LUCIANA RODRIGUES DA CUNHA

GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF
Lactobacillus gasseri ISOLATED FROM A NEWBORN INFANT

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS-BRASIL
2011

LUCIANA RODRIGUES DA CUNHA

GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF *Lactobacillus gasseri* ISOLATED FROM A NEWBORN INFANT

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae*.

APROVADA: 18 de março de 2011.

Pesq. Cláudia Lúcia de O. Pinto (Coorientadora)	**Prof. Paulo César Stringheta (Coorientador)**
Profª Elisabeth Neumann	**Pesq. Marcelo Bonnet Alvarenga**

Profª Célia Lúcia de Luces Fortes Ferreira

(Orientadora)

To my parents

Abel Fernandes da Cunha e Lúcia M. Rodrigues da Cunha

for their unconditional love and constant support

ACKNOWLEDGMENTS

BIOGRAPHY

LUCIANA RODRIGUES DA CUNHA was born on November 23, 1980 in Viçosa, Minas Gerais, Brazil. She obtained a Bachelor's degree in Food Engineering at Federal University of Viçosa, Viçosa, Minas Gerais, Brazil, on January 2004. At the same year, she was admitted by the Graduate Program in Food Science and Technology at the Department of Food Technology of the Federal University of Viçosa and received a fellowship of National Council of Technological and Scientific Development (CNPq). Luciana completed her Master degree in Food Science and Technology with a minor in Food Science and Food Microbiology, in 2006. Following that, she was admitted by the same Graduate Program at the Department of Food Technology of the Federal University of Viçosa, and her studies were funded by the National Council of Technological and Scientific Development (CNPq). In 2009, she was awarded a scholarship from Foundation Coordination for Improvement of Higher Education Personnel (CAPES) to conduct part of her Doctoral studies at the Department of Food, Bioprocessing and Nutrition Sciences of North Carolina State University (NCSU), Raleigh, North Carolina, USA under the direction of Dr. Todd R. Klaenhammer.

LIST OF CONTENTS

CHAPTER I CHARACTERIZATION OF *Lactobacillus gasseri* ISOLATES FROM A BREAST-FED INFANT **60**

LIST OF FIGURES

LIST OF TABLE

LIST OF ABBREVIATIONS

GRAS - Generally Recognized as Safe

LAB – Lactic Acid Bacteria

FAO/WHO – Food and Agriculture Organization and World Health Organization

HMB – Human Milk Bank

UFVCC – Universidade Federal de Viçosa Culture Collection

NCK – North Carolina Klaenhammer Culture Collection

MIC – Minimum Inhibitory Concentration

PFGE – Pulsed Field Gel Electrophoresis

CDS - Coding DNA Sequence

GAMOLA - Global Annotation of Multiplexed On-site bLasted DNA-sequences

BLAST - Basic Local Alignment Search Tool

TA System – Toxin-Antitoxin System

EPAMIG – Empresa de Pesquisa Agropecuária de Minas Gerais

RESUMO

CUNHA, Luciana Rodrigues da, D.Sc.,Universidade Federal de Viçosa, março de 2011. **Caracterização genotípica e fenotípica de *Lactobacillus gasseri* isolatedos de recém nascidos.** Orientadora: Célia Lúcia de Luces Fortes Ferreira. Coorientadores: Cláudia Lúcia de Oliveira Pinto and Paulo César Stringheta.

O objetivo deste estudo foi caracterizar novas estirpes de *Lactobacillus gasseri* isoladas de recém-nascidos saudáveis para potencial uso como probiótico em bancos de leite humano no Brasil. Este estudo foi dividido em três fases. Na primeira, realizou-se a caracterização genotípica das estirpes com a identificação dos isolados ao nivel de espécie e avaliação da diversidade genética entre eles). Caracterização fenotípica também foi realizada considerando os aspectos funcionais e de segurança recomendados pela FAO/OMS para a validação de novas culturas probióticas. Avaliou-se trinta estirpes isoladas de fezes de recém-nascidos quanto à resistência a antibióticos, atividade hemolítica, tolerância a sais biliares, presença de plasmídeos e antagonismo a patógenos Gram-positivos e Gram-negativos. Com base na diversidade genética (PFGE), resistência a antibióticos e perfil plasmidial, três estirpes (NCK2140, 2141 e 2142) foram selecionadas e avaliadas quanto à resistência ao suco gástrico e pancreático, capacidade de adesão à mucina, fibronectina, e às linhagens de células intestinais humanas, Caco-2 e HT-29. Na segunda fase, os quatro plasmídeos identificados na estirpe NCK2141 foram sequenciados pela técnica de *shotgun*, anotados por meio do GAMOLA[®] software e os genes resultantes foram identificados por meio do software Artemis[®]. Posteriormente, análises BLAST foram realizadas para confirmar as anotações *in silico*. Na terceira fase, avaliou-se a capacidade das estirpes selecionadas aderirem à fibronectina imobilizada e a influência dessa proteina sobre as caracteristicas probióticas da célula como, resposta ao estresse digestivo e habilidade de adesão. Os trinta isolados foram identificados como *Lactobacillus gasseri* por meio do sequenciamento do 16S rDNA, e vinte nove deles foram idênticos pela técnica de Eletroforese em Campo Pulsado (PFGE) utilizando-se as enzimas de restrição Sma I e Apa I. O isolado NCK2142 apresentou-se estritamente relacionado aos outros, diferindo-se apenas por uma única banda no gel. Todas as 30 estirpes não apresentaram atividade hemolítica e carreavam três plasmídeos, exceto a estirpe NCK2141, que apresentou um quarto plasmídeo, codificando um sistema de transporte que confere resistência à múltiplas drogas (lmrB). Essa mesma estirpe

mostrou maior tolerância a bile (0.5%) e resistência a eritromicina, cefalotina e oxacilina. Todas as estirpes apresentaram antagonismo frente aos patógenos avaliados sendo a inibição assossiada apenas à produção de ácidos orgânicos. Os três isolados selecionados NCK2140, 2141 e 2142 foram resistentes ao suco gástrico e ao suco pancreático e apresentaram boa capacidade de adesão *in vitro* a mucina, fibronectina e às linhagens de células humanas do câncer de cólon, Caco-2 e HT-29. Os quatro plasmídeos identificados, pTRK1023, 1024, 1025 e 1026 apresentaram morfologia circular, replicação por mecanismo teta e 36, 7, 20 e 46 *open reading frames* (ORF), respectivamente. Os conteúdos de G+C foram consistentes com aqueles encontrados em outras estirpes de *Lactobacillus* (37 - 40%), exceto o pTRK1025 que foi de 44,57%. Algumas propriedades funcionais como, proteínas de ligação ao colágeno (pTRK1026), biossíntese de lantibióticos (pTRK1023) e transporte de carboidratos (pTRK1023 e pTRK1026) foram identificados nos plasmídeos, as quais podem promover vantagens competitivas para as células hospedeiras. Mecanismo de manutenção celular como, Sistema de Separação e Toxina-Antitoxina também foi observado, explicando a dificuldade de cura dos plasmídeos. O gene *lmrB* identificado no pTRK1024, é associado à resistência a alguns antibióticos, no entanto, o experimento de clonagem não confirmou a relação do mesmo com a maior resistencia à eritromicina, cefalotina e oxacilina apresentada pelo isolado NCK2141 carreador desse plasmideo. Por questões de segurança, essa estirpe não pode ser utilizada como um probiótico. Maior adesão *in vitro* foi observada à fibronectina quando as estirpes NCK2140, 2141 e 2142 foram cultivadas em ágar MRS e incubadas em condições de anaerobiose. Observou-se também que os mutantes *fbp* (NCK2147 e NCK2148) tiveram menor aderência à fibronectina imobilizada e à linhagem celular humana HT-29. A inativação do gene *fbp* não influenciou a susceptibilidade das estirpes recombinates ao suco gástrico.

ABSTRACT

CUNHA, Luciana Rodrigues da, D.Sc.,Universidade Federal de Viçosa, March, 2011. **Genotypic and phenotypic characterization of *Lactobacillus gasseri* isolated from a newborn infant**. Adviser: Célia Lúcia de Luces Fortes Ferreira. Co-advisers: Cláudia Lúcia de Oliveira Pinto and Paulo César Stringheta.

The objective of this study was to characterize *Lactobacillus gasseri* strains isolated from a healthy newborn infant for potential use as a probiotic in human milk banks in Brazil. This study was divided into three phases. The first examines the genotypic (identification at the species level and evaluation of genetic diversity among the isolates) and phenotypic characterization of the strains with regard to safety and functional properties currently recommended by FAO/WHO for validation of new probiotic strains. Thirty strains were isolated from breast fed newborn stools and were evaluated for resistance to antibiotics, hemolytic activity, bile tolerance, antagonism toward selected Gram-positive and Gram-negative pathogens and presence of plasmids. Based on the results from PFGE, antibiotic resistance and plasmid profiles, three strains (NCK2140, 2141 and 2142) were selected and evaluated for resistance to small intestine and gastric juices, ability to adhere to mucin, fibronectin, Caco-2 and HT-29 cell lines. In the second phase, the four plasmids identified in the strain NCK2141 were sequenced by the shotgun sequencing technique and were annotated by GAMOLA® software. The resulting genes and CDS designations were viewed using Artemis® software. BLAST was carried out for each designated gene to confirm and to refine the *in silico* annotations. In the third phase, the ability of the selected strains to adhere to fibronectin binding protein and the effect of growth conditions on the adhesion were examined. It was also investigated the functional role of this protein in the adhesion and stress response of the cell. In this study, all thirty isolates were identified as *Lactobacillus gasseri* through 16S rDNA sequencing. Pulsed-Field Gel Electrophoresis (PFGE) using restriction enzymes Sma I and Apa I revealed that 29 of the *L. gasseri* were identical; one isolate (NCK2142) was closely related, but exhibited a distinctive DNA fingerprint. All 30 strains harbored 3 plasmids, with one strain (NCK2141) that showed strong tolerance to 0.5% of bile and harbored a unique fourth plasmid encoding a putative multidrug resistance transporter protein (lmrB). No hemolytic activity or antagonism, beyond acid inhibition was observed. The three selected strains NCK2140, 2141 and 2142

showed strong resistance to small intestinal and gastric juices, and adhered *in vitro* to mucin, fibronectin and two intestinal epithelial cell lines, Caco-2 and HT-29. The complete nucleotide sequence of the plasmids (pTRK1023, 1024, 1025, and 1026) revealed that they are circular and contain 36, 7, 20 and 46 open reading frames, respectively. All four plasmids are predicted to replicate by the theta mechanism, and have G+C contents consistent with those found in other lactobacilli strains (37-40%), with the exception of pTRK1025 (44.57%). The plasmids appear to encode functional properties, such as collagen binding (pTRK1026), lantibiotic biosynthesis (pTRK1023) and carbohydrate transport (pTRK1023 and pTRK1026) that may provide competitive advantages to the plasmid-carrying cells. Cell maintenance mechanisms (partitioning and TA system) were also observed, thus explaining the failure to cure these plasmids. Plasmid pTRK1024 harbored a putative *lmrB* gene, an ATP-binding cassette-type multidrug resistance transporter protein, which has been associated with clindamycin and lincomycin resistance. Cloning experiments did not correlate the erythromycin, cephalothin and oxacillin resistance of NCK2141 to LmrB. However, for safety reasons, strain NCK2141 should not be used as a probiotic. Significantly higher adherence to fibronectin was observed when the strains NCK2140, 2141 and 2142 were grown on MRS agar under anaerobic condition. It was also observed a reduction of *in vitro* adherence of *fbp* mutants (NCK2147 and NCK2148) to immobilized fibronectin and HT-29 intestinal epithelial cells. The inactivation of the *fbp* locus did not influence the gastric juice susceptibility of the mutant strains.

1 - INTRODUCTION

Breastfeeding is recognized worldwide as nurturing and protective to the developing infant. Human milk supplies the nutrients and energy needs of the newborn and protects them against infections. Despite the numerous benefits conferred by this human milk (HM), many children are deprived from its consumption because their mothers cannot breastfeed them. Therefore, human milk banks have a fundamental role as an important food source for such newborns.

Brazil is a world reference in the Human Milk Banking with more than 290 banks currently under operation. In 2010, the 152,813 thousand liters of HM collected provided protection to more than 139,000 newborns. Almost all of these children were prematurely born and were not able to breast feed. Furthermore, it is estimated that 7,000 Brazilian children would die every year if they were not breastfed properly, which emphasizes the importance of the human milk banks (HMB). Pasteurization ($65^{o}C$ x 30 min^{-1}) insures the microbial safety of the milk deposited in the HMB. However, studies from Borba et al (2003) indicated that this heat treatment reduces, inactivates or eliminates some prebiotic constituents, which are essential for establishing *Bifidobacterium* and other desirable lactic acid bacteria (LAB) in the intestinal tract of the newborn, therefore compromising the formation of a beneficial intestinal microbiota for them.

The development of a beneficial microbiota contributes to the health of the host. The gut microbiota acts as an important intestinal immune-modulator, not only educating the naive infant immune system but also serving as an important source of non inflammatory immune stimulators throughout life in healthy individuals. In addition, the enteric microbiota can secrete molecules that inhibit host pathogens, metabolize compounds that harm the host to less toxic substances and produce a range of bioactive compounds such as conjugated linoleic acid, and short chain fatty acids that may play a role in protection from lifestyle illnesses such cancer, obesity and cardiovascular disease.

Given the importance of probiotic bacteria in the formation of a beneficial microbiota and, since the heat treatment is an indispensable practice in HMB, a possible alternative to overcome any undesirable effects of this heat treatment, could be through the addition of well selected endogenous intestinal LAB strains into the

pasteurized milk provided in the milk banks. With this in mind, 30 LAB strains were isolated in previous work in our laboratory of Lactic Acid Bacteria, from the target population, healthy newborn fully breast fed.

In order to claim that a bacterial strain is a potential probiotic, in 2002, the Food and Agriculture Organization (FAO) and World Health Organization (WHO) established guidelines with some safety and functional criteria. According to this document, all probiotic strains need to be properly identified at the genus and species level using current and internationally scientific practices. In addition, they also need to be evaluated for resistance to bile, antagonism toward pathogens, ability to adhere to intestinal epithelial cells, tolerance to small intestine and gastric juices, hemolytic activity and resistance to antibiotics. The importance of assessing the antibiotic resistance profile pattern of new isolates is to limit the use of probiotic cultures harboring plasmids containing transferable antibiotic-resistance genes.

Given the above background and the possible implementation of lactobacilli strains in human milk banks, the objective of this study was to characterize recently isolated lactobacilli from the stool of a newborn infant to select suitable strains for potential use as a probiotic culture in human milk banks in Brazil. To this end, the study was divided in three phases. In the first one the objective was to evaluate some safety and functional aspects currently recommended by FAO/WHO and select those strains with desirable probiotic characteristics for being further evaluated in vivo studies. In the second phase, the goal was to sequence all those plasmids harbored by the selected strains in the first phase to verify if they contained transferable antibiotic-resistance genes. Finally, the third phase aimed to investigate the effect the growth condition on the adhesion of three selected *Lactobacillus gasseri* strains to immobilized fibronectin binding protein and the functional role of this protein in the adhesion and stress response of the cell.

2 - LITERATURE REVIEW

2.1 - Human Milk and Child Health

Human milk is considered the ideal food for the newborn. It has a balanced nutritional composition, containing the right amount of fatty acids, lactose, water and amino acids for newborn digestion, brain development and growth (Willians and Stehlin, 2005), and it is enough to supply all the nutritional needs of newborns during the first six month of life (Workgroup on Breastfeeding, 1997). In addition, it has more than 45 bioactive compounds, which contribute to the maturation of the newborn gastrointestinal tract (Kunz, 1999).

Besides providing optimal nutrition to infants, human milk contains a multitude of immunological components, which are transferred from the mothers to infant via breastfeeding. About 80 percent of the cells in breast milk are macrophages, cells that kill bacteria, fungi and viruses (Willians and Stehlin, 2005). In addition to macrophages, human milk contains proteins such as lactoferrin, lysozyme and casein, lipids, oligosaccharides, enzymes, prostaglandins, growth factors, hormones, and cells that work in many different ways to prevent infections and modulate the immune system. This natural immune protection is not available to artificially fed infants (Hanson, 2007). Studies have been shown that breastfed babies are less susceptible to certain diseases, such as bacterial meningitis, bacteremia, diarrhea, respiratory tract infections, necrotizing enterocolitis, otitis, urinary tract infections (Singhal et al 2002), hypercholesterolemia, and asthma (Chulada et al 2003) than not breastfed infants. These special properties of human milk also provide long-term protection from many diseases seen at higher rates in artificially fed infants, including an increased risk of obesity, type 1 and 2 diabetes, and childhood leukemia (Hamosh, 2001). In addition, the protective effect of human milk against HIV postnatal transmission in humans (Walter et al 2009) and in the reduction of the progression of the disease (Coutsoudis et al 2003; Tozzi et al 1990) also has been reported.

Despite the numerous benefits conferred by human milk, many children are deprived of it because their mothers cannot breastfeed them. Therefore, human milk banks have a fundamental role as an important food source for such newborns (Giugliani, 2002).

2.2 - Human Milk Banks

Human milk banks (HMB) are specialized centers established for encouraging breastfeeding and for collecting, screening, processing, storing and distributing donated human milk (colostrum, transition milk and mature human milk). They must be linked to a maternity or hospital and are responsible for quality control of the milk.

Brazil is a world reference in the Human Milk Banking with 283 banks under current operation (Fiocruz, 2010). In 2007, 1,350 million liters of human milk were collected through the Nacional Net of Human Milk providing protection to more than 107,000 newborns. Almost all these children were prematurely born and were not able to breast feed. Furthermore, it is estimated that 7,000 Brazilian children would die every year if they were not breastfed properly (Bom dia Brazil, 2008) which emphasizes the importance of the human milk banks (HMB).

As evidenced in literature, human milk can be easily contaminated and vehicle of various infectious diseases, such as AIDS, herpes, rubella, hepatitis B and C (Goldin, 1997). Thus, for safety reasons, all milk is pasteurized (65°C x 30 min^{-1}). However, studies from Borba et al (2003) indicated that this heat treatment reduces, inactivates or eliminates some prebiotic constituents. The prebiotic character of human milk is important to establishing *Bifidobacterium* and other desirable probiotic lactic acid bacteria (LAB) in the newborn´s intestinal tract. A possible way to augment any undesirable effects of heat treatment could be through the addition of well selected endogenous intestinal probiotic strains into the pasteurized milk provided in the milk banks.

2.3 - Probiotics - History and definitions

The concept of probiotics was founded in 1908 when Elie Metchnikoff suggested the benefits of lactic bacteria in the prolongation of life (Metchnikoff, 1908). The term "probiotic" was introduced for the first time in 1965 by Lilly and Stillwell to nominate substances of microbiological origin which stimulated the growth of other microorganisms. This term was introduced to differentiate probiotics from antibiotics. In 1989, Fuller emphasized the need for viability and defined probiotics as live organisms which beneficially affect the host by improving its intestinal balance. In 1999, Dunne et al brought up the question of security and defined probiotics as non-pathogenic microorganisms, which when ingested exerted positive influences on health and physiology of the host. With the understanding of

physiological and therapeutical properties of these microorganisms, the definition has been suffering alterations until today and there is still no consensus, however the definition most utilized internationally (Grupta and Garg, 2009; Petrof, 2009; Sanders, 2009; Reid, 2008; Pineiro and Stanton, 2007) is that they are live microorganisms which when administered in adequate concentrations benefit the health of the host (World Health Organization [WHO] and Food and Agriculture Organization [FAO], 2002). In Brazil, the department which regulates probiotic products is the ANVISA (National Agency for Sanitary Vigilance), and according to this Agency probiotics are live microorganisms capable of improving intestinal microbial equilibrium, producing beneficial effects to the health of the individual.

According to the last two definitions, microorganisms must be alive to exert beneficial effects. However, the scientific basis of this affirmation has been questioned by some authors, since there are studies showing that non-viable microorganisms (inactivated by heat or UV radiation), products or even bacterial DNA may exert heath benefits to the host in specific situations (Kataria et al 2009). Zhang et al (2005) reported that thermally inactivated *Lactobacillus rhammnosus* GG promoted a reduction in IL-8 (interleukin-8) production induced by TNFα (tumor necrosis factor alpha) in Caco-2 cells. Xiao et al (2003) administered capsules of active or thermal inactivated *Lactobacillus acidophilus* LB to 64 and 59 patients with chronic diarrhea, respectively. After four weeks of treatment, it was verified that the inactive probiotic form was significantly more effective in the combat of diarrhea. Children (3-24 months old) with acute diarrhea and moderate degrees of dehydration also showed clinical improvement after receiving thermally inactivated *L. acidophilus* (Simakachorn et al 2000). Besides the ability to modulate the immune system and combat diarrhea, it has also been reported that inactivated probiotic bacteria have potential to adhere to intestinal cells (Chauviere et al 1992).

Another aspect to be considered in the definition proposed by the FAO/WHO and ANVISA is the specificity of the host. The definition is broad and does not mention the origin of the strain. For many authors (Staton et al 2003; Dunne et al 2001, Klaenhammer and Kullen, 1999, Charteris et al, 1998) this is an important criterion and if the probiotic was destined for human consumption it must be of human origin. Thus, these microorganisms would have a greater chance of competing with resident microbiota and establish themselves in the intestinal tract of the host (Morelli, 2000). However, there are reports showing the beneficial effects promoted

by *Saccharomyces boulardii*, isolated from lychee (*Litchi chinensis Soon*) in the treatment of diarrheas associated to antibiotics and *Clostridium difficile* (Kotowska et al 2005; Vanderhoof & Young, 2002), ulcerative colitis (Guslandi et al 2000), irritated bowel syndrome (Maupas et al 1983) and Crohn's disease (Guslandi et al 2000).

2.4 - Principal Probiotic Microorganisms

The microorganisms most commonly used as a probiotics are producers of lactic acid such as lactobacilli and bifidobacteria (Ferreira, 2003; Kopp-Hoolihan, 2001). *Enterococcus faecium* and *Enterococcus faecalis* have also been employed as functional supplements in foods and their utilization is permitted in Brazil by the ANVISA. However, there exists a concern on the part of the scientific community in relation to utilization of these bacteria as probiotics due to the evidences relating these microorganisms to cases of bacteremia (Gasser et al 1994), septicemia (Poh et al 2006; Schaberg et al 1991), urinary infections (Poh et al 2006; Lopes et al 2005; Schaberg et al 1991) and endocarditis (Adam et al 1999). Moreover, virulence factors such as hemolysins, gelatinases and surface proteins (Jahangiri et al 2010; Mundy et al 2000), as well as conjugative plasmids encoding antibiotic resistance genes (Eaton & Gasson, 2001) have also been encountered in these bacteria.

2.4.1 - Genus *Lactobacillus*

Lactobacillus are Gram positive microorganisms, nonspore-forming rods or coccobacilli, deprived of flagella and are facultative anaerobic. They possess optimal pH and temperature of growth in the range of 5.5 – 6.3 and 30 – 40ºC respectively (Gomes and Malcata, 1999), and lower G+C content (Carr et al 2002). This genus is amply distributed in the environment, especially in the intestinal tract (Wall et al 2007; Reuter 2001), mouth (Smith et al 2001), vagina (Martin et al 2008) and stomach (Ryan et al 2008) of humans and other animals, as well as in plants, organic material, sewage and fermented or deteriorated foods (Bernardeau et al 2006; Axelsson, 1989).

Taxonomically, the genus *Lactobacillus* belongs to the phylum Firmicutes, class of the *Bacillus*, the order *Lactobacillales* and family *Lactobacillaceae* (Garrity et al 2004). The genus was described for the first time by Beijerinck in 1901, where the species were grouped based on phenotypic characteristics (optimal growth temperature and fermentation of hexoses). Later, they were reclassified as obligatory

homofermentative, facultative heterofermentative and obligatory heterofermentative (Kandller and Weiss, 1986). Obligatory homofermentative lactobacilli include those which exclusively ferment glucose in lactic acid and do not ferment pentoses or gliconate. The obrigatory heterofermentatives include lactobacilli which ferment hexoses in lactic acid, acetic acid and/or ethanol and carbon dioxide, being that the production of gas from glucose is a distinctive characteristic of these bacteria. The facultative heterofermentatives include *Lactobacillus* which ferment hexoses in lactic acid and may produce gas from gliconate, but not from glucose. These microorganisms also ferment pentoses by activity of an induced phosphoketolase to produce lactic and acetic acid (Vásques et al, 2005).

The genus *Lactobacillus* is phylogenetically diverse (Figure 1) and with development of molecular techniques and genetic characterization, this was reorganized and new groups were identified, based on the 16S rRNA region (Collins et al 1991). Currently, in accordance with data obtained from the NCBI (National Center of Biotechnology Information, USA), there are 195 different species of *Lactobacilli*, including subspecies.

2.4.1.1 - *Lactobacillus gasseri*

Lactobacillus gasseri are obligate saccharoclastic, homofermentative organism, with optimum growth at 35 to 38ºC, and forms small rods with rounded ends from 0.6 to 0.8 by 3 to 5 μm in size. *L. gasseri* is considered one of the true autochthonous species of the human intestinal "probiome," defined as commensal intestinal bacteria considered to have a beneficial influence on human health (Azcarate-Peril et al 2008). Depending on consumption habits and geographic location, *L. gasseri* has been determined to be one of the *Lactobacillus* species native to the human gastrointestinal tract (GIT) of neonates (Wall et al 2007) and adults (Reuter, 2001). *L. gasseri* has also been described as a common member of the vaginal (Martin et al 2008) and oral *Lactobacillus* biota (Smith et al 2001), and it has been proposed that the oral cavity acts as a reservoir and source of intestinal lactobacilli (Dal Bello et al 2006).

L. gasseri was routinely classified as "*L. acidophilus*" since morphologically it differs only slightly from *L.acidophilus* and cannot be distinguished from *L. acidophilus* by the classical taxonomic characteristics, such as carbohydrate utilization, lactic acid isomer produced, etc. (Lauer and Kandler, 1980). In 1980, *L.*

gasseri was differentiated by DNA/DNA hybridization patterns from *L. acidophilus* and named after Francis Gasser, who studied lactate dehydrogenases of *Lactobacillus* species (Gasser and Mandel, 1968). Today, the complete genome of *L. gasseri* ATCC 33323 are deposited in the Bacterial Genome Database at the National Center for Biotechnology Information (NCBI) and was sequenced by the Department of Energy-Joint Genome Institute in collaboration with the Lactic Acid Bacteria Genomics Consortium (LABGC) (Klaenhammer et al 2002).

Figure 1: Phylogenetic tree showing the relationships among the species of the Family Lactobacillaceae, including genera *Lactobacillus* (abbreviated with *"L"* in the tree), *Paralactobacillus* and *Pediococcus* (abbreviated with ``*P*`` in the tree). Extracted from Felis et al 2009.

2.5 - Probiotics and beneficial effects to health of children

2.5.1 - Modulation of the intestinal microbiota

The relation between a balanced intestinal microbiota and health of the host has been documented in literature. The enteric microbiota not only functions as a barrier, but is involved in the immune response, inhibition of pathogen growth and synthesis of bioactive compounds, such as conjugated linoleic acid, γ-aminobutíric acid and short chain fatty acids, which exert an important role in the protection against diseases including cancer, obesity and cardiovascular diseases (Wall et al 2009). Analogously, when there is a breakdown of intestinal homeostasis, and pathogenic and/or opportunistic microorganisms are excessive, the enteric microbiota may also contribute to the development of diseases (Kelly et al 2007). Therefore, this suggests that predominance of a beneficial microbiota is associated with the promotion of metabolism, nutrition and health of the host (Mackie et al 1999).

Probiotics may promote the modulation of the intestinal microbiota by competition for epithelium adhesion sites and nutrients, production of antimicrobial compounds and stimulation of the immune system (Sobko et al 2006).

2.5.1.1 - Production of antimicrobial compounds

Organic acids

Lactobacilli and bifidobacteria produce metabolites, such as acetic and lactic acid, which possess an inhibitory effect on growth of pathogenic microorganisms (Servin, 2004). This effect is related to the non-dissociated form of the acid (Podolak et al 1996), which being lipophilic and apolar passively diffuse through the membrane (Kashket, 1987) and promote acidification of the cellular cytoplasm. Therefore, the photoelectrochemical gradient is collapsed promoting interruption of the substrate transport system to the cell (Snijders et al 1985) and impairment of the metabolic function of the microorganism. Acetic acid has a greater inhibitory effect than lactic acids for the same pH value due to its pKa (pKa=5.76) being greater than that of lactic acid (pKa=3.86), resulting in greater concentrations of the non-disassociated form in the bacterial cytoplasm (Vasseur et al 1999).

Not only does it exert its activity by means of reducing the pH and its non-disassociated form, but lactic acid also acts in permeabilization of the membrane in

Gram-negative bacteria (Alakomi et al 2000) and as a chelation agent, capturing elements such as iron, essential for bacterial growth (Presser et al 1997).

Studies *in vitro* have shown organic acids to be the principal inhibiting agent to growth of pathogenic bacteria, as *Escherichia coli* (Maragkoudakis et al 2006; Forestier et al 2001; Ogawa et al 2001; Dembeté et al 1998), *Listeria monocytogenes* (Dembeté et al 1998), *Salmonella typhimurium* (Maragkoudakis et al 2006; Keersmaecker et al 2006; Forestier et al 2001), *Clostridium difficile* (Forestier et al 2001), *Helicobacter pylori* (Maragkoudakis et al 2006; Midolo 1995) and *Staphylococcus aureus* (Dembeté et al 1998).

Bacteriocin and/or molecules similar to bacteriocin

Bacteriocin are antimicrobial compounds of proteic origin, ribosomally synthesized and produced by bacteria, and are generally active against species intimately related with the producing strain (Klaenhammer, 1988; Tagg et al 1976). However, some bacteriocin are capable of inhibiting growth of phylogenetically distant microorganisms, as for example, *Clostridium botulinum*, *Bacillus* sp, *Enterococcus faecalis*, *Listeria monocytogenes* and *Staphylococcus aureus* (Tichaczek et al 1992). These compounds were principally characterized in gram-negative bacteria, in which colicins (*Escherichia coli*) are the most studied (Lazdunski, 1988). Posteriorly, it was found that Gram-positive bacteria also produce these compounds and within this class, lactic acid bacteria have been amply explored for utilization of their bacteriocin in the conservation of foods (Cleveland et al 2001).

These molecules act promoting collapse of the membrane potential by means of electrostatic bonds with the phospholipids (negatively charged). These bonds are favored since the majority of bacteriocins are amplified and cationic. After bonding, the hydrophobic portion of the bacteriocin is inserted in the membrane forming pores. Formation of these pores allow for the exit of ions, principally potassium and magnesium, promoting dissipation of the promoter force, compromising synthesis of macromolecules and production of energy, resulting in cell death (Montville et al 1995).

Lactobacillus and *Bifidobacterium* are microorganisms capable of producing bacteriocins and their substances have been cited as responsible for growth inhibition of various pathogenic bacteria. Lozo et al (2004) identified bacteriocin (Bac217) produced by *Lactobacillus paracasei subsp. paracasei* BGBUK2-16, which showed

inhibitory activity on growth of *Staphylococcus aureus* and *Bacillus cereus*. Yildirim and Johnson (1998) identified bacteriocin (bifidocina B) produced by *Bifidobacterium bifidum* with inhibitory effects on growth of some species of the genera *Listeria, Bacillus, Enterococcus, Lactobacillus, Leuconostoc* and *Pediococcus*. Gassericin A, a bacteriocin produced by *Lactobacillus gasseri* LA39 isolated from feces of newborns, inhibited growth of *Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus* (Itoh et al 1995). Ten Brink et al (1994) identified bacteriocin produced by *Lactobacillus salivarus* M17 (salivaricin B), which present inhibitory activity on growth of *Listeria monocytogenes, Bacillus cereus* and *Enterococcus ssp*. The authors identified another bacteriocin produced by *Lactobacillus acidophilus* M46 (acidocin B), which has an inhibitory effect on *Clostridium speorogenes*.

Lactobacillus and *Bifidobacterium* may also antagonize growth of pathogens by the production of lipid or protein compounds of low molecular weight. Collado et al (2005) encountered inhibition of *Helicobacter pylori* by protein compounds, thermostable and sensitive to proteases, produced by *Bifidobacterium*. The growth of *Giardia intestinalis* and its bonding to Caco-2 human colon adenocarcinoma cells lines was significantly reduced by antimicrobial substances of low molecular weight (La1) produced by *Lactobacillus johnsonii* (Perez et al 2001). Liévin et al (2000) attributed the antagonism of bifidobacteria on growth of *Salmonella typhimurium* to production of lipophilic molecules of low molecular weight (<3500Da).

Production of H_2O_2

Some lactic acid bacteria produce hydrogen peroxide in the presence of oxygen as a result of the action of flavoprotein oxidase or peroxides (NADH). Its antimicrobial effect on various pathogens is the result of the oxidation of sulfhydryl groups, promoting inactivation of various enzymes. Moreover, this substance may promote alteration in permeability of the cell membrane by peroxidation of lipids in the membrane and damages to DNA by formation of free radicals such as (O^{2-}) and hydroxyl (OH^-) (Byezkowski and Gessner, 1988). (Equation 1).

$O_2 + 1e^- \rightarrow O_2^-$ (superoxide radical)

$O_2 + 1e^- + 2H^+ \rightarrow H_2O_2$ (hydrogen peroxide) Eq. 1

$H_2O_2 + 1e^- \rightarrow OH^- + OH^{\cdot}$ (hydroxyl radical)

$OH^{\cdot} + 1e^- + H^+ \rightarrow H_2O$
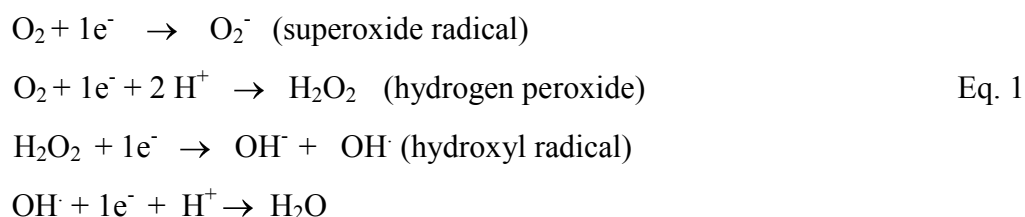
In the membrane peroxidation process, the free hydroxyl radical captures one hydrogen atom from the unsaturated fatty acid of the phospholipid membrane and is transformed in energy. The fatty acid, in the presence of oxygen, generates a free radical and initiates a chain reaction which will destroy the cell membrane. The damaged membrane loses its flexibility and its barrier functions allowing the entrance of calcium into the cell and activating the phospholipases. These enzymes continue to damage the cell since they attack the lysosomal membrane and liberate its enzymes, accelerating degradation. In the DNA, the peroxide radical reacts with the iron ions present in the molecule, producing the hydroxyl radical. This principally attacks the pyrimidine in the bond with deoxyribose, rupturing the sugar-phosphate bond and liberating the free bases of the nucleotides (Silva, 2003).

Lactobacillus plays an important role in maintaining vaginal health and preventing the growth of pathogens including *Escherichia coli* and *Gardnerella vaginalis* by the production of acid and H_2O_2. *Lactobacillus crispatus* and *Lactobacillus jensenii* inhibit growth of gonococci *in vitro* by the production of H_2O_2 (Amant et al 2002). Aroutcheva et al (2001), while examining the antimicrobial activity of twenty-one strains of *Lactobacillus spp.* of vaginal origin, observed that approximately 80% of these produced H_2O_2, lactic acid and bacteriocins, and were effective in inhibiting the growth of *Gardnerella vaginalis*, *Lactobacillus brevis*, *Lactobacillus salivarius* and *Lactobacillus gasseri in vitro*.

2.5.1.2 - Competition for nutrients and adhesion sites

The adhesion of pathogens to the intestinal mucus is considered the first step in the infective processes (Finlay and Falkon, 1997). It is mediated by bacterial adhesins which recognize specific receptors of the mucus. Studies have shown that some probiotic bacterial possess adhesins similar to those of the pathogens (Neesser et al 2000), allowing for adhesion to intestinal sites, making them unavailable to the pathogenic microorganisms (Reid and Hammond, 2005).

Collado et al (2007b) verified inhibition of adhesion of pathogenic bacteria, as *Bacteroides vulgatus* DSM 1447, *Clostridium histolyticum* DSM 627, *Clostridium difficile* DSM 1296, *Escherichia coli* K2, *Enterobacter aerogenes* DSM 30053, *Listeria monocytogenes* ATCC 15313, *Salmonella enterica serovar Typhimurium* ATCC 12028, *Staphylococcus aureus* DSM 20231 to human intestinal mucus by probiotic bacteria (*Lactobacillus rhamnosus GG* [ATCC 53103], *L. rhamnosus* LC705, *L. casei, Shirota* [Yakult], *L. fermentum* ME3, *L. acidophilus* NCFM, *L. plantarum* Lp-115, *L. salivarius* Ls-33, *Bifidobacterium longum* 46, *B. lactis* Bb12, *B. lactis* 420, *B. breve*) isolated from commercial milk products. The authors also reported that these not only inhibited adhesion, but the probiotic bacteria were capable of removing the pathogens already adhered to the intestinal mucus.

In another study performed by the same group, the authors verified inhibition of pathogenic bacteria adhesion (*Bacteroides vulgatus* DSM 1447, *Clostridium histolyticum* DSM 627, *C. difficile* DSM 1296, *Escherichia coli* K2, *Listeria monocytogenes* ATCC 15313, *Salmonella enterica* serovar Typhimurium ATCC 12028, *Staphylococcus aureus* DSM 20231) to the human intestinal mucus by the combination of probiotic bacteria *Lactobacillus rhamnosus* GG (ATCC 53103), *L. rhamnosus* LC705 (DSM 7061), *B. breve* 99 (DSM 13692) and *B. lactis* Bb12 (DSM 10140). The authors also found that the probiotic bacteria in the form of a pool promote greater inhibition to adhesion that when alone (Collado et al 2006). However, the researchers, suggested the individual evaluation of each probiotic strain for later selection of the pool since adhesion level of these bacteria to the intestinal cells are dependent on genus, species and even the strain (Collado et al 2007b; Collado et al 2006).

Lee et al (2003) verified a reduction in the adhesion levels of *E. coli* and *Salmonella* sp. to the intestinal mucus (isolated from the feces of healthy humans) and in Caco-2 human colon adenocarcinoma cell line when incubated together with *Lactobacillus rhamnosus GG* and *Lactobacillus casei* Shirota. *Lactobacillus johnsonii* LJ1 and *Lactobacillus casei* Shirota significantly inhibited adhesion of *Salmonella typhimurium* to the human intestinal mucus (Tuomola et al 1999).

The probiotic bacterial also inhibited growth of pathogens by competitive utilization of nutrients in the gastrointestinal tract. These microorganisms metabolize substrates, such as sugars, vitamins, minerals, proteins, as well as ingredients partially

degraded by the digestive enzymes of the host, making them unavailable to the pathogen (Gibson et al 2005).

2.5.1.3 - Stimulation of the immune system

The intestine represents the largest lymphoid organ of the human body, being responsible for 80% of the immunological response. This action is due to the presence of antibodies and various immunocompetent cells dispersed on the inner wall and epithelium, or organized in structures, which play fundamental role in the antigenic presentation and elaboration of the immune response to the microorganisms and proteins of the diet (Morais and Jacob, 2006).

In vitro studies and animal and human models suggest that probiotics may stimulate the immune system. This effect is attributed to the capacity of these strains to interact with the Peyer's patches and the intestinal epithelial cells, stimulating the Ig A producing B cells and the migration of intestinal T cells (Perdigon and Holgado, 2000). It has also been shown that probiotics favor unexpected phagocytic activity of the alveolar macrophages, suggesting systemic action by secretion of mediators which stimulate the immune system (Cross, 2002). Increase in the secretory IgA serum levels and production of macrophages are extremely important in the immune response of the intestine since this is the first line of non-specific defense of the organism (Vanderhoof, 2001). Schiffrin et al (1997) in a randomized double blind study administered of supplemented or non-supplemented (control) fermented milk with *Bifidobacterium bifidum* Bb12 (1×10^{10} CFU/day) and *Lactobacillus acidophilus* LA1 (7×10^{10} CFU/day) to 28 adults (23 – 62 years old) for 3 weeks and reported a significant increase in phagocytic activity and in the serum levels of granulocytes and monocytes, but not in lymphocytes or T cell activity. Fukushima et al (1998) administered a formula supplemented with *Bifidobacterium bifidum* Bb12 (10^9 CFU/day) to Japanese children (15-31 months). After 21 days of treatment a significant increase was verified in levels of Ig A in the feces of the infants fed with the probiotic strain. The authors concluded that this increase in Ig A may increase the resistance of mucus to intestinal infections.

2.5.2 - Maturation of the Intestinal Microbiota

The gastrointestinal tract includes a complex microbiota with more than 500 bacterial species. This is sterile when we are born, but soon becomes colonized by

microorganisms present in the environment and in the mother (Mitsuoka, 1982). Roughly 24 hours after birth, the feces of most newborns already contain bacteria, such as coliforms, *Enterococcus, Clostridium* and *Sthaphylococcus* in various proportions (Figure 2), whose growth is favored at the beginning of colonization in function of the positive oxido-reduction potential in the intestine.
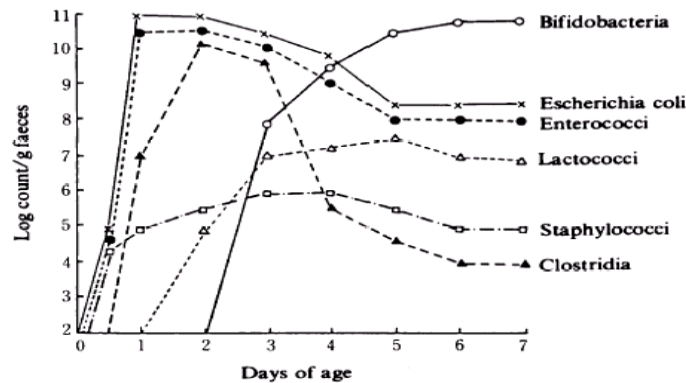
Figure 2: Changes in the intestinal microbiota in babies from birth to 7 days (Mitsuoka, 1989).

Although some of these microorganisms belong to species with pathogenic potential, they do not cause harm to the host (Morelli, 2008). The first colonizers consume the oxygen and produce new metabolics, preparing the intestinal environment for the establishment of strictly anaerobic bacteria, as for example *Bifidobacterium, Clostridium* and *Bacteroides*, which play an important role in maturation of the intestinal tract of the new-born (Adlerberth and Wold 2009; Morelli, 2008).

Three to four days after birth, bacteria of the genus *Bifidobacterium* begin to proliferate and predominate. In response to the increase of these bacteria, coliforms, *Enterococcus* and putrefactive bacteria are restricted and diminish gradually, probably by the reduction of nutrients and low oxido-reduction potential of the environment (Mitsuoka, 1978). The bacterial microbiota of the intestinal tract stabilizes in the first week of the newborns, with predominance of bifidobacteria, believing that this predominance has an effect on prevention of intestinal infections (Mitsuoka, 1989).

