Scuola di Dottorato per il Sistema Agro-alimentare
Doctoral School on the Agro-Food System

cycle XXVIII

S.S.D: AGR/16

FOOD SAFETY AND QUALITY IN DEVELOPING COUNTRIES: THE ROLE OF LACTIC ACID BACTERIA

Coordinator: Ch.mo Prof. Antonio Albanese

Candidate: Matteo Angri

Matriculation n.: 4110924

Tutor: Ch.mo Prof. Pier Sandro Cocconcelli

Academic Year 2014/2015

Summary

GENERAL INTRODUCTION

1. FOOD SAFETY AND FOODBORNE DISEASES: A PROBLEM FOR THE DEVELOPING COUNTRIES

Food safety can be recognized as an integral part of the food security, of the allowing environment for reducing hunger and malnutrition, and the inclusion of food safety with nutrition, results from the complex relationship between food and health (Unnevehr, 2015).

The planet population is still affected by a wide range of food-related challenges, like food diseases, vitamin and mineral deficiencies. In fact unsafe food causes both acute and chronic illness, and reduces the availability of nutrients, in particular for the poorest and most vulnerable consumers (Conference & Food, 2014).

In 1996 the World Food Summit declares: "Food security exists when all the people, at all times, have physical, social and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life" (Unnevehr, 2015). The food hazards are not only a problem related to the human health, but lead also to the food losses and availability reduction for the food insecure populations, exacerbating the difficulty of food access. Finally, food safety may also play a role in producing livelihoods, as smallholders seek to meet requirements in high value markets (Conference & Food, 2014).

Given the recognized importance of improved food safety for food security, it is relevant to understand how it can be addressed for food insecure populations. In the last two decades a remarkable progress in the application of science and in the management to this issue has been accomplished (Unnevehr, 2015).

Food safety risks contribute to the burden of illness in developing countries. For example unsafe food represents the cause of both acute or chronic illness and reduces the bioavailability of natural nutrients, in particular for the vulnerable and poorest consumers (Conference & Food, 2014).

The food hazards can really lead to food losses and reduce food availability for all the food insecure community. Foodborne and malnutrition still remain one the

most important responsible causes of death among infants and children under 5 years of age worldwide each year.

Estimates say that each year lose their lives about 13 million children in developing countries due to food biologically contaminated and problems related to malnutrition (Motarjemi, Käferstein, Moy, & Quevedo, 1993). Young people and children are the most susceptible to foodborne diseases, and this is due to greater ease in contracting intoxications or infections, causes of illnesses and in certain cases even of death in serious circumstances (Motarjemi et al., 1993).

In children aged around 5 years, diarrhea caused by foodborne causes represents one of the greater problems that afflict the public health. For the last 20 years in developing countries, approximately 1.5 billion of diarrhea cases have been recorded, remaining more or less constant (Käferstein, 2003). Pathogens involved in foodborne diseases seem to be various and little is known about their real contribution to the development of illnesses in the poorest areas.

Indeed not much is known regarding the high-risk food being the source of disease transmission and finally there are no significant data regarding the real nature and extension of food safety in developing countries. But it is possible to consider the data collected and the researches carried out in developed countries about the causes of foodborne diseases, and thus apply them in the emerging countries. Many and various are the pathogens identified causing illness through food, like *Escherichia coli, Salmonella* spp., *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Clostridium perfringens* and other (Käferstein, 2003) (Motarjemi et al., 1993).

The interaction between infectious disease and bad nutrition is by now accepted, and is almost ascertained that the food infection can determine an incorrect metabolic development of children during growth. A study developed by L.J. Mata (Director of Institute of Health Research, University of Costa Rica in San Jose),

investigated the effect of different diseases in relation to the nutrition status of young people (Fig. 1).

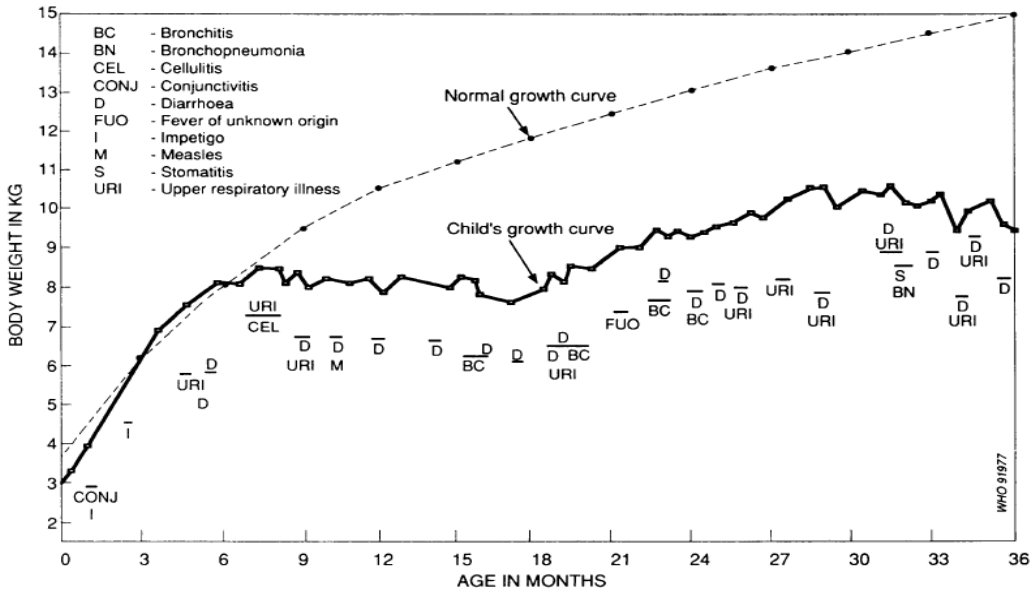


Figure 1: Effect of food disease on nutritional child status. The horizontal bars indicate how long it lasts the disease. Source: Y.Motarjiemi et al. Contaminated weaning food: a major risk factor for diarrhea and associated malnutrition. Bulletin Bulletin of the World Health Organization, 71 (1): 79-92 (1993).

It is plausible to assume that in the case the infant is infected from some pathogen bacteria that cause diarrhea o other disease, the nutrients are drained and lost from the body, thus causing malnutrition and consequently a lowered resistance to the foodborne diseases (Käferstein, 2003).

While the understanding of food contamination risk exposure in the incidence of illness still remains limited, various studies are suggesting that microbial hazards are important in a wide range of food, like raw milk for example (Arimi, Koroti, Kang'ethe, Omore, & McDermott, 2005). Another important food hazard is represented by the spoilage of the food, such as the development of filamentous fungi in food that are able to produce mycotoxin. For example, aflatoxin exposure through food consumption, which is more prevalent in the tropical areas, is

associated to immune suppression and higher rates of illness, as well as child stunting (Unnevehr & Grace, 2013). Mycotoxins have attracted worldwide attention for the significant losses associated with their impact on human and animal health, and consequent national economic implications.

So it is assumed that the inhabitants of developing countries are experiencing heavy dietary exposure to food-borne mycotoxins (Bankole & Adebanjo, 2003).

2. MALNUTRITION AND VITAMINE DEFICIENCE

In developing countries malnutrition still remains one of the main problems for public health, especially in areas marked by enormous problems of poverty such as South Asia and sub-Saharan Africa, where access to food is a huge challenge

Often diets are low in macronutrients (proteins, carbohydrates and fats), in micronutrients (electrolytes, minerals and vitamins) or even in both of them.

The majority of the population that is suffering from malnutrition related issues (about 827 million) lives in developing countries, where the percentage of malnourishment is about 14.3% (FAO / IFAD / WFP, 2013).

Malnutrition has to be seen like a cofactor of different main causes, like secondary diseases, but the risk of death is proportional to the increase of the level of malnutrition (Walton & Allen, 2011). The problem of malnutrition is not tied only to the inability of rural households to self-produce healthy and safe food for a proper diet, but also depends on other factors closely related to each other, first of all, the poverty that often affects most rural communities (Müller & Krawinkel, 2005). But it is important to define the term of malnutrition, that is a physical pathological situation critically determined by a medical condition that is established when the nutritional needs are not met in time. In other words we can define malnutrition as the deficiency of one or more essential food components in a balanced and healthy diet: a shortage of vitamins in the diet, for example, may lead to the onset of the condition of chronic malnutrition.

Diets with low content in vitamins may cause a greater susceptibility to diseases such as chronic diarrhea, peumonia, measles and other diseases that can often lead to death (Fitzpatrick et al., 2012). Vitamins are organic compounds present in small amounts in certain foods, commonly recognized as principles nutrients given the inability of the human body to synthesize them. However they are extremely important for the normal development in the physical well-being, especially during the early years of life. They play a role in many biochemical reactions, but they are not considered as a source of energy but they do facilitate the metabolic reactions (Lukaski, 2004). Inappropriate consumption is now globally recognized as one of the major promoters of diseases and in some cases of death, especially in children under 5 years old, as well as of mental disability and metabolic issues during the growth phase. The dietary reference intakes are actually certified (Table 3). The deficiency in vitamins is a problem that particularly affects the developing countries where the causes of death and childhood infections are often caused by low doses of vitamins in the diet, compromising the normal metabolic development (Black, 2003). Vitamins are therefore of paramount importance for life and only through a proper diet human being is able to absorb the right amount of different vitamins needed.

The vitamin malnutrition in developing countries mainly affects children between the 6th and the 24th month because breast milk alone is unable to meet the needs of vitamins. The normal weaning and feeding is not often sufficient to cover the needs in vitamins for both (Thu et al., 1999), and the risks associated with a low dose of vitamins in the diet are many and often leading to the premature death of children (about 55%). Currently it is estimated that about 200 million children under 5 years suffer from disorders related to avitaminosis (Branca & Ferrari, 2002).

3. THE FOLIC ACID

GENERAL CONSIDERATION

Folic acid belongs to the water-soluble vitamins of the B group and is mainly found in vegetables and fruits, above all in green leafy vegetables, legumes, and in certain fermented products. Studies of folates started early in the 18th century when they began to study the effects of vitamin deficiencies within the poorer social classes, but the first actual description of the role of folate in the diet took place in 1931 in a study realized by Wills.

Through a study of anemia in tropical India, he emphasized for the first time the negative effects of a diet low in folate, but this group of vitamins was given the name of folic acid only in 1941 when it was isolated from spinach leaves (folium = leaf (Lat)and was used as a substrate for growth of a strain of *Streptococcus faecalis* (History & Folic, 2001). As folate can not be directly synthesized, diet plays a key role in the daily requirement of this vitamins. Daily doses of folate intake have been establishedfor a long time and depend on the sex and age of the individual, with a further difference in the case of pregnant women: in that case, the RDA (Recommended Dietary Allowance) increases to compensate for the physiological needs of folic acid in favor of the fetus. Currently it is recommended that an RDA for an adult is approximately 400µg/d, which rises up to 600µg/d for pregnant women (Molloy, 2001; Bailey,

Group	Adequate intake[2]	Recommended dietary allowance
	µg of DFE/d	
Infants		
0–5	65	
6–11	80	
Children and adolescents		
1–3		150
4–8		200
9–13		300
14–18		400
Adults		
≥19		400
Pregnant women		
All ages		600
Lactating women		
All ages		500

Table 1: Dietary reference intakes of folate estimated for all age and sex categories (DFE is a term adopted by the National Academy of Sciences for adjust the value of folate in food) (Source Bailey, 1999).

1999; Allen, 2008; Mosley *et al.*, 2009) and this value is confirmed by the Food and Nutrition Board (FNB, 1998). This value was based on the assumptions that 100-200 g of folate per day is required to maintain tissue folate levels and that only 25-50% of dietary folate is absorbed. Since polyglutamyl folate is the predominant form in foods, absorption was thought to be limited by the requirement for hydrolysis to monoglutamyl folate prior to absorption (Reisenaãœer & Halsted, 1987). Adequate folate intake is vital for cell division and homeostasis and that is due to the essential role of folate coenzymes in nucleic acid synthesis, methionine regeneration, and in the shuttling, oxidation and reduction of one-carbon units required for normal metabolism and regulation.

Folic acid consists of the union of a molecule of p-aminobenzoic acid to a ring pteridinico and a single molecule of glutamic acid . In order to be absorbed into the body it has first to undergo one or more reductions in natural bioactive forms, as tetrahydrofolate (THF) or 5-MeTHF-monoglutamate, which represent active forms that will be subsequently used in important metabolic processes, such as DNA Synthesis Cycle and the Methylation Cycle (plays a crucial role during the formation of the first reagent methyl, l 'S-adenosylmethionin) devolving dozens of methyltransferase enzyme present in the cells and involved in methylation of lipids, in the structure of the proteins (Crider, Bailey, & Berry, 2011). Folic acid is composed of a pteridine ring linked to a para-aminobenzoic acid molecule and a glutamic acid molecule (Fig. 2).

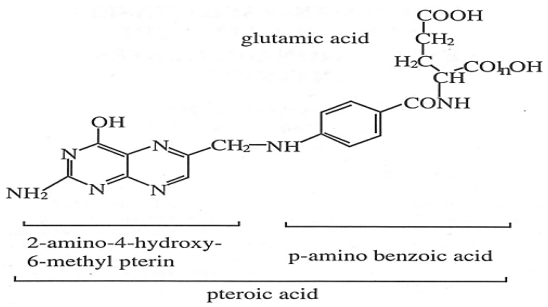


Figure 2: Folate Chemical Structure. Folates are a group of heterocyclic compounds based on the 4-[(pteridin-6-ylmethyl)amino]benzoic acid skeleton, conjugated with one or more L-glutamate units.

Absorption is made by a saturable, pH-dependent, active transport process, and once absorbed, folate is transported to the liver, where it is metabolized to polyglutamate forms prior to storage or reconverted to monoglutamates and thus released into the blood circulation predominantly in the form of 5-methyltetrahydrofolate (5-MTHF). Folate is also released into bile, where it is subsequently reabsorbed via enterohepatic circulation. The main circulating form of the vitamin must be reduced and reconjugated to the polyglutamate form before it can be metabolically active. Folate is essential for the transfer and utilization of one-carbon units for amino acid metabolism, synthesis of purines and pyrimidines (precursors of DNA and RNA synthesis), and methylation reactions.

BIOSYNTHESIS OF FOLIC ACID

Prokaryotic and eukaryotic beings require reduce folate cofactor ad acceptor donor of one-carbon units in different biosynthesis process, including the formation of methionine, purines and thymine. Animal are not able to synthetize directly folate so the diet play a crucial role for the daily supplementation. Instead, in a different way, plants, fungi, certain protozoa, and several archaea and bacteria can synthesize folates, likely through the same general biosynthetic pathway (Fig. 3).

The folate molecule contains one pterin moiety, originating from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP), bound to para-aminobenzoic acid (pABA, vitamin B10). Thus, de novo biosynthesis necessitates both the precursors, DHPPP and pABA. The latter can be produced by plants and bacteria starting from the pentose phosphate pathway. Erythrose 4-phosphate and phosphoenolpyruvate undergo the shikimate pathway to ultimately lead to chorismate, which serves as a branching point toward the biosynthesis of aromatic amino acids and pABA. Chorismate is converted via aminodeoxychorismate synthase (EC 2.6.1.85) into 4-amino-4-deoxychorismate. Subsequently, pyruvate is cleaved by 4-amino-4-deoxychorismate lyase (EC 4.1.3.38) to give pABA, which ultimately serves for

folate biosynthesis. The biosynthesis of DHPPP proceeds via the conversion of guanosine triphosphate (GTP) in four consecutive steps. The first step is catalyzed by GTP cyclohydrolase I (EC 3.5.4.16) and involves an extensive transformation of GTP, to form a pterin ring structure. Following dephosphorylation, the pterin molecule undergoes aldolase and pyrophosphokinase reactions, which produce the activated pyrophosphorylated DHPPP.

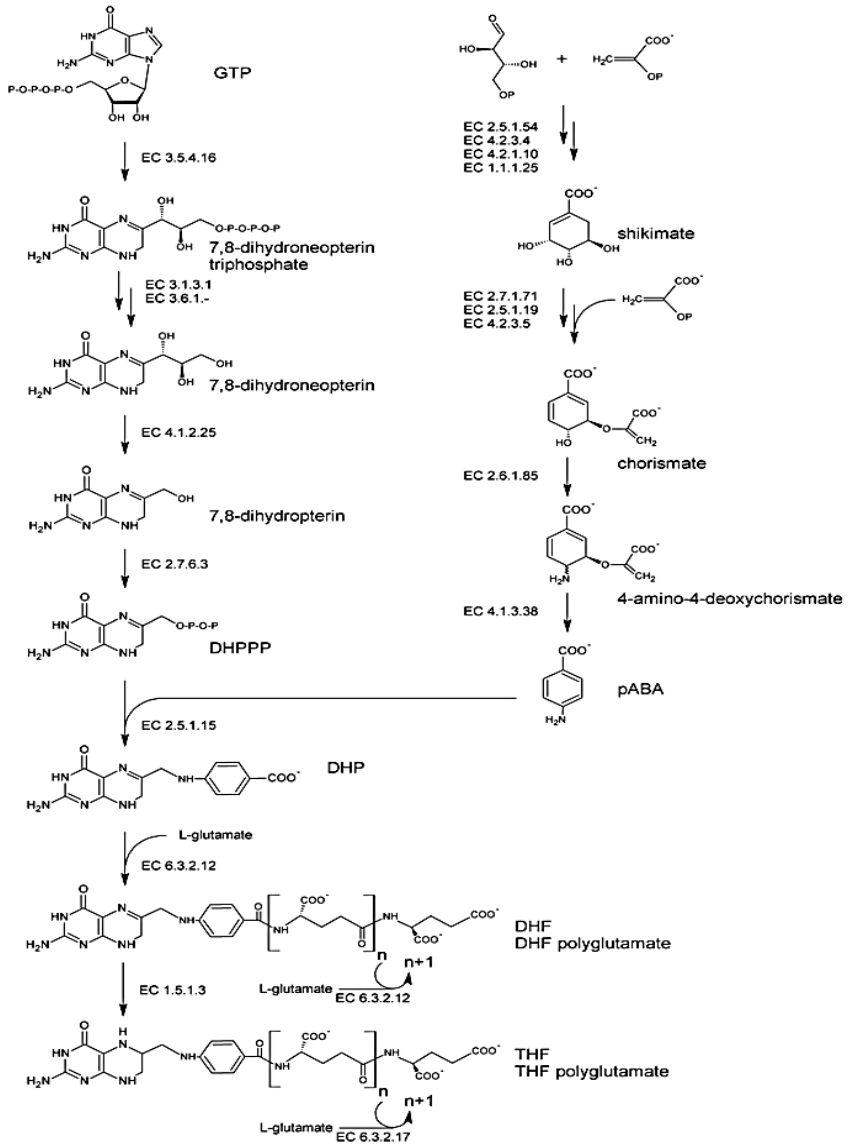


Figure 3: Pathway of de novo bacterial biosynthesis of folate. Abbreviations: GTP, guanosine triphosphate; DHPPP, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; pABA, para-aminobenzoic acid; DHP, 7,8-dihydropteroate; DHF, dihydrofolate; THF, tetrahydrofolate

Folate biosynthesis continues with the formation of a C–N bond joining DHPPP to pABA. This condensation reaction, catalyzed by dihydropteroate synthase (EC 2.5.1.15), yields 7,8-dihydropteroate (DHP). DHP is glutamylated by dihydrofolate synthase (EC 6.3.2.12) giving dihydrofolate (DHF). Then, it is reduced by DHF reductase (EC 1.5.1.3) to the biologically active cofactor tetrahydrofolate (THF) and subjected to the addition of multiple glutamate moieties by folylpolyglutamate synthase (EC 6.3.2.17) to yield THF-polyglutamate. Polyglutamilation may take place also before the occurrence of the reduction step, being catalyzed by DHF synthase or, in many bacteria, by a bifunctional enzyme which is responsible for both EC 6.3.2.12 and EC 6.3.2.17 activities (Rossi, Amaretti, & Raimondi, 2011).

FOLIC ACID DEFICIENCES

Emerging evidence supports a number of roles for folate in maintaining health, from maternal and fetal health in pregnancy through childhood to preventing chronic disease in middle and old age, and a deficient or a low status of folate is not uncommon even otherwise in well-nourished countries (Mcnulty, Pentieva, Hoey, Strain, & Ward, 2012).

Pregnancy is well recognized as a time when folate requirement is increased to sustain the increased demand for folate related to rapid cell replication and growth of fetal, placental and maternal tissue.

Low maternal folate status during pregnancy and lactation remains a significant cause of maternal morbidity in some communities. The folate status of the neonate tends to be protected at the expense of maternal stores; nevertheless, there is mounting evidence that inadequate maternal folate status during pregnancy may lead to low infant birthweight, thereby conferring risk of developmental and long-term adverse health outcomes. Moreover, folate-related anemia during childhood and adolescence might predispose children to further infections and disease (a.M., P.N., L.C., J.M., & J.L., 2008).

While folate deficiency has been acknowledged for decades as the most common cause of macrocytosis in pregnancy, the vitamin is now known to play a much more profound and wide-ranging role in maintaining healthy reproduction.

The causes related to low dosage of folate may depend on factors concerning "genetic polymorphism" (polymorphism of some genes can influence the natural folate metabolism, as discussed by Finnel *et al.*), to problems associated with "alcoholism" (the Ethanol plays a role as an antagonist in the intestinal absorption of folate) (Allen 2008), for the loss of folate "during the processes of preparation of food" (it is assumed that any loss of about 50% - 80 of folate during the cooking process of the plant and vegetables) (McKillop *et al.*, 2002; Ruiz-Rodriguez *et. al*, 2008), for the use of "certain medications" (anti-malarial treatments, used in developing countries, they act as antagonists during the absorption of folate) (Allen, 2008; Carmel, 2008), but the major cause linked to folate deficiency is mainly linked to the "poorest folate diet". Folate deficiency results in reduced DNA biosynthesis and thereby in reduced cell division, leading to the major clinical manifestation of the deficiency, megaloblastic anemia. The deficiency also results in elevated plasma total homocysteine concentrations as functioning of the methylation cycle is impaired (Hoey, Mcnulty, Duffy, & Hughes, 2015).

Studies show that low levels of folate for long periods, can be related to neurodegenerative diseases such as *Alzheimer's* and *Parkinson's*, and caused by degenerative problems at the neural level (Mattson, 2003; Benoist, 2008; Swodon, 2000; Black, 2008; Rosenberg, 2008). Further studies also associate the low value of folate to disorder (especially in adults) such as *depression* and *schizophrenia* (Mattson, 2003; Black, 2008).

But the more assumed related disease of folate deficiencies is the NTD (Neural Tube Defect), which is a common complex congenital malformation of the central nervous system resulting from failure of the neural tube closure during embryogenesis. The prevalence of NTDs varies widely between 1 and 10 per 1,000

births, depending on geographic region and ethnical grouping, making them one of the most frequent congenital malformations. The relationship between folate deficiencies and NTD was just acquired on the early ’60s.

In the Latest ‘90s, with the introduction of mandatory National Food Fortification program, began the first Nation Program against NTDs (Grains and cereals were enriched with Folic Acid at a concentration of 1.4 mg/kg in order to increase the average consumption of women of reproductive age by around 0.1 mg per day) but only later, with the introduction of daily doses, it came to establish the recommended value of 600 µg/d. The NTD is one of the major cause of death and morbidity, and it is estimated that approximately among the 300,000 children born with this syndrome, each year 41,000 of them do not survive to the first year of life. These estimates mean that the NTD is the third leading cause of congenital diseases for global importance (Blecowe *et al.*, 2010).

According to a study by Barber *et al.*, the causes of NTDs are due to the role of folate in the synthesis of nucleotide. In fact during the embryo development, the rapidity of cell division for the development of the neural tube requires the synthesis of a large amount of nucleotides that is to facilitate the replication of DNA. It can therefore be assumed that the neuroepithelial cells do not have an adequate supply of nucleotides, then in the absence of folate, cell division happens much slower, resulting in a failure to complete the development of the neural tube (Barber *et al*, 1999).

FOLIC ACID IN FERMENTED FOOD

Methods such as fortification have been proven to be very useful in reducing health problems associated with folate mal-intake. However, it was recently shown that high-level intakes of chemically synthesized folate might have some adverse health effects (Powers, 2007). A natural and easy way to increase the folate content in food is represented by a fermentation fortification process. Fermentation

fortification is a new concept which can be improved in several ways: 1) bacterial strain selection, 2) delivery engineering and 3) metabolic engineering. There is a growing interest in using lactic acid bacteria (LAB) for improvement of the nutritional value of fermented food products and/or for adding health benefits to these products. Milk is not a food rich in folic acid. But, many dairy products are processed using microbial fermentations in which folate can be synthesized, significantly increasing folate concentrations in the final product. In most regions of the world, yogurt constitutes the main portion of the per capita consumption of fermented milk products; nevertheless, cultured milk, cultured buttermilk, and cultured cream are also commonly consumed in European (Iyer, Tomar, Uma Maheswari, & Singh, 2010). In addition to several attractive sensory aspects attributed to these fermented milk products, the public is becoming increasingly aware of their nutritional and physiological properties. Among dairy products, fermented milks are considered as a potential matrix for folate fortification because folate binding proteins of milk improve folate stability and the bioavailability of both 5-methyltetrahydrofolate and folic acid may be enhanced. However, due to the potential risks of fortification with folic acid, the elaboration of fermented milks containing elevated levels of natural form folates would be a better suited alternative (Rossi et al., 2011).

The modified Food Guide Pyramid released in 2005 by the United States Department of Agriculture (USDA) recommends the consumption of at least 3 servings of milk products as part of a healthy daily diet. Taking into account this recommendation, and considering that a normal serving consists of 240 ml, currently available fermented milk products could contribute significantly to the reference daily intake of folates. This intake could be even higher if properly selected starter cultures capable of producing elevated levels of natural folates were used in the elaboration of these products (LeBlanc, de Giori, Smid, Hugenholtz, & Sesma, 2007).

4. LACTIC ACID BACTERIA: A FUNCTIONAL FOOD INGREDIENTS

The Lactic Acid Bacteria (LAB) is microorganisms which can be found in many environments, and many of them are now usually used in the food production, for preservation and for the modification of the organoleptic characteristics. Many strains of LAB are among the most important groups of microorganisms used in the food and feed industries and them will been used in food preservation and for the modification of the organoleptic characteristics of foods, for example flavors and texture (Soomro, Masud, & Anwaar, 2002; Florou-Paneri, Christaki, & Bonos, 2013). Various strains of LAB can be found in dairy products (yoghurt, cheese), fermented meats (salami), fermented vegetables (olives, sauerkraut), sourdough bread, etc. (Caplice & Fitzgerald, 1999). Moreover, nowadays, LAB is playing an important role in different industries for the synthesis of chemicals, pharmaceuticals, or other useful products. Also, the biotechnological production of lactic acid has recently reported that offers a solution to the environmental pollution. In figure 4 are schematically reported the different "attitude" of LAB and their use like functional ingredients for many different uses.

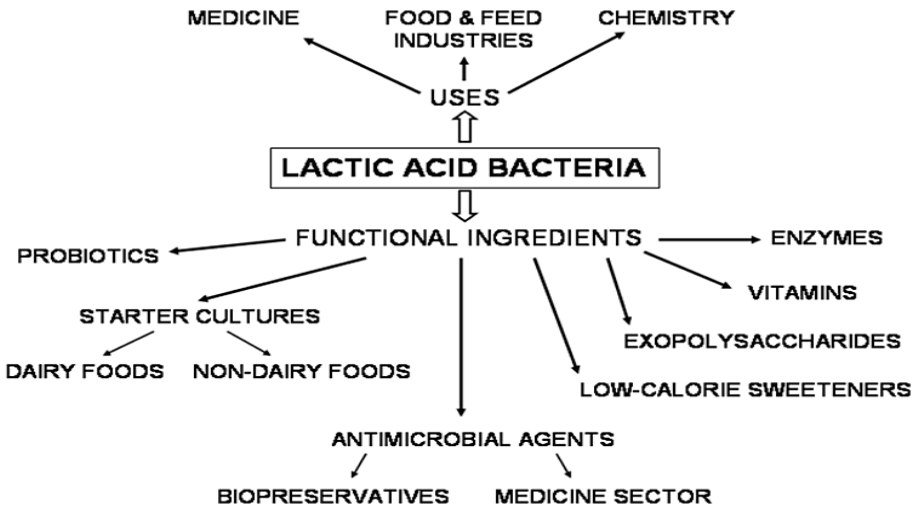


Figure 4: Schematically representation of different attitude of LAB like functional ingredients in the industries (Florou-Paneri et al., 2013)

The increasing demand of consumer's for natural products and functional food, in relation with human health, have determine an recent increasing use of LAB as source of probiotics, starter cultures, antimicrobial agents, vitamins, and others products.

Probably the use like probiotic source represents the more interesting function of LAB for the industries.

Since probiotics can colonize the gastrointestinal tract and exert their beneficial effect long term, without requiring continuous medical intervention, probiotics are considered as an alternative solution to antibiotics due to the increasing spread of antibiotic resistance and the need for treatment cost reduction (Soomro et al., 2002). Microorganisms considered as commercial probiotics are mainly of the Lactobacillus genus with over one hundred species recognized, for example*: L. acidophilus*, *L. rhamnosus*, *L. reuteri*, *L. casei*, *L. plantarum*, *L. bulgaricus*, *L. delbrueckii*, *L.helveticus* and Lactobacilli are Generally Recognized As Safe (GRAS) organisms (O'Sullivan, Ross, & Hill, 2002; Käferstein, 2003; Samelis, Kakouri, Rogga, Savvaidis, & Kontominas, 2003; Cui et al., 2012; Sangmanee & Hongpattarakere, 2014). The main mechanisms of action of probiotic bacteria by which they improve mucosal defenses of the gastrointestinal tract include 1) *antimicrobial activity*, 2) *enhancement of mucosal barrier function against ingested pathogens* and 3) *immunomodulation* (specific strains of probiotics might influence the innate and the acquired immune system, thus playing an important role in human diseases).

Another important role of LAB is representing by their use like starter food cultures. In fact, LAB for a long time have been applied as starter cultures in fermented foods and beverages, because they can improve nutritional, organoleptic, technological and shelf-life characteristics (W. Holzapfel, 1997; Caplice & Fitzgerald, 1999; Soomro et al., 2002; Valyasevi & Rolle, 2002; Bourdichon et al., 2012; Lefeber, Papalexandratou, Gobert, Camu, & De Vuyst, 2012; Florou-Paneri

et al., 2013). LAB initiates a rapid and adequate acidification of the raw materials, through the production of various organic acids from carbohydrates. Lactic acid is the most abundant, followed by acetic acid, whilst LAB can also produce ethanol, bacteriocins, aroma compounds, exopolysaccharides and some enzymes. Earlier the production of fermented foods and beverages was obtained on a spontaneous fermentation, but nowadays the direct addition of selected LAB starter cultures to the food matrix was preferred by the food industry, thanks to the high degree of control over the fermentation process and the standardization of the final product (Muhialdin & Hassan, 2011; Florou-Paneri et al., 2013). Traditionally, LAB has been used in the fermentation of dairy products, as a simple and safe way of preserving such foods. The main species of LAB that can potentially be used as starter cultures in dairy products belong to the *Lactobacillus* spp. (*L. acidophilus*, *L. lactis*, *L. casei*, *L. plantarum*, *L. rhamnosus*, *L. reuteri*, *L. delbrueckii* subsp. *bulgaricus*) or to the *Enterococcus* spp. (*E. faecalis*, *E. faecium*). The dairy products represent also as ideal vehicle for the probiotic delivering and yogurt or fermented milk represent the most important dairy product produced using through the use of LAB starter cultures, representing a functional interaction between probiotic and starter cultures (Delavenne et al., 2013; Li et al., 2013; Lollo et al., 2013). But LAB find an important role also during the not dairy product production, like in some fermented meat and fish product (salami, sausages and smoked salmon for example), during some fermented vegetables preparation (fermented vegetable juice of tomatoes, cabbage, red beet, carrot and celery), and also like starter cultures in silages (it is based on natural fermentation, where LAB ferment water-soluble carbohydrates into organic acids, mainly lactic acid or acetic and formic acids, under anaerobic conditions. Inoculation of LAB is often used as silage additive to enhance lactic acid fermentation and among the LAB genera frequently used are *Lactobacillus plantarum*, *Enterococcus faecium*, *Pediococcus acidilactici*, *Pediococcus pentoseceus* and *Lactobacillus acidophilus*).

In the last years the food industries are focusing their attention on the use of some LAB as producers of antimicrobial compounds, representing the natural way for the food preservation without use of synthetic chemical product. Bacteriocins represent the most interesting antimicrobial compound and will be largely discussed below. Finally the LAB can represent a source of vitamins, in the following thesis it will be discussed the folate production by the use of selected LAB during the milk fermentation. But folate did not represent the single vitamin produced by LAB, vitamin B12 or cobalamin, vitamin K2, riboflavin and thiamine are also produced by Lactobacilli (Caplice & Fitzgerald, 1999; LeBlanc et al., 2007; Santos, Wegkamp, de Vos, Smid, & Hugenholtz, 2008; Rossi et al., 2011; Laiño, Juarez del Valle, Savoy de Giori, & LeBlanc, 2013; Massa, 2014). For conclude, Lactic Acid Bacteria are a very promising sources for novel products and applications, especially those that can satisfy the increasing consumer's demands for natural products and functional foods. They can be used in the diet of humans and animals, with particular role in their health status.

5. FOLIC ACID PRODUCTION BY LACTIC ACID BACTERIA:

Lactic Acid Bacteria (LAB) with ability to produce folate belong to the *Lactobacillus* spp. (*L. lactis, L. plantarum, L. bulgaricus*), *Streptococcus* spp. and *Enterococcus* spp. Nevertheless, some lactobacilli strains (*L. gasseri, L. salivarius, L. acidophilus* and *L. johnsonii*). They can be used as both starter cultures and probiotics. It has been reported that some starter culture and probiotic lactobacillus in non-dairy foods utilize more folate than the amount they produce. For this reasons the food industry is focusing the on the strategy to select and use folate producing probiotic strains, to accomplish fermented products with elevated amounts of "natural" folate concentrations, without increasing production cost, although increasing health benefits (Florou-Paneri et al., 2013)

The potential application of the LAB to produce folate has been intensively investigated. Strains of gastrointestinal microbiota and strains of fermented food could be used as microbial starter for folate food fortification. Recently, the genome sequence of an increasing number of strains of *Lactobacillus* and LAB has provided a major contribution to the knowledge of folate biosynthesis by these bacteria (de Crécy-Lagard, El Yacoubi, de la Garza, Noiriel, & Hanson, 2007).

A key role in the folate metabolic production by LAB is played by the pABA synthesis. In fact, the enzymes which are necessary for the conversion into pABA are lacking. Thus, it is expected that lactobacilli are generally unable to produce folate in the absence of pABA. Diversely, pABA supplementation should be unnecessary in the phylogenetically related genera *Lactococcus* and *Streptococcus*, since all the sequenced lactococci and streptococci, with rare exceptions, possess all the genes for both shikimate pathway and chorismate conversion into pABA (Journal & Biolowxl, 1974).

Therefore, *L. plantarum*, *L. sakei*, *L. delbrueckii*, *L. reuteri*, *L. helveticus*, and *L. fermentum* are expected to produce DHPPP and may be considered as potential folate producers if they are cultured in the presence of pABA (Republic, 2002).

More recently, dozens of strains of LAB have been screened for folate production. Unlike the strains of *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, and *Streptococcus thermophilus*, the strains of *Lactobacillus* are generally unable to produce folate with the exception of *L. plantarum* (Sybesma, Starrenburg, Tijsseling, Hoefnagel, & Hugenholtz, 2003).

The strains of *Lactococcus lactis* and *Streptococcus thermophilus* were demonstrated to produce folate, to accumulate the vitamin within the cells and to excrete it into the medium. The extent of vitamin production, the partitioning between accumulation and excretion, and the form in which the vitamin is occurred (e.g., the number of glutamate residues, and the association to formyl or methenyl groups) mostly depended on the strain and, in some cases, were influenced by

culture conditions, such as the pH, the growth rate and the presence of pABA. On the contrary, the strains of *Lactobacillus* consumed folate with the exception of *L. plantarum*. With hindsight, these observations are in agreement with the presence or the lack of the genes for folate biosynthesis, as predicted from the sequenced genome. Several attempts were carried out to exploit strains of *Lactobacillus* for folate fortification of fermented dairy products, but the use of lactobacilli alone is likely to deplete the folate levels of the fermented product. Nonetheless, folate production and utilization is additive in mixed cultures of *St. thermophilus* and Lactobacilli. Thus, increased folate levels in yoghurt and fermented milk are possible through judicious selection of inoculum species, even though the folate levels remain relatively low in terms of recommended daily intake (Crittenden, Martinez, & Playne, 2003).

For these reasons, combining genome-based metabolic models with growth experiments on minimal media is fundamental to unravel the authentic metabolic capabilities and nutritional requirements of bacteria and to reveal inconsistencies between the predictions and their actual behavior (Rossi et al., 2011)

6. LACTIC ACID BACTERIA AS ADJUST CULTURES FOR SAFE FOOD

There is an increasing trend for food containing probiotic cultures with protective ability. Consumers may be more concerned about safety in food than in any other products, including medicines. As we entered the new millennium, people have become aware that, in order to spend a healthy life, diet style plays a role in preventing diseases and in promoting health (Castellano, Belfiore, Fadda, & Vignolo, 2008).

Fermentation of various food stuffs by lactic acid bacteria (LAB) is one of the oldest forms of biopreservation practiced by mankind. Bacterial antagonism has been recognized for over a century but in recent years scientific community has paid more attention to this phenomenon , particularly to the use of various strains

of lactic acid bacteria (Castellano et al., 2008). One important attribute of many LABs is their ability to produce antimicrobial compounds called bacteriocins. In recent years , the interest in these compounds has grown substantially due to their potential usefulness as natural substitute for chemical food preservatives in the production of foods with enhanced shelf life and/or safety (Messens & De Vuyst, 2002). There is a growing consumer awareness of the link between diet and health. Recent scientific evidence supports the role of probiotic LAB in mediating many positive health effects. Traditional probiotic dairy strains of lactic acid bacteria have a long history of safe use and most strains are considered commensal microorganisms with no pathogenic potential (Soomro et al., 2002).

- ***BACTERIOCINS PRODUCTION BY LACTIC ACID BACTERIA***

During the last few years, a large number of new bacteriocins produced by lactic acid bacteria (LAB) have been identified and characterized. The bacteriocins produced by Gram-positive bacteria like LAB are small peptides, 3-6 kDa, in size, although there are some exceptions (Aly, T, N, & Alfred, 2006).

LAB-bacteriocins comprise a heterogeneous group of physicochemical diverse ribosomal-synthesized peptides or proteins showing a narrow or broad antimicrobial activity spectrum. Bacteriocins are classified into separate groups such as the lantibiotics (Class I); the small (<10 kDa) heat-stable posttranslational unmodified non-lantibiotics (Class II), further subdivided in the pediocin-like and anti-*Listeria* bacteriocins (subclass IIa), the two-peptide bacteriocins (subclass IIb), and the *sec*-dependent bacteriocins (subclass IIc); and the large (>30 kDa) heat-labile non-lantibiotics (Class III). Most bacteriocins characterized to date belong to Class II and are synthesized as precursor peptides (pre-pro-bacteriocins) containing an N-terminal double-glycine leader peptide, which is cleaved off concomitantly with externalization of biologically active bacteriocins by a dedicated ABC-transporter and its accessory protein (Cintas et al., 2001). Most bacteriocins act on

sensitive cells by destabilization and permeabilization of the cytoplasmic membrane through the formation of transitory portion complexes or ionic channels that cause the reduction or dissipation of the proton motive force (PMF). Bacteriocin producing LAB strains protect themselves against the toxicity of their own bacteriocins by the expression of a specific immunity protein which is generally encoded in the bacteriocin operon.

Group I: Modified bacteriocins (the lantibiotics)		Group II: Unmodified bacteriocins	
Type A	Type B	One peptide bacteriocins	Two peptide bacteriocins
Nisin	NK	Pediocin-like bacteriocins	Lactococcin G
Lactocin S		Pediocin PA1, Leucocin A,	Lactacin F
Lacticin 481		Sakacin P, Curvacin A,	Plantaricin E/F
Carnocin UI 49		Mesentericin Y105,	Plantaricin J/K
Cytolysin		Carnobacteriocin BM1, Carnobacteriocin B2, Enterocin A, Piscicolin 126, Bavaricin MN, Piscicocin V1a	Lactobin A Plantaricin S Pediocin L50 Thermophilin 13
		Nonpediocin- like bacteriocins: Lactococcin A and B, Crispacin A, Divergicin 750, Lactococcin 972, AS-48 , Enterocin B, Carnobacteriocin A	

Figure 5: Antimicrobial peptides (peptide-bacteriocins) produced by lactic acid bacteria (Nissen-Meyer, Oppegård, Rogne, Haugen, & Kristiansen, 2010).

Bacteriocin production in LAB is frequently regulated by a three-component signal transduction system consisting of an induction factor (IF), and histidine protein kinase (HPK) and a response regulator (RR) (Schillinger, Geisen, & Holzapfel, 1996; Cintas et al., 2001; Aly et al., 2006; Trias, 2008).

The pathogens and fungi present in the food can have not only a consequence for the human health, through foodborne disease, but can be also an economic loss occurring due to the action of some microorganism causing post-production rot (Gourama, 1997).

The control of the food spoilage may contribute to limit the growth and colonization of pathogens microorganisms. The use of the LAB like bio-control of the food spoilage is a likely prevention method and shows some important advantages: that can be used in place of the products chemically synthesized and does not affect human health (Trias, 2008), but the mechanism of action of different antimicrobial compounds by LAB are not completely understood yet.

- ***CHARACTERISTICS AND BENEFITS OF BACTERIOCINS PRODUCED BY LACTIC ACID BACTERIA***

The bacteriocins produced by LAB have peculiar traits which make them suitable to use as food bio-preservative, like: 1) Their protein nature (they will inactivated by proteases enzymes in the gastrointestinal tract, making them safe for the human health); 2) Nontoxic and generally non immunogenic; 3) Thermos resistant (generally the antimicrobial activity remain after heat treatment like pasteurization and sterilization); 4) Are affecting most of the Gram-positive bacteria (nowadays study are investigating to their activity against Gram-negative bacteria to); 5) Genetic determinants generally located in plasmid facilitating genetic manipulation to increase the variety of natural peptides; 6) Usually act on the bacterial cytoplasmic membrane having no cross resistance with antibiotics (Schillinger, et al. 1996; Kuipers, De Ruyter, Kleerebezem, & De Vos, 1998; Cintas et al., 2001; McAuliffe, 2001; Vaughan, Eijsink, O'Sullivan, O'Hanlon, & Van Sinderen, 2001; Eijsink et al., 2002; O'Sullivan et al., 2002; Aly et al., 2006; Cui et al., 2012; Yang, Fan, Jiang, Doucette, & Fillmore, 2012; Florou-Paneri et al., 2013; C.L. Gerez, Torres, Font de Valdez, & Rollán, 2013).

Some benefits deriving by using LAB bacteriocins as food bio preservatives are: 1) Capacity to extend the food shelf life; 2) The risk of transmission of food born pathogenic bacteria will be reduced; 3) Amelioration of economic losses due to food spoilage; 4) The use of LAB bacteriocin determine the no addition of

synthetic food preservatives; 5) Decrease of the intensity of heat treatments resulting in better preservation of food nutrients and sensory properties of the food; 6) Possibility to the production of new "novel" food with higher water content (Dobson, Cotter, Ross, & Hill, 2012).

There are at least three ways in which bacteriocins can be incorporated into a food to ameliorate its safety: 1) By using a purified or semi-purified bacteriocin preparation as food ingredient; 2) By introducing an ingredient that has earlier been fermented with a bacteriocin producing strain; 3) By using a bacteriocin-producing culture in fermented products to produce the bacteriocin in situ (Florou-Paneri et al., 2013). Also, bacteriocin production can contribute to the probiotic functionality of intestinal LAB, while in certain cases may be directly responsible for it, with respect to either beneficially modulating the gut microbiota or inhibiting some gastrointestinal pathogenic bacteria. Finally bacteriocins derived by LAB, can cover a broad field of applications (in the food industry and in medical sector) mainly in combination with other treatments to increase their effectiveness (Soomro et al., 2002; Aly et al., 2006; Dobson et al., 2012).

7. USE OF LAB TO INHIBIT MOULD FOOD SPOILAGE

Filamentous fungi and yeasts are common spoilage organisms of food products (Cappa & Cocconcelli, 2001; Al-Kadamany, Khattar, Haddad, & Toufeili, 2003; Shephard, 2008; Lee et al., 2014). Fungi in food represent a potential danger for public health and may cause major economic losses. Spoilage of food by these microorganisms has been a global issue and has entailed a great loss in the economy for stored, prepared food and feeding systems. This fact is more relevant in the tropical countries because of the high temperature and moisture which are suitable conditions for the fungal grow. It is the major cause decreasing the shelf life of many foods (Muhialdin & Hassan, 2011). It is estimated that between 5 and 10% of the world's food production is lost due to fungal deterioration (Filtenborg,

Frisvad, & Thrane, 1996; Schnürer & Magnusson, 2005). They are common spoilage organisms of food products, like milk, cheese, fermented meat, bread, and stored crop, as documented by Filtenborg et al. (1996). *Penicillium* and *Aspergillus* species are spoilage organisms from a wide range of different food and *Fusarium* are often founded in cereals products. Different *Penicillium* species frequently occur in foods stored under cold conditions, and *P. roqueforti* and *P. commune* commonly spoil in milk products (Filtenborg et al., 1996; Carla Luciana Gerez, Torino, Rollán, & Font de Valdez, 2009). In addition, fungi may be responsible for off flavours and synthesis of mycotoxins and allergenic compounds. Therefore more than 400 mycotoxins are well known produced from different fungi (Filtenborg et al., 1996). Mycotoxinogenic fungi such as *Aspergillus, Fusarium* and *Penicillium* are serious hazards for the human health. Six classes of mycotoxins frequently encountered in different food systems are: aflatoxins, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone (Blagojev, Škrinjar, Vesković-Moračanin, & Šošo, 2012).

In order to control the mould growth in food, it would be suggested to adopt several techniques. Drying, freeze-drying, cold storage, modified atmosphere storage, and heat treatments are all means of physical methods of food preservation. But also chemical products have preservative functions, thus organic acid acetic, lactic, benzoic, propionic and sorbic acid are used for food preservation (Brul & Coote, 1999). Finally there are different types of antibiotics that have antifungal activity, like the natamicin, used for prevention of the fungi growth in cheese (Schnürer & Magnusson, 2005). But the use of chemical products, especially antibiotics, determines an increasing number of species become resistant to antibiotics, and fungi are not excepted (C.L. Gerez et al., 2013). There is a great risk that the resistance phenomenon will increase in the future because of the frequent use of antibiotics or chemical preservatives. But the general public consumers demand a reduced use of the chemical products in food, however

requiring a high quality, preservative free and a long shelf life. For such a reason, the use of LAB selected strains with inhibition ability, seems to be a promising alternative to chemical preservatives (Schnürer & Magnusson, 2005). LAB has a long history of use of bio-preservatives for food and feed storage; now it is known that they are able to produce different antimicrobial compounds that are able to control spoilage fungi. Their preserving effects mainly relate to the production of organic acid such as lactic acid and acetic acid, hydrogen peroxide, competition for nutrients, production of bacteriocins and proteins or proteinaceous compounds (Cintas et al., 2001; O'Sullivan, Ross, & Hill, 2002; Schnürer & Magnusson, 2005; Muhialdin & Hassan, 2011). The precise mechanism of antifungal activity is difficult to be elucidated , and this is due to the complex and commonly synergistic interaction between different compounds (Niku-Paavola, Laitila, Mattila-Sandholm, & Haikara, 1999; Castellano et al., 2008;Tiwari et al., 2009; Crowley, Mahony, & van Sinderen, 2013; Cortés-Zavaleta, López-Malo, Hernández-Mendoza, & García, 2014). The fungal inhibitory metabolites produced by LAB are different. In the figure (Fig. 5) below are showed a summary of the antifungal compounds produced by LAB. The compounds produced by LAB are: (1) *Organic acids*: they are the main products of the LAB during the fermentation. They are lactic acid and acetic acid and other acids depending on the strain of LAB, and the mechanism of action is described in literature (Piard & Desmazeaud, 1991). The acid can diffuse through the membrane of the organism target and then reduce the pH and lock the metabolic activity. These types of compound, generally linked with the bacteriocin activity, are able to inhibit the growth of some food pathogens and show ability against some filamentous fungi too. Corsetti, Gobbetti, & Smacchi, (1996), describe the activity of these compounds against *Fusarium, Penicillium, Aspergillus* and *Monilia*. (2) *Bacteriocins:* we have just described previously the different types of bacteriocin involved in the inhibition ability against mould. Bacteriocins are proteinaceous antibacterial compounds, which

constitute a heterologous subgroup of ribosomal synthesized antimicrobial peptides. In general these substances are cationic peptides that display hydrophobic or amphiphilic properties and the bacterial membrane is in most cases the target for their activity. Depending on the producer organism and classification criteria, bacteriocins can be classified into several groups (Aly et al., 2006).

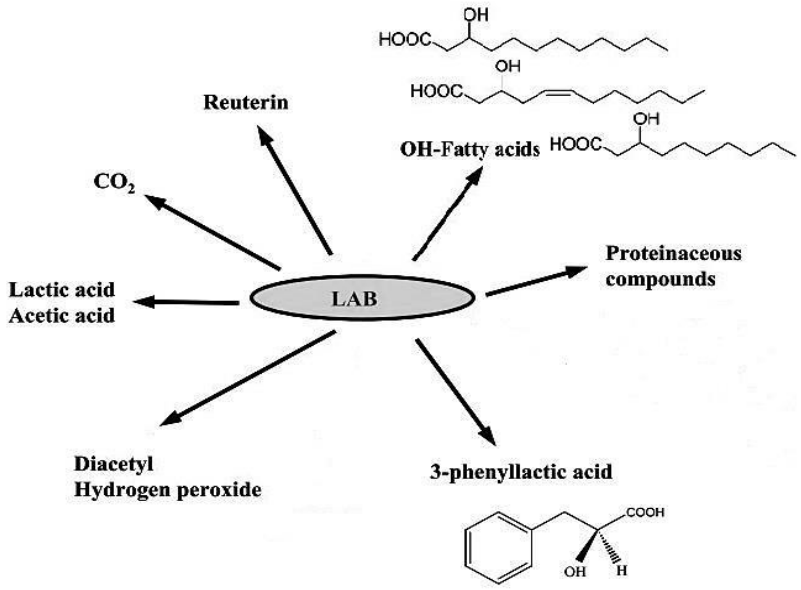


Figure 6: Summary of current knowledge of the complex antifungal activities of lactic acid bacteria.

(3) *Carbone dioxide:* Carbon dioxide CO2 is one of products produced by hetero-fermenters LAB. The activity of CO2 is due to two factors firstly, it creates anaerobic condition and replaces the existent molecular oxygen in the products and secondly, CO2 has antimicrobial activity and this activity is important in the vegetable fermentation to prevent the growth of spoilage fungi (Naidu, Bidlack, & Clemens, 1999). (4) *Hydrogen peroxide:* Hydrogen peroxide (H2O2) is produced by most of the LAB when oxygen is available. Hydrogen peroxide is well studied and the modes of action are well known. It is related to the strong oxidizing effect on the bacterial cell, and to the destruction of basic molecular structures of cellular proteins (Schnürer & Magnusson, 2005). (5) *Reuterin: It* is a product of some

strains of lactic acid bacteria (LAB) from the fermentation of the glycerol, and is produced by *L. reuteri.* *It* Is active against many kinds of microorganisms including Gram-positive, Gram-negative bacteria, yeasts and fungi (Chung & Axelsson, 1989). (6) *Phenyl lactic acid: it* is a special organic acid produced by certain LAB and shows antifungal activity. Many authors, Gerez et al. (2013), Crowley et al. (2013), Schnürer & Magnusson (2005), and others, report that the phenyl lactic acid isolated from LAB had a inhibition ability against pathogens bacteria and mould spoilage.

