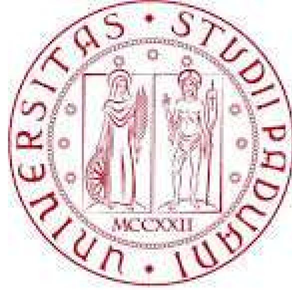

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Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente

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SCIENZE ANIMALI E AGROALIMENTARI

CICLO XXXII

Characterization and Assessment of Health-related Probiotic Properties of Newly Isolated Lactic Acid Bacteria and Study of their Technological Potential by *In-silico*, *In-vitro*, and *In-vivo* Approaches

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Somehow I can't believe that there are any heights that can't be scaled by a man who knows the secrets of making dreams come true. This special secret, it seems to me, can be summarized in four Cs. They are curiosity, confidence, courage, and constancy, and the greatest of all is confidence. When you believe in a thing, believe in it all the way, implicitly and unquestionable.

Walt Disney

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ABSTRACT

Lactic Acid Bacteria (LAB) are the most frequently probiotics used. Within this functional group, *Streptococcus thermophilus* is a thermophilic species widely used as a starter culture for a huge number of dairy products. Besides being rapid acidifiers, many *S. thermophilus* strains can produce and release folate during growth. *S. macedonicus* is another homofermentative lactic acid bacterium (LAB) which was first isolated from a typical Greek cheese obtained by natural fermentation in Macedonia. Some *Streptococcus macedonicus* strains have revealed some interesting properties, such as proteolytic activity, production of bacteriocins against food pathogens, production of exopolysaccharides and tolerance to stress associated with food processing. Lactobacilli also as a member of LAB identified as GRAS and used as a commercial starter in fermented dairy products or as probiotics related to human health. They can play significant roles in different ways such as inhibition of pathogens, anti-cancer activity and different vitamins production in human. Given this point, this thesis has aimed to identify and select some new potential probiotic-technological strains of LAB which recently were isolated from different sources through genomic and physiological studies to be used in human health and food industry. All the strains were evaluated for different technological aspects such as acidification activity and fermentation on different sugars. The genomes of strains were sequenced and applied for *in-silico* analysis to get enough information about their safety and application in technology and human health. On the other side, the interesting strains from technological part were selected to be evaluated for different *in-vitro* probiotic properties such as antibiotic susceptibility, hemolytic activity, resistance to simulated gastrointestinal conditions, bile salts hydrolysis activity, different vitamins production, adhesion to HT-29 human epithelial cell and anti-cancer activity against colorectal cancer cells (HT-29). Based on results from *in-vitro* probiotic characterization, strains *Lactobacillus paracasei* DTA81 and *S. thermophilus* TH982 were chosen for *in-vivo* experiments using the laboratory mice. The results of this study revealed that strain *L. paracasei* DTA81 was found to possess *in-vitro* and *in-vivo* probiotic properties besides lowering the blood cholesterol and Light Density Lipid (LDL) as well as Fasting Blood Sugar (FBS). *Lactobacillus paracasei* DTA81 had already indicated interesting technological traits such as growing on all different sugars including some prebiotics as well.

INTRODUCTION

1.1. Lactic Acid Bacteria (LAB) and Food Technology

The category of Lactic Acid Bacteria or (LAB) was described in early 1900 in order to group all bacteria able to coagulate milk. The earliest production of fermented foods was based on spontaneous fermentation due to the development of the microorganisms naturally present on foods. Later on, the first isolation of lactic acid bacteria from naturally fermented foods to obtain a pure culture was done by Pasteur in 1873 and he began to develop the first starter for the production of cheese. Nowadays, the use LAB associated with food technology has been increasingly evolving to produce a broad range of fermented products from milk, meat, fish and vegetables, not only to obtain a fermented product but also to increase its nutritional values (1). The market for fermented foods has reached an economic value of more than 100 billion Euros a year (2).

Lactic acid bacteria are Gram-positive, non-motile, non-spore-forming, obligate anaerobic or aero-tolerant with low G+C content that ferment sugars according to different metabolic processes, producing mainly or exclusively lactic acid. All the microorganisms belonging to this group are very demanding regarding the nutrients and require complex substrates for growth. LAB were also recently proposed as functional starters. Functional starter cultures are starters that possess functional properties, meaning they contribute to food safety and confer health and nutritional advantages to the consumers (1).

LAB are being applied to produce different types of fermented foods during the last decades and have obtained the GRAS status (Generally Regarded As Safe) by the Food and Drug Administration authority (3). We can classify the LAB in four different categories according to their functional properties: food safety, traits improving product characteristics, properties related to technological aspects and characteristics bringing beneficial influence on human health.

Regarding food protection potential, most LAB show antimicrobial activity by production of organic molecules. Production of organic acids such as lactic acid, acetic acid, formic acid, phenyl lactic acid and so on by LAB has revealed a strong bacteriostatic activity. Besides, the production of specific bacteriocins by LAB against other microbes displayed a significant effect on food preservation (4).

LAB strains also play an important role to enhance the texture of dairy products by producing polysaccharides that can increase the viscosity and firmness of dairy products (5). They can also modify the aroma of the final product in different ways such as by production of lactic acid, lipolytic activities or producing aromatic compounds. On the other hand, homofermentative LAB produce lactic acid through pyruvate. Pyruvate can be used to generate many metabolites like acetate, ethanol, diacetyl, and acetaldehyde that contribute to the typical, pleasant flavor of some fermented products (6). This trait can allow the design of new low-calorie food products avoiding the additional synthetic aromas. For instance, LAB strains activity can produce ethanol by removing highly caloric sugars by fermentation (7). We have seen that bacterial enzymes also can play a significant role in complex food digestion in the human gut (8). They can contribute to reduce the toxic effect of some complex nutrients such as lactose and galactose in fermented milk for people who suffer from lactose and galactose intolerance. They are also able to produce a valuable amount of vitamins in fermented products, especially B-group, that can enhance the value of fermented foods (9).

1.1.1. Taxonomy

Orla-Jensen was the first person who proposed the classification of LAB according to the basis of phenological and morphological characteristics (10). By developing of biochemical and molecular methods after the Second World War, the taxonomy of LAB was changed. Figure 1 indicates the present phylogenetic relationships among LAB by Holzapfel (11).

Currently, the LAB are a heterogeneous group of bacteria sharing some common metabolic properties. The most important feature is their ability to produce lactic acid as the final product of the fermentation. The most important genera constituting the LAB group are *Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Oenococcus, Enterococcus* and *Leuconostoc* (12).

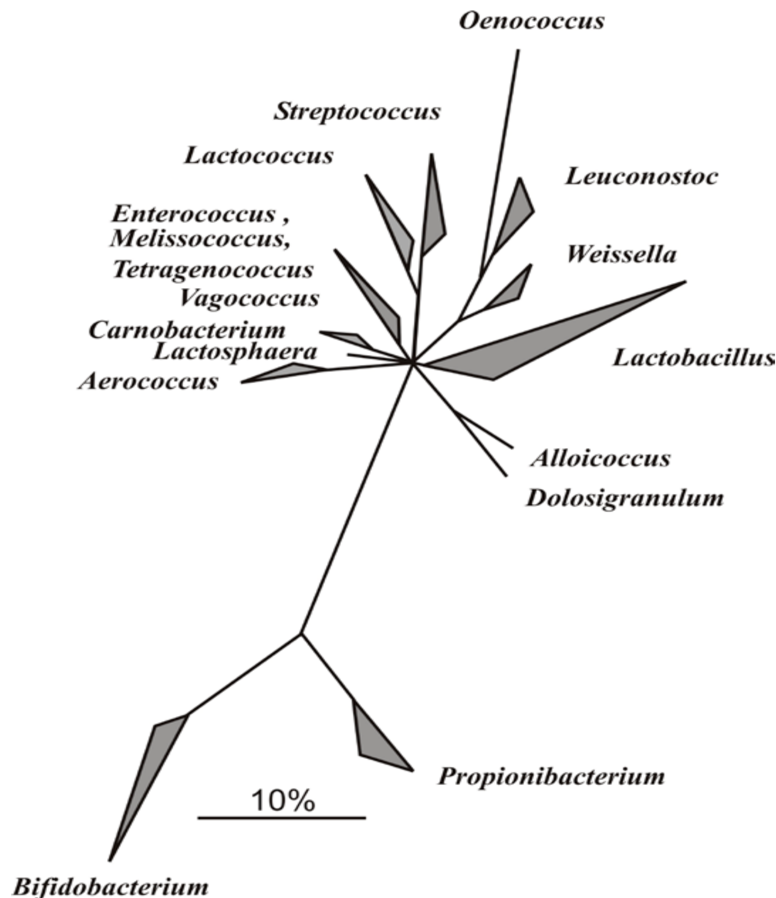

Figure 1: Phylogenetic tree, based on the comparative analysis of the 16S rRNA sequence showing the main phylogenetic groups of LAB with a low percentage of G+C and unrelated gram-positive: Bifidobacterium and Propionibacterium (11).

1.1.2. *Lactobacillus*

The genus *Lactobacillus* consists of more than 180 species and encompasses a broad variety of microorganisms includes a great number of Generally Recognized As Safe (GRAS) species (13). The genus *Lactobacillus* belongs to the phylum Firmicutes, class Bacilli, order Lactobacillales, and family Lactobacillaceae. Members of the genus *Lactobacillus* are gram-positive, rod-shaped, non motile, non-spore forming, anaerobes (strict or aerotolerant) , acid-tolerant, and negative to oxidase and catalase tests. The optimal growth temperature and pH of this genus can vary from 30 – 40 °C and 5.5 – 6.2, respectively. But they are able to grow at a temperature range from 5 to 53° C and at pH range between 3 and 8 (14, 15). This genus, in general, is very demanding regarding the nutrients for growth and these bacteria could be found in a variety of foods such as dairy products, grain products, meat, fish products, beer, wine, fruits and fruit juices, pickled vegetables, mash, sauerkraut, silage, and sourdough. They are

part of the natural microbioma of the human gut and genital tracts as well (16). Due to the high biodiversity present inside the genus, *Lactobacillus* species were classified over the years according to different criteria. The most famous classification is based on metabolism which divides *Lactobacillus* species into 3 different groups:

1) Obligate homofermentative which includes: *L. acidophilus*, *L. delbruesckii, L. helveticus* and *L. salivarius.* This group can ferment and convert almost 85% of hexoses to lactic acid by using the Embden-Meyerhof-Parnas pathway (EMP) or glycolysis. This kind of fermentation is defined by the formation of fructose - 1- 6- diphosphate (FDP), that is split by an FDP aldolase into dehydroxyacetonephosphate (DHAP) and glyceraldehydes-3-phosphate (GAP). The later product then is converted to pyruvate which is then reduced to lactic acid. They usually don't ferment pentoses and gluconate.

2) Facultative heterofermentative, which includes: *L. casei, L. curvatus, L. plantarum* and *L. sakei.* The facultative heterofermentative group also ferments hexoses to lactic acid via EMP pathway, however they produce different metabolites such as acetate, ethanol or formic acid under glucose limitation. Moreover, they are able to ferment pentoses by usin the phosphoketolase. The final product of this kind of fermentation could be ethanol or acetate, besides lactate.

3) Obligate heterofermentative which includes: *L. brevis, L. buchneri, L. fermentum,* and *L. reuteri.* They ferment hexoses exclusively via the phosphogluconate pathway and produce lactate, acetate or ethanol, and CO_2. This fermentation is defined by primary dehydrogenation steps with the formation of 6-phosphogluconate, followed by decarboxylation. The residual pentose-5-phosphate is split via phosphoketolase into GAP and acetyl phosphate. GAP is metabolized in the same principle as for the glycolytic pathway, resulting in lactic acid formation. In case of absence of electron acceptor, acetyl phosphate is reduced to ethanol via acetyl CoA and acetaldehyde.

L. rhamnosus

L. rhamnosus belongs to the facultative heterofermentative group of lactobacilli (17). It is gram-positive, rod-shaped, non-spore forming, anaerobe, acid-tolerant, and negative for oxidase and catalase test. It is a typical colonizer of the human gut, in particular of the gastrointestinal tract.

L. rhamnosus has received great attention among researcher for its beneficial properties to human health. It is normally considered a probiotic species since it possesses many properties such as resistance to gastrointestinal juice, the ability to adhere to the intestinal tract, to inhibit potentially pathogenic species of microbes, helping weight loss in obese women and protecting the colon (18). It was also reported that *L. rhamnosus* can be used against common causes of traveler's diarrhea (18).

L. rhamnosus is considered safe by EFSA for food production and human consumption and it has been involved in the preparation of fermented foods such as meat and milk (19).

L. paracasei

L. paracasei is another important member of the *Lactobacillus* genus that belongs to the facultative heterofermentative group which has the ability to produce both lactic acid and acetic acid. There was a controversial debate regarding the classification of *L. paracasei* in the past whether it is a subspecies of *L. casei* or should be recognized as a separate species. Today *L. paracasei* is considered a separated species that is typical of the human gastrointestinal tract and is also present in many kinds of cheese. Technologically speaking, it grows very well in cheese during ripening and its heat-resistant and remarkable proteolytic activity is well known (20). *L. paracasei* like *L. rhamnosus* is considered safe by EFSA for food production and human consumption (21).

In addition to being a well-known starter for food products, the probiotic potential of this species has also been well studied. Particularity, it was proved that this species has the strong ability to produce branched short chain lipids, which play a beneficial role in the protection of the integrity of the colon epithelium. This means that they have been associated with the inhibition of inflammatory effect and prevent oxidative damage (22). Regarding the physiological aspects, *L. paracasei* grows very well at a temperature range of 10 to 37 °C. It does not appear to grow perfectly at temperatures above 40 °C (23).

1.1.3. *Streptococcus*

The genus *Streptococcus* consists of more than 99 recognized species, many of which are involved in human or animal diseases. The *Streptococcus* genus is positioned inside the phylum Firmicutes, class Bacilli, order Lactobacillales, and family Streptococcaceae.

Streptococci are gram-positive, cocci shaped that tend to form pairs or cell chains of different length. Cells have spherical-ovoid or coccobacillary shape, with a size of less than 2 µm. They are non-spore forming, immobile and negative for catalase and oxidase tests with the exception of *S. didelphis* that shows positive response to the catalase test.

Streptococci require several nutrients for growth. They are able to utilize carbohydrates to synthesize mainly lactic acid without any production of gas. They also produce formate and acetate in the shortage of glucose in the environment. They have been considered as the largest aero-tolerant anaerobes that have the optimal growth temperature of 37°C. However, they usually can grow in a temperature range between 20-42°C.

Among streptococci, *S. thermophilus* and *S. macedonicus* are the only ones adapted to the dairy environments and can normally be isolated from dairy products such as cheese, yogurt and milk (24).

S. thermophilus

S. thermophilus is the most frequently used microorganism in the dairy industry and in particular it is used for the preparation of yogurt in combination with *L. delbrueckii* subsp. *bulgaricus* (18). *S. thermophilus* is a homofermentative, aerotolerant thermophilic bacterium with an optimal growth temperature from 37 to 42°C (25). The taxonomic status of *S. thermophilus* is coming from streptococcal species of the "viridians" group that is divided into five subgroups including *S. mutans*, *S. alginosus, S. sanguinis, S. mitis* and *S. salivarius*. It is phylogenetically close to *S. salivarius* and before being defned as species it was identified as *S. salivarius* subsp. *thermophilus.*

An interesting point about this bacterium is that it prefers disaccharides such as sucrose and lactose to utilize for energy production rather than monosaccharides. Moreover, some strains are able to metabolize galactose which is released from the cell after lactose breakdown (26).

Regarding food production, it is used exclusively in fermented dairy products, particularly for its ability to cidify mik rapidly ad high tempertures (19). Its metabolic activity influences the aromatic characteristics and the texture respectively by the ability to metabolize citrate and production of EPS. It is also responsible for acetaldehyde production in yogurt.

S. thermophilus is also present in the human microbiome and can produce at least five different types of bacteriocins. The production of these molecules is highly affected by environmental

factors especially the presence of salt (26). *S. thermophilus* is also important for folate production. The capability to produce folate is an interesting trait for a potential probiotic strain since it has been demonstrated that consume of folate-producing bacteria can increase plasma folate concentration in humans (27). Folate is an important factor in the human diet, being involved in essential functions of cell metabolism such as DNA replication, repair, and methylation and synthesis of nucleotides. Several studies report that folate deficiency is quite widespread among people, particularly in women. Given its historical use to obtain numerous dairy products such as cheeses and fermented kinds of milk, *S. thermophilus* is recognized as a GRAS organism in the United States and QPS according to EFSA in Europe (26).

S. macedonicus

S. macedonicus is a homofermentative LAB which has been isolated from a typical Greek cheese obtained by natural fermentation in Macedonia, Greece (28). Recently it has been reclassified as *S. gallolyticus* subsp. *macedonicus* (29). *S. macedonicus* is a mesophilic bacterium that can also grow at the temperature around 40 °C. It is a moderately acidophilus and can tolerate pH between 5 and 8 with an optimum pH of 6.

Technologically speaking, *S. macedonicus* is a moderately acidifying bacterium and possesses a weak proteolytic activity. However, it is known for its lipolytic activity and production of exopolysaccharides (EPS) which improve the texture of dairy products. Moreover, it produces a noticeable amount of aromatic components as a result of citrate metabolism that can be considered another technological trait of this species (30).

S. macedonicus strains are able to produce bacteriocins called macedocins. that can inhibit a broad range of bacteria, including *Clostridium tyrobutyricum* and other food spoilagemicroorganisms and pathogens such as *Bacillus cereus* and *Listeria monocytogenes* (31). Interestingly, it was reported that macedocin from *S. macedonicus* can inhibit some other lactic acid bacteria such as *L. sakei* subsp. *sakei* (31).

Overall, although this species is currently under study for its properties to be used in the food industry, the presence of some potentially pathogenic traits such as relation with cases of endocarditis, colorectal cancer, bacteremia, and meningitis still needs to be completely clarified in order to obtain GRAS and QPS status (32).

1.2. Technological Aspects

Lactic acid bacteria live in a broad variety of environments, such as milk, vegetables, cereals and meat products (12).

The nature of LAB makes them strong competitors, both for the ability to acidify food environments, and also for the production of a variety of molecules such as bacteriocins or other antimicrobial molecules that are also applied in the food industry (33). Their metabolic simplicity is a key factor which is mostly related to the need for complex precursors necessary to satisfy their nutritional needs and this is what makes them powerful competitors. The LAB usually require several nutrients, including carbon sources, amino acids, vitamins, nucleic acids and mineral salts to grow.

The main technological aspect which defines LAB is their capability to produce lactic acid by fermenting different sugars. However, it is not the only technological benefit from this group of bacteria. In addition to the production of lactic acid, they have been also applied in food industry for their proteolytic activity, lipolytic activity, ability to synthesize a wide range of compounds such as organic acids, peptides, antimicrobial agents, aromatic compounds by metabolism of citrate and production of exopolysaccharides that improve the texture of food.

1.2.1. Sugars Fermentation and Acidification

LAB are widely used as starters to produce fermented foods,thanks to their lactose fermentation ability that also contributes to milk acidification together with effects on cheese flavor, cheese texture, and cheese safety. The produced acid is required to favour coagulation, promote syneresis that controls the moisture, prevention of pathogenic and spoilage bacteria and so on (34). If we do not provide the essential acidic environment during cheese making by a suitable starter, the final product would be an unpleasant product soft, soapy, fruity and bitter. *S. thermophilus* is considered the second most important commercial species of LAB in the industry after *L. lactis,* with a market value around 40 billion US$ and over 10^{21} areingestedcells by humans annually (26). Indeed, *S. thermophilus* is commonly used as a natural starter for the manufactory of many Italian cheeses such as Fontina, Grana Padano, Mozzarella, Pecorino Toscano, and many other, particularly sodt and semi-hard types of cheese (35). Anyway, the main role of *S. thermophilus* in milk fermentation is to provide rapid acidification (36).

Carbohydrates, in general, are the main and primary energy source for fermentation and acidification of LAB. For the transport within the cell, different systems exist in bacteria. The

main system is the PTS (Phospho Transferase System). All sugars must turn to monomer forms before getting used inside the bacterial cell and for this reason some LAB have developed the ability to produce a glycolytic extracellular activity. By considering this point, a fermentation process can be done by two different paths depending on whether the bacterium is a homofermentative or heterofermentative. The homofermenters lead a fermentation with a production of lactic acid and the heterofermenters conduct a fermentation producing in addition to lactic acid, ethanol, acetic acid, and CO□. Homofermentative LAB such as *Lactococcus, Streptococcus, Pediococcus, Enterococcus* and some species of *Lactobacillus* use glycolysis for the production of pyruvate which is later converted into lactic acid by lactate dehydrogenase (37, 38).

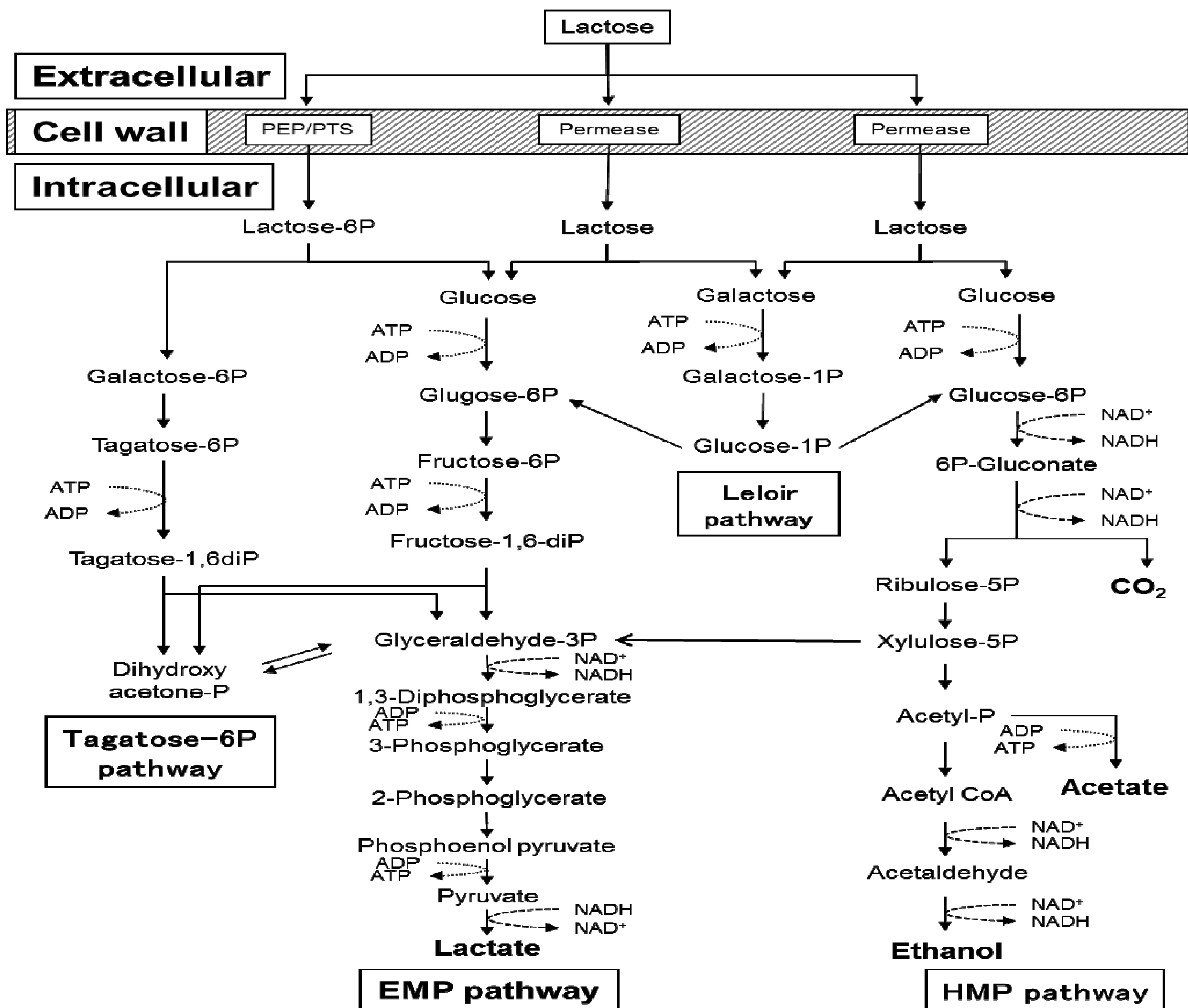

Figure 2: Pathway of lactose fermentation in lactic acid bacteria (39).

On the other hand, there is an alternative pathway when galactose is the main energy source. When galactose is imported into the cell by a specific permease, it would take the Leloir pathway to obtain lactic acid as the final product. In galactose-positive strains, the galactose is metabolized by the Leloir pathway, that includes four different enzymes, namely galactose mutarotase (GalM), galactokinase (GalK), galactose-1-phosphate uridylyltransferase (GalT) and UDP-glucose 4-epimerase (GalE) (40) As it was earlier mentioned, it has been reported by different studies that under special conditions such as limited availability of carbon source, the homolactic metabolism may change into an acid-mixed fermentation with production of formic acid, acetic acid, ethanol and CO_2 in combination with lactic acid (41–44).

The heterofermenters genera such as *Leuconostoc, Oenococcus* and some species of *Lactobacillus* ferment the sugars according to the phosphoketolase pathway (PKP) where the fermentation of the pentoses leads to the final production of lactic acid and acetic acid from pyruvate and acetyl-phosphate respectively. However, hexoses are converted into lactic acid, ethanol, and CO_2 (12) (figure 2).

In general, we homofermenters are most frequently related to foods such as dairy products, meat, and sauces with high salt content, whilst heterofermentatives are more present in fermented vegetable products such as wine, beer, cider, cereals, sourdough and sauerkraut (33).

1.2.2. Exopolysaccharides (EPSs) production

EPSs are produced by plants, algae, fungi, and bacteria (45). Among different EPS producing microbes, LAB have gained particular attention due to their application in the food industry and the related health. They play a key role in food production in different ways such as by controlling viscosity, improving texture, improving mouthfeel, freeze-thaw stability, being used in low calories food products, dietary fibers products and so on (45, 46). On the other hands, they can be useful to human health by providing some beneficial effects such as anti-cancer, anti-ulcer, antioxidant potential, cholesterol-lowering activity, and immune-stimulating properties (47). LAB are used in a huge variety of food products like dairy products, meat products, and vegetables to increase their preservation, sensory characteristics and nutritional value and several others.

EPSs are usually long chains of different repeating units of sugars such as rhamnose, galactose, and glucose (48). Regarding the structure of EPS, they are very different in molecular mass, molecular size, charge, and as a consequence, in their rheological properties. EPSs from LAB are

highly variable polymers that can be classified following their monomer compositions. And grouped into homo-exopolysaccharides (HoPS) or hetero-exopolysaccharides (HePS) (49).

HoPS are mainly produced in species of the *Weissella* genus and have been well described. However, the majority of EPSs produced by LAB are HePS constituted of 3 to 8 repeating units of two or more monosaccharides (51). HePS are produced by a large number of mesophilic and thermophilic LAB. Mesophilic LAB include *L. lactis* subsp. *lactis*, *L. rhamnosus, L. lactis* subsp. *cremoris*, *L. sakei*, and *L. casei*. On the other hand, thermophilic LAB contain *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. helveticus* and *S. thermophilus* (52).

Biosynthesis of EPSs by LAB is a complicated process which requires a large number of enzymes and proteins (Figure 3). Generally, it has been categorized into four different steps initiating with sugar transport into the cytoplasm, synthesis of sugar-1P, polymerization of repeating units precursors and lastly EPSs transport outside the cell. The related genes involving the production of EPSs can be located both on plasmid or on chromosomal DNA (53). Basically, information for EPSs synthesis in mesophilic LAB strains is located on plasmids (53) while in thermophilic *Streptococcus* and *Lactobacillus* strains it located on chromosomal DNA (49, 54). Consequently, EPS production in mesophilic LAB is less stable than in thermophilic strains due to plasmid location of the EPSs gene clusters and presence of mobile insertion sequences (51). Overall, the EPS genes in LAB are mostly found on plasmids rather than on the chromosome (55).

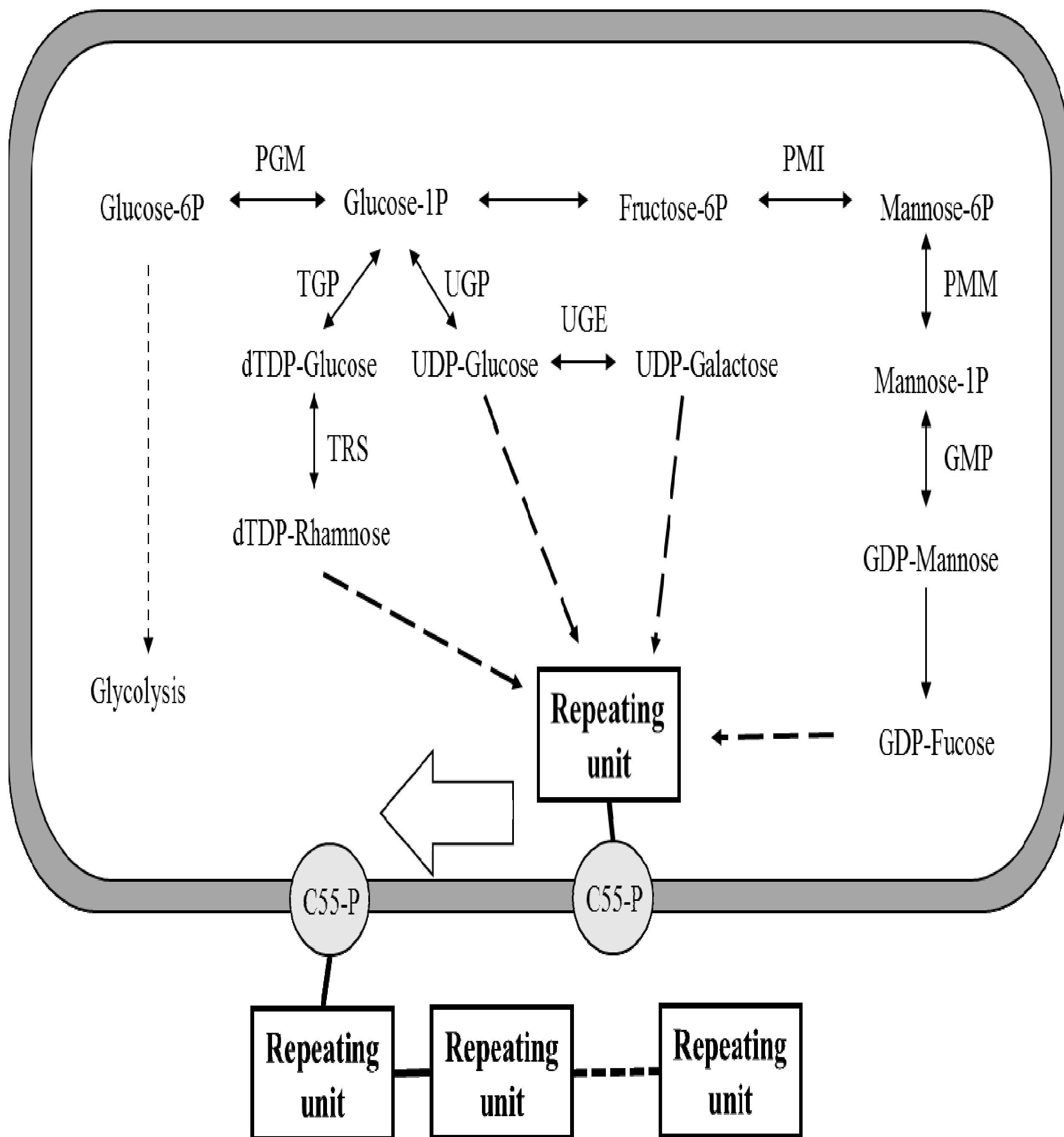

Figure 3: Outline of biosynthesis of hetero exopolysaccharides. PGM: α-phosphoglucomutase, UGP: UDP-glucose pyrophosphorylase, UGE: UDP-galactose 4-epimerase, TGP: dTDP-glucose pyrophosphorylase, TRS: dTDP-rhamnose synthetic enzyme system, PMI: phosphomannoisomerase, PMM: phosphomannomutase, GMP: GDP-mannose pyrophosphorylase (39).

1.2.3. Proteolytic activity

It is well known that LAB are able to able to synthesize a variable number of amino acids depending on species and strain. Therefore, proper growth for most LAB depends on a protein profile of the growth medium that can cover their amino acid requirements. Fortunately, the protein profile of milk and dairy products satisfies the essential needs for growth and that is why LAB are widespread in this kind of product.

Proteolytic activity of LAB is another important technological trait. In cheese manufacturing, the degradation of caseins by lactic acid bacteria plays a crucial role since amino acids resulting from proteolytic activity are precursors of specific flavor compounds such as various alcohols, aldehydes, acids, esters, and sulfur compounds. On the other side, milk proteins could be act as allergens in people suffering from casein intolerance. This kind of problem can be reduced or removed by exploiting the proteolytic activity of LAB during fermentation (56).

LAB can utilize caseins by producing proteolytic enzymes which can degrade them into peptides and later a transport system and a set of peptidases to convert peptides into free amino acids (12).

The first step usually is done by Cell-Envelope Proteinases (CEP) enzymes. Five different types of these enzymes have been observed in LAB, namely PrtP from *L. lactis* and *L. paracasei*, PrtH from *L. helveticus*, PrtR from *L. rhamnosus*, PrtS from *S. thermophilus*, and PrtB from *L. bulgaricus* The genes related to these enzymes can be both plasmid-encoded, like PrtP from *L. lactis,* or genome-encoded, like PrtR from *L. rhamnosus* (57–61).

The transportation of peptides into the cell is obtained by using the Opp transporter system that belongs to the ATP-binding cassettes that mediate the uptake of peptides into the cell (62). Soon afterintroduction, peptides are degraded by different peptidase such as endopeptidases, aminopeptidases, and the X-prolyl dipeptidyl aminopeptidase that are the first enzymes to hydrolyze the oligopeptides (63).

1.2.4. Lipolytic Activity

The use of lipases in the food industry can have different effects, e.g. on texture and flavor. The first studies about the lipolytic activity of LAB had revealed a weak production of this kind of enzymes (64). However, it was later reported that some LAB such as *L. plantarum* are able to produce large amounts of lipases that can be applied in many different industries such as fermented meat products for the development of flavor and aroma (65, 66). In general, LAB

strains play a significant role in maturation in fermented meat products by producing various extracellular lipolytic enzymes.

Lipase enzymes for technological uses have been isolated from different species of plants, animals, and microbes (46). However, microbial enzymes, especially from bacteria and fungi are the most common lipases for the industry, and easy cultivation of microbes and easy extraction procedures (67). Microbial lipases have also shown wide diversity regarding their enzymatic properties and substrate specificity which make them very interesting for the industry (46).

1.2.5. Citrate metabolism

Citrate metabolism by LAB is another technological trait that can have either negative or positive effects. Citrate exists in many fermented food products such as those from milk, vegetables, and fruit and it is used as additive in the production of fermented sausages as well. Among bacteria, citrate metabolism has been deeply addressed in LAB due to its link with aroma production. Diacetyl is one of the most well-known 4-carbon (C4) compounds from citrate metabolism for its buttery aroma. However, this molecule is detrimental in products such as wine and beer.

Citrate metabolism also gives a great opportunity to citrate positive microorganisms to use this carbon source for their growth. Basically, LAB ferment the citrate under strict anaerobic condition (68). By consuming the lactose during cheese production, citrate could be a carbon source usable by the bacteria to support their growth during cheese ripening (47). Generally, citrate metabolism under acidic conditions releases 4-carbon (C4) compounds, namely diacetyl, acetoin, and 2,3-butanediol. Among these C4 compounds, diacetyl and acetoin are known to confer nutty and buttery aromatic notes. LAB such as *L. lactis* subsp. *lactis, L. plantarum, O. oeni* some *Leuconostoc* and some *Enterococcus* are known to produce diacetyl and acetoin (46).

