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Área de Tecnologia de Alimentos

Efeito do leite probiótico fermentado na resposta imune celular em cólon de camundongos BALB/c

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Tese para obtenção do grau de
DOUTOR

Orientador:
Profa. Dra. Maricê Nogueira de Oliveira

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Comissão Julgadora
da
Tese para obtenção do grau de Doutor

Profa. Dra. Maricê Nogueira de Oliveira
orientador/presidente

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4º. examinador

São Paulo, 15 de Outubro de 2012.

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SUMMARY

LIST OF TABLES

LIST OF FIGURES

SYMBOLS AND NOMENCLATURE

°C	Graus Celsius
ab	Antibody
APC	Antigen Presenting Cell
CD3	Cluster Differenciation 3
CD4	Cluster Differenciation 4
CD5	Cluster Differenciation 5
CD8	Cluster Differenciation 8
CD11	Cluster Differenciation 11
CFU	Colony Formation Unity
DC	Dendritic Cell
FBM	Fermented Bifido Milk
FBMHT	Fermented Bifido Milk Heat Treated
FITC	Fluorescein
FMO	Fluorescence Minus One
GIT	Gastrointestinal Tract
HE	Hematoxilin eosin
IgA	Imunoglobulin A
IgD	Imunoglobulin D
IgM	Imunoglobulin M
IL-2	Interleucin 2
IL-4	Interleucin 4
IL-5	Interleucin 5
IL-6	Interleucin 6
IL-9	Interleucin 9
IL-10	Interleucin 10
IL-13	Interleucin 13
IL-25	Interleucin 25
IL-33	Interleucin 33
IP	Intra peritoneal
LAB	Lactic Acid Bacteria
log	Logaritm

mg	Microgram
min	Minute
mL	Mililiter
N	Number
NF-κB	Nuclear Factor kappa B
NK	Natural Killer
PC	Phagocitic Capacity
PE	Phicoeritrin
pIgR	Polymeric Immunoglobulin Receptor
PP	Peyer Patch
SPF	Specif Pathogen-Free
TCR	T Cell Receptor
TGF-β	Transformiggrow factor beta
Th1	T helper 1
Th2	T helper 2
Th9	T helper 9
Th17	T helper 17
Th22	T helper 22
TLR	Toll Like Receptor
TNF-α	Tumor Necrosis Factor alpha
UFBM	Unfermented Bifido Milk
UHT	Ultra Hight Temperature
Γ-INF	Gama Interferon
µg	Microgram
µm	Micrometer

ABSTRACT

Functional food industry is in expansion mainly due to probiotic and prebiotic products. Studies have shown some probiotic strains develop immune modulation effect, however, these results are controversial and the mechanisms are not been well understood. Although, some probiotic strains increase IL-10 and γ-INF release modulating immune response, this response is weaker in probiotic strains when compared to pathogenic Gram-positive bacteria. The major aim of the present study was to assess the effect of probiotic fermented milk in cellular immune response of Balb/c mice colon. The specific objectives were: (i) to determine the immunomodulation of the milk added of probiotic in normal mice; (ii) to identify the cellular types implied in immune specific response and, (iii) to colocalize them in histological sections. Besides, the analyze and comparation of the probiotic resistance upon *in vitro* gastrointestinal and bioactive metabolites release in fermented or unfermented bifido milk using the same matrix, probiotic strain and probiotic dose in CFU. mL^{-1} were conducted. Dairy products were prepared in which variable form of technological appliance were: (i) milk, (ii) water, (iii) unfermented milk, (iv) fermented milk, and (v) fermented and heat treatment milk, all using *Bifidobacterium* subsp. *lactis* HOWARU HN019 strain in the same concentration. The skimmed milk and water were used as controls. The immune effects were evaluated by histological sections and the lymphocytic infiltrated was analyzed by flow citometry and histology.

Key words: Matrix-mucosa-probiotic interaction, bifidobacterium, fermented milk, immune modulation, B-1 cell.

RESUMO

O principal crescimento na indústria de alimentos funcionais corresponde ao dos produtos probióticos e prebióticos. A literatura mostra efeitos imunomoduladores de certas cepas probióticas, contudo, os resultados são às vezes controversos e os mecanismos implicados ainda são pouco elucidados. Sabe-se, no entanto que algumas cepas de probióticos aumentam significantemente a liberação de IL-10 e γ-INF modulando a resposta imune, além destas respostas serem de forma mais branda relacionada às bactérias Gram-positivas probióticas do que às Gram-positivas patogênicas. O presente trabalho teve como objetivo geral estudar o efeito do leite probiótico fermentado na resposta imune celular em cólon de camundongos BALB/c. Os objetivos específicos foram: (i) determinar o efeito imunomodulador do leite adicionado de probiótico em camundongos normais, (ii) identificar os tipos celulares implicados na resposta imune específica por citometria de fluxo e, (iii) colocalizá-los nos cortes histológicos. Simultaneamente, a análise e a comparação da resistência do probiótico à digestão gastrintestinal *in vitro* e a produção de metabólitos bioativos de acordo com os deferentes produtos foi realizada. Foram preparados leites nos quais as variáveis estudadas foram a tecnologia empregada para a produção das formulações (a) leite; (b) água, (c) leite não fermentado; (d) leite fermentado; (e) leite fermentado seguido de pasteurização, usando a mesma concentração da cepa comercial *Bifidobacterium animalis* subsp. *lactis* HOWARU HN019. O leite desnatado e a água foram usados como controles.

Palavras chaves: Interação matriz-mucosa-probiotico, bifidobactéria, leite fermentado, imuno modulação, células B-1.

INTRODUCTION AND OBJECTIVES

The immune response is initialized after exposition of foreign antigens or suffer tecidual injury initializing the inflammatory process starting adaptive response, maintaining the homeostasis control when have persistent injuries. Besides that, the immune homeostasis unbalance provides severe inflammation process and uncontrolled tecidual damage and disease (Fang Yan & Polk, 2011).

Humans, like animals, live in continuum healthy association between Gastrointestinal Tract (GIT) and microorganisms. The main benefit of these symbioses is the resistance increase of host infection diseases (Sullivan & Nord, 2002). On the other hand, the microbiota composition could be affected for many food and environment factors that increase digestive disorders or diseases susceptibility to the host.

In 1907, Metchnikoff had demonstrated that intake of fermented products could reestablished the gut microbiota and promote beneficial effects to humans and animals. Nowadays, the recent research showed the key effect of microbiota in maintains and increase quality life. Some workers showed that health animals with complete microbiota are more resistant to infections than that deprived of microbiota (Perdigon, Galdeano, Valdez, & Medici, 2002). Moreover, a lot of studies (Ibnou-Zekri, Blum, Schiffrin, & von der 5 Weid, 2003; Lodinova-Zadnikova, Cukrowska, & Tlaskalova-Hogenova, 2003; Mercenier, Pavan, & Pot, 2003; Reid & Burton, 2002) showed that probiotic food promote many favorable effects like activate immune response (Paturi, Phillips, & Kailasapathy, 2008), reestablish of colon microbiota balance, treatment of some

urogenital and gut infections (Isolauri, Kirjavainen, & Salminen, 2002), risk reduction of allergy, cancer and ulcerous (Lodinova-Zadnikova et al., 2003). The probiotic microorganisms applied for mainly functional food are lactic acid bacteria (LAB), especially *Lactobacillus* ssp. and *Bifidobacterium* ssp. (Borriello et al., 2003; Sullivan & Nord, 2002).

Probiotic activity is strain specific, further all, the beneficial effect attributed to a specific strain could not be attributed to another without test, even thought they belongs to the same specie (Holzapfel, Haberer, Geisen, Björkroth, & Schillinger, 2001).

The bifidobacteria, *Bifidobacterium* genera, had shown a dependent culture medium cellular polymorphism (bifido or raminhosus) like N-acetilglucosamine, alanine and calcium dependence. Many bifidobacteria species and strains with different functional properties could colonize the human gut (Matto et al., 2004). The bifidobacteria are Gram-positive, immobile, no sporulated, anaerobic (some species could tolerate the oxygen), catalase negative (except *B. indicum* and *B. asteroides*) and sacarolitics. Their “ecologic niches” are the human gut, the oral cavity and the animal GIT (Ventura, van Sinderen, Fitzgerald, & Zink, 2004).

In order to achieve the beneficial effects of probiotic strain, *Lactobacillus* or *Bifidobacterium*, even another one, should be stay in concentration of 10^7 viable cells per gram at gut (Stanton et al., 2001). This concentration depends on the food matrix and quantity daily consumed. However, recent studies showed that some probiotics not viable could exert similar effect to immune system (Bautista-Garfias, Ixta-Rodriguez, Martinez-Gomez, Lopez, & Aguilar-

Figueroa, 2001; Kankaanpaa, Sutas, Salminen, & Isolauri, 2003; Lammers et al., 2003; Ruas-Madiedo, Hugenholtz, & Zoon, 2002).

The immune response modulation by protiocs promoted an increased researcher interests beside the fact that infections, allergies and immune deficiencies are in focus in human public health (Chiang, Sheih, Wang, Liao, & Gill, 2000; Cunningham-Rundles et al., 2000). Earlier studies showed that some bifidobacteria are able to stimulated the immune function activating macrophages, IgA production, anti tumoral effects, allergy reduction and a complex regulation net that reflects the overlap of adaptive over innate immunity and shown the interaction between microbiota and immune system to mntain the gut homeostasis (Sawa et al., 2011).

The *in vitro, ex vivo* and *in vivo* methodology are applied in animals and rarely in man to analyze the immunomodulation effect promoted by probiotics in oral tolerance, allergies and infections. This information could be an immunotherapy alternative or prevention therapy to treat the immune pathologies and abnormalities (Calder & Kew, 2002; Noverr & Huffnagel, 2004; Ouwehand, Salminen, & Isolauri, 2002).

The main mechanism of action attributed to probiotics is the control of pathogens microorganisms through production of antimicrobial substances, competitive exclusion of nutrients and places, interaction with indigenous microbiota and immune modulation. Unfortunately, the cellular and molecular mechanisms that probiotic affect the indigenous microbiota still uncovered (Isolauri, Sutas, Kankaanpaa, Arvilommi, & Salminen, 2001; Perdigon, Locascio, Medici, Holgado, & Oliver, 2003; Uronis et al., 2011; Vasiljevic &

Shah, 2008). New studies are necessary to elucidate those observations.

Since the necessity to elucidate the cellular and molecular mechanism, which the probiotics are involved to promote immunomodulation in host, this study aimed to evaluate fermented and unfermented probiotic milk and fermented probiotic milk followed by heat treatment in immune response in healthy BALB/c colon mice using the strain *Bifidobacterium animalis* subsp. *lactis* HOWARU HN019.

The specific objectives were:

(i) Developed probiotic products stable and with at least 10^7 CFU.mL^{-1} counts of probiotic bacteria:

- *Bifidobacterium animalis* subsp. *lactis* HOWARU HN019 unfermented milk (UFBM);
- *Bifidobacterium animalis* subsp. *lactis* HOWARU HN019 fermented milk (FBM);
- *Bifidobacterium animalis* subsp. *lactis* HOWARU HN019 fermented milk followed heat treatment (FBMHT);

(ii) Analyze and compare the probiotic resistance upon *in vitro* gastrointestinal and bioactive metabolites release in fermented or unfermented bifido milk using the same matrix, probiotic strain and probiotic dose in CFU.mL^{-1}.

(iii) Determine the immune modulator effect promoted by *Bifidobacterium animalis* subsp. *lactis* HOWARU HN019 fermented milk or not and fermented followed pasteurization in healthy isogenic BALB/c mice.

CHAPTER 1 – PROBIOTICS AND IMMUNE SYSTEM: AN OVERVIEW

ABSTRACT

The major attribute of the mucosal immune system is the ability to discriminate between harmful pathogens and the harmless members of the microbiota of gastrointestinal, pulmonary, nasopharyngeal, oral, ocular, and genitourinary tracts. Of the various mechanisms involved, the numerous and complex interactions between the microbiota and the local immune system found in the mucosa play a first-line role. The roles of probiotic bacteria do not have a clear image yet indeed it is also well documented. Hence, are described some studies analyzing clinical effects, with variable levels of proof, suggesting a hypothesis of the mechanisms of action, through which these effects may occur.

1.1. INTRODUCTION

The immune response is initiated by innate immunity following exposure to foreign substances or tissue injury. Innate immunity exerts protective roles in host homeostasis in part by priming adaptive immune responses and inducing inflammation. However, the unbalanced immune response may lead to severe inflammation, uncontrolled tissue damage and disease (Yan & Polk, 2011).

Humans live in symbiosis with a diverse community of micro-organisms, these symbionts can be mutualists (benefiting themselves and the host), commensals (benefiting just themselves) or pathogens (benefiting themselves by harming the host) (Reid et al., 2011).

Probiotics are live microorganisms that when administrate in adequate amounts confer health benefit on the host (FAO/WHO, 2002). The benefits include immunomodulation, antagonistic activity towards gastrointestinal pathogens through bacteriocin production (Gotteland, Brunser, & Cruchet, 2006), effects on cholesterol and lactose metabolism, antimutagenic and anticarcinogenic properties (T. Vasiljevic & N.P. Shah, 2008). Sensing of the intestinal microbiota by the host, mucosal immune system plays significant roles in maintaining intestinal homeostasis and inducing systemic protective responses (Yan & Polk, 2011).

This review focuses on the actual knowledge about probiotic effect and the possible mechanisms involved in modulation of acquired and innate immunity.

1.2. IMMUNE SYSTEM

The complexity of the immune system includes two major components: innate and adaptive immunity, which work in concert to protect us from external and internal injury (T. Vasiljevic & N.P. Shah, 2008).

1.2.1. *Innate immune system*

The innate immune system acts as the first line of defense against pathogens without specificity. The major characteristic is the quickness of response. Cells participating in innate immunity react rapidly to challenge by infectious agents, allowing for early protection of the host, succeeding an inflammatory reaction in an attempt to eliminate the invading agent. The

phagocytic cells, like neutrophils, monocytes (MO) and macrophages (Mø) are the main players in the innate immune response and are able to produce cytokines recruiting other inflammatory cells. Natural Killer (NK) plays a role in immunological surveillance and reacts to the presence of virus infected cells in the early stages of infection by killing the infected target cell. Dendritic cells (DCs), along with macrophages and monocytes, provide an interface between the innate and adaptive immune systems as professional antigen-presenting cells (APCs) (Delcenserie et al., 2008).

Discrimination between self and non-self has to be realized by innate immune cells (Delcenserie et al., 2008). This is achieved partly, by an evolutionary-conserved family of cell surface and cytosolic receptors, referred as toll-like receptors (TLRs), which function in microbial recognition. The ability of TLRs to discriminate between pathogens and commensals is not clear yet, however, these complex regulatory systems, derived both from host and bacterial origin, appear to reinforce and support this balance. Host factors that modulate and alter TLR-mediated signaling have recently been defined and are thought to control the level of immune activation. Similarly, certain gut bacteria are also recognized to suppress unnecessary inflammatory responses, thereby helping to maintain immune homeostasis. Their relative contribution to these regulatory processes is currently unknown. The host transcription factor, nuclear factor kappa B (NF-κB) has been consistently identified as an important target molecule for bacterial regulation. NF-κB, which is also essential for immune activation, is an important therapeutic target for the treatment of inflammatory bowel diseases (Kelly & Conway, 2005).

1.2.2. *Adaptive immune system*

In contrast, the adaptive system is acquired through interactions with the environment. It is subject to induction, anticipation (immune memory) and clonal expansion (T. Vasiljevic & N. P. Shah, 2008). Lymphocytes B and T are the essential players in the adaptive immune response and can provide more effective protection against pathogens through their ability to recognize and remember an impressive number of antigens.

Uncommitted helper T cells can be induced to different towards T helper 1 (Th1), Th2, Th17 and regulatory (Treg) phenotypes according to the local cytokine millieu. Th1 cells produce pro-inflammatory cytokines like IFNγ, TNFα, lymphotoxin (TNFβ) and IL-2, while Th2 cells produce the cytokines IL-4, IL-5, IL-6, IL-9 and IL-13. Th9, Th17 and Th22, another T helper cells participate in Th1 and Th2 differentiation (Wisniewski & Borish, 2011; Afzali et al, 2007). The cytokines produced by Th1 cells stimulate the phagocytosis and destruction of microbial pathogens while Th2 cytokines such as IL-4 generally can stimulate the production of antibodies directed toward large extracellular parasites. IL-5 stimulates eosinophil responses, also part of the immune response toward large extracellular parasites. On the negative side, Th1 pathway seems to be involved in organ-specific autoimmune diseases such as arthritis and multiple sclerosis while Th2 pathway is seen as underlying allergy. Th1 differentiation is reliant on IFN−γ and IL-12 whereas Th2 development relies on IL- 4 (Delcenserie et al., 2008; Wisniewski & Borish, 2011). Finally, the presence of a further subset of CD4+ T helper cells with pro-inflammatory properties, called Th17, are characterized by the production of IL-17 and differentiation by TGF-β and IL-6 cytokines (Bettelli et al., 2006). The balance between Th1 and Th2 cytokine

production can determine the direction and outcome of an immune response. A true balance between Th1 and Th2 profiles can be difficult to maintain, as Th1 and Th2 cells inhibit each other. However, the regulatory T cells, a minor population of $CD4^+$ T cells (~10%) that co-express CD25 are crucial for the control of autoreactive T cells, can also intervene to block either Th1 or Th2 activity or both (Wisniewski & Borish, 2011).

1.3. MUCOSAL IMMUNE SYSTEM

The knowledge about the influence of the resident microbiota on mucosal immune function and gut health has become well recognized in the past decade (Macpherson et al, 2011) as an active dialogue between the symbionts microorganisms and the host mucosal immune system (Dogi & Perdigon, 2006; Macpherson & Harris, 2004). This cross talk elicits different host responses to commensal and pathogenic bacteria, which can be variably labeled symbionts or pathobionts, having a profound effect in different animal model systems (Macpherson et al, 2011).

Symbionts bacteria may even share molecular patterns recognized by toll-like receptors (TLRs), which can recognize patterns associated mainly with pathogens (Janeway & Medzhitov, 2002). The healthy host is able to elicit a balance mucosal immune response against luminal antigens and to maintain a “physiological state of inflammation” in the gut, but it is also capable of responding to invading commensal organisms or pathogens. In the healthy host the penetration of the commensal bacteria is usually prevented by the physical barrier afforded by the intestinal epithelium and by the immune cells associated with the mucosa, which are highly adapted to the presence of the normal

microbiota. If the commensal microorganisms invade the host tissues, the innate immune mechanisms contribute to their rapid clearance, but when pathogens enter the intestine, innate and adaptive mechanisms are coordinately stimulated to respond to the danger signals (Janeway & Medzhitov, 2002), which are highly adapted to the presence of the normal microbiota. If the commensal microorganisms invade the host tissues, the innate immune mechanisms contribute to their rapid clearance, but when pathogens enter the intestine, innate and adaptive mechanisms are coordinately stimulated to respond to the danger signals (Janeway & Medzhitov, 2002).