8. USE OF LAB TO INHIBIT THE PATHOGEN FOOD CONTAMINATION

Foodborne diseases are a consequence of food pathogens that affect hundreds of thousands of people every year. Moreover the several use of antibiotics led to the development of resistance strains (World Health Organization, 2014).

The use of LAB can be a solution as like as against food spoilage fungi. The antimicrobial effects against food pathogens are more or less the same as the ones looked for the food mould spoilage: organic acids, hydrogen peroxide, carbon dioxide, diacetyl, broad-spec- Lactic acid bacteria lack true catalase to break trump antimicrobials such as reuterin and the pro-down the hydrogen peroxide generated in the production of bacteriocins (Bourdichon et al., 2012).

The production of one or more antimicrobial active metabolites is part of the complex mechanism by which a culture becomes established in the presence of other competing organisms. Understanding these mechanisms provides a valuable key towards 'biological' approaches in food preservation (W. H. Holzapfel, Geisen, & Schillinger, 1995).

The compounds produced by LAB that inhibit the pathogens growth, are the same as for the fungi inhibition, and they are: (1) *Organic acids:* Lactic acid, the characteristic fermentation product of LAB may reduce pH to a level where

putrefactive (e.g. *clostridia* and *pseudomonas*), pathogenic (e.g. *Salmonellae* and *Listeria* spp.) and toxicogenic bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum*) will either be inhibited or destroyed (W. H. Holzapfel et al., 1995). (2). Hydrogen peroxide is produced by a number of LAB in the presence of molecular oxygen together with lactate, pyruvate and NADH by flavin enzymes (Kandler, 1983). Undesired *Hydrogen peroxide* bacteria such as *Pseudomonas* spp. As for other metabolic products, the tolerable amount of H2O2 is dependent on the product type and situation, and may be detrimental to sensory quality of e.g. processed meats (Nakajima et al., 2003; Jones, Hussein, Zagorec, Brightwell, & Tagg, 2008)

(3) *Enzymes:* Relative to other metabolites, enzymes produced by food-grade bacteria and especially LAB, are of little direct consequence to food preservation. Lipolytic activity, important in ripening of cheese, may, however, affect the production of fatty acids with antimicrobial properties (Bourdichon et al., 2012). (4) *Reuterin:* or 3-hydroqpropionaldehyde, is produced from glycerol, dependent of coenzyme B, by *Lactobacillus reuteri*. It shows broad-spectrum antimicrobial activity, probably by inhibition of ribonucleotide reductase, and has been suggested for biopreservation of fish and meat, using *L. reuteri* (Arqués, Rodríguez, Nuñez, & Medina, 2011). (5) *Bacteriocin:* bacteriocin production by LAB is the most interested compound produced by this microorganism, not only for ability to inhibit the filamentous fungi development, but also for the useful antimicrobial bio preservation against food pathogens. A recent definition of bacteriocin produced by LAB, suggested that they should be regarded as "extracellular released primary or modified product of bacterial ribosomal synthesis, which have a relatively narrow spectrum of bactericidal activity" (Caplice & Fitzgerald, 1999). They should include at least some strains of the same species as the producer of the bacteriocin and against which the producer has some activity of self-protection (Jack, Tagg, & Ev, 1995). The action target of bacteriocins is the cytoplasmic membrane and they

have more effect against Gram-positive bacteria (Venema, Venema, & Kok, 1995; Jack et al., 1995; Caplice & Fitzgerald, 1999; O'Sullivan et al., 2002). In the context of the food fermentation, the most important target includes spoilers like *Clostridium* and heterofermative lactobacilli and foodborne pathogens like *Listeria monocytogenes, Staphylococcus* spp., *Clostridium, Bacillus* spp. and *Enterococcus* (Aly et al., 2006). The most studied bacteriocin used to prevent the growth of pathogens bacteria is the *Nisin,* produced by *L. lactis* subsp. *lactis.* It can prevent the development of pathogens like *Bacillus* and *Clostridium* (Okereke & Montville, 1991).

Harris, Fleming, & Klaenhammer (1992), Abee, Rombouts, Hugenholtz, Guihard, & Letellier (1994), Benech, Kheadr, Laridi, Lacroix, & Fliss (2002), de Carvalho, Mantovani, & Vanetti (2007) and other authors demonstrated the activity of the Nisin and bacteriocin class I against *L monocytogenes*, *C. tyrobutyricum* and *Staphylococcus aureus.*

Class II bacteriocin represent a group composed by pediocin-like bacteriocin with anti-listeria activity and many studies report the inhibition of *L. monocytogenes*, but also of *B. cereus*, in fermented meat and milk product. Various studies are testing the ability of this class of bacteriocins against a huge number of pathogen strains, including *L. innocua*, *Clostridium* spp. and *Staphylococcus* spp. (Diep, Håvarstein, & Nes, 1996; Vincent G H Eijsink et al., 1998; Ferchichi et al., 2001; Cintas et al., 2001; V. G H Eijsink et al., 2002; Cotter, Ross, & Hill, 2005; Aly et al., 2006; Deegan, Cotter, Hill, & Ross, 2006; Drider, Fimland, Hechard, McMullen, & Prevost, 2006).

9. AIMS OF THE WORK

This thesis is focused on the study of selected LAB with nutritional and inhibition ability, able to improve quality and safety in fermented food, in particular as regards the dairy food.

The thesis specifically

- Analyzed the production of folic acid by *St. thermophilus* in fermented milk, as a bio fortifying system of folate.
- Investigated the ability of selected LAB to prevent fungal food spoilage and food pathogenic bacteria
- Proteomic analysis of selected LAB genome for the characterization of bacteriocins production.

BIBLIOGRAPHY

. S. a. H., . T. M., & . K. A. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

a.M., M., P.N., K., L.C., B., J.M., S., & J.L., M. (2008). Effects of folate and vitamin B12 deficiencies during pregnancy on fetal, infant, and child development. *Food and Nutrition Bulletin*, *29*(2 SUPPL.), S101–S111. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed8&NEWS=N&AN=18709885

Abee, T., Rombouts, F. M., Hugenholtz, J., Guihard, G., & Letellier, L. (1994). Mode of action of nisin Z against Listeria monocytogenes scott a grown at high and low temperatures. *Applied and Environmental Microbiology*, *60*(6), 1962–1968.

Adams, M. R., & Nicolaides, L. (1997). Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control*, *8*(5-6), 227–239. doi:10.1016/S0956-7135(97)00016-9

Al-Kadamany, E., Khattar, M., Haddad, T., & Toufeili, I. (2003). Estimation of shelf-life of concentrated yogurt by monitoring selected microbiological and physicochemical changes during storage. *LWT - Food Science and Technology*, *36*(4), 407–414. doi:10.1016/S0023-6438(03)00018-5

Aly, S., T, O. C. a, N, B. I. H., & Alfred, T. S. (2006). Bacteriocins and lactic acid bacteria - a minireview. *African Journal of Biotechnology*, *5*(May), 678–683. doi:10.5897/AJB05.388

Arimi, S. M., Koroti, E., Kang'ethe, E. K., Omore, A. O., & McDermott, J. J. (2005). Risk of infection with Brucella abortus and Escherichia coli O157:H7 associated with marketing of unpasteurized milk in Kenya. *Acta Tropica*, *96*(1), 1–8. doi:10.1016/j.actatropica.2005.05.012

Arqués, J. L., Rodríguez, E., Nuñez, M., & Medina, M. (2011). Combined effect of reuterin and lactic acid bacteria bacteriocins on the inactivation of food-borne pathogens in milk. *Food Control*, *22*(3-4), 457–461. doi:10.1016/j.foodcont.2010.09.027

Bankole, S. a, & Adebanjo, a. (2003). Mycotoxins in food in West Africa : current situation and possibilities of controlling it. *Journal of Biotechnology*, *2*(September), 254–263. doi:10.4314/ajb.v2i9.14833

Benech, R. O., Kheadr, E. E., Laridi, R., Lacroix, C., & Fliss, I. (2002). Inhibition of Listeria innocua in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Applied and Environmental Microbiology*, *68*(8), 3683–90. doi:10.1128/AEM.68.8.3683

Black, R. (2003). Editorial Micronutrient deficiency — an underlying cause of morbidity and mortality, *81*(03808), 3808.

Blagojev, N., Škrinjar, M., Vesković-Moračanin, S., & Šošo, V. (2012). Control of mould growth and mycotoxin production by lactic acid bacteria metabolites. *Romanian Biotechnological Letters*, *17*(3), 7219–7226.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *International Journal of Food Microbiology*, *154*(3), 87–97. doi:10.1016/j.ijfoodmicro.2011.12.030

Branca, F., & Ferrari, M. (2002). Impact of micronutrient deficiencies on growth: The stunting syndrome. *Annals of Nutrition and Metabolism*, *46*(SUPPL. 1), 8–17. doi:10.1159/000066397

Brul, S., & Coote, P. (1999). Preservative agents in foods: Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, *50*(1-2), 1–17. doi:10.1016/S0168-1605(99)00072-0

Caplice, E., & Fitzgerald, G. F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, *50*(1-2), 131–149. doi:10.1016/S0168-1605(99)00082-3

Cappa, F., & Cocconcelli, P. S. (2001). Identification of fungi from dairy products by means of 18S rRNA analysis. *International Journal of Food Microbiology*, *69*(1-2), 157–160. doi:10.1016/S0168-1605(01)00587-6

Castellano, P., Belfiore, C., Fadda, S., & Vignolo, G. (2008). A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Science*, *79*(3), 483–499. doi:10.1016/j.meatsci.2007.10.009

Cavera, V. L., Arthur, T. D., Kashtanov, D., & Chikindas, M. L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *International Journal of Antimicrobial Agents*, 7–14. doi:10.1016/j.ijantimicag.2015.07.011

Chung, T., & Axelsson, L. (1989). In vitro studies on reuterin synthesis by Lactobacillus reuteri. *Microbial Ecology in …*, *2*, 137–144. doi:10.3109/08910608909140211

Cintas, L. M., Herranz, C., HernÁndez, P. E., Casaus, M. P., Nes, I. F., & HernÁndez, P. E. (2001). Review: Bacteriocins of Lactic Acid Bacteria. *Food Science & Technology International*, *7*(4), 281–305. doi:10.1106/R8DE-P6HU-CLXP-5RYT

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*(1), 1–20. doi:10.1016/S0168-1605(01)00560-8

Conference, I., & Food, U. N. (2014). Food safety must accompany food and nutrition security-WHO Proceedings. *Nutrition and Food Safety*, *6736*(14), 1–4. doi:10.1016/S0140-6736(14)62037-7

Corsetti, a., Gobbetti, M., & Smacchi, E. (1996). Antibacterial activity of sourdough lactic acid bacteria: isolation of a bacteriocin-like inhibitory substance fromLactobacillus sanfranciscoC57. *Food Microbiology*, *13*(6), 447–456. doi:10.1006/fmic.1996.0051

Cortés-Zavaleta, O., López-Malo, a, Hernández-Mendoza, a, & García, H. S. (2014). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *International Journal of Food Microbiology*, *173*, 30–5. doi:10.1016/j.ijfoodmicro.2013.12.016

Crider, K. S., Bailey, L. B., & Berry, R. J. (2011). Folic Acid Food Fortification—Its History,

Effect, Concerns, and Future Directions. *Nutrients*, *3*(3), 370–384. doi:10.3390/nu3030370

Crittenden, R. G., Martinez, N. R., & Playne, M. J. (2003). Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. *International Journal of Food Microbiology*, *80*(3), 217–222. doi:10.1016/S0168-1605(02)00170-8

Crowley, S., Mahony, J., & van Sinderen, D. (2013). Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends in Food Science & Technology*, *33*(2), 93–109. doi:10.1016/j.tifs.2013.07.004

Cui, Y., Zhang, C., Wang, Y., Shi, J., Zhang, L., Ding, Z., Cui, H. (2012). Class IIa bacteriocins: Diversity and new developments. *International Journal of Molecular Sciences*, *13*(12), 16668–16707. doi:10.3390/ijms131216668

de Carvalho, a. a. T., Mantovani, H. C., & Vanetti, M. C. D. (2007). Bactericidal effect of bovicin HC5 and nisin against Clostridium tyrobutyricum isolated from spoiled mango pulp. *Letters in Applied Microbiology*, *45*(1), 68–74. doi:10.1111/j.1472-765X.2007.02150.x

de Crécy-Lagard, V., El Yacoubi, B., de la Garza, R. D., Noiriel, A., & Hanson, A. D. (2007). Comparative genomics of bacterial and plant folate synthesis and salvage: predictions and validations. *BMC Genomics*, *8*(Figure 2), 245. doi:10.1186/1471-2164-8-245

Delavenne, E., Ismail, R., Pawtowski, A., Mounier, J., Barbier, G., & Le Blay, G. (2013). Assessment of lactobacilli strains as yogurt bioprotective cultures. *Food Control*, *30*(1), 206–213. doi:10.1016/j.foodcont.2012.06.043

Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin Production: a Probiotic Trait? *Applied and Environmental Microbiology*, *78*(1), 1–6. doi:10.1128/AEM.05576-11

Eijsink, V. G. H., Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H., & Nes, I. F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *81*(1-4), 639–654. doi:10.1023/A:1020582211262

Filtenborg, O., Frisvad, J. C., & Thrane, U. (1996). Moulds in food spoilage. *International Journal of Food Microbiology*, *33*(1), 85–102. doi:10.1016/0168-1605(96)01153-1

Fitzpatrick, T. B., Basset, G. J. C., Borel, P., Carrari, F., DellaPenna, D., Fraser, P. D., … Fernie, A. R. (2012). Vitamin deficiencies in humans: can plant science help? *The Plant Cell*, *24*(2), 395–414. doi:10.1105/tpc.111.093120

Florou-Paneri, P., Christaki, E., & Bonos, E. (2013). Lactic Acid Bacteria as Source of Functional Ingredients. *Lactic Acid Bacteria-R& D for Food, Health and Livestock Purposes*, 589–614.

Gerbaldo, G. a, Barberis, C., Pascual, L., Dalcero, A., & Barberis, L. (2012). Antifungal activity of two Lactobacillus strains with potential probiotic properties. *FEMS Microbiology Letters*, *332*(1), 27–33. doi:10.1111/j.1574-6968.2012.02570.x

Gerez, C. L., Torino, M. I., Rollán, G., & Font de Valdez, G. (2009). Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control*, *20*(2), 144–148. doi:10.1016/j.foodcont.2008.03.005

Gerez, C. L., Torres, M. J., Font de Valdez, G., & Rollán, G. (2013). Control of spoilage fungi by

lactic acid bacteria. *Biological Control*, *64*(3), 231–237. doi:10.1016/j.biocontrol.2012.10.009

Gourama, H. (1997). Inhibition of Growth and Mycotoxin Production of Penicillium By Lactobacillus Species. *Food Science & Technology-Lebensmittel-Wissenschaft & Technologie*, *30*(3), 279–283.

Gupta, R., & Srivastava, S. (2014). Antifungal effect of antimicrobial peptides (AMPs LR14) derived from Lactobacillus plantarum strain LR/14 and their applications in prevention of grain spoilage. *Food Microbiology*, *42*, 1–7. doi:10.1016/j.fm.2014.02.005

Harris, L. J., Fleming, H. P., & Klaenhammer, T. R. (1992). Novel paired starter culture system for sauerkraut, consisting of a nisin-resistant Leuconostoc mesenteroides strain and a nisin-producing Lactococcus lactis strain. *Applied and Environmental Microbiology*, *58*(5), 1484–1489. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=195629&tool=pmcentrez&rendertype=abstract

History, T. H. E., & Folic, O. F. (2001). Historical Review.

Hoey, L., Mcnulty, H., Duffy, M. E., & Hughes, C. F. (2015). EURRECA — Estimating Folate Requirements for Deriving, *8398*(October), 1040–1050. doi:10.1080/10408398.2012.742858

Holzapfel, W. (1997). Use of starter cultures in fermentation on a household scale. *Food Control*, *8*(5-6), 241–258. doi:10.1016/S0956-7135(97)00017-0

Holzapfel, W. H., Geisen, R., & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology*, *24*(3), 343–362. doi:10.1016/0168-1605(94)00036-6

Iyer, R., Tomar, S. K., Uma Maheswari, T., & Singh, R. (2010). Streptococcus thermophilus strains: Multifunctional lactic acid bacteria. *International Dairy Journal*, *20*(3), 133–141. doi:10.1016/j.idairyj.2009.10.005

Jack, R. W., Tagg, J. R., & Ev, M. I. R. (1995). Bacteriocins of Gram-Positive Bacteria, *59*(2), 171–200.

Jones, R. J., Hussein, H. M., Zagorec, M., Brightwell, G., & Tagg, J. R. (2008). Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. *Food Microbiology*, *25*(2), 228–234. doi:10.1016/j.fm.2007.11.001

Journal, T. H. E., & Biolowxl, O. F. (1974). Folate Coenzymes faecalis*. *Cell*, 6–7.

Käferstein, F. (2003). Foodborne diseases in developing countries: aetiology, epidemiology and strategies for prevention. *International Journal of Environmental Health Research*, *13 Suppl 1*(July 2015), S161–S168. doi:10.1080/0960312031000102949

Kuipers, O. P., De Ruyter, P. G. G. a, Kleerebezem, M., & De Vos, W. M. (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology*, *64*(1), 15–21. doi:10.1016/S0168-1656(98)00100-X

Laiño, J. E., Juarez del Valle, M., Savoy de Giori, G., & LeBlanc, J. G. J. (2013). Development of a high folate concentration yogurt naturally bio-enriched using selected lactic acid bacteria. *LWT - Food Science and Technology*, *54*(1), 1–5. doi:10.1016/j.lwt.2013.05.035

Lan, W., Chen, Y., Wu, H., & Yanagida, F. (2012). Bio-protective potential of lactic acid bacteria isolated from fermented wax gourd. *Folia Microbiologica*, *57*(2), 99–105. doi:10.1007/s12223-012-0101-1

LeBlanc, J., de Giori, G., Smid, E. J., Hugenholtz, J., & Sesma, F. (2007). Folate production by lactic acid bacteria and other food-grade microorganisms. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, *1*, 329–339.

Lee, S. C., Billmyre, R. B., Li, A., Carson, S., Sykes, S. M., Huh, E. Y., … Heitman, J. (2014). Analysis of a Food-Borne Fungal Pathogen Outbreak: Virulence and Genome of a Mucor circinelloides Isolate from Yogurt. *mBio*, *5*(4), e01390–14–. doi:10.1128/mBio.01390-14

Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., & De Vuyst, L. (2012). On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. *Food Microbiology*, *30*(2), 379–92. doi:10.1016/j.fm.2011.12.021

Lewus, C. B., Kaiser, a., & Montville, T. J. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Applied and Environmental Microbiology*, *57*(6), 1683–1688.

Li, H., Liu, L., Zhang, S., Uluko, H., Cui, W., & Lv, J. (2013). Potential use of Lactobacillus casei AST18 as a bioprotective culture in yogurt. *Food Control*, *34*(2), 675–680. doi:10.1016/j.foodcont.2013.06.023

Lollo, P. C. B., de Moura, C. S., Morato, P. N., Cruz, A. G., Castro, W. D. F., Betim, C. B., … Amaya-Farfan, J. (2013). Probiotic yogurt offers higher immune-protection than probiotic whey beverage. *Food Research International*, *54*(1), 118–124. doi:10.1016/j.foodres.2013.06.003

Lukaski, H. C. (2004). Vitamin and mineral status: effects on physical performance. *Nutrition*, *20*(7-8), 632–644. doi:10.1016/j.nut.2004.04.001

Massa, S. (2014). Lactobacillus plantarum as a Strategy for an In situ Production of Vitamin B2. *Journal of Food & Nutritional Disorders*, *s1*(01), 1–5. doi:10.4172/2324-9323.S1-004

McAuliffe, O. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews*, *25*(3), 285–308. doi:10.1016/S0168-6445(00)00065-6

Mcnulty, H., Pentieva, K., Hoey, L., Strain, J. J., & Ward, M. (2012). Nutrition Throughout Life : Folate, *82*(5), 348–354. doi:10.1024/0300-9831/a000130

Messens, W., & De Vuyst, L. (2002). Inhibitory substances produced by Lactobacilli isolated from sourdoughs - A review. *International Journal of Food Microbiology*, *72*(1-2), 31–43. doi:10.1016/S0168-1605(01)00611-0

Motarjemi, Y., Käferstein, F., Moy, G., & Quevedo, F. (1993). Contaminated weaning food: a major risk factor for diarrhoea and associated malnutrition. *Bulletin of the World Health Organization*, *71*(1), 79–92.

Muhialdin, B. J., & Hassan, Z. (2011). Annals . Food Science and Technology BIOPRESERVATION OF FOOD BY LACTIC ACID BACTERIA AGAINST 1 .

INTRODUCTION Lactic acid bacteria (LAB) are Gram-positive microorganisms , prefer anaerobic conditions but are aero-tolerant , catalase negative , cocci or.

Müller, O., & Krawinkel, M. (2005). Malnutrition and health in developing countries, *173*(3), 2000–2003.

Naidu, a. S., Bidlack, W. R., & Clemens, R. a. (1999). Probiotic Spectra of Lactic Acid Bacteria (LAB). *Critical Reviews in Food Science and Nutrition*, *39*(1), 13–126. doi:10.1080/10408699991279187

Nakajima, H., Toba, T., Ito, A., Kudo, S., Sato, S., & Sato, Y. (2003). The Screening of Hydrogen Peroxide-Producing Lactic Acid Bacteria and Their Application to Inactivating Psychrotrophic Food-Borne Pathogens. *Current Microbiology*, *47*(3), 231–236. doi:10.1007/s00284-002-3993-1

Niku-Paavola, M. L., Laitila, a, Mattila-Sandholm, T., & Haikara, a. (1999). New types of antimicrobial compounds produced by Lactobacillus plantarum. *J. Appl. Microbiol.*, *86*(1), 29–35. doi:10.1046/j.1365-2672.1999.00632.x

Nissen-Meyer, J., Oppegård, C., Rogne, P., Haugen, H. S., & Kristiansen, P. E. (2010). Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins. *Probiotics and Antimicrobial Proteins*, *2*(1), 52–60. doi:10.1007/s12602-009-9021-z

O'Sullivan, L., Ross, R. P., & Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie*, *84*(5-6), 593–604. doi:10.1016/S0300-9084(02)01457-8

Okereke, a, & Montville, T. J. (1991). Bacteriocin-mediated inhibition of Clostridium botulinum spores by lactic acid bacteria at refrigeration and abuse temperatures. *Applied and Environmental Microbiology*, *57*(12), 3423–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=183991&tool=pmcentrez&rendertype=abstract

Onilude, a a, Fagade, O. E., Bello, M. M., & Fadahunsi, I. F. (2005). Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels. *Journal of Biotechnology*, *4*(December), 1404–1408.

Piard, J. C., & Desmazeaud, M. (1991). Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Le Lait*, *71*(5), 525–541. doi:10.1051/lait:1991541

Powers, H. J. (2007). Folic acid under scrutiny. *The British Journal of Nutrition*, *98*(4), 665–666. doi:10.1017/S0007114507795326

Reisenaãœer, a N. N. a I., & Halsted, C. H. (1987). Issues and Opinions In Nutrition Human Folate Requirements, *45*(October 1986), 600–602.

Republic, C. (2002). Biosynthesis of Folates by Lactic Acid Bacteria and Propionibacteria in Fermented Milk. *Food Science*, *22*(5), 175–181.

Rossi, M., Amaretti, A., & Raimondi, S. (2011). Folate Production by Probiotic Bacteria. *Nutrients*, *3*(12), 118–134. doi:10.3390/nu3010118

Røssland, E., Andersen, G. I., Langsrud, T., & Sørhaug, T. (2003). Inhibition of Bacillus cereus by strains of Lactobacillus and Lactococcus in milk, *89*, 205–212. doi:10.1016/S0168-1605(03)00149-1

Rouse, S., Harnett, D., Vaughan, a., & Sinderen, D. Van. (2008). Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *Journal of Applied Microbiology*, *104*, 915–923. doi:10.1111/j.1365-2672.2007.03619.x

Samelis, J., Kakouri, a., Rogga, K. J., Savvaidis, I. N., & Kontominas, M. G. (2003). Nisin treatments to control Listeria monocytogenes post-processing contamination on Anthotyros, a traditional Greek whey cheese, stored at 4°C in vacuum packages. *Food Microbiology*, *20*(6), 661–669. doi:10.1016/S0740-0020(03)00018-2

Sangmanee, P., & Hongpattarakere, T. (2014). Inhibitory of multiple antifungal components produced by Lactobacillus plantarum K35 on growth, aflatoxin production and ultrastructure alterations of Aspergillus flavus and Aspergillus parasiticus. *Food Control*, *40*, 224–233. doi:10.1016/j.foodcont.2013.12.005

Santos, F., Wegkamp, a., de Vos, W. M., Smid, E. J., & Hugenholtz, J. (2008). High-Level Folate Production in Fermented Foods by the B12 Producer Lactobacillus reuteri JCM1112. *Applied and Environmental Microbiology*, *74*(10), 3291–3294. doi:10.1128/AEM.02719-07

Schillinger, U., Geisen, R., & Holzapfel, W. H. (1996). Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends in Food Science & Technology*, *7*(5), 158–164. doi:10.1016/0924-2244(96)81256-8

Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology*, *16*(1-3), 70–78. doi:10.1016/j.tifs.2004.02.014

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *25*(2), 146–151. doi:10.1080/02652030701567442

Soomro, a. H., Masud, T., & Anwaar, K. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

Stoyanova, L. G., Ustyugova, E. A., Sultimova, T. D., Bilanenko, E. N., Fedorova, G. B., Khatrukha, G. S., … Pirogovskaya, B. (2010). New Antifungal Bacteriocin-Synthesizing Strains of Lactococcus lactis ssp . lactis as the Perspective Biopreservatives for Protection of Raw Smoked Sausages Department of Microbiology , Faculty of Biological , M . V . Lomonosov Moscow State University Len, *5*(4), 477–485.

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, M. H. N., & Hugenholtz, J. (2003). Effects of Cultivation Conditions on Folate Production by Lactic Acid Bacteria. *Metab. Eng.*, *69*(8), 4542–4548. doi:10.1128/AEM.69.8.4542

Thu, B. D., Schultink, W., Dillon, D., Gross, R., Leswara, N. D., & Khoi, H. H. (1999). Effect of daily and weekly micronutrient supplementation on micronutrient deficiencies and growth in young Vietnamese children. *The American Journal of Clinical Nutrition*, *69*(1), 80–86.

Tiwari, B. K., Valdramidis, V. P., O' Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen,

P. J. (2009). Application of Natural Antimicrobials for Food Preservation. *Journal of Agricultural and Food Chemistry*, *57*(14), 5987–6000. doi:10.1021/jf900668n

Trias, R. (2008). *Lactic acid bacteria as bioprotective agents against foodborne pathogens and spoilage microorganisms in fresh fruits and vegetables*. Retrieved from http://www.tdx.cat/handle/10803/7932

Unnevehr, L. (2015). Food safety in developing countries: Moving beyond exports. *Global Food Security*, *4*, 24–29. doi:10.1016/j.gfs.2014.12.001

Unnevehr, L., & Grace, D. (2013). Tackling Aflatoxins : An Overview of Challenges and Solutions, (November), 3–4.

Valyasevi, R., & Rolle, R. S. (2002). An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: Scope for their improvement. *International Journal of Food Microbiology*, *75*(3), 231–239. doi:10.1016/S0168-1605(01)00711-5

Vaughan, a., Eijsink, V. G. H., O'Sullivan, T. F., O'Hanlon, K., & Van Sinderen, D. (2001). An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. *Journal of Applied Microbiology*, *91*(1), 131–138. doi:10.1046/j.1365-2672.2001.01365.x

Walton, E., & Allen, S. (2011). Malnutrition in developing countries. *Paediatrics and Child Health*, *21*(9), 418–424. doi:10.1016/j.paed.2011.04.004

Wong, H., & Chen, Y. (1988). Effects of Lactic Acid Bacteria and Organic Acids Germination of Bacillus cereus Growth and. *Microbiology*, *54*(9), 2179–2184.

World Health Organization. (2014). Antimicrobial resistance. *Who*, *317*(September), 609–610. Retrieved from http://www.who.int/mediacentre/factsheets/fs194/en/#

Yang, E., Fan, L., Jiang, Y., Doucette, C., & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express*, *2*(1), 48. doi:10.1186/2191-0855-2-48

Yang, R., Johnson, M. C., & Ray, B. (1992). Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology*, *58*(10), 3355–3359.

CHAPTER I

FOLIC ACID PRODUCTION BY SELECTIVE *Streptococcus thermophilus* STRAINS CULTURE IN FERMENTED MILK: NATURAL BIO-ENRICHING MILK FORTIFICATION

ABSTRACT

Humans cannot synthesize folic acid (B-group vitamin), and it must be obtained exogenously through the dietary sources naturally rich in folic acid. Milk is a food with a low content of folic acid (26 µg/L), but some Lactic Acid Bacteria (LAB) species are able to produce folate during the fermentation, greatly increasing the content, being a natural resource for the folate in milk naturally bio-enriched. But it is not sufficiently known about the production, and the variability of folate enriches in milk by LAB starter strains. The concentration of folate in fermented milk varies greatly and, is closely dependent on the selection of the starter strain used, and about the fermentation condition. In this study, *St. thermophilus* starter was used for the fermented milk production ,and was analyzed the folate production during the fermentation period. *St. thermophilus* was grown in milk and extracellular folate productions were evaluated. Combination with *L. bulgaricus*, analysis on the pH dependence and the difference of folic acid production between medium culture and milk was analyzed, and finally tested the variability on the folate bio-enriching of milk. By their ability in the production of folate in milk, starter strains analyzed in these studies can be used for the production of fermented milk bio-enriched in natural folic acid, as an alternative to fortification with chemical folic acid.

Key words: Folates, Lactic Acid Bacteria, Folic Acid, *L. Bulgaricus*, *St. thermophilus*, Bio-enrichment

INTRODUCTION

Folic acid represents an essential nutritional component. It is involved in important metabolic pathways, in the one-carbon transfer reactions for example, like DNA replication, purine and pyrimidine biosynthesis, in some amino acids interconversion, in the biosynthesis of nucleic acids and other vitamins(Republic, 2002; Crittenden, Martinez, & Playne, 2003; Laiño, Juarez del Valle, Savoy de Giori, & LeBlanc, 2013).

Folate belongs to the water-soluble vitamins of the B group and is found in a wide variety of food. This vitamin is not synthesized in the human body and the diet plays a crucial role in the daily requirement. The DFE (Dietary Folate Equivalent) was established and for the adults varies between 200 and 400 µg/die, for an increased requirement up to 600 µg/die for the pregnant women (Molloy, (Benoist, 2014; Carmel, 2014; Molloy, Kirke, Brody, Scott, & Mills, 2008).

There is an increased risk of folate deficiency, especially in the elderly, in children and in pregnant women, because their intake of food are lower than a regular vitamin daily intake and for a consume of a lower variety of food (Laiño et al., 2013; Allen, 2014). Folate deficiencies may contribute to the etiology of important diseases such as Alzheimer, cancer, cardiovascular disease and, first of all, to the neural tube defect (NTD) (Mills & Signore, 2004; Ulrich, 2006; Rofail, Colligs, Abetz, Lindemann, & Maguire, 2011; Baggott, Oster, & Tamura, 2012; Imbard, Benoist, & Blom, 2013). Mandatory national fortification program has been adopted, for the first time, in the late 1990s in USA, Canada, Costa Rica and other countries, in order to decrease the NTD cases in each countries (Quinlivan, 2003; Mills & Signore, 2004; Crider, Bailey, & Berry, 2011; Ricks et al., 2012). Studies, however, have shown that high intakes of chemically synthesized form of folate, can cause adverse health effects such as the masking of the early haematological manifestations of vitamin of group B, especially B12, deficiency, alteration in the activity of the hepatic dihydrofolate reductase enzyme, or be the source of cancer

promoter (Ulrich, 2006; Bayston, R, Russel A, Wald N.J, 2007; Bailey & Ayling, 2009; Baggott et al., 2012). It is known then as the folic acid intake of organic origin can also be a suitable solution to overcome problems arising from taking folic acid chemically synthesized. The natural source of that naturally founded in food or produced by some microorganisms, do not cause health problems for the consumers (Lin & Young, 2000; Crittenden, Martinez, & Playne, 2003; Santos, Wegkamp, de Vos, Smid, & Hugenholtz, 2008). LAB (Lactic Acid Bacteria) has the ability, some strains more than other ones, to synthesize folic acid during the fermentation process, but it is strictly dependent on species, strains, growth time, pH and cultivation conditions (Sybesma, Starrenburg, Tijsseling, Hoefnagel, & Hugenholtz, 2003; Wegkamp, 2008; Hugenschmidt, Schwenninger, Gnehm, & Lacroix, 2010; Iyer, Tomar, Uma Maheswari, & Singh, 2010; Rossi, Amaretti, & Raimondi, 2011; Laiño et al., 2013). Fermentation with selected starter bacteria can be an easy system for increasing the folate concentration in food with naturally low folate, such as milk, that is not considered a primary source for natural folic acid. Bacteria can be used in the procession of many dairy products, across the fermentation process in which folate could be naturally synthesized. Fermented milk contains a three-fold increase in folate concentrations compared to non-fermented milk (Wouters, Ayad, Hugenholtz, & Smit, 2002). Yogurt or fermented milk is defined as "coagulated milk produced by lactic acid fermentation through the action of *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* (*St. thermophilus*) from milk (pasteurized or concentrated milk) with or without additions (milk powder, skim milk powder, etc.). The microorganisms in the final product must be viable and abundant". There is a symbiosis between these two strains during the milk fermentation, as in the stimulation of *L. bulgaricus* through formic acid produced by *St. thermophilus,* or in the provision of peptides by *L. bulgaricus* to *St. thermophilus* (Laiño et al., 2013). This largely depends on the starter strain culture used for the production of

fermented milk, but recently known that *St. thermophilus* is able to produce folic acid in milk during fermentation, and it can be used from the *L. bulgaricus* for production of more folate (Republic, 2002). The optimization of fermentation process, using selected starter culture able to produce folate in milk can be a promising approach for the natural enriching system. The objective of this study was to select the more capable folate producer of *St. thermophilus* in milk, and how it can modify with the combination with *L. bulgaricus,* in order to obtain fermented milk bio-enriched in folate.

- **Aim of the study**

The present research is focused on the analysis of the production of folate in fermented milk through the use of selected Lactic Acid Bacteria starter cultures. In other words the research is pointed on the practical evaluation of the use of selected LAB as food folate bio-enrichers, representing the right answer to the increase demand by the consumers for the natural food without chemical addition.

MATERIALS AND METHODS

- **MICROORGANISM AND GROWTH CONDITIONS**

A total of 60 strains of *St. thermophilus* and *L.delbrueckii* ,obtained from the culture bank collection of UCSC Piacenza (Università Cattolica del Sacro Cuore di Piacenza, Italy) were assayed for the folate production in fermented milk. All strains were stored in freezer-dried or in deep frozen forms. After microscopic examination, cultures were used for the analysis. *L. delbrueckii* were grown without agitation in MRS broth (de man-Rogosa-Sharpe, Oxoid) at 37°C in over-night, and *St. thermophilus* in M17 broth (Oxoid) at 42°C in over-night without agitation. The samples obtained were used as inoculum in sterilized milk (Oxoid),

and incubated at 37°C and 42°C for *L. delbrueckii* and *St. thermophilus* respectively, until coagulation occurred (8, 12 or 48 hours of fermentation).

- **FERMENTED MILK**

A total of 60 *Streptococcus thermophilus* and 9 *Lactobacillus* (L.) *delbrueckii* subsp. *bulgaricus* were used for this study. Reconstituted non-fat powder milk (500 mL) (Oxoid Skim Milk Powder) was heated 120°C for 15 min, representing the optimal substrate for the folate analysis, given his minimal low content in natural folate. After pasteurization the reconstituted milk was cooled to 40°C and inoculated with the selected yogurt strain, growth in broth and put in the thermostat. After the fermentation process, the coagulated milk obtained was used like inoculum for the subsequent test for folate determination in milk. The milk inoculated with *St. thermophilus* was incubated at 42°C and those one with *L. delbrueckii* at 37°C, until fermentation occurred. Folate concentration and pH were determined.

- **FERMENTED MILK SAMPLE PREPARATION**

Fermented milk samples (500µL) were aseptically withdrawn at different time of incubation and added/mixed with 500µL of protection buffer (0.1 M phosphate buffer, pH 6.8 containing 1.5 W/V of ascorbic acid to prevent oxidation and degradation), for the folate evaluation. The mixture obtained was vortexed and the resulting sample was boiled (100°C) for 5 min to precipitate the proteins and release folate from binding proteins present in fermented milk and immediately centrifuged (10000 x g for 6 min). The sample obtained was collected and stored at -20 for the total folates determination.

- **FOLATE DETERMINATION IN FERMENTED MILK**

Determination of folate concentration in milk was performed using a modified microbiological assay (Tamura, 1998; Hugenschmidt, Schwenninger, Gnehm, & Lacroix, 2010; Laiño, Juarez del Valle, Savoy de Giori, & LeBlanc, 2013) using *Lactobacillus casei* subsp. *rhamnosus* (*L. rhamnosus)* ATCC7459 as the indicator strain. In the test, is possible to calculate the production of folic acid, in base at the growth/development of the indicator strain. The strain, previously stored at -40 °C, before using for the assay, was inoculated in fresh MRS and incubated in thermostat for 24 hours at 37°C. After the correct development of the strain, aliquot of 1mL have been made and washed 3 times in saline solution, re-suspended in the original volume and used to inoculate 2% v/v of fresh FACM (Folic Acid Casei Medium, Difco), and incubated at 37°C for 24 hours. This last step was repeated to clean the strain (for depleting folate reserves in the indicator strain), and the second culture was used to perform the folate determination. The frozen samples were defrosted at room temperature in absence of light and processed kipping attention to work in controlled light condition. All the samples were diluted with the protection buffer and 5 µL of them were added to 195 µL of protection buffer, and 125 µL of the mixture obtained were placed in triplicate to 96 well sterile microplate. The indicator strain (*L. rhamnosus* ATCC7459) grown in FACM as described previously, was inoculated 4% v/v in 10 mL of 2 x FACM, and fraction of them (125 µL) were added to each well, previously filled with the sample of strain selected for the test. Each plate covered and stored at 37°C for 24/48 hours. After this incubation period, each plate was read with optical density (OD) at 620 nm microplate reader. In each plate a standard curve was realized using standard solution of folic acid diluted in a rage from 0.0078 to 10 ng mL^{-1}, for a total of 12 standard wells in order to obtain values within the range of the standard curve. To obtain the final concentration of folate product by the single strain, the value obtained from the optical analysis was expressed in µg/L.

- **STATISTICAL ANALYSIS**

Each data obtained from the results was measured in triplicate. All the results were expressed as means ± p-value. Statistical analysis was performed using ANOVA test for Windows Excel 2012, and differences were considered statistically significant at $p < 0.05$

RESULTS

- **Screening of folate production strains of *St. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in culture medium and milk**

Fifty-seven strains of *St. thermophilus* and 9 strains of *L. delbrueckii* were screened for their ability to produce folate in culture medium. Figure 1 are showing the folate production by *St. thermophilus* strains grown in M17 broth over night at 42°C. The figure shows differences on the folate concentration in the supernatant, from 21 µg/L to 1081 µg/L. UC9071 and UC9062, showing a higher performance in M17 broth, compared with other ones that are showing a lower capacity in the extracellular folate. These results support the fact that the vitamin production is strictly strain-dependent.

St. thermophilus UC9064, UC9050, UC8542 and UC9071 strains that shown the highest performance (strains that have highlighted the greater constancy in the production), were inoculated in milk for testing their ability to produce folate.

The fermentation process was performed incubating at 42°C overnight the inoculated raw milk. Milk contains lower concentration of folate (average value of 26 µg/L). Figure 2 shows that the *St. thermophilus* strains chosen were able to increase the folate levels up to ten times he was of raw milk. While difference in production of folate between UC9071 and UC9050 strains, grown in M17 was observed in milk the folate levels were more homogeneous.

The folate production by *L. delbrueckii* subsp. *bulgaricus* in MRS broth medium after incubation at 37°C overnight were also analyzed. Figure 3 is showing the folate level produced by *L. delbrueckii* subsp. *bulgaricus*. These *Lactobacillus* strains are low producer of extracellular folate, or even metabolize it, as shown by the negative values in results.

The production of folate in milk by *L. delbrueckii* subsp. *bulgaricus* was also analyzed. Some strains remain as low producers (Figure 4), such as UC8092 and UC8087.

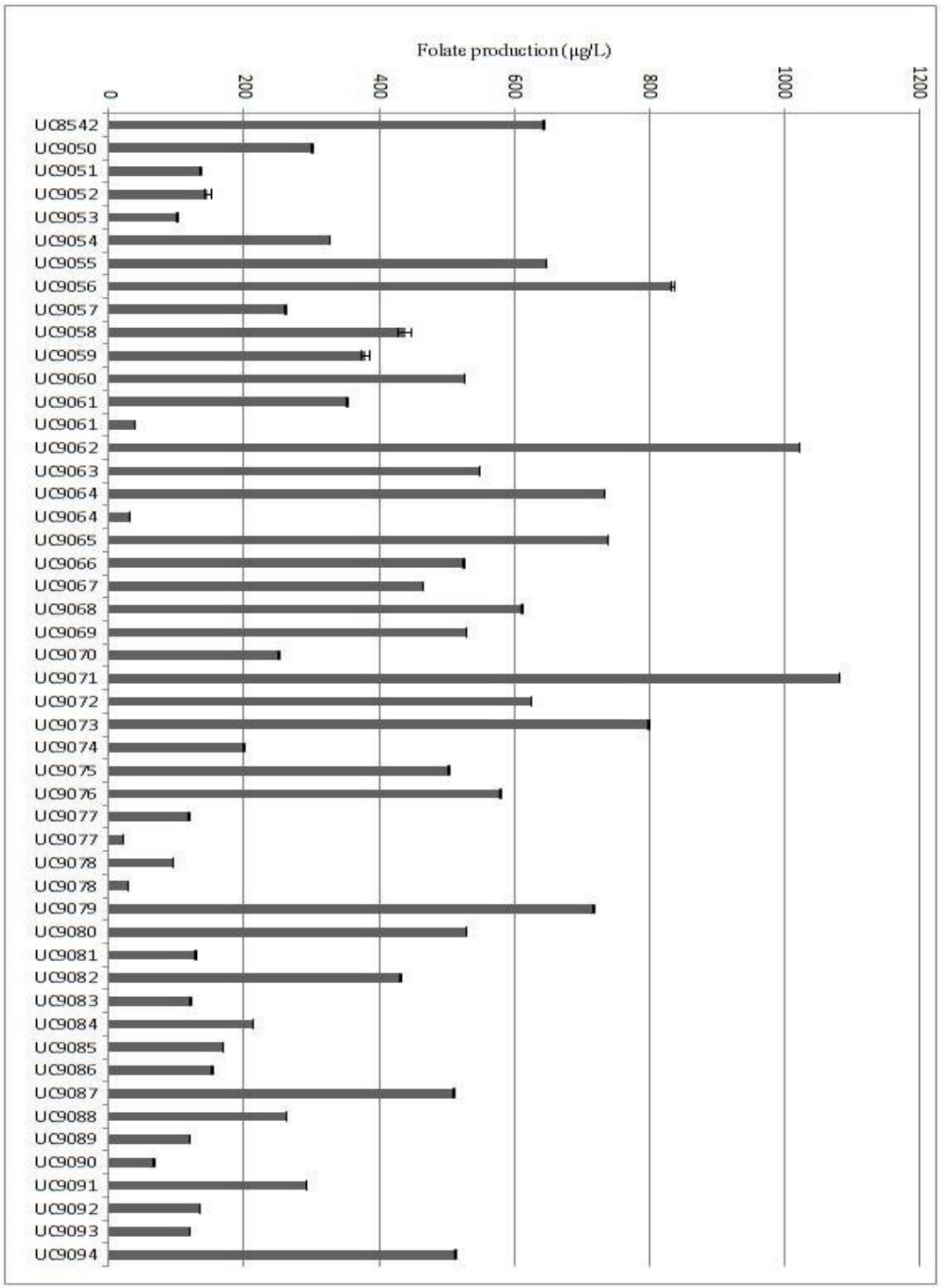


Figure 1: Folate production by *St. thermophilus* in M17 broth medium at 42°C. All the data are obtained through triplicate measure for a statistical significance approach ($p < 0.05$). Error bars indicate standard deviations.

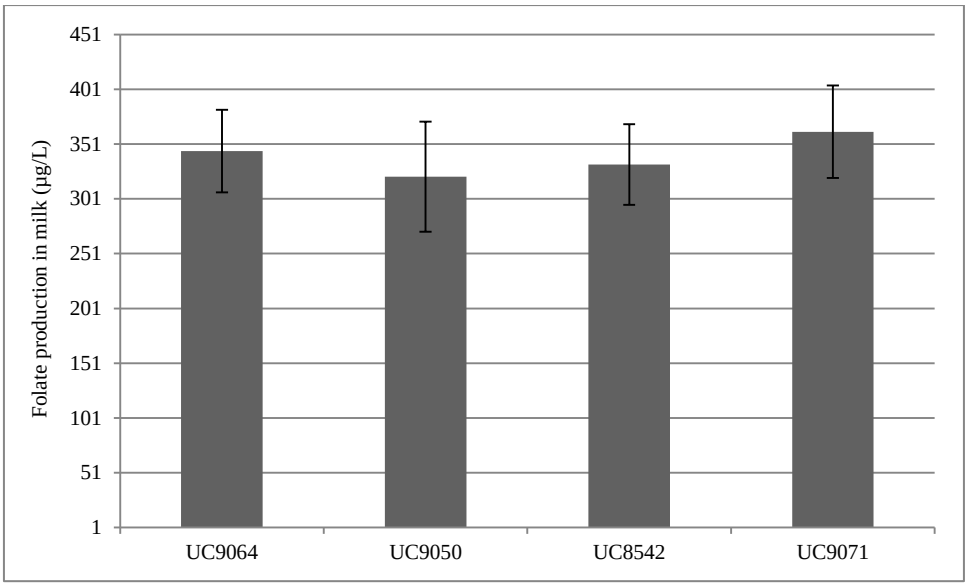


Figure 2: Folate production by *St. thermophilus* in milk. The data was collected by inoculum of the strain in raw milk and incubate for overnight at 42°C for stimulate the fermentation process. All the sample were made in triplicate for a statistical significance (p<0.05). Error bars indicate standard deviations.

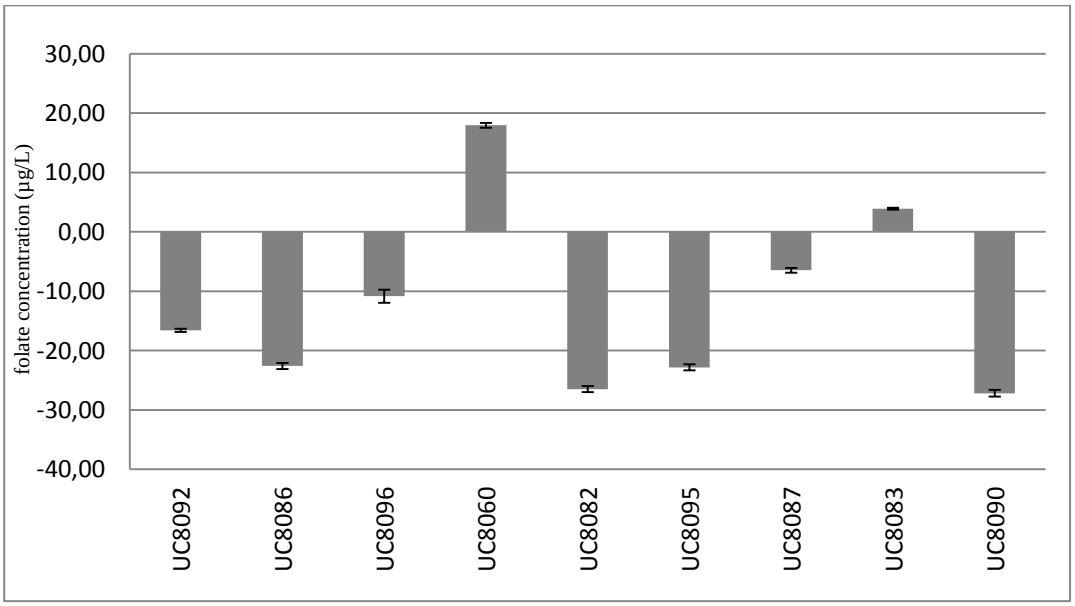


Figure 3: Production of folate by strains of *L. delbrueckii* growth in MRS broth at 37°C in overnight. All the data are obtained through triplicate for a statistical significance (p<0.05). Error bars indicate standard deviations.

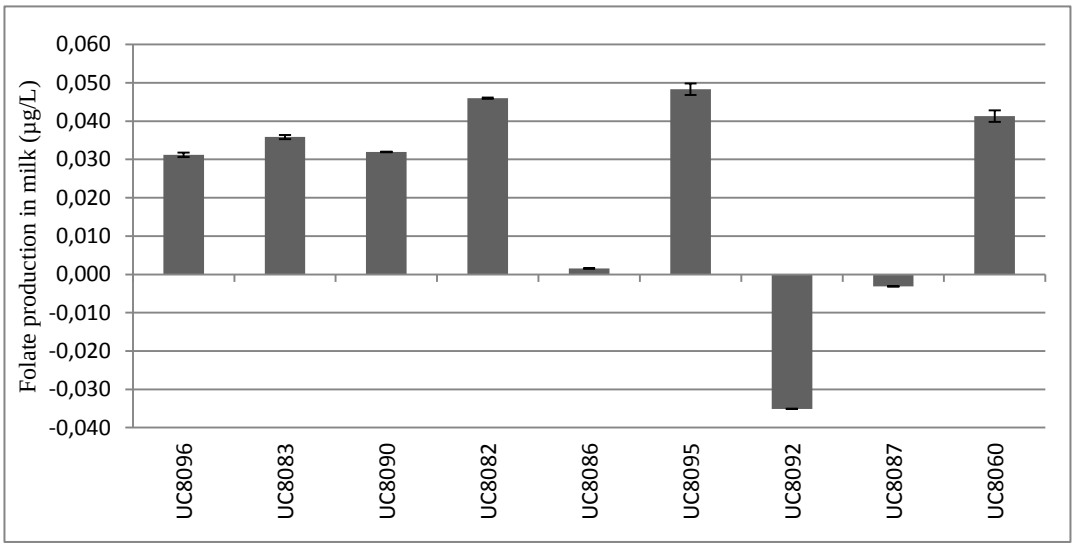


Figure 4: Folate production in milk obtained with selected starter strains of *L. delbrueckii* after an overnight fermentation at 37°C. All the data are obtained through triplicate for a statistical significance ($p<0.05$). Error bars indicate standard deviations.