1.3. Probiotics

The word "probiotic" means "for life" which is coming from the Latin ord "pro" and the Greek word "bios". It is generally associated with microorganisms that have beneficial effects on human and animals. At the beginning of the 20th century, the exact role of gut natural flora was unknown. Eli Metchnikoff, a Russian scientist winner of the Nobel Prize, was the first scientist that observed an amazing connection between health and consumption of yogurt produced by fermentative activity of *Lactobacillus* strains. The rising interest of health care, the constant increase in life expectancy and the steady desire to improve the quality of life have been the

encouraging factors for research and improvement in the area of functional foods. Despite the concept of functional foods has been suggested long time ago by Hippocrates and his motto "Let food be your medicine", quite recently the researchers with the evidence achieved started to consider the hypothesis that diet plays a fundamental role in the modulation of important body functions in humans and animals. Bioactive components from fermented foods and prebiotics molecules represent the central pillar, due to their long-standing tradition of safe use and the supposed positive effects. The fermentation of dairy products represents one of the oldest methods to enhance food preservation. The roots of fermented milks can be found before the Phoenician era in the Middle East. Metchnikoff was the first scientist that published a text, “The Prolongation of Life” regarding the conceivable positive effects of bacteria found in fermented milk on human health (69). On the other side, Henry Tissier, a French scientist, discovered bifidobacteria in the feces of breast-fed infants. He also found a significant connection between bacteria present in stools and health condition of children. The stools of children affected by diarrhea had a lower number of specific bacteria (Y shaped morphology) than the stools of healthy children (70). However, Lilly and Stillwell in 1965 used for the first time the word “probiotic”. They called as “probiotics” the substances secreted by one microorganism which stimulated the growth of another. Finally, in 1974, Parker defined probiotics as “organisms and substances which contribute together to intestinal microbial balance” which has been the closest definition to that even by the WHO. In 2001, an expert panel commissioned by Food and Agriculture Organization (FAO) and the World Health Organization (WHO) defined probiotics as “live microorganisms, which, when administered in adequate amounts confer a health benefit on the host”. Nowadays, this definition is widely adopted. However, some part of this definition has been a controversial matter like the phrase of "adequate amounts" which has been the unsolved question. In fact, the level of microorganism that confers a positive effect on humans depends on the strain and the specific health benefit. In Italy, the Ministry of Health regulated the use of the term probiotic for food and food supplements to 10^9 cells as a minimum number of viable bacteria which should be administered per day.

1.3.1. Bacterial species used as probiotics

According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) definition, probiotics can be consisting of any live microbes. However, among all the microbes, bacteria are the most studied and characterized probiotics, and lactic acid bacteria

(LAB) are the most frequently used, mainly species belonging to the *Lactobacillus* and *Bifidobacterium* genera (Table 1).

Besides *Lactobacillus* and *Bifidobacterium*, other LAB also have been used in dairy-based products. They mostly belong to *Enterococcus*, *Lactococcus, Streptococcus* and *Pediococcus* genera. On the other side, the use of yeasts in the commercial probiotic is not common. However, *Saccharomyces cerevisiae* var. *boulardii* is the only one that is used as probiotic (71–73).

Table 1: The most used probiotic species are listed.

Lactobacillus spp.	*Bifidobacterium* spp.	Other spp.
L. acidophilus	*B. bifidum*	*E. coli*
L. casei	*B. breve*	*S. boulardii*
L. crispatus	*B. infantis*	*S. thermophilus*
L. delbrueckii subsp. bulgaricus	*B. longum*	*E. francium*
L. fermentum	*B. lactis*	*Propionibacterium*
L. gasseri	*B. animalis*	*Pediococcus*
L. johnsonii	*B. adolescentis*	*Leuconostoc*
L. paracasei	*B. essensis*	
L. reuteri	*B. laterosporus*	
L. rhamnosus		
L. helveticus		
L. lactis		
L. sporogens		

1.3.2. Safety Aspects

In order to evaluate the probiotic properties of the strains, the experts from FAO, WHO and European Food Safety Authority (EFSA) have established a specific guideline. According to this guideline, the new strains that are supposed to be used as probiotic in the food industry, after receiving the safe status, should be capable of surviving passage through the gastrointestinal tract and also have the ability to adhere and proliferate inside the human gut. This means they must be

resistant to gastric juices and be able to grow in the presence of bile and above all, they have to confer some health benefits to humans (74). Actually, a broad variety of microorganisms or their enzymes have been used to enhance technical aspects such as flavor, taste, texture, and shelf-life or to give beneficial effects on human health. Therefore, EFSA has also established an assessment method with the aim of producing safe products in the food industry. The safety aspects of microbes proposed as probiotics has always been a controversial issue of regulatory authorities. The European Community issued a proper legislation containing specific criteria such as their classification, antibiotic resistance and blood hemolytic activity to be evaluated before using these strains commercially (75).

1.3.3. Classification and Taxonomy of Strains

The safety aspects of microbes proposed as probiotics has always been an important issue for regulatory authorities. Therefore, it has been strongly recommended in Europe to select probiotics strains among the microbes that are already in the Qualified Presumption of Safety (QPS) group introduced by EFSA in 2007. The QPS is an evaluation of safety based on reasonable evidence. If the evaluation of a microorganism concludes that it does not raise any safety concerns, that microorganism will be granted the "QPS status". All the microorganisms belong to the QPS list do not need to undergo the full safety assessment and they need to undergo limited safety examinations, such as antibiotic resistance, blood hemolytic activity and biogenic amine production. On the other side, microorganisms that do not belong to the QPS list must undergo a full safety assessment.

In order to grant the QPS status to a specific microorganism, it has to meet the following criteria:

1) The taxonomy of strain should be clearly determined.

2) There should be enough knowledge regarding any scientific literature, history of use, industrial applications, ecological data, and clinical data to establish its safety.

3) The absence of pathogenic properties must be demonstrated. Also, must be evaluated the production of enterotoxins, cytotoxins or the capability to invade epithelial cells.

4) The purpose of use must be described very well.

Since probiotic properties are strain depended, it has been recommended that identification be performed by phenotypic tests followed by genetic identification, using different methods such as DNA/DNA hybridization or 16S RNA sequencing to be sure about the taxonomy of the related strains. Figure 4 shows the scheme for evaluation of QPS status according to EFSA (76).

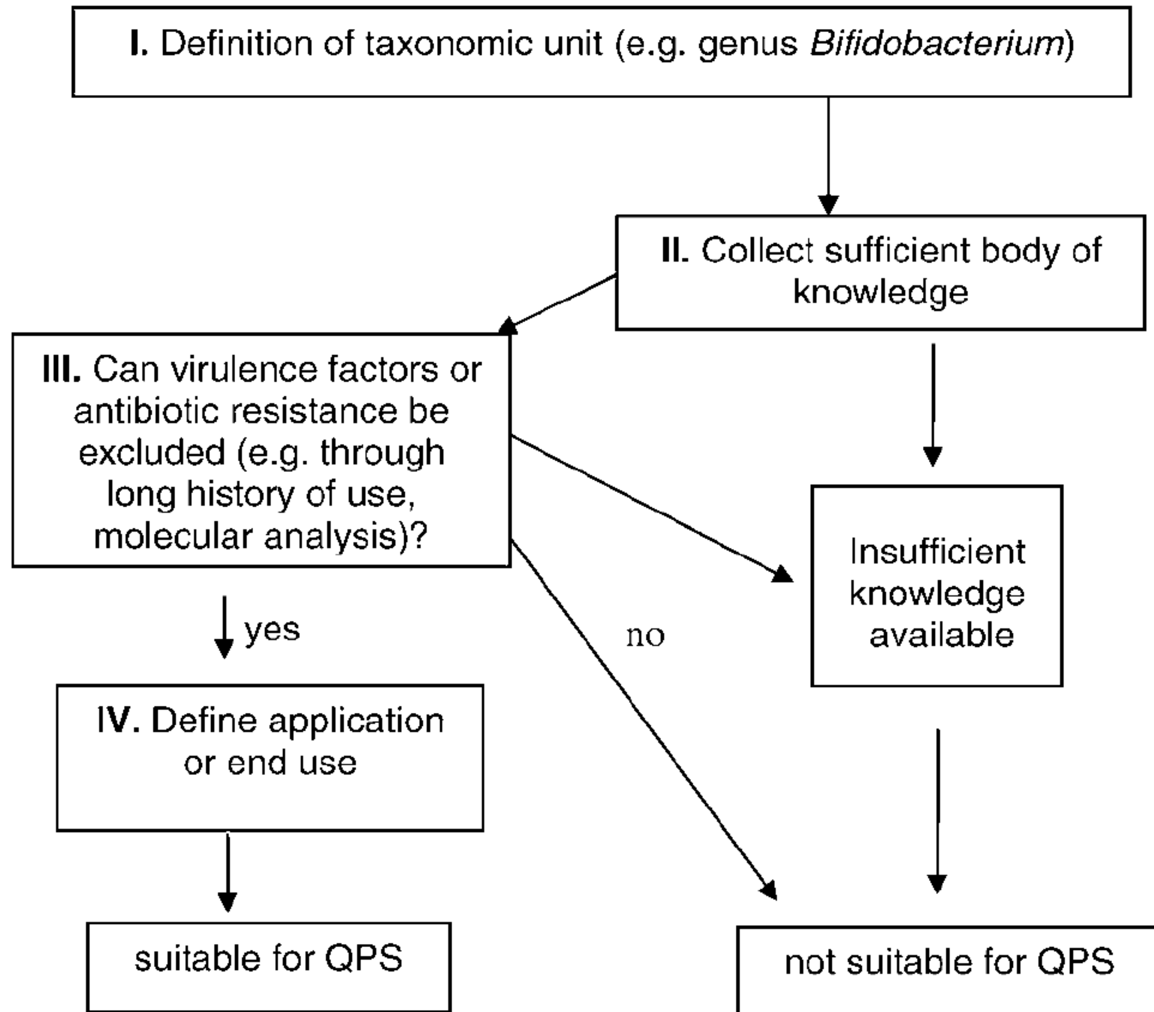

Figure 4: Decision tree for QPS status determination according to EFSA (76).

1.3.4. Antibiotic Resistance

Antibiotic resistance is the most important safety issue that needs be considered during the assessment of new strains even when you work with QPS bacteria, since this characteristic is strain dependent. Therefore, it has been strongly recommended to perform the antibiotic susceptibility examination in order to be sure that the proposed probiotic bacteria do not carry any transmissible antibiotic resistance genes.

Antibiotic resistance is present in some lactic acid bacteria, including probiotic ones and this resistance could be related to chromosomal, transposon or plasmid located genes (77).

In order to diagnosis the antibiotic resistance, the conventional methods are based on the evaluation of bacterial growth when they are exposed to the antimicrobial agents. The mechanisms of antibiotic resistance can be categorized into four different groups (78).

1) Reduction of the cell wall permeability that limits or prevents the entrance of the antibiotics.

2) Efflux mechanism that exists among some bacteria and can release the antibiotics from the cell before they reach an effective concentration.

3) Enzymatic deactivation mechanism that some bacteria use to denature or change the structure of the antibiotics.

4) Modification of the target mechanism that helps the target site retaining its function in the cell without allowing the antibiotics to bind to it.

Among all 4 different mechanisms, the decrease of the cell wall permeability and the modification of the target site are generally considered as intrinsic or natural resistance.

However, bacteria can acquire resistance by gaining exogenous DNA or by mutation of indigenous genes (79). The acquired resistance can be transferred easily when the responsible genes that code for the resistance are on mobile genetic elements, such as plasmids and transposons elements. In other words, when the specific antibiotic resistance is seen among all the strains of specific species, we usually considered this resistance as ‘intrinsic resistance' or natural resistance. Contrary, when a strain of a typically sensitive species is resistant to a specific antibiotic, it is generally referred to ‘acquired resistance’. This acquired antibiotic resistance can happen due to the added transmissible genes from other bacteria or the mutation of indigenous genes (80). Figure 5 provides a scheme for antimicrobial resistance assessment of bacterial strains (81). The Minimum Inhibitory Concentration (MICs) which is mentioned in figure 5 defines the lowest concentration of an antibiotic that prevents the visible growth of a bacterium. The cut-off values are set for the same species or genus (79).

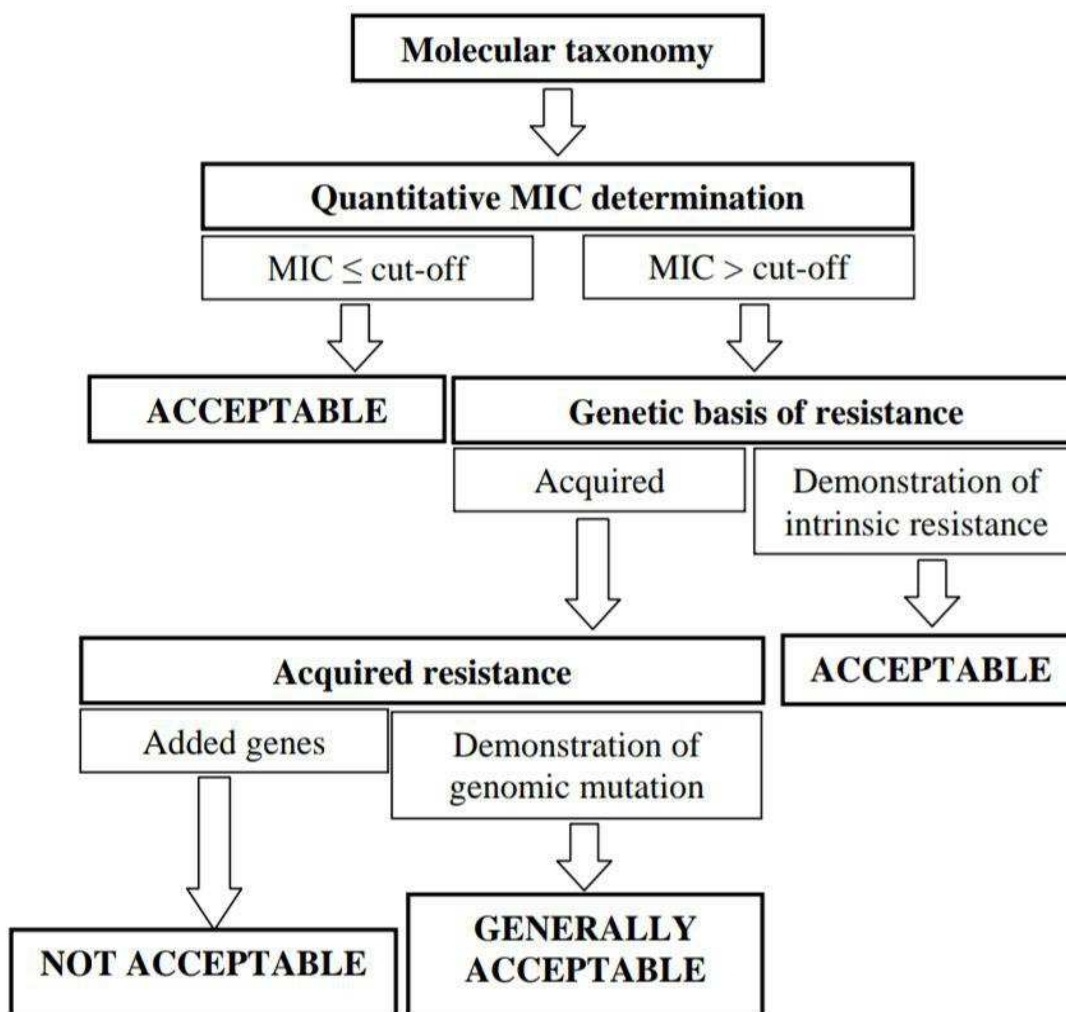

Figure 5: Proposed scheme for antimicrobial resistance assessment of the bacterial strains used as a feed or food additive (81).

1.3.5. Blood Hemolytic Activity

The lack of beta-hemolytic activity is clearly one of the most important safety aspects to be considered for a food-grade strain. Indeed, *in-vitro* assessment of hemolytic activity on blood agar medium even for bacterial species that are considered safe is very important. Literally, hemolytic activity refers to the breakdown of the membrane of erythrocytes by a specific bacterial protein, named hemolysin. The hemolysin can integrate into the erythrocytes membrane and produce a hole throughout the membrane. There are three different types of hemolytic activity in bacteria, namely alpha, beta, and gamma. Alpha hemolytic activity, which is known as partial decomposition of the hemoglobin, refers to hydrogen peroxide production by the

bacterium that causes hemoglobin oxidation. In the alpha hemolysis group, the membrane of the erythrocytes is left intact. The second group is the beta-hemolytic activity that is known as a complete decomposition of the hemoglobin of the erythrocytes by hemolysin action. Several human pathogens posses alfa or beta hemolysins. The third type, that has no hemolytic activity, is named gamma hemolysis. The LAB are a large group of bacteria, that show either alpha or gamma hemolytic ativity.

1.3.6. Biogenic Amines Production

Biogenic amines are produced by conversion of amino acids into amines by some bacteria in many foods, especially in fermented ones such as cheeses, wines, and beer. Although a low amount of biogenic amines can be tolerated by humans, the ingestion of high levels of these molecules, specifically histamine and tyramine, can provoke food intoxication (82). LAB are the main biogenic amines producers in fermented foods. *O. oeni*, *L. hilgardii* and *P. parvulus* as examples of species responsible for histamine accumulation in wine products. Tyramine, which is another important biogenic amine, is the most abundant detected in cheese and fermented meat products produced mainly by LAB strains of *Enterococcus* and *Lactobacillus*. On the other side, *E. faecalis, E. faecium* and *E. durans* are considered very strong tyramine producers. Some other LAB such as *L. brevis* and *L. hilgardii* from wine, *L. fermentum* and *L. paracasei* from cheese, meat and sausage have shown to be able to produce putrescine as well (83, 84).

Among all, histamine and tyramine were the most frequent biogenic amines produced by LAB. For this reason, histamine and tyramine production by a food-grade or probiotic strains should be low or absent. Biogenic amines usually are considered heat stable molecules, therefore difficult to inactivate by thermail treatments. For this reason, biogenic amines formation should be avoided by the use of good hygiene in raw and processed food (85). Biogenic amines can be categorized as heterocyclic, like histamine and tryptamine, aliphatic like putrescine, cadaverine, spermine, and spermidine or aromatic such as tyramine and phenylethylamine, according to their chemical structure; they can also be divided into monoamines (tyramine and phenylethylamine) and diamines (putrescine and cadaverine) by their number of amine groups (86).

The normal way for biogenic amines production is the decarboxylation of free amino acids by decarboxylases produced by bacteria. By removing the alpha carboxyl group from the specific amino acid, we can produce its related biogenic amines. The names of many biogenic amines are

given based on the names of their originating amino acids. As an example, the decarboxylation of tyrosine yields tyramine or histidine produces histamine (87).

1.3.7. Functional Aspects

Human Gastro-Intestinal Tract (GIT) and Related Bacteria

Microbiologically speaking, the human gut can be divided into three major parts, namely stomach, intestine (small intestine) and colon (large intestine). The gut microbiota is a broad collection of microbial species that are normal inhabitants of the human GIT. The stomach has very few numbers of bacteria due to the extremely acidic conditions.

The intestinal microbiota contains bacteria which belong to different phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria. This distribution can vary from adult to infant. In adults, the dominant Phylum is Firmicutes whilst in the infants the most frequent is the Actinobacteria, especially the genus of *Bifidobacterium* (88). In the human GIT according to different physical and chemical conditions, three distinct categories of bacterial species can be observed, including:

1) Indigenous microflora present in large numbers, such as *Bacteroides* and *Bifidobacterium*.

2) Indigenous microflora present in moderate or small number, like Enterobacteriaceae, *Streptococcus,* and *Lactobacillus*.

3) Ephemeral microflora which is normally in few numbers and comes from other parts of the body (e.g. *Staphylococcus*) or from the environment (e.g. *Bacillus*, *Corynebacterium*).

Physical and chemical conditions such as pH, Eh, and secretions like pancreatic enzymes play the main role in the selection of the gut microbiota.

The lumen of the human stomach has the lowest number of bacteria due to the low pH. However, there are some bacteria that reside in the mucosal layer that overlies the gastric epithelium. *H. pylori* resists to the gastric conditions due to ammonia production. This Gram-negative bacterium uses its flagella to move through the gastric mucus layer and attach to epithelial cells. By these mechanisms *H. pylori* can easily colonize the stomach (89).

Figure 6 indicates the microbial density of human Gastro-intestinal tract from the stomach (10^2 cells/ml) to the distal ileum (10^8 cells/ml) to the colon (10^{10}-10^{11} cells/ml) respectively. The *Lactobacillus* species, together with *Streptococcus* species dominate the upper part of the small intestine among LAB (90).

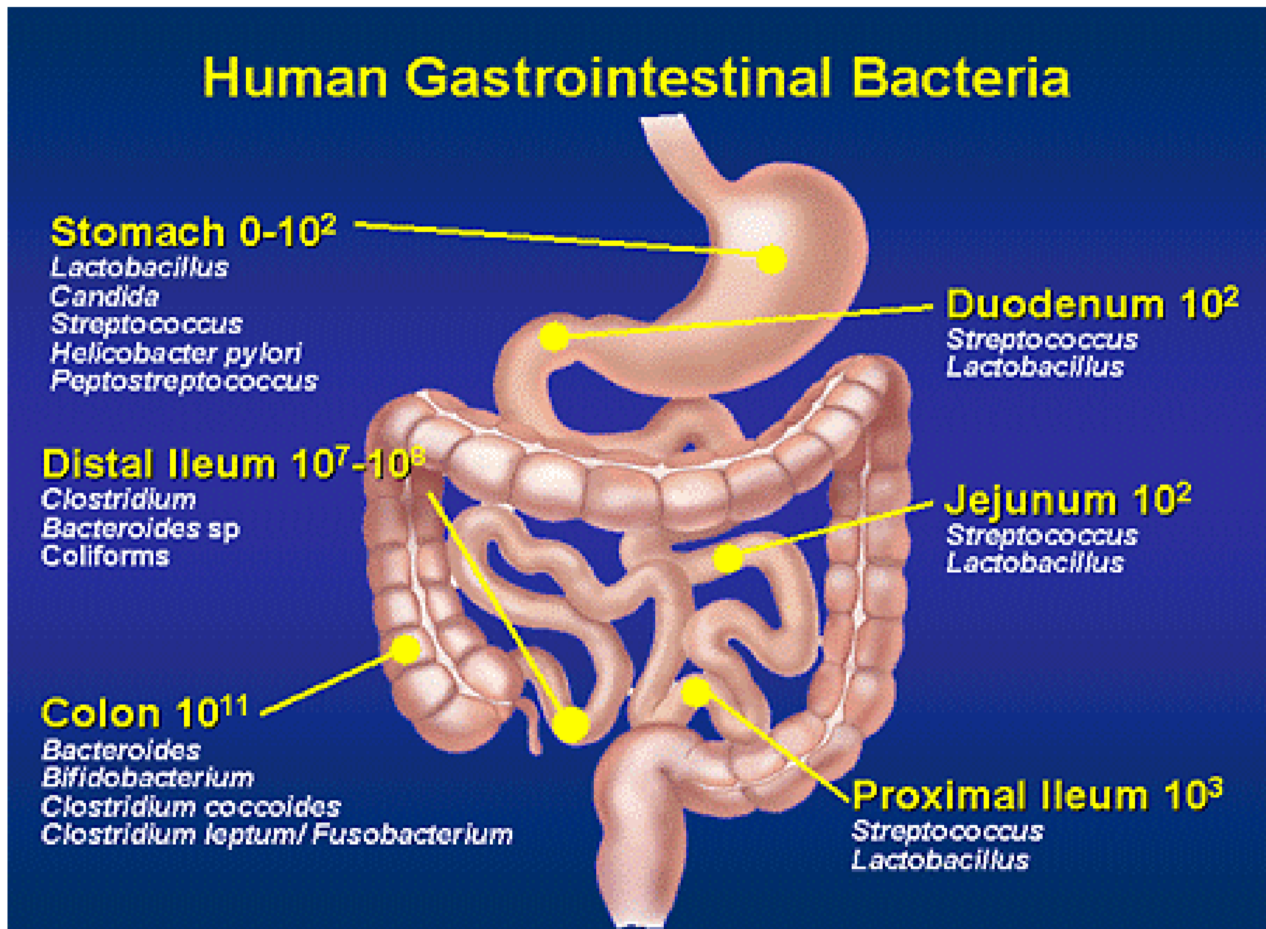

Figure 6: Scheme of the human GI - tract microbiota

The gut microbiota can play a significant role by fermenting and utilizing many disaccharides and polysaccharides such as starches as well as dietary fibers like pectins and xylans that are indigestible for humans due to lack of specific enzymes (91). Moreover, many studies demonstrated that the gut microbiota can mediate the immune response and protect the host from inflammatory diseases in different ways, e.g. by inducing IgA production (92–94) and maintain the homeostasis of different T cell such as T (Treg), T helper 1 (TH1), and 17 (TH17) cells (95). On the other side, gut microbes can possess some metabolic functions such as synthesis of vitamins, lactate, ethanol, succinate, valerate, caproate, isobutyrate, 2-methyl butyrate, and isovalerate that act on the intestinal epithelium (96); for instance butyrate represents the preferred energy source for epithelial cells (97).

Nowadays, it is well known that the *Streptococcus* and *Lactobacillus* species occupy different parts of the human GIT. Table 2 indicates species of LAB most frequently found in different part of the human GIT (98-110).

Table 2: Distribution of *Streptococcus* and *Lactobacillus* species in different parts of the human GIT

Oral cavity	Stomach	Small intestine	Feces	Colon epithelial biopsies
L. paracasei	*L. gasseri*	*L. gasseri*	*L. gasseri*	*L. plantarum*
L. fermentum	*L. ruminis*	*L. reuteri*	*L. paracasei*	*L. rhamnosus*
L. rhamnosus	*L. reuteri*	*L. rhamnosus*	*L. ruminis*	*L. paracasei*
L. plantarum	*S. thermophilus*	*S. thermophilus*	*L. reuteri*	
L. gasseri			*L. plantarum*	
S. thermophilus			*L. salivarius*	
			L. sakei	

Regarding the presence of *Streptococcus* species in the human GIT, only few studies are available (98, 99). They all suggest that in the small intestine microbiota of healthy humans, *Streptococcus* should be present in a large number. Other studies also reported that *Streptococcus* species are present not only in the small intestine but also in the microbiota of the stomach, esophagus, throat, and oral cavity (100, 101).

As regards *Lactobacillus* species, it has been reported as one of the dominant organisms in the human gut (102). However, after improvement of anaerobic culturivation techniques, lactobacilli were also reported in very few numbers in human fecal microbiota. Most studies report averages of around 10^6 CFU/gr of *Lactobacillus* spp. (103–105).

Generally, *Lactobacillus* strains are the dominating genus in infants delivered vaginally in comparison with infants delivered by caesarean (106, 107). This indicates that *Lactobacillus* strains are a major component of the urogenital ecosystem of healthy women (108, 109).

Among *Lactobacillus* species, *L. rhamnosus*, *L. paracasei*, *L. fermentum*, *L. gasseri* and, at lower frequencies, *L. plantarum*, *L. delbrueckii,* and *L. reuteri* are the most frequently seen bacteria in fecal samples of infants during the first year of life (110).

On the other hand, *L. rhamnosus, L. paracasei,* and *L. fermentum* have been isolated from saliva (111) while *L. gasseri, L. reuteri,* and *L. ruminis* were found in the gastric mucosa (112). Regarding the small intestine, *Lactobacillus* was found to be the dominant bacterial genus in the upper part of the small intestine (113, 114). (115).

Gastro-intestinal Juice Tolerance

Generally, probiotics should be resistant to human gastrointestinal condition. They have to show a good resistance to enzymes in the oral cavity and stomach, low pH (around 1.5 to 3), and to bile in the intestinal tract. Regarding low pH, it has been demonstrated that F_0F_1-ATPase in Gram-positive bacteria confers the essential resistance to acidic condition (116). The F_0F_1-ATPase is a multiple-subunit enzyme containing a catalytic portion which is called F_1 assimilating the α, β, γ, δ, and ε subunits for ATP hydrolysis and an integral membrane subunit called F_0 containing a, b, and c subunits, which functions as a membranous channel for proton translocation (117). On the other side, the liver is the organ in which the bile acids are synthesized from cholesterol and are sent to the gallbladder and secreted, in the conjugated form, into the duodenum during the digestion. Bile salts can affect probiotic cell membrane functionality because of its detergent-like function that breaks lipids and fatty acids (118). Bile-Salt Hydrolases (BSHs) activity of probiotic bacteria has also been a controversial issue. Some probiotic bacteria have the ability to deconjugate the bile-salt secreted from the liver (119). However, this can be considered as a negative effect. Although BSH is somehow related to intestinal survival of probiotics and cholesterol lowering in the human host, however, it cannot be considered as a desirable property for probiotics, since de-conjugated bile salts could have many undesirable effects for the human host (120).

Adherence Ability

Adherence ability of probiotic bacteria to intestinal epithelium is considered another important characteristic. Probiotic bacteria can attach to the human epithelium by producing some components such as adhesins, polysaccharides, and proteins which play a major role in the adherence process (121).

A mucus layer in the human intestine contains glycolipids, mucins, and large and highly glycosylated proteins which covers the epithelial cells. This combination of proteins and mucine usually protects against undesirable colonization by pathogens. Generally, bacteria that try to

attach to mucus are rapidly removed. Even so, LAB species are common inhabitants of the mucus (122, 123).

In LAB species there are different mechanisms of adhesion:

- Mucus-binding proteins: are extracellular proteins reported in *L. reuteri* 1063 (Mub) (124), *L. acidophilus* NCFM (125), and *L. plantarum* WCFS1 (Msa) (126).
- Surface layer proteins (S-layers): represent 10-15% of the total proteins of the cell wall of *Lactobacillus* species and mediate adhesion to intestinal epithelial cells (125, 127–129). Some examples are , CbsA in *L. crispatus* JCM 5810 (130, 131), Slp of *L. helveticus* R0052 (127), SlpA in *L. brevis* ATCC 8287 (129, 132), and SlpA in *L. acidophilus* NCFM (125).

Peculiar proteins of *L. johnsonii* also have reported some mediating adhesion activity. It was reported in 2006 by Granato and colleagues that *L. johnsonii* La1 NCC 533 posesses the elongation factor Tu (EF-Tu), a guanosine nucleotide-binding protein involved in protein synthesis in the cytoplasm. The EF-Tu of *Lactobacillus johnsonii* La1 is localized at the bacterial surface and probably mediates the adhesion to human intestinal cells and mucins. Moreover, *L. johnsonii* La1 possesses the heat shock protein GroEL on its surface which normally works as a mediator of protein folding but it can also promote the adhesion to the GIT (133).

In *S. thermophilus* it has been reported that the presence of lactose can enhance the fermentative activity and lead to a higher level of luminal lactate which subsequently works to modulate the host epithelium. Therefore, activation of enzymes involved in sugars metabolism constitutes the metabolic signature of *S. thermophilus* in the intestine and favors the interaction with the colon epithelium (134).

Non-protein factors can also play a role in the adhesion ability. Other surface factors such as Lipoteichoic acids (LTAs) and EPS promote LAB adhesion ability. Lipoteichoic acids are negatively charged due to the presence of polyol phosphate polymers. They possess a glycolipid anchored in the membrane and a polyglycerophosphate chain extended into the cell wall (135). On the other side, exopolysaccharides are long-chain polysaccharides consisting of branched, repeating units of sugars that loosely attach to the cell surface or are secreted into the environment. As an example, *L. acidophilus* CRL639 has shown the production of different types of exopolysaccharides which modulate its adhesion ability (136).

Antimicrobial Activity

The antimicrobial activity of LAB is due to the production of some substances such as hydrogen peroxide, organic acids, carbon dioxide, reuterin and reutericyclin, and bacteriocins (137).

Organic acids are considered the most common antimicrobial compounds produced by the LAB. Among them, lactic and acetic acid are the most important ones. They penetrate into the cytosol and affect the metabolic functions by reducing the internal pH and dissipating the membrane potential (138, 139).

Reuterin and reutericyclin produced by *L. reuteri* have shown a strong antimicrobial activity. Reutericyclin is a tetrameric acid derivative and reuterin is a mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of β-hydroxypropionaldehyde. They revealed a strong inhibitory activity in particular against Gram-negative bacteria, fungi, and protozoa (140).

Hydrogen peroxide produced by LAB also can play a significant role in antimicrobial activity. The oxidizing effect of hydrogen peroxide is very strong and can affect bacterial cells and cell proteins (141).

Carbon dioxide produced by LAB also provides an anaerobic environment that can stop the growth of obligatory aerobic cells. It can also inhibit the enzymatic decarboxylations of fungi and affect membrane permeability (141).

Bacteriocins are the most important and effective compounds produced by LAB that can inhibit bacterial cells. They are antibacterial proteinaceous substances. They can be categorized into two general different types according to their spectrum: Bacteriocins with the narrow spectrum (active against specific species) and bacteriocins with broad spectrum (active across genera). They mostly inhibit low G+C Gram-positive species (142). Bacteriocins are classified into 3 classes:

Class I: Lantibiotics.

Class II: Small heat-stable bacteriocins which are divided into four subclasses; class IIa: *Listeria*-active bacteriocins; class IIb: two-peptide complexes; class IIc: the *sec*-dependent bacteriocins; class IId: unclassified small heat-stable non-lanthionine bacteriocins.

Class III: Large heat-labile bacteriocins.

Bacteriocins can play an important role regarding antimicrobial activity by inhibiting pathogens acting as "killing peptides" (143), informing other bacteria through quorum sensing and signaling the immune system of host by their "signaling peptides" (144–147).

1.3.8. Health benefits

Probiotics can be considered for their health benefits in the human body. They can be used in the prevention or treatment of several human disorders. Health benefits of probiotics can be seen locally, in the gastrointestinal tract, or systemically, throughout the body.

Prevention of diarrhea

Diarrhea has always been a major health problem worldwide. Overall, we have three different types of diarrhea that not only affect endemically people from developing countries, but it is also a serious problem in developed countries.

The first type is related to microbial pathogens: rotavirus have been reported as the most importrant cause but other microbes such as enterovirus, adenovirus, *Vibrio, Campylobacter, Salmonella*, *Shigella,* and enterotoxigenic *E. coli* also are responsible for many diarrheas in the world. Probiotic bacteria such as *L. rhamnosus GG* (148)*,* and *B. lactis* BB-12 (149) have demonstrated strong prevention effect against acute diarrhea in children while *L. reuteri* SD2222 has proved the ability to treat acute diarrhea (150).

The second type of diarrhea is associated with antibiotic treatments (AAD that influence not only the pathogens but also the indigenous gut microflora. A major issue is the prevalence of *C. difficile*, a bacterium highly resistant to many antibiotic that ca proliferate in the gut during antibiotic therapy. Consumption of probiotics during the antibiotic treatments period can help to restore the microflora balance in the GIT. The most common probiotics regarding AAD are *S. boulardii, Bifidobacterium* spp, and *L. rhamnosus* (151). The third type of diarrhea is known as “travelers’ diarrhea”. The traveler’s diarrhea (TD) happens in many people who travel to high-risk areas of tropical and semitropical regions of the Latin America, southern Asia, and Africa (152). Bacteria such as *E. coli, Salmonella, Aeromonas, Shigella, Campylobacter, and* non-cholera *Vibrio* are responsible for more than 60% of TD in those regions. Probiotic bacteria such as *L. acidophilus, B. bifidum,* and *L. bulgaricus* have shown strong inhibitory activity against some of the abovementioned bacteria in both *in vitro* and *in vivo* studies (153–155).

Anti-Colorectal Cancer Activity

In 1990, Kubota discovered a significant relationship between bacteria population and colon cancer incidence. The incidence of colon cancer was at the lowest when the number of *Bifidobacteria* cells was at the highest. On the other side, it was seen that when the population of *C. perfringens* was the lowest, the incidence of colon cancer was also at the lowest. In fact, *C.*

perfringens possesses high levels of 7α-dehydroxylase that is considered as an important enzyme in the conversion of secondary bile acids from primary bile acids in the colon. It has been demonstrated that secondary bile acids play a role as tumor promoters in the gut. Probiotic strains can inhibit and reduce the number of *C. perfringens* in the colon by bacteriocin production and hence limit the formation of these bile acids.

Moreover, many studies indicate that some probiotics can regulate cell apoptosis by intrinsic and extrinsic pathways, which are potentially critical mechanisms in the prevention of colon cancer. Some *L. paracasei* and *L. casei* strains showed effective anticancer activity by upregulating the expression of apoptotic genes *BAX, BAD, caspase3, caspase8*, and *caspase9* and by downregulating the expression of the *BCl-2* gene (156), while other *L. casei* induced up-regulation of TRAIL protein expression (157), known to selectively induce apoptosis in many tumor cell lines without affecting normal cells and tissues, thus appearing as a promising therapeutic drug (158).

In 2012, Chen and colleagues found that administration of *L. acidophilus* reduced the severity of colorectal carcinogenesis in mice. In addition, *L. reuteri* can prevent colorectal cancer by down-regulating nuclear factor-kappaB (NF-κB)-dependent gene products which regulate cell proliferation (Cox-2, cyclin D1) and survival (Bcl-2, Bcl-xL) (26).

Other studies have reported that exopolysaccharides of *L. acidophilus* and *L. rhamnosus* were playing a role as anti-tumorigenic against HT-29 colon cancer cells since they can induce the activation of autophagic cell death (159).

Probiotic strains also can play a key role to inhibit colon cancer by antioxidant activity. It was shown that Reactive Oxygen Species (ROS) can increase the possibility of colon cancer and probiotic strains by enzymatic mechanisms such as coupled NADH oxidase/peroxidase system and catalase can play a significant role in diminishing these compounds (160, 161). The homofermentative *Lactobacillus* species indicated high antioxidant activity whereas this characteristic appeared to be strain-dependent among heterofermentative strains (162, 163).

