agar media, bacterial cells were collected at stationary phase of growth and their ability to hydrolyze different fractions of milk proteins was assessed at pH 7.2.

Strains *E. faecalis* AN1, A121, A124, A1221, A1232, A71 were able to hydrolyze all casein fractions. *Lb. paracasei* A581 was unable to hydrolyze α_S-caseins at all, displaying specific activity only to β-casein. *Lb. helveticus* A75 showed proteolytic activity different to what was observed during growth in UHT skim milk. In non-prolifirating cells system this strain presented higher activity and was able to hydrolyze all casein fractions. This difference in proteolytic activity level could be due to pH and substrate difference used in both systems. It is possible, that proteases produced by this strain are more active at neutral pH.

The evolution in time of Na-caseinate hydrolysis by studied strains is shown in Fig. 3.7 and the HPLC profiles of released peptides are shown in Fig 3.8. Caseins degradation was detectable after a short incubation period (3 h) and continued up to 24 h of incubation. All strains hydrolyzed β-casein more efficiently than α_{S1}-and α_{S2}-caseins. Strain *E. faecalis* AN1 displayed the highest proteolytic activity and hydrolyzed totally all caseins after 9 h incubation. Strain *E. faecalis* A1232 displayed the lowest proteolytic activity among studied *Enterococci* strains. Hydrolysis of β-casein was observed after 3 h incubation with almost total degradation after 24 h in all strains studied. After 3 h of incubation, hydrolysis of α_{S1}-casein was very low (except for strain *E.faecalis* AN1). Strains *E. faecalis* A1232 and *Lb. helveticus* A75 were unable to hydrolyze it entirely (70% and 60% hydrolysis respectively) even after 24 h incubation. In case of other strains almost total (98%) hydrolysis of α_{S1}-and α_{S2}-caseins was observed after 24 h incubation.

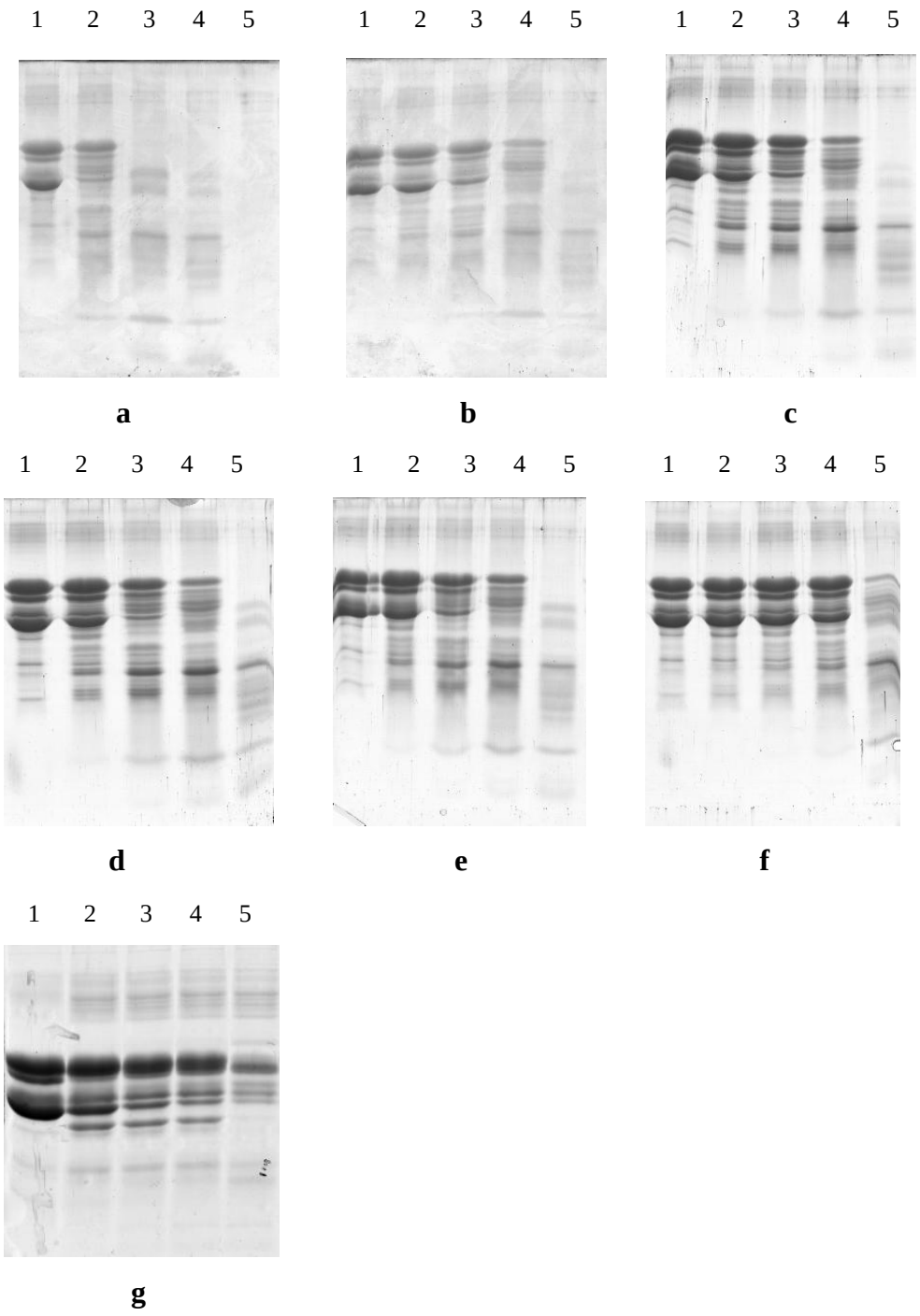


Figure 3.7. SDS-PAGE profile of Na-caseinate hydrolyzed by strains in non-proliferating cells system at 37 ºC (from **a** to **g**: strains *E. faecalis* AN1, A71, A121, A1221, A124, A1232 and *Lb. helveticus* A75 respectively). Lane 1: control (substrate incubated in the absence of cells), lanes 2 to 5: samples (substrate incubated in the presence of cells) taken after 3, 6, 9 and 24 h incubation with substrate

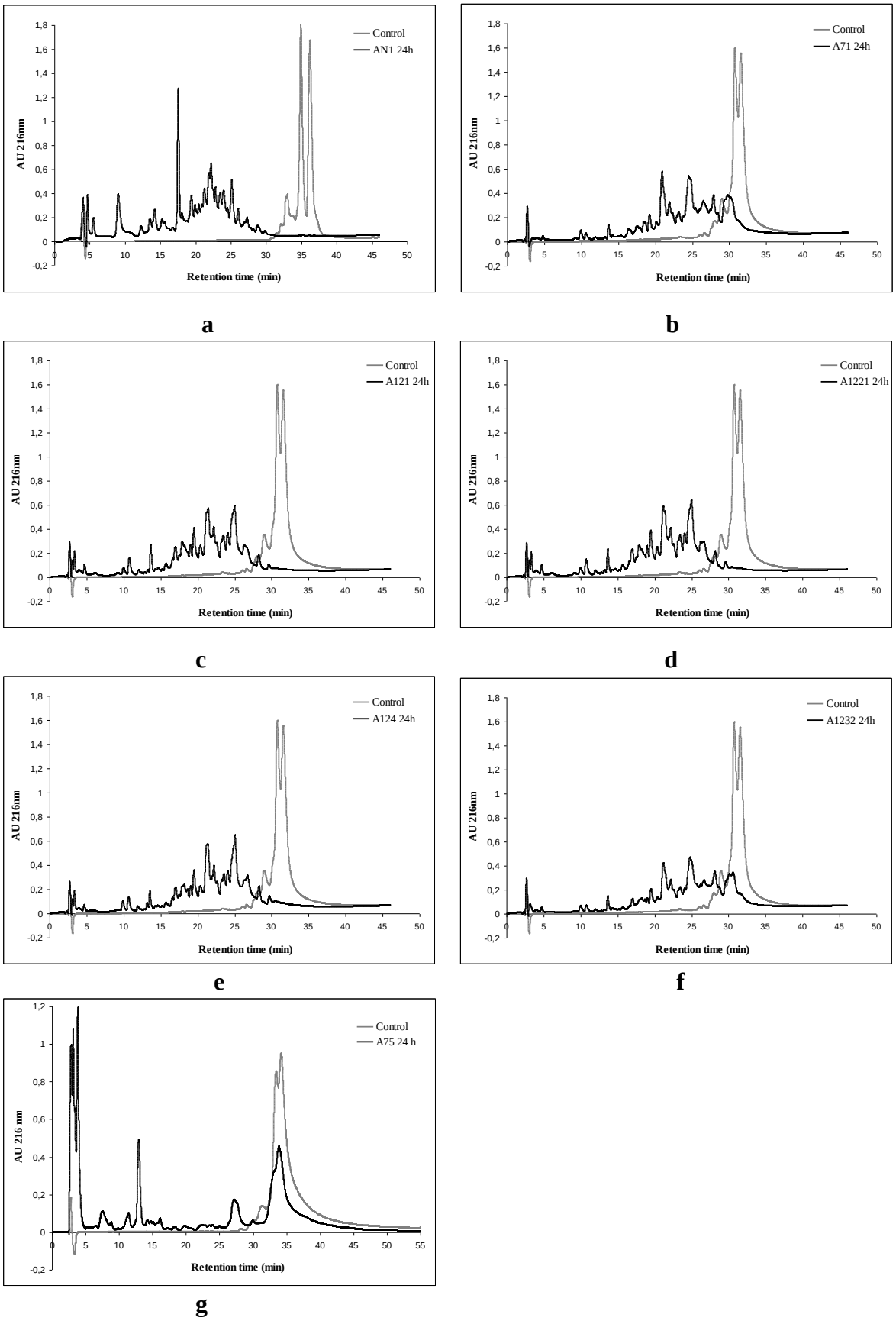


Figure 3.8. RP-HPLC profile of peptides released from Na-caseinate (24 h incubation at 37 ºC) by proteases of isolated strains (from **a** to **g**: strains *E. faecalis* AN1, A71, A121, A1221, A124, A1232 and *Lb. helveticus* A75 respectively). Control: grey line; hydrolysate: black line

Lb. paracasei A581 was not able to hydrolyze α_S-caseins at all, displaying specific activity only to β-casein (Fig. 3.9 **a** and **b**). The similar specificity toward β-casein was also observed during its growth in UHT skim milk. In non-proliferating cells system this strain was able to hydrolyze to some extent β-casein fraction of Na-caseinate. When pure β-casein was used as substrate, total hydrolysis was observed after 24 h; however, the presence on the gel of big peptides could not be confirmed. Similar results were observed in a study of Kojik et al. (1995), where the proteolytic activity of two studied *Lb. paracasei* strains was also specific toward β-casein.

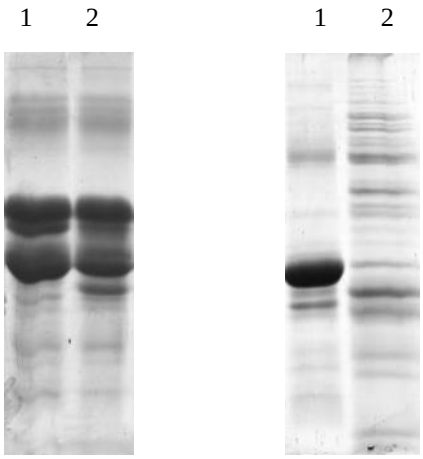
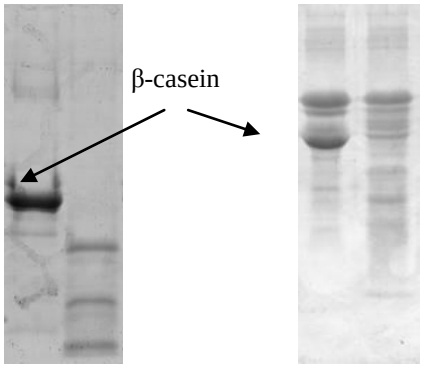


Figure 3.9. SDS-PAGE profile of Na-caseinate (**a**) and β-casein (**b**) hydrolyzed by strain *Lb. paracasei* A581 in non-proliferating cells system (Lane 1 - control (substrate incubated in the absence of cells), lane 2 - sample (substrate incubated in the presence of cells) taken after 24 h incubation at 37 ºC with substrate

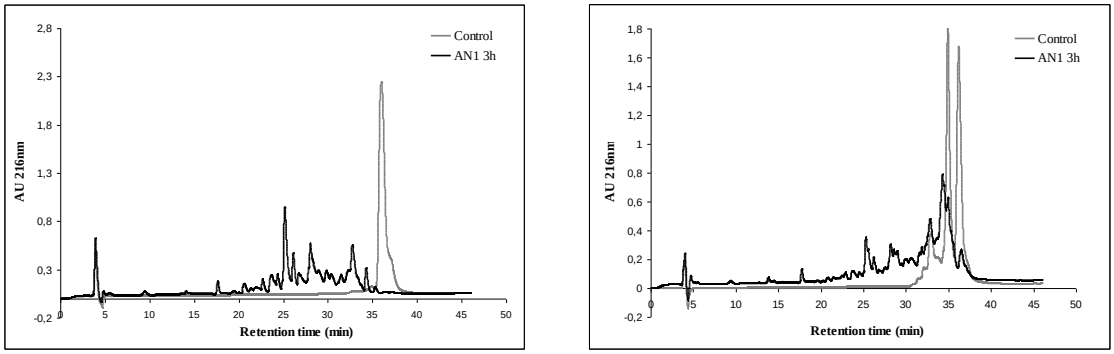
In our study, it was observed that the time course of caseins hydrolysis by proteinases produced by the studied strains was also dependent on substrate used. For example, hydrolytic activity of *Enterococcus faecalis* AN1 toward ß-casein was higher when purified ß-casein was used as substrate and total hydrolysis of this protein was observed after 3 h incubation (Fig 3.10 a and Fig. 3.11 a). However, when total caseins were used as a substrate, total hydrolysis of ß-casein was not reached at the same time point (Fig 3.10 b and Fig 3.11 b).

Something similar was observed also in the case of *Lb. helveticus* A75. In contrast to results obtained with Na-caseinate (Fig. 3.7 g and 3.8 g), the strain was able to hydrolyze after 24 h incubation α_{S1}- and β-caseins entirely when they were used in pure form (Fig. 3.12 a, b). It is possible that the differences in the observed level of hydrolysis might be due to the different conformations of individual caseins when they are isolated or included inside whole casein.