- **Influence of fermentation time on the folate production in milk by *St. thermophilus* and *L. delbrueckii* subsp. *bulgaricus***

The entire samples were analyzed in triplicate to perform the statistical significance of each data collected. Looking to the figure 5 is possible to evaluate the time trend of the folate production by *St. thermophilus* (UC9050, UC9071, UC9064 and UC8542) strains, observing that there is a marked production of folate during the log phase (first 8 hours). The folate trend continues to rise up to 18 hours, is possible to see the maximum production of folic acid. Generally during the 8 hours of fermentation the vitamin concentration increases up more than 100%, underling the higher performance of the selected strains using for the test. *St. thermophilus* UC9071 was the higher folate producer in milk, increasing the folate concentration up to 72%, after18h of fermentation comparing with the values obtained at 8 hours of fermentation. After the 18 h of fermentation the trend of the production has a significant decrease, that probably depending on the use of the folate for the metabolic activity of the strains. But interesting fact is the trend that increase significantly after the 24 hours of fermentation, registering a buildup of 54% on the

case of *St. thermophilus* UC9071. All the producing strains, generally exhibited the same trend during the fermentation: the UC9064 shown a positive trend in the first ON fermentation, during with the folate concentration increase up to 63 %, as the UC8542 strain (71%). The trend was negative after 24 hours, when a decrease of folate concentration of 29% occurs. Differently UC8542 continued to increase the production about 4%. Both strains showing a positive trend after 48 hours which recorded a positive percentage production of 24 and 74 % respectively. Different condition is the UC8050 that shown a positive trend during ON (increment of 32%) and a continuous negative trend on the 24 and 48 hours (reduction of 38 and 4%), showing a different attitude than the other selected strains. Less standardized are the result obtained thought the inoculum of *L. delbrueckii* in milk figure 6. Five of nine strains showed a negative trend during the fermentation process, along the 48 hours of incubation, and some of them (UC8092 and UC8087) were negative producers. Noteworthy, *L. delbrueckii* UC8096, that registered a positive trend, with a drop in the production after the ON, was able to rise up the folate concentration up more than 100% after 24 and 48 hours.

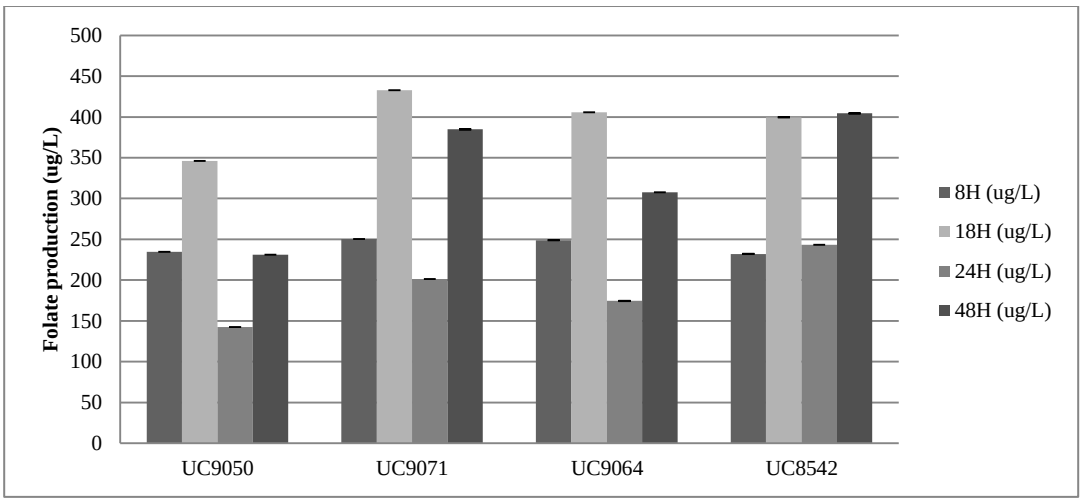


Figure 5: Analysis of folate production by *St. thermophilus* in milk in different fermentation process in four different time-step's: 8H (8 hours), 18H (18 hours), 24H (24 hours) and 48H (48 hours). Error bars indicate standard deviations. All the data are obtained through triplicate for a statistical significance ($p<0.05$).

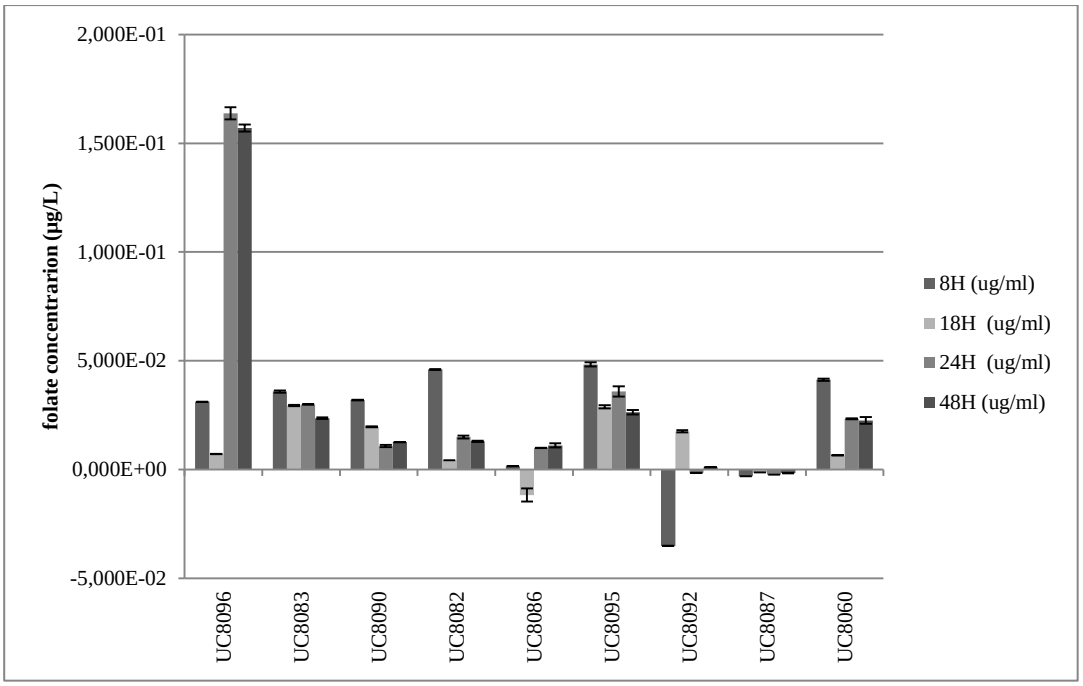


Figure 6: Analysis of folate production by *L. delbrueckii* subsp. *bulgaricus* recorded in milk during the fermentation process in four different time-step's: 8H (8 hours), 18H 18 hours), 24H (24 hours) and 48H (48 hours). Error bars indicate standard deviations

- **Influence of using mixed culture of St. thermophilus and L. delbrueckii on the folate production.**

To evaluate the possible difference in the folate production, strains of *L. delbrueckii* subsp. *bulgaricus* (UC8082 and UC8092) were mixed with strain on *St. thermophilus* (UC9050, UC9071, UC9064 and UC8542), and if they are able to increase it. For this characterize the test was performed using one of the worst strain (UC8082) and one of the mediocre producer (UC8092) of *L. delbrueckii* subsp. *bulgaricus*, previously shown, mixed with the selected strain of *St. thermophilus* (Figure 7).

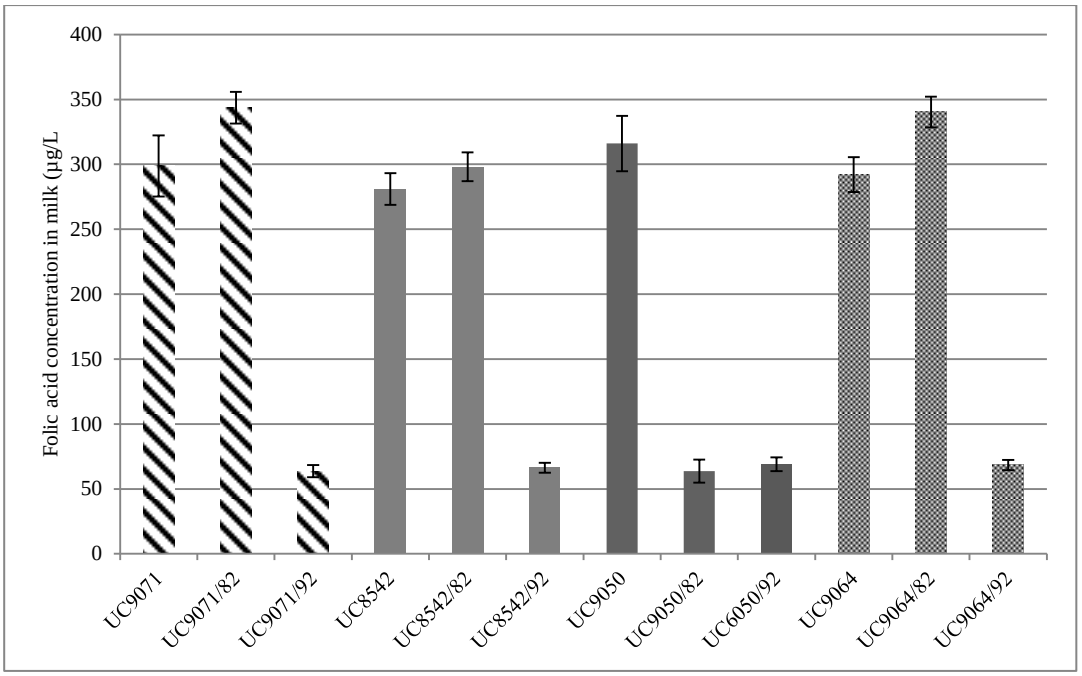


Figure7: folic acid concentration in milk produced by strains combiantion of *St. thermophilus* and *L. delbrueckii.* Folate analysis after overnight incubation at 42°C. Error bars indicate standard deviations

Results obtained show that strain of *L. delbrueckii* subsp. *bulgaricus* UC8082 tends to have a positive effect on the strain of *St. thermophilus*, determining in 3 cases out of four and increasing of folate extracellular produced. Contrarily the strain UC8092, that shown previously a worse production, it causes a decrease in folic acid produced in all the strains of *St. thermophilus.* Finally was tested the attitude on the folate production by *St. thermophilus* in yogurt, using two different selected strain of *L. delbrueckii* subsp. *bulgaricus* with general positive production of folic acid, or rather UC8096 and UC8095. In figure 8 is shown the results of the combination, demonstrate a different production if compared with the findings viewed in figure 7. Selected strain of *L. delbrueckii* subsp. *bulgaricus* in this case can have a different interaction with *St. thermophilus*, not going to adversely affect

the final production of folic acid in milk, even with strains less tolerant as the UC9050.

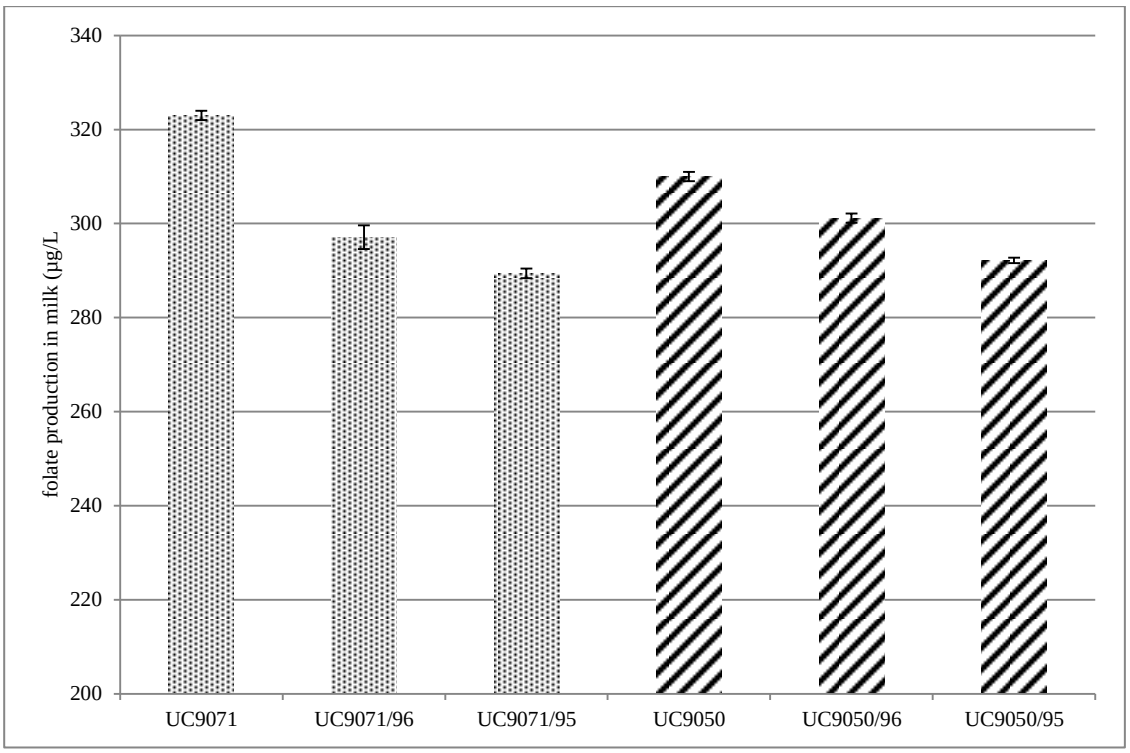


Figure 8: folic acid concentration in milk produced by strains combiantion of *St. thermophilus* and *L. delbrueckii.* Folate analysis after overnight incubation at 42°C. Error bars indicate standard deviations

DISCUSSION:

Although milk is considered one of the most nutritious food with the concentration of folic acid is around 20 – 50 µg/L (LeBlanc, de Giori, Smid, Hugenholtz, & Sesma, 2007; Laiño, Juarez del Valle, Savoy de Giori, & LeBlanc, 2013) lower than the WHO recommended daily intake of folic acid (400 µg/L per day by adults and 200 µg/L per day by children) (Hoey, Mcnulty, Duffy, & Hughes, 2015). The microbial enrichment, using selected bacterial strain able to increase the folate concentration in milk, is a promising alternative comparing to the chemical

enrichment, further recalling the attention of consumers who are increasingly opposed to the use of chemicals in food. During the fermentation process, folate producer's lactic acid bacteria (LAB) may increase of concentration of folate in milk, representing also a very simple system of food bio enrichment. Different LAB has been shown the ability to synthesize natural folate and is now known that the ability to produce this B group vitamin by yogurt starter LAB strains (*St. thermophilus* and *L. delbrueckii*) is very variable and is strictly strain-dependent (Crittenden, Martinez, & Playne, 2003; LeBlanc et al., 2007; Hugenschmidt, Schwenninger, Gnehm, & Lacroix, 2010). *L. delbrueckii* are known as folate consumers too (Crittenden et al., 2003), but some of them have lower ability to produce folate in medium previously enriched with folic acid. The absence of dairy fermented product with natural enrichment in folic acid, the production of folate on fermented milk was examined after the selection of different yogurt starter cultures; different strains of *St. thermophilus* and *L. delbrueckii*, were combined between them to evaluate the possible synergies, the ability of folate production by *St. thermophilus* in co-culture with *L. delbrueckii* and how the *L. delbrueckii* strains may affect the final levels of folate in milk.. The time during milk fermentation with the maximum folate production was determinate. Our results demonstrate that the combination of different yogurt starter cultures determine a wide variation on the level of folate production. Looking to the first results is possible to note how the strain of *St. thermophilus* produce folate in M17 medium and how they differ among them, empathizing the role of the strain depending on folate production. All the 57 strains selected were able to produce a certain amount of folic acid, but only 31 of them showed a higher attitude on the production of this B group vitamin (for estimation it has been taken into account the value of >200 µg/L, corresponding to the WHO recommended daily intake of folic acid). Different attitude, instead, for the strain of *L. delbrueckii*, that shown a lower ability on the production of folate. Only two strains of *L. delbrueckii* (UC8060 and UC8087) revealed a positive

activity in MRS medium, showing how difficult it is for these strains been to produce folic acid in medium that contain little or no initial concentrations.

Was then analysed the ability of folic acid production in milk by strains of *St. thermophilus* and the four strains with greater aptitude and homogeneity in the production have been selected for the research activities (UC9064, UC9050, UC8542 and UC9071), showing a higher attitude more than other strains of the same species evaluated in previously report (Lin & Young, 2000; Republic, 2002; Crittenden et al., 2003; Sybesma, Starrenburg, Tijsseling, Hoefnagel, & Hugenholtz, 2003; LeBlanc et al., 2007; Iyer, Tomar, Uma Maheswari, & Singh, 2010; Rossi, Amaretti, & Raimondi, 2011), demonstrating the great variability of production between similar strains and how little is known about the real production of folate in milk by LAB.

L. delbrueckii su strains in milk showed a different behaviour when compared with the production in MRS medium, probably due to the presence of slight folic acid naturally present in milk, that can induce a little production from this strains, although the concentration detected in milk cannot been considered significant at the nutritional level.

Little is known about the timing production of folate during the fermentation, for this reason in this study was evaluated the production during the classical fermentation process in milk by LAB, trying to demonstrate the moment of maximum production. Different times point were evaluated during 48 hours of incubation at 42°C and 37°C for *St. thermophilus* and *L. delbrueckii* respectively for the first overnight hours (16/18 hours), at 30°C during the following 24 and 48 hours after milk coagulation (evaluation of folate production on room temperature and in low fermentation condition).

The strain of *St. thermophilus* showed an inconstant trend on the folate production during the first 48 hours of fermentation but generally positive. It can be also noted that the higher production is registered during ON (overnight), after which the

concentration tends to decrease (similar results was founded by Hugenschmidt, Schwenninger, Gnehm, & Lacroix, 2010). This diminution will be determinate by the metabolic use of folate product by the same strain.

The results obtained for strains of *L. delbrueckii* instead show a progressive diminution of the folate concentration during the 48 hours of analysis emphasizing their difficulties on the folate synthesis and also, how reported previously by Crittenden et al. (2003), some strains of this specie depletes all the available folate in milk (single positive case is that of the strain UC8096 that while producing low amounts, however, tends to maintain a positive trend during 48).

St. thermophilus and *L. delbrueckii* are the LAB strains commonly used as starter for the production of yogurt. It is therefore wanted to analyze the production of folate in milk ferment using mixed LAB selected in order to assess the possible synergism positive or negative bacteria when grown in the same environment.

Given the high consumption of folic acid by *L. delbrueckii* is expected a substantial decrease of folate. To this end, was then selected two strains of *L. delbrueckii* with low production capacity (UC8082 and UC8092), mixed with *St. thermophilus* strains for evaluate the hypothetic decrease of folate produced by *St. thermophilus.* But the results have shown a different trend: the presence of the LAB strain UC8082 stimulate the 90% of the cases to produce folate, causing an increase in final concentration of folate. The effect of stimulation and positive synergy between labs was previously analyzed and theorized by (Rossi, Amaretti, & Raimondi, 2011) but clearly visible in the results obtained in these analyzes.

Different results, however, was obtained using strains of *L. delbrueckii* with positive activity in milk. Strains UC8096 and UC8095 were mixed with strains of *St. thermophilus* UC9071, which demonstrated positive ability to produce folic acid in milk in one case even when in inoculated in mixture, and the strain UC9050 which instead showed a bad synergism in the two previous experiments. In these cases the folate depleting by the strains of *L. delbrueckii* wasn’t so higher than as

expected, in the entire sample tested, showing a positive synergism between the different strains, and underlining the importance of using strains of LAB attentively selected for their ability to produce or not to deplete folate in milk.

Concentrations that occur when the strains are inoculated in the mixture are, as possible see from the data, lower compared to values obtained when strains of *St. thermophilus* are grown individually. Is possible to affirm, after these results, that among the species tested, the strains ST demonstrate greater dominance in the production of folate in milk, as reported also from (Republic, 2002). Although the results obtained of folate in these experiments are still recorded above 200 µg/L (above the minimum daily dose recommended) or rather more than 100% compared to folate naturally present in raw milk.

- **Conclusion**

With this study want to demonstrate the ability on the production of folic acid by strains of *St. thermophilus* in fermented milk, as natural system for bio-enriching in milk of this important micronutrient. The property in the extracellular production was seen that is strictly strain dependent. Factors like time of incubation were analyzed and can influence the final production. Important results obtained, it was the synergism in milk of *St. thermophilus* and *L. delbrueckii*. Little is known about the production of folate by *L. delbrueckii* in milk (are generally recognized like folate consumers and not folate producers), but results obtained in this study report a little but positive production, shown a moderate ability on the folate production, probably dictated by the lower presence of natural folate in milk. Has been so far little studied the synergism of the two species of starter yogurt LAB when used both in milk for the production of fermented milk. In this study is shown that *L. delbrueckii* can have a bad relationship with *St. thermophilus* for the production of folate, given them the high use of this vitamin for their metabolic process,

determining in some case a higher decreasing of folate extracellular in milk. But a careful preliminary analysis, it can be useful for determining the strains of *L. delbrueckii* less demanding in folic acid, and with slight production capacity. In fact, the results show how the use of strains of the *L. delbrueckii* selected must not go too far to affect the production of folic acid by *St. thermophilus*, thus ensuring a high content of folate in fermented milk. The selection of the strain for the use on the food production it will be a mandatory step. This kind of bio-enrichment system, the use of selected lactic acid bacteria, could be introduced as an efficient tool to increase the folate amount in food that are naturally scarce in folic acid, and to prevent folate deficiencies, without having use products of synthetic origin, in countries where folic acid are rare to find in the poorest diet.

BIBLIOGRAPHY

. S. a. H., . T. M., & . K. A. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

a.M., M., P.N., K., L.C., B., J.M., S., & J.L., M. (2008). Effects of folate and vitamin B12 deficiencies during pregnancy on fetal, infant, and child development. *Food and Nutrition Bulletin*, *29*(2 SUPPL.), S101–S111. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed8&NEWS=N&AN=18709885

Abee, T., Rombouts, F. M., Hugenholtz, J., Guihard, G., & Letellier, L. (1994). Mode of action of nisin Z against Listeria monocytogenes scott a grown at high and low temperatures. *Applied and Environmental Microbiology*, *60*(6), 1962–1968.

Adams, M. R., & Nicolaides, L. (1997). Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control*, *8*(5-6), 227–239. doi:10.1016/S0956-7135(97)00016-9

Al-Kadamany, E., Khattar, M., Haddad, T., & Toufeili, I. (2003). Estimation of shelf-life of concentrated yogurt by monitoring selected microbiological and physicochemical changes during storage. *LWT - Food Science and Technology*, *36*(4), 407–414. doi:10.1016/S0023-6438(03)00018-5

Aly, S., T, O. C. a, N, B. I. H., & Alfred, T. S. (2006). Bacteriocins and lactic acid bacteria - a minireview. *African Journal of Biotechnology*, *5*(May), 678–683. doi:10.5897/AJB05.388

Arimi, S. M., Koroti, E., Kang'ethe, E. K., Omore, A. O., & McDermott, J. J. (2005). Risk of infection with Brucella abortus and Escherichia coli O157:H7 associated with marketing of unpasteurized milk in Kenya. *Acta Tropica*, *96*(1), 1–8. doi:10.1016/j.actatropica.2005.05.012

Arqués, J. L., Rodríguez, E., Nuñez, M., & Medina, M. (2011). Combined effect of reuterin and lactic acid bacteria bacteriocins on the inactivation of food-borne pathogens in milk. *Food Control*, *22*(3-4), 457–461. doi:10.1016/j.foodcont.2010.09.027

Bankole, S. a, & Adebanjo, a. (2003). Mycotoxins in food in West Africa : current situation and possibilities of controlling it. *Journal of Biotechnology*, *2*(September), 254–263. doi:10.4314/ajb.v2i9.14833

Benech, R. O., Kheadr, E. E., Laridi, R., Lacroix, C., & Fliss, I. (2002). Inhibition of Listeria innocua in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Applied and Environmental Microbiology*, *68*(8), 3683–90. doi:10.1128/AEM.68.8.3683

Black, R. (2003). Editorial Micronutrient deficiency — an underlying cause of morbidity and mortality, *81*(03808), 3808.

Blagojev, N., Škrinjar, M., Vesković-Moračanin, S., & Šošo, V. (2012). Control of mould growth and mycotoxin production by lactic acid bacteria metabolites. *Romanian Biotechnological Letters*, *17*(3), 7219–7226.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., … Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *International Journal of Food Microbiology*, *154*(3), 87–97. doi:10.1016/j.ijfoodmicro.2011.12.030

Branca, F., & Ferrari, M. (2002). Impact of micronutrient deficiencies on growth: The stunting

syndrome. *Annals of Nutrition and Metabolism*, *46*(SUPPL. 1), 8–17. doi:10.1159/000066397

Brul, S., & Coote, P. (1999). Preservative agents in foods: Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, *50*(1-2), 1–17. doi:10.1016/S0168-1605(99)00072-0

Caplice, E., & Fitzgerald, G. F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, *50*(1-2), 131–149. doi:10.1016/S0168-1605(99)00082-3

Cappa, F., & Cocconcelli, P. S. (2001). Identification of fungi from dairy products by means of 18S rRNA analysis. *International Journal of Food Microbiology*, *69*(1-2), 157–160. doi:10.1016/S0168-1605(01)00587-6

Castellano, P., Belfiore, C., Fadda, S., & Vignolo, G. (2008). A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Science*, *79*(3), 483–499. doi:10.1016/j.meatsci.2007.10.009

Cavera, V. L., Arthur, T. D., Kashtanov, D., & Chikindas, M. L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *International Journal of Antimicrobial Agents*, 7–14. doi:10.1016/j.ijantimicag.2015.07.011

Chung, T., & Axelsson, L. (1989). In vitro studies on reuterin synthesis by Lactobacillus reuteri. *Microbial Ecology in …*, *2*, 137–144. doi:10.3109/08910608909140211

Cintas, L. M., Herranz, C., HernÁndez, P. E., Casaus, M. P., Nes, I. F., & HernÁndez, P. E. (2001). Review: Bacteriocins of Lactic Acid Bacteria. *Food Science & Technology International*, *7*(4), 281–305. doi:10.1106/R8DE-P6HU-CLXP-5RYT

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*(1), 1–20. doi:10.1016/S0168-1605(01)00560-8

Conference, I., & Food, U. N. (2014). Food safety must accompany food and nutrition security-WHO Proceedings. *Nutrition and Food Safety*, *6736*(14), 1–4. doi:10.1016/S0140-6736(14)62037-7

Corsetti, a., Gobbetti, M., & Smacchi, E. (1996). Antibacterial activity of sourdough lactic acid bacteria: isolation of a bacteriocin-like inhibitory substance fromLactobacillus sanfranciscoC57. *Food Microbiology*, *13*(6), 447–456. doi:10.1006/fmic.1996.0051

Cortés-Zavaleta, O., López-Malo, a, Hernández-Mendoza, a, & García, H. S. (2014). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *International Journal of Food Microbiology*, *173*, 30–5. doi:10.1016/j.ijfoodmicro.2013.12.016

Crider, K. S., Bailey, L. B., & Berry, R. J. (2011). Folic Acid Food Fortification—Its History, Effect, Concerns, and Future Directions. *Nutrients*, *3*(3), 370–384. doi:10.3390/nu3030370

Crittenden, R. G., Martinez, N. R., & Playne, M. J. (2003). Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. *International Journal of Food Microbiology*, *80*(3), 217–222. doi:10.1016/S0168-1605(02)00170-8

Crowley, S., Mahony, J., & van Sinderen, D. (2013). Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends in Food Science & Technology*, *33*(2), 93–109. doi:10.1016/j.tifs.2013.07.004

Cui, Y., Zhang, C., Wang, Y., Shi, J., Zhang, L., Ding, Z., Cui, H. (2012). Class IIa bacteriocins: Diversity and new developments. *International Journal of Molecular Sciences*, *13*(12), 16668–16707. doi:10.3390/ijms131216668

de Carvalho, a. a. T., Mantovani, H. C., & Vanetti, M. C. D. (2007). Bactericidal effect of bovicin HC5 and nisin against Clostridium tyrobutyricum isolated from spoiled mango pulp. *Letters in Applied Microbiology*, *45*(1), 68–74. doi:10.1111/j.1472-765X.2007.02150.x

de Crécy-Lagard, V., El Yacoubi, B., de la Garza, R. D., Noiriel, A., & Hanson, A. D. (2007). Comparative genomics of bacterial and plant folate synthesis and salvage: predictions and validations. *BMC Genomics*, *8*(Figure 2), 245. doi:10.1186/1471-2164-8-245

Delavenne, E., Ismail, R., Pawtowski, A., Mounier, J., Barbier, G., & Le Blay, G. (2013). Assessment of lactobacilli strains as yogurt bioprotective cultures. *Food Control*, *30*(1), 206–213. doi:10.1016/j.foodcont.2012.06.043

Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin Production: a Probiotic Trait? *Applied and Environmental Microbiology*, *78*(1), 1–6. doi:10.1128/AEM.05576-11

Eijsink, V. G. H., Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H., & Nes, I. F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *81*(1-4), 639–654. doi:10.1023/A:1020582211262

Filtenborg, O., Frisvad, J. C., & Thrane, U. (1996). Moulds in food spoilage. *International Journal of Food Microbiology*, *33*(1), 85–102. doi:10.1016/0168-1605(96)01153-1

Fitzpatrick, T. B., Basset, G. J. C., Borel, P., Carrari, F., DellaPenna, D., Fraser, P. D., Fernie, A. R. (2012). Vitamin deficiencies in humans: can plant science help? *The Plant Cell*, *24*(2), 395–414. doi:10.1105/tpc.111.093120

Florou-Paneri, P., Christaki, E., & Bonos, E. (2013). Lactic Acid Bacteria as Source of Functional Ingredients. *Lactic Acid Bacteria-R& D for Food, Health and Livestock Purposes*, 589–614.

Gerbaldo, G. a, Barberis, C., Pascual, L., Dalcero, A., & Barberis, L. (2012). Antifungal activity of two Lactobacillus strains with potential probiotic properties. *FEMS Microbiology Letters*, *332*(1), 27–33. doi:10.1111/j.1574-6968.2012.02570.x

Gerez, C. L., Torino, M. I., Rollán, G., & Font de Valdez, G. (2009). Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control*, *20*(2), 144–148. doi:10.1016/j.foodcont.2008.03.005

Gerez, C. L., Torres, M. J., Font de Valdez, G., & Rollán, G. (2013). Control of spoilage fungi by lactic acid bacteria. *Biological Control*, *64*(3), 231–237. doi:10.1016/j.biocontrol.2012.10.009

Gourama, H. (1997). Inhibition of Growth and Mycotoxin Production of Penicillium By Lactobacillus Species. *Food Science & Technology-Lebensmittel-Wissenschaft & Technologie*, *30*(3), 279–283.

Gupta, R., & Srivastava, S. (2014). Antifungal effect of antimicrobial peptides (AMPs LR14) derived from Lactobacillus plantarum strain LR/14 and their applications in prevention of grain spoilage. *Food Microbiology*, *42*, 1–7. doi:10.1016/j.fm.2014.02.005

Harris, L. J., Fleming, H. P., & Klaenhammer, T. R. (1992). Novel paired starter culture system for sauerkraut, consisting of a nisin-resistant Leuconostoc mesenteroides strain and a nisin-producing Lactococcus lactis strain. *Applied and Environmental Microbiology*, *58*(5), 1484–1489. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=195629&tool=pmcentrez&rendertype=abstract

History, T. H. E., & Folic, O. F. (2001). Historical Review.

Hoey, L., Mcnulty, H., Duffy, M. E., & Hughes, C. F. (2015). EURRECA — Estimating Folate

Requirements for Deriving Dietary Reference Values EURRECA, *8398*(October), 1040–1050. doi:10.1080/10408398.2012.742858

Holzapfel, W. (1997). Use of starter cultures in fermentation on a household scale. *Food Control*, *8*(5-6), 241–258. doi:10.1016/S0956-7135(97)00017-0

Holzapfel, W. H., Geisen, R., & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology*, *24*(3), 343–362. doi:10.1016/0168-1605(94)00036-6

Iyer, R., Tomar, S. K., Uma Maheswari, T., & Singh, R. (2010). Streptococcus thermophilus strains: Multifunctional lactic acid bacteria. *International Dairy Journal*, *20*(3), 133–141. doi:10.1016/j.idairyj.2009.10.005

Jack, R. W., Tagg, J. R., & Ev, M. I. R. (1995). Bacteriocins of Gram-Positive Bacteria, *59*(2), 171–200.

Jones, R. J., Hussein, H. M., Zagorec, M., Brightwell, G., & Tagg, J. R. (2008). Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. *Food Microbiology*, *25*(2), 228–234. doi:10.1016/j.fm.2007.11.001

Journal, T. H. E., & Biolowxl, O. F. (1974). Folate Coenzymes faecalis*. *Cell*, 6–7.

Käferstein, F. (2003). Foodborne diseases in developing countries: aetiology, epidemiology and strategies for prevention. *International Journal of Environmental Health Research*, *13 Suppl 1*(July 2015), S161–S168. doi:10.1080/0960312031000102949

Kuipers, O. P., De Ruyter, P. G. G. a, Kleerebezem, M., & De Vos, W. M. (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology*, *64*(1), 15–21. doi:10.1016/S0168-1656(98)00100-X

Laiño, J. E., Juarez del Valle, M., Savoy de Giori, G., & LeBlanc, J. G. J. (2013). Development of a high folate concentration yogurt naturally bio-enriched using selected lactic acid bacteria. *LWT - Food Science and Technology*, *54*(1), 1–5. doi:10.1016/j.lwt.2013.05.035

Lan, W., Chen, Y., Wu, H., & Yanagida, F. (2012). Bio-protective potential of lactic acid bacteria isolated from fermented wax gourd. *Folia Microbiologica*, *57*(2), 99–105. doi:10.1007/s12223-012-0101-1

LeBlanc, J., de Giori, G., Smid, E. J., Hugenholtz, J., & Sesma, F. (2007). Folate production by lactic acid bacteria and other food-grade microorganisms. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, *1*, 329–339.

Lee, S. C., Billmyre, R. B., Li, A., Carson, S., Sykes, S. M., Huh, E. Y., Heitman, J. (2014). Analysis of a Food-Borne Fungal Pathogen Outbreak: Virulence and Genome of a Mucor circinelloides Isolate from Yogurt. *mBio*, *5*(4), e01390–14–. doi:10.1128/mBio.01390-14

Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., & De Vuyst, L. (2012). On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. *Food Microbiology*, *30*(2), 379–92. doi:10.1016/j.fm.2011.12.021

Lewus, C. B., Kaiser, a., & Montville, T. J. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Applied and Environmental Microbiology*, *57*(6), 1683–1688.

Li, H., Liu, L., Zhang, S., Uluko, H., Cui, W., & Lv, J. (2013). Potential use of Lactobacillus casei AST18 as a bioprotective culture in yogurt. *Food Control*, *34*(2), 675–680. doi:10.1016/j.foodcont.2013.06.023

Lollo, P. C. B., de Moura, C. S., Morato, P. N., Cruz, A. G., Castro, W. D. F., Betim, C. B., Amaya-Farfan, J. (2013). Probiotic yogurt offers higher immune-protection than probiotic whey beverage. *Food Research International*, *54*(1), 118–124. doi:10.1016/j.foodres.2013.06.003

Lukaski, H. C. (2004). Vitamin and mineral status: effects on physical performance. *Nutrition*, *20*(7-8), 632–644. doi:10.1016/j.nut.2004.04.001

Massa, S. (2014). Lactobacillus plantarum as a Strategy for an In situ Production of Vitamin B2. *Journal of Food & Nutritional Disorders*, *s1*(01), 1–5. doi:10.4172/2324-9323.S1-004

McAuliffe, O. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews*, *25*(3), 285–308. doi:10.1016/S0168-6445(00)00065-6

Mcnulty, H., Pentieva, K., Hoey, L., Strain, J. J., & Ward, M. (2012). Nutrition Throughout Life : Folate, *82*(5), 348–354. doi:10.1024/0300-9831/a000130

Messens, W., & De Vuyst, L. (2002). Inhibitory substances produced by Lactobacilli isolated from sourdoughs - A review. *International Journal of Food Microbiology*, *72*(1-2), 31–43. doi:10.1016/S0168-1605(01)00611-0

Motarjemi, Y., Käferstein, F., Moy, G., & Quevedo, F. (1993). Contaminated weaning food: a major risk factor for diarrhoea and associated malnutrition. *Bulletin of the World Health Organization*, *71*(1), 79–92.

Muhialdin, B. J., & Hassan, Z. (2011). Annals . Food Science and Technology BIOPRESERVATION OF FOOD BY LACTIC ACID BACTERIA AGAINST 1 . INTRODUCTION Lactic acid bacteria (LAB) are Gram-positive microorganisms , prefer anaerobic conditions but are aero-tolerant , catalase negative , cocci or.

Müller, O., & Krawinkel, M. (2005). Malnutrition and health in developing countries, *173*(3), 2000–2003.

Naidu, a. S., Bidlack, W. R., & Clemens, R. a. (1999). Probiotic Spectra of Lactic Acid Bacteria (LAB). *Critical Reviews in Food Science and Nutrition*, *39*(1), 13–126. doi:10.1080/10408699991279187

Nakajima, H., Toba, T., Ito, A., Kudo, S., Sato, S., & Sato, Y. (2003). The Screening of Hydrogen Peroxide-Producing Lactic Acid Bacteria and Their Application to Inactivating Psychrotrophic Food-Borne Pathogens. *Current Microbiology*, *47*(3), 231–236. doi:10.1007/s00284-002-3993-1

Niku-Paavola, M. L., Laitila, a, Mattila-Sandholm, T., & Haikara, a. (1999). New types of antimicrobial compounds produced by Lactobacillus plantarum. *J. Appl. Microbiol.*, *86*(1), 29–35. doi:10.1046/j.1365-2672.1999.00632.x

Nissen-Meyer, J., Oppegård, C., Rogne, P., Haugen, H. S., & Kristiansen, P. E. (2010). Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins. *Probiotics and Antimicrobial Proteins*, *2*(1), 52–60. doi:10.1007/s12602-009-9021-z

O'Sullivan, L., Ross, R. P., & Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie*, *84*(5-6), 593–604. doi:10.1016/S0300-9084(02)01457-8

Okereke, a, & Montville, T. J. (1991). Bacteriocin-mediated inhibition of Clostridium botulinum spores by lactic acid bacteria at refrigeration and abuse temperatures. *Applied and Environmental Microbiology*, *57*(12), 3423–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=183991&tool=pmcentrez&rendertype=abstract

Onilude, a a, Fagade, O. E., Bello, M. M., & Fadahunsi, I. F. (2005). Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels. *Journal of Biotechnology*, *4*(December), 1404–1408.

Piard, J. C., & Desmazeaud, M. (1991). Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Le Lait*, *71*(5), 525–541. doi:10.1051/lait:1991541

Powers, H. J. (2007). Folic acid under scrutiny. *The British Journal of Nutrition*, *98*(4), 665–666. doi:10.1017/S0007114507795326

Reisenaãœer, a N. N. a I., & Halsted, C. H. (1987). Issues and Opinions In Nutrition Human Folate Requirements, *45*(October 1986), 600–602.

Republic, C. (2002). Biosynthesis of Folates by Lactic Acid Bacteria and Propionibacteria in Fermented Milk. *Food Science*, *22*(5), 175–181.

Rossi, M., Amaretti, A., & Raimondi, S. (2011). Folate Production by Probiotic Bacteria. *Nutrients*, *3*(12), 118–134. doi:10.3390/nu3010118

Røssland, E., Andersen, G. I., Langsrud, T., & Sørhaug, T. (2003). Inhibition of Bacillus cereus by strains of Lactobacillus and Lactococcus in milk, *89*, 205–212. doi:10.1016/S0168-1605(03)00149-1

Rouse, S., Harnett, D., Vaughan, a., & Sinderen, D. Van. (2008). Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *Journal of Applied Microbiology*, *104*, 915–923. doi:10.1111/j.1365-2672.2007.03619.x

Samelis, J., Kakouri, a., Rogga, K. J., Savvaidis, I. N., & Kontominas, M. G. (2003). Nisin treatments to control Listeria monocytogenes post-processing contamination on Anthotyros, a traditional Greek whey cheese, stored at 4°C in vacuum packages. *Food Microbiology*, *20*(6), 661–669. doi:10.1016/S0740-0020(03)00018-2

Sangmanee, P., & Hongpattarakere, T. (2014). Inhibitory of multiple antifungal components produced by Lactobacillus plantarum K35 on growth, aflatoxin production and ultrastructure alterations of Aspergillus flavus and Aspergillus parasiticus. *Food Control*, *40*, 224–233. doi:10.1016/j.foodcont.2013.12.005

Santos, F., Wegkamp, a., de Vos, W. M., Smid, E. J., & Hugenholtz, J. (2008). High-Level Folate Production in Fermented Foods by the B12 Producer Lactobacillus reuteri JCM1112. *Applied and Environmental Microbiology*, *74*(10), 3291–3294. doi:10.1128/AEM.02719-07

Schillinger, U., Geisen, R., & Holzapfel, W. H. (1996). Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends in Food Science & Technology*, *7*(5), 158–164. doi:10.1016/0924-2244(96)81256-8

Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology*, *16*(1-3), 70–78. doi:10.1016/j.tifs.2004.02.014

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *25*(2), 146–151. doi:10.1080/02652030701567442

Soomro, a. H., Masud, T., & Anwaar, K. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

Stoyanova, L. G., Ustyugova, E. A., Sultimova, T. D., Bilanenko, E. N., Fedorova, G. B., Khatrukha, G. S., … Pirogovskaya, B. (2010). New Antifungal Bacteriocin-Synthesizing

Strains of Lactococcus lactis ssp . lactis as the Perspective Biopreservatives for Protection of Raw Smoked Sausages Department of Microbiology , Faculty of Biological , M . V . Lomonosov Moscow State University Len, *5*(4), 477–485.

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, M. H. N., & Hugenholtz, J. (2003). Effects of Cultivation Conditions on Folate Production by Lactic Acid Bacteria. *Metab. Eng.*, *69*(8), 4542–4548. doi:10.1128/AEM.69.8.4542

Thu, B. D., Schultink, W., Dillon, D., Gross, R., Leswara, N. D., & Khoi, H. H. (1999). Effect of daily and weekly micronutrient supplementation on micronutrient deficiencies and growth in young Vietnamese children. *The American Journal of Clinical Nutrition*, *69*(1), 80–86.

Tiwari, B. K., Valdramidis, V. P., O' Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of Natural Antimicrobials for Food Preservation. *Journal of Agricultural and Food Chemistry*, *57*(14), 5987–6000. doi:10.1021/jf900668n

Trias, R. (2008). *Lactic acid bacteria as bioprotective agents against foodborne pathogens and spoilage microorganisms in fresh fruits and vegetables*. Retrieved from http://www.tdx.cat/handle/10803/7932

Unnevehr, L. (2015). Food safety in developing countries: Moving beyond exports. *Global Food Security*, *4*, 24–29. doi:10.1016/j.gfs.2014.12.001

Unnevehr, L., & Grace, D. (2013). Tackling Aflatoxins : An Overview of Challenges and Solutions, (November), 3–4.

Valyasevi, R., & Rolle, R. S. (2002). An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: Scope for their improvement. *International Journal of Food Microbiology*, *75*(3), 231–239. doi:10.1016/S0168-1605(01)00711-5

Vaughan, a., Eijsink, V. G. H., O'Sullivan, T. F., O'Hanlon, K., & Van Sinderen, D. (2001). An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. *Journal of Applied Microbiology*, *91*(1), 131–138. doi:10.1046/j.1365-2672.2001.01365.x

Walton, E., & Allen, S. (2011). Malnutrition in developing countries. *Paediatrics and Child Health*, *21*(9), 418–424. doi:10.1016/j.paed.2011.04.004

Wong, H., & Chen, Y. (1988). Effects of Lactic Acid Bacteria and Organic Acids Germination of Bacillus cereus Growth and. *Microbiology*, *54*(9), 2179–2184.

World Health Organization. (2014). Antimicrobial resistance. *Who*, *317*(September), 609–610. Retrieved from http://www.who.int/mediacentre/factsheets/fs194/en/#

Yang, E., Fan, L., Jiang, Y., Doucette, C., & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express*, *2*(1), 48. doi:10.1186/2191-0855-2-48

Yang, R., Johnson, M. C., & Ray, B. (1992). Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology*, *58*(10), 3355–3359.

CHAPTER II

FUNGAL MILK SPOILAGE INHIBITION BY LACTIC ACID BACTERIA

ABSTRACT

Chemical preserving compounds are traditionally used in the agro-food industry during food preparation and processing. Recently the interest of consumers in natural food without synthetic preservatives is increased and therefore the use of selected Lactic Acid Bacteria (LAB) with antibacterial ability, may represent a valid bio-preservative alternative. Moulds are among the most common causes of spoilage in milk products and can determine economic loss and problem for the consumers health, through the production of mycotoxins. The present study investigate the antifungal activity of two selected LAB, *Lactobacillus plantarum* UC491 and *Lactobacillus rhamnosus* UC8490, against two mould, *Penicillium brevicompactum* UC7075 and *Penicillium roqueforti* UC8544. For determine the inhibitory activity of the selected LAB, as bio-preservatives in fermented milk, the selective strains were inoculated in raw milk prior to the addition of *P. roqueforti* and *P. brevicompactum* and the inhibitory activity was monitored after incubation at 30°C. Different combination of LAB in fermented milk were tested to perform the thesis about the inhibition efficiency. Antimicrobial assay and statistical approach (probabilistic model of multinomial logistic regression) were used for the inhibitory evaluation. Results shown that the selected LAB have a potential inhibitory effect against mould in fermented milk. The ease of use and the high performance of LAB can represent a viable and economic alternative for the food preservative.

Key words: Fungal food spoilage, Inhibitory activity by Lactic Acid Bacteria, *Penicillium* spp., *L. plantarum* and *L. rhamnosus*

INTRODUCTION

Filamentous fungi are capable to grow on all kinds of food products, including cereals, meats, fruits, vegetables and dairy product. Moulds are important food spoilage organisms and causing extensive economic losses for the food industries, and can be the cause of health risks for the consumers for toxicity (production of aflatoxins) and pathogenicity of some species (susceptible individual can suffer of infections or allergies caused by moulds). For these reasons, food industries use several strategies to extend the food's shelf life such as the use of modified atmosphere during the packaging, heat treatments, food irradiation with infrared rays or microwaves, and finally adding chemical preservatives (sorbic, benzoic and propionic acids) (Adams & Nicolaides, 1997; E Delavenne, Mounier, Déniel, Barbier, & Le Blay, 2012; Gerez, Torres, Font de Valdez, & Rollán, 2013; Belguesmia et al., 2014; Emilie Delavenne et al., 2015). The use of synthetic food preservative agents has been increasingly unpopular with the consumers, who look for food that are not containing them and, also, some authors reported high mutation frequencies of target microorganisms with the use of some common antifungal agents, determining an increase resistance (Cortés-Zavaleta, López-Malo, Hernández-Mendoza, & García, 2014). In the recent years the use of bio-preservation, such as the use of selected microorganisms and/or the metabolite produced by them to prevent spoilage, and to extend the shelf life, has determined an increased interest for the consumers that causes an increase on the demand for reducing the use of chemically-synthesized products for food preservation (Magnusson, 2003; Schnürer & Magnusson, 2005; Wang, Yan, Wang, Zhang, & Qi, 2012; Cortés-Zavaleta et al., 2014). Lactic acid bacteria (LAB) have been used for long time like bio-preservation organisms in foods preventing the growth of spoilage microorganisms through the production of bioactive molecules such as organic acids, fatty acids, hydrogen peroxide and bacteriocins. Lactic acid bacteria are gram-positive bacteria, catalase-negative, nonsporulating, usually non-motile

rods and cocci that utilize fermentative carbohydrates and form lactic acid as the major end product (Onilude, Fagade, Bello, & Fadahunsi, 2005). This bacteria are widely used in food and feed fermentation, contributing to the hygienic safety, storage stability and attractive sensory proper-ties (Aly, T, N, & Alfred, 2006; Gerbaldo, Barberis, Pascual, Dalcero, & Barberis, 2012). For this reason are generally regarded as safe (GRAS), and have an important role in the preservation of foods and fermented food products (Florou-Paneri, Christaki, & Bonos, 2013) In the last years efforts has been directed to harness the antifungal activity by LAB in order to reduce the food spoilage (O'Sullivan, Ross, & Hill, 2002; Jones, Hussein, Zagorec, Brightwell, & Tagg, 2008; Rouse, Harnett, Vaughan, & Sinderen, 2008; Carla Luciana Gerez, Torino, Rollán, & Font de Valdez, 2009; Muhialdin & Hassan, 2011; Cheong et al., 2014). Different studies and screening have been made to identifying LAB with antifungal proprieties isolated from difference food source, such as vegetables, cereals, and dairy food (Corsetti, Gobbetti, and Smacchi 1996; Strain et al. 2000; Gerez et al. 2009, 2013). The interest of LAB increased for their application in the food production, especially for that strain able to limit the fungal growth and consequently able to reduce the mycotoxin production, in this particular case of aflatoxigenic fungi (Yang et al. 2012; Gerbaldo et al. 2012). The interest of bacteriocins/antimicrobial peptides production by LAB increased in the last years and has been reported or is involved in the preservation of many processed and natural food. Their usage in the food industry has helped to reduce the addition of synthetic preservatives and intense heat treatments, thus, resulting in foods which are more naturally preserved and richer in nutritional properties (Yang, Johnson, and Ray 1992; Schillinger, Geisen, and Holzapfel 1996; Cintas et al. 2001; Soomro, Masud, and Anwaar 2002; O'Sullivan et al. 2002; Aly et al. 2006; Abriouel et al. 2011). The effect of antifungal activity through the use of culture supernatant of various lactobacilli strains against few fungi has been studied and worked out by various workers (Strain et al. 2000; Messens and De Vuyst

2002; Magnusson 2003; Aly et al. 2006; alerio et al. 2009; Delavenne et al. 2012; Li et al. 2012; Sorrentino et al. 2013; Gupta and Srivastava 2014; Sangmanee and Hongpattarakere 2014; V Essia Ngang et al. 2014). The present study was carried out to examine the development of common spoilage fungi in yogurt (*Penicillium brevicompactum* UC7075 and *Penicillium roqueforti* UC8544) and the ability of selected LAB strains *Lactobacillus plantarum* UC8491 and *Lactobacillus rhamnosus* UC8490 with antifungal activity to preserve the quality of fermented milk as bio protective antifungal strains. The present study was developed to study the use of selected LAB strain with antifungal activity as bio preservatives, for maintain stable the quality of fermented milk for one week at room temperature without using chemical preservative.

- **Aim of the study**

The present study is focused on the analysis of the ability of selected LAB strain to inhibit fungal milk spoilage. Through the results obtained, the present work want to evaluate the possibility to use selected LAB as natural bio preservatives in dairy products.

MATERIALS AND METHODS

- **MICROORGANISM AND CULTURES CONDITIONS**

Lactobacillus plantarum UC8491, *Lactobacillus rhamnosus* UC8490, were previously isolated by food and stored in MRS agar (de man-Rogosa-Sharpe, Oxoid) supplemented with glycerol (20%v/v) at -120°C, and routinely inoculated or cultivated in growth medium. Lactic Acid Bacteria yogurt starter culture, ten *Lactobacillus delbrueckii* subsp. *bulgaricus* (stored in MRS agar, supplemented with glycerol 20%v/v, at -120°C) and 19 *Streptococcus thermophilus* (stored in

M17 agar Oxoid, supplemented with glycerol 20%v/v, at -120°C), were used for yogurt/fermented milk preparation. *Penicillium brevicompactum* UC7075 and *Penicillium roqueforti* UC8544, stored at 4°C in Malt Extract Agar (Oxoid), were used for developing the inhibition test in this study. All the strains mentioned are stored in the culture collection of UCSC Piacenza (Università Cattolica del Sacro Cuore di Piacenza, Italy). *Lactobacillus plantarum* UC8491 *Lactobacillus rhamnosus* UC8490 and all the *L. delbrueckii* subsp. *bulgaricus* UC8096 were reactivated without agitation in MRS broth (de man-Rogosa-Sharpe, Oxoid) at 37°C in over-night, and the *St. thermophilus* strains in M17 lactose broth (Oxoid) at 42°C in over-night without agitation. At growth occurred the sample strains were passed three times in specific broth for stabilizing the strain before the analysis test. *Penicillium brevicompactum* UC7075 and *Penicillium roqueforti* UC8544 were reactivated in RB agar (Rose Bengal chloramphenicol agar-base, Oxoid) or Malt Extract Agar at 30°C for 3/5 days and used as inoculum in milk once grown (spores suspension were extract after culture grown and stored in peptone water at 4°C).