All probiotic strains can also increase the TNF-α, interferon-γ (IFN-γ), and the regulatory cytokine IL-10. *L. acidophilus*, *L. casei,* and *B. longum* exert immunomodulatory and antitumor effects by suppressing the proliferation of tumor cells (164). *L. casei* Shirota also showed strong anti-metastatic effects on tumor cells suppressing chemically-induced carcinogenesis (Takagi *et*

al., 2001). In addition, the administration of *L. casei* Shirota increased NK cell cytotoxicity which delays tumor onset or suppresses tumor incidence (165).

Lactose Intolerance

Lactose intolerance is a serious digestive disorder for people who have lost the ability to digest lactose into its constituents, glucose, and galactose. A low level of the enzyme β-galactosidase usually causes the "Lactose intolerance" disease. This enzyme has the highest activity at birth but in some people it declines after weaning. The symptoms such as bloating, flatulence, and watery stool are a result of unabsorbed lactose (166).

Probiotic strains can play a beneficial role in two different ways: on one side, producing the fermented foods which have lower lactose concentration due to the lactase activity of probiotics; on the other side, probiotics can increase lactase levels in the small intestine (167).

Cholesterol Lowering Effect

Probiotic strains can help humans reducing the risk of coronary heart diseases caused by a high level of cholesterol in the blood (168). They can reduce the level of cholesterol in human body in many ways such as by deconjugation of bile *via* bile salt hydrolysis activity, by binding cholesterol to their cellular surface and then incorporating it into the cellular membrane, by co-precipitation of cholesterol with deconjugated bile (169), and by cholesterol conversion to coprostanol (170).

The main mechanism is related to bile salts hydrolysis activity. Bile salts hydrolase is the enzyme that catalyzes the hydrolysis of glycine- and/or taurine-conjugated bile salts into amino acid and free bile acids. They are less reabsorbed from the intestinal lumen and usually excreted in the feces.

Inhibitory Activity Against *H. pylori*

During the last decades *H. pylori* infection has been a frequent disorder in humans. It was reported that more than 60% of people in the world are carrying these bacteria in their stomach whixhis responsible for diseases such as peptic ulcers, type B gastritis, and gastric cancer. In addition, it causes iron-deficiency anemia, idiopathic thrombocytopenic purpurea, and vitamin B_{12} deficiency. *H. pylori* is a Gram-negative pathogenic bacterium that survives under low pH and gastric conditions thanks to its urease enzyme that can hydrolyze urea into carbon dioxide and ammonia to increase the pH in the stomach. This bacterium also can colonize the gastric

epithelium due to its flagella (171), and reduce the production of gastric mucin by suppressing the expression of MUC5AC and MUC1 genes (172). On the other side, probiotic strains such as *L. rhamnosus* GG and *L. plantarum* 299v can increase the expression of the abovementioned genes and secretion of mucin (173, 174).

Moreover, it has been reported that *L. reuteri* strains JCM 1081 and TM 105 are able to inhibit the attachment of *H. pylori* by inhibiting bacteria binding to glycolipid receptors asialo-GMI and sulfatide (175).

Treatment of Irritable Bowel Syndromes (IBS)

Irritable bowel syndrome (IBS) is another human disorder that causes symptoms like stomach cramps, bloating, diarrhea, constipation and flatulence (176). Administration of probiotics revealed to be an effective treatment for this disorder that results in modulation of microbiota composition. *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. brevis* Bb99, and *P. freudenreichii*, are the strains most frequently used (176). In particular, it has been reported that a mixed culture of *B. brevis* Bb99, *L. rhamnosus* LC705, *L. rhamnosus* GG, and *P. freudenreichii* is able to decrease *Ruminococcus torques* and stabilize *C. thermosuccinogenes* levels in the feces from IBS patients (177).

Immunomodulatory Effects

It has been demonstrated that probiotic strains are able to modulate the human immune system in different ways. They can increase the number of natural killer cells, neutrophils, macrophages, and T-helper lymphocytes. It has been demonstrated under *in-vivo* condition in rats that *L. casei* is able to increase the population of T-helper lymphocytes significantly (178). In addition, in other studies probiotics have shown a strong ability to increase the number of natural killer cells, neutrophils and macrophages that are considered the first line of defense in our body (179).

Probiotics such as *Bifidobacterium* and *Lactobacillus* strains have shown the ability to modify gene expression of mucins, nuclear factors, and interleukins carrying on an anti-inflammatory response. They can also play a significant role in the interaction with the surface of antigen-presenting cells by downregulating pro-inflammatory genes and upregulating other anti-inflammatory genes (180).

On the other side, the expression of cytokines in the human body has been the most frequent study related to the immunomodulatory effect of probiotics. Several studies demonstrated an increase in pro-inflammatory cytokines such as IL-12 and tumor necrosis factor-alpha (TNF-α)

in the presence of probiotics. In 2000, Haller and colleagues found that human peripheral blood mononuclear cells treated with *L. johnsonii* and *L. sakei* increase the IFN-γ and IL-12 levels, while IL-10 did not seem to increase (181).

In 2015, Wang and colleagues showed that dialysis patients treated with *B. bifidum*, *B. catenulatum*, *B. longum,* and *L. plantarum* indicated a decrease in the serum levels of proinflammatory cytokines TNF-α, IL-5, and IL-6, while levels of serum of IL-10 significantly increased (182).

Probiotics can also affect the antigen-specific immune response against infections; especially, their act by increasing the immunoglobulin A (IgA) level that is considered the first line of human immune system defense against pathogenic bacteria and viruses. For instance, in 2014, Kikuchi and colleagues showed that *L. plantarum* can increase IgA level of Peyer's patch (PP) cells (183). On the other side, probiotics have shown anti-inflammatory and immunomodulatory activity related to control of inflammation and allergic reactions in the human body. They play a significant role by keeping the proper balance between Th1 and Th2 cells, which provides protection against allergies (184, 185). Probiotics have also been able to act by decreasing the inflammatory immune response to food antigens (186).

Vaginitis Inhibitory Activity

The urinary tract infections can be caused by pathogenic bacteria and fungi that climb from the vagina and sometimes bladder urothelium (187, 188). The common symptoms for vaginitis consist of dysuria, pyuria, frequency, and painful micturition, and rarely hematuria. A common urogenital disorder in women worlwide is bacterial vaginosis. The pH of the vagina is mostly related to the vagina microflora, and varies between 3.5 and 4.5 during the period between puberty and menopause. The predominant vaginal microflora consists of *L. casei, L. fermentum, L. acidophilus*, and *L. iners* that play an important role in vaginal healthy (189). There is a direct correlation between the presences of a vaginal microflora in healthy women and the absence in patients with infections. The vaginal microbiota can keep the vaginal environment acidic by production of lactic acid and preserve the vagina from pathogenic microbes attack. Plenty of things can disturb this balance such as taking broad-spectrum antibiotics, spermicides, hormones, and dietary habits.

Overall, women's vagina can get infected by pathogenic Gram-negative anaerobic bacteria such as *Mycoplasma hominis, Gardnerella vaginalis, E. coli* and *Mycoplasma curtisii,* or can be subjected to yeast infection by *C. albicans* (190, 191)**.**

Probiotic strains such as *L. acidophilus*, *L. rhamnosus* GR-1, and *L. fermentum* RC-14 have shown to be beneficial by replacing the microflora in a unhealthy vagina and by occupation of specific attachment sites at the epithelial surface of the urinary tract. Moreover, they maintain a low pH and can produce antimicrobial compounds, such as acids and hydrogen peroxide (187).

1.4. Prebiotics

For the first time In 1995, Gibson and Roberfroid defined a prebiotic as "A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/ or activity of one or a limited number of bacteria in the colon, and thus improves host health" (192). Later on, FAO in 2008 defined a prebiotic as a "Non-viable food component that confers a health benefit on the host associated with modulation of the microbiota" (176). This definition is currently approved by the European Food Safety Authority.

According to the definition by FAO, any food that reaches the human colon can be a candidate for prebiotic. In fact, the critical requirement for prebiotic compounds is be able to pass through the small intestine to the large bowel without being broken down and to be available just for probiotic bacteria and not to others.

In general, the dose of 3 gr per day of these products is recommended to guarantee a positive effect on humans. To be considered a prebiotic, a substance has to meet some criteria, such as resistance to gastric acidity and mammalian enzymes, sensitivity to fermentation by gut bacteria, and ability to enhance the viability of beneficial microorganisms (193).

Prebiotics can be obtained from differet foods such as garlic, chicory root, leek, onions, wheat, and artichokes by enzymatic hydrolysis, e.g. extraction of oligofructose from inulin. All oligosaccharides such as glucooligosaccharides, maltooligosaccharides xylo-oligosaccharides, glycooligosaccharides, lactitol, isomalto- oligosaccharides, stachyose, and raffinose are non-digestible carbohydrates (194).

Fructooligosaccaharides (FOS) are among the most frequently prebiotics used nowadays. They are made by fructose units that are linked by β (2→1) bonds in which the number of fructose units ranges from 2 to 7 and they often end up with a glucose unit. They are found naturally in

plants such as garlic, asparagus, banana, onion, chicory, garlic, asparagus, banana, and artichoke (195).

Inulin is famous prebiotic which made by long chain-terminating glucosyl moieties and a repetitive fructosyl moiety which is linked by β-(2,1) bonds and it usually found in some crops including onions, leeks, garlic, banana, wheat, Jerusalem artichoke, and chicory (196, 197). Inulin also is completely resistant to saliva and to small intestine enzymes.

Galactooligosaccharides (GOS) are polysaccharide produced by enzymatic treatment of lactose by β-galactosidase to produce several oligomers of (198). GOS consist of a galactose chain attached to a single glucose molecule, which varies in chain length (2–8 monomers). They have β (1→4), β (1→2) and β (1→6) linkages (199, 200).

It has been proposed recently that some monosaccharides such as arabinose, xylose, and xylitol can also be considered as prebiotic, since some probiotics such as *Lactobacillus* and *Bifidobacterium* are able to digest them while pathogenic bacteria are not..

1.5. Project outline

This thesis was aimed to identify and select some new potential probiotic and technological strains of lactic acid bacteria, from a collection of 46 strains recently isolated from different sources, through genomic and physiological studies. These strains were evaluated for some technological aspects, since the species considered in this study are important in the food industry, particularly regarding fermented foods production. Their genomes were also sequenced and studied by different *in-silico* analyses to get information about their genetic potential. Some promising strains resulting from the technological studies were chosen for *in-vitro* probiotic characterization, including antibiotic susceptibility, hemolytic activity, resistance to simulated gastrointestinal conditions, bile salts hydrolytic activity, antimicrobial activity, folate production, adhesion to human epithelial cells and anti-cancer activity against human colorectal cancer cells. Based on results from *in-vitro* characterization, two strains, namely *L. paracasei* DTA81 and *S. thermophilus* TH982 were chosen for *in-vivo* experiments on laboratory mice to get information about their health benefits in the host. Therefore, 32 laboratory mice were considered and fed with probiotic strains for six weeks. Then all animals were evaluated for food consumption and weight gaining, blood biochemical analysis, oral glucose tolerance test (OGTT), survival of probiotics after passage through the GIT, 16S metagenomics analysis of gut microbiome, and immunomodulatory effects. This confirmatory *in-vivo* analysis allowed us to collect several dataabout the tested strains and in particular to identify *L. paracasei* DTA81 as a strong probiotic bacterium possessing also interesting technological traits.

Chapter 1

Genomic Study and Technological Properties of Newly Isolated Lactic Acid Bacteria

Lactic acid bacteria (LAB) are one of the most important group of microorganisms widely applied for the production of dairy product, beverages, fermented foods, and as probiotics (201). In general, the genus *Lactobacillus* consists of over 180 species and encompasses a broad variety of microorganisms that includes the highest number of GRAS species (Generally Recognized As Safe) (13). The *Lactobacillus* genus includes both homofermentative strains and facultative heterofermentative ones that can produce both lactic acid and acetic acid from fermentation thereby decreasing the pH of the food. In addition to being a well-known starter for food products, the probiotic potential of this genus has also been studied very well. For instance, *L. rhamnosus* has received great attention among researchers for its beneficial properties to human health. It is normally considered as probiotic since it possesses many properties such as resistance to gastrointestinal juice, ability to adhere to the intestinal tract, inhibition of potentially pathogenic species of microbes, capability to help weight loss in obese persones and protection of the colon (18). It was also reported that *L. rhamnosus* can be used against common causes of traveler's diarrhea (18).

Streptococcus is a genus containing more than 100 species, among which *S. thermophilus* is the only non-pathogenic species that has technological importance in dairy food productions, known since 1919. Moreover, *S. thermophilus* has been considered as an important bacterium in the dairy industry because of acidifying capabilities and some antimicrobial activities related to production of organic molecules such as lactic acid, acetic acid and formic acid. *S. thermophilus* has the ability to acidify the milk rapidly by first breaking lactose into glucose and galactose and then producing lactic acid by fermenting glucose, thus lowering the pH, an important feature that significantly affects microbial development in all environments, including food (202, 203). *S. macedonicus* is another species of the genus which was identified and isolated from some dairy foods including some Italian kinds of cheese (204–206). *S. macedonicus* strains show some interesting properties, such as proteolytic activity, production of bacteriocins against food pathogens, production of exopolysaccharides, and tolerance to stress associated with food processing (30, 207). These traits make *S. macedonicus* a promising species, suitable for further studies for applications in food productions (208). Some *S. macedonicus* strains have already been evaluated as starter cultures in cheese-making trials (30, 209, 210). The aim of this part of the study was to comparatively evaluate some technological and physiological differences between *S. thermophilus* and *S. macedonicus* strains as well as a study of probiotic potential of *L.*

rhamnosus and *L. paracasei* in relation to their use in food productions as starter cultures. Their ability to consume some sugars commonly existing in foods, along with the respective growth kinetics, was evaluated as well.

2.2. MATERIALS AND METHODS

2.2.1. Strains used and growth condition

The *S. thermophilus* and *S. macedonicus* strains applied in this study were already isolated and identified from dairy environments in Italy and were part of the collection of the Department of Agronomy Food Natural Resources Animals and Environment. The genome of *S. thermophilus* strains had been already sequenced and studied and they have been applied for bioinformatics analysis in comparison with *S. macedonicus*. Regarding *S. macedonicus*, some strains were genome sequenced and applied for genomic analysis and *in silico* study in this work. As regard *L. rhamnosus* and *L. paracasei* strains, they were previously isolated from stool of infants aged between 7 and 21 days from different hospitals in Rio de Janeiro (RJ, Brazil), were identified at species level by 16 rDNA sequencing and RAPD analysis (211) and grouped into 9 clusters according to a RAPD similarity profile percentage of more than 80%. Later on, in this study they were checked out to the further identification using a new molecular method to discriminate species belonging to the *L. casei* group, namely *L. casei*, *L. paracasei* and *L. rhamnosus*, that was recently proposed based on a multiplex PCR assay targeting the mutL gene (212). All strains used in the present work have been listed in table 1.1. The strains were kept at − 80 °C in BHI broth (Oxoid, UK) containing glycerol (25% v/v) and sub-cultured two times, prior to use, in M17 medium containing 0.5% lactose for Streptococcus species and MRS medium for *Lactobacillus* species (Oxoid, UK). Cultures were incubated for 24 h at 37 °C.

2.2.2. Molecular identification of strains belonging to the *Lactobacillus casei group*

DNA was extracted by using one colony from the MRS agar plate and according to (212). DNA yield and purity were assessed by NanoDrop 2000c (Thermo Fischer Scientific,

Wilmington, DE, USA). The multiplex PCR assay was done using the primer pairs and PCR conditions described previously (212). The type strains *L. paracasei* subsp. *paracasei* DSM 5622, *L. casei* DSM 20011 and *L. rhamnosus* DSM 20021 were used as reference for the species

and *L. plantarum* subsp. *plantarum* DSM 20174 was used as a negative control. Results were visualized by gel electrophoresis on SYBR Safe stained 1.5% agarose gel.

Table 1.1: Bacterial strains used in the present work.

Strain	Geographical origin	Isolation matrix	Reference/source
S. thermophilus TH1436	Friuli Venezia Giulia	Goat Raw milk	(213)
S. thermophilus TH1435	Friuli Venezia Giulia	Goat Raw milk	(213)
S. thermophilus TH1477	Veneto	Cow Raw milk	(214)
S. thermophilus 1F8CT	Veneto	Curd from cow raw milk	(214)
S. thermophilus TH982	Campania	Buffalo mozzarella curd	(214)
S. thermophilus TH985	Campania	Buffalo mozzarella whey	(214)
S. thermophilus M17PTZA496	Valle d'Aosta	Fontina cheese (cow)	(215)
S. thermophilus MTH17CL396	Valle d'Aosta	Fontina cheese (cow)	(215)
S. macedonicus 8SP	Trentino Alto Adige	Curd of Spressa cheese	(208)
S. macedonicus 19AS	Veneto	Natural milk culture for Asiago cheese (cow)	(216)
S. macedonicus 62AS	Veneto	Natural milk culture for Asiago cheese (cow)	(208)
S. macedonicus 27MV	Veneto	Monte Veronese cheese (cow)	(216)
S. macedonicus 203MA	Veneto	"Malga" cow cheese	Veneto Agricoltura*
S. macedonicus 211MA	Veneto	"Malga" cow cheese	(216)
S. macedonicus 33MO	Veneto	Curd of Morlacco cheese (cow)	(213)
L. paracasei DTA72	Rio de Janeiro	Infant feces	(217)
L. paracasei DTA76	Rio de Janeiro	Infant feces	(217)
L. rhamnosus DTA79	Rio de Janeiro	Infant feces	(217)
L. paracasei DTA81	Rio de Janeiro	Infant feces	(217)
L. paracasei DTA83	Rio de Janeiro	Infant feces	(217)
L. paracasei DTA96	Rio de Janeiro	Infant feces	(217)
L.rhamnosus DTA93	Rio de Janeiro	Infant feces	(217)
L. rhamnosus DTA105	Rio de Janeiro	Infant feces	(217)

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2.2.3. Acidification test

An aliquot of 0.1 ml of overnight bacterial culture from all strains in M17 and MRS broth was added into 10 ml of skim milk and then, it was incubated at 37 °C for 24 h. Later on, 1% of this culture was transferred into 40 ml of skim milk and divided into 4 tubes for different times (10 ml for each tube) and incubated at 37 °C to be fermented. Four different times were selected to

measure the pH of the medium (0, 3, 6, and 9 h). The pH was immediately measured with the pH-meter regarding time zero (Sigma precision Mettler Toledo MP225).

2.2.4. Fermentation on different sugars

Fermentation on different sugars was done by using microtiter plate incubator and reader (Spark 10M, Tecan GmbH, Grödig, Austria) and 96-wells microtiter plates (Sigma SIAL0596, MO, USA). To start the experiment ten ml of overnight cultures from different strains of *S. thermophilus, S. macedonicus, L. rhamnosus,* and *L. paracasei* were centrifuged at 10,000×g for 10 min. 5 ml of PBS was applied to wash the pellets twice and they were resuspended in another 5 ml of PBS. On the other side, 10% (w/v) stock solution of different sugars namely lactose, glucose, galactose, fructose, xylose, and inulin were prepared and sterilized by using 0.22 µm filters. Later on, they were added to MRS medium and M17 medium to 1% final concentration and then incubated at 37 °C for 24 h. Growth was measured by an automatic reading of absorbance at 600 nm (OD600) every 30 min. All experiments were performed with three biological and four technical replicates. Blank and negative controls were inserted in all experiments. The Gompertz model (218) was used to growth data to estimate the growth parameters, namely, maximum cell number at stationary phase (Nmax), maximum growth rate (µmax), and lag phase duration (λ). In addition, the genome of sequenced *Streptococcus* strains were applied for bioinformatics analysis and genome content study to compare *S. thermophilus* and *S. macedonicus* strains regarding different sugars consumption. Therefore, eight *S. thermophilus* and four *S. macedonicus* genomes were screened using RAST (Rapid Annotation System Technology) (219).

2.2.5. Inhibitory activity

The inhibitory activity on different strains was tested by using the disc diffusion agar method against six different indicator strains, namely *Pseudomonas fluorescens* DSM50090, *E. coli* DH1, *Staphylococcus xylosus* DSM20266, *Bacillus amyloliquefaciens* DSM7, *Staphylococcus aureus* DSM20231, *Bacillus subtilis* DSM10, according to Maragkoudakis et al., (2013) with the following modifications. All *Streptococcus* and *Lactobacillus* strains were cultured from the stock two times prior to the assay in 10 ml of MRS and M17 broth containing 0.5% lactose respectively and incubated at 37 °C for 24 h. After incubation, all tubes were centrifuged, and the supernatants were collected, the pH was adjusted to 7.0 with NaOH 1 M, to remove the possible

inhibitory effect of acidic pH, and sterilized through 0.22 μm filters. Then the supernatants were lyophilized and four different concentrations were prepared as follow; 200, 100, 50, and 25 mg/ml. On the other side, indicator strains were cultured using BHI broth and incubated at 37 °C for 24 h. To perform the disc diffusion test, sterile paper discs (6 mm diameter, cat. n. 185006, Biolife, Italy) were immersed into the related supernatants for 5 min and then placed at 37 °C for 4 h, until they were completely dried. The suspensions of the indicator strains were also adjusted at 1 McFarland turbidity and were streaked on BHI agar medium. The discs were then placed on the surface of the plates and incubated at 37 °C for 24 h. After incubation, plates were checked out for the presence of inhibition haloes. Commercial chloramphenicol discs (30 mg) and paper discs soaked with 20 μl of distilled water were used as positive and negative controls, respectively. The experiment was repeated three times.

2.2.6. Genomic analysis of *S. macedonicus* strains

The extraction of genomic DNA, sequencing and annotation strategies for *S. macedonicus* 33MO, 19AS, 27MV and 211MA are reported by Vendramin et al. (2014) and Treu et al. (2017). The CGView Server (223) was used to generate a graphical map of the *S. macedonicus* strains used in this study. For comparative analysis, whole-genome sequences of 6 *S. macedonicus*, 6 *S. gallolyticus*, 5 *S. equinus* and 8 *S. thermophilus* strains available in Genbank (NCBI) were downloaded (Supplementary Table 2). A fragmented all-against-all comparison in TBLASTX mode was carried out with Gegenees software (224), setting the parameters to 200/100 (frag-size/slide-size). An unrooted phylogenetic tree was computed using SplitsTree4 using the neighbor-joining (NJ) method (225). Moreover, a comparative genomic study considering the total number of proteins among the *S. macedonicus* strains used in this study, *S. macedonicus* ACA-DC 198 and a representative strain of the *S. gallolyticus* group (*S. gallolyticus* ICDDRB-NRC-S1) was conducted. Bacterial proteins were clustered using the web platform Orthovenn with 1e-5 and 1.5 of cutoff e-value and inflation value, respectively. Singletons were extracted and functionally annotated using the Customizable Web Server for Fast Metagenomic Sequence Analysis (WebMGA) with default parameters (226). Proteins assigned to a specific Cluster of Orthologous Group (COG) were manually checked for the identification of conserved domains using the Conserved Domains Database (CDD) (227). *In-silico* safety assessment of *S. macedonicus* was performed based on the complete genome of each strain. The detection of antibiotic resistance genes was conducted using the Comprehensive Antibiotic Resistance

Database (CARD) selecting perfect, strict and loose hits, and considering sequences with low quality/coverage (228). The IslandViewer 4 (229) was used for identification and visualization of genomic islands, whereas the presence of genes associated with bacterial pathogenesis was checked against the virulence factors database (VFDB) (230). The PHAge Search Tool (PHASTER) web server (231) was used to predict prophage regions, whereas restriction-modification systems (R-M) were determined in the REBASE genomes database (232).

2.2.7. Statistical analysis

The SigmaPlot software version 12.0 (Systat Software, San Jose, CA) was used for statistical analysis. The normality of each trait was tested using the Shapiro–Wilk normality. After the normality test, data were analyzed for statistical significance using analysis of variance (ANOVA) followed by Tukey's test. Data with a non-normal distribution were analyzed by non-parametric test (Dunn's Method).

2.3. RESULTS AND DISCUSSION

2.3.1 Molecular identification of *Lactobacillus* isolates

Thirty-five isolates belonging to this genus, previously isolated from infant stools, were identified at the species level by 16 rDNA sequencing and RAPD analysis (211) and grouped into 9 clusters according to RAPD similarity profile percentage of more than 80%. A molecular method to discriminate species belonging to the *L. casei* group, namely *L. casei, L. paracasei* and *L. rhamnosus*, was recently proposed based on a multiplex PCR assay targeting the *mutL* gene (212). Its application on one strain chosen from each branch of the above-mentioned cluster allowed to identify six *L. paracasei* (DTA72, DTA76, DTA81, DTA83, DTA93, and DTA96) and two *L. rhamnosus* (DTA79 and DTA105) (Fig. 1.1).

2.3.2. Acidification test

Among technological characterization of LAB, the acidification ability is one of the most important traits. The ability to decrease the pH in all abovementioned strains were measured during the first 9 hours of growth which is the most appropriate period regarding cheese making. The experiment was performed in skim milk and the pH was measured every 3 hours. According to the results (Table 1.2), none of the *Lactobacillus* showed good acidification ability as the best one was *L. paracasei* DTA81 that reached a pH of 6.45 after 9 h. On the other side, *S.*

thermophilus strains indicated good potential regarding pH reduction. All strains reached pH 5.5 after 9 h while two strains, namely TH1436 and TH1435 showed very strong potential by reaching values below 4.6, which is the value required for caseins coagulation and for growth inhibition of most food pathogens. As regard *S. macedonicus* strains, they showed poor ability in acidification as they could not decrease the pH below 5.3 after 9 h. Rapid acidification is a priority in the development of starter cultures for fermented milk products. It is essential for coagulation and prevention or reduction of growth of adventitious microflora (233). The result of this study confirms the unsuitability of *L. rhamnosus,* and *L. paracasei* strains as fermentation starters, as it is reported in many works in literature. Lactic Acid Bacteria (LAB) are traditionally used in cheese manufacturing where the starter LAB (SLAB) mainly participate in the fermentation process, whereas the non-starter LAB (NSLAB) are implicated in other activities related to cheese ripening. For instance, *L. acidophilus, L. casei* and *L. rhamnosus* are generally used as additional cultures due to their poor fermentation kinetics and the scarce sensory properties of fermented milk

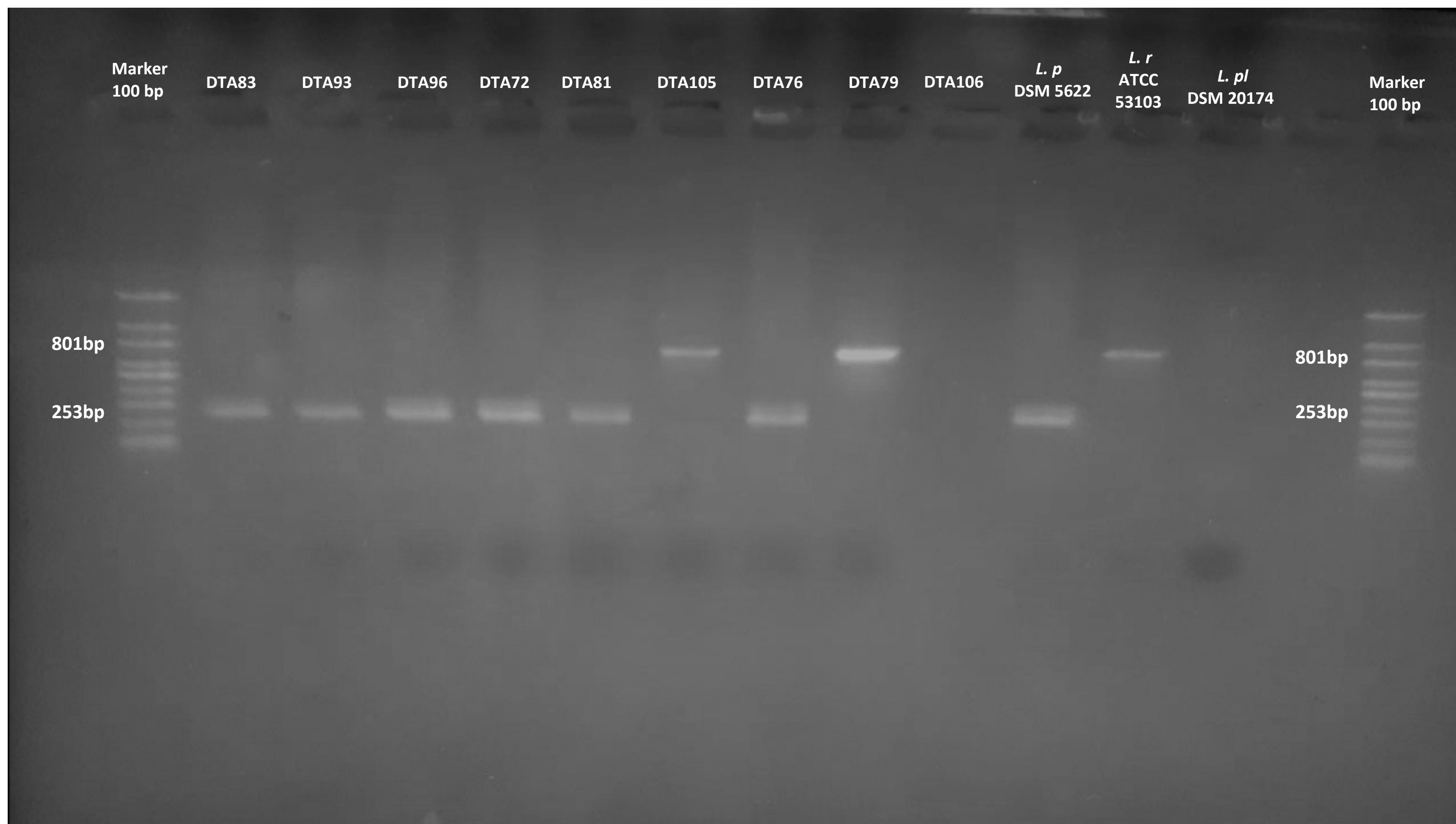


Fig 1.1: Multiplex PCR amplification results of *L. casei* group reference and newly isolated strains related to the *mutL*-targeting gene. Predicted amplicon size was 253 bp for *L. paracasei* (*Lp*, DSM 5622) and 801 bp for *L. rhamnosus* (*Lr*, ATCC 53103). Negative control was represented by *L. plantarum* (*L.pl*, ATCC 14917).

obtained when they are used as starters (234–236). On the other side, *S. thermophilus* is widely applied in starter cultures with the aim of growing rapidly and decreasing the pH, because changes in pH induce modifications in the bacterial population composition (203, 220). Although much less is known about *S. macedonicus*, several studies have been done to study its characterization and especially it's potential to decrease the pH rapidly. However, in all the cases tested they have shown weak to moderate acidification ability as they showed in our study (30).

Table 1.2: Acidification ability of different LAB.

	Strains	pH (time 0)	pH (3h)	pH (6h)	pH (9h)	ΔpH
S. thermophilus						
	1F8CT	6.41	6.42	6.21	5.94	0.47
	M17PTZA496	6.48	6.32	5.92	5.41	1.07
	MTH17CL396	6.47	6.17	5.75	5.52	0.95
	TH982	6.4	6	5.43	5	1.4
	TH985	6.51	6.43	6.12	5.4	1.11
	TH1435	6.48	5.91	4.71	4.3	2.18
	TH1436	6.44	6	4.81	4.6	1.84
	TH1477	6.43	6.11	5.41	5.1	1.33
S. macedonicus						
	8SP	6.42	6.23	6.01	5.91	0.51
	19AS	6.27	6.24	6.05	5.94	0.33
	27MV	6.22	6.21	6.03	5.93	0.29
	203MA	6.28	6.24	6.02	5.74	0.54
	62AS	6.26	6.11	5.98	5.82	0.44
	33MO	6.36	6.27	6.1	5.97	0.39
	211MA	6.11	6.11	5.92	5.72	0.39
L. paracasei						
	DTA72	6.65	6.58	6.61	6.51	0.14
	DTA76	6.6	6.58	6.61	6.54	0.06
	DTA79	6.6	6.58	6.57	6.48	0.12
	DTA81	6.66	6.53	6.53	6.45	0.21
	DTA83	6.65	6.59	6.58	6.57	0.08
	DTA96	6.65	6.58	6.54	6.48	0.17
L. rhamnosus	DTA93	6.59	6.59	6.54	6.53	0.06
	DTA105	6.65	6.55	6.52	6.46	0.19

2.3.2. Sugars utilization

Carbohydrates, in general, are the main and primary energy source for fermentation and acidification of LAB. While for different *Lactobacillus* species and *S. thermophilus* several works assessed the consumption of glucose, lactose, and fructose (237), few data are available for *S. macedonicus*. In the present work, six *L. paracasei*, two *L. rhamnosus*, seven *S. macedonicus*, and eight *S. thermophilus* strains, isolated from different sources in Italy and Brazil, were comparatively studied to evaluate their growth by using different sugars provided in the medium as the only energy source. The following sugars were tested: lactose, galactose, glucose, fructose, xylose, and inulin. Lactose is the main sugar in dairy products, from which the *S. thermophilus* and *S. macedonicus* strains used in this work were isolated and are adapted to live. Galactose also exists in milk following its exportation from cells of strains not able to metabolize it after lactose hydrolysis (238, 239). This sugar can cause many problems, such as browning of heat-treated products (e.g., Mozzarella in pizza preparation), cheese fractures due to CO2 overproduction, and toxic effects on people who are suffering by galactosemia, which is a genetic disease disturbing galactose metabolism (240). Glucose and fructose as simple sugars have been also checked due to they are consumed by most of LAB and can be applied for fermented food products. Xylose is the basic constituent of xylooligosaccharides (XOS), molecules that have great prebiotic potential (241). Inulin is a fructan, a polymer of fructose, widespread in many plant materials. This complex molecule, which is indigestible for humans and many bacteria composing the human natural flora, is used by probiotic bacteria, mainly lactic acid bacteria, for growth in the bowel and it is therefore considered a prebiotic (242). The growth curves of *Lactobacillus* species, *S. thermophilus*, and *S. macedonicus* strains on MRS and M17 media supplemented with different sugars as the sole energy source during 24 h are reported in Figures 1.2, 1.3, and 1.4. In Table 1.3, the main growth parameters, namely, λ, µmax, and Nmax, calculated by using the Gompertz growth model, are reported for lactose, galactose, glucose, and fructose. By considering the negative control information, only *Lactobacillus* strains that exceeded an OD600 of 0.6 were considered able to use the supplemented sugar and are inserted in the table. Regarding *Streptococcus* strains, this value was about 0.2 according to negative control data. Strains with lower OD600 values than cut off, probably utilize small amounts of energy sources present in MRS and M17 media without sugars supplement.

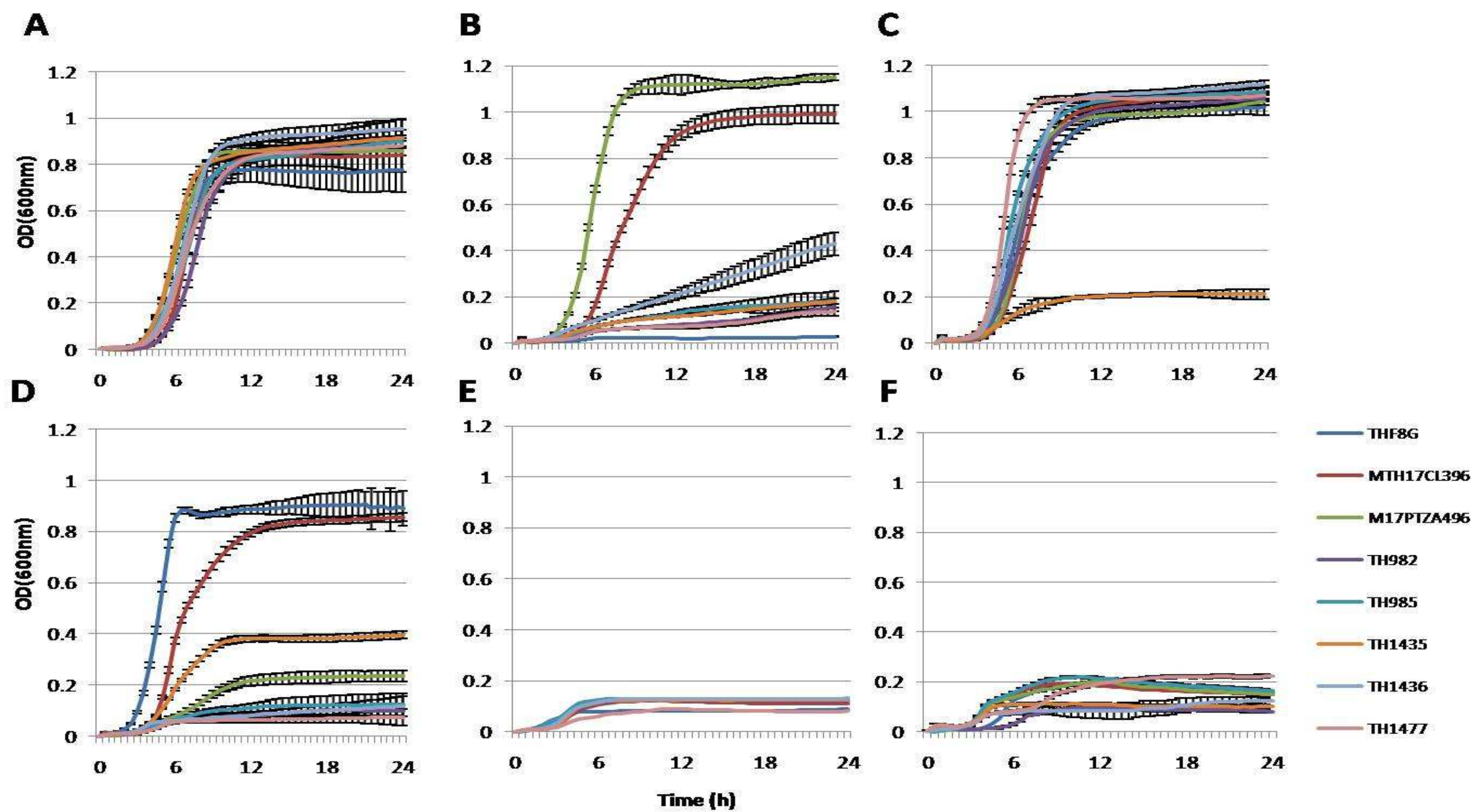


Fig. 1.2: Growth curves of *S. thermophilus* strains on selected carbohydrates as follow: A: Lactose, B: Galactose, C: Glucose, D: Fructose, E: Xylose, F: Inuline.

