CYPRUS UNIVERSITY OF TECHNOLOGY

FACULTY OF GEOTECHNICAL SCIENCES AND ENVIRONMENTAL MANAGEMENT

Doctoral Dissertation

DONKEY MILK MICROBIOTA: ISOLATION AND CHARACTERIZATION FOR POTENTIAL APPLICATIONS

Maria Aspri

Limassol, March, 2017

CYPRUS UNIVERSITY OF TECHNOLOGY

FACULTY OF GEOTECHNICAL SCIENCES AND ENVIRONMENTAL MANAGEMENT

DEPARTMENT OF AGRICULTURAL SCIENCES, BIOTECHNOLOGY AND FOOD SCIENCE

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Approval Form

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“Let food be the medicine and medicine be the food”

Hippocrates, 4 B.C.

LIST OF PUBLICATIONS

JOURNAL PUBLICATIONS

- Papademas, P., Parmaxi, I., & Aspri, M. (2015). Probiotic, Antimicrobial, Antioxidant and Sensory properties of Fermented donkey milk with *Lactobacillus fermentum* ME-3 and *Lactobacillus acidophilus* (ATCC 4356). *BAOJ Microbiology*, *1*(004).
- Malissiova, E., Arsenos, G., Papademas, P., Fletouris, D., Manouras, A., Aspri, M., Nikolopoulou, A., Giannopoulou, A., & Arvanitoyannis, I. S. (2016). Assessment of donkey milk chemical, microbiological and sensory attributes in Greece and Cyprus. *International Journal of Dairy Technology*, *69*(1), 143-146.
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- Aspri, M., Bozoudi, D., Tsaltas, D., Hill, C., & Papademas, P. (2017). Raw donkey milk as a source of *Enterococcus* diversity: Assessment of their technological properties and safety characteristics. *Food Control*, *73*, 81-90.
- Aspri, M., Field, D., Papademas, P., Cotter, P.D., Ross, P., Hill, C., (2017). Application of bacteriocin-producing *Enterococcus faecium* isolated from donkey milk, in the biocontrol of *Listeria monocytogenes* in fresh whey cheese (Accepted- *International Dairy Journal*).
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BOOK CHAPTER

- Papademas, P and Aspri, M (2014) Dairy Pathogens: Characteristics and Impact pp 69- 114. In: Dairy Microbiology: a practical approach, Ed Papademas, P. CRC Press, USA

ORAL PRESENTATIONS

- The biodiversity Lactic Acid Bacteria isolated from raw donkey milk and their characteristic and probiotic properties. 7th IDF Symposium on Sheep, Goat and other non-cow milk, Limassol, Cyprus, 23-25/03/2015.
- Assessment of the functionality of fermented donkey milk, 1st International Multidisciplinary Conference on Nutraceuticals and Functional Foods, Kalamata, Greece 7-9 July 2016.

POSTER PRESENTATIONS

- Growth and survival of *Lb. fermentum* ME-3 and *Lb. acidophilus* ATCC 4356 in donkey milk for the production of a probiotic milk drink. 5th IDF Symposium on Science and Technology of fermented milk, Melbourne, Australia, 03-07/03/2014
- The microbiological, chemical characteristics and functional properties of donkey colostrum and milk. Mediteranska hrana in prehrana, Izola, Slovenia,16-17/10/2015
- Technological characteristics of the LAB isolated from the Cyprus goat breed "Machairas": a preliminary study. 7th IDF Symposium on Sheep, Goat and other non-cow milk, Limassol, Cyprus, 23-25/03/2015.

ABSTRACT

Milks from non traditional animal species (i.e., donkey, camel, and buffalo) are recently gaining momentum mainly due to the fact that they are considered suitable to supplement the needs of special population groups (i.e., infants, the elderly). Research on donkey milk has dramatically increased over the past few years; therefore, this study aims to critically summarize the current research, characterizing the microbiota diversity of donkey milk and finally offering an insight into its unique functional properties, namely, antimicrobial, antioxidant and ACE-inibitory activities. The purpose of this project was to provide general information about donkey milk produced in Cyprus by characterizing and identifying the microbiota diversity of raw donkey milk, for potential application into the production of a nutraceutical fermented donkey drink. The project consists of four main parts.

There has been a trend recently to isolate wild-type strains from natural sources for use as starter cultures in food fermentation. Therefore, the first part of the project has concentrated on the study of the diversity, technological and the safety aspects of LAB isolated from raw donkey milk, in order to determine their potential to be used as starters/adjuncts cultures in dairy products. Gram-positive, catalase negative bacteria (257) were isolated using selective microbiological media from eleven raw milk samples, collected over seven months from a donkey farm in Cyprus. All isolates were identified by phenotypic and molecular methods. Organisms identified with partial 16S rDNA sequence analysis were classified within the genus of *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Enterococcus*. Following identification, the predominant LABs were assessed for technological properties; acidification capacity, proteolytic, lipolytic and autolytic activities and production of exopolysaccharides (EPS) and diacetyl. Furthermore, in order to assess their suitability as starter/adjunct cultures, their susceptibility to antibiotics, the absence of virulence factors, the lack of haemolytic activity and production of biogenic amines were also investigated. The safety profile of the isolates revealed that their great majority were susceptible to clinically important antibiotics (i.e. vancomycin) and production of biogenic amines (i.e. tyramine) while the presence of some virulence genes occurred in a few isolates.

For the second part of the project a total of 77 isolates isolated from donkey milk, selected upon their technological and safety properties were screened for their

antimicrobial activity against several spoilage and foodborne pathogenic bacteria. Amongst them, 3 *E. faecium* strains showed antimicrobial activity against specific *L. monocytogenes, S. aureus* and *B. cereus* strains. Mass spectrometry analysis demonstrated that all *enterococci* used in this study produced peptides with masses consistent with those for enterocins A and B which was also confirmed by PCR amplification. The cell free supernatant of the identified bacteriocin-producing enterococci were equally active over a wide range of pH and heat treatments making them excellent candidates for potential applications in bio-preservation. Furthermore, bacteriocins produced by these strains were tested for their capability to control post-processing contamination and growth of *L. monocytogenes* in experimentally contaminated fresh whey cheese produced in Cyprus during refrigerated storage. A strain of *E. faecium* was considered bactericidal while the other two were classified as bacteriostatic.

As there is increasing demand for probiotics, one of the questions investigated in this study was; can probiotic candidates be isolated from raw donkey milk? 77 isolates were tested for their survival at low pH. Then, the isolates that showed the highest survival rates (9) were selected for further characterization; i.e. resistance to bile salts, adhesion (BATH test), autoaggregation, coaggregation and bile salt hydrolysis.

The last part of the project was concentrated on the production of a functional fermented donkey milk beverage rich in bioactive peptides, with ACE-inhibitory, antioxidant and antimicrobial activities. LAB isolated previously from raw donkey milk (9) were evaluated for their ability to produce fermented milk rich in ACE-inhibitory, antioxidant and antimicrobial activities. The antioxidant peptide capacity was determined using two antioxidant assays while antihypertensive capacity was evaluated by measuring the capacity to inhibit ACE *in vitro* and the antimicrobial activity by using well diffusion assay against important foodborne pathogens. An important limiting factor of the large-scale diffusion of food carrying potential bioactivities is the bioavailability of the peptides responsible of such bioactivities. The main factors influencing the bioavailability of peptides are the resistance to digestion enzymes and the absorption by the intestinal epithelium. Therefore, the bioavailability of the bioactive peptides were evaluated using an *in vitro* digestion model. All activities were elevated when milk was fermented while a further significant increase was observed after simulated gastrointestinal digestion. The milk that was fermented with *E. faecium* DM33 exhibited

the strongest antioxidant activity and the highest antimicrobial activity. The highest ACE-inhibitory activity was observed in milk fermented with *Lb. casei* DM214.

In conclusion, the main contribution of this PhD thesis was to provide new knowledge about the microbiota diversity of LAB population presented in raw donkey milk. This research demonstrates that natural environments are rich in diversity and therefore could be considered as a valuable source of bacterial strains. Moreover, this project provides evidence that raw donkey milk is an excellent source of wild LAB that are able to grow well and produce fermented donkey milk with ACE-inhibitory, antimicrobial or antioxidant activities. Therefore, results of our study, illustrate that there indigenous strains of LAB showing interesting technological and potential probiotic properties that could potentially be utilized further by the food industry in the fields of food microbiology (i.e. biosafety/bio-preservation) or in dairy technology (i.e. fermented dairy products). Moreover, this project demonstrated that donkey milk fermented with LAB can be considered as a potential functional food.

Keywords: Donkey milk, LAB, Bacteriocins, Probiotics, Bioactive Peptides

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LIST OF ABBREVIATIONS

ABC:	ATP binding cassette
ABTS:	2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid
ACE:	Angiotensin-Converting Enzyme
AD:	Adsorption-Desorption
AMPs:	Antimicrobial Peptides
APD:	Antimicrobial Peptide Database
ATP:	Adenosine Triphosphate
BHA:	Butylated Anisole
BHI:	Brain Heart Infussion
BHT:	Butylated Hydroxytoluene
BLAST:	Basic Local Alignment Search Tool
BP:	Baird Parker
CAMP:	Collection of AntiMicrobial Peptide
CEP:	Cell-Envelope Proteinase
CitP:	Citrate Permease
CMP:	Caseinomacropeptide
CMPA:	Cow Milk Protein Allergy
DGGE:	Denaturing gradient gel electrophoresis
DNA:	Deoxyribonucleic acid
DPC:	Dairy Products Research Center Moorepark
DPPH:	2,2-diphenyl-1-picrylhydrazyl
DRBC:	Dichloran Rose Bengal Chloramphenicol Agar
EDTA:	Ethylene-diamineteraacetic Acid
EFSA:	European Authority of Food Safety

EPS:	Exopolysacharides
ET:	Electron Transfer
FFAs:	Free Fatty Acids
FRAP:	Ferric Reducing Antioxidant Power
GAP:	Glyceraldehyde Phosphate
GIT:	Gastrointestinal Tract
GRAS:	Generally Recognized As Safe
HAT:	Hydrogen Atom Transfer
HHP:	High Hydrostatic Pressure
HPK:	Histidine Protein Kinase
IBS:	Irritable Bowel Syndrome
IEC:	Ion Exchange Chromatography
IF:	Induction Factor
ISO:	International Organization for Standardization
LAB.:	Lactic Acid Bacteria
LDHs:	Lactate Dehydrogenases
LDL:	Low Density Lipoprotein
LF:	Lactoferrin
LPS:	Lipopolysacharides
LPS:	Lipopolysaccharides
MALDI-TOF MS:	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectroscopy
MRS:	de Man, Rogosa and Sharpe Agar
MS:	Mass Spectrometry
NADH:	Nicotinamide Adenine Dinucleotide

NSLAB	Non-Starter Lactic Acid Bacteria
OAD:	Oxaloacetate Decarboxylase
OD:	Optical Density
OPA:	o-phthaldialdehyde
Opp:	Oligopeptide Transport System
ORAC:	Oxygen Radical Absorbance Capacity
OXA:	Oxaloacetate
PBMCs:	Peripheral Blood Mononuclear Cells
PBS:	Phosphate Buffer Saline
PCA:	Plate Count Agar
PCA:	Plate Count Agar
PCA:	Plate Count Agar
PCR:	Polymerase Chain Reaction
PEF:	Pulsed Electric Field
PET-PTS:	Phosphoenolpyruvate-dependent Phosphotransferase System
PFGE:	Pulsed-Field Gel Electrophresis
PGHs:	Peptidoglycan Hydrolases
PMF:	Proton Motive Force
PUFAs:	Polyunsaturated Fatty Acids
QPS:	Qualified Presumptions of Safety
QSAR:	Quantitative Structure activity Relationship
RADP:	Random Amplification of Polymorphic DNA
RNA:	Ribonucleic Acid
ROS:	Reactive Oxygen Species
RP-HPLC:	Reversed-phase High–Performance Liquid Chromatography

RR:	Response Regulator
RSM:	Reconstinued Skim Milk
SDS PAGE:	Sodium Dodecyl Sulfate Polyacrylamide GelElectrophoresis
SDS:	Sodium Dodecyl Sulphate
SFAs:	Saturated Fatty Acids
SGF:	Simulated Gastric Fluids
SHR:	Spotaneously Hypertensive Rats
SIF:	Simulated Intestinal Fluids
SSF:	Simulated Salivary Fluids
TA:	Tributyrin Agar
TCA:	Trichloroacetic Acid
TEAC:	Trolox Equivelant Antioxidant Capacity
TFA:	Trifluoroacetic Acid
TRAP:	Total Radical Trapping Antioxidant Parameter
TSB:	Tryptone Soya Brotha
UHT:	Ultra High Temperature
UHT:	Ultra High Temperature
UPLC:	Ultra High Liquid Chromatography
USA:	United States of America
VRBGA:	Violet Red Bile Glucose Agar
WSE:	Water Soluble Extract

1 Introduction

Milk has been a staple part of our diet since the agricultural revolution as it supplies our body with important nutrients and confers numerous health benefits; it plays a critical role in nutrition and health. Milk's importance for human nutrition has been extensively researched and is very well documented. However in recent years, bovine milk and its derived products have suffered poor public perception. Therefore, milks from non-traditional animal species are gaining interest mainly due to the fact they are considered to supplement the needs of special population groups such as infants and the elderly. Donkey milk, with its unique composition, nutritional and potential bio-functional properties, could be used as a valuable alternative, especially for sensitive populations i.e. infants and the elderly and also for people that suffer from allergic symptoms of bovine milk consumption (Jirillo et al., 2014). Since 2005, the number of published research papers regarding donkey milk has increased to almost 30 per year, and the great majority of them are by Italian researchers, as it becomes evident that donkey milk could open new frontiers in terms of added-value functional dairy products.

The chemical composition and nutritional properties of donkey's milk have been widely investigated, however information regarding the natural microbiota of this milk is limited. Although, there are some studies on donkey's milk microbiota, they mainly focused on the detection of pathogenic bacteria (Cavallarin et al., 2015; Conte et al., 2012; Pilla et al., 2010; Salimei et al., 2004; Sarno et al., 2012). LAB of donkey milk are not fully characterized, and only few studies refer to the isolation and identification of single strains (Murua et al., 2013; Nazzaro et al., 2008; Sa et al., 2011). The beneficial microbiota of donkey milk represented by LAB is a potential source of bacterial to be used in dairy technology. However, the transformation of donkey milk by fermentation is not easy and more research for elucidating the process is needed. Thus, this study aims in isolating, characterizing the LAB microbiota of donkey milk, as well as offering an insight of the special characteristics of this milk, while proposing some new research grounds for new donkey milk products.

1.1 Research Question and Hypothesis

Could donkey milk serve as a medium/reservoir for isolation of beneficial lactic acid bacteria?

Therefore, our working hypothesis was that donkey milk could be used for the isolation of LAB that possess possible technological, probiotic properties and antimicrobial activities (i.e. bacteriocin production). Furthermore, these LAB could be used for the production of fermented donkey milk with functional properties (i.e. antimicrobial, antioxidant and ACE-inhibitory activities).

1.2 Research Objectives

Over the last decades, there has been a continuous increase in the consumption of functional foods and the market of these products is flourishing. According to literature donkey milk has a unique composition which includes high levels of whey protein, lactose and minerals, and a low amount of fat; a composition similar to human milk and is claimed to have special therapeutic properties. The general objective of this study is to provide information about donkey milk produced in Cyprus that can be potentially used for isolation of beneficial microbial strains to be used for the production of a functional drink.

In order to achieve this goal, this study has focused in four different areas. The first part of the study aimed at characterising the microbial biodiversity of donkey milk by isolating the LAB population. The second part focused on the study of the antimicrobial activity of LAB isolates against indicator bacteria and to investigate the presence of bacteriocins with antimicrobial activity against indicator bacteria. The third part of this project examined the technological and probiotic properties of isolated strains including acidification activity, proteolytic activity, tolerance against acid and bile, haemolysis and antibiotic resistance. The last part of the study focused on ACE-inhibitory, antioxidant and antimicrobial activities of donkey milk fermented with selected bacterial isolates.

The specific aims of the study are:

- To collect indigenous donkey milk samples during spring and summer period, in order to determine the predominant microbial groups

- To isolate and characterize new strains of lactic acid bacteria (LAB) from donkey milk with promising potential for technological exploitation by using both phenotypic, molecular, physiological and biochemical methods
- To the examine antimicrobial activity of LAB isolates against indicator bacteria and to investigate the presence of bacteriocins from LAB with antimicrobial activity against indicator bacteria
- To screen strains of LAB for (a) technological properties (proteolytic activity, acidification activity and (b) probiotic characteristics (tolerance to bile concentrations and low pH, haemolytic activity and antibiotic sensitivity).
- To produce a fermented donkey milk with isolated LAB and compare the angiotensin-converting enzyme (ACE)-inhibitory, antimicrobial and antioxidant activities with raw donkey milk.

1.3 Significance of the study

This is the first comprehensive project on the characterization of LAB strains isolated from raw donkey milk. The findings of the study will be important in order to show that donkey milk can be used as a medium for the isolation of beneficial microbial strains that can be used for the production of a functional drink. At the moment, despite the great interest in donkeys' milk, no commercial fermented products are available, such as lactic-alcoholic beverages Koumiss and Airag produced from mares' milk. Therefore, this research work provides a baseline data on the production of a fermented donkey milk. The study can also add value to donkeys beyond the known value accustomed by society such as transportation, pack and ridding. Finally, it can serve as a starting point for other researchers to investigate further the nutritional, microbiological, medical and cosmetic properties of donkey milk.

2 Literature Review

2.1 Donkey Milk

2.1.1 Introduction

Milk has been a staple part of the human diet since the agricultural revolution, as it supplies the body with important nutrients, hence playing a critical role both in nutrition and in health. However, in recent years, individuals affected by intolerances and allergic symptoms derived from consuming cow's milk and its products have increased in numbers leading those affected to look for alternatives (Jirillo et al., 2010). Avoiding cow's milk is imperative in the diet of cow milk protein allergy (CMPA) subjects and donkey milk can be used as an alternative ingredient in the "solid food-based diet" or after the first year of life of sensitive infants. It is important to stress those donkey milk nutritional deficiencies (i.e., fat content) need to be integrated in the diet.

The donkey, *Equus africanus asinus*, is a domesticated member of the Equidae family. The domestication of the donkey began about 6000 BC in present-day Libya, starting from one or two subspecies of African wild asses (*E. africanus*). Over the centuries, donkeys have spread in Asia, India, South America, and south Europe. Donkeys have a lifespan of 30–50 years and have historically been used for riding, as a pack animal for short-distance transport, and as a draft animal. Other uses include milk production (used in human nutrition and in cosmetic industry), meat production (very limited in some countries), onotherapy (a method of using contact and educational techniques with donkeys to help people with challenges), and finally for recreational purposes such as riding and ecotourism.

In the 19th century, upper-status society consumed donkey milk, but poor families saved it for a sick child or a weakened old person. During this time, donkey's milk started to be regularly used in maternity hospitals and used to feed infants. Furthermore, until the beginning of the 20th century, donkey's milk was marketed for the feeding of orphan infants, unhealthy children, ill people, and the elderly. It is further explained that because of the traditional use of donkey's milk, farms of donkeys were set up in Italy, France, Belgium, Switzerland, and Germany during the start of the 20th century. The

success of these farms that exploit donkey's milk attests to the product's ancestral reputation and the significance of its vital properties (Tesse et al., 2009).

Nowadays, about 43 million donkeys were reported in 2014, with this figure rising over the last 10 years. The great majority are present in Africa (44.3%) and Asia (38.6%) of the total population, while the Americas and Europe represent 15.9% and 1.2%, respectively (FAOSTAT, 2014). On a country basis, Ethiopia has 7.4 million heads followed by China (6.0 million), Pakistan (4.9 million), and Mexico (3.3 million), while in Europe, Spain has 140,000, Portugal 115,000, Greece 35,000, Italy 24,900, and France 15,000 (FAOSTAT, 2014). Some researchers believe that the actual numbers are somewhat higher since many donkeys go unaccounted for.

The aim of this part is to critically review recent research on donkey's milk and offer "food for thought" for future research, opening new frontiers in terms of added-value functional dairy products.

2.1.2 Donkey's milk production

Donkey milk production differs greatly from that of other dairy species, especially in terms of milk supply. As with all mammals, lactation is triggered by birth. Interestingly, an investigation in Sicily showed that the local latitude determined small photoperiod oscillations between different seasons, and under these conditions the donkeys have a continuous reproductive cycle (Giosue et al., 2008). According to Salimei et al., (2004) there is a difference in the milk yield obtained when donkeys were milked twice a day (morning and afternoon). Results show that morning milking yields statistically lower milk than the milk yield during the afternoon milking, 549.2 mL vs. 949.3 mL, respectively. Also studies carried out by D'Alessandro and colleagues (2007; 2009; 2012) have investigated the effects of daily milking on donkey milk production. The studies showed that the highest milk yield corresponded to three milkings per day every 3 hours, while daily milking regimen of six milkings per day did not increase milk production and had a negative influence on the health of the mammary gland. Alabiso et al., (2009) also showed that the highest milk yield can be obtained with three milkings per day compared to two per day, with an increase in milk fat content, too. Martini et al., (2014) estimated that the average milk yield remains constant for up to 8–10 months of lactation. Moreover, some authors showed that milk production also can be affected

by both breed and the breeding season. For instance, the donkeys that gave birth in an autumn–winter period yielded more milk than donkeys foaling in spring–summer period since seasonal thermal stress can have detrimental effect on the quantity and quality of milk (Cosentino et al., 2012)

2.1.3 Chemical composition of donkey milk

The main milk components of mammals are water, fat, protein, carbohydrates, minerals, and vitamins, which differ significantly both quantitatively and qualitatively between species. Species in the same taxonomic order such as equids tend to produce milk with quite similar composition. Donkey's milk composition resembles that of human milk rather than dairy animals (e.g., cow, buffalo, sheep, goat, and camel). More precisely, donkey and human milk have similar lactose, total protein, and whey protein contents. Caseins are present in donkey milk but at a much lower content than in cow's milk (see Table 1). Donkey milk shows a homogeneous nutritional profile that is particularly important since it will be utilized by sensitive populations (infants and elderly) (Guo et al., 2007). The reason why donkey milk is so similar in macro-composition to that of human milk is still unclear, especially as donkey and humans are not phylogenetically related (Uniacke-Lowe et al., 2010).

Table 1: Nutritional composition and pH values in different milk types

Components g/100g	***Donkey***	***Bovine***	***Human***
Total Solids	8.8-11.7	12.5-13.0	11.7-12.9
Fat	0.3-1.8	3.5-3.9	3.5-4.0
Lactose	5.8-7.4	4.4-4.9	6.3-7.0
Ash	0.3-0.5	0.7-0.8	0.2-0.3
Protein	1.5-1.8	3.1-3.8	0.9-1.7
Casein	0.64-1.03	2.46-2.80	0.32-0.42
Whey Proteins	0.49-0.80	0.55-0.70	0.68-0.83
pH	7.0-7.2	6.6-6.8	7.0-7.5

Reference: Guo et al., 2007

2.1.3.1 Proteins

As presented in Table 1, the content of total protein of donkey milk is quite low (1.5–1.8 g/100 g) when compared to bovine milk (3.1–3.8 g/100 g) and is closer to the

corresponding human milk (0.9–1.7 g/100 g). The protein fraction of donkey milk is rich in whey proteins, which represent 35–50% of the nitrogen fraction, while in bovine milk they represent only 20%. Specifically, the ratio of casein to serum proteins is 52:37 and varies among the lowest limits for human and higher for cow (Guo et al., 2007). This condition gives human milk the special property of forming a soft curd during digestion in the infants' gut. The softness of the curd is due to the lower ratio of soluble calcium (El-Agamy, 2007). This condition may explain why in parts of the world donkey milk is used as human milk substitutes for bottle-fed infants. On the other hand, both ovine and buffalo milk give a hard curd, which of course is preferred in cheese making. Donkey's milk three major whey proteins are α-La (1.80 mg/ml, 22.56%), β-Lg (3.75 mg/ml, 29.85%), and lysozyme (1.00 mg/ml, 21.03%) (Fantuz et al., 2001; Salimei et al., 2004). In addition to these three, also present are immunoglobulins (Igs) (11.5%), blood serum albumin (BSA) (6.2%), and lactoferrin (4.5%). All percentages above represent (%) of total whey protein content.

Tidona et al., (2014) studied the casein micelle size in donkey milk by photon correlation spectroscopy, showing a wide range of variability among the individual milk samples (257.5 ± 4.9–330.1 ± 1.6 nm, with an average value of 298.5 ± 18.9 nm). The variability is independent of the isoelectric focusing protein pattern and is correlated to the lactation stage. Lactation stage was associated to micelle sizes and total protein content; i.e., longer lactation period decreased micelle size and total protein content. Tidona et al., (2014) also state that human casein micelles are very small at 64 nm, compared with donkey casein micelles.

As reported by Salimei et al., (2004) and Ivankovic et al., (2009) the protein content of donkey milk is not affected by the numbers of milking and breeding conditions but is affected significantly during the lactation period, where particularly it decreases during the first 6 months. After this period, its percentage remains constant (1.50%) (Guo et al., 2007). Milk proteins and their fractions are summarized in Table 2.

Table 2: Casein and whey protein composition of non-protein nitrogen content and casein micelle size in different milk types

Proteins (g/kg)	***Donkey***	***Bovine***	***Human***
Total casein	**7.8**	**26**	**2.4**
α_{s1}- casein	nq	10.7	0.77
α_{s2}- casein	nq	2.8	nq
β- casein	nq	8.6	3.87
κ- casein	nq	3.1	0.14
γ- casein	nq	0.8	nq
Total whey proteins	**5.8**	**6.3**	**6.2**
β-lactoglobulin	3.3	3.2	n/a
α-lactoglobulin	1.9	1.2	2.5
Serum albumin	0.4	0.4	0.48
Proteose petone	-	0.8	nq
Immunoglobulins	1.30	0.80	0.96
$IgG_{1.2}$	nq	0.65	0.03
IgA	nq	0.14	0.96
IgM	nq	0.05	0.02
Lactoferrin	0.37	0.10	1.65
Lysozyme	1.00	neg	0.34
NPN (mg/L)	455	266	454
Casein Micelle Size (nm)	100-200	182	64

neg=negligible nq = not quantified

References: Guo et al., 2007; Uniacke-Lowe et al., 2010

Tidona et al., (2014) also studied the digestibility of donkey milk protein and they reported that after 30 min of gastric digestion, a reduction in total milk proteins occurs, while 77% of proteins resisted degradation. At the end of the treatment, when duodenal enzymes were present, 30% of proteins were undigested. After 60 minutes of digestion, the proteins that were completely broken down were lactoferrin and caseins, whereas lysozyme and α-lactalbumin were the most resistant proteins to the human digestive enzymes (Tidona et al., 2014).

2.1.3.2 Total amino acids

The total amino acid composition of donkey milk was investigated by Guo et al., (2007) after acid hydrolysis and as is reported in Table 3, donkey milk has noticeably higher levels of serine, glutamate, arginine, and valine and much less cysteine. The content of seven out of the eight essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, valine) is also higher than that of bovine milk.

Table 3: Amino acid composition of different milk types

Amino acid (g AA /100g protein)	***Donkey***	***Bovine***	***Human***
Aspartic acid	8.9	7.8	8.3
Serine	6.2	4.8	5.1
Glutamic acid	22.8	23.2	17.8
Glycine	1.2	1.8	2.6
Histidine	2.3	3.0	2.3
Arginine	4.6	3.3	4.0
Threonine	3.6	4.5	4.6
Alanine	3.5	3.0	4.0
Proline	8.8	9.6	8.6
Cystine	0.4	0.6	1.7
Tyrosine	3.7	4.5	4.7
Valine	6.5	4.8	6.0
Methionine	1.8	1.8	1.8
Lysine	7.3	8.1	6.2
Isoleucine	5.5	4.2	5.8
Leucine	8.6	8.7	10.1
Phenylalanine	4.3	4.8	4.4
Tryptophan	n.d.	1.5	1.8
Essential AA	38.2	37.5	40.7

n.d.: not determined

Reference: Guo et al., 2007

2.1.3.3 Fat and fatty acids

Fat content of donkey milk ranges from 0.28% to 1.82%. Breed, milking strategy, and technique seem so far to be the main factors affecting the donkey milk fat content (Guo

et al., 2007). The fatty acid profile of donkey milk has been extensively studied by Martemucci and D'Alessandro, (2012), Martini et al., (2014), Salimei and Fantuz, (2013) and Gastaldi et al., (2010). The lipid fraction is comparable to that of human milk and is characterized by high levels of essential fatty acids and low saturated fatty acids (SFAs) (Gastaldi et al., 2010). In Table 4, a comparison is made between milks (i.e., < donkey, bovine, and human milk). Bovine milk has the highest proportion of SFA from all reported species (67.7–74%). Donkey and horse milks have similar fatty acid profiles, although donkey milk showed a higher concentration of SFAs compared to horse's milk while the latter has a higher content of monounsaturated fatty acids (Gastaldi et al., 2010). More specifically, butyric (C4:0), caproic (C6:0), and lauric (C12:0) acid contents were consistent with horse's milk data; however, lower contents for caprylic (C8:0) and capric acid (C10:0) were observed (Salimei et al., 2004).

Donkey milk is rich in polyunsaturated fatty acids (PUFAs), which also predominate in human milk (15–20%), with a high percentage of linoleic acid (essential in that PUFAs are not synthesized by the organism), a low ω-6 to ω-3 ratio (LA/ALA ratio), low and advantageous values of atherogenic and thrombogenic indices, and high level of unsaturated/SFA content. All of the above parameters seem to have a positive effect in human diet by immune-stimulant properties, cholesterol-lowering agents, preventing the formation of blood clots, and minimizing the risk of coronary heart disease, hypertension, and thrombosis (Gastaldi et al., 2010).

Table 4: Fatty acid composition of different milk types

Fatty acids (g /100g)	***Donkey***	***Human***	***Bovine***
C4:0	0.32-0.6	0.19	3.90
C6:0	0.28-1.22	0.15	2.50
C8:0	8.52-12.8	0.46	1.50
C10:0	18.65-20.42	1.03	3.20
C12:0	10.67-15.9	4.4	3.60
C14:0	5.77-10.59	6.27	11.10
C14:1 n5	0.22-0.88	0.80	0.41
C15:0	0.32-0.57	0.43	1.20
C16:0	11.47-29.17	22.00	27.90
C16:1	2.37-3.93	3.29	1.50
C17:0	0.22-0.52	0.60	0.58
C17:1	0.27-0.73	0.37	0.36
C18:0	1.12-3.91	8.06	12.20
C18:1 n9	9.7-22.15	31.30	17.20
C18:2 n6 (LA)	8.15-15.17	10.85	1.40
C18:3 n3(ALA)	6.32-16.33	1.03	1.80
C20:0	0.12	0.44	0.35
C20:5	0.27	0.12	0.09
C22:0	0.05	0.12	0.20
C22:6	0.30	0.25	0.01
SFA % total fatty acids	46.7-67.7	39.41-42.24	55.7-72.8
MUFA % total fatty acids	15.3-35.0	44.30-45.11	22.7-30.3
PUFA % total fatty acids	15.2-30.5	15.48	2.4-6.3
PUFA n3 % total fatty acids	9.45-9.64	1.27-2.19	nc
PUFA n6 % total fatty acids	11.57-13.09	11.17-14.1	nc

ALA = alpha linolenic acid, LA=linoleic acid, nc=not calculated

Reference: Claeys et al., (2014); Salimei & Fantuz, (2012); Uniacke-Lowe & Fox, (2011)

2.1.3.4 Lactose

The lactose content of donkey milk ranges from 6 to 7% and is higher than that of cow milk (4.1–4.4%). The high lactose content promotes the osteogenesis processes, facilitates the intestinal absorption of calcium and phosphorus, and influences the mineral accumulation in bone structure, which helps in the prevention of osteoporosis.

In addition to this, lactose is also responsible for the good taste of donkey milk (Iacono et al., 1992).

2.1.3.5 Vitamins

Milk can be considered as an almost complete food for newborns, since it contains the recommended amounts of essential nutrients; its whey fraction is a great source of water-soluble vitamins. Additionally, fat-soluble vitamins are present in its lipid fraction (Tafaro et al., 2007). The total vitamin content of milk varies, depending on the mother's diet (water-soluble vitamins are more influenced from mother's diet than fat-soluble vitamins) and vitamin status (Claeys et al., 2014). The high content of vitamins present in donkey's milk makes it an excellent nutritional food with a beneficial effect on human nutrition and health (Cunsolo et al., 2007; Tafaro et al., 2007).

Vitamin levels of different milks are presented in Table 5. Specifically, Vitamin B12 (cobalamin) is responsible for maintaining healthy nerve cells and helps in producing DNA and RNA is present in much higher concentration than bovine and human milks. Additionally, other vitamins of the B-complex [with the exception of niacin (vitamin B3)], such as thiamine (vitamin B1) and riboflavin (vitamin B2) are higher in donkey milk than in human milk. Donkey milk has a lower amount of vitamin A and E when compared to bovine and human milk. The reported total vitamin C content present in donkey milk represents the recommended daily intake of vitamin C for children aged 6–12 months (Gubic et al., 2014).

Table 5: Vitamin content of different milk types

Vitamins (mg/ L)	*Donkey*	*Bovine*	*Human*
Vitamin A	0.017	0.32-0.50	0.3-0.7
Vitamin E	0.051	0.98-1.28	3-8
Vitamin C	3.5-5.0	0.94	50-100
Vitamin B1	0.41	0.37	0.003-0.015
Vitamin B2	0.64	1.8	0.4-06
Vitamin B3	0.74	0.9	1.7
Vitamin B12	1.10	0.004	0.5

Reference: Salimei & Fantuz, (2012); Uniacke-Lowe & Fox, (2011)

2.1.3.6 Minerals and trace elements

The importance of minerals in human nutrition is well known, because they play a fundamental role in growth and skeletal structure development. The mineral and trace element composition of donkey milk is very close to that of human except that donkey milk has higher levels of calcium and phosphorus, but the Ca-P ratio is similar (see Table 6). Regarding essential trace elements, donkey milk contains similar concentrations of Zn, Co, and I, with human milk, whereas Fe, Cu, and Se concentrations are lower. The milk produced in the first month of lactation, when it is the only nutritional source for the foal, contained the highest levels of mineral elements, which may be related to the fast growth stage of the foal. During the lactation, there is a significant decrease in the composition of minerals in milk, which could be explained by the concomitant decline of casein amount since those minerals are mainly associated to the casein micelles (Fantuz et al., 2012; Giosue et al., 2008).

Table 6: Minerals and trace element content of different milk types

Minerals (mg/L)	***Donkey***	***Bovine***	***Human***
Ca	330-1140	122	278
P	320-650	119	140
K	240-747	152	530
Na	100-268	58	180
Mg	40-83	12	35
Ca/P	1.72	-	1.7
Fe	0.43-2.66	0.08	0.72
Zn	1.23-3.19	0.53	1-3
Cu	0.08-0.30	0.06	0.2-0.4
Mn	trace	0.02	0.003-0.006
Ti	0.0773	0.111	0.025
Rb	0.3391	-	-
Sr	0.8817	0.417	0.06
Mo	0.0045	0.022	0.017
Cs	0.00049	-	-
Pb	0.0032	-	-
Se	0.00446	0.01	0.1-0.2
Co	0.00049	0.0005	0.0001-0.0002
I	0.0749	0.1-0.9	0.062

Reference: Bilandžić et al., (2014); Darragh & Lonnerdal (2011); Fantuz et al., (2012); (2013); (2015); Potorti et al., (2013); Salimei & Fantuz, (2012); Uniacke-Lowe & Fox, (2011)

2.1.4 Microbiological quality of donkey milk

The growing interest in donkey milk as a food for sensitive consumers, such as infants with allergies or immunocompromised elderly people, implies strict regulations for food

safety. In this regard, the European Union laid down specific rules for the production of raw milk from any mammal (Regulations EC 852 & 853/2004), and restrictions are applied by Member States for safety reasons. The EC Regulation 853/2004 would allow the sale of donkey's milk under the clause "other milk producing species," where the total bacterial plate count is less than 1,500,000 cfu/ml at 30 °C, for raw donkey milk intented to be used for products made from raw donkey milk and not for direct human consumption (Colavita et al., 2011). Very recently, Greek authorities have passed on specific legislation for the use of equine (donkey and horse) milk for human consumption, while in Italy specific requirements are introduced at regional levels for selling raw (or pasteurized) donkey milk with no homogeneity in quality standards (plate count agar (PCA) ranges from 25,000 to 500,000 cfu/mL) (Colavita et al., 2011). In Cyprus, the local veterinary services are preparing to pass legislation on donkey milk quality to be implemented in 2017.

The microbiological data of raw donkey milk reported in literature show a quite low total plate count; mean of total plate count of donkey milk is 2.40–5.87 log cfu/ml (Chiavari et al., 2005; Colavita et al., 2011; Coppola et al., 2002; Salimei et al., 2004; Zhang et al., 2008). The reason for the lower microbial count might be due to the good health record of donkeys, the excellent natural anatomical position of the udder, (Salimei & Fantuz, 2012) the smaller size of the udder and therefore limit exposure of the teats to bacterial contamination, (Doreau & Martin-Rosset, 2011) as well as to the presence of natural antimicrobial components, such as lysozyme, immunoglobulins, lactoferrin, and lactoperoxidase.

Moreover, regarding the presence of foodborne pathogens such as *Salmonella* spp., *E. coli* 0157, *Listeria monocytogenes*, *Bacillus cereus*, and *Camplylobacter* spp., these have never been reported in literature except for the presence of *B. cereus* and *Staphylococcus* spp (Cavallarin et al., 2015; Conte et al., 2006; Conte & Passantino., 2009; Conte et al., 2012; Malissiova et al., 2016; Pilla et al., 2010; Salimei et al., 2004; Sarno et al., 2012). In addition, some human pathogenic strains of *Streptococcus* were detected in raw donkey milk, even though raw donkey milk has not been associated with outbreaks so far (Verraes et al., 2014). Regarding the LAB microbiota of donkey milk, studies are limited. According to some studies, the LAB content of donkey milk ranges between 1.0 and 4.2 log cfu/ml, but only few LAB isolates have been identified

(Carminati et al., 2014; Chiavari et al., 2005; Coppola et al., 2002; Saric et al., 2012; Zhang et al., 2008). At 20 °C, LAB, yeasts/molds, and coliforms are reported to grow well in donkey milk (Zhang et al., 2008). The high percentage of coccus-shaped species may be due to the high lysozyme content in donkey's milk. According to Neviani et al., (1991) LAB cocci are more resistant to lysozyme than lactobacilli, and moreover among lactobacilli, the lysozyme sensitivity is species or strain specific; for instance, thermophilic species are more sensitive than hetero-fermentative mesophilic lactobacilli.

Additionally, a novel bacterial strain, LCJO2T, was isolated on R2A agar from donkey milk powder. Its proposed name is *Asinibacterium lactis*. This bacterium is Gram-negative, non-motile, non-spore-forming, and rod-shaped and produces yellow-pigmented colonies. It represents a novel genus and species in the family Chitinophagaceae (Lee et al., 2013).

2.1.5 Chemical contamination and potential toxic elements

The chemical contaminants that could affect milk are persistent organic pollutants (organochlorine pesticides) from soil and polychlorinated biphenyls derived from industrial emissions. A recent study revealed that donkey milk did not pose any toxicological risk of persistent organic pollutants for consumers (Di Bella et al., 2014). Residues of these pollutants may accumulate in donkeys through contaminated food and inhaled air, stored in fat-rich tissues of the animal, and then excreted into the milk. Potorti et al., (2013) revealed that donkey milk is rich in selenium, zinc, copper, and iron, while it has low amounts of manganese and chromium. These results did not postulate any dangerous residues for human health. Indeed, Hg and As are within the permitted range, while Cd and Pb were infrequently reported (and only for children), above the European benchmark of cow milk (0.02 mg kg−1). However, in a recent study by Fantuz et al., (2015) the authors reported lower concentrations of As, Cd, and Pb, when compared to the results of Potorti et al., (2013) probably highlighting the expected variability of pollutants originating from the environment.

2.1.6 Health-related functional properties of donkey milk

A balanced diet plays an important role in preventing diseases and in promoting well-being (Martemucci & D'Alessandro, 2012). The therapeutic properties of donkey's milk are known since ancient times, but nowadays research focuses on its nutrient composition and potential health effects (Gubic et al., 2014). The milk is becoming increasingly important in Europe, especially in France, Italy, Hungary, and the Netherlands (Martini et al., 2014; Salimei and Fantuz, 2012).

Functional properties of donkey milk as classified in Figure 1 are mainly attributed to (a) antimicrobial activities, (b) immunomodulating activities, and (c) hypoallergenicity due to the presence of milk constituents in substantial quantities such as immunoglobulins, lysozyme, lactoferrin, Ω3-fatty acids, bioactive peptides, and favorable casein:whey protein ratio (Nazzaro et al., 2010).

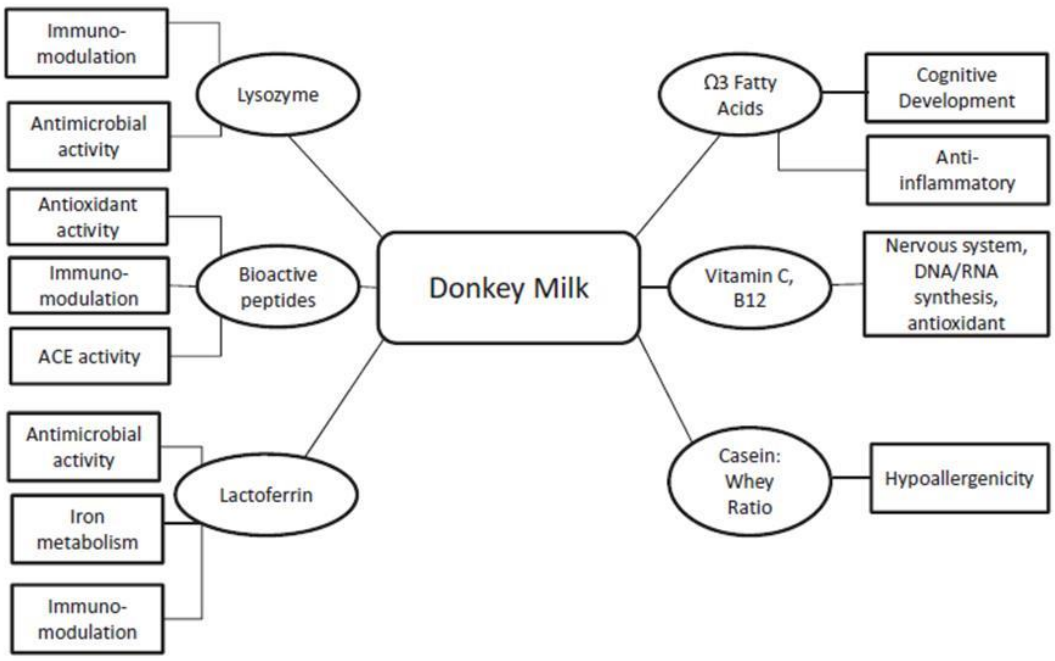


Figure 1: Main functional properties of donkey milk

2.1.6.1 Antimicrobial activity

Donkey milk contains several antimicrobial proteins, namely lysozyme, lactoferrin, and lactoperoxidase, which have the ability to inhibit the growth of a broad spectrum of bacteria. Additionally, they have the ability to reduce the incidence of gastrointestinal

infections in the digestive tract or by defending against infections of the mammary gland.

Lysozyme is an enzyme present in donkey milk with two variants namely, A and B, which differ by three amino acids substituted at different positions. It is practically absent in other milk-producing species (cow, goat, sheep), while in human milk the concentration of lysozyme is reported as 0.2–0.34 g/L (Table 2). According to Guo et al., (2007) the concentration of lysozyme in donkey milk is (1.0 g/L), consistent with the reports of Civardi et al., (2002a), Miranda et al., (2004) and Salimei et al., (2004).

The high lysozyme content of donkey milk may be responsible for the low bacterial count reported almost comprehensively in literature and also makes this milk suitable to prevent or reduce gastrointestinal infections of infants. Donkey milk has been used as an alternative to egg lysozyme in cheese making for the inhibition of spore-forming clostridia strains causing late blowing in hard Italian cheeses (Cosentino et al., 2013; Cosentino et al., 2015; Galassi et al., 2012). Additionally, lysozyme has other important functions (i.e., strong microbial inhibitory activity, inactivation of certain viruses, anti-inflammatory, and anti-tumor activities), while it stimulates the immune system in early childhood (Mao et al., 2009; Monti et al., 2007; Salimei et al., 2004; Zhang et al., 2008).

Lactoferrin is an iron-binding protein and acts as an antimicrobial agent by hydrolyzing glycosidic bonds of mucous polysaccharides in bacterial cell walls. Moreover, according to Uniacke-Lowe et al., (2010), the possible synergistic Lf-Lyz action is due to that Lf binds oligosaccharides in the outer bacterial membrane, thereby opening ‘pores’ for Lyz to disrupt glycosidic linkages in the interior of the peptidoglycan matrix. Donkey’s milk concentration of lactoferrin is reported to be higher than that of bovine milk (Table 2).

The isolation of a bacteriocin-producing strain of *L. paracasei* from donkey milk was reported (Sa et al., 2011). The bacteriocin was found to possess antimicrobial properties against several pathogenic bacteria (*Salmonella typhi, Pseudomonas aeruginosa, E. coli*). Similarly, it was reported by Murua et al., (2013) that an isolate of *L. plantarum* from donkey milk produced a bacteriocin (LP08AD) inhibiting the growth of LAB, food spoilage bacteria, and pathogens (*Listeria monocytogenes, Enterococcus faecium*, and *Lactobacillus curvatus*).

The aforementioned antimicrobial activity and/or immune-modulating effect of donkey milk will be further discussed and could give answers to the fact that donkey milk was traditionally used as a natural remedy to treat pertussis (whooping cough) (Westermarck, 2013). This bacterial infection is caused by *Bordetella pertussis* and affects infants (<3 months old) and young children. Pertussis (whooping cough) is a very serious disease that can cause pneumonia, seizures, and ultimately death. In the USA, there are approximately 40,000 annually reported cases of pertussis, and in the period 2000–2012 there were 255 deaths (221 out of 255 were infants <3 months olds) (CDC, 2014a). A report by Centers for Disease Control and Prevention (CDC,2014b) shows that worldwide it is estimated that there are 16 million cases and 195,000 children deaths mainly in the developing countries where vaccination rates are low.

2.1.6.2 Bioactive peptides in donkey milk

In addition to bioactive milk proteins (casein and whey proteins), there are many bioactive peptides that are present in the amino acid sequence of the milk proteins (Korhonen & Pihlanto, 2006). Even though the proteomic profile of donkey milk was extensively studied, only few studies focused on the bioactivity of donkey milk (Chianese et al., 2010; Criscione et al., 2009; Cunsolo et al., 2011; Polidori & Vincenzetti, 2012; Piovesana et al., 2015; Vincenzetti et al., 2012).

In a study by Nazzaro et al., (2010) donkey milk was acidified and hydrolyzed by pepsin in order to yield additional antimicrobial components other than lysozyme. In this study, the inhibitory activity of donkey milk after hydrolysis was tested by the inhibition halo test against different pathogenic microorganisms such as *B. cereus*, *S. aureus*, *Enterococcus faecalis*, and *E. coli*. Hydrolyzed milk was active against S. *aureus* and *E. faecalis* in a dose-dependent manner. Regarding the *B. cereus* strains, hydrolyzed milk shows also dose-dependent effectiveness, but the inhibitory activity was different between the strains, which also demonstrate a strain-dependent activity within the same species. The most resistant strain was *E. coli*. The reported findings highlight the presence of biomolecules generated by the hydrolysis of milk proteins that may contribute to its antimicrobial activity.

In another study by Tidona et al., (2011) donkey milk showed to be a good source of antimicrobial peptides, released during in vitro digestion. Results of this study showed

that raw and digested donkey milk inhibit the growth of the pathogens tested. A significant reduction was observed against *E. coli*, followed by *L. monocytogenes*, while no effect was observed against *B. cereus*. However, the inhibitory effect was noticeably higher for the digested samples compared to raw milk samples; thus, the antimicrobial activity might result from a synergistic effect of peptides released by gastrointestinal enzymes together with intact proteins, as lysozyme.

Additionally, Bidasolo et al., (2012) in an in vitro study simulating gastrointestinal digestion with pepsin and a mixture of pancreatic proteases of donkey milk, identified one β-casein derived peptide with potent angiotensin-converting enzyme (ACE)-inhibitory activity. Brumini et al., (2013) studied the antiviral activity of various fractions of donkey's milk proteins against Echovirus type 5, which affects the gastrointestinal tract as the primary organ (Civardi et al., 2002b). Brumini et al., (2013) found that the maximum antiviral effects of all the protein fractions tested was shown by the whey proteins (whey) in relation to low-molecular-weight whey protein fractions (β-Lg and α-La). The results show that different protein fractions in donkey milk, possibly acting synergistically, exert an antiviral action in Echovirus type 5 and help to prevent infections by gastrointestinal viruses in humans.

Furthermore, donkey milk was found to contain growth factors and hormones such as human-like leptin, insulin-like growth factor 1, ghrelin, and triiodothyronine T3. These molecules play a direct role in metabolism, body composition, and in regulating food intake (Salimei & Fantuz, 2012).

In two recent studies by Piovesana et al., (2015) and Chozzi et al., (2016), peptides isolated from donkey milk showed ACE-inhibitory and antioxidant activity. Particulary in the study carried out by Piovesana et al., (2015), 35% and 67% of the isolated peptides showed antioxidant and ACE-inhibitory activity, respectively. In addition Chozzi et al., (2016), isolated two novel endogenous antioxidant peptdes and two novel ACE-inhibitory peptides from donkey milk.

2.1.6.3 Immunomudation activity

Lactobacillus rhamnosus is naturally present in human microbiota, and it plays an important part of human health; it balances the gut environment and provides local and systemic immunomodulation. Peng et al., (2014) assessed the immunological function

of donkey milk and *L. rhamnosus* ZDY114 in mice, with a focus on cellular, humoral, and nonspecific immunity. This synergistically enhances the functions of an immune system in mice by promoting splenic lymphocyte transformation, accelerating the removal of carbon particle and strengthening the NK cell activity (Peng et al., 2014). Further research has to be done to illustrate whether this cooperation between *L. rhamnosus* and donkey milk exists on human immune system too. Donkey milk induced the expression of activation cell surface molecules (CD25 used to track disease progression and CD69 a signal transmitting receptor in lymphocytes) on peripheral blood mononuclear cells (PBMCs) and also has the ability to induce the release of interleukins such as IL-2, IFN-γ, IL-6, TNF-α, and IL-1β from lymphocytes and macrophages (Mao et al., 2009). These cytokines contribute to the immunoenhancing mechanism of donkey milk. In addition, donkey milk induces nitric oxide release from PBMCs (Tafaro et al., 2007). All these immunological activities expressed by donkey milk may be useful in the prevention of atherosclerosis, by acting as a vasodilator and an effective agent in preventing pathogens or their products from occurring (Tafaro et al., 2007).

2.1.6.4 Hypoallergenicity

Food allergy refers to an abnormal reaction of the recipient immune system occurring each time food is ingested, even in small quantities. Food allergy is commonly present in infancy and in early childhood. Interestingly, infants develop food allergy in the same order as foods have been introduced into their diet. The main allergens responsible for more than 85% of food allergies are proteins present in milk, egg, peanuts, tree nuts, shellfish, wheat, sesame seed, and soy (Crittenden & Bennett 2005). As the name Cow Milk Protein Allergy (CMPA) implies, this is an abnormal immunological response to cow milk proteins in certain individuals. CMPA is one of the most common food allergies. In developed countries, cow's milk proteins are the first foreign proteins given to infants (Host & Halken, 2014). It affects 2–7% of children less than 6 months of age and decreases into adulthood to an incidence of 0.1–0.5% (Polidori & Vincenzetti 2013a). Allergic reaction to cow's milk is driven by various immunological mechanisms such as (a) immediate IgE-mediated hypersensitivity reaction (whereas symptoms will occur within the 30 minutes after food ingestion) or (b) delayed non-IgE mediated reaction (symptoms will start hours–days after food ingestion) (Caffarelli et al., 2010;

Muraro et al., 2002; Swar, 2011). The main allergens in cow's milk are caseins (αs1- and β-caseins) followed by β-lactoglobulin and α-lactalbumin, although the latter occurs to a minor extent (Host and Halken, 2014). The therapeutic strategy to respond to CMPA is the total elimination of cow milk. Milk represents an important source of nutrients until the age of 2, it should not be eliminated from the diet, and therefore is important to use milks from different species. The selected milk needs to offer nutritional adequacy and low allergenicity and should be palatable and reasonably priced. Even though, in the case of CMPA, the recommended first choice is hypoallergenic formula, the use of milk from other species should be supervised by pediatricians.

The low allergenicity of donkey's milk is mainly due to the low casein content (Vincenzetti et al., 2008, Vincenzetti et al., 2011). Donkey milk is easily digestible because of its high whey protein content (Tidona et al., 2014). It has low fat content and low calorific value as previously mentioned. In order to improve the overall energy and fat content of donkey milk, medium-chain triglycerides could be used as a supplement (Salimei and Fantuz, 2012). Fat supplementation could also apply to lyophilized donkey milk, and according to Vincenzetti et al., (2011) the nutritional characteristics of freeze dried milk remain basically unchanged when compared with raw donkey milk. The first clinical evidence reported by Iacono et al., 1992 suggests that infants with food allergy could consume fat-supplemented donkey milk (approximately 210–250 mL donkey milk kg^{-1} body weight d^{-1}). It is evident that new clinical studies must be designed in order to confirm or update reported results.

2.1.7 Dairy Technology

2.1.7.1 Fermenting activity

Due to its unique properties, processing of donkey milk into traditional dairy products is difficult. For example, cheese cannot be easily manufactured due to the lack of firm curd on renneting. Donkey milk has the ability to form a weak coagulum under acidic conditions, and therefore its ability is utilized for the production of yogurt-type products with probiotic and therapeutic properties. The only significant fermented product from horse milk available in the market is koumiss. Koumiss (Kumys) is widely consumed in Russia, Mongolia, and Kazakhstan, primarily for its therapeutic and nutritive value.

Traditionally, koumiss was made from fresh raw milk mixed with a mixture of bacteria and yeasts. The milk was held for fermentation (3–8 hours) in a leather sack called a "turdusk." Microbial population mainly belongs to the *Lactobacillus* genus (L. *delbrueckii subsp. bulgaricus, L. casei*), *Lactococcus* genus (*Lactococcus lactis subsp. lactis*), and *Streptococcus* species, whereas yeast species are mainly *Saccharomyces* and *Candida* species. The LAB and yeasts are responsible for acid, ethanol, and carbon dioxide production, respectively. During the mixing and maturation process, it is essential to control the levels of acidity and alcohol content, which is accomplished by adding more milk (Uniacke-Lowe, 2011).

Donkey milk could also be valorized as a good base ingredient for functional food preparations (fermentation) due to low initial microbiological load and high lysozyme content. Coppola et al., (2002) reported that the high content of lactose in donkey milk favors the growth of probiotic lactobacilli. The authors incubated pasteurized donkey milk with the probiotic *L. rhamnosus* (AT 194, GTI/1, and GT 1/3) and found that the strains remained viable after 15 days at 4 °C and at low pH 3.7–3.8. Carminati et al., (2014) isolated and identified *Lb. paracasei, Lb. brevis, Lb. salivarius*, and *Lb. plantarum*. The high lysozyme content of donkey milk did not influence the growth of the strains and also did not affect the acidification activity of the strains. *L. rhamnosus* inhibits the growth of most harmful bacteria in the intestine and acts as a natural preservative in yoghurt and other dairy products in order to extend the shelf-life.

Chiavari et al., (2005) produced fermented beverages from donkey milk using the probiotic bacterial strains *Lactobacillus rhamnosus* AT 194, CLT 2/2, and *Lactobacillus casei* LC 88. In all cases, they found that the strains remained viable even after 30 days of storage, and the lysozyme activity was unchanged with respect to initial values. Fermented beverage made by *L. casei* strain developed a more acceptable and balanced aroma, while milk fermented with *L. rhamnosus* strains gave a boiled vegetable/acidic taste and a smell of grasses and animal odors. Similar results were obtained with other probiotic strains of LAB, namely, *L. acidophilus* ATCC 4356 and *L. fermentum* ME-3 (Papademas et al., 2015). Texture and flavor of fermented horse and donkey milk, on the other hand, may be a constraint to the acceptability of the products, so that fortification with Na-caseinate, pectin, and threonine or the addition of flavors can enhance the rheological and sensory quality (Chiavari et al., 2005). However, the

production of fermented milks by means of a standardized manufacturing protocol should be considered crucial for consumers and markets, according to Di Cagno et al., (2004).

2.1.7.2 Thermal treatments

Donkey milk's availability, as already discussed, is rather limited, and, therefore, preservation for extending storage is of great importance. At the moment, donkey milk is available in the market as nonprocessed (raw) and heat treated (i.e., pasteurized and ultrahigh temperature (UHT)). Additionally, donkey milk has been stored in other forms (i.e., frozen, freeze-dried (lyophilized), and powdered).

Donkey milk is mainly consumed for its health-related benefits; hence, the effect that processing has on the valuable components should be assessed. Polidori and Vincenzetti, (2013b) studied the effect of thermal treatments on the protein fraction of donkey milk, reporting that raw and freeze-dried milk had comparable quality characteristics (i.e., lysozyme and vitamin C contents). Lysozyme was active even after powdering or freezing donkey milk, while vitamin C content overall decreased during frozen storage for 3 months. Thermal treatments of donkey milk at 63 °C/30min and 72 °C/15s did not significantly affect the immunoglobulin content (IgA, IgG) when compared to raw milk (unpublished data, Papademas). Further to the above-mentioned changes occurring in donkey milk constituents, thermal treatment increased furosine formation during milk powder production, while α-tocopherol content was decreased by 41% when donkey milk was heated to 90 °C/1 min (Salimei and Fantuz, 2012). Vitamin C is also partially damaged during milk powder production (Salimei and Fantuz, 2013). Additionally, lipid peroxidation of unsaturated fatty acids of donkey milk is of concern when technologies and processes for extending donkey milk shelf-life are applied (Salimei and Fantuz, 2012). Moreover, Salimei and Fantuz, (2013) discuss the effect of heat treatment on lysine content of powdered horse milk, which was found to be lower than raw or freeze-dried milk.

2.2 Lactic Acid Bacteria (LAB)

2.2.1 Introduction – Historical Background

LAB play a significant role in food, agricultural and clinical applications. The concept of LAB as a group of organisms developed at the beginning of the 1900s (Liu et al., 2014). LABs were amongst the very first bacteria studied. The first bacteria pure culture (‘*Bacterium lactis*, now known as *Lactococcus lactis’*) was isolated in 1873 by Joseph Lister (Teuber, 1995). LABs are the most important group of bacteria and are widely distributed in the nature. They are the habitants of mucosal surfaces, particularly the gastrointestinal tract of humans and animals, oral cavity, vagina but also associated with soil, vegetables, plants and fermented food products such as grains, cereals, milk, dairy and meat (Wedajo, 2015).

2.2.2 Classification

In the past, there was an agreement between scientists that LABs form a uniform bacterial group, which was used synonymously with “milk-souring organisms” which means that bacteria can cause fermentation and coagulation of milk (Axelsson, 1998). This is still true, even though LABs now comprise a phylogenetically ubiquitous and heterogeneous group of bacteria (Axelsson, 1998). The classical classification of LABs was mainly relied on examination of their phenotypic, morphological and biochemical characteristics. Nowadays, with the introduction of molecular biology methods, LAB taxonomy had many revisions. The most recent taxonomy of LABs includes the following genera: *Aerococcus, Carnobacterium, Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus,* and *Vagococcus* (Khalid, 2011).

LABs are found in two distinct phyla, namely Firmicutes and Actinobacteria. Within the Firmicutes, the following genera are included: *Aerococcus, Alloiococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Symbiobacterium, Tetragenococcus, Vagococcus* and *Weissella,* which are all low guanine–cytosine content organisms (31–49 %) (Liu et al., 2014). LAB in Actinobacteria phylum includes only species of *Bifidobacterium* genus.

Figure 2 shows the phylogenetic tree of LABs based upon 16S rRNA sequence comparison.

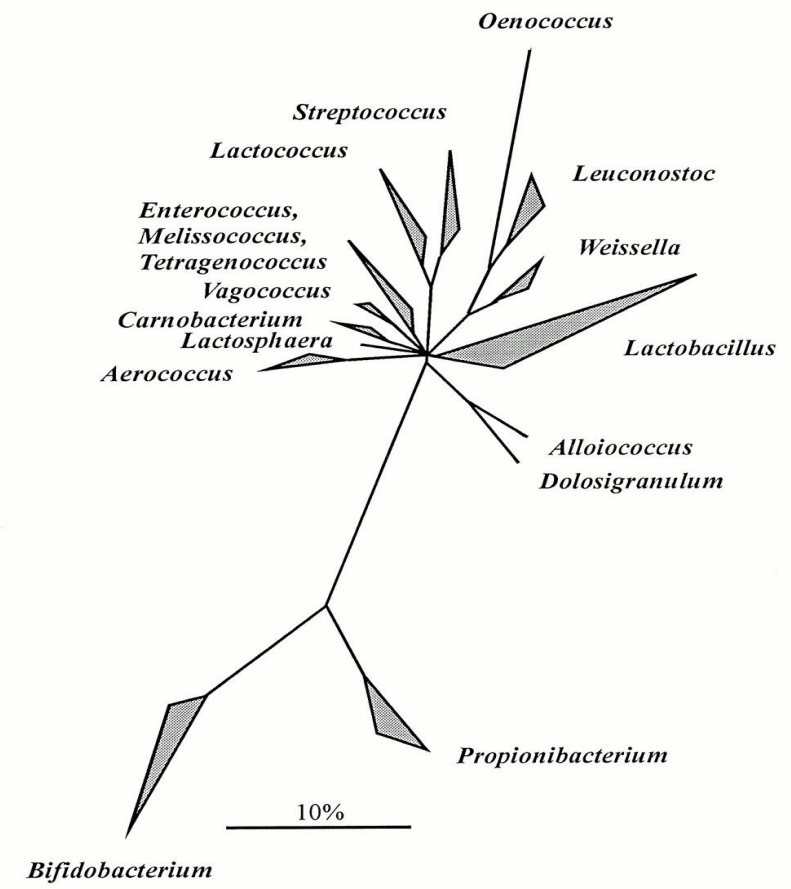


Figure 2: Phylogenetic tree of LAB based on 16S rRNA sequence analysis (Holzapfel et al., 2001)

2.2.3 General Characteristics

LABs are non-spore-forming, catalase negative, gram-positive bacteria, coccus (*Leuconostoc*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Oenococcus*, *Vagococcus*, *Aerococcus*, *Pediococcus*, and *Tetragenococcu*) or rod (*Lactobacillus* and *Carnobacterium*) shaped. They ferment carbohydrates into lactic or acetic acid and carbon dioxide. The cell morphology of the rod group of LAB is long, slender rods to coccobacilli, with variable size and range between 0.5-1.2×1.0-11.0 μm, and cells arrange in chains. The coccus group has spherical cell shape ranging with a diameter range of 0.5-3.5 μm, which occur singly or in pairs, chains and tetrads. The genus

Weissella is an exception, as it can have both cocci and rods. Moreover, cells can also divide in two perpendicular directions in a single plane to form tetrads, which is used as a distinctive characteristic in the differentiation of the cocci.

Aerococcus, *Pediococcus*, and *Tetragenococcus* belong to tetra-forming group. Bifidobacteria have similar characteristics to lactobacilli, except that they are irregularly shaped rods and their growth requirements are stricter. They are classified as obligate anaerobes because they die in the presence of oxygen, which also complicates their viability when added to food and nutritional products. Due to the low energy yield, LABs often grow more slowly when compared to other bacteria and also produce small colonies with a diameter range from 1-3 mm which are also differed in color and shape between LAB genera. Examples of colony morphology of some LABs are presented in Table 7.

Table 7: Colony morphology of LABs

Genus	**Medium**	**Colony morphology**	**Colony size (diameter)**	**Cultivation time and growth conditions**
Leuconostoc	MRS	Greyish-white smooth and round convex	1.0-1.5 mm	48 hours –anaerobic 25 ^{o}C
Streptococcus	M17	Circular entire, convex, opaque shiny smooth	2.0 mm	48 hours, anaerobic 37 ^{o}C
Enterococcus	M17	Smooth circular	1.0-2.0 mm	24hours, anaerobic 35-37 ^{o}C
Pediococcus	MRS	Grayish-white, convex, circular and entire	1.0 mm	24hours, anaerobic, 37 ^{o}C
Lactobacillus	MRS	White, smooth and convex	1.0-2.0 mm	48 hours, anaerobic, 37 ^{o}C

They are typically facultative anaerobes, meaning that they can produce ATP energy in the presence of oxygen, but in the presence of anaerobic conditions they switch to fermentation. Members of the LAB are usually subdivided into two distinct groups based on the end product of carbohydrate (glucose) metabolism (Ross et al., 2002). The homofermentative group consisting of *Lactococcus*, *Pediococcus*, *Enterococcus*,

Streptococcus and some lactobacilli utilize the glycolytic pathway which converts glucose into lactic acid. On the other hand, heterofermentative bacteria utilize the phosphoketolase pathway and metabolize glucose to lactic acid, CO_2, ethanol or acetic acid. This category includes *Leuconostoc*, *Weissella* and some lactobacilli.

The ideal temperature for their growth is between 35-38 ^{o}C with 37 ^{o}C be the optimum for most of them. However, it depends on the specific species and subtype. According to their optimum growth temperature, LABs are classified into mesophilic and thermophilic. Mesophilic LABs have an optimum growth temperature between 20-30 ^{o}C while thermophilic between 30-45 ^{o}C. Therefore, growth at certain temperatures is a criterion used to distinguish cocci LAB. For example, *Enterococci* can grow well at both 10 ^{o}C and 45 °C, while *Lactococci* and *Vagococci* grow at 10 ^{o}C, but they don't grow at 45 ^{o}C and *Streptococci* do not grow at 10 ^{o}C and the growth at 45 ^{o}C is species dependent (Axelsson, 1998).

They are acid tolerant with the ideal pH values for growth to be slightly acidic (pH 5.5-6.0). Tolerance to acid and/or alkaline conditions is also an important biochemical characteristic to distinguish LAB. For example, *Enterococci* are able to grow at both high and low pH. Table 8 shows the main phenotypic, biochemical and physiological characteristics of LABs.

Table 8: Morphological and biochemical differential characteristics of LABs

Genera	**Characteristics**								
	Shape	***Tetrad formation***	***CO_2 from glucose***	***10 ^{o}C***	***45 ^{o}C***	***6.5% NaCl***	***pH 4.4***	***pH 9.6***	***Type of Lactic Acid***
Enterococ.	C	-	-	+	+	+	+	+	L
Tetrageonoc.	C	+	-	+	-	+	V	+	L
Vagococ.	C	-	-	+	-	-	V	-	L
Pediococ.	C	+	-	V	V	V	+	-	D,L,DL
Lactob.	R	-	V	V	V	V	V	-	D,L,DL
Leucon.	C	-	+	+	-	V	V	-	D
Oenococ.	C	-	+	+	-	V	V	-	D
Weissela	C/R	-	+	+	-	V	V	-	D,DL
Lactococ.	C	-	-	+	-	-	V	-	L
Streptococ.	C	-	-	-	V	-	-	-	L

+ positive, - negative, V: variable, C: Cocci, R: Rods

Reference: Axelsson, 1998

2.2.4 Taxonomy

2.2.4.1 Lactobacillus

Lactobacillus is the largest group and currently contains over 221 species and 29 subspecies (LPSN, 2016). This heterogeneous group of LABs includes Gram-positive, rod-shaped, strictly fermentative, non-endospore forming bacteria which grow well in anaerobic environments, although they are aerotolerant (Salveti et al., 2012). Lactobacilli have been isolated from different sources such as the intestinal tract of mammals, plants, raw milk and sewerage (Salveti et al., 2012). The range of DNA mol% G+C of *lactobacillus* is between 32-53%, which is responsible for the heterogeneity of this group. *Lactobacillus* can be subdivided into three sub-groups based on sugar fermentation, namely facultative heterofermentative (Group I), obligated heterofermentative (Group II) and obligated homofermentative (Group III) (Bernardeau et al., 2008). *Lactobacilli* from Group I ferment hexoses to lactic acid and pentoses to lactic acid and acetic acid, and gas is not produced from glucose, but from gluconate. Examples of Group I strains are *L. casei* and *L. plantarum*. Group II bacteria produce carbon dioxide, lactic acid, acetic acid and/or ethanol from hexoses, and produce gas from glucose. Examples include *L. fermentum, L. brevis* and *L. keferi*. *Lactobacilli* from Group III do not ferment gluconate or pentose, but ferment glucose to lactic acid. Representatives of this group include *L. delbrueckii* and *L. acidophilus*.

2.2.4.2 Streptococcus

The genus *Streptococcus* was among the earliest group of bacteria recognized by microbiologists due to their involvement in a large number of human and animal diseases (Hardie & Whiley, 1997). In 1884, Rosebbach was introduced for the first time the generic name *Streptococcus* to describe the chain-forming, coccus shaped bacteria associated with wound infections (Hardie & Whiley, 1997). The *Streptococci* bacteria are Gram-positive, catalase-negative, anaerobic, aerotolerant, coccus-shaped cells grouped in linear chains (Delorme, 2008; Stiles & Holzapfel, 1997). At the moment

over 116 species and 22 subspecies of *Streptococcus* are recognized (LPSN, 2016). Most are found in the mouth and respiratory tract of vertebrates, while some are pathogens. *S. thermophilus* is the only specie from this genus that "generally recognized as safe" (GRAS) and is used as a dairy starter (De Vuyst & Tsakalidou, 2008; Delorme, 2008).

2.2.4.3 Lactococcus

In 1985 the genus *Lactococcus* was suggested by Schleifer and colleagues after reclassification of species from the genera *Lactobacillus* and *Streptococcus* through chemotaxonomic analysis and 16S rRNA sequencing (Casalta & Montel, 2008). *Lactococci* are coccus-shaped, Gram-positive, non-motile that are homofermentative and produce exclusively L (+) lactic acid from glucose (Casalta & Montel, 2008). Currently the genus *Lactococcus* consists of 12 species and 4 subspecies, (LPSN, 2016). *Lactococci* are generally isolated from plant surfaces and animal skin. Mesophilic *lactococci* are often isolated from raw milk due to contamination from the environment and equipment used during milking (Casalta & Montel, 2008). They can be used in starter cultures in dairy fermentation because firstly of their acidification activity, and secondly for their contribution to the texture and flavor by producing exopolysaccharides and aromatic compounds (alcohols, ketones, aldehydes).

2.2.4.4 Leuconostoc

Leuconostoc was first described by Van Tieghem in 1878 (Hemme & Foucaud-Scheunemann, 2004). They are heterofermentative cocci that are sometimes oval or even short rods and occur in pairs or short chains. *Leuconostoc* are Gram positive, non-motile, catalase negative, non-spore-forming, facultative anaerobes and catalase negative (Hemme & Foucaud-Scheunemann, 2004; Ogier et al., 2008). All the species in this genus are resistant to the antibiotic vancomycin, a useful characteristic for isolation of these bacteria (Hemme & Foucaud-Scheunemann, 2004; Ogier et al., 2008). These organisms form small, gray, flat colonies on agar media. *Leuconostoc* can be distinguished from most *lactobacilli* by their inability to produce ammonia from arginine and by forming D-lactate from glucose. Currently the genus *Leuconostoc* consists of 24 species and 7 sub-species (LPSN, 2016). Most of the *Leuconostoc* strains favor growth between 4 – 10 ^{o}C and also grow at 30 ^{o}C, but no growth occurs at 45 ^{o}C

(Hemme & Foucaud-Scheunemann, 2004). Although the members of the genus *Leuconostoc* are classified as opportunistic pathogens, they are GRAS for use in food fermentations. *Leuconostoc* are used in industrial dairy starters, but most often these bacteria disseminate to dairy environments and are often present in traditionally prepared fermented milks and as non-starter LAB (NSLAB) in raw milk cheeses (Hemme & Foucaud-Scheunemann, 2004; Ogier et al., 2008). *Leuconostoc* species are responsible for a buttery aroma, which was a desirable characteristic of many dairy products (Hemme and Foucaud-Scheummann 2004). However, some *Leuconostoc* species can have a negative effect by producing undesirable compounds (biogenic amines) or dextran in a sugar fermentation process which are resulting in food spoilage (Ogier et al., 2008).

2.2.4.5 Pediococcus

The genus *Pediococcus* was first described by Balcke in 1884 (Holzaphel & Franz, 2006). Fifteen species of *Pediococcus* are currently recognized (LPSN, 2016). They are Gram-positive, non-motile, oxidase negative, and catalase negative cocci that divide at right angles in two planes, resulting in tetrad morphology. *Pediococci* are facultative aerobic homofermenters which produce lactic acid as the major product of glucose fermentation. Some species in this genus can withstand extreme environmental conditions, such as high temperatures, pH and NaCl concentrations. These bacteria are often isolated from plants, a variety of fermented foods such as sauerkraut, fermented sausages and as spoilage microbes from beer (Stiles & Holzapfel, 1997).

2.2.4.6 Bifidobacterium

In 1899, at the Institute Pasteur Tissier described the first member of *Bifidobacteria* which has been isolated from the faeces of breast-fed-infants and he named it, *Bacillus bifidus* (Arunachalam, 1999). In 1924 Orla-Jensen proposed an independent genus, Bifidobacterium (Lee & O'Sullivan, 2010). They are Gram positive, strictly anaerobic, non-motile, non-spore forming, high in G+C (42-67%) catalase negative bacteria that are classified into the phylum of *Actinobacteria* (Martinez et al., 2013). They have a rod like appearance but vary in size and tend to be clubbed with branch, forming the ‘y’ or “v” rod shaped patterns. Presently there are 56 species and 10 subspecies included in the

genus *Bifidobacterium (*LPSN, 2016). *Bifidobacteria* are typically found in the gastrointestinal tract (GIT) and faeces of humans and animals (Lee & O'Sullivan, 2010).

2.2.4.7 Enterococcus

The first description of *enterococci* occurred by Thiercelin in 1899 as a new Gram-positive diplococcus (Lebreton et al., 2014). Later, in 1903 Thiercelin and Jouhaud include this bacterium in the new genus *Enterococcus,* with the type species *Enterococcus proteiformis* (Javed, 2011). Then in 1906, Andrewes and Horder based on its ability to form short or long chains renamed it as *Streptococcus faecalis* (Bhardwaj et al., 2008). The species name 'faecalis' was suggested because of their close resemblance to strains isolated from the human intestine (Franz et al., 1999). Development of a serological typing system for *streptococci* by Lancefield in 1933 showed that strains of 'faecal origin' possessed the group D antigen (Bhardwaj et al., 2008). Then in 1937, Sherman proposed a new taxonomic classification scheme for *streptococci* separated it into four divisions' designated pyogenic, viridans, lactic and 'enterococcus'. The '*enterococcus*' group include *Streptococcus faecalis, Streptococcus faecium, Streptococcus bovis* and *Streptococcus equinus* as the 'enterococcal' strains (Khan et al., 2010). In 1984, Schleifer and Kilpper-Bälz using DNA homology studies, demonstrate that *Streptococcus faecalis* and *Streptococcus faecium* were sufficiently distinct from other *streptococci*. Therefore, the *streptococci sensu lato* were subdivided into three different genera: *Streptococcus sensu stricto, Enterococcus* and *Lactococcus* (Klein, 2003).

Enterococci are coccus shaped, Gram-positive, facultative anaerobic, oxidase and catalase negative, non-spore-forming, that occurs singly, in chains or in pairs (Giraffa, 2003). They are homofermenters and produce L (+) lactic acid from glucose and are also able to metabolize amino acids and citrate (Ogier & Serror, 2008). Until now 55 enterococcal species and 2 subspecies have been identified (LPSN, 2016). *E. faecalis* and *E. faecium* are commonly isolated from feces as they are inhibitors of the GIT in humans and animals. *Enterococci* enter the food and dairy environment from other primary habitats such as feces, soil, plants and water (Franz et al., 1999; Giraffa, 2003; Moreno et al., 2006; Ogier & Serror, 2008). The reason for their adaptability to various environments is that these bacteria can grow in high salinity (6.5%), extreme pH (4.0 - 9.6), temperatures between 10 – 45 ^{o}C and survive 30 min of heating at 60 ^{o}C.

2.2.4.7.1 Dual role of enterococci

The genus *Enterococcus is* a controversial group of LAB. This controversial nature of *Enterococci* has prompted an enormous pro/contra groups in scientific papers and reviews in recent years (Moreno et al., 2006). Unlike to other LAB, *enterococci* are not considered as GRAS and their presence in water are regarded as an indicator of fecal contamination (Ogier & Serror, 2008).

2.2.4.7.1.1 Beneficial and technological properties of enterococci

On the one hand, *enterococci* play an important role in cheese technology. Specifically, they play an important role in the ripening of artisanal cheeses such as Cheddar, Feta, Cebreino, Water-buffalo mozzarella, Hispanico and Vecano, probably through proteolysis, lipolysis, esterolysis and diacetyl production due citrate breakdown, hence contributing to their typical taste and flavor (Moreno et al., 2006; Ogier & Serror, 2008). The cheese *enterococci* usually originate from the different types of milk used to produce cheeses. Moreover, apart from their technological properties, many strains of *enterococci*, especially *E. faecalis* and *E. faecium* produce bacteriocins (enterocins) which are very active against *L. monocytogenes*, *C. perfringens* and *S. aureus (*Khan et al., 2010).

2.2.4.7.1.2 Pathogenicity of enterococci

On the other hand, *enterococci* are considered as emerging pathogens for humans and some of them they have been associated with a number of clinical human infections such as meningitis, bacteremia, urinary tract infections and endocarditis (Higuita & Huycke, 2014). They are considered the third most common cause of hospital-acquired infections after *E. coli* in the USA and the fourth in Europe (Ogier & Serro, 2008). Most enterococcal human infections are caused by *E. faecalis* (more than 80%), while *E. faecium* is associated with the majority of the remaining infections (20%) (Higuita & Huycke, 2014). The resistance to a wide variety of antibiotics and the presence of virulence genes are a cause of concerns and also contributing to pathogenesis of enterococci.

- **Antibiotic Resistance**

Antibiotic multi-resistance has been more commonly reported for *E. faecalis* due to its notorious ability to acquire and transfer antibiotic resistance genes (Gomes et al., 2010). Antibiotic resistance of *enterococci* can be divided into two types: inherent or intrinsic and acquired. Inherent or intrinsic resistance indicates a usual resistance in all or most of the strain of the same species. *Enterococci* are intrinsically resistance to following antibiotics: semi-synthetic penicillinase resistance penicillins, cephalosporins, low levels of aminoglycosides, clindamycin (Higuita & Huycke, 2014). Acquired resistance is a consequence from either mutation in the existing DNA or acquisition of new DNA. Examples of enterococcal acquired resistance include resistance to chloramphenicol, erythromycin, tetracycline, penicillin, high levels of clindamycin and aminoglycosides, high levels of β-lactams, fluoroquinolones and glycopeptides, such as vancomycin (Franz et al., 2003). The most significant concern for human infections is vancomycin resistance *enterococci* (Marothi et al., 2005). The two most important vancomycin-resistance phenotypes are the VanA which is associated with a high level of inducible resistance to vancomycin and cross resistance to teicoplanin and the VanB which is usually displaying variable levels of inducible resistance only to vancomycin (Giraffa, 2002).

Many studies have been made in order to compare the antibiotic resistance profile of different *enterococci* according their source of isolation such human, animal or food. Results showed that human or animal isolates have the highest antibiotic resistance followed by food isolates (Ogier & Serror 2008). However, only few of the food isolates were resistant to the clinically important antibiotics such as ampicillin, penicillin, and vancomycin.

- **Virulence factors**

A virulence factor is an effector molecule that enhances the ability of a microorganism to cause disease beyond the basic to the species background (Moreno et al., 2006). For *enterococci* to cause infection, the first step involves the colonization of the host tissue, resistance to both host specific and non-specific defense mechanisms, and finally produce pathological changes either directly or indirectly (Franz et al., 1999). The most

common virulence factors of enterococci include aggregation substance (AS), enterococcal-extracellular surface protein (esp), the enterococcal endocarditis antigens (EfaA), collagen binding protein (Ace), gelatinase (GelE), hyaluronidase (Hyl) and cytolysin (Cyl) (Franz et al., 2003). Table 9 shows the most important virulence factors and their association with disease development identified in *enterococci.*

Table 9: Examples of enterococcal virulence factors and their association with stage of disease

Virulence Factor	**Associated Disease**
Aggregation substance (AS)	Aids in binding to host cells
Cytolysin (Cyl)	Eukaryotic cell toxin, lyses immune cells (evasion of host immune response)
Gelatinase (Gel)	Hydrolyses biological peptides (collagen, fibrin)
Enterococcal surface proteins (Esp)	Adhesin, promotes colonizations, associated with antibiotic resistance
Adhensin to collagen (Ace)	Adhession to extracellular matrix, role in translocation
Endocarditis antigen from *E. faecalis* and *E. faecium* ($EfaA_{fs}$ and $EfaA_{fm}$)	Plays a role in endocarditis
Hyaluronidase (Hyl)	Hydrolyses hyaluronic acid, role in traslocation

AS is a surface-bound protein responsible for bacterial aggregation (Upadhyaya et al., 2008). AS contribute to virulence both by promoting plasmid encoded virulence factors and antibiotic resistance dissemination and by facilitating colonization through the promotion of adhesion to extracellular matrix and other proteins, host cells and also to plastic polymers and invasion of cells and tissue damage during infection (Semedo et al., 2003). Esp is a cell-wall associated protein is related with adhesion to eukaryotic cells, colonization and evasion of the immune response of the host and also play some role in antibiotic resistance (Fisher & Philips, 2009). Furthermore, Esp is contribute to enterococcal biofilm formation, which could lead to resistance to environmental stresses (Upadhyaya et al., 2008). Cytolysin (also called haemolysin) is a bacterial toxin, where the genes for the production are located on pheromone-responsive plasmids (Franz et al., 2001). Cytolysins are responsible for lysing a broad range of eukaryotic and prokaryotic cells, while *gelE* acts on collagenous material in tissues, facilitates in invasion and involved in the hydrolysis of gelatin, collagen, haemoglobin and other

bioactive peptides (Bhardwaj, 2008).Hyaluronidase is a cell surface-associated enzyme which cleaves the mucopolysaccharide moiety of connecting tissues or cartilage and digests hyaluronic acid (Bhardwaj, 2008). This enzyme is believed to act as 'spreading factor' and therefore facilitate the spread of enterococci and their toxins through host tissue. Sex pheromones are small peptides which promote the acquisition of plasmid DNA and facilitates the conjugation mediated uptake of antibiotic resistance and virulence traits (Franz et al., 2003). As in the case with antibiotic resistance, virulence factors are mainly detected among clinical enterococcal isolates followed by food isolates and then starter isolates (Eaton & Gasson, 2001). Moreover, *E. faecalis strains* generally harbour more and multiple virulence traits than *E. faecium.*

- **Production of biogenic amines**

The presence of biogenic amines in food is also a concern for the food industry and regulatory agencies. The toxicity threshold for amines is due to many different factors, such as the health of the person and individual sensitivity. In most cases, consumption of food containing biogenic amines does not lead to intoxication because amine-destroying enzymes in the digestive tract prevent their uptake in the blood (Shalaby, 1996). As much as 1 mmol histamine and 3 mmol tyramine can be consumed without noticeable effects (Giraffa et al., 1997). However, when amine degradation is impaired, smaller quantities may cause food poisoning. Decarboxylating bacteria can find suitable conditions to proliferate and produce biogenic amines during cheese ripening. The amount of amines produced depends mainly on the concentration of available amino acid precursors. The prolific growth of *enterococci* in milk and milk products may leads to the formation of significant levels of biogenic amines. However, the only biogenic amine produced by *enterococci* isolated from dairy products is tyramine (Linares et al., 2011).

2.2.5 Metabolism of LAB

Metabolic activities of LAB are important for their survival and growth. The three main pathways of LAB metabolism are the conversion of carbohydrates-lactose (glycolysis), fat (lipolysis) and caseins (proteolysis). Glycolysis involves the conversion to lactate but a fraction of the intermediate pyruvate can alternatively be converted to various flavour compounds such as diacetyl, acetoin, acetaldehyde or acetic acid. Lipolysis results in

the generation of free fatty acids (FFAs), which can are key flavour constituents and also can be precursors of flavour compounds such as methyl ketones, secondary alcohols, esters and lactones. However, the most important biochemical pathway is proteolysis which involves the degradation of proteins into amino acids.

This section is not intended to provide an in-depth review of metabolic pathways of LAB, but to give a brief outline of the most important metabolic pathways.

2.2.5.1 Carbohydrate (lactose) metabolism

Most LABs are able to grow in milk due to their ability to utilize lactose, as a carbon source. Lactose is transported inside the bacteria cell either via the phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) or by lactose permease systems (Shiby & Mishra, 2013). During the transport in the cell membrane, lactose translocated via PEP-PTS system is phosphorylated lactose phosphate, which is then cleaved by phospho-β galactosidase to yield glucose and galactose or galactose 6-phosphate (Shiby & Mishra, 2013). LAB utilize sugars to form lactic acid by either the homofermentative pathway (glycolysis or Embden-Meyerhof-Parnas pathway) and heterofermentative pathway (the pentose phosphate pathway) (Khalid, 2011). Figure 3 shows the lactose metabolism using heterofermentative and homofermentative pathway.