- **PRODUCTION OF YOGURT SAMPLES**

Reconstituted non-fat powdered milk (Oxoid Skim Milk Powder) was sterilized at 120°C for 15 min. After pasteurization the reconstituted milk was rapidly cooled to 45°C before inoculation with the selected yogurt strain and moulds mentioned previously, and put in the thermostat. Fermentation was carried out at 42°C for overnight when the analysis involves the use of the strain of *St. thermophilus*, otherwise the milk sample were stored at 37°C in overnight to promote the LAB growth. Once occurred all the inoculation with the mould strains, the milk sample were stored finally at 30°C for one week.

- **QUANTIFICATION OF LAB STRAINS AND FILAMENTOUS FUNGI**

For assessing the growth of each strain (LAB and moulds) used during the tests, CFU counting process was used. At the end of each fermentation/incubation time predetermined, yogurt samples were 10-fold diluted in sterile 0.1% peptone water and 100µl of each samples were plated in triplicate in the selected growth agar for each different strain. Different culture temperature and time incubation were used to favourite the right development of microorganisms.

After the incubation period, the growth colonies were counted. Respectively to this methodology, for the mould development in yogurt, an observation of the surface protocol was used to evaluate the quantitative development of moulds in fermented milk.

- **ASSESSMENT OF ANTIFUNGAL ACTIVITY**

The fungal spoilage in milk was evaluated testing the growth ability of two fungal strains, *P. roqueforti* UC8544 and *P. brevicompactum* UC7075 in fermented milk obtained with different combination of starter strains of *St. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* selected for their ability to increase the acidity in milk. This first step was fundamental for evaluating if the fungi strains were able to develop in high fermented milk, stressing their ability to have a natural resistance to acidity (Vivier, Rivemale, Ratomahenina, & Galzy, 1992; Carrillo-Inungaray et al., 2014). The LAB starter strain was previously inoculated tree times in selected medium and used like inoculum in milk. Milk samples containing the *St. thermophilus* strains were incubated at 42°C in overnight, whereas samples with *L. delbrueckii* subsp. *bulgaricus* at 37°C in overnight, to perform the fermentation. The samples obtained of fermented milk (10 mL) from each bacterial strain, were used as inoculum for the subsequent test. pH in milk was monitored during one week and the strains with the best attitude in acidification were used to test the ability of fungi to grow in an acid environment. Strains of *St. thermophilus* and *L.*

delbrueckii subsp. *bulgaricus* were combined to obtain fermented milk, that will be inoculated with 1% of suspension spores of the mould (10^7 spores/mL), and finally incubated at 30°C for one week. CFU were counted and test of variance (ANOVA) was performed. This step was important for determining if the acidity will constitute a good barrier against fungi spoilage development in milk. Subsequent antifungal property in milk by selected LAB was carried out using strains of *L. plantarum* UC8491 and *L. rhamnosus* UC8490 tested for their ability to inhibit the growth of fungi spoilage. This LAB was tested in plate to evaluate their performance on the inhibition activity through initial test on MRS agar (De Man Ragosa, Oxoid). Samples of 10 µL of *L. plantarum* UC8491 and *L. rhamnosus* UC8490 (growth in overnight at 37°C in MRS broth), were crawled on MRS agar plate and put at 37°C for overnight. After the LAB growth the plates were then overlaid with 7ml of malt extract soft agar (1.5% malt agar, Difco™) containing 0.1 mL ($1x10^6$ spores/mL) of *Penicillium brevicompactum* and *Penicillium roqueforti* and incubated aerobically at room temperature (30°C) for 3-5 days, or until an uniform growth of the moulds was observed. The inhibition zones were recorder around the LAB spot. This was an important step for the analysis for having a first confirmation about the inhibition capacity by the two LAB strains selected. The subsequent step was that to evaluate the development of antifungal activity of the selected LAB in fermented milk, and during storage at 30°C for one week, for determining the possible long shelf life fermented milk. Samples of pasteurized milk (reconstituted non-fat dried milk, 10% v/v, Oxoid) was inoculated with starter strain St. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, that previously shown the worst attitude to prevent fungal development in milk, and incubated at 42°C in overnight until fermentation occurred. Other samples were inoculated with the inhibitory LAB to confront the different results. At fermentation occurred strain of *Penicillium roqueforti* UC8544 and *Penicillium brevicompactum* UC7075 were inoculated at 1% v/v and the samples were

incubated at 30°C for one week. After the storage period, CFU rate of all the microorganism strain used in the experiment were recorded using selected growth medium for each specie and strain, and pH measures were made.

- **EVALUATION OF QUALITATIVE INHIBITION OF FUNGI SPOILAGE IN FERMENTED MILK USING THE YOGURT PLATE ASSAY**

The qualitative evaluation of the development of spoilage fungi strains were evaluated in fermented milk in absence and presence of LAB strains with antifungal activity. A specific protocol was created for the analysis. Square sterile plates with wells were used to perform the methodology and to obtain a good number of replicates for a statistical relevance (each plate correspond at three replicate for each sample). Each well was filled with 2ml of pasteurized milk (reconstituted non-fat powder milk, 10% v/v, Oxoid), subsequently inoculated with antifungal LAB strains UC8491 and UC8490 alone, to test their ability on inhibition, without other LAB. In other wells the reconstituted milk were inoculated with the yogurt starter strains (St. *thermophilus* ST07 and *L. delbrueckii* subsp. *bulgaricus* UC8096) and the two antifungal strains, to analyse the possible positive or negative interaction with different LAB strain. All the plates were incubated in overnight at 42°C, to perform the milk fermentation. At the fermentation occurred, moulds strains (*P. roqueforti* UC8544 and *P. brevicompactum* UC7075) were inoculated in different dilution ($1x10^{-1}$, $1x10^{-2}$, $1x10^{-3}$, $1x10^{-4}$, $1x10^{-5}$, $1x10^{-6}$ and $1x10^{-7}$), in order to monitor where it will have the greatest degree of inhibition. The inhibitor strains were inoculated at 1% and 3% of v/v, for evaluate the possible differences on the inhibition rate. When the inoculum of all the microorganisms occurred, the square plate were incubated at 30°c for one week, for performing the moulds development. After the growth period the plate were analysed for evaluating the growth rate of the inoculated fungi, with visual observation of the surface of single well, giving a percentage of growth to each

well, from 0 (complete inhibition occurred, or rather 0% of mould development) to 4 (100% of the well surface were occupied by the mould, corresponding to a complete failure on the inhibition by the LAB). Statistical approach was used to analyse the results, using a probabilistic model (cumulative logit).

- **STATISTICAL ANALYSIS : PROBABILISTIC MODEL OF MULTINOMIAL LOGISTIC REGRESSION**

For the statistical analysis, a probabilistic model based on a "Multinomial Logistic Regression" was used, for analysing the results recorded during the qualitative protocol described above. The statistical analysis try to determine the inhibition property of the *L. plantarum* UC8491 and L. *rhamnosus* UC8490 strains against the fungal spoilage in milk, through a growth/non-growth model, adjusted using a logistic regression. With this model, is trying to describe the probability of *P. roqueforti* and P. *brevicompactum* UC7075 to grow or not, conditioned by the presence of *L. plantarum* UC8491 and *L. rhamnosus* UC8490 in different concentration and if they are using alone or like secondary inoculum in a fermented milk containing LAB yogurt starter, testing their bio-protection. Following the specific logistic regression model in present Eq, (Gortmaker, Hosmer, & Lemeshow, 1994; Agresti, 2002, 2011; Massaguer & Saraiva, 2011).

$$P(Y \leq j|x) = \pi 1(x) + \cdots + \pi j(x),$$
$$j = 1, \dots, j.$$

Where $P(Y \leq j|x)$ can be seen like the probability of growth or non-growth of the mould and the cumulative logit transformation of $P(Y \leq j|x)$ is:

$$Logit[P(Y \leq j|x)] = log \frac{P(Y \leq j|x)}{1P(Y \leq j|x)}$$

After fitting the logistic regression model, critical prediction of the variable were made, with a 0.05% of probability level.

RESULTS

- **INHIBITORY ACTIVITY BY LACTIC ACID BACTERIA IN FERMENTED MILK AGAINST *Penicillium* spp.**

In order to assess the ability to inhibit the fungal spoilage, two selected LAB strain, *L. plantarum* UC8491 and *L. rhamnosus* UC8490, were tested against two mould strains (*P. roqueforti* UC8544 and *P. brevicompactum* UC7075), commonly responsible of milk spoilage.

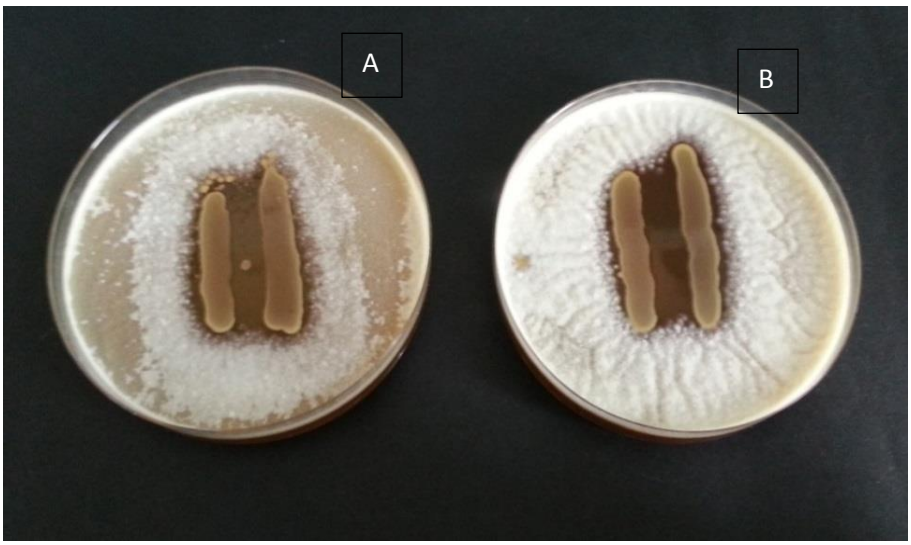


Figure 1: fungal *Penicillium brevicompactum* UC7075 inhibition attitude in MRS by *L. plantarum* UC8491 (A) and *L. rhamnosus* UC8490 (B)

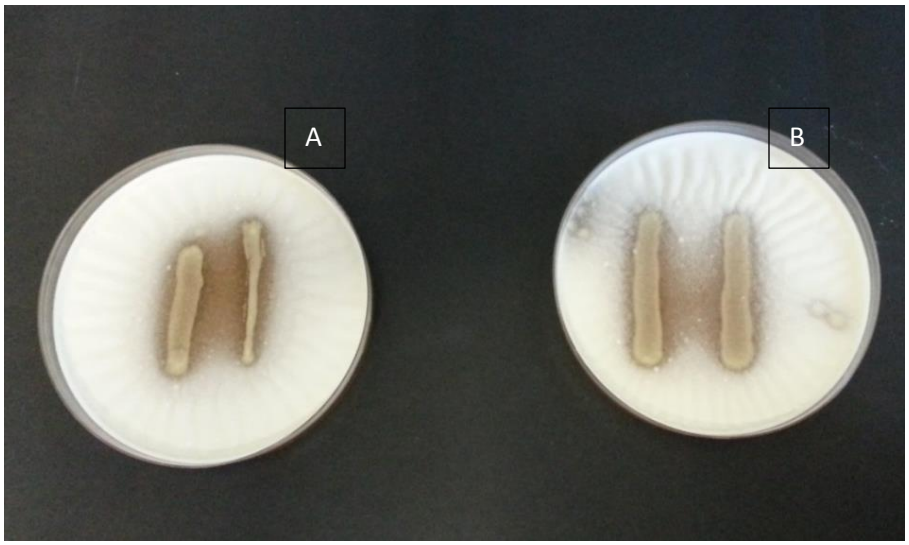


Figure 2: Fungal *Penicillium roqueforti* UC8544 inhibition attitude in MRS by *L. plantarum* UC8491 (A) and *L. rhamnosus* UC8490 (B)

In figure 1 and 2, is possible to observe the results obtained, pointing up the antifungal activity performed of two LAB, by the evident inhibition zones. The subsequent step was that to determine the inhibition attitude in fermented milk. It was decide to test the antifungal property in fermented milk obtained by the combined of *St. thermophilus* UC9064 and *L. delbrueckii* subsp. *bulgaricus* UC8082, that shown previously (Figure 1) the worst results against the fungal growth. The fermented milk was inoculated ($1x10^7$ CFU/mL) with the two yogurt starter strains (UC9064 and UC8082) and with the selected strains with antifungal ability, alone or combined. At the end of the fermentation step, overnight at 42°C, the different samples were inoculated with $1x10^7$ spores/mL of the fungal spoilage and stored at 30°C for one week, and then the CFU of the LAB and moulds was collected. In Figures 3 and 4 can be seen the data of the trend of CFU for individual strains, LAB and moulds, collected in fermented milk after a week of storage at 30°C.

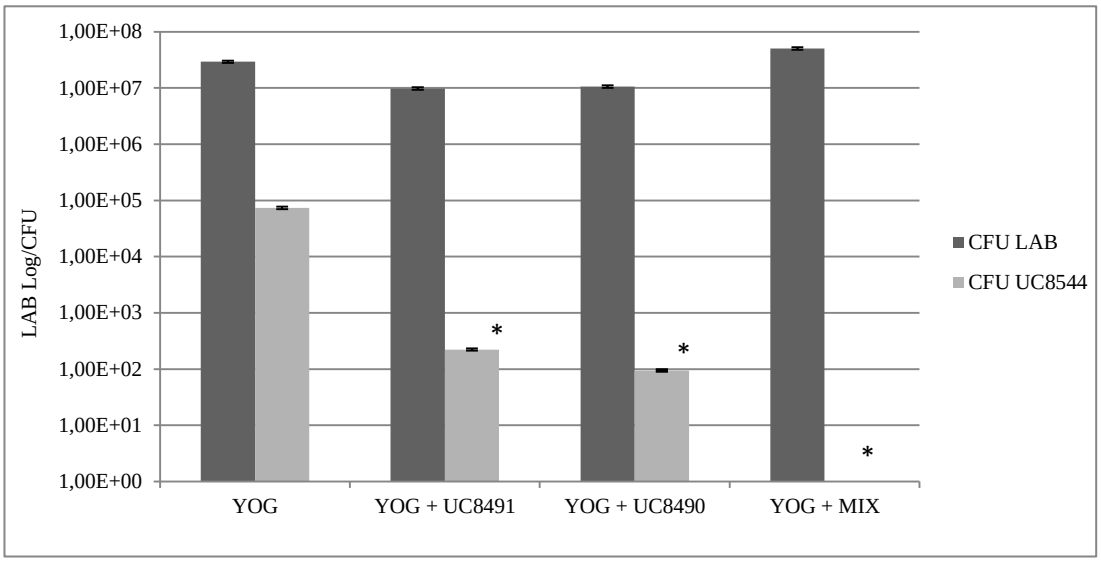


Figure 3: Analysis of the inhibition ability by selected LAB strain UC8491 and UC8490. The test compare the different attitude of the LAB strain against *P. roqueforti* UC8455 Different samples was created: YOG (fermented milk created only with *St. thermophilus* UC9064 and L. bulgaricus UC8082); YOG +UC841 (fermented milk created with *St. thermophilus* UC9064 and L. bulgaricus UC8082 with the addition of the strain *L. plantarum* UC8491); YOG + UC8490 (fermented milk created with *St. thermophilus* UC9064 and L. bulgaricus UC8082 with the addition of the strain *L. rhamnosus* UC8490) and YOG +MIX (fermented milk created with *St. thermophilus* UC9064 and *L. bulgaricus* UC8082 with the addition of the two strains antifungal property). The data collected was recorded in triplicate. ANOVA test realize with a significance value of 0,04. Statistically significantly different from the control YOG are indicated with *. Error bars indicate standard deviations.

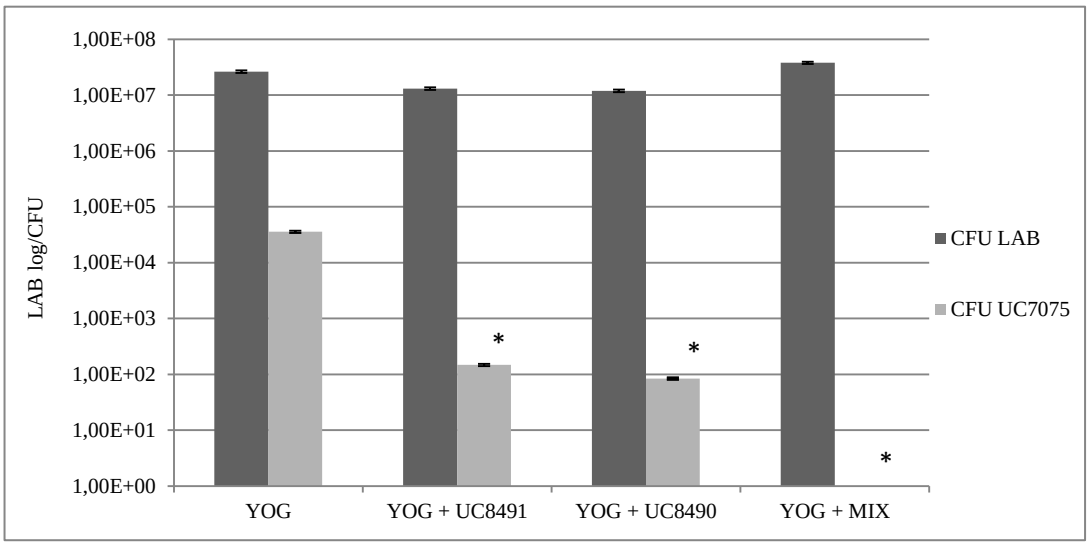


Figure 4: Analysis of the inhibition ability by selected LAB strain UC8491 and UC8490. The test compares the different attitude of the LAB strain against *P. brevicompactum* UC7075. Different samples was created: YOG (fermented milk created only with *St. thermophilus* UC9064 and *L. bulgaricus* UC8082); YOG +UC841 (fermented milk created with *St. thermophilus* UC9064 and *L. bulgaricus* UC8082 with the addition of the strain *L. plantarum* UC8491); YOG + UC8490 (fermented milk created with *St. thermophilus* UC9064 and L. bulgaricus UC8082 with the addition of the strain *L. rhamnosus* UC8490) and YOG +MIX (fermented milk created with *St. thermophilus* UC9064 and *L. bulgaricus* UC8082 with the addition of the two strains antifungal property). The data collected was recorded in triplicate. ANOVA test realize with a significance value of 0,01. Statistically significantly different from the control YOG are indicated with *. Error bars indicate standard deviations.

The figures refer to the value of inhibition, evaluated according to the number of mould spores per mL, in relation to the presence or absence of inhibitors strains in milk, assessed in CFU/mL. It is noted that there is a significant difference between the values obtained from the fermented milk without inhibitors (YOG, fermented milk created only with the starter strains UC9064 and UC8082), compared to yogurt they are added the selected strains with anti-spoilage activity (YOG + UC8491, YOG +UC8490 and YOG + MIX). The values of spores/mL relative to the moulds found in fermented milk without inhibitors (YOG) after a week, hovering around $1x10^4$ spores/mL, while the LAB are around 1x10.7 CFU/mL (keeping a constant value of initial inoculation). Compared to the initial inoculum of mould (1x107 spores/mL), it can be assumed to a partial inhibition of the mould, with a decrease in the value of the spores of about 3 log. The results, however, change when the fermented milk are inoculated with inhibitors strains *L. plantarum* UC8491 (YOG + UC8491) or *L. rhamnosus* UC8490 (YOG + UC8490), both individually or in mixture (YOG + MIX). In all cases, however, is noted as the decrease of the spores of the mould after a week is clear, marking the strong effect of bio-protection of the strains inhibitors, with a further decrease of approximately 3 logs compared to values obtained in the fermented milk with only LAB starter strains. Given the possible inhibitory effect determined by the pH, previously hypothesized with the results obtained with starter strains, but especially in the search for an effect of inhibition determined by the production of bacteriocins by strains UC8491 and UC8490, have measured the pH values of individual samples (YOG, YOG + UC8491, YOG + UC8490 and YOG + MIX), visible in Figures 5 and 6, where is making a relationship between the pH values measured at one week of incubation at 30 ° C and the number of spores/mL of mould present.

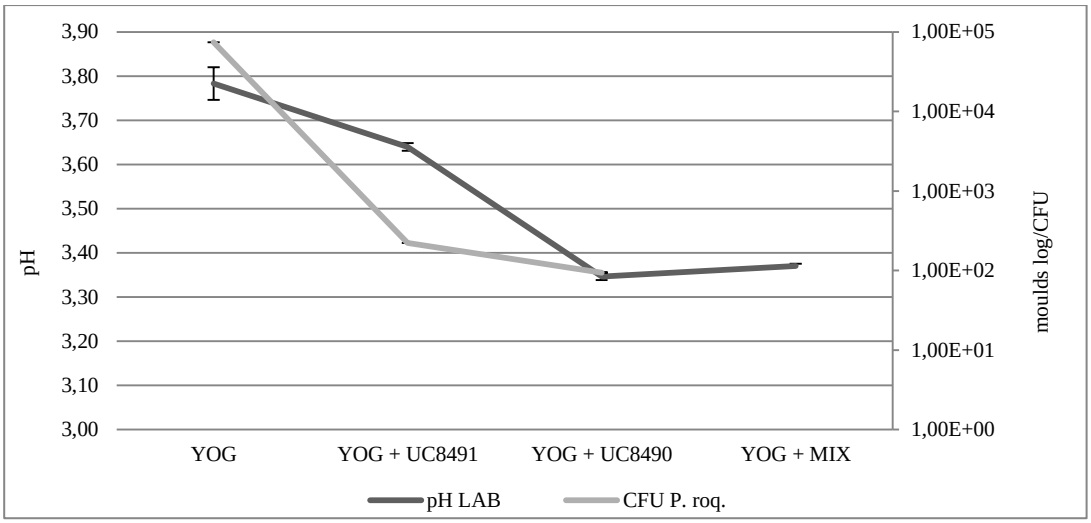


Figure 5: Analysis of the possible relationship about pH trend of the fermented milk produced with different combination of LAB (YOG (fermented milk created only with *St. thermophilus* UC9064 and *L. bulgaricus* UC8082); YOG +UC841 (fermented milk created with *St. thermophilus* UC9064 and *L. bulgaricus* UC8082 with the addition of the strain *L. plantarum* UC8491); YOG + UC8490 (fermented milk created with *St. thermophilus* UC9064 and L. bulgaricus UC8082 with the addition of the strain *L. rhamnosus* UC8490) and YOG +MIX (fermented milk created with *St. thermophilus* UC9064 and *L. bulgaricus* UC8082 with the addition of the two strains antifungal property), and the inhibition of *P. roqueforti* UC8455. Error bars indicate standard deviations.

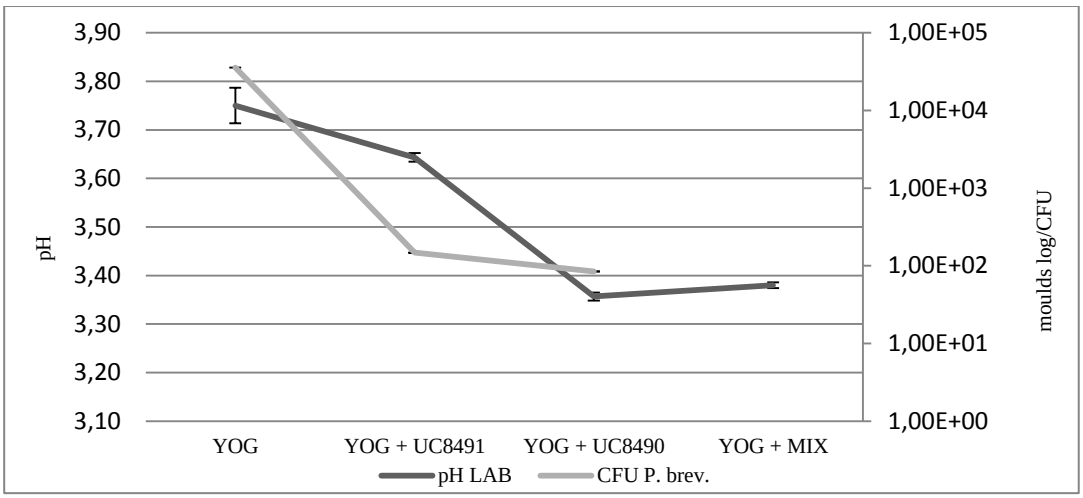


Figure 6: Analysis of the possible relationship about pH trend of the fermented milk produced with different combination of LAB (YOG (fermented milk created only with *St. thermophilus* UC9064 and *L. bulgaricus* UC8082); YOG +UC841 (fermented milk created with *St. thermophilus* UC9064 and *L. bulgaricus* UC8082 with the addition of the strain *L. plantarum* UC8491); YOG + UC8490 (fermented milk created with *St. thermophilus* UC9064 and L. bulgaricus UC8082 with the addition of the strain *L. rhamnosus* UC8490) and YOG +MIX (fermented milk created with *St. thermophilus* UC9064 and *L. bulgaricus* UC8082 with the addition of the two strains antifungal property), and the inhibition of *P. brevicompactum* UC7075. Error bars indicate standard deviations.

Can be seen how the decrease in the number of mould spores follow the increase in the acidity. The pH values of the recorded data show different degrees of acidity according to the sample, but the differences are minimal between the various samples, with a mean deviation of ± 0.4 pH between the highest value and the lowest. As seen previously strains *P. roqueforti* UC8455 and *P. brevicompactum* UC7075, however, tend to have a certain resistance to the lower pH, also observed in the latest images (the spore recorded after one week at 30°C in the sample of fermented milk created only with the starter strain, YOG, remain stable around $1x10^4$ spore/mL). Taking into account the results obtained with the inoculation of the inhibitors strains in yogurt (YOG+UC8491 and YOG+UC8490), is visible an inhibition activity, although there are a pH similar to samples containing only the starter strains (YOG). This can lead to the hypothesis that the inhibitory effect is not due exclusively to the production of lactic acid during the fermentation, and then lowering the pH, but rather the production of bacteriocins or other antifungal compounds by strains UC8491 and UC8490, thus determining a lack of development of moulds.

- **QUALITATIVE EVALUATION OF THE INHIBITION PROCESS IN FERMETED MILK**

To qualitatively assess the interaction between the two Lactic Acid Bacteria (*L. plantarum* UC8491 and *L. rhamnosus* UC8490), and the fungal culture was developed. Different concentration and mixtures of the microorganisms were used during the protocol. In each plate, was possible to create three replicate for single test, that to have a relevant statistical approach.

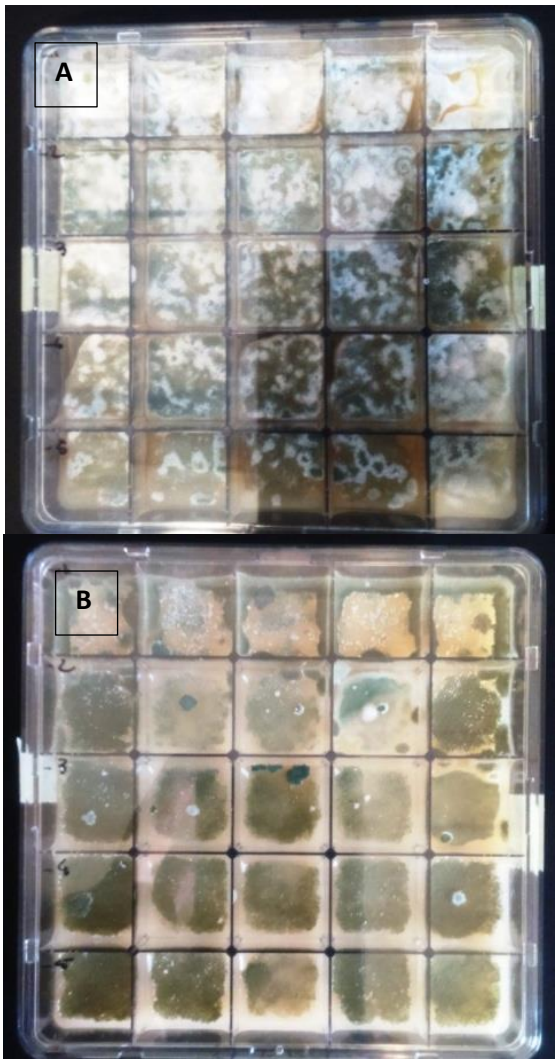


Figure 7: Example of square plates used for the fermented milk assay test effectuated with strain of *P. roqueforti* UC8455 (A) and *P. brevicompactum* UC7075 (B) (inoculum at 1%v/v) in fermented milk obtained using only the yogurt starter strains *St. thermophilus* UC9064 and *L. delbrueckii* subsp. *bulgaricus* UC8082.

Figure 7 (A and B) shows the constant development of moulds *P. roqueforti* UC8455 and *P. brevicompactum* UC7075 inoculated at 1% v/v in fermented milk obtained only with the bacteria starter, in decreasing dilution (from the top to down). From left to right instead can be seen the five replicates carried out for each dilution.

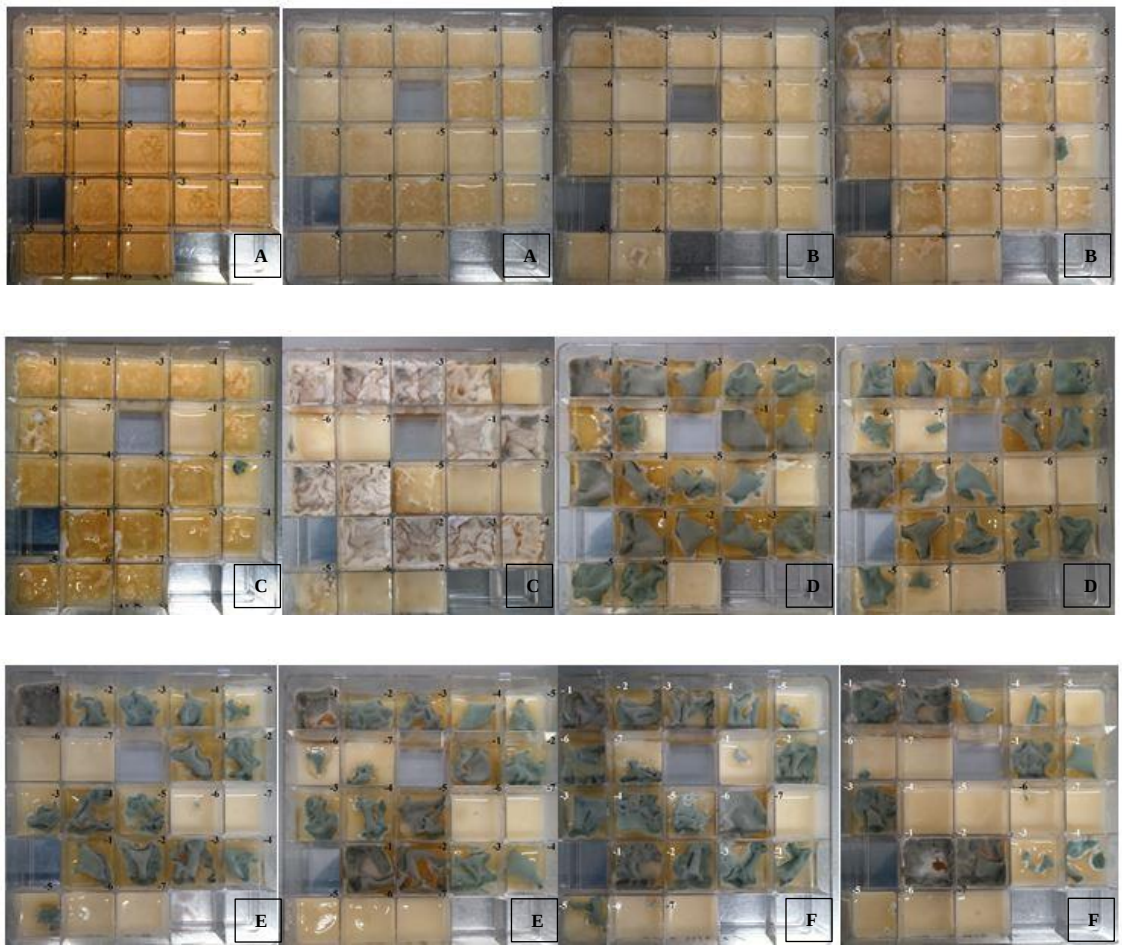


Figure 8: Images about the adoption of the square plate used during the fermented milk assay for testing the inhibition ability by *L. plantarum* UC8491 and *L. rhamnosus* UC8490 against *P. roqueforti* UC8455 In the following pictures is possible to see the difference inhibition attitude about the two LAB strains, if they are inoculated alone, in combination and at different percentage of inoculum (Images A: L. plantarum UC8491 at 1% and 3%; B: *L. rhamnosus* UC8490 at 1% and 3%; C: *L. plantarum* UC8491 and L. rhamnosus UC8490 inoculated in combination at 1% and 3%; D: *L. plantarum* UC8491 inoculated in combination at the yogurt starter strains at 1% and 3%; E: L. *rhamnosus* UC8490 inoculated in combination at the yogurt starter strains at 1% and 3%; F: *L. plantarum* UC8491 and *L. rhamnosus* UC8490 inoculated in combination at the yogurt starter strains at 1% and 3%). The same test was performed for the strain of *P. brevicompactum* UC7075 (images not showed).

Figure 8 is showing the results performed by using the strain *L. plantarum* UC8491 and *L. rhamnosus* UC8490 against the mould strain *P. roqueforti* UC8455 in fermented milk (the pictures were showed as an example of the test. Not all the pictures are showed in the following study). After one week at 30°C the plate were

evaluated through the evaluation system previously described and the results are showed on the Table 3 and Table 4. The results shown a constant and homogenous inhibition operated by *L. plantarum* UC8491 and *L. rhamnosus* UC8490 on the two mould, *P. roqueforti* UC8455 (Table 1) and *P. brevicompactum* UC7075 (Table 2). During the test different percentage of inoculum of inhibitory LAB were made to stress the inhibition ability and to evaluate if the modified concentration will determine different level of inhibition. Also combination with LAB yogurt starter *St. thermophilus* UC8455 and *L. delbrueckii* subsp. *bulgaricus* UC8096 were tested to demonstrate the inhibition attitude against fungal spoilage operated by the LAB with inhibitory ability in fermented milk. Different dilution of the mould were inoculated, for evaluate when the inhibition operated by the LAB is more efficient. Results shown that the highest inhibition is observed when the strain *L. plantarum* UC8491 and *L. rhamnosus* UC8490 are inoculated alone, above all when they're inoculated at 1% v/v, in fact especially for *L. rhamnosus* UC8490 at 3% v/v are showing not a constant inhibitory ability. Similar results are obtained when the inhibitory LAB are inoculated together at 3% v/v. In both case (in the test against *P. roqueforti* UC8455 and *P. brevicompactum* UC7075) the total or partial inhibition are starting only at the dilution $1x10^{-5}$, showing the difficult inhibition ability operated by the LAB, underling that the combination at higher concentration of this bacteria have a negative influence on the bio-protection of fermented milk. Overall, however, the results have a positive trend and showing a strong attitude to inhibition by the two strains of LAB. Less positive and unpredictable are the results obtained when LAB *L. plantarum* UC8491 and *L. rhamnosus* UC8490 are mixed with LAB yogurt starter, during the preparation of fermented milk. The resistance to inhibition of the mould is greater, and as it begins to have a positive value only at the decreasing concentration of mould in milk (on average, the inhibiting effect is positive at a dilution $1x10^{-5}$). The obtained results also differ greatly depending on the mould inoculated into milk, showing a different capability of resistance to

the inhibitory activity of the LAB. In particular, the strain of *P. roqueforti* UC8455 shows a level of resistance greater than the strain of *P. brevicompactum* UC7075, which appears to be more susceptible to the influence of inhibitory LAB even when it is at higher concentrations ($1x10^{-3}$), as shown by the results obtained from the sample YOG + MIX 3% (Table 2). The results obtained show, however, how the effect of inhibitors becomes decisive (when mixed with the bacteria starter) when the concentration of the moulds is around to 1x10-5, whereas at higher concentrations the inhibition tends to be milder. On these results it was also performed a statistical study based on a regression multinomial (cumulative logit), as reported below.

% LAB /CFU *P. roqueforti* UC8455	10^{-1}			10^{-2}			10^{-3}			10^{-4}			10^{-5}			10^{-6}			10^{-7}		
YOG	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
L. plantarum 1%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. plantarum 3%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. rhamnosus 1%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
L. rhamnosus 3%	2	1	1	1	0	0	0	0	0	0	0	0	0	0	1	3	0	1	0	1	0
Mix 1%	2	0	2	2	2	2	2	1	1	2	1	1	2	2	2	3	2	3	0	1	3
Mix3%	4	4	4	4	4	4	4	4	4	4	4	4	1	2	2	1	0	0	0	0	0
yog + *L. plantarum* 1%	4	4	4	4	4	4	4	4	4	4	4	3	3	3	1	3	3	3	2	1	0
yog + *L. plantarum* 3%	4	4	4	4	4	4	4	4	4	3	3	3	1	3	3	2	0	1	1	0	0
yog + *L. rhamnosus* 1%	4	3	3	2	3	3	3	2	3	3	3	3	2	3	2	0	0	0	0	0	0
yog + *L. rhamnosus* 3%	4	4	4	4	4	4	3	3	3	2	3	3	2	3	0	1	0	0	1	0	0
yog + mix 1%	4	1	4	4	4	4	4	4	3	3	4	3	2	3	2	2	2	1	2	0	0
yog + mix 3%	4	4	4	4	3	4	3	4	2	2	0	3	0	0	1	1	1	0	0	0	0

0	0%
1	25%
2	50%
3	75%
4	100%

Table 1: Analysis about the inhibition process performed by *L. plantarum* UC8491 and *L. rhamnosus* UC8490, inoculated in different concentration (1% and 3% v/v), alone or in combination against fungal spoilage *P. roqueforti* UC8455 The test took place in different fermented milk, obtained using only the selected bacteria having the inhibition ability or in a common fermented milk inoculated with starter yogurt strains (YOG) Each well was analysed and the inhibition rate was assessed based on the percentage of the development of moulds (from 0% to 100%).

% LAB/CFU *P. brevicompactum* UC7075	10^{-1}			10^{-2}			10^{-3}			10^{-4}			10^{-5}			10^{-6}			10^{-7}		
YOG	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
L. plantarum 1%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. plantarum 3%	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	0	0
L. rhamnosus 1%	1	0	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
L. rhamnosus 3%	0	0	0	1	3	2	3	4	4	4	4	4	0	0	0	0	0	0	0	0	0
Mix 1%	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Mix3%	4	4	4	4	4	4	4	4	4	4	4	0	0	0	0	0	0	0	0	0	0
yog + *L. plantarum* 1%	4	4	4	4	4	4	4	4	4	4	3	4	1	1	2	1	0	1	1	0	0
yog + *L. plantarum* 3%	4	4	4	4	4	4	4	4	4	0	4	0	0	0	0	0	0	0	0	0	0
yog + *L. rhamnosus* 1%	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	1	0	0	1	0	0
yog + *L. rhamnosus* 3%	4	4	4	4	4	4	4	4	4	4	4	0	0	0	0	0	0	0	0	0	0
yog + mix 1%	4	4	4	4	4	4	4	4	4	3	4	3	3	0	0	0	1	0	0	0	0
yog + mix 3%	4	4	4	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0	0%
1	25%
2	50%
3	75%
4	100%

Table 2: Analysis about the inhibition process performed by *L. plantarum* UC8491 and *L. rhamnosus* UC8490, inoculated in different concentration (1% and 3% v/v), alone or in combination against fungal spoilage *P. brevicompactum* UC7075. The test took place in different fermented milk, obtained using only the selected bacteria having the inhibition ability or in common fermented milk inoculated with starter yogurt strains (YOG). Each well was analysed and the inhibition rate was assessed based on the percentage of the development of moulds (from 0% to 100%).

From the previous table it was possible to perform the following statistical analysis.

***Penicillium brevicompactum* UC7075**					
Coefficients:	**Value**	**St.err**	**t**	**pvlaues**	**Sign. level**
dilution (10^{-7})	-3,7738	0,8003	-4,72	0,000	***
dilution (10^{-6})	-3,2287	0,7263	-4,45	0,000	***
dilution (10^{-5})	-2,6617	0,6686	-3,98	0,000	***
dilution (10^{-3})	1,604	0,6307	2,54	0,011	*
dilution (10^{-2})	1,8821	0,6350	2,96	0,003	**
dilution (10^{-1})	1,4401	0,6338	2,27	0,023	*
L.plantarum 1%	-17,7423	0,0000		0,000	***
L.plantarum 3%	-0,3266	0,8250	-0,40	0,692	
L.rhamnosus 3%	1,3069	0,7662	1,71	0,088	.
MIX 1%	-1,295	0,9492	-1,36	0,173	
MIX 3%	3,2287	0,8322	3,88	0,000	***
YOG+*L.plantarum* 1%	4,8896	0,8700	5,62	0,000	***
YOG+*L.plantarum* 3%	2,7881	0,8131	3,43	0,001	***
YOG+*L.rhamnosus* 1%	5,3268	0,9157	5,82	0,000	***
YOG+*L.rhamnosus* 3%	3,2287	0,8322	3,88	0,000	***
YOG+MIX1%	3,6753	0,8271	4,44	0,000	***
YOG+MIX3%	1,1254	0,7951	1,42	0,157	

A

***Penicllium roqueforti* UC8455**					
Coefficients:	**Value**	**St.err**	**t**	**pvlaues**	**Sign. level**
dilution (10^{-7})	-3,9065	0,6289	-6,21171	0,000	***
dilution (10^{-6})	-2,1034	0,5555	-3,78627	0,000	***
dilution (10^{-5})	-1,3694	0,5039	-2,71775	0,007	**
dilution (10^{-3})	0,7345	0,495	1,48376	0,138	
dilution (10^{-2})	1,5919	0,527	3,02089	0,003	**
dilution (10^{-1})	1,975	0,5554	3,55633	0,000	***
L.plantarum 1%	-11,1847	253,3032	-0,04416	0,965	
L.plantarum 3%	-11,1847	253,3032	-0,04416	0,965	
L.rhamnosus 3%	2,7962	1,1763	2,37703	0,017	*
MIX 1%	5,3424	1,188	4,49704	0,000	***
MIX 3%	7,4014	1,2216	6,05895	0,000	***
YOG+*L.plantarum* 1%	8,6389	1,267	6,81841	0,000	***
YOG+*L.plantarum* 3%	7,5115	1,219	6,16192	0,000	***
YOG+*L.rhamnosus* 1%	5,8302	1,1833	4,92721	0,000	***
YOG+*L.rhamnosus* 3%	6,6715	1,1968	5,57427	0,000	***
YOG+MIX1%	7,8225	1,2384	6,31678	0,000	***
YOG+MIX3%	5,9744	1,1872	5,03214	0,000	***

B

Table 3: Statistical results obtained through logit regression model. The "*" signify the level off significance. The tables refer to the study conducted on the ability of inhibition by the LAB on fungi *P. brevicompactum* UC7075 (A) and *P. roqueforti* UC8544

Tables 3 A and B show the statistical study carried out on the results obtained by the qualitative study of the inhibition. For the study was put the interaction between the LAB strains against the fungal strains and levels of dilutions of the latter. As a

reference for the study took into account the level of inhibition "4", this corresponds to the level of minimum inhibition activity. Negative values refer to how they approach the results of inhibition at the level value lost as a reference. Greater is the negative value, greater is the distance of the result from the reference level. The asterisks ("*") correspond instead to the degree of significance of the results. The greater the number of asterisks, the greater the degree of significance as reported in the following table (Table 4):

Signif. codes	
0	“***”
0.001	“**”
0.01	“*”
0.05	“.”
0.1	“
1	///

Table 4: Grade levels of statistical significance used in the study, to decrease the number of asterisks decreases the significance value of the results obtained

- **DISCUSSION**

Fungal spoilage is one of the main cause of substantial economic losses for milk products and might also been regarded as source of mycotoxins, involving public health problems (Ledenbach & Marshall, 2010). For these reasons, LAB (Lactic Acid Bacteria) may be considered an important alternative for the conservation of milk products, used in a bio-conservation system. In this study fifteen experimental fermented milk were prepared using different combination of yogurt starter strains selected for their ability in decreasing pH. Two common fungal milk spoilages, *P. roqueforti* UC8455 and *P. brevicompactum* UC7075 were inoculated, to assess

their ability to grow in fermented milk with low pH. The amounts of spore/mL after one week at 30°C were measured. From the first results obtained, it evinced that the spoilage fungi tend to conserve a resistance to the fermentation, according to their ability to develop in presence of low pH (Vivier, Rivemale, Ratomahenina, & Galzy, 1992; Carrillo-Inungaray et al., 2014). These results put on evidence that fermented milk/yogurt produced using only starter strain, even if able to decrease the average pH under 4, and the absence of some fungal preservative determine the development of spoilage, in according to other study that recorded the low pH tolerance by *Penicillium* spp. (Vivier, Rivemale, Ratomahenina, & Galzy, 1992; Gourama, 1997; Gerez, Torino, Rollán, & Font de Valdez, 2009; Sorrentino et al., 2013; Dhakar, Sharma, & Pandey, 2014). At this point, two selected LAB strains with antifungal activity were tested in fermented milk: *L. plantarum* UC8491 and *L. rhamnosus* UC8490. Strains of *L. plantarum* and *L. rhamnosus* are being studied by several research groups, thanks precisely to their already well-known antifungal ability, mainly related to their attitude on antimicrobial compound production, first of all the bacteriocins production, in fermented food (Strain et al., 2000; Prema, Smila, Palavesam, & Immanuel, 2008; Yang & Chang, 2010; Yang, Kim, & Chang, 2011; Gerbaldo, Barberis, Pascual, Dalcero, & Barberis, 2012; S Crowley, Mahony, & van Sinderen, 2012; Wang, Yan, Wang, Zhang, & Qi, 2012; Sarah Crowley & Bottacini, 2013; Sorrentino et al., 2013; Wang et al., 2013; Coman et al., 2014; Cortés-Zavaleta, López-Malo, Hernández-Mendoza, & García, 2014; Delavenne et al., 2014; Gupta & Srivastava, 2014; Ryu, Yang, Woo, & Chang, 2014; Sangmanee & Hongpattarakere, 2014; Trivedi, Jena, & Seshadri, 2014). In consideration of the above, in this study, it was therefore sought to test the inhibitory activity and thus the production of bacteriocins possible, in yogurt, by *L. plantarum* UC8491 and *L. rhamnosus* UC8490. The LAB was tested in vitro against suspension of the mould *P. brevicompactum* UC7075 and *P. roqueforti* UC 8455. The present test clearly show the antifungal activity of *L. plantarum* UC8491

and *L. rhamnosus* UC8490 on fungal suspension of *P. roqueforti* UC8455 and *P. brevicompactum* UC7075. The results agree with those reported by (Trias, 2008; Gomah, Ragab, & Bullerman, 2010; Gerbaldo, Barberis, Pascual, Dalcero, & Barberis, 2012; Trias, 2008; Gerbaldo et al., 2012), who assayed the effect of *Lactobacillus* spp. in vitro antifungal effects. Other researchers are obtained similar results in the in vitro growth fungal control (Magnusson, 2003; Magnusson, Ström, Roos, Sjögren, & Schnürer, 2003; Sjögren, Magnusson, Broberg, Schnürer, & Kenne, 2003; Schnürer & Magnusson, 2005; Muhialdin & Hassan, 2011; Ryan et al., 2011; Wang, Yan, Wang, Zhang, & Qi, 2012). As mentioned previously and as demonstrated by other study, LAB strains are producers of organic acids, bacteriocins and, in some case, hydrogen peroxide (M.V. Leal-Sánchez, R. Jiménez-Díaz, A. Maldonado-Barragán, A. Garrido-Fernández, 2002) (Gerbaldo et al., 2012), the presence of these substances in culture media can inhibit the development of fungal spoilage, as observed in the assay performed in the present study. Lactic acid acetic is the main products during the fermentation of carbohydrates by LAB, and are able to diffuse through the membrane of target organisms in their hydrophobic undissociated form and then reduce cytoplasmic pH, causing cell destruction (Dalié, Deschamps, & Richard-Forget, 2010; Gerbaldo et al., 2012; Gerez, Torres, Font de Valdez, & Rollán, 2013). Moreover several authors have reported that some LAB are able to antifungal metabolites that are sensitive to proteolytic enzyme, such as the bacteriocins production (Rouse, Harnett, Vaughan, & Sinderen, 2008; Muhialdin & Hassan, 2011; Lan, Chen, Wu, & Yanagida, 2012; Crowley, Mahony, & van Sinderen, 2013; Li et al., 2013). From another point of view, the strong inhibition activity can be attributed to a high competition between LAB and the fungi species in strictly conditions. Inhibition ability was tested in a fermented milk to test the LAB for their applicability like bio-preservative during the fermented milk production, because yet little is known the use of LAB during dairy products (Strain et al., 2000; Gougouli, Kalantzi,

eletsiotis, & Koutsoumanis, 2011; Delavenne et al., 2013; Li et al., 2013). The LAB strains selected for their ability in inhibiting mould (UC8491 and UC8490), have been at this point mixed with yogurt starter from that have shown the lower natural aptitude to inhibit the growth of moulds in milk (*St. thermophilus* UC9064 and *L. delbrueckii* subsp. *bulgaricus* UC8096), as well as to monitor the inhibitory potential of LAB *L. plantarum* UC8491 and *L. rhamnosus* UC8490. As noted starters in some way have a more or less inhibitory activity, determined probably by the degree of the pH. It can therefore hypothesize a synergistic inhibitory action determined by an increase in acidity in the load of the starter strains (lactic acid production) assembly to inhibitory action of the inhibitors bacteria, thanks to the metabolic products previously exposed, as exposed also by (Delavenne et al. , 2013, 2014). In fact, as can be seen from the results obtained in the milk, from the initial value of the inoculated mould (1x107 spores / mL), there is a decrease of approximately 3 log determined only by the acid action by bacteria starter, which increases for more 4 log when are added to the strains *L. plantarum* UC8491 and *L. rhamnosus* UC8490, supporting further as stated above. That there is an additional inhibitory action in addition to the action resulting from dropping the pH can also be seen with further tests carried out in this study. It is known that as the pH can be a constraint to the development of moulds (Carrillo-Inungaray et al., 2014), but can also see how the acidity values obtained from combinations of LAB in fermented milk used for the tests, all fall within a range very thin (values between ± 3.7 and ± 3.3). From these results it is then possible to envisage a further inhibitory action on fungi carried by *L. plantarum* UC8491 and *L. rhamnosus* UC8490 and not exclusively linked to the lowering of the pH. Finally the tests that aimed to identify the quality of inhibition, developed in this study (test with the square plates), shown that the inhibitory action is greater when the inhibitory strains are inoculated alone in fermented milk and not mixed with the yogurt starter strains. Is seen in fact how the effect of inhibition of decreases drastically with increasing

concentration of LAB, both in the mixture which inoculated individually. The strains inoculated at 1% v/v have a capacity of inhibition greater than when inoculated at 3% v/v, which is further reduced when they are mixed together (MIX 1% v/v and MIX 3% v/v). It is therefore clear how the degree of inhibition is closely linked to the concentration value of the strains inhibitors. The antagonistic effect, against moulds, is further reduced when the strains of *L. rhamnosus* UC8491 and *L. plantarum* UC8490 are mixed with the yogurt bacteria starter. The high concentration of LAB of different species in this case limits/mask the action of inhibition operated by strains the two strains of LAB, probably caused by systems of interaction between the bacteria linked to the mechanism of quorum sensing (Kuipers, De Ruyter, Kleerebezem, & De Vos, 1998; Bassler, 1999; Jersey & Jersey, 2001; Kleerebezem, 2004; Waters & Bassler, 2005). If the action of inhibition occurs especially when the lactic acid bacteria are found in lower concentrations, it should not be seen as a negative result, but rather it can be an important and positive point of view, especially for possible future use in food production, which then translates into a lower cost of use/production, as estimated also for other LAB strains in similar studies (Belguesmia et al., 2014). However, from these results, the inhibition effect is also evident in the case of mixtures of LAB, providing coverage to the development of fungi, without the use of chemical additives. In conclusion, the strains *L. plantarum* UC8491 and *L. rhamnosus* UC8490 can be seen as important and promising bio-protective strains in fermented milk. Furthermore, the inhibition capacity has been maintained for the period of storage at 30°C, then in situations conducive to fungal growth, thus emphasizing the capacity of the high inhibitory capacity of the two strains of LAB. Through the statistical study carried out using a probabilistic model of the multinomial regression (logit) carried out with the use of the software "R", it became possible to study the significance of the quantitative results obtained. The use of the multinomial regression is a method of analysis which ensures the obtaining of

results even in situations in which the variables of the data obtained are not quantitative, but rather qualitative or as a visual expression of a particular effect (Massaguer & Saraiva, 2011). With the following study is known as the inhibition values are statistically significant, in fact the higher the value of inhibition, the greater are detached from the result of "complete lack of inhibition" taken as a reference value. The study relates the degree of inhibition of the moulds with the strain of LAB used and its concentration of inoculum. At the decrease of the concentration of mould in the milk, the greater the degree of significance obtained. To conclude is possible to say that the results are all statistically significant, supporting the inhibitory effect of lactic acid bacteria and also the fermented milk protocol used in this study offers the opportunity to obtain statistically useful results, starting from qualitative data (it is the first time you used a statistical study on the potential for inhibition of lactic acid bacteria on the fungal spoilage, based on the use of a regression multinomial "logit").