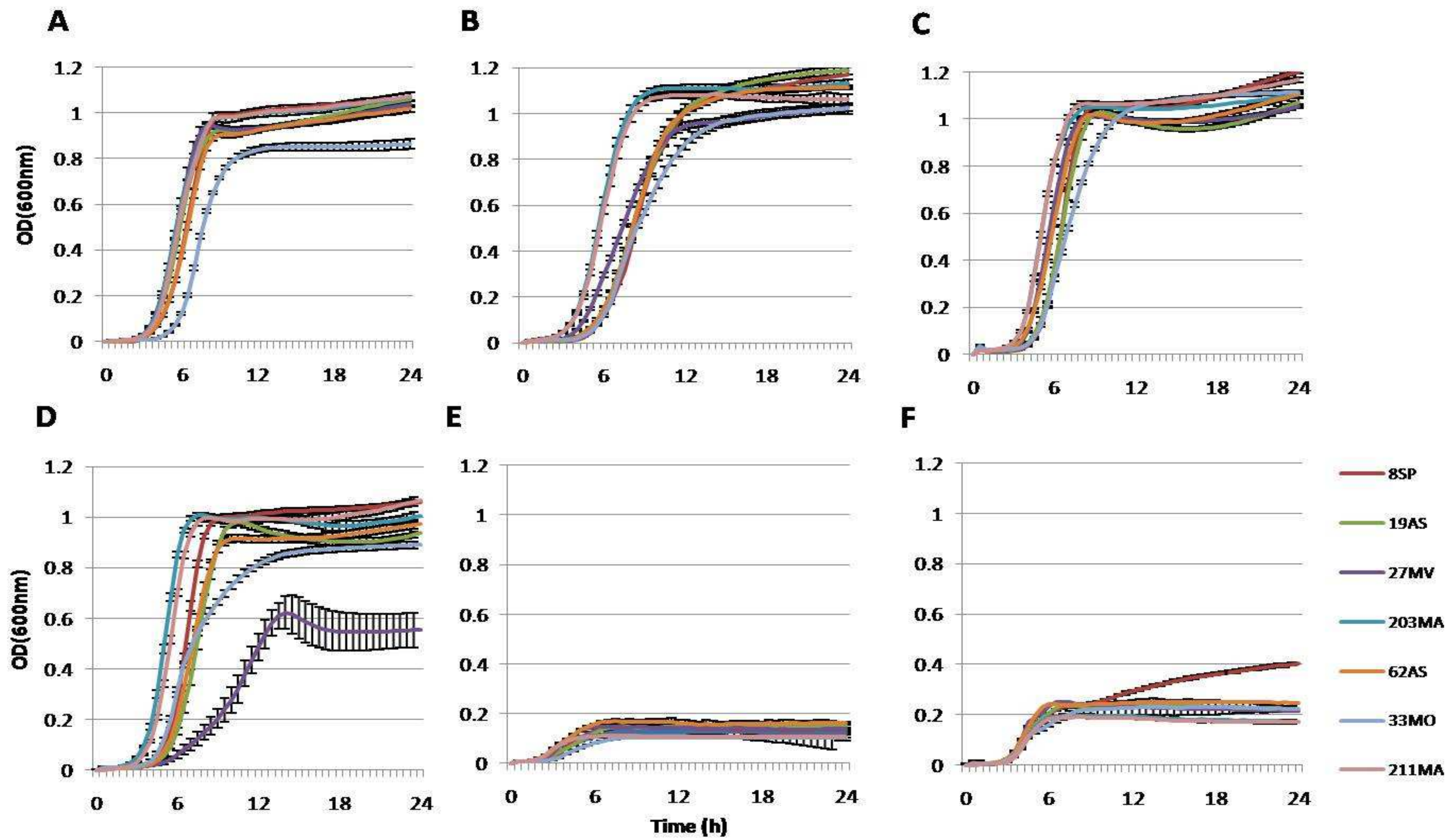


Fig. 1.3: Growth curves of *S. macedonicus* strains on selected carbohydrates as follow: A: Lactose, B: Galactose, C: Glucose, D: Fructose, E: Xylose, F: Inuline

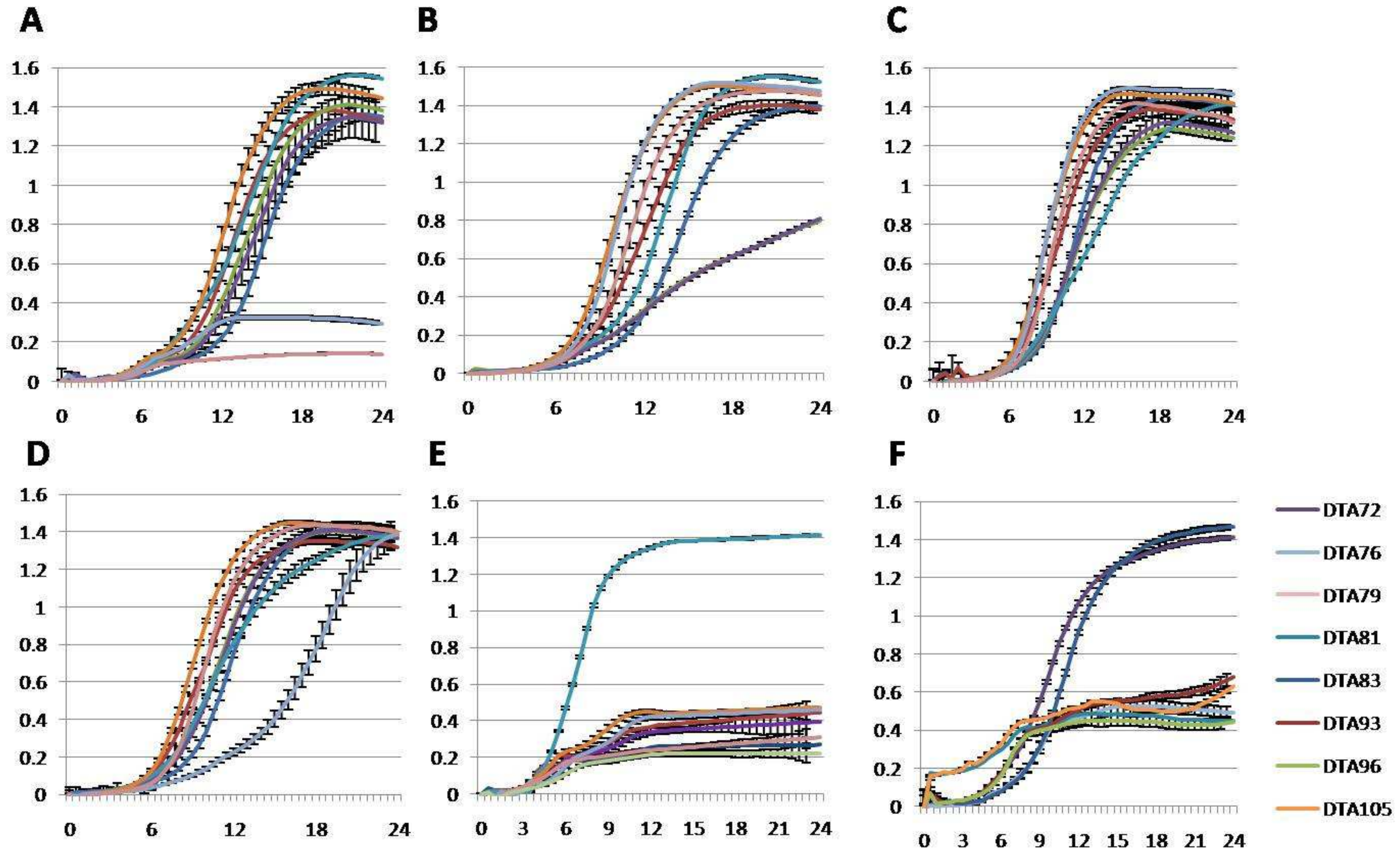


Fig. 1.4: Growth curves of *Lactobacillus* strains on selected carbohydrates as follow: A: Lactose, B: Galactose, C: Glucose, D: Fructose, E: Xylose, F: Inuline.

All *Streptococcus* strains grew pretty well on lactose, but they showed significantly different parameters according to the Gompertz growth model. The *S. macedonicus* strains showed a shorter lag phase and reached a higher number of cells at the stationary phase. The strain 33MO was the only one among *S. macedonicus* strains that had a different behavior in terms of growth. Indeed it revealed growth parameters much similar to those of *S. thermophilus*, although genomic analysis indicated no differences regarding gene content for lactose/galactose uptake and utilization among the four *S. macedonicus* strains analyzed (Table 1.4). However, compared to the *S. thermophilus* strains used in this study, *S. macedonicus* strains had a higher number of genes regarding different carbohydrates utilization, that could be correlated to the better growth observed. This result shows that, since the main technological purpose of a starter culture is to rapidly colonize and acidify the medium, *S. macedonicus* strains appear to be the best choice. The situation is different within the genus *Lactobacillus*. Most of the *L. paracasei* strains are capable of metabolizing lactose with the exception of strain DTA76 which indicated difficulty in growth, while for the two *L. rhamnosus* strains tested the behaviors were different. Strain DTA79 was able to utilize lactose pretty well where strain DTA105 could not use it as a source of energy. This indicates how the streptococcal species can be widely used to obtain fermented milk-based products, unlike the *Lactobacillus* genus, with few exceptions. Furthermore, by analyzing the kinetics, the *L. paracasei* strains appear to have a long lag phase, on the other hand, the stationary population reaches higher cell concentrations (Nmax). Regarding galactose, *S. macedonicus* strains showed very strong ability as they all were able to grow on galactose with very similar kinetics, apart from slight differences in the lag phases. As regard the *Lactobacillus* genus, except for the strain DTA72 which was not able to grow on galactose, all the others utilized the galactose although not as efficiently as as *S. macedonicus*. Among all different species tested in this study, *S. thermophilus* showed the weakest growth as only three *S. thermophilus* strains; namely, MTH17CL396, M17PTZA496, and TH1436 were able to grow on galactose. This is a very interesting result aiming at reducing galactose present in food that advises choosing *S. macedonicus* as starter cultures. Regarding growth kinetics, due to the high variability of *S. thermophilus* strains, statistical analysis did not evidence significant differences. As regards glucose, it was consumed with similar kinetics by all strains of different species and no statistically significant differences between their growth parameters were found. The only exception was *S. thermophilus* TH1435 that failed to utilize glucose.

Table 1.3: Growth parameters calculated by the Gompertz model.

	Strains	Lactose			Galactose			Glucose			Fructose		
		λ (h)	μ_{max} (h^{-1})	N_{max} (OD_{600})	λ (h)	μ_{max} (h^{-1})	N_{max} (OD_{600})	λ (h)	μ_{max} (h^{-1})	N_{max} (OD_{600})	λ (h)	μ_{max} (h^{-1})	N_{max} (OD_{600})
S. thermophilus	1F8CT	2.06	1.92	0.84				2.52	1.89	0.94	1.58	2.04	0.91
	M17PTZA496	3.48	2.78	0.8	3.9	1.59	0.86	3.68	1.69	1.01	3.24	1.92	0.75
	MTH17CL396	2.24	2.7	0.81	1.7	1.85	1.18	3.53	1.79	0.98	2.48	1.27	0.19
	TH982	2.94	2.08	0.82				2.94	1.67	0.98			
	TH985	1.94	1.92	0.8				2.88	2	1.01			
	TH1435	1.98	2.22	0.87							1.38	1.28	0.4
	TH1436	2.25	1.69	0.96	1.13	0.7	0.2	2.57	1.69	1.04			
	TH1477	2.3	1.49	0.9				2.16	2	1.11			
S. macedonicus	8SP	0.2	2	1.04	3.89	1.59	1.02	4.15	2	1.07	3.65	2.13	1.06
	19AS	0.05	2.78	0.95	3.04	1.43	1.08	3.88	1.82	1.02	3.23	1.61	1.09
	27MV	0.24	2.5	0.96	2.03	1.61	0.94	3.4	2.1	1	3.06	0.83	0.72
	203MA	0.67	2.22	1.09	1.47	1.67	1.18	2.87	2.1	1.05	1.41	2.01	1.07
	62AS	0.24	2.38	0.97	2.32	1.28	1.22	3.11	1.79	1.01	1.87	1.67	1.15
	33MO	2.93	1.7	0.97	3.13	1.33	0.98	3.8	1.59	1.02	1.86	1.59	0.89
	211MA	1.09	2	1.09	1.43	1.54	1.16	1.98	1.92	1.11	0.68	2.38	1.07
L. paracasei	DTA72	3.70	1.65	0.84	2.63	1.50	0.52	0.11	1.15	1.97	1.56	1.38	2.51
	DTA76				0.93	0.97	2.84	0.70	0.50	1.67	0.58	1.47	0.48
	DTA79				1.08	0.77	1.96	0.28	0.61	1.62	0.37	0.77	1.92
	DTA81	0.38	0.84	1.05	0.27	1.32	1.66	0.17	1.10	1.22	0.21	1.20	1.59
	DTA83	5.03	1.48	0.44	5.43	1.50	1.05	5.61	1.13	1.67			
	DTA93	2.29	1.41	1.16	2.33	1.31	1.69	4.19	1.03	1.41	1.54	1.13	1.76
L. rhamnosus	DTA96	1.79	1.43	0.99	4.24	1.46	0.42	0.91	1.07	2.01	1.85	1.34	2.19
	DTA105	0.97	1.09	1.59	0.98	0.79	2.37	0.39	0.65	1.67	0.17	0.86	1.83

Table 1.4: Genes assigned to carbohydrate metabolism by RAST analysis.

Strains	Sucrose *	Maltose/ maltodextrin *	Trehalose **	Lactose/ galactose **	Lactose	Fructo-oligosaccharides (FOS) /Raffinose	Beta-glucoside	Fructose *
S. macedonicus 211MA	6	20	0	30	0	16	22	7
S. macedonicus 33MO	6	22	0	23	0	16	21	7
S. macedonicus 27MV	6	27	0	37	0	16	27	7
S. macedonicus 19S	6	20	0	23	0	16	20	7
S. thermophilus M17PTZA496	6	18	10	10	2	0	0	7
S. thermophilus TH1477	5	15	5	11	3	0	0	10
S. thermophilus TH1436	5	15	12	10	2	0	0	10
S. thermophilus TH1435	5	0	11	0	0	0	0	0
S. thermophilus TH985	5	13	12	10	2	0	0	7
S. thermophilus TH982	5	13	11	11	2	0	0	10
S. thermophilus MTH17CL396	5	0	10	11	2	0	0	10
S. thermophilus 1F8CT	5	17	11	10	2	0	0	7

* Utilization; ** uptake and utilization

In this study, *S. macedonicus* strains were generally very efficient sugar utilizers, with the partial exception of strain 27MV which grew much slower than the others. As found for glucose, *S. thermophilus* strains showed a heterogeneous behavior, since only two strains could grow very well on fructose and showed significant differences with other species. *S. thermophilus* TH1435, the only one also unable to use glucose, grew very slow even on fructose, probably due to the lack of fructose uptake and utilization genes, observed only in this strain (table 1-4). Regarding *L. paracasei* and *L. rhamnosus*, they have shown good ability in growing on fructose and indicated kinetics similar to *S. macedonicus* without any significant difference. On the other side, none of the *Streptococcus* strains tested was able to grow significantly neither on xylose nor on inulin while *L. paracasei* DTA83 and *L. paracasei* DTA72 were able to use inulin as sole carbon source and *L. paracasei* DTA81 was the only bacterium able to use xylose as sole carbon source. This can be considered a positive trait for *Streptococcus* strains that are intended to be used as starter cultures, because prebiotic molecules should not be consumed by starters, rather they should reach intact the gut to feed the intestinal microbiota. On the other side, utilizing complex sugars such as inulin (prebiotic) by *Lactobacillus* strains also can be a positive character to consider them as probiotics and not as starter culture. Overall, *S. macedonicus* strains reached the same population density and had similar growth rates on all the four sugars but all strains started growing clearly sooner (lower λ) on lactose. Moreover, all strains were able to grow on all the four sugars, while we could not see this situation in *S. thermophilus* and *Lactobacillus* strains.

2.3.3. Inhibitory activity

Inhibitory activity against some deleterious bacteria due to the production of organic acids, short chain fatty acids, and bacteriocins are desirable functional properties for strains to be used in food technology. Thermophilin is a famous bacteriocin produced by *S. thermophilus* that showed strain-dependent activity against *L. monocytogenes* and *S. aureus* (243). *S. macedonicus* strains have been reported to produce macedocins endowed with anticlostridial activity (30). Paracin 1.7 is also a bacteriocin synthesized by *L. paracasei* HD1-7 that showed strain-dependent inhibitory activity against *L. monocytogenes.* However, in the present study, the growth of six indicator bacteria was not inhibited by any of the concentrated cell-free supernatants obtained from the tested strains.

2.3.4. Genomic analysis of *S. macedonicus* strains

Chromosome alignment of *S. macedonicus* isolated in Italy and phylogenomic tree

Undoubtedly, genome sequencing and analysis of probiotic potential candidates have become mandatory in the last years mainly in terms of biosafety aspects and possible mode of action (244). In this study, although *in-silico* analyses have been conducted considering all sequenced Italian *S. macedonicus* strains, a special focus was given to *S. macedonicus* 211MA as a result of interesting features obtained from the phenotypic assays such as strong adhesion to human epithelial cells. We performed a full chromosome alignment through BLASTN comparisons using *S. macedonicus* 19AS, 27MV, 211MA and 33MO genomes. The analysis revealed segments well preserved and shared among all the strains, although genomic regions poorly conserved can be identified. Overall, we observed higher homology at nucleotide level between *S. macedonicus* 33MO and 19AS (Figure 1.5). As reported by Papadimitriou et al. (2014), strain-specific regions are indicative of gene loss during the evolution of *S. macedonicus* when compared to *S. gallolyticus* due to selective pressures. Indeed, all Italian *S. macedonicus* strains were isolated from different cheeses produced in the Veneto region and, consequently, evolved under diverse cheese making processes (246). A phylogenomic tree was constructed based on the complete genome sequences available for all *S. macedonicus* strains, *S. thermophilus* isolated in Northern Italy, *S. gallolyticus* and from representants of the *Streptococcus bovis*/*Streptococcus equinus* complex (SBSEC). As evidenced in figure 1.6, six major groups were formed, with *S. macedonicus* strains inserted in two different ones. Interestingly, considering the Italian isolates, *S. macedonicus* 211MA was placed with *S. macedonicus* ACA-DC 198 and *S. gallolyticus* ICDDRB-NRC-S1, reflecting the close phylogenetic relationship among the strains. In fact, Sarker et al. (2016) demonstrated the tight similarity between *S. macedonicus* ACA-DC 198 and *S. gallolyticus* ICDDRB-NRC-S1 genomes based on a phylogenetic tree constructed considering the single nucleotide polymorphisms (SNPs) found in each strain. The similarities among these strains are also demonstrated by *in-vitro* and *in-vivo* assays. *S. macedonicus* ACA-DC 198 was successfully recovered from stool samples during a safety *in vivo* trial using a murine model (248), whereas *S. gallolyticus* ICDDRB-NRC-S1 was isolated from human feces (247). Both results are in accordance with those obtained for *S. macedonicus* 211MA, which has demonstrated high cell viability after exposure to simulated gastrointestinal conditions and strong adhesion potential to epithelial cells.

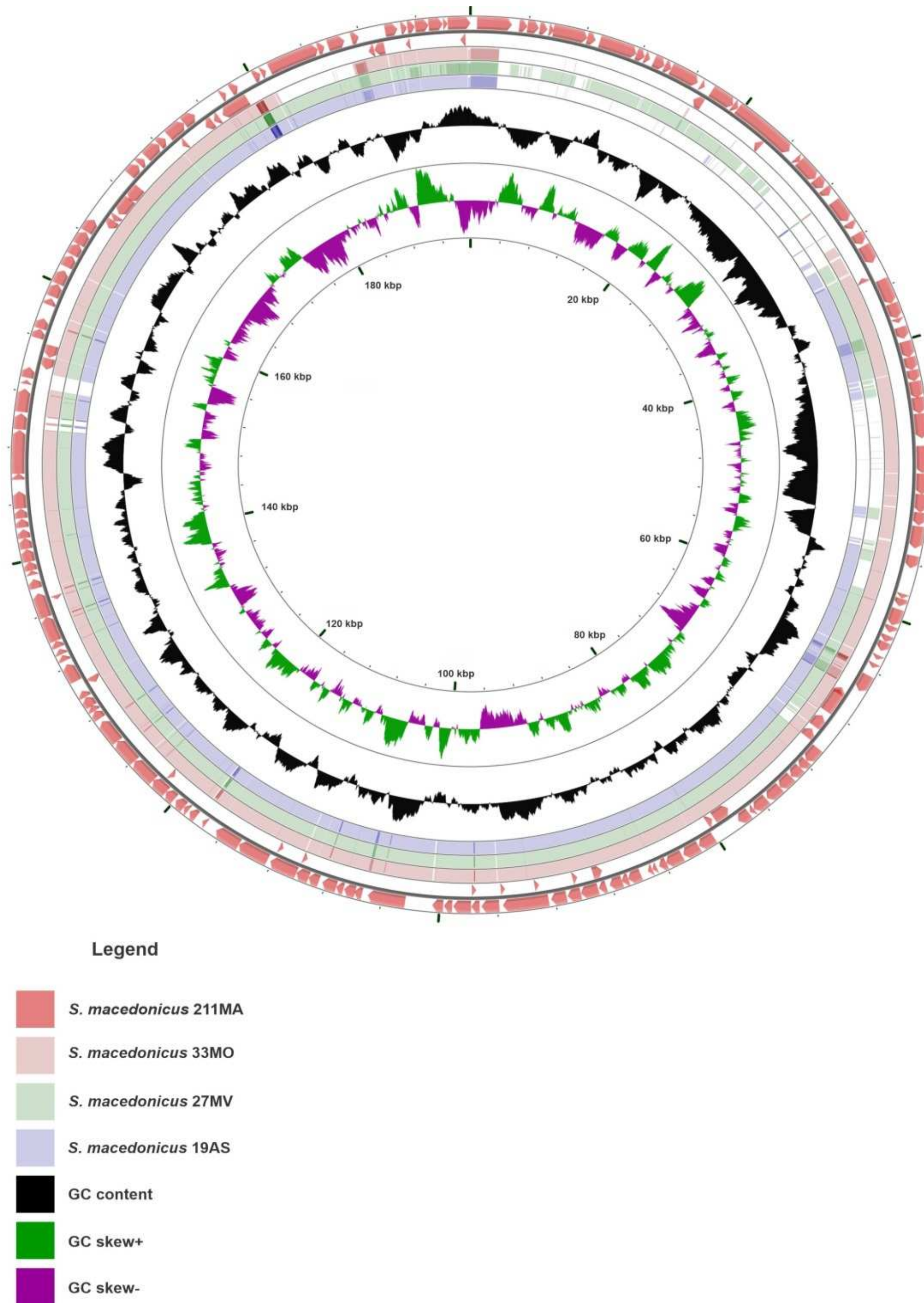


Fig. 1.5: Genome map of *S. macedonicus* strains isolated in northern Italy. *S. macedonicus* 211MA coding sequences (CDSs), *S. macedonicus* 33MO, S. macedonicus 27MV and 19AS open reading frames (ORFs) are reported in circles from the outside inwards, as well as GC content, GC skew+ and GC skew−. The visualization shows GC content and skews information only for *S. macedonicus* 211MA. Regions with different color intensity reflect the level of similarity amongst genome traits after BLAST analysis.

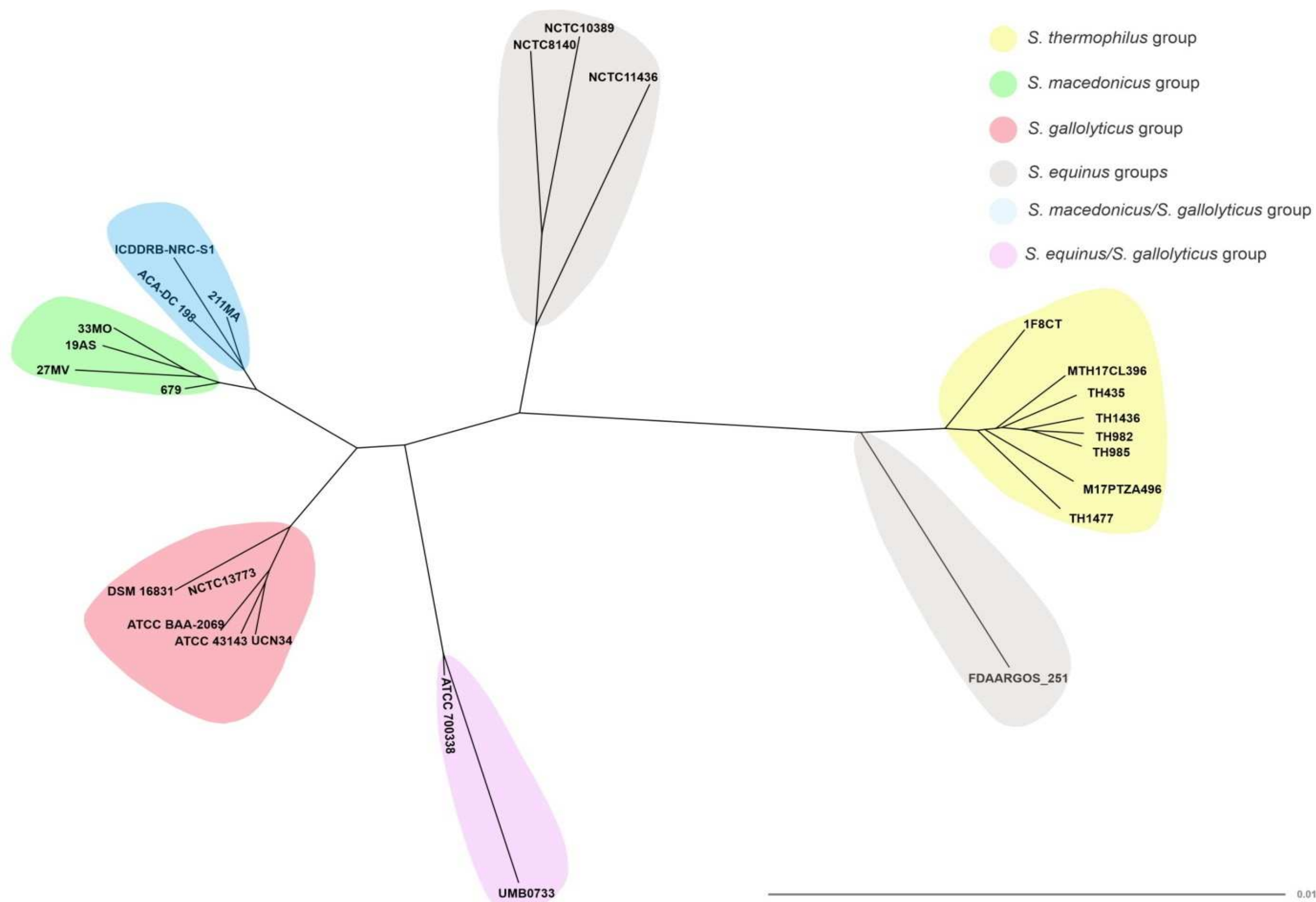


Fig. 1.6: Phylogenomic tree constructed using the whole genome sequence of of 5 *S. equinus*, 6 *S. gallolyticus*, 7 *S. macedonicus* and 8 *S. thermophilus* genomes. GenBank accession numbers are reported in Supplementary Table 1. The scale bar represents a 0.01% difference on the average tBLASTx score. Different clusters are highlighted with different colors.

Core genome analysis and single-copy genes functional clusterization

In order to conduct the pan- and core-genome study, bacteria were selected according to the cluster obtained from the phylogenomic tree (*S. macedonicus* and *S. macedonicus*/*S. gallolyticus* groups). Analysis with Orthovenn revealed that the six species form 2130 orthologous clusters (2123 at least containing two species) and 7 single-copy gene clusters. The core genome contains 1415 orthologous proteins (figure 1.7), a value quite similar to that obtained for *S. gallolyticus*, *S. pasteurianus* and *S. macedonicus* (245). We also extracted the set of genes specific to single genomes (singletons) for all six strains, which might represent a source of uncharacterized proteins conferring selective advantages (249, 250). *S. macedonicus* 211MA displayed the lowest content of annotated singletons, whereas *S. gallolyticus* ICDDRB-NRC-S1 the highest (table 1.5). The high number of proteins related to mobile genetic elements (mobilome) is evidence of the natural competence capability observed in the genus *Streptococcus*, as well as the high rate of bacteriophage transduction events (251). Indeed, Phaster predicted prophage regions in all the strains analyzed (item Y). With regards to single-copy genes coding for proteins grouped into the transcription class, a common set of transcriptional factors (TF) was identified across the isolates, mostly controlling virulence factors expression (e.g., HipB, AcrR and AraC). The third most abundant group of singletons is classified into the replication group, which is in practical terms essential for the preservation of genome stability via bacterial DNA repair systems (252). Together, both classes can confer advantages regarding competition strategies used by lactic acid bacteria (LAB) in adverse environments. Although not included among the most abundant categories, an in-depth investigation of the singletons annotated to *S. macedonicus* 211MA revealed the presence of a glycine betaine/choline-binding transporter annotated as OpuBC (table 1-6). Interestingly, this coding sequence is part of the ABC transporter complex OpuCABCD, involved with the bacterial genetic response to osmolarity changing of the environment, and was also predicted in *S. macedonicus* ACA-DC 198 (245, 253). The *opuCABCD* operon was not identified in other *S. macedonicus* genomes publicly available. As described for *L. monocytogenes* strains LO28 and ScottA, the elimination of OpuC drastically impaired bacteria adhesion in mice intestine after oral ingestion (254). In spite of the fact that few reports about the real role of *opuCABCD* are available in LAB, its presence in *S. macedonicus* 211MA could be associated with the strong epithelial cell adhesion showed by this strain.

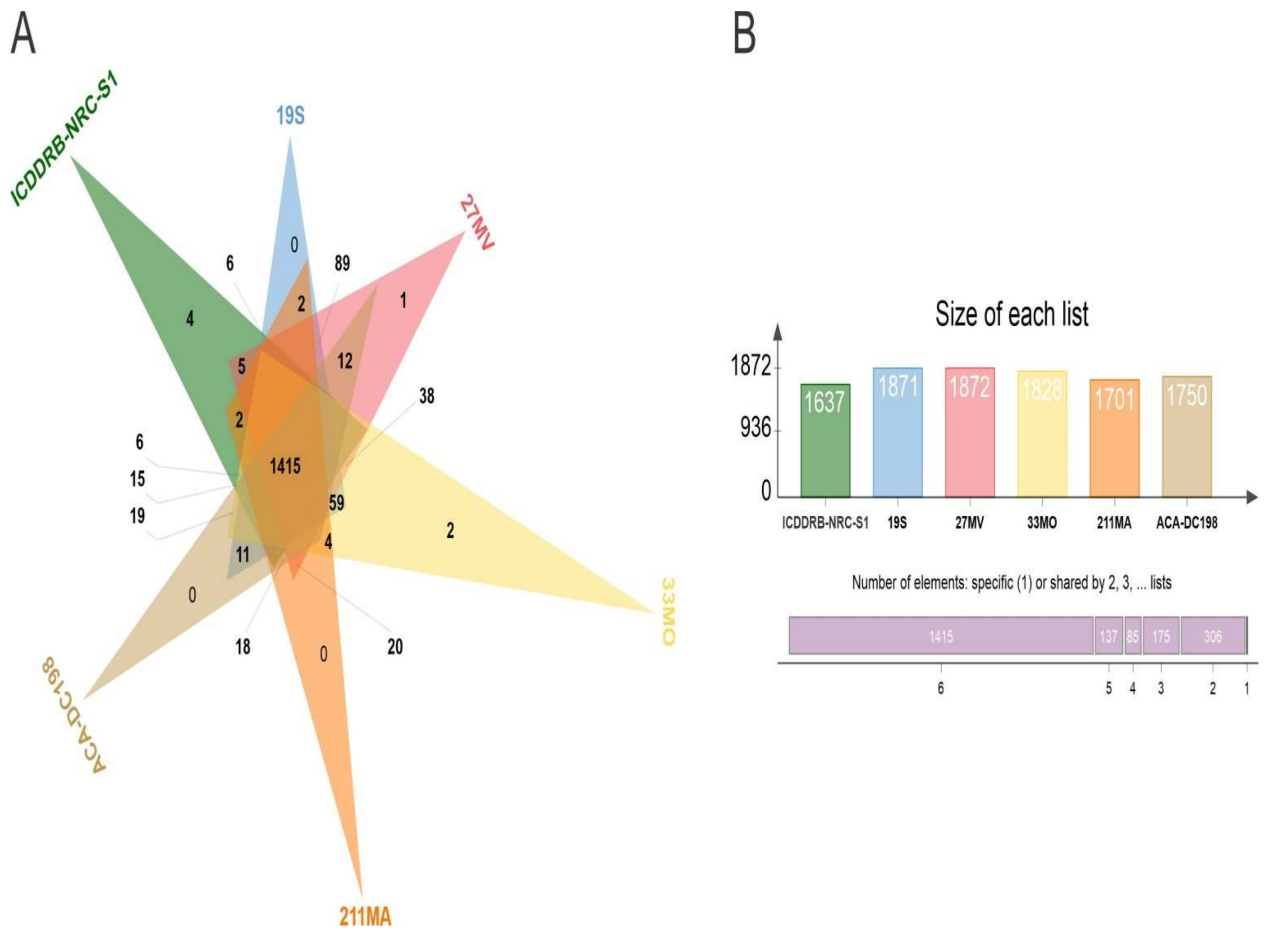


Fig. 1.7: Pan- core-genome analysis conducted with OrthoVenn. A – Venn diagram showing the distribution of common gene families (orthologous clusters) among *S. macedonicus* strains (211MA, 27MV, 19AS, 33MO and ACA-DC 198) and *S. gallolyticus* ICDDRB-NRC-S1. B – The number of clusters for each strain is shown in the bars. The values of shared or single orthologous are shown in the purple boxes.

Table 1.5: Singleton relation for each sequenced Italian *S. macedonicus*, *S. macedonicus* ACA-DC 198 and *S. gallolyticus* ICDDRB-NRC-S1 after orthologous analysis. Proteins were clustered according to the Conserved Domain Database (CCD) classes. Asterisks represents the presence of a specific protein with domain attributed to different CDD classes, whereas and # represent, respectively, hashtag indicates absence of proteins grouped in that class.

