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Thesis title

Safety aspects and beneficial features of lactobacilli isolated from Lebanese Baladi goat milk

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Abstract

The microbiological and physicochemical parameters of Lebanese goat milk from the “Baladi” breed were evaluated and twenty-eight lactobacilli were isolated and examined *in vitro* for their probiotic potential. Initially, *Lactobacillus* isolates were examined for safety issues and exhibited diverse susceptibility to commonly used antibiotics while none was hemolytic. Subsequently, Isolates showed variable antimicrobial activity towards a range of spoilage and pathogenic bacteria. Regarding their performance in conditions simulating the human gastrointestinal tract, all isolates remained unaffected at pH 3 and in the presence of bile salts (0.5% (w/v)) for 3 hours. Based on their survival at pH 2.5 for 3 hours, 10 isolates were selected for the adhesion assay. Low adhesion was observed to HT-29 and Caco-2 cells. Co-cultivation of THP-1 cells with specific isolates indicated a tendency for anti-inflammatory modulation shown by an increase in IL10 mRNA levels. Further analysis for probiotic properties indicated partial bile salt hydrolase activity for all isolates (n=28). Isolates were identified by 16S rRNA sequence and were affiliated to the *Lactobacillus casei* group. Overall, by applying *in vitro* tests, a select number of presumptive *Lactobacillus rhamnosus* strains showed promising probiotic features from the Lebanese Baladi goat milk. This is the first report about safety and beneficial characterization of *Lactobacillus* strains isolated from Baladi goat milk from Lebanon.

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CHAPTER 1: Introduction

1.1 Lebanon: geography, topography and climate

Lebanon is a small country of 10,452 square kilometers of surface on the Mediterranean Sea surrounded from the north and east by Syria and from the south by Palestine (Figure 1). It is a mountainous and coastal country, which consists of a narrow strip of territory 217 km long (north-south) and 40 to 80 km wide (east-west) and a fairly rugged relief.

Lebanon is divided into eight governorates (Mohafazat): Beirut, North Lebanon, Akkar, Mount Lebanon, Bekaa, Baalbeck-Hermel, South Lebanon and Nabatieh. It consists of two mountain ranges: Mount Lebanon and Anti-Lebanon (border with Syria).

Lebanon has a temperate Mediterranean climate, with hot, dry summers and cold, wet winters. Compared to its geographical surroundings, it enjoys relatively high precipitation in winter, concentrated between November and March, while the summer is very dry, especially in Baalbeck-Hermel and Bekaa, the two plains which constitute the main farming areas: the Bekaa plain (between the two mountain ranges) and the Akkar plain (to the north, along the Syrian border).

Figure 1: Map of Lebanon

The Bekaa plain is characterized by an average altitude of 900 meters, covering 4,000 square kilometers, which consist of more than the third of the country's surface. The plain extends over 120 km with a width ranging from 8 to 14 km. The southern part, called by the Lebanese "the West Bekaa" because of the north-east-south-west axis of the plain, is bounded by Jabal Gharbi in the west and Mount Hermon in the east and it is crossed by the Litani river.

1.2 Goat farming sector in Lebanon and Baladi Goat breed

Goat farming is well developed in the marginal areas and especially in the Bekaa valley. According to the General Census of Agriculture (2012) established by the Ministry of Agriculture and the FAO, the total number of goat in Lebanon is 403860 heads distributed on 5847 breeders of whom, 1141 breeders do not invest agricultural land and cover 29% of the goat population. The average herd size is 69. The number of female goats is 241467 heads, representing 60% of the total herd, and an average of 41 heads per breeder. The Bekaa valley contains the quarter of Lebanon's goat population (25% of the national population).

The local goat population consists mainly (96.8%) of the indigenous breed "*Baladi*" (Figure 2) (also named Black or Mamber) characterized by its rusticity and ability to withstand the heat and drought of the region (Chedid et al., 2018a; Serhan and Mattar, 2018). It is a medium-sized breed (40 to 50 kg live weight) characterized by a great heterogeneity of colors. However, its dominant color remains the plain black, with often tawny spots above the eyes. Some other breed are also available and constitute a very small percentage, less than 5%, of the total goat population and consist mainly of a local breed named Damascus or Chami or foreign breeds such as Alpine and Saanen (Hamadeh et al., 1996; Hajj, 1999; Ministry of Agriculture-FAO, 2004). The Chami or Damascus breed is native to Syria as its name shows, but it is also found in Egypt and Turkey (LTIC, 2003). This large breed (60 to 70 kg live weight) is characterized morphologically by a uniform buff color. Alpine and Saanen goats selected for their milk production are imported from France by some NGOs (LTIC, 2003).

Figure 2: The Baladi breed

The adopted breeding system, especially for the breed Baladi, is the extensive pastoral system, relying on pastures and crop residues as its main source of feed (Chedid et al., 2018a). Shifts from transhumance system to a more sedentary and crop based one are starting to appear and some commercial farms are starting to be based using mainly Damascus, Saanen and Alpine in intensive breeding with high milk production potential (Chedid et al., 2018b). Extensive breeding is more likely to value the least productive areas of the country and the most arid which are very rich in spring after the snow melts and becomes poor in June after being used intensively. For this reason, an original and complementary organization has been established between farmers from the plain that can provide crop residues (Wheat, potato...) and shepherds, applying horizontal or semi-nomadic transhumance systems, which allows a significant availability of feed for flocks of these systems.

1.3 Goat milk production

Goats are reared for the production of milk and meat. Goat milk production of the Baladi breed relying on extensive breeding is seasonal, and milking season is from March to August. Milk is generally collected twice per day, in the early morning and in the evening, and sold to private collectors. The local breed Baladi has a low milk productivity, the production estimation is 189 kg for 180 days of milking (Serhan and Mattar, 2018), but has an excellent adaptation to difficult environments (Iñiguez, 2004). On the other hand, Damascus and foreign breeds have a higher milk productivity reaching respectively 270 and 600 kg of milk for 250-270 days of milking (Keskin, 2002; Mioc et al., 2008).

Goat milk production in Lebanon has increased from 21.2 (2008) to 34 (2010) thousand tons (Serhan and Mattar, 2018). Moreover, a growth of 76% in caprine dairy production accounting for over 54 million Euros has been recorded in 2014 accompanied with a rising consumer demand for goat dairy products (Chedid et al., 2018a). Milk is almost entirely transformed, in a traditional way, to cheese and other dairy products like Laban (Tamime and Robinson, 2007), Labneh (Serhan et al., 2016), Ayran and other more typical products like Kishk (Salameh et al., 2016), Ambariss and Darfieh cheese (Serhan and Mattar, 2013). Laban, Labneh and Ayran are fresh products to be consumed, with an optimal usage time of three weeks and are highly appreciated by the Lebanese consumer. Physicochemical and organoleptic and sensory properties of these artisanal products are defined by natural starters and traditional methods of production passed from generations to generations (Serhan and Mattar, 2018).

Due to the milk seasonal production, many preservation methods of caprine dairy products are used: goat Labneh and Ambarees are usually shaped into small balls and conserved in glass jars with olive oil, also Ambarees can be frozen for later use. Darfieh cheese is conserved in olive oil as well, Baladi goat cheese is preserved in brine (salty water), and Kishk is well-maintained as powder to be used in soup and other recipes. These types of production with a long shelf life provide to the farmers a fairly satisfactory income throughout the year.

The Lebanese market is still largely driven by imports. Lebanese cheese imports registered 32,000 tons in 202 and full dairy exports amounted to 420 tons in 2002 (Serhan and Mattar, 2018). A scientific and technical contribution and a restructuring of the Lebanese and especially

goat dairy sector are decisive for the valorization of the internal production of dairy products and the stimulation of the goat sector.

1.4 General characteristics of *Lactobacillus*

Most of the bacteria used for the fermentation of milk are called lactic acid bacteria (LAB) due to the fact that they mainly produce lactic acid by the catabolism of lactose. LAB are organotrophic prokaryotic cells forming a group heterogeneous consisting of cocci and bacilli. They are Gram-positive bacteria, catalase negative, do not produce spores and usually non-motile (König and Fröhlich, 2009; Prasirtsak et al., 2013; Laranjo et al., 2017).

The genus *Lactobacillus* represents the largest family of LAB (Canchaya et al., 2006; Mangia et al., 2019). It belongs to the group of Firmicutes, to the class of Bacilli, to the order of Lactobacillales and to the Lactobacillaceae family (Killer et al., 2014).

Qualitatively, it is the most important group of LAB. It was created for the first time by Beijerinck in 1901, it includes over 200 species with an extreme diversity on the phylogenetic, phenotypic and ecological level (Goldstein et al., 2015; Sun et al., 2015) This diversity is due to the variation in guanine/cytosine content (G/C) which varies between 30 and 55% depending on the species (De Vos et al., 2009). Lactobacilli are non-spore-forming, Gram-positive, non-motile, thin rods varying in length from long to short (Slover and Danziger, 2008). They were originally grouped taxonomically according to their major carbohydrate metabolism, as homofermentative (group A), facultatively heterofermentative (group B) or obligately heterofermentative lactobacilli (group C) (Sun et al., 2015). Lactobacilli belonging to group A ferment hexoses almost exclusively (>85 %) to lactic acid via the Embden-Meyerhof-Parnas pathway (EMP) or glycolysis; pentoses and gluconate are not fermented. Lactobacilli of group B ferment hexoses to lactic acid via EMP and are able to degrade pentoses and gluconate via an inducible phosphoketolase, an enzyme of the pentose phosphate (PP) pathway, with a resulting production of acetic acid, ethanol and formic acid under glucose limitation. Finally, the group C lactobacilli possess a fructose-bisphosphate aldolase B, but not phosphoketolase, and they metabolize pentoses and hexoses exclusively via phosphogluconate pathway (corresponding to the first part of the PP) and produce lactic acid, ethanol (or acetic acid) and CO2 (Salvetti et al., 2012). The

optimum growth temperature of lactobacilli lies between 30 and 40°C but they can grow at temperatures ranging from as low as 5°C to an upper limit of 53°C depending on the species (Ahmed et al., 2006) and according to their growth temperature, lactobacilli are classified into mesophilic or termophilic species. Lactobacilli optimal pH growth is between 4.5 and 6.4; they can also tolerate high salt concentrations (6.5%) (De Vos et al., 2009). Lactobacilli have an extensive habitat and are found in many biotopes: water, soil, milk and dairy products, plants, meat products, fish, beer, wine and fruit. Lactobacilli are, among other things, an important part of the human microbiota and animal. In healthy humans, they are found throughout the digestive system: from the mouth to the colon (Bernardeau et al., 2005). The bacteria that occupy a niche in the GIT are true residents or autochthonous (i.e., found where they are formed). Other bacteria are just “get a lift” through the gut and are allochthonous (i.e., formed in another place) (Pithva et al., 2012).

1.5 *Lactobacillus* in milk and dairy products

Lactobacillus species are important in the dairy industry, since they are used as starters (SLAB) or non-starter lactic acid bacteria (NSLAB) for the production of fermented products. *Lactobacillus* spp. along with coccal LAB, act early during the first stage of cheese making. Their action is mainly linked to two aspects of their metabolism: 1- The production of lactic acid by lactic fermentation of sugars. 2- Hydrolysis of proteins and especially caseins by proteases, enzyme located in the outer cell wall which affects the curd formation. The fermentative activity is therefore important from a technological point of view but also for the control of pathogenic microorganisms (Gobbetti et al., 2018).

The raw milk is a natural growth substrate for *Lactobacillus* which constitutes the majority of the NSLAB present in most fermented dairy product. It is well-known that specificity and typicality of raw milk cheeses is due mainly to NSLAB composed especially of mesophilic *Lactobacillus* species (*L. casei subsp. paracasei, L. plantarum, L. rhamnosus, L. curvatus, L. brevis, L. fermentum*) found in raw milk (De Angelis et al., 2001; Lafarge et al., 2004; Bernardeau et al., 2005; Folli et al., 2017). NSLAB may enter adventitiously from the milk but they may also come from the cheese making environment.

They play an important role in cheese ripening since they are highly adapted to stress conditions during cheese production (heat-related, osmotic, oxidative and acidic stress) through many mechanisms. They contribute in the development of good organoleptic properties of dairy products mainly due to their proteolytic and lipolytic activities. Some NSLAB co-metabolize citrate and fermentable carbohydrates. The concomitant utilization of hexoses, pentoses and citrate produces an excess of pyruvate that is converted into acetoin, 2,3-butanediol, and diacetyl which protect the cells against low pH as well as contribute to cheese flavor. They increase the content of peptides, free amino acids and free fatty acids in the cheese matrix that contributes to flavor intensity, increasing aroma and reducing bitterness and harshness, as well as accelerates cheese ripening (Rehman et al., 2000; Castro-Sowinski, 2016). A study by Mangia et al. (2008) showed that mesophilic lactobacilli along with lactococci seemed responsible for a balanced lipolytic, proteolytic and fermentative activity that conferred desired attributes to the experimental cheese and, remarkably, reduced significantly the waste production. Many authors have studied Cheddar cheese made from raw or pasteurised milk and generally agree that Cheddar cheese made from raw milk is more intensely flavored and ripens faster than cheese made from pasteurised milk (Rehman et al., 2000). The NLSAB *L. casei* and *L. rhamnosus*, mostly isolated from different Caciocavallo Cheeses, used in the production of Caciocavallo Palermitano cheese generated a different aromatic profile from that of the other strains and from the control and were able to produce typical flavor compounds as 2,3-butanedione and 2-butanone, 3-hydroxy (Guarrasi et al., 2017).

1.5.1 *Lactobacillus* in bioconservation

Bioconservation (or biopreservation) can be defined as the extension of shelf life and food safety by the use of natural or controlled microbiota and/or their antimicrobial compounds. One of the most common forms of food biopreservation is fermentation. Traditionally, foods have been protected against spoiling by natural processes of fermentation (Ananou et al., 2007; Mangia et al., 2019). The increase use of artificial chemical preservatives in food and the consumer's high demand for safe and minimally processed foods has led to the need for finding safer alternatives

in food preservation (Muhaildin et al., 2013). The use of LAB and or their metabolites for food preservation is generally accepted by consumers as something “natural” and “health-promoting”. Among LAB, addition of *Lactobacillus* culture to food is an approach in food preservation, it inhibits food spoilage bacteria by producing growth inhibiting substances like bacteriocins, lactic acid etc. (Pithva et al., 2012).

1.5.2 *Lactobacillus* as probiotics

Other than their importance in the fermentative process and in the bioconservation of dairy products, various strains of LAB are considered as probiotics and have a number of well-established benefits on the human health. They can improve lactose digestion, play a role in preventing and treating diarrhea, act on the immune system and help the body to resist and fight infection (De Vrese and Marteau, 2007; Ashraf and Shah, 2014).

LAB strains, have been considered as the most suitable candidates as probiotics, and dairy products are the most widely used food carriers to deliver probiotics (Shokryazdan et al., 2014; Linares et al., 2017) Among the known health-promoting or probiotic microorganisms, the probiotic LAB species are most often belonging to *Bifidobacterium* spp. and *Lactobacillus* spp. (Linares et al., 2017).

1.5.2.1 Concept of probiotics

The concept of probiotics is far from being new. It was born from the observations made by the Russian biologist Metchnikoff in 1907, winner of the Nobel Prize in Medicine and Physiology (Gordon, 2008). He has linked the "unusual" longevity of some rural populations in Bulgaria with their high consumption of fermented milk products (Sule et al., 2014). He hypothesized that lactobacilli were important for human health, longevity, and promoted yogurt and other fermented foods as healthy ((Pithva et al., 2012). It was after these observations that he began to modify the flora of his patients by giving them milk acidified with a strain of *Lactobacillus delbrueckii* subspecies *bulgaricus*, a species traditionally used for yogurt making. Metchnikoff was the first to suggest that consumption of LAB in these fermented milks could have a beneficial effect on health. FAO/WHO experts in 2001 (Food and Agriculture Organization) have

issued an official definition of probiotics: "live microorganisms that, when administered in sufficient quantity, confer beneficial effects on the health of the host" (Fijan, 2014).

1.5.2.2 Criteria for selecting probiotics strains

The definition given to probiotics is broad since it includes all microorganisms that might be bacteria, yeasts, protozoa, *archaea* and viruses. Criteria have therefore been proposed by different authors for the purpose of selecting potentially probiotic strains (Shewale et al., 2014).

These criteria are divided into three categories: safety, functional and technological criteria (Table 1) (Bussarin and Rakshit 2006; Nagpal et al., 2012; Shewale et al., 2014).

Table 1: Main criteria used for the selection of probiotic strain

Safety criteria	• History of non-pathogenicity and non-invasion of the epithelium intestinal • Strain of human or food origin • Strain characterized by phenotypic and genotypic techniques • Precise taxonomic identification • No possible transmission of resistance genes to antibiotics
Functional criteria	• Tolerance to acidity, bile and digestive enzymes • Production of antimicrobial substances (bacteriocins, acids organic, hydrogen peroxide or other inhibiting compounds) and antagonism towards pathogens • Bile salt hydrolase activity • Adhesion to intestinal cells and persistence in the intestinal tract • Immunomodulation • Ability to produce beneficial effects on the health of the host
Technological criteria	• Stability during manufacturing processes and in the finished product • Preservation of probiotic properties after production • No modification of the organoleptic qualities of the finished product

Among the criteria related to safety, first, it must be non-pathogenic and be recognized as safe. The taxonomic identification of the strain is an important step in establishing new potentially probiotic strains. Each strain must be identified by reliable molecular techniques and confronted with an updated nomenclature (FAO/WHO, 2002). Sequencing of 16S RNA is a very reliable method commonly used for the identification of probiotic strains. In the latter case, it is recommended that the technique be combined with biochemical and phenotypic tests to ensure compliance of the strain. The origin of the strain is also an important condition as the specific interaction with the host is maximized when it comes from the same habitat (Donelli et al., 2013).

Concerning the functional criteria, it must have the ability to survive and grow in the physiological conditions of the digestive tract, as well as have a good tolerance to the acidic pH found in the stomach and bile salts encountered in the duodenum (Boke et al., 2010). Adhesion to epithelial cells of the intestine is often cited as a selection criterion. The importance of this feature is evidenced by the fact that many probiotics do not colonize the intestine and therefore need to attach themselves to have their beneficial effect (Ouwehand and Salminen, 2003). The production of antimicrobial compounds is also considered an important selection criterion (Ghanbari and Jami, 2013). Indeed, the production of these compounds can improve the competitiveness of the producing strains against strains naturally present in the digestive tract and inhibit potentially present pathogens (Dobson et al., 2012).

Finally and from a technological point of view, probiotic strains should survive to food processing and biological stresses, which include extremes in temperature, pH, as well as osmotic, oxidative stresses, and bacteriophage attack. They must have several criteria such as ease of cultivation while maintaining their biological properties and stability during production and storage processes. They should not even have adverse effects on the taste or aroma of the product and should not increase the acidification during its shelf-life (Koskin and Rakshit, 2006; Mills et al., 2011).

Health Canada has approved the following bacterial species, when delivered in food at a level of 1×10^9 colony forming units (CFU) per serving, as probiotics: *Bifidobacterium* (*adolescentis, animalis, bifidum, breve* and *longum*) and *Lactobacillus* (*acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus* and *salivarius*) (Hill et al., 2014); The Italian Ministry of Health has regulated the use of probiotic bacteria in the food sector and confirmed the use of the word probiotic for food and food supplements under certain conditions, including a minimum number of viable cells (1×10^9 CFU) administered per day, a full genetic characterization of the probiotic strain and a demonstrable history of safe use in the Italian market (Ministero della Salute, 2013). A viable concentration should be present at the end of the product life cycle (Soccol et al., 2010)

Dairy products are good probiotic vectors for humans (Mocanu and Botez, 2012). Several factors have been claimed to affect the viability probiotic cultures in fermented milk products. During

the production and storage of fermented products, factors such as acidity, dissolved oxygen and redox potential can affect the growth and survival of probiotics (Shah, 2000). In order to obtain the required viable count of probiotics at the end-of-life of the product, the mortality that will occur during storage must be taken into account.

For probiotics to develop in dairy products that already contain lactic cultures, antibiosis should be avoided and, ideally, a symbiosis should be established between lactic ferment and probiotics. Thus, care must be taken to choose probiotic strains compatible with lactic cultures (Champagne et al., 2005). Probiotics grow slowly relatively to lactic cultures in a dairy environment. It is therefore necessary to use means that will increase and maintain high concentrations of probiotics (McComas and Gilliland, 2003).

Lactobacillus are the most commonly used microorganisms as probiotics because of the perception that they are desirable members of the intestinal microflora and because these bacteria are considered GRAS (generally recognized as safe) and/or included in the QPS (Quality Presumption of Safety) list (Shokryazdan et al., 2014; Mangia et al., 2019). However, the increasing isolation of LAB in clinical infections (Aguirre and Collins, 1993, Kochan et al., 2011) and the increase of antimicrobial resistant strains by gene transfer impose the study of the safety aspect (hemolytic activity, susceptibility to antibiotics) of the probiotic lactobacilli strains (FAO/WHO, 2002).

Mechanisms based on *in vivo* and *in vitro* studies have been proposed (Table 2) to explain the major modes of action associated with probiotics, including lactobacilli, to prevent colonization and growth of pathogenic microorganisms.

Table 2: Probiotic effect and mechanisms of action of probiotic lactobacilli (De Vrese and Marteau, 2007; Lebeer et al., 2008; Liong, 2008; Ashraf and Shah, 2014; Quinto et al., 2014; Wedajo, 2015; Rocha-Ramirez et al., 2017)

Probiotic effect	Mechanisms of action
Improvement of lactose digestion	Secretion of lactase, an enzyme capable of digesting lactose often deficient in the digestive tract of the host, especially in people who are called "lactose intolerant"
Reduction of food allergies	Decrease in the protein flow by reducing the permeability of the intestinal membrane
Reduction of the blood cholesterol level	Deconjugation of bile salts
Stabilization of the intestinal flora	Competition with pathogenic bacteria at the level of adhesion to the receptors
Inhibition of pathogenic or undesirable germs	Production of metabolites such as organic acids (pH decrease), hydrogen peroxide and production of antibacterial substances such as bacteriocins
Reduction of the risk of diarrhea	Inhibit the growth and the metabolic activity as well as the adhesion to intestinal cells of enteropathogenic bacteria
Prevention of the colon cancer	Stimulation of the immune system Production of antimutagenic compounds Altering the differentiation process of tumor cells
Stimulation of the intestinal immune system of the host	Improving the intestinal barrier influencing the activity of the innate immune cells associated to the gut Regulation of cytokine expression Effects on phagocytosis Modulation of dendritic cells

Aim of the study

The Lebanese dairy industry is made up of many small factories where good hygiene practices are not always well applied, consequently milk quality is not always guaranteed. The local dairy sector in Lebanon runs the risk of being supplanted by the abundant imported supply of dairy products which are perceived to have better hygiene and quality and being tastier than the local ones. Thus, Lebanese dairy products must be reorganized and restructured in order to have the chance to compete with the imported products, otherwise it might become out of market one day.

