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Probiotic features of autochthonous lactobacilli

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Abstract

Probiotics are as defined “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2001). Probiotics regroup a wide variety of bacterial species and genera; lactobacilli and bifidobacteria are of the main studied bacteria for probiotic use. In this study, we investigated the probiotic characteristics of autochthonous lactobacilli strains isolated from traditional Sardinian cheese. Results showed that six *Lactobacillus plantarum* strains and one *Lactobacillus casei* strain were able to resist the upper gastrointestinal tract stress and inhibit *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enterica* growth. Strains also had good adherence to intestinal epithelial cells (HT-29) and proved susceptibility to antibiotics. Furthermore, all strains were capable of inhibiting NFκB activation *in vitro* proving immune modulation capacities. Together probiotic features and strains origin are of great importance to traditional and Protected Denomination of Origin (PDO) cheese manufactures; the use of probiotic autochthonous starter cultures increases the production of “functional” traditional cheeses.

To Salwa and my parents

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Introduction

1. Lactic acid bacteria

1.1 History

Lactic acid fermentation has long been used by numerous cultures around the world to improve storage qualities and nutritive value of perishable foods such as milk, vegetables, meat fish and cereals. Lactic acid bacteria (LAB) are the main actors of this process. The nomenclature was first designated to milk coagulating and fermenting bacteria or by other words, to bacteria capable of producing lactic acid from lactose. In 1919 the family name "*Lactobacteriaceae"* was first applied by Orla-Jensen to the group of bacteria mainly capable of producing lactic acid or secondary products such as acetic acids, alcohol and carbon dioxide.

LAB are Gram positive, catalase negative (Fooks et al., 1999), non-spore forming, carbohydrate fermenting lactic acid producing bacteria. They were initially subdivided into four genera: *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Lactobacillus* but currently combines the genera: *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Holzapfel et al., 2001; Stiles and Holzapfel, 1997).

LAB were traditionally used to produce fermented foods and were first isolated in milk (Carr et al., 2002; Premi et al., 1972). LAB can be found nowadays in a large variety of foods and fermented products; meat, vegetables, beverages and bakery products (Aukrust and Blom, 1992; Caplice and Fitzgerald, 1999; Gobbetti and Corsetti, 1997; Harris et al., 1992; Liu, 2003; Lonvaud-Funel, 2001; O'Sullivan et al., 2002). Nevertheless, their presence is not limited to food and food stuff environments; in fact, LAB have been detected in soil, water and sewage (Holzapfel et al., 2001), human body (Boris et al., 1998; Carroll et al., 1979; Elliott et al., 1991; Martin et al., 2003; Ocana et al., 1999a; Reid, 2001; Schrezenmeir and de Vrese, 2001) and animals (Fujisawa and Mitsuoka, 1996; Fuller and Brooker, 1974; Gilliland et al., 1975; Klijn et al., 1995; Premi et al., 1972; Schrezenmeir and de Vrese, 2001).

1.2 LAB classification

LAB strains are generally classified based on their morphology, glucose fermentation, and growth at different temperatures.

Morphology classification remains unchanged since Orla-Jensen (1919). Even though it's questionable in bacterial taxonomy, it's still of great importance. LABs are divided into 2 major groups: rods and cocci.

Glucose fermentation under standard conditions is also a classification factor of LAB. Thus they are classified into two groups: homofermentative bacteria (*L. acidophilus, L. delbrueckii, L. helveticus,* and *L. salivarius*), which produce two lactates out of one glucose, and heterofermentative bacteria (*L. casei, L. curvatus, L. plantarum* and *L. sakei*) that produce lactic acid , acetic acid and carbon dioxide out of glucose (Caplice and Fitzgerald, 1999; Jay, 2000; Kuipers et al., 2000). Gas production test from glucose will distinguish between the groups (Sharpe, 1979).

Lactobacilli are generally classified into three metabolic groups relying on biochemical and physiological properties; obligatory homofermentative lactobacilli which ferment hexoses to lactic acid, facultative heterofermentative which ferment both hexoses and pentoses and finally obligatory heterofermentative which ferment hexoses to lactate, acetic acid and carbon dioxide. Within these groups species are classified given their phylogenic relationship.

1.3 *Lactobacillus* genus

The genus *Lactobacillus* is the largest group among the LAB. At present it includes more than 180 species and 15 subspecies (Felis and Dellaglio, 2007). *Lactobacillus* species have a wide variety of phenotypes and physiological properties. This diversity is due to the definition of its members as rod-shaped LABs.

Lactobacilli are naturally associated with a large variety of nutritive-rich plant and animal derived environments; oral cavity, intestine, vagina, plant products and fermented foods.

Members of the genus *Lactobacillus* are commonly present as members of microbial communities and have received considerable attention with respect to their putative health

conferring properties as probiotics (Goldin and Gorbach, 1992). Lactobacilli are worldwide industrially used as starters in the manufacturing of fermented milk products. Moreover, strains with probiotic characteristics are included in fresh fermented products or used in capsular health products.

2. Probiotics

Probiotic is a Greek derived word meaning “for life”. The first use of probiotics dates back to a Roman naturalist “Pliney”. He used to recommend drinking fermented milk to treat intestinal problems. This practice was performed well before the discovery of bacteria.

A century ago, Russian scientist Tissier perceived for the first time, that gut microbiota from healthy breast fed infants were dominated by rods with a bifid shapes (*Bifidobacteria*) which were absent in formula fed infants guts suffering from diarrhea, establishing the concept that they played a role in maintaining health (Kechagia et al., 2013). Then in the 1900’s Russian scientist Eli Metchnikoff was intrigued by the longevity of Bulgarian dwellers. After discovering that the latters were drinking fermented yoghurt on daily basis, his studies found for the first time that a probiotic strain called *Lactobacillus bulgaricus* was present in the yoghurt and helped improve their health. It was the first time. In 1908 Metchnikoff received the noble prize in medicine for his work demonstrating that the consumption of beneficial microbes can help treating intestinal diseases. During the shigellosis outbreak in 1917 Nissel isolated a strain of *"Escherichia coli"* from the feces of a soldier who was not affected by the disease and used it in severe gastrointestinal infectious salmonellosis and shigellosis cases with great success. In 1920, Kulp and Rettger experiments seemed to show that *Lactobacillus bulgaricus* could not live in the intestine, and was damaged in the stomach by gastric acids. Thus, he lately demonstrated that naturally existent gut bacteria could be effective as probiotics, restoring normal bacteria colonization. In 1920, Rettger experiments seemed to show that Metchnikoff’s *L. bulgarius* could not live in the intestine, and was destroyed in the stomach by gastric acids. Thus, he lately demonstrated that naturally existent gut bacteria could be effective as probiotics, restoring normal bacteria colonization (Alcaide et al., 2003).

Probiotics were first defined in 1953 by Kollath as all organic and inorganic food complexes in contrast to harmful antibiotics. Not long after, in 1965 Lilly and Stillwell defined them as "microorganisms promoting the growth of other microorganisms". Several other definitions were lately proposed and failed to clearly define probiotics until 1992, when Havenaar and Veld defined probiotics as "mono- or mixed cultures of live microorganisms which, when applied to animal or human, beneficially affect the host by improving the properties of the indigenous microflora". In 1999, probiotics have also been defined by the European Commission Concerted Action on Functional Food Science in Europe as "viable preparations in foods or dietary supplements to improve the health of humans and animals". The current definition of probiotics dates to 2001 when the world health organization (WHO) and the food and agriculture organization (FAO) referred to probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001).

2.1 Probiotic selection criteria

A probiotic strains must be above all Generally Recognized as Safe (GRAS) (Havenaar et al., 1992). Almost all the lactobacillus genus is considered GRAS therefore safe for human consumption; in fact it has been used for years as starter cultures of foods fermentation (Salminen, 1998). Once consumed, probiotics must reach the lower intestinal tract to extract their benefit. Thus, they must tolerate the upper gastrointestinal tract (GIT) stress, during their path.

- **Acidity tolerance**

Before reaching the intestinal tract, probiotic bacteria must first survive the transit through the stomach (Dunne et al., 2001). The stomach secretion of gastric juices is the first defense against ingested microorganisms. In fact, cells lining the stomach produces around two liters of gastric juices daily with a pH that could be as low as pH 1.5 (Lankaputhra and Shah, 1995) and as high as pH 6 or more after meals, in general it averages between pH 2.5 and pH 3.5 (Huang and Adams, 2004). This daily production provides a normally-effective, high-acid barrier against entrance of viable bacteria into the GIT. Food nature affects its transit time in the stomach; it varies between 2 to 4 hours in normal cases but can be reduced to 20 minutes in case of liquids (Neumann et al., 1995).

Therefore, all probiotic candidates must have a tolerance to low pH values. Acid tolerance tests consists in evaluating viability of candidate strains after exposing them to low pH in a buffer solution or medium for a period of time. Previous studies showed that human isolated lactobacilli were able to tolerate gastric acidity and function effectively (Dunne et al., 2001). It also showed the better resistance of *Lactobacillus* to stomach condition than Bifidobacteria. Furthermore, (Conway et al., 1987) demonstrated that enteric lactobacilli species were more resistant to gastric conditions than yogurt producing *Lactobacillus* species. Some *Lactobacillus* showed different behaviors under different conditions as for *L. rhamnosus* GG which was able to survive at pH 1 and remained viable at pH 3 (Goldin and Gorbach, 1992). *L. plantarum* and *L. paracasei* are very acid resistant strains; works of Jacobsen et al. (1999) and Papamanoli et al. (2003a) demonstrated their capacity of survival after 1 and 4 hours of low pH exposure. Corcoran et al. (2005) evaluated the survival capacity of some *Lactobacillus* strains in simulated human stomach pH conditions; five strains were tested in simulated gastric juices with a pH of 2 for 90 minutes. The best survival rate was detected with the *L. rhamnosus* strain with 9 log cfu.mL^{-1}. On the other hand *L. paracasei* NFBC 338 was undetectable after 30 minutes.

Nowadays, probiotics are incorporated in many dairy products and deserts; a large variety of yoghurts, cheeses, milks and ice creams are commercialized as probiotic products. Thus, low viability in the GIT is still a problem and the use of probiotic from intestinal origins is still hard to

propagate, a high demand on new technologies and highly resistant strains is faced to enhance probiotic viability.

- **Bile salts/acids tolerance**

Bile is composed of aqueous solution of bile acids, cholesterol, phospholipids, and biliverdin pigment, which gives the bile its yellow-green color. It is a detergent-like biological substance synthesized from cholesterol in the liver. Its major physiological function is to facilitate the absorption of lipophilic compounds from the diet, including vitamins and lipids. Around 500 to 700 mL of bile is secreted each day; in fact, bile acids are first synthesized in the liver, from cholesterol, by the hepatocytes cells then secreted in the duodenum by the gallbladder (Hofmann and Roda, 1984). In humans, bile synthesis is regulated by many factors including nutrients, hormones and bile acids (Fuchs, 2003).

Bile acids are divided into two categories: primary and secondary. Primary bile acids are mainly two; cholic acid (CA) and chenodeoxycholic acid (CDCA) (Ridlon et al., 2006), they are sterol compounds synthesized in the liver from cholesterol then conjugated with glycine or taurine before secretion in the small intestine. The ratio of gluco to tauro-conjugates in human bile is usually around 3:1 this value can vary depending on specific individual population or diet composition (Begley et al., 2005). During their transit through the large intestine, primary bile acids are deconjugated by enteric anaerobic bacteria enzymes producing secondary bile acids: deoxycholic acid (DCA) and lithocholic acid (LCA) (Lefebvre et al., 2009; Ridlon et al., 2006).

Bile plays a crucial role in the establishment of the human intestinal microbiota. In fact, bile salts are potent antimicrobial agents; their detergent property dissolves bacterial membranes and only tolerant microbial populations are able to survive in the gut. Therefore, the functionality of probiotics depends on their ability to survive and temporarily persist in this harsh environment. The most important target of bile salts/acids is the bacterial cell membrane. The direct interaction with cell membrane is associated to cytotoxicity. In fact, the higher membrane affinity of hydrophobic salts/acids makes them more toxic than the hydrophilic ones. Free bile acids are weak acids that can

pass across the lipid bilayer without the need of any membrane transporter and inhibiting the cell (Kurdi et al., 2003; Kurdi et al., 2000).

On the other hand, it is also noticeable the influence of gut microbiota on bile salts composition during intestinal transit. The most important modification is the deconjugation of primary bile salts at the C-24 amid bond. This deconjugation is carried out by bile salt hydrolase (BSH) enzymes common to many of the of the members of the intestinal microbiota including *Lactobacillus* and *Bifidobacterium* which release glycine or taurine from the steroid nuclei, rendering the free bile acid (Begley et al., 2006). This effect is well noticed in the human feces; being solely produced by microbial metabolism the 60% rate of DCA and LCA is a clear evidence of the active microbial community (Ridlon et al., 2006). In 2002, the WHO added the bile salts/acids tolerance to the list of selection criteria of a probiotic strain.

It is believed that bile deconjugation is a detoxification mechanism and BSH plays an important role in the bile tolerance characteristic of probiotic organisms in the GIT (Lundeen and Savage, 1992). Deconjugated bile acids happen to be more effective against Gram positive strains than Gram negatives, Vowden et al. (1986) suggested that BSH enzymes are detergent shock proteins that protect lactobacilli from bile toxic effects, a major advantage of non-BSH producing bacteria. Recently, (Begley et al., 2006) reported a relation between BSH activity and bile tolerance; BSH mutants were more sensitive to bile with a major growth decrease.

- **Antimicrobial activity**

Food borne pathogens are the center of interest of antimicrobial studies too. *Listeria monocytogenes* is a ubiquitous Gram-positive, non-spore forming, facultative anaerobic rod which grows between -0.4 and 50˚C. It can be found in raw milk and raw milk products originating from animals or humans intestinal tracts, contaminated water, soil and manure. It causes Listeriosis a potentially fatal disease which occurs most often in immunocompromised individuals, elderly, pregnant women and their unborn fetus. Many studies have shown the capacities of lactobacilli strains to inhibit *L. monocytogenes*; Ennahar et al. (1998) showed that *L. plantarum* WH92 had an antimicrobial effect on *L. monocytogenes* during cheese ripening, Mills et al. (2011) have also

shown the capacity of *Lactobacillus plantarum* LMGP-26358 to inhibit *L. monocytogenes* growth in the intestinal tract.

Salmonella enterica is a Gram negative facultative anaerobic rod-shaped bacterium belonging to the *Enterobacteriaceae* family. It can be found in the intestinal tract of both warm and cold blooded animals. *S. enterica* causes salmonellosis or enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a foodborne infection/intoxication (Todar, 2012). *Lactobacillus* strains have also proven to inhibit *S. enterica* growth in food matrix and the intestine (Abdel-Daim et al., 2013; Jankowska et al., 2008).

Staphylococcus aureus is a facultative anaerobic Gram-positive spherical bacterium, member of the *Staphylococcaceae* family. It is mainly found in the respiratory tract and may be found on the skin, oral cavity and gastrointestinal tract. *S. aureus* can grow at temperatures ranging from 15 to 45˚C and tolerate NaCl concentrations up to 15%. *S. aureus* pathogenicity results in its capacity to produce coagulase; an enzyme capable of coagulating plasma and protect the bacterial cell from phagocytosis. *Lactobacillus* strains have also shown an inhibition effect against *S. aureus*; Ocana et al. (1999b) proved the capacity of *L. paracasei* subsp. *paracasei* to inhibit *S. aureus* by H_2O_2 production while Prince et al. (2012) showed the capacity of *Lactobacillus reuteri* to inhibit *S. aureus* by competitive exclusion.