CDD Class	*S. macedonicus* 211MA	*S. macedonicus* 33MO	*S. macedonicus* ACA-DC 198	*S. macedonicus* 27MV	*S. macedonicus* 19AS	*S. gallolyticus* ICDDRB-NRC-S1
Mobilome: prophages, transposons	InsE, AlpA*	Tra5, InsE, IS285, COG3547	IS285	Tra5, InsE, COG3561, KilAC, COG5527	COG2932, Rve, COG3378, COG3547, COG3617, KilAC, COG5421	COG3378, COG3464, COG3747, YmfN, COG4653, BeeE, YomH, COG4824, PblB, YqbO, COG5412, COG5614, XerC*
Transcription	HipB, AlpA*	AcrR	CitB*, SSL2, PadR, COG2865, BglG	SSL2*, AcrR, XRE, AraC, COG3620	LysR, AcrR, HipB, RpiR, AraC, YdeE	PurR, HepA*
Replication, recombination and repair	#	XerD	Mod	Dcm, NicK	Dcm, DnaC, NicK, RecT, DnaD, XerD	Dcm, RecA, PolA, YhdJ, Udg4, PinR, XerD, XerC*, HepA*
Cell wall/membrane/envelope biogenesis	OpuBC	#	RfaG	COG3942	WcaA, LolD, LytE, Acm, SrtA, OCH1, COG3942, LolE	AmiC, LytE
Defense mechanisms	AhpC, AbiF, AhpF	HsdM, YadH	COG0610, CcmA, SunT, COG3587, LcnDR2	#	#	HsdS, McrA
General function prediction only	EcfA2*	YdhF	#	ProP*, Cof*, LdhA*, WbbJ, COG1545, ImmA*	#	Uup, YbbA, COG3580, COG3581
Carbohydrate transport and metabolism	GlcU, Pgl	#	GlgP, GT2	DAK1, AraJ, ProP*	PulA, BglB	#
Function unknown	#	COG1479, COG1808, CwlO1	#	COG3462	COG2512, CwlO1	HdeD, YrzB, MdpB
Inorganic ion transport and metabolism	FetB, EcfA2*	ClcA, CorA	*	ProP*, ImmA*	ZntA, COG4097	ClcA
Signal transduction mechanisms	#	#	CitB*, COG4585	SPS1, BaeS, Fic, DhaM, SSL2*	#	#
Coenzyme transport and metabolism	#	CoaE	#	Cof*, Sfp, LdhA*	UbiE	MetK
Cell cycle control, cell division, chromosome partitioning	#	#	#	FtsK	Smc, FtsK	Smc, Spo0J
Lipid transport and metabolism	*	FabD	#	PaaJ, PksG	#	YjiL
Energy production and conversion	#	#	Mdh, InsE	LdhA*	#	#
Posttranslational modification, protein turnover, chaperones	YdiL	#	#	-	YdiL	YdiL
Amino acid transport and metabolism	#	#	#	CysE, PropP*	#	#
Nucleotide transport and metabolism		YjhB	#	YjhB	#	#
Intracellular trafficking, secretion, and vesicular transport	#	#	#	VirD2	#	#
Secondary metabolites biosynthesis, transport and catabolism	#	#	#	EntF	#	#
Translation, ribosomal structure and biogenesis	#	#	#	RimI	#	#

Table 1.6: Virulence factors prediction for each sequenced Italian *S. macedonicus*, *S. macedonicus* ACA-DC 198 and *S. gallolyticus* ICDDRB-NRC-S1. Genbank protein accession numbers is showed for each strain. Asteriks indicates absence of proteins grouped in a specific virulence factor class.

Virulence Factor Class	**Virulence Factors**	**Related Genes**	***S. macedonicus* 33MO**	***S. macedonicus* 211MA**	***S. macedonicus* 19AS**	***S. macedonicus* 27MV**	***S. gallolyticus* ICDDRB-NRC-S1**	***S. macedonicus* ACA-DC 198**
Adherence	Agglutinin receptor	Undetermined	KEH52040.1 KEH51921.1	*	WP_099421313.1	*	*	WP_014294881.1
	Fibronectin-binding proteins	*fbp54*	KEH52565.1	WP_099412341.1	WP_039670682.1	WP_039670682.1	WP_039670682.1	WP_014294346.1
	Sortase A	*srtA*	KEH52500.1 KEH51412.1	WP_014294568.1	WP_039670802.1 WP_099390785.1 WP_099421016.1	WP_039670802.1 WP_099390534.1 WP_099390785.1	WP_014294568.1	WP_014294568.1
	Streptococcal lipoprotein rotamase A	*slrA*	KEH51811.1	WP_014295056.1	WP_014295056.1	WP_014295056.1	WP_014295056.1	WP_014295056.1
	Streptococcal plasmin receptor/GAPDH	*plr/gapA*	KEH51616.1	WP_014295288.1	WP_014295288.1	WP_014295288.1	WP_014295288.1	WP_014295288.1
Enzyme	Streptococcal enolase	*eno*	KEH51897.1	WP_014294917.1	WP_014294917.1	WP_014294917.1	WP_014294917.1	WP_014294917.1
Immune evasion	Capsule	Undetermined	KEH51458.1 KEH52321.1 KEH52323.1 KEH52324.1 KEH52079.1 KEH52082.1 KEH52083.1 KEH52084.1 KEH52080.1	WP_014295403.1 WP_039670925.1 WP_039670927.1 WP_039670928.1 WP_039670942.1 WP_099390353.1 WP_099412025.1 WP_099412313.1 WP_099412314.1	WP_014294412.1 WP_014294795.1 WP_014294800.1 WP_014295403.1 WP_039670929.1 WP_099390353.1 WP_099390829.1 WP_099421125.1 WP_099421128.1 WP_099421129.1 WP_099421130.1 WP_099421284.1 WP_099421286.1	WP_014294412.1 WP_039670929.1 WP_099390349.1 WP_099390350.1 WP_099390351.1 WP_099390353.1 WP_099390355.1 WP_099390659.1 WP_099390828.1 WP_099390829.1 WP_099390832.1WP _099390950.1 WP_099390965.1	WP_039670929.1 WP_058621336.1 WP_058621550.1 WP_058621551.1 WP_058621554.1 WP_058621702.1 WP_058621703.1 WP_058621705.1	WP_014294410.1 WP_014294412.1 WP_014294413.1 WP_014294417.1 WP_014294418.1 WP_014294792.1 WP_014294795.1 WP_014294797.1 WP_014294799.1 WP_014294800.1 WP_014294801.1 WP_014295403.1
Protease	C3-degrading protease	*cppA*	KEH51587.1	WP_014295261.1	WP_014295261.1	WP_014295261.1	WP_014295261.1	WP_014295261.1
	Serine protease	*htrA/degP*	KEH53077.1	WP_014295515.1	WP_014295515.1	WP_014295515.1	WP_014295515.1	WP_014295515.1
	Trigger factor	*tig/ropA*	KEH52943.1	WP_014293924.1	WP_014293924.1	WP_014293924.1	WP_014293924.1	WP_014293924.1
Toxin	Cytolysin	*cylR2*	KEH53095.1	WP_012961332.1	WP_012961332.1	WP_012961332.1 WP_099390813.1	WP_012961332.1	WP_012961332.1
Antiphagocytosis	Capsule	*cpsI*	KEH52327.1	WP_099412311.1	WP_014334589.1	*	WP_058621700.1	WP_014294416.1
Surface protein anchoring	Lipoprotein diacylglyceryl transferase	*lgt*	*	WP_014334283.1	*	WP_014334283.1	*	*

Mobile genetic elements, restriction-modification systems (R-M) and virulence factors (VF) prediction

Among the four *S. macedonicus* genotypes, 211MA displayed the shortest size considering all the clusters, which reflects an undergoing gene decay process for this strain (figure 1.8). Overall, Italian *S. macedonicus* strains possess similar values of predicted GIs when compared with *S. macedonicus* ACA-DC 198, however, a remarkably high percentage of genes acquired by HGT (~ 18.19% the size of the bacterial chromosome) was detected, reflecting the exposure to donors of laterally transmissible genes via transduction, conjugation and natural transformation. In terms of viral sequences (prophages) on *S. macedonicus* chromosomes from Italy, *S. macedonicus* 27MV possesses the highest number of viral sequences integrated (4 incomplete and 1 questionable prophage), whereas *S. macedonicus* 33MO the lowest amount (1 incomplete prophage region). Only 1 intact prophage was observed and has been found in *S. macedonicus* 19AS. According to Papadimitriou et al. (2007), *S. macedonicus* shows an increased ability to resist to bacteriophages infections due to the involvement of bacterial restriction-modification (R-M) systems. The lack of *S. macedonicus* phage genomes deposited on the Genbank database up to date, as well as the identification of *S. thermophilus* bacteriophages sequences on *S. macedonicus* CRISPR modules (256), reinforce the role of *S. thermophilus* viruses in *S. macedonicus* ecology. After R-M components prediction, the presence of genes involved in all three types of R-M (2 type III, 2 type II and 1 type III) was uniquely identified in *S. macedonicus* 33MO and could be considered responsible for the low number of phage proteins annotated in this strain. As described by Papadimitriou et al. (2012), the presence of both types of R-M systems was previously identified only in *S. macedonicus* ACA-DC 198. Finally, we used the VFanalyzer to predict potential virulence factors (VF) for each strain, especially concerning the presence of bacterial gene clusters associated with adherence capability in *S. macedonicus* 211MA (table 1.6). Contrary to what expected, when compared with other strains (*S. macedonicus* strains and *S. gallolyticus* ICDDRB-NRC-S1), *S. macedonicus* 211MA showed the lowest content of genes involved in adherence, demonstrating an attenuated virulence capability. Our analysis suggests that a "core of virulence factors" is shared among *S. macedonicus* strains and contains *fbp54* (fibronectin-binding), *srtA* (sortase A), *slrA* (Streptococcal lipoprotein rotamase A), *plr*/*gapA* (Streptococcal plasmin receptor/GAPDH), *eno* (Streptococcal enolase) and *cppA* (C3-degrading protease).

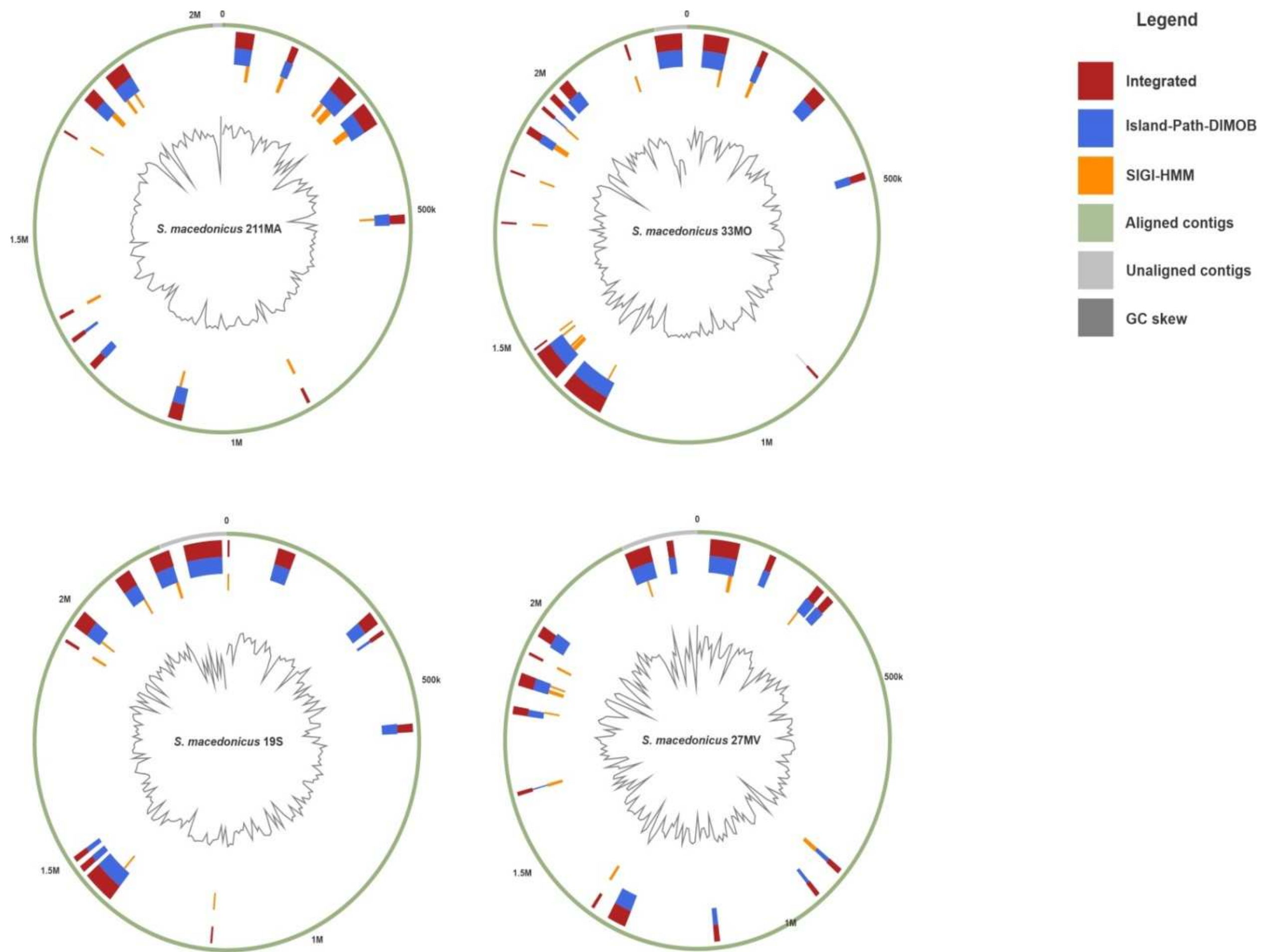


Fig. 1.8: Circular visualization of predicted of genomic islands (GIs) in Italian *S. macedonicus* strains. Blocks are colored according to the prediction method.

Chapter 2

In-vitro Probiotic Properties and Cytotoxic Activity of Lactic Acid Bacteria

According to the WHO/FAO definition, probiotics are “live micro-organisms which, when ingested in adequate amounts, confer a health benefit on the host” (149). The increase in knowledge on probiotic bacteria has led to develop food products that can confer health benefits beyond basic nutrition. Probiotic- containg foods represent a tremendous functional food available on the market worldwide, projected to reach a value of US $46.55 billion by 2020 (258). During the past years, the probiotic potential of many LAB has been studied since these bacteria they are usually recognized as safe for human consumption (i.e. GRAS). Although *Bifidobacterium, Pediococcus, Lactococcus,* and *Enterococcus* genera contain probiotic strains, , most probiotic bacteria on the market nowadays belong to the genus *Lactobacillus*. This genus includes more than 170 species and it is recognized as a taxonomically complex group (259, 260). Lactobacilli are normally found in nature and can be isolated from different matrices, such as plant material (261), fermented foods (262), soil (263) and human gut (211, 264). Many LAB are present in the human body and they are part of the normal microbiota of the human gut. They can play significant roles in different ways such as inhibition of pathogens, anti-tumor activity and different vitamins production in human. Some others can play fundamental roles in the production of fermented foods and for this reason; they are consumed in considerable amounts by people. Besides the safety aspects such as resistance to antibiotics, biogenic amines, and blood hemolytic activity, there are some other criteria to evaluate a strain as probiotic including survival to the human gastrointestinal conditions, adherence ability to the intestinal epithelial cells, possible antimicrobial potential against pathogens, and prevention of colon cancer. Moreover, technological properties such as viability during processing, phage resistance, good sensory properties, and stability in production and during storage would be desirable (265, 266). Generally, most probiotic strains do not possess good technological traits and must therefore be added to fermented foods together with the technological strains. Therefore, the identification of new probiotics from species known to possess technological properties is always desirable (267–270). *S. thermophilus* is a thermophilic LAB that is used as starter in many dairy products, being considered the second most important species of industrial LAB after *L. lactis*. The technological importance of *S. thermophilus* is mainly related to its ability to decrease the pH rapidly, thus being considered a fast acidifier. This feature of *S. thermophilus* can induce modifications in bacterial (220) and also yeast (271) population composition which is particularly related to food safety, since most pathogenic bacteria grow very slowly or not at all at acidic pH. *S.*

thermophilus is also very famous for the production of folate, which is a necessary component of the human diet (272). During the last decades, many LAB have been evaluated for the ability to produce folate and in some fermented dairy products, a considerable amount of folate (up to 110 µg/l) was found due to the activity of LAB (273). For instance, of the two species present in yogurt, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, only the latter was reported to product folate. It was demonstrated that the consumption of food containing folate-producing bacteria can increase plasma folate concentration in humans (273). On the other side, special attention has been given to the role of probiotic bacteria in the prevention of colon cancer (274). Probiotic strains can be beneficial regarding cancer prevention in different ways such as by their immunomodulatory effects or by expression of different genes involved in cell transformation, migration and invasion, and this property is strain dependent (275). Therefore, assessment of cytotoxic activity against different cancer cells could be a very interesting feature of newly isolated probiotic bacteria. The aim of this part of the thesis was to evaluate and select new potential probiotic strains among species of *S. thermophilus, S. macedonicus, L. paracasei,* and *L. rhamnosus* which already had indicated good technological properties (see chapter 1). Therefore, this study examined the capability to withstand the transit through the gastrointestinal tract and the ability to hydrolyze bile salts, the absence of hemolytic activity, production of biogenic amines, and transmissible antibiotic resistance and finally, we looked for health-related traits, namely the production of extracellular riboflavin (vitamin B2), folic acid (vitamin B9), cobalamin (vitamin B12), and the ability to attach and inhibit the growth of human HT-29 colorectal adenocarcinoma cells.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial strains and standard growth conditions

The strains of *S. thermophilus, S. macedonicus, L. paracasei,* and *L. rhamnosus* used in this study are listed in Table 1.1. *L. rhamnosus* GG (ATCC 53103) which is a well-known commercial probiotic strain was included in most tests for probiotic properties as reference strain. All strains were routinely grown at 37 °C in MRS medium for lactobacilli and M17 medium (Difco, United States) containing 0.5% lactose for streptococci, unless otherwise stated. Each strain was also sub-cultured three times prior to its use.

3.2.2. Antibiotic susceptibility test

Antibiotic susceptibility test was performed by applying the agar overlay diffusion method, according to the National Committee for Clinical Laboratory Standards (276). According to the European Food Safety authority (277) recommendations, fourteen antibiotics, namely erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), penicillin G (10 IU), streptomycin (10 µg), tetracycline (30 µg), trimethoprim (5 µg), ampicillin (10 µg), amoxicillin (10 µg), cephalexin (30 µg), chloramphenicol (30µg), ciprofloxacin (5µg), cloxacillin (5µg), and vancomycin (30 µg) were selected to conduct the antibiotic susceptibility test. All strains were cultured from the stock three times prior to assay in 10 ml of MRS or M17 broth, and then they were incubated at 37 ˚C for 24h. Four ml of MRS and M17 soft agar inoculated with 200 µl of overnight strain cultures were used to overlay the plates containing 16 ml of MRS and M17 media to give a final concentration of about 10^7 cells/ml. After solidification, antibiotic-containing disks (Liofilchem, Italy) were placed on the surface and plates were incubated at 37 ˚C for 24h. Finally, inhibition halo diameters were measured and compared to the values proposed by (278) to score strains as resistant, intermediate or susceptible. The test was performed in triplicate. *E. coli* ATCC 25922 was used as quality control of the antibiotic disks.

3.2.3. Hemolytic activity test

Fresh cultures of *S. thermophilus, S. macedonicus, L. paracasei,* and *L. rhamnosus* strains were streaked on MRS and M17 plates containing 5% (w/v) of sheep blood (Thermo Fisher Scientific, United States), incubated at 37 ˚C for 48 h and then checked for the presence of hemolytic haloes. *S. aureus* ATCC 6538 and *L. rhamnosus* GG were included as positive and negative control, respectively (279). The experiment was repeated three times with three technical replicates each.

3.2.4. Biogenic amines production

Production of histamine and tyramine by LAB strains were determined using a defined decarboxylase medium according to (280) with some modifications. Five grams of tryptone, 0.2 g $MgSO_4$, 0.05 g MnSO4, 0.04 g FeSO4, 0.1 g CaCO3, 8g beef extract, 4 g yeast extract, 0.5 g tween 80, and 0.06 g bromocresol purple were dissolved in 1 l of deionized water, the pH of the medium was adjusted to 5.3 and autoclaved at 121°C for 10 min. On the other side, LAB Strains were cultured in MRS and M17 broth for 24 h, then pellets were washed three times with sterilized PBS, transferred to tubes containing the decarboxylase medium and incubated at 30 ˚C

for 5 days. After incubation, 200 µl of each culture were used to inoculate sterile tubes containing 2 ml of defined decarboxylase medium plus the specific amino acid L-histidine or L-tyrosine at 0.5% final concentration. Then all tubes were incubated for 3 days at 30 °C to determine biogenic production activity. Conversion of the medium color from yellow to purple was considered as positive response. The medium without amino acid addition was used as negative control. The experiment was performed with three technical replicates.

3.2.5. Resistance to simulated gastrointestinal conditions

The resistance of LAB strains to simulated human gastro-intestinal conditions was examined as previously described (281) with the following modifications. First, the basic juice was prepared for the gastrointestinal assay which was later used for the preparation of both gastric and intestinal juices. The basic juice contained (per liter) potassium chloride, 1.12 g; sodium chloride, 2.0 g; calcium chloride, 0.11 g; potassium dihydrogen phosphate, 0.4 g. It was sterilized by autoclaving at 121 °C for 15 min. The artificial gastric juice was prepared 1 h prior to use, by adding (per liter) 0.26 g swine pepsin (Sigma-Aldrich, United States) and 3.5 g swine mucin (Sigma-Aldrich, United States). The pH was adjusted to 2.5 with 1 N HCl, filter sterilized and then added to the gastrointestinal basic juice. Aliquots of 100 µl of bacterial cells suspensions obtained after three subcultures in MRS and M17 broth for 24 h were transferred to 900 µl of artificial gastric juice and incubated at 37 °C for 1 h with agitation at 200 rpm. After incubation, the microbial viability was evaluated by the micro drop technique.

The intestinal juice contained (per liter) 1.95 g pancreatin (Sigma-Aldrich, St. Louis, MI, United States), 3 g ox-bile extract (Sigma-Aldrich, St. Louis, MI, United States), and 0.1 g lysozyme (Sigma-Aldrich, St. Louis, MI, United States). The pH was adjusted to 8.0 with 1 N sodium bicarbonate and the medium was filter sterilized. As regard intestinal condition, after gastric juice incubation, 1 ml of intestinal solution was added, and the incubation was continued at 37 °C with agitation for further 3 and 5 h. Microbial viability was evaluated at each time point by the micro drop technique. The experiment was repeated three times with three technical replicates each.

3.2.6. Bile salts hydrolysic activity

Fresh LAB cultures were streaked on MRS or M17 plates containing 0.5% taurodeoxycholic acid (Sigma-Aldrich, St. Louis, MI, United States). Bile salt hydrolytic activity was evaluated after 48

h of incubation at 37 ˚C by checking the presence of deoxycholic acid precipitation haloes around positive colonies and into the surrounding medium. MRS and M17 plates without taurodeoxycholic acid were used as negative controls, whereas *L. mesenteroides* SJRP 55 was used as a positive control (267).

3.2.7. Extracellular vitamins production

Extracellular vitamins production by LAB strains was testedfor riboflavin (vitamin B2), folate (vitamin B9), and cobalamin (vitamin B12). Folate and riboflavin were quantified by using Folic Acid Casei medium (HIMEDIA laboratories, Mumbai, India) and Riboflavin Assay medium (Difco, Livonia, Michigan, United State), respectively. *L. rhamnosus* ATCC 7469 was used as indicator strain to measure both folate and riboflavin. As regards cobalamin, Vitamin B12 Assay medium (Sigma-Aldrich, St. Luis, Missouri, United State) and *L. leichmannii* ATCC 7830 as indicator strain were used to measure the cobalamin produced by different strains. Increasing amounts of vitamins determine a proportional increase in the growth of the indicator strains.

The indicator strains were prepared in advance by growing them in AOAC medium (Difco, United States) at 37 ˚C for 24 h. After incubation, cultures were centrifuged and the pellets washed twice with 10 ml of sterile 0.85% NaCl. Finally, cells were resuspended in 10 ml of 0.85% NaCl and diluted 1:100 for folate measurement and 1:10 for cobalamin and riboflavin respectively. Later, 50 µl aliquots were used to inoculate the assay tubes.

The strains to be tested for production of vitamins were grown in a chemically defined medium (282) without the vitamin under test (folic acid, riboflavin or cobalamin) at 37 ˚C for 24 h. After centrifugation, 1 ml of supernatant was collected and added to the tube containing 5 ml of the specific medium (Folic Acid Casei medium, Riboflavin Assay medium, or Vitamin B12 Assay medium, depending on the vitamin tested) and 4 ml of deionized water, to give a final volume of 10 ml. Tubes were autoclaved at 121 ˚C for 5 min, then cooled down at room temperature. Each tube was inoculated with 50 µl of indicator strains, prepared as described above. After incubation at 37 ˚C for 24 h, the optical density at 620 nm was measured and the results interpreted according to the standard curve by considering the dilution factor of the supernatants.

The standard curve was obtained according to the manufacturer's instruction. The experiment was repeated twice with three technical replicates each.

3.2.8. Adherence ability to HT-29 cells

Adherence ability to HT-29 cancer cells was tested as previously described (283), with the following modifications. HT-29 cells were cultured in DMEM medium (Gibco BRL, United States) supplemented with 1% antibiotics mixture of penicillin/streptomycin (Gibco BRL, United States) and 10% of heat-inactivated fetal bovine serum (Gibco BRL, United States). Aliquots of 3 ml containing 1.5×10^5 cells/ml were seeded on six-well Corning tissue culture plates and incubated at 37 ˚C in 5% CO_2 humid atmosphere until a complete monolayer was achieved. Changing of the medium was done every 48 h until getting a complete monolayer of the cell. Then the medium was discarded from the wells, plates were washed twice with sterile PBS and filled with fresh antibiotic-free DMEM medium. Plates were then incubated at 37 ˚C in 5% CO_2 atmosphere for 30 min before adding the bacterial cells. The adherence ability of LAB strains was evaluated by inoculating 120 µl of bacterial culture, suspended in antibiotic-free DMEM medium, at a concentration of about 1×10^8 CFU/ml and incubating at 37 ˚C for 3 h in 5% (v/v) CO_2 atmosphere. Then, plates were washed four times with PBS to release unbound bacteria. After washing, the fixation step was carried out by using 3 ml of methanol and incubating at room temperature for 10 min. Then the methanol was removed and 3 ml of Giemsa stain solution (1:20) (Merck, Darmstadt, Germany) were added to the wells and again incubated at room temperature for 30 min to stain the cells. After staining, the wells were washed until no color was visible in the washing solution. Then all plates were dried at 37˚C and evaluated under an optical microscope at 1000× magnification. The attached bacterial cells were counted in 20 random microscopic fields for each test and they were scored as non-adhesive when less than 40 bacteria were present in 20 fields, adhesive when containing 41–100 bacteria in 20 fields, and strongly adhesive when more than 100 bacteria were counted in 20 fields. The experiment was repeated three times with three technical replicates each.

3.2.9. Cytotoxic activity against HT-29 cells

The cytotoxic activity against HT-29 colorectal cancer cells of lactobacilli and streptococci stains was evaluated through the MTT [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium-bromide] tetrazolium reduction assay (284) with some modifications. All strains were cultured in MRS or M17 media and incubated at 37 ˚C for 24 h. After incubation, the supernatants were collected, adjusted to pH 7.0 with 1 N NaOH, lyophilized and serially diluted in DMEM at the following concentrations: 125, 250, 500, 750, 1000, 2000, 4000, and 8000 µg/ml. On the other

side, aliquots of 100 µl of HT-29 cell in DMEM medium containing 1.2×10^5 cells/ml were inoculated in the wells of 96-well microplates. When 50% confluence was achieved, the medium was removed and the cells were treated with 100 µl of filtered supernatant from lactobacilli and streptococci cultures at different concentrations and cells were incubated at 37 °C for 48 h under 5% CO2 atmosphere. After incubation, 20 µl of PBS containing 5 mg/ml MTT were added to each well and incubated for further 4 h. After that, 100 µl of pure DMSO (Sigma-Aldrich, United States) were added to each well to dissolve formazan crystals by stirring for 20 min at 200 rpm. Then MTT reduction was measured as absorbance at 570 nm using a microplate reader (Spectra Max M5, Molecular Devices, United States). Moreover, cells treated with MRS and M17 alone were used as negative control while cells treated with 3% DMSO were considered as positive controls. The experiment was repeated two times with three technical replicates each.

3.2.10. Biofilm inhibitory activity

The ability of the *Lactobacillus* strains to inhibit biofilm formation by *E. coli* DSM 30083^T and *L. innocua* DSM 20649^T was evaluated as previously described (285), with some modifications. *E. coli* DSM 30083T and *Listeria innocua* DSM 20649T were cultured on the hydrophobic surface of a 24-well polystyrene plate. The biofilm inhibitory activity was evaluated in two different conditions, namely competition and exclusion. In the competition condition, the *Lactobacillus* strains were co-cultured with *L. innocua* or *E. coli* at a concentration of 10^7 CFU/mL in a 24-well plate and incubated at 37 °C for 18 h. In the exclusion condition, *Lactobacillus* cell suspensions containing 10^7 CFU/ mL were inoculated inside 24-wells plate and incubated for 18 h at 37 °C. After incubation, the wells were washed three times with PBS, and inoculated with *L. innocua* or *E. coli* cell suspensions at the same concentration (10^7 CFU/mL) and incubated for further 18 h at 37 °C. Wells inoculated with *L. innocua* or *E. coli* alone were used as controls. Following incubation, each well was washed three times with PBS to remove nonadherent bacterial cells. Biofilms were collected using a sterile swab and cells were serially diluted using sterile PBS. All dilutions were plated on BHI (DIFCO, Maryland, USA) containing 1.5% LiCl for *L. innocua* and VRBA medium (DIFCO, Maryland, USA) for *E. coli*. Plates were incubated at 37 °C for 48 h and then colonies were counted. The level of inhibition was determined by comparing the values of the co-inoculated cultures with those containing only *L. innocua* or *E. coli*. The experiment was repeated three times.

3.2.11. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's test was used as post hoc analysis. The IC50 (half maximal inhibitory concentration), which represents the dose necessary to inhibit half of the cells, was calculated by non-linear regression using the GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA, United States).

3.3. RESULTS AND DISCUSSION

3.3.1. Antibiotic susceptibility test

Probiotic bacteria usually are selected by considering their inability to transfer possible antibiotic resistance genes to human pathogenic bacteria. Therefore antimicrobial susceptibility has become one of the most important characteristics to assess (286). Although acquired antibiotic resistance is an undesirable characteristic of probiotic bacteria, however, intrinsic resistance could be quietly favorable to the host, to favourintestinal microbiota restoration after antibiotic treatments (278). The antimicrobial susceptibility of *Lactobacillus* strains used in this study is reported in table 2.1. All strains were resistant to cephalexin (CL), ciprofloxacin (CIP), gentamycin (CN), kanamycin (K), streptomycin (S), trimethoprim (TM), and vancomycin (VA) while all were susceptible to ampicillin (AMP), chloramphenicol (C), erythromycin (E), and tetracycline (TE). Antibiotic susceptibility is one of the crucial criteria regarding the safe use of probiotic strains. In fact, antibiotic-resistant bacteria used as probiotics may carry antibiotic resistance genes which could be transferred to pathogenic bacteria (287). The *Lactobacillus* genus displays a range of antibiotic resistance naturally, but in most case, antibiotic resistance is not belonging to the transmissible type and therefore it doesn't usually create a safety concern (288). Several species of *Lactobacillus* including *L. paracasei* and *L. rhamnosus* are intrinsically resistant to vancomycin (289). *Lactobacillus* has a high natural resistance level to aminoglycosides: kanamycin, gentamycin, and streptomycin (290). For instance, the resistance towards kanamycin and streptomycin in *L. delbrueckii* was conferred by the occurrence of the *aph* (3′)-IIIa and *ant* (6) genes, while resistance against inhibitors of nucleic acid synthesis, such as trimethoprim, seems to be intrinsic (80). On the other hand, *Lactobacillus* is usually sensitive to inhibitors of protein synthesis such as chloramphenicol, erythromycin, and tetracycline (80, 290–292). The result of antibiotic susceptibility tests in this study shows that all resistance to different antibiotics are considered as natural (intrinsic resistance) and all *Lactobacillus* strains

could be considered safe regarding this issue. Regarding *S. thermophilus* strains, antimicrobial susceptibility data are reported in table 2.2. All strains were susceptible to cephalexin, chloramphenicol, erythromycin, amoxicillin, ampicillin, penicillin G, tetracycline, and vancomycin, while all were resistant to streptomycin, kanamycin, and trimethoprim. According to previous studies (80) and the guidelines by EFSA (277), *S. thermophilus* strains are generally resistant to aminoglycosides antibiotics such as gentamicin, kanamycin, streptomycin, and trimethoprim. Therefore, such resistance is normally referred to as intrinsic and not transmissible. Regarding ciprofloxacin and cloxacillin, *S. thermophilus* strains indicated different behaviors: all were susceptible to ciprofloxacin and cloxacillin except TH1435 for ciprofloxacin and TH1435 and TH985 for cloxacillin, respectively that evidenced intermediate resistance. These results show that the resistances found in this study can be considered natural (intrinsic) and therefore not dangerous for human usage.

Table 2.1: Antibiotic susceptibility of *Lactobacillus* strains measured as diameters (mm) of inhibition haloes.

Antibiotics	Amount	Strains							
	(µg)	DTA72	DTA76	DTA79	DTA81	DTA83	DTA93	DTA96	GG
Amoxicillin	10	S	S	MS	S	S	S	MS	MS
Ampicillin	10	S	S	S	S	S	S	S	S
Cephalexin	30	R	R	R	R	R	R	R	R
Chloramphenicol	30	S	S	S	S	S	S	S	S
Ciprofloxacin	5	R	R	R	R	R	R	R	R
Cloxacillin	5	R	R	MS	MS	MS	MS	MS	MS
Erythromycin	15	S	S	S	S	S	S	S	S
Gentamycin	10	R	R	R	R	R	R	R	R
Kanamycin	30	R	R	R	R	R	R	R	R
Penicillin G	10 IU	MS	MS	MS	MS	S	MS	S	S
Streptomycin	10	R	R	R	R	R	R	R	R
Tetracycline	30	S	S	S	S	S	S	S	S
Trimethoprim	5	R	R	R	R	R	R	R	R
Vancomycin	30	R	R	R	R	R	R	R	R

Susceptibility is indicated with S, moderate susceptibility with MS and resistance with R. Cutoff values are taken from Charteris et al. (1998).

Table 2.2: Antibiotic susceptibility of S. *thermophilus* strains measured as diameters (mm) of inhibition haloes.

Antibiotic	Amount (µg)	Strains								
		1F8CT	MTH17CL396	M17PTZAMT496	TH982	TH985	TH1435	TH1436	TH1477	GG
Amoxicillin	10	S	S	S	S	S	S	S	S	MS
Ampicillin	10	S	S	S	S	S	S	S	S	S
Cephalexin	30	S	S	S	S	S	S	S	S	R
Chloramphenicol	30	S	S	S	S	S	S	S	S	S
Ciprofloxacin	5	S	S	S	S	S	MS	S	S	R
Cloxacillin	5	S	S	S	S	MS	MS	S	S	MS
Erythromycin	15	S	S	S	S	S	S	S	S	S
Gentamycin	10	R	S	S	S	S	S	R	S	R
Kanamycin	30	R	R	R	R	R	R	R	R	R
Penicillin G	10 IU	S	S	S	S	S	S	S	S	S
Streptomycin	10	R	R	R	R	R	R	R	R	R
Tetracycline	30	S	S	S	S	S	S	S	S	S
Trimethoprim	5	R	R	R	R	R	R	R	R	R
Vancomycin	30	S	S	S	S	S	S	S	S	R

Susceptibility is indicated with S, moderate susceptibility with MS and resistance with R. Cutoff values are taken from Charteris et al. (1998).

As regard *S. macedonicus* strains*,* the antimicrobial susceptibility to 14 relevant antibiotics is reported in Table 2.3. All *S. macedonicus* strains were susceptible to erythromycin, ciprofloxacin, cloxacillin, penicillin G, tetracycline, amoxicillin, ampicillin, cephalexin, chloramphenicol, and vancomycin. On the other side, all strains were resistant to streptomycin, kanamycin, and trimethoprim. Regarding gentamycin, all *S. macedonicus* strains were resistant, with the only exception of strain 27MV. Regarding information on antibiotic susceptibility in *S. macedonicus*, only limited data are available (208, 293). Resistance to kanamycin, streptomycin, and trimethoprim could be considered natural since they are found for all *S. macedonicus* strains without exceptions. Considering the few data available on *S. macedonicus*, Zoumpopoulou et al., (2008) found a strain resistant to kanamycin, while no information was previously available on streptomycin and trimethoprim. Regarding gentamycin, in a study by Ozteber, (294) 6 *S. macedonicus* strains out of 11 were reported to be resistant to gentamycin. These data, compared with those presented in other studies, indicate that resistance to gentamycin can be considered acquired in *S. macedonicus* species. Therefore, it needs to be investigated for the presence of an added gene in the genome. Regarding *S. macedonicus* strains used in this study, no added genes

were detected when the genome was analyzed by *in-silico* approach and they can be therefore considered safe to be used.

Table 2.3: Antibiotic susceptibility of S. *macedonicus* strains measured as diameters (mm) of inhibition haloes.

