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TESE

Efeito da Pasteurização Sobre Proteínas Bioativas do Leite Humano e Identificação de Lactobacilos de Origem Humana: Caracterização da Atividade Probiótica *in vitro*

André Fioravante Guerra

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ANDRÉ FIORAVANTE GUERRA

Sob a Orientação da Professora
Rosa Helena Luchese

Tese submetida como requisito parcial para obtenção do grau de **Doutor em Ciências,** no Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Área de Concentração em Ciência de Alimentos

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RESUMO

GUERRA, André Fioravante. **Efeito da pasteurização sobre proteínas bioativas do leite humano e identificação de lactobacilos de origem humana: Caracterização da atividade probiótica *in vitro*.** 2018. 120 p. Tese (Doutorado em Ciência e Tecnologia de Alimentos). Instituto de Tecnologia, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ. 2018.

O objetivo desta pesquisa foi avaliar o impacto do tratamento térmico praticado nos bancos de leite sobre proteínas bioativas do leite humano; identificar genotipicamente e selecionar cepas de lactobacilos de origem humana; determinar o efeito de fatores de estresse sobre a susceptibilidade de lactobacilos à agentes antibióticos; e avaliar suas propriedades tecnológicas, funcionais e de segurança, visando a utilização como probióticos. A atividade de glutationa peroxidase (GPx), lisozima (Lis) e perfil proteico do leite humano foi monitorado ao longo de 6 meses de lactação, antes e após ~~a~~ pasteurização lenta a 62,5 °C por 30 minutos (LTLT). Os lactobacilos foram identificados por sequenciamento da região 16S do rDNA e agrupados por *clusters* de similaridade genética através do RAPD-PCR. Foram avaliadas alterações na susceptibilidade de um representante de cada *cluster* de lactobacilos frente a 12 antibióticos de uso humano, assim como a sobrevivência em leite fermentado por até 45 dias de estocagem a 7 °C, antes e após passagem pelas condições gastrointestinais *in vitro* (CGI). No quadragésimo quinto dia de estocagem, também foi mensurado o pH, a acidez titulável e a produção de peróxido de hidrogênio. Uma cepa representativa das duas espécies mais comumente isoladas do intestino de lactentes (*Lactobacillus rhamnosus* DTA 79 e *Lactobacillus paracasei* DTA 83) foi selecionada para avaliação e utilização como potenciais probióticos. Para isso, testou-se as propriedades probióticas e de segurança como inibição da formação de biofilmes por patogênicos, ausência de hemólise alfa ou gama, sensibilidade à Lis, além da susceptibilidade aos agentes antibióticos e sobrevivência às CGI. O potencial tecnológico foi caracterizado através da determinação da capacidade de acidificação do leite em crescimento axênico ou em co-cultura com *Streptococcus thermophilus* (TH 895 e TH 1435), pelas propriedades cinéticas de coagulação do leite e pela capacidade de sobrevivência em sorbet e sherbet, em pastilhas probióticas e em leite fermentado adicionado ou não de mel de abelha (5 % p/v) pasteurizado (78 °C/6 min). LTLT reduziu o conteúdo de LF, Igs e a atividade de GPx do leite humano. Em adição, a atividade de GPx foi maior no colostro e no leite de transição e reduziu naturalmente ao longo de 6 meses de lactação. Em contraste, LTLT não afetou a atividade de Lis do leite humano. O estresse ocasionado pela passagem às CGI ou pela fermentação e estocagem do leite fermentado, alteraram a susceptibilidade de lactobacilos aos antibióticos que atuam sobre a parede celular ou sobre a síntese proteica e de ácidos nucleicos. Conclui-se que LTLT reduz o potencial antimicrobiano e antioxidante do leite humano por reduzir a atividade de GPx, imunoglobulinas e LF. Condições de estresse microbiano alteram a susceptibilidade de lactobacilos à antibióticos e podem induzir a transferência horizontal de genes de resistência. O leite de vaca constitui boa matriz para veicular lactobacilos probióticos (DTA 79 e DTA 83), mas redução na viabilidade foi observado ao longo da validade comercial em sorbet (base de água), mostrando não ser uma boa matriz para esta finalidade. Mel de abelha confere fatores de crescimento e proteção para ~~a~~ lactobacilos durante à passagem pelas CGI. Pastilhas probióticas possuem potencial para reduzir a candidíase oral em pessoas na terceira idade, efetividade foi observada *in vitro*, mas estudos *in vivo* ainda precisam ser conduzidos para comprovação da eficácia.

Palavras chave – probióticos, potencial, matrix alimentícia

ABSTRACT

GUERRA, André Fioravante. **Effects of holder pasteurization on human milk bioactive proteins and identification of *lactobacilli* from human origin: *In vitro* probiotic activity characterization.** 2018. 120 p. Thesis (Doctorate degree in Food Science and Technology). Institute of Technology, Federal Rural University of Rio de Janeiro, Seropédica, RJ. 2017.

The aim of this research was to evaluate the impact of the heat treatment practiced in milk banks (MB) on bioactive molecules of human milk; isolate and identify genotypically and select lactobacilli strains from human origin; to evaluate the effect of microbial stress factors, milk fermentation, cold storage and *in vitro* gastrointestinal conditions (GIC) on the lactobacilli antibiotic susceptibility; besides to evaluate its technological, functional, and safety properties aiming its use as probiotics. The activity of glutathione peroxidase (GPx), lysozyme (Lys), and electrophoretic protein profile of human milk samples were monitored during six months of lactation before and after Holder Pasteurization (HoP). Lactobacilli were identified by Gram and catalase test, 16S rDNA sequencing, and grouped in 9 clusters of genetic similarity by RAPD-PCR method. Changes in the susceptibility of a representative of each cluster of lactobacilli against 12 antibiotic agents for human use, as well as their microbial survival in fermented milk, were investigate in three moments: inoculated milk (IM), fermented milk (FM), and after 45 days cold (7 °C) storage (CSM) before and after *in vitro* GIC. At 45 days of storage, the hydrogen potential (pH), and titratable acidity, and hydrogen peroxide (H_2O_2) production were also measured. A representative strain of each cluster of most commonly lactobacilli isolated from the intestines of infants (*Lactobacillus rhamnosus* DTA 79 and *Lactobacillus paracasei* DTA 83) were selected for evaluation and use as probiotics. For this, they were assessed for safety and probiotic properties such as inhibition of biofilm formation by pathogens, presence or absence of alpha or gamma hemolysis, sensitivity to Lys, as well as its susceptibility to antibiotics and GIC survival. Technological potential was assessed by milk acidification potential either in axenic growth or co-cultivation with *Streptococcus thermophilus* (TH 895 and TH 1435), by the kinetic properties of milk coagulation, and by microbial survivability in ice cream bar or ice-lolly, in probiotic tablets, and in fermented milk added or not of pasteurized (78 °C/6 min) honey bee (5% w/v). HoP reduced the content of lactoferrin (LF), immunoglobulin (Igs) and GPx of human milk. In addition, GPx was higher in colostrum and transitional milk, naturally reducing during 6 months of lactation. In contrast, HoP did not affect human milk Lys activity. Thirty-five lactobacilli were isolated from infant stools and 25 were naturally associated with bifidobacteria. In general, stressful conditions such fermentation and cold storage of the fermented milk increased antibiotic susceptibility over time while, GIC reduced their susceptibility HoP reduces antimicrobial and antioxidant potential of human milk by inactivating GPx, (Igs) and LF. Changes in the antibiotic susceptibility of lactobacilli was observed in this research and this can increase the risk of horizontal induction gene resistance transfer. Cow's milk is a suitable matrix for delivering both DTA 79 or DTA 83 strains, but reduction in viability was observed over commercial shelf-life in sherbet and sorbet (water-based), showing that it will not be a good matrix for this purpose. Probiotic tablets have potential to reduce oral candidiasis in elderly, as shown by their effectiveness *in vitro* tests, but *in vivo* studies shall be further performed.

Key word – probiotic, potential, food matrix

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INTRODUÇÃO GERAL

Microrganismos probióticos influenciam positivamente a saúde, especialmente pela capacidade de inibir patógenos e estimular o sistema imune. O leite materno (LM) é um alimento funcional e possui fatores prebióticos, isto é, capazes de estimular a prevalência de bactérias probióticas, principalmente bifidobactérias, no trato intestinal nos primeiros meses de vida.

Probióticos são alimentos funcionais microbianos. Alimentos funcionais são semelhantes em aparência ao alimento consumido como parte da dieta usual, porém além das funções nutricionais básicas, são capazes de produzir efeitos metabólicos ou fisiológicos importantes para manutenção da boa saúde física, mental e no auxílio à redução do risco de doenças crônico-degenerativas.

O LM é fonte de muitos componentes multifuncionais, como substâncias antioxidantes e antimicrobianas, sendo o primeiro alimento prebiótico disponível para consumo humano. Desta forma, além dos compostos antimicrobianos que inibem o crescimento de patógenos, o LM também possui substâncias que estimulam o crescimento de bactérias probióticas.

Porém, em alguns casos, sua oferta pelas mães não é possível e diante destas circunstâncias, criaram-se bancos de leite humano (BLH). O LM ordenhado é submetido, nos BLH, ao processo conhecido como pasteurização lenta (62,5 ºC/30 min). Este processo visa à eliminação dos microrganismos patogênicos e a redução dos deteriorantes a um nível aceitável. Tal fato, pode resultar em perda de atividade de substâncias termo sensíveis.

O consumo de microrganismos probióticos se faz necessário durante toda a vida de um indivíduo. Há várias formas de veicular estes microrganismos, mas a mais comum é utilizando matrizes alimentícias para esta finalidade. Para isso, os probióticos devem manter-se viáveis até chegarem à luz intestinal, onde ainda devem desempenhar função benéfica ao hospedeiro.

A disbiose da microbiota intestinal é um problema cada vez mais frequente e tem como consequência várias doenças correlacionadas. Uma nova abordagem relaciona o consumo de prebióticos, probióticos e simbióticos na modulação e atividade de microbiota intestinal, que atuam especialmente na exclusão competitiva de patógenos e estimulação do sistema imunológico. Desta forma, uma alimentação saudável é essencial para manutenção da boa saúde.

Bifidobacterium e *Lactobacillus* são os gêneros mais importantes para manutenção de uma boa saúde humana, e o tipo de alimentação pode influenciar positivamente o estabelecimento e modulação em prol de uma microbiota benéfica na luz intestinal. Ambos, bifidobactérias e lactobacilos, são beneficiados em ambientes com baixo potencial redox (E_h) e, consequentemente, pelas substâncias antioxidantes presentes naturalmente no leite humano. Portanto, o conhecimento sobre o potencial prebiótico do leite humano *in natura* e pasteurizado a 62,5 °C por 30 minutos (LTLT) é de suma importância para prever os benefícios transmitidos aos lactentes quando alimentados ao peito ou com leite dos bancos. Aumentar o conhecimento sobre a composição da microbiota intestinal, assim como a necessidade nutricional e possíveis interações microbianas, especialmente à exclusão de patógenos, é crucial para modular beneficamente este bioma

Apesar dos indubitáveis benefícios provenientes dos microrganismos probióticos, se faz necessário aprofundar o conhecimento acerca das condições de segurança dos probióticos. Microrganismos podem expressar mecanismos de resistência a antibióticos, sob condições de estresse gerado por fatores intrínsecos e extrínsecos desfavoráveis. Desta forma, a resistência gênica aos antibióticos conferida pelas condições de estresse poderia resultar em futuras transferências horizontais destes genes para microrganismos patogênicos ou indesejáveis. Os microrganismos probióticos atuariam como verdadeiros bancos de genes de resistência.

OBJETIVOS

Geral

Estudar o impacto da pasteurização sobre moléculas bioativas do leite humano e avaliar propriedades funcionais, tecnológicas e de segurança de novas cepas probióticas de origem humana.

Específicos

- Avaliar o impacto do tratamento térmico praticado nos bancos de leite (LTLT) sobre a atividade de glutationa peroxidase e de lisozima, além de verificar alterações no perfil proteico pela técnica do SDS-PAGE;

- Separar, re-isolar lactobacilos e bifidobactérias naturalmente associados e identificá-los à nível de gênero e espécie;

- Avaliar o efeito do estresse causado pela fermentação, armazenamento de leites fermentados e passagem pelas condições gastrointestinais *in vitro* sobre a susceptibilidade de lactobacilos à agentes antibióticos;

- Avaliar as propriedades tecnológicas, funcionais e de segurança para uso como probióticos das cepas mais promissoras;

- Desenvolver produto alimentício e suplemento alimentar com a utilização das cepas mais promissoras.

CHAPTER I
HOLDER PASTEURIZATION OF HUMAN MILK AFFECTS SOME BIOACTIVE PROTEINS

ABSTRACT

The aim of this research was to investigate the effect of Holder pasteurization (HoP) on the protein profile and glutathione peroxidase (GPx) and lysozyme (Lys) activities in human milk. Human milk samples were analyzed over six months of lactation, before and after HoP, for GPx, Lys, and protein electrophoresis profile. HoP caused changes in lactoferrin (LF) and immunoglobulin-protein profiles. HoP did not affect Lys, but reduced GPx activity. In addition, GPx activity which is high in colostrum and transitional milk was naturally reduced over the 6-month lactation period. In contrast, HoP did not affect human milk Lys activity. Considering that besides its critical cellular antioxidant role in protecting the organism from oxidative damage, GPx decreases redox potential (E_h) of milk stimulating the growth of anaerobic microorganism, such as the probiotic *Bifidobacterium,* it is concluded that an important part of its function has been lost. This antioxidant compounds should be replaced by human milk banks after the HoP step to recover lost functionality. Otherwise, an alternative technology to HoP, which can yield better retention of human milk properties, should be employed by milk banks to eliminate the risk of transmission of infectious agents.

Key words: bifidogenic factor, probiotic, health

RESUMO

O objetivo desta pesquisa foi investigar o efeito da pasteurização lenta (LTLT) do leite humano no perfil protéico e na atividade de glutationa peroxidase (GPx) e de lisozima (Lis). As amostras de leite humano foram analisadas ao longo de seis meses de lactação, antes e depois da LTLT, quanto GPx, Lis e perfil proteico eletroforético. LTLT causou alterações nos perfis proteícos de lactoferrina (LF) e imunoglobulinas (Igs), não afetou a atividade da Lis, mas reduziu a atividade da GPx. Além disso, a atividade de GPx que mostrou-se alta no colostro e leite de transição, foi naturalmente reduzida ao longo do período de lactação de 6 meses. Considerando que, além de seu papel crítico como antioxidante celular na proteção do organismo contra o dano oxidativo, a GPx reduz o potencial redox (*Eh*) de leite estimulando o crescimento de microrganismos anaeróbicos, como *Bifidobacterium* probiótico. Conclui-se que uma parte importante de sua função é perdida após LTLT. Estes compostos antioxidantes devem ser readicionados pelos bancos de leite humano após a etapa de pasteurização para recuperar a funcionalidade perdida. Caso contrário, uma tecnologia alternativa de pasteurização que possa produzir uma melhor retenção de propriedades do leite humano, deve ser empregada pelos bancos de leite para eliminar o risco de transmissão de agentes infecciosos.

Palavras-chave: fator bifidogênico, probiótico, saúde

1 INTRODUCTION

Human milk is recognized not only as a food but also as a dynamic biologic system. This complex fluid provides nutrients, bioactive compounds, and immune factors such as immunoglobulins (Ig), lactoferrin (LF), and lysozyme (Lys). Feeding human milk is the best method to nourish and protect the newborn from infectious diseases (TOBBACK et al., 2017).

Bioactive compounds from human milk, such as short-chain oligosaccharides, LF, and Lys promote the growth of beneficial gut microbiota, such as species of the genera *Bifidobacterium* and *Lactobacillus* (THONGARAM et al., 2017). Sometimes, mothers discontinue breastfeeding soon after partum because milk is in short supply or unavailable, and the infant cannot be nourished exclusively by this optimum source of nutrition (KRONBORG et al., 2015). In this situation human milk banks are essential, as they provide milk from donor mothers, which is a better alternative to infant formula. Food safety is ensured by Holder pasteurization (HoP), also called LTLT method (62.5 °C for 30 min). The pasteurization step is mandatory to inactivate pathogenic bacteria and viruses and reduces spoilage to an acceptable level when delivery to impaired individuals or medical institutions is considered. However, heat treatment may reduce the content of some bioactive compounds in human milk (LIMA et al., 2017).

Human milk may prevent many illnesses and diseases, such as ear infections, gastrointestinal infections, severe lower respiratory tract infections, atopic diseases (allergies, hay fever, asthma, and dermatitis), obesity, cardiovascular diseases, childhood leukemia, and sudden infant death syndrome (LÖNNERDAL, 2017; MOUKARZEL & BODE, 2017). Governments usually spend a lot money treating these diseases, and these resources could be better applied to other health sectors. Additionally, gut colonization by *Bifidobacterium* and *Lactobacillus* is largely related to a well-functioning immune system, especially the production of IgA, IgG, and IgM (TALJA et al., 2014). Poor immunity is one of the main causes of childhood death. Improving the quality of the milk from the banks is essential to ensure a healthier life for the newborn and to reduce expenditure on disease treatment (KATKE et al., 2015).

HoP adversely affects bioactive compounds in human milk, including LF, unlike HTST treatment (KLOTZ et al., 2017). Human milk is a dynamic enzymatic fluid, and pasteurization may cause protein denaturation (OLIVEIRA et al., 2016). LF is an iron-binding glycoprotein, and its bacteriostatic and bactericidal effects on Gram-positive and Gram-negative bacteria are well known. It is known that some lactic acid bacteria, including probiotic bifidobacteria and lactobacilli, are resistant to its antibacterial effects. Moreover, LF-binding protein in bifidobacteria is not only involved in growth stimulation mechanisms but could also play other roles (ODA et al., 2014).

Information about glutathione peroxidase (GPx) activity in prokaryotes is scarce. The GPx gene and synthesis pathways have not been detected in anaerobes, including bifidobacteria. Lowering of the redox potential by GPx may stimulates the growth of bifidobacteria (ŽIVKOVIĆ et al., 2015). GPx may be depleted by heat treatment; therefore, pasteurization may reduce the growth-promoting potential of human milk. Supplementation of GPx to these bacteria may protect them from oxidative stress (LADERO & SÁNCHEZ, 2017) and as a sulfur and nutrient source.

The aim of this research was to investigate the effect of Holder pasteurization on the protein profile and GPx and Lys activities in human milk.

2 LITERATURE REVIEW

2.1 Human Milk

Breastfeeding is an unequalled way of providing ideal food to the healthy growth and development of infants; it is also an integral part of the reproductive process with important implications for the health of mothers. Review of evidence has shown that, on a population basis, exclusive breastfeeding for 6 months is the optimal way of feeding infants. Thereafter infants should receive complementary foods with continued breastfeeding up to 2 years of age or beyond (WHO, 2017).

Benefits to infant from human milk fed is either by nutritional importance or source of beneficial microorganisms. Human milk contains a diverse array of bioactives and is also a source of bacteria for the developing infant gut. Genera shared between infant feces and human milk samples accounted for 70–88% of the total relative abundance in infant faces samples, supporting the hypothesis of vertical transfer of bacteria from milk to the infant gut. In addition, identical strains of *Bifidobacterium breve* and *Lactobacillus plantarum* were isolated from the milk and feces of one mother-infant pair. Vertical transfer of bacteria via breastfeeding may contribute to the initial establishment of the microbiota in the developing infant gut (MURPHY et al., 2017).

Another important human milk property to infant are those related with immunological protection. Breast milk is a rich source of SIgA with lesser amounts of IgG and IgM. JÄRVINEN et al. (2014) assessed mother's milk samples for ß-lactoglobulin, casein, and specific IgA and IgG by ELISA. Although studies have reported no consistent association between total and food-specific IgA levels in breast milk and development of allergic disease in older children. The etiology of low breast milk IgA is unknown but unrelated to maternal atopy. The Figure 1.1 shows macronutrient (g/dL) and energy (kcal/dL) composition of human milk.

Table 1.1 - Macronutrient (g/dL) and energy (kcal/dL) composition of human milk from specific references

Author (year), n	Protein	Fat	Lactose	Energy
	Mean (± 2 SD)	Mean (± 2 SD)	Mean (± 2 SD)	Mean (± 2 SD)
Term infants, 24-hour collection, mature milk				
Nommsen et al (1991), n=58	1.2 (0.9, 1.5)	3.6 (2.2, 5.0)	7.4 (7.2, 7.7)	70 (57, 83)
Donor human milk samples				
Wojcik et al (2009), n=415	1.2 (0.7, 1.7)	3.2 (1.2, 5.2)	7.8 (6.0, 9.6)	65 (43, 87)
Michaelsen et al (1990), n=2553	[a] 0.9 (0.6, 1.4)	[a] 3.6 (1.8, 8.9)	[a] 7.2 (6.4, 7.6)	[a] 67 (50,115)
Representative values of mature milk, term infants				
Reference standard	0.9	3.5	6.7	65 to 70
Preterm, 24-hour collection, first 8 weeks of life				
Bauer & Gerss (2011)				
Born <29 weeks, n=52	2.2 (1.3, 3.3)	4.4 (2.6, 6.2)	7.6 (6.4, 8.8)	78 (61, 94)
Born 32-33 weeks, n=20	1.9 (1.3, 2.5)	4.8 (2.8, 6.8)	7.5 (6.5, 8.5)	77 (64, 89)
Preterm donor milk				
Hartmann (2012), n=47	1.4 (0.8, 1.9)	4.2 (2.4, 5.9)	6.7 (5.5, 7.9)	70 (53, 87)

[a] Median (lower bound)

Source - BALLARD and MORROW (2013).

Breast milk is the natural first food for babies, it provides all the energy and nutrients that the infant needs for the first months of life, and it continues to provide up to half or more of a child's nutritional needs during the second half of the first year, and up to one-third during the second year of life (WHO, 2017). The Table 1.2 shows major bioactive factors from human milk.

Table 1.2 - Major bioactive factors from human milk (continues)

Component	**Function**	**Reference**
Cells		
Macrophages	Protection against infection, T-cell activation	Jarvinen, 2002, Yagi, 2010, Ichikawa, 2003
Stem cells	Regeneration and repair	Indumathi, 2012
Immunoglobulins		
IgA/sIgA	Pathogen binding inhibition	Van de Perre, 2003, Cianga, 1999; Brandtzaeg, 2010; Kadaoui, 2007; Corthësy, 2009; Hurley, 2011; Agarwal, 2010; Castellote, 2011
IgG	Anti-microbial, activation of phagocytosis (IgG1, IgG2, IgG3); anti-inflammatory, response to allergens (IgG4)	Cianga, 1999; Agarwal, 2010
IgM	Agglutination, complement activation	Brandtzaeg, 2010; Van de Perre, 1993; Agarwal, 2010
Anti-microbial		
Lactoferrin	Acute phase protein, chelates iron, anti-bacterial, anti-oxidant	Adamkin, 2012; Sherman, 2004; Manzoni, 2009; Hirotani, 2008; Buccigrossi, 2007; Velona, 1999
Lactadherin/ MFG E8	Anti-viral, prevents inflammation by enhancing phagocytosis of apoptotic cells	Stubbs, 1990; Kusunoki, 2012; Aziz, 2011; Shi, 2004; Chogle, 2011; Baghdadi, 2012; Peterson, 1998; Newburg, 1998; Shah, 2012; Miksa, 2006; Komura, 2009; Miksa, 2009; Wu, 2012; Matsuda, 2011; Silvestre, 2005
Metabolic hormones		

Adiponectin	Reduction of infant BMI and weight, anti-inflammatory	Martin, 2006; Newburg, 2010; Woo, 2009; Woo, 2012; Ley, 2011; Dundar 2010; Ozarda, 2012; Savino, 2008; Weyerman, 2006
Leptin	Regulation of energy conversion and infant BMI, appetite regulation	Savino, 2008; Savino, 2012a; Savino 2012b; Palou, 2009; Weyermann, 2006
Ghrelin	Regulation of energy conversion and infant BMI	Savino, 2008; Savino, 2012; Dundar 2010
Oligosaccharides & glycans		
HMOS	Prebiotic, stimulating beneficial colonization and reducing colonization with pathogens; reduced inflammation	Newburg, 2005; Morrow, 2005; DeLeoz, 2012; Marcoba, 2012; Kunz, 2012; Ruhaak, 2012; Bode, 2012
Gangliosides	Brain development; anti-infectious	Wang B, 2012
Glycosaminoglycans	Anti-infectious	Coppa, 2012; Coppa 2011
Mucins		
MUC1	Block infection by viruses and bacteria	Ruvoen-Clouet, 2006; Liu, 2012; Sando, 2009; Saeland, 2009; Yolken, 1992
MUC4	Block infection by viruses and bacteria	Ruvoen-Clouet, 2006; Liu, 2012; Chaturvedi, 2008

Source - BALLARD and MORROW (2013) (adapted).

Large mortality rate due to artificial babies feeding led to the need to stock human milk to nourish infants when the mother cannot breastfeed them. HoP method performed at 62.5 °C for 30 minutes was taken by the technical standard of human milk banks (HMB) to guarantee food safety. This process kills all pathogenic microorganisms and reduces spoilages to an acceptable level. However, heating may inactivate bioactive compounds of human milk.

There are at least three phases in human milk: colostrum, transitional, and mature. However, human milk changes its composition during lactation time. Until the 7^{th} day of lactation, it is named as colostrum; between 7^{th} to 15^{th}, it is named as transitional milk; and after 15^{th}, it is mature human milk. Over lactation day there is also modification in its composition, so milk of the first feeding is different from the second, and so on. There is also difference during the same feeding. Due to all these modifications in human milk composition, it is extremely important to obtain a standardized collection protocol in working researches.

2.2 Human Milk Banks (HMB)

The first human milk bank opened in Vienna, Austria, in 1909. Milk banking in Brazil began in 1943 in Rio de Janeiro – Fernandes Figueira Institute. HMB around world have been continued until the 1980s when many banks closed because of the fear of HIV transmission (KIM & UNGER, 2010). Instead of, Brazil encouraged to discovery new method for qualitative and quantitative expansion of human milk banks, and in this way the HMB have new role in the Brazil public health.

> *"A existência de normas para a doação de leite humano remonta à época do Império, quando a preocupação com a saúde da criança levou D. Pedro II a outorgar legislação disciplinando os "serviços de ama-de-leite".*
> *Os Bancos de Leite Humano - BLHs - no Brasil começaram a surgir no final dos anos trinta e, até 1981, quando da implantação do Programa Nacional de Incentivo ao Aleitamento Materno - PNIAM, não chegaram a constituir fator de representatividade em saúde pública.*
> *A partir daí, os BLHs apareceram de forma expressiva, como resultado não esperado. Em 1984, tornaram-se motivo de preocupação para o PNIAM devido à proliferação desordenada, sem atender a objetivos e procedimentos uniformes, constatando-se entre outros fatos, a compra e venda do leite humano, a sua troca por cestas alimentícias e a falta de controle de qualidade do produto.*
> *O sistema de saúde não tinha ingerência na implantação nem no funcionamento dos Bancos, chegando mesmo a desconhecê-los. Cite-se como exemplo dessa afirmativa que, por ocasião da primeira tentativa de levantamento dessas unidades, uma secretaria estadual de saúde arrolou todas as instituições bancárias, confundindo-as com Bancos de Leite.*
> *Por outro lado, a comunidade científica detinha vasto conhecimento sobre o leite materno, mas pouco era conhecido sobre o leite humano ordenhado.*
> *Diante dessa realidade, o PNIAM reuniu técnicos que se encontravam à frente dos BLHs para, depois de trocar experiências, construir normas que abalizassem a conduta dessas unidades e permitissem a garantia de procedimentos uniformes, desde a coleta até a distribuição, oferecendo um produto com qualidade sob o ponto de vista de saúde pública.*
> *Outro item importante, que necessitava de definição muito clara, era a clientela a ser beneficiada, inclusive por ordem de prioridade.*
> *O resultado do esforço de aproximadamente três anos foi concluído com a assinatura da Portaria MS nº 322/88, a qual contemplou todas as etapas de implantação e funcionamento de Bancos de leite Humano e tornou o Brasil o primeiro país a possuir tal instrumento legal.*
> *A divulgação do conteúdo da referida portaria, caracterizando os BLHs como polos de incentivo ao aleitamento materno, buscará também obter o comprometimento institucional das entidades que decidirem adotar estes serviços, em manter práticas facilitadoras da amamentação."*
>
> BRAZIL (1995)

HMB is a specialized center responsible for promoting breastfeed. It performs collection, processing (including heat treatment) and quality control of colostrum, transition milk and mature human milk. Milk is further distribution, under medical prescription or nutritionist.

Collection Post (CP) is a unit designed to make collection of colostrum, transitional milk and mature milk. It should provide an adequate physical area and all the necessary technical conditions, being able to be fixed or mobile, but obligatorily linked to a HMB (BRAZIL, 1995).