Escherichia coli is the head of the *Enterobacteriaceae* family. It is a facultative anaerobic Gram negative rod shape bacteria. It is found in the GIT of warm-blooded animals and it's one of the first colonizers of GIT. *E. coli* colonizes the GIT just a few hours after birth and becomes the most predominant facultative organism during the rest of it. *E. coli* is generally harmless in its environment, its pathogenicity appears in immunocompromised hosts or where the normal gastrointestinal barriers are breached (Kaper et al., 2004) which causes eric/diarrhoeal disease, urinary tract infections and sepsis/meningitis. On the other hand, lactobacilli have also proven to inhibit *E. coli* in different environments as for the study of Riaz et al. (2010).

Lactobacillus strains are historically used as antimicrobial agents for food preservation. Their preservative action in food and beverage systems is attributed to the combined action of antimicrobial metabolites and organic acids produced during fermentation.

Nonetheless, it can also be attributed to the reduction of pathogens activity in the host through immune modulation (Matsuzaki and Chin, 2000) or competitive inhibition of microbial adhesion sites (Puertollano et al., 2007), this part will be detailed later.

Until this moment, *Lactobacillus* antimicrobial activity could be attributed to one, or more, of four antimicrobial compounds: organic acids, hydrogen peroxide, carbon dioxide/diacetyl and bacteriocins.

- *Organic acids*

Fermentation reduces the amount of available carbohydrates and results in a range of small molecular mass organic molecules that exhibit antimicrobial activity, the most common being lactic, acetic and propionic acid (Blom and Mortvedt, 1991). Homofermentation of hexose produces lactic acid. In contrast, heterofermentation produces equimolar amounts of lactic acid, acetic acid/ethanol and carbon dioxide (Klingenberg, 1987). It is believed that acids antimicrobial activity is due to their interference with cell membrane potential, reducing intracellular pH, inhibiting active transports and inhibiting different metabolic functions. In general, weak acids are more active at low pH than at neutral one. Compared to lactic acid, acetic acid has a better antimicrobial activity against yeast moulds and bacteria (Blom and Mortvedt, 1991). This is due to the higher pKa of Acetic acid (4.75) compared to lactic acid (3.86). The weak acid antimicrobial activity is assumed to be due to its undissociated molecule (Eklund, 1983). The undissociated form of the organic acid diffuse across the cell membrane once inside the cytoplasm, the acid is dissociated due the neutral pH (Padan et al., 1981). Many hypotheses have been made in attempt to explain this behavior; Salmond et al. (1984) suggested that growth inhibition is due to the release of protons in the cytoplasm causing the acidification and dissipation of membrane pH gradient, while Russell (1992) suggested that the antimicrobial activity is due to the anion accumulation reducing the macromolecules synthesis and affects cell membrane transports, simultaneously LAB reduce anion accumulation effects by the reduction of cytoplasm pH.

❖ *Hydrogen peroxide*

LAB are capable of producing hydrogen peroxide (H_2O_2) in the presence of oxygen, it is due to the activity of flavoprotein oxidases, NADH peroxidases, NADH oxidases and α-glycerophosphate oxidase. LAB do not produce catalase in absence of a heme, furthermore the elimination systems of H_2O_2 are less actives than the ones leading to its accumulation. Fountain et al. (1996) suggested that H_2O_2 does not accumulate *in vivo* due to the peroxidases, flavoproteins and pseudocatalase activities. H_2O_2 is strongly capable of oxidizing bacterial cells; in fact, sulfhydryl groups of both cell proteins and membrane lipids can be oxidized (Schlegel 1985). Lactoperoxidase, an enzyme found in saliva and milk, can enhance H_2O_2 antimicrobial effect in natural conditions; it catalyze the oxidation of thiocyanate by H_2O_2 generating hypothiocyanate, a possible cause of structural and membrane damages (Kamau et al., 1990). However, glycolysis blocking is considered the main antimicrobial effect of H_2O_2. Hydrogen peroxide production is quantified measuring the oxidation of subtracts in the presence of H_2O_2 and peroxidase. Strains growth medium, before food inoculation, plays an important role in the production of H_2O_2; in fact, *L. delbruekii* subsp. *lactis* produced more H_2O_2 after preparation in MRS (De Man, Ragosa and Sharpe) medium (Jaroni and Brashears, 2000).

❖ *Carbon dioxide and diacetyl*

Lactic acid bacteria hexose heterofermentation is the main source of carbon dioxide; many other metabolic pathways also produce carbon dioxide during fermentation (Gottschalk 1986). Carbon dioxide main antimicrobial activity is due to the creation of an anaerobic environment as well as its capacity to disrupt the lipid bilayer permeability (Lindgren and Dobrogosz 1990). On the other hand, diacetyl major role is the determination of the aroma and flavor of the final product; it is also believed that it has an antimicrobial activity against Gram- bacteria by binding to its arginine binding protein and preventing its use (Jay 1986).

❖ *Bacteriocins*

Many lactic acid bacteria are capable of producing ribosomal antimicrobial compounds called Bacteriocins (Jack et al. 1995). Bacteriocins are active antimicrobial compounds against many closely related species, food borne pathogens and Gram+ spoilage microorganisms (Tagg J. R. et al. 1976). Their natural origin and wide range of use gave them a great importance as natural food preservatives. Bacteriocins in general have a low molecular weight, active at low pH, thermostable and have a wide spectrum of activity. Nisin is one of the most common bacteriocin encountered; it has a GRAS status rewarded after 25 years of safe use in many European countries (Phumkhachron P. et al. 2010). Two other identified bacteriocins are reuterin and 2-pyrolidone-5-carboxylic acid (PCA). Reuterin is known for its activity against sulfhydryl enzymes and ribonucleotide binding subunit thereby disrupts DNA synthesis (Dobrogosz et al. 1989). LAB bacteriocins are classified into four classes according to the works of Klaenhammer (1993) and Nes et al. (1996); (i) lantibiotics, (ii) small hydrophobic heat-stable peptides (<13000 D), (iii) large heat-labile proteins (>30000) and (iv) complex bacteriocins.

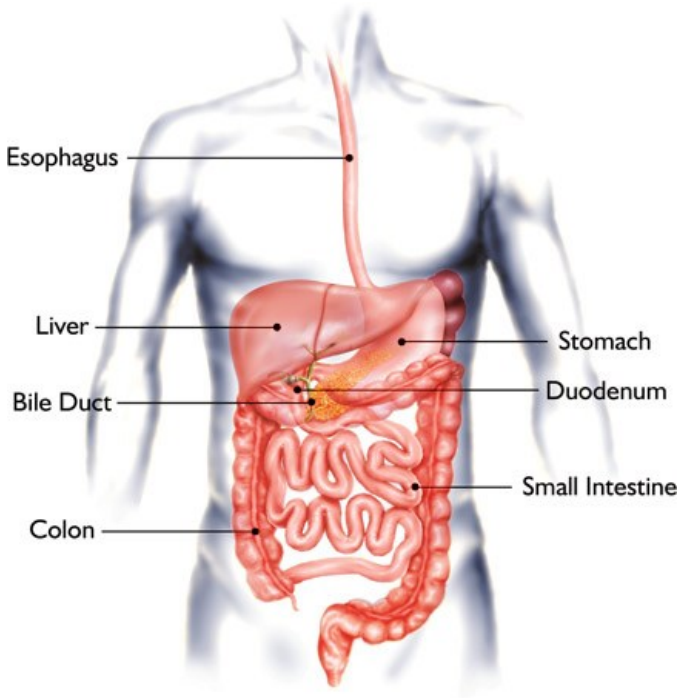

Figure 1. Food flow in the digestive tract. All foods, including probiotics, are assimilated through the mouth and make their way to the stomach through the esophagus. Probiotics are first exposed to the low pH in the stomach for about 3 hours, and then continue their movement downwards the duodenum where they are exposed to bile salts synthesized in the liver. Once in the small intestine moving toward the colon, probiotics adherence capacity and antimicrobial activities are put into test, enabling then to deliver their probiotic effects.

- **Bacterial antibiotic resistance**

Antibiotics are a major tool utilized by the health care industry to fight bacterial infections (Mathur S. 2005 paper). Antibiotics are classified in different groups depending on their mechanism of action: cell wall synthesis inhibitors, protein synthesis inhibitors, DNA synthesis inhibitors, RNA synthesis inhibitors, mycolic acids synthesis inhibitors and folic acid synthesis inhibitors.

Cell wall synthesis inhibitors are grouped into the Beta lactam class; it is one of the most commonly used antibiotic groups. β-lactam class regroups all antibiotic agents containing β-lactam ring in their molecular structures as Penicillin G, Vancomycin, Ampicillin and Amoxicillin (Holten and Onusko, 2000). It inhibits cell wall synthesis by inactivating enzymes located in the bacterial

cell membrane; it interfere with penicillin binding proteins (PBPs), inhibit peptidoglycan synthesis and generally lead to cell death. Bacterial cell wall contains peptidoglycan autolysis enzymes which are normally regulated to allow peptidoglycan breakdown at growing points, β-lactams inhibition of cell wall synthesis leads to an autolytic reaction which initiates a cell death program (Stephen and Calderwood, 2014). β-lactams were originally used for treatments against Gram positive bacteria, thus development of broad-spectrum β-lactam antibiotics allowed their use against Gram negative bacteria.

Protein synthesis inhibitors target mainly the bacterial ribosome. They inhibit ribosome function either by interfering in messenger RNA translation or by blocking the formation of peptide bonds (Lambert, 2012) on the 30S and 50S ribosome subunits respectively. Therefore, protein synthesis inhibitors are divided into anti-30S ribosomal subunit and anti-50S ribosomal subunit.

The Anti-30S ribosomal subunit group includes the Aminoglycosides and Tetracycline classes. Aminoglycosides class includes gentamicin, streptomycin, kanamycin and many other antibiotics. Aminoglycosides inhibit bacterial protein synthesis by pleiotropic actions that lead to the alteration of translation at diverse steps including initiation, elongation and termination (Davies, 1991). On the other hand, antibiotics of the tetracycline class such as tetracycline, bind to the 30S subunit at the A site and impairs the stable binding of aminoacyl-transfer (t)RNA to the bacterial ribosomal A-site (Chopra and Roberts, 2001).

Finally, the anti-50S ribosomal subunit group includes macrolides, chloramphenicol and lincosamide classes. Macrolides such as erythromycin bind to the 23S rRNA, close to the peptidyl transferase center of the 50S subunit and block extension of the peptide chain leading to the dissociation of peptidyl-tRNA (Leclerc, 2010). Chloramphenicol averts protein chain elongation by binding to specific nucleotides of the domain V of the 23S rRNA and preventing peptide bond formation (Lambert, 2012). Lastly lincosamide, such as clindamycin, also bind to 23S portion of the 50S subunit causing a premature dissociation of peptidyl-tRNA.

Antibiotic resistance is a type of resistance at which a microorganism is able to survive exposure to an antibiotic (Karapetkov et al., 2011). The overwhelming use of antibiotics has played a significant role in the outspread of antibiotic resistance bacteria (Ashraf and Shah, 2011). The bacterial capacity to resist to antibiotics could pose a major threat to human and animal health.

Additional antibiotic resistance is still encouraged with the use of antibiotics in livestock animal-feed for human consumption. The greatest threat to the use of antibiotics is the emergence and spread of resistance in pathogenic bacteria that can no longer be treated by previous successful regimens (Mathur and Singh, 2005).

In 2008 the European Food Safety Association (EFSA) Panel on Additives and Products or Substances used in Animal Feed defined three types of antibiotic resistance; natural or innate (Danielsen and Wind, 2003), acquired and mutational (Tenover, 2006).

Natural or innate resistance results' thrive of the microorganism in presence of the antimicrobial agent; it is not horizontally transferable and poses no problem to non-pathogenic bacteria (Mathur and Singh 2005). On the other hand, acquired antibiotic resistance could be horizontally acquired to other microorganisms by gene transfer or genome mutation.

EFSA also defined the microbiological breakpoints of the most commonly used antibiotics and recommended a verification of absence of transferable antibiotic resistance.

- **Adherence to epithelial cells**

Good adhesive probiotics were originally the center of interest due to the belief that good adherent strains could colonize the intestine more easily, especially the small intestine where food flow rates are relatively high (Ouwehand et al., 1999); adhesion to intestinal mucosa was of great importance. Nevertheless, studies showed that microorganisms do not colonize the GIT permanently; in fact they were undetectable in feces after 1 or 2 weeks from ingestion (Saarela et al., 2002). Therefore, probiotics persistence or transit colonization is evaluated rather than colonization.

Adherence to intestinal mucosa ensures temporary colonization of the gut; it is an essential property for health benefits expression (Forestier et al., 2001). In fact, the adherence to host tissue mainly depends on cell surface proteins composition of both cell and host surfaces. The *Lactobacillus* genus is one of the most studied genera at this concern; Lactobacilli are can adhere to vaginal epithelial cells (Andreu et al., 1995; McLean and Rosenstein, 2000; Osset et al., 2001; Redondo-Lopez et al., 1990); intestinal mucus (Gusils et al., 2003; Kirjavainen et al., 1998; Matsumura et al., 1999; Rojas and Conway, 1996; Roos and Jonsson, 2002; Roos et al., 2000;

Tuomola et al., 1999); intestinal carcinoma cells (Granato et al., 1999; Kirjavainen et al., 1999) and extracellular matrix (ECM) components (Aleljung et al., 1991; Harty et al., 1994; Mcgrady et al., 1995; Nagy et al., 1992; Toba et al., 1995). Despite the extensive study of Lactobacilli adherence capacity, no detailed knowledge of the adhesion mechanism is clear; the ECM is a macromolecular layer underlaying the epithelial and endothelial cells and surrounding connective tissue cells (Westerlund and Korhonen, 1993). ECM is generally unavailable to binding due to the covering epithelial and endothelial layers, though tissues disorder can expose ECM and allow bacterial binding, colonization and infection (Nallapareddy et al., 2000). The exposure of ECM increases the risk of pathogenic bacteria binding to host cell, therefore probiotic bacteria compete with pathogens for the same binding receptors and can occupy potential binding sites (Styriak et al., 2003).

- **Probiotics and host immune system**

The intestinal tract complex is of great importance to the host immune system. By its nature, the GIT is the most exposed part of the human body to antigens. It must distinguish between invasive microorganisms and harmless antigens, such as food proteins and commensal bacteria (Mowat et al., 2003). Human mucosa surfaces are the main accesses points of human pathogens and intestinal mucosa is no exception; therefore, strong immune system is required for the maintenance of healthy balance. The intestinal mucosa is composed of one thick upper layer called the epithelium or epithelial layer, it separates the lumen from the underlying layer the lamina propria. The lamina propria is a sterile connective tissue containing various immune cells (Bron et al., 2012), while the epithelial layer plays a double role in nutrient absorption and passage blockage of non-luminal components such as bacteria and food components that could induce pro-inflammatory responses (O'Hara et al., 2006).

