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DEPARTMENT OF AGRICULTURAL SCIENCE

PhD Thesis in

Agricultural and Food Sciences

XXX cycle

Study of the dynamics of microbial communities in fermented foods and selection and use of functional cultures

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Preface

Fermented foods and beverages have become part of the human diet through civilizations' culinary traditions all over the world. The fermentation has represented the most widespread way for food preservation since prehistory. Today is known that fermented foods from different food sources are characterized by complex microbial communities that play needful and specific role of pro-technological interest improving the food safety, the shelf-life, and the sensory profile of the end product. Moreover, in the last decades the demand by consumers for foods able to improve the human health is growing. In that context, fermented foods containing probiotic microorganisms cover a large slice in the sector of functional foods. With the purpose to satisfy goals of pro-technological and functional interest, microbial ecosystem of fermented foods has been object of study since many years (Bokulich and Mills, 2012). Today the investigation on microbial diversity of fermented foods through dependent-culture methods are becoming less common to make room for culture-independent methods. The latter are characterized by higher speed, accuracy and ability to detect the presence of non-dominant and/or viable-but-non-cultivable microbial groups during the fermentation and the ripening (Bokulich and Mills, 2012). The next generation sequencing (NGS) techniques fall in the category of culture-independent methods. NGS techniques are characterized by high-throughput sequencing after DNA or RNA extraction directly from food matrices, followed by the polimerase chain reaction (PCR) amplification of gene coding for rRNA 16S (Ercolini, 2013; Mayo et al., 2014; Polka et al., 2015). In recent years NGS techniques have greatly supported the study of microbial diversity, the evolution of the microbiota during food processing, the monitoring of selected starter and probiotic cultures and the survival of pathogen and spoilage microorganisms during food manufacturing and ripening, and the forecast of product shelf-life (Mayo et al., 2014). The overall aim of the research consists to improve fermented food quality, allowing the standardization of traditional fermented foods processing and at the same time maintaining the typical characteristics of the products. The goals were pursued through the study of microbial ecosystem of fermented foods and the monitoring of starter and pathogenic bacteria by NGS techniques, and the isolation and characterization of starter and probiotic cultures and their application in food matrices. The goals were achieved according to the research plan reported in **Figure 1**.

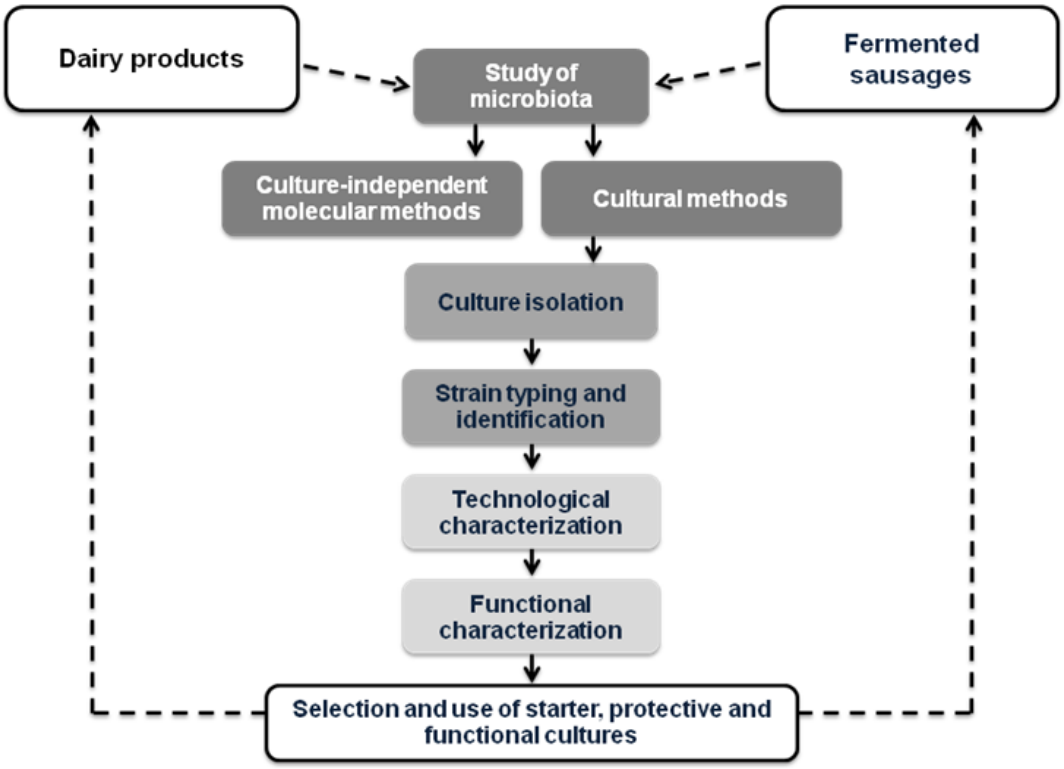

Figure 1. Flow chart of PhD thesis research project

The first step of the PhD project had the objective to study the microbial diversity and the evolution of the microbiota during fermented food production. On this purpose, the **Chapter 2** deals with the study of the dynamics of bacterial communities during manufacture and ripening of traditional Caciocavallo of Castelfranco cheese, and also to investigate if different cows' feedings could affect microbial evolution during cheese production, and sensory profile of the cheese. The study of the dynamics of cheese bacterial communities was carried out by both 16S rRNA gene pyrosequencing and viable counts.

The **Chapter 3** has as main topic the isolation and selection of novel lactic acid bacteria (LAB) strains from Caciocavallo of Castelfranco cheese. The isolated LAB were subjected to a first screening to determine the acid production in milk. Based on this attitude, the LAB were divided into starter LAB (SLAB) and non starter LAB (NSLAB). SLAB were assayed for activities of pro-technological interest: proteolytic and lipolytic abilities, diacetyl production, and decarboxylase activity. Low acidifying NSLAB were investigated to their probiotic and functional abilities: survival under simulated gastrointestinal tract conditions, radical scavenging activity,

bile salts tolerance and deconjugation ability, use of prebiotic fibers as alternative carbon sources, antibiotic susceptibility, and cholesterol-lowering activity.

In the **Chapter 4** *Lb. paracasei* LIL4T, one among the NSLAB strains isolated during Caciocavallo cheese production, was tested to assess its cholesterol lowering ability on human colorectal adenocarcinoma Caco-2 cell line, due to its promising attitude as probiotic strain and for its ability to assimilate the cholesterol in liquid media as showed in the Chapter 2. This study addressed through the accomplishment of four main activities: 1) evaluate the effect of *Lb. paracasei* LIL4T on the expression of genes involved in cholesterol transport and metabolism in Caco-2 cells; 2) cholesterol uptake by Caco-2 cell incubated with *Lb. paracasei* LIL4T; 3) effect of *Lb. paracasei* LIL4T on cholesterol efflux through apical and basolateral compartment of Caco-2 cells; and 4) the ability of *Lb. paracasei* LIL4T to adhere on Caco-2 monolayer, a crucial skill for probiotic bacteria.

The **Chapter 5** deals with studying the microbiota and monitoring the evolution of added starter cultures and the survival of pathogen and spoilage microorganisms during food manufacturing and ripening. Specifically, the Chapter 5 refers to the study on the inhibition of *Listeria monocytogenes* by bacteriocin producing *Lactobacillus curvatus* 54M16 as starter and protective culture. The first part of the work consisted to develop co-culture experiments by incubating *Lb. curvatus* 54M16 producer of sakacins X, T and P with *Listeria monocytogenes*. The growth capacity of the co-cultured bacteria and the antimicrobial power of *Lb. curvatus* 54M16 against the phatogen under different temperature and nutrient media composition were evaluated. Moreover, the behavior of *Listeria monocytogenes* and of *Lactobacillus curvatus* 54M16 co-inoculated in fermented sausages, and the potential impact on product safety were studied.

In conclusion, critical synthesis and conclusions of the main findings achieved from the entire research project were reported in **Chapter 6**.

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CHAPTER 1

Introduction

1.1 Study the microbiota of fermented foods: from culture-dependent methods to next-generation sequencing techniques

Fermented foods have widespread in diets of world populations since the born of human civilizations, representing 20% of the total food consumed in the world (Kwon, 1994). The fermentation method was born in ancient times with the necessity to preserve foods from spoilage and to extend their shelf-life. With time, the production of fermented foods increased due to their sensory and nutritional properties compared with the corresponding unfermented products.

The term fermented foods is generally referred to those products of different origin (animal or vegetal) and nature (liquid to solid) that are the result of controlled or spontaneous microbial and enzymatic activities. Several biochemical reactions are involved into the transformation of raw materials in products safe and organoleptically acceptable to consumers. According to Marco et al. (2017), the fermentation process can be classified on the basis of primary metabolites produced by specific microorganisms: alcohol and carbon dioxide by yeast, acetic acid by *Acetobacter*, lactic acid by *Leuconostoc, Lactobacillus* and *Streptococcus*, propionic acid by *Propionibacterium*, and ammonia and fatty acids by *Bacillus* and molds. Due to their complex microbiota, fermented foods are often the result of a combination of these biochemical transformations.

The progress in science and advances in foods technology act as a double-cut weapon for fermented foods production. On one hand, innovations in food production allow the diversification of fermented foods and the production of certain traditional fermented foods at larger scale by improving the formulation, and enhancing their biofunctionality (Tamang, 2010; Rao et al., 2006). On the other hand, the accelerated industrialization in food production cause an excessive standardization of fermented foods, reducing the diversity of bacterial communities and specific biochemical transformations, leading to the loss of typical organoleptic characteristics of traditional products (Marco et al., 2017).

Many interests and reasons led to study still today microbial ecosystems of fermented foods: follow natural fermentation process, control fermentation

dynamics, monitor the fate of starter cultures or probiotics, detect and monitor food-borne pathogens and spoilage microorganisms. Over the last decades, food microbial ecology has dramatically revolutionized the way to study the microbiota of fermented foods and these changes are in constant evolution (Solieri et al., 2013). Before 90s, the study of food microbial ecosystems was based on cultivation of microorganism using culture-dependent techniques that need to cultivate microorganisms before identify them. The cultivation-based methods are not able to identify the complete microbiota of foods as microbial cells are not always able to grow on synthetic media, determining failure or imprecise detection of some species or genera. The limitations of molecular methods based on the cultivation of microorganisms lead to underestimating microbial diversity. In fact, microorganisms in low concentrations may undergo the competition by dominant species that preventing the detection and subsequent identification of sub-dominant microorganisms. This could give a strongly simplified and distorted vision of fermented food ecosystems (Mayo et al., 2014; van Hijum et al., 2013). According to Cocolin and Ercolini (2008), most of these issues have been solved by the introduction of culture-independent methods based on the extraction of microbial DNA or RNA directly from food matrices without any previous cultivation steps. These techniques are characterized by higher speed, accuracy and ability to detect the presence of non-dominant and/or viable-but-non-cultivable microbial groups during the fermentation and ripening (Bokulich and Mills, 2012). The use of culture-independent techniques rather than culture-dependent methods has advantages and disadvantages. Our results showed that the use of both the approaches allows to: 1) describe the physiological potential of isolated microorganisms; 2) control the microbiota by culture-based methods; 3) characterize microbial communities and determine their diversity and abundance by using culture-independent techniques (Ndoye et al., 2011). In the field of culture-independent techniques, the study of microbial diversity in the last years has performed by using high-throughput sequencing (HTS) approaches through new sequencing techniques that are collectively known as next-generation sequencing (NGS) techniques. NGS techniques are characterized by high-throughput sequencing after DNA or RNA extraction directly from food matrix followed by PCR amplification of a “microbial barcode”. Microbial barcodes commonly used are 16S rRNA coding gene for bacteria, and ITS or 18S rRNA coding genes for fungi. These DNA regions are

chosen because have two fundamental requisites: (i) are contained in all microbial species to differentiate; (ii) contain conserved regions and hypervariable regions, which are often conserved within microorganisms of the same species but allow the discrimination among different species. Therefore, these characteristics led to widespread the use of these microbial barcodes determining a continuous enrichment of sequence databases. NGS techniques are distinguishable from others culture-independent methods in post amplification analysis methods, even though all culture-independent methods have the common goal to detect DNA heterogeneity. In NGS techniques, the direct sequencing of rRNA amplicons replace the electrophoretic approach of post amplification analysis that characterize fingerprinting-based ecology studies as the PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) (Cocolin and Ercolini, 2015). From direct sequencing of rRNA amplicons are obtained sequences identified by comparing them with sequences available in databases. After the identification, the sequences are collocated in a specific operational taxonomic unit (OTU) defined as cluster of sequences within a given similarity cut-off (Mayo et al., 2014) that represents a microbial entity. The number of sequences included into the same OTU defines the relative abundance of each microbial entity in the sample. Therefore, this approach allows to give a quantitative information about genera and species that coexist into food microbial ecosystem analyzed. NGS platforms involve mainly four types of technologies: 454 Pyrosequencing (Roche), Illumina (Illumina), SOLiD and IonTorrent (Life Technologies). These technologies differ from sequencing method, length of reads, number of sequences, accuracy and cost, and thus are chosen based on the complexity of fermented food to investigate and the purpose of the study. Following are briefly described the principles of 454 Pyrosequencing and Illumina that are two technologies used in this research work.

Pyrosequencing method is based on the dosage of the pyrophosphate released after the attachment of a deoxyribonucleotide triphosphate (dNTP) to the polymerized DNA strand. This sequencing technique consists of following main steps (**Figure 1.1**).

1. *Library construction.* The step consists on the PCR amplification of the DNA region target, such as hypervariable region V1-V3 of rRNA 16S gene, performed using specific primers named “fusion primers”. Fusion primers are complementary to the target region and contain: adapters-454 (A for forward primer, B for reverse

primer), a short key sequence, and a sample-specific barcode sequence defined MID (**Figure 1.2**). Barcoded amplicons are pooled by creating a single library and then used as templates for emulsion PCR (emPCR).

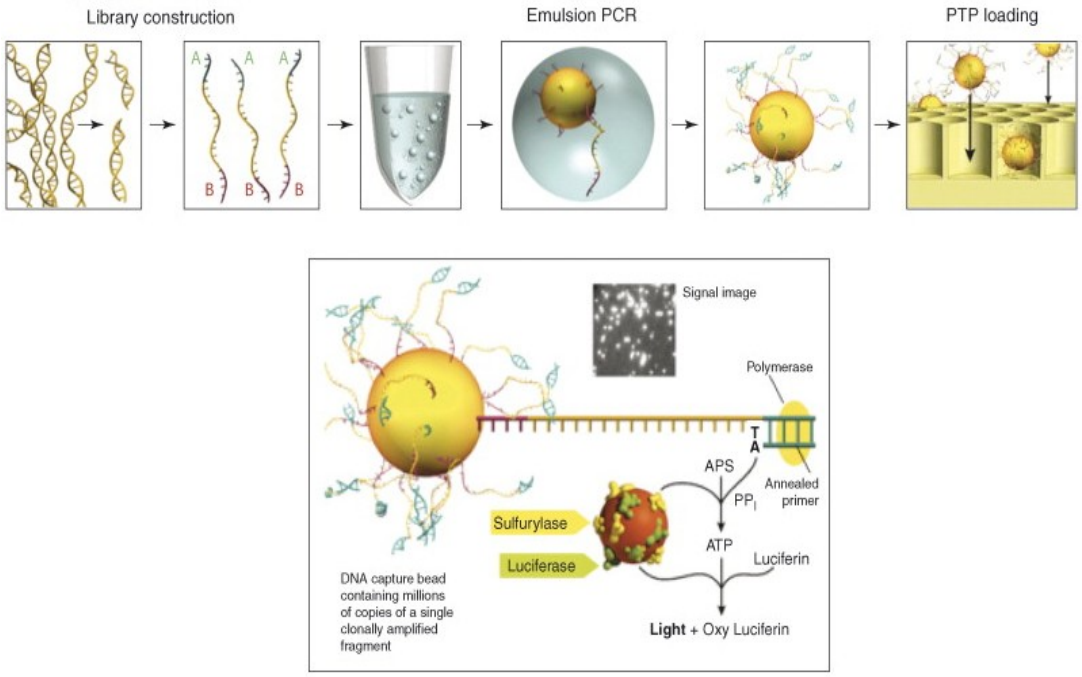

Figure 1.1. 454 pyrosequencing (www.roche.com). Library construction, emPCR, PTP loading and pyrosequencing reaction.

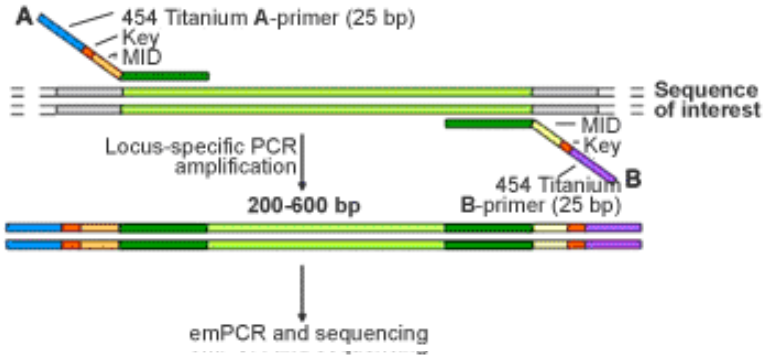

Figure 1.2. Fusion primer structure: adapters-454 (A for forward primer, B for reverse primer) - key sequence - MID.

2. *emPCR.* The amplicon library is subjected to a second amplification, where the emulsion in oil leads the formation of micro-reactors drops. Each micro-reactor contains one Capture Bead bound via adapter B to a single DNA template. At the end

of the process, each drop will have from 10 to 50 million copies of double-strand DNA (dsDNA) from the same fragment. After removing the beads without DNA, follows DNA denaturation of dsDNA and the recovery of Capture Beads with single-strand DNA (ssDNA).

3. *Pyrosequencing.* The beads are loaded into Pico Titer Plate (PTP) containing 1.6 million wells. Each well are able to contain one Capture Bead and reagents required for pyrosequencing. One of the four dNTPs is added cyclically on the surface of the PTP. DNA polymerase catalyses the addition of such base only if it is complementary to the DNA template causing the release of inorganic pyrophosphate (PPi). PPi produced is transformed into ATP by solforilase that uses ASP as substrate. The ATP allows the conversion of luciferin to oxyluciferine by luciferase producing a light signal detected by a photosensitive camera (CCD). Since the nucleotide order addition is known, it is possible to determine the sequence of DNA measuring the intensity of the light signal that is proportional to the number of nucleic acids incorporated. For example, a double light signal means that 2 nucleic acids added during the same cycle have been incorporated (repetition of the same base on the template). Conversely, a null signal indicates that the added NTP in that cycle is not complementary to the DNA template. At present, 454 pyrosequencing can generate up to 1.2 million reads longer than 1000 bp.

The Illumina Platform is based on the principle of reversible chain termination and the technique consists of three phases.

1. *Library construction.* Genomic DNA is fragmented and oligo-adapters are added to both the ends of each fragment for the subsequent amplification. The amplification takes place on a flow-cell, a plate on which specific oligonucleotides complementary to oligo-adapters are bonded at the ends of DNA fragments (**Figure 1.3**).

2. Bridge PCR. The oligo-adapters of DNA fragments bind the complementary oligonucleotide on the surface of the flow cell, forming bridge structures. At this point, DNA polymerase incorporates nucleotides to form double-stranded bridges, that are then denatured leaving a single-stranded template anchored to the substrate (**Figure 1.4**). After several cycles of the "Bridge PCR", millions of groups (clusters) are created, each with millions of copies of the same copy as the original DNA molecule and the reverse strand. Therefore, it is necessary clean the clusters from antisense fragments before proceeding with the sequencing.

3. Sequencing. Each sequencing cycle involves the DNA polymerase and the four dNTPs bonded to a fluorescent marker and to a reversible terminator. The marker reacts differently to the laser excitation when is bonded to each of the four dNTPs allowing the identification of the sequenced base. For each cluster on the flow cell, the emitted fluorescence is captured and collected in an image. After incorporation of dNTPs, the reversible terminator and the fluorescent marker are removed to enable the sequencing of the next base. Finally, all the reagents are washed away and new flux of four dNTPs bonded with a fluorescent marker and a reversible terminator are added, and the second base image is acquired (**Figure 1.5**).

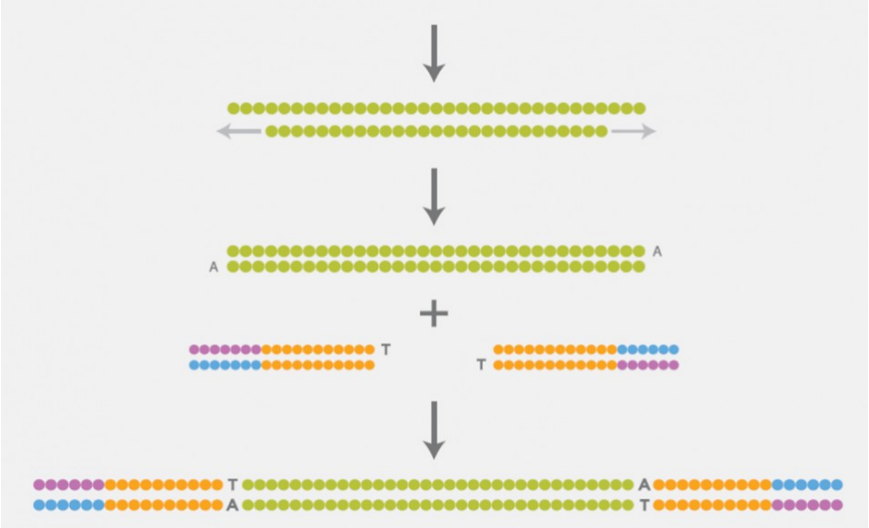

Figure 1.3. Preparation of DNA library. (www.illumina.com)

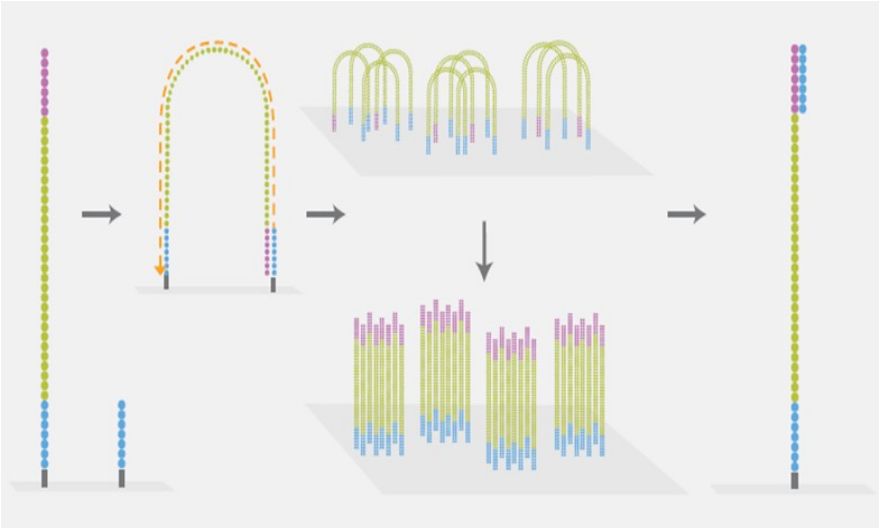

Figure 1.4. Clonal amplification. (www.illumina.com)

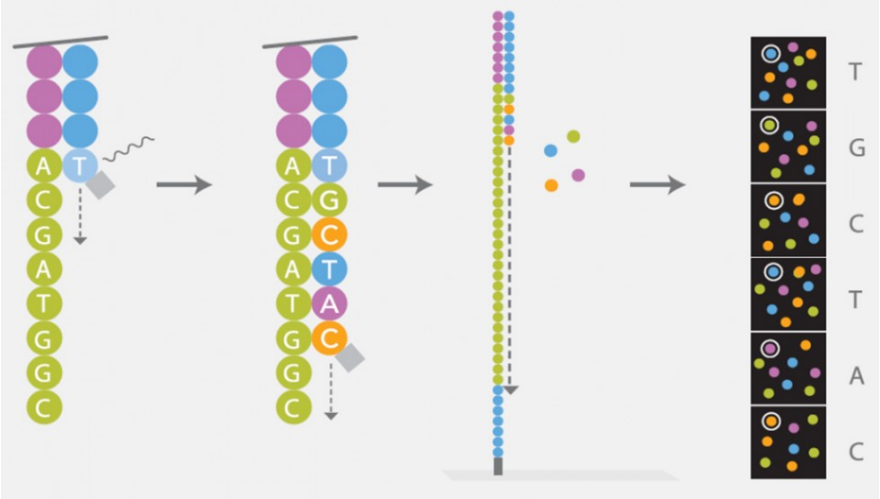

Figure 1.5. Sequencing. (www.illumina.com)

Compared to 454 pyrosequencing, the platform Illumina has lower cost, provides higher coverage by generating up to one billion of short reads (100 pb) characterized by low taxonomic resolution. Moreover, the platform Illumina is very likely to detect rare or underrepresented sequences (Bigot et al., 2015).

The **Table 1.1** summarizes main recent pubblications in which the composition and dynamics of bacterial ecosystems in cheeses and fermented meat products have been studied or re-approached by NGS techniques. Both 454 pyrosequencing and Illumina platforms were widely used to study the diversity and dynamics of bacterial populations during the manufacture and ripening of traditional cheeses, including Italian hard cheeses (De Filippis et al., 2014; Bassi et al., 2015); Italian semi-hard pasta filata cheeses (De Pasquale et al., 2014; De Filippis et al., 2016; Giello et al., 2017); Mozzarella cheese (De Filippis et al., 2014); Danish raw milk cheeses (Masoud et al., 2011); Irish cheeses (Quigley et al., 2012). Main findings of these projects highlighted the influence of the ripening stages on LAB species domination; while the study of Irish artisanal cheeses by 454 pyrosequencing allowed the detection of genera such as *Faecalibacterium*, *Prevotella*, and *Helcococcus* never found and associated before with traditional cheeses (Quigley et al., 2012). More are also works with the purpose to observe the influence of heat treatments on milk and curd or the influence of the temperature during the ripening, and on the composition and succession of cheese-associated microbiota. This is the case observed by De Filippis et al. (2016) where an increase of the abundance of *Lb. casei* and *Lb.*

buchneri directly related with the ripening temperature was observed. Moreover, the abundance of the mentioned NSLAB was significantly higher in the cheese core compared to the rind.

Table 1.1. Recent studies on microbiota of cheeses and fermented meat products by NGS approaches

Food sample	Sequenced region	Sequencing platform	Main results	Reference
Dairy products				
Caciocavallo Silano PDO cheese	V1-V3 16S from cDNA	Illumina	Ripening driven by NSLAB *Lb. casei* and *Lb. buchneri;* NSLAB increase with ripening temperature.	De Filippis et al., 2016
Mozzarella (M), Grana Padano (GP), Parmigiano Reggiano (PR) cheeses	V1-V3 16S; *lacS* gene from genomic DNA	454 GS Junior	GP-PR curd fermentation driven by *Lb. delbrueckii* and *Lb. helveticus; S. thermophilus* dominates M whey starters and curd.	De Filippis et al., 2014
Caciocavallo Pugliese cheese	V1-V3 16S from cDNA	454 FLX	*S. thermophilus* dominates curd; *Lb. casei* group drives the ripening.	De Pasquale et al., 2014
Caciocavallo of Castelfranco TFP cheese	V1-V3 16S from genomic DNA	454 GS Junior	*S. thermophilus* dominates from raw milk to cheese and *Lb. casei* group becomes the subdominant population at the end of ripening; reduction of *Pseudomonas* sp. after curd fermentation.	Giello et al., 2017
Danish raw milk cheeses	V3-V4 16S from genomic DNA and cDNA	454 FLX	Overall, the abundance of *S. thermophilus* decrease with the ripening; *Lactococcus* or *Lactobacillus* genera dominate in the final stage of ripening.	Masoud et al., 2011
Irish artisanal cheeses	V4 16S from genomic DNA	454 FLX	*Faecalibacterium*, *Prevotella*, and *Helcococcus* never detected before in cheese; influence of the animal source of milk and pasteurization on cheese microbiota.	Quigley et al., 2012
Spoiled Grana Padano cheese		Illumina	Cheeses with high abundance of *S. thermophilus* and *Lb. rhamnosus* spoiled by *C. tyrobutyricum*; abundance of *Lb. delbrueckii* among *Lactobacillus* spp, related to the spoiler *C. butyricum*; lysozime reduces *C. tyrobutyricum* in favour of *C. butyricum.*	Bassi et al., 2015
Fermented meat products				
Llama sausage	V3-V4 16S from genomic DNA	Illumina	Highest bacterial diversity in artisanal production; prevalence of *Lactobacillus*	Fontana et al., 2015

			and *Leuconostoc* in pilote production; artisanal production characterized by *Lactobacillus, Pseudomonas, Acinetobacter* and *Leuconostoc.*	
Salame Piacentino PDO	V3-V4 16S from genomic DNA	Illumina	*Lb. sakei* dominated the early ripening stage of salami with starter and the end product without starter; more homogeneous distribution of species in final salami with starter; high abundance of lactobacilli at first days of ripening (*Lb. coryniformis, Lb. curvatus, Lb. oris, Lb. graaminis, Lb. vaginalis*)	Połka et al., 2015
Fermented sausages	V3-V4 16S from cDNA	Illumina	Fermented sausages inoculated with bacteriocin producing *Lb. curvatus* strain showed lower levels of spoilage-associated genera: *Pseudomonas*, *Brochothrix*, *Carnobacterium*, *Psychrobacter;* lower levels of *Listeria* in fermented sausages co-inoculated with *Listeria* and *Lb. curvatus* compared to those inoculated only with *Listeria*.	Giello et al., 2018
Piemontese traditional salami	V1-V3 16S from cDNA	454 GS Junior	Highest abundance of *Lb. sakei* during the ripening; *P. fragi, Photobacterium sp., Brochothrix thermosphacta* found in meat batter and sporadically at low levels during the ripening.	Greppi et al., 2015

NGS techniques allow to highlight possible correlations among microbial species or genera and their mutual influence in the abundance or absence/presence when co-existing in the same ecosystem. Bassi and coworkers (2015) found an ecological relationships among species in spoiled Grana Padano cheese: high abundance of *S. thermophilus* and *Lb. rhamnosus* was correlated by the presence of *C. tyrobutyricum* as spoiler species, whereas cheese with high abundance of *Lb. delbrueckii* among *Lactobacillus* spp., was found related to the spoiler *C. butyricum*.

Current approaches consist also to check the efficacy of starter and protective cultures when added in both cheese and fermented meat products. On this purpose, Giello et al. (2018) found that fermented sausages produced by adding bacteriocin producing *Lb. curvatus* 54M16, showed lower levels of spoilage-associated genera such as *Pseudomonas*, *Brochothrix*, *Carnobacterium* and *Psychrobacter.* In the same

study, lower significant levels of the OTU corresponding to *Listeria* sp. in fermented sausages co-inoculated with *Listeria monocytogenes* and *Lb. curvatus* 54M16 were found, compared to fermented sausages contaminated only with *Listeria monocytogenes*. In another study, bacterial diversity in Salame Piacentino PDO, performed by Illumina platform, revealed the impact of starter cultures on bacteria dynamics during the ripening. *Lb. sakei* dominated the early ripening stage of salami produced by adding starter cultures, whereas salami produced without starter were dominated by *Lb. sakei* at the end of ripening only (Połka et al., 2015).

In this research project the use of both NGS techniques and culture-dependent methods to investigate the microbial diversity of fermented foods provided a complete overview of microbial populations that occur in fermented foods. Culture-independent methods, as NGS techniques, are able to describe the bacterial ecosystem at species level, which are hard to highlight using culture-based methods. Nevertheless, isolation and characterization are still needed to date because represent the unique way to select strains to use as starter, protective, functional and probiotic cultures in fermented foods. On this purpose, one of the goals of the research is focused on the screening of LAB strains and their selection for abilities of pro-technological, protective and probiotic interest for the industry of fermented foods.

1.2 Starter cultures in fermented foods

Microorganisms play a primary role in food transformations as they are able to modify physicochemical and organoleptic characteristics of raw materials thanks to their metabolic activity. Moreover, starter cultures ensure an extended shelf-life to end products, conferring aromatic characteristics appreciated by consumers.

By definition, starter cultures are composed by living and metabolically active microorganisms used to exploit their metabolism and to achieve specific technological goals. Starter cultures have been introduced in the industry of fermented foods to start fermentation and to ensure the technological outcome required. The selection of strains for the formulation of starter cultures has been based for a long time mainly on the ability of strains to carry out biochemical processes required by various processing technologies. In addition, starter cultures contribute to inhibit the growth of unwanted microorganisms such as spoilage and pathogenic microorganisms. The development of biotechnologies and the best

knowledge of the microbial metabolism involved in fermentation processes, have allowed to highlight the technological potential of strains used as starter cultures.

Starter cultures are formulated as single or mixed strains, especially for their adaptive ability to substrates or to raw materials. The selection of these strains is performed to evaluate: interactions between the selected culture and microbial populations of the product, the behavior of starter strains under process conditions, the interactions with food substrate on which starter cultures must act. Main objectives that starter cultures must reach to be used for fermented foods production, such as dairy and fermented meat products, comprise the growth inhibition of unwanted microorganisms (spoilers and pathogens), and the obtaining of specific organoleptic characteristics of the end product. Starter cultures are chosen following safety criteria, and technological and economic efficiency (Lòpez, 2000). In details, starter cultures are used in the production of fermented foods to: improve organoleptic characteristics; reduce processing time; standardize quality; ensure hygiene and safety.

Pure cultures, isolated from microbial populations of typical fermented foods exhibit a wide variety of metabolic activities that vary within each strain (Holzapfel, 2002), including:

- growth rate;
- ability to adapt to particular substrates as food matrices;
- ability to degrade antinutritional compounds;
- ability to exhibit antimicrobial activity;
- ability to produce aromatic compounds;
- ability to dominate autochthonous microflora.

Commercial starter cultures generally originate from food matrices or from the same fermentation process in which they are then applied. Therefore, environmental conditions, the ability to adapt, and the repeated use of specific utensils can contribute to the selection of microbial populations of typical fermentation processes.

LAB are naturally present in raw materials and are used during food productions as starter and/or protective cultures. Starter LAB are largely used in the production of a wide variety of fermented foods such as dairy products, fermented vegetable, fermented meat products, bakery products, contributing in various ways in determining characteristics and stability of end products. Most LAB species are considered Food-Grade organisms and the qualified presumption of safety (QPS)

status was attributed. LAB have several pro-technological abilities, closely related to their metabolic activity, such as:

- acidifying power to drive the fermentation process;
- lipolytic, proteolytic, thickening and flavoring activities to improve organoleptic characteristics;
- antioxidant, probiotic and antagonistic activities to ensure food safety and to promote health benefits.

LAB induce through the fermentation a rapid acidification of raw materials by the production of organic acids (Wood et al., 1995), mainly lactic acid and other compounds such as: ethanol; acetic acid (prevents spoilage and affects the aroma); exopolysaccharides (increase viscosity and improve the texture), bacteriocins (bacteriostatic and bactericidal actions). Microbial antagonism by LAB is realized not only by the competition for nutrients by microbial populations that living in the same food ecosystem, but it is also performed through the production by LAB of oligopeptides with antimicrobial action and named bacteriocins. Bacteriocins are oligopeptides with antimicrobial activity against different bacterial species, but not against the producer microorganism. Bacteriocins show bacteriostatic and bactericidal activities against pathogenic or alterative microorganism phylogenetically related to the producer microorganism (Burdock et al., 2004). Bacteriocins are biologically active at low pH values, are thermostable and their target is the cellular membrane of Gram-positive bacteria on which the formation of pores causes cellular lysis. The contact between the oligopeptide and the target microorganism is necessary so that bacteriocin shows antimicrobial action by bullet-target mechanism (1:1) with reduced spectrum of activity (Pèrez et al., 2014).

LAB play a crucial role during transformation and storage of dairy products, as they are able to drive the fermentation, to inhibit undesired and pathogenic microflora, and to show acidifying power, flavoring and probiotic activities. During milk fermentation, the decrease in pH affects various aspects of the cheese making process, such as hygienc quality, texture and flavor of the product. During cheesemaking, lactic acid production influences milk coagulation and the final cheese texture. Moreover, the decrease in pH by the production of lactic acid indirectly affects the aroma. In fact, the lactic acid influences the proteolytic activity

and other biochemical reactions involved in the formation of aromatic compounds. Aromatic molecules produced during the cheesemaking are the result of biochemical reactions that have as substrates main milk components: lactose, proteins and lipids (Marilley and Casey, 2004). Starter LAB cultures for dairy industry can be classified according to their function, their growth temperature and their composition.

Starter LAB can be classified as mesophilic or thermophilic depending on temperatures that occur during cheesemaking. Mesophilic LAB grow and produce lactic acid at optimum temperatures around 30°C although they tolerate maximum fermentation temperatures of 38-40°C. Main mesophilic LAB species used as starter cultures are*: Lactococcus lactis* subsp. *lactis, Lc. lactis* subsp. *lactis* biovar. *diacetylactis*, *Lc. lactis* subsp. *cremoris*, *Leuconostoc lactis,* and *Leuconostoc mesenteroides* subsp. *cremoris.* Thermophilic LAB grow at optimum temperatures around 42°C although they tolerate processing temperatures between 30-55°C and main species used in dairy industry are: *Streptococcus thermophilus*, *Lactobacillus delbrueckii,* and *Lactobacillus helveticus.* Both mesophilic and thermophilic starter LAB are used in association to produce some type of dairy products (Parente and Cogan, 2004). Their use in combination is characteristic of most Swiss cheeses and Italian cheeses such as Parmigiano Reggiano, Pecorino Romano, Provolone, Montasio and other cheeses where technology requires high cooking temperatures and high acidification of the curd (De Felip, 2001; Mucchetti and Neviani, 2006).

Nowadays, there is still the interest to select potential starter cultures among indigenous strains isolated from raw milk and traditional cheeses in order to preserve typical organoleptic characteristics and at the same time to standardize and increase cheese productions.

The use of starter LAB in fermented meat products has encountered many difficulties than those found in dairy industry, due to inhomogeneous and solid texture of the meat. Despite the presence of natural microflora in meat batter, spontaneous fermentations that characterize artisanal fermented sausages are carried out by an occasional or inadequate microflora that is not sufficiently competitive and vigorous. In fact, natural microflora can severely compromise sensory quality of fermented sausages by the production of acetic acid, giving undesiderable acidity to products, and of CO_2, that causes the formation of alveoli into the sausage. Moreover, natural and non-competitive microflora is not able to dominate the fermentation and to inhibit spoilage and pathogenic bacteria. LAB are used as starter cultures in

fermented sausages production because dominate the fermentation process having a faster growth capacity, that allow them to compete with other natural microflora genera. During the maturation of fermented sausages, the presence of salt, the consequent reduction of the water activity and the decrease of oxygen concentration, favor the development of *Micrococcaceae* and LAB (Comi et al., 1996). The fermented sausage is one of the most complex among food matrices, and thus starter LAB must be able to develop in typical ecological conditions of meat batter (Ammor and Mayo, 2007). Starter LAB are mainly selected for their ability to decrease rapidly the pH of the meat batter favouring: inhibition of pathogenic bacteria (*Listeria monocytogenes, Staphylococcus aureus, Salmonella, Escherichia coli),* improving food safety; inhibition of spoilage bacteria (*Pseudomonas*, *Enterobacteriaceae*) that allows to extend the shelf-life of the product and to preserve sensory characteristics; creation of environmental conditions that favor biochemical processes that develop molecules responsible of new sensory properties (Lücke, 2000). LAB perform their inhibitory activities, metabolizing carbohydrates and releasing natural preservatives such as alcohols, lactic acid and acetic acid, as well as CO_2 (Hansen, 2002). Lactic acid and other organic acids exert in non-dissociated form a bacteriostatic effect. In fact, organic acids penetrate into the bacterial wall and inhibit intracellular metabolic activities (Holzapfel et al., 1995) with particular effect against Gram-negative pathogenic bacteria (Holzapfel, 2002).
Starter LAB cultures for the production of fermented sausages are usually composed by most isolated species from meat products, such as *Lb. sakei*, *Lb. curvatus*, *Lb. plantarum*, *Lb. pentosus*, *Lb. casei*, *Pediococcus pentosaceus* and *Pediococcus acidilactici* (Ammor and Mayo, 2007). During sausage ripening the lysis of bacterial cells causes the release of intracellular enzymes with specific proteolytic activities that contribute to enrich the aromatic profile of the end product (Baruzzi et al., 2006). Moreover, starter LAB are able to deaminate amino acids and produce ethanol, acetone, diacetyl, volatile acids, and other alcohols that influence the aroma and flavor of the product (Alvarez-Martin et al., 2008).

1.3 Probiotic bacteria

According to the FAO/WHO definition, probiotics are identified as "live microorganisms which, when administered in adequate amounts, confer an health benefit on the host" (FAO/WHO, 2001). The term probiotic comes from Greek "*pro*"

+ "*bios*" and literally means "in favor of life" (Hamilton-Miller et al., 2003). At the beginning of the 20th century, the Russian biologist Elie Metchnikoff suggested that "the dependence of intestinal microflora on ingested food would allow to adopt specific measures in order to modify the intestinal microflora by replacing harmful bacteria with useful bacteria". In this regard, he assumed that the longevity of Bulgarian and Caucasian shepherds was attributed to the massive yoghurt consumption, as bacteria involved in the production of fermented lactic drinks inhibited the putrefactive fermentation at the intestinal tract level. Subsequent studies confirmed Metchnikoff's theory. Cohendy (1906) after the administration of acidified milk with *Lactobacillus delbrueckii* subsp. *bulgaricus* to subjects suffered of putrefactive intestinal fermentation, found a decrease in metabolites produced from putrefactive processes. In 1922 Rottger and Cheplin demonstrated that milk added with *Lb. acidophilus* had therapeutic effects on indigestion symptoms. Shortly after in the East, the Japanese scientist Shirota (1930) isolated one lactobacillus strain able to survive after gastrointestinal transit. The strain was identified as *Lb. casei* Shirota and has been used for the production of fermented milk drink marketed as "Yakult" since 1935. The term probiotic, with the current meaning, has been used since 1991 when the scientist Fuller used it to indicate "a microbial supplement that added to the food favors the host by improving its intestinal balance" (Fuller, 1991).

To gain probiotic status, microorganisms must satisfy safety, functional and technological criteria (FAO/WHO, 2001), and the principal of them are showed in **Figure 1.6**. The essential criterion for the selection of probiotic microorganisms is the assessment of their safety for the consumer and assignment of QPS microorganism appellation. For this purpose, an accurate taxonomic identification (species/strain) is needed. According to Saarela et al. (2000) in order to ensure safety, probiotics should must be common inhabitant of the target organism and, therefore, they must be of human origin. However, the use of certain yeasts belonging to *Saccharomyces cerevisiae* species proved that microbial strains of non-human origin may also be used as probiotics and may show beneficial effects to human health (Ouwehand et al., 2002). Selection criteria include that probiotic microorganisms must do not carry transmissible antibiotic resistance genes. In addition, bacterial cells in adequate number must be able to: survive after gastrointestinal transit; tolerate gastric juice, bile acid, and duodenal juice; be

resistant to digestive enzymes action; adhere and colonize the epithelial surface of gastrointestinal tract.

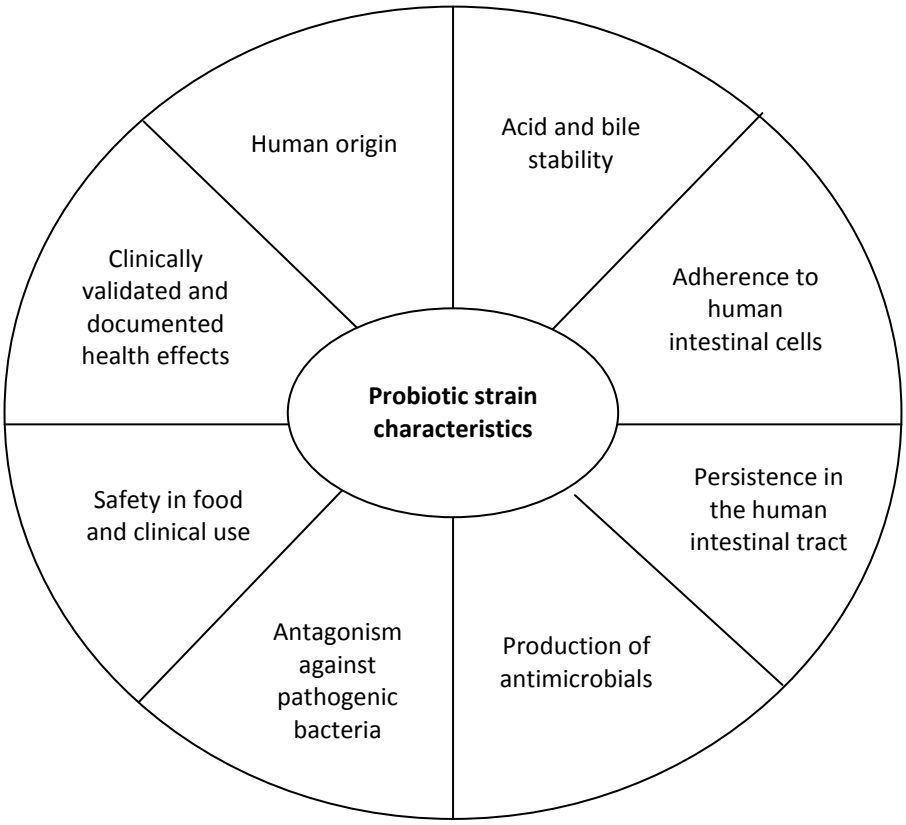

Figure 1.6. Principal criteria to select probiotic strains for human use (Saarela et al., 2000).

To exert positive effects on human health, assumed probiotic microorganisms must be alive, non-inactivated, and ingested at concentration at least of 10^9 colony forming units (cfu) per day, although their intake amount is not mentioned in the FAO/WHO definition. In regards to functional aspects, probiotics must show the ability to exert one or more health benefits clinically documented such as:

- Prevention of infections by interference, exclusion or antagonism against pathogenic microorganisms (Saarela et al., 2000; Ouwehand et al., 2002);
- production of antimicrobial compounds such as bacteriocins, hydrogen peroxide, organic acids (Ouwehand et al., 1999);
- prevention/reduction of intestinal inflammatory response through regulation/modulation of local immune response (Ouwehand et al., 2002; Saarela et al., 2000);

- prevention/reduction of certain adverse reactions to foods such as alleviation of symptoms from lactose intolerance (Ouwehand et al., 2002);
- anticarcinogenic and antimutagenic activity (Saarela et al., 2000);
- serum cholesterol lowering activity (Ooi and Liong, 2010);
- reduction in the incidence and duration of diarrhea (Hickson, 2011; Goldenberg et al., 2013);
- maintenance of the intestinal mucosa integrity (Klaenhammer and Kullen, 1999);
- improve the bioavailability of minerals (Scholz-Ahrens et al., 2007).

Functional activities of probiotics and related health benefits due to their consumption are schematized in **Figure 1.7**.

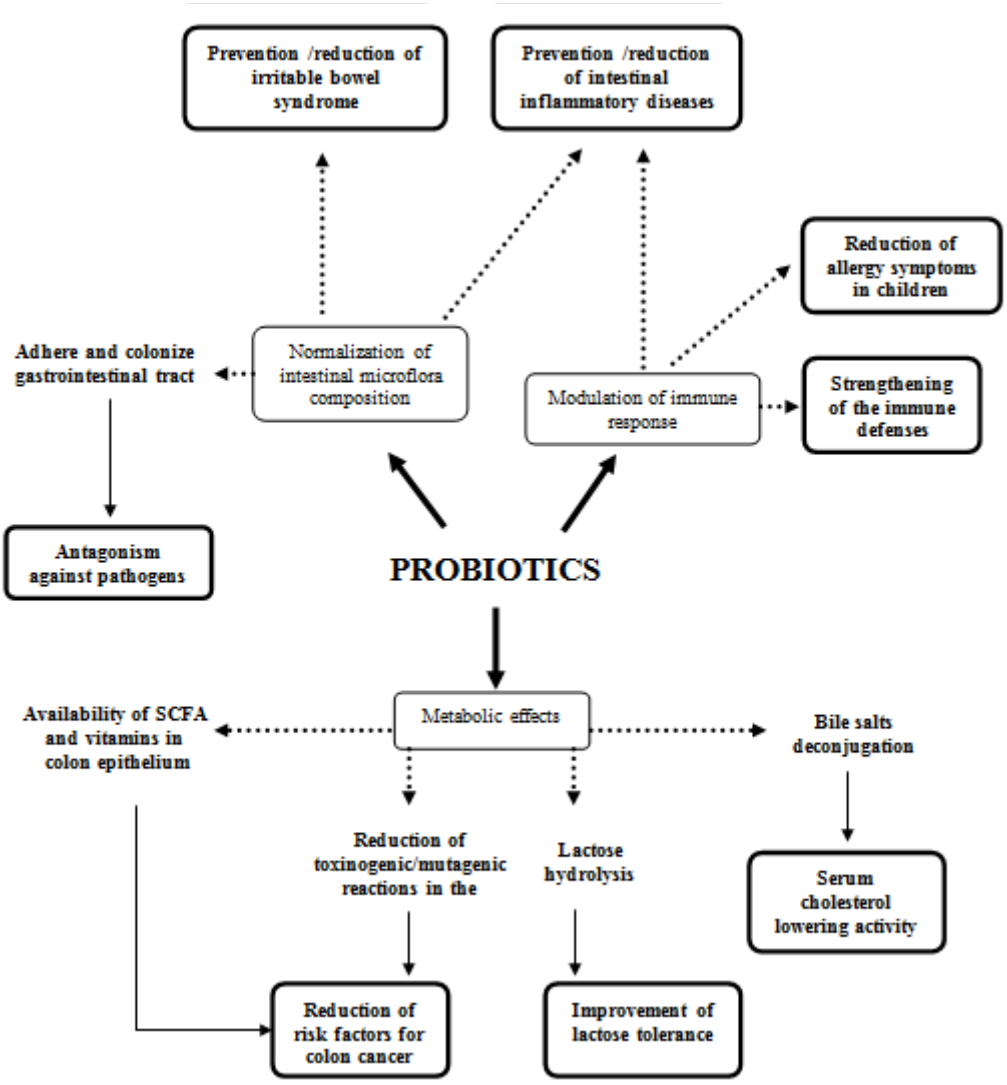

Figure 1.7. Health benefits after the consumption of probiotics (Saarela et al., 2000).