HMB shall promote, protect, and support breastfeeding; optimize over donation of milk; perform collecting operations, selection/classification, processing, clinical control, quality control and delivery human milk in accordance with current law. License to operate HMB is conditional on the appointment of a local. Fernandes Figueira Institute (FFI) is the reference center to others HMB in Brazil. HMB staff should be legally-qualified by senior professionals to take responsibility for medical care and food technology. The operation must be conditional upon a legally qualified higher-level coordinator. National Commission of Human Milk Banks (NCHMB) should provide technical to advice child's health and breastfeeding; assess policy on HMBs including their economic and financial aspects; discuss guidelines; identify needs and coordinate the production of technical and scientific documents; assist in the monitoring of activities; participate in the redirection of strategies; support the articulation process, mobilize and sensitize sectors of Government and civil society for the development of actions inherent to the theme; and to propose measures on matters submitted to it and by the members of the commission (BRAZIL, 2006).

In 1988, the Health Ministry provided the minimum conditions necessary for the operation of HMB and establishments handling colostrum, transitional milk and mature human milk. According to this law, nursing mothers who: carry infectious-contagious diseases will be considered unfit for donation, at medical discretion; use drugs or drugs that are excreted through milk and that promote side effects on recipients; undergoing chemotherapeutic or radiotherapeutic treatment; show signs of malnutrition or are considered unfit for other reasons. Raw milk, collected in a room suitable for this purpose, may be pre-stored under freezing for 5 days or refrigerated at 5 °C for 24 hours.

Milk must be frozen transported and refrigerated below 10 °C. All milk collected must be Holder pasteurized and stored at the HMB. The coolers and freezers must be used exclusively for this aim. After HoP step, human milk has due until 48 hours (refrigerated milk), until 6 months (frozen milk), and until 1 year (lyophilized milk). Regarding the structure, the human milk banks and collection points should have: a simplified center of sterilized material; men's and women's toilets with 3.2 m², with a minimum linear dimension of 1.6 m; sanitary facilities for the disabled; deposit of cleaning material (minimum area of 2 m^2); manage room; kitchen pantry; clinic area; demonstration room to health education (BRAZIL, 2006).

There are in Brazil 217 HMB and 125 CP, distributed by region as follows (Table 1.3):

Table 1.3 – Distribution Human Milk Banks (HMB) and Collection Points (CP) in Brazil

Region	HMB/CP
Midwest	28/6
Distrito Federal	15/3
Goiás	5/1
Mato Grosso	3/2
Mato Grosso do Sul	5/0
Northeast	50/59
Alagoas	5/1
Bahia	6/2
Ceará	9/17
Maranhão	4/1
Paraíba	6/23
Pernambuco	10/7
Piauí	1/2
Rio Grande do Norte	6/5
Sergipe	3/1
North	15/28
Acre	1/0
Amapá	1/2
Amazonas	3/23
Pará	5/0
Rondônia	1/1
Roraima	1/0
Tocantins	3/2
Southeast	96/75
Espírito Santo	7/4
Minas Gerais	14/26
Rio de Janeiro	18/8
São Paulo	57/37
South	32/22
Paraná	10/15
Rio Grande do Sul	9/1
Santa Cararina	13/6
Brazil	221/190

Source - REDE BRASILEIRA DE BANCOS DE LEITE HUMANO (2005)

2.3 Holder Pasteurization

According to the Technical Standards for the HMB of the Fernandes Figueira Institute (FFI), the pasteurization of milked human milk is:

> "*tratamento térmico, conduzido a 62,5° C por 30 minutos, aplicado ao leite humano ordenhado, com o objetivo de inativar 100% dos microrganismos patogênicos e 99,99% da microbiota saprófita, equivalendo a um tratamento15D para inativação térmica da Coxiella burnetti"* (ALMEIDA et al., 2005).

Pre-heating time is defined by the Technical Standards for the HMB of the Instituto Fernandes Figueira (IFF), as: *"período compreendido entre o momento da colocação dos*

frascos a serem pasteurizados no banho-maria até a estabilização da temperatura do banho em 62,5ºC (ALMEIDA et al., 2005)*."*

Guidelines to pasteurize milk by Holder method is follow:

"Regular o banho maria à temperatura de tratamento (62,5ºC) e esperar que o mesmo se estabilize; carregar o banho maria com a capacidade máxima de frascos por ela comportado (os frascos deverão conter o mesmo volume de leite humano ordenhado; deverão estar termicamente estabilizados a uma mesma temperatura, recomenda-se utilizar 5º C; o frasco que ocupar a posição central do banho-maria deverá conter um termômetro para medir a temperatura do leite e funcionará como controle; o bulbo do termômetro deverá estar posicionado no ponto frio do frasco, que situa na parte central, a uma altura equivalente a 2/3 da coluna de líquido, medido da superfície em relação ao fundo); iniciar a marcação do tempo imediatamente após carregar o banho-maria com todos os frascos, o tempo final de pré-aquecimento será determinado quando a temperatura final do frasco controle atingir 62,5º C; os frascos deverão ser agitados manualmente ou em intervalos regulares de 5 minutos; iniciar a marcação do tempo de letalidade térmica (30 minutos) a partir do momento que a temperatura do leite humano atingir a marca de 62,5º C; transcorrido os 30 minutos de letalidade térmica, promover o resfriamento dos frascos até que o leite humano atinja uma temperatura igual ou inferior a 5º C, o resfriamento dos frascos pode ser obtido através de resfriadores automáticos ou pela imersão dos mesmos em um banho contendo água e gelo" (ALMEIDA et al., 2005).

HoP affect bioactive compounds of human milk. Tables 1.4 and 1.5 show compounds affected significantly by HoP reported by PEILA et al. (2016) and OGUNDELE (2000)**,** respectively.

Table 1.4 - Compounds affected significantly by HoP (consensus on results) (contimues)

Compounds	**Effect of HoP**
IgA	Reduction (20%–62%)
sIgA	Reduction (20%–50%)
IgM	Reduction (50%–100%)
IgG	Reduction (23%–100%)
IgG4	Reduction (% not reported)
Lactoferrin	Reduction (35%–90%)
Lipase	Complete loss
Alkaline phosphatase	Complete loss
Amylase	Reduction (15%)

Compounds	Effect of HoP
Insulin, Adiponectin	Reduction (% not reported)
Arginine, leucine	Increase
Aspartate	Reduction (% not reported)
Glutamine	Increase (% not reported)
Ascorbic + Dehydroascorbic	Reduction (12%)
Ascorbic Acid	Reduction (16.2%–26%)
B6	Reduction (15%)
Glutathione, Glutathione peroxidase activity, Total antioxidant capacity	Reduction (% not reported)
Lactulose	Increase (% not reported)/(Not detected in all samples)
Nucleotide monophosphate content	Increase (% not reported)

Source – PEILA et al. (2016) – adapted.

Table 1.5 – Major anti-microbial factors in human milk

Compounds	Effect against micorbes	Effect of heat
Secretory IgA	Trans-luminar transport, neutralization, antigen binding, complement activation	After 30 min. stable at 56 °C, some loss at 62.5 °C; destroyed by sterilization
IgG	Neutralization, activation of complement	After 30 min. stable at 56 °C, some loss at 62.5 °C; destroyed by sterilization
IgM	Neutralization, activation of complement	After 30 min. stable at 56 °C, some loss at 62.5 °C
Bifidobacterium growth factor	Bacteriostatic	Stable on sterilization
Lysozyme, lactoperoxidase	Bacteriostatic, anti-inflammatory	Some loss at 56 °C for 30 min; destroyed by sterilization for 15 min
Complement	Lysis, opsonization, viral neutralization	Destroyed after 30 min at 56 °C
Lactose	Increased calcium bio-availability and *Lactobacillus* growth	Stable at 85 °C for 30 min
Lactoferrin	Bacteriostatic (bind iron)	66% destroyed after 30 min at 62.5 °C
Gangliosides, glycoconjugates	Receptor-like functions	Stable on sterilization
Cytokines	Immuno-modulation, anti-allergy	Unknown
Lipid products	Lysis	Stable on sterilization
Milk cells	Phagocytosis, imune-modulation, antibory production	Destroyed after 30 min at 62.5 °C

Source - OGUNDELE (2000).

2.4 Bioactive Compounds of Human Milk

Breastmilk are ideal and normative stand arts for infant feeding and nutrition. Proper nutrition in infancy is essential for normal growth, resistance to infections, long-term adult health, and optimal neurologic and cognitive development. Breastfeeding has short and long-term advantages for infant neurodevelopment due to the immunological component in the structure. Also, it has short and long-term advantages for infant digestive system therefore breast milk has the components appropriate for the enzyme structure of the newborn baby. Additionally, adequate of milk intake can be assessed by voiding and stooling patterns of the infant. A well-hydrated infant voids six to eight times a day. The mean feeding frequency during the early weeks postpartum is 8 to 12 times per day so it can meet all the needs the first 6 months for baby of mothers who do not have a health problem (OGREN et al., 2017). The composition of human milk is dynamic with significant change from colostrum, transitional to mature milk, between preterm and term milk and with interindividual and intraindividual variation. Specifically, HMOs show interindividual variation relative to the total number of HMOs and individual HMOs varying with mother's Lewis blood group and secretor status (AQUILANO et al., 2014).

2.5 Glutathione

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) that is synthesized in the cytosol from the precursor amino acids glutamate, cysteine and glycine (Figure 1.1). The cell contains millimolar concentrations of GSH (up to 10 mM) that is maintained in this reduced form by a cytosolic NADPH-dependent reaction catalyzed by glutathione reductase (CHAKRAVARTHI et al., 2006; AQUILANO et al., 2014) (Figure 1.2).

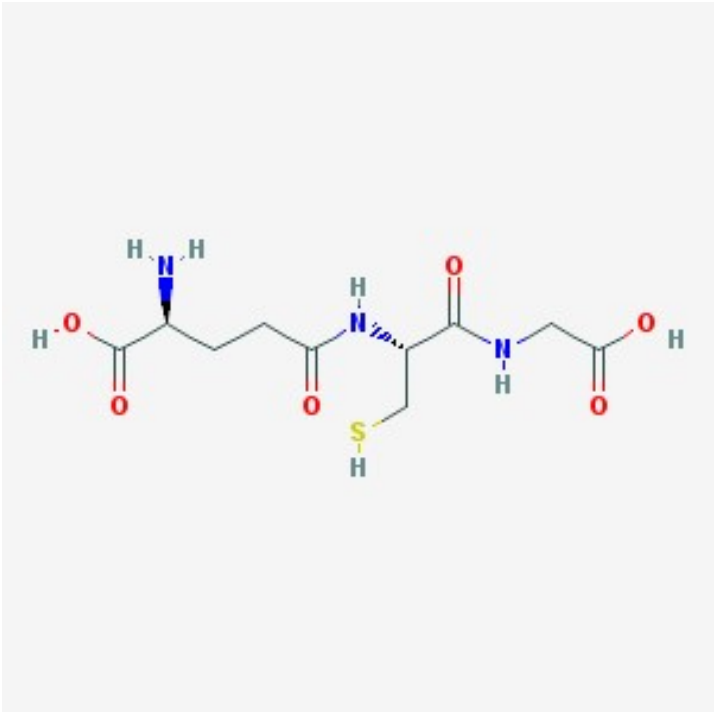


Figure 1.1 – 2D structure of glutathione.
Source - NCBI (2017b)

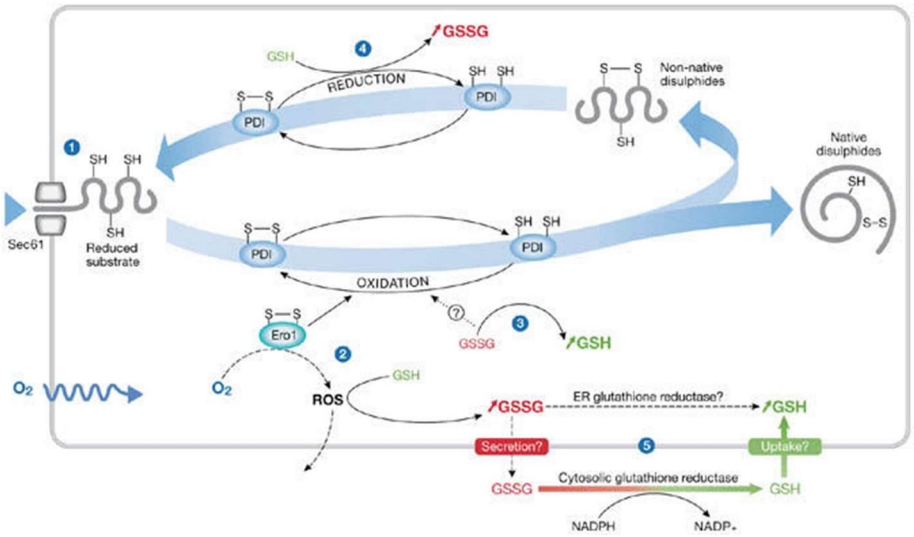


Figure 1.2 – Reduced (GSH) or oxidized (GSSG) glutathione production from protein disulphide bonds (PDI).
Source - CHAKRAVARTHI et al. (2006).

New research techniques have expanded the understanding about potential for human milk's effect on the infant that will never be possible feed by milk formulas. Human milk microbiome directly shapes the infant's gut microbiome, while the human milk oligosaccharides drive the growth of these microbes within the gut. New techniques such as genomics, metabolomics, proteomics, and glycomics are being used to describe this symbiotic relationship. Human milk is a dynamic source of nutrients and bioactive factors; unique in providing for the human infant's optimal growth and development. The growing infant's immune system has a number of developmental immune deficiencies placing the infant at increased risk of infection (AQUILANO et al., 2014).

2.6 Lactoferrin

Lactoferrin is about 80-kDa glycoprotein composed of 703 amino acid residues and is a member of the transferrin family. LF is a breast milk glycoprotein with protective effects against neonatal infections. Same role related with LF are: bacteriostatic, bactericidal activity, immunomodulatory activity, cell proliferation and differentiation, iron uptake (LÖNNERDAL et al., 2017). LF is a sialic acid-rich, iron-binding milk glycoprotein, known to have multifunctional health benefits, including its ability to modulate immune function and facilitate iron absorption, as well as its antibacterial and anti-inflammatory actions (WANG, 2016).

Mothers with higher LF concentration in colostrum has higher values in the following 2 months. High maternal income and multiple gestation were significantly associated with higher LF levels; in contrast, maternal peripartum infections and male neonatal gender were associated with lower LF levels (TURIN et al., 2017).

VILLAVICENCIO et al. (2017) made a compilation of 70 qualified articles from 29 countries with publication dates ranging from 1976 to 2015 that colostrum has the highest LF levels, but they decrease with days postpartum. No other factor has been consistently associated with LF concentration. A major limitation of most published studies is the small sample size and the different methods used to measure LF concentration. Therefore, there is a need for large, multicenter studies with standardized study design, sample collection, and LF measurement methods to identify clinically significant factors associated with LF expression in breast milk, which will help promote exclusive breastfeeding in preterm infants. The Figure 1.3 shows 3D LF structure.

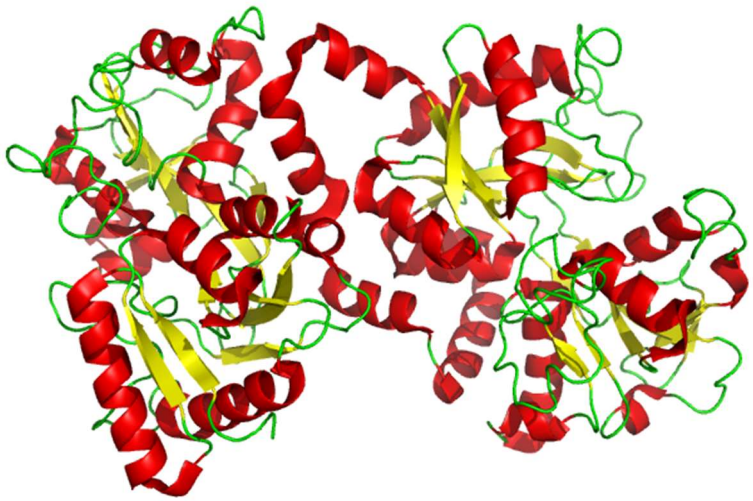


Figure 1.3 – 3D lactoferrin structure
Source - (NCBI, 2017a)

2.7 Lysozyme

Lys, (1,4-β-N-acetylmuramidase) is an enzyme that plays an important role in the prevention of bacterial infections. It does this by attacking a specific component of certain bacterial cell walls, peptidoglycan. Peptidoglycan is composed of the repeating amino sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), crosslinked by peptide bridges. Lys acts by hydrolyzing the bond between NAG and NAM, increasing the bacteria's permeability and causing bacterial burst. Lys is widely distributed in plants and animals. Human Lys is expressed in the mucous membranes of the nasal cavity and tear ducts. It is also found in saliva, tears, milk, cervical mucus, leukocytes, and kidney tissue. The majority of the Lys used in researches is purified from hen egg whites (BMRB, 2017).

Alexandre Fleming in 1922 discovered the Lys by see that after his nasal mucus fell in a Petri dish with bacteria, it died, and a transparent halo appeared. At first, his discovery was enthusiastic due to subsequent therapeutic application and biotechnological production of Lys. However, in 1928, Fleming discovered the penicillin, a potent antibiotic that discouraged him to advance working with Lys.

Jollès and Canfield in 1963, even without any connection between their researches, described the primary structure of human milk Lys, it had 129 amino acids. When it was compared with bird Lys, there was an insert and a deletion in the sequence, the most likely position being at residue # 48 and 100, respectively.

Primary structure of Lys has a single polypeptide chain with 4 pairs of cysteine bound by disulfide bond. The Figure 1.4 shows the primary structure of chicken egg Lys. Lysozymes are categorized into five classes, hen egg-white Lys is the best characterized of all (IMOTO, 2017).

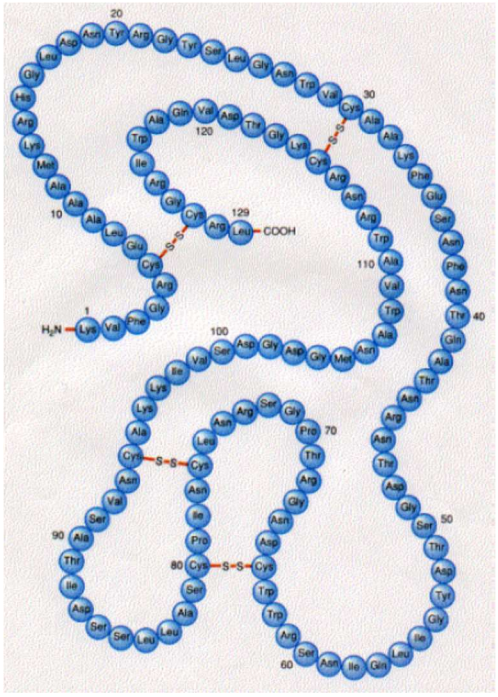


Figure 1.4 – Primary structure of hen egg-white lysozyme.
Source - SIMOES et at., 2006

Secondary structure of Lys has 5 helical regions (three alpha helices and others are intermediate). In the alpha helices, there are extensions of the amino acid radicals out and extension of the carbonyl groups of each peptide bond parallel to the helix axis, pointing to the NH group of the amino acid chain. Lys also presents five regions of beta layers. These consist of side-by-side (often anti-parallel) chains pairs being stabilized by hydrogen bonds between the hydrogen atoms attached to the nitrogens of each peptide bond and the carbonyl oxygen atom of the peptide bonds of the adjacent peptide chain. The Figure 1.5 shows tertiary structure of hen egg Lys (SIMÕES et al., 2006).

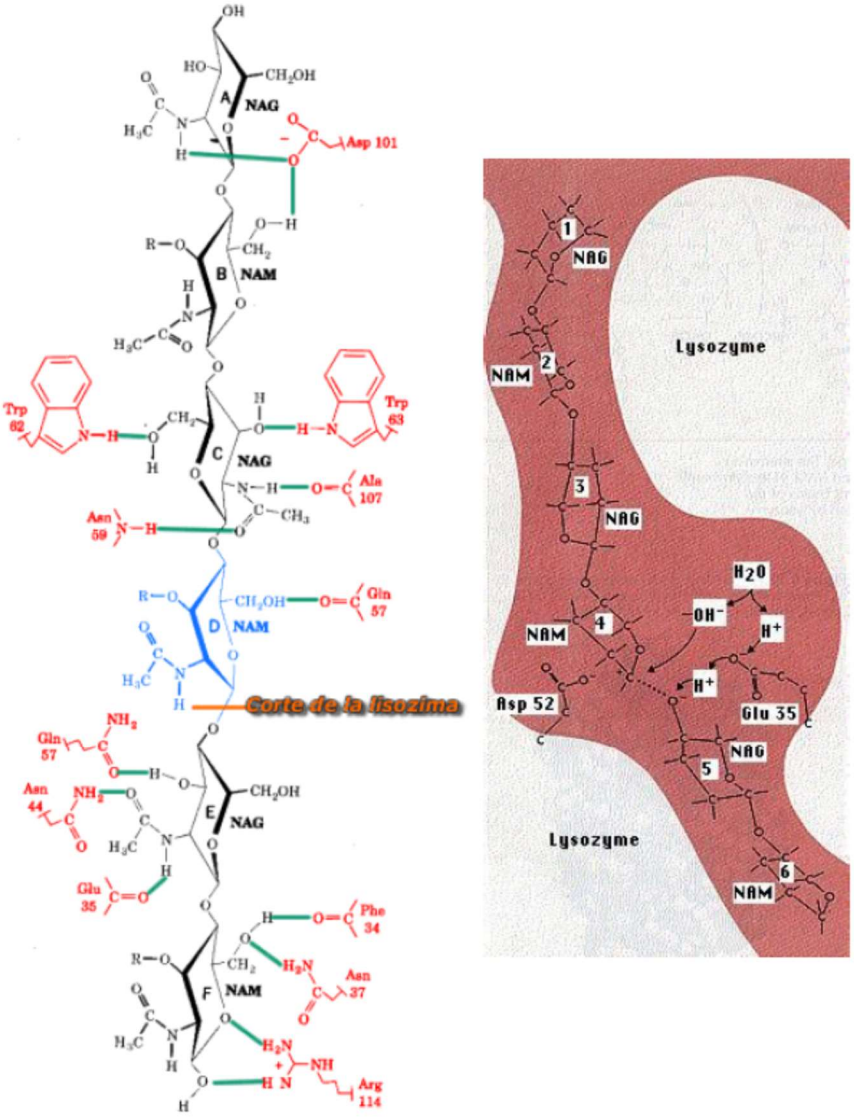


Figure 1.5 - Secondary structure of hen egg-white lysozyme.
Source - SIMOES et at., 2006

Tertiary structure, Lys is a globular protein and has five regions of beta layers. It is shown in the Figure 1.6.

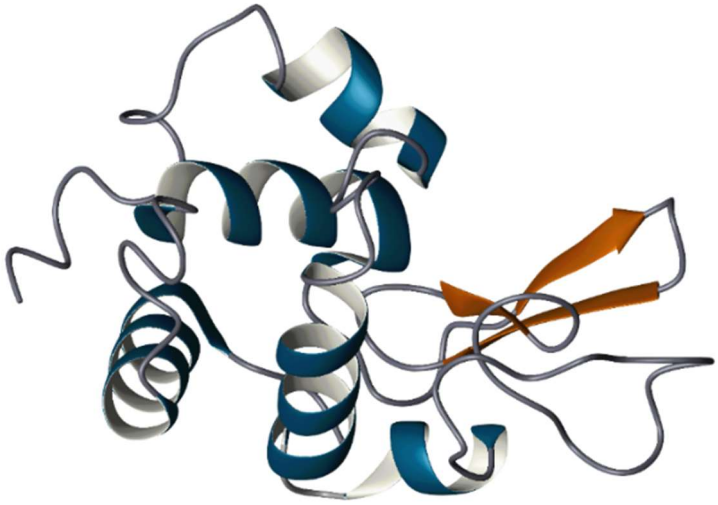


Figure 1.6 - Tertiary structure of hen egg-white lysozyme.
Source - BMRB (2017)

SALTON (1952) started the study of the sensitivity of microorganisms to Lys showing that the cell wall of *Micrococcus lisodeikticus*, today *Micrococcus luteus*, was broke by Lys. Since then, this strain has been used to perform Lys quantification by the plate diffusion method.

Bactericidal action of egg Lys occurs in place between amino acids 98 and 112, but the active site is located between amino acids 107 and 110. Interactions between enzyme and substrate occur through six hydrogen bonds and 40 Van der Waals. Other possible interactions occur through molecular conformations (BLAKE et al., 1965; HELD & VAN SMAALEN, 2014).

3 MATERIAL AND METHODS

3.1 Human Milk Samples

GPx activity and SDS-PAGE was performed on a total of 21 samples of breast milk taken from a healthy mother over a period of 6 months after term delivery, with 3–4 collections each month (Table 1.6). Lys activity was measured in 30 samples taken from the HMB of IFF-Fernandes Figueira Institute (Rio de Janeiro) and two others donated by healthy individuals. Nipples were cleaned with saline solution and a paper towel, and milk was collected by hand expression. The samples were immediately frozen to −18 °C and taken to the laboratory on ice. After thawing at 7 °C for 9 h, the milk was transferred in equal volumes to sterile test tubes.

Table 1.6 – Human milk samples over 6-months of lactation

Months	Days
1	0
	3
	12
	28
2	35
	37
	51
	56
3	62
	67
	87
4	94
	101
	110
	113
5	123
	134
	145
6	165
	166
	179

3.2 Holder Pasteurization Procedure

Breast milk samples were pasteurized by the LTLT method (HoP), according to the HMB Technical Standards. Samples were heat treated by immersion in a thermostatically controlled, stirred water bath. When the water temperature stabilized at 62.5 °C, the tubes were immersed in the bath so that the water level was 2 cm above the milk level. A tube containing the same amount of milk was used as a control, and a thermometer was inserted into the tube. The tubes were held in the water bath at pasteurization temperature for 30 min and shaken at regular intervals (3 min) (Figure 1.7). Thereafter, samples were immediately cooled to 5 °C in an ice bath.

Figure 1.7 – Holder pasteurization (HoP) of human milk.

3.3 Glutathione Peroxidase Activity

GPx activity was measured in samples of raw and pasteurized milk (21 of each) from an individual donor by using a Glutathione Peroxidase Assay Kit (703102, Cayman Chemical, EUA). A boiled milk sample was used as a negative control, in addition to the positive and negative controls provided with the assay kit. The reaction was started by addition of 20 µl of cumene hydroperoxide to all wells, and the absorbance was read in a plate reader (Uniscience, Multiskan FC, Thermo Scientific) at 340 nm (Figure 1.8). After linear regression of time and absorbance values, the angular coefficient was recovered and substituted into Equation 1 to calculate GPx activity.

$$GPxactivity = \frac{\Delta AC}{0.00373} x \frac{0.19}{0.02} \qquad \text{Eq. 1}$$

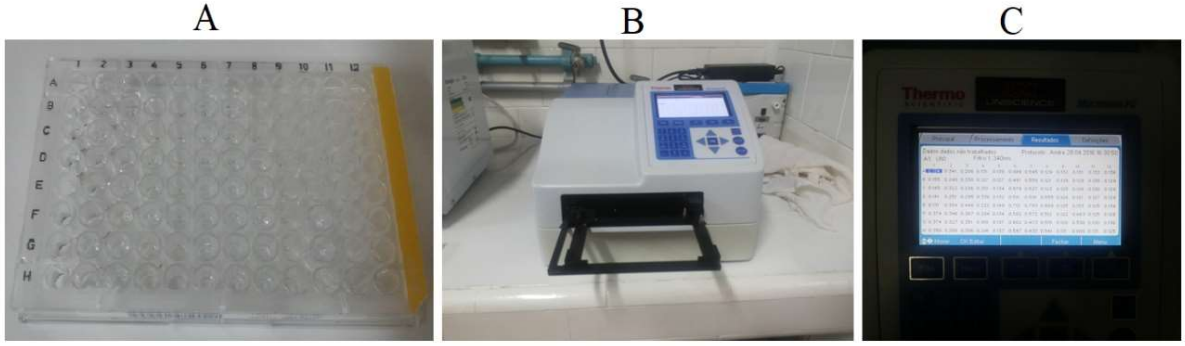


Figure 1.8 – Glutathione peroxidase active assay. A – microtiter plate. B;C – plate reader (Uniscience, Multiskan FC, Thermo Scientific).

3.4 Protein Profile Assay

Milk aliquots (100 µl) were mixed with sample buffer (200 µl) to obtain the protein electrophoresis profile and kept frozen (−18 °C) until use. Different proteins were identified by SDS-PAGE analysis in a Bio-Rad vertical PROTEAN II xi cell (Hercules, CA, USA). Stacking and running gels were prepared by using 8 % and 12 % (w/v) acrylamide solutions, respectively. A 100 V electric current was used throughout the entire running time (8 h). Gels were stained with Coomassie Brillant Blue. Molecular weight standards from Bio-Rad were run in parallel and used to estimate the protein amount in each band by comparison (LAEMMLI, 1970).

3.5 Lysozyme Activity Measurement

The lysoplate method, with *Micrococcus luteus* (ATCC 4698; INCQS 356) as indicator, was used to measure Lys activity. Melted MRS agar (Himedia, Mumbai, India) was inoculated with a fresh suspension of the indicator microorganism to a final concentration of approximately 10^6 cfu/ml and plated in Petri dishes. Five wells were dug in each plate with a sterile manual borer (6.8 mm) and filled with 100 µl milk sample. After incubation at 36 °C for 48 h, the inhibition halo formed from the well border was measured with a caliper.