Most of the intestinal cells (80%) are called columnar cells which absorb nutrients and present many metabolic functions. Other neighboring intestinal cells form tight junctions in order to maintain selective impermeable-barrier function. Furthermore, Paneth and goblet cells are also found in the epithelium; their role resides in the innate immune response of the host. In fact, Paneth cells are responsible of the production of antimicrobial components that prevent contact of

microorganisms with the proliferative cells in the crypts (Bevins and Salzman, 2011), while goblets cells are responsible of mucin production, forming a protective mucus layer to the epithelium (McCracken and Lorenz, 2001). Combined, the intestinal epithelial barrier, the innate immune system and mucus layer reduces the bacterial load at the interface between the lumen and epithelium (Bron et al., 2012).

Gut-associated lymphoid tissue (GALT) of the mucus layer, more specifically Peyer's patches, is largely associated to immune responses. In effect, Peyer's patches contain follicle covered by specialized follicle-associated epithelium with microfold cells (M cells) forming an antigen entrance into the follicle. Peyer's patches and follicles encompass connective tissue and immune cells simultaneously; antibodies producing B cells or antigen-presenting cells (APCs), macrophages, dendritic cells (DCs) and T cells. Macrophages and DCs are mainly considered phagocytes though the difference in their mode of action; macrophages remove cellular debris and pathogens and help initiate the adaptive immune system as APCs, while DCs are more specialized APCs that control both innate and adaptive immune systems (Kelsall, 2008). Immature DCs found in the mucus layer can also activate as Nuclear Factor-κB (NFκB) pathway. T cells on the other hand, are divided into T helper (Th) and regulatory T cells (T_{Reg}). T_{Reg} cells are major producers of interleukins (IL) mainly anti-inflammatory IL-10 and contribute in the regulation of T cell-mediated immune responses (Barnes and Powrie, 2009). The complex reaction of the epithelium, macrophages and DCs towards intestinal microbiota leads to intestinal homeostasis (Sansonetti, 2008).

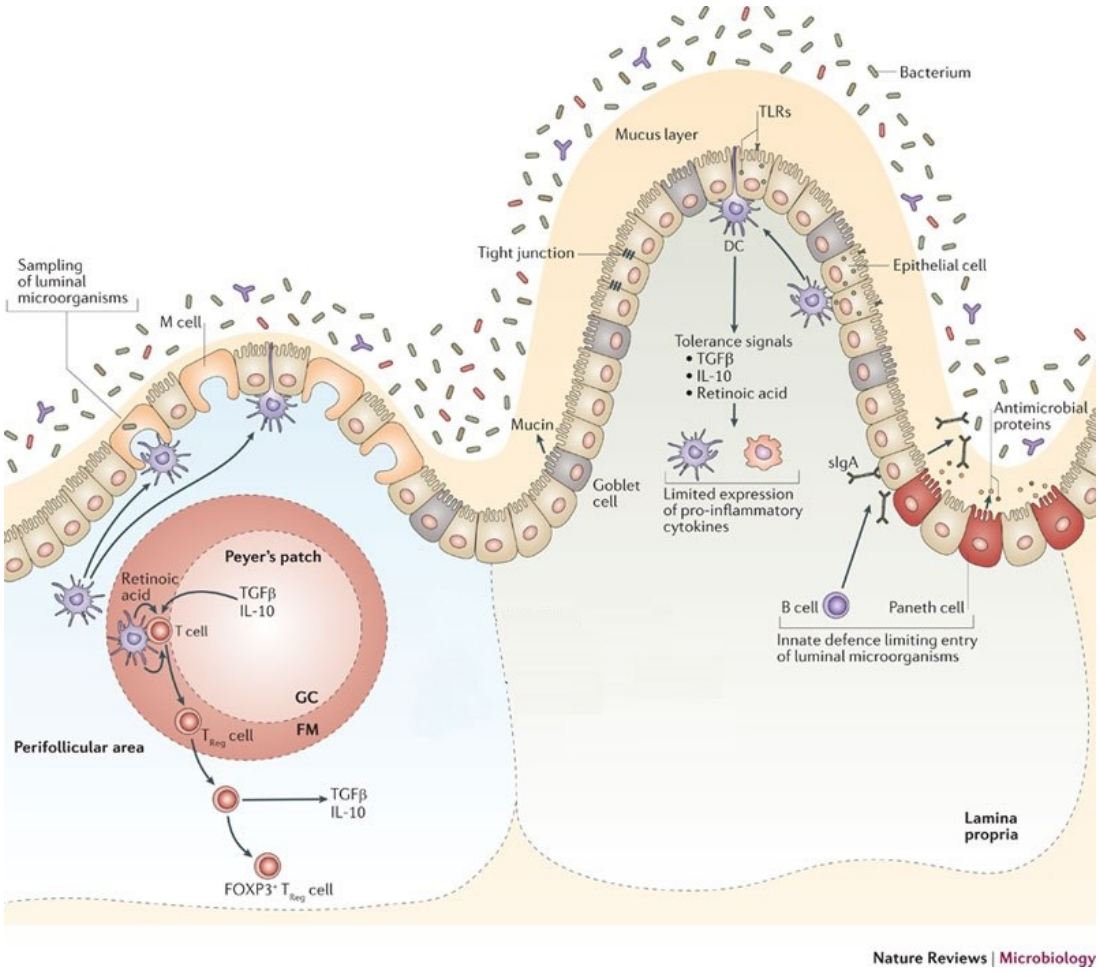

Figure 2. Host and intestinal microorganisms interaction; in general intestinal microorganisms are either tolerated or fought in the intestinal tract. The tolerance is mainly due to appropriate innate defense mechanisms which limits microbial flow into the intestinal tissues. In fact, intestinal epithelial cells form a physical barrier that separates luminal microorganisms from the intestinal tissues and control homeostasis. Mucus and antimicrobial proteins production by goblet and Paneth cells respectively as well as B cells secretory immunoglobulin A (sIgA) also limits bacterial exposure to epithelial cells. Innate immune cells (Dendritic cells (DCs) and macrophage) are generally activated through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), their activation induce antimicrobial pathways, activation of T helper 1/17 cells and adaptive immune cells. In fact, DCs present antigen to T cells in the Peyer's patches and cause the differentiation of regulatory T (TReg) cell populations regulated by interleukin-10 (IL-10), transforming growth factor-β (TGF β) and retinoic acid (Bron et al., 2012).

Several studies have shown that different bacterial strains can exert beneficial probiotic effects on the host by influencing its immune system, thereby modulating immune responses (Taverniti and Guglielmetti, 2011). Beneficial probiotics effects includes enhancing the intestinal epithelial cells function, protecting against physiologic stress, modulating cytokine secretion profiles, influencing T-lymphocyte populations, and enhancing antibody secretion (Thomas and Versalovic, 2010). Studies have revealed that probiotic host communication is due to the modulation of key signaling pathways such as NFκB, Mitogen-Activated Protein Kinases (L.P. MAPK) and Peroxisome Proliferator-Activated Receptors gamma (PPARγ) to either enhance or suppress activation and influence downstream pathways. Both viable and non-viable microorganisms are capable of influencing host immune system (Kataria et al., 2009) via a large variety of bacterial factors, however clear mechanisms of action are still uncertain.

Microorganisms and host first interact at the Intestinal epithelial cells (IEC) level. It is the first defense line of the host and plays a major role in the communication with commensal microbes and probiotics. In fact, probiotics can affect IECs in different ways; increase mucin production (Mack et al., 2003; Mack et al., 1999); enhance barrier function (Czerucka et al., 2000; Resta-Lenert and Barrett, 2003; Seth et al., 2008; Zyrek et al., 2007); induce heat shock protein production (Schlee et al., 2008; Wehkamp et al., 2004); inhibit pathogens (Castagliuolo et al., 1999) and modulate pathways signaling.

- *Nuclear factor kappa-light-chain-enhancer of activated B cells*

NFκB is one of the most probiotic influenced pathway, it can be activated by a wide range of stimuli and leads to an adaptive immune response. NFκB inactive form is found in the cytoplasm bounded to nuclear factor kappa light polypeptide gene enhancer inhibitor IκB molecule; once a stimulus signal is detected IκB is phosphorylated by IκB kinase enzyme preparing it for ubiquitination. As soon as NFκB unbound from IκB it migrates into the nucleus, bind target promoters and activate transcription of effector genes (Thomas and Versalovic, 2010). NFκB activation regulates the expression of host cytokines, chemokines, and adhesion molecules genes (Lawrence, 2009). Probiotics can interfere in many stages of the NFκB activation regulating with that downstream secretion of pro-inflammatory molecules. In fact, studies have shown the capacity

of probiotics to inhibit IκB poly-ubiquitination and proteasomal degradation (Neish et al., 2000) consequently NFκB nucleus migration and genes expression (Ma et al., 2004; Petrof et al., 2009). Alternatively, studies have also shown the capacity of probiotics to stimulate NFκB hence promote cytokines production and immune response (Haller et al., 2002; Ruiz et al., 2005).

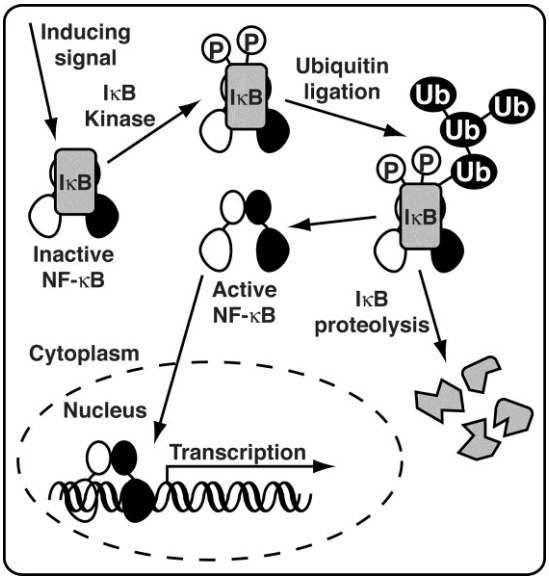

Figure 3. NFκB activation and gene expression. IκB phosphorylation is triggered by the inducing signal and catalyzed by the IκB kinase. Ubiquitin ligation soon follows phosphorylation and active NFκB is released. The latter is then transferred to the nucleus where it binds to promoters and activates target genes (San Diego state university, structural biochemistry laboratory2013).

❖ *Transforming growth factor beta*

On the other hand, transforming growth factor β (TGF-β) is a pleiotropic cytokine with potent regulatory and inflammatory activity (Sanjabi et al., 2009). It plays a vital role in the development, tissue homeostasis and diseases development (Schmierer and Hill, 2007). The probiotics inhibition role of TGF-β can limit gut inflammations. In fact, TGF-β binds to serine and threonine kinase receptors type II (TGF-β RII) and type I (TGF-β RI) membrane receptors leading to formation of hetero-complex receptors where TGF-β RII phosphorylates threonine and serine residues in the TTSGSGSG motif of TGF-β RI and activates it (Wrighton et al., 2009). Once activated, Smad molecules start nuclear translocation while target genes are transcripted (Li and Flavell, 2008). TGF-β plays an important role in the induction of peripheral tolerance; it is essential for the survival of naïve T cells and maintains peripheral tolerance by inhibiting the proliferation and differentiation of self-reactive CD4+ and CD8+ T cells (Sanjabi et al., 2009). Under inflammatory conditions, TGF-β can also promote inflammation and boost autoimmune response; in fact it leads to the differentiation of T helper 17 and Treg cells in the presence of IL-6 (Korn et al., 2009), while it promotes IL-9 and IL-10 producing T cells in the presence of IL-4 (Veldhoen et al., 2008).

❖ *Indoleamine-pyrrole 2,3-dioxygenase*

Finally Indoleamine 2,3-dioxygenase (IDO) is an intra-cellular tryptophan-degrading enzyme responsible of immunosuppressive mechanism. Its expression is induced by endotoxin and interferon-γ (INF-γ) (Mellor and Munn, 2004) thus its immunomodulatory effects are still not completely clear. Munn et al. (1999) proposed that tryptophan starvation have similar effect on T cells and bacteria; it provokes cell cycle perturbations and makes cells more sensitive to apoptosis. In effect, lower level of tryptophan leads to unresponsiveness state of peripheral T cells by activating amino acid-sensitive general control non-depressible 2 (GCN2) stress kinase pathway with uncharged tRNA (Munn et al., 2005). Other studies showed that tryptophan downstream metabolites as kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid are toxic to lymphocytes (Frumento et al., 2002; Terness et al., 2002); they can cause apoptosis and

differentiation of naive CD4+ T cells into immunosuppressive Treg cells (Chen et al., 2003). IDO role has been the center of interest in allergic response, antimicrobial activity and tumor tolerance (Mellor and Munn, 2004). In fact, reduction of free tryptophan, high expression of IDO and accumulation of kynurenines leads to localized immunosuppression.

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Aim of study

LAB have a long history in foods fermentation and preservation from spoilage microorganisms. They also have beneficial effects to the host therefore called probiotic LABs. Most of them belong to the *Lactobacillus* and *Bifidobacterium* species. Mesophilic lactobacilli in particular constitute a great part of the microbial community in traditional PDO Cheese. The autochthonous microbiota of these latter cheeses originates from raw milk, the manufacturing environment and specific cheese-making procedures and exerts a significant influence on the biochemical and organoleptic characteristics of cheeses, while contributing to the development of their typical features. That is why the official rules and regulations which apply to the manufacturing of Sardinian PDO cheeses foresee that only natural LAB derived from each production area can be used in the respective cheese making processes.

The objective of this study was to select *Lactobacillus* strains with probiotic activity that could be used as starter cultures in traditional and PDO cheese productions.

The research was divided into three major parts:

i) Isolation of lactobacilli strains;

ii) Tolerance of the upper gastrointestinal stress and safety assessment;

iii) Intestinal behavior assessment.

Material and methods

1. Lactobacilli isolation

Thirty five traditional raw milk Sardinian cheese samples were used for strains isolation during this experiment. Cheese samples were collected after 60 days of ripening. The isolation procedure was performed as soon as samples reached the laboratory. Briefly, ten grams of each sample were collected and homogenized in 90 mL of ¼ ringer solution (Oxoid, Milan, Italy) using a "Sewar stomacher 400". Homogenized samples were then serial diluted before streak plate method. One mL of sample's dilutions was plated into Man, Rogosa and Sharpe (MRS, Microbiol Cagliari. IT) soft agar (0.75% agar) pH 6.2±0.2, the use of this medium aimed to isolate the lactobacilli of cheese samples. Plates were then incubated in jars under anaerobic conditions using the Anaerogen system® (Oxoid, Milan Italy) for 48 hours at 37°C. Isolates shape morphology was then observed on optical microscope (phase contrast microscopy, Zeiss, Gottingen, Germany); only rod-shaped isolates were then purified by successive sub-culturing on MRS agar plates.

Light microscopy was then used for the evaluation of Gram reaction. Strains were grown on MRS broth medium at 37 °C for 24 hours in preparation for the test. Strains were then stained according to the Gram procedure. Lactobacilli stains are known to be Gram positive bacteria, therefore a blue-purple color must be observed after Gram staining. Gram positive isolates were then selected.

Many microorganisms are capable of producing catalase enzyme. This enzyme allows them to break down hydrogen peroxide into water and oxygen causing by that the formation of visible gas bubbles. Since lactobacilli strains are known to be catalase negative bacteria thus, catalase test was lastly performed. Overnight culture of strains was prepared in MRS broth at 37 ˚C for 8 hours previously to test. 3% Hydrogen peroxide solution was then added to 1 mL of overnight culture and gas bubbles formation was observed. Non-bubble forming strains only were selected.