- **CONCLUSION**

The fungal food spoilage still represent a serious problem for the food preservation, human health and an economic loss for the food industries. (Filtenborg et al., 1996; Rouse, Harnett, Vaughan, & Sinderen, 2008). Selected Lactic Acid Bacteria may represent the future for the natural food preservation, thanks for their natural ability on the antimicrobial compound's, such as benzoic and formic acid, hydrogen peroxide and, more important, bacteriocin (Stoyanova et al., 2010; Gerbaldo, Barberis, Pascual, Dalcero, & Barberis, 2012; Gupta & Srivastava, 2014). In the present study the ability of selected LAB *Lactobacillus plantarum* UC8491 and *Lactobacillus rhamnosus* UC8490 to inhibit mould food spoilage growth in milk was investigated. Results shown an high inhibitory activity against two common milk mould (*Penicillium brevicompactum* and *Penicillium roqueforti*), demonstrating the importance of selected LAB for the bio-protection of milk and

dairy food. Statistical approach was extremely important to understand and describe the inhibitory rate. In conclusion, the study are showing the positive use of LAB for the food protection, but further study are needed to largely understand and characterize the inhibitory action operated by selected LAB.

BIBLIOGRAPHY

. S. a. H., . T. M., & . K. A. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

a.M., M., P.N., K., L.C., B., J.M., S., & J.L., M. (2008). Effects of folate and vitamin B12 deficiencies during pregnancy on fetal, infant, and child development. *Food and Nutrition Bulletin*, *29*(2 SUPPL.), S101–S111. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed8&NEWS=N&AN=18709885

Abee, T., Rombouts, F. M., Hugenholtz, J., Guihard, G., & Letellier, L. (1994). Mode of action of nisin Z against Listeria monocytogenes scott a grown at high and low temperatures. *Applied and Environmental Microbiology*, *60*(6), 1962–1968.

Adams, M. R., & Nicolaides, L. (1997). Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control*, *8*(5-6), 227–239. doi:10.1016/S0956-7135(97)00016-9

Al-Kadamany, E., Khattar, M., Haddad, T., & Toufeili, I. (2003). Estimation of shelf-life of concentrated yogurt by monitoring selected microbiological and physicochemical changes during storage. *LWT - Food Science and Technology*, *36*(4), 407–414. doi:10.1016/S0023-6438(03)00018-5

Aly, S., T, O. C. a, N, B. I. H., & Alfred, T. S. (2006). Bacteriocins and lactic acid bacteria - a minireview. *African Journal of Biotechnology*, *5*(May), 678–683. doi:10.5897/AJB05.388

Arimi, S. M., Koroti, E., Kang'ethe, E. K., Omore, A. O., & McDermott, J. J. (2005). Risk of infection with Brucella abortus and Escherichia coli O157:H7 associated with marketing of unpasteurized milk in Kenya. *Acta Tropica*, *96*(1), 1–8. doi:10.1016/j.actatropica.2005.05.012

Arqués, J. L., Rodríguez, E., Nuñez, M., & Medina, M. (2011). Combined effect of reuterin and lactic acid bacteria bacteriocins on the inactivation of food-borne pathogens in milk. *Food Control*, *22*(3-4), 457–461. doi:10.1016/j.foodcont.2010.09.027

Bankole, S. a, & Adebanjo, a. (2003). Mycotoxins in food in West Africa : current situation and possibilities of controlling it. *Journal of Biotechnology*, *2*(September), 254–263. doi:10.4314/ajb.v2i9.14833

Benech, R. O., Kheadr, E. E., Laridi, R., Lacroix, C., & Fliss, I. (2002). Inhibition of Listeria innocua in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Applied and Environmental Microbiology*, *68*(8), 3683–90. doi:10.1128/AEM.68.8.3683

Black, R. (2003). Editorial Micronutrient deficiency — an underlying cause of morbidity and mortality, *81*(03808), 3808.

Blagojev, N., Škrinjar, M., Vesković-Moračanin, S., & Šošo, V. (2012). Control of mould growth and mycotoxin production by lactic acid bacteria metabolites. *Romanian Biotechnological Letters*, *17*(3), 7219–7226.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *International Journal of Food Microbiology*, *154*(3), 87–97. doi:10.1016/j.ijfoodmicro.2011.12.030

Branca, F., & Ferrari, M. (2002). Impact of micronutrient deficiencies on growth: The stunting

syndrome. *Annals of Nutrition and Metabolism*, *46*(SUPPL. 1), 8–17. doi:10.1159/000066397

Brul, S., & Coote, P. (1999). Preservative agents in foods: Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, *50*(1-2), 1–17. doi:10.1016/S0168-1605(99)00072-0

Caplice, E., & Fitzgerald, G. F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, *50*(1-2), 131–149. doi:10.1016/S0168-1605(99)00082-3

Cappa, F., & Cocconcelli, P. S. (2001). Identification of fungi from dairy products by means of 18S rRNA analysis. *International Journal of Food Microbiology*, *69*(1-2), 157–160. doi:10.1016/S0168-1605(01)00587-6

Castellano, P., Belfiore, C., Fadda, S., & Vignolo, G. (2008). A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Science*, *79*(3), 483–499. doi:10.1016/j.meatsci.2007.10.009

Cavera, V. L., Arthur, T. D., Kashtanov, D., & Chikindas, M. L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *International Journal of Antimicrobial Agents*, 7–14. doi:10.1016/j.ijantimicag.2015.07.011

Chung, T., & Axelsson, L. (1989). In vitro studies on reuterin synthesis by Lactobacillus reuteri. *Microbial Ecology in …*, *2*, 137–144. doi:10.3109/08910608909140211

Cintas, L. M., Herranz, C., HernÁndez, P. E., Casaus, M. P., Nes, I. F., & HernÁndez, P. E. (2001). Review: Bacteriocins of Lactic Acid Bacteria. *Food Science & Technology International*, *7*(4), 281–305. doi:10.1106/R8DE-P6HU-CLXP-5RYT

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*(1), 1–20. doi:10.1016/S0168-1605(01)00560-8

Conference, I., & Food, U. N. (2014). Food safety must accompany food and nutrition security-WHO Proceedings. *Nutrition and Food Safety*, *6736*(14), 1–4. doi:10.1016/S0140-6736(14)62037-7

Corsetti, a., Gobbetti, M., & Smacchi, E. (1996). Antibacterial activity of sourdough lactic acid bacteria: isolation of a bacteriocin-like inhibitory substance fromLactobacillus sanfranciscoC57. *Food Microbiology*, *13*(6), 447–456. doi:10.1006/fmic.1996.0051

Cortés-Zavaleta, O., López-Malo, a, Hernández-Mendoza, a, & García, H. S. (2014). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *International Journal of Food Microbiology*, *173*, 30–5. doi:10.1016/j.ijfoodmicro.2013.12.016

Crider, K. S., Bailey, L. B., & Berry, R. J. (2011). Folic Acid Food Fortification—Its History, Effect, Concerns, and Future Directions. *Nutrients*, *3*(3), 370–384. doi:10.3390/nu3030370

Crittenden, R. G., Martinez, N. R., & Playne, M. J. (2003). Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. *International Journal of Food Microbiology*, *80*(3), 217–222. doi:10.1016/S0168-1605(02)00170-8

Crowley, S., Mahony, J., & van Sinderen, D. (2013). Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends in Food Science & Technology*, *33*(2), 93–109. doi:10.1016/j.tifs.2013.07.004

Cui, Y., Zhang, C., Wang, Y., Shi, J., Zhang, L., Ding, Z., Cui, H. (2012). Class IIa bacteriocins: Diversity and new developments. *International Journal of Molecular Sciences*, *13*(12), 16668–16707. doi:10.3390/ijms131216668

de Carvalho, a. a. T., Mantovani, H. C., & Vanetti, M. C. D. (2007). Bactericidal effect of bovicin HC5 and nisin against Clostridium tyrobutyricum isolated from spoiled mango pulp. *Letters in Applied Microbiology*, *45*(1), 68–74. doi:10.1111/j.1472-765X.2007.02150.x

de Crécy-Lagard, V., El Yacoubi, B., de la Garza, R. D., Noiriel, A., & Hanson, A. D. (2007). Comparative genomics of bacterial and plant folate synthesis and salvage: predictions and validations. *BMC Genomics*, *8*(Figure 2), 245. doi:10.1186/1471-2164-8-245

Delavenne, E., Ismail, R., Pawtowski, A., Mounier, J., Barbier, G., & Le Blay, G. (2013). Assessment of lactobacilli strains as yogurt bioprotective cultures. *Food Control*, *30*(1), 206–213. doi:10.1016/j.foodcont.2012.06.043

Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin Production: a Probiotic Trait? *Applied and Environmental Microbiology*, *78*(1), 1–6. doi:10.1128/AEM.05576-11

Eijsink, V. G. H., Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H., & Nes, I. F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *81*(1-4), 639–654. doi:10.1023/A:1020582211262

Filtenborg, O., Frisvad, J. C., & Thrane, U. (1996). Moulds in food spoilage. *International Journal of Food Microbiology*, *33*(1), 85–102. doi:10.1016/0168-1605(96)01153-1

Fitzpatrick, T. B., Basset, G. J. C., Borel, P., Carrari, F., DellaPenna, D., Fraser, P. D., Fernie, A. R. (2012). Vitamin deficiencies in humans: can plant science help? *The Plant Cell*, *24*(2), 395–414. doi:10.1105/tpc.111.093120

Florou-Paneri, P., Christaki, E., & Bonos, E. (2013). Lactic Acid Bacteria as Source of Functional Ingredients. *Lactic Acid Bacteria-R& D for Food, Health and Livestock Purposes*, 589–614.

Gerbaldo, G. a, Barberis, C., Pascual, L., Dalcero, A., & Barberis, L. (2012). Antifungal activity of two Lactobacillus strains with potential probiotic properties. *FEMS Microbiology Letters*, *332*(1), 27–33. doi:10.1111/j.1574-6968.2012.02570.x

Gerez, C. L., Torino, M. I., Rollán, G., & Font de Valdez, G. (2009). Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control*, *20*(2), 144–148. doi:10.1016/j.foodcont.2008.03.005

Gerez, C. L., Torres, M. J., Font de Valdez, G., & Rollán, G. (2013). Control of spoilage fungi by lactic acid bacteria. *Biological Control*, *64*(3), 231–237. doi:10.1016/j.biocontrol.2012.10.009

Gourama, H. (1997). Inhibition of Growth and Mycotoxin Production of Penicillium By Lactobacillus Species. *Food Science & Technology-Lebensmittel-Wissenschaft & Technologie*, *30*(3), 279–283.

Gupta, R., & Srivastava, S. (2014). Antifungal effect of antimicrobial peptides (AMPs LR14) derived from Lactobacillus plantarum strain LR/14 and their applications in prevention of grain spoilage. *Food Microbiology*, *42*, 1–7. doi:10.1016/j.fm.2014.02.005

Harris, L. J., Fleming, H. P., & Klaenhammer, T. R. (1992). Novel paired starter culture system for sauerkraut, consisting of a nisin-resistant Leuconostoc mesenteroides strain and a nisin-producing Lactococcus lactis strain. *Applied and Environmental Microbiology*, *58*(5), 1484–1489. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=195629&tool=pmcentrez&rendertype=abstract

History, T. H. E., & Folic, O. F. (2001). Historical Review.

Hoey, L., Mcnulty, H., Duffy, M. E., & Hughes, C. F. (2015). EURRECA — Estimating Folate

Requirements for Deriving Dietary Reference Values EURRECA, *8398*(October), 1040–1050. doi:10.1080/10408398.2012.742858

Holzapfel, W. (1997). Use of starter cultures in fermentation on a household scale. *Food Control*, *8*(5-6), 241–258. doi:10.1016/S0956-7135(97)00017-0

Holzapfel, W. H., Geisen, R., & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology*, *24*(3), 343–362. doi:10.1016/0168-1605(94)00036-6

Iyer, R., Tomar, S. K., Uma Maheswari, T., & Singh, R. (2010). Streptococcus thermophilus strains: Multifunctional lactic acid bacteria. *International Dairy Journal*, *20*(3), 133–141. doi:10.1016/j.idairyj.2009.10.005

Jack, R. W., Tagg, J. R., & Ev, M. I. R. (1995). Bacteriocins of Gram-Positive Bacteria, *59*(2), 171–200.

Jones, R. J., Hussein, H. M., Zagorec, M., Brightwell, G., & Tagg, J. R. (2008). Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. *Food Microbiology*, *25*(2), 228–234. doi:10.1016/j.fm.2007.11.001

Journal, T. H. E., & Biolowxl, O. F. (1974). Folate Coenzymes faecalis*. *Cell*, 6–7.

Käferstein, F. (2003). Foodborne diseases in developing countries: aetiology, epidemiology and strategies for prevention. *International Journal of Environmental Health Research*, *13 Suppl 1*(July 2015), S161–S168. doi:10.1080/0960312031000102949

Kuipers, O. P., De Ruyter, P. G. G. a, Kleerebezem, M., & De Vos, W. M. (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology*, *64*(1), 15–21. doi:10.1016/S0168-1656(98)00100-X

Laiño, J. E., Juarez del Valle, M., Savoy de Giori, G., & LeBlanc, J. G. J. (2013). Development of a high folate concentration yogurt naturally bio-enriched using selected lactic acid bacteria. *LWT - Food Science and Technology*, *54*(1), 1–5. doi:10.1016/j.lwt.2013.05.035

Lan, W., Chen, Y., Wu, H., & Yanagida, F. (2012). Bio-protective potential of lactic acid bacteria isolated from fermented wax gourd. *Folia Microbiologica*, *57*(2), 99–105. doi:10.1007/s12223-012-0101-1

LeBlanc, J., de Giori, G., Smid, E. J., Hugenholtz, J., & Sesma, F. (2007). Folate production by lactic acid bacteria and other food-grade microorganisms. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, *1*, 329–339.

Lee, S. C., Billmyre, R. B., Li, A., Carson, S., Sykes, S. M., Huh, E. Y., Heitman, J. (2014). Analysis of a Food-Borne Fungal Pathogen Outbreak: Virulence and Genome of a Mucor circinelloides Isolate from Yogurt. *mBio*, *5*(4), e01390–14–. doi:10.1128/mBio.01390-14

Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., & De Vuyst, L. (2012). On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. *Food Microbiology*, *30*(2), 379–92. doi:10.1016/j.fm.2011.12.021

Lewus, C. B., Kaiser, a., & Montville, T. J. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Applied and Environmental Microbiology*, *57*(6), 1683–1688.

Li, H., Liu, L., Zhang, S., Uluko, H., Cui, W., & Lv, J. (2013). Potential use of Lactobacillus casei AST18 as a bioprotective culture in yogurt. *Food Control*, *34*(2), 675–680. doi:10.1016/j.foodcont.2013.06.023

Lollo, P. C. B., de Moura, C. S., Morato, P. N., Cruz, A. G., Castro, W. D. F., Betim, C. B., Amaya-Farfan, J. (2013). Probiotic yogurt offers higher immune-protection than probiotic whey beverage. *Food Research International*, *54*(1), 118–124. doi:10.1016/j.foodres.2013.06.003

Lukaski, H. C. (2004). Vitamin and mineral status: effects on physical performance. *Nutrition*, *20*(7-8), 632–644. doi:10.1016/j.nut.2004.04.001

Massa, S. (2014). Lactobacillus plantarum as a Strategy for an In situ Production of Vitamin B2. *Journal of Food & Nutritional Disorders*, *s1*(01), 1–5. doi:10.4172/2324-9323.S1-004

McAuliffe, O. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews*, *25*(3), 285–308. doi:10.1016/S0168-6445(00)00065-6

Mcnulty, H., Pentieva, K., Hoey, L., Strain, J. J., & Ward, M. (2012). Nutrition Throughout Life : Folate, *82*(5), 348–354. doi:10.1024/0300-9831/a000130

Messens, W., & De Vuyst, L. (2002). Inhibitory substances produced by Lactobacilli isolated from sourdoughs - A review. *International Journal of Food Microbiology*, *72*(1-2), 31–43. doi:10.1016/S0168-1605(01)00611-0

Motarjemi, Y., Käferstein, F., Moy, G., & Quevedo, F. (1993). Contaminated weaning food: a major risk factor for diarrhoea and associated malnutrition. *Bulletin of the World Health Organization*, *71*(1), 79–92.

Muhialdin, B. J., & Hassan, Z. (2011). Annals . Food Science and Technology BIOPRESERVATION OF FOOD BY LACTIC ACID BACTERIA AGAINST 1 . INTRODUCTION Lactic acid bacteria (LAB) are Gram-positive microorganisms , prefer anaerobic conditions but are aero-tolerant , catalase negative , cocci or.

Müller, O., & Krawinkel, M. (2005). Malnutrition and health in developing countries, *173*(3), 2000–2003.

Naidu, a. S., Bidlack, W. R., & Clemens, R. a. (1999). Probiotic Spectra of Lactic Acid Bacteria (LAB). *Critical Reviews in Food Science and Nutrition*, *39*(1), 13–126. doi:10.1080/10408699991279187

Nakajima, H., Toba, T., Ito, A., Kudo, S., Sato, S., & Sato, Y. (2003). The Screening of Hydrogen Peroxide-Producing Lactic Acid Bacteria and Their Application to Inactivating Psychrotrophic Food-Borne Pathogens. *Current Microbiology*, *47*(3), 231–236. doi:10.1007/s00284-002-3993-1

Niku-Paavola, M. L., Laitila, a, Mattila-Sandholm, T., & Haikara, a. (1999). New types of antimicrobial compounds produced by Lactobacillus plantarum. *J. Appl. Microbiol.*, *86*(1), 29–35. doi:10.1046/j.1365-2672.1999.00632.x

Nissen-Meyer, J., Oppegård, C., Rogne, P., Haugen, H. S., & Kristiansen, P. E. (2010). Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins. *Probiotics and Antimicrobial Proteins*, *2*(1), 52–60. doi:10.1007/s12602-009-9021-z

O'Sullivan, L., Ross, R. P., & Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie*, *84*(5-6), 593–604. doi:10.1016/S0300-9084(02)01457-8

Okereke, a, & Montville, T. J. (1991). Bacteriocin-mediated inhibition of Clostridium botulinum spores by lactic acid bacteria at refrigeration and abuse temperatures. *Applied and Environmental Microbiology*, *57*(12), 3423–8. Retrieved from

Onilude, a a, Fagade, O. E., Bello, M. M., & Fadahunsi, I. F. (2005). Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels.

Journal of Biotechnology, *4*(December), 1404–1408.

Piard, J. C., & Desmazeaud, M. (1991). Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Le Lait*, *71*(5), 525–541. doi:10.1051/lait:1991541

Powers, H. J. (2007). Folic acid under scrutiny. *The British Journal of Nutrition*, *98*(4), 665–666. doi:10.1017/S0007114507795326

Reisenaãœer, a N. N. a I., & Halsted, C. H. (1987). Issues and Opinions In Nutrition Human Folate Requirements, *45*(October 1986), 600–602.

Republic, C. (2002). Biosynthesis of Folates by Lactic Acid Bacteria and Propionibacteria in Fermented Milk. *Food Science*, *22*(5), 175–181.

Rossi, M., Amaretti, A., & Raimondi, S. (2011). Folate Production by Probiotic Bacteria. *Nutrients*, *3*(12), 118–134. doi:10.3390/nu3010118

Røssland, E., Andersen, G. I., Langsrud, T., & Sørhaug, T. (2003). Inhibition of Bacillus cereus by strains of Lactobacillus and Lactococcus in milk, *89*, 205–212. doi:10.1016/S0168-1605(03)00149-1

Rouse, S., Harnett, D., Vaughan, a., & Sinderen, D. Van. (2008). Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *Journal of Applied Microbiology*, *104*, 915–923. doi:10.1111/j.1365-2672.2007.03619.x

Samelis, J., Kakouri, a., Rogga, K. J., Savvaidis, I. N., & Kontominas, M. G. (2003). Nisin treatments to control Listeria monocytogenes post-processing contamination on Anthotyros, a traditional Greek whey cheese, stored at 4°C in vacuum packages. *Food Microbiology*, *20*(6), 661–669. doi:10.1016/S0740-0020(03)00018-2

Sangmanee, P., & Hongpattarakere, T. (2014). Inhibitory of multiple antifungal components produced by Lactobacillus plantarum K35 on growth, aflatoxin production and ultrastructure alterations of Aspergillus flavus and Aspergillus parasiticus. *Food Control*, *40*, 224–233. doi:10.1016/j.foodcont.2013.12.005

Santos, F., Wegkamp, a., de Vos, W. M., Smid, E. J., & Hugenholtz, J. (2008). High-Level Folate Production in Fermented Foods by the B12 Producer Lactobacillus reuteri JCM1112. *Applied and Environmental Microbiology*, *74*(10), 3291–3294. doi:10.1128/AEM.02719-07

Schillinger, U., Geisen, R., & Holzapfel, W. H. (1996). Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends in Food Science & Technology*, *7*(5), 158–164. doi:10.1016/0924-2244(96)81256-8

Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology*, *16*(1-3), 70–78. doi:10.1016/j.tifs.2004.02.014

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *25*(2), 146–151. doi:10.1080/02652030701567442

Soomro, a. H., Masud, T., & Anwaar, K. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

Stoyanova, L. G., Ustyugova, E. A., Sultimova, T. D., Bilanenko, E. N., Fedorova, G. B., Khatrukha, G. S., Pirogovskaya, B. (2010). New Antifungal Bacteriocin-Synthesizing Strains of Lactococcus lactis ssp . lactis as the Perspective Biopreservatives for Protection of Raw Smoked Sausages Department of Microbiology , Faculty of Biological , M . V . Lomonosov

Moscow State University Len, *5*(4), 477–485.

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, M. H. N., & Hugenholtz, J. (2003). Effects of Cultivation Conditions on Folate Production by Lactic Acid Bacteria. *Metab. Eng.*, *69*(8), 4542–4548. doi:10.1128/AEM.69.8.4542

Thu, B. D., Schultink, W., Dillon, D., Gross, R., Leswara, N. D., & Khoi, H. H. (1999). Effect of daily and weekly micronutrient supplementation on micronutrient deficiencies and growth in young Vietnamese children. *The American Journal of Clinical Nutrition*, *69*(1), 80–86.

Tiwari, B. K., Valdramidis, V. P., O' Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of Natural Antimicrobials for Food Preservation. *Journal of Agricultural and Food Chemistry*, *57*(14), 5987–6000. doi:10.1021/jf900668n

Trias, R. (2008). *Lactic acid bacteria as bioprotective agents against foodborne pathogens and spoilage microorganisms in fresh fruits and vegetables*. Retrieved from http://www.tdx.cat/handle/10803/7932

Unnevehr, L. (2015). Food safety in developing countries: Moving beyond exports. *Global Food Security*, *4*, 24–29. doi:10.1016/j.gfs.2014.12.001

Unnevehr, L., & Grace, D. (2013). Tackling Aflatoxins : An Overview of Challenges and Solutions, (November), 3–4.

Valyasevi, R., & Rolle, R. S. (2002). An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: Scope for their improvement. *International Journal of Food Microbiology*, *75*(3), 231–239. doi:10.1016/S0168-1605(01)00711-5

Vaughan, a., Eijsink, V. G. H., O'Sullivan, T. F., O'Hanlon, K., & Van Sinderen, D. (2001). An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. *Journal of Applied Microbiology*, *91*(1), 131–138. doi:10.1046/j.1365-2672.2001.01365.x

Walton, E., & Allen, S. (2011). Malnutrition in developing countries. *Paediatrics and Child Health*, *21*(9), 418–424. doi:10.1016/j.paed.2011.04.004

Wong, H., & Chen, Y. (1988). Effects of Lactic Acid Bacteria and Organic Acids Germination of Bacillus cereus Growth and. *Microbiology*, *54*(9), 2179–2184.

World Health Organization. (2014). Antimicrobial resistance. *Who*, *317*(September), 609–610. Retrieved from http://www.who.int/mediacentre/factsheets/fs194/en/#

Yang, E., Fan, L., Jiang, Y., Doucette, C., & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express*, *2*(1), 48. doi:10.1186/2191-0855-2-48

Yang, R., Johnson, M. C., & Ray, B. (1992). Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology*, *58*(10), 3355–3359.

CHAPTER III

FOODBORNE PATHOGENIC BACTERIA INHIBITION IN MILK BY SELECTED LACTIC ACID BACTERIA WITH ANTIMICROBIAL PROPERTIES

ABSTRACT

Chemical preservatives have been traditionally used during the manufacturing of processed products. However the continuous growing of consumers for fresh and natural products makes it necessary to search for alternative and natural compounds. In this context, food industries have been widely investigating and using Lactic Acid Bacteria as natural preservative, due to their ability to acidify and to produce antibacterial compounds. Is nowadays known that Lactic Acid Bacteria are able to prevent the grown of pathogenic bacteria in fermented food, representing a greater alternative to the synthetically compounds for increase the food shelf-life and to prevent human foodborne. The present study are focused to analyze the antibacterial activity of two selected LAB, *Lactobacillus plantarum* UC491 and *Lactobacillus rhamnosus* UC8490 during milk fermentation, against three pathogenic bacteria (*Listeria monocytogenes* L211, *Bacillus cereus* EELA72 and *Salmonella infantis* EELA72) and one non-pathogenic bacteria (*Listeria innocua* L113). For analyze the inhibitory activity performed by the selected LAB, commercial yogurt were inoculated with the pathogens and the bacteria development were analyze. Subsequent the results obtained were compared with them obtained in fermented milk obtained with inoculum of selected LAB. Antimicrobial assay and CFU count were used. Results shown an effective inhibitory performance carried out by the two LAB during milk fermentation process, underling the potential of LAB in the prevention of pathogenic foodborne bacteria.

Key words: Foodborne pathogenic bacteria, *Listeria* spp., *Bacillus cereus, Salmonella infantis,* Inhibitory activity by Lactic Acid Bacteria, *L. plantarum* and *L. rhamnosus,* fermented milk.

INTRODUCTION

Fermented foods are largely consumed widely worldwide, and commercially produced cultured milk is now popular with the consumers. Fermented food consumption have many advantages including enhanced nutritional value, digestibility, therapeutic benefits and finally, safety against pathogens foodborne (Caplice & Fitzgerald, 1999). The food fermentation is one of oldest food processing technologies for food preservation, known to man. Actually consumers are concerned about the use of chemical preservatives in food production, and about the possible health problem related to that (Aly et al., 2006). Artificial drying, vacuum packaging and ionizing are other different type of food conservation process. But this untreated food can be harbor for different foodborne pathogens, which can grow under refrigeration or without oxygen. A solution for this problem is the use of microorganism, especially bacterial strains, and their production of antimicrobial compound during the fermentation process (Lewus, Kaiser, & Montville, 1991; Yang, Johnson, & Ray, 1992; Cintas et al., 2001; Aly et al., 2006). It must to also remember that there are many parts of the world where the preservation role is still played by the fermentation process, underling the importance of this common food process (Caplice & Fitzgerald, 1999). Lactic acid bacteria (LAB) play the predominant role in this food process and are naturally used in a wide range of food production such us fish, meat, cereals, milk, fruits and vegetables. Through their action, LAB, confer desirable sensory characteristics and improve quality and safety. LAB are also able to inhibit the development and the survival of the spoilage microbiota and foodborne pathogens that can contaminate the raw material (Adams & Nicolaides, 1997). The antimicrobial activities performed by the LAB have been the subjects of numerous studies, but is clear that this bacteria strain play a very important role in the production of safe food. Their principal inhibitory effect are produced through the fermentative pathway that they use to generate cellular energy, producing organic acids, principal lactic, with a

consequence decrease of the pH (commonly to 3.5 – 4.5 pH). Acidity is the most important inhibitory agent, but another anti-microbial factors are now receiving a great interest by the scientific world. The most important anti-microbial compounds are the bacteriocins (Lewus et al., 1991; Jack, Tagg, & Ev, 1995; Schillinger, Geisen, & Holzapfel, 1996; Ennahar, Sonomoto, & Ishizaki, 1999; Cintas et al., 2001; Aly et al., 2006; Nandakumar & Talapatra, 2014; Cavera, Arthur, Kashtanov, & Chikindas, 2015). Strain of *L. plantarum* and *L. rhamnosus* were been study for their ability on bacteriocin productions (Diep, Håvarstein, & Nes, 1996; Niku-Paavola, Laitila, Mattila-Sandholm, & Haikara, 1999; M.V. Leal-Sánchez, R. Jiménez-Díaz, A. Maldonado-Barragán, A. Garrido-Fernández, 2002; Wilson, Sigee, & Epton, 2005; Dal Bello et al., 2007; Prema, Smila, Palavesam, & Immanuel, 2008; E. J. Yang, Kim, & Chang, 2011; Crowley, Mahony, & van Sinderen, 2012; Coman et al., 2014; Gupta & Srivastava, 2014; Sorrentino et al., 2013; Wang et al., 2013; Song, Zhu, & Gu, 2014; Delavenne et al., 2015). In this study the antimicrobial effect of *L. plantarum* UC8491 and *L. rhamnosus* UC8490, against three foodborne pathogenic species *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella* spp. and one non-pathogenic strain *Listeria innocua* was investigated.

- **Aim of the study**

The present study is focused on the analysis of the ability of selected LAB strain to inhibit foodborne pathogens in milk, with the purpose to evaluate the practical use of LAB as natural bio preservatives in dairy products against foodborne pathogens.

MATERIALS AND METHODS

- **Microorganisms and culture conditions:**

Lactobacillus plantarum (UC8491), *Lactobacillus rhamnosus* (UC8490) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*,) (ATCC15808), were grown in MRS agar (de man-Rogosa-Sharpe, Oxoid), instead the strain of *Streptococcus thermophilus* (UC9050) in M17 agar (Oxoid). Three pathogenic strains *Listeria monocytogenes* (L211), *Bacillus cereus* (EELA72), *Salmonella infantis* (EELA72) and one nonpathogenic strain *Listeria innocua* (L113) were used during the analysis. These four strains were stored at -120°C, in TSA (Tryptone Soya Agar, LabM).

- **Cultivation of bacterial strains for yogurt inoculation:**

Lactobacillus plantarum (UC8491), *Lactobacillus rhamnosus* (UC8490) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (ATCC15808) were reactivated without agitation in MRS broth (LabM) at 37°C in over-night. The *St. thermophilus* strain UC9050 was reactivated in M17 lactose broth (LabM) at 42°C in over-night without agitation. The *Listeria* spp. strains (L211 and L113) were reactivated in TSB (LabM), the *Bacillus cereus* (EELA72) and the *Salmonella infantis* (EELA72) strains were reactivated in BHI (LabM) and stored in overnight at 37°C. At growth occurred the samples strains were passed three times in specific agar plate for stabilize the strain before the analysis test.

- **Production of yogurt/fermented milk samples**

Reconstituted non-fat powder milk (Oxoid) was sterilized at 90°C for 30 min. After pasteurization the reconstituted milk was rapidly cooled to 45°C before inoculation with the selected yogurt strain and pathogens mentioned previously. Samples of 35 mL of milk were prepared before the inoculum in sterile 50 mL tubes. Once

controlled the stability for each strain in medium broth, *L. plantarum* UC8491, *L. rhamnosus* UC8490, *St. thermophilus* UC9050 and *L. delbrueckii* subsp. *bulgaricus* ATCC15808, were inoculated in reconstituted non-fat powder milk at 1% v/v (10 mL of total volume of each samples) and used after the fermentation (18 hours at 42°C for *St. thermophilus* strain and 37°C for the LAB strains). Subsequent fermented milk preparation was made, according to the following LAB combination:

a. YC (Classic Yogurt): *St. thermophilus* UC9050 (1% v/v) + L. *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC15808 (1% v/v)
b. YP: Classic Yogurt + *L. plantarum* UC8491 (1% v/v)
c. YR: Classic Yogurt + *L. rhamnosus* UC8490 (1% v/v)
d. YMIX: Classic Yogurt + *L. plantarum* UC8491 + *L. rhamnosus* UC8490 (1% v/v)

Actively growing cultures strains of pathogens were subsequent added separately to the inoculated yogurt. Fermentation was carried out at 42°C for promote the LAB growth. Before their inoculation, the pathogenic strains were adjusted (after 18 hours stored at 37°C) with a spectrophotometer analysis, for obtain an OD of 0.16 to 0.21at 625nm wavelength, to achieve a homogeneous concentration of approximatively 10^8 CFU/mL for each strains. Once obtained the correct concentration, milk samples of 35 mL v/v were prepared with the LAB combination showed previously and with the pathogens strains (1% v/v for each strain), following the present combination:

Samples	*L. monocytogenes* L211	*L. innocua* L113	*B. cereus* EELA72	*S. infantis* EELA72
YC T0	YC LM T0	YC LI T0	YC BC T0	YC SAL T0
YP T0	YP LM T0	YP LI T0	YP BC T0	YP SAL T0
YR T0	YR LM T0	YR LI T0	YR BC T0	YR SAL T0
YMIX T0	YMIX LM T0	YMIX LI T0	YMIX BC T0	YMIX SAL T0
YC T8	YC LM T8	YC LI T8	YC BC T8	YC SAL T8
YP T8	YP LM T8	YP LI T8	YP BC T8	YP SAL T8
YR T8	YR LM T8	YR LI T8	YR BC T8	YR SAL T8
YMIX T8	YMIX LM T8	YMIX LI T8	YMIX BC T8	YMIX SAL T8
YC T12	YC LM T12	YC LI T12	YC BC T12	YC SAL T12
YP T12	YP LM T12	YP LI T12	YP BC T12	YP SAL T12
YR T12	YR LM T12	YR LI T12	YR BC T12	YR SAL T12
YMIX T12	YMIX LM T12	YMIX LI T12	YMIX BC T12	YMIX SAL T12

Table 1: Different combinations of the bacterial strains used for the preparation of the of fermented milk samples. Samples of fermented milk (YC, YP, and YR, YMIX) were inoculated with strains of pathogens LM (*L. monocytogenes* L211), LI (*L. innocua* L113), BC (*B. cereus* EELA72) and SAL (*S. infantis* EELA72) and monitoring the growth of bacteria occurred in three time step (T0, T8 and T12).

Subsequently, the samples of 35 mL of milk inoculated with the strain, in according with the different combination, were incubated at 42°C for perform the fermentation process.

- **Persistence of pathogens strain in fermented milk**

The growth rate of pathogens strain was analyzed in raw milk and in commercial fermented milk, following the procedure previously described. Pasteurized milk and common fermented milk were stored at 42°C for to promote the fermentation carried out by the LAB, for 18 hours and subsamples (1 mL) were collected at three time step for monitor the growth rate of each bacteria. Raw milk was instead stored at 30°C for 18 hours. Viable counts were determined by decimal dilution and planted in the appropriate selective agar medium. In order to determine the survival trend of pathogenic strains, at each time step (T0, T8 and T12) were made serial dilutions of each sample (1 mL) and subsequent plating in selective medium were carried out in order to determine the value of CFU/mL for each bacterial strain. Selective medium agar were used, M17 agar (LabM) for the strains of *St. thermophilus*, MRS agar (LabM) for the strain of *L. plantarum*, *L. rhamnosus* and *L. delbrueckii* subsp. *bulgaricus*, BHI agar (LabM) for the strain of *Bacillus cereus*, Salmonella agar (HIMEDIA) for the strain of *Salmonella infantis* and finally

Harlequin Listeria Chromogenic agar (ISO) (LabM) for the strain of *Listeria monocytogenes* and *Listeria innocua*. The plates were incubated at 37 ° C for 18 hours to perform the development. pH was determined at three time steps (T0, T8 and T12) using a pH meter, after calibrating using standard buffers at pH 4 and 7.

- **STATISTICAL ANALYSIS:**

All the trials were repeated three times. The results obtained were performed using a one-way analysis of variance (ANOVA) on the replicates at 95% of significance. Significant results were referring to $p < 0.05$.

RESULTS AND DISCUSSION

- **Pathogens bacteria growth and survival in yogurt**

This study, aim to analyzed the ability of LAB strains to produce antimicrobial compounds in fermented milk. The firts stept was to test the bacteria growth ability in milk and incubated for 12 hours 30° C. The results obtained are showed in the Table 2, a uniform development was observed for all strains in raw milk. As milk is an optimum growth environment for pathogenic strains *L. monocytogenes* L211, *L. innocua* L113, *S. infantis* EELA72 and *B. cereus* EELA72 reached after 12 hours, a rate a growth of $1x10^6$ CFU7mL. The milk pH (6.5 – 6.7) and the incubation temperature (30 ° C), in fact confer an excellent growth environment for pathogenic bacteria, as already reported by Adams & Nicolaides (1997). In particular, there is an increase in the concentration (CFU/mL) of almost 3 log for the strain *B. cereus* EELA72, that passing from ±1x103 to ± 1x107, an increase of almost 4 log. Other strains, *L. monocytogenes* L211, *L. innocua* L113 and *S. infantis* EELA72, show a positive development, but less pronounced, but still showing an increase in their concentration of about 1 log. These early findings are supported by previous work carried out on these pathogenic strains, which stresses the importance of the fermentation process as a system of inhibiting the

development of pathogenic bacteria metabolic (Wong & Chen, 1988; Leyer & Eric, 1992; Røssland, Andersen, Langsrud, & Sørhaug, 2003; T, Langsrud, & Sørhaug, 2005; Wilson, Sigee, & Epton, 2005; De Keersmaecker et al., 2006; Trias, 2008; Y. Yang, 2008; Ladeuze, Lentz, Delbrassinne, Hu, & Mahillon, 2011; E. Yang, Fan, Jiang, Doucette, & Fillmore, 2012).

Pathogens \ time of incubation	T0	T8	T12
***Listeria monocytogenes* L211**	3,88E+05	1,00E+06	1,00E+06
***Listeria innocua* L113**	3,68E+05	1,00E+06	1,00E+06
***Salmonella infantis* EELA72**	3,56E+05	1,00E+06	1,00E+06
***Bacillus cereus* EELA72**	4,00E+03	1,00E+06	1,00E+06

Table 2: Growth rate (CFU/mL) of pathogenic strains in raw milk. The data was collected in triplicate for each sample during different time step (T0: time of inoculum; T8: after eight hours of fermentation; T12: after 12 hours of fermentation) and stored at 30°C. All the results are statistically significant ($p < 0.05$).

The growth kinetiks and the intereaction among differents pathogenics strains *L. monocytogenes* L211, *L. innocua* L113, *S. infantis* EELA72 and *B. cereus* EELA72 and yogurth starter cultures was investigated. Commercial yogurt was used to inoculate pasteurized raw milk to produce experimental yogurt, characterized by an acidic pH values (Figure 1), with an average of about 3.8 after 12 hour of fermentation. Pathogenic strains were inoculated to a concentration of 1% v/v ($1x10^8$ CFU/Ml) in samples of 35 mL of yogurt and finally stored at a temperature of 42°C to facilitate the milk fermentation. It was carried out withdrawals of subsamples of 1mL, in three time intervals (T0, T8 and T12) for the monitoring of the development of pathogenic strains during the three fermentation steps.

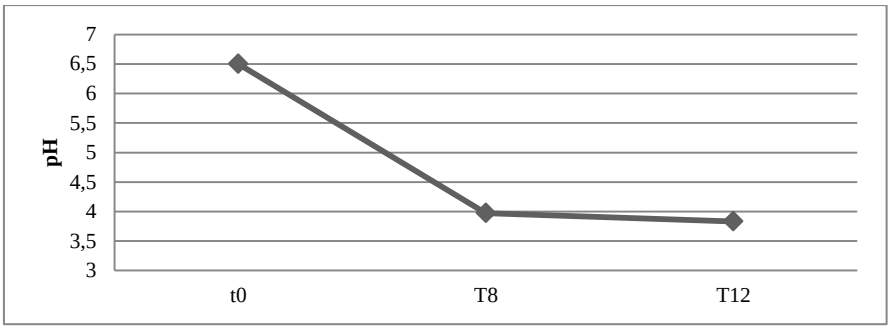


Figure 1: pH trend of commercial fermented milk. All the data were collected in triplicate in three different time steps to monitor the development acidity during the fermentation process. Error bars indicate standard deviations.

Pathogens (CFU/mL)/ time of incubation	T0	T8	T12
LM L211	9,30E+05	9,00E+03	< 10
LI L113	1,30E+06	2,93E+05	< 10
SAL EELA72	4,10E+05	< 10	< 10
BC EELA72	1,05E+04	5,00E+01	< 10

Table 3: Resistance (log CFU/mL) of pathogenic strains (LM: *Listeria monocytogenes* L211; LI: *Listeria innocua* L113; SAL: *Salmonella infantis* EELA72; BC: *Bacillus cereus* EELA72) in commercial fermented milk. The data was collected in triplicate for each sample different time step (T0: time of inoculum; T8: after eight hours of fermentation; T12: after 12 hours of fermentation). The results are statistically significant ($p < 0,05$).

Table 3 shows the results obtained. As expected, differently from the results obtained in raw milk, the numbers (CFU/mL) of pathogenic decreased with the progress of the fermentation process. Strains most affected by the sudden lowering of pH, are *Listeria monocytogenes* L211 (-2 log CFU/mL in the first eight hours and -6 log CFU/mL at 12 hours of fermentation, $p < 0.05$) and *Salmonella infantis* EELA72 (-5 log CFU/mL in eight hours and -6 log CFU/mL in 12 hours, $p<0.05$), showing a gradual but steady decline in their concentration, since to the total inhibition occurred at 12 hours of fermentation. This inhibitory effect is certainly to be attributed to the rapid drop of pH during fermentation milk and the high sensitivity to acidification especially by the strains of *Listeria monocytogenes* L211 and *Salmonella infantis* EELA72. The strong sensitivity to acidification by *Listeria* and *Salmonella* strains was also highlighted by other previous studies (Lewus,

Kaiser, & Montville, 1991; Leyer & Eric, 1992; Adams & Nicolaides, 1997; Tadesse et al., 1999; Messens & De Vuyst, 2002; Wilson, Sigee, & Epton, 2005; De Keersmaecker et al., 2006; Mufandaedza, Viljoen, Feresu, & Gadaga, 2006; Trias, 2008; Yang, Fan, Jiang, Doucette, & Fillmore, 2012; Tulini et al., 2014). The strain of *B. cereus* EELA72, showed a greater survival durign the fermentation of milk. There is indeed a considerable decrease in the concentration, of ± 4 log CFU/mL in the ones eight hours, which however remains stable even after 12 hours of fermentation. One would expect a total inhibition, dictated by the strong decrease of the pH, which in this case does not take place. Studies show that the pH is a limiting factor to the development of the species, attributing to 4.9 the value of pH useful in the normal growth of *B. cereus*, as also supported by further studies (Wong & Chen, 1988; Little & Knøchel, 1994; Wong, 1997; Røssland et al., 2003; T, Langsrud, & Sørhaug, 2005; Shen, Yu, & Chou, 2008). Other study speculate that the survival ability of certain strains of *B. cereus* in milk during the fermented to a pH of 3.8, are probably determine by an speed spore production during the early stages of the fermentation (Røssland, Andersen, Langsrud, & Sørhaug, 2003), but in the current study no spore were observed in samples. It is noted that the strain of *L. innocua* L113, showing the greater capacity of resistance to acidity. In fact, after 12 hours of fermentation is visible only a reduction of ± 1 log, underlining the strong adaptation to low pH, as further studies also show this supposed adaptation /resistance to low pH by some strains of *L. innocua* (Phan-Thanh, Mahouin, & Aligé, 2000; the Marc et al., 2002). We note, however, from these first results, when compared with those obtained from raw milk, as the process of fermentation and then the acidifying activity of lactic acid bacteria present in the commercial fermented milk, is already a natural obstacle to the normal development of some pathogenic bacteria, which otherwise they would find in milk an excellent growth environment.

- **Inhibition of pathogen bacteria in fermented milk by selected LAB: *L. plantarum* UC8491 and *L. rhamnosus* UC8490**

L. plantarum UC8491 and *L. rhamnosus* UC8490 were selected for their ability to produce anti-microbial compounds associated with the ability of acidification of milk during fermentation, to assess the capacity of inhibition of the pathogenic bacteria in milk. After grown in milk, the strains were inoculated in pasteurized milk, at concentration of 1% v/v, (Lp and Lr) or mixed (MIX). A strain of *St. thermophilus* UC9050 and *L. delbrueckii* subsp. *bulgaricus* (*L.bulgaricus*) ATCC15085, were used as yogurt starter bacteria for the production of fermented milk samples. Figures 2 shows the pH trend in milk, after inoculation of the two selected strains of LAB, and it tend to decrease rapidly in the early stages of fermentation, especially in the first eight hours, where the pH drops an average of two degrees with respect to the initial value of the milk. It is also noted how the two strains of LAB have a different attitude to acidify; in fact, the strain of *L. rhamnosus* UC8490, after the first eight hours of fermentation, continues its process of production of lactic acid, lowering further the degree of pH, reaching the threshold of 3.5 pH after 12 hours of fermentation.

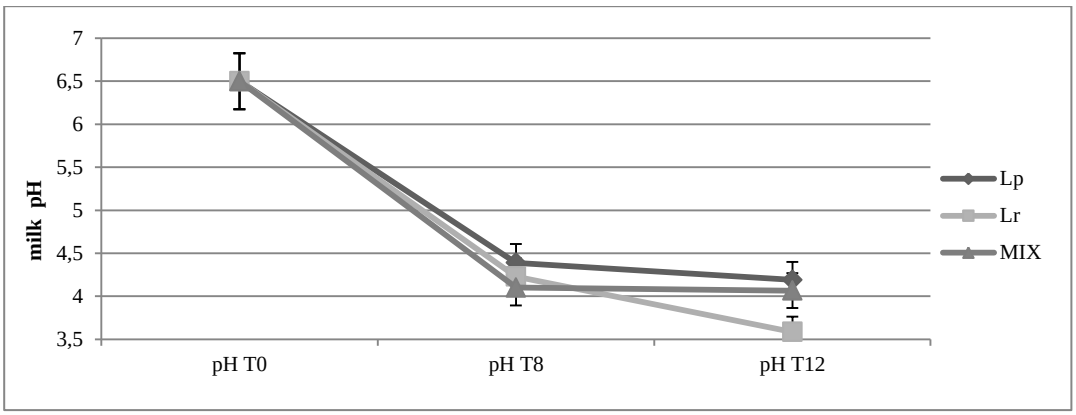


Figure 2: pH trend of fermented milk produced with selected LAB strains (Lp: *L. plantarum* UC8491; Lr: *L. rhamnosus* UC8490; MIX: both lab strain inoculated simultaneously). Data were collected in triplicate. Error bars indicate standard deviations.

L. plantarum UC8491 strain, however tends to maintain a value of acidity constant, even after 12 hours of fermentation, stopping the degree of acidity of around 4.1. Finally, the value of acidity of the fermented milk product with both strains of LAB, is stable around 4.09, probabily depending by the sinergism of the two strains. At the same time it was examined the development (CFU/mL) of individual strains in milk (Figure 3), during the 12 hours of fermentation. The development of the strain *L. plantarum* UC8491 displays a horizontal trend, maintaining the fixed concentration around 1x107 CFU/mL, with a slight drop in the number of CFU after 12 hours. On the contrary *L. rhamnosus* UC8490 shows a positive progression, showing how the milk represents a good growth environment for this strain. It is known in fact an increase of 2 log (from 1x107 CFU/mL to 1x109 CFU/mL) after 12 hours of fermentation. This increase in concentration can also justify the increase in the value of acidity, previously shown.

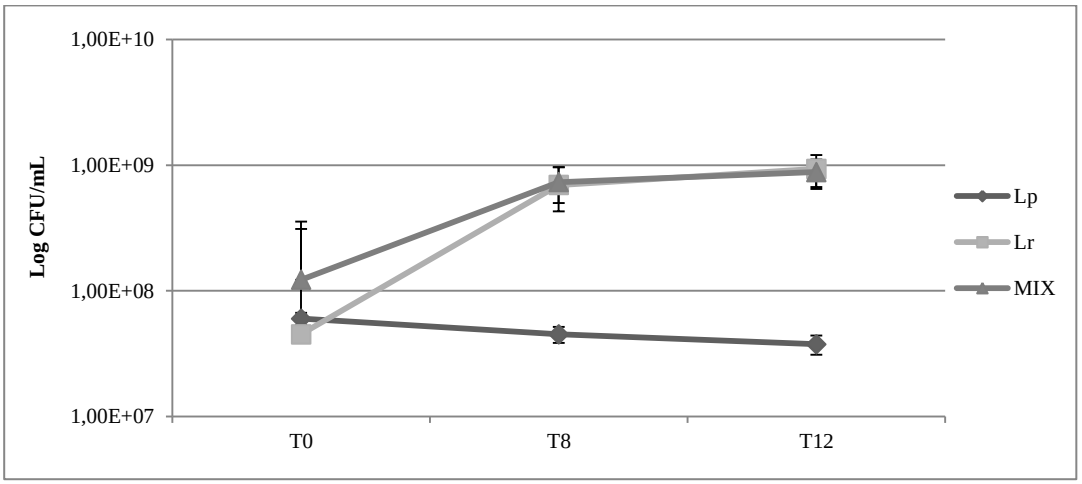


Figure 3: Trend of the development of the LAB strains selected (Lp: *Lactobacillus plantarum* UC8491; Lr: *Lactobacillus rhamnosus* UC8490; MIX: both lab strain inoculated simultaneously). The data were collected in triplicate (statistically significant $p < 0.05$). Error bars indicate standard deviations.