The particular characteristics of soluble, particulate antigens and pathogens will affect the gut immune response in relation to the way that they initiate the interaction with the immune system. At least three different routes exist for the uptake of luminal antigens: DC, specialized M cells from the Peyer's patches (PP), and individual M cells found in the villous epithelium (Neutra et al., 2001). The anatomical location of the immune cells from the innate response and the way by which these cells acquire antigens are crucial in determining the nature of the subsequent responses. Many attempts have been made to understand the gut immunomodulation by pathogenic bacteria but not the mechanisms involved in the modulation of the gut immune system by commensal bacteria and by nonpathogenic microorganisms present in many foods included in the daily diet (Galdeano, de LeBlanc, Vinderola, Bonet, & Perdigon, 2007).

Mucosal epithelial cells form an efficient barrier, which prevents antigens from environmental pathogens from gaining access to the host milieu. Flagellated microorganisms, including symbionts, trigger epithelial homeostatic

chemokine responses that recruit immune cells of the innate immune system to the epithelium and lamina propria of the intestine to link the innate or/and the adaptive immune response (Rumbo et al., 2004). It has also been shown that commensal bacteria can activate TLR signals (Iwasaki & Medzhitov, 2004). TLR signals are essential, not only for response to pathogens (Netea, Van der Graaf, Van der Meer, & Kullberg, 2004) but also to maintain the intestinal barrier function (Rakoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004). Cario, Gerken, & Podolsky (2005), had shown that intestinal epithelial cells express several TLRs, including TLR2 and TLR4, *in vitro* and *in vivo*. As the frontline of the mucosal immune system, the intestinal epithelium constantly is exposed to large amounts of various TLR ligands that appear to coexist in the intestinal mucosa. To maintain mucosal homeostasis, inflammatory responses are suppressed toward symbionts, leading to the phenomenon of tolerance or ignorance in the healthy gut.

1.3.1. *Gut immune system*

In the gut, immune response induced by commensal bacteria, the antigen presentation from the luminal microbiota, leads to the generation of large quantities of local immunoglobulin A (IgA) without induction of systemic immunity (Neutra et al., 2001). IgA is the most abundantly produced immunoglobulin in the body; it is mainly secreted as a dimer across the epithelial cell layer through a specialized transport system. Classical experiments showed that IgA+ B cells are induced in the Peyer's patches and circulate through the mesenteric lymphatics to enter the blood stream via the thoracic duct and home back to the intestinal mucosa. Similar recirculation also

occurs with many intestinal T cells. Studies on the functional importance of secreted IgA, show that it can neutralize viruses or intraluminal toxins or during transport via the polymeric immunoglobulin receptor (pIgR). This presumably accounts for only a tiny proportion of the IgA, as the comparisons between germ-free and specific pathogen-free (SPF) mice show that the abundance of intestinal IgA-secreting plasma cells depends on the presence of commensal bacteria. Despite this, we have evidences that the role of IgA is to prevent commensal bacterial penetration or to limit the growth of bacteria and their densities in the lumen of the intestine. Initial studies of the mechanisms of IgA induction and the cytokine requirements were carried out using cell culture systems. These showed that TGF-β and IL-4 promoted the switch from surface IgM to IgA expression and that IL-2, IL-6, and IL-10 worked in a synergistic fashion. Experiments in which cellular components (B and T lymphocytes and dendritic cells) were purified from different secondary lymphoid structures and reconstituted *in vitro* showed that the IgA switch was much more efficient when leukocytes—especially dendritic cells—were derived from Peyer's patches than from other cellular sources. This suggests that IgA+ B cell induction takes place locally within the mucosa, although the system is primitive in terms of T independence and the superfluity of compartmentalized B, T, and follicular zones within the intestinal lymphoid follicles (Macpherson et al. 2011).

The earliest site of B cell production is the fetal liver, but after birth B cells are produced in both the bone marrow and pleuropericardial cavities of mice. The progeny of these different sites can be distinguished according to their surface markers: B1 cells from the pleuropericardium stain strongly for IgM, Mac-1, and CD5 (B1a), but weakly for B220 and IgD. The situation is reversed

for B2 cells from the bone marrow, in which strong B1 markers stain weakly and vice-versa. B1 cells are a major source for IgM antibodies specific for bacterial cell wall components. The surface markers that characterize B1 and B2 cells are down regulated as plasma cells differentiate, but the relative contribution of each has been assessed indirectly by reconstituting radiation chimeras with allotypically marked bone marrow and peritoneal leukocytes. In most cases, this has shown that B1 cells are the source of up to half the secretory intestinal IgA, although much lower proportions (≤10–15%) have also been found after recolonization of gnotobiotic chimeras in which neonatal antibody depletion preceded reconstitution. The reconstitution experiments in TCRβ−/− δ−/− mice showed that peritoneal B1 cells reconstituted most of the T independent IgA. The interpretation of these reconstitution experiments relies on the independence of the adult B1 and B2 lineages; this is in itself controversial, as in immunoglobulin transgenic and “knock-in” mice B cells can be generally distributed with B1 or B2 phenotypes predominating depending on their B cell receptor specificity and surface density rather than site of origin. However, in an independent approach a substantial contribution of B1 cells to intestinal IgA production was also detected in MHC class II−/− mice, where antigen-specific intestinal IgA was abrogated when the strain was made deficient of Bruton kinase (*xid*), which causes deficiency of B1a cells (MacPherson & Uhr, 2004).

The endogenous intestinal microbiota exerts a beneficial effect by creating a natural line of defense against infection and adverse environmental conditions. Certain physiopathological and environmental conditions are known to be able to alter the composition and metabolism of the intestinal microbiota to a greater or lesser degree. Antibiotics, changes in dietary habits and stress can

all result in changes in the composition and/or metabolism of the intestinal microbiota, which could affect the physiological parameters of the host such as the immune system.

1.4. PROBIOTICS

Probiotics have been with us for as long as people have eaten fermented milks (around 10000 years ago), but their association with health benefits dates from the Metchnikoff studies in the 1900´s (Fuller, 1991). Recommendations by (FAO/WHO, 2002) working group on the evaluation of probiotics in food, suggest the definition: “probiotics is live microorganisms that when administered in adequate amounts confer a health benefit on the host”. Consequently, a wide variety of genus and species could be considered potential probiotics, commercially, however, the most widespread genus are lactic acid bacteria (LAB) (Vasiljevic & Shah, 2008).

In order to survive, probiotic bacteria entering by the mouth must be resistant to pH, bile acid, proteolytic enzymes, antimicrobial peptides, intestinal peristalsis, and luminal secretory IgA blocking (Galdeano et al., 2007; Perdigon, Medina, Vintini, & Valdez, 2000; Tuomola, Crittenden, Playne, Isolauri, & Salminen, 2001).

Lactic Acid Bacteria (LAB) are usually described as Gram-positive microorganisms, devoid of cytochromes and preferring anaerobic conditions but are aero-tolerant, acid-tolerant, and strictly fermentative, producing lactic acid as a main product (Stiles & Holzapfel, 1997). The most important genera are: Lactobacillus, Lactococcus, Enterococcoccus, Streptococcus, Pediococcus,

Leuconostoc, and Bifidobacterium. Members of the LAB are usually subdivided into two distinct groups based on their carbohydrate metabolism. The homofermentative group consisting of Lactococcus, Pediococcus, Enterococcus, Streptococcus and some lactobacilli utilize the Embden–Meyerhof–Parnas (glycolytic) pathway to transform a carbon source chiefly into lactic acid. Heterofermentative bacteria, in turn, produce equimolar amounts of lactate, CO_2, ethanol or acetate from glucose exploiting phosphoketolase pathway. Members of this group include Leuconostoc, Weissella and some lactobacilli. The species belonging to Enterococcus genus are frequently found in traditional fermentations and may be included as a component of some mixed starters (Vasiljevic & Shah, 2008).

As demonstrated by Ley, Peterson, & Gordon (2006), using culture-independent molecular methods, dietary factors can lead to long-term changes. This general stability is made possible by the recognition and tolerance of the infant acquired microbiota by the gut immune system (Ouwehand, Salminen, & Isolauri, 2002). Comparative studies of adults with varying degrees of relatedness have shown that host genotype is more important than diet, age, and lifestyle in determining the composition of the gut microbiota (Hopkins, Sharp, & Macfarlane, 2001; Zoetendal, Ben-Amor, Akkermans, Abee, & de Vos, 2001).

It was previously thought that to have an effect on the immune system, the probiotic strains must remain viable. In 2007, (Galdeano et al., 2007) demonstrated that this fact is true only for some strains. For *Lactobacillus delbrueckii* subsp*. bulgaricus*, viability was not necessary for the induction of positive cells producing cytokines, although the number of positive cells was

comparatively lower than the number obtained with viable *L. delbrueckii* subsp. *bulgaricus* organisms. The viability was critical for determining the time of residence in the gut with differences between viable and nonviable probiotic bacteria administration; nonviable bacteria were cleared more rapidly. The probiotic bacteria must remain in the gut at least 48 to 72 h to be effective; that is the time required for any particulate antigen to induce gut immunostimulation (Galdeano et al., 2004; Galdeano et al., 2007). This fact is a very important finding, indicating the importance of daily administration in a dose established for each probiotic bacterium to have an adjuvant effect without the induction of oral tolerance (Galdeano et al., 2007).

1.4.1. Gut Microbiota

The biofilm-like architecture of the mucosal microbiota, in close contact with the underlying gut epithelium, facilitates beneficial functions including nutrient exchange and induction of host innate immunity. Fecal samples are often used to investigate the intestinal microbiota because they are easily collected. However, the degree to which composition and function of the fecal microbiota differ from mucosal microbiota remains unclear (Eckburg et al., 2005). With the development of methods for identifying gut microbiota that do not require culturing (i.e., molecular fingerprinting and ecological statistical approaches), a much more thorough and reliable assessment of the gut microbiota is now possible (Gill et al., 2006; Palmer, Bik, DiGiulio, Relman, & Brown, 2007; Bogsan et al., 2011).

The sequencing of 16S ribosomal RNA (rRNA) genes from amplified bacterial nucleic acid extracted from fecal material or mucosal samples has

greatly facilitated the identification and classification of bacteria (Macfarlane & Macfarlane, 2004). The study of entire microbial communities using metagenomic approaches based on these molecular methods has revealed a much greater diversity in the bacterial and archaeal domains than was previously thought to exist and has helped determine the community structure of several other previously unknown ecosystems (Frank & Pace, 2008; Gill et al., 2006).

Using these techniques, investigators have estimated that the gastrointestinal tract in an adult human contains approximately 10^{12} microorganisms per milliliter of luminal content and harbors approximately 500 to 1000 distinct bacterial species (Eckburg et al., 2005; Gill et al., 2006; Ley et al., 2005). Frank et al (2007) suggests that this number is in fact much higher, with at least 1800 genera and between 15,000 and 36,000 species of bacteria. The Human Microbiome Project has analysed the largest cohort and set of distinct, clinically relevant body habitats so far. The diversity and abundance of each habitat's signature microbes to vary widely even among healthy subjects, with strong niche specialization both within and among individuals. Although a general consensus about the phylum level composition in the human gut is emerging, the variation in species composition and gene pools within the human population is less clear. (Arumugam et al., 2011; Huttenhower et al., 2012).

Despite our limited understanding of the composition of the indigenous gut microbiota, evidence suggests that it is established within the first year of life (Palmer et al., 2007) and that the transformation to adult-type microbiota is likely triggered by multiple host and external factors (Mackie, Sghir, & Gaskins,

1999), including the effects of the microbiota itself, developmental changes in the gut environment, and transition to an adult diet. The gut microbiota of the infant has long been thought to resemble that of the mother because most bacterial species are acquired during the birthing process. However, this paradigm has been brought into question by recent evidence obtained using molecular techniques showing that children's stool samples do not resemble those of their parents more than those of other adults (Mackie et al., 1999; Palmer et al., 2007). The gut microbiota remains remarkably constant after transformation to adult-type microbiota; however, transient changes can occur.

1.5. HOW PROBIOTICS WORKS ON IMMUNE SYSTEM?

Probiotic bacteria, including bifidobacteria, are largely used as live components of many functional foods (Ventura, van Sinderen, Fitzgerald, & Zink, 2004). However, despite their increased use, little is known about whether or how probiotics impact on indigenous microbiota or indeed on the host. Recently, the setting up of simplified and defined model systems, i.e. colonization of axenic mice with specific bacteria, has provided a valid tool to study functional properties and operating principles of human gut microbial communities. In this context, the combination of in silico reconstructions of microbial metabolism based on transcriptional profiles and whole genome transcriptional profiling of laser capture microdissected intestinal mucosa from germ-free and colonized mice has provided valuable information in order to dissect how resident gut bacteria and probiotic bacteria influence each other and the host (Sonnenburg, Chen, & Gordon, 2006; Turroni, Ribbera, Foroni, van Sinderen, & Ventura, 2008).

The increase in the number of IgA-producing cells was the most remarkable property induced by probiotic microorganisms or by fermented milk yogurt (Macpherson, Geuking, & McCoy, 2011; Perdigon, Galdeano, Valdez, & Medici, 2002). The IgA B cells induced in the Peyer's patches circulate through the mesenteric lymphatic nodes to enter into the blood via the thoracic duct and return to the intestinal mucosa, repopulating distant mucosal sites. Similar recirculation also occurs with intestinal T cells. Some probiotic microorganisms are also able to increase the IgA cycle, and this effect is dose dependent (De Moreno de LeBlanc & Perdigon, 2005; Rachid et al., 2002).

Some probiotic bacteria can act as adjuvants of the mucosal and systemic immune response (Perdigon et al., 1990; Perdigon et al., 2002). The stimulation with probiotic bacteria induced signals on epithelial and immune cells that evoked different patterns of cytokines in the intestine depending on the dose administered (Galdeano, de LeBlanc, & Perdigon, 2004; Vinderola, Matar, & Perdigon, 2005). The quantity of these microorganisms to achieve the adjuvant effect in the mucosal or systemic immune response was 10^8 to 10^9 CFU.day^{-1} (Galdeano et al., 2007; Vitini et al., 2000).

In the analyses of the profiles of cytokines induced by some LAB, the most remarkable effect was the increase in TNF-α, IFN-γ and in IL-10 for all the probiotic strains assayed. This effect was obtained without increasing the inflammatory response was found. However, the induction of TNF-α by the probiotic bacteria would be necessary to initiate the cross talk between the immune cells associated with the lamina propria and the intestinal epithelial cells. IFN-γ would also play a physiological role; it has been demonstrated that this cytokine is necessary for the maturation of some immune cells, such as

dendritic cells, and also controls their cellular proliferation at the intestinal level (Rumbo et al., 2004).

Kelly & Conway (2005), demonstrated that probiotic microorganisms are able to induce a gut mucosal immune response which requires the bacteria to interact with the epithelial and immune cells in the gut to induce the network of signals involved in an immune response. Probiotic bacteria may arrive in the intestine along routes, which correspond with the different pathways to the internalization of antigens. These bacteria (as whole cells or as antigenic fragments) must interact with the M cells in the Peyer's patches, with gut epithelial cells, or with the associated immune cells. After contact with these cells, the release of cytokines is induced to up- or down-regulate the immune response.

Probiotic bacteria could be also internalized through M cells in the Peyer's patches or may be sampled by dendritic cells as whole cells or their antigenic fragments (Galdeano et al., 2004; Perdigon et al., 2002). They may be captured by other DC or macrophages associated with the lamina propria to increase the signals to the epithelial cells and/or other immune cells. There is scientific evidence that the uptake of nonpathogenic bacteria or their fragments by macrophages or dendritic cells in the lamina propria is possible through direct sampling of luminal antigens for dendritic cells (Kaisho & Akira, 2002; Lee, Puong, Ouwehand, & Salminen, 2003).

Other mucosal immune mechanisms, such as the Th1 cell response, can be modulated by probiotic bacteria, this was demonstrated in pathological processes such as (i) allergy (Isolauri, 2001), (ii) in inflammatory bowel disease - treatment of patients with mild to moderate Ulcerative Colitis, witch not

responding to conventional therapy, using probiotic mixture VSL#3 results in a combined induction of remission/response rate of 94% in patients that completed the study; 77% of patients responded when analyzed in an intent to treat fashion with no adverse events noted (Bibiloni et al., 2005; de LeBlanc & Perdigon, 2004), (iii) in colon cancer - yogurt can inhibit tumor progression and promote the modulation of immune response and cellular apoptosis (de Moreno de LeBlanc, Matar, Farnworth, & Perdigon, 2006; de LeBlanc & Perdigon, 2004). Perdigón *et al.* (2005) suggested that under physiological conditions the probiotic bacteria interact with the epithelial cells and preferentially with the immune cells from the innate immune system, reinforcing this barrier (Galdeano & Perdigon, 2004; Vinderola et al., 2005). When they interact with cells from Peyer's patches, they can induce an increase of the IgA cycle (de Moreno de LeBlanc & Perdigon, 2005).

In human studies, production of cytokines (Aattouri, Bouras, Tome, Marcos, & Lemonnier, 2002; SolisPereyra, Aattouri, & Lemonnier, 1997), phagocytic activity (Schiffrin, Brassart, Servin, Rochat, & DonnetHughes, 1997), modulation of antibodies antibody production (Perez et al., 2010; Wroblewska, Kaliszewska, Malinowska, & Troszynska, 2011) and the activity of NK cells (Dong, Rowland, Tuohy, Thomas, & Yaqoob, 2010; Fink, Zeuthen, Ferlazzo, & Frokiaer, 2007) increase with consumption of yoghurts. In young children, post-vaccinal response in terms of secretory IgA is increased following consumption of certain strains of lactobacilli and bifidobacteria (Fang, Elina, Heikki, & Seppo, 2000; Yan & Polk, 2011).

It has also been demonstrated that probiotics are able to modulate lymphocyte proliferation *in vitro* (Kirjavainen, Ouwehand, Isolauri, & Salminen,

1998; Rodes et al., 2011) as well as the production of both specific antibodies and non-specific antibodies (Vitini et al., 2000) in the mouse. The results of *ex vivo* and *in vitro* studies also show that probiotics modulate cytokine production (Pessi et al., 2001).

The available data indicate that probiotics may exert immunostimulatory action by enhancing post-vaccinal humoral response (Isolauri, Joensuu, Suomalainen, Luomala, & Vesikari, 1995) in normal individuals, or by restoring (at least partially) depressed function seen for example in elderly subjects (Gill & Rutherfurd, 2001; Gill, Rutherfurd, & Cross, 2001; Gill, Rutherfurd, Prasad, & Gopal, 2000).

Clinical observations and observations in rodent models with spontaneous colitis show that the normal flora is involved in triggering of intestinal inflammation in colitis (Madsen, Doyle, Jewell, Tavernini, & Fedorak, 1999; Wallace et al., 2011) and that the ingestion of lactobacilli and bifidobacteria may result in partial remission of colitis (Gionchetti et al., 2000; Gionchetti et al., 1998).

Medici, Vinderola, Weill, & Perdigon (2005), investigating the protective capacity of the oral administration of fermented milk containing probiotic strains (*L. casei*, *L. bulgaricus*, and *S. thermophilus*) in a murine (BALB/ c mice) model demonstrated that the protection against enteroinvasive *E. coli* infection may be associated with an enhance of the intestinal mucosa immunity.

1.6 CONCLUSIONS

The most important mechanisms involved in the gut immune stimulation by probiotic microorganisms are the clonal expansion of B-lymphocyte IgA^+ and the innate immune response. The magnitude of such stimulation did not enhance the inflammatory immune response. They induced up-or down-regulation of the innate response in order to maintain the intestinal homeostasis (Galdeano et al., 2007).