Analysis of variance (ANOVA) following by Fisher's test ($P > 0.05$) and Tuckey's test ($P > 0.05$) was used to analyze the significative differences among group means of GPx and Lys activity, respectively.

4 RESULTS AND DISCUSSION

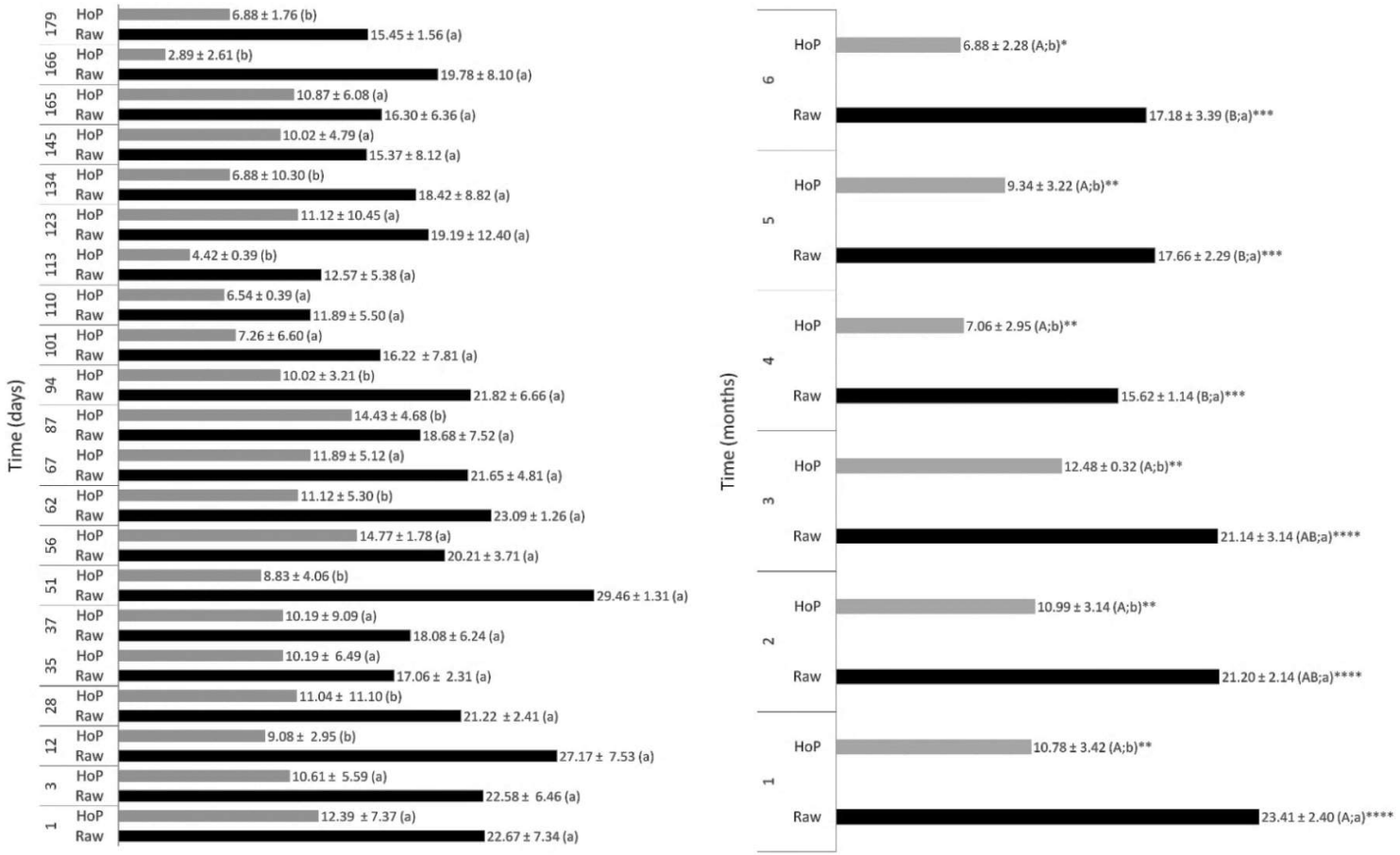
4.1 Glutathione Peroxidase Activity

HoP reduced GPx activity of human milk, and this was coupled with an observed natural reduction in GPx concentration throughout 6-months lactation period. The rate of GPx decline increased after the third month of lactation. Due to the low level of GPx in pasteurized milk, no difference ($P > 0.05$) in GPx activity was observed over time. GPx activity in raw milk at the end of the 6-month period was higher than that of pasteurized milk in the first month of lactation (Figure 1.8). Similar findings were observed by MARINKOVIC et al. (2016) which reported that pasteurization caused a significant drop in the activity of the major antioxidative enzymes superoxide dismutase and glutathione peroxidase (Figure 1.9).

Redox potential (E_h) of human milk is mainly due to superoxide dismutase, catalase, and GPx activities. Therefore, the change in E_h is correlates directly to change in GPx activity. Antioxidative properties of human milk refer to the sum of activities derived from active antioxidative enzymes (superoxide dismutase, glutathione system– glutathione peroxidase (GPx), and reductase), nonenzymatic antioxidants (such as ascorbate), and other bioactive factors (e.g. urate) (MARINKOVIC et al., 2016).

A low E_h is necessary for growth, survival, and viability of bifidobacteria. Therefore, in addition to lowering the antioxidant activity in milk, the HoP process also causes a loss of prebiotic activity in human milk (FISER et al., 2015; LADERO & SÁNCHEZ, 2017). The best-known milk bifidogenic factors are galactooligosaccharides, which are not affected by HoP, but GPx may serve a prebiotic function, especially in early life.

A low maternal selenium status in pregnancy was found to be associated with an increased risk of infant infection during the first 6 weeks of life and a lower psychomotor score at 6 months (VARSI et al., 2017). GPx activity and selenium in the colostrum secreted by mothers of preterm infants was significantly greater than the selenium content of milk secreted by the same mothers at 21 and 42 days of lactation. Mature milk produced by mothers of very preterm infants on d 21 of lactation contained significantly greater enzyme activity ($P < 0.05$) than milk produced by mothers of term infants at the same stage of lactation (ELLIS et al., 1990).



Significative difference is indicated by different lowercase letters for lactation time (per day or per month) and by different capital letters for heat treatment (raw or Holder pasteurized), in the same Figure (left or right). Asterisks arrange samples (raw and pasteurized) from lowest to highest (* < ** < *** < ****). All statistic treatments were performed by Fischer's test ($P < 0.05$).

Figure 1.9 - Glutathione peroxidase activity (µg/ml) from raw and Holder pasteurized (HoP) human milk per day (left) and per month (right) **(**mean ± standard deviation).

HoP adversely affects bioactive compounds in human milk, including LF, unlike HTST treatment (KLOTZ et al., 2017). Human milk is a dynamic enzymatic fluid, and pasteurization may cause protein denaturation (OLIVEIRA et al., 2016). LF is an iron-binding glycoprotein, and its bacteriostatic and bactericidal effects on Gram-positive and Gram-negative bacteria are well known. It is known that some lactic acid bacteria, including probiotic bifidobacteria and lactobacilli, are resistant to its antibacterial effects. Moreover, LF-binding protein in bifidobacteria is not only involved in growth stimulation mechanisms but could also play other roles (ODA et al., 2014).

4.2 Protein Profiles – SDS-Page

SDS-PAGE protein profiles were similar in raw and pasteurized milk (Figures 1.10A and 1.10B). However, in the first month of lactation, the abundance of proteins between 60.89 kDa and 114.50 kDa was higher than in the following months, suggesting higher amounts of proteins like LF and Ig.

A reduction in the abundance of these proteins was observed over time, and this reduction was higher following heat treatment of milk. LF is a glycoprotein with antimicrobial activity and is part of a nonspecific defense system in human milk. The concentration of this protein is higher in colostrum and decreases naturally over the lactation period (KAISER et al., 2017).

Human milk immune components are unique and important for the development of the newborn. Milk processing at the HMB, however, causes partial destruction of immune proteins. After milk processing, IgA, IgM, and LF mean concentrations were reduced by 30.0 %, 36.0 %, and 70.0 %, respectively. Reduction of biological activity was mainly attributed to pasteurization for IgA and LF; the first freezing/thawing processes before pasteurization showed no significant reduction difference between mean concentrations of IgA and LF but showed a significant effect on IgM concentration, and the second freezing/thawing procedure only showed a significant effect on IgA (ARROYO et al., 2017).

Same LF also can be lost over thawed storage. RAOOF et al. (2016) studied the effect of 3 and 6 months storage human milk at −20 °C, the average decrease in LF concentrations was 55% and 65%, respectively. The bioactivity of LF also decreased significantly over 6 months.

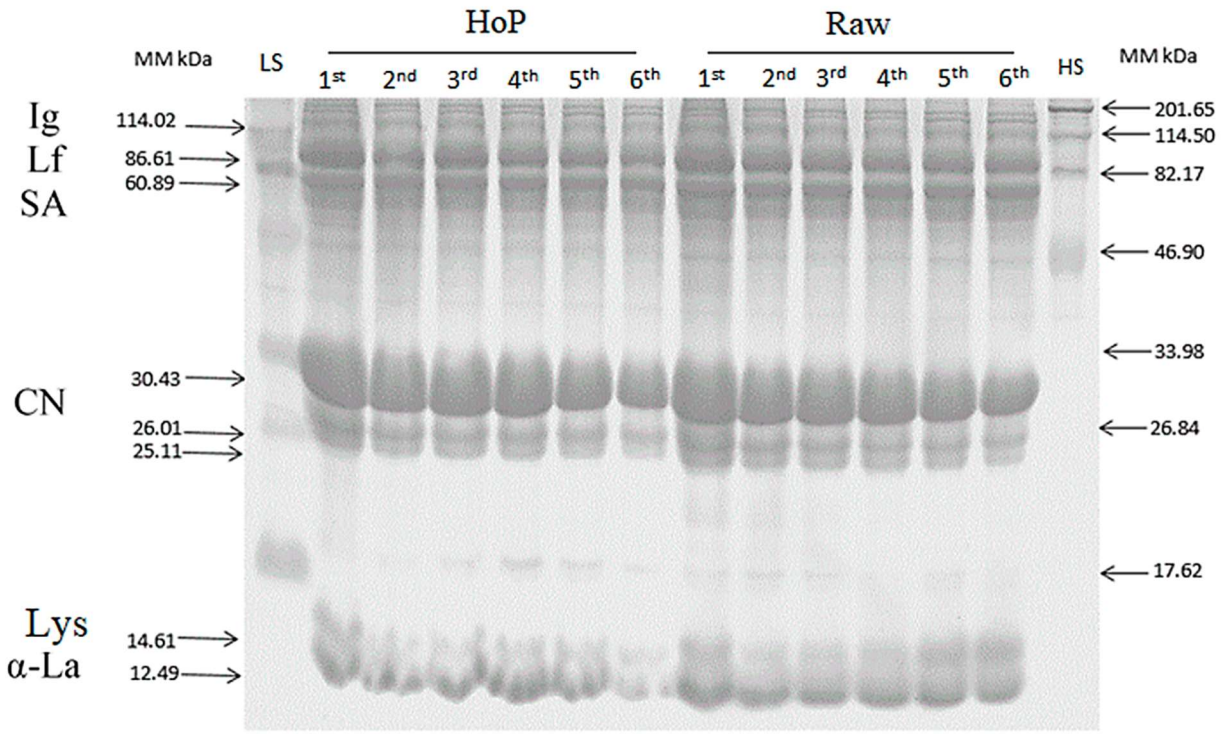


Figure 1.10A – Sodium dodecyl sulfate-PAGE protein profiles from Holder pasteurized (HoP) and raw human milk over 6 lactation months. Ig (immunoglobulins), Lf (lactoferrin), SA (serum albumin), CN (caseins), Lys (lysozyme), α-La (Alpha lactalbumin). Molecular weight standards were of low standard (LS) and high standard (HS) masses.

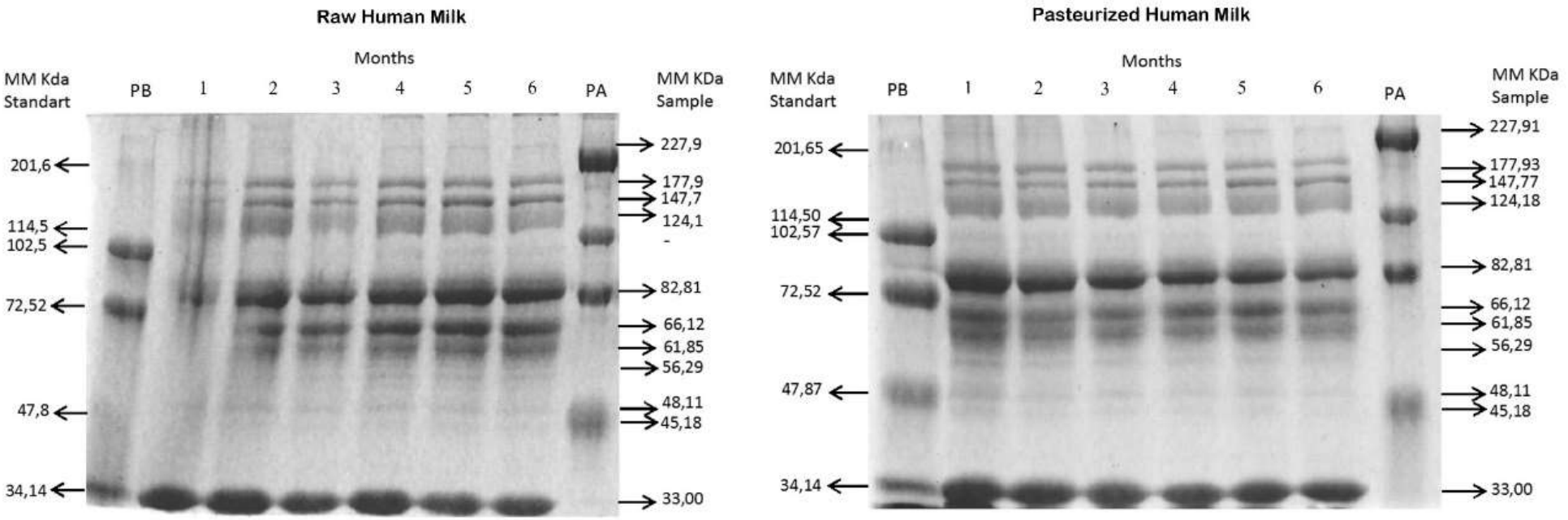


Figure 1.10B– Sodium dodecyl sulfate-PAGE protein profiles from Holder pasteurized (HoP) and raw human milk over 6 lactation months. Ig (immunoglobulins), Lf (lactoferrin), SA (serum albumin), CN (caseins), Lys (lysozyme), α-La (Alpha lactalbumin). Molecular weight standards were of low standard (LS) and high standard (HS) masses.

4.3 Lysozyme Active Measurement

Lys activity was not affected by HoP (Table 1.7).

Table 1.7 - Lysozyme activity from 32 human milk samples before (raw) or after Holder pasteurization (HoP).

	Lysozyme Activity (µg/ml) (Mean ± Standard Deviation)	
Donor Code	Before HoP	After HoP
1	390.65 ± 33.13	392.86 ± 3.83
2	225.01 ± 11.48	293.48 ± 27.58
3	213.97 ± 3.83	213.97 ± 3.83
4	183.05 ± 44.11	187.47 ± 13.79
5	317.77 ± 19.88	328.81 ± 3.83
6	**	1.95 ± 3.83
7	269.18 ± 23.27	213.97 ± 15.30
8	251.52 ± 17.53	229.43 ± 16.67
9	280.23 ± 53.96	222.80 ± 19.13
10	304.52 ± 19.88	324.40 ± 58.89
11	313.35 ± 3.83	260.35 ± 21.30
12	266.97 ± 20.24	240.47 ± 3.83
13	227.22 ± 21.30	242.68 ± 13.79
14	200.72 ± 7.65	191.89 ± 0.00
15	266.97 ± 10.12	278.02 ± 36.89
16	194.09 ± 10.12	145.51 ± 57.38
17	196.30 ± 19.13	130.05 ± 21.30
18	269.18 ± 13.79	306.73 ± 13.79
19	187.47 ± 3.83	238.26 ± 13.25
20	1.95 ± 3.83	37.29 ± 13.79
21	255.93 ± 10.12	253.72 ± 3.83
22	244.89 ± 28.88	269.18 ± 20.24
23	167.59 ± 33.35	180.84 ± 42.08
24	308.94 ± 10.12	269.18 ± 16.67
25	77.04 ± 21.30	26.25 ± 17.53
26	269.18 ± 25.08	68.21 ± 16.67
27	77.04 ± 10.12	79.25 ± 23.89
28	116.80 ± 36.49	90.29 ± 10.12
29	**	6.37 ± 0.00
30	202.93 ± 13.79	152.13 ± 19.88
D1	196.30 ± 3.83	158.76 ± 26.50
D2	278.02 ± 17.53	271.39 ± 6.63
Mean ± Standard Deviation	225.16 ± 80.95a	197.06 ± 100.47a

The same letter in the same column indicates no significant difference by Tukey's test ($P < 0.05$). (**) Absence of lysozyme activity.

However, SOUSA et al. (2014) reported a reduction of Lys activity after HoP using the turbidimetric method with *Micrococcus luteus* (ATCC 4698) as an indicator microorganism. Nevertheless, it is well accepted that heating causes changes in the conformation of Lys without affecting its antimicrobial activity. It is also known that Lys activity is dependent on ionic strength and pH (optimal 5.2). The turbidimetric procedure measures enzyme activity in up to 3 minutes of contact and may not reflect bioactivity in the human body. HMB should add bioactive compounds inactivated by HoP, such as GPx, to recover the lost functionality of milk.

Transitional milk has decreasing amounts of protein and Igs and increasing lactose and fat and water-soluble vitamins resulting in a higher caloric density of the milk to meet the infant's growth demands while the quantities of bioactive factors declines over time. The composition of mature milk remains constant after 6 weeks through the remainder of the lactation period. The amount of Igs and LF in milk decreases over the first 3–4 months, while the amount of Lys increases (BUTTE et al., 1984; CACHO & LAWRENCE, 2017).

COSENTINO et al. (2016) studied raw and pasteurized jenny milk Lys against *Bacillus megaterium*, *Bacillus mojavensis*, *Clavibacter michiganensis*, *Clostridium tyrobutyricum*, *Xanthomonas campestris*, and *Escherichia coli* after condensation at 40 and 20% of the initial volume. pasteurization did not affect the concentration or antimicrobial activity of Lys in jenny milk, except for *B.* mojaventis, which showed resistance to Lys in milk samples subjected to heat treatments. Moreover, Lys in jenny milk showed antimicrobial activity similar to synthetic antibiotics versus some Gram-positive strains and also versus the Gram-negative strain *Xanthomonas campestris*.

5 CONCLUSIONS

HoP does not affect Lys activity in human milk but reduces the content of LF and immunoglobulins. It also reduces glutathione peroxidase, which is an important antioxidant and may play a role in modulating the gut microbiota of infants.

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CHAPTER II
UNCOUPLING AND IDENTIFICATION OF NATURALLY ASSOCIATED POTENTIAL PROBIOTIC STRAINS FROM HUMAN ORIGIN

ABSTRACT

The aim of this research was to isolate bifidobacteria and lactobacilli naturally associated from human gut microbiome. Associated bifidobacteria and lactobacilli strains were isolated from infant's stools of up to 21 days old by directed plating in a modified MRS medium. Associated microorganisms were uncoupled by sonication. *Lactobacilli* grew easily in axenic cultures on MRS agar, but bifidobacteria growth was very poor showing much dependency of its pair suggesting a mutualist type of interaction. Lactobacilli were further identified by 16S rDNA sequencing and grouped by genetic similarity clusters by RAPD-PCR method. It is concluded that association with lactobacilli is very important to the establishment of bifidobacteria and its maintenance into gut microbiome. Thus, probiotic food makers must consider finding correct strains for mixed starters associations in order to improve bifidobacteria performance.

Key-words – gut microbiome, interaction, survival

RESUMO

O objetivo desta pesquisa foi isolar bifidobactérias e lactobacilos naturalmente associados do microbioma intestinal humano. Cepas de bifidobactérias e lactobacilos associadas foram isolados de fezes de lactentes de até 21 dias de idade por semeadura direta em agar MRS modificado. Os microorganismos associados foram desacoplados por sonicação. As cepas de lactobacilos cresceram facilmente em cultura axênica em ágar MRS, mas o desenvolvimento de bifidobactérias foi negligenciável, mostrando muita dependência de seu par e sugerindo uma interação do tipo mutualista. Os lactobacilos foram posteriormente identificados por sequenciamento da região 16S do rRNA e agrupados em dendogramas por similaridade genética pelo método RAPD-PCR. Conclui-se que a associação com lactobacilos é muito importante para o estabelecimento de bifidobactérias e sua manutenção no microbioma intestinal. Assim, os fabricantes de alimentos probióticos devem considerar encontrar cepas corretas para associações de culturas mistas visando melhorar o desempenho de bifidobactérias

Palavras-chave – microbioma intestinal, interação, sobrevivência

1 INTRODUCTION

The first observation of the benefic role played by some selected bacteria is attributed to Eli Metchnikoff, the Russian born Nobel Prize winner by its work at the Pasteur Institute at the beginning of the last century. Metchnikoff suggested that "*the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes.*" WHO (2006) and FAO (2006) define probiotics as: "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*."

Probiotic microorganisms can be used in several types of products, including foods, drugs, and dietary supplements. Species of *Lactobacillus* and *Bifidobacterium* are most often used as probiotics, but the yeast *Saccharomyces cerevisiae* and some *Escherichia coli* and *Bacillu*s species have also been employed. Lactic acid bacteria, including species of *Lactobacillus*, which have been used for preservation of fermented food for thousands of years, can serve a dual function by acting as agents of food fermentation and, in addition, potentially imparting health benefits. Strictly speaking, however, the term "probiotic" should be reserved for live microorganisms that have been shown in controlled human studies to impart a health benefit. Fermentation of food provides characteristic of taste profiles and lowers the pH, which prevents contamination by potential pathogens. Fermentation is a process globally applied in the preservation of a range of raw agricultural materials (cereals, roots, tubers, fruit and vegetables, milk, meat, fish, etc).

Probiotic bacteria can perform distinct functions in the gut, such as modulation of gut microbiota by competitive exclusion. Probiotic competitive exclusion can occur by preventing pathogenic microorganisms adhesion in the gut mucin (globet cells), epithelial cells, as well as by reducing colonization by pathogens due to probiotic production of antimicrobial substances (BRON et al., 2017). *Lactobacillus* species are found in environments rich in available carbohydrates such as vegetables and milk sources, but also in human and animal cavities (CAMPANARO et al., 2014). *Lactobacillus paracasei* species are commonly used in probiotic dairy products, such as yogurt, cheese, fermented milk, and ice cream (STEFANOVIC et al., 2017).

FAO/WHO recommended three-stage to evaluate the functional efficiency foods including: safety assessment in *in vitro* and *in vivo* experiments (phase I); evaluation in the Double-Blind, Randomized, Placebo-Controlled trial (phase 2); and post-approval monitoring (phase III). It is noted that along with the ability to obtain statistically significant results of the evaluation, there are practical difficulties of conducting the experiments related to duration, costs, difficulties in selection of target biomarkers and populations. The promising approach for assessing the functional efficacy of a functional food is the concept of nutrigenomics. It examines the link between the human diet and the characteristics of his genome to determine the influence of food on the expression of genes and, ultimately, to human health. Nutrigenomic approaches are promising to assess the impact of probiotics in healthy people. The focusing on the nutrigenomic response of gut microbial community and its individual populations (in this regard the lactobacilli can be very informative) was proposed (MARKOVA IU & SHEVELEVA, 2014). MUNOZ-QUEZADA et al. (2013) isolated and identified novel strains of lactic acid bacteria with probiotic properties from the feces of exclusively breast-fed infants. Amount species identified were *Lactobacillus paracasei*, *Lactobacillus rhamnosus,* and *Bifidobacterium breve*. The strain identification was confirmed by sequencing the 16S-23S rRNA intergenic spacer regions. The authors did not report about lactobacilli and bifidobacteria interactions.

Bifidobacteria represent one of the dominant microbial groups that occur in the gut of various animals, being particularly prevalent during the suckling period of humans and other mammals. The abundance of bifidobacterial saccharolytic features in human microbiomes supports the notion that metabolic accessibility to dietary and/or host-derived glycans is a potent evolutionary force that has shaped the bifidobacterial genome (MILANI et al., 2015). AIZAWA et al. (2016) reported that patients with major depressive disorder (MDD) have lower *Bifidobacterium* and/or *Lactobacillus* compared to controls. The authors report about a possible association between bifidobacteria and lactobacilli in the gut microbiota with MDD.

2 MATERIAL AND METHODS

2.1 Lactobacilli and Bifidobacteria Isolation

Stool samples were collected from healthy infants aged between 7 to 21 days old assisted by the Human Milk Bank (HMB) and the Intensive Care Unit (ICU) of the Neonatal Fernandes Figueira Institute, FIOCRUZ, Rocha Faria State Hospital. Samples (about 1g) were collected in duplicate on pre-weighed sterile plastic tubes and immediately placed in plastic bags with ice. A hand pump was used for removed atmospheric air. Decimal serial dilution was performed in anaerobic buffer Wilkins-Chalgren broth (Oxoid, Basingstoke, United Kingdom). Aliquot (100 µl) of each dilutions were plated both on Lamvab agar for lactobacilli isolation (HARTEMINK et al., 1997) and modified MRS (Difco, France) according to HARMSEN et al. (2000), for bifidobacteria. Plates were incubated at 36 °C for 48 hours under partial anaerobic conditions and for up to 4–5 days in an anaerobic workstation (AW200SG, ELECTROTEK, England) with a gas mixture of 10 % H_2, 10 % CO_2, and 80 % N_2 for lactobacilli and bifidobacteria, respectively (Figure 2.1). Characteristics colonies was pre-identified by Gram stained and catalase test.