Microbial strains used as probiotics belong to several genera: *Lactobacillus, Bifidobacterium, Propionibacterium, Bacillus, Enterococcus, Saccharomyces* (Ouwehand et al., 2002). Among these genera, probiotic *Lactobacillus* strains show good resistance to *in vivo* stress and show desirable technological properties. These features explain the frequent use of *Lactobacillus* strains since early approaches to probiotic foods. The **Table 1.2** shows the most used probiotic *Lactobacillus* strains for the production of main yogurts, fermented milk drinks and of food supplements. *Bifidobacteria* strains are commonly used as probiotics although less than lactobacilli. In fact, *Bifidobacteria* strains are sensitive to the oxygen and are nutritionally exigent, thus more difficult to use in probiotic foods. Benefits attributed to probiotic bacteria are not species-specific but depend strictly on the strain (Ouwehand et al., 2002).

Table 1.2. Most used probiotic *Lactobacillus* strains and their functional characteristics (Villani, 2016)

Functional properties and clinical effects	***Lb. johnsonii* LA-1**	***Lb. casei* Shirota, *Lb. casei* 114-001 Immunitas**	***Lb. rhamnosus* GG**
Human origin	[a]+	+	+
Acid and bile tolerance	+	+	+
Production of antimicrobial compounds	+	+	+
Adhesion to intestinal mucosa	+	[b]+/-	+
Colonization of intestinal epithelium	+	+/-	+
Diarrhea prevention	+	+	+
Modulation of intestinal microflora	+	+	+
Reduction of symptoms from lactose intolerance	+	+	+
Commercial product	Yogurt LC-1, food supplements	Fermented milk drink Yakult (Shirota), drinking yogurt Actimel (Immunitas)	Drinking yogurt, food supplements

a: +, possessed property; b:+/- variable property among strains

1.3.1 Benefits of probiotics on human health

Main beneficial effects on human health after the ingestion of probiotic strains are summarized in the **Figure 1.7**. The gastrointestinal tract hosts a complex microflora that influences the host's health through several functions such as the prevention of intestinal infection and the digestion enhancement. Microorganisms ingested with food cross the lower intestinal tract through the mouth and during their transit are exposed to stress factors that affect their survival. In the stomach, ingested microorganisms undergo cellular stress due to the environmental pH (about 2), whereas bile salts secreted in the small intestine reduce cell viability causing the lysis of the cell membrane (Succi et al., 2005). The survival to gastrointestinal tract is the first requirement that a probiotic strain must have to reach the intestine alive and metabolically active and to carry out activities and related health benefits discussed below.

Modulation of immune response

The gastrointestinal tract plays a key role in the immune response. Structures responsible of these functions are represented by the lymphoid tissue distributed in the intestinal mucosa known as GALT (Gut Associated Lymphoid Tissue). The adhesion and colonization of the intestinal epithelium by probiotics determines the stimulation of mechanisms linked to local production of IgA and of IgM that reinforce systemic immune responses. Probiotics influence lymphoid cells through two types of interactions that are correlated or unrelated to the adhesion ability of bacterial cells. Such interactions can be established between the lymphoid tissue and the microbial cell and/or its fragments and/or its metabolites produced *in situ* (Ouwehand et al., 1999). The immunomodulation is related to the adhesion ability, as probiotic bacteria link phagocytic cells such as macrophages and neutrophils. For example, macrophages possess specific receptors capable of recognizing galactose residues on the surface of the bacterial cell stimulating phagocytosis process and thus the immune response. The immunomodulation not related to the adhesion capacity of probiotics could be induced by: peptidoglycan fragments from the digestion of the bacterial cell; modulating intestinal microflora; modifying intestinal permeability (Ouwehand et al., 1999). Studies on various probiotic strains have shown their immunomodulatory capabilities. Strains *Lb. johnsonii* LJ1 and *Lb. salivarius* UCC118 were able to stimulate IgA production in the intestinal mucosa and to

increase phagocytic activity. Moreover, *Lb. rhamnosus* GG and *Bf. lactis* Bb12 significantly improved clinical symptoms in children with food allergy (Saarela et al., 2000).

Cholesterol-lowering activity

Epidemiological studies have shown that blood cholesterol levels higher than recommended normal levels are associated with the development of cardiovascular disease (CVD) recognized as major causes of mortality in Western countries. Clinical studies report that the increase of 1 mmol/l above the normal blood cholesterol level (> 5.2 mmol/l) causes an increase of CVD risk by 35%, while reductions of 1% of blood cholesterol reduce CVD risk by 2-3% (Miremadi et al., 2014). There are drugs such as statins that can effectively reduce cholesterol levels in the blood, but they are responsible for several side effects. For this reason, the use of functional foods containing probiotic microorganisms has been promoted and widespread thanks to the ability of probiotics in preventing CVDs by reducing blood total cholesterol (Miremadi et al., 2014). Mechanisms that explain the ability to reduce blood cholesterol levels by probiotic LAB are proposed by Tahri and coworkers in 1995. In the study, microorganisms were able to low cholesterol levels by two principal mechanisms: the absorption of cholesterol on bacterial surface and the co-precipitation of cholesterol with deconjugated bile salts. Certain LAB strains are able to deconjugate bile salts through some active BSH (bile salt hydrolase). Bile salts in deconjugated form are less soluble and less absorbable by the intestine, thus they are rapidly excreted out from the intestinal tract. To replace excreted bile salts, liver-like synthesis of bile salts increases starting from cholesterol that is the natural precursor of bile salts; consequently, levels of blood cholesterol decrease.

Prevention/alleviation of symptoms from allergic diseases of food origin

In the first years of life, the endogenous intestinal microflora is the main stimulus to the development and maturation of an efficient immune system in humans (Isolauri et al., 2001). In Western countries, cases of allergic disorders are increasing, especially in children. Among the causes, excessive hygiene of Western populations would cause a reduced exposure to microorganisms and, consequently, a reduced appearance of infectious phenomena in the early years of children's life. This led to a low maturation of both immune system and gastrointestinal barrier in children, causing inflammation and alteration of the permeability of the intestinal mucosa

(Salminen et al., 1998). Consumption of probiotics has been shown to be effective in relieving inflammatory responses due to food antigens in allergic people. Such benefit may be due to the stimulation by probiotics on the production of certain anti-inflammatory cytokines and to control allergy inflammation at intestinal level (Ouwehand et al., 2002). In addition, potential prevention of atopic dermatitis by probiotic bacterial action has been demonstrated. In an important study, probiotic microorganisms were administered for 6 months, before and after the birth, to children with high risk of atopic disease. In these subjects, the prevalence of the disease was reduced by 50% compared with children that received placebo treatment. (Ouwehand et al., 2002).

Antagonism against pathogens

In order to exert positive effects on intestinal flora, probiotic strains should be able to show antagonistic activity against pathogenic bacteria as *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* through different mode of action:

- production of antimicrobial compounds such as organic acids, hydrogen peroxide, bacteriocins. The most widely produced antimicrobial substances are organic acids, particularly lactic acid and acetic acid; other low molecular weight substances such as hydrogen peroxide, diacetyl, carbon dioxide and secondary metabolites can show a broad inhibition spectrum against pathogenic bacteria such as *Salmonella, Escherichia coli, Clostridium* and *Helicobacter.*
- competitive mechanisms such as competing for nutrients and co-aggregation ability. The latter represents the ability of probiotic cells to bind pathogen cells; in this way probiotics are more able to inhibit pathogens, producing antimicrobial substances close to pathogen cells (Reid et al., 1988).

Treatment of inflammatory bowel disease

Inflammatory bowel disease (IBD) includes disorders characterized by inflammation, ulcerations and abnormal narrowing of the gastrointestinal tract, resulting in abdominal pain, diarrhea and gastrointestinal bleeding (Hanauer, 2006). Crohn's disease and ulcerative colitis are the main examples of IBD. In healthy people, the immune system exhibits tolerance to the endogenous microflora at intestinal mucosa level, reacting only when pathogenic microorganisms proliferate excessively (Marteau and Boutron-Ruault, 2002). In susceptible individuals, IBD emerges when the immune system is not able to tolerate the endogenous microflora, causing chronic

obstructive wounds and inflammation of the intestinal mucosa (Marteau and Boutron-Ruault, 2002). Several preparations based on probiotic microorganisms including lactobacilli, bifidobacteria, *Escherichia coli* or *Saccharomyces,* on both humans and animals with IBD have been tested. The positive effect of tested probiotics appears to be due to both *in vivo* expression of some intrinsic metabolic properties and to the ability to modulate endogenous microflora and to stimulate the immune system (Shanahan, 2002; Marteau and Boutron-Ruault, 2002).

Reduction of risk factors for colon cancer

In the last decades the attention to antimutagenic and anticancerogenic activities of probiotic microorganisms is constantly growing. Mutagenic compounds are commonly ingested with foods or they can be produced under certain stress conditions caused by viral or bacterial infections. Some epidemiological researches have shown a correlation between the intake of fermented milk-based foods and the reduced incidence of colon cancer (Hirayama and Rafter, 2000). Antimutagenic and anticancerogenic properties of probiotic bacteria, ingested with foods or constituting gastrointestinal microflora, have been studied since many years. Mechanisms by which probiotic bacteria can prevent the onset of colorectal cancer are (Scheinbach, 1998):

- suppression of carcinogenic and of pro-cancerous compounds through link, block and removal by probiotics;
- growth inhibition of bacteria able to convert procarcerogenic compounds into carcinogens molecules through their enzymatic activity, such as some species belonging to *Bacteroides* genus;
- lowering intestinal pH resulting in modification of the activity of the microflora and the solubility of bile acids;
- influence on the transit time of faeces in the colon, resulting in faster expulsion of the mutagenic molecules present in the stools;
- immune system stimulation.

Probiotic lactobacilli seem contribute to the reduction of faecal levels of certain mutagenic bacterial enzymes as β-glucuronidase, nitroreductase and azotoreductase. LAB show lower fecal enzymatic activity than coliforms, clostrides and bacteroides. Moreover, LAB can inhibit the growth of bacteria whose enzymatic activity converts

procarcerogenic compounds into carcinogens molecules by competing with such microbial groups and by producing acids, peroxides and bacteriocins.

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CHAPTER 2

Dynamics of bacterial communities during manufacture and ripening of traditional Caciocavallo of Castelfranco cheese in relation to cows' feeding

2.1 INTRODUCTION

In the world there are thousands of varieties of cheese produced both industrially that at artisanal level (Fox et al., 2004). Italy has a long history in the production of a large variety of cheeses some of which have been awarded Protected Designation of Origin and Protected Geographical Indication labels (http://europa.eu). In South of Italy, there are many small producers making artisanal semi-hard pasta-filata cheeses that fall under the common name "Caciocavallo". These products, which are prepared without using selected starter cultures to ensure their characteristic organoleptic properties, are not usually produced on a large-scale basis but, rather, are sold on local markets as "traditional products". Like most cheeses, traditional Caciocavallo is the result of biochemical, microbiological, physical, and sensorial changes occurring in raw milk during curd fermentation and cheese ripening in appropriate environmental conditions. However, different production technologies exist, that differ in the origin of the raw material (from cows, goats, buffalos and ewes), starter cultures (Natural Whey Cultures (NWCs) or selected starter or without starter addition) curd (whether cooked or not) and cheese ripening conditions (from a few months up to one year in defined or not defined conditions of temperature and relative humidity) that leading to different types of Caciocavallo (De Angelis and Gobbetti, 2011). Regardless from the milk origin, these ripened cheeses are traditionally fermented by the use of NWCs prepared from drained whey of a previous manufacture that was incubated at temperatures ranging from 45°C to 10°C up to 24 hours. Gobbetti et al. (2002) have reported that microbial composition of the NWCs used for the manufacture of Caciocavallo Pugliese was composed of the species *Lactobacillus delbrueckii*, *Lb. fermentum*, *Lb. gasseri*, *Lb. helveticus*, *Streptococcus thermophilus* and *Weissella viridescens*. Culture-independent analysis of sixty-three NWCs for the manufacture of Caciocavallo Silano cheese revealed the occurrence of mainly thermophilic lactic acid bacteria such as *Lb. delbrueckii*, *Lb. helveticus* and *S. thermophilus* and occasionally of *Lactococcus lactis* (Ercolini et al., 2008). These complex consortia of multi-strain microorganisms are considered of

great importance for the typical flavour and aroma of traditional products (De Filippis et al., 2014; Parente and Cogan, 2004). During traditional Caciocavallo making procedures, curd ripening takes place under whey until the conditions of pH make the curd suitable to be stretched in hot water. In many cases, the curd is cooked by heating whey at temperatures up to 60°C. The oval flask-like shaped cheese was salted in brine and commonly ripened for 1-3 months.

As previously published in many studies on conventional and molecular microbiology, fermentation and ripening of Caciocavallo cheese are processes dominated by lactic acid bacteria (LAB). Moreover, it is well known that many genera and species belonging to the group of starter LAB (SLAB) or non starter LAB (NSLAB) are involved in the development of texture, colour, flavour and favouring the hygienic condition of the product, inhibiting pathogenic or spoilage microorganisms by acidification or by production of antimicrobial compounds (De Pasquale et al., 2014; Di Grigoli et al., 2015; Gobbetti et al., 2002; Piraino et al., 2005; Settanni et al., 2012).

Until a few years ago, most of the studies that described the succession of microbial groups during milk fermentation and cheese ripening relies on culture-dependent methods associated with a variety of molecular techniques for genetic identification and differentiation of community member, by isolation, on suitable culture media, of the cultivable microorganisms that are not always representative of the complex cheese microbiota ecosystem (Neviani et al., 2013).

Over the years, there has been significant progress in the analysis of microbial communities without their cultivation, through the application of techniques such as PCR-DGGE (Ercolini, 2004) until the current and emerging high-throughput sequencing (HTS) of 16S rRNA gene amplicons, which it is much more sensitive for detecting the entire microbial community including sub-dominant populations (Ercolini, 2013).

By few years is spreading a deep remake of microbial diversity by HTS of many cheeses that were previously studied by culture dependent and independent methods (Ercolini et al., 2012; De Filippis et al., 2014; Masaud et al., 2011; Quigley et al., 2012) such as Caciocavallo Pugliese (De Pasquale et al., 2014).

Deep-sequencing approach allows a reliable knowledge of dynamics of cheeses microbial communities that is of great importance particularly for traditional cheeses manufactured from complex raw materials, where standardization is needed to assure

the achievement of technological objectives in the respect of their typical characteristics.

Composition of raw milk microbiota can be influenced by the combination of several factors including seasonal variation (Callon et al., 2007), outside and inside grazing animal feeding systems (Hagi et al., 2010), geographical farm location (Bonizzi et al., 2009), bedding and milking procedures. Consequently, according to cheese varieties and cheesemaking parameters (partial skimming, thermal treatments, type of starter, time and temperature of acidification and ripening), the conditions under which milk is produced might affect the final quality of cheese.

In an increasingly competitive market of dairy products, small dairy farms forced to pursue competitive strategies focused on product quality. In this context, the traditional food product (TFP) represent an important opportunity. *Caciocavallo of Castelfranco* is designated as an Italian "pasta filata" cheese that has been recognized as TFP (MIPAF Ministerial Decree 07/18/2000, confirmed by MIPAF Ministerial Decree 05/08/2001 and 14/06/2002).

On the basis of these premises, the objective of this work was to study the dynamics of bacterial communities during manufacture and ripening of traditional Caciocavallo cheese and to evaluate the possible correlation between cheese microbiota and silage and hay cows' feeding.

2.2 MATERIALS AND METHODS

2.2.1 Cows' feed and experimental design

The experiment took place in a dairy cattle farm (useable agricultural area of 50 ha) located in Fortore Mountains (Benevento, Campania region, southern Italy). The farm raised 70 Italian Friesian cows and produced Caciocavallo of Castelfranco cheese exclusively from milk produced on farm.

On May 2014, a 7-ha dryland field (690 m asl) was sown with *Sorghum sudanese* (cv Hermes, Hi-Gest technology, Alforex®). At harvesting time, four months later, forage from 3.5 ha was cut, dried in the field for 3 days (25.5% of dry matter) and preserved as hay in large (400 kg) round bales. Due to light precipitation and cloudy conditions during the harvest period, the forage remained in the field 4-5 days. Forage from the other 3.5 ha was cut by using a tractor-mounted mower-conditioner and immediately ensiled (20% DM) in plastic bags (700 kg) without additives. The bales and the bags were stored in covered barns until the feeding trial started (March 2015).

Forty-four lactating Holstein-Friesian cows were divided into Silage and Hay groups balanced for days in milk (169±116 vs. 165±109 d, respectively for Silage and Hay groups), milk yield (23.4±8.2 vs. 23.7±7.4 kg/head/d), milk fat (4.4±0.8 vs. 4.5±0.9) and milk protein (3.4±0.5 vs. 3.6±0.4). Cows' groups were bred in two separated free stall pens with concrete floor and equipped with feed manger, drinker, and covered paddock. Cows were milked twice per day (05:00 and 17:00) and managed for feeding likewise. Hay group was fed a total mixed ration based on sorghum hay, whereas cows belonging to Silage group were fed by sorghum silage-based ration in which 6 kg of sorghum hay were added in order to ensure an adequate level of fibers and to avoid the risk ruminal acidosis (Agabriel, 2007). Silage and Hay diets compositions are reported in **Table 2.1.**

Hay and Silage feeds samples were ground in an hammer mill with a 1-mm screen to determine Dry Matter and Crude Protein (N*6.25) according to procedures of the AOAC (2000). Neutral detergent fiber (NDF) was estimated accordingto Van Soest et al. (1991), and then corrected for residualacid-insoluble ash. Feeds energy values were determined according to INRA (1989), as Milk Forage Unit. Cows were fed once per day (15:00) providing approximately 10% orts for ad libitum consumption. During the day, an unlimited access to the feed was ensured by re-approaching the cows to the ratio several times. Cows were fed 10 days with the proper diet before to

bulk morning milk on three consecutive days. Milk samples were collected separately for Hay and Silage groups.

Table 2.1. Ingredients, chemical composition (mean ±SD) and energy value (as Milk Forage Unit) of diets (Giello et al., 2017)

Diet	Ingredient[a] (kg/head/d as-fed)	Chemical Composition
Hay		
Sorghum silage	0	-
Sorghum hay	11.0	-
Commercial concentrate	12.0	-
Crude protein (% DM[2])	-	17.3±0.14
Neutral Detergent Fibre (% DM)	-	35.1±0.21
Milk Forage (Unit/kg DM)	-	0.89±0.01
Silage		
Sorghum silage	22.0	-
Sorghum hay	6.0	-
Commercial concentrate	12.0	-
Crude protein (% DM[b])	-	16.5±0.11
Neutral Detergent Fibre (% DM)	-	39.2±0.57
Milk Forage (Unit/kg DM)	-	0.85±0.01

a: the source for dietary ingredients took place in a dairy cattle farm (useable agricultural area of 50 ha) located in the Fortore Mountains, Benevento, Campania region, southern Italy.
b: DM, Dry Matter

2.2.2 Cheese manufacturing and sampling schedule

Caciocavallo of Castelfranco was produced with raw milk from cows fed with hay and silage based diets. Cheese manufacture was carried out in the industrial plant located in Castelfranco in Miscano (Benevento, Campania region, Italy). For each of two types of diet, three cheeses making trials (H: hay cheeses and S: silage cheeses) were carried out during three consecutive days. Each trial was performed in duplicate by using identical cheesemaking techniques.

The cheesemaking process is reported in **Figure 2.1**. Vats were filled with about 100 L each of raw milk. After heating at 37°C, the milk was inoculated with 1% of natural whey culture (NWC) and added of liquid calf rennet (20 mL/100 L). NWCs were obtained from a previous day of cheesemaking from milk of H and S diets by storing the whey at room temperature until its use.

A first coarse cut of the curd was carried out after milk coagulation (about 30 minutes), followed by a partial extraction of the whey. After about 10 minutes the curd was cut to obtain particles of about 1 cm and cooked at 50°C per 30 minutes. About 60% of whey was extracted and the curd was allowed to mature under whey at

37°C until reaching pH suitable for the manual (mechanically) stretching in hot water at 80°C. Produced cheeses (about 3 Kg) were salted in brine (25% NaCl) for 36 hours and then ripened at 16-17°C and relative humidity of 60-70% for 60 days.

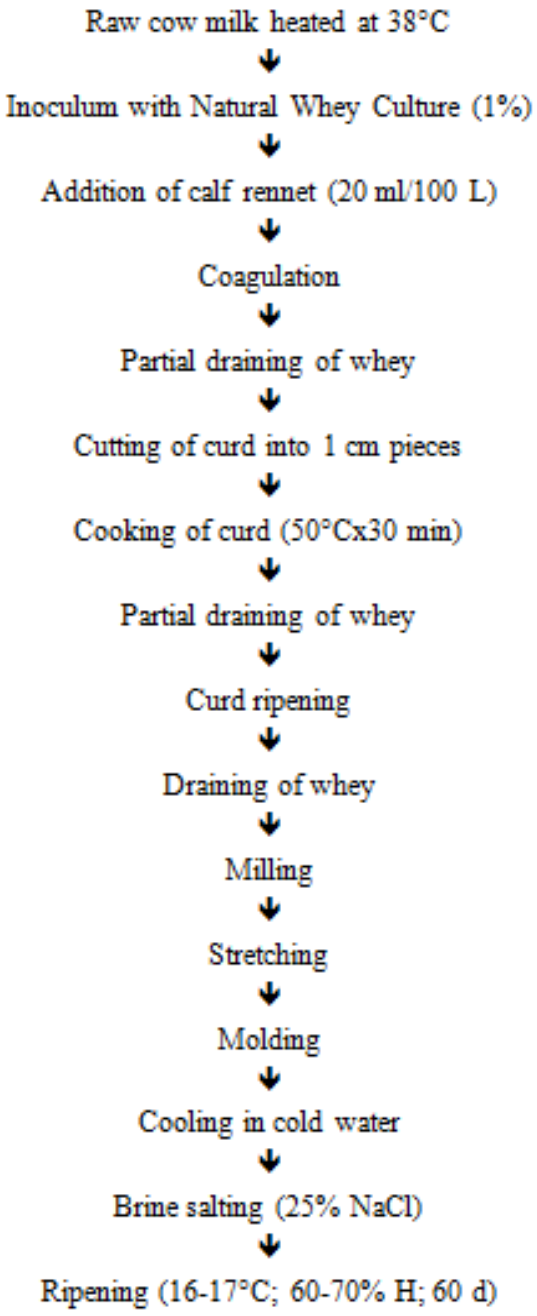

Figure 2.1. Manufacturing procedure for Caciocavallo of Castelfranco cheese (Giello et al., 2017).

For each trial, two replicates of the following samples were collected: hay and silage milk (HM and SM); Natural Whey Cultures (HNWC and SNWC); hay and silage curd immediately after coagulation (HCC and SCC); hay and silage curd at end of

ripening (HCS and SCS); hay and silage cheeses after 1, 30 and 60 days of ripening (HC1, HC30, HC60 and SC1, SC30, SC60).

The samples were transported at laboratory at 4°C and analysed immediately for microbiological analysis or frozen at -80°C prior to DNA extraction.

2.2.3 Microbiological analysis and pH measurement

Twenty-five grams of solid samples (curd immediately after coagulation and cheeses without the crust) were suspended in 225 mL of sterile sodium citrate (2% w/v) solution and homogenized for 2 min with a stomacher (Lab-Blender 400 Seward Medical, London, UK); whereas 10 mL of the liquid samples (M and NWC) were suspended in 90 mL of sterile quarter-strength Ringer's solution (Oxoid, Basingstoke, UK). Furthermore, all samples were decimal serially diluted in Ringer's solution (Oxoid, Basingstoke, UK) and appropriate dilutions were plated by spreading plate method on Petri dishes. Microbial enumerations were carried out in duplicate.

For microbiological counts were used the following media and incubation conditions. Plate count agar (Oxoid) incubated at 30°C for 48 h to determine mesophilic bacteria; tryptone bile X-glucuronid (TBX, Oxoid) agar incubated at 37°C for 2 h and then at 44°C for 46 h to determine presumptive *Escherichia coli*; presumptive thermophilic and mesophilic coccus LAB counted on M17 agar (Oxoid) with lactose 1% for 48 h at 42°C and at 30°C, respectively; presumptive thermophilic and mesophilic rod LAB counted on mMRS-BPB agar [deMan-Rogosa Sharpe (MRS) agar (Oxoid) at pH 6.5 supplemented with 0.05% L-cysteine/HCl (Sigma-Aldrich, St Louis, MO, USA) and with 0.002% bromophenol blue] prepared according to Lee and Lee (2008) incubated under anaerobiosis (Anaerogen kit, Oxoid) for 48 h at 37°C and at 25°C, respectively; Slanetz & Bartley agar (Oxoid) at 37°C for 2 h and then at 44°C for 70 h to determined presumptive enterococci; yeasts and moulds counted on dichloran rose-bengal chloramphenicol agar (SIMAD, Italy) after incubation at 25°C for 72 h.

The pH measurements were performed. The analysis were carried out by insering a FC2320 pH electrode directly into the samples (Hanna Instruments, Italy).

2.2.4 DNA isolation, PCR sequencing and bioinformatics

Microbial DNA extraction from dairy samples was carried out by using BiosticTMBacteremia DNA isolation kit (MO BIO Laboratories, Inc. Carlsbad, CA). For three trials of H and S production of Caciocavallo cheese, the extraction protocol was applied to the pellet (12,000 x *g*) from 20 mL of liquid samples (M and NWC) or from 20 mL of homogenates from 5-fold dilution of solid samples (CC, CS, C1, C30, C60) in one quarter-strength Ringer's solution (Oxoid, Milano, Italy).

Bacterial diversity was evaluated by pyrosequencing of the amplified V1-V3 region (amplicon size, 520 bp). Amplification was carried out by using the primers Gray28f (59-TTTGATCNTGGCTCAG-39) and Gray519r (59-GTNTTACNGCGGCKGCTG-39) as described by Ercolini et al. (2012). 454 adaptors were included in the forward primer, followed by a 10-bp sample-specific multiplex identifier (MID). Each PCR mixture (final volume, 50 μL) contained 60 ng of template DNA, 0.4 M of each primer, 0.50 mmol/L of each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, 5 μL of 10x PCR buffer, and 2.5 U of native *Taq* polymerase (Invitrogen, Milano, Italy). The following PCR conditions were used: 94°C for 2 min, 35 cycles of 95°C for 20 s, 56°C for 45 s, and 72°C for 5 min, and a final extension cycle at 72°C for 7 min. PCR products were purified twice by AgencourtAMPure kit (Beckman Coulter, Milano, Italy) and quantified using Plate Reader AF2200® (Eppendorf, Milan, Italy) prior to further processing as reported by De Filippis et al. (2014). The amplicon pool was used for pyrosequencing on GS Junior platform (454 Life Sciences, Roche Diagnostics, Milan, Italy) according to the producer's protocol using Titanium chemistry. Sequences are available at the Sequence Read Archive (project SRP070077).

A first filtering of the results was performed by using 454 amplicon signal processing, and sequences were then analyzed by using QIIME 1.8.0 software (Caporaso et al., 2010). After the split library script was performed with QIIME, reads were excluded from the analysis if they had an average quality score of 20, if they were 300 bp, and if there were ambiguous base calls. Sequences that passed the quality filter were denoised, and singletons were excluded. Operational taxonomic units (OTUs) were defined by 97%; the taxonomy assignment and alpha and beta diversity analyses were performed by using QIIME, as previously described (De Filippis et al., 2013). Alpha- and beta-diversity were evaluated through QIIME as previously described (De Filippis et al., 2013, 2014).

The influence of cow's diets on the bacterial population was assessed by the OTU taxonomy tables generated by QIIME used to perform Adonis, Anosim and ANOVA statistical tests by using compare_category.py and otu_category_significance.py scripts of QIIME. Moreover, the same OTUs tables were used to draw a pseudo heatmap by using the software TMeV v. 4.8 (Ercolini et al., 2013).

2.2.5 Sensory analysis

Sensory analysis was carried out on Caciocavallo cheeses at 30 and 60 days of ripening. A quantitative descriptive analysis method (Murray et al., 2001) was used to assess the products, according to procedures described elsewhere (Esposito et al., 2014). A panel was composed of eight judges that were trained in the assessment of the intensity of sensory stimuli and were involved in the development of the attributes to generate a specific vocabulary for Caciocavallo cheese. A single score card with 2 appearance, 6 odor/flavor, 4 taste, and 3 texture descriptors was compiled **(Table 2.3).** Attributes were evaluated by rating the samples on 100-mm unstructured lines with anchor points at each end (0 = absent and 100 = very strong). Tests were performed in sensory booths. The panellists were not provided with any information regarding the samples to be tasted. Cheese cube samples (1 cm^3) were served in random order.

2.2.6 Statistical analysis

Statistical analysis were performed by using Statistical Analysis System package version 9.2 (SAS, 2010). The results of the microbial count were elaborated by general linear model (GLM) procedure considering as independent variables the effect of the diet (F: H, S) and the nature of the sample (L: M, NWC, CC, CS, C1, C30, C60). Moreover, the influence of the variables interaction (F*L) was assessed. The Tukey's test ($P < 0.05$) was used to compare mean values among microbial groups to determine differences due to F, L and F*L at $P < 0.05$ significance level.
Sensory attributes were analysed by ANOVA observing the effect of feeding (F: H, S), cheese ripening (R: C30, C60), judges (8), and replication (3 batches of production) and their first order interactions, using SAS software package version 9.2 (SAS, 2010).

2.3 RESULTS AND DISCUSSIONS

2.3.1 Microbiological analysis

The effect of feeding (F: H, S), nature of the sample (L: M, NWC, CC, CS, C1, C30, C60) and their interaction F*L are reported in **Table 2.2**. Except for enterococci, all the microbial groups were significantly affected by the type of sample (L) ($P< 0.001$), as likewise shown in the study of Di Grigoli et al. (2015) during the ripening of a traditional pasta filata cheese.

The evolution of mesophilic and thermophilic lactobacilli, mesophilic streptococci and presumptive *E. coli* was influenced by the variable feeding (F), whereas the interaction of two explicative variables (F*L) significantly affected mesophilic lactobacilli only ($P <0.05$).

Mean results of viable counts of the microbial population and pH values of hay (H) and silage (S) samples during cheese making and ripening of Caciocavallo of Castelfranco are reported in **Table 2.2**. The evolution of microbial groups during cheese making and ripening of Caciocavallo of Castelfranco reflected similar trend as already observed for other semi-hard pasta filata cheeses dominated by LAB (Settanni et al., 2012; De Pasquale et al., 2014; Di Grigoli et al., 2015).

H and S raw cows' milk (M) had similar pH values and mean counts comparing all microbial groups investigated, with slightly higher number of presumptive mesophilic and thermophilic cocci **(Table 2.2)**. As reported in previous works, microbiological quality of milk was affected by technological conditions such as temperature and storage time, environment, and equipment for dairy production (Settanni et al., 2012; Di Grigoli et al., 2015). Furthermore, animal nutrition and milk's animal sources, playing a key role on the composition and the evolution of microbiota during cheese production (Zhang et al., 2015; Quigley et al., 2012). Particularly, LAB normally contaminate milk and their number may vary from a few hundreds to thousands when milk is not quickly processed (De Pasquale et al., 2014).

Presumptive mesophilic and thermophilic cocci were also dominant populations in all the samples of HNWC and SNWC, reaching average values higher than 8.0 Log cfu mL^{-1}. Moreover most of microbial groups in HNWC and SNWC, did not shown statistically different values **(Table 2.2).**

During curd acidification, presumptive mesophilic lactobacilli remained constant in H production until the curd was ready to be stretched; instead, in S production a decrease of about one Log cfu mL^{-1} or g^{-1} was observed. On the contrary, mean

values of presumptive thermophilic lactobacilli remained almost constant throughout curd acidification (5.30-5.70 Log cfu g^{-1}) in both H and S productions. Mean counts of presumptive mesophilic and thermophilic cocci LAB increased in both H and S productions until the end of curd acidification reaching levels higher than 8.0 Log cfu g^{-1} **(Table 2.2).**

Mean counts of enterococci decreased from milk (4.92 Log cfu g^{-1} and 5.06 Log cfu g^{-1} in H and S productions, respectively) to the end of curd acidification (3.22 Log cfu g^{-1} and 3.40 Log cfu g^{-1} in H and S productions, respectively).

In other pasta filata cheese such as and Caciocavallo Palermitano (Settanni et al., 2012) and Caciocavallo Pugliese (De Pasquale et al., 2014) a similar trend on the evolution of LAB during curd acidification was observed.

The mean number of presumptive *E. coli* increased in both H and S productions until the end of curd acidification reaching levels of 2.06 and 2.68 Log cfu g^{-1}, respectively (**Table 2.2**).

After curd stretching and salting phases (samples HC1 and SC1, Table 2.2), mean values of all microbial groups remain almost stable except for enterococci that increase more than one Log cfu g^{-1} in H production, whereas in S production, slight decrease of mesophilic lactobacilli and thermophilic cocci (about 0.5 Log cfu g^{-1}) and decrease of more than one Log cfu g^{-1} of thermophilic lactobacilli was observed. Similarly to the H production, an increase of mean values of enterococci (about 0.5 Log cfu g^{-1}) was observed in S production.

During ripening (from 1 to 60 days), in both H and S productions, mean counts of presumptive mesophilic and thermophilic lattobacilli progressively increased reaching the highest value after 60 days and a similar increasing trend was observed in the study of Coppola et al. (2003) on traditional Caciocavallo cheese. Instead, a progressive decrease of presumptive mesophilic and thermophilic streptococci was observed. Enterococci slightly decreased during ripening of H production (about 0.8 Log cfu g^{-1}), while remained almost constant in S ripening (about 4.0 Log cfu g^{-1}) as previously described for other cheeses produced from raw milk (De Pasquale et al., 2014). The presence of presumptive enterococci in raw milk depends strongly by hygienic condition during milking process, during manipulations by workers and by equipments in contact with milk (Randazzo et al., 2006). Nevertheless, in recent studies on artisanal cheese of Mediterranean area, enterococci could contribute to the formation of characteristic aroma and flavour of traditional cheeses (Foulquié

Moreno et al., 2006; Abriouel et al., 2008). The concentration of presumptive enterococci from milk to cheese remains almost unchanged probably thanks to their thermoresistance required during cooking and stretching phases (Giannino et al., 2009). Decrease trend of presumptive *Escherichia coli* sp. was probably due to the reduction of pH and water activity during cheese making. In addition, the high temperature during manufacturing seemed to play a key role to prevent the development of *Escherichia coli* sp. (Masoud et al., 2012). In fact, the detection of *Escherichia coli* sp. in the curd after maturation and the radical absence in C1 samples would seem attributed partly to the high temperature of the spinning stage (80°C), to the salting in brine and to low ripening temperature (16-17°C). Other factors could prevent the development of pathogenic bacteria as *Escherichia coli* sp. are the competition for nutrients with LAB and their ability to ferment lactose and leading to the pH reduction (Settanni et al., 2012).

Yeasts and moulds mean values increased in both H and S productions during 60 days of ripening and attained the highest value in S cheese samples (4.29 Log cfu g^{-1}).

Finally, after curd stretching, salting phases and ripening, presumptive *E. coli* disappeared in both H and S productions.

Decreasing trend of pH from milk to the end of curd acidification was observed, reaching average values of 5.11 in H production and 5.33 in S productions, whereas at the end of cheese ripening the pH was of about 5.21 and 5.39 in H and S cheeses, respectively.

Table 2.2. Evolution of microbial populations (Log cfu mL^{-1} or g^{-1}) and pH during manufacture and ripening of Caciocavallo of Castelfranco (Giello et al., 2017)

Samples[A]	Growth media[B] and pH								
	PCA	**mMRS 25°C**	**mMRS 37°C**	**M17 30°C**	**M17 42°C**	**S-B**	**DRBC**	**TBX**	**pH**
Hfeeding									
M	5.62±0.28	4.86±0.38^{a}	5.33±0.21^{abc}	6.03±0.33^{b}	5.97±0.40^{c}	4.92±0.58	nd	1.38±0.99^{abc}	6.79
NWC	nd	4.61±0.78^{a}	5.57±0.45^{abc}	8.50±0.18^{a}	8.48±0.06^{a}	4.04±0,43	nd	1.17±0.23^{ab}	4.44
CC	nd	4.89±0.55^{ab}	5.38±0.44^{abc}	6.53±0.20^{b}	6.56±0.09^{ce}	4.79±0.39	nd	1.21±0.22^{ab}	6.70
CS	nd	4.86±0.45^{a}	5.49±0.27^{abc}	7.75±0.33^{abc}	8.16±0.27^{abd}	3.82±0.20	nd	2.06±0.58^{bcd}	5.11
C1	nd	4.82±0.44^{a}	5.53±0.32^{abc}	8.03±0.60^{ac}	8.01±0.23^{abd}	5.08±0.05	3.22±0.59^{ac}	<1^{a}	5.28
C30	nd	7.03±0,84^{cd}	6.78±0.99^{cd}	7.55±0.60^{abc}	7.50±0.65^{bdf}	4.40±0.47	3.51±0.23^{ab}	<1^{a}	5.22
C60	nd	7.92±0.64^{d}	7.58±0.41^{d}	6.99±0.85^{bc}	5.80±0.50^{c}	4.20±0.32	3.83±0.58^{ab}	<1^{a}	5.21
S feeding									
M	5.81±0.45	5.57±0.31^{abc}	5.64±0.34^{abc}	6.08±0.32^{b}	6.01±0.45^{c}	5.06±0.43	nd	1.72±0.61^{abc}	6.74
NWC	nd	4.61±0.81^{a}	5.04±0.61^{ab}	8.44±0.15^{a}	8.29±0.36^{ab}	5.60±0.59	nd	2.11±0.40^{cd}	4.49
CC	nd	5.55±0.25^{abc}	5.59±0.23^{abc}	6.63±0.25^{b}	6.59±0.16^{cef}	5.57±0.24	nd	1.68±0.26^{abc}	6.76
CS	nd	4.78±0.75^{a}	5.71±0.46^{abc}	8.44±0.60^{a}	8.83±0.66^{a}	3.40±0.40	nd	2.68±0.47^{d}	5.33
C1	nd	4.33±0.58^{a}	4.44±0.63^{a}	8.55±0.79^{a}	8.38±0.16^{ab}	3.98±0.36	2.18±0.61^{c}	<1^{a}	5.29
C30	nd	5.43±0.65^{abc}	5.59±0.63^{abc}	7.51±0.68^{abc}	7.37±0.82^{def}	3.74±0.88	3.91±0.40^{ab}	<1^{a}	5.29
C60	nd	6.57±0.46^{bcd}	6.48±0.70^{bcd}	6.84±0.99^{bc}	6.46±0.93^{c}	3.99±0.91	4.29±0.47^{b}	<1^{a}	5.39
Statistical significance[C]									
F	ns	*	**	ns	*	ns	ns	**	nd
L	nd	***	***	***	***	ns	***	***	nd
F*L	nd	*	ns	ns	ns	ns	ns	ns	nd

The values are the means ± SD obtained from duplicate plates of three independent trials. Means within a column with different letters (a to f) are significantly different ($P < 0.05$); nd, not determined.

A: H, hay; S, silage; M, milk; NWC, natural whey culture; CC, curd immediately after coagulation; CS, curd at end of acidification; C1, C30 and C60, cheese after 1, 30 and 60 days of ripening. B: PCA for mesophilic bacteria; mMRS 25°C for mesophilic bacilli LAB; mMRS 37°C for thermophilic bacilli LAB; M17 30°C for mesophilic cocci LAB; M17 42°C for thermophilic cocci LAB; S-B for enterococci; DRBC for yeasts and moulds; TBX for *Escherichia coli*. C: F, feeding; L: sample; F*L: interaction; P value: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, not significant; nd, not determined.

2.3.2 16S rRNA-based analysis of bacterial communities

Culture-independent analysis was carried out by 16S-based HTS analyses to define bacterial genera and species and their relative abundance in traditional Caciocavallo of Castelfranco. The analysis of bacterial communities of 42 hay and silage samples during cheese making and ripening of Caciocavallo of Castelfranco were performed by 16S rRNA gene pyrosequencing. The number of OTUs, Chao1 and Shannon indices, and Good's estimated sample coverage (ESC) are reported in **Table 2.4**. After 454 amplicon processing, 170,795 raw sequence reads were obtained, 128,014 of which passed filters by using QIIME 1.7.0 software, with an average length of 447 bp. More than 90% of the microbial diversity was described in all samples as explained by Good's coverage, excepted for HM3, SM1 and HM1 raw milk samples. Moreover, samples richness varied among samples from 208 to 2030 OTUs.

Bacterial sequences belonged to four different phyla: *Firmicutes* (89.6%), *Proteobacteria* (9.4%), *Actinobacteria* (0.4%) and *Bacteroidetes* (0.3%), accounting more than 99% of the total bacterial abundance, followed by *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*. *Firmicutes* ranged from 15.5% to 100% of relative abundance in all samples resulting dominant in 88% of them. *Proteobacteria* was in 24 samples with relative abundance ranging from 0.1% to 82.8%, among which in five samples was dominant. After QIIME analysis, 537 OTUs were identified but only 28 showed a relative abundance higher than 0.5% in at least one sample **(Table 2.5).**

Raw cows' milk samples showed the highest diversity at genus and species level in three trials of both H and S production. The distribution of 11 OTUs with relative abundance higher than 0.5% in at least two samples is shown in **Figure 2.2**. All OTUs, except *Staphyloccoccus*, *Staphyloccoccus aureus*, *Lactococcus garvieae*, *Streptococcus parauberis* and the family of *Enterobacteriaceae* were present in all milk samples. *Streptococcus thermophilus* (24.23 to 59.96%) and *Lactococcus lactis* (21.46 to 41.09%) were the main species found in HM samples; whereas SM samples were dominated by *Lactococcus lactis* (10.02 to 42.53%), *Lactococcus* (5.61 to 17.55%) and *Acinetobacter* sp. that reaches in the trial 2 relative abundance of 38.26%. Moreover, in HM and SM samples, *Pseudomonas* sp. had a relative abundance ranging from 2.62% to 18.88%, whereas *Acinetobacter johnsonii*, *Corynebacterium*, *Pseudomonas fragi* and *Lactococcus raffinolactis* showed relative abundance up to 2.79%, 2.37%, 4.39% and 8.41%, respectively **(Figure 2.2**). In HM

and SM samples *Acinetobacter* sp. become the dominant OTU as previously observed in the case of refrigerated milk (Raats et al., 2011).

Table 2.4. Number of sequences (reads), operational taxonomic units (OTUs) and estimated sample coverage (ESC) for 16S rRNA amplicons analyzed in this study. Shannon index, Chao1 and ESC were calculated by QIIME at distance level of 3% (Giello et al., 2017)

Feed[a]	Sample[b]	Reads	Shannon	Chao1	OTUs	ESC (%)
	M1	2805	3.09	646.03	241	94
	M2	3760	5.79	1350.75	590	90
	M3	11046	8.14	4235.18	2030	89
	NWC	2587	3.03	495.45	208	95
Hay	CC	10054	5.62	2896.63	1331	92
	CS	12583	5.14	3713.75	1391	93
	C1	7326	3.36	1086.77	476	96
	C30	8646	4.25	1404.77	744	95
	C60	9290	6.34	2725.83	1316	92
	M1	2292	6.99	1433.38	558	84
	M2	6253	5.03	1607.25	749	93
	M3	2748	7.03	1742.94	684	83
	NWC	2816	3.13	685.59	248	94
Silage	CC	5752	4.75	2028.88	791	91
	CS	8984	5.28	3274.12	1262	90
	C1	8648	3.35	1152.74	541	96
	C30	8966	3.62	1266.61	611	96
	C60	13458	6.30	4537.84	2015	90

a: H, hay; S, silage.

b: M, milk; NWC, natural whey culture; CC, curd immediately after coagulation; CS, curd at end of acidification; C1, C30 and C60, cheese after 1, 30 and 60 days of ripening.

Table 2.5. Incidence of OTUs in samples from three trials for hay (H) and silage (S) production of Caciocavallo cheese (Giello et al., 2017)

	Incidence of OTU (%)																	
	H Samples[a]									S Samples[a]								
	M1	**M2**	**M3**	**NWC**	**CC**	**CS**	**C1**	**C30**	**C60**	**M1**	**M2**	**M3**	**NWC**	**CC**	**CS**	**C1**	**C30**	**C60**
Acinetobacter spp.	5.90	0.82	21.9	0	2.29	0	0	0	0	2.01	68.26	2.62	0	2.14	0	0	0	0
Acinetobacter johnsonii	0.79	0.03	2.79	0	0.25	0	0	0	0	0	0	2.73	0	0.97	0	0	0	0
Arthrobacter psychrolactophilus	0	0	0.81	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Carnobacterium spp.	0	0	0.60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Corynebacterium spp.	0.29	0.72	0.19	0	0	0	0	0	0	1.41	2.03	2.37	0	0	0	0	0	0
Enterobacteriaceae	0.03	0.03	0.81	0	0.79	0	0	0	0	0	0	5.82	0	3.97	0	0	0	0
Gammaproteobacteria	0	0	0.50	0	0	0	0	0	0	0.04	0.35	0.11	0	0	0	0	0	0
Janthino bacterium lividum	0	0	0.65	0	0	0	0	0	0	0	0.02	0.25	0	0	0	0	0	0
Lactobacillus spp.	0	0	0	0	0	0	0.94	0	6.29	0	0	0	0	0	0	1.12	0	3.55
Lb. casei group	0	0	0	0	0	0	6.84	0	28.40	0	0	0	0	0	0	4.37	0	9.38
Lb. delbrueckii	0	0	0	0	0	2.19	0	0	0.68	0	0	0	0	0	1.56	0	0	0.65
Lb. helveticus	0	0	0	0	0	0.69	0	0	0	0	0	0	0	0	5.26	0	0	0
Lactococcus spp.	4.18	8.86	12.26	0	0	0	0	0	0	7.20	2.19	20.67	0	0	0	0	0	0
L. garvieae	0.43	0	0.26	0	0	0	0	0	0	4.24	1.02	1.11	0	0	0	0	0	0
L. lactis	15.03	71.09	6.46	0	0	0	0	0	0	52.53	0.02	33.70	0	0	0	0	0	0
L. raffinolactis	2.00	5.11	5.03	0	0	0	0	0	0	2.49	0.10	8.41	0	0	0	0	0	0
Leuconostoc spp.	0	0	0.10	0	0	0	0	0	0	1.00	0	0.04	0	0	0	0	0	0
Pseudomonas spp.	9.11	5.11	35.57	0	16.93	0.19	0	0	0	2.62	13.56	15.87	0	20.37	0.73	0	0	0
P. fragi	1.19	1.46	0.88	0	0	0	0	0	0	0.39	0.14	1.75	0	0	0	0	0	0
Staphylococcus spp.	0.03	0	0.01	0	0	0	0	0	0	1.22	1.42	0.04	0	0	0	0	0	0
S. aureus	0	0.16	0.02	0	0	0	0	0	0	3.50	1.50	1.72	0	0	0	0	0	0
S. sciuri	0	0.03	0	0	0	0	0	0	0	1.31	0	0	0	0	0	0	0	0
Streptococcus parauberis	0.88	1.46	0.88	0	0	0	0	0	0	1.57	0.02	0.36	0	0	0	0	0	0
S. thermophilus	59.96	4.23	4.42	100.00	76.33	93.93	99.52	92.16	62.13	9.34	13.03	1.06	99.89	69.36	88.29	99.49	94.02	78.72

a: H, hay; S, silage; M, milk; NWC, natural whey culture; CC, curd immediately after coagulation; CS, curd at end of acidification; C1, C30 and C60, cheese after 1, 30 and 60 days of ripening. Only OTUs with a relative abundance higher 0.5 in at least one samples are included.

The kind and the abundance of bacterial populations that composed raw milk microbiota can be influenced by several sources of microbial contamination such as teat surfaces, milking procedures, air, dust, animal feeding systems (Montel et al. 2014), and animal feed (Driehuis, 2013). Nevertheless, few studies have investigated on the possible correlation between different types of animal diet and microbial composition of milk (Zhang et al., 2015). In this regard, restrictions or prohibitions on the use of silage as dairy cows feeding to produce cheeses labelled as PDO (Protected Designation of Origin), are due to an open debate about the effects of silages on chemical, physical, microbiological and technological characteristics of cheese (Driehuis, 2013).

Adonis and Anosim statistical tests highlighted significant differences between microbial populations in milk samples from two different diets. In addition, significant diversity of the microbiota in milk replicates within the same diet group was found **(Figure 2.2)**, suggesting that such differences should not related to the diet.

Bacterial populations in NWC samples, during curd acidification and cheese ripening of H and S production are shown in **Figure 2.3**. Statistical comparison of microbiota showed no significant differences of three samples during the same day of production ($P > 0.05$).

Streptococcus thermophilus was the unique species that dominate all HNWC and SNWC samples.

NWCs were obtained from previous days of cheesemaking from milk of H and S diets and their microbial composition was influenced by microbial composition of the milk, cheese technology and the storage conditions of the milk (Parente and Cogan, 2004). Moreover, populations of LAB in NWCs vary depending on the kind of artisanal pasta-filata cheese where *S. thermophilus* was always among the dominant species (Coppola et al., 2001; Ercolini et al., 2008; De Filippis et al., 2016).

The distribution of the OTUs during curd acidification and cheeses ripening of H and S productions with relative abundance higher than 0.5% in at least two samples are shown in **Figure 2.3**. Statistical comparison of microbiota of three individual samples in the same trial showed no significant differences ($P >0.05$). In the curd at beginning of acidification (CC), *S. thermophilus* was the most abundant OTU both in H and S samples, with relative abundance of 76.33% and 69.36%, respectively.