Finally, presumptive *Lactobacillus* (rod-shaped, Gram positive, catalase negative) isolates were stored at -80°C in broth media and 20% of glycerol (v/v) until further tests.

2. Acidity tolerance

Good probiotic sources should withstand at least pH 3.0 (Fernandez et al., 2003), therefore, a general screening method was initially used to limit strains number in further tests. Strains were first grown in pH 3 MRS broth tubes, acidified with 0.1M HCl, for 24 hours. Growth rates were then visually evaluated on a scale from 0 to 4 where 0 represents no growth, 1 low growth, 2 medium growth, 3 good growth and 4 very good growth. Strains with 2 or higher growth rate were then selected for further testing.

After the general screening, selected isolates acidity tolerance was evaluated for 3 hours at pH 2.5 and pH 2. Plate count method was used for that purpose. Overnight cultures were prepared as described above. One hundred µL (1%) of strain's overnight culture were inoculated in 9.9 mL of MRS broth pH 2.5 and incubated at 37 ˚C for 3 hours. Vital counts were performed at T_0 (starting time), $T_{1.5}$ (after 1.5 hour) and T_3 (after 3 hours) by plate pour technique on unmodified MRS agar plates. Appropriate dilutions were done before inoculation and plates were incubated at 37 °C under anaerobic conditions for 48 h. Colonies were then counted for viability evaluation. Tolerant strains were retested at pH 2 following the same protocol. All experiments were done in triplicate; untolerant strains were used as negative control (N.C.).

3. Bile salts/acids tolerance

Lactobacilli's bile tolerance was assessed after 3 hours of exposure to 0.5% of bile salts. MRS broth supplemented with bile salts (Oxoid Milan Italy) was used during this test. Plate count method was used, as described above, to assess strain's viability at T_0, $T_{1.5}$ and T_3. 0.5% tolerant strains were retested with 1% of bile salts following the same protocol. All experiments were done in triplicate; untolerant strains were used as negative control (N.C.).

4. Molecular identification of isolates

- **DNA extraction**

Total DNA of presumptive *Lactobacillus* isolates was extracted using the ArchivePure DNA Yeast & Gram-+ Kit (5 PRIME GmbH, Hamburg, Germany) according to the manufacture instructions. The concentration and purity of DNA was assessed by spectrophotometric measurements using a LvisPLATE SpectroSTAR Nano (BMG Labtech, Ortenberg, Germany).

- **16S rDNA sequencing**

Single strain DNA was extracted as reported above and then used to amplify a 1500 bp fragment of the 16s ribosomal rRNA gene containing the V1-V4 variable region using the universal primer (Invitrogen) W001 and W002 as previously described by Goupil-Feuillerat et al. (1997). The amplicon were subsequently sequenced after being purified with QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. Sequencing was performed with the W001 and W002 primers at BMR Genomics, University of Padova, Italy (http://www.bmr-genomics.it). An average of 800 bp per sequence were obtained and compared with those in the GenBank database using the BLAST program (http:// www.ncbi.nih.gov/BLAST/) (Altschul et al., 1997), and with those in the Ribosomal Database project (http://rdp.cme.msu.edu/edu/index.jsp).

5. Antimicrobial activity

Strains antimicrobial activity was tested against four of the most common food-borne pathogens: *Listeria monocytogenes* DSMZ 20.600, *Salmonella enterica* DSMZ 13772, *Staphylococcus aureus* DSMZ 20231, and *Escherichia coli* DSMZ 30083. All pathogen strains were refreshed in NB (Nutriment Broth, Oxoid Milan Italy) expect the *Listeria monocytogenes* which was refreshed in BHI (Brain Heart Infusion, Oxoid Milan Italy) medium.

Antimicrobial activity was first assessed using the agar spot test described by Fleming et al. (1985) with minor variations. Briefly, strain's overnight cultures were spotted on MRS agar plates (2% bacteriological agar, Oxoid Milan Italy) and incubated for 24 hours at 37˚C under anaerobic conditions allowing the spots to grow. Pathogen strains were then inoculated at the 1% v/v in NB or BHI soft (0.7% bacteriological agar) mediums and poured over the MRS plates on which the lactobacilli were grown. Plates were reincubated at 37˚C for 12 hours and inhibition zones were measured around spots. Inhibition was considered positive if the width of the clear zone was 0.5 cm or larger.

The well diffusion test was then used following the method described by Herreros et al. (2005), in order to understand whether the antimicrobial activity is due to organic acids, bacteriocin production or hydrogen peroxide. In details 1 mL of indicator microorganisms was inoculated in 15 mL of MRS soft media, poured into petri dishes and left to dry. Once solidification is achieved, three wells of 5 mm (diameter) were cut into each plate. Strains' cell-free supernatant (CFS) were then prepared from overnight cultures; cultures were first centrifuged at 14,000 rpm for 5 min (centrifuge type) then filtered through 0.22µm syringe sterile filter (Millipore). 35 µL of unadjusted CFS were then added to the first well. pH of the remaining CFS was then adjusted to pH 6.5 using NaOH 1 M eliminating by that the potential inhibition effect of present organic acids. A volume of 35 µL of CFS pH 6.5 was then added to the second well. Finally, the neutralized CFS was treated with catalase (1mg mL^{-1}) at 25°C for 30 min in order to eliminate H_2O_2 effect then filtered and added to the third well. Plates were then incubated anaerobically at 37°C for 24 hours. Inhibition zones were measured after incubation; inhibition in the third well is an indicator of bacteriocin production.

6. Antibiotic susceptibility

Strains were tested against ten of the most commonly used antibiotics in human medicine and animal production. Antibiotic resistance was first assessed by the disc diffusion method described by Florez et al. (2005). Previously prepared discs of chloramphenicol (30 μg), clindamycin (2 μg), penicillin G (10 μg), amoxicillin (2 μg), erythromycin (15 μg), tetracycline (30 μg), ampicillin (10 μg), kanamycin (30 μg), gentamycin (10 μg) and vancomycin (30 μg) (Oxoid Milan Italy) were used during this test. A volume of 100 μL of freshly grown bacterial cultures (10^8 cfu mL^{-1}) was spread on MRS agar plates and allowed to dry. Antibiotic discs were then applied on plate's surfaces and incubated anaerobically at 37˚C for 48 h. Inhibition zones were measured after incubation and used as an indication of susceptibility and resistance.

Minimal inhibition concentration (MIC) of resistant strains was then calculated using the broth micro-dilution method according to the ISO L.c. 10932:2012 standard. Antibiotic stock solutions were first prepared with a concentration of 1024 μg mL^{-1}. Stock solutions were then diluted, in LAB susceptibility test medium (LSM) broth, into a series of concentrations ranging from 0.25 to 1024 μg mL^{-1}. Overnight cultures were then used to prepare bacterial inocula for the test; briefly, after centrifugation at 12 000 rpm for 10 min, bacterial colonies were resuspended in sterile saline solution until an optical density OD_{625} of 0.2, corresponding to 3 x 10^8 cfu.mL^{-1}, and diluted 1:500 in LSM broth. 50 μL of diluted inocula were added to each desired well in the 96-well micro-dilution plate previously containing 50 μL of desired antibiotic solution. Plates were then incubated at 37°C for 48 h. After incubation, growth was determined by OD_{625} for each antibiotic by comparing it with positive control. MIC was determined as the lowest concentration of an antibiotic in which visible growth was inhibited. MIC values were then compared to MIC breakpoints of lactobacilli defined by Panel on Additives and Products or Substances used in Animal Feed (EFSA, 2012).

7. Adherence to epithelial cells

HT-29 human colon carcinoma cells were used during this study to evaluate strains adherence capacity to human epithelial cells. Cells were routinely cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (sigma) supplemented with 10% fecal calf serum (FCS), penicillin (50 U mL^{-1}), streptomycin (50 U mL^{-1}) and 2 mM glutamine.

HT-29 monolayer plates were prepared 48 hours before adhesion testing; HT-29 were seeded in 24 well plate (type) in order to have of 2 x 10^5 cells in 500 µL RPMI medium per well and incubated at 37˚C and 5% CO_2.

Overnight cultures of lactobacilli grown in MRS medium were centrifuged for 10 min at 12000 rpm, and then resuspended in Phosphate Buffer Solution (PBS) until an OD_{600} of 0.5 in preparation for the test. Vital counts were then determined at T_0 by plating bacterial suspension on MRS agar plates and incubating it at 37˚C for 48 hours.

HT-29 monolayer plates were washed twice with PBS pH 7.4 before the addition of 500 µL of bacterial suspension into each well. Plates were then centrifuged for 10 min at 1400 rpm and incubated in 5% CO_2 for 1 hour. HT-29 cells were lysed, after incubation, with PBS and triton 0.1% (v/v) solution. Appropriate dilutions were made, plated on MRS agar plates and incubated (T_1) at 37˚C anaerobically for 48 hours. Adhesion rate was calculated from T_0 and T_1 vital counts. Experiments were done in triplicate.

8. Immune modulation

Strains immune modulation capacity was assessed using three HT-29 clone cell lines carrying chromosomally located luciferase reporter gene under the control of three promoters:

- Nuclear factor kappa B (NFκB)
- Transforming growth factor beta (TGFβ)
- Indoleamine-pyrrole 2,3-dioxygenase (IDO)

Cell lines were grown and maintained before testing in RPMI medium supplemented with 10% of FCS, penicillin (50 U mL^{-1}) and streptomycin (50 U mL^{-1}) and then seeded in 96 well plates

24 hours before immune modulation tests; briefly, 90 µL of $5x10^4$ cells were added to each well then incubated at 37˚C and 5% CO_2 (Kacy et al. 2009).

On the other hand, overnight cultures were prepared on the eve of the tests, strains were then harvested in exponential growth phase ($OD_{600} \cong 0.5$), centrifuged for 10 min at 12 000 rpm, washed twice PBS and finally resuspended in antibiotic free RPMI medium to a final concentration of $5x10^6$ cells.mL^{-1}.

Once strains suspensions were ready, 10 µL were added to plate wells as well as 10 µL of stimulator; tumor necrosis factor alpha (TNFα) was used for NFκB tests, interleukin 1 (IL1) for TGFβ tests and interferon (IFN) for IDO tests. Plates were then incubated for 6 hours at 37˚C and 5% CO_2. Once the incubation was done, 50 µL of each well were replaced with 50 µL of Neolite reagent (source) and Luminesce was measured using TECAN infinite M200 device. Strains activity was confronted with positive and negative controls within the plates.

Intact bacterial strains were first assessed for immune modulation capacities. Once anti-inflammatory effects were detected, further tests were conducted in order to investigate the basis of this activity. Strains supernatant activity was first evaluated in search of secreted metabolites effects. Far along, heat inactivated strains (15 min at 70˚C) were also evaluated seeking surface components effects and finally trypsin treated strains (Trypsin 15 min at 37˚C) were evaluated in search of surface components with peptidic bonds effect.

Experiments were done in triplicates. Wells of HT-29 cells and its stimulator were considered positive controls and wells of HT-29 were considered negative control.

9. Statistical analysis

Results of the microbiological data were transformed into respective decimal logarithms to fit a normal distribution of value. Data were subjected to one-way ANOVA to investigate separately the effect of low pH and bile salts on strains as well as to evaluate their antimicrobial activity and immune modulation capacities. When the effect was significant ($P<0.05$), differences between means were separated by Tukey-Kramer test of multiple comparisons. Correlation coefficients among different variables were also calculated. Variance analysis and correlations between counts of the microbial groups were determined with the aid of the program StatGraphic centurion XV.

Results

1. Isolation of presumptive *Lactobacillus* strains

Thirty five traditional raw milk Sardinian cheese samples were collected from twelve different Sardinian regions representing the entire island. First samples were received on the 11th of June while the last ones were received on the 16th of July; isolation was performed upon reception. A total of 220 isolates were first isolated on selective growth medium.

Isolated strains were then Gram and catalase tested and a total of 156 presumptive *Lactobacillus* strains were finally selected, purified and conserved in the laboratory collection at -80˚C awaiting probiotic characterization.

2. Acidity tolerance

In total thirty four strains were selected after pH 3 screening (results not shown). Selected strains were retested at pH 2.5 for 3 hours. Viable counts were used as growth indicator. Results showed (Table 1) that most of the strains had a similar behavior, their viable counts decreased gradually with the exposure period except seven presumptive *Lactobacillus* strains: 103, 109, C1E5, DC2, DC3, MBM and MAP. 103, 109 and C1E5 strains showed a stable behavior at pH 2.5; viable counts slightly decreased from 9.15, 9.39, 8.37 log cfu mL^{-1} to 8.8, 8.17 and 8.16 log cfu mL^{-1} respectively maintaining a relatively high viability rate among other strains. On the other hand MAP and MBM strains were both able to grow at pH 2.5 from 7.38 and 7.68 log cfu mL^{-1} to 7.74 and 8.22 log cfu mL^{-1} respectively. Finally, DC2 and DC3 seemed to be unharmed at pH 2.5; both strains grew normally during the 3 hours exposure period starting at 8.2 and 7.7 log cfu.mL^{-1} respectively reaching 9.4 log cfu mL^{-1} by the end of the test.

TABLE 1. Viable counts of 35 *Lactobacillus* strains during 3 hours at pH 2.5.

	Viable count (log cfu mL^{-1})									
Bacterial strain	**0h**			**1.5h**			**3h**			**Statistical Groups**
N.C.	7.545	±	0.061	4.301	±	0.078	2.342	±	0.111	X
VSO2 m8	6.771	±	0.041	0.000	±	0.000	0.000	±	0.000	X
VSO1 m1	7.142	±	0.018	7.093	±	0.039	5.021	±	0.108	X
DSMZ3 m4	6.841	±	0.007	6.671	±	0.034	4.684	±	0.028	X
VSO1 m2	6.389	±	0.027	6.199	±	0.046	4.985	±	0.026	X
DSMZ5 m3	7.227	±	0.028	6.757	±	0.037	4.696	±	0.013	XX
VSO2 m1	6.682	±	0.519	6.223	±	0.096	5.872	±	0.032	XX
VSO3 m8	6.790	±	0.025	6.409	±	0.058	5.677	±	0.046	XX
DSMZ3 m5	7.082	±	0.020	6.826	±	0.028	5.287	±	0.013	XX
DSMZ2 m5	7.082	±	0.133	6.826	±	0.055	5.287	±	0.012	XX
VSO1 m4	7.427	±	0.038	6.835	±	0.147	5.176	±	0.152	XX
QB2 m1	7.292	±	0.015	7.008	±	0.009	5.907	±	0.014	XX
DSMZ1 m4	7.223	±	0.087	6.850	±	0.006	5.541	±	0.064	XX
VSO2 m4	7.071	±	0.014	7.111	±	0.061	5.266	±	0.031	XX
QB2 m3	7.218	±	0.059	7.363	±	0.018	5.356	±	0.021	XX
VSO2 m5	7.148	±	0.006	7.114	±	0.022	6.038	±	0.007	XX
QB3 m2	7.271	±	0.073	6.920	±	0.025	5.914	±	0.022	XX
QB1 m3	7.429	±	0.096	7.515	±	0.027	5.285	±	0.034	XX
AP2 m4	7.265	±	0.014	7.158	±	0.021	5.894	±	0.012	XX
GM2 m2	7.641	±	0.170	7.301	±	0.048	6.165	±	0.172	XX
VSO2 m7	7.552	±	0.068	7.716	±	0.042	6.008	±	0.007	XX
GM2 m3	7.549	±	0.072	7.468	±	0.024	6.308	±	0.267	XX
QB3 m2	7.322	±	0.034	7.418	±	0.196	5.497	±	0.180	XX
QB2 m1	7.446	±	0.171	6.359	±	1.782	4.990	±	0.563	XX
VSO2 m5	7.158	±	0.127	7.320	±	0.124	5.391	±	0.059	XX
VSO1 m6	6.103	±	0.588	6.227	±	0.026	0.000	±	0.000	XX
VSO1 m2	7.219	±	0.030	6.889	±	0.185	5.589	±	0.368	XX
VSO2 m4	7.190	±	0.039	6.939	±	0.026	6.149	±	0.032	XX
MAP	7.386	±	0.021	7.792	±	0.031	7.745	±	0.030	XX
MBM	7.683	±	0.021	8.092	±	0.031	8.224	±	0.030	XX
C1E5	8.372	±	0.046	7.922	±	0.046	7.362	±	0.036	XXX
DC3	7.747	±	0.214	7.803	±	0.553	9.400	±	0.257	XXXX
109	9.399	±	0.055	7.981	±	0.076	8.179	±	0.257	XXX
DC2	8.290	±	0.070	8.750	±	0.079	9.400	±	0.057	XX
103	9.154	±	0.103	9.011	±	0.101	8.806	±	0.100	X

N.C.: Negative control. Results are expressed as mean ± standard deviation (n = 6).
Multiple statistical comparison groups identified as X's columns (95% Tukey test).