With the improvement of molecular techniques, an additional group of anaerobic bacteria was identified: *Ruminococcus*, which has been encountered in large numbers in the intestinal tract of children fed with maternal milk (Favier et al 2003). The role of these microorganisms in the health of infants is still little

understood, but it is known that they are stimulated by prebiotics (Konstantinov et al 2003) and that they produce ruminocin A, a bacteriocin effective in inhibiting growth of different species of *Clostridium.*

During weaning, when an adult diet is introduced to the child, a bacillary microbiota, Gram negative, similar to adults becomes predominant. The number of *Bifidobacterium* diminishes, being surpassed by *Bacteroides,* eubacterium and *peptostreptococci.* As time passes, in the third age, the concentration of *Bifidobacterium* diminished even further and some undesirable groups such as clostridia, including *Clostridium perfringens* increase significantly, as well as lactobacilli, s*treptococci* and *enterobacteriaceae* (Figure 3) (Mitsuoka, 1996).
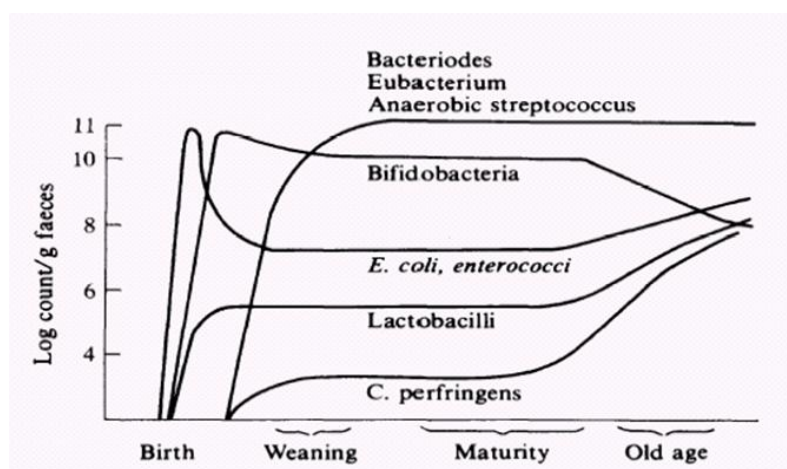
Figure 3: Changes in the intestinal microbiota with age (Mitsuoka, 1989).

The bifidobacteria species also suffer alterations with the advancing of age, where the species of *Bifidobacterium infantis* and *Bifidobacterium breve*, which are typical of newborns, are succeeded by *Bifidobacterium adolescentis. Bifidobacterium longum* endures throughout the life of the host, and for this reason, is one of the most sought for integration in functional foods (Mitsuoka, 1990).

These alterations are amply influenced by the composition of the diet of the child (maternal milk x formulas), type of birth (cesarean section x normal birth), gestational age, antibiotic therapy, state of health of the mother and hospitalization (Adlerberth and Wold 2009; Morelli, 2008).

Of the factors mentioned above, the type of birth and feeding have the greatest influence on development of microbiota of the newborn (Wall, et al 2009; Morelli, 2008). In a normal birth, infants are colonized first by the fecal and vaginal

microbiota of the mother, however, in those born by cesarean section, the maternal microbiota does not have a strong influence in the initial contamination (Neu and Douglas-Escobar, 2008), where the infants are first exposed to bacteria of the hospital environment (doctors, nurses, equipment and other newborns) [Reinhardt et al, 2009, Morelli, 2008]. According to Morelli (2008), infants born by cesarean section possess a smaller concentration of bifidobacteria and *Bacteroides fragilis*, and greater numbers of *Clostridium difficile* than those of vaginal birth. Grounlund et al (1999) observed a lower concentration of *Bacteroides fragilis* and later colonization by *Bifidobacterium* and *Lactobacillus* in newborns of cesarean section birth.

In relation to the effect of the diet, traditionally, the microbiota of the newborns feed with breast milk has been considered to possess a greater number of *Bifidobacterium* and lower concentration of facultative anaerobes, including *Streptococci, Staphylococci, Enterococci, Lactobacilli* and *Enterobacteria*, while the microbiota of newborns fed with formulas is more diverse and includes bacteria of the group Bacteroides, *Clostridium* and Enterobacteriaceae (Wall, et al 2009). Some authors attributed this difference to the presence of secretory IgA, lysozyme, lactoferrin, nucleotides and bifidogenic factors in breast milk which stimulate the growth of bifidobacteria and inhibit the pathogenic microbiota (Coppa et al 2004). Additionally, breast milk appears to be a continuous source of bacteria for the intestinal tract of the infant (Martin et al 2008). Bacteria with probiotic potential have been isolated in breast milk, for example, *L. gasseri, L. rhamnosus, L. plantarum, L. fermentum, B. Breve, B. Adolescentis* and *B. Bifidum* (Martin et al 2008). However, the effect of the diet on intestinal microbiota composition, more specifically, on the predominance of bifidobacteria in breastfeed newborns still controversial. Some authors encountered no difference between the two types of feeding, and suggested that modern formulas supplemented with prebiotics would increase the number of bifidobacteria in the intestine to values similar to those encountered in breastfeed babies (Adlerberth, et al 2009).

The gestation time also has an influence on enteric microbiota composition. Due to the immaturity of the intestinal tract, premature newborns remain for long time in Neonatal Intensive Care Units and under frequent use of broad spectrum antibiotics, limiting the establishment of the beneficial micriobiota and allowing the development of pathogens (Mshvildadze, et al 2008). Antibiotics negatively affect the

composition of intestinal microbiota of the infant by decreasing the number of anaerobic bacteria [bifidobacteria and Bacteroides] (Martin et al 2008).

The environment is another factor which infers on intestinal colonization of newborns. In developed countries, it is believed that the rigorous aseptic practices at birth and the immediate care, resulting in low environmental contamination, may affect intestinal colonization pattern (Martin and Walker, 2006). The hygiene hypothesis, which is based on the practices of good sanitation conditions in developed countries, diminishes exposure of newborns to microorganisms, resulting in reduced microbial immune stimulation. On the other hand, infants born in poorer countries are exposes to successive contaminations with pathogenic bacteria, thus increasing the risk of infectious diseases (Kalliomaki and Walker, 2005). One study performed in Pakistan accompanied the microbiota of infants delivered by vaginal birth at home, and a premature and intense colonization of *E. coli* of the environment was observed (Alderth et al 1998).

Besides the maturation and modulation of enteric microbiota, probiotics has also shown an important role in the prevention and treatment of gastrointestinal disturbances.

2.5.3 - Probiotics and Gastrointestinal Diseases

2.5.3.1 - Diarrhea

In Brazil and other regions of the world, infant mortality due to acute and persistent diarrhea, malnutrition and dehydration among children younger than 5 has decreased in the last decades. A study performed by the Brazilian Department of Heath revealed a 93.9% decrease in infant death caused by diarrhea in the last 25 years. With this reduction, the problem is no longer the second leading cause of infant mortality (24.3% in 1980) in the country and is now in fourth (4.1% in 2005), of a total of six principal causes (Brazil, 2009).

Although evidence has shown a decline in the mortality rate, diarrhea continues to be the second leading cause of death in the world among children (WHO, 2009). In 2008, nine million infants died and approximate 40% of these deaths were caused by pneumonia and diarrhea (Wardlaw et al 2010). Annually, roughly 1.5 million children die from diarrhea, which kills more than AIDS, malaria and measles together (WHO, 2009). Despite the high mortality rate, in developed countries as in

Brazil, its importance is also related to the impact of the diseases of the population, brought by its harm to human health and affecting infant development as well as the society due to the costs generated by the demand for medical care services and hospitalizations. Therefore, prevention of diarrhea is a great challenge to public health.

Therapies with probiotics have shown to be effective both in prevention as well as cure of acute diarrhea of different etiologies.

2.5.3.1.1 - Antibiotic Associated Diarrhea (DAA)

Antibiotic Associated Diarrhea (DAA) is an intestinal disturbance which affects 5-15% of patients subjected to antibiotic therapy and results from disequilibrium in the enteric microbiota causes by action of the antibiotics (Bartlett, 2002).

Treatment with probiotics has shown to reduce the incidence and severity of DAA as indicated in various studies. One of the pioneering studies performed in this area was conducted by Vanderhoof et al 1999, who administered *Lactobacillus GG* to 210 children between 6 months and 10 years old who had received antibiotics for treatment of respiratory infections. The use of probiotics resulted in a significant reduction (26% to 8%) in the frequency of diarrhea and in the duration of the disease. In a similar study, Arvola et al 1999 administered *Lactobacillus rhamnosus* GG (2 x 10^{10} CFU) in capsule two times a day to 167 children with respiratory infections. Again, the use of the probiotic resulted in a reduction (16% to 5%) of diarrhea occurrence. Corrêa et al (2005) verified the effect of formulas supplemented with *Bifidobacterium lactis* and *S. thermophilus* in the prevention of diarrhea associated to antibiotics in 157 children between 6 and 36 months old. The percentage of children who developed the disease in the next 30 days after implementation of the antibiotic for respiratory disturbances was significantly lower in the group receiving the probiotic strain (16.3%) than in the control (31.2%).

However, not all studies support these results. Thomas et al 2001 administered *Lactobacillus* GG (2 x 10^9 CFU/day) or placebo during 14 days to 167 patients subjected to antibiotic therapy. The authors observed no difference in the rate of diarrhea occurrence between the placebo (29.9%) and the group receiving the probiotic strain (29.3%). Similar results were also obtained in study performed by Tankanow, 1999.

2.5.3.1.2 - Diarrhea associated with Rotavirus infection

Since its discovery in 1970, rotavirus has been considered one of the greatest causers of infections associated to diarrhea in children throughout the world (Maldonado et al 1990). In developing countries, this microorganism is responsible for severe cases of dehydration associated to infectious diarrheas, causing the death of more than 440 thousand people per year. In 2003, it was estimated that 1205 children die daily from diarrhea caused by the rotavirus, where 80% of these are infants in undeveloped countries (Parashar et al 2003).

Studies performed with probiotic microorganisms have shown promising results in the treatment and prevention of acute diarrhea, as well as reducing the severity and duration of infections promoted by the rotavirus in children (Isolauri, 2003; Szajewska and Mrukowict, 2001).

- Guandalini et al (2001) administered oral rehydration supplemented or not (control) with *Lactobacillus* GG ($1x\ 10^{10}$ CFU/ 250 mL) to children (1-36 months old) and reported a reduction in the duration of diarrhea in the group which received the probiotic strain. The authors also verified that in children with rotaviral infection, the duration of diarrhea was 76.6 ± 41.6h in the control group and 56.2 ± 16.9 h in the group given L. *rhamnosus* GG.

- Guarino et al (1997) administered oral rehydration supplemented or not (control) with *Lactobacillus* GG for 6 days to 100 children (3 to 36 months old). The authors observed a reduction in the duration of diarrhea from 6 to 3 days in children who received the probiotic strain.

- Majamma et al (1995) administered 2.8×10^9 CFU of *Lactobacillus* GG and 10^9 CFU of *Lactobacillus casei* in capsule to 49 children ($4 - 35$ months old) with acute gastroenteritis promoted by the rotavirus, showing symptoms for more than 7 days. After 5 days of treatment, a reduction in diarrhea was observed along with increase in rotavirus specific IgA, IgG, Ig M and secretory IgA.

- Saaverdra et al (1994) reported significant reduction in diarrhea promoted by rotavirus in children ($5 - 24$ months old) administered formula supplemented with *Bifidobacterium lactis* (1.9×10^8 UFC/g) and *Streptococcus thermophilus* (0.14×10^8 UFC/g).

- Isolauri et al (1991) administered lyophilized *Lactobacillus GG* (single dose of 10^{10}-^{11} CFU) or added in fermented milk (two times per day 10^{10}-^{11} CFU) to

children (4 to 45 months old) for 5 days. The authors reported a reduction in the duration of diarrhea caused by rotavirus in the groups which received the probiotic strain (1.4 days) compared to the control (2.4 days) which received only pasteurized milk.

It has been demonstrated that the promoting effect of *L. rhamnosus* is dose dependent. Fang, et al (2009) randomly administered three different concentrations (0 CFU/day, 2 x 10^8 CFU/day and 6 x 10^8 CFU/day) of *L. rhamnosus* 35 (Lcr35) to 23 children with rotaviral gastroenteritis. After three days of treatment, a significant reduction (86%) was verified in the count of rotavirus in the feces only in the group which received the greatest dose of the probiotic (6 x 10^8 CFU/day). The authors suggest a minimal dose of de 6 x 10^8 CFU/day for 3 days in order to obtain the protective effect caused by this probiotic strain.

Clinical studies have also shown that probiotics are as effective in the control of rotaviral gastroenteritis as conventional medicines. Teran et al (2009) evaluated *in vivo* the effectiveness of the drug nitazoxanide, utilized in the treatment of infections caused by protozoa and worms, and probiotics for combating diarrhea caused by rotavirus. The children (28 days to 24 months old) were divided randomly into 3 groups. The first group received 15 mg/Kg/day nitazoxanide two times per day for 3 days. The second group received 1.25 x 10^9 CFU/g of probiotics (*L. acidophilus, L. rhamnosus, B. longum* and *Sacharomyces boulardii*) two times per day for 5 days. The third group (control) received only an oral or systemic rehydration solution. The authors reported a significant reduction (p=0.017) in the average hospitalization period of the patients who received nitazoxanide (81h) and probiotics (72h) compared to the control group (108h). Similarly, the average duration of diarrhea was reduced (p=0.009) in children who received the medication (54h) and probiotics (48h) compared to the control group (79h), demonstrating that the probiotics were as effective as nitazoxanide in the treatment of diarrhea caused by rotavirus. The probiotic effect has been attributed to the reduction in propagation of the rotavirus, modulation of the intestinal microbiota, normalization of intestinal permeability and increase in the expression of mucins and IgA secretory cells (Isolauri, 2004).

Although various studies have demonstrated the efficiency of probiotic strains in the control of rotaviral gastroenteritis, more studies are necessary to confirm this protective effect since the same results were not verified by other authors (Misra et al 2009; Sarker et al 2005; Costa-Ribeiro, et al 2003).

2.5.3.2 - Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is a serious intestinal infection which attacks premature newborns admitted in Neonatal Intensive Care Units (ICU). It is characterized by abdominal distensions, bilious vomiting, diarrhea with the presence of blood, lethargy, respiratory apnea and thermal instability (Caplan and Jilling, 2000). This disease has been reported in $10 - 25\%$ of premature newborns admitted to the Neonatal ICU and may affect 33 to 50% of all low birth weight babies. The mortality rate is high $(20 - 30\%)$ among all that developed the disease, and those that survive, may continue with sequels, such as short bowel syndrome and intestinal obstruction (Glass et al 1991).

Necrotizing enterocolitis has a multifactorial origin and is caused by intestinal and immunological immaturity, infection, hypoxia, hyperosmolar enteral feeding and by the altered composition of intestinal microbiota. The contribution of each of these factors is still unknown, however, it is known that retarding the installation of normal microbiota favors enteritis by the lack of protective bacteria and reduced development of the local and systemic immune system (Kliegman and Walsh, 1987). Studies have shown that low birth weight premature babies delivered by caesarean section always require intensive care and only receive breast milk after several days. The process by which microorganisms, for example lactobacilli, are ingested during the vaginal birth and propagated by breast milk does not occur in these children (Gewolb et al 1999). Thus, they are exposed to pathogenic bacteria, such as *Salmonella, Clostridium, Escherichia, Streptococcus* and *Staphylococcus* which colonize the intestine and increase the risk of necrosis. Considering the association of the development of predominantly beneficial intestinal microbiota with impediment of NEC (Hoy et al 1990), the administration of probiotics has been suggested by some authors with the intent of optimizing development of these microbiota and consequently protecting newborns from infectious diseases.

Hoyos, 1999 was the first to propose the utilization of probiotics in a study performed in Columbia in 1999. The author administered 2.5 x 10^8 CFU/day of *Bifidobacterium infantis* to 1237 newborns and verified a reduction of 60% in the indices of mortality and occurrence of NEC.

In the study conducted by Bin-Num et al (2005) in Israel, newborns weighing less than 1500g received 10^9 UFC/day of *Bifidobacterium infantis, Bifidobacterium*

bifidum and *Streptococcus thermophilus*. The authors verified that the probiotic reduced both the incidence (14 to 1%) and severity of NEC in newborns.

In India, in a randomized controlled double blind study, 186 newborns with a gestational age of less than 32 weeks and weighing less than 1500g were feeding (two times per day) with breast milk supplemented or not (control, n=95) with 2.5×10^9 CFU/dose of *Bifidobacterium infantis, Bifidobacterium bifidum, Bifidobacterium longum* and *Lactobacillus acidophilus*. The authors verified that enteral administration of probiotics significantly reduce the time of hospitalization and death caused by NEC in very low birth weight newborns (Samanta et al 2009).

In a recent randomized controlled and double blind study performed at the Institute of Medicine Fernandes Figueira in Brazil, 231 premature newborns with body weights from 750 to 1499g received pasteurized breast milk supplemented or not (control, n-112) with *Lactobacillus casei* and *Bifidobacterium breve* (3.5×10^7 to 3.5×10^9 CFU) for a period of 30 days, or until improvement or death, considering that which occurred first. The authors reported (4 cases) of NEC only in the control group and concluded that oral supplementation with *B. breve* and *L. casei* reduced the occurrence of NEC in newborns (Braga et al 2011).

Although the majority of publications suggest a protective effect of probiotic bacteria in the prevention of NEC and their secure use, some authors have reported the onset of sepsis in children who received the probiotic strain (Lin et al 2008, Wagner et al 1997), thus suggesting caution in the use of probiotics in immunocompromised children.

2.5.3.3 - Probiotics and Alergies

The allergy, manifested in the form of topical eczema, allergic rhinitis and asthma, is an excessive immune response to substances foreign to the organism. Currently, it has been considered one of the chronic disorders most common in children of developed countries (Taylor, 2007). It is believed that this increase is related, in part, to good sanitary conditions and infrastructure of these sites, reducing exposure of the children to commensal microorganisms and other organisms closely associated with humans throughout their evolutionary history (Rook, 2007; Guarner, et al 2006). Such microorganisms, including helminthes and saprophytic bacteria, may be recognized by the immune system of children as harmless commensals, thus triggering immune responses against themselves and other foreign substances in the

air or food (Rook, 2007). Thus, exposure to certain microorganisms in early life may prevent the subsequent development of immune-mediated diseases, such as allergies, asthma and atopic dermatitis (Hsieh et al 2008). One evidence of this fact, which is about children whose parents adopt an anthroposophic lifestyle, with restricted use of antibiotics, antipyretics and vaccinations, and make use of vegetables spontaneously fermented by *Lactobacillus* have lower incidence of allergies and microbiota rich in bifidobacteria and lactobacilli (Alm et al 2002).

It is believed that the composition of colonizing microorganisms is also critical for proper development of a balanced immune response. Studies conducted with children from countries with high and low incidences of allergic diseases have shown differences in the colonizing species, with a reduction of lactobacilli, bifidobacteria and enterococci and increase in clostridia and *Staphylococcus aureus* in children from countries with high incidences of allergic disorders (Bottcher et al 2000). Studies have also shown that breastfeeding infants have a greater concentration of bifidobacteria and lower incidence of allergies than those given formulas (Watanabe et al 2003). These findings suggest that certain microorganisms can direct the allergenic potential of an individual, and that changes in the profile of bacteria in early life may possibly change the potential for allergies (Furries et al 2005). Therefore, probiotics have been considered an important alternative to modify the intestinal microbiota early in life, thus preventing the development of allergic disorders in childhood (Huurre, et al 2008).

2.5.3.3.1 - Eczema and atopic dermatitis

Abrahamsson et al (2007), in a randomized double-blind, placebo-controlled study administered *Lactobacillus reuteri* ATCC 55730 daily to 188 mothers during 36 gestational weeks and to their respective babies for a year. The authors found decreased levels of IgE-associated eczema during the second year of life.

In a randomized, double-blind, placebo-controlled, study conducted by Kalliomäki et al (2007) in Finland, *Lactobacillus rhamnosus* GG was administered to pregnant women with a history of atopic eczema, rhinitis or asthma and to their babies for six months. The authors verified a significant reduction (50%) in the incidence of atopic eczema in children who received the probiotic strain.

Kalliomäki et al (2003 and 2001) administered *L. rhamnosus* GG to 159 mothers with atopic diseases during the entire gestational period and their respective

babies for 6 months after birth. The infants were accompanied for 2 years (Kalliomäki et al 2001) and 4 years (Kalliomäki et al 2003). The authors found that the incidence of atopic dermatitis was 23% in children who received the probiotic and 46% in the placebo group in both experiments (2 and 4 years).

Isolauri et al (2000) administered whey hydrolysate supplemented or not (control) with *Bifidobacterium lactis* and *Lactobacillus rhamnosus* GG to 27 breastfed newborns with atopic eczema. After two months of treatment, the authors found that the SCORAD (Scoring Atopic Dermatitis - index used to evaluate the severity of dermatitis) was significantly lower in the groups receiving the probiotic strains compared to the control group.

Majamaa and Isolauri (1997) showed that children with atopic dermatitis and cow's milk allergy, when fed with a whey hydrolysate formula supplemented with *Lactobacillus* GG for a period of 30 days, showed a reduction of more than 50% in the indices of atopic dermatitis compared with the placebo group.

2.5.3.4 - Probiotics and Respiratory infections

In a study conducted by Hatakka et al (2001), milk supplemented or not (control) with *Lactobacillus rhamnosus* GG was administered to 571 healthy children (1-6 years old) for 18 days. The authors found a 17% relative reduction in the number of children with respiratory and respiratory tract infections. The authors also observed a 19% reduction in antibiotic use for treatment of respiratory infections.

It is believed that the protective effect of probiotic bacteria is related to mechanisms of the intestinal mucosal barrier and the imbalance Th1 and Th2 cells to several antigens (Vaarala, 2002). The cytokines of Th2 cells (IL-4, IL-5, IL10 and IL-13) predominate in allergic responses and are responsible for the production of IgE antibodies and activation of eosinophils (Heyman and Menard, 2002). *Lactobacillus* inhibits the production of Th2 cytokines, and thereby controls the allergic response.

However, the role of probiotics in allergy prevention remains controversial since other authors have not confirmed this effect. In an Australian study conducted by Abrahamson et al (2007), 178 newborns from mothers with a history of allergy received *Lactobacillus acidophilus* LAVRI-A1 or placebo for 6 months. After the treatment period there was no difference in the incidence of atopic dermatitis between groups receiving the probiotic (23/89) and placebo (20/88). The authors found that after 12 months, the rate of sensitization was significantly higher in the probiotic

group (P = 0.03), suggesting that treatment with *L. acidophilus* increased the risk of cow's milk allergy (P = 0.012).

Additionally, it is not clear whether all probiotic strains are able to prevent the development of allergies and if this effect could be extended to all age groups. Studies have shown that some strains of *L. reuteri*, isolated from breast milk, induced the production of tumor necrosis factor (TNF) by the macrophages; however, other strains inhibited the production of the same compound (Lin et al 2008; Rook, 2007). Helin et al (2002) administered *L. rhamnosus* GG to adults and adolescents and found that the probiotic strain did not prevent pollen allergy in these individuals.

2.5.3.5 - Probiotics and obesity

Childhood obesity is among the most important problems nowadays (Owen et al 2005) due to its health implications, including diabetes mellitus type 2, cardiovascular disease, pulmonary hypertension, apnea, reflux, cancer, among others (Ogden and Leibel, 2007). This disease is the result of changes in energy balance, i.e., the form in which the body regulates absorption, use and storage of energy. Genetic factors combined with social behavior, cultural and environmental factors directly influence the energy balance and consequently body weight (Dibaise, et al 2008). Studies have suggested that the intestinal microbiota also exerts an influence on the weight of the individual, and thus it is a factor for the development of obesity, since it directly affects nutrient acquisition and energy regulation. By means of energetic economization, the undigested substrates that reach the colon are metabolized by indigenous bacteria to form monosaccharaides and short chain fatty acids. These compounds are more easily absorbable by the human body and transported to the liver where they are converted into more complex lipids. Concomitantly, the microbiota regulates the expression of host genes that promote the storage of energy absorbed by the adipocytes (Backhed et al 2004). Evidence of this has been shown by some authors. Cani et al (2009) found that consumption of oligofructose (prebiotics) by rats resulted in increased concentration of bifidobacteria and reduced levels of lipopolysaccharides (LPS) in the plasma. This compound has been linked to the development of obesity, and increase in its concentration in the plasma has been reported in rats fed with high-fat diets (Cani et al 2007). Turnbaugh et al (2008) transferred the microbial communities of obese and lean rats to germ-free rats and verified a greater accumulation of fat in those who received the intestinal microbiota

originating from the obese rats. Kalliomäki et al (2008) found that obese children had lower concentrations of *Bifidobacterium* and higher numbers of *Staphylococcus* than infants of normal weight. Backhed et al (2004) found that germ-free rats possessed 40% less body fat than those with a normal microbiota. The authors also found that by transferring the intestinal microbiota from normal rats to germ-free rats, the body fat content increased by 60% in two weeks.

These studies indicate that manipulation of the intestinal microbiota by administration of probiotics may reduce susceptibility to obesity and alter the metabolism of the body in positive directions in pediatric practice (Hsieh and Versalovic, 2008). Kondo et al (2010) administered *Bifidobacterium breve* B-3 (108 to 109 CFU/day) for 8 weeks to rats with obesity induced by a high fat diet. After treatment, the authors found that the probiotic suppressed the accumulation of weight and epididymal fat and promoted improvement in the levels of serum total cholesterol, glucose and insulin. Increase in the concentration of *Bifidobacterium breve* in the feces of the evaluated animals was also reported.

However, the role of probiotics in the prevention of obesity is still controversial since there are studies showing weight gain in the groups which were administered with probiotic (Chouraqui et al 2008; Guandalini et al 2000).

2.5.4 - Selection of probiotic bacteria

The benefits conferred by probiotic bacteria have led to the search for new strains to be added to infant formulas, with the intent of stimulating intestinal colonization similar to that found in healthy newborns, and thus prevent development of chronic gastrointestinal diseases and disorders. As reported above, the potential beneficial effects promoted by probiotic bacteria are species and even strain specific. Moreover, the benefits provided by traditional probiotic bacteria cannot be extrapolated to the new isolates and, therefore a careful and individualized evaluation of these microorganisms should be performed.

In 2002, the World Health Organization [WHO] and Food and Agriculture Organization [FAO], provided a guide for evaluation of probiotics in foods. In this document, items of safety and functionality that need to be evaluated before the incorporation of new strains in the human food chain are listed.

2.5.4.1 - Aspects of functionality

The organism should present: i) resistance to gastrointestinal barriers ii) ability to adhere to cells and intestinal tissues, iii) antagonism to pathogens.

2.5.4.1.1 - Survival of probiotic bacteria against gastrointestinal barrier

Probiotic bacteria, when ingested, must be resistant to enzymes of the oral cavity, such as lysozyme (Fuller, 1992), and the gastric acidity of the stomach, which secretes hydrochloric acid and enzymes. More than two liters of gastric juices are secreted per day, promoting a pH of 1.5. This low pH, combined with the antimicrobial action of pepsin, represents an effective barrier to the entrance of bacteria in the intestinal tract (Morelli, 2000). Evidence of the destructive action of this environment was shown by Conway et al 1987. The authors found that lactic acid bacteria used in the production of yoghurt were significantly more sensitive to human gastric juices than enteric lactobacilli.

After resisting acidic stress in the stomach, bacteria must have the ability to resist the toxicity of bile salts and pancreatin in the intestine. Bile salts, major components of bile, consist of a ring structure of cholesterol linked to amino acids (glycine or taurine) by an amide bond. The amphiphilic nature of these conjugated compounds promotes their ability to act as emulsifiers, thus facilitating the absorption of dietary lipids. Likewise, bile salts may act on cell membrane lipids of bacteria, destroying them (Begley et al 2005). Some microorganisms in the intestinal tract are capable of hydrolyzing the amide bond, creating unconjugated bile salts which can also damage the cell membrane of the bacteria, causing their death (Kurdi et al 2006). Some studies have shown that these compounds can also cross the cell membrane, and in cytoplasm damage DNA and protein (Prieto et al 2004; Leverrier et al 2003).

Lactobacillus and *Bifidobacterium* are generally resistant to bile salts (Kroll et al 2010; Cukrowska et al 2009; Charteris et al 1998) and the level of resistance varies between species (Delgado et al 2007; Du Toit, et al 1998; Chateau et al 1994; Gilliland, 1984). According to Du Toit, et al (1998), this difference is due to the presence of hydrolases, which act to reduce the toxic effects of conjugated bile salts.

Membrane transport systems related to the efflux of bile have also been reported as defense mechanisms. Pfeiler and Klaenhammer (2009) found induced expression of ATPase subunits in the ABC transporter system (BAL 1679-1680) in

Lactobacillus acidophilus NCFM growing in MRS supplemented with 0.5% bile. These genes were annotated as being of the multidrug resistance transporter (MDR), a class of transport membranes able to reverse the toxic effects of bile and certain antibiotics by pumping these compounds out of the cell.

Given all these barriers present in the gastrointestinal tract, it is essential that the probiotic bacteria develop resistance mechanisms which enable them to reach their site of action viable and in sufficient numbers to promote beneficial effects to the host.

2.5.4.1.2 - Antagonism to pathogens

In order to have an impact on the intestinal microbiota, probiotics should create an environment unfavorable to the growth of pathogens, protecting the host from diseases (Saarela et al 2000). As already reviewed in this article, Lactobacilli and bifidobacteria produce metabolites such as organic acids, hydrogen peroxide and bacteriocins, which inhibits the growth of pathogens (Servin, 2004).

2.5.4.1.3 - Adherence to intestinal tissues

The gastrointestinal tract, especially the small intestine, is a dynamic environment with peristaltic movements and high rate of cell renewal, a fact which hinders the persistence of non-adhered bacteria in this environment. Thus, the ability to adhere is an important characteristic of probiotic bacteria, since not only does it allow the permanence in the intestinal tract, studies have shown that it is also related to inhibition of pathogens (competition for adhesion sites) and modulation of the immune response (Collado et al 2007; Vesterlund et al 2006; Coconnier and Servin 2003; Moreli, 2000).

In vitro cell models, involving human intestinal cells (HT-29, Caco-2), mucins and extracellular matrices (laminin and fibronectin) have been used extensively to study mechanisms of adhesion of enteropathogenic bacteria (Coconier et al 1992) and the ability of probiotic microorganisms to adhere to the intestinal tissues (Cammarota et al 2009; Provencio Munoz et al 2009; Horie et al 2005; Styriak et al 2003; Blum and Renieri, 1999).

HT-29 and Caco-2 are human colon adenocarcinoma cell line which express morphological and physiological characteristics of normal human enterocytes (Brassart et al 1998). Although they are of colonic origin, the use of these cellular

models also promote a good approximation of adhesion in the small intestine due to formation of a monolayer of polarized cells expressing intestinal microvilli on the apical surface and with enzymes and transport proteins common to that environment (Lenaerts et al 2007, Quarona and Hochman, 1996).

Fibronectin is a dimeric glycosylated protein (454 kDa) present in soluble form in blood plasma and in the immobilized form on the surfaces and extracellular matrices (ECM) of the host cells. It is found inserted in the mucus, potentially exposed to the intestinal lumen in cases of trauma, infection or inflammation (Styriak et al 2003; Lorca et al 2002). Adherence of pathogenic bacteria to ECM has been associated with several infections (Ljungh and Wadstrom, 1995). Thus, probiotics must be able of competing with pathogens for the same receptors, making them unavailable in the intestine (Neesser et al 2000).

Mucins are high molecular weight glycoproteins, synthesized and secreted by epithelial cells of various organs, including the intestine. They are characterized by large molecular size, high carbohydrates concentrations and O-glycosidic bonds between N-acetylgalactosamine and serine/threonine molecules in the peptide backbone (Forstner et al 1994). Different genes related to the mucin have been identified, and among those found in humans, MUC2 and MUC3 appear to predominate in ileocolonic mucins. MC2 contains 23 amino acid repeated peptides rich in threonine and proline residues, highly glycosylated and flanked by cysteine-rich domains (Gum et al 1997). This is expressed in goblet cells in the small and large intestines (Chang et al 1994), where the main mucin is expressed in the colon (Van Klinken et al 1995). MC3 has periodic tandem repetitions of 17 amino acids rich in threonine and serine residues and is expressed in goblet and enterocyte cells of the small intestine (Chang et al 1994).

2.5.4.2 - Technological Aspects

The technological aspects are important criteria to be evaluated since probiotic bacteria in the carrier food must withstand the processing and storage conditions, not promoting changes in texture and sensory characteristics of the product, as well as remain active and viable to exert their beneficial effects on the host (Vasiljevic and Shah, 2008; Ferreira, 2003; Huis't Veld; Shortt, 1996). The viability of these strains is dependent on multiple factors such as pH, presence of competitive microorganisms, storage temperature and presence of inhibitory bacteria compounds in the food matrix

(Kurmann and Rasic, 1991). Some probiotic strains, especially bifidobacteria, are more sensitive to pH and oxygen. Thus, microencapsulation has been considered a good alternative (Kim et al 1996). This technique consists of encapsulating the solid, liquid or gas materials in extremely small capsules, which can release the contents in a controlled manner and under specific conditions (Ubbink and Krüger, 2006).

2.5.4.3 - Aspects of Safety

Bifidobacteria and lactobacilli have a long history of safe use in human and animal diets (Shortt, 1999). However, in recent years, some reports have associated probiotic lactic acid bacteria with clinical pathology (Ishibashi and Yamazaki, 2001). Pathological situations in which the presence of these strains were identified are rare and estimated at 0.05% - 0.4% of cases of endocarditis and bacteremia. However, it is unlikely that these microorganisms are the causative agents of these diseases (Gasser, 1994). These occurrences have increased interest and questioning of the safety of probiotics, especially with regard to new strains being introduced into the human food chain. Security aspects such as genetic identification of the probiotic strain, presence of virulence factors, antibiotic resistance, toxicity and bacterial translocation must all be evaluated (FAO / WHO, 2001; Saarela, 2000).

2.5.4.3.1 - Identification of probiotic strain

According to a document established by FAO/WHO, 2001, all probiotic strains must be properly identified to the species level using current and internationally accepted methods. Generally, these are based on deoxyribonucleic acid. Furthermore, probiotic bacteria must be properly named in accordance with current nomenclature. The genetic diversity among strains of the same species should be evaluated by pulsed field gel electrophoresis (PFGE). Randomly Amplified Polymorphic DNA (RAPD) may also be used; however, the results are less reproducible.

The specificity of the probiotic is another factor that must be taken into consideration. Probiotics that are intended for humans must be isolated from the microbiota of healthy individuals and those intended for animals should therefore be isolated from the respective animal species (Borriello et al 2003).

2.5.4.3.2 - Determination of virulence factors

Virulence factors, such as haemolytic and gelatinase activities, are common mechanisms among various pathogens. Hemolysin production enables them to acquire iron ions, thus causing anemia and edema in the host (Vesterlund et al 2007). Iron is a micronutrient that acts as a cofactor in various reactions, and it is thus necessary for the growth of these microorganisms (Husain, 2008). *Lactobacilli* are reported as an exception among living organisms, because there was no need for iron to grow; this is considered an ecological advantage in the natural environment where they compete with pathogenic bacteria (Elli et al 2000). However, some studies have shown hemolysin producing lactobacillus strains (Maragkoudaki et al 2006; Baumgartner et al 1998). According to Elli et al (2000), this behavior may be related to the demand for iron ions by some probiotic strains for the metabolism of purine and pyrimidine in an environment with a limited source of specific nucleotides. Therefore, it is essential to examine the hemolytic activity of new potential probiotic isolates.

Gelatinase is an extracellular zinc metalloprotease capable of hydrolyzing gelatin, collagen, casein, hemoglobin and other bioactive peptides. These proteases are normally found associated with inflammation and virulence in humans and animals (Kanemitsu et al 2001).

2.5.4.3.3 - Resistance to antibiotics

The assessment of antimicrobial susceptibility of new isolates aims to prevent the indication of strains resistant to antibiotics, and the possibility of spreading this resistance to pathogenic bacteria in the intestinal tract of host (Robredo et al 2000; Austin et al 1999). The indiscriminate use of antimicrobials for human beings and animals, as well as the use of these agents as growth promoters has promoted the selection of resistant strains, causing serious problems in the treatment of bacterial infections (Smith, 2001).

The food chain has been indicated as one of the principal routes of transmission of resistance genes to the human commensal microbiota (Witte, 2000; Teuber et al 1999), and although lactic acid bacteria are GRAS (Generally Recognized as Safe) these bacteria may contribute to the transfer of resistance genes (Ammore et al 2007). Huys et al (2004) reported resistance to tetracycline in *Enterococcus* isolated from cheeses in Europe. According to the author, this resistance may be linked to tet (M) genes found in all strains, which also carry

conjugative transposons. Cataloluk and Gogebaken (2004) reported the existence of tet (M) and erm (B) genes in the majority (61.9%) of lactobacilli (*L. acidophilus, L. gasseri, L. plantarum* and *L. crispatus*) isolated from human and dairy product, in Turkey. Perreten et al (2001) identified plasmid (pK214) carrying antibiotic resistance genes (tet [S], str, and cat) in *Lactobacillus lactis ssp. lactis* isolated from cheese made with raw milk. The authors also found that this plasmid possesses a typical lactococcica replication region.

Among lactic acid bacteria, special attention has been given to the probiotics, since they being used in the large-scale for integration with food products (Ammore et al 2008), as well as ingested daily in large numbers.

Probiotic bacteria naturally exhibit a wide variation in antimicrobial resistance (Klein, 2010; Charteris et al 1998), which may be intrinsic or acquired. Intrinsic resistance is based on the natural characteristics of the microorganism, such as physiology and structural peculiarities of the strain (characteristics of the cell wall or loss of active function of the antibiotic) and unlikely to be transmitted to other microorganisms (Wright, 2005). Heterofermentative Lactobacilli, for example, are intrinsically resistant to vancomycin (Ammore, et al 2008; Hamilton-Miller and Shah, 1998) due to the presence of D-Ala-D-lactate in their peptidoglycan rather than the dipeptide D-Ala-D -Ala (Klein et al 2000). In contrast, acquired resistance may be transferred horizontally between bacteria and results of mutations in the bacterial genome or acquisition of resistance genes by means of conjugation processes (gene transfer requires cell-cell contact, differing from other processes), transduction (genetic transfer assisted by bacteriophages) and transformation (uptake of soluble DNA from the medium by donor cells) [Mathur and Singh, 2005]. Transposons may also contribute to gene transfer. In transposition, the segments can "jump" or transfer themselves from one DNA molecule (plasmid, chromosome) to another (plasmid, chromosome, phage) within a single cell (Tortora et al 2000).

The contribution of these different mechanisms is unknown, but it is believed that conjugation is the main pathways for the spread of resistance (Salyers, 1995), since many of these genes have been found in conjugative plasmids and transposons. Furthermore, conjugation allows the transfer of DNA between phylogenetically distant bacteria, while transformation and transduction typically occur between organisms of the same species (Matheur and Singh, 2005).

In the conjugation process, the plasmids (DNA fragments often times circular and double stranded, that can replicate independently of the genetic material of the host cell) can self transfer from one cell to another. In this process, a plasmid DNA strand is transferred from the receptor to donor cell. This DNA serves as a template for synthesis of the complementary strand and at the end of the process; both the donor and recipient organism have the plasmid, thus contributing to the spread of resistance (Snyder and Chapness, 2003, Brooks et al 2000).

Plasmids encoding resistance to tetracycline, erythromycin, chloramphenicol, streptomycin and lincomycin have been reported in *L. reuteri* (Lin et al 1996), *Lactobacillus fermentum* (Fons et al 1997), *Lactobacillus acidophilus* (Vescovo et al 1982) and *L. plantarum* (Daniels, 2002), isolated from meats, silage and feces. However, they are rarely found in *Bifidobacterium* and other species of *Lactobacillus* (Ammore et al 2007).

The determination of resistance to antibiotics is verified *in vitro* by tests of agar dilution, disk diffusion, E-test and microdilution (through which the MIC [minimum inhibitory concentration] is normally calculated). The different methods employed, combined with the different experimental conditions (incubation time and temperature, amount of inoculum, culture medium) complicate the direct comparison of results with those available in literature (Ammor et al 2007).

2.5.5 - Legislation on probiotics

The ANVISA (National Agency for Sanitary Vigilance) is an agency under the Brazilian Ministry of Health and is responsible for establishing standards, proposing, monitoring and implementing policies, guidelines and actions of Sanitary Vigilance, and also grants registration of probiotic products in Brazil. This agency is advised by CTCAF (Techno-scientific Advisory Commission on Foods with Allegations of Functional Properties and/or Health), which has the function of supporting the management of foods and toxicology in decisions related to that subject. Currently, the following regulations related to probiotics are in effect:

Resolution RDC n.º 2, of January 7, 2002 - Technical Regulation of Bioactive substances and Probiotic isolates with allegations of functional and/or health properties. This resolution was created in order to standardize the procedures to be adapted for safety assessment, registration and commercialization of Bioactive Substances and Probiotic Isolates with the allegation of functional and/or health

properties. Under this regulation, the probiotic product should have no medicinal or therapeutic purpose, and should be safe for human consumption without the need for guidance and/or medical care, unless directed towards specific population groups.

Resolution RDC n.º 17, January 30, 1999 - Technical Regulations for Risk Assessment and Food Safety. According to this resolution, product safety should be proven by means of clinical studies, biochemical, nutritional and/or physiological and/or toxicological studies in experimental animals.

Resolution RDC n.º 18, April 30, 1999 - Technical Regulations for Analysis and Verification of Functional Properties and/or Health Claims in Food Labeling. According to the ANVISA, functional property claims are those related to the metabolic or physiological roles that the nutrient or non-nutrient has on growth, development, maintenance and other normal functions of the human body. Allegation of health properties is that which states, suggests or implies the existence of a relationship between food or ingredient with the disease or health-related condition. The allegation may refer to the maintenance of general health, the physiological role of nutrients and non-nutrients, and disease risk reduction. Health allegations referring to cure or disease prevention are not permitted.

Currently, ANVISA has 1 claim approved for probiotics products "The (state species of microorganism) (probiotics) contributes to the balance of intestinal flora. Its consumption should be associated with a balanced diet and healthy lifestyle". There are still no approved health claims.

Resolution RDC n.º 19, April 30, 1999 – Technical Regulation of Procedures for Registration of Foods with Allegations of Functional and/or Health Properties on their Labels.

Resolution RDC n.º 323, November 10, 2003 – Technical Regulation for Registration, Alteration and Revalidation of Probiotic Medical Registrations. In this case, the probiotic is no longer treated as a food but as a medicine. The probiotic strains are concentrated and available in the form of capsules. According to the regulation, the applicant must present a report on the resistance to the gastrointestinal tract and viability of microorganisms in conditions of the intestine, as well as demonstrate the probiotic potential and safe use of the microorganism for a period of no less than three years.

2.6 - REFERENCES

Abrahamsson, T.R., Jakobsson, T., Bottcher, M.F., Fredrikson, M., Jenmalm, M.C., Bjorksten, B. et al (2007). Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. **Journal of Allergy and Clinical Immunology**. 119, 1174–1180.

Adams, M.R. (1999). Safety of Industrial Lactic Acid Bacteria. **Journal of Biotechnology**. 68, 171-178.

Adlerberth, I., Wold, A. (2009). Establishment of the gut microbiota in Western infants. **Acta paediatrica**. 98,229-238.

Alakomi, H.L., Skytta, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I.M. (2000). Lactic acid permeabilizes gram-nagative bacteria by disrupting the outer membrane. **Applied Environmental Microbiol**. 66, 2001-2005.

Alderth I, Jalil F, Carlsson B, Mellander L, Hanson LA, Larsson P, Khalil, K., Wold, A.E. (1998). High turnover rate of *Escherichia coli* strains in the intestinal flora of infants in Pakistan. **Epidemiol infect**. 121, 587-598.

Alm, J.S., Swartz, J., Bjorksten, B., Engstrand, L., Engstrom, J., Kuhn, I., Lilja, G., Mollby, R., Norin, E., Pershagen, G., Reinders, C., Wreiber, K., and Scheynius, A. (2002). An anthroposophic lifestyle and intestinal microflora in infancy. **Pediatric Allergy and Immunology**. 13, 402–411.