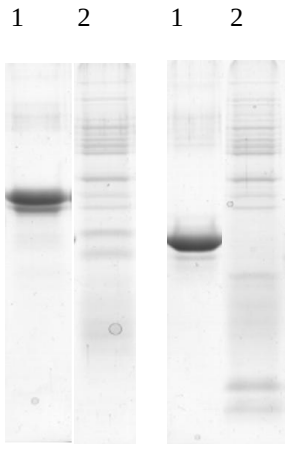
a **b**

Figure 3.10. SDS-PAGE profile of ß-casein (**a**) and Na-caseinate (**b**) hydrolyzed by *E. faecalis* AN1 in non-proliferating cells system after 3 h of incubation at 37 ºC. Lane 1: control (substrate incubated in the absence of cells), lane 2: samples (substrate incubated in the presence of cells)



a **b**

Figure 3.11. RP-HPLC profile of β-casein (**a**) and Na-caseinate (**b**) hydrolysed by *E. faecalis* AN1 during 3h incubation at 37 ºC. Control: grey line; hydrolysate: black line



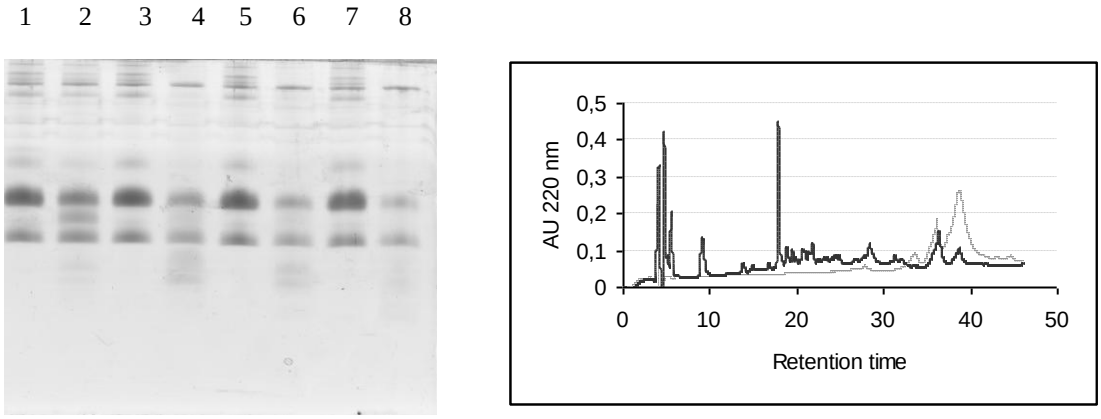
a **b**

Figure 3.12. SDS-PAGE of α_{S1}- (**a**) and β-caseins (**b**) hydrolysis after 24 h incubation with *Lb. helveticus* A75 at 37 ºC. Lane 1: control (substrate incubated in the absence of cells), lane 2: samples (substrate incubated in the presence of cells)

According to the substrate specificity, it can be concluded that proteinases of *E. faecalis* strains and *Lb. helveticus* A75 remind the lactococcal PIII type proteinases and the lactococcal PI type proteinase of strain *Lb. paracasei* A581.

Hydrolysis of denaturated whey proteins resulted in BLG degradation only by proteases produced by strain *E. faecalis* AN1. In case of other strains, hydrolysis of beta-lactoglobulin (BLG) was not observed (data not shown). In contrast, the hydrolysis of BLG by all studied *E. faecalis* strains was observed in UHT skim milk. Observed differences in substrate specificities for BLG in two studied LAB systems could be due to different factors. They could include differences in conditions and temperatures of denaturation of substrate whey proteins and of UHT milk, effect of casein micelles and other components of milk on denaturation and formation of BLG-ALA aggregates and also effect of pH differences in two studied systems on protease activities.

Kinetics of whey proteins hydrolysis by strain *E. faecalis* AN1 is shown in Fig. 3.13.a. This strain started to hydrolyze BLG after 3 h of incubation (22% of hydrolysis), but even after 24 h incubation total degradation of BLG did not occur (47% of hydrolysis). RP-HPLC analysis of denaturated whey proteins hydrolyzed by strain *E. faecalis* AN1 after 24 h incubation is shown in Fig. 3.13.b. Hydrolysis of BLG generated mostly medium-sized peptides with hydrophobic and moderate hydrophilic properties.



a **b**

Figure 3.13. Hydrolysis of denaturated whey proteins in non-proliferating cells system by strain *E. faecalis* AN1

(**a**) Tricine-SDS-PAGE. Lanes 1, 3, 5, 7: controls (substrate incubated in the absence of cells for 3, 6, 9 and 24 h, respectively); lanes 2, 4, 6, 8: samples (substrate incubated at 37 ºC in the presence of cells for 3, 6, 9 and 24 h respectively).

(**b**) RP-HPLC profile of peptides released after 24 h incubation with substrate at 37 ºC. Control: grey line; hydrolysate: black line

3.3. API ZYM

API ZYM test was performed only for *E. faecalis* strains in order to determine the enzymatic profiles of studied strains and to check the relevant technological properties of these strains for dairy industry. On the Fig. 3.14 is presented the profile of enzymatic activities of *E. faecalis* strains studied by API ZYM kit.

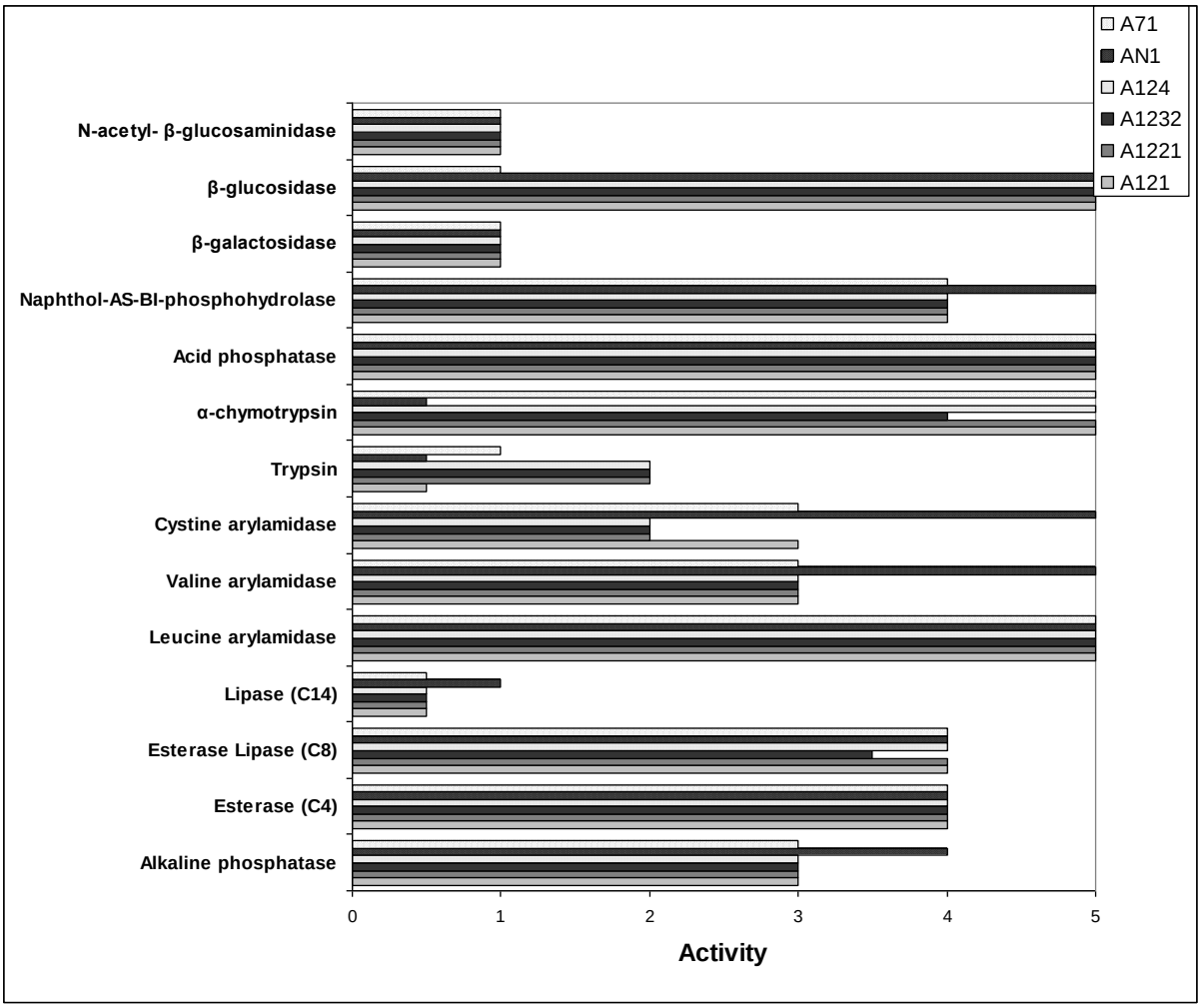


Figure 3.14. The profile of enzymatic activities of *E. faecalis* strains studied by API ZYM kit*

* The results were graded from 0 to 5 by comparison of the observed color with the color reaction chart. A value of 0 corresponded to negative reaction and 5 to a reaction of maximal intensity. The approximate number of free nmol hydrolyzed substrate is obtained from the color strength: 0 - no activity; 1 - liberation of 5 nmol; 2 - 10 nmol; 3 - 20 nmol; 4 - 30 nmol; 5 - 40 nmol.

All strains showed the C-4 esterase and C-8 esterase and lipase activities, but at the same time very low C-14 lipase activities. These results are in agreement with previously published data about *Enterococci*, what indicates also that the esterolytic systems of *Enterococci* are more efficient than lipolytic. This allows them to hydrolyze efficiently milk fat (Sarantinopoulos et al.,

2001; Giraffa et al., 2003). C-4 Esterase and C-8 esterase/lipase activities are important features, which give specific tastes to some cheeses (Tsakalidou et al., 1993). Investigated strains showed also good phosphohydrolytic and acid phosphatase activities, what is very important for the hydrolysis of phosphopeptides during the cheese ripening (Centeno et al., 1999; Franz et al., 2003).

3.4. Influence of different factors on proteolytic activity

3.4.1. Effect of pH and temperature on proteolytic activities

Determination of pH influence on hydrolysis of caseins showed that the proteinases produced by *Lb. helveticus* A75 hydrolyze substrate in a broad range of pH values. No significant differences were observed in Na-caseinate hydrolysis degree at pH 5.4, 6.0, 6.6 and 7.2 (Fig. 3.15, Lanes 2, 4, 6, 8 respectively). Activity decreased at pH 8.0 (lane 10). It can be concluded that the optimal pH values for activities of proteinases of this strain in assayed system are evolving between the low acidic and neutral pH.

In previous studies Tsakalidou et al. (1999) and Fira et al. (2001) also showed that proteolytic activity of *Lactobacilli* was higher at neutral pH range. However, the temperature optima varied in their studies depending on strain.

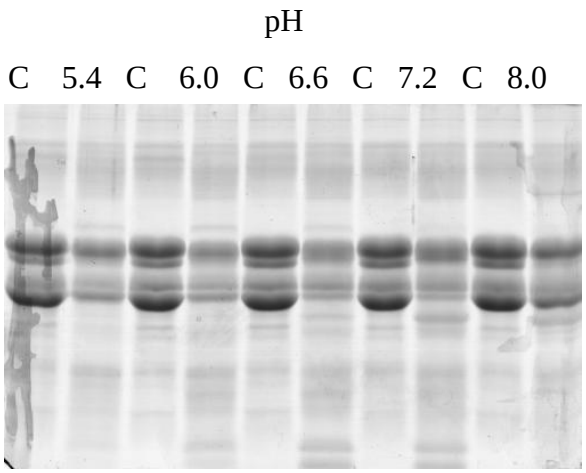
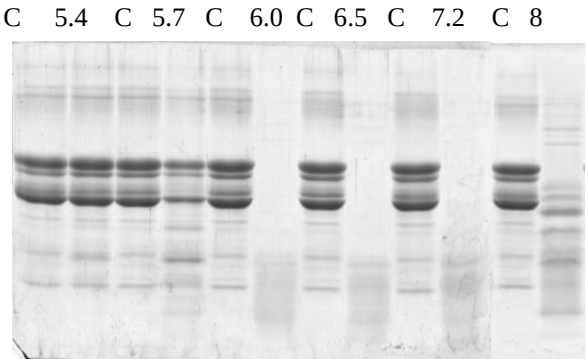


Figure 3.15. Effect of pH on proteolytic activity of *Lb. helveticus* A75: controls (C) – substrate (Na-caseinate) without cells and samples – substrate mixed with cells, incubated at different pH values (24h incubation)

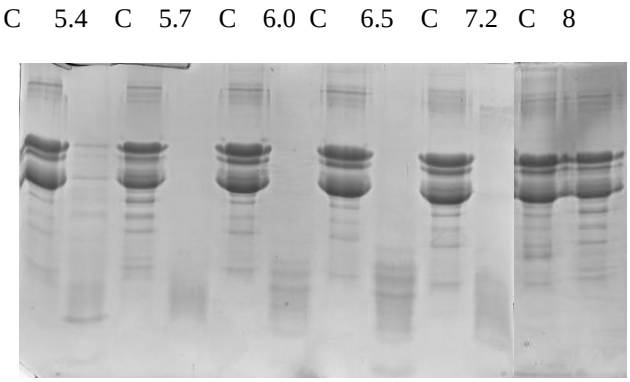
For *E. faecalis* strains maximal protein degradation was observed at the pH range 6.0 - 7.2. Further increase of pH led to decrease the proteolysis level. At pH 5.7 activity and at pH 5.4 hydrolysis was observed only for strains *E. faecalis* AN1 (Fig 3.16 a) and A121 (Fig 3.16 b). It can be concluded that the optimal pH values for hydrolytic activities of the strains in assayed

system are in the neutral pH range. Other authors also found that optimal conditions for proteolytic enzymes of *E. faecalis* isolates were at neutral pH range (El-Ghaish et al., 2010).

Determination of pH influence on β-casein hydrolysis by *Lb. paracasei* A581 showed that the proteinases produced by strain hydrolyze this substrate at a broad range of pH values. No significant differences were observed in substrate hydrolysis levels at pH 5.4, 5.7, 6.0, 6.6 and 7.2 (Fig. 3.17). Activity was totally reduced at pH 8.0. It can be concluded that the optimal pH values for proteinases activity of the strain in assayed system are in the low acidic, neutral pH range.



a



b

Figure 3.16. Effect of pH on proteolytic activity of *E. faecalis* AN1 (**a**) (9h incubation) and *E. faecalis* A121 (**b**) (24 h incubation): controls (C) – substrate (Na-caseinate) without cells and samples – substrate mixed with cells, incubated at different pH values

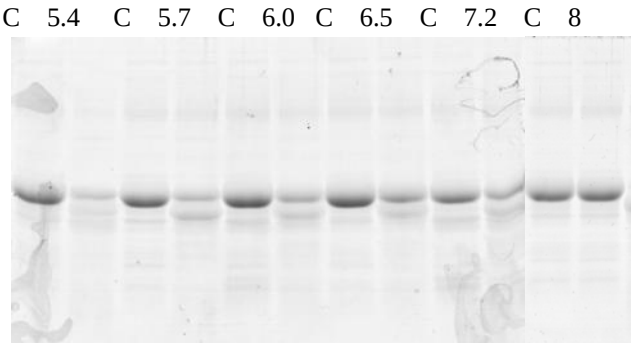


Figure 3.17. Effect of pH on proteolytic activity of *Lb. paracasei* A581: controls (C) – substrate (β-casein) without cells and samples – substrate mixed with cells, incubated at different pH values (9 h incubation)

In a parallel experiment, the temperature optima of hydrolysis were determined. Whole cells of the studied strains were incubated with substrate for 24 h at various temperatures (20, 30, 37, 45 and 55 °C) and samples were analyzed by SDS-PAGE. Optimal temperature for hydrolysis of caseins for *Lb. helveticus* A75 was found to be 37 °C (Fig. 3.18). At 20 and 30 °C hydrolysis level was also efficient, however at 45 °C it was very low and at 55 °C no hydrolysis of the substrate was observed at all.

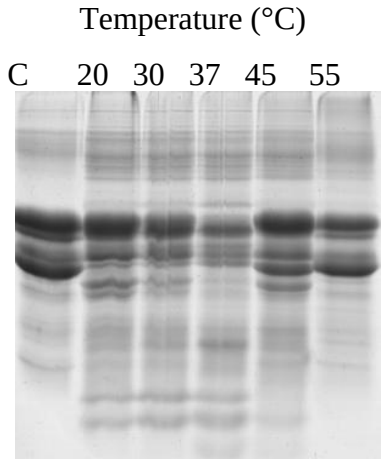


Figure 3.18. Effect of temperature on proteolytic activity of *Lb. helveticus* A75: controls (C) – substrate (Na-caseinate) without cells and samples – substrate mixed with cells, incubated at different temperatures during 24 h

For *Lb. paracasei* A581 no significant difference in substrate hydrolysis was observed at 30, 37 and 45 °C, however, *Lb. paracasei* A581 strain was unable to efficiently hydrolyze Na-caseinate at 55 °C (Fig. 3.19).