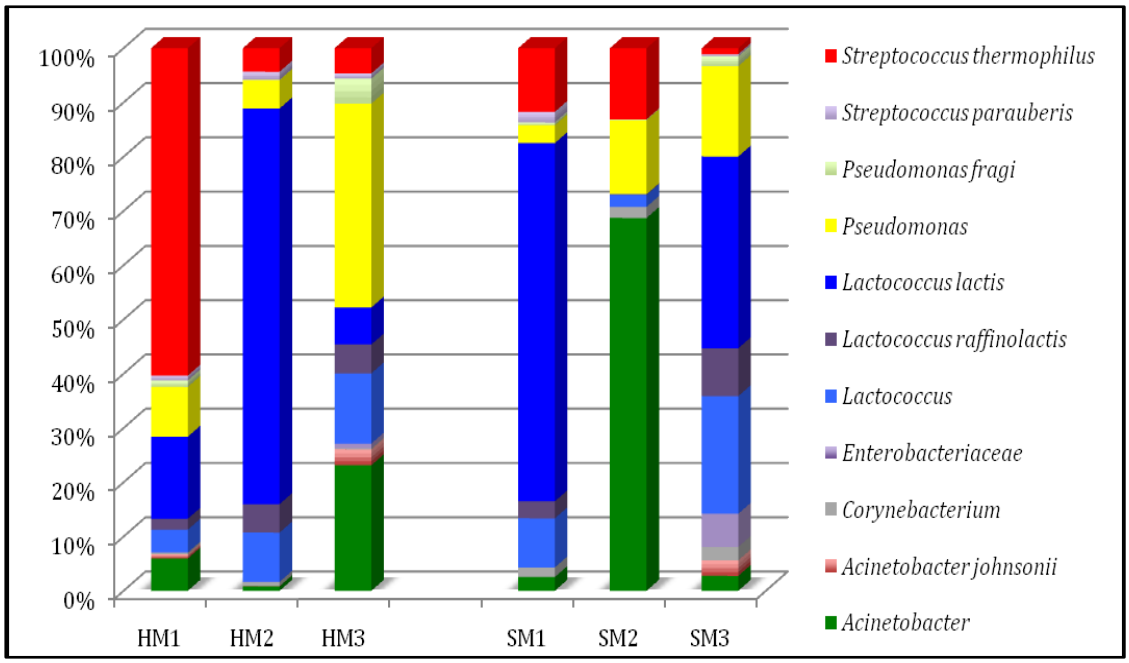

Figure 2.2. Incidence of OTUs at family, genus and species level detected by pyrosequencing analysis of 16S rRNA of all DNA from raw cows' milk (M) in three trials for hay (H) and silage (S) production of Caciocavallo cheese. Only OTUs with a relative abundance above 0.5% in at least two samples are included (Giello et al., 2017).

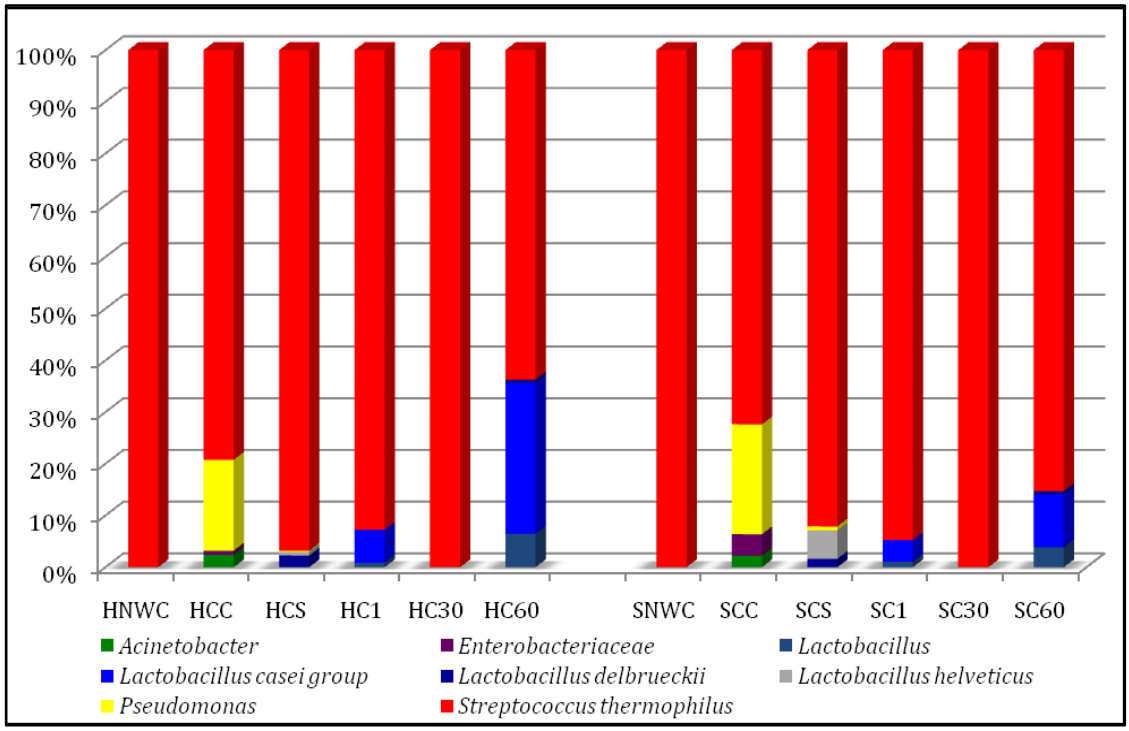

Figure 2.3. Incidence of OTUs at family, genus and species level detected by pyrosequencing analysis of 16S rRNA of all DNA from samples of natural whey culture (NWC), curd immediately after coagulation (CC); curd at end of acidification (CS); cheese after 1, 30 and 60 days of ripening (C1, C30 and C60) in the three trials for hay (H) and silage (S) production of Caciocavallo cheese. Only OTUs with a relative abundance above 0.5 in at least two samples are included (Giello et al., 2017).

Pseudomonas sp. was found to relative high abundance of about 16% and 20% in HCC and SCC samples, respectively; whereas *Acinetobacter* sp. and *Enterobacteriaceae* were found at low incidences. Bacterial profile of curds at end of acidification (HCS and SCS samples) changed by reducing the relative abundance of *Pseudomonas* sp. to very lower percentage while *S. thermophilus* still persisted at high abundance levels. Moreover, *Lb. helveticus* and *Lb. delbrueckii* although at low abundance, emerged in CS samples while other bacteria previously detected in low abundance were not found **(Figure 2.3).**

After curd stretching, moulding and salting, in cheeses ripened for 30 days *S. thermophilus* still dominated (more than 99%) and all other genera were not detectable **(Figure 2.3).**

At end of ripening (60 days) *S. thermophilus* decreased to abundances of 62 and 78% in H and S Caciocavallo cheeses, respectively, while *Lb. casei* group reaches values of 28.40% and 9.38%. *Lactobacillus* sp. and *Lb. delbrueckii* were also found, although at low frequency **(Figure 2.3).**

The evolution of the microbiota switching from thermophilic to mesophilic bacterial species is typical during cheese ripening. According to a recent study of De Filippis et al. (2016), the increase of mesophilic lactobacilli during Caciocavallo cheese production is correlated to the presence of aroma compounds as result of the up regulation of genes associated to ripening-related activities from species belonging to *Lb. casei* group.

In agreement with a previous study, after curd fermentation dominating phylum was *Firmicutes;* whereas relative abundance of *Proteobacteria* decreased (Aldrete-Tapia et al., 2014). In general during the cheesemaking, particularly after fermentation, a reduction of OTUs was observed. This trend was common in other studies on the diversity and dynamics of the microbiota during cheese manufacturing using pyrosequencing (Masoud et al., 2011; Ercolini et al., 2012; Quigley et al., 2012; Ercolini et al., 2013).

The distribution of the samples to describe the composition of the microbiota was reported in **Figure 2.4** by PCoA plot based on the weighted UniFrac distances. The position of milk samples on the plot is the proof of their complex microbiota regardless of animal feeding. On the other hand, fermented samples (NWCs, curd before stretching and cheeses) were characterized by lower level of diversity in their microbiota **(Table 2.4)**, and all together form a specific cluster and were clearly

separated from the milk samples **(Figure 2.4)**. Considering the explicative variable feeding, Adonis and Anosim statistical tests confirmed significant differences between milk replicates within the same diet group ($P < 0.05$ for HM samples and $P < 0.01$ for SM samples) and between H and S diet groups ($P < 0.001$); whereas fermented samples did not show significant differences within three cheesemaking trials and between two animal diets. Same views to describe the distribution of the samples based on the composition of the microbiota were drawn in the pseudo-heatmap (**Figure 2.5**) where the complex microbiota and heterogeneity of milk samples compared to the fermented samples was confirmed.

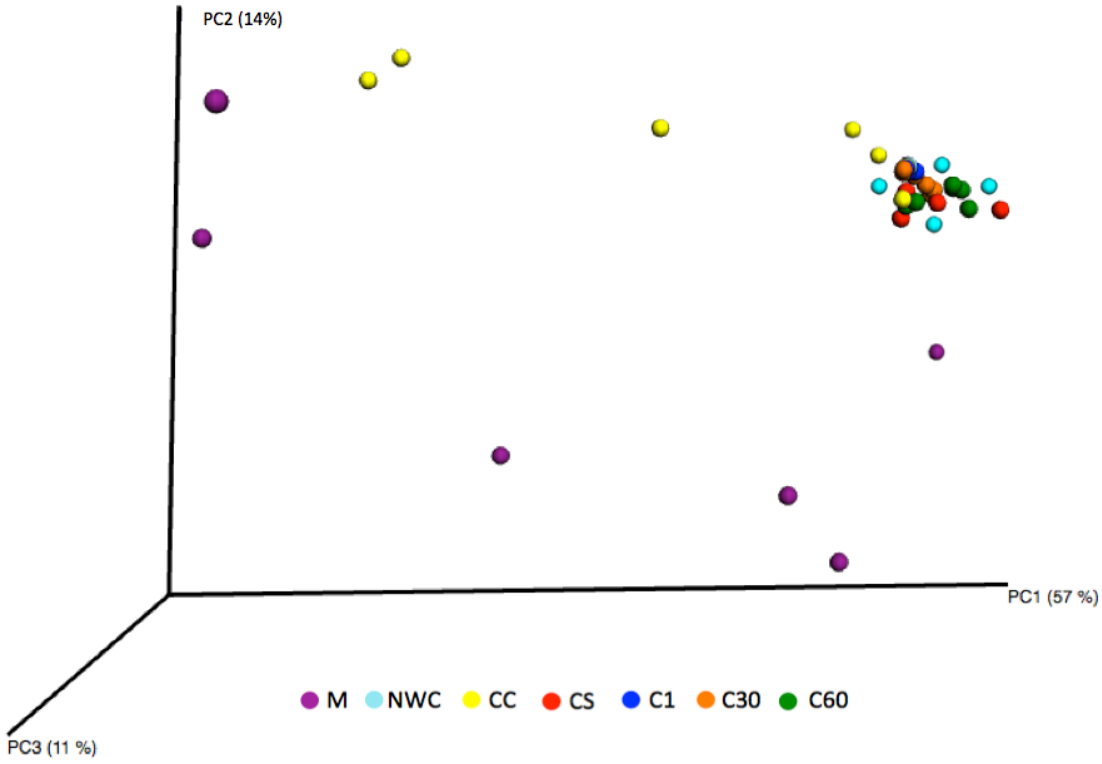

Figure 2.4. Principal coordinates analysis (PCoA) graphic for weigted UniFrac results of bacterial ecosystem at genus and species level. Samples were assessed on the basis of the type (M: milk; NWC= natural whey cultures; CC: curd after coagulation; CS: curd at the end of acidification; C1, C30 and C60: cheese after 1, 30 and 60 days of ripening) (Giello et al., 2017)

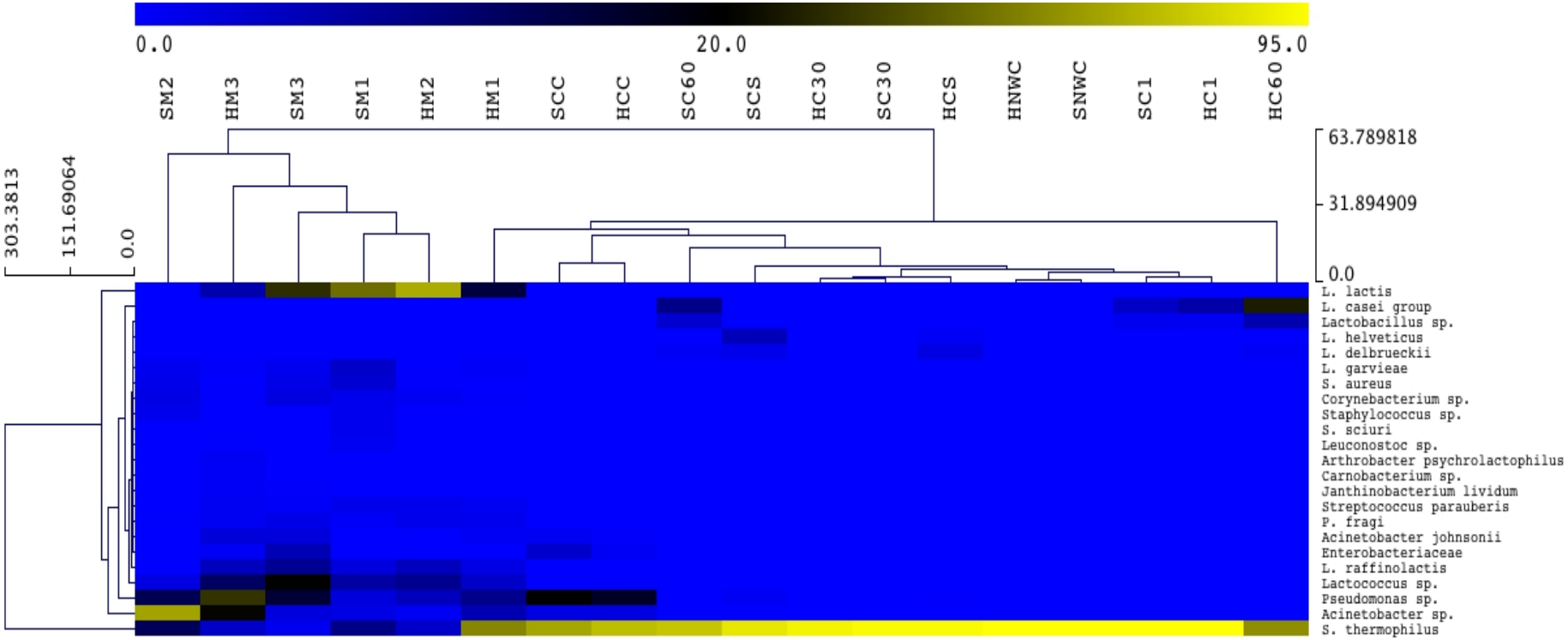

Figure 2.5. Pseudo-heatmap depicting the distribution (%) at family, genus and species level of samples of milk (M1, M2, M3), natural whey culture (NWC), curd immediately after coagulation (CC); curd at end of acidification (CS); cheese after 1, 30 and 60 days of ripening (C1, C30 and C60) for hay (H) and silage (S) production of Caciocavallo cheese. Only OTUs with a relative abundance >0.5% in at least one sample were included. Clustering of samples was obtained using Euclidean distance and the average linkage method. The color key defines the percentages of OTUs in the samples.

2.3.3 Sensory analysis

The sensory analysis was carried out on cheese samples at 30 and 60 dayS of ripening. The statistical analysis did not show significant interactions between two explicative variables (diet and ripening time) on the assessor and replication factors; this led to consider the training program adequate to reach a good reliability of the panel, as each product was evaluated equally in different replications and/or by different assessors. Sensory profiles of cheeses are reported in **Table 2.3**. Except for Overall Flavor, Milk (odor/flavor), and Springiness (texture), all attributes were influenced by the diet (D) and the ripening time (R). Particularly, Yellow attribute was significant higher for S-cheese compared to H-cheese ($P < 0.001$); whereas considering the effect of the ripening time, Yellow attribute tended to be higher for cheeses at 60 days than at 30 day ($P < 0.10$) regardless to the diet. Overall odour showed higher intensity at 60 days of ripening than at 30 days ($P < 0.05$), whereas Butter ($P < 0.05$), Grass ($P < 0.01$), and Hay ($P < 0.05$) were higher for S-cheeses compared to H-cheese. Moreover, Overall odour, Overall flavour and Grass odour/flavour showed significant interaction between diet and ripening variables (D*R) ($P < 0.01$) that increasing significantly during the ripening (R) in S-cheeses and reducing from 30 to 60 days H-cheeses. The intensity of taste attributes (Salty, Sour, Bitter, Umami) were higher in H-cheeses than S-cheeses ($P < 0.001$). In addition, Bitter and Umami showed significant D*R interaction by different trends: Bitter increased significantly from 30 to 60 days in H-cheeses ($P < 0.001$), whereas Umami increased in S-cheese only ($P < 0.001$). Among texture attributes, tenderness and oiliness were more pronounced in S-cheeses and by increasing ripening time, both attributes decreased in S-cheese only ($P < 0.001$ and < 0.05, respectively). Moreover, D*R interaction was statistically significant for Tenderness and Oiliness attributes ($P < 0.001$ and < 0.05, respectively). In general, sensory analysis indicated that many attributes were affected by diet (D) and ripening time (R).

Table 2.3. Sensory profile (Least square means ± S.E.) of Caciocavallo cheese as affected by diet (D; Silage vs. Hay) within ripening time (R; 30 d vs. 60 d) (Giello et al., 2017)

	30 d ripening		60 d ripening		Significance		
	Silage	Hay	Silage	Hay	D	R	D*R
Appearance							
Yellow	51.92^{a}± 2.20	35.98^{b}± 3.23	56.37^{a}± 3.11	42.38^{b}± 3.11	0.001	0.10	NS
Uniformity	80.46^{b} ±2.06	92.14^{ac}± 2.13	88.17^{c}± 2.06	84.04^{b} ± 2.06	0.10	NS	0.001
ODOR/FLAVOUR							
Overall Odor	53.0^{b}± 1.98	61.29^{a}± 2.05	63.50^{a}± 1.98	58.58^{a}± 1.98	NS	0.05	0.01
Overall Flavor	44.00^{a}± 2.46	49.93^{b}± 2.56	53.75b^{b}± 2.46	44.46^{a}± 2.46	NS	NS	0.01
Milk	37.45 ± 2.90	37.84 ± 3.00	36.33 ± 2.89	30.66 ± 2.89	NS	NS	NS
Butter	20.54^{a}± 2.14	18.49^{a}± 2.22	26.12^{b}± 2.14	17.25^{a}± 2.14	0.05	NS	NS
Grass	12.83^{a}± 1.51	12.32^{a}± 1.57	19.54^{b}± 1.51	9.62^{a}± 1.51	0.01	NS	0.01
Hay	19.12^{b}± 2.31	13.35^{a}± 2.40	19.67^{b}± 2.31	15.33^{a}± 2.31	0.05	NS	NS
TASTE							
Salty	32.12^{a}± 1.77	22.41^{b}± 1.84	40.25^{c}± 1.77	27.50^{d}± 1.77	0.001	0.001	NS
Sour	24.62^{a}± 1.51	15.78^{b}± 1.57	18.46^{c}± 1.51	14.08^{cb}± 1.51	0.001	0.05	NS
Bitter	31.08^{a}± 2.08	12.81^{b}± 2.16	29.00^{b}± 2.08	24.71^{cb}± 2.08	0.001	0.05	0.01
Umami	14.79^{a}± 1.82	11.95^{a}± 1.89	27.83^{b}± 1.82	14.46^{a}± 1.82	0.001	0.001	0.01
TEXTURE							
Tenderness	75.00^{a}± 2.11	51.78^{b}± 2.19	55.12^{b}± 2.11	48.83^{b}± 2.11	0.001	0.001	0.001
Springiness	39.37 ± 3.87	44.90 ± 4.02	39.37 ± 3.87	39.17 ± 3.87	NS	NS	NS
Oiliness	36.75^{a}± 2.36	21.99^{b}± 2.45	24.92^{b}± 2.36	22.67^{b}± 2.36	0.001	0.05	0.05

$^{a-c}$Means within a row with different superscript letters differ at P <0.05.

2.4 CONCLUSIONS

In conclusion, the results showed that *S. thermophilus* was the unique species that dominate from raw milks to fermented intermediates and cheese in both H and S cheese production. Moreover, the differences in the taxonomic structure of the microbiota of milk within diet groups were not related to silage and hay cows'feeding. Overall, quantitative descriptive analysis highlights the influence of feeding and ripening time on sensory profile of Caciocavallo cheese. Hence, there appears the possibility to modify the sensory quality of this cheese by choosing the most appropriate combination of diet and ripening time.

Notes

This chapter reports contents of the original research articles "Dynamics of bacterial communities during manufacture and ripening of traditional Caciocavallo of Castelfranco cheese in relation to cows' feeding" by Giello, M., La Storia, A., Masucci, F., Di Francia, A., Ercolini, D., Villani F. (2017), and published by Elsevier on Food Microbiology 63, 170-177.

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CHAPTER 3

Selection of lactic acid bacteria isolated from Caciocavallo of Castelfranco cheese: technological and probiotic characteristics

3.1 INTRODUCTION

Cheese is one of the oldest product among fermented foods known by humans, used as way to concentrate and preserve over time the nutritional value of milk. Cheese productions were probably discovered by chance and were practiced knowing little about fermentative processes, and by empirical methods adopted to select useful microorganisms from raw materials and from the environment. Even today, some traditional cheeses are produced exploiting microbial populations that contaminate raw materials and production plants (Hansen, 2002).

The discovery of microorganisms has allowed to drive food fermentations using useful bacteria known as starter cultures, i.e. bacterial cultures isolated, selected, and characterized for their specific metabolic activities with the purpose to guarantee the outcome of the process and to improve organoleptic characteristics and food safety. Biotechnology developments in food sciences and the increased knowledge on the microbial metabolism involved in fermentation processes can contribute with great influence to both the achievement of industrial goals and the conservation of those characteristics that preserve typicality of the product (Villani, 2016). Lactic acid bacteria (LAB) are food grade microorganisms to which the status GRAS (generally recognised as safe) was attributed. Afterwards, the European Food Safety Authority (EFSA) has coined the status of qualified presumption of safety (QPS). Similarly to GRAS concept, the EFSA includes most LAB species into QPS status which is aimed to allow the use of LAB with QPS status in foods, after demonstrating the safety of bacteria by ensuring the absence of resistance to antibiotics, the absence of pathogenicity, and other safety requirements. LAB are widely used as starter cultures to lead the production of fermented foods by speeding up the fermentation and ensure the success of the production thanks to their pro-technological abilities. The use of LAB has an important role in food industry by contributing to food safety and by offering technological, nutritional and organoleptic benefits as well as having positive effects on the consumer's health. Moreover, in recent years, as a result of changing lifestyles and habits, foods have undergone significant changes, placing greater emphasis on the relationship between food and health. As result, the interest

increased for certain kind of foods that, in addition to their nutritional values, have also beneficial effects on body functions, improving the health of the consumer and reducing the risk of diseases (Caplice and Fitzgerald, 1999). These foods are known as functional foods and this classification includes products adjunct with probiotic bacteria, among which LAB stains are the most common bacteria used as probiotics (Rivera Espinoza and Gallardo-Navarro, 2010). Probiotics are defined by the FAO/WHO as live microorganisms, which, when administered in adequate amounts, confer health benefits on the host by improving the intestinal microbial balance (FAO/WHO, 2002). LAB take up a large slice among probiotic microorganisms, as they are able to: prevent or reduce several gastrointestinal disorders and correlated disease such as food allergies (Marco et al., 2006; Saxelin et al., 2005), inhibit the gut colonization by pathogenic bacteria (Macfarlane and Cummings, 2002), reinforce the immune system (Bezirtzoglou and Stavropoulou, 2011), act for anticarcinogenic and antimutagenic prevention (de Moreno de LeBlanc and LeBlanc, 2014). In order to confer beneficial effects, probiotics should be able to pass and survive through gastrointestinal environment, adhere and colonize the intestinal mucosa and show all requisites to be considered as QPS microorganisms.

The aims of the present study consisted on the isolation of novel LAB strains from Caciocavallo of Castelfranco cheese, a traditional semi-hard pasta filata cheese produced in the South of Italy, and the investigation of their pro-technological properties and probiotic potential through series of *in vitro* tests.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of lactic acid bacteria during Caciocavallo cheesemaking

As described by Giello et al. (2017) and as reported in the Chapter 2 of the present thesis, samples taken during Caciocavallo of Castelfranco production were analyzed to determine microbiological populations: hay and silage milk, HM and SM; hay and silage Natural Whey Cultures, HNWC and SNWC; hay and silage curd immediately after coagulation, HCC and SCC; hay and silage curd at end of ripening, HCS and SCS; hay and silage cheeses after 1, 30 and 60 days of ripening, HC1, HC30, HC60 and SC1, SC30, SC60. After microbiological analysis, presumptive LAB were isolated by picking up five to ten colonies randomly from the highest plates dilution of MRS and M17 agar for each sample. A total of 325 isolates were identified as Gram-positive by KOH method (Gregersen, 1978) and catalase-negative by using 3% (v/v) H_2O_2. Presumptive rods LAB (79 mesophilic and 73 thermophilic) and presumptive cocci LAB (111 mesophilic and 62 thermophilic) were purified by successive streakings on MRS (rod LAB) and M17 agar (cocci LAB), and they were then checked microscopically. Pure cultures were stored at -20°C in their optimal medium broth supplemented with 20% (v/v) glycerol. Before each assay, isolates were activated by two sequential incubations overnight in 10 mL of the appropriate broth. Briefly, rod LAB were inoculated at 1% in MRS broth (at 25°C mesophiles and 37°C thermophiles); whereas cocci LAB were inoculated at 1% in M17 broth (at 30°C mesophiles and at 42°C thermophiles).

3.2.2 Acid production in milk

To evaluate acid production in milk, 325 overnight LAB cultures were centrifuged at 5000 x *g* for 10 min and pellets washed once and suspended in 10 mL of Ringer's solution (Oxoid). Bacterial suspensions were inoculated at 3% (v/v) in 10 mL of 10% (w/v) sterile reconstituted skim milk (RSM, Oxoid) and incubated at 37°C in a water-bath for 24 h to allow the isolates to adapt at milk environment. Skim milk samples coagulated after 24 h were used to inoculate at 3% (v/v) 10 mL of RSM then incubated at 37°C in a water-bath for 6 h. During the incubation for 6 h, the pH of samples was measured every 30 minutes using pH meter HI 221 (Hanna Instruments, Italy). All isolates able to lower the milk pH at 5.2 within 6 h at 37°C were considered as acidifying isolates. Results were expressed as the time that each isolate

takes to lower the pH of skim milk to 5.2. The assay was carried out by three replicates for each isolate and un-inoculated skim milk was used as control sample. Results were reported as mean of three replicates ± standard deviation (SD).

3.2.3 Screening of lactic acid bacteria for technological characterization

Only isolates able to lower the milk pH at 5.2 within 6 h were then screened to study the following activities of pro-technological interest: proteolytic and lipolytic activities, diacetyl production, and decarboxylase activity.

3.2.3.1 Determination of proteolytic activity

Before perform the test, rod and cocci LAB isolates were activated as described in the paragraph 3.2.1. To evaluate proteolytic activity, overnight cultures were centrifuged at 5000 x *g* for 5 min and pellets washed once and suspended in 10 mL of phosphate buffered saline pH 7.0 (PBS). Bacterial suspensions were inoculated at 2% (v/v) in 10 mL of 10% (w/v) RSM and incubated at 37°C in a water-bath for 24 h. After that, coagulated skim milk samples were used to inoculate at 2% (v/v) 10 mL of RSM and incubated at 37°C in a water-bath for 6 h. Coagulated milks were stored at 15°C for 30 days. Proteolytic activity was assayed at 0, 7, 15, 20 and 30 days of incubation at 15°C by using o-phthaldialdehyde (OPA) method described by Church et al. (1983) with minor modifications. After the incubation period at 15°C, 5 mL of coagulated milks were mixed with 10 mL of 0.75 N trichloroacetic acid (TCA) and left at room temperature for 10 min. Mixtures were then filtered by using filter paper (Wathman #2). The concentration of free amino groups in the filtrates was determined using OPA solution prepared mixing: 0.04 g o-phtaldialdehyde (Sigma-Aldrich, Italy), 1 mL methanol, 25 mL 0.1 M sodium thetraborate, 2.5 mL 20% sodium-dodecil-sulphate (SDS, Sigma-Aldrich), 0.1 mL β-mercaptoethanol (Sigma-Aldrich), and deionized water to 50 mL. Therefore, 50 µL of filtrate solution were mixed with 1 mL of OPA solution and left to react for 2 min before read the absorbance at 340 nm using the spectrophotometer (BioSpectrometer® Series, Eppendorf). Results were expressed as optical density (OD) read at 340 nm. The assay was carried out by three replicates for each isolate and un-inoculated skim milk was used as control sample. Results were reported as mean of three replicates ± standard deviation (SD).

3.2.3.2 Determination of lipolytic activity

All LAB isolates with good acid production in milk were tested for lipolytic activity in whole milk. After the activation in appropriate nutritive broth, 50 mL of pasteurized whole milk (further treated at 80°C for 5 min) were inoculated with 3% of overnight culture of each isolate and incubated in water-bath for 6 h at 37°C. Coagulated milks were then stored at 15°C for 24 days. Lipolytic activity was assayed at 3, 15, and 24 days of incubation at 15°C by titration method as described by Casaburi et al. (2005) with some adaptations. At each time, 10 mL of milk samples were mixed vigorously for 2 min with 5 mL of petroleum ether (Merck & Co. Inc., NJ, USA) to allow lipids extraction. Mixtures were then centrifuged at 1000 x *g* for 10 min and upper phases rich in lipid, including free fatty acids, were taken and titrated with NaOH 0.1 N in ethanol and by using 1% phenolphthalein ethanol solution as indicator. The percentage of free fatty acids to express lipolytic activity was calculated as follows:

$$\%\ \text{palmitic acid} = a \times N \times 25{,}6) / g$$

where: *a* corresponds to mL of NaOH used for titration; *N* is the normality of NaOH used for titration; 25.6 is the equivalent weight of palmitic acid; *g* the quantity of milk fat.

The assay was carried out by three replicates for each isolate and un-inoculated whole milk sample was used as control. Results were reported as mean of three replicates ± standard deviation (SD).

3.2.3.3 Detection of diacetyl production

To evaluate diacetyl production, overnight cultures of acidifying isolates were inoculated at 3% in pasteurized whole milk and incubated at 37°C for 6 h and then stored at 15°C for 18 h. At the end of the storage, milk samples were centrifuged at 5000 x *g* for 10 min and 4 mL of the upper phase was taken and mixed with 2 mL of a solution composed by α-naphthol (1% w/v) (Sigma-Aldrich, Italy) and KOH (16% w/v). The mixture was incubated in water-bath at 30°C for 30 minutes and diacetyl production was indicated by the formation of a red ring at the top of the mixture (Franciosi et al., 2009). Results were expressed as ratio between the height of the red ring and the height of the whole mixture in the tube. The assay was carried out by

three replicates for each isolate and un-inoculated whole milk was used as control sample. Results were reported as mean of three replicates ± standard deviation (SD).

3.2.3.4 Decarboxylase activity

Overnight cultures of acidifying LAB isolates were centrifuged at 5000 x *g* for 10 min and the pellets washed once and suspended in 10 mL of PBS pH 7.0. Bacterial suspensions were standardized at about $1.0x10^6$ cfu mL^{-1} by using McFarland standards. Ten microliters of each bacterial suspension were spotted on plates containing Improved medium which composition was previously described by Bover-Cid and Holzapfel (1999) and containing one of the following amino acids that produce biogenic ammines by decarboxylation: histidine (precursor of histamine), lysine (produces cadaverine), and tyrosine (precursor of tyramine). The plates were incubated for 72 h at optimal temperatures of the isolates (25°C for mesophilic lactobacilli; 37°C for thermophilic lactobacilli; 30°C for mesophilic cocci LAB; 42°C for thermophilic cocci LAB). Isolates were spotted in duplicate on plates containing Improved medium with amino acids and without amino acids (as negative control). After incubation, decarboxylating isolates produced purple halo in the yellow medium with histidine or lysine, while on plates containing Improved medium with tyrosine, precipitates disappeared around colonies of decarboxylating isolates.

3.2.4 Probiotic and functional characterization of lactic acid bacteria

The screening to evaluate probiotic and functional abilities of LAB isolates was started by studying the survival to gastrointestinal transit of 28 selected starter LAB (SLAB) previously screened for their pro-technological potential. After that, survival screening was performed on lactobacilli with low acid production (lactobacilli not able to lower the pH of RSM at 5.2 within 6 h). All isolates that showed % of survival higher or equal to 85% were screened to study the following activities of probiotic and functional interest: bile salt tolerance; Bile Salt Hydrolase (BSH) activity; growth with prebiotic fibres; cholesterol assimilation.

3.2.4.1 Survival to gastrointestinal transit assay

Twenty-eight starter LAB (SLAB), previously selected for pro-technological characteristics, and 38 low acidifying lactobacilli were tested to assess their tolerance to simulated gastrointestinal transit. Strains' survivability was performed according to Ricciardiet al. (2015) with slight modifications as follows. After growth in appropriate nutritive broth for 16 hours, culture broths were centrifuged at 5000 x *g* for 10 min and the pellets washed in sterile Ringer's solution (Oxoid, Italy). Two milliliters of cell suspensions were centrifuged at 13000 x *g* for 5 min and resulting pellets were suspended in 2 mL of simulated saliva (SS: 86 mmol L^{-1} NaCl, 7 mmol L^{-1} KCl, 45 mmol L^{-1} $NaHCO_3$, 100 mg L^{-1} lysozyme (Sigma), pH 6.90) and incubated at 37°C for 5 min. Bacterial cells were harvested by centrifugation and re-suspended in 2 mL of simulated gastric juice (SGJ: 86 mmol L^{-1} NaCl, 7 mmol L^{-1} KCl, 45 mmol L^{-1} $NaHCO_3$, 3 g L^{-1} pepsin, pH 2.5) and incubated at 37°C for 120 min under constant stirring at 200 rpm in order to simulate peristaltic movements. Bacterial suspensions were then centrifuged and pellets re-suspended in 2 mL of simulated pancreatic juice (SPJ: 22 mmol L^{-1} NaCl, 3.2 mmol L^{-1} KCl, 76 mmol L^{-1} $NaHCO_3$, 0.5% porcine bile (Sigma), 0.1% pancreatin (Sigma), pH 7.5) and incubated at 37°C for 120 min at 200 rpm. All simulated juices were autoclaved at 121 °C for 15 min and enzymatic solutions were added by filtersterilizing using 0.45-µm membrane filters (Merk Millipore, Italy). Isolates were counted before the incubation in SS and at the end of simulated gastrointestinal transit. Bacterial counts were performed in duplicate by *pour plate* method and plates were incubated at optimal growth temperatures (25°C for mesophilic lactobacilli; 37°C for thermophilic lactobacilli; 30°C for mesophilic cocci LAB; 42°C for thermophilic cocci LAB) for 48 hunder anaerobic conditions. *Lb. rhamnosus* GG was used as reference strain.

The survival after gastrointestinal transit was expressed as percentage of survival by using the following equation:

$$\% \text{ survival} = [\beta/\alpha\ ] \times 100$$

where α is the Log cfu mL^{-1} of the assayed isolate before treatment and β is the Log cfu mL^{-1} of the same isolate after the gastrointestinal transit. Three independent experiments were carried out and for each experiment two replicates were performed. Results were reported as mean of six replicates ± standard deviation (SD).

Only isolates that showed % survival higher or equal to 85% were screened to study the following probiotic and functional abilities: bile salt tolerance; Bile Salt Hydrolase (BSH) activity; growth with prebiotic fibres; cholesterol assimilation.

3.2.4.2 Assays for bile salts tolerance and Bile Salt Hydrolase (BSH) activity

Minimal inhibitory concentration (MIC) of a mix of bile salts (oxgall, Sigma-Aldrich), of taurocholic acid sodium salt (TCA), of sodium taurodeoxycholate (TDCA), of glycocholic acid sodium salt (GCA), and of sodium glycodeoxycholate (GDCA) (Sigma-Aldrich) was evaluated following the method of Pennacchia et al. (2004). Briefly, 10 µL of overnight culture (about 10^7 cfu mL^{-1}) of each strain were spotted on MRS agar plates containing the mix of bile salts or the singular bile salt (0%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5%). Plates were incubated in anaerobiosis at 37°C for 4 days. MIC was defined as the lowest concentration of bile salts that causes total inhibition on the growth of colonies in the spots. The test was carried out in duplicate for all isolates and for each type of bile salts.

According to Chen et al.(2014), growth kinetics of lactobacilli with and without 0.3% of oxgall were monitored. MRS-THIO broth (MRS with 0.2% sodium thioglycollate), prepared with and without 0.3% oxgall, was placed in microplate and inoculated at 1% with each strain. Bacterial growth in the control (MRS-THIO broth without oxgall) and in the test cultures (MRS-THIO broth with oxgall) was monitored at 37°C every hour until an increase of absorbance at 590 nm of 0.3 units was reached. Results were expressed as lag time (LT) defined as the difference in hours between the control (bacterium incubated in MRS-THIO broth without oxgall) and the test (the same bacterium incubated in MRS-THIO broth with oxgall) to increase the absorbance of 0.3 units. For both MIC and growth rate assays, *Lb. rhamnosus* GG was used as reference strain. The tests were performed by two replicates for each of three independent experiments.

Moreover, Bile salt hydrolase (BSH) activity was detected using the method of Michael et al. (2016) with slight modifications. After autoclaving, MRS agar was added with filter-sterilized solution of TDCA (Sigma-Aldrich) up to a concentration of 0.3% (w/v). MRS agar plates (as negative controls) and MRS agar plates containing 0.3% TDCA were pre-reduced anaerobically at 37°C for 24 h. Overnight cultures were streaked on plates and spotted on sterile filter disks placed on the agar surface. Plates were incubated at 37°C for 48-72 h in anaerobiosis. After the

incubation, BSH-positive strains formed white precipitate around the colonies or white opaque colonies.

3.2.4.3 Assay for growth rate with prebiotic fibres

The use of prebiotic fibres by LAB was evaluated by monitoring the growth kinetics of the strains in modified MRS broth, where glucose was replaced with inulin or fructoligosaccharides (FOS) as carbon source. MRS broth without carbohydrates was autoclaved at 121°C for 15 min and carbon sources (inulin or FOS) were added by filter-sterilizing using 0.22-µm membrane filters to a final concentration of 2% (w/v). MRS broth with glucose was used as positive control. Broths with fibers or with glucose were inoculated at 2% (v/v) with bacterial suspension prepared by suspending the bacterial pellet in 10 mL of 0.1 M PBS pH 6.9. Growth kinetics were monitored using a microtiter reader by reading the optical density at 650 nm (OD_{650}) every hour during 72 hours of incubation at 37°C in anaerobiosis (by covering the wells with paraffin). According to Ferrando et al.(2016), the equation to calculate the growth cell rate is described follows:

$$\mu = \ln_f OD_{650} - \ln_0 OD_{650} / t_f - t_0$$

where: µ, specific speed rate; $_f OD_{650}$, final optical density; $_0OD_{650}$, initial optical density; t_f, final time; t_0 initial time.

The assay was carried out in triplicate for each strain in both fibers, and results reported as mean of three replicates ± SD.

3.2.4.4 Evaluation of antibiotic resistance

Non-starter lactobacilli were tested for their susceptibility against the following antibiotics: inhibitors of protein synthesis such as gentamycin (30 µg), chloramphenicol (10 µg), tetracycline (30 µg), erythromycin (5 µg), clindamycin (2 µg), streptomycin (25 µg), and kanamycin (30 µg); inhibitors of cell wall synthesis as ampicillin (10 µg). The test was performed by using disk diffusion method with antibiotic disks (Oxoid, Italy) according to the Clinical and Laboratory Standards Institute (1999) with some modifications as described by Charteris et al. (1998). Overnight MRS broth cultures were standardized at an optical density of 0.1 at 590 nm and used as inoculum. Antibiotic disks were placed on MRS agar plates that were then overlaid with 8 mL of MRS agar soft (0.75% (w/v) agar) seeded with 0.2 mL of

standardized inoculum of tested strains. The plates were incubated in anaerobiosis at 37°C for 24 h. After incubation, the diameter (mm) of inhibition zone, including the diameter of the disk, was measured. The antibiotic resistance was interpreted according to the classification provided by Charteris et al. (2001) that classify the strains as resistant (R), moderately sensitive (MS), and sensitive (S) on the basis of the diameter of the inhibition zone. Experiments were performed in duplicate and *Lb. rhamnosus* GG was used as reference strain.

3.2.4.5 Determination of antioxidant activity

Supernatants and cell suspensions, used to test the antioxidant activity, were prepared by adapting the procedure used by Muganga et al. (2015) with some modifications. Bacterial cultures growth in MRS broth at 37°C for 24 h, were centrifuged at 5000 x *g* for 10 min. Bacterial pellets were washed twice in phosphate-buffered saline (PBS: 0.80% NaCl, 0.02% KCl, 0.02% KH_2PO_4, and 0.22% Na_2HPO_4, pH 7.4) and cell concentration was standardized at about 10^9 cells/mL using McFarland standards obtaining the standardized cell suspension (CS).

To achieve the supernatant from skim milk (S-SM), the strains were previously inoculated at 2% in sterilized RSM (10% w/v) with 1% glucose and 0.5% yeast extract and incubated for 24 h at 37°C. Therefore, coagulated enriched skim milks were used to inoculate RSM (10% w/v) at 2% and incubated for 24 h at 37 °C. After the incubation, coagulated skim milks were centrifuged at 5000 x *g* for 10 min and the pH of supernatants adjusted at pH 7.4. Lastly, to remove precipitates after neutralization, supernatants were centrifuged again at 13000 x *g* for 10 min and then S-SMs were used for the test.

The antioxidant ability of non-starter lactobacilli, by using α,α-Diphenyl-β-Picrylhydrazyl (DPPH) radical, was analyzed by the method performed by Chen et al. (2014) with slight modifications. Four-hundreds-forty-five microliters of CS or S-SM and 555 µL of DPPH solution 0.2 mM in ethanol were mixed in a cuvette and allowed to react for 30 min in the dark. PBS was used as blank solutions, whereas PBS (for CS) or supernatant at pH 7.4 from acidified skim milk with lactic acid (for S-SM) were used as control samples. Sample analyses were performed in duplicate. Scavenged DPPH was monitored by measuring the decrease in absorbance at 517 nm and the radical scavenging activity was defined as percentage inhibition by the following equation according to Ankolekaret al. (2012):

$$\% \text{ inhibition} = (A_{control} - A_{sample})/A_{control} * 100$$

where $A_{control}$ means the absorbance at 517 nm read in the control sample; A_{sample} is the absorbance at 517 nm read in the sample. The assay was carried out in triplicate and results reported as mean of three replicates ± SD.

3.2.4.6 Investigation on cholesterol assimilation

Cholesterol assimilation by non-starter lactobacilli was assessed by adding 300 µg/mL of water-soluble cholesterol PEG-600 (Sigma-Aldrich) in the following media: MRS broth according to Tomaro et al. (2014); MRS broth with 0.3% oxgall (Michael et al., 2016); PBS (0.80% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.22% Na_2HPO_4, pH 7.4) with 3.5 g/L glucose and 0.3 % oxgall (Miremadi et al., 2014); simulated intestinal fluid (0.85% (w/v) NaCl, 6.8 g/L KH_2PO_4, 1.5 g/L Oxgall, 3.5 g/L glucose, and 10 g/L pancreatin, pH 6.8) (Tomaro et al., 2014). A stock solution 3 mg/mL of water-soluble cholesterol PEG-600 (Sigma-Aldrich) was prepared, sterilized through 0.22 µm filter (Millipore, Corp., Bedford, MA, USA) and added to the media to obtain a final concentration of 300 µg/mL of cholesterol. Overnight cultures of non-starter lactobacilli and of *Lb. rhamnosus* GG (as reference strain) were inoculated at 1% (v/v) to each cholesterol-medium and incubated at 37°C for 24 h under anaerobic conditions. Cholesterol concentrations before and after the incubation were determined according to the protocol by Rudel and Morris (1973) with some modifications. Overnight bacterial cultures were centrifuged at 5000 x *g* for 10 min at 4°C. Five-hundred microliters of the supernatants were transferred in glass tubes and then added with 500 µl KOH (33% w/v) and 1 mL of absolute ethanol. The solutions were vortexed for 60 sec and heated at 37°C for 15 min in a water-bath. After cooling at room temperature for 15 min, the solutions were added with 1 mL of distilled water and with 1.5 mL of hexane followed by vortexing for 60 sec. The mixtures were left at room temperature for 15 min to allow the separation of immiscible liquid phases. Five-hundred microliters of the upper hexane phases were transferred in glass tubes followed by fast evaporation under nitrogen flow. Subsequently, dry residues were dissolved and mixed in 0.5 mL of o-phthalaldehyde (OPA, Sigma) solution (0.5 mg/mL in glacial acetic acid; prepared and stored in the dark 2 h before the use) and left to react 10 min at room temperature. Two-hundred-fifty microliters of sulphuric acid (98%, Sigma) were then added and the mixture

vortexed for 60 sec followed by resting for 20 min at room temperature. The absorbance was measured at 550 nm using UV-spectrophotometer (BioSpectrometer, Eppendorf) on the mixtures obtained from samples immediately after the inoculum of strains in the each cholesterol-medium, and on the mixtures obtained from same samples at the end of the incubation period (37°C for 24 h under anaerobic conditions). A standard curve of absorbance was prepared using the following cholesterol concentrations (μg/mL): 0; 31; 25; 62.5; 125; 250 and 500 (R^2=0.9979).
The ability of non-starter lactobacilli and of *Lb. rhamnosus* GG to assimilate cholesterol was determined as follows:

% assimilated cholesterol =

$$[\text{cholesterol } (\mu g/mL)_{0h} - \text{cholesterol } (\mu g/mL)_{24h} / \text{cholesterol } (\mu g/mL)_{0h}] \times 100$$

Samples and standards were assayed in triplicate and results reported as mean of three replicates ± SD.

3.2.5 Molecular identification of SLAB and non-starter lactobacilli

For molecular identification of SLAB and non-starter lactobacilli, DNA extraction was performed following the method described by Marmur (1961) with slight modifications. Two milliliters of overnight culture in MRS broth were centrifuged at 12000 x *g* for 1 min and the pellet was washed once with 1.5 mL of ET (50 mM Tris-HCl, 5 mM EDTA, pH 8). After the centrifugation at 12000 x *g* for 5 min, bacterial pellet was suspended in 0.5 mL ET and 70 μL lysozyme (50 mg/mL) and incubated for 1 h at 37°C before adding 100 μL of 10% SDS (Sigma) and 3 μL of Pronase E (20 mg/mL, Sigma). The mixture was shaken 3-4 times by inverting the tube and then incubated at 37°C for 30 min. After cooling the sample in ice bath for 5 min, 700 μL of ammonium acetate 5 M were added. The mixture was then shaken 10 times by inverting the tube and centrifuged at 12000 x *g* for 5 min at 4°C. The supernatant was precipitated with one volume of isopropanol, gently shaken and incubated at -18°C for 30 min. After the centrifugation at 12000 x *g* for 20 min, the resulting pellet was air dried and suspended in 20 μL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) and in 1 μL of RNAse solution (10 mg/mL) followed by incubation at 37°C for 30 min. DNA templates were used to identify SLAB isolates at species level by sequencing of 16S rRNA and at strain typing by repetitive sequence-based

PCR (rep-PCR). Non-starter lactobacilli needed for their identification of additional molecular assays such as sequencing of Hsp60 gene and species-specific PCR. All amplifications were carried out in a Thermal Cycler (Mastercycler, Eppendorf).

3.2.5.1 Strain typing by repetitive sequence-based PCR (rep-PCR)

The rep-PCR was performed to detect interspecific strain variability by amplification of repetitive sequences spread in the bacterial genome by using the primer $(GTG)_5$ 5'-GTGGTGGTGGTGGTG-3' (Gevers et al., 2001) to allow the discrimination at strain level through different DNA-fingerprintings which are specific and unique for each strain. Selected 28 SLAB and 19 non-starter lactobacilli were analyzed to discriminate different strains and rep-PCR was performed in a total volume of 25 µl containing 50 ng of DNA template, 2.5 µL of *Taq* DNA polymerase 10X buffer (Invitrogen, Italy), 1.75 µL of 50 mM $MgCl_2$, 1 µL of a deoxynucleoside triphosphate mix (10 mM each), 0.15 µL of primer $(GTG)_5$ (0.1 mM) and 0.5 µL of 5 U/µL *Taq* DNA polymerase (Invitrogen). PCR conditions consisted of an initial DNA melting (95°C for 4 min) followed by 35 cycles (94°C for 1 min; 40°C for 1 min; 72°C for 1 min) and final cycle for 8 min at 72°C. PCR amplicons were separated by agarose (1.75%, w/v) gel electrophoresis at 100 V for 2 h and visualized with UV trans-illuminator UVI doc HD2 (Uvitech, UK).