Antibiotic	Amount (µg)	Strains							
		8SP	**19AS**	**27MV**	**203MA**	**62AS**	**33MO**	**211MA**	**GG**
Amoxicillin	10	S	S	S	S	S	S	S	MS
Ampicillin	10	S	S	S	S	S	S	S	S
Cephalexin	30	S	S	S	S	S	S	S	R
Chloramphenicol	30	S	S	S	S	S	S	S	S
Ciprofloxacin	5	S	S	S	S	S	S	S	R
Cloxacillin	5	S	S	S	S	S	S	S	MS
Erythromycin	15	S	S	S	S	S	S	S	S
Gentamycin	10	R	R	S	R	R	R	R	R
Kanamycin	30	R	R	R	R	R	R	R	R
Penicillin G	10 IU	S	S	S	S	S	S	S	S
Streptomycin	10	R	R	R	R	R	R	R	R
Tetracycline	30	S	S	S	S	S	S	S	S
Trimethoprim	5	R	R	R	R	R	R	R	R
Vancomycin	30	S	S	S	S	S	S	S	R

Susceptibility is indicated with S, moderate susceptibility with MS and resistance with R. Cutoff values are taken from Charteris et al. (1998).

3.3.2. Hemolytic activity test

β-hemolytic activity is one of the main safety concerns besides antibiotic resistance that must be assessed for new isolates. Indeed, *in-vitro* investigation of β-hemolytic activity on Blood Agar medium even for bacterial species that are recognized GRAS is strongly recommended (176). In this study, none of the LAB strains indicated β-hemolytic activity while *S. aureus* ATCC6538, used as positive control, clearly showed β-hemolytic activity.

3.3.3. Biogenic amines production

Amino acids decarboxylation by bacteria and production of biogenic amines can be found in many foods, particularly in fermented ones such as beer, wines, and cheeses (295). Although low levels of biogenic amines, in general, could be tolerable by people, the ingestion of high amounts of these molecules , especially tyramine and histamine, can provoke food intoxication (82). Regarding the LAB strains used in this study, a qualitative analysis was done for tyramine and

histamine, which are the most common biogenic amines produced by LAB. The analysis indicated that among *S. thermophilus* strains, only *S. thermophilus* MTH17CL396 was able to produce both tyramine and histamine, while *S. thermophilus* TH1436 produced only tyramine. As regard to *S. macedonicus, L. paracasei,* and *L. rhamnosus* strains, none of them were able to produce biogenic amines or could produce very low amounts that were not enough to visually change the color of the medium to purple (83, 296). From the number of studies on biogenic amines production by LAB (83, 296) it can be deduced that this ability is strain dependent and should be evaluated for different strains of the same species. Regarding *S. thermophilus* MTH17CL396 and TH1436 that were able to produce tyramine and histamine, *in-silico* analysis was performed to look for genes related to biogenic amines production in the genome. Results indicate that among *S. thermophilus* strains, seven out of eight strains possess the histidine decarboxylation cluster *hdc,* however histamine production was detected only in *S. thermophilus* MTH17CL396. This result shows that there is a weak correlation between production of histamine and presence of the hdc gene, which is in accordance with (296). It should be also mentioned that production of histamine in *S. thermophilus* can be affected by environmental conditions, as proved by (297) that found hdcA expression upregulated under particular conditions, such as 2% NaCl. Regarding tyramine producing strains, tdcA was not detected in any of the *S. thermophilus* strains used in this work. This result shows that other gene(s) could be involved in this pathway, as hypothesized for hdcA (296).

3.3.4. Resistance to simulated gastrointestinal conditions

Resistance and survival to the gastrointestinal juices during passage through the human gut is the key factor for the probiotic strains to benefit the host (298). Many studies have investigated the susceptibility of different species of LAB to gastrointestinal conditions (278, 299). The human stomach has usually pH around 1.3 to 2.5 during fasting and can reach up to 4.5 soon after a meal (300). Therefore, pH 2.5 was chosen to assess the LAB ability to tolerate gastric juice. Many studies reported survival of different *Lactobacillus* strains at that pH (301). However, the survival of *Streptococcus* strains has been a controversial issue among the researchers. Indeed, it is very well known that probiotic properties are strain specific and this gives a strong motivation to keep seeking better strains (302). In our study, this assessment was done in two successive steps. First, the LAB strains were inoculated and incubated in artificial gastric juice for 60 min

and then immediately transferred into the intestinal juice for 180 min (short incubation) followed by further 120 min (prolonged incubation, total 300 min).

Lactobacillus strains indicated a very good resistance to gastric juice (Fig. 2.1), as viability decrement was always below 1 log and strains DTA72 and DTA83 revealed the lowest viability loss, together with the commercial strain GG. These results on the resistance of *Lactobacillus* strains to gastric juice in the presence of mucin and pepsin at pH 2.5 are comparable with data available in the literature (278, 299). *S. thermophilus* strains also showed good viability regarding gastric juice since the reduction was less than 1 log in most of the cases and strain TH985 resultede the most resistant (Fig. 2.2). On the other side, *S.macedonicus* strains indicated very weak resistance to gastric juice as six out of eight strains indicated viability decrements above 1 log (Fig. 2.3). *S.macedonicus* 8SP showed the highest viability loss, equal to 3.9 log reductions, among the LAB tested.

Regarding incubation in gastrointestinal juice, after 180 min all *Lactobacillus* strains showed a statistically significant reduction, with the only exception of DTA79 that did not show any significant decrease after 180 min in gastrointestinal juice. As regard to prolonged incubation (300 min) in gastrointestinal juice, strains showed a further significant decrease in viability, with the exception of strains DTA105 and DTA96 that maintained the same level of viability. *S. thermophilus* strains also showed very good resistance to intestinal juice as well. Strain TH985 was the most resist strain after 180 min while it had a dramatic decrease after prolonged 300 min incubation. On the other side, we have seen very good resistance to intestinal juice both after 180 and 300 min from *S. macedonicus* strains as none of the strains had more than 1 log viability loss during the interaction with intestinal juice. Indeed, *S. macedonicus* strains were the most resistant strains regarding intestinal juice among all LAB tested in this study.

The resistance of *Lactobacillus* species to the gastrointestinal condition has been reported by different studies (303, 304). Such resistance could be due to the preservation of internal pH, functionality, and integrity of cell membrane, and existence of bile salts efflux pumps (305–307). Different studies also reported the survival of *S. thermophilus* strains after passing through the human gastrointestinal tract. Some studies have reported that they could not recover *S. thermophilus* from human feces (308) while, on the other side, Brigidi (309) could recover *S. thermophilus* strains, from stool samples of 10 healthy subjects who had been treated orally for 3 days. In another study by (310), they reported a significant recovery of viable *S. thermophilus* in

human stool after consumption of yogurt. As regard to *S. macedonicus,* our results were in accordance with results obtained by (311) that reported a huge reduction in cell viability of *S. macedonicus* strains after exposure to pH 3. Regarding intestinal condition, *S. macedonicus* strains were reported quite resistant to bile salts (293, 311). According to our results, some newly isolated strains indicated better performances in comparison with the commercial *L. rhamnosus* GG. Indeed, most *S. thermophilus, L. rhamnosus, L. paracasei* strains indicated just a slightly lower resistance to the gastric juice (Fig. 2-1); however, *L. rhamnosus* GG showed much less tolerance to the intestinal juice (1.5-log decrease), especially after prolonged incubation with 3.4-log decrease. Besides, considering that probiotics are mainly used in food or milk-based products, it is worth mentioning that some works demonstrated how food composition can play a significant role in protecting these bacteria due to the protein, fat and other compositions (278, 299).

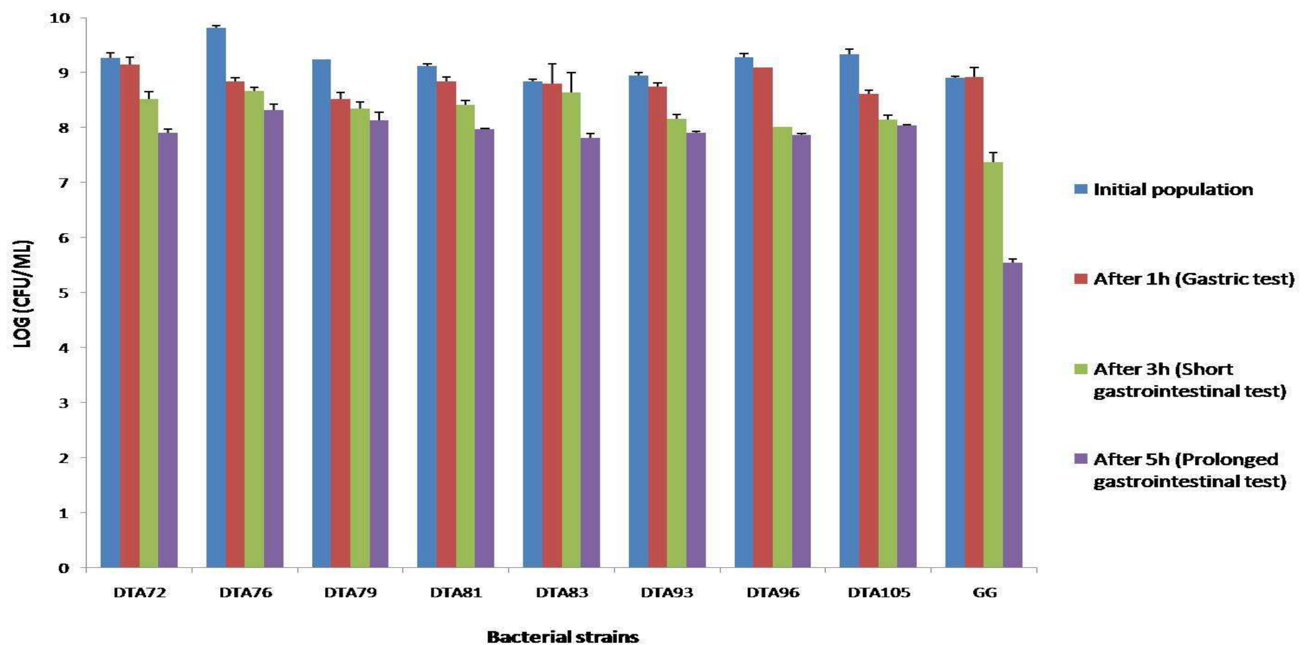


Fig.2.1: Survival of *Lactobacillus* strains and *L. rhamnosus* GG during exposure to *in vitro* simulated gastrointestinal conditions. Results are expressed as the mean ± SD (n=3).

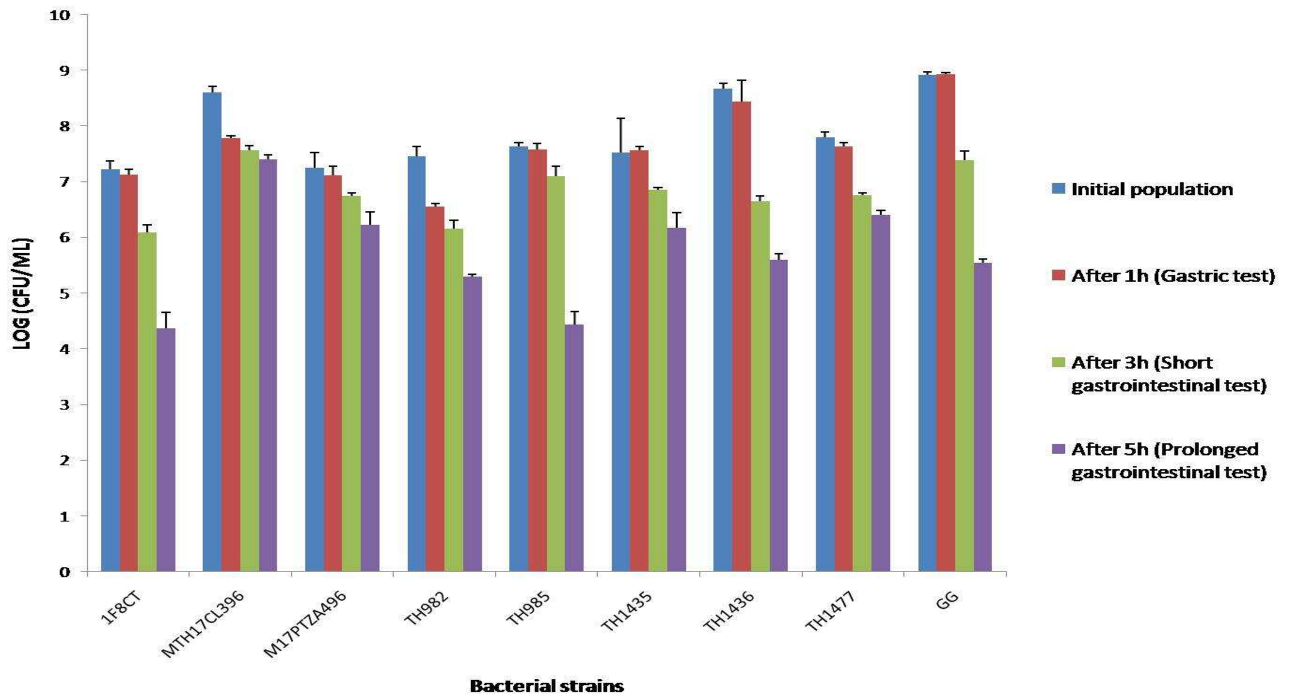


Fig.2.2: Survival of *S. thermophilus* strains and *L. rhamnosus* GG during exposure to *in vitro* simulated gastrointestinal conditions. Results are expressed as the mean ± SD (n=3).

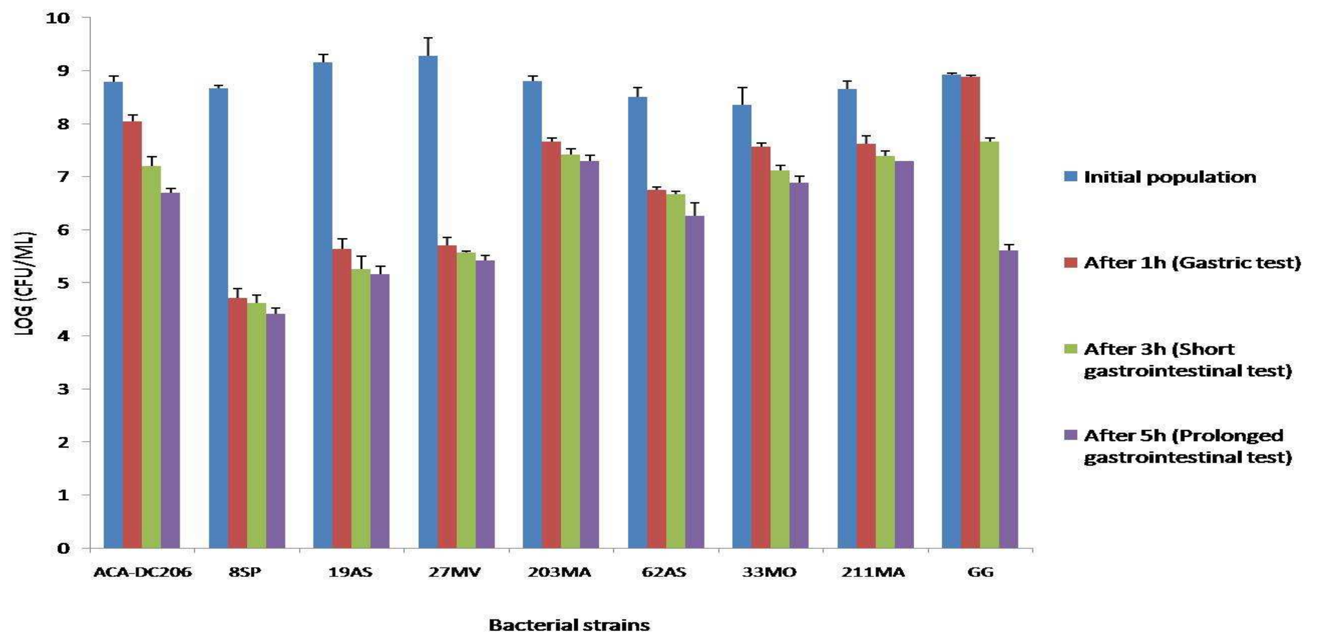


Fig.2.3: Survival of *S. macedonicus* strains and *L. rhamnosus* GG during exposure to *in vitro* simulated gastrointestinal conditions. Results are expressed as the mean ± SD (n=3).

3.3.5. Bile salts hydrolytic activity

None of the tested *L. rhamnosus, L. paracasei, S. thermophilus, S. macedonicus* strains showed bile salt hydrolytic (BSH) activity when grown on MRS and M17 agar containing 0.5% taurodeoxycholic acid. BSH activity of probiotics has been source of a controversial debate during the last years. Although BSH is somewhat related to intestinal survival of probiotics and cholesterol lowering in the human host, however, it is not an absolute desirable property for probiotics, since de-conjugated bile salts could have many undesirable effects for the human host (120, 312).

3.3.6. Extracellular vitamins production

Extracellular production of riboflavin (vitamin B2), folate (vitamin B9), and cobalamin (vitamin B12) by different LAB strains were evaluated using microbiological assays. Extracellular vitamin concentration was monitored after 24 h of bacterial growth to describe the trend of its production in the studied strains. Production of riboflavin was not detected in any of the tested LAB. As regards folate, all *S. thermophilus* strains were able to produce it in different amounts while *L. rhamnosus, L. paracasei, S. macedonicus* strains did not show any production of folate (table 2-4). Extracellular folate production by *S. thermophilus* strains in this study ranged from 5.06 to 147.67 ng/ml. Strains M17PTZA496 and TH982 gave the highest values, i.e., 147.67 and 95 ng/ml, respectively that was higher than that values found in the literature (272). The ability to produce folic acid is one of great interest for a potential probiotic strain, since it has been demonstrated that consume of folate-producing probiotics can increase plasma folate concentration in humans (27). Folate is an important factor in the human diet, being involved in essential functions of cell metabolism such as DNA replication, repair, and methylation and synthesis of nucleotides. Several works report that folate deficiency is quite widespread among people, particularly in women (313). Recently, some studies reported that high-folate diets can work against cardiovascular diseases (314) and some forms of cancer (315). Different LAB species and strains have different abilities in folate production. *Lactobacillus* strains normally do not produce folate with the exception of *L. plantarum*, *L. lactis*, while *S. thermophilus* strains are usually considered strong producer of folate (272, 316). Regarding cobalamin, *L. rhamnosus* was the only species that did not produce any detectable amount of cobalamin. On the other side, four strains of *L. paracasei,* seven strains of *S. thermophilus,* and 3 strains of *S. macedonicus* were able to produce cobalamin in a low amount (Table 2.4).

Table 2.4: Different vitamins production detected using the microbiological assay. Results are expressed as the mean ± SD (n=3).

		Vitamin production		
		Riboflavin (ng/mL)	Folate (ng/mL)	Cobalamin (pg/mL)
S. thermophilus				
	1F8CT	-	17.00 ± 1.73	10.00 ± 0.03
	MTH17CL396	-	14.66 ± 2.30	5.00 ± 0.02
	M17PTZA496	-	147.30± 6.65	7.50 ± 0.02
	TH982	-	95.00 ± 5.29	12.50 ± 0.04
	TH985	-	22.66 ± 2.30	7.50 ± 0.00
	TH1435	-	28.66 ± 1.52	7.50 ± 0.00
	TH1436	-	18.33 ± 1.15	7.50 ± 0.02
	TH1477	-	25.33 ± 1.15	-
S. macedonicus				
	8SP	-	-	-
	19AS	-	-	-
	27MV	-	-	-
	203MA	-	-	12.50 ± 0.01
	62AS	-	-	-
	33MO	-	-	7.50 ± 0.01
	211MA	-	-	12.50 ± 0.01
L. paracasei				
	DTA72	-	-	7.00 ± 0.04
	DTA76	-	-	-
	DTA79	-	-	-
	DTA81	-	-	7.00 ± 0.03
	DTA83	-	-	-
	DTA96	-	-	2.50 ± 0.03
L. paracasei	DTA93	-	-	7.50 ± 0.05
	DTA105	-	-	-

3.3.7. Adherence ability to HT-29 cells

The capability to attach to human intestinal cells is a very important characteristic for probiotic bacteria to stably colonize the host gut. Results of the adhesion test for all *Lactobacillus* strains are reported in Table 2.5. In Figure 2.4 we can observe some images of *Lactobacillus* strains adhesion to HT-29 colorectal cancer cells. According to the results, strains DTA79, DTA81, and DTA93, strongly adhered to HT-29 cells whilst strains DTA76 and DTA96 indicated adhesive characteristic and strains DTA72, DTA83 and DTA105 were non-adhesive. The *in-vitro* adherence ability of probiotics to HT-29 cells line has been frequently considered during the last

decades (317–319). Two different ways have been reported by which bacteria can attach and interact with cell surfaces, i.e. specific or non-specific. The non-specific is a consequence of the physicochemical properties of the cell wall, especially its outer constituents (320) and depends on the hydrophobic properties of the surfaces and on the balance of electrostatic interactions (321). On the other hand, specific attachment is related to the recognition of a particular site or ligand by a receptor on the bacterial surface (320). Many lactobacilli possess this specific interactions, and the adhesion ability of *Lactobacillus* strains has been related to this specific interactions (322, 323).

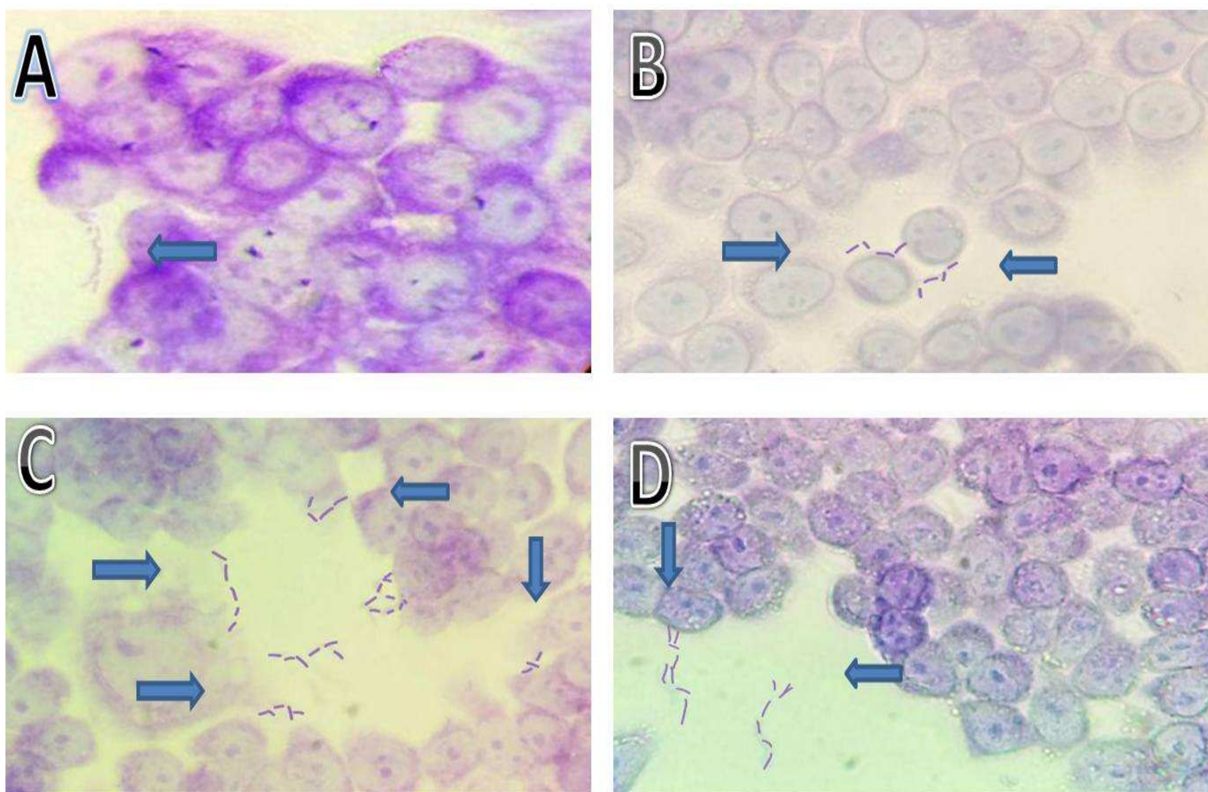


Figure 2.4: Adhesion of *Lactobacillus* strains to HT-29 cells observed under the optical microscope (1000X). Strains: A) GG, B) DTA79, C) DTA81, D) DTA93.

In our study, three out of eight lactobacilli, namely strains DTA79, DTA81, and DTA93, indicated strong adherence to HT-29 cells line; among these, *L. paracasei* DTA81 showed a dramatically strong adherence ability, about ten times higher and stronger than that of the commercial strain *L. rhamnosus* GG, thus indicating it as a very interesting probiotic candidate.

Table 2.5: Adhesion potential of *Lactobacillus* strains. Scores are the average number of adhering cells in 20 microscopic fields ± SD (*n* = 3).

Strain	Adhesion score	Category
L. paracasei DTA72	25.5 ± 2.3	Non-adhesive
L. paracasei DTA76	46.4 ± 4.02	Adhesive
L. rhamnosus DTA79	359.1 ± 7.2	Strongly adhesive
L. paracasei DTA81	4044.0 ± 10.2	Extremely adhesive
L. paracasei DTA83	20.6 ± 1.9	Non-adhesive
L. paracasei DTA93	294.5± 5.2	Strongly adhesive
L. paracasei DTA96	41.1± 2.7	Adhesive
L. rhamnosus DTA105	28.6 ± 3.5	Non-adhesive
L. rhamnosus GG	420.8 ± 8.1	Strongly adhesive

Regarding adherence ability of *S. thermophilus* strains to HT-29 colorectal cancer cells, results are reported in table 2.6. Figure 2.5 also shows their adhesion to HT-29 colorectal cancer cells and its related array. According to the results, strains MTH17CL396, M17PTZA496, TH982, TH985, TH1435, and TH1436 were strongly adhesive whilst the others indicated a non-adhesive character. Moreover, strains MTH17CL396, M17PTZA496, TH982, and TH985 showed no significant difference ($P < 0.05$) in adhesion score with respect to the commercial *L. rhamnosus* GG. Adherence ability of different *S. thermophilus* strains has been reported in several studies (134, 324). Extracellular polysaccharides production and strong cell surface hydrophobicity were reported as the main reasons for this characteristic in bacteria (325, 326). Indeed, a previous study (50) reported that strains MTH17CL396, M17PTZA496, and TH982 are good producers of exopolysaccharides. On the other side, many studies reported that GIT survival of probiotics following oral administration can be directly connected to the colonization of the intestine by attaching to the epithelium (309, 327, 328). Another study (134) revealed that the presence of lactose enhanced the fermentative activity of *S. thermophilus* leading to a higher level of luminal lactate which subsequently acts to modulate the host epithelium.

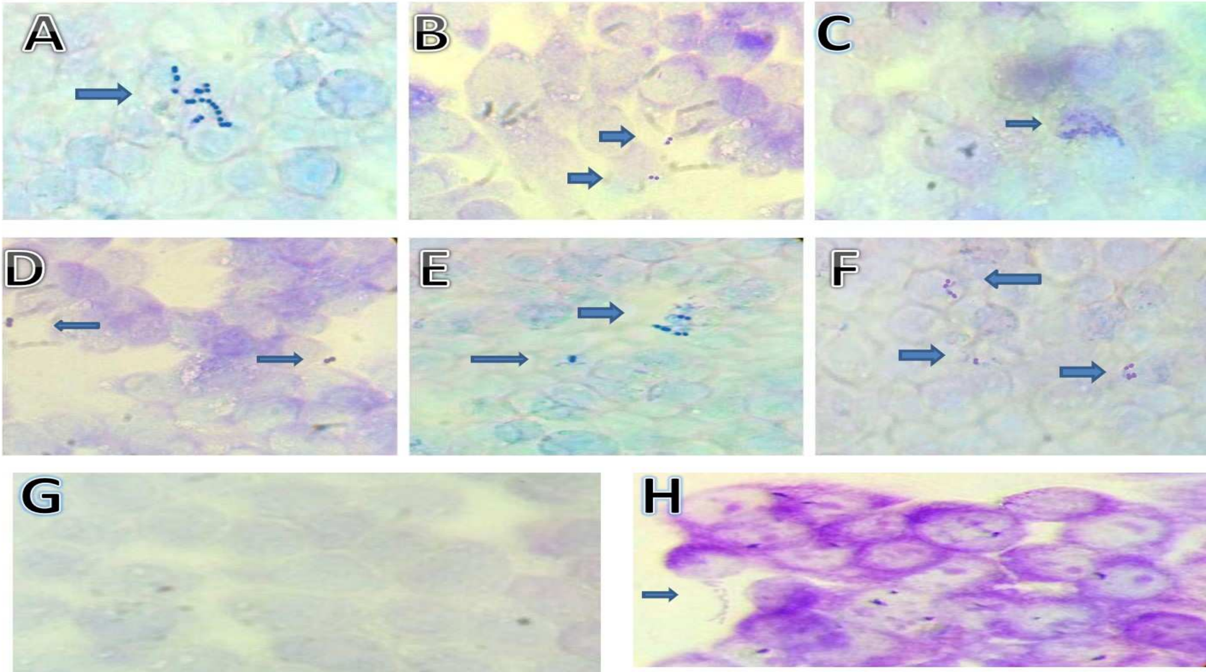


Figure 2.5: Adhesion of *S. thermophilus* cells on HT-29 cell cultures observed under a light microscope (100X). **Strains: A)** *S. thermophilus* MTH17CL396, **B)** *S. thermophilus* M17PTZA496, **C)** *S. thermophilus* TH982, **D)** *S. thermophilus* TH985, **E)** *S. thermophilus* TH1435, **F)** *S. thermophilus* TH1436, **G)** Blank HT-29 cell line, **H)** *L. rhamnosus* GG

Therefore, activation of enzymes involved in carbohydrate metabolism constitutes the metabolic signature of *S. thermophilus* in the GIT and favors the interaction with the colon epithelium.

Table 2.6: Adhesion potential of *S.thermophilus* strains. Scores are the average number of adhering cells in 20 microscopic fields ± SD (n = 3).

Strains	Adhesion score	Category
S. thermophilus 1F8CT	14.8 ± 2.3	Non-adhesive
S. thermophilus MTH17CL396	383.9 ± 8.0	Strongly adhesive
S. thermophilus M17PTZA496	363. 3± 8.5	Strongly adhesive
S. thermophilus TH982	500.3 ± 6.0	Strongly adhesive
S. thermophilus TH985	456.1 ± 7.6	Strongly adhesive
S. thermophilus TH1435	506.1 ± 8.1	Strongly adhesive
S. thermophilus TH1436	1062.3 ± 9.1	Strongly adhesive
S. thermophilus TH1477	11.6 ± 1.9	Non-adhesive
L. rhamnosus GG	420.8 ± 8.1	Strongly adhesive

With regard to *S. macedonicus*, strains 203MA and 211MA were strongly adhesive while strain 8SP indicated normal adhesive characteristic and other *S. macedonicus* strains were non-adhesive (Table 2.7 and Fig. 2.6). There are very limited studies on the adhesion ability of *S. macedonicus* to intestinal epithelial cells in the literature. In a study by (329), a weak adhesion to HT-29 cells was reported for *S. macedonicus* strains. Bacterial cell surface structure plays a significant role in their adhesion ability. For instance, extracellular polysaccharides production or surface proteins such as pili can increase this ability (330, 331).

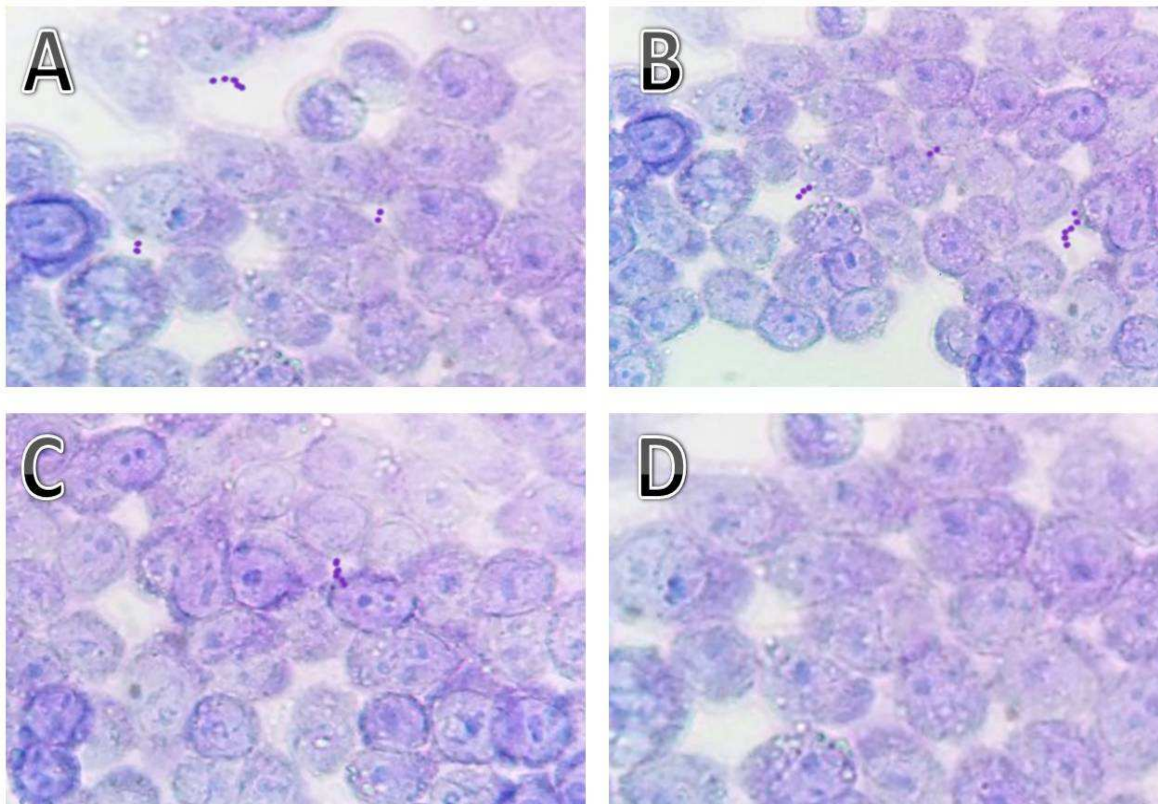


Figure 2.6: Adhesion of *S. macedonicus* cells on HT-29 cell cultures observed under a light microscope (100X). **Strains: A)** *S. macedonicus* 211MA, **B)** *S. macedonicus* 19AS, **C)** *S. macedonicus* 27MV, **D)** Blank HT-29 cell line.

On the other hand, the presence of the operon pil3 in *S. macedonicus* ACA-DC 198 was reported by (245). In *S. gallolyticus* which is phylogenetically close to *S.macedonicus*, pil3 plays a significant role in attachment to HT29 cells and different adhesion ability coul.d be related to the different level of pili encoding-gene expression in different strains (332).

Table 2.7: Adhesion potential of *S. macedonicus* strains. Scores are the average number of adhering cells in 20 microscopic fields ± SD (*n* = 3).

Strain	Adhesion score	Category
S. macedonicus 8SP	99.8 ± 6.7	Adhesive
*S. macedonicus*19as	73 ± 5.4	Adhesive
S. macedonicus 27MV	77.8 ± 4.2	Adhesive
S. macedonicus 203MA	1308.6 ± 6.5	Strongly adhesive
S. macedonicus 62AS	33.5 ± 3.9	Non-adhesive
S. macedonicus 33MO	38.6 ± 3.0	Non-adhesive
S. macedonicus 211MA	1229.4 ± 9.6	Strongly adhesive
L. rhamnosus GG	420.8 ± 8.1	Strongly adhesive

3.3.8. Cytotoxic activity against HT-29 cells

Colorectal cancer is a disease that mostly has been studied by Caco-2 and HT-29 cells that can cause death widely in the world. Strains *L. rhamnosus* DTA79, *L. paracasei* DTA93, DTA96, DTA81, and *S. thermophilus* MTH17CL396, M17PTZA496, TH982, which had indicated good adhesion activity to HT-29 cells and revealed good probiotic potential were examined for anti-cancer activity. According to the results reported in Table 2.8, some lactobacilli supernatants indicated strong anti-cancer activity against HT-29 cancer cells. In comparison with commercial probiotic strains, no significant difference ($P < 0.05$) was found between *L. paracasei* DTA93 and *L. rhamnosus* GG when tested with multiple comparison tests (Tukey's test). The *L. paracasei* DTA96 was the only strain that did not show any anti-proliferative activity. *L. paracasei* DTA81 showed the strongest anti-cancer activity with 39.4%±0.05, 34.1%±0.03, 21.5%±0.02 at concentrations of 2000, 4000, 8000 µg/mL respectively (table 2-8). However, the half minimal inhibitory concentration (IC50) value indicated that there is no significant difference between *L. paracasei* DTA93, *L. paracasei* DTA81 and the commercial strain *L. rhamnosus* GG (table 2.9). Many studies have reported during the last decades the anti-cancer activity of lactobacilli such as *L. acidophilus* and *L. casei* against the proliferation of tumor cells (164). A recent study by Haghshenas (333) reported that production of metabolites such as bioactive peptides by lactobacilli can play an important role in cytotoxicity by linking to pre-carcinogenic molecules, carcinogenic enzymes or mutagenic compounds or by exerting some immunomodulatory effect (334). On the other hand, *L. casei* induced up-regulation of TRAIL protein expression (157), known to selectively induce apoptosis in many tumor cell lines without

affecting normal cells and tissues, thus appearing as a promising therapeutic drug (158). In addition, they showed good antiproliferative activity against cervix cancer (HeLa) cells by upregulating the expression of apoptotic genes namely, caspase3, caspase8, caspase9, BAX, and BAD (156).