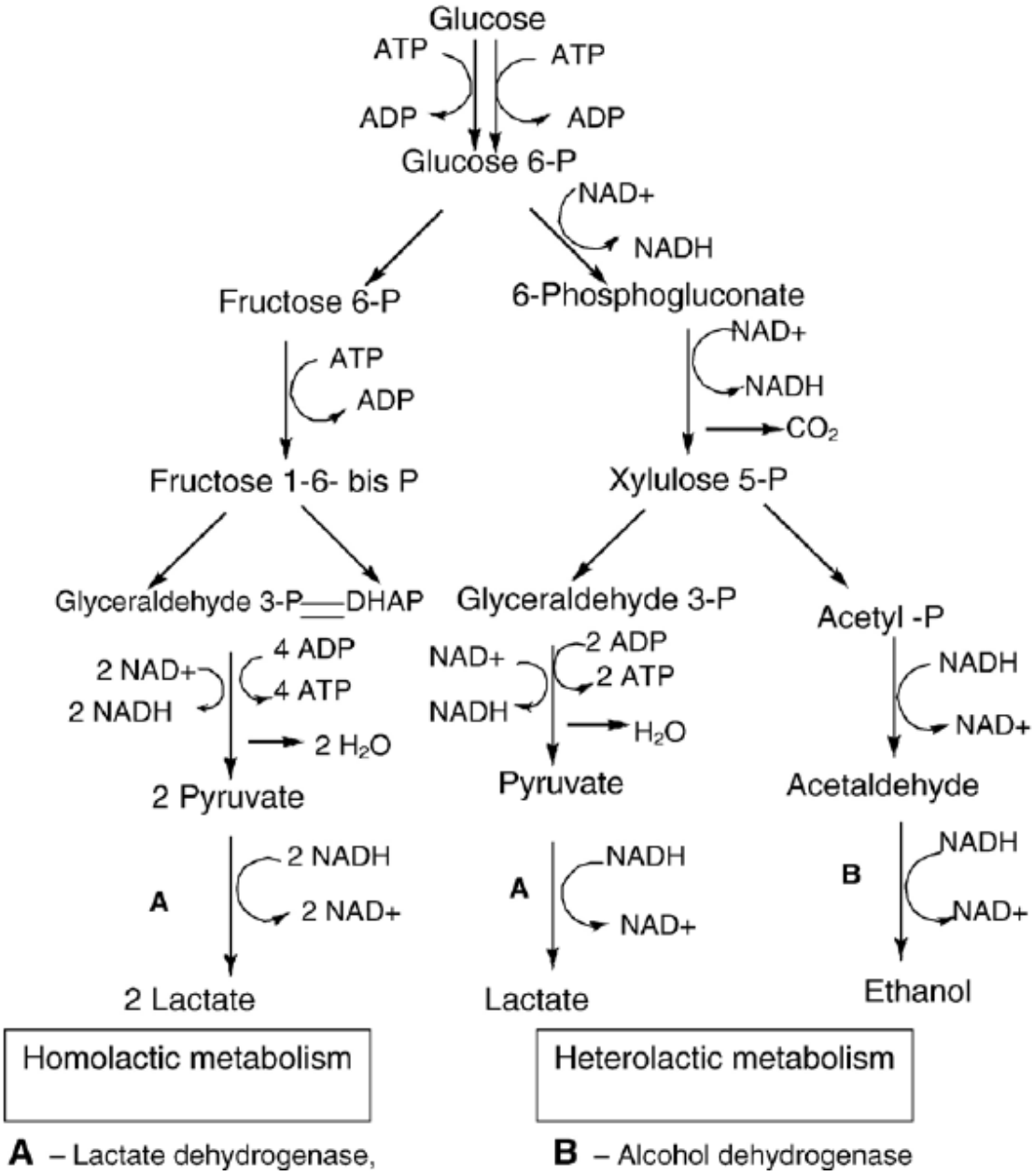


Figure 3: Lactose metabolism (Reddy et al., 2008)

The homofermentative pathway, results in the transformation of one mole glucose to two moles of pyruvate through the Embden–Meyerhof–Parnas pathway, which yields lactic acid. The first step is the conversion of glucose. It is characterized by the formation of fructose-1-6-bisphospha, which is split by an FDP aldolase into dehydroxyacetonephosphate and glyceraldehydes-3-phosphate. Then glyceraldehydes-3-phosphate is converted to pyruvate. Intracellular redox balance can be maintained through the oxidation of NADH, associated with pyruvate reduction to produce lactic acid by the action of lactate dehydrogenases (LDHs). This process yields two moles of

ATP and 2 moles of lactic acid per mole glucose. Representative homofermentative/ homolactic LAB genera include *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, and group I lactobacilli (Pörtner, 2016).

Obligate heterofermenters lack aldolase and must divert the flow of carbon through a different series of reactions. This pathway is called 6-phosphogluconate/ phosphoketolase (6-PG/PK). In this pathway, heterofermentative LAB from one mole of glucose which is initially dehydrogenated to 6-phosphogluconate and followed by decarboxylation to yield one mole of CO2, produced lactate and either acetic acid or ethanol and ATP (Pörtner, 2016). Then, the remaining pentose-5-phosphate formed is cleaved into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. GAP is further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. Obligate heterofermentative LAB include *Leuconostoc*, *Oenococcus*, *Weissella*, and group III lactobacilli (Pörtner, 2016).

2.2.5.2 Citrate metabolism

LAB citrate metabolism has been extensively studied due to its aroma compound production such as diacetyl which is contribute to the buttery aroma of dairy products (Laëtitia, 2014). *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, few *Leuconostoc* ssp., few *Enterococcus* spp., *Lactobacillus plantarum* and *Oenococcus oeni* are known as diacetyl and acetoin producers (Laëtitia, 2014). The first metabolic step which is common in both homo and heterofermentative LAB involves the internalization of the molecule followed by its breakdown to obtain pyruvate. During the first step, citrate is taken up by the citrate permease (CitP), a member of the 2-hydroxycarboxylate family transporters (Laëtitia, 2014). Following up the uptake of citrate, citrate lyase enzyme break down citrate into into acetate and oxaloacetate (OxA). Finally, OxA is decarboxylated by the oxaloacetate decarboxylase (OAD) into pyruvate and carbon dioxide. Pyruvate can also be catabolised to different compounds such as acetate, formate, acetaldehyde, ethanol, alanine, diacetyl and 2,-butanediol. Figure 4 shows the citrated metabolism of LAB.

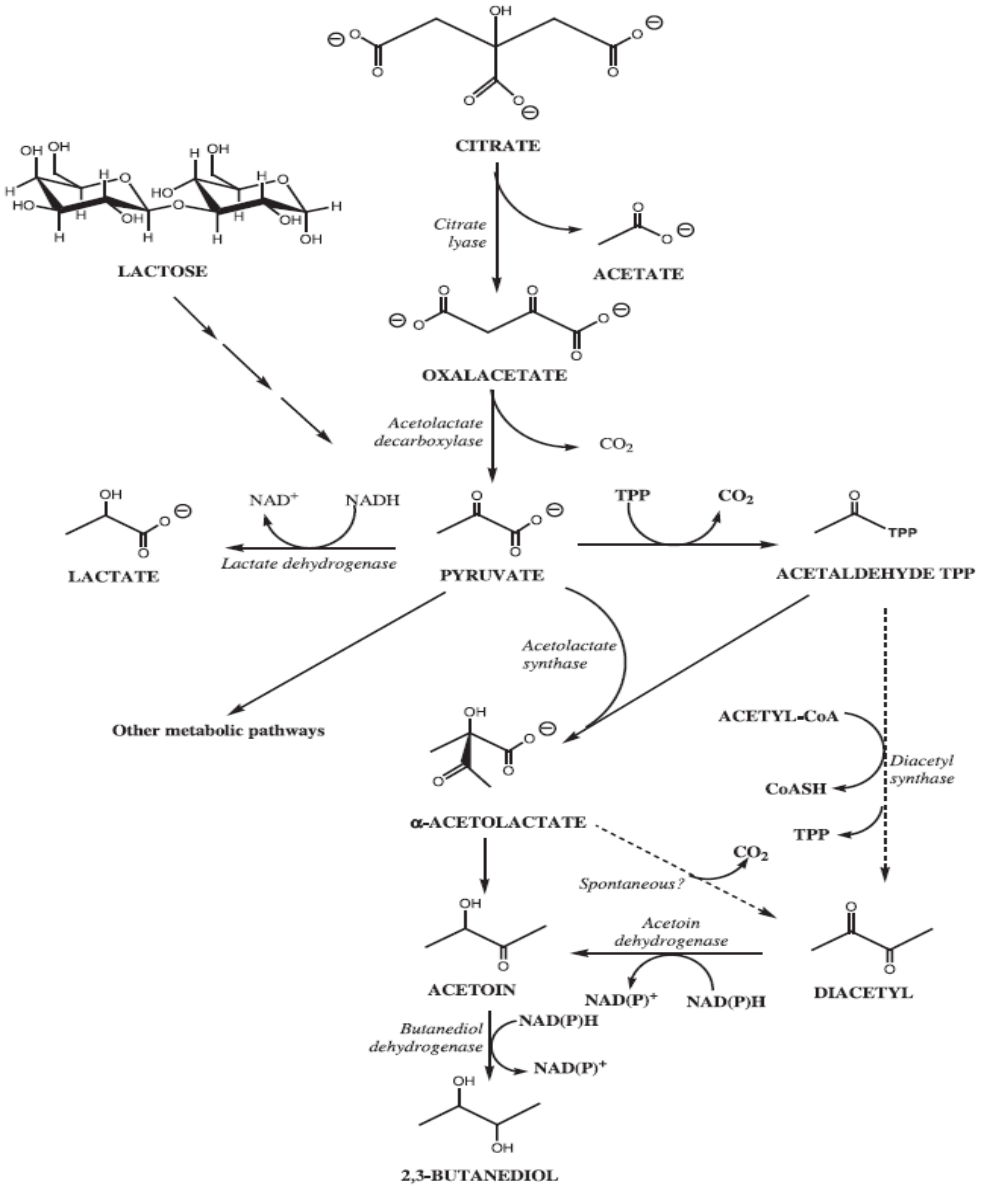


Figure 4: Citrate Metabolism (Laëtitia, 2014)

2.2.5.3 Nitrogen metabolism (proteolysis)

LAB has gained interest due to their proteolytic activities, which are playing an important role in cheese maturation, and particularly in the development of aroma, flavor and texture (Murtaza et al., 2013). Proteolysis involves the breakdown of proteins into smaller peptides and amino acids by the action of proteinases and peptidases. Therefore, the proteolytic system of LAB is important as a mean of making protein, peptide, and amino acids available for bacterial growth, but this system can also form the rheological and organoleptic properties of fermented foods (Saeed & Salam, 2013).

Proteinase also helps to reduce the allergic properties of milk and milk products for infants who can lead to a severe nutritional problem of protein-energy deficiency (Yuan & Furuta, 2003). Furthermore, during proteolysis, bioactive peptides can also release which are in an inactive state within the sequence of milk proteins.

It is generally accepted that LAB are poorly proteolytic (Sarantinopoulos et al., 2001). Due to their high nutritional requirements LAB require external nitrogen sources for growth to high numbers because of their limited ability to synthesize amino acids (McSweeney, 2004). In a medium containing large concentrations of protein but small amounts of free amino acids such as milk, LAB developed a complex proteolytic system in order to degrade milk caseins as the major source of amino acids to smaller peptides and free amino acids which are necessary for their growth. Caseins contains all the necessary amino acids for the growth of LAB in milk but actually less than 1% of the total casein constituents are required (Kunji et al., 1996).

The proteolytic system of LAB (Figure 5) consists of: (i) cell-envelope proteinase (CEP or lactocepin or PrtP) that initiate the degradation of casein into oligopeptides; (ii) intracellular proteinases; (iii) transport system that is involved in the uptake of peptides and amino acids across the cell wall and (iv) intracellular peptidases that hydrolyse further the oligopeptides to amino acids and (iv) different enzymes that convert liberated amino acids into various components (Kunji et al., 1996; McSweeney, 2004).

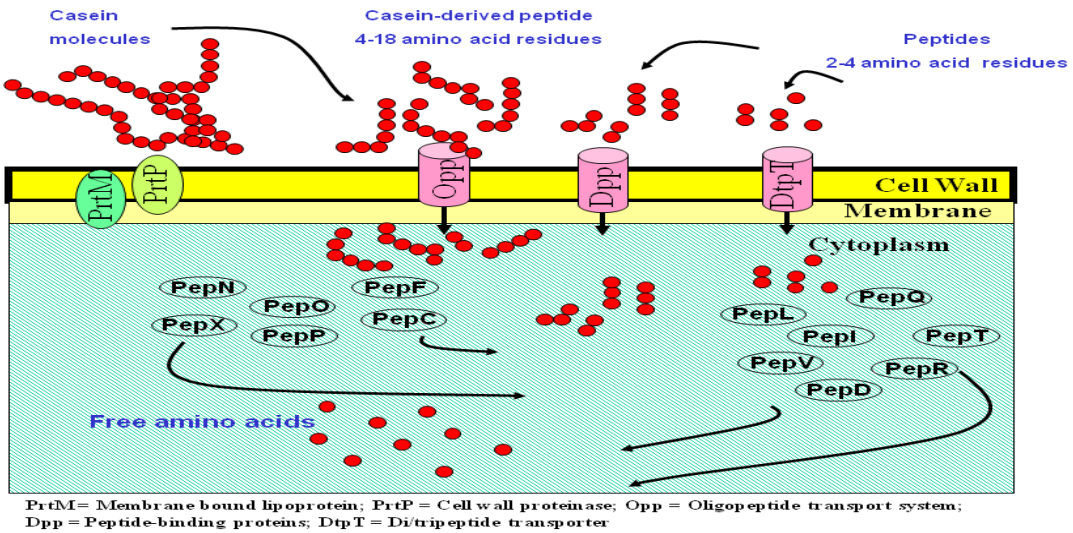


Figure 5: Proteolytic System of LAB (Pinto et al., 2012)

2.2.5.3.1 Proteinases

Numerous studies have shown that hydrolysis of caseins is initiated by single CEPs (Griffiths & Tellez, 2013). Five different types of enzymes were cloned and characterized from LAB, including PrtP from *L. lactis* and *L. paracasei*, PrtH from *L. helveticus*, PrtR from *L. rhamnosus*, PrtS from *S. thermophilus*, and PrtB from *L. bulgaricus* (Savijoki et al., 2006). CEPs have a strong preference for hydrophobic caseins which is also the most abundant proteins in milk (Savijoki et al., 2006). Generally, LAB proteinases are classified into two groups based on their specificity to degrade αs1-, β- and κ-caseins, which are generally indicated as PI and PIII (Rao et al., 1998). The PI type proteinase is particularly active at the C-terminus of β-casein (β-CN) and to lesser extent on αs1-CN and κ-CN (McSweeney, 2004). It is also responsible for the release of bitter peptides, which are highly resistant to the hydrolytic activity of chymosin and microbial proteinases, giving the bitter taste to the cheese. PIII type proteinase degrades κ-CN and αs1-CN and also cleaves β-casein, but at different sites from the PI (McSweeney, 2004).

2.2.5.3.2 Amino acid and peptide transport systems

The second most important step of proteolytic system is the transportation of degradation products derived from the caseins (peptides) into the cell. Three functional peptide transport systems have been described in literature for LAB: DtpT (hydrophilic di- and tripeptide, oligopeptide transport system (Opp) and DtpP or Opt (hydrophobic di-tri peptide transport system (Sinz & Schwab, 2012). Opp is required ATP instead of proton motive force and transports peptides from 4 up to 18 amino acid residues (Kunji et al., 1996). Opp is a five component ABC system which includes a peptide binding protein (OppA), two integral membrane proteins (OppB and OppC), and two ATP-binding proteins (OppD and OppF) (Kunji et al., 1996). OppA act as a receptor for the uptake and transport of peptides across to the membrane-bound proteinase. OppB and OppC recognized as highly hydrophobic proteins and facilitate the translocation of oligopeptides through the cell membrane. The ATP-binding proteins, OppD and OppF couple the hydrolysis of ATP and allow the translocation of the peptides across the membrane. DtpT is a membrane bound transporter and is responsible for the transport of hydrophilic di-and tri-peptides and is a proton motive force- dependent transporter (Hagting et al., 1994). The Opt system transports hydrophobic di-and tripeptides and is

energized by ATP or energy-rich phosphorylated intermediates and it belongs to the ABC transporter superfamily.

2.2.5.3.3 Peptidases

After protein degradation by CEPs, peptides released are taken up into the cells and are further degraded to smaller peptides and amino acids by the action of several peptidases with different specificities (Kunji et al., 1996). Generally, peptidases of LAB are named according to the substances which they hydrolyze. These include endopeptidases (degrade oligopeptides), aminopeptidases (release amino acids from the N-terminal ends of oligopeptides, important for the development of flavour in fermented milk products), dipeptidases (hydrolyse dipeptides), tripeptidases (hydrolyse tripeptides) and proline specific peptidases (Christensen et al., 1999).

2.2.5.4 Lipid metabolism

Lipid metabolism involves the break-down of lipid into fatty acids by lipases (intracellular or extracellular). Moreover, fatty acids can also be metabolized to other flavor components, such as methyl-ketones and esters. Lipolysis of milk fat by LAB is one of the most important biochemical changes which contribute to the development of cheese flavor (McSweeney & Sousa, 2000). Different studies showed that the kind of milk can directly affect the flavor of the cheese. For example cheeses made from skim milk, or milk in which milk fat had been replaced by other lipids did not develop correct flavor (Collins et al., 2003).

Therefore, lipolysis is particularly extensive in hard Italian cheese varieties, surface ripened (smear) cheese and blue mold chesses. On contrary in Cheddar, Gouda and Swiss cheeses extensive lipolysis is considered undesirable characteristic whereas high levels of fatty acids in these cheeses can lead to rancidity (Hassan et al., 2013). The principal lipids of milk are triacylglycerides, which comprise up to 98% of the total lipid fraction (Collins et al., 2003). Despite the presence of etserolytic/ lipolytic enzymes which are capable of hydrolyzing various esters of fatty acids, and tri-, di- and mono-acylglycerides, lactic acid bacteria considered to have low lipolytic activity. Lipolytic enzymes of lactic acid bacteria can be classified into esterases and lipases based on the following characteristics: 1) the length of the hydrolyzed acyl ester chain, 2) the physicochemical nature of the substrate, and 3) the enzyme kinetics (Wolf et al.,

2009). Esterases hydrolyze acyl ester chains between 2 to 8 carbon atoms, while lipases hydrolyze esters with chains of 10 or more carbon atoms. Also the esterases hydrolyze soluble substrates in aqueous solution while lipases hydrolyze emulsified substrates. The enzyme kinetics of esterases and lipases also differ. Esterases have classical kinetics Michaelis-Menten type kinetics, and lipases exhibit surface Michaelis-Menten kinetics (Collins et al., 2003). In general, lipolytic enzymes are specialized on the exterior ester bonds of tri- or diglycerides (sn-1 position and sn-3). Lipases in cheese originate from six sources: the milk, rennet preparation (rennet paste), starter, adjunct starter, non-starter bacteria and possiblt their addition as exogenous lipases (McSweeney & Sousa, 2000).

2.2.6 Biochemical properties of technological interest

LAB is the most important bacteria for food fermentations whether is relating to dairy products or other foods, such as fermented meat products, fermented vegetables, fermented foods and the wine. They have a long and safe history in food applications. The cultures used in fermentations should cover some specifications in terms of both safety and technological performance. Moreover, depending on the particular application (type of food) some specific biochemical- technological properties of these strains such as acidification, proteolytic, lipolytic activity are also important. In this part, the biochemical properties of technological interest of LAB strains, to be used as starter or adjunct cultures in food fermentations will be discussed. Figure 6 shows the most important biochemical pathways for flavor formation.

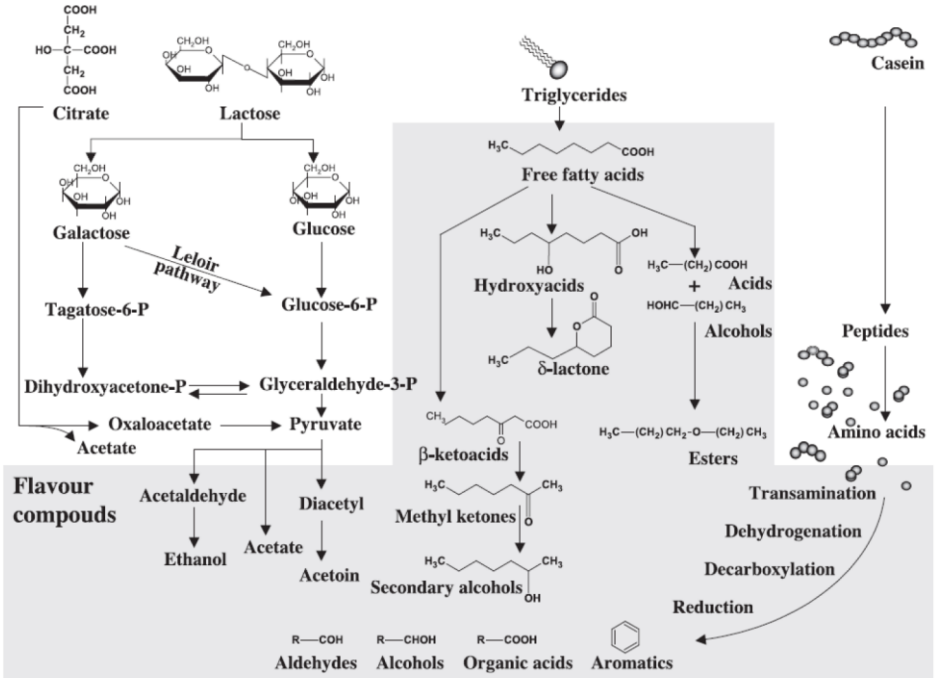


Figure 6: Biochemical pathways leading to the formation of flavor compounds. The grey surface indicated compounds with a flavor note (Marilley & Casey, 2004)

2.2.6.1 Acid production

Acid production is an important characteristic that results in the development of appealing sensory attributes in the fermented products, especially cheeses. Normally milk has an initial pH of around 6.6. Therefore, a rapid decrease in pH during the initial stages of cheese production is essential since it is necessary for milk coagulation and the prevention or reduction of the growth of undesirable microflora (Sarantinopoulos et al., 2001).

2.2.6.2 Proteolytic and peptidolytic activities

Proteolysis is considered as one of the most important and complex biochemical events involved in manufacturing of many fermented dairy products such as during cheese ripening. For example, casein breakdown softens cheese texture, which facilitates the release of flavour compounds when the cheese is consumed (Broadbent & Steele, 2005). In addition to the production of flavor peptides, undesirable bitter-tasting peptides can also be generated which could lead to off-flavour formation. The liberation of free amino acids can also directly affect flavours such as glutamate and aspartate residues enhance flavour and are the taste stimulants. The products of amino acid catabolism,

which may arise via decarboxylation, domination, 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 (Broadbent & Steele, 2005).

2.2.6.3 Lipolytic and esterases activity

Lipolysis results in the formation of free fatty acids, which can be precursors of flavor compounds such as methyl-ketones, secondary alcohols, esters and lactones and also can directly affect the cheese ripening process, and texture (Collins et al., 2003). However, LABs, in general, contribute very little to lipolysis.

2.2.6.4 Autolysis

Bacterial autolysis results from the enzymatic degradation of the bacteria cell wall peptidoglycan by endogenous peptidoglycan hydrolases (PGHs) called autolysins (Lortal & Chapot-Chartier, 2005). Autolysis of LAB plays an important role in dairy fermentation and is of special interest regarding their use as starter cultures. Controlling and increasing starter LAB autolysis is considered as an essential factor in order to control and accelerate cheese ripening.

2.2.6.5 Diacetyl production

Milk contains about 1.5 g/L of citrate, most of which is lost in the whey during cheese making (Hassan et al., 2013). The presence of citrate in cheese curd is of great importance since it may be metabolized to a number of volatile flavours such as succinate and diacetyl by citrate-positive *lactococci*, *enterococci* and *leuconostoc* (Sarantinopoulos et al., 2001). Succinate is a flavour enhancing compound specific for several cheese varieties, like Swiss-type Cheddar cheeses. In addition to the role of flavour formation, diacetyl is also known to have inhibitory activity against some food spoilage microorganisms. Most LAB strains can decarboxylate α-acetolactate to acetoin by α-acetolactate decarboxylase whereas some LAB strains do not have the responsible enzyme, resulting in accumulation of α-acetolactate and production of diacetyl in dairy

products which is responsible for the sweet buttery aroma in buttermilk, cultured sour milk and some yoghurts (Saeed & Salam, 2013).

2.2.6.6 Exopolysaccharide (EPS) production

LAB can be also used in the production of fermented dairy products due to their ability to produce EPS. They can provide the fermented food with a proper rheology, texture, stability, reduction of syneresis and mouth feel. EPS also offer a low calorie and low cost substitute for the production of smooth and creamy yogurt instead of fat, protein, sugars, or stabilizer (Welman & Maddox, 2003). However, the industrial application of EPS produced by LAB is affected by oxygen, pH, temperature, and medium constituents, such as orotic acid and the carbon source (Saeed & Salam, 2013). Moreover, EPS production by LAB offers protection to the cell against desiccation, phagocytosis, phage attack, osmotic stress, antibiotics or toxic compounds, adhesion to surfaces and formation of biofilms that facilitate colonisation to various ecosystems (Patel & Prajapat, 2013). These characteristics offer an advantage to EPS producing LAB to withstand technological stresses and survive and colonize the gastrointestinal tract. Further, EPS may induce positive physiological responses such as anti-tumour, anti-ulcer, immunomodulating and cholesterol lowering effects (Patel & Prajapat, 2013).

2.2.7 Identification methods

A range of methods have been developed for the identification of LAB. The identification methods can be divided into two basic groups, phenotypic and genotypic. Since precise identification of LAB is a prerequisite of their application as commercial starter cultures in the fermentation industry or as probiotic candidates, a detailed identification and characterization of LAB is essential. Therefore, the polyphasic approach is suggested for the isolation, identification and characterization of new LAB. The polyphasic approach is based on the combination of different methodologies such as phenotypic, genotypic and phylogenetic methods.

2.2.7.1 Phenotypic methods

Phenotypic methods are mainly based on morphological examinations (shape), biochemical, and physiological tests. Even though phenotypic methods are more

laborious, time consuming and also can yield ambiguous results and are quite unreliable, these methods are still the standard in bacterial systematics. Classification of LAB to genus level is based on microscopic examination using Gram staining. In respect to the reaction to Gram stain, bacteria can be divided into two major groups; Gram positive and Gram negative organisms. LAB belongs to the Gram positive group. In order to obtain more accurate results in the classification at species level, additional biochemical tests are necessary, such as carbohydrate fermentation profiles, haemolysis test, extracellular polysaccharide formation, utilization of growth factors, enzymatic activities, growth response in milk media, growth at different temperatures, salt concentrations and serological assays (Temmerman et al., 2004).

2.2.7.2 Molecular methods

Contrary to classical methods, genotypic identification using molecular tools provide more accurate, rapid and reproducible results. A range of molecular methods have been used for the identification of LAB such as pulse field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis, PCR-denaturing gradient gel electrophoresis/ PCR-DGGE, PCR-RFLP and DNA sequencing (Lawson & Tsaltas, 2014; Mohania et al., 2008; Temmerman et al., 2004).

2.2.8 LAB fermentation and health benefits

Fermentation is a very old traditional food processing technology with earliest records dating back to 6000 BC. Fermentation can be defined as a desirable process in which a food product is subjected to biochemical modifications resulting from the metabolic activities of microorganisms (usually LAB) and their enzymes. There are two types of fermentation, homolactic and heterolactic. 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. Fermentation ensures not only increased shelf life and microbial safety of the food product through the production of lactic acid, hydrogen peroxide and bacteriocins, but may also improve the nutritional value and digestibility of food products. Furthermore, fermentation helps in the reduction of toxic substances such as in the fermentation of cassava tubers *(Manihot esculentum*) which contains toxic cyanogens (Caplice & Fitzgerald, 1999).

Of all fermented foods produced with LAB, fermented dairy products are the most important worldwide, both by production volume and value. Fermented dairy products can exhibit a wide variety of textures such as liquids (kefir), semi-solid and solid products (yoghurt). Fermentation of milk with LAB leads to specific organoleptic characteristics (taste, aroma, texture and flavor) of the final product which is principally related to LAB acidifying and proteolytic activity.

Numerous scientific papers and review articles have reported the health benefits associated with the consumption of fermented dairy products (Fernández et al., 2015; Marco et al., 2017). Some of the proposed health benefits are thought to be conferred by live bacteria (probiotics) contained in the final products. Foods containing probiotic bacteria are currently categorized as functional foods and they are gaining widespread popularity and acceptability worldwide. Health benefits probiotics include alleviation of symptoms of lactose intolerance, treatment of diarrhea, anti-carcinogenic properties, anti-mutagenic activity, and reduction in blood cholesterol and improvement in immunity. The importance of probiotics in human health is considered further in the following section.

2.3 Probiotics

2.3.1 Introduction

The notion that food could serve as medicine was first conceived thousands of years ago by the Greek philosopher and father of medicine, Hippocrates, who once wrote: 'Let food be thy medicine, and let medicine be the food' (Chow, 2002). Over the past several years, the focus of nutritional studies has shifted from nutrient deficiency diseases to optimizing health and prevents chronic diseases. However, nowadays, the concept of food having specific health benefits beyond its nutritional value reborn as 'functional foods'. The term functional food was first used in Japan, where the concept of food designed to be medically beneficial to the consumer evolved during the 1980s (Siro et al., 2008). Functional food can be defined as food or dietary components that may provide a health benefit beyond the basic function of provide nutrients (Cencic & Chingwaru, 2010).

One of the most promising areas for the development of functional foods lies in the use of probiotics in order to increase the number of advantageous bacteria in the intestinal tract. This section will describe the history and significance of probiotics as well as their selection criteria and the challenges of producing a viable probiotic product. The various health benefits of probiotics and their mechanism of action will also be highlighted.

2.3.2 History and definition of probiotics

In the early 1900s, microbiologist at the Pasteur Institute in Paris and Nobel Prize winner Elie Metchnikoff began to elucidate the mechanisms by which these bacteria confer health benefits, and promoted their ability to increase human longevity (Mackowiak, 2013). He observed that the longevity of Bulgarians peasants was associated with the consumption of large quantities of fermented milks. This observation has led to the believe that fermentation by lactic acid bacteria results in improved gastrointestinal function and nutrient absorption, as well as stimulation of the immune system and intestinal microbiota balance. Although many LABs are applied commercially for the production of probiotic foods, the two most commonly used genera are *Lactobacillus* and *Bifidobacterium.* Some of the most common microorganisms used as probiotics (Table 10) are *L. acidophilus, L. plantarum, L. casei, L. casei* subsp. *rhamnosus, L. delbreuckii* subsp. *bulgaricus, L. fermentum, L. reuteri, L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, Bifidobacterium bifidum, S. salivarius* subsp. *thermophilus, E. faecalis* and *E. faecium* (Collins et al., 1998).

Table 10: The most commonly used species of LAB in probiotic preparations

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	***Enterococcus* spp.**	**Other LAB**
L. acidophilus	*B. bifidum*	*Ent. faecalis*	*S. remoris*
L.casei	*B. adolescentis*	*Ent. faecium*	*S. salivarius*
L. delbrueckii subsp bulgaricus	*B. animalis subsp animalis*		*L. lactis subsp lactis*
L. cellobiosus	*B. infantis*		*L. mesenteroides*
L. cuvatus	*B. thermophilum*		*S. thermophillus*
L. fermentum	*B. longum*		*Sporolactobacillus inulinus*
L. lactis	*B. animalis subsp lactis*		*L. lactis subsp cremoris*
L. plantarum	*B. breve*		*P. acidilactisi*
L. reuteri			
L. brevis			
L. amylovorus			
L. salivarus			
L. rhamnosus			
L. paracasei			
L. gasseri			
L. johnsonii			
L. helveticus			
L. gallinarum			
L. crispatus			

Reference: Leroy et al., 2008

The word probiotic is derived from the Greek “pro bios” which means ‘for life’ (Bagchi, 2014). The term probiotic was first defined by Kollath in 1953 to denote all organic and inorganic food complexes in contrast to harmful antibiotics (Gogineni et al., 2013). Then Lilly and Stillwell, (1965) describe probiotics as ‘substances secreted by one microorganism that stimulate the growth of another' (Franz et al., 2014). The probiotic definition has evolved over the years as more scientific knowledge and better understanding on its relationship between intestinal health and general well-being has been gained (Table 11). Parker in 1974 defined probiotics as organisms and substances that contribute to the balance of the intestinal microbiota. Then Roy Fuller modified the

definition in 1989 as 'live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance. Havenaar and Huis, (1992) described probiotics as mono or mixed cultures of live microorganism which, when applied to animal or man, affects beneficially the host by improving the properties of the indigenous microbiota. Salminen et al. (1998) defined probiotic as food that contain live bacteria that are beneficial to health. However, the widely accepted definition of probiotics is 'live microorganisms which confer a beneficial health effect on the host when administered in adequate amounts' (FAO/ WHO, 2002).

Table 11: Some of the definitions of probiotics commonly cited over the years

Year	Description
1953	Probiotics are common in vegetable food as vitamins, aromatic substances, enzymes and possibly other substances connected with vital processes
1954	Probiotics are opposite of antibiotics
1955	Deleterious effects of antibiotics can be prevented by probiotic therapy
1965	A substance secreted by one microorganism which stimulates the growth of another
1971	Tissue extracts which stimulate microbial growth
1973	Compounds that build resistance to infection in the host but do not inhibit the growth of microorganisms in vitro
1974	Organisms and substances that contribute to intestinal microbial balance
1992	Live microbial feed supplement which beneficially affects the host animal by improving microbial balance
1992	Viable mono- or mixed cultures of live microorganisms, which applied to animals or man, have beneficial effect on the host by improving the properties of the indigenous microflora
1996	Live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host
1996	Living microorganisms which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition
1999	Microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host
2001	A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effect in this host
2002	Live microorganisms that when administrated in adequate amount confer a health benefit on the host

Reference: Vasijevic & Shah, 2008

2.3.3 Probiotic Market

Probiotic microorganisms are available for direct or indirect human consumption in three different for formats, culture concentrate to be added to a food (dried or deep-freeze form), food products (fermented or non-fermented), and dietary supplements (drug products in powder, capsule or tablet forms) (Mortazavian et al., 2012). The most popular way is the consumption of probiotic bacteria through food products.

The demand of probiotic products is growing very rapidly due to increased awareness of consumers about the impact of food on health. Today probiotic products comprise between 60% and 70% of the total functional food market (De Prisco & Mauriello, 2016). At the moment, the largest markets for functional foods are the United States, followed by Europe, and then Japan, most of which comprising by functional dairy foods (Marsh et al., 2014). Over half of the probiotic market is occupied by foods, with supplements (30-40 %) and < 10 % pharmaceuticals (Bansal et al., 2016). The global market for probiotic foods and drinks was around 24.8 billion euros in 2011 and is estimated to get over 31.1 billion euros in 2015 and is predicted to reach around 40 billion euros by 2018 (Buriti et al., 2016). Table 12 shows the most important commercial probiotic products on the European market.

Table 12: Commercial probiotic products on the European market

Type of Product	Trade name	Probiotic microorganisms
Fermented milk with high viscosity	Bifisoft, Bifidus, Bioghurt, Biofit, BiofardePlus, Biola, Biologic Bifidus, Cultura Dofilus, Dujat Bio Aktiv, Ekologisk Jordgubbs Yoghurt, Fit&Aktiv, Fjall Yoghurt, Gaio Dofilus, Gefilac, Gefillus, LC1, Probiotisches Joghurt, ProViva, Verum, Vifit Vitamel, Vitality	*L. acidophilus, L. acidophilus LA5, L. rhamnosus (LGG, LB21 and 271), L. casei, L. casei L19, L. johnsonii, L. plantarum 299v, L. reuteri, Lactococcus lactis spp lactis L1A, B. bifidum, B. animalis ssp. lactis BB-12, B. animalis ssp. animalis*
Fermented milk with low viscosity (cultured buttermilk, yoghurt drink, dairy drink)	A-fil, Actimel, Aktifit, Bella Vita, Bifidus, Biofit, Biola, Casilus, Cultura, Emmifit, Fundo, Gaio, Gefilac, Kaiki Actifit, LC1 Go, Onaka, ProViva, Pro X, Verum, ViktVaktama, Litality, Le'Vive+, Yakult, Yoco Acti-fit	*L. acidophilus, L. acidophilus LA5, L. rhamnosus (LGG, LB21 and 271), L. casei (F19, 431, Imunitas, Shirota), L. johnsonii, L. plantarum 299v, L. reuteri, L. fortis, Lactococcus lactis spp lactis L1A, B. bifidum, B. animalis ssp. lactis BB-12, B. animalis ssp. animalis, B. longum BB536*
Non-fermented dairy products (milk, ice cream)	Gefilus, God Halsa, RELA, Vivi Vivo	*L. rhamonosus LGG, L. plantarum 299v, L. reuteri*

2.3.4 Selection criteria for probiotics

Selecting the suitable probiotic strain is very important. Figure 7 shows selection criteria for a probiotic strain. The first requirement of selecting probiotics is that they should be safe and produce desirable health benefits. Moreover, they must be present in adequate amounts and remain viable during processing, storage and also survive and colonize the GI tract. Therefore, there are many in vitro tests for the screening of potential probiotic strains. The selection criteria of a strain to use as probiotic can be categorized in four basic groups (FAO/WHO, 2002):

- General aspects (origin and identity)
- Safety
- Functional features
- Technological aspects

Strains which have these properties can be used for the development of probiotic food products. However, these tests are not fully adequate to predict the functionality in the human body (FAO/WHO, 2002). Therefore, often, in vitro studies are combined with *in vivo* studies and clinical trials to investigate the reliability of the above parameters and also to confirm the beneficial properties of probiotic strains, such as improving the health of consumers, of any disease symptoms and general quality of life.

The clinical evaluation of a probiotic is composed of four stages. In the first stage, the safety of probiotics is addressed, the second stage examines the activity of the strains along with any side effects, in the third stage their effectiveness and in the last stage are monitored on the basis of epidemiological data (FAO / WHO, 2002).

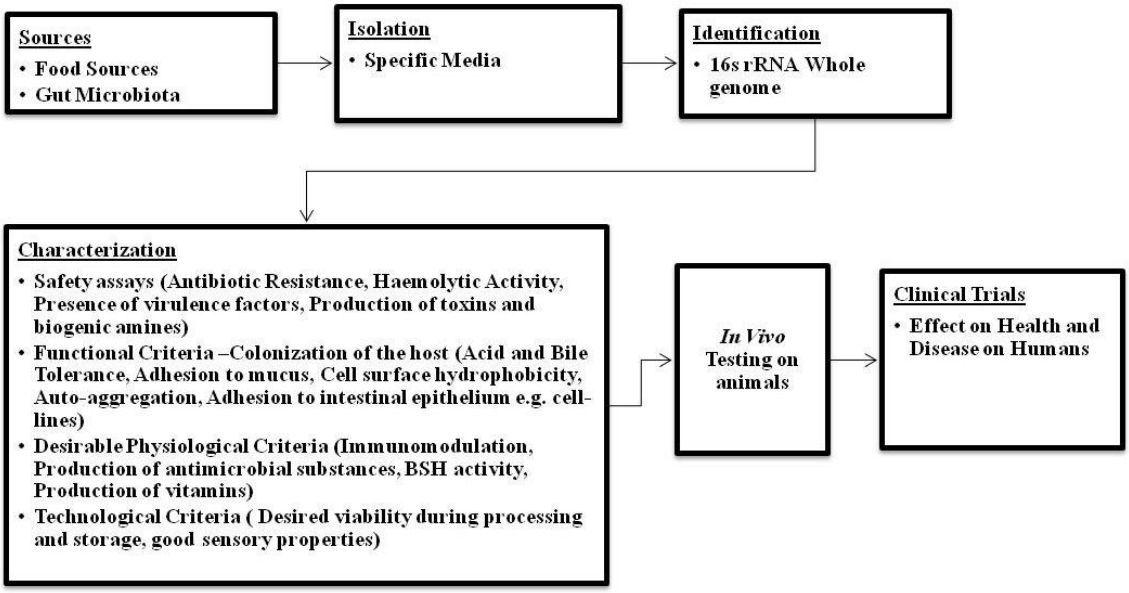


Figure 7: Selection criteria for probiotics

In general LAB strains must have the following properties in order to consider as probiotic candidates (Saarela et al., 2000):

- The strain should be recognized as GRAS. This means that the strain must be of human origin and genetically stable and also free of antibiotic resistance especially to clinically important antibiotics.
- The strain must be able to survive and colonize the GIT. The probiotic strain should be resistant to gastric acidity and able to proliferate in the presence of bile salts.

- Selected strains should be able to exert health benefits *in vivo* and the efficacy of probiotics must be demonstrated in carefully designed and managed double-blinded, completely randomized and placebo controlled human studies.
- The strain should be able to adhere and colonize the gastrointestinal tract. This improves its persistence and allows its multiplication in the intestine and may favour the competitive exclusion of potential pathogens of the mucosal surfaces.
- Probiotic strain must has good technological properties in order to be manufactured and incorporated into food products without losing functionality and viability or creating unpleasant flavours or textures.
- It must be capable of being prepared on a large scale. It is also important to remain viable and active in the specific delivery vehicle. Moreover, another critical point is to meet the requirements of a minimum of 10^6 CFU/ml at the expiry date.
- Probiotics must be safe during food consumption and clinical use, even for immunocompromised individuals.

2.3.4.1 General aspects (origin and identity)

The first step in selecting a probiotic strain is the determination of its taxonomic classification, which may give an indication of the origin, habitat and physiology of the strain (Wedajo, 2015). A FAO/WHO (2006) group of experts reported that is not the source of probiotic organism, but the specificity of probiotic action that is important. The source can be from a human origin like human large intestine, small intestine, or a breast milk, animal origin, food source like a raw milk or fermented food. The reason of selecting strains with human origins based on the fact that research has shown that strains isolated from the gut of a human being are more likely to adhere to the human intestinal wall than others and more likely to safe (Shewale et al., 2014). As a result, strains derived from human gastrointestinal are most commonly tested for probiotic properties. If a strain is able to colonize the intestine, that means remains there for longer, so it can act. It is also known that the beneficial effects of probiotic microorganisms depend on each member individually, without the need to form a common trait for all strains belonging to the same species. For this reason, the accurate identification at the strain level is more than necessary. Therefore, it is recommended to utilize a combination of phenotypic and molecular methods to achieve the accurate

identification, classification and typing. The primary identification criteria used for phenotypic characterization of strains are cell morphology, determination of metabolites, enzyme activity and the ability to utilize a sugar. Molecular tools have also been developed for identifying the probiotics based on the analysis of nucleic acids and other macromolecules because of high potential provided by using the PCR amplification, hybridization with DNA and RNA and DNA Sequence Encoding 16SrRNA are also being used for the identification (Yadav & Shukla, 2015).

2.3.4.2 Safety

Before incorporation into food products, probiotic strains should be carefully tested for safety. The strain should be considered as GRAS and should be followed Qualified Presumptions of Safety (QPS) considering by the European Food Safety Authority (EFSA). An important criterion for a probiotic strain is that it needs to be intrinsically resistant to antibiotics, and the resistance should not be transmissible or inducible (Gueimonde et al., 2013). The main concern among LAB is the antibiotic resistance within the genus of *Enterococcus*, particularly the resistance to vancomycin (Gueimonde et al., 2013). It must have no history of association with diseases, non-pathogenicity and not deconjucating bile salts. Recognizing the importance of ensuring safety, even in bacteria that are recognized as GRAS, the FAO / WHO (2002) recommends to examine the probiotic candidate strains with a series of tests, which include:

- Determination of antibiotic resistance
- Determination of certain metabolic activities (e.g. lactase production)
- Verification of side effects in clinical trials
- Post market surveillance of adverse incidents on consumer health

2.3.4.3 Functional characteristics

Functional properties are also important for the selection of a probiotic strain. The probiotic strain should be able to survive the acidic conditions, the presence of bile salts in GIT and proliferate in the intestine in order to be effective in therapeutic actions and carry on normal metabolic activity after consumption (Mitropoulou et al., 2013). Probiotic strains are delivered in the food system, where they begin their journey to the lower intestinal tract via the mouth. Therefore, in order for probiotic strains to survive

this journey must be resistant to the enzymes like lysozyme in the oral cavity. Then the probiotic strains will pass through the stomach and they will enter the upper intestinal tract which contains bile. Therefore, a prerequisite for the use of a strain as a probiotic is to be able to survive in its passage through the stomach to reach the intestine. The secretion of gastric acid from the stomach mucosa is an important barrier for the inhibition of potential pathogens that enter the body with food consumption (Dunne et al., 2001). The pH of an the empty stomach is usually 1.5-2 but after the food consumption rises to 4-5 and maintained at that level for 2-4 hours. Therefore, all probiotic strains must have a tolerance to low pH. The pH tolerance of probiotic strains can be evaluated by exposing them to low pH in a buffer solution or medium for a specific period of time, during which the number of surviving bacteria is calculated. Equally important for a probiotic strain candidate is its resistance to bile salts. Bile salts are synthesized in the liver from cholesterol, concentrated in the gall bladder, from which are secreted into the duodenum (Begley et al., 2006). Tolerance to bile salts can be evaluated by exposing them into different concentrations of bile salts in a suitable medium and monitoring cell survival during incubation. Another important characteristic is the adherence and colonization to intestinal epithelial cells and/ or mucus in order to promote the gut resistance time, pathogen exclusion and host and immune system interactions (Fontana et al., 2013). Adhesion to the intestinal mucosa may prevent the probiotic cells being washed out and therefore, enabling temporary colonization, immune modulation and competitive exclusions of pathogens (Lahtinen et al., 2009). In order to produce enzymes, lactic acids, vitamins and natural antibiotics, the probiotic strain must be adhered to the intestinal wall, colonize and multiply. Over the last years, the Caco-2 cell line has been used extensively as an ideal model for the determination of the adhesion capacity of probiotic strains. Caco-2 cells form a continuous homogeneous monolayer that resembles that of human mature enterocytes in the small intestine and they also form crypts which are typical structures of the epithelial monolayer (Fontana et al., 2013).

Antimicrobial activity is another important selection criterion for probiotics in order to control undesirable bacteria and pathogens. Antimicrobial compounds produced by LAB include organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins.

However, the production of bacteriocins by LAB with specific inhibitory activity against closely related species is the most extensively studied.

2.3.4.4 Technological characteristics

Probiotics must have good technological properties so that it can be manufactured and incorporated into food products without losing functionality and viability or creating unpleasant flavours and textures (Mattila-Sandholm et al., 2002). For example, the rate of acid development is a critical criterion for the selection of the probiotics in milk-fermented products. A rapid acid production in raw milk not only helps to prevent the growth of unwanted microorganisms but is also essential for the aroma, texture, and flavor development in the end product. Moreover, they must be capable of being prepared in a viable manner and on a large scale since it is also very important for probiotics to be stable, viable and active in the specific delivery vehicle during manufacture and storage. This is important, as any probiotic product sold with health claims must meet the criterion of a minimum of 10^6 CFU/ml at the expiry date; because the minimum suggested daily intake is 10^8-10^9 cells (Mattila-Sandholm et al., 2002).

2.3.5 Probiotic preparation and viability

Most of the definitions used for probiotics stress the importance of adequate numbers of administered bacteria. The benefits of probiotic bacteria are largely dependent on their ability to survive, colonize and multiply in the host (Bhat et al., 2015a). If enough viable bacteria do not reach the target site, the probiotic product would not be useful. Many of the probiotic microorganisms used in food and drink products do not survive for long enough to confer a health benefit on the host. Several review articles on probiotics have mentioned studies that highlight the loss in viability of probiotic bacteria during different stages in their manufacture, storage and after ingestion (Shori, 2016). Therefore, the major challenge for administering a useful probiotic product is to maintain viability, which is a prerequisite for achieving health benefits via microorganisms as the medicinal efficacy of probiotic food products depends upon the number of viable and active cells per gram or millilitre of the products at the moment of consumption (Iravani et al., 2015)

The above fact is highlighted by WHO/FAO (2002), which has established the criterion that any food sold with health claims based on the addition of probiotics must contain at

least 10^6 to 10^7 cfu/ml of viable probiotic bacteria. There are several stages where the viability of probiotic bacteria is susceptible. Firstly, the bacteria have to survive the processing stage. Following this, if the probiotic bacteria are to be administered in food, they have to endure the storage period or shelf life of the food in which they are delivered. Finally, upon ingestion, they have to survive the acidic conditions of the stomach as well as the bile salts in the small intestine, before reaching the lower portions of the gastrointestinal tract where they will provide beneficial effects.

The main factors that affect the viability and activity of probiotic cultures include environmental, food and processing parameters such as pH, titratable acidity, molecular oxygen, water activity, presence of salt, sugar and chemicals like hydrogen peroxide, bacteriocins, artificial flavouring**,** coloring agents, heat treatment, rate and proportion of inoculation, strain species, incubation temperature, packaging materials and conditions, storage methods and conditions (Champagne et al., 2005; Ross et al., 2005; Tripathi & Giri, 2014). Moreover, apart from the production and storage factors, probiotic survival can also be affected by the acidity of the stomach, bile salts, enzymes such as lysozyme present in the intestine, toxic metabolites including phenols produced during digestion, bacteriophage, antibiotics and anaerobic conditions.

Several attempts have been made to improve the viability of probiotics in different food products during their production until the time of consumption. Strategies to improve viability of probiotic organisms include the appropriate selection of acid and bile resistant strains, use of oxygen impermeable containers, two-step fermentation, microencapsulation, spray-drying, freeze-drying and incorporation of micronutrients such as peptides and amino acids (Ross et al., 2005; Sarkar, 2010; Tripathi & Giri, 2014).

2.3.6 Health benefits

Health benefits of probiotics can be found locally (in GIT) or systemically (throughout the body). Potential health benefits may result from the growth and action of the probiotics during the manufacturing of probiotic foods, while some may result from the growth and action of probiotics in the intestinal tract (Ranadheera et al., 2010). Probiotics provide a number of health benefits. Examples of therapeutic applications of probiotics include prevention of infantile diarrhea, urogenital diseases, osteoporosis,

food allergy and atopic diseases; reduction of antibody-induced diarrhea; alleviation of constipation and hypercholesterolemia; control of inflammatory bowel diseases; and protection against colon and bladder cancer (Nagpal et al., 2012). Figure 8 presents the health benefits and the mechanism of action of probiotics.

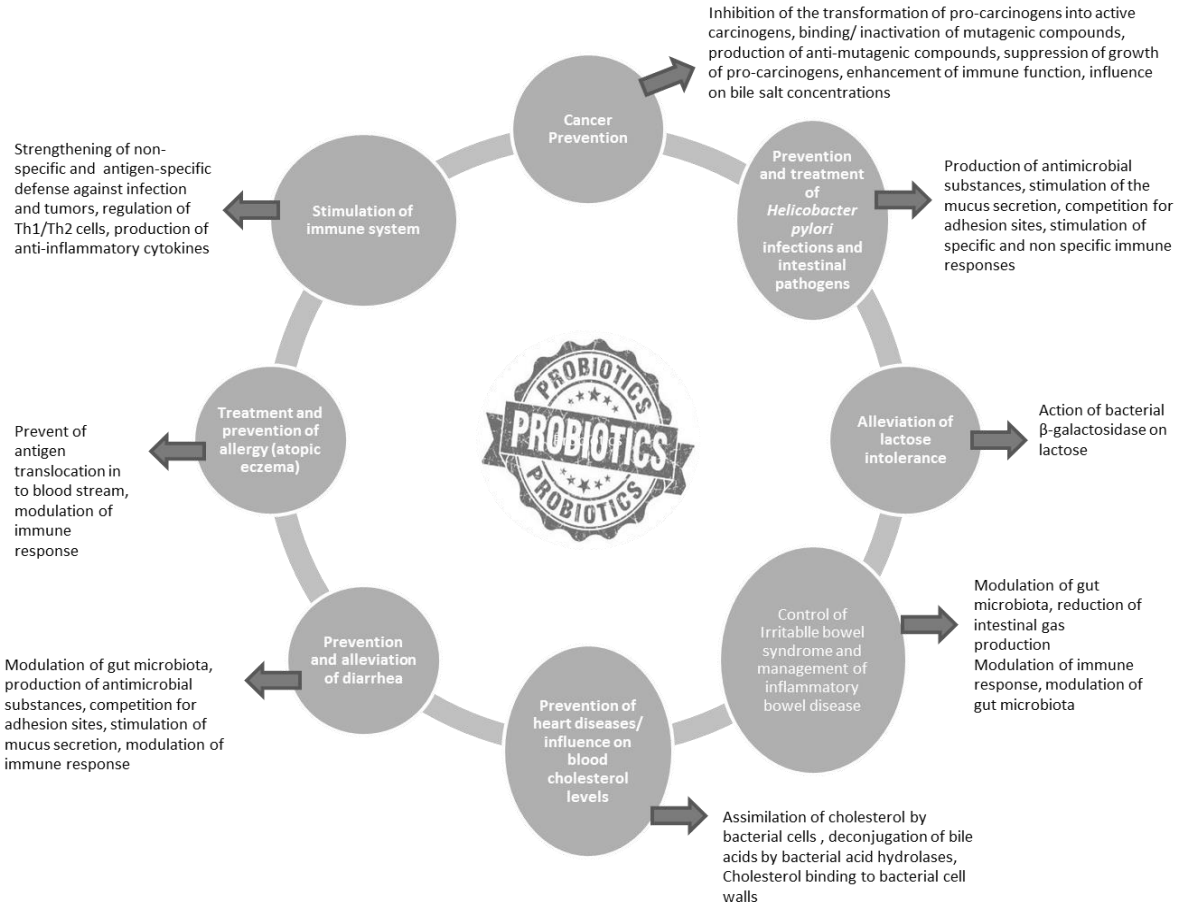


Figure 8: Health Benefits and mechanism of action of probiotics

2.3.6.1 Diarrhea

One of the most popular health application of probiotics is for the prevention and management (decrease duration) of acute viral and bacterial diarrhea, including also antibiotic associated diarrhea, *Clostridium difficile* diarrhea, rotavarious infection and traveler's diarrhea (Parvez et al., 2006). A number of strains such as *Lactobacillus GG*, *Lactobacillus reuteri*, *Saccaromyces boulardii*, *Bifidobacteria* spp. and others, have been shown to have a significant effect on diarrhea (Parvez et al., 2006).

Antibiotic associated diarrhea occurs usually between 2-3 weeks after antibiotic therapy due to an imbalance of intestinal bacterial microbiota, for example the normal

microflora may be suppressed during the antimicrobial therapy and can favour both infection by exogenous pathogens and overgrowth by endogenous pathogens, especially those of *Clostridium difficile*, *Candida albicans*, *Salmonella* spp., and *Klebsiellia caytoca* (Hickson, 2011). Moreover, because of the decreased activity of bacteria in the gut, complex carbohydrates are not completely broken down leading to osmotic water secretion (Iannitti & Palmieri, 2010). Different studies demonstrate that administration of probiotics can prevent or cure intestinal infections caused by *Cl. difficile* by restoring the balance of intestinal microflora. However, most of the studies suggest that there is some benefit such as reduction in duration or pain when using probiotics to treat antibiotic associated diarrhea, however, further research is needed to identify the probiotics with the greatest efficacy towards different types of antibiotics. The most common probiotics that have been used in clinical trials for the treatment of antibiotic associated diarrhea are *L. rhamnosus* GG and *Saccharomyces boulardii* , *L. acidophilus* and *B. bifidum*, *L. casei* DN 114 001 (Hempel et al., 2012; Pochapin, 2000). Another type of diarrhea is traveler's diarrhea which affects the healthy travelers not only in developing countries but also in Europe and most of the cases are due to bacterial pathogens (Gismondo et al., 1999). One of the reasons tourists become susceptible to disease is that travel (stress, jet lag, unfamiliar food) can disrupt the natural defense mechanisms of the body against infections by disturbing the normally protective bacteria in the intestines (Takahashi et al., 2007). Probiotics have proven to be a promising therapeutic strategy for some forms of traveler's diarrhea as they act by inhibiting pathogen attachment, enhancing the immune response and assisting in re-establishing normal microbiota (McFarland, 2007). Several studied indicated that several probiotics such as *Saccharomyces boulardii* and a mixture of *L. acidophilus* and *B. bifidum* had a remarkable efficacy in the prevention and treatment of traveler's diarrhea. Rotaviral diarrhea is the most common cause of diarhea in children worldwide and affects mainly in infants aged 6 months to 2 years (Thapar & Sanderson, 2004). The effect of various probiotic strains such as *Sac. boulardii, L. acidophilus, L. rhamnosus,* and *B. longum* on rotaviral diarrhea has been investigated by double-blind, placebo-controlled randomized studies (Das et al., 2016; Lee et al., 2015). Recent review on probiotic efficacy on diarrhea have been published by Guarino et al., (2015).

2.3.6.2 Lactose intolerance

Worldwide several people are expertise lactose malabsorption and the frequency of the disorder will increase with age (De Vrese et al., 2001). Lactose intolerance is caused by the deficiency of the enzyme lactase (β-galactosidase) which is important for lactose metabolism in digestive tract. Therefore, lactose is not completely hydrolyzed into its component monosaccharides, glucose and galactose. This decline in activity ends up in lactose malabsorption which can causes flatulence, bloating, abdominal pain and cramps, and moderate to severe diarrhea, from 30 minutes to 2 hours after the consumption of milk or lactose- containing products (Vonk et al., 2012). Different studies demonstrated that the addition of certain starter cultures to milk products, allows the lactose intolerant people to consume those products (eg. yoghurt or other fermented dairy products) without the usual rise of breath hydrogen or associated symptoms (De Vrese et al., 2001). The beneficial effects of probiotics on lactose intolerance could be explained as follow. Firstly, the lactose concentration in the fermented dairy products is lower due to the high lactase activity of bacterial preparations used in the production and secondly, the amount of active lactase enzyme enters the small intestine through the consumption of fermented dairy products is higher and the presence of this enzyme may lead to lactose hydrolysis and improved lactose tolerance (De Vrese et al., 2001).

2.3.6.3 Cancer

Consumption of probiotics have been shown to exert anticarcinogenic, antigenotoxic and antimutagenic effects and they have received much attention recently (Yu & Li, 2016). Generally, cancer is caused by mutation or activation of abnormal genes that control cell growth and division. Several experimental studies showed that probiotic intake may be help to prevent initiation of cancer. For instance Hirayama and Rafter, (2000) demonstrated that consumption of probiotics reduced colon cancer incidence (Hirayama & Rafter, 2000). Moreover Tavan et al., (2002) demonstrated the ability of lactobacilli and bifidobacteria to decrease the genotoxic activity of certain chemical compounds (Tavan et al., 2002). Detailed reviews on probiotic efficancy on cancer have been published by Maleki et al., (2016); So et al., (2017); Yu and Li (2016).

The mechanism by which probiotics exert antimutagenicity and anticarcinogenicity health effects is not clearly understood but might be due to decrease the exposure to

chemical carcinogens by: (i) detoxifying ingested carcinogens; (ii) altering the metabolic activity of intestinal microbiota and thereby decreasing populations or metabolic activities of bacteria that may generate carcinogenic compounds; (iii) producing metabolic products (e.g. butyrate) which improve a cell's ability to die when it should die (a process known as apoptosis or programmed cell death); (iv) producing compounds that inhibit the growth of tumour cells; or (v) enhancing the host's immune response to better defend against cancer cell proliferation (Commane et al., 2005; Zoumpopoulou et al., 2016).

2.3.6.4 Cholesterol reduction

Cholesterol has an important role and is essential for many functions in the human body. Cholesterol is an important component of cell membranes and nervous system and is the precursor for synthesis of certain hormones and vitamins (steroids, Vitamin D) (Daliri & Lee, 2015). However, elevated levels of total blood cholesterol or other blood lipids are a well documented risk factor for the development of atherosclerosis which leads to cardiovascular disease in modern industrialized countries (Kumar et al., 2012). Different studies evaluated the effect of probiotic strains on the serum cholesterol levels but the evidence is not overwhelming (Ooi & Liong, 2010; Parvez et al., 2006; Thushara et al., 2016). The mechanism by which probiotics affect cholesterol levels is still unknown. Some hypotheses about the mechanism include that : the bacteria may bind or incorporate cholesterol directly into the cell membrane or the bile salt hydrolysis enzymes deconjugate and precipitate the bile salts which are more likely to be exerted resulting in increased cholesterol breakdown (Ishimwe et al., 2015; Pavlović et al., 2012).

2.3.6.5 Allergies

Over the last years, the prevalence of allergic diseases (atopic dermatitis, asthma, allergic rhinitis) has increased especially in Western countries (Toh et al., 2012). The term allergy is defined as a hypersensitivity reaction initiated by immunologic mechanisms and based on the immunological mechanisms involved, is divided into IgE-mediated allergy or non-IgE-mediated allergy (Collado et al., 2009). Probiotics may have positive effects on allergies by improving mucosal barrier function and boosting stimulation of the immune system (MacFarlane & Cummings, 2002). Different studies

using LAB such as *L. rhamnosus GG* demonstrated that administration of probiotics can reduced the incidence of allergies (Cuello-Garciat al., 2015). Probiotic bacteria may also be helpful in alleviating allergy symptoms associated with proteins in milk. The positive effect of probiotics is possibly due to the ability of probiotic bacteria to break down milk proteins into smaller amino acids and peptides, thereby alleviating atopic dermatitis symptoms (Toh et al., 2012). Probiotic bacteria have further proved to regulate anti-inflammatory cytokines, such as interleukin-10, in children with atopic eczema (Pessi et al, 2000). Detailed reviews on probiotic efficacy on allergies have been published by Forsberg et al., (2016); Tang et al., (2015); West et al., (2016).

2.3.6.6 Hypertension

Hypertension is a major risk factor for the development of cardiovascular diseases. Probiotics have also been studied for their effect in blood pressure control and this has been demonstrated by animal and clinical studies that showed antihypertensive effects of probiotic ingestion (Lye et al., 2009). At the moment, the ability of probiotics to reduced blood pressure has been demonstrated through the fermentation and proteolysis of milk proteins in order to release ACE-inhibitory peptides. A recent review published by Upadrasta and Madempudi, (2016), demonstrated the efficacy of probiotics for the treatment of hypertension.

2.3.6.7 Helicobacter pylori treatment

H. pylori is a Gram-negative rod bacterium which colonize the human gastric mucosa (Collado & Sanz, 2009). Several *in vitro* and *in vivo* studies demonstrated that probiotic bacteria may inhibit the gastric colonization, growth and activity of *H. pylori*, which is associated with gastritis, peptic ulcers and gastric cancer (Ruggiero, 2014). The consistent results among these clinical trials indicate that probiotics were beneficial in decreasing gastritis and bacterial loads, but not lead to the eradication of *H. pylori*. However the use of probiotics in association with antibiotic treatments increased eradication rates and also may decreased side effects due to the antibiotics (Cremonini et al., 2001). Several mechanisms regarding the effect of probiotics on *H. pylori* have been suggested including production of antimicrobial substances, enhanced gut barrier function and competition for adhesion sites (Patel et al., 2014). Detailed reviews on

probiotic efficacy for the treatment of *H. pylori* have been published by Patel et al., (2014) and Ruggiero, (2014).

2.3.6.8 Irritable bowel syndrome

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder and is characterized by abdominal pain, bloating, and change in bowel habit, with an absence of any overt mucosal abnormality and flatulence (Andresen & Baumgart, 2006). Chrohn's disease and ulcerative colitis are the main clinical phenotypes and characterized by inflammation that affects the colon and the small intestine (Damaskos & Kolios, 2008). The etiology of the disease is still not well understood, but genetic predisposition and normal intestinal microbiota are thought to be the most important factors (Andresen & Baumgart, 2006). Therefore, modifying the composition and activity of the normal microbiota may help to reduce the incidence of the disease. At the moment there is still no universally satisfactory curative treatment for IBS. It is believed that the use of probiotics, it helps to reduce the symptoms of IBS. Moreover, the use of probiotics during pregnancy may have prophylactic benefits for neonates at high risk of developing IBS in later life (Barouei et al., 2009). Different studies using LAB such as *B. infantis*, *L. rhamnosus* GG can be effective to reduce symptoms of IBS such as bloating, abdominal pain, constipation, and flatulence. Several mechanisms are proposed by which probiotics help to reduce the symptoms of IBS and include increasing the number of beneficial bacteria the GIT and reducing pro- and anti-inflammatory cytokines (Dai et al., 2013). Detailed reviews on the efficacy for probiotics on IBS have been published by Dai et al., (2013), Distrutti et al., (2016) and Quigley, (2015).

2.3.6.9 Other health benefits

Other health benefits of probiotics include vitamin production and enhancement of mineral bioavailability and absorption, protection against vaginal or urinary tract infections, relieving anxiety and depression, prevention and treatment of obesity and diabetes and inhibiting decalcification of the bones in elderly people.

2.3.7 Mechanism of action

In order to understand how probiotics work, it is essential to know a little about the physiology, microbiology of the gastrointestinal tract and as well as the digestive process. The digestive process begins as soon as food enters the mouth. The process of chewing increases the surface area of food particles, making the food more susceptible to the digestive enzymes, including those in saliva. Then the food is travelled in the stomach where, the microbes present in the GIT have the potential to act in a favorable, a deleterious or a neutral manner. Then microbes in the small intestine and in the large intestine complete the digestion process. Varying numbers and composition of microbial species are found throughout the gastrointestinal tract. In total, the human gut microbiota contains about 10^{13} to 10^{14} bacterial cells (Vinderola et al., 2011). These bacteria are acquired rapidly after birth (Vinderola et al., 2011). Different number and type of bacteria reside in different parts of the gastrointestinal tract. The stomach is highly acidic (pH 1-2) due to the presence of hydrochloric acid and containes less than 10^3 cells/gm of bacteria. The prominent bacterial population in this region comprises of *Lactobacilli* and *Streptococci* (Prakash et al., 2011). The small intestine (duodenum, jejunum and ileum) has a higher pH (6-7). *Lactobacilli*, *E. coli* and *Enterococcus* occupied this region, with populations ranging from 10^4 to 10^7 cells/gm. The large intestine and colon are the richest in terms of bacterial cells, with cell numbers ranging from 10^{10} to 10^{12} cells/gm. Bacteria living in the large intestine include LAB, *Bacteroides* and *Bifidobacterium* species.

The mechanisms of action of probiotics are fully understood. This is a problem for EFSA, which recently refused the health claims of marketed probiotics, because of the lack of sufficient scientific evidence. This is due to the fact that most probiotics are tested in vitro or in animal model which results in difficulties to extrapolate these results to human. Moreover, the mechanisms of action may vary from one probiotic strain to another and are, thus making the investigation of the responsible mechanisms of action a very difficult and complex task (O'Hara & Shanahan, 2007). Probiotic bacteria benefit human being in a variety of manners for example by releasing antimicrobial substances in the body such as organic acids and bacteriocins, resisting colonization of harmful bacteria, stopping pathogens from attaching to GIT, modulating immune responses, enhancing intestinal barrier function, or altering the gut microbiota, degradation of toxin

receptor and by blocking the adhesion sites. In order for probiotics to exert beneficial effects, however, they must have suitable environmental conditions in the human body as well as the initial means of delivery and growth therein (O'Hara & Shanahan, 2007). Figure 9 shows the potential mechanisms of action of probiotics.

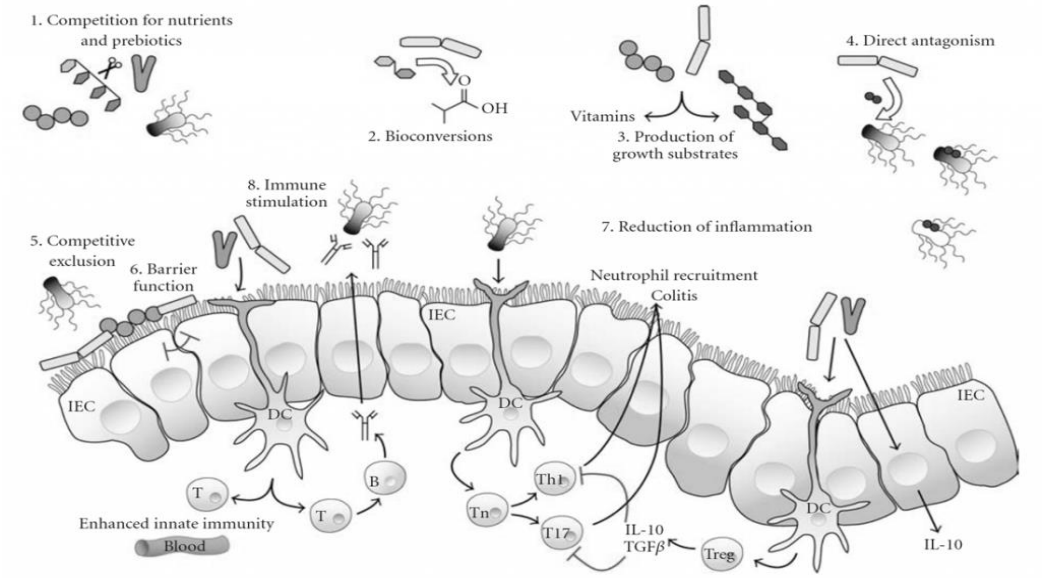


Figure 9: Probiotic mechanism of action (O' Toole & Cooney, 2008 .)

2.3.8 Adverse effects and safety of probiotics

Although beneficial effects are the most common outcome in clinical trials and *Lactobacillus* and *Lactococcus* have GRAS status, opportunistic infections resulting from probiotic administration have also been reported especially with immune-compromised people (Sanders et al., 2010). Moreover, despite the widespread use of probiotics, there are some theoretical risks regarding their safety use (Salminen et al., 1998). These include the potential for transmigration and the fact that colonization with probiotics may have a negative impact on gastrointestinal physiology and function, including metabolic and physiologic effects (Sanders et al., 2010). Another negative effect is the transfer of antibiotic resistance genes within the gastrointestinal tract among probiotic bacteria and the host's commensal bacteria (Doron and Snydman, 2015; Sharma et al., 2014;).

2.3.9 *Enterococci* as probiotics

In contrast to probiotic *Lactobacillus* and *Bifidobacterium* strains, there are relatively few published studies that deal with the effectiveness of enterococcal strains as probiotics. One of the best studied probiotic *Enterococcus* strain is *E. faecium SF68* which is promised to be effective for the treatment of children diarrhea or antibiotic associated diarrhea and is considered an alternative treatment to antibiotics (Franz et al., 1999). The strain was originally isolated in Sweden and patented in Switzerland and other countries (Franz et al., 2011). Its effectiveness for the treatment of intestinal disorders is probably due to the fact that the strain itself is a commensal of the gastrointestinal tract and that it has a short lag phase and generation time (approx. 20 min under optimal condition) (Franz et al., 2011). The strain is moderately resistant to antibiotics and is able to inhibit in vitro the growth of *E. coli, Salmonella* spp., *Shigella* spp. and *Enterobacter* spp. In addition, it is resistant to low pH values, tolerant to bile salts and individuals show a high tolerance to it without side effects (Franz et al., 2011). Moreover, *E. faecium SF 68* has also been studied as a probiotic supplement in animal feed and has been used in a dry dog food where it significantly enhanced cell-mediated and humoral immune functions in dogs (Moreno et al., 2006). Another probiotic preparation which contains *E. faecalis* together with *B. mesentericus* and *Cl. butyricum*, the Bio-three (TOA Pharmaceutical Lts, Japan) is also effective for the treatment of acute diarrhea and reduced also the associate inflammatory response, the severity of diarrhoea and length of hospital stay in children (Chen et al., 2010). Another enterococci probiotic product is the probiotic yoghurt Gaio, which produced in Denmark. The product contains a specific probiotic culture Causido which consists of two strains of *S. thermophillus* together with one strain of *E. faecium*. This probiotic has been claimed to be hypocholesterolaemic in the short-term (Agerholm-Larsen et al., 2000), but long-term reduction of LDL-cholesterol levels was not demonstrated (Richelsen et al., 1996). In another study, Rossi et al., (1999) used the strain *E. faecium* CRL 183 in combination with *Lactobacillus jugurti* for the development of a novel fermented soymilk product with potential probiotic properties, especially for cholesterol reduction. *E. faecium* PR88 (*E. faecium* Fargo 688\ from Quest International, Naarden, The Netherlands) is also a probiotic strain. Allen et al., (1996) demonstrated its consumption led to alleviation of the symptoms of irritable bowel syndrome in humans.

This strain was also used for a started adjunct culture the manufacture of a probiotic Cheddar cheese. The addition of the strain to the cheese did not affect its composition and texture, but the flavor of the cheese was influenced positively (Gardiner et al., 1999a; Gardiner et al., 1999b). Another commercial probiotic product which contains an *E. faecium* strain is Walthers ECOFLOR (Walthers Health Care, Den Haag, The Netherlands). According to the manufacturer, the probiotic product is effective against diarrhea, has anticarcinogenic properties, produce enterocins which are active towards *L. monocytogenes*, and possible reduce LDL-cholesterol level (Moreno et al., 2006). Additionally, Symbioflor is another enterococcal probiotic product which is produced by SymbioPharm (Herborn, Germany) and is used for immune regulation to combat recurrent, chronic sinusitis or bronchitis (Franz et al., 2011).

2.4 Bacteriocins

2.4.1 Introduction

LABs as well as other bacteria are capable of producing substances (bacteriocins, toxins, bacteriolytic enzymes, bacteriophages) that may be inhibitory either for themselves or for other bacteria. Bacteriocins are one of the most important and studied bacterial defense system. Consumer demands for natural, minimally processed and free of pathogens foods increased the interest for LAB bacteriocins. Bacteriocins produced by LAB genera have gained increased interest from the food industry for their potential use as additives with GRAS status in improving food safety and quality (Mills et al., 2011).

The purpose of this section is to provide an overview of bacteriocins produced by LAB, their biosynthesis, mode of action and applications before addressing a detailed review of the bacteriocins produced by enterococci species and their applications.

2.4.2 History and definition

Colicins were the first bacteriocins identified by Andre Gratia in 1925 and they got their name because they killed *E. coli* (Gillor et al., 2008). Colicins are a diverse group of antibacterial proteins, which kill closely related bacteria by inhibition of cell wall synthesis, permeabilizing the target cell membrane or by inhibiting RNase or DNase

activity (Cleveland et al., 2001). In 1928, Rogers and Whittier reported that Gram-positive bacteria also produce these 'colicin-like' substances. They observed that some lactococcal strains had an inhibitory effect on the growth of other lactic acid bacteria and later, in 1947, Mattick and Hirsh concentrated an inhibitory substance isolated from a strain of *Lactococcus lactis* subsp. *lactis*, termed nisin (Cotter et al., 2005). Nisin was initially purified and marketed in England in 1953 (Deegan et al., 2006). The same year, the term bacteriocin was proposed for antimicrobial peptides produced by microorganisms by Jacob et al., 1953 (Jack et al., 1995). Then, in 1969, was considered to be safe for food use by the Joint FAO/WHO Expert Committee on Food Additives.

Bacteriocins are defined as ribosomally synthesized proteins or peptides produced by both Gram-negative and Gram-positive bacteria strains that exhibit an antagonistic effect toward other bacteria strains and species (Cotter et al., 2005). Bacteriocins show antimicrobial activity against food spoilage and pathogenic bacteria. These target organisms can be either closely-related species or bacteria from diverse genera (Deegan et al. 2006). BACTIBASE is an open-access online database designed for the documentation of bacteriocins and the number of entries continues to grow. It is developed by the Functional Proteomics & Alimentary Bio-preservation Unit at Institute of Applied Biological Sciences Tunis (ISSBAT), Tunisia in collaboration with Nutraceuticals and Functional Foods Institute (INAF), Laval University, Canada (Bali et al., 2016). It gives information about the physicochemical, structural, microbiological and taxonomic characteristics of the bacteriocins. The BACTIBASE database contains 229 bacteriocin sequences (December, 2016), most of which are produced by Gram positive bacteria mainly LAB.

2.4.3 Bacteriocins VS antibiotics

Bacteriocins are often confused in the literature with antibiotics (Cleveland et al., 2001). This confusion might limit their use in food applications from a legal standpoint. Therefore, it is important to distinguish bacteriocins from antibiotics. The most important differences are summarized in Figure 10. The most important difference between bacteriocins and antibiotics is that bacteriocins have a relatively narrow killing spectrum and restrict their antimicrobial activity to strains in the same or closely related species, while antibiotics have a wider spectrum of inhibitory activity (Cotter et al.,

2013). Moreover, bacteriocins differ from antibiotics by the fact that bacteriocins are proteinaceous compounds which makes them easily digestible by proteases in the human digestive tract. In addition, all classes of bacteriocins are ribosomally synthesized and produced during the exponential growth phase, only class I is post-translationally modified to produce the active form. On the other hand antibiotics are usually secondary metabolites and also are not ribosomally synthesized (Cotter et al., 2013).

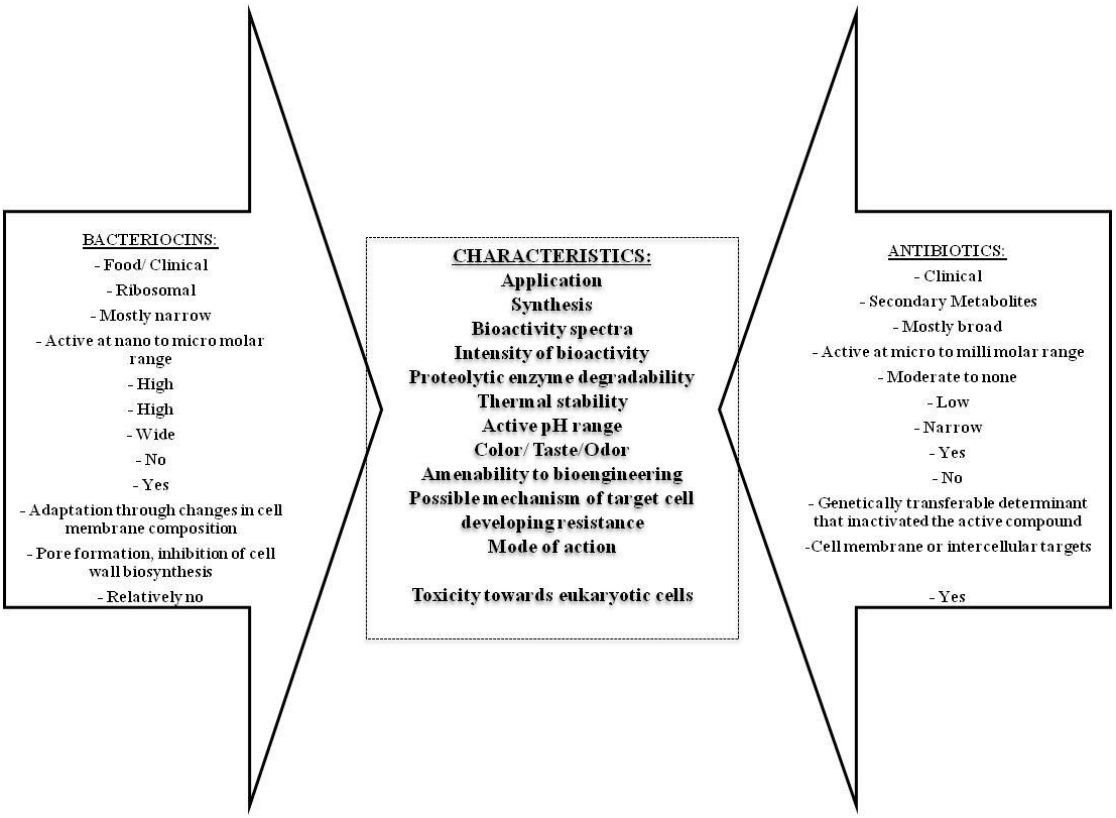


Figure 10: Bacteriocins VS antibiotics

2.4.4 Classification

Most of the bacteriocins produced by LAB are small, cationic, hydrophobic, or amphiphilic peptides composed of 19 to 60 amino acid residues (Cleveland et al., 2001). The classification of bacteriocins is very controversial. The bacteriocins from LAB have been classified based on their producers, molecular weight, primary structures and mode of actions (Cotter et al., 2005; Klaenhammer, 1993; Nes et al., 1996; Rea et al., 2011; Zouhir et al., 2010).

Klaenhammer, (1993) divided LAB bacteriocins into four classes. Class I bacteriocins (lantibiotics) were characterized as lanthionine-containing bacteriocins that undergo extensive post-translational modifications. Class II bacteriocins were heat-stable peptides and could be further divided into three subgroups. Class III was composed by large and heat-labile proteins, while class IV bacteriocins were defined as protein complexes composed of protein and chemical moieties. This classification scheme was later modified. The fourth class was abolished, and criteria for dividing class II bacteriocins were changed on the basis of leader differences (Nes et al., 1996). Later, according to their specific secretion pathways, the number of subclasses of class II bacteriocins was expanded to six (Van Belkum & Stiles 2000). Bacteriocins were once divided into only two main groups: class I (lanthionine-containing bacteriocins/ lantibiotics) and class II (the non-lanthionine bacteriocins). In this proposal, the large heat-labile class III bacteriocins were renamed as bacteriolysins, i.e. non-bacteriocin lytic proteins (Cotter et al., 2005). Zouhir et al., (2010) proposed a new structure-based classification was proposed. G+ bacteriocins were classified into 12 groups based on their amino acid sequences. Rea et al., (2011), based on the classification scheme of Cotter et al., (2005), presents a new classification scheme. Rea et al., (2011), agreed that Gram positive bacteriocins could be divided in two classes. Class I which covers all post-translationally modified bacteriocins from G+ bacteria, whereas the class II and bacteriolysins retained the same (Rea et al. 2011). In addition, specific classification schemes have been proposed for bacteriocins produced by the genus *Enterococcus* and *Bacillus* (Franz et al., 2007; Abriouel et al., 2011). In this review, LAB bacteriocins will be divided into 3 classes: class I (lantibiotics), class II (unmodified heat-stable bacteriocins), and class III (large heat-labile bacteriocins) or bacteriolysins based on recent classification schemes (Cotter et al., 2005; Rea et al., 2011). Figure 11 shows the classification of LAB bacteriocins and their characteristics.

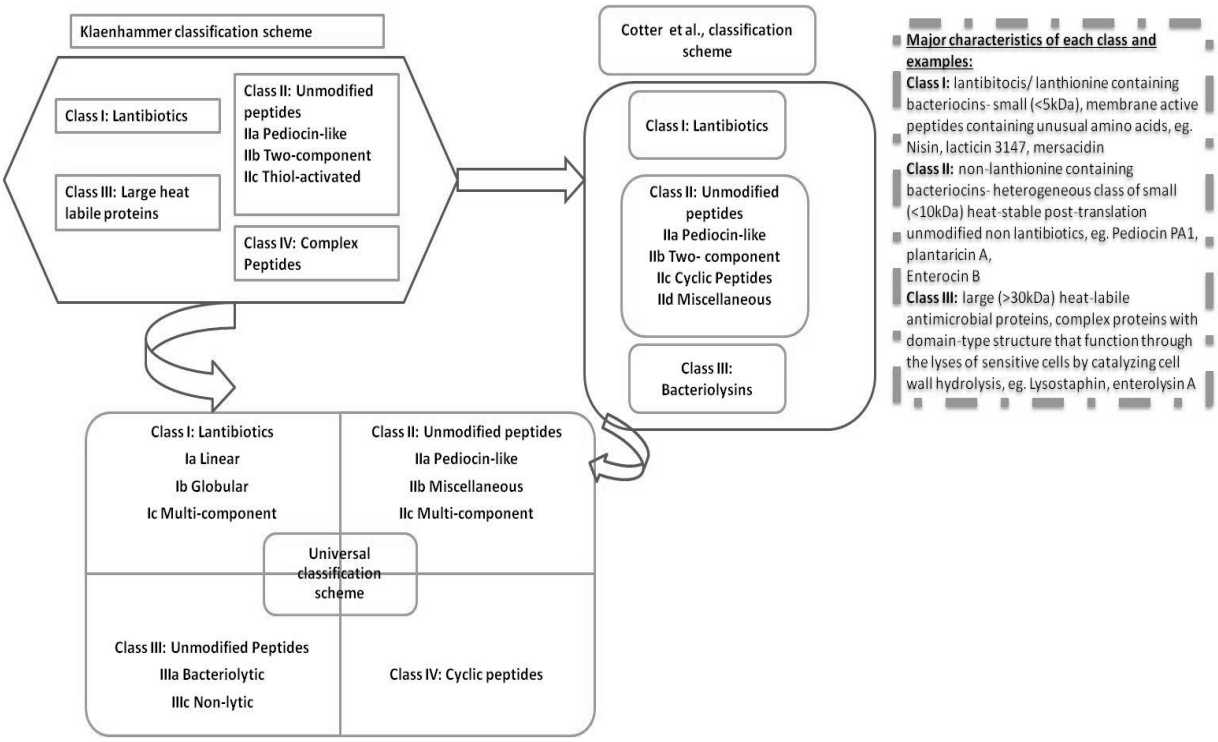


Figure 11: Bacteriocin classification

2.4.4.1 Class I

Class I bacteriocins, termed lantibiotics, are small (<5 kDa), membrane-active and heat-stable peptides, with 19 to 50 amino acids (Field et al., 2010). These bacteriocins are characterized by their unusual thioether amino acids, like lanthionine, methylxanthine, dehydrobutyrine and dehydroalanine which are introduced by post translational enzymatic modification (Field et al., 2010). In this class, the most well-known and studied bacteriocin is nisin which has been isolated from *Lactococcus lactis* (Cleveland et al., 2001). The Class I bacteriocins was further subdivided into Class Ia and Class Ib. Class Ia bacteriocins consist of cationic and hydrophobic peptides that form pores in target membranes and have a flexible structure compared to the more rigid Class Ib (Cleveland et al., 2001). Class Ib, labyrinthopeptins are named as a results of their "labyrinthine" structure and they are distinguished by the presence of labionin, a previously unidentified carbacyclic post-translationally modified amino acid (Field et al., 2010). However, as the number of lantibiotics increased and their characteristics were elucidated, this classification became unclear. Recently, a new classification scheme was proposed by Rea et al., (2011). Class I bacteriocins are divided into four subgroups on the basis of the modification pathway and antimicrobial activity (a) the

lantibiotics/lantipeptides, (b) the labyrinthopeptins and (c) the sactibiotics. Class Ia bacteriocins (lantibiotic/ lantipepetides) are further divided into four subgroups. Lantibiotics like nisin, epidermin and Pep5 are considered as type I lantibiotics which undergo modifications by two enzymes, LanB for a dehydration of Thr and Ser residues and LanC for lanthionine ring formation. On the other hand, type II lantibiotics, such as lacticin 481, mersacidin, and actagardin are modified by one large bifunctional enzyme, LanM which exhibits both dehytrase and cyclase activity. Two-peptide lantibiotics like lacticin 3147, lactocin S and haloduracin are also included in this class. Type III lantibiotics were described as lanthionine-containing peptides without antimicrobial activity. This type grouped on the basis of related modification enzymes. SapB, SapT and AmfS belong to type III, as their modification enzymes share homology to the C-terminal domain of LanM. The type IV lantibiotics possess novel lanthionine synthetases called LanL. For this type the term "lantipeptides" can be used as they have clear lantibiotic characteristics in their structures and biosynthetic means, but their antimicrobial activities are still unknown.