Amant, D.C., Valentin-Bon, I.E. And Jerse, A.E. (2002). Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. **Infection and Immunity**. 70, 7169–7171.

Ammor, M.S., Florez, A.B., Van Hoek, A.H., Reyes-Gavilan, C.G., Aarts, H.J., Margolles, A., Mayo, B. (2008). Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. **Journal of Molecular Microbiol and Biotechnology**. 14, 6-15.

Ammor, M.S., Florez, A.B., Mayo, B. (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. **Food Microbiology**. 24, 559-570.

ANVISA. Resolução RDC n.º 17, de 30 de janeiro de 1999. Available at http://elegis.anvisa.gov.br/leisref/public/showact.php?id=108. Acessed 10/11/2010.

ANVISA. Resolução RDC n.º 18, de 30 de abril de 1999. Available at http://elegis.anvisa.gov.br/leisref/public/showact.php?id=109. Acessed 10/11/2010.

ANVISA. Resolução RDC n.º 19, de 30 de abril de 1999. Available at http://elegis.anvisa.gov.br/leisref/public/showact.php?id=110. Acessed 10/11/2010.

ANVISA. Resolução RDC n.º 2, de 7 de janeiro de 2002. Available at http://elegis.anvisa.gov.br/leisref/public/showact.php?id=1567. Acessed 10/11/2010.

ANVISA. Resolução RDC n.º 323, de 10 de novembro de 2003. Available at http://www.anvisa.gov.br/legis/resol/2003/rdc/323_03rdc.htm. Acessed 10/11/2010

Aroutcheva, A., Gariti, D., Simon, M., Shott, S., Faro, J., Simoes, J.A., Gurguis, A. and Faro, S. (2001). Defense factors of vaginal lactobacilli. **American Journal of Obstetrics and Gynecology**. 185, 375–379.

Arvola T, Laiho K, Torkkeli S, et al (1999). Prophylactic *Lactobacillus* GG reduces antibiotic-associated diarrhea in children with respiratory infections: A randomized study. **Pediatrics**. 104(5): e64.

Austin, D. J., Kristinsson, K. G., Anderson, R. M. (1999). The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. **Proceedings of the National Academy of Sciences of the United States of America**. 96, 1152–1156.

Axelson LT, Chung TC, Dobrogosz WJ, Lindgren SE. (1989). Production of a broad spectrum antimicrobial substance by *Lactobacillus reuteri*. **Microbial Ecology in Health and Disease**. 8, 131–136.

Azcarate-Peril, M.A., Altermann, E., Goh, Y.J., Tallon, R., Sanozky-Dawes, R.B., Pfeiler, E.A., O'Flaherty,S., Buck, L. Dobson,A., Duong,T., Miller, M.J., Barrangou, R., Klaenhammer, T.R (2008). Analysis of the Genome Sequence of *Lactobacillus gasseri* ATCC 33323 Reveals the Molecular Basis of an Autochthonous Intestinal Organism. **Applied and Environmental Microbiology**. 4610–4625.

Backhed, F., Ding, H., Wang, T.,Hooper, L.V., Gou, Y.K., Nagv, A., Semenkovich, C.F., Gordon, J.I. (2004). The gut microbiota as an environmental factor that regulates fat storage. **Proceedings of the National Academy of Sciences of the United States of America**. 101(44), 15718-15723.

Bartlett JG. (2002). Clinical practice. Antibiotic-associated diarrhea. **New England Journal of Medicine**. 346, 334–339.

Baumgartner, A., Kueffer, M., Simmen, A. and Grand, M. (1998). Relatedness of *Lactobacillus rhamnosus* strains isolated from clinical specimens and such from food-stuffs, humans and technology. **Lebensmittel-Wissenschaft und-Technologie**. 31, 489–494.

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

Beijerinck, M.W., (1901). Sur les ferments lactiques de l'industrie. **Archives Néerlandaises des Sciences Exactes et Naturelles** (Section 2). 6, 212–243.

Berg, R. (1980). Inhibition of *Escherichia coli* translocation from the gastrointestinal tract by normal cecal flora in gnotobiotic or antibiotic decontaminated mice. **Infection and Immunity.** 29, 1073-1081.

Berg, R.D. (1992). Translocation and the indigenous gut flora. **Scientific basis of probiotic concept**. 55-85.

Berg, R.D. (1995). Bacterial Translocation from Gastrointestinal Tract. **Trends in Microbiology**, vol.3 n° 4, 149-154.

Berg, R.D., Garlington, A.W. (1979). Translocation of certain indigenous bacteria from the gastrointestinal tract to mesenteric lymph nodes and other organs in a gnotobiotic mouse model. **Infection and Immunity**. 23, 403-411.

Bernardeau M., Guguen M. and Vernoux J.P. (2006). Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. **FEMS Microbiology Reviews**. 30, 487–513.

Bin-Num et al (2005). Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. **Journal of Pediatrics**. 147, 192-196.

Blum, S., Reniero, R., Schiffrin, E.J., Crittenden, R., Mattila-Sandholm, T., Ouwehand, A.C., Salminen, S., Von Wright, A., Saarela, M., Saxelin. M., Collin, K., Morelli, L. (1999). Adhesion studies for probiotic: need for validation and refinement. **Trends and Food Science Technology**. 10, 405-410.

Bom dia Brasil. Available at WWW. URL: https://www.medplan.com.br/materias/2/7059.html. Last update 07/29/2008.

Borriello, S.P., Hammes, W.P., Holzapfel, W., Marteau, P., Schrenmeir, J., Vaara, M., Valtonen, V. (2003). Safety of Probiotics That Contain Lactobacilli or Bifidobacteria. **Clinical Infectious Diseases**. 36, 775-780.

Bottcher M.F., Nordin E.K., Sandin A, Midtvedt T, Bjorksten B. (2000). Microflora-associated characteristics in faeces from allergic and nonallergic infants. **Clinical and Experimental Allergy**. 30,1590–1596.

Braga, T.D., da Silva, G.A., de Lira, P.I., de Carvalho, L.M. (2011). Efficacy of *Bifidobacterium breve* and *Lactobacillus casei* oral supplementation on necrotizing enterocolitis in very-low-birth-weight preterm infants: a double-blind, randomized, controlled trial. **The American Journal of Clinical Nutrition**. 93, 81-86.

Brassart D, Schiffrin E, Rochat F, Offord E.A., Macé C. & Neeser J.R., (1998). The future of functional foods: Scientific basis and future requirements. **Lebensmittel Technol**. 7–8, 258–266.

Brook, I., Macvittie, T.J., and Walker, R.I., (1984). Recovery of aerobic and anaerobic bacteria from irradiated mice. **Infection and Immunity**. 46, 270–271.

Brooks, G.F., Butel, J.S. & Morse, S.A. (2000). **Microbiologia Médica**, 21 ed., Rio de Janeiro: Guanabara Koogan S.A., p. 117-141.

Byezkowski, J., Gessner, T. (1988). Biological role of superoxide íon-radical. **International Journal of Biochemistry**. 20, 569-580.

Cammarota, M., Rosa, M., Stellavato, A., Lamberti, M., Marzaioli, I., Giulianoa, M. (2009). *In vitro* evaluation of Lactobacillus plantarum DSMZ 12028 as a probiotic: Emphasis on innate immunity. **International Journal of Food Microbiology**. 135, 90-98.

Cani, P.D, Amar, J., Iglesias, M.A., et al (2007). Metabolic endotoxemia initiates obesity and insulin resistance. **Diabetes**. 56(7),1761-1772.

Cani, P.D., Possemiers, S., Van de Wiele, T., Guiot, Y., Everard, A., et al (2009). O Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. **Gut**. 58,1091-1103.

Caplan, M. S., & Jilling, T. (2000). Neonatal necrotizing enterocolitis: Possible role of probiotic supplementation. **Journal of Pediatric Gastroenterology and Nutrition**, *30*(Suppl 2), S18–S22.

Carr, F.J., Chill, D., Maida, N. (2002). The lactic acid bacteria: a literature survey. **Critical Reviews in Microbiology**. 28, 281-310.

Cataloluk O. & Gogebakan B. (2004). Presence of drug resistance in intestinal lactobacilli of dairy and human origin in Turkey. **FEMS Microbiology Letters**. 236, 7–12.

Chang, S.K., Dohrman, A.F., Basbaum, C.B., Ho, S.B., T. Tsuda, T., Toribara, N.W., Gum, J.R. and Kim, Y.S. (1994). Localization of mucin (MUC2 and MUC3) messenger RNA and peptide expression in human normal intestine and colon cancer. **Gastroenterology**. 107,28–36.

Charteris, W.P., Kelly, P.M., Morelli, L., Collins, JK. (1998). Ingredient selection criteria for probiotic microorganisms in functional dairy foods. **International Journal of Dairy Technology**. 51(4), 123-36.

Chateau, N., Deschamps, A.M., Hadh-Sassi, A. (1994). Heterogeneity of bile salts resistance in the *Lactobacillus* isolates from a probiotic consortium. **Letters Applied Microbiology**. 18, 42-44.

Chauvière, G., Coconnier, M.-H., Kernéis, S., Fourniat, J. and Servin, A.L., (1992). Adhesion of *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. **Journal of General Microbiology**. 138, pp. 1689–1696.

Chouraqui, J.P., Grathwohl, D., Labaune, JM., Hascoet, J.M., Montgolfier, I., Leclaire, M., Giarre, M., Steenhout, P. (2008). Assessment of the safety, tolerance, and protective effect against diarrhea of infant formulas containing mixtures of probiotics or probiotics and prebiotics in a randomized controlled trial. **American Journal of Clinical Nutrition** 87, 1365–1373.

Chulada, P.C., Arabes S.J. Jr, Dunson, D., Zeldin, D.C. (2003). Breast-Feeding and the prevalence of asthma and wheeze in children: analyses from the third National Healt and Nutrition Examination Survery. **Allergy and Clinical Immunology**. v. 111, p.328-336.

Cleveland, J., Monteville, T.J., Nes, I.F., Chikindas, M.L. (2001). **International Journal of Food Microbiology**. 71, 1-20.

Coconnier, M.H., Klaenhammer, T.R., Kerneis, S., Bernet, M.F., and Servin, A.L (1992). Protein-mediated adhesion of *Lactobacillus acidophilus* BGF04 on human enterocyte and mucus secreting cell lines in culture. **Applied environmental Microbiology**. 58, 2034-2039.

Collado, M.C., González, A., González, R., Hernández, M., Ferrús, M.A., Saz, Y. (2005). Antimicrobial peptides are among the antagonistic metabolites produced by

Bifidobacterium against *Helicobacter pylori*. **International Journal of Antimicrobial Agents**. 25, 385-391.

Collado, M.C., Jalonen L., Meriluoto, J., Salminen, S. (2006). Protection mechanism of probiotic combination against human pathogens: *in vitro* adhesion to human intestinal mucus. **Asia Pacific Journal of Clinical Nutrition** 15(4), 570-575.

Collado, M.C., Meriluoto, J., Salminen, S. (2007). Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. **Letters in Applied Microbiology** 45, 454–460.

Collado, M.C., Surono, I.S., Meriluoto, J. and Salminen, S. (2007b) Potential probiotic characteristics of *Lactobacillus* and *Enterococcus* strains isolated from traditional dadih fermented milk against pathogen intestinal colonization. **Journal of Food Protection**. 70, 700–705.

Collins, M.D., Rodrigues, U.M., Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A., Williams, A.M. And Wallbanks, S. (1991). Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of the 16S rRNA. **FEMS Microbiology Letters**. 77, 5–12.

Conway, B.R., Gorbash, S.L., Goldin, B.R. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. **Journal of Dairy Science**.70, 1-12.

Coppa, G.V., Stefano, B., Morelli, L., Soldi, S., Orazio, G. (2004). The first prebiotics in humans: human milk oligosaccharides. **Journal Clinical Gastroenterology**. 38, 80-82.

Correa N.B.O., Filho, L.A.P, Penna, F.J.M., Lima, F.M.L.S., Nicoli, J. (2005). A randomized formula controlled trial of *Bifidobacterium lactis* and *Streptococcus thermophilus* for prevention of antibiotic-associated diarrhea in infants. **Journal Clinical Gastroenterology**. 39,385–389.

Costa-Ribeiro, H., Ribeiro, T.C.M., Mattos, A.P., Valois, S.S., Neri, D.A., Almeida, P. Cerqueira, C.M., Ramos, E., Young, R.J., Vanderhoof, J.A. (2003). Limitations of probiotic therapy in acute, severe dehydrating diarrhea. **Journal of Pediatric Gastroenterology and Nutrition**. 36, 112–115.

Coutsoudis, A., Pillary, K., Spooner, E., Kuhn, L., Coovadia, H.M. (2005). Influence of infant-feeding patterns on early mother-to-chil transmission of HIV-1, 2003. In: **American Academic of Pediatrics**. Breastfeeding and the use of human milk. **Pediatrics**, v. 115, nº2.

Cross, M.L. (2002). Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. **FEMS Immunology and Medical Microbiology**. 34, 245–253.

Cukrowska, B., Motyl, I., Kozakova, H., Schwarzer, R.K., Gorecki, R.K., Klewicka, E. (2009). Probiotic *Lactobacillus* Strains: in vivo and *in vitro* Studies. **Folia Microbiology**. 54, 533-537.

Dal Bello, F., and C. Hertel. (2006). Oral cavity as natural reservoir for intestinal lactobacilli. **Systematic and Applied Microbiology**. 29:69–76

Danielsen, M. (2002). Characterization of the tetracycline resistance plasmid pMD5057 from *Lactobacillus plantarum* 5057 reveals a composite structure. **Plasmid**. 48, 98–103.

Delgado, S., O`Sullivan, E., Fitzgerald, G., Mayo, B. (2007). Subtractive Screening for Probiotic Properties of Lactobacillus Species from the Human Gastrointestinal Tract in the Search for New Probiotics. **Food Microbiology and Safety**, 72, Nº 8. M310-M315.

Dembélé, T., Obdrzálek, V., Votava M. (1998). Inhibition of bacterial pathogens by lactobacilli. **Zentralbl Bakteriol**. 288(3), 395-401.

DiBaise, J.K., Zhang, H., Crowell, M.D., Krajmalnik-Brown, R., Decker, G.A., Rittmann, B.E. (2008). Gut Microbiota and Its Possible Relationship With Obesity. **Mayo Clinical Proceedings**. vol. 83 Nº 4, 460-469.

Dunne, C., Murphy, L., Flyin, S., O'Mahony, L., O'Halloran, S.,Feeney, M., Morrisey, D., Thorton, G., Fitzgerald, G., Daly, C.,Kiely, B., Quigley, E. M. M., O'Sullivan, G. C., Shanahan, F., & Collins, K. (1999). Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. **Antonie van Leeuwenhoek**, 76, 279–292.

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

Du-Toit, M., Franz, C., Schillinger, U., Warles, B., Holzappfel, W. (1998). Characterization and selection of probiotic lactobacilli for a preliminary minipig-feeding trail and their effect on serum cholesterol level and faeces moisture contents. **International Journal of Food Microbiology**. 40, 93-104.

Eaton, T.J., Gasson, M.J (2001). Molecular Screening of *Enterococcus* Virulence Determinants and Potential for Genetic Exchange between Food and Medical Isolates. **Applied Environmental Microbiology**. 67(4), 1628–1635.

Elli, M., Zink, R., Rytz, A., Reniero, R., Morelli, L. (2000). Iron requirement of *Lactobacillus spp*. in completely chemically defined growth media. **Journal of Applied Microbiology**. 88, 695-703.

Fang, SB., Lee, HC., Hu, JJ., Hou, SY., Liu, HL., Fang, HW. (2009). Dose-dependent effect of *Lactobacillus rhamnosus* on quantitative reduction of faecal rotavirus shedding in children. **Journal of Tropical Pediatrics**. Vol. 55, Nº. 5. 297-301.

Favier, C.F., De Vos, W.M., Akkermans, A.D. (2003). Development of bacterial and bifidobacterial communities in feces of newborn babies. **Anaerobe**. 9, 219-229.

Felis, G.E., Dellaglio, F., Torriani, S. (2009). Genomics of probiotic bacteria, p. 681-723.In D. Charalampopoulos and R. A. Rastall, Robert (ed.), **Prebiotics and Probiotics Science and Technology**, vol. 2. Springer, LLC., New York.

Ferreira, C.L.L.F. (2003). Prebioticos e Probióticos: Atualização e Prospecção Ed. Suprema Gráfica e Editora, 1ªed, Viçosa-MG.

Finlay, B.B. & Falkow, 5. (1997). Common themes in microbial pathogenicity revisited. **Microbiology and Molecular Biology Reviews**. 61, 136-169.

Fiocruz. Fundação Oswaldo Cruz. RedeBLH. (2010). www.fiocruz.br//redeBLH/cgi/cgilua.exe/sys/start/htm?tpl=home&id=ce.

Fons, M., Hege, T., Ladire, M., Raibaud, P., Ducluzeau R., Maguin, E. (1997). Isolation and characterization of a plasmid from Lactobacillus fermentum conferring erythromycin resistance. **Plasmid**. 37, 199–203.

Food and Agriculture Organization of the United Nations And World Health Organization. (2002). Guidelines For The Evaluation of Probiotics. In: Food And Agriculture Organization For The United Nations And World Health Organization Working Group Report, On Line.

Forestier, C., De Champs, C., Vatoux, C. and Joly, B.(2001). Probiotic activities of Lactobacillus casei rhamnosus: *in vitro* adherence to intestinal cells and antimicrobial properties. **Research in Microbiology**. 152, 167–173.

Forstner, J.F., and Forstner, G.G. (1994). Gastrointestinal mucus. In: Physiology of the Gastrointestinal Tract (3rd ed.), edited by L. R. Johnson. New York: Raven, p. 1255–1283.

Fukushima, Y., Kawata, Y., Hiroyoshi, H., Terada, A., Mitsuoka, T. (1998) Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children **International Journal of Food Microbiology**. 42, 39–44

Fuller R. (1992). History and development of probiotics. In Probiotics. The Scientific Basis, pp. 1-8. Chapman & Hall, London.

Fuller, R. (1989). Probiotic in man and animals. Journal of Applied Bacteriology. Oxford, 6, 365-378.

Furrie, E. (2005). Probiotic and Allergy. **Proceedings of the Nutrition Society**. 64, 465–469.

Garrity, G.M., Bell, J.A., and Lilburn, T.G. (2004). Taxonomic Outline of the Procaryotes. Bergey's Manual of Systematic Bacteriology, 2nd edition, Springer-Verlag, New York.

Gasser, F. (1994). Safety of lactic-acid bacteria and their occurrence in human clinical infections. **Bulletin de L'Istitut Pasteur**, 92, 45-67.

Gasser, F., Mandel, M. (1968). Deoxyribonucleic acid base composition of the genus *Lactobacillus*. **Journal of Bacteriology**. 96, 580–588.

Gibson, G.R., McCartney, A.L., Rastall, R.A., (2005). Prebiotics and resistance to gastrointestinal infections. **British Journal of Nutrition**. 93, S31–S34.

Gilliland, S.E., Staley, T.E. and Bush, L.J. (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. **Journal of Dairy Science**. 67, 3045–3051.

Giugliani, E. R. J. (2002). Rede Nacional de Bancos de Leite Humano do Brasil: Tecnologia para Exportar. **Jornal de Pediatria** v. 78, n°3.

Glass R.I., Lew J.F., Gangarosa R.E., LeBaron C.W., Ho M.S. (1991). Estimates of morbidity and mortality rates for diarrheal diseases in American children. **Journal of Pediatrics**. 118, S27–S33.

Goldin J. (1997). Unnatural constituints of breast milk: medication, lifestyle, pollutants, viruses. **Early Human Development**, v. 49: S29-S43. (Suppl.).

Gomes, A.M.P., Malcata, F.X. (1999). *Bifidobacterium* spp. and *Lactobacillus acidophilus* biological, biochemical, technological and therapeutical properties relevant for use as probiotics. **Trends in Food Science & Technology**. 10, 139-157.

Gronlund, M.M et al (1999). Fecal microflora in health infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. **Journal of Pediatric Gastroenterology and Nutrition**. V.28, N^0 1, 19-25.

Grupta, V., Garg, R. (2009). Probiotic. **Indian Journal of Medical Microbiology**. 27, 202-209.

Guandalini, S. et al (2000). *Lactobacillus* GG administered in oral rehydration solution to children with acute diarrhea: a multicenter European trial. **Journal of Pediatric Gastroenterology and Nutrition**. 30, 54–60.

Guandalini, S., Pensabene, L., Zikri, A., Duas, A.J. et al (2001). *Lactobacillus GG* administered in oral rehydration solution to children with acute diarrhea: A multicenter European trial. **Journal of Pediatric Gastroenterology and Nutrition**. 30, 54-60.

Guarino, A., Canani, R.B., Spagnuolo, M.I., Albano, F., Di Benedetto, L. (1997). Oral bacterial therapy reduces the duration of symptoms and of viral excretion in children with mild diarrhea. **Journal of Pediatric Gastroenterology and Nutrition**. 25,516–519.

Guarner, F., Bourdet-Sicard, R., Brandtzaeg, P., Gill, H.S., McGuirk, P., van Eden, W., Versalovic, J., Weinstock, J.V., Rook, G.A. (2006). Mechanisms of disease: the hygiene hypothesis revisited. **Nature Clinical Practice Gastroenterology and Hepatology**. 3, 275–284.

Gum, J.R., Ho, J.J.L., Pratt, W.S., Hicks, J.W., Hill, A.S., Vinall, L.E., Roberton, A.M., Swallow, D.M. and Kim, Y.S. (1997). MUC3 human intestinal mucin. Analysis of gene structure, the carboxyl terminus, and a novel upstream repetitive region. **The Journal of Biological Chemistry**. 272, 26678–26686.

Guslandi, M., Mezzi G., Sorghi, M., et al (2000). *Saccharomyces boulardii* in maintenance treatment of Crohn's disease. **Digestive Diseases and Sciences**. 45,1462– 1464.

Hamilton-Miller, J.M., Shah, S. (1998). Vancomycin susceptibility as an aid to the identification of lactobacilli. **Letters in Applied Microbiology**. 26, 153–115.

Hamosh M. Bioactive factors in human milk. (2001). **Pediatric Clinics of North America**. 48(2):69-86.

Hanson L. The role of breastfeeding in the defense of the infant. (2007). In: Hale T, Hartmann PE, editors. Textbook of Human Lactation (1st ed). Amarillo, TX: Hale Publishing. p. 159-92.

Hatakka, E., Savilahti, A., Ponka, J.H., Meurman, T., Poussa, L.N., Saxellin, M. and Korpela, R. (2001). Effect of long term consumption of probiotic milk on infection in children attending day care centres. **British Medical Journal**. 322, 1318–1319.

Helin, T., Haahtela, S., Haahtela, T. (2002). No effect of oral treatment with an intestinal bacterial strain, *Lactobacillus rhamnosus* (ATCC 53103), on birch-pollen allergy: a placebo-controlled double-blind study. **Allergy**. 57(3), 243–246.

Heyman, M., Ménard, S. (2002). Probiotic microorganisms: how they affect intestinal pathophysiology. **Cellular and Molecular Life Sciences**. 59, $N^0$7, 1151-1165.

Horie, M., Murakami, T., Sato, T., Tarusawa, Y., Nakamura, S, Toba, T. (2005). Anaerobic induction of adherence to laminin in *Lactobacillus gasseri* strains by contact with solid surface. **Current Microbiology**. 51(4), 275-82.

Hoy, C., Millar, M.R., MacKay, P., Godwin, P.G.R., Langdale. V., Levene. M.I. (1990). Quantitative changes in faecal microflora preceding necrotizing enterocolitis in premature neonates. **Archives of Disease in Childhood**. 65, 1057–1059.

Hoyos, A.B. (1999). Reduced incidence of necrotizing enterocolitis with enteral administration of *Lactobacillus acidophilus* and *Bifidobacterium infantis* to neonates in an intensive care unit. **International Journal of Infectious Disease**. 3 , 197-202.

Hsieh, M., Versalovic, J. (2008). The human microbioma and probiotics: Implications for pediatrics. **Current Problems in Pediatric and Adolescent Health Care**. 309-327.

Huis In´Tveld, J.H.J., Shortt, C. (1996). Selection criteria for probiotic microorganisms. In: Leeds, A.R., Rowland, I.R. Ed. Gut Flora and Health – past, present and future. Internacional Congress and Symposium 219, Royal Society of Medicine Press Limited p.27-36.

Husain, S. (2008). Effect of ferric iron on siderophore production and pyrene degradation by *Pseudomonas fluorescens* 29L. **Current Microbiology**. 57, 331–334.

Huurre, A., Kalliomäki, M., Rautava, S., Rinne, M., Salminen, S., Isolauri, E. (2008). Mode of Delivery – Effects on Gut Microbiota and Humoral Immunity. **Neonatology**. 93, 236–240.

Huys, G., D'Haene. K., Collard. J.M. & Swings. J. (2004). Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. **Applied Environmental Microbiology**. 70, 1555–1562.

Ishibashi, N., Yamazaki, S. Probiotics and Safety. (2001). **The American Journal of Clinical Nutrition**. 73 (suppl), 465S-70S.

Isolauri, E. (2003). Probiotics for infectious diarrhea. **Gut**. 52, 436–7.

Isolauri, E., Arvola, T., Sütas, Y., Moilanen, E. and Salminen, S. (2000), Probiotics in the management of atopic eczema. **Clinical & Experimental Allergy**. 30, 1605–1610.

Isolauri, E., Juntunen, M., Rautanen, T., Sillanaukee, P., Koivula, T. (1991). A human *Lactobacillus* strain (*Lactobacillus casei* sp strain GG) promotes recovery from acute diarrhea in children. **Pediatrics**. 88, 90-7.

Isolauri, E., Salminen, S., Ouwehand, A.C. (2004). Probiotic. **Best Practice Research Clinical Gastroenterology**. London 18, 299-313.

Itoh, T., Fujimoto, Y., Kawai, Y., Toba, T., Saito, T. (1995). Inhibition of food-borne pathogenic bacteria by bacteriocins from *Lactobacillus gasseri*. **Letters in Applied Microbiology**. 21, 137– 141.

Jahangiri, S., Talebi, M., Eslami, G., Pourshafie, M.R. (2010). Prevalence of virulence factors and antibiotic resistance in vancomycin-resistant *Enterococcus faecium* isolated from sewage and clinical samples in Iran. **Indian Journal of Medical Microbiology**. 28, 337-41.

Kalliomaki, M., Collado, M.C, Salminen, S., Isolauri, E. (2008). Early differences in fecal; microbiota composition in children may predict overweight. **The American Journal of Clinical Nutrition**. 87, 534-538.

Kalliomaki, M., Isolauri, E. (2003). Role of intestinal flora in development of allergy. **Current Opinion in Allergy and Clinical Immunology**. 3, 15-20.

Kalliomaki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., Isolauri, E. (2001). Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. **Lancet**. 357, 1076-1079.

Kalliomäki, M., Salminen, S., Poussa, T., Isolauri, E. (2007). Probiotics during the first 7 years of life: a cumulative risk reduction of eczema in a randomized, placebo-controlled trial. **The Journal of Allergy and Clinical Immunology**. 119. 1019–1021.

Kalliomaki, M., Walker, W.A. (2005). Physiology and pathology interactions of bacteria with gastrointestinal epithelium. **Gastroenterology Clinics of North America**. v.34. No3, 383-399.

Kandler, O. and Weiss, N. (1986). Genus *Lactobacillus*. In: Sneath, P.H.A.,Mair, N.S., Sharpe,M.E., Holt, J.G. (Eds.), Bergey'sManual of Systematics Bacteriology, vol. 2. Williams and Wilkins, Baltimore M.D., USA, 1209–1234.

Kanemitsu, K., Nishino, T., Kunishima, H. (2001). Quantitative determination of gelatinase activity among enterococci. **Journal of Microbiological Methods**. 47, 11-16.

Kashket, E.R., (1987). Bioenergetics of lactic acid bacteria: Cytoplasmic pH and osmotolerance. **FEMS Microbiology Reviews**. 46, 233-244.

Kataria, J., Li, N., Wynn, J.L., Neu, J. (2009). Probiotic microbes: do they need to be alive to be beneficial? **Nutrition Reviews**. 67(9), 546-550.

Keersmaecker, S.C.J., Verhoeven, T.L.A., Desair, J., Marchal, K., Vanderleyden, J., Nagy, I. (2006). Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimirium* due to accumulation of lactic acid. **FEMS Microbiology Letter** 259, 89-96.

Kelly, D., King, T., Aminov, R. (2007). Importance of microbial colonization of the gut in early life to the development of immunity. **Mutation Research**, 622, 58-69.

Kim, I.K. Baek, Y.J. Yoon, Y.H. (1996). Effects of rehydration and immobilisation in Ca-alginate on the survival of *Lactobacillus casei* and *Bifidobacterium bifidum*. **Korean Journal of Dairy Science**. **18**, 193–198.

Klaenhammer, T. R. (1988). Bacteriocins of lactic acid bactéria. **Biochemie**. 70, 337-349.

Klaenhammer, T., E. Altermann, F. Arigoni, A. Bolotin, et al (2002). Discovering lactic acid bacteria by genomics. **Antonie van Leeuwenhoek**. 82:29–58.

Klaenhammer, T.R., Kullen, M.J (1999). Selection and design of probiotics. **International Journal of Food Microbiology**. 50, 45-57.

Klein, G. (2010). Antibiotic Resistance and Molecular Characterization of Probiotic and Clinical Lactobacillus Strains in Relation to Safety Aspects of Probiotics. **Foodborne Pathogens and Disease**. 1-15.

Klein, G., Hallmann, C., Casas, I.A., Abad, J., Louwers, J., Reuter, G. (2000). Exclusion of van A, van B and van C type glycopeptideo resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as a probiotic by polymerase chain reaction and hybridization methods. **Journal of Applied Microbiology**. 89, 815-824.

Kliegman, R.M., Walsh, M.C. (1987) Neonatal necrotizing enterocolitis: pathogenesis, classification and spectrum of illness. **Current Problems in Pediatrics**. 17, 213–288

Koll, P., Mandar, R., Smidt, I., Hutt, P., Truusalu, K., Mikelsaar, R., Shchepetova, J., Krogh-Andersen, K., Marcotte, H., Hammarstrom, L., Milkelsaar, M. (2010). Screening and Evaluation of Human Intestinal Lactobacilli for the Development of Novel Gastrointestinal Probiotics. **Current Microbiology**. 61(6), 560-566.

Kondo, S., Xiao, Jz., Satoh, T., Odamaki, T., Takahashi, S., Sugahara, H., Yaeshima, T., Iwatsuki, K., Kamei, A. (2010). Antiobesity Effects of *Bifidobacterium breve*

Strain B-3 Supplementation in a Mouse Model with High-Fat Diet-Induced Obesity. **Bioscience, Biotechnology, and Biochemistry.** Vol. 74, N°. 8, 1656-1661

Konstantinov, S.R., Zhu, W.Y., Williams, B., Tamminga, S., de Vos, W.M., Akermans, A.D., (2003). Effect of fermentable catbohydrates on piglets faecal bacterial communities as revealed by denaturating gradient gel electrophoresis analysis of 16S ribosomal DNA. **FEMS Microbiology Ecology**.43, 225-235.

Kopp-Hoolihan, L, (2001). Prophylactic and therapeutic uses of probiotics: A review. **Journal of American Dietetic Association**. 101, 229-238 quiz 239-241.

Kotowska, M., Albrecht, P., and Szajewska, H. (2005). *Saccharomyces boulardii* in the prevention of antibioticassociated diarrhoea in children: a randomized double blind placebo-controlled trial. **Alimentary Pharmacology and Therapeutics**. 21,583-590.

Kunz, C., Rodriguez-Palmero, M., Koletzko, B., Jensen, R. (1999). Nutritional and biochemical properties of human milk, part I: general aspects, proteins and carbohydrates. **Clinics in Perinatology**. v. 26, 307-333.

Kurdi, P., Kawanishi, K., Mizutani, K. and Yokota, A. (2006). Mechanism of growth inhibition by free bile acids in *Lactobacilli* and Bifidobacteria. **The Journal of Bacteriology**. 188,1979-1986.

Kurmann, J.A. and Rasic, J.L. (1991) . The health potential of products containing bifidobacteria. In: Therapeutic Properties of Fermented Milks. R.K. Robinson, Editor, Elsevier Applied Food Science Series . pp. 117–158. London .

Lara-Villoslada, F., Sierra, S., Martin, R., Delgado, S., Rodriguez, J.M., Olivares, M., Xaus, J. (2007). Safety assessment of two probiotic strains, *Lactobacillus coryniformis* CECT5711 and *Lactobacillus gasseri* CECT5714. **Journal of Applied Microbiology**. 103, 175-184.

Lauer, E., and O. Kandler. (1980). *Lactobacillus gasseri* sp., a new species of the subgenus *Thermobacterium*. **Zentralbl Bakteriol**. Mikrobiol.Hyg. Abt. 1 Orig. C 1:75–78.

Lazdunski, C.J. (1988). Pore-forming colicins: synthesis, extracellular release, mode of action, immunity. **Biochimie** 70, 1291–1296.

Lee, Y.K., Puong, K.Y., Ouwehand, A.C. and Salminen, S. (2003) Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. **Journal of Medical Microbiology**. 52, 925–930.

Lenaerts, K., Bouwman, F.G., Lames, W.H., Renes, J., Mariman, E.C. (2007). Comparative proteomics analysis of cell lines and scraping of the human intestinal ephitelium. **BMC Genomics**. 8, 1-14.

Leverrier, P., Dimova, D., Pichereau, V., Auffray, Y., Boyaval, P. et al (2003). Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii*: physiological and proteomic analysis. **Applied Environmental Microbiology**. 69,3809-18.

Liévin, V., Peiffer, I., Hudault, S., Rocht, F., Brassart, D., Neeser, J. R., Servin, A.L. (2000). Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. **Gut**, 47, 646-652.

Lilly DM, Stillwell RH.(1965). Growth promoting factors produced by probiotics. **Science**. 147, 747-8.

Lin, F., Fung, Z.F., Wu, C.L., Chung, T.C. (1996). Molecular characterization of a plasmid borne (pTC82) chloramphenicol resistance determinant (cat-Tc) from Lactobacillus reuteri G4. **Plasmid**. 36, 116–124.

Lin, H.C., Hsu, C.H., Chen, H.L. et al (2008). Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial. **Pediatrics**.122, 693–700.

Ljungdahl, M., Lundholm, M., Katouli, M., et al (2000). Bacterial translocation in experimental shock is dependent on the strains in the intestinal flora. Scandinavian **Journal of Gastroenterology**. 35,389–397.

Ljungh, A. and Wadström, T. (1995). Binding of extracellular matrix proteins by microbes. **Methods in Enzymology**. 253, 501–514.

Lopes, F. S., Simoes, M., Tenreiro, R., Figueiredo, M.J.J., Crespo, B.M.T (2006). Activity and expression of a virulence factor, gelatinase, in dairy enterococci. **International Journal of Food Microbiology**. 112, 208–214.

Lorca, G., Torino, M.I., deValdez, G.F. and Ljungh, A. (2002). Lactobacilli express cell surface proteins which mediate binding of immobilised collagen and fibronectin. **FEMS Microbiology Letters**. 206, 31–37.

Lozo, J., Vukasinovic, M., Strahinic, I., Topisirovic, L., (2004). Characterization and antimicrobial activity of bacteriocin 217 produced by natural isolate *Lactobacillus paracasei subsp. paracasei* BGBUK2–16. **Journal of Food Protection**. 67, 2727–2734.

Mackie, R.I., Sghir, A., Gaskins, H.R. (1999). Developmental microbial ecology of teh neonatal gastrointestinal tract. **The American Journal of Clinical Nutrition**. v.69, 1035S-1045S.

Majamaa, H., Isolauri, E. (1997). Probiotics: a novel approach in the management of food allergy. **Journal of Allergy and Clinical Immunology**. 99, 179–185.

Majamaa, H., Isolauri, E., Saxelin, M. and Vesikari, T. (1995). Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. **Journal of Pediatric Gastroenterology and Nutrition**. 20, 333-338.

Maldonado, Y., Yolken, R. (1990). Rotavirus. **Bailliere Clinical Gastroenterology**. Vol.4, 609-625.

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

Martin, C.R., Walker, W.A. (2006). Intestinal Immuno defenses and the inflammatory response in necrotizing enterocolitis. **Semin in Fetal and Neonatal Medicine**. V.11. n.5, 369-377.

Martin, R., Soberon, N., Vaneechoutte, M., Florez, A.B., Vazquez, F., Suarez, J.E. (2008). Characterization of indigenous vaginal lactobacilli from health women as probiotic candidates. **International Microbiology**. 11, 261-266.

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

Maupas, J., Champemont, P., and Delforge, M. (1983). Treatment of irritable bowel syndrome with *Saccharomyces boulardii*: a double-blind, placebo-controlled-study. **Medicine Chirurgie Digestives**. 12, 77-79.

Metchnikoff, E. (1908). The prolongation of life. Optimistic studies New York: Putman's Sons; p. 161-83.

Midolo, P.D., Lambert, J.R., Hull, R., Luo F., Grayson, M.L.(1995). *In vitro* inhibition of Helicobacter pylori NCTC 11637 by organic acids and lactic acid bacteria. **Journal of Applied Bacteriology**. 79, 475-479.

Misra, S., Sabui, T.K., Pal, N.K. (2009). A Randomized Controlled Trial to Evaluate the Efficacy of *Lactobacillus* GG in Infantile Diarrhea. **The Journal of Pediatrics**. 155, 129-132.

Mitsuoka, T. (1982). Recente trends in research on intestinal flora. **Bididobacteria and Microflora**, v.1, p.3-24.

Mitsuoka, T. (1989). Microbes in the intestine- our lifelong partners Yakult Honsha Co., Std Tokyo. 104 p.

Mitsuoka, T. (1990). Bifidobacteria and their role in human health. **Journal of Industrial Microbiology**. 6, 263-268.

Mitsuoka, T. (1996). Intestinal flora and human health. **Asia Pacific Journal Clinical and Nutrition**. V.1, p.2-9.

Mitsuoka, T., (1978). Intestinal bacteria and health, Tokyo: Harcourt Brace Jovarnovich Japan, 1978, 208p.

Montville, T.J., Winkowski, K., Ludescher, R.D., (1995). Models and mechanisms for bacteriocin action application. **International Dairy Journal**. 5, 797-814.

Morais, M.B., Jacob, C.M. (2006). The role of probiotics and prebiotics in pediatric practice. **Journal of Pediatrics**. 82, S189-97.

Morelli, L. (2000). *In vitro* selection of probiotic Lactobacilli: A critical appraisal. **Current Issues Intestinal Microbiology**. 1(2), 59-67.

Morelli, L. (2008). Fecal Microflora in Healthy Infants Born by Different Methods of Delivery: Permanent Changes in Intestinal Flora After Cesarean Delivery. **Journal of Pediatric Gastroenterology & Nutrition**. v28, 19-25.

Mshvildadze, M., Neu, J. and Mai, V. (2008). Intestinal microbiota development in the premature neonate: establishment of a lasting commensal relationship. **Nutrition Reviews**. 66, 658–663.

Mundy, L.M., Sahm, D.F., Gilmore, M.S. (2000). Relationship between enterococcal virulence and antimicrobial resistance. **Clinical Microbiology Review**. 13, 513–522.

Munoz-Provencio, D., Perez-Martınez, G. and Monedero, V. (2009). Characterization of a fibronectin-binding protein from *Lactobacillus casei* BL23. **Journal of Applied Microbiology**. 108, 1050–1059.

Neeser, J.R., Granto, D., Rouvet, M., Servin, A., Teneberg, S., and. Karlsson, K.-A. (2000). *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. **Glycobiology**. 10, 1193–1199.

Neu, J., Douglas-Escobar, M. (2008). Necrotizing enterocolitis: pathogenisis, clinical care and prevention. In Neu, Journal of Gastroenterology and Nutrition: Neomatology Questions and Controversis. Philadelphia: Saunders.

Ogawa, M., Shimizu, K., Nomoto, K., Takahashi, M., Watanuki, M., Tanaka, R., Tanaka, T., Hamabata, T., Yamasaki, S. And Takeda, Y. (2001). Protective effect of *Lactobacillus casei* ssp Shirota on Shiga toxin- producing *Escherichia coli* O157:H7 infection in infant rabbits. **Infection Immunity**. 69, 1101–1108.

Ogden, C.L., Yanovski, S.Z., Carrol, M.D., Flegal, K.M. (2007). The epidemiology of obesity. **Gastroenterology.** 132(6), 2087-2102.

Orla-Jensen, S. (editor) (1919). The Lactic Acid Bacteria. Host, Copenhagen, pp. 1–196.

Owen, C., Martin, R., Whincup, P., Smith, D., Cook, D. (2005). The effect of infant feeding on the risk of obesity across the life course: a quantitative review of published evidence. **Pediatrics**. 115, 1367–77.

Parashar, U.D., Gibson, C.J., Bresee, J.S., Glass, R.I. (2003). Rotavirus and severe childhood diarrhea. Emerging Infections Disease. Vol 12. n2. www.cdc.gov.

Perdigón, G., Holgado, A.P.R. (2000). Mechanisms involved in the immunostimulation by lactic acid bacteria. In: FULLER, R.; PERDIGÓN, G. Probiotics 3: Immunodulation by the Gut Microflora and Probiotics. Dordrecht : Kluwer Academic, 213-233.

Pérez, P.F., Minnaard, J., Rouvet, M., Knabenhans, C., Brassart, D., De Antoni, G.L., Schiffrin, E.J (2001). Inhibition of *Giardia intestinalis* by Extracellular Factors from Lactobacilli: an *In Vitro* Study. Applied and Environmental Microbiology**. V. 67, No. 11, 5037-5042.**

Perreten, V., Schwarz, F., Teuber, M., Levy, S.B. (2001). Mdt(A), a new efflux protein conferring multiple antibiotic resistance in Lactococcus lactis and *E. coli*. **Antimicrobial Agents Chemotherapy**. 45, 1109-1114.

Petrof, E.O. (2009). Probiotics and Gastrointestinal Disease: Clinical Evidence and Basic Science. **Antiinflammatory Antiallergy Agents and Medical Chemotherapy**. 8(3), 260–269.

Pfeiler, E.A., Klaenhammer, T.R. (2009). Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. **Applied Environmental Microbiology**. 75(18), 6013-6.

Pineiro, M., Staton, C. (2007). Probiotic Bacteria: Legislative Framework – Requirements to evidence basis. **The Journal of Nutrition**. 137, 850S-853S.

Podolak, P.K., Zayas, J.F., Kastner, C.L., Fung, D.Y.C. Inhibition of Listeria monocytogenes and Escherichia coli O157:H7 on beef by application of organic acids. **Journal of Food Protection**. 59, 370-373, 1996.

Poh, C. H., Oh, H. M. L. & Tan, A. L. (2006). Epidemiology and clinical outcome of enterococcal bacteraemia in an acute care hospital. **Journal of Infection**. 52, 383–386.

Presser, K.A., Ratkowsky, D.A., Ross, T. (1997) Modelling the growth rate of Escherichia coli as a function of pH and lactic acid concentration. **Applied Environmental Microbiology**. 63, 2355-2360.

Prieto, A.I., Ramos-Morales F. and Casadesus, J. (2004). Bile-induced DNA damage in *Salmonella enterica*. **Genetics**.168, nº4, 1787-1794.

Quaroni, A., Hochman, J. (1996). Development of Intestinal Cell Culture Models for Drug Transport and Metabolism Studies. **Advanced Drug Den Review**. 22, 3–52.

Reid, G., Hammond, J.A. (2005). Probiotics some evidence of their effectiveness. **Can Fam Physician**. 51, 1487-1493.

Reid, G.R. (2008). Probiotics and prebiotics: progress and challenges. **International Dairy Journal**. 18, 969–975.