Finally, strains were also retested at pH 2. Most strains were unable to survive even after 1.5 hour. Thus it was noticeable that 6 out of 7 pH 2.5 resistant strains were able to resist for at least 1.5 hour while 3 stains were able to resist for 3 hours (Table 2). In fact, L.c. 109 was the only strain unable to resist after 1.5 hour of exposure, while MAP, MBM and 103 had 4.283, 3.594 and 4.473 log cfu mL^{-1} viable counts respectively after 1.5 hour. Only three strains were able to survive after 3 hours of exposure; DC2, DC3 and C1E5. The latters showed a higher resistance to pH 2 with viable counts of 4.48, 3.54 and 6.09 log cfu mL^{-1} after 1.5 hour and 3.28, 3.62 and 5.36 log cfu mL^{-1} after 3 hours respectively (Table 2). Statistical analysis showed that growth was significantly different among strains ($P < 0.05$) still more among time; significant differences were noticed comparing strains 0h, 1.5 and 3h viable counts. Statistical differences were also noticed among strains growth evolution rates; C1E5 had the slower growth decrease among other strains followed by DC2 and DC3 respectively, while MAP, MBM, 103 and 109 had similar growth decrease behaviors (Figure 4).

TABLE 2. Viable counts of 7 *Lactobacillus* strains during 3 hours at pH 2.

Bacterial strain	Viable count (log cfu mL^{-1}) 0h	1.5h	3h	P value (strains)	P value (time)
MAP	7.353 ± 0.032	4.283 ± 0.446	0.000 ± 0.000	*	†
MBM	7.066 ± 0.106	3.594 ± 0.207	0.000 ± 0.000	*	†
DC2	6.962 ± 0.049	4.481 ± 0.030	3.282 ± 0.024	*	†
DC3	7.398 ± 0.032	3.542 ± 0.063	3.627 ± 0.304	*	†
103	7.148 ± 0.032	4.473 ± 0.253	0.000 ± 0.000	*	†
109	6.890 ± 0.035	0.000 ± 0.000	0.000 ± 0.000	*	†
C1E5	7.482 ± 0.029	6.096 ± 0.069	5.361 ± 0.100	*	†

Results are expressed as mean ± standard deviation (n = 9)

*: Strains growth values significantly different (P <0.05)

†: Single strain growth values significantly different (P <0.05) during time

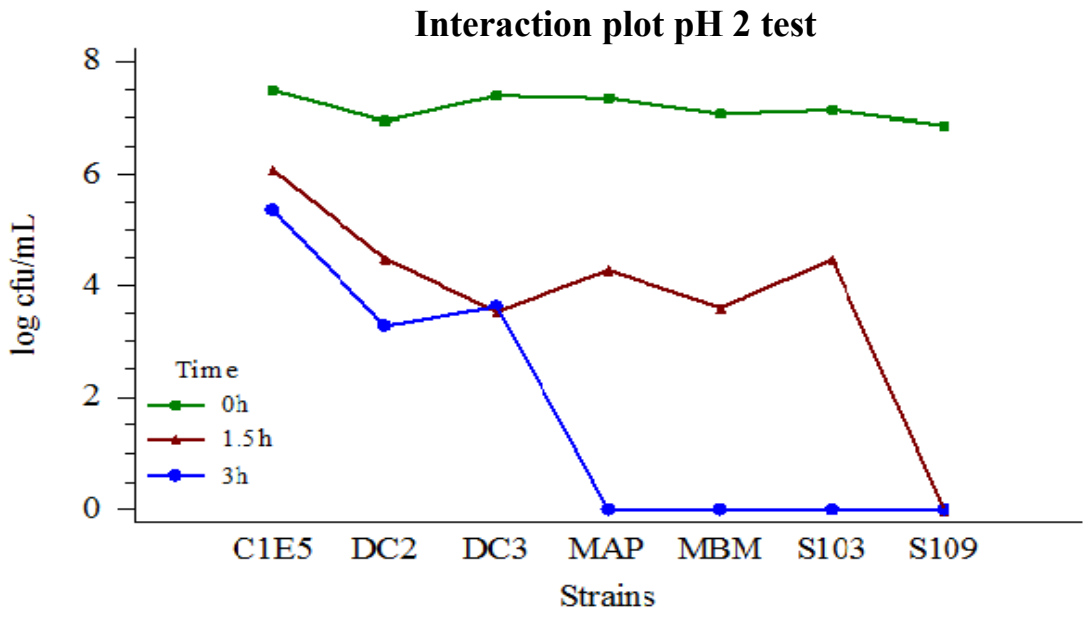

Figure 4. pH 2 multiple comparisons Tukey-Kramer test interaction plot; comparison of strains viable growth evolution during time.

3. Bile salts/acids tolerance

All strains were first tested in MRS broth supplemented with 0.5% bile salts for 3 hours. Results surprisingly showed the intolerance of most of tested strains except the seven pH resistant strains (Table 3). In fact, the same pH 2.5 resistant strains showed the highest viable counts after 3 hours of exposure to 0.5% bile salts. Results show that the most resistant strains were MAP, DC3 and C1E5 with final viable counts of 4.98, 4.04 and 4.08 log cfu mL^{-1} respectively. While 109 in contrast, was the only strain which was unable to survive the 3 hours exposure. Statistical analysis showed that growth was significantly different among strains and time ($P < 0.05$); significant differences were noticed comparing strains 0h, 1.5h and 3h viable counts. All strains showed significant growth variation rates differences; C1E5 was the only strain showing growth signs, MAP was also the only strain showing slight growth variation, while L.P. DC3 showed the slower growth decrease rate, followed by 103, L.P. MBM and 109 respectively (Figure 5).

TABLE 3. Viable counts of 7 *Lactobacillus* strains during 3 hours with 0.5% bile salts.

Bacterial strain	Viable count (log cfu mL^{-1})			P value (strains)	P value (time)
	0h	1.5h	3h		
MAP	5.054 ± 0.041	5.059 ± 0.078	4.988 ± 0.135	*	†
MBM	5.052 ± 0.085	3.077 ± 0.054	1.991 ± 0.015	*	†
DC2	3.817 ± 0.051	4.490 ± 0.021	3.977 ± 0.100	*	†
DC3	4.915 ± 0.035	4.543 ± 0.173	4.044 ± 0.057	*	†
103	4.593 ± 0.062	3.405 ± 0.073	3.365 ± 0.034	*	†
109	3.059 ± 0.241	2.552 ± 0.066	0.000 ± 0.000	*	†
C1E5	3.627 ± 0.046	3.992 ± 0.038	4.084 ± 0.175	*	†
N.C.	4.471 ± 0.081	2.454 ± 0.151	0.000 ± 0.000	*	†

N.C.: Negative control. Results are expressed as mean ± standard deviation (n = 9)

*: Strains growth values significantly different ($P < 0.05$)

†: Single strain growth values significantly different ($P < 0.05$) during time

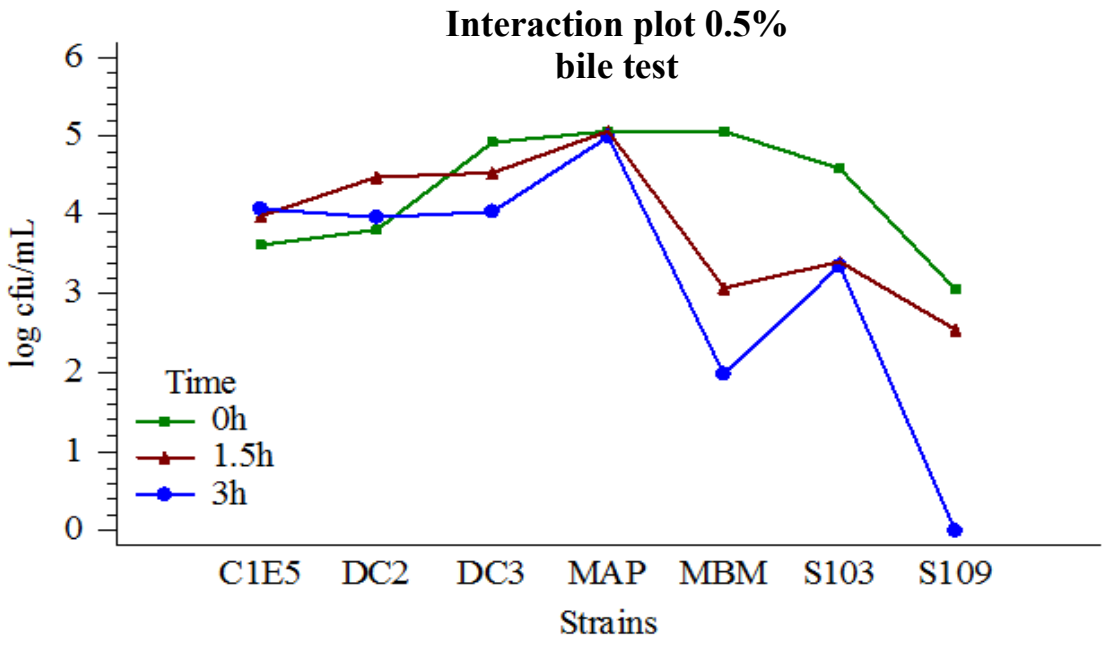

Figure 5. 0.5% bile salts multiple comparisons Tukey-Kramer test interaction plot; comparison of strains viable growth evolution during time.

All seven strains were retested with 1% bile salts for 3 hours (Table 4). Most strains were able to tolerate stress for 1.5 hour with a decrease in viable counts. Still, three strains were able to tolerate bile salts for 3 hours; in fact, DC2, DC3 and C1E5, had viable counts of 4.88, 2.35 and 4.96 log cfu mL^{-1} respectively. Statistical analysis showed significant growth differences among strains and time ($P < 0.05$). Even more, significant differences were noticed among strains growth evolution rates; C1E5 and DC2 had the slowest growth decrease among others followed by DC3, while. MAP, MBM, 103 and 109 showed similar behaviors (Figure 6).

TABLE 4. Viable counts of 7 *Lactobacillus* strains during 3 hours with 1% bile salts.

Bacterial strain	Viable count (log cfu mL^{-1})									P value (strains)
	0h			1.5h			3h			
MAP	5.161	±	0.026	2.365	±	0.066	0.000	±	0.000	*
MBM	5.588	±	0.032	2.910	±	0.075	0.000	±	0.000	*
DC2	5.116	±	0.036	4.883	±	0.012	4.887	±	0.019	*
DC3	5.459	±	0.025	4.815	±	0.020	2.359	±	0.039	*
103	4.379	±	0.032	2.254	±	0.020	0.000	±	0.000	*
109	4.262	±	0.035	2.437	±	0.027	0.000	±	0.000	*
C1E5	5.962	±	0.047	5.212	±	0.014	4.946	±	0.111	*
N.C.	4.554	±	0.100	0.887	±	0.015	0.000	±	0.000	*

N.C.: Negative control. Results are expressed as mean ± standard deviation (n = 9)

*: Strains growth values significantly different (P <0.05)

†: Single strain growth values significantly different (P <0.05) during time

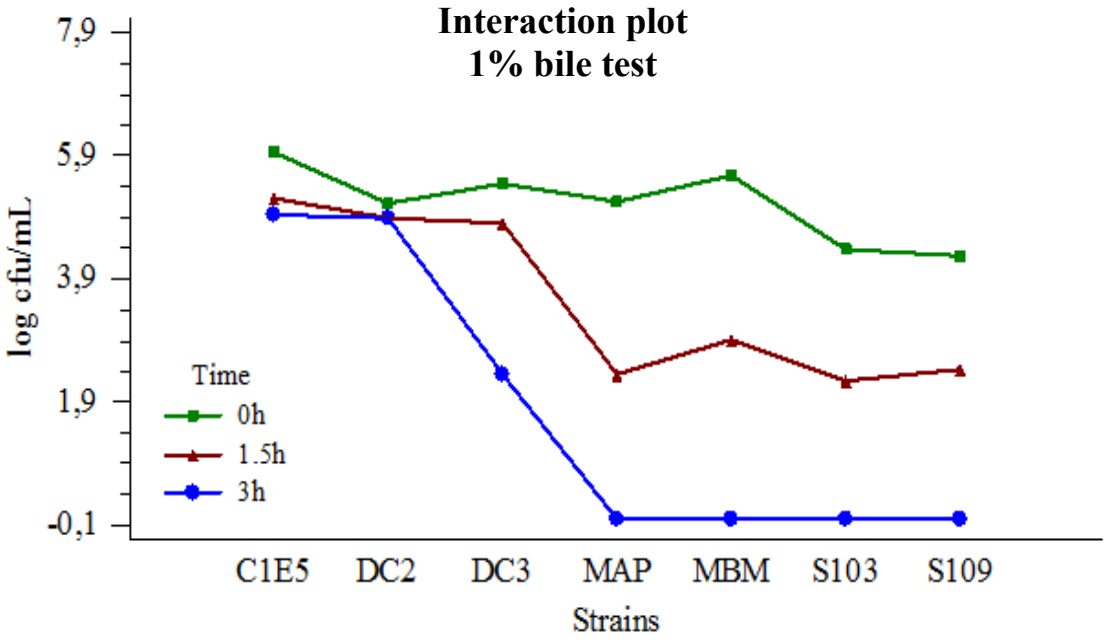

Figure 6. 1% bile salts multiple comparisons Tukey-Kramer test interaction plot; comparison of strains viable growth evolution during time

4. Molecular identification of isolates

After acidity and bile salts tolerance tests strains number was limited to seven. Total DNA of selected strains was extracted and the 16S rDNA was sequenced for species identification. Sequencing results showed that all strains belonged to *Lactobacillus plantarum* (*L.p.)* species except "L.c. 109" which belongs to the *Lactobacillus casei* (*L.c.*) specie. Therefore, during this thesis stains will be referred at as L.p. 103, L.p. DC2, L.p. DC3, L.p. C1E5, L.p. MBM, L.p. MAP and L.c. 109.