2.4.4.2 Class II

Class II bacteriocins contain small (<10 kDa), between 36 and 57 amino acids, heat-stable, non-lanthionine-containing peptides (Cleveland et al., 2001). Class IIa bacteriocins are probably the most important and well-studied bacteriocins among the unmodified AMPs. Class IIa bacteriocins are regarded to possess a narrow antimicrobial spectrum. They are particularly effective against the foodborne pathogen *Listeria* strains. Recently, some class IIa bacteriocins have been found to be effective against both G+ and G- bacteria, such as *Campylobacteri jejuni* and *Escherichia coli* O157:H7 (Drider et al., 2006; Franz et al., 2007). Unlike lantibiotics, these bacteriocins do not undergo post-translational modifications. Class II unmodified bacteriocins were originally subdivided into 3 groups: IIa pediocin-like, IIb two-peptide, and IIc thiol-activated bacteriocins (Klaenhammer, 1993). Since then, there have been several alternative classifications about class II bacteriocins, but all of them agreed and identified the two major subclasses, i.e. the pediocin-like antilisterial bacteriocins (class IIa) and two-component bacteriocins (class IIb) (Nes et al., 1996; Drider et al., 2006). In addition to the two established subclasses, class IIc cyclic bacteriocins and class IId non-pediocin-like single linear peptides were suggested and retained in a review of class

II bacteriocins (Cotter et al., 2005; Nissen-Meyer et al., 2009). In this review, we follow the classification by Cotter et al., (2005), which subdivide class II bacteriocins into 4 subgroups. Class IIa, Listeria-active or anti-listerial peptides, are 'pediocin-like' peptides and are characterized by a well conserved Tyr-Gly-Asn-Gly-Val-Xaa-Cys consensus sequence at their N-terminal. Class IIa bacteriocins have two cysteines forming an S-S bond in the N terminal half of the peptide. Class IIb bacteriocins are composed of two different peptides, both of the peptides are required for their antimicrobial activity, such as lactococcin G , lacticin F and plantarixins EF and JK . Class IIc bacteriocins are cyclic peptides whose N-and C- terminal are linked by a covalent bond. Enterocin AS-48 produced by different enterococci strains was the first discovered class IIc bacteriocin (Martinez-Bueno et al., 1994). Other LAB-derived class IIc bacteriocins include gassericin A, uberolysin from *Streptococcus uberis* and lactocyclicin Q from *Lactococcus* spp. (Sawa et al., 2009; Wirawan et al., 2007). Class IId, is suggested to contain all the other bacteriocins that they don't have any sequence similarities with the other Class II bacteriocins. This group includes single-peptide non-pediocin-like linear bacteriocins. The best characterized example of this group is lactococcin A produced by *L. lactis* strains.

2.4.4.3 Class III

Class III bacteriocins are secreted by the bacterial preprotein translocase (sec-pathway) and are large (>30kDa), heat-labile proteins (Nes et al., 1996). In their domain-type structures, different domains function differently for translocation, binding to receptor, and antimicrobial activity (Cotter et al., 2005). This group is not well characterized and also most of the classification schemes are emphasize class I and class II bacteriocins. Moreover Cotter et al., 2005 refused to recognize these heat labile proteins as bacteriocins (bacteriolycins), since these proteins were considered to degrade the targeted cell wall leading to cytolysis. Some examples of Class III bacteriocins produced by LAB are helveticin J produced by *Lactobacillus helveticus* 481 and enterolysin A from *Enterococcus faecalis* LMG 2333.

2.4.5 Mode of action

The potential application of bacteriocins produced by lactic acid bacteria as food preservatives requires a detailed knowledge of their bactericidal mode of action with the

cytoplasmic cell membrane as the primary target. Most of the bacteriocins produced by lactic acid bacteria appear to have the same mechanism of action, namely depleting the proton motive force (PMF) in the target cells through the formation of discrete pores in the phospholipids bilayer of the cell cytoplasmic membrane (Abee, 1995). These pores alter the membrane permeability, thus disturbing membrane transport and resulting in the uncontrolled efflux of ATP, amino acids and essential ions (Mg++ and K+) (Abee, 1995). This uncontrolled flow of substances in and out of the cell subsequently inhibits the energy production and biosynthesis of proteins or nucleic acids.

Moreover some class I bacteriocins such as nisin have a dual mode of action. Nisin is a membrane active bacteriocin, therefore it kills bacteria by using the membrane bound peptidoglycan precursor lipid II as a docking molecule, a step which facilitates two bactericidal activities, the inhibition of peptidoglycan biosynthesis and membrane pore formation which results in the leakage of small molecules from the cytoplasm and subsequent loss of membrane potential and finally cell death (Field et al., 2007).

Regarding class IIa bacteriocins the presence of amphiphilic segments, their water solubility and membrane-binding ability suggest that this class of bacteriocins act primarily by permeabilizing the membranes of susceptible microorganisms, through the formation of pore complexes following a 'barrel-stave' model (Ennahar et al., 2000). The first step is believed to be an interaction with the membrane surface of the target cell which is mediated by an electrostatic binding between the positively charged and polar residues. Then hydrophobic interactions would occur between the hydrophobic/amphiphilic domains with in the C-terminal half of the bacteriocin and the lipid acyl chains. This step is vital for pore formation in the cell membrane. After membrane permeabilization, pore formation leads to the total or partial dissipation of the proton motive force, depletion of intracellular ATP and leakage of amino acids and ions (Diep et al., 2007).

2.4.6 Inhibitory spectrum

In general, LAB bacteriocins tend to be active against a wide range of mostly closely related Gram-positive bacteria (Jack et al., 1995). On the other hand, Gram-negative bacteria, yeast and fungi are generally resistant to LAB bacteriocins because they contain lipopolysaccharides (LPS) on the outer membrane which makes their cell wall

are far less permeable than those of Gram-positive bacteria (Jack et al., 1995). However there are some treatments of Gram-negative bacteria which can cause destabilization of the layer and therefore make them susceptible to nisin. These treatments/methods include exposure to chelating agents such as EDTA, sublethal heat, osmotic shock and freezing (Galvez et al., 2007).

The majority of class I bacteriocins have a fairly wide inhibitory spectrum. They not only inhibit closely related bacteria, such as species from the genera *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus*, and *Streptococcus*, but are also effective against less closely related Gram-positive bacteria, such as *L. monocytogenes, Staphylococcus aureus, Bacillus cereus*, and *Clostridium botulinum*. In addition, bacteriocins from Class I, such as nisin and thermophilin 13, prevent out-growth of spores of *B. cereus* and *C. botulinum* (Chen & Hoover, 2003). On the other hand class II and III bacteriocins have relatively narrow antimicrobial activity spectra and seems to inhibit primarily *Listeria* strains and only closely related gram-positive bacteria. In general, members of the genera *Enterococcus, Lactobacillus, Pediococcus, Leuconostoc* are sensitive to class IIa bacteriocins, and members of genus *Lactococcus* are resistant (Chen & Hoover, 2003). An exception from class II bacteriocins is the pediocin PA-1 which has a broad antimicrobial spectrum and can inhibit some less closely related Gram-positive bacteria, such as *S. aureus* and vegetative cells of *Clostridium* spp. and *Bacillus* spp (Gillor et al., 2008). Recently, some class IIa bacteriocins have been found to be effective against both G+ and G- bacteria, such as *Campylobacteri jejuni* and *Escherichia coli* O157:H7 (Drider et al., 2006; Svetoch & Stem, 2010).

2.4.7 Biosynthesis

Bacteriocins are ribosomally synthesized and the genes necessary for their production and immunity are usually arranged as operon clusters (Nes et al., 1996). These operons can be located on conjugative transposable elements (nisin), on the host chromosome (subtilin) and also on plasmids (cytolysin) (McAuliffe et al., 2001). Bacteriocins are primarily synthesized as biologically inactive precursor or pre-peptides and contain an N-terminal extension or leader sequence that is attached to the C-terminal propeptide. The leader peptide keeps the bacteriocin inactive and is speculated to protect the producing organism from being killed by its own bacteriocin.

The lantibiotic biosynthesis operons usually contain structural genes coding the prepeptide (LanA - the abbreviation lan refers to homologous genes of different lantibiotic gene clusters), genes encode enzymes that are responsible for modification reactions (LanB,lanC, LanM, lan D and lanJ), a gene encoding serine protease which is responsible for removal of the leader peptide (LanP), the ABC (ATP-binding cassette) peptide transporter, superfamily transport protein involved in peptide translocation (LanT), regulatory proteins (LanR, lank, lanQ, lanX) and immunity genes (LanI, FEG) encoding proteins that protect the producer from the producer lantibiotic (Field et al., 2007).

According to Drider et al., (2006), at least four genes are required for the synthesis and secretion of class II bacteriocins which are the structural bacteriocin gene, encoding a pre bacteriocin; (ii) the immunity gene, encoding an immunity protein that protects the bacteriocin producer from its own bacteriocin; (iii) a gene encoding an ABC-type transport protein (ATP-binding cassette) r necessary for secretion; and (iv) a gene encoding a membrane bound accessory protein that is essential for export.

The biosynthetic pathway of lantibiotic follows a general scheme for bacteriocin production and transport. Lantibiotics are synthesized as biologically inactive pre-peptides consisting of an N-terminal leader peptide attached to the C-terminal pro-peptide (Field et al., 2015). During the maturation process, the leader peptide is removed and the C-terminal pro-peptide is modified to the active lantibiotic. Serine, threonine and cysteine residues in the propeptide region are undergoing extensive post-translational modification reactions to form lanthionine (Lan) or methyl- lanthionine (MeLan) (McAuliffe et al., 2001). Following the modification reactions, the modified pre-lantibiotics undergo proteolytic processing to remove the leader peptide, leading to activation of lantibiotic either before or after export from the cell, resulting in the mature active peptide and is exported from the cell through a dedicated ABC-transporter and its accessory protein (Nes et al., 1996). In order to protect the bacteriocin producers from being killed by their own secreted bacteriocins, bacteria have a self-protective system termed immunity. This is accomplished by immunity proteins which are co-produced with the bacteriocins (Drider et al., 2006). Self-protection of lantibiotic-producing strains is typically mediated by the immunity proteins (LanI) and/or a dedicated ABC transport protein system composed of LanFEG (McAuliffe et al., 2001).

The biosynthesis of lantibiotics is often regulated in order to maintain a proper balance between production and immunity (Field et al., 2015). This regulation is often mediated via two-component regulatory systems; a membrane bound histidine protein kinase (HPK), and a cytoplasmic response regulator (RR).

Unlike the lantibiotics, class II bacteriocins do not undergo extensive post-translational modifications (Perez et al., 2015). Most class II bacteriocins are synthesized primarily in the form of a pre-peptide or a biologically inactive pre-bacteriocin. This compound contains a sequence from 18 to 27 amino acids and a characteristic double-glycine proteolytic processing site at the N-terminus, 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 (Perez et al., 2015). The presence of the leader peptide at the N-terminus of pre-bacteriocin makes the peptide inactive thus protecting the producing cell. The two glycines present in the sequence are responsible for recognition by the pre-bacteriocin transport system (Ennahar et al., 2000). Following synthesis of the biologically inactive pre-peptide, cleavage of the N-terminal leader sequence. After recognizing the pre-peptide, the leader amino acid sequence of bacteriocin is removed and then the active peptide/bacteriocin is secreted into the extracellular medium through a dedicated ABC transporter and its accessory protein (Ennahar et al., 2000). As with lantibiotics, immunity proteins are responsible for conferring self-immunity to class II bacteriocins. Unlike the lantibiotics, the immunity proteins for the type IIa bacteriocins are located almost exclusively in the cytoplasm, with less than 1% associated with the membrane (Ennahar et al., 2000). Quorum sensing mechanisms for regulating the production of class IIa bacteriocins are usually termed as three component regulatory systems (Drider et al., 2006). The 3-component regulatory system typically includes the two component system which composed of a transmembrane HPK and cytoplasmic RR and induction factor (IF), which is acts as a signal to induce the transcription of bacteriocin genes.

2.4.8 Bacteriocin resistance mechanism

Once a new antimicrobial agent is described, and proven to be safe and effective against pathogens, it is crucial to evaluate the potential risks of resistance development upon prolonged exposure to it (Cotter et al., 2013). As found with therapeutic antibiotics in

the environment, bacteriocin-resistant mutants do occur. A detailed review concern bacteriocin resistance has been published recently by de Freire Bastos and Coelho, (2015). The resistance of spontaneous or induced mutants to bacteriocins may be related to changes in membrane and cell wall, such as alterations in the electrical potential, fluidity, membrane lipid composition and load or cell wall thickness, or even a combination of all factors (de Freire Bastos & Coelho, 2015). The exact mechanism of bacteriocin resistance is not fully understood. According to Collins et al., (2012), the mechanisms involved in bacteriocin resistance can be divided into two groups: acquired resistance and innate resistance. Moreover, according to Van Schaik et al., (1999), these mutational changes on the cell surface of the resistant strain may occurred following cell exposure to low concentrations of bacteriocins or as part of an adaptive response to some other stress. For instance, Abee, (1995) showed that the resistance of *L. monocytogenes* to nisin is related to variation in fatty acid composition of cell membranes, reducing the concentration of phospholipids, hindering the formation of pores. The mechanism of resistance to subclass IIa bacteriocins appears to be linked to reduced expression of mannose permease of the phosphotransferase system (Kjos et al., 2011).

2.4.9 Methods for determination of bacteriocin activity

Several steps are involved for the isolation, purification, and identification of novel bacteriocins. The first step involves the screening of bacteriocin producing strains from a large number of isolates isolated from different sources such as humans, food, plants. There are many assays for detecting bacteriocin production. Most are based on the diffusion of bacteriocins through solid or semisolid culture media to inhibit growth of sensitive indicator strains (Parente et al., 1992; Papagianni et al., 2006).

Some examples of bacteriocin assays are the spot-on-law assay, whereas the putative bacteriocin producer is spotted on an agar medium and incubated overnight to develop colonies. Then, the colonies are overlaid with a sensitive indicator organism and incubated to develop zones of inhibition. Another method is the well diffusion assay, where the supernatants from the putative bacteriocin producing microorganism placed in the wells cut into the agar inoculated with the indicator strain. The clear zones of inhibition around the colonies or wells can indicate bacteriocin production and is

measure as the degree of inhibition. However, there are other antagonistic compounds produced by LAB in addition to bacteriocins such as lactic acid, hydrogen peroxide, which can cause problems during the assays. The results of these methods are generally qualitative. In addition to culture based methods, a molecular approach is important either for confirmation purpose or for the detection of bacteriocin. Moreover, in order to avoid characterizing and purifying bacteriocins that have been previously isolated and characterized, a good approach is to first screen by PCR the producer strain using known"bacteriocin-specific" primers.

2.4.10 Bacteriocin purification

Due to the great diversity of bacteriocins there is not one single purification method or general protocol that has been used. Therefore, there is a wide range of purification protocols for LAB bacteriocins. Adequate purification of bacteriocins is necessary for their characterization. As most of LAB bacteriocins are produced in low amounts, it is crucial to concentrate the supernatant that contains the antimicrobial substance (Pingitore et al., 2007). Therefore, the first step of purification is the concentration of culture supernatant. Different protocols have been used for the concentration step such as salt precipitation with ammonium sulphate, acid precipitation, and adsorption-desorption, organic solvent extraction or hydrophobic matrix such as amberlite XAD-16 (Parada et al., 2007; Pingitore et al., 2007). The above step produces a partially purified extract of bacteriocins. Then these concentrated extracts must be subjected to a subsequent purification step such as ion exchange chromatography (IEC), hydrophobic interaction, reverse-phase C18 solid phase extraction, adsorption-desorption (AD), reverse-phase high-perfomance liquid chromatography (RP-HPLC), and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) (Parada et al., 2007; Pingitore et al., 2007). Table 13 shows examples of purification steps for some enterocins.

Table 13: Examples of purification steps for some enterocins

Organism	Bacteriocin	Purification scheme		
		Volume reduction/ concentration	**Final purification**	**References**
E. faecalis S-48	Enterocin AS-48	-	CEX, GF, RP-HPLC	Galvez et al., 1989
E. faecalis BFE 1071	Enterocin 1071A	Ammonium sulphate precipitation	CEX	Balla et al., 2000
E. faecalis BFE 1071	Enterocin 1071B	Ammonium sulphate precipitation	CEX	Balla et al., 2000
E. faecalis WHE 96	Enterocin 96	Ultrafiltration	CEX, RP-HPLC	Izquierdo et al., 2009
E. faecium CTC 492	Enterocin A	Ammonium sulphate precipitation	CEX, HIC, RP-HPLC	Aymerich et al., 1996
E. faecium T 136	Enterocin B	XAD-16	CEX, HIC, RP-HPLC	Casaus et al., 1997
E. faecium CRL 35	Enterocin CRL 35	Ammonium sulphate precipitation	GF, CEX, RP-HPLC	Farias et al., 1996
E. faecium P13	Enterocin P	Ammonium sulphate precipitation	GF, CEX, HIC, RP-HPLC	Cintas et al., 1997
E. faecium L50	Enterocin L50A	XAD-16	CEX, HIC, RP-HPLC	Cintas et al., 2000
E. faecium L50	Enterocin L50B	XAD-16	CEX, HIC, RP-HPLC	Cintas et al., 2000
E. faecium L50	Enterocin Q	XAD-16	CEX, HIC, RP-HPLC	Cintas et al., 2000

CEX: Cation Exchange, GF: Gel Filtration, RP-HPLC: Reverse-Phase High-Performance Liquid Chromatography, HIC: Hydrophobic Interaction

2.4.11 Bacteriocin characterization

Bacteriocin characterization assays provide the necessary information about the newly identified bacteriocin and also helps to establish optimal conditions for future applications of bacteriocins in food products. Characterization can be divided into biochemical and physiological characteristics. Biochemical characteristics include the resistance of bacteriocins against heat, the sensivity to pH, enzymes, organic enzymes

and determination of the molecular weight while physiological characteristics involve the bacteriocin mode of action, growth and bacteriocin production kinetics (Parada et al., 2007). For example, thermal stability of bacteriocins is determined usually at pasteurization and sterilization temperatures. If the bacteriocin retains its activity after heat treatment is characterized as heat stable. Then, the bacteriocin stability is tested against a wide range of pH usually from 2-10, as acid tolerant bacteriocins are more preferable for applications in food bio preservation. For enzymatic sensitivity a range of enzymes can be utilized such as proteolytic, hydrolytic and lipolytic enzymes. This assay demonstrates the proteinateous nature of bacteriocin and also the sensitivity to proteolytic enzymes is important for ensuring that bacteriocins are safe if they are ingested (Parada et al., 2007). Then, the molecular mass of bacteriocin is determined using usually Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The final step for the complete characterization of the bacteriocins are to determine the amino acid sequence followed by the analysis of the gene cluster involved in the production and immunity of bacteriocin. These characteristics are important for the preliminary characterization of bacteriocins and to assess their stability in food systems, the conditions for optimum bacteriocin activity and also contribute to the classification of the bacteriocin (Parada et al. 2007).

2.4.12 Bacteriocins produced by *enterococci*

The first enterocin purified to homogeneity was the enterocin AS-48 produced by *E. faecalis* S-48 and was defined as a cyclic peptide antibiotic (Martínez-Bueno et al., 1994). Since then, the number of characterized bacteriocins produced by *Enterococci* is increasing. Bacteriocins produced by enterococci are called enterocins and they generally belong to class II bacteriocins (Giraffa, 1995). Enterocins are heat stable, and also are stable over a wide range of pH. As some of the criteria used to classify bacteriocins (structure or genetic characteristics) are not applicable to enterocins Franz et al., (2007) proposed a new classification scheme especially for enterocins.

According to Franz et al., (2007), enterocins are divided into four major classes (Figure 12). Class I consisting of post-translationally modified lantibiotic enterocins. An example is the two-component cytolysin from *E. faecalis*. Class II (non lanthibiotic enterocins) consisting of linear, unmodified peptides having molecular mass less than

10 kDa. Class II is subdivided into three subclasses: the pediocin family of enterocins (IIa), enterocins synthesized without a leader peptide (IIb) and linear enterocins that do not belong to the pediocin family (IIc). Enterocins under subclass IIa show strong antilisterial activity. Class IIa enterocins have a conserved N-terminal sequence, Tyr-Gly-Asn-Gly-Val, and two cysteines forming an S-S bridge in the N-terminal half of the peptide (Javed et al., 2011). Examples of class IIa enterocins Bacteriocin 31, Enterocin A, Enterocin CRL35 and Enterocin P. Class IIb enterocins are composed of two polypeptide chains and both peptides are required for full biological activity and their primary amino acid sequences are also different. This subclass includes many bacteriocins that lack the YGNGVXC motif and are synthesized as leaderless peptides which require dedicated export systems (Franz et al., 2007). Examples of Class IIb are Enterocin RJ-11, Enterocin EJ97 Enterocin Q, Enterocin L50A and Enterocin L50B. Subcall IIc contains enterocins that cannot be included in the other subclasses such Enterocin B, Enterocin 1071A and Enterocin 1071B. Class III is consisting of cyclic peptides, like Enterocin AS-48 from *E. faecalis*. Class IV includes the large, heat labile proteins such as Enterolysin A. APPENDIX I shows all the known enterocins isolated until now as deposit in BACTIBASE.

Bacteriocins produced by enterococci are particularly active against pathogenic bacteria, such as *Listeria*, *Clostridium* and *Staphylococcus* (Franz et al., 2007). The anti-*Listeria* activity may be explained by the fact that enterococci and listeriae are phylogenetically closely related (Moreno et al., 2006).

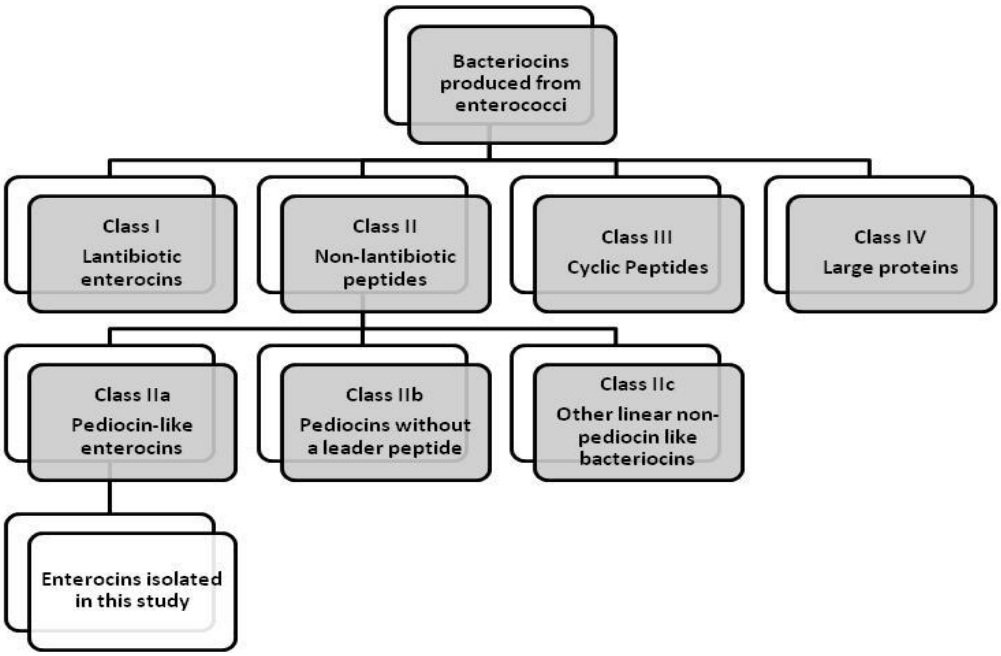


Figure 12: Classification of enterocins

2.4.12.1 Examples of enterocins isolated from food sources

In 1994, Olasupo and his colleagues isolated an *E. faecium* NA01 strain from Nigerian fermented skimmed cow milk ('wara') which produces a bacteriocin (designated enterocin 01). This bacteriocin showed inhibitory activity towards *Lactobacillus, Enterococcus* and *Listeria* strains. The bacteriocin was inactivated by α-chymotrypsin and proteinase K, but not by trypsin and pepsin, and also inhibitory activity was stable at 100 ^{o}C for 5 min and at pH 2.0-6.0.

Villani et al., (1993) isolated *E. faecalis* 226, from natural whey cultures utilized as starters in the manufacture of mozzarella cheese from water-buffalo milk. The strain produces enterocin 226NWC which has inhibitory towards *L. monocytogenes*, *L. innocua*, *E. faecalis* and *E. casseliflavus*. Enterocin 226NWC has a bactericidal effect. The bacteriocin is stable at 90 ^{o}C for 30 min, with 50% activity remaining after 30 min at 100 ^{o}C. The peptide is completely inactivated after autoclaving (121 ^{o}C for 15 min) and after treatment with α-chymotrypsin, trypsin, pronase E, papain and proteinase K. Treatment with pepsin, lysozyme, catalase, lipase (type VII) and α- amylase also have no effect on the antibacterial activity of enterocin 226NWC.

A bacteriocin-producing strain *E. faecium* CRL 35 was isolated from regional Argentinean cheese (Tafi cheese) by Farias et al., (1994, 1999) and produces a

bacteriocin called Enterocin CRL35. The bacteriocin has a molecular weight of 3500Da and showed antimicrobial activity against food-borne pathogens like *S. aureus* and *L. monocytogenes*. Enterocin CRL 35 has also antiviral activity against thymidine-kinase (tk+) and deficient (tk-) strains of herpes simplex (HSV) type 1 and 2 in Vero and BHK-21 cells (Wachsman et al., 1999). The activity of Enterocin CRL 35 remains stable at extreme pH values, heat treatment, and different storage conditions, but that it showed sensitivity to protease enzymes.

Another bacteriocin producing enterococci strain (*E. faecium* CTC 492) was isolated from fermented Spanish sausages by Aymerich et al., (1996). The bacteriocin was named Enterocin A which belongs to class IIa bacteriocins, pediocin-like bacteriocin with a molecular weight of 4833 Da. The bacteriocin is active against *Listeria* spp., *E. faecalis* and *Pediococcus* spp.

The same year, Franz et al., (1996) isolated from black olives which an *E. faecium* BFE 900 strain which also produced a bacteriocin that named Enterocin 900. The bacteriocin showed inhibitory activity against *L. sakei*, *C. butyricum*, *C. perfringens* and *L. monocytogenes*. Enterocin 900 is heat-stable even at sterilization temperature (121 °C for 15 min), showed activity against a wide pH range and is inactivated by trypsin, proteinase K, α- chymotrypsin and pepsin but not by α-amylase, catalase or other non-proteolytic enzymes.

Casaus et al., (1997) observed that *E. faecium* strain T136 isolated from Spanish dry fermented sausages, manufactured with no added starter cultures, produced two bacteriocins, enterocin A, and a new bacteriocin termed enterocin B. Enterocin B is a small non-pediocin-like class IIc bacteriocin. The bacteriocin is secreted *sec*-independently and is a one-peptide bacteriocin lacking the YGNGVXaaC-motif. The bacteriocin's prepeptide includes a 53- residue sequence and a double-glycine leader sequence of 18 residues in the putative N-terminus. Enterocin B has a molecular weight of 5462.2 Da. The bacteriocin is heat stable and retained some activity after incubation at 100 ^{o}C for 20 min. Bacteriocin B are bactericidal and inhibit spoilage and foodborne pathogens such as *C. tyrobutyricum*, *C. sporogenes*, *Propionibacterium* spp., *L. monocytogenes*, and *S. aureus.*

In another study by Cintas et al., (1997) *E. faecium* P13 isolated from Spanish dry-fermented sausages, produces a bacteriocin which was named Enterocin P. Enterocin P

is an amphipatic, cationic pediocin-like peptide composed of 44 amino acids and has a molecular weight of 4493 Da. Enterocin P showed antimicrobial activity against *L. monocytogenes*, *S. aureus*, *C. perfringens*, *C. botulinum*, *E. faecalis*, *S. carnosus*, *C. sporogenes*, *C. tyrobutyricum*, and *Propionibacterium* spp. Enterocin P remains active when heated for 15 min at 121 °C and at extreme pH values and also its activity is not affected during freeze-thawing, lyophilization, and long term storage at −20 and 4 °C.

E. faecium 6T1 isolated from Spanish style fermented green olives produced a bacteriocin which was named Enterocin I (Floriano et al., 1998). Enterocin I showed antimicrobial activity against a range of food-borne and spoilage Gram-positive bacteria such as *E. faecalis, Bacillus* spp., *Clostridium* spp., *Listeria* spp., *Pediococcus* spp. and *Propionibacterium* spp. Enterocin I remains stable when heated for 5 min at 100 °C but was partially inactivated in autoclave temperature. Enterocin I consists of 44 amino acids and has a molecular size of 5 kDa.

E. faecium WHE 81 isolated from Munster cheese produced also an enterocin which was termed Enterocin 81 (Ennahar et al., 1998). The bacteriocin showed a narrow inhibitory spectrum and is mainly inhibited the growth of some enterococcal strains, *L. innocua, L. seeligerii* and *L. monocytogenes.*

Another two bacteriocin producing *E. faecium* strains AA13 and G16 have been isolated from a traditional typical Spanish dry-fermented sausage manufactured with no added starter cultures (Herranz et al., 1999). Both strains showed antimicrobial activity against *L. monocytogenes*, *S. aureus, C. perfringens* and *C. botulinum*, with *E. faecium* AA13 to show greater antimicrobial spectrum of activity. Both strains produced heat stable bacteriocins even at autoclave temperatures and remain active in a wide range of pH, from 2 to 11. However, both strains produced the known enterocin P.

Another already known bacteriocin, enterocin A, was produced by an *E. faecium* EFM01 from cheese which produced Enterocin A (Ennahar & Deschamps, 2000). Moreover, Enterocin A was also produced by *E. faecium* N15 strain isolated from nuka (Japanese rice-bran paste) (Losteinkit et al., 2001).

Moreno et al., (2002) isolated two bacteriogenic *E. faecium* strains B1 and B2 from a Malaysian mold-fermented product tempeh. Both strains producing enterocins with antimicrobial activity against *Carnobacterium divergens, E. faecalis, L. brevis, C.*

piscicola, L. pentosus and *Paralactobacillus selangorensis, L. monocytogenes, B. pumilus, Micrococcus luteus* and *L. innocua*. The bacteriocins were named Enterocin B1 and Enterocin B2, respectively. Both bacteriocins were lost their activity after treatment with α-amylase, proteinase K, α-chymotrypsin, trypsin and pepsin but were not affected by catalase, lysozyme and lipase and also when they heated at 121 °C for 20 min at alkaline pH.

In 2005, Achemchem et al., isolated a bacteriogenic strain *E. faecium* F58 from a soft farmhouse goat's cheese, Jben, made in Morocco which was made without adding starter cultures. The strain produced a bacteriocin which was called enterocin F58, and was active against several foodborne pathogens and spoilage bacteria such as *L. innocua, L. monocytogenes, S. aureus, B. subtilis, B. cereus, C. tyrobutyricum, C. perfringens* and *Brochothrix.*

Another bacteriocin-producing strain of *E. faecium* MMT21 isolated from Tunisian rigouta cheese by Ghrairi et al., (2008) which is also showed antimicrobial activity against *L. monocytogenes, S. aureus, E. faecalis* and *B. cereus*. Moreover the activity of the enterocin was stable when heated for 15 min at 100 °C and after incubation at pH values ranging from 2 to 10.

Javed et al., (2010) isolated an *E. faecium* IJ-31 from a butter sample which showed to produce a bacteriocin that is able to inhibit the growth of *L. monocytogenes, B. subtilis* and *B. cereus*. The enterocin retained its activity even after heating at 121 °C for 15 min and also remained stable to a wide range of pH values, from 4 to 10.

In a study carried out by Liu et al., (2011), *E. faecium* LM-2 isolated from "Byaslag", a traditional cheese in Inner Mongolia of China produced an enterocin LM-2 with a broad antimicrobial activity against the genera *Listeria, Staphylococcus, Bacillus, Clostridium, Salmonella* and *Pseudomonas.* The enterocin was remain stable at heat treatment and over a broad pH values. The strain was also produced enterocin P and L50.

Rivas et al., (2012) isolated from ewe's milk and cheese three bacteriocin producing *Enterococcus* strains (TW15, TW20 and TW22), that they showed antimicrobial activity against *L. monocytogenes, L. innocua* and *S. aureus*.

E. faecium AQ71 isolated from Azerbaijani Motal cheese showed antimicrobial activity against selected LAB, *L. monocytogenes* and *B. cereus* strains (Ahmadova et al., 2013).

Moreover, the strain showed also other probiotic properties such as resistant to physiological concentrations of bile salts and good auto-aggregation ability as well as co-aggregation ability with L. monocytogenes.

Enterocin T, a novel bacteriocin produced by *Enterococcus* sp. 812 isolated from fresh broccoli described by Chen et al., (2013). Enterocin T has a molecular mass of 4,521.3 Da, belongs into class IIa bacteriocins is heat stable and showed antimicrobial activity towards *L. monocytogenes* and some *Lactobacillus* spp. However, it had no inhibitory activity towards *E. coli* and *Vibrio vulnificus*.

In another study by Chang et al., (2013), a novel bacteriocin produced by *E. faecium* D081821, isolated from the traditional Taiwanese fermented food dochi (fermented black beans). Enterocin TW21 is a member of class IIa bacteriocin and shows great inhibitory ability against *L. monocytogenes*.

Enterococcus faecalis F4-9 isolated from Egyptian salted-fermented fish produces a novel bacteriocin, termed enterocin F4-9 (Maky et al., 2015). Enterocin F4-9 has a molecular mass 5,516.6 Da and showed antimicrobial activity against some Gram-positive food spoilage bacteria, including *Enterococcus* strains and *B. coagulans* and *E. coli* JM109.

Moreover in a study carried out by Gao et al., (2016), *E. faecalis* L11 isolated from Chinese traditional fermented cucumber, produced a bacteriocin termed enterocin L11. It showed a wide range of antimicrobial activity and is mainly active against Gram-positive bacteria, including *B. subtilis, S. aureus, L. monocytogenes, Sarcinaflava*, L. *acidophilus, L. plantarum, L. delbrueckii subsp. delbrueckii, L. delbrueckii subsp. bulgaricus* and *S. thermophilus*, but also some Gram-negative bacteria including *Salmonella typhimurium, E. coli* and *Shigella flexneri*.

A novel enterocin (enterocin T1) was also produced *E. faecium* T1 isolated from Chinese Tibet cheese (Liu et al., 2016). It has a molecular weight of 4629 Da. Enterocin T1 was stable at 80–100 °C and over a wide pH range, pH 3 to 10. Enterocin T1 showed antimicrobial activity against a range of Gram-negative and Gram-positive bacteria including *P. putida, P. aeruginosa, P. fluorescens, E. coli, Salmonella typhimurium, Shigella flexneri, Shigella sonnei, S. aureus, L. monocytogenes*.

2.4.13 Application of bacteriocins

Several bacteriocins especially LAB bacteriocins offer potential applications in food preservation. Some of the benefits regarding the use of bacteriocins in food include an extended shelf life of foods and therefore reduce economic losses due to food spoilage, provide extra protection during temperature abuse conditions, permit the application of less severe heat treatments without compromising food safety: better preservation of food nutrients and vitamins, as well as organoleptic properties of foods, and satisfy consumers demands for minimally-processed and fresher foods without chemical preservatives (Gálvez et al., 2007).

According to Schillinger et al., (1996), the most common approaches for the application of bacteriocins in food biopreservation are:

- Inoculation of food with LAB (protective culture) that produce bacteriocin in the products (*in situ* production). Therefore, the ability of the LAB to grow and produce bacteriocin in the products is crucial for its successful use.
- Addition of purified or semi-purified bacteriocins as food preservatives.
- Use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing.

However, before a bacteriocin is considered for food application should meet some of the following safety and functional requirements (Schillinger et al., 1996). Firstly, the bacteriocin producing strain should preferably have QPS (qualified presumption of safety) status, should be heat stable, active against pathogenic or spoilage bacteria, effective in the food vehicle and also should not pose any health risks such as virulence and antibiotic resistance. Another important factor to consider will be the economic aspects or cost of using a bacteriocin in foods (Javed, 2009). One way to reduce the cost is to determine the optimum parameters for the production of a bacteriocin. For economical use in food, the bacteriocins have to be produced in large amounts and preferably by growing the strains in media containing food grade ingredients (Yang & Ray, 1994).

For in situ bacteriocin production, bacteriocinogenic strains can be used as direct starter cultures or as co-cultures in combination with a starter culture. Foods can also be

supplemented with bacteriocins ex situ produced that can be added in the form of raw concentrates obtained by cultivation of the producer strain in a food-grade substrate (such as milk or whey). However, by incorporating purified bacteriocins to the food and beverages might be not attractive to the industry, since in this form bacteriocins may have to be labelled as an additive like other preservatives and regulatory approval might be necessary (Deegan et al., 2006).

Both the chemical composition and the physical conditions of food can affect the efficacy of bacteriocins in food systems. Some of the factors include pH, cell concentration, lipid content, proteolytic enzymes, limited diffusion in solid matrixes, temperature, narrow spectrum of antimicrobial activity as most of them are not active against Gram-negative bacteria, lower levels of production and bacteriocin-resistant bacteria (Abee et al., 1995; Devlieghere et al., 2004). Therefore, in order to increase the effectiveness of bacteriocins as biopreservatives, one way is to combine them with other antimicrobial hurdles such as organic acids, chelating agents or essential oils (Deegan et al., 2006). Moreover, combination of non-thermal treatments such as HHP and pulsed electric field (PEF) with bacteriocins is also an economical and effective way to increase the shelf life of the food and also to extend the spectrum of antimicrobial activity to Gram-negative microorganisms which are protected by the presence of an outer cell membrane (O'Connor et al., 2015).

Nisin is the best studied bacteriocin that is being commercially produced and used. It is a permitted food additive (E234) in more than 50 countries including the US and Europe. It is produced by the fermentation of milk using *Lactococcus lactis*, and is sold as a commercial preparation under the trade name of Nisaplin by Aplin and Barret Ltd. (U.K.) (Gharsallaoui et al., 2016). It has been approved by the World Health Organization for use in foods since 1968, but it has been gained approval from the U.S. Food and Drug Administration since 1988 (Delves-Broughton et al., 1996). The primary applications for Nisaplin® include dairy products (processed cheese and cheese spreads, direct acidified cheeses, pasteurised dairy desserts and fresh and recombined milk), liquid egg, dressings and sauces, high moisture/reduced fat foods (baby foods), canned foods, crumpets and the processing of fermentation products (Gharsallaoui et al., 2016). Nisaplin® is manufactured by a controlled fermentation of *Lactococcus lactis* which

produces nisin. Nisaplin® inhibits a broad range of Gram-positive bacteria, including clostridia, *Bacillus*, *Listeria* and lactic acid bacteria (Gharsallaoui et al., 2016).

2.4.13.1 Application of bacteriocins in dairy products

The most important food borne pathogens in the dairy industry are those which are capable to survive and grow in raw materials as well as the manufacturing and ripening process of some cheeses. The main pathogenic bacteria of concern are *L. monocytogenes*, *S. aureus*, *E. coli*, and *Salmonella spp*. (Oliver et al., 2005; Papademas & Aspri, 2014). Moreover spoilage of cheeses such as Emmental and Gouda due to gas formation by *C. tyrobutyricum* is also of great economical concern (Galvez et al., 2008). Therefore, most of the applications of bacteriocins in milk and dairy products were focused on the control/ inhibition of *Listeria* or *Clostridium.* Figure 13 shows the most important applications of bacteriocins in dairy products

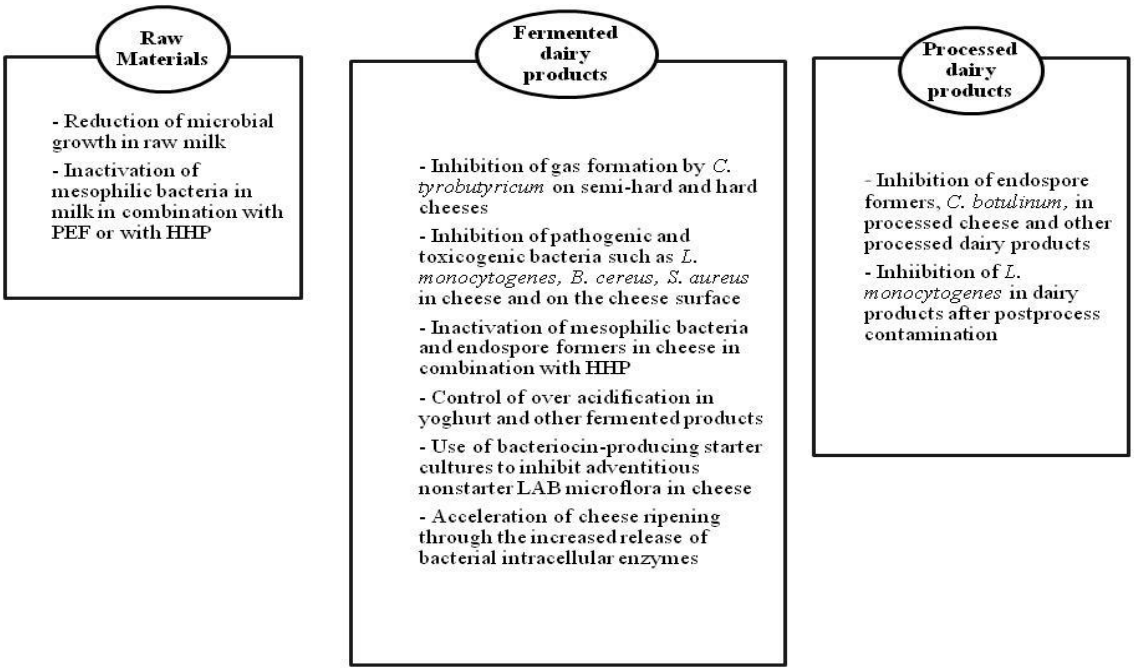


Figure 13: Examples of application of bacteriocins in dairy products

Nisin or Nisaplin is one of the first bacteriocins that have been used commercially and is used widely in the dairy industry. The earliest application of nisin in dairy products was to prevent the spoilage of *C. tyrobutyricum* which is responsible for gas blowing in cheese (Delves-Broughton et al., 1996). Other applications of nisin in dairy products include processed cheese products (block cheese, and spread cheeses), cheese products (cottage cheese, Camembert), in order to prevent proliferation of surviving endospore

bacteria such as *C. botulinum*, or to prevent the growth of post-process contaminating bacteria such as *L. monocytogenes* (Delves- Broughton et al., 1996; Sobrino-Lopez & Martin-Belloso, 2008). Moreover, nisin has also found successful applications in many other pasteurized products such as chilled desserts, flavored milk, clotted cream or canned evaporated milks (Galvez et al., 2008). Nisin was also used in combination with other hurdle technologies. For example the combination of nisin with high hydrostatic pressure (HHP), strongly reduced the levels of bacteria associated with milk spoilage such as *E. coli, S. aureus, L. innocua, L. monocytogenes* (Garcıa-Graells et al., 1999) and is also useful for the inactivation of endospores and mesophilic bacteria in cheese (Lopez-Pedemonte et al., 2003). Moreover, in skim milk, whey, or simulated milk ultrafiltrate media, nisin antimicrobial activity was increased when it combined with pulsed electric fields (PEFs) against several bacteria such as *L. monocytogenes, S. aureus, B. cereus,* and *E. coli* (Galvez et al., 2008). Furthermore, nisin immobilized in polyethylene/polyamide packaging or in sodium caseinate films was also shown to reduce the population of LAB, *Listeria* and *S. aureus* in cheeses (Cao-Hoang et al., 2010; Scannell et al., 2000). However, the application of nisin in cheese fermentation may interfere with growth of starter cultures, and have detrimental effects on acidification and/or aroma formation. Therefore, in order to protect starter cultures from the detrimental action of nisin during cheese production and also to enhance nisin stability, nisin Z encapsulated into liposomes was successfully tested and found to inhibit *Listeria* in cheddar cheese (Benech et al., 2002; 2003).

Lacticin 3147 is another lactococcal bacteriocin with a high potential for application in the preservation of dairy products (Sobrino-Lopez & Martin-Belloso, 2008). Lacticin 3147 was isolated from an Irish kefir grain used for making buttermilk; *L. lactis* subsp. lactis DPC3147 (Ross et al., 1999; Ryan et al., 1996). Addition of lacticin 3147 powder in infant milk formulation, natural yoghurts and cottage cheesed was shown to rapidly inactivate *L. monocytogenes* and reduce the viable cell numbers of *S. aureus* (Morgan et al., 2001). Apart from the addition of lacticin 3147 as a powder, the application of a lacticin 3147 producing strain can also reduce the levels of *Listeria* or to inhibit the growth of clostridia on smear ripened cheese (O'Sullivan et al., 2006; Martinez-Cuesta et al., 2010). As with other bacteriocins, lacticin 3147 can also be used in combination with hurdle technologies. In a study by Morgan et al., 2000 showed that combination of

lacticin 3147 with HHP increased the antimicrobial activity against *L. monocytogenes* and *S. aureus* in milk and whey (Morgan et al., 2000).

Although pediocins have been tested mainly in meat products, pediocin applications in dairy products is very limited due to given the poor adaptation of *Pediococcus* to dairy substrates (Galvez et al., 2008). Some successful applications of pediocins in dairy products include milk and cheese products in order to control the growth of *L. monocytogenes* (Rodriquez et al., 2002).

Other bacteriocins of interest in the preservation of dairy products are the propionicins. Microgard is approved in certain countries for commercial use as an ingredient mainly in dairy products such as cottage cheese and yogurt (Galvez et al., 2008). Propionicin PLG-1 is a bacteriocin produced by *Propionibacterium* thoenii P127 was shown to kill or inhibit several psychrotrophic spoilage or pathogenic bacteria in milk such *L. monocytogenes, Yersinia enterocolitica,* and *Corynebacterium* spp. (Lyon et al., 1993). Jenseniin G is another bacteriocin produced by the dairy *Propionibacterium jensenii (thoenii)* P126. According to Weinbrenner et al., (1997) jenseniin G can be applied on dairy products especially yoghurt to prevent over acidification or post acidification in order to decrease the sour taste because of its inhibitory activity to *Lactobacillus delbrueckii ssp. bulgaricus*.

Moreover, bacteriocins produced by thermophilic *streptococci* are also found applications in dairy products for the control of *Listeria* or to inhibit the growth *C. tyrobutyricum* (Galvez et al., 2008). One last example for application of bacteriocins to control food borne pathogens and spoilage bacteria in dairy foods is variacin. For example application variacin in the form of a milk-based ingredient inhibited the growth of *B. cereus* in chilled dairy products, vanilla, and chocolate desserts in a concentration-dependent way (O'Mahony et al., 2001).

2.4.13.2 Examples of application of enterocins in food products

Currently there are investigations to exploit the food preservative properties of many other bacteriocins such as enterocins. Enterococci are common in dairy foods (especially in traditional fermented products such as cheeses). Most of the bacteriocinogenic enterococci can grow well in milk and cheese products and therefore produce bacteriocins (Gálvez et al., 2008). This property makes them good candidates

as adjunct cultures for protection against foodborne pathogens. Most of the enterocins have anti-listeria properties and have a wider spectrum of activity than other bacteriocins (nisin and pediocin). Many studies have demonstrated the application of enterocins in dairy products.

An early study by Parente and Hill, 1992, showed the bacteriocin from *E. faecium* DPC1146 had a rapid bactericidal effect on *L. monocytogenes* in milk. In another study by Giraffa et al., (1995), *E. faecium* 7C5 strain along with a thermophilic starter, composed of *S. thermophilus* and *L. delbrueckii subsp. bulgaricus* control the growth of *L. inoccua* in Taleggio cheese. Then, Nunez et al., (1997) demonstrated that *E. faecalis* INIA 4 is able to produce enterocin 4 and was successfully control the growth of *L. monocytogenes* during the manufacture and storage of Mexican Manchego cheese. Furthermore, an increase in enterocin activity was recorded in the cheese during the first week.

Lauková and colleagues also demonstrated the effectiveness of enterocin CCM 4231 against various pathogens in selected food products especially dairy products. Addition of enterocin CCM 4231 at a concentration of 3200 AU/ml in soy milk, inhibit completely the growth of *L. monocytogenes* Ohio was observed after 24h, but only partial inhibition of *S. aureus* was observed (Lauková & Czikková, 1999). However, the addition of enterocin in Sunar® milk (milk nourishment for suckling babies), reduced *S. aureus* SA1 by three-fold after one day of incubation (Lauková et al., 1999). In the same study enterocin CCM 4231 was added into a yoghurt product, whereas a significant inhibition of the SA1 strain was observed after 3.5h of addition and also decreased the viable cells of *L. monocytogenes* Ohio strain (Lauková et al., 1999). Furthermore, enterocin CCM 4231 was added during Saint-Paulin cheese preparation and also was found to inhibit the growth of *Listeria* without affecting the traditional fermentation and ripening process (Lauková et al., 2001). The antilisteria activity of enterocin CCM 4231 was also tested in "Bryndza" a traditional Slovak dairy cheese prepared from sheep milk to control the growth of *L. innocua* (artificially inoculated) and *E. coli, S. aureus* and *enterococci* which are present as natural contaminants. Immediately after enterocin addition the counts of the *L. innocua* were reduced. Furthermore *enterococci* and *staphylococci* were totally eliminated and *E. coli* counts reduced by 1 fold (Lauková and Czikková, 2001).

Farıas et al., (1999), also demonstrated that addition of enterocin CRL35 as additive during goat cheese making reduced the growth of Listeria during the whole period of ripening without affecting the cheese quality.

The bacteriocinogenic strain *E. faecium* FAIR-E 198 isolated from Greek feta cheese failed to produce bacteriocin when used as an adjunct starter for Feta cheese production (Sarantinopoulos et al., 2002). In contrast to *E. faecium* FAR-E 198, *E. faecium* DPC 1146 and *E. faecium* RZS C5 were able to produce bacteriocions and be effective in the control of *Listeria* during cheddar cheese manufacture (Moreno et al., 2003).

In another study, in a nonfat hard cheese, addition of *E. faecalis* A-48-32 inhibits the growth of *B. cereus* (Munoz et al., 2004). Addition of the same strain in skim milk also inhibits the growth of *B. cereus* and *S. aureus.* However the addition of the strain in cheese, inhibit the growth of *S. aureus* but to a lesser extent (Munoz et al., 2007).

Another example of a bacteriocin producing enterococci is *E. faecium* F58 that has been isolated from goat's milk. It used as starter for the preparation of Jben, a Moroccan fresh cheese which was successfully reduced completely the growth of *L. monocytogenes* (Achemchem et al., 2006).

In a study by Liu et al., (2008), the enterocin A producing ability was transferred to a *Lactococcus lactis* MG1614 strain by introducing the plasmid pEnt02. Then the strain used as a co-culture along with a parent L. lactisENT− strain for the preparation of cottage cheese in order to control the growth of *L. monocytogenes*. The results of the study showed that *L. monocytogenes* levels dropped below detectable limits within 2 days in the samples prepared with the L. lactisENT+ strain, while in control cheese prepared without the enterocin A producing strain L. monocytogenes could still be detected even after 10 days.

An *E. faecium* WHE 81 strain which produces enterocins A and B, along with two other class IIa bacteriocins was able to completely inhibit the growth of *L. monocytogenes* in Munster cheese, a red surface ripened soft cheese with no effect on the ripening flora or pigmented bacteria (Izquierdo et al., 2009).

In another study by Pingitore et al., (2012) two cheese isolated enterococci *E. muntii* CRL 35 and *E. faecium* ST88Ch tested for their capability to control growth of *L. monocytogenes* 426 in experimentally contaminated fresh Minas cheese during

refrigerated storage. The results of the study showed that both *enterococci* can effectively control the growth of *L. monocytogenes* in fresh Mina's cheese during refrigerated storage. However *E. mundtii* CRL35 presented a more evident inhibitory effect than *E. faecium* ST88Ch.

In a recent study carried out by Cardenas et al., 2016, *E. faecium* CECT 8849, inoculated in fermented milk and cheese, showed a reduction in *Listeria* growth.

The results of these studies demonstrated the efficacy of enterocins in dairy foods in order to control *Listeria*, *Staphylococcus* and other important food borne pathogens. Some of the examples that demonstrated the successful applications of bacteriocins as food preservatives are listed in Table 14.

Table 14: Enterocins application in dairy products

Producing strain	Enterocin produced	Product tested	Typical target organisms	References
E. faecium DPC1146	Enterocin DPC 1146	Milk	*L. monocytogenes*	Parente and Hill, 1992
E. faecium 7C5	Undefined bacteriocin	Taleggio (Italian soft smear cheese)	*L. innocua*	Giraffa et al., 1995
E. faecalis INIA 4	Enterocin 4	Manchego cheese	*L. monocytogenes*	Nunez et al., 1995
E. faecium CCM 4231	Enterocin CCM 4231	Soy milk, skimmed milk, yoghurt, Bryndza (traditional Slovak dairy product), Saint- Paulin cheese, Sunar	*S. aureus, L. monocytogenes, L. innocua, E. coli*	Laukova et al., (1999;2001)
E. faecium CRL 35	Enterocin CRL 35	Goat cheese	*L. monocytogenes*	Farias et al., 1999
E. faecium FAIR-E 198	Enterocin A and P	Feta cheese	*L. monocytogenes*	Sarantinopoulos et al., 2003
E. faecium RZS C5	Enterocin RZS C5	Cheddar cheese	*L. monocytogenes*	Moreno et al., 2003
E. faecium DPC 1146	Enterocin DPC 1146	Cheddar cheese	*L. monocytogenes*	Moreno et al., 2003
E. faecalis A-48-32	Enterocin AS-48	Non-fat hard cheese	*B. cereus*	Munoz et al., 2004
E. faecium F58	Enterocin L50A and B	Goat's milk and Jben	*L. monocytogenes*	Achemchem et al., 2006
E. faecalis A-48-32	Enterocin AS-48	Skimmed milk and non-fat unripened soft cheese	*S. aureus*	Munoz et al., 2007
Lactococcus lactis MG1614	Enterocin A	Cottage cheese	*L. monocytogenes*	Liu et al., 2008
E. faecium WHE 81	Enterocin A and B	Munster cheese	*L. monocytogenes*	Izquierdo et al., 2009
E. muntii CRL 35 and *E. faecium* ST88Ch	Enterocin CRL 35, Enterocin ST88Ch	Minas cheese	*L. monocytogenes*	Pingitore et al., 2012
E. faecium CECT 8849	Enterocin CECT 8849	Fermented milk and cheese	*L. monocytogenes*	Cardenas et al., 2016

2.4.14 Bacteriocin bioengineering

The gene encoded and ribosomal synthesized nature of bacteriocins, offers them potentially significant advantages for the implementation of modern cutting-edge bioengineering strategies in order to alter their biological, chemical and physical properties (Balciunas et al., 2013). Different strategies have been employed in order to mutate specific amino acid in lantibiotics. One strategy is the introduction and expression of additional copies of genes encoding bacteriocin biosynthes/production and regulation to facilitate its overproduction in a strain (Balciunas et al., 2013). Another strategy to improve bacteriocin producing strains is to conjugate multiple large bacteriocin-encoding plasmids into a single strain while maintaining the food grade status of the recipient strain. This offers the advantage to the strain to kill the undesired bacteria more effectively than the wild type (Field et al., 2010; O'Sullivan et al., 2003). It is also possible to achieve this goal through the amplification and cloning of lantibiotic-encoding genes into shuttle vectors and heterologous production in other strains (O'Shea et al., 2013). The development of heterologous expression systems for bacteriocins offers a number of advantages over the natural host system (Marti et al., 2003). These include the possibility of achieve higher levels of bacteriocin production, broadened inhibitory spectrum and increased efficacy with certain ecosystems since the effectiveness of bacteriocins can be limited by a range of factors, such as a narrow activity spectrum, spontaneous loss of bacteriocinogenicity, poor adaptation of the natural host to food environments, deleterious sensorial effects caused by growth of the natural host in foods and/or the emergence of bacteriocin-resistant bacteria. Moreover heterologous production of LAB bacteriocins opens up the possibility of expressing antimicrobial properties to strains of technological interests (starter cultures) or probiotic cultures (Marti et al., 2003).

2.4.15 Bacteriocins contribution to probiotic functionality

Over the last few years there has been growing evidence that bacteriocin production confers a number of advantages on probiotic strains. Bacteriocin production may contribute to probiotic functionality in different ways. Firstly, the ability of a strain to produce bacteriocins may help the strain to establish itself in a new niche and also survive in the gut environment. Secondly, bacteriocins can act as inhibitor competitors

and therefore inhibit the growth of pathogens and alter the composition of the gut microbiota. Finally, bacteriocins can act as signaling peptides through quorum sensing in the intestinal environment and therefore involve in the modulation of the immune system. Detailed reviews of the role of bacteriocins in probiotic functionality haves been published by Dobson et al., (2012); Gillor et al., (2008) and Hegarty et al., (2016).

2.5 Milk Bioactive Peptides

2.5.1 Introduction

Nutraceuticals, a term combines two "nutrition" and "pharmaceutical", is a food or a dietary supplement that provides medical or health benefits including the prevention and treatment of diseases (Penner et al., 2005).

A functional food essentially provides a health benefit beyond the basic nutrition, whereas nutraceutical is to describe an isolated or purified and concentrated molecular extract from food that has bioactive compounds (Pinto et al., 2012).. Milk has been the most widely consumed liquid food source for humans since ancient times. Milk is a complex medium containing a variety of nutrients, minerals and vitamins as well as other molecules with functional or bioactive properties. Milk is especially rich in proteins that are naturally divided into two classes: 1) the major milk proteins which include caseins (α, β and κ caseins) and two whey proteins (α-lactalbumin and β-lactalbumin), and 2) minor milk proteins including lysozyme, lactoferrin, lactoperoxidase and immunoglobulins (Madureira et al., 2007; Benkerroum, 2008). Milk is considered to be a complete functional food, devised by nature for the protection and development of a newborn mammal (Donnet-Hughes et al., 2000). Among the numerous nutritional and health benefits of milk consumption, milk proteins have gained an enormous interest for being a 'complete' protein as they provide all nine essential amino acids (leucine, isoleucine, valine, phenylalanine, tryptophan, histidine, threonine, methionine, lysine) required by humans. Even though dietary proteins are known to pose wide range of nutritional and functional properties for a long time, the last few decades, there is an increasing interest in the research of food-derived bioactive peptides, especially on those from milk proteins. The first report for milk bioactives peptides is by Mellander in 1950; when he discovered that milk casein derived phosphorylated peptides enhance vitamin D-independent bone calcification in rachitic

infants (Bhat et al., 2015b). Since then, numerous bioactive peptides have been identified from different food sources such as milk and dairy products, meat, fish and plants.

Bioactive peptides are defined as 'specific protein fragments that have a positive impact on body functions and conditions and may ultimately influence health (Urista et al., 2011). Among the numerous bioactive peptides isolated up to now, increasing interest is focused on milk-derived bioactive peptides.

Bioactive peptides usually contain 3 to 20 amino acid residues per molecule (Haque et al., 2008). Bioactive peptides are inactive or latent within the sequence of the parent protein, and can be released in three ways: a) through digestion/hydrolysis with commercial proteolytic enzymes, b) during gastrointestinal digestion through the action of digestive enzymes or of the microbial enzymes of the intestinal flora and c) during food processing such as milk fermentation with proteolytic starter/adjunct cultures (Korhonen & Pihlanto, 2006). Two factors can influence the type of the generated bioactive peptide from a particular protein: the primary sequence of the protein source and the specificity of the enzyme(s) used to generate the peptides (Harnedy & FitzGerald, 2012). Once bioactive peptides are liberated, may be absorbed via the intestine and then enter the blood circulatory system intact, where they exert physiological (systemic) effects, or they may produce local effects in the digestive tract (Erdmann et al, 2008). Upon oral administration, bioactive peptides may affect the major body systems such as the cardiovascular, digestive, immune and nervous systems, also can reduced the risk of various lifestyle related diseases (hypertension) (Korhonen & Pihlanto, 2006). A wide range of activities has been described for bioactive peptides such as lowering blood pressure, opioid, antimicrobial, mineral binding, antioxidant, antithrombotic, immune-modulatory and anticancer effects and also many of them pose multifunctional properties as they can exert more than one biological effect (Meisel, 1997).

This section will focus on bioactive peptides derived from milk proteins, with emphasis on their production, occurrence in fermented dairy products, and potential health benefits.

2.5.2 Methods for the production of bioactive peptides

Milk bioactive peptides can be produced in the following ways: (a) enzymatic hydrolysis by digestive enzymes such as pepsin, trypsin (b) fermentation of milk with proteolytic starter cultures, (c) proteolysis by enzymes derived from microorganisms or plants.

2.5.2.1 Gastrointestinal digestion and enzymatic hydrolysis

Bioactive peptides may be released *in vivo* during gastrointestinal digestion. These bioactive peptides are mostly the result of the degradation of casein due to the action of digestive enzymes such as pepsin, trypsin or chymotrypsin.

Enzymatic hydrolysis is one of the most common, fastest and safest processes for the production of bioactive peptides from food products (de Castro & Sato, 2015). Some studies have conducted hydrolysis with single digestive proteases or combinations of pepsin, trypsin, and chymotrypsin. Moreover, proteolytic enzymes isolated from LAB have been successfully employed to release bioactive peptides from milk proteins such Flavourzyme derived from *Aspergillus oryzae*, proteinase K, from *Tritirachium album* and Actinase-E , derived from *Actinomyces* ssp.

2.5.2.2 Food processing-fermentation

The process of fermentation carried out by microorganisms has been known and used in dairy production for many thousands of years in order to extend the consuming period of milk (Szwajkowska et al., 2011). Therefore, fermentation of milk proteins with proteolytic starter culture is another method for the production of protein hydrolysates. During microbial fermentation, milk proteins are broken down to bioactive peptides through the action of bacterial proteases and peptidases. An important factor in milk fermentation that affects the nature of the synthesized peptide is the type of the starter/ adjunct bacterial culture. The most common bacterial cultures used to generate milk derived peptides include bacterial cultures of *Lactobacilli* spp., *Lactococci* spp and *Enterococci* spp. (Fitzgerald and Murray, 2006).

An alternative strategy used by food industry in order to increase the bioactivity and content of the generated peptides is to ferment milk with a proteolytic starter LAB culture along with food grade enzyme (Hafeez et al., 2014). For example, fermentation

of fresh low-fat milk with a commercial starter culture mixture of five LAB accompanied by hydrolysis with microbial protease (prozyme 6) enhanced the ACE-inhibitory activity of the whey fraction (Chen et al., 2007). Flavourzyme, derived from *Aspergillus oryzae*, is another food grade protease used to facilitate milk fermentation in combination with LAB starter cultures. Addition of Flavourzyme also increases the peptide concentration in the fermented products (Ahtesh et al., 2016; Pihlanto et al., 2010; Shi et al., 2016).

2.5.3 Bioactivities investigated in the present study

The main health related properties described for milk-derived peptides includes opiate, antithrombotic, antihypertensive, immunomodulating, antioxidative, antimicrobial, anticancer, mineral carrying and growth-promoting properties (Figure 14). However, this study focuses on the three bioactivities: radical scavenging activity, angiotensin I-converting inhibition and antimicrobial activity, which will be described in detail in the following section.

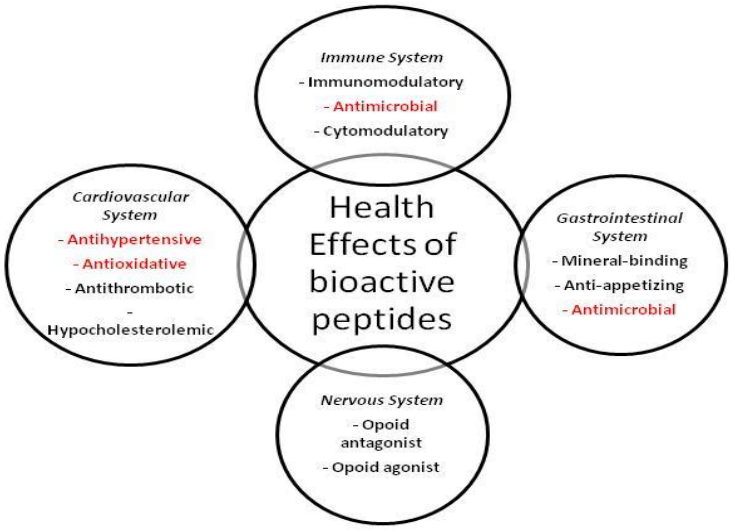


Figure 14: Health benefits of bioactive peptides

2.5.3.1 Antimicrobial peptides

Resistance to clinically important antibiotics has become a major health problem and cause serious problem in the treatment of infections, especially in immunocompromised

individuals. The indiscriminate and excess use of antimicrobial drugs and also the genetic mutation capacity of the bacteria are the most important factors in the rise of antibiotic resistance in microorganisms (de Castro et al., 2015). This clearly highlights the need for new natural's sources of antimicrobial compounds which has enormous potential because they have characteristics such as low toxicity and high specificity. Peptides with antimicrobial properties are widely distributed in nature and are essential to the immune system. They are playing a vital role in the first line of defense against colonization by exogenous microbial pathogens, and they play a fundamental role in regulating bacterial populations on the mucosa and other epithelial surfaces (de Castro et al., 2015). Most antimicrobial peptides are short amino acid chains and are composed mainly of cationic and hydrophobic amino acids (Dziuba & Dziuba, 2014). Milk is a rich natural source of antimicrobial proteins and peptides, capable of exerting antimicrobial activities comparable to antibiotics (Lopez-Exposito & Recio, 2008). Antimicrobial milk derived peptides have the advantage due to their precise infected cell targeting, broad spectrum nature, safe and economical source with vast industrial potential (Agyei & Danquah, 2011).

The first discovery of a milk-derived antimicrobial peptide was made by Jones and Simms 1930. The antibacterial factor was called lactenin resulted from treatment of milk with rennet and it showed antibacterial activity against scarlet fever *Streptococcus* (Jones & Simms 1930). The total antibacterial effect in milk is greater than the sum of the individual contributions of immunoglobulin and non-immunoglobulin defense proteins such as lactoferrin, lactoperoxidase, lysozyme, and (Brumini et al., 2016). This may be due to the synergistic activity of naturally occurring proteins and peptides in addition to peptides generated from inactive protein precursors (Brumini et al., 2016).

Lysozyme, whose content is particularly rich in the milk of humans and donkeys, catalyzes the breakdown of peptidoglycan polymers of bacterial cell wall, resulting in the lysis of the sensitive bacteria (Benkerroum, 2008).

Lactoperoxidase is a heme-containing glycoprotein and is one of the most abundant and heat stable enzymes in many mammalian milk systems (Seifu et al., 2005).It catalyzes the oxidation of thiocyanate and some halides (I-, Br- but not Cl-) by hydrogen peroxide to generate antimicrobial products kill or inhibit the growth of many bacteria species by

oxidizing the cell membrane of microorganisms, which results in a loss of structure and leads to cell lysis and death (Boots & Floris, 2006).

Lactoferrin (LF) is a well-characterized iron-binding antimicrobial whey glycoprotein discovered in 1939, capable of binding two molecules of Fe^{3+} per protein molecule, which belongs to transferrin family (González-Chávez et al., 2009). LF exhibits both bacteriostatic and bactericidal activity against a range of Gram-positive (*L. monocytogenes, S. aureus)* and Gram-negative (*E. coli* O157:H7, *Salmonella Typhimurium*) bacteria as well as viruses (RNA and DNA, enveloped or non-enveloped viruses) and fungi (*Rhodotorularubra)* by a number of different mechanisms (Séverin et al., 2005). Originally the antimicrobial activity is due to its ability chelate iron thereby depriving potential pathogens of this essential nutrient. Further studies showed that it may act as a more general chelator, releasing lipopolysaccharides (LPS) from the outer membrane of gram negative bacteria with an associated increase in membrane permeability which leads to the death of microorganism (Ellison & Giehl, 1991).

2.5.3.1.1 Mechanism of action

The action of antimicrobial peptides derived from milk proteins is likely to be membrane-lytic, with specificity for prokaryotic cell membranes. The mode of action of antimicrobial peptides has been extensively investigated and it has been shown that an amphiphilic, mostly α-helical formation, and an overall net positive charge is proposed to initiate the interaction with the bacterial surface and the peptides are believed to facilitate the peptide to enter the membrane interior (Floris et al., 2003; Gobbetti et al., 2004). The antimicrobial activity of these peptides is believed to result from the severe disruption of microbial membranes, which leads to ion and metabolite leakage, depolarization, disruption of membrane coupled respiration and finally cell death (Hayes et al., 2007). The first step in this interaction is the initial attraction between the antibacterial peptide and the target cell membrane. In the case of the Gram-negative bacteria insertion of the peptides into the outer membrane structure by hydrophobic interactions leads to a disturbance on the outer membrane and permeabilizes this membrane to other peptide molecules. As a result, peptides arrive at the cytoplasmic membrane in a process driven by electrostatic and hydrophobic interactions (Floris et al., 2003).

Many antimicrobial peptides have been derived from a variety of milk proteins including α-lactoglobulin, αs1-casein, α-lactalbumin and κ-casein and several inhibit Gram-positive and Gram- negative microorganisms. A detailed review on antimicrobial peptides derived from milk has been published recently by Mohanty et al., (2016). Examples of antimicrobial peptides derived from milk proteins presented in APPENDIX II.

2.5.3.1.2 Examples of casein and whey derived antimicrobial peptides

The first discovery of a milk-derived antimicrobial peptide was made by Jones and Simms 1930. Lactenin resulted from treatment of milk with rennet and showed antimicrobial activity against streptococci (Jones & Simms 1930). Later, a group of basic, glycosylated and high-molecular weight milk derived polypeptides termed casecidins were identified following heated and rennin-treated casein (Lahov & Regelson 1996). These peptides showed antimicrobial activity against *S. aureus, B. subtilis, Diplococcus pneumonia* and *Streptococcus pyogenes* (Hayes et al., 2007; Lahov et al., 1996).

Isracidin is another antimicrobial peptide identified into the sequence of bovine as1-casein (Benkerroum, 2010). Isracidin is derived from αs1-casein by chymosin action and corresponded to the N-terminal fragment, as1-casein f(1–23) (Hayes et al., 2007). This peptide found to inhibit the *in vitro* growth of lactobacilli and other Gram –positive bacteria such as *S. aureus* and *L. monocytogenes*, but only at high concentrations (Lopez-Exposito et al., 2006). In another study, Hayes et al. (2006) studied the production of three peptides generated by *Lactobacillus acidophilus* DPC6026 fermentation of bovine αs1-casein (Caseicin A, B and C) which have common features with isracidin and they were active against emerging food borne pathogens such *Escherichia coli* O157:H7, *Enterobacter sakazakii* and *S. aureus,* suggesting the possible bio-protective applications of these antimicrobial peptides in infant milk formula. Moreover, isracidin may be useful to protect the udder of sheep and cow against mastitis (Lahov & Regelson, 1996).

Another cationic peptide released by hydrolysis of the bovine αs1-casein with pepsin and corresponds to amino acid residues (f99–109) of bovine as1-casein has been isolated and identified by McCann et al., (2006). This peptide has shown a broad

spectrum of activity against Gram-positive and Gram- negative bacteria such as *Salmonella typhimurium*, *E. coli*, *Salmonella enteritidis* and *Citrobacter freundii*, *B.subtilis* and *L. innocua* (McCann et al., 2006). As this peptide was derived from digestion of bovine casein with pepsin, it might be released in the stomach and therefore can potentially provide some protection against microbial infection in the gastrointestinal tract (Lopez-Exposito and Recio, 2008).

The first described antimicrobial peptide in the sequence of αs2-casein is known as casocidin-I. It is a 39 amino-acid fragment, corresponding to amino acid residues 165–203 and can inhibit the growth of *E. coli* and *S. carnosus*, *B. subtilis*, *S. epidermidis*, *E. faecium*,and *Rhodotorula rubra* (Zucht et al., 1995). It is a heat and acid stable peptide isolated from boiled and acidified milk followed by different treatments to remove fat and most of the remaining high molecular weight proteins (Benkerroum, 2010) . Zucht et al., (1995) suggested the use of casocidin-I in infant formulae to provide infants with antibacterial peptides that can be influence the composition of the intestinal microflora. Moreover, it can be used in the treatment of various dermatitis and mucosal infections of different origins (bacteria, fungi or parasites) as well as diarrheic gastroenteritis (Benkerroum, 2010). In addition, pepsin digestion of bovine as2-casein rendered two potent antibacterial peptides, f(164-179), which is part of the previously identified casocidin-I, and another one at the C-terminus of the protein, f(183e207) (Recio & Visser, 1999). Both fragments showed an important antimicrobial activity against Gram-positive and Gram negative bacteria such as *E. coli*, *B. cereus* and *Streptococcus thermophilus*. Furthermore, McCann et al., (2005) have also identified five antibacterial peptides derived from chymosin digestion of bovine αs2-casein. The four of these antimicrobial peptides had not been reported earlier. However, the other one matched exactly one of the peptides previously isolated from the pepsin hydrolysate of bovine as2-casein and considered to be the fragment containing the active domain f(183- 207) (Recio and Visser 1999). They were active against a wide variety of Gram-positive and also Gram negative bacteria such as *L. monocytogenes, L. innocua, B. subtilis, Salmonella typhimurium, Salmonella enteridis* and *E. coli* (McCann et al., 2005). Three of these pepides (f (181- 207), f (175-207) and f (164-207)) inhibited sensitive Gram-positive bacteria as effectively as nisin and lactoferricin B, therefore they have a high potential to be used as food grade preservatives (Benkerroum, 2010).

Kappacin is an antimicrobial peptide derived from κ-casein. Kappacin corresponds to nonglycosylated and phosphorylated form of bovine caseinomacropeptide (CMP) f(106e169), which exhibited growth-inhibitory activity against Gram-positive and Gram-negative bacteria and is also resistant against proteolytic enzymes (Malkoski et al., 2001). Kappacin has been reported to show the ability to bind enterotoxins and to prevent viral and bacterial adhesion such as the binding of cariogenic bacteria to oral surfaces (Benkerroum, 2010). The mechanism of kappacin to limit gastrointestinal tract infection in the developing neonate is that the release of kappacin in stomach results in increased sensitivity of bacteria to gastric acid by collapsing essential transmembrane cation gradients (Jabbari et al., 2012). In fact, kappacin has already been commercially available for dental care application by the Cooperative Research Centre for Oral Health Science (Australia) as KappacinTM or as KappaZinTM. Furthermore, kappacin was subjected to hydrolysis with endoproteinase Glu-C, given that the peptide Ser (P)149 k-casein-A (138*158) was found to be active against *Streptococcus mutans*, E. *coli*, and *Porphyromonas gingivalis* (Lopez-Exposito & Recio, 2008;). The antimicrobial role of k-casein also involves a pentapeptide, f(17-21), called k-casecidin, which has been identified from the trypsin digestion of bovine κ- casein (Kamau et al., 2010). This antimicrobial peptide showed inhibitory activity against pathogenic bacteria such as *S. aureus, E. coli*, and *Salmonella typhimurium*. However, k-casecidin was found to display cytotoxic activity towards some mammalian cells, including human leukemic cells lines, probably due to apoptosis (Matin & Otani, 2002). Other six peptides derived from a peptic digest of k-casein with antibacterial activity against *L. innocua*, *Salmonella carnosus and E. coli* reported by Lopez-Exposito and Recio et al., (2006). Of the peptides identified, the most active corresponded to k-casein f(18–24), f(139–146) and f(30–32).

Similar to the caseins, whey proteins can also be a source of antimicrobial peptides Another important antimicrobial peptide is lactoferricin, LF f(17–41), a bioactive peptide generated *in vitro* upon enzymatic cleavage of lactoferrin with pepsin in a region distinct from its iron-binding sites (Wakabayashi et al., 2003). LFcin exhibits a broad antimicrobial spectrum including gram-negative bacteria, gram-positive bacteria, yeast, filamentous fungi, protozoa and viruses (Jones et al., 1994). Apart from its antibacterial activity, LFcin has been demonstrated to possess antifungal, immune-

modulatory, antitumoral and antiviral properties. The peptides derived from LF hydrolysates can be useful for clinical applications because of their immunomodulatory effects or for chemoprevention of carcinogenesis. Moreover, according to Lopez-Exposito et al., (2008), LF hydrolysates can also be used in oral care and as food preservative. Furthermore, in the sequence of bovine lactoferrin a new antimicrobial peptide has been identified, Lactoferrampin (f265-284), which has shown broad-spectrum activity against the yeast *Candida albicans* and many gram positive and negative bacteria such as *B. subtilis, E. coli* and *Pseudomonas aeruginosa* but not against the fermenting bacteria, *Actinomyces naeslundii* , *Porphyromonas gingivalis* , *Streptococcus mutans* and *Streptococcus sanguis* (van der Kraan et al., 2005). Lactoferrampin plays a crucial role in membrane mediated activities of lactoferrin. Moreover, chimerisation of LFcin and lactoferrampin can increases the antimicrobial activity against several microorganisms, such as *Candida albicans*, and *Burkholderia pseudomallei* (Puknun et al., 2013).

Furthermore, several peptides obtained by enzymatic digestion of α-lactalbumin and β-lactoglobulin have also shown antimicrobial activity against Gram-positive bacteria such as *E. coli* JM103 (Lopez-Exposito and Recio, 2006).

2.5.3.2 Antihypertensive peptides

Hypertension defined as a sustained increase in blood pressure and is considered as a major risk factor in the development of a number of cardiovascular diseases such as stroke and coronary infarction (Beltrán-Barrientos et al., 2016). Hypertension or high blood pressure is a major health problem worldwide, affecting approximately 25% of the adult population and is estimated that this percentage will be increased by 4% by the year 2025, representing a total of 1.56 billion people (Chockalingam et al., 2006). Normal blood pressure is in the range of 100–140 mmHg (systolic) and 60–90 mmHg (diastolic). A person is considered to be hypertensive when SBP >140 mmHg or DBP >90 mmHg 140/90 mmHg) (Fekete et al., 2016). A first recommendation for management is to address lifestyle habits, especially diet (salt reduction) and physical exercises. According Dietary Approaches to Stop hypertension (DASH) diet, sodium restrictions to less than 1500 mg per day, consumption of fresh fruits and vegetables, whole grains, lean meats, and low fat or fat-free dairy products have been prescribed (Jauhiainen & Korpela, 2007). However, in cases where such changes are ineffective or

insufficient, drug treatments may be prescribed. Pharmacological drugs are effective treatments, but are often accompanied by undesirable side effects. According to the American Heart Association, it was estimated that in 2030, $274 billion in the U.S. would be spent on hypertension-related health care, medication, and missed work days. Therefore, there is a need for alternative approaches to address hypertension, ideally focusing on its prevention. Antihypertensive bioactive peptides are a suitable alternative since they are relatively cheaper to produce with none or less attendant side effects. Increased consumer awareness of the relationship between diet and health supports investigation into the possibilities of functional foods and dietary modifications including foods that contain bioactive substances. In this regard, milk proteins and peptides provide compelling clinical evidence to reduce blood pressured with no reported adverse effects (Huth et al., 2006). This potential bioactivity has stoked the interest of researchers to develop functional foods that both prevent from hypertension and medication-related side effects.

The first well known study of ACE was isolated in 1956 from horse plasma, and found that the enzyme had significant effect converting the decapeptide angiotensin I to the octapeptide angiotensin II (Riordan, 2003). Angiotensin I-converting (ACE, peptidyldipeptide hydrolase, EC 3.4.15.1) is a multifunctional ectoenzyme that plays a critical role in regulation of blood pressure (Li et al., 2004). It is a chloride-dependent zinc metalloprotease, and a dipeptidyl carboxypeptidase that hydrolyzes carboxy terminal dipeptides from oligopeptide substrates (Li et al., 2004). It is part of both the Renin-Angiotensin System and the Kinin-Nitric Oxide system that control sodium balance, body fluid volumes and arterial pressure via membrane bound receptors located on different body tissues including the brain, heart, lungs, liver, pancreas, intestine and vascular epithelial cells (Fitzgerald et al., 2004). ACE-inhibitory peptides plays a crucial role in the regulation of blood pressure. It is responsible for the conversion of angiotensin I to the potent octapeptide vasoconstrictor angiotensin II by cleavage of angiotensin I – via removal of two amino acid residues (His-Leu) from the C-terminal end in the renin-angiotensin system, and catalyzes degradation of the nonapeptide bradykinin, a blood pressure-lowering nonapeptide in the kallikrein-kinin system (Li et al., 2004). Angiotensin II binds to angiotensin II type 1 (AT1) receptor which is a member of the G protein- coupled-receptor superfamily, which plays various roles in

vasoconstriction, aldosterone synthesis and secretion from the adrenal cortex, which leads to sodium retention and reabsorption (Jauhiainen & Korpela, 2007). Hence, inhibition of ACE enzyme results in an overall antihypertensive effect by lowering the blood pressure. Due to the multifunctional property of ACE, it may also influence different regulatory systems involved in blood pressure modulation, immune-defense and nervous activities (Fitzgerald & Meisel, 2003; Gobbetti et al., 2000).

2.5.3.2.1 Examples of ACE-inhibitor peptides

As mentioned previously, ACE is known to play an important physiological role in blood pressure control and cardiovascular function, which has led to increased interest in the study and exploitation of ACE inhibitors as potential medical treatments for antihypertensive patients. Captopril, the first successful ACE inhibitor used in clinical practice, is a sulfhydryl reagent (Cushman & Ondetti, 1999). Since that, several ACE-inhibitor drugs have been synthesized such as enalapril, cilazapril, fosinopril, quainapril, and trandolapril (Ibrahim et al., 2016). However, these drugs have several undesirable side effects such as cough, exanthema, taste alterations, skin rashes, gastric problems and edema of the lips (Ibrahim et al., 2016). Thus, due to their side effects have increases the interest to identify ACE inhibitors from natural sources with little or no side effects for use as substitutes for the synthetic ACE inhibitor drugs.

The first available ACE-inhibitory peptide was isolated from venom of *Bothrops jararaca* snake as naturally occurring peptides (Gobbetti et al., 2000).

Until now many ACE-inhibiting peptides have been derived from many sources such as plant, milk, fish, meat, egg, and soy proteins. The first antihypertensive peptide isolated and identified from food, particularly from bovine milk was described in 1982 by Maruyama and Suzuki (1982) and was named CEI12.

Fermentation of milk by LAB or yeast starter cultures can results in the production of ACE-inhibitory peptides. However, the type of the starter culture can influence their production. The majority of milk protein derived ACE-inhibitors have moderate inhibitory potencies, usually within an IC50 range from 100 – 500 μmol/L (Hayes et al., 2007). Different types of fermented milk and dairy products have been studied for the production of ACE inhibitor peptides (Rai et al., 2015). Some examples include different varieties of cheese, sour milk, dahi, kefir, koumiss, sheep milk yogurt,

fermented camel milk and goat milk. ACE inhibitory properties of some of these products has also been proved by animal experiments and clinical trials Table 15 shows examples of fermented dairy products as well as the starter cultures used for their production with ACE inhibitor peptides. These peptides are not only produced during fermentation but also during storage of fermented product (Rai et al., 2015).

One of the most studied dairy products for production of ACE inhibitor peptides is yoghurt due to its probiotic properties. Many studies demonstrated the production of ACE-inhibitory peptides in yoghurt fermented with different LAB strains or by using other milk types such as sheep milk (Papadimitriou et al., 2007; Politis & Theodorou, 2016; Shakerian et al., 2015). In a study carried out by Ashar and Chand, (2004), production of ACE inhibitor peptides was also demonstrated in dahi, which is Indian type fermented yoghurt. Also in another study by Padghan et al., (2016) demostrated that lasi, an Indian fermented milk yoghurt also contains ACE inhibitor peptides. ACE-inhibitory activities have also been reported in herbal yoghurts fermented with mixed starter consisting *Bifidobacterium bifidum* Bb- 12, *Lactobacillus acidophilus* LA-5, *Lactobacillus casei* LC-01 and *Streptoccus thermophilus* Th-4 (Amirdivani & Baba, 2011).

Moreover, fermentation of milk with both yeast and LAB has also resulted in the generation of ACE inhibitor peptides. These types of products are generally sour to taste and have an alcoholic flavour (Rai et al., 2015). Examples of these products are Calpis, kefir and koumiss (Chaves-López et al., 2012; Namakura et al., 1995; Quiros et al., 2005).

In addition to the traditional fermented milk products ACE inhibitor peptides are also isolated in milk fermented with other LAB strains such as fermentation of UHT milk with *L. delbrueckii subsp. bulgaricus* SS1 and *L. lactis subsp. cremoris* FT4, *bovine milk fermented with L. helveticus* LBK-16H, fermentation of milk with *E. faecium strains,* probiotic fermented milk by *Bifidobacterium bifidum* MF 20/5, cow milk fermented with *E. durans* and *Lactobacillus acidophilus,* camel milk fermented by *Lactobacillus rhamnosus* and bovine skim milk fermented by *E. faecalis,* fermentation of milk with *Lactobacillus helveticus* (Chen et al., 2015; Gobbeti et al., 2000; Gonzalez-Gonzalez et al., 2013; Gutiez et al., 2013; Quiros et al., 2007).

Table 15: ACE-Inhibitory peptides isolated from milk fermented with LAB

Source of peptide	Employed bacterial strains	Reference
Milk	Several strains of LAB	Hernandez-Ledesma et al., 2005
Milk	*Enterococcus faecalis*	Muguerza et al., 2006
Milk	*Enterococcus faecalis*	Quiros et al., 2007
Milk	*Streptococcus thermophilus, Lactobacillus bulgaricus*	Tsai et al., 2008
Milk	*Bifidobacterium bifidum* MF 20/5	Gonzalez-Gonzalez et al., 2013
Milk	*Lactococcus lactis* strains	Rodríguez-Figueroa et al., 2013
Milk	*Lactobacillus helveticus*	Chen et al., 2015
Milk	Non-starter lactobacilli	Solieri et al., 2015
Milk	*Kluyveromyces marxianus* Z17	Li et al., 2015
Milk	*Lactobacillus plantarum* strains	Aguilar-Toalá et al., 2016
Skim milk	*Lactobacillus helveticus JCM1004*	Pan et al., 2005
Skim milk	Several strains of LAB	Pihlanto et al., 2010
Skim milk	Different bacterial cultures	Gonzalez-Gonzalez et al., 2011
Skim milk	*Enterococcus faecalis*	Gutiez et al., 2013
Skim milk	*Lactobacillus helveticus*	Ahtesh et al., 2016
UHT skim milk	*Lactobacillus delbrueckii*subsp. *bulgaricus* SS1 and *Lactococcus lactis*subsp. *cremoris* FT4	Gobbetti et al., 2000
Fresh low-fat milk	Several strains of LAB	Chen et al., 2007
Sour milk	*Lactobacillus helveticus* and *Saccharomyces cerevisiae*.	Nakamura et al., 1995
Casein	*Lactobacillus helveticus CP790*	Maeno et al., 1996
Yoghurt like product	*Lactobacillus helveticus CPN4*	Yamamoto et al., 1999
Casein-enriched milk	*L. helveticus* strains	Leclerc et al., 2002
Caprine kefir	Different bacterial cultures	Quiros et al., 2005
Sheep milk yoghurt	Different bacterial cultures	Papadimitriou et al., 2007
Yoghurt	Different bacterial cultures	Donkor et al., 2007
Fresh low-fat milk	Several strains of LAB	Chen et al., 2007
Koumiss	Different bacterial cultures	Chen et al., 2010
Camel and Bovine Milk	*Lactobacillus rhamnosus* PTCC 1637	Moslehishad et al., 2013

2.5.3.2.2 Types of ACE-inhibitor peptides

ACE inhibitory peptides can be divided into three groups, depending on their inhibitory activity following pre-incubation with ACE (Iroyukifujita et al., 2000). The first group of ACE inhibitory peptides is called “true inhibitor type” peptides. Inhibitor-type peptides are ACE inhibitory peptides whose activity is not significantly altered as the peptides bind to the enzyme, blocking the activity of ACE without being modified, thus preventing the binding of the natural substrate (Ang I) to the active site. The second groups of peptides, known as “substrate type” peptides are hydrolyzed by ACE resulting in peptides with weaker inhibitory activity. Finally, the third group is called “pro-drug type” inhibitors. The peptides in this group, once incubated with ACE, are converted to “true inhibitor type” peptides by ACE or proteases of the digestive tract. *In vivo* studies have demonstrated that only peptides belonging to the groups of true inhibitor type or pro-drug type reduce the systolic blood pressure after oral administration in spontaneously hypertensive rats (SHR).