Reinhardt, C., Reigstad, C.S. Backhed, F. (2009). Intestinal microbiota during infancy and its implications for obesity. **Journal of Pediatric Gastroenterology and Nutrition**. 48, 249–256.

Reuter, G. (2001). The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. **Current Issues Intestestinal Microbiology**. 2, 43–53.

Robredo, B., Singh, K.V., Torres, C. and Murray, B.E. (2000): Streptogramin resistance and shared pulsed-field gel electrophoresis patterns in vanA-containing Enterococcus faecium and Enterococcus hirae isolated from humans and animals in Spain. **Microbial Drug Resistance**. 6, 305–311.

Rook, G.A. (2007). "The hygiene hypothesis and the increasing prevalence of chronic inflammatory disorders." **Transaction of the Royal Society of Tropical Medicine and Hygiene.** 101, 1072-4.

Ryan, K.A., Jayaraman, T., Daly, P., Canchaya, C., Curran, S., Fang, F., Quigley, E.M., O`Toole, P.W (2008). Isolation of lactobacilli with probiotic properties from the human stomach. **Letters in Applied Microbiology**. 47, 269-274.

Saarela, M., Mogensen, G., Fonden, R., Matto, J., Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. **Journal of Biotechnology**. 84, 197-215.

Saavedra, J.M., Bauman, N.A., Oung, I., Perman, J.A., Yolken, R.H. (1994). Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. **Lancet**. 344, 1046-1049.

Salyers, A.A. Shoemaker, N.B. Stevens, A.M. Li, L.Y. (1995). Conjugative transposons: and unusual and diverse set of gene transfer elements. **Microbiology Reviews**. 4, 579–590.

Samanta, M., Sarkar, M., Ghosh, P., *et al* (2009). Prophylactic probiotics for prevention of necrotizing enterocolitis in very low birth weight newborns. **Journal of Tropical Pediatrics**. 55, 128–31.

Sanders, M.E. (2009). How do we know when something called probiotic is really a probiotic? A guideline for consumers and health care professionals. **Functional Food Reviews.** 1, 3-12.

Sarker, S. et al, (2005). Lactobacillus paracasei strain ST11 has no effect on rotavirus but ameliorates the outcome of nonrotavirus diarrhea in children from Bangladesh. **Pediatrics**. 116, 221–228.

Schaberg, D.R., Culver, D.H., Gaynes, R.P., (1991). Major trends in the microbial etiology of nosocomial infection. **American Journal of Medicine**. 91 (Suppl. 3B), 72S– 75S.

Schiffrin, E.J., Brassart, D., Servin, A.L., Rochat, F., Donnet-Hughes, A. (1997). Imune modulation of bloood leukocytes in humans by lactic acid bacteria: criteria for strains selection. **American Journal of Clinical Nutrition**. 66, 515-520.

Servin, A. L. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. **Microbiology Reviews**. 28, 405-440.

Servin, A.L., Coconnier, M.H. (2003). Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. **Best Practice and Research Clinical Gastroenterology**. 17, 741-754.

Shortt, C., (1999). The probiotic century: historical and current perspectives. **Trends in Food Science and Technology**. 10, 411-417.

Shu, Q., Zhou, J.S.., Rutherfurd, K.J., Birtles, M.J., Prasad, J., Gopal, P.K., Gill, H.S. (1999). Probiotic Lactic Acid Bactéria (*Lactobacillus acidophilus* HN017, *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019) have no adverse affects on the health of mice. **International Dairy Journal**. 9, 831-836.

Silva, C.H.P.M., (1999). Bacteriologia: um texto ilustrado, Teresópolis: Eventos, p. 107-119, 1999.

Silva, T.M. O que são radicais livres. (2003). Acesso em 15 Fev. 2006. Disponível em <www.medstudents.com.br/content/resumos/trabalhos_radicais_livres>.

Simakachorn, N., Pichaipat, V., Rithipornpaisarn, P., Kongkaew, C., Tongpradit, P., Varavithya, W.(2000). Clinical evaluation of the addition of lyophilized, heat-killed *Lactobacillus acidophilus* LB to oral rehydration therapy in the treatment of acute diarrhea in children. **Journal of Pediatrics Gastroenterology and Nutrition**. 30, 68–72.

Singhal, A, Farooqi, I.S, O'rahilly, S., Cole, T.J, Fewtrell, M., Lucas, A. (2002). Early nutrition and leptin concentrations in later life. **American Journal of Clinical Nutrition**. v. 75, 993-999.

Smith, S.I., Aweh, A.J. Coker, A.O. Savage, K.O. et al (2001). *Lactobacilli* in human dental caries and saliva. **Microbios**. 105:77–85.

Snijders, J.M., Van Logtestijn, J.G., Mossel, D.A.A., Smulders, F.J.M. (1985). Lactic acid as a decontaminant in slaughter and processing procedures. **Veterinary Quarterly**. 7, 27-282.

Snyder, L., Champness, W. (2003). Molecular Genetics of Bacteria. Second Ed. ASM Press. Washington, USA.

Sobko, T., Huang, L., Midtvedt, T., Norin, E., Gustafsson, L.E., Norman, M., Jansson, E.A., Lundber, J.O. (2006). Generation of NO by probiotic bacteria in the gastrointestinal tract. **Free Radical Biology and Medicine**. 41: 985-91.

Staton, C., Desmond, C., Coaklen, M., Collins, J.K., Fitzgerald, G., Ross, R.P. (2003). Challenges facing development of probiotic containing functional foods. In: Farnworth, E.R. Ed. Handbook of fermented functional foods. Boca Raton: CRC Press 27-58.

Staton, C., Gardiner, G., Muhan, H., Collins, K., Fitzgerald, G., Lyneh, P.B., Ross, R.P (2001). Market potential for probiotics. **American Journal of Clinical Nutrition**. 73, 476S-83S.

Steffen, E.K., Berg, R.D., and Deitch, E.A.J. (1988). **Infection Disease**. 157, 1032-1037, 1988.

Styriak, I., Nemcova, R., Chang, Y.H. and Ljungh, A. (2003). Binding of extracellular matrix molecules by probiotic bacteria. **Letters in Applied Microbiology**. 37, 329–333.

Szajewska, H., Mrukowicz, J.Z, (2001). Probiotics in the tratament and prevention of acute infectious diarrhea in infants and children. **Jornal of Pediatric Gastroenterology and Nutrition.** 33, p 17-25.

Tagg, J.R., Dajani, A.S., Wannamaker, L.W., (1976). Bacteriocins of Gram positive bacteria. **Bacteriology Reviews**. 40, 722-756.

Tankanow RM, Ross MB, Ertel IJ, Dickinson DG,McCormick LS, Garfinkel JF. (1990). A double-blind, placebo-controlled study of the efficacy of Lactinex in the

prophylaxis of amoxicillin-induced diarrhea. DICP – **The annals of pharmacotherapy**. 24(4):382–4.

Taylor, AL., Dunstan, JA., Prescott, SL.(2007). Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: a randomized controlled trial. **Journal of Allergy and Clinical Immunology**. 119, 184–91.

Ten Brink, T., Minekus, M., Van der Vossen, J.M.B.M., Leer, R.j., Husìnt Veld, J.H.J (1994). Antimicrobial activity of lactobacilli: preliminary characterization and optimization of production of acidocin B, a novel bacteriocin produced by *Lactobacillus acidophilus* M46. **Journal of Applied Bacteriology**. 77, 140-148.

Teran, C.G., Teran-Escalera, C.N., Villarroela, P. (2009). Nitazoxanide vs. probiotics for the treatment of acute rotavirus diarrhea in children: a randomized, single-blind, controlled trial in Bolivian children. **International Journal of Infectious Diseases**. V.13, 518-523.

Teuber, M., Meile, L., Schwarz, F. (1999). Acquired antibiotic resistance in lactic acid bacteria from food. **Antonie van Leeuwenhoek**. 76, 115–137.

Thomas, M.R., Litin, S.C., Osmon, D.O., et al (2001). Lack of effect of *Lactobacillus* GG on antibiotic-associated diarrhea: a randomized, placebo-controlled trial. **Mayo Clinic Procedure**.76, 883–889.

Tichaczek, P.S., Nissen-Meyer, J., Nes, I.F., Vogel, R.F., Hammes, W.P. (1992). Characterization of the bacteriocins curvacin A from *Lactobacillus curvatum* LTH1174 and sakacin P from *Lactobacillus sake* LTH673. Sys. Applied Microbiology. 15, 460-468.

Tissier, H., (1899). Le *Bacterium coli* et le réaction chromophilr d' Escherich. C. R. Soc. Biol. 51, 943.

Tortora, G.J., Funke, B.R., Case, C.L. (2000). Microbiologia. Ed. Artes Médicas Sul, 6ªed, Porto Alegre.

Tozzi, A.E, Pezzotti, P., Greco, D. (1990). Does breastfeeding delay progression to AIDS in HIV-infected children? **AIDS**. v.4:1, 293-400.

Tuomola, C., Crittenden, R., Playne, M. Isolauri, E., Salminen, S. (2001). Quality assurance criteria for probiotic bacteria. **American Journal of Clinical Nutrition**. 73, 393S-8S.

Tuomola, E.M., Ouwehand, A.C., Salminen, S.J (1999). The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. **FEMS Immunology e Medical Microbiology**. 26, 137-142.

Turnbaugh, P.J., Backhed, F., Fulton, L., Gordon, J.I. (2008). Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome, Cell Host Microbe 3, 213–223.

Ubbink, J. Krüger, J. (2006). Physical approaches for the delivery of active ingredients in foods. **Trends in Food Science and Technology**. v. 17, n. 5, p. 244-254.

UNICEF, WHO. (2009). Diarrhoea: why children are still dying and what can be done. New York: United Nations Children's Fund, 2009.

Vaarala, O. (2002). The gut immune system and type 1 diabetes. **Annal of New York Academy of Science**. 958, 39-46.

Van Klinken, B.J., Tytgat, K.M.A.J., Buller, H.A., Einerhand, A.W.C., Dekker, J. (1995). Biosynthesis of intestinal mucins: MUC1, MUC2, MUC3 and more. **Biochemestry Society Transactions**. 23, 814–818, 1995.

Vanderhoof, J.A. (2001). Probiotic: Future direction. **American Journal of Clinical Nutrition**. 73, 1152S-5S

Vanderhoof, J.A., Whitney, D.B., Antonson, D.L. et al (1999). *Lactobacillus* GG in the prevention of antibiotic-associated diarrhea in children. **Journal of Pediatrics**. 135, 564–568.

Vanderhoof, J.A., Young, R.J. (2002). Probiotics in pediatrics. **Pediatrics**.109 :956 – 958

Vasiljevic, T. and Shah, N.P. (2008). Probiotics—From Metchnikoff to bioactives. **International Dairy Journal.** 18, 714– 728, 2008.

Vásquez, A., Molin, G., Pettersson, B., Antonsson, M. and Ahrne, S. (2005). DNA-based classification and sequence heterogeneities in the 16S rRNA genes of Lactobacillus casei/paracasei and related species. **Systematic and Applied Microbiology.** 28, 430-441.

Vasseur, C., Bavarel, L., Hebraud, M., Labadie, J. (1999). Effect of osmotic alkaline, acid or thermal stresses on the growth and inihibition of *Listeria monocytogenes*. **Journal of Applied Microbiology**. 86, 469-476.

Vescovo, M., Morelli, L., Bottazzi, V. (1982). Drug resistance plasmids in *Lactobacillus acidophilus* and *Lactobacillus reuteri*. **Applied Environmental Microbiology**. 43, 50–56.

Vesterlund, S., Karp, M., Salminen, S. and Ouwehand, A.C. (2006). *Staphylococcus aureus* adheres to human intestinal mucus but can be displaced by certain lactic acid bacteria. **Microbiology**. 152, 1819–1826.

Vesterlund, S., Vankerckhoven, V., Saxelin, M., Goossens, H., Salminen, S., Ouwehand, A.C. (2007). Safety assessment of *Lactobacillus* strains: Presence of putative risk factprs in faecal, blood and probiotic isolates. **International Journal of Food Microbiology**. 116, 325-331.

Wagner, RD., Pierson, C., Warner, T., et al (1997). Biotherapeutic effects of probiotic bacteria on candidiasis in immunodeficient mice. **Infection Immunology**. 65, 4165–72

Wall, R., Fitzgerald, G., Hussey, S. Ryan, T. et al (2007). Genomic diversity of cultivable *Lactobacillus* populations residing in the neonatal and adult gastrointestinal tract. **FEMS Microbiology Ecology**. 59, 127–137.

Wall, R., Ross, R.P., Ryan, C.A., Hussey, S., Murphy, B., Fitzgerald, G.F., Staton, C. (2009). Role of gut microbiota in early infant development. **Clinical Medicine Pediatrics**. 3, 45-54.

Walter, J., Ghosh, M.K., Kuhn, L., Semrau, K., Sinkala, M., Kankasa, C.,Thea, D.M., Aldrovandi, G.M. (2009). High Concentrations of Interleukin 15 in Breast Milk Are Associated with Protection against Postnatal HIV Transmission. **Infection Disease**. 15; 1498–1502.

Wardlaw. T., Salama, P., Brocklehurst, C., Chopra, M., Mason, E. (2010). Diarrhoea: why children are still dying and what can be done. **Lancet**. 375, 870-872.

Watanabe, S., Narisawa, Y., Arase, S., Okamatsu, H., Ikenaga, T., Tajiri, Y. et al (2003). Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. **Journal of Allergy and Clinical Immunology**. 111, 587–591.

Wells, C.L. (1990). Relationship between intestinal microecology and the translocation of intestinal bacteria. **Antonie van Leeuwenhoek**. 58, 87–93.

Willians, R., Stehlin, I. (2005). Breast milk or formula: making the right choice for your baby. Adapted from FDA consumer.

Witte, W. (2000). Selective pressure by antibiotic use in livestock. **International Journal of Antimicrobial Agents.** 16, S19–S24.

Work Group on Breastfeeding American Academy of Pediatrics. (1997). Breastfeeding and the use of human milk. **Pediatrics.** v. 100, 1035-1039.

Wright, A.V, (2005). Regulating the Safety of Probiotics. **The European Approach. Current Pharmaceutical Design**. 11, 17-23.

Xiao, SD., Zhang, DZ., Lu, H et al (2002). Multicenter randomized controlled trial of heat killed Lactobacillus acidophilus LB in patients with chronic diarrhea. **Chinese Journal of Digestive Disease**. 3, 167-171.

Yildirim, Z., Johnson, M.G., (1998). Characterization and antimicrobial spectrum of bifidocin B, a bacteriocin produced by *Bifidobacterium bifidum* NCFB 1454. **Journal of Food Protection.** 61, 47-51.

Zhang, L., Li, N., Caicedo, R., Neu, J. (2005). Alive and Dead Lactobacillus rhamnosus GG Decrease tumor necrosis factor-α-induced interleukin-8 production in Caco-2 cells. **Nutritional Immunology**. 1752-1756.

Zhou, J.S., Shu, Q., Rutherfurd, K.J., Prasad, J., Gopal, P.K., Gill, H.S. (2000). Acute oral toxicity and bacterial translocation studies on potencially probiotic strains of Lactic acid bacteria. **Food and Chemical Toxicology**. 38, 153-161.

3 - EXPERIMENTAL HYPOTHESES

The problem exposed earlier motivated the construction of the following experimental hypotheses tested in this study.

I) **Newly *Lactobacillus gasseri* isolates do not automatically share the GRAS status of traditional Lactic Acid Bacteria.**

The hypothesis was tested according to the following objectives:
- To identify all isolates at the species level;
- To evaluate the antibiotic resistance and hemolytic activity for all isolates;
- To investigate the presence of plasmids in all strains.

II) *Lactobacillus gasseri* **strains isolated from the same child could be genetically identical.**

The hypothesis was tested according to the following objectives:
- To evaluate the genetic diversity among the 30 isolates through the PFGE technique.

III) **Potential probiotic strains might be tested for important traits that will confer ability of the strain to survive and reach the target site and infer the functionality expected.**

The hypothesis was tested according to the following objectives:
- To evaluate the tolerance of *L. gasseri* to 0.25 and 0.5% oxgall;
- To evaluate the resistance of *L. gasseri* to small intestine and gastric juices.
- To evaluate the antagonism of *L. gasseri* toward pathogenic bacteria, such as *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Staphylococcus aureus*;
- To evaluate the ability of *L. gasseri* to adhere to Caco-2 and HT-29 cell lines;
- To examine the ability of *L. gasseri* to adhere to mucin and to extra-intestinal matrix cellular fibronectin binding protein.

IV) **The antagonism of *Lactobacillus gasseri* toward pathogenic bacteria depends mainly on the substances secreted into the medium. These metabolites can be hydrogen peroxide, proteins or organic acids.**

The hypothesis was tested according to the following objectives:

- To elucidate the chemical nature of the of the inhibition.

V) ***Lactobacillus gasseri* strains can harbor plasmids containing transferable antibiotic resistance genes.**

The hypothesis was tested according to the following objective:

- To sequence all plasmids to verify the presence of antibiotic resistance genes.

VI) ***lmrB* gene can confer *Lactobacillus gasseri* resistance to erythromycin, oxacillin and cephalothin**

The hypothesis was tested according to the following objectives:

- To investigate if the *lmrB* gene encoding a ATP-binding cassette-type multidrug resistance transporter protein was responsible for the erythromycin, oxacillin and cephalothin resistance showed by *Lactobacillus gasseri* NCK2141.

VII) **Growth conditions may influence in the ability of *L. gasseri* to adhere to epithelial intestinal cells.**

The hypothesis was tested according to the following objective:

- To evaluate the effect of the growth condition in the ability of the *L. gasseri* to adhere to fibronectin binding protein.

VIII) **Some genes have more functions that are apparent from their annotation or placement in an operon and, therefore, may influence other cell functions, which can directly affect important probiotic attributes.**

The hypothesis was tested according to the following objectives:

- To investigate the functional role of the fibronectin binding protein in the adhesion and the stress response of the cell;

- To knockout the *fbp* gene in the chromosomal of *L. gasseri* and compare the adherence ability of *fbp* mutants and parental strains to immobilized fibronectin-binding protein and HT-29 cell lines;

- To evaluate the resistance of *L. gasseri fbp* mutants to gastric juice and compare with their parental strains.

CHAPTER I

CHARACTERIZATION OF *Lactobacillus gasseri* ISOLATES FROM A BREAST-FED INFANT

ABSTRACT

The potential health benefits of probiotic bacteria have led to the isolation of new microbial strains for incorporation into food, dairy, or medical products. However, newly isolated candidate probiotic organisms do not automatically share the "generally recognized as safe" (GRAS) status of traditional Lactic Acid Bacteria (LAB). Before their introduction into food products, the safety of new isolates has to be evaluated. The objective of this study was to characterize LAB isolates from the stool of a newborn infant, and evaluate their safety and probiotic potential, *in vitro*. Thirty colonies were identified as *Lactobacillus gasseri* through sequencing of 16S rDNA. Pulsed Field Gel Electrophoresis (PFGE) using restriction enzymes Sma I and Apa I revealed that 29 of the *L. gasseri* were identical; one isolate was closely related, but exhibited a distinctive DNA fingerprint. All 30 *L. gasseri* were evaluated for resistance to antibiotics, oxgall tolerance, hemolytic activity and antagonism toward selected Gram-positive and Gram-negative pathogens. All 30 strains harbored 3 plasmids, with one strain that showed strong tolerance to 0.5% of bile and harbored a unique fourth plasmid encoding a putative multidrug resistance transporter protein (lmrB). For all thirty strains no hemolytic activity or antagonism, beyond acid inhibition was observed. Three selected strains UFVCC1083 (NCK2140), 1091 (NCK2141) and 1112 (NCK2142) showed strong resistance to small intestinal and gastric juices and adhered *in vitro* to mucin and two intestinal epithelial cell lines, Caco-2 and HT-29. This study identified and characterized recently isolated *L. gasseri* strains from faeces of a breast fed infant as potential probiotic candidates for pediatric use, such as in the human milk banks in Brazil.

Key words: Probiotic safety, *Lactobacillus gasseri,* antibiotic resistance

RESUMO

Os inúmeros benefícios conferidos por bactérias probióticas têm comandado o isolamento de novas estirpes para incoporação em produtos alimentícios e fármacos. Entretanto, os novos isolados não automaticamente possuem o status GRAS (Geralmente reconhecida como segura) das bactérias láticas tradicionais (BAL). Dessa forma, aspectos de segurança necessitam ser avaliados antes da incorporação de novas estirpes na cadeia alimentar humana. O objetivo desse estudo foi caracterizar BAL isoladas de fezes de recém nascidos e avaliar a segurança e potencial probiótico das mesmas *in vitro*. Os trinta isolados foram identificados como *Lactobacillus gasseri* por meio do sequenciamento do 16S rDNA e vinte nove deles foram idênticos pela técnica de Eletroforese em Campos Pulsados (PFGE), utilizando-se as enzimas de restrição Sma I e Apa I. O isolado NCK2142 apresentou-se estritamente relacionado aos outros, diferindo-se apenas por uma única banda no gel. As 30 estirpes de *L. gasseri* foram avaliadas quanto a resistência a antibióticos, atividade hemolítica, tolerância ao oxgall, presença de plasmídeos e antagonismo a patógenos Gram-positivos e Gram-negativos. Todas as 30 estirpes não apresentaram atividade hemolítica e carreavam três plasmídeos, exceto a estirpe NCK2141, que apresentou um quarto plasmideo, codificando um sistema de transporte de resistência à multiplas drogas (lmrB). Essa mesma estirpe mostrou maior tolerância a bile (0.5%) e resistência a eritromicina, cefalotina e oxacilina. Todas as estirpes apresentaram antagonismo frente aos patógenos avaliados sendo a inibição assossiada apenas à produção de ácidos orgânicos. Com base na diversidade genética (PFGE), resistência a antibióticos e perfil plasmidial, três estirpes (NCK2140, 2141 e 2142) foram selecionadas e avaliadas quanto à resistência ao suco gástrico e ao suco do intestino delgado, capacidade de adesão à mucina, fibronectina, e às células humanas do câncer de cólon Caco-2 e HT-29. Os três isolados selecionados NCK2140, 2141 e 2142 foram resistentes ao suco gástrico e ao suco do intestino delgado e apresentaram boa capacidade de adesão *in vitro* a mucina, fibronectina e às células humanas do câncer de cólon, Caco-2 e HT-29. Este estudo identificou e caracterizou novas estirpes de *L. gasseri* isoladas de fezes de recém nascidos com potencial de uso como probiótico em bancos de leite humano no Brasil.

Palavras-Chave: Probiótico, seguranças, *Lactobacillus gasseri,* resistência a antibióticos

1 - INTRODUCTION

The relationship between balanced microbiota and host health has been well documented. The gut microbiota acts as an important intestinal immune-modulator, not only educating the naïve infant immune system but also serving as an important source of non inflammatory immune stimulators throughout life in healthy individuals (Tuohy et al, 2003). In addition, the enteric microbiota can secrete molecules that inhibit host pathogens, metabolize compounds that harm the host to less toxic substances and produce a range of bioactive compounds such as conjugated linoleic acid, and short chain fatty acids that may play a role in protection from lifestyle illnesses such as cancer, obesity and cardiovascular disease (Wall, et al 2009). However, when this balance is interrupted, the gut microbiota can contribute to the pathogenesis of certain diseases (Kelly et al 2007).

Application of probiotics ("live microorganisms that, when administered in adequate amounts, confer a health benefit on the host", FAO/WHO, 2002) can support the maintenance of a balanced microbiota (Schrezenmeier and Vrese, 2001). Those microorganisms prevent the growth of undesirable microorganisms by producing inhibitory compounds such as organic acids and bacteriocins (Gonzales-Martines et al 2003), competing for nutrient and epithelium adhesion sites (Collado et al 2006), and modulating immune response (Forsythe and Bienenstock, 2010; Round, 2009; Griffin et al 2002). Lactobacilli are frequently used as probiotic cultures, and promising data have been reported in the prevention of colon cancer (Commane et al 2005), reduction of serum cholesterol (Lin and Chen, 2000), lactose digestion (Griffin et al 2002), metabolism of proteins and vitamins (Berg, 1980; Young and Huffman, 2003) stimulation of the immune system (Round, 2009; Isolauri, 2001; Kalliomaki et al 2001) and protection of children from allergy and intestinal infections (Collado et al 2006; Harsharnijit, 2003).

Breastfeeding is recognized worldwide as nurturing and protective to the developing infant. (Schandler et al 2003). Despite the numerous benefits conferred by this human milk, many children are deprived of it because their mothers cannot breastfeed them. Therefore, human milk banks have a fundamental role as an important food source for such newborns (Giugliani, 2002).

Brazil is a world reference in Human Milk Banking with 283 banks under current operation (Fiocruz, 2010). In 2007, 1,350 million liters of human milk were collected through the National Human Milk Bank Network providing protection to

more than 107,000 newborns. Almost all these children were prematurely born and were not able to breast feed. Furthermore, it is estimated that 7,000 Brazilian children would die every year if they were not breastfed properly (Bom dia Brazil, 2008) which emphasizes the importance of the human milk banks (HMB). Pasteurization ($65^{o}C$ x 30 min^{-1}) insures the microbial safety of the milk deposited in the HMB. However, studies from Borba et al (2003) indicated that this heat treatment reduces, inactivates or eliminates some prebiotic constituents. The prebiotic character of human milk is important to establishing *Bifidobacterium* and other desirable lactic acid bacteria (LAB) in the newborn´s intestinal tract. A possible way to augment any undesirable effects of heat treatment could be through the addition of well selected endogenous intestinal LAB strains into the pasteurized milk provided in the milk banks.

Lactobacilli are generally regarded as safe due to their long history of safe use in fermented foods and their presence in the normal intestinal microbiota of humans (Tuohy et al, 2003). Newly isolated LAB do not automatically share the GRAS status of traditional Lactic Acid Bacteria (LAB), and strains must be fully characterized taxonomically, phenotypically and genetically.

In order to achieve this goal, the objective of this study was to characterize recently isolated lactobacilli from the stool of a newborn infant to select suitable strains for potential use as a probiotic culture in human milk banks in Brazil.

2 - MATERIALS AND METHODS

2.1 - Bacterial strains and growth conditions

Thirty bacterial strains used in this study were isolated using NPNL - Neomycin, Paromomycin, Nalidixic acid and Lithium chloride agar from stools of a Brazilian newborn infant (cesarean birth, 45 days old). The strains were deposited in the culture collection of the Department of Food Science and Technology, Federal University of Viçosa (UFVCC). Three of them, UFVCC1083 (NCK2140), UFVCC 1091 (NCK2141) and UFVCC1112 (NCK2142) are deposited in the Klaenhammer culture collection at North Carolina State University. The provisional identification of the strains was based on the Gram-positive rod-shaped non-sporing cell morphology, biochemical tests with the API 50 CHL system (BioMerieux-Massy, France) and negative catalase reaction. The strains were maintained at $-80^{o}C$ and they were

propagated at 37ºC statically in MRS (DeMan et al 1960) broth (Difco Laboratories, Inc., Detroid, MI) under aerobic conditions or anaerobically on MRS agar (1.5% [wt/vol], Difco).

2.2 - Bacterial identification

To analyze the 16S rDNA gene sequence, polymerase chain reactions (PCR) were performed using a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) using universal primers: plb16 (5'AGAGTTTGATCCTGGCTCAG 3') and mlb16 (5' GCTGCTGGCACGTAGTTAG 3'), previously described by Kullen et al (2000). Genomic DNA was extracted using the ZR Fungal / Bacterial DNA Kit (Zymo research, USA) according to the manufacturer's protocol. Primers were synthesized by Integrated DNA Technology (Coralville, IA). PCR was carried out over 30 cycles (initial denaturation at 95 °C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and polymerization at 72°C for 1 min) with a final 10-min polymerization step at 72°C. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA), quantified using a NanoDrop ND 1000 spectrophotometer and sequenced at Davis Sequencing (Davis, CA). Sequencing of the 16S rDNA gene PCR product was performed in both forward and reverse directions with the plm16 and mlb16 primers. The 16S rDNA gene sequence was examined for similarity with deposited sequences using the Blast N program available through the National Centre for Biotechnology Information (NCBI) (http:/www.ncbi.nlm.nih.gov/).

2.3 - Pulsed-Field Gel Electrophoresis (PFGE)

2.3.1 - Preparation of genomic DNA in agarose blocks

Bacterial genomic DNA was prepared from 1.2 ml of overnight culture in MRS broth, using a modified method from Tanskanen *et al* (1990). Cells were centrifugally harvested, washed (TE 1X buffer) and then suspended in 500 µL of TE 1X buffer. Three hundred microliters (300µl) of standardized cells ($0.7{\leq}A_{600nm}{\leq}1.35$) was mixed with 300 µL of low-melting-temperature agarose (1.2% wt/vol; Bio-Rad Laboratories, Hercules, CA) at 55ºC, dispensed into molds (Bio-Rad Laboratories) and then allowed to solidify. Cells immobilized in agarose blocks were incubated 18h at 37ºC in 5 ml of lysis buffer containing 100 m*M* EDTA, 1 *M* NaCl, 6.0 m*M* Tris-

HCl (pH 7,6), 1 mg/ml lysozyme (Sigma, St. Louis, MO) and 100µl mutanolysin (Sigma). Treatment with proteinase K solution (10 mL; Sigma) was performed overnight at 50ºC in 250 mM EDTA pH 8.0, 1% lauroyl-sarcosine and 100 µg/ml proteinase K. The plugs were washed twice at 55°C for 15 min with 55°C sterile water and four times with 5 ml of 55°C TE buffer (10 mM Tris and 1 mM EDTA, pH 7.6).

2.3.2 - Restriction enzyme digestion and PFGE.

Washed plugs were digested overnight at room temperature (RT) with 250 µl of restriction solution (7.5µl of SmaI / ApaI [Roche Applied Science, Indianapolis, Ind]; 25 µl of restriction enzyme buffer [Roche] and 217.5 µl of sterile water). Plugs were cut using a coverslip, equilibrated with TBE (Tris-borate-EDTA) for 30 min and placed in the wells of a 1.1% agarose gel. Electrophoresis was carried out for 18 hr with a CHEF Mapper (Bio-Rad) at 6.0 V/cm with an angle of the 120º and initial switch time of 2 s to a final switch time of 20 s at 14°C in 0.5X TBE (Tris-borate-EDTA) running buffer. The agarose gel was stained with ethidium bromide (0.5 µg/ml) in 0.5X Tris-Borate-EDTA buffer for 1 h. DNA bands were visualized using White/UV Transilluminator, TMW-20, UVP, LLC, photographed with a AlphaImager Camera 70-12704-00 and analyzed with a AlphaEase FC, version 4.1.0, software (Alpha Innotech Corporation, CA).

2.4 - Testing of Hemolytic Activity and Bile Tolerance

Hemolysis was tested by the modified method of Eaton and Gasson (2001). Briefly, a single line of *Lactobacillus* culture (grown in MRS broth for 18h) was streaked onto MRS agar supplemented with 7% sheep blood and incubated for 48 h at 37°C under anaerobic conditions. Zones of clearing around colonies indicate hemolysin production. *Staphylococcus aureus* (ATCC 6538) was used as a positive control.

The bile tolerance of *Lactobacillus* strains was evaluated by inoculating overnight cells (2%) into wells (96-well microplate) containing 200 µl of MRS (control) and MRS supplemented with 0.25 and 0.5% Oxgall (wt/vol; Difco). The growth curves were automatically monitored by determining the changes in absorbance (A_{600}) as a function of time using a FLUOStar OPTIMA microtiter plate reader (BMG Labtech, Cary, NC). Each point represents the mean of two independent

cultures, each with triplicate wells containing individual cultures. Results were expressed as percent of growth in relation to the control.

2.5 - Antagonistic activity against pathogens

The antagonistic properties of the 30 *Lactobacillus gasseri* toward pathogens *in vitro* were analyzed by the spot method described by Harris et al (1989). Briefly, overnight *Lactobacillus gasseri* (grown in MRS broth) were individually spotted (7 µl) onto the surface of an MRS agar plate and incubated at 37ºC for 18-24 h under anaerobic conditions. The plates were overlaid with 10 mL of BHI (Brain Heart Infusion, Difco) soft agar (0.75% agar) seeded with 10 µl of stationary-phase indicator bacteria. After overnight incubation at 37ºC under aerobic condition, the plates were examined for zones of inhibition in the indicator cell lawn.

Salmonella typhimurium SL1344, *Listeria monocytogenes* ATCC19115, *Staphylococcus aureus* RN4220 and *Escherichia coli* ATCC11229 were used as indicators. All the pathogenic bacteria were propagated three times in BHI at 37ºC before the assay.

In order to verify the nature of the inhibitory agent (bacteriocin or organic acid) nine different proteases: proteinase K (P2308), bacillus protease (P5380), bovine protease (P4630), papain (P4762), pronase E type XIV (P5147), chymotrypsin (7762), trypsin (T8003), fungal protease (P2143) and pepsin (P7012) at a concentration of 1 mg/ml. All proteases were obtained from Sigma, USA. In order to test for organic acid effects, sodium bicarbonate (0.2%) and different concentrations of sodium hydroxide (0, 01N, 0.1 N, 1N, 2N and 10N) were spotted around colonies of *L. gasseri*. The plates were kept at room temperature for 1 h, overlaid with 10 mL of BHI soft agar (0.75% agar) containing 10^6 CFU/ml of indicator bacteria, incubated overnight 37ºC under aerobic conditions and examined for inhibition of indicator growth.

For the bacteriocin assay *Lactobacillus johnsonii* ATCC33200; *Lactobacillus delbrueckii ssp. bulgaricus* ATCC 4797 and *Lactobacillus acidophilus* (NCFM) were used as indicators. They were propagated three times in MRS broth at 37ºC before the antagonistic assay.

2.6 - Testing of Antibiotic Susceptibility

All 30 *Lactobacillus gasseri* isolates were screened for resistance against 14 clinically relevant antibiotics (gentamicin [10 μg], amikacin [30 μg], ciprofloxacin [5μg], merophenen [10 μg], penicillin [10 UI], ampicillin [10 μg], amoxycillin [100 μg], vancomycin [30 μg], sulphonamide [300 μg], oxacillin [1 μg], erythromycin [15 μg], cephtriaxone [30μg], cephalexin [30 μg] and cephalothin [30 μg]) by the agar disk diffusion method described by Bawer (1966). All the antibiotic discs were purchased from Sensifar (Cefar Diagnóstica, São Paulo, Brazil). Diameters of the inhibition zones were measured and the resistance to each antibiotic was graded according to supplier's specifications as resistant (R), intermediate (I) or sensitive (S). The assay was performed in three independent trials.

For sulphonamide, oxacillin, erythromycin, cephtriaxone, cephalexin and cephalothin the MIC (Mininmum inhibitory concentration) for each strain was evaluated using a slightly modified version of the microdilution broth method developed by Jones et al (1985). Briefly, overnight cells (grown in MRS broth) were centrifuged, washed (PBS pH 7.4), standardized ($A_{600nm} = 1$) and inoculated (2%) into each well (96-well microplate) containing MRS (control) and MRS plus antibiotic. Cells were checked for growth after 24 h at 37ºC.

2.7 - Plasmid analysis

Aliquots (1 ml) of log-phase *Lactobacillus gasseri* cultures (6 to 8h incubation) were centrifuged and cells were washed twice with PBS (pH 7.4). The final pellet was resuspended in 200μl of 25% sucrose solution containing 30 mg/ml of lyzozyme and 3μl of mutanolysin (10U/μl) and plasmid DNA extracted by the method described by O'Sullivan and Klaenhammer (1993). Plasmid DNA from strain UFVCC 1091 was extracted using the Qiagen Large-Construct Kit and sequenced in the Genome Sciences Laboratory at North Carolina State University (http://gsl.cals.ncsu.edu/). Four large contigs from the sequencing results were identified as possible plasmids based on Blast search results of contig ORFs. Those sequences were circularized by designing PCR primers reading out from the sequence ends and then sequencing the resulting PCR product from each putative potential plasmid (data not shown). The complete sequence from the plasmids was annotated by an extended version of GAMOLA[®] (Alterman and Klaenhammer, 2003) and

analyses were done with Artemis® software (version 11). In order to verify if all other 29 *L. gasseri* strains harbored the plasmids found in the UFVCC 1091 strain, PCR reactions were performed using specific primers for each plasmid (Table 1). PCR was carried out over 30 cycles (initial denaturation at 94 °C for 5 min, denaturation at 94°C for 30 sec, annealing at 46°C for 30 sec, and polymerization at 72°C for 1 min) with a final 10-min polymerization step at 72°C.

Table 1: Primers tested in the study.

Primer	Sequence
plas1_f	5`AAGGCGTTAATACCACTTTG 3`
plas1_r	5`GAATGCCATTGTCATATGTC 3`
plas2_f	5`TCACTACAAGCTTCTTCTAC 3`
plas2_r	5`CAGGAAGTATTTGGCTCATC
plas3_f	5`AACTCCTTGCACCTACATTG 3`
plas3_r	5`TGGAGGGATCGTTATGTC 3`
plas4_f	5`GTGTGCTATCACTAACTACC 3`
plas4_r	5`GTTCGATCGCCACCGTGTAG 3

2.8 - Tolerance to simulated gastric and small intestinal juices

Simulated gastric and small intestinal juices were prepared as described by Goh and Klaenhammer (2010). Briefly, cells were grown overnight from a 1% inoculum in MRS. Aliquots (1 ml) were centrifuged, and the cell were washed twice with sterile water. The cell suspension (0.2 ml) was mixed with 1 ml of freshly prepared simulated gastric juice (0.5% [wt/vol] NaCl solution containing 3 g/liter pepsin [Fisher Scientific, Pittsburg, PA], pH 2.0) or small intestinal juice (0.5% NaCl solution containing 1 g/liter pancreatin [Sigma] and 3 g/liter Oxgall [Difco], pH 8.0) and incubated at 37ºC. Viable cell count was determined by plating onto MRS at 30-min or 1-h intervals.

2.9 - Adherence assay

Epithelial adherence assays were performed as described previously by Goh et al (2009) using MRS grown stationary phase cells (16 h of growth).

Caco-2 and HT-29 – Aliquots (5 ml) of overnight cells were pelleted, washed (twice with PBS pH 7.4), resuspended in PBS and adjusted to an $A_{600nm} = 1$ (~1 x 10^8 CFU/ml) prior to adding into each well containing a Caco-2 or HT-29 monolayer. Plates were incubated for 1h at 37ºC and washed five times with PBS pH 7.4 (1 ml /well). Adhered cells were recovered by treating each well with 1 ml of 0.05% Triton X-100 solution for 15 min with agitation (Caco-2) or 0.25% trypsin at 37ºC for 10 min followed by 10 min at room temperature with shaking (HT-29). Cell suspensions were diluted and plated onto MRS agar to enumerate adhered cells.

Mucin – Mucin (Type III from porcine stomach; Sigma) was diluted in PBS (pH 7.4) at 10 mg/ml. The protein solutions were coated onto Nunc Maxisorp 96-well microplate wells (100 µl/well) by incubation at 4ºC overnight. After two washes with PBS, the wells were blocked with 2% bovine serum albumin (BSA) solution (Invitrogen) for 2h at 37ºC, followed by two additional washes with PBS to remove excess BSA. Washed (PBS buffer [pH 5.0]) and adjusted ($A_{600nm} = 1$ [1 x 10^8 CFU/ml]) overnight cells were added (100 µl) to each protein-coated well. After 1h incubation at 37ºC, the wells were washed five times with PBS pH 6.0 (200 µl/well) and cells were recovered by treating each well with 100 µl of 0.05% Triton x-100 solution for 15 min with agitation. Cell suspensions were diluted and plated onto MRS agar to enumerate adhered cells. All adherence experiments were performed with at least two independent cultures each with quadruplicate wells containing individual substrate layers. All data were submitted to analysis of variance (ANOVA). Tukey test compared media among treatments and differences were judged to be statistically significant when P value was <0.05.

3 - RESULTS

3.1 - Bacterial identification and Pulsed-Field Gel Electrophoresis (PFGE)

The thirty (30) isolates from NPNL agar were identified as *Lactobacillus gasseri* through sequencing of 16S rDNA amplicons generated using PCR primers: plb16 5`AGAGTTTGATCCTGGCTCAG 3` and mlb16 5` GGCTGCTGGCACGTAGTTAG 3`. Twenty nine (29) of the *L. gasseri* were identical by inspection of PFGE banding using restriction enzymes Sma I (Figure 1)

and Apa I (data not shown). The UFVCC 1112 (↓) strain was identical to the other 29, except for a single band polymorphism (white arrow, Figure 1).
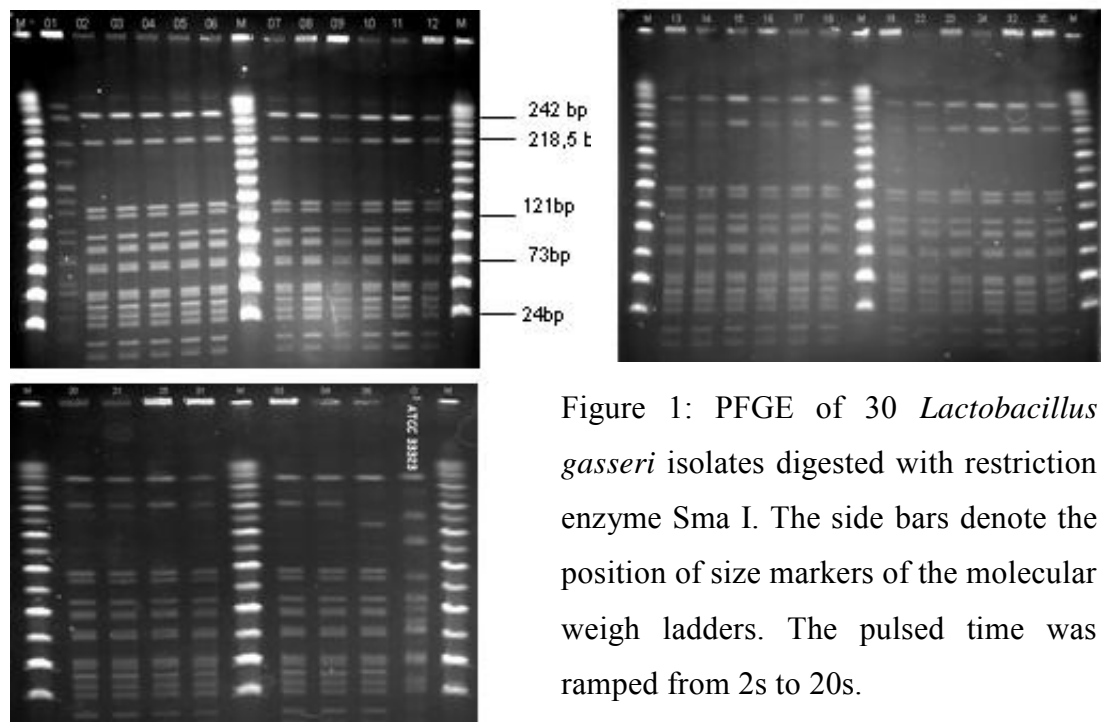
Figure 1: PFGE of 30 *Lactobacillus gasseri* isolates digested with restriction enzyme Sma I. The side bars denote the position of size markers of the molecular weigh ladders. The pulsed time was ramped from 2s to 20s.

3.2 - Testing of Hemolytic Activity and Bile Tolerance

None of the 30 tested lactobacilli caused lysis of erythrocytes of sheep blood, whereas complete lysis (β-hemolysis) was caused by the positive control *Staphylococcus aureus* ATCC 6538 (data not shown).