5. Antimicrobial activity

After pH and bile salts tolerance tests, strains number was limited to seven out of 156 strains. Those latter were then subjects of further probiotic characterization in order to explore their probiotic features. Antimicrobial capacity of strains was first assed using the agar spot test technique against two Gram positive (*L. monocytogenes* and *S. aureus*) and two Gram negative (*S. enterica* and *E. coli*) pathogens. Results showed that four out of seven strains were able to inhibit *L. monocytogenes* (Figure 7). L.p. C1E5, L.p. DC3, L.p. MBM and L.p. MAP which had inhibition diameters of 0.76, 0.88, 0.75 and 0.61 cm respectively while L.p.103, L.c. 109 and L.P.DC2 inhibition diameters were lower than 0.5 cm. Even more, all strains were capable of inhibiting *S. enterica*, *S. aureus* and *E. coli*. Both L.p. 103 and L.c. 109 were the best inhibiting strains of *S. enterica* with an inhibition zone diameter of 1.067 cm while L.p. DC3 was the best inhibiting strain of *E. coli* with an inhibition diameter of 1.33 cm. Lastly, L.p. C1E5 was the best inhibiting strains of *S. aureus* with an inhibition zone diameter of 0.89.

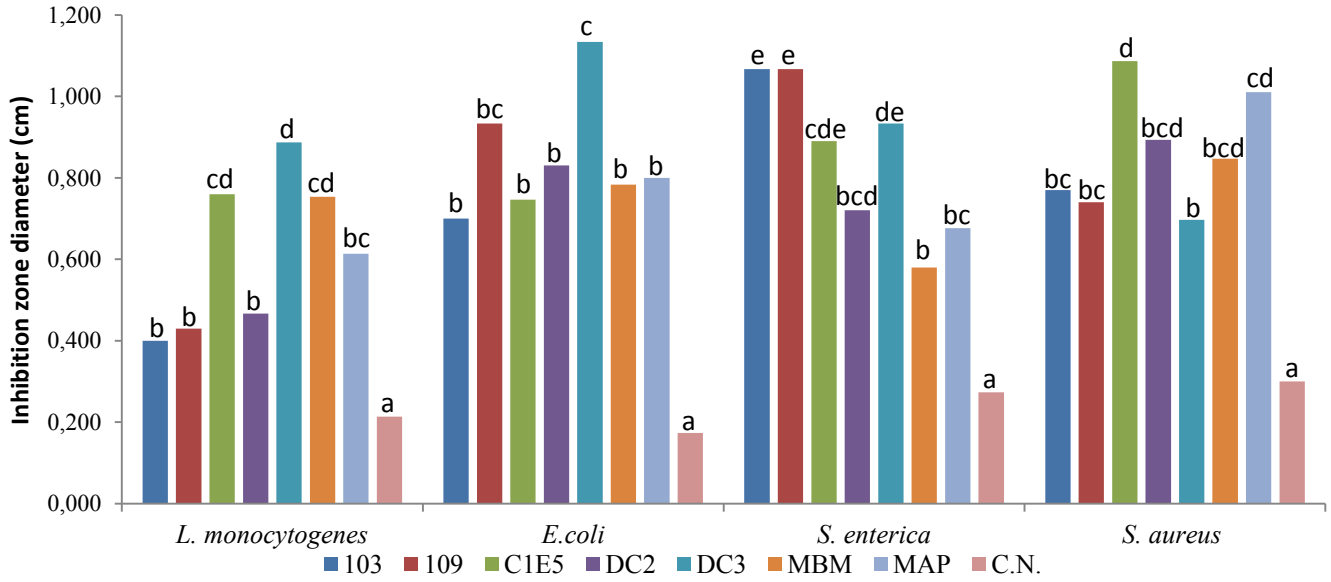

Figure 7. Antimicrobial inhibition zone diameter (cm) of seven *Lactobacillus* strains against *L. monocytogenes*, *E. coli*, *S. enterica* and *S. aureus*. Different letters represent significant statistical difference.

Strains antimicrobial activity was revaluated with the well diffusion method. All strains showed inhibition zones in the filtered supernatant well confirming that the activity is due to a secreted compound in the supernatant. Alternatively, no inhibition zones were noticed in the second well indicating that the antimicrobial activity is due to secreted organic acids.

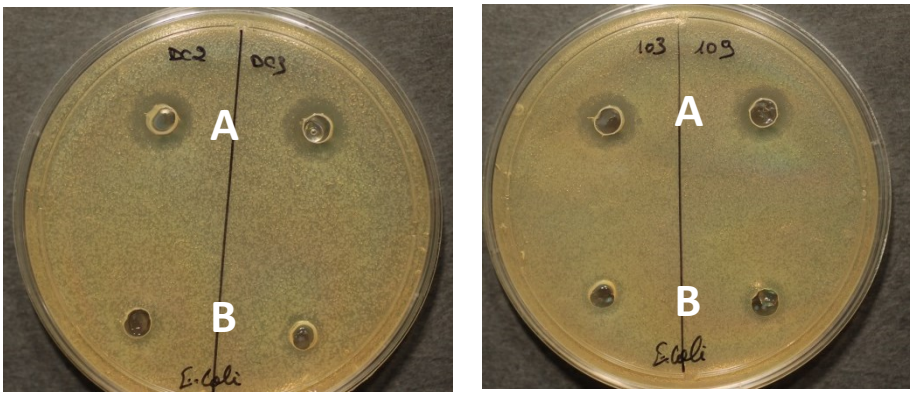

Figure 8. Well diffusion antimicrobial activity test against *E. coli* strain; A) positive results of L.p. DC2 and L.p. DC3 unmodified supernatant wells, B) negative results of L.p. DC2 and L.p. DC3 neutralized supernatant wells.

6. Antibiotic susceptibility

All selected strains were tested for antibiotic susceptibility, using both disc diffusion and MIC determination methods for 10 of the most commonly used antibiotics. Antibiotic disc diffusion test indicated that all strains were susceptible to chloramphenicol (30 µg), clindamycin (2 µg), penicillin G (10 µg), amoxicillin (2 µg), erythromycin (15 µg) (Figure 10a), tetracycline (30 µg) and ampicillin (10 µg). In fact, strains were unable to grow next to the antibiotic discs, forming an inhibition zone. Strains were more sensible to chloramphenicol than other antibiotics, inhibition zones diameter ranged from 1.27 cm for L.p. C1E5 and 1.5 cm for L.p. MBM (Figure 9). On the other hand, strains were less sensible to clindamycin with inhibition zone diameters between 0.3 cm and 1.23 cm for L.p. DC3 and L.p. 103 respectively.

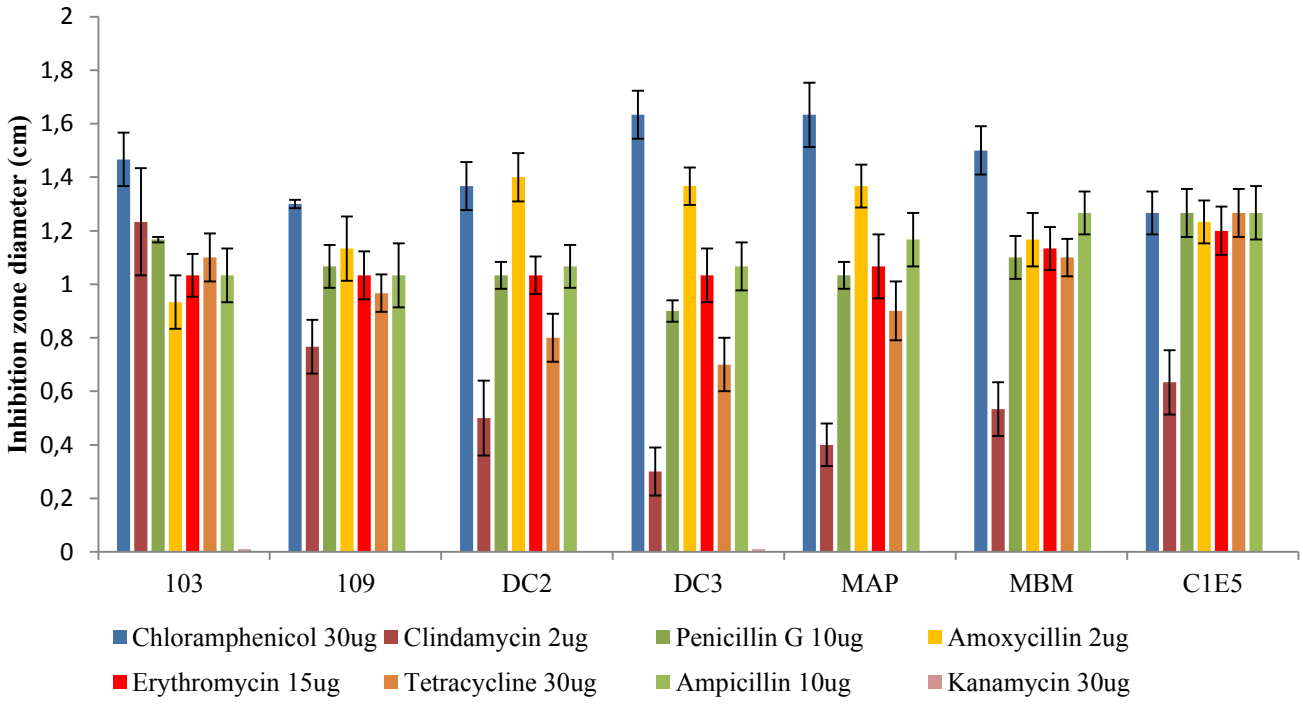

Figure 9. Antibiotic susceptibility test. Inhibition zone diameter (cm) of all seven lactobacilli strains and nine tested antibiotics.

Finally, strains were resistant to three tested antibiotics: kanamycin (30 µg), gentamycin (10 µg) and vancomycin (30 µg) (Figure 10b). No measurable inhibition zone was observed on plates, therefore antibiotics MIC were calculated. Results showed that all strains were resistant to vancomycin (Figure 11); a range of concentrations from 0.25 µg to 128 µg were unable to inhibit strains growth. Then again, strains were sensible to gentamycin and kanamycin. Kanamycin MIC was 16 µg for both L.p. DC2 and L.c. 109 and 32 µg for the rest of the strains, whereas gentamycin MIC was 1 µg for L.p. 103, L.p. C1E5 and L.p. DC2 and 2 µg for the rest of the strains

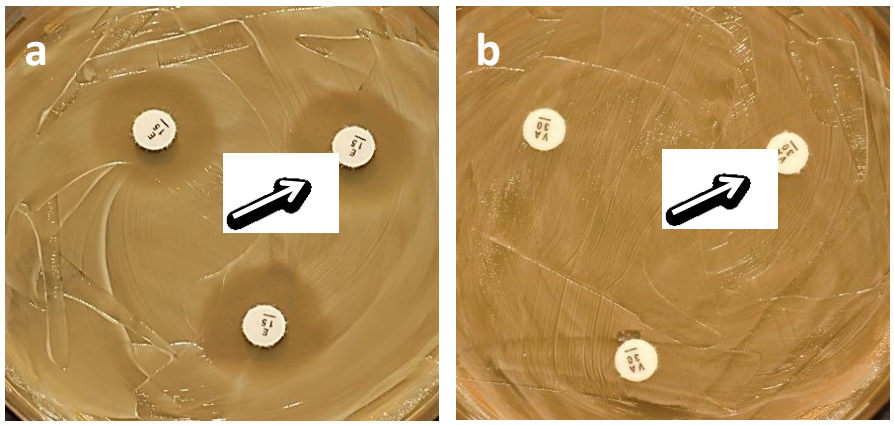

Figure 10. Antibiotic disc diffusion test results of *L. casei* 109. Susceptibility to erythromycin (a) and resistance to vancomycin (b).

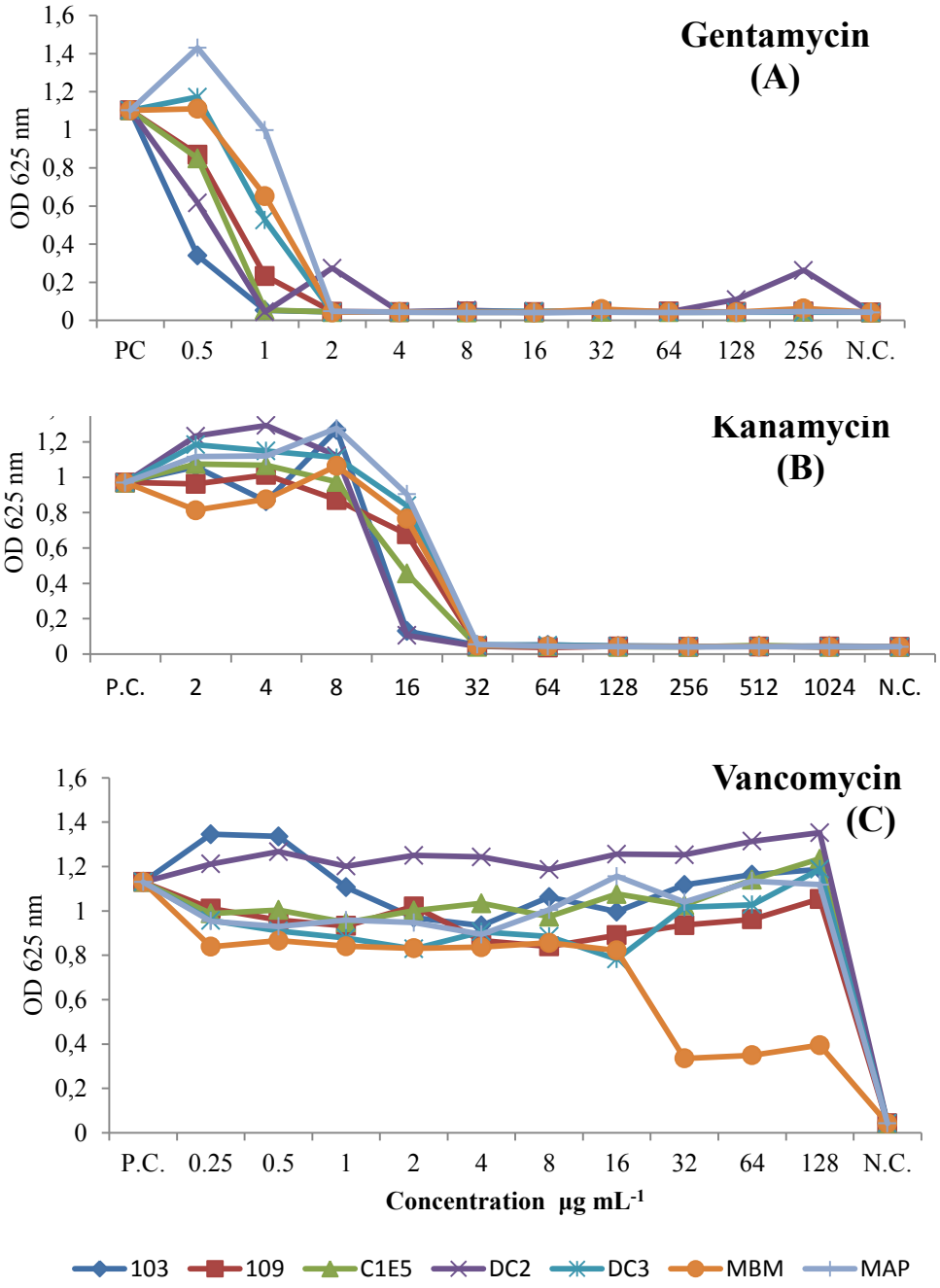

Figure 11. MIC determination test. *Lactobacillus* growth rates (OD_{625}) at different concentrations (µg mL^{-1}) of gentamycin (A), kanamycin (B) and vancomycin (C). P.C.: positive control, N.C.: negative control.