The results of human and animal studies clearly suggest that lactic acid bacteria exert immunomodulatory effects. Nevertheless, while the range of experimental conditions and markers studied provide a convincing bibliographical image, it is still imprecise concerning the modes and precise degree of these effects. In particular, it appears that the exact nature of these immunomodulatory effects is largely dependent on the strains of microorganisms used and the host environment.

Chapter 2 – Differences between fermented and unfermented bifido milk: Technological approach changes the microorganism resistance upon *in vitro* gastrointestinal digestion and bioactive molecules release

ABSTRACT

Development of dairy products containing bifidobacteria is one of the main focus in food industry as health benefits attributed to this probiotic is related to its survival through gut intestinal tract and to its role in stimulating the immune system and preventing microbial gastroenteritis. The aim of this study was to analyze and compare the probiotic resistance upon *in vitro* gastrointestinal and bioactive metabolites release in fermented or unfermented bifido milk using the same matrix, probiotic strain and probiotic dose in CFU. mL^{-1}. Two technological processes were employed using skim milk UHT: (i) Fermentation: conducted 37°C until milk reach pH 4.7 controlled by CINAC system until pH 4.7 - Fermented bifido milk (FBM), and (ii) Addition of probiotic culture: after inoculation product was stored in refrigerator at 4°C - Unfermented bifido milk (UFBM).

Lactic matrix protects *B. lactis* HN019 through stomach acidity, assuring the correct probiotic counts at gut entrance. The FBM had shown a viability of 5.11 log UFC.mL^{-1} when UFBM had not viability after *in vitro* gastrointestinal digestion in products after 24 h of cold storage whilst there were respectively 5.17 log UFC.mL^{-1} (FBM) and 4.81 log UFC.mL^{-1} (UFBM) after 7 days of cold storage. Employing different technologies slightly affected the distribution of fatty acids in the products. Moreover, fermentation could bio transform some FA in bioactive compounds as shown in the little increase observed in linoleic acid

and conjugated linoleic acid. Although, it was noted a little increase in monounsaturated fatty acids and saturated fatty acids in fermented product and slight higher contents of polyunsaturated fatty acids in unfermented products, carbon chain length was not significantly affected by fermentation in bifido milks. Control milk and unfermented bifido milk showed the same peptides even after 7 days of storage. The present study shows that fermentation of milk by *B. lactis* HN019 increased bioactive peptides. In this study, it is possible to suggest that opioids, either as agonists, antagonists peptides are formed due to fermentation process, increasing the source of bioactive peptides. Finally, some storage modified peptides and the Increased antibacterial activity.

Keywords: *Bifidobacterium animalis* subsp. *lactis* HN019, fermented milk, digestion, bioactive metabolites.

2.1. INTRODUCTION

Development of dairy probiotic products is the main focus in food industry. Regarding the benefits of dairy functional foods, milk is known, beyond its nutritional properties, to contain some bioactive compounds that may enhance health (Szwajkowska et al., 2011).

Bifidobacteria are natural members of the human intestinal microbiota, in which they occur at concentrations of 10^9 to 10^{11} cells per mL of feces, and represent up to 91% of the total gut population during the early stages of life (Sanchez et al, 2006). Interest of bifidobacteria for human health is related to

their survival through gut intestinal tract and to their role for stimulating the immune system and for preventing microbial gastroenteritis (Foligne et al., 2007; Hols et al., 2005). Furthermore, sub lethal bile concentrations can also trigger a physiological adaptive response in bifidobacteria (Kurdi, et al., 2003). Biogenic compounds, as bioactive peptides or fat acids, produced by bifidobacteria were shown to be a possible mechanism for their health enhancing properties (Oh et al., 2003; Gobbetti et al. 2010)

There are many studies describing the effects of probiotics in man, from both a preventative and a therapeutic standpoint. The expected beneficial characteristics of potential probiotic strains encompass besides the physiological, immunological, metabolic and genetic traits, also, importantly, are the technological aspects. Moreover, probiotic activity is not changed just due to strain specificity but also by the technological process used and matrix in which it is delivery (Sánchez, Reyes-Gavila, Margolles, & Gueimonde, 2009).

Based on pioneering studies by Metchnikoff (1907) and Tissier (1906), the notion that ingested live bacteria could have a beneficial effect has been developed and pared down into the idea of “probiotics”, a term that has generated several definitions over time. There currently appears to be a consensus concerning the definition published by an expert committee of the FAO and WHO, which states that *probiotics* are “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002).

Fermented milks are the main vehicles of probiotics (TAMIME, 2002), and the inclusion of bifidobacteria in these products is a challenge. Besides health benefits, production of fermented milks containing bifidobacteria has

been focalized as these probiotic bacteria growths slowly in milk due their absence in essential proteolytic activity (Oliveira et al., 2001; Gopal et al, 2005. Furthermore, some important characteristics expected of probiotic strains according to Mercenier, Pavan and Pot (2003) and beyond others are: (i) capable of survival, proliferation and metabolic activity in the target site, which implies resistance to gastric acid bile; ability to persist, albeit for short periods, in the gastrointestinal tract, ability to compete with the resident flora and (ii) Viability in high populations.

Few data is available in literature, to our knowledge, regarding the effects of technological approach i.e. fermenting or not the milk by bifidobacteria and its association with possible health benefits. The aim of this study was to analyze and compare the probiotic resistance upon *in vitro* gastrointestinal and bioactive metabolites release in fermented or unfermented bifido milk using the same matrix, probiotic strain and probiotic dose in $CFU.mL^{-1}$.

2.2. MATERIALS AND METHODS

2.2.1. Experimental Procedure

Bifidobacterium animalis subsp. *lactis* HN019 (DuPont-Danisco, Madison, USA) was the probiotic strain used in this study. In brief, UHT skim milk (Molico®, Nestlé, Araçatuba, SP, Brazil). Afterwards, milk base was inoculated with 1 mL of probiotic culture to obtain the same 9.00 $\log_{10}.UFC.mL^{-1}$ counts of microorganism in each final product. Two technological processes were employed: (i) Fermentation: conducted at 37°C until milk reach pH 4.7 controlled by CINAC system (*Cynetique d'acidification,* Ysebaert, Frépillon,

France) (Spinnler & Corrieu, 1989; Florence et al., 2012). At pH 4.7, the fermentation was stopped by rapid cooling in an ice bath until 10 °C. Fermented bifido milk (FBM) was stored in refrigerator at 4°C, and (ii) Addition of probiotic culture: after inoculation product - Unfermented bifido milk (UFBM), was stored in refrigerator at 4°C. Both products were dispensed in 60 mL polietilene tereftalate cups (PET 60 mL).

Probiotics counts were determined before fermentation (D0), after 24 hours (D1) and 7 days (D7) after preparation and storage at 4°C (Dave & Shah, 1996; Saccaro et al, 2011).

2.2.2. *In vitro* evaluation of gastrointestinal survival

In vitro evaluation of gastrointestinal survival of *B. lactis* HN019 – gastric and enteric, was conducted according to Baruzzi et al. (2011) with adaptations. Briefly, in order to analyze the survival capacity of probiotic bacterium *B. animalis* subsp. *lactis* HN019 in milk prepared according to different technological processes - FBM and UFBM at D1 and D7 cold storeged - three phases of digestion were carried out. Initially, for gastric digestion, the samples were suspended in ortophosforic acid (200 mM pH 1.93 at room temperature). Porcine pepsine (Sigma-Aldrich, São Paulo, Brasil) was added to buffer at 16,000 $U.mL^{-1}$ for two hours at 37 °C under 150 rpm agitation. Subsequently, before entrance to phase enteric I, samples were washed twice and centrifuged at 5500g during 10 min at 4°C in (Centrifuge Eppendorf 5810/5810R, Hanppange, NY, USA). The pellet was dissolved in porcine pancreatine (6 $g.L^{-1}$, Sigma pr. num. P7545, 8× USP specification activity), diluted in TRIS buffer (0.1

M base TRIS, pH 8.39 at ambient temperature corresponding to pH 8.00 a 37 °C) and maintained during two hours at 37 °C under 150 rpm agitation. At the end of phase enteric I, samples were washed twice (5500g, 10 min, 4°C). Finally, phase enteric II was initiated suspending the pellet in sterile saline 0.09% solution containing 0.1% of porcine bile salts (Sigma-Aldrich, São Paulo, Brazil) and 0.3 % bovine bile salts (Sigma-Aldrich, São Paulo, Brazil) diluted in the ratio 1:10 in MRS Broth culture media, incubated for one hour under 50 rpm stirring at 37 °C. Samples were collected at the end of each phase, and enumeration of probiotic viable cells conducted. Survival was expressed as concentrations attained at different intestinal sites or the percentage of the number of ingested microorganisms.

2.2.3. Enumeration of probiotic viable cells

B. lactis was enumerated by pour plate in RCA (Oxoid, Basingstoke, UK) added with 2 µg/mL of dicloxacillin (pH 7.1) and 0.3 $g.L^{-1}$ aniline blue (InLab, São Paulo, Brazil) incubated at 37°C for 72 h under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK) (Saccaro et al., 2011).

2.2.4. Biogenic compounds determination

2.2.4.1. Fatty acids

The fermented and unfermented bifido milk where submitted to lipid extraction according to ISO method 14156 (ISO, 2001), which is dedicates method for extraction or separation of lipids and liposoluble compounds from

milk and milk products. Briefly, fatty acids methyl esters (FAME) of milk lipids were prepared by transesterification according to ISO method 15884 (ISO, 2002), that consists in a base-catalyzed methanolysis of the glycerides, followed by a neutralization with crystalline sodium hydrogen sulfate to avoid saponification of esters. Analyses of FAME were carried out in a gas chromatograph, model 3400CX (Varian, Walnut Creek, Ca., USA) equipped with a split-injection port, a flame-ionization detector and a software package for system control and data acquisition (model Star Chromatography Workstation version 5.5). Injections were performed in a 30 m long fused silica capillary column with 0.25 mm internal diameter, coated with 0.25 μm Chrompack CP-Wax 52CB (ChromTech, Apple Valley MN, USA). Helium was used as carrier gas at a flow rate of 1.5 $mL.min^{-1}$ and a split ratio of 1:50. The injector temperature was set at 250°C and the detector at 280°C. The oven temperature was initially set at 75 °C for 3 min, then programmed to increase to 150 °C at a rate of 37.5 °C min^{-1}, and then to 215 °C at a rate of 3 °C min^{-1} (Luna et al., 2004). Samples (1 μL) were injected manually after a dwell-time of ca 2s. Qualitative fatty acid composition of the samples was determined by comparing the retention times of the peaks with those of standards 05632 and 189-19 (Sigma, Chemical Co., St Louis, MO, 210 USA). The relative content of each FAME was calculated from the area of each peak, and expressed as a percentage, according to the official method CE 1-62 (AOCS, 1997). Results were grouped and expressed as percentages of short chain fatty acids (SCFA - C4:0 and C6:0), medium chain fatty acids (MCFA - C8:0 to C15:0), long chain fatty acids (LCFA - C16:0 to C18:3), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA),

according to Ackman (2007). Control milk, FBM and UFBM at D1 preparation were analyzed in triplicate.

2.2.4.2. Peptides

To analyze the potentially bioactive peptides present in milk, UFBM and FBM the samples were microfiltered (0.20 micrometre, Milippore, Billerica, MA, USA). Skimmed milk was used as control (Molico, Nestlé, São Paulo, Brazil). One milliliter of the filtrate was stored in a freezer at -80 ° C until analysis by mass spectrometry. Central Analítica - Chemistry Institute of São Paulo University, performed the analysis. In brief, peptides were analyzed on HPLC coupled to a mass analyzer type ion trap LC-MS (Bruker Daltonics MicroTOF) with time-of-flight analyzer Esquire ESI-MS (n) low resolution. For analysis, the samples are thawed at room temperature to be injected into the C18 column at flow rate of 1 $mL.min^{-1}$. The peptides were eluted thought 0.01 min gradient 5% B 30 min 60% B, 35 min 100% B, 40 min 100% B, 5 min 45% B, where B is 100% acetonitrile, adjusted to a wavelength of 280 nm.

2.2.5. Statistical analysis

Experimental procedure was carried out in two independent assays i.e. the experiment was replicated twice on different days. All analyses were conducted in duplicate. Data were analyzed using the one-way ANOVA procedure using Statistica version 8.0 (StatSoft Inc., Yulsa, USA). The differences between means were detected by Tukey test. In all analyses significance was considered $P \leq 0.05$.

2.3. RESULTS AND DISCUSSION

2.3.1. *B. lactis* HN019 survival in the product and after *in vitro* simulation of gastrointestinal digestion

Counts of *B. lactis* HN019 before fermentation (D0), after 24 hours (D1) and 7 days (D7) of storage at 4°C in fermented bifido milk (FBM) or unfermented bifido (UFBM) are shown Table 2.1. These data have shown that although counts were significant different before fermentation or addition of probiotic at D0 ($P \leq 0.05$), equal counts of viable bifidobacteria in each product – UFBM and FBM, during the seven days of storage were achieved.

Table 2.1. Number of viable cells ($CFU.mL^{-1}$) ± standard deviation of *B. lactis* HN019 in fermented bifido milk (FBM) and unfermented bifido milk (UFBM) before fermentation and during 7 days storage at 4°C.

Product	D0	D1	D7
FBM	8.72 ± 0.49^{b}	9.52 ± 0.85^{a}	9.53 ± 0.90^{a}
UFBM	9.94 ± 0.05^{a}	9.21 ± 0.30^{a}	9.00 ± 0.29^{a}

Same letters in the same column showed statistics similarity ($P \leq 0.05$). N=4.

D0: before fermentation; D1: 24 hours after fermentation; D7: 7 days after fermentation.

During the course of gastrointestinal transit, probiotic bacteria undergo drastic physiological stress - acidity, presence of digestive enzymes and bile salts, which significantly affect their survival. Figure 2.1 presents the counts of *B. lactis* HN019 after *in vitro* gastrointestinal digestion of FBM. At D1, *B. lactis* HN019 in FBM showed decrease of 2.24 log UFC.mL^{-1} after gastric digestion maintaining more than 75% of survival ratio. After enteric digestion phases I and II, counts were respectively 5.92 log UFC.mL^{-1} and 5.11 log UFC.mL^{-1}. At the end of gastrointestinal digestion the cells viability suffered a decrese of 45.52% ofsurvival ratio. At D7, the probiotic bacteria suffered an adaptation in the product, and may resist to digestion of enteric phases I and II, presenting respectively 6.93 log UFC.mL^{-1} (75.08%), 6.40 log UFC.mL^{-1} (69.34%) and 5.17 log UFC.mL^{-1} (56.01%) of viable cells count respectively.

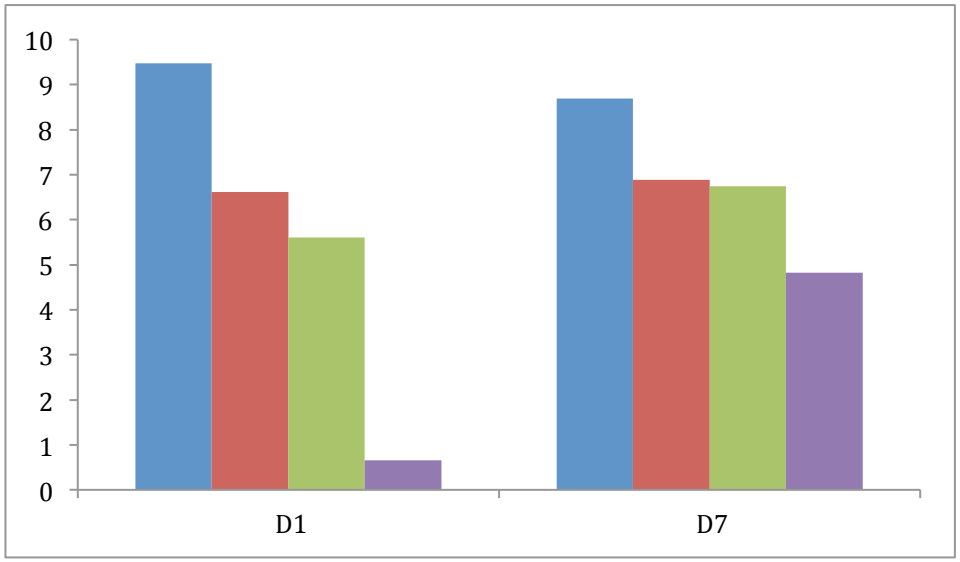

Figure 2.1. *B. lactis* HN019 counts (log UFC.mL^{-1}) after *in vitro* digestion of FBM after 24 hours (D1) and 7 days (D7) of storage at 4°C.

Similar resistance of throughout of gastrointestinal digestion could be observed in UFBM (Figure 2.2). At D1, the unfermented *B. lactis* HN019 milk had suffer a reduction of 31.89% (6.45 log UFC/mL) after gastric digestion. After enteric phases I and II, counts were respectively 6.45 log UFC.mL^{-1} and 5.73 log UFC.mL^{-1}. At the end of gastrointestinal digestion there were 60.51% of viable *B. lactis* HN019 cells. At D7, counts of probiotic bacteria in UFBM after gastric, enteric digestion phases I and II were respectively 6.92 log UFC.mL^{-1} (79.81%), 6.73 log UFC.mL^{-1} (77.62%) and 4.81 log UFC.mL^{-1} (55.48%).

At end of gastrointestinal digestion counts of *B. lactis* HN019 in FBM resulted in decrease of 4.27 log of viable cells to product digested after 24h of storage at 4°C. In the contrast, UFBM *B. lactis* HN019 survival at D7 were 5.49 log highest than D1 after digestion process and showed a decrease of 3.86 log of viable cells.

These data confirm that the lactic matrix protects *B. lactis* HN019 trough stomach acidity, assuring the correct probiotic counts at gut entrance. Cold storage promoted a probiotic adaptability that could better resist to enteric digestion and be delivered in appropriate amounts to colon. These data suggests that the probiotic resistance to *in vitro* gastrointestinal digestion is not related to technology applied, but the protection and adaptability of the matrix in resisting of digestion process (Sanchez, 2012). Finally, it is considered that survival of a microorganism within the gastrointestinal tract is necessary to allow the organism in question to exert an effect upon its host although bacterial lysis in the intestinal milieu can release biologically active substances.

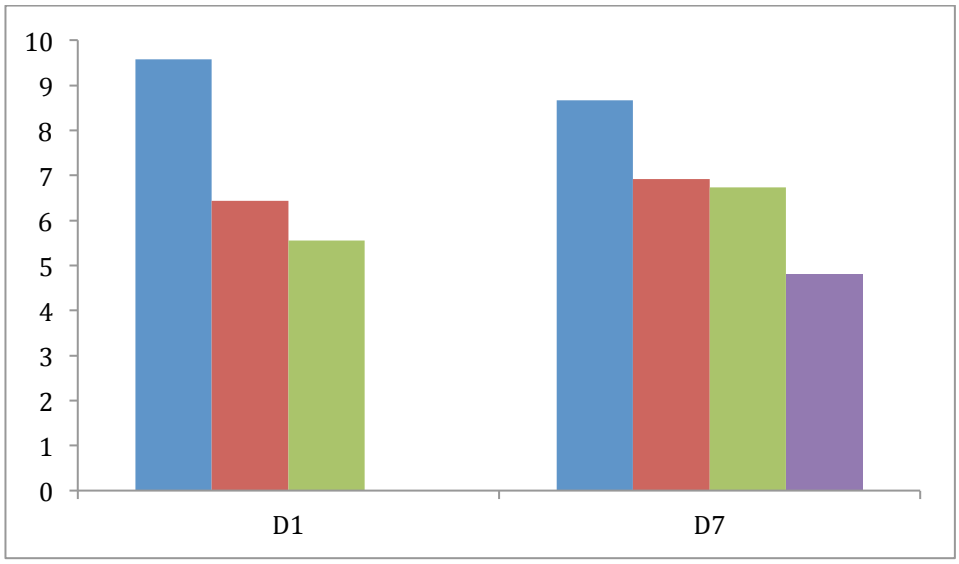

Figure 2.2. *B. lactis* HN019 counts (log UFC/mL) after *in vitro* digestion of UFBM after 24 hours (D1) and 7 days (D7) of storage at 4°C.