The majority of the *L. gasseri* isolates were tolerant to 0.25% oxgall (Figure 2). Isolate UFVCC 1096 was the least tolerant to bile (15% growth compared to the control without bile) while UFVCC 1098, the most tolerant (47% of control). In the presence of 0.5% oxgall, most of the strains did not grow very well. Interestingly, strain UFVCC 1091 showed the highest growth (66% of control) (Figure 2).
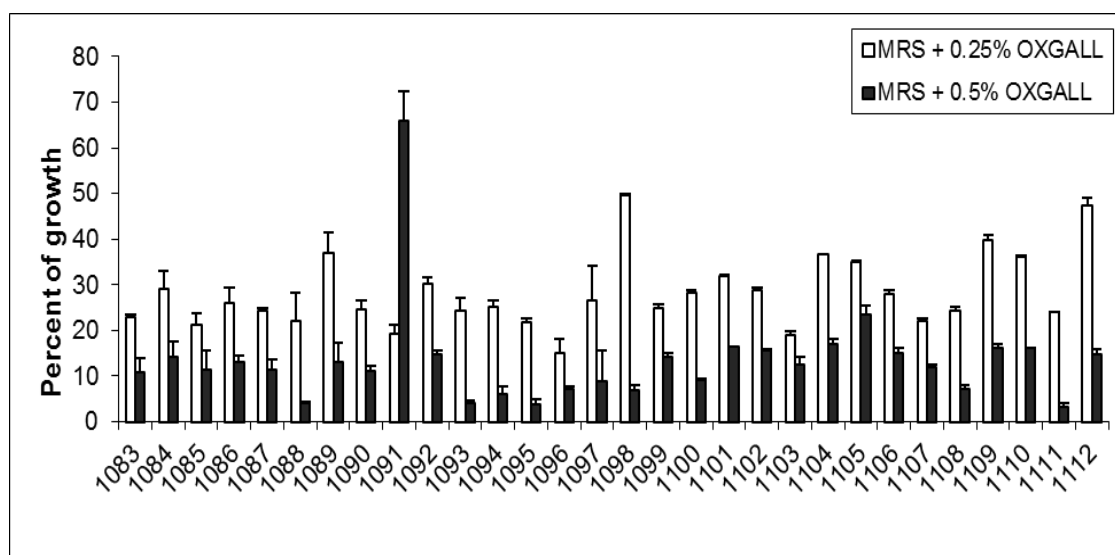
Figure 2: Percent of growth (A$_{600nm}$) after 24 h in the presence of oxgall compared to the control (MRS medium without bile). Each column represents the mean of two replicates. The bars represent the standard deviation.

3.3 - Antagonistic activity against selected pathogens

All isolates of *L. gasseri* exhibited antagonistic activity against *Escherichia coli* ATCC 11229 (Figure 3a), S*almonella typhimurium* SL 1314, *Listeria monocytogenes* ATCC19115 and *Staphylococcus aureus* RN 4220 *(data not shown).* The inhibition was attributed to the action of organic acids since the inhibitory effect was completely reversed by the addition of sodium hydroxide around the colonies on test plates.

None of the thirty *L. gasseri* strains exhibited bacteriocin activity against the indicator organisms *Lactobacillus johnsonii* ATCC33200, *Lactobacillus delbrueckii ssp bulgaricus* ATCC 4797, and *Lactobacillus acidophilus* NCFM. The proteases tested had no effect on the inhibition of *E. coli* ATCC11229 (Figure 3a) or on the other pathogens tested (not shown). Positive controls confirmed protease-sensitive bacteriocin production by *L. acidophilus* NCFM (Figure 3b).
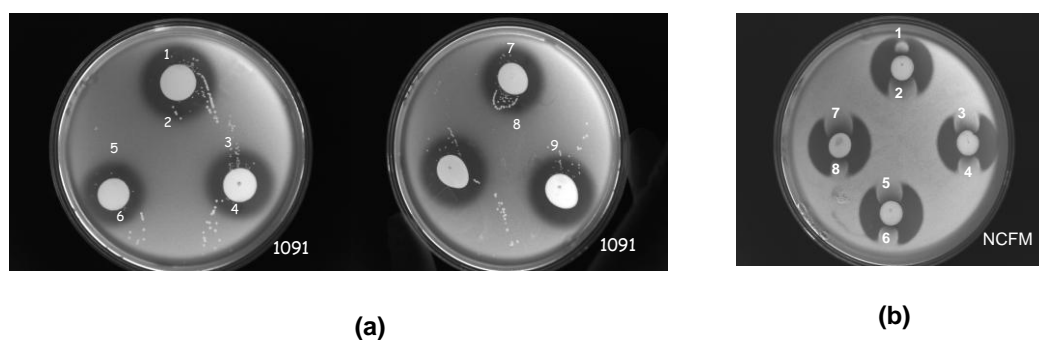
Figure 3: (a) Inhibition of *E. coli* by *Lactobacillus gasseri* (UFVCC1091). (b) Inhibition of *L. bulgaricus* by NCFM (used as a control in this assay). The numbers show the proteases (1mg/ml) dropped (5 µl) around the colony: 1 - Proteinase K; 2- *Bacillus* protease; 3- Bovine; 4- Papain; 5- Pronase E type XIV; 6- Chymotrypsin; 7- Trypsin; 8- Fungal; 9- Pepsin .

3.4 - Antibiotic Susceptibility

Using agar disc diffusion, all isolates were resistant to gentamicin (10 µg), amikacin (30 µg), and ciprofloxacyn (5µg) and sensitive to merophenen (10 µg), penicillin (10 UI), ampicillin (10 µg), amoxycillin (100 µg) and vancomycin (30 µg) (data not shown). The susceptibility to sulphonamide (300 µg), oxacillin (1 µg), erythromycin (15 µg) cephtriaxone (30µg), cephalexin (30 µg) and cephalothin (30 µg) was variable. For these antibiotics the MIC for each strain was evaluated (Table 2).

Table 2: MIC values of *L. gasseri* to human therapeutic antibiotics.

Strains (UFVCC)	Antibiotic				
	Ceftriaxone	Cephalotin	Cephalexin	Erythromycin	Oxacillin
1083 to 1090; 1092 to 1112	< 1.25	< 0.125	1	< 0.0625	1
1091	< 1.25	1	1	8	3.5

MIC – Minimum Inhibitory Concentration (mg/mL). The first column shows all the 30 *L. gasseri* (from 1083 to 1112) grouped according to MIC values found for each one.

All the strains were equally sensitive to ceftriaxone (MIC <1.25 mg/mL), and cephalexin (MIC <1 mg/mL). However, strain UFVCC 1091 showed higher MIC values for cephalotin, erythromycin and oxacillin (Table 2).

3.5 - Plasmid content and selection of strains for further studies

Plasmids were identified in all of the 30 *L. gasseri* isolates (data not shown). Three plasmids were detected by agarose gel electrophoresis in strain UFVCC 1091 (Figure 4a). However sequencing the plasmid DNA pool from UFVCC 1091 revealed 4 plasmids (pTRK1023 [43.105 kb]; pTRK1024 [9.751 kb]; pTRK1025 [28.174 kb] and pTRK26 [50.122 kb]). However only three of the plasmids were found in the other 29 strains. When PCR was performed on genomic DNA from each *L. gasseri* isolate using primers designed to amplify a unique fragment from each of the plasmids, only 3 of the plasmids were found (pTRK1023, pTRK1025, pTRK1026).

(a) (b)
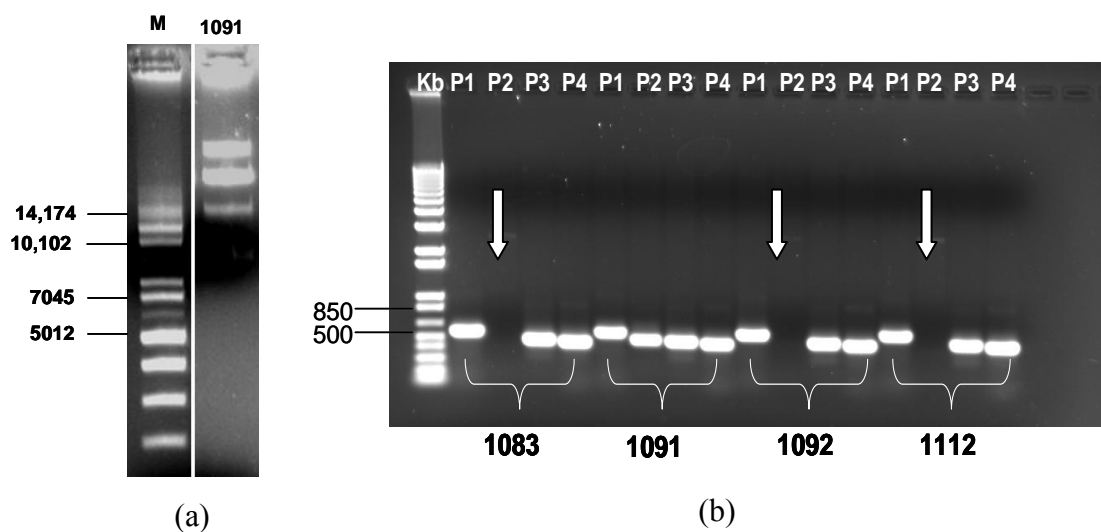
Figure 4: (a) Agarose gel electrophoresis plasmid profile of *Lactobacillus gasseri* UFVCC1091. The side bars denote supercoiled size markers. (b) PCR products using specific primer pairs (P1 [pTRK1023], P2 [pTRK1024], P3 [pTRK1025] and P4 [pTRK1026]) designed to amplify each of the four plasmids of UFVCC1091. 1Kb Plus DNA Ladder. The arrows show P2 (pTRK1024) missing in strains UFVCC1083, 1092 and 1112. The electrophoretic profile of strains UFVCC1083, 1091, 1092 and 1112 are shown. This result was similar for all 29 strains.

Based on the results from PFGE, antibiotic resistance and plasmid profiles, the 30 isolates were divided into three groups (Table 3) and one strain of each group (UFVCC1083, 1091 and 1112) was chosen for further characterization.

Table 3: *Lactobacillus gasseri* grouped according to their intrinsic characteristics.

Strains (UFVCC)	PFGE	Plasmids
1083 to 1090 1092 to 1111	*	3
1091	*	4
1112	**	3

* Identical strains based on PFGE technique using restriction enzymes SmaI and ApaI

** Closely related strains to group 1 but with a distinctive DNA fingerprint.

3.6 - Tolerance to simulated gastric and small intestinal juices

The tested strains (UFVCC1083, 1091 and 1112) showed high tolerance to small intestinal juice (Figure 5a) after 4 hours and to gastric juice (Figure 5b) after 90 minutes of exposure. Strain UFVCC1112 was more resistant to gastric juice (Figure 14 [b]).

(a)   (b)
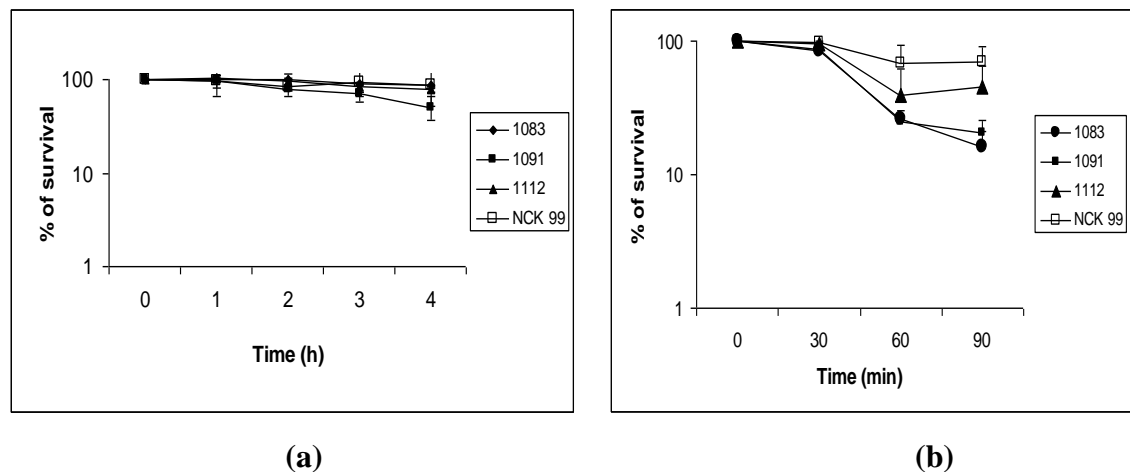
Figure 5: Survival of stationary phase cells of *Lactobacillus gasseri* in simulated small intestinal juice at pH 8.0 (Panel a) and gastric juice at pH 2.0 (Panel b). Percent survival represents viable cells (CFU/mL) remaining after exposure at the time points indicated versus pre-treatment (time 0). The data represent the means of two independent replicates.

3.7 - Adherence assay

L. gasseri UFVCC1083, 1091 and 1112 were evaluated for their capacity to bind immobilized mucin, Caco-2 cells and HT-29 (Figure 6). The binding capacity of these strains was comparable to that shown by the positive control strain *L. gasseri* NCK99, a strain with good adhesion properties (Kleeman and Klaenhammer, 1982). For mucin and Caco-2 cells we also compared the adherence of the cells cultured in static MRS broth or MRS agar under anaerobic conditions.

The three isolates grown in static MRS broth exhibited around 50% and 60% of the adhesion capacity of the control strain, respectively to mucin (Figure 6a) and Caco-2 (Figure 6b). There was no difference (P> 0.05) in the adherence level between the cells grown in static MRS broth and MRS agar under anaerobic conditions, although the percent adherence trended slightly higher for the cells grown on MRS agar.

The strains also showed a strong capacity to adhere to HT-29 cells (Figure 6c), in particular UFVCC1083 had 50% more adhesion ability than the control strain NCK 99.

(a)   (b)   (c)
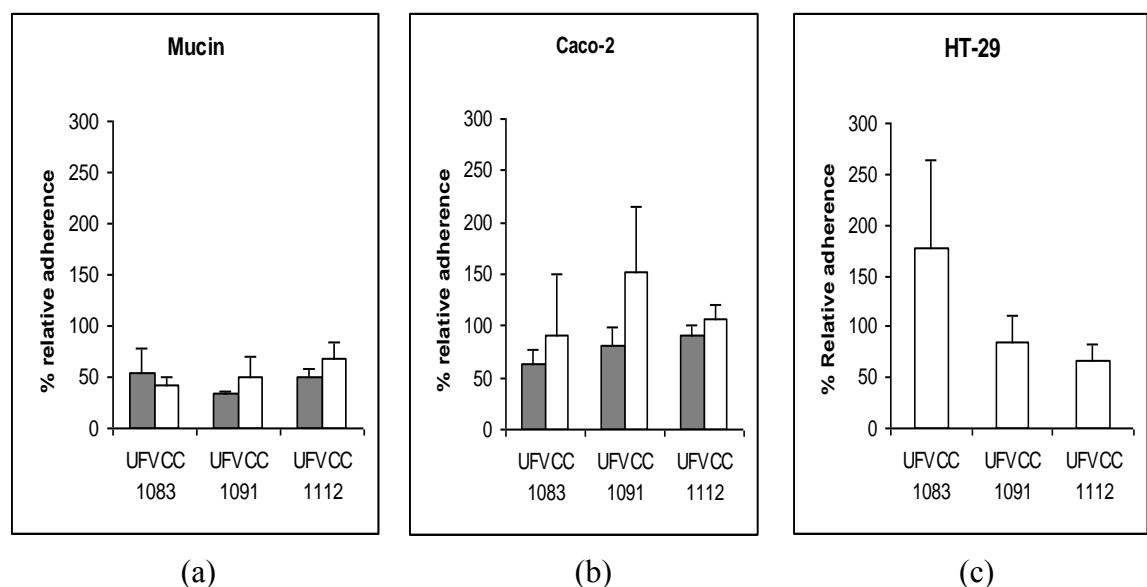
Figure 6: Adherence of *Lactobacillus gasseri* UFVCC1083, 1091 and 1112 to mucin (Panel a), Caco-2 cells (Panel b) and HT-29 (Panel c) *in vitro*. The bacteria grown in static MRS broth in ambient atmospheric (filled) and on MRS agar under anaerobic conditions (open). The data represent the means ± standard errors of the means for two independent replicates.

4 - DISCUSSION

This study was the first phase of a multi-phase study aimed to obtain and characterize *Lactobacillus* strains isolated from a healthy newborn infant for potential use as a probiotic in human milk banks in Brazil.

In order to claim that a bacterial strain is a potential probiotic, the FAO/WHO (2002), has established guidelines with safety and functional criteria. According to this document, all probiotic strains need to be properly identified at the genus and species level using current and internationally scientific practices, generally deoxyribonucleic acid (DNA)-based, and properly named according to current nomenclature. In this study, we screened and identified 30 intestinal *Lactobacillus gasseri* strains from a newborn infant stool sample. Among them, 29 were identical by PFGE using Sma I (Figure 1) and Apa I enzyme (data not shown). One strain (UFVCC1112) was differentiated from the others, by a single band polymorphism. This result is not surprising, since all the strains were isolated from the same child. Although the 29 *L. gasseri* were identical by PFGE, phenotypic assays were performed to verify how similar or different the strains were.

Lactobacillus gasseri are natural inhabitants of the human gastrointestinal tract (GIT) of neonates (Wall et al 2007) and adults (Reuter, 2001), and some reports also have shown this microorganism among oral biota (Smith et al 2001), vaginal (Martin et al 2008) and stomach (Ryan et al 2008) microbiota. Although they are autochthonous human intestinal members (Azcarate-Peril et al 2008) and some strains have a long history of safe use, new strains do not automatically command the GRAS status of traditional ones. Hence, new probiotic bacteria must be fully characterized and a safety assessment conducted that includes antibiotic resistance and hemolytic activity (FAO/WHO 2002).

The importance of assessing the antibiotic resistance profile pattern of new isolates is to limit the use of probiotic cultures harboring transferable antibiotic-resistance genes. In our study, the 30 *L. gasseri* were screened for resistance against 14 clinically relevant antibiotics by the agar disk diffusion method. All isolates were resistant to gentamicin (10 μg), amikacin (30 μg), ciprofloxacyn (5μg), and sensitive to merophenen (10 μg), penicillin (10 UI), ampicillin (10 μg), amoxycillin (100 μg) and vancomycin (30 μg). Aminoglycosides (amikacin and gentamicin) are amino sugars joined to a hexose nucleus in a glycosidic linkage. The drug binds to the 30S

ribosomal subunit at aminoacyl tRNA (aa-tRNA) acceptor site (A) on the 16S ribosomal RNA (rRNA), affecting protein synthesis by induction of codon misreading and inhibition of translocation (Mingeot-Leclercq et al 1999). Generally, lactobacilli have been shown to be resistant to aminoglycosides (Hummel et al 2007; Danielsen and Wind, 2003; Charteris et al 1998). This resistance has been considered to be intrinsic (Danilesen and Wind, 2003) and is due to the absence of cytochrome-mediated electron transport, which mediates drug uptake (Charteris et al, 2001). In addition, it was shown that when lactobacilli were grown in medium containing bile (a multifaceted stressor, which disrupts the cell membrane and causes damage to DNA and protein [Begley et al 2005]), they became more sensitive to aminoglycosides, suggesting that membrane impermeability plays an important role in this intrinsic aminoglycoside resistance (Elkins and Mullis, 2004). Therefore, there is apparently no risk that this resistance could be transferred to pathogenic/opportunistic bacteria in the gut.

Ciprofloxacin is a new 6-fluoro-7-piperozino-4 quinolone that acts by inhibiting the enzymes DNA gyrase and topoisomerase IV, thereby disrupting DNA replication (Levine et al 1998). It is a broad spectrum antibacterial drug against Gram-negative bacteria, and most Gram-positive bacteria are susceptible or moderately susceptible. However, Krõll et al (2010) found resistance to ciprofloxacin (MIC > 32) for all four *L. gasseri* isolated from stools of children 1-2 years old. Martin, et al (2008) analyzing antibiotic susceptibility (by agar dilution technique) of 45 vaginal lactobacilli strains (21 *L. crispatus*, 17 *L. jensenii*, 6 *L. gasseri* and 1 *L. plantarum*) detected resistance for ciprofloxacin for all strains tested. Similar results also were reported by Hummel et al (2007) and Danilesen and Wind (2003). Akin to the aminoglycosides, this resistance has been found to be intrinsic in lactobacilli (Charteris et al 2001, Klein et al 2000). The susceptibility to sulphonamide (300 μg), oxacillin (1 μg), erythromycin (15 μg) cephtriaxone (30μg), cephalexin (30 μg) and cephalothin (30 μg) was variable and strain dependent. For these antibiotics the MIC for each strain was evaluated by microdilution broth method. All the strains were sensitive at the same level to ceftriaxone (MIC <1.25 mg/ml) and cephalexin (MIC <1 mg/ml).

All the strains were equally susceptible to cephalotin, erythromycin and oxacillin, with exception for the strain UFVCC 1091, which showed higher MIC values for these 3 antibiotics (Table 2). Through plasmid sequencing and PCR

analysis it was verified that 29 strains of *L. gasseri* harbored 3 plasmids (pTRK1023, pTRK1025 and pTRK1026), whereas UFVCC 1091 harbored a fourth plasmid (Figure 4b). Plasmid pTRK1024 harbored an ORF with similarity to an *lmrB* gene encoding an ATP-binding cassette-type multidrug resistance transporter protein. This gene had been reported in *Bacillus subtilis* (Kumano et al, 1997) and in a multi copy plasmid harbored by *Corynebacterium glutamicum* (Kim et al, 2001). In both cases, the lmrB gene was responsible for the lincomycin and clindamycin resistance. Those antibiotics were not among the antibiotics tested in this current research, but we observed that the strain UFVCC1091, harboring the plasmid encoding lmrB gene, showed higher MIC values for erythromycin, oxacillin and cephalotin (Table 2). Cloning experiments did not correlate the resistance to those antibiotics to lmrB (data not shown). Kim et al (2001) also did not detect a difference in sensitivity to erythromycin in the wild type and lmrB mutant strains. Therefore, we suggest that the resistance to erythromycin, oxacillin and cephalothin observed with strain UFVCC 1091 may be chromosomally encoded and for safety reasons this strain should not be considered as a candidate probiotic. None of the other 3 plasmids (pTRK1023, pTRK1025 and pTRK1026) were found to encode transmissible antibiotic resistance genes.

Hemolysis is a common virulence factor among pathogens, facilitating iron availability to the microorganism and causing anaemia and edema in the host (Vesterlund et al 2007). Iron is a micronutrient that acts as a cofactor for several enzymes, thus required for growth of these microorganisms (Husain, 2008). Lactobacilli are capable of growing without iron and this is considered an ecological advantage in the natural environment, where they compete with pathogenic bacteria (Elli et al 2000). However, some studies (Maragkoudaki et al 2006; Baumgartner et al 1998,) have shown lactobacilli strains with hemolytic activity. According to Elli et al (2000), this hemolytic activity may be involved in the iron requirement of bacteria for pyrimidine and purine metabolism in an environment with limited specific nucleotide sources. Hemolytic activity was not detected among the 30 lactobacilli tested. These results are in agreement with those found by Krõll et al (2010) who also detected no hemolytic activity in 93 lactobacilli strains isolated from Estonian and Swedish children (1-2 years old). Similarly, Vesterlund et al (2007) did not detect hemolytic activity in lactobacilli isolated from 52 clinical samples, 44 healthy adult feces samples, or 15 dairy products. These observations corroborate the results found

by Maragkoudakis et al (2006), who detected no hemolytic activity in 29 lactobacilli isolated from dairy products.

Bile salts are one of the major components of bile and consist of a cholesterol-derived ring structure that is amide linked to an amino acid, either glycine or taurine. The amphipathic nature of conjugated bile salts gives them the ability to emulsify the lipid membranes of bacterial cells (Begley et al 2005), inhibiting many gastrointestinal aerobic and anaerobic bacteria (Jonsson et al 1995). Some microbes in the GIT have the ability to deconjugate bile salts, which can damage bacterial membranes leading to cell death (Kurdi et al 2006). Therefore, ability to survive the action of bile salts is an important characteristic of probiotic bacteria. Goldin and Gorbach (1992) suggest that to be effective, probiotic microorganisms should be able to grow in the presence of 0.15 - 0.3% of oxgall. In our study, all *L. gasseri* isolates withstood 0.25% oxgall (Figure 2). Growth of the majority of *L. gasseri* isolates decreased at 0.5% oxgall, except for strain UFVCC1091 (Figure 2). It has been shown that intestinal lactobacilli are usually resistant to bile salts (Krõll et al 2010; Cukrowska et al 2009; Charteris et al 1998), and the level of this resistance varies largely among lactobacilli strains (Delgado et al 2007; Du-Toit et al 1998; Chateau et al 1994; Gilliland, 1984). According to Du-Toit et al (1998) varying levels of bile resistance among members of the same species of enteric lactobacilli may be due to the presence of bile salt hydrolase (BSH), but this is not the case for *L. acidophilus* (McAuliffe et al 2005). UFVCC 1091 grew better in 0.5% of oxgall than in 0.25%. This behavior may be correlated with the presence of a unique plasmid (9.75 kb) with the putative lmrB gene encoding a ATP-binding cassete-type multidrug resistance transporter proteins. Pfeiler and Klaenhammer (2009) showed that bile at the concentration of 0.5% induced the expression of ATPase subunits of an ABC transporter (LBA 1679-1680). These genes were annotated as multidrug resistance (MDR) transporter, a class of transporter that can act as a defense mechanism against inhibitory compounds by extruding a wide variety of structurally dissimilar substrates from the cytoplasm, including antibiotics, bile salts and peptides.

Lactobacilli produce metabolites such as organic acids, fatty acids, hydrogen peroxide and bacteriocins, which can inhibit the growth of pathogenic bacteria (Servin, 2004). In our study, 30 *L. gasseri* were assayed for antimicrobial activity against four pathogenic bacteria. All of them exhibited antagonistic activity against *Escherichia coli* ATCC11229 (Figure 3a), S*almonella typhimurium* SL1344, *Listeria*

monocytogenes ATCC19115 and *Staphylococcus aureus* RN4220 (not shown). The inhibition was the result of the action of organic acids, since the inhibitory substance was completely reversed by the addition of sodium hydroxide. In this study, none of the thirty *L. gasseri* strains exhibited bacteriocin activity against the indicator organisms used (*Lactobacillus johnsonii*, *Lactobacillus delbruechii ssp bulgaricus*, *Lactobacillus acidophilus*) and to four pathogens used in the study. These results are in agreement with Martin et al (2008), Maragkoudakis et al (2006) and Martin et al (2005) that also did not detect bacteriocin production among commensal lactobacilli isolates.

At this stage in this study, all the 30 L. *gasseri* strains were divided into three groups (Table 3) and one strain from each group (UFVCC1083, 1091 and 1112) were chosen to evaluate their ability to adhere to mucin and human intestinal epithelial cells (Caco-2 and HT-29), and their tolerance to digestive stresses. In this work, strains UFVCC 1083, 1091 and 1112 were highly tolerant to artificial small intestinal juices (Figure 5a) after 4 hours of exposure and matched the tolerance level of the control *L. gasseri* NCK99. These results are in agreement with those obtained by other researchers (Fernandez et al 2003; Jacobsen et al 1999; Charteris et al 1998; Du Toit et al 1998) who also found that most lactobacilli were resistant to small intestinal juices. Strains UFVCC1083, 1091 and 1112 were also tolerant to gastric juice at pH 2 (Figure 5b) showing less than a 1 log of reduction after 90 minutes of exposure. Dunne et al (2001) also found *Lactobacillus* strains resistant to human gastric juice. These *in vitro* results suggest that these human isolates could successfully transit the human stomach and reach the intestinal environment.

Another important property for potential probiotic strains is their ability to adhere to the intestinal mucosa, because adherence may influence the residence time of the bacteria in the intestinal tract (Servin and Coconnier, 2003). The capacity of some probiotic strains to inhibit pathogenic colonization and/or invasion and to modulate the immune response has also been related to their ability to adhere to intestinal mucus and/or epithelial cells (Servin and Coconnier 2003). *In vitro* cellular models involving mucin and human intestinal epithelial cell lines HT-29 and Caco-2 (derived from colon carcinomas) have been used to assess the adhesive properties of probiotic strains. These methods mimic the morphological and physiological characteristic of human enterocytes (Blum et al 1999) in the small intestine by forming homogeneous cell monolayers (Lenaerts et al 2007). In this study, *L. gasseri*

UFVCC1083, 1091 and 1112 demonstrated the ability to adhere to mucin, Caco-2 and HT-29 cells. There was no significant difference (P>0.05) in the adherence level between cells that were grown in static MRS broth and those grown in MRS agar under anaerobic conditions, although adherence was slightly elevated for agar cultures for the latter treatment. Spencer and Chesson (1994) found significantly better attachment for *L. fermentum, L. brevis, and L. salivarus* to porcine enterocytes when the cells were grown on agar. Significantly higher adherence levels have been also found for eight *L. gasseri* and one *L. johnsonii* to laminin (Horie et al 2005) when grown on solid medium.

5 - CONCLUSION

This study has identified and characterized three new *L. gasseri* isolates that were obtained from the feces of a breast feeding infant. Genomic characterization of the *L. gasseri* isolates indicated that they are largely identical except for a single genome polymorphism for UFVCC 1112 by PFGE and the presence of a unique plasmid in isolate UFVCC 1091 (NCK 2141). Since UFVCC 1091 was resistant to erythromycin, oxacillin and cephalothin this strain is not indicated to be used as a probiotic.

After broad phenotypic analysis, the non-antibiotic resistant strains UFVCC 1083 (NCK 2140) and UFVCC 1112 (NCK 2142) were found *in vitro* to possess some desirable probiotic properties. These bacteria will be further evaluated in vivo to elucidate their potential health benefits and their application as a probiotic in the pediatric area, such as in the human milk banks in Brazil.

6 - REFERENCES

Altermann, E., and T. R. Klaenhammer. (2003). GAMOLA: a new local solution for sequence annotation and analyzing draft and finished prokaryotic genomes. **OMICS** 7:161–169.

Ascarate-Peril, M.A., Altermann, E., Goh, Y., Tallon. R., Sanosky-Dawes, R.B., Pfeiler, E.A., O`Flaherty, S., Buck, L., Dobson, A., Duong, T., Miller, M.J., Barrangou, R., Klaenhammer, T.R (2008). Analysis of the Genome Sequence of *Lactobacillus gasseri* ATCC 33323 reveals the molecular Basis of an Autochthonous Intestinal Organism. **Applied and Environmental Microbiology**. V74, No 15, 4610-4625.

Baumgartner, A., Kueffer, M., Simmen, A. And Grand, M. (1998). Relatedness of *Lactobacillus rhamnosus* strains isolated from clinical specimens and such from food-stuffs, humans and technology. **Lebensmittel-Wissenschaft und-Technologie**. 31, 489–494.

Bawer, A.W., Kirly. W. M. M, Sherris, J. C., Turk, M. (1966). Antibiotic susceptibility testing by standardized single disk method. **American Journal of Clinical Pathology**. 45, 493-493.

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

Blum, S., Reniero, R., Schiffrin, E.J., Crittenden, R., Mattila-Sandholm, T., Ouwehand, A.C., Salminen, S., Von Wright, A., Saarela, M., Saxelin. M., Collin, K.., Morelli, L. (1999). Adhesion studies for probiotic: need for validation and refinement. **Trends in Food Science and Technology**. 10 (12), 405-410.

Bom dia Brasil: https://www.medplan.com.br/materias/2/7059.html. Accessed July, 2008.

Borba, L. M., Castro, L.C.V., Fanceschine, S.C.C., Ferreira, C.L.L.F. (2003). Composição do Leite Humano e Microbiota Predominantemente Bífida de Lactente em Aleitamento Materno Exclusivo. **Nutrire.** 25 (1), 135-154.

Charteris, W.p., Kelley, P.M., Morelli, L., Collins, J.K (2001). Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. **Journal of Food Protection**. 64, 2007-2014.

Charteris, W.P., P.M. Kelyy, L. Morelli, And J. K. Collins. (1998). Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. **Journal of Applied Microbiology**. 84, 759-68

Chateau, N., Deschamps, A.M., Hadh- Sassi, A. (1994). Heterogeneity of bile salts resistance in the Lactobacillus isolates from a probiotic consortium. **Letters Applied Microbiology.** 18, 42-44.

Collado, M.C., Jalonen, L., Meriluoto, J., Salminen, S. (2006). Protection mechanism of probiotic combination against human pathogens: *in vitro* adhesion to human intestinal mucus. **Asia Pacific Journal of Clinical Nutrition**. 15(4), 570-575

Commane, D., Hughes, R., Shortt, C., Rowland, I. 2005. The potential mechanisms involved in the anti-carcinogenic action of probiotics. **Mutation Research**. 591, 276-289.

Cukrowska, B., Motyl, I., Kozakova, H., Schwarzer, R.K., Gorecki, R.K., Klewicka, E. (2009). Probiotic *Lactobacillus* Strains: *in vivo* and *in vitro* Studies. **Folia Microbiology**. 54, 533-537.

Danielsen, M., Wind, A.A (2003) Susceptibility of *Lactobacillus ssp.* to antimicrobial agents. **International Journal of Food Microbiology**. 82, 1-11.

Delgado, S., O`Sullivan, E., Fitzgerald, G., Mayo, B. (2007). Subtractive Screening for Probiotic Properties of Lactobacillus Species from the Human Gastrointestinal Tract in the Search for New Probiotics. **Food Microbiology and Safety**. 72, No 8. M310-M315.

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

Du-Toit, M., Franz, C., Schillinger, U., Warles, B., Holzappfel, W. (1998). Characterization and selection of probiotic lactobacilli for a preliminary minipig-feeding trail and their effect on serum cholesterol level and faeces moisture contents. **International Journal of Food Microbiology**. 40, 93-104.

Eaton, T.J., Gasson, M.J. (2001). Molecular screening of Enterococcus virulence determinats and potential for genetic exchange between food and medical isolates. **Applied Environmental Biology**. 67, 1628-1635.

Elkins, C. A., Mullis, L. M., (2004). Bile-mediated sensitivity in *Lactobacillus* species likely results from increased membrane permeability attributable to cholic acid. **Applied Environmental Microbiology**. 70, 7200-7209.

Elli, M., Zink, R., Rytz, A., Reniero, R., Morelli, L. (2000). Iron requirement of *Lactobacillus* spp. in completely chemically defined growth media. **Journal of Applied Microbiology**. 88, 695-703.

Fernandez, M.F., Boris, S., Barbés, C. (2003). Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. **Journal Applied Microbiology**. 94, 449-455.

FIOCRUZ. Fundação Oswaldo Cruz. RedeBLH. (2010). www.fiocruz.br//redeBLH/cgi/cgilua.exe/sys/start/htm?tpl=home&id=ce.

Food and Agriculture Organization of the United Nations And World Health Organization. (2002). Guidelines For The Evaluation Of Probiotics In Food And Agriculture Organization For The United Nations And World Health Organization W Orking Group Report, On Line.

Forsythe P, Bienenstock, J. (2010). Immunomodulation by commensal and probiotic bacteria. **Immunological Investigation**. 39(4-5), 429-48.

Gilliland, S.E., Staley, T.E. And Bush, L.J. (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. **Journal of Dairy Science**. 67, 3045–3051.

Giugliani, E.R.J. (2002). Rede Nacional de Bancos de Leite Humano do Brasil: Tecnologia para Exportar. **Jornal de Pediatria**. 78 (3), 183-184.

Goh, Y.J, Azcárate-Peril, M.A., O'Flaherty, S., Durmaz, E., Valence, F., Jardin, J., Lortal, S., Klaenhammer, T.R. (2009). Development and application of a upp-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. **Applied Environmental Microbiology**. 75(10), 3093-105.

Goh, Y.J, Klaenhammer, T.R. (2010). Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM . **Applied Environmental Microbiology**.76(15), 5005-12.

Goldin, B.R., Gorbach, S.L. (1992) Probiotics for humans. In Probiotics in the Scientific Basis, ed. Fuller, R., pp. 355-376. London: Chapman & Hall. ISBN 0-412-40850-3.

González-Martínez, B.E., Treviño, M.G., Jiménez-Salas, Z. (2003). Bacteriocinas de probióticos. **Revista de la Facultad de Salud Pública y Nutrición**. 4 (2).

Griffin, I.J., Davila, P.M., Abrams, S.A. (2002). Non-digestible oligosaccharides and calcium absorption in girls with adequates calcium intakes. **British Journal of Nutrition**. 87, 187-191.

Harris, L.J, Daescheyl MA, Stiles, M.E, Klaenhammer, T.R (1999). Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. **Journal of Food Protection.** 52, 384–887.

Harsharnjit, S.G. (2003). Probiotics to enhance anti-infective defences in the gastrointestinal tract. **Best Practive & Research Clinical Gastroenterology.** 17 (5), 755-773.

Horie M, Murakami T, Sato T, Tarusawa Y, Nakamura S, Toba T. (2005). Anaerobic induction of adherence to laminin in *Lactobacillus gasseri* strains by contact with solid surface. **Current Microbiology**. 51(4), 275-82.

Hummel, A.S., Hertel, C., Holzapfel, W.H., Franks, C.M.A.P. (2007). Antibiotic Resistance of Starter and Probiotic Strains of Lactic Acid bcateria. **Applied and Environmental Microbiology**. 73 (3), 730-739.

Husain, S. (2008). Effect of ferric iron on siderophore production and pyrene degradation by *Pseudomonas fluorescens* 29L. **Current Microbiology**. 57, 331–334.

Isolauri, E. (2001). Probiotics: effects on immunity. **American Journal of Clinical Nutrition**. 73, 444-450.

Jacobsen, C. N., V. Rosenfeldt-Nielsen, A. E. Hayford, P. L. Moller, et al (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus spp*. by *in vitro* techniques and evaluation of the colonization ability of fiveselected strains in humans. **Applied Environmental Microbiology**. 65, 4949–4956.

Jones, R.N., Ballow, C.H., Biedenbach, D.J., Deinhart, J.A., Schentag, J.J. (1998). Antimicrobial activity of quinupristin-dalfopristin (RP 59500, Synercid®) tested against over 28,000 recent clinical isolates from 200 medical centers in the United States and Canada. **Diagnostic Microbiology and Infectious Disease**. 30, 437–51.

Jonsson, G., Midvedt., A.C., Norma, A., Midvedt, T. (1995). Intestinal microbial bile acid transformation in healthy children. **Journal of Pediatric Gastroenterology and Nutrition**. 20, 394-402.

Kalliomaki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., Isolauri, E. (2001). Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. **Lancet**. 357, 1076-1079.

Kelly,D., King, T., Aminov, R. (2007). Importance of microbial colonization of the gut in early life to the development of immunity. **Mutation Research**. 622, 58-69.

Kim, H., Kim, Y., Lee, M., Lee, H.(2001). Gene lmrB of *Corynebacterium glutamicum* Confers Efflux-mediated Resistance to Lincomycin. **Molecules and Cells**. 12 (1), 112-116

Kleeman, E.G., Klaenhammer, T.R. (1982). Adherence of *Lactobacillus* species to human fetal intestinal cells. **Journal of Dairy Science**. 65(11), 2063-2069.

Klein, G., Hallmann, C., Casas, I.A., Abad, J., Louwers, J., Reuter, G. (2000). Exclusion of van A, van B and van C type glycopeptideo resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as a probiotic by polymerase chain reaction and hybridization methods. **Journal of Applied Microbiology**. 89, 815-824.

Kroll, P., Mandar, R., Smidt, I., Hutt, P., Truusalu, K., Mikelsaar, R., Shchepetova, J., Krogh-Andersen, K., Marcotte, H., Hammarstrom, L., Milkelsaar, M. (2010). Screening and Evaluation of Human Intestinal *Lactobacilli* for the Development of Novel Gastrointestinal Probiotics. **Current Microbiology**. 61 (6), 560-566.

Kullen, M.J., Sanozky-Dawes, R.B., Crowell, D.C., Klaenhammer, T.R. (2000). Use of the DNA sequence of variable region of the 16s rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. **Journal Applied Microbiology**. 89(3), 511-516.

Kumano, M., Tamakoshi, A., and Yamane, K. (1997). A 32 kb nucleotide sequence from the region of the lincomycin-resistance gene (22°–25°) of the *Bacillus subtilis* chromosome and identification of the site of the lin-2 mutation. **Microbiology**. 143, 2775–2782.

Kurdi, P., K. Kawanishi, K. Mizutani, and A. Yokota. (2006). Mechanism of growth inhibition by free bile acids in Lactobacilli and Bifidobacteria. **Journal of Bacteriology**. 188, 1979-1986.

Lenaerts, K., Bouwman, F.G., Lames, W.H., Renes, J., Mariman, E.C. (2007). Comparative proteomics analysis of cell lines and scraping of the human intestinal ephitelium. **BMC Genomics**. 8, 1-14.

Levine C, Hiasa H, Marians KJ. (1998). DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities. Biochimica et Biophysica Acta (BBA). 1400, 29-43.

Lin, S. Y., Chen, C. T., (2000). Reduction of cholesterol by *Lactobacillus acidophilus* in culture broth. **Journal Food Drug Anal**. 8, 97-102.

Maragkoudakis, P.A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., Tsakalidou, E. (2006). Probiotic potential of *Lactobacillus* strains isolated from dairy products. **Internation Dairy Journal**. 16, 189-199.

Martin, R., Olivares, M., Martin, M.L., Fernandez, L., Xaus, J., Rodriguez, J.M. (2005). Probiotic Potential of 3 Lactobacilli Strains Isolated From Breast Milk. **Journal of Human Lactation**. 21(1), 8-17.

Martin, R., Soberon, N., Vaneechoutte, M., Florez, A.B., Vazquez, F., Suarez, J.E. (2008). Characterization of indigenous vaginal lactobacilli from health women as probiotic candidates. **International Microbiology**. 11, 261-266.

McAuliffe, O., Cano, R.J., Klaenhammer, T.R. (2005). Genetic analysis of two bile salt hydrolase activities in *Lactobacillus acidophilus* NCFM. **Applied Environmental Microbiology**. 71(8), 4925-4929.

Mingeot-Leclercq, M-P, Glupczynski, Y, Tulkens, PM. (1999). Aminoglycosides: Activity and resistance. **Antimicrobial Agents and Chemotherapy**. 43 (4), 727-737.

O'Sullivan, D.J. Klaenhammer, T.R. (1993). Rapid mini-prep isolation of high quality DNA from *Lactococcus* and *Lactobacillus* species. **Applied Environmental Microbiology**. 59, 2730-2733

Pfeiler, E.A., Klaenhammer, T.R. (2009). Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. **Applied Environmental Microbiology**. 75(18), 6013-6.

Reuter, G. (2001). The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. **Current Issues Intestinal Microbiology**. 2 (2), 43–53.

Round JL, Mazmanian SK. (2009). The gut microbiota shapes intestinal immune responses during health and disease. **National Review of Immunology**. 9, 313-323.

Ryan, K.A., Jayaraman, T., Daly, P., Canchaya, C., Curran, S., Fang, F., Quigley, E.M., O`Toole, P.W (2008). Isolation of lactobacilli with probiotic properties from the human stomach. **Letters in Applied Microbiology**. 47, 269-274.

Schandler, R.J., Hurst, N.M., Lau, C. (1999). The use of human milk and breast-feeding in premature infants. **Clinics in Perinatology**. 26, 357-379.

Schrezenmeier, J., De Vrese, M. (2001). Probiotics, prebiotics and Synbiotics – approaching a definition. **American Journal of Clinical Nutrition**. 73 (2), 361S-364S.

Servin, A. L. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. **Microbiology Reviews**. 28, 405-440.

Servin, A.L., Coconnier, M.H (2003). Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. **Best Practice and Research: Clinical Gastroenterology.** 17, 741-754.

Smith, S. I., A. J. Aweh, A. O. Coker, K. O. Savage, et al 2001. *Lactobacilli* in human dental caries and saliva. **Microbios**. 105, 77–85.

Spenser, R.J., Chesson, A., (1994). The effect of *Lactobacillus spp.* on the attachment of enterotoxigenic *Escherichia coli* to isolated porcine enterocytes. **Journal of Applied Bacteriology**. 77, 215-220.