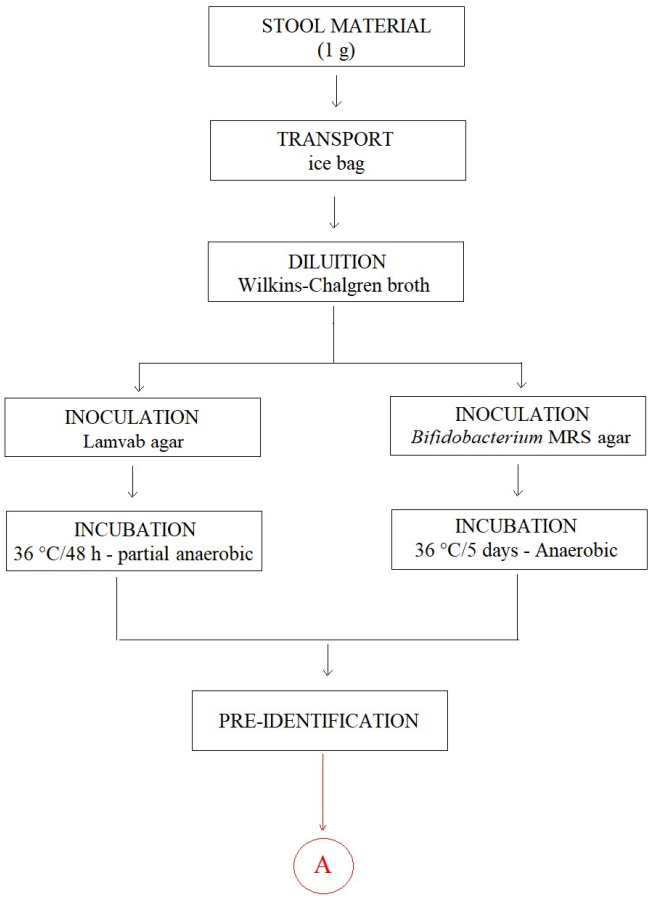


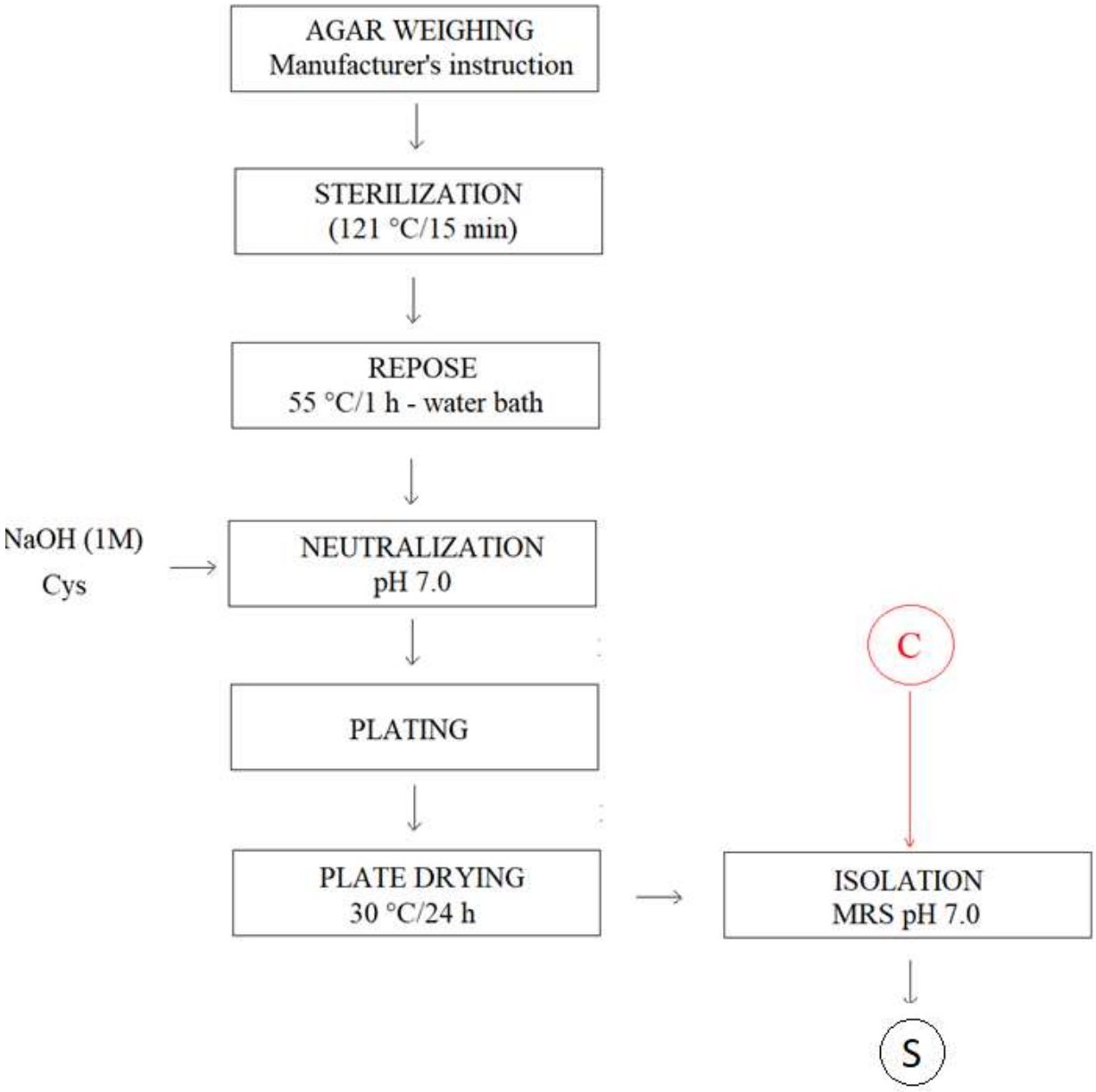
Figure 2.1 – Isolation of potential probiotic lactic acid bacteria – procedure

2.2 Uncoupling Natural Associated Bifidobacteria and Lactobacilli

Associated cultures of lactobacilli and bifidobacteria were kept frozen in MRS broth (HiMedia, Mumbai, India) added of 15% of sterile glycerol (Vetec, Brazil). After thawing (7 °C), the cultures were streaked on ~~in~~ a modified MRS agar (HiMeida, Mumbai, India) containing 0.1 % of cysteine (Vetec, Brazil) and pH adjusted to 7.0 with NaOH (1M) after sterilization (121

°C/15 min). Plates were incubated under anaerobiosis (GasPakTMEZ, Becton, Dickinson and Company, USA) for up to 5 days. Several isolated colonies (4-5 units) were transferred to Eppendorff tubes containing 1 ml of phosphate buffer pH 6.5 added of 500 mg/l cysteine (Vetec, Brazil) (PBC) ~~phosphate buffer PBC~~. A sonicator (UNIQUE, São Paulo, Brazil) working at 30% of its total power for 30 seconds was used to uncouple naturally associated bifidobacteria and lactobacilli. An ice bath was used to prevent sample from heating.

Sonicated cultures were streaked again on modified MRS agar pH 7.0 followed by incubation under anaerobiosis (GasPakTMEZ, Becton, Dickinson and Company, USA) for 5 days (Figure 2.2). Genotypic identification was performed by sequencing of the 16S rDNA region. Pure cultures of lactobacilli and bifidobacteria were kept frozen (-18 °C) ~~frozen~~ in MRS broth added of sterile glycerol (15 %).



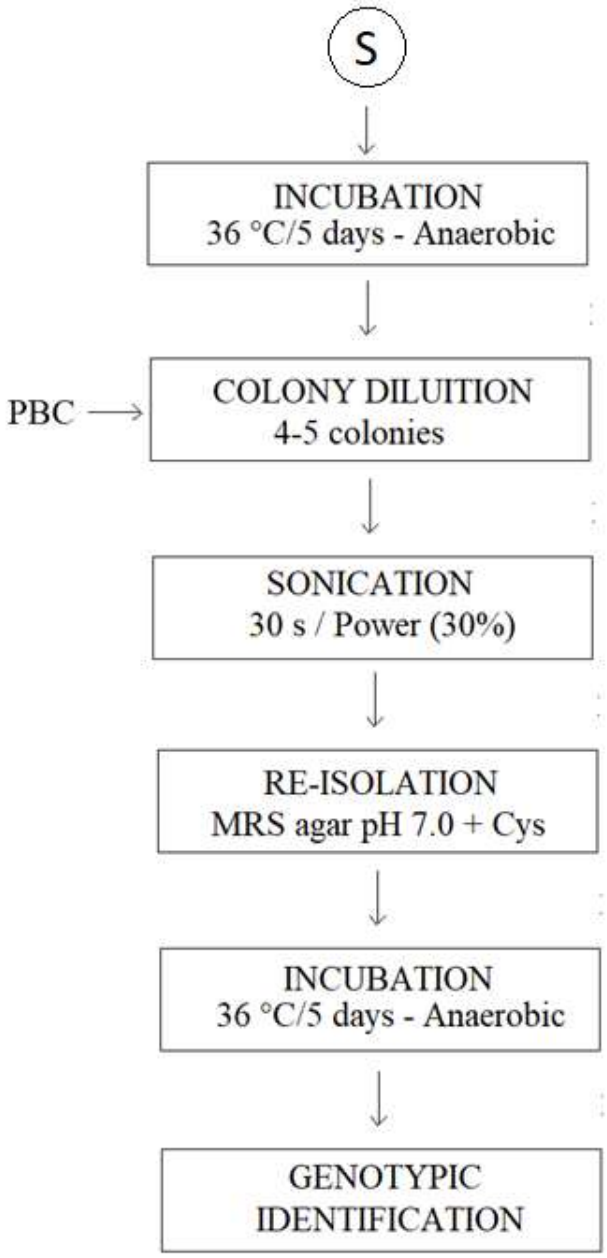
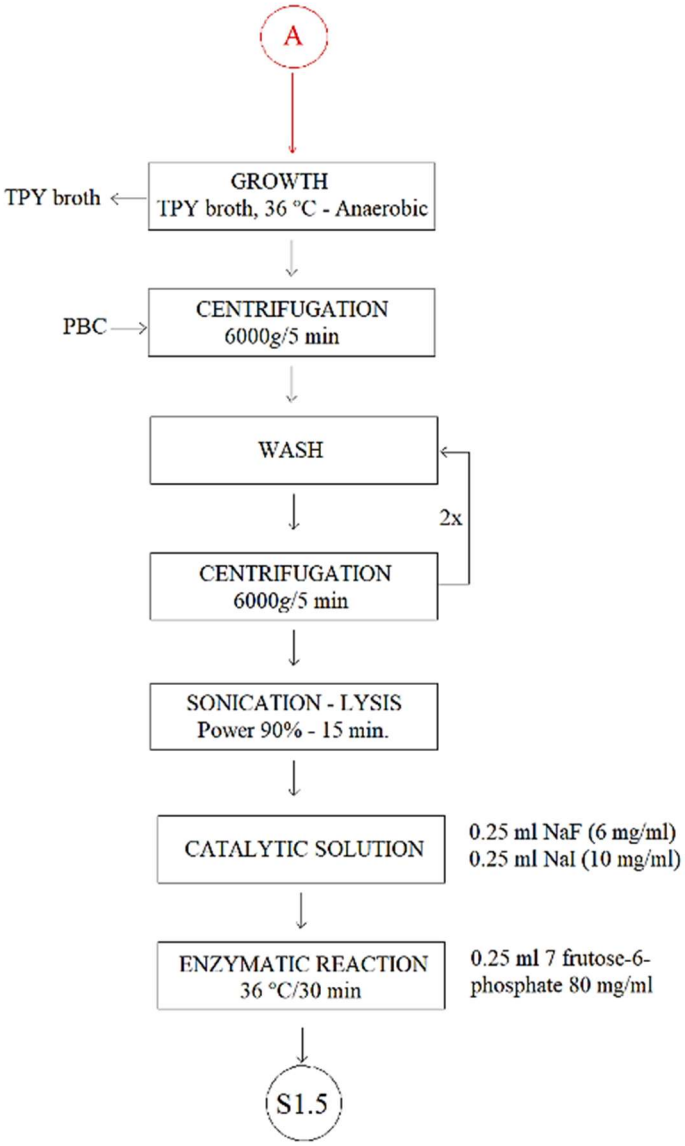


Figure 2.2 – Procedure used to uncouple naturally associated bifidobacteria and lactobacilli. PBC – Phosphate buffer pH 6.5 + 500 mg/l cysteine (Vetec, Brazil). Cys – cysteine (Vetec, Brazil).

2.3 Genus-level Identification of Bifidobacteria

The technique described by Scardovi (1986) was used for identification of genus bifidobacteria. Briefly, presumptive bifidobacterial isolates were grown overnight anaerobically (AW200SG, Electrotek, England) at 36 °C in TPY broth (Trypticase, Phytone, Yeast Extract) followed by centrifuging (2K15, Sigma Laborzentrifugen, Germany) at 6000*g* for 6 minutes and washed twice with PBC. Cell lysis was done by sonication (UNIQUE, São Paulo, Brazil) for 15 minutes, working at 90 % of total power and probe 5 mm inserted into cellular suspension. An ice bath was used to prevent sample heating. Immediately after cell lysis, 0.25 ml of catalytic reaction solution (NaF at 6 mg/ml and NaI at 10 mg/ml, Sigma-Aldrich, S. Louis, Mo, USA) and 0.25 ml of enzymatic substrate (7 fructose-6-phosphate at 80

mg/ml, Sigma-Aldrich, S. Louis, Mo, USA) were added, followed by incubation in a thermostatically controlled, stirred water bath, at 35 °C for 30 minutes. Enzymatic reaction was stopped with 1.5 ml of HCl-hydroxylamine (Sigma-Aldrich, S. Louis, Mo, USA) at 139 mg/ml previously neutralized to pH 6.5 with NaOH (1 M). The mixture was kept quiet at room temperature for 10 minutes followed by addition of 1 ml trichloroacetic acid at 15% and 1 mL of hydrochloric acid at 4M (Sigma-Aldrich, S. Louis, Mo, USA). Finally, 1 ml ferricyanide hexahydrate at 5% (Sigma-Aldrich, S. Louis, Mo, USA) in hydrochloric acid (Vetec, Brazil) was added for color development. Reddish violet color development immediately after shaking, reveal presence of fructose-6-phosphoketolase, a *Bifidobacterium* genus exclusive enzyme (Figure 2.3).



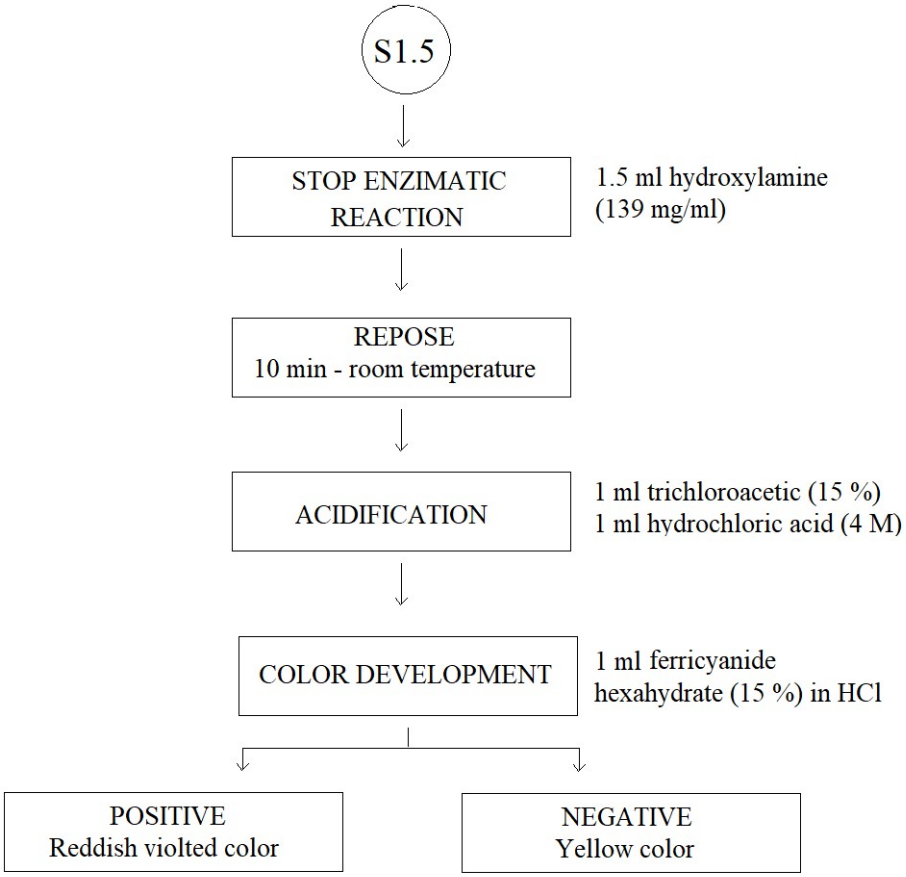


Figure 2.3 – Detection of fructose-6-phosphoketolase activity in presumptive bifidobacteria cultures.

2.4 Genotypic Identification of Lactobacilli by Sequencing of the 16S rDNA Region

DNA of axenic cultures was extracted with 50 µl of lysis solution (0.25% SDS and 0.05 mol/L NaOH). After mixing for 60 seconds, the mixture was treated at 95 °C for 15 min in a thermocycler (T100, BioRad, Italy) for cell lysis, followed by the centrifugation at 10.512*g* for 10 minutes (5427 R Centrifuge, Eppendorf). Volumes of 10 µL of supernatants were diluted in 90 µl of ultrapure water for molecular biology (Sigma-Aldrich) and used as a template for PCR.

Reaction mixtures for Polymerase Chain Reaction (PCR) contained dNTPs (Invitrogen), 200µmol/l; "AGAGTTTGATCCTGGTCAG" and "AAGGAGGTGATCCAGCCGCA" primers (MWG Biotech), 1µmol l^{-1}; Taq Polymerase (Amersham), 1U; DNA template, 2µl of the diluted lysate; ultrapure water for molecular biology (Sigma-Aldrich) to a final volume of 25µl.

The reaction was initiated with hot start and after a common one cycle initial denaturation step (94 ºC/300s.), the amplification program was 35 cycles at: 94 °C for 30 s; 56 °C for 30 s; 72 °C for 60 s and a single final extension step 72 °C for 300 s.

Subsequently, 10µl of PCR product was mixed with 10µl of bromophenol blue buffer and applied to agarose gel using 1.2% TAE (Tris-Acetate EDTA) as running buffer. The run started at 80V/5 minutes, followed by 100V/40 minutes. The gel was then immersed in ethidium bromide solution (10mg ml^{-1}) for 1 hour and photographed in a photo documentator (LAS ImageQuanti 500, GE Healthcare Life Science, UK). After checking the amplification, the PCR product was dried in the thermal cycler at 50 °C for 10 minutes and sequenced as described by ANDRIGHETTO et al. (2001).

The sequences of the 16S rDNA region were search with BLAST against the nucleotide database in NCBI. The results of the sequences were confirmed using RAPD-PCR analysis (Figure 2.4).

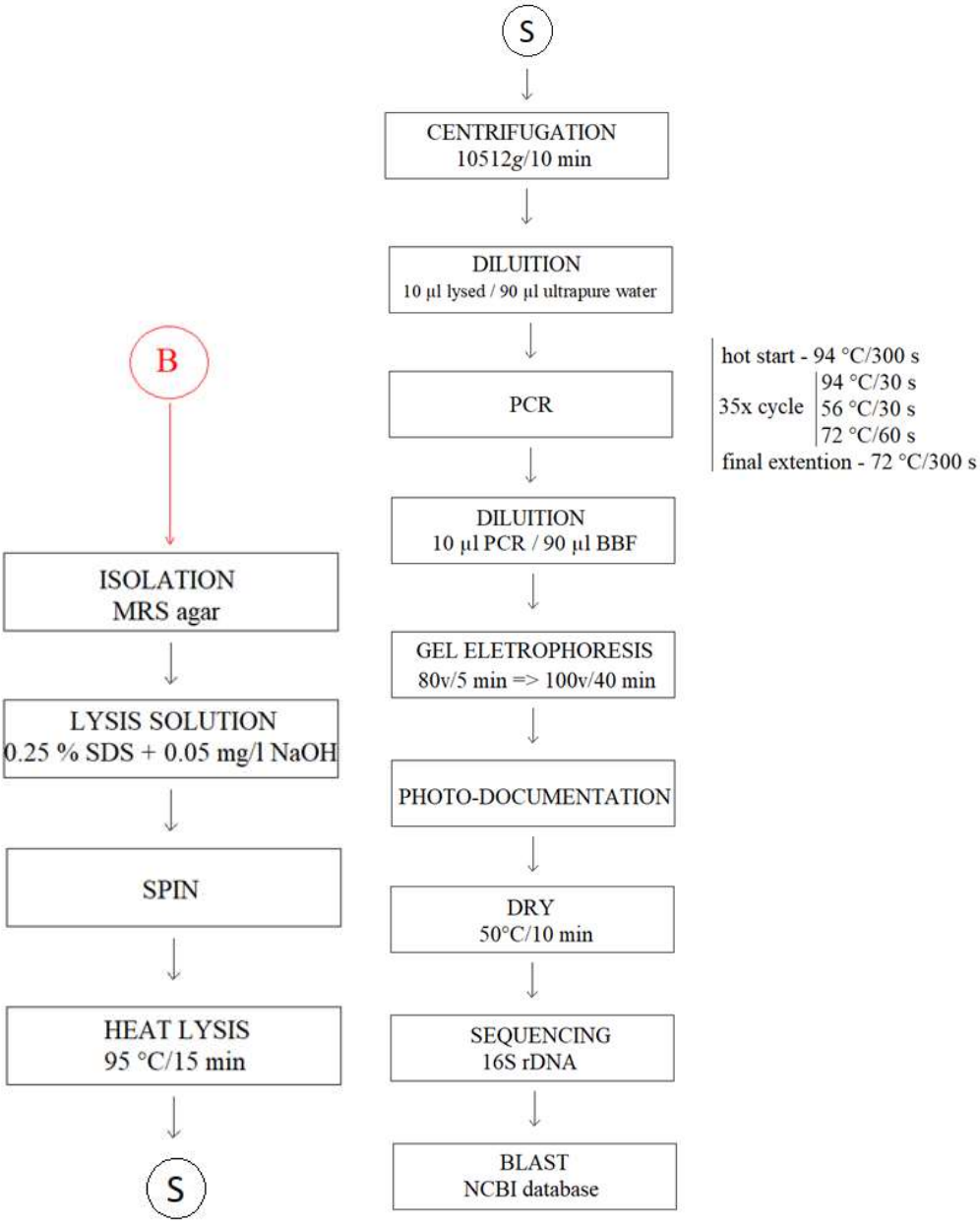


Figure 2.4 - Genotypic identification of lactobacilli by sequencing of 16S rDNA region - procedure

2.5 RAPD-PCR Assay

DNA for RAPD–PCR analysis was extracted, as described in 2.4 section. Amplification reactions were performed according Andrighetto et al., (2001), and the amplification products were separated by electrophoresis on agarose gel (15 g/l) in 0.5 x TBE buffer (0.45 mmol/l-Tris–HCl, 0.45 mmol/l-boric acid, 1 mmol/l EDTA, pH 8.3). Cluster analysis of RAPD–PCR profiles (Table 2.6) were analysed using the GelCompar software version 4.1 (Applied Maths, Kortrijk, Belgium) (Figure 2.5).

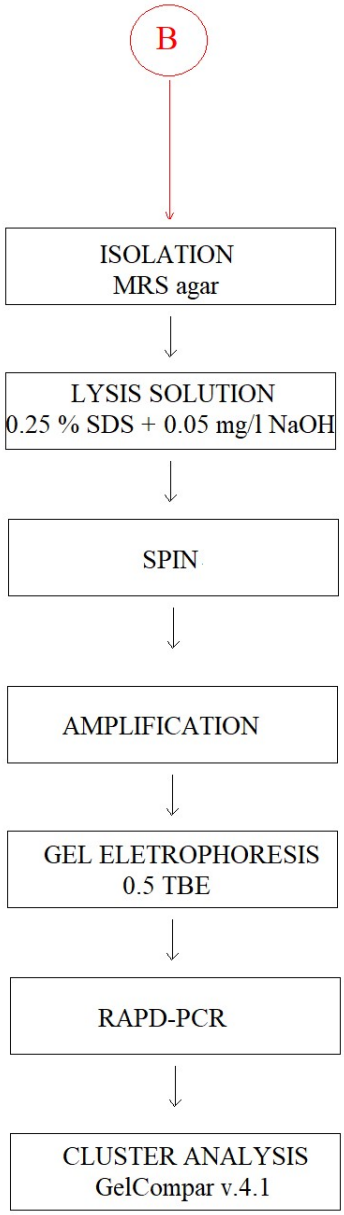


Figure 2.5 - RAPD-PCR assay – procedure

3 RESULTS AND DISCUSSION

Bifidobacterium are heterofermentative, but do not produce CO_2. The enzyme responsible for the metabolism of hexoses into acetic acid and lactic acid is fructose-6-phosphate phosphoketolase, which is also used as a taxonomic marker for identification of these bacteria at the genus level, also known as the bifidus pathway (BIAVATI et al., 1992). Even when cultures were still associated with lactobacilli, it was possible to observe the presence of bifidobacteria by fructose-6-phosphate phosphoketolase positive reactions (Figure 2.6)

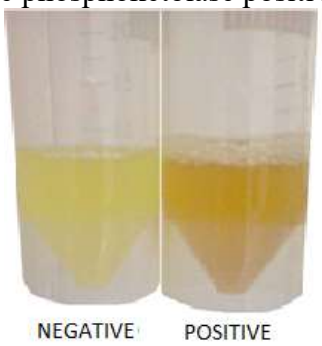


Figure 2.6 - Fructose-6-phosphate phosphoketolase positive and negative reactions. **Source** – Guerra (2013)

Lactobacilli were easily purified from bifidobacteria, but bifidobacteria is a sensible microorganism, and often it does not grow in ordinary MRS agar, unless the medium pH is adjusted to 7.0. Until now only *Bifidobacterium bifidum* species have been identified after uncoupled from *lactobacilli* by sonication and isolation on modified MRS agar (Figure 2.7).

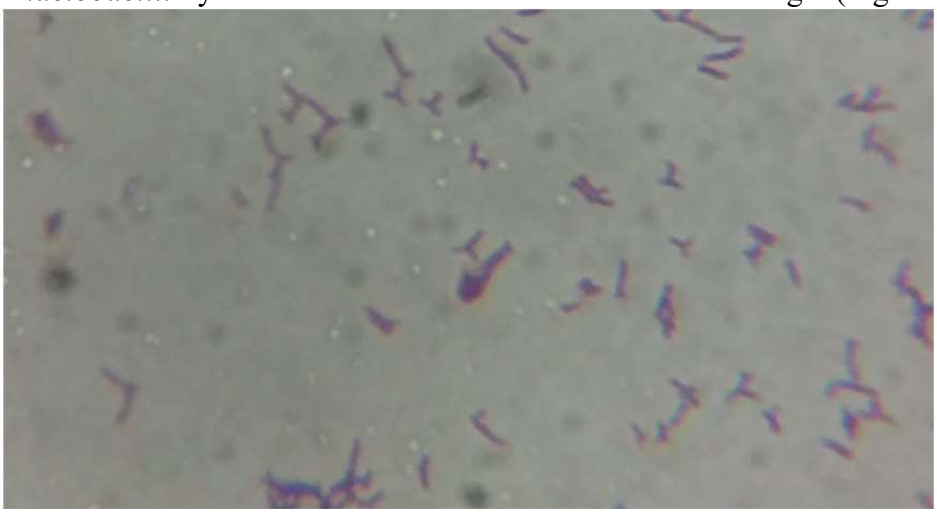


Figure 2.7 –. Gram stain of *Bifidobacterium bifidum* after sonication and isolating in modified MRS agar (0.1% of cysteine and pH 7.0).

A pool of *Lactobacillus* sp. strains was isolated from newborn without antibiotic-therapy history. Thirty-five strains were organized into 9 different genetic profiles (Table 2.1 and Figure 2.8) and were observed through the ramifications and respective percentages of similarity among the strains in the phylogenetic tree. RAPD-PCR method were suitable to give information on the genetic relationship between these strains, establishing the variability within the same species and among *Lactobacillus* species, which were confirmed by 16S highly

conserved genome sequencing. This achievement of identification and intraspecific differentiation of *Lactobacillus* species by the RAPD-PCR technique was also observed in studies conducted by ANDRIGHETTO et al. (2001), and TSAFRAKIDOU et al. (2016). Probiotics from human origin can developed better in human biome (RAVISHANKAR & RAI, 2015). However, this attribute is not enough to guarantee the probiotic safety. Three essential safety attributes were considered in our study, (1) the strains were isolated from newborns without antibiotic-therapy history, (2) the strains did not present hemolytic activity, (3) and they did not display transmissible antibiotic resistances, only their intrinsic ones.

Martim et al. (2009) also used fructose-6-phosphate phosphocetolase method and 16S rRNA gene sequencing for elucidate if breast milk contains bifidobacteria and whether they can be transmitted to the infant gut through breastfeeding. Bifidobacteria were present in 8 milk samples and 21 fecal samples. *Bifidobacterium breve*, *B. adolescentis*, and *B. bifidum* were isolated from milk samples, while infant feces also contained *B. longum* and *B. pseudocatenulatum*. Between infant feces and human milk samples, genus *Bifidobacterium* accounted for 70–88% of the total relative abundance in infant fecal samples, supporting the hypothesis of vertical transfer of bacteria from milk to the infant gut. In addition, identical strains of *Bifidobacterium breve* and *Lactobacillus plantarum* were isolated from the milk and feces of one mother-infant pair. Vertical transfer of bacteria via breastfeeding may contribute to the initial establishment of the microbiota in the developing infant gut (MURPHY et al., 2017).

In general, bifidobacteria strains assimilate more diverse carbohydrate compared to lactobacilli species. Several bifidobacteria strains were able to metabolize xylo-oligosaccharide, arabinoxylan, maltodextrin, galactan and carbohydrates containing fructo-oligosaccharide (FOS) components. In contrast, maltodextrin, galactan, arabinogalactan and galactomannan did not support robust growth ($\geq$0.8 $OD_{600\,nm}$) of any of the *Lactobacillus* strains (MANDAL et al., 2015).

Table 2.1 - Group lactobacilli strains from infant's stools

Genetic profiles	Strains
1	DTA 72, 73, 74 (*L. rhamnosus*)
2	DTA 75, 76, 77, 78 (*L. rhamnosus*)
3	DTA 79 (*L. rhamnosus*)
4	DTA 80, 81 (*L. paracasei*)
5	DTA 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, (*L. paracasei*)
6	DTA 92, 93, 94 (*L. paracasei*)
7	DTA 95, 96, 97, 98, 99, 100, 101, 102, 103 (*L. paracasei*)
8	DTA 104, 105 (*L. fermentum*)
9	DTA 106 (*L. fermentum*)

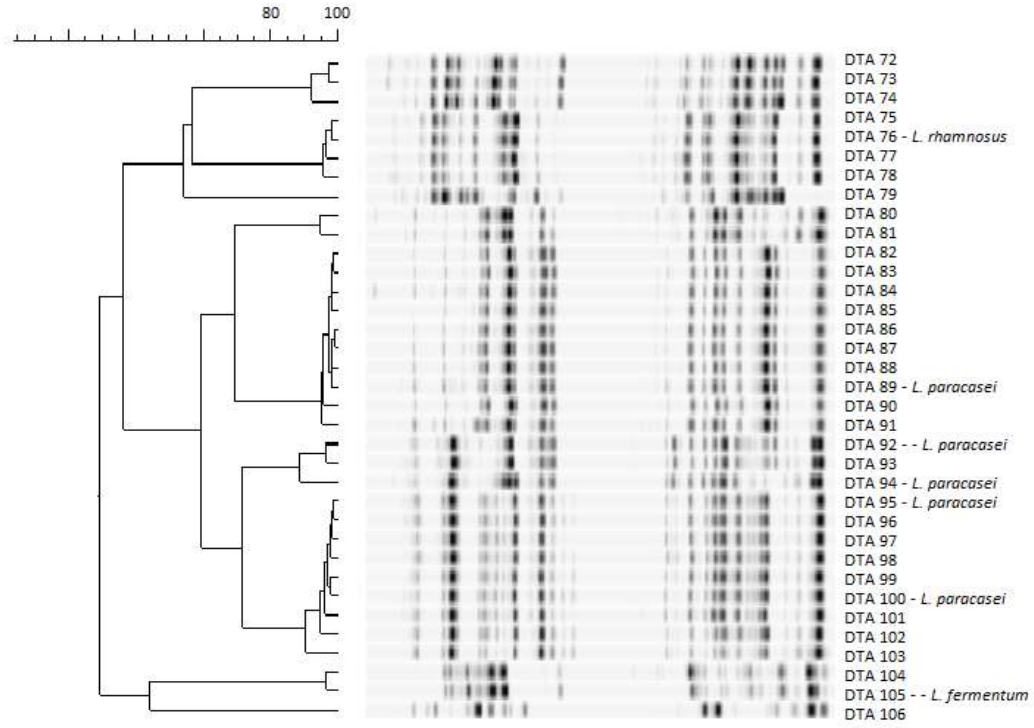


Figure 2.8 - Cluster analysis of RADP-PCR profiles obtained of 35 isolates from stool samples of infants aged between 7 to 21 days. The amplification patterns were analyzed using the software GelCompar 4.1 (Applied Maths).