7. Adherence to epithelial cells

Overall, adherence to epithelial cells varied among strains (Table 5). L.p. C1E5 was the adherent strain; 16.46% of bacteria were adherent after 1.5h of conincubation with HT-29 cells while L.c. 109 was the second best adherent strain with 14.30% of adherence. L.p. DC3, L.p. MAP, L.p. 103, L.p. DC2 and L.p. MBM were less adherent compared to previous strain with adhesion percentage of 9.79%, 9.59%, 8.23%, 4.96% and 7.15% respectively

TABLE 5. *In vitro* adherence test of seven *Lactobacillus* strains to intestinal epithelial cells.

Bacterial strain	Count 0h	Count 1.5h	Number of bacterial cells attached to 1 HT-29	Adherence percentage (%)
L.p.103	2.82E+08	3.20E+07	160	8.23
L.c. 109	4.20E+08	6.41E+07	320	14.30
L.p. C1E5	3.78E+08	8.45E+07	422	16.46
L.p. DC2	2.63E+08	1.68E+07	84	4.96
L.p. DC3	2.93E+08	3.70E+07	185	9.79
L.p. MBM	3.65E+07	2.57E+07	129	7.15
L.p. MAP	2.79E+08	3.38E+07	169	9.59

Results are expressed as mean (n = 3) log cfu mL^{-1} and percentage (%)

4.1 Immune modulation

All seven selected *Lactobacillus* strains were tested for their capacity to inhibit NFκB, TGFβ activation and IDO expression. All strains were capable of reducing NFκB activation after TNF induction (Figure 12); the inhibition percentage varied among strains; 18.8% for L.P. MAP, 20.4% for L.P. MBM, 20.7% for L.c. 109, 21.5% for L.p. C1E5, 23.1% for L.p. DC3, 24.3% for L.p. 103 and 25.6% for L.p. DC2 The results confirmed the immune modulation capacity of tested *Lactobacillus* strains, thus further tests were done in order to define its nature. In all, supernatants, heat treated and trypsin treated bacteria were tested.

Strains supernatants were unable to reduce NFκB inhibition; in fact most strains lost their capacity except L.P. MAP. Inhibition percentages decreased dramatically for L.p. 103, L.c. 109, L.p. C1E5, L.p. DC2, L.p. DC3 and L.p. MBM with values of 5.67%, 6.5%, 5.9%, 8.8%, 7.7% and 5.9% respectively, while L.p. MAP maintained an inhibition percentage of 16.09% (Figure 12).

On the other hand, heat treated bacteria behavior was in contrast with those of the supernatants. In fact, all strains maintained an inhibition activity except L.p. MAP. Inhibition percentages varied among strains; 12.7% for L.p. 103, 19.2% for L.p. MBM, 19.5% for L.p. DC3 and 19.9% for L.c. 109, 22.9% for L.p. DC2 and 23.1% for L.p. C1E5, while L.p. MAP inhibition was limited to 3.6% (Figure 12).

Finally, trypsin treated bacteria showed different behaviors. It was noticed that only L.p. DC2 and L.p. C1E5 conserved a high rate of inhibition with 19.2% and 16% respectively, while L.p. 103, L.p. DC3, L.p. MBM and L.p. MAP kept a shy inhibition rate of 4.04%, 5.34%, 10.3% and 3.5% respectively. On the other hand L.c. 109 completely lost its activity (Figure 12).

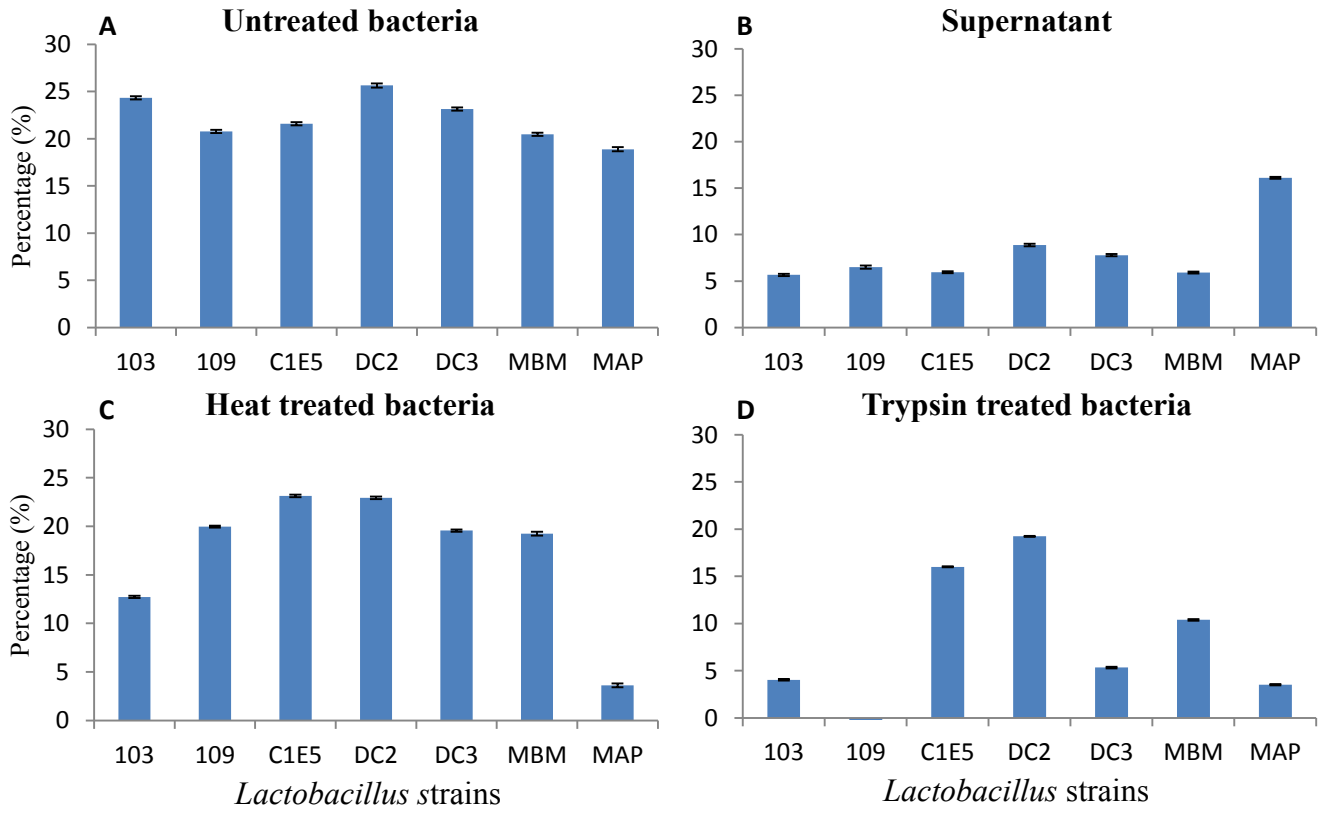

Figure 12. Strains NFκB inhibition. Inhibition percentage (%) of untreated bacteria (A), supernatant (B), heat treated bacteria (C) and trypsin treated bacteria (D). Shown results are the average of 6 experiences with 3 repetitions each.

Strains were also tested for their capacity to inhibit TGFβ activation. Different behaviors were noticed among strains (Figure 13); L.p. DC2 was unable to inhibit TGFβ activation, while L.p. 103 and L.p. C1E5 inhibition percentages were limited to 4.7% and 9.7% respectively, L.p. DC3 had the highest inhibition percentage with 15.3% followed by L.p. MBM with 14.4%, L.p. MAP with 11.9% and L.c. 109 with 11.7%.

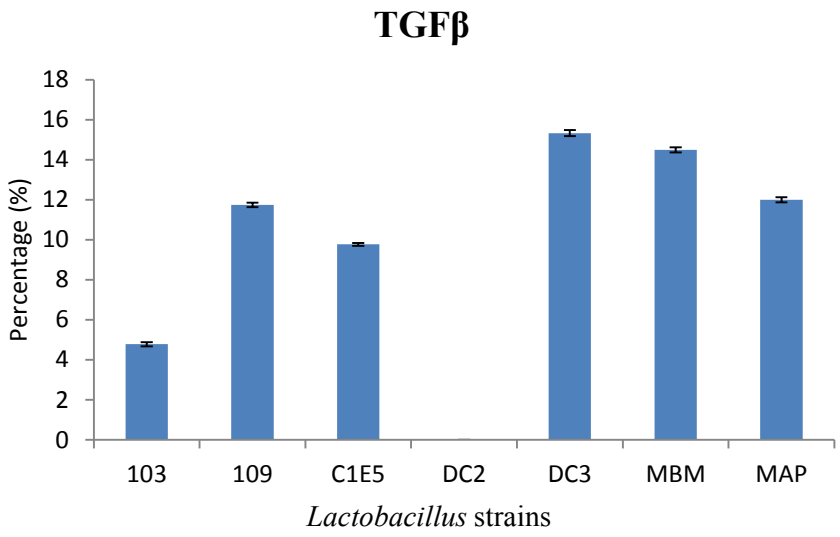

Figure 13. Strains TGFβ inhibition percentage (%) of untreated bacteria. Shown results are the average of 6 experiences with 3 repetitions each.

Ultimately strains activation capacity of IDO was also assessed. Results have shown slight activity of some strains (Figure 14). In fact, L.p. DC2, L.p. DC3 and L.p. MAP had no activity at all, while L.p. 103, L.p. C1E5 and L.p. MBM activation percentage were 1.5%, 6.2% and 3% respectively, only L.c. 109 had an activation percentage of 10.4%.

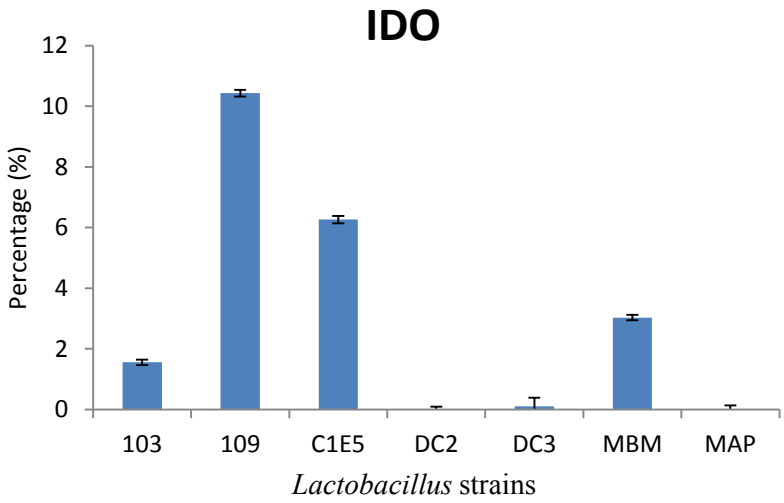

Figure 14. Strains IDO inhibition percentage (%) of untreated bacteria. Shown results are the average of 6 experiences with 3 repetitions each.

Discussions

Strains probiotic evaluation requires extensive *in vitro* and *in vivo* investigation. Many *in vitro* models can simulate with good approximation the strains surviving abilities in the GIT and confer a health benefit to the host. Such tests include investigations of the resistance to gastric acidity, bile salts and the adherence to human epithelial cells using the cell lines, the immune-modulating effects, the antibiotic resistance profile, the antimicrobial activity against pathogens, as well as competition with them for the sites of adhesion (Tannock, 2005).

Tolerance of the upper gastrointestinal tract stress

One of the main properties of probiotic bacteria is its survival capacity in different digestive conditions, mainly low pH at the stomach level. The latter can reach values as low as pH 1.5 and a good probiotic source should withstand at least pH 3.0 (Fernandez et al., 2003) *Lactobacillus* strains are naturally equipped to create and withstand acidic pH values, an important technological characteristic of bacteria associated with dairy products production (van de Guchte et al., 2012). This adaptation allows them prepare for stomach acidity stress.

In the present study, after screening 156 strains at pH 3, thirty four strains were selected based on their acidity tolerance capacity. All selected strains showed viability after 1.5 and 3 hours of exposure at pH 2.5, while seven strains maintained higher growth rate than others. Such behavior at low pH is similar to many previous studies testing other *Lactobacillus* strains from different origins for probiotic use. *L. reuteri* BFE 1058 and *L. johnsonii* BFE 106 isolated from pig faeces showed growth capacities after exposure to pH 3 (du Toit et al., 1998); *L. fermentum* SK5 was also able to survive at pH 4 and pH 3 (Kaewnopparat et al., 2013). Furthermore, twenty nine out of forty four *Lactobacillus* strains were capable to tolerate pH 2.5 after 4 hours of incubation (Jacobsen et al., 1999). Reduced viability at pH 2 has also been described; works of (Huang and Adams, 2004) and (Zarate et al., 2000) have showed growth reduction of potential probiotic *Bifidobacteria* at pH 2. Finally, some studies have also proven *Lactobacillus* capacity to survive at pH 1.5 (Haller et al., 2001) and pH 1 (Conway et al., 1987).

Based on the results of this study, pH 2.5 tolerant strains were selected for further probiotic testing, even though L.c. 109, a previously identified *L. casei* species, was unable to tolerate pH 2.5 for 1.5 hours it was included within the selection. Such behavior was previously described (Mathara

et al., 2004); strains of the *L. casei* group, isolated from the Maasai traditional fermented milk "kule naoto", were more susceptible to gastric acidity than other *Lactobacillus* species. However survival of ingested bacteria in the stomach is obviously influenced by the buffering capacity of food components. Probiotic lactic acid bacteria are mostly applied in fermented dairy products and milk substances which may provide a protective matrix enhancing survival of bacteria in the gastric juice (Ouwehand et al., 2001). Similar protective effects have been demonstrated with skim milk added to human gastric juice (Conway et al., 1987), milk and whey proteins added to simulated gastric juice (Charteris, 1998) and cheese added to porcine gastric juice (Gardiner et al., 1999). Such behavior was also observed *in vivo*, Lick et al. (2001) showed that *L. delbrueckii* and *S. thermophilus*, two poorly surviving strains *in vitro*, exhibited high survival rates *in vivo*. Consequently, it seems more appropriate to evaluate low pH tolerance with strains incorporated in the final product.

Meanwhile, in order to reach the colon in viable state, strains must cope with bile salts stress in the upper small intestine (Ruiz et al., 2013). Bacterial probiotic investigators have previously determined a testing concentration of 0.3% or 0.15% of bile salts (w/v) (Chou and Weimer, 1999; Fernandez et al., 2003; Zarate et al., 2000), thus in this study strains were tested with 0.5% and 1% of bile salts.

The determination of strains bile salt tolerance during this study showed that *L. plantarum* strains were more tolerant than *L. casei* strain (L.c. 109), this behavior has been previously described by many studies proving bacterial species or genus specific behaviors (Begley et al., 2005; Charteris, 1998; Zarate et al., 2000). Nonetheless, the tested concentration of 0.5 % bile salts is within the range of the physiological concentrations of bile and has been used previously by other researchers to mimic the small intestine environment (Burns et al., 2011). Various *Lactobacillus* strains from human and non-human origins, such as *L. casei*, *L. paracasei*, *L. plantarum* and *L. acidophilus*, showed tolerance to such concentration (Burns et al., 2008; Corsetti et al., 2008; Vinderola et al., 2002), whereas fewer strains showed tolerance to 1%; *L. rhamnosus L. acidophilus* and *L. casei* (Mishra and Prasad, 2005).