Tanskanen, E. I., D. L. Tulloch, A. J. Hillier, and B. E. Davidson. (1990). Pulsed-field gel electrophoresis of SmaI digests of lactococcal genomic DNA, a novel method of strain identification. **Applied Environmental Microbiology**. 56, 3105–3111.

Tuohy, K.M., Probert, H.M., Smejkal, C.W., Gibson, G.R. (2003). Using probiotic and prebiotics to improve gut health. **Drug Discovery Today**. 8, 692-700.

Vesterlund, S., Vankerckhoven, V., Saxelin, M., Goossens, H., Salminen, S., Ouwehand, A. C. (2007). Safety assessment of *Lactobacillus* strains: Presence of putative risk factprs in faecal, blood and probiotic isolates. **International Journal of Food Microbiology**. 116, 325-331.

Wall, R., G. Fitzgerald, S. Hussey, T. Ryan, et al (2007). Genomic diversity of cultivable *Lactobacillus* populations residing in the neonatal and adult gastrointestinal tract. **FEMS Microbiology Ecology**. 59:127–137.

Young, R.J., Huffman, S. (2003). Probiotic Use in Children . **Journal of Pediatrics and Health Care**. 17, 277-283.

CHAPTER II

SEQUENCE ANALYSIS OF FOUR PLASMIDS OF *Lactobacillus gasseri* NCK2141 ISOLATED FROM A NEWBORN INFANT

ABSTRACT

Lactobacillus gasseri NCK2141 is a human intestinal probiotic candidate strain, and was found to contain 4 plasmids. The complete nucleotide sequence of pTRK1023 (43,105 bp), 1024 (9,751 bp), 1025 (28,174 bp) and 1026 (50,122 bp) revealed they are circular and contain 36, 7, 20 and 46 open reading frames, respectively. All four plasmids are predicted to replicate by the theta mechanism and have G+C contents consistent with those found in other lactobacilli strains (37-40%), with the exception of pTRK1025 (44.57%). The plasmids appear to encode functional properties, such as collagen binding (pTRK1026), lantibiotic biosynthesis (pTRK1023) and carbohydrate transport (pTRK1023 and pTRK1026) that may provide competitive advantages to the plasmid-carrying cells. However, bacteriocin activity was not observed in our previous study. Cell maintenance mechanisms (partitioning and TA system) were also observed, thus explaining the failure to cure these plasmids. Plasmid pTRK1024 harbored a putative *lmrB* gene, an ATP-binding cassette-type multidrug resistance transporter protein, which has been associated with clindamycin and lincomycin resistance. Cloning experiments failed to correlate the erythromycin, cephalothin and oxacillin resistance of NCK2141 to LmrB. However, for safety reasons, NCK2141 should not be used as a probiotic in the pediatric area. The *lmrB* gene has not been previously described in *Lactobacillus gasseri* plasmids, possibly reflecting horizontal gene transfer in the gut.

Key words: plasmid, probiotic, *lmrB*, *Lactobacillus gasseri*

RESUMO

Lactobacillus gasseri NCK2141 é uma estirpe potencialmente probiótica isolada do trato intestinal de um recém nascido saudável e carreia 4 plasmídeos circulares: pTRK1023 (43,105 bp), 1024 (9,751 bp), 1025 (28,174 bp) e 1026 (50,122 bp) que contêm respectivamente, 36, 7, 20 e 46 open reading frames. Replicação do tipo theta foi proposta para os quatro plasmideos. Os conteúdos de G+C foram concistentes com aqueles encontrados em outras estirpes de *Lactobacillus* (37 - 40%), exceto o pTRK1025 que foi de 44,57%. Algumas propriedades funcionais como, proteínas de ligação ao colágeno (pTRK1026), transporte de carboidratos (pTRK1023 e pTRK1026) e biossíntese de lantibióticos (pTRK1023) foram identificados nos plasmídeos, as quais podem promover vantagens competitivas para as células hospedeiras. Entretanto, a produção de bacteriocina não foi observada em nossos estudos anteriores. Mecanismo de manutenção celular como, sistema de separação e toxina-antitoxina também foi observado, explicando a dificuldade de cura dos plasmídeos. O gene *lmrB* identificado no pTRK1024 codifica para uma bomba de efluxo a múltiplas drogas. Entretanto, o experimento de clonagem não confirmou a relação desse gene com a maior resistencia à eritromicina, cefalotina e oxacilina apresentada pelo isolado NCK2141 carreador desse plasmídeo. Por questões de segurança, essa estirpe não será utilizada como probiótico. Esse foi o primeiro trabalho relatando a presença do gene *lmrB* em plasmideos isolados de *Lactobacillus gasseri*, sugerindo transferência horizontal de genes no trato intestinal humano.

Palavras-Chave: plasmídeos, probiótico, lmrB, *Lactobacillus gasseri*

1 - INTRODUCTION

Lactic acid bacteria (LAB) are gram positive, acid tolerant, non-sporulating rods or coccobacilli, without flagella, and with low G+C content (Hugenholtz, 1998). Among the genera comprising the LAB, *Lactobacillus* is the largest genus, with 197 species (including subspecies) described to date (February, 2011; http://www.ncbi.nlm.nih.gov/taxonomy). They are nutritionally fastidious facultative anaerobes, and can be divided into three groups based on fermentation characteristics: obligately homofermentative, facultatively heterofermentative and obligately heterofermentative (Gomes and Malcata, 1999; Hammes and Vogel, 1995). Taxonomically, the *Lactobacillus* genus belongs to the phylum Firmicutes, class *Bacillus*, order *Lactobacillales* and family *Lactobacillaceae* (Garrity et al 2004).

Lactobacilli are amply distributed in the environment, especially in the intestinal tract (Wall et al 2007; Reuter 2001), mouth (Smith et al 2001), vagina (Martin et al 2008) and stomach (Ryan et al 2008) of humans and animals, as well as in plants, sewage and fermented foods (Bernardeau et al 2006; Axelsson, 1989). They are generally regarded as safe (GRAS) due to their long history of safe use in fermented foods and their presence in the normal intestinal microbiota of humans (Tuohy et al, 2003), where *Lactobacillus gasseri* are one of the most commonly found inhabitants (Wall et al 2007).

Lactobacillus gasseri are obligate saccharoclastic, homofermentative organisms, with optimum growth at 35 to 38ºC. They are probiotic bacteria ("live microorganisms that, when administered in adequate amounts, confer a health benefit on the host", FAO/WHO, 2002) with health promoting effects, such as immunomodulation (Olivares et al 2006b), suppression of *Helicobacter pylori* induced interleukin-8 production (Tamura et al 2006), improvement of immune parameters involved in the allergic response in children and in intestinal health (water content of feces, stool volume and frequency) in adults (Olivares et al 2006a). These benefits have led to the search for new strains to be added to food products as well as infant formulas. In 2002, the World Health Organization [WHO] and Food and Agriculture Organization [FAO], established a guide for evaluation of probiotics in foods. This document outlines safety parameters, such as antibiotic resistance patterns, which should be evaluated before the incorporation of new strains in the human food chain. The use of probiotic cultures harboring plasmids encoding transferable antibiotic-resistance genes is unsafe.

Plasmids, autonomously replicating extrachromosomal genetic elements, are widely present in the genus *Lactobacillus*. Plasmids encoding resistance to tetracycline, erythromycin, chloramphenicol, streptomycin and lincomycin have been reported in *L. reuteri* (Lin et al 1996), *Lactobacillus fermentum* (Fons et al 1997), *Lactobacillus acidophilus* (Vescovo et al 1982) and *L. plantarum* (Danielsen, 2002), isolated from meats, silage and human feces. However, little information has been documented about plasmids in *L. gasseri*. To our knowledge a few reports have been published, including Luchansky, et al 1991 (26.5 Kb cryptic plasmid in strain ADH), Roussel et al 1993 (linear plasmid) Majhenic et al 2003 (pK7 in strain K7), Martim et al 2008 (uncharacterized plasmid) and Ito, et al 2009 (conjugative plasmid pLgLA39 carrying genes for the production of and immunity to the circular bacteriocin Gassericin A). Furthermore, only one plasmid, pLgLA39, has been entirely sequenced (http://www.ncbi.nlm.nih.gov/nuccore/AB436615).

This study presents the complete sequence of the plasmids pTRK1023, pTRK1024, pTRK1025 and pTRK1026 from *Lactobacillus gasseri* NCK2141 (UFVCC1091). Following analysis of the plasmid sequences, the putative *lmrB* gene (ATP-binding cassete-type multidrug resistance transporter protein) harbored by pTRK1024 was cloned into vector pTRK989 and expressed in closely related erythromycin sensitive strains NCK2140 and 2142 to determine whether this gene was responsible for the erythromycin resistance observed in NCK2141.

2 - MATERIALS AND METHODS

2.1 - Bacterial strain, media and growth condition

The bacterial strains, primers and plasmids used in this study are summarized in Table 1. *Lactobacillus gasseri* strains were propagated in MRS broth (Difco Laboratories, Inc., Detroit, MI) supplemented as needed with chloramphenicol (5µg/ml) at 37ºC. For cloning experiments, recombinant strains were selected anaerobically on MRS agar at 37ºC with 5µg/ml of chloramphenicol (Cm) (Sigma-Aldrich, St. Louis, MO). *Escherichia coli* strains were propagated in Luria-Bertani broth (Difco Laboratories, Inc.) supplemented with 20µg/ml of chloramphenicol at 37ºC. *E. coli* MC1061 recombinants were selected on BHI (Brain Heart Infusion, Difco) agar with 10 µg/ml of chloramphenicol.

2.2 - DNA manipulation, plasmid isolation and sequencing

Genomic DNA was extracted using a ZR Fungal/Bacterial DNA Kit (Zymo Research Corporation, Irvine, CA) according to the manufacturer's protocol. Plasmid DNA from strain NCK2141 was extracted using the Qiagen Large-Construct Kit (Qiagen Inc., Valencia, CA) and sequenced in the Genome Sciences Laboratory at North Carolina State University (http://gsl.cals.ncsu.edu/). A library was prepared according to the manufacturer's instructions, and sequenced with the Genome Sequencer FLX (Roche Applied Science, Indianapolis, IN) using 454 sequencing with the GS FLX Titanium chemistry.

DNA homologies were determined using the Basic Local Alignment Search Tool (BLAST) at the National Library of Medicine (NLM). http://www.ncbi.nlm.nih.gov/ (Zhang, et al 2000). Plasmid sequences were circularized by designing PCR primers (Table 1) reading out from the sequence ends and then sequencing the resulting PCR product from each putative potential plasmid. Primers were synthesized by Integrated DNA Technology (Coralville, IA) and the PCR reactions were carried out with an initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 46°C for 30 sec, and polymerization at 72°C for 1 min; and a final 10-min polymerization step at 72°C. One percent agarose gels were run at 100.0 V/cm for 1 h and stained using ethidium bromide (0.5μg/ml, Amresco, Solon, OH). DNA bands were visualized using a White/UV Transilluminator, TMW-20 (UVP, Inc., Upland, CA), photographed with an AlphaImager Camera 70-12704-00 and analyzed with a AlphaEase FC, version 4.1.0, software (Alpha Innotech Corporation, CA).

For cloning purposes, plasmid DNA was extracted using the Qiaprep Spin Miniprep Kit (Qiagen Inc.) for *E. coli* and by the method described by O'Sullivan and Klaenhammer (1993) for *Lactobacillus*.

2.3 - Sequence annotation

The complete sequence from the plasmids was annotated by an extended version of the GAMOLA® software package (Alterman and Klaenhammer, 2003), and the resulting genes, and CDS designations were viewed using Artemis® software (version 11, Wellcome Trust Sanger Institute, Cambridge, UK). Further analysis using BLAST was carried out for each designated gene to confirm and refine the *in silico*

annotations. Clone Manager 9 software (Scientific and Educational Software, Cary, NC) was used for primer design and in silico plasmid manipulation.

2.4 - Blunt cloning

For cloning in *E. coli*, a fragment containing the putative *lmrB* gene was amplified from *L. gasseri* NCK2141 genomic DNA using the lmrB_f and lmrB_r primers (Table 1). PCR amplicons were generated using Pfu Ultra II Fusion HS DNA polymerase (Stratagene Corp., La Jolla, CA), based on the manufacturer's instructions. The amplified product (1.7 kb) was purified with a QIAquick PCR Purification Kit (Qiagen Inc.), quantified using a NanoDrop ND 1000 spectrophotometer (NanoDrop Products, Wilmington, DE), and converted to 5′-phosphorylated blunt-end DNA with the End-It™ DNA End-Repair Kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's instructions. The product was purified and concentrated using the Zymo Kit DNA Clean & Concentrator™-5 Kit (Zymo Research Corporation, Irvine, CA).

2.4.1 - Restriction enzyme digestion of pTRK989

Plasmid pTRK989 containing the constitutive P6 promoter was used as a shuttle vector. Plasmid pTRK989 was constructed with a XbaI deletion and religation of pTRK696 (Sturino and Klaenhammer, 2004). Vector pTRK989 was digested using a blunt end cutting enzyme EcoICRI (New England Biolabs, Beverly, MA) at 37ºC for 3h. Super SAP (USB, Santa Clara, CA) was used to dephosphorylate the digested vector. The vector DNA was extracted with the Zymo DNA Clean & Concentrator ™ - 5 Kit (Zymo Research Corporation) and quantified using a NanoDrop ND 1000 spectrophotometer.

Table 1: Bacterial strains, primers and plasmids used in this study.

Strain or plasmid	Relevant characteristic	Reference or source
Lactobacillus gasseri UFVCC1083 (NCK2140)	Human intestinal isolate harboring pTRK1023, pTRK1025 and pTRK1026.	Cunha, et al 2010
Lactobacillus gasseri UFVCC1091 (NCK2141)	Human intestinal isolate harboring pTRK1023, pTRK1024, pTRK1025 and pTRK1026. Erm[r]	Cunha, et al 2010
Lactobacillus gasseri UFVCC1112 (NCK2142)	Human intestinal isolate harboring pTRK1023, pTRK1025 and pTRK1026.	Cunha, et al 2010
Lactobacillus gasseri NCK 2144	Electrotransformant of UFVCC1083 with pTRK1027	This study
Lactobacillus gasseri NCK 2145	Electrotransformant of UFVCC1112 with pTRK1027	This study
E. coli NCK2147	Residing host for pTRK989	This study
E. coli MC1061	Cloning host strain	Casdaban and Cohen, 1980
Plasmids		
pTRK1023	43.105 Kb; isolated from *L. gasseri* NCK2141	This study
pTRK1024	9.751 Kb; isolated from *L. gasseri* NCK2141. Contain lmrB[*] gene.	This study
pTRK1025	28.174 Kb; isolated from *L. gasseri* NCK2141	This study
pTRK1026	50.122 Kb; isolated from *L. gasseri* NCK2141	This study
pTRK989	Cm[r]. Rolling circle shuttle vector containing P6 promoter	This study
pTRK1027	Derivative of pTRK 989 containing lmrB gene from pTRK 1024. Cm[r].	This study
Primers		
Analysis of the complete sequence of the plasmids		
Plas1_f	AAGGCGTTAATACCACTTTG	Chapter 1
Plas1_r	GAATGCCATTGTCATATGTC	Chapter 1
Plas2_f	TCACTACAAGCTTCTTCTAC	Chapter 1
Plas2_r	CAGGAAGTATTTGGCTCATC	Chapter 1
Plas3_f	AACTCCTTGCACCTACATTG	Chapter 1
Plas3_r	TGGAGGGATCGTTATGTC	Chapter 1
Plas4_f	GTGTGCTATCACTAACTACC	Chapter 1
Plas4_r	GTTCGATCGCCACCGTGTAG	Chapter 1
PCR analysis and DNA sequencing of lmrB		
lmrB_f	GTGCCAATGATTAGCATCTAGG	This study
lmrB_r	ACTGCTTTACAAGCTGAATAG	This study
Sau_f	TGCTGAAGAGCATCTCATTG	This study
Sau_r	CCCGTTAGTTGAAGAAGGTT	This study

* lmrB gene - ATP-binding cassete-type multidrug resistance transporter protein. Erm[r] – resistant to erythromycin; Cm[r] – resistant to chloramphenicol. NCK - North Carolina Klaenhammer) Type Culture Collection.

2.4.2 - Ligation and transformation

The polished PCR product and digested vector pTRK989 were ligated overnight at 16ºC using T4 DNA ligase (New England Biolabs, Inc. Beverly, MA) according to the manufacturer's instructions. The ligation mix was then transformed into *E. coli* MC1061 with selection on BHI agar containing Cm. Competent cells of *E. coli* were prepared by rubidium chloride method (Hanahan, 1985). Constructs were confirmed by plasmid DNA digestion and sequencing of PCR amplicons generated with the Sau_f and Sau_r primers designed from the vector DNA flanking the insertion site (Table 1). The resulting recombinant plasmid, pTRK1027 (Figure 1), carrying the *lrmB* gene, was electroporated into *L. gasseri* NCK2140 and NCK2142. Electroporation was performed using cold 0.2 mm cuvettes with the pulser settings 2.5KV/cm, 25μF and 400Ω in a Bio-Rad Gene Pulser electroporator (Hercules, CA). Recombinants were selected on MRS agar supplemented with chloramphenicol. Clones were confirmed by digesting the plasmid DNA with Xho I.

2.5 - Phenotypic assay - Antibiotic susceptibility

The assay for antibiotic resistance was performed by calculating the MIC (Minimum inhibitory concentration) for each strain using a slightly modified version of the microdilution broth method (Jones et al 1985). Briefly, overnight cells (grown in MRS broth) were centrifuged, washed with PBS at pH 7.4, standardized to $A_{600nm} = 1$, and inoculated (2%) into each well of a 96-well microplate containing MRS (control) and MRS plus antibiotic. Cells were verified for growth after 24 h and 48 h at 37ºC.
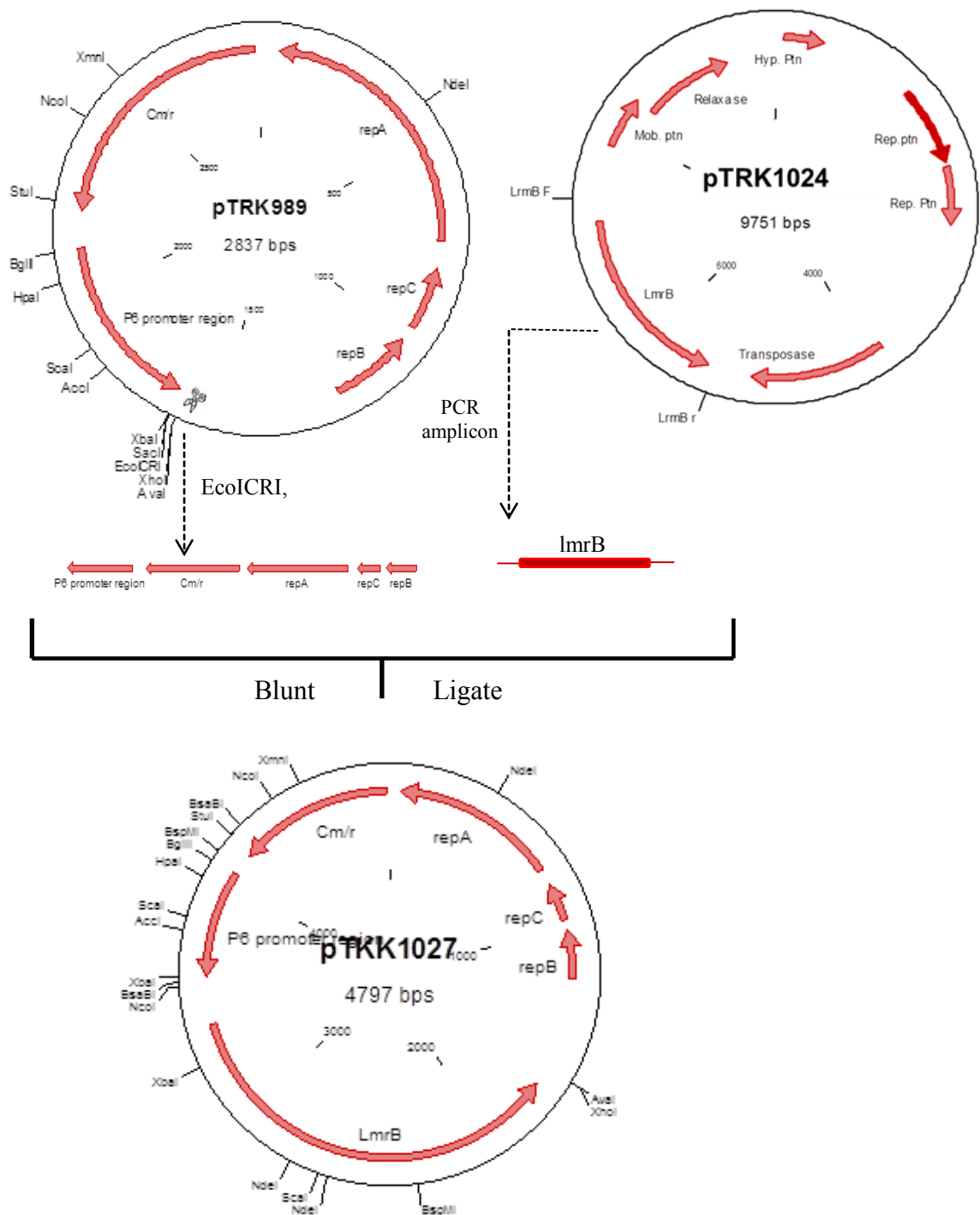
Figure 1: Construction strategy and restriction enzymes sites of pTRK1027 vector.

3 - RESULTS AND DISCUSSION

3.1 - Plasmid DNA analysis and sequencing

The three closely related *Lactobacillus gasseri* strains NCK2140, 2041 and 2042 used in this study were isolated from a Brazilian newborn infant stool and identified at the species level through sequencing of 16S rDNA (Cunha, et al 2010). They were previously characterized, and shown to have desirable probiotic properties, such as resistance to simulated gastric and small intestinal juices, antagonistic activity against pathogenic bacteria (*Escherichia coli* ATCC11229, *Salmonella typhimurium* SL1344, *Listeria monocytogenes* ATCC19115 and *Staphylococcus aureus* RN4220), ability to adhere to mucin, Caco-2 and HT-29 cell lines, and tolerance to 0.5% of oxgall (Chapter 1). Among the three stains, NCK2141 proved to have a different antibiotic resistance profile, with higher resistance to erythromycin, oxacillin and cephalothin. All three strains had the same plasmid profiles, but with an additional plasmid in NCK2141. The plasmid content of NCK2141 was sequenced, and four large contigs from the sequencing were identified as possible plasmids based on BLAST search results showing homology with plasmid sequences. Each of the contigs was circularized when PCR products amplified using plas1, plas2, plas3 and plas4 forward and reverse primers, respectively, (Table 1) joined the two ends of each sequence (Figure 2a-d). The sequence and genetic organization of the four plasmids are discussed based on putative functional regions, and the more interesting ORFs (open reading frame) are discussed in detail.

3.2 - General features of the plasmids

The maps of the four plasmids are presented in Figure 2, and annotated ORFs are shown in Tables 2, 3, 4, and 5. Plamid pTRK1023 is 43,105 bp, with G+C content of 31.1% and 36 potential ORFs. Plamid pTRK1024 is 9,751 bp with G+C content of 32.61% and 7 potential ORFs. Plasmid pTRK1025 is 28,174 bp, with G+C content of 44.57%, which was slightly higher than that observed in pLgLA39 isolated from *Lactobacillus gasseri* LA39 (39% [Ito et al 2009]) and other plasmids reported in lactobacilli strains (37-40% [Zhang, et al 2008; Sudhamani, et al 2008; Sorvig et al 2005; Daming et al 2003; Danielsen, 2002]). This finding suggests that *Lactobacillus gasseri* NCK2141 could have acquired this plasmid or part of it, by horizontal gene transfer. It was predicted to encode 20 putative ORFs. Plasmid pTRK1026 is

is 50,122 bp, with G+C content of 36.72% and 46 potential ORFs.

Figure 2: Physical and genetic map of plasmid pTRK1023 (a), pTRK1024 (b), pTRK 1025 (c) and pTRK1026 (d) of *Lactobacillus gasseri* NCK2141. Each ORF is numbered, and arrows indicate positions and directions of predicted ORFs. ORF numbers refer to Tables 2, 3, 4, and 5.

Table 2: ORF analysis of the native plasmid pTRK1023 from *L. gasseri* NCK2141 with best matches to sequences in the public databases.

ORF	Size (aa)	Proposed function [a]	Source strain	E-value	% amino acid identity	GenBank Accession Nº (protein database)
1	83	Conserved Hypotherical Protein	*L. gasseri* JV-V03	1e-41	100	ZP_07058965.1
2	362	Replication initiator ptn A	*L. gasseri* JV-V03	0	99	ZP_07058964.1
3	261	Replication associated protein Rep B	*L. gasseri* JV-V03	4e-150	99	ZP_07058963.1
4	729	Ribonucleotide-diphosphate reductase subunit alpha	*L. helveticus* DPC 4571	0	99	YP_001578042.1
5	141	Ribonucleotide-diphosphate subunit gamma	*L. gasseri* JV-V03	2e-76	100	ZP_07059007.1
6	340	Ribonucleotide-diphosphate reductase subunit beta	*L. gasseri* JV-V03	0	100	ZP_07059004.1
7	85	3-Oxoacyl-(acyl-carrier protein reductase	*L. helveticus* DSM 20075	2e-14	98	ZP_05753170.1
8	195	Resolvase family site specific recombinase	*L. helveticus* DSM 20075	2e-106	97	ZP_05753511.1
9	405	Transposase	*L. crispatus* CTV-05	7e-43	79	ZP_07789873.1
10	464	Transposase	*L. helveticus* DPC 4571	7e-158	91	YP_001576946.1
11	100	Conserved Hypotherical Protein	*L. gasseri* JV-V03	4e-49	100	ZP_07058999.1
12	268	Replication associated protein Rep B	*L. gasseri* JV-V03	5e-155	100	ZP_07058998.1
13	528	Abc family ATP binding cassete transporter	*L. helveticus* DSM 20075	0	99	ZP_05752313.1
14	523	Abc transporter ATPase and permeases proteins	*L. gasseri* JV-V03	0	98	ZP_07058993
15	72	Conserved Hypotherical Protein	*L. gasseri* JV-V03	3e-32	98	ZP_07058992.1
16	73	Conserved Hypotherical Protein	*L. gasseri* JV-V03	2e-34	100	ZP_07058991.1
17	110	Single-strand binding protein	*L. gasseri* JV-V03	4e-59	99	ZP_07058869.1
18	288	Xre fam – transcriptional Regulator	*L. gasseri* 33323	9e-157	98	YP_819582.1
19	419	Major facilitator family permease	*L. gasseri* 33323	0	97	YP_819583.1
20	164	Conserved Hypotherical Protein	*L. gasseri* JV-V03	1e-91	100	ZP_07058987.1
21	292	Conserved Hypotherical Protein	*L. gasseri* JV-V03	3e-169	99	ZP_07058985.1
22	196	Possible transcriptional Regulator	*L. gasseri* JV-V03	1e-110	100	ZP_07058984.1
23	203	Integrase	*L. gasseri* JV-V03	1e-116	100	ZP_07058983.1
24	295	AAA ATPase	*L. gasseri* JV-V03	4e-168	100	ZP_07058982.1
25	295	Alpha/beta fold family hydrolase	*L. gasseri* JV-V03	1e-169	100	ZP_07058980.1
26	490	Possible Acetyl-coa synthetase	*L. gasseri* JV-V03	0	99	ZP_07058979.1
27	455	Conserved Hypotherical Protein	*L. gasseri* JV-V03	0	100	ZP_07058977.1
28	261	Hypothetical Protein HMPREF 0514_11733	*L. gasseri* JV-V03	3e-146	100	ZP_07058976.1
29	280	Lantibiotic biosynthesis protein	*L. gasseri* JV-V03	5e-156	100	ZP_07058975.1
30	844	Lantibiotic dehydratase superfamily protein	*L. gasseri* JV-V03	0	99	ZP_07058974.1
31	585	Conserved Hypotherical Protein	*L. gasseri* JV-V03	0	99	ZP_07058973.1
32	229	Bacterioferritin comigratory protein	*L. gasseri* JV-V03	2e-123	99	ZP_07058972.1
33	374	Conserved Hypothetical Protein	*L. antri* DSM 16041	6e-20	90	ZP_05744681.1
34	703	Nicking enzyme (Tra A) – like	*L. paracasei ssp. paracasei*	0	98	ABA_12817.1
35	75	Conserved Hypothetical Protein	*L. gasseri* JV-V03	1e-32	98	ZP_07058968.1
36	273	Conserved Hypothetical Protein	*L. gasseri* JV-V03	2e-67	94	ZP_07058966.1

[a] Blast top hit as of February 2011.

101

Table 3: ORF analysis of the native plasmid pTRK1024 from *L. gasseri* NCK2141 with best matches to sequences in the public databases.

ORF	Size (aa)	Proposed function [a]	Source strain	E-value	% amino acid identity	GenBank Accession N° (protein database)
1	229	Conserved Hypothetical protein	*Enterococcus faecim*	1e-55	89	ZP_05674584.1
2	245	Plasmid replication protein Rep 3	*E. faecim*	2e-138	99	ZP_05660508.1
3	178	Plasmid replication protein	*E. faecim* E980	1e-80	97	YP_691719.1
4	403	Transposase	*E. faecim*	0	100	ADO_66841.1
5	567	LmrB	*Lactococcus lactis subs. lactis*	4e-149	51	NP_861550.1
6	159	Bacterial mobilization ptn	*E. faecim*	3e-86	97	ZP_05660516.1
7	304	Relaxase superfamily	*E. hirae*	8e-178	99	ABG47456.1

[a] Blast top hit as of February 2011.

Table 4: ORF analysis of the native plasmid pTRK1025 from *L. gasseri* NCK2141 with best matches to sequences in the public databases.

ORF	Size (aa)	Proposed function [a]	Source strain	E-value	% amino acid identity	GenBank Accession N° (protein database)
1	223	Transposase	*Lb. fermentum* ATCC 14931	2e-124	99	ZP_03945789.1
2	282	Transposase	*Lb. fermentum* IFO 3956	3e-164	99	ADJ41366.1
3	226	Putative ATPase	*Lb. casei str.* Zhang	4e-117	93	YP_002268568.1
4	63	Conserved Hypothetical protein	*Lb. brevis ssp gravenensis*	1e-27	100	ZP_03940860.1
5	93	Addiction module antitoxin, Rel B/Din J	*Lb. oris* PB13-T2-3	8e-45	97	ZP_07729431.1
6	372	Replication Initiator protein	*Lb. gasseri*	0	99	BAH_15383.1
7	50	Hypothetical protein HMPREF0539_3116	*Lb. rhamnosus* LM52-1	5e-19	94	ZP_04442584.1
8	233	ParA_like	*Lb. paracasei subs. Paracasei*	4e-132	99	ABA_12835.1
9	95	Conserved Hypothetical protein	*Lactobacillus antri* DSM 1604	8e-48	100	ZP_05746648.1
10	94	DNA damage inducible protein J	*Lb. brevis* ATCC 3671	2e-43	94	YP_796396.1
11	278	Hypothetical protein	*Lb. paracasei subs. Paracasei*	5e-119	100	ABA_12838.1
12	150	Hypothetical protein LCA2H_p038	*Lb. casei* str Zhang	2e-79	100	YP_002268602.1
13	114	PemI like protein	*Lb. salivarus* UCC118	1e-42	99	YP_163762.1
14	405	Conserved Hypothetical protein	*S. oralis* ATCC 35037	2e-47	32	ZP_06611683.1
15	130	Exported protein	*S. equi ssp equi* 4047	0.085	30	YP_002747085.1
16	1482	DNA Segregation ATPase	*E. faecalis* D55	0	49	ZP_05562990.1
17	386	Conserved Hypothetical protein	*Streptococcus sp.*	2e-60	36	ZP_06059481.1
18	1137	Predicted protein	*E. faecim*	1e-44	36	ZP_05668392.1
19	143	LysM domain	*S. parasanguinis* F0405	5e-31	80	ZP_07727015.1
20	686	Nicking enzyme Tra A protein	*Lb. gasseri*	0	95	YP_002474161.1

[a] Blast top hit as of February 2011.

Table 5: ORF analysis of the native plasmid pTRK1026 from *L. gasseri* NCK2141 with best matches to sequences in the public databases.

ORF	Size (aa)	Proposed function [a]	Source strain	E-value	% amino acid identity	GenBank Accession Nº (protein database)
1	395	Transposase	*Lb. fermentum* CECT 5716	0	98	ADJ_41664.1
2	443	Oxidoreductase, pyridine nucleotide-disulfide	*Scardovia inopinata* F0304	0	99	ZP_06756422.1
3	614	Permease of major facilitator superfamily	*Lb. paracasei ssp. paracasei*	0	99	YP_003329285.1
4	191	Transcriptional regulator family protein	*Lb. gasseri* JV-V03	2e-88	99	ZP_07059206.1
5	261	Putative plasmid partition	*Lb. salivarus* UCC 118	9e-138	91	YP_163776.1
6	432	Maturase	*Lb. crispatus* JV –V01	2e-152	81	ZP_07791491.1
7	131	Flavin nucleotide binding protein	*Lb. crispatus* JV –V01	6e-57	91	ZP_03996518.1
8	140	Conserved hypothetical protein	*Lb. antri* DSM 16041	2e-71	94	ZP_05746657.1
9	94	ORF 12	*Lb. Sakei*	5e-41	90	NP_862277.1
10	195	Hypothetical protein pLP9000_03	*Lb. Plantarum*	6e-16	96	NP_631994.1
11	233	Par A like	*Lb. paracasei subs. paracasei*	8e-122	95	ABA_12835.1
12	50	Hyp. Ptn. HMPREF0539_3116	*Lb. rhamnosus* LMS2-1	2e-18	92	ZP_04442584.1
13	367	Putative replication initiator protein	*Lb. salivarus* UCC 118	0	92	YP_163743.1
14	99	Conserved Hypothetical protein	*Lb. ultunensis* DSM 16047	1e-34	84	ZP_04011571.1
15	53	Hypothetical protein HMPRGF0514-1982	*Lb. gasseri* JV-V03	5e-17	96	ZP_07059213.1
16	277	Hypothetical protein LSL-2034	*Lb. salivarus* UCC 118	5e-55	43	YP_163812.1
17	204	Conserved hypothetical protein	*Lb. gasseri* JV-V03	1e-115	99	ZP_07059215.1
18	96	Conserved hypothetical protein	*Lb. gasseri* JV-V03	3e-40	92	ZP_07059216.1
19	75	Conserved hypothetical protein	*Lb. ultunensis* DSM 16047	7e-33	98	ZP_04011567.1
20	686	Nickase	*Lb. ultunensis* DSM 16047	0	97	ZP_04011566.1
21	125	Large conductance mechanosensitive channel	*Lb. Johnsonii*	8e-54	90	NP_965615.1
22	397	Hypothetical protein LA2_00995	*Lb. amylovorus* GRL 1112	7e-131	57	YP_004030991.1
23	186	Resolvase	*Lb. plantarum ssp. plantarum*	5e-86	85	YP_003927887.1
24	685	Conserved hypothetical protein	*S. mitis* ATCC 6249	7e-123	39	ZP_07463232.1
25	432	Type I restriction modification system restriction subunit	*Lb. brevis subsp. gravesensis* ATCC 27305	1e-84	85	ZP_03940814.1
26	240	ABC family binding cassete transporter	*Lb. ultunensis* DSM 16047	4e-123	98	ZP_04011555.1
27	305	ABC family binding cassete transporter	*Lb. ultunensis* DSM 16047	6e-176	99	ZP_04011556.1
28	111	Conserved hypothetical protein	*Lb. antri* DSM 16041	3e-58	99	ZP_05746654.1
29	261	Mut T/ Nudex family hydrolase	*Lb. brevis subsp. gravesensis* ATCC 27305	5e-111	76	ZP_03939123.1
30	475	Possible nicotinate phosphoribosyltransferase	*Lb. brevis subsp. gravesensis* ATCC 27305	0	85	ZP_03939124.1
31	452	Transposase IS4 family protein	*Lb. fermentum* 28-3-CHN	4e-113	95	ZP_05864732.1
32	293	Conserved hypothetical protein	*Lb. reuteri* CF-48-3A	0.067	34	ZP_03975334.1
33	196	Site specific recombinase	*Lb. ultunensis* DSM 16047	7e-109	99	ZP_04011558.1
34	314	Macrolide biosynthetic ptn AvrD family	*Lactococcus lactis subsp. lactis* KF 147	6e-26	34	YP_003353672.1
35	258	Conserved hypothetical protein	*Streptococcus equi subs. Zooepidemicus*	2e-90	64	YP_002745388.1
36	230	ABC family binding cassette transporter	*Lb. ultunensis* DSM 16047	1e-126	97	ZP_04011546.1
37	503	ABC family binding cassette transp. permease ptn	*Lb. ultunensis* DSM 16047	0	96	ZP_04011547.1
38	-	Unknown	-	-	-	-
39	92	Putative transcriptional regulator	*Lb. rhamnosus* HN001	2e-18	56	ZP_03212967.1
40	122	Hyp. Ptn. HMPREF9459-00383	*Streptococcus anginosus*	7e-38	62	ZP_08013395.1
41	430	Nickase like protein	*Tetragenococcus halophilus*	2e-43	62	YP_001789018.1
42	96	Hhypothetical protein LSL_2035	*Lb salivarus* UCC 118	3e-9	91	YP_163813.1
43	278	Beta-carotene 15,15`monoxigenase	*Lb. gasser*i JV-V03	1e-139	91	ZP_07059214.1
44	228	Conserved hypothetical protein	*Lb. gasser*i JV-V03	1e-125	97	ZP_07059208.1
45	876	Collagen Binding A precursor protein	*Lb. casei* str Zhang	0	84	YP_002268596.1
46	247	Conserved hypothetical protein	*Lb. casei* str Zhang	4e-128	99	YP_002268595.1

3.3 - Putative replication functions

The regions most likely to encode replication initiator proteins were identified in pTRK1023 as ORF2 and 3, in pTRK1025 as ORF6 and in pTRK1026 as ORF13, by similarity to replication factors through BLAST searches. The predicted Rep A protein of all these ORFs, except ORF3, contains the conserved domain RepA_N (pfam06970) and exhibits 70%, 98% and 92% (ORF2, ORF6 and ORF13, respectively) identity to the initiator protein in the plasmid pSF 118-44 (Genbank Accession Number AF488832) of *Lactobacillus salivarus* UCC118. Plasmid pSK 118-44 is predicted to replicate via a theta replication mechanism (Fang et al 2008), and according to the similarities found, we also suggest a theta replication for pTRK1023, 1025 and 1026. ORF2 in pTRK1023, ORF6 in pTRK1025 and ORF13 in pTRK1026 display 63-67% identity to each other (Figure 3). ORF3 in pTRK1023 shows 99% amino acid identity with the RepB protein from *Lactobacillus gasseri* JV-V03 and a conserved domain ParA that is implicated in chromosomal segregation.

```
RepA pSK118-44    1 ----------mtkstnfnyyeadnvygalffqfpkvlmygeqykhlssdaklaymvlkdrleyslrnnwvdeeghvyftftnqelenlfnchnqkvvkikkeletahllmqkqlqfnpktkkmepnrlylskldvkatdv
RepA pTRK1023     1 vyqsrkgvfimtkstnfnfieeekayatrffqfpqvliygsqyktlsdsaklqymilhdrldyslknnwvdednrvyfiftnqeltdlmqvskkkvirvkkelenqqllfqktmhfnpktqkmepnrlyladlevtatdi
RepA pTRK1025     1 vcqsrkgvfimtkstnfnyyeadnvygalffqfpkvlmygeqykhlssdaklaymvlkdrleyslrnnwvdeqqhvyftftnqelenlfnchnqkvvkikkeletahllmqkqlqfnpktkkmepnrlylskldvkatdv
RepA pTRK1026     1 ----------mdqanfkfyqadnvygalffqfpkalmygqqyrkladtaklaymvlkdrleyslrnbwideeghvyfiftnqelmdlfncskpklanvkkelekagllyqkanhfnpqtqkmepnrlylaeldmqstdv

RepA pSK118-44  131 ylrgedspkvsqtldtsgnvkitlpqetaqtldtsgnvkitlphksadetaqtldtsgnvkithnlykdyknnidtnrydidtqkldfsttnfspaeieaqnrdlvkhadefltdednglpvflepeavqllsfvcrtpq
RepA pTRK1023   141 ytksrq--qeprtldtresvkteporkfvktldtresvk-tepar---kfvktldtresvktshnl--dytnnidtnrydidtqkldfstanfspaeieaqnrdlvkhandfltdednglpvflepeavqllsfvcrtph
RepA pTRK1025   141 ylrgedspkvsqtldtsgnvkitlpqetaqtldtsgnvkitlphksvdetaqtldtsgnvkithnlykdyk-nidtnrydidtqkldfstanfspaeieaqnrdlvkhadefltdednglpvflepeavqllsfvcrtpq
RepA pTRK1026   130 ylrgeyvqkepqalatseslknrpsretvetlatseslknrpsrksvddtpqtfatseslknrbdl--dkeqvldtnrymidtqkldfsttnfspaeieqqnhdlvbhandfltdedsglpvflepeavqllsfvcrtpq

RepA pSK118-44  271 qmrrfigiilnakyavekehkdlgvvilddpdlkkmtktlrryfnalrsdekhikmvenylygtmqnlfgvvwmqqaareyaakhprrt-------------ekw------------
RepA pTRK1023   273 qmrrfigiilnakyrvekdhkdigvviplddeelkplmtkalrryfnalrsdekhikmvenylygtmqnlfgiwnkqaarkyaakhpeeq-------------sadne-------------
RepA pTRK1025   280 qmrrfigiilnakyavekehkdlgvvilddpdlkkmtktlrryfnalrsdekhikmvenylygtmqnlfgvvwnqqaakdyaakhpeee-------------ksadndnaglyy----
RepA pTRK1026   268 qmrrfigiilnakykvekdhrdigvyiplddeelkplmtktlrryfnalrsnekhikdvenylygtmqnlfgiwnkqaareyaakhpeernahnedfwdpnvfqkvhrsflndqtkqgs
```

Figure 3: Areas of significant similarity among RepA pSK118-44 (standard) and RepA protein from pTRK1023, pTRK1025 and pTRK 1026. The green areas show high matches at the same base position.

In pTRK1024, the ORFs 2 and 3 are predicted initiator proteins. ORF2 has been annotated as replication protein Rep B, belonging to the Rep 3 superfamily and to the Cluster of Orthologous Groups (COG) 5527, which is involved in the initiation of plasmid replication. RepB proteins possess nicking-closing (topoisomerase I) like activity. ORF2 shows the highest amino acid identity to the RepB protein from *Enterococcus faecium* (99% identity) and considerable homology to the Rep E protein from plasmid pS86 isolated from *Enterococcus faecalis* (41% identity), which is predicted to replicate via a theta replication mechanism (Martinez-Bueno et al 2000).

ORF3 was found to encode a Winged helix-turn-helix (WHTH) DNA-binding domain of the GntR family of transcriptional regulators. It presented highest amino acid similarity to a replication protein from *Enterococcus faecium* (97% identity). Although pTRK1024 does not contain a predicted RepA protein, which is typical of theta replicating plasmids, it may replicate by the theta mechanism, since not high, but considerable similarity was found between pTRK1024 and the theta replicating plasmid pS86.