Sofu (2017) compared several methods for lactic acid bacteria identification. By means of rep-PCR, which is the analysis of repetitive sequences that are based on 16S ribosomal RNA (rRNA) gene sequence, it is possible to conduct structural microbial community analyses such as Restriction Fragment Length Polymorphism (RFLP) analysis of different sizes of DNA fragments resulting from cutting with enzymes, Random Amplified Polymorphic DNA (RAPD) polymorphic DNA amplified randomly at low temperatures and Amplified Fragment-Length Polymorphism (AFLP)-PCR of genomic DNA fragments. Besides, in the recent years, non-culture-based molecular methods such as Pulse Field Gel Electrophoresis (PFGE), Denaturing Gradient Gel Electrophoresis (DGGE), Thermal Gradient Gel Electrophoresis (TGGE), and Fluorescence In-situ Hybridization (FISH) have replaced classical methods once used for the identification of LAB. Pyrosequencing as Next Generation Sequencing (NGS) will be one of the most important to identification of lactic acid bacteria.

Soltan Dallal et al. (2017) identified acid lactic bacteria by their physiological features (e.g., gas production from glucose, arginine hydrolysis, CO_2 production from glucose in MRS broth, carbohydrate fermentation), growth at temperatures of 15°C, 30°C, and 45°C in MRS broth for 3 days, besides Gram staining and catalase test. These results were compared with those of PCR analyses of the 16S rDNA region. The probiotic characteristics of these bacteria were studied using acid and bile tolerance.

4 CONCLUSION

Association with lactobacilli is very important to the establishment of bifidobacteria and its maintenance into gut biome. Therefore, food maker must consider these interactions on formulating starters to improve bifidobacteria growth in probiotic foods.

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CHAPTER III
STRESS-INDUCED CHANGES IN LACTOBACILLI ANTIBIOTIC SUSCEPTIBILITY

ABSTRACT

The aim of this research was to evaluate possible changes in lactobacilli antibiotic susceptibility due to stress-induced by metabolites from milk fermentation process and/or gastrointestinal conditions (GIC). Fermentation process was performed in reconstituted skimmed milk powder (12 %) at 36 °C for 18 hours, following by storage at 7 °C for up to 45 days. Antimicrobial susceptibility tests were performed on MRS agar by disk diffusion method, with 12 antibiotic agents for human use at three different moments: inoculated milk (IM), soon after milk fermentation (FM) and storage fermented milk (SFM) on the 45th day. Hydrogen peroxide, titratable acidity and pH were also measured in SFM. Lactobacilli survivability was evaluated by drop plate technique on MRS agar both in FM and SMF. Stress-induced by metabolites of milk fermentation caused more statistically significant-changes ($P<0.05$) in the level of antibiotic susceptibility than GIC. However, most of these statistically significant-changes make them more susceptible to antibiotic agent, instead CGI makes lactobacilli less susceptible. Changes in lactobacilli antibiotic susceptibility was observed in this research and this can increase the risk of horizontal gene resistance transfer.

Key-words – probiotic culture, carry back, extracellular vesicles

RESUMO

O objetivo desta pesquisa foi avaliar possíveis alterações na susceptibilidade de lactobacilos à agentes antibióticos em consequência do estresse ocasionado pelos metabólitos produzidos na fermentação e/ou pelas condições gastrointestinais (CGI). A fermentação foi conduzida a 36 °C por 18 horas em leite desnatado em pó reconstituído (12 %), seguido de estocagem (7 °C) por 45 dias. Alterações na susceptibilidade de lactobacilos à 12 agentes antibióticos de uso humano foi investigada pelo método de difusão em disco usando ágar MRS em três momentos diferentes: no leite inoculado (LI), logo após a fermentação do leite (LF) e no 45° dia de estocagem do leite fermentado (LFS). Peróxido de hidrogênio, pH, acidez titulável e produção de peróxido de hidrogênio também foram mensurados no LFS. O estresse devido aos metabólitos da fermentação induziu mais alterações significativas ($P<0,05$) que as CGI. No entanto, a maioria dessas alterações deixaram os lactobacilos mais susceptíveis aos antibióticos, ao contrário, CGI os deixaram menos susceptível. Alterações na susceptibilidade de lactobacilos à antibióticos foi observada nesta pesquisa e isso pode aumentar o risco de transferência horizontal de genes de resistência.

Palavras-chave – culturas probióticas, transferência horizontal de genes de resistência, *carry back*, *extracellular vesicles*

1 INTRODUCTION

Among the probiotic safety properties, the antibiotic susceptibility is an important criterion for selection of probiotic strains, although most of studies, poorly performs this property. Microorganisms can change their antibiotic susceptibility induced by microbial stressful conditions. Either storage or gastrointestinal condition such as acid, peroxide, bile salt, etc., could induce such changes (MARINO et al., 2017).

Increasing the consumption of probiotic foods is related to healthy eating habits that prevent disease and illness (SEBASTIÁN DOMINGO, 2017; WILBURN & RYAN, 2017). Probiotics intended for improving the gut health shall perform this function *in loco*. Until probiotics reach the gut lumen, besides stressful conditions of food matrices, they still go through to gastric and intestinal stressful substances, such as lactic and acetic acid, hydrogen peroxidase and other substances (GÄNZLE, 2015). Probiotic microorganisms can become a reservoir of antibiotic resistant genes in the human gut. These resistant genes can be transferred to pathogens that share the same gut microbiome, thus may resulting in serious clinical ramifications (ZHENG et al., 2017). Often bacteria changes plasmids by themselves, which can encode resistance genes. Thus, antibiotic bacterial resistance is carried mainly by plasmid transfer (RICHARDSON, 2017). Strains with resistance plasmids should not be used as human or animal probiotics, as they are able to transmit resistance factors to pathogenic bacteria, making it difficult to cure infections

A common mechanism used by bacteria to reduce the effects of antibiotics is to acquire or increase the expression of drug efflux pumps. These pumps expel drugs from the cytoplasm, limiting their ability to access their target (DEC et al., 2017; RICHARDSON, 2017).

Regulatory agencies standardize at least 10^6 cfu/ml of live probiotic lactobacilli for yogurt and fermented milk (SHORI, 2015). For this reason, probiotic strains should carry on the fermentation process alone or together with starter cultures. Furthermore, probiotic cultures should be from human origin, since they are naturally adapted to live in gut microbiome (RAVISHANKAR & RAI, 2015).

Antibiotic resistance was reported to occur when a drug loses partially or totally its effectiveness on bacterial growth inhibition. Bacteria become “resistant” and keep multiplying in the presence of therapeutic levels of the antibiotics. Bacteria, when replicates even in the presence of the antibiotics, are called a resistant bacteria (ZAMAN et al., 2017). The Table 3.1 shows the main bacterial mechanism of drug resistance of common antibiotic agent for human use.

Table 3.1 – Main mechanism of drug resistance of common antibiotic agent for human use

Antibiotic class	Example(s)	Mode(s) of resistance
P-Lactams	Penicillins, Cephalosporins, Penems, Monobactams	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, Streptomycin, Spectinomycin	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, Teicoplanin	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, Tigecycline	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromycin	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Carbon-Oxygen lyase, acetylation, efflux, altered target
Oxazolidinones	Linezolid	Efflux, altered target
Phenicols	Chloramphenicol	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	Efflux, altered target
Sulfonamides	Sulfamethoxazole	Efflux, altered target
Rifamycins	Rifampin	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Altered target
Cationic peptides	Colistin	Altered target, efflux

Source - ZAMAN et al. (2017)

Human consumption of probiotic and symbiotic foods has increased in last years. Microbial safety tests are of primary importance due possibility of bacteria transfer horizontally resistance to pathogens and commensal gut microbiota. Specific antibiotic resistance determinants carried on mobile genetic elements, such as tetracycline resistance genes, have often been detected in the typical probiotic genera, and hence constitute a reservoir of resistance for potential food or gut pathogens, thus representing a serious safety issue. Plasmid-associated antibiotic resistance, which occasionally occurs, is a matter of concern as its use as probiotics can be detrimental owing the possibility of the resistance spreading to harmful microorganisms inhabiting the same niche. Further, presence of transferable antibiotic resistance genes even to a less innocuous member of the gut microbial community poses a safety hazard and needs to be considered (SHARMA et al., 2014).

More than 50 % of all plasmids can be transferred by conjugation, but some authors have suggested that conjugation is not a major mechanism responsible for the persistence of plasmids (LOPATKIN et al., 2017) show that common conjugal plasmids, even when costly, are indeed transferred at sufficiently high rates to be maintained in the absence of antibiotics in *Escherichia coli*. The authors suggest that reducing antibiotic use alone is likely insufficient for reversing resistance.

Bacterial can become resistant to antibiotic either by "mutational resistance" or "horizontal gene transfer". In general, mutations resulting in antimicrobial resistance alter the antibiotic action via one of the following mechanisms: *i)* modifications of the antimicrobial target (decreasing the affinity for the drug), *ii)* a decrease in the drug uptake, *iii*) activation of efflux mechanisms to extrude the harmful molecule, or *iv)* global changes in important metabolic pathways via modulation of regulatory networks. Horizontal gene transfer mechanism by: *i*) transformation (incorporation of naked DNA), *ii*) transduction (phage mediated) and, *iii*) conjugation (bacterial "sex"). The most important mobile genetic elements are plasmids and transposons, both of which play a crucial role in the development and dissemination of antimicrobial resistance among clinically relevant organisms. One of the most efficient mechanisms for accumulating antimicrobial resistance genes is represented by integrons, which are site-specific recombination systems capable of recruiting open reading frames in the form of mobile gene cassettes (MUNITA & ARIAS, 2016).

The Figure 3.1 shows the main mechanisms of antibiotic agent on microorganism.

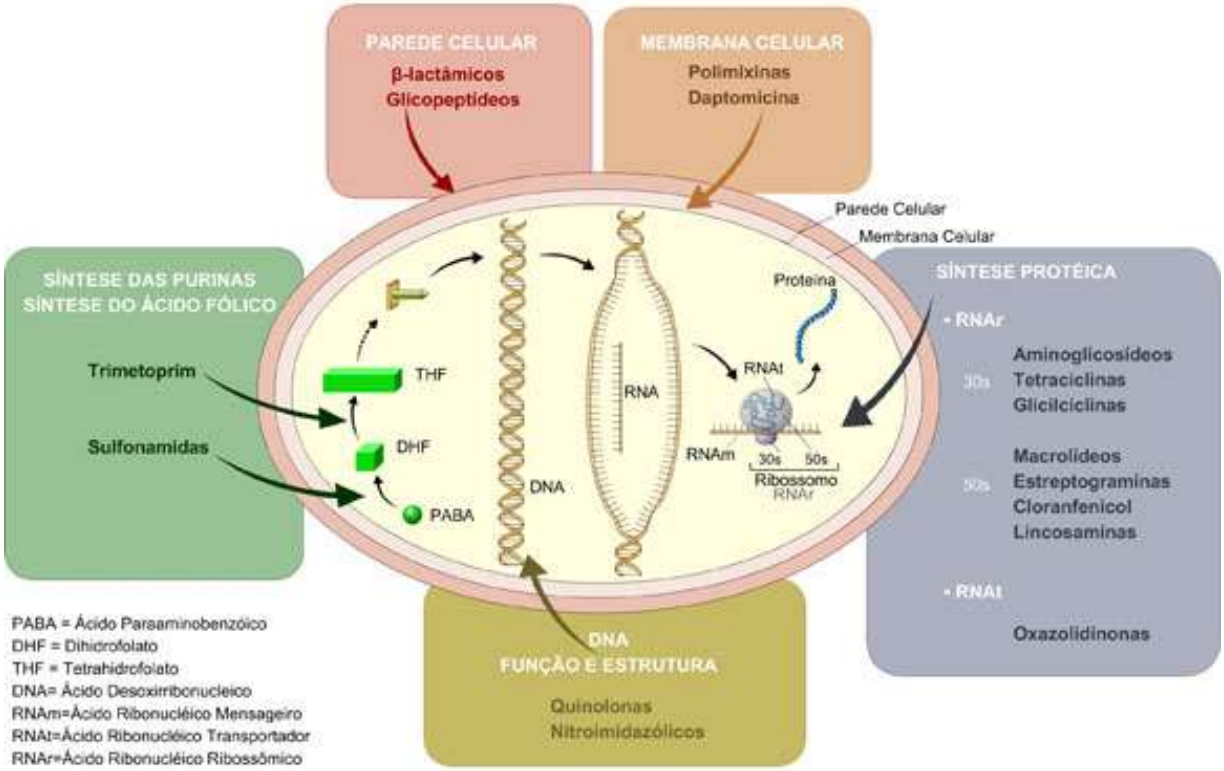

Figure 3.1 – Action mechanisms of antibiotics on microorganism.
Source - Anvisa

Recently, Jiang et al. (2017) reported a new bacterial antibiotic resistance pathway. The authors support the existence of ancient and, possibly, recent transfers of antibiotic resistance genes from antibiotic-producing actinobacteria to proteobacteria. This new mechanism is reported as 'carry-back'.

From an evolutionary perspective, bacteria use two major genetic strategies to adapt to the antibiotic "attack", *i*) mutations in gene(s) often associated with the mechanism of action of the compound, and *ii)* acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT).

2 MATERIAL AND METHODS

2.1 *Lactobacillus* Strains

Lactobacillus cultures were isolated, identified and grouped by clusters of genetic similarity as described in the Chapter 2 (2.1, 2.4, and 2.5 Sections). Cluster analysis of RADP-PCR revealed 4 clusters of *L. paracasei*, 3 clusters of *L. rhamnosus*, and 2 clusters of *L. fermentum.* One lactobacilli representative of each cluster, totalizing 9 strains, were used, as follow: *L rhamnosus* (DTA 73, DTA 76, DTA 79), *L. paracasei* (DTA 81, DTA 83, DTA 92, DTA 97), and *L. fermentum* (DTA104, DTA 106).

2.2 Fermented Milk

Reconstituted milk (12 %) was prepared from skimmed milk powder and delivered in glass bottles (300 ml), followed by sterilization at 110 °C for 10 minutes. Each bottle was inoculated with *ca* 10^6 cfu/ml of each lactobacilli strain (Figure 3.2). Fermentation was carried out at 36 °C for 18 hours, followed by storage (7 °C) for up to 45 days.

Figure 3.2 – Bottles of fermented milk by the different lactobacilli strains.

Three moments have been considered for monitoring the stressful metabolites produced by lactobacilli in the fermented milks: inoculated milk (IM), soon after milk fermentation (FM), and stored fermented milk (SMF) on the 45th day. For the purpose hydrogen peroxide (Peroxide Test, Merck, France), titratable acidity, and pH were measured in the SFM (Figure 3.3).

Figure 3.3 – Measurement of stressful metabolites produced by lactobacilli during the milk fermentation. A - pH measurement (KR20, Akrom, Brazil). B - titratable acidity. C - hydrogen peroxidase (Peroxide Test, Merck, France).

2.3 Simulation of Gastrointestinal Conditions

Gastrointestinal condition (GIC) simulation was performed as reported by FAVARIN et al. (2015) with some modifications. Gastrointestinal base juice (GBJ) was formulated as follows: calcium chloride (0.11 g/l); potassium chloride (1.12 g/l), sodium chloride (2.0 g/l), and potassium dihydrogen phosphate (0.4 g/l). This solution was sterilized at 121 °C for 15 minutes.

Artificial gastric juice (AGJ) was freshly prepared by adding to the GBJ, 3.5 g/l of swine mucin (Sigma-Aldrich, S. Louis, Mo, US), and 0.26 g/l of swine pepsin (Sigma-Aldrich, S. Louis, Mo, USA). The pH was adjusted to 2.0 with HCl (1 M). Aliquots (100 µl) of fermented milk (FM) or stored fermented milk (SFM) were transferred to Eppendorf tube with 900 µl of AGJ and incubated in the anaerobic workstation (Electrotec, England) with artificial atmosphere (80 % N_2, 10 % CO_2, and 10 % H_2) at 36 °C for 45 minutes, with gentle shaking.

Subsequently, artificial intestinal juice (AIJ) was obtained by adding to the AGJ, 3 g/l of bile salt (Ox bile, Merck, Darmstadt, Germany), 1.95 g/l of pancreatin, and 0.1 g/l of egg white lysozyme (Sigma-Aldrich, S. Louis, Mo, USA). The pH was adjusted to 7.0 with sodium bicarbonate solution (1M) and incubated in the anaerobic workstation (Electrotec, England) with artificial atmosphere (80 % N_2, 10 % CO_2, and 10 % H_2) at 36 °C for 180 minutes, with gentle shaking (Figure 3.4).

Lactobacilli survivability was evaluated on MRS agar using the drop plate technique in the FM and SFM before and after GIC. Results were statistically analyzed by variance test (ANOVA) followed by Dunnett 's test with 95% of confidence using XLSTAT software 7.5.

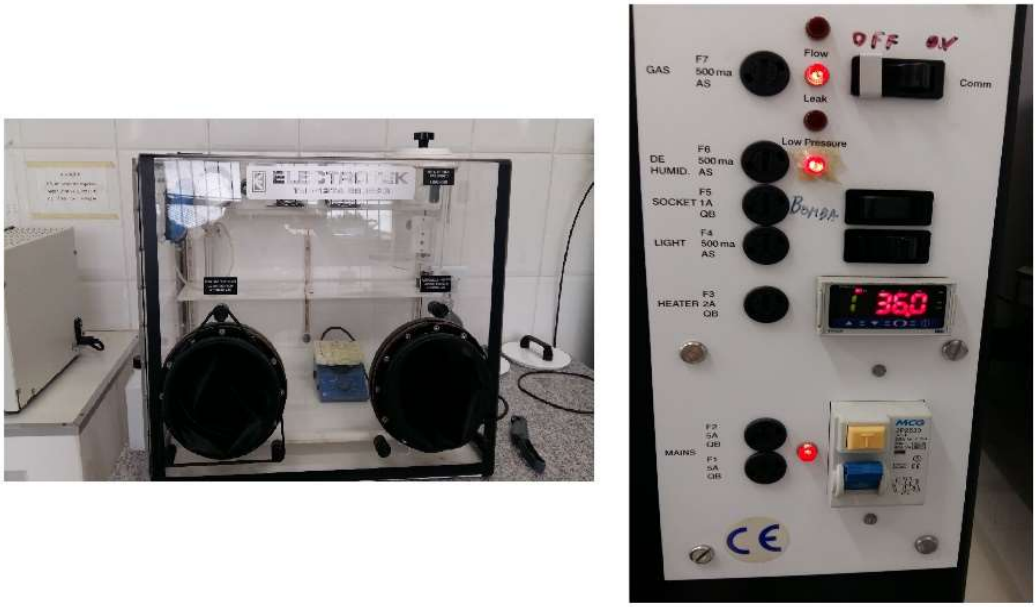

Figure 3.4 – Anaerobic workstation (Electrotec, England) used to incubate the tubes with gastrointestinal solution. Artificial vacuum atmosphere used: 80 % N_2, 10 % CO_2, 10 % H_2. Temperature: 36 °C. Humidity: 70%.

2.4 Antimicrobial Susceptibility

Antimicrobial susceptibility tests were performed by agar disk diffusion method (BAUER et al., 1966) using MRS agar (HiMedia, Mumbai, India). Aliquots (100 µl) of IM, FM, and SFM from each lactobacilli strain (total of 9 strains), before and after GIC were spread on the agar surface with a Drigaslky spreader. Dried discs (CECON, São Paulo) impregnated with antibiotics agents for human use: AMC - amoxicillin + clavulanic acid – 30 mcg; NAL - nalidixic acid – 30 mcg; VAN - vancomycin – 30 mcg; KAN - kanamycin – 30 mcg; CIP – ciprofloxacin – 5 mcg; AMP - ampicillin – 10 mcg; DOX - doxycycline – 30 mcg; GEN - gentamicin – 10 mcg; ERI - eritromicin – 15 mcg, CLO - chloramphenicol – 30 mcg; PEN – penicillin G – 10 U.I.; TET - tetracycline – 30 mcg, were placed on top of the agar and lightly

press down with a sterile tweezer. Plates were incubated at 36 °C for 48 hours and the diameter of growth inhibition zones surrounded the discs were measured with a caliper (including disc) (Figures 3.5). Results were compared with the susceptibility level proposed by VLKOVÁ et al. (2006). Variance test (ANOVA) followed by Fischer's test ($P<0.05$) was used to verify the significative changes amount IM and FM and SFM or before and after GIC.

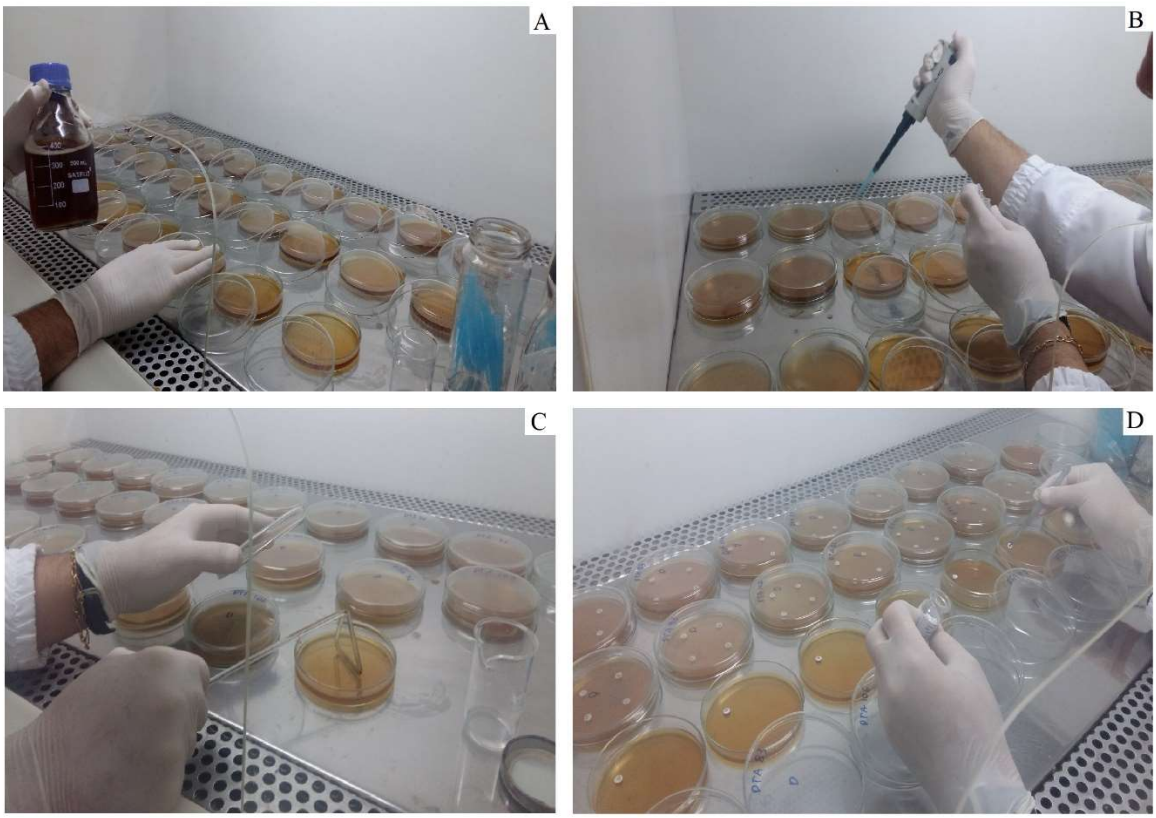

Figure 3.5 – Antibiogram susceptibility test. A – MRS plating. B – inoculation. C - handling a Drigaslky spreader. D – antibiotic discs placing.

3 RESULTS AND DISCUSSION

3.1 Stressful Compounds Produced by Lactobacilli in Fermented Milk

All lactobacilli were able to acidify the milk lowering the pH to below 4.8, within 18 hours at 36 °C. *L. rhamnosus* DTA 79 showed the lowest acidification capacity, instead *L. paracasei* DTA 97 was the most acidifying. Regard to production of hydrogen peroxide, out-off *L. rhamnosus* DTA 76, all lactobacilli produced up to 2 μg/ml (Table 3.2). Hydrogen peroxide and lactic acid are two stressing metabolites produced during the fermentation of milk by lactic acid bacteria.

There was no significant reduction in the lactobacilli survivability during 45 days of storage at 7 °C, but reductions were observed when they were exposed to GIC. Although, there was no cell death during the storage, some cellular injury happened. When FM was exposed to GIC there were four significant reductions (*L. rhamnosus* DTA 73, 76, 79, and *L. paracasei* DTA 92), but when SFM was exposed to GIC the reductions increased by seven (*L. rhamnosus* DTA 73, 76, 79, *L. paracasei* DTA 81, 92, and *L. fermentum* DTA 104, 106).

L. rhamnosus are more sensitive than *L. paracasei* and *L. fermentum* to GIC. All strains of *L. rhamnosus* reduced their survivability both in FM and SFM. *L. fermentum* (DTA 104, 106) and some *L. paracasei* strains only reduced in the SFM. Two *L. paracasei* (DTA 83, 97) strains did not reduce their survivability either in the FM or FMS.

Overlapping two or more stress condition can increased the cellular stress. Cells became more sensitive during the storage of FM, but cell death only occurs to GIC exposition. When probiotics are carried by food matrices, the overlap of stress conditions should be considered as probiotic feature.

However, even with loss of cell viability, probiotic strains could still play probiotic functions. New approach has successfully effective against influenza virus when heat-killed *Lactobacillus kunkeei* YB38 was orally administrate (ASAMA et al., 2017).

Table 3.2 - Stressful compounds produced by each *Lactobacillus* representative of the cluster and microbial survivability.

				log cfu/ml ± SD survival of lactobacilli strain			
				FM		SFM	
Lactobacilli strain	*pH value ± SD*	*% lactic acid value ± SD*	*mg/l H2O2 value*	*Before GI*	*After GI*	*Before GI*	*After GI*
L. rhamnosus DTA 73	4.05 ± 0.03	2.210 ± 0.006	2	8.78 ± 0.05	7.63 ± 0.05 *	8.76 ± 0.02	6.50 ± 0.60 *
L. rhamnosus DTA 76	4.38 ± 0.05	2.156 ± 0.159	0	8.45 ± 0.12	7.48 ± 0.50 *	8.25 ± 0.19	7.12 ± 0.56 *
L. rhamnosus DTA79	4.78 ± 0.02	1.814 ± 0.045	2	8.59 ± 0.08	7.76 ± 0.30 *	8.50 ± 0.28	6.98 ± 0.01 *
L. paracasei DTA 81	4.13 ± 0.01	2.210 ± 0.045	2	9.00 ± 0.07	8.26 ± 0.39	8.02 ± 0.04	7.14 ± 0.64 *
L. paracasei DTA 83	4.30 ± 0.02	1.989 ± 0.013	2	9.20 ± 0.11	8.81 ± 0.16	9.08 ± 0.05	7.86 ± 0.13
L. paracasei DTA 92	4.07 ± 0.02	2.637 ± 0.038	2	8.19 ± 0.65	6.83 ± 0.20 *	8.51 ± 0.01	6.71 ± 0.04 *
L. paracasei DTA 97	4.04 ± 0.05	2.250 ± 0.000	0.5	8.85 ± 0.05	8.27 ± 0.56	8.79 ± 0.00	8.22 ± 1.00
L. fermentum DTA 104	4.33 ± 0.02	1.881 ± 0.013	2	8.66 ± 0.22	8.18 ± 0.41	8.70 ± 0.02	7.55 ± 0.06 *
L. fermentum DTA 106	4.35 ± 0.03	2.115 ± 0.013	0.5	8.75 ± 0.03	7.83 ± 0.20	8.58 ± 0.10	7.41 ± 0.65 *

* Indicate significative difference (P<0.95) by the Dunnett's test between before (control) and after GI, for the same microorganism (same row) and for the same sample (FM or FMS). SD - Standard Deviation. GI - *in vitro* gastrointestinal conditions. FM - Fermented milk (freshly produced). SFM – Stored fermented milk on the after 45th days at 7 °C.