3.2.5.2 Sequencing of 16S rRNA

DNA template was used for 16S rRNA gene sequencing using primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (*Escherichia coli* positions 8-17 and 1540-1524, respectively) (Weisburg et al., 1991). PCR conditions consisted of an initial template melting (95°C for 3 min) followed by 30 cycles of denaturation (94°C for 1 min), annealing (54°C for 45 s) and extension (72°C for 2 min) plus one additional elongation cycle for 7 min at 72°C. Reaction mix composition (50 µL) were performed including 50 ng of the DNA template, 5.0 µL of *Taq* DNA polymerase 10X buffer (Invitrogen, Italy), 2.5 µL of 50 mM $MgCl_2$, 0.5 µL of a deoxynucleoside triphosphate mix (25 mM each), 0.1 µL of each primer (0.1 mM) and 0.5 µL of 5 U/µL *Taq* DNA polymerase (Invitrogen). PCR products were run in agarose gel (1.5% w/v) for 90 min at 100V and then purified by using a QIAquick PCR purification kit (Qiagen S.p.A., Milan) following the manufacturer's protocol. Sequencing data of 16S were viewed using

PC FinchTV software (http://www.geospiza.com/finchtv/) and DNA similarity analysis was performed using the BLAST (bl2seq) program at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.5.3 Hsp60

To allow the identification of the species among *Lactobacillus casei* group, the polymorphism within the hsp60 (groEL, encoding a 60-kDa heat shock protein) gene was evaluated. A 650-bp fragment internal to the hsp60 gene was amplified as previously described (Blaiotta et al., 2008) using oligonucleotide primers H279 (5'-GAATTCGAIIIIGCIGGIGA(TC)GGIACIACIAC-3') and H280 (5'-CGCGGGATCC(TC)(TG)I(TC)(TG)ITCICC(AG)AAICCIGGIGC(TC)TT-3'),
where Inosine (I) was used to minimize the degeneration of the primers. To carry out the amplification, PCR reaction mix (50 µL) was performed including 50 ng of the DNA template, 5.0 µL of *Taq* DNA polymerase 10X buffer (Invitrogen, Italy), 2.5 µL of 50 mM $MgCl_2$, 0.5 µL of a deoxynucleoside triphosphate mix (25 mM each), 0.125 µL of each primer (0.1 mM), and 0.5 µL of 5 U/µL *Taq* DNA polymerase (Invitrogen). PCR conditions consisted of an initial template melting (95°C for 3 min) followed by 40 cycles (94°C for 30 s; 35°C for 1 min; 72°C for 1 min) plus one additional elongation cycle for 10 min at 72°C. PCR amplicons were separated by agarose (2%, w/v) gel electrophoresis at 100 V for 2 hand then purified by using QIAquick PCR purification kit (Qiagen S.p.A., Milan) by following the manufacturer's protocol.
Sequencing data of *hsp60* were viewed using PC FinchTV software (http://www.geospiza.com/finchtv/) and DNA similarity analysis was performed using the BLAST (bl2seq) program at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.5.4 Species-specific PCR

To discriminate non-starter lactobacilli strains at species level, a multiplex PCR was performed by species-specific primers designed by Ventura et al. (2003) with minor modifications. Amplification reactions were performed in a total volume of 50 µl containing 20 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, deoxynucleoside triphosphate mix (500 µM each), 10 pmol each of primers PAR (5'-GACGGTTAAGATTGGTGAC-3'), CAS (5'-ACTGAAGGCGACAAGGA-3'), and

RHA (5'-GCGTCAGGTTGGTGTTG-3'), 50 pmol of primer CPR (5'-CAANTGGATNGAACCTGGCTTT-3'), 2.5 U of *Taq* DNA polymerase (Invitrogen, Italy), and 100 ng of template DNA. PCR conditions consisted of an initial template melting (95°C for 5 min) followed by 30 cycles (95°C for 30 s; 52°C for 1 min; 72°C for 1.5 min) and one elongation cycle for 7 min at 72°C. PCR amplicons were separated by agarose (2%, w/v) gel electrophoresis at 100 V for 90 min and then visualized with UV trans-illuminator UVI doc HD2 (Uvitech, UK).

3.2.6 Statistical analysis

All data are expressed as means ± standard deviation (SD) and were analysed by one-way analysis of variance (ANOVA) or t-test. Comparisons of mean results were carried out using post-hoc Tukey's test at $P \leq 0.05$ significance level. Statistical analyses were performed using Stata software package version 13 (Stata, 2013).

3.3 RESULTS AND DISCUSSIONS

3.3.1 Milk fermentation ability

To evaluate the acid production in milk, 325 LAB isolates among lactobacilli and streptococci were grown in skim milk. All isolates able to lower the pH of the milk at 5.2 within 6 h at 37°C were classified as acidifying LAB. According to the established criteria, among 325 isolates only 28 LAB (about 9%) were able to lower pH at 5.2 within 6 h (**Table 3.1 and Figure 3.1**). In detail, 28 acidifying isolates were composed by: 4 mesophilic lactobacilli, 4 thermophilic lactobacilli, 6 mesophilic streptococci and 14 thermophilic streptococci. On a total of 28 isolates, were able to acidify the skim milk within 4 h mostly of the presumptive streptococci: five streptococci isolated from curd at the end of ripening (HCS and SCS), one streptococcus isolated from Natural Whey Culture (HNWC), one streptococcus isolated from curd after coagulation (HCC), one streptococcus isolated from cheese after one day of ripening (HC1), two streptococci isolated from cheese after 30 days of ripening (HC30) and four streptococci isolated from cheese at the end of ripening (HC60 and SC60). Conversely, just two lactobacilli isolated from the curd at the end of ripening (SCS) were able to acidify the skim milk within 4 h. Regardless of the source from which LAB were isolated, the bacteria isolated on M17 and presumptively belonging to *Streptococcus* genus, were the fastest LAB to lower pH to 5.2 than presumptive lactobacilli. These results are in agreement with Franciosi et al. (2009) and Marino et al. (2003). In their works, the strains belonging *Streptococcus* genus showed the fastest acidifying power in milk compared to other LAB genera tested in the same studies. In this study, acid production by 28 selected LAB was higher than the LAB strains isolated from dairy products studied in previous works (Garabal et al.,2008; Turchi et al., 2011; Zuo et al., 2014; Franciosi et al., 2009; Marino et al., 2003) confirming that 28 acidifying isolates could be promising starter cultures for Caciocavallo of Castelfranco cheese production as regards the acidifying activity as crucial factor for cheesemaking process. The **Table 3.1** shows that about 45% of acidifying isolates were isolated from the curd at the end of ripening (HCS and SCS) while the rest of isolates came from cheese after 1, 30 and 60 days of ripening, from the curd after coagulation and from natural whey culture. Moreover, about 59% of 28 acidifying LAB were isolated from samples coming from milk of cows fed with hay based diet, while remaining 12 acidifying

LAB were isolated from samples coming from milk of cows fed with silage based diet. During cheesemaking process, curd acidification plays a key role to allow the stretching of the curd in hot water. For this reason, it was chosen to evaluate the acid production in milk of LAB isolates by setting a reachable pH values at 5.2 that represent a suitable value for the stretching step (Esposito et al., 2014; Piraino et al., 2005). The time to reach pH of the milk at 5.2 was set at 6 h because overly long acidification time causes the elongation of the manufacturing time and excessive syneresis of the curd (Speranza et al., 2014). Moreover, rapid acidification prevents the growth of food-borne and pathogenic microorganisms and play a key role on the formation of aroma, texture, and flavor of the cheese (Akabanda et al., 2014).

Table 3.1. SLAB selected for their acid production in milk

Isolate[a]	Diet[b]	Sample[c]	LAB group[d]
CFL10T	H	CS	TL
CFL7M	H	CS	ML
CFL9M	H	CS	ML
CFS10T	H	CS	TS
CFS11T	H	CS	TS
CFS12T	H	CS	TS
CFS17M	H	CS	MS
CFS9M	H	CS	MS
CIL18M	S	CS	ML
CIL19M	S	CS	ML
CIL4T	S	CS	TL
CIS10T	S	CS	TS
CIS18M	S	CS	MS
FF3S2T	H	C30	TS
FF3S4T	H	C30	TS
FF6S12M	H	C60	MS
FF6S17M	H	C60	MS
FFL6T	H	C1	TL
FFS5T	H	C1	TS
FI3L10T	S	C30	TL
FI3S12T	S	C30	TS
FI3S2T	S	C30	TS
FI3S6T	S	C30	TS
FI6S16T	S	C60	TS
FI6S7T	S	C60	TS
LIFS11T	H	CC	TS
SFS9T	H	NWC	TS
SIS7M	H	NWC	MS

a: isolate's identification code.
b: H, cows' hay based diet; S, cows' silage based diet.
c: samples source of LAB isolates. NWC, natural whey culture; CC, curd after coagulation; CS, curd after stretching; C1, cheese at one day of ripening; C30 cheese at 30 days of ripening; C60, cheese at 60 days of ripening.
d: TL, thermophilic lactobacilli isolated from MRS agar plates incubated at 37°C; ML, mesophilic lactobacilli isolated from MRS agar plates at 25°C; TS, thermophilic streptococci isolated from M17 agar plates at 42°C; MS, mesophilic streptococci isolated from M17 agar plates at 30°C.

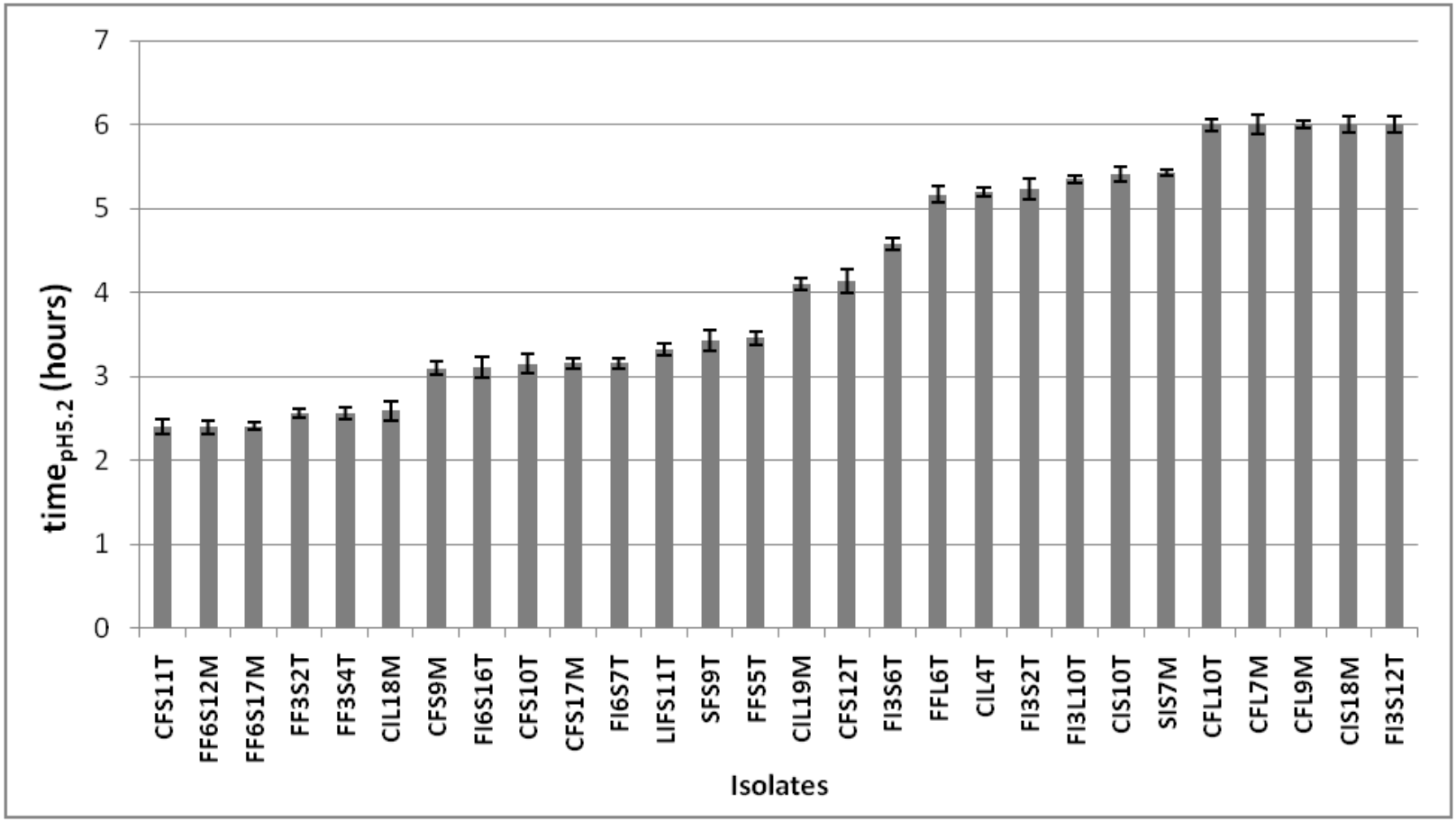

Figure 3.1. Acidifying LAB ($t_{pH5.2} \leq 6$ h) isolated during Caciocavallo of Castelfranco production. Results are the mean of three replicates expressed as hours ± SD spent by LAB isolates to lower the pH of skim milk at values of 5.2.

3.3.2 Technological characterization of the isolates

3.3.2.1 Proteolytic activity

The results on proteolytic activity of 28 potential SLAB were shown in **Table 3.2.** Proteolytic activity of each isolate was detected at 0, 7, 15, 20 and 30 days of incubation at 15°C in skim milk to simulate times and temperatures of cheese ripening process. The results were expressed as values of absorbance measured at 340 nm. The **Table 3.2** shows that groups of isolates are characterized by different trends on proteolytic activity during the incubation period. In this regard, several studies have demonstrated that the hydrolysis of proteins seems to be time dependent and species or strain specific among LAB (Hati et al., 2017; Akabanda et al., 2014). Most of isolates (6 lactobacilli and 12 streptococci) determined an increase of amino groups released over time until the 15^{th} or 20^{th} day at which was observed a decrease of optical density values to then increase or remain almost constant until the end of incubation (30 days). The decrease of amino acids and peptides between 15^{th} or 20^{th} day of incubation could be due to the utilization by LAB of milk proteins as their primary source of essential amino acids with growth-stimulating action (Atanasova et al., 2014). The increasing trend on proteolytic activity after 20 days of incubation showed from some SLAB, can be explained because it has been found that many LAB peptidases are of intracellular origin and were released only after cell lysis contributing to hydrolyze milk proteins (Nieto-Arribas et al., 2010; Atanasova et al., 2014; Frau et al., 2016). Five isolates (FI3S2T, FI6S16T, FI6S7T, SFS9T, SIS7M) showed the highest OD values after 7 or 15 days of incubation after that the concentration of free amino groups decreased until the end of incubation period. Only isolates CIL19M (lactobacillus) and CIS10T (streptococcus) did not show proteolytic activity at any incubation time; whereas LIFS11T, CIL4T and FF3S2T were the unique three isolates to show a linear increase in proteolytic activity for all the incubation period (**Table 3.2**). These last results are in agreement with the work of Hati et al. (2017) where LAB strains inoculated in skim milk or soymilk (*S. thermophilus* MD2, *Lb. helveticus* V3 and NCDC 292, *Lb. rhamnosus* NS6 and NS4, *Lb. bulgaricus* NCDC 09, and *Lb. acidophilus* NCDC 015 and NCDC 298) were characterized by an increase in proteolytic activity over the whole incubation time.
The isolate FI3L10T showed the highest release of amino groups showing OD value of 0.877 at 30 days (**Table 3.2**), followed by isolates FFL6T, CFS10T and CFL10T.

Proteolytic activity by starter bacteria plays a key role on cheese quality because the quantity and the quality of peptides and amino acids released by peptidases impact on flavor or act as precursors of molecules that determine the overall cheese flavor, texture and taste (Zuo et al., 2014; Nieto-Arribas et al., 2010; Akabanda et al., 2014; Frau et al., 2016).

Table 3.2. Proteolytic activity of 28 SLAB

Isolate[a]	LAB group[b]	A_{340} at days[c]				
		0	7	15	20	30
CFL10T	TL	0.020	0.036	0.156	0.102	0.228
CFL7M	ML	0.024	0.126	0.157	0.119	0.163
CFL9M	ML	0.014	0.082	0.178	0.112	0.158
CFS10T	TS	0.012	0.012	0.028	0.004	0.245
CFS11T	TS	0.013	0.078	0.034	0.020	0.051
CFS12T	TS	0.019	0.046	0.020	0.054	0.069
CFS17M	MS	0.025	0.082	0.148	0.061	0.115
CFS9M	MS	0.022	0.065	0.105	0.069	0.074
CIL18M	ML	0.025	0.153	0.148	0.086	0.092
CIL19M	ML	0.010	0.015	0.011	0.007	0.002
CIL4T	TL	0.026	0.035	0.027	0.059	0.130
CIS10T	TS	0.010	0.017	0.014	0.016	0.016
CIS18M	MS	0.025	0.147	0.035	0.033	0.039
FF3S2T	TS	0.021	0.020	0.011	0.014	0.060
FF3S4T	TS	0.014	0.017	0.048	0.016	0.028
FF6S12M	MS	0.020	0.052	0.015	0.070	0.063
FF6S17M	MS	0.021	0.069	0.044	0.088	0.095
FFL6T	TL	0.030	0.294	0.410	0.310	0.460
FFS5T	TS	0.030	0.114	0.147	0.076	0.128
FI3L10T	TL	0.029	0.427	0.543	0.281	0.877
FI3S12T	TS	0.024	0.037	0.038	0.024	0.037
FI3S2T	TS	0.025	0.080	0.085	0.071	0.057
FI3S6T	TS	0.017	0.044	0.032	0.041	0.063
FI6S16T	TS	0.015	0.031	0.056	0.046	0.031
FI6S7T	TS	0.013	0.545	0.113	0.068	0.082
LIFS11T	TS	0.018	0.056	0.056	0.059	0.077
SFS9T	TS	0.019	0.240	0.069	0.045	0.090
SIS7M	MS	0.016	0.539	0.099	0.088	0.040

a: Isolate's identification code.
b: TL, thermophilic lactobacilli isolated from MRS agar plates incubated at 37°C; ML, mesophilic lactobacilli isolated from MRS agar plates at 25°C; TS, thermophilic streptococci isolated from M17 agar plates at 42°C; MS, mesophilic streptococci isolated from M17 agar plates at 30°C.
c: values of absorbance (340 nm) read at 0, 7, 15, 20 and 30 days of skim milk incubation at 15°C. The results are means of three independent assays; SD ranged between 0.002 and 0.023.

3.3.2.2 Lipolytic activity

Lipolytic activity of 28 SLAB were carried out by titration method and the results are reported in the **Figure 3.2** as grams of palmitic acid hydrolyzed by LAB isolates from milk fat. The figure shows that all LAB isolates, previously selected for their acid production in milk, were able to hydrolyze milk fat with an increasing trend over the time of incubation. Nevertheless, only three streptococci (CFS11T, CFS12T, FF6S17M) showed lipolytic ability starting from the third day of incubation with % of palmitic acid of 0.29, 0.25 and 0.37, respectively. On the basis of lipolytic activity at 24 days, the isolates were classified as low lipolytic (% palmitic acid $\leq$ 0.55%), medium lipolytic (% palmitic acid between 0.56% and 0.85%); strong lipolytic (% palmitic acid $\geq$ 0.86%). Therefore, 3 lactobacilli and 6 streptococci were classified as low lipolytic; 3 lactobacilli and 12 streptococci were classified as medium lipolytic; 2 lactobacilli (CFL7M and CIL19M) and 2 (CFS10T and FF6S12M) streptococci were classified as strong lipolytic. The results obtained by Ben Moussa et al. (2008) on lipolytic ability of some LAB strains isolated from Tunisian raw milk, showed the % of oleic acid ranged from a minimum of 0.09% by *Lc. lactis* 279 and a maximum of 0.3% by *Lb. brevis* 270 after 7 days of incubation. These results seem to be in line with the ones obtained in our study. Lipolysis consisting of partial degradation of the lipid fraction which plays a primary role in the formation of aroma during cheesemaking. Moreover, fatty acids released through further oxidative reactions, act as precursors of other aromatic molecules such as esters and aldehydes (Frau et al., 2016).

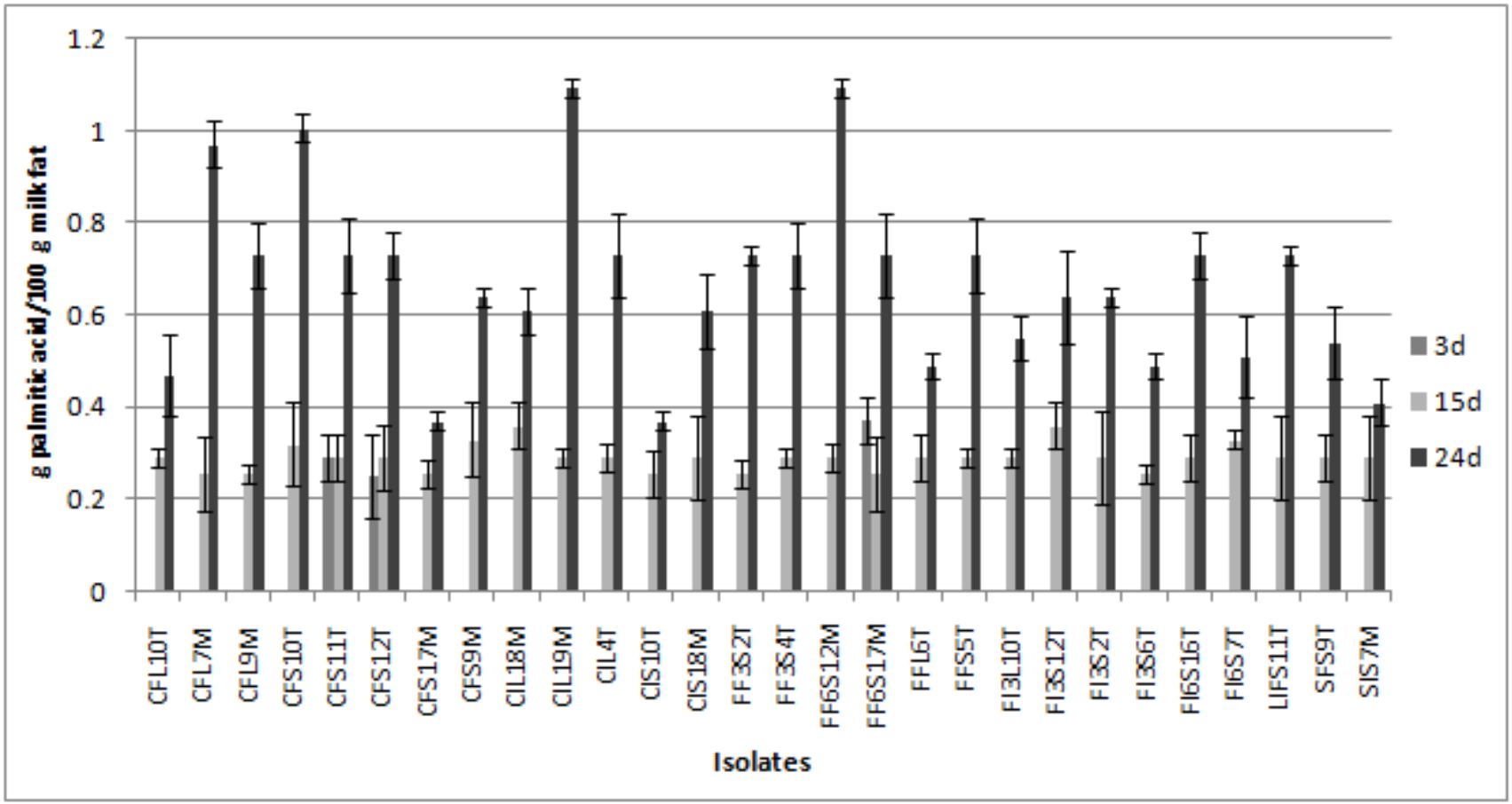

Figure 3.2. Lipolytic activity of acidifying LAB ($t_{pH5.2} \leq 6h$) isolated during Caciocavallo of Castelfranco production. The results are the mean of three replicates expressed as grams of palmitic acid produced by hydrolysis of 100 g of milk fat. Lipolytic activity was measured after 3 days (3d), 15 days (15d) and 24 days (24d) of incubation at 15°C in whole milk.

3.3.2.3 Diacetyl production

The ability by LAB to generate flavor compounds in dairy products is one of the fundamental requisites that starter cultures must possess since significantly influence sensory quality of the end-product (Hugenholtz, 1993). Diacetyl is an aromatic compound generated by certain LAB as end product of citrate metabolism (Marilley and Casey, 2004).

Diacetyl production by 28 potential SLAB were shown in **Figure 3.3**, where the results were expressed as ratio between the height of the red ring formed on the top of the solution (indicates diacetyl production) and the height of the whole solution in the tube. Based on the value of the ratio, LAB isolates were classified as high (≥ 0.251 cm), medium (from 0.101 cm to 0.250 cm) or low (up to 0.1) diacetyl producers. The **Figure 3.3** shows that 4 lactobacilli and 6 streptococci were classified as low diacetyl producers; 2 lactobacilli and 4 streptococci were medium diacetyl producers; 1 lactobacillus and 6 streptococci were high diacetyl producers. Contrariwise, 18% of screened isolates (1 lactobacillus and 4 streptococci) were not able to produce diacetyl in whole milk. These results suggest that diacetyl production in our study could be strain dependent as demonstrated in previous works of Franciosi et al. (2009) and Zuo et al. (2014)

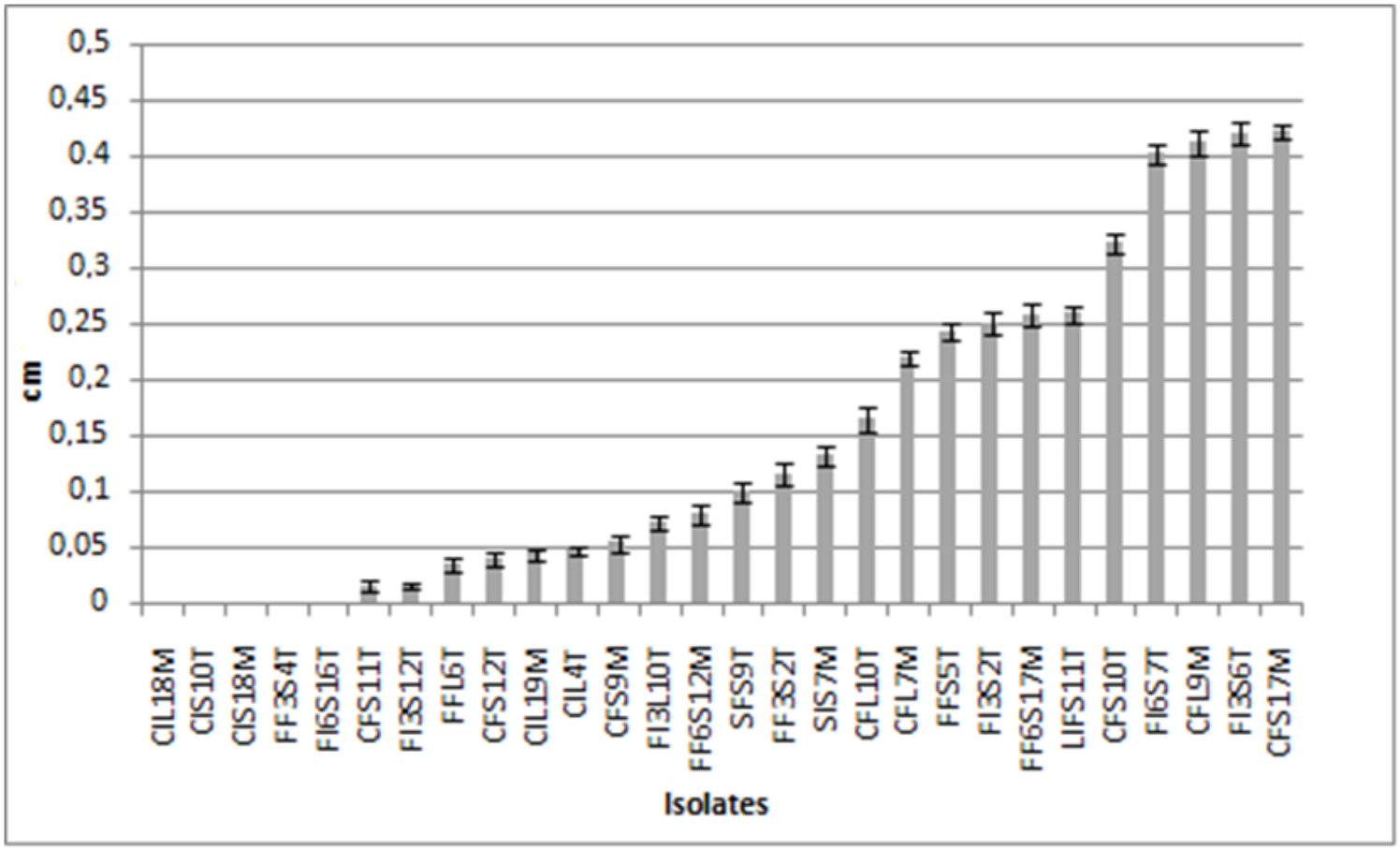

Figure 3.3. Diacetyl production by acidifying LAB isolated during Caciocavallo of Castelfranco production. The results were expressed as mean ± SD of ratio between the height (cm) of the red ring and the height (cm) of the whole mixtures in the tubes. The assay was carried out by three replicates for each isolate.

3.2.3.4 Decarboxylase activity

Biogenic amines are organic basic compounds generated by degradation of certain amino acids through decarboxylase enzyme possessed by microorganisms found in foods of several origins. It has been reported that the ingestion of high levels of biogenic amines through foods causes toxicological effects of varying gravity (Brink et al., 1990). Moreover, biogenic amines are associated with poor hygiene of food and acting as indicator of food alteration (Karmas, 1981; Vidal-Carouet al., 1990). None of the potential SLAB were able to generate biogenic amines in presence of corresponding amino acid precursors, thus showing a positive characteristic in the selection of starter cultures to use for cheesemaking.

3.2.3.5 SLAB molecular identification

Molecular identification of SLAB at strain level was performed by rep-PCR able to detect interspecific strain variability by amplification of repetitive sequences spread in the bacterial genome by using the primer $(GTG)_5$ (Gevers et al., 2001). The **Figure 3.4** and the **Table 3.3** show DNA-fingerprintings by rep-PCR for each of 28 SLAB isolates selected for their acid production in milk and assayed for pro-technological properties. Molecular identification of the strains at species level was performed by analyzing genomic sequences coding for 16S ribosomal RNA. The **Table 3.3** shows DNA % similarity of strains with the corresponding accession numbers found by BLAST (bl2seq) program at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). As reported in the **Table 3.3**, 20 isolates were identified as *Streptococcus thermophilus*, two isolates belonging to *Lactococcus lactis* subsp. *lactis*, one isolate was identified as *Lc. lactis* subsp. *cremoris*, three isolates as *Lb. delbrueckii* subsp. *bulgaricus*, one isolate as *Lb. helveticus,* and one isolate as *Leuconostoc citreum*. Moreover, 20 isolates identified as *S. thermophilus* were characterized by three different DNA-fingerprintings (letters i, h and g) (**Table 3.3**). Therefore, starting from 28 SLAB isolates, 11 strains characterized by different DNA-fingerprinting were obtained.

Table 3.3. Molecular identification of SLAB by 16S rRNA sequencing

Isolate[a]	Figure 3.4		Species[d]	Similarity %[d]	Accession Number[d]
	Code[b]	Profile[c]			
CFL10T	3	*m*	*Lb. helveticus*	99	KT 428592.1
CFL7M	*1*	*a*	*Lc. lactis* subsp. *lactis*	100	NC 013656.1
CFL9M	*2*	*b*	*Lc. lactis* subsp. *cremoris*	99	NC 008527.1
CFS10T	*10*	*h*	*S. thermophilus*	99	NZCP 011217.1
CFS11T	*11*	*i*	*S. thermophilus*	99	NC 017927.1
CFS12T	-	*i*	*S. thermophilus*	99	NC 017927.1
CFS17M	-	*i*	*S. thermophilus*	99	NC 017927.1
CFS9M	*9*	*g*	*S. thermophilus*	100	NZCP 011217.1
CIL18M	*4*	*c*	*Lc. lactis* subsp. *lactis*	99	NC 013656.1
CIL19M	*5*	*d*	*Leuconostoc citreum*	99	NC 010471.1
CIL4T	*6*	*l*	*Lb. delbrueckii* subsp. *bulgaricus*	99	NC 014727.1
CIS10T	-	*i*	*S. thermophilus*	99	NC 017927.1
CIS18M	-	*i*	*S. thermophilus*	99	NC 017927.1
FF3S2T	-	*i*	*S. thermophilus*	99	NC 017927.1
FF3S4T	-	*i*	*S. thermophilus*	99	NC 017927.1
FF6S12M	-	*i*	*S. thermophilus*	99	NC 017927.1
FF6S17M	-	*i*	*S. thermophilus*	99	NC 017927.1
FFL6T	*7*	*e*	*Lb. delbrueckii* subsp. *bulgaricus*	99	NC 014727.1
FFS5T	-	*i*	*S. thermophilus*	99	NC 017927.1
FI3L10T	*8*	*f*	*Lb. delbrueckii* subsp. *bulgaricus*	99	NC 014727.1
FI3S12T	-	*i*	*S. thermophilus*	99	NC 017927.1
FI3S2T	-	*i*	*S. thermophilus*	99	NC 017927.1
FI3S6T	-	*i*	*S. thermophilus*	99	NC 017927.1
FI6S16T	-	*i*	*S. thermophilus*	99	NC 017927.1
FI6S7T	-	*i*	*S. thermophilus*	99	NC 017927.1
LIFS11T	-	*i*	*S. thermophilus*	99	NC 017927.1
SFS9T	-	*i*	*S. thermophilus*	99	NC 017927.1
SIS7M	-	*i*	*S. thermophilus*	99	NC 017927.1

a: see Table 3.1

b: Numeric codes of each strain used to identify the isolates in the Figure 3.4. -, profiles not shown in Figure 3.4

c: Each letter (from “a” to “m”) corresponds to a DNA-fingerprinting.

d: Molecular identification of SLAB by 16S rRNA sequencing

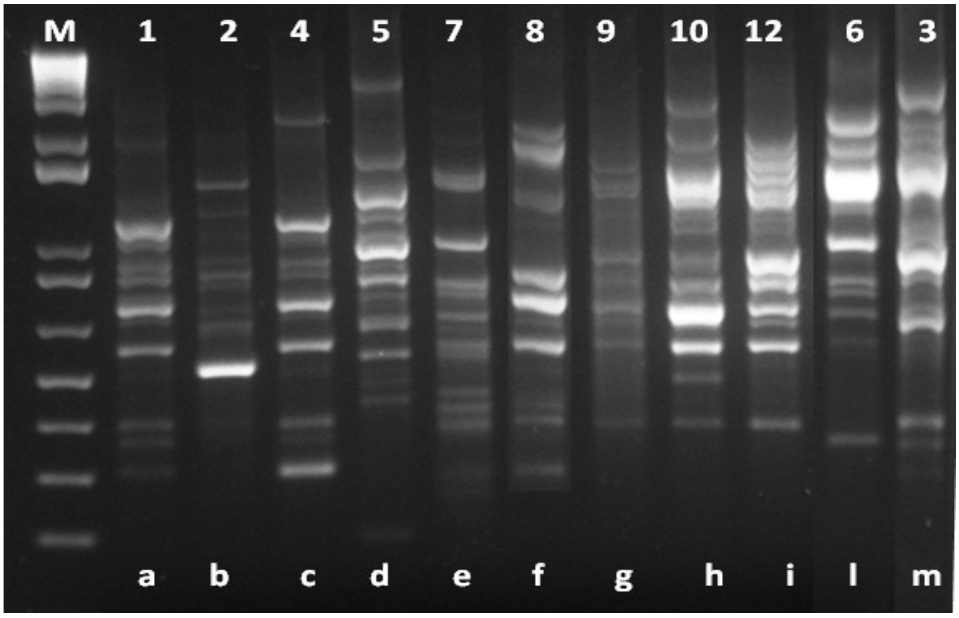

Figure 3.4. DNA-fingerprintings of 28 SLAB isolates by rep-PCR. Different strains are indicated with different letters (from a to m). M: molecular marker 1 Kb plus DNA Ladder (Invitrogen, Italy). Numeric codes on the top of each strain profile corresponding to the strains reported in the Table 3.3.

3.3.3 Survival under simulated gastrointestinal tract conditions

Microorganisms to be considered as probiotic should be able to survive and colonize the gastrointestinal tract and to provide benefits to human health. In order to select LAB resistant to gastrointestinal transit, 28 SLAB previously selected for pro-technological characteristics, and 38 non-acidifying lactobacilli were tested to assess their tolerance to simulated gastrointestinal juices. Survival ≥ 85% could be a proper range to select bacteria resistant to gastrointestinal transit, as has been reported by the current literature (Ricciardi et al., 2015).

The results on the survival of 28 SLAB after gastrointestinal transit (5 min in salivary juice, 2 hours in gastric juice and 2 hours in duodenal juice) are listed in **Table 3.4** where it shows that *S. thermophilus* CFS11T did not survive to gastrointestinal transit; whereas remaining 10 strains showed a survival % ranged between 30% and 80.8%. Therefore, none of SLAB were able to survive under gastrointestinal conditions to an acceptable survival level. Overall results suggest that starter lactobacilli seem tolerate better the gastrointestinal environment than *S. thermophilus* strains tested.

Table 3.4. Survival of selected SLAB to simulated gastrointestinal transit

Species	Strain	%S*
Streptococcus thermophilus	CFS11T	$0^{a} \pm 0.0$
Leuconostoc citreum	CIL19M	$30.0^{b} \pm 0.6$
Lb. delbrueckii subsp. *bulgaricus*	FI3L10T	$33.3^{b} \pm 0.8$
S. thermophilus	CFS9M	$35.5^{bc} \pm 0.4$
S. thermophilus	CFS10T	$42.1^{c} \pm 0.5$
Lb. helveticus	CFL10T	$45.5^{c} \pm 1.2$
Lactococcus lactis ssp. cremoris	CFL9M	$66.6^{d} \pm 1.3$
Lc. lactis subsp. *lactis*	CIL18M	$68.0^{d} \pm 1.8$
Lb. delbrueckii subsp. *bulgaricus*	FFL6T	$79.2^{e} \pm 2.0$
Lc. lactis subsp. *lactis*	CFL7M	$79.8^{e} \pm 1.4$
Lb. delbrueckii subsp. *bulgaricus*	CIL4T	$80.8^{e} \pm 2.2$

*% survival: $\% S = [\beta/\alpha] \times 100$ where α, Log cfu mL^{-1} of the assayed strain before gastrointestinal transit; β, Log cfu mL^{-1} of the strain after incubation under the test conditions. The results are expressed as mean ± SD of three independent experiments.

[a-e]Means within a column with different superscript letters differ at $P < 0.05$.

The sources from which 38 non-acidifying lactobacilli were isolated during the production of Caciocavallo cheese and their results on survival to simulated digestion are shown in **Table 3.5.** The results highlight that 7 on 38 isolates were not able to survive after gastrointestinal transit (% survival of 0 log cfu mL^{-1}) whereas the survival of remaining 31 isolates ranged between 64.7% and 100%. Among 31 isolates, about 61% (19 isolates) showed % of survival ≥ 85% and the highest survival was observed by isolates FF3L9T, LIL4T, FFL21T and FF3L16M (% survival of 100, 98.7, 98.6 and 97.6, respectively).

Promising probiotic bacteria to perform their functional ability beneficial for human health, must be able to survive in hostile gastrointestinal environment (Sanders, 2003). Gastric and instestinal juices act as the highest hurdles for the survival of ingested probiotics (Liong and Shah, 2005). Contrariwise, more recent studies observed that beneficial effects for human health could be showed also by taking dead probiotic cells, indicating that the ability to give beneficial effects to the host are not an exclusive prerogative just of live probiotic cells (Adams, 2010). However, despite the loss of viability of some SLAB and NSLAB, we did not consider to study the isolates that did not survive to simulated digestion or with % survival < 85%. The variability of results on the resistance to gastrointestinal transit by LAB showed by previous studies, was affected by several factors such as the method used for the

assay, growth phase at which the strains were tested (lag phase, log phase, stationary phase) and growth strain conditions (Whitehead et al., 2008; Zago et al., 2011; Van Bokhorst-vande Veen et al., 2012; Bove et al., 2013). Differently from our work, significant studies on viability to gastrointestinal transit used different approach based on treating bacteria with a single assays, i.e. stressing bacteria with gastric juice and with intestinal juice individually to know how each fluid can impact on probiotic survival (Solieri et al., 2014; Muganga et al., 2015). These studies highlighted that most of *Lactobacillus* strains are more susceptible to gastric fluid, while they resist better at intestinal juice, as emerged also from the study by Casarotti and Barretto Penna (2015) based on multiple and consecutive stress-based assays.

As mentioned above, only isolates that showed % survival higher or equal of 85% were identified and screened to study the following probiotic and functional abilities.

Table 3.5. Source of non-acidifying lactobacilli isolated during Caciocavallo of Castelfranco production and their survival to simulate gastrointestinal transit

Isolate[A]	Diet[B]	Sample[C]	LAB group[D]	% S[E]
CIL16M	S	CS	ML	$0^{a} \pm 0.0$
CIL11M	S	CS	ML	$0^{a} \pm 0.0$
CIL15M	S	CS	ML	$0^{a} \pm 0.0$
CIL7M	S	CS	ML	$0^{a} \pm 0.0$
FF3L17M	H	C30	ML	$0^{a} \pm 0.0$
FFL3T	H	C1	TL	$0^{a} \pm 0.0$
FIL5T	S	C1	TL	$0^{a} \pm 0.0$
FI6L13T	S	C60	TL	$64.7^{b} \pm 0.8$
FF6L14T	H	C60	TL	$65.4^{b} \pm 0.5$
FF3L3T	H	C30	TL	$73.5^{bc} \pm 0.7$
FF3L5M	H	C30	ML	$74.0^{bc} \pm 1.1$
FF3L18M	H	C30	ML	$74.1^{bc} \pm 1.2$
LIL8T	S	CC	TL	$80.9^{c} \pm 1.4$
FFL15M	H	C1	ML	$81.5^{c} \pm 2.2$
FI6L6T	S	C60	TL	$82.0^{c} \pm 1.7$
FF6L1T	H	C60	TL	$82.1^{c} \pm 0.5$
FF6L13T	H	C60	TL	$82.2^{c} \pm 2.0$
FF6L6T	H	C60	TL	$83.1^{cd} \pm 0.9$
FF3L9M	H	C30	ML	$83.8^{cd} \pm 1.5$
FIL2M	S	C1	ML	$84.8^{cd} \pm 2.3$
FF3L13T	H	C30	TL	$85.1^{cd} \pm 2.1$
FF6L7T	H	C60	TL	$85.8^{d} \pm 1.5$
FF6L17T	H	C60	TL	$86.1^{d} \pm 0.9$
FF6L3T	H	C60	TL	$86.8^{d} \pm 2.6$
FF3L4T	H	C30	TL	$88.3^{d} \pm 1.1$

FF3L13M	H	C30	ML	$88.8^{de} \pm 2.3$
FF3L3M	H	C30	ML	$89.9^{de} \pm 1.3$
FF3L1M	H	C30	ML	$91.9^{de} \pm 1.8$
FF3L6M	H	C30	ML	$94.4^{e} \pm 2.0$
FF6L8T	H	C60	TL	$94.8^{e} \pm 2.5$
FI3L12T	S	C30	TL	$95.9^{e} \pm 1.6$
FI3L1T	S	C30	TL	$96.0^{ef} \pm 1.5$
FF3L17T	H	C30	TL	$96.0^{ef} \pm 2.2$
FF3L14T	H	C30	TL	$96.2^{ef} \pm 1.0$
FF3L16M	H	C30	ML	$97.6^{ef} \pm 2.6$
FFL21T	H	C1	TL	$98.6^{g} \pm 2.8$
LIL4T	S	CC	TL	$98.7^{g} \pm 1.6$
FF3L9T	H	C30	TL	$100^{h} \pm 1.9$
Lb. rhamnosus GG[f]	-	-	-	$100^{h} \pm 0.7$

A: Isolate's identification code.

B: H, cows' hay based diet; S, cows' silage based diet.

C: Samples source of LAB isolates. CC, curd after coagulation; CS, curd after stretching; C1, cheese after one day of ripening; C30 Cheese after 30 days of ripening; C60, cheese after 60 days of ripening.

D: TL, thermophilic lactobacilli isolated from MRS agar plates incubated at 37°C; ML, mesophilic lactobacilli isolated from MRS agar plates incubated at 25°C.

E: % survival: % S = [β/α] × 100 where α, Log cfu mL^{-1} of the assayed strain before gastrointestinal transit; β, Log cfu mL^{-1} of the strain after incubation under the test conditions. The results are expressed as mean ± SD of three independent experiments. [a-h]Means within a column with different superscript letters differ at $P < 0.05$.

F: *Lb. rhamnosus* GG used as reference strain.

3.3.4 Molecular identification of non-starter lactobacilli

To allow the discrimination at strain level, rep-PCR was performed to detect interspecific strain variability by amplification of repetitive sequences spread in the bacterial genome by using the primer $(GTG)_5$ (Gevers et al., 2001). The **Figure 3.5a** and **Table 3.6** show DNA-fingerprintings of 19 non-starter lactobacilli isolates with % survival after gastrointestinal transit ≥ 85%. Different strains are indicated with different letters. It was found that 7 couple of isolates showed the same DNA-fingerprinting indicated with the letters b, c, d, e, h, i and l. Then, starting from 19 isolates, 12 strains identified by different DNA-fingerprinting were obtained.

Molecular identification of strains at species level was performed by analyzing genomic sequences coding for 16S ribosomal RNA. In the **Table 3.6** were showed DNA % similarity of strains with the corresponding accession numbers found by BLAST program (bl2seq) at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All strains showed sequences of 16S rRNA referable to *Lb. casei* and *Lb. paracasei* with the same DNA % similarity. Interestingly, according to our results on survival to simulated digestion and on

identification by 16S rRNA gene sequencing, Sumeri et al. (2012) found that among several NSLAB strains isolated from two type of cheese, the most resistant strain to gastrointestinal transit belonged to *Lb. casei*/*Lb. paracasei* group.

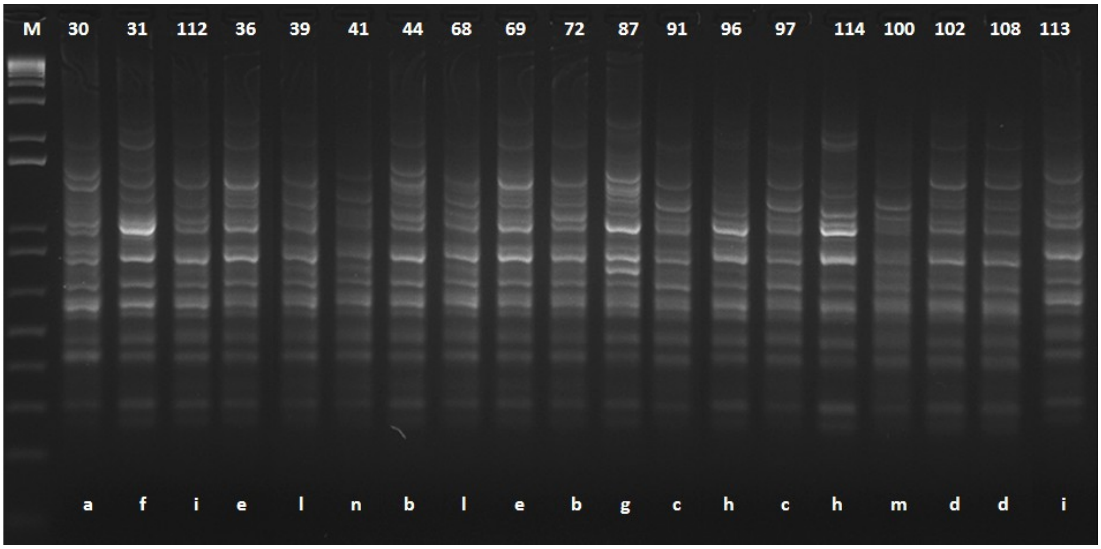

Figure 3.5a. DNA-fingerprintings by rep-PCR of 19 non starter lactobacilli with % survival after gastrointestinal transit ≥ 85%. Different strains are indicated with different letters (from a to n). M: molecular marker 1 Kb plus DNA Ladder (Invitrogen, Italy). Numeric codes on the top of each strain profile corresponding to the strains reported in the Table 3.6.

Table 3.6. Molecular identification of non-starter lactobacilli by 16S rRNA sequencing

Isolate[a]	Figure 3.5a		Species[d]	Similarity%[d]	Accession Number[d]
	Code[b]	Profile[c]			
FF3L13T	30	*a*	*Lb.casei/ Lb. paracasei*	99/99	NC014334.2/ NCPZ013921.1
FF3L1M	44	*b*	*Lb casei/ Lb. paracasei*	99/99	NC014334.2/ NCPZ013921.1
FF3L9T	72	*b*	*Lb. casei/ Lb. paracasei*	99/99	NC014334.2/ NCPZ013921.1
FF3L4T	91	*c*	*Lb. casei/ Lb. paracasei*	99/99	NC014334.2/ NCPZ013921.1
FF3L14T	97	*c*	*Lb. casei/ Lb. paracasei*	99/99	NC014334.2/ NCPZ013921.1
FF3L17T	102	*d*	*Lb. casei/ Lb. paracasei*	99/99	NC014334.2/ NCPZ013921.1
FF6L17T	108	*d*	*Lb. casei/ Lb. paracasei*	99/99	NC014334.2/ NCPZ013921.1
FF3L16M	36	*e*	*Lb. casei/ Lb. paracasei*	99/99	NC014334.2/ NCPZ013921.1
FI3L12T	69	*e*	*Lb. casei/ Lb. paracasei*	99/99	NC014334.2/ NCPZ013921.1
FF6L7T	31	*f*	*Lb. casei/*	99/99	NC010999.1/

			Lb. paracasei		NCPZ013921.1
LIL4T	87	*g*	*Lb. casei/*	99/99	NC014334.2/
			Lb. paracasei		NCPZ013921.1
FIL2M	96	*h*	*Lb. casei/*	99/99	NC014334.2/
			Lb. paracasei		NCPZ013921.1
FFL21T	114	*h*	*Lb. casei/*	99/99	NC014334.2/
			Lb. paracasei		NCPZ013921.1
FI3L1T	112	*i*	*Lb. casei/*	99/99	NC014334.2/
			Lb. paracasei		NCPZ013921.1
FF3L13M	113	*i*	*Lb. casei/*	99/99	NC014334.2/
			Lb. paracasei		NCPZ013921.1
FF3L3M	39	*l*	*Lb. casei/*	99/99	NC014334.2/
			Lb. paracasei		NCPZ013921.1
FF6L3T	68	*l*	*Lb. casei/*	99/99	NC014334.2/
			Lb. paracasei		NCPZ013921.1
FF6L8T	100	*m*	*Lb. casei/*	99/99	NC014334.2/
			Lb. paracasei		NCPZ013921.1
FF3L6M	41	*n*	*Lb. casei/*	99/99	NC014334.2/
			Lb. paracasei		NCPZ013921.1

a: see Table 3.5

b: Numeric codes of each strain used to identify isolates in the Figure 3.5a.

c: Each letter (from a to n) corresponds to DNA-fingerprinting.

d: Molecular identification of non-starter lactobacilli by 16S rRNA sequencing

As widely reported in literature (Ward and Timmins, 1999; Desai et al., 2006), species members of the group called "*Lb. casei* group", to which belong *Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus* and *Lb. zeae* species, are difficult to differentiate because of their high similarity in 16S rRNA sequences. Therefore, it was necessary conduct species-level identification using target genes with greater discriminating power, suitable for phylogenetic and taxonomic identification within closely related species as for those belonging to "*Lb. casei* group". In this study sequencing data of *hsp60* gene (groEL, encoding a 60-kDa heat shock protein) were not able to discriminate species *Lb. casei/Lb. paracasei* among non-starter lactobacilli strains (data not shown), as well as was obtained for the analysis of 16S rRNA sequences. Therefore, *tuf* multiplex assay by species-specific primers designed by Ventura et al. (2003) was performed. As shown in **Figure 3.5b**, all non-starter lactobacillus strains showed specie-specific profile that allow to identify them as belonging to *Lb. paracasei* species by generating two distinct amplicons of 240 bp and 520 bp, as well as for *Lb. paracasei* LMG P-21380 (line P). *Lb. casei* Shirota showed three bands (700, 520 and 350 bp) as expected (lines C), whereas *Lb. rhamnosus* GG generated one PCR product of about 520 bp (line GG).