3.4 - Transposable elements

Transposable genetic elements are important motors of genetic variability. They are components of the extensive bacterial horizontal gene pool which also includes plasmids, and can constitute a relatively large proportion of both eukaryote and prokaryote genomes (Craig et al 2002). Transposons are DNA sequences that can hop, or transpose, from one location to another through a mechanism catalyzed by transposases, which are enzymes that cut the donor DNA at the ends of the transposon and then insert the transposon into the target DNA. The site-specific recombinase enzymes (resolvase) also are important in this process. They promote the resolution of cointegrates by recognizing the *res* sequence that occurs in one copy of the transposon (Snyder and Champness, 2003). In this study, seven transposases with conserved domains were found on the four plasmids using BLAST homology searches. The predicted transposases ORF4 in pTRK1024 and ORF1 in pTRK1026 contain the conserved domains for the transposase mutator family (pfam 00872) and COG3328, which are characteristic of transposases. They showed high homology to *Enterococcus faecim* (100%) and *Lactobacillus fermentum* (98%) transposases, respectively. ORF10 in pTRK1023 and ORF2 in the pTRK1025 contain the conserved domains PHA02517 (putative transposase ORFB) and Tra5, and a motif, rve (pfam00665) that is found in transposase sequences and inactive derivatives involved in DNA replication, recombination, and repair. They showed high homology to *Lactobacillus helveticus* DPC4571 (91%) and *Lactobacillus fermentum* IFO 3956 (99%) transposases, respectively. In addition, ORF9 (COG2963) in pTRK1023, ORF1 in pTRK1025 and ORF31 in pTRK1026 (motif transposase 11 [pfam 01609]) have been also identified as putative transposases.

Roles as resolvases or site specific recombinases (ORF 33) have been identified for ORF 8 in pTRK1023 and ORFs 23 and 33 in pTRK1026. Analyses of

the amino acid sequences of those ORFs revealed the presence of COG1961 and COG2452 groups, which are related to site specific recombinase/DNA invertase and site specific integrase/resolvase activity, respectively. A putative integrase enzyme was also identified in pTRK1023 (ORF23) which encodes integrase/recombinase domains and a phage-integrase motif (pfam 00589).

3.5 - Mobilization

Two ORFs (6 and 7) of pTRK1024 were predicted to be proteins related to DNA transfer. ORF 6 was identified as a bacterial mobilization protein containing a conserved domain Mob C, which belongs to the relaxase group. It is a 159 amino acid sequence and is most homologous to a mobilization protein of *Enterococcus faecium* (97% identity). Similarly, ORF 7 was identified as a relaxase containing a motif, pfam 03432, which is related with bacterial conjugation. It exhibited high homology to a relaxase protein of *Enterococcus hirae* (99% identity). DNA relaxases are the key enzymes in the initiation of conjugative transfer and operate by catalyzing the cleavage of a specific phosphodiester bond in the plasmid **nic** site within **oriT** in a strand- and site-specific manner (Núñez and La Cruz, 2001).

Nicking enzymes were also identified in pTRK1023 (ORF34), pTRK1025 (ORF20) and pTRK1026 (ORF20). All of them encoded a conserved nickase domain MobA_MobL (pfam 03389), which includes the MobA protein from *E. coli* plasmid RSF1010, and the MobL protein from the *Thiobacillus ferroxidans* plasmid pTF1. These mobilization proteins are essential for specific plasmid transfer. ORF34, ORF20 (pTRK 1025) and ORF20 (pTRK 1026) exhibited most homology to the nickase of *Lactobacillus paracasei ssp. paracasei* (98% identity), *Lactobacillus gasseri* (95% identity) and *Lactobacillus ultunensis* DSM 16047 (97% identity) respectively.

3.6 - Partitioning

To avoid being lost from dividing cells, plasmids carry partitioning systems that ensure at least one copy of the plasmid segregates into each daughter cell during cell division. The par systems consist of a *cis*-acting site, parS, and two genes parA and parB encoding *trans*-proteins (Snyder and Champness, 2003). In this study, three ORFs (ORF 8 in pTRK1025 and ORFs 5 and 11 in pTRK1026) were identified as putative Par A proteins. All of them contain two conserved ParA domains and one

ATPase (COG1192) domain, which are involved in chromosomal segregation. ORF8 (pTRK1025) and ORFs 5 and 11 (pTRK1026) exhibited most homology to ParA protein of *Lactobacillus paracasei ssp. paracasei* (99% identity), *Lactobacillus salivarus* UCC118 (91% identity) and *Lactobacillus paracasei ssp. paracasei* (95% identity), respectively.

In addition to the partitioning mechanism, some bacterial plasmids have other maintenance systems to ensure their distribution, and among them is the plasmid addiction system, also known as the TA (toxin-antitoxin) system (Gerdes et al 2005). The TA loci have been grouped into seven two-component gene families plus one three-component system (Pandey and Gerdes, 2005). Among them, there are RelBE loci of *Escherichia coli* and Pem loci of plasmid R1 and R100 from *E. coli* (Gerdes, 2005). The molecular basis of this mechanism requires the function of two genes: one specifying a stable toxin and other coding for a unstable factor, which prevents the lethal action of the toxin (Jensen and Gerdes, 1985). The inherent instability of the antitoxin leads to activation of the toxin in plasmid free cells. Therefore, the presence of a TA system results in increased plasmid maintenance (Gerdes, 2000). In this study, two putative genes were found to belong to TA system in the pTRK1025. ORF5 was annotated as an addiction module antitoxin RelB/DinJ, similar to the RelB-like protein from *Lactobacillus gasseri*. ORF13 was identified as a PemI (plasmid emergency maintenance) like-protein, presenting most homology to PemI protein from *Lactobacillus salivarus* UCC118 (99% identity). Although a toxin gene has not been identified, the presence of the two anti-toxins in the pTRK1025 may explain why we were unable to cure this plasmid (data not shown) after successive subcultures (40 times) followed by incubation at 45ºC. The TA system was previously identified in plasmids isolated from other *Lactobacillus* species, such as LgLA39 from *Lactobacillus gasseri* (Ito et al 2009), pSF118-20 and pSF118-44 from *Lactobacillus salivarus* UCC118 (Fang et al 2008), and p256 from *Lactobacillus plantarum* (Sorvig et al 2005).

3.7 - Lantibiotic biosynthesis

The lantibiotics are a group of ribosomally synthesised, post-translationally modified peptides containing unusual amino acids, such as lanthionine, β-methyllanthionine and a number of dehydrated residues (Guder et al 2000; van Kraaij et al 1999). They belong to bacteriocin class I (Klaenhammer, 1993). Nisin is the

most well-known representative of this class, has GRAS status and has been approved for use in the preservation of foods in several countries. In this study, ORF29 in the pTRK1023 was annotated as a putative lantibiotic biosynthesis protein, which demonstrates highest homology to a lantibiotic biosynthesis protein from *Lactobacillus gasseri* JV-V03. Three other genes encoding proteins related to lantibiotic biosynthesis were identified in the same plasmid. The ORFs 13 and 14 were annotated as ABC transporter proteins, which are essential for externalization of the bacteriocin (Havarstein and Diep, 1995), and the ORF30 has been annotated as a lantibiotic dehydratase protein, which catalyzes the site-specific dehydratation of serine and threonine residues to give acid dehydroalanine and dehydrobutyrine residues, respectively (Weil et al 1990; Sahl and Bierbaum, 1998). ORF13 showed highest homology to the ABC family ATP binding cassete transporter from *Lactobacillus helveticus* DPC4571, and contains several domains related to ABC type multidrug transport, bacteriocin/lantibiotic export and posttranslational modification. ORF14 was most homologous to the ABC transporter ATPase and permease protein from *Lactobacillus gasseri* JV-V03, and contains various domains related to sugar transport, ABC type multidrugs transport, bacteriocin/lantibiotic export, and phosphate and cation transport. Despite the identification of these putative lantibiotic biosynthesis genes, bacteriocin production by *Lactobacillus gasseri* NCK2141 was not observed in our previous study *in vitro* (Chapter 1).

3.8 - Putative transport regions

ORFs 19 and 22 in pTRK1023 and ORFs 3, 4, 26, 27, 36, 37 and 39 in pTRK1026 have been identified as putative transport proteins. ORFs 19 (pTRK1023) and ORF3 (pTRK1026) display high identities with the permease protein of the major facilitator superfamily (MFS) from *Lactobacillus gasseri* ATCC 33323 (97% identity) and *Lactobacillus paracasei ssp. paracasei* (99% identity), respectively. One conserved domain was observed in ORF19, namely the H^+ antiporter domain (2A0121). Major facilitator superfamily (pfam 07690) and drug resistance transporter domains were identified in ORF3.

The ORFs 26, 27, 36 and 37 in pTRK1026 demonstrate high homology to the ABC family binding cassette transporter from *Lactobacillus ultunensis* DSM 16047 (>96% identity; Table 5). Conserved domains related to transport of lipids, cobalt, drugs, amino acids, phosphate and metallic anions were observed in ORFs 27 and 36.

Two conserved domains have been also identified in the ORF37, which are related with antimicrobial transport and lipoprotein release.

ORF22 in pTRK1023 and ORFs 4 and 39 in pTRK1026 are homologous to transcriptional regulator proteins from *Lactobacillus gasseri* JV-V03 (100% identity), *Lactobacillus gasseri* JV-V03 (99% identity) and *Lactobacillus rhamnosus* HN001 (56% identity). Putative transcriptional regulator conserved domains were observed in ORF22 in pTRK1023 and ORFs 4 and 39 in pTRK1026.

The presence of numerous ORFs encoding putative permease proteins of the major facilitator superfamily and ABC transporter proteins can promote a competitive advantage to the plasmid-carrying cells in environments with limited nutritional substrates.

3.9 - Collagen adhesion

Adherence ability is an important characteristic of probiotic bacteria, since it not only maintains the bacteria in the intestinal tract (at least temporarily), but also promotes immunomodulation and pathogen exclusion (Collado et al 2007; Vesterlund et al 2006; Servin and Coconnier, 2003). Lactobacilli can adhere either directly to the epithelium or to a variety of proteins present in the extracellular matrix (ECM), such as laminin, collagen, and fibronectin, which are shed into the mucus or can be exposed to the intestinal lumen in case of trauma, infection or inflammation (Lorca et al 2002; Styriak et al 2003). Collagen consists of a group of high-molecular mass glycoproteins and is the major constituent of the ECM (Patti and Hook, 1994; Lorca et al 2002). In this study, the 876 aa ORF 45 of pTRK1026 is identified as a putative collagen binding A precursor protein that contains a collagen-binding-B conserved domain, which mediates bacterial adhesion to collagen. Repeats of the Cna protein B-type domain were also identified. This domain is found in the *Staphylococcus aureus* collagen-binding surface protein and does not mediate collagen binding, a function carried out by the collagen-bind region (Deivanayagam et al 2000). The sequence is most homologous to the collagen Binding A precursor protein of *Lactobacillus casei* Zhang (84% identity). Since *Lactobacillus gasseri* NCK2141 is a human intestinal isolate, the collagen binding protein present in pTRK1026 may confer an advantage for this microorganism to persist in the gut and to interact with the host.

3.10 - **Multidrug resistance transporter (lmrB)**

ORF5 of the plasmid pTRK1024 has been identified as a putative *lmrB* gene encoding an ATP-binding cassette-type multidrug resistance transporter protein. It is a 567 aa sequence with 6 predicted transmembrane helices (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and is most homologous to the *lmrB* gene of *Lactococcus lactis ssp lactis* (51% identity). Conserved domains were also observed, most of them related with multidrug transport, bacteriocin/lantibiotic export, ABC transport permease/ATP binding, and carbohydrate, amino acid and cation transport.

In *Lactococcus lactis,* the *lmrB* gene was identified in a 5.6 Kb plasmid and has been shown to be involved with secretion and immunity of two bacteriocins, LsbA (a hydrophobic peptide that is initially synthesized with an N-terminal extension) and LsbB (relatively hydrophilic protein synthesized without an N-terminal leader sequence) produced by this strain (Gajic et al 2003). In addition to conferring insensitivity towards antimicrobial peptides produced by bacteria, *lmrB* also has been shown to confer resistance to eukaryotic antimicrobial peptides such as magainin II and cecropin P1 (Gajic, 2003). These compounds kill bacterial cells at very low concentration by causing depolarization of bacterial membranes (Westerhoff et al 1989) or by changing membrane permeability followed by the formation of physical holes (Yang et al 2000). LmrB is one of the five multidrug resistance transporters (LmrA [van Veen et al 1996], LmrCD [Lubelski et al 2004], LmrP [Bolhuis et al 1995] and MdtA [Perreten et al 2001]) described in *Lactococcus lactis*. LmrB has homology to LmrA of *L. lactis* MG1363 (van Veen et al 1996), to prokaryotic ABC transporters of *B. subtilis*, *Staphylococcus aureus*, *E. coli*, *Campylobacter jejuni*, and *Haemophilus influenzae* and to eukaryotic ABC transporters, *e.g.* human multidrug resistance P-glycoprotein, which is one of the causes of failure of chemotherapeutic treatment in human cancer (Gottesman et al 1995). Extensive biochemical and functional studies have been carried out for LmrA showing that it confers resistance to anticancer drugs, cytotoxic agents (colchicine), DNA intercalators (ethidium bromide), toxic peptides (nigericin, valinomycin) and antibiotic compounds belonging to the classes of aminoglycosides, lincosamides, macrolides, quinolones and tetracyclines (Putman et al 2000).

The *lmrB* gene has also been reported in *Bacillus subtilis* (Kumano et al, 1997), where it was predicted to confer resistance to lincomycin, and in a multi copy

plasmid harbored by *Corynebacterium glutamicum* (Kim et al, 2001). In *C. glutamicum* the *lmrB* gene was responsible for the lincomycin and clindamycin resistance. *Corynebacterium glutamicum lmrB* expression strains showed 9-fold increase in resistance to lincomycin compared to that of the parental strain. It is believed that *lmrB* confers such resistance by effluxing lincomycin using an energy dependent efflux process (Kim et al 2001).

We have recently isolated and characterized thirty *Lactobacillus gasseri* strains for possible use as probiotics in the pediatric area (Chapter 1). Among the 30 closely related original *Lactobacillus gasseri* isolates from a breast fed infant, NCK2141 was the only strain that harbored plasmid pTRK1024, and interestingly, this strain showed a different antibiotic resistance profile, with higher MIC (minimum inhibitory concentration) values to erythromycin, oxacillin and cephalothin than all of the other strains. In order to verify if the *lmrB* gene harbored in pTRK1024 was responsible for the erythromycin resistance in NCK2141, this gene was cloned into pTRK989 and transformed into *L. gasseri* NCK2140 and 2142, two of the isolates lacking pTRK1024 and resistance to the three antibiotics.

3.10.1 - Construction of lmrB recombinants

The *lmrB* gene fragment (~ 1.7 Kb) was amplified by PCR, and subsequently cloned into pTRK 989, which encodes a P6 promoter sequence. The recombinant plasmid (Figure 4), pTRK1027, carrying the *lrmB* gene was electroporated into *L. gasseri* NCK2140 and NCK2142.
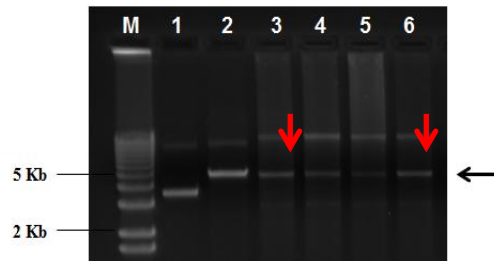
Figure 4: Verification of recombinant plasmids by restriction enzyme digestion with XhoI. Lane M, 1 kb plus DNA ladder marker. Lane 1, undigested pTRK1027 isolated from *E. coli* MC1061 (4.8 kb). Lane 2, digested pTRK1027 isolated from *E. coli* MC1061 (control). Lanes 3 and 4, digested pTRK1027 isolated from *L. gasseri* NCK2144 isolates. Lanes 5 and 6, digested pTRK1027 isolated from *L. gasseri* NCK2145 isolates. The black and red arrows indicate the XhoI digested plasmids.

3.10.2 - Phenotypic assay

The antibiotic resistance assay was performed using the strains NCK2141 (control) and NCK2144 (pTRK1027 in NCK2140) and 2145 (pTRK in NCK2142). The assay was performed as described in the materials and methods section. After 24 h of incubation at 37ºC, no difference in the sensitivity to erythromycin, oxacillin and cephalothin was observed (data not shown). These results are in agreement with those found by Kim et al (2001) that also did not detect a difference in sensitivity to erythromycin in the wild type and *lmrB* mutant strains. Therefore, we suggest that the resistance to erythromycin, oxacillin and cephalothin observed with strain NCK2141 may be chromosomally encoded.

4 - CONCLUSIONS

In this study we have shown the complete sequence of 4 plasmids (pTRK1023, 1024, 1025 and 1026) from *Lactobacillus gasseri* NCK2141, which was isolated from a breast-fed infant. Few similarities were evident among the plasmids, thus a common origin from an ancestral plasmid cannot be assumed. The plasmids appear to encode functional properties, such as collagen binding (pTRK1026), lantibiotic biosynthesis (pTRK1023) and carbohydrate transport (pTRK1023 and pTRK1026) that can provide a competitive advantage to the plasmid-carrying cells. However, bacteriocin production by NCK2141 was not observed *in vitro*. Putative cell maintenance mechanisms (partitioning and TA systems) were also observed, providing a possible explanation for the inability to cure these plasmids. Three of the plasmids (pTRK1023, pTRK1025 and pTRK1026) were shared with two other closely related *L. gasseri* strains isolated from the same infant, NCK2140 and NCK2142. Plasmid pTRK1024 was unique to NCK2141, which differed from the other two strains in its resistance to erythromycin, cephalotin and oxacillin. This plasmid harbored a putative *lmrB* gene encoding an ATP-binding cassette-type multidrug resistance transporter protein, which has been related to clindamycin and lincomycin resistance. However, when the lmrB gene was cloned and expressed *in trans*, it did not confer antibiotic resistance to closely related NCK2140 or NCK2142, which lack pTRK1024. Regardless of the source of its antibiotic resistance, due to the possibility of transfer of antibiotic resistance in human hosts, NCK2141 should not be considered as a candidate probiotic. This study contributes for the probiotic characterization of NCK2140 and NKC2142, and also increases our knowledge about plasmids in *Lactobacillus gasseri*.

5 - REFERENCES

Altermann, E., Klaenhammer, T.R. (2003). GAMOLA: a new local solution for sequence annotation and analyzing draft and finished prokaryotic genomes. **OMICS** 7, 161–169.

Axelson, L.T., Chung, T.C., Dobrogosz, W.J., Lindgren, S.E. (1989). Production of a broad spectrum antimicrobial substance by *Lactobacillus reuteri*. **Microbial Ecology and Health Disease**. 8, 131–136.

Bernardeau M., Guguen, M., Vernoux, J.P. (2006). Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. **FEMS Microbiology Reviews**. 30, 487–513.

Bolhuis, H., Poelarends, G., van Veen, H.W., Poolman, B., Driessen, A.J., Konings, W.N (1995). The lactococcal lmrP gene encodes a proton motive force-dependent drug transporter. **Journal of Biological Chemistry**. 270: 26092–26098.

Casdaban, M., Cohen, S. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. **Journal of Molecular Biology**. 138, 179-207.

Collado, M.C., Meriluoto, J., Salminen, S. (2007). Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. **Letters in Applied Microbiology**. 45, 454–460.

Craig, N.L., Craigie, R., Gellert, M., Lambowitz, A.M. (2002). Mobile DNAII, ASM Press, Washington, DC.

Cunha, Luciana Rodrigues., Ferreira, Celia L. L. F., Sanozky-Dawes, Rosemary B., Durmaz, Evelyn, Klaenhammer, Todd R. (2010). *Lactobacillus gasseri* Isolated from a Newborn Infant. 110th General Meeting of the American Society for Microbiology. San Diego, Ca. May 23-27.

Daming, R., Yinyu, W., Zilai, W., Jun, C., Hekui, L., Jingye, Z. (2003). Complete DNA sequence and analysis of two cryptic plasmids isolated from *Lactobacillus plantarum*. **Plasmid**. 50, 70-73.

Danielsen, M. (2002). Characterization of the tetracycline resistance plasmid pMD5057 from Lactobacillus plantarum 5057 reveals a composite structure. **Plasmid**. 48, 98–103.

Deivanayagam, C.C., Rich, R.L., Carson, M., Owens, R.T., Danthuluri, S., Bice, T., Hook, M., Narayana, S.V. (2000). Novel fold and assembly of the repetitive B region of the *Staphylococcus aureus* collagen-binding surface protein. **Structure**. 8, 67-78.

Fang, F., S. Flynn, Y. Li, M. J. Claesson, J. P. van Pijkeren, J. K. Collins, D. van Sinderen, and P. W. O'Toole. (2008). Characterization of endogenous plasmids from *Lactobacillus salivarius* UCC118. **Applied Environmental Microbiology**. 74, 3216–3228.

Fons, M., Hege, T., Ladire, M., Raibaud, P., Ducluzeau R., Maguin, E. (1997). Isolation and characterization of a plasmid from *Lactobacillus fermentum* conferring erythromycin resistance. **Plasmid**. 37, 199–203.

Food and Agriculture Organization of the United Nations And World Health Organization. 2002. Guidelines For The Evaluation Of Probiotics In Food And Agriculture Organization For The United Nations And World Health Organization Working Group Report, On Line.

Gajic, O., Buist, G., Kojic, M., Topisirovic, L., Kuipers, O.P., Kok, J. (2003). Novel mechanism of bacteriocin secretion and immunity carried out by lactococcal multidrug resistance protein. **The Journal of Biological Chemistry**. 178, 34291-34298.

Garrity, G.M., Bell, J.A., and Lilburn, T.G. (2004). Taxonomic Outline of the Procaryotes. Bergey's Manual of Systematic Bacteriology, 2nd edition, Springer-Verlag, New York.

Gerdes, K., S. K. Christensen, and A. Lobner-Olesen. (2005). Prokaryotic toxin-antitoxin stress response loci. **National Review of Microbiology**. 3, 371–382.

Gomes, A.M.P., Malcata, F.X. (1999). *Bifidobacterium* spp. and *Lactobacillus acidophilus* biological, biochemical, technological and therapeutical properties relevant for use as probiotics. **Trends in Food Science & Technology**. 10, 139-157.

Gottesman, M. M., Hrycyna, C. A., Schoenlein, P. V., Germann, U. A., Pastan, I. (1995). Genetic analysis of the multidrug transporter. **Annual Review of Genetics**. 29, 607-649.

Guder, A., Wiedemann, I. and Sahl, H.-G. (2000) Post-translationally modified bacteriocins the lantibiotics. **Biopolymers**. 55, 62-73.

Hammes, W.P., Vogel, R.F. (1995). The genus *Lactobacillus*: The Genera of Lactic Acid Bacteria. V2. (Wood BJB & Holzapfel, W.H, eds). 19-54. Blackie Academic & Professional Glasgow, UK.

Hanahan, D. (1985). Techniques for transformation of *E. coli*, p. 109–135. In D. M. Glover (ed.), DNA cloning: a practical approach, vol. 1. IRL Press Ltd., Oxford, England.

Havarstein, L.S., Diep, D.B., Nes, I.F. (1995). A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. **Molecular Microbiology**. 16, 229-240.

Hugenholtz, P. (1998). The genera of Lactic Acid Bacteria. Blackie Academic & Professional, London, UK.

Ito, Y., Kawai, Y., Arakawa, K., Honme, Y., Sasaki, T., Saito, T. (2009). Conjugative plasmid from *Lactobacillus gasseri* LA39 that carries genes for production of and

immunity to the circular bacteriocin gassericin A. **Applied and Environmental Microbiology**. 75 (19), 6340-6351.

Jensen, R. B., Gerdes, K. (1995). Programmed cell death in bacteria: proteic plasmid stabilization systems. **Molecular Microbiology**. 17, 205-210.

Jerdes, K. (2000). Toxin-antitoxin modules may regulate synthesis of macromolecules during nutritional stress. **Journal of Bacteriology**. 182. 561-572.

Jones, R. N., Ballow, C. H., Biedenbach, D. J., Deinhart, J.A., Schentag, J.J. (1998). Antimicrobial activity of quinupristin-dalfopristin (RP 59500, Synercid) tested against over 28,000 recent clinical isolates from 200 medical centers in the United States and Canada Diagnostic. **Microbiology and Infectious Disease**. 30, 437–51.

Kim, H., Kim, Y., Lee, M., Lee, H.(2001). Gene lmrB of *Corynebacterium glutamicum* Confers Efflux-mediated Resistance to Lincomycin. **Molecular Cells**. Vol. 12, No. 1, pp. 112-116

Klaenhammer, T.R (1993). Genetic of bacteriocins produced by lactic acid bacteria. **FEMS Microbial**. 20, 39-85.

Kumano, M., Tamakoshi, A., and Yamane, K. (1997). A 32 kb nucleotide sequence from the region of the lincomycin-resistance gene (22°–25°) of the *Bacillus subtilis* chromosome and identification of the site of the lin-2 mutation. **Microbiology**. 143: 2775–2782.

Lin, F., Fung, Z.F., Wu, C.L., Chung, T.C. (1996). Molecular characterization of a plasmid borne (pTC82) chloramphenicol resistance determinant (cat-Tc) from *Lactobacillus reuteri* G4. **Plasmid**. 36, 116–124.

Lorca, G., Torino, M.I., deValdez, G.F. and Ljungh, A. (2002). Lactobacilli express cell surface proteins which mediate binding of immobilised collagen and fibronectin. **FEMS Microbiology Letters**. 206, 31–37.

Lubelski, J., Mazurkiewicz, P., van Merkerk, R., Konings, W.N., Driessen, A.J. (2004) ydaG and ydbA of Lactococcus lactis encode a heterodimeric ATP-binding cassette-type multidrug transporter. **Journal of Biological Chemistry**. 279: 34449–34455.

Luchansky, J. B., M. C. Tennant, and T. R. Klaenhammer. (1991). Molecular cloning and deoxyribonucleic acid polymorphisms in *Lactobacillus acidophilus* and *Lactobacillus gasseri*. **Journal of Dairy Science**. 74:3293–3302.

Majhenic, A. C., B. B. Matijasic, and I. Rogelj. (2003). Chromosomal location of the genetic determinants for bacteriocins produced by *Lactobacillus gasseri* K7. **Journal of Dairy Research**. 70:199–203.

Martin, R., Soberon, N., Vaneechoutte, M., Florez, A.B., Vazquez, F., Suarez, J.E. (2008). Characterization of indigenous vaginal lactobacilli from health women as probiotic candidates. **International Microbiology**. 11, 261-266.

Martinez-Bueno, M., Valdivia, E., Galvez, A., Maqueda, M. (2000). pS86, A New Theta-Replicating Plasmid from *Enterococcus faecalis*. **Current Microbiology**. 41, 257–261.

Núnez, B., de la Cruz, F. (2001). Two atypical mobilization proteins are involved in plasmid CloDF13 relaxation. **Molecular Microbiology**. 39**,** 1088-1099.

Olivares, M., M. A. Díz-Ropero, N. Góez, F. Lara-Villoslada, S. Sierra, J. A. Maldonado, R. Martin, E. Lóez-Huertas, J. M. Rodriguez, and J. Xaus. (2006a). Oral administration of two probiotic strains, *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711, enhances the intestinal function of healthy adults. **International Journal of Food Microbiology**. 107**,** 104-111.

Olivares, M., M. P. Díz-Ropero, N. Góez, F. Lara-Villoslada, S. Sierra, J. A. Maldonado, R. Martin, J. M. Rodriguez, and J. Xaus. (2006). The consumption of two new probiotic strains, *Lactobacillus gasseri* CECT 5714 and *Lactobacillus coryniformis* CECT 5711, boosts the immune system of healthy humans. **International Microbiology**. 9**,** 47-52.

O'Sullivan, D.J. And T.R. Klaenhammer. (1993). Rapid mini-prep isolation of high quality DNA from *Lactococcus* and *Lactobacillus* species. **Applied Environmental Microbiology**. 59, 2730-2733.

Pandey, D., Gerdes, K. (2005). Toxin- antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. **Nucleic Acids Research**. 55, 78–89.

Patti, J. M., and M. Höök. (1994). Microbial adhesins recognizing extracellular matrix macromolecules. **Current Opinion in Cell Biology**. 6**,** 752-758.

Perreten, V., Schwarz, F.V., Teuber, M., Levy, S.B (2001) Mdt(A), a new efflux protein conferring multiple antibiotic resistance in *Lactococcus lactis* and *Escherichia coli*. **Antimicrobial Agents and Chemotherapy**. 45, 1109–1114.

Putman, M., van Veen, H.W., Degener, J.E., Konings, W.N. (2000). Antibiotic resistance: era of the multidrug pump. **Molecular Microbiology**. 36, 772–773.

Reuter, G. (2001). The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. Curr. Issues Intest. Microbiol. 2, 43–53.

Roussel, Y., Colmin, C., Simonet, J.M,, Decaris, B. (1993). Strain characterization, genome size and plasmid content in the *Lactobacillus acidophilus* group (Hansen and Mocquot). **Journal of Applied Bacteriology**. 74(5), 549-56.

Ryan, K.A., Jayaraman, T., Daly, P., Canchaya, C., Curran, S., Fang, F., Quigley, E.M., O`Toole, P.W (2008). Isolation of lactobacilli with probiotic properties from the human stomach. **Letters in Applied Microbiology**. 47, 269-274.

Sahl, H.G., Bierbaum, G. (1998). Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. **Annual Review of Microbiology**. 52, 41–79.

Servin, A.L., Coconnier, M.H. (2003). Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. **Best Practice and Research: Clinical Gastroenterology**. 17, 741-754.

Smith, S.I., Aweh, A.J., Coker, A.O., Savage, K.O., Abosede, D.A., Oyedeji, K. S. (2001). *Lactobacilli* in human dental caries and saliva. **Microbios**. 105, 77–85.

Snyder, L., Champness, W. (2003). Molecular Genetics of Bacteria. ASM press. Washington, D.C. 2ed. 566p.

Sorvig, E., Skaugen, M., Naterstad, K., Eijsink, V.G.H., Axelsson, L. (2005). Plasmid p256 from *Lactobacillus plantarum* represents a new type of replicon in lactic acid bacteria, and contains a toxin-antitoxin-like plasmid maintenance system. **Microbiology**. 151, 421-431.

Sturino, J.M., Klaenhammer, T.R. (2004). **Applied and Environmental Microbiology**. 70 (3), 1735-1743.

Styriak, I., Nemcova, R., Chang, Y.H. and Ljungh, A. (2003). Binding of extracellular matrix molecules by probiotic bacteria. **Letters in Applied Microbiology**. 37, 329–333.

Sudhamani, M., Ismaiel, E., Geis, A., Batish, V., Heller, K.J. (2008). Characterization of pSMA23, a 3.5Kbp plasmid of *Lactobacillus casei*, and application for heterologous expression in *Lactobacillus*. **Plasmid**. 59, 11-19.

Tamura, A., H. Kumai, N. Nakamichi, T. Sugiyama, R. Deguchi, A. Takagi, and Y. Koga. (2006). Suppression of *Helicobacter pylori*-induced interleukin-8 production *in vitro* and within the gastric mucosa by a live *Lactobacillus* strain. **Journal of Gastroenterology and Hepatology.** 21, 1399–1406.

Tuohy, K.M., Probert, H.M., Smejkal, C.W., Gibson, G.R. (2003). Using probiotic and prebiotics to improve gut health. **Drug Discovery Today**. 8, 692-700.

Van Kraaij, C., de Vos, W.M., Siezen, R.J. and Kuipers, O.P. (1999). Lantibiotics: biosynthesis, mode of action and applications. **National Product Reports**. 16, 575-587.

Van Veen, H.W., Margolles, A., Muller, M., Higgins, C.F., Konings, W.N. (2000). The homodimeric ATP-binding cassette transporter LmrA mediates multidrug transport by an alternating two-site (two-cylinder engine) mechanism. **The EMBO Journal**. 19, 2503–2514.

Vescovo, M., Morelli, L., Bottazzi, V. (1982). Drug resistance plasmids in *Lactobacillus acidophilus* and *Lactobacillus reuteri*. **Applied Environmental Microbiology**. 43, 50–56.

Vesterlund, S., Karp, M., Salminen, S. and Ouwehand, A.C. (2006). *Staphylococcus aureus* adheres to human intestinal mucus but can be displaced by certain lactic acid bacteria. **Microbiology**. 152, 1819–1826.

Wall, R., G. Fitzgerald, S. Hussey, T. Ryan, et al (2007). Genomic diversity of cultivable *Lactobacillus* populations residing in the neonatal and adult gastrointestinal tract. **FEMS Microbiology Ecology**. 59, 127–137.

Weil, H.P. Beck-Sickinger, A.G. Metzger, J. Stevanovic, S.Jung, G. Josten, M. Sahl, H.G. (1990). Biosynthesis of the lantibiotic Pep5. Isolation and characterization of a prepeptide containing dehydroamino acids. **European Journal Biochemistry**. 194, 217–223.

Westerhoff, H. V., Juretic, D., Hendler, R. W. and Zasloff, M. (1989). Magainins and the disruption of membrane-linked freeenergy transduction. **Proceedings of the National Academy of Science of the United States of America.** 86, 6597-6601.

Yang, L., Weiss, T. M., Lehrer, R. I. and Huang, H. W. (2000). Crystallization of antimicrobial pores in membranes: magainin and protegrin. **Biophysical Journal**. 79, 2002-2009.

Zhang, W., Yu, D., Sun, Z., Chen, X., Bao, Q., Meng, H., HU, S., Zhang, H. (2008). Complete nucleotide sequence of plasmid plca36 isolated from *Lactobacillus casei* Zhang. **Plasmid**. 60, 131-135.

Zhang, Z., Schwartz, S., Wagner, L., Miller, W. (2000). "A greedy algorithm for aligning DNA sequences". **Journal of Computational Biology**. 7(1-2), 203-14.

CHAPTER III

FUNCTIONAL ANALYSIS OF FIBRONECTIN BINDING PROTEIN IN *Lactobacillus gasseri*

ABSTRACT

Fibronectin is a dimeric glycosylated protein present in soluble form in blood plasma and in the immobilized form on the surfaces and extracellular matrices (ECM) of the host cells. It is an important target for bacterial attachment and therefore, represents a distinguishing feature for selection of probiotic bacteria. There are numerous researches describing the attachment of lactic acid bacteria to fibronectin, however, little is known about the growth condition and the functional role of this protein in the probiotic properties. We have analyzed the effect of the growth condition on the adhesion of three (NCK2140, 2141 and 2142) selected strains of *Lactobacillus gasseri* to immobilized fibronectin binding protein and the functional role of this protein in the adhesion and stress response of the cell. Mutant were constructed using the site-directed chromosomal integration system and phenotypic assays were undertaken with parental strains (NCK2140 and NCK2141) and *fbp*⁻ mutants (NCK2147 and NCK2148) to assign a function for this gene. Significantly higher adherence to fibronectin was observed when the bacteria were grown on MRS agar under anaerobic condition compared to bacteria grown in MRS broth. It was also observed a reduction of *in vitro* adherence of *fbp* mutants (NCK 2147 and NCK 2148) to immobilized fibronectin and HT-29 intestinal epithelial cells. The inactivation of the *fbp* locus did not influence the gastric juice susceptibility of the mutant strains. These results indicate a role for *fbp* in adherence to immobilized fibronectin and HT-29 cell lines. Further studies will be performed to explore the role of this protein in the stress tolerance.

Key words: Fibronectin-binding protein, probiotic, adhesion

RESUMO

Fibronectina é uma proteína dimérica, glicosilada presente na forma solúvel no plasma sanguíneo e, na forma imobilizada na superfície e matrizes extracelulares (MEC) das células do hospedeiro. Essa proteína é um alvo importante para adesão bacteriana e portanto, representa uma ferramenta imprescindível para seleção de bactérias probióticas. Há vários estudos demostrando a capacidade de bactérias láticas aderirem à fibronectina, no entanto, pouco é conhecido sobre a influência das condições de crescimento e o papel funcional dessa proteína nas propriedades probióticas. Afim de obter mais informações, analisou-se o efeito das condições de crescimento de três estirpes de *L. gasseri* (NCK2140, 2141 e 2142) sobre a adesão à fibronectina e o papel funcional dessa proteina na capacidade de adesão e resposta ao estresse digestivo. Mutantes foram construidos utilizando-se o sistema site-directed chromosomal integration. Ensaios fenotípicos foram realizados com as cepas parentais (NCK2140 e NCK2141) e mutantes *fbp*⁻ (NCK2147 e NCK2148). Maior adesão *in vitro* foi observada à fibronectina quando as estirpes NCK2140, 2141 e 2142 foram cultivadas em ágar MRS e incubadas em condições de anaerobiose. Observou-se também, que os mutantes *fbp* (NCK2147 e NCK2148) tiveram menor habilidade de aderir a fibronectina imobilizada e a célula humana do câncer de cólon HT-29. A inativação do gene *fbp* não influenciou a susceptibilidade das estirpes recombinates ao suco gástrico. Estes resultados indicam o papel do gene *fbp* na adesão à fibronectina imobilizada e à célula humana do câncer de cólon, HT-29. Mais estudos deverão ser realizados para verificação do papel dessa proteina na tolerância ao estresse digestivo.

Palavras – chave: Fibronectina, probiótico, adesão

1 - INTRODUCTION

The Food and Agriculture Organization (FAO) and World Health Organization (WHO) defined probiotics as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). *Lactobacillus* is frequently used as a probiotic and promising data have also been accumulated on the benefits of the consumption of this genus as single or as a combined cultures in the prevention of colon cancer (Commane et al 2005), reduction of serum cholesterol (Lin & Chen, 2000), increase of digestion of lactose in lactose intolerant hosts (Griffin et al 2002), metabolism of proteins and vitamins (Berg, 1980; Young & Huffman, 2003) stimulation of the immune system (Round, 2009; Isolauri, 2001; Kalliomaki et al 2001) and in the protection of children from allergy and intestinal infections (Collado et al 2006; Harsharnijit, 2003).

Due to the benefits, substantial efforts have been concentrated in the past years to isolate and identify new strains of lactobacilli to add in food products and several requirements have been proposed for screening of new strains. Among them, bacterial adhesion to the intestinal epithelium is considered one major criterion for probiotic selection, since it may influence the residence time of the bacteria in the intestinal tract (Servin and Coconnier 2003) and the ability of the strains to inhibit pathogenic bacteria by competition and blocking of their binding sites at the mucosa (Collado et al 2007; Servin and Coconnier 2003).

In vitro cell models, involving human intestinal cells (HT-29, Caco-2), mucins and extracellular matrices (laminin and fibronectin) have been used extensively to study the ability of probiotic microorganisms to adhere to the intestinal tissues (Cammarota et al 2009; Provencio Munoz et al 2009; Horie et al 2005; Styriak et al 2003; Blum and Renieri, 1999). Fibronectin is a dimeric glycosylated protein (454 kDa) present in soluble form in blood plasma and in the immobilized form on the surfaces and extracellular matrices (ECM) of the host cells. It is found inserted in the mucus, potentially exposed to the intestinal lumen in cases of trauma, infection or inflammation (Styriak et al 2003; Lorca et al 2002). It is an important target for bacterial attachment in many pathogens, such as *Streptococcus pneumoniae* and *Streptococcus pyogenes*, where fibronectin-binding proteins are important pathogenic factors (Holmes et al 2001; Jedrzejas 2007; Molinari et al 1997).

There are numerous works describing the attachment of lactic acid bacteria to fibronectin, but information about the functional role of this protein in the probiotic properties is limited. The development of a site-directed lactococcal chromosomal integration system by Law et al (1995) involving the simultaneous use of a broad-host-range nonreplicative pWV01-derived vector (Ori$^+$ RepA$^-$) or so-called pORI-based vector and a temperature-sensitive helper plasmid, pVE6007 (Maguin et al 1992), that provides *repA in trans* for conditional replication of the pORI-based plasmids has permitted to elucidate the role of several genes to probiotic traits. This gene knockout strategy was adapted for use in *L. acidophilus* and *L. gasseri* with an alternate helper plasmid, pTRK669, which provides a higher permissive temperature range for thermophilic lactobacilli (Russel and Klaenhammer, 2001). This system has been used successfully with inactivation of different genes related to probiotic properties, such as acid tolerance (Azcarate-Peril et al 2004), transport and catabolism of fructooligosaccharide (Barrangou et al 2003) and adhesion factors (Buck et al 2005). In order to study the functional role of the fibronectin-binding protein in the probiotic attributes of *Lactobacillus gasseri*, the objective of the present study was to knockout the *fbp* gene in the chromosome of *L. gasseri* NCK2140 and NCK2141 and investigate how this gene affects the adherence ability and stress response. We also study the effect of the growth condition of these probiotics strains on the adhesion to immobilized fibronectin. The *L. gasseri* NCK2141 and NCK2142 were isolated from a Brazilian newborn infant stool and identified at species level through sequencing of 16S rDNA (Cunha, et al 2010). They were previously characterized and presented desirable probiotic properties, such as resistance to simulated gastric and small intestinal juices, antagonistic activity against pathogenic bacteria (*Escherichia coli* ATCC11229, *Salmonella typhimurium* SL1344, *Listeria monocytogenes* ATCC19115 and *Staphylococcus aureus* RN4220), tolerance to 0.5% of oxgall and ability to adhere to mucin, Caco-2 and HT-29 cell lines (Chapter 1). They were also found to contain 3 plasmids, except NCK2141 that additionally harbored a unique plasmid encoding an ATP-binding cassette-type multidrug resistance transporter protein (LmrB), which has been related with clindamycin and lincomycin resistance. In addition, NCK2141 showed different phenotypic behavior from the other *Lactobacillus gasseri* strains, presenting higher resistance to oxgall and higher MIC values to erythromycin, oxacillin and cephalothin. For safety reasons, NCK2141 will

not be used as a probiotic candidate. However, we decided to further investigate this strain for its unique plasmid.

MATERIALS AND METHODS

1.1 - Bacterial strains, media and growth conditions

The bacterial strains, primers and plasmids used in this study are summarized on Table 1. The lactobacilli strains were propagated in MRS (DeMan et al 1960) broth (Difco Laboratories, Inc., Detroit, MI) statically under aerobic condition or on MRS agar (1.5% [wt/vol]; Difco) under anaerobic condition at 37ºC or 42ºC, as indicated below. Recombinants strains were selected in the presence of 2 µg/ml of erythromycin (Erm) (Sigma-Aldrich, St. Louis, MO) and/or 5 µg/ml of chloramphenicol (Cm) (Sigma) when appropriate. *Escherichia coli* strains were grown in brain heart infusion (BHI; Difco) medium at 37ºC with aeration. *E. coli* EC101 was propagated in the presence of 40 µg/ml of kanamycin (Kn) and, when necessary, Erm was added at a final concentration of 150 µg/ml.

For the adhesion assay, *L. gasseri* strains were grown on MRS agar (0.1 ml) under anaerobic conditions or in MRS broth in under aerobic condition at 37ºC for 18–20 h, after two consecutive subcultures in MRS broth under aerobic condition. The bacterial cells were collected by centrifugation from the liquid culture or by spreader from the plate culture. A total volume of 5 ml of phosphate-buffered saline (PBS pH 7.4) were added to the MRS agar plate to recover the cells, followed by centrifugation. After washing with PBS two times, the bacterial cells were finally resuspended in PBS at a concentration of 1.0×10^8 CFU/ml and were used for the adherence assay. For recombinant *L. gasseri* strains, adherence assays were performed using cells grown in MRS broth supplemented with 2µg/ml of erythromycin.

1.2 - DNA manipulations and sequence analysis

Genomic DNA of *L. gasseri* was extracted using a Mo Bio UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA). Plasmid DNA from *E. coli* was isolated using a QIAprep Spin miniprep kit (Qiagen Inc., Valencia, CA). PCR primers (Table 1) were synthesized by Integrated DNA Technologies (Coralville, IA) and, when appropriate, restriction sites were designed into the 5' ends

of the primers to facilitate cloning steps. PfuUltra II Fusion HS DNA polymerase (Stratagene Corp., La Jolla, CA) were used to generate PCR amplicons for cloning purposes and Choice-Taq Blue DNA polymerase (Denville Scientific Inc., Metuchen, NJ) was used for screening *E. coli* recombinants.