3.2 Changes in Lactobacilli Susceptibility to Antibiotics due to Storage of Fermented Milk: Before and After Gastrointestinal Conditions Simulation

The Tables 3.3 and 3.4 show the induced susceptibility on *L. rhamnosus* due to processing conditions, storage time, and after stress condition that mimic gastrointestinal transit.

Table 3.3 - Antibiotics that act by inhibiting or interfering with cell wall synthesis a or by altering the permeability of the cell membrane: induced susceptibility on *Lactobacillus rhamnosus* due to processing condition and after stress condition that mimic gastrointestinal transit (mean and Standard deviation).

Antibiotic agent	Matrice Condition		DTA-73	DTA-76	DTA-79
AMC	Inoculated Milk (IM)	bGI	44.88 ±5.13 B;*	34.95 ±6.58 a;B;*	36.35 ±1.34 B;*
		aGI	43.50 ±2.12 B;*	25.45 ±4.31 b;B;*	40.43 ±2.58 B;*
	Fermented Milk (FM)	bGI	47.23 ±1.80 B;*	36.03 ±4.91 a;B;*	36.43 ±0.60 B;*
		aGI	45.28 ±3.92 B;*	27.43 ±2.23 b;B;*	38.85 ±1.98 B;*
	Stored Fermented Milk (SFM)	bGI	61.38 ±4.28 A;*	54.73 ±3.36 a;A;*	42.58 ±0.95 A;*
		aGI	53.03 ±1.52 A;*	46.60 ±0.99 b;A;*	45.33 ±1.17 A
AMP	Inoculated Milk (IM)	bGI	30.00 ±0.85 *	28.30 ±1.41 B;*	30.33 ±1.45 a;B;*
		aGI	27.50 ±1.70 *	23.93 ±2.72 B;*	13.40 ±1.56 b;B
	Fermented Milk (FM)	bGI	33.30 ±0.78 *	27.15 ±0.42 B;*	29.43 ±0.60 a;B;*
		aGI	28.33 ±0.46 *	26.93 ±1.52 B;*	12.20 ±1.06 b;B
	Stored Fermented Milk (SFM)	bGI	30.60 ±0.78 *	35.00 ±0.71 A;*	39.05 ±0.42 A;*
		aGI	30.83 ±1.10 *	33.05 ±2.26 A;*	38.88 ±1.52 A;*
PEN	Inoculated Milk (IM)	bGI	45.78 ±0.67 *	29.80 ±0.28 b;*	29.45 ±0.07 *
		aGI	43.48 ±2.09 *	38.03 ±7.60 a;*	28.00 ±0.78 *
	Fermented Milk (FM)	bGI	44.10 ±1.13 *	29.80 ±0.28 b;*	31.68 ±1.17 *
		aGI	42.03 ±0.18 *	38.03 ±7.60 a;*	29.43 ±0.60 *
	Stored Fermented Milk (SFM)	bGI	46.25 ±1.06 *	34.30 ±0.64 *	34.85 ±0.21 *
		aGI	40.15 ±1.63 *	35.40 ±0.64 *	34.60 ±0.85 *
VAN	Inoculated Milk (IM)	bGI	***	***	***
		aGI	***	***	***
	Fermented Milk (FM)	bGI	***	***	***
		aGI	***	***	***
	Stored Fermented Milk (SFM)	bGI	***	***	***
		aGI	***	***	***

Different capital letters in the column indicate significant difference amount aGI (inoculated milk, and fermented milk, and stored fermented milk) or bGI (inoculated milk, and fermented milk, and storage fermented milk); for only one lactobacilli strain and only one antibiotic. Different lower-case letters in the column indicate significant difference between inoculated milk (aGI and bGI), or fermented milk (aGI and bGI), or stored fermented milk (aGI and bGI); for only one lactobacilli strain and only one antibiotic. All by Fischer's test at 0.95 of reliability. Asterisks compare the results with the susceptibility level (*** Resistant; ** Intermediate; * Sensible) proposed by Vlkovà et al. (2006). (aGI) after (bGI) before *in vitro* gastrointestinal condition. Disc diameter = 6mm. AMC, amoxicillin + clavulanic acid; AMP, ampicillin; PEN, penicillin, VAN, vancomycin.

Table 3.4 - Antibiotics that act by inhibiting protein synthesis or nucleic acid synthesis: Induced Susceptibility on *Lactobacillus rhamnosus* due to processing conditions and after stress condition that mimic gastrointestinal transit (mean and Standard deviation).

Antibiotic agent	Matrice Condition		DTA-73	DTA-76	DTA-79
CIP	Inoculated Milk (IM)	bGI	18.50 ±4.24 B;**	22.15 ±0.07 a;A;**	14.20 ±2.83 ***
		aGI	13.50 ±4.10 B;**	14.40 ±2.97 b;***	15.20 ±1.41 ***
	Fermented Milk (FM)	bGI	19.60 ±1.06 B;**	20.25 ±0.14 a;A;**	14.35 ±1.27 ***
		aGI	16.78 ±1.73 B;**	14.65 ±1.13 b;***	15.43 ±0.60 ***
	Stored Fermented Milk (SFM)	bGI	25.50 ±1.77 A;*	12.30 ±1.63 B;***	11.85 ±0.85 ***
		aGI	20.88 ±0.32 A;**	11.73 ±1.03 ***	12.78 ±1.16 ***
DOX	Inoculated Milk (IM)	bGI	33.00 ±0.42 B;*	33.60 ±1.41 a;AB;*	35.45 ±2.90 B;*
		aGI	31.55 ±5.87 B;*	24.20 ±1.13 b;B;*	36.48 ±1.45 *
	Fermented Milk (FM)	bGI	29.60 ±1.77 B;*	31.80 ±1.63 B;*	31.23 ±1.73 b;B;*
		aGI	31.75 ±1.56 B;*	27.18 ±2.51 B;*	38.30 ±2.19 a;*
	Stored Fermented Milk (SFM)	bGI	48.45 ±0.92 a;A;*	35.00 ±1.91 A;*	41.95 ±1.34 A;*
		aGI	36.05 ±2.30 b;A;*	36.50 ±0.99 A;*	40.53 ±0.67 *
GEN	Inoculated Milk (IM)	bGI	12.25 ±3.04 ***	11.55 ±1.34 A;***	10.40 ±0.00 ***
		aGI	9.25 ±4.45 AB;***	10.35 ±1.63 ***	8.93 ±3.01 ***
	Fermented Milk (FM)	bGI	13.73 ±1.80 ***	6.75 ±0.21 B;***	9.08 ±0.74 ***
		aGI	12.60 ±0.35 A;***	6.80 ±1.13 ***	10.65 ±0.85 ***
	Stored Fermented Milk (SFM)	bGI	6.00 ±0.00 ***	6.00 ±0.00 ***	9.60 ±0.64 ***
		aGI	8.75 ±0.00 B;***	6.00 ±0.00 ***	9.78 ±1.73 ***
ERI	Inoculated Milk (IM)	bGI	46.65 ±1.41 *	39.00 ±3.39 *	35.85 ±0.35 *
		aGI	48.80 ±1.13 A;*	42.90 ±0.99 *	31.88 ±8.80 B;*
	Fermented Milk (FM)	bGI	46.43 ±1.66 *	39.00 ±3.39 *	35.35 ±0.71 *
		aGI	44.80 ±0.28 AB;*	42.90 ±0.99 *	31.93 ±4.28 B;*
	Stored Fermented Milk (SFM)	bGI	47.20 ±1.70 a;*	40.90 ±2.05 *	40.28 ±1.80 *
		aGI	36.53 ±1.84 b;B;*	38.65 ±1.70 *	42.75 ±3.82 A;*
CLO	Inoculated Milk (IM)	bGI	43.43 ±0.60 *	33.85 ±1.06 *	33.65 ±0.64 *
		aGI	42.20 ±0.85 AB;*	25.13 ±2.44 B;*	35.28 ±0.04 *
	Fermented Milk (FM)	bGI	43.98 ±0.04 *	33.85 ±1.06 *	31.85 ±1.41 *
		aGI	45.93 ±0.46 A;*	25.13 ±2.44 B;*	33.48 ±0.67 *
	Stored Fermented Milk (SFM)	bGI	43.83 ±1.17 a;*	34.48 ±0.67 *	36.15 ±1.56 *
		aGI	36.25 ±1.41 b;B;*	36.40 ±0.64 A;*	35.43 ±0.67 *
TET	Inoculated Milk (IM)	bGI	33.13 ±0.18 AB;*	43.45 ±1.63 A;*	36.40 ±3.11 *
		aGI	36.15 ±0.35 *	39.33 ±0.88 *	33.40 ±4.53 *
	Fermented Milk (FM)	bGI	35.43 ±0.81 A;*	43.45 ±1.63 A;*	35.40 ±0.64 *
		aGI	35.00 ±0.57 *	39.33 ±0.88 *	35.85 ±0.07 *
	Stored Fermented Milk (SFM)	bGI	30.80 ±1.70 B;*	38.25 ±1.98 B;*	38.10 ±0.78 *
		aGI	35.45 ±1.63 *	37.20 ±1.06 *	38.58 ±0.60 *
KAN	Inoculated Milk (IM)	bGI	***	***	***
		aGI	***	***	***
	Fermented Milk (FM)	bGI	***	***	***
		aGI	***	***	***
	Stored Fermented Milk (SFM)	bGI	***	12.20 ± 0.28 ***	***
		aGI	***	11.25 ± 1.70 ***	***
NAL	Inoculated Milk (IM)	bGI	***	***	***
		aGI	***	***	***
	Fermented Milk (FM)	bGI	***	***	***
		aGI	***	***	***
	Stored Fermented Milk (SFM)	bGI	***	21.18 ± 1.45 **	***
		aGI	***	19.25 ± 0.42 **	***

Different capital letters in the column indicate significant difference amount aGI (inoculated milk, and fermented milk, and stored fermented milk) or bGI (inoculated milk, and fermented milk, and stored fermented milk); for only one lactobacilli strain and only one antibiotic. Different lower-case letters in the column indicate significant difference between inoculated milk (aGI and bGI), or fermented milk (aGI and bGI), or stored fermented milk (aGI and bGI); for only one lactobacilli strain and only one antibiotic. All by Fischer's test at 0.95 of reliability. Asterisks compare the results with the susceptibility level (*** Resistant; ** Intermediate; * Sensible) proposed by Vlkovà et al. (2006). (aGI) after (bGI) before *in vitro* gastrointestinal condition. Disc diameter = 6mm. CIP, ciprofloxacin; DOX, doxacycline; GEN, gentamicin; ERI, erythromycin; CLO, chloramphenicol; TET, tetracycline; KAN, kanamycin; NAL, nalidixic acid.

L. rhamnosus DTA 73 increased its AMC, CIP, DOX, ERI (aGI) susceptibility and reduced its GEN (aGI), ERI (aGI), CLO (aGI), TET (bGI) susceptibility due to stress caused by fermentation metabolites. Stress due to GIC increased its ERI (FM) and CLO (FM) susceptibility. There was no AMP, PEN, KAN, NAL changing susceptibility.

L. rhamnosus DTA 76 increased its DOX, CLO (aGI), KAN, NAL susceptibility and reduced its CIP (bGI), GEN (bGI), TET (bGI) susceptibility due to stress caused by fermentation metabolites. Stress due to GIC increased its AMP (IM; FM; FMS), PEN (IM; FM) susceptibility and reduced its CIP (IM; FM), DOX (IM) susceptibility.

L. rhamnosus DTA 79 increased its AMC, AMP, DOX (bGI), ERI (aGI) susceptibility due to stress caused by fermentation metabolites. Stress due to GIC increased its AMP (IM; FM) susceptibility and reduced its DOX (FM) susceptibility.

There were two significative changes at the susceptibility level proposed by Vlakovà et al. (2006): *L. rhamnosus* DTA 73 increased its NAL, CIP (bGI) susceptibility in SFM.

Acid and peroxides produced during fermentation of milk tended *L. rhamnosus* to increase their susceptibility level to antibiotics that act on the cell membrane. Throughout storage, such substances are doing injury on the cell membrane. This trend was not observed for antibiotics acting at the DNA or for the stress caused by GIC (Tables 3.3 and 3.4).

The Tables 3.5 and 3.6 show the induced susceptibility on *L. paracasei* due to fermentation process, storage time, and stress condition that mimic gastrointestinal transit.

Table 3.5 - Antibiotics that act by inhibiting or interfering with cell wall synthesis a or by altering the permeability of the cell membrane: induced susceptibility on *Lactobacillus paracasei* due to processing condition and after stress condition that mimic gastrointestinal transit (mean and Standard deviation).

Antibiotic agent	Matrice Condition		DTA-81	DTA-83	DTA-92	DTA-97
AMC	Inoculated Milk (IM)	bGI	41.85 ±10.39 *	44.05 ±0.28 *	37.25 ±1.34 a;B;*	37.45 ±3.04 a;B;*
		aGI	42.08 ±0.74 *	45.93 ±0.81 *	28.20 ±0.14 b;B;*	28.05 ±0.92 b;B;*
	Fermented Milk (FM)	bGI	40.10 ±0.35 *	45.30 ±0.64 *	35.73 ±0.67 a;B;*	36.60 ±1.34 a;B;*
		aGI	40.53 ±1.59 *	45.50 ±0.71 *	29.03 ±0.04 b;B;*	28.58 ±0.04 b;B;*
	Stored Fermented Milk (SFM)	bGI	41.88 ±0.46 *	44.90 ±0.57 *	47.28 ±0.74 A;*	48.50 ±1.27 A;*
		aGI	41.05 ±0.07 *	42.15 ±1.63 *	44.58 ±0.60 A;*	44.88 ±0.32 A;*
AMP	Inoculated Milk (IM)	bGI	30.35 ±3.04 *	34.95 ±0.99 *	29.95 ±0.35 b;B;*	30.00 ±0.85 B;*
		aGI	31.50 ±7.21 *	36.33 ±0.67 *	32.40 ±8.49 a;*	27.50 ±1.70 *
	Fermented Milk (FM)	bGI	29.93 ±0.11 *	33.30 ±0.64 *	28.48 ±0.25 B;*	33.30 ±0.78 AB;*
		aGI	25.93 ±1.31 *	31.05 ±0.28 *	28.23 ±1.10 *	28.33 ±0.46 *
	Stored Fermented Milk (SFM)	bGI	29.93 ±0.39 *	29.90 ±0.49 *	38.23 ±2.51 A;*	38.20 ±1.70 a;A;*
		aGI	31.40 ±1.48 *	29.23 ±0.88 *	32.73 ±0.39 *	31.00 ±1.41 b;*
PEN	Inoculated Milk (IM)	bGI	31.75 ±2.05 B;*	36.40 ±0.78 *	30.30 ±2.69 *	29.25 ±1.20 a;*
		aGI	35.15 ±0.21 *	33.80 ±1.48 *	28.20 ±0.28 B;*	23.10 ±3.25 b;C;*
	Fermented Milk (FM)	bGI	32.70 ±0.35 AB;*	37.77 ±1.16 *	31.80 ±2.40 *	33.30 ±0.49 *
		aGI	34.40 ±0.64 *	33.75 ±1.27 *	27.08 ±0.67 B;*	28.70 ±10.25 B;*
	Stored Fermented Milk (SFM)	bGI	37.18 ±1.59 A;*	31.43 ±1.10 *	31.38 ±0.53 b;*	32.00 ±1.41 b;*
		aGI	32.95 ±2.19 *	34.23 ±1.10 *	47.95 ±2.05 a;A;*	43.60 ±1.84 a;A;*
VAN	Inoculated Milk (IM)	bGI	***	***	***	***
		aGI	***	***	***	***
	Fermented Milk (FM)	bGI	***	***	***	***
		aGI	***	***	***	***
	Stored Fermented Milk (SFM)	bGI	***	***	***	***
		aGI	***	***	***	***

Different capital letters in the column indicate significant difference amount aGI (inoculated milk, and fermented milk, and stored fermented milk) or bGI (inoculated milk, and fermented milk, and stored fermented milk); for only one lactobacilli strain and only one antibiotic. Different lower-case letters in the column indicate significant difference between inoculated milk (aGI and bGI), or fermented milk (aGI and bGI), or stored fermented milk (aGI and bGI); for only one lactobacilli strain and only one antibiotic. All by Fischer's test at 0.95 of reliability. Asterisks compare the results with the susceptibility level (*** Resistant; ** Intermediate; * Sensible) proposed by Vlkovà et al. (2006). (aGI) after (bGI) before *in vitro* gastrointestinal condition. Disc diameter = 6mm. AMC, amoxicillin + clavulanic acid; AMP, ampicillin; PEN, penicillin, VAN, vancomycin.

Table 3.6 - Antibiotics that act by inhibiting protein synthesis or nucleic acid synthesis: Induced Susceptibility on *Lactobacillus paracasei* due to processing conditions and after stress condition that mimic gastrointestinal transit (mean and Standard deviation).

Antibiotic agent	Matrice Condition		DTA-81		DTA-83		DTA-92		DTA-97	
CIP	Inoculated Milk (IM)	bGI	13.20 ±1.56	***	19.25 ±0.35	A;**	17.95 ±1.20	a;B;**	18.50 ±4.24	a;B;**
		aGI	15.35 ±4.31	***	17.63 ±0.88	A;**	11.95 ±0.78	b;B;***	13.50 ±4.10	b;B;***
	Fermented Milk (FM)	bGI	13.25 ±0.85	***	17.48 ±1.45	AB;**	20.30 ±0.35	a;B;**	19.60 ±1.06	a;B;**
		aGI	16.50 ±1.34	***	14.13 ±0.18	A;***	11.70 ±0.92	b;B;***	16.78 ±1.73	b;B;***
	Stored Fermented Milk (SFM)	bGI	11.48 ±1.10	***	12.05 ±0.21	B;***	30.03 ±0.32	A;*	26.23 ±0.39	a;A;*
		aGI	10.60 ±1.06	***	10.63 ±0.53	B;***	28.33 ±0.95	A;*	22.58 ±1.03	b;A;**
DOX	Inoculated Milk (IM)	bGI	33.10 ±3.25	B;*	37.98 ±0.74	A;*	33.60 ±2.69	b;B;*	33.00 ±0.42	B;*
		aGI	29.25 ±2.90	B;*	36.50 ±0.71	*	40.03 ±0.81	a;B;*	31.55 ±5.87	B;*
	Fermented Milk (FM)	bGI	33.30 ±0.78	B;*	33.90 ±2.48	AB;*	29.60 ±0.21	b;B;*	29.60 ±1.77	B;*
		aGI	30.05 ±4.10	B;*	35.05 ±2.55	*	38.48 ±0.67	a;B;*	31.75 ±1.56	B;*
	Stored Fermented Milk (SFM)	bGI	38.68 ±0.60	A;*	32.15 ±0.42	b;B;*	40.68 ±0.95	b;A;*	39.05 ±0.42	A;*
		aGI	41.68 ±0.46	A;*	38.95 ±1.13	a;*	47.00 ±1.84	a;A;*	38.53 ±1.24	A;*
GEN	Inoculated Milk (IM)	bGI	10.00 ±2.12	***	15.13 ±0.18	***	7.70 ±2.26	***	12.25 ±3.04	***
		aGI	8.35 ±2.62	B;***	14.13 ±0.18	***	8.40 ±1.70	***	9.25 ±4.45	***
	Fermented Milk (FM)	bGI	10.03 ±0.61	***	13.13 ±0.18	***	6.75 ±1.06	***	13.73 ±1.80	***
		aGI	11.13 ±0.18	B;***	13.43 ±2.02	***	6.00 ±0.00	***	12.60 ±0.35	***
	Stored Fermented Milk (SFM)	bGI	6.00 ±0.00	***	11.63 ±0.81	***	6.00 ±0.00	***	6.00 ±0.00	***
		aGI	15.88 ±4.99	A;***	11.23 ±2.58	***	8.00 ±2.83	***	6.00 ±0.00	***
ERI	Inoculated Milk (IM)	bGI	35.20 ±1.13	B;*	41.25 ±0.35	*	33.45 ±2.90	B;*	34.30 ±2.97	B;*
		aGI	33.25 ±1.77	B;*	42.70 ±0.78	A;*	36.55 ±2.19	B;*	37.25 ±1.77	B;*
	Fermented Milk (FM)	bGI	36.73 ±1.52	B;*	39.33 ±0.46	*	31.20 ±1.77	B;*	35.55 ±2.90	AB;*
		aGI	32.55 ±0.57	B;*	38.23 ±0.32	AB;*	34.08 ±2.09	B;*	37.88 ±0.04	B;*
	Stored Fermented Milk (SFM)	bGI	50.20 ±2.62	a;A;*	36.83 ±2.09	*	40.78 ±0.32	b;A;*	40.90 ±0.49	b;A;*
		aGI	39.63 ±1.24	b;A;*	32.88 ±2.30	B;*	47.08 ±3.85	a;A;*	48.95 ±0.07	a;A;*
CLO	Inoculated Milk (IM)	bGI	31.90 ±0.57	*	35.48 ±0.18	*	29.50 ±4.10	B;*	30.00 ±2.26	b;B;*
		aGI	33.50 ±0.14	*	37.35 ±3.54	A;*	30.80 #####	B;*	37.95 ±0.35	a;*
	Fermented Milk (FM)	bGI	31.65 ±1.70	*	31.08 ±3.43	*	30.45 ±0.85	B;*	31.38 ±1.59	b;B;*
		aGI	30.05 ±0.14	*	35.85 ±5.66	AB;*	34.03 ±2.30	B;*	36.95 ±0.00	a;*
	Stored Fermented Milk (SFM)	bGI	34.75 ±1.63	*	31.05 ±0.49	*	39.20 ±0.28	A;*	38.95 ±0.42	A;*
		aGI	30.25 ±1.06	*	29.00 ±0.00	B;*	41.90 ±0.99	A;*	36.75 ±0.35	*
TET	Inoculated Milk (IM)	bGI	34.65 ±4.31	B;*	44.63 ±0.88	*	43.45 ±1.63	a;A;*	32.00 ±1.13	*
		aGI	31.53 ±1.45	*	44.10 ±1.27	A;*	36.65 ±2.76	b;*	36.15 ±1.63	*
	Fermented Milk (FM)	bGI	34.74 ±1.19	B;*	44.68 ±3.08	*	41.23 ±1.31	AB;*	30.43 ±0.81	b;*
		aGI	33.90 ±1.34	*	43.90 ±0.14	A;*	38.25 ±2.26	*	36.63 ±0.04	a;*
	Stored Fermented Milk (SFM)	bGI	39.00 ±1.34	a;A;*	42.50 ±0.71	*	35.98 ±5.62	B;*	30.28 ±0.39	b;*
		aGI	32.03 ±0.11	b;*	39.13 ±1.59	B;*	35.00 ±0.00	*	37.53 ±0.74	a;*
KAN	Inoculated Milk (IM)	bGI	***		***		***		***	
		aGI	***		***		***		***	
	Fermented Milk (FM)	bGI	***		***		***		***	
		aGI	***		***		***		***	
	Stored Fermented Milk (SFM)	bGI	***		***		***		***	
		aGI	***		***		***		***	
NAL	Inoculated Milk (IM)	bGI	***		***		***		***	
		aGI	***		***		***		***	
	Fermented Milk (FM)	bGI	***		***		***		***	
		aGI	***		***		***		***	
	Stored Fermented Milk (SFM)	bGI	***		***		***		***	
		aGI	***		***		***		***	

Different capital letters in the column indicate significant difference amount aGI (inoculated milk, and fermented milk, and stored fermented milk) or bGI (inoculated milk, and fermented milk, and stored fermented milk); for only one lactobacilli strain and only one antibiotic. Different lower-case letters in the column indicate significant difference between inoculated milk (aGI and bGI), or fermented milk (aGI and bGI), or stored fermented milk (aGI and bGI); for only one lactobacilli strain and only one antibiotic. All by Fischer's test at 0.95 of reliability. Asterisks compare the results with the susceptibility level (*** Resistant; ** Intermediate; * Sensible) proposed by Vlkovà et al. (2006). (aGI) after (bGI) before *in vitro* gastrointestinal condition. Disc diameter = 6mm. CIP, ciprofloxacin; DOX, doxacycline; GEN, gentamicin; ERI, erythromycin; CLO, chloramphenicol; TET, tetracycline; KAN, kanamycin; NAL, nalidixic acid.

L. paracasei DTA 81 increased its PEN (bGI), DOX, GEN (aGI), ERI, TET (bGI) susceptibility due to stress caused by fermentation metabolites. Stress due to GIC did not changes any susceptibility profile to cell-wall acting antibiotic but increased its ERI and TET susceptibility only SFM. There was no AMC, AMP, VAN, CIP, CLO, KAN, NAL changing susceptibility.

L. paracasei DTA 83 did not modify its susceptibility to cell-wall acting antibiotic due the stress caused either by fermentation metabolites or GIC. However, it reduced its susceptibility to DNA-acting antibiotics (CIP, DOX (bGI), ERI (aGI), CLO (aGI), TET (aGI)).

L. paracasei DTA 92 increased its AMC, AMP (bGI), PEN (aGI), CIP, DOX, ERI susceptibility and reduced its TET (bGI) susceptibility due to the stress caused by fermentation metabolites. Stress due to GIC increased its AMP (IM), PEN (SFM), DOX (IM, FM; SFM), ERI (SFM) susceptibility and reduced its AMC, IM, FM, (IM) susceptibility.

L. paracasei DTA 97 increased its AMC, AMP (bGI), PEN (aGI), CIP, DOX, ERI, CLO (bGI) susceptibility due to the stress caused by fermentation metabolites. Stress due to GIC increased its PEN (SFM), ERI (SFM), CLO (IM, FM), TET (IM; FM) susceptibility and reduced its AMC (FM), AMP, PEN susceptibility.

Antibiotic susceptibility profile can be changed during the storage of fermented milk. *L. paracasei* DTA 97 increased its PEN (IM) resistance after GIC but reduced it after 45 of storage (SFM).

Antibiotic susceptibility level proposed by Vlkovà et al. (2006), *L. paracasei* DTA 83 changed its CIP (bGI) susceptibility from intermediate to resistant after both in the FM and SFM. *L. paracasei* DTA 92 changed its CIP susceptibility from intermediate to resistant after GIC in IM and FM. *L. paracasei* DTA 97 changed its CIP susceptibility from intermediate to resistant in IM and FM after GIC, and from sensitive to intermediate in SFM (Tables 3.5 and 3.6)

The Tables 3.7 and 3.8 show the induced susceptibility on *L. fermentum* due to fermentation process, storage time, and stress condition that mimic gastrointestinal transit.

Table 3.7 - Antibiotics that act by inhibiting or interfering with cell wall synthesis a or by altering the permeability of the cell membrane: induced susceptibility on *Lactobacillus fermentum* due to processing condition and after stress condition that mimic gastrointestinal transit (mean and Standard deviation).

Antibiotic agent	Matrice Condition		DTA-104		DTA-106	
AMC	Inoculated Milk (IM)	bGI	43.25 ±1.34	*	34.80 ±0.71	a;B;*
		aGI	42.78 ±0.74	B;*	26.93 ±1.03	b;B;*
	Fermented Milk (FM)	bGI	39.95 ±0.42	*	33.38 ±1.38	a;B;*
		aGI	41.50 ±0.71	B;*	24.48 ±1.45	b;B;*
	Stored Fermented Milk (SFM)	bGI	42.90 ±0.07	*	38.98 ±3.15	A;*
		aGI	46.20 ±3.11	A;*	38.55 ±0.78	A;*
AMP	Inoculated Milk (IM)	bGI	32.50 ±8.77	*	32.35 ±2.76	a;B;*
		aGI	33.60 ±7.21	*	41.83 ±1.80	b;A;*
	Fermented Milk (FM)	bGI	35.40 ±3.46	*	30.93 ±1.87	B;*
		aGI	35.45 ±3.11	*	30.93 ±1.31	B;*
	Stored Fermented Milk (SFM)	bGI	31.30 ±0.92	*	38.80 ±1.41	a;A;*
		aGI	32.10 ±0.92	*	28.98 ±0.04	b;B;*
PEN	Inoculated Milk (IM)	bGI	27.35 ±1.77	b;B;*	30.75 ±0.49	a;*
		aGI	36.60 ±1.98	a;*	19.40 ±2.12	b;B;*
	Fermented Milk (FM)	bGI	27.20 ±1.06	B;*	29.23 ±0.81	a;*
		aGI	29.88 ±4.07	*	19.28 ±0.88	b;B;*
	Stored Fermented Milk (SFM)	bGI	37.40 ±2.83	A;*	31.25 ±1.70	*
		aGI	33.83 ±2.23	*	32.25 ±0.99	A;*
VAN	Inoculated Milk (IM)	bGI	***		***	
		aGI	***		***	
	Fermented Milk (FM)	bGI	***		***	
		aGI	***		***	
	Stored Fermented Milk (SFM)	bGI	***		***	
		aGI	***		***	

Different capital letters in the column indicate significant difference amount aGI (inoculated milk, and fermented milk, and stored fermented milk) or bGI (inoculated milk, and fermented milk, and stored fermented milk); for only one lactobacilli strain and only one antibiotic. Different lower-case letters in the column indicate significant difference between inoculated milk (aGI and bGI), or fermented milk (aGI and bGI), or stored fermented milk (aGI and bGI); for only one lactobacilli strain and only one antibiotic. All by Fischer's test at 0.95 of reliability. Asterisks compare the results with the susceptibility level (*** Resistant; ** Intermediate; * Sensible) proposed by Vlkovà et al. (2006). (aGI) after (bGI) before *in vitro* gastrointestinal condition. Disc diameter = 6mm. AMC, amoxicillin + clavulanic acid; AMP, ampicillin; PEN, penicillin, VAN, vancomycin.