Table 2.8: Anti-cancer effect of lyophilized supernatants collected from lactobacilli cultures on HT-29 cancer cells after 48 h.

Supernatant	HT-29 cells viability (%)					
concentration (µg/mL)	DTA79	DTA81	DTA93	DTA96	GG	MRS medium
125	97.48 ± 0.28	97.81 ± 0.03	70.20 ± 0.01	98.20 ± 0.05	65.80 ± 0.01	90.27 ± 0.10
250	74.94 ± 0.12	97.51 ± 0.05	63.00 ± 0.03	86.90 ± 0.06	63.90 ± 0.09	91.67 ± 0.03
500	65.56 ± 0.07	93.10 ± 0.11	59.98 ± 0.07	88.30 ± 0.11	58.90 ± 0.02	90.03 ± 0.05
1000	57.33 ± 0.05	53.27 ± 0.07	47.42 ± 0.01	84.70 ± 0.06	54.10 ± 0.02	89.47 ± 0.28
2000	46.64 ± 0.03	39.42 ± 0.05	44.79 ± 0.10	89.20 ± 0.05	48.20 ± 0.02	88.61 ± 0.19
4000	37.78 ± 0.00	34.09 ± 0.03	40.48 ± 0.01	90.50 ± 0.05	41.10 ± 0.03	82.63 ± 0.20
8000	31.27 ± 0.07	21.48 ± 0.02	28.56 ± 0.02	88.80 ± 0.24	29.10 ± 0.02	89.44 ± 0.06

Table 2.9: IC_{50} of *Lactobacillus* strains against HT-29 cell line (All values are mean ± SD of 2 experiments).

Strains	IC_{50} (mg/mL)[1]
L. rhamnosus DTA79	1.96 ± 0.20
L. paracasei DTA81	1.40 ± 0.25
L. paracasei DTA93	1.30 ± 0.13
L. rhamnosus GG	1.42 ± 0.12

[1] IC_{50}: half minimal inhibitory concentration

Regarding *S. thermophilus,* HT-29 cells were significantly inhibited by MTH17CL396, M17PTZA496, and TH982 compared to the untreated cancer cells (Table 2.10). For all different concentrations, no significant difference ($P < 0.05$) was found between *S. thermophilus* M17PTZ396 and *L. rhamnosus* GG when tested in a multiple comparison test (Tukey's test). The half minimal inhibitory concentration (IC50) value revealed that there is no significant difference between *S. thermophilus* M17PTZ396 and the commercial strain *L. rhamnosus* GG, while values

from *S. thermophilus* M17PTZA496 and TH982 were lower (table 2.11). According to the results of our study, all three *S. thermophilus* strains showed an anti-proliferative effect on HT-29 cancer cells (Table 2.10). To exclude that such activity could be due to the lactic acid molecules produced by all *S. thermophilus* strains examined, it is worth noticing that strain MTH17CL396, which showed the best anti-cancer activity, has the lowest acidification ability among the *S. thermophilus* strains tested, as previously reported (Vendramin et al., 2017). Mechanisms such as binding and degrading carcinogens, boosting the host's immune response, producing anti-mutagenic compounds, and altering the physiochemical conditions in the colon have been reported as to how LAB can inhibit colon cancer (169, 335). In addition, it has been demonstrated that probiotics can reduce the level of some dangerous enzymes such as azoreductase, β-glucuronidase, glycosidase, and nitroreductase in the human body that can convert the precarcinogens into active carcinogens (308, 336).

Table 2.10: Anti-cancer effect of lyophilized supernatants collected from *S. thermophilus* strains cultures on HT-29 cancer cells after 48 h.

Supernatant concentration (μg/mL)	HT-29 cells viability (%)				
	M17PTZA396	M17PTZA496	TH982	GG	M17 growth medium
125	75.0 ± 0.02	93.7 ± 0.02	98.3 ± 0.01	65.8 ± 0.01	98.2 ± 0.05
250	65.2 ± 0.04	78.0 ± 0.07	71.1 ± 0.03	63.9 ± 0.09	86.9 ± 0.06
500	58.4 ± 0.12	65.3 ± 0.05	71.0 ± 0.05	58.9 ± 0.02	88.3 ± 0.11
1000	45.5 ± 0.07	62.5 ± 0.04	58.5 ± 0.01	54.1 ± 0.02	84.7 ± 0.06
2000	43.4 ± 0.05	61.8 ± 0.01	58.7 ± 0.05	48.2 ± 0.02	89.2 ± 0.05
4000	40.2 ± 0.03	58.8 ± 0.02	54.7 ± 0.04	41.1 ± 0.03	90.5 ± 0.05
8000	37.6 ± 0.03	57.6 ± 0.01	46.0 ± 0.04	29.1 ± 0.02	88.8 ± 0.24

Table 2.9: IC_{50} of *S. thermophilus* strains against HT-29 cell line (All values are mean ± SD of 2 experiments

Probiotic strains	IC_{50}(mg/ml)[1]
S. thermophilus MTH17CL396	0.9 ± 0.2
S. thermophilus M17PTZA496	- ± -
S. thermophilus TH982	5.08 ± 0.4
L. rhamnosus GG	1.42 ± 0.4

[1] IC_{50}: half minimal inhibitory concentration

3.3.9. Biofilm inhibitory activity

Biofilm inhibitory activity of lactobacilli against *E. coli* and *L. innocua* was evaluated using two different approaches. *L. innocua* was selected because it is physiologically very close to the pathogen *L. monocytogenes*, which has strong ability to form biofilm and represents a serious problem for surfaces and industrial settings (337). *E. coli* is an abundant bacterium in the human gut, particularly in the small intestine, and the possibility to displace it represents for a strain a good probiotic potential. Fig. 2.6 indicates the outcome of the biofilm inhibitory activity achieved by inoculating a *Lactobacillus* strains first and subsequently either *E. coli* (Fig. 2.6A) or *L. innocua* (Fig. 2.6B) (exclusion test) and inoculating simultaneously one *Lactobacillus* strain together with either *E. coli* (Fig. 2.6A) or *L. innocua* (Fig. 2.6B) (competition test). In exclusion method, all *Lactobacillus* strains showed inhibitory activity by reducing the number of adhered *E. coli* and *L. innocua* cells to a different extent; however *L. paracasei* DTA81 and DTA93 indicated the highest inhibitory effects by 1.05 and 0.80 log cell reductions, respectively on *E. coli* and 0.58 and 0.60 logs, respectively on *L. innocua*. On the other side, similar results were seen in the competition method where strains DTA81 and DTA93 still were the best biofilm inhibitors by 0.78 and 0.65 log cells reduction, respectively on *E. coli* and 0.29 and 0.42 log cells reduction, respectively on *L. innocua.* The strong inhibitory effect of DTA81 and DTA93 can be related to their strong adhesion ability shown in the HT-29 attachment test. For all lactobacilli, the exclusion effect was always equal or superior to the respective competition one, with the only exception of *L. paracasei* DTA83 that showed good inhibitory activity on *E. coli* during the competition test (0.52 log decrease) but produced a negligible exclusion effect (0.03 log). Similar behavior was shown by DTA83 on another *E. coli* strain, namely ATCC25922 (211), therefore it is worth noticing that this inhibitory effect could be due to some strain-specific antimicrobial activity, such as bacteriocin production, rather than to biofilm activity. This idea is also reinforced by the fact that the same effect was not obtained on *L. innocua* and that *L. paracasei* DTA83 showed the lowest performance to the adhesion to HT-29 cells test.

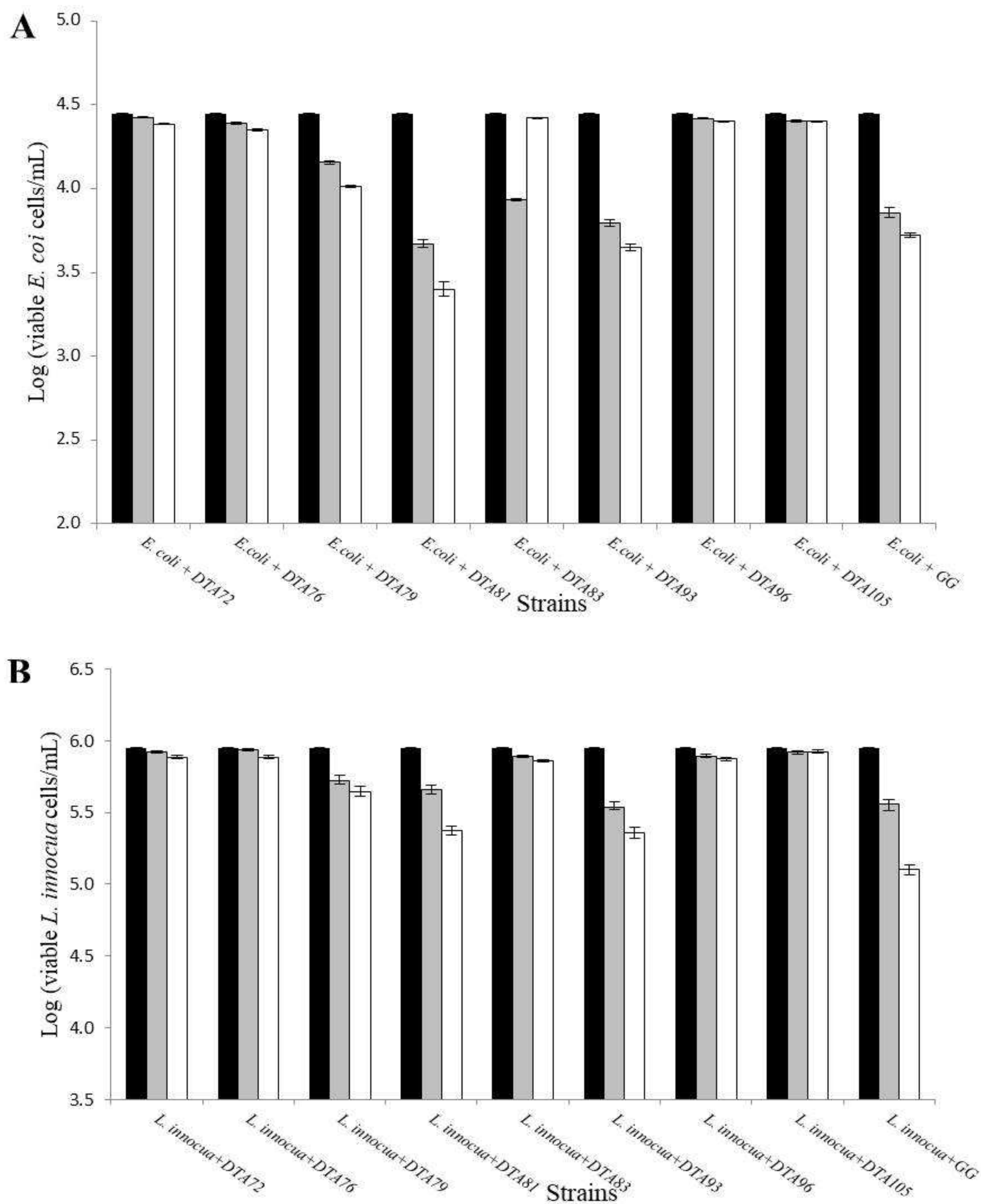


Figure 2.7: Biofilm inhibitory activity of *Lactobacillus* strains against *E. coli* (A) and *L. innocua* (B) in competition and exclusion tests. Results are express as mean ± SD (n = 3) of *E. coli* and *L. innocua* viable cells. Black bars: *E. coli* and *L. innocua* population alone; grey bars: *E. coli* and *L. innocua* after competition test; white bars: *E. coli* and *L. innocua* after exclusion test.

Chapter 3

In-vivo Probiotic Properties and Related Health Benefits on Laboratory Mice

According to the last definition of probiotic by of the FAO/WHO (Food and Agriculture Organization/World Health Organization), probiotics are "non-pathogenic, viable microorganisms that when administrated in adequate amounts, are able to reach and colonize the Gastro-Intestinal (GI) tract and to confer health benefits to the host (338)". Probiotic foods represent a tremendous functional food available on the market worldwide, projected to reach a value of US $ 46.55 billion by 2020 (258). During the past years, the probiotic potential of many LAB have been studied since they are GRAS microorganisms and can therefore be safely used in foods. Probiotic consumption can benefit human health mainly by modulation of the immune system, affecting the gut microbial composition and production of antimicrobial substances that can contribute to reduce deleterious bacteria and promote the stability of beneficial microbes (339–341). Many studies have revealed the influence of gut microbiota on metabolic disorders and obesity in humans (342, 343). A recent study by Turnbaugh (344) indicated that energy homeostasis and metabolism of the host can be directly influenced by gut microbiota. They have shown that transferring of gut microbiota from obese mice can result in gaining more weight in comparison with a lean mouse when the gut microbiota was transfer to germ-free mice. Nowadays, hypercholesterolemia is reported as a common human disorder which is mostly related to cardiovascular disease (CVD) and coronary heart disease (CHD) (345). Many *in-vitro* and *in-vivo* studies recently reported that probiotics such as *Lactobacillus* and *Bifidobacterium* strains can have beneficial effects on serum lipid profiles (346, 347). Probiotics can also reduce blood cholesterol in different ways such as utilizing prebiotics to produce short-chain fatty acids in the human gut that can further inhibit hepatic cholesterol synthesis and will result in reduction of blood lipids (348) or in a different way, probiotics can assimilate cholesterol directly and reduce its presence in the human gut. Therefore, many probiotic bacteria have been proposed and used as food supplements to reduce the rate of hypercholesterolemia in human (349). In another study by Shimizu et al, they have proved that ingestion of LAB for 4 weeks can result in lowering the blood LDL and triglycerides significantly (350). It has been demonstrated that the probiotic strains are able to modulate the human immune system in different ways. Expression of cytokines in the human body has been the most frequent study related to the immunomodulatory effect of probiotics. Several studies demonstrated an increase in pro-inflammatory cytokines such as IL-12 and tumor necrosis factor-alpha (TNF-α) in the presence of probiotics. In 2000, Haller and colleagues found that human peripheral blood mononuclear cells treated with *L.*

johnsonii and *L. sakei* increase the IFN-γ and IL-12, while the level of IL-10 does not seem to increase (181). In 2015, Wang and colleagues showed that the dialysed patients treated with *B. bifidum*, *B. catenulatum*, *B. longum,* and *L. plantarum* indicated a decrease of serum levels of proinflammatory cytokines TNF-α, IL-5, and IL-6, while levels of serum of IL-10 significantly increased (182). Probiotic strains also can exert some anti-cancer activity by increasing the TNF-α, interferon-γ (IFN-γ), and the regulatory cytokine IL-10. *L. acidophilus*, *L. casei,* and *B. longum* possess immunomodulatory and antitumor properties acting by suppressing the proliferation of tumor cells and decreasing their survival (164). *L. casei* Shirota also have shown strong anti-metastatic effects on tumor cells by suppressing chemically-induced carcinogenesis (351). In addition, the administration of *L. casei* Shirota increased NK cell cytotoxicity which delays tumor onset or suppresses tumor incidence (165). In previous *in-vitro* studies, we have characterized two probiotic potential strains, namely *L. paracasei* DTA81 and *S. thermophilus* TH982 which were found to possess interesting probiotic properties and anti-cancer activity. In particular, strain DTA81 revealed amazingly strong adherence ability to HT-29 cells line. Therefore, these two strains were chosen to be further investigated regarding anti-cancer activity through immunomodulatory effect and anti-obesity activity using an *in-vivo* approach.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial strains and growth conditions

L. paracasei DTA81 and *S. thermophilus* TH982 were routinely grown using MRS medium for *L. paracasei* DTA81 and M17 medium (Difco, United States) containing 0.5% lactose for *S. thermophilus* TH982 and then incubated at 37 °C for 24 h. For *in-vivo* assays, overnight cultures were centrifuged (5,000×g for 5 min), washed two times with sterile PBS, and resuspended in skim milk (10%) to the final concentration of about 10^{10} CFU/mL.

4.2.2. Animals

Thirty-two male laboratory mice, four weeks old, were collected from the Animal House at the Biological Sciences Center of the Universidade Federal de Viçosa to be used in anti-obesity study and immunomodulatory effect indiced by probiotics. All thirty-two mice were housed in a controlled environment (temperature 22 °C, humidity 55±5 %) with 12 h light/dark cycle and they received food and sterilized distilled water (Nuvilab®, São Paulo, Brazil) *ad libitum* except at sampling time when access to food was restricted. Mice body weight (weekly) and food

consumption (daily) were also recorded. Throughout the study, all mice were treated according to the National Research Council guide for the care and use of laboratory animals (352).

4.2.3. Diets and experimental design

All thirty-two animals were left for a week with conventional diet (353) and then were turned to high-fat-diet (HFD) prepared as described in table 3.1. The animals were randomly selected and divided into four different experimental groups (eight animals per group) as group 1 (HFD + skim milk), group 2 (HFD + *L. paracasei* DTA81), group 3 (HFD + *S. thermophilus* TH982), and group 4 (HFD + water). The animals from groups 2 and 3 were treated daily with one oral administration of 100 µl (approx. 10^{10} CFU/mL) of the appropriate probiotics dissolved in skim milk (10%) via gavage while groups 1 and 4 received the same amount of skim milk and water respectively as negative controls for six continuous weeks. After six weeks of treatment, all animals were used for evaluation of food consumption and weight gaining, blood biochemical analysis, oral glucose tolerance test (OGTT), survival of probiotics after transition through the GIT, 16S metagenomics analysis of gut microbiome, and immunomodulatory effects.

Table 3.1: Composition of basal diets for conventional and high-fat diet (g/100g).

Ingredients (g $100g^{-1}$)	Conventional Diet	High-fat Diet
Corn starch	46.56	-
Fat (lard)	-	31.7
Casein	14	25.8
Dextrinized starch	15.5	16.2
Sucrose	10	8.9
Soybean oil	4	3.2
Microfine cellulose	5	6.5
Mineral mix	3.5	1.3
Vitamin mix	1	1.3
L-cystine	0.18	0.39
Choline bitartrate	0.25	0.3
Potassium citrate	-	2.1
Calcium phosphate	-	1.7
Calcium carbonate	-	0.7
Energy density (kcal/g)	3.76	5.17

4.2.4. Oral Glucose Tolerance Test (OGTT)

Oral glucose tolerance test (OGTT) was performed after six weeks of treatment according to (354) with some modifications. A solution of D-glucose (2g/kg) was prepared and given to each animal using gavage after overnight fasting condition (12 h) and blood was collected from the tail at time 0, 30, 60, and 120 min after oral glucose dosing. The concentration of glucose in blood serum was recorded using Comfort Curve Strips (Roche) and ACCU-CHEK Advantage Glucometer and the GTT was determined by calculating the area under the curve.

4.2.5. Probiotics enumeration after transitioning through the GIT

Survival of probiotics after transition through the GIT was evaluated in the middle (after three weeks) and at the end of the study (after six weeks). Three mice from groups fed with skim milk, *L. paracasei* DTA81, and *S. thermophilus* TH982 were randomly selected and their feces were collected, weighed and resuspended in 10 ml sterilized PBS and serially diluted using the same solution. Then they were plated on MRS medium for *L. paracasei* DTA81 and M17 medium containing 0.5% lactose for *S. thermophilus* TH982 supplemented with kanamycin (64μg/mL) and incubated at 37 °C for 48 h. For the control group (skim milk), both media were used to enumerate the existing LAB cells. Resistance to kanamycin of both strains used in this study had been determined in previous studies (217, 355). After incubation, microbial viability and colony-forming units were determined and reported per gram of wet feces. In addition, five colonies were also randomly taken from each group and investigated for further microscopic and biochemical analysis. It is worth mentioning that the same mice were selected for evaluation in the middle and at the end of the experiment and when feces from the control mice were evaluated on antibiotic-containing M17 and MRS plates, no colonies were observed.

4.2.6. Blood biochemical analysis

After six weeks of treatment, the animals were anesthetized using ketamine (Imalgène, 200mg/kg) and Rompun (Xylasine, 20mg/kg) diluted in NaCl 0.9% and blood samples were collected and centrifuged at 700×g for 10 minutes to obtain the serums that that were immediately examined for total cholesterol, high density lipoprotein (HDL), triglyceride, glutamate-oxaloacetate transaminase (GOT), and glutamate-pyruvate transaminase (GPT) by using Bioclin®, (Diagnostics®, Belo Horizonte, Brazil) and auto analyzer equipment (COBAS MIRA Plus, Roche Diagnostic Systems, Branchburg, NJ). Low density lipoprotein (LDL) was also calculated according to the method of Friedwald et al. (356).

4.2.7. Immunomodulatory effect in colon tissue

The BD CBA Human Th1/Th2/Th17 Cytokine Kit II (BD Biosciences, CA, USA) and BD FACSVers flow cytometer were used to quantitatively measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-17 (IL-17), Tumor Necrosis Factor (TNF), and Interferon-γ (IFN-γ) protein levels in the mice colon samples. To perform local inflammatory cytokine analysis, colon tissue from each animal was collected and the samples were prepared according to the Kit instruction to be further analyzed by BD FACSVers flow cytometer.

4.2.8. 16S metagenomic analysis of gut microbiome

Four mice from each group (*L. paracasei* DTA81, *S. thermophilus* TH982, skim milk, and water) were randomly chosen at the beginning and at the end of the experiment and their feces collected in three days in a row, weighed and total DNA extracted by using the DNeasy PowerSoil Microbial Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were assessed by using 1% agarose gel electrophoresis and Spark 10M (Tecan Trading AG, Männedorf, Switzerland), respectively. The V3-V5 regions of the 16S rDNA genes were PCR amplified and sequenced using an Illumina MiSeq desktop sequencer (Eurofin Genomics Germany GmbH, Ebersberg, Germany) producing 350 bp paired-end (PE) reads. Then 16S rDNA sequenced reads were analyzed using the CLC (version 11.0.1, Qiagen, Hilden, Germany). After that, operational taxonomic units (OTUs) with relative abundance were created for further analysis.

4.2.9. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA). Tukey's test was used as *post hoc* analysis by the GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA, United States). In general, results were considered significantly different when P values were lower than 0.05. The indications * meaning $P < 0.05$, ** meaning $P < 0.01$, *** meaning $P < 0.001$ were used to indicate the level of confidence during the statistical analyses.

4.3. RESULTS AND DISCUSSION

4.3.1. Weight gain and Oral Glucose Tolerance Test (OGTT)

Although after six weeks of experiment the treated mice did not show any significant difference regarding the weight gain, however, weekly monitoring of animals weight showed a lower

average weight in animals treated with *L. paracasei* DTA81 (fig. 3.1 A/B). The influence of probiotic treatments on plasma glucose at selected intervals (0, 30, 60, and 120 min) after six weeks is indicated in (fig. 3.1 C/D). Regarding the Fasting Blood Sugar (FBS), a significant glucose reduction was recorded among the animals treated with *L. paracasei* DTA81 when compared with the control groups fed with skim milk (P < 0.001) or water (P < 0.05) (fig. 3.1 C). However, after receiving the glucose, no significant difference was seen among the groups regarding the glucose tolerance test (fig. 3.1 D).

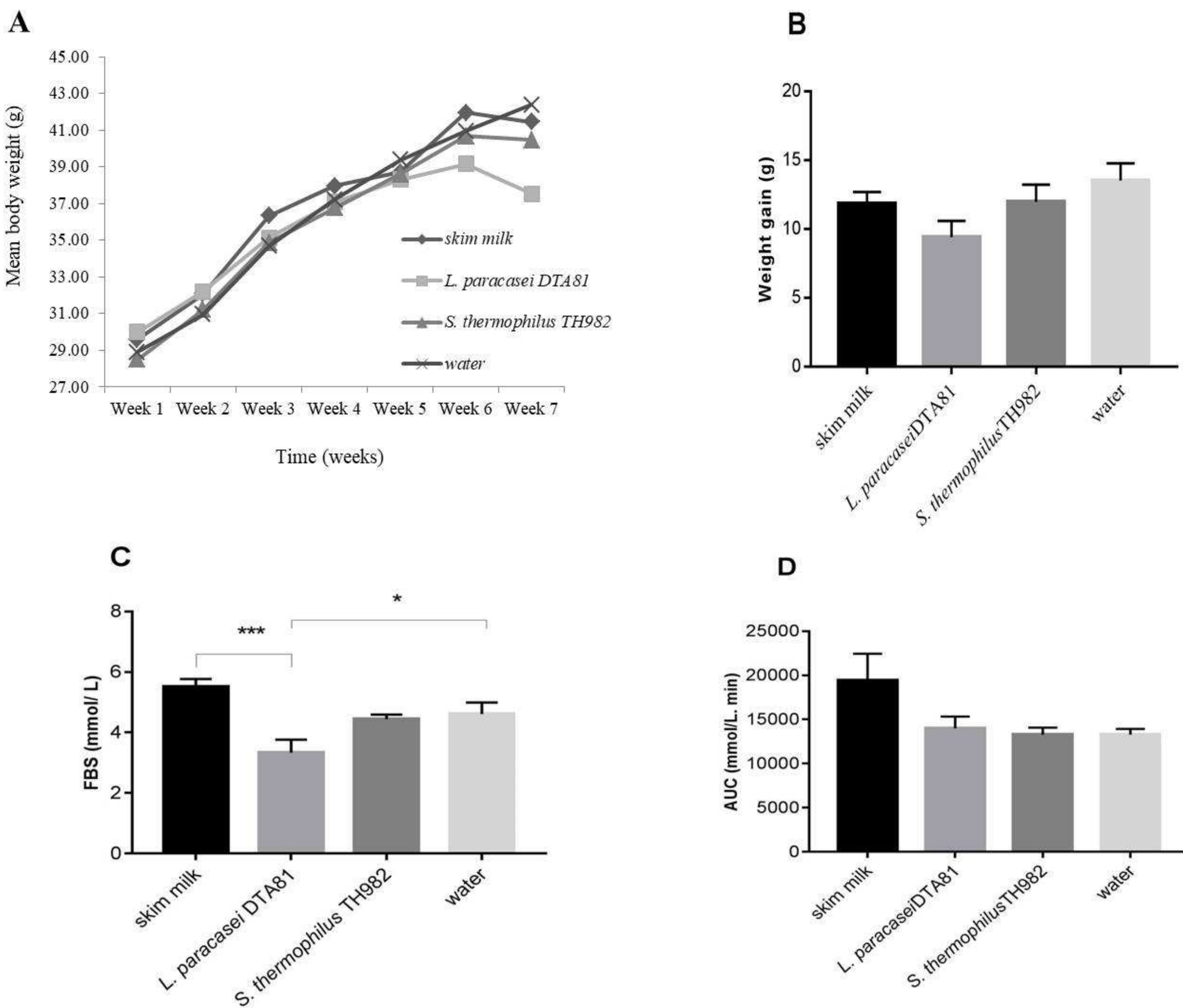


Fig. 3.1: Effect of probiotic consumption on body weight, Fasting Blood Sugar (FBS), and Glucose Tolerance Test. (A) mean body weight; (B) weight gain; (C) Fasting Blood Sugar (FBS); (D) Glucose Tolerance Test. Results are expressed as means ± SEM (n=8).

4.3.2. Probiotics enumeration after passage through the GIT

Survival of probiotics after transition through the GIT was evaluated in the middle and at the end of the experiment and the result is presented in table 3.2. Both probiotic strains *L. paracasei* DTA81 and *S. thermophilus* TH982 dispersed in skim milk were administered (10^9 CFU/day) to mice and enumerated from collected feces using their resistance to kanamycin (64µg/mL). After 21 days (in the middle) of treatment, the probiotic-treated animals were found to excrete approximately Log 8.50 and Log 7.74 CFU/g fecesfor *L. paracasei* DTA81, and *S. thermophilus* TH982 respectively, while no colonies were observed from feces of the control animals evaluated on antibiotic-containing M17 and MRS plates. After 42 days (at the end) of treatment, we did not see any significant changes in the number of retrieved cells for *L. paracasei* DTA81 (Log 8.67) and *S. thermophilus* TH982 (Log 7.71). Many studies have reported survival of different *Lactobacillus* strains (301) . Indeed, it is very well known that probiotic traits are strain specific and it gives a strong motivation to keep seeking better strains (302). In our study, *L. paracasei* DTA81 indicated a better resistance to gastrointestinal environment in comparison with *S. thermophilus* TH982. The resistance of *Lactobacillus* species to the gastrointestinal condition has been reported by different studies (303, 304), that seems to be linked to the preservation of internal pH, functionality, and integrity of cell membrane, and existence bile salt efflux pumps (305–307). Survival of *Streptococcus* strains is less ascertained. Some studies were not able to recover *S. thermophilus* from human feces (308) while, Brigidi (Brigidi et al., 2003) and Mater (Mater et al., 2005) reported isolation of *S. thermophilus* strains from feces samples of people who had received the cells orally ;).

Table 3.2: Bacterial cell enumeration after transitioning through the GIT

	Log10 bacterial cell numbers at time intervals	
	Middle of the treatment (Day 21)	End of the treatment (Day 42)
Skim milk (MRS medium)*	0	0
Skim milk (M17 medium)*	0	0
L. paracasei DTA81 (MRS medium)	8.50 ± 0.16	8.67 ± 0.23
S. thermophilus TH982 (M17 medium)	7.74 ± 0.24	7.71 ± 0.29

*MRS and M17 media were used to detect *L. paracasei* DTA81 and *S. thermophilus* TH982 respectively.

4.3.3. Blood biochemical analysis

Blood biochemical analysis after six weeks of treatment is reported in Figure 3.2. Parameters such as total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglyceride, glutamate-oxaloacetate transaminase (GOT), and glutamate-pyruvate transaminase (GPT) were evaluated among different groups. As indicated in figure 3.2, the total cholesterol and low density lipoprotein (LDL) in the group treated with *L. paracasei* DTA81 showed a significant reduction in comparison with the other groups after six weeks of treatment while there was no significant difference between the skim milk and water groups (controls). However, regarding triglyceride, glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT) and high density lipoprotein (HDL), we have not seen any significant difference among the groups. Although there was a significant difference ($P< 0.05$) between the *L. paracasei* DTA81 group and water group regarding high density lipoprotein (HDL), however, no statistically significant difference was detected between *L. paracasei* DTA81 group and skim milk group. It has been reported that addition of probiotics in gut microbiota can lead to be a potential therapeutic strategy for metabolic disorders such as hypercholesterolemia and obesity (357). Probiotics can reduce blood cholesterol in different ways such as utilizing prebiotics to produce short-chain fatty acids in human gut that can further inhibit hepatic cholesterol synthesis and will result in reduction of blood lipids or, probiotics can assimilate cholesterol directly thus eliminating cholesterol from the human gut (346–348). Although many studies have shown that probiotic consumption can be beneficial for the improvement of hypercholesterolemia, however, the consequence of probiotic consumption in patients who consume probiotics still remained unclear (358). In another study by (350), they could demonstrate that consumption of probiotics in elderly and in hypercholesterolemic patients can be more effective than in youngsters and in individuals with normal lipid levels. The outcome of our study indicates that consumption of *L. paracasei* DTA81 in mice can lead to statistically significant reduction of total cholesterol and low density lipoprotein (LDL) without any significant effect on high density lipoprotein (HDL) that can be very interesting and useful in people who suffer from cardiovascular disease (CVD) and coronary heart disease (CHD).

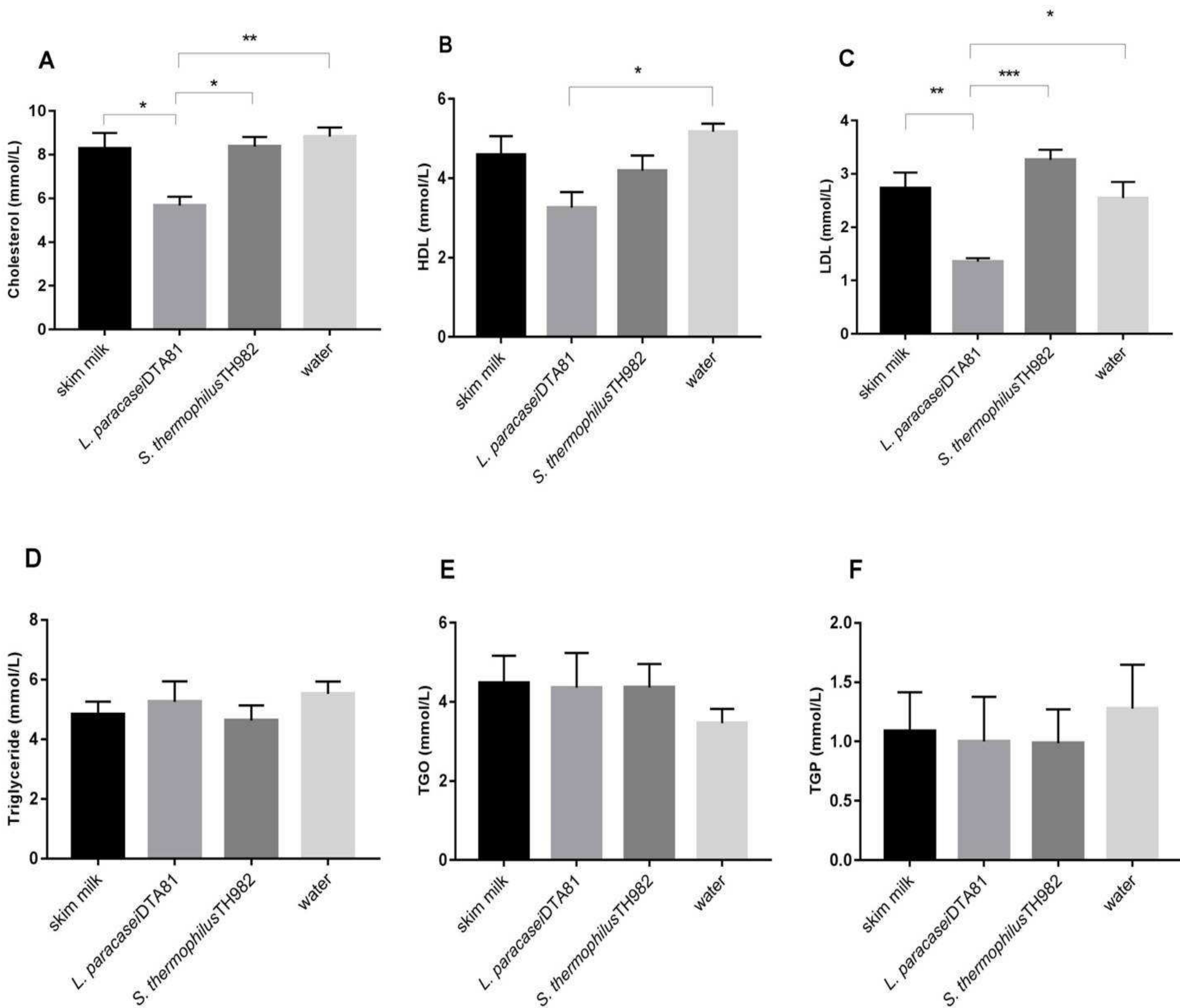


Fig. 3.2: Effect of different treatments on blood parameters. (A) Total cholesterol; (B) High density lipoprotein (HDL); (C) Low density lipoprotein (LDL); (D) Triglyceride; (E) Glutamate-oxaloacetate transaminase (GOT); (F) Glutamate-pyruvate transaminase (GPT); Results are expressed as means ± SEM (n=8).

4.3.4. Inflammatory cytokine analysis in colon tissue

Comparison of colon tissue levels of Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-17 (IL-17), Tumor Necrosis Factor (TNF), and Interferon-γ (IFN-γ) after six weeks of probiotic consumption among the groups is summarized in figure 3.3. The level of cytokines did not reveal any significant difference among the different groups. Cytokines are proteins that are produced by cells and serve as molecular messengers between cells. As determinants and modulators of immune pathology, cytokines play a key regulatory role among the many components of the animal immune system (Lin and Karin,

2007). Cytokine production is largely dependent on the di□erentiation state of T-cells, which can be divided into two di□erent types according to the pattern of cytokine production (Deng et al., 2013). Among the cytokines determined in the present study, IL-2, TNF-α, and IFN-γ are produced by T helper 1 cells and play an important role in the cell-mediated immune response. By contrast, IL-4, IL-6 and, IL-10 are secreted by T helper 2 cells and enhance humoral immunity (Kikuchi and Crystal, 2001). Overproduction or inappropriate production of certain cytokines by the body can result in disease. For instance, overproduction of interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-alpha) are involved in inflammation and tissue destruction. Occasionally, it has been reported that insertion of external bacterial cells inside the human body leads to inappropriate production of certain cytokines which can cause some inflammatory diseases. In our study, none of the strains made a change in the level of cytokines production in the colon. Therefore, they can be considered safe regarding inflammatory diseases.