2.3.2. Biogenic compounds released in the products

2.3.2.1. *Bioactive fatty acids*

It has been demonstrated that the dietary intake of benefic fatty acids and probiotics may impact on the modulation of microbiota and consequently handing on health benefits on the host (Bogsan et al., 2011).

The fatty acids profile in FBM and UFBM are shown in Figures 2.3, 2.4 and 2.5. It could be seen that the main fatty acids in the products were C16:0, palmitic acid and C18:1 (Oleic acid). Significant differences were observed in fatty acids profile of both products ($P \leq 0.05$) for C8:0 (Caprilic acid), C10:0 (Caproic acid), C14:0 (Miristoleic acid), C16:0 (Palmitic acid), C17:0

(Heptadecanoic acid), C18:1 (Oleic acid), C18:2 (Linoleic acid), 20:1 (Eicoseinoic cis-11 acid) and 20:3 (Eicosatrienoic cis 8,11,14 acid). Conjugated linoleic acid (CLA) was detected in amounts of 0.93% (FBM) and 0.91 % (UFBM), with significant differences ($P \leq 0.05$). However, both products presented similar amounts of α-linolenic acid (ALA) ($P \leq 0.05$).

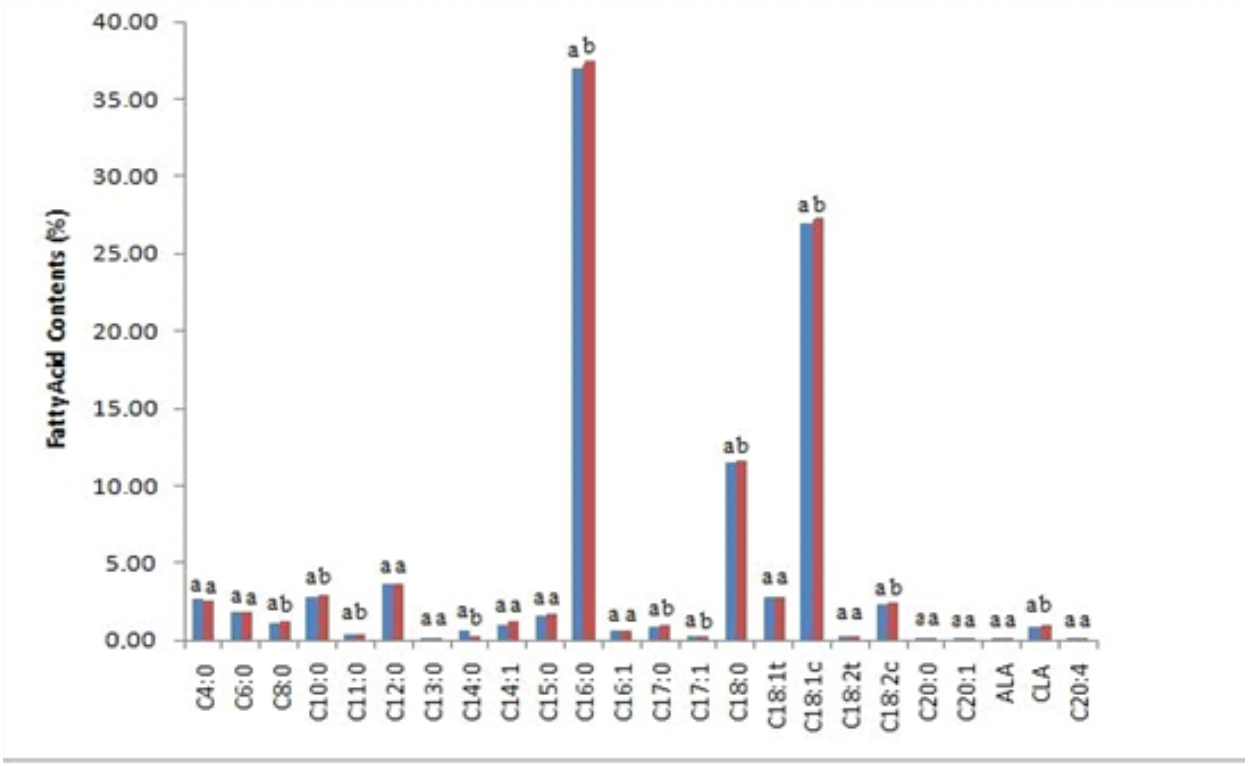

Figure 2.3. Fatty acids profile (%) in fermented bifido milk (FBM) and unfermented bifido milk (UFBM). Means (N = 6) with different letters in the same bar are significantly different. Tukey test $P \leq 0.05$.

CLA in fermented milk and dairy products should provide “functional” aspects as inhibition of initiation of carcinogenesis process, effects on anti-atherogenic, anti-adipogenic, anti-diabetogenic and anti-inflammatory activities, beneficial regulatory effects on immune function, and alters the low-density

lipoprotein/high-density lipoprotein cholesterol ratio (Florence et al, 2009). Moreover, some previous research showed enhancement of CLA (Oh et al., 2003; Bisig et al., 2007, Florence et al., 2009 and Oliveira et al., 2009) and ALA levels (Espirito Santo et al., 2010 and 2012) using lactic acid bacteria and bifidobacteria in yoghurt-like products.

Saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in fermented bifido milk (FBM) and unfermented bifido milk (UFBM) could be seen in Figure 2.4. When results were grouped, significant differences were observed in fatty acids amounts when comparing both products ($P \leq 0.05$).

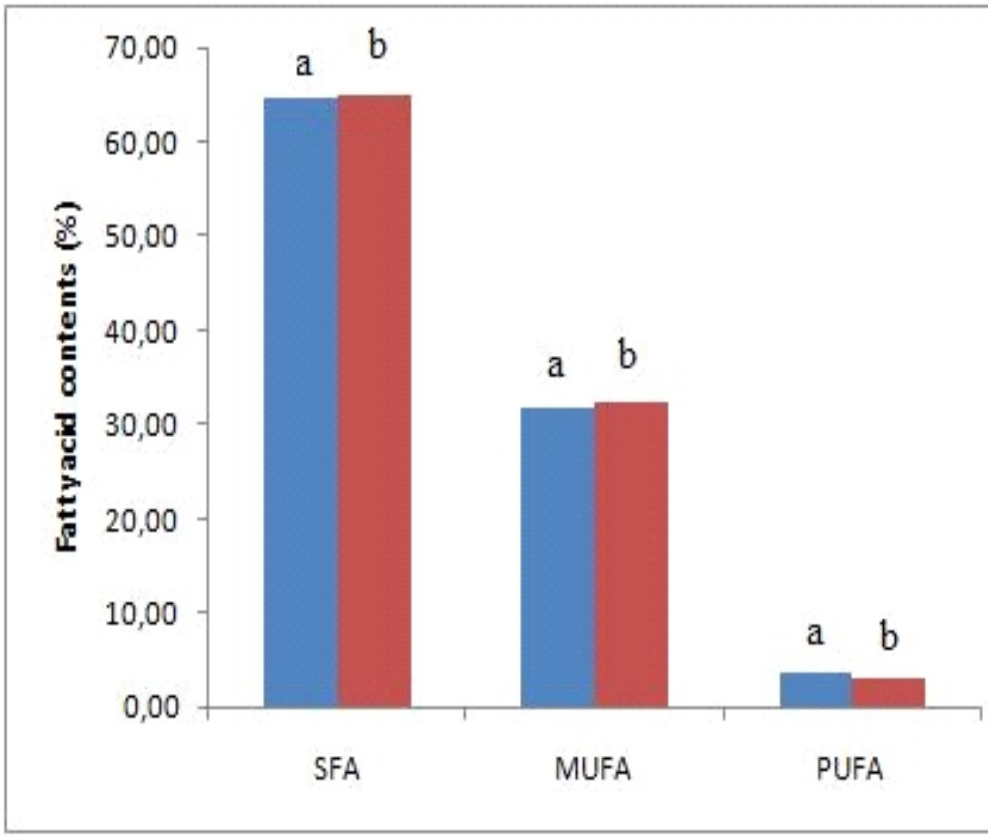

Figure 2.4. Saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in fermented bifido milk (FBM) and unfermented bifido milk (UFBM). Means (N = 6) with different letters in the same bar are significantly different. Tukey test $P \leq 0.05$.

Ackman (2007) characterized the fat acids in short chain fat acid (SCFA) the fat acids with C2 to C4, medium chain fat acid (MCFA) that from C6 to C12 and long chain fatty acid (LCFA) that from C14 until C24. In this study, means of SCFA were 4.42 in both products whilst means of MCFA were respectively 12.54% and 12.44% in FBM and UFBM. Finally, LCFA were in average 87.57% in both products (Figure 2.5). These results agree with Florence at al. (2012), which demonstrated that MCFA concentration decrease and LCFA increase during fermentation process.

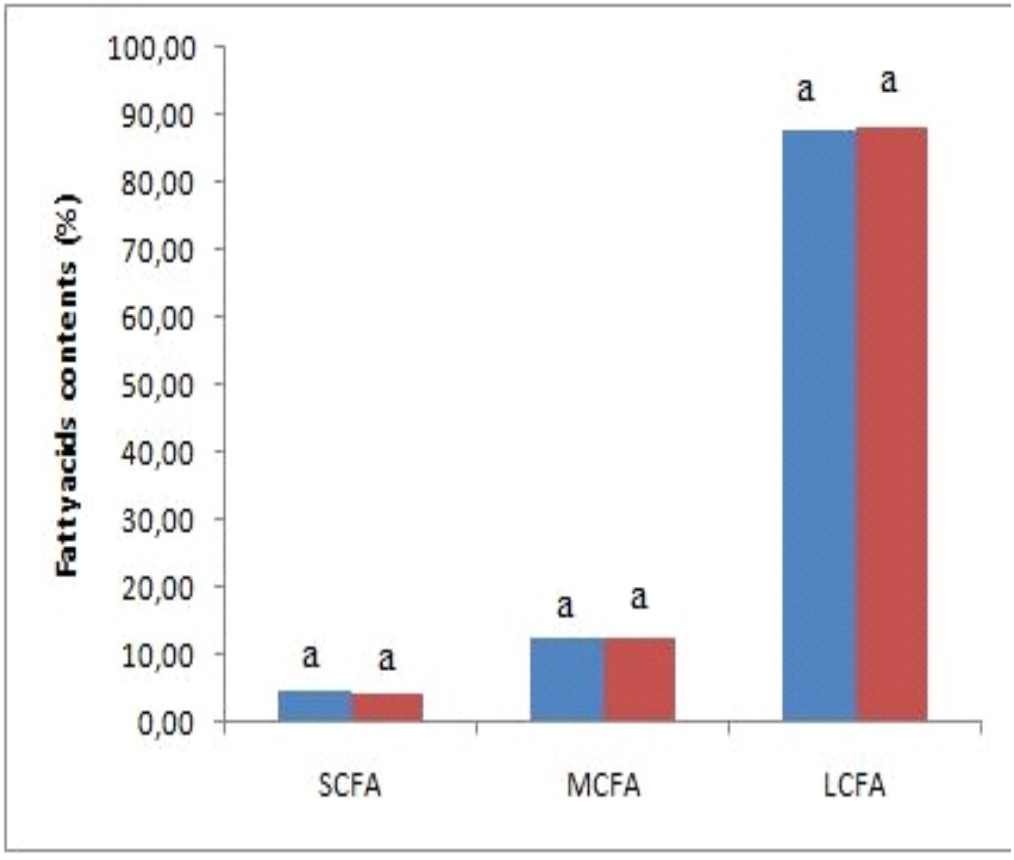

Figure 2.5. Short chain (SCFA), median chain (MCUFA) and long chain (LCFA) fatty acids in fermented bifido milk (FBM) and unfermented bifido milk (UFBM). Means (N = 6) with different letters in the same bar are significantly different. Tukey test $P \leq 0.05$.

These data showed that employing different technologies slightly affected the distribution of fatty acids in the products. Moreover, fermentation may biotransform some FA in bioactive compounds as shown in the little increase observed in linoleic acid (C18:2), an essential FA precursor of linolenic (ω-3) and arachidonic acid (ω-6) (Figure 2.3) as well as in CLA. Although, it was noted a little increase of MUFA and SFA in fermented product and slight higher contents of PUFA in unfermented products (Figure 2.4). The carbon chain length was not significantly affected by fermentation in bifido milks (Figure 2.5).

2.3.2.2. *Bioactive peptides*

Bioactive substances of food origin are considered to be dietary components that exert a regulatory activity in the human organism, beyond basic nutrition (Han et al, 2012). Bioactive peptides derived from milk protein are described to promote a lot of physiologic responses. Mostly, the milk proteins are latent until released and activated after digestive enzymes' hydrolysis or through action of proteolic microorganisms in food processing. These peptides have 3-20 amino acids (aa) residues per molecule and your activity is relationship to aa composition and sequencing. Some peptides show multifunctional properties such as opioid, antihypertensive, antimicrobial, antithrombotic, immunomodulating, and metal-binding activities (Hajirostamloo, 2010). Moreover, milk contains Angiotensin I-Converting enzyme (ACE-I) and cell modulation peptides that are important to anti-inflamatory actions (Rosa et al, 2012).

The combination of cation exchanger and filtration were the appropriate

method for purification and fractionation of the proteins in fermented milk. These use of the cation exchanger made possible separation of uncharged and anionic compounds such as sugars or lactic acid. A typical LC-MS chromatogram of the control milk could be seen in Figure 2.6.

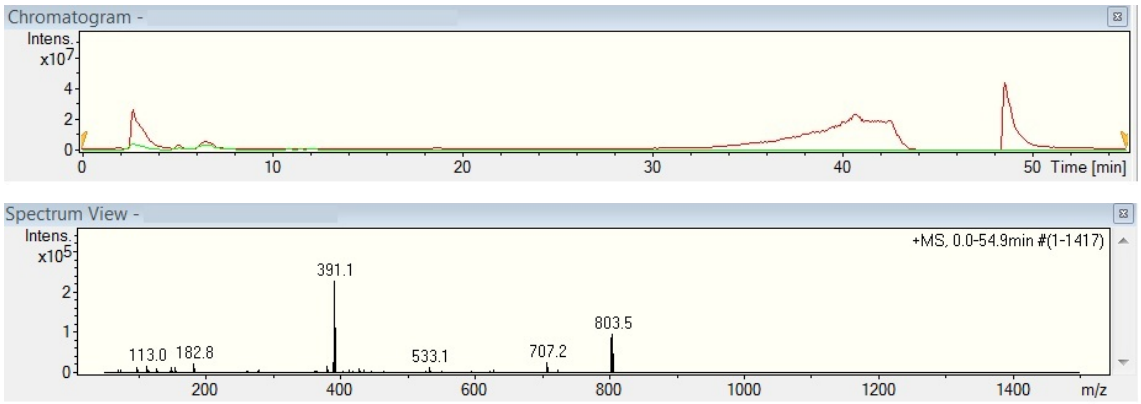

Figure 2.6 - LC-MS chromatogram and peak spectrum of the control milk analyzed by software ESI compass 1.3 for micrOTOF/maXis Data Analysis version 4.0 SP3 (Build 275), copyright 1993-2010 Bruker Daltonik GmbH.

Table 2.2 shows the biopeptides profile presented in probiotic milk products during storage determined by HPLC LC-MS. The possible sequences and activities were compared with the literature (Kunda et al. 2012)**.** Control milk and unfermented bifido milk showed the same peptides even after 7 days of storage. The present study shows that fermentation of milk by *B. lactis* HN019 increased bioactive peptides. Nevertheless, according to the literature, little is known about peptides formed by bifido fermentation; most reports concerns data about yoghurt starters' bacteria. In this study, it was clearly demonstrated that opioids, antagonist and agonist peptides are formed due to

fermentation process, increasing the source of bioactive peptides. Finally, storage modified some peptides and increased the antibacterial activity.

The unfermented bifido milk is not able to change the peptides of matrix milk, and milk fermented by *B. lactis* HN019 improved biogenic compounds release.

Table 2.2. Peptides detected by LC-MS HPLC in milk, fermented and unfermented milk, probable sequence and function according to literature.

Product	MW of peptides detected by HPLC (Da)	MW of reported peptides (Da)	Probable sequence (Kunda et al. 2012)	Probable Function (Kunda et al. 2012)
CM	113.0	--	--	--
	182.8	--	--	--
	391.1	391.1	Phe-Leu-Leu	ACE-I
	533.1	--	--	--
	707.1	--	--	--
	803.5	802.5	--	Cell Modulation
			Lys-Ala-Val-Pro-Tyr-Pro-Gln	
FBM – D1	98.9	--	--	--
	182.8	--	--	--
	391.1	391.1	Phe-Leu-Leu	ACE-I
	485.8	--	--	--
	549.8	--	--	--
	628.4	627.3	--	Opioid agonist
	707.2	--	Tyr-Leu-Gly-Tyr-Leu	--
	770.5	771.3	--	Opioid antagonist
	803.5	802.5	Ser-Arg-Tyr-Pro-Ser-Tyr	Cell Modulation
			Lys-Ala-Val-Pro-Tyr-Pro-Gln	

FBM D7	–	98.9 182.8 391.1 451.7 549.8 628.4 707.2 770.5 803.5	-- -- 391.1 904.4 -- 627.3 -- 771.3 802.5	-- -- Phe-Leu-Leu Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val -- Tyr-Leu-Gly-Tyr-Leu -- Ser-Arg-Tyr-Pro-Ser-Tyr Lys-Ala-Val-Pro-Tyr-Pro-Gln	-- -- ACE-I Antibacterial -- Opioid agonist -- Opioid antagonist Cell Modulation
UFBM D1	–	113.0 182.8 391.1 533.1 707.1 803.5	-- -- 391.1 -- -- 802.5	-- -- Phe-Leu-Leu -- -- -- Lys-Ala-Val-Pro-Tyr-Pro-Gln	-- -- ACE-I -- -- Cell Modulation
UFBM D7	–	113.0 182.8 391.1 533.1 707.1 803.5	-- -- 391.1 -- -- 802.5	-- -- Phe-Leu-Leu -- -- -- Lys-Ala-Val-Pro-Tyr-Pro-Gln	-- -- ACE-I -- -- Cell Modulation

Abbreviations: Control Milk (CM); Fermented Bifido Milk (FBM); Unfermented Bifido Milk (UFBM); 24 hours after preparation (D1); seven days after cold storage (D7).

2.4. CONCLUSIONS

The survival capacity of bifidobacteria and the production of bioactive compounds is a promising area of research. The potential health benefits of milk protein-derived peptides and fat acids have been a subject of highest commercial interest to functional foods health-promoting. Finally, knowledge about the most beneficial compounds of functional dairy foods starts needs to be elucidated and assets more research.