The maintenance of product quality depends on a large number of factors, related to the physicochemical and microbiological characteristics of the raw material used, the types of LAB added and to the manufacturing technology.

Due to the limited literature on Lebanese goat milk and its natural bacterial ecosystem, the aim of the thesis is, first to evaluate the physicochemical and microbiological features of Lebanese raw goat milk and secondly, to study the safety and beneficial aspects of the *Lactobacillus* isolated strains. This work could contribute to the improvement of traditional methods of fermentation: at the farmer's scale, some of these isolated strains could have potential uses in the improvement of traditional fermentation technologies and at the industrial scale, indigenous strains of bacteria could have applications in the areas of health, nutrition and bioconservation.

CHAPTER 2: Physicochemical and microbiological characterization of Baladi goat milk and *Lactobacillus* identification

2.1 Introduction

Goat farming is an important part from the economy point of view in many countries, especially in the Mediterranean and Middle East region (Lad et al., 2017). In Lebanon, small ruminant hold a key role within the economy of marginal regions and contribute to the financial gain of the population involved in pastoral activities (Chedid et al., 2018b). Adaptability of goats to harsh climates makes them suitable for landless and marginal farmers (Zenebe et al., 2014). Goat milk and products are manufactured and consumed since many centuries and produced traditionally in small farms (Lad et al., 2017). Popularity of goat milk and goat milk products is increasing worldwide because of their organoleptic and nutritional properties (Da Silva et al., 2016). Production and processing of goat milk can provide a profitable alternative to cow milk due to its specific composition, taste, texture, flavor and its natural and healthy aspects when ingested as part of daily diet (Terzic-Vidojevic et al., 2013). Goat milk health benefits such as better digestibility, high calcium and high essential amino acids content compared to cow and sheep milks have been reported (Perin and Nero, 2014). Furthermore, goat milk contains similar amount of vitamin B6 and pantothenic acid, more niacin (about 3.5-fold), but less vitamin B12 (about 4-fold) and folic acid (approximately 6-fold) than bovine milk. Folic acid deficiency is one of the main charges against the use of goat milk as a product for infant nutrition. To overcome this issue, folate bio-enrichment of goat dairy products was achieved using native folate producing starter cultures (Sanna et al., 2005).

Moreover, people who are suffering from anemia, osteoporosis and malabsorption, are advised to consume goat milk (Lad et al., 2017). It has also been proven to improve the state of health and wellness of the human body and to reduce the risk of developing disease, especially allergies (Lai et al., 2016).

Goat milk has a very rich and complex autochthonous microbiota including LAB (*Lactococcus lactis, Lactobacillus acidophilus, L. reutei, L. plantarum, L. casei, L. paracasei, L. bulgaricus, L. lactis, Bafidobacterium bifidum, B. longum, B. lactis, Streptococcus thermophilus, Enterococcus faecium, E. feacalis*…) which play an important role in the fermentation of food (Perin and Nero, 2014; Mittu and Girdhar, 2015). Goat products have a distinctive and relatively strong flavor compared to cow milk and the indigenous flora is the main factor responsible of the aroma and

taste of the final product and varies in intensity in artisanal products versus mass-produced goat cheese products (Serhan and Mattar, 2018).

Microbial component of raw milk is influenced by many factors e.g.: season, location, pasture, animal's health and more generally by the hygiene practices implemented in the farm and milk collection and storage used for transformation and which has a decisive influence on the species and the level of microbial contamination (Mangia et al., 2016).

The European Union legislation with the implementation of the Hygiene package has defined a series of hygiene rules to be applied throughout the food chain (from field to table) in order to guarantee the safety of food for consumer protection. In Lebanon, the current legislation is old and restrictive hygiene rules are not well applied especially in small farms, consequently goat milk quality is not always excellent and turned out to be a reservoir of undesirable and pathogenic bacteria as *Staphylococcus*, Enterobacteria, *Clostridium* and many others. Ministry of Public Health in Lebanon showed an increase in the number of reported cases of food poisoning from 43 in 2002 to 373 in 2004 and some of these cases were related to the consumption of homemade cheese (Serhan and Mattar, 2018).

Physicochemical and microbiological quality of Lebanese goat milk has received very little attention to date. The use of commercial LAB cultures and pasteurized milk for industrial cheese production has led to the loss of flavor and a reduction in the diversity of dairy microflora (Terzic-Vidojevic et al., 2013). Given the increased demand on goat milk and goat milk products, a better knowledge of the microbiological quality and physicochemical composition of goat milk of the Baladi breed will contribute to the improvement of the quality of raw goat milk in Lebanon. Furthermore, isolation and screening of LAB from natural processes have always been the most powerful mean for obtaining useful cultures for commercial use (Terzic-Vidojevic et al., 2013). Few studies about goat milk cheese, which report about the biodiversity of LAB exist. Thus, a better study of the LAB microbiota in general and *Lactobacillus* species in particular is essential for a future use of this matrix for the production of fermented products.

2.2 Material and Methods

2.2.1 Milk sampling

Sample of goat's raw milk from the Baladi breed were collected from six farms (Table 3) adapting the extensive breeding in the West Bekaa region (Lebanon) between June and July 2016. Milking was manual and samples were collected in sterile containers from the evening milking and stored on ice until delivered to the laboratory. Milk was analyzed within 12 hours of storage at 4°C.

Table 3: Characteristics of the six farms

Farm	Date	Farm owner	Region	Total number/ Number of milking goat	Breeding	Nutrition
A	12/6/16	Mahmoud El Kaabi	Amik	300/250	Extensive	Pasture
B	19/6/16	Mohamad El Nabaa	Kab Elias	370/105	Extensive	Pasture
C	17/7/16	Manei Oukla	Kab Elias	100/75	Extensive	Crop residue (wheat and potato)
D	17/7/16	Oukla Oukla	Kab Elias	150/75	Extensive	Crop residue (wheat and potato)
E	24/7/16	Najib Chrenik	Jeb Janine	300/150	Extensive	Crop residue (wheat and potato)
F	24/7/16	Mohamad Chrenik	Jeb Janine	350/100	Extensive	Crop residue (wheat and potato)

2.2.2 Microbiological analyses

Volume of 1 mL of milk were taken from each sample, 6-fold diluted in a physiological sterile solution (9 mL) and plated on the specific culture media for each kind of groups of bacteria. Samples were examined for total microbial count (TMC) on Plate count agar (PCA, Biolab) at

30°C for 48h in aerobic conditions, total coliforms by Brilliant green bile broth 2% (BGBB, Liofilchem) at 37°C for 48h in aerobic conditions using Durham tubes for gas detecting and at 44°C for fecal coliforms in the same conditions. *Staphylococcus* spp. were counted on Baird Parker agar (BPA, Biolab) supplemented with Egg Yolk Tellurite Emulsion (Avonchem) at 37°C for 48h in anaerobic conditions using Gas-Pack (Oxoid) and presumptive colonies of coagulase positive staphylococci (CPS) were assayed for coagulase activity using the Staphylase test (Oxoid). Presence or absence of *Salmonella* were detected following two steps: 1- An enrichment using Selenite broth (SB, Liofilchem) at 44°C for 24h in aerobic conditions 2- Incubation on Shigella agar (SS, Biolab) at 37°C in the same conditions. Presumptive *Clostridium perfringens* were counted on Tryptose Sulphite Neomycin Agar (TSN, Himedia) at 44°C for 24 h in anaerobic conditions. Yeasts were quantified using Yeast Extract-Peptone-Dextrose Agar (YPDA, Bio Basic) at 30°C for 48h in aerobic conditions. LAB were enumerated on Man Rogosa Sharpe agar (MRS, Himedia) for presumptive lactobacilli and on M17 agar (Himedia) for Coccal-shaped LAB at 37°C for 48h in anaerobic conditions using the pour plate technique. After incubation, petri dishes with 30-300 colonies were used for enumeration (CFU mL^{-1}).
Petri dishes with 30-300 colonies were used for enumeration and number of bacteria was expressed as colony forming units (CFU) per milliliter of milk. Total and fecal coliforms were quantified using the MPN method.

2.2.3 Physicochemical analyses

Milk samples pH value was measured using a pH meter (Hanna Instruments). Physical characteristics (density and freezing point) and milk constituents (fat, solid non fat and protein) were determined by milk analyser Milkana, Kam 98-2A, (Ekomilk, Milk Analyser) in triplicata.

2.2.4 Isolation of *Lactobacillus* spp.

Selected colonies (n=28) of presumptive *Lactobacillus* spp. on MRS agar medium were tested for Gram stain, shape morphology and catalase production. Homo- and hetero-fermentative activities were determined using Durham tubes. Gram positive, catalase negative and rod-shaped colonies

were purified by repeated streaking on MRS agar and stored using the same medium of isolation with 20% (v/v) of glycerol (Avonchem, UK) at -80°C for further identification and characterization.

2.2.4.1 Molecular identification

<u>DNA extraction</u>

DNA was extracted from 2 mL of overnight culture as described by Georgalaki et al. (2017) with minor modifications. Cell pellet was washed twice with PBS, heated at 65°C for 10 min and centrifugated (10,000×g, 5 min). After discarding the supernatant, 100 μL of lysozyme (50 mg mL^{-1}; Sigma, St. Louis, MO, USA) in TE buffer, 10 μL of mutanolysin (5U μL^{-1}, Sigma) and 10 μL of RNase A solution (Sigma) are added to the pellet. The suspension were incubated at 37°C for 30 min and shacked often to avoid the pelletization of the cells. Then, 500 μL of a GES reagent composed of 5 mol L^{-1} guanidium thiocyanate (Sigma),100 mmol L^{-1} EDTA at pH 8 and 0.5% (w/v) sarcosyl were added and the solution were cooled on ice for 5 min. After cooling, 250 mL cold ammonium acetate (7.5 mol L^{-1}; Sigma) were added and the samples were held on ice for 10 min. A volume of 500 μL of chloroform was added and well mixed. Samples were centrifuged (13,000×g, 5 min at 4°C) and supernatants were transferred to new Eppendorf tubes. A volume of 0.54 of cold isopropanol was added, tubes were mixed by inversion and stored at -20°C for an overnight. The next day, tubes were centrifugated (12500×g, 10 min at 4°C) and supernatants were discarded. Pellets were washed twice with 70% (v/v) of ethanol and washes were separated by a centrifugation (12500×g, 10 min at 4°C). A final centrifugation with the same conditions was realized, the supernatant were discarded and all the drops of ethanol were taken off using a micropipette. DNA pellet were dried by incubation at 37°C for 3 min and than at room temperature until the total evaporation of ethanol and re-suspended in 30 mL of TE buffer and stored at 4°C for at least 1 hour before PCR use. DNA is checked qualitatively on a 0.8% (w/v) agarose gel electrophoresis and quantitatively using a spectrophotometer.

<u>16S rDNA gene sequence analysis</u>

Selected isolated were identified by 16S rRNA sequencing. Amplification was performed in 50 μL final PCR reaction volume containing 2 μL 16S F (5’-GGA GAG TTA GAT CTT GGC TCA

G-3’) and 2 µL 16S R (5’-AGA AAG GAG GTG ATC CAG CC-3’) primers, 200ng DNA and One Taq Quick-Load 2x Master Mix (New England BioLabs Inc., Ipswich, MA, USA). The PCR amplification was carried out in a Mastercycler Gradient thermal cycler (Eppendorf, Germany) according to the following program: initial denaturation at 94°C for 2 min, amplification for 30 cycles with denaturation at 94°C for 30s, primer annealing at 56°C for 30s, and primer extension at 72°C for 80s, followed by a final extension at 72°C for 5 min. The products were separated electrophoretically at 90V on a 1% (w/v) agarose gel. The 1 Kb DNA ladder (Invitrogen, Carlsbad, CA, USA) was used for comparison. For sequence analysis of the 16S rRNA gene, the PCR products were purified using the Nucleospin gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions and sequenced (Eurofins Genomics, Wien, Austria). Searches in the GenBank database were performed with the BLAST program to determine the closest known relatives of the partial 16S rRNA gene sequences obtained (http://www.ncbi.nlm.nih.gov/BLAST).

2.2.5 Statistical analysis

Results of microbiological counts were log10 transformed to normalize the data. The significance of difference between means of farms for each microbial groups and each physicochemical parameter, was determined by Anova single factor ($P < 0.05$). When the effect was significant ($P < 0.05$), differences between means were separated by Tukey-Kramer test ($P < 0.05$).

2.3 Results and Discussion

In the study, goat milk samples were collected from six farms in the West Bekaa region which include a large part of the small ruminant farmers and population and which is largely understudied in comparison to other Lebanese region such as North Bekaa (Chedid et al., 2018b).

2.3.1 Microbiological characterization of goat milk

Microbial count of the six farms is listed in table 4. Values of TMC for the six farms varied from 3.87 to 5.39 log CFU mL^{-1}. The limit value for TMC according to the Regulation (EC) No 853/2004 is set to be $< 1.5\ 10^6$ CFU mL^{-1}. This limit was not exceeded in any of the farms. Tabet et al. (2016), showed higher values (6.92 log CFU mL^{-1}) for the same breed elevated extensively in Ashkout, a Lebanese region situated in the Mount-Lebanon. Studies on other breeds in other countries showed microbial values similar to the average (4.94 log CFU mL^{-1}) obtained for the six farms (Foschino et al., 2002; Kyozaire et al., 2005; Suguna et al., 2012). Farm A and B showed the lowest values without any significant difference. Farm C and D showed the highest values without also any significant difference. Values of the total microbial count of farm E and F were very close without any significant difference, occupying an average position within the six farms. Comparing statistically ($P < 0.05$) all farms, they can be associated into three groups; from the less contaminated to the most: group 1 including farm A and B, group 2 including farm D, E and F and group 3 including farm C and D. Total microbial count is an indication of the sanitary conditions under which the food was produced (Ombarak and Elbagory, 2017). It provides information on the hygienic quality of raw milk. It is considered to be the determining factor in the shelf life of fresh milk. It is the most sought-after flora in microbiological analyzes.

Total and fecal coliforms are indicators of fecal contamination of livestock products, and their presence was highlighted by the gas bubbles which appeared in the Durham tubes using the BGBB medium after incubation at 37°C.

Total coliform were absent in Farm A and fecal coliform were absent in farm A, B and D. Farm C and D showed the lowest values for total coliforms with 0.85 and 0.60 log CFU mL^{-1} respectively with no significant difference ($P > 0.05$) with farm A, while farm B was the most

contaminated with 4.04 log CFU mL^{-1} forming a single group. Values of farm E and F were much close as for TMC counts with 2.66 and 2.46 log CFU mL^{-1} respectively with no significant difference. The average (3.29 log CFU mL^{-1}) obtained for the six farms in the study is considered as an intermediate value comparing to values obtained in other studies for the same and different breed (Foschino et al., 2002; Kyozaire et al., 2005; Suguna et al., 2012; Lai et al., 2016; Tabet et al., 2016). Fecal coliform was detected only in farm C (0.85 log CFU mL^{-1}), E (1.64 log CFU mL^{-1}) and F (1.08 log CFU mL^{-1}). Statistical analyses ($P < 0.05$) divided the farms into two groups: group 1 including farm A, B, C, D and F and group 2 including farm C, E and F. The search for microorganisms indicating contamination of faecal origin makes it possible to judge the hygienic state of the samples. High coliform counts are an indication of lack of hygiene in the farms and during milking: an unclean udder, unsanitary milking practices, or milk contamination in the container (Ombarak and Elbagory, 2017). Kyozaire et al. (2005) obtained the highest coliforms count in the herd under the extensive system and contribute this contamination to the water used for goats and to hand-milking procedure.

Baird-Parker agar medium supplemented with Egg Yolk Tellurite Emulsion was used to enumerate CPS in the samples. Characteristic black colonies surrounded by a clear zone were selected and subjected to coagulase test. *Staphylococcus aureus* among CPS is one of the predominant causes of food poisoning worldwide, and a particular concern to the dairy industry (Merz et al., 2016). *Staphylococcus aureus* may access milk bulk either by direct excretion from the udder with clinical and subclinical staphylococcal mastitis, or by faecal contamination. Spread of infection can occur through milkers' hands, washcloths, teat cup liners, and flies causing udder infections accompanied by an increase in the permeability between the blood compartment and the milk which has for consequence a change in the milk composition (Kousta et al., 2010). As goat and sheep milk are often used for traditional, unpasteurized products such as raw milk cheeses, they represent a potential source of staphylococcal food poisoning (Merz et al., 2016). Values of S*taphylococcus* spp. for farm A, B, C and E were intermediate between all the six farms ranging from (2.98 to 3.21 log CFU mL^{-1}) with no significant difference. Farm D, showed the lowest value (2.63 log CFU mL^{-1}) and farm F the highest value (4.21 log CFU mL^{-1}) and were significantly different from each other and from the remaining farms. Average value for

all farms was 3.56 log CFU mL^{-1} similar to other studies from other breeds showing values of 4.15 log CFU mL^{-1} (Ombarak and Elbagory, 2017), 2.95 log CFU mL^{-1} (Suguna et al., 2012) and 3.11 log CFU mL^{-1} (Foschino et al., 2002). Results of Tabet et al. (2016) for the same breed showed absence of coagulase positive staplylococci and an average of 4.82 log CFU mL^{-1} for coagulase negative staphylococci. However, *Staphylococcus aureus* poses a risk with respect to staphylococcal food poisoning when concentration levels are higher than 5 log CFU g^{-1} and this limit was not exceeded in any of the farms (Kousta et al., 2010).

Microbiological analysis of *Salmonella* spp. did not show any evidence of contamination, which is in accordance with the Regulation (EC) No 2073/2005.
The results obtained concerning the absence of salmonella in milk are consistent with those of Tabet et al. (2016) but in contrast for what was stated by Lai et al. (2016) that Salmonella is commonly detected in raw goat milk. The main source of contamination would be fecal excretion, dissemination of the bacterium into the environment and then contamination of the skin of the udders and milking equipment (Cortés et al., 2006).

Presumptive *Clostridium perfringens* was detected in farm E (0.30 log CFU mL^{-1}) only. *C. perfringens* is associated with diverse environments including soils, food, sewage, and as a member of the gastrointestinal (GI) tract microbial community (i.e., microbiota) of both diseased, and non-diseased humans and animals. Clostridia are able to survive in the environment and contaminate any type of food or material if the conditions of hygiene and sterilization are not respected (Xiao et al., 2012; Kiu and Hall, 2018).

Farm C and D showed the lowest values for yeast with 2.58 and 2.38 log CFU mL^{-1} respectively while farm F was the most contaminated with 3.72 log CFU mL^{-1}. Values of farm A and B were similar (3.06 log CFU mL^{-1}) and close to farm E (3.09 log CFU mL^{-1}). Statistical analyses ($P < 0.05$) divide the farms into three groups: group 1 including farm A, B and E, group 2 including farm C and D, group 3 including farm F. No moulds were detected. Average value (3.2 log CFU mL^{-1}) of yeast count for the six farms is considered acceptable comparing to other results obtained from other studies (Foschino et al., 2002; Suguna et al., 2012). Concerning yeasts spoilage in dairy products, little data is available. It was defined that yeasty and fermented off-

flavors in cheese were detected when yeasts grew at populations equal or above 5 to 6 log CFU g^{-1}. The milk contamination with yeast and mold is inevitable, especially in case of manual milking and could be responsible for modifying the organoleptic properties of the milk. Until now, more than 60 yeast species have been identified as spoilage agents of dairy products (García et al., 2004; Garnier et al., 2017).

As already mentioned, microbial component of raw milk is influenced by many factors and milk can easily get contaminated and spoiled due to poor hygienic conditions maintained at 'on farm' levels or due to improper handling, inadequate storage and transport conditions of milk (Suguna et al., 2012). There are a series of factors behind the difficulties in managing the sanitary quality in the farms. These factors include the low level of production per head, small flocks size, poor milking facilities, poor water supply, dirty teats and udder, hand-milking and consequently long milking times, conditions under which the herds or flocks are raised, adverse climatic conditions and the spread of production over a wide geographic area (Ombarak and Elbagory, 2017). On the other hand, adaptation of the breed to a specific environment and its tolerance to severe climatic conditions could be a factor of resistance to bacterial contamination. An epidemiological survey was conducted on a sampling of a Lebanese goat population in order to determine the prevalence of infection with the Caprine arthritis encephalitis virus (CAEV) in Lebanon. Local herds were less affected than imported breeds. This was explained by the fact that indigenous breeds are more resistant and tolerant than other breeds with regard to CAEV infection (Tabet et al., 2015).
A study achieved by Kyozaire et al. (2005), comparing extensive, intensive and semi-intensive system and pipeline, bucket and hand milking, showed that dairy goat farming under the extensive production, where hand-milking is applied, can be adequate for the production of safe raw goat milk. Goat milk handling is very primitive with almost no cooling devices for the collected milk, and very poor hygienic conditions linked to the cleaning and disinfection of the utensils, which means poor control over zoonotic diseases. Very few farmers have the innovation capacity to follow the correct milking and handling procedures and only few large holdings with large investment and very modern facilities in Lebanon follow the international norms and standards of milking, handling, hygiene and control of quality (Serhan and Mattar, 2018). The implementation of basic principles of public health practice in dairy routines, may, however, be

difficult to achieve. Training for farmers should, nevertheless, be carried out to ensure the use of hygienic practices to enhance good milk hygiene principles and alleviating the problem of food insecurity in these communities (Kyozaire et al., 2005).

Providing adequate details on the prevalence of pathogenic or spoilage microorganisms in goat milk might be useful to identify and implement appropriate HACCP (Hazard Analysis Critical Control Point) ISO 22000 International Standards Organization, or British Retail Consortium (BRC) along with good GAP and GMP (good agricultural and manufacturing practices) at the farm level, to benefit both consumers and the dependent dairy industry. GMP defines the series of general measures to be implemented by the food industries to ensure the safety of the process and conformity of food products to precise guidelines such as: processes necessary for primary production, design of the premises and equipment, training, documentation and consumer awareness, hygiene of the handling personnel and sanitation and maintenance practices (Suguna et al., 2012; Serhan and Mattar, 2018).