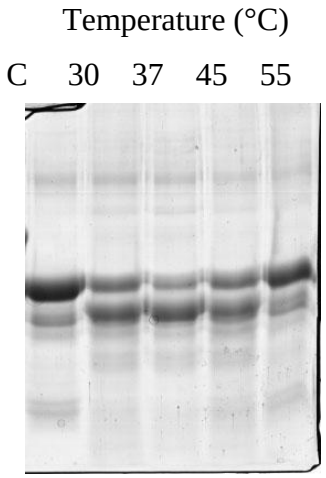


Figure 3.19. Effect of temperature on proteolytic activity of *Lb. paracasei* A581: controls (C) – substrate (β-casein) without cells and samples – substrate mixed with cells, incubated at different temperatures during 24 h

Optimal temperature for hydrolysis of caseins by *E. faecalis* strains was found in the range of 37- 45 °C, however, hydrolysis was observed in all assayed temperatures. In the Fig. 3.20 we present the results of this experiment with the strain of *E. faecalis* AN1.

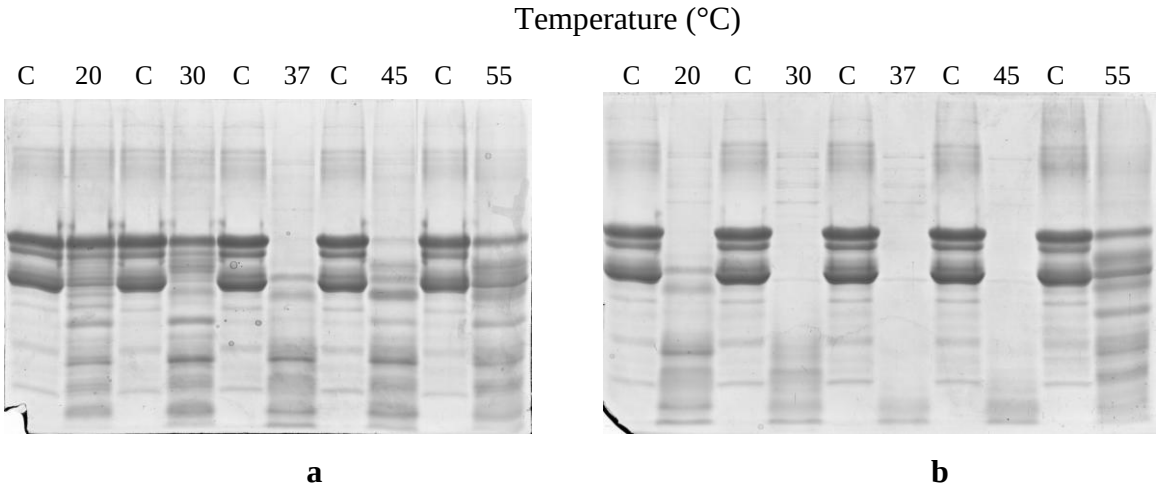


Figure 3.20. Effect of temperature on proteolytic activity of *E. faecalis* AN1: controls (C) – substrate (Na-caseinate) without cells and samples – substrate mixed with cells, incubated for 6 h (**a**) and 24 h (**b**) at different temperatures

3.4.2. Effect of inhibitors

Study of effects of inhibitors on proteolytic activities displayed similar results for all *E. faecalis* strains. Taking in account this, we will present results on the example of strain *E. faecalis* AN1. Proteolytic activity was strongly inhibited in the presence of EDTA (Fig. 3.21, lane 6) indicating the presence of metalloproteases. Inhibition of proteinase activity in the

presence of EDTA has been previously reported in case of some *Enterococci* (El-Ghaish et al., 2010). Specific inhibitor of serine type proteases – PMSF slightly inhibited activity (Fig. 3.21, lane 3), what is in disagreement with previously published data about LAB proteases (Fira et al., 2001; Tsakalidou et al., 1999). Protease activity was also reduced to some extent in the presence of iodoacetic acid (Fig. 3.21, lane 5), a specific inhibitor of cysteine proteinases. Similar results were obtained by other authors (Tsakalidou et al., 1999). It can be concluded that the most active proteases produced by the studied *E. faecalis* strains are metalloproteases, but the presence of other types of proteases involved in the proteolytic activity of the investigated strains was also detected.

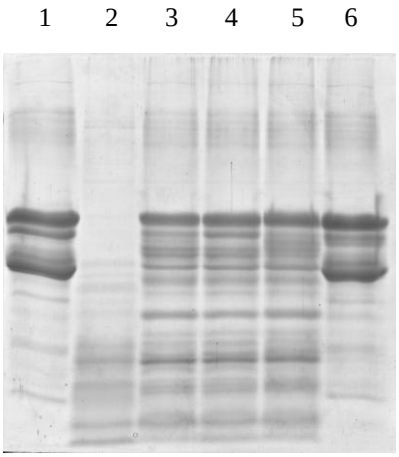


Fig. 3.21. SDS-PAGE of Na-caseinate (lane 1) hydrolyzed by strain *E. faecalis* AN1 (at 37 ºC during 24 h) in the presence of PMSF (lane 3), isopropanol (lane 4), iodoacetic acid (lane 5) and EDTA (lane 6) as compared to the control performed in the absence of inhibitors (lane 2)

In case of *Lb. helveticus* A75 proteinase activity was totally inhibited after incubation with PEFABLOC, known to inhibit serine proteases (Fig. 3.22, lane 3). This is in agreement with previously published data about LAB proteases (Fira et al., 2001; Tsakalidou et al., 1999), which are in most of the cases serine proteases. On the other hand, inhibitor of metalloproteases - EDTA, also strongly affected enzymatic activity resulting in total inhibition of substrate hydrolysis (Fig. 3.22, lane 4). No inhibition was observed in the presence of iodoacetic acid (Fig. 3.22, lane 5), a specific inhibitor of cysteine proteinases. The same results were obtained for strain *Lb. paracasei* A581 (Fig 3.23), which showed inhibition in the presence of PEFABLOC and EDTA (lanes 3 and 4 respectively). The inhibition by iodoacetic acid was not observed for this strain neither (Fig 3.23, lane 5).

Several studies support the hypothesis of the presence of at least two types of proteinases at the cell surfaces of some *Lactobacilli* (Stefanitsi et al., 1995; Gilbert et al., 1997) and inhibition of proteinase activity in the presence of EDTA has been already reported for some of them (Martín-Hernandez et al., 1994).

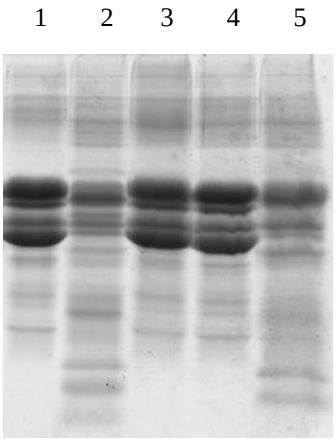


Figure 3.22. SDS-PAGE of Na-caseinate (lane 1) hydrolyzed by strain *Lb. helveticus* A75 (at 37 ºC during 24 h) in the presence of PEFABLOC (lane 3), EDTA (lane 4), iodoacetic acid (lane 5) as compared to the control performed in the absence of inhibitors (lane 2)

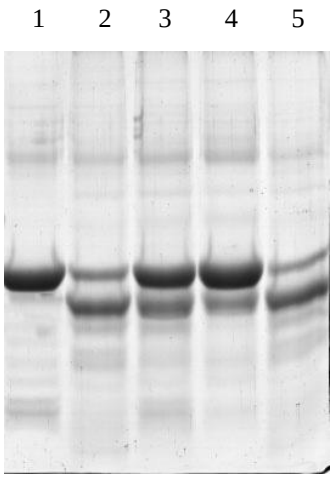


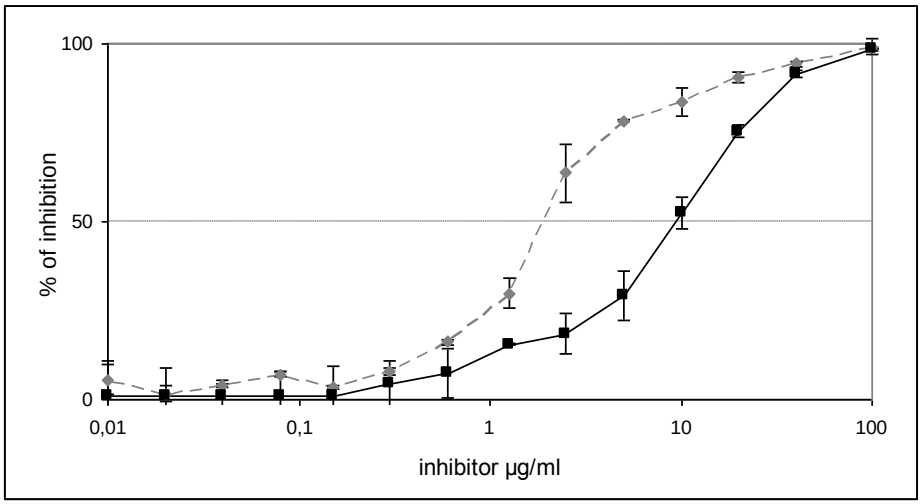
Figure 3.23. SDS-PAGE of β-caseinate (lane 1) hydrolyzed by strain *Lb. paracasei* A581 (at 37 ºC during 24 h) in the presence of PEFABLOC (lane 3), EDTA (lane 4), iodoacetic acid (lane 5) as compared to the control performed in the absence of inhibitors (lane 2)

3.5. Competitive F-ELISA

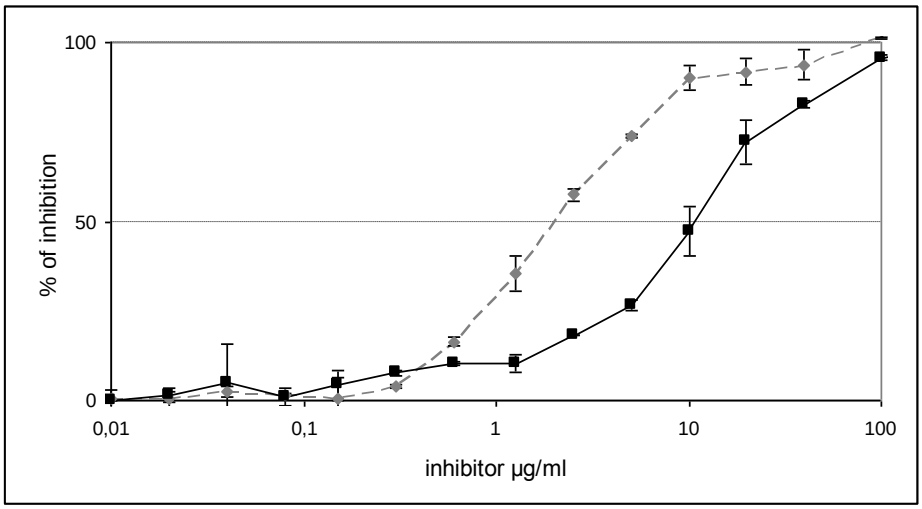
The effect of proteolysis of α_{S1}- and β-caseins by *Lb. helveticus* A75 and also α_{S1}-casein by *E. faecalis* AN1 on the IgE binding ability of caseins was studied using an ELISA inhibition assay. The series of sera used were different for each strain and were collected at different times. These strains were selected on the basis of their proteolytic activity profile.

In the case of *Lb. helveticus* A75 a pool of 8 sera from CMA patients was used. The titre of IgE specific α_{S1}- and β-caseins was determined in each sera sample by direct ELISA assay in the BIA-FIP laboratory. Data presented in Fig. 3.24 show the inhibition of binding of IgE to

intact α_{S1}- (a) and β- (b) caseins, or to their hydrolysates. It was observed that the binding of serum IgE by hydrolysates was considerably weaker than binding by intact caseins, indicating that α_{S1}- and β-caseins hydrolysed by *Lb. helveticus* A75 are less recognised than intact proteins by IgE from CMA patients. For example, at the concentration of 5 μg/mL, 80% and 74% of total IgE were bound by intact α_{S1}- and β-casein, respectively, while only 30% and 26% were bound by their hydrolysates. The calculated IC_{50} values were 2.01 and 2.28 μg/mL for α_{S1}- and β-casein, respectively. These values increased till 9.08 and 13.65 μg/mL, respectively, for their hydrolysates obtained by action of proteinases of *Lb. helveticus* A75 (Table 3.3). This means that the binding of hydrolyzed α_{S1}- and β-caseins was about 5-6 times lower than that of the intact proteins.



a



b

Figure 3.24. Inhibition (%) of anti-α_{S1}-(**a**) and anti-β-caseins (**b**) IgE in the pool of sera. A comparison between native proteins and their hydrolysates with *Lb. helveticus* A75. Native proteins: grey line; hydrolysates: black line

Table 3.3. IC_{50} (μg/mL) values for native and hydrolyzed by *Lb. helveticus* A75 caseins

Caseins	IC_{50} (μg/mL)	
	Native protein	Hydrolyzed protein
α_{S1}-casein	2.01 ± 0.075	9.08 ± 0.266
β-casein	2.28 ± 0.044	13.65 ± 0.092

From the curves of inhibition in Fig. 3.24, it can be seen that with the increase of inhibitor concentration, the difference in the IgE binding ability between intact and hydrolysed proteins decreased progressively. Consequently, even if SDS-PAGE showed total degradation of α_{S1}- and β-caseins after 24 h of incubation (See Fig. 3.12) with whole cells of *Lb. helveticus* A75, this degradation might only be partial, leaving peptides long enough to bind the antibodies. Even if the release of proteases and peptidases from the bacterial cells results in more effective hydrolysis of milk proteins with higher possibility for cleavage of epitopes, it is also possible that by further cleavage of the peptides into smaller peptides and amino acids by peptidases, some hidden epitopes or linear epitopes could be exposed. Some authors reported that there was no linear correlation between the degree of hydrolysis of milk proteins and the antigenicity of whey proteins during the fermentation (Bu et al., 2010; Ehn et al., 2005).

The proteolytic activity of strain *Lb. helveticus* A75 and effect of milk caseins hydrolysis by this strain in their immunoreactivity are detally described in our article – Ahmadova et al., article in press.

The effect of proteolysis of α_{S1}-casein by strain *E. faecalis* AN1 on their IgE binding ability was studied using an ELISA inhibition assay using a pool of 8 sera from CMA patients. The series of sera used was different from that used for *Lb. helveticus* A75. In Fig. 3.25 we present the binding inhibition of IgE to intact α_{S1}-casein and to its hydrolysate by the strain *E. faecalis* AN1. It was observed that the binding of serum IgE by this hydrolysate was considerably weaker than binding of IgE by intact caseins, indicating that hydrolysis of α_{S1}-casein by this strain led to decrease of its recognition by IgE from CMA patients. The calculated IC_{50} values were 0.19 and 535.35 μg/mL for α_{S1}-casein and its hydrolysate, respectively. This means that the binding of hydrolyzed α_{S1}-casein was about 2800 times lower than that of the intact proteins and it is almost 500 times lower than that was observed for *Lb. helveticus* A75. From the curves of inhibition in Fig. 3.25, it can be observed that the difference in the IgE binding ability between intact and hydrolysed proteins was very high till the concentration of inhibitor was 100 μg/mL. With the increase of inhibitor concentration more than 100 μg/mL, this difference progressively decreased. SDS-PAGE shows a total degradation of α_{S1}-casein after 24 h of incubation (Fig. 3.26) with whole cells of *E. faecalis* AN1. However this degradation might

be only partial, leaving some peptides long enough to bind the antibodies and it is possible that with the increase of concentration the probability of IgE to bind these peptides increases also.