2.5.3.2.3 Structure correlation of ACE inhibitor peptides

ACE can work on a wide range of peptide substrates, and appears to have a broad specificity. The mode of action of the majority of ACE inhibitory peptides is thought to be as competitive inhibitors of ACE (Iwaniak et al., 2014). Some characteristics of the peptide structures influence positively the inhibitory activity and the mode of action of these peptides. Potential ACE-inhibitor peptides are relatively small in size and usually contain 2-12 amino acids residues in their sequence, even though larger peptides have identified to show ACE inhibitory activity (Iwaniak et al., 2014). This is due to the ability of small peptides to pass the gastrointestinal tract, easily absorbed and reach the peripheral target sites (Quiros et al., 2005).

ACE has two domains (N and C), each of which contains an active site with a His-Glu-X-X-His. The His residues are considered to participate in Zn binding and Glu residue in the catalytic mechanism. The primary structural feature that affects ACE-inhibitory activity is the C-terminal tripeptide sequence and thus these peptides interact with s1, s1' and s2 ' subunits at the active site of ACE (Tavares & Malcata, 2013). ACE prefers to interact with substrates or inhibitors with hydrophobic amino acid residues (e.g. Trp, Tyr, Phe) at the C- terminal tripeptide positions and with hydrophobic branched

aliphatic amino acid such as valine, isoleucine and arginine at the N-terminal (Li et al., 2004). Generally, aliphatic, basic and aromatic residues are preferred in the penultimate positions, while aromatic proline and aliphatic residues are preferred in theultimate positions. Moreover the presence of hydrophobic Pro residue at the last or in the third position in the C-terminal tripeptide favors binding of peptide to enzyme, and therefore influence positively the ACE inhibitory activity of the peptide (Tavares & Malcata, 2013). Furthermore, positively charged guanidine and ε-amine group derived from Arg and Lys are also increased the ACE inhibitory activity of the potential peptides (Iwaniak et al., 2014).

Recently computational methods such as the ulitilization of quantitative structure activity relationship (QSAR) model have been applied in order to study information relating chemical structure of peptides to potential bioactivities (Wu et al., 2006a; Wu et al., 2006b). According to different studies that have been used QSAR models indicated that dipeptides were composed of amino acid residues with bulky and hydrophobic side chains and, while tripeptides usually contained an aromatic amino acid residue in the first position from the C-terminal, a positively charged amino acid residue in the second position, and an hydrophobic amino acid residue in the third position (Wu et al., 2006a). For tetrapeptides, tyrosine and cysteine are preferred for the first position from the C-terminus, histidine, tryptophan and methionine are usually found on the second position while isoleucine, leucine, valine and methionine are favourable for the third position and tryptophan for the fourth position (Wu et al., 2006b).

2.5.3.2.4 In vitro methods to measure ACE-inhibitory activity

Antihypertensive activity is generally evaluated *in vitro* by measuring the inhibition of ACE. There are several methods to determine the ACE-inhibitory activity including spectophotometry, bioassays, flourometric assays, and HPLC (Chen et al., 2013). The most commonly utilized substrate for ACE is Hippuryl-His-Leu (HHL), and the methods measure the catalytic activity of ACE to produce hippuric acid from the substrate (Cushman and Cheung, 1971). From the ACE activity in the absence and the presence of an inhibitor, the percent ACE inhibition can be deduced. When this is done for different concentrations of inhibitor, the IC_{50} value, which is the concentration of inhibitor needed to reduce the ACE activity to half of its initial value, can be calculated. ACE-inhibitory activity is usually measured in terms of IC50 (inhibitory substance

concentration required to inhibit 50 % of ACE activity) (Iwaniak et al., 2014). Low IC50 values mean that a small concentration of inhibitory substance is required to produce enzyme inhibition, so that substance displays a potent inhibitory activity. In the widely used spectrophotometric method of Cushman and Cheung, (1971) the hippuric acid and histidyl-leucine (HL), which results from the hydrolysis of the ACE-specific substrate HHL by ACE, released is measured at 228nm after its extraction with ethyl acetate, as both hippuric acid and HHL absorb light of similar wavelength. The hippuric acid can also be measured by HPLC assays avoiding the extraction step (Vermeirssen et al., 2002). However, 2-furanacryloyl-phenylalanyl-glycylglycine (FAPGG) can also be used as substrate for ACE (Holmquist et al., 1979). This method has also been applied in microtiter plates (Vermeirssen et al., 2002). Moreover, substrates such as o-aminobenzoylglycyl-pnitrophenylalanylproline are designed for fluorometric assays (Sentandreu & Toldrá, 2006).

However, comparison of IC50 values in literature is hampered by the use of different substrates, different sources of ACE, different determination methods, different assay conditions and different ways of calculation.

2.5.3.2.5 Commercial applications of ACE inhibitor peptides

According to Meisel, (2005), efficacy, safety and fine sensory properties are important factors for the consumer acceptance of bioactive peptides/foods. Until now, only few ACE inhibitor peptides have been successfully commercialized as bioactive supplements and have been introduced to food matrix, such as sour milk with brand name Calpis, produced by Calpis Food Industry co, Ltd, Tokyo, Japan and Evolus® commercialized by Valio Ltd, Valio, Finland, known to contain IPP and VPP (Korhonen et al., 2009). Another commercial product is BioZate® produced by DAVISCO foods international, INC, Eden Prairie, USA contains hypotensive whey protein hydrolysates (Pins & Keenan, 2002).

2.5.3.3 Antioxidant peptides

Antioxidants play a vital role in both food systems as well as in the human body to reduce oxidative processes (Sindhi et al., 2013). In foods antioxidants are useful to maintain food quality and increased the shelf life that can be affected by lipid oxidation. Lipid oxidation can cause deterioration in food quality, a reduction in the shelf-life and

nutritional value of a food product, by producing undesirable flavors and toxic substances, while the consumption of foods containing lipid oxidation products has been linked to various diseases, including cancers, diabetes and cardiovascular disease (Brandelli et al., 2015). Free radicals are defined as reactive chemical species that contains an unpaired electron in the outer orbita and can be generated through normal cell metabolism within the body during respiration (Phaniendra et al., 2015). The human body is equipped with endogenous antioxidants that help to protect tissues and organs from oxidative damage caused by reactive oxygen species (ROS), such as superoxide anion (O2-) and hydroxyl (OH-) radicals, which are molecules that can damage proteins, mutate DNA, oxidize membrane phospholipids and modify low-density lipoproteins (LDL) (Pihlanto, 2006). However, in certain circumstances the endogenous defence system fails to protect the body against reactive radicals and this result in oxidative stress, which can induce cell damage and death, including lipids, membranes and proteins resulting in the development of chronic and degenerative diseases such as cancer, arthritis, aging, Alzheimer's, diabetes mellitus autoimmune disorders, and cardiovascular and neurodegenerative diseases (Pham-Huy & Pham-Huy, 2008). Therefore, one way to prevent oxidative stress and its negative effects is the consumption of dietary antioxidant peptides that can act as free radical scavengers and prevent lipid oxidation. Dietary intake of antioxidant compounds can reinforce the body's oxidant status and help to maintain a balanced condition in terms of oxidant/antioxidant in the body (Chakrabarti et al., 2014).

In order to prevent oxidative damage as well as lipid oxidation in foods, synthetic antioxidants such as butylated anisole (BHA) and butylated hydroxytoluene (BHT) are applied to many food products (Lobo et al., 2010). However, due to their side effects, interest on natural antioxidants such as bioactive peptides with no or little side-effects has gained interest in the last years. In addition to the well-known and most studied dietary antioxidants such as vitamin C, E, polyphenols and carotenoids, several recent studies have shown that antioxidant peptides can be released from food proteins such as milk, egg, rice, meat and fish. Antioxidant peptides can be released from food proteins during protein hydrolysis using proteolytic enzymes, food processing or during microbial fermentation, as well as during gastro-intestinal digestion of food proteins.

2.5.3.3.1 Mechanism of action

The exact mechanisms for the antioxidant activities of food derived peptides are not fully understood, but several studies have demonstrated the ability of peptides to inhibit lipid peroxidation, remove free radicals, chelate metal ions and eliminate reactive oxygen species (de Castro et al., 2015; Liu et al., 2016). As with the other bioactive peptides, the antioxidant activity of the peptides is influenced by the molecular mass, the amino acid composition, structure, sequence and hydrophobicity (Zou et al., 2016).

Antioxidant peptides usually consist of 5–11 amino acids, including, proline, histidine, tyrosine, tryptophan and hydrophobic amino acids and a molecular weight lower than 1000 Dalton (Sah et al., 2016; Zou et al., 2016). The amount of histidine, cysteine, proline, methionine, and aromatic amino acids has been reported to contribute to the antioxidant activity of food peptides (Udenigwe & Aluko, 2012). Structure-function studies using a number of synthetic peptides demonstrates that the histidine amino acid residue contains an imidazole ring which can participate in hydrogen atom transfer and single electron transfer reactions to neutralize free radicals or bind metal ions and therefore contributes to increased metal ion chelation, quenching of active oxygen, and scavenging of hydroxyl radical (Udenigwe & Aluko, 2012). The presence of hydrophobic amino acids such as proline and leucine, to the N-terminus of the peptides also increases the antioxidative activity of the peptides (Liu et al., 2016). Apart from enhancing cellular uptake, hydrophobic amino acids are important for enhancement of the antioxidant properties of peptides since they can increase the accessibility of the antioxidant peptides to hydrophobic cellular targets such as fatty acids of biological membranes, in order to limit the oxidative damage as unsaturated fatty acids in cell membranes are very susceptible to oxidative damage by free radical and oxygen species (Udenigwe and Aluko, 2012). Furthermore, the presence of the aromatic amino acids such as phenylalanine, tyrosine, and tryptophan which have phenolic, indole and imidazole groups, respectively can act as proton donors to electron deficient radicals and efficiently scavenge them (Zou et al., 2016).

2.5.3.3.2 In vitro antioxidant assays

Several *in vitro* assays have been developed to test the antioxidant activity of food peptides such as (2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay

(DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) assay (ABTS), oxygen radical absorbing capacity assay (ORAC), ferric reducing antioxidant power (FRAP), hydroxyl radical scavenging and inhibition of low density lipoprotein (LDL) oxidation (Alam et al., 2013). Currently, there is no official antioxidant assay due to the complex nature of antioxidants, therefore is essential to use different assays for the evaluation of antioxidant capacity of potential antioxidant peptides. On the basis of the chemical reactions, the antioxidant assays can be classified into two groups: assays based on hydrogen atom transfer (HAT) and assays based on electron transfer (ET) (Moharram & Youssef, 2014). However, the results obtained from the different antioxidant assays are hardly comparable due to the different mechanisms, redox potentials, pH and solvent dependencies, of each assay. HAT assays include the ORAC, total radical trapping antioxidant parameter (TRAP) (Apak et al., 2013). The HAT-based assays apply a competitive reaction scheme and generally involve the use of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant (Apak et al., 2013). ET assays include the Trolox equivalent antioxidant capacity (TEAC), FRAP, DPPH and ABTS (Apak et al., 2013). These methods are non-competitive and measure the capacity of an antioxidant to reduce an oxidant, which changes colour when reduced where the degree of colour change is correlated with the sample's antioxidant activity (Huang and Prior, 2005). The most commonly applied antioxidant assays for the evaluation of antioxidant activity in proteins and peptides from foods are DPPH, and ABTS (Gülçin, 2012). DPPH assay works based on the reduction of DPPH in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H+ by the reaction. DPPH dissolved in methanol (purple solution) and is incubate in dark for specific time with the mixture of the peptides. Then the solution is reduced by the antioxidant peptides to the corresponding pale yellow hydrazine which is proportional to the antioxidant capacity of investigated peptides (Alam et al., 2013). The reducing ability of the antioxidant can be evaluated by monitoring the absorbance decrease at 515-517 nm. The DPPH, however, is a test that is more affected by the composition of the peptides, the abundance in free amino acids, the size and the molecular weight of the peptides, the effect of the solvent / solubility of the peptide due to steric hindrance that is created between the radical and the same amino acid residues (Prior et al., 2005). It is also a test which does not allow evaluating the hydrophilic antioxidants because it is conducted in ethanol. On the other hand, in the ABTS assay,

ABTS radicals are used as probes and oxidants. ABTS assay measures the relative ability of antioxidant to scavenge the ABTS generated in aqueous phase as compare with a trolox standard (Alam et al., 2013). ABTS radicals are generated after the incubation of ABTS with a strong oxidizing agent such as potassium persulfate. This radical is able to scavenge electrons from antioxidant peptides and reduced the intensity of the blue green colour of ABTS solution (Prior et al., 2005). Decoloring process is monitored by measuring the aborbance at 734 nm. The ABTS assay instead allows studying both hydrophilic and lipophilic antioxidants and it is not affected by steric hindrance.

2.5.3.4 Other bioactivities

Various other types of bioactive peptides have been derived from milk including immunomodulatory, opioid, antithrombotic and mineral binding peptides. Immunomodulatory milk peptides affect both the immune system and cell proliferation responses. For example they can enhance immune cell functions, such as lymphocyte proliferation, natural killer cell activity, antibody synthesis and cytokine regulation (Park et al., 2015). Opioid peptides have opiate-like properties and have affinity for an opiate receptor and reported to play a central role in the nervous network within human body, which encoded by genes that are responsible for generating opiate-like activity and it can exhibit similarity mode of action of morphine (Meisel & FitzGerald, 2000). Some of the functions of these peptides are to increase analgesic action, moderate social behavior, stimulate endocrine secretions, increase GI transient time and thereby inhibiting intestinal peristalsis and motility (Meisel & FitzGerald, 2000). Antithrombotic peptides interfere with the formation of thrombin which is a pathological condition that results in clots formation in arteries by inhibited platelet aggregation and preventing fibrinogen binding with blood platelets (Mills et al., 2011). Mineral-binding peptides are collectively called caseinophosphopeptides, as they are derived from digests of caseins are able to form soluble organophosphate salts functioning as mineral carriers, particularly for calcium (Rutherfurd-Markwick & Moughan, 2005).

2.5.4 Digestion and absorption of bioactive peptides

Some bioactive peptides can express their activity directly on the gastrointestinal tract but the majority of them have to be able to pass through the intestinal wall in order to enter the blood stream and reach target organs inside the body (Urista et al., 2011). In order to exert the desired biological activity *in vivo*, peptides from food sources should reach target sites in the body without losing their activity, these peptides should be remain stable during the digestion process (resistant to the digestion enzymes) or should produce bioactive peptide fragments upon the GI digestion process. Hence, this is a major challenge to the success of bioactive peptides as functional food ingredients, as proteolysis during gastric transit can destroy their biological activity, previously detected using *in vitro* assays (Hayes et al., 2007). Therefore, it is really important to understand firstly the physiology of digestion of proteins and peptides in the human gastrointestinal tract, in order to understand the mechanisms determining the bioavailability of bioactive peptides *in vivo*.

In the human's digestive system, the main sites for the digestion of proteins and peptides are the mouth, stomach and the small intestine. Food proteins are extensively hydrolysed at all stages of GI digestion. The human GIT secretes a variety of enzymes, peptidases, which function synergistically to cleave polypeptide chains into free amino acids and small peptides. Due to their high substrate specificity, they play a major role in regulating the release of bioactive peptides from food proteins. The ingested food, after mastication in the mouth, passes into the stomach where the pH is in the range of 1.5-3. The digestion of food proteins begins in the stomach via pepsins which is activated in an acidic pH environment. The pepsins catalyze hydrolysis of peptide bonds involving Phe, Tyr and Leu which results in the production of long polypeptides, short oligopeptides, and some free amino acids (Erickson and Kim, 1990).

Then the digested products enter into the first part of the small intestine, the duodenum and further digested by pancreatic proteases, trypsin, α-chymotrypsin, elastase, and carboxypeptidase A and B which are activated in an alkaline environment wherein gastric pepsins are inactivated, hydrolyzed the polypeptides released from the gastric phase further into oligopeptides and free amino acids (Segura-Campos et al., 2011). The oligopeptides produced can undergo a second hydrolysis by a number of brush border

peptidases resulting in a mixture consisting of free amino acids and di- and tri-peptides (Segura-Campos et al., 2011).

After digestion, bioactive peptides can either produce local effects in the gastrointestinal tract or be absorbed through the intestine in order to enter the blood circulation intact and exert systemic effects (Segura-Campos et al., 2011).The small intestine is the principal site of absorption of the end products of protein digestion. Dipeptides and tripeptides can be absorbed intact through the epithelia membrane with the help of a peptide-specific transport system, while oligopeptides with more than 4 amino acid residues and proteins are transferred through other routes such as pinocytosis or paracellular channels, depending on their size and hydrophobicity (Roberts et al., 1999). However, peptides that act in the gastrointestinal tract, for example cholesterol-binding, do not have to be absorbed to exert their biological effect.

2.5.4.1 Use of in vitro methods to assess protein digestibility and release of peptides

As we discussed above, the quality of the protein, as well as its essential amino acid composition, the digestibility, the ability to release amino acids and peptides with biological activities during digestion and the bioavailability of amino acids are vital when considering the nutritional quality and health benefits of food proteins. Therefore, studies on digestion and absorption of food proteins in humans are essential in order to understand the physiological, functional and nutritional roles of proteins. However, due to ethical reasons and the time and cost involved, it is extremely difficult to carry out such research involving human participants. Thus, there is an increasing need to develop *in vitro* gastrointestinal digestion models that could mimic the human digestion processes. *In vitro* methods offer an appealing alternative to human and animal studies. They can be simple, rapid, and low in cost, without any ethical concerns (Minekus et al., 2014). In the last years a great diversity of *in vitro* gastrointestinal digestion models incorporating the multi-phase nature of the digestive processes, to mimic the passage the food into the stomach and then into the gut, have been developed or adapted for assessing digestibility of food allergens and proteins (Hur et al., 2011). In addition, simulated gastro-intestinal digestion tries to mimic in vivo physiological conditions, taking into account the presence of digestive enzymes and their concentrations, pH, digestion time, and salt concentrations, among other factors (Minekus et al., 2014).

However, there is substantial variability among the conditions, such as the number and type of steps included in the digestion sequence (mouth, stomach, small intestine, large intestine), the composition of the digestive fluids used in each step (enzymes, salts, buffers, biological polymers, and surface-active components) (Hur et al., 2011). Moreover, another important factor that hampered the ability to compare results across the different studies using in vitro digestion models is that there is great differentiation in the inclusion of various digestion stages and whether the chosen conditions are static with constant concentrations of enzymes and bile acids at each step of digestion or dynamic with varying enzyme concentrations and pH that represents the transport of digested food (Hur et al., 2011). The most frequently utilized enzymes and other biological molecules used within in vitro digestion models were pepsin, pancreatin, trypsin, chymotrypsin, peptidase, α- amylase, lipase, bile salt, and mucin (Hur et al., 2011). An *in vitro* digestion models should consider three main stages of the physiological digestion processing: (i) processing in the mouth, (ii) processing in the stomach (cumulative to the mouth) and (iii) processing in the duodenum (cumulative of mouth and stomach) (Wickham et al., 2009). Thus, a consensus concerning the basic parameters and stages of digestion would be relevant in order to harmonize the various *in vitro* digestion models. Therefore, recently within the COST Infogest network (which is an international network joined by more than 200 scientists from 32 countries working in the field of digestion), proposed a general standardized and practical static digestion method based on physiologically relevant conditions that can be applied for various endpoints, which may be amended to accommodate further specific requirements (Minekus et al., 2014) (Figure 15).

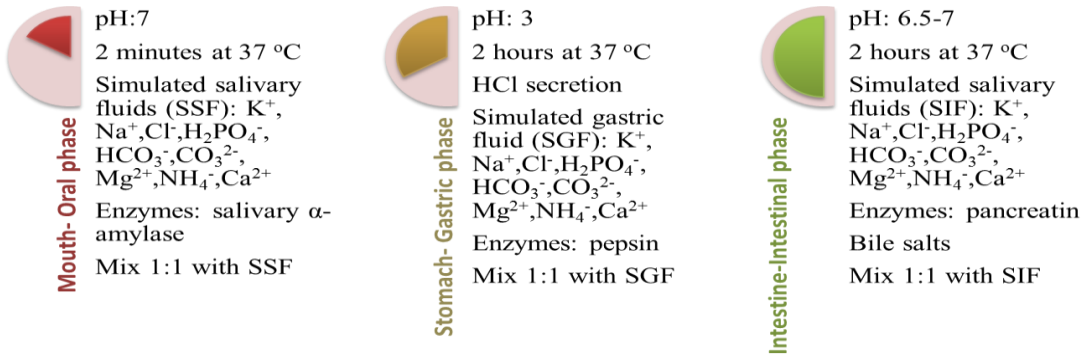


Figure 15: *In vitro* human digestion model according to Minekus et al., (2014)

2.5.5 Methods used to isolate and characterize bioactive peptides

The classical approach to identifying and processing bioactive peptides is presented in Figure 16. This represents the most widely used method for the discovery of bioactive peptides from food proteins. This approach involves the selection of the appropriate food protein source followed by *in vitro* protein digestion by the proteolytic action of endogenous enzymes, exogenous enzymes (e.g., hydrolysis by digestive enzymes), or by food technological processes (such as ripening and fermentation, followed by fractionation and purification of the resulting protein hydrolysates (Capriotti et al., 2016). Then, peptides are tested for biological activity and the actual peptide sequence(s) of the active fraction(s) are identified by MS analysis. Then the selected peptides are chemically synthesized, and bioactivity assays are conducting in order to confirm their bioactivity. Even though the classical approach is still the most used, it still has some major drawbacks. This approach is time consuming as it requires intensive sample preparation (fractionation, purification). Moreover, there is a limit to the number of peptide species that can be studied at a time and gives low yield of isolated peptides (Agyei et al., 2016). Another important drawback is that it doesn't give any prior indication of the types of peptides that can be expected from a particular food protein and also there is the possibility that individually potent peptides may not be discovered (Agyei et al., 2016).

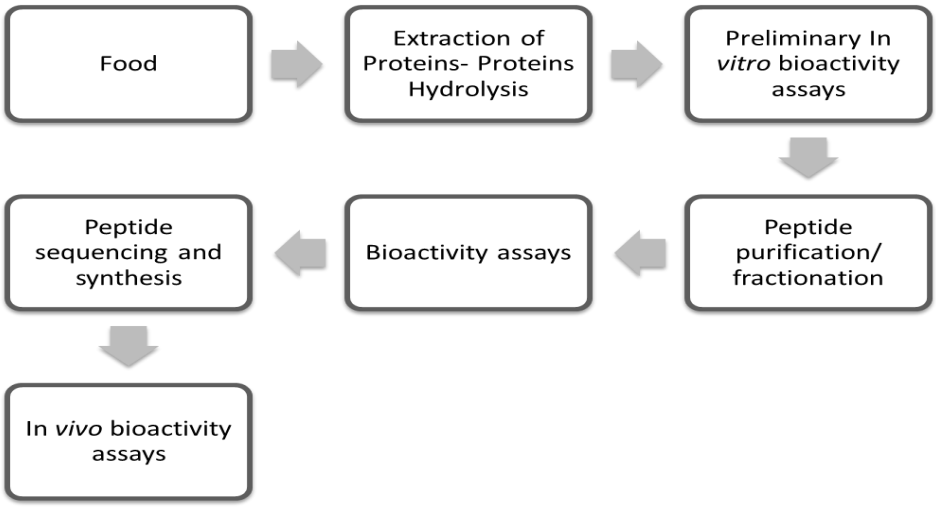


Figure 16: Classical approach for the identification of food-derived bioactive peptides

Based on physical and chemical characteristics of the peptides, the isolation of bioactive peptides from milk has been carried out using various methods including ultrafiltration, acid and isoelectric precipitation and several types of chromatography (Korhonen &

Pihlanto 2006). After isolation and purification, the identification of peptides is usually carried using Mass spectrometry (MS) (Tandem MS, Electrospray ionization tandem MS, matrix assisted laser desorption/ionization –time of flight MS) combined with a high-resolution separation technique such as ultra high pressure liquid chromatography (UPLC).

To overcome the major drawbacks of the classical approach, computer based (*in silico*) approaches have recently been introduced towards the discovery of bioactive peptides encrypted in food proteins (Capriotti et al., 2016). Using *in silico* approach, important information on bioactive peptides can actually be predicted from food proteins of known sequence quickly and effectively before to wet-laboratory synthesis (Agyei et al., 2016).

The most important steps of *in silico* approach are summarized below. The first step of this approach is to consult an online protein database such as BIOPEP whereas the desired protein sequences are selected. This is followed by the *in silico* digestion of the proteins with the use of the appropriate proteolytic enzymes of characteristic specificity that will release the desired peptides. The next step is the characterization of the peptides resulted from *in silico* proteolysis, in order to identify structural properties, and potential biological activities, including toxicity and allergenicity. If the predicted properties obtain are satisfactory, wet laboratory synthesis is followed. This approach for the production of bioactive peptides, allows the discovery of bioactive peptides from several proteins and proteolytic enzymes with a high level of accuracy even before synthesis of the peptides, and also is less laborious intensive and relatively cheaper (Carrasco-Castilla et al., 2012; Minkiewicz et al., 2008).

The bioinformatic approach involves the use of information provided by various databases, such as BIOPEP, PepBank, PeptideDB, the antimicrobial peptide databases (APD2) and the Collection of AntiMicrobial Peptide (CAMP), to describe a biological activity to the identified peptides (Capriotti et al., 2016). For example, BIOPEP database include antibacterial peptides, cytokines and growth factors peptide hormones, and toxin/venom peptides, antithrombotic peptides, anticancer, immunomodulating and antioxidant peptides whereas APD2 and CAMP are limited to antimicrobial peptides, such as antiviral, antifungal, antibacterial, and antiparasitic peptides (Minkiewicz et al., 2008). As the number of bioactive peptides derived from milk and dairy products is

increasing, Théolier et al., (2014) developed a specific database, named MilkAMP, which contains valuable information on antimicrobial peptides of dairy origin, including microbiological and physicochemical data (Théolier et al., 2014). Since occurrence does not necessarily indicate liberation of the cryptic peptides, bioinformatic softwares can also be used to simulate proteolytic specificities of enzymes in order to generate profiles of peptides *in silico* (Udenigwe et al., 2014). Examples of *in silico* proteolysis tools include BIOPEP "enzyme action" tool, ExPASy PeptideCutter and PoPS (Agyei et al., 2016). The peptides resulting from *in silico* proteolysis can then be matched with bioactive peptides in databases for predetermined bioactivities.

2.5.6 Commercial and potential use of bioactive peptides in functional foods

Bioactive peptides have gained great interest in recent years due to their potential applications in functional foods and nutraceuticals (Korhonen & Pihlanto, 2007). At the moment, milk proteins are the best source of bioactive ingredients but until recently the commercial production of milk derived bioactive peptides has been limited by a lack of suitable large-scale technologies (Korhonen & Pihlanto, 2007). Table 16 lists commercially available dairy products which contain bioactive peptides.

Commercial products containing bioactive peptides already have been introduced worldwide such as dairy foods (Calpis, Evolus, Peptide Soup EX), toothpaste and chewing gum (MI PasteTM, Trident Xtra CareTM), bioactive ingredients (Capolac MM0525, Lacprodan, RecaldentTM, Ameal peptide) and food supplements (Ameal bp, PeptACE, Fish Peptide, Vasotensin) (Agyei et al., 2016; Korhonen, 2009; Li-Chan, 2015).

The two fermented sour milks, Calpis® and Evolus® are based on antihypertensive tripeptides Val-Pro-Pro and Ile-Pro- Pro. The Japanese product Calpis is a soft drink made of skim milk fermented with a culture containing *L. helveticus* and *S. cerevisiae* while Evolus produced in Finland by Valio is milk fermented using *L. helveticus* LBK-16 H strain (Korhonen et al., 2009). In addition to the reduction of blood pressure via the inhibition of the renin-angiotensin system, long term consumption of these peptides is associated with the improvement of the vascular function of hypertensive subjects by reducing arterial stiffness (Korhonen et al., 2009). Other products containing

antihypertensive peptides include C12 peptides (DMV, Netherlands) and BioZate (Davisco, USA) (López-Fandiño et al. 2006). PeptoPro® is a sport drink (protein shake) obtained by the cleavage of caseins using a new and patented debitterizing technology, which supply energy and fast muscle refuelling by stimulating the production of insulin (Tidona et al., 2009). Another commercial product which associated with many health benefits such as better sleep, improvement of memory and learning, improvement of depression and anxiety is BioPure-Alphalactalbumin produced by Davisco (Tidona et al., 2009).

Table 16: Commercial dairy products and ingredients with bioactive peptides

Product, manufacturer	**Product type**	**Functional effect**
Calpis, Calpis Co., Japan	Sour milk	Lowering of blood pressure
Evolus, Valio Oy, Finland	Fermented milk product enriched with calcium	Lowering of blood pressure
Biozate, Davisco, USA	Hydrolyzed isolate of whey proteins	Lowering of blood pressure
BioPURE-GMP, Davisco, USA	Whey proteins isolate	Prevent dental caries, influence blood coagulability, antiviral, antibacterial
BioPURE-Alphalactalbumin, Davisco, USA	Whey proteins isolate	Helps sleep and memory
PRODIET F200/Lactium, Ingredia, France	Flavored milk product, confectionery, capsules	Stress release
C12, DMV International, Holland	Component hydrolysate	Lowering of blood pressure
Capolac, Arla Foods Ingredients, Sweeden	Component hydrolysate	Helps mineral absorption
PeptoPro, DSM Food Specialties, Norway	Component hydrolysate	Improvement of muscle strength and physical exercise capacity
Casein DP Peptio Drink, Kanebo, Japan	Drink	Lowering of blood pressure
Tekkotsu Inryou	Drink	Helps mineral absorption
Glutamine peptides, DMV International, Holland	Milk protein hydrolysate	Immunomodulating properties

However, the large-scale production of products containing bioactive peptides has been limited due to the lack of suitable large scale technologies. Therefore, several

approaches have been designed to increase the oral delivery of bioactive peptides (Renukuntla et al., 2013). Current trends include the chemical modification of the proteins, genetic cloning and the expression of bioactive peptides via the use of bacterial and fungal vectors in order to increase the production of bioactive peptides (Renukuntla et al., 2013). Moreover, different challenges concerning bioactive peptides incorporation into food products and even pharmaceuticals such as toxicity, allergenicity, interaction with other food components must also be studied (Carrasco-Castilla et al., 2012).

2.5.7 Safety and regulation of bioactive peptides

Most studies on milk bioactive peptides has focused on efficacy and not on safety (Schaafsma, 2009). In addition, efficacy data are mainly based on *in vitro* data and animal model studies. Even though food derived peptides have great potential as food additives, they may not be suitable in all food applications as it is important to consider the safety and toxicity of the protein. At the moment most of the studies indicate lack of toxicity or any side effects in humans (Phelan et al., 2009) The lack of toxicity can be explained by the fact that these peptides are similar to those from digestion of food proteins and also for their production usually food grade enzymes are applied (Schaafsma, 2009). A detailed review about the safety of milk derived peptides in terms of allergenicity, cytotoxicity, genotoxicity and mutagenicity has been published recently by Beltrán-Barrientos et al., (2016).

Dietary proteins and protein hydrolysates have the GRAS status in the United States. However, no specific legislation exists about the use of bioactive peptides as dietary supplements or as food ingredients in most EU countries (Schaafsma, 2009). In EU, food derived peptides are fall under the regulation of novel foods, EC 258/97/ CE. Novel foods are defined in Europe as foods and food ingredients that were not used for human consumption to a significant degree within the European Community before 15 May 1997 and must fall in one of the available categories (Commission of the European Communities, 1997, 2008). Of these categories, the last two categories ‘foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating of breeding practices and having a history of safe use’ and ‘foods and food ingredients to

which a production process has been applied, where that process gives rise to significant changes in the composition or structure of the food or food ingredients, which affects its nutritional value, metabolic effect or level of undesirable substances' are mainly applied to milk derived peptides. Regarding the nutritional and health claims made on food products require authorization under the Regulation EC 1924/2006, in order to ensure the effective functioning of the internal market while providing a high level of consumer protection. Health claims should only be authorized for use in the community after a scientific assessment of the highest possible standard. EFSA is responsible for assessing the scientifically based evidence. Finally, the safety of bioactive peptides is regulating under the Aricle 8 of Regulation (EC) No. 1925/2006 (Schaafsma, 2009).

3 Materials and Methods

3.1 Isolation of LAB from donkey milk: Assessment of their technological properties and safety characteristics

3.1.1 Chemicals and Reagents

All chemicals and reagents used were of the highest analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified. PCR primers and other molecular biologicals (PCR reaction mixtures, Taq DNA polymerase) were obtained from Thermo-Fisher Scientific (USA).

3.1.2 Microbiological media and media preparation

The microbial media used in the cultivation, isolation and enumeration of the microorganisms were purchased from Oxoid Ltd (Basingstoke, Hampshire, UK), Merck (Darmstadt, Germany) and Difco BD Diagnostic Systems (Sparks, MD, USA). The microbial media and diluents were prepared following the manufacturers instruction by dissolving a known weight of the media powder in the required quantity of distilled water and sterilized by autoclaving at 121°C for 15 mins. The desired pH of each media was checked and if necessary adjusted before autoclaving. Sterilised media was stored at 4ºC prior to use.

3.1.3 Collection of milk samples

Milk samples were collected from the "Golden Donkeys Farm" in Larnaca area, Cyprus. In particular, sampling was conducted from March to October 2013 and a total of eleven samples were collected by manually milking 8 Jennies. The bulk milk samples were collected in sterilized 250 mL containers, placed in cool-boxes and immediately transported to the laboratory at 4 ^{o}C and maintained at this temperature for analysis within 12 h. Care was taken by milk handlers to avoid cross contamination (gloves, disinfection of teats).

3.1.4 Microbiological analysis of donkey milk samples

The samples were evaluated for total aerobic bacteria, *Enterobacteriaceae*, LAB, yeasts and molds and *Staphylococci*, by the standard pour plate method after serial dilutions in saline solution (0.85% w/v). Table 17 shows the growth media, incubation time, temperature and method used for each evaluated group of microorganisms.

3.1.4.1 Sample preparation- homogenization and serial dilutions

Samples (10 ml) were homogenized with 90 ml sterile saline solution (8.5g NaCl, 1000 ml distilled water, pH 7.0 ± 0.2) and serially diluted. The pour plate method of Harrigan and McCance (1986) was used for all the analyses except for yeasts and molds count, which was done according to the spread plate method. Triplicate plates were prepared per dilution. All counts were reported as $\log_{10}$ colony-forming units per ml ($\log_{10}$ cfu/ml) of donkey milk.

3.1.4.2 Enumeration of microorganisms

Total aerobic bacteria

One ml of the appropriate dilutions was used to inoculate pour plates using plate count agar (PCA). The plates were incubated at 30 ºC for 72 hours.

Enumeration of lactic acid bacteria (LAB)

One ml of the appropriate dilutions was used to inoculate pour plates of MRS (deMan Rogosa Sharpe) agar, MRS acidified to pH 5.7 and M17 agar. The plates were incubated anaerobically in Anaerobic jars at 30 or 45 ºC for 3 days.

Enumeration of yeasts and molds

0.1 ml of the appropriate dilutions was used to inoculate spread plates of dichloran rose-bengal chloramphenicol agar (DRBC). The plates were then incubated at 25 ºC for 5 days, followed by colony counting of plates.

Enumeration of enterobacteriacae

One ml of the appropriate dilutions was used to inoculate pour plates of violet red glucose agar (VRGA). The plates were then incubated at 37 ºC for 24 hours after which the colonies on plates colonies were counted.

Enumeration of staphylococci

One ml of the appropriate dilutions was used to inoculate pour plates of baird-parker agar (BP). The plates were then incubated at 37 ºC for 48 hours after which the colonies on plates colonies were counted.

Detection of Listeria monocytogenes

Twenty-five mls of each sample sample was aseptically taken, blended for 2 min in 225 ml of Half Fraser broth and incubated at 37 °C for 24 h. One milliliter of primary enrichments was transferred to 9 ml of Frazer broth and incubated at 37 °C for 24-48 h. Secondary enrichments were streaked on Oxford agar and PALCAM agar and incubated at 37 °C for 48 h. Enrichment broths and selective agars were supplemented according to the manufacturer's guidelines. The plates were examined for typical *Listeria* colonies (black colonies with black sunken centers).

Detection of Salmonella spp.

Twenty-five mls of each sample sample was aseptically taken, blended for 2 min in 225 ml of Buffered Peptone Water and incubated at 37 °C for 24 h. One milliliter of primary enrichments was transferred to Muller Kauffman broth Rappaport Vasiliades broth and incubated at 37 °C or 41.5 °C respectively, for 24 h. One loopful from selective enriched broth was streaked onto plates of XLD and Brilliant Green agars and incubated at 37 ºC for 24 h. The plates were examined for the presence of typical colonies of Salmonella spp.

Table 17: Methods used for microorganisms enumeration

Microorganism	Growth Media	Incubation conditions	Reference Method
Total Aerobic Bacteria	PCA (Merck, Darmstadt, Germany)	30°C/ 72h	ISO 4833-2, (2013)
Enterobacteriacae	VRBGA (BD, Sparks, MD)	37°C/24h	ISO 21528-2, (2004b)
Yeast and Molds	DRBC (BD)	25°C/5 days	ISO 6611, (2004a)
Staphylococci	BP (Oxoid, Basingstoke, UK)	37°C/48h	ISO 6888-1, (1999)
LAB	MRS,	37 °C/72h*	ISO 15214, (1998)
	MRS pH 5.7	30 °C/72h*	
	M17 (Oxoid)	45 °C/48h and then 20°C/4 days	

*Anaerobic conditions were achieved by using sachets (CampyGen, Oxoid, Basingstoke, UK)

3.1.5 Physicochemical and chemical analyses of donkey milk samples

3.1.5.1 pH and titratable acidity

The pH of the donkey milk samples was determined by potentiometry. The pH meter glass probe was first calibrated using standard buffers at pH 4 and 7 before being used to measure the sample pH. Sample pH was measured by submerging the tip of the probe into the sample for ~1-2 min until a stable reading was registered on the pH meter scale. Measurements were done in triplicates and average values were reported.

Titratable acidity was measured by titrating 10 ml of donkey milk sample in conical flask, to which 3-4 drops of Phenolphthalein solution was added. The mixture was then titrated with 0.1M NaOH solution until faint pink color observed. The percentage of lactic acid was calculated by:

Titrable acidity (as % lactic acid) = (V_{NaOH}*0.1M NaOH*90/10).

3.1.5.2 Fat content

Fat content of samples was determined by Gerber method. The butyrometer was filled with 10 ml H_2SO_4. Donkey milk sample (10.75 ml) was added into a butyrometer and they placed in water bath at 65 ^oC for 10 min. Then 1 ml amyl alcohol was added. After that, the butyrometers were centrifuged in Gerber centrifuge for 5 min and after centrifuging the butyrometer was placed in a water bath at 65° C for 5 minutes before

the reading was taken. The oil level was read as percentage oil in donkey milk from butyrometer vessel (ISO 488-2008).

3.1.5.3 Protein content

Protein content of donkey milk samples was determined by ISO 8968-2:2001 for total nitrogen. Milk samples (around 5 ± 0.1 g) was accurately weighed, wrapped in nitrogen-free paper, and then placed in a 250 ml digestion tube. A solution comprised of a copper catalyst of 15 g K2SO4 and 0.04 g anhydrous CuSO4, 3 g of pumice, and 20 mL of concentrated sulfuric acid was added to the flask, to begin the digestion. The digestion tubes along with the metal rack were then placed into the unheated digestion block (Digestor 2508, FOSS Analytical A/S, Hillerod, Denmark). As the first step, the digestion block was initially brought stepwise to a temperature of 200˚C, then to 300˚C, and finally to 420˚C. The samples were then digested for 60 minutes or until the samples were green and clear. The tubes with digested samples were removed from the block and cooled for 25 min at room temperature. The Kjeldahl digests were distilled using an autodistillation unit (Kjeltec 8100,). The method used a 40% NaOH solution to generate an alkaline distillation environment for producing ammonia vapor and 4% boric acid solution to collect the distilled ammonia. The boric acid solution also contained 0.001% bromocresol green and 0.0007% methyl red indicators to indicate the endpoint during subsequent titration with standardized hydrochloric acid. The collected ammonia in boric acid solution was titrated with 0.1 N HCl to the endpoint and volume of titrant consumed was recorded to the nearest 0.1 mL (VA). The titrant required to titrate the reagent blank (VB) was also recorded. Percentage nitrogen was calculated using the following equation:

Wn =[1.4007 x (Vs – VB) x N]/W,

Where Wn = nitrogen content of sample, expressed as a percentage by mass; VS = volume in ml of the standard hydrochloric acid used for sample; VB = volume in ml of the standard hydrochloric acid used for blank test; N = Normality of the standard hydrochloric acid expressed to four decimal places; W = mass of test portion in g, expressed to nearest 0.1 mg.

Percentage crude protein (CP) was calculated using the following equation: Protein (%) = %N× 6.38.

3.1.5.4 Total solids

In this procedure, a known quantity of milk is dried on a boiling water bath. Subsequently sample is dried in hot air oven at 102 ±2°C and from the weight of the residue, the total solids content in milk is determined.

A porcelain crucible is heated in the drying oven at least 1 hour. Then, the dish is transferred to a desiccator and allowed to cool to room temperature (at least 30 mins) and weigh to the nearest 0.1 mg (A). Then 2 g of donkey milk samples are weighted in the dish (B). The crucible is then transferred into a hot air oven adjusted to 102±2 ºC, for 2 hours.then the crucile is removed from the oven, cooled into a desicator and weighted again (C). The procedure was repeated until the difference in the two consecutive weighing does not exceed 2 mg. Total solid content was calculated using the following equation:

Total solid content= [(C-A)/B]x100

3.1.6 Lysozyme content of donkey milk samples

Lysozyme was quantified according to a sensitive fluorescence-based method using EnzChek® kit (Molecular Probes). Lysozyme activity is proportional to fluorescence release from fluorescently labeled *Micrococcus lysodeikticus* cell walls. DQ Lysozyme substrate stock suspension (1.0 mg/ml) and the 1000 U/ml lysozyme stock solution were prepared according to the manufacturer.

3.1.6.1 Preparation of lysozyme standard curve

8 wells were filled with 50 µl of 1X reaction buffer, 50 µl of the 1000 U/ml stock solution of lysozyme were added to the first well, mixed by pipetting, then 50 µl were transferred to the second well. This process was repeated from one well to the next, except 50 µl from the mixture in the seventh well was discarded and nothing were added to the eighth well. Thus, the lysozyme concentration will range from 500 U/ml to 0 U/ml in the 50 ml volumes, for a range of 250 U/ml to 0 U/ml in the final 100 ml volumes (units according to the conditions stated from the manufacturer).

3.1.6.2 Lysozyme assay

Experimental samples were diluted in 1X reaction buffer and 50 µL of the appropriate dilution were placed in each well. The DQ lysozyme substrate working suspension was prepared by diluting the 1 mg/ml stock suspension 20-fold in 1X reaction buffer. 50 µl volumes were used for each reaction. The final concentration of the DQ lysozyme working suspension was twofold lower in the final reaction buffer. Starting the reaction 50 µl of the DQ lysozyme substrate working suspension was added to each microplate well containing the standard curve and experimental samples. The samples incubated at 37 ^{o}C for 30 min, protected from light. The fluorescence was measured using a fluorescence microplate reader with fluorescein filter (Tecan Austria GmbH). Digestion products from the DQ lysozyme substrate showed an absorption maximum at 494 nm and a fluorescence emission maximum at 518 nm. Correction for background fluorescence was done by subtracting the value derived from the no-enzyme control. Lysozyme activity levels of the experimental samples were determined from the standard curve. The assay was carried out in triplicates.

3.1.7 Isolation of LAB

The colonies between 30 and 300 on each petri dish were counted as total LAB. Representative LAB strains were isolated from MRS, acidified MRS and M17 agars, according to different morphological characteristics (i.e size, shape and/or color). Purity of the isolates was checked by streaking on MRS and M17 agar, respectively, followed by microscopic examination. Stock cultures of identified strains of LAB were stored in MRS or M17 broth using 40% (v/v) glycerol as cryoprotective agent at 80 ^{o}C.

3.1.8 Biochemical, physiological and phenotypic characterization of the strains

The purified isolates were examined by cell morphology applying Gram staining, and catalase tests, as recommended in Bergey's Manual of Determinative Bacteriology (Holt, Krieg, Sneath, Staley, & Williams, 1994). For Gram staining, a single colony was picked with sterile wire loop. The colony was emulsified in a drop of distilled water on a clean slide and spread out to make a thin film. The slide was air dried and the smear was fixed by passing through a flame three times. The smear was then stained with

Crystal violet solution for one minute and rinsed rapidly with tap water. Gram iodine solution was added and left for one minute, then poured off and the slide was left to dry. The slide was washed with 95% ethanol and was then rinsed with tap water and stained with safranin for 30 seconds and was then washed well and air dried. The smear was then examined microscopically. The Gram positive organisms appeared violet in color and Gram negative organisms were pink in color. Catalase activity was determined by transferring 24 h culture from MRS to a slide glass. Rapid gas formation after dropping 3% hydrogen peroxide (H_2O_2) indicated a positive result. Oxidase test was done using Identification Sticks (Oxoid Ltd., Basingstoke, Hampshire, UK) coated with a dye N-tetramethyl paraphenylenediamine dihydrochloride, to detect the presence of cytochrome 'C' oxidase which is responsible for the oxidation of the dye. The oxidase sticks were smeared on pure colonies and observe for colour change. Development of purple color within 10-30 sec indicates positive reaction whereas no color change indicates negative reaction. Gram-positive, catalase and oxidase negative rods and cocci were presumptively identified as LAB.

Further classification was done according to the biochemical criteria described by (Harrigan & McCance, 1976), such as growth at various temperatures (15 ^{o}C, 30 ^{o}C, 37 ^{o}C and 45 ^{o}C), salt concentrations (2, 4, 6.5, 8% w/v NaCl), pH (4.4, 6, 8 and 9.6) and litmus milk reduction. For their ability to grow at different temperatures, isolated strains inoculated (1% v/v) in MRS/M17 broth and incubated at the appropriate temperature. OD was measured every hour up to 24 or 48 hrs. For their ability to grow at different salt concentrations or pH, MRS/ M17 were adjusted to different salt concentrations or pH levels. 1% (v/v) of overnight cultures was inoculated into the different test media and incubated at 37 ^{o}C. The optical density was measured every hour up to 24 hrs. The litmus milk reduction test was performed by inoculating 1% (v/v) inoculum in 5 ml of sterilized litmus milk and incubated at 37 ± 1°C for 7 days. Gas production and nature of the curd formed (coagulum) and color changes were recorded daily for 7 days. More specifically, the following changes in the medium were recorded:

- Acid production shown by a change in the colour of the litmus from light purple to pink and clotting of the milk (acid clot).
- Reduction of the litmus and loss of colour.

- Coagulation of the milk as a result of proteolytic enzyme activity affecting the casein, the litmus colour remaining light purple (sweet clot).
- Hydrolysis of casein as a result of proteolytic enzyme activity causing clearing and loss of opacity in the milk (peptonisation).
- Utilisation of citrate in the milk medium resulting in the production of an alkaline medium shown by colour change to a deep purple colour.

3.1.9 Molecular identification of the strains

The identification of the isolates at genus and species level was confirmed by applying molecular techniques. Bacterial DNA from each strain was obtained by lysis of cells at 96 ^{o}C for 10 min with IGEPAL CA-630 (Sigma-Aldrich). 16S F: 50 TGCCTAATACATGCA0 3 and R: 50 CTTGTTACGACTTCA0 3 (Eurofins, MWG Operon, Ebesberg, Germany) were used to amplify the 16S rRNA gene fragments of LAB isolates, using a thermal cycler (PTC-200 Peltier, MJ Research, Inc., Watertown, MA, USA). The concentration of the DNA extracts obtained was measured by spectrophotometry. A 1 µl sample was pipetted onto the pre-cleaned end of the lower measurement pedestal (receiving fibre) of the NanoDrop spectrophotometer (ND1000, England) whilst the second optic cable (sampling fibre) was open. The sampling arm was closed and spectral measurement performed using NanoDrop software v.3 (NanoDrop Technologies Inc., England). Sample carryover between successive measurements was minimised by wiping the sample from the pedestals using a clean buffer. TAE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was used as the blank. PCR reactions contained template DNA, 1X KOD PCR reaction buffer, 200 mM of each dNTP, 2.5 mM MgCl2, 0.5 mM of each primer, 1 ml KOD Hot Start DNA polymerase (Novagen), and distilled water was added to a final volume of 50 ml according to the manufacture instructions. The PCR conditions were an initial denaturing step for 2 min at 95 ^{o}C followed by 34 cycles of denaturation at 95 ^{o}C for 20 s, annealing at 50 ^{o}C for 10 s, and elongation at 70 ^{o}C for 30 s, followed by a final extension step of 7 min at 72 ^{o}C. Amplification products were separated by electrophoresis on 1.5% (w/v) agarose gel, in 1X TAE buffer, stained with Midori green (Anachem, UK) and visualized under UV light. A 100 bp DNA ladder (Invitrogen) was used as a marker. The PCR products were purified using a commercial Purification kit

(Purelink PCR, Invitrogen, USA) following the manufacturer's instruction. The purified PCR products were sent to Source Bioscience (Dublin, Ireland) for Sanger sequencing. Sequences were then compared to those in the GenBank database using the BLAST algorithm (http://www.ncbi.nih.gov). The identities of the isolates were determined on the basis of the highest matching (similarity ≥97%).

3.1.10 Technological properties

3.1.10.1 Acidification activity

Acidifying activity of the strains was determined according to the ISO 26323, (2009), as well as a pH meter (Hanna Instruments, Padova, Italy). Tubes containing 10 mL of sterile skimmed milk (RSM 10% w/v; Oxoid) were inoculated (1% v/v) with fresh overnight cultures (10^8 cfu/ml) and incubated at 37 ^oC. Titratable acidity and pH was determined immediately after inoculation (0 h), at 6 and 24 h. The analysis was carried out in triplicate using non-inoculated skim milk as negative control. The acidification rate was calculated as ΔpH; ΔpH =pH (at time 24 h)-pH (zero time) (Ayad et al., 2004). Regarding titratable acidity, the data were expressed as mL lactic acid per 100 mL RSM (10% w/v).

3.1.10.2 Proteolytic activity

3.1.10.2.1 Skim milk agar

Extracellular proteolytic activity was determined according to the method of Franciosi et al., (2009). Two microliters of fresh overnight cultures (10^8 cfu/ml) were spotted onto the surface of an agar medium composed of 10% (w/v) skim milk powder (Oxoid) and 2% (w/v) agar (Oxoid) and incubated at 37 ^oC for 4 days. Proteolytic activity was indicated by a clear zone around the colonies. The analysis was carried out in duplicates.

3.1.10.2.2 O-Pthaldialdehyde Assay (OPA)

The proteolysis of the LAB strains was also determined by the o-phthaldialdehyde (OPA) method, described by Church et al., (1983).

Preparation of OPA reagent:

The OPA solution was made by combining the following reagents: 25 ml of 100 mM sodium tetraborate, 2.5 ml of 20% (w/w) sodium dodecyl-sulphate (SDS), OPA (40mg dissolved in 1 ml of methanol) and 100 µl of β-mercaptoethanol. The final volume was made up to 50 ml using dH2O. OPA reagent is light-sensitive and thus must be protected from light sources during preparation and during the assay. This reagent was prepared fresh and used within 2 hours of preparation.

OPA Assay:

Strains were sub-cultured twice in MRS broth for 24 h at 30 ºC (1 % v/v inoculum). 10 ml of sterilized RSM (Oxoid) was inoculated with 2 % of the above mentionated culture and incubated at 37 ºC overnight. A sample of 5 ml of this culture was added to 1 ml of water, 10 ml of Trichloroacetic Acid (TCA) (0.75 N), mixed and rested for 15 minutes at ambient temperature. Then the suspension was mixed again and filtrated with a Whatman paper n. 1, and a sample of 50 µL of this filtrate was added directly to 1.0 ml of o-phtaldialdehyde (OPA) reagent in a quartz cuvette. The solution was mix briefly by inversion of the cuvette and rested for 2 min at ambient temperature. The absorbance at 340 nm was measured using Infinite PRO 200, Tecan, Switzerland. All the assays were carried out in triplicate.

Glycine standard curve:

For construction of the standard curve, a series of standard solutions of glycine were prepared in sterilized skim milk. The glycine standards were prepared and treated in the same manner for samples for each OPA assays. The results were expressed as mM glycine g/L according to a standard curve constructed with known glycine concentrations.

3.1.10.3 Lipolytic activity

Lipolytic activity was tested according to the method described by Leuschner et al., (1997). Tested strains were grown overnight at 37 ^{o}C in MRS broth. A loopful of fresh culture was placed on tributyrin agar (TA). Plated were incubated at 37 ^{o}C for 4 days and observed daily for halo formation around the colonies. Lipolytic activity was recognized through the formation of clear halos around colonies on TA plates resulting

from hydrolysis of the ester bonds in the triglyceride tributyrin and the subsequent release of butyric acid. The analysis was carried out in duplicates.

3.1.10.4 EPS production

EPS production from lactose was determined following the method of Cogan et al., (1997) by qualitatively measuring the degree of "stringiness" of cultures which had been grown in RSM (10% w/v) (Oxoid) at 37 ^{o}C for 18 h. The culture was regarded as being EPS positive if the coagulated culture could be teased into a string with an inoculating loop. The analysis was carried out in duplicates.

3.1.10.5 Autolytic activity

Autolysis of the cells was measured in triplicate according to Mora et al., (2003). The strains were grown in MRS broth (Oxoid) for 24 h at 37 ^{o}C to reach an OD600 nm 0.8-1. The cells were washed in potassium phosphate buffer (50 mmol^{-1}, pH 6.5) and re-suspended in the same buffer to an OD_{600} nm of 0.6-0.8 and incubated at 30 ^{o}C. The degree of autolysis was expressed as the percentage decrease in the OD_{600} nm 24 h which was defined as follows: (A0 - At) x 100/A0 where A0: initial absorbance, and At: absorbance measured after 24 h of incubation.

Autolysis was ranked in accordance to the activity level of each genus: *lactobacilli*; good 70 – 96, fair 40 – 69, poor 0 – 39; and *enterococci*; good 35 – 66, fair 24 – 34, poor 0 - 22 as described by Ayad et al., (2004).

3.1.10.6 Diacetyl production

Diacetyl production was determined in duplicate according to King, (1948). Fresh overnight cultures (1% v/v) were inoculated in 10 mL of UHT milk and incubated at 37 ^{o}C for 24 h. One milliliter of each cell suspension was combined with 0.5 mL of a-naphthol (1% w/v) and KOH (16% w/v) (Sigma-Aldrich) and incubated at 30 ^{o}C for 10 min. Diacetyl production is indicated by the formation of a red ring at the top of the tubes. The intensity of the colour developed within 10 minutes was noted and assigned a score objectively using the scale below. The corresponding amount of diacetyl, in mg/100 ml of milk, is indicated in parentheses.

Score Colour intensity:

0 no pink colour (< 0.5 mg/100ml)

1 slightly pale pink (0.5 – 3 mg/100ml)

2 pale pink (3.1 – 10 mg/100ml)

3 red (10.1 – 30 mg/100ml)

4 dark red (> 30 mg/100ml)

3.1.11 Safety characteristics

3.1.11.1 Antibiotic susceptibility testing

The strains were grown overnight in MRS broth at 37 ^{o}C. For antibiotic resistant profile analysis, 20 mL of MRS agar was inoculated with revitalized strains (1% v/v) and allowed to solidify. Antibiotic disks (Oxoid) containing the antibiotics, erythromycin (15 mg/mL), rifampicin (30 mg/mL), streptomycin (10 mg/mL), tetracycline (30 mg/mL), penicillin (30 mg/mL), vancomycin (5 mg/ mL), gentamicin (10 mg/mL), ampicillin (2 mg/mL), trimethoprime/ sulphamethoxazole (5 mg/mL) and chloramphenicol (30 mg/mL) were then placed onto each agar plate. The plates were incubated at 37 ^{o}C for 24 h. The occurrence of a clear zone of inhibition around the disk indicated that the strain was susceptible to the antibiotic tested. The analysis was performed in triplicate and the results expressed as diameter of clear zone (mm) around the antibiotic disk. Based on the inhibition zone size, the results were interpreted as resistant (R), intermediate resistant (IR), or susceptible (S) to the antimicrobial agents according to the cut-off values and clinical breakpoints proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015) while the breakpoints of the Clinical and Laboratory Standards Institute (CLSI, 2015) were used for those antibiotics not included in EUCAST. *Enterococcus faecalis* ATCC 29212 was used as positive control.

3.1.11.2 Biogenic amine production

The production of biogenic amines by the selected LAB isolates was evaluated according to Joosten and Northolt, (1989). Decarboxylase production was induced by two consecutive transfers of 0.5 mL aliquots of the cultures into MRS broth (Oxoid) supplemented with pyridoxal-5- phosphate at 0.005% (w/v) (Sigma Aldrich) with purpose to promote enzyme induction and with each one of the biogenic amine precursors at 0.1% (w/v): tyrosine free base (for tyramine), histidine monohydrochloride

(for histamine), and ornithine monohydrochloride (for putrescine) (Sigma-Aldrich). Each culture was incubated 37 ^{o}C for 24 h, followed by streaking onto decarboxylase agar supplemented with each biogenic amine precursor 1% (w/v) as described above. Plates without amino acids served as controls. The plates were incubated at 37 ^{o}C for 3-7 days and positive results were recorded by color changing from yellow to purple or tyrosine precipitate disappeared around the colonies (Bover-Cid & Holzapfel, 1999).

3.1.11.3 Virulence activity using phenotypic and genotypic tests

Cultures of all the selected isolates were subjected to phenotypical tests to identify their virulence activity according to Perin et al., (2014). Gelatinase production was verified by spotting 1 μL aliquots of the 24 h cultures onto the surface of Luria Bertani (LB) agar (Oxoid) supplemented with 3% (w/v) gelatin (Oxoid) and incubated at 37 °C for 72 h. After incubation, the plates were maintained at 4 °C for 4 h and the hydrolysis of gelatin was recorded by the formation of opaque halos around the colonies. Hemolytic activity was assessed by streaking the cultures onto trypticase soy agar (Oxoid) supplemented with defibrinated horse blood at 5% (v/v) and incubated at 37 °C for 48 h. The hemolysis formed by each isolate was classified as total or β-hemolysis (clear halos around the colonies), partial or α-hemolysis (greenish halos around the colonies), and absent or γ-hemolysis. Lipase production was assessed by spotting 1 μL of cultures onto LB plates (Oxoid) supplemented with $CaCl_2$ (Sigma-Aldrich, at 0.2%, w/v) and Tween 80 (Sigma-Aldrich, at 1%, v/v) and incubated at 37 °C for 72 h. The formation of clear halos around the colonies was recorded as lipase production. DNAse was identified by spotting 1 μL aliquots of the cultures onto the surface of DNAse methyl green agar (Oxoid), and incubated at 37 °C for 72 h. Positive results were identified by the formation of clear halos around the colonies.

For checking the presence of genes encoding virulence, antibiotic resistance and amino acid decarboxylase activity, DNA was extracted using Bacterial DNA kit (Invitrogen) according to the manufacturer's instructions. Then the DNA concentration was estimated using NanoDrop2000 (ThermoFisher Scientific). The presence of the virulence, antibiotic resistance and biogenic amine related genes was determined using multiplex PCR for the following genes: gelE (gelatinase), cylA (cytolysin), hyl (hyaluronidase), asa1 (aggregation substance), esp (enterococcal surface protein), efaA (endocarditis antigen), ace (adhesion of collagen), vanA and vanB (both related to

vancomycin resistance), and genes for amino acid decarboxylases: hdc1 and hdc2 (both related to histidine decarboxylase), tdc (tyrosine decarboxylase), and odc (ornithine decarboxylase). The amplification products were analyzed by 1.5% agarose gel electrophoresis in 1X TAE buffer. Gels were stained with SYBR Safe DNA gel stain (Invitrogen, USA) and visualized under UV light. Primer sequences, annealing temperatures and fragment sizes are described in Table 18. The DNA of the reference strain *Enterococcus faecium* ATCC 29212 was used as positive control in the corresponding PCR reactions.

Table 18: List of primers used in this study

Target gene	Primers	Annealing Temp. (◦C)	Fragment size (bp)	Reference
Gelatinase (*gelE*)	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	47	213	Vankerckhoven et al. (2004)
Hyluronidase (*hyl*)	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	53	276	
Cytolysin (*cylA*)	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	52	688	
Aggregation substance (*asa1*)	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	50	375	
Enterococcal surface protein (*esp*)	AGATTTCATCTTTGATTCTTG AATTGATTCTTTAGCATCTGG	4	510	
Endocartidis antigen (*efaA*)	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC	57	688	Martín-Platero et al., (2009)
Adhesion of collagen protein (*ace*)	GAATTGAGCAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC	48	1008	
Vancomycin Resistance (*vanA*)	TCTGCAATAGAGATAGCCGC GGAGTAGCTATCCCAGCATT	52	377	
Vancomycin Resistance (*vanB*)	GCTCCGCAGCCTGCATGGACA ACGATGCCGCCATCCTCCTGC	60	529	
Histidine decarboxylase (*hdc*)	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	46	367	De las Rivas et al., (2005)
Histidine decarboxylase (*hdc2*)	AAYTCNTTYGAYTTYGARAARGARG ATNGGNGANCCDATCATYTTRTGNCC	50	534	
Tyrosine decarboxylase (*tdc*)	GAYATNATNGGNATNGGNYTNGAYCARG CCRTARTCNGGNATAGCRAARTCNGTRTG	55	924	
Ornithine decarboxylase (*odc*)	GTNTTYAAYGCNGAYAARCANTAYTTYGT ATNGARTTNAGTTCRCAYTTYTCNGG	54	1446	

3.2 Bacteriocin production from strains isolated from donkey milk

3.2.1 Bacterial strains and culture conditions

A total of 77 LAB strains previously isolated from donkey milk, and selected on the basis of their acidification and proteolytic activity, were included in this study. These strains were cultivated routinely on MRS broth at 37 °C and stored at 4 °C on agar slants.

The bacterial strains used as indicator organisms for the evaluation of antimicrobial activities and their growth conditions are listed in Table 19. The selected strains were chosen in order to represent a diverse range of Gram positive bacteria including foodborne spoilage and pathogenic bacteria. All cultures were streaked for purity on the appropriate media and permanent stocks were maintained in 40% glycerol at -20 °C and -80 °C.

Table 19: Bacterial strains used as indicators organisms

Indicator Strains	**Origin/ Characteristics**	**Growth Conditions***
Bacillus cereus DPC 6086	DPC culture Collection	BHI, 37°C
Bacillus cereus DPC 6089	DPC culture Collection	BHI, 37°C
Enterococcus faecium DPC 1146	DPC culture Collection	GM17, 37°C
Enterococcus faecium DPC 5119	DPC culture Collection	GM17, 37°C
Lactobacillus acidophilus 4356	ATCC Type Strain	MRS+1% lactose+20mM $CaCl_2$, 42°C
L*actobacillus bulgaricus* 1373	UCC Culture Collection/Bacteriocin sensitive indicator	MRS+1% lactose+20mM $CaCl_2$, 42°C
Lactobacillus bulgaricus HE	UCC Culture Collection/Bacteriocin sensitive indicator	MRS+1% lactose+20mM $CaCl_2$, 42°C
Lactobacillus helveticus UCC 505	UCC Culture Collection/Bacteriocin sensitive indicator	MRS+1% lactose+20mM $CaCl_2$, 42°C
Lactococcus cremoris IP 5	UCC Culture Collection/Bacteriocin sensitive indicator	GM17, 30°C

Lactococcus cremoris KH 745	UCC Culture Collection/Bacteriocin sensitive indicator	GM17, 30°C
Lactococcus lactis 275	UCC Culture Collection/Bacteriocin sensitive indicator	GM17, 30°C
Lactococcus lactis 303	UCC Culture Collection/Bacteriocin sensitive indicator	GM17, 30°C
Lactococcus lactis DPC 4268	DPC culture Collection/Bacteriocin sensitive indicator	GM17, 30°C
Lactococcus lactis HP	UCC Culture Collection/Bacteriocin Sensitive indicator	GM17, 30°C
Lactococcus lactis MG 1363	UCC Culture Collection/Bacteriocin Sensitive indicator	GM17, 30°C
Listeria monocytogenes 33013	UCC Culture Collection/Massachusetts Outbreak 1985 (*L. mono* Scott A)	BHI, 37°C
Listeria monocytogenes 33104	UCC Culture Collection/Clinical Isolate California Outbreak 1985 (*L. mono* F2365)	BHI, 37°C
Listeria monocytogenes 33410	UCC Culture Collection/ Clinical Isolate California Outbreak 1985 (*L. mono* F4565)	BHI, 37°C
Listeria monocytogenes 33411	UCC Culture Collection/Clinical Isolate Halifax, Canada Outbreak 1981 (L. mono Ts50)	BHI, 37°C
Listeria monocytogenes 33413	UCC Culture Collection/Food Isolate United Kingdom outbreak (*L. mono* Ts45)	BHI, 37°C
Listeria monocytogenes 33423	UCC Culture Collection/Food Isolate	BHI, 37°C
Listeria monocytogenes CD 1078	UCC Culture Collection/Chicken Isolate	BHI, 37°C
Listeria monocytogenes LO28	UCC Culture Collection/ Clinical isolate (Faeces of healthy pregnant woman)	BHI, 37°C
Micrococcus luteus DSM1790	German Collection of Microorganisms and Cell Cultures(DSMZ)/Bacteriocin sensitive indicator	TSB with 0.3% (w/v) yeast extract, 37°C
Staphylococcus aureus SA 113	UCC Culture Collection/Representative staphylococcal organism in model virulence studies	BHI, 37°C
Staphylococcus aureus NCDO 1499	National Collection of Dairy Organisms	BHI, 37°C
Staphylococcus aureus	UCC Culture Collection	BHI, 37°C

5971		
Staphylococcus aureus DPC 5243	DPC culture Collection/ Mastitis-associated indicator organism	BHI, 37°C
Staphylococcus aureus DPC 5247	DPC culture Collection/ Mastitis-associated indicator organism	BHI, 37°C
Staphylococcus aureus Newman	UCC Culture Collection	BHI, 37°C
Staphylococcus aureus RF 122	UCC Culture Collection /Represents the most common *S. aureus* clone derived from bovine mastitis worldwide	BHI, 37°C
Streptococcus agalacticae ATCC 13813	ATCC Type Strain/Lancefield GrpB	TSB with 0.3% (w/v) yeast extract, 37°C
Streptococcus dysgalacticae ATCC 43078	ATCC Type Strain/ Quality control strain, cow isolate, Lancefield GrpC	TSB with 0.3% (w/v) yeast extract, 37°C
Steptococcus uberis ATCC 700407	ATCC Reference Strain/ Quality control strain for API products (API 78-11-025)	TSB with 0.3% (w/v) yeast extract, 37°C
*All microbiological media purchased from Oxoid, Basingstoke, UK DPC: Dairy Products Research Centre Moorepark		

3.2.2 Antimicrobial activity assay

Two different antagonism assays were performed, i.e. spot-on-lawn assay and well diffusion assays according to Field et al., (2008). For the initial screen, 74 strains stored in 96 well plates were spotted on GM17 agar plates using a 96 pin replicator (Boekel) and allowed to grow overnight at the appropriate temperature. Following overnight growth the strains were subjected to UV radiation for 30 min prior to overlaying with GM17, BHI or TSB agar (0.75% w/v agar) seeded with the appropriate indicator. Zone size was calculated as the diameter of the zone of clearing minus the diameter of bacterial growth (5mm) (Table 21). Bacterial candidates of interest, bacterial colonies that displayed antimicrobial activity were also tested using well-diffusion assays. For well diffusion assays molten agar was cooled to 48 ^{o}C, and 50 µl culture of the indicator strain, grown to the early stationary phase in the appropriate medium, was added to 20 ml media. The plates were allowed to solidify and dry before making wells (4.6 mm in diameter) in the seeded plates. Aliquots (50 µl) of the cell free supernatant of the isolated strains obtained by centrifugation at 16000 rpm for 15 min at 4 °C were dispensed into the wells and the plates incubated at the appropriate temperature. To

eliminate the possibility of a zone of inhibition due to acid production, all culture supernatants in both assays were adjusted to pH 7.0 with 1 N NaOH and filter-sterilized (0.45 μm). Zone size was calculated as the diameter of the zone of clearing minus the diameter of the well (4.6 mm), using an electronic caliper with digital display.

3.2.3 Effect of proteolytic enzymes

Sensitivity to proteolytic enzymes was tested according to the method described by Ryan et al., (1996) with slight modifications. Briefly, the enzymes were pepsin, α-chymotrypsin, trypsin and pronase (Sigma-Aldrich Chemie, Steinheim, Germany) were dissolved in sterile 100 mM Tris-HCl-10 mM $CaCl_2$ to final concentrations of 100 μg/ml, at their optimum pH i.e. 8.2, 7.8, 7.8 and 7.0, respectively. All enzyme solutions were filter sterilized. 50 μl aliquots of cell-free supernatant of bacteriocin producing strains and 50 μl of each enzyme were added into two separate wells that were 1 cm apart on GM17 agar plates seeded with *E. faecium* DPC 5119 as the indicator strain (most bacteriocin-sensitive strain). All plates were incubated overnight at 37 °C. Protease sensitivity was observed as a half-moon-shaped zone of inhibition.

3.2.4 Effect of pH, organic solvent and heat treatment

The stability of antimicrobial-containing cell free supernatant of the selected strains following exposure to a variety of treatments was assessed. The pH of the cell free supernatant was measured using a pH meter (Hannah Instruments, RI, USA) and adjusted to a range of pH 2-8 using dilute 0.5 M HCl and 1 M NaOH and kept at room temperature for 5 hours in order to assess the pH range over which the antimicrobials were active. Negative controls consisted of buffers at the different pH values used. To assess temperature stability, samples were heat treated at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C , 90 °C and 100 °C for 20 mins each. Also autoclave temperature (121 °C) for 15 min was used. To determine the sensitivity of the supernatants to solvents, 300 μl of supernatant was combined with 700 μl ethanol, 70% acetonitrile, methanol, and isopropanol, respectively. Following treatments the level of antimicrobial activity retained was assessed via well diffusion using *E. faecium* DPC 5119 as indicator.

3.2.5 Effect of medium composition on bacteriocin production

The effect of different concentrations of medium ingredients on bacteriocin production was evaluated using two different media, BHI and TSB with glucose. Well diffusion assays of cell free supernatant were carried out as above.

3.2.6 Bacteriocin Purification

Bacteriocin purification was performed by reverse phase (RP)-HPLC according to method described by Field et al., (2008), with slight modifications. MRS broth was clarified, before autoclaving at 121 °C for 15 minutes, by passing through a column containing isopropanol washed Amberlite XAD16 beads (Sigma- Aldrich) (a nonionic chelating resin that adsorbs and releases ionic species through hydrophobic and polar interactions) to remove hydrophobic constituents of the media prior to growth of the producing strain. These constituents would interfere with bacteriocin binding to the resin, thus the removal of such constituents aids in higher yields of bacteriocin during the purification process. Then, MRS broth inoculated with 1% of an overnight culture of producing strain and incubated overnight at 37 °C. The culture was centrifuged at 7000 rpm for 20 min at 4 °C. The supernatant was decanted and passed through 60 g of pre equilibrated Amberlite XAD16 beads (Sigma-Aldrich). The beads were washed with 500 ml 30% ethanol (v/v) and eluted with 250 ml 70% isopropanol containing 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich). The bacteriocin antibacterial activity after each step was assayed as described above with *E. faecium* DPC5119 as the sensitive indicator. The isopropanol was removed through rotary evaporation (Buchi R-300, Switzerland) and the sample pH adjusted to 4 before being applied to a 10 g Phenomenex SPE C-18 column pre-equilibrated with methanol and then water. The columns were washed with 120 ml of 30% ethanol and the inhibitory activity was eluted in 60 ml of 70% isopropanol 0.1% TFA. 10 ml of each sample was concentrated to 2 ml through rotary evaporation, and this was subsequently applied to a Phenomenex C12 reverse phase (RP)-HPLC column (Jupiter 4 m proteo 90 A, 250 x 10.0 mm, 4μm) previously equilibrated with 25% acetonitrile, 0.1% TFA. To facilitate this, a gradient of 30–80% acetonitrile containing 0.1% TFA was developed, from 5 to 40 min at a flow rate of 3 ml/min. Absorbance was monitored at a wavelength of 214 nm. 50 μl of eluted fractions (9 to 40 mins and collected at 1 min intervals) were evaluated for their

antibacterial activities against the indicator strain *L. inoccua* UCC 504 and *E. faecium* DPC 5119.

The molecular weight of biologically active fractions was measured by a MALDI-TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) according to the procedure described by Cotter et al., (2006). Antimicrobial activity of the purified bacteriocin was tested using well diffusion assays as described above.

3.2.7 Bacteriocin inhibitory assay in culture broth

Effect of purified bacteriocin on sensitive cells was studied according to Favaro et al., (2014) with some modifications. Briefly, 10 ml of the appropriate broth was inoculated with 1% (v/v) of the overnight cultures of selected indicator strains: *B. cereus* DPC 6086, *L. monocytogenes* 33411, *L. monocytogenes* 33423, *L. monocytogenes* CD 1078, *S. aureus* DPC 5243, S. *aureus* 5971 and *S. aureus* RF 122 respectively and incubated for 3 h at 37 ^{o}C. Twenty microliters of purified bacteriocin were added to the culture and OD_{620} was recorded every hour for next 24 h. Controls represented growth of the indicator strains without the addition of purified bacteriocin. The assay was carried out in triplicates.

3.2.8 PCR amplification of bacteriocin genes

PCR of genomic DNA of bacteriocin producing strains was performed following lysis of cells in 10% Igepal CA-630 (Sigma-Aldrich Chemie, Steinheim, Germany) at 94 °C for 10 min. PCR amplification was carried out with specific primers encoding the 5'- and 3'- terminal regions of the structural genes encoding the mature portions of the different enterocins listed in Table 20, using the conditions described by De Vuyst et al., (2003). Oligonucleotide primers were obtained from Sigma-Aldrich. Amplification reactions (35 cycles) were performed on a PTC-200 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). The reaction mixture (50 µl) consisted of 20.75 µl PCR- quality water, 5 µl of 10X PCR Reaction buffer, 0.25 µl of 5U Taq DNA, 2 µl of (25 mM) $MgCl_2$, 8 µl of dNTPS (10 mM), 2 µl of each primers and 10 µl of template DNA. The presence and molecular size of the PCR products were analysed by electrophoresis on 1.5% (w/v) agarose gels in 1X TAE buffer. The gel was run at 100 V for approximately 30 min, using 100 bp ladders as the molecular weight standard. Agarose gel was stained

in 100 ml 1X TAE buffer containing 5 µl of midori green. The gel was photographed under UV light.

Table 20: Specific primers for PCR amplification of enterocin structural genes

Gene	Primer sequences (5' to 3')	Product size bp	References
Enterocin A	F:GGT ACC ACT CAT AGT GGA AA R:CCC TGG AAT TGC TCC ACC TAA	138	Aymerich et al., 1996
Enterocin B	F: CAA AAT GTA AAA GAA TTA AGA TCG R: AGA GTA TAC ATT TGC TAA CCC	201	Casaus et al., 1997
Enterocin P	F: GCT ACG CGT TCA TAT GGT AAT R: TCC TGC AAT ATT CTC TTT AGC	87	Cintas et al., 1997
Enterocin HF	F: GGA AAA ATT AAC TGT GAA AGA AAT R: TTA ACC TTT CCA CCC TGC TTT TC	174	This study
Enterocin L50A	F: CCA TGG GAG CAA TCG CAA AA R: AAG CTT AAT GTT TTT TAA TCC ACT CAA T	135	Batdorj et al., 2006
Enterocin L50B	F: ATG GGA GCA ATC GCA AAA TTA R: TAG CCA TTT TTC AAT TTG ATC	252	Cintas et al., 1998
Enterocin Q	F: ATGAATTTTCTTAAAAATGG TATCGCAAAATG R: TTAACAAGAAATTTTTTCCCATGGCAA	105	Cintas et al., 2000
Enterocin AS-48	F: GAG GAG TAT CAT GGT TAA AGA R: ATA TTG TTA AAT TAC CAA	339	De Vuyst et al., 2003
Enterocin 31	F: CCT ACG TAT TAC GGA AAT GGT R: GCC ATCG TTG TAC GGA AAT GGT	135	De Vuyst et al., 2003

3.2.9 Challenge test

3.2.9.1 Sample preparation and inoculum

To study the ability of bacteriocin producing enterococci to control *L. monocytogenes* in cheese, two independent batches of cheeses containing 9 containers for each trial were conducted as below:

A Anari cheese not artificially contaminated (control)

B Anari cheese with *L. monocytogenes* 33413(control)

C Anari cheese with *E. faecium* DM33 (control)

D Anari cheese with *E. faecium* DM224 (control)

E Anari cheese with *E. faecium* DM270 (control)

F Anari cheese with *E. faecium* DM33 and *L. monocytogenes*

G Anari cheese with *E. faecium* DM224 and *L. monocytogenes*

H Anari cheese with *E. faecium* DM270 and *L. monocytogenes*

I Anari cheese with *E. faecium* DM118 bacteriocin negative and *L. monocytogenes*

Fresh unsalted Anari cheese (200 g portions, in plastic containers with a lid) was purchased directly from the manufacturer on the first day of their shelf life. In trial A, the cheese not artificially contaminated was assessed at the beginning of the test in order to determine the total viable counts on plate count agar (PCA) and absence of *L. monocytogenes* (Oxford Agar) and *enterococci* (MRS).

Overnight cultures (10 ml) at 37 °C in BHI broth of *L. monocytogenes* 33413 and in MRS broth for *E. faecium* DM33, DM224 and DM270 were centrifuge at 12000 rpm for 15 min at 4 °C. The supernatant was decanted and the pellet washed twice with PBS and re-suspend in 10 ml of the same buffer. The final suspensions were submitted to serial dilutions and plated on BHI for *Listeria* or MRS agar for *enterococci* in order to determine the number of viable cells. Then, cheese samples were artificially contaminated with *L. monocytogenes* (3 log cfu/g) and bacteriocin producing strains (6 log cfu/g). The volume of the inoculum injected into each container was 5 ml and thoroughly homogenized by means of a sterile spatula to be evenly distributed in the cheeses. All samples were stored at 4 ^{o}C for 9 days. The study was limited to nine days, in accordance with shelf-life recommendations by the manufacturer.

3.2.9.2 Bacterial counts and pH

After 2 h of inoculation and then after 1,3,6 and 9 days of storage 10 g (in triplicate) of each sample was aseptically weighted and diluted in 90ml of sterile saline solution (0.85% w/v NaCl) and homogenized for 120 s in a stomacher, prior to the preparation of 1/10 serial dilutions for microbiological analysis. The following microbial parameters were analysed; aerobic mesophilic plate counts were determined on plate count agar (PCA, Oxoid, Basingstone, Hampshire, UK), incubated at 30 ^{o}C for 72 h (ISO, 2013); LAB were grown in MRS agar incubated at 37 ^{o}C for 48h, and *L. monocytogenes* were

counted by spread plating on Oxford agar (Oxoid) and incubating at 37 ^{o}C for 48h. Especially for Oxford Agar 1 ml (0.25 ml x 4 times) samples from the first dilution were also spread on four respective agar plates to reduce the lowest detection limit of the analysis to 1 log cfu/g of cheese. At the day of inoculation, for *L. monocytogenes* enumeration, culture enrichment was used. The enrichment step was done according to ISO 11290-2 (ISO, 1998) with some modifications, whereas 25 g of cheese samples added to 225 ml of Half Fraser and incubated at 37 ^{o}C for 24h. Then 1 ml of the enrichment was plated (0.25 ml x 4 times) on Oxford Agar and incubated at 37◦C. Microbial counts results were converted to log cfu/g and the means and standard deviations (n=3) were calculated.

At each sampling for bacterial counts, 10 g of cheese samples were homogenized in 0.1% peptone water and the pH was measured using pH meter (Hannah Instruments, USA).

3.2.9.3 Bacteriocin detection in cheese samples

Bacteriocin production was evaluated in all samples according to method described by Ryan et al., (1996). Bacteriocin activity in cheese samples during storage was determined as follows. Cheese samples were mixed with equal volumes of distilled water in a stomacher for 15 min. The resulting suspension was heated to 80 ^{o}C for 10 min. Then aliquots of 50 µl were dispensed in wells, and bacteriocin activity was assayed as outlined above.

3.3 Probiotic potential

3.3.1 Resistance to low pH

Resistance to pH 3.0 was evaluated by monitoring bacterial growth on 96 well plates according to the method of Chenoll et al., (2011) with some modification. Bacterial cells from overnight (18 h) cultures were harvested by centrifugation (10,000g, 5 min, 4 ^{o}C), washed twice with PBS buffer (pH 7.2) before being resuspended in MRS broth of pH 3.0. Resistance was assessed in triplicates by measuring the absorbance (OD_{600}) using spectrophotometer (Infinite PRO 200, Tecan, Switzerland) in hourly intervals for 3 h at 37 ^{o}C, which reflects the time food spends in the stomach. The survival

percentage was calculated as follows: % survival = Abs_t/control × 100, where Abs_t is the absorbance at different times.

3.3.2 Bile salt tolerance

The ability of the isolates to grow in the presence of bile salts was determined according to García-Ruiz et al., (2014). Bacterial cells from fresh overnight (18 h) cultures were inoculated (1% v/v) in MRS broth containing 0.3% (w/v) bile salts (Sigma- Aldrich). The absorbance (OD_{600}) was measured using spectrophotometer (Infinite PRO 200, Tecan, Switzerland) in hourly intervals for 4 h at 37 ^{o}C which reflects to the time food spends in the small intestine. The survival percentage was calculated as follows: % survival = Abs_t/control × 100, where Abs_t is the absorbance at different times. Samples without addition of bile salts were used as controls. Assays were carried out in triplicate.

3.3.3 Cell hydrophobicity assay (Microbial adhesion to hydrocarbons)

The degree of hydrophobicity of the strains to two hydrocarbonds hexadecane and xylene was determined by employing the method described by Rosenberg et al., (1980) with slight modifications. Cultures were grown in MRS broth at 37 ºC for 24 h and centrifuged at 7500 rpm for 6 min at 4 ^{o}C. Cells were washed twice in Phospate buffer saline (0.1M PSB, pH 7.2) and re-suspended in the same buffer. The absorbance at 600 nm was measured. Then 3 ml of cell suspension was mixed with 1 ml of the each hydrocarbon. After a 10 min pre-incubation at room temperature, the two phase system was mixed thoroughly by vortexing for 2 min. The two phases were allowed to separate for 30 min at room temperature and absorbance of the aqueous phase was measured at 600 nm. Hydrophobicity determinations were done in triplicate. Affinity to hydrocarbons (hydrophobicity) was reported as the average percentage of 3 replicates, according to the following equation Hydrophobicity% = $[(A_0-A_{final})/A_0] \times 100$, where A_0 and A_{final} are the absorbance before and after mixing with the hydrocarbons, respectively. Assays were carried out in triplicate. Lyophilised culture of *Lactobacillus acidophilus* LA5 (Christian Hansen, Hørsholm, Denmark) was used as the positive control.

3.3.4 Auto-aggregation assay

Aggregation of the bacterial cultures was screened using a spectrophotometric assay, as described by Collado et al., (2008). Overnight cultures were centrifuged and washed twice with 0.1M PBS (pH 7.2) buffer and suspended in the same buffer. The absorbance of the supernatant was measured at 600 nm. For the auto-aggregation assay, each bacterial suspension was vortexed for 10 s and incubated at room temperature for 5 hours. At each interval, the growth was measured at OD_{600} nm. Auto-aggregation percentage was determined by A_{600} according to the following equation: Auto-aggregation %=1- (At/Ao)x100 At represents the absorbance at different times (t=1,2,3,4,5 hrs) and A0 the absorbance t =0. Assays were carried out in triplicate. Lyophilised culture of *Lactobacillus acidophilus* LA5 (Christian Hansen, Hørsholm, Denmark) was used as the positive control.

3.3.5 Co-aggregation assay

The co-aggregation test was performed using bacterial suspensions prepared as described for the auto-aggregation analysis, according to method described by Collado et al., (2008). For the coaggregation assay, equal volumes (500 µl) of potential probiotic strain and pathogen strains (*Listeria monocytogenes* 33413, *Staphylococcus aureus* RF 122) were mixed, vortexed for 10 s and incubated at room temperature for 5 h. The absorbance at 600 nm of the suspensions was measured after mixing, 2 and after 5 hours of incubation at room temperature. The percentage of coaggregation was calculated using the following equation: Coaggregation%= $[(A_{pat} + A_{probio})/2 - (A_{mix})/(A_{pat} + A_{probio})/2] \times 100$,

where A_{pat} and A_{probio} represent A_{600} of the separate bacterial suspensions in control tubes and A_{mix} represents the absorbance of the mixed bacterial suspension at different times tested. Assays were carried out in triplicate. Lyophilised culture of *Lactobacillus acidophilus* LA5 (Christian Hansen, Hørsholm, Denmark) was used as the positive control.

3.3.6 Bile salt hydrolase activity

The ability of potentially probiotic isolated strains to hydrolyse bile salt was assayed using a plate test according to the method described by Bhardwaj et al., (2010). For this,

10 µl of an overnight culture was spotted onto MRS agar plates (pH 6.5) containing 0.5% (w/v) sodium salt of taurodeoxycholic acid and 0.37 g/l $CaCl_2$. Plates were incubated anaerobically at 37 ^oC for 72 hours. A strain was considered bile salt hydrolase positive if a white zone of precipitation occurred which surrounded the colony. Assays were carried out in triplicate. Lyophilised culture of *Lactobacillus acidophilus* LA5 (Christian Hansen, Hørsholm, Denmark) was used as the positive control.