LAB occur naturally as indigenous microflora in raw milk and are predominant, when selected, contribute to an increase in the functional value of goat milk (Junior et al., 2015; Da Silva et al., 2016). LAB were enumerated in raw milk samples from the six farms using M17 agar for coccal-shaped LAB and MRS agar for rod-shaped LAB.

Counts showed high variability between farms: the size of the population ranged from 1.60 to 4.19 log CFU mL^{-1} of raw milk for both rod-shaped and coccal-shaped LAB with an average of 3.46 and 3.66 log CFU mL^{-1} respectively and significant differences were observed between all the samples. To our knowledge, this work is the first study to present the diversity of LAB in Lebanese raw goat milk from the Baladi breed. Previous studies by Serhan et al. (2009) and Dib et al. (2012) on isolation of LAB from Lebanese goat dairy products (Laban, Labneh, Ambarise, Darfieh, Keshek and Shanklish) showed the highest average value of LAB for Darfiyeh cheese with 7 log CFU g^{-1} after 20 days of ripening. Cheriguene et al. (2007) obtained an average of 6.7 log CFU mL^{-1} of LAB in Algerian goats'milk. Comparison of the results obtained in this study with literature shows low content of LAB in goat milk from Baladi breed, this might be due to many factors such as nutrition.

Table 4: Microbial groups (Log CFU mL^{-1}) in raw goat milk samples from the six farms

Microbial groups	**Farms**						**Average**
	A	**B**	**C**	**D**	**E**	**F**	
Total microbial count	3.87 ± 0.01^{a}	3.93 ± 0.10^{a}	5.39 ± 0.20^{b}	5.14 ± 0.04^{bc}	4.79 ± 0.20^{c}	4.76 ± 0.16^{c}	4.94 ± 0.57
Total coliform	0.00 ± 0.00^{a}	4.04 ± 0.07^{b}	0.85 ± 0.59^{a}	0.60 ± 0.45^{a}	2.66 ± 0.04^{c}	2.46 ± 0.11^{c}	3.29 ± 1.40
Fecal coliform	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.85 ± 0.59^{ab}	0.00 ± 0.00^{a}	1.64 ± 0.09^{b}	1.08 ± 0.58^{ab}	1.00 ± 0.64
***Staphylococcus* spp.**	3.05 ± 0.11^{a}	3.13 ± 0.09^{a}	3.21 ± 0.13^{a}	2.63 ± 0.05^{b}	2.98 ± 0.11^{a}	4.21 ± 0.02^{c}	3.56 ± 0.49
***Salmonella* spp.**	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00
Presumptive *C. perfringens*	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.79 ± 1.06^{b}	0.00 ± 0.00^{a}	1.00 ± 0.67
Yeast	3.06 ± 0.01^{a}	3.06 ± 0.23^{a}	2.58 ± 0.08^{bc}	2.38 ± 0.12^{c}	3.09 ± 0.15^{a}	3.72 ± 0.09^{d}	3.20 ± 0.43
Rod-shape LAB	3.00 ± 0.02^{a}	1.95 ± 0.03^{b}	2.56 ± 0.03^{c}	1.78 ± 0.05^{d}	1.60 ± 0.03^{e}	4.19 ± 0.03^{f}	3.46 ± 0.89
Coccal-shaped LAB	3.91 ± 0.01^{a}	3.36 ± 0.03^{b}	1.60 ± 0.00^{c}	2.14 ± 0.08^{d}	3.04 ± 0.03^{e}	4.19 ± 0.01^{f}	3.66 ± 0.92

Results are expressed as mean ± standard deviation (n = 6)
[a,b,c] Values in the same row with different superscripts differ significantly ($P < 0.05$)

2.3.2 Physicochemical characterization of goat milk

The quality and chemical composition of goat milk are directly related to breed (indigenous or selected), lactation stage and physiological and genetic aspects of the animal. In addition, goat milk characteristics may vary as a result of diet composition and environmental conditions of each country (Peres et al., 2016). The physicochemical characteristics of goat milk allow their use in a wide range of products besides fluid milk, that can be consumed either raw, pasteurized or UHT, such as cheese, butter, yogurt, ice-cream and sweets, amongst others (Da silva et al., 2016).

The pH values of milk samples were as follow: 6.72, 6.78, 6.65, 6.62, 6.66 and 6.67 respectively for the six farms (A, B, C, D, E and F). They are close to the normal pH of goat fresh milk. The average obtained 6.68 ± 0.05is in line with the standards and milk samples were stored in good conditions.

Physicochemical parameters of the six farms are listed in table 5. Fat content is the more quantitatively and qualitatively variable component of milk, depending on lactation stage, season, breed, genotype and feeding. Total protein is one of the main quality criteria applied to goat and sheep milk payment in many countries (Raynal-Ljutovac et al., 2008; Abbas et al., 2014). Protein content may also vary among species, and according to animal and lactation stage. Goat milk has some particular properties that confer technological advantages in comparison to cow milk, such as alpha s1-casein, resulting in softer gel products, a higher water holding capacity and a lower viscosity. Goat milk has smaller size of fat globules that confer technological advantages in comparison to cow milk like providing a smoother texture in derived products. Goat milk contains a somewhat lower amount of caseins and so high proportion of serum proteins which is the first reason normally given to explain the greater digestive utilization made of goat milk protein than of cow milk protein. (Raynal-Ljutovac et al., 2008; Vaquil and Rathee, 2017).

Fat content varies between 2.55 and 3.99% and shows significant differences ($P < 0.05$) between farms. Protein content varies between 3.01 and 3.69% without any significant differences between farms ($P > 0.05$). Farm D represents the highest value for both contents. Tabet et al. (2016) showed higher values 4.31% and 4.00 for the same breed. Fat content for Alpine,

Toggenburg, Saanen and LaMancha breeds in Mclnnis et al. (2015) varied (2.6 to 3.4%) closely to the results obtained and protein content (2.3 to 3.1%) was slightly lower.

Solid non fat content varies between 7.89 and 8.69% without any significant differences between farms ($P > 0.05$). Farm B and D represent the highest value and farm C the lowest one.

Total solids content varies between 10.86 and 12.68% without any significant differences between farms ($P > 0.05$). Total solids content is the sum of solid non fat and fat content. Farm D already showed the highest values for both parameters, simultaneously has the highest value of total solids between farms. Due to its low value of fat content, farm C shows the lowest value for total solids.

Density values were very similar between farms matching 1.02 for farm C and 1.03 for the others farms and within normal range (Gabas and Cabral, 2012).

Table 5: Physicochemical parameters of raw goat milk samples from the six farms

Physicochemical parameters	Farms						Average
	A	B	C	D	E	F	
Fat (%)	2.66 ± 0.03^{a}	3.57 ± 0.11^{bcd}	2.97 ± 0.59^{ac}	3.99 ± 0.18^{b}	2.55 ± 0.14^{a}	2.75 ± 0.08^{ad}	3.08 ± 0.52
Protein (%)	3.49 ± 0.10^{a}	3.67 ± 0.14^{a}	3.01 ± 0.91^{a}	3.69 ± 0.12^{a}	3.61 ± 0.05^{a}	3.39 ± 0.24^{a}	3.48 ± 0.23
Solid non fat (%)	8.49 ± 0.12^{a}	8.69 ± 0.17^{a}	7.89 ± 1.11^{a}	8.69 ± 0.14^{a}	8.63 ± 0.06^{a}	8.36 ± 0.29^{a}	8.46 ± 0.28
Total solids (%)	11.16 ± 0.14^{a}	12.26 ± 0.28^{a}	10.86 ± 1.70^{a}	12.68 ± 0.20^{a}	11.18 ± 0.17^{a}	11.11 ± 0.32^{a}	11.54 ± 0.68
Density	1.03 ± 0.00^{a}	1.03 ± 0.00^{a}	1.02 ± 0.00^{a}	1.03 ± 0.00^{a}	1.03 ± 0.00^{a}	1.03 ± 0.00^{a}	1.03 ± 0.00

Results are expressed as mean ± standard deviation (n = 6)
^{a,b,c} Values in the same row with different superscripts differ significantly ($P < 0.05$)

2.3.3 Identification of *Lactobacillus* spp.

MRS medium (used in anaerobic conditions) is selective for *Lactobacillus* but some species of LAB belonging to the genera of *Leuconostoc* and *Pediococcus* can also grow on this medium. Moreover, it does not allow the growth of other LAB such as *Carnobacterium*, because of the presence of acetate, and *Bifidobacteria* that require the addition of cysteine to the culture medium. Since lactobacilli are rod-shaped, microscopic examination of isolates helps to eliminate coccal-shape LAB corresponding to the genera *Leuconostoc* and *Pediococcus*. On each of the petri plate used for the enumeration, colonies were classified into categories according to their macroscopic appearance (shape, size, color and texture). In each category a colony was randomly selected as representative among those observed to carry out the first orientation tests. Selected isolates were subjected to microscope examination (form and arrangement), Gram staining and catalase test.

Based on the preliminary identification, 28 isolates resulted Gram positive and catalase negative, presumptive *Lactobacillus* and then selected for the species identification stage (Kumar and Kumar, 2015).

One of the criteria for identifying *Lactobacillus* species is the study of their fermentation type. No gas production was detected in the Durham tubes for all isolates and thus can be identified as presumptive homofermentative.

According to the report of the joint FAO/WHO expert consultation on evaluation of probiotics in food, it is necessary to know the genus and species of the probiotic strain (FAO/WHO, 2002).

Unlike traditional phenotypic methods, molecular methods have been widely used for the identification of prokaryotic organisms and for phylogenetic and taxonomic studies. Several molecular biological techniques for community analysis have emerged over the past decade, and most take advantage of the molecular phylogeny derived from 16S rRNA comparative sequence analysis (Friedrich and Lenke, 2006). The 16S rRNA gene sequence is about 1,550 bp long and is composed of both variable and conserved regions which are specific for each bacterial species (Clarridge, 2004). PCR amplification of the 16S rRNA gene fragment is an effective technique that provides a rapid tool for the identification of bacterial isolates (Jenkins et al., 2012). The 16S rRNA gene sequence has been determined for a large number

of strains. GenBank, the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of 16S rRNA gene. This means that there are many previously deposited sequences against which to compare the sequence of an unknown strain species (Clarridge, 2004). In the study, this molecular technique was used to confirm the identity of presumptive *Lactobacillus* isolates. The 16S rRNA sequence of *Lactobacillus* isolates shows high degree of similarity (99-100%) to the 16S rRNA of *Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus paracasei* and *Lactobacillus zeae* (Table 6). These four *Lactobacillus* species are reported to belong to the same taxonomic grouping and to have close phylogenetic relationship (Coudeyras et al., 2008; Sardaro et al., 2016). Further experiments such as Randomly Amplified Polymorphic DNA-polymerase chain reaction (RAPD-PCR) and species-specific PCR are necessary in order to discriminate these species (Mangia et al., 2016).

Table 6: Percentages of homology of sequence obtained from *Lactobacillus* isolates to reference strains in GenBank

***Lactobacillus* isolates**	**% of homology**	**Species**
A2	*Lactobacillus rhamnosus*	100%
E90	*Lactobacillus rhamnosus*	100%
E97	*Lactobacillus rhamnosus*	100%
E98	***Lactobacillus rhamnosus*** ***Lactobacillus casei*** ***Lactobacillus paracasei***	**99%**
E99	*Lactobacillus rhamnosus*	100%
F70	*Lactobacillus rhamnosus*	100%
F71	*Lactobacillus rhamnosus*	100%
F72	*Lactobacillus rhamnosus*	100%
F74	*Lactobacillus rhamnosus*	100%
F75	*Lactobacillus rhamnosus*	100%
F76	*Lactobacillus rhamnosus*	100%
F77A	*Lactobacillus rhamnosus*	100%
F77B	*Lactobacillus rhamnosus*	100%
F78	*Lactobacillus rhamnosus*	100%
F79	*Lactobacillus rhamnosus*	100%
F80	*Lactobacillus rhamnosus*	100%
F81	*Lactobacillus rhamnosus*	100%
F82	***Lactobacillus rhamnosus*** ***Lactobacillus paracasei*** ***Lactobacillus casei*** ***Lactobacillus zeae***	**99%**
F83	***Lactobacillus paracasei*** ***Lactobacillus paracasei subsp. tolerans*** ***Lactobacillus rhamnosus***	**100%**
F84	*Lactobacillus rhamnosus*	100%
F85	*Lactobacillus rhamnosus*	100%
F86	*Lactobacillus rhamnosus*	100%
F87	*Lactobacillus rhamnosus*	100%
F88A	*Lactobacillus rhamnosus*	100%
F88B	***Lactobacillus rhamnosus*** ***Lactobacillus paracasei*** ***Lactobacillus casei*** ***Lactobacillus paracasei subsp. tolerans*** ***Lactobacillus zeae***	**99%**
F91	*Lactobacillus rhamnosus*	100%
F92	*Lactobacillus rhamnosus*	100%
F93	***Lactobacillus rhamnosus*** ***Lactobacillus zeae*** ***Lactobacillus paracasei*** ***Lactobacillus casei*** ***Lactobacillus paracsei subsp. tolerans***	**99%**

CHAPTER 3: Safety aspects and beneficial features of *Lactobacillus* isolates

3.1 Introduction

Microorganisms used as probiotics must meet a criterion of major health; absence of any pathogenicity. Lactobacilli have been implicated in recent years in many infectious complications. Endocarditis and bacteremia have been reported as the most common pathologies associated with lactobacilli (Wallet et al., 2002; Cannon et al., 2005; Tommasi et al., 2008; Fradiani et al., 2010). But many other infections have been described as cases of intestinal ischemia with fatal outcomes in patients suffering from acute pancreatitis (Besselink et al., 2008), liver abscesses (Rautio et al., 1999; Cukovic-Cavka et al., 2006), meningitis (Robin et al., 2010; Schmidt et al., 2011) or cases of septic arthritis (Chanet et al., 2007). Thus, being the cause of many infections and diseases, *Lactobacillus* probotic strains safety has to be assessed such for hemolytic activity and antibiotic resistance (Mathur and Singh, 2005; Aquilanti et al., 2007; Kochan et al., 2011; Li et al., 2017).

The determination of the hemolytic activity is one of the safety requirements of use of LAB in food or as probiotics. Probiotics are living organisms and may be theoretically responsible for side effects such as risks of systemic infection.

Another important aspect of the safety of LAB strains intended for Human use is the profile of antibiotic resistance. Antibiotics are one of the most important therapeutic classes and have revolutionized human medicine. The "official" discovery of the first antibiotics penicillin was totally random; in fact, in 1928, Sir Alexander Fleming observed an inhibition of the growth of staphylococci by a fungus, *Penicillium.* He then speculates that this fungus is capable of synthesizing a substance with antibacterial properties, which he calls "penicillin". Subsequently, many other antibiotic molecules were discovered leading to the development of this therapeutic class, allowing treating many infections (Nikaido, 2009).

From the beginning of the clinical use of antibiotics, bacterial strains resistant to these molecules appeared. Indeed, Fleming has already launched a warning about the excessive use of penicillin since 1945 during his speech at the Nobel Prize award ceremony.

For each new class of antibiotics developed and commercialized, resistant bacterial strains emerged. This phenomenon has been amplified by the misuse of antibiotics for half a century. Indeed, high consumption and misuse of these molecules are at the origin of the emergence and diffusion of resistance.

Bacterial resistance to an antibiotic is of genetic origin and this resistance can be natural or acquired. The distinction between the natural and acquired resistance to antimicrobial drugs is of great importance. Acquired resistance poses a high risk of transmission of resistance to pathogenic and commensal intestinal bacteria (Ishibashi and Yamazaki, 2001).

The genes of resistance are part of the genetic patrimony of the bacteria. Natural resistance is a character present in all strains belonging to the same species. Any bacterial species can be naturally resistant to one or more classes of antibiotics. This type of resistance is detected from the first studies carried out on antibiotics to determine its activity and contribute to defining its antibacterial spectrum. Intrinsic resistance is permanent, stable and transmitted to progeny (Vertical transmission) in cell division but it is not generally transferable from a genus to another (Horizontal transmission) (Mathur and Singh, 2005).

Strains previously sensitive to an antibiotic can develop a resistance to this antibiotic which involves chromosomal or extra-chromosomal genetic changes. Acquired resistance is usually caused from bacterial mutation or may carry plasmid encoding of antibiotics resistance genes and only concerns certain strains within a species normally susceptible to the antibiotic in question. It usually has a low risk of horizontal transmission when resistance is a result of a chromosomal mutation. On the other hand, acquired resistance is considered to have a higher potential for horizontal diffusion of antibiotic resistance when resistance genes are present in mobile genetic elements (plasmids and transposons). Horizontal gene transfer contributes to the spread of antibiotic resistance through the exchange of genetic material across genera, which increases the potential for harmful, antibiotic resistant bacteria to develop. This property raises the question whether resistance genes can be transferred by probiotics to the endogenous flora or to pathogens (Courvalin, 2006; Munita and Arias, 2016; Von Wintersdorff et al., 2016).

The appearance of a resistance gene in a bacterium can result from several mechanisms (Mathur and Singh, 2005; Nikaido, 2009):

- Mutations on chromosomal genes, spontaneous or induced:

- Spontaneous mutations: this is a spontaneous, rare and hereditary change that will affect the nucleotide sequence of the bacterial genome;
- Induced mutations: adaptation of a bacterium to unfavorable conditions for its growth (in particular the presence of antibiotics).

- Acquisition of resistance genes from other strains:

- By conjugation: a gene is transferred from one bacterium to another via plasmids or transposons (inter-species transfer, often from non-pathogenic bacteria of the environment);
- By transduction: a gene is transferred from one bacterium to another via a bacteriophage (intra-species transfer);
- By transformation: transfer of naked DNA between two bacteria (intra- or inter-species transfer).

Bacteria can collect multiple resistance traits over time and can become resistant to many different families of antibiotics (Magiorakos et al., 2011).

In the agri-food industry, LAB are used in both manufacturing and preservation of food products of animal and vegetable origin. In addition to their technological features as acidifying activity, ability to improve the flavor and texture of foods, LAB play an important role in the biopreservation of food through different mechanisms such as nutritional competition and production of metabolites during fermentation like the production of organic acids, hydrogen peroxide, carbon dioxide, diacetyl, broad-spectrum of antimicrobials compounds such as reuterin and the production of bacteriocins (Jacobsen et al., 2003; Vermeiren et al., 2004; Lee et al., 2006; Ananou et al., 2007; Kaktcham et al., 2012).

Bioconservation is a new conservation approach based on the use of methods involving natural and/or organic preservatives and which is henceforth advocated in the food industry. These preservatives usually have a microbial origin or part of the intrinsic structures of the food and contribute to its conservation. Biopreservation uses antagonistic microorganisms as well as their metabolites to inhibit or destroy the undesirable microorganisms in food (Rodgers, 2001). As already mentioned, fermented foods are a good example of products using biopreservation and this, by the growth and the metabolism of LAB and the conditions they impose in this type of food (low pH, competition, etc…), therefore, lactic bacteria are the essential actors of this biopreservation.

The lactic and acetic acids produced by these bacteria during fermentation provide important antimicrobial functions. These organic acids act at two levels:

- Direct action: organic acids passively diffuse through the bacterial membrane in their undissociated form. Acidify the cytoplasm after dissociation and inhibit the cellular functions of acid-sensitive pathogens bacteria which affect their viability (Lavermicocca et al., 2008).
- Indirect action: due to the tolerance of lactobacilli to acidity, in an acid environment, their bacterial competitiveness is favored compared to other bacteria (Servin, 2004, Tejero-Sarinena et al., 2012).

Lactobacilli are catalase-negative and some strains can accumulate hydrogen peroxide. This compound has long been recognized as an important agent of the antibacterial activity of lactobacilli and able to inhibit many pathogens (Dasari et al., 2014; Oldak et al., 2017). The formation of hydrogen peroxide is due to the action of oxidases and superoxide dismutase (Kelley et al., 2010).

The bacteriocins produced by LAB are antimicrobial substances with variable molecular weight. They have an inhibitory activity against bacteria close to the producing strain and their spectrum of action is usually narrow. The best known are: Nisin, diplococcin, acidophilin and bulgarican (Ogunbanwo et al., 2003, Dortu and Thonart, 2009). Most bacteriocins produced by LAB share the same mode of action, based on the formation of pores in the membrane of the target bacterium (Kumari et al., 2009).

In the health sector, certain specific LAB are used as probiotics (Gill and Holley, 2003; Sieladie et al., 2011) and in the treatment many disease as already discussed in the introduction. Probiotics can be classified into four categories: The first category contains species of the genus *Lactobacillus*. The second category is composed of Bifidobacterium species. The third group of probiotics includes other coccal-shaped LAB such as *Enterococcus* and *Streptococcus*. As for the fourth group, it consists of non-lactic acid bacteria (Table 7).

Table 7: List of microorganisms considered as probiotics (Holzapfel et al., 2001; Fijan, 2014)

Groups	Species
Lactobacillus species	*L. acidophilus, L. amylovorus, L. brevis, L. casei, L. crispatus, L. delbrueckii subsp. bulgaricus, L. fermentum, L. gallinarum, L. gasseri, L. johnsonii, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus*
Bifidobacterium species	*B. adolescentis, B. animalis, B. bifidum, B. breve, B. infantis, B. lactis, B. longum*
Other LAB	*Enterococcus faecalis, Enterococcus faecium, Enterococcus durans, Lactococcus lactis, Leuconstoc mesenteroides, Pediococcus acidilactici, Sporolactobacillus inulinus, Streptococcus thermophilus*
Non lactic acid bacteria	*Bacillus coagulans, Bacillus cereus, Bacillus subtilis, Escherichia coli Nissle, Saccharomyces boulardii*

In order to be functional and beneficial to health and to comply with the definition of probiotics, microorganisms must survive, persist temporarily in the digestive tract and show an activity that must result in positive effects for the host (Vasiljevic and Shah, 2008).

To be effective, probiotics strains must come alive to the site of their action, namely the gut and resist during their passage to the conditions of the stomach such as acidity (Dunne et al., 2001). During fasting, stomach pH can go down very low to arrive to 1.5 and transit time can be from 1 to 3 hours depending on the individual and his diet which can drastically affect the bacterial growth and viability.

Lactobacilli are naturally well adapted to acidic pH (Van de Guchte et al., 2002). During lactic fermentation, they produce and accumulate in their environment acidic compounds that make the environment acidic and unfavorable to the growth of other bacteria (Servin, 2004). In these circumstances, lactobacilli are protected by inducible mechanisms giving them acid stress tolerance. This tolerance increases during the exponential growth of the bacterium therefore suggesting their adaptation and their operating at low pH after ingestion when meeting another acidic environment, the stomach (Van de Guchte et al., 2002).