Finally it was observed that of *L. rhamnosus* UC8490 tends to affect the pH value even when mixed with the strain of *L. plantarum* UC8491, highlighting the feature

to acidify the growth environment, resulting in a positive synergy and it represent an important criterion for the evaluation of LAB strains to be used as bio-protective against the development of pathogenic bacteria, as evidenced by other authors (Abriouel, Franz, Ben Omar, & Gálvez, 2011; Coman et al., 2014; Delavenne et al., 2015. In this study it was also tested the antibacterial activity of *Lactobacillus* strains inoculated individually or mixed in a ratio of 1:1 in milk with pathogen bacteria and the growth trend was measured. In Table 4 are reported the results obtained. It is notable from the beginning how the values of inoculum are lower by almost 1 log if compared with the results obtained during the previously test in fermented milk. The presence of the two selected LAB *L. plantarum* UC8491 and *L. rhamnosus* UC8490, maybe represent the cause of the CFU decline of the pathogens bacteria. The inhibitory effect is visible more markedly after eight hours, especially on *L. monocytogenes* L211 (Table 4/A), *S. infantis* EELA72 (Table 4/B) and on the strain of *B. cereus* EELA72 (Table 4/D). It can be seen that there is a share of total inhibition after only a few hours, highlighting the low resistance of the three strains in the presence of pathogenic strains of LAB *L. plantarum* UC8491 and *L. rhamnosus* UC8490. The results are confirmed by measurements made after 12 hours of fermentation, which confirm the inhibiting effect operated by the two LAB on the development of these three pathogenic bacteria. Different results were instead obtained with the strain of L. *innocua* L113 (Table 4/C) showing a high resistance to inhibition stronger respects to previous strains pathogenic. Can you see how after eight hours the value of CFU/mL is almost equal to the initial inoculum, underlining the strong adaptable to the fermentation of this strain. It is noted, however, a decrease in the concentration of about 1 log (± 1x105 CFU/mL to ± 1x104 CFU/mL) when in the fermented milk are mixed the two LAB in combination (Table 4/C MIX), highlighting the possible synergy in the inhibition by the two Lactobacillus. After 12 hours, however, the effect of inhibition shows even more marked on the strain of *L. innocua* L113, in fact it may

be notice a total action of inhibition against the *Listeria* strain, both in the case in which the LAB strains are inoculated alone (Lp and Lr) or when they are inoculated into mixture (MIX). From the results obtained we concluded that the presence of the selected *Lactobacillus* strains are decisive for the effects of inhibition desired during milk fermentation. Furthermore, by comparing the results of pH, it will not be notice a difference so significant as to attribute the effect of inhibition to the only milk acidification, but it can be assumed that the effect of inhibition is related to the production of bacteriocins capable of determining the failure to develop pathogenic bacteria (Leyer & Eric, 1992; Soomro, Masud, & Anwaar, 2002; El Soda, Ahmed, Omran, Osman, & Morsi, 2003; De Keersmaecker et al., 2006; Sieuwerts et al., 2008; Trias, 2008; E Delavenne, Mounier, Déniel, Barbier, & Le Blay, 2012; Gerbaldo, Barberis, Pascual, Dalcero, & Barberis, 2012; S Crowley et al., 2012; Li et al., 2013; Sarah Crowley & Bottacini, 2013; Wang et al., 2013; Belguesmia et al., 2014; Coman et al., 2014; Cortés-Zavaleta, López-Malo, Hernández-Mendoza, & García, 2014; Emilie Delavenne et al., 2013, 2015). Through this study it is therefore possible to state that the use of LAB bacteria selected strain for their antibacterial activity, can be seen as a positive solution during the production of fermented foods, avoiding the use of products of chemical synthesis, which currently represents one aspect considered very important by consumers. The easy of reproduction and the low cost for the maintenance of these LAB strains can finally also be accepted from an economic point of view within the chain of food production. This study demonstrated that a rapid decrease of the pH during the early log phase of the fermentation is strongly connected with the decrease of the initial concentration of the foodborne pathogens. The acidity represents a first obstacle for the development of the strains, but acid resistance was demonstrated.

		T0	T8	T12
	Lp LM	1,57E+05	< 10	< 10
	Lr LM	1,56E+05	< 10	< 10
A	**MIX LM**	1,59E+05	< 10	< 10

		T0	T8	T12
	Lp SAL	1,62E+05	< 10	< 10
	Lr SAL	1,54E+05	< 10	< 10
B	**MIX SAL**	1,65E+05	< 10	< 10

		T0	T8	T12
	Lp LI	1,80E+05	1,75E+05	< 10
	Lr LI	1,95E+05	1,87E+05	< 10
C	**MIX LI**	1,94E+05	1,00E+04	< 10

		T0	T8	T12
	Lp BC	1,80E+04	< 10	< 10
	Lr BC	2,40E+04	< 10	< 10
D	**MIX BC**	2,00E+04	< 10	< 10

Table 4: Analysis of the resistance by *Listeria monocytogenes* L211 (A), *Salmonella infantis* EELA72 (B), *Listeria innocua* L113 (C) and Bacillus cereus EELA72 (D). The pathogens were inoculated in fermented milk obtained with the inoculum selected LAB strains *Lactobacillus plantarum* UC8491 (Lp) and *Lactobacillus rhamnosus* UC8490 (Lr), inoculated alone or in combination (MIX). The data were collected in triplicate in three different time step for evaluate the survival trend of pathogens during the fermentation. Anova test for statistical analysis were performed with significant results p<0.05.

CONCLUSIONS

The inhibitory action of Lactic Acid Bacteria can be due to the production and accumulation of different metabolite such us lactic and acetic acids, ethanol and carbon dioxide. Additionally, many LAB are also capable to produce antimicrobial compounds like formic and benzoic acids, hydrogen peroxide, diacetyl, acetoin and bacteriocins (Yang et al., 1992; Cintas et al., 2001; Cleveland, Montville, Nes, & Chikindas, 2001; Aly et al., 2006; Cui et al., 2012). The inhibitory activities of LAB against Gram positive pathogens have been mostly shown to be due to the bactericidal effect of protease sensitive bacteriocins (Cavera, Arthur, Kashtanov, & Chikindas, 2015). In conclusion, the results obtained from the present study demonstrate the effective antimicrobial activity of the selected LAB (*L. plantarum* and *L. rhamnosus*) species during the early stages of the fermentation in milk, against foodborne pathogens. According to other studies, a large number of LAB strains with different bioactive potentials, especially in the form of the antimicrobial properties have been identified in different fermented food (Adams & Nicolaides, 1997; Caplice & Fitzgerald, 1999; Onilude, Fagade, Bello, & Fadahunsi, 2005; Trias, 2008 Lan, Chen, Wu, & Yanagida, 2012). This study wants to highlight the importance of the use of selected LAB strain for the bio-preservation of food and the future important role in the food biopreservation. Although further studies are needed to be able to fully understand the ability of certain LAB strains in preventing the growth of pathogenic bacteria.

BIBLIOGRAPHY

. S. a. H., . T. M., & . K. A. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

a.M., M., P.N., K., L.C., B., J.M., S., & J.L., M. (2008). Effects of folate and vitamin B12 deficiencies during pregnancy on fetal, infant, and child development. *Food and Nutrition Bulletin*, *29*(2 SUPPL.), S101–S111. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed8&NEWS=N&AN=18709885

Abee, T., Rombouts, F. M., Hugenholtz, J., Guihard, G., & Letellier, L. (1994). Mode of action of nisin Z against Listeria monocytogenes scott a grown at high and low temperatures. *Applied and Environmental Microbiology*, *60*(6), 1962–1968.

Adams, M. R., & Nicolaides, L. (1997). Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control*, *8*(5-6), 227–239. doi:10.1016/S0956-7135(97)00016-9

Al-Kadamany, E., Khattar, M., Haddad, T., & Toufeili, I. (2003). Estimation of shelf-life of concentrated yogurt by monitoring selected microbiological and physicochemical changes during storage. *LWT - Food Science and Technology*, *36*(4), 407–414. doi:10.1016/S0023-6438(03)00018-5

Aly, S., T, O. C. a, N, B. I. H., & Alfred, T. S. (2006). Bacteriocins and lactic acid bacteria - a minireview. *African Journal of Biotechnology*, *5*(May), 678–683. doi:10.5897/AJB05.388

Arimi, S. M., Koroti, E., Kang'ethe, E. K., Omore, A. O., & McDermott, J. J. (2005). Risk of infection with Brucella abortus and Escherichia coli O157:H7 associated with marketing of unpasteurized milk in Kenya. *Acta Tropica*, *96*(1), 1–8. doi:10.1016/j.actatropica.2005.05.012

Arqués, J. L., Rodríguez, E., Nuñez, M., & Medina, M. (2011). Combined effect of reuterin and lactic acid bacteria bacteriocins on the inactivation of food-borne pathogens in milk. *Food Control*, *22*(3-4), 457–461. doi:10.1016/j.foodcont.2010.09.027

Bankole, S. a, & Adebanjo, a. (2003). Mycotoxins in food in West Africa : current situation and possibilities of controlling it. *Journal of Biotechnology*, *2*(September), 254–263. doi:10.4314/ajb.v2i9.14833

Benech, R. O., Kheadr, E. E., Laridi, R., Lacroix, C., & Fliss, I. (2002). Inhibition of Listeria innocua in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Applied and Environmental Microbiology*, *68*(8), 3683–90. doi:10.1128/AEM.68.8.3683

Black, R. (2003). Editorial Micronutrient deficiency — an underlying cause of morbidity and mortality, *81*(03808), 3808.

Blagojev, N., Škrinjar, M., Vesković-Moračanin, S., & Šošo, V. (2012). Control of mould growth and mycotoxin production by lactic acid bacteria metabolites. *Romanian Biotechnological Letters*, *17*(3), 7219–7226.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P. Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *International Journal of Food Microbiology*, *154*(3), 87–97. doi:10.1016/j.ijfoodmicro.2011.12.030

Branca, F., & Ferrari, M. (2002). Impact of micronutrient deficiencies on growth: The stunting syndrome. *Annals of Nutrition and Metabolism*, *46*(SUPPL. 1), 8–17. doi:10.1159/000066397

Brul, S., & Coote, P. (1999). Preservative agents in foods: Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, *50*(1-2), 1–17. doi:10.1016/S0168-1605(99)00072-0

Caplice, E., & Fitzgerald, G. F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, *50*(1-2), 131–149. doi:10.1016/S0168-1605(99)00082-3

Cappa, F., & Cocconcelli, P. S. (2001). Identification of fungi from dairy products by means of 18S rRNA analysis. *International Journal of Food Microbiology*, *69*(1-2), 157–160. doi:10.1016/S0168-1605(01)00587-6

Castellano, P., Belfiore, C., Fadda, S., & Vignolo, G. (2008). A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Science*, *79*(3), 483–499. doi:10.1016/j.meatsci.2007.10.009

Cavera, V. L., Arthur, T. D., Kashtanov, D., & Chikindas, M. L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *International Journal of Antimicrobial Agents*, 7–14. doi:10.1016/j.ijantimicag.2015.07.011

Chung, T., & Axelsson, L. (1989). In vitro studies on reuterin synthesis by Lactobacillus reuteri. *Microbial Ecology in …*, *2*, 137–144. doi:10.3109/08910608909140211

Cintas, L. M., Herranz, C., HernÁndez, P. E., Casaus, M. P., Nes, I. F., & HernÁndez, P. E. (2001). Review: Bacteriocins of Lactic Acid Bacteria. *Food Science & Technology International*, *7*(4), 281–305. doi:10.1106/R8DE-P6HU-CLXP-5RYT

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*(1), 1–20. doi:10.1016/S0168-1605(01)00560-8

Conference, I., & Food, U. N. (2014). Food safety must accompany food and nutrition security-WHO Proceedings. *Nutrition and Food Safety*, *6736*(14), 1–4. doi:10.1016/S0140-6736(14)62037-7

Corsetti, a., Gobbetti, M., & Smacchi, E. (1996). Antibacterial activity of sourdough lactic acid bacteria: isolation of a bacteriocin-like inhibitory substance fromLactobacillus sanfranciscoC57. *Food Microbiology*, *13*(6), 447–456. doi:10.1006/fmic.1996.0051

Cortés-Zavaleta, O., López-Malo, a, Hernández-Mendoza, a, & García, H. S. (2014). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *International Journal of Food Microbiology*, *173*, 30–5. doi:10.1016/j.ijfoodmicro.2013.12.016

Crider, K. S., Bailey, L. B., & Berry, R. J. (2011). Folic Acid Food Fortification—Its History,

Effect, Concerns, and Future Directions. *Nutrients*, *3*(3), 370–384. doi:10.3390/nu3030370

Crittenden, R. G., Martinez, N. R., & Playne, M. J. (2003). Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. *International Journal of Food Microbiology*, *80*(3), 217–222. doi:10.1016/S0168-1605(02)00170-8

Crowley, S., Mahony, J., & van Sinderen, D. (2013). Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends in Food Science & Technology*, *33*(2), 93–109. doi:10.1016/j.tifs.2013.07.004

Cui, Y., Zhang, C., Wang, Y., Shi, J., Zhang, L., Ding, Z., … Cui, H. (2012). Class IIa bacteriocins: Diversity and new developments. *International Journal of Molecular Sciences*, *13*(12), 16668–16707. doi:10.3390/ijms131216668

de Carvalho, a. a. T., Mantovani, H. C., & Vanetti, M. C. D. (2007). Bactericidal effect of bovicin HC5 and nisin against Clostridium tyrobutyricum isolated from spoiled mango pulp. *Letters in Applied Microbiology*, *45*(1), 68–74. doi:10.1111/j.1472-765X.2007.02150.x

de Crécy-Lagard, V., El Yacoubi, B., de la Garza, R. D., Noiriel, A., & Hanson, A. D. (2007). Comparative genomics of bacterial and plant folate synthesis and salvage: predictions and validations. *BMC Genomics*, *8*(Figure 2), 245. doi:10.1186/1471-2164-8-245

Delavenne, E., Ismail, R., Pawtowski, A., Mounier, J., Barbier, G., & Le Blay, G. (2013). Assessment of lactobacilli strains as yogurt bioprotective cultures. *Food Control*, *30*(1), 206–213. doi:10.1016/j.foodcont.2012.06.043

Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin Production: a Probiotic Trait? *Applied and Environmental Microbiology*, *78*(1), 1–6. doi:10.1128/AEM.05576-11

Eijsink, V. G. H., Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H., & Nes, I. F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *81*(1-4), 639–654. doi:10.1023/A:1020582211262

Filtenborg, O., Frisvad, J. C., & Thrane, U. (1996). Moulds in food spoilage. *International Journal of Food Microbiology*, *33*(1), 85–102. doi:10.1016/0168-1605(96)01153-1

Fitzpatrick, T. B., Basset, G. J. C., Borel, P., Carrari, F., DellaPenna, D., Fraser, P. D., … Fernie, A. R. (2012). Vitamin deficiencies in humans: can plant science help? *The Plant Cell*, *24*(2), 395–414. doi:10.1105/tpc.111.093120

Florou-Paneri, P., Christaki, E., & Bonos, E. (2013). Lactic Acid Bacteria as Source of Functional Ingredients. *Lactic Acid Bacteria-R& D for Food, Health and Livestock Purposes*, 589–614.

Gerbaldo, G. a, Barberis, C., Pascual, L., Dalcero, A., & Barberis, L. (2012). Antifungal activity of two Lactobacillus strains with potential probiotic properties. *FEMS Microbiology Letters*, *332*(1), 27–33. doi:10.1111/j.1574-6968.2012.02570.x

Gerez, C. L., Torino, M. I., Rollán, G., & Font de Valdez, G. (2009). Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control*, *20*(2), 144–148. doi:10.1016/j.foodcont.2008.03.005

Gerez, C. L., Torres, M. J., Font de Valdez, G., & Rollán, G. (2013). Control of spoilage fungi by lactic acid bacteria. *Biological Control*, *64*(3), 231–237. doi:10.1016/j.biocontrol.2012.10.009

Gourama, H. (1997). Inhibition of Growth and Mycotoxin Production of Penicillium By Lactobacillus Species. *Food Science & Technology-Lebensmittel-Wissenschaft & Technologie*, *30*(3), 279–283.

Gupta, R., & Srivastava, S. (2014). Antifungal effect of antimicrobial peptides (AMPs LR14) derived from Lactobacillus plantarum strain LR/14 and their applications in prevention of grain spoilage. *Food Microbiology*, *42*, 1–7. doi:10.1016/j.fm.2014.02.005

Harris, L. J., Fleming, H. P., & Klaenhammer, T. R. (1992). Novel paired starter culture system for sauerkraut, consisting of a nisin-resistant Leuconostoc mesenteroides strain and a nisin-producing Lactococcus lactis strain. *Applied and Environmental Microbiology*, *58*(5), 1484–1489. History, T. H. E., & Folic, O. F. (2001). Historical Review.

Hoey, L., Mcnulty, H., Duffy, M. E., & Hughes, C. F. (2015). EURRECA — Estimating Folate Requirements for Deriving Dietary Reference Values EURRECA *8398*(October), 1040–1050. doi:10.1080/10408398.2012.742858

Holzapfel, W. (1997). Use of starter cultures in fermentation on a household scale. *Food Control*, *8*(5-6), 241–258. doi:10.1016/S0956-7135(97)00017-0

Holzapfel, W. H., Geisen, R., & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology*, *24*(3), 343–362. doi:10.1016/0168-1605(94)00036-6

Iyer, R., Tomar, S. K., Uma Maheswari, T., & Singh, R. (2010). Streptococcus thermophilus strains: Multifunctional lactic acid bacteria. *International Dairy Journal*, *20*(3), 133–141. doi:10.1016/j.idairyj.2009.10.005

Jack, R. W., Tagg, J. R., & Ev, M. I. R. (1995). Bacteriocins of Gram-Positive Bacteria, *59*(2), 171–200.

Jones, R. J., Hussein, H. M., Zagorec, M., Brightwell, G., & Tagg, J. R. (2008). Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. *Food Microbiology*, *25*(2), 228–234. doi:10.1016/j.fm.2007.11.001

Journal, T. H. E., & Biolowxl, O. F. (1974). Folate Coenzymes faecalis*. *Cell*, 6–7.

Käferstein, F. (2003). Foodborne diseases in developing countries: aetiology, epidemiology and strategies for prevention. *International Journal of Environmental Health Research*, *13 Suppl 1*(July 2015), S161–S168. doi:10.1080/0960312031000102949

Kuipers, O. P., De Ruyter, P. G. G. a, Kleerebezem, M., & De Vos, W. M. (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology*, *64*(1), 15–21. doi:10.1016/S0168-1656(98)00100-X

Laiño, J. E., Juarez del Valle, M., Savoy de Giori, G., & LeBlanc, J. G. J. (2013). Development of a high folate concentration yogurt naturally bio-enriched using selected lactic acid bacteria. *LWT - Food Science and Technology*, *54*(1), 1–5. doi:10.1016/j.lwt.2013.05.035

Lan, W., Chen, Y., Wu, H., & Yanagida, F. (2012). Bio-protective potential of lactic acid bacteria isolated from fermented wax gourd. *Folia Microbiologica*, *57*(2), 99–105. doi:10.1007/s12223-012-0101-1

LeBlanc, J., de Giori, G., Smid, E. J., Hugenholtz, J., & Sesma, F. (2007). Folate production by lactic acid bacteria and other food-grade microorganisms. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, *1*, 329–339.

Lee, S. C., Billmyre, R. B., Li, A., Carson, S., Sykes, S. M., Huh, E. Y., Heitman, J. (2014). Analysis of a Food-Borne Fungal Pathogen Outbreak: Virulence and Genome of a Mucor circinelloides Isolate from Yogurt. *mBio*, *5*(4), e01390–14–. doi:10.1128/mBio.01390-14

Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., & De Vuyst, L. (2012). On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. *Food Microbiology*, *30*(2), 379–92. doi:10.1016/j.fm.2011.12.021

Lewus, C. B., Kaiser, a., & Montville, T. J. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Applied and Environmental Microbiology*, *57*(6), 1683–1688.

Li, H., Liu, L., Zhang, S., Uluko, H., Cui, W., & Lv, J. (2013). Potential use of Lactobacillus casei AST18 as a bioprotective culture in yogurt. *Food Control*, *34*(2), 675–680. doi:10.1016/j.foodcont.2013.06.023

Lollo, P. C. B., de Moura, C. S., Morato, P. N., Cruz, A. G., Castro, W. D. F., Betim, C. B., Amaya-Farfan, J. (2013). Probiotic yogurt offers higher immune-protection than probiotic whey beverage. *Food Research International*, *54*(1), 118–124. doi:10.1016/j.foodres.2013.06.003

Lukaski, H. C. (2004). Vitamin and mineral status: effects on physical performance. *Nutrition*, *20*(7-8), 632–644. doi:10.1016/j.nut.2004.04.001

Massa, S. (2014). Lactobacillus plantarum as a Strategy for an In situ Production of Vitamin B2. *Journal of Food & Nutritional Disorders*, *s1*(01), 1–5. doi:10.4172/2324-9323.S1-004

McAuliffe, O. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews*, *25*(3), 285–308. doi:10.1016/S0168-6445(00)00065-6

Mcnulty, H., Pentieva, K., Hoey, L., Strain, J. J., & Ward, M. (2012). Nutrition Throughout Life : Folate, *82*(5), 348–354. doi:10.1024/0300-9831/a000130

Messens, W., & De Vuyst, L. (2002). Inhibitory substances produced by Lactobacilli isolated from sourdoughs - A review. *International Journal of Food Microbiology*, *72*(1-2), 31–43. doi:10.1016/S0168-1605(01)00611-0

Motarjemi, Y., Käferstein, F., Moy, G., & Quevedo, F. (1993). Contaminated weaning food: a major risk factor for diarrhoea and associated malnutrition. *Bulletin of the World Health Organization*, *71*(1), 79–92.

Muhialdin, B. J., & Hassan, Z. (2011). Annals . Food Science and Technology BIOPRESERVATION OF FOOD BY LACTIC ACID BACTERIA AGAINST 1 . INTRODUCTION

Müller, O., & Krawinkel, M. (2005). Malnutrition and health in developing countries, *173*(3), 2000–2003.

Naidu, a. S., Bidlack, W. R., & Clemens, R. a. (1999). Probiotic Spectra of Lactic Acid Bacteria (LAB). *Critical Reviews in Food Science and Nutrition*, *39*(1), 13–126. doi:10.1080/10408699991279187

Nakajima, H., Toba, T., Ito, A., Kudo, S., Sato, S., & Sato, Y. (2003). The Screening of Hydrogen Peroxide-Producing Lactic Acid Bacteria and Their Application to Inactivating Psychrotrophic Food-Borne Pathogens. *Current Microbiology*, *47*(3), 231–236. doi:10.1007/s00284-002-3993-1

Niku-Paavola, M. L., Laitila, a, Mattila-Sandholm, T., & Haikara, a. (1999). New types of antimicrobial compounds produced by Lactobacillus plantarum. *J. Appl. Microbiol.*, *86*(1), 29–35. doi:10.1046/j.1365-2672.1999.00632.x

Nissen-Meyer, J., Oppegård, C., Rogne, P., Haugen, H. S., & Kristiansen, P. E. (2010). Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins. *Probiotics and Antimicrobial Proteins*, *2*(1), 52–60. doi:10.1007/s12602-009-9021-z

O'Sullivan, L., Ross, R. P., & Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie*, *84*(5-6), 593–604. doi:10.1016/S0300-9084(02)01457-8

Okereke, a, & Montville, T. J. (1991). Bacteriocin-mediated inhibition of Clostridium botulinum spores by lactic acid bacteria at refrigeration and abuse temperatures. *Applied and Environmental Microbiology*, *57*(12), 3423–8. Retrieved from

Onilude, a a, Fagade, O. E., Bello, M. M., & Fadahunsi, I. F. (2005). Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels. *Journal of Biotechnology*, *4*(December), 1404–1408.

Piard, J. C., & Desmazeaud, M. (1991). Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Le Lait*, *71*(5), 525–541. doi:10.1051/lait:1991541

Powers, H. J. (2007). Folic acid under scrutiny. *The British Journal of Nutrition*, *98*(4), 665–666. doi:10.1017/S0007114507795326

Reisenaãœer, a N. N. a I., & Halsted, C. H. (1987). Issues and Opinions In Nutrition Human Folate Requirements, *45*(October 1986), 600–602.

Republic, C. (2002). Biosynthesis of Folates by Lactic Acid Bacteria and Propionibacteria in Fermented Milk. *Food Science*, *22*(5), 175–181.

Rossi, M., Amaretti, A., & Raimondi, S. (2011). Folate Production by Probiotic Bacteria. *Nutrients*, *3*(12), 118–134. doi:10.3390/nu3010118

Røssland, E., Andersen, G. I., Langsrud, T., & Sørhaug, T. (2003). Inhibition of Bacillus cereus by strains of Lactobacillus and Lactococcus in milk, *89*, 205–212. doi:10.1016/S0168-1605(03)00149-1

Rouse, S., Harnett, D., Vaughan, a., & Sinderen, D. Van. (2008). Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *Journal of Applied Microbiology*, *104*, 915–923. doi:10.1111/j.1365-2672.2007.03619.x

Samelis, J., Kakouri, a., Rogga, K. J., Savvaidis, I. N., & Kontominas, M. G. (2003). Nisin treatments to control Listeria monocytogenes post-processing contamination on Anthotyros, a traditional Greek whey cheese, stored at 4°C in vacuum packages. *Food Microbiology*, *20*(6), 661–669. doi:10.1016/S0740-0020(03)00018-2

Sangmanee, P., & Hongpattarakere, T. (2014). Inhibitory of multiple antifungal components produced by Lactobacillus plantarum K35 on growth, aflatoxin production and ultrastructure alterations of Aspergillus flavus and Aspergillus parasiticus. *Food Control*, *40*, 224–233. doi:10.1016/j.foodcont.2013.12.005

Santos, F., Wegkamp, a., de Vos, W. M., Smid, E. J., & Hugenholtz, J. (2008). High-Level Folate Production in Fermented Foods by the B12 Producer Lactobacillus reuteri JCM1112. *Applied and Environmental Microbiology*, *74*(10), 3291–3294. doi:10.1128/AEM.02719-07

Schillinger, U., Geisen, R., & Holzapfel, W. H. (1996). Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends in Food Science & Technology*, *7*(5), 158–164. doi:10.1016/0924-2244(96)81256-8

Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology*, *16*(1-3), 70–78. doi:10.1016/j.tifs.2004.02.014

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *25*(2), 146–151. doi:10.1080/02652030701567442

Soomro, a. H., Masud, T., & Anwaar, K. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

Stoyanova, L. G., Ustyugova, E. A., Sultimova, T. D., Bilanenko, E. N., Fedorova, G. B., Khatrukha, G. S., … Pirogovskaya, B. (2010). New Antifungal Bacteriocin-Synthesizing Strains of Lactococcus lactis ssp . lactis as the Perspective Biopreservatives for Protection of Raw Smoked Sausages Department of Microbiology , Faculty of Biological , M . V . Lomonosov Moscow State University Len, *5*(4), 477–485.

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, M. H. N., & Hugenholtz, J. (2003). Effects of Cultivation Conditions on Folate Production by Lactic Acid Bacteria. *Metab. Eng.*, *69*(8), 4542–4548. doi:10.1128/AEM.69.8.4542

Thu, B. D., Schultink, W., Dillon, D., Gross, R., Leswara, N. D., & Khoi, H. H. (1999). Effect of daily and weekly micronutrient supplementation on micronutrient deficiencies and growth in young Vietnamese children. *The American Journal of Clinical Nutrition*, *69*(1), 80–86.

Tiwari, B. K., Valdramidis, V. P., O' Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of Natural Antimicrobials for Food Preservation. *Journal of Agricultural and Food Chemistry*, *57*(14), 5987–6000. doi:10.1021/jf900668n

Trias, R. (2008). *Lactic acid bacteria as bioprotective agents against foodborne pathogens and*

spoilage microorganisms in fresh fruits and vegetables. Retrieved from http://www.tdx.cat/handle/10803/7932

Unnevehr, L. (2015). Food safety in developing countries: Moving beyond exports. *Global Food Security*, *4*, 24–29. doi:10.1016/j.gfs.2014.12.001

Unnevehr, L., & Grace, D. (2013). Tackling Aflatoxins : An Overview of Challenges and Solutions, (November), 3–4.

Valyasevi, R., & Rolle, R. S. (2002). An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: Scope for their improvement. *International Journal of Food Microbiology*, *75*(3), 231–239. doi:10.1016/S0168-1605(01)00711-5

Vaughan, a., Eijsink, V. G. H., O'Sullivan, T. F., O'Hanlon, K., & Van Sinderen, D. (2001). An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. *Journal of Applied Microbiology*, *91*(1), 131–138. doi:10.1046/j.1365-2672.2001.01365.x

Walton, E., & Allen, S. (2011). Malnutrition in developing countries. *Paediatrics and Child Health*, *21*(9), 418–424. doi:10.1016/j.paed.2011.04.004

Wong, H., & Chen, Y. (1988). Effects of Lactic Acid Bacteria and Organic Acids Germination of Bacillus cereus Growth and. *Microbiology*, *54*(9), 2179–2184.

World Health Organization. (2014). Antimicrobial resistance. *Who*, *317*(September), 609–610. Retrieved from http://www.who.int/mediacentre/factsheets/fs194/en/#

Yang, E., Fan, L., Jiang, Y., Doucette, C., & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express*, *2*(1), 48. doi:10.1186/2191-0855-2-48

Yang, R., Johnson, M. C., & Ray, B. (1992). Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology*, *58*(10), 3355–3359.

CHAPTER IV

ANALYSIS AND CHARACTERIZATION OF BACTERIOCIN PRODUCTION BY *Lactobacillus plantarum* UC8491 STRAIN

ABSTRACT

Lactic Acid Bacteria (LAB) with ability to inhibit food-spoilage are recently buying greatest interest for the food industry. Since, the antifungal activity of LAB remains poorly understood, need to be continuously study to develop new commercial formulations applicable to the control of food-borne yeasts and moulds. The whole genome of a bacteriocin producing strain *Lactobacillus plantarum* UC8491 able to inhibit foodborne pathogenic bacteria and spoilage fungi in fermented food (cheese, yogurt and vegetable products) was sequenced. The draft genome has a size of 3.30 Mb and contain 334 subsystems, according to the RAST analysis. The presence of the plantaricin biosynthesis cluster containing the pln genes organized in five operons: plnABCD; plnEFI; plnJKLR, plnMNOP, and plnGHSTUV in the genome of *L. plantarum* UC8491 was confirmed. A bottom-up proteomic approach was carried out through QTOF mass spectrometry from cell free supernatant and from a band excised on Tricine-SDS-PAGE gel corresponding to the halo of *Penicillium* inhibition. Peptides were filtered through a 10 kDa membrane, reduced and alkylated, then separated via nanoscale CHIP liquid chromatography in reverse mode, and analyzed in data-dependent tandem mass spectrometry. Spectra were processed in SpectrumMill versus Bactibase and Bagel database. The analysis of the fraction ≤10 kDa resulted in the identification of several peptides belonging to bacteriocins (in particular class II) or related proteins, such as Bioactive peptide 1; acidocin, pyiocin/carocin, lantibiotic, ABC transporter, ATP-binding protein, Enterocin AS-48 and divergicin 750. Peptides with homology to colicins were also identified. This results are in accordance with the Bagel database analysis, showing the presence in the genome of *L. plantarum* UC 8491 of more than 30 small ORF from putative bacteriocin from Class IIb and IIc diffretent than plantaricin. Results obtained in this study revealed that this strain

produces additional antimicrobial peptides, probably responsible of its antifungal activity.

Key words: Bacteriocin, *L. plantarum*, Tricine-SDS-PAGE, QTOF mass spectrometry, antifungal activity

INTRODUCTION

Antimicrobial peptides application, such as bacteriocins from lactic acid bacteria (LAB) targeting food pathogens has received a great attention during the last decades.. Bacteriocins are ribosomal synthesized substances of proteinaceous nature produced by bacteria that can kill or inhibit the development of other microorganisms. This mainly happens with related bacteria but, in a few cases also with filamentous fungi (Falguni, Shilpa, & Mann, 2010; Stoyanova et al., 2010; Gerbaldo et al., 2012; Cortés-Zavaleta et al., 2014;Gupta & Srivastava, 2014;). Numerous Lactic Acid Bacteria in fermented food are able to produce bacteriocins (Cleveland, Montville, Nes, & Chikindas, 2001; Batdorj et al., 2006), and have been the focus of extensive research in recent years, due to their potentials as bio-preservatives (Holzapfel, Geisen, & Schillinger, 1995; Cintas et al., 2001; Vaughan, Eijsink, O'Sullivan, O'Hanlon, & Van Sinderen, 2001). The LAB bacteriocins are classified in four main classes based on common, main structural characteristics. The most important belongs to the I and II class. Bacteriocins of I classification are named "lantibiotics"; they are small (< 5 kDa) membrane-active peptides, containing post-translationally modified amino acids. The most studied bacteriocin of this class is the Nisin (McAuliffe, 2001). Bacteriocins belonging to class II are small, heat-stable, non lanthionine-containing peptides. The latter, are sub-groups organised: class IIA characterized by *Listeria*-active peptides; class IIB, consisting in small cationic peptides without specific homology sequence. The activity of these bacteriocins consists of associations of two different peptides (Batdorj et al., 2006). This bacteriocins sub-class II produced by LAB, is the most promising bacteriocins candidate for food preservation. This, due to better performances in terms of biological activity and physiochemical proprieties than most bacteriocins from other classes (Lewus, Kaiser, & Montville, 1991; R. Yang, Johnson, & Ray, 1992; Vincent G H Eijsink et al., 1998; Cleveland et al., 2001; Cintas et al., 2001;

Vaughan et al., 2001; Soomro, Masud, & Anwaar, 2002; O'Sullivan, Ross, & Hill, 2002; V. G H Eijsink et al., 2002; Aly, T, N, & Alfred, 2006; Ammor, Tauveron, Dufour, & Chevallier, 2006; Castellano, Belfiore, Fadda, & Vignolo, 2008; Tomé, Pereira, Lopes, Gibbs, & Teixeira, 2008; Cui et al., 2012; E. Yang, Fan, Jiang, Doucette, & Fillmore, 2012a, 2012b; Fontana, Cocconcelli, Vignolo, & Saavedra, 2015). Several researches revealed that LAB strains are able to produce more than one bacteriocin (Cleveland et al., 2001; Vaughan et al., 2001 Aly, T, N, & Alfred, 2006; Prema, Smila, Palavesam, & Immanuel, 2008). An organism that produces bacteriocin has a selective advantage over their competitors in the natural environment. Furthermore some bacteriocin produced by LAB, inhibit closely related species and are highly effective against food-borne pathogens and numerous gram-positive spoilage microorganisms. An approach to introduce bacteriocin in the food industry is to use live cultures. These have the ability to produce the antimicrobial peptide *in situ*. Same techniques are applied in the same field, to improve safety in the final food product (O'Sullivan et al., 2002). Bacteriocins have considerably attracted great interest as natural food preservatives in recent years. Related to that, the consumption of food that has been developed with chemical preservative has increased the consumers' concerns creating a higher demand for natural and minimally processed food. The aim of this study is to define the activities of bacteriocin produced by *Lactobacillus plantarum* UC8491 against food borne and food spoilage microorganism in fermented food.

- **Aim of the study**

To identify and characterize the bacteriocins produced by *Lactobacillus plantarum* UC8491.

MATHERIALS AND METHODS

- **Bacteria and culture media**

The *Lactobacillus plantarum* UC8491, was grown in MRS (Oxoid),at 37°C for 18 hours. Strain of *Penicillium brevicompactum* UC7075 and *Penicillium roqueforti* UC8455, used as indicator strains, were grown in Rose Bengal Agar (Oxoid) for 3/5 days at 30°C. After development, spore were obtained and stored in peptone water at 4°C.

- **Cell-free supernatant (CFS)**

After LAB strain reactivation, 250 mL of sterile MRS was inoculated with 1% v/v with the supernatant previously obtained, and stored for 48 hours at 37°C, without shaking the liquid. Then aliquots of 50 ml were taken and centrifuge at 15.000 rpm for 10 min. The CFS obtained was stored at -80°C for 12 hours. After the freezing step, sterile frozen samples of CFS were lyophilized and stored at -20°C for subsequent test.

- **Antimicrobial activity assay**

Three different antimicrobial essays were performed in the following study. First was used an *Agar Plate Model Assay*: the inhibitory activity by *L. plantarum* UC8491 was tested against food spoilage fungi *P. brevicompactum* UC7075 and *P. roqueforti* UC8455, in MRS plate. Aliquot of 10µL of CFS were spotted on MRS agar plate and store at room temperature until dry, then, 7 mL of Malt Soft Agar containing an suspension of mould spore (50 µL) were spilled on the plate. Once the soft agar dried, the plates were incubated at 30 °C until the mould growth (3 – 5 days) and the inhibition halo around the spot were evaluated. The second antimicrobial test was a *Cheese Plate Model Assay*: aliquot of 10µL of CFS were crawled on sterile cheese slice and subsequent double aliquot of 10µL of moulds

spore suspensions were crawled creating cross. Once the aliquot dried, the cheese slice plate were incubated at 30°C for 3 – 5 days, and the inhibition activity operated by the LAB strain observed on the surface of the plate. *Tricine-SDS-Gel Plate Model Assay* was the third antimicrobial activity tested: once obtained the tricine gel migration according to the protocol (described below), the tricine gel it is transferred on a sterile square plate and Soft Malt Agar (0.75%) containing 50 µL of mould spore suspension it is spilled (overlaying) on the gel and live at room temperature until dry. One dried the plate are incubated for 18 hours at 37°C and inhibition halo are visible on the migration protein zone on the tricine gel.

- **Tricine-SDS-PAGE and bacteriocin activity assay**

The molecular mass of bacteriocins were estimated in a tricine-SDS-PAGE system as described by Schägger and Von Jagow (1987), using 10-20% precasting Tris-Tricine gel. Electrophoresis was performed in vertical gels (80 mm x 70 mm x 0.75 mm) in a Mini-protean II cell (Bio-Rad Laboratories, Italy) at 35 mA for 3 h**.** To determine the apparent molecular mass of bacteriocins, a low-molecular mass protein marker ranging from 2 to 250 KDa (Precision Plus Protein Dual Xtra Standart, Bio-Rad) was used. The gels were fixed in 45% methanol–10% acetic acid in water for 30 min at room temperature and washed for six times (15 min each) with deionized water to remove SDS. The gel was used for direct detection of antimicrobial activity like described previously.

- **Protein characterization**

Culture medium of *Lactobacillus plantarum* UC8491 was loaded into Centrisart I centrifuge tubes (Sartorius Stedim Biotech GmbH, Germany) to filter proteins and peptides with a cut-off of 10 kDa. Protein fractions were measured with the Bio-Rad protein assay kit using bovin γ-globulin as standard, following to the manufacturer's instructions. Fifty micrograms of proteins were then reduced with

dithiothreitol, alkylated with iodoacetamide and then digested with Trypsin (Promega, Madison WI, USA) at 37 °C overnight.

- **Tandem MS analysis and proteins inference**

Tryptic peptides were analysed by a shotgun MS/MS approach using a hybrid quadrupole-time-of-flight (Q-TOF) mass spectrometer (6550 IFunnel Q-TOF, Agilent Technologies, CA, USA), with a nano LC Chip Cube source (Agilent Technologies, CA, USA). The chip consisted of a 40-nL enrichment column (Zorbax 300SB-C18, 5 µm pore size) and a 150 mm separation column (Zorbax 300SB-C18, 5 µm pore size) coupled to an Agilent Technologies 1200 series nano/capillary LC system and controlled by the Mass Hunter Workstation Acquisition (version B.04). A volume of 8 µL was injected per run, loading peptides onto the trapping column at 4 µL min^{-1} in 2% (v/v) acetonitrile and 0.1% (v/v) formic acid. After enrichment, the chip was switched to separation mode and peptides were backflush eluted into the analytical column, during a 150 min acetonitrile gradient (from 3% to 70% v/v in 0.1% formic acid) at 0.6 µl min^{-1}. The mass spectrometer was used in positive ion mode and MS scans were acquired in data-dependent mode over a mass range from 300 to 1700 m/z at 3 spectra s^{-1}.

Precursor ions were selected for auto-MS/MS at an absolute threshold of 1000 and a relative threshold of 0.01%, with a maximum of 20 precursors per cycle (enabling active exclusion after 2 spectra, with release after 0.2 min.

Analysis of MS/MS spectra for peptides identification was performed by protein database with Spectrum Mill MS Proteomics Workbench (Rev B.04; Agilent Technologies). Auto MS/MS spectra were extracted from raw data accepting a minimum sequence length of 3 amino acids. Merging scans with the same precursor within a mass window of ±0.4 mass-to-charge ratio in a time frame of ±30 s. Search parameters were Scored Peak Intensity (SPI) ≥ 50%, precursor mass tolerance of ± 10 ppm and product ions mass tolerance of ± 20 ppm.

Carbamidomethylation of cysteine was set as fixed modification and trypsin was selected as enzyme for digestion, accepting 2 missed cleavages per peptide.
Auto thresholds were used for peptide identification in Spectrum Mill, to achieve a target 1% false discovery rate. A label-free quantitation, using the protein summed peptide abundance, was carried out after identification.

- **Genome sequencing analysis**

The complete genome of bacteriocin producer strains *L. plantarum* UC8491 was sequenced using the Illumina Genome Hiseq 1000. Velvet software 1.1.04 was used for the countings assembled. Both genomes were annotated using RAST server. Specific software, such as Bagel, was used to the search of putative genes coding for bacteriocin.

RESULTS

- **Antimicrobial activity**

The first set of experiments was targeted to determinate if *Lactobacillus plantarum* UC8491 has the capacity to limit the growth of filamentous fungi. As shown inFig. 1, in a cheese plate assay, the growth of this bacteria species limited two food spoilage species, *P. brevicompactum* UC7075 *and P. roqueforti* UC8455. To check if the antimicrobial compound(s) produced by *L. plantarum* UC8491 are secreted in the medium, an experiment was conducted with the concentrated filtered supernatant (CFS). As reported in Figure 2 A/B the CFS inhibited the two studied fungal cultures.

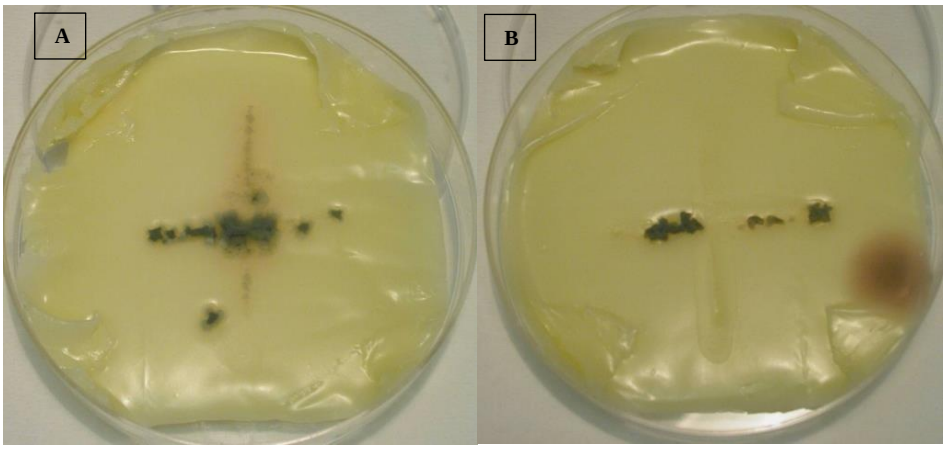


Figure 1: Inhibitory effect by *L. plantarum* UC8491 on growth of *P. roqueforti* UC8455 (A) and *P. brevicompactum* UC7075 (B), through the cheese plate assay. The inhibition effect is visible were the LAB strain were inoculated (central line).

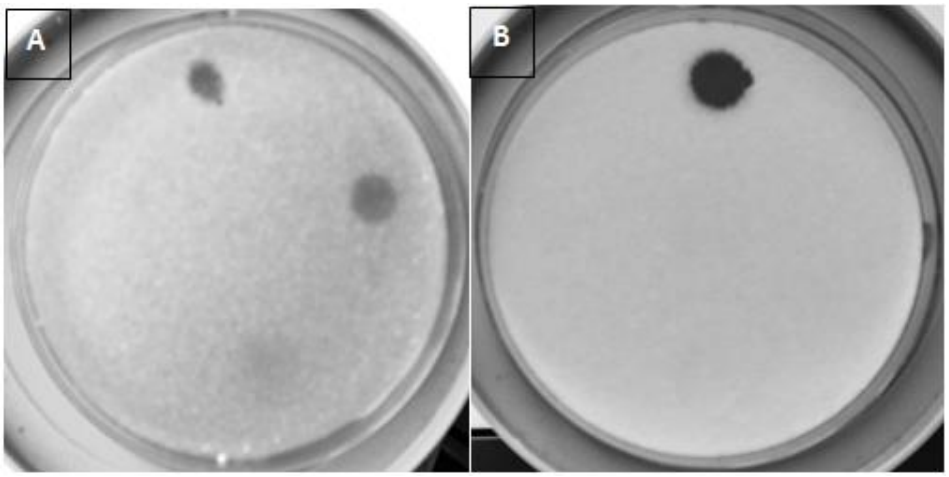


Figure 2: Halo derived for the inhibition activity performed by the use of CFS of *L. plantarum* UC8491 against fungal spoilage strain *P. roqueforti* UC8455 (A) and *P. brevicompactum* UC7075 (B), in agar plate model. Test to examine the level of inhibition.

To identify the chemical nature the *L. plantarum* UC8491 CFS, a Tricine SDS PAGE, in condition designed to separated peptides, was carried out (Fig. 3).

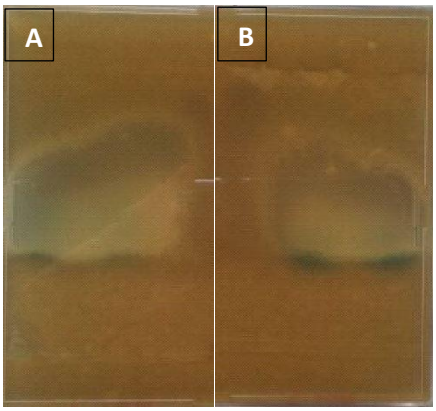


Figure 3: Inhibition grow test of *P. roqueforti* UC8455 (A) and *P. brevicompactum* UC7075 (B) on Tricine-SDS-Page loaded with *L. plantarum* UC8491 Cell Free Supernatant

The presence of a halo in the Tricine-SDS-Page gel, indicates that the inhibitory activities are effectuated by peptides with a range of molecular weight between 2.5 and 7 kDa, produced by the LAB strain *L. plantarum* UC8491. This analysis confirms the thesis that the strain has the capacity to produce antimicrobial compounds with a lower weight, like bacteriocins.

- **Whole genome sequencing**

The genomic DNA from *L. plantarum* UC8491 was sequenced trough a whole-genome shotgun strategy with an Illumina Genome analyser Hiseq1000. Quality-filtered reads were assembled using the Velvet software (version 1.1.04), which generated 62 contigs. Open reading frames (ORFs) were predicted using Glimmer 3.02. Functional annotation was done by merging the results obtained from the RAST Server, BLAST, tRNA scan-SE 1.21 and RNAmmer 1.2.The draft genome has a size of 3.30 Mb and contains 266 subsystems, according to the RAST analysis (Figure 4).

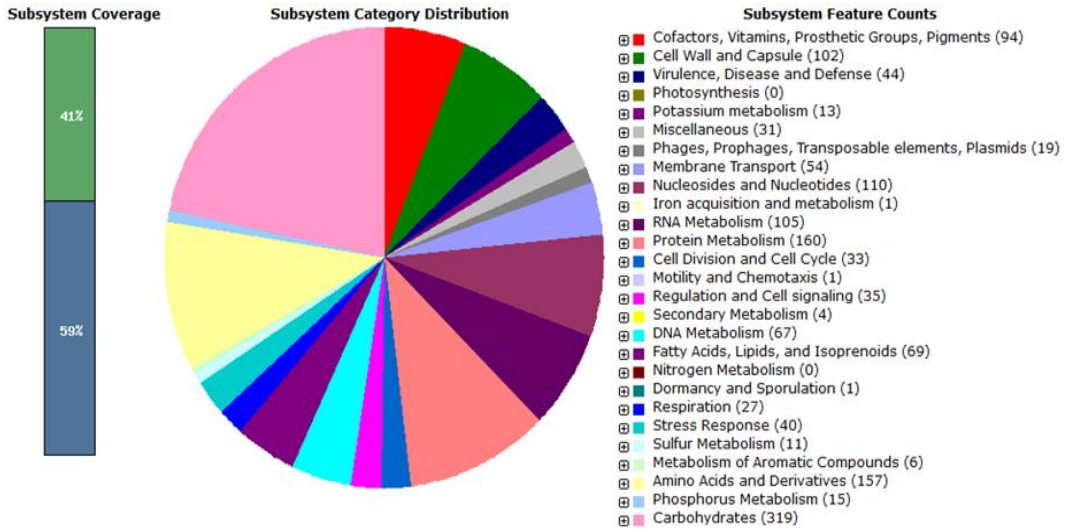


Figure 4: Results obtained by RAST analysis on *L. plantarum* UC 8491 genome

The presence of the plantaricin biosynthesis cluster containing the *pln* genes organized in five operons (*plnABCD; plnEFI*; *plnJKLR*, *plnMNOP*, and *plnGHSTUV)* in the genome of *L. plantarum* UC8491 was confirmed when compared with complete genome from *L. plantarum* WCFS1 present in the RAST database (Figure 5).

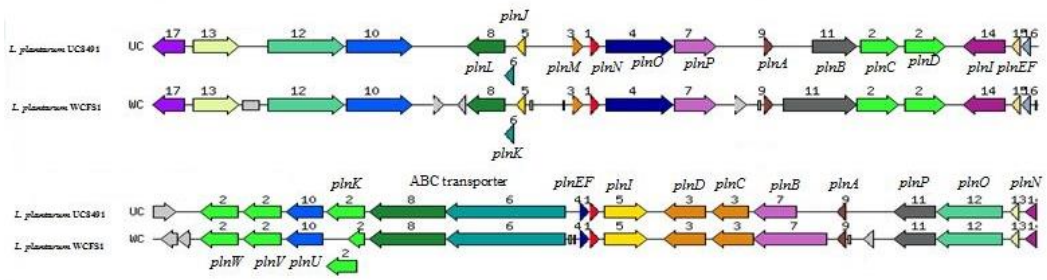


Figure 5: Genome sequences of *L. plantarum* UC8491 confronted with the genome sequences of *L. plantarum* WCFS1. The analysis confirms the presence of bacteriocins (Plantaricin) in the genome of *L. plantarum* UC8491.

Data reported in Figure 5 demonstrate that the *L. plantarum* UC8491 strain is a bacteriocin producer. The comparison with the genome of another strain of *L. plantarum*, present in the database, confirms that the inhibition activity (previously shown) is performed by the presence of gene bacteriocins productions. Moreover, 12 *pln* gene were found: *plnA*, *plnB*, *plnC*, *plnD, plnEF, plnI, plnL, plnM*, *plnN*, *plnO*, *plnP*, *plnJ* and *plnK*. These are all genes implicate on the plantaricin production, with different functions (Table 1).

Target gene	Function	Target gene	Function
plnA	Induction factor	*plnL*	Putative immunity protein
plnB	Histidine kinase	*plnJ*	Prebacteriocin
plnC	Response regulator	*plnK*	Prebacteriocin
plnD	Response regulator	*plnM*	Unknown function
plnE	Prebacteriocin	*plnN*	Putative Prebactercion
plnF	Prebacteriocin	*plnO*	Glycosyl trasnferase
plnI	Immunity	*plnP*	Protease CAAX family

Table 1: *pln* gene individuated through the genome analysis of *L. plantarum* UC8491 and corresponding function

- **Proteomic analysis**

A bottom-up proteomic approach was carried out through QTOF mass spectrometry. Two different samples were used: the cell free supernatant after protein precipitation and ultra-filtering with a Cut-Off of 10 kDa. From the band excised from the Tricine-SDS-PAGE gel, corresponding to the halo of *Penicillium* inhibition. Peptides were reduced and alkylated, then separated via nanoscale CHIP liquid chromatography in reverse mode. Lately analysed in data-dependent tandem mass spectrometry. Spectra were processed in Spectrum Mill versus Bactibase and Bagel3 (bacteriocin mining tools). The analysis of the fraction ≤ 10 kDa resulted in

the identification of several peptides belonging to bacteriocins (in particular class IIc) or related proteins, such as Bioactive peptide 1, acidocin, pyiocin/carocin, lantibiotic, ABC transporter, ATP-binding protein and several peptides with homology to colicins were also identified (Table 2).