Chapter 3 – FERMENTED OR UNFERMENTED PROBIOTIC MILK: TECHNOLOGICAL APPROACH CHANGES THE IMMUNE ACTIVATION

ABSTRACT

The physiological benefits attributed to Bifidobacteria are their ability to physically interfere with the adhesion of pathogenic species to surfaces of intestinal cells and their ability to enhance the host immune function that is believed to be a result of their metabolic activity. Functional foods are the mainly delivery form of probiotics, but the differences between fermented or unfermented product in bifido health benefits are rarely focused. The aim of this study was to analyze and compare the immune effect in gut mucosa promoted by different food technological process applied using the same matrix and the same probiotic strain. BALB/c mice were fed *ad libitum* with fermented or unfermented bifido milks for two weeks. The colon was analyzed by histology and the immune pattern by flow cytometry and immunofluorescence.

Probiotic efficacy is changed through many factors not just due to strain specificity but also by the technological process used, like fermentation and moreover by the matrix in which is delivery. These work had shown differences in mucosal morphology and immunity promoted by different food technological process using the same matrix and the same probiotic strain - fermented or unfermented bifido milk - in health BALB/c mice, suggesting that changes in functionality of bifidobacteria and/ or the metabolites produced by fermentation process, is the key to improve beneficial effect in the host gut mucosa

throughout increase in mucus and cellularity production, changes in immune pattern and preservation of mucosal epithelia in health Balb/c mice.

Keywords: Fermented milk, Immunomodulation, B1 cells, matrix-probiotic-mucosa interaction, *Bifidobcaterium animalis* subsp. *lactis* HN019.

3.1. INTRODUCTION

Since 2002, probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002) without attention about the interactions of these microorganisms with matrix where they are delivered or the changes undergone by the fermentation process. The probiotic health benefits are strain specific, some authors have been documented in randomized clinical trials, such effects as shortening diarrhea of children, relieving the symptoms of atopic eczema and alleviating allergic rhinitis (Ouwehand et al., 2008).

Bifidobacterium are suggested to be beneficial for human health, and strains from these genera are often used as probiotics (Collado, Isolauri, Salminen, & Sanz, 2009), for the physiological benefits attributed to the ability to physically interfere with the adhesion of pathogenic species to surfaces of intestinal cells and their ability to enhance the host immune function what is believed to be a result of their metabolic activity.

The probiotic activity is strain specific and could act in differentially towards at intestinal mucosa, by secreting antimicrobial products, resisting colonization of pathogens, enhancing and maintaining barrier function,

modulating the epithelial cell signal transduction and throughout innate and adaptive immunomodulation, but the exactly mechanism of action is not fully understood (Collado et al., 2009).

Functional foods are the mainly delivery form of probiotics, but the differences between fermented or unfermented product in bifido benefits are not intensely focused. The probiotic activity is not changed just for the strain specificity but also by the technological process used and matrix in which bacteria is delivery (Sánchez, Reyes-Gavila, Margolles, & Gueimonde, 2009). The aim of this study was to analyze and compare the immune effect in gut mucosa promoted by different food technological process applied using the same matrix – skimmed milk, and the same probiotic strain - *Bifidobacterium animalis* subsp. *lactis* HN019.

3.2. MATERIALS AND METHODS

3.2.1. Product design

Fermented and unfermented milk were produced with *Bifidobacterium animalis* subsp. *lactis* HN019 (Dupont-Danisco, Madison, USA), skimmed milk and water was used as control. In brief, skim milk UHT (Molico®, Nestlé, Araçatuba, SP, Brazil) was purchased from local market. Afterwards, milk base was inoculated with 1 mL of probiotic culture to obtain the same 9.00 $\log_{10}$.CFU.mL^{-1} counts of microorganism in each final product. Three technological processes were employed: (i) Fermented bifido milk (FBM): fermentation was conducted at 37°C until milk reach pH 4.7 controlled by

CINAC system (*Cynetique d'acidification,* Ysebaert, Frépillon, France) (Spinnler & Corrieu, 1989) according to (Saccaro et al, 2009); fermented milk was stored in refrigerator at 4°C; (ii) Unfermented bifido milk (UFBM): after inoculation product was stored in refrigerator at 4°C, and (iii) Fermented bifido milk heat treated (FBMHT): fermentation was conducted as described in (i) and afterward the product was heat treated at 60°C during 40 minutes in Thermomix. Probiotic´s enumeration was carried out in each product during animal trial in order to assure recommended dose (WGO, 2009) i.e. counts higher than 8.00 log_{10}.CFU.mL^{-1} in UFBM and FBM, and destruction of bacteria in FBMHT.

3.2.2. Animals and protocol design

Eight-week-old male BALB/c mice were fed with commercial food and fermented or unfermented bifido milks were administrated *ad libitum* for two weeks. UHT milk (Molico®, Nestlé, Araçatuba, SP, Brazil) and water were used as control. The weight was measured daily. Protocol design has been approved by the institutional animal care and use committee from Pharmaceutical Sciences Faculty, São Paulo University (CEEA/FCF/14/2009 – protocol n. 210), and was replicated twice.

3.2.2.1. Histological sections

The mice were sacrificed in CO_2 chamber (Insight Equipamentos, Ribeirão Preto) and the large intestine tissues were prepared for histological studies, fixed in formaldehyde, dehydrated using a graded series of ethanol and xylene, and embedded in paraffin. The colon was sectioned and stained by hematoxilin-eosin (HE) and alcian blue (AB) coloration. The histological analyses were performed in a blind fashion. Colon was analyzed by light

microscopy (Olympus America Inc., Center Valley, PA, USA). The sections were digitally processed using photometrics methodology (Photometrics system coolSNAPcf and software metaVue v. 6,3r7, Photometrics, Tucson, AZ, USA). Four different areas were considered for each lamina, and the percentages of threshold areas were calculated.

3.2.2.2. Evaluation of Peyer´s Patches

The small intestine was collected and the Peyer´s Patches were counted manually.

3.2.2.3. Flow citometry of gastrointestinal tract (GIT)

The small and large intestine were minced and incubated for 90 min at 37 °C in digestion buffer containing 0.7 $mg.mL^{-1}$ collagenase IV (Sigma-Aldrich, St. Louis, USA). Large particulate matter was removed by passing cell suspension through a small loose nylon wool plug and the cells were analyzed. Dentric cells, macrophages, CD3+CD4+ cells, CD3+CD8+ cells and B-2 cells and B-1 cells were determined by flow cytometry using FACSCanto II (Becton Dickinson, San Jose, USA). In order to determine the membrane markers, the cells were labeled with Mabs against mouse CD11b-APC (e- biosciense, San Diego, USA), IgM–PE (e- biosciense, San Diego, USA), IgD-PE (BD Pharmingen, San Jose, USA) e CD5- PerCy5 (BD Pharmingen, San Jose,

USA), CD3-PE (BD Pharmingen, San Jose, USA), CD4-APC (BD Pharmingen, San Jose, USA), CD8a- FITC (BD Pharmingen, San Jose, USA), CD11c-FITC (eBioscience, San Diego, USA) and F4/80-PerCP (eBioscience, San Diego, USA). FlowJo was used for analysis of flow cytometry data. To distinguish auto fluorescent cells from cells expressing low levels of individual surface marker were established upper thresholds for auto fluorescence by staining samples with fluorescence-minus-one (FMO) control stain sets (Herzenberg et al. 2006). In these sets, a reagent for a channel of interest is omitted.

3.2.2.4. Immunofluorescence of colon

The number of IgA positive cells, macrophages and dendritic cell were determined on histological slices using a direct immunofluorescence assay. After deparaffinization using xylene and rehydration in a decreasing gradient of ethanol, paraffin sections (4 µm) were incubated with a 1:100 dilution of α-chain monospecific antibody conjugated with FITC (Sigma, St Louis, MO, USA), CD11b-FITC (eBioscience, San Diego, USA), F4/80-PE (eBioscience, San Diego, USA), CD11c-FITC (eBioscience, San Diego, USA), TLR-4-FITC (eBioscience, San Diego, USA) for 1h30min and observed with a fluorescent light microscope. The number of fluorescent cells was counted in 30 fields at 1000× magnification and results were expressed as the number of positive fluorescent cells per ten fields of vision (de Moreno de LeBlanc et al. 2008).

3.2.2.5. Phagocytic Index

The phagocytic index were measured as described by Oda et al. (Oda, Kubelka, Alviano, & Travassos, 1983). Briefly, peritoneal cells were collected from mouse abdominal cavity by repeated lavage with 5 mL of RPMI-1640 medium (Sigma). Cells (2×10^6 cel.mL^{-1}) were dispensed on 24 wells plate with glass cover slips and incubated at 37 °C for 24 hours. The culture supernatants were then aspirated to remove the non-adherent cell fraction. Adherent cell monolayers were rinsed with RPMI and subsequently covered with supplemented medium plus 10µg.mL^{-1} zymosan particules. Cultures were maintained at 37 °C in 5% CO_2 for 4 hours. The glass cover slips were stained by giemsa (Newprov, Brasil).

The Phagocytic Index (PI) was determined by Phagocytic Capacity (PC) multiplied by the number of phagocyted particles in each phagocyte cell (P), like the formula **PI=PCxP**.

3.2.3. Statistical analyses

All results are representative of at least two independent experiments with similar results. Data were analyzed with Statistica version 8.0 (StatSoft Inc., Yulsa, USA). ANOVA was performed to compare the means of two groups and Kruskal–Wallis test for comparison of three or more groups. Log-rank test

was used to compare the difference in survival. A $P<0.05$ was considered significant.

3.3. RESULTS AND DISCUSSION

Probiotic designed products used during the *in vivo* protocol presented counts higher than 9.00 log_{10}.UFC.mL^{-1} in UFBM and FBM, and destruction of bacteria in FBMHT (table 3.1).

Table 3.1. Viability through 7 days of storage at 4°C.

Sample	D0	D1	D7
UFBM	9,94±0,05[a]	9,21±0,30[a]	9,00±0,29[a]
FBM	8,72±0,49[b]	9,52±0,85[a]	9,53±0,90[a]
FBMHT	8,72±0,49[b]	-	-

Same letters in the same column showed statistics similarity ($P \leq 0.05$).

UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat treatment; D0: after inoculation; D1: 24 hours after fermentation; D7: 7 days after fermentation.

3.3.1. Differences in histology in gut mucosa through technological approaches

Histology of the colon performed to evaluate if the effect of technology processes employed in probiotic products – UFBM, FBM and FBMHT - interfere in integrity of intestinal mucosa (Grzeskowiak, 2011) are presented in Figures 3.1 and 3.2. FBM promoted about 15% an increase on cellular influx and restored the epithelium (Figures 3.1c and 3.3) when compared with the controls and an increase about 7% when compared with the UFBM (Figures 3.1d and 3.3). The UFBM showed a little destruction on epithelium (Figure 3.1d), probably by mucus decrease when compared to FBM and CM (Figure 3.2d and 3.3), the weight lose in UFBM group could be promoted by decrease in capacity to nutrients absorption and changes in microbiota interaction (Acheson & Luccioli, 2004)

The metabolites produced during fermentation or changes bacterial functionality stimulating gut barrier, by establishing a low grade of inflammation (Perdigon et al., 2002) and increase in mucus production (Grzeskowiak et al 2011).

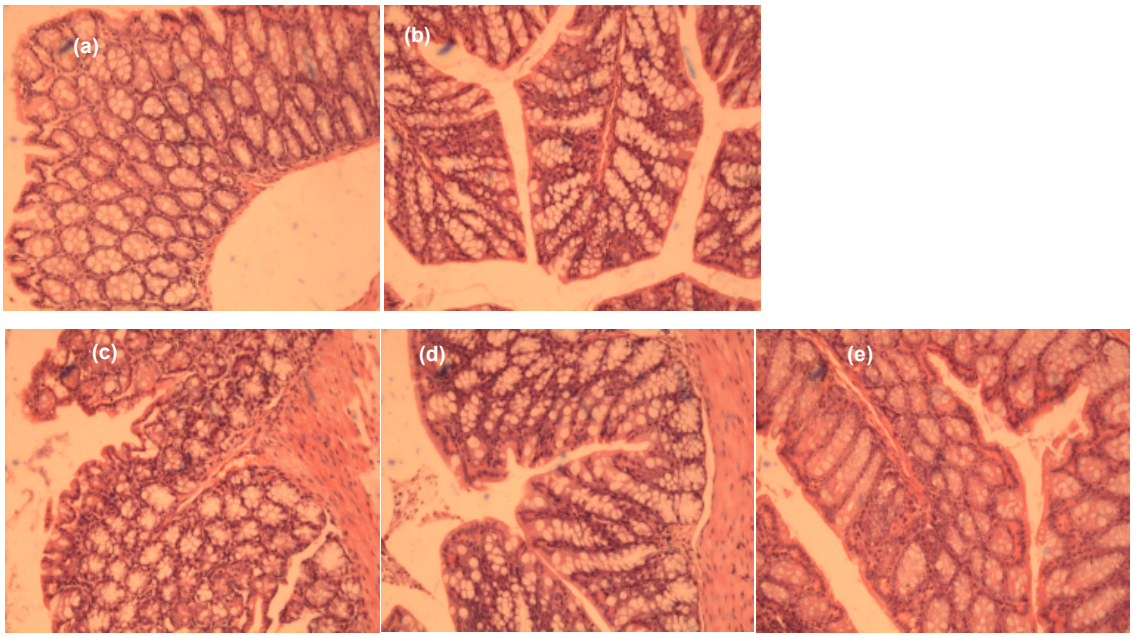

Figure 3.1. Histologic sections of colon stained by Hematoxylin-eosin. Analyzed by Olympus BX60 microscope with lens 10X/0.30 Ph1 UplanFI and condenser of 1.25X Photometrics coolSNAPcf through the system and software version 6.5r7 metaVue. (a) CW; (b) CM; (c) UFBM; (d) FBM; (e) FBMHT. Abbreviations: CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat-treated.

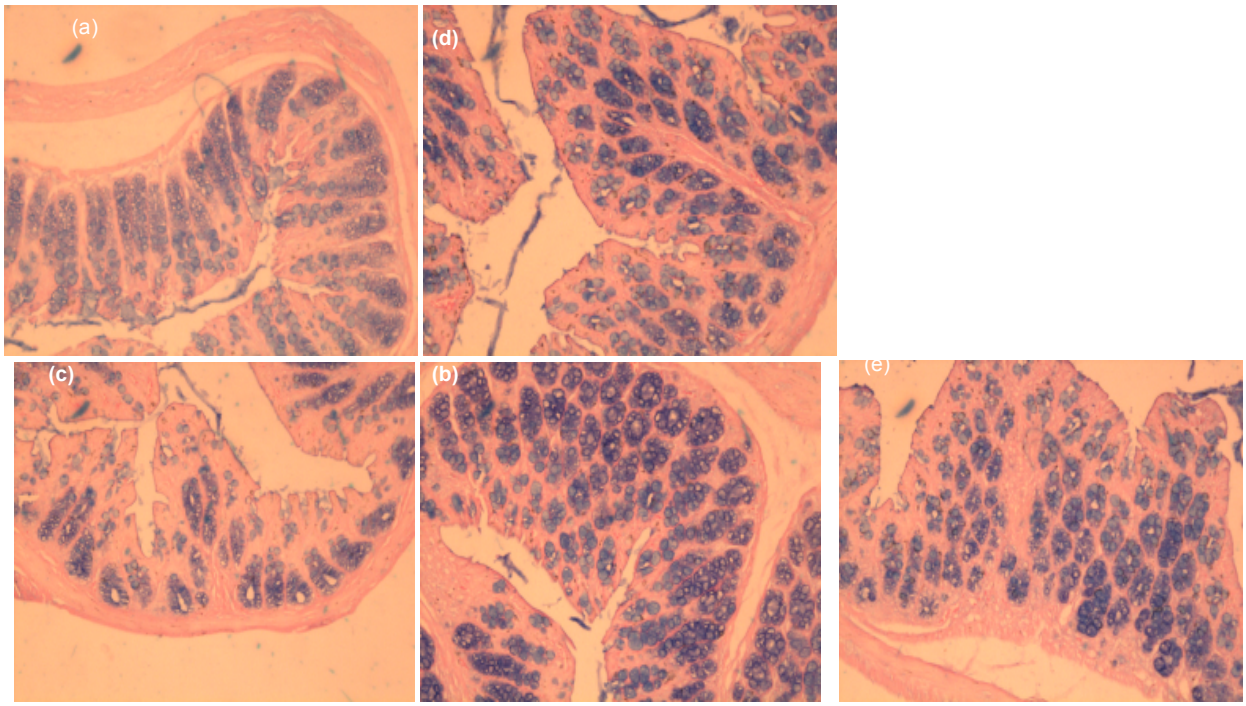

Figure 3.2. Histologic sections of colon stained by Alcian Blue. Analyzed by Olympus BX60 microscope with lens 10X/0.30 Ph1 UplanFI and condenser of 1.25X Photometrics coolSNAPcf through the system and software version 6.5r7 metaVue. (a) CW; (b) CM; (c) UFBM; (d) FBM; (e) FBMHT. Abbreviations: CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat treated

The digital images analyzed had shown a similar cellular infiltration in animals that consumed water, milk and unfermented milk ($P \leq 0,05$) when compared to animals that consumed fermented bífido milk and fermented bífido milk followed by heat treatment (Figure 3.3). These data suggest that in order to enhance mucus production and to start basal inflammation, the metabolites produced during the *B. lactis* HN019 fermentation or the modification of this probiotic functionality are required and play a key role in the interaction between host and functional foods.

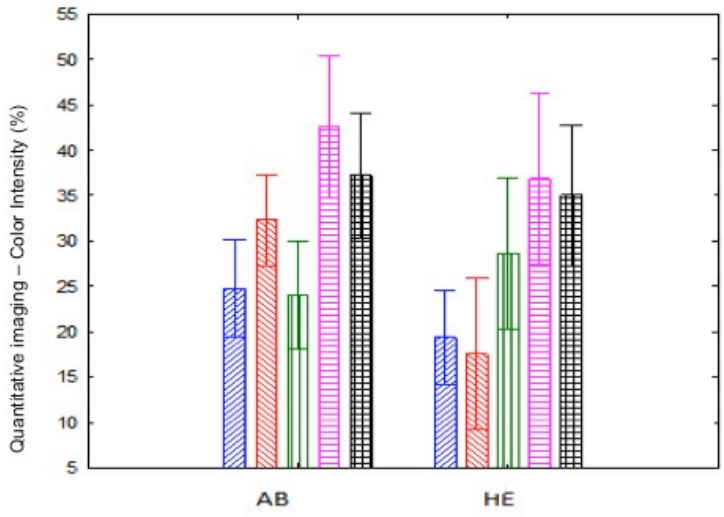

Figure 3.3. Cellular infiltrate and mucus production in BALB/c mice colon after 14 days products intake (N=5). Abbreviations: CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat treated. Tukey test (*P*<0,05).

Control water; Control milk; UFBM; FBM; FBMHT

3.3.2. Unfermented bifido milk promotes decrease in body weight

Despite the discussion about probiotics increase or not animal body weight (Bogsan et al. 2011), the interference of technology process employed in body weight development was considered. Body weight increased when mice consumed FBM during 14 days similar behavior was observed in control mice groups having

water and milk ($P \leq 0.05$). The animals that consumed UFBMHT had shown significant weight decrease ($P \leq 0.05$) when compared with controls, probably by destruction of epithelial mucosa and worst absorption of nutrients (Figure 3.4).