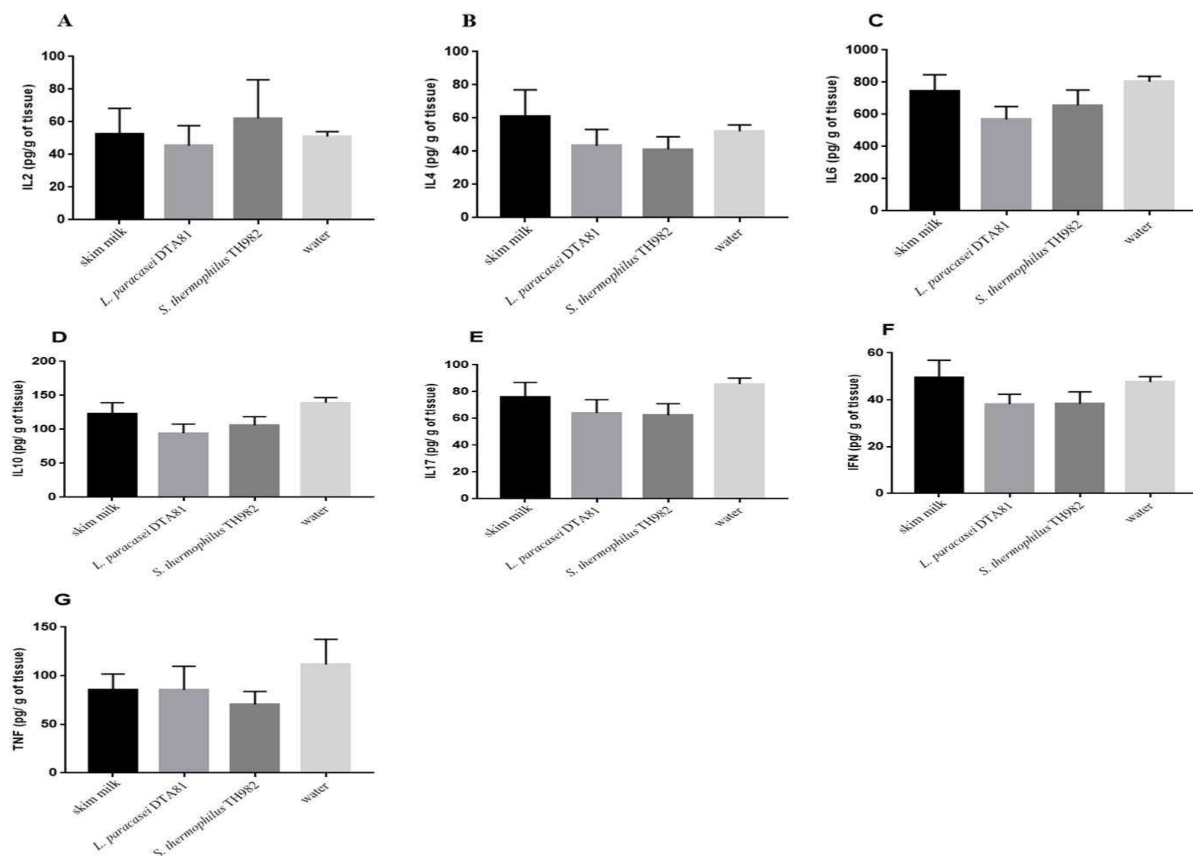
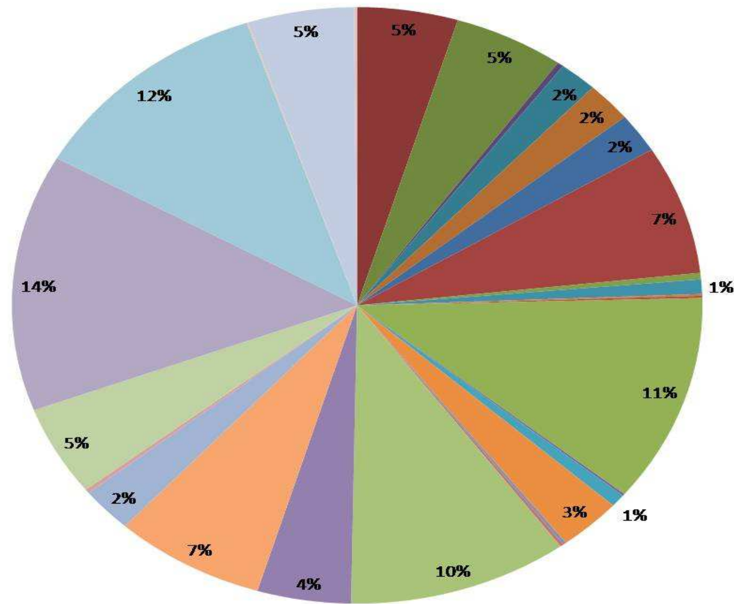


Fig. 3.3: Effect of different treatments on local cytokines. (A) *Interleukin-2*; (B) *Interleukin -4*; (C) Interleukin -6; (D) Interleukin -10; (E) Interleukin -17; (F) Interferon gamma; (G) Tumor necrosis factor. Results are expressed as means ± SEM (n=8).

4.3.5. 16S metagenomics analysis of the gut microbiome

After the clustering process (97% similarity threshold), a total of 79 OTUs were identified. However, only OTUs > 0.1% were considered as the most abundant and used for further analyses. At the phylum level, Firmicutes (78%) was the most predominant, which is in accordance with studies that investigated the gut microbiota in mice (359, 360). Figure 3.4 shows the microbial diversity in both conditions of normal diet (beginning) and high-fat diet (at the end) in all mice used from group 4 (water). At the beginning of the experiment and before starting the high-fat diet, the most abundant genera detected were *Helicobacter* (26%), *Ruminococcus* (11%), and *Oscillospira* (10%) respectively, whilst, after feeding the animals with high-fat diet (after six weeks), the microbial composition ranking changed indicating *Allobaculum* (22%), *Lactobacillus* (21%), and *Bifidobacterium* (12%) as the most abundant genera. Several *in-vivo* studies have reported that a high-fat diet can increase the total colon anaerobic microflora (361–363). In our study, we noticed that high-fat diet increased the abundance of anaerobic genera such as *Allobaculum* and *Clostridium* from 0.002% and 0.09% to 22% and 0.20% respectively. This result is in line with other studies that reported that a high-fat diet can increase colon anaerobic bacteria, such as *Clostridium* (361, 364). Studies in rats also indicated that consumption of a high-fat diet results in more propionate and acetate producing bacteria, including Clostridiales, Bacteroidetes, and Enterobacteriales (353). On the other side, in our study after six weeks of high-fat diet, the presence of *Helicobacter* dramatically decreased from 26% to 9%. In a recent study by (353) related to associations between dietary patterns and *H. pylori* infection, they reported that the high-carbohydrate/sweet diet was positively associated with the prevalence of *H. pylori* infection ($P < 0.001$), while the high-protein/cholesterol diet was associated with a lower prevalence of *H. pylori* infection. Other studies report that the presence of vitamin D can decrease the prevalence of *H. pylori* infection since it can play role as central regulator of host defense against infections (365) and high-protein/cholesterol food (animal foods) are very rich in vitamin D (365, 366).



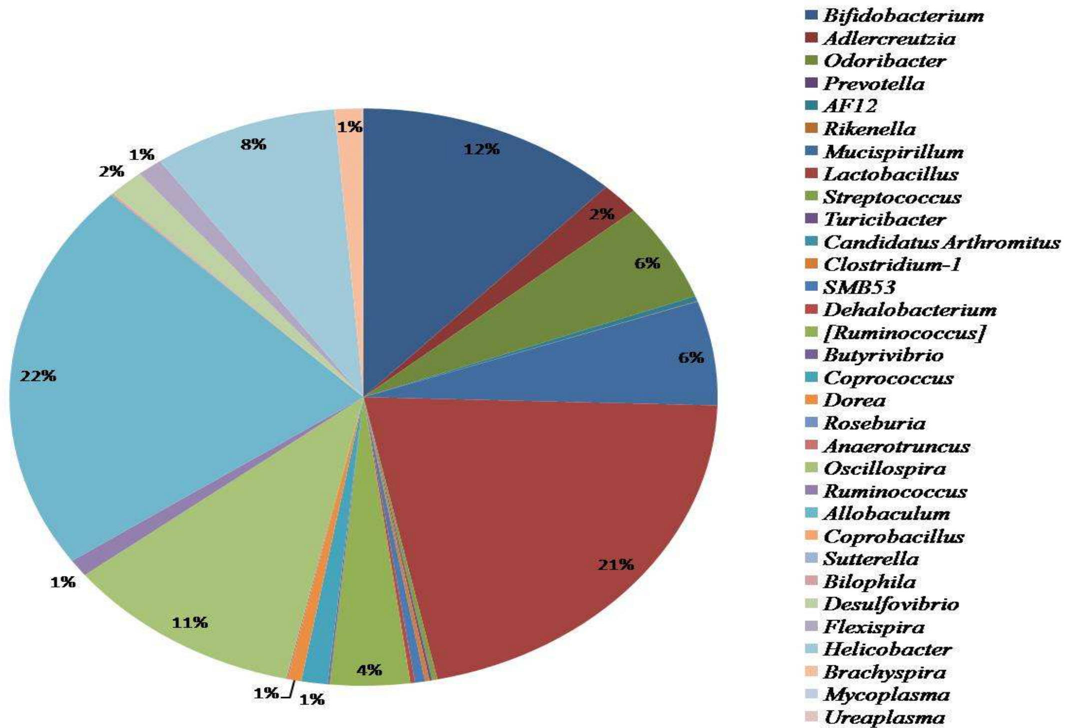


Fig. 3.4: Gut microbiota composition in response to dietary fats. (**A**) Relative abundance of gut microbiota related to normal diet on the genera level. (**B**) Relative abundance of gut microbiota related to high-fat-diet on the genera level.

Figure 3.5 indicates the average microbial diversity following different probiotic treatments and controls at the genus level. After six weeks of treatment, the abundance of the genus *Odoribacter* in both groups treated with probiotics *L. paracasei* DTA81 and skim milk increased in comparison with the same before the treatment, whilst the other groups did not show any change. On the other side, in the group treated with *L. paracasei* DTA81, we had detected some changes after six weeks. Interestingly, the abundance of *Alistipes*, *Bacteroides*, and *Butyrivibrio* genera increased whilst in the other groups we did not see something similar. Surprisingly, the group treated with *L. paracasei* DTA81 had already shown cholesterol-lowering activity in blood biochemical analysis. The heatmap also shows a comparison of gut microbiome among the different replicates of different treatments at genus level (figure 3.6).

In a study on the characterization of the gut microbial community of obese patients following a weight-loss intervention using whole metagenome shotgun sequencing, it was shown that participants, who were able to loose weight effectively over two years, had a microbiota enriched in *Alistipes* genus (367). In another study by (368), the authors indicated that the administration of the probiotic *B. pseudocatenulatum* CECT 7765 significantly increased the proportion of the Rikenellaceae family, particularly of the *Alistipes* genus which has been linked to anti-obesity activity in children. Over the last years, several studies were dedicated to discovering the role of gut microbiota in human health and disease, such as obesity (344, 369), inflammatory bowel diseases (370), type 2 diabetes (371), liver cirrhosis (372), and atherosclerosis (373, 374). Papers reporta very complicated interaction between intestinal microbiota, their metabolites, and their related effect on human healths (375). Besides diet, genetics and other parameters, gut microbiota have been proposed to affect directly cholesterol metabolism and play a significant role in different pathways including bile acid metabolism, cholesterol conversion into coprostanol or cholesterol entrapment.

As regards bile acids metabolism, several bacterial genera, including *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Lactobacillus and Enterococcus* have been reported having BSH activity (376, 377). Bile salts deconjugation activity by bacteria can play a significant role in making these molecules much less soluble and less efficiently reabsorbed. This results in higher excretion of free bile acids into the stool and subsequent reduction of cholesterol (378). Interestingly, in the present study, the group that had already shown cholesterol-lowering ability (*L. paracasei* DTA81), showed an increased presence of the *Bacteroides* genus after six weeks of treatment.

Conversion of cholesterol to coprostanol by gut microbiota was first reported in the 1930s (379). There are two different pathways described for this transformation, including direct stereospecific reduction of the 5, 6-double bond of cholesterol and indirect transformation with at least three steps forming cholestenone and coprostanone as intermediates (379). Among intestinal bacteria , *Eubacterium*, *Bifidobacterium*, *Lactobacillus*, and *Peptostreptococcus* are those mostly related to cholesterol reduction (380, 381). Generally, absorption of cholesterol takes place in the upper small intestine which is mostly populated by the *Lactobacillus* genus (381). Quite recently, new groups of bacteria belonging to the *Rikenellaceae* (*Alistipes* genus), *Lachnospiraceae* and *Ruminococcaceae* have been associated with high coprostanol levels in healthy humans (382).

On the other side, gut microbiota can reduce cholesterol level by assimilating and entrapping this molecule into bacterial membranes (383, 384). There are plenty of species of *Lactobacillus* and *Bifidobacterium* that have shown cholesterol assimilation during *in-vitro* experiments and it has been reported that this ability is strictly strain dependent (385, 386).

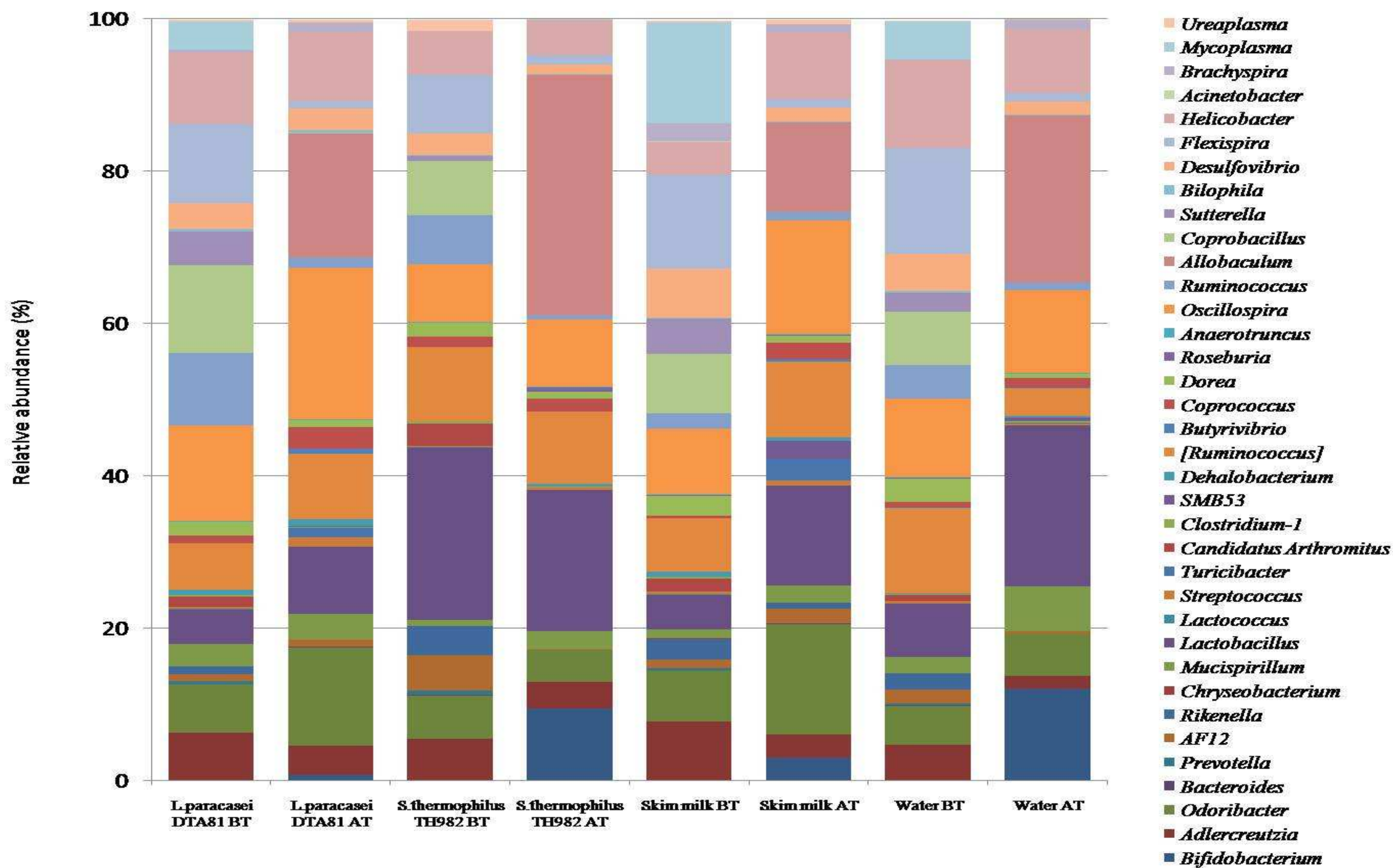


Fig. 3.5: Relative abundance of gut microbiota related to different control and probiotic treatments on the genera level. BT: Before treatment. AT: After treatment.

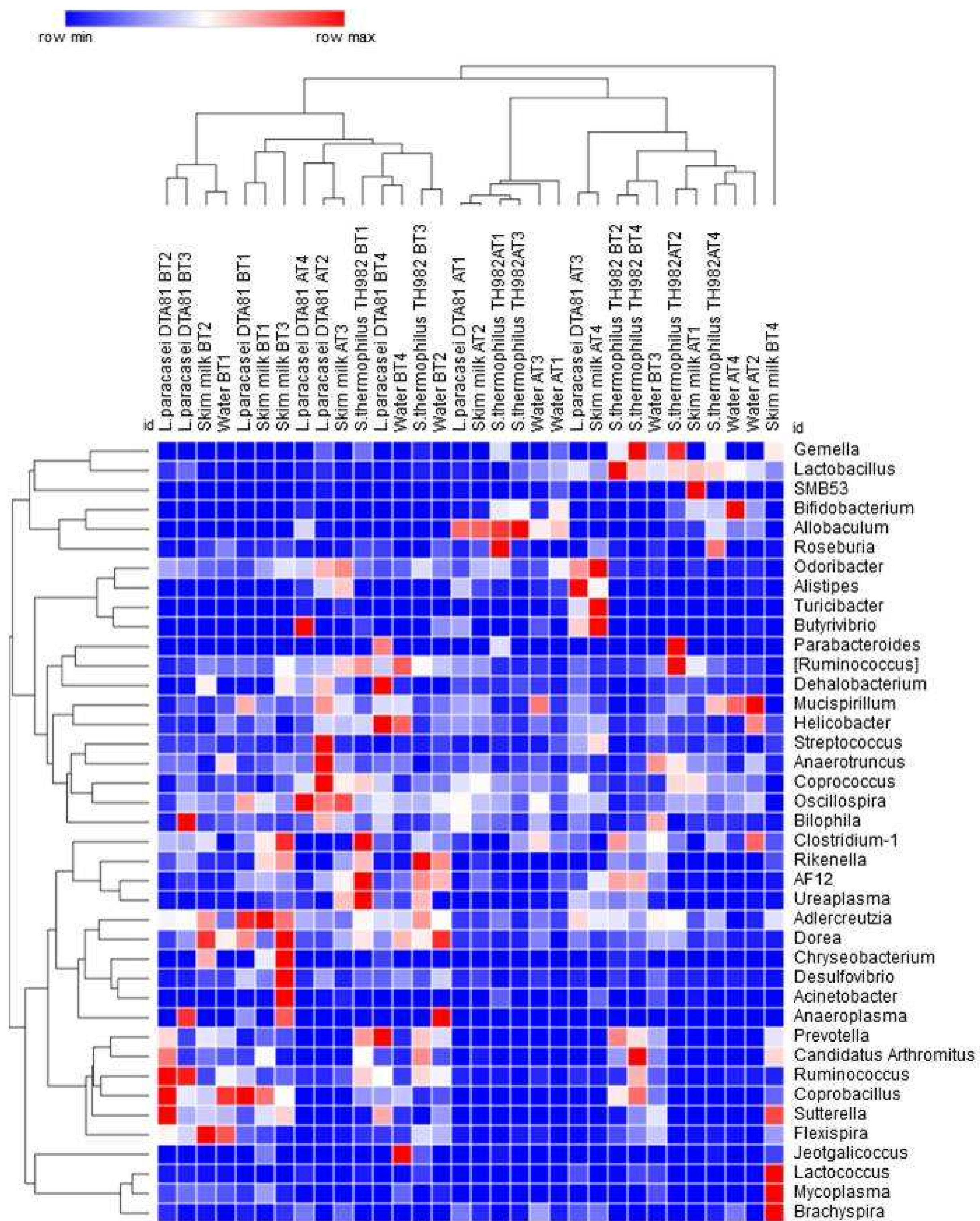


Fig. 3.6: Heatmap shows the comparison of gut bacteria on OUT genera level among the different replicates of different treatments.

CONCLUSIONS

Lactic acid bacteria (LAB) are one of the most important microbial groups widely used for the production of, fermented foods, beverages and used as probiotic. Lactobacilli have the capability to produce lactic acid alone (homofermentatives) or in combination with ethanol or acetic acid (heterofermentatives) by fermentation thus decreasing decrease the pH of the food. In addition to being well-known starters for food products, the probiotic potential of this genus has also been very well studied. For instance, *L. rhamnosus* has received great attention among researchers for its beneficial properties to human health. It is normally considered as probiotic since it possesses many properties such as resistance to gastrointestinal juice, the ability to adhere to the intestinal tract, to inhibit potentially pathogenic species of microbes, to help weight loss in obese people and protecting the colon. It was also reported that *L. rhamnosus* can be used in the competition against common causes of traveler's diarrhea.

S. thermophilus is the only non-pathogenic species of the genus that has technological importance in fermented food productions, known since 1919. Moreover, *S. thermophilus* has been considered an important bacterium in the dairy industry because of its acidification ability and some antimicrobial activities which are related to organic molecules such as lactic acid, acetic acid, formic acid. *S. thermophilus* can acidify the milk rapidly by breaking of lactose into glucose and galactose and producing lactic acid thus rapidly lowering the pH, an important feature that significantly affects microbial development in all environments, including foods. *S. thermophilus* is also well-known for the production of folate, which is a necessary component of the human diet. During the last decades, many LAB have been evaluated for the ability to produce folate and in some fermented dairy products, a considerable amount of folate present is due to the activity of LAB. *S. macedonicus* is another species of the *Streptococcus* genus which was identified and isolated quite recently from some dairy foods including several Italian kinds of cheese. Some *S. macedonicus* strains evidence some interesting properties, such as proteolytic activity, production of bacteriocins against food pathogens, production of exopolysaccharides and tolerance to stress associated with food processing. These traits make *S. macedonicus* a promising species, suitable for further studies for applications in food productions and also for its use as probiotic. Some *S. macedonicus* strains have already been evaluated as starter cultures in cheese-making trials. Overall, although *S. macedonicus* is generally considered a non-pathogenic species, but the lack of genome sequences available up to few years ago in public databases has

so far limited genetic *in-silico* analyses to predict their safety, technological and probiotic potentialities. Recently, some studies have reported some strains possessing probiotic properties.

Given all these points, this thesis has aimed to characterize and assess some health-related probiotic characteristics, such as anti-cancer and cholesterol-lowering properties, of newly isolated LAB strains and to study their technological potential using *in-silico*, *in-vitro*, and *in-vivo* approaches. All strains were evaluated for different technological properties, including acidification activity and fermentation on different sugars. The genomes of the strains were sequenced and applied to *in-silico* analysis to get information about their safety and possible application in technology and on human health. On the other side, the interesting strains from the technological part were selected to be evaluated for different *in-vitro* probiotic properties such as antibiotic susceptibility, hemolytic activity, resistance to simulated gastrointestinal conditions, bile salts hydrolysis activity, vitamins production, adhesion to human epithelial cell and anti-cancer activity against colorectal cancer cells (HT-29). Besides, based on information from the technological part and *in-vitro* probiotic assessment, two strains were chosen for *in-vivo* experiments using laboratory mice. For this purpose, 32 laboratory mice were used and treated with probiotic strains for six weeks. After that, all animals were used for evaluation of food consumption and weight gaining, blood biochemical analysis, oral glucose tolerance test (OGTT), the survival of probiotics after transitioning through the GIT, 16S metagenomics analysis of gut microbiome, and immunomodulatory effect.

According to *in-silico* and *in-vitro* information, two strains of *Lactobacillus* (*L. paracasei* DTA81 and DTA93) two strains of *S. thermophilus* (M17PTZA496 and TH982) and one strain of *S. macedonicus* (211MA) were found to possess interesting probiotic and technological properties. Some traits resulted very close to and in some cases superior to those of the widespread commercial probiotic strains *L. rhamnosus* GG that has been used as a reference in this thesis. In particular, *L. paracasei* DTA81 showed a remarkable adherence ability to HT-29 adenocarcinoma cell lines which resulted about ten times stronger than that of the commercial strain *L. rhamnosus* GG and, to our knowledge, represents the highest level reported to date for this type of cells. Besides, both *L. paracasei* DTA81 and *L. paracasei* DTA93 were able to effectively inhibit colorectal cancer cells (HT-29) and biofilm formation by other bacteria. Moreover, *S. thermophilus* M17PTZA496 and TH982 evidenced interesting *in-vitro* probiotic properties such as anti-cancer activity and production of folic acid (Vitamin B9). As regards *S.*

macedonicus, strain 211MA was the only one evidencing good properties. Considering all information related to technological and probiotic properties, the best strains namely *Lactobacillus paracasei* DTA81 and *S. thermophilus* TH982 were selected for *in-vivo* evaluation on laboratory mice.

Finally, the outcome of current dissertation showed that strain *L. paracasei* DTA81 was found to possess strong probiotic properties related to ability to lower blood cholesterol and Low Density Lipid (LDL) as well as Fasting Blood Sugar (FBS). Therefore, this strain can be considered as a new promising probiotic strain with a lot of potential interesting health benefits. Evidently, the final assessment shoul be a human trial to confirm the maintenance of these properties in humans.

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Appendix
A) Media and solutions

TAE buffer (50X)
Tris base 242 g, Acetic Acid 57.1 ml of, EDTA 100 ml of 0.5 M (pH 8.0), water to 1L.

PBS buffer
NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM, pH 7.4.

M17 broth
Pancreatic digest of casein 5.0 g/l, soy peptone 5.0 g/l, beef extract 5.0 g/l, yeast extract 2.5 g/l, ascorbic acid 0.5 g/l, $MgSO_4$ 0.25 g/l, disodium-β –glycerophosphate 19.0 g/, pH 6.9.

MRS broth
Beef extract 10 g/l, yeast extract 5 g/l, dextrose 20 g/l, Na Ac 5 g/l, polysorbate 80 1 g/l, KH_2PO_4 2 g/l, ammonium citrate 2 g/l $MgSO_4$ 0.1 g/l, $MnSO_4$ 0.05 g/l, pH 6.5 Baird Parker broth Enzymatic digest of casein 10 g/l, beef extract 5 g/l, yeast extract 1 g/l, LiCl 5g/l, glycine 12 g/l, Na pyruvate 10 g/l, enriched with egg yolk 30%, potassium tellurite 0.15%, pH 7.0

Brain Heart Infusion Broth
Brain heart infusion 17.5 g/l, enzymatic digest of gelatin 10 g/l, dextrose 2 g/l NaCl 5 g/l, Na_2HPO_4 2.5 g/l, pH 7.4.

Violet Red Bile Agar (VRBA)
Peptic digest of animal tissue 7g/l, Yeast extract 3 g/l, Sodium chloride 5 g/l, Bile salts mixture 1.5 g/l, Lactose 10 g/l, Neutral red 0.03 g/l, Crystal violet 0.002 g/l, Agar 15 g/l, Final pH (at 25°C) 7.4±0.2.

Chemically-define medium (CDM)
Lactose 5.0 g/l, Na acetate 1.0 g/l, NH_4 citrate 0.6 g/l, KH_2PO_4 3.0 g/l,K_2HPO_4 2.5 g/l, Urea 0.24 g/l, ascorbic acid 0.5 g/l, pyridoxamine HCl $0.8*10^{-3}$ g/l, nicotinic acid $0.1*10^{-3}$ g/l, riboflavine $0.05*10^{-3}$ g/l, Ca-pantothenate $0.1*10^{-3}$ g/l, thiamine HCl $0.005*10^{-3}$ g/l, $MgCl_2$ $6H_2O$ 0.16 g/l, $CaCl_2$ $2H_2O$ 0.01g/l, aspartic acid 0.46 g/l, asparagine 0.46 g/l, glutamic acid 0.40 g/l, glutamine 0.39 g/l, lysine 0.44 g/l, arginine 0.13 g/l, histidine 0.15 g/l, proline 0.68 g/l, phenylalanine 0.28 g/l, tryptophane 0.05 g/l, methionine 0.13 g/l, alanine 0.24 g/l, valine 0.33 g/l, leucine 0.48 g/l, isoleucine 0.22 g/l, glycine 0.18 g/l, serine 0.34 g/l, threonine 0.23 g/l, cysteine 0.25 g/l, tyrosine 0.29 g/l, pH 6.4.

Folic Acid Casei Medium

Charcoal treated pancreatic digest of casein 10.0 g/l, dextrose 40.0 g/l, Na Ac 40.0 g/l, dipotassium phosphate 1.0 g/l, monopotassium phosphate 1.0 g/l, DL-tryptophan 0.2 g/l, L-asparagine 0.6 g/l, L-cysteine HCL 0.5 g/l, adenine sulfate 10.0 mg/l, guanine HCL 10.0 mg/l, uracil 10.0 mg/l, xanthine 20.0 mg/l, polysorbate 80 0.1 g/l, glutathione (reduced) 5.0 mg/l, MgSO4 0.2 g/l, NaCl 20.0 mg/l, FeSO4 20.0 mg/l, MnSO4 15.0 mg/l, riboflavin 1.0 mg/l, p-aminobenzoic Acid 2.0 mg/l, pyridoxine HCl 4.0 mg/l, thiamine HCl 400.0 µg/l, Ca pantothenate 800.0 µg/l, nicotinic acid 800.0 µg/l, biotin 20.0 µg/l. pH 6.7.

Riboflavin Assay Medium

Dextrose 20.0 g/l, Sodium acetate 15.0 g/l, Vitamin assay casamino acids 10.0 g/l, Dipotassium phosphate 1.0 g/l, Monopotassium phosphate 1.0 g/l, L-asparagine 0.6 g/l, DL-tryptophan 0.2 g/l, L-cystine0.2 g/l, Magnesium sulfate USP 0.4 g/l,Adenine sulfate 20.0 mg/l, Guanine HCl 20.0 mg/l, Uracil 20.0 mg/l, Xanthine 20.0 mg/l, Ferrous Sulfate 20.0 mg/l, Manganese sulfate (monohydrate) 20.0 mg/l, NaCl 20.0 mg/l, Pyridoxine HCl 4.0 mg/l, Pyridoxal HCl 4.0 mg/l, p-aminobenzoic acid 2.0 mg/l, Calcium pantothenate 800.0 µg/l, folic acid 800.0 µg/l, nicotinic acid 800.0 µg/l, Thiamine HCl 400.0 µg/l, Biotin 1.0 µg/l. pH 6.8.

Cobalamin Assay Medium

D(+)-Glucose anhydrous 40.0 g/l, Casein hydrolysate "Vitamin-free" 15.0 g/l, L-Asparagine 0.2 g/l, L-Cystinium chloride 0.2 g/l, L-Cystine 0.4 g/l, DL-Tryptophan 0.4 g/l, Adenine 0.02 g/l, Guanine 0.02 g/l, Uracil 0.02 g/l, Xanthine 0.02 g/l, 4-Aminobenzoic acid 0.002 g/l, L(+)-Ascorbic acid 4.0 g/l, D(+)-Biotin (Vitamin H) 0.00001 g/l, Calcium D(+)-pantothenate 0.001 g/l, Folic acid 0.0002 g/l, Nicotinic acid 0.002 g/l, Pyridoxal hydrochloride 0.004 g/l, Pyridoxamine hydrochloride 0.0008 g/l, Riboflavin 0.001 g/l, Thiaminium dichloride 0.001 g/l, Potassium phosphate dibasic 1.0 g/l, Iron(II)sulfate 0.02 g/l, Potassium phosphate monobasic 1.0 g/l, Magnesium sulfate 0.4 g/l, Manganese(II) sulfate 0.02 g/l, Sodium acetate anhydrous 20.0 g/l, Sodium chloride 0.02 g/l, Final pH 5.6 +/- 0.2 at 25°C.

Dulbecco's Modified Eagle Medium (DMEM)

Calcium chloride dihydrate 265.000 mg/l, Ferric nitrate nonahydrate 0.100 mg/l, Magnesium sulphate anhydrous 97.720 mg/l, Potassium chloride 400.000 mg/l, Sodium chloride 6400.000 mg/l, L-Arginine hydrochloride 84.000 mg/l, L-Cystine dihydrochloride 62.570 mg/l, L-Glutamine 584.000 mg/l, L-Histidine hydrochloride monohydrate 42.000 mg/l, L-Isoleucine 105.000 mg/l, L-Leucine 105.000 mg/l, L-Lysine hydrochloride 146.000 mg/l, L-Methionine 30.000 mg/l, L-Phenylalanine 66.000 mg/l, L-Serine 42.000 mg/l, L-Threonine 95.000 mg/l, L-Tryptophan 16.000 mg/l, L-Tyrosine disodium salt 103.790 mg/l, L-Valine 94.000 mg/l, Choline chloride 4.000 mg/l, D-Ca-Pantothenate 4.000 mg/l, Folic acid 4.000 mg/l, Nicotinamide 4.000 mg/l, Pyridoxal hydrochloride 4.000 mg/l, Riboflavin 0.400 mg/l, Thiamine hydrochloride 4.000 mg/l, i-Inositol 7.200 mg/l, D-Glucose 4500.000 mg/l, Phenol red sodium salt 15.900 mg/l.

B) Bioinformatic tools and database

Here are reported sources of the bioinformatic tools and database cited in the main text.
BAGEL4
BAGEL is a webbased bacteriocin mining tool http://bagel.molgenrug.nl/index.php/bagel3

BLAST
Basic Local Alignment Search Tool BLAST finds regions of similarity between biological sequences http://blast.ncbi.nlm.nih.gov/Blast.cgi

MG RAST
MG-RAST is an open source, open submission web application server that suggests automatic phylogenetic and functional analysis of metagenomes.

RAST
Rapid Annotation using Subsystem Technology RAST is a fully-automated service for annotating complete or nearly complete bacterial and archaeal genomes. http://rast.nmpdr.org/

CLC
User-friendly bioinformatics software that allows for comprehensive analysis of your NGS data, including whole genome and transcriptome de novo assembly, targeted resequencing analysis, variant calling, ChIP-seq and DNA methylation (bisulfite sequencing analysis).

UniProt
UniProt provides a comprehensive, high-quality and freely accessible resource of protein sequence and functional information. http://www.uniprot.org.

CG View
The CGView Server generates graphical maps of circular genomes that show sequence features, base composition plots, analysis results and sequence similarity plots. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2447734/.

Gegenees
Gegenees' uses a fragmented alignment approach to facilitate the comparative analysis of hundreds of microbial genomes. The genomes are fragmented and compared, all against all, by a multithreaded BLAST control engine. http://www.gegenees.org/.

Orthovenn

OrthoVenn is a web platform for comparison and annotation of orthologous gene clusters among multiple species. No installation or registration is required. It works on any operating system with a modern browser and Javascript enabled. http://www.bioinfogenome.net/OrthoVenn/.

Web MGA

WebMGA provides users with rapid metagenomic data analysis using fast and effective algorithms. All the tools behind WebMGA were implemented to run in parallel on our local computer cluster. Users can access WebMGA through web browsers or programming scripts to perform individual analysis or to configure and run customized pipelines. WebMGA is freely available at http://weizhongli-lab.org/webMGA.

CARD

The Comprehensive Antibiotic Resistance Database is a bioinformatic database of resistance genes, their products and associated phenotypes. https://card.mcmaster.ca/.

VFDB

The virulence factor database (VFDB) is an integrated and comprehensive online resource for curating information about virulence factors of bacterial pathogens. Since its inception in 2004, VFDB has been dedicated to providing up-to-date knowledge of VFs from various medically significant bacterial pathogens. http://www.mgc.ac.cn/VFs/main.htm.

PHASTER

PHASTER (PHAge Search Tool Enhanced Release) is a significant upgrade to the popular PHAST web server for the rapid identification and annotation of prophage sequences within bacterial genomes and plasmids. While the steps in the phage identification pipeline in PHASTER remain largely the same as in the original PHAST, numerous software improvements and significant hardware enhancements have now made PHASTER faster, more efficient, more visually appealing and much more user friendly. http://phaster.ca/.