The *in vitro* methods of studying the resistance of strains to conditions simulating the stomach condition generally rely on bacterial survival measured primarily by enumeration on agar

culture media following exposure to low pH. Authors suggest that probiotic strains must be resistant to a pH of 2.5 in a culture medium for 3 hours (Ammor and Mayo, 2007).

Several studies have shown that various strains belonging to the species: *L. rhamnosus, L. acidophilus, L. brevis, L. casei, L. plantarum and L. fermentum* have tolerance to gastric juice conditions (Khalil et al., 2007; Xiaodong et al., 2009; Kirtzalidou et al., 2011; Zoumpopoulou et al., 2017).

In the small intestine, bile salts tolerance is an important factor contributing to the survival of probiotics. Bacteria that survive the acidic conditions of the stomach must then deal with the detergent action of bile salts released in the duodenum after ingestion of fatty meals. Bile resistance tests rely on survival and counting of bacteria after exposure to oxgall (Huang and Adams, 2004). Oxgall, a derivative of bile bovine, is very frequently used for these tests at a concentration of 0.3% corresponding to the physiological concentration estimated in the human intestine (Begley et al., 2005; Hu et al., 2018).

Several studies have shown that intestinal probiotics such as lactobacilli have developed resistance to the detergent action of bile salts. One of the mechanisms of this resistance is the deconjugation of bile salts through the Bile Salt Hydrolase (BSH) enzyme. Many researchers demonstrated the role of bile salts hydrolases (BSH) of probiotics in the reduction of serum cholesterol. Hence, the research of probiotics with BSH activities is also considered as an additional criterion for the selection of probiotics (Bemmo et al., 2017). The BSH enzyme catalyzes the hydrolysis of conjugated bile salts with glycine or taurine to acid amines residues and free bile salts which has the effect of reducing the solubility of the bile and its detergent activity (Begley et al., 2005, Hamon et al., 2011). Deconjugated bile acids are excreted more rapidly from the intestinal tract than those which are conjugated. Thus, free bile acids are excreted from the body through feces and the synthesis of new bile salts from serum cholesterol as substrate leads to a reduction in the total concentration of cholesterol in the blood (Bemmo et al., 2017).

Another mechanism responsible for the resistance of lactobacilli to bile salts is the extrusion of bile. This mechanism is achieved through Multidrug Resistance System (MDR). MDRs are thus responsible for the resistance to many toxic compounds such as antibiotics, organic solvents, detergents and bile salts. Many studies have demonstrated the importance of MDR

systems in bile salts resistance in different strains of *L. acidophilus* (Pfeiler and Klaenhammer, 2009) and *L. reuteri* ATCC 55730 (Whitehead et al., 2008).
In order to exercise their beneficial effects, probiotics must adhere to intestinal mucus or epithelial cells and persist in the intestine (Collado et al., 2005; Xiaodong et al., 2009). The longer a bacterium spends time in the gastrointestinal tract, the more likely it is to have a beneficial effect on the host. The ability of probiotics to adhere to mucosal surfaces prevents their rapid evacuation by intestinal contraction and after peristaltic discharge of the digest. The ability to adhere to mucus or epithelial cells determines the intestinal residence time and therefore the capacity of colonization of the gastrointestinal tract. Adherent bacteria can stay in the intestine longer than normal transit would allow, unlike non-adherent bacteria. The flow rate is relatively greater in the small intestine compared to the large intestine and for this adhesion is more important for establishing bacterial populations in the small intestine.
Several beneficial effects of probiotic bacteria are directly related to the adhesion capacity. Adhesion is important for immunomodulation because only adherent bacteria are in contact with the immune cells of the epithelium. Moreover, adhesion is the first defense mechanism against the invasion of pathogenic bacteria (Ouwehand and Salminen, 2003).
It is based on the realization of a set of *in vitro* tests then *in vivo* using cells of animal or human origin (Palomares et al., 2007; De Los Reyes-Gavilan et al., 2011). *In vitro* methods generally comprise three steps: incubation of the bacterial cells with the adhesion substrate to allow the bacteria to adhere to the target; leaching of non-adhered bacteria; enumeration of the adhered bacteria. The culture of human cell lines of intestinal origin is widely used for adhesion tests. The most commonly used cell types in probiotic research are Caco-2 (ATCC HTB-37) and HT-29 (ATCC HTB-38), both isolated from human colon adenocarcinoma. Their resemblance to epithelial cells made them the main models of the human small intestine epithelium and the most widely used supports for adhesion (Nowak and Motyl, 2017).

Among the health claims conferred on probiotic strains, immune modulation of LAB has continued to be a subject of growing interest.
The microbiota plays a vital role in the maturation of the immune system and is therefore essential for the health of the host. An imbalance in the microbiota level is observed in various pathologies such as diarrhea, Crohn's disease, and ulcerative colitis. Taking antibiotics also changes the composition of the microbiota. This results in a decrease of the diversity of

the bacterial species, but also in a malfunction of the immune system. Consequently, microbiota control seems important in maintaining the intestinal homeostasis in adults and the maturation of the immune system in children. Thus, the intestinal epithelium provides the first line of defense of the host by distinguishing between commensal bacteria and pathogenic microorganisms. Probiotics stimulate the immune system by inducing the production of anti-inflammatory cytokines and by stimulating the innate immune system and more precisely the dendritic cells (Ouwehand et al., 2002; Preidis et al., 2009; Garrett et al., 2010; Oelschlaeger, 2010; Bron et al., 2011). In particular, lactobacilli form a source of potential modulators of the immune system. It has been demonstrated that specific *Lactobacillus* strains can modulate host immunity, which positively correlates with enhanced resistance to various viral and bacterial infections. Lactobacilli can inflect the production of cytokines that are involved in the regulation, activation, growth and differentiation of immune cells (Wells, 2011). Interleukin 10 (IL10) is a pluripotent cytokine and the most important anti-inflammatory cytokine found within the human immune response (De Moreno de Leblanc et al., 2011). IL10 is produced by many cell populations. Its main biological function seems to be the limitation and termination of inflammatory responses and the regulation of differentiation and proliferation of several immune cells such as T cells, B cells, natural killer cells, antigen-presenting cells, mast cells, and granulocytes (Asadullah et al., 2003).

Nitric oxide (NO) is a pleiotropic and short-lived free radical, implicated in a vast number of physiological processes. NO is produced by NOS (NO synthase) using L-arginine and molecular oxygen as substrates, and yielding NO and L-citrulline as products. Beneficial properties of NO have been observed in the regulation of vascular relaxation, platelet aggregation, neurotransmission, cellular respiration, and the modulation of immune responses. There are three known NOS isoforms. The two constitutively expressed isoforms are neuronal NOS (nNOS or NOS1), located predominantly in neurons and eNOS/constitutive NOS (NOS3), expressed predominantly in endothelial cells. These enzymes are termed calcium-dependent, as their activity is regulated by intracellular calcium levels. The third isoform, inducible NOS (iNOS or NOS2), is not constitutively expressed, but its expression can be induced in tissues undergoing inflammatory responses (Hickey, 2001; Ibiza and Serrador, 2008).

The NO produced by iNOS can exert protective and toxic effect depending on its concentration. Low amount of NO preserves the cellular integrity and mediate anti-inflammatory effects in the early phase of inflammation. But in the advanced stage of inflammatory process, excess production of NO by iNOS cause tissue injury (Korhonen et al., 2001). *Lactobacillus rhamnosus* has been found to induce nitric oxide production in macrophages and a human colon epithelial cell line. This nitric oxide production was mediated through the induction of inducible nitric oxide synthetase which is considered to be a pro-inflammatory event. Interestingly, some bacteria, for example *Lactobacillus farciminis*, are able to reduce nitrite to nitric oxide, at least *in vitro* conditions. If their nitrite-reducing properties persist *in vivo*, such bacteria are promising in terms of reducing colonic inflammation (Fioramonti et al., 2003).

Cyclooxygenases (COXs) are a family of myeloperoxidases located at the luminal side of the endoplasmic reticulum and nuclear membrane. The COX catalyzes the conversion of arachidonic acid to prostaglandins. COX exists in three isoforms, COX-1, COX-2 and COX-3. COX-1 is a housekeeping enzyme, which is expressed constitutively in many tissues, and involved in the production of prostaglandins important for homeostasis and mediating certain cellular physiological functions ranging from cell proliferation to angiogenesis, platelet aggregation due to thromboxane production. COX-2 is the inducible form and rapidly induced in many cell types by cytokines, mitogens, and endotoxins during inflammation and facilitates inflammatory response (Chandrasekharan et al., 2002; Devi and Rashmi, 2017). Furthermore, COX-2 expression is associated with increased epithelial cell proliferation, decreased apoptosis, and increased cell invasiveness, whereas chronic inhibition of COX activity by nonsteroidal anti-inflammatory drugs has been associated with chemopreventative effects on colon cancer. Consequently, the identification of the pathways and regulatory elements that control COX-2 expression in general and in intestinal cells in particular is a subject of major interest (Otte and Mahjurian-Namari, 2009). Many studies have described the effect of *Lactobacillus* strains in COX-2 regulation. *Lactobacillus acidophilus*, has been shown to slightly upregulate COX-2 expression in an intestinal epithelial cells culture system (Nurmi et al., 2005), whereas this strain has been reported to downregulate *Helicobacter pylori* induced COX-2 expression in the gastric epithelium of Mongolian gerbils (Brzozowski et al., 2006).

Furthermore, *Lactobacillus casei* has been shown to downregulate COX-2 expression in a rodent trinitrobenzenesulphonic acid (TNBS) colitis model (Peran et al., 2007).

3.2 Material and Methods

3.2.1 Hemolytic activity

Hemolytic activity of *Lactobacillus* isolates was evaluated on Columbia agar plates supplemented with 5% (v/v) sheep blood (Oxoid) and incubated at 37 °C for 48h. Thereafter, the plates were observed and classified based on lysis activities of red blood cells in the media around and under the colonies. Strains that produced green zones are considered α-hemolysis, clear zones are ß-hemolysis and no zones are γ-haemolysis. Only strains with γ-haemolysis are considered as safe (Padmavathi et al., 2018).

3.2.2 Susceptibility to antibiotics

Antibiotics resistance of *Lactobacillus* isolates was firstly assessed by the disc diffusion method according to Landeta et al. (2013) with slight modifications. A suspension from fresh overnight cultures in MRS soft agar medium with a density of McFarland 0.5 in buffered saline was inoculated on Mueller-Hinton agar plates containing the antibiotics disc tetracycline (TE30), chloramphenicol (C30), ampicillin (AMP10), kanamycin (K30), clindamycin (DA2), vancomycin (VA30), amoxicillin (AML2), gentamicin (CN10), erythromycin (E15), penicillin (P10) and spectinomycin (SH10) (Oxoid, UK). Resistance or susceptibility to antibiotics according to the interpretative criteria of CLSI (Clinical and Laboratory Standards Institute, 2015) was assessed by measuring the zone of inhibition in mm of bacterial growth after incubation for 24 h at 37°C.

Minimal inhibition concentration (MIC) of resistant isolates was then calculated using the broth micro-dilution method according to Maragkoudakis et al. (2006) with slight modifications. Overnight bacterial cultures (8-9 log CFU mL^{-1}) that show resistant to an antibiotic were inoculated (1%) in Mueller-Hinton broth supplemented with this specific antibiotic at various final concentration (ranging from 2 to 1024 mg L^{-1}) and examined for growth using a microplate reader OD at 610nm after 24h of incubation at 35°C. MIC was determined as the lowest concentration of an antibiotic in which visible growth was inhibited and MIC values were compared to MIC breakpoints of *Lactobacillus* defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2018).

3.2.3 Antimicrobial activity

An agar spot test was used to detect antimicrobial activity against *Salmonella enterica* DSMZ 13772, *Staphylococcus aureus* DSMZ 20231*, Escherichia coli* DSMZ 30083, *Enterococcus faecalis* STAA (Sezione di Scienze e Tecnologie Ambientali e Alimentari), *Enterococcus faecium* STAA and four kinds of *Listeria monocytogenes* (DSMZ 20600, LB STAA, LC STAA, LE STAA). Overnight cultures of pathogens bacteria were plated with the specific medium soft agar (Brain heart infusion medium for *Listeria monocytogens*, M17 for the *Enterococcus* species and Nutrient Broth for the others pathogens) and solidified at room temperature. *Lactobacillus* isolates were spotted on pathogens plates and incubated at 37°C for 24h. Inhibition was considered positive if the width of the clear zone was 5 mm or larger.

Isolates of *Lactobacillus* with positive results were further tested using a well diffusion assay. Plates with pathogen bacteria were prepared as for the agar spot test. After solidification, two wells were performed on each plate. Overnight *Lactobacillus* isolates were centrifugated and the cell-free supernatant was added to first second well. The supernatant was then adjusted to pH 6.5 using 1M NaOH to eliminate the effect of lactic acid, treated with catalase to a final concentration of 0.5 mg mL^{-1} at 25°C for 30 min to exclude potential inhibition by hydrogen peroxide, filtered using 0.22μm syringe sterile filter and added to the second well. Plates were then incubated at 37°C and inhibition zones were measured after 24 hours and 48 hours of incubation.

3.2.4 Acidity tolerance

Lactobacillus strains with probiotics characteristics should be able to resist to low pH. Therefore, 1% of overnight cultures were inoculated in different MRS broth tubes acidified with 0.1M HCl to pH 3. Tubes were incubated at 37°C and vital counts were performed at t0 (starting time), t1.5 (after 1.5 hour) and t3 (after 3 hours) using the appropriate dilutions with the plate pour technique on MRS soft agar plates. Plates were incubated at 37°C for 48h. Isolates that were able to resist to pH 3 were further tested at pH 2.5 using the same technique. All experiments were done in triplicate.

3.2.5 Bile salts tolerance

Lactobacillus strains were tested for their tolerance to bile salts. Overnight cultures (1%) were inoculated in different MRS broth tubes adjusted to 0.5% (w/v) of bile salts (LP0055, Oxoid). Tubes were incubated at 37°C and vital counts were performed at t0 (starting time), t1.5 (after 1.5 hour) and t3 (after 3 hours) using the appropriate dilutions with the plate pour technique on MRS soft agar plates. Plates were incubated at 37°C for 48h. All experiments were done in triplicate.

3.2.6 Bile Salt Hydrolase Activity

Bile salt hydrolase activity was detected as described by Shehata et al. (2016) with minor modifications. Overnight cultures were spotted or streaked on MRS agar (Biokar, France) plates containing 0.5% (w/v) of sodium salt of taurodeoxycholic acid (TDCA, T0875, Sigma-Aldrich, St. Louis, MO, USA) and incubated anaerobically at 37 °C for 48h. A precipitation zone surrounding the colonies indicates a bile salt hydrolase activity of the strains.

3.2.7 Adherence to epithelial cells

Adhesion ability was examined using a collagen-based 96-well microplate assay, as well as two human colon adenocarcinoma cell lines (HT-29 and Caco-2 cells).

The collagen based assay was used as a first screening of the capacity of adhesion of the isolates as described previously by Zoumpopoulou et al. (2017) with slight modifications. MRS broth were inoculated (2%) from overnight cultures and immediately 100 µL were transferred into 96-well collagen-coated microplates (Cellcoat®, Collagen Type I, Greiner Bio-One GmbH, Frickenhausen, Germany) and incubated at 37°C for 24h. Supernatant was discarded and the cells were washed using ½ strength Ringer solution. A volume of 100 µL of Gram’s crystal violet solution (Merck, Darmstadt, Germany) was added per well for 15min and then adhered cells were washed with Ringer solution and re-suspended in modified biofilm dissolving solution (MBDS prepared previously from SDS dissolved with 40% (v/v) ethanol in H_2O to a final concentration of 10% (w/v) for 5 min at room temperature. Supernatant were transferred to a new 96-well microplate in order to measure the absorbance at 600 nm.

Adhesion to cell line HT-29 and Caco-2 cells were screened according to Zoumpopoulou et al. (2017). HT-29 and Caco-2 cells were procured from the Laboratory of Medical Microbiology, Hellenic Pasteur Institute, Athens, Greece. Upon arrival to the laboratory, cells were incubated at 37 °C and 5% CO_2 for 30 minutes. After the incubation time, cells were seeded (1×10^5 cells per well) in 12-well tissue culture plates (CELLSTAR®, Greiner Bio-One GmbH, Frickenhausen, Germany) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with L-glutamine, 100 U mL^{-1} penicillin/100 µg mL^{-1} streptomycin, and 10 or 15% (v/v) fetal bovine serum (FBS) for HT-29 or Caco-2, respectively. Cell cultures were incubated at 37 °C and 5% CO_2 and cells were detached using a trypsin (0.25% (w/v)) solution. Plates were incubated for 3 and 5 days for HT-29 and Caco-2 cells respectively and medium was changed daily. After the respective days of incubation, overnight bacterial cultures (8 log CFU mL^{-1}) were washed with PBS (pH 7.4) and re-suspended in fresh DMEM without antibiotics and FBS to achieve a final concentration of 10^8 CFU mL^{-1}. Medium in the 12-well culture plates was discarded, cell monolayers were washed twice with PBS Dulbecco solution (Biochrom GmbH) and co-cultured with 1 mL of the above bacterial cell suspension for 2 h at 37 °C. The bacterial suspension was then aspirated and cell monolayers were washed twice with PBS and then 1 mL of trypsin solution was added. For bacterial enumeration (CFU mL^{-1}), trypsin solutions with detached eukaryotic cells were serially diluted and plated in MRS agar medium to calculate the % adhesion from the percentage of viable bacteria compared to their initial population added per well.

Bacterial enumeration (CFU mL^{-1}) was determined at t0 (starting time) for the overnight bacterial cultures and t2 (after 2 hours) for the trypsin solutions with detached eukaryotic cells, by plate pour technique in MRS agar using the appropriate dilutions and incubated at 37˚C for 48h.

For both test, *Lactobacillus plantarum* ACA-DC 2640 and ACA-DC 4039 were used as positive control.

3.2.8 Immunomodulation

The methods used and described below are according to Zoumpopoulou et al. (2017). THP-1 (TIB-202) human monocytes (ATCC, Rockville, MD, USA) were cultured in RPMI-1640 (Biochrom GmbH) containing 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 10%

(v/v) FBS at 37 °C and 5% CO2. Cell number never exceeded 2×10^5 cells mL^{-1} in the cultures. THP-1 cells were plated in 6-well plates (10^6 cells per well) in culture medium without antibiotics and 100 ng mL^{-1} phorbol-12-myristate-13-acetate (PMA, Sigma- Aldrich) were added in each well before incubation at 37 °C for 24 h. Subsequently, 5×10^7–10^8 CFU of bacterial cultures were re-suspended in 0.3 mL RPMI-1640 and added to the wells (bacteria:THP-1 cells ratio ranging from 50:1 to 100:1). RPMI-1640 medium (0.3 mL) was used as control. Gentamycin (Biochrom GmbH) was also added at a final concentration of 250 µg mL^{-1}. RNA extraction was performed after a 4 h co-culture of THP-1 cells with the bacteria. RNA extraction from THP-1 cells was performed using the TRI Reagent® (Sigma-Aldrich) and first-strand cDNA synthesis was performed with the first-strand cDNA Synthesis Kit (Sigma-Aldrich). qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, London, UK) was used in the real-time PCR experiments that were performed in a MX3000P cycler (Stratagene, La Jolla, CA, USA). Relative expression of interleukin 10 (IL10), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX2) gene expression was estimated with the $2^{-\Delta\Delta Ct}$ method, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference gene. Sequences of primers used are presented in table 8.

Table 8: Sequences of primers used in the Immunomodulation experiment

Primer	**Sequence**
IL10 (F)	CACCCACTTCCCAGGCAACC
IL10 (R)	TCTCAGACAAGGCTTGGCAACC
iNOS (F)	CCCAGCCTCAAGTCTTATTTCCTC
iNOS (R)	GCACTCAGCAGCAAGTTCCATC
COX2 (F)	CCTGTGCCTGATGATTGC
COX2 (R)	CTGATGCGTGAAGTGCTG
GAPDH (F)	GAGTCCACTGGCGTCTTC
GAPDH (R)	GCATTGCTGATGATCTTGAGG

3.2.9 Statistical analysis

Results of CFU mL^{-1} counts were log10 transformed to normalize the data. The significance of difference between different treatments and the control was determined by Student's t-test ($P < 0.05$).

3.3 Results and Discussion

3.3.1 Safety aspects

Safety profile of LAB used as probiotics is determined by their hemolytic activity and antibiotic susceptibility (Joint FAO/WHO, 2002).

Lactobacillus strains used in food fermentation or as probiotics must be essentially incapable of causing lysis of red blood. Hemolysis remains one of the main virulence factors among pathogenic bacteria but *Lactobacillus* are generally non-hemolytic in nature. None of the *Lactobacillus* isolates (28) showed any clear transparent or greenish zone when grown in Columbia sheep blood agar, surrounding their colonies, and thus were found to be γ-hemolytic or non-hemolytic in accordance with other studies confirming their non-hemolytic in nature (Ambalam et al., 2013; Zoumpopoulou et al., 2017).

Susceptibility to antibiotics of *Lactobacillus* isolates was tested using both disc diffusion and MIC determination methods for the 11 most commonly used antibiotics: tetracycline, chloramphenicol, ampicillin, kanamycin, clindamycin, vancomycin, amoxicillin, gentamicin, erythromycin, penicillin and spectinomycin.

Antibiotic disc diffusion test results were as follow (Table 9):

- All isolates (n=28; 100%) were susceptible to tetracycline (TE30), chloramphenicol (C30), ampicillin (AMP10) and erythromycin (E15).
- One isolate (3.6%) F82 for clindamycin (DA2) and F92 for penicillin (P10) showed intermediate results while the others (n=27; 96.4%) were susceptible.
- Varied results were observed for amoxicillin (AML2); two isolates (7.2%) were susceptible (F75 and F88A), 8 isolates (28.6%) showed intermediate results (A2, E90, E97, E98, E99, F77A, F82, F91) and the remaining 18 isolates (64.2%) were resistant (F70, F71, F72, F74, F76, F77B, F78, F79, F80, F81, F83, F84, F85, F86, F87, F88B, F92, F93).
- All isolates (n=28; 100%) were resistant to kanamycin (K30), vancomycin (VA30), gentamicin (CN10) and spectinomycin (SH10).