3.4 Production of a functional fermented donkey milk drink

3.4.1 Bacterial cultures and growth conditions

The bacterial strains used in this study are listed in Table 21. The selection of the LAB strains was based on their technological, probiotic potential, bacteriocin production and safety profile. All strains were maintained as frozen stock at -80 °C in MRS or BHI broth supplemented with 40% glycerol. Prior to the experimental use, the cultures were twice propagated in MRS or BHI medium and incubated at 37 ^oC for 24 h.

Table 21: Bacteria strains used in this study

Microorganism	**Growth conditions**
Bacillus cereus DPC6089	BHI, 37 °C
E. coli NCTC9000	BHI, 37 °C
Enterococcus faecium DM18	MRS, 37 °C
Enterococcus faecium DM224	MRS, 37 °C
Enterococcus faecium DM270	MRS, 37 °C
Enterococcus faecium DM33	MRS, 37 °C
Enterococcus gallinarum DM150	MRS, 37 °C
Enterococcus lactis DM237	MRS, 37 °C
Enterococcus mundii DM246	MRS, 37 °C
Lactobacillus casei DM214	MRS, 37 °C
Lactobacillus Helveticus JM1004	MRS, 37 °C
Leuconoctoc mesenteroides DM236	MRS, 37 °C
Listeria monocytogenes 33413	BHI, 37 °C
Listeria monocytogenes CD 1078	BHI, 37 °C
Salmonella typhimurium NCTC12023	BHI, 37 °C

Staphylococcus aureus DPC 5247	BHI, 37 °C
Staphylococcus aureus RF122	BHI, 37 °C

3.4.2 Production of fermented milks

Fermented milk was prepared in triplicates as previously described (Muguerza et al., 2006). The process flow diagram for the production of fermented donkey milks is shown in Figure 17. Briefly, pre-cultures of the strains were prepared in sterile donkey milk incubated overnight at 37 ^{o}C to reach an initial bacterial concentration of 10^{7}–10^{8} colony forming units cfu/ml. A 3% (v/v) of corresponding pre-culture was added to sterile donkey milk and fermentation was carried out during 48 h at 37 ^{o}C (until pH drops to 4.6). Each fermented product was prepared with a single bacterial strain. Bacterial growth through fermentation was determined by plate count on MRS agar after incubation at 37 ^{o}C for 48 h. The fermentation process was stopped by pasteurization of the fermented milk at 75 ^{o}C for 1 min. At the end of the fermentation, the pH of the fermented milk was directly measured with a pH-meter (Hanna, pH meter).

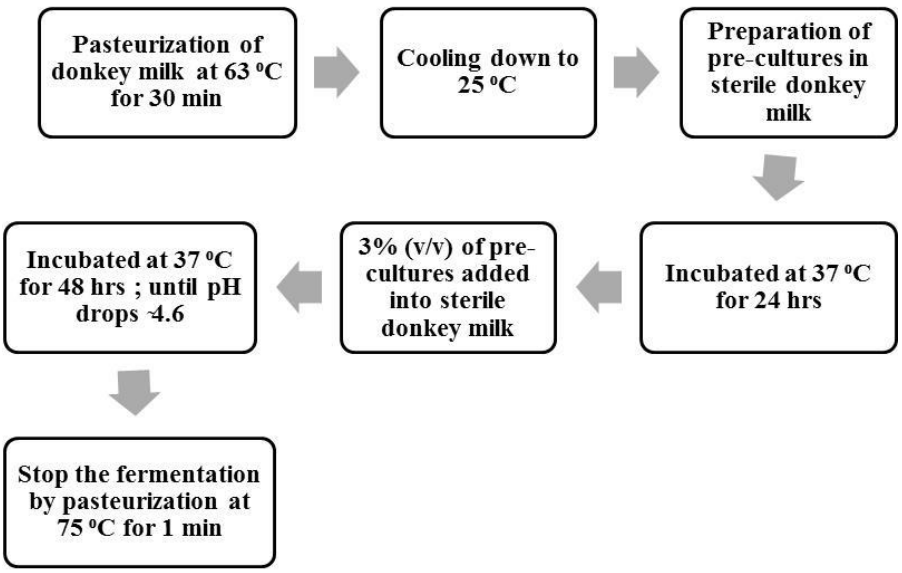


Figure 17: Flow diagram for the production of fermented donkey milk with starters isolated from raw donkey milk

3.4.3 Preparation of the sample extracts

The water-soluble extract of the fermented milk was obtained by centrifugation at 20000 *g* for 20 min at 4 ^{o}C and by filtration through a Whatman no. 40 filter. The supernatants from milk fermentation samples were stored at -20 °C for further analysis.

3.4.4 *In vitro* digestion of samples

Samples were subjected to *in vitro* digestion according to the protocol recently described by Minekus et al., (2014). This model was developed by the COST action FA1005 INFOGEST and is based on human gastrointestinal physiologically relevant conditions.

The protocol is based on the use of 3 simulating fluids, saliva at pH 7 (SSF), gastric juice at pH 3 (SGF) and intestinal (duodenal) juice at pH 7 (SIF). The simulating fluids are prepared by dissolving different salts and acid-base solutions as reported in the following tables (Table 24 and Table 25).

Table 22: Composition of stock solutions of simulated digestion fluids

			SSF pH7		SGF pH3		SIF pH7	
Constituent	**Stock conc.**		**Vol. of stock**	**Conc. in SSF**	**Vol. of stock**	**Conc. in SSF**	**Vol. of stock**	**Conc. in SSF**
	g/L	**mol/L**	**mL**	**mmol/L**	**mL**	**mmol/L**	**mL**	**mmol/L**
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.3	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H2O)_6$	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	-	-
$CaCl_2(H_2O)_2$	44.1	0.3		1.5		1.5		0.6
HCl	6		0.09	1.1	1.3	15.6	0.7	8.4

SSF: Simulated Salivary Fluids; SGF: Simulated Gastric Fluid; SIF: Simulated Intestinal Fluid.

The concentrations correspond to 400ml and final volume was up to 500 ml after addition of the enzymes, bile and $CaCl_2(H_2O)_2$, during in vitro digestion. The necessary volume of $CaCl_2(H_2O)_2$ was added to the final mixture of the digestion medium to prevent precipitation.

Table 23: Enzymatic solutions used in this study. They prepared by dissolving the enzymes in the proper stimulating fluid

Simulated digestion human phase	Enzyme	Concentration, U/ml* or mM**
oral	α-amylase	1500*
gastric	Pepsin	25000*
intestinal	Pancreatin	800*
intestinal	Bile	160**

Oral phase (final ratio of food to SSF of 50 : 50 (w/v)). 13 g of donkey milk samples were mixed with 875 μL of SSF electrolyte stock solution and minced together. Then, 125 μL salivary a-amylase solution of 1500 U/ml made up in SSF electrolyte stock solution (α-amylase from porcine pancreas Type VIB, ≥10 units/mg solid) is added. Then 6.25 μL of 0.3 M $CaCl_2$ $(H_2O)_2$ and 243.5 μL of water were added and thoroughly mixed. The sample was incubated for 2 minutes at 37 °C at 150 rpm in an orbital shaker (Stuart, UK).

Gastric phase (final ratio of food to SGF of 50 : 50 (v/v)). Oral bolus (14.25 ml) was mixed with 1.875 mL of SGF electrolyte stock solution. Then, 400 μL porcine pepsin stock solution of 25000 U/mL made up in SGF electrolyte stock solution (pepsin from porcine gastric mucosa, powder, ≥250 units/mg solid, P7000 Sigma) is added, followed by 1.25 μL of 0.3 M CaCl2, 50 μl of 1 M HCl to reach pH 3.0 and 173.75 μl of water. The sample was placed into the shaker and incubated for 2 h at 37 °C at 150 rpm.

Intestinal phase (final ratio of gastric chyme to SIF of 50 : 50 (v/ v)). Gastric chyme (16.75 ml) is mixed with 2.75 mL of SIF electrolyte stock solution, 1.25 mL of a pancreatin solution 800 U/ml made up in SIF electrolyte stock solution based on trypsin activity (Pancreatin from porcine pancreas, 8 × USP specifications, P7545 Sigma), 625 μL fresh bile (160 mM in fresh bile), 10 μL of 0.3 M $CaCl_2$, 37.5 μL of 1 M NaOH to reach pH 7.0 and 327.5 μL of water. The sample was incubated on an orbital shaker for 2 h at 37 °C, 150 rpm.

pH was monitored and adjusted, if necessary, at 7, 3 and 7, for the oral, gastric and duodenal phase, respectively. The reaction was stopped by heating the test tubes for 15 minutes at 95°C. Then samples were centrifuged for 40 minutes at 4 °C and separated from pellets. The aqueous supernatant was used for the following experiments. Control

digestions without samples were carried out as well. Samples were stored at -18 °C until analyzed. *In vitro* digestion was carried out in triplicates for each sample.

3.4.5 Determination of protein content

The protein concentration was determined in triplicate using Qubit Protein Assay Kit (Invitrogen). A working solution containing 0.5% of fluorophore (QUBIT Protein Reagent, 200x concentrate in PEG) in sample buffer (QUBIT Protein Buffer Invitrogen Molecular Probes) was dosed in eppendorf of 1.5 ml volume (QUBIT, Molecular Probes assay tubes), respectively 198 µl for the samples tubes and 190 µl for the three peptides standards tubes. Then, 2 µl of each sample and 10 µl of each peptide standard were added (Quant-it Protein Standard #1,#2 and #3, Component C, D and E respectively 0, 200 and 400 ng/ul, in TE buffer, contains 2mM azide, Q33212) in the respective working solution eppendorfs. After vortexing and 15 min dark incubation at room temperature, the peptide concentration was measured by QUBIT fluorimeter, Invitrogen, Molecular Probes.

3.4.6 Bioactivities of samples

3.4.6.1 Antioxidant activity

The antioxidant activity of ferment d milks before and after *in vitro* human digestion were evaluated using ABTS and DPPH assay.

*3.4.6.1.1 ABTS *2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) Assay*

The antioxidant activity of fermented milk samples and samples after simulated digestion was assayed according to the method described by Re et al., (1999) with some modifications. 2,2′-Azino-bis (3-ethylbenzothiazoline- 6-sulphonic acid) radical cation (ABTS•+) was produced by dissolving 7 mM ABTS stock solution with 2.45 mM potassium persulphate and keeping the mixture in the dark at room temperature for 12–16 h before use to provoke the formation of ABTS radical. The solution was then diluted in water to reach an absorbance of 0.70 + 0.02 at 734 nm. 25 µl of sample or trolox (6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid) as positive control was added to 975 mL of diluted ABTS•+ solution and incubated at 30 °C for 6 min.

Then scavenging of the ABTS•+ radical was measured spectrophotometrically (Infinite PRO 200, Tecan, Switzerland) in absorbance at 734 nm. A solvent blank was run with each assay (negative control). All determinations were carried out in triplicate, and their average was used as a datum point. A calibration curve was plotted of absorbance reduction and concentration of the Trolox standard. The ABTS scavenging capacity of the test samples was expressed as Trolox equivalent antioxidant capacity (TEAC) in mM per 100 ml of sample (mM TEAC/100mL).

3.4.6.1.2 DPPH (2, 2 diphenyl - 1 -picryl hydrazyl) Assay

The antioxidant activity of samples before and after gastrointestinal digestion was analyzed using DPPH Assay given by (Brand-Williams et al., 1995) with some modifications. The DPPH was used at a concentration of 60 mol/L, dissolved in methyl alcohol. The prepared solution was used only on the day of analysis. In the dark, aliquots of 0.1 mL sample were transferred to test tubes with 3.9 mL radical DPPH (60 mol/L DPPH solution) and homogenized by shaking and incubated for 30 min in the dark, at room temperature. The absorbance of each sample at 517 nm was measured spectrophotometrically (Infinite PRO 200, Tecan, Switzerland). Methanol was used as a blank, while DPPH solution in methanol served as control. The Trolox standard curve was used for determination of Trolox equivalent antioxidant capacity (TEAC) and the results were expressed as Trolox equivalent antioxidant capacity (TEAC) in mM per 100 ml of sample (mM TEAC/100mL). The standard curve was performed using DPPH concentrations from 0 to 60 mol/L. The experiment was performed in triplicate.

3.4.6.2 Antimicrobial activity

A well diffusion assay was performed according to Field et al., (2008) using the indicator strains listed in Table 23. For well diffusion assays molten agar was cooled to 48 °C, and 50 µl culture of the indicator strain, grown to the early stationary phase in the appropriate medium, was added to 20 ml media. The plates were allowed to solidify and dry before making wells (4.6 mm in diameter) in the seeded plates. Aliquots (50 µl) of the cell free supernatant of the isolated strains obtained by centrifugation at 16000 rpm for 15 min at 4 °C were dispensed into the wells and the plates incubated at the appropriate temperature. Zone size was calculated as the diameter of the zone of clearing minus the diameter of the well (4.6 mm).

3.4.6.3 Determination of angiotensin-I-converting enzyme (ACE)-inhibitory activity

ACE inhibitory activity was determined according to Gonzalez-Gonzalez et al., (2011) with some modifications. Briefly, a solution of hyppuryl–histidil–leucine (HHL, 5 mM) was prepared in sodium phosphate buffer (NaPB, 0.1 M, pH 8.2) containing NaCl (0.3 M). ACE (Angiotensin Converting Enzyme from rabbit lung, 1 unit/ml, EMD Millipore) was prepared (1 U/mL) with potassium phosphate buffer (KPB, 0.01 M, pH 7) containing NaCl (500 mM). For each analysis, to 40 µl of sample, 100 µl of HHL and 10 µl of ACE solutions were added in an Eppendorf. Samples were incubated for 60 min at 37 °C, then 125 µl of HCl (1N) were added to stop the reaction. Each sample was analyzed in double and the procedure was repeated for all the different sample dilutions The concentration of HA produced at the end of the reaction was determined by UV-high performance liquid chromatography (HPLC-UV) (Alliance 2695 separation module, Waters, Billerica, MA, USA) equipped with absorbance detector mod. 2487 (Waters). The conditions were the following: column, KNAUER Eurospher II 100-3 C18 P, 150 x 2 mm; gradient elution, eluent A, water with 0.1% formic acid and 0.2% acetonitrile; eluent B, acetonitrile with 0.1% formic acid; gradient, 0-10 min, 100% A; 10-32.50 min, linear to 100% B; 32.50-47.50 min, isocratic 100% B; 47.50-48.50 min, linear to 100% A; 48.50-60 min, isocratic 100% A; column temperature, 35°C; injection volume, 10 µl; run time, 60 min; flow rate, 0.2 mL/min; detector UV, λ = 228. Data were processed with the Empower software (Waters Co., Milford, MA, USA). Captopril (Sigma-Aldrich Company, Dorset, UK) was used as a positive control. The percentage of ACE inhibition (ACEi%) was calculated by subtracting the HA produced in the presence of the inhibitors to the HA produced in absence of inhibitors (under the same conditions) as shown below:

$$ACEi\% = 100 \times ([HA_{Control}]-[HA_{Sample}])/[HA_{Control}]$$

where ACEi% is the inhibition percentage of ACE, HA_{Sample} is the concentration of the released HA (in the presence of inhibitor), $HA_{Control}$ is the concentration of HA in the control blank (without inhibitor). On account of the matrix effect of milk samples, as described in Gonzalez-Gonzalez et al., (2011), we used a maximum diluted sample as a blank. For HA quantification, an external calibration line was used, derived from

standard solutions of HA at different concentrations (from 1000 to 10 μM, y=35102x + 189229, r^2 = 0.999).

IC50 was calculated according to the ACEi% dilution curve, in which % inhibition was plotted as a function of sample protein concentration.

3.4.7 Peptide profiling by mass spectrometry analysis

3.4.7.1 UPLC-ESI-MS-MS

UPLC/ESI-MS analyses were performed with an UPLC/ESI-MS system (UPLC Acquity Waters equipped with a single quadrupole mass spectrometer Waters Acquity Ultraperformance). Conditions were as follows: column, RP ACQUITY UPLC BEH 300 C18 (1.7 μm, 2.1 x 150 mm, Waters); gradient elution: eluent A, water with 0.1% formic acid and 0.2% acetonitrile, eluent B, acetonitrile with 0.1% formic acid; gradient: 0-7 min 100% A, 7-50 min linear from 100% A to 50% A, 50-52.6 min isocratic 50% A, 52.6-53 min linear from 50% A to 0% A, 53-58.2 min isocratic 0% A, 58.2-59 min linear from 0% A to 100% A, 59-72 min isocratic 100% A. LC parameters: flow rate, 0.2 ml/min; analysis time, 72 min; column temperature, 35°C; sample temperature, 18°C; injection volume, 2 μl for digested samples (with 7 minutes of solvent delay) and 4 μl for extracted samples (with no solvent delay). MS parameters: Full Scan mode, acquisition time 7-58.2 min; ionization type, ESI + (positive ions); scan range, 100-2000 m/z; capillary voltage, 3.2 kV; cone voltage, 30 V; source block temperature, 150°C; desolvation temperature, 300°C; cone gas flow, 100 l/h; desolvation gas flow, 650 l/h. Data were acquired and analyzed by MassLynx 4.0 software (Waters Co., Milford, MA, USA).

3.4.7.2 HPLC-LTQ-ORBITRAP MS

The samples (both digested and non-digested) were diluted 1:10 with distilled water, then analyzed by HPLC-LTQ-ORBITRAP XL using a C18 Aeris peptide 3.6u XB - C18 150 x 2.1 mm column and a gradient elution; eluent A was water with 0.2% formic acid and eluent B was acetonitrile with 0.2% formic acid, gradient as follows: 0-5 min isocratic 98% A, 5-54 min linear from 98% to 55% A, 54-55 min from 55% to 10 % A, 55-60 min isocratic 10% A, 60-61 min linear from 10% to 98% A, 61-70 min isocratic 98% A. The analysis parameters were: flow 0.2 ul/min; analysis time 70 min; column

temperature 40°C; sample temperature: 18 °C; injection volume 1 µl; acquisition time 60 min; solvent delay 3 min; ionization type positive ions; scan event 1 from 250 to 2000 m/z (first acquisition) and from 100 to 2000 m/z (second acquisition); spray voltage 3.5 kV; cone voltage 13 V; source temperature 275 °C. Tune: peptidi 200 µl LTQ Tune; Sheath Gas Flow Rate (arb.): 40; Aux Gas Flow Rate (arb.): 10; Sweep Gas Flow Rate (arb.): 10; Tube Lens: 70 V. Chromatographic signals were integrated using the Xcalibur Software. Data were processed by PEAKS 7.5 software for LC-MS/MS data analysis to provide peptides sequences identification and the analogous Proteom Discoverer (version 1.4).

3.5 Statistical analysis

All experiments were carried out in triplicates excepti lipolytic, autolytic activity and diacetyl production. The data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The data were subjected to one-way analysis of variance (ANOVA) to determine the differences of samples. Significant differences were compared by Duncan test on the level of $P < 0.05$.

4 Results and discussion

4.1 Isolation of LAB from donkey milk: Assessment of their technological properties, safety characteristics and probiotic potential

4.1.1 Microbiological analysis of donkey milk samples

Table 24 shows the microbiological results of the 11 milk samples for total viable microorganisms, LAB, yeasts and molds, *Staphylococcus aureus*, *Enterobacteriaceae*, *Listeria monocytogenes* and *Salmonella* spp. of raw donkey milk. The average numbers of LAB in raw donkey milk samples, as estimated from the bacterial counts on M17, acidified MRS and MRS pH 6.2 agar media, was 3.4 $\log_{10}$ cfu/mL (Table 26). These values are in agreement with the average range reported in the literature for donkey milk (Carminati et al., 2014; Chiavari et al., 2005; Coppola et al., 2002; Saric et al., 2012; Zhang et al., 2008). The total viable microorganisms were 3.83 $\log_{10}$ cfu/mL, while *S. aureus* and *Enterobacteriaceae* were around 10^3 cfu/mL. Yeasts and molds were detected only in 2 samples with an average of 3.86 $\log_{10}$ cfu/mL. The pathogens *Salmonella* spp. and *Listeria monocytogenes* were not detected in any of the samples.

Table 24: Counts of different microbiological groups present in donkey milk samples (n=11, x±SD)

Parameters	Log cfu/ml
Enterobacteriaceae	3.2 ± 0.24
Total Microbial Count	3.8 ± 0.37
S. aureus	2.96 ± 1.36
Yeast and Moulds	3.86 ± 0.05
LAB	3.4 ± 0.25
Listeria monocytogenes	N.D.
Salmonella spp.	N.D.

4.1.2 Physicochemical and chemical analyses of donkey milk samples

The results of analyses of raw donkey milk samples are shown in Table 25, and results are in line with other studies (Guo et al. 2007; Malissiova et al., 2016; Medhammar et

al. 2012; Polidori & Vincenzetti, 2013b; Salimei and Fantuz 2012). The pH of the samples ranged from 7.03 to 7.60 with a mean level of 7.31±0.14, which is in accordance with the literature (Guo et al., 2007; Malissiova et al., 2016; Polidori & Vincenzetti, 2013b; Salimei et al., 2004). This suggests that the pH value may not be influenced by possible breed variation for donkeys. As an indicator of milk quality, milk acidity is used to measure and monitor such processes as making cheese and yogurt. Accordingly, due to the buffering capacity of protein and milk salts, fresh cow milk exhibits an initial acidity of 0.14 to 0.16 when titrated with 0.1 N NaOH (Gemechu et al., 2015). In this study, the acidity value obtained from donkey milk was in the range of 0.05 to 0.09% (lactic acid) which is lower than cow milk acidity. The average fat content of donkey milk samples was also similar to the values reported by Salimei et al., (2004) for jennies (0.42% + 0.13), machine milked.

The protein content of donkey milk was 0.47±0.08 g/ 100 mL, which is lower than the protein content of donkey milk reported in literature (1.72 g/ 100 ml) by Salimei et al., (2004). There is evidence in the literature that protein content of donkey milk is influenced by the breed, age, lactation stage, the lactation and foaling season (Giosue et al., 2008; Guo et al., 2007; Salimei et al., 2004).

The mean dry matter observed in current donkey milk study was of 8.73 + 0.11 % which is consistent with the values reported in the literature for donkey milk (Chiavari et al., 2005; Guo et al., 2007; Salimei et al., 2004).

Table 25: Characteristics of donkey milk samples

Chemical composition of donkey milk	**Mean value of 11 samples ± SD**
pH	7.31 ± 0.14
Acidity (%)	0.061±0.016
Fat (%)	0.42 ± 0.13
Protein (%)	0.47±0.08
Total Solids (%)	8.73±0.11
Lactose (%)	7.04±0.19

Donkey milk is characterized by low fat contents, high pH value and low acidity (Table 27). Fat is the most variable component of donkey milk, ranging from 0.1 g/100 mL

(Salimei et al., 2004) to 1.8 g/100 mL (Guo et al., 2007) in different reports, being affected from both lactation stage and milking technique (Cosentino et al., 2013; Giosue et al., 2008; Ivankovic et al., 2009; Salimei et al., 2004). Donkey milk has higher pH and lower acidity than bovine milk due to the lower concentration of casein and phosphate. In general, the chemical composition of donkey milk is considered as favorable for the production of fermented milks (i.e. low fat and low protein). Moreover, donkey milk composition is similar to human milk and could be used for a variety of purposes (Tesse et al., 2009). However, any suggestion to use donkey milk as alternative milk for infants should take into consideration its low fat and energy content. The low donkey milk fat concentration and its consequent low energy content (1.7 kj/kg) are the main limits to its use in nutrition of children allergic to cow's milk protein, in their first year of life, since recommended dietary allowances may not be reached, unless adequate supplementation is provided (D'Auria et al., 2011). On the other hand, this feature makes donkey milk a hypo-caloric and highly digestible food for consumers with specific dietary requirements, such as athletes and elderly people.

4.1.3 Lysozyme content of donkey milk samples

The assay measures lysozyme activity on *Micrococcus lysodeikticus* cell walls, which are labeled to such a degree that the fluorescence is quenched. Lysozyme action can relieve this quenching; yielding a dramatic increase in fluorescence that is proportional to lysozyme activity.

Figure 18 displays the standard curve of the fluorescence-based activity assay. The linear regression equation and the correlation coefficient of the standard curve were $y = 0.3637x + 8504$ and $R^2 = 0.9907$. The mean lysozyme activity value measured for the eleven samples was 6027.55±840.63 U/ml. Our results were in accordance with other studies carried out by Gubić et al., (2014); Guo et al., (2007); Pilla et al., (2010); Šarić et al., (2012); Šarić et al., 2014; Vincenzetti et al., (2011).

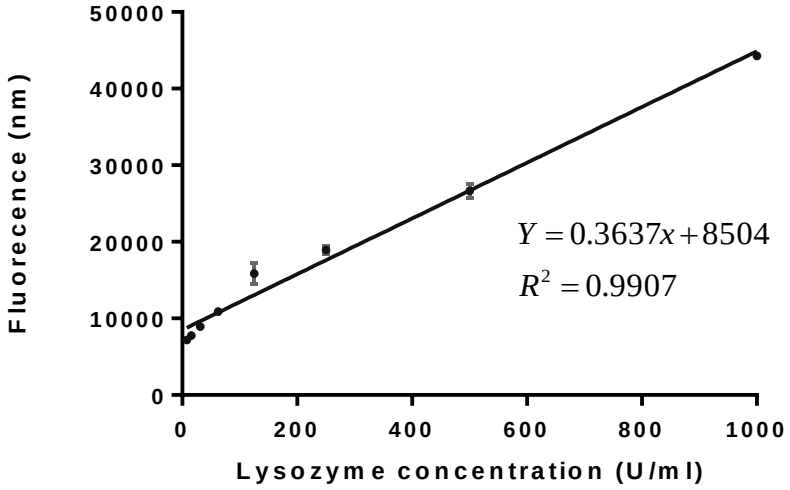


Figure 18: Lysozyme standard curve

The large amount of lysozyme in donkey milk may be useful not only to prevent intestine infections in infants, but also responsible for the low bacterial count as reported by Salimei et al., (2004), giving to donkey's milk the peculiarity to preserve their organoleptic and microbiological characteristics unchanged for a long time (Šarić et al., 2012; Zhang et al., 2008). In fact it has been observed in a donkey milk sample stored at refrigeration system for over 10 days that the organoleptic characteristics, pH and microbiota showed no significant changes (Polidori & Vincenzetti, 2007). At this regard, Zhang et al., (2008) showed that the absence of *Salmonella* and *Shighella* strains and a growth reduction of *S. choleraus* and *S. disenteriae* in donkey's milk can be attributed to the activity of lysozyme and other antimicrobial molecules such as lactoperoxidase system, lactoferrin, immunoglobulin and free fatty acids. Also Tidona et al. (2011) showed antimicrobial effect of donkey's milk on selected pathogenic bacteria with a significant reduction growth of *E. coli* during its stationary phase and inhibition of *L. monocytogenes* 2230/92 in a dose dependent way. The interest for lysozyme is also motivated by its role in mitigating the inflammation of the epidermis and scalp, which justifies the dermatological use of donkey's milk carried out since ancient Rome. In addition, lysozyme has also other physiological functions, including inactivation of certain virus, immunoregulatory activity, anti-inflammatory and anti-tumor activity (Ibrahim & Aoki, 2003). In fact, in Mao et al., (2009) study, a high content of lysozyme in donkey's milk may contribute to its anti-proliferative and anti tumor effects on A549 human long cancer cells in vitro. Considering the numerous

benefits of donkey milk, including its healthy-promoting characteristic and probiotic effect, Chiavari et al., (2005) and Coppola et al., (2002) suggested the possibility of using donkey's milk for probiotic purposes. Donkey's milk could be valorised as a very good base for a fermented milk beverage, since it proved to be a good growth medium for probiotic lactobacilli (*L. rhamnosus* and *L. casei*) because of its initial low microbial count, high lactose content and mainly high lysozyme content (Coppola et al., 2002). In fact it must be noted that lysozyme can be considered an indirect "bifidogenic factor" and so a vehicle for the consumption of probiotic bacteria. However the high lysozyme content in donkey's milk did not affect the probiotic strains viability during the storage and so, only partially it influenced the growth of the strains tested without also any significant effect on their acidifying activity (Chiavari et al., 2005; Coppola et al., 2002). Moreover, the elevated lysozyme activity may explain the low incidence of mastitis in donkeys, that usually follows physical injuries to the glands or drying off (Conte et al., 2006). Donkey milk lysozyme, as well as equine and canine counterpart, belongs to C-type calcium-binding lysozyme and is able to bind calcium ions; this binding leads to more stable complex with an enhanced antimicrobial activity (Wilhelm et al., 2009). Recently, Šarić et al., (2014) reported that donkey milk shows a calcium-dependent activity against *E. coli*. In donkey milk, two genetic variants of lysozyme (A and B), both containing 129 amino acids (gij126613; gij126614), and with molecular weight 14,632 Da that differ in three amino acid substitutions at positions 48, 52, and 61 have been described so far (Cunsolo et al., 2007; Herrouin et al., 2000).

4.1.4 Isolation of LAB

4.1.5 Biochemical, physiological and phenotypic characterization of the strains

The average of LAB population in the raw donkey milk produced in Cyprus was 3.4 $\log_{10}$ cfu/ml. A total of 270 colonies were randomly picked from plates containing between 30 and 300 colonies of the eleven samples of raw donkey milk. Plated were examined by eye, and the different colony types were individually picked. They were propagated twice and streaked on MRS broth to obtain the pure cultures (Figure 19).

Figure 19: Pure cultures of LAB isolated from raw donkey milk

The initial isolation and identification was based on morphological appearance, oxidase and catalase test. After conducting some preliminary tests (Gram staining and catalase), a total of 257 isolates were picked for further identification. All the 257 isolates were found to be Gram positive, oxidase and catalase negative cocci and rods. The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to its simplicity. In performing catalase test, no bubbles were observed indicating that the isolated bacteria are catalase negative and could not mediate the decomposition of hydrogen peroxide (H_2O_2) to produce carbon-dioxide (CO_2). According to Salminen et al., (2004), LAB are Gram positive rod or cocci shaped bacteria and are catalase negative. All the isolates were characterized further using biochemical and physiological tests. Physiological test involved examining the influence of temperature, pH and salt concentration on the growth of LAB isolates. The examination of the influence of temperature was aimed to understand the type of bacteria as the result of isolation, whether it belongs to psychrophilic, mesophilic or thermophilic groups. Cellular growth was indicated by measuring OD after incubated for 24-48 hours on MRS broth. According to the phenotypic, biochemical and physiological identification 189 of 257 isolated strains were presumptively identified as *enterococci* on the basis of phenotypic characterization, grew well in pH 4.4-8.0 and pH 9.6, in the presence of 2-8% (w/v) NaCl and at temperatures of 15-45 °C. All *enterococci* strains were fermentative without gas production and produced acid in litmus milk with curd

formation. The three *Leuconostoc* strains (DM92, DM115 and DM236) grew at 15 °C but not at 45 °C, grew at pH 4.4-8.0 but not pH 9.6. They grew in the presence of 2-4% NaCl and they were fermentative with gas production, produced acid from litmus milk and coagulate litmus milk weakly. Members of the genus *Lactobacillus (*DM214 and DM259) - rod shaped grew at 15 °C, in the presence of 2-4% NaCl and at pH 4.4-8.0 and produce acid from litmus milk with clot formation (Figure 20). None of the strains were able to grow at 45 °C, 6.5% NaCl or pH 9.6. The strains that belonged to *Streptococcus* spp. showed growth in 2-4% NaCl and at pH 5-8 but which did not grow at 45 °C, in 6.5% NaCl and at pH 4.4 and 9.6. All *streptococci* strains were fermentative without gas production and produced acid in litmus milk with curd formation.

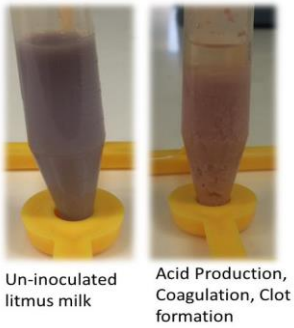


Figure 20: Litmus milk test

In summary, according to the phenotypic, physiological and biochemical identification (Table 26) 189 out of 257 isolated strains were presumptively identified as *Enterococci*, 63 belonged to *Streptococcus* spp., 3 belongs to the genus of *Leuconostoc*, and 2 to the genus of *Lactobacillus*.

Table 26: Phenotypic and physiological characteristics of lactic acid bacteria isolated from raw donkey milk (+: Positive reaction; - Negative Reaction). Total number of isolates in each product are given in brackets

Characteristics	***Enterococcus* spp (189 strains)**	***Streptococcus* Spp (63 strains)**	***Leuconostoc* spp (3 strains)**	***Lactobacillus* spp (2 strains)**
Gram Reaction	+	+	+	+
Cellular morphology	coccoid	coccoid	coccoid	rods
Catalase Test	-	-	-	-
Oxidase Test	-	-	-	-
Growth at 15◦C	+	+	+	+
Growth at 30◦C	+	+	+	+
Growth at 45◦C	+	-	-	-
Growth at 2% (w/v) NaCl	+	+	+	+
Growth at 4% (w/v) NaCl	+	+	+	+
Growth at 6.5 % (w/v) NaCl	+	-	-	-
Growth at 8% NaCl	+	-	-	-
Growth at pH 4.4	+	-	+	+
Growth at pH 6	+	+	+	+
Growth at pH 8	+	+	+	+
Growth at pH 9.6	+	-	-	-
Growth in litmus milk	Produce acid in litmus milk clot formation-coagulation of litmus milk	Produce acid in litmus milk/ clot formation-coagulation of litmus milk	Produce acid in litmus milk/ gas production/ clot formation-coagulation of litmus milk	Produce acid in litmus milk- clot formation-coagulation of litmus milk

+: positive; -: negative

4.1.6 Molecular identification of the strains

According to the molecular identification and the 16S RNA gene sequencing, *Enterococcus* prevailed, representing 74% of the isolates. The species of *Enterococcus* identified were *E. faecium* (44%, 112/189), *E. faecalis* (17%, 44/189), *Enterococcus thailanticus/lactis* (6%, 15/189), *Enterococcus hirae* (3%, 7/189), *Enterococcus durans*

(2%, 5/189), *Enterococcus mundtii* (1%, 2/189), *Enterococcus gallinarum* (1%, 4/189). Members of the genera *Streptococcus*, *Leuconostoc* and *Lactobacillus* were also detected (24%-63/257, 1%-3/257 and 1%-2/ 257, respectively), comprising the species *Streptococcus gallolyticus*, *Streptococcus macedonicus, Leuconostoc mesenteroides* and *Lactobacillus paracasei* (all of the isolates) (Figure 21). Appendix III shows the ID of each isolate using 16S rDNA sequencing. The microbial population of raw donkey milk reveals that coccus-shaped LAB were the predominant group which is an agreement with the results obtained by Carminati et al. (2014). This is possibly related to high lysozyme content in donkey's milk, as LAB cocci are more resistant to lysozyme than lactobacilli (Neviani et al., 1991). Our observations are in agreement with the few studies available on the characterization of microbial population of donkey's milk, where the only *lactobacilli* identified belonged to the species *Lb. paracasei*, *Lb. brevis, Lb. salivarius* and *Lb. plantarum* (Murua et al., 2013; Nazzaro et al., 2008; Sa et al., 2011).

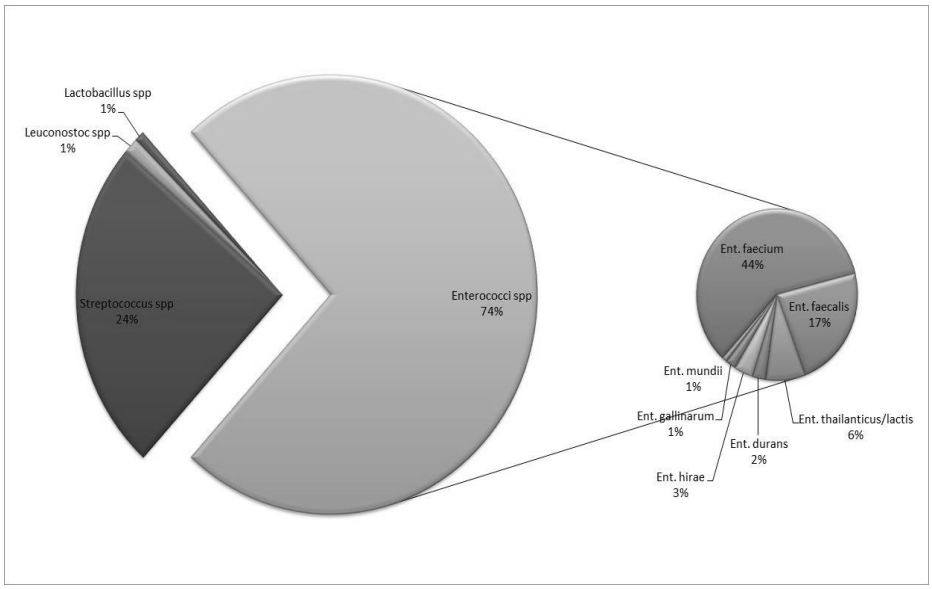


Figure 21: Percentage of genes and species distribution of lactic acid bacteria isolated from raw donkey milk according to 16sRNA sequencing

4.1.7 Technological properties

One of the aims of this study was to select LAB from natural sources, in this case donkey milk, as possible candidates to be used as starter or adjunct cultures for the production of a fermented functional donkey drink. For this reason, the isolates (*Enterococci, Lactobacilli* and *Leuconostoc*) were investigated for the most important

technological properties such as acidification activity, proteolytic activity, EPS production, autolytic activity, lipolytic activity and diacetyl production. APPENDIX IV shows in detail the technological properties for each strain.

4.1.7.1 Acidification activity

To select a starter culture for lactic fermentation of donkey milk, the strains were characterized on the basis of acid production ability. The acidity increased during the fermentation, and there was variability in acidification rate between the different strains used to inoculate the milk (Figure 22). Strain are considered fast, medium and slow when a ΔpH of 0.4 units is reached in 3, 3–5 or >5 h, respectively (Ayad et al. 2004) using skim milk as a substrate. For the production of fermented dairy drinks the rate of acid development is a critical factor for dairy industry, which results from the metabolism of milk lactose to lactic acid. The fast acidifying strains are good candidate in the dairy fermentation process as primary starter organisms, whereas, the poor acidifiers strains can be used as adjunct cultures depending on their other important properties, such as proteolysis and autolysis. Moreover, the resultant lowering in pH prevents the growth of undesirable microorganisms such as spoilage and pathogenic bacteria and is also responsible for aroma and flavor development of the end product. For this aim, pH and lactic acid production were monitored for all the isolates for 6 and 24 hours of incubation at 37 °C (Figure 25). A good mesophilic fast acid producing starter culture will reduce the pH of the milk from its initial value of 6.6 to 5.3 in 6 h at 30 ^{o}C (Cogan et al., 1997). The initial pH of milk was 6.90 units.

The highest acidifying activity was observed for the 2 *Lactobacillus paracasei* strains. Even though that the 2 *Lactobacillus* strains differed in their ability to reduce the pH of milk initially and there were strains that did not change the pH of milk at 6 h. Nevertheless, after 24 h incubation they reduced the pH of milk below 5. *Lactobacillus casei* may ferment lactose through a b-galactosidase activity, but some strains also show a b-phospho-galactosidase activity (Herrero et al., 1996). Rapid acid production abilities indicated that they were the most suitable starter candidates for dairy applications.

Within the *enterococci* isolates only 15 out of the 189 strains tested reduced the pH of milk to <6.0 (5.59–5.99) after 6 h of growth. After 24 h, ~38% of the strains had lowered the milk pH <5, with pH values ranged from 4.97 to 4.50. In addition 5% of *enterococci* did not reduced the pH of the milk <6. In this respect, enterococcal isolates

could be considered slow or medium acidifiers, as reported elsewhere (Sarantinopoulos et al., 2001). An acidifying potential in skim milk with a pH lowering to about 4-5 after 24 h fermentation has also been reported in *enterococci* strains isolated from artisan Italian cheese (Giraffa, 2003; Foulquie-Moreno et al., 2003). *Enterococci* occur as NSLAB in various, especially artisanal, cheeses produced in the southern Europe from goat, ewe, water buffalo or bovine milk. Since *enterococci* may dominate the NSLAB of many cheeses, it is supposed that they can positively contribute to the flavor development during cheese ripening. As a consequence, *enterococci* may improve the sensory characteristics of the final product (Cogan et al., 1997; De Angelis et al., 2001).

Low acidifying activity was obtained for the *Leuconostoc* assayed as compared to the values reported for *Enterococcus* and *Lactobacillus* isolates. The 3 *Leuconostoc mesenteroides* strains did not reduced the pH <5 in 24 h. This fact is in agreement with the hetero-fermentative metabolism of this genus and is consistent with the results reported by other authors (Ayad et al., 2004; Garabal et al., 2008). For this reason, Server-Busson et al., (1999) suggested that *Leuconostoc* isolates should be used combined with acid producing LAB as starters or starter adjuncts.

The difference observed from one LAB species to another was explained by Badis et al., (2004). The acidifying activity of each strain is related to its specific capacity to break down the carbon and nitrogen substrates in the medium and the capability to assimilate the nutrients essential for growth. On occasion, differences are also due to the presence or absence of nutrient transport systems (Albenzio et al., 2001).

Results of titratable acidity are in the range of 0.5-0.34% (expressed as % of lactic acid) after 24 h. This is an agreement with the results of pH, as most of the strains were slow or medium acidifiers. Regarding the values of titratable acidity, the isolated strains are capable of producing a mild acid flavor to the fermented milk product. Therefore, as the aim of the current study is to isolate LAB in order to be used for the production of fermented donkey milk, 74 strains of LAB (72 *enterococci* and 2 *lactobacillus*) that showed considerable acidification activity (drop pH below 5) after 24 hours of incubation at 37 °C has been used for further investigation.

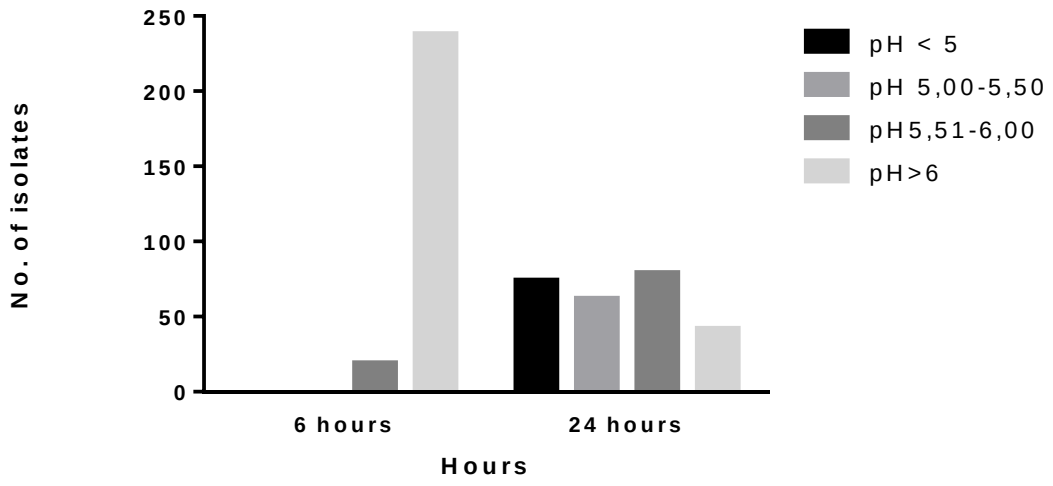


Figure 22: Number of isolated reduced the pH at 6 and 24 h

4.1.7.2 Proteolytic activity

The proteolytic system of LAB is essential for the optimal growth in milk through the release of proteolytic enzymes and is also involved in the development of organoleptic properties of different fermented milk products (Axelsson, 1998; Christensen et al., 1999). Only 1%–2% of milk proteins undergo proteolysis during milk fermentation and the principal milk protein is casein, but limited degradation of whey proteins may also occur (Szwajkowska et al., 2011; Griffiths & Tellez, 2013). LABs have a complex system of proteases and peptidases, which allow them to use milk casein as a source of amino acids and nitrogen. Intra- and inter-specific variability in proteolysis is commonly reported for isolates from natural sources (Franciosi et al., 2009). Determination of proteolytic activity on skim milk agar was used as a qualitative method for preliminary characterization of the strains. On the basis of the area of the halo, the strains were classified as highly proteolytic and non-proteolytic. Skim milk agar is an effective and rapid medium to detect the extracellular cell-bound proteinases as shown by a clear zone surrounding the colonies. According to Fquiri et al., (2016), a strain is called proteolytic if it has a zone of lysis of diameter between 15 and 21 mm. According to this from the 74 LAB isolates, only 18 (24%) strains gave clear zones between 15-21mm after incubation at 37 °C, while 40 strains (54%) gave a clear zone between 2-14 mm (Figure 23). The proteolytic activity of strains as a selection criterion for the manufacture of fermented milks may not be as crucial as it is for cheese

production (i.e. flavour evolution during maturation, texture development), but proteolytic strains could lead to the formation of peptides with bioactive properties (i.e. antimicrobial, antioxidant and anti-hypertensive) during milk fermentation (Korhonen, 2009).

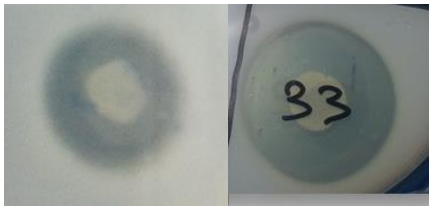


Figure 23: Example of a proteolytic assay with a positive result indicated by a clear halo around the colony

The second method used for the determination of proteolytic activity of the strains was OPA. The OPA-based spectrophotometric assay is very good method for detecting released α-amino groups, which result from the proteolysis of milk proteins and gives a direct measurement of proteolytic activity. The proteolytic activity of all the strains is presented in APPENDIX IV. Samples were taken at 6 and 24 hours. The proteolytic activity of these bacterial cultures were calculated from a calibration curve obtained from dilution of glycine in milk is expressed in mM Gly/L of milk after subtraction of values for the uninoculated control RSM (Figure 24).

The proteolytic activity measured using the OPA method ranged between 0.15 and 18.45 mM Gly/L after 24 h of incubation at 37 °C. It was shown that this activity varied among the strains. The amount of liberated amino groups and peptides increased significantly during fermentation from 6 to 24 h for all the strains.

The highest proteolytic activity corresponded to *E. faecalis* DM117 (18.45 ± 1.80 mM Gly/L), followed by *E. faecium* DM223 (15.23 ± 1.16 mM Gly/L). Our results confirm data previously reported by other authors indicating that *enterococci*, in general, were more proteolytic than the other LAB groups isolated (Khedid et al., 2008; Sarantinopoulous et al., 2002). The 2 *Lactobacillus paracasei* strains (DM214 and DM259) presented a proteolytic activity of 10.42 and 1.43 mM Gly/L respectively. The proteolytic activity values of *lactobacillus* were similar to those reported by other authors (Garabal et al., 2008; Herreros et al., 2003). Also, Bonomo & Salzano, (2013) reported medium to high proteolytic activity in 41% 46% of *Lb. paracasei* isolated from Pecorino di Filiano cheese.

No clear relationship was established between the proteolytic and the acidifying activities of the LAB isolates obtained in the present study and appeared rather as the characteristic properties of each strain, which has also been observed in other studies (Durlu-Ozkaya et al., 2001; Fortina et al., 2008). Thus, strains with the strongest acidifying abilities (*Lb. paracasei* DM214) did not exhibit the highest proteolytic activities and there were strains with very low acidifying but high proteolytic activity (e.g. *E. faecalis* DM117).

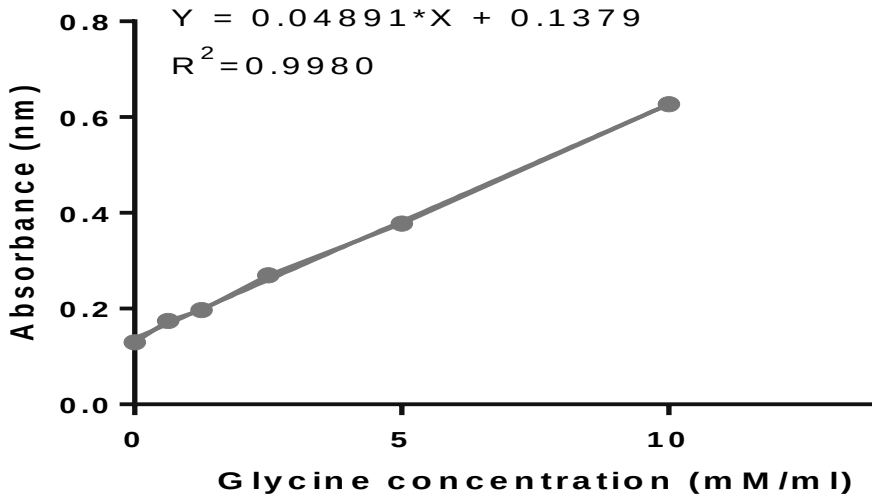


Figure 24: OPA standard curve using glycine

4.1.7.3 Lipolytic activity

Lipolysis is an important process mainly in cheese ripening due to its role in the development of flavor and texture of the final product (Morandi et al., 2006). This is achieved by enzymatic hydrolysis of triglycerides to fatty acids and, which can be precursors of flavor compounds such as methyl ketones, secondary alcohols, esters and lactones.

The results obtained in this study using tributyrin agar confirmed that both *lactobacilli* and *enterococci* have generally low lipolytic activity, as none of the tested strains gave a zone. Our results are in aggrement with other studies demonstrated that LAB are generally weak lipolytic (Giraffa, 2003; Hulak et al., 2016; Sarantinopoulos et al., 2001). One possible explanation for the absence of lipolytic activity of the tested strains is maybe due to the fact that donkey milk fat is very low compared to other milks (Malissiova et al., 2016). In contrast to the desirable effect of lipolysis in hard Italian

cheeses and blue mold cheeses, high lipolysis in fermented milks is undesirable. Therefore, the low lipolytic activity of tested isolates can be considered as an advantage, since only a slight lysis of the milk fat is enough to induce aroma production without giving a rancid flavor to the final product (Herrero et al., 1996).

4.1.7.4 EPS production

Regarding EPS production, 27 strains out of 74 (36%) were capable to produce EPS. The production of EPS is considered an important feature for the selection of LAB as starter cultures in dairy technology and especially in fermented milks, since they act as texturizers and stabilizers therefore creating smooth creamy products (Parente & Cogan, 2004). The production of EPS, can be considered as an advantage for these strains, as it can be used for the fermentation of donkey milk which is low in fat, in order to obtain a better mouth feeling (such as better viscosity, smooth texture and creaminess). For example, EPS-producing stains are used as starter cultures for the production of Scandinavian cultured milks in order to give their characteristic body and texture (viscous and a ropy/stringy body). Moreover the use of EPS-producing bacteria can help to reduce the cost by replacing the use of hydrocolloid additives in fermented products. Apart from their role in improving the rheology of fermented dairy products, the LAB produce EPS probably as a protective function in their natural environment such as against desiccation, phagocytosis, phage attack, osmotic stress, antibiotics or toxic compounds (Patel & Prajapat, 2013). This is a beneficial trait for probiotics in their endeavor to colonize the gastrointestinal track.

4.1.7.5 Autolytic activity

The autolytic activity was evaluated in order to quantify the ability of the strains to release flavor precursors (Al-Saleh et al., 2014). The autolytic activities of LAB strains were different among strains, and were classified into three groups; poor, fair, and good, according to the autolytic capacity of each genus as described by Ayad et al., (2004). Both *Lactobacillus paracasei* DM214 and DM259, displayed fair autolytic activity (63.78% and 61.94%, respectively). Among the *enterococci* 14% showed the highest percentage of autolysis ranged from 35% to 66% whereas 15% had a fair autolytic activity (24-34%) and the rest of strains (71%) showed poor autolysis (0-22%) (Figure 25).

Lactobacilli showed higher autolysis rate compared to *enterococci*. These results are in agreement with several authors (Dako et al., 1995), that indicated that *lactobacillus* autolysed more rapidly than *enterococcus* strains. Autolytic activity is an interesting property of LAB since autolysis affects the release of intracellular proteolytic and lipolytic enzymes which is affect the flavor and aroma during cheese ripening (Ayad et al., 2004; Franciosi et al., 2009). Therefore, autolytic activity can be considered as a desirable trait in some matrices of dairy products such as cheese. However, according to Kenny et al., (2006), the use of intermediate autolytic strain for the production of cheddar cheese, gave a better flavor than the cheddar cheese made with the most autolytic strain which is also has a higher degree of proteolysis.

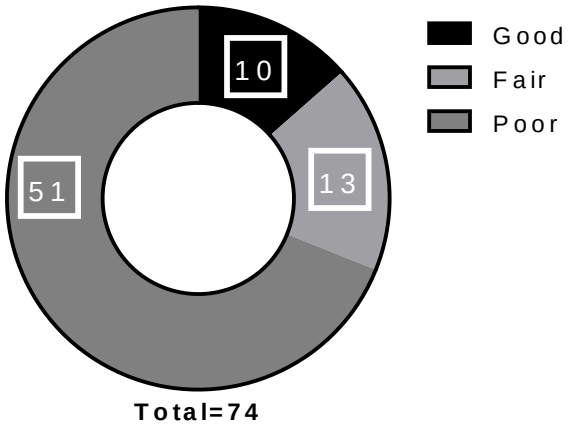


Figure 25: Autolytic activity of LAB isolates

4.1.7.6 Diacetyl production

Diacetyl (2,3-butanedione) is a volatile compound generated as an end-product of citrate metabolism by certain LAB and contributes directly to flavor formation, giving the characteristic butter aroma in fermented milks (Rincon-Delgadillo et al., 2012). The results obtained in this study showed that 56 out of 74 (76%) were capable in producing diacetyl. The ability to produce diacetyl from citrate was found to be species and strain dependent, both lactobacilli and 75% of enterococci produced diacetyl. This result was in agreement with other authors (Bonomo & Salzano, 2013; Giraffa, 2003; Hemme & Foucaud-Scheunemann, 2004; Moreno et al., 2006). Also, Domingos-Lopes et al., (2017) found that both genera produced high diacetyl production compared to the other

genera of LAB such as *Leuconostoc*. From these, only 17 gave a pale pink color which is corresponding to 3.1-10 mg/100ml diacetyl production, while the other 39 gave a slightly pale pink which is corresponds to 0.5-3 mg/100ml diacetyl production (Figure 26). This finding assists us in screening for LAB that might contribute in flavor evolution of fermented milks.

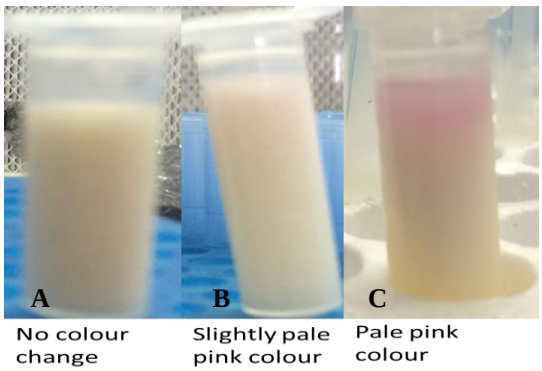


Figure 26: Example of diacetyl production of LAB isolates (A: uninoculated control; B: slightly pale pink; C: pale pink)

4.1.8 Safety characteristics

Enterococci could be relevant as starter cultures in several artisanal foods, being responsible for the production of peculiar typical characteristics. However, the virulence potential of *enterococci* needs a proper characterization of wild strains, to verify their adequacy to be used as biopreservatives (Moraes et al., 2012). Therefore, it seemed needful to test the our *enterococci* strains for their antibiotic resistance, virulence factors profile and production of biogenic amines. Safety profile for each strain is presented in APPENDIX V.

4.1.8.1 Antibiotic susceptibility testing

Figure 27 & 28 shows the susceptibility percentage according to the zones of inhibition of the 72 tested *enterococcus* isolates to the different antibiotics tested. Isolates were considered resistant when they showed inhibition zones (in mm) higher than the breakpoints established by the EUCAST, 2015 (Table 27). Wherever EUCAST has not set breakpoints for a specific antibiotic, those proposed by the CLSI, 2015 were used (Table 27). The most important factor for the safety evaluation of enterococci is their resistance to glycopeptides such as vancomycin. In this study, all strains except 3 (4%

of the isolates) were sensitive to vancomycin. Results are consistent with other studies of *enterococci* isolated from different types of food sources (Carasi et al., 2014; Favaro et al., 2014). Moreover, *enterococci* are considered intrinsically resistant to beta-lactam antibiotics (Lopes et al., 2005). The results obtained in the present study are not in accordance with this generalization, as all tested isolates were sensitive to ampicillin and 46% of them to penicillin. On the other hand, there were studies with similar to ours results (Valenzuela et al., 2008; 2009; 2010). The use of chloramphenicol for human treatment is not frequent due to its side effects, and the use in animal husbandry was also banned in Europe in 1994 (Peters et al., 2003). *Enterococci* isolates were sensitive to chloramphenicol in their entirety (Cariolato et al., 2008). A high percentage of *enterococci* was classified as intermediate (42%) or resistant (50%) and only 8% of them were sensitive to erythromycin, as reported previously (Mannu et al., 2003; Peters et al., 2003). The resistance to erythromycin as a representative of the macrolide antibiotics is a matter of concern, while macrolides are common substitutes used in patients with a penicillin allergy (Barbosa et al., 2014). *Enterococci* are usually described as having intrinsic resistance to aminoglycosides, such as gentamicin and streptomycin, and high levels of resistance was already described in strains obtained from dairy products (Mathur & Singh, 2005). Results of this study confirmed that 100% of the isolates were resistant to both of the above mentioned antibiotics. This is in agreement with Cariolato et al., (2008) who showed that 100% of *enterococci* isolates were resistant to streptomycin. Hammad et al., (2015), showed that all *enterococci* were resistant to gentamicin and streptomycin. Rifampicin is a broad-spectrum antibiotic used to treat tuberculosis. As shown in Fig. 3, 43%, 49% and 8% of the isolates were resistant, intermediate and sensitive, respectively. The wide use of tetracycline in husbandry activities is a possible explanation for the high level of tetracycline resistance frequently found among *enterococci* (Busani et al., 2004). In this study, 54% isolates were resistant to this antibiotic while 33% were intermediate and 13% sensitive.

Table 27: Breakpoints established by the European Committee on Antimicrobial Susceptibility (EUCAST, 2015) or by the Clinical and Laboratory Standards Institute (CLSI, 2015)

Class	Antibiotic	Symbol	Disc concentration	EUCAST/ CLSI Breakpoints (mm)		
				S	I	R
Aminoglycosides	Gentamycin	GEN	10 µg	≥15	13-14	≤12
	Streptomycin	S	30 µg	≥15	12-14	≤11
β-Lactams	Ampicillin	AMP	2 µg	≥10	9	≤8
Penicillin	Penicillin G	P	10 µg	≥15	n.a.	≤14
Phenicols	Chloramphenicol	C	30 µg	≥18	13-17	≤12
Glycopeptides	Vancomycin	VAN	5 µg	≥12	n.a.	<12
Macrolides	Erythromycin	E	15 µg	≥23	14-22	≤13
Sulphonamides/ Pyrimidines	Sulphamethoxazole/ Trimethropime	STX	5 µg	≥17	13-16	≤12
Tetracyclines	Tetracycline	TET	30 µg	≥19	15-18	≤14
Rifamycin	Rifampicin	RF	30 µg	≥20	17-19	≤16

S: Sensitive; I: Intermediate; R: Resistant

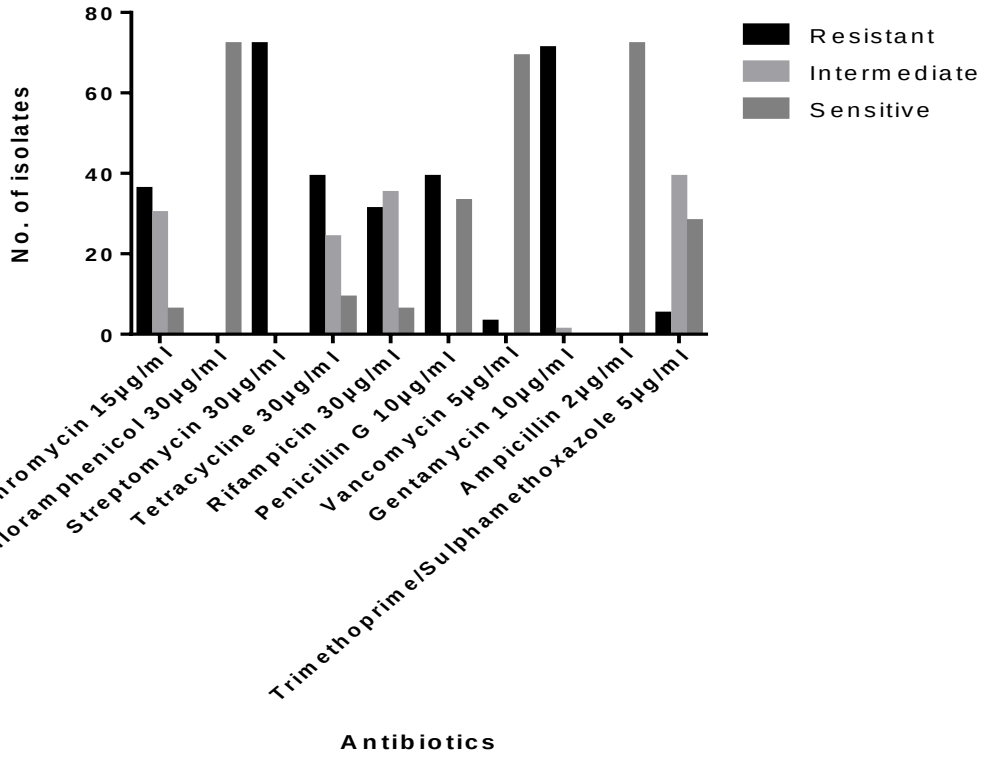


Figure 27: No of LAB isolates that are sensitive, intermediate and resistant against 10 antibiotics

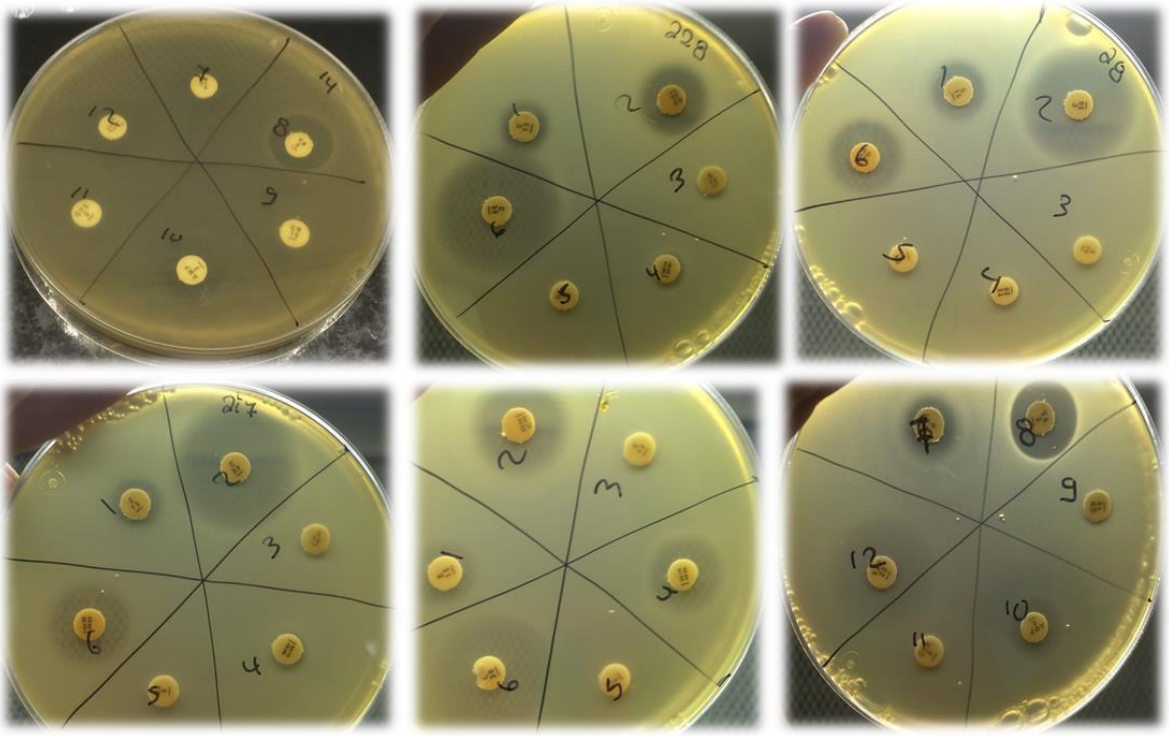


Figure 28: Examples of antibiotic disc diffusion assay

4.1.8.2 Biogenic amine production

Biogenic amines occur in different types of food, most frequently as a result of fermentation. None of the isolates showed decarboxylase activity on histidine or ornithine in the Joosten and Northold decarboxylase medium. However, decarboxylase activity on tyrosine was exhibited by 75% of the strains after 7 days of incubation (Figure 29). The results of this study are in consistence with previous studies showing that the only biogenic amine formed by enterococci is tyramine (Bover-Cid & Holzapfel, 1999; Giraffa, 1995; Psoni et al., 2006). Tyramine has been shown to be responsible for health problems, such as headache, hypersensitive reactions with antidepressive drugs, after consumption of ripened cheese. Large quantities of tyramine may affect the well-being of susceptible consumers and values of 100-800 mg/kg have been reported as toxic doses in food (Santos, 1996).

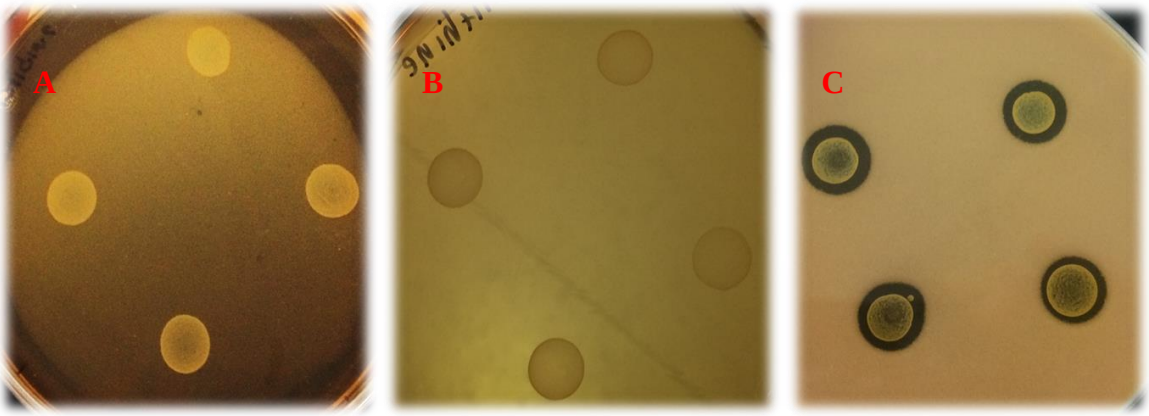


Figure 29: Biogenic amine production (A: histidine, B: ornithine, C: tyrosine-positive)

4.1.8.3 Virulence activity using phenotypic and genotypic tests

The verification of virulence factors in *Enterococcus* spp. even for those isolated from food sources by molecular and phenotypic methods is needful due to the risk of genetic transfer since these genes are usually located in conjugative plasmids (Eaton & Gasson, 2001). The presence of virulence factors in *enterococci* can greatly contribute to enhance the severity of hospital infections. According to phenotypic tests, only 2 (DM151 & DM154) out of 72 showed b-haemolysis and 1 showed a-haemolysis (DM157) (Figure 30). Additionally, gelatinase, DNase and lipase activity were not detected in none of the isolates by plate screening assay, which is in accordance with the results of Franz et al., (2001) and Mannu et al., (2003).

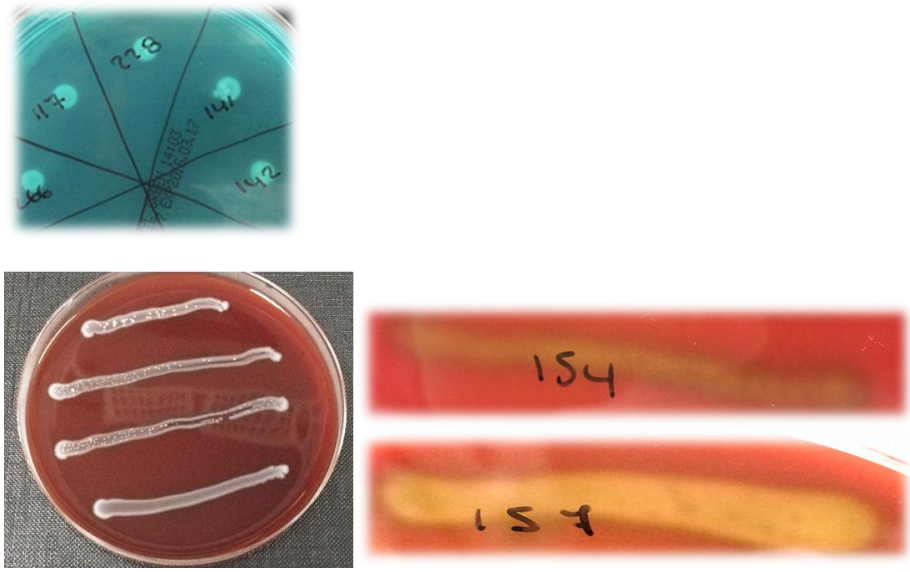
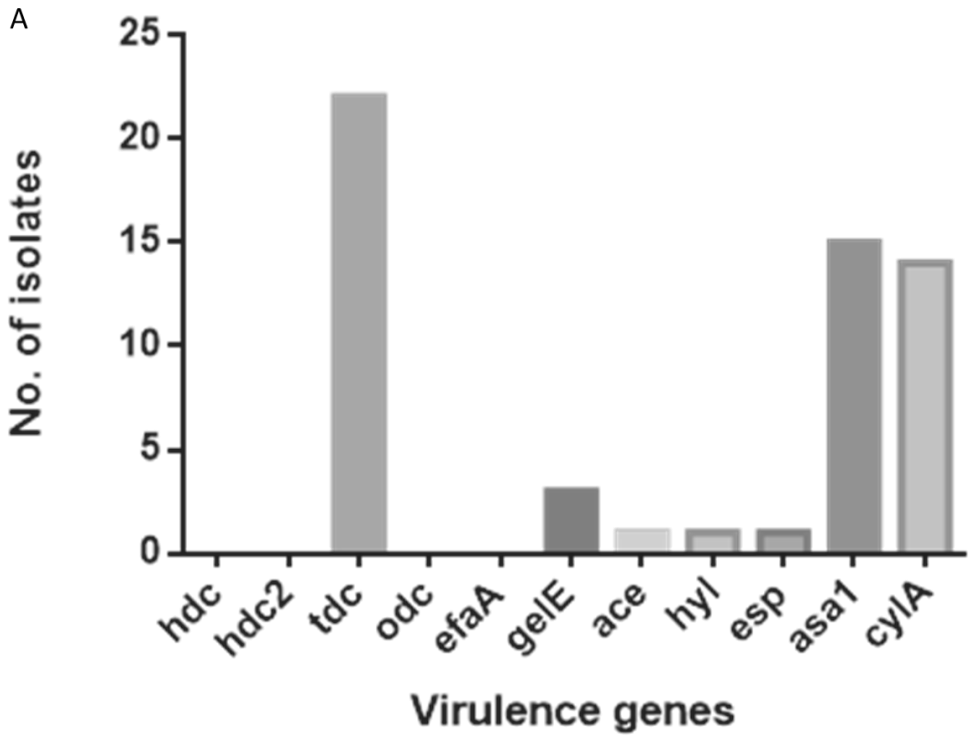


Figure 30: Phenotypic tests for enterococcus pathogenicity

Moreover, the isolates were tested for the presence of genes encoding potential virulence factors. Results on virulence genes posses by the *enterococci* are shown in Figure 31. Three (4.2%) isolates were positive for *gelE* gene, while did not produce gelatinase in phenotypic tests, which is common as it reported previously (Cariolato et al., 2008; Eaton & Gasson, 2001; Mannu et al., 2003). According to Eaton and Gasson, (2001), the loss of gelatinase activity during *in vitro* tests can be attributed to the high influence of *gelE* gene expression by the environmental and culture conditions in the laboratory. Moreover, the presence of *gelE* gene might not be enough for gelatinase activity, as the complete fsr operon seems to be necessary for its expression (Lopes et al., 2006). However, the fsr operon seems to be easily damaged, lost or suffers from deletions, mainly during the freezing of the cells in the laboratory. Haemolysin production can increase the severity of enterococcal infections and the presence of genes involved in haemolysin/ cytolysin production is considered as a risk factor. Regarding *cylA* gene only 14 (19.4%) isolates gave a positive result, but according to phenotypical tests, a-haemolysis and b-haemolysis was observed in 1 and 2 isolates respectively. Therefer *cyl* can be considered as a silent gene where its gene expression can be influenced by the environmental factors and conditions used for phenotypic tests (Eaton & Gasson, 2001). The genes *esp*, *efaA* and *ace* are related to the production of different substances involved in colonization and adhesion at biotic and non-biotic surfaces, and to evasion of the host immune system. The genes *esp* and *ace* were found in 1.4% of isolates, while *efaA* gene was not detected in none of the isolates. Moreover, only 1 isolate gave a positive result for *hyl*, related to the production of hyaluronidase which facilitates the spread of toxins and bacteria throughout the host tissue by causing tissue damage. Aggregation substance (*asa1*) was presented in 15 (20.8%) isolates. *Asa1* is a sex pheromone plasmid-encoded surface protein, which promotes the conjucative transfer of sex pheromone plasmids by formation of mating aggregates between donor and recipient cells. Antibiotic resistance to clinically important antibiotics for enterococcal infections is a serious problem. None of the isolates was positive for *vanA* and *vanB* genes. This is an agreement with the results of Franz et al., (2001) reporting that *vanA* and *vanB* genes have not been found frequently in *enterococci* isolates from food sources. The safety profile of *enterococci* isolated from donkey milk, revealed that the majority of them were susceptible to the most clinically relevant antibiotics such as vancomycin. Finally to complete the safety-status analysis,

the *enterococci* were screended for the presence of three amino decarboxylase genes. This is a feature shared by many LAB that produce biogenic amines in food. Regarding the presence of genes for the production of histidine (*hdc1* and *hdc2*), ornithine (*odc*) and tyrosine (*tdc*) decarboxylase, no amplification occurred for either hdc1 and hdc2 or odc. However, the *tdc* gene was presented in 22 (30.6%) isolates. Phenotypic tests confirmed the genetic results since the production of biogenic amines was detected only for tyrosine.

Moreover, the presence of some virulence genes such as *gelE, asa1, ace* and *esp* genes in some isolates, cannot be considered as a negative trend since these genes have been also found in commercial *enterococci* starter cultures with a long history of safe use (Eaton & Gasson, 2001). The presence of proteins encoded for these virulence genes may be considered as a benefit for the bacteria since they were associated with colonization abilities and therefore enable the bacterium to colonize and proliferate in the gastrointestinal track and reveal its probiotic properties (Cebrian et al., 2012).



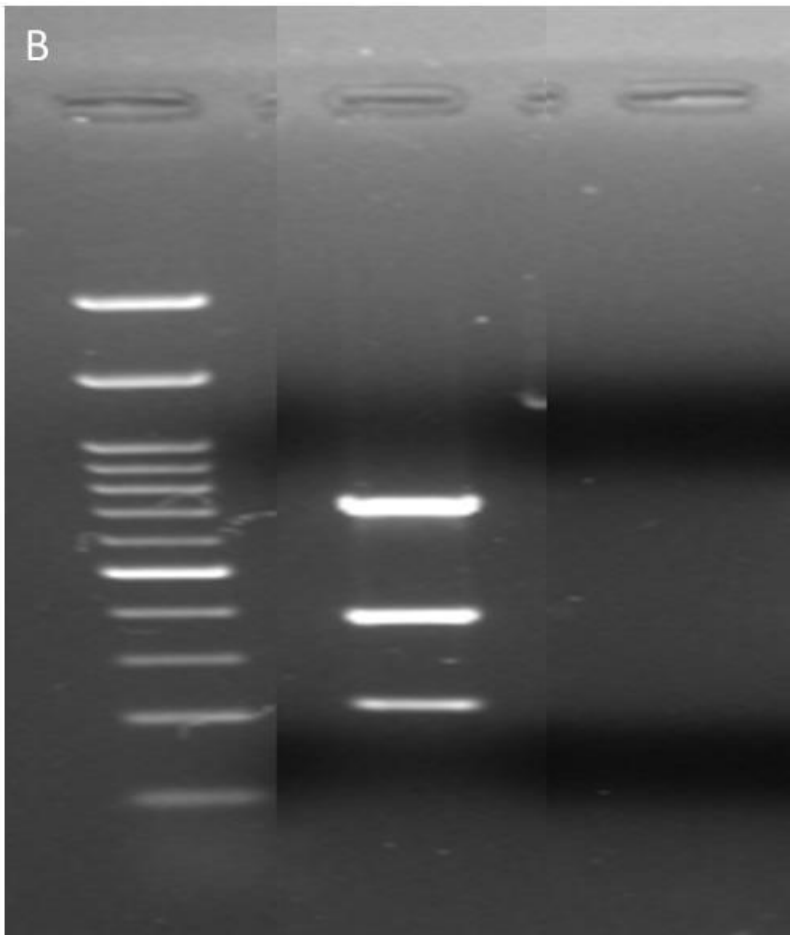


Figure 31: A) Distribution of virulence factors among Enterococci isolated from donkey milk; B) Multiplex PCR for *gelE* (213bp), *asa1* (375bp) and *cylA* (688bp) genes, Lane 1: Molecular weight marker 100bl, Lane 2: *E. faecalis* ATCC 29212 (positive control), Lane 3: *E. faecium* DM33

4.2 Bacteriocin production from strains isolated from donkey milk

4.2.1 Antimicrobial activity assays

Isolates (74) tested for technological properties plus the three *Leuconostoc mesenteroides* strains collected from donkey milk were screened for antimicrobial activity by spot on law and agar well diffusion assay. Only 15 isolates (19.5%) displayed antibacterial activity against at least one indicator strain. Of these, three isolates, *E. faecium* DM33, *E. faecium* DM224 and *E. faecium* DM270, were selected for further analyses based on their antimicrobial activity against more than four indicator strains such as *B. cereus* DPC 6089, *L. monocytoges* 33104, *L.*

monocytogenes 33413 and *S.aureus* RF 122. Our results are concordant with previous investigations in that production of bacteriocins with activity against *Listeria* spp. is a common characteristic of enterococci isolated from different food sources (Cocolin et al., 2007; Ghrairi et al., 2008; Moreno et al., 2006; Rehaiem et al., 2016). This can be related to the close phylogenetic relationship of enterococci and listeriae (Laukova et al., 2001). Moreover, the inhibitory activity of enterocins has also been demonstrated against *S. aureus* and *B. cereus* (Chen et al., 2007; Ghairi et al., 2008; Munoz et al., 2004).

4.2.2 Effect of proteolytic enzymes

In order to establish the proteinaceous nature of the antimicrobial substances produced by these three strains, their sensitivity to a variety of proteolytic enzymes was examined. The enzymes α-chymotrypsin, trypsin and pronase were shown to eliminate the antimicrobial activity derived from cell free supernatant of all three strains, but the antimicrobial activity was not inactivated by pepsin (Figure 32). Similar results have recorded for most enterocins produced by *E. faecalis* and *E. faecium* (Cocolin et al., 2007; Franz et al., 1996; Ghrairi et al., 2008; Parente and Hill, 1992).

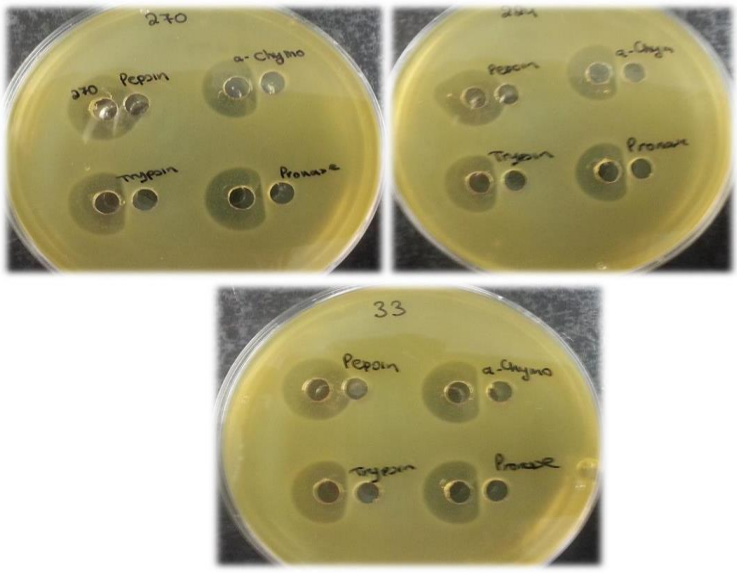


Figure 32: Effects of the proteases pepsin, α-chymotrypsin, trypsin and pronase on the antibacterial activity of cell free supernatant of *Enterococcus faecium* DM33, DM224 and DM 270.

4.2.3 Effect of pH, organic solvents and heat treatments

The effects of pH, organic solvent and heat treatment on the antibacterial activity of cell free supernatant produced by the three strains of interest are presented in Table 28. The cell free supernatant from all 3 strains remained fully active over the whole pH range utilized in the study (pH2-8) (Figure 33). The pH stability of the bacteriocins produced in this study is not unique, as similar stability of bacteriocins produced by *E. faecium* was observed over a wide pH range in previous studies (Favaro et al., 2014; Franz et al., 1996; Park et al., 2003). The pH stability over a wide range provides them with a great potential as natural preservatives for foods and fermented products where products are acidified or pH levels decreases due to natural LAB present as well in non-acid foods (Franz et al., 1996).

The bacteriocin-containing supernatant also retained a considerable degree of activity after high temperature treatments as determined by well diffusion assays. Inhibitory activity did not decrease even following autoclave temperature (121 °C) for 15 min (Table 30). Heat stability is a common property of bacteriocins produced by *enterococcus* (Yildirim et al., 2014). Moreover, it is a very important trait should the bacteriocin be developed for use as a food preservative, since many food-processing procedures involve a heating step (Lee et al., 1999).

Table 28: Effect of temperature, pH, organic solvents on the antibacterial activity present in *E. faecium* DM33, DM224 and DM270 culture supernatants. Values are the mean of triplicate zone of inhibitions using well assays (mm) ± SD; in (parenthesis) is given the retained activity

	Strains		
Parameters	**DM33**	**DM224**	**DM270**
pH2	14.57±0.02[b] (79%)	12.94±0.05[a] (81%)	15.89±0.23[c] (89%)
pH3	14.97±0.16[b] (82%)	12.80±0.12[a] (80%)	16.12±0.34[c] (90%)
pH4	15.64±0.21[b] (85%)	12.96±0.01[a] (81%)	16.13±0.10[c] (91%)
PH5	15.55±0.09[b] (85%)	13.43±0.03[a] (84%)	16.53±0.29[c] (93%)
pH6	15.46±0.01[b] (84%)	13.21±0.05[a] (83%)	15.91±0.35[b] (89%)
pH7	15.47±0.17[b] (84%)	13.43±0.33[a] (84%)	15.90±0.06[c] (89%)
pH8	15.46±0.04[b] (84%)	13.11±0.18[a] (82%)	15.94±0.13[c] (89%)
30°C	15.91±0.03[b] (88%)	14.00±0.11[a] (88%)	16.83±0.20[c] (94%)
40°C	16.23±0.19[b] (88%)	14.04±0.42[a] (88%)	16.85±0.35[b] (95%)
50°C	16.25±0.11[b] (89%)	14.62±0.09[a] (82%)	16.57±0.21[c] (93%)
60°C	15.99±0.32[b] (87%)	14.37±0.12[a] (90%)	17.09±0.23[c] (96%)
70°C	16.08±0.07[b] (88%)	14.19±0.04[a] (89%)	16.53±0.09[c] (93%)
80°C	16.07±0.03[b] (88%)	14.19±0.08[a] (89%)	16.62±0.12[c] (93%)
90°C	15.94±0.01[b] (87%)	13.89±0.20[a] (87%)	16.60±0.19[c] (93%)
100°C	15.95±0.14[b] (87%)	13.87±0.06[a] (87%)	17.74±0.09[c] (100%)
121°C	14.00±0.22[a] (76%)	14.31±0.09[a] (90%)	16.02±0.08[b] (90%)
Methanol (70%)	13.10±0.11[b] (71%)	11.93±0.22[a] (75%)	13.88±0.07[c] (78%)
Acetonitrile (70%)	13.55±0.03[b] (74%)	11.45±0.17[a] (72%)	11.69±0.07[a] (66%)
Isopropanol (70%)	14.10±0.17[b] (77%)	12.10±0.04[a] (76%)	14.30±0.11[b] (80%)
Ethanol (70%)	13.14±0.27[b] (72%)	11.72±0.16[a] (73%)	13.70±0.04[c] (77%)
Control	18.35±0.28	15.95±0.12	17.82±0.12

Prior to attempts for purification via HPLC, the antimicrobial activity of cell free supernatants from the three strains were examined in a variety of organic solvents that are often used during peptide purification procedures and HPLC. Stability in organic solvents is important in the isolation and extraction of bacteriocins from the liquid culture supernatants. Moreover, elution of the bacteriocin from polymeric adsorbent materials (e.g. Amberlite XAD-16), Phenyl Sepharose and Reverse Phase columns requires high concentrations of some organic solvents like isopropanol, ethanol,

methanol, and acetonitrile. It was observed that activity was retained when mixed with various organic solvents (Figure 33). In addition, the resistance of enterocin DM33, DM224 and DM270 to organic solvent confirms that lipid moiety is not responsible for the antimicrobial activity (Yildirim et al., 2014). Therefore, it may find application in high-fat foods such as cheeses.