Table 1: Bacterial strains, primers and plasmids used in this study.

Strain or plasmid	Genotype or characteristics*	Reference or source
L. gasseri NCK2140	Human intestinal isolate, also named UFVCC1083	Cunha et al 2010
L. gasseri NCK2141	Human intestinal isolate, also named UFVCC1091	Cunha et al 2010
L. gasseri NCK2142	Human intestinal isolate, also named UFVCC1112	Cunha et al 2010
L. gasseri NCK2148	*fbp* mutant	This study
L. gasseri NCK2147	*fbp* mutant	This study
L. gasseri ADH	Human intestinal isolate; also named NCK99 (MS02). Control strains for adherence assay	Kleeman and Klaenhammer, 1982
L. gasseri ATCC33323	Human instestinal isolate. Standard strain used to design primers to amplify *fbp* gene from NCK2141.	Klaenhammer et al 2002
E. coli EC101 (NCK1831)	RepA⁺ JM101; Kmʳ; *repA* from pWV01 integrated in chromosome; host for pORI-based plasmids.	Law et al 1995
Plasmids		
pTRK1028	pORI19 with a modified copy of LGAS1011 gene cloned into EcoRI/XbaI sites	This study
pTRK669	Ori (pwv01), Cmʳ, RepA⁺	Russel and Klaenhammer, 2001
pORI19	Emʳ Ori⁺ (pWV01), *lacZ'*, replicates only with *repA* provided *in trans*	Law et a., 1995
Primers (5' to 3')		
Construction of mutants		
PC1_f	GTAATATCTAGAGTTATTCAGGGCATAATGTAGC	This study
PC1_r	AACACCTGATGAACAAGATG	This study
PC2_f	CATCTTGTTCATCAGGTGTTCTTGTTCGAATGGTTGATAG	This study
PC2_r	TAAGTAGAATTCATAACCCATCTGCCATTAAG	This study
PCR and sequencing analysis of integration e/or deletion targets		
Fibdel_f	CAACGTAGATTACGTGATAG	This study
Erm_323	CAAAACGCTCATTGGCATTA	Goh and Klaenhammer unpublished
Fibdel_r	GCTTCTTCTGGGCTTATATC	This study
Erm_615	TTCCTGAGCCGATTTCAAAG	Goh and Klaenhammer, unpublished

*Restriction enzyme sites for XbaI (TCTAGA) and EcoRI (GAATTC) are underlined.

PCR products were analyzed on 1% agarose gels and purified using a QIAquick gel extraction kit (Qiagen). Sequencing was performed by Eton Bioscience Inc, (Cary, NC). Restriction enzymes (Roche Molecular Biochemicals, Indianapolis, IN) and T4 DNA ligase (New England Biolabs, Beverly, MA) were used according to the manufacturers' recommendations. *E. coli* chemically competent cells were prepared and transformed according to method described by Hanahan (1985). Electrocompetent *L. gasseri* were prepared as described by Walker et al (1996) with some modification (Goh et al 2009). Briefly, stationary-phase cells were inoculated into MRS broth (2% inoculum) and grown for 3 h (A_{600}, 0.1 to 0.2). A filter-sterilized penicillin G stock solution was then added to obtain a final concentration of 10 mg/ml, and the culture was incubated for 2 h at 37ºC before it was harvested.

Sequence analysis was performed using Clone Manager 6.0 (Scientific and Educational Software). Deduced protein sequences were compared with the nonredundant protein database using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

1.3 - Construction of *L. gasseri fbp* mutants

The fibronectin binding protein from *L. gasseri* NCK2141 was sequenced by amplification of chromosomal DNA using primers designed based on the *L. gasseri* ATCC 33323 genome sequence (Genbank accession no. CP000413). Insertion mutations were constructed using the pORI-based gene replacement system (Russell and Klaenhammer, 2001, Law et al, 1995). Briefly, *L. gasseri* NCK2141 chromosomal DNA was used as a template to amplify a 797 bp and 697 bp DNA segment flanking the region upstream and downstream of the insertion target, respectively, using PC1_f/PC1_r and PC2_f/PC2_r primers pairs (Table 1). Both purified PCR products were joined and amplified via splicing by overlap extension PCR (SOE-PCR) (Horton et al 1989), using 10 ng of each PCR product as amplification template in a 50 μl PCR with the PC1_f and PC2_r primer pair (Table 1). The purified SOE-PCR product was digested with EcoRI and XbaI and subsequently ligated into similarly digested pORI19 integration vector. Ligation mixtures were transformed into *E. coli* EC101 with selection on BHI agar containing Kn, Erm, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactopyranoside (IPTG). Plasmid constructs were confirmed by DNA sequencing and the resulting recombinant plasmid, pTRK1028 (Figure 1), carrying

the flanking regions of LAGS1011 were electroporated into *L. gasseri* NCK2140 and NCK2141, previously transformed with the temperature-sensitive helper plasmid pTRK669. Three Erm^r Cm^r transformants carrying both plasmids were propagated overnight once at 37°C in MRS broth plus 2 μg/ml of erythromycin and 2 μg/ml of chloramphenicol. The culture was then transferred three times at 42°C (1% inoculum) in MRS broth plus 2 μg/ml of erythromycin. Chromosomal integrants with Erm^R Cm^s phenotype were selected by replica plating onto MRS agar supplemented with Erm or 5μg/ml Cm. Chromosomal integration was confirmed by PCR of integration junctions using fibdel_f/Erm 323 and Erm 625/fibdel_r primers pair (Table 1). Two Erm^r Cm^s pTRK1028 integrants were selected and propagated in MRS broth with Erm at 37°C overnight, followed by three transfers (1% inoculum) in MRS broth without Erm. To recover plasmid-free recombinants, the cultures were plated at 10^{-7} and 10^{-8} dilutions on MRS agar, followed by replica plating on MRS agar with or without Erm to screen for Erm sensitive colonies. Double recombinants were screened by colony PCR using the fbpdel_f/fbpdel_r primer pair (Table 1), that specifically anneals to the flanking region of the *fbp* gene.

1.4 - Adherence assay

The fibronectin-binding protein assay was performed as described previously by Goh et al 2009 using MRS grown stationary phase cells (16 h of growth).

Immobilized Fibronectin-binding assay - Fibronectin (from human plasma; Sigma) was diluted in 50 mM carbonate-bicarbonate buffer pH 9.6 to a final concentration of 10 μg/ml. The protein solutions were coated onto Nunc Maxisorp 96-well microplate wells (100 μl/well), followed by incubation at 4ºC overnight. After two washes with PBS (pH 7.4), the wells were blocked with 2% bovine serum albumin (BSA) solution (Invitrogen) for 2h at 37ºC, followed by two additional washes with PBS to remove excess BSA. Washed (PBS buffer [pH 5.0]) and adjusted (OD$_{600nm}$ = 1 [~ 1 x 10^8 CFU/ml]) overnight cells were added (100 μl) to each protein-coated well. After 1 h incubation at 37ºC, the wells were washed five times with PBS pH 6.0 (200 μl/well) and cells were recovered by treating each well with 100 μl of 0.05% Triton X-100 solution for 15 min with agitation. Cell suspensions were diluted and plated onto MRS agar to enumerate adhered cells that were expressed by [CFU/ml at t-1 h / CFU/ml at t-0] x 100). The experiment was performed with at least four independent cultures, each with quadruplicate wells

containing individual substrate layers. For *fbp* knockout purpose, the assay was performed with two independent cultures each with quadruplicate wells containing individual substrate layers. *Lactobacillus gasseri* NCK99 (Kleeman and Klaenhammer, 1982) was used as a control strain. A Student`s t-test compared media among treatments and differences were judged to be statistically significant when P value was < 0.05.

HT-29 adherence assay – Aliquots (1 ml) of adjusted cell (A_{600nm} ~1 [1 x 10^8 CFU/ml]) were added into each well containing a HT-29 monolayer. Plates were incubated for 1h at 37ºC under ambient atmosphere and washed five times with PBS pH 7.4 (1 ml/well). Adhered cells were recovered by treating each well with 1 ml of 0.25% trypsin solution (Invitrogen) at 37ºC for 10 min, followed by 10 min at room temperature with shaking. Cell suspensions were diluted and plated onto MRS agar to enumerate adhered cells (expressed by [CFU/ml at t-1 h/CFU/ml at t-0] x 100). The assay was performed with two independent cultures each with quadruplicate wells containing individual substrate layers.

1.5 - Tolerance to simulated gastric juice

Survival in simulated gastric juice was performed as described by Goh and Klaenhammer, 2010, Callan et al 2008 and Bitan-Banin et al 2003. Briefly, cells were grown overnight from a 1% inoculum in MRS. Aliquots (1 ml) were centrifuged, and the cell were washed twice with sterile water. The cell suspension (0.2 ml) was mixed with 1 ml of freshly prepared simulated gastric juice (0.5% [wt/vol] NaCl solution containing 3 g/liter pepsin [Fisher Scientific, Pittsburg, PA], pH 2.0) and incubated at 37ºC. Viable cell count was determined by plating onto MRS at 30-min intervals.
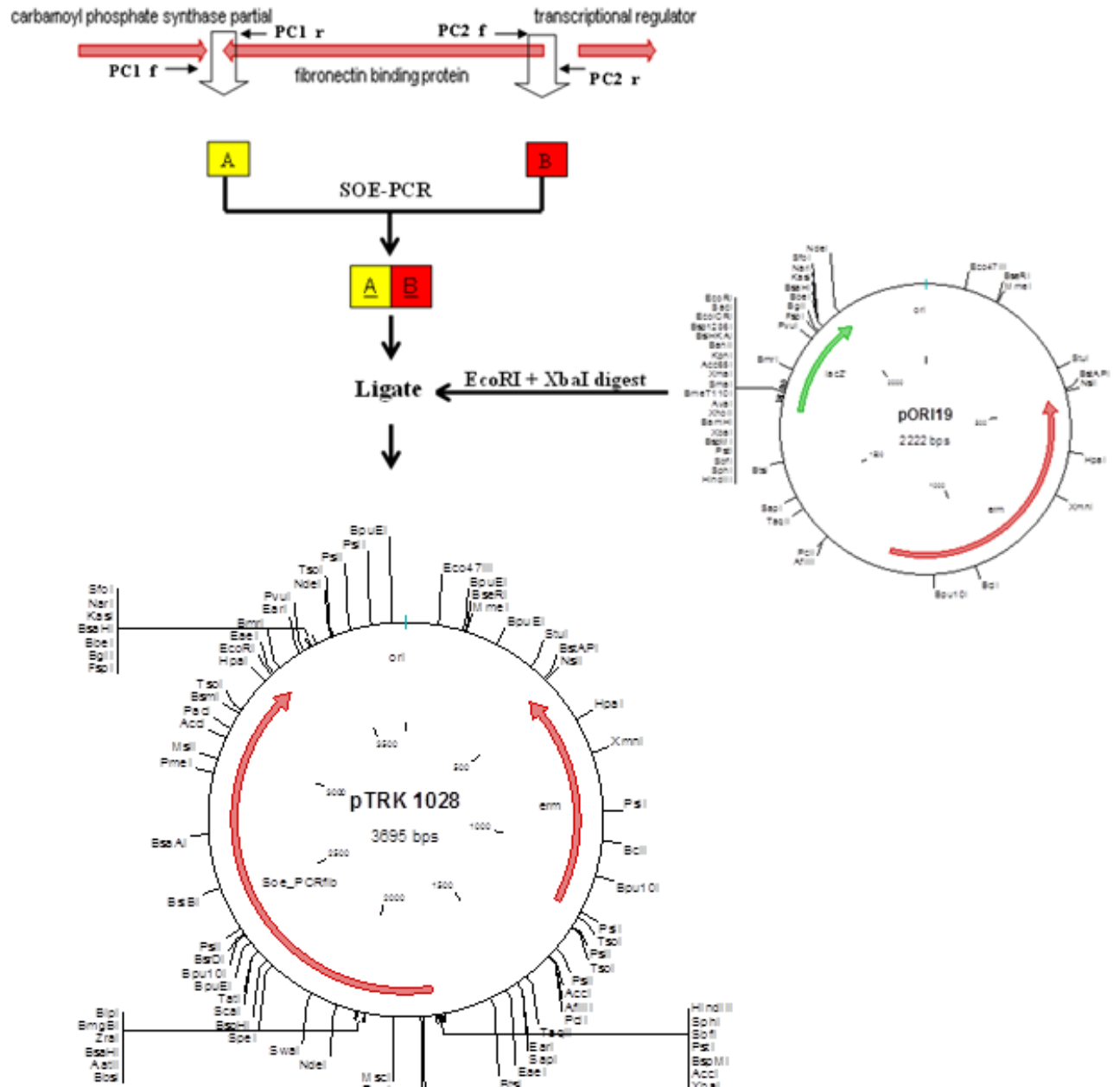
Figure 1: Construction of the pTRK1028 integration vector. *ori*, origin of replication of pWV01; *erm*, gene encoding Em resistance; MCS, multiple cloning sites from pUC19. Only unique restriction sites are shown (Adapted from Goh et al 2009).

2 - RESULTS

2.1 - Adherence to immobilized fibronectin and effect of the growth condition on the adhesion

Due to importance of adhesion in the selection of probiotic cultures, the capacity of NCK 2140, 2141 and 2142 to bind to immobilized fibronectin binding protein after growth on static MRS broth and on MRS agar under anaerobic condition was investigated. The adhesion of the remaining strains was comparable to that shown by the positive control strain *Lactobacillus gasseri* NCK99, a strain with good adhesion properties (Kleeman and Klaenhammer, 1982). It was observed that all the selected strains had good adherence to immobilized fibronectin binding protein when compared to the control strain NCK99 (Figure 2). Higher (P< 0.05) levels of adhesion were observed when the *L. gasseri* strains were cultured anaerobically on agar plates (Figure 2).
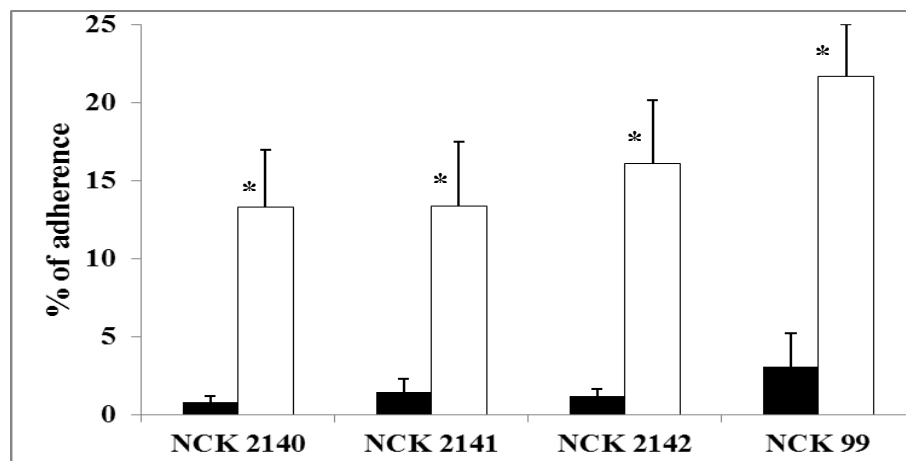

Figure 2: Adherence of *Lactobacillus gasseri* NCK2140, 2141, 2142 and the control strain NCK99 to fibronectin *in vitro*. The bacteria grown in static MRS broth in ambient atmosphere (black bars) and on MRS agar under anaerobic condition (white bars) were used for adherence testing at a concentration of 1.0 x 10^8 cells/ml. Bacterial cells were exposed to microwells coated with fibronectin for 1 h at 37ºC, followed by plating on MRS agar medium for enumeration of adhered bacterial cells. The data represent the means ± standard errors of the means for four independent replicates. A Student`s t-test indicated that the results are significantly different (*) at a value of P<0.05 for adherence of the strain grown in MRS broth compared to the same strain grown in MRS agar.

2.2 - Characterization of Fibronectin-binding protein from *L. gasseri* NCK2141

Due to the good ability of the strains to bind to fibronectin, we decided to investigate the functional role of this protein in the adhesion and stress response of the cell. The putative fibronectin-binding protein encoding gene from NCK2141, LGAS1011, was sequenced and has a G+C content of 32.15% and 564 amino acid residues. LGAS1011 is flanked by a carbamoyl phosphate synthase (upstream) and a transcriptional regulator gene (downstream). Protein blast analysis of LGAS1011 against the nonredundant database (NCBI) demonstrated high sequence homology to a fibronectin binding protein from *Lactobacillus gasseri* ATCC33323 (100% identity; GenBank accession number YP_814825.1), *Lactobacillus johnsonii* (91% identity, GenBank accession nᵒ YP_003293133.1), *Lactobacillus helveticus* DPC 4571 (72% identity; GenBank accession nᵒ. YP_001577562.1), *Lactobacillus amylovorus* GRL1112 (72% identity; GenBank accession nᵒ. YP_004032033.1), *Lactobacillus crispatus* MV-3A-US (71% identity; GenBank accession nᵒ. YP_06019393.1) and *Lactobacillus acidophilus* NCFM (71% identity, GenBank accession nᵒ YP_104018.1) demonstrating that this protein is highly conserved among lactobacilli strains. Moderate sequence homology was also observed in species other than lactobacilli, such as *Enterococcus faecium* (44% identity, Genbank Accession n⁰ ZP_05664289.1) and *Pediococcus acidilactis* (46% identity, Genbank Accession n⁰ ZP_07367271.1).

2.3 - Construction of *fbp* mutants

Two mutant strains, NCK2147 and 2148, with integration in the LGAS1011 gene of NCK2141 and NCK2140, respectively, were successfully constructed using a pORI based gene replacement system (Russel and Klaenhammer, 2001 and Law et al, 1995). The initial goal was to delete the *fbp* gene, but it was not possible to obtain a pure population of double recombinants carrying solely the Δ*fbp* allele (Figure 3). For this reason, we decided just to knockout the *fbp* gene by integration of the pTRK1028 vector in the target gene (*lgas*1011) in order to study the functional role of this gene in the cell stress response and adherence to intestinal cells, *in vitro*. The mutants (NCK2147 and NCK2148) were compared with the parent strains NCK2140 and NCK2141 in subsequent phenotypic assays.
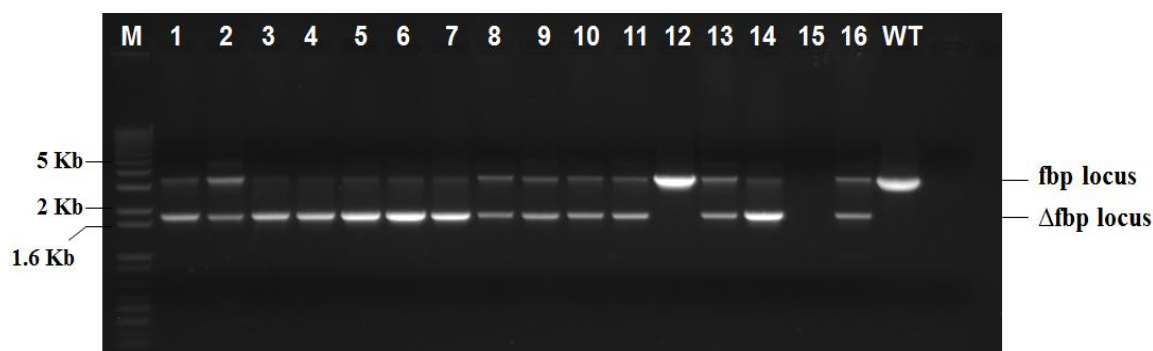
Figure 3: Colony PCR analysis of 16 selected Erm-sensitive double recombinants. Lane M, DNA size marker (1 Kb plus); lane 1 to 8, selected NCK2148 isolated Erm-sensitive recombinants; lane 9 to 16, selected NCK2147 isolated Erm sensitive recombinants; Lane WT, parent strain NCK 1041 (control). The expected amplicon sizes generated from wild-type and Δ*fbp* genotypes are approximately 3.311 kb and 1.8 kb, respectively.

2.4 - Phenotypic analysis of the *fbp* insertion mutants

2.4.1 - Survival to simulated gastric juice

The ability of NCK2148 (Fig 4a) and NCK2147 (Fig 4b) to survive during gastric transit was assessed *in vitro* by exposure to simulated gastric juice at pH 2. The *fbp* mutants showed high tolerance to gastric juice after 90 minutes of exposure and, presented less than 1 log of reduction. Thus, inactivation of the *fbp* locus did not influence the gastric juice susceptibility of the NCK2148 and NCK2147 strains.
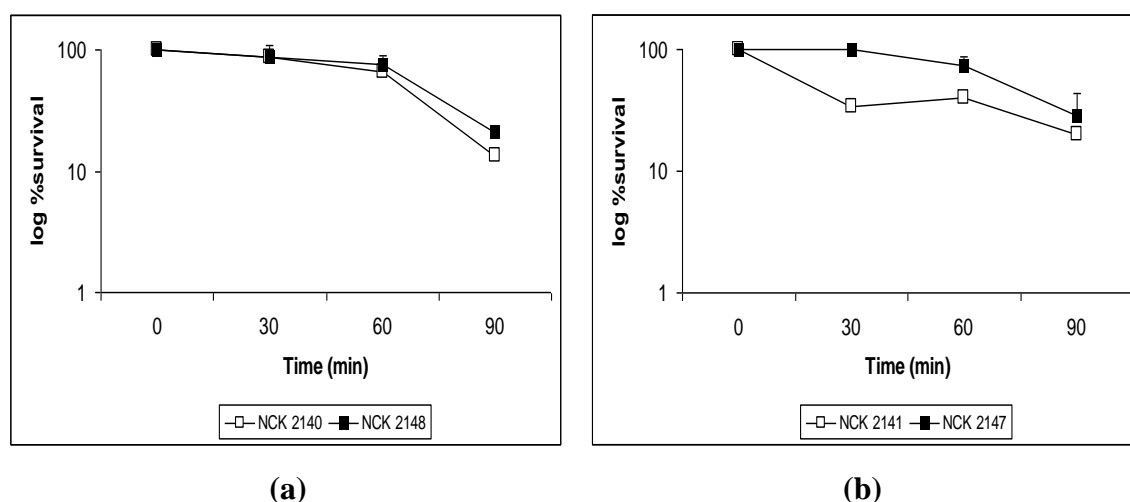
(a)   **(b)**

Figure 4: Survival of stationary phase cells of *Lactobacillus gasseri* NCK 2140 (a) and NCK 2141 (b) (open square) compared with their respective fbp mutants NCK 2148 (a) and NCK 2147 (b) (dark square) in simulated gastric juice at pH 2.0. Percentage of survival represents viable cells (CFU/ml after treatment at various time points versus before treatment (time 0). The data are the means ± standard errors of the means for two independent replicates.

2.4.2 - Adherence ability of NCK 2147 and NCK 2148

To examine the effect of fibronectin gene knockout on the adherence ability, the stationary-phase culture of NCK 2148 and NCK 2147 were exposed to fibronectin and HT-29 monolayers for 1h at 37ºC. A reduction of 20% and 30% in adherence of NCK 2148 (NCK 2140::pTRK 1028) to fibronectin (Figure 5a) and HT-29 (Figure 5b) was observed, respectively, compared to the control strain NCK 2140. The NCK 2147 mutant showed an approximately 40% decrease in adherence to fibronectin and HT-29 compared to that of NCK 2141 (parental reference strain). Therefore, the fibronectin gene knockout resulted in a reduced ability of the mutant strains to adhere to fibronectin and HT-29 cells.

133

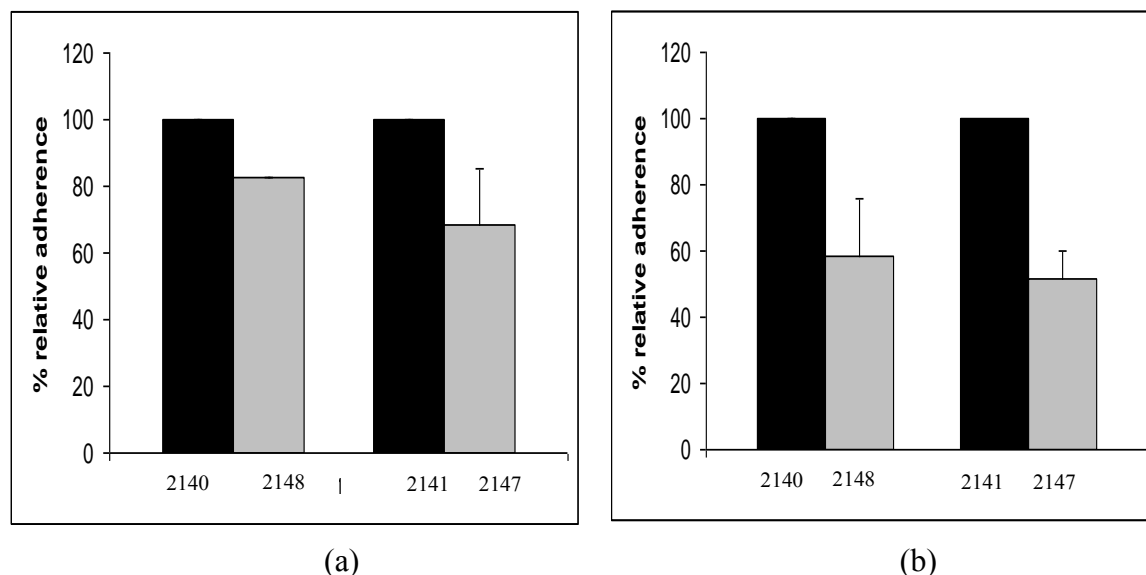(a)   (b)

Figure 5: Adherence of *Lactobacillus gasseri* NCK 2148 (NCK 2140::pTRK2148) and NCK 2147 (NCK 2141::pTRK2148) relative to their respective control NCK 2140 and NCK 2141 to fibronectin (a) and HT-29 (b) *in vitro*. The bacteria grown in static MRS broth in ambient atmosphere were used for adherence test at a concentration of 1.0×10^8 cells/ ml. Bacterial cells were exposed to HT-29 monolayers, or microwells coated with fibronectin for 1 h at 37ºC, washed, and plated on MRS agar medium for enumeration of adhered bacterial cells. The data represent the means ± standard errors of the means for two independent replicates.

3 - DISCUSSION

L. gasseri is one of the most common inhabitants detected in the human intestinal tract (Wall et al 2007) and has attracted much attention as probiotic bacteria for its beneficial effects on the human health (Olivares et al 2009; Olivares et al 2006a; Olivares, et al 2006b). Much of these benefits are due to the ability of the probiotic strain to adhere to the intestinal mucosa, since it will assure the colonization of mucosal surfaces, at least transiently, thus interfering with pathogen binding and permitting an efficient interaction with the immune system cells (Vesterlund et al 2007). It has been shown that *L. gasseri* strains have good adherence properties (Horie et al 2005; Greene and Klaenhammer, 1994), however, little is known about the effect of growth conditions on the adhesion. In this current study, the selected probiotic strains NCK2140, 2141 and 2142 showed strong ability to adhere to immobilized fibronectin after growth on a solid substrate under anaerobic conditions.

However, low adherence was observed when the strains were cultured in static liquid MRS medium. These results are in agreement with those found by Horie et al (2005) that also observed significantly higher adherence to fibronectin for *L. gasseri* strains from different origins after growth on solid surface under anaerobic atmosphere. Induction of adhesion of cells grown on solid medium has been reported in intestinal lactobacilli other than *L. gasseri*. Spenser and Chesson (1994), Jonsson et al (2001) and Horie et al (2005) also reported induction of adhesion to porcine enterocytes (*L. fermentum*, *L. brevis* and *L. salivarus*), immobilized pig mucus (*L. reuteri*) and to laminin (*L. johnsonii*), respectively, after growth on MRS agar under anaerobic conditions. The reason for this phenomenon is unclear. Some authors suggested regulation by the level of environmental oxygen or carbon dioxide (Fogg and Caparon, 1997). Others suggested regulation by sensory process, other than quorum sensing, as inducers of these changes in adhesiveness (Horie et al 2005). Therefore, more studies need to be done to elucidate the mechanism involved in the induction of adhesion in *L. gasseri* strains after growth in MRS agar under anaerobic conditions.

Using the pORI based gene replacement system, it was possible to knockout the *fbp* gene, although deletion was unsuccessful. Maybe, this gene in *L. gasseri* strains is involved in other essential function in the cell besides adhesion. The *fbp* insertion mutants exhibited reduced adherence to HT-29 cell line and immobilized fibronectin, *in vitro*. These results are in agreement with those found by Munoz-Provencio et al (2009) and Buck et al (2005) that also found decrease in binding to immobilized fibronectin by *L. casei* BL23 and *L. acidophilus* NCFM *fbp* mutants, respectively. Buck et al (2005) also found a significant reduction of the *fbp* mutant on Caco-2 cells attachment. These results suggest that fibronectin-binding protein participates in adhesion to epithelial cells *in vitro*, and could potentially serve as an adhesion factor in vivo that participates in the interaction with the host`s intestinal epithelial cells and fibronectin component of the ECM.

On the other hand, inactivation of *fbp* did not affect the tolerance of the NCK2047 and NCK2048 mutant strains to gastric juice. The comparison of this finding is difficult because it is the first paper reporting the role of *fbp* gene in stress tolerance of the probiotic bacteria. Others authors have studied the functional role of some genes related to adherence in stress tolerance. O`Flaherty and Klaenhammer (2010) investigated the role of a putative cell surface protein (myosin cross reaction reactive, LBA649) in stress tolerance. The authors found that the ΔLBA0649 mutant

was more sensitive to porcine bile, showed longer lag phase and slower growth in sodium lactate, sodium acetate and sodium chloride than the parental *Lactobacillus acidophilus* NCFM strains. Goh et al 2009 deleted the *apf* gene encoding an aggregation-promoting-like factor in *L. acidophilus* NCFM and showed that besides a reduction in the adherence of Caco-2 epithelial cells, mucin and fibronection *in vitro*, the mutant was also more susceptible to bile, sodium dodecyl sulfate (SDS), simulated small intestinal juice and gastric juice when compared to the parental strain. These results suggest that some genes have more than obvious function that are apparent from their annotation or placement in an operon and, therefore may influence other function in the cell, which can directly affect important probiotic bacteria attributes.

4 - CONCLUSION

This study showed a strong induction of *Lactobacillus gasseri* adherence to immobilized fibronectin after growth on MRS agar under anaerobic condition. The reason for this phenomenon is unknown and further study needs to be done to elucidate the mechanisms involved in this induction.

It was found that the fbp gene from *Lactobacillus gasseri* participates in cell attachment to immobilized fibronectin and HT-29 cells. It is an important, but not the only factor contributing to fibronectin and HT-29 binding in NCK2140 and NCK2141, since the inactivation of this gene did not prevented the total adherence in HT-29 cell lines and immobilized fibronectin.

The *fbp* gene did not affect the tolerance of *Lactobacillus gasseri* strains to gastric juice and additions assays, such as tolerance to small intestinal juice, oxgall, SDS among others should be done to further explore the functional role of this gene in such stress responses.

5 - REFERENCES

Azcarate-Peril, M. A., E. Altermann, R. L. Hoover-Fitzula, R. Cano, and T. R. Klaenhammer. (2004). Identification and inactivation of genetic loci involved with *Lactobacillus acidophilus* acid tolerance. **Applied Environmental Microbiology**. 70, 5315–5322.

Barrangou, R., E. Altermann, R. Hutkins, R. Cano, and T. R. Klaenhammer. (2003). Functional and comparative analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. **Proceedings of National Academy of Sciences of the United States of America**. 100, 8957–8962.

Berg, R. (1980). Inhibition of *Escherichia coli* translocation from the gastrointestinal tract by normal cecal flora in gnotobiotic or antibiotic decontaminated mice. **Infection and Immunology**. 29, 1073-1081.

Bitan-Banin, G., R. Ortenberg, and M. Mevarech. (2003). Development of a gene knockout system for the halophilic archaeon Haloferax volcanii by use of the pyrE gene. **Journal of Bacteriology**. 185, 772–778.

Blum, S., Reniero, R., Schiffrin, E.J., Crittenden, R., Mattila-Sandholm, T., Ouwehand, A.C., Salminen, S., Von Wright, A., Saarela, M., Saxelin. M., Collin, K.., Morelli, L. (1999). Adhesion studies for probiotic: need for validation and refinement. **Trends and Food Science Technology**. 10, 405-410.

Buck, B. L., E. Altermann, T. Svingerud, and T. R. Klaenhammer. (2005). Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. **Applied Environmental Microbiology**. 71, 8344–8351.

Callanan, M., Kaleta, P., O'Callaghan, J., O'Sullivan, O., Jordan, K., McAuliffe, O., Sangrador-Vegas, A., Slattery, L., Fitzgerald, G.F., Beresford, T., Ross, P. R. (2008). Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. **Journal of Bacteriology**. 190, 727–735.

Cammarota, M., Rosa, M., Stellavato, A., Lamberti, M., Marzaioli, I., Giulianoa, M. (2009). *In vitro* evaluation of *Lactobacillus plantarum* DSMZ 12028 as a probiotic: Emphasis on innate immunity. **International Journal of Food Microbiology**. 135, 90-98.

Collado, M.C., Jalonen, L., Meriluoto, J., Salminen, S. (2006). Protection mechanism of probiotic combination against human pathogens: *in vitro* adhesion to human intestinal mucus. **Asia Pacific Journal of Clinical Nutrition.** 15(4), 570-575

Commane, D., Hughes, R., Shortt, C., Rowland, I. (2005). The potential mechanisms involved in the anti-carcinogenic action of probiotics. **Mutation Research**. 591, 276-289.

Cunha, Luciana Rodrigues., Ferreira, Celia L. L. F., Sanozky-Dawes, Rosemary B., Durmaz, Evelyn, Klaenhammer, Todd R. (2010). *Lactobacillus gasseri* Isolated from a Newborn Infant. 110th General Meeting of the American Society for Microbiology. San Diego, Ca. May 23-27.

Fogg, G.C., Caparon, M.G. (1997). Constitutive expression of fibronectin-binding in *Streptococcus pyogenes* as a result of anaerobic activation of rofA. **Journal Bacteriology.** 179, 6172-6180.

Food and Agriculture Organization of the United Nations And World Health Organization. (2002). Guidelines For The Evaluation Of Probiotics In Food And Agriculture Organization For The United Nations And World Health Organization Working Group Report.

Goh, Y.J, Azcárate-Peril, M.A, O'Flaherty, S., Durmaz E, Valence F, Jardin J, Lortal S, Klaenhammer TR. (2009). Development and application of a upp-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. **Applied Environmental Microbiology**. 75 (10), 3093-105.

Goh, Y.J, Klaenhammer, T.R. (2010). Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. **Applied Environmental Microbiology**. 76 (15), 5005-12.

Greene, J.D., Klaenhammer, T.R. (1994). Factors Involved in Adherence of Lactobacilli to Human Caco-2 Cells. **Applied and Environmental Microbiology**. 60 (12), 4487-4494

Griffin, I.J., Davila, P.M., Abrams, S.A. (2002). Non-digestible oligosaccharides and calcium absorption in girls with adequates calcium intakes. **British Journal of Nutrition**. 87, 187-191.

Harsharnjit, S.G. (2003). Probiotics to enhance anti-infective defences in the gastrointestinal tract. **Best Practive & Research Clinical Gastroenterology.** 17 (5), 755-773.

Holmes, A.R., McNab, R., Millsap., K.W., Rohde, M., Hammerschmidt, S., Mawdsley, J.L., Jenkinson, H.F. (2001). The pavA gene of *Streptococcus pneumonia* encodes a fibronectin-binding protein that is essencial for virulence. **Molecular Microbiology**. 41, 1395-1408.

Horie, M., Murakami, T., Sato, T., Tarusawa, Y., Nakamura, S., Toba, T. (2005). Anaerobic induction of adherence to laminin in *Lactobacillus gasseri* strains by contact with solid surface. **Current Microbiology.** 51(4), 275-82.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., Pease, L.R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. **Gene**. 77, 61–68.

Isolauri, E. (2001). Probiotics: effects on immunity. **American Journal of Clinical Nutrition**. 73, 444-450.

Jedrzejas, M.J. (2007). Unveiling molecular mechanisms of bacterial surface proteins: Streptococcus pneumonia as a model organism for structural studies. **Cellular and Molecular Life Sciences**. 64, 2799-2822.

Jonsson, H., Strom, E., Roos, S. (2001). Addition of mucin to growth medium triggers mucus-binding activity in different strains of Lactobacillus reuteri *in vitro*. **FEMS Microbiology Letters**. 204, 19-22.

Kalliomaki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., Isolauri, E. (2001). Probiotics in primary prevention of atopic disease: a randomized placebo-controlled trial. **Lancet**. 357, 1076–1079.

Klaenhammer, T.R., Altermann, E., Arigoni, F., Bolotin, A., Breidt, F., Broadbent, J., Cano, R., Chaillou, S., Deutscher, J., Gasson, M., van de Guchte, M., Guzzo, J., Hartke, A., Hawkins, T., Hols, P., Hutkins, R., Kleerebezem, M., Kok, J., Kuipers, O., Lubbers, M., Maguin, E., McKay, L., Mills, D., Nauta, A., Overbeek, R., Pel, H., Pridmore, D., Saier, M., van Sinderen, D., Sorokin, A., Steele, J., O'Sullivan, D., de Vos, W., Weimer, B., Zagorec, M., Siezen, R. (2002). Discovering lactic acid bacteria by genomics. **Antonie van Leeuwenhoek**. 82, 29–58.

Kleeman, E.G., Klaenhammer, T.R. (1982). Adherence of *Lactobacillus spp*. to human fetal intestinal cells. **Journal of Dairy Science**. 65, 2063–2069.

Law, J., Buist, G., Haandrikman, A., Kok, J., Venema, G., Leenhouts, K. (1995). A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. **Journal of Bacteriology**. 177, 7011–7018.

Lin, S.Y., Chen, C.T. (2000). Reduction of cholesterol by *Lactobacillus acidophilus* in culture broth. **Journal of Food and Drug Analysis**. 8, 97-102.

Lorca, G., Torino, M.I., deValdez, G.F., Ljungh, A. (2002). Lactobacilli express cell surface proteins which mediate binding of immobilised collagen and fibronectin. **FEMS Microbiology Letters**. 206, 31–37.

Maguin, E., Duwat, P., Hege, T., Ehrlich, D., Gruss, A. (1992). New thermosensitive plasmid for gram-positive bacteria. **Journal of Bacteriology**. 174, 5633–5638.

Molinari, G., Talay, S.R., Valentin-Weigand, P., Rohde, M., Chlatwal, G.S. (1997). The fibronecting-binding protein from *Streptococcus pyogenes*, SfbpI, is involved in the internalization of group A streptococci by epithelial cell. **Infection and Immunology**. 65, 1357-1363.

Munoz-Provencio, D., Perez-Martinez, G., Monedero, V. (2009). Characterization of fibronectin-binding protein from *Lactobacillus casei* BL23. **Journal of Applied Microbiology**. 108, 1050-1059.

O'Sullivan, M.G., Thornton, G.M., O'Sullivan, G.C., Collins, J.K. (1992). Probiotic bacteria: myth or reality? **Trends in Food Science and Technology**. 3, 309–14.

O'Flaherty, S.J., Klaenhammer, T.R.(2010). Functional and phenotypic characterization of a protein from Lactobacillus acidophilus involved in cell morphology, stress tolerance and adherence to intestinal cells. **Microbiology.** 156, 3360-7.

Olivares, M., M. A. Díz-Ropero, N. Góez, F. Lara-Villoslada, S. Sierra, J. A. Maldonado, R. Martin, E. Lóez-Huertas, J. M. Rodriguez, and J. Xaus. (2006a). Oral administration of two probiotic strains, *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711, enhances the intestinal function of healthy adults. **International Journal of Food Microbiology**. 107**,** 104-111.

Olivares, M., M. P. Díz-Ropero, N. Góez, F. Lara-Villoslada, S. Sierra, J. A. Maldonado, R. Martin, J. M. Rodriguez, and J. Xaus. (2006b). The consumption of two new probiotic strains, *Lactobacillus gasseri* CECT 5714 and *Lactobacillus coryniformis* CECT 5711, boosts the immune system of healthy humans. **International Microbiology.** 9**,** 47-52.

Round, J.L., Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. **Nature Review Immunology**. 9, 313-323.

Russell, W. M., Klaenhammer, T.R. (2001). Efficient system for directed integration into the Lactobacillus acidophilus and Lactobacillus gasseri chromosomes via homologous recombination. **Applied Environmental Microbiology**. 67, 4361–4364.

Servin, A.L., Coconnier, M.H. (2003). Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. **Best Practice and Research: Clinical Gastroenterology**. 17, 741-754.

Spencer, R.J., Chesson, A. (1994). The effect of *Lactobacilllus* spp. on the attachment of enterotoxigenic Escherichia coli to isolated porcine enterocytes. **Journal of Applied Bacteriology**. 77, 216-220.

Styriak, I., Nemcova, R., Chang, Y.H., Ljungh, A. (2003). Binding of extracellular matrix molecules by probiotic bacteria. **Letters in Applied Microbiology**. 37, 329–333.

Vesterlund, S., Vankerckhoven, V., Saxelin, M., Goossens, H., Salminen, S., Ouwehand, A.C. (2007). Safety assessment of Lactobacillus strains: presence of putative risk factors in faecal, blood and probiotic isolates. **International Journal of Food Microbiology**. 116, 325-331.

Wall, R., Fitzgerald, G., Hussey, S., Ryan, T., Murphy, B., Ross, P., Stanton, C. (2007). Genomic diversity of cultivable *Lactobacillus* populations residing in the neonatal and adult gastrointestinal tract. **FEMS Microbiology Ecology**. 59, 127–137.

Young, R.J., Huffman, S. (2003). Probiotic Use in Children. **Journal of Pediatric Health Care**. 17, 277-283.

GENERAL CONCLUSION

This study has identified and characterized three new *L.gasseri* strains isolated from the feces of a breast feeding infant. Genomic characterization of the *L. gasseri* isolates indicated that they are largely identical except for a single genome polymorphism for UFVCC1112 (NCK2142) by PFGE and the presence of a unique plasmid encoding a putative ATP-binding cassette-type multidrug resistance transporter protein in isolate UFVCC1091 (NCK2141). Since UFVCC1091 was resistant to erythromycin, oxacillin and cephalothin this strain is not indicated to be used as a probiotic.

After broad phenotypic analysis, the non-antibiotic resistant strains UFVCC 1083 (NCK2140) and UFVCC1112 (NCK2142) were found *in vitro* to possess desirable probiotic properties, such as resistance to simulated gastric and small intestinal juices, antagonistic activity against pathogenic bacteria (*Escherichia coli* ATCC11229, *Salmonella typhimurium* SL1344, *Listeria monocytogenes* ATCC19115 and *Staphylococcus aureus* RN4220), ability to adhere to mucin, fibronectin, Caco-2 and HT-29 cell lines, and tolerance to 0.5% of oxgall. In addition, all strains were found to contain three plasmids harboring functional properties, such as collagen binding, lantibiotic biosynthesis and carbohydrate transport that may provide competitive advantages to the plasmid-carrying cells. These bacteria will be further evaluated in vivo to elucidate their potential health benefits and their application as a probiotic in the pediatric area, such as in the human milk banks in Brazil.

The resistance to erythromycin, oxacillin and cephalothin observed with strain UFVCC1091, emphasizes the importance of assessing the antibiotic resistance profile pattern of new isolates before approving them as a probiotic. Thereby limiting the incorporation of probiotic cultures harboring potential transferable antibiotic-resistance genes in the human food chain.