According to our knowledge it is the first study about the ability of *E. faecalis* to decrease the immuno-reactivity of caseins. Most of the research in this area was performed on *Lactobacillus* strains. Moreover, most of experiments about the ability of LAB to reduce the antigenic activities of milk proteins were performed on whey proteins and skim milk (Kleber et al., 2006; Bu et al., 2010). There are only few studies about the effect of LAB fermentations on immuno-reactivity of caseins. Recently El-Ghaish et al. (2011a) found that after hydrolysis of α_{S1}-casein with *Lb. fermentum* IFO3956 the recognition and the binding of this casein to IgE from the pooled sera of 18 patients with cow's milk allergy was reduced significantly. The studied bacterial strain hydrolyzed α_{S1}-casein in many sites including the epitopes triggering the allergic reactions against α_{S1}-casein.

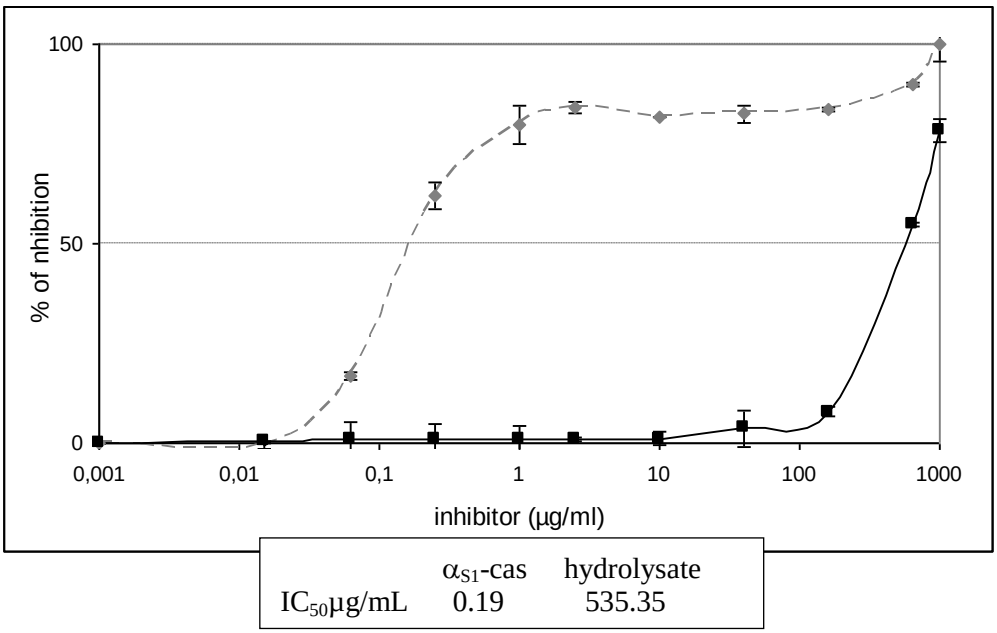


Figure 3.25. Inhibition (%) of anti-α_{S1}-IgE in the pool of sera. A comparison between native α_{S1}-casein and its hydrolysate with *E. faecalis* AN1. Native protein: grey line; hydrolysates: black line

Figure 3.26. SDS-PAGE of α_{S1}-casein hydrolyzed by strain *E. faecalis* AN1 (24 h incubation at 37 ºC): Lane 1 – control (native protein), lane 2 – hydrolysate

Results obtained in this study clearly indicate that the hydrolysis of α_{S1}- and β-caseins by studied strains significantly decreases the IgE binding capacity. This weaker binding of IgE could lead to weaker allergic reactions. Consequently, the hydrolysis of α_{S1}- and β-caseins by these strains could attenuate allergic reactions. However, further *in vitro* and *in vivo* studies are necessary before making any conclusions about potential application of the studied strain in dairy industry.

3.6. LC/MS/MS analyses

The hydrolysate of α_{S1}-casein by strain *E. faecalis* AN1 was selected for LC/MS/MS analysis based on the results obtained with competitive ELISA. In Figure 3.27 we present the sequence of bovine α_{S1}-casein (199 amino acids). This primary structure of α_{S1}-casein contains as reported by Chatchatee et al. (2001), 8 epitopes defined as 5 major plus 3 minor IgE-binding epitopes. The major epitopes are the following peptide fragments: N17-K36, K83-K102, L109-L120, N139-F153 and Y159-T194. The minor epitopes are: E39-S48, E69-Q78 and M123-K132. These epitopes are marked on figure by grey colour. Similar results were reported by Wang et al. (2010) stating that the major epitopes are the peptides F28-I44, Q78-L95, L109-S122 and A163-F179. Cerecedo et al. (2008) observed using peptide microarray, a single region differentially recognized by the reactive IgE group (peptide F28-E50). The reported new epitopes were already observed by Chatchatee et al. (2001).

Mass spectrometry analyses of hydrolysate of α_{S1}-casein obtained after the incubation with cells of *E. faecalis* AN1 for 24 h at 37 °C revealed the presence of 18 peptides of different sizes, ranging from 6 to 36 amino acids and included 8 phosphorylated peptides (L40-Q52, L109-L122, L40-E55, Y104-R119, K36-M54, L101-R119, D56-Y91, I57-S62) (Table 3.4, Fig. 3.27).

Most of the cleavages occurred after proline and after charged amino acids (E, K Q R, D). It was previously reported that cell-envelope proteinases from LAB have a strong preference for hydrophobic sites on caseins (Swaisgood, 1982).

The cleavage sites of proteolysis were situated on the all identified epitopes of α_{S1}-casein, except one phosphorylated epitope (E69-Q78) that resisted hydrolysis. However another phosphorylated epitope (E39-K58) was subjected to the hydrolytic action of *E. faecalis* AN1.

Table 3.4. LC-MS/MS spectrometry identification of the major peptides released from α_{s1}-casein by proteolytic activity of *E. faecalis* AN1

Experimental mass (Da)	**Peptide sequence**	**Sequence assignment**
763.1657	DIKQME	56-61
806.2221	APFPEVF	26-32
907.1271	NSEKTTMP	190-197
525.1991	IKHQGLPQE	6-14
631.2090	IKHQGLPQEVL	6-16
752.8502	IKHQGLPQEVLNE	6-18
784.7354	LSKDIGSESTEDQ	40-52
837.3349	LEIVPNSAEERLHS	109-122
861.3990	IHAQQKEPMIGVNQE	127-141
866.4628	IKHQGLPQEVLNENL	6-20
950.0879	LSKDIGSESTEDQAME	40-55
976.4222	YKVPQLEIVPNSAEER	104-119
1121.3092	KVNELSKDIGSESTEDQAM	36-54
774.3831	LKKYKVPQLEIVPNSAEER	101-119
1268.1704	TDAPSFSDIPNPIGSENSEKTTMP	174-197
1309.5946	PSFSDIPNPIGSENSEKTTMPL	177-199
1520.3358	DIKQMEAESISSSEEIVPNSVEQKHIQKEDVPSERY	56-91
509.6158	IKEMEAES	57-62

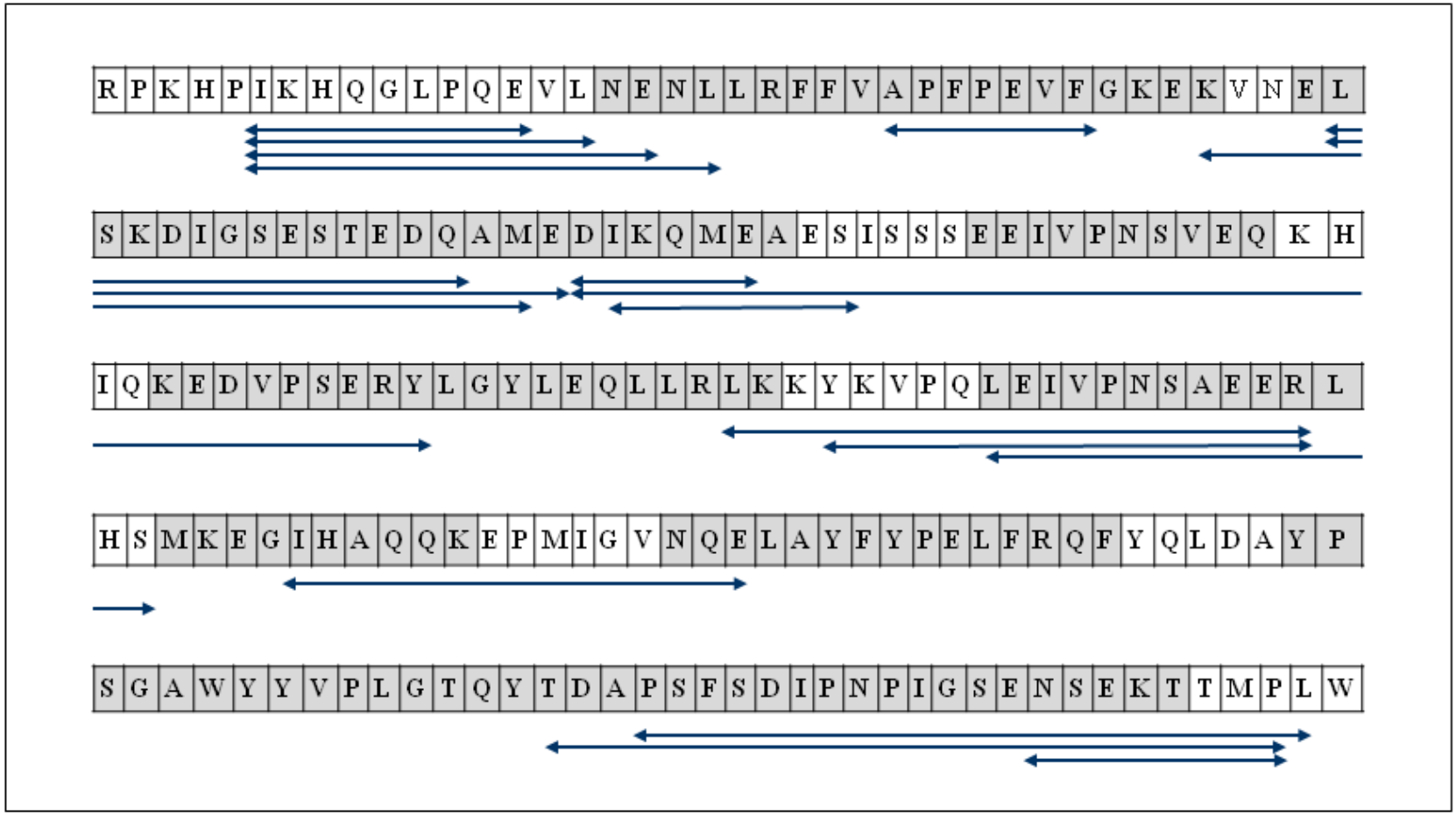


Figure 3.27. Primary sequence of α_{S1}-casein and location on the sequence of identified peptides released by the proteolytic action of *E. faecalis*AN1 after 24 h incubation

3.7. Amplification of proteinase genes by PCR

The presence of proteinase genes in *Lb. helveticus* A75 and *Lb. paracasei* subsp. *paracasei* 581 was analyzed by PCR, using primers specific for different types of CEPs. In case of *Lb. helveticus* A75 PCR amplicon of the expected size (1034 bp) was obtained only with the pair of primers P23 / P25 that targets the active site of the *prtH* gene (Fig. 3.28). Nucleotide sequence analysis of this fragment showed more than 99% of identity with the corresponding active site region from *Lb. helveticus* CNRZ32 (GenBank: AF133727.1).

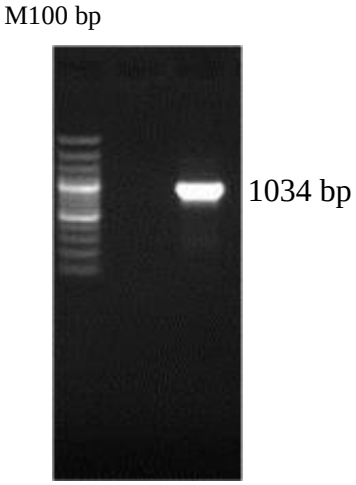


Figure 3.28. PCR detection of *prtH* gene. 1.5% agarose gel with amplified PCR product

In contrast to other LAB, *Lb. helveticus* possesses several proteinases genes and their distribution is strain-dependent. For *Lb. helveticus* CNRZ32, a minimum of two genes encoding the CEPs, *prtH* and *prtH2*, has been reported and two others, *prtH3* and *prtH4*, remain putative (Smeianov et al., 2007; Broadbent et al., 2008). Both proteinases have been characterized and it has been shown that *prtH2* is ubiquitous, whereas the presence of *prtH* is strain-dependent (Genay et al., 2009; Pederson *et* al., 1999). Although the *prtH* and *prtH2* genes were both shown to be expressed in dairy matrices, it is still difficult to distinguish the activity and specificity of each CEP. However, some authors reported that hydrolysis rate of α_{S1}-casein was the criterion distinguishing the strains containing one or both genes of proteases and was higher in the presence of *prtH* (Sadat-Mekmene et al., 2011).

In case of *Lb. paracasei* subsp. *paracasei* 581 no PCR products were obtained with any set of used primers recognizing *prt* genes of LAB.

The several sets of primers were designed for detection of proteinase genes in studied *E. faecalis* strains (Table 2.2). Results obtained from PCR reactions with these primers are presented in Table 3.5. PCR amplicons of the expected size were obtained with the primers *Ser*E1, coding serine proteinases and with the primers *Met*E11, *Met*E21 and *Met*E23, which

encode metallo-proteases of *E. faecalis*. No amplification product was obtained with the primer set *Ser*E7.

Results of PCR amplification of proteinase genes agree well with the data of the experiment obtained with inhibitors, where we observed inhibition of proteolytic activities of isolated *E. faecalis* strains with inhibitors of serine- and metallo-proteases. However, it is important to highlight, that it is first study of enterococcal proteases carried out on a molecular level.

Table 3.5. PCR amplification of proteinase genes in *E. faecalis* strains

Gene	**Strains**					
	A121	A124	A1232	A1221	AN1	A71
*Ser*E1	+[2]	+	+	+	+	+
*Ser*E7	-[1]	-	-	-	+	-
*Met*E11	+	+	+	+	+	+
*Met*E21	+	+	+	+	+	-
*Met*E23	+	+	+	+	+	+

1. "- " - gene is absent
2. "+ " - gene is present

There are very limited information about the proteolytic system of *Enterococci*, since the main objects of previous studies were proteinases of *Lactococci* and *Lactobacilli* despite the fact that *Enterococci* play important roles in the dairy products. They occur as NSLAB in a variety of cheeses, especially artisanal cheeses produced from raw or pasteurized milk (Suzzi et al., 2000). High proteolytic activity differentiated *E. faecalis* isolates from the other *Enterococci* (Suzzi et al., 2000; Sarantinopoulos et al., 2001; Psoni et al., 2006; Veljovic et al., 2009). Due to their proteolytic and lipolytic activities as well as to their ability to metabolize citrate (Tsakalidou et al., 1993; Sarantinopoulos et al., 2001), they may contribute to the sensorial and textural properties of the cheeses.

3.8. Isolation and identification of BLIS producing LAB

A collection of 431 isolates (Table 3.1) from traditional Azerbaijani dairy products was screened for the presence of bacteriocin like inhibitory substances (BLIS). Preliminary screening by deferred antagonism test resulted in 46 isolates (32 cocci and 14 bacilli) from 9 cheese samples, which showed inhibition zones on the lawn on agar media with indicator strain. The antimicrobial activity of these strains was further tested by well diffusion method. Only 12 isolates (10 cocci and 2 bacilli) showed inhibition of indicator organism by this method, which indicates that inhibitory activities of other isolates, were due to the acid production. All isolates

were tested for the presence of catalase and Gram test and preliminarily were identified as LAB. The inhibitory activity of these isolates is shown on Fig. 3.29.