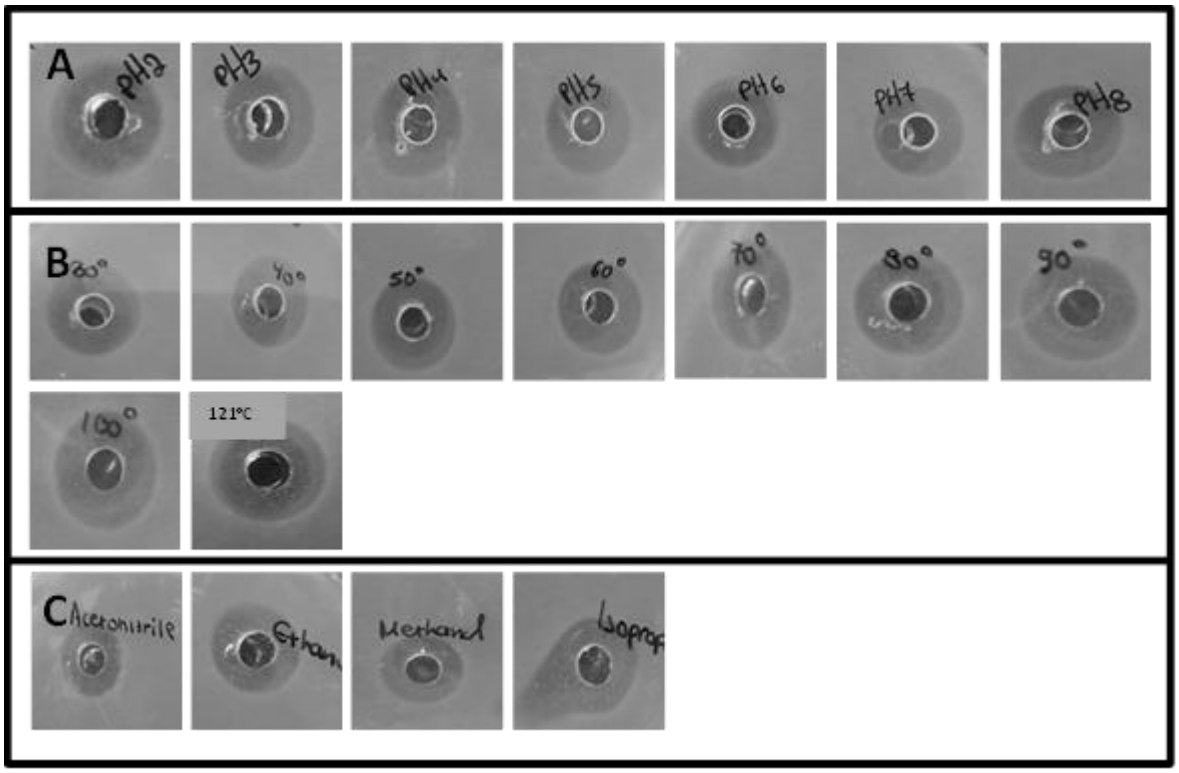


Figure 33: (A) Effect of pH, (B) Heat Treatment, (C) Organic Solvent, on the antimicrobial activity of CFS of strain DM33

4.2.4 Effect of medium composition on bacteriocin production

The culture conditions and composition of the growth medium are very important for the production of individual bacteriocins. Several types of media have been evaluated by numerous authors to improve bacteriocin synthesis because these peptides are not always produced in standard or enriched culture media. LAB are fastidious microorganisms that require rich media containing milk, whey ultrafiltrate, or complex synthetic media such as MRS or M17 for growth. Therefore, the isolation of a peptide(s) in rich medium supernatant is an additional problem, making the purification of the bacteriocin a relatively complicated protocol.

In experiments with different growth media, the results indicated that the composition of medium greatly influences the production of bacteriocin by *enterococci*. Indeed, of the three growth media tested, MRS was deemed to be the most suitable medium for bacteriocin production. All strains produced low levels of bacteriocin activity when grown in BHI. In contrast, no antimicrobial activity was observed when grown in TSB. The low activity levels recorded in BHI broth and TSB broth suggest that specific nutrients required for bacteriocin production are absent in these media.

4.2.5 Purification of bacteriocins

The bacteriocin produced by each strain was purified and separated by adsorption onto a C18 silica column, followed by RP-HPLC, resulting in the chromatogram shown in Figure 34. Subsequent matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis revealed an associate mass of approximately 4830Da and 5465Da for each strain, but a definitive mass for Ent P could not be detected.

The molecular weights of enterocins A, B, and P have previously been reported, using mass spectrometry: 4828-Da for enterocin A from *E. faecium* CTC 492 (Aymerich et al., 1996), 5465.2 Da for enterocin B from *E. faecium* T 136 (Casaus et al., 1997), and 4.5 kDa for enterocin P from *E. faecium* P 13 (Cintas et al., 1997). The analysis of the mass spectrometry data revealed that all *enterococci* isolates produced both enterocins A and B simultaneously. Even though this combination of bacteriocins was also observed by other researches (Park et al., 2003; Poeta et al., 2007; Strompfová et al., 2008), this combination of genes has not been reported for any donkey isolate.

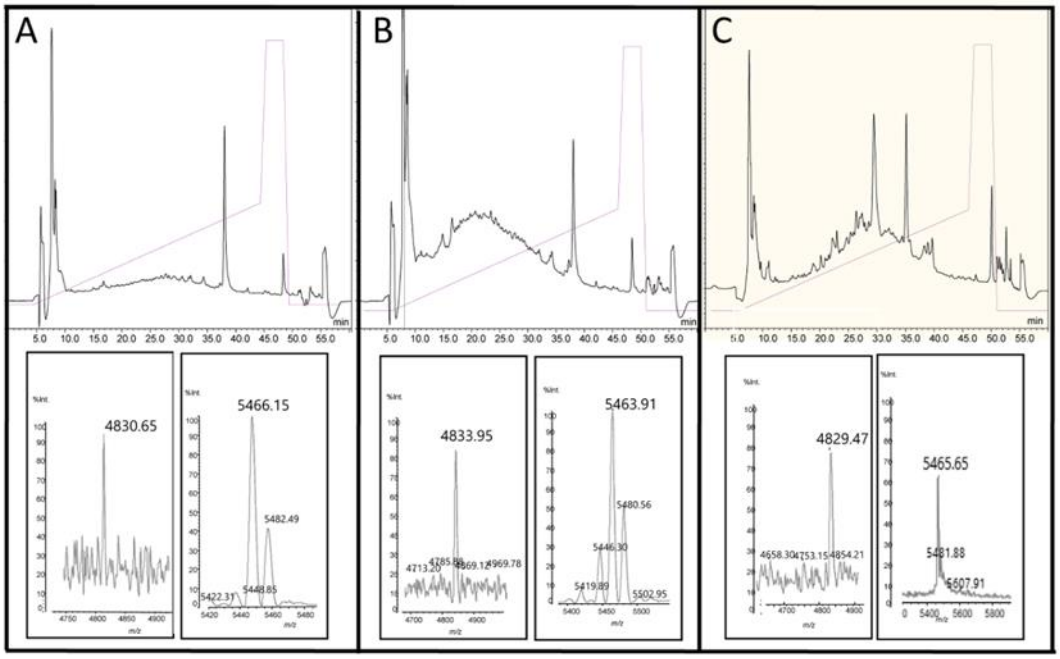


Figure 34: RP-HPLC profile (top panel) and Matrix- assisted laser desorption/ionization time-of-flight MS data (bottom panel) of partially purified extracts produced by (A) *E. faecium* DM33,(B) *E. faecium* DM224 and (C) *E. faecium* DM270.

4.2.6 Inhibitory spectrum of purified bacteriocins

The inhibitory spectrum of purified bacteriocin extracts from the enterococcal strains DM33, DM224 and DM270 as assessed by the agar-well diffusion method are presented in Table 29 and Figure 35. Inhibitory activity was directed against several food spoilage bacteria and foodborne pathogens, including LAB, *L. monocytogenes, S. aureus* and *B. cereus*. Strain DM33 and strain DM270, exhibited almost identical inhibitory spectra, including *L. monocytogenes* but not against any of the *S. aureus* strains tested. In contrast, strain DM224, exhibited a much broader range of antimicrobial activity. In addition to foodborne pathogens, antimicrobial activity was also detected against the natural microbiota present in fermented dairy products such as some *Lactobacillus, Lactococcus, and Streptococcus*. Importantly, no activity was apparent against other strains of *lactobacilli*, i.e. *Lb. bulgaricus* HE, *Lb. bulgaricus* 1373 and *Lb. acidophilus* 4356, that can often be used as commercial probiotic cultures. The inhibitory spectrum of the three bacteriocin producing strains is in aggrement with other studies which also showed a strong activity against *Listeria* and limited antagonistic activity against

mesophilic dairy starter cultures. From an application point of view, a bacteriocin that antagonizes undesirable bacteria with no effect on useful starter and nonstarter LAB would be suitable as a food preservative (Rehaiem et al., 2010).

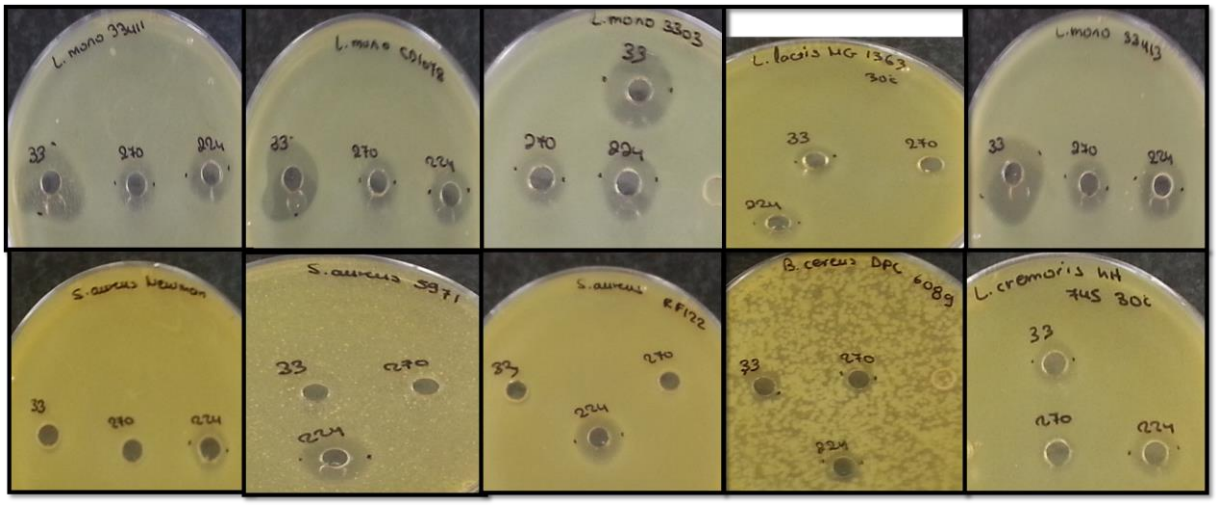


Figure 35: Antimicrobial activity of LAB isolates against different indicator strains

Table 29: Inhibitory spectrum of purified bacteriocins. Values are the mean of triplicates of zone of inihibitions using well assays (mm). (-) No antimicrobial activity (mm)

Strains	*E. faecium* DM 33 (mm)	E. faecium DM 224 (mm)	E. faecium DM 270 (mm)
B. cereus DPC 6086	-	12.22±0.38	-
B. cereus DPC 6089	11.34±0.17^{c}	10.55±0.22^{b}	9.81±0.08^{a}
E. faecium DPC 1146	-	-	-
Lb. acidophilus ATCC 4356	-	-	-
Lb. bulgaricus 1373	-	-	-
Lb. bulgaricus HE	-	-	-
Lb. helveticus UCC 505	21.95±0.11^{c}	17.10±0.29^{a}	19.19±0.08^{b}
L. cremoris IP 5	7.10±0.19^{a}	13.79±0.03^{b}	7.33±0.03^{a}
Lactococcus cremoris subsp cremoris KH	10.90±0.12^{b}	12.40±0.06^{c}	7.30±0.16^{a}
Lactococcus lactis subsp lactis 275	-	10.73±0.15	-
Lactococcus lactis subsp lactis 303	17.02±0.04^{c}	13.93±0.19^{a}	14.86±0.12^{b}
Lactococcus lactis subsp cremoris HP	15.10±0.09^{b}	13.71±0.06^{a}	15.76±0.24^{c}
Lactococcus lactis subsp cremoris	9.22±0.06^{b}	11.17±0.04^{c}	8.75±0.14^{a}
L. mono 33013	17.84±0.10^{c}	14.85±0.24^{b}	12.60±0.05^{a}
L. mono 33104	18.70±0.04^{c}	15.35±0.16^{b}	13.80±0.12^{a}
L. mono 33410	18.52±0.09^{c}	15.68±0.13^{b}	13.78±0.27^{a}
L. mono 33411	18.32±0.27^{c}	15.67±0.02^{b}	13.1±0.19^{a}
L. mono 33413	21.06±0.01^{c}	15.65±0.11^{b}	14.96±0.31^{a}
L. mono 33423	19.87±0.14^{c}	14.90±0.07^{b}	13.28±0.11^{a}
L. mono CD 1078	19.20±0.01^{c}	13.67±0.10^{b}	11.66±0.02^{a}
M. luteus DSM 1790	-	10.64±0.13	-
S. aureus NCDO 1499	-	13.20±0.22	-
S. aureus 5971	-	14.83±0.08	-
S. aureus DPC 5243	-	14.15±0.17	-
S. aureus DPC 5247	-	13.19±0.14	-
S. aureus Newman	-	12.34±0.20	-
S. aureus RF122	-	14.31±0.11	-
S. dysgalacticae ATCC 43078	-	15.71±0.04	-
S. uberis ATCC 700407	-	-	-

Different letters in the same row indicate significant statistical differences ($P<0.05$) as analyzed by Duncan test.

4.2.7 Bacteriocin inhibitory assay in culture broth

As observed from the results reported in Table 29, all three bacteriocin-containing extracts possess bactericidal activity against all of the *L. monocytogenes* strains utilized in this study. Importantly, these isolates included *L. monocytogenes* 33104 (Also known as F2365) associated with an epidemic outbreak of listeriosis in 1985, *L. monocytogenes* 33413 (also known as Ts45) from a UK food outbreak in 1988), *L. monocytogenes* 33013 (also known as Scott A, Massachusetts outbreak 1983) as well as *L. monocytogenes* 33411 (Food outbreak, Canada 1981) and *L. monocytogenes* 33410 associated with an outbreak in California in 1985 (Clayton et al., 2011). While deferred antagonism and well diffusion assays represent important tools in the detection and characterisation of bacteriocin producing strains, they are end point assays and cannot reveal the more subtle details of the impact of an antimicrobial on bacterial viability that are apparent when growth curve analysis is performed. Here, a most profound delay in growth was observed for *L. monocytogenes* CD 1078, *L. monocytogenes* 33423 and *L. monocytogenes* 33411 when in the presence of purified extracts from DM33, DM270 and DM224 (Figure 36). Similar findings were previously reported for other bacteriocins produced by *E. faecium* strains (Favaro et al., 2014; Schirru et al., 2012). Additionally growth curve analysis of S. *aureus* RF 122, *B. cereus* DPC 6086, *S. aureus* DPC 5243, and *S. aureus* 5971, indicated the purified extracts had a bacteriostatic effect when compared to the untreated control. Noticeably, the purified extract derived from DM224 proved to have the maximum inhibitory effect against *S. aureus* RF 122 and *S. aureus* 5971 as observed by the increased lag phase compared to the purified extracts from DM33 and DM270, in agreement with the findings from deferred antagonism assays.

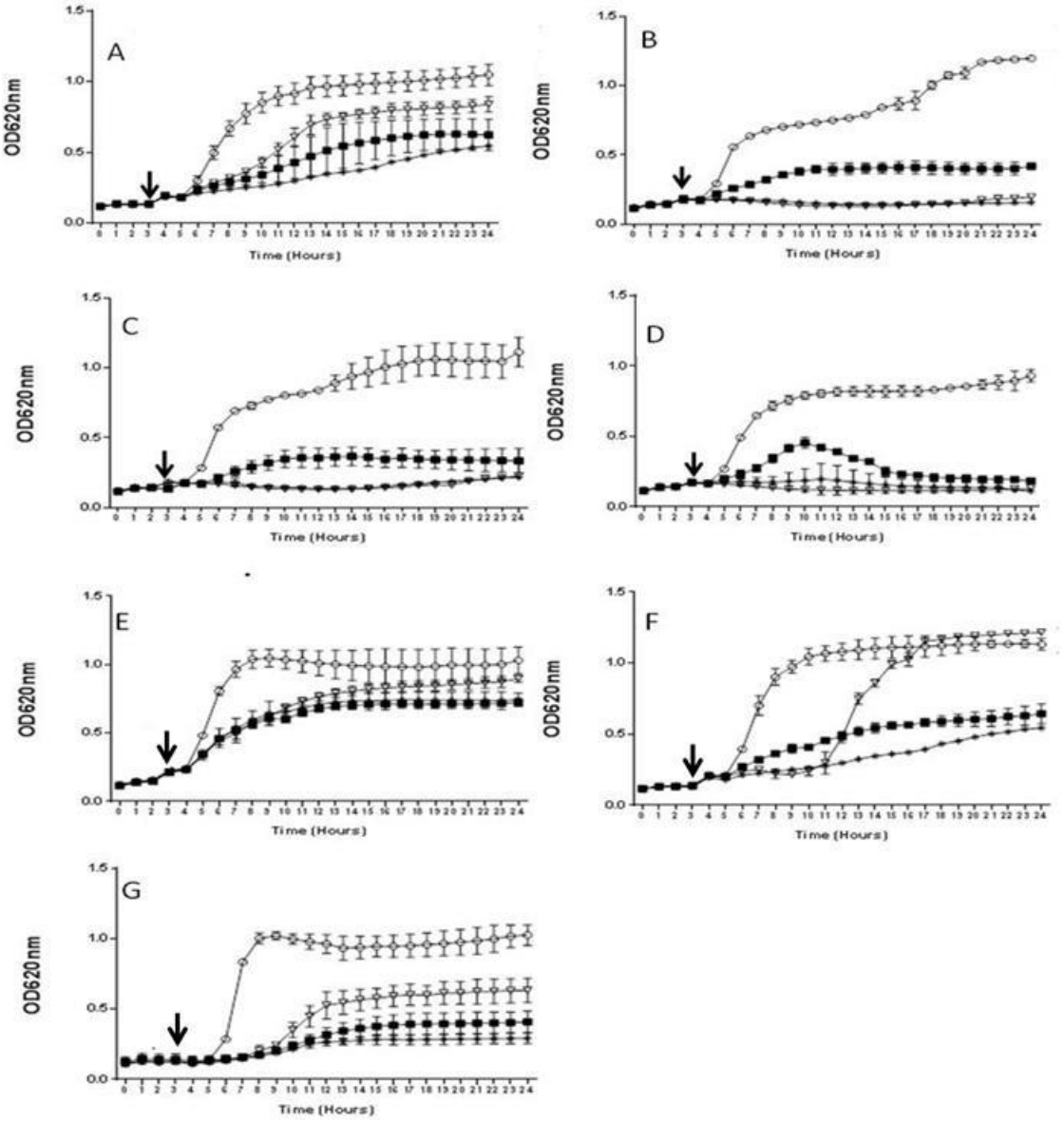


Figure 36: Growth of (A) *B. cereus* DPC 6086, (B) *L. monocytogenes* 33423, (C) *L. monocytogenes* 33411, (D) *L. monocytogenes* CD1078, (E) *S. aureus* DPC 5243, (F) *S. aureus* RF122 and (G) *S. aureus* 5971 in BHI broth, without (open circle) and in the presence of purified extracts of *E. faecium* DM 33 (open triangle), *E. faecium* DM 224 (diamond) and *E. faecium* DM 270 (closed square). Arrows indicate the point at which the bacteriocins were added.

4.2.8 PCR amplification of bacteriocin genes

The DNA of the three strains was further screened by PCR for the presence of known enterocin structural genes, using specific primers. The agarose gel electrophoresis revealed three different amplified fragments of 138bp, 201bp and 87bp (Figure 37).

These fragments corresponded with PCR signals for enterocin A, B and P, respectively, whereas the other primers used did not yield any visible bands. Enterocins HF, L50A/B, Q, AS-48 and enterocin 31 genes were not found in any of the three strains. It has been previously reported that one LAB strain can carry more than one bacteriocin gene (Poeta et al., 2007).

According to (Ishibashi et al., 2012) multiple enterocins-producing strains are thought to be more effective and might show a wider range of inhibition than a single bacteriocin producer. Additionally, in populations where the dominant bacteriocin producing strain produces multiple bacteriocins, the development of resistant bacteria could be slowed down (Tessema et al., 2009). However, the occurrence of several enterocin structural genes in *enterococcus* isolates does not always associate with a higher bacteriocin activity in their supernatants, and not all enterocin genes express it at the same time (Casaus et al., 1997).

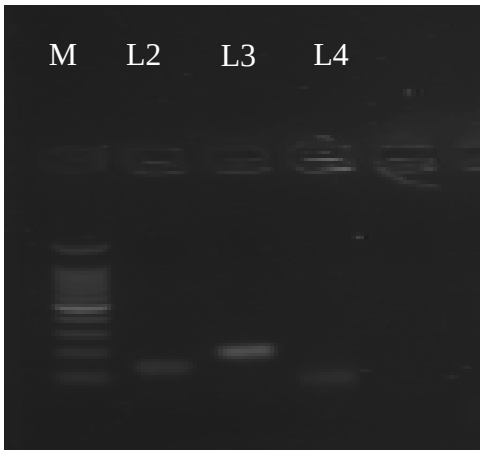


Figure 37: Amplification of bacteriocin genes from isolate DM33 on 1.5% agarose gel; M:100bp DNA marker, L2: *entA*, L3: *entB*, L4: *entP*

4.2.9 Challenge test

4.2.9.1 Bacterial counts and pH

The antimicrobial activity of bacteriocin-producing strains against *L. monocytogenes* 33413 in Anari cheese during storage is illustrated in Table 30 and Figure 38. Microbiological analysis of control cheese showed no detectable levels of *L. monocytogenes* 33413 and *Enterococci* while the initial total viable count (TVC) was measured at 0.7 log cfu/g. In the cheeses experimentally contaminated with 3 log cfu/g

of *L. monocytogenes* 33413, in the absence of bacteriogenic culture, *L. monocytogenes* 33413 grew well reaching 6.50 log cfu/g and 7.38 log cfu/g after 6 and 9 days respectively, under refrigeration. The excessive growth of *L. monocytogenes* 33413 in control cheese is consistent with previous studies indicating a high growth potential of bacterial pathogens in whey cheeses (Samelis et al., 2003).

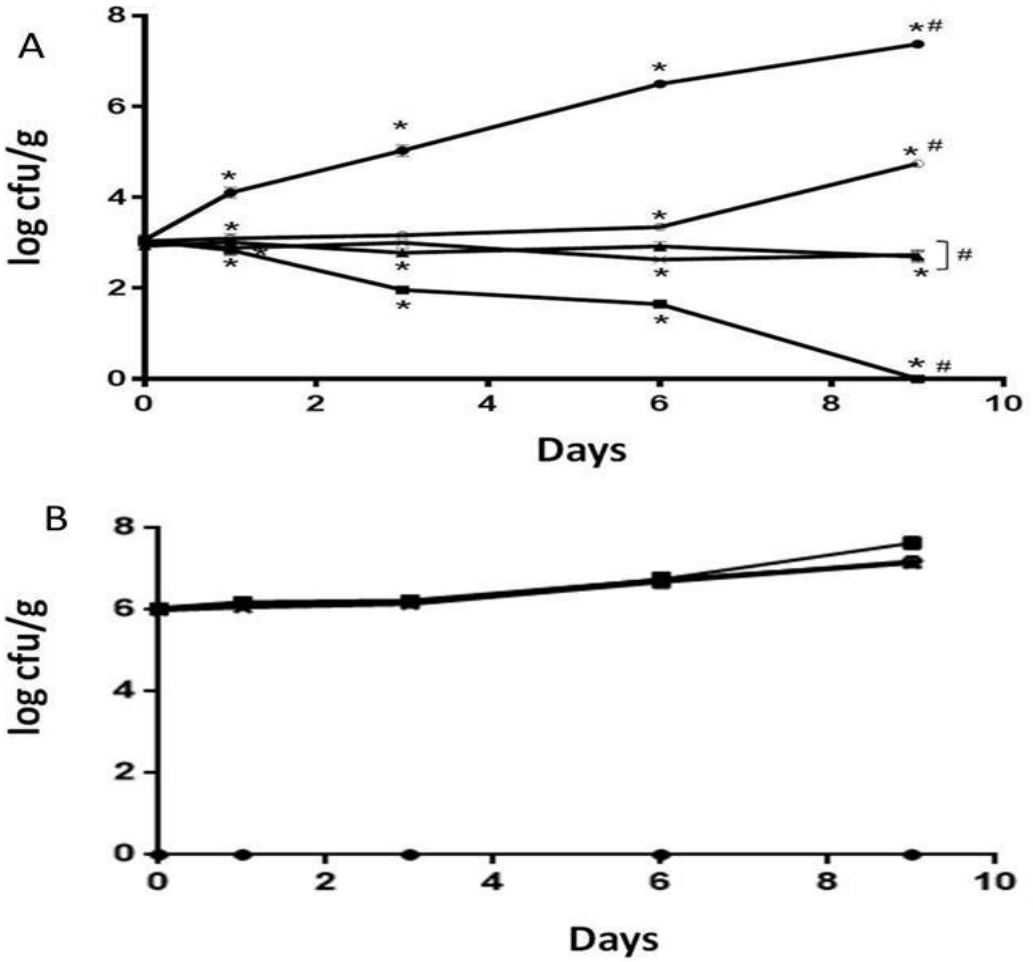


Figure 38: A) *L. monocytogenes* count (mean log CFU/g ± SD) B) enterococci population (mean log CFU/g ± SD) during the storage of Anari cheese manufactured with bacteriocin-producing lactic acid bacteria and a non-bacteriocin producing control lactic culture; control cheese *L. monocytogenes* 33413 (closed circle), *E. faecium* DM 33 (closed square), *E. faecium* DM 224 (closed triangle), *E. faecium* DM 118 (open diamond) and *E. faecium* DM 270 (cross) . * Statistically significant difference ($P < 0.05$) of the same strain during storage. # Statistically significant difference ($P < 0.05$) between strains at day 9.

Table 30: *L. monocytogenes* and LAB counts (mean log CFU/g ± SD) during the storage of fresh Anari cheese inoculated with bacteriocin producing enterococci and a non-bacteriocin producing enterococci.

Sample	*L. monocytogenes 33413*					*Enterococci*				
	Storage time (days)					Storage time (days)				
	0	**1**	**3**	**6**	**9**	**0**	**1**	**3**	**6**	**9**
A	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
B	3.06± 0.11$^{A, a}$	4.10±0 .11$^{E, b}$	5.03± 0.12$^{E, c}$	6.50± 0.06$^{E, d}$	7.38± 0.08$^{D, e}$	<10	<10	<10	<10	<10
F	3.04± 0.11$^{A, e}$	2.84±0 .05$^{A, d}$	1.96± 0.08$^{A, c}$	1.65± 0.08$^{A, b}$	ND$^{A, a}$	6.06± 0.06$^{A, a}$	6.21±0 .04$^{D, b}$	6.25± 0.04$^{D, b}$	6.75± 0.13$^{A, c}$	7.65± 0.12$^{B, d}$
G	2.93± 0.06$^{A, b}$	3.01±0 .09$^{CD, b}$	2.78± 0.09$^{B, a}$	2.92± 0.10$^{C, b}$	2.70± 0.12$^{B, a}$	6.05± 0.04$^{A, a}$	6.18±0 .04$^{CD, b}$	6.20± 0.03 $^{CD, b}$	6.76± 0.13$^{A, c}$	7.16± 0.06$^{A, d}$
H	3.04± 0.06$^{A, c}$	2.88±0 .15$^{BC, b}$	3.00± 0.07$^{C, bc}$	2.63± 0.08$^{B, a}$	2.74± 0.10$^{B, a}$	5.98± 0.06$^{A, a}$	6.06±0 .08$^{A, ab}$	6.13± 0.05 $^{A, b}$	6.67± 0.11$^{A, c}$	7.15± 0.05$^{A, d}$
I	3.03± 0.06$^{A, a}$	3.09±0 .10$^{D, bc}$	3.17± 0.04$^{D, c}$	3.35± 0.07$^{D, d}$	4.75± 0.08$^{C, e}$	6.01± 0.06$^{A, a}$	6.12±0 .08$^{BC, b}$	6.18± 0.07 $^{BC, b}$	6.72± 0.15$^{A, c}$	7.20± 0.04$^{A, d}$

ND=Not detected
Different lowercase letters in the same row indicate significant statistical differences ($p<0.05$) during storage
Different uppercase letters in the same column indicate significant statistical differences ($p<0.05$) between the strains

L. monocytogenes 33413 counts were reduced at different levels for all the trials where the cheese had been inoculated with *Enterococci* regardless of the strain used. However, the highest reduction in *L. monocytogenes* 33413 counts was observed for cheese artificially inoculated with *E. faecium* DM33. After 6 days of storage at 4 °C *L. monocytogenes* 33413 counts were reduced by more than 4 logs compared to the cheese inoculated with *L. monocytogenes* 33413 without the bacteriocin producing srain, and by the 9th day of storage, no *L .monocytogenes* 33413 were recovered, indicating the bactericidal nature of DM33. In contrast, both *E. faecium* DM270 and DM224 inhibited the growth of *L. monocytogenes* 33413, therefore a bacteriostatic action was observed. After 9 days of storage at 4 °C, the counts of *L. monocytogenes* 33413 remained the same as the initial inoculum. Furthermore, the counts of *L. monocytogenes* 33413 in the cheese that was inoculated with a bacteriocin-negative *E. faecium* DM118 strain

reached 4.75 log cfu/g after 9 days of storage. This confirmed that the inhibition of *L. monocytogenes* 33413 in cheese was due to enterocin production.

Cell counts of the bacteriocin producing bacteria were also recorded during storage at 4 °C. The results illustrate (Table 30) that all the added *Enterococci* maintained their viability in Anari cheese during the 9 days of storage under refrigeration, despite the presence of *L. monocytogenes* 33413. The pH values determined during the storage of cheese samples at 4 °C showed no major differences in pH between control (pH 6.74) and artificially contaminated cheeses (pH 6.68-6.74). Moreover, no visible changes in the physical characteristics (texture or colour) of the cheeses containing the bacteriocin producing strains were observed.

According to different studies the application of bacteriogenic strains in cheese to control the growth of *L. monocytogenes* depends on the strain and the type of cheese. Izquierdo et al., (2009) reported that *E. faecium* WHE 81 is capable of controlling *L. monocytogenes* in Munster cheese due to bacteriocin production. In another study, the growth of *L. monocytogenes* 426 in Minas Frescal cheese was inhibited due to the presence of *E. mundtii* CRL 35, a bacteriocinogenic strain, up to 12 days at 8 °C (Pingitore et al., 2012).

Our results showed that amongst the tested *enterococci, E. faecium* DM33 demonstrated a good potential for application in Anari cheese for the control of *L. monocytogenes* 33413. Results from safety evaluation reveal that this strain is negative for gelatinase, aggregation substance, extracellular surface protein, vancomycin resistance genes (*vanA, vanB*) and biogenic amine production (tyramine, histidine, ornithine) indicating that this strain is safe for application in cheese. Even though the safe application of *enterococci* in food products has also been demonstrated by other researchers, another way to apply this strain in food products is through heterologous expression in more safer strains such as *Lactobacillus* or *Lactococcus* strains (GRAS) (Franz et al., 2011; Ogier & Serror, 2008).

4.2.9.2 Bacteriocin detection in cheese samples

Bacteriocin activity in cheese inoculated with the different *enterococci* strains was detected only in the cheese inoculated with the *E. faecium* DM33 throughout the storage time (10.4±0.4 mm), while for *E. faecium* DM270 bacteriocin activity detected after 6

days of storage at 4 °C (9.6±0.2 mm). No bacteriocin activity was detected for *E. faecium* DM224. Regarding the strains DM270 and DM224, an explanation for the late bacteriocin production in the first case, and the lack of bacteriocin activity in the second case by the well assay method, could be attributed to the fact that the production of these two enterocins was reduced in a natural food system such as milk compared to the activity obtained in broth (Sarantinopoulos et al., 2002). The effectiveness of a pure bacteriocin-producing culture in a food product depends on the capability of the added strain to grow and produce the bacteriocin under the fermentation conditions. For this reason the production of a certain bacteriocin in laboratory media (*in vitro*) does not imply its effectiveness in a natural food system. The factors that might affect its activity include the food structure and composition and their interaction with bacteriocins (Cocolin et al., 2007). Moreover, these two strains showed lower antimicrobial activities in the broth tests as well as in well assays (Figure 40, Table 31) than strain DM33.

4.3 Probiotic Potential

A number of probiotic products currently on the market include some enterococcal, *lactobacillus* and *leuconostoc* preparations and for this reason we tested some properties recommended as desirable for a probiotic microorganism, including pH, bile tolerance, auto-aggregation and co-aggregation ability, cell hydrophobicity and bile salt hydrolase activity. The first requirement for a probiotic bacterium is its ability to survive transport to the active site in which its beneficial action is expected. Hence, the bacteria destined to benefit intestinal functions must survive passage through the hydrolysis in the human oral cavity, the acidic environment of the stomach and intestinal conditions.

4.3.1 Acid tolerance

In order to exert their beneficial effects in the host, probiotics must remain alive during both ingestion and their transit prior to reaching the large intestines. Acid tolerance of bacteria is important not only for withstanding gastric stresses, but also a prerequisite for their use as dietary adjuncts and enables strains to survive for longer period of time in high acid carrier food without larger reduction in humans (Shehata et al., 2016). Probiotics need to tolerate the acidic conditions of the stomach with pH between 1.5 and 3.0 in order to successfully pass through the stomach and small intestine. Although

stomach pH can be as low as 1.0, in most in vitro assays, pH 3.0 has been preferred (Haddadin et al., 2004). The mean resident time of food in the stomach is 3 hours, and hence assays are normally run for that long. The strains (77 isolates, 72 *enterococci*, 3 *leuconostoc* and 2 *lactobacilli*) were analyzed *in vitro* for their ability to survive under acidic conditions. Table 31 shows the results of the 9 strains used for further characterization. The selection of the strains was based on the percentage of survival after 3 h at pH 3. The strains showed the highest percentage of survival from each genus plus the three bacteriocin producing strains were used for further characterization.

The capability of bacteria to survive acidic conditions in the *in vitro* gastrointestinal model of this study were arbitrarily defined as not resistant (survival of <50% of cells), weakly resistant (survival of 50-75% of cells), moderately resistant (survival of 75-90% of cells), and strongly resistant (>90% survival of cells). In this study, all strains isolated from donkey milk survived in all times tested (1, 2 and 3h) at pH 3, which is considered to be the standard values of acid tolerance of probiotic cultures.

Table 31: Survival rate (%) of LAB isolates under acidic conditions (pH 3) during 3 hrs of incubation (Data are Mean Values ± SD, n=3)

Isolate	pH 3		
	1 h	2 h	3 h
DM18	99,42±0.85^{a}	88,73±0.61^{b}	70,32±0.43^{c}
DM33	99,74±0.30^{a}	79,34±0.74^{d}	73,13±0.27^{b}
DM150	86,23±0.38^{c}	79,25±0.70^{d}	73,24±0.27^{b}
DM214	88,21±0.72^{b}	77,25±0.64^{e}	71,82±0.51^{bc}
DM224	87,72±0.42^{b}	81,16±0.16^{cd}	66,34±2.21^{d}
DM236	76,63±0.61^{d}	76,42±0.56^{e}	70,91±0.98^{c}
DM237	76,44±0.20^{d}	70,48±1.00^{f}	64,93±0.16^{d}
DM246	88,33±0.12^{b}	81,74±0.70^{c}	70,94±0.56^{c}
DM270	99,81±0.32^{a}	99,30±0.77^{a}	75,32±0.74^{a}

$^{a-f}$ Means in the same column with different lowercase letters differ significantly ($p<0.05$)

Of all the 77 strains, 60 strains were weakly resistant after the incubation period with survivability between 50-75%, and only 2 strain demonstrated moderately resistance at pH 3 with survival range between 75-90%. The strains showed the highest resistances

were the bacteriocin producing *E. faecium* DM33 and DM270, followed by *L. paracasei* DM214.

Regarding the *enterococci* population 76% (55 of 72 strains), were resistant to pH 3. More than half of the strains showed survival more than 80% after two hour's exposure to this low pH. This exemplifies that *enterococci* are actually quite acid resistant. This feature, together with the heat resistance and salt tolerance of *enterococci*, is well known for these bacteria and is one of the reasons often cited for the robust nature of these bacteria (Franz et al., 1999; Giraffa et al., 2003). This resistance of low pH was also expected for *enterococci*, as these bacteria naturally occur in the gastrointestinal tract, or can be transmitted from environmental sources or food sources to the human gastrointestinal tract.

Both *lactobacilli* were resistant at pH 3 with a survival range between 66-72 %. Similar results have been previously reported by some authors evaluating the acid tolerance of *Lb. paracasei* strains from different fermented foods (Dunne et al., 2001; Leite et al., 2015; Maragkoudakis et al., 2006; Solieri et al., 2014).

Moreover, this study showed that all 3 strains of *L. mesenteroides* DM092, DM115 and DM236 can survive against the stress conditions assayed in this study. Previous studies reported that *L. mesenteroides* subsp. *mesenteroides* isolated from different sources were able to grow up and survive at low pH levels (Argyri et al., 2013; Benmechernene et al., 2013).

However, acid tolerance of the bacterial strains could be improved by some natural protectors within the product consumed, such as proteins and fats found in many dairy products (Livney, 2010). It is important to point out that the *in vitro* trials involving pH, bile salts, and NaCl tolerance cannot predict patterns of behavior in the human body. This is because most methodologies used to analyze the potential probiotic strains in stressful conditions are static models, which cannot foresee the gradual changes of pH values and bile salts in the GIT such as the presence of different enzymes and the peristaltic movements (Papadimitriou et al., 2015).

4.3.2 Bile salt tolerance

Resistance to bile salts is an important criterion for the selection of probiotic bacteria, which is a prerequisite for the colonization and metabolic activity of the strain in the small intestine of the host. Bile salts are surface-active chemicals produced in the liver

from the catabolism of cholesterol (Soomro & Masud, 2012). Bile entering the duodenal section of the small intestine was found to reduce survival of bacteria. This is probably due to the fact that all bacteria have cell membranes consisting of lipids and fatty acids which are very susceptible to destruction by bile salts which have detergent characteristics. Hence, the success of a probiotic also depends on the selected strain possessing bile-resistance qualities (Jin et al., 1998). Bile salts play an important role in the defense mechanisms of the gut; its inhibitory effects depend on the concentrations of this. Although the bile concentration of the human gastrointestinal tract varies and depends on ethnic group, physiological conditions, and gender, the mean intestinal bile concentration is believed to be 0.3 %, and the retention time is suggested to be 4 hours (Dunne et al., 2001; Prasad et al., 1998). By applying the same bile salt concentration (0.3 % w/v), the ability of all the selected isolates (9) to resist bile salts was revealed after 4 hours of incubation at 37 ^{o}C (Table 32). The results showed that all strains remain viable after culturing in 0.3 % concentration of bile salts after 4 hours of incubation. Two strains (DM236 and DM237) showed 100% survival, while the minimum survival (53.53%) was observed for *E. gallinarum* DM150 at 0.3% (w/v) of bile salt.

All *enterococci* used in this study showed a good survival rate except *E. gallinarum* DM150 which is in accordance with previous studies (Ahmadova et al., 2013; Hosseini, et al., 2009). *Enterococci* are well known to be commensals of the gastrointestinal tract of human and animals, and in this ecological niche, these bacteria come in contact and interact with bile salts. Thus, it is not surprising to find *Enterococcus* spp. resistant to bile salts (Franz et al., 2001).

Table 32: Survival rate (%) of LAB isolates in 0.3% (w/v) bile concentration during 4 hrs of incubation (Data are Mean Values ± SD, n=3)

Isolates	Bile concentration (0.3% w/v)			
	1 h	2 h	3 h	4 h
DM18	99,65±0.51[a]	63,60±0.56[f]	59,98±0.63[e]	87,79±0.18[b]
DM33	100,00±0.28[a]	93,84±0.48[a]	74,09±0.59[a]	87,23±0.32[b]
DM150	99.72±0.12[a]	75,41±0.74[d]	61,10±0.74[d]	53,53±0.28[f]
DM214	100,00±0.14[a]	80,25±0.21[b]	44,87±0.41[g]	59,86±0.12[e]
DM224	100,00,±0.11[a]	77,20±0.72[c]	65,11±0.45[c]	80,07±0.70[c]
DM236	99.84±0.26[a]	67,83±0.51[e]	67,51±0.12[b]	100,00±0.56[a]
DM237	93,12±0.43[c]	61,50±0.18[g]	68,42±0.16[b]	100,00±0.49[a]
DM246	96,53±0.18[b]	67,04±0.20[e]	49,44±0.18[f]	63,16±0.15[e]
DM270	100.00 ±0.27[a]	64,07±0.41[f]	49,78±0.21[f]	72,00±0.86[d]

[a-f] Means in the same column with different lowercase letters differ significantly ($p<0.05$)

L. mesenteroides (DM236) showed 100% survival at 0.3% bile salt concentration which is accordance with the results published by Diana et al., (2015). However, Allameh et al., (2012) and Todorov et al., (2008) reported that bile salt affected the growth rate of *L. mesenteroides* subsp. *mesenteroides* and limited its viability. *Lb. paracasei* DM214 showed also resistance in MRS broth containing bile salts at 0.3%, indicating that it possessed a bile tolerance property which is in agreement with previous studies (Guo et al., 2009; Floros et al., 2012; Zoumpopoulou et al., 2008).

4.3.3 Cell hydrophobicity assay

Hydrophobicity is one of the physicochemical properties that can facilitate the first contact between the microorganisms and the host cells (Shobharani & Agrawal, 2011). The bacterial cell surface contains proteins and polysaccharides with hydrophobic and hydrophilic properties (carboxylic groups and Lewis acid–base interactions), which are responsible for the adhesion of bacteria to surfaces (de Paula et al., 2015). Bacterial adhesion determines the colonization capability of a microorganism. Through adhesion ability and colonization of tissues, probiotic microorganisms can prevent pathogen access by steric interactions or specific blockage on cell receptors (Otero et al., 2004). The determination of microbial adhesion to hydrocarbons as a way to estimate the

ability of a strain to adhere to epithelial cells is a valid qualitative phenomenological approach (Collado et al., 2008). Hydrophobicity is directly related to concentration of carbon in hydrocarbon form and inversely related to oxygen concentration or to the nitrogen/phosphate ratio (Boonaert & Rouxhet, 2000). Greater hydrophobicity of cells is postulated in greater attractive forces and higher levels of adhesion (Marin et al., 1997). Many studies showed that the presence of (glycol-) proteinaceous material at the cell surface resulted in higher hydrophobicity; whereas, hydrophilic surfaces were associated with the presence of polysaccharides (Abdulla et al., 2014). Although hydrophobicity may assist in adhesion, it is not a prerequisite for strong adhesion to human intestinal cells. Cell surface hydrophobicity assays do not measure the intrinsic microbial cell surface hydrophobicity, but rather the bacterial partitioning to a certain hydrophobic substrate (i.e., xylene).

The selected strains were subjected to hydrophobicity testing towards different hydrocarbons i.e. n-hexadecane and xylene. Tests were done in triplicate. The results are shown in Table 33. Hydrophobicity of selected strains was arbitrarily defined as weak (0-30% hydrophobicity), moderate (31-60% hydrophobicity), and strong (61-100% hydrophobicity).

Results in Table 33 revealed that hydrophobicity of the 9 isolates toward hexadecane were lower than that in xylene, an apolar solvent, demonstrating hydrophobic cell surface of these strains. The percentages of hydrophobicity ranged from 2.15 to 28.22% for hexadecane and 27.62 to 40.76% for xylene. *E. faecium* DM33 showed the highest hydrophobicity against xylene (40.76%) which is the same as the control strain LA-5, while *E. faecalis* DM237 showed the lowest hydrophobicity (27.62%). 2 out of 9 strains showed weak hydrophobicity and the other 7 isolates moderate hydrophobicity towards xylene. On the other hand, all tested strains have shown weak hydrophobicity towards hexadecane. However, these low levels of *in vitro* percent cell surface hydrophobicity values might be sufficient for *in vivo* adhesion, since *in vitro* analyses do not necessarily correlate *in vivo* analyses. This is in accordance with a results obtained by other researchs (Bhardwaj et al., 2010; Wijaya, 2003). The variation in hydrophobicity to n-hexadecane has been reported in other probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* and is due to the fact that the adhesion depends upon the origin of strains as well as surface properties (De Ambrosini et al., 1998).

However, these results should be interpreted with caution because the adherence feature to intestines does not necessarily mean an *in vivo* adhesion would occur (Bautista-Gallego et al., 2013). Moreover, cell surface hydrophobicity is strain-specific and the presence of different nutrients or carrier food matrices may influence the expression of adhesion genes in the microorganisms (de Paula et al., 2015).

Table 33: Hydrophobicity (%) against xylene and hexadecane of LAB isolates. (Data are Mean Values ± SD, n=3)

Isolate	Xylene	Hexadecane
DM18	$31,84\pm2.85^{c}$	$14,00\pm3.59^{cd}$
DM 33	$40,76\pm0.05^{a}$	$20,85\pm0.28^{b}$
DM 150	$35,99\pm3.75^{bc}$	$23,23\pm4.54^{ab}$
DM 214	$36,70\pm3.13^{b}$	$2,15\pm0.69^{e}$
DM 224	$30,49\pm0.10^{c}$	$12,47\pm0.67^{d}$
DM 236	$37,26\pm5.64^{b}$	$19,36\pm0.44^{bc}$
DM 237	$27,62\pm0.20^{d}$	$19,47\pm0.06^{bc}$
DM 246	$34,81\pm2.05^{c}$	$28,22\pm9.36^{a}$
DM 270	$32,12\pm0.02^{c}$	$21,39\pm0.47^{b}$
LA5*	40.21+0.88	19.35+0.72

[a-f] Means in the same column with different lowercase letters differ significantly ($p<0.05$)

*LA5 is positive control and not part of means differences

4.3.4 Auto-aggregation

Auto-aggregation ability has been suggested to be an important property of many bacterial strains used as probiotics, which can help probiotic cultures to adhere to the oral cavity, GIT, and urogenital tract and modulate the immune system in the GIT (de Paula et al., 2015). The aggregating bacteria may achieve an adequate mass to form biofilm or adhere to the mucosal surfaces of the host and exert their benefic functions. In general LAB with aggregation ability and hydrophobicity cell surface could be more capable to adhere to intestinal cells.

To evaluate the potential adhesion properties of LAB isolates, we studied their autoaggregation ability, a bacterial trait that can be predictive of adhesiveness of

probiotic bacteria. The sedimentation rate of the strains tested was measured over a period of 5 h.

Wang et al., (2010) classified that bacterial strains with at least 40% of aggregation as having good auto-aggregation properties while strains with less than 10% are considered to have weak auto-aggregation. The results showed that 44% of the tested strains showed good auto-aggregation with values higher than 50% while the other 56% exhibited a moderate autoaggregation phenotype, with values ranging from 34.90 to 36.74%.

As seen in Table 34, auto-aggregation of LAB isolates increased with increasing the incubation period. Among the isolates, DM214 showed the strongest auto-aggregation ability (59.04± 1.46 %) after 5 h of incubation. This results indicate that *Lb. paracasei* DM214 is potentially high capable of adhering to epithelial cells and mucosal surfaces.

The auto-aggregation property of the probiotic candidates is important since it is related to the type and the amount of surface layer protein that contributes to the bacterial adhesion onto the intestinal cell wall; a prerequisite attribute in providing beneficial health effects to the host (Bao et al., 2010). Several studies had also demonstrated that auto-aggregation ability of LAB ceases to exist if their surface layer protein had been removed (Tuo et al., 2013).

Table 34: Autoaggregation (%) of LAB isolates during 5 hrs. Data are Mean Values ± SD, n=3)

Isolate	1	2	3	4	5
DM18	3,07±0.05^{b}	3,07±0.08^{e}	20,12±0.38^{c}	29,58±0.24^{a}	36,74±0.91^{d}
DM 33	1,52±0.12^{c}	2,96±0.01^{e}	18,98±0.41^{d}	27,10±0.56^{b}	34,90±0.75^{d}
DM 150	4,86±0.01^{a}	5,17±0.26^{d}	23,10±0.15^{b}	27,32±0.87^{b}	55,20±0.88^{b}
DM 214	2,98±0.11^{b}	7,33±0.04^{c}	28,73±0.24^{a}	30.74+0.41^{a}	59,04±1.46^{a}
DM 224	2,95±0.06^{b}	2,89±0.14^{ef}	19,77±0.22^{c}	29,07±0.71^{a}	50,24±0.94^{c}
DM 236	2,84±0.21^{b}	16,80±0.04^{a}	19,40±0.37^{cd}	27,17±0.65^{b}	35,61±1.22^{d}
DM 237	0,54±0.06^{e}	9,78±0.08^{b}	17.89±0.21^{e}	20.33±0.48^{c}	35,15±0.94^{d}
DM 246	1,08±0.17^{d}	9,61±0.10^{b}	19,93±0.04^{c}	27,21±0.14^{b}	35,09±1.05^{d}
DM 270	1,10±0.28^{d}	2,59±0.11^{f}	19,64±0.19^{c}	29,00±0.38^{a}	50,15±0.96^{c}
LA5*	3.12+0.06	23.41+1.12	38.21+0.84	42.12+0.79	57.75+1.22

$^{a-f}$ Means in the same column with different lowercase letters differ significantly ($p<0.05$)

*LA5 is positive control and not part of means differences

4.3.5 Co-aggregation

The ability to co-aggregate is a desirable property for probiotics in health promoting foods (Collado et al., 2008). The organisms with the ability to co-aggregate with other bacteria can help to prevent colonization by invading foodborne pathogens (Collado et al., 2007). This characteristic can increase the competition of receptor epithelial intestine cells and potentially decrease the presence of undesired microorganism in the intestine due the production of antimicrobial compounds or other factors, blocking the dissemination of pathogens to other attachment sites, and enhancing the competition in the GIT pathogens (Collado et al., 2007). Moreover, these characteristics can increase the immune system and protect the host against undesired pathogens.

The results of co-aggregation of 9 LAB isolates in the presence of *L. monocytogenes* 33413 and *S. aureus* RF122 separately at 2 h and 5 h of incubation at 37 °C are shown in Table 35. All potential probiotic strains tested showed coaggregation abilities with the *L. monocytogenes* 33413. *Lb. paracasei* DM214 exhibited the highest coaggregation ability with *L. monocytogenes* 33413 (52.65%) and the lowest coaggregation potential with *L. monocytogenes* 33413 was exhibited by *E. faecalis* DM237 (18.01%). The coaggregation abilities of tested strains with *S. aureus* RF 122 were lower and also 3 strains showed no coaggregation. The percentage of coaggregation was demonstrated to be strain-specific and dependent on incubation time as previous demonstrated by Collado et al., (2008).

The coaggregation potential of selected LAB portrayed their better antimicrobial potential because probiotic bacteria with coaggregation ability have been observed to contain high ability to kill undesirable bacteria due to the synthesis of antimicrobial substances in close proximity to them. Furthermore, it has been suggested that inhibitor- or bacteriocin producing lactic acid bacteria, which coaggregate with pathogens, may constitute an important host defense mechanism against infection (Collado et al., 2008).

Table 35: Co-aggregation (%) of LAB isolates with two pathogens at 2 and 5 hrs of incubation. Data are Mean Values ± SD, n=3)

Isolate	*L. monocytogenes* 33413		*S. aureus* RF 122	
	2 h	**5 h**	**2 h**	**5 h**
DM18	25,37±0.15^{d}	28,17±0.13^{cd}	14,94±0.22^{b}	17,01±0.25^{d}
DM33	27,05±0.56^{c}	29,24±0.34^{c}	22,73±0.12^{a}	27,38±0.22^{a}
DM150	19,72±0.39^{e}	19,76±0.39^{de}	2,16±0.81^{c}	2,58±0.32^{e}
DM214	48,68±0.28^{a}	52,65±0.21^{a}	23,25±0.19^{a}	25,06±0.34^{b}
DM224	35,58±0.18^{b}	38,85±0.23^{b}	14,23±0.24^{b}	17,50±0.22^{d}
DM236	26,22±0.21^{cd}	27,10±0.50^{cd}	ND	ND
DM237	16,85±0.31^{f}	18,01±0.36^{e}	ND	ND
DM246	19,59±0.18^{e}	20,22±0.22^{e}	22,24±0.34^{a}	23,74±0.28^{c}
DM270	18,87±0.21^{e}	19,57±0.48^{e}	ND	ND
LA5*	52.34±0.45	65.18±1.28	20.49±0.65	21.72±0.12

$^{a-f}$ Means in the same column with different lowercase letters differ significantly ($p<0.05$)

ND: Not Detected

*LA5 is positive control and not part of means differences

4.3.6 Bile salt hydrolase activity

The ability of probiotic strains to detoxify bile salt by producing BSH enzyme activity has often been included among the criteria for probiotic strain selection (Noriega et al., 2006). Bile salt hydrolases are intracellular enzymes that detoxify bile by deconjugating bile acids. Many studies have been carried out to demonstrate the reduction of cholesterol concentrations in human blood using bile salt hydrolases from LAB strains, but it has not yet been proved (Kumar et al. 2013). Deconjugation of bile salts by LAB increases the demand for cholesterol which, in turn, prompts the synthesis of more bile salts in the liver. This process may lead to a reduction in serum cholesterol (Kumar et al. 2012). Current data suggest that this activity could maximize the intestinal survival and persistence of probiotic strains increasing the overall beneficial effects associated with the strain (Begley et al., 2006). Marteau et al., (1995) reported that though bile salt hydrolase activity is considered an important criterion for the selection of a probiotic organism, the potential risks associated with BSH activity, i.e. conversion of primary to secondary bile salt in the intestine which is procarcinogenic, should be kept in mind.

When the potentially probiotic strains were spotted onto MRS agar plates supplemented with the sodium salt of taurodeoxycholic acid and $CaCl_2$, a white zone of precipitation occurred when these strains were BSH-positive (Figure 39).

Our results indicated that all tested strains, except *E. faecium* DM224, were not able to deconjugate bile salts and thus the majority of the breakdown products of the dehydroxylating activity by strains may be precipitated and excreted in feces (Begley et al., 2006). Moreover, most BSH activity relates to strains that have been isolated from the intestines or feces of mammals' rich environment, and non-conjugated bile acids. Strains of other environments, such as milk or vegetables-environment in which bile salts are absent, usually do not possess bile salt hydrolase activity (Begley et al., 2006) as we have also observed in this study. Moreover, the strains which did not exhibit BSH activity were able to survive at different bile salts concentrations, confirming that the two activities are not correlated each other, which is in accordance with previous reported data (Moser & Savage, 2001). Poor bile salts hydrolase activity has already been detected in other LAB (Cebrian et al., 2012; Fortina et al., 2008; Hosseini et al. 2009).

Figure 39: The detection of BSH activity using a plate assay (Isolate DM224, positive)

4.4 Assessing the possibility of producing a potential functional fermented donkey drink

4.4.1 Fermentation of donkey milk with selected LAB isolates

All the LAB strains were tested in sterile donkey milk to determine their fermentative capacity and to evaluate the production of bioactive peptides with antioxidant, antimicrobial and ACE inhibitor activity. Their fermentative performance was assessed at regular intervals up to 48 h after the inoculum based on the production of lactic acid as primary metabolite and the pH decline. The growth was also assessed by determining viable cell counts.

All strains tested in this study were able to grow in the heat treated donkey milk under the applied conditions. These milk products were produced from heat treated milk under sterile conditions as a measure to exclude any possible interference caused by contaminant microorganisms. The decrease in pH for all fermented milk samples is shown in Figure 40. After inoculum, the values of pH were in the range of 6.68 to 6.71. All strains reached pH value of about 4.6 (4.36-4.68) after 48 h of growth. The cell counts at the end of fermentation ranged from 7.5 log CFU/ml to 8.3 log CFU/ml, confirming that all the strains carried out fermentation.

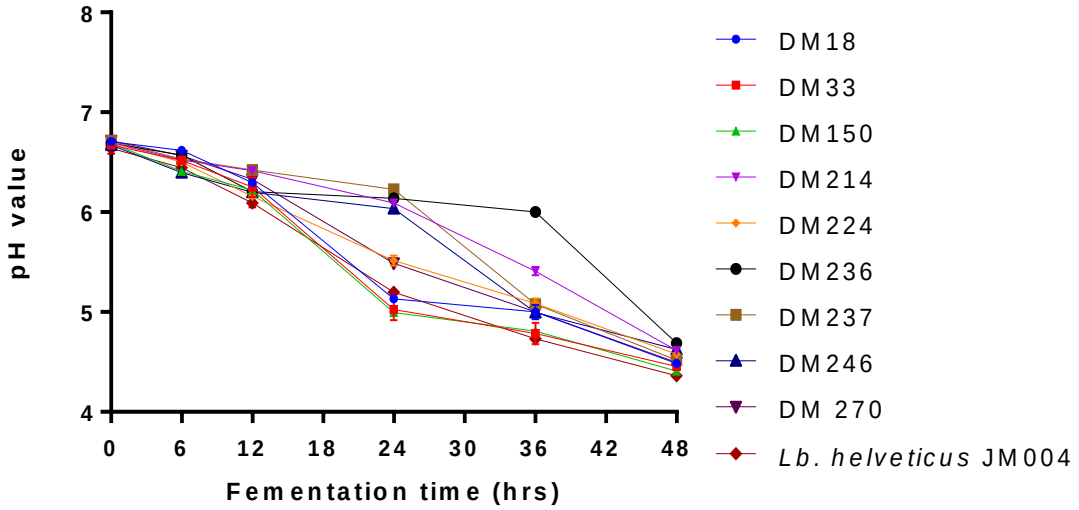


Figure 40: pH of donkey milk samples during fermentation at 37 ◦C; Vertical bars represent standard errors (n=3)

4.4.2 Bioactivities of fermented donkey milk

4.4.2.1 Antioxidant activity

In the last decade, the production of peptides having antioxidant properties by enzymatic hydrolysis of proteins or microbial fermentation has been extensively reported in several studies. Milk is considered as an interesting source of proteins able to generate by enzymatic hydrolysis or during microbial fermentation peptides displaying antioxidant activity (Pihlanto, 2006; Power et al., 2013). Protein hydrolysates can contain antioxidant peptides that can protect the human body by scavenging free radicals, such as reactive oxygen species, and also increase shelf life of foodstuffs by retarding the process of lipid peroxidation through hydrogen atom or electron transfer mechanisms (Pihlanto, 2006). Functional foods with such natural antioxidants are interesting since they can be potentially employed without the toxic side effects associated with the use of synthetic equivalents. Also, antioxidants from protein hydrolysates might confer nutritional value besides functional/physiological properties, which are additional advantages over the synthetic counterparts. Antioxidant activity can be demonstrated by various mechanisms and reactions, such as radical scavenging, reductive capacity, prevention of chain initiation and binding of transition metal ion catalysts, and inhibition of lipid peroxidation (Frankel & Meyer, 2000), so it is necessary to evaluate antioxidant activity by different methods. In this study, the antioxidant activity of the water-soluble extracts (WSE) of fermented milk products as well as samples after gastrointestinal digestion was evaluated by the ABTS and DPPH radical assays. The DPPH radical scavenging method is widely used to evaluate antioxidant activities, because of its simplicity and rapidity compared with other methods (Milardovic et al., 2006). The principle of the assay is based on the reduction of ethanolic DPPH solution in the presence of a hydrogen-donating antioxidant, leading to the formation of non-radical form DPPH-H. The antioxidant is able to reduce the stable radical DPPH from purple to yellow-colored diphenylpicrylhydrazine. The radical ABTS is reduced with concomitant conversion to a colourless product in the presence of antioxidants with hydrogen-donating or chain-breaking properties. Both assays indicate the ability of substances to act as electron donors or H atom donors in free radical reactions.

Based on ABTS radical scavenging activity, the antioxidant activity of the WSE of unfermented raw and pasteurized donkey milk was 46,41 and 45,30 mM Trolox/100ml, respectively. After fermentation, the antioxidant activity of all fermented milks was increased which is an agreement with other studies (Virtanen, 2007). A further increased in the antioxidant activity was observed for all the samples after *in vitro* gastrointestinal digestion which is an agreement with other studies that demonstrated that the antioxidant properties of casein and whey proteins could be increased through hydrolysis with many digestive enzymes as some peptides possess stronger antioxidant capacity than others (Hernandez-Ledesma et al., 2005; Mandalari et al., 2009; Power et al., 2013). Gastrointestinal digestion is of major importance in the bioavailability of antioxidant capacity of milk. This demonstrates that that the compounds with antioxidant activity present in these extracts are resistant to the reaction of the enzymes active during gastrointestinal digestion. Furthermore, these enzymes appear to produce new compounds with antioxidant activity, which increases the bioactivity after digestion. The antioxidant activity was not directly influenced by the proteolytic activity of the strains, as *Lb. heleveticus* showed the highest proteolytic activity but low antioxidant activity. While, *E. faecium* DM33 showed an intermediate proteolytic activity, but exhibited the highest antioxidant activity in both assays. It can therefore be concluded that radical scavenging activity is more dependent on certain specific proteolytic enzymes of bacterial strains comparing to high proteolytic state of fermented product (Virtanen et al. 2007). Overall, our results revealed that various starter cultures had different influences on the antioxidant activity of fermented milk products, measured by either ABTS or DPPH method.

As shown in Figure 41, the WSE of fermented donkey milks and digested samples exhibited much lower antioxidant capacities as measured by DPPH assay relative to ABTS assay. Even though, there was a relatively weak correlation observed between the results for DPPH and ABTS (r^2=0.44 and r^2=0.68 respectively), both assays rank the samples in a quite similar order. This may be because of different radical scavenging mechanisms, different peptides present in the samples capable of reacting and quenching different radicals, and the sensitivity of two assay methods in hydrophilic and lipophilic antioxidant systems. The same results were previously reported by Mirzaei et al., (2015) & Tang et al., (2010). The difference between the DPPH and

ABTS is mainly due to the fact that DPPH can be dissolved only in organic media (methanol), which can lead to precipitation of proteins (Tang et al., 2010). On the other hand, ABTS is a water-soluble radical monocation and can be dissolved in both aqueous and organic media. Therefore, the antioxidant activity of both hydrophilic and lipophilic compounds can be measured with ABTS assay (Tang et al., 2010). Our results are in agreement with these findings. Thus, we can suggest that the ABTS method was more appropriate than the DPPH assay for the measurement of antioxidant activity of fermented donkey milk samples.

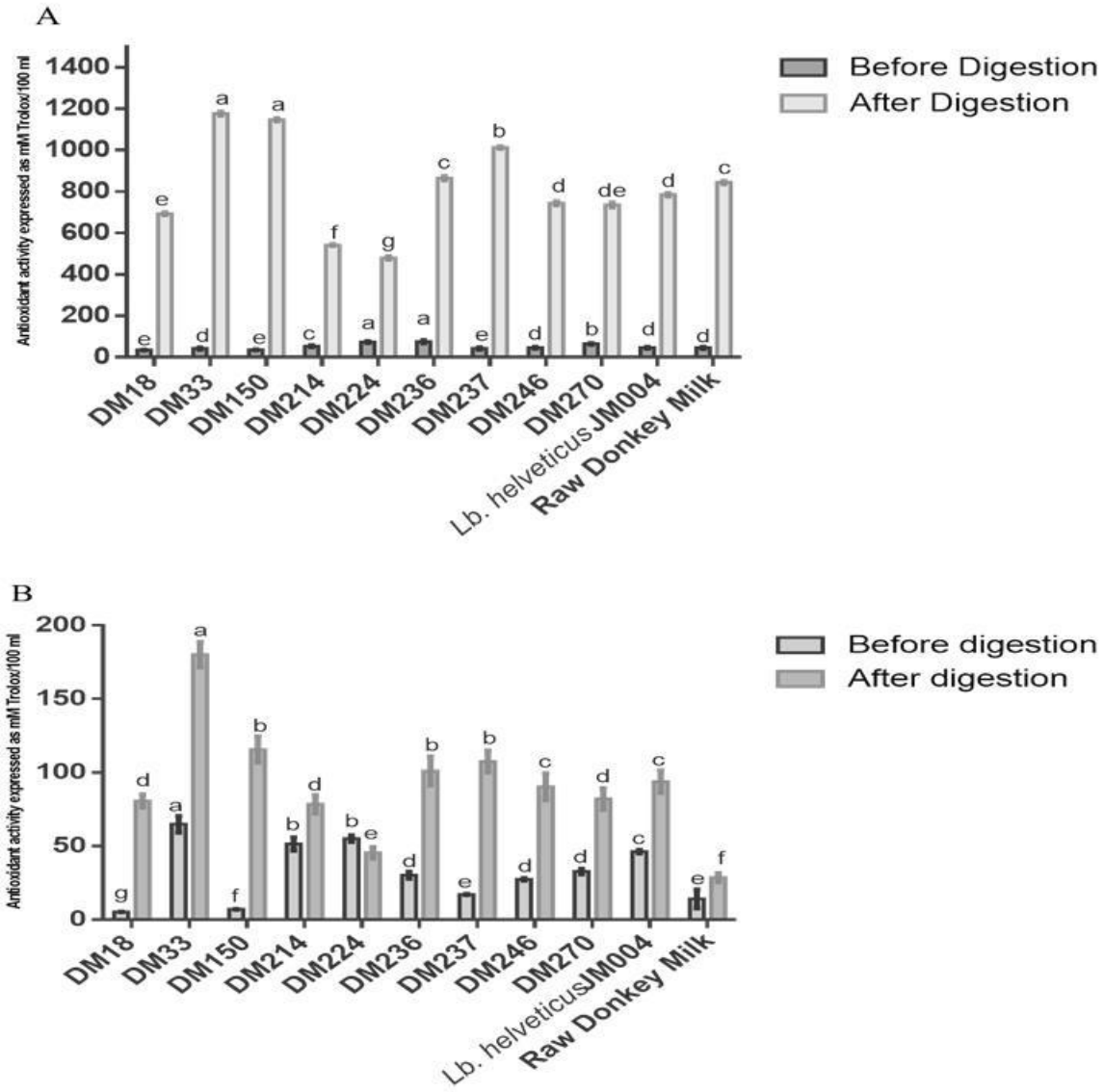


Figure 41: Antioxidant activity of milk samples using A) ABTS assay and B) DPPH assay; Vertical bars represent standard errors (n=3); [a-f] Means in the same column with different lowercase letters differ significantly ($p<0.05$)

4.4.2.2 Antimicrobial activity

Despite the large numbers of antibiotics available currently, the growing bacterial resistance against many conventional antibiotics in recent decades has directed the investigation of alternative compounds. In addition, the use of natural antimicrobial compounds has received great attention due to consumer demands for minimally processed food. Thus, inhibitory activities of WSPE of fermented donkey milk samples before and after *in vitro* gastrointestinal digestion were evaluated against two gram-negative (*E. coli, Salmonella typhimurim*) and three gram-positive (*S. aureus, L. monocytogenes and B. cereus*) bacteria, and the findings are presented in Table 36.

The antimicrobial activity was higher for fermented milk samples after digestion. Regarding the fermented donkey milk samples before digestion, inhibition was observed only against *L. monocytogenes* 33413. On the other hand, the antimicrobial activity of fermented samples after *in vitro* gastrointestinal digestion was markedly higher against *L. monocytogenes*, and was also observed against *S. aureus* and *B. cereus*. The results are suggesting that antimicrobial proteins resisted to the action of digestive enzymes or that antimicrobial fragments of fermented donkey milk were released or both. Likewise, antimicrobial activity in donkey milk samples digested with human gastrointestinal enzymes has been reported (Tidona et al., 2011). This antimicrobial activity in donkeys' milk might be partially due to the high level of lactoferrin and lysozyme; the latter is reported to resist the degradation by gastrointestinal enzymes. In conclusion, this study has highlighted that donkey milk fermented with LAB contains different protective antimicrobial factors, including peptides released during the digestion process that can exert a beneficial impact on gut health, particularly for the low immune defence system of children, elderly and the convalescent.

Antimicrobial peptides derived from milk proteins usually possess an amphiphilic and cationic character, which appears to be significant for their mechanism of action, since it is proposed that electrostatic bonding between the peptides and the bacterial membranes (negatively charged) is the initial stage of the pore formation process leading to cell death. Milk protein-derived antimicrobial peptides usually show a broad range of activity against foodborne pathogens or spoilage microorganisms. However, from our results, only Gram-positive bacteria were inhibited (Table 38). As the cell

envelope of Gram negative bacteria is both structurally and functionally more complex than that of Gram-positive bacteria, these differences in bacterial membrane composition might have implications for the mode of action and the bacterial specificity of these antibacterial compounds. Antimicrobial peptides generated from food proteins (particularly milk) present the great advantage of being produced from harmless and inexpensive sources. Hence there is a growing interest in the utilization of these peptides, for instance, as food grade bio-preservatives or as health-promoting food supplements in the food industry.

Table 36: Antimicrobial activity of femented milk samples agaist indicator strains, (values: Means±SD)

Fermented donkey milk samples posterior digestion	**Indicator bacterial Strains**						
	***S. aureus* DPC5247**	***S. aureus* RF122**	***L. monocytogenes* 1078**	***L. monocytogenes* 33413**	***B. cereus* 6089**	***E. coli* 9001**	***Sal. typhimurium* 12023**
DM 224	10.37±0.18	9.83±0.11	11.43±0.10	10.37±0.05	9.35±0.08	ND	ND
DM 237	8.55±0.11	8.58±0.33	10.22±0.07	10.57±0.16	9.03±0.18	ND	ND
DM 236	9.01±0.04	9.51±0.09	11.56±0.12	9.37±0.04	9.33±0.10	ND	ND
DM 270	11.44±0.10	11.43±0.04	10.91±0.07	9.85±0.16	9.63±0.06	ND	ND
DM 246	ND	ND	ND	ND	ND	ND	ND
DM 33	10.21±0.13	12.74±0.02	13.33±0.07	13.42±0.14	9.32±0.10	ND	ND
JM1004	10.04±0.17	11.89±0.10	10.65±0.29	10.59±0.11±	7.95±0.20	ND	ND
DM 150	8.37±0.08	10.56±0.21	11.51±0.23	ND	9.37±0.17	ND	ND
Raw donkey milk	ND	ND	10.09±0.19	8.59±0.08	8.53±0.05	ND	ND
DM 18	9.82±0.12	8.63±0.04	12.15±0.16	11.53±0.05	8.02±0.09	ND	ND
DM 214	ND	9.75±0.07	12.4±0.11	9.24±0.16	8.57±0.12	ND	ND
Fermented donkey milk samples prior							

digestion							
DM 224	ND	ND	9.6±0.06	ND	ND	ND	ND
DM 237	ND	ND	9.95±0.08	12.72±0.12	ND	ND	ND
DM 236	ND	ND	ND	9.58±0.07	ND	ND	ND
DM 270	ND	ND	10.95±0.12	ND	ND	ND	ND
DM 246	ND	ND	11.58±0.19	9.01±0.13	ND	ND	ND
DM 33	ND	ND	10.58±0.07	ND	ND	ND	ND
JM1004	ND	ND	ND	ND	ND	ND	ND
DM 150	ND	ND	13.64±0.08	ND	ND	ND	ND
Raw donkey milk	ND	ND	ND	ND	ND	ND	ND
DM 18	ND	ND	13.81±0.23	12.93±0.02	ND	ND	ND
DM 214	ND	ND	12.06±0.05	ND	ND	ND	ND

4.4.2.3 ACE-inhibitory activity

Hypertension is considered as one of the major risk factors to develop cardiovascular diseases. Together with the correct lifestyle and diet, the consumption of fermented milks enriched with bioactive peptides could be an important help to prevent hypertension. Antihypertensive peptides inhibit ACE (peptidyl-dipeptide hydrolase; EC 3.4.15.1). ACE is a multifunctional ectoenzyme that is located in different tissues and plays an important physiological role in the renin-angiotensin, kallikrein-kinin, and immune systems (FitzGerald et al., 2004). The enzyme is responsible for the increase in blood pressure by converting the inactive angiotensin-I to the potent vasoconstrictor, angiotensin-II, as well as degrading bradykinin, which has a significant vasodilator activity (Iwaniak et al., 2014). Therefore, inhibition of this enzyme proves to be an effective strategy for prevention and treatment of high blood pressure, although it may also influence different regulatory systems involved in inmmunodefense and nervous system activity. In the present study, LABs isolated from raw donkey milk were screened for the capacity of generating ACE-inhibitory peptides during milk

fermentation and upon in vitro gastrointestinal digestion. Regarding the ACE-inhibitory activity of fermented milk samples, only the samples subjected to in vitro gastrointestinal digestion were analyzed. The protein concentration of the whey fraction was determined by the Quibid method and the IC50 was also calculated as micrograms per milliliter, which is the required amount of hydrolysate needed to inactivate 50% of the ACE activity: the lower the IC50 value the higher the ACE-inhibitory activity of the milk fraction. Captopril (Sigma Aldrich Company, Dorset, UK), a commercially available drug used to treat hypertension through ACE inhibition, was used in the present study as a positive control. We measured the IC50 of captopril to test the correct activity of the enzyme preparation, and we found a value of 0.023 mM, in agreement with the values reported in the literature (Murray, Walsh, & FitzGerald, 2004).

The activity of the enzyme varies with the concentration of the sample (Figure 42). In particular Figure 42 shows the chromatograms of 3 samples (pink without dilution, green 1/100 and black 1/1000). By increasing the dilution, the concentration of ACE- is decreases the effect of the peptides on the enzyme which is then able to exert its activity by releasing more HA from HHL substrate.

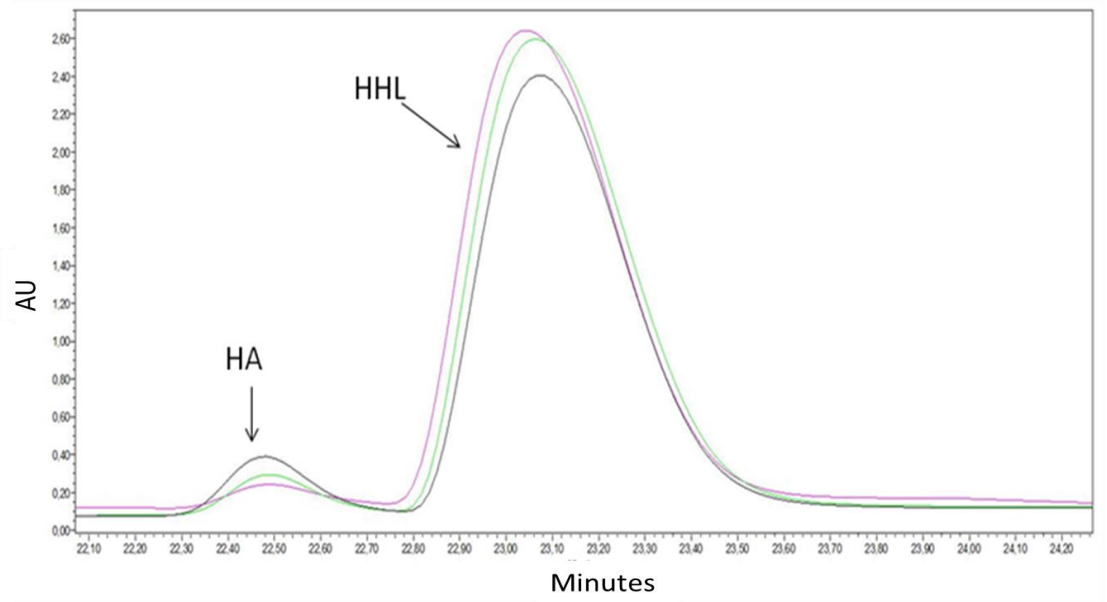


Figure 42: Enzyme activity in different sample concentrations

The IC50 of donkeys' milk digest in our study was found to be 2.14 ± 1.48 mg/mL which is lower than the IC50 value (273.0 ± 27.9 mg/mL) obtained by Bisadolo et al., (2012). This is probably due to the different peptide digestion model and the consequent

peptide profile observed. In our case, the use of the consensus model by Minekus could be very important for future comparisons with standardized digestion procedures. Therefore, it has to be confirmed that donkeys' milk might be considered as a source of bioactive peptides able to inhibit a key enzyme in blood pressure regulation.

More interestingly, in the case of fermented samples, lower IC50 values are observed (Table 37), which in most cases are far lower than the value measured for raw milk. Moreover, there was a significant (P<0.05) difference in IC50 values between the bacterial strains, indicating potential interesting differences in the quality of ACE-inhibitory peptides produced by the organisms. As an example, Figure 43 shows the in vitro ACEI activity of milk samples fermented by some bacteria strains isolated in this study versus the peptide concentration of dilutions in mg/ml. By comparing the peptide profiles from the different fermented samples, these results should be linked to differences in the proteinase and peptidase specificities of the starter cultures, which may affect the milk protein breakdown to various extents and thus can yield a wide range of peptides with functional properties (Gonzalez- Gonzalez et al., 2011). Moreover, also the rate of degradation of peptides by the cultures during fermentation could be another important parameter (Donkor et al., 2007). Although it has been reported that the concentration of ionic Ca and the pH of the final product could affect the ACE inhibition activity (Gonzalez-Gonzalez et al., 2011), in the present study the pH of the fermented donkey milk samples was at the same for all the samples (pH = 4.6) and the obtained values are in agreement with those measured by Gonzalez-Gonzalez et al., (2011).

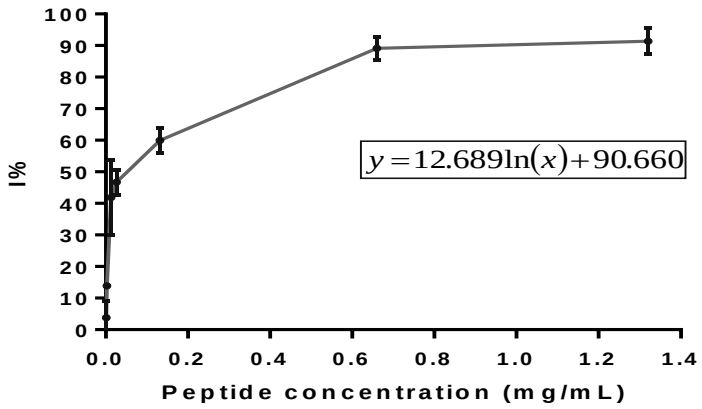


Figure 43: Dose-response curve for the fermented donkey milk DM214. Vertical bars represent standard errors (n=3)

Under the experimental conditions of this study, the lowest ACE-inhibitory activity was obtained for milk fermented with *E. faecium* DM270, with an IC50 value of 2.71 mg/ml, indicating that a large amount of milk would be required to inhibit 50% of the enzyme activity. The highest ACE inhibition was measured for donkey milk fermented with *Lb. casei* DM214, and *Enterococcus lactis* DM237 with IC50 of 0.04 and 0.06 mg/ml respectively. The same results were reported by other authors (Nejati et al., 2013; Pihlanto et al., 2010), and *L. casei* 279 or *L. casei* LAFTI L26 were successfully used as adjunct starters for the manufacture of a Cheddar cheese with increased ACE-inhibitory activity (Ong et al., 2007). Unexpectedly, *Lb. helveticus* JM1004 milk had an IC50 of 1.58 mg/mL which is considerably higher than that obtained with other strains used in this study and also from the reported IC50 values (0.16–1.1 mg/mL) of other studies (Fuglsang et al., 2002; Leclerc et al., 2002). A good ACE-inhibitory activity value was also measured for the digested donkey milk sample fermented by *Enterococcus faecium* DM33, which showed the most differentiated and abundant peptide profile by LTQ-Orbitrap analysis (data not shown).

Although it is clear that the observed ACE-inhibitory activities are related to a complex contribution of several potentially active sequences, it could be interesting in the near future to more deeply investigate and identify the most relevant peptide sequences in order to eventually identify the most effective producers among the strains and the most effective conditions to boost the concentration levels of these compounds.

Results of ACE-inhibitory activity of fermented donkey milk samples after *in vitro* gastrointestinal digestion are very interesting since the potential hypertensive effect of ACE-inhibitory compounds depends on their ability to reach the organs, i.e. where they exert their bioactivity. Testing gastrointestinal digestion is important to determine whether these bioactive compounds maintain their activity after passing through the human digestive system and remain intact. Furthermore, this digestion can generate novel bioactive compounds from other inactive molecules present in the extracts.

Table 37: IC_{50} values (mg/ml) for fermented milk samples after *in vitro* digestion (values: Means±SD)

Donkey milk sample	**IC_{50} mg/ml**
Raw donkey milk	2.14±1.48
Lb. helveticus JM004	1.17±0.15
DM18	0.17±0.09
DM33	0.35±0.01
DM150	0.15±0.20
DM214	0.04±0.01
DM224	0.54±0.38
DM236	0.27±0.35
DM237	0.06±0.03
DM246	0.41±0.33
DM270	2.71±2.52

4.4.3 Peptide profiling

Mass spectrometry is a powerful analytical technique for both protein and peptide analysis because it can rapidly and reliably identify the components of complex matrices. The most popular analysers in proteomics are ion traps, triple quadrupoles, time of-flight and orbitrap. A complete sequence of the peptides of interest is often achieved by using tandem MS (MS/MS), by fragmentation of a selected precursor peptide ion to generate specific fragment ions for sequence elucidation to identify these peptides, spectra are scanned against protein-sequence databases using search algorithms.

Donkey milk proteins have been studied and characterized and sequences of the main casein and serum proteins are available on protein data banks (Bidasolo et al., 2012; Salimeri et al., 2004; Vincenzetti et al., 2008). Phenotypic variability and polymorphism has also been investigated (Criscione et al., 2009). Interest is related to the similarity between donkey and human milk in terms of protein amounts and ratio between caseins and serum proteins that makes possible to use it as an efficient human milk substitute. Moreover, potential lower allergenicity has aroused attention in recent years. An interesting feature not yet investigated for donkey milk is the occurrence of bioactive peptides, largely studied in particular in the case of cow milk and related dairy products. Bioactive peptides, encrypted in milk protein sequences, may be released by

fermentation and gastrointestinal digestion and may exert several different biological function, both in the intestinal lumen and, if adsorbed, also towards different target organs in the body.

The peptidome of donkey milk, potential bioactivities have been recently investigated both in the raw milk and upon simulated gastrointestinal digestion to verify the potential release of antihypertensive and antimicrobial sequences (Bidasolo et al., 2012; Chiozzi et al., 2016; Tidona et al., 2011). In particular, Bisadolo et al., (2012) identified peptides sequences, deriving mainly by β-casein, and proved the occurrence of ACE-inhibitory peptides by in vitro test, showing for the donkey milk digest a total value of IC_{50}= 273.0 ± 27.9 μg/mL and also synthesizing one of the most abundant identified sequence VAPFPQPVVP with an IC_{50} = 48.8 ± 2.3 mM. Tidona et al., (2011) showed, instead, the antimicrobial activity of undigested and digested donkey milk towards different microbial species.

In the present study, we applied a consensus digestion model to raw and fermented donkey milk samples, the latter obtained by fermentation with different bacteria previously isolated from donkey milk.

The sample extracts of raw and fermented donkey milk samples before and after the digestion process have been analysed first in UPLC-ESI-MS, focussing on peptides with a MW up to 10 kDa. The efficiency of the digestion process was evaluated comparing the protein and peptide profile of raw and fermented donkey milk samples before and after the digestion process. The peptide profile of digesta from raw and fermented donkey milk samples is different from that of the extract before digestion and absolutely richer than that obtained for non-digested samples. A typical UPLC-MS peptide chromatographic profile of a digested sample compared with that of the corresponding extract is reported in Figure 44.

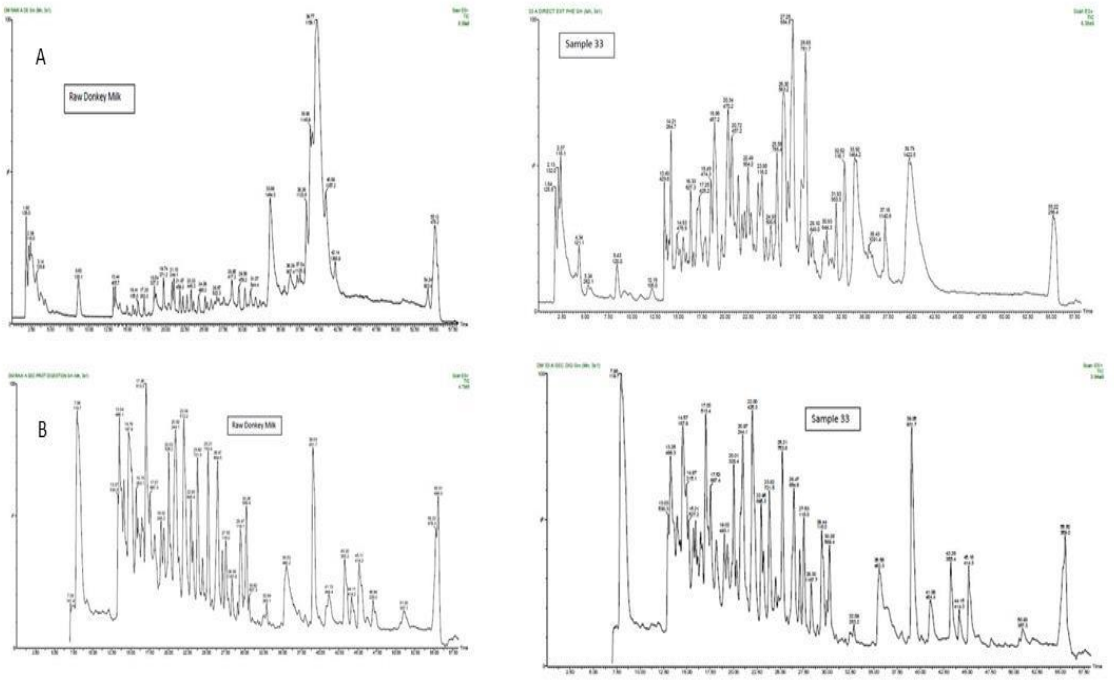


Figure 44: Total Ion Chromatograms obtained in UPLC-ESI-MS raw donkey milk and fermented donkey milk DM33 samples a) before digestion and b) after digestion

The cleavage of peptide bonds by digestive proteases results into the release of peptides of various length and free amino acids. The peptide profile of digested milk samples was analysed in order to identify the peptides regarded as the most significant (obtained by UPLC-ESI-MS): on account of the large number of peptides and the great complexity of the chromatogram, the identified peptides are those corresponding to the most intense signals. The identification was carried out matching the results obtained by LTQ-Orbitrap analyses. Peptide identification was performed by Proteome Discoverer software, matching the experimental mass spectra of each peptide with those of peptides of the same mass obtained from donkey protein sequences retrieved from Uniprot database.

In accordance to results reported by others, the highest number of identified peptides was derived for β-CN, followed by α-s1 and α-s2 caseins, whereas among serum proteins most peptides derives from β-lactoglobulin I and II, because of its higher susceptibility to cleavage in comparison to a_{s1}-CN and a_s2-CN. It is more unstructured and more accessible to enzymes, being therefore hydrolysed more easily (Chang et al.,

2013). A small number of peptide sequences were identified from lactalbumin and lysozyme, in agreement with their lower susceptibility of these proteins to the proteolytic action of LAB and to its relative resistance to gastrointestinal digestion for their high compact formation (Quiros et al., 2005). In addition, those proteins were described as more resistant to gastrointestinal digestion proteolysis than caseins, whose susceptibility to proteolysis increases with pre-treatments as heating or fermentation (Kopf-Bolanz et al., 2014). In addition, in agreement with Tidona et al., (2011) peptides originating by the hydrolysis of κ-casein have been also identified.

One of the reported sequences has been already identified by Tidona et al., (2011) whereas shorter fragments deriving by the same position and other peptides have been identified in this work, mainly originating from the mid- and N-terminal part of the protein.