Resistance of *Lactobacillus* species to vancomycin and to aminoglycosides (gentamicin, kanamycin and spectinomycin), is well known to be intrinsic and considered as safe (Zhou et al., 2005; Ammor et al., 2007; Caggia et al., 2015). Thus, MIC for amoxicillin was performed on *Lactobacillus* isolates (26) that showed intermediate and resistance results when tested using the antibiotic disc diffusion method. According to the breakpoints set by EUCAST (2018), *Lactobacillus* species are considered resistant for a MIC value > 8 mg L^{-1} and susceptible for a MIC value < 4 mg L^{-1}. All 26 isolates tested were susceptible with MIC breakpoint < 4 mg L^{-1}.

In agreement with the results obtained, studies have reported that lactobacilli are generally resistant to aminoglycosides, susceptible to antibiotic inhibitors of protein synthesis, such as chloramphenicol, erythromycin, clindamycin and tetracycline (Zhou et al., 2005; Ammor et al., 2007) and susceptible to antibiotics bacterial wall inhibitors like beta-lactams such as penicillin and ampicillin (Danielsen and Wind, 2003; Coppola et al., 2005; Kacem and Karam, 2006; Ammor et al., 2007). Intrinsic resistance to aminoglycosides is attributed to the absence of cytochrome-mediated electron transport, enabling antibiotic uptake (Charteris et al., 2001).

The resistance profile of *Lactobacillus* isolates seems to be interesting. Perhaps, this is related to the fact that these isolates have been collected in rural areas where farmers don't seek the assistance of veterinary, what limits the use of antibiotics and therefore limits the emergence of acquired resistance. In addition, the resistance observed to vancomycin, gentamicin, kanamycin and spectinomycin seem to be natural. Intrinsic resistance is generally not horizontally transferable, and poses no risk in non-pathogenic bacteria (Mathur and Singh, 2005).

In addition, natural resistance of lactobacilli to a range of antibiotics clinically important allows their use as "safe" probiotics in therapy with combination to antibiotics against bacterial infections. Despite the fact that resistance to aminoglycosides is commonly considered as intrinsic (i.e. not transferable), further studies are necessary to assess the potential transferability of resistance genes.

Table 9: Antibiotics susceptibility of *Lactobacillus* isolates by the disc diffusion test

Isolates	Antibiotics										
	TE 30	**C30**	**AMP10**	**K30**	**DA2**	**VA30**	**AML2**	**CN10**	**E15**	**P10**	**SH10**
A2	S	S	S	R	S	R	I	R	S	S	R
E90	S	S	S	R	S	R	I	R	S	S	R
E97	S	S	S	R	S	R	I	R	S	S	R
E98	S	S	S	R	S	R	I	R	S	S	R
E99	S	S	S	R	S	R	I	R	S	S	R
F70	S	S	S	R	S	R	R	R	S	S	R
F71	S	S	S	R	S	R	R	R	S	S	R
F72	S	S	S	R	S	R	R	R	S	S	R
F74	S	S	S	R	S	R	R	R	S	S	R
F75	S	S	S	R	S	R	S	R	S	S	R
F76	S	S	S	R	S	R	R	R	S	S	R
F77A	S	S	S	R	S	R	I	R	S	S	R
F77B	S	S	S	R	S	R	R	R	S	S	R
F78	S	S	S	R	S	R	R	R	S	S	R
F79	S	S	S	R	S	R	R	R	S	S	R
F80	S	S	S	R	S	R	R	R	S	S	R
F81	S	S	S	R	S	R	R	R	S	S	R
F82	S	S	S	R	I	R	I	R	S	S	R
F83	S	S	S	R	S	R	R	R	S	S	R
F84	S	S	S	R	S	R	R	R	S	S	R
F85	S	S	S	R	S	R	R	R	S	S	R
F86	S	S	S	R	S	R	R	I	S	S	R
F87	S	S	S	R	S	R	R	R	S	S	R
F88A	S	S	S	R	S	R	S	R	S	S	R
F88B	S	S	S	R	S	R	R	R	S	S	R
F91	S	S	S	R	S	R	I	R	S	S	R
F92	S	S	S	R	S	R	R	R	S	I	R
F93	S	S	S	R	S	R	R	R	S	S	R
% S	100	100	100	0	96.4	0	7.2	0	100	96.4	0
% I	0	0	0	0	3.6	0	28.6	3.6	0	3.6	0
% R	0	0	0	100	0	100	64.2	96.4	0	0	100

TE: Tetracycline; C: Chloramphenicol; AMP: Ampicillin; K: Kanamycin; DA: Clindamycin; VA: Vancomycin; AML: Amoxicillin; CN: Gentamicin; E: Erythromycin; P: Penicillin; SH: Spectinomycin
S: Susceptible; I: Intermediate; R: Resistant

3.3.2 Antimicrobial activity

LAB are used in the fermentation and bioconservation of food due to their antimicrobial activity and production of metabolites with inhibitory activity, such as organic acids, against certain pathogenic strains. Antimicrobial activity is also considered an important criterion for potential probiotic strains (Shewale et al., 2014). The increasing level of antibiotics resistance as discussed in chapter 3 is the highlight aspect of 21st century. Metabolites generated by LAB with antimicrobial activity against pathogens bacteria can be the best alternatives to antibiotics use (Zahid et al., 2015) and traditional dairy products are a source of new antimicrobial strains.

Lactobacillus isolates were examined for antimicrobial activity against food spoilage and pathogenic bacteria. Target strains include Gram-negative bacteria: *Salmonella enterica* and *Escherichia coli* and Gram-positive bacteria: *Staphylococcus aureus, Enterococcus faecalis*, *Enterococcus faecium* and *Listeria monocytogenes.*

Results of agar spot test (Figure 3) were as follow: all isolates (28) were active against *Listeria monocytogenes LB* and *Enterococcus faecalis*, eight isolates (A2, E90, E99, F86, F87, F88A, F88B, F93) were active against *Listeria monocytogenes LE*, eight isolates (F70, F71, F72, F74, F75, F76, F77A, F77B) were active against *Listeria monocytogenes* DSMZ 20600, six isolates (F72, F74, F75, F76, F77A, F77B) were active against *Staphylococcus aureus* DSMZ 20231 and five isolates (A2, E90, E97, E98, E99) were active against *Escherichia coli* DSMZ 30083 (clear zone > 5 mm). No activity was detected against *Listeria monocytogenes LC*, *Enterococcus faecium* and *Salmonella enterica.*

Antimicrobial activity of the isolates with positive results was revaluated with the well diffusion assay. Growth of pathogens was inhibited by the cell-free supernatant which confirms the production of antimicrobial agent by the lactic isolates in the medium. Many studies have shown that the extracellular fraction contains substances responsible for this interaction (Metlef and Dilmi-Bouras, 2009). *Listeria monocytogenes LB* was inhibited by 14 *Lactobacillus* isolates (2 mm < Diameter < 22 mm), *Listeria monocytogenes LE* was inhibited by 8 *Lactobacillus* isolates (18 mm < Diameter < 26 mm), *Listeria monocytogenes* DSMZ 20600 was inhibited by seven *Lactobacillus* isolates (16 mm < Diameter < 24 mm), *Staphylococcus aureus* was inhibited by six *Lactobacillus* isolates (2 mm < Diameter < 8

mm), *Escherichia coli* was inhibited by five *Lactobacillus* isolates (6 mm < Diameter < 12) and no inhibition was observed for *Enterococcus faecalis* (Table 10).

No inhibition at all was observed when the pathogens were grown in the presence of cell-free supernatants adjusted to pH 6.5 and treated with catalase, hence inhibition effects cannot be explained by hydrogen peroxide or bacteriocin action and are most probably due to the production of organic acids along with the low pH. The lowering of pH due to organic acids (especially lactic and acetic acids) produced by lactobacilli in the gut has a bactericidal or bacteriostatic effect (Maragkoudaki et al., 2006; Argyri et al., 2013).

Antibacterial activity was more pronounced against Gram-positive bacteria than Gram-negative bacteria. Onda et al. (2003) suggest that gram-positive bacteria are generally more sensitive to bactericidal LAB. Zoumpopoulou et al. (2008) tested the antimicrobial activity of *Lactobacillus fermentum* ACA-DC 179 and *Lactobacillus plantarum* ACA-DC 287 against 507 indicator strains (Gram-positive: 443 strains and Gram-negative: 64 strains) and no activity was detected against any of the Gram-negative bacteria. The *in vitro* inhibitory capacity of LAB against pathogenic organisms appears to be a good probiotic property, as it can play a role in preserving quality hygiene of foodstuffs (Ammor et al., 2006).

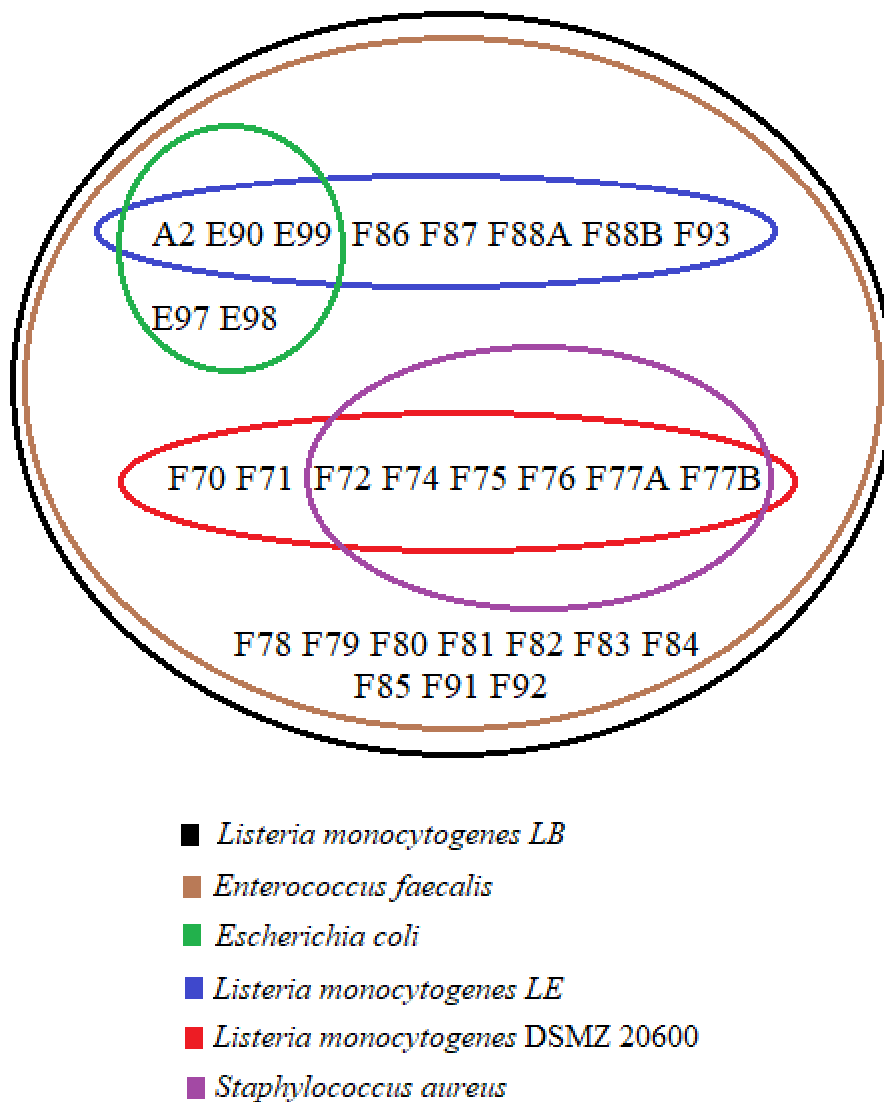

Figure 3: Antimicrobial activity of *Lactobacillus* isolates against pathogenic bacteria using the agar spot test

Table 10: Antimicrobial activity (inhibition zone diameter in mm) produced by supernatants of *Lactobacillus* isolates against pathogens strains with the well diffusion assay

Isolates	*Listeria monocytogenes*			*S. aureus*	*E. faecalis*	*E. coli*
	LB	***LE***	**DSMZ 20600**			
A2	14	26	n.t.	n.t.	0	8
E90	0	18	n.t.	n.t.	0	9
E97	10	n.t.	n.t.	n.t.	0	12
E98	14	n.t.	n.t.	n.t.	0	6
E99	12	18	n.t.	n.t.	0	6
F70	24	n.t.	18	n.t.	0	n.t.
F71	0	n.t.	22	n.t.	0	n.t.
F72	0	n.t.	16	6	0	n.t.
F74	0	n.t.	20	8	0	n.t.
F75	0	n.t.	24	6	0	n.t.
F76	0	n.t.	22	6	0	n.t.
F77A	2	n.t.	22	6	0	n.t.
F77B	22	n.t.	0	2	0	n.t.
F78	2	n.t.	n.t.	n.t.	0	n.t.
F79	16	n.t.	n.t.	n.t.	0	n.t.
F80	6	n.t.	n.t.	n.t.	0	n.t.
F81	10	n.t.	n.t.	n.t.	0	n.t.
F82	10	n.t.	n.t.	n.t.	0	n.t.
F83	0	n.t.	n.t.	n.t.	0	n.t.
F84	0	n.t.	n.t.	n.t.	0	n.t.
F85	0	n.t.	n.t.	n.t.	0	n.t.
F86	0	20	n.t.	n.t.	0	n.t.
F87	0	22	n.t.	n.t.	0	n.t.
F88A	0	22	n.t.	n.t.	0	n.t.
F88B	0	22	n.t.	n.t.	0	n.t.
F91	12	n.t.	n.t.	n.t.	0	n.t.
F92	7	n.t.	n.t.	n.t.	0	n.t.
F93	0	18	n.t.	n.t.	0	n.t.

n.t.: Agar spot test negative, isolate not tested with the well diffusion assay

3.3.3 Acidity tolerance

LAB survival in low pH is very important for tolerating the first stress in the human body which is the gastric acid (Ji et al., 2015). As already mentioned above, the pH in human stomach ranges from 1.5 during fasting, to pH 4.5 after a meal, and food ingestion can take up to 3 h. Since fermented products have a pH of 4.5 and where LAB can survive easily, tolerances of strains to lower pH (3 and 2.5) are examined (Papadimitriou et al., 2015).

Lactobacilli are naturally well adapted to low pH levels (Van de Guchte and al., 2002) by their production of organic acids during lactic fermentation. By therefore, in humans, after ingestion, lactobacilli encounter another acidic environment, the stomach, and some strains can adapt to the very low pH of the stomach (Van de Guchte et al., 2002, Nanatani and Abe, 2011).

All *Lactobacillus* isolates (28) were able to resist at pH 3, and no viability loss was detected. Isolates were retested at pH 2.5 for 3 hours of exposure (Table 11) and results were as follow: after 1.5 hour of exposure, three lactobacilli isolates (E90, E99 and F75; 10.7 % of all lactobacilli tested) showed less than 2 log CFU mL^{-1} reduction, followed by seven isolates (A2, F70, F71, F72, F79, F82 and F91; 25%) with a viability decline between 2 and 3 log CFU mL^{-1}. The viability loss of 11 lactobacilli isolates (E97, E98, F80, F81, F83, F85, F86, F87, F88A, F92 and F93; 39.28%) was more pronounced reaching for some isolates between 3 to 5 log CFU mL^{-1} of reduction while seven isolates (F74, F76, F77A, F77B, F78, F84 and F88B; 25%) were totally inhibited. Maragkoudakis et al. (2006) reported similar results: at pH 3 the viability of *Lactobacillus* strains was unaffected and when exposed to lower pH for even only 1h only six out of 29 maintained some viability.

After 3 hours of exposure to pH 2.5, ten isolates (A2, E90, E99, F71, F72, F75, F79, F85, F86, and F87; 35.71%) were able to resist with a viability loss ranging from 2.03 log CFU mL^{-1} for F75 to 5.43 log CFU mL^{-1} for A2. Significant differences were noticed on seven isolates of the ten latter comparing viable counts of control and pH 2.5 after 3 hours of incubation at 37°C (Figure 4).

Table 11: Viable counts of *Lactobacillus* isolate at pH 2.5 during 3 hours of exposure

Isolates	Viable count (Log CFU mL^{-1})		
	0h	1.5h	3h
A2	7.77 ± 0.10	4.90 ± 0.61	2.34 ± 0.26
E90	7.87 ± 0.24	6.18 ± 0.65	4.54 ± 1.25
E97	7.11 ± 0.01	2.41 ± 0.07	0.00 ± 0.00
E98	7.03 ± 0.05	3.40 ± 0.02	0.00 ± 0.00
E99	7.99 ± 0.11	6.44 ± 0.12	5.07 ± 0.56
F70	7.13 ± 0.01	4.86 ± 0.01	0.00 ± 0.00
F71	7.92 ± 0.09	5.42 ± 0.86	3.78 ± 0.52
F72	7.91 ± 0.09	5.74 ± 0.06	3.56 ± 0.43
F74	6.98 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
F75	8.00 ± 0.00	6.66 ± 1.00	5.97 ± 3.14
F76	7.00 ± 0.04	0.00 ± 0.00	0.00 ± 0.00
F77A	6.88 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
F77B	6.90 ± 0.07	0.00 ± 0.00	0.00 ± 0.00
F78	6.85 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
F79	7.96 ± 0.11	5.14 ± 1.06	3.40 ± 1.85
F80	7.01 ± 0.03	2.75 ± 0.06	0.00 ± 0.00
F81	6.97 ± 0.00	2.99 ± 0.06	0.00 ± 0.00
F82	7.09 ± 0.03	4.57 ± 0.01	0.00 ± 0.00
F83	7.16 ± 0.02	3.89 ± 0.04	0.00 ± 0.00
F84	6.91 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
F85	8.01 ± 0.16	4.35 ± 0.22	3.06 ± 0.18
F86	7.86 ± 0.13	3.19 ± 0.32	2.65 ± 0.23
F87	8.06 ± 0.10	4.26 ± 0.01	3.46 ± 0.29
F88A	6.99 ± 0.00	3.10 ± 0.25	0.00 ± 0.00
F88B	6.87 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
F91	6.99 ± 0.02	4.05 ± 0.01	0.00 ± 0.00
F92	6.94 ± 0.01	3.22 ± 0.03	0.00 ± 0.00
F93	7.03 ± 0.05	3.84 ± 0.01	0.00 ± 0.00

Results are expressed as mean ± standard deviation (n = 6)

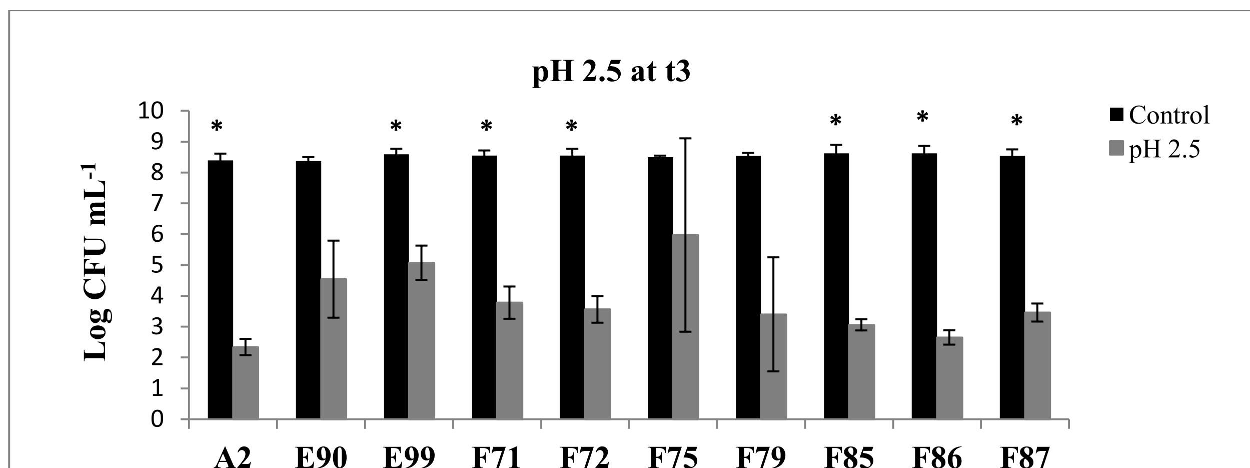

Figure 4: Viable counts (Log CFU mL^{-1}) of the ten resistant isolates to pH 2.5 after 3 hours of incubation at 37°C. Asterisks denote statistically significant differences ($P < 0.05$) between control and pH 2.5

3.3.4 Bile salts tolerance and bile salt hydrolase activity

After passing through the stomach, the strains arrive to the duodenum where bile is secreted. At this level, some components of bile, including bile acids such as cholic acid, seriously compromise the viability of ingested bacteria. Bile tolerance is one of the characteristics sought when probiotic bacteria are selected so that as many bacteria as possible cross the duodenum in the direction of their site of action, while remaining viable and able to multiplicate.

Tolerance to bile salts is essential for LAB to survive in the small intestine where bile concentration ranges from 0.1% to 0.3%. So it is necessary for a probiotic strain to be able to grow and survive in the presence of at least 0.3% of bile salts concentration (Divya et al., 2012; Azat et al., 2016).

When exposed to 0.5% (w/v) of bile salts for 3h all *Lactobacillus* retained their viability and even viable counts of 21 isolates slightly increased comparing t0 to t3 (Table 12). Several studies have shown the ability of lactobacilli to survive in conditions mimicking the juice of the small intestine of man. Resistance cases to bile salts, similar to the results obtained, have been observed with strains belonging to the species: *L. plantarum, L. paracasei, L. delbrueckii, L. rhamnosus, L. acidophilus* and *L. salivarius* (Köll et al., 2008; Guo et al., 2010; Zoumpopoulou et al., 2017).

The ability of bacteria to produce bile salt hydrolase is one of the selection criterions of probiotics due to its capacity of managing hypercholesterolaemia (Kumar et al. 2012; Sornplang and Piyadeatsoontorn, 2016). *Lactobacillus* isolates exhibited a partial bile salt hydrolase activity, precipitating halos around the colonies differentiated colony morphology on MRS agar plate supplemented with TDCA in comparison with the control MRS agar plates (Figure 5). Similar results in Argyri et al. (2013) were observed.