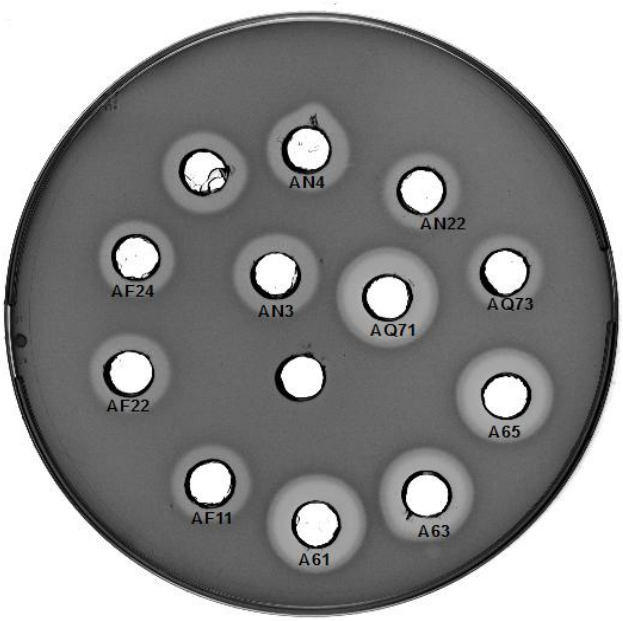


Figure 3.29. Agar plates indicating antimicrobial activity of LAB isolates against indicator strain *Lb. brevis* F145

Antimicrobial activity of these isolates was further tested against different indicator strains. Results are presented in Table 3.6.

Table 3.6. Antimicrobial activity of obtained LAB isolates

Indicator strains	**LAB isolates**											
	AN2	AN3	AN4	AN22	A61	A63	A65	AQ71	AQ73	AF11	AF22	AF24
Lb. bulgaricus 340	+[1]	+	+	+	+	+	+	+	+	+	+	+
Lb. brevis F145	+	+	+	+	+	+	+	+	+	+	+	+
Lb. brevis F1.114	-[2]	-	-	-	+	-	-	+	-	-	-	-
Lb. brevis F1106	-	-	-	-	+	-	-	+	-	-	-	-
Listeria. innocua CIP 80.11	-	-	-	-	+	-	-	+	-	-	-	-

1. "+" - presence of inhibition
2. "-" – absence of inhibition

As we can see from the above Table 3.6, 10 of tested 12 isolates showed narrow inhibitory activities, which are directed against *Lb. bulgaricus* 340 and *Lb. brevis* F145. The supernatants from the cultures of these isolates showed very low heat stability (almost total decrease of activity after 5 min boiling) and also activities at narrow pH range (from pH 5 till pH 7) (data not shown). Other two isolates named A61 and AQ71 showed broad activity spectra and resistance to pH and heating (see results in the part "Effect of pH, temperature, enzymes and different chemicals on antimicrobial activity"). These isolates were selected for further study.

The source of isolate AQ71 was Motal cheese ripened in the sheepskin during 3 months in the presence of 3% NaCl. The isolate A61 was isolated from white cheese ripened during 1 month. 16S rDNA fragment sequencing resulted in identification of isolate AQ71 as *E. faecium*. In case of another isolate A61 the 16S rDNA fragment sequencing gave the same similarity level with *Lb. sakei* and *Lb. curvatus* (99%). Thus, we used species-specific primers to identify this strain to the species level.

Results of DNA amplification of strain *Lb. curvatus* A61 with species-specific primers supported previously obtained data with 16S rDNA sequencing and resulted in amplification product with the primers specific for *Lb. curvatus* species (Fig. 3.30 a). With another primer, specific for *Lb. sakei*, the amplification product only for positive control was obtained (Fig. 3.30 b).

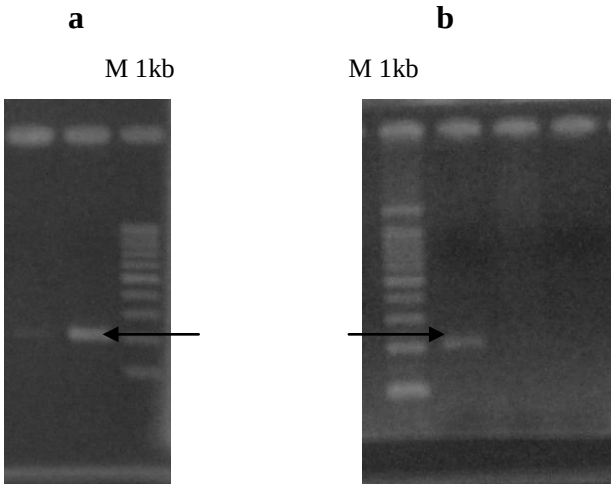


Figure 3.30. Agarose gels (1%) with amplification products, obtained with species-specific primers

3.9. Inhibitory activity spectra

Inhibitory spectra of studied strains were determined against different LAB, food-spoilage and pathogenic strains (Table 3.7). Both *E. faecium* AQ71 and *Lb. curvatus* A61 showed activity against closely related species such as *Enterococci* and *Lactobacilli*, and inhibited the growth of *Lb. bulgaricus* 340, 3 strains of *Lb. brevis* and *E. faecium.* However, the

inhibition of other LAB strains, such as *Lb. paracasei*, strains of *Lactococcus lactis* and *Lb. sakei* was observed only for strain *E. faecium* AQ71. *Lb. curvatus* A61 did not inhibit any of aforementioned strains. Both of the strains showed inhibitory activities against most of the tested *Listeria monocytogenes* strains and also against tested *Bacillus cereus* strain. No activity was observed against strains of *Salmonella, E. coli* and other studied bacterial strains.

Table 3.7. Inhibitory activity spectra of studied strains

Test organism	Growth conditions	Origin	Activity	
			A61	AQ71
Bacteria				
Lactobacillus paracasei subsp. paracasei DF60	MRS, 30 °C	FM, USP[1]	-[7]	+[6]
Lactococcus lactis subsp. lactis DF04	MRS, 30 °C	FM, USP	-	+
Lactobacillus sakei 2a	MRS, 30 °C	FM,USP	-	+
Lactobacillus sakei 15221	MRS, 30 °C	ATCC[2]	-	+
Lactococcus lactis subsp. cremoris R704	MRS, 30 °C	Chr.Hansen	-	+
Lactobacillus bulgaricus 340	MRS, 37 °C	Rhodia Food Tex. Fr	+	+
Lactobacillus brevis F145	MRS, 37 °C	INRA[3]	+	+
Lactobacillus brevis F1.144	MRS, 37 °C	INRA	+	+
Lactobacillus brevis F1106	MRS, 37 °C	INRA	+	+
Enterococcus faecium HKLHS	BHI, 37 °C	INRA	+	+
Staphylococcus aureus CIP 53154	BHI, 37 °C	ONIRIS[4]	-	-
Staphylococcus aureus 25923	BHI, 37 °C	ATCC	-	-
Listeria innocua CIP 80.11	BHI, 37 °C	ONIRIS	+	+
Listeria ivanovii	BHI, 37 °C	INRA	+	+
Listeria monocytogenes EGDe107776	BHI, 37 °C	ONIRIS	+	+
Listeria monocytogenes 7644	BHI, 37 °C	ATCC	-	-
Listeria monocytogenes 104	BHI, 37 °C	FM, USP	-	-
Listeria monocytogenes 724	BHI, 37 °C	FM, USP	+	+
Listeria monocytogenes 211	BHI, 37 °C	FM,USP	-	-
Listeria monocytogenes 506	BHI, 37 °C	FM, USP	+	+
Listeria monocytogenes 409	BHI, 37 °C	FM, USP	+	+
Listeria monocytogenes 302	BHI, 37 °C	FM,USP	+	+
Listeria monocytogenes 103	BHI, 37 °C	FM, USP	+	+
Listeria monocytogenes 620	BHI, 37 °C	FM, USP	+	+
Listeria monocytogenes 703	BHI, 37 °C	FM,USP	+	+
Listeria monocytogenes 106	BHI, 37 °C	FM, USP	+	+

Listeria monocytogenes 603	BHI, 37 °C	FM, USP	+	+
Listeria monocytogenes 607	BHI, 37 °C	FM, USP	+	+
Listeria monocytogenes 711	BHI, 37 °C	FM,USP	-	-
Salmonella typhimurium 14028	BHI, 37 °C	ATCC	-	-
Salmonella enteritidis 13076	BHI, 37 °C	ATCC	-	-
Salmonella enteritidis 13076	BHI, 37 °C	ATCC	-	-
Bacillus cereus 11778	BHI, 37 °C	ATCC	+	+
Klebsiella pneumoniae CIP 53153	BHI, 37 °C	ONIRIS	-	-
Escherichia coli 23355 (BAS)	LB, 37 °C	ATCC	-	-
Yeast				
Saccharomyces cerevisiae DSH213.83	YPD, 30 °C	ONIRIS	-	-
Candida pseudotropicalis	YPD, 30 °C	INRA	-	-
Fungi				
Penicillium roqueforti	PDA, 30 °C	LUBEM[5]	-	-
Fusarium CBS 1385	PDA, 30 °C	INRA	Strong inhibition	-
Cladosporium	PDA, 30 °C	LUBEM	Strong inhibition	-

1. FM, USP – Laboratory of Food Microbiology, University of Sao Paulo, Sao Paulo, Brazil
2. ATCC - American Type Culture Collection.
3. INRA - Institut National de la Recherche Agronomique, Nantes, France
4. ONIRIS - Ecole Nationale Nantes Atlantique Vétérinaire, Agroalimentaire et de l'Alimentation, Nantes, France
5. LUBEM – Laboratoire Universitaire de Biologie et d'Ecologie Microbienne, Brest, France
6. '+' – presence of the inhibition zone
7. '-' – no inhibition

The co-culture in dual slab agar of *Lb. curvatus* A61 with several fungi displayed inhibition of the growth mold in the same extent for *Cladosporium* and *Fusarium* ssp., however, no antifungal activity was observed against *Penicillium roqueforti*. Other strain *E. faecium* AQ71, did not exhibit antifungal activity.

The observed antilisterial activities exhibited by the studied strains suggest their potential application in biopreservation of fermented foods and preventing listeriosis. Moreover, the strong inhibition of fungal growth caused by *Lb. curvatus* A61 could be of a great interest. The data characterizing LAB antifungal metabolites or their mechanisms are very scarce (Schnurer and Magnusson, 2005). Identification of the antifungal compounds in LAB may give an additional mean for food biopreservation.

3.10. Effect of pH, temperature, enzymes and different chemicals on antimicrobial activities

Bacteriocins detected in the CFSs of *E. faecium* AQ71 and *Lb. curvatus* A61 were stable after heating at 100 ºC for 30 min (Table 3.8). No loss of activities was observed after autoclaving for 15 min at 121ºC. Activities of both strains were also stable over a wide range of pH from 2 to 10. the CFSs from the studied strains were treated with catalase, α-amylase, lipase and proteolytic enzymes. The bacteriocin activities of strains totally disappeared after 1 h incubation with proteolytic enzymes, what indicates their proteinaceous nature. Catalase, lipase and α-amylase had no effect on the bacteriocin activities.

Table 3.8. Effects of heating, pH, enzymes and different chemicals on the antibacterial activity in culture supernatants

Treatments	**Antimicrobial activity**	
	A61	**AQ71**
Heating		
50 °C 30 min	+[1]	+
100 °C 5 min	+	+
100 °C 15 min	+	+
100 °C 30 min	+	+
121 °C 15 min	+	+
pH		
3-10	+	+
Enzymes		
Protease TypeX	-[2]	-
Pronase E	-	-
Protease Type VIII	-	-
α-Chymotrypsin Type II		
Catalase	+	+
Lipase Type VII	+	+
α-amylase	+	+
Chemicals		
SDS	+	+
Triton X-20	+	+
Triton X-80	+	+
Triton X-100	+	+
β-mercaptoetanol	+	+
Na-EDTA	+	+
NaCl	+	+

1. '+' – presence of the inhibition zone
2. '-' – no inhibition

Effect of different chemicals on bacteriocin activities in cell-free supernatants of studied strains revealed, that TritonX-20, Triton X-80, Triton X-100, β-mercaptoetanol Na-EDTA, SDS and NaCl did not modify the inhibitions of the test organism with the treated supernatants. No inhibition was caused by controls. These results indicate that the bacteriocin is resistant to tensioactive compounds and salt, evidencing a good potential to survive in the gastrointestinal tract.

The thermostability of bacteriocins produced by studied could be a very useful characteristic for their application as food preservatives, because many food-processing procedures involve robust heating steps. pH resistance also may be useful in acid as well as in nonacid foods.

3.11. Kinetics of bacteriocin production

Bacteriocin production by studied strains was tested during the growth in MRS media at 30 and 37 °C. The optimal temperature for bacteriocin production was found to be 30 °C for *Lb. curvatus* A61 and 37 °C for *E. faecium* AQ71 (Fig. 3.31 and 3.32). *Lb. curvatus* A61 started to produce bacteriocin (200 AU/mL) at 3h growth point of logarithmic growth phase. Maximum production (3200 AU/mL) was reached at the end of the exponential phase. However, it was decreased when strain entered stationary phase (1600 AU/mL). After 24 h incubation very low level of activity (200 AU/mL) was detected (Fig. 3.31).

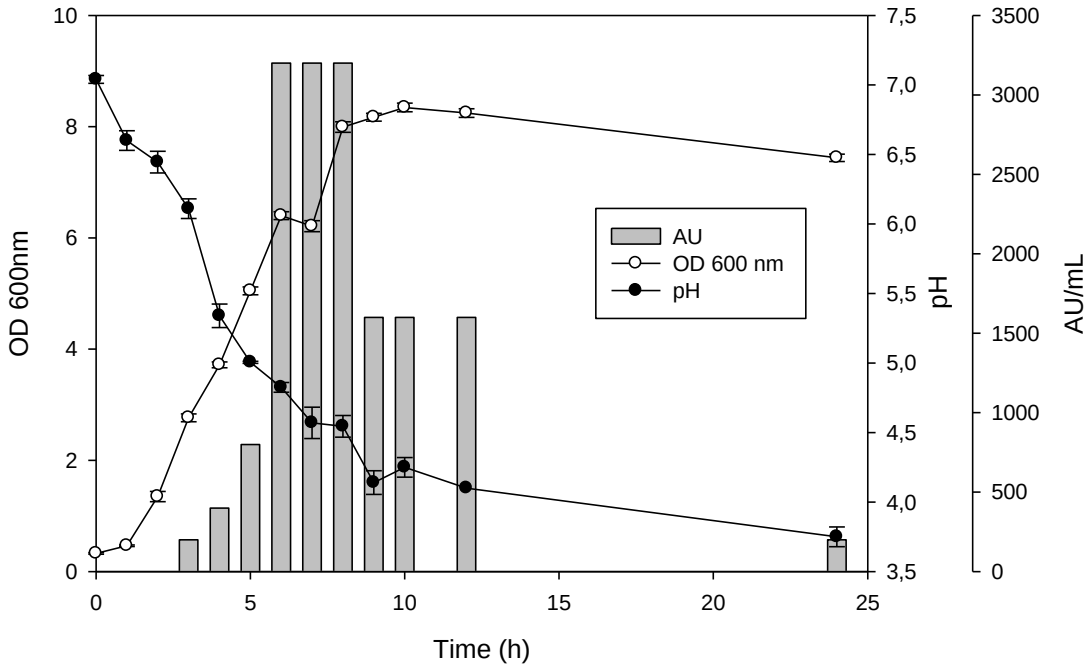


Figure 3.31. Kinetics of growth, acidification and bacteriocin production by *Lb. curvatus* A61

Similar results were observed for *E. faecium* AQ71. This strain started to produce bacteriocin in the early exponential phase (2 h incubation, 200 AU/mL) and maximal production of bacteriocin was observed after 5 h incubation (3200 AU/mL), when the strain was at the end of exponential growth phase. Some decrease of activity was also observed in stationary growth phase (Fig. 3.32).