Peptide profile was determined before and after the gastrointestinal digestion process: large differences were observed both on the number and the identity of the sequences, as expected. The difference is quite large and, in general, the amount of identified sequences is lower after digestion. As an example, comparing the peptide profile of raw donkey milk and milk fermented by DM 33, a total of 221 (raw) and 439 (DM 33) peptide sequences were identified by LTQ-Orbitrap before digestion. Upon digestion, a total of 124 (raw milk) and 246 (DM 33) peptide sequences were identified. Thus, digestion has a strong influence on the peptide profile, producing a high reduction of the number of different sequences. As an example, considering peptides originating by β-casein, the most hydrolyzed protein, before digestion a total of 116 (raw) and 315 (DM 33) sequences were identified, whereas only 39 (raw) and 78 (DM 33) were found upon digestion, respectively. As expected, digestion also induces a more homogeneous composition of the peptide profile, owing to the overwhelming effect of the digestive enzyme. Indeed, the percentage of common sequences changed a lot: before digestion the two considered samples only share 22% and 8% of the peptide profile, respectively. Percentages grew to 69% and 35% respectively upon digestion, thus showing that digestion process tend to smooth the differences between the different sample, although fermented milk seems to give a richer peptide profile, owing to the pre-digestion exerted by bacteria that favor the subsequent degradation by digestive enzymes. The proteolytic

sites of digestive enzymes produce a homogenization of the general characteristics of the digesta, although some differences were maintained.

As a further comparison, in the case of the other studied microorganisms we can see that, in the case of β-casein, for example DM 214, DM 237 and DM 150 share with the other already mentioned raw milk and DM 33 fermented milk 50%, 57% and 57% of their peptide profile, respectively.

It is worth noting that, generally, the lower percentage of similarity is due to the occurrence of other sequences in addition to those already shared with the other samples and that, in many cases, some differences are related to the presence of one or two more amino acid residues on the amino or carboxy terminal sides, showing a slightly different degree of progression in the action of amino- and carboxy-peptidases.

If we compare the effect of digestion on these samples, we can see that the effect is relevant both in terms of MW and number of residues: a comparison between the average MW and residues for the casein proteins for raw and DM 33 fermented milk before and after digestion is reported in Figure 45.

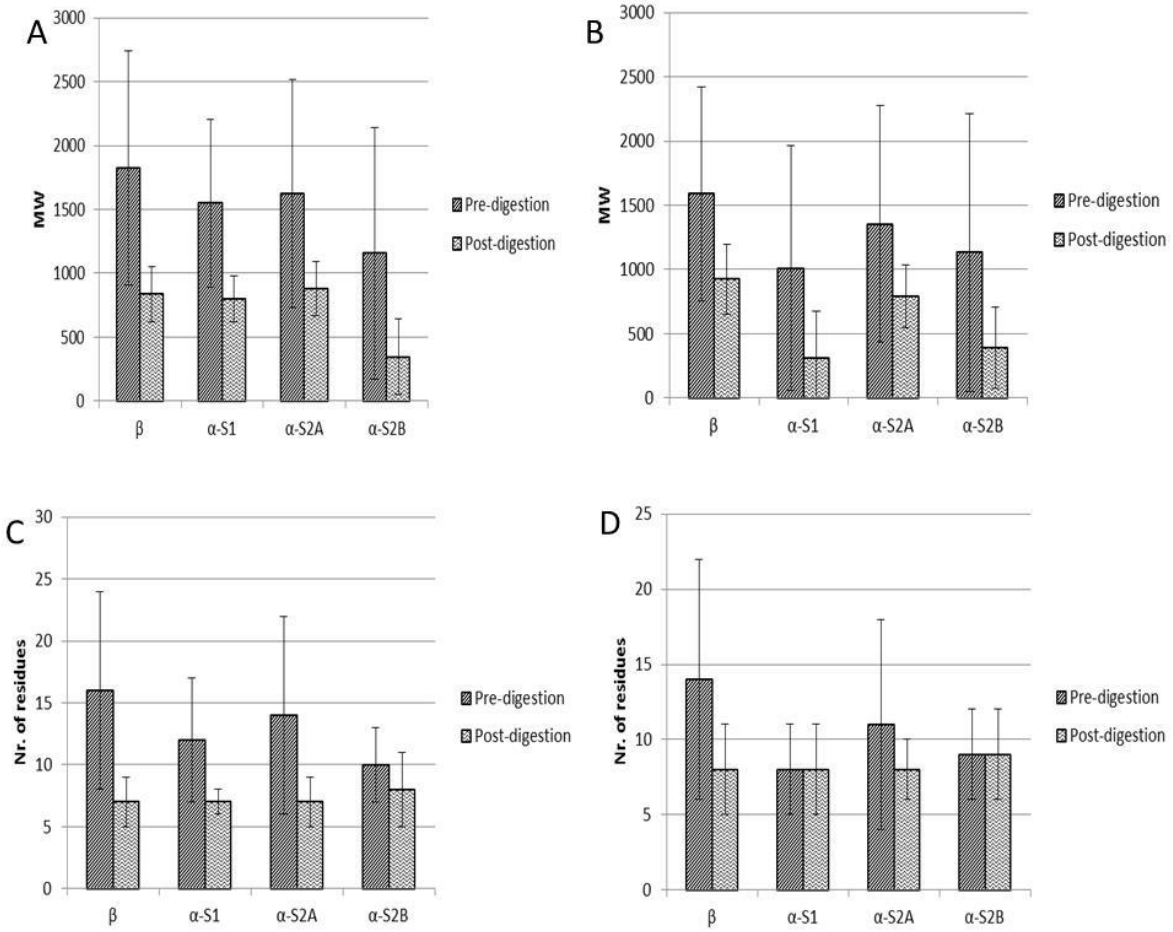


Figure 45: A) Change in the average MW of β-casein peptides before and after the digestion process for raw milk, B) Change in the average MW of casein peptides before and after the digestion process for DM 33 fermented milk, C) Change in the average no. of residues of β-casein peptides before and after the digestion process for raw milk and D) Change in the average no. of residues of casein peptides before and after the digestion process for DM 33 fermented milk

As far as the characterization of the entire peptide profile, the different sequences identified deriving from the most abundant casein and serum proteins for the above mentioned fermented samples in comparison with raw milk digesta are reported in APPENDIX VI. Sequence coverages of the relative proteins on the base of the sample with the richest peptide profile (digesta from DM 33) are reported below (Table 38).

Table 38: Sequence coverages found in donkey milk proteins after gastrointestinal digestion.

β-casein (D2EC27_EQUAS)	MKILILACLV ALALA**REKEE LNVSSETVES** LSSNEPDSSS EE**SITHIN**KE KVQK**FKHEGQ QQ**R**EVEHQDK** ISR**FVQPQPV** VYPY**AEPVPY AVVPQ**NI**LPL AQPPIVPFLQ PEIM**EVSQA**K ETLLPK**RKVM PF**LKSPIVPF** SE**RQILNPTN GENLRLPVHL IQPFMHQVPQ S**LLQT**LMLPS QPVLSPPQS**K **VAPFPQPVVP YPQRDTPVQA** FLL**YQDPQLG LTGEFDPATQ PI**VPV**HNPVI V**
α-s1 casein (CASA1_EQUAS)	RPK**LPHRHPE IIQNEQDSR**E KVLKERKFPS F**ALHTPR**EE**Y INELN**RQREL LKEKQKDEHK EY**LIEDPEQQ E**SSSTSSSEE VVPINTEQKR IPREDMLYQH TLEQLRRLSK Y**NQLQLQAIY AQEQLIR**MKE NSQRKPM**RVV NQEQA**Y**FYLE PFQPSYQLDV YP**YAAW**FHPA Q**IMQHVAY**SP FHDT**AKL**IAS ENSEKTDIIP EW**
α-s2 casein A (CASA2_EQUAS)	MKFFIFTCLL AVALAKHNME HRSSSEDSVN ISQEKFKQEK **YVVIPTSK**ES ICSTSCEEAT RNINEMES**AK FPTEV**YSSSS SSEESAKFPT EREEK**EVEEK** HHLKQLNKIN QFYEKLNFLQ YLQALRQPR**I VLTPW**DQTK**T GASPFIPIVN** T**EQLFTSEEI PKKTVDMEST EVV**TEK**TELT EEEKN**YLKLL NKINQYYEKF TLPQYFK**IVH QHQTTMDPQS H**SKTNS**YQII PV**LRYF
α-s2 casein B (C1L3G3_EQUAS)	MKFFIITCLL AVALAKHEIK HVSSSEESTN ISQEK**YKQDN NVA**FQTSQES SSGSSSEETT DSLTDEKEHH SSSEEFTSIS QEKTSK**KTVD MGSTEIFPEE IELSDEEKN**Y LKQLKQL**VKI NPK**FPSPQY**F QAVHPQQIPM SPWN**RSKENT YPFIITLRNF
β-lactoglobulin I (LACB1_HORSE)	MKCLLLALGL ALMCGIQA**TN IPQTMQDLDL QEVAGKWHSV AMAASDISLL DSESAPLRVY IEKLRPTPED NLEIILREGE NK**GCAEKKIF **AEKTESPAE**F **KINYLDEDTV** FALDTDYKNY LFLCMKN**AAT PGQSLV**CQYL AR**TQMVDEEI MEK**FRRA**LQP LPG**RVQIVPD LTRMAERCRI
β-lactoglobulin II (D6QX31_EQUAS)	MKCLLLALGL SLMCGNQA**TD IPQTMQDLDL QEVAGRWHSV AMVASDISLL DSESAPL**R**VY VEELRPTPEG NLEIILREGA NHV**CVERNIV AQ**KTEDPAV**F TVNYQGERKI SVLDTDYAHY MFFCVGPPLP S**AEHGTVCQY LARTQKVDEE V**MEKFSR**ALQ PLPGHVQIIQ DPSGGQE**RCG F
lactalbumin I (LALBA_EQUAS)	KQFTKCELSQ VLKSMDGY**KG VTLPEWI**CTI **FHSSG**YDTQT IVKNNGKTEY GL**FQINN**KMW CRDNQILPSR NICGISCNK**F LDDDLTDDVM** CAK**KILDSEG ID**Y**WLAHKPL** CSEKLEQWLC EEL

It is also worth noting that coverage of the proteins is very high, demonstrating an enzyme hydrolyzing activity all along the protein sequence, which is further boosted by the fermentation.

In comparison with the work by Tidona et al., (2011) in which human gastric and duodenal juices were used, although different sequences were common, deriving from all the examined proteins, it can be observed that, generally, fragments in the digesta of Tidona et al., (2011) are longer and, in many cases, they contain the sequences found by

us, as if a lower degree of digestion was reached in those experimental conditions in comparison with the present ones.

In comparison with the already cited paper by Bisadolo et al., (2012), in this work, a higher number of sequences have been identified (Table 39). For instance, in the digesta of raw milk, we found 39 peptides from β-casein, 12 α_{s1}, 13 α_{s2}, 14 β-LgI, 10 β-LgII and 13 α-lactalbumin compared to 30, 2, 2, 4, and 3 found respectively by Bidasolo et al., (2012) Of course, an even higher number of sequences were identified in the digesta of fermented donkey milk, in particular in the case of DM 33.

As a general remark, the majority of identified peptides has less than 20 amino acids residues, which is the typical maximum size of bioactive peptides. It has to be underlined that the profiling by LTQ-Orbitrap doesn't allow making a photograph of smaller peptides such as di-, tri- and somewhat tetrapeptide, which are better identified using tandem mass spectrometry instrumentation. Nevertheless, this fraction also readily undergo to further hydrolysis to free amino acids by the action of dipeptidyl and tripeptidyl peptidases in the gastrointestinal lumen.

Table 39: Number of identified sequences in digested donkey milk samples

Proteins	**Samples**										
	Raw DM	**JM 1004**	**DM 18**	**DM 33**	**DM 150**	**DM 214**	**DM 224**	**DM 236**	**DM 237**	**DM 246**	**DM 270**
β-casein accession number: D2EC27_EQUAS	39	41	52	78	45	53	56	34	47	44	42
as1 casein accession number: CASA1_EQUAS)	12	8	12	30	8	11	15	0	3	12	11
as2 casein accession number: CASA2_EQUAS	13	10	13	26	10	14	17	7	10	13	12
as2 casein B accession number: C1L3G3_EQUAS	11	2	14	17	2	9	19	0	0	12	3
β-lactoglobulin I accession number: LACB1_HORSE	14	17	23	38	21	24	20	22	21	21	15
β-lactoglobulin II accession number: D6QX32_EQUAS	10	12	15	26	15	15	12	13	15	16	11
α-lactalbumin accession number: LALBA_EQUAS	13	12	15	14	11	15	15	7	12	18	14
serum albumin accession number: F7BAY6_HORSE e P35747	10	5	0	9	0	0	3	10	4	0	9
Lysozyme C accession number: P11375|LYSC_EQUAS	0	3	4	6	2	4	3	3	2	2	2
κ-casein accession number: F0V6V5_EQUAS	0	0	4	9	0	0	0	2	0	0	3

4.5 Selection of strains

In summary, this research study started with 257 isolates and ends with selected two LAB isolates, *E. faecium* DM33 and *Lb. paracasei* DM214 which can serve as potential cultures for the development of probiotic or functional foods. The selection of these two strains was based on their technological, safety, probiotic and functional properties (Table 40). Regarding their technological properties, especially for the production of fermented milk, both strains reduced the pH to 4.6 after 24 h of incubation at 37 °C, produced EPS and showed a considerable proteolytic activity.

As per safety concerns, potential probiotic bacteria should not showed haemolytic activity or antibiotic resistance. The one strain (DM214) is belonging to the risk 1 group because of their long history of safe use and they did not have transferable antibiotic resistance, which implies their acceptability according to the guidelines of the EFSA, while the second strain (DM33), did not show any antibiotic resistance to most clinically important antibiotics or haemolytic activity, when tested according to EFSA guidelines.

One of the beneficial effects of probiotic consumption is attributed to the ability of the probiotic bacteria to inhibit the growth of foodborne pathogens or spoilage bacteria.

At the time of infection, the host initiates the immune system functioning. Probiotic bacteria work synergistically with the host immune system and helps maintain the intestinal barrier integrity, or breaks down the toxins produced by the pathogens, or creates a low pH environment which is unfavourable for the growth of pathogens, or it produces metabolites. Their antimicrobial activity is often due to the secretion of organic acids, hydrogen peroxide or bacteriocins. The antimicrobial activity profile shows that strain DM33 was capable to produced bacteriocins which inhibit the growth *L. monocytogenes*. The cell free supernatant of the identified bacteriocin-producing *enterococci* were equally active over a wide range of pH and heat treatments making them excellent candidates for potential applications in bio-preservation. Moreover, when the strain tested for its capability to control post-processing contamination and growth of *Listeria monocytogenes* in experimentally contaminated fresh whey cheese produced in Cyprus during refrigerated storage, a bactericidal effect was observed.

Table 40: Technological, safety, probiotic and functional properties of two selected LAB for potential applications

	***E. faecium* DM33**	***Lb. paracasei* DM214**
Technological properties		
Acidification activity (pH after 24 hours)	4.93±0.01	4.33±0.00
Proteolytic activity	0.48±0.19	10.42±0.54
EPS production	Positive	Positive
Autolysis	13.30±0.08	63.48±0.24
Diacetyl	Positive	Positive
Safety profile	No antibiotic resistance, haemolytic activity and no virulence factors	GRAS status
Potential probiotic properties		
Bacteriocin production	Positive	Negative
Acid tolerance (%)	73.1±0.27	71.8±0.51
Bile salt tolerance (%)	87.23±0.32	59.86±0.12
Hydrophobicity (%)	40.76±0.05	36.70±3.13
Autoaggregation (%)	34.90±0.75	59.04±1.46
Coaggregation (%) with L. monocytogenes 33413	29,24±0.34	52,65±0.21
Coaggregation (%) with S. aureus RF122	27,38±0.22	25,06±0.34
BSH activity	Negative	Negative
Functional properties		
Antioxidant activity	Positive	Positive
Antimicrobial activity	Positive	positive
ACE- inhibitory activity (IC_{50} mg/ml)	0.35±0.01	0.04±0.01

The first criterion of this study for selection of new potentially probiotic strains was the ability of the strains to survive under simulated gastrointestinal conditions. This ability should ensure that these strains reach the small intestine, which is the intended site of action. According to the guidelines for the evaluation of probiotics in food reported by a Joint FAO/WHO working group, two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile salts. Both strains were able to survive gastrointestinal conditions, while a lower survival rate was observed for DM214 in the

presence of 0.3% bile salts. This indicates that they, especially strain DM33, may reach the site of action, the small intestines unharmed.

The bacterial adhesion with the gut cells is considered to be a significant requirement for a probiotic culture to bring health effects over an extended period. The potential of bacteria to adhere and colonize in the gut is measured by their cell surface hydrophobicity and aggregation properties, respectively. Both strains showed cell surface hydrophobicity of 37-41% with xylene, while a lower percentage of hydrophobicity observed in the case of n-hexadecane. After adherence, the probiotic culture should be able to aggregate and colonize in the gut for sustaining health promoting effect. Strain DM214 (59.04±1.46%) showed the strongest auto aggregation and coaggregation ability of all the tested strains.

Furthermore, both strains were used as starter cultures for the production of potential functional fermented donkey milk. The acidification rate observed in donkey milk used for fermentation was lower than that observed in reconstituted skim milk. The differences in the acidification rate could be attributed to the high concentration of lysozyme in donkeys' milk which may interfere with the growth activity of starter cultures, and also to the different buffering capacity of donkey milk due to differences in the proportion of proteins and certain salts in both milks and finally natural pH differs between bovine and donkey milk (Bornaz et al., 2010). The milk that was fermented with *E. faecium* DM33 exhibited the strongest antioxidant activity and the highest antimicrobial activity, while the highest ACE-inhibitory activity was observed in milk fermented with *Lb. casei* DM214.

Our results showed that both strains can serve as a potential probiotic or adjunct culture in the development of nutraceutical or probiotic food products for therapeutic or prophylactic applications after proper animal and human clinical studies.

5 General conclusion

In the present study an attempt has been made to isolate most efficient potential probiotic LAB from raw donkey milk, their screening, identification, assessment of essential probiotic attributes and finally their application as nutraceutical agent in preparation of nutritionally enriched functional fermented donkey milk drink.

The major findings of the work include:

In total, 270 bacterial isolates were isolated from donkey milk and their morphological, phenotypical and biochemical characteristics were studied. Out of all isolated bacteria, 257 isolates showed catalase test negative and were tentatively identified as *Lactobacilli, Leuconostoc, Enterococcus* and *Streptococcus* and selected for preliminary screening. All these 257 isolates were screened for their safety and technological properties directly related to the manufacture of fermented products such as their ability to acidify milk, proteolytic, lipolytic and autolytic activity as well as the production of EPS and diacetyl. The predominant LABs associated with donkey milk possess technological properties, with potentials for the development of starter/bioprotective cultures to be used for the production of fermented donkey milk with consistent quality and safety.

Among them, 74 isolates were further selected on the basis of their technological and safety properties and further evaluated for the production of antimicrobial compounds using well diffusion method. Among the isolates, 3 LAB belong to *Enterococcus* genus (DM33, DM224 and DM270) showed antimicrobial activity against more than one indicator strain and therefore used for further characterization. In addition the present study provides strong evidence that bacteriocin-producing *enterococci* can perform efficiently in the control of *L. monocytogenes* in fresh whey cheese during refrigerated storage.

In the present study, after screening 74 strains at pH 3, 9 strains were selected based on their acidity tolerance capacity and they were further characterised for their potential probiotic and functional properties. All selected strains showed viability at a different degree, after 3 hours of exposure at pH 3. Meanwhile, in order to reach the colon in viable state, strains must cope with bile salts stress in the upper small intestine. The determination of strains bile salt tolerance during this study showed that all strains were able to tolerate 0.3% bile concentration. Moreover, all the strains showed in vitro cell

hydrophobicity towards xylene, cellular autoaggregation and coaggregation with *L. monocytogenes*, at a different level. Furthermone, only one strain DM224 showed BSH activity. Results of this study have shown that each strain presented individual characteristics, which may contribute to their 'probiotic' health-promoting effects.

Finally, this research investigated the presence of bioactive peptides in fermented donkey milk. All samples were screened for antioxidant, antimicrobial activity against foodborne pathogens and inhibition of the activity of ACE. All fermented products produced peptides that had varying degrees of bioactivity. *In vitro* digestion of the fermented donkey milk products generally resulted in stronger antimicrobial, antioxidant and ACE-inhibitory activity compared with undigested fermented products. Donkey milk fermented with *E. faecium* DM33 exhibited the strongest antioxidant activity and the highest antimicrobial activity, while the highest ACE-inhibitory activity was observed in milk fermented with *Lb. casei* DM214.

It may be concluded that LAB isolated from donkey milk showed desirable technological and probiotic attributes for their successful use in food products to exert their nutritional as well as therapeutic benefits and could successfully be exploited to prepare novel functional foods/nutraceuticals as in the present study. All of them at a different level were found highly acceptable vehicles to deliver the probiotic health effects and also to produce bioactive peptides. The data obtained from the screening tests indicates that isolates *E. faecium* DM33 and *Lb. paracasei* DM214, have promising potential for further development of a novel fermented donkey milk product with potential probiotic and functional properties.

6 Future work

Fermented dairy products play an important role in human diet. Continuous development of new functional foods is the response of science and industry to the increased consumer awareness regarding health and the role of foods for improving quality of life. This study demonstrated that donkey milk is a good natural source for isolation of LAB, with potential applications in food industry, such as production of new probiotic or functional fermented dairy beverages or for food bio-preservation.

The discovery of new bacteriocins from LAB is of high importance in food industry in order to meet the consumer demands for safer foods. The current study shows that LAB from raw donkey milk can exhibit antimicrobial activity against a number of common foodborne pathogens. However, further investigations to identify the minimum inhibitory concentration (MIC) of each species in order to be successfully applied as bio-preservatives in food products such as dairy products need to be done. As the bacteriocin producing strains isolated in this study belong to *enterococcus* genus, a heterologous expression in safer strains such as *Lactobacillus* or *Lactococcus* strains (GRAS) can also be carried out; even though the safe application of *enterococci* in food products has also been demonstrated. Sensory analysis would also be required to determine whether the LAB and/or their antimicrobial compounds would impart undesirable flavours to the dairy products.

LAB isolated from donkey milk showed potential probiotic properties. While knowledge of some potential probiotic strains for their acid, bile tolerance, BSH activity, cell surface hydrophobicity, auto-aggregation and co-aggregation has been ascertained as well as antimicrobial activity, further work is still needed on improving viability of probiotic strains in commercial products and determining the concentration of probiotic bacteria for daily consumption in order to achieve health benefits. Also, the effect of the probiotic organisms on the quality of the donkey milk, and their contributions to the development of flavors and organoleptic properties during storage will be also useful to study.

The potential health benefits of milk protein-derived peptides have been a subject of growing commercial besides scientific interest in the context of health promoting functional foods. A number of potential ACE-inhibitory, antioxidant and antimicrobial peptides were isolated and in this study. Future directions of this research could include

conducting mixed fermentations using synergistic bacteria with varying degrees of proteolytic activity that could result in the production of larger amounts of peptides that may be potentially bioactive. Further studies are already in progress in order to identify the peptides that are responsible for these bioactivities. Also, research could include synthesizing the promising peptides identified in this study by solid phase peptide synthesis and verified their bioactivities. Furthermore, nuclear magnetic resonance (NMR) could be used to determine the structure-activity relationship of the potentially novel ACE inhibitory, antioxidant and antimicrobial peptides. In addition to these functional properties, a more detailed investigation of other bioactivities such as anti-inflammation, immunomodulation, cholesterol lowering can also be carried out. Also, many scientific, technological and regulatory issues, however, must be resolved before these peptides can be optimally exploited for human nutrition and health. The physiological function of these peptides needs to be established *in vivo* through clinical trials with animal and human studies.

As the sensory characteristics of donkeys' milk are quite different from those of cows' milk, for instance donkey milk is more translucent, less white and tastes sweeter, a sensory evaluation of the new fermented donkey milk products needs to be carried out in order to optimize consumer acceptability of new fermented milk formulations. In this study, we proposed a fermentation strategy of donkey milk. However, future studies are needed in order to reduce the fermentation time (i.e using fast acidifying starter cultures) and also according to a study carried out by Di Cagno et al. (2004) fortification of donkey milk with bovine sodium caseinate, pectin and threonine may improve the rheological and sensory characteristics. Finally, the chemical, physical, microbiological and organoleptic changes of the developed fermented products during storage days need to be evaluated.

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APPENDIX I

Enterocins isolated from enterococcus spp (BACTIBASE; http://www.bactibase.pfba-lab-tun.org)

Strain	Bacteriocin	Target Strains	Molecular Mass (Da)
Enterococcus avium	Avicin A (class IIa)	*Carnobacterium divergens, Carnobacterium piscicola, Enterococcus avium, Enterococcus faecalis, Enterococcus maldoratus, Lactobacillus rhamnosus, Lactobacillus sakei, Leuconostoc lactis, Leuconostoc mesenteroides, Listeria innocua, Listeria monocytogenes, Pediococcus acidilactici, Pediococcus pentosaceus*	4288
Enterococcus columbae	Columbicin A (Lantibiotic)		6211.2
Enterococcus durans	Durancin Q Durancin TW-49M	*Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae, Lactobacillus alimentarius, Lactobacillus kimchii, Lactobacillus plantarum, Lactobacillus sake, Lactococcus lactis, Geobacillus stearothermophilus, Bacillus subtilis, Bacillus coagulans , Bacillus circulans*	7277.62 5246.92
Enterococcus faecalis	Enterocin 96 (Class II)	*Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae, Enterococcus pseudoavium, Enterococcus sulfureus, Enterococcus saccharolyticus, Enterococcus columbae, Lactobacillus plantarum,Lactobacillus acidophilus, Lactobacillus sakei, Lactobacillus paracasei, Lactococcus lactis, Lactococcus lactis subsp. Cremoris, Leuconostoc mesenteroides , Bacillus subtilis, Bacillus cereus, Listeria innocua, Listeria monocytogenes, Staphylococcus xylosus, Staphylococcus aureus, Salmonella Typhimurium, Salmonella Infantis, Klebsiella pneumonia, Serratia liquefaciens, Proteus vulgaris, Enterobacter cloacae, Escherichia coli.*	5493.92
Enterococcus faecalis	Bacteriocin 31 (Unclassfied)	*Enterococcus hira, Enterococcus faecium, Listeria monocytogenes*	4955.33
Enterococcus faecalis	Enterocin 1071A (Unclassfied)		4285.16
Enterococcus faecalis	Enterocin SE-K4 (Class IIa)		5376.9
Enterococcus faecalis	Enterolysin A (class III)	*Lactobacillus sakei, Lactobacillus brevis, Lactobacillus curvatus, Lactococcus cremoris, Lactococcus lactis, Pediococcus pentosaceus, Pediococcus acidilactici, Enterococcus*	34524.69

		faecium, Enterococcus faecalis, Listeria innocua, Listeria ivanovii, Bacillus subtilis, Bacillus cereus , Staphylococcus carnosus, Propionibacterium jensenii	
Enterococcus faecalis	Enterocin EJ97 (Unclassfied)	*Bacillus circulans, Bacillus coagulans, Bacillus megaterium, Bacillus stearothermophilus, Bacillus subtilis, Paenibacillus macerans, Enterococcus faecalis, Enterococcus faecium, Listeria grayi, Listeria innocua, Listeria ivanovii, Listeria monocytogenes, Listeria murrayi, Listeria welshimeri, Staphylococcus aureus*	5340
Enterococcus faecalis	Enterocin W alfa (Class IIb)	*Bacillus coagulans, Bacillus circulans,Listeria innocua, Pediococcus pentosaceus, Enterococcus faecalis, Lactococcus lactis, Lactobacillus sakei*	3256
Enterococcus faecalis	Enterocin W beta (Class IIb)	*Bacillus coagulans, Bacillus circulans, Listeria innocua, Pediococcus pentosaceus, Enterococcus faecalis, Lactococcus lactis, Lactobacillus sakei*	2728
Enterococcus faecium	Enterocin Q (Class IIc)	*Lactobacillus sakei, Enterococcus faecium*	3970.31
Enterococcus faecium	Enterocin P (Class IIa)	*Lactobacillus sakei, Enterococcus faecium*	4648.91
Enterococcus faecium	Enterocin 7A	*Clostridia,propionibacteria Listeria monocytogenes Lactobacillus sakei, Enterococcus faecium, Pediococcus acidilactici, Pediococcus pentosaceus*	5177.18
Enterococcus faecium	Enterocin A (Class IIa)	*Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae, Lactobacillus plantarum, Lactobacillus sakei, Lactococcus lactis, Bacillus coagulans, Bacillus subtilis, Listeria innocua, Pediococcus spp., Listeria monocytogenes*	4851.38
Enterococcus faecium	Enterocin B (class IIc)	*Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae, Lactobacillus plantarum, Lactobacillus sakei, Bacillus coagulans, Bacillus subtilis*	5484.14
Enterococcus faecium	Bacteriocin E50-52 (Unclassfied)	*Campylobacter jejuni, Yersinia enterocolitica, Yersinia pseudotuberculosis, Staphylococcus aureus, Staphylococcus epidermidis, Listeria monocytogenes, Listeria grayi, Listeria innocua, Listeria ivanovii, Listeria denitrificans, Escherichia coli O157:H7, Salmonella spp., Shigella dysenteriae*	4144.4
Enterococcus faecium	Enterocin-HF (class IIa)		4328.1
Enterococcus faecium	Enterocin Xalpha (Class IIb)	*Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae, Lactobacillus plantarum, Lactobacillus sakei, Lactococcus lactis ssp. cremoris, Listeria innocua*	4420

Enterococcus faecium	Enterocin Xbeta (Class IIb)	*Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae, Lactobacillus plantarum, Lactobacillus sakei, Bacillus circulans, Bacillus coagulans, Bacillus subtilis, Listeria innocua*	4068
Enterococcus faecium	Bacteriocin T8 (class IIa)	*Enterococcus faecalis, Propionibacterium spp*	4220.36
Enterococcus faecium	Enterocin NKR-5-3B (Ent53B)	*Bacillus coagulans, Bacillus subtilis, Kocuria rhizophila, Listeria innocia, Pediococcus pentosaceus, Enterococcus faecalis, Lactococcus lactis, Lactobacillus plantarum, Lactobacillus sakei*	6316
Enterococcus hirae	Hiracin JM79 (Class IIc)	*Lactobacillus helveticus, Lactobacillus curvatus, Lactobacillus bulgaricus, Lactobacillus sakei,- Pediococcus pentosaceus, Enterococcus faecium, Enterococcus faecalis, Propionibacterium acidipropionici, Clostridium tyrobutiricum, Listeria monocytogenes, Listeria ivanovii, Listeria seeligeri, Listeria welshimeri, Listeria grayi, Staphylococcus aureus*	5093
Enterococcus mundtii	Mundticin (class IIa)	*Listeria monocytogenes, Clostridium botulinum, Carnobacterium, Enterococcus, Lactobacillus, Leuconostoc,- Pediococcus*	4308.55
Enterococcus mundtii	Mundticin KS (Unclassfied)		4967.44
Enterococcus mundtii	Enterocin CRL35 (Mundticin KS) (Class IIa)		4308.55
Enterococcus mundtii	Mundticin L (class IIA/YGNGV)		4290
Enterococcus spp	Enterocin E-760 (class IIb)	*Staphylococcus aureus, Staphylococcus epidermidis, Listeria monocytogenes, Campylobacter sp, Salmonella Enteritidis, Salmonella Choleraesuis, Salmonella typhimurium, Salmonella gallinarum, Escherichia coli, Escherichia coli O157:H7, Yersinia enterocolitica, Yersinia pseudotuberculosis, Citrobacter freundii , Klebsiella pneumonia, Shigella dysenteriae, Campylobacter jejuni*	6198.95

APPENDIX II

Milk-derived antimicrobial peptides

Antimicrobial peptide	Source	Amino acid sequence	Antibacterial activity	Isolation	Reference
Lactenin	α-casein	Unavailable	Pathogenic enterococci	Rennet	Jones and Simms, 1930
Casecidins	Bovine α_{s1}-casein	Unavailable	Gram- positive bacteria (*Sarcina* spp., *Bacillus subtilis, Diplococcus pneumonia, Streptococcus pyogenes*)	Chymosin	Lahov and Regelson, 1996
Isracidin f(1-23)	Bovine α_{s1}-casein	R_1PKHPIKHQGLP QEVLNENLLRF$_{23}$	Gram- positive and gram-negative bacteria	Chymosin	Hill et al., 1974
Caseicin A α_{s1}-CN f (21-29)	Bovine α_{s1}-casein	I_{21}KHQGLPQE$_{29}$	Gram- positive and gram-negative bacteria (*E. Coli, Ent. Sakazakii, L. bulgaricus, L. innocua, S. mutants*)	Fermentation with *Lactobacillus acidophilus*	Hayes et al., 2006
Caseicin B α_{s1}-CN f (30-37)	Bovine α_{s1}-casein	V_{30}LNENLLR$_{37}$	Gram- positive and gram-negative bacteria (*E. Coli, Ent. Sakazakii, L. bulgaricus, L. innocua, S.*	Fermentation with *Lactobacillus acidophilus*	Hayes et al., 2006

			mutants)		
Caseicin c α_{s1}-CN f (195-208)	Bovine α_{s1}-casein	SDIPNPIGSENSEK	*L. innocua*	Fermentation with *Lactobacillus acidophilus*	Hayes et al., 2006
Casocidin-I f(150-188)	Bovine α_{s2}-casein	K_{150}TKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVYQHQK_{188}	Gram- positive and gram-negative bacteria (*E. Coli, S. carnosus, B. subtilis, S. epidermidis, E. faecium, Rhodotorularubra*	Trypsin	Zucht et al., 1995
f(164-179)	Bovine α_{s2}-casein	LKKISQRYQKFALPQY	Several gram-positive and gram- negative bacteria (*E. coli, B. cereus, Streptococcus thermophilus*)	Pepsin	Recio and Visser, 1999
f(183-207)	Bovine α_{s2}-casein	YQHQKAMKPWIQPKTKVIPYVRYL	Several gram-positive and gram- negative bacteria (*E. coli, B. cereus, Streptococcus thermophilus*)	Pepsin	Recio and Visser, 1999
f(181-207)	Bovine α_{s2}-casein	K_{181}TVYQHQKAMKPWIQPKTKVIPYVRYL_{207}	Gram-positive and gram-negative bacteria (*L. monocytogenes, L. innocua, B. subtilis, Salmonella typhimurium, Salmonella enteridis, E. coli*	Chymosin	McCann et al., 2005
f(180-207	Bovine α_{s2}-casein	L_{180}KTVYQHQKAMKPWIQPKTKVIPYVRYL_{207}	Gram- positive and gram-negative bacteria (*L. monocytogenes, L. innocua, B. subtilis, Salmonella typhimurium, Salmonella*	Chymosin	McCann et al., 2005

			enteridis, E. coli		
f(175-207	Bovine α_{s2}-casein	A_{175}LPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL$_{207}$	Gram- positive and gram-negative bacteria (*L. monocytogenes, L. innocua, B. subtilis, Salmonella typhimurium, Salmonella enteridis, E. coli*	Chymosin	McCann et al., 2005
f(172-207	Bovine α_{s2}-casein	Q_{172}KFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL$_{207}$	Gram- positive and gram-negative bacteria (*L. monocytogenes, L. innocua, B. subtilis, Salmonella typhimurium, Salmonella enteridis, E. coli*	Chymosin	McCann et al., 2005
f(164-207)	Bovine α_{s2}-casein	L_{164}KKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL$_{207}$	Gram- positive and gram-negative bacteria (*L. monocytogenes, L. innocua, B. subtilis, Salmonella typhimurium, Salmonella enteridis, E. coli*	Chymosin	McCann et al., 2005
Casecidin15 f(193-207)	β-casein	Y_{193}QEPVLGPVRGPFPI$_{207}$	*E. coli*	Chymotrypsin and proteinase K	Birkemo et al., 2009
Casecidin15 f(193-209)		Y_{193}QEPVLGPVRGPFPIIV$_{209}$	*E. coli*	Chymotrypsin and proteinase K	Birkemo et al., 2009
Kappacin f(106-169)	Bovine κ-casein	A_{106}IPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDS*PEVIESPPEINTVQVTSTAV$_{169}$	Gram- positive and gram-negative bacteria (*Streptococcus mutants, Porphyromonas gingival*)	Chymosin	Malkoski et al., 2001
f(42-49)	Bovine κ-casein	Y_{42}YQQKPVA$_{49}$	Gram-positive and gram-negative bacteria	Pepsin	Lopez-Expozito et al., 2006

			(*L. innocua, S. carnosus, E.coli, Streptococcus marcescens*)		
f(28-30	Bovine κ-casein	$I_{28}QY_{30}$	Mainly Gram-negative bacteria (*E.coli, Streptococcus marcescens*)	Pepsin	Lopez-Expozito et al., 2006
f(162-169)	Bovine κ-casein	$V_{162}QVTSTAV_{169}$	Mainly Gram-positive bacteria (*L. innocua, S. carnosus*)	Pepsin	Lopez-Expozito et al., 2006
f(141-146)	Bovine κ-casein	$S_{141}TVATL_{146}$		Pepsin	Lopez-Expozito et al., 2006
f(18-24)	Bovine κ-casein	$F_{18}SDKIAK_{24}$		Pepsin	Lopez-Expozito et al., 2006
f(30-32)	Bovine κ-casein	$Y_{30}VL_{32}$	Mainly Gram-positive bacteria (*L. innocua, S. carnosus*)	Pepsin	Lopez-Expozito et al., 2006
f(118-121)	Bovine κ-casein	$E_{118}IPT_{121}$	Mainly Gram-negative bacteria (*E.coli, Streptococcus marcescens*)	Pepsin	Lopez-Expozito et al., 2006
f(139-146)	Bovine κ-casein	$V_{139}ESTVALT_{146}$	Gram-positive and gram-negative bacteria (*L. innocua, S. carnosus, E.coli, Streptococcus marcescens*)	Pepsin	Lopez-Expozito et al., 2006
f(64-75)	Bovine κ-casein	$P_{64}AAVRSPAQILQ_{75}$	Gram-positive and gram-negative bacteria (*L. innocua, S. carnosus, E.coli, Streptococcus marcescens*)	Pepsin	Lopez-Expozito et al., 2006
LF f(17-41/42)	Bovine lactoferrin	FKCRRWQWRMKKLGAPSITCVRRAF/A	Gram-positive and gram-negative bacteria	Pepsin or chymosin	Bellamy et al., 1992; Dionysius and Milne, 1997
LF f(1-16)S-	Bovine	APRKNVRWCTIS	*Micrococcus*	Pepsin	Recio and

S(45-48)	lactoferrin	QPEWCIRA	*flavus*		Visser, 1999
LF f(1-11)S-S (17-47)	Bovine lactoferrin	APRKNVRWTIFK CRRWQWRMKKL GAPSITCVRRAFA LECIR	*Micrococcus flavus*	Pepsin	Recio and Visser, 1999
LF f(1-16)S-S(43-48)	Bovine lactoferrin	APRKNVRWCTIS QPEWLECIRA	Gram-positive and gram-negative bacteria	Pepsin	Dionysius and Milne, 1997
LF f(1-42)S-S(43-48)	Bovine lactoferrin	APRKNVRWCTIS QPEWFKCRRWQ WRMKKLGAPSIT CVRRAFALECIR A	*E. coli*	Pepsin	Dionysius and Milne, 1997
LF f(1-16)S-S(17-48)	Bovine lactoferrin	APRKNVRWCTIS QPEWFKCRRWQ WRMKKLGAPSIT CVRRAFALECIR A	*E. coli*	Pepsin	Hoek et al., 1997
a-La f(1-5)	Bovine α-lactoglobulin	EQLTK	Gram-positive bacteria	Trypsin	Pellegrini et al., 1999
a-La f(17-31)S-S(109-114)	Bovine α-lactoglobulin	GYGGVSLPEWV CTTFALCSEK	Gram-positive bacteria	Trypsin	Pellegrini et al., 1999
a-La f(61-68)S-S(75-80)	Bovine α-lactoglobulin	CKDDQNPHISCD KF	Gram-positive bacteria	Chymotrypsin	Pellegrini et al., 1999
a-La f(117-121)	Bovine α-lactoglobulin	KVGIN	Mainly Gram-negative bacteria	Pepsin	Theolier et al., 2013
b-lg f(15-20)	Bovine β-lactoglobulin	VAGTWY	Gram-positive bacteria	Trypsin	Pellegrini et al., 2001
b-lg f(25-40)	Bovine β-lactoglobulin	AASDISLLDAQS APLR	Gram-positive bacteria	Trypsin	Pellegrini et al., 2001
b-lg f(78-83)	Bovine β-lactoglobulin	IPAVFK	Gram-positive bacteria	Trypsin	Pellegrini et al., 2001

b-lg f(92-100)	Bovine β-lactoglobulin	VLVLDTDYK	Gram-positive bacteria	Trypsin	Pellegrini et al., 2001
b-lg f(14-18)	Bovine β-lactoglobulin	KVAGT	Mainly Gram-positive bacteria	Pepsin	Theolier et al., 2013
b-lg f(123-125)	Bovine β-lactoglobulin	VRT	Mainly Gram-positive bacteria	Pepsin	Theolier et al., 2013
b-lg f(50-54)	Bovine β-lactoglobulin	PEGDL	Mainly Gram-negativebacteria	Pepsin	Theolier et al., 2013
b-lg f(143-146)	Bovine β-lactoglobulin	LPMH	Weakly inhibitory to Gram-positive and gram-negative bacteria	Pepsin	Theolier et al., 2013
b-lg f(134-136)	Bovine β-lactoglobulin	EKF	Weakly inhibitory to Gram-positive and gram-negative bacteria	Pepsin	Theolier et al., 2013
b-lg f(147-149)	Bovine β-lactoglobulin	IRL	Mainly Gram-positive bacteria	Pepsin	Theolier et al., 2013

APPENDIX III

ID of each isolate using 16s RNA sequencing

Isolate	ID using 16s RNA sequencing	Isolate	ID using 16s RNA sequencing
DM002	*Streptococcus macedonicus*	**DM023**	*Streptococcus gallolyticus*
DM003	*Enterococcus gallinarum*	**DM024**	*Streptococcus gallolyticus*
DM004	*Streptococcus gallolyticus*	**DM025**	*Streptococcus macedonicus*
DM005	*Enterococcus thailanticus/lactis*	**DM026**	*Enterococcus faecium*
DM006	*Enterococcus faecium*	**DM027**	*Enterococcus faecium*
DM007	*Streptococcus macedonicus*	**DM028**	*Enterococcus thailanticus*
DM008	*Streptococcus macedonicus*	**DM029**	*Enterococcus faecium*
DM009	*Streptococcus gallolyticus*	**DM030**	*Streptococcus gallolyticus*
DM010	*Enterococcus faecium*	**DM031**	*Enterococcus faecium*
DM011	*Streptococcus gallolyticus*	**DM032**	*Enterococcus faecalis*
DM012	*Enterococcus thailanticus/lactis*	**DM033**	*Enterococcus faecium*
DM013	*Enterococcus hirae*	**DM034**	*Enterococcus faecium*

DM014	*Enterococcus faecium*	**DM035**	*Enterococcus faecalis*
DM015	*Enterococcus faecium*	**DM036**	*Enterococcus faecium*
DM016	*Streptococcus gallolyticus*	**DM037**	*Enterococcus faecium*
DM017	*Enterococcus faecium*	**DM038**	*Streptococcus gallolyticus*
DM018	*Enterococcus faecium*	**DM039**	*Enterococcus faecium*
DM019	*Enterococcus thailanticus/lactis*	**DM040**	*Streptococcus gallolyticus*
DM020	*Streptococcus macedonicus*	**DM041**	*Enterococcus hirae*
DM021	*Streptococcus macedonicus*	**DM042**	*Enterococcus faecium*
DM022	*Enterococcus durans*	**DM043**	*Enterococcus faecium*
DM044	*Streptococcus macedonicus*	**DM077**	*Streptococcus gallolyticus*
DM046	*Streptococcus gallolyticus*	**DM080**	*Enterococcus faecalis*
DM047	*Enterococcus faecium*	**DM082**	*Enterococcus faecium*
DM049	*Enterococcus faecalis*	**DM083**	*Enterococcus faecium*
DM051	*Enterococcus faecalis*	**DM084**	*Enterococcus thailanticus/lactis*
DM054	*Enterococcus faecalis*	**DM085**	*Streptococcus gallolyticus*
DM056	*Enterococcus faecalis*	**DM086**	*Enterococcus faecium*
DM057	*Enterococcus faecium*	**DM087**	*Enterococcus faecalis*
DM058	*Streptococcus macedonicus*	**DM088**	*Enterococcus faecium*
DM060	*Enterococcus faecalis*	**DM089**	*Enterococcus faecium*
DM061	*Enterococcus faecium*	**DM090**	*Streptococcus gallolyticus*
DM062	*Enterococcus faecium*	**DM091**	*Enterococcus hirae*
DM063	*Enterococcus faecalis*	**DM092**	*Leuconostoc mesenteroides*
DM064	*Enterococcus faecium*	**DM093**	*Enterococcus faecalis*
DM065	*Enterococcus faecium*	**DM094**	*Enterococcus faecium*
DM066	*Streptococcus gallolyticus*	**DM095**	*Enterococcus faecium*
DM067	*Enterococcus faecium*	**DM096**	*Streptococcus gallolyticus*
DM068	*Enterococcus faecium*	**DM097**	*Enterococcus faecalis*
DM069	*Streptococcus gallolyticus*	**DM098**	*Enterococcus faecium*
DM071	*Enterococcus hirae*	**DM099**	*Enterococcus faecium*
DM072	*Enterococcus faecalis*	**DM100**	*Enterococcus faecium*
DM073	*Streptococcus gallolyticus*	**DM101**	*Streptococcus macedonicus*
DM074	*Enterococcus thailanticus/lactis*	**DM102**	*Enterococcus faecalis*
DM075	*Enterococcus faecalis*	**DM103**	*Enterococcus faecium*
DM076	*Enterococcus faecium*	**DM104**	*Enterococcus faecium*
DM105	*Enterococcus faecium*	**DM130**	*Streptococcus gallolyticus*

DM106	*Enterococcus faecalis*	**DM131**	*Enterococcus faecalis*
DM107	*Streptococcus gallolyticus*	**DM132**	*Streptococcus gallolyticus*
DM108	*Enterococcus faecium*	**DM133**	*Enterococcus faecalis*
DM109	*Enterococcus faecium*	**DM134**	*Enterococcus faecalis*
DM110	*Enterococcus faecium*	**DM135**	*Streptococcus gallolyticus*
DM111	*Enterococcus hirae*	**DM136**	*Enterococcus thailanticus*
DM112	*Enterococcus faecalis*	**DM137**	*Enterococcus faecium*
DM113	*Enterococcus faecalis*	**DM138**	*Enterococcus thailanticus/lactis*
DM114	*Streptococcus macedonicus*	**DM139**	*Enterococcus faecium*
DM115	*Leuconostoc mesenteroides*	**DM140**	*Streptococcus macedonicus*
DM116	*Enterococcus faecalis*	**DM141**	*Enterococcus durans*
DM117	*Enterococcus faecalis*	**DM142**	*Enterococcus faecium*
DM118	*Enterococcus faecium*	**DM143**	*Enterococcus faecalis*
DM119	*Enterococcus faecalis*	**DM144**	*Enterococcus thailanticus*
DM120	*Streptococcus gallolyticus*	**DM145**	*Enterococcus faecalis*
DM121	*Enterococcus faecium*	**DM146**	*Enterococcus faecium*
DM122	*Enterococcus faecium*	**DM147**	*Enterococcus faecalis*
DM123	*Streptococcus gallolyticus*	**DM148**	*Streptococcus macedonicus*
DM124	*Enterococcus durans*	**DM149**	*Enterococcus faecalis*
DM125	*Enterococcus faecium*	**DM150**	*Enterococcus gallinarum*
DM126	*Enterococcus faecalis*	**DM151**	*Enterococcus gallinarum*
DM127	*Enterococcus faecium*	**DM152**	*Enterococcus faecalis*
DM128	*Streptococcus gallolyticus*	**DM153**	*Streptococcus gallolyticus*
DM129	*Enterococcus faecium*	**DM154**	*Enterococcus hirae*
DM155	*Streptococcus gallolyticus*	**DM181**	*Enterococcus thailanticus*
DM156	*Streptococcus gallolyticus*	**DM182**	*Enterococcus faecalis*
DM157	*Enterococcus gallinarum*	**DM183**	*Enterococcus faecium*
DM158	*Enterococcus faecalis*	**DM184**	*Enterococcus faecium*
DM159	*Enterococcus faecium*	**DM185**	*Enterococcus faecalis*
DM161	*Enterococcus faecium*	**DM186**	*Streptococcus gallolyticus*
DM162	*Enterococcus faecalis*	**DM187**	*Enterococcus faecium*
DM163	*Enterococcus faecium*	**DM188**	*Enterococcus mundii*
DM164	*Enterococcus faecium*	**DM189**	*Enterococcus thailanticus*
DM165	*Streptococcus gallolyticus*	**DM190**	*Streptococcus gallolyticus*
DM166	*Enterococcus faecium*	**DM191**	*Enterococcus faecium*

DM167	*Enterococcus faecalis*	**DM192**	*Enterococcus thailanticus*
DM168	*Streptococcus gallolyticus*	**DM193**	*Enterococcus faecalis*
DM169	*Enterococcus faecalis*	**DM194**	*Enterococcus thailanticus*
DM170	*Enterococcus faecium*	**DM195**	*Streptococcus macedonicus*
DM171	*Enterococcus faecium*	**DM196**	*Enterococcus faecium*
DM172	*Enterococcus faecalis*	**DM197**	*Enterococcus faecium*
DM173	*Streptococcus gallolyticus*	**DM198**	*Enterococcus faecalis*
DM174	*Streptococcus gallolyticus*	**DM199**	*Streptococcus gallolyticus*
DM175	*Enterococcus faecalis*	**DM200**	*Enterococcus faecium*
DM176	*Enterococcus faecium*	**DM201**	*Enterococcus faecium*
DM177	*Enterococcus faecalis*	**DM202**	*Enterococcus faecium*
DM178	*Enterococcus faecium*	**DM203**	*Enterococcus durans*
DM179	*Enterococcus faecalis*	**DM204**	*Enterococcus faecium*
DM180	*Streptococcus macedonicus*	**DM205**	*Enterococcus faecium*
DM206	*Streptococcus gallolyticus*	**DM231**	*Streptococcus macedonicus*
DM207	*Enterococcus faecium*	**DM232**	*Enterococcus faecium*
DM208	*Enterococcus hirae*	**DM233**	*Enterococcus faecium*
DM209	*Enterococcus faecium*	**DM234**	*Enterococcus faecium*
DM210	*Enterococcus faecium*	**DM235**	*Enterococcus faecium*
DM211	*Enterococcus faecium*	**DM236**	*Leuconostoc mesenteroides*
DM212	*Streptococcus macedonicus*	**DM237**	*Enterococcus faecalis*
DM213	*Enterococcus faecalis*	**DM238**	*Enterococcus faecium*
DM214	*Lactobacillus paracasei*	**DM239**	*Enterococcus faecium*
DM215	*Enterococcus faecium*	**DM240**	*Streptococcus macedonicus*
DM216	*Enterococcus faecium*	**DM241**	*Enterococcus faecium*
DM217	*Enterococcus thailanticus/ lactis*	**DM242**	*Enterococcus faecium*
DM218	*Streptococcus gallolyticus*	**DM243**	*Enterococcus faecium*
DM219	*Enterococcus faecium*	**DM244**	*Enterococcus faecium*
DM220	*Streptococcus gallolyticus*	**DM245**	*Streptococcus gallolyticus*
DM221	*Enterococcus faecium*	**DM246**	*Enterococcus mundii*
DM222	*Enterococcus faecium*	**DM247**	*Streptococcus gallollyticus*
DM223	*Enterococcus faecium*	**DM248**	*Streptococcus gallollyticus*
DM224	*Enterococcus faecium*	**DM249**	*Streptococcus gallollyticus*
DM225	*Enterococcus faecium*	**DM250**	*Streptococcus gallollyticus*
DM226	*Streptococcus macedonicus*	**DM251**	*Enterococcus faecium*

DM227	*Enterococcus faecium*	**DM252**	*Enterococcus faecium*
DM228	*Enterococcus thailanticus*	**DM253**	*Enterococcus durans*
DM229	*Enterococcus faecium*	**DM254**	*Enterococcus faecium*
DM230	*Enterococcus faecium*	**DM255**	*Streptococcus macedonicus*
DM256	*Enterococcus faecium*	**DM264**	*Enterococcus faecium*
DM257	*Enterococcus faecium*	**DM265**	*Enterococcus faecium*
DM258	*Enterococcus faecium*	**DM266**	*Enterococcus faecium*
DM259	*Lactobacillus paracasei*	**DM267**	*Enterococcus faecium*
DM260	*Enterococcus faecium*	**DM268**	*Enterococcus faecium*
DM261	*Streptococcus macedonicus*	**DM269**	*Streptococcus gallollyticus*
DM262	*Enterococcus faecium*	**DM270**	*Enterococcus faecium*
DM263	*Enterococcus faecium*		

APPENDIX IV

Technological Properties of LAB isolates

Isolate	Acidifying activity						Proteolytic activity	Proteolytic activity (OPA test) ppm glycine		Lipolytic activity	Autolytic activity %	EPS production	Diacetyl production
Strains	6 h			24 h				6h	24h				
	pH	ΔpH	TA (%)	pH	ΔpH	TA (%)							
Lactobacillus paracasei													
DM214	6,17±0.01	0,48±0.01	0,14±0.03	4,33±0.00	2,32±0.00	0,38±0.04	++	2,25±0,07	10,42±0,54	-	63,78±0.24	+	+
DM259	6,04±0.01	0,53±0.01	0,14±0.02	4,50±0.02	2,07±0.02	0,30±0.01	++	1,32±0,02	1,43±0,01	-	61,94±0.38	-	+
Leuconostoc mesenteroides													
DM092	6,38±0.03	0,22±0.03	0,10±0.04	6,02±0.01	0,58±0.01	0,12±0.01							
DM115	6,29±0.01	0,27±0.01	0,12±0.01	5,21±0.01	1,4±0.01	0,18±0.02							
DM236	6,10±0.01	0,56±0.01	0,14±0.02	5,02±0.02	1,72±0.02	0,28±0.02							
Enterococci													
Enterococcus durans													
DM022	6,42±0.01	0,30±0.01	0,10±0.04	6,15±0.01	0,57±0.01	0,12±0.03							
DM124	6,10±0.03	0,42±0.03	0,12±0.01	5,85±0.02	0,67±0.02	0,12±0.07							
DM141	6,28±0.02	0,35±0.02	0,10±0.01	4,83±0.02	1,80±0.02	0,12±0.02	+	0,17±0,02	9,13±0,32	-	3,42±0.06	+	+
DM203	5,93±0.05	0,73±0.05	0,14±0.06	4,95±0.02	1,71±0.02	0,28±0.02	++	0,12±0,02	0,15±0,03	-	20,72±0.51	+	+
DM253	6,04±0.03	0,53±0.03	0,14±0.03	4,58±0.01	1,99±0.02	0,32±0.02	+	0,10±0,03	1,02±0,03	-	39,30±0.14	-	-
Enterococcus faecalis													

DM032	6,48±0.01	0,14±0.01	0,10±0.01	6,27±0.01	0,35±0.01	0,12±0.01	
DM035	6,25±0.02	0,48±0.02	0,12±0.04	5,94±0.01	0,79±0.01	0,12±0.02	
DM049	6,33±0.00	0,37±0.00	0,11±0.03	6,2±0.02	0,50±0.02	0,11±0.02	
DM049	6,29±0.00	0,39±0.00	0,12±0.02	6,07±0.03	0,61±0.03	0,12±0.01	
DM054	6,41±0.01	0,33±0.01	0,12±0.02	5,93±0.03	0,81±0.03	0,14±0.02	
DM056	6,38±0.01	0,31±0.01	0,10±0.03	6,18±0.02	0,51±0.02	0,14±0.01	
DM060	6,15±0.02	0,53±0.02	0,12±0.01	5,99±0.01	0,69±0.01	0,16±0.01	
DM063	6,30±0.03	0,29±0.03	0,10±0.04	5,73±0.02	0,86±0.02	0,22±0.02	
DM072	6,68±0.01	0,05±0.01	0,08±0.02	5,94±0.01	0,79±0.01	0,18±0.03	
DM075	6,22±0.01	0,45±0.01	0,12±0.03	5,33±0.01	1,34±0.01	0,20±0.05	
DM080	6,51±0.00	0,23±0.00	0,09±0.02	6,18±0.02	0,56±0.02	0,14±0.02	
DM087	6,44±0.02	0,26±0.02	0,12±0.01	5,57±0.02	1,13±0.02	0,22±0.01	
DM093	6,35±0.01	0,31±0.01	0,12±0.01	5,75±0.01	0,91±0.01	0,30±0.01	
DM097	6,31±0.04	0,37±0.04	0,12±0.02	5,69±0.01	0,99±0.01	0,18±0.02	
DM102	6,43±0.01	0,26±0.01	0,10±0.03	5,90±0.04	0,79±0.04	0,14±0.02	
DM106	6,32±0.01	0,33±0.01	0,12±0.02	5,85±0.05	0,80±0.05	0,16±0.04	
DM112	6,44±0.02	0,25±0.02	0,12±0.01	5,22±0.02	1,47±0.02	0,26±0.01	
DM113	6,62±0.02	0,23±0.02	0,08±0.00	5,17±0.02	1,68±0.02	0,24±0.03	
DM116	6,14±0.01	0,35±0.01	0,12±0.01	5,13±0.01	1,36±0.01	0,30±0.05	
DM117	6,24±0.04	0,28±0.04	0,10±0.01	5,95±0.00	0,57±0.00	0,26±0.01	
DM119	6,62±0.01	0,18±0.01	0,08±0.00	5,92±0.04	0,88±0.04	0,12±0.01	
DM126	6,63±0.01	0,24±0.01	0,08±0.00	5,95±0.01	0,92±0.01	0,28±0.02	

DM131	6,28±0.01	0,32±0.01	0,10±0.01	5,95±0.01	0,65±0.01	0,18±0.03							
DM133	6,70±0.02	0,19±0.02	0,08±0.03	5,15±0.02	1,74±0.02	0,22±0.02							
DM134	6,38±0.03	0,33±0.03	0,08±0.02	5,81±0.01	0,90±0.01	0,22±0.02							
DM143	6,35±0.02	0,44±0.02	0,10±0.01	5,35±0.01	1,44±0.01	0,32±0.01							
DM145	6,51±0.01	0,18±0.01	0,08±0.01	5,07±0.02	1,62±0.02	0,30±0.01							
DM147	6,31±0.01	0,42±0.01	0,10±0.00	5,67±0.02	1,06±0.02	0,14±0.05							
DM149	6,22±0.03	0,49±0.03	0,12±0.00	5,86±0.01	0,85±0.01	0,12±0.01							
DM152	6,19±0.01	0,53±0.01	0,12±0.01	5,25±0.03	1,47±0.03	0,28±0.04							
DM158	6,25±0.01	0,49±0.01	0,10±0.02	5,45±0.04	1,29±0.04	0,28±0.03							
DM162	6,19±0.01	0,44±0.01	0,12±0.01	5,86±0.00	0,77±0.00	0,18±0.01							
DM167	6,33±0.02	0,44±0.02	0,10±0.01	5,44±0.02	1,33±0.02	0,12±0.01							
DM169	6,30±0.01	0,37±0.01	0,10±0.02	5,97±0.01	0,70±0.01	0,16±0.03							
DM172	6,35±0.01	0,38±0.01	0,08±0.00	6,17±0.04	0,56±0.04	0,16±0.01							
DM175	6,37±0.03	0,32±0.03	0,10±0.01	5,29±0.02	1,40±0.02	0,12±0.02							
DM177	6,29±0.02	0,47±0.02	0,10±0.01	5,91±0.01	0,85±0.01	0,20±0.02							
DM179	6,36±0.01	0,33±0.01	0,10±0.02	6,18±0.02	0,51±0.02	0,18±0.04							
DM182	6,24±0.02	0,34±0.02	0,10±0.01	5,52±0.02	1,06±0.02	0,28±0.01							
DM185	6,35±0.02	0,35±0.02	0,10±0.01	6,04±0.01	0,66±0.01	0,16±0.01							
DM193	6,62±0.01	0,23±0.01	0,08±0.01	5,17±0.03	1,68±0.03	0,24±0.05							
DM198	6,43±0.01	0,29±0.01	0,12±0.01	5,62±0.01	1,10±0.01	0,18±0.01							
DM213	6,23±0.04	0,40±0.04	0,12±0.02	5,49±0.01	1,14±0.01	0,14±0.01							
DM237	5,93±0.01	0,73±0.01	0,14±0.01	4,83±0.01	1,83±0.01	0,30±0.02	+	0,76±0,01	2,71±0,17	-	15.46±0.12	-	+

Enterococcus faecium													
DM006	6,70±0.01	0,15±0.01	0,10±0.03	5,53±0.01	1,32±0.01	0,16±0.02							
DM010	6,69±0.02	0,12±0.02	0,08±0.01	5,24±0.04	1,57±0.01	0,18±0.01							
DM014	6,16±0.02	0,48±0.02	0,08±0.01	4,97±0.01	1,67±0.01	0,26±0.03	++	0,31±0,10	2,39±0,08	-	39.44±0.78	-	+
DM015	6,20±0.01	0,58±0.01	0,10±0.01	4,62±0.01	2,16±0.01	0,14±0.01	++	0,23±0,02	1,41±0,03	-	21.14±0.18	-	-
DM017	6,21±0.01	0,27±0.01	0,10±0.00	5,03±0.02	1,45±0.02	0,18±0.01							
DM018	6,38±0.01	0,25±0.01	0,08±0.01	4,77±0.04	1,86±0.04	0,22±0.02	-	0,10±0,02	1,17±0,13	-	23.19±0.56	-	-
DM026	6,41±0.00	0,33±0.00	0,12±0.01	5,93±0.03	0,81±0.03	0,14±0.01							
DM027	6,24±0.03	0,28±0.03	0,12±0.01	5,78±0.01	0,74±0.01	0,14±0.01							
DM029	6,49±0.01	0,39±0.01	0,08±0.00	5,71±0.02	1,17±0.02	0,30±0.04							
DM031	6,68±0.02	0,18±0.02	0,08±0.01	5,23±0.01	1,63±0.01	0,16±0.01							
DM033	6,03±0.02	0,64±0.02	0,14±0.02	4,93±0.01	1,74±0.01	0,20±0.01	++	0,11±0,01	0,48±0,19	-	13.30±0.08	+	+
DM034	6,22±0.03	0,47±0.03	0,12±0.01	6,03±0.02	0,66±0.02	0,28±0.01							
DM036	6,15±0.03	0,30±0.03	0,12±0.02	5,31±0.02	1,09±0.02	0,12±0.02							
DM037	6,17±0.05	0,27±0.05	0,12±0.01	5,40±0.01	1,04±0.01	0,18±0.01							
DM039	6,14±0.01	0,30±0.01	0,12±0.01	4,94±0.02	1,50±0.02	0,18±0.01	+	0,15±0,07	2,59±0,03	-	26.27±0.45	+	+
DM042	6,41±0.04	0,46±0.04	0,08±0.01	5,83±0.01	1,04±0.01	0,18±0.03							
DM043	6,02±0.01	0,45±0.01	0,12±0.02	4,93±0.01	1,54±0.01	0,16±0.01	-	0,42±0,04	2,24±0,11	-	22.22±0.09	-	+
DM047	6,32±0.01	0,41±0.01	0,12±0.01	5,89±0.02	0,84±0.02	0,16±0.04							
DM057	6,27±0.01	0,41±0.01	0,12±0.01	5,55±0.01	1,13±0.01	0,10±0.01							
DM061	6,34±0.03	0,34±0.03	0,12±0.02	6,02±0.01	0,66±0.01	0,12±0.01							
DM062	6,32±0.02	0,41±0.02	0,12±0.01	5,06±0.03	1,67±0.03	0,12±0.01							

DM064	6,59±0.01	0,13±0.01	0,08±0.00	5,91±0.02	0,81±0.02	0,24±0.05							
DM065	6,34±0.02	0,40±0.02	0,12±0.01	5,59±0.01	1,15±0.01	0,14±0.01							
DM067	6,49±0.01	0,14±0.01	0,10±0.01	5,73±0.01	0,90±0.01	0,16±0.03							
DM068	6,21±0.02	0,47±0.02	0,12±0.02	5,47±0.02	1,21±0.02	0,14±0.02							
DM076	6,44±0.01	0,24±0.01	0,12±0.02	5,40±0.02	1,28±0.02	0,18±0.02							
DM082	6,28±0.01	0,36±0.01	0,12±0.01	5,85±0.01	0,79±0.01	0,14±0.01							
DM083	6,52±0.01	0,12±0.01	0,10±0.01	5,49±0.04	1,15±0.04	0,14±0.01							
DM086	6,45±0.05	0,22±0.05	0,12±0.03	5,36±0.02	1,31±0.02	0,14±0.03							
DM088	6,36±0.01	0,30±0.01	0,12±0.01	5,66±0.01	1,00±0.01	0,20±0.05							
DM089	6,38±0.02	0,38±0.02	0,12±0.01	5,66±0.01	1,10±0.01	0,16±0.01							
DM094	6,30±0.02	0,36±0.02	0,12±0.01	5,51±0.01	1,15±0.01	0,16±0.01							
DM095	6,31±0.01	0,37±0.01	0,12±0.02	5,73±0.01	0,95±0.01	0,18±0.02							
DM098	6,19±0.01	0,47±0.01	0,12±0.01	5,88±0.02	0,78±0.02	0,14±0.01							
DM099	6,39±0.03	0,32±0.03	0,10±0.01	5,78±0.01	0,93±0.01	0,12±0.01							
DM100	6,17±0.01	0,47±0.01	0,12±0.02	6,12±0.01	0,52±0.01	0,16±0.03							
DM103	6,31±0.01	0,34±0.01	0,12±0.01	5,58±0.05	1,07±0.05	0,12±0.01							
DM104	6,40±0.00	0,27±0.00	0,12±0.01	5,17±0.01	1,50±0.01	0,16±0.02							
DM105	6,41±0.01	0,28±0.01	0,12±0.01	5,93±0.02	0,76±0.02	0,18±0.02							
DM108	6,22±0.02	0,48±0.02	0,12±0.02	5,56±0.01	1,14±0.01	0,16±0.01							
DM109	6,43±0.01	0,29±0.01	0,12±0.01	5,62±0.01	1,10±0.01	0,18±0.01							
DM110	5,93±0.02	0,62±0.02	0,14±0.03	4,72±0.02	1,83±0.02	0,20±0.05	-	1,10±0,05	3,93±0,72	-	7.27±0.06	-	+
DM118	6,33±0.01	0,48±0.01	0,10±0.01	4,95±0.04	1,86±0.04	0,18±0.02	-	0,44±0,21	18,45±1,80	-	29.63±0.53	-	+

DM121	6,19±0.01	0,67±0.01	0,12±0.01	4,93±0.01	1,93±0.01	0,12±0.01	-	0,28±0,02	0,62±0,04	-	16.48±0.12	-	+
DM122	6,64±0.04	0,18±0.04	0,08±0.00	5,72±0.03	1,10±0.03	0,26±0.06							
DM125	6,07±0.02	0,42±0.02	0,12±0.01	4,95±0.01	1,54±0.01	0,12±0.01	+	0,54±0,11	6,35±0,67	-	8.15±0.09	+	-
DM127	6,21±0.01	0,18±0.01	0,12±0.02	5,27±0.02	1,12±0.02	0,12±0.03							
DM129	6,42±0.01	0,31±0.01	0,08±0.01	4,88±0.04	1,85±0.04	0,16±0.01	+	1,13±0,12	2,89±0,08	-	20.48±0.56	-	+
DM137	6,15±0.03	0,64±0.03	0,12±0.01	4,86±0.01	1,93±0.01	0,30±0.05	-	0,23±0,03	1,01±0,07	-	19.18±0.67	-	+
DM139	5,99±0.01	0,47±0.01	0,14±0.02	4,65±0.01	1,81±0.01	0,34±0.04	+	0,20±0,01	0,60±0,07	-	13.22±0.14	+	+
DM142	6,04±0.02	0,54±0.02	0,12±0.01	4,75±0.02	1,83±0.02	0,32±0.01	+	0,48±0,01	0,60±0,13	-	24.83±0.83	-	+
DM146	6,30±0.02	0,29±0.02	0,10±0.02	5,73±0.01	0,86±0.01	0,22±0.02							
DM159	6,19±0.01	0,53±0.01	0,12±0.01	4,73±0.06	1.99±0.06	0,16±0.01	++	0,73±0,06	7,80±0,19	-	20.28±0.41	-	+
DM161	6,50±0.01	0,29±0.01	0,08±0.00	5,44±0.01	1,35±0.01	0,30±0.01							
DM163	6,33±0.01	0,35±0.01	0,10±0.01	6,15±0.01	0,53±0.01	0,16±0.04							
DM164	6,20±0.01	0,47±0.01	0,12±0.01	5,79±0.01	0,88±0.01	0,12±0.01							
DM166	6,68±0.01	0,13±0.01	0,08±0.01	5,95±0.01	0,86±0.01	0,14±0.01							
DM170	6,27±0.02	0,40±0.02	0,10±0.02	6,21±0.03	0,46±0.03	0,16±0.02							
DM171	6,44±0.01	0,27±0.01	0,08±0.01	5,45±0.01	1,26±0.01	0,12±0.01							
DM176	6,27±0.05	0,43±0.05	0,10±0.01	5,26±0.01	1,44±0.01	0,20±0.03							
DM178	6,22±0.01	0,47±0.01	0,10±0.01	5,58±0.02	1,11±0.02	0,16±0.01							
DM183	6,29±0.03	0,35±0.03	0,08±0.01	5,59±0.01	1,05±0.01	0,16±0.02							
DM184	6,44±0.02	0,27±0.02	0,08±0.01	5,93±0.02	0,78±0.02	0,16±0.01							
DM187	6,38±0.01	0,30±0.01	0,10±0.02	5,52±0.01	1,16±0.01	0,22±0.06							
DM191	6,44±0.00	0,24±0.00	0,10±0.01	6,28±0.05	0,40±0.05	0,12±0.01							

DM196	5,99±0.01	0,73±0.01	0,14±0.03	4,78±0.01	1,94±0.01	0,26±0.01	+	0,87±0,04	1,66±0,02	-	13.24±0.11	+	-
DM197	6,06±0.02	0,51±0.02	0,14±0.02	4,72±0.03	1,85±0.03	0,28±0.05	+	0,12±0,01	0,24±0,04	-	15.92±0.26	-	+
DM200	5,98±0.04	0,59±0.04	0,14±0.01	4,67±0.01	1,90±0.01	0,30±0.01	-	0,13±0,03	0,33±0,07	-	11.02±0.06	-	+
DM201	6,00±0.01	0,57±0.01	0,14±0.01	4,72±0.01	1,85±0.01	0,28±0.02	+	0,22±0,05	1,38±0,14	-	50.84±1.15	-	+
DM202	5,95±0.01	0,59±0.01	0,16±0.03	4,85±0.05	1,69±0.05	0,28±0.09	-	1,01±0,07	7,26±0,20	-	14.71±0.05	-	+
DM204	6,01±0.02	0,56±0.02	0,14±0.01	4,58±0.01	1,99±0.01	0,28±0.01	+	0,74±0,13	1,50±0,02	-	18.75±0.09	-	+
DM205	6,01±0.01	0,69±0.01	0,14±0.01	4,56±0.04	2,14±0.04	0,32±0.03	+	0,09±0,02	1,03±0,01	-	12.57±0.45	+	+
DM207	6,41±0.01	0,18±0.01	0,12±0.01	5,45±0.01	1,14±0.01	0,18±0.01							
DM209	6,17±0.03	0,40±0.03	0,12±0.01	5,64±0.01	0,93±0.01	0,14±0.02							
DM210	5,87±0.01	0,81±0.01	0,16±0.02	4,93±0.01	1,75±0.01	0,24±0.03	+	0,43±0,19	3,41±0,11	-	8.22±0.18	-	+
DM211	6,21±0.02	0,22±0.02	0,10±0.01	5,38±0.01	1,05±0.01	0,16±0.03							
DM215	6,02±0.01	0,62±0.01	0,14±0.02	4,87±0.02	1,77±0.02	0,32±0.05	+	0,87±0,04	1,19±0,14	-	14.51±0.21	+	+
DM216	6,07±0.01	0,60±0.01	0,14±0.02	4,64±0.02	2,03±0.02	0,30±0.04	++	1,14±0,09	1,90±0,17	-	18.12±0.45	-	+
DM219	6,01±0.03	0,69±0.03	0,14±0.01	4,75±0.01	1,95±0.01	0,28±0.01	+	0,14±0,05	0,21±0,01	-	39.12±0.83	-	+
DM221	5,94±0.01	0,75±0.01	0,14±0.01	4,95±0.02	1,74±0.02	0,26±0.02	+	0,07±0,04	1,26±0,06	-	7.47±0.12	-	+
DM222	6,10±0.01	0,57±0.01	0,14±0.03	4,91±0.01	1,76±0.01	0,28±0.03	++	0,31±0,06	3,24±0,16	-	22.01±0.74	+	+
DM223	6,02±0.02	0,63±0.02	0,14±0.01	4,84±0.01	1,81±0.01	0,32±0.06	++	0,08±0,01	15,23±1,16	-	12.35±0.18	+	+
DM224	6,50±0.01	0,03±0.01	0,10±0.01	4,66±0.01	1,87±0.01	0,30±0.02	+	1,71±0,02	5,15±0,37	-	22.10±0.63	+	+
DM225	6,48±0.02	0,23±0.02	0,10±0.01	4,73±0.02	1,98±0.02	0,28±0.02	+	0,10±0,02	1,99±0,17	-	16.57±0.11	-	+
DM227	6,32±0.01	0,26±0.01	0,12±0.02	5,95±0.04	0,63±0.04	0,14±0.02							
DM229	6,28±0.01	0,39±0.01	0,12±0.01	5,03±0.01	1,64±0.01	0,18±0.01							
DM230	6,23±0.01	0,34±0.01	0,12±0.01	5,10±0.02	1,47±0.02	0,24±0.02							

DM232	6,04±0.02	0,58±0.02	0,14±0.02	4,92±0.01	1,70±0.01	0,28±0.02	+	1,73±0,05	3,14±0,29	-	23.89±0.29	-	+
DM233	6,09±0.02	0,56±0.02	0,14±0.04	4,93±0.01	1,72±0.01	0,28±0.03	+	0,15±0,03	0,81±0,11	-	9.51±0.18	+	+
DM234	6,11±0.01	0,34±0.01	0,12±0.01	4,80±0.02	1,65±0.02	0,16±0.01	++	0,16±0,03	1,51±0,02	-	4.40±0.09	+	+
DM235	6,32±0.04	0,26±0.04	0,12±0.01	5,27±0.02	1,31±0.02	0,22±0.02							
DM238	6,44±0.01	0,25±0.01	0,12±0.01	5,01±0.02	1,68±0.02	0,26±0.04							
DM239	6,27±0.03	0,39±0.03	0,12±0.02	5,06±0.04	1,60±0.04	0,26±0.03							
DM241	6,20±0.01	0,47±0.01	0,12±0.01	5,08±0.02	1,59±0.02	0,26±0.02							
DM242	6,43±0.01	0,29±0.01	0,12±0.01	5,06±0.01	1,66±0.01	0,24±0.01							
DM243	6,17±0.01	0,41±0.01	0,12±0.01	4,75±0.02	1,83±0.02	0,24±0.02	+	0,10±0,05	0,84±0,03	-	18.36±0.29	-	+
DM244	6,03±0.01	0,54±0.01	0,14±0.02	4,96±0.02	1,61±0.02	0,30±0.05	+	0,45±0,02	1,01±0,04	-	17.75±0.41	-	+
DM252	6,20±0.01	0,38±0.01	0,10±0.02	4,66±0.01	1,92±0.01	0,28±0.02	-	0,60±0,05	0,93±0,02	-	22.83±0.32	-	+
DM254	6,44±0.01	0,13±0.01	0,10±0.01	4,54±0.01	2,03±0.01	0,30±0.02	-	0,35±0,04	0,76±0,02	-	13.25±0.18	-	-
DM256	6,07±0.01	0,51±0.01	0,14±0.04	4,71±0.03	1,87±0.03	0,28±0.01	+	0,30±0,05	0,55±0,04	-	40.04±1.02	-	+
DM258	5,84±0.01	0,69±0.01	0,14±0.01	4,79±0.01	1,74±0.01	0,26±0.01	-	0,15±0,01	1,03±0,03	-	21.51±0.19	-	+
DM260	6,50±0.02	0,05±0.02	0,12±0.01	4,80±0.01	1,75±0.01	0,26±0.04	+	0,34±0,02	2,00±0,24	-	25.23±0.41	-	-
DM262	5,72±0.04	0,87±0.04	0,16±0.02	4,50±0.02	2,09±0.02	0,30±0.06	+++	0,15±0,05	1,78±0,07	-	38.95±0.84	-	-
DM263	6,25±0.01	0,29±0.01	0,12±0.01	4,90±0.01	1,64±0.01	0,22±0.03	-	0,98±0,10	1,12±0,02	-	18.02±0.56	-	+
DM264	6,07±0.02	0,46±0.02	0,12±0.02	4,84±0.04	1,69±0.04	0,28±0.04	+	0,71±0,04	1,46±0,16	-	24.99±0.15	+	+
DM265	6,17±0.01	0,41±0.01	0,12±0.01	4,86±0.01	1,72±0.01	0,24±0.02	-	0,31±0,03	0,58±0,02	-	15.74±0.09	-	+
DM266	6,07±0.01	0,46±0.01	0,12±0.01	4,64±0.02	1,89±0.02	0,30±0.03	-	0,13±0,04	0,37±0,02	-	33.63±0.58	-	+
DM267	5,81±0.03	0,78±0.03	0,14±0.03	4,51±0.02	2,08±0.02	0,32±0.03	-	0,20±0,01	0,50±0,04	-	31.75±0.63	-	-
DM268	6,51±0.01	0,02±0.01	0,10±0.01	4,76±0.03	1,77±0.03	0,26±0.02	+	0,60±0,05	1,22±0,03	-	20.96±0.34	-	-

DM270	6,50±0.02	0,03±0.02	0,12±0.02	4,65±0.01	1,88±0.01	0,32±0.05	+	0,44±0,01	0,92±0,01	-	24.63±0.27	-	+
Enterococcus gallinarum													
DM003	6,22±0.02	0,50±0.02	0,12±0.01	5,96±0.01	0,76±0.01	0,12±0.02							
DM150	6,38±0.01	0,23±0.01	0,08±0.00	4,93±0.04	1,68±0.04	0,14±0.02	+	0,19±0,02	7,90±0,34	-	22.84±0.24	+	+
DM151	6,22±0.01	0,44±0.01	0,10±0.02	4,85±0.01	1,81±0.01	0,28±0.04	+	0,34±0,10	0,74±0,03	-	26.96±0.49	+	-
DM157	6,21±0.03	0,51±0.03	0,10±0.01	4,93±0.01	1,79±0.01	0,16±0.03	+	0,93±0,05	2,50±0,19	-	45.30±1.65	+	-
Enterococcus hirae													
DM013	6,27±0.02	0,22±0.02	0,12±0.01	5,68±0.04	0,81±0.04	0,14±0.02							
DM041	6,24±0.01	0,27±0.01	0,10±0.01	5,34±0.02	1,17±0.02	0,12±0.01							
DM071	6,34±0.02	0,31±0.02	0,12±0.02	5,44±0.03	1,21±0.03	0,14±0.01							
DM091	6,19±0.02	0,71±0.02	0,12±0.01	4,79±0.01	2,11±0.01	0,16±0.03	+	0,16±0,02	0,22±0,04	-	59.06±1.22	+	+
DM111	6,12±0.04	0,46±0.04	0,14±0.03	4,76±0.02	1,82±0.02	0,26±0.02	+	0,70±0,08	2,28±0,27	-	19.10±0.74	-	-
DM154	6,24±0.02	0,34±0.02	0,12±0.01	4,94±0.02	1,64±0.02	0,18±0.01	+	0,55±0,01	0,63±0,11	-	5.10±0.09	+	-
DM208	5,88±0.01	0,66±0.01	0,12±0.01	4,76±0.01	1,78±0.01	0,26±0.04	-	0,54±0,07	2,60±0,09	-	10.17±0.41	+	+
Enterococcus mundii													
DM188	6,25±0.01	0,33±0.01	0,10±0.01	4,86±0.02	1,72±0.02	0,18±0.03	+	0,42±0,04	1,23±0,12	-	7.94±0.07	+	+
DM246	5,59±0.02	0,99±0.02	0,14±0.02	4,97±0.03	1,61±0.03	0,12±0.01	++	0,61±0,03	0,70±0,04	-	15.13±0.16	-	-
Enterococcus thailanticus/lactis													
DM005	6,32±0.01	0,40±0.01	0,08±0.00	5,63±0.01	1,09±0.01	0,14±0.02							
DM012	6,35±0.01	0,29±0.01	0,10±0.01	5,60±0.02	1,04±0.02	0,14±0.01							
DM019	6,38±0.03	0,16±0.03	0,10±0.01	5,86±0.02	0,68±0.02	0,32±0.04							
DM028	6,12±0.01	0,54±0.01	0,12±0.02	4,88±0.01	1,78±0.01	0,18±0.01	+	0,63±0,03	2,97±0,06	-	19.12±0.83	-	+

DM074	6,22±0.02	0,45±0.02	0,12±0.01	5,49±0.01	1,18±0.01	0,12±0.02							
DM084	6,35±0.02	0,31±0.02	0,12±0.01	5,56±0.01	1,10±0.01	0,18±0.03							
DM136	6,69±0.03	0,17±0.03	0,08±0.00	4,70±0.01	2,16±0.01	0,24±0.03	++	1,38±0,11	1,83±0,46	-	46.44±1.24	+	+
DM138	6,03±0.01	0,44±0.01	0,12±0.01	4,60±0.02	1,87±0.02	0,28±0.06	++	0,13±0,02	0,15±0,03	-	8.43±0.77	-	-
DM144	6,39±0.01	0,50±0.01	0,10±0.01	4,70±0.01	2,19±0.01	0,18±0.01	++	0,87±0,10	1,28±0,08	-	3.77±0.15	-	+
DM181	6,22±0.02	0,36±0.02	0,10±0.02	4,97±0.02	1,61±0.02	0,16±0.02	-	0,07±0,01	0,33±0,02	-	27.49±0.67	-	+
DM189	6,29±0.01	0,32±0.01	0,10±0.01	4,82±0.02	1,79±0.02	0,28±0.07	++	0,13±0,02	1,13±0,11	-	46.20±0.98	+	+
DM192	6,35±0.02	0,35±0.02	0,10±0.01	4,96±0.02	1,74±0.02	0,12±0.01	+	0,76±0,02	1,30±0,23	-	20.26±0.14	-	+
DM194	6,05±0.02	0,62±0.02	0,14±0.03	4,82±0.05	1,85±0.05	0,28±0.05	+	0,13±0,01	0,36±0,01	-	21.21±0.22	+	+
DM217	5,88±0.01	0,76±0.01	0,16±0.02	4,75±0.03	1,89±0.03	0,34±0.02	++	0,36±0,09	0,78±0,03	-	20.44±0.46	+	-
DM228	6,07±0.01	0,56±0.01	0,14±0.01	4,91±0.01	1,72±0.01	0,26±0.02	+	0,80±0,09	1,82±0,09	-	16.85±0.11	-	-

APPENDIX V

Safety profile of LAB isolates

Isolate	Virulence genes							Antibiotic resistant genes		Biogenic amines genes				Antibiotics										Biogenic Amines		
	gelE	*hyl*	*cylA*	*asa1*	*esp*	*efaA*	*ace*	*vanA*	*vanB*	*hdc*	*hdc2*	*tdc*	*odc*	E	C	S	TET	RF	P	VAN	GEN	AMP	STX	His	Tyr	Orn
DM014	-	-	-	-	-	-	-	-	-	-	-	+	-	R	S	R	R	R	R	S	R	S	R	-	+	-
DM015	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	I	I	S	S	R	S	S	-	-	-
DM018	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	R	I	I	S	S	R	S	S	-	-	-
DM028	-	-	-	-	-	-	-	-	-	-	-	+	-	R	S	R	R	I	R	S	R	S	S	-	+	-
DM033	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	R	I	S	S	R	S	S	-	-	-
DM039	-	-	-	-	-	-	-	-	-	-	-	+	-	I	S	R	R	R	R	S	R	S	S	-	+	-
DM043	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	I	S	R	S	R	S	S	-	-	-
DM091	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	I	I	S	S	R	S	S	-	+	-
DM110	-	-	-	-	-	-	-	-	-	-	-	+	-	I	S	R	R	I	S	S	R	S	I	-	+	-
DM111	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	R	S	S	S	R	R	S	S	-	+	-
DM118	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	I	I	S	S	R	S	I	-	-	-
DM121	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	S	S	S	R	S	S	-	-	-
DM125	-	-	-	-	-	-	+	-	-	-	-	-	-	R	S	R	I	I	S	S	R	S	R	-	+	-
DM129	-	-	+	+	-	-	-	-	-	-	-	-	-	I	S	R	R	I	R	S	S	S	I	-	-	-

DM136	-	-	-	-	-	-	-	-	-	-	-	+	-	R	S	R	R	R	R	S	R	S	R	-	+	-
DM137	+	-	+	+	-	-	-	-	-	-	-	+	-	I	S	R	I	I	S	S	R	S	I	-	+	-
DM138	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	R	R	R	S	R	S	I	-	+	-
DM139	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	S	R	S	R	S	R	-	+	-
DM141	-	-	-	-	-	-	-	-	-	-	-	+	-	R	S	R	R	R	R	S	R	S	I	-	+	-
DM142	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	R	R	S	R	S	I	-	-	-
DM144	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	R	R	R	R	S	R	S	S	-	+	-
DM150	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	I	I	S	S	R	S	S	-	-	-
DM151	+	-	+	+	-	-	-	-	-	-	-	-	-	R	S	R	S	I	S	R	R	S	S	-	+	-
DM154	+	-	+	+	-	-	-	-	-	-	-	-	-	S	S	R	S	I	S	R	R	S	S	-	+	-
DM157	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	S	R	S	S	R	S	I	-	+	-
DM159	-	-	+	+	-	-	-	-	-	-	-	+	-	R	S	R	R	R	R	S	R	S	I	-	+	-
DM181	-	-	+	+	-	-	-	-	-	-	-	+	-	I	S	R	I	I	S	S	R	S	I	-	+	-
DM188	-	-	-	-	-	-	-	-	-	-	-	+	-	R	S	R	R	R	R	S	R	S	I	-	+	-
DM189	-	-	+	+	-	-	-	-	-	-	-	-	-	R	S	R	R	I	R	S	R	S	S	-	+	-
DM192	-	-	+	+	-	-	-	-	-	-	-	+	-	I	S	R	R	R	R	S	R	S	I	-	+	-
DM194	-	-	+	+	-	-	-	-	-	-	-	+	-	R	S	R	R	R	R	S	R	S	R	-	+	-
DM196	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	I	R	S	R	S	I	-	+	-
DM197	-	-	+	+	-	-	-	-	-	-	-	+	-	I	S	R	R	I	S	S	R	S	I	-	+	-
DM200	-	-	+	+	-	-	-	-	-	-	-	-	-	I	S	R	R	I	R	S	R	S	I	-	+	-
DM201	-	-	+	+	-	-	-	-	-	-	-	+	-	I	S	R	R	R	R	S	R	S	I	-	+	-