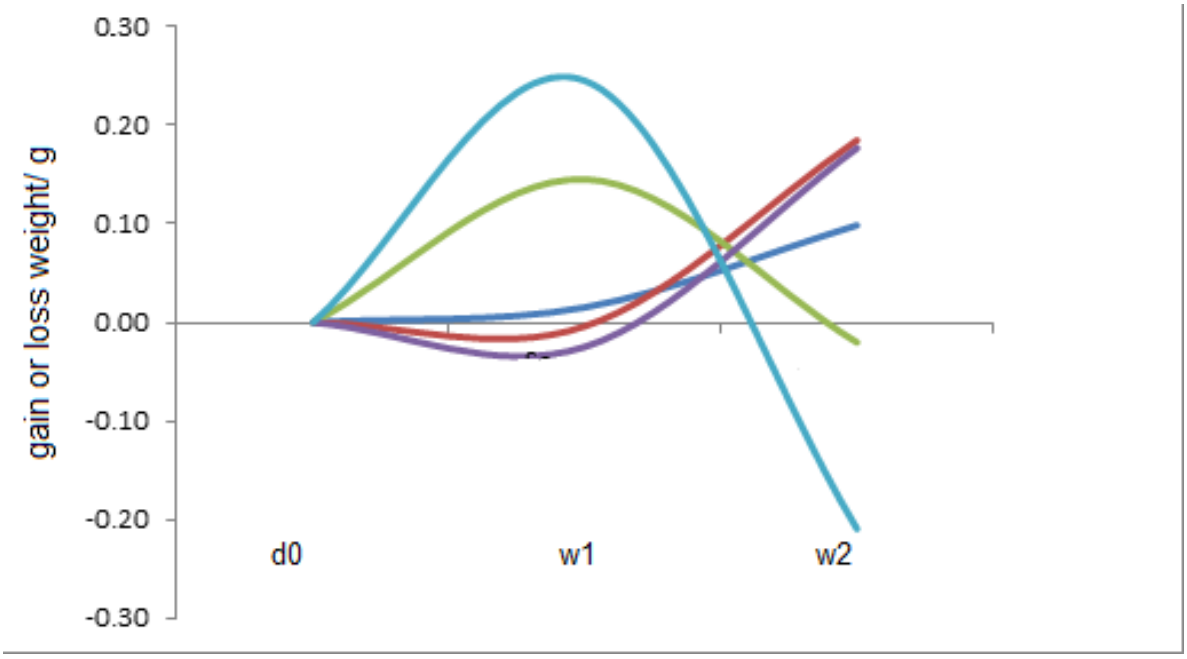

Figure 3.4. Weight curve showing the differences between standardized averages of initial and final weight of Balb/c mice during 14 days of administration of W (—), M (—), UFBM (—), FBM (—) and FBMHT (—). Abbreviations: W: control water; M: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat treated.

These data suggest that not just the viable microorganism or metabolites produced during fermentation are able to perform the best interaction between probiotic and host, but a combination of both. Therefore, the purpose of probiotic utilization needs be clearly before the probiotic administration to obtain the best result, because the technology, matrix and strain employed could to change all desired effects.

3.3.3. Differences in immune activation in gut mucosa through technological approaches

The mucosal immune system has an excellent IgA response to the presence of commensal intestinal bacteria, but this is separate from the systemic immune response. Immune responses to commensals are, therefore, a function of exposure to the organisms (Macpherson & Uhr, 2004).

Payer's Patches (PP) are clusters of lymph nodes located in the small intestine. The main function is the presentation of antigens to initiate the immune function. They mainly trigger the differentiation of plasmocytes that secrete IgA to delivery through gut mucosa to intestinal lumen the sIgA. The purpose of sIgA is mucosa protection from indigenous microbiota and from food contaminated with pathogenic microbes. The PP number could be modified through extrinsic factors like nutrition, stress and age (Kruiningen et al, 2002).

Considering the differences showed in mucosa preservation and cellular infiltrate from the products described before, the number of PP were affected as evidenced in Figure 3.5.

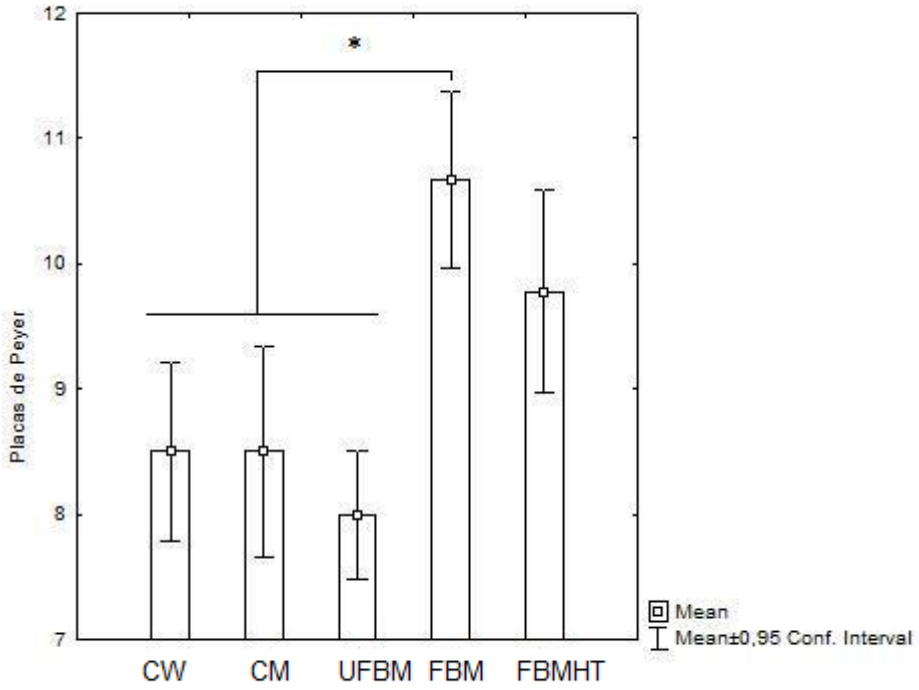

Figure 3.5. Number of Peyer's patches in BALB/c mouse after products intake during 14 days (N=5). Abbreviations: CW: Control water; CM: Control milk; UFBM: Unfermented bifido milk; FBM: Fermented bifido milk; FBMHT: fermented bifido milk heat treated. Tukey test (P<0.05).

The number of PP in BALB/c mice small intestine fed with FBM during 14 days had a significant increase (P≤0.05) compared with CW, CM and UFBM. These data collaborate with the hypothesis that not just the viable microorganism or metabolites produced during fermentation are able to perform the best interaction between probiotic and host, but a combination of both. The increased in cellularity and in PP's numbers in FBM mice group let to investigate the immune pattern which data are shown in Figure 3.6.

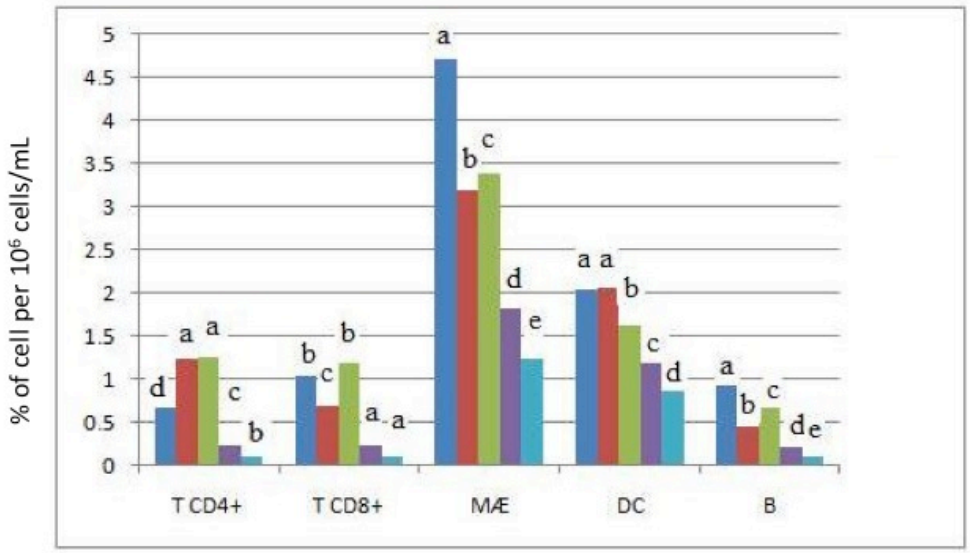

Figure 3.6. Profile of immune cells in intestinal mucosa of BALB/c mice fed with CW (■), CM (■), UFBM (■), FBM (■) and FBMHT (■) analyzed by flow citometry. Abbreviations: CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat treated; T CD4+: cells T helper; T CD8+: T cells citotoxics; DC: dendritic cells, MAE: macrofages and B: B cells.

The percentage of T cell had shown the same ratio between helper T cell (CD3+ CD4+) and cytotoxic T cell (CD3+ CD8+) which is in accordingly to literature (Vitini, Alvarez *et al.*, 2000; Galdeano e Perdigon, 2004). Indeed, when these populations were compared between the different groups it could be observed a significant increase in percentage of immune cells of UFBM comparing with FBM and FPBM ($P<0.05$). However, when the macrophages and DC were analyzed, the UFBM had shown a decrease in % number of cells.

The same effect was observed concerning B cells (IgM+ e IgD+). These results allow us to infer that BALB/c mice consuming UFBM activated the adaptative immunity when the animals having FBM do not.

Table 3.2. Phagocytic Index of peritoneal macrophage stimulated by zimozan particules.

Group	**PC(%)**	**PI (%)**
CM	22.38[a]	1.00[a]
UFBM	35.77[b]	1.95[b]
FBM	15.34[c]	1.55[c]
FBMHT	5.77[d]	0.20[d]

PC: Phagocytic capacity; PI: Phagocytic Index; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk and FBMHT: fermented bifido milk heat treated. Means (N = 5) with different superscript letters in the same column differ significantly ($P < 0.05$).

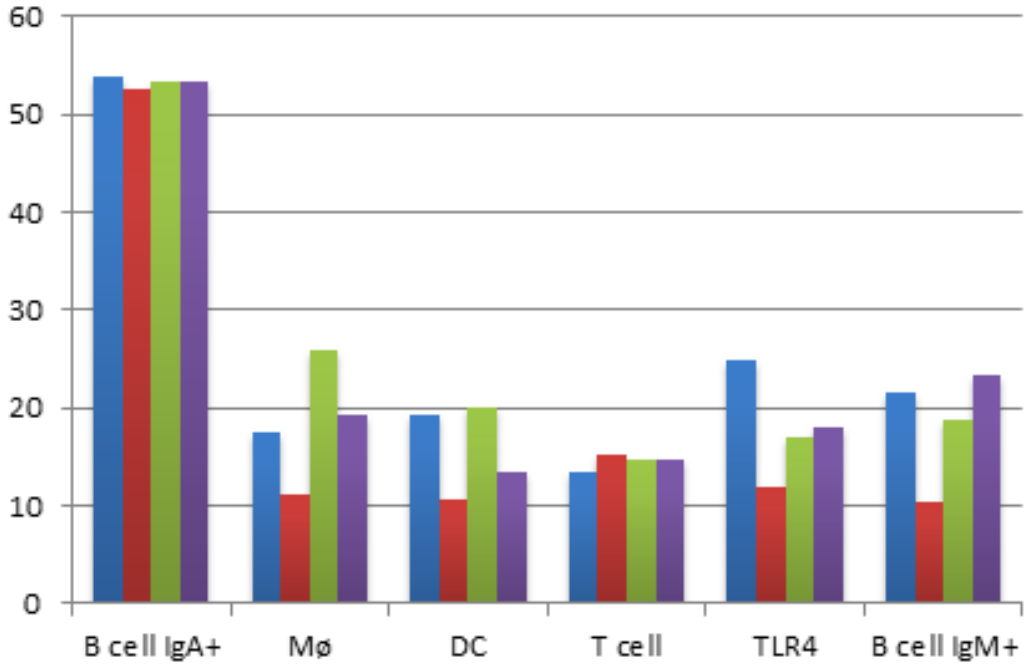

Figure 3.7. Number of positive cells per ten fields of vision in intestinal mucosa of BALB/c mice fed with CM ( ), UFBM ( ), FBM ( ) and FBMHT ( ) analyzed per colon immunohistochemistry (N=6). Abbreviations: CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat treated

The literature showed the macrophages and DC percentage increase in peripheral blood (GILL e RUTHERFURD, 2001), but in intestinal mucosa these values are not well described. Analyzing the innate immunity trough DC and macrophages, colon macrophages and DC had shown an increase when FBM was consumed comparing with control milk; in contrast, they had a significant decrease when UFBM was consumed (Figure 3.7). These results could explain the effect in phagocytic index i.e. when DC where in low levels the phagocytic activities are impaired (Table 3.2).

De Moreno De Leblanc *et al.* (2008) showed that in animals that consumed *L. casei* fermented milk had an increase in bifidobacteria population and a significant increase in IgA+ plasmocytes. In spite of this, in the present study IgA+ plasmocytes nor T cell present significant difference between the groups when IgM+ plasmocytes and TLR4+ cells had shown an increase expression in FBM group (Fig. 3.7). These data could infer that FBM stimulate the innate immunity when UFBM do not.

B1 cells (B-1a and B-1b) are a major source for antibodies specific for bacterial cell wall components that liberates IgA without T cell activation (Macpherson *et al.*, 2004).

Subtypes of B cells were also analyzed and the results could be seen in Figures 3.8 and 3.9.

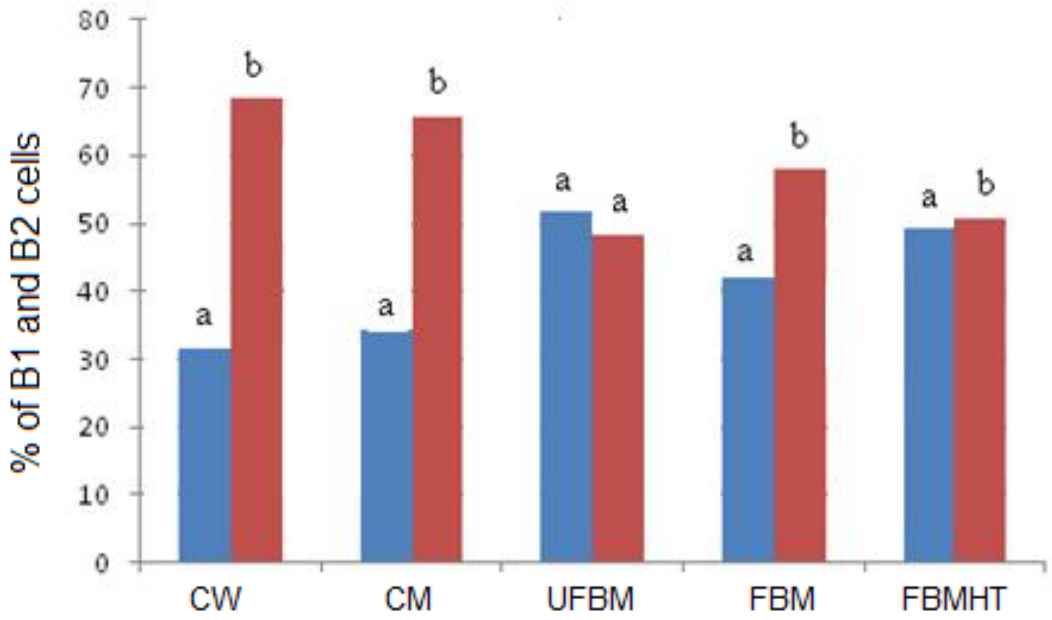

Figure 3.8. Distribution of B cell subtypes - B1 (■) and B2 (■), in gut mucosa of BALB/c mice fed with CW, CM, UFBM, FBM and FBMHT during 14 days. Abbreviations: CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat treated; B1: cells B IgM+ IgD+ CD5+; B2: cells B IgM+ IgD+ CD5+.

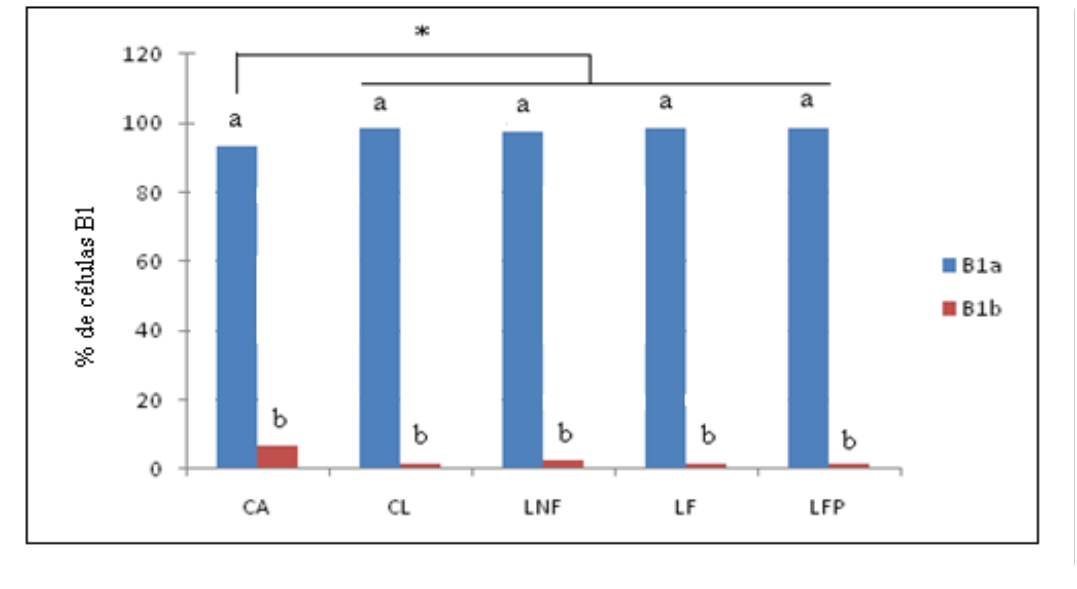

Figure 3.9. B-1 cells distribution - B1a ( ) and B1b ( ), in gut mucosa of BALB/c mice fed with CW, CM, UFBM, FBM and FBMHT during 14 days. Abbreviations: CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; B1a- B cell IgM+ IgD+ CD5+, B1b – B cell IgM+ IgD+ CD5-.

These data suggest a change in B cell activation pattern (Figures 3.8 and 3.9). When the control water and milk showed a B1 predominance (VITINI *et al.*, 2000; GALDEANO e PERDIGON, 2004), FBM and FBMHT showed a decrease in B2:B1 relationship; in contrast B1 maintained the prevalence in all products except to UFBM, where the B2 population is over than B1, changing the immune pattern. So, it could be postulated that metabolites or changes in bacteria functionality promoted by fermentation process represent a key factor to signaling the B cell activation pattern. These fact is emphasized when the FBM intake is observed, because the B cells T independent activation (B1) are

maintained when compared with UFBM or FBMHT, that had an adaptive immunity activation through increase in B cell T dependent activation (B2). What believes be antigenic presentation absence of food antigens (VITINI *et al.*, 2000; GALDEANO e PERDIGON, 2004) could have a relationship to changes in activation pattern and could be related to PP increased observed in Figure 3.5.