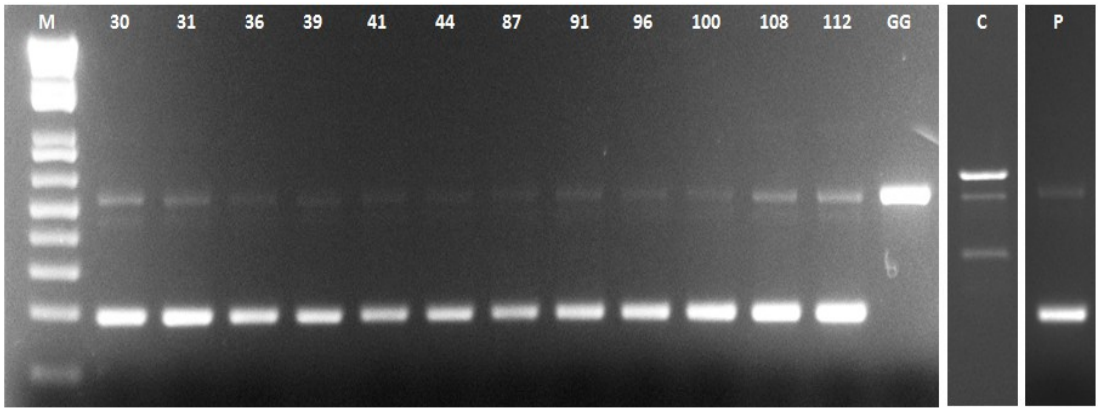

Figure 3.5b. Amplification products obtained from *tuf* multiplex assay. Lane M, 1 Kb plus DNA ladder (Invitrogen, Italy). Numeric codes on the top of each strain profile corresponding to strains reported in the Table 3.6. Lane C, *Lb. casei* Shirota; lane P, *Lb. paracasei* LMG P-21380.

3.3.5 Probiotic and functional characterization of non-starter lactobacilli

3.3.5.1 DPPH radical inhibition

DPPH radical scavenging method is a valid technique to evaluate LAB antioxidant activity thanks to its rapidity, sensitivity and reproducibility compared to other methods (Milardovic et al., 2006).

Antioxidant activity of non-starter lactobacilli were evaluated on supernatants from skim milk inoculated with strains (S-SM) and on cell suspensions after growing the strains in MRS broth (CS). The **Figure 3.6** shows DPPH radical inhibition by 12 strains previously selected for their resistance to gastrointestinal transit. CS samples showed % radical inhibition of DPPH starting from 4.90% up to 18.10% (strain FF3L6M). The antioxidant activity of S-SM samples from 12 strains ranged between 25.90% and 62.10% (**Figure 3.6**). CS samples from 7 strains did not show antioxidant activity. Moreover, radical inhibition was significantly higher for S-SM samples than CS samples (P value < 0.05) for strains that showed antioxidant activity with S-SM. The best antioxidant strain was FIL2M with % inhibition of 62.10% and 4.90% for S-SM and CS, respectively. Other researchers have studied antioxidant activity of LAB strains by using DPPH method and the most significant results were carried out by Zhang et al. (2011) and Chen et al. (2014). In both studies, scavenging DPPH radical was tested on cell suspensions of LAB strains and on cell-free extracts obtained by lysis of bacterial cells. Contrariwise with our results, antioxidant activity

of cell suspensions was higher than that of cell-free extracts for all tested strains in both studies.

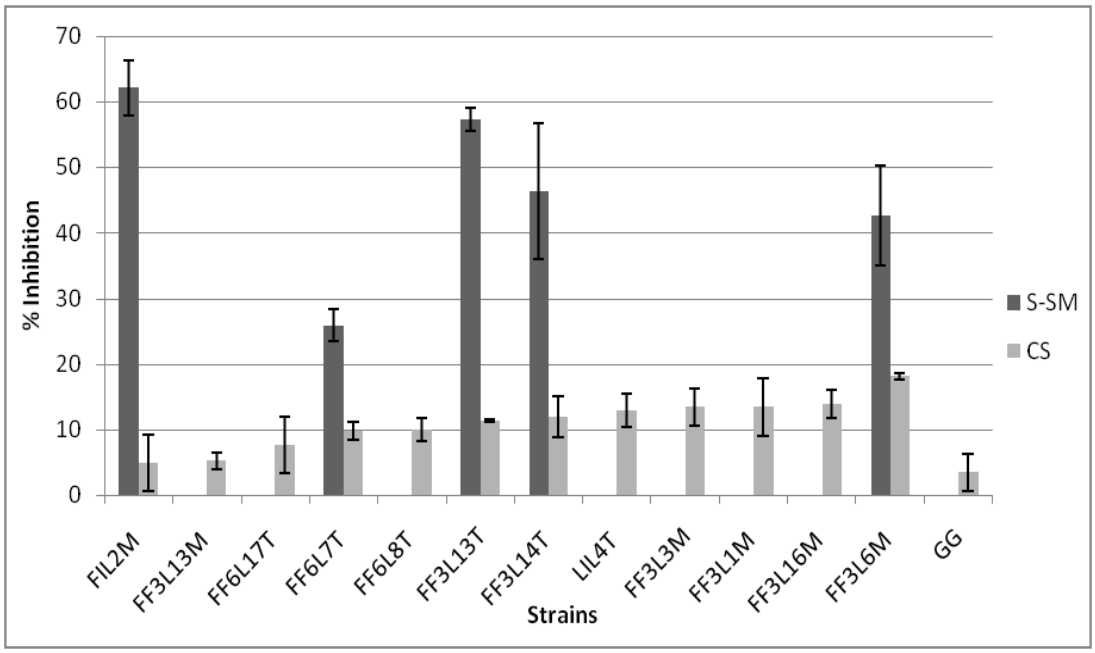

Figure 3.6. Scavenging DPPH rate of S-SM and CS of non-starter lactobacilli selected for their survival to gastrointestinal transit. The results are the mean of three replicates expressed as % scavenging DPPH inhibition± SD.

3.3.5.2 Bile salts tolerance and deconjugation

Bile salts tolerance is considered one of the primary requisites that probiotic culture must possess to survive in the intestine and to express its health benefits. In a previous study it has been calculated that the concentration of bile salts in the human intestine is about 0.3% (w/v) (Guo et al., 2012). With the purpose to test bile salts tolerance of 12 non-starter lactobacilli, MIC test was carried out to evaluate the capability to withstanding at increasing concentrations of mixture of bile salts (oxgall, Sigma-Aldrich) or of single bile acids (TDCA, TCA, GCA, GDCA) **(Table 3.7)**. According to the observation of Pennacchia et al. (2004), as oxgall or single bile acids concentration increased in plates, bacterial growth on the spots became less and less homogeneous and colonies become increasingly distanced and smaller due to bile salts inhibitory effect. Our results suggest that, except for the strain FF3L4T, the MIC of GCA was of 0.1-0.2% for all strains, suggesting that GCA was the limiting bile acid at which strains were more sensible. Afterwards, growth kinetics of strains with and without 0.3% of oxgall are shown in the **Table 3.8**. Eight among tested non-starter lactobacilli showed time required to increase the absorbance of 0.3

units in MRS-THIO broth with 0.2% or 0.3% of oxgall above 48 hours. When the same strains were incubated without oxgall, the increase of 0.3 units in absorbance was reached in times under 10 hours. Therefore, for these 8 strains the latency time was above 48 h. The strains FF3L13T, FF6L7T and LIL4T showed latency times of 4 h, 2 h and 1 h, respectively with 0.2% oxgall, and of 6 h, 2 h and 2 h, respectively with 0.3% oxgall. Only the strain FIL2M did not show delay on growth when incubated with oxgall compared to the control (growth without oxgall) (**Table 3.7**). Moreover, our results showed significantly differences in latency time among tested strains (P value < 0.05) for both 0.2% and 0.3% concentrations of oxgall. Chen et al. (2014) found that among strains tested for bile salts tolerance, three *Lb. casei* strains (2W, BD-II and Zhang) showed latency times of 2.32 h, 3.76 h and 1.08 h confirming that bile tolerance could be strain-specific as confirmed by our results. Likewise, Li et al. (2015) confirmed strain-specific bile tolerance observing that 9 *Lb. plantarum* strains showed different sensitiveness after growing with bile salts.

Table 3.7. Bile salts minimal inhibitory concentration (MIC) of non-starter lactobacilli

Species	Strain[a]	MIC[b]				
		Oxgall	TCA	TDCA	GCA	GDCA
Lb. paracasei	FF3L13T	0.5	> 0.5	> 0.5	> 0.5	0.2
Lb. paracasei	FF6L7T	0.5	> 0.5	> 0.5	> 0.5	0.2
Lb. paracasei	FF3L16M	> 0.5	> 0.5	> 0.5	> 0.5	0.2
Lb. paracasei	FF3L3M	0.5	> 0.5	> 0.5	> 0.5	0.2
Lb. paracasei	FF3L6M	0.5	> 0.5	> 0.5	> 0.5	0.2
Lb. paracasei	FF3L1M	0.5	> 0.5	> 0.5	> 0.5	0.2
Lb. paracasei	LIL4T	> 0.5	> 0.5	> 0.5	> 0.5	0.2
Lb. paracasei	FF3L4T	>0.5	>0.5	>0.5	> 0.5	>0.5
Lb. paracasei	FIL2M	0.4	> 0.5	> 0.5	> 0.5	0.2
Lb. paracasei	FF6L8T	0.5	> 0.5	> 0.5	> 0.5	0.2
Lb. paracasei	FF6L17T	> 0.5	> 0.5	> 0.5	> 0.5	0.1
Lb. paracasei	FF3L13M	0.5	> 0.5	> 0.5	> 0.5	0.2
Lb. rhamnosus	GG	> 0.5	> 0.5	> 0.5	> 0.5	0.2

a: see Table 3.5

b: MIC, the lowest concentration (%) of bile salts that causes a total inhibition on the growth of colonies in the spots. TCA, taurocholic acid sodium salt; TDCA, sodium taurodeoxycholate; GCA, glycocholic acid sodium salt; GDCA, sodium glycodeoxycholate (Sigma-Aldrich).

Table 3.8. Bile salts tolerance of non-starter lactobacilli

Species	Strain[A]	$h_{0.3}$[B]			LT (h)[C]	
		0.2%	0.3%	CT	0.2%	0.3%
Lb. paracasei	FF3L13T	11	13	7	4^{a}	6^{e}
Lb. paracasei	FF6L7T	7	7	5	2^{b}	2^{b}
Lb. paracasei	FF3L16M	> 48	> 48	9	> 48^{d}	> 48^{d}
Lb. paracasei	FF3L3M	> 48	> 48	10	> 48^{d}	> 48^{d}
Lb. paracasei	FF3L6M	> 48	> 48	8	> 48^{d}	> 48^{d}
Lb. paracasei	FF3L1M	> 48	> 48	8	> 48^{d}	> 48^{d}
Lb. paracasei	LIL4T	6	7	5	1^{bc}	2^{b}
Lb. paracasei	FF3L4T	> 48	> 48	5	> 48^{d}	> 48^{d}
Lb. paracasei	FIL2M	5	5	5	0^{c}	0^{c}
Lb. paracasei	FF6L8T	> 48	> 48	8	> 48^{d}	> 48^{d}
Lb. paracasei	FF6L17T	> 48	> 48	9	> 48^{d}	> 48^{d}
Lb. paracasei	FF3L13M	> 48	> 48	9	> 48^{d}	> 48^{d}
Lb. rhamnosus	GG	5	7	4	1^{b}	3^{ab}

A: see Table 3.5

B: time (h) required to increase the absorbance (600 nm) of 0.3 units in MRS-THIO broth without oxgall (CT) or with 0.2% or 0.3% oxgall.

C: LT, latency time that is the delay (h) of bacterial growth in presence of oxgall calculated as the difference between the time (h) required to increase the absorbance (600 nm) of 0.3 units with oxgall (0.2% or 0.3%) and the time required to increase the absorbance (600 nm) of 0.3 units without oxgall (CT). Values within columns with different superscript letters (a-e) differ at $P < 0.05$.

Non-starter lactobacilli resistant to gastrointestinal transit were tested for the presence of Bile Salt Hydrolase (BSH) enzyme. BSH is involved in the deconjugation of bile salts that allow bacteria to be protected from conjugated bile salts of which toxicity to bacteria is known (De Smet et al., 1995). Except for strain LIL4T, that showed a weak BHS activity to TDCA (**Figures 3.7a and 3.7b**), all strains tested did not show precipitation zones around the colonies or growth of opaque colonies on MRS agar with TDCA, suggesting the lack ability to deconjugate bile salts. Some of strains, although not carrying BSH activity, exhibited high tolerance to bile salts. Studies by Schmidt et al. (2001) showed that the resistance to bile salts by lactobacilli may not be related to BSH activity as well as showed by Solieri et al. (2014) that found NSLAB strains able to tolerate bile salts but not to deconjugate them.

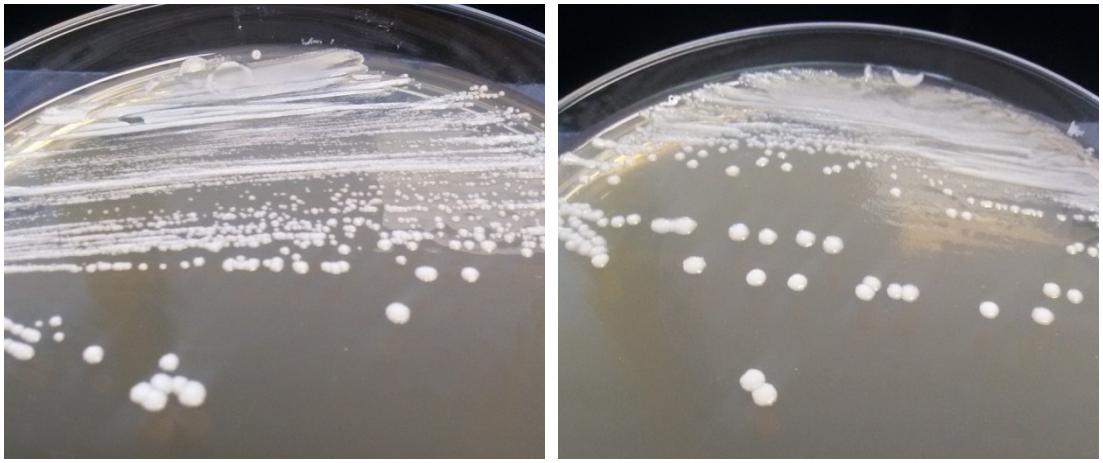

Figure 3.7a (left) weak BSH activity after incubation of the strain LIL4T in MRS agar + 0.3% (w/v) TDCA by showing white opaque colonies compared to the **Figure 3.7b** (right) where LIL4T shows white polished colonies grown on MRS agar without TDCA.

3.3.5.3 Growth on prebiotic fibres

Prebiotic is a non-digestible carbohydrate by humans that promotes the modulation of the gut flora favoring its colonization by several *Lactobacillus* and *Bifidobacterium* strains able to enzymatically hydrolyse prebiotics in the caeco-colon (Delzenne, 2003) and improving the human health by inhibition of gut pathogens, lowering serum cholesterol, prevention of colon cancer, stimulation of calcium and other minerals absorption and enhancing the immune response (Cummings and Macfarlane, 2002; Marteau and Boutron-Ruault, 2002).

FOS and inulin are respectively oligomers and polymers of fructose with the generic structure, α-D-Glu- (1-2)-[β-D-Fru-1-2-]$_n$.

Non-starter lactobacilli were tested for their ability to use prebiotic fibers, such as inulin and FOS as alternative carbon sources. The **Table 3.9** shows the specific speed rate of the strains grown with glucose, inulin or FOS. The strains showed specific speed rate with glucose starting from 0.129 to 0.177. LIL4T was the unique strain able to grow with inulin and FOS as alternative carbon source with specific speed rates of 0.192 and 0.151, respectively. The reason why FOS and inulin did not promote the growth of the other 11 strains may be due to their chain length and structure. In fact, the longer chains of carbohydrates the slower fermentability by bacteria (Van Laere et al., 2000). In a previous screening on 25 strains of *Lb. plantarum*-group, *Lb. brevis, Lb. paracasei*-group regarding their ability to ferment

FOS, inulin and other prebiotics, a species-related behaviour of fermentation was observed (Pennacchia et al., 2006). Strains of *Lb. plantarum*-group were able to ferment FOS but not the inulin; *Lb. brevis* strains were not able to ferment inulin while fermented FOS with very slow rate. *Lb. paracasei*-group strain EL7 was the only able to ferment inulin and FOS with similar grow rate of the control media (with glucose and sucrose).

Table 3.9 Growth of non-starter lactobacilli strains with prebiotic fibres

Species	Strain[a]	μ[b]		
		glucose	inulin	FOS
Lb. paracasei	FF3L13T	0.142±0.01	[b]0	0
Lb. paracasei	FF6L7T	0.133±0.01	0	0
Lb. paracasei	FF3L16M	0.134±0.00	0	0
Lb. paracasei	FF3L3M	0.129±0.01	0	0
Lb. paracasei	FF3L6M	0.143±0.00	0	0
Lb. paracasei	FF3L1M	0.138±0.00	0	0
Lb. paracasei	LIL4T	0.177±0.01	0.192±0.01	0.151±0.00
Lb. paracasei	FF3L4T	0.147±0.01	0	0
Lb. paracasei	FIL2M	0.137±0.00	0	0
Lb. paracasei	FF6L8T	0.134±0.00	0	0
Lb. paracasei	FF6L17T	0.144±0.01	0	0
Lb. paracasei	FF3L13M	0.133±0.00	0	0

a: see Table 3.5

b: μ, specific speed rate; $\mu = \ln_f OD_{650} - \ln_0 OD_{650} / t_f - t_0$

3.3.5.4 Antibiotic susceptibility

Agar disk diffusion method was used to determine susceptibility to 8 antibiotics (ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, and tetraclycine). All 12 strains were sensitive to ampicillin (AMP), tetraclycine (TE), gentamycin (CN), and moderately sensitive to clindamycin (DA) (**Table 3.10** and **Figures 3.8a**, **3.8b**). Furthermore, all strains including the reference strain *Lb. rhamnosus* GG, displayed resistance to kanamycin (K) by showing none halo of inhibition around the disks. All strains showed moderate susceptibility to streptomycin, except for two streptomycin resistant strains FF3L1M and FF3L13M, whereas FF3L4T was the only strain susceptible to streptomycin and chloramphenicol but showed resistance to erythromycin. In general, 75% of strains showed the same antibiotic susceptibility profile of the reference strain *Lb.*

rhamnosus GG (**Table 3.10**). Moreover, the most susceptible strain to antibiotics was FF3L4T.

Table 3.10. Antibiotic susceptibility of non-starter lactobacilli

Strain	Antibiotic[a]							
	AMP	**K**	**DA**	**TE**	**CN**	**C**	**S**	**E**
FF3L13T	S[b]	R[b]	MS[b]	S	S	MS	MS	MS
FF6L7T	S	R	MS	S	S	MS	MS	MS
FF3L16M	S	R	MS	S	S	MS	MS	MS
FF3L6M	S	R	MS	S	S	MS	MS	MS
FF3L3M	S	R	MS	S	S	MS	MS	MS
FF3L1M	S	R	MS	S	S	MS	R	MS
LIL4T	S	R	MS	S	S	MS	MS	MS
FF3L4T	S	R	MS	S	S	S	S	R
FIL2M	S	R	MS	S	S	MS	MS	MS
FF6L8T	S	R	MS	S	S	MS	MS	MS
FF6L17T	S	R	MS	S	S	MS	MS	MS
FF3L13M	S	R	MS	S	S	MS	R	MS
GG[c]	S	R	MS	S	S	MS	MS	MS

a: AMP, ampicillin (10 µg); K, kanamycin (30 µg); DA, clindamycin (2 µg); TE, tetraclycine (30 µg); CN, gentamycin (30 µg); C, chloramphenicol (10 µg); S, streptomycin (25 µg); E, erythromycin (5 µg).
b: Susceptibility expressed as R (resistant), MS (moderately susceptible), or S (susceptible).
c: *Lb. rhamnosus* GG as reference strain.

Lactobacillus spp. are generally susceptible to inhibitors of cell wall synthesis as penicillin and β-lactamase, but are often resistant to cephalosporins and vancomycin. At the same time, lactobacilli are usually sensitive to low concentrations of many inhibitors of protein synthesis, such as chloramphenicol, macrolides, lincosamides, and tetracycline, but are often resistant to aminoglycosides (Gueimonde et al., 2013). Our results partially corroborate the study of Radulović and coworkers (2010) that found four autochthonous *Lb. paracasei* strains, isolated from traditional Serbian cheese, susceptible to chloramphenicol, ampicillin, tetracycline, and resistant to streptomycin and kanamycin. In accordance with our results, in a previous screening on potential probiotic NLAB isolated from ripened Parmigiano Reggiano cheese (Solieri et al., 2014), 18 *Lb. paracasei* strains and strains belonging to species *Lb. rhamnosus*, *Lb. casei*, *Lb. harbinensis,* and *Lb. fermentum*, showed sensitivity to ampicillin, tetracycline, chloramphenicol, anderythromycin. Furthermore, all *Lb. paracasei* strains were resistant to streptomycin. On the other hand, previous studies reported that LAB are usually resistant to the class of aminoglycoside antibiotics

(such as streptomycin, kanamycin, and gentamycin) where the reduced sensibility may be due to low cell membrane permeability (Charteriset al., 1998; Katla et al., 2001; Danielsen and Wind, 2003).

Safety concerns on the use of antibiotic resistant strains as probiotic, arise due to the possibility of transferring antibiotic resistant genes to intestinal pathogens (Curragh and Collins, 1992). On this purpose, determination of antibiotic resistant genes location could contribute in safely application of antibiotic resistant probiotic LAB (Radulovićet al., 2010). Antibiotic resistance of lactobacilli is non-transmissible to other bacteria because is chromosomally encoded (Charteris et al., 2001; Katla et al., 2001; Danielsen and Wind, 2003). Particularly, the resistance of lactobacilli to aminoglycosides is chromosomally encoded and is attributed to the lack of a cytochrome involved into the antibiotic uptake (Monteagudo-Mera et al., 2012).

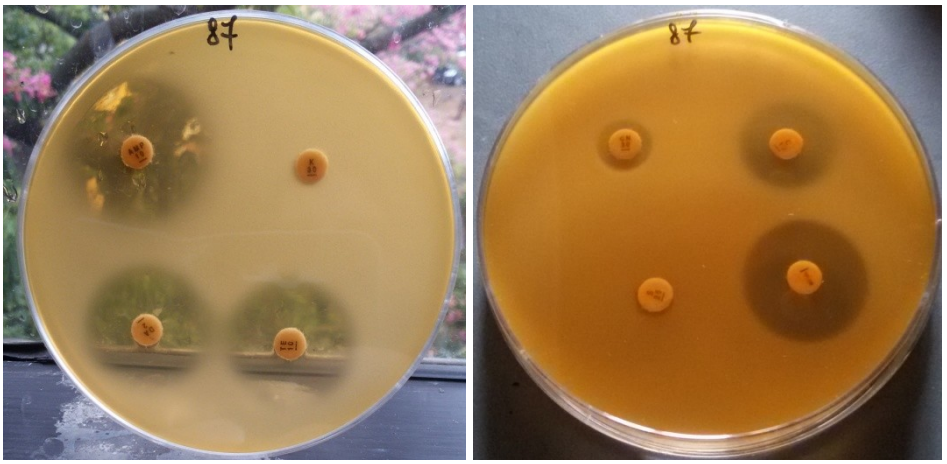

Figure 3.8a (left) Antibiotic susceptibility of *Lb. paracasei* LIL4T to AMP, K, DA and TE displayed as halo of inhibition in MRS agar plates after 24 h of incubation at 37°C in anaerobiosis. **Figure 3.8b** (right) Antibiotic susceptibility of *Lb. paracasei* LIL4T to CN, C, S and E in the same conditions described above.

3.3.5.5 Cholesterol removal activity

An excess of serum cholesterol is associated with cardiovascular disease considered one of the major causes of mortality in the world. Many *in vitro* and/or *in vivo* studies have demonstrated cholesterol-lowering activity of probiotic LAB strains (Michael et al., 2016, 2017; Gorenjak et al., 2014; Huang and Zheng, 2010; Huang et al., 2010, 2013; Yoon et al., 2011, 2013), even if the mechanisms involved into reduction of serum cholesterol levels by probiotic LAB are still partially unknown.

The results of cholesterol assimilation ability by 12 non-starter lactobacilli isolated from caciocavallo of Castelfranco cheese in MRS broth containing 300 µg/mL of cholesterol are reported in **Figure 3.9.** The percentage of cholesterol assimilation by strains ranged from 10.4% (strain FF6L6T) to 31.3% (strain LIL4T); whereas FF3L13M, FF3L6M and FF3L1M were not able to assimilate cholesterol in the medium. The reference strain *Lb. rhamnosus* GG assimilated 35.3% of cholesterol initially added in the medium. The results regarding the ability to assimilate cholesterol by strains in MRS broth supplemented with 0.3% oxgall and in simulated intestinal fluid are reported in **Figure 3.10.**

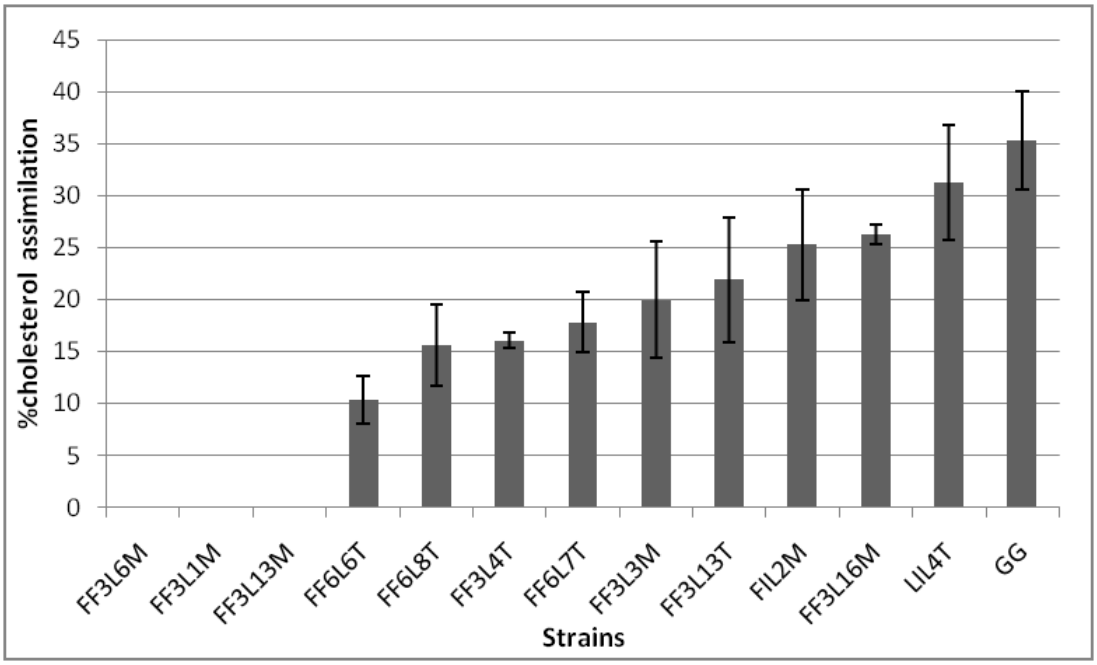

Figure 3.9. Cholesterol removal ability by non-starter lactobacilli in MRS broth. % cholesterol assimilation = [cholesterol (µg/mL)$_{0h}$ - cholesterol (µg/mL)$_{24h}$ / cholesterol (µg/mL)$_{0h}$] ×100. The results are the mean of three replicates expressed as % cholesterol assimilated by strains ± SD.

This assay was performed on those strains that in previous assay on bile salts tolerance, showed fast growth in MRS-THIO broth with 0.3% of oxgall (FF3L13T, FF6L7T, LIL4T and FIL2M) (see **Table 3.7**). These 4 strains showed varying cholesterol assimilation percentage: 0%, 13.1%, 22.2% and 1.62% in MRS broth supplemented with oxgall respectively for the strains FF3L13T, FF6L7T, LIL4T and FIL2M. Cholesterol assimilation percentages when the strains FF3L13T, FF6L7T, LIL4T and FIL2M were incubated in simulated intestinal fluid were of 9.4%, 51.2%, 68.4% and 15.8%, respectively. Analyzing the results on cholesterol assimilation by non-starter lactobacilli in the three different media, it may notice that cholesterol

assimilation percentage was lower in MRS broth than MRS broth supplemented with oxgall for all tested strains, while the assimilation in simulated intestinal fluid was higher than in MRS broth supplemented with oxgall (**Figures 3.9** and **3.10**).

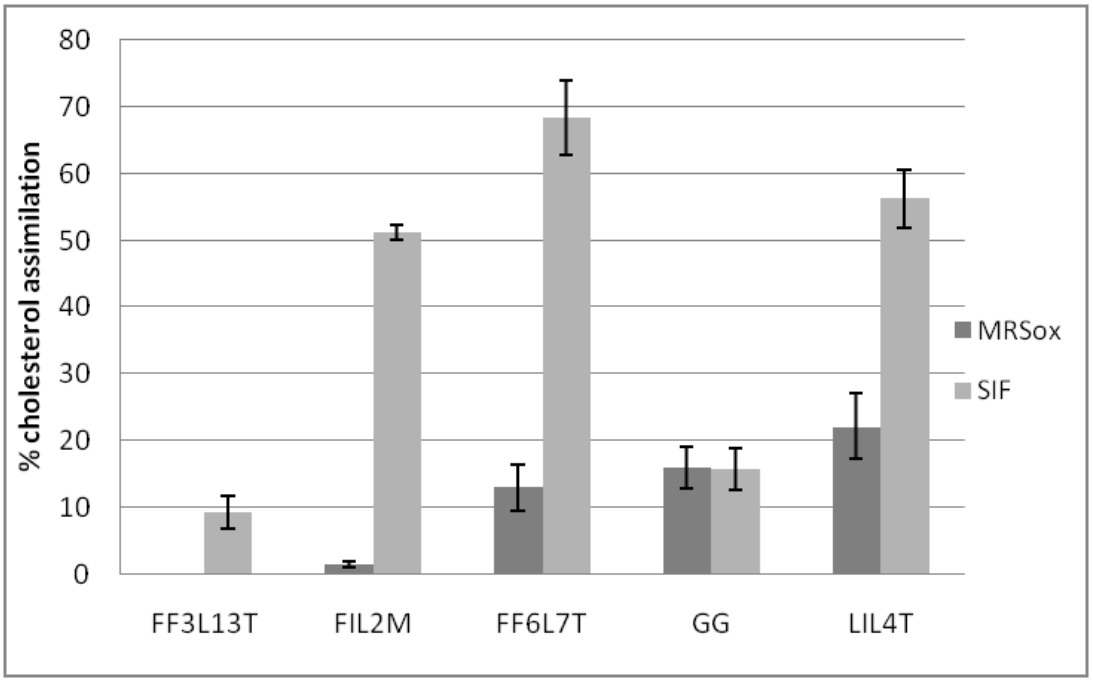

Figure 3.10. Cholesterol removal ability by non-starter lactobacilli in MRS broth supplemented with 0.3% oxgall (MRSox, dark grey bars) and in simulated intestinal fluid (SIF, light grey bars). % cholesterol assimilation = [cholesterol $(\mu g/mL)_{0h}$ - cholesterol $(\mu g/mL)_{24h}$ / cholesterol $(\mu g/mL)_{0h}$] ×100. The results are the mean of three replicates expressed as % cholesterol assimilated by strains± SD.

Therefore, these results suggest that cholesterol assimilation may influenced by growth media composition, such as the presence or not of bile salts. Tomaro and coworkers (2014) tested the cholesterol assimilation ability of 11 strains of lactobacilli belonging to *Lb. reuteri, Lb. fermentum, Lb. rhamnosus, Lb. acidophilus* and *Lb. plantarum* species. Cholesterol assimilation rates recorded by strains in MRS broth ranged from 13% to 39%, while in simulated intestinal fluid ranged between 11% and 73%. Therefore, these results are in agreement with ours since cholesterol assimilation ranges in MRS broth and in simulated intestinal fluid are approximately the same.

3.4 CONCLUSIONS

The strains listed in **Table 3.11** reported SLAB isolated from Caciocavallo of Castelfranco that have shown the best results in pro-technological activities performed in this study, and that led to consider them as promising starter cultures for the production of type caciocavallo cheese or for other cheeses. In the specific case of Caciocavallo cheese production, since the curd maturation takes place in 4-5 hours and the cheese is ripened for 60 to 90 days, strains of *S. thermophilus* FFS5T and CFS10T can be proposed to drive the cheesemaking thanks especially to their good acid production in milk and suitable proteolytic and lipolytic abilities. In any case, it is necessary confirm the results carried out from *in vitro* selection, directly in food system. Therefore, further studies are needed in order to validate technological activities of selected SLAB strains during the experimental production of Caciocavallo cheese.

Moreover, experimental results obtained in this work provide a general screening of non-starter lactobacilli isolated from Caciocavallo of Castelfranco cheese, some of which might be considered potentially probiotic considering the results carried out from *in vitro* screening. The strains listed in **Table 3.12** reported non-starter lactobacilli isolated from Caciocavallo of Castelfranco cheese that have shown the best results in functional activities performed, and that leading to consider them as promising probiotics. Due to good results showed in functional tests and for the ability to assimilate cholesterol in liquid media, *Lb. paracasei* LIL4T was tested to assess its cholesterol lowering ability on human colorectal adenocarcinoma Caco-2 cell line (Chapter 4).

Table 3.11. Strains selected as potential starter cultures for Caciocavallo cheese production

Species	Strain[a]	Acid production[b]	Lipolytic activity[c]	Proteolytic activity[d]	Diacetyl production[e]
S. thermophilus	CFS10T	3.15±0.10	1.0±0.03	0.245±0.016	0.322±0.007
S. thermophilus	FFS5T	3.34±0.11	0.75±0.02	0.128±0.009	0.243±0.01

a: see Table 3.1

b: acid production in milk ($t_{pH5.2} \leq 6h$) expressed as hours ± SD spent by strains to lower pH of skim milk to 5.2.

c: lipolytic activity of strains expressed as grams of palmitic acid produced by hydrolysis of 100 g of milk fat by strains after 24 days of incubation at 15°C in whole milk.

d: proteolytic activity of strains expressed as values of absorbance (340 nm) read after 30 days of incubation at 15°C in skim milk.

e: diacetyl production expressed as mean ± SD of ratio between the height (cm) of the red ring and the height (cm) of whole mixture in the tube.

Table 3.12. Strains selected as potential probiotic cultures

Species	Strain[a]	%S [b]	A.A. of S-SM[c]	Growth oxgall[d]	Growth inulin/FOS[e]	Chol. Ass. in SIF[f]
Lb. paracasei	FF6L7T	85.8±1.5	25.9±2.9	2	0/0	68.4±9.2
Lb. paracasei	FIL2M	84.8±2.3	62.1±4.8	0	0/0	51.2±2.7
Lb. paracasei	LIL4T	98.7±1.6	0	2	0.19±0.01/0.15±0.0	56.3±8.3

a: see Table 3.5

b: %S, % survival = [β/α] × 100 where α, Log cfu mL^{-1} of assayed strain before gastrointestinal transit; β, Log cfu mL^{-1} of strain after incubation under test conditions.

c: % scavenging DPPH inhibition of supernatant from fermented skimmed milk.

d: latency time that is the delay (h) of bacterial growth in presence of 0.3% oxgall calculated as the difference between the time required to increase the absorbance (600 nm) of 0.3 units with 0.3% oxgall and the time required to increase the absorbance of 0.3 units without oxgall.

e: μ, specific speed growth rate.; $\mu = \ln_f OD_{650} - \ln_0 OD_{650}/t_f - t_0$

f: Cholesterol removal ability by NSLAB strains in simulated intestinal fluid (SIF). % cholesterol assimilation = [cholesterol $(\mu g/mL)_{0h}$ - cholesterol $(\mu g/mL)_{24h}$ / cholesterol $(\mu g/mL)_{0h}$] × 100.

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CHAPTER 4

Involvement of *Lactobacillus paracasei* 87 on cholesterol lowering mechanisms in Caco-2 enterocytes

4.1 INTRODUCTION

Nowadays, cardiovascular disease (CVD) represents one of the major causes of death in industrialized countries. Several epidemiologic studies impute hypercholesterolemia as one among main risk factors for CVD (Gyarfas et al., 2006; Kearney et al., 2004; Pereira and Gibson, 2002) together with hypertriglyceridemia, hypertension, hyperglycemia, chronic inflammation and Type 2 diabetes mellitus (Roger et al., 2012). Conventional approaches to prevent hypercholesterolemia are based on the daily administration of statins, able to inhibit *de novo* synthesis of cholesterol, or on the intake of cholesterol absorption inhibitors (Buckley and Ramji, 2015; Michael et al., 2012; Taylor et al., 2013). However, taking of these drugs causes more or less serious side effects in different body systems (Banach et al., 2015; Abel and Feher, 2009; Buettner and Lecker, 2008). The need to replace cholesterol-lowering drugs and the contemporary large diffusion of CVD and of correlated risks into the world population, led to act by the cure of risk factors, and especially on their prevention. Healthy life styles and eating habits based on the use of substances with proved lowering serum cholesterol ability, such as soluble fibers and other prebiotics, omega-3 fats, antioxidants, plant sterols, monounsaturated fatty acids and probiotic bacteria, are adopted as ways to prevent CVD's risk factors. LAB represent a large slice among microorganisms with probiotic properties, particularly strains and species belonging to *Lactobacillus* genus able to prevent or reduce several gastrointestinal disorders and correlated disease, such as food allergies (Marco et al., 2006; Saxelin et al., 2005) .

In the last years is increasing the interest on probiotic bacteria as tool to reduce serum cholesterol level. On this purpose, many *in vitro* and/or *in vivo* studies to evaluate cholesterol-lowering activity of LAB strains have been done (Michael et al., 2016, 2017; Gorenjak et al.,2014; Huang and Zheng, 2010; Huang et al., 2010, 2013; Yoon et al., 2011, 2013), even if the mechanisms involved into the reduction of serum cholesterol levels by probiotic LAB are still partially unknown. However, several studies have highlighted that probiotic bacteria, particularly LAB genera, are involved on cholesterol-lowering activity through several mechanisms involved into

cholesterol homeostasis and cholesterol metabolism of the host. Enzymatic deconjugation of bile acids is one of the best known way that LAB use to reduce cholesterol serum levels. In fact, some LAB strains are capable to assimilate cholesterol and/or deconjugate bile salts through some active Bile Salt Hydrolase enzymes (BSH) (Scheinbach, 1998). The best colonization and survival in the intestine is showed by BSH positive lactobacilli than BSH negative ones, suggesting that BSH activity may be considered as crucial attribute to select probiotic lactobacilli (De Smet et al., 1995).

Bile salts in deconjugated form are less soluble and less absorbable by intestinal tract; therefore, they are easily and rapidly excreted by faeces. This involves to replace excreted bile salts by increasing *de novo* bile synthesisby liver, starting from circulating cholesterol that is natural precursor of bile salts. Consequently, reducing intestinal absorption, cholesterol levels in the blood decrease (De Smet et al., 1994; Usman, 1999). Several studies have shown that probiotic bacteria, including lactobacilli, may modulate key intestinal cholesterol transport-related proteins by regulating their gene expression (Michael et al., 2016, 2017; Gorenjak et al., 2014; Huang and Zheng, 2010; Huang et al., 2010, 2013; Yoon et al., 2011, 2013). Main proteins involved in cholesterol transport in the intestine are: Niemann-Pick C1-like 1 protein (NPC1L1), ATP-binding cassette transporter G5/8 (ABCG5/8), ATP-binding cassette transporter-1 (ABCA-1) and HMG-CoA reductase (HMCGR). Main cholesterol transporters and their localization in enterocytes are shown in **Figure 4.1.**

NPC1L1 transporter is localized at brush border of intestinal enterocytes and plays key role on cholesterol uptake by taking up free cholesterol from the intestinal lumen into epithelial cells (Altman et al., 2004). Ezetimibe is NPC1L1 inhibitor that reduces the cholesterol absorption and is used to treat hypercholesterolemia (Sudhop et al., 2002). As well as NPC1L1, transporters ABCG5 and ABCG8 are highly expressed on the brush border of intestinal enterocytes and are found to promote intracellular cholesterol efflux from the inside of the enterocyte back to intestinal lumen (Berge et al., 2000; Hui and Howles, 2005). An overexpression of ABCG5/ABCG8 genes may promote the excretion of biliary cholesterol and limit the cholesterol absorption from the intestine. By contrast, mutations in these genes may contribute to sitosterolemia (Shmitz et al., 2001). ABCA-1 uses apolipoprotein-AI (Apo-AI) as cholesterol acceptor and is highly expressed on basal membrane of the enterocyte. ABCA-1 is responsible of cholesterol efflux together with ABCG5 and ABCG8. ABCA-1

promote the cholesterol efflux to extracellular Apo-AI, which thus determines the reduction of intracellular cholesterol levels. Downregulation on the expression of ABCA-1 in mice may impede the absorption of cholesterol into intestinal enterocytes (Drobnik et al., 2001). HMGCR may promote the atherosclerosis through sequential mechanisms by causing the increase of the catabolism of plasma LDL (Alaarg et al., 2017). One of inhibition way mainly widespread to inhibit HMGCR function is by using cholesterol-lowering drugs known as statins.

The objective of this study was to investigate whether *Lb. paracasei* 87 impact cholesterol efflux and uptake and regulates genes for transporters involved into cholesterol homeostasis and metabolism using human colorectal adenocarcinoma Caco-2 cells as intestinal cell model. *Lb. paracasei* 87 known also as *Lb. paracasei* LIL4T (see Table 3.5 in Chapter 3) was isolated during Caciocavallo of Casterlfranco cheese production, particularly from a sample of curd after coagulation from milk of cows fed with a based silage diet. *Lb. paracasei* 87 was classified as low acidifying strain and assayed for properties of probiotic and functional interest. Due to its promising attitude as probiotic strain and for its ability to assimilate cholesterol in liquid media, *Lb. paracasei* 87 was tested to confirm its cholesterol lowering ability on Caco-2 cells.

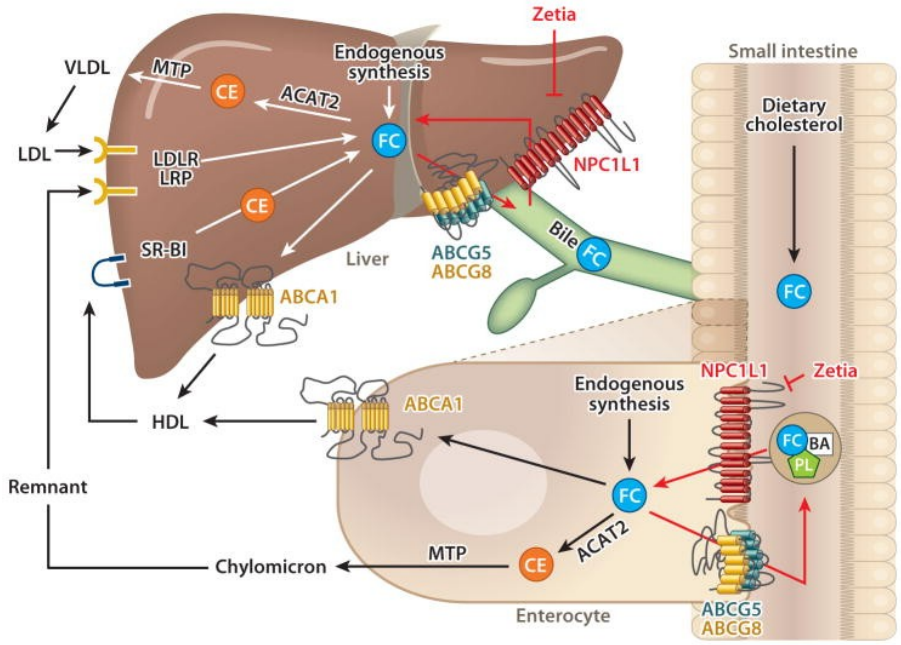

Figure 4.1. Cholesterol transport in the small intestine and liver. FC: free cholesterol; BA: bile salts; PL: phospholipids (Jia et al., 2011).

4.2 MATERIALS AND METHODS

4.2.1 Caco-2 cell culture conditions

The human colorectal adenocarcinoma Caco-2 cell line used for the assays were obtained from American Type Culture Collection (ATCC, Middlesex, UK). Cell line maintenance and sub-culturing were carried out following good Caco-2 cell culture practices (Natoli et al., 2012). Briefly, Caco-2 cells were grown in 75 cm^2 flasks (Corning Inc., USA) in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose (Biosera, UK) and supplemented with: 1% (v/v) of MEM non essential amino acids (NEAA; Sigma, UK); 10% (v/v) of foetal bovine serum (FBS, Labtech, UK) heat inactivated at 60°C for 10 minutes before use; 100 U/mL penicillin; 100 µg/mL streptomycin. Cell cultures were incubated at 37°C in 5% CO_2 and 90% humidity and sub-cultured in well plates at about $5x10^5$ cells/cm^2 when reached 80% confluence in the flask. Caco-2 cells were maintained for 18 to 21 days at 37°C in 5% CO_2 and 90% humidity to ensure their polarization. The medium was changed three times per week. One week before the assays, Caco-2 cells were fed with supplemented DMEM lacking of antibiotics. The assays were performed with cells from passages 4 to 14.

4.2.2 Standardization of bacteria for assays with Caco-2 cells

Lb. paracasei 87 and *Lb. plantarum* CUL66 were grown in MRS broth for 18 hours at 37°C and in anaerobic conditions. *Lb. plantarum* CUL66 is a probiotic strain kindly given by Cultech Ltd (Port Talbot, UK) and was used as reference strains for gene expression analysis, cholesterol uptake and efflux assays. Culture broths of each strain were centrifuged at 5495 x *g* for 10 min and bacterial pellets were washed once in sterile PBS. Cellular pellets of both strains were then suspended in DMEM with 4.5 g/L glucose, 1% (v/v) NEAA, 10 mMN-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) and standardized at the following concentrations (cfu mL^{-1}): $2.0x10^8$, $1.0x10^8$, $5.0x10^7$, $1.0x10^7$, $5.0x10^6$, $1.0x10^6$, $1.0x10^5$, and $1.0x10^4$. For both the strains, 1 mL of each standardized culture broth was co-incubated on Caco-2 cell monolayer for 6 h at 37°C in 5% CO_2 and 90% humidity. After the incubation, the supernatants were removed and pH measured. For the assays performed with Caco-2 cells, the highest bacterial concentrations that did not cause a decrease of pH under 6.5 were chosen to avoid damages to Caco-2 cells.

Standardized strains were assayed alone or combined in ratio 1:1 (v/v) for all tests described below. For each assay pH of supernatants were checked at the end of the incubation period.

4.2.3 Adhesion to Caco-2 cell monolayer assay

Adhesion ability of the strains was performed on 24-well tissue culture plates (Costar, UK) containing Caco-2 monolayers of cells grown for 21 days. The assay was performed by the method of García-Cayuela et al. (2014) with minor modifications. Caco-2 monolayers were washed twice in warm phosphate buffered saline (PBS, pH 7.4) to allow the removal of antibiotics. Meanwhile, 18 hours cultures of *Lb. paracasei* 87 and *Lb. plantarum* CUL66 were centrifuged (5495 x *g* for 10 min) and washed in PBS. Bacterial pellets were suspended in warm non-supplemented DMEM and bacterial concentration adjusted at about $1.0x10^8$ cfu mL^{-1} before the incubation with Caco-2 monolayers for 1 h at 37°C and 5% CO_2. After incubation, the supernatants were removed and Caco-2 monolayers were washed three times with PBS to remove non-adhered bacteria. After washing, Caco-2 monolayers were lysed by adding 0.05% trypsin-EDTA solution (ThermoFischer Scientific, UK) for 5 min at 37°C. The number of adhered bacterial cells was estimated after plating 10-fold dilutions on MRS agar (Oxoid, UK) for 48-72 h of incubation at 37 °C. The results were expressed as: 1) as the number of adhering bacteria per mm^2 of Caco-2 monolayer; 2) as the number of adhering bacteria per one Caco-2 epithelial cell; 3) as the percentage of adhering bacteria calculated as follows:

$$\% \text{ adhesion} = (\text{cfu mL}^{-1}\ [b_f]/\text{cfu mL}^{-1}\ [b_i]) \times 100$$

Where b_f, adhered bacteria; b_i, added bacteria.

For each strain, three independent experiments were carried out, and for each experiment, three replicates per strain were performed.

4.2.4 Viability of Caco-2 cells exposed to bacteria

Viability assay was performed on 24-well tissue culture plates (Costar, UK) containing Caco-2 monolayers of cells grown for 21 days. Simultaneously with gene expression assay, the viability of Caco-2 monolayers co-incubated with bacterial strains was evaluated according to the method of Gorenjak et al. (2014). Caco-2 monolayers were incubated with *Lb. plantarum* CUL66 (2.0×10^8) or *Lb. paracasei* 87 (5.0×10^7 cfu mL^{-1}) or with a mixture of both the strains in DMEM (plus 4.5 g/l glucose, 1% (v/v) NEAA and 10 mM HEPES) for 6 hours. After the incubation, Caco-2 monolayers were washed with warm PBS, added with 100 µl of 0.1% crystal violet in 2% (v/v) ethanol and left to react for 5 min. Cell monolayers were washed once with warm sterile deionized water and left dry at room temperature overnight. After adding 100 µl of 10% acetic acid to each well, the plates were placed on a horizontal orbital shaker for 1 h. Viability was measured spectrophotometrically at 595 nm by microplate reader 680 (Bio-rad) to detect the crystal violet retained in living Caco-2 cells control (incubated without bacteria) and comparing it with absorbance of living Caco-2 cells co-incubated with bacteria. The assay was performed in triplicate and the results expressed as a percentage of the control, that has been assigned as 100%.