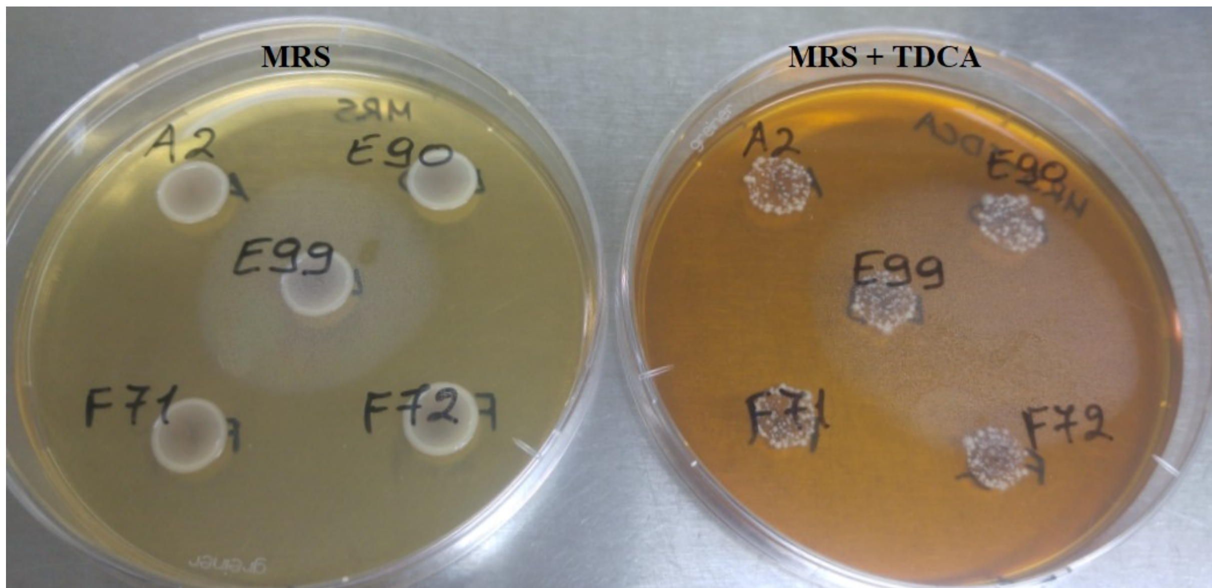

Figure 5: Bile salt hydrolase activity test: MRS supplemented with TDCA showing precipitating halos around the colonies comparison with the control MRS agar plates

As already discussed, bile salt hydrolase activity contribute to bile salts tolerance. Partial bile salt hydrolase activity observed on the 28 *Lactobacillus* isolates may explain their resistance to bile salts.

Taking into account the results of viability of isolates at pH 2.5, statistical analyses comparing viable counts of control and 0.5% (w/v) of bile salts after 3 hours of incubation at 37°C of the ten most resistant isolates at pH 2.5 (A2, E90, E99, F71, F72, F75, F79, F85, F86, F87) showed significant differences for only two isolates F72 and F86 (Figure 6).

Table 12: Viable counts of *Lactobacillus* isolate with 0.5% (w/v) bile salts during 3 hours of exposure

Isolates	Viable count (Log CFU mL^{-1})			Survival in the presence of 0.5% (w/v) bile salts (Δlog CFU mL^{-1} t0-t3)
	0h	1.5h	3h	
A2	6.26 ± 0.10	6.40 ± 0.03	6.60 ± 0.03	0.34 ± 0.19
E90	6.19 ± 0.16	6.42 ± 0.02	6.51 ± 0.07	0.31 ± 0.21
E97	6.42 ± 0.06	6.16 ± 0.08	6.15 ± 0.16	–0.28 ± 0.19
E98	6.36 ± 0.06	6.41 ± 0.03	6.64 ± 0.10	0.28 ± 0.16
E99	6.19 ± 0.16	6.36 ± 0.08	6.36 ± 0.02	0.17 ± 0.15
F70	6.29 ± 0.01	6.41 ± 0.08	6.69 ± 0.02	0.40 ± 0.20
F71	6.20 ± 0.03	6.30 ± 0.07	6.62 ± 0.03	0.41 ± 0.21
F72	6.26 ± 0.02	6.61 ± 0.04	6.82 ± 0.02	0.57 ± 0.28
F74	6.41 ± 0.03	6.66 ± 0.09	6.82 ± 0.03	0.41 ± 0.21
F75	6.23 ± 0.13	6.35 ± 0.01	6.61 ± 0.01	0.38 ± 0.22
F76	6.52 ± 0.08	6.45 ± 0.12	6.79 ± 0.01	0.27 ± 0.15
F77A	6.40 ± 0.03	6.58 ± 0.02	6.80 ± 0.03	0.40 ± 0.20
F77B	6.45 ± 0.01	6.61 ± 0.06	6.80 ± 0.01	0.34 ± 0.17
F78	6.39 ± 0.20	6.68 ± 0.06	6.82 ± 0.05	0.43 ± 0.28
F79	6.22 ± 0.04	6.36 ± 0.12	6.51 ± 0.03	0.29 ± 0.15
F80	6.42 ± 0.06	6.27 ± 0.08	6.74 ± 0.04	0.32 ± 0.17
F81	6.56 ± 0.02	6.68 ± 0.07	6.79 ± 0.01	0.23 ± 0.12
F82	6.39 ± 0.18	6.42 ± 0.13	6.72 ± 0.12	0.33 ± 0.23
F83	6.39 ± 0.08	6.22 ± 0.01	6.27 ± 0.13	–0.12 ± 0.13
F84	6.76 ± 0.03	6.31 ± 0.01	6.53 ± 0.02	–0.24 ± 0.12
F85	6.24 ± 0.14	6.49 ± 0.00	6.59 ± 0.02	0.35 ± 0.21
F86	6.45 ± 0.09	6.54 ± 0.13	6.67 ± 0.05	0.22 ± 0.14
F87	6.34 ± 0.04	6.46 ± 0.06	6.57 ± 0.10	0.23 ± 0.13
F88A	6.42 ± 0.09	5.95 ± 0.05	6.13 ± 0.05	–0.29 ± 0.16
F88B	6.72 ± 0.05	6.70 ± 0.01	6.81 ± 0.02	0.09 ± 0.06
F91	6.55 ± 0.06	5.90 ± 0.00	6.23 ± 0.05	–0.32 ± 0.17
F92	6.45 ± 0.01	6.27 ± 0.06	6.34 ± 0.02	–0.11 ± 0.06
F93	6.41 ± 0.01	6.19 ± 0.16	6.35 ± 0.11	–0.05 ± 0.08

Results are expressed as mean ± standard deviation (n = 6)

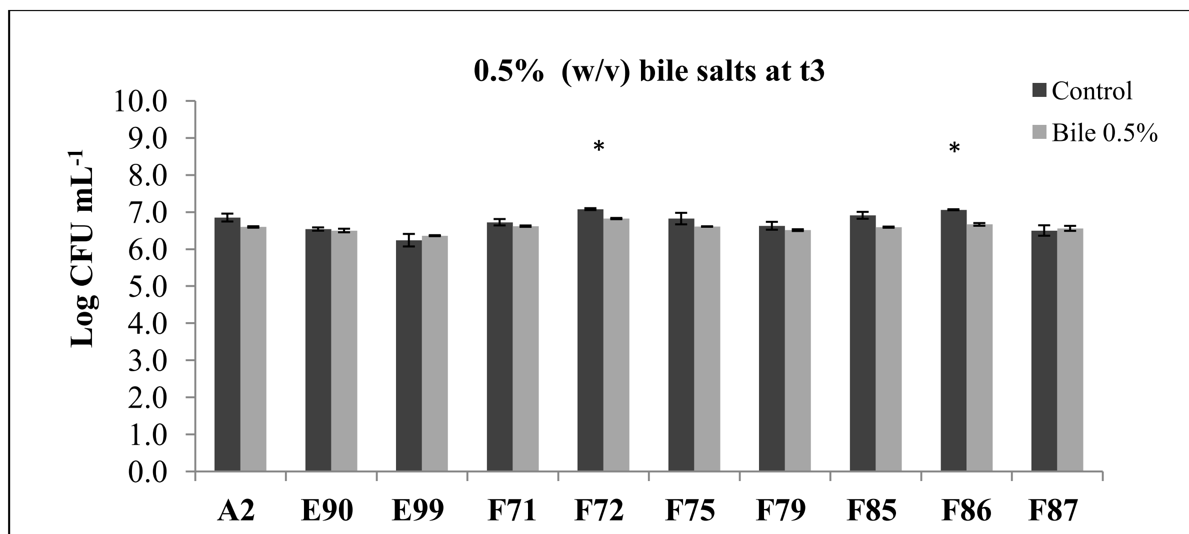

Figure 6: Viable counts (Log CFU mL^{-1}) in 0.5% (w/v) bile salts of the ten resistant isolates to pH 2.5 after 3 hours of incubation at 37°C. Asterisks denote statistically significant differences ($P < 0.05$) between control and 0.5% (w/v) bile salts

3.3.5 Adherence to epithelial cells

As the study of epithelial cell adhesion *in vivo* is difficult to achieve, *in vitro* adhesion is the most used model (Gu et al., 2008). In the study used epithelial cells of human origin Caco-2 and HT-29 cells were used.

Ten *Lactobacillus* isolates (A2, E90, E99, F71, F72, F75, F79, F85, F86 and F87) that showed acidity tolerance to pH 2.5 after 3 hours of exposure were tested for adherence properties. Selected *Lactobacillus* isolates were evaluated by first a collagen-based 96-well microplate assay. *Lactobacillus plantarum* ACA-DC 2640 and ACA-DC 4039 were included as controls, because they were reported to show high adhesion ability (Zoumpopoulou et al., 2017).

Absorbance values OD_{600nm} obtained for the ten selected *Lactobacillus* (Table 13) were considered low comparing to the results showed by Zoumpopoulou et al. (2017). Adhesion capacity of *Lactobacillus* isolates to HT-29 and Caco-2 cells were tested and relatively low adhesion was also observed with a percentage of adhesion < 1% for both adenocarcinoma cell lines (Table 13).

The adhesion values to collagen-coated plates (OD_{600nm}) of *L. plantarum* ACA-DC 2640 and *L. plantarum* ACA-DC 4039 used as positive control, reported in literature (Zoumpopoulou et

al., 2017) (0.799 ± 0.182 and 0.513 ± 0.152) are higher than values obtained in this study (0.328 ± 0.065 and 0.218 ± 0.057) respectively.

Same observation was detected for adhesion of positive control strains to adenocarcinoma cells: Zoumpopoulou et al. (2017) revealed a percentage of adhesion to Caco-2 cells ranging from 5.56 to 9.97% and to HT-29 32.28% and 20.07% for *L. plantarum* ACA-DC 2640 and ACA-DC 4039 respectively. While in this study percentage of adhesion to both adenocarcinoma cells were much lower for both lactobacilli strains: 3.94% and 3.65% for Caco-2 and 8.98% and 2.88 for HT-29 for *L. plantarum* ACA-DC 2640 and ACA-DC 4039 respectively.

Although models used for *in vitro* adhesion assays represent well the *in vivo* situation, more concisely, Caco-2 cell cultures express biologically significant proteins similarly to those expressed in small intestinal scrapings *in vivo* studies and grow in culture forming an homogeneous and polarized cell monolayer, which resembles mature human enterocytes in the small intestine, *in vivo* studies are required to confirm *in vitro* results. Some comparative evaluations of the *in vitro* adhesion ability of probiotic strains were in agreement with *in vivo* results and other in disagreement (Ouwehand and Salminen, 2003; Laparra and Sanz, 2009). The adhesion experiments of *Bifidobacterium longum* strains (BB536 and ATCC 15707) to Caco-2 cells were in agreement with the *in vivo* intestinal colonization; on the other hand, *in vitro* adhesion test revealed an underestimation of the bacterial adhesion of *Bifidobacterium animalis* to Caco-2 cells *in vivo*. These differences between the *in vitro* and *in vivo* situation could be because of the lack of suitability of a unique model system to predict adhesion ability of every strain (Laparra and Sanz, 2009).

Moreover, no definite correlation appears to exist between *in vitro* adhesion and pathogens exclusion. Good adhesion to host tissue may also be a potentially negative property, especially to damaged tissue, and could be the first step in pathogenesis even though, this conclusion is far from being applicable to *Lactobacillus* species. Non adherent strains do not exist and bacteria will always bind to a given substratum (Ouwehand and Salminen, 2003).

Table 13: Adhesion of the ten selected *Lactobacillus* isolates to collagen-coated plates (OD_{600nm}) and to Caco-2 and HT-29 human intestinal epithelial cells *in vitro*

Isolates	Adhesion to collagen-coated plates (OD_{600nm})	Caco-2 Viable count (Log CFU mL^{-1})			HT-29 Viable count (Log CFU mL^{-1})		
		0h	2h	Percentage of Adhesion	0h	2h	Percentage of adhesion
A2	0.051 ± 0.006	7.87 ± 0.06	5.35 ± 0.26	0.3	8.01 ± 0.05	4.96 ± 0.12	0.09
E90	0.047 ± 0.002	7.87 ± 0.07	5.56 ± 0.46	0.49	8.07 ± 0.07	5.39 ± 0.35	0.21
E99	0.051 ± 0.002	7.98 ± 0.02	5.55 ± 0.44	0.37	8.09 ± 0.07	5.04 ± 0.11	0.09
F71	0.049 ± 0.001	7.78 ± 0.11	5.49 ± 0.57	0.52	8.05 ± 0.03	5.04 ± 0.11	0.10
F72	0.054 ± 0.004	7.83 ± 0.20	5.62 ± 0.49	0.62	8.15 ± 0.05	5.09 ± 0.13	0.09
F75	0.050 ± 0.001	7.89 ± 0.02	5.53 ± 0.27	0.44	8.00 ± 0.03	5.63 ± 0.51	0.42
F79	0.049 ± 0.001	7.98 ± 0.04	5.34 ± 0.43	0.23	7.94 ± 0.08	3.66 ± 1.98	0.01
F85	0.048 ± 0.002	8.05 ± 0.07	5.51 ± 0.44	0.29	8.01 ± 0.11	4.83 ± 0.16	0.07
F86	0.052 ± 0.002	8.05 ± 0.07	5.39 ± 0.50	0.22	8.06 ± 0.02	4.92 ± 0.04	0.07
F87	0.054 ± 0.007	8.09 ± 0.10	5.41 ± 0.50	0.21	8.09 ± 0.01	4.65 ± 0.23	0.04
2640	0.328 ± 0.065	8.22 ± 0.03	6.81 ± 0.07	3.94	8.08 ± 0.12	7.03 ± 0.42	8.98
4039	0.218 ± 0.057	8.12 ± 0.03	6.68 ± 0.56	3.65	8.18 ± 0.06	6.64 ± 0.13	2.88

Results are expressed as mean ± standard deviation (n = 6)

3.3.6 Immunomodulation

Based on the results obtained for the acidity tolerance test and considering that all the isolates belong to the same taxonomic grouping, four *Lactobacillus* isolates (A2, E90, F75 and F86) were selected for immunomodulation test belonging to three different farms. Changes in IL10, iNOS and COX2 mRNA levels were assessed in human monocytes co-cultured with the *Lactobacillus* isolates (Figure 7).

All tested isolates were able to increase IL10 expression without significant difference. High level of IL10 indicates a tendancy for anti-inflammatory modulation of THP-1 cells (Zoumpopoulou et al., 2017).

COX2 mRNA levels were statistically downregulated by A2 isolate and remained statistically unaffected with the resting isolates. iNOS expression was statistically upregulated when THP-1 cells were co-cultured with E90 and remained unaffected with the other isolates. Given the pathophysiological role of abnormally high levels of COX-2 and iNOS and their putative involvement in intestinal inflammation and carcinogenesis, A2, F75 and F86 might be considered as a therapeutic alternative in the regulation of these enzymes in the intestinal epithelium (Ibiza and Serrador, 2008; Otte and Mahjurian-Namari, 2009).

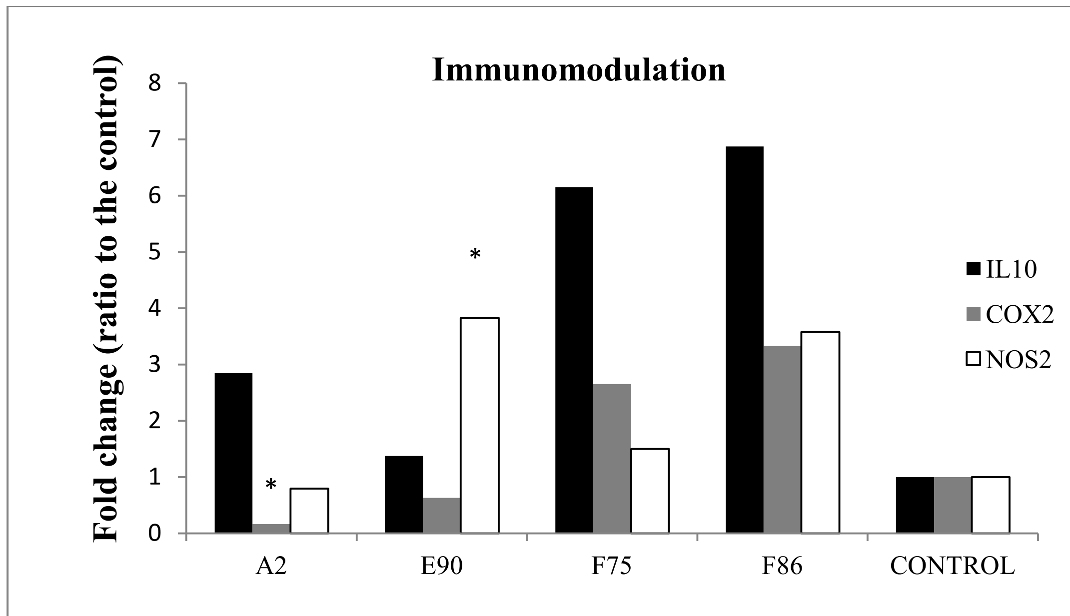

Figure 7: *In vitro* immonumodulation properties of four selected *Lactobacillus* isolates in human THP-1 cells after co-culture for 4h. Asterisks denote statistically significant differences in comparison to the untreated control for $P < 0.05$

Conclusions

Lebanese goat sector represents an underestimated resource which at the same time has great potential for an increase of milk production and dairy products. Efforts for the development of the cattle herd are deployed, but this is not the case for goat and ewe species, however, better adapted to the agro-climatic conditions of the region.

Recently, biopreservation has become a topic of interest. This technique is used as an alternative to chemical additives for increasing self-life storage and enhancing safety of food by using natural microflora and their antimicrobial products. Moreover, the beneficial effects of live bacteria, termed probiotics, on human health are increasingly being promoted during the last years.

Results of the microbiological analyses on raw milk were not very satisfactory and an improving of the hygienic conditions and sanitary measures in the farms are fundamental.
Overall, *in vitro* tests revealed a number of lactobacilli possessing promising probiotic features and could be used as adjunct and probiotic culture for dairy industry.

Obtained results open further perspectives to elaborate on microbial biodiversity aspects of Lebanese goat milk for instance: complete the genotypic identification and study the technological potential of *Lactobacillus* isolates, investigate *in vivo* the potential food safety benefits of *Lactobacillus* isolates and identify and study the safety status of coccal-shaped LAB isolated.

References

Abbas HM., Hassan FAM., Abd el-Gawad M. and Enab AK. 2014. Physicochemical Characteristics of Goat's Milk. *Life Science Journal* **11** 307-317

Aguirre M. and Collins MD. 1993. Lactic acid bacteria and Human clinical infection. *Journal of Applied Bacteriol*ogy **75** 95-107

Ahmed T., Kanwal R. and Ayub N. 2006. Influence of temperature on growth pattern of *Lactococcus lactis*, *Streptococcus cremoris* and *Lactobacillus acidophilus* isolated from camel milk. *Biotechnology* **5** 481-488

Ambalam P., Ramoliya JM., Dave JM. and Vyas BRM. 2013. Safety assessment of potential probiotic strains *Lactobacillus rhamnosus* 231 and *Lactobacillus rhamnosus* V92 in mouse model. *International Journal of Bioassays* **2** 333-337

Ammor S., Tauveron G., Dufor E. and Chevalier I. 2006. Antibacterial activity of lactic acidbacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility 1-Screening and characterization of antibacterial compound. *Food Control* **17** 454-461

Ammor MS. and Mayo B. 2007. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production. *Meat Science* **76** 138-146

Ammor MS., Florez AB. and Mayo B. 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology* **24** 559-570

Ananou S., Maqueda M., Martinez-Bueno M. and Valdivia E. 2007. Biopreservation, an ecological approach to improve the safety and shelf-life of foods. In "Communicating Current Research and Educational Topics and Trends in Applied Microbiology", (A. Mendez-Vilas, Ed.), pp. 475-486. Formatex, Badajoz, Spain

Aquilanti L., Garofalo C., Osimani A., Silvestri G., Vignaroli C. and Clementi F. Isolation and molecular characterization of antibiotic-resistant lactic acid bacteria from poultry and swine meat products. *Journal of Food Protection* **70** 557-565

Argyri AA., Zoumpopoulou G., Karatzas KAG., Tsalakidou E., Nychas GJE., Panagou EZ. and Tassou CC. 2013. Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiology* **33** 282-291

Ashraf R.. and Shah NP. 2014. Immune System Stimulation by Probiotic Microorganisms. *Critical Reviews in Food Science and Nutrition* **54** 938-956 https://doi.org/10.1080/10408398.2011.619671

Asadullah K., Sterry W. and Volk HD. 2003. Interleukin-10 therapy–Review of a new approach. 2003. *Pharmacological Reviews* **55** 241-269

Azat R., Liu Y., Li W., Kayir A., Lin DB., Zhou WW. and Zheng XD. 2016. Probiotic properties of lactic acid bacteria isolated from traditionally fermented Xinjiang cheese. *Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)* **17** 597-609

Begley M., Gahan CGM. And Hill C. 2005. The interaction between bacteria and bile. *FEMS Microbiology Reviews* **29** 625-651

Bemmo KUL., Kaktcham PM., Momo KCH., Foko KEM., Zambou NF., Wang RY., Zhu T. and Yin L. 2017. Bile salt hydrolase and antimicrobial activities of three bile resistant probiotics *Lactobacillus plantarum* strains isolated from Cameroonian artisanal fermented milk Journal of Microbiology and Biotechnology research **7** 21-30

Bernardeau M., Guguen M. and Vernoux JP. 2005. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. *FEMS Microbiology Reviews* **30** 487-513

Besselink MG., van Santvoort HC., Buskens E., Boermeester MA., van Goor H., Timmerman HM., Nieuwenhuijs VB., Bollen TL., van Ramshorst B., Witteman BJ., Rosman C., Ploeg RJ., Brink MA., Schaapherder AF., Dejong CH., Wahab PJ., van Laarhoven CJ., van der Harst E., van Eijck CH., Cuesta MA., Akkermans LM. and Gooszen HG. 2008. Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. *Lancet* **371** 651-659