Table 3.8 - Antibiotics that act by inhibiting protein synthesis or nucleic acid synthesis: Induced Susceptibility on *Lactobacillus fermentum* due to processing conditions and after stress condition that mimic gastrointestinal transit (mean and Standard deviation).

Antibiotic agent	Matrice Condition		DTA-104		DTA-106	
CIP	Inoculated Milk (IM)	bGI	13.85 ±2.33	***	23.35 ±0.35	b;*
		aGI	13.50 ±4.10	***	29.58 ±0.88	a;*
	Fermented Milk (FM)	bGI	13.45 ±1.56	***	26.40 ±0.78	*
		aGI	13.80 ±1.63	***	28.40 ±1.34	*
	Stored Fermented Milk (SFM)	bGI	12.80 ±2.13	***	26.14 ±2.45	*
		aGI	13.00 ±2.08	***	27.65 ±0.47	*
DOX	Inoculated Milk (IM)	bGI	32.55 ±4.60	B;*	33.75 ±3.32	B;*
		aGI	30.88 ±1.80	B;*	27.53 ±1.45	B;*
	Fermented Milk (FM)	bGI	34.80 ±5.87	B;*	33.58 ±2.93	B;*
		aGI	32.68 ±1.66	B;*	28.23 ±0.39	B;*
	Stored Fermented Milk (SFM)	bGI	49.28 ±0.88	a;A;*	43.08 ±1.31	A;*
		aGI	42.00 ±0.07	b;A;*	42.45 ±1.56	A;*
GEN	Inoculated Milk (IM)	bGI	8.90 ±0.14	B;***	6.05 ±0.07	B;***
		aGI	10.80 ±0.42	B;***	6.25 ±0.21	B;***
	Fermented Milk (FM)	bGI	11.83 ±1.17	B;***	6.00 ±0.00	***
		aGI	11.50 ±1.63	B;***	6.00 ±0.00	***
	Stored Fermented Milk (SFM)	bGI	20.13 ±0.67	A;**	11.15 ±0.57	A;***
		aGI	15.98 ±2.02	A;***	9.95 ±0.21	A;***
ERI	Inoculated Milk (IM)	bGI	32.45 ±4.45	B;*	37.50 ±1.56	B;*
		aGI	31.80 ±9.05	B;*	33.83 ±2.30	B;*
	Fermented Milk (FM)	bGI	28.75 ±1.20	B;*	33.20 ±6.01	B;*
		aGI	30.25 ±1.84	B;*	32.45 ±2.12	B;*
	Stored Fermented Milk (SFM)	bGI	49.15 ±0.35	A;*	49.80 ±1.56	a;A;*
		aGI	49.23 ±0.39	A;*	41.50 ±1.34	b;A;*
CLO	Inoculated Milk (IM)	bGI	37.95 ±0.35	A;*	31.95 ±1.91	*
		aGI	33.13 ±3.50	*	29.83 ±1.03	*
	Fermented Milk (FM)	bGI	37.50 ±0.71	AB;*	30.88 ±3.01	*
		aGI	35.40 ±2.19	*	28.83 ±1.17	*
	Stored Fermented Milk (SFM)	bGI	32.20 ±0.35	*	33.05 ±0.57	*
		aGI	32.03 ±0.04	B;*	34.58 ±0.95	*
TET	Inoculated Milk (IM)	bGI	38.65 ±6.43	a;*	32.75 ±0.78	a;B;*
		aGI	33.10 ±2.97	b;AB;*	22.98 ±1.03	b;B;*
	Fermented Milk (FM)	bGI	40.68 ±3.22	a;*	32.13 ±1.03	a;B;*
		aGI	32.63 ±3.08	b;B;*	20.90 ±0.78	b;B;*
	Stored Fermented Milk (SFM)	bGI	42.73 ±1.66	a;*	42.23 ±1.52	a;A;*
		aGI	37.00 ±2.47	b;A;*	32.93 ±1.03	b;A;*
KAN	Inoculated Milk (IM)	bGI	***		***	
		aGI	***		***	
	Fermented Milk (FM)	bGI	***		***	
		aGI	***		***	
	Stored Fermented Milk (SFM)	bGI	***		***	
		aGI	***		***	
NAL	Inoculated Milk (IM)	bGI	***		***	
		aGI	***		***	
	Fermented Milk (FM)	bGI	***		***	
		aGI	***		***	
	Stored Fermented Milk (SFM)	bGI	***		***	
		aGI	***		***	

Different capital letters in the column indicate significant difference amount aGI (inoculated milk, and fermented milk, and stored fermented milk) or bGI (inoculated milk, and fermented milk, and stored fermented milk); for only one lactobacilli strain and only one antibiotic. Different lower-case letters in the column indicate significant difference between inoculated milk (aGI and bGI), or fermented milk (aGI and bGI), or stored fermented milk (aGI and bGI); for only one lactobacilli strain and only one antibiotic. All by Fischer's test at 0.95 of reliability. Asterisks compare the results with the susceptibility level (*** Resistant; ** Intermediate; * Sensible) proposed by Vlkovà et al. (2006). (aGI) after (bGI) before *in vitro* gastrointestinal condition. Disc diameter = 6mm. CIP, ciprofloxacin; DOX, doxacycline; GEN, gentamicin; ERI, erythromycin; CLO, chloramphenicol; TET, tetracycline; KAN, kanamycin; NAL, nalidixic acid.

L. fermentum DTA 104 increased its AMC (aGI), PEN (bGI), DOX, GEN, ERI, TET susceptibility and reduced its CLO (bGI) susceptibility due to stress caused by fermentation metabolites. Stress due to GIC increased its PEN (IM) and reduced its DOX (SFM) and TET (IM; FM; SFM) susceptibility. There was no AMP, PEN, KAN, NAL changing susceptibility.

L. fermentum DTA 106 increased its AMC, AMP (bGI), PEN (aGI), DOX, GEN, ERI TET susceptibility and reduced its AMP (aGI) susceptibility. Stress due to GIC increased its CIP (IM) and AMP (IM) susceptibility and reduced its AMC (IM; FM), PEN (IM; FM), ERI (SFM), TET (IM; FM; SFM) susceptibility. There was no VAN, KAN, and NAL changing susceptibility. There was also no changing at the susceptibility level proposed by Vlkovà et al. (2006) (Tables 3.7 and 3.8).

Stress-induced by metabolites of milk fermentation caused significant changes in antibiotic susceptibility than GIC. Metabolites carry on 50,5 % of significant changes, against 18 % of GIC changes. However, most of significant changes due to metabolites of milk fermentation make lactobacilli more susceptible (34,3 %) to antibiotic agents. On the contrary, GIC make lactobacilli less susceptible (11,7 %). Statistically significant-changes ($P < 0.05$) in antibiotic susceptibility occurred due to stress-induced either by fermented metabolites and GIC (Figures 3.6 and 3.7).

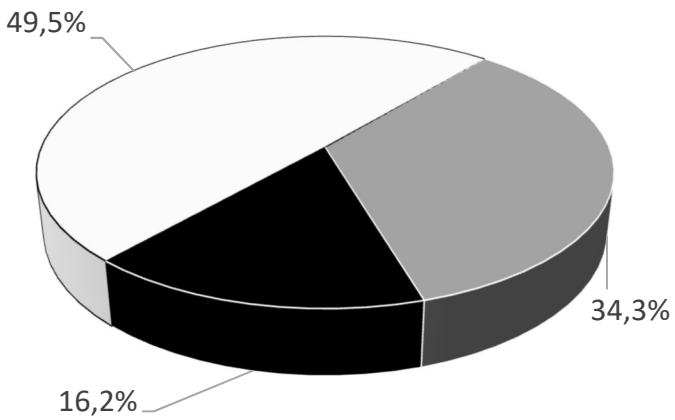

Figure 3.6 – Percentage of statistically significant-changes in the level of antibiotic susceptibility of *Lactobacillus* due to stress-induced by fermentation metabolites during the storage (7 °C). Fischer's test at 0.95 of confidence.
Increase antibiotic susceptibility level (■), reduction antibiotic susceptibility level (■), no significance (□).

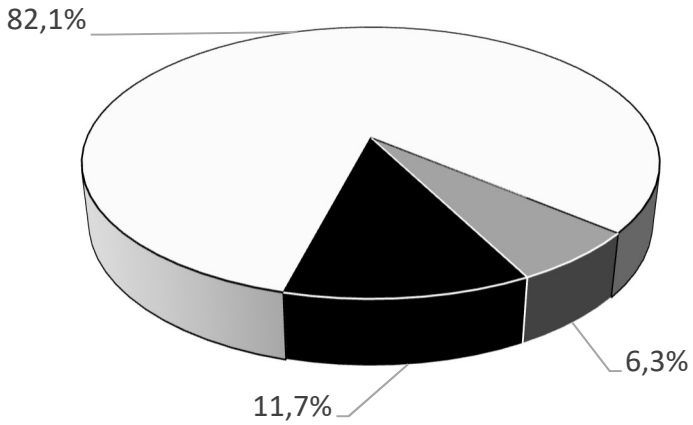

Figure 3.7 – Percentage of statistically significant-changes in the level of antibiotic susceptibility of *Lactobacillus* due to stress-induced by *in vitro* gastrointestinal conditions (B). Fischer's test at 0.95 of confidence.
Increase antibiotic susceptibility level (■), reduction antibiotic susceptibility level (■), no significance (□).

During selection of probiotic microorganisms is desirable to choose strains with high survivability levels during passage through gut conditions. Another point of view, these probiotic microorganisms can be a reservoir of resistance genes and transfer them to pathogenic microorganisms (SOMMER et al., 2017). Although the resistance genes seem to be correlated mainly with phenotypic resistance, some molecular resistance was also reported (DEC et al., 2017).

Peptidoglycan consists of glycan chains made of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) that are linked via β-1,4 bonds is the main component of the Gram-positive cell wall. In Lactic acid bacteria, the amino acid sequence of the peptide is L-Ala-γ-D-Glu-X-D-Ala, while the third amino acid (X) is a di-amino acid. Often L-Lys (e.g., in *L. lactis* and most lactobacilli) but can also be meso-diaminopimelic acid (mDAP) (e.g., in *L. plantarum*) or L-ornithine (e.g., in *L. fermentum*). Vancomycin-resistant lactobacilli is due presence of D-lac residues. Cross-linking between neighboring stem peptides takes place between the D-Ala in position four of one peptide chain and the diamine acid in position three (4-3 cross-link) of another chain. A direct cross-connection is seen in mDAP-type PG, which is typically found in Gram-negative bacteria, but which is also present in *L. plantarum*. Others lactic acid bacteria, the Lys-type PG is found and includes an interpeptide bridge made of one D-amino acid (e.g., D-Asp or D-Asn in *L. lactis, L. casei*, and most lactobacilli) or several L-amino acids (e.g., $L\text{-}Ala_2$ or $L\text{-}Ala_3$ in *Streptococcus thermophilus*) (CHAPOT-CHARTIER, 2014).

Beyond of three well-known antibiotic resistance mechanisms such as transformation, transduction and conjugation, another bacterial gene transfer mechanism also may occur by extracellular vesicles. With bacterial death, several membranes fragments may contain all sorts of molecules, such as proteins and DNA (ROBBINS & MORELLI, 2014; DOMINGUES & NIELSEN, 2017). They may contain DNA encoding antibiotic resistance. Other bacteria can absorb them and become antibiotic resistant. Probiotic strain can carry on resistance gene to gut biome just by death during the fermentation step.

Jiang et al. (2017) described a new model showing how bacteria acquire antibiotic resistance and it is originated from the microorganism that produces the antibiotic. Antibiotic resistance genes originate from the same place as the antibiotic compounds, i.e. from a group

of soil bacteria called Actinobacteria. Gram-negative pathogen injects its DNA into the Actinobacteria by conjugation. This make they exchanging gene information. But sometimes Gram-negative bacteria can also use this mechanism to inject DNA into unrelated Gram-positive bacteria like Actinobacteria. If these bacteria containing resistance genes, after the bacterial dies, the recombinant DNA is released into the environment. Then, this recombinant DNA is taken for another bacterium conferring resistance to it. This mechanism was called by the authors as "carry back". When probiotic microorganisms are ministered to improve gut health, an inevitably contact happens between probiotics and Gram-negative bacteria in the gut microbiome. From this contact, an antibiotic resistance gene manufacturer can be born. This is another important property to be checked when new probiotic strains are found.

Hydrogen peroxide and acid-stressful produced by lactobacilli during milk fermentation can changing of cell membrane metabolism (HUANG et al., 2016). Over milk storage, there are increase of susceptibility level because these substances are gradually making injury on the cell wall. Another negative effect of hydrogen peroxide is related with injury on gut epithelium (XU et al., 2016). However, some authors reported that lactic acid and hydrogen peroxide-producing by lactobacilli strain may contribute to the maintenance of a normal and homeostatic microbiota, especially vaginal microbiota (TACHEDJIAN et al., 2017; WANG et al., 2017).

Changes were observed in antibiotic susceptibility of lactobacilli due to SFM and/or CGI. These results show the need for further studies to ensure that probiotic cultures are antibiotic susceptible, and more attention should be done when selecting probiotic strains for dairy processes. Despite the indisputable beneficial effects of the probiotics, they can carry transferable antibiotic resistance genes to pathogenic or commensal species (ABRIOUEL et al., 2015).

SHARMA et al. (2016) researched antibiotic susceptibility using disc diffusion method of 45 antibiotics against 30 lactobacilli strains (*Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus plantarum,* and *Lactobacillus fermentum)*. Most of the isolates were found to exhibit multiple resistance against some of the most commonly used antibiotics. The isolates showed elevated resistance level toward nalidixic acid, vancomycin, kanamycin, teicoplanin, co-trimoxazole, amikacin, streptomycin, norfloxacin, cefepime and nitrofurantoin. Besides, isolates displayed a low level of resistance toward tobramycin, gentamicin, ampicillin, cefaclor, methicillin, penicillin, tetracycline, levofloxacin, azithromycin, chloramphenicol, amoxyclav, sulbactam, oxacillin, ofloxacin, ciprofloxacin, cloxacillin and novobiocin. All the isolates were found to be susceptible against cefatrixone, ceftazidime, cefadroxil, cefotaxime, cephalothin, cefoperazone and netillin, whereas none of the strains showed resistant to clindamycin, erythromycin, linezolid, quinupristin/dalfopristin and doxycycline.

4 CONCLUSIONS

- Stressful substances from either fermented milk or GIC can lead changing on antibiotic susceptibilityof lactobacilli,

- Most of lactobacilli have increased resistance after passage through gastrointestinal condition

- Despite of indisputable health-beneficial effects of probiotic microorganisms, further studies are needed to clarify the possibility of horizontal gene transfer to gut pathogenic or commensal species.

- More careful research is needed when studying the susceptibility of probiotic strains to antibiotics related to gastrointestinal conditions

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CHAPTER IV
BIO-ACTIVITY, SAFETY, AND TECHNOLOGICAL PROPERTIES OF NOVEL *LACTOBACILLUS*: APPLICATION IN FUNCTIONAL PRODUCTS

ABSTRACT

The aim of this research was to evaluate the functional, safety, and technological properties of novel lactobacilli strains from human origin. *Lactobacillus rhamnosus* DTA 79 and *Lactobacillus paracasei* DTA 83 from infant's stool aged up to two weeks, were evaluated for functional (resistance to lysozyme and pathogen biofilm inhibition,) and safety (antibiotic susceptibility and blood hemolysis) features either before and after *in vitro* gastrointestinal condition (GIC). Lactobacilli technological potential was assessed by milk acidification profile in axenic growth and co-cultured with *Streptococcus thermophilus* (TH 895 or TH 1435), milk coagulation kinetics properties, microbial survivability in ice cream bar and ice-lolly and in probiotic tablets. Both lactobacilli strains inhibited *Escherichia coli*, *Salmonella* Typhimurium and *Candida albicans* biofilms by competition and exclusion mechanisms, but by displacement mechanism they were only able to inhibit *E. coli* and *C. albicans*. All lactobacilli strains were resistant up to 3000 μg/ml of egg white lysozyme, were not alfa or gamma-hemolytic and they were susceptible to 6 antibiotic agents for human use. Stress due to GIC did not affect any these probiotic properties. Microbial survivability in ice cream bar and ice-lolly was assessed for up 21 days of frozen storage (-18 °C). Viability was maintained in ice cream bar, but there was a reduction of almost 2.0 logs in ice-lolly. There was a mutualistic type association between *L. rhamnonus* DTA-79 and *S. thermophilus* TH 895 speeding up the milk acidification to pH 5.2, showing potentiality for the starter-cultures development. Fermented milk added with honey bee (5 %) provides protection to *L. rhamnosus* DTA 79 survivability to GIC, against *L. paracasei* DTA 83 did not need any protector factor to survive the GIC. Therefore, *L. rhamnosus* DTA 79 and *L. paracasei* DTA 83 are safe strains for human consumption and have some probiotic properties. In addition, they have potential to acidify milk and honey provide protection to lactobacilli survivability during GIC. Fermented milk and ice cream bar are a suitable matrix to delivery both lactobacilli strains ice-lolly is an unsuitable frozen-dessert to vehicle the *L. paracasei* DTA 83. Probiotic tablets have potential to reduce oral candidiasis in elderly, they are effective *in vitro* tests, but *in vivo* studies shall be further performed.

Key words - specificity, application, functional food, probiotic strain

RESUMO

O objetivo desta pesquisa foi avaliar as propriedades tecnológicas, funcionais e de segurança de novas cepas de lactobacilos isolados de origem humana intencionadas para o consumo humano. *Lactobacillus rhamnosus* DTA 79 e *Lactobacillus paracasei* DTA 83 isolados de material fecal de crianças de até duas semanas de idade, foram avaliados quanto propriedades probióticas funcionais (resistência à lisozima e inibição de biofilmes de microrganismos patogênicos) a de segurança (susceptibilidade à antibióticos e hemólise) antes e após condições gastrointestinais *in vitro* (CGI). O potencial tecnológico das cepas de lactobacilos foi avaliado através do perfil de acidificação do leite em crescimento axênico ou co-cultivado com *Streptococcus thermophilus* (TH 895 ou TH 1435), cinética da coagulação do leite, sobrevivência em sorbet, sherbet e em pastilhas probióticas. A viabilidade foi mantida em sherbet, mas ocorreu redução de quase 2 logs em sorbet. *L. rhamnosus* DTA 79 e *L. paracasei* DTA 83 reduziram a formação de biofilmes de *Escherichia coli, Salmonella* Typhimurium e *Candida albicans* pelos mecanismos de competição e exclusão, mas somente desacoplaram E. *coli* e *C. albicans*. As cepas de lactobacilos foram resistentes até 3000 μg/mL de lisozima de ovo, não apresentou hemólise alfa ou beta e foram susceptíveis à 6 antibióticos para uso

humano. O estresse devido CGI não afetou nenhuma propriedade probiótica das cepas. A associação entre as cepas *L, rhamnosus* DTA 79 e *S. thermophilus* TH 895 foi do tipo mutualística, mostrando potencial possibilidade para desenvolvimento de culturas *starter*. Leite fermentado adicionado de mel de abelha (5 %) forneceu proteção para *L. rhamnosus* DTA 79 sobreviver ao CGI, ao contrário *L. paracasei* DTA 83 não precisou de qualquer fator de proteção para sobreviver ao CGI. Portanto, *L. rhamnosus* DTA 79 e *L. paracasei* DTA 83 são seguras para consumo humano e apresentaram algumas propriedades probióticas. Em adição, estas culturas têm potencial para acidificar o leite de vaca e mel de abelha pode ser utilizado como protetor à CGI. Leite fermentado e sherbet são matrizes adequadas para veicular ambas as cepas DTA 79 e DTA 83, em contrapartida não é aconselhável utilizar sorbet para veicular *L. paracasei* DTA 83. As pastilhas probióticas possuem potencial para reduzir a candidíase oral em pessoas na terceira idade, elas foram efetivas nos testes *in vitro*, mas estudos *in vivo* precisam ser conduzidos para comprovação da eficácia.

Palavras chave - especificidade, aplicação, alimento funcional, cepa probiótica

1 INTRODUCTION

Fermented foods have unique functional properties imparting some health benefits to consumers due to presence of functional microorganisms, which have probiotics properties, antimicrobial, antioxidant, peptide production, etc. Health benefits of some global fermented foods are synthesis of nutrients, prevention of cardiovascular disease, prevention of cancer, gastrointestinal disorders, allergic reactions, diabetes, immune system stimulation; reduction of serum cholesterol levels; production of important enzymes; increased availability of free amino acids and vitamins, especially riboflavin, niacin, thiamine, vitamin B6, vitamin B12 and folic acid. Moreover, the probiotics can increase the availability of lactase, fatty acids, absorption of minerals; decreased the lactose intolerance and colon cancer; and potentiated of the immune response among other (TAMANG et al., 2016; LEBLANC et al., 2017).

Selected probiotic and symbiotic dairy products may be used in the nearest future to prevent a variety of health disorders. Current studies suggest that lactic acid bacteria may exert anti-obesity and anti-diabetic effects in the hosts and thus can play a crucial role in human health care. At the same time, research in the rheological and physicochemical properties of ice cream as well as its applications are also on the increase. These applications face certain hurdles including technological, as well as consumer acceptability of new functional foods may be influenced by culture, ethics or religion (EVIVIE et al., 2017).

When harvesting the best strains to new probiotic food, the main beneficial criteria sought in probiotic strains are: resistant to gastrointestinal, antimicrobial activity, adherence in intestinal mucosa, absence of pathogenicity and infectivity history, bile salts metabolic activity, absence of haemolytic activity and of the genes that transmit resistance to antimicrobials, reduction of biofilm formation by potential pathogenic microorganisms, resistance to lysozyme, and not cause "off-flavour" in food (SHEWALE et al., 2014; THAKUR et al., 2016). Furthermore, the interaction between probiotics and food should be also considered in the selection of *compatible* strains. For example, BLAIOTTA et al. (2017) found good interaction between two probiotic lactobacilli strains (*L. rhamnosus* LbGG and SP1) with cheese samples. They show a remarkable tolerance to acid-gastric and duodenal stresses, for up to 40 days of ripening at 4 °C, keeping a viability higher than 10^8 cfu/g. Moreover, the odor of the cheese enriched with these two probiotic strains appeared more acidic, creamy, buttery, and characterized by a pleasant matrix deconstruction, resulting in a *Stracchino*-like product.

Sometimes, cultures isolated of dairy products have properties different from that found in commercial starter culture. Han et al. (2016), studied 19 high exopolysaccharide producing strains of *Streptococcus thermophilus* which were isolated from traditional Chinese fermented dairy products. *S. thermophilus* zlw TM11 (indigenous strain) was combined with *Lactobacillus delbrueckii* subsp. *bulgaricus*. The exopolysaccharides of these fermented yogurt were equal or better than that of two commercial starter cultures. In addition, the yogurt fermented with indigenous strain had the lowest syneresis (8.5%) and better texture and sensory attributes than that one made with two commercial starter cultures.

1.1 Selection of Probiotic Strains

Starter cultures must be carefully selected, since the ability of microbial cultures to produce bioactive metabolites is generally a strain-dependent trait and varies considerably among strains within the same species. The yield of bioactives synthesis and the concentration of such compounds in dairy products is another critical strain-dependent factor. In this regard, the dose of bioactive ingested with the corresponding food product should remain over the minimum required to meet the human requirements and/or have the claimed therapeutic level on the consumer, according to existing clinical recommendations and studies. An open question

when using co-cultures or strain combinations is their interaction in terms of nutrient availability, bacterial growth, as well as the bioactive production yield. In some cases, metabolites (i.e., vitamins etc.) produced by one of the strains could be consumed by the other strains, thus decreasing the final content in food (LINARES et al., 2017). The Table 4.1 shows main probiotic species applied as probiotic products.

Table 4.1 - Main probiotic species applied commercially in food and drug

Genera	species
Lactobacillus	*acidophillus* *johnsonii* *plantarum* *rhamonosus* *reuteri* *fermentum* *brevis* *lactis* *cellobiosus* *paracasei* *helveticus*
Bifidobacterium	*lactis* *pseudocatenulatus* *bifidus* *infantis* *longum* *thermophilus* *adolescentis*
Streptococcus	*intermedius* *salivarius* *cremoris* *lactis*
Aspergillus	*niger* *oryzae*
Leuconostoc	*mesenteroides*
Pediococcus	*acidilactici*
Enterococcus	*faesium*
Lactococcus	*lactis*
Saccharomyces	*boulardii*
Propionibacterium	*freudenreichii*

Source – Meybodi and Mortazaviam (2017).

Markova and Sheveleva (2014) reported about regulation of probiotic food products in various countries. Besides the strain-specificity effect, the minimum criteria used for probiotics in food products are: need to identify a probiotic at genus, species, and strain levels, using the high-resolution techniques; viability and the presence of a sufficient number in the product at the end of shelf life; proof of functional characteristics inherent to probiotic strains, in controlled experiments.

FAO/WHO recommended three-stage procedure to evaluate functional food efficiency that includes: safety assessment in *in vitro* and *in vivo* experiments (phase I); evaluation in the

Double-Blind, Randomized, Placebo-Controlled trial (phase II); and post-approval monitoring (phase III). It is noted that along with the ability to obtain statistically significant results of the evaluation, there are practical difficulties of conducting it due to duration, costs, difficulties in selection of target biomarkers and populations. The promising approach for assessing the functional efficacy is the concept of nutrigenomics. It examines the link between the human diet and the characteristics of his genome to determine the influence of food on the expression of genes and, ultimately, to human health. Nutrigenomic approaches are promising to assess the impact of probiotics in healthy people.

1.2 Interactions Between Probiotic Strains and Host

Probiotic microorganisms are increasingly incorporated into food matrices to confer proposed health benefits on the consumer. It is important that the health benefits, sensory properties, shelf-life and probiotic survival in the gastrointestinal tract of these products are carefully balanced as they determine functionality and drive consumer acceptance. Results indicate that carrier matrices have a significant impact on the quality of probiotic products. Matrix components, such as proteins, carbohydrates and flavoring agents are shown to alter probiotic efficacy and viability. However, only a limited number of studies have specifically addressed the effects of carrier matrices on the product-parameters; most studies seem to focus solely on the strain-specific effects of probiotic microorganisms (FLACH et al., 2017).

The Table 4.2 shows some applications of lactic acid bacteria strains

Table 4.2 – Some applications of lactic acid bacteria strains

LAB Strain(s)	**Applications**
Lactobacillus rhamnosus	Reduction of dental caries risk
Lactobacillus casei	Mitigation of Type II diabetes and obesity
Bifidobacterium longum SPM 1207 *Lactobacillus spp.*	Lowering of cholesterol and low-density lipoproteins
Lactobacillus spp.	Inhibition of Listeria monocytogenes and Clostridium spp.
Lactobacillus reuteri *Lactobacillus plantarum*	Antifungal agents
Lactobacillus casei *Lactobacillus plantarum*	Drug-delivery vehicles
Lactobacillus acidophilus *Lactobacillus salivarius* *Lactobacillus rhamnosus* *Lactobacillus brevis* *Lactobacillus casei*	Immune system modulation and mental health
Lactobacillus spp.	Reduction of mycotoxins in fermented maize products

Source - Mokoena et al. (2016)

Commercial probiotics products for human consumption are available in two main forms, including food products and drug supplements. In drug supplements, tablets or capsules, very high amounts of viable probiotic cells (about 10^{10} cfu/ml) are carried through the body, while in food products the cells are added into carrier foods or applied as starter probiotics in fermented foods. A series of complex interactions and interventions is carried out in food matrices that might adversely affect the viability of initially inoculated probiotics in food before

consumption. Therefore, the matrix and process engineering in food probiotic production is nowadays an advanced and developed science and technology. However, production and maintenance of probiotic in food products are considerably more difficult than the drug ones (NEYBODI and MORTAZAVIAM, 2017). Presently, dose levels are not well defined. It is apparent that, in some cases, the beneficial effect is derived from metabolites of the added bacteria or from the action of the added bacteria on the food matrix. Then, action does not depend on bacteria being alive when consumed (FARNWORTH & CHAMPAGNE, 2016). Analyzing these two points of view, supplements with probiotic strain despite of large number of viable cells, they have no interaction between probiotic and foods for the production of metabolites important for health. The Table 4.2 shows the main applications of lactic acid bacteria strains.

SANDERS et al. (2013) reported that advantage between probiotic and diseases will be achieved by optimizing strain, dose and product formulations, including protective commensal species; matching these formulations with selectively responsive subpopulations; and identifying ways to manipulate diet to modify bacterial profiles and metabolism. The Table 4.3 shows some strains of lactic acid bacteria, bifidobacteria and propionibacteria with potential to biosynthesize health-promoting compounds in fermented dairy products.