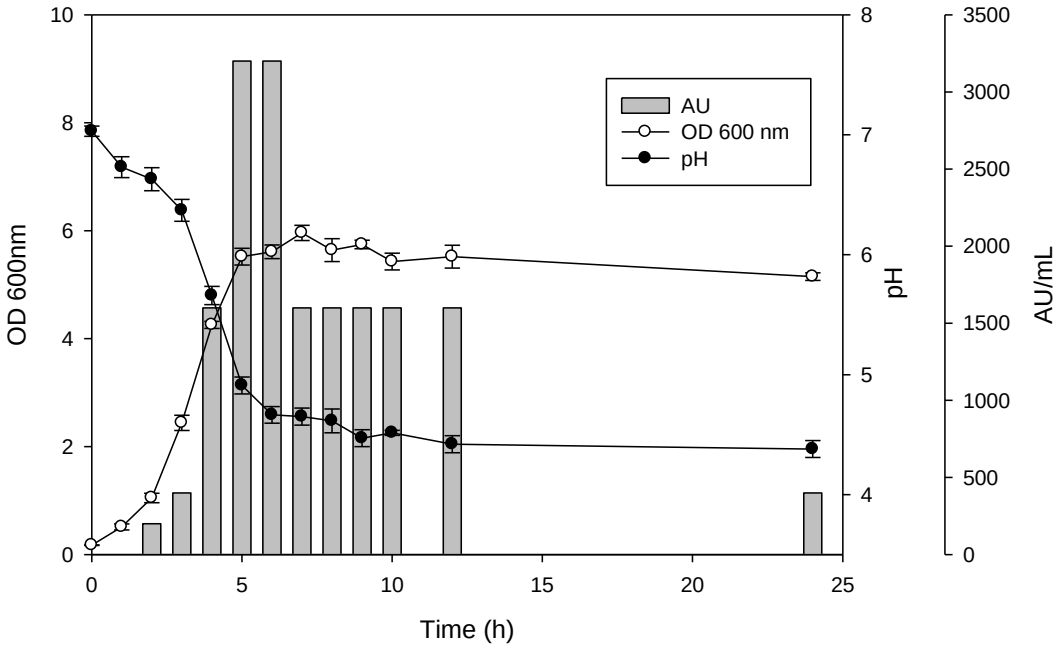


Figure 3.32. Kinetics of growth, acidification and bacteriocin production by *E. faecium* AQ71

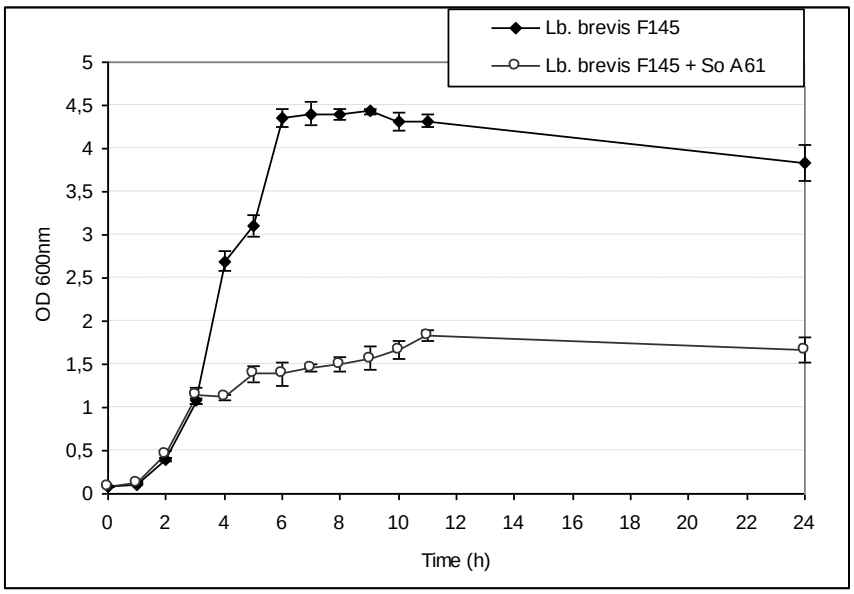
Obtained results are in agreement with the results obtained for other bacteriocins (Hadji-Sfaxi et al., 2011; Todorov and Dicks, 2009). Most of the studied bacteriocins were produced during the exponential growth phase with the greatest production occurring at the end of the exponential, or at the beginning of the stationary phase.

3.12. Effects of bacteriocin-containing cell-free supernatants on the growth of indicator strains

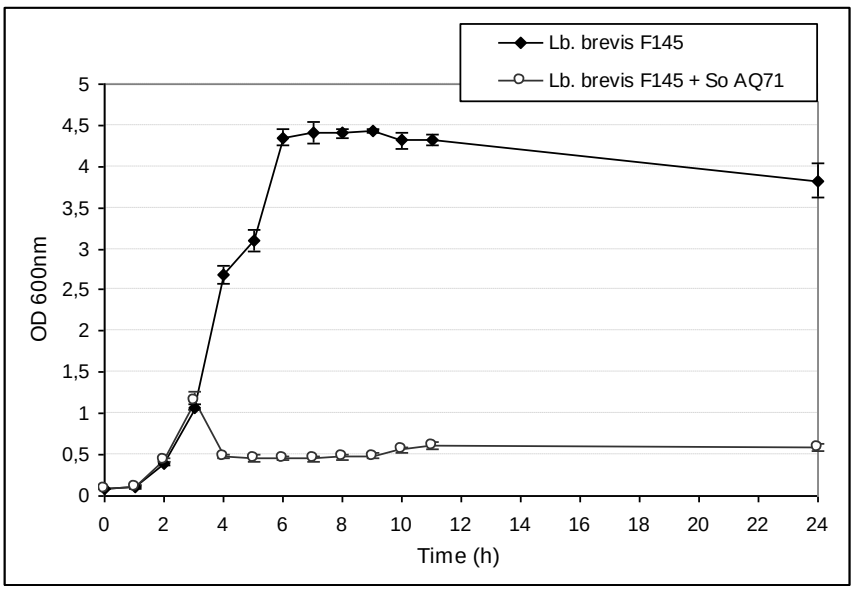
The addition of bacteriocin containing CFSs obtained from overnight cultures of studied strains, to cell cultures of *Lb. brevis* F145 and *L. monocytogenes* 506 in early exponential growth phase (3 h-old-culture) led to the inhibition of the cell growth (Fig. 3.33 and 3.34). Supernatant of strain *Lb. curvatus* A61 exhibited bacteriostatic mode of action against both of the studied indicator strains. After addition of supernatant of this strain to the cultures of indicator strains no cell lysis was observed, as the OD did not decrease. However the growth of indicator strains was inhibited (Fig. 3.33 a and 3.34 a).

Effect of cell-free supernatant of strain *E. faecium* AQ71 was strain dependent. The bacteriocin of this strain caused lysis of the cells of *Lb. brevis* F145 (Fig 3.33 b), as we observed some decrease of the OD after addition of it. It seems, that strain exhibit some kind of bactericidal effect against this indicator micro-organism. However, effect of addition of supernatant of this strain to the cell culture of *L. monocytogenes* 506 was similar to that obtained with the supernatant of *Lb. curvatus* A61 (Fig 3.34 b).

Viability loss recorded after 24 h was concomitant with an optical density decrease (data not shown).

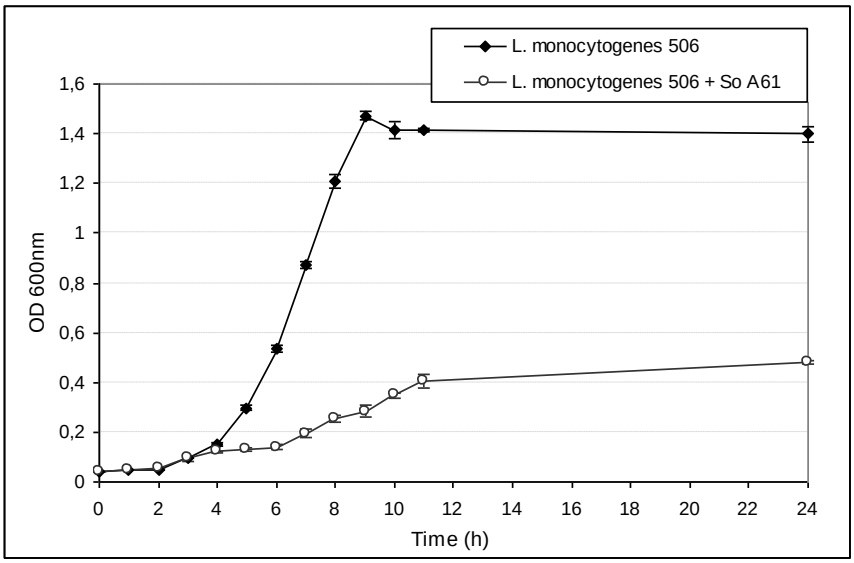


a

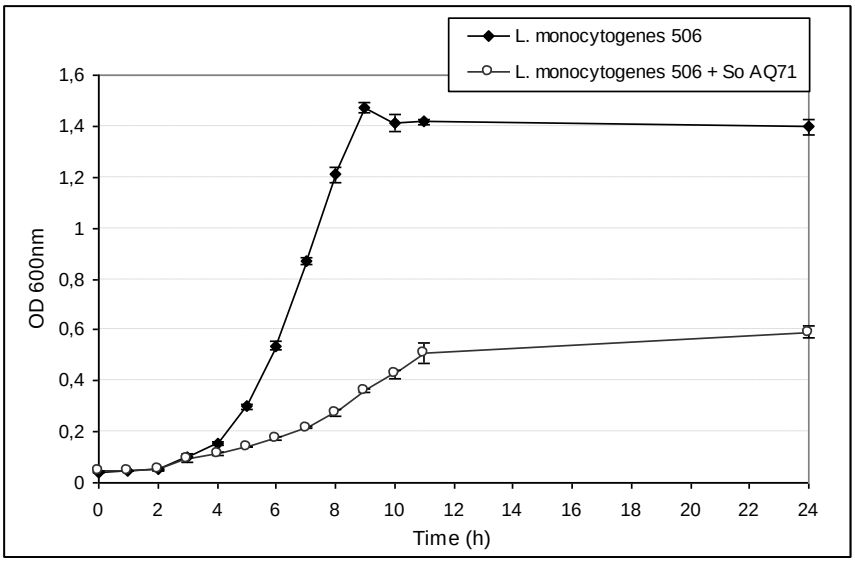


b

Figure 3.33. Effects of bacteriocin-containing cell-free supernatants of strains *Lb. curvatus* A61 (**a**) and *E.faecium* AQ71 (**b**) on the growth of *Lb. brevis* F145



a



b

Figure 3.34. Effects of bacteriocin-containing cell-free supernatants of strains *Lb. curvatus* A61 (**a**) and *E. faecium* AQ71 (**b**) on the growth of *L. monocytogenes* 506

Bacteriocins may possess a bactericidal or bacteriostatic mode of action on sensitive cells. This mode of action depends on several factors, such as bacteriocin dose and degree of purification, physiological state and the type of indicator strains, as well as the experimental conditions (Cintas et al., 2001).

3.13. Adsorption of bacteriocins on indicator strains

Several mechanisms are essential for the action of bacteriocins. The first step necessary for the action of bacteriocin is the adsorption on the cell surface of the sensitive micro-organism. In the current experiment we studied the ability of bacteriocins of tested LAB strains to adsorb

on the cells of different *L. monocytogenes* strains. Obtained results clearly indicate, that adsorption level of partially purified bacteriocins produced by studied strains was sufficiently high (Fig. 3.35). However, we also observed some adsorption on the resistant strain of *L. monocytogenes* L211.

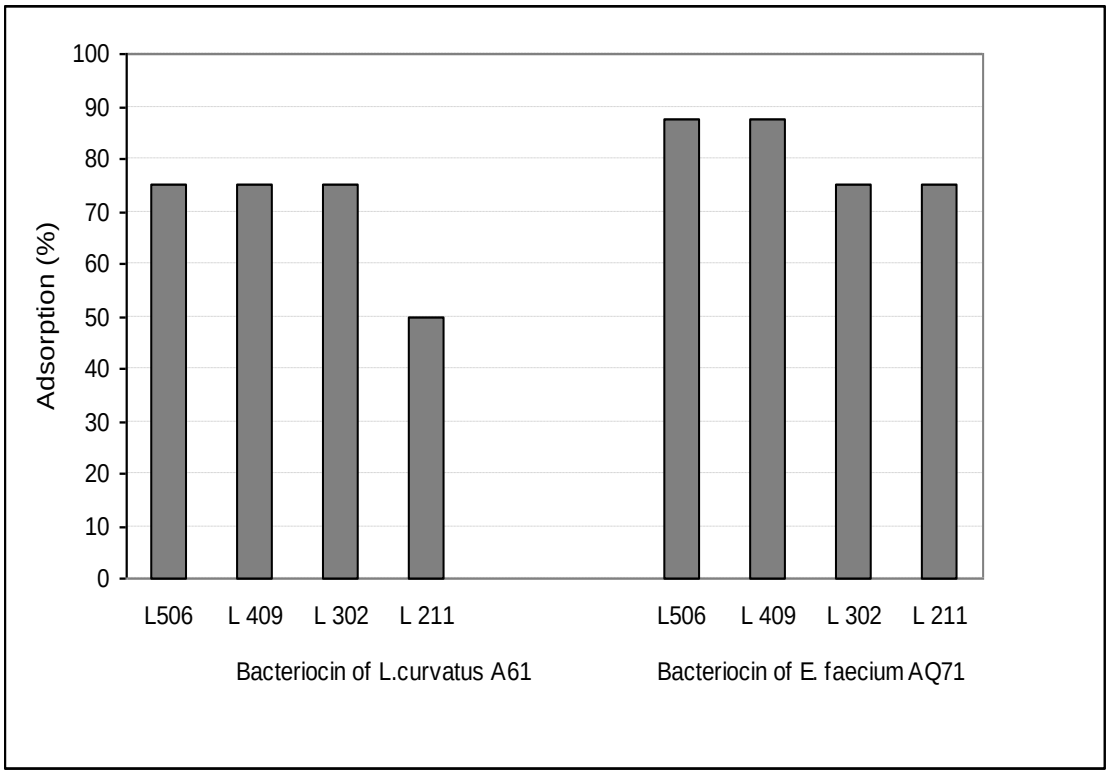


Figure 3.35. Adsorption studies of bacteriocins on the cells of different *Listeria monocytogenes* strains

According to Mayr-Harting et al. (1972) the adsorption of bacteriocin molecules to the target cells could occur by several mechanisms, that include "non specific" adsorption, which will not lead to death of the cell. Irreversible adsorption to specific lethal receptors, which can cause the destruction of cells and irreversible adsorption to specific non-lethal receptors, occupation of which by bacteriocin will not lead to the death of the cell. Thus, the adsorption of bacteriocins of studied strains to the cells of sensitive and resistant *Listeria monocytogenes* strains could occur by the same mechanism, or by different mechanisms, that could explain the high level of adsorption to both type of strains.

3.14. Auto-aggregation and co-aggregation

Aggregation is an important feature for biofilm formation. However, co-aggregation between LAB and other cells, especially *L. monocytogenes*, may be considered as a positive

characteristic, as it is one of the steps required for the elimination of nondesirable strains from the gastrointestinal tract (GIT) (Todorov and Dicks 2008). Auto-aggregation and co-aggregation are strain-specific and most probably involve species-specific surface proteins.