Boke H., Aslim B. and Alp G. 2010. The role of resistance to bile salts and acid tolerance of exopolysaccharides (epss) produced by yogurt starter bacteria. *Archives of Biological Science* **62** 323-328

Bron PA., van Baarlen P. and Kleerebezem M. 2011. Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. *Nature Reviews Microbiology* **10** 66-78

Brzozowski T., Konturek PC., Mierzwa M., Drozdowicz D., Bielanski W., Kwiecien S., Konturek SJ., Stachura J., Pawlik WW. and Hahn EG. 2006. Effect of probiotics and triple eradication therapy on the cyclooxygenase (COX)-2 expression, apoptosis, and functional gastric mucosal impairment in Helicobacter pylori-infected Mongolian gerbils. *Helicobacter* **11** 10-20

Bussarin K. and Rakshit SK. 2006. Microbial and processing criteria for production of probiotics. *Food Technology and Biotechnology* **44** 371-379

Caggia C., De Angelis M., Pitino I., Pino A. and Randazzo CL. 2015. Probiotic features of *Lactobacillus* strains isolated from Ragusano and Pecorino Siciliano cheeses. *Food Microbiology* **50** 109-117

Canchaya C., Claesson MJ., Fitzgerald GF., Sinderen D. and O'Toole PW. 2006. Diversity of the genus *Lactobacillus* revealed by comparative genomics of five species. *Microbiology* **152** 3185-3196

Cannon JP., Lee TA., Bolanos JT. and Danziger LH. 2005. Pathogenic relevance of *Lactobacillus*: a retrospective review of over 200 cases. *European Journal of Clinical Microbiology & Infectious Diseases* **24** 31-40

Castro-Sawinski S. 2016. Microbial models: from environmental to industrial sustainability 348pp

Champagne CP., Gardner NJ. and Roy D. 2007. Challenges in the addition of probiotic cultures to foods. *Critical Reviews in Food Science and Nutrition* **45** 61-84

Chandrasekharan NV., Dai H., Roos KLT., Evanson NK., Tomsik J., Elton TS. and Simmons DL. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: Cloning, structure, and expression. *Proceedings of the National Academy of Sciences* **99** 13926-13931

Chanet V., Brazille P., Honore S., Michel M., Schaeffer A. and Zarrouk V. 2007. *Lactobacillus* septic arthritis. *Southern Medical Journal* **100** 531-532

Charteris WP., Kelly PM., Morelli L. and Collins JK. 2001. Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. *Journal of Food Protection* **64** 2007-2014

Chedid M., Tawk ST., Chalak A., Karam S. and Hamadeh SK. 2018a. The Lebanese Kishk: A traditional dairy product in a changing local food system. *Journal of Food Research* **7** http://dx.doi.org/10.5539/jfr.v7n5p16

Chedid M., Tourrand JF., Jaber LS. and Hamadeh SK. 2018b. Farmers' perception to change and adaptation strategies of small ruminant systems in the West Bekaa of Lebanon. *Small Ruminant Research* **167** 16-21

Cheriguene A., Chougrani F., Bekada AMA., El Soda M. and Bensoltane A. 2007. Enumeration and identification of lactic microflora in Algerian goats' milk. *African Journal of Biotechnology* **6** 1854-1861

Clarridge J. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews* **17** 840-862

Clinical and Laboratory Standards Institute 2015. M100-S25 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement Wayne, PA 19087 USA

Collado MC., Gueimonde M., Hernandez M., Sanz Y. and Salminen S. 2005. Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and its role in enteropathogen exclusion. *Journal of Food Protection* **68** 2672-2678

Coppola R., Succi M., Tremonte P., Reale A., Salzano G. and Sorrentino E. 2005. Antibiotic susceptibility of *Lactobacillus rhamnosus* strains isolated from Parmegiano Reggiona cheese. *Lait* **85** 193-204

Cortés C., De la Fuente R., Contreras A., Sánchez A., Corrales JC., Martinez S. and Orden JA. 2006. A survey of *Salmonella* spp and *Campylobacter* spp in dairy goat faeces and bulk tank milk in the Murcia region of Spain. *Irish Veterinary Journal* **59** 391-393

Coudeyras S., Marchandin H., Fajon C. and Forestier C. Taxonomic and Strain-Specific Identification of the Probiotic Strain *Lactobacillus rhamnosus* 35 within the *Lactobacillus casei* Group. *Applied And Environmental Microbiology* **74** 2679-2689

Courvalin P. 2006. Antibiotic resistance: the pros and cons of probiotics. *Digesitive and Liver Disease* **38** 261-265

Cukovic-Cavka S., Likic R., Francetic I., Rustemovic N., Opacic M. and Vucelic B. 2006. *Lactobacillus acidophilus* as a cause of liver abscess in a NOD2/CARD15-positive patient with Crohn's disease. *Digestion* **73** 107-110

Da Silva FFP., Biscola V., LeBlanc JG. and Franco BDG. 2016. Effect of indigenous lactic acid bacteria isolated from goat milk and cheeses on folate and riboflavin content of fermented goat milk. *Food Science and Technology* **71** 155-161

Danielsen M. and Wind A. 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *International Journal of Food Microbiology* **82** 1-11

Dasari S., Shouri RND., Wudayagiri R. and Valluru L. 2014. Antimicrobial activity of *Lactobacillus* against microbial flora of cervicovaginal infections. *Asian Pacific Journal of Tropical Disease* **4** 18-24

De Angelis M., Corsetti A., Tosti N., Rossi J., Corbo MR. and Gobbetti M. 2001. Characterization of non-starter lactic acid bacteria from italian ewe cheeses based on phenotypic, genotypic, and cell wall protein analyses. *Applied and Environmental Microbiology* **67** 2011-2020

De Los Reyes-Gavilan CG., Suarez A., Fernandez-Garcia M., Margolles A., Gueimonde M. and Ruas-Madiedo P. 2011. Adhesion of bile-adapted *Bifidobacterium* strains to the HT29-MTX cell line is modified after sequential gastrointestinal challenge simulated *in vitro* using human gastric and duodenal juices. *Research in Microbiology* **162** 514-519

De Moreno de Leblanc A., Del Carmen S., Zurita-Turk M., Rocha CS., Van de Guchte M., Azevedo V., Miyoshi A. and LeBlanc JG. 2011. Importance of IL-10 modulation by probiotic microorganisms in gastrointestinal inflammatory diseases. International *Scholary Research Notices: Gastroenterology* http://dx.doi.org/10.5402/2011/892971

De Vos P., Garrity GM., Jones D., Krieg NR., Ludwig W., Rainey FA., Schleifer KH. And Whitmanet WB. 2009. Genus *Lactobacillus, Bacillus and Listeria. In* : « Bergey's manual of systematic bacteriology - The Firmicutes » Vol 3. Springer éd., New York. pp.19-511

De Vrese M. and Marteau PR. 2007. Probiotics and Prebiotics : Effects on Diarrhea. *The Journal of Nutrition* **137** 803-811

Devi PUM. and Rashmi HK. 2017. Probiotic conjugated linoleic acid inhibits COX-2 inflammatory pathway. *Journal of Pharmacy Research* **11** 767-774

Dib H., Hajj Semaan E. , Mrad R., Ayoub J., Choueiry L., Moussa H. and Bitar G. 2012. Identification et évaluation de l'effet probiotique des bactéries lactiques isolées dans des fromages caprins traditionnels *Lebanese Science Journal* **13** 43-58

Divya JB., Varsha KK. And Nampoothiri KM. 2012. Newly isolated lactic acid bacteria with probiotic features for potential application if food industry. *Applied Biochemistry and Biotechnology* **167** 1314-1324

Dobson A., Cotter PD., Ross RP. and Hill C. 2012. Bacteriocin production: a probiotic trait? *Applied and Environmental Microbiology* **78** 1-6

Donelli G., Vuotto C. and Mastromarino P. 2013. Phenotyping and genotyping areboth essential to identify and classify a probiotic microorganism. Microbial Ecology in Health and Disease **24** http://dx.doi.org/10.3402/mehd.v24i0.20105

Dortu C. and Thonart P. 2009. Les bactériocines des bactéries lactiques : caractéristiques et intérêts pour la bioconservation des produits alimentaires. *Biotechnologie, Agronomie, Société et Environement* **13** 143-154

Dunne C., O'Mahony L., Murphy L., Thornton G., Morrissey D., O'Halloran S., Feeney M., Flynn S., Fitzgerald G., Daly C., Kiely B., O'Sullivan GC., Shanahan F. and Collins JK. 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *The American Journal of Clinical Nutrition* **73** 386-392

European Committee on Antimicrobial Susceptibility Testing 2018. Breakpoint table for interpretation of MICs and zone diameters Version 8.1 http://www.eucast.org

FAO/WHO 2002. Report of a Joint FAO/WHO expert consultation on guidelines for the evaluation of probiotics in food. World Health Organization and Food and Agriculture Organization of the United Nations, London Ontario, Canada

Fioramonti J., Theodorou V. and Bueno L. 2003. Probiotics: what are they? What are their effects on gut physiology? *Best Practice & Research Clinical Gastroenterology* **17** 711-724

Fijan S. 2014. Microorganisms with Claimed Probiotic Properties: An Overview of Recent Literature. *International Journal of Environmental Research and Public Health* **11** 4745-4767

Folli C., Levante A., Percudani R., Amidani D., Bottazzi S., Ferrari A., Rivetti C., Neviani E. and Lazzi C. 2017. Toward the identification of a type I toxin-antitoxin system in the plasmid DNA of dairy *Lactobacillus rhamnosus*. *Journal of Scientific Reports* 7: 12051 https://doi.org/10.1038/s41598-017-12218-5

Foschino R., Invernizzi A., Barucco R. and Stradiotto K. Microbial composition, including the incidence of pathogens, of goat milk from the Bergamo region of Italy during a lactation year. *Journal of Dairy Research* **69** 213-225

Fradiani PA., Petrucca A., Ascenzioni F., Di Nucci G., Teggi A., Bilancini S. and Cipriani P. 2010. Endocarditis caused by *Lactobacillus jensenii* in an immunocompetent patient. *Journal of Medical Microbiology* **59** 607-609

Friedrich U. and Lenke J. 2006. Improved enumeration of Lactic Acid Bacteria in mesophilic dairy starter cultures by using multiplex quantitative Real-Time PCR and flow cytometry-fluorescence In Situ Hybridization. *Applied and Environmental Microbiology* **72** 4163-4171

Gabas AL. and Cabral RAF. 2012. Density and rheological parameters of goat milk. *Ciência e Tecnologia de Alimentos* **32** 381-385

García T., Mayoral B., González I., López-Calleja I, Sanz A., Hernández PE., and Martín R. 2004. Enumeration of yeasts in dairy products: a comparison of immunological and genetic techniques. *Journal of Food Protection* **67** 357–364

Garnier L., Valence F. and Mounier J. 2017. Diversity and control of spoilage fungi in dairy products: an update. *Microorganisms* **4** 42 https://doi.org/10.3390/microorganisms5030042

Garrett WS., Gordon JI. and Glimcher LH. 2010. Homeostasis and inflammation in the intestine. *Cell* **140** 859-870

General Census of Agriculture 2012. النتائج العامة للإحصاء الزراعي الشامل ٢٠١٠. Ministry of Agriculture and FAO

Georgalaki M., Zoumpopoulou G., Mavrogonatou E., Driessche GV., Alexandraki V., Anastasiou R., Papadelli M., Kazou M., Manolopoulou E., Kletsas D., Devreese B., Papadimitriou K. and Tsakalidou E. 2017. Evaluation of the antihypertensive angiotensin converting enzyme inhibitory (ACE-I) activity and other probiotic properties of lactic acid bacteria isolated from traditional Greek dairy products. *International Dairy Journal* **75** 10-21

Ghanbari M. and Jami M. 2013. Lactic acid their bacteriocins: a promising approach to seafood biopreservation. In: Kongo M (ed). *Lactic Acid Bacteria-R & D for Food, Health and Livestock Purposes* 381-404

Gill AO. and Holley RA. 2003. Interactive inhibition of meat spoilage and pathogenic bacteria by lysozyme, nisin and EDTA in the presence of nitrite and sodium chloride at 24 °C. *International Journal of Food Microbiology* **80** 251-259

Gobbetti M., Di Cagno R., Calasso M., Neviani E., Fox PF. and De Angelis M. 2018. Drivers that establish and assembly the lactic acid bacteria biota in cheeses. *Trends in Food Science & Technology* **78** 244-254

Goldstein EJ., Tyrrell KL. and Citron M. 2015. *Lactobacillus* Species: Taxonomic Complexity and Controversial Susceptibilities. *Clinical Infectious Diseases* **60** 98-107

Gordon S. 2008. Elie Metchnikoff: Father of natural immunity. *European Journal of Immunology* **38** 3257-3264

Gu RX., Yang ZQ., Li ZH., Chen SL. and Luo ZL. 2008. Probiotic properties of lactic acid bacteria isolated from stool samples of longevous people in regions of Hotan, Xinjiang and Bama, Guangxi, China. *Anaerobe* **14** 313-317

Guarrasi V., Sannino C., Moschetti M., Bonanno A., Di Grigoli A. and Setttanni L. 2017. The individual contribution of starter and non-starter lactic acid bacteria to the volatile organic compound composition of Caciocavallo Palermitano cheese. *International Journal of Food Microbiology* **259** 35-42

Guo XH., Kim JM., Nam HM., Park SY. and Kim JM. 2010. Screening lactic acid bacteria from swine origins for multistrain probiotics based on *in vitro* functional properties. *Anaerobe.* **16** 321-326

Hajj E. 1999. Enquête sur l'élevage caprin au Liban. *La Chèvre* **230** 37-40

Hamadeh SK., Shomo F., Nordblom T., Goodchild A. and Gintzburger G. 1996. Small ruminant production in Lebanon's Bekaa Valley. *Small Ruminant Research* **21** 173-180

Hamon E., Horvatovich P., Izquierdo E., Bringel F., Marchioni E., Aoudé-Werner D. and Ennahar S. 2011. Comparative proteomic analysis of *Lactobacillus plantarum* for the identification of key proteins in bile tolerance. *BMC Microbiology* 11:63 https://doi.org/10.1186/1471-2180-11-63

Hickey MJ. 2001. Role of inducible nitric oxide synthase in the regulation of leucocyte recruitment. *Clinical Science* **100** 1-12

Hill C., Guarner F., Reid G., Gibson GR., Merenstein DJ., Pot B., Morelli L., Canani RB., Flint HJ., Salminen S., Calder PC. and Sanders ME. 2014. Expert consensus document. The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology and Hepatology **11** 506-514

Holzapfe WH., Haberer P., Geisen R., Björkroth J. and Schillinger U. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. *The American Journal of Clinical Nutrition* **73** 365-373

Hu PL., Yuan YH., Yue TL. And Guo CF. 2018. Bile acid patterns in commercially available oxgall powders used for the evaluation of the bile tolerance ability of potential probiotics. *PLOS One* **13** https://doi.org/10.1371/journal.pone.0192964

Huang Y. and Adams MC. 2004. *In vitro* assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *International Journal of Food Microbiol*ogy **91** 253-260

Ibiza S. and Serrador JM. 2008. The role of nitric oxide in the regulation of adaptive immune responses. *Immunología* **27** 103-117

Iñiguez L. 2004. Goats in resources-poor systems in the dry environments of West Asia, Central Asia and the Inter-Andean valleys. *Small Ruminant Research* **51** 137-144

Ishibashi N. and Yamazaki S. 2001. Probiotics and safety. *The American Journal of Clinical Nutrition* **73** 465-470

Jacobsen T., Budde BB. and Koch AG. 2003. Application of Leuconostoc carnosum for biopreservation of cooked meat products. *Journal of Applied Microbiology* **95** 242-249

Jenkins C., Ling C., Ciesielczuk H., Lockwood J., Hopkins S., McHugh T., Gillespie S. and Kibbler C. 2012. Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. *Journal of Medical Microbiology* **61** 483-488

Ji K., Jang NY. and Kim YT. 2015. Isolation of lactic acid bacteria showing antioxidative and probiotic activities from *Kimchi* and infant feces. *Journal of Microbiology and Biotechnology* **25** 1568-1577

Júnior WLGA., Ferrari IS., Souza JV., Silva CDA., Costa MM. and Dias FSD. 2015. Characterization and evaluation of lactic acid bacteria isolated from goat milk. *Food Control* **53** 96-103

Kacem M. and Karam NE. 2006. *In vitro* preselection criteria for probiotic *Lactobacillus plantarum* strains of fermented olives origin. *International Journal of Probiotics and Prebiotics* **1** 27-32

Kaktcham PM., Zambou NF., Tchouanguep FM., El-soda M. and Choudhary MI. 2012. Antimicrobial and safety properties of lactobacilli isolated from two Cameroonian traditional fermented foods. *Scientia Pharmaceutica* **80** 189-203

Kelley EE., Khoo NKH., Hundley NJ., Malik UZ., Freeman BA. and Tarpey MM. 2010. Hydrogen Peroxide is the Major Oxidant Product of Xanthine Oxidase. *National Institutes of health* **48** 493-498

Keskin M. 2002. Effect of rearing systems on kid performance, lactation traits and profitability of Shami goats. Journal of Applied Animal Research **22** 267-271

Khalil R., El-Halafawy K., Mahrous H., Kamaly K., Frank J. and El Soda M. 2007. Evaluation of probiotic potential of lactic acid bacteria isolated from faeces of breast-fed infants in Egypt. *African Journal of Biotechnol*ogy **6** 939-949

Killer J., Havlik J., Vloka E., Rada V., Pechar R., Benada O., Kopecny J., Kofronova O. and Sechovcova H. 2014. *Lactobacillus* rodentium sp. nov., from the digestive tract of wild rodents. *International Journal of Systematic and Evolutionary Microbiology* **64** 1526-1533

Kirtzalidou E., Pramateftaki P., Kotsou M. and kyriacou A. 2011. Screening for lactobacilli with probiotic properties in the infant gut microbiota. *Anaerobe* **17** 440-443

Kiu R. and Hall LJ. 2018. An update on the human and animal enteric pathogen *Clostridium perfringens*. *Emerging Microbes & Infections* 7:141 https://doi.org/10.1038/s41426-018-0144-8

Kochan P., Chmielarczyk A., Szymaniak L., Brykczynski M., Galant K., Zych A., Pakosz K., Giedrys-Kalemba S., Lenouvel E. and Heczko PB. 2011. *Lactobacillus rhamnosus* administration causes sepsis in a cardiosurgical patient--is the time right to revise probiotic safety guidelines? *Clinical Microbiology and Infection* **17** 1589-1592

Köll P., Mandar R., Marcotte H., Leibur E., Mikelsaar M., and Hammarstrom L. 2008. Characterization of oral lactobacilli as potential probiotics for oral health. *Oral Microbiology Immunology* **23** 139-147

König H. and Fröhlich J. 2009. Lactic acid bacteria. In Biology of Microorganisms on Grapes, in Must and in Wine ed. König H., Unden G. and Fröhlich J. 3-29. Berlin Heidelberg: Springer-Verlag

Korhonen R., Korpela R., Saxelin M., Maki M., Kankaanranta H. and Moilanen E. 2001. Induction of nitric oxide synthesis by probiotic *Lactobacillus rhamnosus* GG in J774 macrophages and human T84 intestinal epithelial cells. *Inflammation* **25** 223-232

Kosin B. and Rakshit SK. 2006. Criteria for Production of Probiotics, *Food Technology and Biotechnology* **44** 371-379

Kousta M., Mataragas M., Skandamis P. and Drosinos E, 2010. Prevalence and sources of cheese contamination with pathogens at farm and Processing levels. *Food Control* **21** 805-815

Kumar R., Grover S. and Batish VK. 2012. Bile salt hydrolase (Bsh) activity screening of lactobacilli: *In vitro* selection of indigenous *Lactobacillus* strains with potential bile salt hydrolysing and cholesterol-lowering ability. *Probiotics and Antimicrobial Proteins* **4** 162-172

Kumar A. and Kumar D. 2015. Characterization of *Lactobacillus* isolated from dairy samples for probiotic properties. *Anaerobe* **33** 117-123

Kumari A., Makeen K., Garg AP., Marotta F., Gupta C. and Divya. 2009. Effet of the bacteriocin produced by *Lactococcus lactis subsp. lactis* CCSUB202, on mode of action of *Lactococcus lactis subsp. lactis* MTCC3038. *International Journal of Probiotics and Prebiotics* **4** 205-210

Kyozaire JK., Veary CM., Petzer IM. and Donkin EF. 2005. Microbiological quality of goat's milk obtained under different production systems. *Journal of the South African Veterinary Association* **76** 69-73

Lad SS., Aparnathi KD. Mehta B. and Velpula S. 2017. Goat milk in human nutrition and health – A review. *International Journal of Current Microbiology and Applied Science* **5** 1781-1792

https://doi.org/10.20546/ijcmas.2017.605.194

Lafarge V., Ogier JC., Girard V., Maladen V., Leveau JY., Gruss A. and Delacroix-Buchet A. 2004. Raw cow milk bacterial population shifts attributable to refrigeration. *Applied and Environmental Microbiology* **70** 5644-5650

Lai CY., Fatimah AB., Mahyudin NA., Saari N. and Zaman MZ. 2016. Physico-chemical and microbiological qualities of locally produced raw goat milk. *International Food Research Journal* **23** 739-750

Landeta G., Curiel JA., Carrascova AV., Munoz R. and de las Rivas B. 2013. Technological and safety properties of lactic acid bacteria isolated from Spanish fry-cured sausages. *Meat Science* **95** 272-280

Laparra JM. and Sanz Y. 2009. Comparison of *in vitro* models to study bacterial adhesion to the intestinal epithelium. *Letters in Applied Microbiology* **49** 695-701

Laranjo M., Elis M. and Fraqueza MJ. 2017. The use of starter cultures in traditional meat products. *Hindawi Journal of Food Quality* Article ID 9546026 https://doi.org/10.1155/2017/9546026

Lavermicocca P., Valerio F., Lonigro SL., Di Leo A. Visconti A. 2008. Antagonistic activity of potential probiotic lactobacilli against the ureolytic pathogen *Yersinia enterocolitica*. *Current Microbiology* **56** 175-181

Lebeer S., Vanderleyden J. and De Keersmaecker S. 2008. Genes and molecules of lactobacilli supporting probiotic action. *Microbiology and Molecular Biology Reviews* **72** 728-764