4.2.5 Gene expression analysis

Gene expression analysis was performed on 24-well tissue culture plates (Costar, UK) containing Caco-2 monolayers of cells grown for 21 days. Caco-2 monolayers were washed three times with warm PBS and the samples were carried out as follows: sample 87 (washed caco-2 monolayer, water-soluble cholesterol PEG-600 (Sigma-Aldrich) at final concentration of 70 µg/ml, *Lb. paracasei* 87standardized in supplemented DMEM at final cell number of 5.0×10^7 cfu mL^{-1}); sample CUL66 (washed caco-2 monolayer, cholesterol at final concentration of 70 µg/ml, *Lb. plantarum* CUL66 standardized in supplemented DMEM at final cell number of 2.0×10^8 cfu mL^{-1}); sample MIX (washed caco-2 monolayer, cholesterol at final concentration of 70 µg/ml, *Lb. paracasei* 87 and *Lb. plantarum* CUL66 standardized in supplemented DMEM at final concentration of 5.0×10^7 cfu mL^{-1} and 2.0×10^8 cfu mL^{-1}, respectively). Caco-2 monolayers incubated with cholesterol and without bacteria were used as control samples. The plates were incubated for 6 h at 37°C and

5% CO_2 and 90% humidity. After that, Caco-2 monolayers were washed three times with warm PBS pH 7.4 and the isolation of total RNA was performed using Ribozol (Amresco LLC, USA) in accordance with the supplier instructions. High RNA quality was confirmed by 2% agarose gel run at 100V for 1 h. Good quality undegraded RNA produces two distinct bands corresponding to the 28S rRNA and 18S rRNA in intensity ratio 2:1. The concentration of isolated RNA was determined using Nanodrop ND-2000 (Thermo Scientific, USA).

Reverse transcription was performed for 1 μg of RNA using High Capacity cDNA reverse transcription Kit (Life technologies, UK) following the manufacturer's instruction. Quantitative PCR (qPCR) was performed in LighyCycler® 96 (Roche, UK) starting from 50 ng of cDNA and using iTag SYBR Green SuperMix (Bio-Rad, UK) and 50 nM of the following gene specific sets of primers (Eurofins Genomics, Germany): 5'-TCTTCCCCTTCCTTGCCATT-3' and 5'-CGGCAGGGTAATTGTTGAGG-3' for NPC1L1 (Michael et al., 2016); 5'-CCCAAGGGACTCCGGGGTCA-3' and 5'-GACCCATGGACCCTCCGGGG- 3' for ABCG-5 (Alemany et al., 2013); 5'-GCCGCCCTCTTGTTCATG-3' and 5'-TAACATTTGGAGATGACATCCAGAA-3' for ABCG-8 (Kim et al., 2013); 5'-TTTCTCAGACAACACTTGACCAAGTA-3' and 5'-GGTTTTTGTGTAATGAGAGGTCTTTTAA-3' for ABCA-1 (Kim et al., 2013); 5'-GACCTTTCCAGAGCAAGCAC-3' and 5'-TCAACAAGAGCATCGAGGGT-3' for HMGCR (Michael et al., 2016) and 5'-ACTCTTCCAGCCTTCCTTCC-3' and 5'-CGTACAGGTCTTTGCGGATG-3' for β-actin (Michael et al., 2016). The RT-qPCR program consisted of: 5 min at 95°C followed by 40 cycles of 15 s at 94°C, 15 s at 60°C and 30 s at 72°C. Relative transcript levels were determined using $2^{-\Delta\Delta Ct}$ method between each target gene and β-actin as housekeeping gene. In order to check the presence of contaminating genomic DNA, negative controls (replacing cDNA samples with the corresponding RNA samples) were run in qPCR. At the end of RT-qPCR, a melting curve analysis was carried out to ensure the presence of single major peaks corresponding to amplification products. PCR products were subjected to gradual increase in temperature whereby at a particular temperature the two DNA stands dissociate and drop in fluorescence is measured. Plotting the change in fluorescence against the change in temperature allows for a graph where each peak corresponding to the melting temperature of the product. Therefore, one peak

resulting from melting peak analysis indicates one product has been formed, while the melting temperature gives information on the specificity of the PCR product.
Three independent experiments were carried out, and for each experiment, threereplicates per sample were performed.

4.2.6 Cholesterol uptake assay

Cholesterol uptake assay was performed on 24-well tissue culture plates (Costar, UK) of Caco-2 monolayers 21-days-old using the method previously described by Michael et al. (2016). To perform cholesterol uptake assay, Caco-2 monolayers were washed three times with warm PBS pH 7.4 and the following samples were carried out: sample 87 (washed Caco-2 monolayer, *Lb. paracasei* 87 standardized in supplemented DMEM at final concentration of 5.0 x 10^7 cfu mL^{-1}); sample CUL66 (washed Caco-2 monolayer and *Lb. plantarum* CUL66 standardized in supplemented DMEM at final concentration of 2.0 x 10^8cfu mL^{-1}); sample MIX (washed Caco-2 monolayer, *Lb. paracasei* 87 and *Lb. plantarum* CUL66 standardized in supplemented DMEM at final concentration of 5.0 x 10^7 cfu mL^{-1} and 2.0 x 10^8 cfu mL^{-1}, respectively); sample CT (washed Caco-2 monolayer without strains). After an incubation for 5 hours at 37°C, each well was added with [4-^{14}C]-cholesterol at final concentration of 0.5 μCi for 1 h. For intracellular lipid extraction, supernatants were discarded and Caco-2 monolayers were washed three times with cold DMEM and incubated for 30 min with 1.0 mL of icedhexane:isopropylalcohol:water (3:2:0.1, v/v/v). Each mix of solvents containing radiolabelled cholesterol was added with 10 mL of Opti-Fluor® (PelkinElmer, Inc., USA) and vortexed. The radioactivity of the samples was measured using the liquid scintillation analyser Tri-Carb 2800TR (PelkinElmer, Inc., USA) and expressed as disintegrations per min (DPM). Caco-2 cell fractions from each well were solubilised by adding 1 mL of 0.2 M NaOH for 30 min at 37°C and total cellular protein levels determined using BCA protein assay kit (Life Technologies, Paisley, UK) in accordance with the manufacturer protocol. DPM values from supernatants were normalised to protein levels of Caco-2 cell monolayers. Cholesterol uptake was expressed as percentage comparing normalized DPM values of samples with normalized DPM values of untreated control (Caco-2 monolayer without strains). Normalized DPM values of untreated control has been set as 100%. The assay was performed by three replicates for each of four independent experiments.

4.2.7 Cholesterol efflux assay

Cholesterol efflux assay was performed on 21-days-old Caco-2 cells grown in 12-well tissue culture plates (Costar, UK) with polycarbonate semi-permeable transwell membranes (0.4 μm pores, Costar, UK). To be sure to work with complete polarized tissue, Caco-2 monolayers were used when the trans-epithelial electrical resistance (TEER) exceeded 750 Ω/cm^2, that in our study fell usually after 21 days of incubation. The TEER was measured using EVOM2 voltmeter with ENDOHM 12 mm electrode (World Precision Instruments, Florida, USA) in accordance with the manufactures instructions.

Cholesterol efflux was performed using the method of Tachibana et al. (2007) adapted then by Michael et al. (2016). Caco-2 monolayers were incubated with 0.5 μCi/well [4-^{14}C]-cholesterol in DMEM (supplemented with FBS 10% (v/v) 4.5 g/L glucose, 1% (v/v) NEAA) for 24 h at 37°C, 5% CO_2 and 90% humidity. After the incubation, apical and basolateral compartments were washed with warm PBS and the apical compartment was added with 1 mM TDCA micelles alone or in combination with *Lb. plantarum* CUL66 (2.0 x 10^8 cfu mL^{-1}) and/or *Lb. paracasei* 87 (5.0 x 10^7 cfu mL^{-1}). Basolateral compartment was added with 10 μg/mL Apo-AI. TDCA micelles were prepared according to the method previously described by Ikeda et al. (2002). TDCA micelles were prepared by mixing in a glass tube 0.6 mM phosphatidylcholine (PC), 1.0 mM of oleic acid (OA) and 6.6 mM of TDCA starting from the following stock solutions: 60 mM PC in chloroform, 100 mM OA in chloroform and 330 mM TDCA in methanol. The mixture was vortexed, the solvents evaporated under a nitrogen flow and the resulting lipid film suspended in DMEM with 4.5 g/L glucose, 1% (v/v) NEAA and 10 mM HEPES and finally sterilized through a 0.4 μm acetate filter (Falcon, UK).

Cholesterol efflux was performed by an incubation for 6 h at 37°C in 5% CO_2 and 90% humidity. After the incubation, the supernatants from both compartments were kept for the analysis, whereas Caco-2 monolayers were washed with warm PBS and solubilised by incubation with 1 mL 0.2 M NaOH for 30 min at 37°C. The radioactivity of apical and basolateral media and of cell fractions was measured using the liquid scintillation analyser Tri-Carb 2800TR (PelkinElmer, Inc., USA) and expressed as DPM. Cholesterol effluxed from cells was expressed as percentage by dividing the radioactivity of the apical or basolateral media by the sum of

radioactivity in the apical medium, basolateral medium and cell fraction. The assay was carried out by three replicates for each of three independent experiments.

4.2.8 Statistical analysis

All data are expressed as means ± standard deviation (SD) and were analysed by one-way analysis of variance (ANOVA). Comparisons of mean results were carried out using post-hoc Tukey's test at $P \leq 0.05$ significance level. Statistical analyses were performed using Stata software package version 13 (Stata, 2013).

4.3 RESULTS AND DISCUSSIONS

4.3.1 Standardization of bacterial concentration

Suitable and highest bacterial concentrations that allow to maintain a neutral pH during the incubation for the assays with Caco-2 cells were found to be of about $2.0x10^8$ cfu mL^{-1} for *Lb. plantarum* CUL66 and of about $5.0x10^7$ cfu mL^{-1} for *Lb. paracasei* 87. *Lb. plantarum* CUL66 was used as reference strain for all assays performed (except for adhesion assay) and it was used at approximately the same concentration as in the work of Michael et al. (2016) of $2.0x10^8$ cfu mL^{-1}, that was found to be the highest bacterial concentration that did not cause a decrease of pH under 6.5. Instead, *Lb. paracasei* 87 seemed to have faster acidification rate than *Lb. plantarum* CUL66 in supplemented DMEM. Therefore, for *Lb. paracasei* 87, the highest concentration that allow to maintain a neutral pH was found to be $5.0x10^7$ cfu mL^{-1}, lower than for CUL66.

4.3.2 Adhesion capability to Caco-2 monolayer

Among functional characteristics that probiotic bacteria must have, it is of high interest the adhesion ability. For this purpose, Caco-2 cell line act as suitable *in vitro* model of human intestinal epithelium. In this study the adhesion ability of *Lb. paracasei* 87 and *Lb. plantarum* CUL66 was performed by determining the number of viable and adhesive bacteria on Caco-2 cell monolayers. The counting assay should be the most appropriate method that allows an accurate and reproducible enumeration of adhesive bacterial cells on the whole Caco-2 cell monolayer (Matijasic et al., 2003). The results on adhesion ability, expressed as percentage of adhesive cells, were about of 4.37% and 4.16%, respectively for *Lb. paracasei* 87 and *Lb. plantarum* CUL66. The values of initially added bacteria and adhesive bacteria expressed in cfu mL^{-1} are reported in **Figure 4.2**. According to Frömmel and coworkers (2013) bacteria can be classified on the basis of their adhesion ability into: non-adherent bacteria (less than 1,000 bacteria/mm^2 of cell monolayer); low-level-adherent bacteria (1,000 to 4,999 bacteria/mm^2); medium-level-adherent bacteria (5,000 to 9,999 bacteria/mm^2); strongly adherent bacteria (>10,000 bacteria/mm^2). One thousand bacteria/mm^2 corresponds at about one adhesive bacterium per Caco-2 cell. Therefore, that classification is in line with one of Candela et al. (2008) that classified bacteria as: non-adhesive bacteria (less than 5 bacteria/Caco-2 cell);

adhesive bacteria (5-40 bacteria/Caco-2 cell); highly adhesive bacteria (> 40 bacteria/Caco-2 cell). Based on these classifications, it may assumed *Lb. paracasei* 87 and *Lb. plantarum* CUL66 as strongly adherent strains showing 51754 bacteria/mm^2 (about 52 bacteria/Caco-2 cell) and 51053 bacteria/mm^2 (about 51 bacteria/Caco-2 cell), respectively.

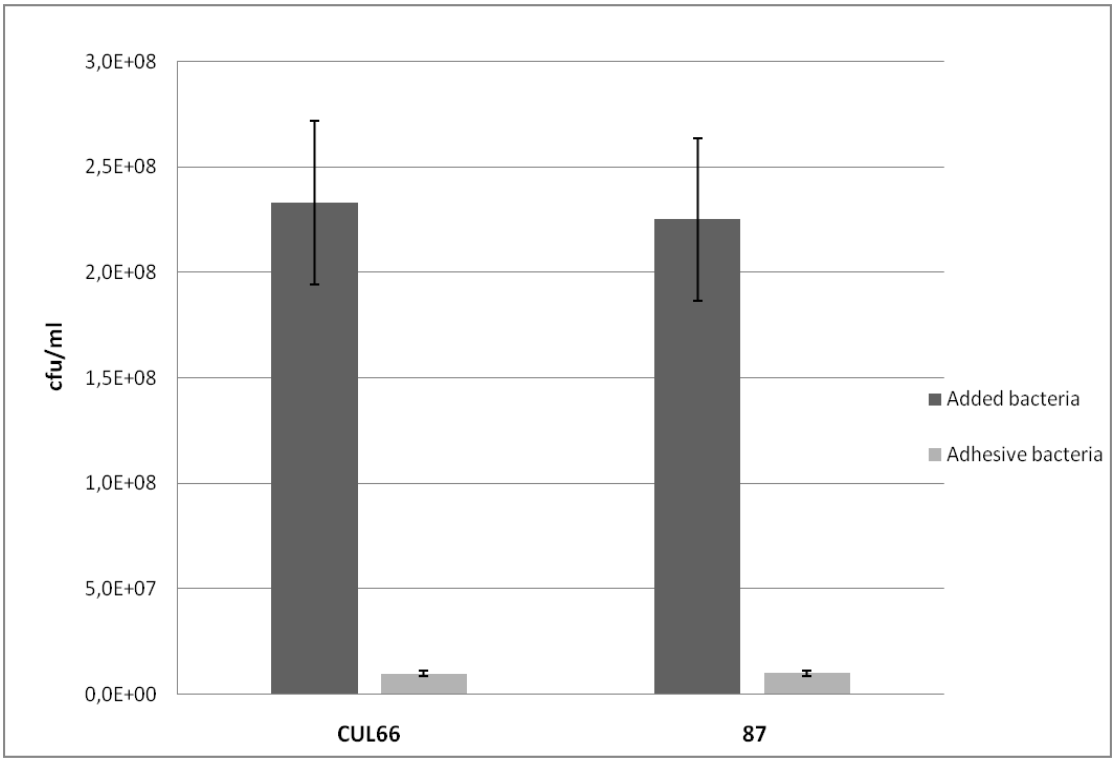

Figure 4.2. Adhesion ability of *Lb. plantarum* CUL66 and *Lb. paracasei* 87 to intestinal Caco-2 cell line. Added bacteria represent the initial number of the strain added and counted before the start of the incubation period. Added *Lb. plantarum* CUL66 was of 2.33x10^8 cfu mL^{-1}; added *Lb. paracasei* 87 was of 2.25x10^8 cfu mL^{-1}. Adhesive bacteria are the number of the strain adhered on Caco-2 cell monolayer after 1 h of incubation. Adhesive *Lb. plantarum* CUL66 was of 9.70x10^6 cfu mL^{-1}; adhesive *Lb. paracasei* 87 was of 9.83x10^6 cfu mL^{-1}.The results are shown as mean values ± standard deviations (±SD) of three independent assays conducted with Caco-2 cells from the same passage.

It is well known that the adhesion ability is strain dependent among the same species, as showed by several studies. The study of García-Cayuela et al. (2014) indicated that the adhesive properties of 5 strains of lactobacilli ranged between 4.98-14.38% while other studies on *Lb. rhamnosus* strains reported percentages of adhered bacterial cells within wide range of values from 0.2% to 20% (Tumola and Salminen, 1998; Gopal et al., 2001; Moussavi and Adams, 2010). Moreover, other factors could influence the adhesive ability, such as the origin and the initial inoculum of the LAB stains (Piątek et al., 2012), the electrostatic interactions, the steric forces and the

bacterial structures (García-Cayuela et al. 2014) or the autoaggregation (Wang et al., 2010). The results of the present study suggest that both *Lb. paracasei* 87 and *Lb. plantarum* CUL66 are classified as strongly adherent strains on Caco-2 cell monolayer showing an high bacterial cells number per one Caco-2 cell. Nevertheless, the percentage of adhesion that is calculated considering the initial number of added bacteria, was not very high compared with the results of the studies mentioned above.

4.3.3 Caco-2 cells viability and gene expression

Caco-2 monolayers co-incubated with bacterial strains were tested to verify cell viability under experimental conditions using crystal violet staining according to the method of Gorenjak et al. (2014). The results showed no significant change on viability of Caco-2 cells when co-incubated with *Lb. paracasei* 87 or *Lb. plantarum* CUL66 or with both the strains **(Figure 4.3)**.

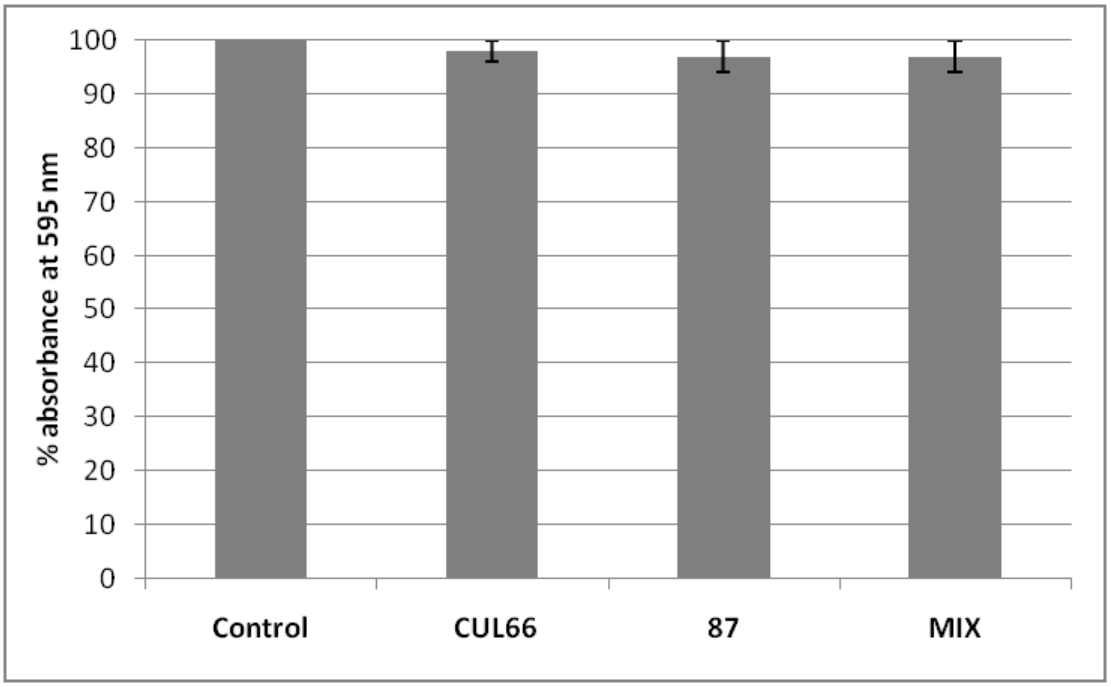

Figure 4.3. Viability of polarized Caco-2 cells exposed to the strains for 6 h at 37°C and 5% CO_2. Control: polarized Caco-2 cells treated with 70 µg/mL cholesterol; CUL66: polarized Caco-2 cells treated with 70 µg/mL cholesterol and exposed to *Lb. plantarum* CUL66 ($2.0x10^8$ cfu mL^{-1}); 87: polarized Caco-2 cells treated with 70 µg/mL cholesterol and exposed to *Lb. paracasei* 87 ($5.0x10^7$ cfu mL^{-1}); MIX: polarized Caco-2 cells treated with 70 µg/mL cholesterol and exposed to *Lb. plantarum* CUL66 ($2.0x10^8$ cfu mL^{-1}) and *Lb. paracasei* 87 ($5.0x10^7$ cfu mL^{-1}) in ratio 1:1 (v/v). Caco-2 cells viability was expressed as percentage of the absorbance at 595 nm that has been assigned as 100% for the Control. The results are expressed as mean values ± SD of triplicate samples from each of three independent assays.

Our results on Caco-2 cells with *Lb. plantarum* CUL66 were in agreement with the previous ones carried out by Michael and coworkers (2016), in which Caco-2 monolayers did not show significant decrease of viability when Caco-2 monolayers were incubated with $1.0x10^8$ cfu mL^{-1} *Lb. plantarum* CUL66 compared to the viability of the control sample (Caco-2 monolayer incubated without the strain).

After the assessment of good viability, Caco-2 monolayers were co-cultured with *Lb. paracasei* 87 and/or *Lb. plantarum* CUL66 to verify any change in the expression of key intestinal cholesterol transport-related proteins NPC1L1, ABCG5/8, ABCA1 and HMGCR.

Before carrying out RT-qPCR analysis, RNA samples quality was assessed. Good quality of undegraded RNA produces in agarose gel two bands for 28S and 18S rRNA with intensity ratio about of 2:1, as was obtained in this study and shown in **Figure 4.4**. Moreover, the melting curve analysis showed that for each set of primers, one major product was formed due to the single peak at the corresponding melting temperature, suggesting the presence of one specific PCR product for each set of primers.

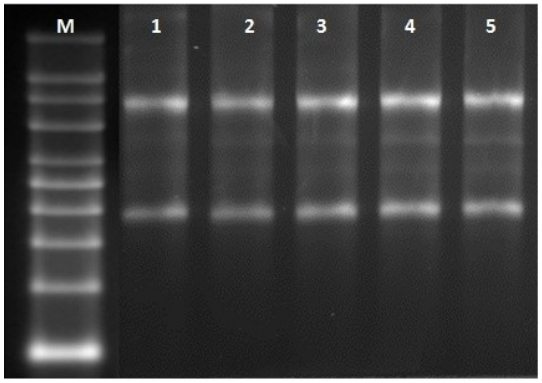

Figure 4.4. Agarose gel for the assessment of RNA quality. M: RNA Millennium Markers™ (Ambion); 1-5 lanes: intact RNA samples where are clearly visible 18S rRNA bands (lower, 1.9 kb) and 28S rRNA bands (upper, 5 kb).

Upon co-culture of the strains with Caco-2 cells for 6 h, no significant changes in gene transcript levels of all the tested genes were observed comparing the control (Caco-2 cells treated with cholesterol alone) with the treated samples (sample 87: Caco-2 cells, cholesterol and $5.0x10^7$ cfu mL^{-1} *Lb. paracasei* 87; sample CUL66: Caco-2 cells, cholesterol and $2.0x10^8$ cfu mL^{-1} *Lb. plantarum* CUL66; sample MIX: Caco-2 cells, cholesterol, mix of standardized *Lb. paracasei* 87 and *Lb. plantarum*

CUL66). The results are reported in **Figure 4.5** as relative gene expression and normalized to β-actin levels. All treated samples (87, CUL66 and MIX) showed decreasing trend on the gene transcript levels of ABCA-1 that is involved into basolateral cholesterol efflux. Similarly, NPC1L1 (key transporter protein in cholesterol uptake) and HMGCR (promotes *de novo* synthesis of cholesterol) showed decreasing trend on their gene expression when Caco-2 cells were incubated with one or both the strains.

Moreover, co-incubation of Caco-2 monolayers with cholesterol (70 μg/mL), *Lb. paracasei* 87 or *Lb. plantarum* CUL66 or mixed strains, resulted in a increasing trend on gene expression of ABCG5 and ABCG8, even if these changing on gene transcript levels were not statistically significant compared to the control sample (Caco-2 cells treated with cholesterol). The results on gene expression carried out on *Lb. plantarum* CUL66 were partially in agreement with the previous study of Michael et al. (2016). Although gene expression assay in this study was made under the same experimental conditions of the work by Michael et al. (2016), and *Lb. plantarum* CUL66 was used at same concentration, in our results *Lb. plantarum* CUL66 did not show similar strong influence on changing gene transcript levels. Moreover, to maintain the same experimental conditions of the major reference study for gene expression (Michael et al. 2016), we had to use our strain *Lb. paracasei* 87 at lower concentration ($5.0x10^7$ cfu mL^{-1}; see paragraph 4.3.1) than CUL66, to avoid a strong acidification of the medium during the incubation with Caco-2 cells. As demonstrated by Michael et al. (2016), changes in gene transcript levels of key intestinal cholesterol transport-related proteins are influenced by threshold viable bacterial cell number co-incubated with Caco-2 monolayers. In fact in their study, *Lb. plantarum* CUL66 significantly influenced gene expression of NPC1L1, ABCG5/8, ABCA-1 and HMGCR when used at $1.0x10^8$ cfu mL^{-1}, but no changes were observed for the same genes when CUL66 was used at $1.0x10^7$ cfu mL^{-1}. Therefore, it will be possible increase the viable bacterial cell number of *Lb. paracasei* 87 to co-incubate with Caco-2 cells over $5.0x10^7$ cfu mL^{-1} and find the potential threshold bacterial number able to significantly influence transcript levels of tested genes. The reduced transcript trends on the expression of NPC1L1 in our study was in accordance with other results (Michael et al., 2016; Michael et al., 2017; Lim et al., 2017; Gorenjak et al., 2014; Yoon et al., 2013; Huang and Zheng, 2010) in which the impact of LAB on cholesterol lowering activity *in vitro* and *in*

vivo was studied. In addition, we also observed a decreasing trend in the expression of ABCA-1 in response to *Lb. plantarum* CUL66, *Lb. paracasei* 87 and both strains together. Lim and coworkers (2017) observed the ability of *Pediococcus acidilactici* LAB4 and *Lactobacillus plantarum* LAB12 to down-regulate ABCA-1 when HT29 cells were pre-treated with LAB prior to cholesterol exposure. The up-regulation of ABCA-1 when HT29 cells were treated with LAB after prolonged exposure to cholesterol was found. The trend of strains 87 and CUL66 to down-regulate HMGCR contrasted the findings of other studies that showed up-regulation of mRNA levels of HMGCR in presence of LAB (Michael et al., 2017; Michael et al., 2016). On the other hand, other results supported the inhibition on gene expression of HMGCR such as in the study of Chen et al. (2016) where HMGCR was down-regulated when HepG2 cells were treated with four LAB strains resulting in inhibit cholesterol *de novo* synthesis. The suppression of HMGCR gene of 2.10 fold change was also showed in the study of Panigrahi et al. (2007) performed on Caco-2 cells treated with a mixed culture of *Escherichia coli* 6-1 and *Lb. plantarum* ATCC 202195.

As regards the gene expression of ABCG5/8 that contribute to apical efflux of cholesterol from the epithelium to intestinal lumen, many studies observed the up-regulation trend of these two genes, as shown on Caco-2 monolayers treated with *Lb. plantarum* CUL66 (Michael et al., 2016) or with Lab4 consortium of probiotics (Michael et al., 2017). In the latter case, the increase of the expression of ABCG5/8 was not statistically significant as obtained from our study.

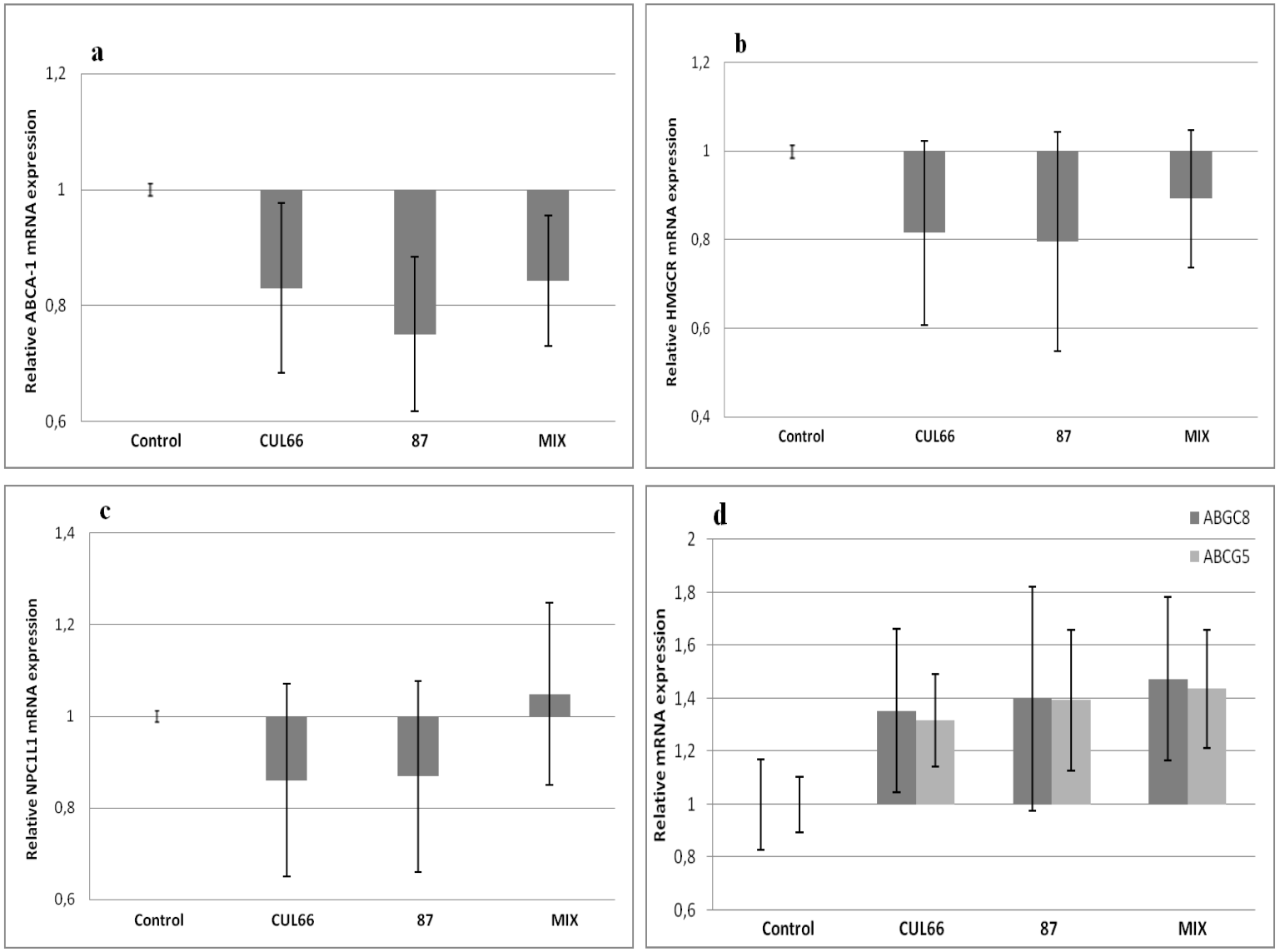

Figure 4.5. Analysis of (a) ABCA-1, (b) HMGCR, (c) NPC1L1 and (d) ABCG5/8 mRNA expression level after 6 h of incubation in polarised Caco-2 cells treated with 70 µg/mL cholesterol (Control) or with cholesterol (70 µg/mL) and *Lb. plantarum* CUL66 (sample CUL66; $2.0x10^{8}$ cfu mL^{-1}) or with cholesterol (70 µg/mL) and *Lb. paracasei* 87 (sample 87; $5.0x10^{7}$ cfu mL^{-1}) or with cholesterol and mixed culture (sample MIX) of *Lb. plantarum* CUL66 and *Lb. paracasei* 87. The results are the mean values of $2^{-\Delta\Delta Ct}$ relative expression ± SD of triplicate samples from each of three independent assays. Gene transcript levels were normalised to β-actin levels and the Control arbitrary set at 1.0.

4.3.4 Investigation on cholesterol uptake reduction

Twenty-one-day polarised Caco-2 cells were treated with *Lb. plantarum* CUL66 or *Lb. paracasei* 87 or with the mixture of both strains for 5 h before adding radiolabelled cholesterol for 1 h. At the end of the incubation, change in cholesterol uptake by Caco-2 cells was measured comparing treated samples with the control (Caco-2 monolayer incubated with radiolabelled cholesterol). Significant changes in the uptake of extracellular radiolabelled cholesterol were observed in response to *Lb. plantarum* CUL66, *Lb. paracasei* 87 and the strains mixed as shown in **Figure 4.6**.

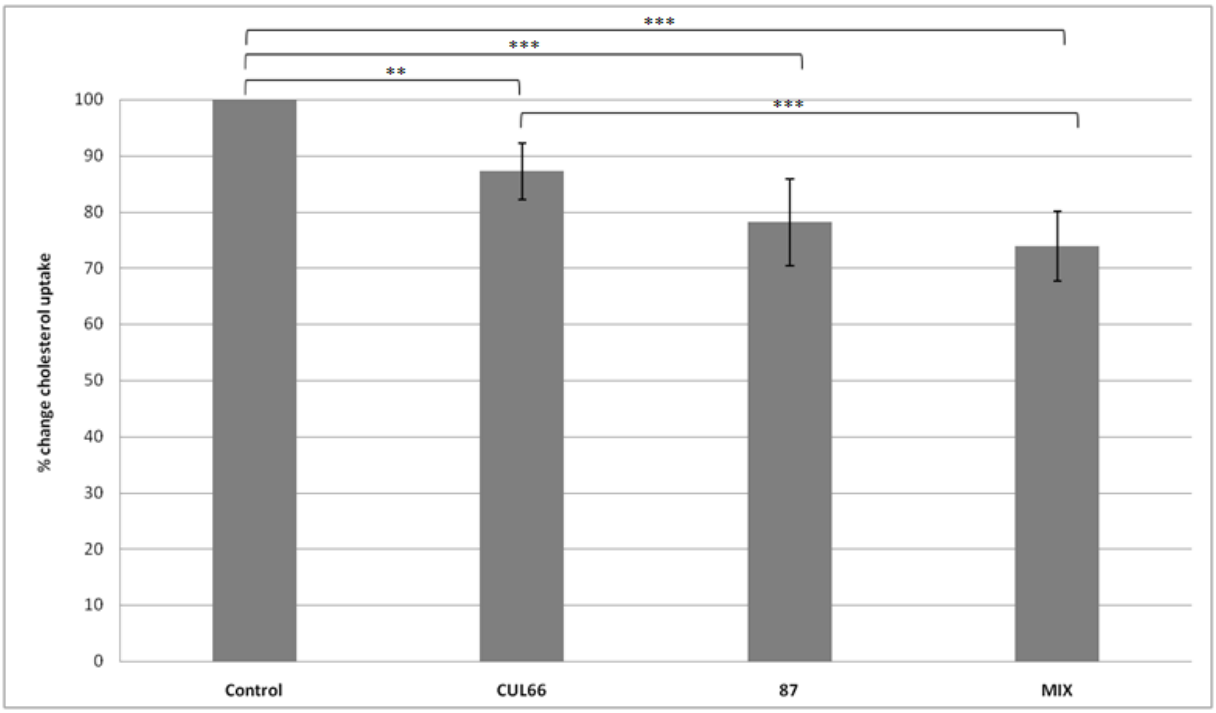

Figure 4.6. Cholesterol uptake by polarized Caco-2 cells. Caco-2 cells were incubated for 5 h with *Lb. plantarum* CUL66 (CUL66) or with *Lb. paracasei* 87 (87) or with mixed culture of *Lb. plantarum* CUL66 and *Lb. paracasei* 87 (MIX) or without strains (Control), prior to the addition of radiolabelled cholesterol for 1 h. Intracellular radioactivity was measured as DPM, normalized to total protein content and expressed as percentage where the Control was arbitrary set at 100%. The results are presentedas mean values ± SD of triplicate samples from each of four independent assays. Statistical analysis was performed using Student's t-test, with ***, $P \leq 0.001$; **, $P \leq 0.01$.

The treatment with *Lb. plantarum* CUL66 inhibited the cholesterol uptake by Caco-2 cells of about 12.74% ($P \leq 0.01$) compared to the control of which the cholesterol uptake level was set arbitrarily at 100%. This result confirmed the ability of *Lb. plantarum* CUL66 to reduce cholesterol uptake by Caco-2 cells as showed in the previous work of Michael et al. (2016), where CUL66 reduced the cholesterol uptake of about 16%. Furthermore, when Caco-2 cells were treated with *Lb. paracasei* 87 or

with the mixture of *Lb. plantarum* CUL66 and *Lb. paracasei* 87, the reduction of cholesterol uptake was reduced of 21.87% ($P \leq 0.001$) and 26.06% ($P \leq 0.001$), respectively. Moreover, significant reduction in cholesterol uptake was observed between Caco-2 cells treated with *Lb. plantarum* CUL66 and with the strains mixed ($P \leq 0.001$), suggesting a possible synergic effect on the inhibition of cholesterol uptake of *Lb. plantarum* CUL66 and *Lb. paracasei* 87 when mixed and incubated together with Caco-2 cells. Despite NPC1L1 mRNA expression, involved into regulation of cholesterol uptake, was not significantly reduced, *Lb. plantarum* CUL66 and *Lb. paracasei* 87 showed a strong ability to reduce the cholesterol uptake by Caco-2 cells. Therefore, our results suggested that the reduction on cholesterol uptake could be due not only to the inhibition of NPC1L1. Other central players on cholesterol uptake could be mechanisms performed by *Lb. plantarum* CUL66 and *Lb. paracasei* 87 such as cholesterol assimilation, binding cholesterol to bacterial walls, cholesterol incorporation into the cellular membrane, co-precipitation with deconjugated bile (Liong and Shah, 2005; Huang and Zheng, 2010). These theories were supported also by Leeber et al. (2008; 2010) who discussed on the key role of the surface molecules of probiotic lactobacilli (peptoglycan, polisaccharides, proteins) involved in the interactions with intestinal human cells.

An opposite case to ours was the study of Michael et al. (2017), where the consortium of probiotics "Lab4" (composed of *Lactobacillus acidophilus* CUL21 and CUL60, *Bifidobacterium bifidum* CUL20 and *Bifidobacterium animalis* subsp. *lactis* CUL34) did not show significantly reduction of cholesterol uptake by Caco-2 cells, despite of the ability of the bacterial culture to inhibit the gene expression of NPC1L1 of about 33%.

4.3.5 Cholesterol efflux of Caco-2 cells exposed to bacteria

To evaluate intracellular cholesterol efflux, Caco-2 cells were grown on semi-permeable transwell inserts to allow the formation of apical and basolateral compartments. According to the unchanged ABCG-5 and ABCG-8 gene expression, an increase of the cholesterol efflux from the epithelium into the intestinal lumen was not observed when Caco-2 cells were treated with *Lb. plantarum* CUL66, *Lb. paracasei* 87 or the strains mixed (**Figure 4.7**).

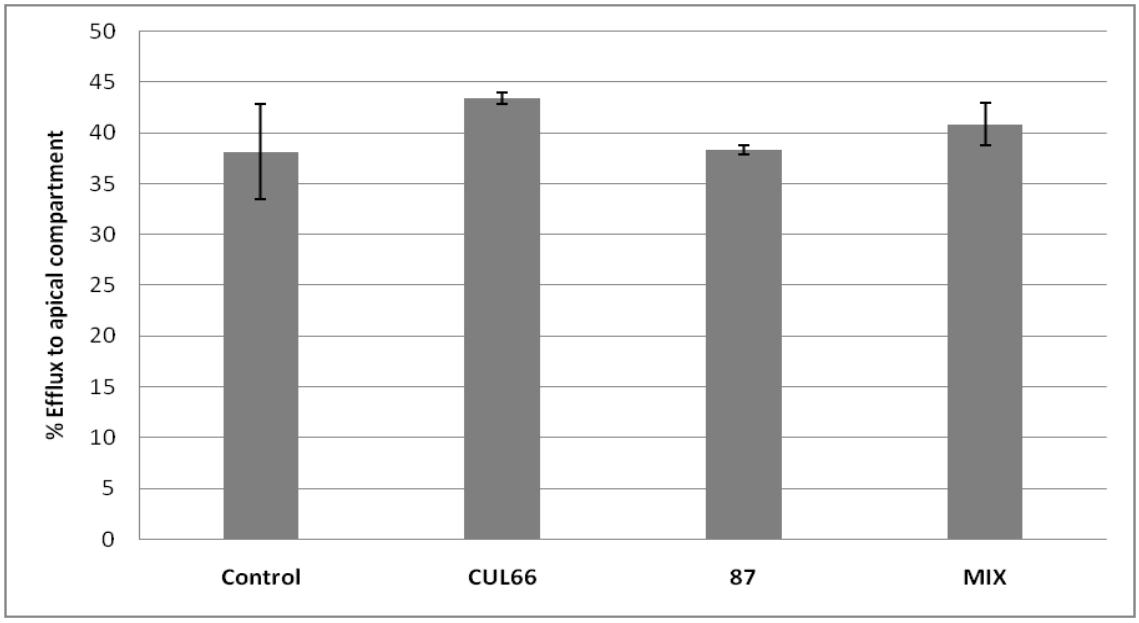

Figure 4.7. Cholesterol efflux into apical compartment of polarized Caco-2 cells untreated (Control), treated with *Lb. plantarum* CUL66 (CUL66) or with *Lb. paracasei* 87 (87) or with mixed culture of *Lb. plantarum* CUL66 and *Lb. paracasei* 87 (MIX). Radioactivity in the apical compartment was measured as DPM and expressed as mean values ± SD of triplicate samples from each of three independent assays.

On the other hand, significant changes in the cholesterol efflux from Caco-2 cells into basolateral compartment were observed in response to *Lb. plantarum* CUL66, *Lb. paracasei* 87 and the strains mixed as shown in **Figure 4.8**. The treatment with *Lb. plantarum* CUL66 caused a decrease of cholesterol efflux to basolateral compartment of about 74.35% ($P \leq 0.001$) compared to the control incubated without the strains. Instead, when Caco-2 cells were treated with *Lb. paracasei* 87 or with the two strains mixed, the reduction of cholesterol efflux to the basolateral compartment was reduced of about 58% ($P \leq 0.01$) and 57.6% ($P \leq 0.001$), respectively. The results on basolateral efflux were not in line with the unchanged ABCA-1 gene expression responsible of cholesterol efflux from the enterocyte to basolateral compartment. Cholesterol efflux in response to *Lb. plantarum* CUL66 was confirmed by Michael et al. (2016) which found unchanging on apical efflux of intracellular radiolabelled cholesterol but a reduction of basolateral cholesterol efflux of about 52% linked to a significant down-regulation on gene expression of ABCA-1. The same case was observed evaluating the effect of a mixed culture of probiotic strains on cholesterol efflux in Caco-2 cells (Michael et al., 2017). In fact, the consortium of probiotics "Lab4" was able to reduce basolateral efflux of cholesterol of 35%

confirming that propriety by down-regulation of mRNA levels of ABCA-1 with 37% of reduction. Therefore, Lab4 did not cause an increase of apical efflux of cholesterol from enterocytes into the intestinal lumen. The study of Yoon et al. (2011) represents the rare case where *Lb. plantarum* NR74, the strain object of study, showed a definite increase in the apical efflux of cholesterol (69.3%) correlated with a significantly increase of ABCG5/8 gene expression, by using non-polarized Caco-2 cells as cell model.

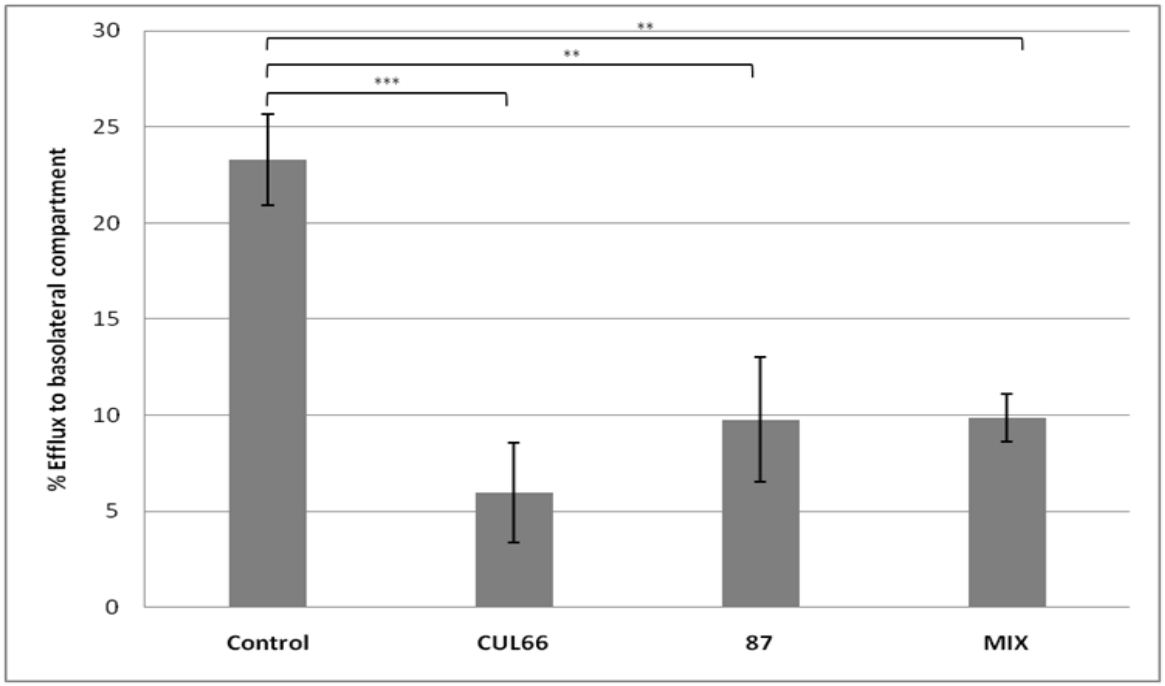

Figure 4.8. Cholesterol efflux into basolateral compartment of polarized Caco-2 cells untreated (Control), treated with *Lb. plantarum* CUL66 (CUL66) or with *Lb. paracasei* 87 (87) or with mixed culture of *Lb. plantarum* CUL66 and *Lb. paracasei* 87 (MIX). Radioactivity was measured as DPM and expressed as mean values ± SD of triplicate samples from each of three independent assays. Statistical analysis was performed using Student's t-test, with ***, $P \leq 0.001$; **, $P \leq 0.01$.

4.4 CONCLUSIONS

These results suggest that *Lb. paracasei* 87 may be an effective probiotic to be use as tool to prevent and fight CVD thanks to its clear cholesterol lowering activity proved *in vitro* by using intestinal cell models. The findings of the present study suggest that *Lb. paracasei* 87 was classified as strongly adherent strain on Caco-2 cell monolayer being one of prerequisite that probiotic bacteria must have to perform its all functional properties. Moreover, *Lb. paracasei* 87 showed marked ability to reduce intestinal cholesterol uptake and basolateral cholesterol efflux that are linked to a reduction of cholesterol level in the blood. This study may contribute to improve the literature on probiotic lactobacilli associated with the cholesterol lowering capability, although further studies are needed to understand what mechanisms *Lb. paracasei* 87 uses to reduce serum cholesterol level through reducing cholesterol uptake and basolateral cholesterol efflux. However, these findings provide the basis to validate the ability of *Lb. paracasei* 87 on the reduction of blood serum cholesterol levels in next *in vivo* studies.

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CHAPTER 5

Impact of *Lactobacillus curvatus* 54M16 on microbiota composition and growth of *Listeria monocytogenes* in fermented sausages

5.1 INTRODUCTION

Despite the application of modern technology and improved control measures during food production, foodborne diseases are still a serious public health problem (Wang et al., 2016). Most (33.7%) of the 4,362 food-borne outbreaks reported in in the European Union (EU) in 2015 were caused by bacterial agents, and *Listeria monocytogenes* was responsible for approximately 2,200 human cases of listeriosis. Moreover, despite a similar notification rate of cases of listeriosis to that of 2014, an increasing trend was recorded in the years 2008-2015 (EFSA, 2016). The pathogen particularly affected the elderly population (age group of over 64-84 years old) and was predominantly associated with ready-to-eat fish and meat products (EFSA, 2016).

The production of fermented sausages is a dynamic process in which biochemical, microbiological, physical and sensorial changes occur in a chopped ground meat mixture during fermentation and ripening in defined conditions of temperature and relative humidity (Villani et al., 2007). Although several hurdles (such as low pH and water activity, salt and nitrites, chemical preservatives, natural and/or starter microflora) lead to products with a generally low sanitary risk, some pathogenic microorganisms, such as *L. monocytogenes,* can adapt to the environment of fermented sausages compromising their safety (De Cesare et al., 2007). Hence, additional barriers are necessary. Several studies indicate that the use of bacteriocins or selected bacteriocin-producers lactic acid bacteria (LAB), in combinations with other preservation methods, can improve the microbiological quality and safety of fermented sausages (Mauriello and Villani, 2012; Balciunas et al., 2013; Vignolo et al., 2015). During the fermentation process, starter and protective cultures dominate the food ecological niche through so-called ‘selfish behavior’. This behaviour contributes to a hostile environment for undesirable pathogenic and spoilage microorganisms and to produce food with high organoleptic standards (Papadimitriou et al., 2015).

The ecosystem of meat fermentations consists of complex, multispecies microbial

populations in which LAB and coagulase-negative staphylococci (CNS) are the dominant microbiota; these microbiotas coexist depending on the product, with enterococci, yeasts and molds (Cocolin et al., 2011; Garriga and Aymerich, 2015). The microbial diversity of fermented sausages has been studied extensively using classical culture dependent and independent methods (Cocolin et al., 2011). In recent years a remake of the microbial communities of many of these products has been done by high-throughput sequencing, revealing improved sensitivity and efficiency in the evaluation of microbial diversity (Połka et al., 2015; Greppi et al., 2015; Fontana et al., 2016). Fermented sausages are traditionally manufactured from complex raw materials. The study of microbiota is essential to understand how meat processors can drive production technology in order to improve products by affecting microbiota activities.