Different values of auto-aggregation were recorded for the studied strains (Fig. 3.36). *Lb. curvatus* A61 showed the high level of auto-aggregation (76%). The value of auto-aggregation was recorded to be 55% for another strain *E. faecium* AQ71. Auto-aggregation values ranged from 31 to 43% for partner *L. monocytogenes* strains. Strains co-aggregated with all partner strains. Strain A61 showed the higher rates of aggregation with *L. monocytogenes* strains compared to strain *E. faecium* AQ71.

It is important to point that selected co-aggregation partners *L. monocytogenes* 506, 409 and 302 were sensitive and *L. monocytogenes* 211 was resistant to bacteriocins of studied strains. Co-aggregation level was higher with sensitive to bacteriocins strains of *L. monocytogenes.*

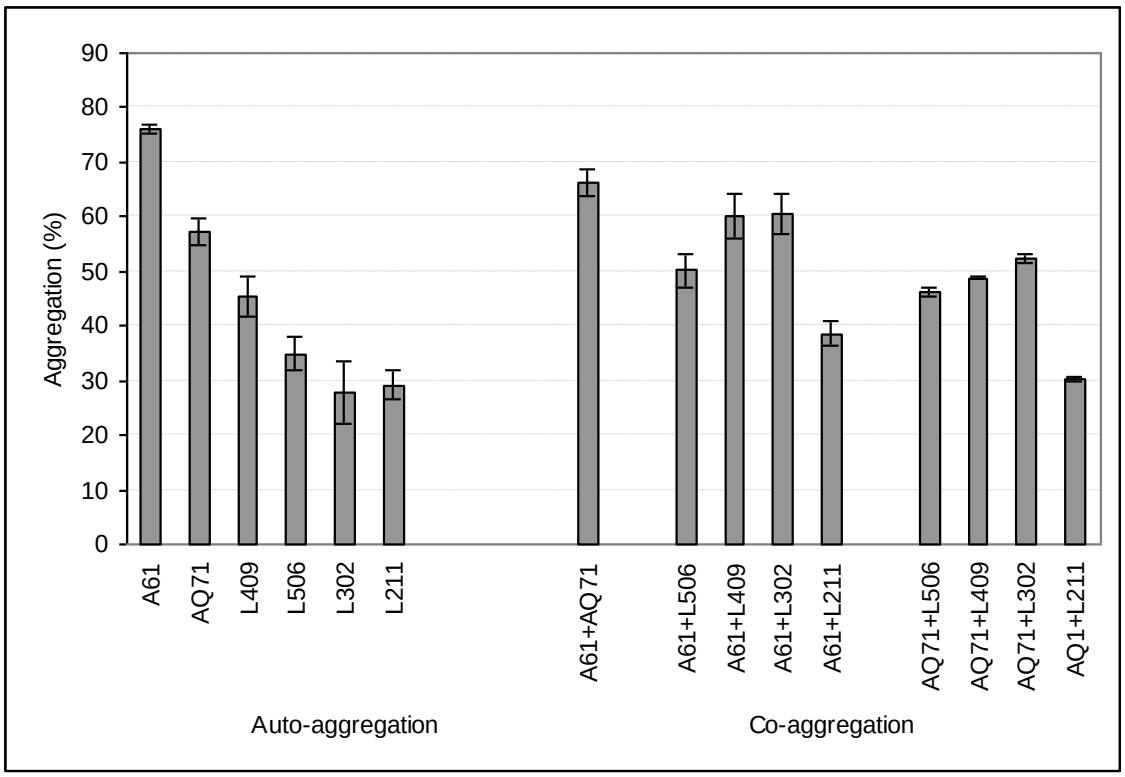


Figure 3.36. Aggregation of *Lb. curvatus* A61 and *E. faecium* AQ71 with different strains of *Listeria monocytogenes*

The low level of co-aggregation with pathogens may play an important role in preventing the formation of biofilms and in this way eliminating the pathogens from the GIT. At the same time, high level of co-aggregation with pathogens can facilitate the action of bacteriocins on the membrane of sensitive cells, thus eliminating them by bactericidal mode of action or preventing

their growth by bacteriostatic mode of action. Co-aggregation level of paired tested LAB strains AQ71 and A61 was 66%. We should mention that these LAB strains were resistant to bacteriocins of each other.

3.15. PCR amplification and sequencing of bacteriocin genes

Various primers specific for bacteriocins of LAB were used for screening of bacteriocin genes in the genome of studied strains (Table 2.3). For *Lb. curvatus* A61 PCR amplicon of the expected size (171 bp) was obtained only with the pair of primers that targets the structural gene of curvacin A *(curA)* (Fig. 3.37).

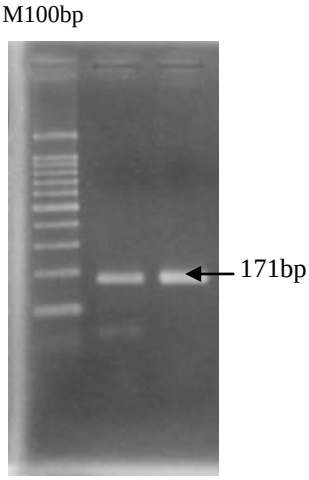


Figure 3.37. PCR detection of *curA* gene. 1.5% agarose gel with amplified PCR product

Nucleotide sequence analysis of this fragment showed 99% of identity with the *curA* gene of *Lb. curvatus* (GenBank: AB292465.1; *Lactobacillus curvatus curA, cuiA* genes for pre-peptide of curvacin A, immunity protein for curvacin A, complete cds, gene – curA position 145..339) (Fig. 3.38).

At the amino acid level, based on reconstruction *via* genetic sequence, *curA* gene detected in *Lb. curvatus* A61 showed 100% identity with Curvacin A (BAF74808.1 ; PDB 2A2B_A) (Fig.3.39).

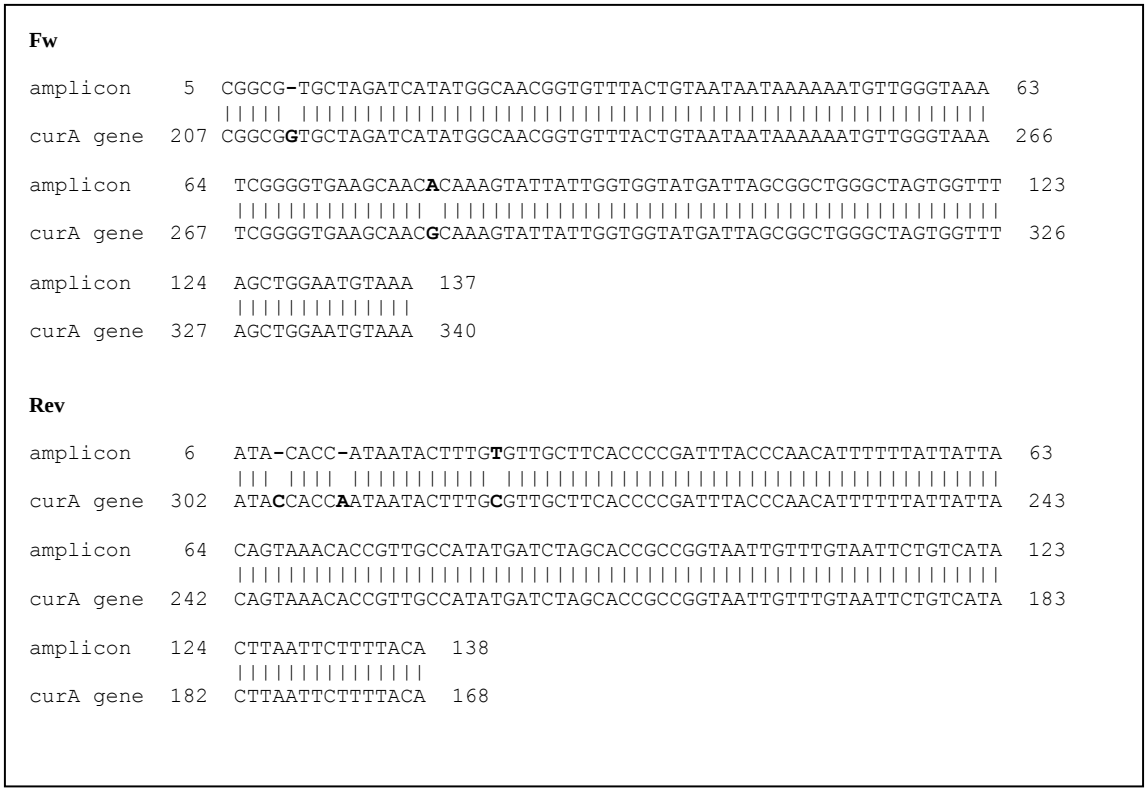


Figure 3.38. Results of sequences alignment (http://blast.ncbi) of obtained *curA* gene amplification product

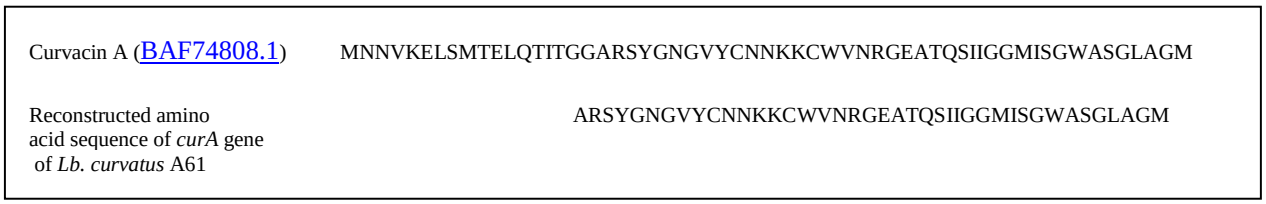


Figure 3.39. Alignment of amino acid sequences of curvacin A

For *E. faecium* AQ71 PCR amplification resulted in detection of 4 bacteriocin genes: *entP*, *entL50A/B* and *entA* (Fig. 3.40). Obtained amplicons were analysed by sequence and amino acid sequence alignments (http://blast.ncbi) based on reconstruction on genetic level, as it was shown before for *curA* gene amplification product of *Lb. curvatus* A61 strain. Nucleotide sequence analysis of amplified PCR products showed 99% of identity of amplicons to corresponding *entP* (AF005726), *entA* (AM746970.1) and *entL50A/B* genes (AJ223633.1).

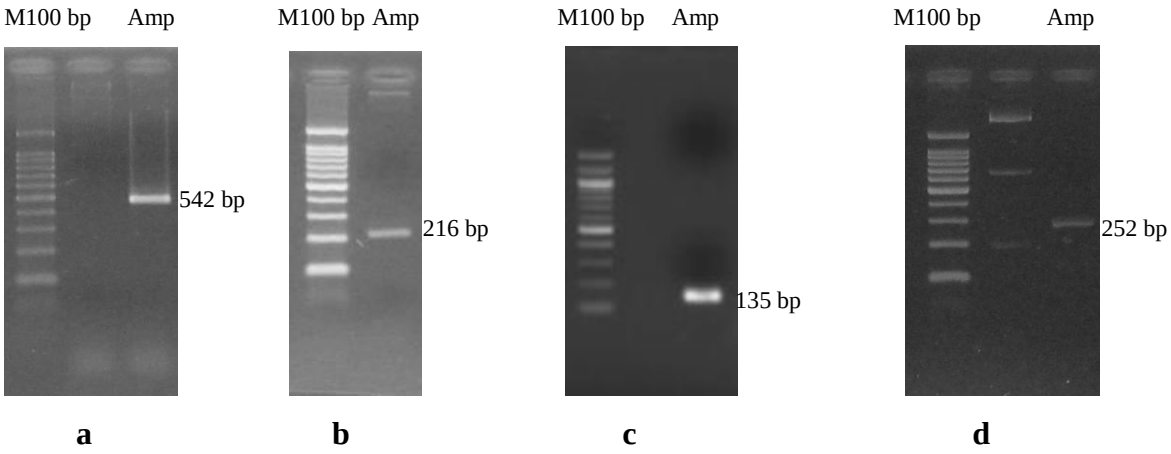


Figure 3.40. PCR detection of enterocin genes (**a**: entA; **b**: entP; **c**: entL50A; **d**: entL50B) in *E. faecium* AQ71. 2.0% agarose gel with amplified PCR products. M100bp – Marker; Amp – amplification product for corresponding gene.

3.16. Probiotic properties and safety of isolated LAB strains

3.16.1. Growth in the presence of bile salts and at different pH values

The ability of isolated proteolytic and bacteriocinogenic LAB strains to grow at different pH values and in the presence of different concentrations of bile salts was tested. Good growth of all tested *Enterococci* strains was recorded in MRS broth with initial pH values of 6.0, 7.0, 9.0 and 11.0 (Table 3.9). At pH 13.0 growth level was low. None of the *Enterococci* strains grow in MRS with initial pH 3.0 and 4.0. Obtained results are in accordance with the literature data about the optimal pH range for *Enterococci* (Giraffa, 2003).

For *Lactobacilli* strains obtained results were different. Strain *Lb. curvatus* A61 was able to grow only in MRS with initial pH 6.0, 7.0 and 9.0. Further decrease or increase of pH inhibited the growth of this strain. *Lb. helveticus* A75 grew well in MRS with initial pH 6.0 and 7.0. When the initial pH of the media was increased till 9.0, or decreased till 5.0 we also observe the growth, however there was delay in entry into the exponential growth phase. At pH 3.0, 4.0 and higher than 9.0 the strain did not grow. *Lb. paracasei* A581 was able to grow in MRS with starting pH ranging from 4.0 till 11.0.

All *Enterococci* strains grew well in the absence and in the presence of oxbile concentrations ranging from 0.2% till 3.0% (Table 3.9). For *Lactobacilli* growth was observed only at concentrations of oxbile 0.2% and 0.3%. *Lb. paracasei* A581 was also able to grow in the presence of oxbile 0.5, 0.6 and 1.0 %, however there was delay in entry into the exponential growth phase. Probiotic strains are considered to have ability of growth in the presence of 0.3% bile salts.

Table 3.9. Growth of studied LAB strains in MRS broth adjusted to different pH values and in MRS broth supplemented with different concentrations of oxbile

LAB strains	Bile salts concentration (%)								pH							
	0	**0.2**	**0.3**	**0.5**	**0.6**	**1.0**	**2.0**	**3.0**	**3.0**	**4.0**	**5.0**	**6.0**	**7.0**	**9.0**	**11.0**	**13.0**
E. faecalis AN1	++	++	++	++	++	++	++	++	-	-	-	++	++	++	++	+
E. faecalis A121	++	++	++	++	++	++	++	++	-	-	-	++	++	++	++	+
E. faecalis A124	++	++	++	++	++	++	++	++	-	-	-	++	++	++	++	++
E. faecalis A1221	++	++	++	++	++	++	++	++	-	-	-	++	++	++	++	+
E. faecalis A1232	++	++	++	++	++	++	++	++	-	-	-	++	++	++	++	+
E. faecalis A71	++	++	++	++	++	++	++	++	-	-	-	++	++	++	++	+
E. faecium AQ71	++	++	++	++	++	++	++	++	-	-	+	++	++	++	++	+
Lb. curvatus A61	++	++	+	-	-	-	-	-	-	-	-	+	++	++	-	-
Lb. helveticus A75	++	+	-	-	-	-	-	-	-	-	+,R	++	++	+,R	-	-
Lb. paracasei A581	++	++	++	+,R	+,R	+,R	-	-	-	+	++	++	++	++	-	-

++ - good growth
+ - low growth
R – retard in entering the exponenthial growth phase

3.16.2. Assessment of resistance to antibiotics

Because of the risks of transmission of antibiotic resistance genes through the food chain to other bacteria, it is necessary to evaluate not only their technological characteristics but also the safety of strains before application in foods. In the present study, antimicrobial susceptibility of all studied LAB isolates was tested with 8 antibiotics.