Acid and bile tolerance represent basic *in vitro* selection criteria for probiotics (Mishra and Prasad, 2005; Morelli, 2007). As already pointed out by various workers in the field *in vitro* studies can only partially mimic the actual in situ conditions in the gut ecosystem (Vizoso Pinto et al.,

2006). Yet, such *in vitro* systems remain powerful tools especially for screening numerous samples. In this study, selected strains behaviors during both low pH and bile salts tolerance tests could partially guarantee their survival in the gastrointestinal tract.

It is noticeable, that L. *plantarum* DC2, DC3 and C1E5 strains were the best tolerant strains during both acid and bile tolerance experiments this property may provide these strains with an advantage over others during future in-vivo study.

Antimicrobial activity

The ability to eliminate pathogenic strains in the host is an important characteristic that probiotic strains should possess in the maintenance of a balanced gut microflora (Ramasamy et al., 2012). Their antimicrobial activity is often due to the secretion of organic acids, hydrogen peroxide or bacteriocins.

Antimicrobial test proved that all strains were able to inhibit both Gram positive and negative pathogens growth with differences in the inhibition rate. Many works have previously documented such an activity; Klinberg et al., (2005) reported *L. plantarum* antimicrobial effect against *E. coli* and *S. enterica*, Nieto-Lozano et al. (2002) and Papamanoli et al. (2003b) both reported *L. plantarum* antimicrobial activity against *L. monocytogenes* and Nieto-Lozano et al. (2002) also reported *L. plantarum* antimicrobial activity against *S. aureus*.

However, strains antimicrobial activity disappeared when the supernatant was buffered to pH 6 and catalase was added; suggesting that the main mechanism mediating the antimicrobial activity is the production of organic acids.

Organic acids do not only fulfill a barrier effect on pathogenic bacteria, but also play a crucial role in maintaining the health of the colon (Cook and Sellin, 1998), they can penetrate the pathogenic bacterial cell and dissociate its inside cytoplasm, causing a decrease of intracellular pH and the accumulation of the ionized organic acid leading to its death (Russell and Diez-Gonzalez, 1998). Many studies have previously reported such an effect; Belicova et al. (2013) revealed that *L. plantarum*, isolated from Slovak Bryndza cheese, organic acids production was enough to inhibit *L. monocytogenes*, *S. aureus* and *S. enterica* growth, Makras et al. (2006) demonstrated that *L. casei* Shirota and *L. rhamnosus* GG antimicrobial activity was solely due to lactic acid production, while

Tejero-Sarinena et al. (2012) showed the antimicrobial effect of *L. plantarum* and *L. casei* against *E. coli* and *S. aureus* was also due to organic acid.

Finally no bacteriocins or bacteriocins-like compounds were produced even though many other studies have reported that *L. plantarum* and *L. casei* antimicrobial activity could be due to them (Aymerich et al., 2000; Enan et al., 1996; Messi et al., 2001).

Strains antibiotic susceptibility and safety

The outspread of antibiotic resistant bacteria have been fueled, during the recent years, by the vast use of antibiotics in the food and feed industries. Many studies suggest that the excessive use of antibiotics could turn commensal bacteria into reservoirs of antibiotic resistant genes; hereafter bacteria used as probiotics, for humans or animals, should not carry any transferable antimicrobial resistance genes (von Wright, 2005). *Lactobacillus* strains are most commonly considered GRAS microorganisms nonetheless they display a wide range of natural antibiotic resistance, in most cases not transmissible (Ashraf and Shah, 2011).

In this study, all strains showed susceptibility to most of tested antibiotics (chloramphenicol, clindamycin, penicillin G, amoxicillin, erythromycin, tetracycline, and ampicillin) except vancomycin, kanamycin and gentamycin. Similar behaviors have previously been reported by many authors: D'Aimmo et al. (2007) stated *L. casei*, *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* resistance to kanamycin, spectinomycin and susceptibility to clindamycin, erythromycin and penicillin. Klare et al. (2007) detailed *L. rhamnosus*, *L. plantarum* and *L. paracasei* susceptibility to penicillin, ampicillin and chloramphenicol whereas Huys et al. (2006) reported *L. plantarum* susceptibility to ampicillin and clindamycin and natural resistance to streptomycin and gentamicin.

MICs of unsusceptible antibiotics were then calculated; results showed that kanamycin MICs of both *L. plantarum* and *L. casei* were lower than the 64 μg mL^{-1} microbiological breakpoint defined by the European Food Safety Agency (EFSA) for both species. Even more, gentamycin MICs of 1 μg $mL^{-1}$and 2 μg mL^{-1}were also lower than the microbiological breakpoints of *L. plantarum* (16 μg mL^{-1}) and *L. casei* (32 μg mL^{-1}) defined by EFSA (2012). It is noticeable, that strains were resistant to higher concentrations of kanamycin and gentamycin during the disc diffusion test than the MIC test this could be explained by lower pH of MRS (pH 6.2 + 0.2) used

during the first test, compared to the LSM (pH 6.7) medium used in the MIC determination (Klare et al., 2005). Lastly, all strains showed resistance to vancomycin, this resistance though is generally considered intrinsic (Blandino et al., 2008; Charteris et al., 1998; Klare et al., 2007); until the present date there has been no indication that vancomycin resistant bacteria can transfer this resistance to other bacteria (D'Aimmo et al., 2007). Antibiotic susceptibility results confirmed strains safety of use as probiotics and starter cultures for different food fermentation processes.

Adherence to epithelial cells

Bacterial adhesion to the intestinal epithelium influences the residence time and the ability of probiotic strains to modulate the immune response and, thereby, to exert health effects in the gut (Servin and Coconnier, 2003). HT-29 cells are derived from colon carcinomas and represent the major cell phenotypes found in the human intestinal mucosa (Gopal et al., 2001) therefore, HT-29 cells were used during this study for strains adherence capacity assessment. Data demonstrate that all tested *Lactobacillus* strains displayed different adhesion behaviors.

During this study, strains adherence rates varied from 4.96% to 16.46%. The most adherent - was the *L. plantarum* C1E5 strain while the only *L. casei*. 109 strain was the second best adherent strain. Different adherence behaviors of commercial probiotic species were previously reported; *L. rhamnosus* and *L. acidophilus* NCFM showed better adherence rates, 19.7% and 16% respectively, than *L. plantarum* (7%) and *L. casei* Shirota (0.6%) when tested with HT-29 cells (Collado et al., 2007). Even more, different adherence behaviors were also reported among the strains belonging to the same species; *L. plantarum* Lp91, *L. plantarum* Lp9 and *L. plantarum* Lp77 were the better adhesive strains (10.2%, 7.4% and 7.3% respectively) compared with *L. plantarum* Lp72 (6.1%), *L. plantarum* Lp75 (4.4%) and *L. plantarum* Lp90 (6.6%) (Raj Kumar Duary, 2010). Lastly, CFU/IEC ratio influence on strains adherence have also been reported; *L. plantarum* and *L. paracasei* had the highest adherence rate of 25% and 27% when incubated at 500:1 compared with 100:1 and 1000:1 ratios (Cammarota et al., 2009).

Such results seem promising many *Lactobacillus* strains with good adhesion profiles have demonstrated probiotic characteristics. *L. rhamnosus* strain DR20 and *L. acidophilus* strain HN017 inhibited *E. coli* strain O157:H7 colonization of the intestinal cell monolayer (Gopal et al., 2001). *L.*

casei rhamnosus GG and *L. casei* Shirota showed steric hindrance behavior toward *E. coli* and *Salmonella* spp. (Y.K Lee and K.Y Puong 2002). *L. acidophilus* strain LB inhibited enterovirulent bacteria adhesion to human intestinal cells (Coconnier et al., 1993), while *L. fermentum* SK5 inhibited the adhesion of both *E. coli* and *G. vaginalis* to HT-29 cells (Kaewnopparat et al., 2013). Likewise, such behavior has also been described for bifidobacteria strains; B. lactis strain DR10 inhibited the binding of *E. coli* strain O157:H7 to intestinal cell monolayer and reduced the invasiveness and pathogenicity of this strain. *Bifidobacterium* spp. strains CA1 and F9 inhibited the entry of *S. enterica* serovar Typhimurium into Caco-2 cells (Lievin et al., 2000). Other studies proved that such activity is dose dependent, *B. breve* 4 and *B. infantis* for instance induced a dose-dependent inhibition of Caco-2 cell invasion by enteropathogenic strains of *E. coli*, *Y. pseudotuberculosis* and *S. typhimurium* (Gopal et al., 2001).

The mechanisms involved in protection against pathogen adhesion have been proposed to be by either non-specific steric hindrance of receptors (Chan et al., 1985; Reid et al., 1987) for pathogens or competition with pathogens for the binding sites (Matijasic et al., 2006) overall it is highly considered strain and pathogen dependent.

Immune modulation

Symbiosis relationships have long been described between the host and its intestinal microbiota. More than 10^{14} bacteria contribute in the maintenance of host intestinal homeostasis (Kaci et al., 2011). Tolerance and inflammatory responses are constantly balanced by bacteria and IECs cross talk.

Selected strains were first tested for their modulation of the innate immune system; down regulation of NFκB dependent transcriptional activity was first assessed. All strains showed an inhibition capacity on TNFα induced NFκB activation. Similar behaviors of other probiotic strains in an inflammatory context have previously been described; NFκB activation and interlukin-8 (IL-8) have been reduced in intestinal epithelial cells models by *Lactobacillus rhamnosus* and *L. casei* (Lin et al., 2009; Tien et al., 2006), *L. rhamnosus* GG and *L. delbrueckii* were capable of reducing IκB degradation and subsequent NFκB translocation into the nucleus (Zhang L et al., 2005; Santos Rocha et al., 2012), *L. casei* proved to reduce NFκB activation by the modulation of several genes

involved in ubiquitination and proteasomal processes while *L. plantarum* WCFS1 was effective at modulating immunity through NFκB and L.p. MAP kinase signaling (van Baarlen et al., 2009).

It is noticeable that strains anti-inflammatory effects were observed using live bacteria, in exponential growth phase, washed before conincubation with the HT29 cells. This procedure allowed us to avoid cell lyse and eliminate potential cytoplasmic compounds released.

On the other hand, supernatants and heat treated bacteria test proved that *L. plantarum* MAP modulation of immune response is due to an excreted active metabolite. Other studies have previously described similar activity *Streptococcus salivarius* and *S. vestibularis* markedly inhibited TNF-α-induced NFκB activation (Kaci et al., 2011), while supernatants of *Bifidocaterium breve* and *S. thermophilus* contained anti-TNF-α metabolites.

As for the six remaining strains (*L. casei* 109, *L. plantarum* 103, DC2, DC3, C1E5 and MBM), no immune modulation activity was observed when strains supernatant was tested, though bacterial cell heat inactivation seemed harmless suggesting that cell surface components are responsible for the anti-inflammatory activity. A number of *Lactobacillus* species have also shown such an activity; *L. reuteri* was capable of inhibiting IL-8 secretion and block NFκB translocation by cell surface components (Ma et al., 2004), *L. acidophilus* ATCC 4536 was capable of preventing NFκB activation by the activation of mitogen-activated protein kinases (L.p. MAPK) through direct contact with epithelial cell and *L. delbrueckii* was capable of preventing IκB phosphorylation via cell surface components (Santos Rocha et al., 2012).

A wide variety of cell surface components from *Lactobacillus* strains can be involved in this immune modulation activity. *L. johnsonii* and *L. acidophilus* inhibited LPS-induced IL-8 secretion via lipoteichoic acid (LTA) (Vidal et al., 2002). Surface proteins of some pathogens can also mediate bacteria host interaction; *Mycobacterium tuberculosis* heat shock proteins use diverse Toll-like receptor (TLR) pathways to activate pro-inflammatory signals (Bulut et al., 2005). Finally, surface lipoproteins can also mediate host immune modulation via TLR 1, 2 and 6 (Shimizu et al., 2005; Shimizu et al., 2008). Therefore, strains cell surface proteins role was assessed. *Lactobacillus* strains were trypsin treated to eliminate surface exposed proteins and applied to HT-29 cells. Results showed that four strains (*L. plantarum* 103, DC3, MBM and *L. casei* 109) lost their NFκB inhibition activity suggesting that surface exposed proteins could be responsible. On the other hand *L.*

plantarum DC2 and C1E5 activity, could probably be due to a non-peptide bond containing component. Therefore, *Lactobacillus* strains inhibited the activation of NFκB, some threw excreted metabolites others threw cell surface components. Thus these *Lactobacillus* strains could be purposed as tools for prevention or treatment of intestinal bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (Cremonini and Talley, 2005; MacDonald and Monteleone, 2005), and pathogens.

Conclusion

Lactobacilli are common and abundantly found in a variety of traditional cheeses produced in Mediterranean part of Europe from raw goats, ewes, or cows' milk. In many, lactobacilli are important for ripening and aroma development. On the other hand, some production specifications of Sardinian PDO cheeses authorize the use of autochthonous LAB cultures, from the area of origin, for milk inoculation in cheese manufacture.

Based on the results *L. plantarum* C1E5 and *L. casei* 109 strains exhibited the characteristics generally considered essential for probiotic organisms. Strains safety and functionality were proven by their resistance to the gastrointestinal conditions (acidity and bile salts), lack of transferable antibiotic resistance, inhibition of undesirable bacteria, good adherence to intestinal cells and promising immune modulation capacities. In this regard, the use of probiotic autochthonous LAB in the cheese making procedure can constitute a successful strategy increasing the production of "functional" traditional cheeses.

Nevertheless, such *in vitro* tests are the first step toward probiotic selection; these strains have to be investigated in more depth for more probiotic characteristics *in vivo*.

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Abbreviations

APC	Antigen-presenting cells
BHI	Brain heart infusion
BSH	Bile salt hydrolase
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CFS	Cell-free supernatant
DC	Dentric cell
DCA	Deoxycholic acid
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EFSA	European Food Safety Authority
FAO	food and agriculture organization
FCS	fecal calf serum
GALT	Gut-associated lymphoid tissue
GCN2	General control non-depressible 2
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
HCl	Hydrogen chloride
HT-29	human colon carcinoma cells
IDO	Indoleamine 2,3-dioxygenase
IEC	Intestinal epithelial cells
IL	Interlukine
INT	Interferon
ISO	International Organization for Standardization
IκB	Nuclear factor kappa light polypeptide gene enhancer inhibitor

LAB	Lactic acid bacteria
LCA	Lithocholic acid
LSM	LAB susceptibility test medium
L.P.	
MAPK	Mitogen-Activated Protein Kinases
MIC	Minimal inhibitory concentration
MRS	Man, Rogosa and Sharpe
N.C.	Negative control
NB	Nutrient broth
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Optical density
PBS	Phosphate buffer solution
PPARγ	Peroxisome Proliferator-Activated Receptors gamma
PRR	Pattern recognition receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
sIgA	Secretory immunoglobulin A
TGF-β	Transforming growth factor β
TGF-β RI	Threonine kinase receptors type I
TGF-β RII	Threonine kinase receptors type II
TH	T helpers
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
WHO	World health organization

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