The FBM and UFBM, like the controls induced an innate response thought increase in macrophages and Dendritic and TLR4 cells in gut mucosa. In contrast, UFBM activate the adaptive immunity, increasing B and T cells and an inversion in B2:B1 cells ratio. Besides, the UFBM decreased regulation of TLR4 expression whilst FBM and FBMHT did not (Figure 3.7). Experiments in which cellular components (B and T lymphocytes and dendritic cells) were purified from different secondary lymphoid structures and reconstituted *in vitro* showed that the IgA switch was much more efficient when leukocytes—especially dendritic cells - were derived from Peyer's Patches than from other cellular sources. This suggests that IgA + B cell induction takes place locally within the mucosa, although the system is primitive in terms of T independence and the superfluity of compartmentalized B, T, and follicular zones within the intestinal lymphoid follicles (Macpherson & Uhr, 2004).

3.4. CONCLUSIONS

In conclusion, data from this study suggest that technological processes changes immune activation pattern. Not all microorganism have the same effect on the host, is not possible to extrapolate the effects found with one probiotic strain to another one more than this, is not possible extrapolate the probiotic effect in one technological process to another one employed, fermented or not, to develop the probiotic product.

4. CONCLUSIONS

The technological differences in viability, stability and fermentation showed in developed products - unfermented bífido milk, fermented bífido milk and fermented bífido milk pasteurized were in accordance with literature; however the effect in gut mucosa was never shown before. These products had shown that immunologic pattern are different for each product, even then they used the same matrix, microorganisms and counts of viable bacteria ($CFU.log^{-1}$).

The increase in mucus and cellular infiltration and the changes in immune pattern had shown that not just the probiotic ingestion but also the presences of their metabolites through fermentation process are the major factor to reached immunomodulation effects.

The technological process changes de pattern of immune activation. The fermentation process induce an innate response trough increase in macrophages and Dendritic cells in gut mucosa whilst the unfermented probiotic activate the adaptive immunity showed by an increase in B and T cells, after all an inversion in B2:B1 cells ratio. These data are important in order to carefully select the probiotic strain and the kind of product - fermented or not, that will be administrate to the host to obtain the desired immunological effect.

5. PERSPECTIVES

This work bring a lot of questions, that will be started to answer troughout:

i. Analysis of the release of cytokines produced by imune cells in the colon.

ii. Analysis of the peptides released in each phase of digestion;

iii. *In vitro* analysis of the effect of these peptides and fatty acids in cultured CACO 2 cells evaluating cytokine.

6. REFERENCES

AATTOURI, N., BOURAS, M., TOME, D., MARCOS, A., & LEMONNIER, D. (2002). Oral ingestion of lactic-acid bacteria by rats increases lymphocyte proliferation and interferon-gamma production. *British Journal of Nutrition, 87*(4), 367-373. doi: 10.1079/bjn2001527

ACHESON, D.W.K., LUCCIOLI, S. (2004). Mucosal immune responses. *Best Practice and Research: Clinical Gastroenterology* 18 (2), pp. 387-404

ACKMAN, R. G.(2007) Application of gas liquid chromatography to lipid separation and analysis: qualitative and quantitative analysis. *Boca Raton: CRC Press*. (Fatty acids in foods and their health implications).

AFZALI, B., LOMBARDI, G., LECHLER, R.I., LORD, G.M. (2007). The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin Exp Immunol. 148*(1):32-46.

AOCS. Official method Ce 1-62: fatty acid composition by gas chromatography, in Official Methods and Recommended Practices of the AOCS. *American Oil Chemists Society, Champaign, IL, USA*, 1997.

ARUMUGAM, M., RAES, J., PELLETIER, E., LE PASLIER, D., YAMADA, T., MENDE, D.R., FERNANDES, G.R., BORK, P. (2011) Enterotypes of the human gut microbiome *Nature 473*, 174–180. doi:10.1038/nature09944

BARUZZI, F., POLTRONIERI, P., QUERO, G. M., MOREA, M. & MORELLI, M. (2011) An in vitro protocol for direct isolation of potential probiotic lactobacilli from raw bovine milk and traditional fermented milks. *Appl Microbiol Biotechnol* 90:331–342 DOI 10.1007/s00253-011-3133-6

BAUTISTA-GARFIAS, C.R.; IXTA-RODRIGUEZ, O.; MARTINEZ-GOMEZ, F.; LOPEZ, M.G.; AGUILAR- FIGUEROA, B. R. Effect of viable or dead Lactobacillus casei organisms administered orally to mice on resistance against Trichinella spiralis infection. *Parasite-Journal De La Societe Francaise De Parasitologie*, v. 8, n. 2, p. S226-S228, 2001.

BETTELLI, E., BAETEN, D., JAGER, A., SOBEL, R. A., & KUCHROO, V. K. (2006). Myelin oligodendrocyte glycoprote in-specific T and B cells cooperate to induce a Devic-like disease in mice. *Journal of Clinical Investigation, 116*(9), 2393-2402. doi: 10.1172/jci28334

BISIG, W., EBERHARD, P., COLLOMB, M., REHBERGER, B. (2007). Influence of processing on the fatty acid composition and the content of conjugated linoleic acid in organic and conventional dairy products - A review. *Lait 87*(1), pp. 1-19

BOGSAN, C. S. B., FLORENCE, A. C. R., PERINA, N., BARBUTI, R. C., NAVARRO-RODRIGUEZ, T., EISIG, J. N., & OLIVEIRA, M. N. (2011). Probiotics intake and metabolic syndrome: A proposal. *Trends in Food Science & Technology, 22*(8), 457-464.

BORRIELLO, S. P.; HAMMES, W. P.; HOLZAPFEL, W.; MARTEAU, P.; SCHREZENMEIR, J.; VAARA, M.; VALTONEN, V. (2203). Safety of probiotics that contain lactobacilli or bifidobacteria. *Clinical Infectious Diseases*, v. 36, n. 6, p. 775-780.

CALDER, P. C.; KEW, S (2002). The immune system: a target for functional foods? *British Journal of Nutrition*, v. 88, p. S165-S176.

CARIO, E., GERKEN, G., & PODOLSKY, D. K. (2005). Endogenous MD-2 glycocomplex resides in the endoplasmic reticulum of intestinal epithelial cells and is differentially regulated in active inflammatory bowel disease. *Gastroenterology,* v.128, n.4, p. A215-A215.

CHIANG, B. L.; SHEIH, Y. H.; WANG, L. H.; LIAO, C. K.; GILL, H. S. (2000) Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (Bifidobacterium lactis HN019): optimization and definition of cellular immune responses. *European Journal of Clinical Nutrition*, v. 54, p. 849-855.

COLLADO, M.C., ISOLAURI, E., SALMINEN, S. & SANZ, Y. (2009). The impact of probiotic on gut metabolism. *Current Drug Metabolism.* v.10, n.1, p. 68-78

CUNNINGHAM-RUNDLES, S.; AHRNE, S.; BENGMARK, S.; JOHANN-LIANG, R.; MARSHALL, F.; METAKIS, L.; CALIFANO, C.; DUNN, A. M.; GRASSEY, C.; HINDS, G.; CERVIA, J Probiotics and immune response. *American Journal of Gastroenterology*, v. 95, n. 1, p. S22-S25, 2000.

DAVE, R. I.; SHAH, N. P. Evaluation of media for selective enumeration of *Streptococcus thermophillus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus* and bifidobacteria. *Journal of Dairy Science*, v. 79, p. 1529-1536, 1996.

DE MORENO DE LEBLANC, A. D. , & PERDIGON, G. (2004), Yogurt feeding inhibits promotion and progression of experimental colorectal cancer. *Medical Science Monitor,* v.10, n.4, p. BR96-BR104.

DE MORENO DE LEBLANC, A. D., & PERDIGON, G. (2005). Reduction of beta-Glucuronidase and nitroreductase activity by yoghurt in a murine colon cancer model. *Biocell,* v.29, n.1, p. 15-24.

RACHID, M.M., GOBBATO, N.M., VALDEZ, J.C., VITALONE, H. & PERDIGON, G. (2002). Effect of yogurt on the inhibition of an intestinal carcinoma by increasing cellular apoptosis. *International Journal of Immunopathology and Pharmacology*. V.15, n.3, p.209-216.

DE MORENO DE LEBLANC, A. D., CHAVES, S., CARMUEGA, E., WEILL, R., ANTOINE, J., PERDIGON, G. (2008). Effect of long-term continuous consumption of fermented milk containing probiotic bacteria on mucosal immunity and the activity of peritoneal macrophages. *Immunobiology*, v. 213, n. 2, p. 97-108.

DE MORENO DELEBLANC, A. D., MATAR, C, FARNWORTH, E., & PERDIGON, G. (2006) Study of cytokines involved in the prevention of a

murine experimental breast cancer by kefir. *Cytokine,* v.34, n. 1-2, p. 1-8. Doi: 10.1016/j.cyto.2006.03.008.

DELCENSERIE, V., MARTEL, D., LAMOUREUX, M., AMIOT, J., BOUTIN, Y., & ROY, D. (2008). Immunomodulatory effects of probiotics in the intestinal tract. *Current Issues in Molecular Biology,* v.10, p. 37-53.

DOGI, C. A., & PERDIGON, G. (2006). Importance of the host specificity in the selection of probiotic bacteria. *Journal of Dairy Research,* v. 73, n. 3, p. 357-366. doi: 10.1017/s0022029906001993

DONG, H., ROWLAND, I., TUOHY, K. M., THOMAS, L. V., & YAQOOB, P. (2010). Selective effects of Lactobacillus casei Shirota on T cell activation, natural killer cell activity and cytokine production. *Clinical and Experimental Immunology,* v.161, n. 2, p. 378-388. doi: 10.1111/j.1365-2249.2010.04173.x

ECKBURG, P.B., BIK, E.M., BERNSTEIN, C. N. PURDON, E., DETHLEFSEN, L. SARGENT, M. RELMAN, D.A.(2005). Diversity of the human intestinal microbial flora. *Science,* v. 308, n. 5728, p. 1635-1638. Doi: 10.1126/science.1110591.

ESPÍRITO SANTO, A.P., CARTOLANO, N.S., SILVA, T.F., SOARES, F.A.S.M., GIOIELLI, L.A., PEREGO, P., CONVERTI, A. & OLIVEIRA, M.N. (2012). Fibers from fruit by-products improve fatty acids profile and increase CLA content and probiotic viability in yoghurts. *International Journal of Food Microbiology*. V. 154, p. 135-144.

ESPÍRITO SANTO, A.P., SILVA, R.C., SOARES, F.A.S.M., Anjos, D., GIOIELLI, L.A., & OLIVEIRA, M.N. (2010). Açai pulp addition improves fatty acids profile and probiotic viability in yogurt. *International Dairy Journal*. v. 20, n.6, p.415-422.

FANG, H., ELINA, T., HEIKKI, A., & SEPPO, S. (2000). Modulation of humoral immune response through probiotic intake. *Fems Immunology and Medical Microbiology, 29*(1), 47-52. doi: 10.1016/s0928-8244(00)00187-5

FAO/WHO. (2002). *Guidelines for the evaluation of probiotics in food: report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food*. London: FAO/WHO.

FINK, L. N., ZEUTHEN, L. H., FERLAZZO, G., & FROKIAER, H. (2007). Human antigen-presenting cells respond differently to gut-derived probiotic bacteria but mediate similar strain-dependent NK and T cell activation. *Fems Immunology and Medical Microbiology, 51*(3), 535-546. doi: 10.1111/j.1574-695X.2007.00333.x

FLORENCE, A. C. R., DA SILVA, R. C., DO ESPIRITO SANTO, A. P., GIOIELLI, L. A., TAMIME, A. Y., & DE OLIVEIRA, M. N. (2009). Increased CLA content in organic milk fermented by bifidobacteria or yoghurt cultures. *Dairy Science and Technology*, 89, 541-553.

FLORENCE, A. C. R., BÉAL, C., SILVA, R. C., BOGSAN, C. S. B., PILLEGGI, A. L. O. S., GIOIELLI, L. A., OLIVEIRA, M. N. (2012). Fatty acid profile, *trans-octadecenoiuc*, α-linolenic and conjugated linoleic and acid contents differed in certified organic and conventional probiotic fermented milks. *Food Chemistry*, *135*(4), pp. 2207-2214

FOLIGNE, B., NUTTEN, S., GRANGETTE, C., DENNIN, V., GOUDERCOURT, D., POIRET, S., et al. (2007). Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. World Journal of *Gastroenterology*, v.13, p. 236-243.

FRANK, D. N., AMAND, A. L. S., FELDMAN, R. A., BOEDEKER, E. C., HARPAZ, N., & PACE, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences of the United States of America,* v.104, n. 34, p. 13780-13785. doi: 10.1073/pnas.0706625104

FULLER, R. (1991). PROBIOTICS IN HUMAN MEDICINE. *Gut,* v. 32, n. 4, p. 439-442.

GALDEANO, C. M., & PERDIGON, G. (2004). Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. *Journal of Applied Microbiology,* v. 97, n. 4, p. 673-681. doi: 10.1111/j.1365-2672.2004.02353.x

GALDEANO, C. M., DE MORENO DE LEBLANC, A. D., VINDEROLA, G., BONET, A. E. B., & PERDIGON, G. (2007). Proposed model: Mechanisms of immunomodulation induced by probiotic bacteria. *Clinical and Vaccine Immunology,* v. 14, n. 5, p. 485-492. doi: 10.1128/cvi.00406-06

GILL, H. S., & RUTHERFURD, K. J. (2001). Immune enhancement conferred by oral delivery of Lactobacillus rhamnosus HN001 in different milk-based substrates. *Journal of Dairy Research,* v. 68, n. 4, p. 611-616. doi: 10.1017/s0022029901005155

GILL, H. S., RUTHERFURD, K. J., & CROSS, M. L. (2001). Dietary probiotic supplementation enhances natural killer cell activity in the elderly: An investigation of age-related immunological changes. *Journal of Clinical Immunology,* v. 21, n. 4, p. 264-271.

GILL, H. S., RUTHERFURD, K. J., PRASAD, J., & GOPAL, P. K. (2000). Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *British Journal of Nutrition,* v. 83, n. 2, p. 167-176.

GILL, S. R., POP, M., DEBOY, R. T., ECKBURG, P. B., TURNBAUGH, P. J., SAMUEL, B. S., NELSON, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *Science,* v. 312, n. 5778, p. 1355-1359. doi: 10.1126/science.1124234

GIONCHETTI, P., RIZZELLO, F., VENTURI, A., BRIGIDI, P., MATTEUZZI, D., BAZZOCCHI, G., CAMPIERI, M. (2000). Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: A double-blind, placebo-controlled trial. *Gastroenterology,* v. 119, n. 2, p. 305-309.

GIONCHETTI, P., RIZZELLO, F., VENTURI, A., MATTEUZZI, D., ROSSI, M., PERUZZO, S., CAMPIERI, M. (1998). Maintenance treatment of chronic pouchitis: A randomised placebo-controlled, double-blind trial with a new probiotic preparation. *Gastroenterology,* v. 114, n. 4, p. G4037.

GOBBETTI, M., DI CAGNO, R., DE ANGELIS, M. (2010) Functional microorganisms for functional food quality. *Critical Reviews in Food Science and Nutrition*. v. 50, n. 8, pp. 716-727

GOPAL, P. DEKKER, J. PRASAD, J. PILLIDGE, C., DELABRE M.L. & COLLETT, M. (2005). Development and commercialisation of Fonterra's probiotic strains. *Australian Journal of Dairy Technology*. v. 60, n. 2 SPEC. ISS., p. 173-182

GOTTELAND, M., BRUNSER, O., & CRUCHET, S. (2006). Systematic review: are probiotics useful in controlling gastric colonization by Helicobacter pylori? *Alimentary Pharmacology & Therapeutics,* v. 23, n. 8, p. 1077-1086. doi: 10.1111/j.1365-2036.2006.02868.x

GRZEŚKOWIAK, L., ISOLAURI, E., SALMINEN, S. & GUEIMONDE, M., (2011). Manufacturing process influences properties of probiotic bacteria. *British Journal of Nutrition*, v. 105, n. 6, pp. 887-894.

HAJIROSTAMLOO, B. (2010). Bioactive component in milk and dairy product. *World Academy of Science, Engineering and Technology.* v. 72 , pp. 162-166

HAN, Y., MA, Q., LU, J., XUE, Y., XUE, C. (2012) Optimisation for subcritical fluid extraction of 17-methyltestosterone with 1,1,1,2-tetrafluoroethane for HPLC analysis. *Food Chemistry*. v. 135, n. 4, pp. 2988-2993

HOLS, P., HANCY, F., FONTAINE, L., GROSSIORD, B., PROZZI, D., LEBLOND-BOURGET, N., et al. (2005). New insights in the molecular biology and physiology of Streptococcus thermophilus revealed by comparative genomics. *FEMS Microbiology Reviews*, v. 29 (Suppl. 3), p. 435-463.

HOLZAPFEL, W. H., HABERER, P., GEISEN, R., BJORKROTH, J., SCHILLINGER, U. Taxonomy and important features of probiotic microorganisms in food and nutrition. *American Journal of Clinical Nutrition*, v. 73, p. 365-373, 2001.

HOPKINS, M. J., SHARP, R., & MACFARLANE, G. T. (2001). Age and disease related changes in intestinal bacterial popular-ions assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. *Gut, 48*(2), 198-205.

HUTTENHOWER, C., GEVERS, D., KNIGHT, R., ABUBUCKER, S., BADGER, J.H., CHINWALLA, A.T., CREASY, H.H., & WHITE, O. (2012). Structure, function and diversity of the healthy human microbiome .*Nature* v. 486, p. 207–214. doi:10.1038/nature11234

IBNOU-ZEKRI, N. *et al.* Divergent patterns of colonization and immune response elicited from two intestinal Lactobacillus strains that display similar properties in vitro. *Infection and Immunity* [S.l.], v. 71, n. 1, p. 428-436, 2003.

ISO. (2001). International Organization for Standarzation. Milk and milk products - Extraction methods for lipids and liposoluble compounds. International Standard *ISO 14156-IDF 172:2001.* 1 ed., 01-06, 2001

ISO. Standard 15884, Milk fat - preparation of fatty acid methyl esters. In: STANDARDIZATION, I. O. F. (Ed.). Geneve, 2002.

ISOLAURI, E., JOENSUU, J., SUOMALAINEN, H., LUOMALA, M., & VESIKARI, T. (1995). Improved Immunogenicity Of Oral Dxrrv Reassortant Rotavirus Vaccine By *Lactobacillus-Casei* GG. *Vaccine,* v. 13, n. 3, p. 310-312.

ISOLAURI, E., KIRJAVAINEN, P.V. & SALMINEN, S. (2002). Probiotics: effects on immunity. *Gut*. V. 50, p. 54-59.

ISOLAURI, E. SUTAS, Y., KANKAANPAA, P., ARVILOMMI, H. & SALMINEN, S. (2001). Probiotics: effects on immunity. *American Journal of Clinical Nutrition.* V.73, n. 2, p. 444S-450S.

IWASAKI, A., & MEDZHITOV, R. (2004). Toll-like receptor control of the adaptive immune responses. *Nature Immunology,* v. *5,* n. 10, p. 987-995. doi: 10.1038/ni1112

JANEWAY, C. A., & MEDZHITOV, R. (2002). Innate immune recognition. *Annual Review of Immunology,* v.*20*, p.197-216.

KAISHO, T., & AKIRA, S. (2002). Toll-like receptors as adjuvant receptors. *Biochimica Et Biophysica Acta-Molecular Cell Research,* v. 1589, n. 1, p. 1-13.