A previous work (Casaburi et al., 2016) highlighted that *Lactobacillus curvatus* 54M16 produced bacteriocins that were active against *Listeria monocytogenes* strains in culture media and that its use as starter culture might contribute to improved quality of traditional fermented sausages. The results suggested the need for further *in vivo* studies of sausages challenged with the pathogen and the bacteriocins-producer strain. Therefore, this work aimed to evaluate the effectiveness of the bacteriocins-producing strain at inhibiting *L. monocytogenes* in vitro co-culture experiments and during the manufacture of fermented sausages. Moreover, the research focused on studying the dynamics of bacterial communities during fermentation and maturation of traditional fermented sausages using 16S rRNA gene sequencing and viable counts methods.

5.2. MATERIALS AND METHODS

5.2.1 Bacterial strains and growth conditions

Bacterial strains, optimal culture conditions and strains source are reported in **Table 5.1**. All cultures were stored at -20°C in appropriate culture media supplemented with 25% (v/v) sterile glycerol.

Table 5.1. Bacterial strains and culture conditions used in the experiments (Giello et al., 2018)

Strain	Media[a]	Incubation (°C)	Source[b]
Lactobacillus curvatus 54M16[c]	MRS broth	30	DAS
Lb. curvatus 20019	MRS broth	30	DSMZ
Listeria monocytogenes 7644	TSBY	30	ATTC
L. monocytogenes OH	TSBY	30	Carminati
L. monocytogenes Scott A	TSBY	30	Carminati
L. monocytogenes Cal	TSBY	30	Carminati
L. innocua 1770	TSBY	30	ATCC

a: TSBY: Tryptone Soy Broth (Oxoid) supplemented with 0.5% yeast extract; MRS broth (Oxoid);
b: DAS: Department of Agricultural Science, Division of Microbiology, University of Naples Federico II; DSMZ: Deutsche SammLung fur Mikroorganismen und Zellkulturen, Braunschweig, Germany; ATCC: American Type Culture collection; Dr. Carminati D., Istituto Sperimentale Lattiero-caseario, Lodi, Italy;
c: Sakacins X, T and P producer strain (Casaburi et al., 2016).

5.2.2 Strain typing of *Listeria* strains

Listeria strains **(Table 5.1)** for co-culture and *in situ* experiments were subjected to RAPD-PCR and rep-PCR genomic fingerprinting.

DNA was extracted from overnight cultures using Insta Gene™ Matrix (Bio-Rad, Milano, Italy) according to the manufacturer's instructions and used as template for RAPD-PCR using primers M13 (Rossetti and Giraffa, 2005), M13R2 (Martín et al., 2005) and rep-PCR using primer $(GTG)_5$ (Gevers et al., 2001). For RAPD-PCR, each 25 µL PCR mix contained 1X PCR Buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM concentrations of each dNTP, 0.8 µM primer (M13 or M13R2), 2 U of Taq DNA polymerase (5U/µL, Invitrogen) and 100 ng of extracted DNA. When primers M13 was used, the amplification process consisted of 2 min of initial denaturation at 94°C; 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 42°C for 20 sec and elongation at 72°C for 2 min; and a final extension cycle at 72°C for 10 min. When primers M13R2 was used, the amplification process consisted of 5 min of

initial denaturation at 94°C; 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 38°C for 1 min and elongation at 72°C for 1 min; and a final extension cycle at 72°C for 10 min.

For Rep-PCR, each 25 µL PCR mix contained 1X PCR Buffer (Invitrogen), 1.75 mM $MgCl_2$, 0.2 mM concentrations of each dNTP, 0.25 µM of primer $(GTG)_5$, 2.5 U of Taq DNA polymerase (5U/µL, Invitrogen) and 100 ng of extracted DNA. The amplification process consisted of 4 min of initial denaturation at 95°C; 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 40°C for 1 min and elongation at 72°C for 1 min; and final extension cycle at 72°C for 8 min.

PCR products were run in 1.5% agarose gel stained with 0.01% SYBR Safe DNA gel stain (Invitrogen) for 90 min at 70 V. RAPD profiles were visualized with UV transilluminator UVIdocHD2 (UVITEC, Cambridge, UK) and were analysed by the Bionumerics software (version 5.1, Applied Maths).

5.2.3 Co-culture experiments

Growth and bacteriocins production by *Lb. curvatus* 54M16 and *Lb. curvatus* DSM 20019 were compared in De Man Rogosa Sharpe (MRS) broth at 15, 20 and 30°C. Viable cell count on MRS agar and bacteriocin activity in arbitrary units (AU mL^{-1}) were measured after incubation for 24 h using the critical dilution assay and the spot-on-lawn method against *L. monocytogenes* and *L. innocua* strains **(Table 5.1)** as previously described (Villani et al., 1995; Casaburi et al., 2016).

For the co-culture experiments, strains OH and Scott A of *L. monocytogenes* (selected on the basis of their sensitivity to the bacteriocins produced by *Lb. curvatus* 54M16 and by their RAPD-PCR profiles, see **Table 5.2**) and two strains of *Lb. curvatus* (54M16, Bac^+ and DSM 20019, Bac^-) were cultivated in appropriate broths at optimal temperatures of incubation **(Table 5.1)** for 16 h. Ten milliliters of each culture of *L. monocytogenes* OH and Scott A were spun in a centrifuge (10,000 x *g* for 10 min at 4°C), and the resulting pellets were suspended into sterile 20 mM phosphate buffer (pH 7.0), then mixed in equal volume to obtain a cell suspension of both strains. Pellets of *Lb. curvatus* strains were washed once with sterile 20 mM phosphate buffer (pH 7.0) and finaly suspended in 10 mL of the same buffer. The cell suspensions of *L. monocytogenes* and *Lb. curvatus* strains were standardized using McFarland standards in order to reach a final inoculum concentration in mono and co-culture of approximately 10^4 cfu mL^{-1} for *L. monocytogenes* and 10^7 cfu mL^{-1}

for *Lb. curvatus*. Ten millilitres of MRS broth were inoculated with standardized cell suspensions for mono-culture and co-culture experiments: A (control), inoculated with the mixture of *L. monocytogenes* OH and Scott A; B, inoculated with *Lb. curvatus* 54M16; C, inoculated with the mixture of *L. monocytogenes* and *Lb. curvatus* 54M16; D, inoculated with *Lb. curvatus* DSM 20019; and E, inoculated with the mixture of *L. monocytogenes* and *Lb. curvatus* DSM 20019. The cultures were incubated at 15°C for five days or at 20°C for 48 h. Two samples were analysed at each time point for three independent replicate experiments.

Table 5.2. Bacteriocins production by *Lb. curvatus* 54M16 co-inoculated with the mixture of *L. monocytogenes* SA and OH (sample C) and RAPD-PCR profiles of the two strains of *L. monocytogenes* (Giello et al., 2018)

Time (h)	15°C			20°C		
	[a]AU mL^{-1} against *L. monocytogenes*		[b]RAPD-PCR profiles	[a]AU mL^{-1} against *L. monocytogenes*		[b]RAPD-PCR profiles
	Strain OH	Strain SA		Strain OH	Strain SA	
0	0	0	[c]ND	0	0	[c]ND
1	0	400	ND	0	800	ND
3	400	800	ND	400	800	ND
5	400	800	ND	400	800	ND
7	800	1600	ND	1600	3200	ND
8	800	1600	ND	1600	3200	ND
24	3200	6400	ND	3200	6400	C, C, C, C, C, D, D
48	6400	12800	C, C, C, C, C, C, C	6400	12800	C, C, C, C, C, C, C, C, D

a:AU (Arbitrary Units) mL^{-1} of neutralized and filter sterilized culture supernatant determined by critical dilution assay against *L. monocytogenes* strains.
b: *L. monocytogenes* OH (profile C) and *L. monocytogenes* Scott A (profile D).
c: not determined.

5.2.3.1 Microbial analysis, bacteriocin production, pH measurement and RAPD-PCR profiles

For each set of mono-culture and co-culture experiments, microbiological counts were performed by preparing serial 10-fold dilutions in sterile quarter-strength Ringer's Solution (Oxoid), and aliquots of 1 or 0.1 ml were poured in or spread on appropriate agar plates in duplicate. *L. monocytogenes* strains were enumerated on agar Listeria according to Ottaviani and Agosti (ALOA, Biolife, Milano, Italy) after incubation at 30°C for 48 h; *Lb. curvatus* strains were enumerated on mMRS-BPB

agar [MRS agar (Oxoid) at pH 6.5 with 0.05% L-cysteine/HCl (Sigma-Aldrich) and 0.002% bromophenol blue] plates (Lee and Lee, 2008) after incubation under anaerobiosis (Anaerogen kit, Oxoid) at 30°C for 48 h. The pH of the cultures was measured with an HI221pH m (Hanna Instruments, Italy).

Bacteriocins activity (AU mL^{-1}) of the cell-free supernatant from the experiment C (*L. monocytogenes* co-cultured with *Lb. curvatus* 54M16) was determined by critical dilution assay and the spot-on-lawn method against *L. monocytogenes* OH and Scott A as described in section 5.2.3.

All analyses were carried out at 0, 1, 2, 3, 5, 7, 8, 24 and 48 h at 20°C and were extended to five days for the experiments at 15°C. For the samples containing *L. monocytogenes* strains (A, C and E), all colonies grown on ALOA plates at the highest dilution and at the end of incubation (24 h and 48 h at 20°C; 48 h and five days at 15°C) were transferred to tryptone soya agar (TSA, Oxoid). The purified cultures were examined for cellular morphology, Gram stain, catalase (H2O2 3% v/v) and oxidase test (Oxidase Strips, Sigma-Aldrich, Milano, Italy), then evaluated by RAPD-PCR using primer M13R2 as described above.

5.2.4 Sausages manufacture

The mixture for sausage preparation was obtained from a meat processor who produces traditional fermented sausages of the Campania region (Italy).

The sausage formulation included (%w/w): meat pork (85); pork back fat (15), NaCl (2.5), black pepper (0.1), glucose (0.3) and lactose (0.3). After chopping and mixing the ingredients, the mixture was transported at a refrigerated temperature to the laboratory of microbiology and divided into four portions: (A) control (uninoculated sausages); (B) sausages inoculated with the mixture of *L. monocytogenes* OH and Scott A to a final concentration of 10^4 cfu g^{-1}; (C) sausages inoculated with *Lb. curvatus* 54M16 (10^7 cfu g^{-1}); (D) sausages inoculated with the mixture of *L. monocytogenes* OH and Scott A (10^4 cfu g^{-1}) and *Lb. curvatus* 54M16 (10^7 cfu g^{-1}). Strains of *L. monocytogenes* and *Lb. curvatus* 54M16 were prepared as described in section 5.2.3. The sausage mixture was mixed and stuffed in natural casings. Sausages (of approximately 250 g each) were ripened in a fermentation chamber (I.C.S., Mod. AS100, San Felice sul Panaro, Modena, Italy) for three days at 20°C and relative humidity (RH) of 75-85% and then for other 25 days at 15°C with a RH of 65-70%. Two independent batches of sausages were used for the experiment. At

casing time and at 3, 7, 14, 21 and 28 days of ripening, three sausages were randomly taken and analysed immediately for microbiological analysis, for pH and water activity (a_w) and for the preparation of bulk sausage stored at -20°C in RNAlater (Ambion, Applied Biosystems, Milan, Italy) for bacterial RNA extraction as described below.

5.2.5 Microbiological analysis, pH and a_w measurement

The casing was aseptically removed, and 10 g of meat were suspended in 90 mL of sterile quarter-strength Ringer's solution (Oxoid, Milano, Italy) and homogenized for 2 min with a stomacher (Lab-Blender 400 Seward Medical, London, UK). LAB were counted on de Man-Rogosa-Sharpe agar (MRS agar, Oxoid) incubated at 30°C for 72 h in anaerobiosis using anaerobic jars with Anaerogen (Oxoid); presumptive *Escherichia coli* were counted on tryptone bile glucuronic medium (TBX, Oxoid) incubated at 37°C for 2 h and then at 44°C for 46 h; coagulase negative Staphylococci were counted on mannitol salt agar (MSA, Oxoid) incubated at 37°C for 48 h; *Enterobacteriaceae* were counted on violet red bile glucose agar (VRBGA, Oxoid) incubated at 37°C for 48 h; *L. monocytogenes* were counted on (ALOA, Biolife, Italy) incubated at 37°C for 48 h; and yeast and moulds were counted on dichloran rose-bengal chloramphenicol agar (DRBC agar, Oxoid) incubated at 25°C for 72 h. Each analysis was done in duplicate.

To detect the presence of wild *L. monocytogenes*, multi-step enrichment procedure described in the ISO 11290-1(ISO, 1996) method was carried out on 25 g of uninoculated sausages mixture (sample A) and on samples inoculated with *Lb. curvatus* 54M16 (sample C). Two replicates of each sample were suspended in 225 mL of half Frazer broth (HFB, Oxoid), homogenized for 2 min in the stomacher and incubated at 30°C for 24 h. Primary selective enrichment (HFB) cultures were streaked onto two plates of ALOA (Biolife, Italy) and 0.1 mL were inoculated in 10 mL of Frazer broth (FB, Oxoid) and incubated at 37°C for 48 h. Secondary selective enrichment (FB) cultures were streaked onto two plates of ALOA (Biolife). Streaked plates from HFB and FB cultures were incubated at 37°C, and the presence of typical colonies were observed after 24 and 48 h.

Measurement of pH was performed using a FC2320 pH electrode inserted directly into the sausage (Hanna Instruments, Italy). Water activity (a_w) was measured using a Hygropalm Rotronic mod. HW3 (International PBI Milano, Italy) at 25 °C.

5.2.6 Identification and strain typing of *L. monocytogenes* from sausages

Typical *L. monocytogenes* colonies from ALOA (Biolife, Italy) plates coming from counts of samples A and D after 14 and 28 days of ripening were streaked on TSA (Oxoid). The purified cultures were examined for cellular morphology, Gram stain, and catalase and oxidase test and were then evaluated by RAPD-PCR, using M13R2 primer as described in Section 5.2.2.

The strains of *Listeria* isolated from sample A (un-inoculated control) were identified by 16S rRNA gene sequencing using primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (*Escherichia coli* positions 8-17 and 1540-1524, respectively) (Weisburg et al., 1991). PCR conditions included an initial template melting (95°C for 3 min), followed by 30 cycles of denaturation (94°C for 1 min), annealing (54°C for 45 s) and extension (72°C for 2 min). The final elongation step occurred for 7 min at 72°C. PCR amplification was performed using Taq DNA polymerase 5U/μL (Invitrogen) in a Mastercycler Nexus PCR machine (Eppendorf).

5.2.7 Antimicrobial activity of *Lactobacillus spp.* isolates

In order to monitor the bacteriocins producing *Lb. curvatus* 54M16 in sample D (where it was co-inoculated with *L. monocytogenes* strains), colonies from the highest dilution of MRS plates at 7, 14 and 28 days were streaked on MRS agar (Oxoid). The purified cultures were examined for cellular morphology, Gram stain, and catalase and stored in MRS broth plus 25% glycerol at -20ºC. Antimicrobial activity of the cell-free supernatant was tested after the growth of lactobacilli isolates in MRS broth at 30°C for 18 h using the critical dilution assay and the spot-on-lawn method against *L. monocytogenes* OH and Scott A and the strains of *L. monocytogenes* isolated in the samples A and D after 14 and 28 days of ripening. Moreover, two antimicrobial-producing isolates from each sampling point were assayed for the detection of bacteriocins genes by PCR amplification as previously described (Casaburi et al., 2016). Briefly, PCR amplification of the genes s*akX*, $sakT_{\alpha}$ and $sakT_{\beta}$ was carried out using the primer set designed by Macwana and Muriana (2012), whereas the specific 186-bp fragment internal to the gene encoding for the sakacin P was amplified using the primers described by Remiger et al. (1996).

Bacteriocins were also extracted directly from sausages during ripening according to the method reported by Corsetti et al. (2004). One-hundred grams of sausage (samples D and C at 7th day) were aseptically weighed and homogenized in 1/10 ratio with 40% of acetonitrile and 0.1% trifluoroacetic acid (v/v) solution for 2 min in stomacher at room temperature. The mixture was centrifuged at 15000 x *g* for 10 min and the supernatant was freeze-dried and re-suspended in 5.5 mL of 10% (v/v) ethanol solution. The antimicrobial activity of extracted bacteriocins was assayed against *L. monocytogenes* OH and Scott A as described above.

5.2.8 RNA extraction and PCR sequencing

Each sausage sample was diluted 1:5 in PBS (phosphate buffered saline, 0.1 M pH 7.4) and homogenised for 1 min with a stomacher (Lab-Blender 400 Seward Medical, London, UK). The suspension was centrifuged at 110 x *g* for 5 min to pellet debris and 5 mL of the supernatant were centrifuged at 12000 x *g* for 5 min. The resulting pellet was immediately stored in 200 µL of RNAlater (Ambion, Foster City, California) at -20°C. RNA extraction was carried out using the Power Microbiome RNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The pellets were washed three times with 1 mL of PBS and lastly suspended in 500 µL of PM1 buffer (provided by the kit). The manufacturer's instructions were followed, but lysozyme solution (50 µL of 50 mg/mL lysozyme, Sigma-Aldrich, St Louis, MO, USA) and a Pronase E (25 µL of 20 mg/mL Pronase E, Sigma-Aldrich) treatments were added at 37°C for 30 min and 65 °C for 15 min, respectively. Co-eluted DNA was digested by repeated treatments with Turbo DNase (Ambion, Foster City, California) for 1 h at 37°C. The absence of DNA was checked by PCR and the treatment repeated if necessary. RNA was quantified using a Nanodrop1000 spectrophotometer (Thermo Scientific, Milan, Italy).

Three hundred nanograms of RNA wasused for the synthesis of complimentary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, California).

The V3-V4 region of the 16S rRNA gene was amplified by using primers S-D-Bact-0341F5'–CCTACGGGNGGCWGCAG and S-D-Bact-0785R5'-GACTACHVGGGTATCTAATCC (Klindworth, et al 2013) and the following PCR conditions: an initial denaturation at 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min.

Amplicon purification, multiplexing and sequencing was carried out as reported in the Illumina 16S Metagenomic Sequencing Library Preparation manual. Sequencing was carried out on a MiSeq platform and using the MiSeq Reagent kit v3, leading to 2x250-bp, paired-end reads.

5.2.8.1 Bioinformatics

Demultiplexed, forward and reverse reads were joined by using FLASH (Magocand Salzberg, 2011). Joined reads were quality trimmed (Phred score < 20) and short reads (< 250 bp) were discarded by using Prinseq (Schmieder and Edwards, 2011). These filters led to a total of 1,058,800 remaining sequences with an average value of 26,470 reads/sample. High quality reads were then imported in QIIME 1.9.1 (Caporaso et al., 2010). Operational taxonomic units (OTUs) were picked through *de novo* approach and uclust method and taxonomic assignment was obtained using the RDP classifier and the Greengenes (McDonald et al., 2012) database, following a previously reported pipeline (De Filippis et al., 2016a). To avoid biases due to different sequencing depths, OTU tables were rarefied at the lowest number of sequences per sample. The 16S rRNA gene sequences produced in this study are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), under accession number SRP100922.

5.2.8.2 Sub-genus diversity of *Lactobacillus*

Reads assigned to *Lactobacillus* genus were extracted and entropy analysis and oligotyping were carried out as described by the developers (Eren et al., 2013). After the initial round of oligotyping high entropy positions were chosen (-C option): 1, 2, 19, 20, 27, 39, 58, 61, 107, 139, 202, 209, 227, 239, 240, 262. To minimize the impact of sequencing errors, we required an oligotype to be represented by at least 100 reads (-M option). Moreover, rare oligotypes present in less than 5 samples were discarded (-s option). These parameters led to 402,351 (84.36%) sequences remaining in the dataset. BLASTn was used to query the representative sequences against the NCBI nr database, and the top hit was considered for taxonomic assignment. Representative oligotypes sequences and *Lb. curvatus* 54M16 16S sequence were aligned using ClustalW, and a phylogenetic tree was built using the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean) in

MEGA6 (Tamura et al., 2013).

5.2.9 Statistical analysis

Microbiological results were analysed by analysis of variance (ANOVA) including the effects of the ripening time (0, 3, 7, 14, 21, 28 days) and of type of samples (A, B, C, and D). Comparisons of mean results were carried out using post-hoc Tukey's test at $P \leq 0.05$ significance level. Analyses were carried outin Stata version 13 (StataCorp, 2013).

OTU abundance tables were imported in R environment for subsequent statistical analyses and visualization (https://www.r-project.org). Permutational Multivariate Analysis of Variance (non-parametric PER-MANOVA) based on Jaccard and Bray Curtis distance matrices was applied with 999 permutations to detect significant differences in the overall microbial community composition, by using the *adonis* function in the *vegan* package. Non-parametric Kruskal-Wallis and pairwise Wilcoxon tests were carried out to find differentially abundant OTUs between the groups. Correction of p-values for multiple testing was performed when necessary (Benjamini and Hochberg, 1995). Alpha-diversity analysis was carried out in QIIME on OTU tables rarefied at 10,025 reads/sample. Principal Component Analysis (PCA) was carried out on the log transformed abundance table by using *dudi.pca* function in *made4* package.

5.3 RESULTS

5.3.1 Selection of strains for co-culture experiments

The *Listeria* spp. to be used in co-culture and *in situ* experiments were evaluated for their sensitivity to bacteriocins produced by *Lb. curvatus* 54M16 and by PCR fingerprinting.

Bacteriocin activity increased with the temperature reaching the highest value of 6400 AU mL^{-1} against *L. monocytogenes* Scott A at 30°C (**Table 5.3)**. As a result of the PCR typing, M13R2 was the only primer that provided different profiles for the five strains of *Listeria* used in this study (**Table 5.3)**.

Table 5.3. *Listeria* spp. strains tested, their sensitivity to bacteriocins produced by *Lb. curvatus* 54M16 and their RAPD-PCR profile (Giello et al., 2018)

Indicator strains	[a]AU mL^{-1} at temperature of			[b]PCR-RAPD profile
	15°C	20°C	30°C	
L. monocytogenes ATCC 7644	1600	1600	3200	A
L. monocytogenes Cal	800	800	1600	A
L. monocytogenes OH	1600	1600	3200	C
L. monocytogenes Scott A	3200	3200	6400	D
L. innocua ATCC 1770	1600	1600	1600	B

a: AU (Arbitrary units) mL^{-1} of neutralized and filter sterilized culture supernatant as determined by critical dilution assay.
b: PCR-RAPD profiles using primer M13R2

5.3.2 Co-culture experiments

Strains OH and Scott A of *L. monocytogenes* and the two strains of *Lb. curvatus* (54M16, Bac^+ and DSM 20019, Bac^-) were used for co-culture experiments.

The antilisterial activity of *Lb. curvatus* 54M16 in co-culture with *L. monocytogenes* Scott A and OH is shown in **Figures 5.1(1) and 5.1(2).** In mono-culture, *L. monocytogenes* strains mixture remained stable at levels ranging from 4.51 to 4.69 Log cfu mL^{-1} within 8 h with an increase of 2.23 Log cfu mL^{-1} after 48 h of incubation at 20°C (**Figure 5.1(1)**). At 15°C, an increase of 0.91 Log cfu mL^{-1} was observed after 48 h (**Figure 5.1(2)**), reaching values of about 8.73 Log cfu mL^{-1} after 120 h (data not shown). When the *L. monocytogenes* strains mixture was co-cultured with the bacteriocins-producing strain of *Lb. curvatus* 54M16, cell counts decreased rapidly in the first five hours (87% of the initial colony count) reaching values <1

Log cfu mL^{-1} at the end of the 48 h of incubation at 20°C. The pH declined from 6.28 to 4.22 during the incubation period and was lower than the value reached in the *L. monocytogenes* mono-culture (**Figure 5.1(1)**). In co-culture at 15°C, both the inhibition of *L. monocytogenes* and decline of pH were lower than the corresponding values at 20°C. The colony count of the strains mixture decreased of about 2.58 Log cfu mL^{-1} after 48 h incubation (about 39% of the initial value), whereas the pH declined to 4.8 (**Figure 5.1(2)**). However, after 5 days of incubation, the number of *L. monocytogenes* decreased below the detectable level (<1 Log cfu mL^{-1}), and the pH reached values around 4.2 (data not shown).

Bacteriocins production by *Lb. curvatus* 54M16 co-inoculated with the mixture of *L. monocytogenes* SA and OH and RAPD-PCR profiles of the two strains of *L. monocytogenes* during the storage period at 15 and 20°C are reported in **Table 5.2.** *L. monocytogenes* Scott A was more sensitive to the bacteriocins than the OH strain. Moreover, RAPD-PCR of the two strains of *L. monocytogenes* during co-culture experiments showed 71.4% and 88.9% presence of the OH strain profile after 24 and 48 h at 20°C, respectively. After 48 h at 15°C, only the profile of *L. monocytogenes* OH was detected (**Table 5.2**). In the sample inoculated only with the mixture of *L. monocytogenes* OH and Scott A, the percentage of the RAPD-PCR profiles of the two strains were approximately the same (data not shown).

The effect of non-bacteriocin-producing strain *Lb. curvatus* DSM 20019 on the growth of *L. monocytogenes* Scott A and OH in MRS broth and on pH changes at 20 and 15°C are shown in **Figures 5.2(1) and 5.2(2).** In co-culture with *Lb. curvatus* DSM 20019 Bac^{-}*, L. monocytogenes* strains mixture was stable at levels ranging from 4.41 to 4.54 Log cfu mL^{-1} during 48 h of incubation at 20°C and the pH decreased to 4.07. In mono-culture, the number of *Listeria* reached values of 8.3 Log cfu mL^{-1} but the pH did not change in the same period of storage (**Figure 5.2(1)**). At 15°C, the strains mixture of *L. monocytogenes* in co-culture experiment increased by approximately 1 Log cfu mL^{-1} after 24 h but decreased to the initial inoculum level (about 4 Log cfu mL^{-1}) at the end of the storage period, whereas the pH reached a value of 4.31. The mono-culture of *L. monocytogenes* increased by approximately 0.9 Log cfu mL^{-1} throughout the 48 h of the storage period (**Figure 5.2(2)**).

Finally, the growth of the two *Lb. curvatus* strains at both at 15 and 20°C was not influenced by the strains mixture of *L. monocytogenes* (**Figures 5.1(1), 5.1(2), 5.2(1) and 5.2(2)).**

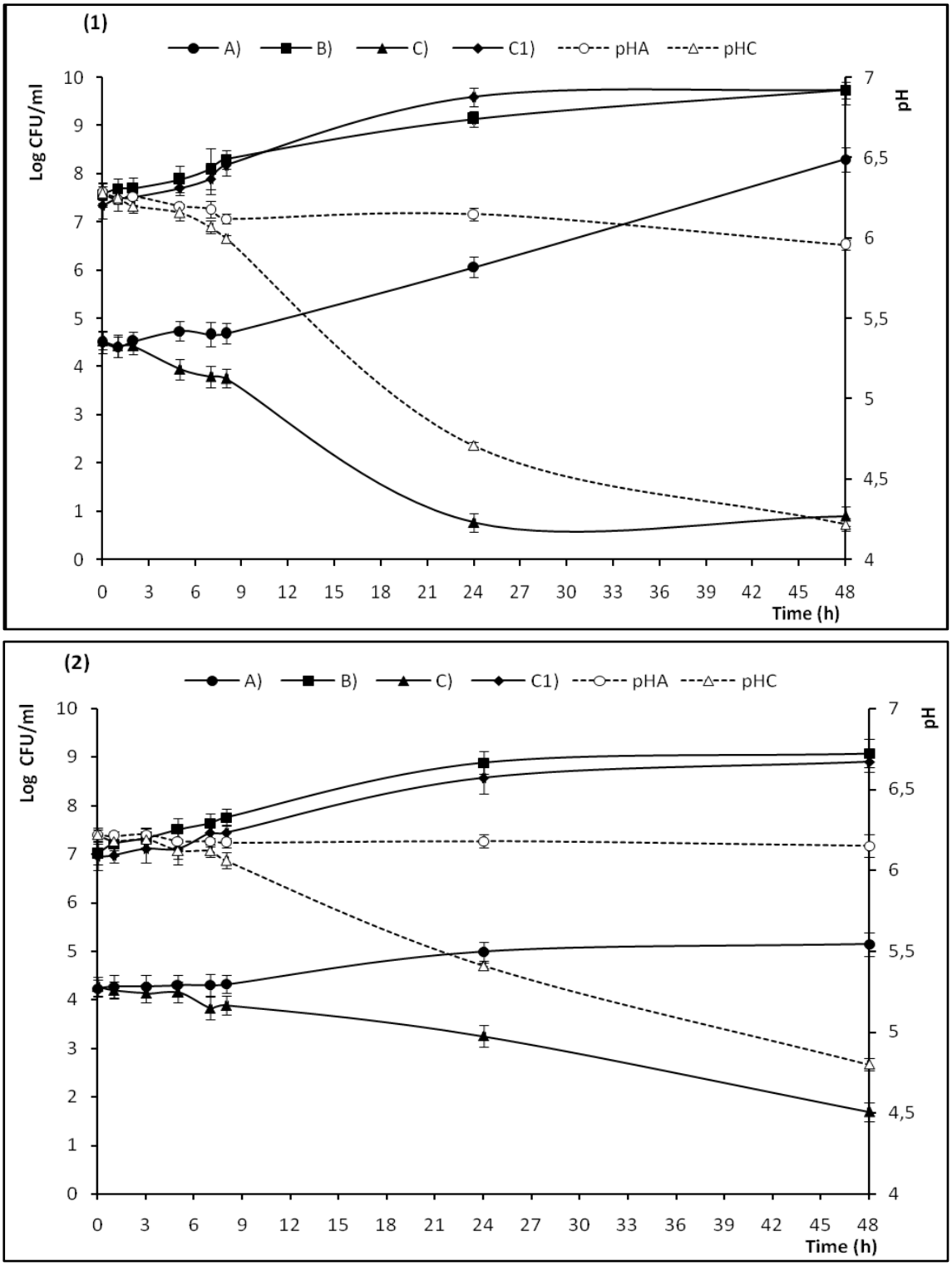

Figure 5.1. Growth of *L. monocytogenes* (mixture of strains OH and Scott A) and *Lactobacillus curvatus* 54M16 in monoculture and co-culture in MRS broth at 20°C (1) and at 15°C (2). A) Log cfu mL^{-1} of *L. monocytogenes* alone; B) Log cfu mL^{-1} of *Lb. curvatus* 54M16 alone; C) Log cfu mL^{-1} of *L. monocytogenes* co-cultured with *Lb. curvatus* 54M16; C1) Log cfu ml^{-1} of Lb. curvatus 54M16 co-cultured with *L. monocytogenes*; pHA and pHC are the values of pH determined in the cultures A and C, respectively. Results are the mean values ± SD (standard deviation) from three independent experiments for each sample (Giello et al., 2018).

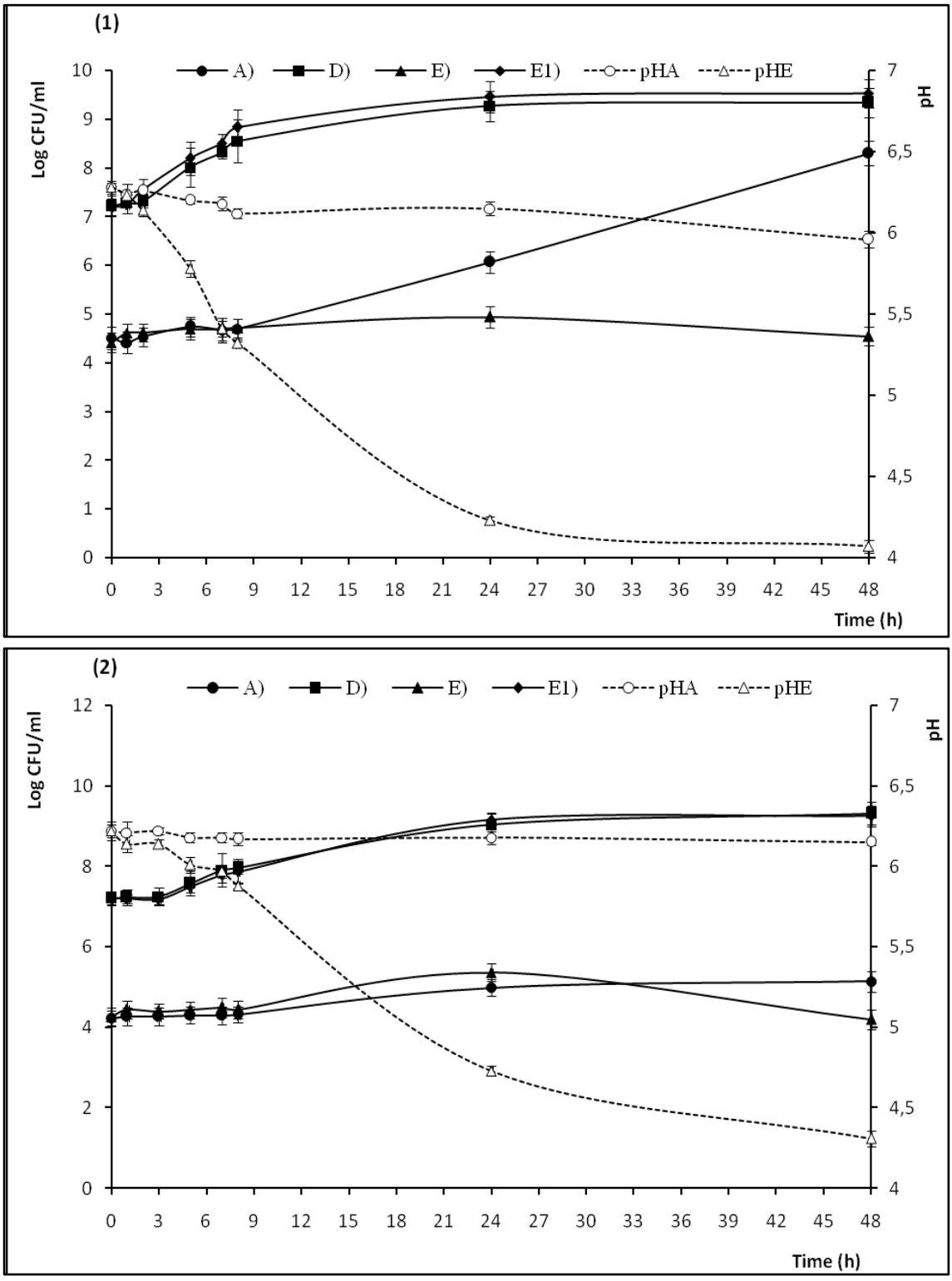

Figure 5.2. Growth of *L. monocytogenes* (mixture of strains OH and Scott A) and *Lactobacillus curvatus* DSM 20019 in monoculture and co-culture in MRS broth at 20°C (1) and at 15°C (2). A) Log cfu mL^{-1} of *L. monocytogenes* alone; D) Log cfu mL^{1} of *Lb. curvatus* DSM 20019 alone; E) Log cfu mL^{-1} of *L. monocytogenes* co-cultured with *Lb. curvatus* DSM 20019; E1) Log cfu mL^{-1} of *Lb. curvatus* DSM 20019 co-cultured with *L. monocytogenes*; pHA and pHE are the values of pH determined in the cultures A and E, respectively. Results are the mean values ± SD (standard deviation) from three independent experiments for each sample (Giello et al., 2018).

5.3.3 Microbial population, pH and a_w during sausages ripening

Meat batter used to produce the sausages was naturally contaminated with *L. monocytogenes* as determined by the enrichment procedure. In non-inoculated sausages (samples A), the counts of wild *L. monocytogenes* were at detectable levels (>1 Log cfu g^{-1}) after 14 days of ripening, reaching values of 1.21 Log cfu g^{-1} and increasing to 1.60 Log cfu g^{-1} at the end of ripening **(Table 5.4).** The pH decreased for up to seven days by 0.47 units, then increased to 5.69 at the end of ripening. The a_w decreased by 0.067 units during the entire process. In sausages co-inoculated with *Lb. curvatus* 54M16 (samples D), *L. monocytogenes* decreased by 1.17 Log cfu g^{-1} in the first three days and by 0.51 Log cfu g^{-1} at the end of ripening from an initial value of 4.22 Log cfu g^{-1}. The pH decreased during fermentation by 0.62 units, then increased to 5.58 at the end of ripening. The levels of *L. monocytogenes* were significantly different between sausages B and D at 3, 7, 21 and 28 days of ripening ($P < 0.05$). The ripening time significantly affected the levels of *L. monocytogenes* in samples B ($P \leq 0.0001$), where it increased by 2 Log cfu g^{-1} after 28 days of ripening **(Table 5.4).** Instead, samples D were not affected by the ripening time. Moreover, in sausages B the, pH was almost constant until the end of the ripening (from 5.83 to 5.74). Finally, when the sausages naturally contaminated with *L. monocytogenes* were inoculated with *Lb. curvatus* 54M16 (samples C), the pathogen was undetectable during the whole ripening process (< 1 Log cfu g^{-1}) and was absent from the 25-g sample also after the enrichment procedure from the seventh day of ripening onward.

The mean values of other microbial groups during the manufacturing and ripening of fermented sausages are reported in **Table 5.5**. All microbial groups were affected by the ripening time.

The number of coagulase negative cocci (CNC) increased in all samples until the 14th day of ripening, then slightly decreased, reaching significantly different values among the samples at the end of ripening ($P < 0.05$). *Enterobacteriaceae* in the sausages without *Lb. curvatus* 54M16 increased until the 14th day (samples A) and 7th day (samples B) of ripening, then decreased to 5.0 Log cfu g^{-1} and 3.84 Log cfu g^{-1}, respectively, at 28 days. In contrast, the initial population was reduced by approximately 63.2% and 36.4%, in C and D, respectively, at the end of ripening. LAB were significantly higher in all sausages inoculated with *Lb. curvatus* 54M16 at each ripening time in comparison to the samples with *L. monocytogenes* alone or the

non-inoculated samples. Particularly, the initial counts of LAB increased by approximately 4.3 Log cfu g^{-1} in samples A and B and by 1.3 Log cfu g^{-1} in sausages C and D at 28 days of ripening. The mean counts of yeast and moulds increased in all samples, reaching significantly different values between samples with (sausages C and D) and without *Lb. curvatus* 54M16 (sausages A and B) at the end of ripening ($P < 0.05$).

In order to monitor the bacteriocins-producing *Lb. curvatus* 54M16 in the samples D, two antimicrobial-producing isolates at each sampling point were assayed for the presence of the s*ak*X, *sak*T_{α}, *sak*T_{β} and *sak*P genes. All isolates showed the 156-bp, 186-bp, 160-bp and 151-bp fragments, corresponding to the structural genes *sak* X, *sak* P, *sakT*$_{\alpha}$ and *sakT*$_{\beta}$, respectively (**Figure 5.3).**

Table 5.4. ***Listeria monocytogenes*** **population (Log cfu g^{-1}) pH and a_w monitored during fermentation and ripening of sausages (Giello et al., 2018)**

Sausages samples	Days[(1)]						SS[(2)]
	0	3	7	14	21	28	
A) Sausages control (not started)							
L. monocytogenes **(Log cfu g^{-1}±SD)**	$<1.0^{a(3)}$	$<1.0^{a(3)}$	$<1.0^{a(3)}$	1.21^{b}±0.31	1.13^{b}±0.19	1.60^{c}±0.60	****
pH	5.83±0.09	5.48±0.07	5.36±0.06	5.52±0.03	5.61±0.05	5.69±0.06	
a_w	0.978±0.03	0.969±0.02	0.967±0.16	0.954±0.08	0.931±0.01	0.911±0.02	
B) Sausages with *Listeria* strains							
L. monocytogenes **(Log cfu g^{-1}±SD)**	4.40^{b}±0.16	5.72^{Ac}±0.17	6.37^{Aa}±0.04	6.57^{a}±0.15	6.52^{Aa}±0.18	6.40^{Aa}±0.23	****
pH	5.83±0.04	5.46±0.06	5.41±0.08	5.56±0.07	5.59±0.06	5.74±0.04	
a_w	nd	nd	nd	nd	nd	nd	
C) Sausages with *Lb. curvatus* 54M16							
L. monocytogenes **(Log cfu g^{-1}±SD)**	<1.0	$<1.0^{(4)}$	<1.0	<1.0	<1.0	<1.0	nd
pH	5.83±0.06	5.19±0.06	5.23±0.04	5.25±0.06	5.36±0.06	5.57±0.07	
a_w	0.979±0.09	0.963±0.02	0.959±0.07	0.942±0.05	0.933±0.02	0.921±0.02	
D) Sausages with *Listeria* and *Lb. curvatus* 54M16							
L. monocytogenes **(Log cfu g^{-1}±SD)**	4.22±0.08	3.05^{B}±0.13	3.38^{B}±0.16	4.19±0.98	4.36^{B}±0.13	3.71^{B}±0.75	ns
pH	5.83±0.06	5.21±0.11	5.24±0.08	5.27±0.10	5.35±0.07	5.58±0.10	
a_w	nd	nd	nd	nd	nd	nd	

1: The data are the mean values ± standard deviation of two batches of sausages. 2:Statistical Significance: different letters in the same row indicate significant differences among times ($P \leq 0.05$); P value: **** $P \leq 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, not significant; nd, not determined. Different capital letters in the columns indicate differences among the samples B and D at each time ($P < 0.05$). 3: *L. monocytogenes* was present after enrichment procedure. 4: *L. monocytogenes* was absent in 25 g after enrichment procedure.

Table 5.5. Microbial populations (Log cfu g^{-1}) monitored during fermentation and ripening of sausages (Giello et al., 2018)

Sausages samples	Microbial populations (Log cfu g^{-1} ± SD) at time (days)[3]						SS[5]
	0	3	7	14	21	28	
A) Sausages control (not started)							
LAB[1]	3.58^{Ab}±0.29	7.36^{Ab}±0.23	8.42^{Ad}±0.30	7.96^{Aa}±0.26	nd[4]	7.90^{Aa}±0.26	****
CNC[2]	3.72^{b}±0.22	6.38^{Ac}±0.06	7.50^{a}±0.39	7.43^{a}±0.28	nd	7.40^{Ca}±0.05	****
Enterobacteriaceae	4.34^{Ab}±0.27	6.36^{Aa}±0.14	5.86^{Ac}±0.31	6.30^{Ba}±0.26	nd	5.00^{Ad}±0.25	****
Yeasts and Moulds	3.0^{Ac}±0.39	4.28^{Ad}±0.31	5.51^{Aa}±0.29	5.67^{ab}±0.22	nd	5.84^{Bb}±0.28	****
B) Sausages with *Listeria* strains							
LAB[1]	3.86^{Ab}±0.31	7.20^{Ac}±0.22	8.25^{Aa}±0.27	8.46^{Ba}±0.25	nd	8.21^{Aa}±0.24	****
CNC[2]	3.67^{b}±0.26	6.30^{Ac}±0.28	7.10^{a}±0.14	7.23^{a}±0.29	nd	6.93^{Ba}±0.21	****
Enterobacteriaceae	5.59^{Ba}±0.26	5.95^{Ba}±0.28	6.13^{Aa}±0.29	4.84^{ABab}±0.22	nd	3.84^{Bb}±0.25	**
Yeasts and Moulds	4.21^{Ba}±0.22	4.27^{Aa}±0.27	6.06^{Bb}±0.26	5.57^{c}±0.28	nd	5.56^{ABc}±0.31	****
C) Sausages with *Lb. curvatus* 54M16							
LAB[1]	7.31^{Bb}±0.20	8.86^{Ba}±0.23	8.98^{Ba}±0.19	8.92^{Ca}±0.22	nd	8.61^{Ba}±0.20	****
CNC[2]	3.72^{b}±0.23	7.11^{Ba}±0.29	7.13^{a}±0.18	7.25^{a}±0.30	nd	6.34^{Ac}±0.25	****
Enterobacteriaceae	4.92^{ABa}±0.22	5.08^{Ca}±0.30	5.04^{Ba}±0.25	4.00^{ABab}±0.28	nd	3.11^{Cb}±0.24	*
Yeasts and Moulds	4.29^{Ba}±0.24	4.03^{Aa}±0.29	5.18^{Cb}±0.28	5.41^{bc}±0.25	nd	5.52^{Ac}±0.23	****
D) Sausages with *Listeria* and *Lb. curvatus* 54M16							
LAB[1]	7.33^{Bb}±0.27	9.00^{Ba}±0.25	9.01^{Ba}±0.22	8.94^{Ca}±0.28	nd	8.67^{Ba}±0.24	***
CNC[2]	3.57^{c}±0.19	6.63^{Aa}±0.28	7.00^{b}±0.24	7.08^{b}±0.22	nd	6.58^{ABa}±0.29	****
Enterobacteriaceae	5.60^{ABa}±0.18	5.45^{Da}±0.25	5.08^{Bb}±0.23	3.30^{Ac}±0.29	nd	2.04^{Dd}±0.22	****
Yeasts and Moulds	4.43^{Bb}±0.30	2.15^{Bc}±0.25	5.56^{Aa}±0.28	5.28^{a}±0.23	nd	5.54^{Aa}±0.29	****

1: LAB, lactic acid bacteria; 2: CNC, coagulase negative cocci;. 3: the data are the mean values± standard deviation of two batches of sausages; 4: not determined; 5: Statistical significance: different letters in the same row indicate significant differences for each microbial group among times ($P \leq 0.05$); P value: **** $P \leq 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$. Different capital letters in the columns indicate differences for each microbial group among the samples at each time ($P < 0.05$).

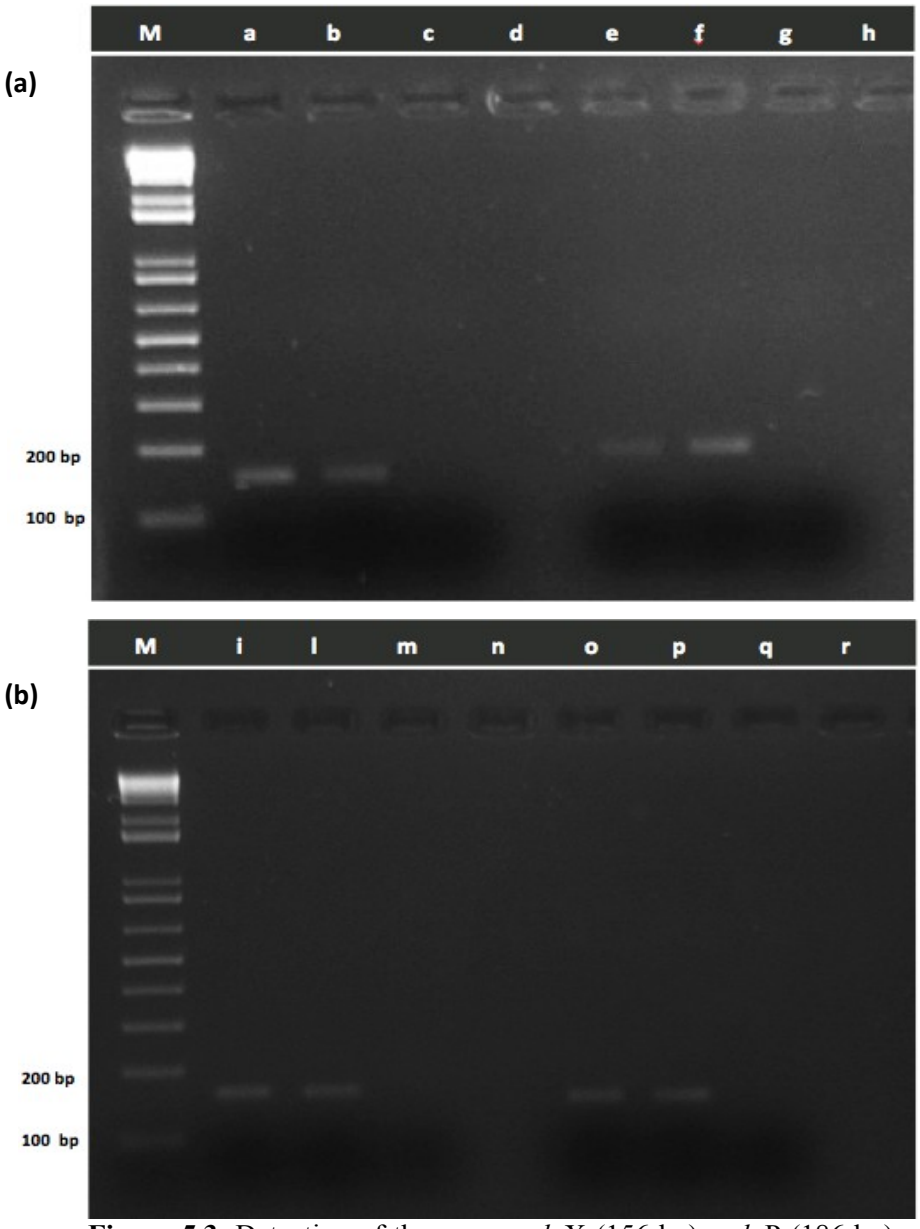

Figure 5.3. Detection of the genes *sak* X (156 bp), *sak* P (186 bp), $sakT_{\alpha}$ (160 bp) and $sakT_{\beta}$ (151 bp), after PCR of genomic DNA from lactobacilli isolated from sausages. (a) M: 1 Kb Plus DNA Ladder. Lanes a and b: *sak* X of two isolates of *Lactobacillus* at 14 and 28 days of ripening; lanes e and f: *sak* P of the same two isolates of *Lactobacillus* at 14 and 28 days of ripening. Lanes c, d, g and h: negative control (no DNA). (b) M: 1 Kb Plus DNA Ladder. Lanes i and l: $sakT_{\alpha}$ of two isolates of *Lactobacillus* at 14 and 28 days of ripening; lanes o and p: $sakT_{\beta}$ of the same two isolates of *Lactobacillus* at 14 and 28 days of ripening. lanes m, n, q and r: negative control (no DNA) (Giello et al., 2018).

5.3.4 Identification, strain typing and bacteriocins sensitivity of *Listeria* isolates from sausages

Presumptive *L. monocytogenes* colonies isolated from ALOA plates from samples A (un-inoculated sausages) and D (sausages inoculated with the mixture of *L. monocytogenes* OH/Scott A and *Lb. curvatus* 54M16) at 14 and 28 days of ripening were identified as belonging to *Listeria monocytogenes* species by 16S rRNA gene sequencing **(Table 5.6)**. *L. monocytogenes* wild-type strains isolated from samples A showed the same RAPD-PCR profile, which was different from that of *L. monocytogenes* OH and Scott A. Conversely, isolates from samples D showed the same RAPD profile of *L. monocytogenes* Scott A **(Table 5.6)**. Moreover, the titre of neutralized and filter sterilized culture supernatant of *Lb. curvatus* 54M16 against the strains of *L. monocytogenes* from samples A and D (400 and 6400 AU mL^{-1}, respectively) confirmed the presence of wild *L. monocytogenes* strains in sample A. During ripening, bacteriocins were also extracted directly from sausages C and D at 7 days (samples C7 and D7, respectively) and assayed against *L. monocytogenes* OH and Scott A. Unlike the results of co-culture experiments (section 5.3.2), *L. monocytogenes* OH and Scott A were less sensitive to the bacteriocins directly extracted from the sausages.