DM202	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	I	I	R	S	R	S	S	-	+	-
DM203	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	R	R	R	S	R	S	I	-	+	-
DM204	-	-	-	-	-	-	-	-	-	-	-	+	-	I	S	R	I	I	S	S	R	S	I	-	+	-
DM205	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	R	R	S	R	S	I	-	+	-
DM208	-	+	-	-	+	-	-	-	-	-	-		-	I	S	R	S	S	S	S	R	S	I	-	+	-
DM210	-	-	+	+	-	-	-	-	-	-	-	+	-	I	S	R	R	I	R	S	R	S	I	-	+	-
DM215	-	-	-	-	-	-	-	-	-	-	-	+	-	R	S	R	R	R	R	S	R	S	I	-	+	-
DM216	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	S	R	R	S	R	S	S	-	+	-
DM217	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	R	R	S	R	S	I	-	+	-
DM219	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	R	R	S	R	S	I	-	+	-
DM221	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	I	I	S	S	R	S	I	-	+	-
DM222	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	S	R	S	R	S	I	-	+	-
DM223	-	-	-	-	-	-	-	-	-	-	-	+	-	R	S	R	R	R	R	S	R	S	S	-	+	-
DM224	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	I	I	S	S	R	S	I	-	-	-
DM225	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	I	I	S	S	R	S	I	-	-	-
DM228	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	R	R	S	R	S	S	-	+	-
DM232	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	S	I	R	S	R	S	I	-	+	-
DM233	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	R	R	S	R	S	I	-	-	-
DM234	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	S	R	R	S	R	S	S	-	-	-
DM237	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	S	R	S	S	R	S	I	-	-	-
DM243	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	I	I	S	S	R	S	S	-	+	-

DM244	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	I	R	R	S	R	S	S	-	+	-
DM246	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	R	I	I	S	S	R	S	S	-	-	-
DM252	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	I	I	S	S	R	S	S	-	+	-
DM253	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	I	R	R	S	R	S	S	-	+	-
DM254	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	I	R	S	R	S	I	-	-	-
DM256	-	-	+	+	-	-	-	-	-	-	-	+	-	I	S	R	R	R	R	S	R	S	S	-	+	-
DM258	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	R	R	S	S	R	S	I	-	-	-
DM260	-	-	-	-	-	-	-	-	-	-	-	+	-	R	S	R	I	R	R	S	R	S	I	-	+	-
DM262	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	R	I	S	S	R	S	S	-	+	-
DM263	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	R	R	R	S	S	R	S	I	-	+	-
DM264	-	-	-	+	-	-	-	-	-	-	-	+	-	I	S	R	I	I	S	S	R	S	I	-	+	-
DM265	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	I	I	R	S	R	S	I	-	+	-
DM266	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	R	I	S	S	R	S	S	-	+	-
DM267	-	-	-	-	-	-	-	-	-	-	-	-	-	R	S	R	R	R	S	S	R	S	I	-	+	-
DM268	-	-	-	-	-	-	-	-	-	-	-	+	-	R	S	R	I	I	S	S	R	S	I	-	+	-
DM270	-	-	-	-	-	-	-	-	-	-	-	-	-	I	S	R	I	I	S	S	R	S	S	-	-	-

-: negative, +: positive, R: Resistant, I: Intermediate, S: Sensitive

APPENDIX VI

Peptides identified in raw and fermented donkey milk samples

Sample 18			
Peptides from beta-casein (parent protein, accession number: D2EC27_EQUAS)			
Peptide	**Mass**	**Length**	**Fragment**
AEPVPY	674.3275	6	85 – 90
APFPQPV	754.4014	7	192 – 198
APFPQPVVP	950.5225	9	192 – 200
APFPQPVVPY	1113.5858	10	192 – 201
APFPQPVVPYP	1210.6385	11	192 – 202
APFPQPVVPYPQ	1338.6971	12	192 – 203
AQPPIV	623.3643	6	101 – 106
AQPPIVP	720.417	7	101 – 107
AQPPIVPF	867.4854	8	101 – 108
AVVPQ	512.2958	5	91 - 95
DTPVQA	629.3021	6	205 – 210
EVEHQ	640.2816	5	64 – 68
FKHEGQ	744.3555	6	55 – 60
FKHEGQQ	872.4141	7	55 – 61
FVQPQPV	813.4385	7	74 – 81
GEFDPATQPI	1073.5029	10	223 – 232
GEFDPATQPIVPV	1368.6925	13	223 – 235
GLTGEFDPA	905.413	9	220 – 228
HQVPQ	607.3078	5	166 – 170
HQVPQS	694.3398	6	166 – 171
ILNPT	556.322	5	145 – 149
KETLLPK	827.5116	7	120 – 126
KSPIVPF	786.4639	7	134 – 140
KVAPFPQPV	981.5647	9	190 – 198
KVAPFPQPVVP	1177.6859	11	190 – 200
KVAPFPQPVVPY	1340.7491	12	190 – 201
LAQPPI	637.3799	6	100 – 105
LAQPPIVPF	980.5695	9	100 – 108
LIQPF	616.3584	5	160 – 164

LKSPIVP	752.4796	7	133 – 139
LKSPIVPF	899.548	8	133 – 140
LMLPSQPV	883.4837	8	176 – 183
LRLPV	596.4009	5	154 – 158
LYQDPQ	762.3548	6	213 – 218
LYQDPQLGLT	1146.592	10	213 – 222
MHQVPQ	738.3483	6	165 – 170
MLPSQPV	770.3997	7	177 – 183
PIVPF	571.337	5	104 – 108
PLAQPPIVPF	1077.6222	10	99 – 108
PQPVVPYPQ	1023.5389	9	195 – 203
PVLSPPQS	823.444	8	182 – 189
REKEEL	802.4185	6	16 – 21
SPIVPF	658.369	6	135 – 140
TLLPK	570.3741	5	122 – 126
TQPIVPV	752.4432	7	229 – 235
VAPFPQ	657.3486	6	191 – 196
VAPFPQPV	853.4697	8	191 – 198
VAPFPQPVVP	1049.5909	10	191 – 200
VAPFPQPVVPY	1212.6543	11	191 – 201
VAPFPQPVVPYP	1309.707	12	191 – 202
VAPFPQPVVPYPQ	1437.7656	13	191 – 203
YQDPQLGLT	1033.5081	9	214 – 222
Peptides from alpha-s1 casein (parent protein, accession number: CASA1_EQUAS)			
ALHTP	537.2911	5	32 – 36
HPEIIQ	735.3915	6	8 – 13
IEDPEQQ	857.3766	7	64 – 70
IIQNEQDSpR	1181.5078	9	11 – 19
LHTPR	622.3551	5	33 – 37
LIEDPEQQ	970.4607	8	63 – 70
RHPEIIQ	891.4926	7	7 – 13
SPFHDT	702.2973	6	179 – 184
SPFHDTA	773.3344	7	179 – 185
TDIIPE	686.3486	6	196 – 201
YLEPF	667.3217	5	148 – 152

YQHTLE	789.3657	6	98 – 103
Peptides from alpha-s2 casein (parent protein, accession number: CASA2_EQUAS)			
ELTEEEKN	990.4506	8	178 – 185
EQLFTSpEEIPK	1399.6272	11	152 – 162
FTSEEIPK	949.4756	8	155 – 162
FTSpEEIPK	1029.4419	8	155 – 162
IVLTPW	727.4269	6	130 – 135
KFPTE	620.317	5	70 – 74
KTVDM	592.2891	5	163 – 167
KTVDMESpTEV	1217.4886	10	163 – 172
LFTSpEEIPK	1142.526	9	154 – 162
LRQPR	668.4081	5	125 – 129
TELTEEEK	977.4553	8	177 – 184
TELTEEEKN	1091.4982	9	177 – 185
TSEEIPK	802.4072	7	156 – 162
alpha-s2 casein B (parent protein, accession number: C1L3G3_EQUAS)			
IELSDEEK	961.4604	8	101 – 108
IELSpDEEK	1041.4268	8	101 – 108
IELSpDEEKN	1155.4696	9	101 – 109
IELSDEEKN	1075.5033	9	101 – 109
YKQDNNVA	950.4457	8	36 – 43
KQDNNVA	787.3824	7	37 – 43
KTVDMGSpTEIFPEE	1661.6896	14	87 – 100
KTVDMGSpTEIFPEEI	1774.7736	15	87 – 101
KTVDMGSpTEIFPEEIEL	2016.9003	17	87 – 103
KTVDM	592.2891	5	87 – 91
KTVDMGSpTEI	1159.4832	10	87 – 96
TVDMGSpTEIFPEE	1533.5946	13	88 – 100
TVDMGSpTEIFPEEI	1646.6786	14	88 – 101
MGSTpEIFPEE	1218.4515	10	91 – 100
Peptides from beta-lactoglobulin I (parent protein, accession number: LACB1_HORSE)			
ALQPLPG	694.4014	7	157 – 163
DEEIME	764.2898	6	147 – 152
DEEIMEK	892.3848	7	147 – 153
DLQEV	602.2911	5	29 – 33

DSESAPL	717.3181	7	51 – 57
EKTESPAE	889.4028	8	92 – 99
KTESPAE	760.3603	7	93 – 99
LLDSESAPL	943.4862	9	49 – 57
LRPTPE	711.3915	6	64 – 69
LRPTPEDN	940.4614	8	64 – 71
LRPTPEDNL	1053.5454	9	64 – 72
LRPTPEDNLE	1182.588	10	64 – 73
LRPTPEDNLEI	1295.6721	11	64 – 74
LRPTPEDNLEII	1408.7561	12	64 – 75
LRPTPEDNLEIIL	1521.8402	13	64 - 76
MVDEEIMEK	1122.4937	9	145 – 153
NAATPGQ	657.3082	7	127 – 133
NAATPGQS	744.3402	8	127 – 134
RPTPEDN	827.3773	7	65 – 71
RPTPEDNLE	1069.5039	9	65 - 73
TNIPQ	571.2966	5	19 – 23
TNIPQT	672.3442	6	19 – 24
VDEEIMEK	991.4532	8	146 – 153
Peptides from beta-lactoglobulin II (parent protein, accession number: D6QX32_EQUAS)			
AEHGTV	612.2867	6	132 – 137
ALQPLPG	694.4014	7	158 – 164
DLQEV	602.2911	5	29 – 33
DSESAPL	717.3181	7	51 – 57
IIQDPSGGQE	1042.493	10	168 – 177
LLDSESAPL	943.4862	9	49 – 57
LRPTPE	711.3915	6	64 – 69
QDPSGGQE	816.325	8	170 – 177
RPTPEGN	769.3718	7	65 – 71
VDEEVMEK	977.4376	8	147 – 154
VEELRPT	842.4498	7	61 – 67
VEELRPTPE	1068.545	9	61 - 69
VEELRPTPEGN	1239.6095	11	61 - 71
YVEELRPTPEGN	1402.6729	12	60 – 71
YVEELRPTPEGNLE	1644.7994	14	60 – 73

Peptides from alpha-lactalbumin (parent protein, accession number: LALBA_EQUAS)			
DDDLTDDV	906.3454	8	82 – 89
DDDLTDDVM	1037.3859	9	82 – 90
DNQILPS	785.3919	7	63 – 69
FLDDDLTDDV	1166.4979	10	80 – 89
FQINN	634.3075	5	53 – 57
ILDSEGID	860.4127	8	95 – 102
KFLDDDLTDDV	1294.5928	11	79 – 89
KGVTLPE	742.4225	7	19 – 25
KGVTLPEW	928.5018	8	19 – 26
KGVTLPEWI	1041.5858	9	19 – 27
KILDSEG	760.3967	7	94 – 100
KILDSEGI	873.4807	8	94 – 101
KILDSEGID	988.5076	9	94 – 102
LAHKPL	677.4224	6	105 – 110
LDDDLTDDV	1019.4294	9	81 – 89
Peptides from k-casein (parent protein, accession number: F0V6V5_EQUAS)			
EVQNQEQPT	1071.4833	9	21 – 29
NNQHMP	739.3072	6	72 – 77
TVIPK	556.3584	5	129 – 133
YARPA	576.3019	5	81 – 85
Peptides from lysozyme C (parent protein, accession number: LYSC_EQUAS)			
DKDLSE	705.3181	6	117 – 122
KAQEM	605.2843	5	13 – 17
LLDDNIDDDI	1159.5244	10	83 – 92
SLANWV	688.3544	6	24 – 29

Sample 33			
Peptides from beta-casein (parent protein, accession number: D2EC27_EQUAS)			
Peptide	**Mass**	**Length**	**Fragment**
GLTGEFDPATQPIVPV	1639.846	16	220-235
LMLPSQPVLSPPQS	1492.796	14	176-189
VAPFPQPVVPY	1212.654	11	191-201
GEFDPATQPIVPV	1368.693	13	223-235

VAPFPQPVVPYPQ	1437.766	13	191-203
QILNPTNGEN	1098.531	10	144-153
LYQDPQLGLT	1146.592	10	213-222
MLPSQPVLSPPQS	1379.712	13	177-189
VAPFPQPVVPYP	1309.707	12	191-202
LMLPSQPVLSPPQ	1405.764	13	176-188
AQPPIVPF	867.4854	8	101-108
YQDPQLGLT	1033.508	9	214-222
TGEFDPATQPIVPV	1469.74	14	222-235
PFMHQVPQ	982.4695	8	163-170
VAPFPQPVVP	1049.591	10	191-200
FKHEGQQQ	1000.473	8	55-62
LMLPSQPV	883.4837	8	176-183
APFPQPVVPYPQ	1338.697	12	192-203
RQILNPTNGEN	1254.632	11	143-153
EVEHQDK	883.4036	7	64-70
VAPFPQPV	853.4697	8	191-198
LPSQPVLSPPQS	1248.671	12	178-189
FVQPQPV	813.4385	7	74-80
FKHEGQQ	872.4141	7	55-61
TLMLPSQPV	984.5314	9	175-183
LYQDPQLG	932.4603	8	213-220
PSQPVLSPPQS	1135.587	11	179-189
LKSPIVPF	899.548	8	133-140
AEPVPY	674.3275	6	85-90
LPSQPVLSPPQ	1161.639	11	178-188
KSPIVPF	786.4639	7	134-140
GEFDPATQPI	1073.503	10	223-232
PLAQPPIVPF	1077.622	10	99-108
PLAQPPIVP	930.5538	9	99-107
MHQVPQS	825.3803	7	165-171
ILNPTNGEN	970.472	9	145-153
LYQDPQLGL	1045.544	9	213-221
KETLLPK	827.5116	7	120-126
FKHEGQ	744.3555	6	55-60

AQPPIVP	720.417	7	101-107
PVHLIQPF	949.5385	8	157-164
QDPQLGLT	870.4447	8	215-222
LNVS$_P$S$_P$ETVES	1223.436	10	21-30
LLYQDPQ	875.4388	7	212-218
SITHIN	683.3602	6	43-48
PVHLIQP	802.4701	7	157-163
RQILNPT	840.4818	7	143-149
MLPSQPV	770.3997	7	177-183
LYQDPQ	762.3548	6	213-218
SPIVPF	658.369	6	135-140
RDTPVQA	785.4031	7	204-210
TLLPK	570.3741	5	122-126
ITHIN	596.3282	5	44-48
REKEEL	802.4185	6	16-21
TQPIVPV	752.4432	7	229-235
LPVHL	577.3588	5	156-160
HNPVIV	677.386	6	236-241
HQVPQS	694.3398	6	166-171
EVEHQD	755.3086	6	64-69
LTGEFDPATQPIVPV	1582.824	15	221-235
LIQPF	616.3584	5	160-164
LQPEIM	729.3731	6	109-114
HQVPQ	607.3078	5	166-170
PLAQPPI	734.4326	7	99-105
DTPVQA	629.3021	6	205-210
LGLTGEFDPA	1018.497	10	219-228
EVEHQ	640.2816	5	64-68
LRLPV	596.4009	5	154-158
VQPQPV	666.3701	6	75-80
YQDPQLG	819.3763	7	214-220
AVVPQ	512.2958	5	91-95
LPLAQPPIVPF	1190.706	11	98-108
APFPQPVVPY	1113.586	10	192-201
LRLPVHL	846.5439	7	154-160

RLPVH	620.3758	5	155-159
RLPVHL	733.4598	6	155-160
ETLLPK	699.4167	6	121-126
PIVPF	571.337	5	104-108
Peptides from alpha-s1 casein (parent protein, accession number: CASA1_EQUAS)			
LIEDPEQQ	970.4607	8	63-70
HPEIIQNEQDSpR	1544.662	12	8-19
IIQNEQDSpR	1181.5078	9	11-19
YLEPFQPS	979.4651	8	148-155
NQLQLQAIYAQEQLIR	1928.048	16	112-127
LPHRHPEIIQ	1238.6884	10	4-13
IEDPEQQE	986.4193	8	64-71
LPHRHPE	884.4617	7	4-10
LPHRHPEIIQNEQDSpR	2047.9589	16	4-19
SPFHDT	702.2973	6	179-184
IASENSEK	876.4188	8	188-195
IEDPEQQ	857.3766	7	64-70
RVVNQEQ	871.4512	7	138-144
RVVNQE	743.3926	6	138-143
YLEPF	667.3217	5	148-152
HPEIIQNE	978.4771	8	8-15
IQNEQDSpR	1068.4237	8	12-19
ALHTP	537.2911	5	32-36
VVNQEQ	715.35	6	139-144
LIEDPEQQE	1099.5033	9	63-71
IASENSE	748.3239	7	188-194
RVVNQEQA	942.4883	8	138-145
FYLEPF	814.3901	6	147-152
YINELN	764.3705	6	40-45
YAQEQ	637.2708	5	120-124
VVNQE	587.2915	5	139-143
YLEPFQPSYQ	1270.586	10	148-157

	9		
HPEIIQ	735.3915	6	8-13
LDVYP	605.306	5	158-162
FHPAQ	598.2863	5	167-171
Peptides from alpha-s2 casein A (parent protein, accession number: CASA2_EQUAS)			
KTVDMESpTEVV	1316.5571	11	163-173
FTSpEEIPK	1029.4419	8	155-162
EQLFTSpEEIPK	1399.6272	11	152-162
TELTEEEKN	1091.4982	9	177-185
FTSEEIPK	949.4756	8	155-162
KTVDMESpTEV	1217.4886	10	163-172
TELTEEEK	977.4553	8	177-184
LFTSpEEIPK	1142.526	9	154-162
TSEEIPK	802.4072	7	156-162
IVHQHQTT	962.4934	8	208-215
TSpEEIPK	882.3735	7	156-162
YVVIPTSpK	985.4885	8	41-48
IVLTPW	727.4269	6	130-135
AKFPTEV	790.4225	7	69-75
HQTTMDPQ	956.4022	8	212-219
IVHQHQTTMDPQ	1433.6721	12	208-219
TGASPFIPI	901.4909	9	140-148
IVHQHQ	760.398	6	208-213
LFTSEEIPK	1062.5597	9	154-162
SEEIPK	701.3596	6	157-162
KTVDM	592.2891	5	163-167
EVEEK	632.3017	5	96-100
MDPQSH	713.2803	6	216-221
YQIIPV	731.4218	6	227-232
TGASPF	578.27	6	140-145
IPIVN	554.3428	5	146-150

alpha-s2 casein B (parent protein, accession number: C1L3G3_EQUAS)			
KTVDMGSpTEIFPEE	1661.6896	14	87-100
KTVDMGSpTEIFPE	1532.647	13	87-99
IELSDEEKN	1075.5033	9	101-109
FQAVHPQQIPM	1294.6492	11	130-140
KTVDMGSpTEI	1159.4832	10	87-96
TVDMGSpTEIFPE	1404.552	12	88-99
IELSpDEEK	1041.4268	8	101-108
YKQDNNVA	950.4457	8	36-43
TVDMGSpTEIFPEE	1533.5946	13	88-100
QIPMSPWN	971.4535	8	137-144
IELSDEEK	961.4604	8	101-108
QIPMSPW	857.4105	7	137-143
QAVHPQQIPM	1147.5808	10	131-140
KQDNNVA	787.3824	7	37-43
QAVHPQ	678.3449	6	131-136
KTVDM	592.2891	5	87-91
VKINPK	697.4486	6	118-123
Peptides from beta-lactoglobulin I (parent protein, accession number: LACB1_HORSE)			
TQMVDEEIMEK	1351.6	11	143-153
NAATPGQSLV	956.4927	10	127-136
VDEEIMEK	991.4532	8	146-153
MVDEEIMEK	1122.4937	9	145-153
WHSVAMAASDISLLDSESAPL	2199.0518	21	37-57
KINYLDEDTV	1208.5924	10	101-110
LRPTPEDNL	1053.5454	9	64-72
LDLQEVAGK	971.5287	9	28-36
AEKTESPAE	960.4399	9	91-99

SLLDSESAPL	1030.5182	10	48-57
LRPTPEDNLE	1182.588	10	64-73
LRPTPEDN	940.4614	8	64-71
LRPTPEDNLEI	1295.6721	11	64-74
LRPTPEDNLEII	1408.7561	12	64-75
TNIPQTMQDLDLQEVAGK	1999.9884	18	19-36
NAATPGQSL	857.4243	9	127-135
ALDTDYKN	938.4345	8	112-119
DLQEVAGK	858.4446	8	29-36
WHSVAMAASDISLLDSESAPLRVYIEK	2987.5061	27	37-63
EKTESPAE	889.4028	8	92-99
LREGENK	844.4402	7	76-82
DLQEVAGKWHSVAMAASDISLLDSESAPL	3039.4858	29	29-57
DSESAPL	717.3181	7	51-57
LRPTPEDNLEIIL	1521.8402	13	64-76
TNIPQ	571.2966	5	19-23
VDEEIME	863.3582	7	146-152
TQMVDEEI	963.4219	8	143-150
VYIEK	650.3639	5	59-63
RPTPEDNLE	1069.5039	9	65-73
ALQPLPG	694.4014	7	157-163
LREGEN	716.3453	6	76-81
TQMVDEEIME	1223.505	10	143-152
DNLEII	715.3752	6	70-75
LDLQEV	715.3752	6	28-33
TNIPQT	672.3442	6	19-24
LDEDTV	690.3072	6	105-110
TNIPQTMQDLDLQEVAGKWHSVAMAASDISLLDSESAPL	4181.0298	39	19-57
ALQPLP	637.3799	6	157-162

Peptides from beta-lactoglobulin II (parent protein, accession number: D6QX32_EQUAS)			
VEELRPTPEGNLE	1481.7361	13	61-73
VEELRPTPEGN	1239.6095	11	61-71
YVEELRPTPEGN	1402.6729	12	60-71
WHSVAMVASDISLLDSESAPL	2227.083	21	37-57
YVEELRPTPE	1231.6084	10	60-69
VYVEELRPTPE	1330.6769	11	59-69
VEELRPTPEGNLEIILR	1977.0895	17	61-77
VEELRPTPE	1068.545	9	61-69
TQKVDEEVMEK	1334.6388	11	144-154
ALQPLPGHVQ	1058.5873	10	158-167
QDPSGGQE	816.325	8	170-177
SLLDSESAPL	1030.5182	10	48-57
ALQPLPGHVQI	1171.6713	11	158-168
ALQPLPGHV	930.5287	9	158-166
TQKVDEEV	946.4607	8	144-151
KTEDPAV	758.381	7	93-99
REGANHV	781.3831	7	77-83
TDIPQTMQDLDLQEVAGR	2028.9786	18	19-36
DSESAPL	717.3181	7	51-57
ALQPLPGHVQIIQDPSGGQE	2083.0698	20	158-177
AEHGTV	612.2867	6	132-137
KTEDPA	659.3126	6	93-98
ALQPLPG	694.4014	7	158-164
LDLQEV	715.3752	6	28-33
ALQPLP	637.3799	6	158-163
LRPTPEGNL	995.54	9	64-72

Peptides from alpha-lactalbumin (parent protein, accession number: LALBA_EQUAS)			
KILDSEGID	988.5076	9	94-102
LDDDLTDDV	1019.4294	9	81-89
FLDDDLTDDV	1166.4979	10	80-89
KGVTLPEWI	1041.5858	9	19-27
KILDSEGI	873.4807	8	94-101
GVTLPEWI	913.4909	8	20-27
KILDSEG	760.3967	7	94-100
FLDDDLTDDVM	1297.5383	11	80-90
ILDSEGID	860.4127	8	95-102
KGVTLPE	742.4225	7	19-25
LAHKPL	677.4224	6	105-110
AHKPL	564.3384	5	106-110
FHSSG	533.2234	5	31-35
FQINN	634.3075	5	53-57
Peptides from k-casein (parent protein, accession number: F0V6V5_EQUAS)			
INNQHMPYQ	1143.5132	9	24-32
VLNSSPR	771.4239	7	4-10
INNQHMPY	1015.4545	8	24-31
LAVLINNQHMPYQ	1539.7867	13	20-32
INNQHMP	852.3912	7	24-30
TVIPK	556.3584	5	82-86
HPRPHPS	826.4198	7	63-69
IAIPPK	637.4163	6	71-76
YARPA	576.3019	5	34-38
Peptides from lysozyme C (parent protein, accession number: LYSC_EQUAS)			
KAQEMDGFGG	1038.444	10	13-22
SLANWV	688.3544	6	24-29
KAQEMD	720.3112	6	13-18
LLDDNIDDD	1046.4404	9	83-91

DKDLSE	705.3181	6	117-122
DKDLS	576.2755	5	117-121
Peptides from serum albumin (parent protein, accession number: ALBU_EQUAS)			
KAPQVSTPT	927.5025	9	437-445
LKPEPDAQ	896.4603	8	139-146
KDDHPNLPK	1062.5458	9	130-138
AEEGPK	629.302	6	592-597
RHPDYS	773.3456	6	360-365
FNDLGEK	821.3919	7	35-41
SEIAH	555.2653	5	29-33
THKDDHPNLPK	1300.6523	11	128-138
KEDDLPSDLPA	1198.5717	11	317-327

Sample 150			
Peptides from beta-casein (parent protein, accession number: D2EC27_EQUAS)			
Peptide	**Mass**	**Length**	**Fragment**
AEPVPY	674.3275	6	85-90
APFPQPV	754.4014	7	192-198
APFPQPVVP	950.5225	9	192-200
APFPQPVVPY	1113.5858	10	192-201
APFPQPVVPYPQ	1338.6971	12	192-203
AQPPIVP	720.417	7	101-107
AQPPIVPF	867.4854	8	101-10
AVVPQ	512.2958	5	91-95
ETLLPK	699.4167	6	121-126
EVEHQ	640.2816	5	64-68
FKHEGQ	744.3555	6	55-60
FKHEGQQ	872.4141	7	55-61
FVQPQPV	813.4385	7	74-80
GEFDPATQPI	1073.5029	10	223-232
GLTGEFDPA	905.413	9	220-228
GLTGEFDPATQPIVPV	1639.8457	16	220-235
HQVPQ	607.3078	5	166-170

KETLLPK	827.5116	7	120-126
KSPIVPF	786.4639	7	134-140
KVAPFPQPV	981.5647	9	190-198
KVAPFPQPVVP	1177.6859	11	190-200
LAQPPI	637.3799	6	100-105
LIQPF	616.3584	5	160-164
LKSPIVP	752.4796	7	133-139
LMLPSQPV	883.4837	8	176-183
LRLPV	596.4009	5	154-158
LSPPQS	627.3228	6	184-189
LYQDPQ	762.3548	6	213-218
LYQDPQLGLT	1146.592	10	213-222
MLPSQPV	770.3997	7	177-183
PIVPF	571.337	5	104-108
PLAQPPIVP	930.5538	9	99-107
PLAQPPIVPF	1077.6222	10	99-108
PVLSPPQ	736.4119	7	182-188
PVLSPPQS	823.444	8	182-189
SPIVPF	658.369	6	135-140
TLLPK	570.3741	5	122-126
VAPFPQ	657.3486	6	191-196
VAPFPQPV	853.4697	8	191-198
VAPFPQPVVP	1049.5909	10	191-200
VAPFPQPVVPY	1212.6543	11	191-201
VAPFPQPVVPYP	1309.707	12	191-202
VAPFPQPVVPYPQ	1437.7656	13	191-203
VQPQPV	666.3701	6	75-80
YQDPQLGLT	1033.5081	9	214-222
Peptides from alpha-s1 casein (parent protein, accession number: CASA1_EQUAS)			
ALHTP	537.2911	5	32-36
ALHTPR	693.3922	6	32-37
IEDPEQQ	857.3766	7	64-70
IIQNEQDSpR	1181.5078	9	11-19
LHTPR	622.3551	5	33-37
LIEDPEQQ	970.4607	8	63-70

SPFHDT	702.2973	6	179-184
YLEPF	667.3217	5	148-152
Peptides from alpha-s2 casein (parent protein, accession number: CASA2_EQUAS)			
FTSEEIPK	949.4756	8	155-162
FTSpEEIPK	1029.4419	8	155-162
IVLTPW	727.4269	6	130-135
KFPTE	620.317	5	70-74
KFPTEV	719.3854	6	70-75
KTVDMESpTEVV	1316.5571	11	163-173
SEEIPK	701.3596	6	157-162
TELTEEEK	977.4553	8	177-184
TELTEEEKN	1091.4982	9	177-185
TSEEIPK	802.4072	7	156-162
alpha-s2 casein B (parent protein, accession number: C1L3G3_EQUAS)			
IELSDEEK	961.4604	8	101-108
QIPMSPW	857.4105	7	137-143
Peptides from beta-lactoglobulin I (parent protein, accession number: LACB1_HORSE)			
ALQPLPG	694.4014	7	157-163
DEEIME	764.2898	6	147-152
DEEIMEK	892.3848	7	147-153
KTESPAE	760.3603	7	93-99
LLDSESAPL	943.4862	9	49-57
LREGEN	716.3453	6	76-81
LRPTPE	711.3915	6	64-69
LRPTPEDN	940.4614	8	64-71
LRPTPEDNL	1053.5454	9	64-72
LRPTPEDNLE	1182.588	10	64-73
LRPTPEDNLEI	1295.6721	11	64-74
LRPTPEDNLEII	1408.7561	12	64-75
MVDEEIMEK	1122.4937	9	145-153
NAATPGQ	657.3082	7	127-133
NAATPGQS	744.3402	8	127-134
RPTPEDN	827.3773	7	65-71
RPTPEDNLEI	1182.588	10	65-74
TNIPQ	571.2966	5	19-23

TNIPQT	672.3442	6	19-24
VDEEIMEK	991.4532	8	146-153
WHSVA	598.2863	5	37-41
Peptides from beta-lactoglobulin II (parent protein, accession number: D6QX32_EQUAS)			
VEELRPTPEGN	1239.6095	11	61-71
YVEELRPTPE	1231.6084	10	60-69
YVEELRPTPEGN	1402.6729	12	60-71
VEELRPTPE	1068.545	9	61-69
QDPSGGQE	816.325	8	170-177
RPTPEGN	769.3718	7	65-71
LLDSESAPL	943.4862	9	49-57
IIQDPSGGQE	1042.493	10	168-177
AEHGTV	612.2867	6	132-137
LRPTPE	711.3915	6	64-69
VEELRPT	842.4498	7	61-67
ALQPLPG	694.4014	7	158-164
KTEDPA	659.3126	6	93-98
EGANHV	625.282	6	78-83
WHSVA	598.2863	5	37-41
Peptides from alpha-lactalbumin (parent protein, accession number: LALBA_EQUAS)			
ALQPLPG	694.4014	7	157-163
DEEIME	764.2898	6	147-152
DEEIMEK	892.3848	7	147-153
KTESPAE	760.3603	7	93-99
LLDSESAPL	943.4862	9	49-57
LREGEN	716.3453	6	76-81
LRPTPE	711.3915	6	64-69
LRPTPEDN	940.4614	8	64-71
LRPTPEDNL	1053.5454	9	64-72
LRPTPEDNLE	1182.588	10	64-73
LRPTPEDNLEI	1295.6721	11	64-74
LRPTPEDNLEII	1408.7561	12	64-75
MVDEEIMEK	1122.4937	9	145-153
NAATPGQ	657.3082	7	127-133
NAATPGQS	744.3402	8	127-134

RPTPEDN	827.3773	7	65-71
RPTPEDNLEI	1182.588	10	65-74
TNIPQ	571.2966	5	19-23
TNIPQT	672.3442	6	19-24
VDEEIMEK	991.4532	8	146-153
WHSVA	598.2863	5	37-41
Peptides from k-casein (parent protein, accession number: F0V6V5_EQUAS)			
NNQHMP	739.3072	6	25-30
TVIPK	556.3584	5	82-86
Peptides from lysozyme C (parent protein, accession number: LYSC_EQUAS)			
KAQEM	605.2843	5	13-17
LLDDNIDDDI	1159.5244	10	83-92
Peptides from serum albumin (parent protein, accession number: ALBU_EQUAS)			
LELDEGYVPK	1161.5917	10	514-523
QDSISGK	733.3606	7	291-297
LKPEPDA	768.4017	7	139-145
KEDDLPSDLPA	1198.5717	11	317-327
AEEGPK	629.302	6	592-597
VEEPK	600.3119	5	404-408
KDDHPNLPK	1062.5458	9	130-138

Sample 214			
Peptides from beta-casein (parent protein, accession number: D2EC27_EQUAS)			
Peptide	**Mass**	**Length**	**Fragment**
AEPVPY	674.3275	6	85 – 90
AQPPIVP	720.417	7	101 – 107
AQPPIVPF	867.4854	8	101 – 108
AVVPQ	512.2958	5	91 – 95
DPATQPIVP	936.4916	9	226 – 234
DTPVQA	629.3021	6	205 – 210
ETLLPK	699.4167	6	121 – 126
EVEHQ	640.2816	5	64 – 68
FKHEGQ	744.3555	6	55 – 60
FKHEGQQ	872.4141	7	55 - 61
FMHQVPQ	885.4167	7	164 – 170

FVQPQP	714.3701	6	74 – 79
FVQPQPV	813.4385	7	74 – 80
HQVPQ	607.3078	5	166 – 170
ILNPT	556.322	5	145 – 149
ITHIN	596.3282	5	44 – 48
KETLLPK	827.5116	7	120 – 126
KSPIVPF	786.4639	7	134 – 140
KVAPFPQPV	981.5647	9	190 – 198
KVAPFPQPVVP	1177.6859	11	190 – 200
KVMPF	620.3356	5	128 – 132
LAQPPI	637.3799	6	100 – 105
LAQPPIVPF	980.5695	9	100 – 108
LIQPF	616.3584	5	160 – 164
LKSPIVP	752.4796	7	133 – 139
LKSPIVPF	899.548	8	133 – 140
LMLPSQP	784.4153	7	176 – 182
LMLPSQPV	883.4837	8	176 – 183
LQPEIM	729.3731	6	109 – 114
LRLPV	596.4009	5	154 – 158
LSPPQS	627.3228	6	184 – 189
LYQDPQ	762.3548	6	213 - 218
MLPSQPV	770.3997	7	177 – 183
PLAQPPI	734.4326	7	99 -105
PLAQPPIVP	930.5538	9	99 – 107
PLAQPPIVPF	1077.6222	10	99 - 108
PVLSPPQS	823.444	8	182 – 189
QPVVPY	701.3748	6	196 – 201
REKEEL	802.4185	6	16 – 21
SPIVPF	658.369	6	135 – 140
TLLPK	570.3741	5	122 – 126
VAPFPQ	657.3486	6	191 – 196
VAPFPQPV	853.4697	8	191 – 198
VAPFPQPVVP	1049.5909	10	191 – 200
VAPFPQPVVPY	1212.6543	11	191 – 201
VAPFPQPVVPYP	1309.707	12	191 – 202

VAPFPQPVVPYPQ	1437.7656	13	191 – 203
VLSPPQ	639.3591	6	183 – 188
VLSPPQS	726.3912	7	183 – 189
VPYPQ	602.3064	5	199 – 203
VQPQPV	666.3701	6	75 – 80
VVYPYA	710.3639	6	80 – 85
YQDPQLGLT	1033.5081	9	214 – 222
Peptides from alpha-s1 casein (parent protein, accession number: CASA1_EQUAS)			
ALHTPR	693.3922	6	32 – 37
IEDPEQ	729.3181	6	64 – 69
IEDPEQQ	857.3766	7	64 – 70
IIQNEQDSpR	1181.5078	9	11 – 19
LHTPR	622.3551	5	33 – 37
LIEDPEQ	842.4021	7	63 – 69
LIEDPEQQ	970.4607	8	63 – 70
SPFHDTA	773.3344	7	179 – 185
TDIIPE	686.3486	6	196 – 201
TDIIPEW	872.428	7	196 – 202
VVNQEQA	786.3871	7	139 - 145
Peptides from alpha-s2 casein (parent protein, accession number: CASA2_EQUAS)			
AKFPTEV	790.4225	7	69 – 75
ELTEEEK	876.4076	7	178 – 184
ELTEEEKN	990.4506	8	178 – 185
FTSEEIPK	949.4756	8	155 – 162
FTSpEEIPK	1029.4419	8	155 – 162
IVLTPW	727.4269	6	130 – 135
KFPTE	620.317	5	70 - 74
KTVDM	592.2891	5	163 – 167
KTVDMESpTEV	1217.4886	10	163 – 172
KTVDMESpTEVV	1316.5571	11	163 – 173
LRQPR	668.4081	5	125 – 129
TELTEEEK	977.4553	8	177 – 184
TELTEEEKN	1091.4982	9	177 – 185
TSEEIPK	802.4072	7	156 – 162
alpha-s2 casein B (parent protein, accession number: C1L3G3_EQUAS)			

FQAVHPQ	825.4133	7	130 – 136
IELSpDEEK	1041.4268	8	101 – 108
IELSpDEEKN	1155.4696	9	101 – 109
KTVDM	592.2891	5	87 – 91
KTVDMGSpTEI	1159.4832	10	87 – 96
KTVDMGSpTEIFPE	1532.647	13	87 – 99
KTVDMGSpTEIFPEE	1661.6896	14	87 – 100
KTVDMGSpTEIFPEEI	1774.7736	15	87 – 101
TEIFPEE	863.3912	7	94 – 100
Peptides from beta-lactoglobulin I (parent protein, accession number: LACB1_HORSE)			
ALQPLP	637.3799	6	157 – 162
ALQPLPG	694.4014	7	157 – 163
DEEIME	764.2898	6	147 – 152
DEEIMEK	892.3848	7	147 – 153
DLDLQE	731.3337	6	27 – 32
DSESAPL	717.3181	7	51 – 57
IFAEK	606.3376	5	89 – 93
KTESPAE	760.3603	7	93 – 99
LREGEN	716.3453	6	76 – 81
LRPTPE	711.3915	6	64 – 69
LRPTPEDN	940.4614	8	64 – 71
LRPTPEDNL	1053.5454	9	64 – 72
LRPTPEDNLE	1182.588	10	64 – 73
LRPTPEDNLEI	1295.6721	11	64 – 74
LRPTPEDNLEII	1408.7561	12	64 – 75
MVDEEIMEK	1122.4937	9	145 – 153
NAATPGQS	744.3402	8	127 - 134
RPTPEDN	827.3773	7	65 – 71
RPTPEDNLE	1069.5039	9	65 – 73
TNIPQ	571.2966	5	19 – 23
TQMVDEEIMEK	1351.6	11	143 – 153
VDEEIME	863.3582	7	146 – 152
VDEEIMEK	991.4532	8	146 – 153
WHSVA	598.2863	5	37 – 41
Peptides from beta-lactoglobulin II (parent protein, accession number: D6QX32_EQUAS)			

AEHGTV	612.2867	6	132 – 137
ALQPLP	637.3799	6	158 – 163
ALQPLPG	694.4014	7	158 – 164
DLDLQE	731.3337	6	27 – 32
DSESAPL	717.3181	7	51 – 57
EELRPTPEGN	1239.6095	11	61 – 71
EGANHV	625.282	6	78 – 83
LRPTPE	711.3915	6	64 – 69
QDPSGGQE	816.325	8	170 – 177
RPTPEGN	769.3718	7	65 – 71
VDEEVMEK	977.4376	8	147 – 154
VEELRPT	842.4498	7	61 – 67
VEELRPTPE	1068.545	9	61 – 69
WHSVA	598.2863	5	37 – 41
YVEELRPTPE	1231.6084	10	60 – 69
Peptides from alpha-lactalbumin (parent protein, accession number: LALBA_EQUAS)			
AHKPL	564.3384	5	106 – 110
DDDLTDDV	906.3454	8	82 – 89
DDDLTDDVM	1037.3859	9	82 – 90
FHSSG	533.2234	5	31 – 35
FLDDDLTDDV	1166.4979	10	80 – 89
FQINN	634.3075	5	53 – 57
GVTLPE	614.3275	6	20 – 25
KGVTLPE	742.4225	7	19 – 25
KGVTLPEW	928.5018	8	19 – 26
KGVTLPEWI	1041.5858	9	19 – 27
KILDSEG	760.3967	7	94 – 100
KILDSEGI	873.4807	8	94 – 101
KILDSEGID	988.5076	9	94 – 102
LAHKPL	677.4224	6	105 – 110
LDDDLTDDV	1019.4294	9	81 – 89
Peptides from lysozyme C (parent protein, accession number: LYSC_EQUAS)			
DKDLSE	705.3181	6	117 – 122
KAQEM	605.2843	5	13 – 17
KAQEMDGFGG	1038.444	10	13 – 22

LLDDNIDDDI	1159.5244	10	83 – 92
Peptides from serum albumin (parent protein, accession number: ALBU_EQUAS)			
AEEGPK	629.302	6	592 – 597
ELDEGYVPK	1048.5077	9	515 – 523
KAPQVSTPT	927.5025	9	437 – 445
KDDHPNLPK	1062.5458	9	130 – 138
LELDEGYVPK	1161.5917	10	514 – 523
LKPEPDA	768.4017	7	139 – 145
LKPEPDAQ	896.4603	8	139 – 146
QDSISGK	733.3606	7	291 – 297
TPVSEK	659.349	6	490 - 495

Sample 224			
Peptides from beta-casein (parent protein, accession number: D2EC27_EQUAS)			
Peptide	**Mass**	**Length**	**Fragment**
APFPQPVVP	950.5225	9	192 - 200
APFPQPVVPYPQ	1338.697	12	192 - 203
AQPPIV	623.3643	6	101 - 106
AQPPIVP	720.417	7	101 - 107
AQPPIVPF	867.4854	8	101 - 108
AVVPQ	512.2958	5	91 - 95
ETLLPK	699.4167	6	121 - 126
EVEHQ	640.2816	5	64 - 68
FKHEGQ	744.3555	6	55 - 60
FKHEGQQ	872.4141	7	55 - 61
FKHEGQQQ	1000.473	8	55 - 62
FMHQVPQ	885.4167	7	164 - 170
FMHQVPQS	972.4487	8	164 - 171
GEFDPATQPI	1073.503	10	223 - 232
HQVPQ	607.3078	5	166 - 170
ILNPT	556.322	5	145 - 149
ITHIN	596.3282	5	44 - 48
KETLLPK	827.5116	7	120 - 126
KVAPF	560.3322	5	190 - 194
KVAPFP	657.3849	6	190 - 195

KVAPFPQP	882.4963	8	190 - 197
KVAPFPQPV	981.5647	9	190 - 198
KVAPFPQPVVP	1177.686	11	190 - 200
KVAPFPQPVVPY	1340.749	12	190 - 201
KVMPF	620.3356	5	128 - 132
LAQPPI	637.3799	6	100 - 105
LAQPPIVP	833.501	8	100 - 107
LAQPPIVPF	980.5695	9	100 - 108
LIQPF	616.3584	5	160 - 164
LKSPIVPF	899.548	8	133 - 140
LMLPSQP	784.4153	7	176 - 182
LMLPSQPV	883.4837	8	176 - 183
LNVSpSETpVES	1223.436	10	21 - 30
LQPEIM	729.3731	6	109 - 114
LRLPV	596.4009	5	154 - 158
LYQDPQ	762.3548	6	213 - 218
LYQDPQLGLT	1146.592	10	213 - 222
MHQVPQ	738.3483	6	165 - 170
MHQVPQS	825.3803	7	165 - 171
MLPSQP	671.3312	6	177 - 182
MLPSQPV	770.3997	7	177 - 183
PLAQPPIVP	930.5538	9	99 - 107
PLAQPPIVPF	1077.622	10	99 - 108
PVLSPPQ	736.4119	7	182 - 188
REKEEL	802.4185	6	16 - 21
RQILNPT	840.4818	7	143 - 149
SPIVPF	658.369	6	135 - 140
TLLPK	570.3741	5	122 - 126
VAPFPQPV	853.4697	8	191 - 198
VAPFPQPVVP	1049.591	10	191 - 200
VAPFPQPVVPY	1212.654	11	191 - 201
VAPFPQPVVPYP	1309.707	12	191 - 202
VAPFPQPVVPYPQ	1437.766	13	191 - 203
VLSPPQS	726.3912	7	183 - 189
VPVHNP	661.3547	6	233 - 238

VQPQPV	666.3701	6	75 - 80
Peptides from alpha-s1 casein (parent protein, accession number: CASA1_EQUAS)			
ALHTP	537.2911	5	32 - 36
ALHTPR	693.3922	6	32 - 37
IEDPEQQ	857.3766	7	64 - 70
IEDPEQQE	986.4193	8	64 - 71
IIQNEQDSpR	1181.508	9	11 - 19
IQNEQDSpR	1068.424	8	12 - 19
LEPFQPS	816.4017	7	149 - 155
LIEDPEQ	842.4021	7	63 - 69
LIEDPEQQ	970.4607	8	63 -70
SPFHDT	702.2973	6	179 - 184
SPFHDTA	773.3344	7	179 - 185
TDIIPE	686.3486	6	196 - 201
TDIIPEW	872.428	7	196 - 202
VVNQEQA	786.3871	7	139 - 145
YLEPF	667.3217	5	148 -152
Peptides from alpha-s2 casein (parent protein, accession number: CASA2_EQUAS)			
AKFPTEV	790.4225	7	69 - 75
ELTEEEKN	990.4506	8	178 - 185
EQLFTSpEEIPK	1399.627	11	152 - 162
FTSEEIPK	949.4756	8	155 - 162
FTSpEEIPK	1029.442	8	155 - 162
IVLTPW	727.4269	6	130 - 135
KFPTE	620.317	5	70 - 74
KFPTEV	719.3854	6	70 - 75
KTVDMESpTEV	1217.489	10	163 - 172
KTVDMESpTEVV	1316.557	11	163 - 173
LFTSpEEIPK	1142.526	9	154 - 162
LTEEEK	747.365	6	179 - 184
LTEEEKN	861.408	7	179 - 185
TELTEEEK	977.4553	8	177 - 184
TELTEEEKN	1091.498	9	177 - 185
TSEEIPK	802.4072	7	156 - 162
TSpEEIPK	882.3735	7	156 - 162

alpha-s2 casein B (parent protein, accession number: C1L3G3_EQUAS)			
DMGSpTEIFPEEI	1446.563	12	90 - 101
ELSpDEEKN	1042.386	8	102 - 109
IELSDEEK	961.4604	8	101 - 108
IELSpDEEK	1041.427	8	101 - 108
IELSpDEEKN	1155.47	9	101 - 109
IPMSPW	729.352	6	138 - 143
KTVDMGSpTEI	1159.483	10	87 - 96
KTVDMGSpTEIFPE	1532.647	13	87 - 99
KTVDMGSpTEIFPEE	1661.69	14	87 - 100
KTVDMGSpTEIFPEEI	1774.774	15	87 - 101
KTVDMGSpTEIFPEEIE	1903.816	16	87 - 102
KTVDMGSpTEIFPEEIEL	2016.9	17	87 - 103
LSDEEK	719.3337	6	103 - 108
QAVHPQ	678.3449	6	131 - 136
QIPMSPW	857.4105	7	137 - 143
TVDMGSpTEIFPE	1404.552	12	88 - 99
TVDMGSpTEIFPEE	1533.595	13	88 - 100
TVDMGSpTEIFPEEIE	1775.721	15	88 - 102
YKQDNNVA	950.4457	8	36 - 43
Peptides from beta-lactoglobulin I (parent protein, accession number: LACB1_HORSE)			
ALQPLPG	694.4014	7	139 - 145
DEEIMEK	892.3848	7	129 - 135
EKTESPAE	889.4028	8	74 - 81
KTESPAE	760.3603	7	75 - 81
LREGEN	716.3453	6	58 - 63
LRPTPE	711.3915	6	46 - 51
LRPTPEDN	940.4614	8	46 - 53
LRPTPEDNL	1053.545	9	46 - 53
LRPTPEDNLE	1182.588	10	46 - 55
LRPTPEDNLEI	1295.672	11	46 - 56
LRPTPEDNLEII	1408.756	12	46 - 57
LRPTPEDNLEIIL	1521.84	13	46 - 58
NAATPGQ	657.3082	7	109 - 115
RPTPEDN	827.3773	7	47 - 53

RPTPEDNLE	1069.504	9	47 - 55
TNIPQ	571.2966	5	1 - 5
TNIPQT	672.3442	6	1 - 6
TQMVDEEIMEK	1351.6	11	125 - 135
VDEEIMEK	991.4532	8	128 - 135
VYIEK	650.3639	5	41 - 45
Peptides from beta-lactoglobulin II (parent protein, accession number: D6QX32_EQUAS)			
AEHGTV	612.2867	6	132 - 137
ALQPLPG	694.4014	7	158 - 164
DSESAPL	717.3181	7	51 - 57
IIQDPSGGQE	1042.493	10	168 - 177
LDSESAP	717.3181	7	50 - 56
LLDSESAPL	943.4862	9	49 - 57
LRPTPE	711.3915	6	64 - 69
LRPTPEGN	882.4559	8	64 - 71
QDPSGGQE	816.325	8	170 - 177
VDEEVMEK	977.4376	8	147 - 154
VEELRPTPE	1068.545	9	61 - 69
VEELRPTPEGN	1239.61	11	61 - 71
Peptides from alpha-lactalbumin (parent protein, accession number: LALBA_EQUAS)			
DDDLTDDV	906.3454	8	82 - 89
DNQILPS	785.3919	7	63 - 69
FLDDDLTDDV	1166.498	10	80 - 89
FQINN	634.3075	5	53 - 57
GVTLPE	614.3275	6	20 - 25
GVTLPEWI	913.4909	8	20 - 27
KGVTLPE	742.4225	7	19 - 25
KGVTLPEW	928.5018	8	19 - 26
KGVTLPEWI	1041.586	9	19 - 27
KILDSEG	760.3967	7	94 - 100
KILDSEGI	873.4807	8	94 - 101
KILDSEGID	988.5076	9	94 - 102
LAHKP	564.3384	5	105 - 109
LAHKPL	677.4224	6	105 - 110
LDDDLTDDV	1019.429	9	81 - 89

Peptides from lysozyme C (parent protein, accession number: LYSC_EQUAS)			
LLDDNIDDDI	1159.524	10	83 - 92
KAQEM	605.2843	5	13 - 17
DKDLSE	705.3181	6	117 - 122
Peptides from serum albumin (parent protein, accession number: ALBU_EQUAS)			
DDHPNLPK	934.4508	8	131 - 138
KAPQVSTPT	927.5025	9	437 - 445
VKEDDLPSDLPA	1297.64	12	316 - 327

Sample 236			
Peptides from beta-casein (parent protein, accession number: D2EC27_EQUAS)			
Peptide	**Mass**	**Length**	**Fragment**
AQPPIVP	720.417	7	101 - 107
AQPPIVPF	867.4854	8	101 - 108
AVVPQ	512.2958	5	91 - 95
FKHEGQ	744.3555	6	55 - 60
FKHEGQQ	872.4141	7	55 - 61
FMHQVPQ	885.4167	7	164 - 170
FMHQVPQS	972.4487	8	164 - 171
FVQPQPV	813.4385	7	74 - 80
GLTGEFDPA	905.413	9	220 - 228
HQVPQ	607.3078	5	166 - 170
ITHIN	596.3282	5	44 - 48
KSPIVPF	786.4639	7	134 - 140
LAQPPI	637.3799	6	100 - 105
LIQPF	616.3584	5	160 - 164
LKSPIVP	752.4796	7	133 - 139
LMLPSQPV	883.4837	8	176 - 183
LRLPV	596.4009	5	154 - 158
LSPPQS	627.3228	6	184 - 189
MHQVPQ	738.3483	6	165 - 170
MLPSQPV	770.3997	7	177 - 183
PLAQPPI	734.4326	7	99 - 105
PLAQPPIVP	930.5538	9	99 - 107
PLAQPPIVPF	1077.622	10	99 - 108

SPIVPF	658.369	6	135 - 140
TLLPK	570.3741	5	122 - 126
VAPFPQ	657.3486	6	191 - 196
VAPFPQPV	853.4697	8	191 - 198
VAPFPQPVVP	1049.591	10	191 - 200
VAPFPQPVVPY	1212.654	11	191 - 201
VAPFPQPVVPYP	1309.707	12	191 - 202
VAPFPQPVVPYPQ	1437.766	13	191 - 203
VLSPPQS	726.3912	7	183 - 189
VPVHNPV	760.4232	7	233 - 239
VQPQPV	666.3701	6	75 - 80
Peptides from alpha-s2 casein (parent protein, accession number: CASA2_EQUAS)			
AKFPTEV	790.4225	7	69 - 75
FTLPQ	604.322	5	200 - 204
FTSEEIPK	949.4756	8	155 - 162
FTSpEEIPK	1029.442	8	155 - 162
KTVDMESpTEVV	1316.557	11	163 - 173
LRQPR	668.4081	5	125 - 129
TSEEIPK	802.4072	7	156 - 162
Peptides from beta-lactoglobulin I (parent protein, accession number: LACB1_HORSE)			
ALQPLPG	694.4014	7	157 - 163
DEEIMEK	892.3848	7	147 - 153
DLQEV	602.2911	5	29 - 33
DSESAPL	717.3181	7	51 - 57
KTESPAE	760.3603	7	93 - 99
LLDSESAPL	943.4862	9	49 - 57
LREGEN	716.3453	6	76 - 81
LRPTPE	711.3915	6	64 - 69
LRPTPEDN	940.4614	8	64 - 71
LRPTPEDNL	1053.545	9	64 - 72
LRPTPEDNLE	1182.588	10	64 -73
LRPTPEDNLEI	1295.672	11	64 - 74
LRPTPEDNLEII	1408.756	12	64 - 75
MVDEEIMEK	1122.494	9	145 - 153
NAATPGQ	657.3082	7	127 - 133

NAATPGQS	744.3402	8	127 - 134
RPTPEDN	827.3773	7	65 - 71
RPTPEDNLE	1069.504	9	65 - 73
TNIPQ	571.2966	5	19 - 23
TNIPQT	672.3442	6	19 - 24
VYIEK	650.3639	5	59 - 63
WHSVA	598.2863	5	37 - 41
Peptides from beta-lactoglobulin II (parent protein, accession number: D6QX32_EQUAS)			
AEHGTV	612.2867	6	132 - 137
ALQPLPG	694.4014	7	158 - 164
DLQEV	602.2911	5	29 - 33
DSESAPL	717.3181	7	51 - 57
IIQDPSGGQE	1042.493	10	168 - 177
LLDSESAPL	943.4862	9	49 – 57
LRPTPE	711.3915	6	64 - 69
QDPSGGQE	816.325	8	170 - 177
QIIQDPSGGQE	1170.552	11	167 - 177
RPTPEGN	769.3718	7	65 - 71
VEELRPTPE	1068.545	9	61 - 69
VEELRPTPEGN	1239.61	11	61 - 71
WHSVA	598.2863	5	37 - 41
Peptides from alpha-lactalbumin (parent protein, accession number: LALBA_EQUAS)			
DDDLTDDV	906.3454	8	82 - 89
FLDDDLTDDV	1166.498	10	80 - 89
KGVTLPE	742.4225	7	19 - 25
KILDSEGID	988.5076	9	94 - 102
LAHKPL	677.4224	6	105 - 110
LDDDLTDDV	1019.429	9	81 - 89
TKCELS	679.321	6	4 - 9
Peptides from k-casein (parent protein, accession number: F0V6V5_EQUAS)			
IAIPPK	637.4163	6	43 - 48
TVIPK	556.3584	5	54 - 58
Peptides from lysozyme C (parent protein, accession number: LYSC_EQUAS)			
DDNIDDDI	933.3563	8	85 - 92
KAQEM	605.2843	5	13 - 17

LLDDNIDDDI	1159.524	10	83 - 92
Peptides from serum albumin (parent protein, accession number: ALBU_EQUAS)			
AEEGPK	629.302	6	592 - 597
FKAET	594.3013	5	525 - 529
KAPQVSTPT	927.5025	9	437 - 445
KDDHPNLPK	1062.546	9	130 - 138
KEDDLPSDL	1030.482	9	317 - 325
KEDDLPSDLPA	1198.572	11	317 - 327
LKPEPDA	768.4017	7	139 - 145
NDLGEK	674.3235	6	36 - 41
QDSISGK	733.3606	7	291 - 297
VKEDDLPSD	1016.466	9	316 - 324

Sample 237			
Peptides from beta-casein (parent protein, accession number: D2EC27_EQUAS)			
Peptide	**Mass**	**Length**	**Fragment**
VAPFPQPVVPY	1212.654	11	191-201
VAPFPQPVVPYPQ	1437.766	13	191-203
AQPPIVPF	867.4854	8	101-108
VAPFPQPVVP	1049.591	10	191-200
VAPFPQPVVPYP	1309.707	12	191-202
KVAPFPQPVVP	1177.686	11	190-200
VAPFPQPV	853.4697	8	191-198
KVAPFPQPV	981.5647	9	190-198
PLAQPPIVP	930.5538	9	99-107
AEPVPY	674.3275	6	85-90
LMLPSQPV	883.4837	8	176-183
LAQPPIVPF	980.5695	9	100-108
YQDPQLGLT	1033.508	9	214-222
MLPSQPV	770.3997	7	177-183
FKHEGQQ	872.4141	7	55-61
KSPIVPF	786.4639	7	134-140
KETLLPK	827.5116	7	120-126
MHQVPQ	738.3483	6	165-170
FKHEGQ	744.3555	6	55-60

PLAQPPIVPF	1077.622	10	99-108
MHQVPQS	825.3803	7	165-171
AQPPIVP	720.417	7	101-107
TLLPK	570.3741	5	122-126
LAQPPI	637.3799	6	100-105
VQPQPV	666.3701	6	75-80
DTPVQA	629.3021	6	205-210
LQPEIM	729.3731	6	109-114
SPIVPF	658.369	6	135-140
LSPPQS	627.3228	6	184-189
HQVPQ	607.3078	5	166-170
LTGEFDPA	848.3915	8	221-228
ITHIN	596.3282	5	44-48
PLAQPPIV	833.501	8	99-106
HQVPQS	694.3398	6	166-171
TQPIVPV	752.4432	7	229-235
LRLPV	596.4009	5	154-158
ETLLPK	699.4167	6	121-126
LIQPF	616.3584	5	160-164
PLAQPPI	734.4326	7	99-105
AVVPQ	512.2958	5	91-95
LMLPSQP	784.4153	7	176-182
EVEHQ	640.2816	5	64-68
PIVPF	571.337	5	104-108
REKEEL	802.4185	6	16-21
RQILNPT	840.4818	7	143-149
ILNPT	556.322	5	145-149
LKSPIVP	752.4796	7	133-139
Peptides from alpha-s1 casein (parent protein, accession number: CASA1_EQUAS)			
LIEDPEQQ	970.4607	8	63-70
ALHTP	537.2911	5	32-36
LHTPR	622.3551	5	33-37
Peptides from alpha-s2 casein (parent protein, accession number: CASA2_EQUAS)			
KTVDMESpTEVV	1316.557	11	163-173
KTVDMESpTEV	1217.489	10	163-172

FTSEEIPK	949.4756	8	155-162
FTSpEEIPK	1029.442	8	155-162
TELTEEEK	977.4553	8	177-184
IVLTPW	727.4269	6	130-135
TSEEIPK	802.4072	7	156-162
ELTEEEKN	990.4506	8	178-185
AKFPTEV	790.4225	7	69-75
KTVDM	592.2891	5	163-167
alpha-s2 casein B (parent protein, accession number: C1L3G3_EQUAS)			
KTVDMGSpTEIFPEE	1661.6896	14	87-100
KTVDMGSpTEIFPE	1532.647	13	87-99
IELSDEEKN	1075.5033	9	101-109
FQAVHPQQIPM	1294.6492	11	130-140
KTVDMGSpTEI	1159.4832	10	87-96
TVDMGSpTEIFPE	1404.552	12	88-99
IELSpDEEK	1041.4268	8	101-108
YKQDNNVA	950.4457	8	36-43
TVDMGSpTEIFPEE	1533.5946	13	88-100
QIPMSPWN	971.4535	8	137-144
IELSDEEK	961.4604	8	101-108
QIPMSPW	857.4105	7	137-143
QAVHPQQIPM	1147.5808	10	131-140
KQDNNVA	787.3824	7	37-43
QAVHPQ	678.3449	6	131-136
KTVDM	592.2891	5	87-91
VKINPK	697.4486	6	118-123
Peptides from beta-lactoglobulin I (parent protein, accession number: LACB1_HORSE)			
MVDEEIMEK	1122.494	9	145-153
LRPTPEDNLEI	1295.672	11	64-74
RPTPEDN	827.3773	7	65-71
LRPTPEDNLE	1182.588	10	64-73
VDEEIMEK	991.4532	8	146-153
RPTPEDNLE	1069.504	9	65-73
LRPTPEDNL	1053.545	9	64-72
DEEIMEK	892.3848	7	147-153

NAATPGQS	744.3402	8	127-134
LRPTPEDN	940.4614	8	64-71
LRPTPEDNLEII	1408.756	12	64-75
DSESAPL	717.3181	7	51-57
LRPTPE	711.3915	6	64-69
LLDSESAPL	943.4862	9	49-57
NAATPGQ	657.3082	7	127-133
KTESPAE	760.3603	7	93-99
VYIEK	650.3639	5	59-63
ALQPLPG	694.4014	7	157-163
TNIPQ	571.2966	5	19-23
WHSVA	598.2863	5	37-41
TNIPQT	672.3442	6	19-24
Peptides from beta-lactoglobulin II (parent protein, accession number: D6QX32_EQUAS)			
VEELRPTPEGN	1239.61	11	61-71
YVEELRPTPE	1231.608	10	60-69
VEELRPTPE	1068.545	9	61-69
IIQDPSGGQE	1042.493	10	168-177
RPTPEGN	769.3718	7	65-71
YVEELRPT	1005.513	8	60-67
VEELRPT	842.4498	7	61-67
DSESAPL	717.3181	7	51-57
LRPTPE	711.3915	6	64-69
LLDSESAPL	943.4862	9	49-57
QDPSGGQE	816.325	8	170-177
AEHGTV	612.2867	6	132-137
QKTEDPA	787.3712	7	92-98
ALQPLPG	694.4014	7	158-164
WHSVA	598.2863	5	37-41
Peptides from alpha-lactalbumin (parent protein, accession number: LALBA_EQUAS)			
FLDDDLTDDV	1166.498	10	80-89
KILDSEGI	873.4807	8	94-101
KILDSEG	760.3967	7	94-100
KGVTLPEWI	1041.586	9	19-27
KGVTLPEW	928.5018	8	19-26

LDDDLTDDV	1019.429	9	81-89
KGVTLPE	742.4225	7	19-25
LAHKP	564.3384	5	105-109
DDDLTDDV	906.3454	8	82-89
LAHKPL	677.4224	6	105-110
AHKPL	564.3384	5	106-110
DNQILPS	785.3919	7	63-69
Peptides from k-casein (parent protein, accession number: F0V6V5_EQUAS)			
INNQHMPYQ	1143.5132	9	24-32
VLNSSPR	771.4239	7	4-10
INNQHMPY	1015.4545	8	24-31
LAVLINNQHMPYQ	1539.7867	13	20-32
INNQHMP	852.3912	7	24-30
TVIPK	556.3584	5	82-86
HPRPHPS	826.4198	7	63-69
IAIPPK	637.4163	6	71-76
YARPA	576.3019	5	34-38
Peptides from lysozyme C (parent protein, accession number: LYSC_EQUAS)			
KAQEM	605.2843	5	13-17
LLDDNIDDDI	1159.524	10	83-92
Peptides from serum albumin (parent protein, accession number: ALBU_EQUAS)			
KAPQVSTPT	927.5025	9	437-445
LKPEPDAQ	896.4603	8	139-146
KDDHPNLPK	1062.5458	9	130-138
AEEGPK	629.302	6	592-597
RHPDYS	773.3456	6	360-365
FNDLGEK	821.3919	7	35-41
SEIAH	555.2653	5	29-33
THKDDHPNLPK	1300.6523	11	128-138
KEDDLPSDLPA	1198.5717	11	317-327

Sample 246			
Peptides from beta-casein (parent protein, accession number: D2EC27_EQUAS)			
Peptide	**Mass**	**Length**	**Fragment**
VAPFPQPVVPY	1212.654	11	191-201

VAPFPQPVVPYPQ	1437.766	13	191-203
VAPFPQPVVPYP	1309.707	12	191-202
YQDPQLGLT	1033.508	9	214-222
AQPPIVPF	867.4854	8	101-108
LYQDPQLGLT	1146.592	10	213-222
VAPFPQPV	853.4697	8	191-198
VAPFPQPVVP	1049.591	10	191-200
LMLPSQPV	883.4837	8	176-183
FVQPQPV	813.4385	7	74-80
KVAPFPQPV	981.5647	9	190-198
LKSPIVPF	899.548	8	133-140
AEPVPY	674.3275	6	85-90
FKHEGQQ	872.4141	7	55-61
AQPPIVP	720.417	7	101-107
KETLLPK	827.5116	7	120-126
KSPIVPF	786.4639	7	134-140
KVAPFPQPVVP	1177.686	11	190-200
LAQPPIVPF	980.5695	9	100-108
PLAQPPIVPF	1077.622	10	99-108
MHQVPQ	738.3483	6	165-170
VAPFPQ	657.3486	6	191-196
MLPSQPV	770.3997	7	177-183
APFPQPVVPYPQ	1338.697	12	192-203
FKHEGQ	744.3555	6	55-60
QDPQLGLT	870.4447	8	215-222
TLLPK	570.3741	5	122-126
SPIVPF	658.369	6	135-140
VQPQPV	666.3701	6	75-80
HQVPQ	607.3078	5	166-170
DTPVQA	629.3021	6	205-210
LAQPPI	637.3799	6	100-105
LQPEIM	729.3731	6	109-114
LSPPQS	627.3228	6	184-189
REKEEL	802.4185	6	16-21
LRLPV	596.4009	5	154-158

PLAQPPI	734.4326	7	99-105
AVVPQ	512.2958	5	91-95
TQPIVPV	752.4432	7	229-235
LIQPF	616.3584	5	160-164
ILNPT	556.322	5	145-149
LYQDPQ	762.3548	6	213-218
LKSPIVP	752.4796	7	133-139
APFPQPVVP	950.5225	9	192-200
Peptides from alpha-s1 casein (parent protein, accession number: CASA1_EQUAS)			
ALHTP	537.2911	5	32-36
HPEIIQ	735.3915	6	8-13
IEDPEQQ	857.3766	7	64-70
IIQNEQDSpR	1181.508	9	11-19
LHTPR	622.3551	5	33-37
LIEDPEQ	842.4021	7	63-69
LIEDPEQQ	970.4607	8	63-70
SPFHDT	702.2973	6	179-184
SPFHDTA	773.3344	7	179-185
TDIIPE	686.3486	6	196-201
VVNQEQA	786.3871	7	139-145
YLEPF	667.3217	5	148-152
Peptides from alpha-s2 casein (parent protein, accession number: CASA2_EQUAS)			
ELTEEEK	876.4076	7	178-184
ELTEEEKN	990.4506	8	178-185
FTSEEIPK	949.4756	8	155-162
FTSpEEIPK	1029.442	8	155-162
IVLTPW	727.4269	6	130-135
KTVDMESpTEV	1217.489	10	163-172
KTVDMESpTEVV	1316.557	11	163-173
LFTSpEEIPK	1142.526	9	154-162
LRQPR	668.4081	5	125-129
TELTEEEK	977.4553	8	177-184
TELTEEEKN	1091.498	9	177-185
TSEEIPK	802.4072	7	156-162
TSpEEIPK	882.3735	7	156-162

alpha-s2 casein B (parent protein, accession number: C1L3G3_EQUAS)			
AVHPQ	550.2863	5	132-136
IELSDEEK	961.4604	8	101-108
IELSDEEKN	1075.503	9	101-109
IELSpDEEKN	1155.47	9	101-109
KQDNNVA	787.3824	7	37-43
KTVDMGSpTEI	1159.483	10	87-96
KTVDMGSpTEIFPEE	1661.69	14	87-100
KTVDMGSpTEIFPEEI	1774.774	15	87-101
LSDEEK	719.3337	6	103-108
QIPMSPW	857.4105	7	137-143
TVDMGSpTEIFPEE	1533.595	13	88-100
YKQDNNVA	950.4457	8	36-43
Peptides from beta-lactoglobulin I (parent protein, accession number: LACB1_HORSE)			
ALQPLPG	694.4014	7	157-163
DSESAPL	717.3181	7	51-57
IFAEK	606.3376	5	89-93
KTESPAE	760.3603	7	93-99
LLDSESAPL	943.4862	9	49-57
LRPTPE	711.3915	6	64-69
LRPTPEDN	940.4614	8	64-71
LRPTPEDNL	1053.545	9	64-72
LRPTPEDNLE	1182.588	10	64-73
LRPTPEDNLEI	1295.672	11	64-74
LRPTPEDNLEII	1408.756	12	64-75
LRPTPEDNLEIIL	1521.84	13	64-76
MVDEEIMEK	1122.494	9	145-153
NAATPGQS	744.3402	8	127-134
RPTPEDN	827.3773	7	65-71
RPTPEDNLE	1069.504	9	65-73
SLLDSESAPL	1030.518	10	48-57
TNIPQ	571.2966	5	19-23
TNIPQT	672.3442	6	19-24
TQMVDEEIMEK	1351.6	11	143-153
WHSVA	598.2863	5	37-41

Peptides from beta-lactoglobulin II (parent protein, accession number: D6QX32_EQUAS)			
AEHGTV	612.2867	6	132-137
ALQPLPG	694.4014	7	158-164
DSESAPL	717.3181	7	51-57
IIQDPSGGQE	1042.493	10	168-177
LLDSESAPL	943.4862	9	49-57
LRPTPE	711.3915	6	64-69
QDPSGGQE	816.325	8	170-177
RPTPEGN	769.3718	7	65-71
SLLDSESAPL	1030.518	10	48-57
VDEEVMEK	977.4376	8	147-154
VEELRPTPE	1068.545	9	61-69
VEELRPTPEGN	1239.61	11	61-71
WHSVA	598.2863	5	37-41
YVEELRPTPE	1231.608	10	60-69
YVEELRPTPEGN	1402.673	12	60-71
YVEELRPTPEGNLE	1644.799	14	60-73
Peptides from alpha-lactalbumin (parent protein, accession number: LALBA_EQUAS)			
AHKPL	564.3384	5	106-110
DDDLTDDVM	1037.386	9	82-90
DNQILPS	785.3919	7	63-69
FLDDDLTDDV	1166.498	10	80-89
ILDSEGID	860.4127	8	95-102
ILDSEGIDY	1023.476	9	95-103
KFLDDDLTDDV	1294.593	11	79-89
KGVTLPE	742.4225	7	19-25
KGVTLPEW	928.5018	8	19-26
KGVTLPEWI	1041.586	9	19-27
KILDSEG	760.3967	7	94-100
KILDSEGI	873.4807	8	94-101
KILDSEGID	988.5076	9	94-102
LAHKP	564.3384	5	105-109
LAHKPL	677.4224	6	105-110
LDDDLTDDV	1019.429	9	81-89
LDDDLTDDVM	1150.47	10	81-90

WLAHKPL	863.5017	7	104-110
Peptides from lysozyme C (parent protein, accession number: LYSC_EQUAS)			
KAQEMDGFGG	1038.444	10	13-22
LLDDNIDDDI	1159.524	10	83-92

Sample 270			
Peptides from beta-casein (parent protein, accession number: D2EC27_EQUAS)			
Peptide	**Mass**	**Length**	**Fragment**
APFPQPV	754.4014	7	192 - 198
APFPQPVVP	950.5225	9	192 - 200
APFPQPVVPYPQ	1338.697	12	192 - 203
AQPPIVP	720.417	7	101 - 107
AQPPIVPF	867.4854	8	101 - 108
AVVPQ	512.2958	5	91 - 95
DTPVQA	629.3021	6	205 - 210
ETLLPK	699.4167	6	121 - 126
FKHEGQ	744.3555	6	55 - 60
FKHEGQQ	872.4141	7	55 - 61
FPQPVVPYPQ	1170.607	10	194 - 203
GEFDPATQPIVPV	1368.693	13	223 - 235
HQVPQ	607.3078	5	166 - 170
KETLLPK	827.5116	7	120 - 126
KSPIVPF	786.4639	7	134 - 140
KVAPFPQPV	981.5647	9	190 - 198
KVAPFPQPVVP	1177.686	11	190 - 200
LAQPPI	637.3799	6	100 - 105
LMLPSQPV	883.4837	8	176 - 183
LQPEIM	729.3731	6	109 - 114
LRLPV	596.4009	5	154 - 158
LSPPQS	627.3228	6	184 - 189
LYQDPQ	762.3548	6	213 - 218
MLPSQPV	770.3997	7	177 - 183
PFPQPVVP	879.4854	8	193 - 200
PIVPF	571.337	5	104 - 108
PLAQPPI	734.4326	7	99 - 105

PLAQPPIVP	930.5538	9	99 - 107
PLAQPPIVPF	1077.622	10	99 - 108
PQPVVPYPQ	1023.539	9	195 - 203
PVLSPPQ	736.4119	7	182 - 188
PVLSPPQS	823.444	8	182 - 189
QDPQLGLT	870.4447	8	215 -222
SPIVPF	658.369	6	135 - 140
TGEFDPA	735.3075	7	222 - 228
TQPIVPV	752.4432	7	229 - 235
VAPFPQ	657.3486	6	191 - 196
VAPFPQPVVP	1049.591	10	191 - 200
VAPFPQPVVPY	1212.654	11	191 - 201
VAPFPQPVVPYP	1309.707	12	191 - 202
VAPFPQPVVPYPQ	1437.766	13	191 - 203
VQPQPV	666.3701	6	75 - 80
Peptides from alpha-s1 casein (parent protein, accession number: CASA1_EQUAS)			
ALHTP	537.2911	5	32 – 36
IEDPEQ	729.3181	6	64 - 69
IEDPEQQ	857.3766	7	64 - 70
IIQNEQDSpR	1181.508	9	11 - 19
IQNEQDSpR	1068.424	8	12 - 19
LEPFQPS	816.4017	7	149 - 155
LIEDPEQ	842.4021	7	63 - 69
LIEDPEQQ	970.4607	8	63 - 70
SPFHDT	702.2973	6	179 - 184
TDIIPE	686.3486	6	196 - 201
VVNQEQ	715.35	6	139 - 144
Peptides from alpha-s2 casein (parent protein, accession number: CASA2_EQUAS)			
EQLFTSpEEIPK	1399.627	11	152 - 162
FTLPQ	604.322	5	200 - 204
FTSEEIPK	949.4756	8	155 - 162
FTSpEEIPK	1029.442	8	155 - 162
IVLTPW	727.4269	6	130 - 135
KFPTE	620.317	5	70 - 74
KTVDM	592.2891	5	163 - 167

KTVDMESpTEV	1217.489	10	163 - 172
KTVDMESpTEVV	1316.557	11	163 - 173
LFTSpEEIPK	1142.526	9	154 - 162
TELTEEEK	977.4553	8	177 - 184
TSEEIPK	802.4072	7	156 - 162
alpha-s2 casein B (parent protein, accession number: C1L3G3_EQUAS)			
IELSDEEK	961.4604	8	101 - 108
KTVDM	592.2891	5	87 - 91
QAVHPQ	678.3449	6	131 - 136
Peptides from beta-lactoglobulin I (parent protein, accession number: LACB1_HORSE)			
ALQPLPG	694.4014	7	139 - 145
DEEIMEK	892.3848	7	129 - 135
EKTESPAE	889.4028	8	74 - 81
LRPTPEDN	940.4614	8	46 - 53
LRPTPEDNLE	1182.588	10	46 - 55
LRPTPEDNLEI	1295.672	11	46 - 56
NAATPGQ	657.3082	7	109 - 115
NAATPGQS	744.3402	8	109 - 116
RPTPE	598.3074	5	47 - 51
RPTPEDN	827.3773	7	47 - 53
RPTPEDNLE	1069.504	9	47 - 55
TNIPQ	571.2966	5	1 - 5
TNIPQT	672.3442	6	1 - 6
VDEEIME	863.3582	7	128 - 134
VDEEIMEK	991.4532	8	128 - 135
Peptides from beta-lactoglobulin II (parent protein, accession number: D6QX32_EQUAS)			
AEHGTV	612.2867	6	132 - 137
ALQPLPG	694.4014	7	158 - 164
EGANHV	625.282	6	78 - 83
IIQDPSGGQE	1042.493	10	168 - 177
QDPSGGQE	816.325	8	170 - 177
RPTPE	598.3074	5	65 - 69
RPTPEGN	769.3718	7	65 - 71
VEELRPT	842.4498	7	61 - 67
VEELRPTPE	1068.545	9	61 - 69

VEELRPTPEGN	1239.61	11	61 - 71
YVEELRPTPEGN	1402.673	12	60 - 71
Peptides from alpha-lactalbumin (parent protein, accession number: LALBA_EQUAS)			
AHKPL	564.3384	5	106 - 110
DDDLTDDV	906.3454	8	82 - 89
DNQILPS	785.3919	7	63 - 69
FLDDDLTDDV	1166.498	10	80 - 89
GVTLPE	614.3275	6	20 - 25
KFLDDDLTDDV	1294.593	11	79 - 89
KGVTLPE	742.4225	7	19 - 25
KGVTLPEW	928.5018	8	19 - 26
KILDSEG	760.3967	7	94 - 100
KILDSEGI	873.4807	8	94 - 101
KILDSEGID	988.5076	9	94 - 102
LAHKP	564.3384	5	105 - 109
LAHKPL	677.4224	6	105 - 110
LDDDLTDDV	1019.429	9	81 - 89
Peptides from k-casein (parent protein, accession number: F0V6V5_EQUAS)			
HPRPHPS	826.4198	7	63 - 69
NNQHMP	739.3072	6	25 - 30
TVIPK	556.3584	5	82 - 86
Peptides from lysozyme C (parent protein, accession number: LYSC_EQUAS)			
DKDLSE	705.3181	6	117 - 122
LLDDNIDDDI	1159.524	10	83 - 92
Peptides from serum albumin (parent protein, accession number: ALBU_EQUAS)			
AEEGPK	629.302	6	592 - 597
DDHPNLPK	934.4508	8	131 - 138
KDDHPNLPK	1062.546	9	130 - 138
KEDDLPSDL	1030.482	9	317 - 325
KEDDLPSDLPA	1198.572	11	317 - 327
LDEGYVPK	919.4651	8	516 - 523
LELDEGYVPK	1161.592	10	514 - 523
VKEDDLPSDL	1129.55	10	316 - 325
VKEDDLPSDLPA	1297.64	12	316 - 327

Sample raw donkey milk			
Peptides from beta-casein (parent protein, accession number: D2EC27_EQUAS)			
Peptide	**Mass**	**Length**	**Fragment**
VAPFPQPVVPY	1212.654	11	191-201
VAPFPQPVVPYPQ	1437.766	13	191-203
VAPFPQPVVPYP	1309.707	12	191-202
VAPFPQPVVP	1049.591	10	191-200
AQPPIVPF	867.4854	8	101-108
LYQDPQLGLT	1146.592	10	213-222
FVQPQPV	813.4385	7	74-80
LMLPSQPV	883.4837	8	176-183
VAPFPQPV	853.4697	8	191-198
AEPVPY	674.3275	6	85-90
YQDPQLGLT	1033.508	9	214-222
LTGEFDPA	848.3915	8	221-228
PLAQPPIVP	930.5538	9	99-107
MHQVPQ	738.3483	6	165-170
KSPIVPF	786.4639	7	134-140
KETLLPK	827.5116	7	120-126
FKHEGQQ	872.4141	7	55-61
VAPFPQ	657.3486	6	191-196
MLPSQPV	770.3997	7	177-183
AQPPIVP	720.417	7	101-107
KVAPFPQPV	981.5647	9	190-198
FKHEGQ	744.3555	6	55-60
FMHQVPQ	885.4167	7	164-170
PLAQPPIVPF	1077.622	10	99-108
KVAPFPQPVVP	1177.686	11	190-200
TLLPK	570.3741	5	122-126
SPIVPF	658.369	6	135-140
ETLLPK	699.4167	6	121-126
LYQDPQ	762.3548	6	213-218
LSPPQS	627.3228	6	184-189
VQPQPV	666.3701	6	75-80
LKSPIVP	752.4796	7	133-139

HQVPQ	607.3078	5	166-170
LIQPF	616.3584	5	160-164
AVVPQ	512.2958	5	91-95
LRLPV	596.4009	5	154-158
REKEEL	802.4185	6	16-21
LQPEIM	729.3731	6	109-114
LAQPPI	637.3799	6	100-105
Peptides from alpha-s1 casein (parent protein, accession number: CASA1_EQUAS)			
LIEDPEQQ	970.4607	8	63-70
IIQNEQDSpR	1181.508	9	11-19
IEDPEQQ	857.3766	7	64-70
ALHTP	537.2911	5	32-36
ALHTPR	693.3922	6	32-37
HPEIIQ	735.3915	6	8-13
YLEPFQPS	979.4651	8	148-155
TDIIPE	686.3486	6	196-201
LIEDPEQ	842.4021	7	63-69
VVNQEQ	715.35	6	139-144
LHTPR	622.3551	5	33-37
SPFHDTA	773.3344	7	179-185
Peptides from alpha-s2 casein (parent protein, accession number: CASA2_EQUAS)			
KTVDMESpTEV	1217.489	10	163-172
FTSpEEIPK	1029.442	8	155-162
FTSEEIPK	949.4756	8	155-162
TELTEEEKN	1091.498	9	177-185
TELTEEEK	977.4553	8	177-184
IVLTPW	727.4269	6	130-135
AKFPTEV	790.4225	7	69-75
TSEEIPK	802.4072	7	156-162
TSpEEIPK	882.3735	7	156-162
KTVDM	592.2891	5	163-167
LRQPR	668.4081	5	125-129
LFTSpEEIPK	1142.526	9	154-162
IPIVN	554.3428	5	146-150
alpha-s2 casein B (parent protein, accession number: C1L3G3_EQUAS)			

KTVDMGSpTEIFPEE	1661.69	14	87-100
IELSpDEEKN	1155.47	9	101-109
KTVDMGSpTEI	1159.483	10	87-96
IELSDEEKN	1075.503	9	101-109
IELSDEEK	961.4604	8	101-108
TVDMGSpTEIFPEE	1533.595	13	88-100
KTVDMGSpTEIFPE	1532.647	13	87-99
TVDMGSpTEIFPE	1404.552	12	88-99
KQDNNVA	787.3824	7	37-43
KTVDM	592.2891	5	87-91
QIPMSPW	857.4105	7	137-143
Peptides from beta-lactoglobulin I (parent protein, accession number: LACB1_HORSE)			
FKINYLDEDTVF	1502.729	12	100-111
VDEEIMEK	991.4532	8	146-153
LRPTPEDNLE	1182.588	10	64-73
RPTPEDN	827.3773	7	65-71
LRPTPEDNL	1053.545	9	64-72
LRPTPEDN	940.4614	8	64-71
DEEIMEK	892.3848	7	147-153
DSESAPL	717.3181	7	51-57
NAATPGQS	744.3402	8	127-134
RPTPEDNLE	1069.504	9	65-73
ALQPLPG	694.4014	7	157-163
QDLDLQE	859.3923	7	26-32
TNIPQT	672.3442	6	19-24
TNIPQ	571.2966	5	19-23
Peptides from beta-lactoglobulin II (parent protein, accession number: D6QX32_EQUAS)			
VEELRPTPEGN	1239.61	11	61-71
YVEELRPTPEGN	1402.673	12	60-71
VEELRPTPE	1068.545	9	61-69
QDPSGGQE	816.325	8	170-177
YVEELRPTPE	1231.608	10	60-69
RPTPEGN	769.3718	7	65-71
AEHGTV	612.2867	6	132-137
DSESAPL	717.3181	7	51-57

ALQPLPG	694.4014	7	158-164
QDLDLQE	859.3923	7	26-32
Peptides from alpha-lactalbumin (parent protein, accession number: LALBA_EQUAS)			
FLDDDLTDDV	1166.498	10	80-89
KILDSEGI	873.4807	8	94-101
DDDLTDDV	906.3454	8	82-89
KGVTLPEWI	1041.586	9	19-27
KILDSEG	760.3967	7	94-100
KGVTLPEW	928.5018	8	19-26
KGVTLPE	742.4225	7	19-25
LAHKP	564.3384	5	105-109
GVTLPE	614.3275	6	20-25
LAHKPL	677.4224	6	105-110
DNQILPS	785.3919	7	63-69
FQINN	634.3075	5	53-57
AHKPL	564.3384	5	106-110
Peptides from lysozyme C (parent protein, accession number: LYSC_EQUAS)			
LLDDNIDDDI	1159.524	10	83-92
KAQEM	605.2843	5	13-17
DDNIDDDI	933.3563	8	85-92
DKDLSE	705.3181	6	117-122
Peptides from serum albumin (parent protein, accession number: ALBU_EQUAS)			
LELDEGYVPK	1161.592	10	514-523
LKPEPDAQ	896.4603	8	139-146
ADFAEDK	794.3446	7	330-336
DDHPNLPK	934.4508	8	131-138
KDDHPNLPK	1062.546	9	130-138
KEDDLPSDLPA	1198.572	11	317-327
LDEGYVPK	919.4651	8	516-523
LKPEPDA	768.4017	7	139-145
QDSISGK	733.3606	7	291-297
AEEGPK	629.302	6	592-597