KANKAANPAA, P., SUTAS, Y., SALMINEN, S., ISOLAURI, E. Homogenates derived from probiotic bacteria provide down-regulatory signals for peripheral blood mononuclear cells. *Food Chemistry*, v. 83, n. 2, p. 269-277, 2003.

KELLY, D., & CONWAY, S. (2005). Bacterial modulation of mucosal innate immunity. *Molecular Immunology,* v. 42, n. 8, p. 895-901. doi: 10.1016/j.molimn.2004.12.003

KELLY, D., CONWAY, S., & AMINOV, R. (2005). Commensal gut bacteria: mechanisms of immune modulation. *Trends in Immunology,* v. 26, n. 6, p. 326-333. doi: 10.1016/j.it.2005.04.008

KIRJAVAINEN, P. V., OUWEHAND, A. C., ISOLAURI, E., & SALMINEN, S. J. (1998). The ability of probiotic bacteria to bind to human intestinal mucus. *Fems Microbiology Letters,* v. 167, n. 2, p. 185-189. doi: 10.1016/s0378-1097(98)00387-5

KRUININGEN, H.J., WEST, A.B., FREDA, B.J. & HOLMES, K.A. (2002). Distribution of Peyer's patches in the distal ileum. *Inflammatory Bowel Diseases*. v. 8, n. 3, pp. 180-185

KUNDA, P.B., BENAVENTE, F., CATALÁ-CLARIANA, S., GIMÉNEZ, E., BARBOSA, J., SANZ-NEBOT, V. (2012). Identification of bioactive peptides in a functional yogurt by micro liquid chromatography time-of-flight mass spectrometry assisted by retention time prediction. *Journal of Chromatography A*, v. 1229 p. 121–128

KURDI, P., H. TANAKA, H. W. VAN VEEN, K. ASANO, F. TOMITA, AND A. YOKOTA. (2003). Cholic acid accumulation and its diminution by short-chain fatty acids in bifidobacteria. *Microbiology* v. 149, p. 2031–2037**.**

LAMMERS, K. M.O.; BRIGIDI, P.O.; VITALI, B.O.; GIONCHETTI, P.O.; RIZZELLO, F.O.; CARAMELLI, E.O.; MATTEUZZI, D.O.; CAMPIERI, M. Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripherical blood mononuclear cells. *FEMS Immunology and Medical Microbiology*, v. 38, p. 165-172, 2003.

LEY, R. E., BACKHED, F., TURBAUGH, P., LOZUPONE, C. A., KNIGHT, R. D., & GORDON, J. I. (2005). Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America,* v. 102, n. 31, p. 11070-11075. Doi: 10.1073/pnas.0504978102.

LEY, R. E., PETERSON, D. A., & GORDON, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell,* v. 124, n. 4, p. 837-848.

LODINOVA-ZADNIKOVA, R., CUKROWSKA, B., TLASKALOVA-HOGENOVA, H. Oral administration of probiotic Escherichia coli after birth reduces frequency of allergies and repeated infections later in life (after 10 and 20 years). *International Archives of Allergy and Immunology*, v. 131, n. 3, p. 209-211, 2003.

LUNA, P., MARTÍN-DIANA, A. B., ALONSO, L., FONTECHA, J., DE LA FUENTE, M. A., REQUENA, T. & JUÁREZ, M. (2004). Effects of milk fat replacement by PUFA enriched fats on n-3 fatty acids, conjugated dienes and volatile compounds of fermented milks. *European Journal of Lipid Science and Technology*, v. 106, p. 417-423.

MACFARLANE, S., & MACFARLANE, G. T. (2004). Bacterial diversity in the human gut. *Advances in Applied Microbiology,* v. 54*,* p. 261-289.

MACKIE, R. I., SGHIR, A., & GASKINS, H. R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. *American Journal of Clinical Nutrition, 69*(5), 1035S-1045S.

MACPHERSON, A. J., & HARRIS, N. L. (2004). Interactions between commensal intestinal bacteria and the immune system. *Nature Reviews Immunology, 4*(6), 478-485. doi: 10.1038/nri1373

MACPHERSON, A. J., & UHR, T. (2004). Compartmentalization of the mucosal immune responses to commensal intestinal bacteria. *Oral Tolerance: New Insights and Prospects for Clinical Application, 1029*, 36-43. doi: 10.1196/annals.1309.005

MACPHERSON, A. J., GEUKING, M. B., & MCCOY, K. D. (2011). Immunoglobulin A: a bridge between innate and adaptive immunity. *Current Opinion in Gastroenterology, 27*(6), 529 - 533.

MADSEN, K. L., DOYLE, J. S., JEWELL, L. D., TAVERNINI, M. M., & FEDORAK, R. N. (1999). Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. *Gastroenterology, 116*(5), 1107-1114.

SZWAJKOWSKA, M, WOLANCIUK, A. , BARŁOWSKA, J., KRÓL, J. & LITWIŃCZUK, Z. (2011). Bovine milk proteins as the source of bioactive peptides influencing the consumers' immune system – a review. *Animal Science Papers and Reports.* v. 29, n. 4, p. 269-280.

MATTO, J., MALINEN, E., SUIHKO, M. L., ALANDER, M., PALVA, A., SAARELA, M. Genetic heterogeneity and functional properties of intestinal bifidobacteria. *Journal of Applied Microbiology*, v. 97, n. 3, p. 459-470, 2004.

MEDICI, M., VINDEROLA, C. G., WEILL, R., & PERDIGON, G. (2005). Effect of fermented milk containing probiotic bacteria in the prevention of an

enteroinvasive Escherichia coli infection in mice. *Journal of Dairy Research,* v. 72, n. 2, p. 243-249. doi: 10.1017/s0022029905000750

MERCENIER, A.; PAVAN, S.; POT, B. Probiotics as Biotherapeutic Agents: Present Knowledge and Future Prospects. *Current Pharmaceutical Design*, v. 9, p. 175-191, 2003

METCHNIKOFF, E. The prolongation of life ; optimistic studies. *The prolongation of life ; optimistic studies*, p. (xx + 343), 1907.

NETEA, M. G., VAN DER GRAAF, C., VAN DER MEER, J. W. M., & KULLBERG, B. J. (2004). Recognition of fungal pathogens by Toll-like receptors. *European Journal of Clinical Microbiology & Infectious Diseases, 23*(9), 672-676. doi: 10.1007/s10096-004-1192-7

NEUTRA, M. R., MANTIS, N. J., & KRAEHENBUHL, J. P. (2001). Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nature Immunology,* v. 2, n. 11, p. 1004-1009.

NOVERR, M. C.; HUFFNAGEL, G. B. Does the microbiota regulate immune responses outside the gut? *Trends in Microbiology*, v. 12, n. 12, p. 562-568, 2004.

ODA, L. M., KUBELKA, C. F., ALVIANO, C. S., TRAVASSOS, L. R. Ingestion of yeast forms of sporothrix-schenckii by mouse peritoneal-macrophages. *Infection and Immunity*, v. 39, n. 2, p. 497-504, 1983.

OH, D. K., HONG, G. H., LEE, Y., MIN, S. G., SIN, H. S., & CHO, S. K. (2003). Production of conjugated linoleic acid by isolated Bifidobacterium strains. *World Journal of Microbiology and Biotechnology*, v. 19, p. 907-912.

OLIVEIRA, M. N., SODINI, I., REMEUF, F., & CORRIEU, G. (2001). Effect of milk supplementation and culture composition on acidification, textural properties and microbiological stability of fermented milks containing probiotic bacteria. *International Dairy Journal*, v. 11 (Suppl. 11-12), p. 935-942.

OLIVEIRA, R. P. S., FLORENCE, A. C. R., SILVA, R. C., PEREGO, P., CONVERTI, A., GIOIELLI, L. A., et al. (2009). Effect of different prebiotics on the fermentation kinetics, probiotic survival and fatty acids profiles in nonfat symbiotic fermented milk. *International Journal of Food Microbiology,* v. 128 (Suppl. 3), p. 467-472.

OUWEHAND, A. C., BERGSMA, N., PARHIALA, R., LAHTINEN, S., GUEIMONDE, M., FINNE-SOVERI, H., STRANDBERG, T., PITKALA, K., SALMINEN, S. Bifidobacterium microbiota and parameters of immune function in elderly subjects. *Fems Immunology and Medical Microbiology*, v. 53, n. 1, p. 18-25, 2008.

OUWEHAND, A. C., SALMINEN, S., & ISOLAURI, E. (2002). Probiotics: an overview of beneficial effects. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 82*(1-4), 279-289.

PALMER, C., BIK, E. M., DIGIULIO, D. B., RELMAN, D. A., & BROWN, P. O. (2007). Development of the human infant intestinal microbiota. *Plos Biology, 5*, 1556-1573.

PATURI, G., PHILLIPS, M., KAILASAPATHY, K. Effect of Probiotic strains Lactobacillus acidophilus LAFTI l10 and Lactobacillus paracasei LAFT1 l26 on systemic immune functions and bacterial translocation in mice. *Journal of Food Protection*, v. 71, n. 4, p. 796-801, 2008.

PERDIGON, G., ALVAREZ, S., DEMACIAS, M. E. N., ROUX, M. E., & HOLGADO, A. P. D. (1990). The oral-administration of lactic-acid bacteria increase the mucosal intestinal immunity in response to enteropathogens. *Journal of Food Protection, 53*(5), 404-410. Perdigon et al., 2002

PERDIGON, G., ALVAREZ, S., DEMACIAS, M. E. N., ROUX, M. E., HOLGADO, A. P. D. Interaction of bifidobacteria with the gut and their influence in the immune function. *Biocell*, v. 27, n. 1, p. 1-9, 2003.

PERDIGON, G., GALDEANO, C. M., VALDEZ, J. C., & MEDICI, M. (2002). Interaction of lactic acid bacteria with the gut immune system. *European Journal of Clinical Nutrition, 56*, S21-S26. doi: 10.1038/sj.ejcn.1601658

PERDIGON, G., MEDINA, M., VINTINI, E., & VALDEZ, J. C. (2000). Intestinal pathway of internalisation of lactic acid bacteria and gut mucosal immunostimulation. *International Journal of Immunopathology and Pharmacology, 13*(3), 141-150.

PEREZ, N., IANNICELLI, J. C., GIRARD-BOSCH, C., GONZALEZ, S., VAREA, A., DISALVO, L., CRAVERO, R. (2010). Effect of probiotic supplementation on immunoglobulins, isoagglutinins and antibody response in children of low socio-economic status. *European Journal of Nutrition, 49*(3), 173-179. doi: 10.1007/s00394-009-0063-5

PESSI, T., ISOLAURI, E., SUTAS, Y., KANKAANRANTA, H., MOILANEN, E., & HURME, M. (2001). Suppression of T-cell activation by Lactobacillus rhamnosus GG-degraded bovine casein. *International Immunopharmacology, 1*(2), 211-218.

RAKOFF-NAHOUM, S., PAGLINO, J., ESLAMI-VARZANEH, F., EDBERG, S., & MEDZHITOV, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell, 118*(2), 229-241.

REID, G., YOUNES, J. A., VAN DER MEI, H. C., GLOOR, G. B., KNIGHT, R., & BUSSCHER, H. J. (2011). Microbiota restoration: Natural and supplemented recovery of human microbial communities. *Nature Reviews Microbiology,* v. *9,* n. 1, p. 27-38. doi: 10.1038/nrmicro2473

RODES, L., PAUL, A., COUSSA-CHARLEY, M., AL-SALAMI, H., TOMARO-DUCHESNEAU, C., FAKHOURY, M., & PRAKASH, S. (2011). Transit Time Affects the Community Stability of Lactobacillus and Bifidobacterium Species in an In Vitro Model of Human Colonic Microbiotia. *Artificial Cells Blood Substitutes and Biotechnology, 39*(6), 351-356. doi: 10.3109/10731199.2011.622280

ROSA, F. T., ZULET, M., MARCHINI, J.S. & MARTINEZ, J. A. (2012). Bioactive compounds with effects on inflammation markers in humans. *International Journal of Food Sciences and Nutrition*, v. 63, n. 6, p. 749–765

RUAS-MADIEDO, P., HUGENHOLTZ, J., ZOON, P. An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. *International Dairy Journal*, v. 12, n. 2-3, p. 163-171, 2002.

RUMBO, M., ANDERLE, P., DIDIERLAURENT, A., SIERRO, F., DEBARD, N., SIRARD, J. C., KRAEHENBUHL, J. P. (2004). How the gut links innate and adaptive immunity. *Oral Tolerance: New Insights and Prospects for Clinical Application, 1029*, 16-21. doi: 10.1196/annals.1309.003

SACARO, D.M., TAMIME, A.Y., PILLEGGI, A.L.O.P.S. & OLIVEIRA, M.N. (2009) The viability of three probiotic organisms grown with yoghurt starter cultures during storage for 21 days at 4 degrees C. *International Journal of Dairy Technology*. v. 62, n. 3, p. 397-404.

SACARO, D.M., HIROTA, C.Y., TAMIME, A.Y., & OLIVEIRA, M.N. (2011). Evaluation of different selective media for enumeration of probiotic micro-organisms in combination with yogurt starter cultures in fermented milk. *African Journal of Microbiology Research*. v. 46, p. 3901-3906.

SANCHEZ, B., RUIZ, L., GUEIMOND, M. RUAS-MADIEDO, P. & MARGOLES, A. (2012). Toward improving technological and functional properties of probiotics in foods. *Trends in Food Science & Technology.* v. 26, n. 1, p. 56e63

SANCHEZ, B., REYES-GAVILAN, C. G. D., MARGOLLES, A. (2006). The F1F0-ATPase of Bifidobacterium animalis is involved in bile tolerance. *Environmental Microbiology*, v. 8, n. 10, p. 1825-1833, 2006.

SÁNCHEZ, B., REYES-GAVILA, C.G., MARGOLLES, A. & GUEIMONDE, M. (2009). Probiotic fermented milks: Present and future. *International Journal of Dairy Technology*.v. 62 n. 4, pp. 472-483

SAWA, S., LOCHNER, M., SATOH-TAKAYAMA, N., DULAUROY, S., BERARD, M., KLEINSCHEK, M., CUA, D., DI SANTO, J. P., EBERL, G. ROR gamma t(+) innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nature Immunology*, v. 12, n. 4, p. 320-U71, 2011.

SCHIFFRIN, E. J., BRASSART, D., SERVIN, A. L., ROCHAT, F., & DONNETHUGHES, A. (1997). Immune modulation of blood leukocytes in humans by lactic acid bacteria: Criteria for strain selection. *American Journal of Clinical Nutrition, 66*(2), S515-S520.Spinnler & Corrieu, 1989

SOLISPEREYRA, B., AATTOURI, N., & LEMONNIER, D. (1997). Role of food in the stimulation of cytokine production. *American Journal of Clinical Nutrition, 66*(2), S521-S525.

SONNENBURG, J. L., CHEN, C. T. L., & GORDON, J. I. (2006). Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host. *Plos Biology, 4*(12), 2213-2226. doi: 10.1371/journal.pbio.0040413

SPINNLER, H. E.; CORRIEU, G. Automatic method to quantify starter activity based on pH measurement. *Journal of Dairy Research*, v. 56, p. 755-764, 1989.

STANTON, C.; GARDINER, G.; MEEHAN, H.; COLLINS, K.; FITZGERALD, G.; LYNCH, P. B.; ROSS, R. P. Market potential for probiotics. *The American Journal of Clinical Nutrition*, v. 73, p. 476S-83S, 2001.

STILES, M. E., & HOLZAPFEL, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology, 36*(1), 1-29.

SULLIVAN, A.; NORD, C. E. The place of probiotics in human intestinal infections. *International Journal of Antimicrobial Agents*, v. 20, n. 5, p. 313-319, 2002.

TAMIME, A. *Dairy Microbiology Handbook: The Microbiology of Milk and Milk Products, 3rd Edition.* Edited by Richard K. Robinson Copyright 0 2002 John Wiley and Sons, Inc. ISBN: 0471-38596-4

TISSIER, H. Treatment of intestinal infection by way of transforming the bacterial flora of the intestine. *Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales*, v. 58, p. 359-361, 1906.

TUOMOLA, E., CRITTENDEN, R., PLAYNE, M., ISOLAURI, E., & SALMINEN, S. (2001). Quality assurance criteria for probiotic bacteria. *American Journal of Clinical Nutrition, 73*(2), 393S-398S.

TURRONI, F., RIBBERA, A., FORONI, E., VAN SINDEREN, D., & VENTURA, M. (2008). Human gut microbiota and bifidobacteria: from composition to functionality. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 94*(1), 35-50. doi: 10.1007/s10482-008-9232-4

URONIS, J. M., ARTHUR, J. C., KEKU, T., FODOR, A., CARROLL, I. M., CRUZ, M. L., APPLEYARD, C. B., JOBIN, C. Gut Microbial Diversity Is Reduced by the Probiotic VSL#3 and Correlates with Decreased TNBS-Induced Colitis. *Inflammatory Bowel Diseases*, v. 17, n. 1, p. 289-297, 2011.

VASILJEVIC, T., & SHAH, N. P. (2008). Probiotics—From Metchnikoff to bioactives. *International Dairy Journal, 18*, 714– 728.

VENTURA, M., VAN SINDEREN, D., FITZGERALD, G. F., & ZINK, R. (2004). Insights into the taxonomy, genetics and physiology of bifidobacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 86*(3), 205-223.

VINDEROLA, G., MATAR, C., & PERDIGON, G. (2005). Role of intestinal epithelial cells in immune effects mediated by gram-positive probiotic bacteria: Involvement of Toll-like receptors. *Clinical and Diagnostic Laboratory Immunology, 12*(9), 1075-1084. doi: 10.1128/cdli.12.9.1075-1084.2005

VITIÑI, E., ALVAREZ, S., MEDINA, M., MEDICI, M., DE BUDEGUER, M. V., & PERDIGON, G. (2000). Gut mucosal immunostimulation by lactic acid bacteria. *Biocell, 24*(3), 223-232.

WALLACE, T. C., GUARNER, F., MADSEN, K., CABANA, M. D., GIBSON, G., HENTGES, E., & SANDERS, M. E. (2011). Human gut microbiota and its relationship to health and disease. *Nutrition Reviews, 69*(7), 392-403. doi: 10.1111/j.1753-4887.2011.00402.x

WISNIEWSKI, J. A., & BORISH, L. (2011). Novel cytokines and cytokine-producing T cells in allergic disorders. *Allergy and Asthma Proceedings, 32*(2), 83-94. doi: 10.2500/aap.2011.32.3428

WROBLEWSKA, B., KALISZEWSKA, A., MALINOWSKA, E., & TROSZYNSKA, A. (2011). Immunoreactivity of transglutaminase cross-linked milk proteins in fermented milk product obtained with Lactobacillus acidophilus. *Postepy Dermatologii I Alergologii, 28*(4), 261-267.

YAN, F., & POLK, D. B. (2011). Probiotics and immune health. *Current Opinion in Gastroenterology*. doi: DOI: 10.1097/MOG.0b013e32834baa4d

ZOETENDAL, E. G., BEN-AMOR, K., AKKERMANS, A. D. L., ABEE, T., & DE VOS, W. M. (2001). DNA isolation Protocols affect the detection limit of PCR approaches of bacteria in samples from the human gastrointestinal tract. *Systematic and Applied Microbiology, 24*(3), 405-410.