Peptide sequence	Protein
(R)YDVAVELQKHVK(T)	Colicin-Ib
(-)EPVVNPPFLQQT(-)	RecName: Full=Bioactive peptide 1; Short=BAP1, partial [Lactobacillus curvatus]
(K)SVSPKYGAKAEQLAR(E)	Colicin-Ib
(K)TFEKYRADINK(K)	Colicin-Ib
(K)WGNAVIGAATPATR(G)	bacteriocin/acidocin LF221B [Lactobacillus gasseri]/porin
(K)VDNTKMALAQNPPK(V)	piocin
(R)DKEITAYKNTLSAQFK(E)	Colicin-Ib
(K)VGEITITPDNSKPGR(Y)	Colicin-Ib
(R)ASVKQCQKTLP(A)	lantibiotic Pep5 [Staphylococcus epidermidis]
(R)HPITAAERKLTEQR(Q)	pyiocin/carocin
(R)YDVAVELQKHVK(T)	colicin
(K)LAVELGKQGTVY(L)	ABC transporter, ATP-binding protein
(I)DWQKPSENKPYP(N)	cell surface protein
(L)AGLLAVNVNLQNIDPL(L)	ABC transporter, ATP-binding protein
(F)INIGISFVIPVY(C)	Transporter, major facilitator family protein
(-)EPVVNPPFLQQT(-)	Bioactive peptide 1
(K)SVSPKYGAKAEQLAR(E)	colicin
(K)GNYPNNTQEPKTGKHV(I)	chitinase [Paenibacillus chitinolyticus]
(I)TKVDGGIIATIPFP(N)	soluble lytic murein transglycosylase-like protein
(E)KLQIFNSGVASIDILQPEV(S)	HAD family hydrolase [Lactobacillus delbrueckii]
(S)FTLPAPTKNAPEAFKK(I)	IpaB/EvcA family protein [Lactobacillus reuteri]
(M)NDGTTKQLPAYMVAAMLGGI(A)	porin. Major intrinsic protein (MIP) superfamily. Members
(N)LVRHDKNIQTVEP(S)	Antimicrobica peptide ABC transporter permease

Table 2: Antimicrobial peptide produced by *L. plantarum* UC8491 corresponding to antimicrobial protein identified by Bactibase database.

Peptides from band excised on Tricine-SDS-PAGE gel, corresponding to the halo of *Penicillium* inhibition, were analysed homology with Enterocin AS-48. Divergicin 750 was found. These results are in accordance with the Bagel database analysis: there is presence in the genome of *L. plantarum* UC8491 (Figure 6) of modified peptide (Table 3) and more than 30 small ORF belonging from putative bacteriocin from Class IIb and IIc (Table 4).

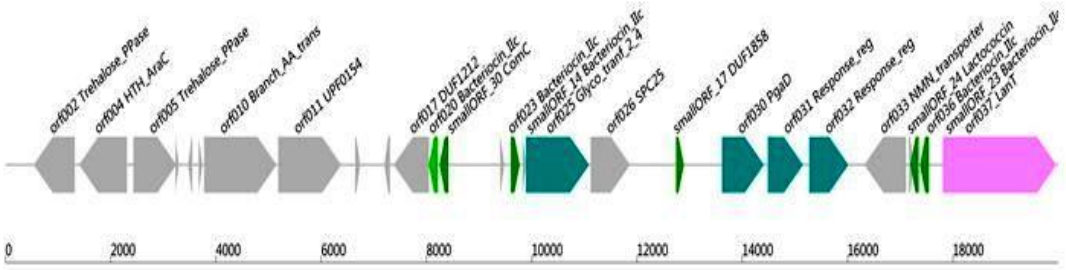


Figure 6: Bacteriocin identification through AOI queryfasta of *L. plantarum* UC8491 performed by BAGEL3.

Type	Protein ID	protein sequence	
ClassII	AOI_1;orf020	MKIKLTVLNEFEELTADAEKNISGGRRSRKNGIGYAIGYAFGAVERAVLGGSRDYNK	Best Hit
ClassII	AOI_1;orf023	MKSLDKIAGLGIEMAEKDLTTVEGGKNYSKTWWYKSLTLLGKVAEGTSSAWHGLG	
ClassII	AOI_1;orf036	LPQKKLAKISGGFNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR	
ClassII	AOI_1;smallORF_14	MAEKDLTTVEGGKNYSKTWWYKSLTLLGKVAEGTSSAWHGLG	
ClassII	AOI_1;smallORF_17	MKIQIKGMKQLSNKEMQKIVGGKSSAYSLQMGATAIKQVKKLFKKWGW	
ClassII	AOI_1;smallORF_23	MLQFEKLQYSRLPQKKLAKISGGFNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR	
ClassII	AOI_1;smallORF_24	MKKFLVLRDRELNAISGGVFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG	
ClassII	AOI_1;smallORF_30	MTVNKMIKDLDVVDAFAPISNNKLNGVVGGGAWKNFWSSLRKGFYDGEAGRAIRR	

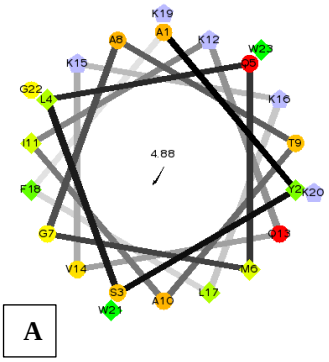
Table 3: Identified putative modified peptide(s) recorded with BAGEL3.

ProteinID	Gene_start	Gene_end	strand	pfam	pfamname	length
AOI_1;orf002	556	1335	-	PF02358.11 [0.00048]	Trehalose_PPase	259
AOI_1;orf004	1418	2326	-	PF00165.18 [2.4e-14]	HTH_AraC	302
AOI_1;orf005	2450	3265	+	PF02358.11 [1e-07]	Trehalose_PPase	271
AOI_1;orf007	3247	3312	+			21
AOI_1;orf008	3486	3566	-			26
AOI_1;orf009	3690	3758	+			22
AOI_1;orf010	3791	5167	+	PF05525.8 [2.8e-130]	Branch_AA_trans	458
AOI_1;orf011	5200	6396	+	PF03672.8 [0.027]	UPF0154	398
AOI_1;orf012	6662	6754	+			30
AOI_1;orf015	7219	7323	-			34
AOI_1;orf017	7396	8064	-	PF06738.7 [0.097]	DUF1212	222
AOI_1;orf020	8061	8234	-	PF10439.4 [0.00018]	Bacteriocin_IIc	57
AOI_1;smallORF_30	8265	8432	-	PF03047.9 [0.00013]	ComC	55
AOI_1;orf022	9417	9494	+			25
AOI_1;orf023	9622	9789	+	PF10439.4 [9.5e-05]	Bacteriocin_IIc	55
AOI_1;smallORF_14	9661	9789	+	PF10439.4 [0.00011]	Bacteriocin_IIc	42
AOI_1;orf024	9843	9914	+			23
AOI_1;orf025	9907	11106	+	PF13704.1 [2.4e-12]	Glyco_tranf_2_4	399
AOI_1;orf026	11137	11883	+	PF06703.6 [0.036]	SPC25	248
AOI_1;smallORF_17	12761	12907	+	PF08984.6 [0.00036]	DUF1858	48
AOI_1;orf030	13632	14426	+	PF13994.1 [0.00011]	PgaD	264
AOI_1;orf031	14502	15170	+	PF00072.19 [2e-10]	Response_reg	222
AOI_1;orf032	15289	16032	+	PF00072.19 [1.1e-09]	Response_reg	247
AOI_1;orf033	16337	17128	-	PF04973.7 [4.2e-05]	NMN_transporter	263
AOI_1;orf034	17186	17275	+			29
AOI_1;smallORF_24	17209	17367	-	PF04369.8 [0.00042]	Lactococcin	52
AOI_1;orf036	17392	17529	-	PF10439.4 [0.00011]	Bacteriocin_IIc	45
AOI_1;smallORF_23	17392	17562	-	PF10439.4 [3.4e-05]	Bacteriocin_IIc	56
AOI_1;orf037	17829	19979	+	PF00437.15 [9.9e-05]	T2SE	716

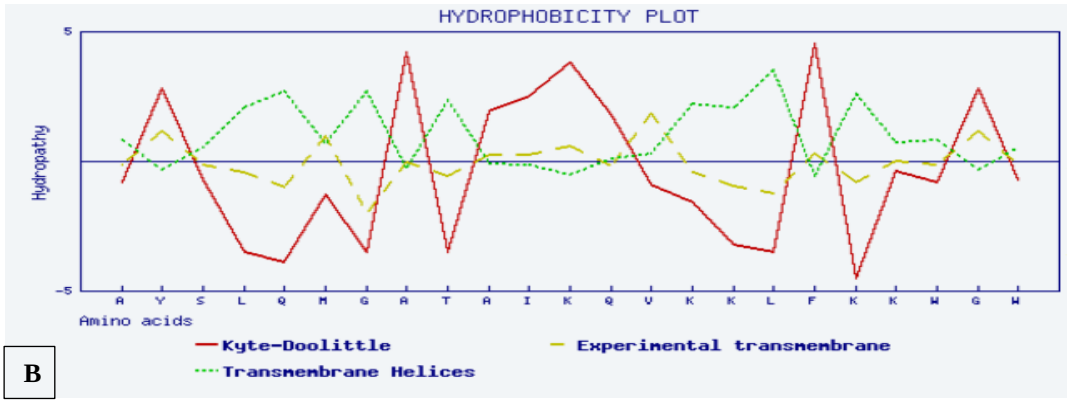
Table 4: Annotation of the context (BAGEL3), evidence the presence of small ORF and modified peptide from putative bacteriocin of Class II

As previously detailed, the detected ORFs in the *L. plantarum* UC8491 genomes are organized into five different operon structures (*plnABCD; plnEFI*; *plnJKLR*, *plnMNOP*, and *plnGHSTUV*). Six bacteriocin group II were detected (PlnA, PlnE,

PlnF, PlnJ, PlnK and PlnN). Of them, five are involved in the plantaricin bacteriocin production. We can conclude that Plantaricin A is a non-lantibiotic one-peptide. As a logical conclusion heat stable bacteriocin may act as pore-forming protein, creating a channel in the cell membrane, with an α-elical secondary structure with low hydrophobicity (Figure 7/A, B and C).



A



B

Figure 7: plantaricin A wheel representation (A) and Hydrophobic plot (B). Red Line: Value according to the hydrophobicity scale of Kyte and Doolitle; Yellow Dashed Plot: Experimentally determined hydrophobicity scale for protein at membrane interface; Green Dotted Dashed Plot: Prediction of transmembrane helices. In this scale more negative value reflect protein hydrophobicity (Source Bactibase)

From the proteomic analysis, other similarly structured peptines -linked to the plantaricin production- were detected. Four peptides (PlnE, PlnF, PlnJ and PlnK)

originate two-peptides bacteriocin PlnE/F and PlnJ/K. These peptides are cationic and adopt a α-elical secondary structure (Figure 8).

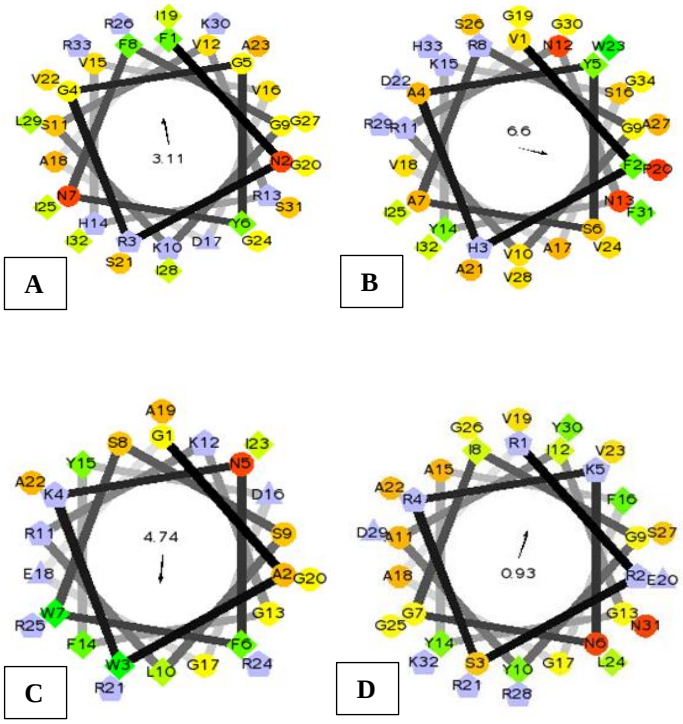


Figure 8: Wheel representation plantaricin peptide PlnE (A), PlnF (B), PlnJ (C) and PlnK (D) (Source Bactibase)

The shotgun Ms/Ms proteomic analysis in the following research was helpful to identify different peptides carrying distinct attitudes in bacteriocin production: colicins, lactococcin, piocin, carocin. Several of them are normally produced by bacterial strains, stranger to the LAB.

DISCUSSIONS

Antimicrobial activity plate assay, cheese plate assay and Tricin-SDS-Page plate assay, show the ability of LAB to inhibit the development of fungal spoilage confirming the putative production on antimicrobial compounds by *L. plantarum* UC8491. The presence of gene encoding to bacteriocin production in the *L. plantarum* UC8491 genome, were confirmed by the results obtained through the proteomic Ms/Ms approach. Further confirmation were obtained when the genome sequence was loaded in the BACTIBASE and BAGEL3 software, and compared with the several genome sequences present in the database. Several studies proof that *L. plantarum* is a LAB able to produce bacteriocin (Massa, 2014; Niku-Paavola, Laitila, Mattila-Sandholm, & Haikara, 1999; Strain et al., 2000; M.V. Leal-Sánchez, R. Jiménez-Díaz, A. Maldonado-Barragán, A. Garrido-Fernández, 2002; Sjögren, Magnusson, Broberg, Schnürer, & Kenne, 2003; Wilson, Sigee, & Epton, 2005; Dal Bello et al., 2007; Prema, Smila, Palavesam, & Immanuel, 2008; Gong, Meng, & Wang, 2010; Yang & Chang, 2010; Yang, Kim, & Chang, 2011; Ramli, Omar, Jin, & Thien, 2012; S Crowley, Mahony, & van Sinderen, 2012; Wang, Yan, Wang, Zhang, & Qi, 2012; Sarah Crowley & Bottacini, 2013; Sorrentino et al., 2013; Wang et al., 2013; Gupta & Srivastava, 2014; Ryu, Yang, Woo, & Chang, 2014; Sangmanee & Hongpattarakere, 2014; Song, Zhu, & Gu, 2014). Latter researches demonstrated the Presence of bacteriocin plantaricin gene clusters, because different ORFs and peptide are strictly involved in the plantaricin production. This bacteriocins class appears to be relatively small. All of the peptides contain a segment that could potentially form an amphiphilic helix. This structure is associated with pore-forming toxins cell membranes channels, through “barrel-stave” mechanisms. (D. B. A. O. Diep, Havarstein, Nissen-meyer, & Nes, 1994; D. B. Diep et al., 1996). (Phoenix, Harris, Daman, & Wallace, 2010). Plantaricin is one of the most common bacteriocin produced by *L. plantarum*

species. Bacteriocins class Iib, and more than 20 different other types, were discovered and purified in different experimentations (Jiménez-Díaz et al., 1995, 1993; Cleveland, Montville, Nes, & Chikindas, 2001; McAuliffe, 2001; Messens & De Vuyst, 2002; Gong et al., 2010; Song et al., 2014). It is now certain that bacteriocin is capable to inhibit a wide range of Gram-positive bacteria (*L. monocytogenes*, *S. aureus*, *M. luteus*, *B. subtilis*, *C. perfringens*, *B. cereus* and some LAB).

Gram-negative microorganisms activity was observed too (Gong et al., 2010; Song et al., 2014). This class of bacteriocin has higher stability in contact with heating and acidic conditions (pH 2.0 – 6.0); this creates the perfect environment to produce bio-preservative and fermented food products with acid pH. Main characteristics of this bacteriocins confer the ability to preserve structure and bactericidial function, even under extreme conditions. This, being an important property in view of its potential use as bio-preservative in food (Vaughan, Eijsink, O'Sullivan, O'Hanlon, & Van Sinderen, 2001; Cintas et al., 2001; O'Sullivan, Ross, & Hill, 2002; Wilson et al., 2005; Aly, T, N, & Alfred, 2006; Fontana, Cocconcelli, Vignolo, & Saavedra, 2015).

According to results, different operons were identified: *plnABCD, pln*EFI, *pln*JKLR, *pln*MNOP and *pln*GHUSTUV.These being involved in the production of plantaricins. Moreover eight open readings obtained are organized into this different operon. The *plnABCD* are probably involved in the production of the bacteriocin Plantaricin A, how suggested by the study of D. B. Diep et al., (1996) and Tai et al., (2015). Plantaricin A is a non-lantibiotic one-peptide bacteriocin, that was the first bacteriocin purified and characterized from the strain *L. plantarum*, and used in the food production (D. B. A. O. Diep, Havarstein, Nissen-meyer, & Nes, 1994).

Studies revealed, however, that the probable function of this peptide is as a pheromone peptide, that induces transcriptions of other *pln* genes; probably gene

codified to plantaricin expression. For these reasons the bacteriocin activity originally attributed to the PlnA, is presumable caused by the interaction with other two-peptides bacteriocins (Tai et al., 2015). This interaction is hypothesized with four other plantaricin peptides (*Pln* E/F and *Pln*J/K) found in the *L. plantarum* UC8491 genome, that will form two different two-peptides bacteriocins (PlnEF and PlnJK) contained in two different operons defined in the present study: *pln*EFI and *pln*JKLR. The antimicrobial activity is depending upon the complementary action of the two peptides PlnE-PlnF and PlnJ-PlnK, which genes are located next to each other. The interaction between complementary peptides is specific. All of these peptides are cationic and adopt an α-helical secondary structure (Hauge, Mantzilas, Eijsink, & Nissen-Meyer, 1999). The four peptide bacteriocin activity was evaluated when combined to form two different two-peptides. Peptides were 10_3 times more active when combined, than when tested individually. Inhibitory activity was evaluated in equal amount of peptides. This confirmed the fact that the structural genes encoding complementary peptides are located next to each other in the same operon and thus are transcribed simultaneously in equal amounts. The combination of the peptide PlnA with PlnEF and PlnJK resulted in an additive antimicrobial effect (D. B. A. O. Diep et al., 1994; Anderssen, Diep, Nes, Eijsink, & Nissen-Meyer, 1998).

As for ORF *pln*GHUSTUV, the first two ORF (*pln*GH) are potentially encoded in two relatively large proteins, and homology searches identified PlnG and PlnH as members of two proteins family:ABC transporter (protein identified by the proteomic analysis in this following study) and their accessory proteins. These are known to be involved in the transport of different substrates across the bacterial membrane (Fatht & Kolter, 1993). The putative ABC transporter PlnG and PlnH have shown a potential percentage of identity to other homologs protein belonging to other bacteriocins, especially lactococcin. As resulted from the proteomic analysis. The other ORFs remained (*pln* S, -T, -U and -V) are predicted to be

hydrophobic and contains PTHs. This suggesting a membrane location. However homology searches and other researches ,showed no similarity with any known protein (D. B. Diep et al., 1996).

ORF *Pln*MNOP also starts with two ORFs, plnMN. Only the PlnN (founded in the *L. plantarum* UC8491) appears to have a double-glycine-type leader and probably contained a frangment wich potentially forms an amphiphilic structure. PlnM differently, appeared to be highly hydrophobic. The last ORFs, PlnO and PlnP encode for relatively large polypeptides. Homology searches of these operon were made but no similarity with other protein was showed. Only the plnO, whose deduced polypeptide contains one of two consensus motifs typical for aspartyl proteases. Such proteins belong to a widely distributed family of proteolytic enzymes known to exist in eukaryotes and viruses but not in bacteria (D. B. A. O. Diep et al., 1994; 1996). This results provide further evidence that strains of *L. plantarum* are able to produce plantaricin. All of them are required for the bacteriocin activity observed during the antimicrobial effect. The previously found operon encodes different bacteriocin-like-peptides with double glycin-type leader; these have no similarity with other protein bacteriocin related. This may be the reason of the different peptides encoding for bacteriocin, not commonly produced by LAB. It is possible to hypothesise the presence of a number of peptide colicin related: a protein produced by some strains of *Escherichia coli.* Nevertheless new information about the latter protein production from others bacteria are emerging. Colicin molecule causes killing after binding to a specific receptor on the outer membrane, being consequently translocated through the cell envelope. (Cascales et al., 2007). Production of colicin by LAB is a little known field, because it is a bacteriocin associated only to *E. coli* strains or similar. Recent researches are demonstrating that, some strains of LAB are able to express the colicin gene. It was demonstrated that colicin can be expressed in various lactic acid bacteria through the use of general protein secretory pathway of other protein or bacteriocin acitivity

(Mccormick, Klaenhammer, & Stiles, 1999). Havarstein, Holo, & Nes, (1994), in their studies report that, through mass spectrophotometry analysis, colicinV leader, shares consensus sequences with peptide leaders that are relatively common among the bacteriocins produced by many LAB. Other authors demonstrated that some lantibiotic bacteriocin secretory pathways, produced by LAB, can express bacteriocin colicin production, after the replacement of some colicin peptide. By the signal peptide of the bacteriocin lantibiotic of LAB (Horn, Ferna, Dodd, Gasson, & Rodríguez, 2004). It is therefore only hypothesised that the strain of LAB used in this research is able to produce colicin, and further detailed studies are necessary. As with colicin, homologous peptides were found which are not commonly produced by strains of *L. plantarum. T*he presence of peptides encoding for the Acidocin, Pyiocin, Lantibiotic PeP5 and others is visible in the results. However these are normally produced by other bacteria strains and species, dissimilar to the bacterial strain used in the following study. It is possible to assert that the sequence similarities to peptides assigned to bacteriocins produced by species other than *L. plantarum*, as supported in this research, were originated from homologous protein (Nandakumar & Talapatra, 2014). There is no certainty that the presence of the peptides determines a similar manifestation of the character to the bacteriocin; however, these results could be used for the development of lactic acid bacteria capable of producing more than one bacteriocin simultaneously. Thereby increasing the usage spectrum in the food industries. In conclusion, the further results highlight how much more needs to be discovered about the production of bacteriocins by strains of LAB. However, it is possible to conclude that the analytical approach supported by LC-MS/MS data acquisition using multiple bioinformatics approach and data mining with different multiple search engines for the global profiling bacteriocins, is newest and more is necessary to read the final data. However the *L. plantarum* UC8491 strain is certainly able to produce

bacteriocins. The strategies resulted in the identification of a wide array of bacteriocin peptides in *L. plantarum* could enhance its potential probiotic benefits.

CONCLUSION

The characterization of bacteriocin production operated by the selected LAB *Lactobacillus plantarum* UC8491, through proteomic analysis, are showing a production of different type of peptide homologue to different bacteriocin, belonging to the Class II bacteriocin. Little is known about the production of different peptide/bacteriocin operated by a single bacteria strain, but gene homologies is hypothesized and the present research are underling the ability of selected *L. plantarum* on the production of bacteriocin. The production of a large spectrum of bacteriocin maybe play, in the future, a crucial role for the food preservation. Finally the present work are confirming the importance of selected LAB for their ability on the production of bacteriocin and for their role for the food bio-preservation.

BIBLIOGRAPHY

. S. a. H., . T. M., & . K. A. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

a.M., M., P.N., K., L.C., B., J.M., S., & J.L., M. (2008). Effects of folate and vitamin B12 deficiencies during pregnancy on fetal, infant, and child development. *Food and Nutrition Bulletin*, *29*(2 SUPPL.), S101–S111. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed8&NEWS=N&AN=18709885

Abee, T., Rombouts, F. M., Hugenholtz, J., Guihard, G., & Letellier, L. (1994). Mode of action of nisin Z against Listeria monocytogenes scott a grown at high and low temperatures. *Applied and Environmental Microbiology*, *60*(6), 1962–1968.

Adams, M. R., & Nicolaides, L. (1997). Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control*, *8*(5-6), 227–239. doi:10.1016/S0956-7135(97)00016-9

Al-Kadamany, E., Khattar, M., Haddad, T., & Toufeili, I. (2003). Estimation of shelf-life of concentrated yogurt by monitoring selected microbiological and physicochemical changes during storage. *LWT - Food Science and Technology*, *36*(4), 407–414. doi:10.1016/S0023-6438(03)00018-5

Aly, S., T, O. C. a, N, B. I. H., & Alfred, T. S. (2006). Bacteriocins and lactic acid bacteria - a minireview. *African Journal of Biotechnology*, *5*(May), 678–683. doi:10.5897/AJB05.388

Arimi, S. M., Koroti, E., Kang'ethe, E. K., Omore, A. O., & McDermott, J. J. (2005). Risk of infection with Brucella abortus and Escherichia coli O157:H7 associated with marketing of unpasteurized milk in Kenya. *Acta Tropica*, *96*(1), 1–8. doi:10.1016/j.actatropica.2005.05.012

Arqués, J. L., Rodríguez, E., Nuñez, M., & Medina, M. (2011). Combined effect of reuterin and lactic acid bacteria bacteriocins on the inactivation of food-borne pathogens in milk. *Food Control*, *22*(3-4), 457–461. doi:10.1016/j.foodcont.2010.09.027

Bankole, S. a, & Adebanjo, a. (2003). Mycotoxins in food in West Africa : current situation and possibilities of controlling it. *Journal of Biotechnology*, *2*(September), 254–263. doi:10.4314/ajb.v2i9.14833

Benech, R. O., Kheadr, E. E., Laridi, R., Lacroix, C., & Fliss, I. (2002). Inhibition of Listeria innocua in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Applied and Environmental Microbiology*, *68*(8), 3683–90. doi:10.1128/AEM.68.8.3683

Black, R. (2003). Editorial Micronutrient deficiency — an underlying cause of morbidity and mortality, *81*(03808), 3808.

Blagojev, N., Škrinjar, M., Vesković-Moračanin, S., & Šošo, V. (2012). Control of mould growth and mycotoxin production by lactic acid bacteria metabolites. *Romanian Biotechnological Letters*, *17*(3), 7219–7226.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *International Journal of Food Microbiology*, *154*(3), 87–97. doi:10.1016/j.ijfoodmicro.2011.12.030

Branca, F., & Ferrari, M. (2002). Impact of micronutrient deficiencies on growth: The stunting syndrome. *Annals of Nutrition and Metabolism*, *46*(SUPPL. 1), 8–17. doi:10.1159/000066397

Brul, S., & Coote, P. (1999). Preservative agents in foods: Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, *50*(1-2), 1–17. doi:10.1016/S0168-1605(99)00072-0

Caplice, E., & Fitzgerald, G. F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, *50*(1-2), 131–149. doi:10.1016/S0168-1605(99)00082-3

Cappa, F., & Cocconcelli, P. S. (2001). Identification of fungi from dairy products by means of 18S rRNA analysis. *International Journal of Food Microbiology*, *69*(1-2), 157–160. doi:10.1016/S0168-1605(01)00587-6

Castellano, P., Belfiore, C., Fadda, S., & Vignolo, G. (2008). A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Science*, *79*(3), 483–499. doi:10.1016/j.meatsci.2007.10.009

Cavera, V. L., Arthur, T. D., Kashtanov, D., & Chikindas, M. L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *International Journal of Antimicrobial Agents*, 7–14. doi:10.1016/j.ijantimicag.2015.07.011

Chung, T., & Axelsson, L. (1989). In vitro studies on reuterin synthesis by Lactobacillus reuteri. *Microbial Ecology in …*, *2*, 137–144. doi:10.3109/08910608909140211

Cintas, L. M., Herranz, C., HernÁndez, P. E., Casaus, M. P., Nes, I. F., & HernÁndez, P. E. (2001). Review: Bacteriocins of Lactic Acid Bacteria. *Food Science & Technology International*, *7*(4), 281–305. doi:10.1106/R8DE-P6HU-CLXP-5RYT

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*(1), 1–20. doi:10.1016/S0168-1605(01)00560-8

Conference, I., & Food, U. N. (2014). Food safety must accompany food and nutrition security-WHO Proceedings. *Nutrition and Food Safety*, *6736*(14), 1–4. doi:10.1016/S0140-6736(14)62037-7

Corsetti, a., Gobbetti, M., & Smacchi, E. (1996). Antibacterial activity of sourdough lactic acid bacteria: isolation of a bacteriocin-like inhibitory substance fromLactobacillus sanfranciscoC57. *Food Microbiology*, *13*(6), 447–456. doi:10.1006/fmic.1996.0051

Cortés-Zavaleta, O., López-Malo, a, Hernández-Mendoza, a, & García, H. S. (2014). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *International Journal of Food Microbiology*, *173*, 30–5. doi:10.1016/j.ijfoodmicro.2013.12.016

Crider, K. S., Bailey, L. B., & Berry, R. J. (2011). Folic Acid Food Fortification—Its History,

Effect, Concerns, and Future Directions. *Nutrients*, *3*(3), 370–384. doi:10.3390/nu3030370

Crittenden, R. G., Martinez, N. R., & Playne, M. J. (2003). Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. *International Journal of Food Microbiology*, *80*(3), 217–222. doi:10.1016/S0168-1605(02)00170-8

Crowley, S., Mahony, J., & van Sinderen, D. (2013). Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends in Food Science & Technology*, *33*(2), 93–109. doi:10.1016/j.tifs.2013.07.004

Cui, Y., Zhang, C., Wang, Y., Shi, J., Zhang, L., Ding, Z.,Cui, H. (2012). Class IIa bacteriocins: Diversity and new developments. *International Journal of Molecular Sciences*, *13*(12), 16668–16707. doi:10.3390/ijms131216668

de Carvalho, a. a. T., Mantovani, H. C., & Vanetti, M. C. D. (2007). Bactericidal effect of bovicin HC5 and nisin against Clostridium tyrobutyricum isolated from spoiled mango pulp. *Letters in Applied Microbiology*, *45*(1), 68–74. doi:10.1111/j.1472-765X.2007.02150.x

de Crécy-Lagard, V., El Yacoubi, B., de la Garza, R. D., Noiriel, A., & Hanson, A. D. (2007). Comparative genomics of bacterial and plant folate synthesis and salvage: predictions and validations. *BMC Genomics*, *8*(Figure 2), 245. doi:10.1186/1471-2164-8-245

Delavenne, E., Ismail, R., Pawtowski, A., Mounier, J., Barbier, G., & Le Blay, G. (2013). Assessment of lactobacilli strains as yogurt bioprotective cultures. *Food Control*, *30*(1), 206–213. doi:10.1016/j.foodcont.2012.06.043

Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin Production: a Probiotic Trait? *Applied and Environmental Microbiology*, *78*(1), 1–6. doi:10.1128/AEM.05576-11

Eijsink, V. G. H., Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H., & Nes, I. F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *81*(1-4), 639–654. doi:10.1023/A:1020582211262

Filtenborg, O., Frisvad, J. C., & Thrane, U. (1996). Moulds in food spoilage. *International Journal of Food Microbiology*, *33*(1), 85–102. doi:10.1016/0168-1605(96)01153-1

Fitzpatrick, T. B., Basset, G. J. C., Borel, P., Carrari, F., DellaPenna, D., Fraser, P. D.,Fernie, A. R. (2012). Vitamin deficiencies in humans: can plant science help? *The Plant Cell*, *24*(2), 395–414. doi:10.1105/tpc.111.093120

Florou-Paneri, P., Christaki, E., & Bonos, E. (2013). Lactic Acid Bacteria as Source of Functional Ingredients. *Lactic Acid Bacteria-R& D for Food, Health and Livestock Purposes*, 589–614.

Gerbaldo, G. a, Barberis, C., Pascual, L., Dalcero, A., & Barberis, L. (2012). Antifungal activity of two Lactobacillus strains with potential probiotic properties. *FEMS Microbiology Letters*, *332*(1), 27–33. doi:10.1111/j.1574-6968.2012.02570.x

Gerez, C. L., Torino, M. I., Rollán, G., & Font de Valdez, G. (2009). Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control*, *20*(2), 144–148. doi:10.1016/j.foodcont.2008.03.005

Gerez, C. L., Torres, M. J., Font de Valdez, G., & Rollán, G. (2013). Control of spoilage fungi by lactic acid bacteria. *Biological Control*, *64*(3), 231–237. doi:10.1016/j.biocontrol.2012.10.009

Gourama, H. (1997). Inhibition of Growth and Mycotoxin Production of Penicillium By Lactobacillus Species. *Food Science & Technology-Lebensmittel-Wissenschaft & Technologie*, *30*(3), 279–283.

Gupta, R., & Srivastava, S. (2014). Antifungal effect of antimicrobial peptides (AMPs LR14) derived from Lactobacillus plantarum strain LR/14 and their applications in prevention of grain spoilage. *Food Microbiology*, *42*, 1–7. doi:10.1016/j.fm.2014.02.005

Harris, L. J., Fleming, H. P., & Klaenhammer, T. R. (1992). Novel paired starter culture system for sauerkraut, consisting of a nisin-resistant Leuconostoc mesenteroides strain and a nisin-producing Lactococcus lactis strain. *Applied and Environmental Microbiology*, *58*(5), 1484–1489.

History, T. H. E., & Folic, O. F. (2001). Historical Review.

Hoey, L., Mcnulty, H., Duffy, M. E., & Hughes, C. F. (2015). EURRECA —Estimating Folate Requirements for Deriving, *8398*(October), 1040–1050. doi:10.1080/10408398.2012.742858

Holzapfel, W. (1997). Use of starter cultures in fermentation on a household scale. *Food Control*, *8*(5-6), 241–258. doi:10.1016/S0956-7135(97)00017-0

Holzapfel, W. H., Geisen, R., & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology*, *24*(3), 343–362. doi:10.1016/0168-1605(94)00036-6

Iyer, R., Tomar, S. K., Uma Maheswari, T., & Singh, R. (2010). Streptococcus thermophilus strains: Multifunctional lactic acid bacteria. *International Dairy Journal*, *20*(3), 133–141. doi:10.1016/j.idairyj.2009.10.005

Jack, R. W., Tagg, J. R., & Ev, M. I. R. (1995). Bacteriocins of Gram-Positive Bacteria, *59*(2), 171–200.

Jones, R. J., Hussein, H. M., Zagorec, M., Brightwell, G., & Tagg, J. R. (2008). Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. *Food Microbiology*, *25*(2), 228–234. doi:10.1016/j.fm.2007.11.001

Journal, T. H. E., & Biolowxl, O. F. (1974). Folate Coenzymes faecalis*. *Cell*, 6–7.

Käferstein, F. (2003). Foodborne diseases in developing countries: aetiology, epidemiology and strategies for prevention. *International Journal of Environmental Health Research*, *13 Suppl 1*(July 2015), S161–S168. doi:10.1080/0960312031000102949

Kuipers, O. P., De Ruyter, P. G. G. a, Kleerebezem, M., & De Vos, W. M. (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology*, *64*(1), 15–21. doi:10.1016/S0168-1656(98)00100-X

Laiño, J. E., Juarez del Valle, M., Savoy de Giori, G., & LeBlanc, J. G. J. (2013). Development of a high folate concentration yogurt naturally bio-enriched using selected lactic acid bacteria. *LWT - Food Science and Technology*, *54*(1), 1–5. doi:10.1016/j.lwt.2013.05.035

Lan, W., Chen, Y., Wu, H., & Yanagida, F. (2012). Bio-protective potential of lactic acid bacteria isolated from fermented wax gourd. *Folia Microbiologica*, *57*(2), 99–105. doi:10.1007/s12223-012-0101-1

LeBlanc, J., de Giori, G., Smid, E. J., Hugenholtz, J., & Sesma, F. (2007). Folate production by lactic acid bacteria and other food-grade microorganisms. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, *1*, 329–339.

Lee, S. C., Billmyre, R. B., Li, A., Carson, S., Sykes, S. M., Huh, E. Y. Heitman, J. (2014). Analysis of a Food-Borne Fungal Pathogen Outbreak: Virulence and Genome of a Mucor circinelloides Isolate from Yogurt. *mBio*, *5*(4), e01390–14–. doi:10.1128/mBio.01390-14

Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., & De Vuyst, L. (2012). On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. *Food Microbiology*, *30*(2), 379–92. doi:10.1016/j.fm.2011.12.021

Lewus, C. B., Kaiser, a., & Montville, T. J. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Applied and Environmental Microbiology*, *57*(6), 1683–1688.

Li, H., Liu, L., Zhang, S., Uluko, H., Cui, W., & Lv, J. (2013). Potential use of Lactobacillus casei AST18 as a bioprotective culture in yogurt. *Food Control*, *34*(2), 675–680. doi:10.1016/j.foodcont.2013.06.023

Lollo, P. C. B., de Moura, C. S., Morato, P. N., Cruz, A. G., Castro, W. D. F., Betim, C. B., Amaya-Farfan, J. (2013). Probiotic yogurt offers higher immune-protection than probiotic whey beverage. *Food Research International*, *54*(1), 118–124. doi:10.1016/j.foodres.2013.06.003

Lukaski, H. C. (2004). Vitamin and mineral status: effects on physical performance. *Nutrition*, *20*(7-8), 632–644. doi:10.1016/j.nut.2004.04.001

Massa, S. (2014). Lactobacillus plantarum as a Strategy for an In situ Production of Vitamin B2. *Journal of Food & Nutritional Disorders*, *s1*(01), 1–5. doi:10.4172/2324-9323.S1-004

McAuliffe, O. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews*, *25*(3), 285–308. doi:10.1016/S0168-6445(00)00065-6

Mcnulty, H., Pentieva, K., Hoey, L., Strain, J. J., & Ward, M. (2012). Nutrition Throughout Life : Folate, *82*(5), 348–354. doi:10.1024/0300-9831/a000130

Messens, W., & De Vuyst, L. (2002). Inhibitory substances produced by Lactobacilli isolated from sourdoughs - A review. *International Journal of Food Microbiology*, *72*(1-2), 31–43. doi:10.1016/S0168-1605(01)00611-0

Motarjemi, Y., Käferstein, F., Moy, G., & Quevedo, F. (1993). Contaminated weaning food: a major risk factor for diarrhoea and associated malnutrition. *Bulletin of the World Health Organization*, *71*(1), 79–92.

Muhialdin, B. J., & Hassan, Z. (2011). Annals . Food Science and Technology BIOPRESERVATION OF FOOD BY LACTIC ACID BACTERIA AGAINST 1 . INTRODUCTION

Müller, O., & Krawinkel, M. (2005). Malnutrition and health in developing countries, *173*(3), 2000–2003.

Naidu, a. S., Bidlack, W. R., & Clemens, R. a. (1999). Probiotic Spectra of Lactic Acid Bacteria (LAB). *Critical Reviews in Food Science and Nutrition*, *39*(1), 13–126. doi:10.1080/10408699991279187

Nakajima, H., Toba, T., Ito, A., Kudo, S., Sato, S., & Sato, Y. (2003). The Screening of Hydrogen Peroxide-Producing Lactic Acid Bacteria and Their Application to Inactivating Psychrotrophic Food-Borne Pathogens. *Current Microbiology*, *47*(3), 231–236. doi:10.1007/s00284-002-3993-1

Niku-Paavola, M. L., Laitila, a, Mattila-Sandholm, T., & Haikara, a. (1999). New types of antimicrobial compounds produced by Lactobacillus plantarum. *J. Appl. Microbiol.*, *86*(1), 29–35. doi:10.1046/j.1365-2672.1999.00632.x

Nissen-Meyer, J., Oppegård, C., Rogne, P., Haugen, H. S., & Kristiansen, P. E. (2010). Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins. *Probiotics and Antimicrobial Proteins*, *2*(1), 52–60. doi:10.1007/s12602-009-9021-z

O'Sullivan, L., Ross, R. P., & Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie*, *84*(5-6), 593–604. doi:10.1016/S0300-9084(02)01457-8

Okereke, a, & Montville, T. J. (1991). Bacteriocin-mediated inhibition of Clostridium botulinum spores by lactic acid bacteria at refrigeration and abuse temperatures. *Applied and Environmental Microbiology*, *57*(12), 3423–8.

Onilude, a a, Fagade, O. E., Bello, M. M., & Fadahunsi, I. F. (2005). Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels. *Journal of Biotechnology*, *4*(December), 1404–1408.

Piard, J. C., & Desmazeaud, M. (1991). Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Le Lait*, *71*(5), 525–541. doi:10.1051/lait:1991541

Powers, H. J. (2007). Folic acid under scrutiny. *The British Journal of Nutrition*, *98*(4), 665–666. doi:10.1017/S0007114507795326

Reisenaãœer, a N. N. a I., & Halsted, C. H. (1987). Issues and Opinions In Nutrition Human Folate Requirements, *45*(October 1986), 600–602.

Republic, C. (2002). Biosynthesis of Folates by Lactic Acid Bacteria and Propionibacteria in Fermented Milk. *Food Science*, *22*(5), 175–181.

Rossi, M., Amaretti, A., & Raimondi, S. (2011). Folate Production by Probiotic Bacteria. *Nutrients*, *3*(12), 118–134. doi:10.3390/nu3010118

Røssland, E., Andersen, G. I., Langsrud, T., & Sørhaug, T. (2003). Inhibition of Bacillus cereus by strains of Lactobacillus and Lactococcus in milk, *89*, 205–212. doi:10.1016/S0168-1605(03)00149-1

Rouse, S., Harnett, D., Vaughan, a., & Sinderen, D. Van. (2008). Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *Journal of Applied Microbiology*, *104*, 915–923. doi:10.1111/j.1365-2672.2007.03619.x

Samelis, J., Kakouri, a., Rogga, K. J., Savvaidis, I. N., & Kontominas, M. G. (2003). Nisin treatments to control Listeria monocytogenes post-processing contamination on Anthotyros, a traditional Greek whey cheese, stored at 4°C in vacuum packages. *Food Microbiology*, *20*(6), 661–669. doi:10.1016/S0740-0020(03)00018-2

Sangmanee, P., & Hongpattarakere, T. (2014). Inhibitory of multiple antifungal components produced by Lactobacillus plantarum K35 on growth, aflatoxin production and ultrastructure alterations of Aspergillus flavus and Aspergillus parasiticus. *Food Control*, *40*, 224–233. doi:10.1016/j.foodcont.2013.12.005

Santos, F., Wegkamp, a., de Vos, W. M., Smid, E. J., & Hugenholtz, J. (2008). High-Level Folate Production in Fermented Foods by the B12 Producer Lactobacillus reuteri JCM1112. *Applied and Environmental Microbiology*, *74*(10), 3291–3294. doi:10.1128/AEM.02719-07

Schillinger, U., Geisen, R., & Holzapfel, W. H. (1996). Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends in Food Science & Technology*, *7*(5), 158–164. doi:10.1016/0924-2244(96)81256-8

Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology*, *16*(1-3), 70–78. doi:10.1016/j.tifs.2004.02.014

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *25*(2), 146–151. doi:10.1080/02652030701567442

Soomro, a. H., Masud, T., & Anwaar, K. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

Stoyanova, L. G., Ustyugova, E. A., Sultimova, T. D., Bilanenko, E. N., Fedorova, G. B., Khatrukha, G. S., … Pirogovskaya, B. (2010). New Antifungal Bacteriocin-Synthesizing Strains of Lactococcus lactis ssp . lactis as the Perspective Biopreservatives for Protection of Raw Smoked Sausages Department of Microbiology , Faculty of Biological , M . V . Lomonosov Moscow State University Len, *5*(4), 477–485.

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, M. H. N., & Hugenholtz, J. (2003). Effects of Cultivation Conditions on Folate Production by Lactic Acid Bacteria. *Metab. Eng.*, *69*(8), 4542–4548. doi:10.1128/AEM.69.8.4542

Thu, B. D., Schultink, W., Dillon, D., Gross, R., Leswara, N. D., & Khoi, H. H. (1999). Effect of daily and weekly micronutrient supplementation on micronutrient deficiencies and growth in young Vietnamese children. *The American Journal of Clinical Nutrition*, *69*(1), 80–86.

Tiwari, B. K., Valdramidis, V. P., O' Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of Natural Antimicrobials for Food Preservation. *Journal of Agricultural and Food Chemistry*, *57*(14), 5987–6000. doi:10.1021/jf900668n

Trias, R. (2008). *Lactic acid bacteria as bioprotective agents against foodborne pathogens and*

spoilage microorganisms in fresh fruits and vegetables. Retrieved from http://www.tdx.cat/handle/10803/7932

Unnevehr, L. (2015). Food safety in developing countries: Moving beyond exports. *Global Food Security*, *4*, 24–29. doi:10.1016/j.gfs.2014.12.001

Unnevehr, L., & Grace, D. (2013). Tackling Aflatoxins : An Overview of Challenges and Solutions, (November), 3–4.

Valyasevi, R., & Rolle, R. S. (2002). An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: Scope for their improvement. *International Journal of Food Microbiology*, *75*(3), 231–239. doi:10.1016/S0168-1605(01)00711-5

Vaughan, a., Eijsink, V. G. H., O'Sullivan, T. F., O'Hanlon, K., & Van Sinderen, D. (2001). An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. *Journal of Applied Microbiology*, *91*(1), 131–138. doi:10.1046/j.1365-2672.2001.01365.x

Walton, E., & Allen, S. (2011). Malnutrition in developing countries. *Paediatrics and Child Health*, *21*(9), 418–424. doi:10.1016/j.paed.2011.04.004

Wong, H., & Chen, Y. (1988). Effects of Lactic Acid Bacteria and Organic Acids Germination of Bacillus cereus Growth and. *Microbiology*, *54*(9), 2179–2184.

World Health Organization. (2014). Antimicrobial resistance. *Who*, *317*(September), 609–610. Retrieved from http://www.who.int/mediacentre/factsheets/fs194/en/#

Yang, E., Fan, L., Jiang, Y., Doucette, C., & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express*, *2*(1), 48. doi:10.1186/2191-0855-2-48

Yang, R., Johnson, M. C., & Ray, B. (1992). Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology*, *58*(10), 3355–3359.

CHAPTER V

ANALYSIS AND CHARACTERIZIATION OF BACTERIOCIN PRODUCTION BY *Lactobacillus rhamnosus* UC8490

ABSTRACT

The consumer's demand for natural food without chemical quality preservative additive, has increased in the last year's, directing the attention of the food industries for the use of natural bio-preservative source. The Lactic Acid Bacteria (LAB) should represent the right answer, with their ability to produce natural proteinaceous compound with antibacterial activity (bacteriocins), during the fermentation process. The production of the bacteriocin still remains poorly understood and need continuous studies, researches and characterization for their directly application during food preparation. In the present study, the whole genome of bacteriocin producing strain *Lactobacillus rhamnosus* UC8490, with ability to inhibit the development of mould food spoilage and foodborne pathogenic bacteria in fermented food (dairy products and vegetable's), was analysed. The draft genome has a size of 3.30 Mb containing 266 subsystems, according to the RAST analysis. A proteomic approach was carried out through a QTOF mass spectrometry from the cell free supernatant (CFS). Peptides were filtered through a 10 kDa membrane, reduced and alkylated, then separated via nanoscale CHIP liquid chromatography in reverse mode, and analyzed in data-dependent tandem mass spectrometry. Spectra were processed in SpectrumMill versus Bactibase, Bagel and UniProt database. During the proteomic analysis, presence of peptide with homology to bacteriocins belonging to Plantaricin group were characterized, confirmed by the presence of amino acid sequence of plantaricin W (PlnW gene). The analysis of the extracted peptide fraction confirm the presence of bacteriocin belonging to the Class II. The results obtained revealed that this strain have the natural ability to produce antimicrobial compound, probably responsible of antibacterial activity.

Key words: Bacteriocin Class II, Plantaricin W, *L. rhamnosus*, QTOF mass spectrometry, antifungal/antimicrobial activity

INTRODUCTION

Anti-microbial properties of some Lactic Acid Bacteria (LAB) allow the extension of food through the fermentation process. The microorganism inhibition responsible for the food spoilage could be attributed to the production of antimicrobial compounds. These can be: organic acids, hydrogen peroxide, antibiotics and bacteriocins (Yang, Johnson, & Ray, 1992; Eijsink et al., 1998; Cleveland, Montville, Nes, & Chikindas, 2001). Different species of *Lactobacillus* utilized during production of manufactured fermented dairy products, inhibit the development of other microorganism- as pathogen bacterial and fungal spoilage- trough the production of anti-bacteria compound or bacteriocins. Bacteriocins are mono or polypeptides with bactericidal or bacteriostatic activity. They react against bacteria that are closely related to the bacteriocin producer strain (Cui et al., 2012; Dobson, Cotter, Ross, & Hill, 2012). Bacteriocins Gram-positive produced microorganism, especially LAB bacteria, display a broad inhibitory spectra with food preservative; moreover they display other therapeutic potential function (Magnusson, Ström, Roos, Sjögren, & Schnürer, 2003; Lan, Chen, Wu, & Yanagida, 2012; Crowley, Mahony, & van Sinderen, 2013). Following this double qualities of LAB, an increased interested in on the use of the LAB has been manifested, due to the bacteriocins production, related to the wide over-prescriptions of antibiotics and possible antibiotics resistance (Cavera, Arthur, Kashtanov, & Chikindas, 2015). A large scale production of bacteria with bacteriocin production ability is required, in order to cultivate bacteriocin as food bio - preservative and as a therapeutic agent. This essay's aim is to analyze the bacteriocin production by the LAB strain *L. rhamnosus* UC8490 related to the bio - protection of fermented food. *Lactobacillus rhamnosus* isn't a widely studied strain. Recently, only a few researchers listed the bacteriocins producers (De Keersmaecker et al., 2006; Sarika, Lipton, & Aishwarya, 2010; Coman et al., 2014;

Delavenne et al., 2015). Bacteriocin *L. rhamnosus* UC8490 is a clinically important LAB (Verdenelli et al., 2009) employ in the production of probiotic strain. Its bacteriocins production ability is relevant in the aim of substituting food chemical preservatives. The use of this strain as bacteriocins producer, in the food production, has yet to be deeply demonstrated. Bacteriocins production and potentials in fermentation process are going to be highlighted in the following disquisition.

- **Aim of the study**

To identify and characterize the bacteriocins produced by *Lactobacillus rhamnosus* UC8490.

MATERIAL AND METHODS

- **Bacteria and medium preparation**

The strain *Lactobacillus rhamnosus* UC8490 selected for his inhibitory activity was let to grow into MRS (de man-Rogosa-Sharpe, Oxoid), at 37°C for 18 hours. Strains of *Penicillium brevicompactum* UC7075 and *Penicillium roqueforti* UC8455 were let to grown in Rose Bengal Agar (Oxoid), for 3/5 days at 30°C. After development, spores were obtained, stored in peptone water at 4°C.

- **Cell-free supernatant (CFS)**

After LAB strain reactivation, 250mL of sterile MRS was inoculated with 1% v/v within the previously obtained supernatant, stored then for 48 hours at 37°C, without stirring/agitating. Aliquots of 50ml were centrifuged at 15.000 rpm for 10 min. The obtained supernatant was separated by the pellet and stored at -80°C for 12 hours. Subsequently sterile frozen samples of CFS were prepared and lyophilized. The lyophilized sample (1,5 mL) was obtained and stored at -20°C for subsequent test.

- **Lyophilized medium bacteriocin analysis**

Culture of *Lactobacillus rhamnosus* UC8490 was loaded into Centrisart I centrifuge tubes (Sartorius Stedim Biotech GmbH, Germany). Proteins and peptides filtered with a cutoff of 10 kDa. Protein fractions were measured with the Bio-Rad protein assay kit using bovin γ-globulin as standard, as manufacturer's instructions. Fifty micro grams of proteins were then reduced in dithiothreitol, alkylated with iodoacetamide and then digested with Trypsin (Promega, Madison WI, USA) at 37 °C overnight.

- **Agar model plate assay**

The inhibition activity of *L. rhamnosus* UC8490 against spoilage fungi *P. brevicompactum* UC7075 and *P. roqueforti* UC8455 was previously tested in MRS plate and in fermented milk. In order to evaluate the effectively inhibition process during fermentation. The Agar plate assay, aliquot of 10µL of supernatant, was crawled on MRS agar plate, at room temperature until dry. 7 mL of Malt Soft Agar containing a suspension of mould spore (50 µL) were poured on the plate. Once the soft agar dried, the plates were incubated at 30 °C, until the mould development (3 – 5 days) and the inhibition were evaluated.