Lee JY., Kim CJ., Kunz B. 2006. Identification of lactic acid bacteria isolated from kimchi and studies on their suitability for application as starter culture in the production of fermented sausages. *Meat Science* **72** 437-445

Li B., Jin D., Evivie SE., Li N., Yan F., Zhao L., Liu F. and Huo G. 2017. Safety assessment of *Lactobacillus* helveticus KLDS1.8701 based on whole genome sequencing and oral toxicity studies. *Toxins* **9** http://dx.doi.org/10.3390/toxins9100301

Linares DM., Gomez C., Renes E., Fresno JM., Tornadijo ME., Ross RP. and Stanton C. 2017. Lactic acid bacteria and bifidobacteria with potential to design natural biofunctional health-promoting dairy foods. *Frontiers in Microbiology* **8** 846 http://doi.org/10.3389/fmicb.2017.00846

Liong MT. 2008. Roles of probiotics and prebiotics in colon cancer prevention: postulated mechanisms and in-vivo evidence. *International Journal of Molecular Sciences* **9** 854-863

Livestock Technical International Cooperation (LTIC), Institut de l'Élevage – Confédération Nationale de l'Élevage 2003. Les filières lait et viande de ruminants au Liban. Compte-rendu d'étude 98 pp

Magiorakos AP., Srinivasan A., Carey RB., Carmeli Y., Falagas ME., Giske CG., Harbarth S., Hindler JF., Kahlmeter G., Olsson-Liljequist B., Paterson DL., Rice LB., Stelling J., Struelens MJ., Vatopoulos A., Weber JT. and Monnet DL. 2011. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection* **18** 268-281

Mangia NP., Murgia MA., Garau G., Sanna M. and Deiana P. 2008. Influence of selected lab cultures on the evolution of free amino acids, free fatty acids and Fiore Sardo cheese microflora during the ripening. *Food Microbiology* **25** 366-377

Mangia NP., Fancello F. and Deiana P. 2016. Microbiological characterization using combined culture dependent and independent approaches of Casizolu pasta filata cheese. *Journal of Applied Microbiology* **120** 329-345

Mangia NP., Saliba L. and Deiana P. 2019. Functional and safety characterization of autochtonous *Lactobacillus paracasei* FS103 isolated from sheep cheese and its survival in sheep and cow fermented milks during cold storage. *Annals of Microbiology* https://doi.org/10.1007/s13213-018-1416-1

Maragkoudakis PA., Zoumpopoulou G., Miaris C., Kalantzopoulos G., Pot B. and Tsakalidou E. 2006. Probiotic potential of *Lactobacillus* strains isolated from dairy products. *International Dairy Journal* **16** 189-199

Mathur S. and Singh R. 2005. Antibiotic resistance in food lactic acid bacteria—a review. *International Journal of Food Microbiology* **105** 281-295

McComas KA. and Gilliland SE. 2003. Growth of probiotic and traditional yogurt cultures in milk supplemented with whey protein hydrolysate. *Journal of Food Science* **68** https://doi.org/10.1111/j.1365-2621.2003.tb07024.x

Mclnnis EA., Kalanetra KM., Mills DA. and Maga E 2015. Analysis of raw goat milk microbiota: Impact of stage of lactation and lysozyme on microbial diversity. *Food Microbiology* **46** 121-131

Merz A., Stephan R. and Johler S. 2016. *Staphylococcus aureus* isolates from goat and sheep milk seem to be closely related and differ from isolates detected from bovine milk *Frontiers in Microbiology* **7** 319 https://doi.org/ 10.3389/fmicb.2016.00319

Metlef S. and Dilmi-Bouras A., 2009. Effet antagoniste de *Lactococcus lactis*, souches extrêmophiles locales, sur des espèces de la flore intestinale résidente. *Revue Nature et Technologie* **1** 33-44

Mills S., Stanton C., Fitzgerald GF., and Ross RP. 2011. Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microbial Cell Factories* **10** 1-19

Ministero della Salute, Commissione unica per la nutrizione e la dietetica 2013. Guidelines on probiotics and prebiotics. *Ministero della Salute* [online], http://www.salute.gov.it/imgs/C_17_pubblicazioni_1016_allegato.pdf

Ministry of Agriculture-FAO 2004. Stratégie de développement agricole du Liban, Ministère de l'agriculture et FAO Projet: « Assistance au Recensement Agricole ». Beyrouth, Liban, 69pp

Mioc B., Pavic V., Barac Z., Prpic Z. and Vnucec I. 2007. Milk yield of some goat breeds in Croatia. *Mljekarstvo* **57** 67-77

Mittu B. and Girdhar Y. 2015. Role of Lactic Acid Bacteria Isolated from Goat Milk in Cancer Prevention. *Autoimmune and Infectious Diseases: Open Access* **1** http://dx.doi.org/10.16966/2470-1025.108

Mocanu GD. and Botez E. 2012. Milk and dairy products: vectors to create probiotic products. Licensee IntechOpen http://dx.doi.org/10.5772/50044

Muhaildin BJ., Hassan Z. and Saari N. 2013. Lactic acid bacteria in biopreservation and the enhancement of the functional quality of bread. IntechOpen Chapter 6 http://dx.doi.org/10.5772/51026

Munita JM. and Arias CA. 2016. Mechanisms of Antibiotic Resistance. *Microbiology Spectrum* **4** https://doi.org/10.1128/microbiolspec.VMBF-0016-2015

Nagpal R., Kumar A., Kumar M., Behare PV., Jain S. and Yadav H. 2012. Probiotics, their health benefits and applications for developing healthier foods. *FEMS Microbioly Letters* **334** 1-15

Nanatani K. and Abe K. 2011. Energy generation coupled with decarboxylation reaction in lactic acid bacteria. *In « Lactic Acid Bacteria and Bifidobacteria: Current Progress in Advanced Research »* Sonomoto and Yokota/ Caister Academic Press éd., Norfolk. United Kingdom. pp. 67-88

Nikaido H. 2009. Multidrug resistance in bacteria. *Annual Review of Biochemistry*. **78** 119-146

Nowak A. and Motyl I. 2017. *In vitro* anti-adherence effect of probiotic *Lactobacillus* strains on human enteropathogens. *Biotechnology and Food Science* **81** 103-112

Nurmi JT., Puolakkainen PA., and Rautonen NE. 2005. *Bifidobacterium Lactis* sp. 420 up-regulates cyclooxygenase (Cox)-1 and down-regulates Cox-2 gene expression in a Caco-2 cell culture model. *Nutrition Cancer* **51** 83-92

Oelschlaeger TA. 2010. Mechanisms of probiotic action–A review. *International Journal of Medical Microbiology* **300** 57-62

Ogunbanwo S.T., Sanni A.I., and Onilude A.A., 2003. Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. *African Journal of Biotechnology* **2** 219-227

Oldak A., Zielinska D., Rzepkowska A. and KoBohyn-Krajewska D. 2017. Comparison of Antibacterial Activity of *Lactobacillus plantarum* Strains Isolated from Two Different Kinds of Regional Cheeses from Poland: Oscypek and Korycinski Cheese. *Hindawi BioMed Research International* Article ID 6820369, 10 pages https://doi.org/10.1155/2017/6820369

Ombarak RA. and Elbagory AM. 2017. Bacteriological quality and occurrence of some microbial pathogens in goat's and ewe's milk in Egypt. *International Food Research Journal* **24** 847-851

Onda T., Yanagida F., Tsuji M., Shinohara T. and Yokotsuka K., 2003. Production and purification of a bacteriocin peptide produced by *Lactococcus* sp. strain GM005, isolated from Miso-paste. *International Journal of Food Microbiology* **87** 153-159

Otte JM. and Mahjurian-Namari R. 2009. Probiotics regulate the expression of COX-2 in intestinal epithelial cells. *Nutrition and Cancer* **6** 103-113

Ouwehand AC., Salminen S. and Isolauri E. 2002. Probiotics: an overview of beneficial effects. *Antoine Van Leeuwenhoek* **82** 279-289

Ouwehand AC. and Salminen S. 2003. *In vitro* adhesion assays for probiotics and their *in vivo* relevance: a review. *Microbial Ecology in Health and Disease* **15** 175-184

Padmavathi T., Bhargavi R., Priyanka PR., Niranjan NR. and Pavitra PV. 2018. Screening of potential probiotic lactic acid bacteria and production of amylase and its partial purification. *Journal of Genetic Engineering and Biotechnology* https://doi.org/10.1016/j.jgeb.2018.03.005

Palomares IC., Pérez-Morales R. and Acedo-Félix E. 2007. Evaluation of probiotic properties in *Lactobacillus* isolated from small intestine of piglets. *Revista Latinoamericana de Microbioliogia* **49** 46-54

Papadimitriou K., Zoumpopoulou G., Foligné B., Alexandraki V., Kazou M., Pot B. and Tsalakidou E. 2015. Discovering probiotic microorganisms: *in vitro*, *in vivo*, genetic and omic approaches. *Frontiers in Microbiology* 6:58 https://doi.org/10.3389/fmicb.2015.00058

Peran L., Camuesco D., Comalada M., Bailon E., Henriksson A., Xaus J., Zarzuelo A. and Galvez J. 2007. A comparative study of the preventative effects exerted by three probiotics, *Bifidobacterium lactis, Lactobacillus casei* and *Lactobacillus acidophilus*, in the TNBS model of rat colitis. *Journal of Applied Microbiology* **103** 836-844

Peres C., Tacito LA. And Rodrigo PL. 2016. An evaluation of the physicochemical, microbiological and sensory properties of goat cheese. *African Journal of Dairy Farming and Milk Production* **3** 120-125

Perin LM. and Nero LA. 2014. Antagonistic lactic acid bacteria isolated from goat milk and identification of a novel nisin variant *Lactococuus lactic*. *BMC Microbiology* 14:36

Pfeiler EA. and Klaenhammer TR. 2009. Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. *Applied and Environmental Microbiology* **75** 6013-6016

Pithva S., Ambalam P., Dave JM. and Vyas BR. 2012. Potential of Probiotic *Lactobacillus* Strains as Food Additives, Food Additive, Prof. Yehia El-Samragy (Ed.), ISBN: 978-953-51-0067-6, InTech, Available from: http://www.intechopen.com/books/food-additive/potential-of-probiotic-lactobacillus-strains-as-food-additives

Prasirtsak B., Tanasupawat S., Boonsombat R., Kodama K. and Thongchul N. 2013. Characterization of lactic producing bacteria from Thai sources. *Journal of Applied Pharmaceutical Science* **3** 33-38

Preidis GA., Hill C., Guerrant RL., Ramakrishna BS., Tannock GW. and Versalovic J. 2011. Probiotics, enteric and diarrheal diseases, and global health. *Gastroenterology* **140** 8-14

Quinto EJ., Jiménez P., Caro I., Tejero J., Mateo J. and Girbés T. 2014. Probiotic lactic acid bacteria: A review. *Food and Nutrition Sciences* **5** 1765-1775 http://dx.doi.org/10.4236/fns.2014.518190

Rautio M., Jousimies-Somer H., Kauma H., Pietarinen I., Saxelin M., Tynkkynen S. and Koskela M. 1999. Liver abscess due to a *Lactobacillus* rhamnosus strain indistinguishable from *L. rhamnosus* strain GG. *Clinical Infectious Diseases* **28** 1159-1160

Raynal-Ljutovac K., Lagriffoul G., Paccard P. Guillet I. and Chilliard Y. 2008. Composition of goat and sheep milk products: An update. *Small Ruminant Research* **79** 57-72

Regulation (EC) No 853/2004. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs. *Official Journal of the European Union* 151 pp

Regulation (EC) No 2073/2005. Commission regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union* 26 pp

Rehman SU., Banks JM., McSweeney PLH. and Fox PF. 2000. Effect of ripening temperature on the growth and significance of non-starter lactic acid bacteria in Cheddar cheese made from raw or pasteurised milk. *International Dairy Journal* **10** 45-53

Robin F., Paillard C., Marchandin H., Demeocq F., Bonnet R. and Hennequin C. 2010. *Lactobacillus rhamnosus* meningitis following recurrent episodes of bacteremia in a child

undergoing allogeneic hematopoietic stem cell transplantation. *Journal of Clinical Microbiology* **48** 4317-4319

Rocha-Ramirez LM., Perez-Solano RA., Castanon-Alonso SL., Guerrero SSM., Pacheco AR., Garibay MG. and Eslava C. 2017. Probiotic *Lactobacillus* strains stimulate the inflammatory response and active human macrophages. *Hindawi Journal of Immunology Research* Article ID 4607491, https://doi.org/10.1155/2017/4607491

Rodgers S. 2001. Preserving non-fermented refrigerated foods with microbial cultures. *Trends Food Science & Technology* **12** 276-284

Salameh C., Banon S., Hosri C. and Scher J. 2016. An overview of recent studies on the main traditional fermented milks and white cheeses in the Mediterranean region. *Food Reviews International* **32** 256-279

Salvetti E., Torriani S. and Felis GE. 2012. The Genus *Lactobacillus*: a taxonomic update. *Probiotics and Antimicrobial Proteins* **4** 217-226

Sanna MG., Mangia NP., Garau G., Murgia MA., Massa T., Franco A. and Deiana P. 2005. Selection of folate-producing lactic acid bacteria for improving fermented goat milk. *Italian Journal of Food Science* **17** 143-154

Sardaro MLS., Levante A., Bernini V., Gatti M., Neviani E. and Lazzi C. 2016. The *spx* gene as a target to identify *Lactobacillus casei* group species in cheese. *Food Microbiology* **59** 57-65

Schmidt M., Maxime V., Pareire F., Carlier R., Lawrence C., Clair B. and Annane D. 2011. A lethal case of meningitis due to *Lactobacillus rhamnosus* as a late complication of anterior cervical spine surgery. *The Journal of Infection* **62** 309-310

Serhan M., Cailliez-Grimal C., Borges F., Revol-Junelles AM., Hosri C. and Fanni J. 2009. Bacterial diversity of Darfiyeh, a Lebanese artisanal raw goat's milk cheese. *Food Microbiology* **26** 645-652

Serhan M. and Mattar J. 2013. Characterization of four Lebanese artisanal goat milk cheeses: Darfiyeh, Aricheh, Shankleesh and Serdale by physico-chemical, microbiological and sensory analyses. *Journal of Food Agriculture and Environment* **11** 97-101

Serhan M., Mattar J. and Debs L. 2016. Concentrated yogurt (Labneh) made of a mixture of goats and cows,milk: Physicochemical, microbiological and sensory analysis. *Small Ruminant Research* **138** 46-52

Serhan M. and Mattar J. 2018. The goat dairy sector in Lebanon. *IntechOpen* Chapter 18 http://dx.doi.org/10.5772/intechopen.70077

Servin AL. 2004. Antagonistic activity of lactobacilli and bifidobacteria againstmicrobial pathogens. *FEMS Microbiology Rev*iews **28** 405-440

Shah NP. 2000. Probiotic bacteria: selective enumeration and survival in dairy foods. *Journal of Dairy Science* **83** 894-907

Shehata MG., El Sohaimy SA., El-Sahn MA. and Youssef MM. 2016. Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. *Annals of Agricultural Science* **61** 65-75

Shewale RN. Sawale PD. Khedkat CD. and Singh A. 2014. Selection criteria for probiotics: a review**.** *International Journal of Probiotics and Prebiotics* **9** 17-22

Shokryazdan P., Sieo CC., Kalavathy R., Liang JB., Alitheen NB., Jahromi MF. and Ho YW. 2014. Probiotic Potential of *Lactobacillus* Strains with Antimicrobial Activity against Some Human Pathogenic Strains. *BioMed Research International* Volume 2014, Article ID 927268, 16 pages http://dx.doi.org/10.1155/2014/927268

Sieladie DV., Zambou NF., Kaktcham PM., Cresci A. Fonteh F. 2011. Probiotic properties of lactobacilli strains isolated from raw cow milk in the western highlands of Cameroon. *Innovative Romanian Food Biotechnology* **9** 12-28

Slover CM. and Danziger L. 2008. *Lactobacillus*: a review. *Clinical Microbiology Newsletter* **30** 23-27

Soccol CR., Vandenberghe LP., Spier MR., Medeiros AB., Yamaguishi CT., Lindner JD., Pandey A. and Thommaz-Soccol V. 2010. The potentia of probiotics. *Food Technology and Biotechnology* **48** 413-434

Sornplang P. and Piyadeatsoontorn S. 2016. Probiotic isolates from unconventional sources: a review. *Journal of Animal Science and Technology* 58:26 https://doi.org/10.1186/s40781-016-0108-2

Suguna M., Bhat R. and Nadiah W. 2012. Microbiological quality evaluation of goat milk collected from small-scale dairy farms in Penang Island, Malaysia. *International Food Research Journal* **19** 1241-1245

Sule J., Korosi T., Hucker A. and Varga L. 2014. Evaluation of culture media for selective enumeration of bifidobacteria and lactic acid bacteria. *Brazilian Journal of Microbiology* **45** 1023-1030

Sun Z., Harris HM., McCann A., Guo C., Argimon S., Zhang W., Yang X., Jeffery IB., Cooney JC., Kagawa TF., Liu W., Song Y., Salvetti E., Wrobel A., Rasinkangas P., Parkhill J., Rea MC., O'Sullivan O., Ritari J., Douillard FP., Paul Ross R., Yang R., Briner AE., Felis GE., de Vos WM., Barrangou R., Klaenhammer TR., Caufield PW., Cui Y., Zhang H. and O'Toole PW. 2015. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. *Nature Communications* 6:8322 http://dx.doi.org/10.1038/ncomms9322

Tabet E., Hosri C. and Abi-Rizk A. 2015. Prévalence et facteurs de risqué de l'arthriteencéphalite caprine virale au Liban. *Revue Scientifique et Technique (International Office of Epizootics)* **34** 3

Tabet E., Mangia NP., Mouannes E., Hassoun G., Helal Z. and Deiana P. 2016. Characterization of goat milk from Lebanese Baladi breed and his suitability for setting up a ripened cheese using a selected starter culture. *Small Ruminant Research* **140** 13-17

Tamime AY. and Robinson RK. 2007. editors. Yogurt Science and Technology. 3rd edition Cambridge, UK: Woodhead Publishing

Tejero-Sarinena S., Barlow SJ., Costabile A., Gibson GR. and Rowland I. 2012. *In vitro* evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. *Anaerobe* **18** 530-538

Terzic-Vidojevic A., Tolinacki M., Nikolic M., Veljovic K., Jovanovic S., Macej O. and Topisirovic L. 2013. Artisanal Vlasina Raw Goat's Milk Cheese: Evaluation and Selection of Autochthonous Lactic Acid Bacteria as Starter Cultures. Food Technology and Biotechnology **51** 554-563

Tommasi C., Equitani F., Masala M., Ballardini M., Favaro M., Meledandri M., Fontana C., Narciso P. and Nicastri E. 2008. Diagnostic difficulties of *Lactobacillus casei* bacteraemia in immunocompetent patients: a case report. *Journal of Medical Case Reports* 2:315

Van de Guchte M., Serror P., Chervaux C., Smokvina T., Ehrlich SD. and Maguin E. 2002. Stress responses in lactic acid bacteria. *Antonie Van Leeuwenhoek* **82** 187-216

Vaquil and Rathee R. 2017. A review on health promoting aspects of goat milk. *The Pharma Innovation Journal* **6** 05-08

Vasiljevic T. and Shah NP. 2008.Probiotics—From Metchnikoff to bioactives. *International Dairy Journal* **18** 714-728

Vermeiren L., Devlieghere F., Debevere J. 2004. Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. *International Journal of Food Microbiology* **96** 149-164

Von Wintersdorff CJH., Penders J., van Niekerk JM., Mills ND., Majumder S., van Alphen LB., Savelkoul PHM. and Wolffs PFG. 2016. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in Microbiology* 7:173 https://doi.org/10.3389/fmicb.2016.00173

Wallet F., Dessein R., Armand S. and Courcol RJ. 2002. Molecular diagnosis of endocarditis due to *Lactobacillus casei* subsp. *rhamnosus*. *Clinical Infectious Diseases* **35** 117-119

Wedajo B. 2015. Lactic Acid Bacteria: Benefits, Selection Criteria and Probiotic Potential in Fermented Food. *Journal of Probiotics & Health* 3:129 https://doi.org/10.4172/2329-8901.1000129

Wells JM. 2011. Immunomodulatory mechanisms of lactobacilli. *Microbial Cell Factories* 10(Suppl 1):S17 http://www.microbialcellfactories.com/content/10/S1/S17

Whitehead K., Versalovic J., Roos S. and Britton RA. 2008. Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. *Applied and Environmental Microbiology* **74** 1812-1819

Xiao Y., Wagendorp A., Moezelaar R., Abee T. and Wells-Bennik MHJ. 2012. A Wide Variety of *Clostridium perfringens* Type A Food-Borne Isolates That Carry a Chromosomal *cpe* Gene Belong to One Multilocus Sequence Typing Cluster. *Applied and Environmental Microbiology* **78** 7060-7068

Xiaodong P., Fenqin C., Tianxing W., Honggang T. and Zhanyu Z. 2009. The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. *Food Control* **20** 598-602

Zahid M., Ashraf M., Arshad M., Muhammad G., Yasmin A. and Hameed HMA. 2015. Antimicrobial activity of bacteriocins isolated from lactic acid bacteria against resistant pathogenic strains. *International Journal of Nutrition and Food Sciences* **4** 326-331

Zenebe T., Ahmed N., Kabeta T. and Kebede G. 2014. Review on medicinal and nutritional values of goat milk. *Academic Journal of Nutrition* **3** 30-39

Zhou JS., Pillidge CJ., Gopal PK. and Gill HS. 2005. Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *International Journal of Food Microbiology* **98** 211-217

Zoumpopoulou G., Foligne B., Christodoulou K., Grangette C., Pot B. and Tsakalidou E. 2008. *Lactobacillus* fermentum ACA-DC 179 displays probiotic potential *in vitro* and protects against trinitrobenzene sulfonic acid (TNBS)-induced colitis and Salmonella infection in murine models. *International Journal of Food Microbiology* **121** 18-26

Zoumpopoulou G., Tzouvanou A., Mavrogonatou E., Alexandraki V., Georgalaki M., Anastasiou R., Papadelli M., Manolopoulou E., Kazou M., Kletsas D., Papadimitriou K. and Tsakalidou E. 2017. Probiotic Features of Lactic Acid Bacteria Isolated froma Diverse Pool of Traditional Greek Dairy Products Regarding Specific Strain-Host Interactions. *Probiotics and Antimicrobial Proteins* **10** 313-322 https://doi.org/10.1007/s12602-017-9311-9