As shown in Table 3.9, all the studied *Enterococci* strains were susceptible to ampicillin (Minimum Inhibitory Concentration (MIC) < 8 μg/mL), gentamicin (MIC < 500 μg/mL) and tetracycline (MIC < 4 μg/mL). They were inhibited at the break-point level by chloramphenicol (MIC = 8 μg/mL). Strain A1232 was resistant to ciprofloxacin (MIC = 8 μg/mL) while other strains were evaluated as sensitive (MIC < 4 μg/mL). Resistance to vancomycin was detected only for strain A71 (MIC = 16 μg/mL). The high resistance to kanamycin (no inhibition at all concentrations used) was observed for all strains. 3.9

Table 3.10. Patterns of antibiotic MIC-s of isolated LAB strains

	Antibiotics susceptibility MIC (μg/mL)[1]							
Strains	AMP	CHL	CIP	VAN	GEN	KAN	PEN	TET
E. faecalis AN1	< 8	= 8	< 4	< 4	< 500	> 32	< 4	< 4
E. faecalis A121	< 8	= 8	< 4	< 4	< 500	> 32	< 4	< 4
E. faecalis A124	< 8	= 8	< 4	< 4	< 500	> 32	< 4	< 4
E. faecalis A1221	< 8	= 8	< 4	< 4	< 500	> 32	< 4	< 4
E. faecalis A1232	< 8	= 8	8	< 4	< 500	> 32	< 4	< 4
E. faecalis A71	< 8	= 8	< 4	16	< 500	> 32	< 4	< 4
E. faecium AQ71	< 8	< 8	< 4	< 4	< 500	ND	< 4	< 4
Lb. curvatus A61	< 8	< 8	< 64	< 4	< 500	ND	< 4	< 4
Lb. helveticus A75	< 8	< 16	< 64	< 8	-	ND	< 4	< 2
Lb. paracasei A581	< 8	< 16	< 32	< 16	-	ND	< 4	< 2

[1] AMP: ampicillin; CHL: chloramphenicol; CIP: ciprofloxacin; VAN: vancomycin; GEN: gentamicin; KAN: kanamycin; PEN: penicillin; TET: tetracycline

Sensitivity of food associated *Enterococci* to β-lactam antibiotics was also observed by other authors (Ben Omar et al., 2004; Veljovic et al., 2009). Vancomycin-resistant *Enterococci* have emerged in the last decade as a frequent cause of nosocomial infections. Only one of the studied isolates was resistant to vancomycin. Low-level resistance to this antibiotic in food isolates was yet observed (Barbosa et al., 2009).

For *Lactobacilli* strains the susceptibility to antibiotic was established according to breakpoints: ampicillin - 2 μg/mL, chloramphenicol - 16 μg/mL, penicillin - 4 μg/mL, tetracycline - 16 μg/mL, ciprofloxacin - 4 μg/mL, vancomycin - 64 μg/mL. Thus, all *Lactobacilli* strains were resistant to ampicillin (MIC < 8 μg/mL) and gentamicin (no susceptibility till 500 μg/mL). For other antibiotics the MIC levels were lower that breakpoints, thus the strains were considered as sensitive. According to the literature, *Lactobacilli* are usually susceptible to ampicillin and chloramphenicol (Ammor et al., 2007; Katla et al., 2001).

3.16.3 Screening for the presence of virulence genes

The potential pathogenicity of the isolated *Enterococci* strains was evaluated investigating the presence of 13 genes encoding virulence factors, referring to virulence determinants studied for enterococcal species (Table 3.10). Cytolysin is a bacterial toxin expressed by some isolates of *E. faecalis* that displays both hemolytic and bactericidal activities. For its production a complex determinant encoding five genes products is necessary (cylA, cylB, cylM, cylLL, cylLs) (Semedo et al., 2003). Results from PCR amplification of cytolysin operon revealed that none of the strains carried *cylA*, *cylLl*, *cylLs*, *cylB* and c*ylM* genes. In other studies, authors also reported the absence of full *cyl* operon in dairy *Enterococci* (Fortina et al., 2008; Martín-Platero et al., 2009).

Genes coding the virulence factors such as the enterococcal surface protein (*esp*) and aggregation substance (*asa1*) also were not detected. For all *E. faecalis* strains, PCR amplification revealed the presence of genes that encode the collagen adhesin gene (*ace*) and the enterococcal antigen (*efaAfs*). Other authors also reported the high incidence of *ace* and *efaA* genes in *E. faecalis* (Martín-Platero et al., 2009; Veljovic et al., 2009). Although the detection of virulence genes may point to virulence potential of food strains, food borne enterococcal infections have never been reported.

The *gelE* determinant was also detected in the studied *E. faecalis* strains. However, at least two of the genes of the *fsr* operon, which is necessary for gelatinase expression, were absent in the genome of all the studied strains. The *gelE* gene encodes a Zn-metalloprotease that has been shown to potentially contribute to *E. faecalis* virulence in some animal models (Qin et al., 2001). An interesting feature of gelatinase is that the presence of the *gelE* gene does not

imply that strains demonstrate a gelatinase activity (Eaton and Gasson, 2001; Semedo et al., 2003). It has been determined that the production of gelatinase, through expression of *gelE*, is induced by a quorum-sensing system in *E. faecalis* encoded by the *fsr* locus (Qin et al., 2001). This *fsr* locus is comprised of *fsrA*, *fsrB* and *fsrC* genes that encode FsrA (response regulator), FsrB (a sensor transducer) and FsrC (auto inducing) proteins, respectively and a complete *fsr* operon is essential for *gelE* expression (Qin et al., 2001; Nakayama et al., 2002). Various *E. faecalis* isolates carrying *gelE* reportedly fail to produce gelatinase, likely due to a deletion of the fsr cluster region (Eaton and Gasson, 2001; Nakayama et al., 2002).

Table 3.11. Patterns of virulence genes of *Enterococci* strains

	Virulence determinants												
Strains	*esp*	*ace*	*asa1*	*cylA*	*cylLl*	*cylLs*	*cylB*	*cylM*	*gel*	*fsrA*	*fsrB*	*fsrC*	$efaA_{fs}$
AN1	–[1]	+[2]	–	–	–	–	–	–	+	+	–	–	+
A121	–	+	–	–	–	–	–	–	+	+	–	–	+
A124	–	+	–	–	–	–	–	–	+	–	–	–	+
A1221	–	+	–	–	–	–	–	–	+	+	–	–	+
A1232	–	+	–	–	–	–	–	–	+	–	–	–	+
A71	–	+	–	–	–	–	–	–	+	+	–	–	+
AQ71	–	+	–	–	–	–	–	–	+	+	–	–	+

1. "- " - gene is absent
2. "+ " - gene is present

PCR amplification of virulence gene did not reveal the presence of any of them in the genome of *E.faecium* AQ71. Amplification products were obtained only for positive control (Table 3.10). Among enterococcal species, according to Eaton and Gasson (2001), *E. faecalis* harbour more of multiple virulence determinants and with much higher frequencies than *E. faecium*. *E. faecium* appears to pose a lower risk for use in foods since their strains are generally free of virulence determinants.

4. CLOSING REMARKS AND CONCLUSIONS

In the present work the screening of 431 LAB isolates from 46 samples of traditionally fermented dairy products obtained from different regions of Azerbaijan for the production of proteolytic enzymes and antimicrobial substances was performed. Search for proteolytic LAB resulted in 6 isolates (cocci) with high proteolytic activity and 2 isolates (bacilli) with lower proteolytic activity. 16S rDNA fragment sequencing resulted in identification of one isolate (A75) as *Lb. helveticus* and another isolate (A581) as *Lb. paracasei* subsp. *paracasei and* one as *E. faecalis*. The phylogenetic diversity *E. faecalis* strains was studied by RAPD-PCR analysis and it was shown that the similarity level between strains was dependent on the source, from where they were isolated.

The screening for the presence of antimicrobial activity resulted in 13 isolates among which only two showed activities not only against closely related LAB strains, but also antilisterial activity. Genotypic identification of these isolates showed that one of them (AQ71) belong to the species *E. faecium* and another (A61) to the species *Lb. curvatus.*

Proteolytic activities of isolated strains were analyzed in two systems: in UHT skim milk and in non-proliferative cells system. Proteolytic activities of identified strains grown in pH uncontrolled conditions in UHT skim milk revealed that among studied microorganisms only *Enterococci* showed remarkable activity and different degrees of α_{S1}-, α_{S2}- and β-caseins and β-lactoglobulin (BLG) hydrolysis by the selected strains was observed. Hydrolysis of α-lactalbumin (ALA) was not observed for any studied LAB.

The ability of the strains to hydrolyze different fractions of milk proteins was also determined in non-proliferative cells system. All *E. faecalis* strains and strain *Lb. helveticus* A75 were able to hydrolyze all casein fractions. *Lb. paracasei* A581 was unable to hydrolyze α_S-caseins at all, displaying specific activity only against β-casein. It was observed that the time course of caseins hydrolysis by proteinases produced by strains was also dependent on substrate used. Hydrolysis of denaturated whey proteins resulted in BLG degradation only by strain *E. faecalis* AN1. In case of other strains, hydrolysis of BLG was not observed (data not shown). In contrast, in UHT skim milk, hydrolysis of BLG was observed for all studied *E. faecalis* strains. Thus, the proteolytic activity and specificity of proteases differ in two studied systems.

Determination of pH influence on casein hydrolysis showed that the proteinases produced by studied strains hydrolyze substrate mainly in the pH range 6.0 - 7.2. Optimal temperature for hydrolysis of caseins by studied strains was found to be 37-45 °C. Study of effects of inhibitors on proteolytic activities showed the inhibition of proteolytic activity by EDTA and PEFABLOC, indicating the presence of metallo- and serine proteases. It seems that strains produce several proteases.

PCR amplification of proteinase coding genes in isolated strains resulted in detection of *prtH* gene *Lb. helveticus* A75 with 99% of identity with the corresponding active site region from *Lb. helveticus* CNRZ32. For *E. faecalis* strains the presence of genes coding metallo- and serine proteinases were detected.

The effect of proteolysis of α_{S1}- and β-caseins by *Lb. helveticus* A75 and also α_{S1}-casein by *E. faecalis* AN1 on their IgE binding ability was studied using a competitive ELISA inhibition assay. It was observed that the binding of serum IgE by hydrolysates was weaker than binding of serum IgE by intact caseins, indicating that α_{S1}- and β-caseins hydrolysed by studied strains are less well recognized by specific IgE from CMA patients than intact proteins. LC/MS/MS analysis of the hydrolysate of α_{S1}-casein by strain *E. faecalis* AN1 resulted in identification of 18 peptides of different sizes, ranging from 6 to 36 amino acids and included 8 phosphorylated peptides. The cleavage sites of proteolysis were situated on the all identified epitopes of α_{S1}-casein, with exception of one phosphorylated epitope (E69-Q78) resisting hydrolysis.

The antimicrobial strains *E. faecium* AQ71 and *Lb. curvatus* A61 showed activity against closely related species such as *Enterococci* and *Lactobacilli* and inhibited the growth of most of the tested *Listeria monocytogenes* strains and *Bacillus cereus* strain. No activity was observed against strains of *Salmonella, E. coli* and other studied pathogens. *Lb. curvatus* A61 displayed also antifungal activity. Activity in cell free supernatants of *E. faecium* AQ71 and *Lb. curvatus* A61 were stable after heating at 100 ºC for 30 min and autoclaving for 15 min at 121 °C and remained stable over a wide range of pH from 2 to 10. The antimicrobial activities of strains totally disappeared after 1 h incubation of cell free supernatants with proteolytic enzymes, what indicates their proteinaceous natures. Catalase, lipase and α-amylase had no effect on the bacteriocin activities. Effect of different chemicals on bacteriocin activities in cell-free supernatants of studied strains revealed, that TritonX-20, Triton X-80, Triton X-100, β-mercaptoetanol Na-EDTA, SDS and NaCl did not influence antimicrobial activities The optimal temperature for bacteriocin production was found to be 30 °C for *Lb. curvatus* A61 and 37 °C for *E. faecium* AQ71.

Several primers specific for bacteriocins of LAB were used for screening of bacteriocin genes in the genome of studied strains. For *Lb. curvatus* A61 PCR the presence of structural gene of curvacin A *(curA)* was determined. For *E. faecium* AQ71 PCR amplification resulted in detection of 3 bacteriocin genes: *entP, entL50A/B* and *entA*. Nucleotide sequence analysis of amplified PCR products showed 99 – 100 % of identity of amplicons to corresponding genes in GeneBank database.

The ability of isolated proteolytic and bacteriocinogenic LAB strains to grow at different pH values and in the presence of different concentrations of bile salts was tested. Studied strains showed good probiotic properties. Assessment of resistance to antibiotics showed, that none of the strains was multi resistant and thus, they are safe for application in dairy industry. The potential pathogenicity of the isolated *Enterococci* strains was evaluated investigating the presence of 13 genes encoding virulence factors, referring to virulence determinants studied in enterococcal species. Results from PCR amplification revealed that none of the strains carried *cylA*, *cylLl*, *cylLs*, *cylB* and c*ylM* genes of *cyl* operon. Genes coding the virulence factors such as the enterococcal surface protein (*esp*) and aggregation substance (*asa1*) also were not detected. For all *E. faecalis* strains, PCR amplification revealed the presence of genes that encode the collagen adhesin gene (*ace*) and the enterococcal antigen (*efaAfs*). The *gelE* determinant was also detected in the studied *E. faecalis* strains. However, at least two of the genes of the *fsr* operon, which is necessary for gelatinase expression, were absent in the genome of all the studied strains.

CONCLUSIONS

1. The screening and study of proteolytic LAB strains from traditional fermented dairy products of Azerbaijan was performed for the first time. Also, the previously started screening of bacteriocinogenic Azerbaijani LAB strains was continued.
2. Ten new LAB strains were isolated, from which 8 were proteolytic and belong to the species *Lb. helveticus* (1 strain), *Lb. paracasei* subsp. *paracasei* (1 strain) and *Enterococcus faecalis* (6 strains). Two strains were producers of bacteriocins and belong to species *E. faecium* and *Lb. curvatus*.
3. Specificity of proteolytic activity of studied LAB strains depended on substrate and on bacterial growth phase. Proteolytic activity was mainly directed towards caseins. The optimal hydrolysis of substrates by studied strains was observed at 37-45 °C and at neutral pH range. The selected strains produce several proteases, mainly metallo- and serine- proteases.
4. The proteolysis of bovine caseins by strains *Lb. helveticus* A75 and *E. faecalis* AN1 decrease their immuno reactivity as a result of destruction of IgE binding linear epitopes.
5. *E. faecium* AQ71 possesses genes, coding enterocins P, L50A/B and A; strain *Lb. curvatus* A61 possesses the structural gene of curvacine A. Antimicrobial substances produced by selected Azerbaijani LAB strains are heat stable and active in broad pH range.
6. The studied bacteriocinogenic LAB strains inhibit growth of closely related LAB species and also of some pathogens, such as *Listeria monocytogenes, Bacillus cereus.* Strain *Lb. curvatus* A61 also has antifungal activity. The bacteriocins mode of action was dependent on test-organism used and was bacteriostatic or bactericidal.
7. The studied strains do not harbour the virulence factors genes, neither the multiresistance to antibiotics, thus they are safe for industrial application. Moreover, studied Azerbaijani LAB strains are resistant to physiological concentrations of bile salts what is one of the main characteristics of probiotic strains.

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