- **Tandem MS analysis and proteins inference**

Tryptic peptides were analyzed by a shotgun MS/MS approach, using a hybrid quadrupole-time-of-flight (Q-TOF) mass spectrometer (6550 IFunnel Q-TOF, Agilent Technologies, CA, USA), with a nano LC Chip Cube source (Agilent Technologies, CA, and USA). The chip consisted of a 40-nL enrichment column (Zorbax 300SB-C18, 5 µm pore size) and a 150 mm separation column (Zorbax 300SB-C18, 5 µm pore size) coupled to an Agilent Technologies 1200 series nano/capillary LC system and controlled by the Mass Hunter Workstation Acquisition (version B.04). A volume of 8 µL was injected per run, loading peptides onto the trapping column at 4 µL min^{-1} in 2% (v/v) acetonitrile and 0.1% (v/v) formic acid. After enrichment, the chip was switched to separation mode and peptides were eluted into the analytical column, during a 150 min acetonitrile gradient (from 3% to 70% v/v in 0.1% formic acid) at 0.6 µl min^{-1}. The mass spectrometer was used in positive ion mode and MS scans were acquired in data-dependent mode over a mass range from 300 to 1700 m/z at 3 spectra s^{-1}.

Precursor ions were selected for auto-MS/MS at an absolute threshold of 1000 and a relative threshold of 0.01%, with a maximum of 20 precursors per cycle (enabling active exclusion after 2 spectra, with release after 0.2 min. Analysis of MS/MS

spectra for peptides identification was performed by protein database searching with Spectrum Mill MS Proteomics Workbench (Rev B.04; Agilent Technologies). Auto MS/MS spectra were extracted from raw data accepting a minimum sequence length of 3 amino acids and merging scans with the same precursor within a mass window of ±0.4 mass-to-charge ratio in a time frame of ±30 s. Search parameters were Scored Peak Intensity (SPI) ≥ 50%, precursor mass tolerance of ± 10 ppm and product ions mass tolerance of ± 20 ppm. Carbamidomethylation of cysteine was set as fixed modification and trypsin was selected as enzyme for digestion, accepting 2 missed cleavages per peptide. Auto thresholds were used for peptide identification in Spectrum Mill, to achieve a target 1% false discovery rate. A label-free quantitation, using the protein summed peptide abundance, was carried out after identification.

- **Genome sequencing analysis**

For the determination of bacteriocin presence in the genome of the LAB strain, the genomic sequence was analyzed using the Illumina Genome Hiseq 1000. Velvet software 1.1.04 was used for counting evaluation. Finally RAST server, BLAST tRNA scan-SE 1.21 and RNAmmer 1 were necessary for the final bacteriocin evaluation; comparing the *L. rhamnosus* UC8490 genome with a recorded genome present in the database, this way founding the gene sequences of bacteriocins.

RESULTS AND DISCUSSIONS

- **Inhibition plate and fermented milk assay**

The images demonstrate the inhibitory effect on the growth of fungi *Penicillium brevicompactum* UC7075 (Figure 1/A) and *Penicillium roqueforti* UC8455 (Figure 1/B): two typical fungi responsible for fungal spoilage in products food, operated

by LAB strain of *Lactobacillus rhamnosus* UC8490. (Rouse, Harnett, Vaughan, & Sinderen, 2008).

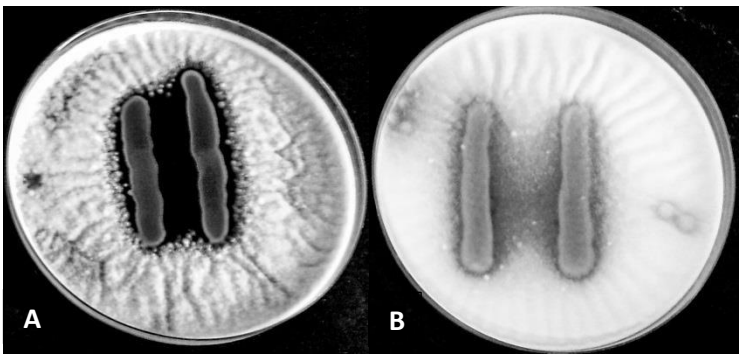


Figure 1: Inhibition effect on fungal spoilage development on MRS plate by *L. rhamnosus* UC8490. Aliquot of 10µL of supernatant were crawled and 7 ml of Malt Agar Soft Agar with fungal spore were spilled. Inhibition halo are visible against *P. brevicompactum* UC7075 (A) and against *P. roqueforti* UC8455 (B), revealing the inhibition activity.

The latter test was advantageous to determine the strain antibacterial activity of LAB. There are visible inhibition zones where the strain of *L. rhamnosus* UC8490 previously grew. The inhibition halos, confirm that the LAB strain is able to inhibit the microorganisms growth, causing a low food quality and causes of food diseases in food consumers (Schnürer & Magnusson, 2005; Ndagijimana et al., 2008; Shephard, 2008; Muhialdin & Hassan, 2011; Blagojev, Škrinjar, Vesković-Moračanin, & Šošo, 2012). To verify that the inhibition effect is related to the antimicrobial compounds production, a bacteriocins research production has been performed, analyzing the genome of the strain *L. rhamnosus* UC8490.

- **Whole genome sequencing**

The genomic DNA of *L. rhamnosus* UC8490 strain was sequenced through a whole- genome shotgun: an Illumina Genome analyzer Hiseq1000. Quality-filtered reads was assembled using the Velvet software (version 1.1.04) generating 89 coatings. Open reading frames (ORFs) were predicted using Glimmer 3.02. Functional annotation by merging the results from the RAST Server, BLAST,

tRNA scan-SE 1.21 and RNAmmer 1.2.The draft genome has a size of 3.30 Mb containing 266 subsystems, according to the RAST analysis (Figure 2).

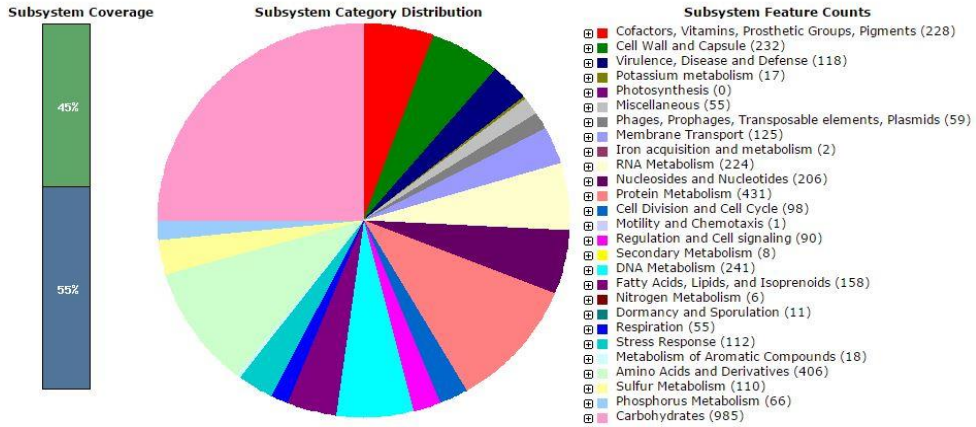


Figure 2: Analysis of the genome of *L. rhamnosus* UC8490 through software RAST. Can be noted the various regions that make up the genome. Can be seen in blue the amplitude of the subsystem concerned in the production of bacteriocins

From the genome analysis, distributions of the composing subsystems are evident. It is deduced that *L. rhamnosus* UC8490 strain is genetically prone to bacteriocin production (Subsystem Virulence, Disease and Defence). Antimicrobial activity of *L. rhamnosus* is nowadays little researched but lately interested in bacteriocin productions within LAB is. Only recently lactobacilli strains interest is arising. Especially in regard to their possible dual attitude (Manuscript, 2009).

- **Proteomic analisys**

To the production of *L. rhamnosus* UC8490 in LAB strain, a bottom-up proteomic approach was applied. Through QTOF mass spectrometry from cell free supernatant, peptides were filtered through a 10 kDa membrane then reduced and alkylated. Finally separated via nanoscale CHIP liquid chromatography in reverse mode and analysed in data-dependent tandem mass spectrometry. Spectra were processed in Spectrum Mill versus Bactibase and Bagel3 database. A first protein analysis showed a peptide presence, homologous to the bacteriocin Plantaricin W

with the following amino acid sequence: SGIPCTIGAAVAASIAVCPTTKCSKRCGKRKK. The amino acid sequence was then subsequently "blasted" with UniProt which confirms the presence of the bacteriocin PlnWβ (Figure 3).

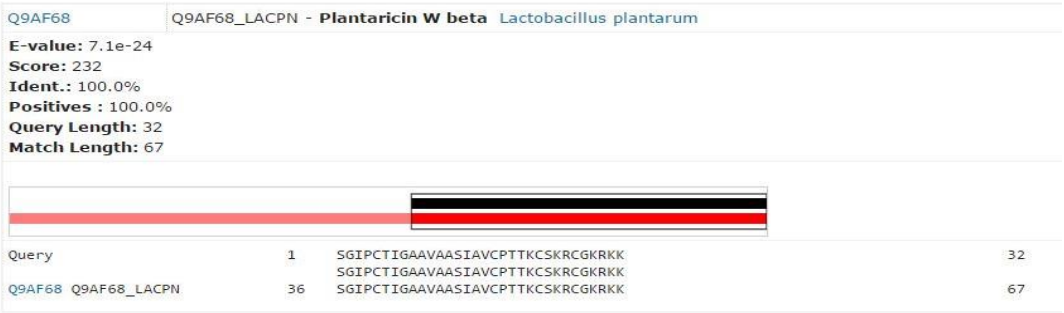


Figure 3: Analysis of the amino acid sequence SGIPCTIGAAVAASIAVCPTTKCSKRCGKRKK obtained by analysis of the genome of the strain *L. rhamnosus* UC8490. The result confirms a homology of 100% with the bacteriocin Plantaricin Wβ

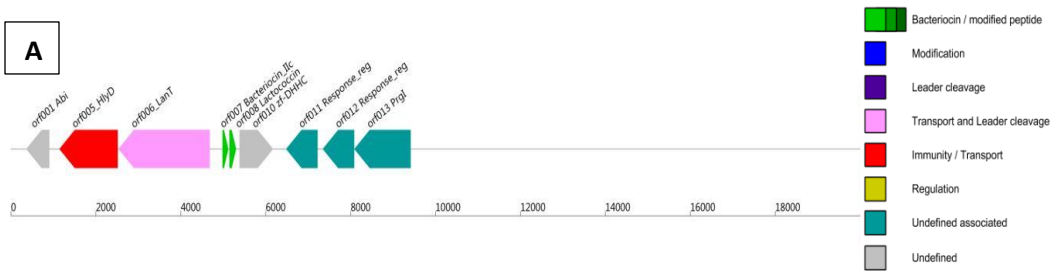
- **Bacteriocin production analysis**

Through the proteomic software such as BAGEL, BACTBIBASE and UNIPROT it was possible to characterize the bacteriocin peptide produced by *L. rhamnosus* UC8490 strain.

PlantaricinW (PlnW), produced by *L. rhamnosus* UC8490 is a new two-peptide bacteriocin generally produced by *Lactobacillus plantarum* strains, but two-peptide bacteriocins have been characterized from many different bacteria, particularly in Lactic Acid Bacteria. (Anderssen, Diep, Nes, Eijsink, & Nissen-Meyer, 1998; Holo, Jeknic, Daeschel, Stevanovic, & Nes, 2001; Nissen-Meyer, Oppegård, Rogne, Haugen, & Kristiansen, 2010). In the PlnW case, it is composed by the peptide Plw*a* and Plw*b*, and is possible to hypothesized that both peptides had an inherent antimicrobial activity (Cintas et al., 1998). In fact the two individual peptides, Plwa (comprising 29 residues) and Plwb (comprising 32 residues), had a sinergical antimicrobial activity, working in 1:1 ratio. Chemical analysis showed that both of

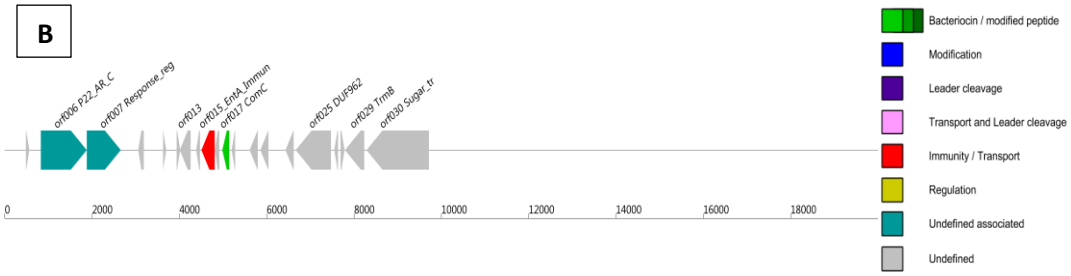
the peptides are lantibiotics, with two unmodified cysteines and one serine residues. Plwa and the Plwb contain only one cysteine residue. This type of lantarcins is constituted with other two-peptide bacteriocins. A new family of bacteriocins, belonging to the type "A" lantibiotics that are linear peptides. Opposed to the circular type "B" lantibiotics (Sahl, Jack, & Bierbaum, 1995). The PlnW structural genes were sequenced and showed to encode prepeptides with similar sequence to two other two-peptide lantibiotics (staphylococcin C55 and lacticin 3147). In the latter study, the founded gene during the chromos analysis of *L. rhamnosus* UC8490 is the plantaricin Wβ (Plwb), as previously showed. This peptide is synthetized with 29 AA leaders, processed by an ABC transporter, sequencly subjected to further proteolytic attack, resulting in the removal of the N-terminal residues (Holo, Jeknic, Daeschel, Stevanovic, & Nes, 2001).

This initial analysis shows the ability of LAB strains of this type, to produce bacteriocins. Unfortunately there is still no detailed knowledge of bacteriocins produced by strains of *L. rhamnosus*. Therefore, a second analysis has been performed, trough BAGEL3 software (Fig. 5/A, B and C). Further confirming the presence of bacteriocin associated peptides.



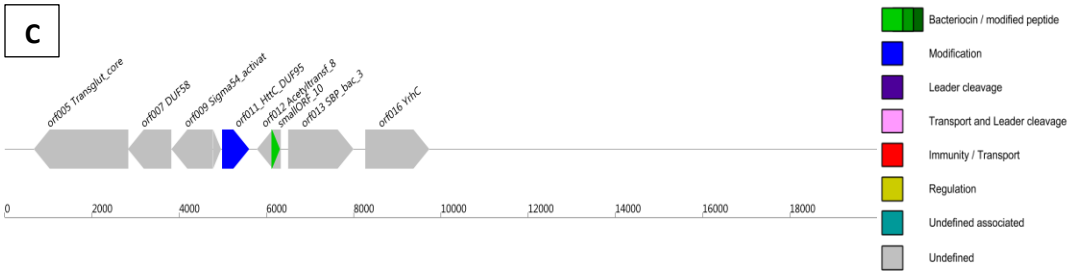
Type	Protein ID	protein sequence
ClassII	AOI_1:orf007	LPQKKLAKISGGFNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR
ClassII	AOI_1:orf008	MKKFLVLRDRELNAISGGVFHAYSARGVRNNYKSAVGPADWVISAVRGFIHG

ProteinID	Gene_start	Gene_end	strand	pfam	pfamname	length	blast_context	reference
AOI_1:orf001	378	923	-	PF02517.11 [1.2e-19]	Abi	181		
AOI_1:orf005	1158	2534	-	PF09429.5 [0.27]	Wbp11	458	P71472_LACPL	
AOI_1:orf006	2550	4700	-	PF01529.15 [5.8]	zf-DHHC	716	Q88ZC2_LACPL	
AOI_1:orf007	5000	5137	+	PF10439.4 [1.7e-05]	Bacteriocin_IIc	45		
AOI_1:orf008	5162	5320	+	PF04369.8 [6.6e-05]	Lactococcin	52		
AOI_1:orf010	5401	6192	+	PF01529.15 [9]	zf-DHHC	263	P71468	
AOI_1:orf011	6497	7240	-	PF00072.19 [1.8e-10]	Response_reg	247	CAA64207.1	
AOI_1:orf012	7359	8102	-	PF00072.19 [3.8e-12]	Response_reg	247	Q48829_LACPL	
AOI_1:orf013	8103	9431	-	PF12666.2 [0.0011]	PrgI	442		



Type	Protein ID	protein sequence
ClassII	AOI_1:orf017	MTKLNEAELSKISGGIGPLVIPVAAILGFLATDAWSHADELVAGVKQGWERS

ProteinID	Gene_start	Gene_end	strand	pfam	pfamname	length	blast_context	reference
AOI_1:orf002	502	579	+			25		
AOI_1:orf006	844	1893	+	PF10548.4 [2.8e-05]	P22_AR_C	349		
AOI_1:orf007	1898	2674	+	PF00072.19 [6e-08]	Response_reg	258	CAA64207.1	
AOI_1:orf008	3075	3200	-			41		
AOI_1:orf009	3645	3716	+			23		
AOI_1:orf012	3955	4035	+			26		
AOI_1:orf013	4028	4273	-			81		
AOI_1:orf014	4400	4489	-			29		
AOI_1:orf015	4523	4825	-	PF12252.3 [8.5e-06]	SidE	100	Q6KCG1_LACSK	
AOI_1:orf016	4826	4930	-			34		
AOI_1:orf017	5000	5158	-	PF03047.9 [0.00024]	ComC	52		
AOI_1:orf018	5216	5293	-			25		
AOI_1:orf020	5626	5811	-	PF10043.4 [8e-05]	DUF2279	61		
AOI_1:orf021	5871	6056	-			61		
AOI_1:orf024	6448	6633	-			61		
AOI_1:orf025	6683	7489	-	PF06127.6 [0.069]	DUF962	268		
AOI_1:orf027	7567	7650	-			27		
AOI_1:orf026	7710	7799	+			29		
AOI_1:orf029	7810	8250	-	PF01978.14 [0.00016]	TrmB	146		
AOI_1:orf030	8305	9732	-	PF00083.19 [1.4e-09]	Sugar_tr	475		



Type	Protein ID	protein sequence	
Head_to_tail_cyclized_peptide	AOI_1:smallORF_10	MTKCSARCSPKLFKVAAKKLISSSAVNVVATHGKKAANESRLVSICSNKSGPSISGRGSASFRS	Best Hit

ProteinID	Gene_start	Gene_end	strand	pfam	pfamname	length	blast_context	reference
AOI_1:orf005	685	2850	-	PF01841.14 [1.9e-29]	Transglut_core	721		
AOI_1:orf007	2843	3835	-	PF01882.13 [4.1e-14]	DUF58	330		
AOI_1:orf009	3840	4784	-	PF00158.21 [1.6e-05]	Sigma54_activat	314		
AOI_1:orf010	4794	4976	+			60		
AOI_1:orf011	5000	5623	+	PF01944.12 [1.3e-24]	DUF95	207		
AOI_1:orf012	5799	6347	-	PF13523.1 [7.2e-07]	Acetyltransf_8	182		
AOI_1:smallORF_10	6133	6327	+			64		
AOI_1:orf013	6518	8014	+	PF00497.15 [7.7e-59]	SBP_bac_3	498		
AOI_1:orf016	8285	9751	+	PF14143.1 [0.00031]	YrhC	488		

Figure 5: Bacteriocins sequences identification, Identified of putative bacteriocin or peptide and Annotation of the context of three (A, B and C) bacteriocins analysis on the genome of *L. rhamnosus* UC8490, made through BAGEL3 database software.

Figures show the identification of three or more bacteriocins. ORF or Small ORF of peptides associated with bacteriocins on the genome of the LAB *L. rhamnosus* UC8490, associated with bacteriocin. Bacteriocin database BAGEL3 was used to perform the results. The detected peptides, with the best hits encoding, for putative bacteriocins, belongs to group II. Among these, we note the most interesting AOI_1: orf008 and AOI_1: orf007 belonging to Plantaricin F and E. These two peptides form a two-peptide bacteriocins called PlnEF. PlnEF ris a bacteriocin formed precisely by two peptides counterparts. The inhibitory ability or antibacterial effect, depends on the synergistic action of both peptides (Anderssen et al., 1998; Hauge, Mantzilas, Eijsink, & Nissen-Meyer, 1999). To highlight the presence of another bacteriocin / peptide AOI_1:orf017, that encodes an enterocin: a commonly bacteriocin produced by strains of *Enterococcus* spp. (Batdorj et al., 2006; Casaus et al., 1997; Cintas et al., 1998; Gong, Meng, & Wang, 2010; Nandakumar & Talapatra, 2014; Tulini et al., 2014) belonging to the classification group of bacteriocins. This kind of bacteriocin classification is not yet defined, amongst bacteria classification. Hypothesis, suggest that enterocine, along with bacteriocins – e.g. staphylococcal cytolysins- should form a separate group. This group might include bactericidal and/or hemolytic active bacteriocin (Cintas et al., 1998). This type of bacteriocins presence includes the one produced by LAB *L. rhamnosus* UC8490 strain. This can be only hypothesized: little is known about bacteriocins production. Recent studies have shown that certain strains of LAB are able to synthesize enterocine (Batdorj et al., 2006), thus ensuring an inhibiting action against pathogenic bacteria development in milk. The cause of presence of different types of bacteriocins by a single strain of LAB is not yet known. Various studies supports the thesis that certain LAB strains have the ability to synthesize different peptides at a genetic level; coding different bacteriocins belonging to diverse groups (O'Sullivan, Ross, & Hill, 2002; Soomro, Masud, & Anwaar, 2002; Batdorj et al., 2006; Dalié, Deschamps, & Richard-Forget, 2010; Nandakumar &

Talapatra, 2014; Trivedi, Jena, & Seshadri, 2014). Nandakumar & Talapatra (2014) show that some strains of LAB are able to produce a different series of bacteriocins or peptides associated with bacteriocins, uncommonly produced by LAB. Among these: Enterocin and Lactococin. Bacteriocins high-inhibitory action and target action can be produced by the same LAB strain. The diverse production of bacteriocins by a single strain is still not defined. In fact the heterogeneity of bacteriocins needs further study. Technologies such as nano LC-MS/MS analysis, could particularly identify the diversity of bacteriocins, since this technique is well suited to the identification of small-molecular-weight and low-abundant bacteriocinogenic peptides (Sarah Crowley et al., 2013). Presence of peptides, obtained through this research, has a high similarity to bacteriocins produced peptides in other species than *L. rhamnosus*. This similarity it is therefore assumed to be caused by the same peptides originating from homologous proteins. Another hypothesis underlying the presence of various peptides generated from a single bacterial strain is that the temperature and the storage conditions could somehow alter the proteins structure (Pasella et al., 2013). Further studies demonstrate that various proteins are susceptible to cold denaturation or air denaturation or at protein-ice interface (Marx, 2012). Also, as pointed out by Nandakumar & Talapatra (2014), the freezing process and subsequent thawing, can cause protein stress. The latter being: changes in ionic strength, water content, protein concentration, and localized pH shifts. Main consequences are denaturation or sensitive proteins aggregation. Even if the cause of protein integrity during the freezing phase is not yet entirely comprehensible. These assumptions emphasize that further studies are necessary to determine the characteristics and the group membership of LAB produced bacteriocins.

CONCLUSIONS

The proteomic analysis of the *Lactobacillus rhamnosus* UC8490 shows a different production of bacteriocin peptide originated by LAB. Little is known about the production capabilities of bacteriocins by *L. rhamnosus* strains. These strains play an important role in food spoilage preservative and probiotic. Bacteriocins characterization, defined into this research, may represent a further step of confirmation. Selected *L. rhamnosus* strains can have a dual role, especially in food production. The first, as probiotic bacteria and secondarily, as bio-protectors, ensuring high food quality and safety.

BIBLIOGRAPHY

. S. a. H., . T. M., & . K. A. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

a.M., M., P.N., K., L.C., B., J.M., S., & J.L., M. (2008). Effects of folate and vitamin B12 deficiencies during pregnancy on fetal, infant, and child development. *Food and Nutrition Bulletin*, *29*(2 SUPPL.), S101–S111. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed8&NEWS=N&AN=18709885

Abee, T., Rombouts, F. M., Hugenholtz, J., Guihard, G., & Letellier, L. (1994). Mode of action of nisin Z against Listeria monocytogenes scott a grown at high and low temperatures. *Applied and Environmental Microbiology*, *60*(6), 1962–1968.

Adams, M. R., & Nicolaides, L. (1997). Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control*, *8*(5-6), 227–239. doi:10.1016/S0956-7135(97)00016-9

Al-Kadamany, E., Khattar, M., Haddad, T., & Toufeili, I. (2003). Estimation of shelf-life of concentrated yogurt by monitoring selected microbiological and physicochemical changes during storage. *LWT - Food Science and Technology*, *36*(4), 407–414. doi:10.1016/S0023-6438(03)00018-5

Aly, S., T, O. C. a, N, B. I. H., & Alfred, T. S. (2006). Bacteriocins and lactic acid bacteria - a minireview. *African Journal of Biotechnology*, *5*(May), 678–683. doi:10.5897/AJB05.388

Arimi, S. M., Koroti, E., Kang'ethe, E. K., Omore, A. O., & McDermott, J. J. (2005). Risk of infection with Brucella abortus and Escherichia coli O157:H7 associated with marketing of unpasteurized milk in Kenya. *Acta Tropica*, *96*(1), 1–8. doi:10.1016/j.actatropica.2005.05.012

Arqués, J. L., Rodríguez, E., Nuñez, M., & Medina, M. (2011). Combined effect of reuterin and lactic acid bacteria bacteriocins on the inactivation of food-borne pathogens in milk. *Food Control*, *22*(3-4), 457–461. doi:10.1016/j.foodcont.2010.09.027

Bankole, S. a, & Adebanjo, a. (2003). Mycotoxins in food in West Africa : current situation and possibilities of controlling it. *Journal of Biotechnology*, *2*(September), 254–263. doi:10.4314/ajb.v2i9.14833

Benech, R. O., Kheadr, E. E., Laridi, R., Lacroix, C., & Fliss, I. (2002). Inhibition of Listeria innocua in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Applied and Environmental Microbiology*, *68*(8), 3683–90. doi:10.1128/AEM.68.8.3683

Black, R. (2003). Editorial Micronutrient deficiency — an underlying cause of morbidity and mortality, *81*(03808), 3808.

Blagojev, N., Škrinjar, M., Vesković-Moračanin, S., & Šošo, V. (2012). Control of mould growth and mycotoxin production by lactic acid bacteria metabolites. *Romanian Biotechnological Letters*, *17*(3), 7219–7226.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., … Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *International Journal of Food Microbiology*, *154*(3), 87–97. doi:10.1016/j.ijfoodmicro.2011.12.030

Branca, F., & Ferrari, M. (2002). Impact of micronutrient deficiencies on growth: The stunting syndrome. *Annals of Nutrition and Metabolism*, *46*(SUPPL. 1), 8–17. doi:10.1159/000066397

Brul, S., & Coote, P. (1999). Preservative agents in foods: Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, *50*(1-2), 1–17. doi:10.1016/S0168-1605(99)00072-0

Caplice, E., & Fitzgerald, G. F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, *50*(1-2), 131–149. doi:10.1016/S0168-1605(99)00082-3

Cappa, F., & Cocconcelli, P. S. (2001). Identification of fungi from dairy products by means of 18S rRNA analysis. *International Journal of Food Microbiology*, *69*(1-2), 157–160. doi:10.1016/S0168-1605(01)00587-6

Castellano, P., Belfiore, C., Fadda, S., & Vignolo, G. (2008). A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Science*, *79*(3), 483–499. doi:10.1016/j.meatsci.2007.10.009

Cavera, V. L., Arthur, T. D., Kashtanov, D., & Chikindas, M. L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *International Journal of Antimicrobial Agents*, 7–14. doi:10.1016/j.ijantimicag.2015.07.011

Chung, T., & Axelsson, L. (1989). In vitro studies on reuterin synthesis by Lactobacillus reuteri. *Microbial Ecology in …*, *2*, 137–144. doi:10.3109/08910608909140211

Cintas, L. M., Herranz, C., HernÁndez, P. E., Casaus, M. P., Nes, I. F., & HernÁndez, P. E. (2001). Review: Bacteriocins of Lactic Acid Bacteria. *Food Science & Technology International*, *7*(4), 281–305. doi:10.1106/R8DE-P6HU-CLXP-5RYT

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, *71*(1), 1–20. doi:10.1016/S0168-1605(01)00560-8

Conference, I., & Food, U. N. (2014). Food safety must accompany food and nutrition security-WHO Proceedings. *Nutrition and Food Safety*, *6736*(14), 1–4. doi:10.1016/S0140-6736(14)62037-7

Corsetti, a., Gobbetti, M., & Smacchi, E. (1996). Antibacterial activity of sourdough lactic acid bacteria: isolation of a bacteriocin-like inhibitory substance fromLactobacillus sanfranciscoC57. *Food Microbiology*, *13*(6), 447–456. doi:10.1006/fmic.1996.0051

Cortés-Zavaleta, O., López-Malo, a, Hernández-Mendoza, a, & García, H. S. (2014). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *International Journal of Food Microbiology*, *173*, 30–5. doi:10.1016/j.ijfoodmicro.2013.12.016

Crider, K. S., Bailey, L. B., & Berry, R. J. (2011). Folic Acid Food Fortification—Its History,

Effect, Concerns, and Future Directions. *Nutrients*, *3*(3), 370–384. doi:10.3390/nu3030370

Crittenden, R. G., Martinez, N. R., & Playne, M. J. (2003). Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. *International Journal of Food Microbiology*, *80*(3), 217–222. doi:10.1016/S0168-1605(02)00170-8

Crowley, S., Mahony, J., & van Sinderen, D. (2013). Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends in Food Science & Technology*, *33*(2), 93–109. doi:10.1016/j.tifs.2013.07.004

Cui, Y., Zhang, C., Wang, Y., Shi, J., Zhang, L., Ding, Z., … Cui, H. (2012). Class IIa bacteriocins: Diversity and new developments. *International Journal of Molecular Sciences*, *13*(12), 16668–16707. doi:10.3390/ijms131216668

de Carvalho, a. a. T., Mantovani, H. C., & Vanetti, M. C. D. (2007). Bactericidal effect of bovicin HC5 and nisin against Clostridium tyrobutyricum isolated from spoiled mango pulp. *Letters in Applied Microbiology*, *45*(1), 68–74. doi:10.1111/j.1472-765X.2007.02150.x

de Crécy-Lagard, V., El Yacoubi, B., de la Garza, R. D., Noiriel, A., & Hanson, A. D. (2007). Comparative genomics of bacterial and plant folate synthesis and salvage: predictions and validations. *BMC Genomics*, *8*(Figure 2), 245. doi:10.1186/1471-2164-8-245

Delavenne, E., Ismail, R., Pawtowski, A., Mounier, J., Barbier, G., & Le Blay, G. (2013). Assessment of lactobacilli strains as yogurt bioprotective cultures. *Food Control*, *30*(1), 206–213. doi:10.1016/j.foodcont.2012.06.043

Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin Production: a Probiotic Trait? *Applied and Environmental Microbiology*, *78*(1), 1–6. doi:10.1128/AEM.05576-11

Eijsink, V. G. H., Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H., & Nes, I. F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *81*(1-4), 639–654. doi:10.1023/A:1020582211262

Filtenborg, O., Frisvad, J. C., & Thrane, U. (1996). Moulds in food spoilage. *International Journal of Food Microbiology*, *33*(1), 85–102. doi:10.1016/0168-1605(96)01153-1

Fitzpatrick, T. B., Basset, G. J. C., Borel, P., Carrari, F., DellaPenna, D., Fraser, P. D., … Fernie, A. R. (2012). Vitamin deficiencies in humans: can plant science help? *The Plant Cell*, *24*(2), 395–414. doi:10.1105/tpc.111.093120

Florou-Paneri, P., Christaki, E., & Bonos, E. (2013). Lactic Acid Bacteria as Source of Functional Ingredients. *Lactic Acid Bacteria-R& D for Food, Health and Livestock Purposes*, 589–614.

Gerbaldo, G. a, Barberis, C., Pascual, L., Dalcero, A., & Barberis, L. (2012). Antifungal activity of two Lactobacillus strains with potential probiotic properties. *FEMS Microbiology Letters*, *332*(1), 27–33. doi:10.1111/j.1574-6968.2012.02570.x

Gerez, C. L., Torino, M. I., Rollán, G., & Font de Valdez, G. (2009). Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control*, *20*(2), 144–148. doi:10.1016/j.foodcont.2008.03.005

Gerez, C. L., Torres, M. J., Font de Valdez, G., & Rollán, G. (2013). Control of spoilage fungi by lactic acid bacteria. *Biological Control*, *64*(3), 231–237. doi:10.1016/j.biocontrol.2012.10.009

Gourama, H. (1997). Inhibition of Growth and Mycotoxin Production of Penicillium By Lactobacillus Species. *Food Science & Technology-Lebensmittel-Wissenschaft & Technologie*, *30*(3), 279–283.

Gupta, R., & Srivastava, S. (2014). Antifungal effect of antimicrobial peptides (AMPs LR14) derived from Lactobacillus plantarum strain LR/14 and their applications in prevention of grain spoilage. *Food Microbiology*, *42*, 1–7. doi:10.1016/j.fm.2014.02.005

Harris, L. J., Fleming, H. P., & Klaenhammer, T. R. (1992). Novel paired starter culture system for sauerkraut, consisting of a nisin-resistant Leuconostoc mesenteroides strain and a nisin-producing Lactococcus lactis strain. *Applied and Environmental Microbiology*, *58*(5), 1484–1489. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=195629&tool=pmcentrez&rendertype=abstract

History, T. H. E., & Folic, O. F. (2001). Historical Review.

Hoey, L., Mcnulty, H., Duffy, M. E., & Hughes, C. F. (2015). EURRECA — Estimating Folate Requirements for Deriving Dietary Reference Values EURRECA — Estimating Folate Requirements for Deriving, *8398*(October), 1040–1050. doi:10.1080/10408398.2012.742858

Holzapfel, W. (1997). Use of starter cultures in fermentation on a household scale. *Food Control*, *8*(5-6), 241–258. doi:10.1016/S0956-7135(97)00017-0

Holzapfel, W. H., Geisen, R., & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology*, *24*(3), 343–362. doi:10.1016/0168-1605(94)00036-6

Iyer, R., Tomar, S. K., Uma Maheswari, T., & Singh, R. (2010). Streptococcus thermophilus strains: Multifunctional lactic acid bacteria. *International Dairy Journal*, *20*(3), 133–141. doi:10.1016/j.idairyj.2009.10.005

Jack, R. W., Tagg, J. R., & Ev, M. I. R. (1995). Bacteriocins of Gram-Positive Bacteria, *59*(2), 171–200.

Jones, R. J., Hussein, H. M., Zagorec, M., Brightwell, G., & Tagg, J. R. (2008). Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. *Food Microbiology*, *25*(2), 228–234. doi:10.1016/j.fm.2007.11.001

Journal, T. H. E., & Biolowxl, O. F. (1974). Folate Coenzymes faecalis*. *Cell*, 6–7.

Käferstein, F. (2003). Foodborne diseases in developing countries: aetiology, epidemiology and strategies for prevention. *International Journal of Environmental Health Research*, *13 Suppl 1*(July 2015), S161–S168. doi:10.1080/0960312031000102949

Kuipers, O. P., De Ruyter, P. G. G. a, Kleerebezem, M., & De Vos, W. M. (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology*, *64*(1), 15–21. doi:10.1016/S0168-1656(98)00100-X

Laiño, J. E., Juarez del Valle, M., Savoy de Giori, G., & LeBlanc, J. G. J. (2013). Development of a high folate concentration yogurt naturally bio-enriched using selected lactic acid bacteria. *LWT - Food Science and Technology*, *54*(1), 1–5. doi:10.1016/j.lwt.2013.05.035

Lan, W., Chen, Y., Wu, H., & Yanagida, F. (2012). Bio-protective potential of lactic acid bacteria isolated from fermented wax gourd. *Folia Microbiologica*, *57*(2), 99–105. doi:10.1007/s12223-012-0101-1

LeBlanc, J., de Giori, G., Smid, E. J., Hugenholtz, J., & Sesma, F. (2007). Folate production by lactic acid bacteria and other food-grade microorganisms. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, *1*, 329–339.

Lee, S. C., Billmyre, R. B., Li, A., Carson, S., Sykes, S. M., Huh, E. Y., … Heitman, J. (2014). Analysis of a Food-Borne Fungal Pathogen Outbreak: Virulence and Genome of a Mucor circinelloides Isolate from Yogurt. *mBio*, *5*(4), e01390–14–. doi:10.1128/mBio.01390-14

Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., & De Vuyst, L. (2012). On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. *Food Microbiology*, *30*(2), 379–92. doi:10.1016/j.fm.2011.12.021

Lewus, C. B., Kaiser, a., & Montville, T. J. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Applied and Environmental Microbiology*, *57*(6), 1683–1688.

Li, H., Liu, L., Zhang, S., Uluko, H., Cui, W., & Lv, J. (2013). Potential use of Lactobacillus casei AST18 as a bioprotective culture in yogurt. *Food Control*, *34*(2), 675–680. doi:10.1016/j.foodcont.2013.06.023

Lollo, P. C. B., de Moura, C. S., Morato, P. N., Cruz, A. G., Castro, W. D. F., Betim, C. B., … Amaya-Farfan, J. (2013). Probiotic yogurt offers higher immune-protection than probiotic whey beverage. *Food Research International*, *54*(1), 118–124. doi:10.1016/j.foodres.2013.06.003

Lukaski, H. C. (2004). Vitamin and mineral status: effects on physical performance. *Nutrition*, *20*(7-8), 632–644. doi:10.1016/j.nut.2004.04.001

Massa, S. (2014). Lactobacillus plantarum as a Strategy for an In situ Production of Vitamin B2. *Journal of Food & Nutritional Disorders*, *s1*(01), 1–5. doi:10.4172/2324-9323.S1-004

McAuliffe, O. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews*, *25*(3), 285–308. doi:10.1016/S0168-6445(00)00065-6

Mcnulty, H., Pentieva, K., Hoey, L., Strain, J. J., & Ward, M. (2012). Nutrition Throughout Life : Folate, *82*(5), 348–354. doi:10.1024/0300-9831/a000130

Messens, W., & De Vuyst, L. (2002). Inhibitory substances produced by Lactobacilli isolated from sourdoughs - A review. *International Journal of Food Microbiology*, *72*(1-2), 31–43. doi:10.1016/S0168-1605(01)00611-0

Motarjemi, Y., Käferstein, F., Moy, G., & Quevedo, F. (1993). Contaminated weaning food: a major risk factor for diarrhoea and associated malnutrition. *Bulletin of the World Health*

Organization, *71*(1), 79–92.

Muhialdin, B. J., & Hassan, Z. (2011). Annals . Food Science and Technology BIOPRESERVATION OF FOOD BY LACTIC ACID BACTERIA AGAINST 1 . INTRODUCTION Lactic acid bacteria (LAB) are Gram-positive microorganisms , prefer anaerobic conditions but are aero-tolerant , catalase negative , cocci or.

Müller, O., & Krawinkel, M. (2005). Malnutrition and health in developing countries, *173*(3), 2000–2003.

Naidu, a. S., Bidlack, W. R., & Clemens, R. a. (1999). Probiotic Spectra of Lactic Acid Bacteria (LAB). *Critical Reviews in Food Science and Nutrition*, *39*(1), 13–126. doi:10.1080/10408699991279187

Nakajima, H., Toba, T., Ito, A., Kudo, S., Sato, S., & Sato, Y. (2003). The Screening of Hydrogen Peroxide-Producing Lactic Acid Bacteria and Their Application to Inactivating Psychrotrophic Food-Borne Pathogens. *Current Microbiology*, *47*(3), 231–236. doi:10.1007/s00284-002-3993-1

Niku-Paavola, M. L., Laitila, a, Mattila-Sandholm, T., & Haikara, a. (1999). New types of antimicrobial compounds produced by Lactobacillus plantarum. *J. Appl. Microbiol.*, *86*(1), 29–35. doi:10.1046/j.1365-2672.1999.00632.x

Nissen-Meyer, J., Oppegård, C., Rogne, P., Haugen, H. S., & Kristiansen, P. E. (2010). Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins. *Probiotics and Antimicrobial Proteins*, *2*(1), 52–60. doi:10.1007/s12602-009-9021-z

O'Sullivan, L., Ross, R. P., & Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie*, *84*(5-6), 593–604. doi:10.1016/S0300-9084(02)01457-8

Okereke, a, & Montville, T. J. (1991). Bacteriocin-mediated inhibition of Clostridium botulinum spores by lactic acid bacteria at refrigeration and abuse temperatures. *Applied and Environmental Microbiology*, *57*(12), 3423–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=183991&tool=pmcentrez&rendertype=abstract

Onilude, a a, Fagade, O. E., Bello, M. M., & Fadahunsi, I. F. (2005). Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels. *Journal of Biotechnology*, *4*(December), 1404–1408.

Piard, J. C., & Desmazeaud, M. (1991). Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Le Lait*, *71*(5), 525–541. doi:10.1051/lait:1991541

Powers, H. J. (2007). Folic acid under scrutiny. *The British Journal of Nutrition*, *98*(4), 665–666. doi:10.1017/S0007114507795326

Reisenaãœer, a N. N. a I., & Halsted, C. H. (1987). Issues and Opinions In Nutrition Human Folate Requirements, *45*(October 1986), 600–602.

Republic, C. (2002). Biosynthesis of Folates by Lactic Acid Bacteria and Propionibacteria in

Fermented Milk. *Food Science*, *22*(5), 175–181.

Rossi, M., Amaretti, A., & Raimondi, S. (2011). Folate Production by Probiotic Bacteria. *Nutrients*, *3*(12), 118–134. doi:10.3390/nu3010118

Røssland, E., Andersen, G. I., Langsrud, T., & Sørhaug, T. (2003). Inhibition of Bacillus cereus by strains of Lactobacillus and Lactococcus in milk, *89*, 205–212. doi:10.1016/S0168-1605(03)00149-1

Rouse, S., Harnett, D., Vaughan, a., & Sinderen, D. Van. (2008). Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *Journal of Applied Microbiology*, *104*, 915–923. doi:10.1111/j.1365-2672.2007.03619.x

Samelis, J., Kakouri, a., Rogga, K. J., Savvaidis, I. N., & Kontominas, M. G. (2003). Nisin treatments to control Listeria monocytogenes post-processing contamination on Anthotyros, a traditional Greek whey cheese, stored at 4°C in vacuum packages. *Food Microbiology*, *20*(6), 661–669. doi:10.1016/S0740-0020(03)00018-2

Sangmanee, P., & Hongpattarakere, T. (2014). Inhibitory of multiple antifungal components produced by Lactobacillus plantarum K35 on growth, aflatoxin production and ultrastructure alterations of Aspergillus flavus and Aspergillus parasiticus. *Food Control*, *40*, 224–233. doi:10.1016/j.foodcont.2013.12.005

Santos, F., Wegkamp, a., de Vos, W. M., Smid, E. J., & Hugenholtz, J. (2008). High-Level Folate Production in Fermented Foods by the B12 Producer Lactobacillus reuteri JCM1112. *Applied and Environmental Microbiology*, *74*(10), 3291–3294. doi:10.1128/AEM.02719-07

Schillinger, U., Geisen, R., & Holzapfel, W. H. (1996). Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. *Trends in Food Science & Technology*, *7*(5), 158–164. doi:10.1016/0924-2244(96)81256-8

Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology*, *16*(1-3), 70–78. doi:10.1016/j.tifs.2004.02.014

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, *25*(2), 146–151. doi:10.1080/02652030701567442

Soomro, a. H., Masud, T., & Anwaar, K. (2002). Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review. *Pakistan Journal of Nutrition*, *1*(1), 20–24. doi:10.3923/pjn.2002.20.24

Stoyanova, L. G., Ustyugova, E. A., Sultimova, T. D., Bilanenko, E. N., Fedorova, G. B., Khatrukha, G. S., … Pirogovskaya, B. (2010). New Antifungal Bacteriocin-Synthesizing Strains of Lactococcus lactis ssp . lactis as the Perspective Biopreservatives for Protection of Raw Smoked Sausages Department of Microbiology , Faculty of Biological , M . V . Lomonosov Moscow State University Len, *5*(4), 477–485.

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, M. H. N., & Hugenholtz, J. (2003). Effects of Cultivation Conditions on Folate Production by Lactic Acid Bacteria. *Metab. Eng.*, *69*(8), 4542–4548. doi:10.1128/AEM.69.8.4542

Thu, B. D., Schultink, W., Dillon, D., Gross, R., Leswara, N. D., & Khoi, H. H. (1999). Effect of daily and weekly micronutrient supplementation on micronutrient deficiencies and growth in young Vietnamese children. *The American Journal of Clinical Nutrition*, *69*(1), 80–86.

Tiwari, B. K., Valdramidis, V. P., O' Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of Natural Antimicrobials for Food Preservation. *Journal of Agricultural and Food Chemistry*, *57*(14), 5987–6000. doi:10.1021/jf900668n

Trias, R. (2008). *Lactic acid bacteria as bioprotective agents against foodborne pathogens and spoilage microorganisms in fresh fruits and vegetables*. Retrieved from http://www.tdx.cat/handle/10803/7932

Unnevehr, L. (2015). Food safety in developing countries: Moving beyond exports. *Global Food Security*, *4*, 24–29. doi:10.1016/j.gfs.2014.12.001

Unnevehr, L., & Grace, D. (2013). Tackling Aflatoxins : An Overview of Challenges and Solutions, (November), 3–4.

Valyasevi, R., & Rolle, R. S. (2002). An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: Scope for their improvement. *International Journal of Food Microbiology*, *75*(3), 231–239. doi:10.1016/S0168-1605(01)00711-5

Vaughan, a., Eijsink, V. G. H., O'Sullivan, T. F., O'Hanlon, K., & Van Sinderen, D. (2001). An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. *Journal of Applied Microbiology*, *91*(1), 131–138. doi:10.1046/j.1365-2672.2001.01365.x

Walton, E., & Allen, S. (2011). Malnutrition in developing countries. *Paediatrics and Child Health*, *21*(9), 418–424. doi:10.1016/j.paed.2011.04.004

Wong, H., & Chen, Y. (1988). Effects of Lactic Acid Bacteria and Organic Acids Germination of Bacillus cereus Growth and. *Microbiology*, *54*(9), 2179–2184.

World Health Organization. (2014). Antimicrobial resistance. *Who*, *317*(September), 609–610. Retrieved from http://www.who.int/mediacentre/factsheets/fs194/en/#

Yang, E., Fan, L., Jiang, Y., Doucette, C., & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express*, *2*(1), 48. doi:10.1186/2191-0855-2-48

Yang, R., Johnson, M. C., & Ray, B. (1992). Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology*, *58*(10), 3355–3359.

GENERAL CONCLUSION

The aim of the present thesis was that to evaluate the use of selected Lactic Acid Bacteria (LAB) to improve quality (through the increasing in folic acid concentration) and safety against food fungal spoilage and foodborne causes, (especially in the dairy food), for the production of food product in the developing countries. For this reason different researches were performed, testing the ability of selected LAB in the production of folic acid and to protect food against the growth of filamentous fungi and food pathogens. Detailed researches were focused on the characterization of the inhibitory compound produced by LAB in milk, for characterize the bacteriocin production during the fermentation process. The economical and easily use of LAB during the food production represent other important further strengths for the food company. Finally together with what has just been stated, the use of bio-product during the food production, such as microorganisms like LAB, can be the answer for the higher demand posted by the consumers for the production of food without ingredients of synthetic origins.

The first part of the thesis was focused on the analysis of the production on folic acid in milk, by selective *St. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Milk is a food with naturally low free folate intake (average 26µg/L), but still play an important role in the diet. In the research it was demonstrated that the folate concentration in milk can been increased by using selected LAB strain with folic acid production ability. *St. thermophilus* represent the strain with the higher ability on the folate production during the fermentation process, but it was shown that the production is still strain dependent and it can change during the early hours of fermentation steps process. It was also demonstrate that the combination with strain of *L. delbrueckii* subsp. *bulgaricus*, for the production of fermented milk, can decrease significantly the folate concentration previously product by the *St. thermophilus* strain, caused by a folate consumption by *L. delbrueckii* subsp. *bulgaricus*. But the results are demonstrating that the pre-selection and an in vitro evaluation of the folate production by LAB represent the better system to find the

right combination of the bacteria to obtain the optimal increasing production of folic acid in milk. Finally, the combination of the two LAB strains can determine a higher final concentration in folate (average increased 100 time's). In the last few years researches shown that the use of folate synthetically produced and use during the food enriching, can be responsible of disease and health problems. The use of LAB with ability to increase the folate amount in milk can represent the right solution for this problem.

The second part of the thesis was focused on the study of the inhibition ability of LAB against fungal food spoilage and pathogens growth in food. In vitro and fermented milk/yogurt assay were performed for the studies. Further studies showed the ability of some LAB to produce lactic acetic, carbon dioxide and other compound able to inhibit the growth of target microorganism. The researchers conducted in the present thesis were pointed on the identification of the production of probable antimicrobial compounds, such as bacteriocins. For that the pH was monitored and the growth rates of the single specie were collected. For the study selected LAB with hypothetical antimicrobial activity were use, *Lactobacillus plantarum* UC8491 and *Lactobacillus rhamnosus* UC8490. The first study was effectuated to evaluate the inhibition activity against two species of *Penicillium* spp., common filamentous fungi responsible of food spoilage. The results shown that the two LAB were able to inhibit the growth of the moulds, both if they was inoculated individually or in combination in fermented milk, showing an higher ability on the mould inhibition. Statistical analysis (probabilistic model of multinomial logistic regression) was made run and the solution demonstrate that all the results obtained were statistical positive, indicating the good inhibitory activity performed by the LAB. pH analysis and inhibition rate of the filamentous fungi give the possibility to speculate the presence of antimicrobial compound, produced by the two LAB strain.

Inhibitory test were performed against food pathogen to. The test were made using the LAB strain *Lactobacillus plantarum* UC8491 and *Lactobacillus rhamnosus* UC8490, used for the test against the fungal spoilage. Preliminary test with commercial fermented milk wad made, and resistance of the pathogens to the fermentation was evaluated. Results shown a different attitude on the resistance on the low pH obtained during the fermentation, from the pathogen strain used during the experiment. Survival rate of the pathogen were subsequently obtained on fermented milk obtained with the inoculum of the two LAB strains, showing a positive inhibitory activity in the first 12 hours of the fermentation process, emphasizing the antimicrobial ability of the two LAB.

The final part of the thesis were focused on the characterization of the antimicrobial compound produced by the LAB strain used during the inhibitory test, *Lactobacillus plantarum* UC8491 and *Lactobacillus rhamnosus* UC8490. Proteomic approach on the genome sequence of the tow LAB was made. The use of different database software was necessary for the evaluation. The results confirm the hypothesis that the LAB selected for the antimicrobial test, have the ability to produce bacteriocin. Different types of bacteriocin were identified. Peptide homologs to plantaricin bacteriocin were identified, but other peptide belonging to bacteriocin not strictly related to the LAB was recorded. The hypothesis for the presence of this peptide was made, according to other authors, but lower is actually known about the presence of homologues peptide encoding for different bacteriocins. Results obtained are important to demonstrate that the LAB strain are able to produce bacteriocin and have the gene to encode peptide of several different bacteriocins with different spectrum of activity, but more studies are necessary to understand all the characteristics of the bacteriocins produced by the lactic acid bacteria.

For conclude, the thesis have shown the probiotic activity of selected LAB strain and their role for the human nutrition, through the production of extracellular

folate. The bio-enrichment of this important micronutrient in milk, can play an important role for the future food production, over all for satisfy the consumer needs. In the present work, also, was shown the ability of LAB to inhibit the growth of fungal spoilage and foodborne pathogen in milk, underling the importance of these bacteria for increase the food safety, without the use of synthetic compounds. But is possible to affirm that, the ability to contrast the growth of pathogens and mould in food, associated with physical food system preservation, can represent the future for the food conservation.

In this thesis it was showed the double attitude of the lactic acid bacteria, the antimicrobial activity (bacteriocin production) and the probiotic function by the folate production. The results obtained confirm the importance of these bacteria for the food production, preservation and for the human health.