Table 5.6. Identification, strain typing and bacteriocins sensitivity of *Listeria* isolates from sausages (Giello et al., 2018)

[1]*L. monocytogenes* isolates	Accession number	[2]PCR-RAPD-profiles	[3]AU mL^{-1}
1D14	NZ_CP007600.1	b	6400
2D14	NZ_CP007600.1	b	6400
2D28	NZ_CP007600.1	b	6400
5D28	NZ_CP007600.1	b	6400
1A28	NZ_CP009242.1	a	400
2A28	NZ_CP009242.1	a	400
5A28	NZ_CP007160.1	a	400
6A14	NZ_CP009242.1	a	400
6A28	NZ_CP009242.1	a	400
OH	[4]nd	c	6400
Scott A	nd	b	12800

1: A (Sausages control not started); D (Sausages with *Listeria* and *Lb. curvatus* 54M16); 14 and 28 indicate the days of ripening from which the *Listeria* were isolated; 2: PCR-RAPD profiles using primer M13R2; 3: AU (Arbitrary units) mL^{-1} of neutralized and filter sterilized culture supernatant of *Lb. curvatus* 54M16 as determined by critical dilution assay against *L. monocytogenes* strains; 4: not determined.

5.3.5 16S rRNA-based analysis of bacterial communities

The microbiota of sausage samples at the beginning of fermentation was similar, regardless of the presence of an *inoculum* **(Figure 5.4)**. Instead, samples inoculated with *Lb. curvatus* 54M16 already clearly clustered apart from un-inoculated samples after 3 days of ripening **(Figure 5.4),** showing that the addition of *Lb. curvatus* strongly strongly impacted the fermented sausage microbiota during fermentation.

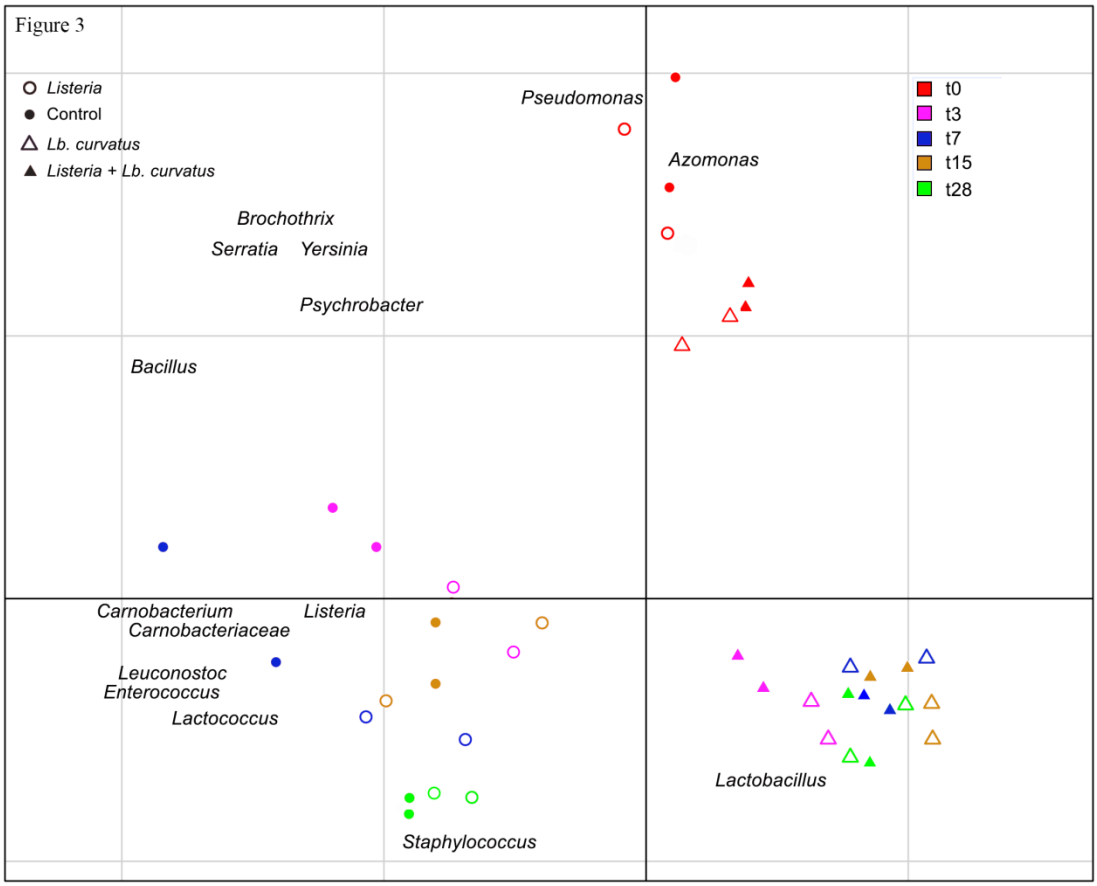

Figure 5.4. Principal Component Analysis (PCA) based on the microbiota composition at genus level. The two principal components were plotted using the vegan package in R. Only genera showing a loading score > 0.7 are shown in the figure. Samples are colored according to the ripening times. Different shapes reflect the four *inocula* tested (Giello et al., 2018).

In particular, samples A and B showed higher levels of spoilage-associated genera, such as *Pseudomonas*, *Brochothrix*, *Carnobacterium* and *Psychrobacter*, potential pathogens (*Yersinia*), and different lactic acid bacteria (*Leuconostoc*, *Enterococcus*, *Lactococcus*, *Streptococcus*, *Pediococcus*) (FDR < 0.05, **Figure 5.5** and **Table 5.7**). The *Lactobacillus* genus dominated the microbiota of fermented sausage samples C and D at 28 days of ripening, which also showed lower microbial diversity compared to samples A and B (FDR < 0.05, **Figure 5.6)**. Moreover, the *inoculum* of *Lb. curvatus* strongly affected *Listeria* abundance: samples co-inoculated with *Listeria*

and *Lactobacillus* (D) showed significantly lower levels of *Listeria* compared to those inoculated only with *Listeria* (B, **Figure 5.7).**

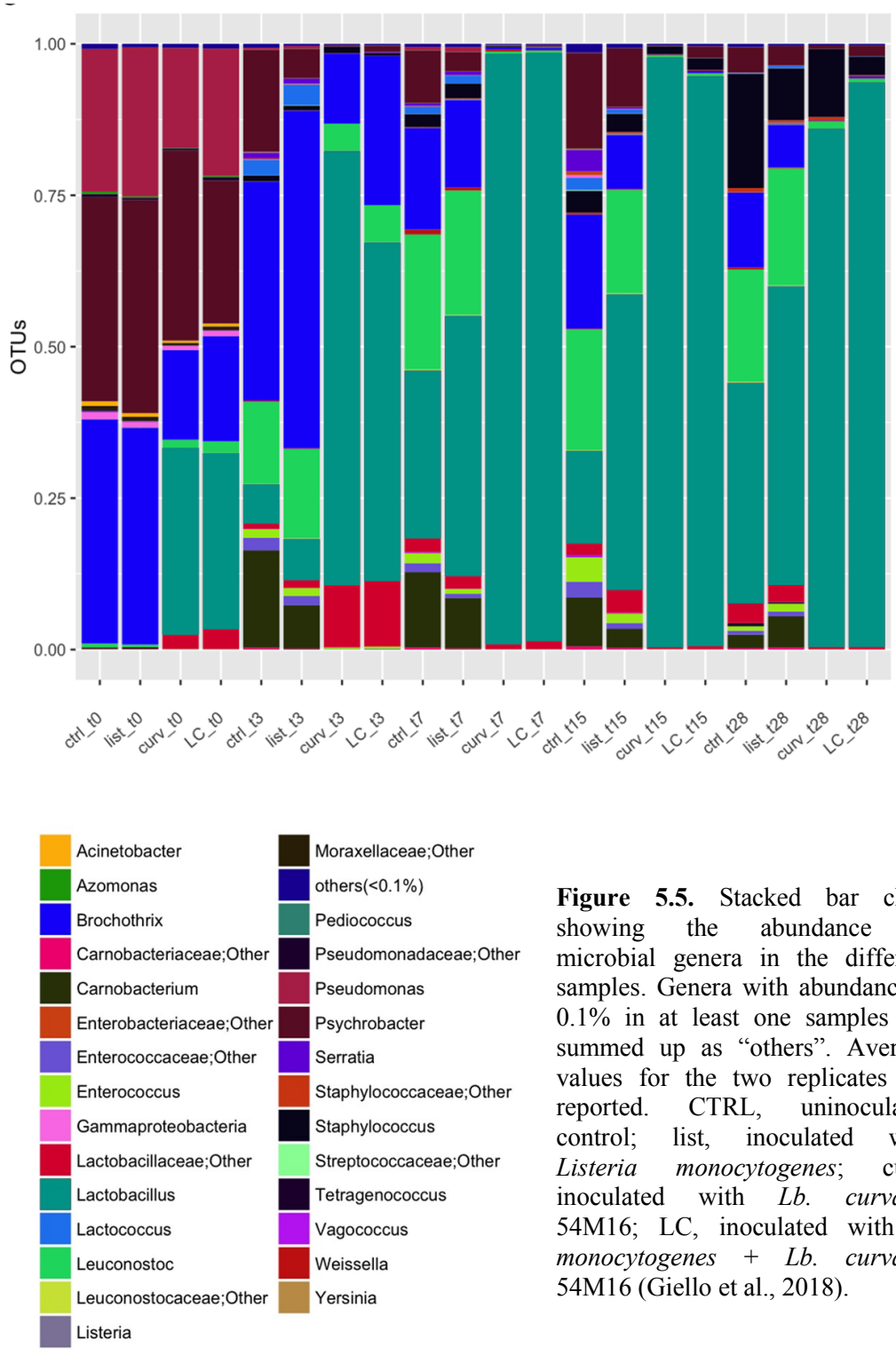

Figure 5.5. Stacked bar chart showing the abundance of microbial genera in the different samples. Genera with abundance < 0.1% in at least one samples are summed up as "others". Average values for the two replicates are reported. CTRL, uninoculated control; list, inoculated with *Listeria monocytogenes*; curv, inoculated with *Lb. curvatus* 54M16; LC, inoculated with *L. monocytogenes* + *Lb. curvatus* 54M16 (Giello et al., 2018).

Table 5.7. Abundance (% of reads) of significantly different* genera in the salami samples at the end of ripening (28 days) (Giello et al., 2018)

Genera	*Sausage samples*			
	Control	***Listeria***	***Lb. curvatus* 54M16**	***Lb. curvatus* 54M16 + *Listeria***
Pseudomonas	0.017[a]	0.018[a]	0.000[b]	0.005[c]
Azomonas	0.005[a]	0.002[a]	0.000[b]	0.000[b]
Psychrobacter	4.187[a]	3.189[a]	0.547[b]	1.752[c]
Yersinia	0.001[a]	0.000[a]	0.000[b]	0.000[b]
Lactococcus	0.154[a]	0.425[a]	0.004[b]	0.042[c]
Streptococcus	0.020[a]	0.008[a]	0.003[b]	0.002[b]
Enterococcus	0.734[a]	1.290[a]	0.023[b]	0.005[c]
Brochothrix	12.339[a]	6.942[a]	0.085[b]	0.282[c]
Leuconostoc	8.696[a]	11.434[a]	0.031[b]	0.030[b]
Lactobacillus	36.469[a]	49.355[a]	85.587[b]	93.222[b]
Pediococcus	0.064[a]	0.038[a]	0.009[b]	0.011[b]
Vagococcus	0.020[a]	0.035[a]	0.002[b]	0.000[b]
Carnobacterium	2.234[a]	5.161[a]	0.003[b]	0.002[b]
Granulicatella	0.008[a]	0.005[a]	0.000[b]	0.000[b]
Bacillus	0.013[a]	0.010[a]	0.002[b]	0.003[b]
Listeria	0.001[a,c]	0.379[b]	0.000[c]	0.000[c]

*Different letters indicate genera with a significant different abundance as detected by pair-wise Wilcoxon tests.

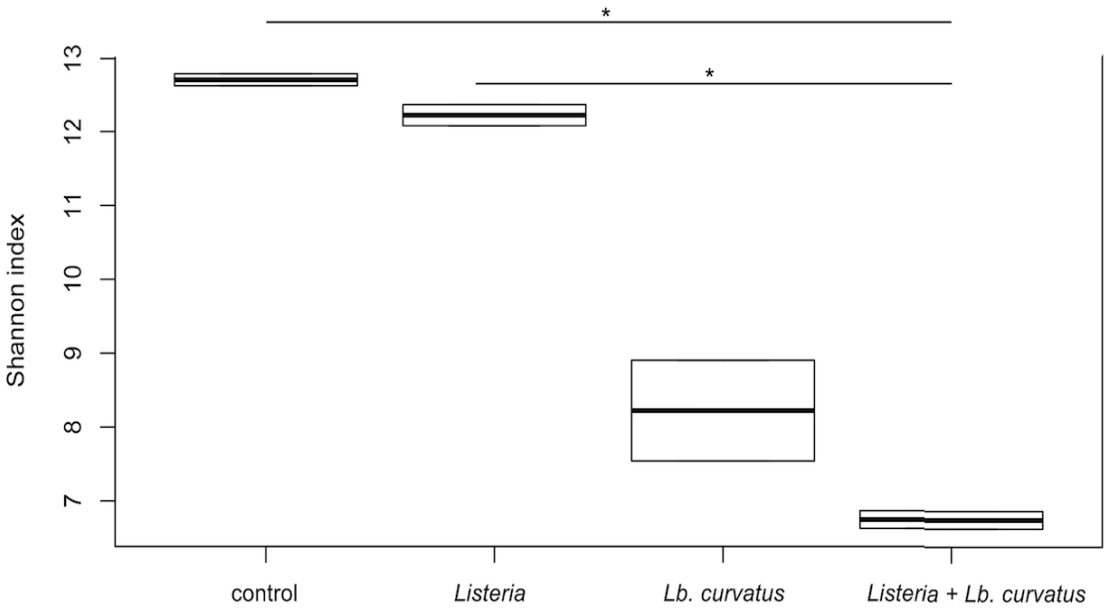

Figure 5.6. Box plots showing the Shannon diversity index in salami samples at the end of the ripening time (28 days). Average values for the two replicates are reported (Giello et al., 2018).

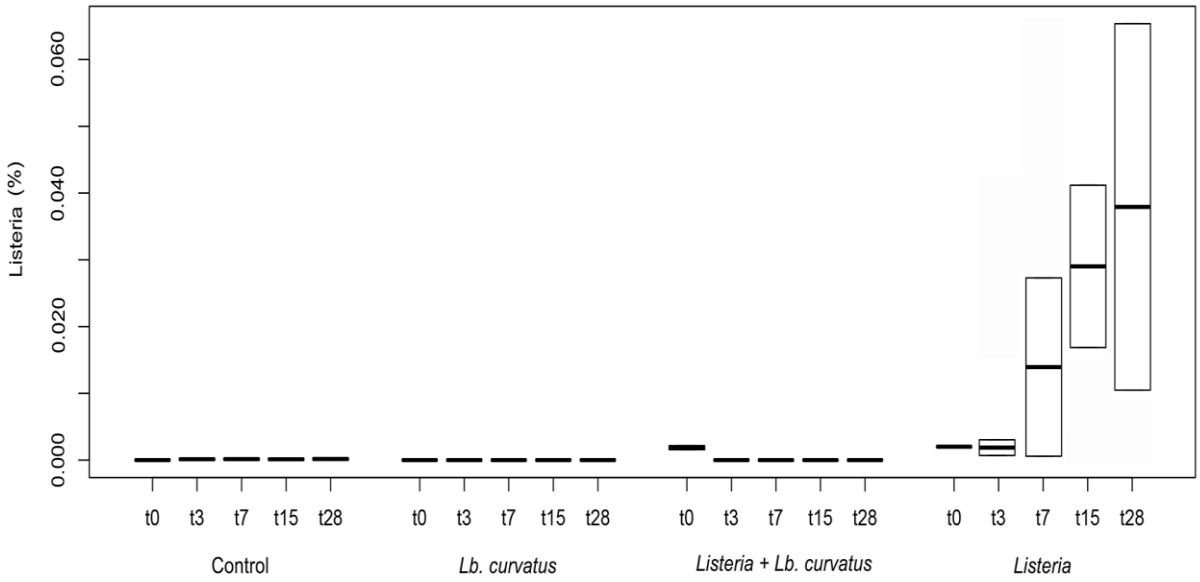

Figure 5.7. Box plot showing the abundance of *Listeria* spp. (B) in the different samples, as detected by HTS. Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.5xIQR from the first and third quartiles, respectively (Giello et al., 2018).

We carried out oligotyping of *Lactobacillus* sequences. Among seventy-five different identified, 25 oligotypes showed a relative abundance higher than 2% **(Table 5.8)**. The *Lactobacillus* oligotype pattern was clearly different in *Lb. curvatus* inoculated samples that clustered apart from A and B **(Figure 5.8)**.

The two *Lactobacillus* oligotypes dominating in A and B (Lb3 and Lb4) were identified as *Lactobacillus sakei* as a best match, whereas Lb1 and Lb2, prevailing in C and D, showed higher similarity with *Lb. curvatus* (**Figure 5.9** and **Table 5.8**). In addition, a phylogenetic tree was built using *Lb. curvatus* 54M16 16S and representative oligotype sequences, and and we observed that the sequence of the inoculated strain showed 100% similarity with oligotype Lb2 over a length of 445 bp (**Figure 5.10**).

Table 5.8. Identification of the most abundant *Lactobacillus* oligotypes (Giello et al., 2018)

***Lactobacillus* oligotype**	**Closest match**	**Identity**	**Query cover**
Lb1	*Lactobacillus curvatus* strain GCU-W-MS1	99.78	100
Lb2	*Lactobacillus curvatus* strain Wikim	100	100
Lb3	*Lactobacillus sakei* strain WiKim49	99.78	100
Lb4	*Lactobacillus sakei* strain 8-5	100	100
Lb5	*Lactobacillus curvatus* strain WiKim38	99.55	100
Lb6	*Lactobacillus curvatus* strain WiKim52	99.78	100
Lb7	*Lactobacillus curvatus* strain WiKim38	99.55	100
Lb8	*Lactobacillus curvatus* strain WiKim38	99.55	100
Lb9	*Lactobacillus curvatus* strain WiKim38	99.78	100
Lb10	*Lactobacillus curvatus* strain WiKim52	99.78	100
Lb11	*Lactobacillus plantarum* strain HFC8	97.76	99.33
Lb12	*Lactobacillus plantarum* strain Lpfg11	97.75	100
Lb19	*Lactobacillus porcinae* strain R-42633	97.08	100
Lb20	*Lactobacillus curvatus* qz1245	98.43	100
Lb24	*Lactobacillus sakei* strain KTNB0024M	99.55	100
Lb29	*Lactobacillus sakei* strain WiKim49	99.55	100
Lb31	*Lactobacillus curvatus* strain ZTR-2	99.78	100
Lb32	*Lactobacillus sakei* strain 8-5	99.55	100
Lb40	*Lactobacillus sakei* strain 8-4	99.10	100
Lb43	*Lactobacillus porcinae* strain R-42633	96.40	100
Lb58	*Lactobacillus plantarum* strain Lpfg69	97.98	100
Lb59	*Lactobacillus plantarum* strain Lpfg68	99.55	100
Lb67	*Lactobacillus sakei* strain 8-10	98.88	100
Lb68	*Lactobacillus curvatus* strain WiKim52	99.55	100

Only oligotype with an average abundance of at least 2% were reported.

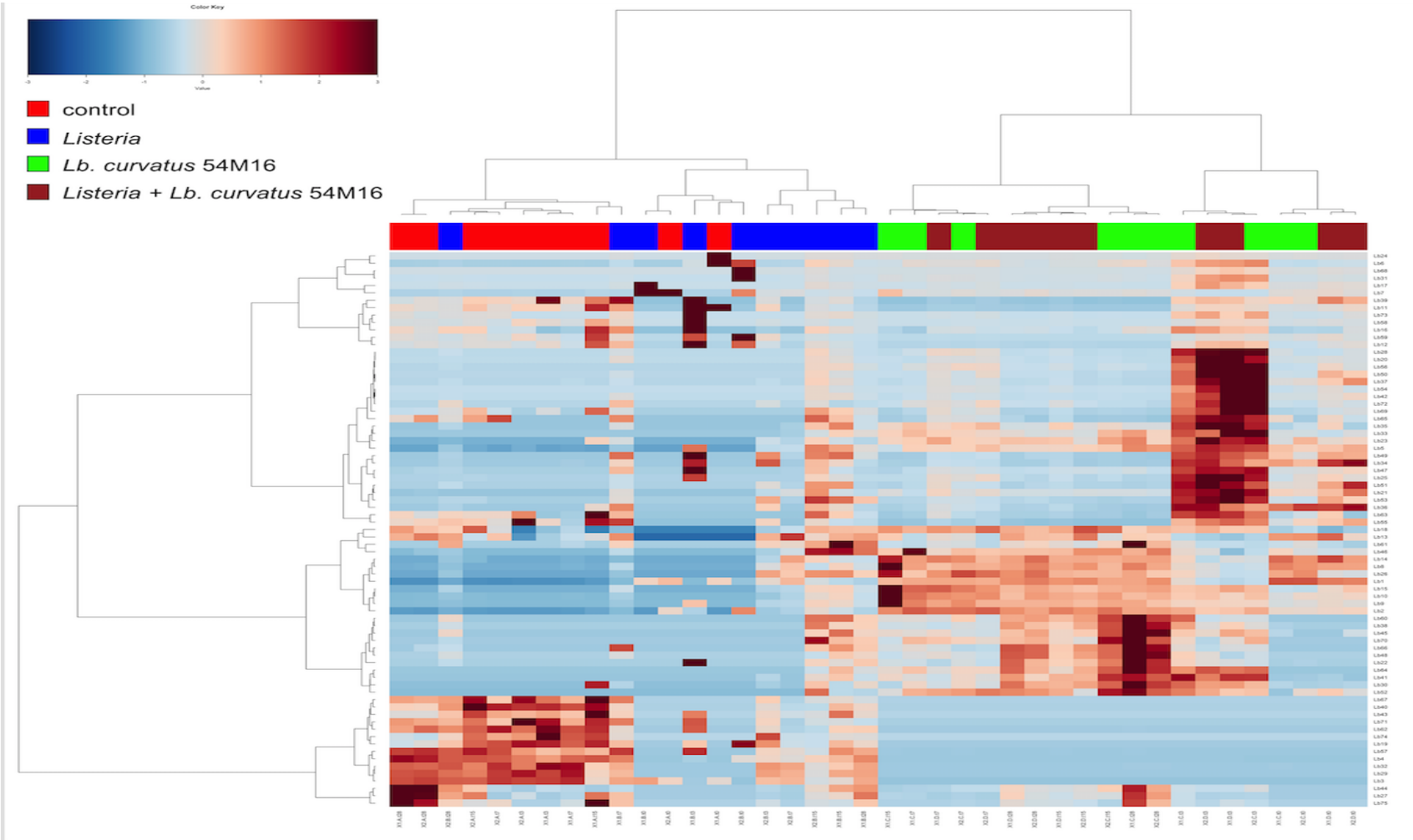

Figure 5.8. Hierarchical Ward linkage clustering of the samples based on the Pearson's correlation coefficient for the abundance of *Lactobacillus* oligotypes. The color scale indicates the scaled abundance of each variable, denoted as the Z-score; red, high abundance; blue, low abundance. Column bar is colored according to the type of *inoculums* (Giello et al., 2018).

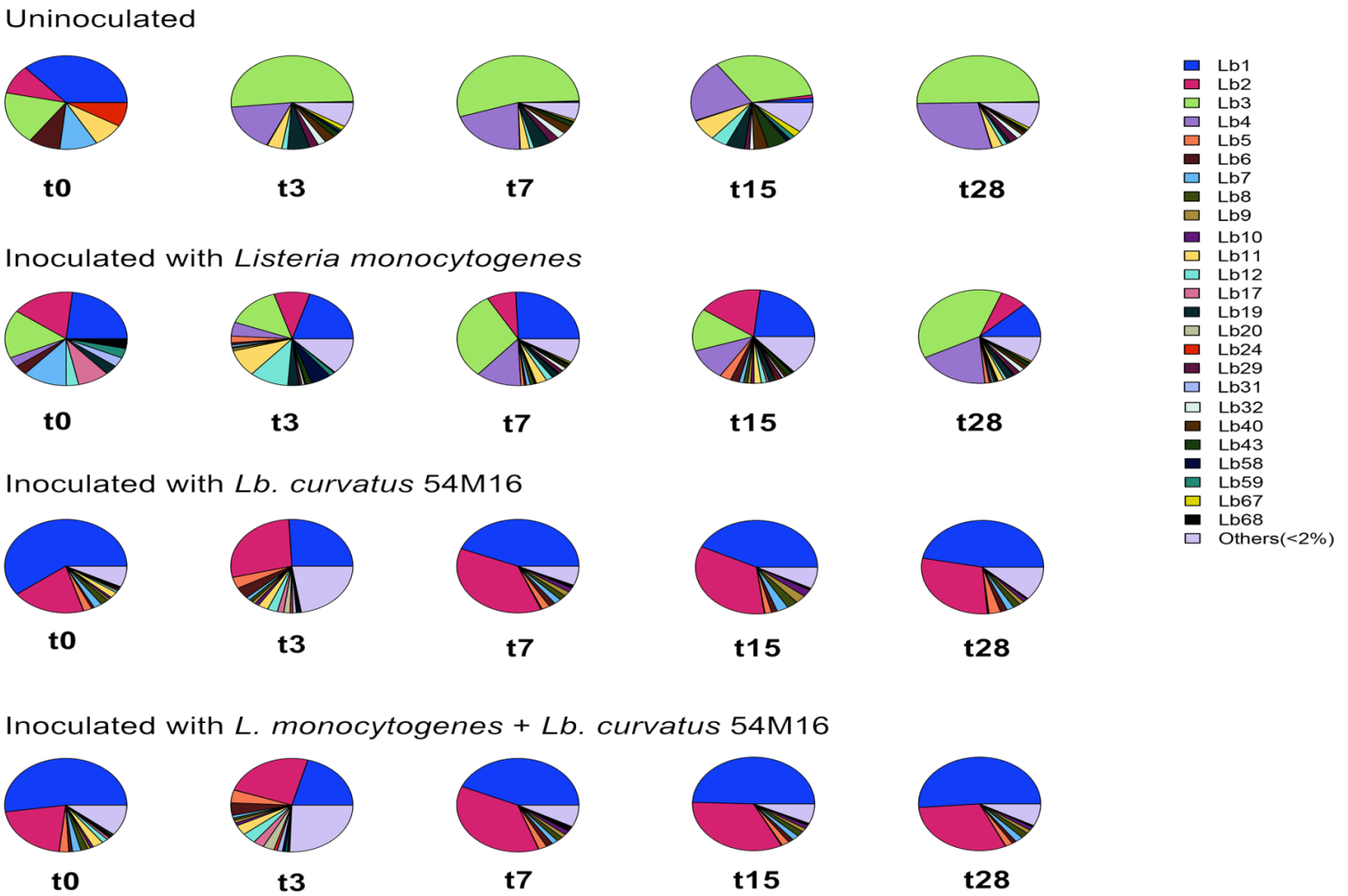

Figure 5.9. Pie charts showing the abundance of *Lactobacillus* oligotypes in the different samples. Oligotypes with abundance < 2% in at least one samples are summed up as "others". Average values for the two replicates are reported (Giello et al., 2018).

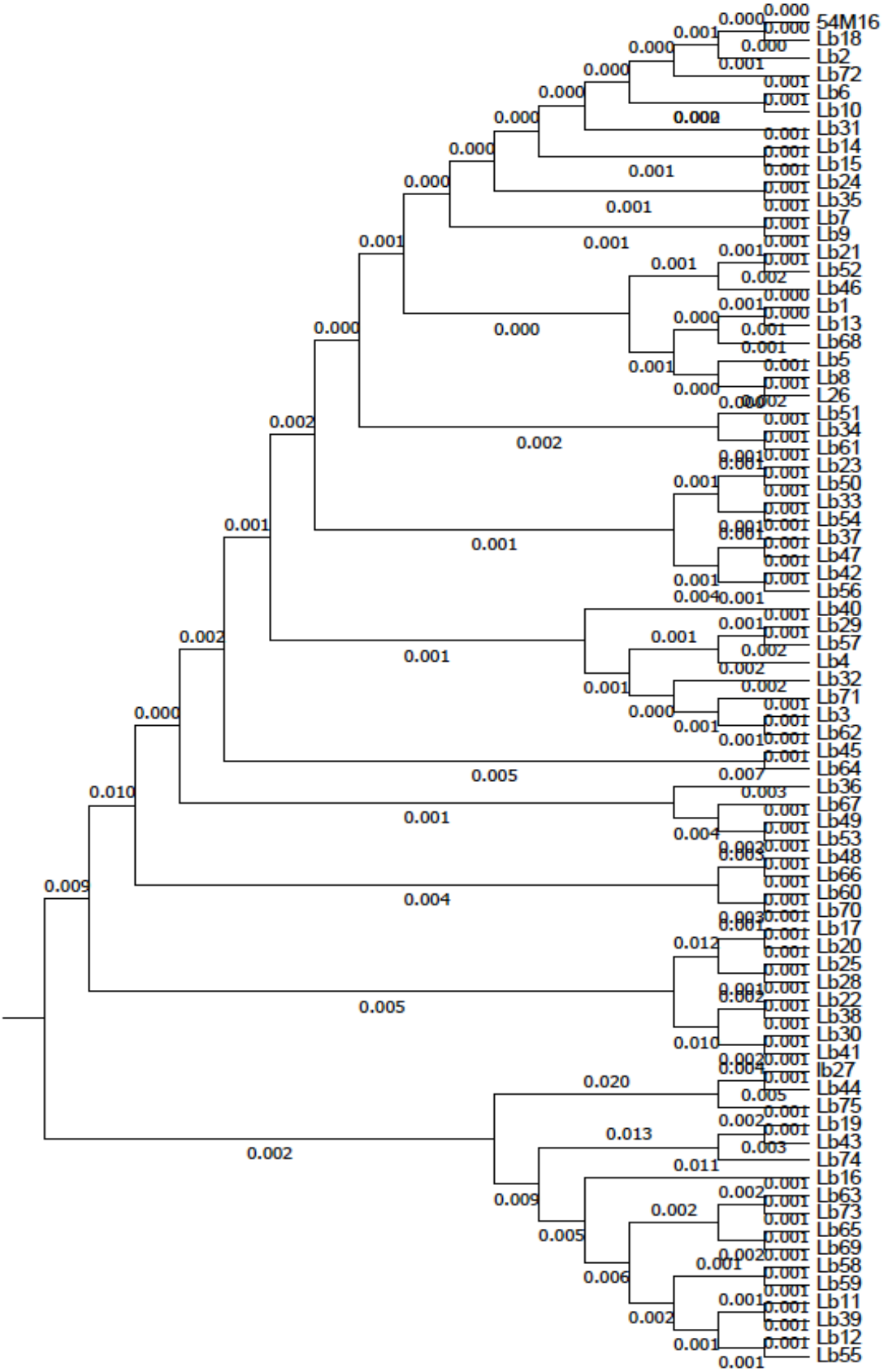

Figure 5.10. Evolutionary relationship among partial 16S rRNA gene sequences (445 bp) of the 75 *Lactobacillus* oligotypes and *Lb. curvatus* 54M16 strain. The evolutionary history was inferred using the UPGMA method (Giello et al., 2018).

5.4 DISCUSSIONS

The selection and use of bacteriocin-producing LAB strains or their bacteriocins as factors in food manufacturing processes is considered an important strategy for the control of foodborne listeriosis (Galvez et al., 2010; Woraprayote et al., 2016).

L. monocytogenes frequently occurs in processed and ready-to-eat foods such as fermented sausages (Petruzzelli et al., 2010; Martins and Germano, 2011; Mataragas et al., 2015) and there is still high interest in its biocontrol (Minayo et al., 2010; Gao et al., 2014; de Souza Barbosa et al., 2015). Levels of *L. monocytogenes* not exceeding 100 cfu g^{-1} have been established for ready-to-eat (RTE) fermented sausages, which are unable to support the growth of the pathogen when their physicochemical parameters accomplish conditions such as pH ≤ 4.4 or aw ≤ 0.92 or a combination of pH ≤ 5.0 and aw ≤ 0.94 (EC Regulation 2073/2005, European Commission, 2005). In 2015, the proportion of non-compliance (limit ≤ 100 cfu g^{-1} of *L. monocytogenes*) observed in fermented sausages, at both processing and retail stages ranged between 0 and 0.64%, and 0-0.27%, respectively, at the EU level (EFSA, 2016).

The aim of this study was to investigate the anti-listerial effectiveness of *Lb. curvatus* 54M16, which produces the bacteriocins *sak X, sak* T_{α}*, sak* T_{β} and *sak P* (Casaburi et al., 2016), as a protective culture in the ripening of fermented sausages.

In the co-culture experiments carried out in this work, *Lb. curvatus* 54M16 reduced the mixture of the two strains of *L. monocytogenes* from 4.54 to 0.9 Log cfu mL^{-1} at 20°C and from 4.27 to 1.69 at 15°C within 48 h **(Figures 5.1(1) and 5.1(2))**. At the same time, the pH values in co-culture declined from 6.28 to 4.22 during the incubation period and were lower than the value observed in the *L. monocytogenes* mono-culture (**Figure 5.1(1)**).

A number of factors may be involved in the interactions between mixed populations in the same ecosystem. These factors include competition for nutrients, production of metabolites (organic acids, diacetyl, hydrogen peroxide, and bacteriocins), signalling molecules and cell-to-cell contact mechanisms. Many studies on *L. monocytogenes* interactions have been done in both in laboratory media and food (Cornu et al., 2011; Mataragas et al., 2010; Mellefont et al., 2008; Pleasants et al., 2001). *L. monocytogenes* inhibition by non-bacteriocinogenic *Carnobacterium piscicola* was due to competition for nutrient, particularly for glucose (Nilsson, et al., 2005), whereas Saraoui et al. (2016) attributed inhibition of the pathogen by non-

bacteriocinogenic *Lactococcus piscium* CNCM I-4031 to cell-to-cell contact mechanisms.

In the present study, to obtain more information on the type of microbial interaction, *L. monocytogenes* was also co-cultured with the non-bacteriocin-producing strain *Lb. curvatus* DSM 20019; the pathogen mixture remained at the initial concentration of about 4 Log cfu mL^{-1} and the pH decreased to 4.07 at 20°C and 4.31 at 15°C throughout the incubation **(Figures 5.2(1) and 5.2(2))**. In agreement with Pleasants et al., (2001), the inhibition of *L. monocytogenes* may be attributed principally to the production of bacteriocins by the producer strain of *Lb. curvatus* 54M16, even if multiple interactions cannot be excluded (Schillinger et al., 1991). The maximum bacteriocins production, and thus the highest antilisterial activity, occurred after 48 h of incubation, corresponding to the beginning of the stationary phase of growth of *Lb. curvatus* 54M16; this timing was also reported for other bacteriocin producing strains (Huang et al., 2016; Han et al., 2013; Castro et al., 2011; Mirhosseini et al., 2013) against foodborne bacteria. Moreover, antibacterial activity of *Lb. curvatus* 54M16 against *L. monocytogenes* Scott A and OH was higher at 20°C than at 15°C within the first 8 h of co-culture, reaching the same AU mL^{-1} at both tested temperatures **(Table 5.2)**. These results are partially in agreement with those of Urso et al. (2006), who did not observe variation in the quantity of sakacin P produced by a strain of *Lb. sakei* at 25°C, 18°C, 14°C and 10°C. Contradictory results have been reported by Messens et al. (2003), who showed the highest bacteriocin production by *Lb. curvatus* LTH 1174 between 20°C and 27°C.

The bacteriocins activity of *Lb. curvatus* 54M16 against *L. monocytogenes* OH and Scott A was also evaluated during the production of fermented sausage. The strains of *L. monocytogenes* were selected based on the ability to perform strain-level monitoring during the process and their sensitivity to the bacteriocins produced by *Lb. curvatus* 54M16. In this study, sausage meat was inoculated with 10^4 cfu g^{-1} of the mixture of *L. monocytogenes* strains. In the sausages that were not inoculated with the bacteriocin-producing strain (sausage B), the pathogen increased by 2 Log cfu g^{-1} after 28 days of ripening. The initial level of pathogens, along with several other conditions (e.g., pH, salt and carbohydrate content, water activity, level of natural and/or starter competitive microorganisms, temperature and water activity during fermentation and ripening) influenced the ability of *L. monocytogenes* to survive in fermented sausages (Puolanne and Petäjä-Kanninen, 2015). In our study,

the lowest pH value was 5.48 after three days of fermentation at 20°C; it remained almost constant thereafter until the end of the ripening. Lower temperatures (< 20°C) usually result in a higher pH (Puolanne and Petäjä-Kanninen, 2015) suggesting the need to raise fermentation temperatures above 20°C in order to make the pH more efficient at controlling *L. monocytogenes* (Mataragas et al., 2015). Moreover, to avoid possible interference in bacteriocin production and activity (Ravyts et al., 2008) in this study, no nitrate and/or nitrite was added to the meat. However, contamination levels as high as 10^4 cfu g^{-1} of *L. monocytogenes* (used in our study) are not commonly found in industry, as already observed in a previous work (Thévenot et al., 2005). Regardless, we are aware that the preference of *L. monocytogenes* strains originating from a meat production environment and from raw meat products would have provided different results; however, the output would be not predictable and would need to be verified by further investigation.

In this study, *L. monocytogenes* co-inoculated with *Lb. curvatus* 54M16 decreased by 2.69 Log cfu g^{-1} at the end of ripening compared to its concentration in the sausages with *L. monocytogenes* alone. However, the count of *L. monocytogenes* co-inoculated with *Lb. curvatus* 54M16 decreased from 4.22 Log cfu g^{-1} to 3.05 Log cfu g^{-1} within the first 3 days of fermentation, then showed a slight increase until the end of ripening (Table 3). A similar trend was observed in the study by Dicks et al. (2004), who investigated the effectiveness of bacteriocin-producing strains of *Lb. plantarum* and *Lb. curvatus* in ostrich meat salami. The resurgence of *L. monocytogenes* was imputed by these authors to reduce bacteriocin efficacy and/or to develop resistance within the *L. monocytogenes* population. Kingcha et al. (2012) have attributed the increase of *L. monocytogenes* during the fermentation of Nham sausages to a reduction of pediocin activity due to the binding of bacteriocin to food matrix and additives and to the degradation of pediocin by proteases. The resumption of *L. monocytogenes* growth during sausage ripeningwas also observed by Ravyts et al. (2008) in a study on the antilisterial activity of bacteriocin-producing *Lb. sakei* CTC494 in Belgian and Italian sausage types. pH values and bacteriocin inhibition by the ingredients used in sausage production technologies were hypothesized by the authors as the main causes of the different effectiveness of the producer strain. Many other authors have previously documented the causes that may affect both bacteriocin production and activity in food matrices (Verluyten et al., 2003; Dortu et al., 2008; Leroy et al., 2005; Aasen et al., 2003; Zhang et al., 2010).

L. monocytogenes can occur at multiple stages of pork meat processing chain lines (Meloni, 2015). Processing environment, raw meat and ingredients and potential post-processing recontamination of the products represent the main causes of contamination of fermented sausages by *L. monocytogenes* (Thévenot et al., 2006). As reviewed in the literature, *L. monocytogenes* is frequently found in raw meat and in minced meat for sausage, reaching prevalence levels of up to 50% (Meloni, 2015; Thévenot et al., 2006). Despite this high incidence, the natural contamination level of the pathogen in minced pork meat intended for sausage manufacture may be very low and in most cases, it is below 100 cfu g^{-1} (Andritsos et al., 2013; Thévenot et al., 2005; Peccio et al., 2003). In our study the level of *L. monocytogenes* found in naturally contaminated batter was < 1 log cfu g^{-1}. The microorganism was able to survive sausage fermentation, reaching a value of 1.60 Log cfu g^{-1} in the final product, which was compliant with the food safety criterion of ≤ 100 cfu g^{-1} of *L. monocytogenes* reflected in EC Regulation 2073/2005 (European Commission, 2005). However, the absence of *L. monocytogenes* that naturally contaminated meat batter when *Lb. curvatus* 54M16 was added to the sausage highlights the anti-listerial effect of bacteriocins produced by *Lb. curvatus* 54M16 under the tested conditions.

Microbial populations and pH during the ripening of the fermented sausages with and without *Lb. curvatus* 54M16 followed similar trends to those previously described by Casaburi et al. (2016), particularly for CNC and *Enterobacteriaceae,* confirming that the addition of *Lb. curvatus* 54M16 in sausages caused a significant reduction in both bacterial groups during the last stages of the ripening.

During ripening, *L. monocytogenes* OH and Scott A showed lower sensitivity to the bacteriocins; moreover, the OH strain was more sensitive to bacteriocins *sak P,*s*ak X*, *sak* T_α and *sak*T_β than *L. monocytogenes* Scott A that was the most sensitive strain in co-culture experiments.

As shown in a previous study (Vaughan et *al.,* 2003), the production of sakacin P is independent from the production of sakacin T and sakacin X, and their production is affected by the growth media (Vaughan et al., 2004). Our results suggest that the production of the *sak P,* s*ak X*, *sak* T_α and *sak*T_β in sausage samples was probably lower than their production in MRS broth. The increased sensitivity of *L. monocytogenes* OH compared to *L. monocytogenes* Scott A in the sausages could be due to the partial inactivation of sakacins X and T or P or their lower production. In

addition, the two *L. monocytogenes* strains could have different sensitivity to each of the four sakacins.

This research was also focused on the study of the dynamics of bacterial communities during the production of fermented sausages, using 16S rRNA gene sequencing. The addition of *Lb. curvatus* 54M16 led to a remarkable change in the microbiota composition. Inoculated samples were dominated by *Lactobacillus*, whereas un-inoculated sausages showed more diverse microbiota in which spoilage-associated bacteria, such as *Brochothrix*, *Psychrobacter, Pseudomonas* and some *Enterobacteriaceae* were metabolically active until the end of ripening. These microorganisms are well known contaminants of fresh meat (De Filippis et al., 2013; Stellato et al., 2016; Nychas et al., 2008; Dougeraki et al., 2012), where they can cause spoilage through the production of volatile compounds associated with off-flavors (Casaburi et al., 2014; Ercolini et al., 2010; Dogan et al., 2003) and other undesirable metabolites (De Filippis et al., 2013b; Durlu-Özkaya et al., 2001). In the inoculated samples, the ripening was carried almost exclusively by *Lactobacillus*, whereas *Leuconostoc* and a small percentage of *Staphylococcus* persisted in un-inoculated sausages. These genera are commonly found in fermented meat (Comi et al., 2005; Cocolin et al., 2001). Polka et al., (2015) reported high diversity within the *Lactobacillus* genus in PDO Salame Piacentino. In addition to *Lb. sakei* and *Lb. curvatus* that are usually isolated during fermentation, an additional 16 species were identified. The olygotyping of *Lactobacillus* genus carried out in this study enabled the detection of 75 sequence types, 25 of which had an abundance > 2%. Nevertheless, they were identified as belonging to only four species (*Lb. sakei*, *Lb. curvatus*, *Lb. porcinae*, and *Lb. plantarum*). Although the same regions of 16S rRNA were analysed in both studies, Polka et al. (2015) used DNA as template, whereas the use of RNA allowed us to avoid the detection of dead cells. *Lactobacillus* identification at the species level was achieved using the oligotyping technique, which decomposes a given taxon into high-resolution units ("oligotypes") by considering the identified nucleotide positions to be the most information-rich (Eren et al., 2013). This approach enables the detection of ecologically meaningful differences within a single genus in gut and environmental microbiota (De Filippis et al., 2016b; Koskey et al., 2014; Eren et al., 2015), and it was recently used for the evaluation of sub-genus diversity of *Pseudomonas* in food (Stellato et al., 2017). In the current study, different *Lactobacillus* oligotype patterns were found in inoculated

and un-inoculated samples, enabling discriminating between closely related *Lb. curvatus* and *Lb. sakei*. In addition, the partial 16S sequence of *Lb. curvatus* 54M16 was identified at high abundance levels in inoculated samples, supporting the validity of this approach in detecting sub-genus diversity.

5.5 CONCLUSIONS

Bacteriocins-producing *Lb. curvatus* 54M16 rapidly inhibited the growth of *L. monocytogenes* in co-culture. Anti-listerial activity was lower during the production of fermented sausages when the pathogen was inoculated at levels of about 4 Log cfu g^{-1}. However, total inhibition of *L. monocytogenes* native to the raw ingredients was achieved over the course of fermentation.

In conclusion, the use of the bacteriocins-producing *Lb. curvatus* 54M16 in fermented sausages could be an important factor in product safety, provided that intrinsic and extrinsic ecophysiological factors are maintained at levels required for the inhibition of pathogens in controlled conditions. This application requires further in-depth studies to understand the best sausage fermentation conditions for the in situ efficacy of both bacteriocins production and activity.

Notes

This chapter reports the contents of the original research articles "Impact of Lactobacillus curvatus 54M16 on microbiota composition and growth of Listeria monocytogenes in fermented sausages" by Giello, M., La Storia, A., De Filippis, F., Ercolini, D., Villani F. (2018) and published by Elsevier on Food Microbiology 72, 1-15.

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CHAPTER 6

General conclusions and future perspectives

Fermentation is a widespread technological process in culinary traditions all over the world, not only because assures the preservation and the safety of products but also because provides foods with excellent sensory and organoleptic characteristics. Moreover, the growing interest in functional foods has led to involve fermented foods as excellent food matrices by which to design functional products. Fermented foods containing probiotic microorganisms, cover a large slice in the sector of functional foods.

This research thesis was finalized on the study of microbial ecosystems of fermented foods using culture-independent molecular methods and cultural methods, the selection of pro-technological and functional strains and their potential use to design safer and healthier products.

The chapters 2 and 5 are focused on the study of microbial ecosystem of a traditional Italian cheese and of a fermented sausage, respectively but with distinct aims.

The former was aimed to study the effect of bovine diet and of cheese production and ripening on microbial community dynamics, from milk to ripened traditional Caciocavallo of Castelfranco cheese. Hay or silage based diets were used to feed the cows, and then molecular analysis was performed to assess the effect of diet on the microbial community during cheesemaking. Sensory analysis of the cheeses was also performed. It has long been known that the bovine diet can influence the quality of dairy products, but the use of 16S rRNA sequencing to assess the effect of diet on microbial community development in the cheese is novel. Contrary to the significant influence of ripening on cheese microbiota, cows' diet did not affect the microbial communities. However, both cheese ripening and diet affected the sensory characteristics of the cheeses. The future work will be focused on the production of traditional Caciocavallo of Castelfranco cheese by adding a formulation of starter LAB strains and non-starter LAB strains isolated from Caciocavallo cheese and selected for their pro-technological or functional properties as reported in the Chapter 3. The next study will include the screening of non starter LAB (selected in the Chapter 3 for their functional characteristics) to evaluate their proteolytic and lipolytic activities and to use them as both aromatic and functional cultures for

cheese production. Moreover, Caciocavallo cheese produced with adjunct starter and non starter cultures will be studied during cheesemaking to evaluate:
- how adjunct cultures impact on autochthonous microflora and on sensory and organoleptic characteristics of Caciocavallo cheese;
- the ability of adjunct starter LAB to drive the fermentation and dominate the bacterial ecosystem;
- the presence of adjunct non starter LAB during cheese ripening and also their influence on proteolysis, catabolic activity, and volatile components analyzed by specific techniques (volatile component analyses, urea-PAGE, reverse-phase high pressure liquid chromatography, free-amino-acid analyses, gas chromatography-mass spectrometry).

Among the selected non starter LAB isolated from Caciocavallo cheese, the strain *Lb. paracasei* LIL4T named also as *Lb. paracasei* 87 was assayed for properties of probiotic and functional interest. Due to its promising attitude as probiotic strain and for its ability to assimilate the cholesterol in liquid media, *Lb. paracasei* 87 was tested to confirm its cholesterol lowering ability on Caco-2 cells (Chapter 4). In this study *Lb. paracasei* 87 showed to lower cholesterol levels by regulating cholesterol transport and metabolism by reducing cholesterol uptake and cholesterol efflux from the enterocyte to basolateral compartment. Nevertheless, any changes in the expression of key intestinal cholesterol transport-related proteins were observed. These results led to deepen the studies with a proteomic approach to make a quantitative analysis of cholesterol transporters when the enterocytes are incubated with *Lb. paracasei* 87. However, meaningful results on cholesterol efflux and cholesterol uptake in Caco-2 cell line provided a significant basis for the design of *in vivo* studies to assess the cholesterol lowering efficacy by *Lb. paracasei* 87.

The Chapter 5 deals one of the topics of this research thesis that regarded the improvement of food quality and safety by the use of selected bacterial culture. On this purpose, the use of bacteriocin-producing strain of *Lactobacillus curvatus* 54M16 as starter and protective culture in the production of fermented sausages was studied. Specifically, the activity of the strain against *Listeria monocytogenes* Scott A and OH, using *in vitro* mono-culture and co-culture experiments and during the production of fermented sausages was investigated. The inhibition of *Listeria monocytogenes* to undetectable levels under *in vitro* conditions was found.

Furthermore, the addition of *Lb. curvatus* 54M16 to sausages inhibited wild-type *Listeria monocytogenes* strain that was found in tested sausages as natural contaminants.On the other hand, the bacteriocins producer strain did not completely inhibit *Listeria monocytogenes* strains (Scott A and OH) inoculated at 4 Log cfu g^{-1} in the sausages. This work shows promise for use of bacteriocin-producing *Lb. curvatus* in the production of sausages to control or suppress the growth of *Listeria monocytogenes* if present at low levels, during sausage production. However, if contamination levels are higher the antilisterial effect is reduced, and is also dependent on the strain.