Table 4.3 - Some strains of lactic acid bacteria, bifidobacteria and propionibacteria with potential to biosynthesize health-promoting compounds in fermented dairy products (it continues)

Bioactive	Producer strain	Food Product	Health effect
Thiamine (B_1)/Riboflavin (B_2)	*Lactobacillus casei* KNE-1	Fermented milk	Vitamin enrichment
	Bifidobacterium infantis CCRC14633	Fermented soymilk	Vitamin enrichment
	Bifidobacterium longum B6	Fermented soymilk	Vitamin enrichment
	Lactobacillus plantarum CRL 2130	Fermented soymilk	Vitamin enrichment
Biotin (Vitamin B_7)	*Lactobacillus helveticus MTCC5463*	Fermented milk	Vitamin enrichment
Cobalamin (Vitamin B_{12})	*Propionibacterium freudenreichii*	Kefir	Vitamin enrichment
	Bifidobacterium animalis Bb12	Fermented milk	Vitamin enrichment
	Lactobacillus reuteri ZJ03	Soy-yogurt	Vitamin enrichment
Folic acid (Vitamin B_9)	*Streptococcus thermophilus* CRL803/CRL415	Yogurt	Vitamin enrichment

Bioactive	Producer strain	Food Product	Health effect
	Lactobacillus bulgaricus CRL871	Yogurt	Vitamin enrichment
	Bifidobacterium lactis CSCC5127	Fermented milk	Vitamin enrichment
	Bifidobacterium infantis CSCC5187	Fermented milk	Vitamin enrichment
	Bifidobacterium breve CSCC5181	Fermented milk	Vitamin enrichment
	Lactobacillus amylovorus CRL887	Fermented milk	Vitamin enrichment
GABA	*Lactobacillus casei* Shirota	Fermented milk	Antidiabetic, blood pressure
	Streptococcus salivarius fmb5	Fermented milk	Antidiabetic, blood pressure
	Lactobacillus plantarum NDC75017	Fermented milk	Antidiabetic, blood pressure
	Lactobacillus brevis OPY-1	Fermented soya milk	Antidiabetic, blood pressure
	Streptococcus thermophilus APC151	Yogurt	Antidiabetic, blood pressure
Bioactive peptides	*Lactobacillus helveticus* Evolus®	Fermented milk	Anti-hypertensive
	Lactobacillus helveticus/S. cerevisiae (Calpis™)	Fermented milk	Anti-hypertensive
	Lactobacillus bulgaricus LB340	Fermented milk/yogurt	Anti-hypertensive, Immunomodulatory
Bacteriocins	*Lactococcus lactis* CNRZ150/TAB50	Camembert/Semihard cheese	Pathogen inhibition
	Lactococcus lactis DPC3147	Cheddar cheese	Pathogen inhibition

Bioactive	Producer strain	Food Product	Health effect
	Lactobacillus acidophilus CH5	Yogurt	Pathogen inhibition
	Pediococcus acidilactici CHOOZIT™	Cheddar/Semihard cheese	Pathogen inhibition
	Lactobacillus plantarum WHE92	Munster cheese	Pathogen inhibition
Conjugated linoleic acid	*Lactococcus lactis* CI4b	Cheddar cheese	Cholesterol lowering
	Lactobacillus rhamnosus C14, *Lactobacillus casei* CRL431, *Streptococcus thermophilus* CRL728, *Bifidobacterium bifidum* CRL1399	Buffalo cheese	Cholesterol lowering
	Lactococcus lactis LMG, *Lactobacillus acidophilus* Lac1, *Lactobacillus plantarum* -2, *Bifidobacterium animalis* Bb12	Fermented buffalo milk	Cholesterol lowering
	Lactobacillus bulgaricus LB430*/Streptococcus thermophilus* TA040	Yogurt	Cholesterol lowering
Exopolysaccharides	*Lactobacillus bulgaricus* OLL1073R-1	Yogurt	Immunostimulatory
	Lactobacillus mucosae DPC 6426	Yogurt/Cheddar cheese	Hypocholesterolemic
	Propionibacterium freudenreichii KG15/KG6	Turkish cheese	Microbiota modulation
	Lactococcus lactis SMQ-461	Cheddar cheese	Microbiota modulation
	Lactobacillus plantarum YW11	Kefir	Microbiota modulation
	Bifidobacterium longum CCUG52486	Yogurt	Immune modulation

Bioactive	Producer strain	Food Product	Health effect
	Streptococcus thermophilus zlwTM11	Yogurt	Microbiota modulation
	Streptococcus thermophilus FD-DVSST-BODY3	Fermented ice-cream	Microbiota modulation

Source - Linares et al. (2017).

Besides its role to help digest dietary lipids, bile acids display antimicrobial activities by damaging the bacterial cell membrane, which is also composed of lipids. The structure of bile acids strongly affects their antimicrobial activities. Recently, researchers found that bile acid is a host factor that regulates composition of gut microbiota in rat cecum. As bile excretion increases on a high-fat diet, bile acid can be a strong selective pressure that determines gut microbiota composition on a high-fat diet, which we call the "bile acid hypothesis." As imbalanced gut microbiota composition on a high-fat diet intake has recently been shown to associate with metabolic disease development (KAWASAKI et al., 2018).

Probiotic may inhibit enteric pathogenic by several mechanisms: i-competitive exclusion, ii-production of inhibitory substances, iii-immune system modulation, and iv-improved barrier function:

i- Probiotics can exclude or reduce the growth of other microorganisms in the gut either through competition for nutrients or adherence space. During competition for nutrients, probiotics produce metabolites such as volatile fatty acids reducing the pH of the gut. Reduction in the pH of the gut makes it an unfriendly environment for pathogens and will thus lead to their inhibition because most of them cannot grow at low pH. Competition for adherence space refers to the situation when the presence of probiotics blocks pathogenic bacteria from colonizing favorite sites such as the gut villi, goblet cells and the colonic crypts. Attachment on the surfaces of gut epithelial cells is a key pathogenic factor of enteric pathogens. At the same time, colonization resistance, through which attachment and multiplication of the pathogens on the gut mucosal membrane is prohibited, is a critical function of the microbiota;

ii- Beyond acids, probiotics can produce other metabolites with antibacterial properties, such as H_2O_2 and bacteriocins, also referred to as non-lactic acid molecules; *Lactobacillus acidophilus* has been reported to produce metabolites such as acidophilin, lactocidin and acidolin, whereas bifidobacteria produces bacteriocin-like substances, all inhibiting bacteria such as *Bacillus*, *Salmonella*, *Staphylococcus* and *Escherichia coli*, *Clostridium perfringens*, *Listeria* sp., among others;

iii- Probiotic can also stimulate the effects of different cells involved in innate and adaptive immunity, such as dendritic cells, macrophages, T cells and B cells, which enhances phagocytosis of gut pathogens. Probiotic strains such as *Lactobacillus rhamnosus* and *Lactobacillus plantarum* adhere to gut-associated lymphoid tissue enhancing both systemic and mucosal immunity. These probiotics strains enhance immunity by up-regulating production of intestinal mucins (MUC2 and MUC3), which disrupts the adherence of pathogens to the gut epithelium, consequently preventing pathogen translocation. Furthermore, they induce expression of TGFβ and interleukins (IL-10 and IL-6) by epithelial cells, which enhances production and secretion of IgA.

iv- Probiotics decrease paracellular permeability, providing innate defense against pathogens and enhancing the physical impeachment of the mucous layer. They are also able to repair this barrier after damage that may have been caused by gut pathogens. As an approach

to repair the gut barrier, probiotics can stimulate mucous secretion, chloride and water secretion and the binding together of submucosa cells by tight junctional proteins (MATHIPA & THANTSHA, 2017).

Probiotics have a key role in the maintenance of immunologic equilibrium in the gastrointestinal tract through the direct interaction with immune cells. Probiotic effectiveness can be species, dose, and disease-specific, and the duration of therapy depends on the clinical indication. There is high-quality evidence that probiotics are effective for acute infectious diarrhea, antibiotic-associated diarrhea, *Clostridium difficile*–associated diarrhea, hepatic encephalopathy, ulcerative colitis, irritable bowel syndrome, functional gastrointestinal disorders, and necrotizing enterocolitis. Conversely, there is evidence that probiotics are not effective for acute pancreatitis and Crohn disease. Probiotics are safe for infants, children, adults, and older patients, but caution is advised in immunologically vulnerable populations (WILKINS & SEQUOIA, 2017).

1.3 Interactions Between Probiotic Strains and Food Matrices

Probiotic strains are incorporated to food products in the following ways: i - probiotic cultures can take part of process; or ii - probiotic cultures can be carried into the final food product; or iii - probiotic cultures can be added together with starter cultures and take part of the fermentation process.

Lactic acid bacteria are naturally present in milk as contaminants from several sources such as udder surface, milking equipment, dairy factory environment, transport and filling operations, storage surfaces. Most of the studies have been focused on the detection and the identification of species, which are mainly involved in the texture and flavor formation of dairy products (PALMIRO POLTRONIERI, 2017). Lactic acid bacteria are considered as microbial cell factories as they can produce diverse types of extracellular polysaccharide. All exopolysaccharides have broad commercial applications due to the versatility in their structural and functional properties. A wide range of exopolysaccharides are produced from lactic acid bacteria including dextran, mutan, alternan, reuteran, inulin, and levan (ZAFAR et al., 2017).

Vasiee et al. (2017) isolated several bacteria from Horreh (typical Iranian cheese). Most of isolated were identified to be *Lactobacillus* (*fermentum*, *plantarum*, and *brevis*) *Weissella cibaria*, *Enterococcus* (*faecium* and *faecalis*), *Leuconostoc* (*citreum* and *mesenteroides* subsp. *mesenteroides*) and *Pediococcus pentosaceus*. Enzymes produced by bacteria during the cheese-manufacture changes physico chemical milk properties and allow new product development.

CÁRDENAS et al. (2014) studied the addition of the *Lactobacillus salivarius* strains in fresh cheese and did not change significantly neither the chemical composition of the cheese nor texture parameters after the storage period, although cheeses manufactured with *L. salivarius* CECT5713 presented significantly higher values of hardness. A total of 59 volatile compounds were identified in the headspace of experimental cheeses, and some *L. salivarius*-associated differences could be identified. All cheeses presented satisfactory results of acceptance after the sensory evaluation. The authors of this study added probiotic culture in ready curd and observed few interactions between probiotic culture and the milk matrix. Consequently, there were no physical chemical changes in milk properties.

Fermented dairy products often are manufactured with lactic acid bacteria such as species from the *Enterococcus*, *Lactobacillus, Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus and Bifidobacterium* genera. However, *Propionibacterium shermanii* and other bacteria including *Brevibacterium linens*, responsible for the flavor of Limburger cheese; and molds (*Penicillium* species) are used in the manufacture of Camembert, Roquefort and Stilton cheeses were also used (MULLAN, 2014).

2 MATERIAL AND METHODS

2.1 Standardization of Inoculum Size and Preparation

Lactobacilli cells suspension obtained after the third subculture in MRS broth (HiMedia, Mumbai, India) was centrifuged and washed twice with 2 ml of phosphate buffer pH 7.2 (PB). Dilutions 1:10, 1:20, 1:50, and 1:100 (v/v) were prepared in PB and the absorbance was read in a spectrophotometer (BEL Photonics, model 1105 SP, USA) at 480 nm wavelength. Simultaneously, viable cells were enumerated by plating aliquots of decimal serial dilutions on MRS agar (HiMedia, Mumbai, India). XY graph was plotted with viable lactobacilli number (cfu/ml) and Optical Densities (OD) to get a linear regression. Working inoculum were obtained by overnight growth of each lactobacilli at 36 °C for 24 hours in MRS (HiMedia, Mumbai, India). Cell suspension were centrifuged (2K15, Sigma Laborzentrifugen, Germany) at 6000*g* for 6 minutes and washed twice with PB. Bacterial pellet was washed twice with PB and finally added of enough PB to get the desirable inoculum.

2.2 Evaluating of Safety and Functional Lactobacilli Probiotic Properties Before and After Stress-inducing by Gastrointestinal Conditions

2.2.1. Simulation of gastrointestinal conditions

Lactobacillus rhamnosus DTA 79 and *Lactobacillus paracasei* DTA 83 inoculum were adjusting to contain *ca* 10^8 cfu/ml. Each culture was divided in two samples and further adjusted to a desirable inoculum number to assess functional (resistance to lysozyme and pathogen biofilm inhibition,) and safety (antibiotic susceptibility and blood hemolysis) features either before and after *in vitro* gastrointestinal condition (GIC).

GIC simulation was performed as reported by FAVARIN et al. (2015) with some modifications. Gastrointestinal base juice (GBJ) was formulated as follows: calcium chloride (0.11 g/l); potassium chloride (1.12 g/l), sodium chloride (2.0 g/l), and potassium dihydrogen phosphate (0.4 g/l). This solution was sterilized at 121 °C for 15 minutes.

Artificial gastric juice (AGJ) was freshly prepared by adding to the GBJ, 3.5 g/l of swine mucin (Sigma-Aldrich, S. Louis, Mo, US), and 0.26 g/l of swine pepsin (Sigma-Aldrich, S. Louis, Mo, USA). The pH was adjusted to 2.0 with HCl (1 M). Aliquots (100 µl) of lactobacilli inoculum (*ca* 10^7 cfu/ml) were transferred to Eppendorf tube with 900 µl of AGJ and incubated in the anaerobic workstation (Electrotec, England) with artificial atmosphere (80 % N_2, 10 % CO_2, and 10 % H_2) at 36 °C for 45 minutes, with gentle shaking.

Subsequently, artificial intestinal juice (AIJ) was obtained by adding to the AGJ, 3 g/l of bile salt (Ox bile, Merck, Darmstadt, Germany), 1.95 g/l of pancreatin, and 0.1 g/l of egg white lysozyme (Sigma-Aldrich, S. Louis, Mo, USA). The pH was adjusted to 7.0 with sodium bicarbonate solution (1M) and incubated in the anaerobic workstation (Electrotec, England) with artificial atmosphere (80 % N_2, 10 % CO_2, and 10 % H_2) at 36 °C for 180 minutes, with gentle shaking.

We have considered a reduction of 1 log (data not show) when a fresh lactobacilli inoculum was submitted to GIC.

2.2.2 Lysozyme resistance assay

Lactobacilli resistance to lysozyme was measured by lysoplate method, using *Micrococcus luteus* (ATCC 4698; INCQS 356) as indicator microorganism. Melted MRS agar (HiMedia, Mumbai, India) was inoculated with a fresh suspension (*ca* 10^6 cfu/ml) either indicator microorganism or lactobacilli inoculum before or after GIC. Five wells were dug in each plate with a sterile manual borer (6.8 mm) and filled with 100 µm of egg white lysozyme

(Sigma-Aldrich, S. Louis, Mo, USA) solution at concentration of 100, 400, 800, 1500 or 3000 µg/ml previously filter (0.22 µm) sterilized. After incubation at 36 °C for 48 h, the inhibition area surround wells were calculated. The results were statistically analyzed by variance test (ANOVA) followed by Fischer's test with 95% of confidence, using XLSTAT software 7.5.

2.2.3 Biofilm inhibition assay

The inhibitory effect of lactobacilli strains on pathogenic biofilm (*Salmonella* Typhimurium DTA 41 and *Candida albicans* DTA 107) or potential food-borne pathogenic biofilms (*Escherichia coli* ATCC 25922) formation, were assessed on hydrophobic surface of flat bottom microtiter (96 wells), as proposed by Woo and Ahn (2013) with some modifications. Lactobacilli were challenged to prevent biofilm formation by three different mechanisms (competition, exclusion, and displacement) and in two different condition (before and after GIC):

i) Competition: lactobacilli inoculum (*ca* 10^7 cfu/ml) before and after GIC was co-cultured with pathogenic, also adjusted to *ca* 10^7 cfu/ml. At the same time, the microplate well was filled with each lactobacilli inoculum (10 µl), pathogenic inoculum (10 µl), MRS broth (40 µl), and casoy broth (40 µl). Microplate was incubated in the anaerobic workstation (Electrotec, England) with artificial atmosphere (80 % N_2, 10 % CO_2, and 10 % H_2) at 36 °C for 24 hours.

ii) Exclusion: lactobacilli inoculum (*ca* 10^7 cfu/ml) before and after GIC was transferred to microplate wells. Each well was was filled with each lactobacilli inoculum (10 µl) and MRS broth (90 µl), followed by incubation in the anaerobic workstation (Electrotec, England) with artificial atmosphere (80 % N_2, 10 % CO_2, and 10 % H_2) at 36 °C for 24 hours. After, wells were rinsed twice with PB to remove nonadherent cells and seeded with pathogenic inoculum (*ca* 10^7 cfu/ml). Now, microplate well was filled with pathogen inoculum (10 µl) and casoy broth (90 µl) and followed by incubation at the same conditions.

iii) Displacement: pathogenic inoculum was adjusted to *ca* 10^7 cfu/ml and transferred to microplate wells. Each well was filled with pathogenic inoculum (10 µl) and casoy broth (90 µl), followed by incubated in the anaerobic workstation (Electrotec, England) with artificial atmosphere (80 % N_2, 10 % CO_2, and 10 % H_2) at 36 °C for 24 hours. After, wells were rinsed twice with PB to remove nonadherent cells and seeded with each lactobacilli inoculum (*ca* 10^7 cfu/ml) before and after GIC. Now, well was filled with lactobacilli inoculum (10 µl) and MRS broth (90 µl) and followed by incubation at the same conditions.

Finally, each well was rinsed twice with PB to remove nonadherent cells and adhered cells were removed with a swab. Monospecies cultures of pathogenic or potential food-borne pathogenic microorganism were used as controls. Enumeration of adhered cells was performed by drop plate technique in selective medium as follows: *E. coli* (Eosin Methylene Blue agar, HiMedia, Mumbai, India; *S.* Typhimurium (Hecktoen Enteric agar, Himedia, Munbai, India), and *C. albicans* (Sabouraud agar, Oxoid, Basingstoke, United Kingdom).

The results were treated by variance analysis (ANOVA) followed by Dunnet's and Fischer's test with 95% of confidence, using XLSTAT software 7.5.

2.2.4 Hemolysis on blood agar assay

Lactobacilli were screened for α or β-hemolytic activity by streaking suspension of lactobacilli before and after to GIC on MRS agar (HiMedia, Mumbai, India) added with 5% defibrinated sheep blood (Eurofarma, Rio de Janeiro, Brazil) and by growth into the agar to find any anaerobic versions of the enzymes to digest blood cells. A sterile Petri dish divided into two compartments was used to perform the test. Positive or negative hemolysis was detected by presence or absence of a gray-green (α-hemolysis) or transparent (β-hemolysis) halo surrounded the lactobacilli growth after 48 hours at 36 °C. Gamma-hemolysis is not detectable by this method.

2.2.5 Antibiotic susceptibility assay

Antimicrobial susceptibility tests were performed by agar disk diffusion method (BAUER et al., 1966) using MRS agar (HiMedia, Mumbai, India). Aliquots (100 µl) of lactobacilli inoculum before and after GIC were spread on the agar surface with a Drigaslky spreader. Dried discs (CECON, São Paulo) impregnated with antibiotics agents for human use: amoxicillin + clavulanic acid (AMC); ciprofloxacin (CIP); cephalexin (CFX); chloramphenicol (CLO); tetracycline (TET); ampicillin (AMP); and nalidixic acid (NAL) were placed on top of the agar and lightly press down with a sterile tweezer. Plates were incubated at 36 °C for 48 hours and the diameter of growth inhibition zones surrounded the discs were measured with a caliper (including disc). Results were expressed as resistant (Ø ≤ 15 mm), intermediate (16 > Ø < 20 mm), or sensitive (Ø ≥ 20 mm) in accordance to what was proposed by (VLKOVÁ et al., 2006).

2.3 Evaluating of Technological Lactobacilli Probiotic Properties

2.3.1 Acidification and monitoring milk, traditional and idealized coagulation kinetics

L. rhamnosus DTA 79 and *L. paracasei* DTA 83 grown overnight in MRS broth (Oxoid, Basingstoke, United Kingdom) at 36 °C were inoculated in a concentration of 1% (v/v) in a thermostats water bath at 35 °C, equipped with steel wells and filled with 1.5 L of pasteurized whole milk (Legnaro, Veneto, Italy), as following:

a) Control: whole milk (1.5 liters)
b) Control + *L. paracasei* – whole milk (1.5 liters) + *L. paracasei* (15 ml)
c) Control + *L. rhamnosus*: whole milk (1.5 liters) + *L. rhamnosus* (15 ml),
d) Control + ½ *L. paracasei* + ½ *L. rhamnosus*: whole milk (1.5 liters) + *L. paracasei* (7.5 ml) + *L. rhamnosus* (7.5 ml),
e) Control + *L. paracasei* + *L. rhamnosus*: whole milk (1.5 liters) + *L. paracasei* (15 ml) + *L. rhamnosus* (15 ml).

At each one-hour time intervals, pH was measured using a portable pHmeter (Crison Basic 25; Crison Instruments SA, Barcelona, Spain), and milk dynamographic profile (LDG) was made to obtain the milk coagulation properties (MCP) in a Formagraph Firm Foss Electric (Hillerod, Denmark). Duplicate aliquots (10 ml) was harvest from each well to obtained LDG profile. Lactobacilli survivability was enumerated on MRS agar (Oxoid, Basingstoke, United Kingdom) by drop plate technique at 36 °C for 48 hours.

Descriptive statistics analysis of milk acidification, coagulation properties, total bacterial counts were used to stablish coagulation model as described by BITTANTE and CECCHINATO (2013).

2.3.2 Interaction between lactobacilli and streptococci strains

Raw cow milk (Legnaro, Veneto, Italia) was sterilized in an autoclave at 110 °C/10 minutes in a glass bottle (500 ml) and quickly cooled in water/ice bath. Each bottle (total of 8 units) received 0.5 ml of working inoculum with *ca* 10^8 cfu/ml of axenic or co-culture, as following:

G1 – *Lactobacillus rhamnosus* DTA 79;
G2 – *Lactobacillus paracasei* DTA 83;
G3 – *Streptococcus thermophilus* TH 895;
G4 – *Streptococcus thermophilus* TH 1435;
G5 – *Lactobacillus rhamnosus* DTA 79 and *Streptococcus thermophilus* TH 895;
G6 – *Lactobacillus rhamnosus* DTA 79 and *Streptococcus thermophilus* TH 1435;
G7 – *Lactobacillus paracasei* DTA 83 and *Streptococcus thermophilus* TH 895;
G8 – *Lactobacillus paracasei* DTA 83 e *Streptococcus thermophilus* TH 1435.

Aliquots (10 ml) from each bottle were followed by incubation at 37 °C for up to 9 hours in a stirred water bath, at 37 °C. At each one-hour time intervals one tube was taken for pH measurement.

2.3.3 Lyophilization of probiotic cultures

Either *Lactobacillus rhamnosu*s DTA 79 or *L. paracasei* DTA 83 were mixed with *Streptococcus thermophilus* (TH 895) to get starter cultures for milk fermentation. For this, cultures were activated by three successive daily growth in MRS or M-17 broth (Oxoid, Basingstoke, United Kingdom) at 36 and 42 °C, respectively for lactobacilli and streptococci. The third growth was obtained in 500 ml of medium. Cellular masses were then harvested by centrifugation at 6000*g* for 6 minutes and each pair of *Lactobacillus* and *Strepatococcus* was mixed at a ratio 60:40 in a tube with 50 ml of skim milk (Oxoid, Basingstoke, United Kingdom). After freezing in liquid nitrogen, the content was lyophilized and packed in a high-density polyethylene pack (10 g).

Started culture was coded as following:
Ls23895Th (*Streptococcus thermophilus* and *Lactobacillus rhamnosus*),
Ls03895Th (*Streptococcus thermophilus* and *Lactobacillus paracasei*).

2.3.4 Probiotic fermented milk production and effect of honey on lactobacilli survivability

Raw milk was purchased in local market (Padova, Veneto, Italia) and fortified with skimmed milk powder (Oxoid, Basingstoke, United Kingdom) to increase the total solids in dry extract up to 15 %. Milk and honey were pasteurized at 84 °C for 1 minute and at 78 °C for 6 minutes, respectively, in a stirred water bath followed by cooling at 37 °C. Ls23895Th or Ls03895Th freeze-dried starter cultures (10 g) were dissolved in pasteurized milk (250 ml) and after incubation at 37 °C for 3 hours, were added to 5 liters of pasteurized milk which contained or not 5% of pasteurized honey Fermentation was performed without any further stirring in high density polyethylene bottles (1000 ml) at 37 °C until pH have reached 4.5.

Fermented milks were cold storaged at 7 °C and lactobacilli survivability was enumerated by drop plate technique both before and after CGI on MRS agar (Oxoid, Basingstoke, United Kingdom) at 36 °C.

Analysis of variance (ANOVA) following by Dunnett's test ($P > 0.05$) was used to compare significant differences in lactobacilli survivability.

2.3.5 Probiotic tablets with viable lactobacilli

L. rhamnosus DTA 79 or *L. paracasei* DTA 83 were grown in 200 ml of MRS broth (HiMedia, Mumbai, India) at 36 °C for 30 hours. Cellular masses were separated from broth by centrifugation at 6000*g* for 6 minutes and washing twice with PB and partially dried at 37 °C for 6 hours. *L. rhamonosus* and *L. paracasei* cellular masses were coded respectively Lr23 and Lp23 and storaged at –18 °C. Gelatin tablets containing 10^8 cfu/tablet of each lactobacilli (Table 4.4) were prepared by a pharmacy (Valença, Rio de Janeiro, Brazil).

Table 4.4 – Probiotic tablet composition

Ingredients	**Composition (%)**
Sorbitol	1,00
Mint flavor	0,30
Magnesium stearate	1,20
Xylitol	3.50
Sucralose	0,15
Gelatin	3.20
Probiotic inoculum	1,00
Sterile water	89.65

Partially dried probiotic inoculum was added in the cooling step of production. They were packed in polypropylene flasks. Lactobacilli survivability was enumerated by drop plate technique on MRS (HiMedia, Mumbai, India) at 36 °C for 48 hours.

Analysis of variance (ANOVA) following by Fisher's test ($P > 0.05$) was used to compare significant differences in the lactobacilli survivability, using XLStat software version 7.5.

2.3.6 Probiotic survivability in ice cream bar and ice-lolly

Ice-lolly was made mixing in a blender, 31.80 % of mineral water at 5 °C with ice (1:1), 40.00 % of purchased acerola pulp (Ice Fruit-Rio, Brazil), 27.10 % of sugar, 1.00 % of probiotic inoculum, 0.02 % of red coloring, and 0.08 % of citric acid. Probiotic culture (*ca* 10^8 cfu ml^{-1}) was added at the last step. It was shaped and frozen in a quick-freezing machine.

Ice cream bar was made mixing 33.00 % of powdered milk (Tangará Foods S/A, Brazil), 33.00 % of purchased acerola pulp (Ice-Fruit-Rio, Brazil), 16.50 % of sugar, 14.36 % of mineral water at 5 °C with ice (1:1), 1.86 % of neutral bind (guar and xanthan gum, corn starch, antimixer and carboxymethylcellulose), 1.00 % of probiotic inoculum, 0.19 % of turmeric dye, and 0.09% of emulsifier. It was shaking and processed in ice cream machine for 15 minutes.

Ten kilograms of ice cream was used to each condition and viable lactobacilli were enumerated for up 21 days at storage at -18 °C by ~~micro~~drop technique on MRS agar (HiMedia, Mumbai, India) at 36 °C for 48 hours. Analysis of variance (ANOVA) following by Fisher's test ($P > 0.05$) was used to compare significative differences in the lactobacilli viability.

3 RESULTS AND DISCUTION

3.1 Evaluating of Safety and Functional Lactobacilli Probiotic Properties Before and After Stress-inducing by Gastrointestinal Conditions

3.1.1 Resistance to egg white lysozyme

L. rhamnosus DTA 79 and *L. paracasei* DTA 83 were resistant for up to 400 μg mL^{-1} of lysozyme. From 800 μg mL^{-1} to 3000 μg mL^{-1}, a slight inhibition was observed, but significantly lower when compared with the indicator microorganism, *Micrococcus luteus* (Figure 2B). There was no difference in the susceptibility of the strains DTA 79 or DTA 83 before or after CGI, but *L. paracasei* DTA 83 was significantly more resistant than *L. rhamnosus* DTA 79 to 800 and 1500 μg/ml of-lysozyme. No significant difference was observed at 3000 μg/ml (Figure 4.1).

Bacterial resistance to lysozyme is an important property to a probiotic strain because gut Paneth cells produce antimicrobial proteins, including lysozyme. Moreover, oral administration of probiotics increases Paneth cells and intestinal antimicrobial activity (CARZOLA et al., 2018). Lysozyme is also a preservative often used in food technology. Shahid Riaz et al. (2015) researched the resistance of lactic acid bacteria strains (*L. plantarum*, *L. rhamnosus*, *L. delbrueckii*, *L. paracasei*, *Weisella spp*, *Enterococcus faecium*, *Enterococcus faecium*, *Weissella paramesenteroids*, and *L. saekei*) at 200 μg/ml, and 300 μg/ml, and 400 μg/ml of lysozyme by Optical Density (OD) at 630nm wavelength. All lactic acid bacteria strains were found to be resistant at all concentrations of lysozyme. YADAV et al. (2016) included probiotic resistance to lysozyme as requisite to produce fermented beverage Raabadi (a summer drink from some regions of India).

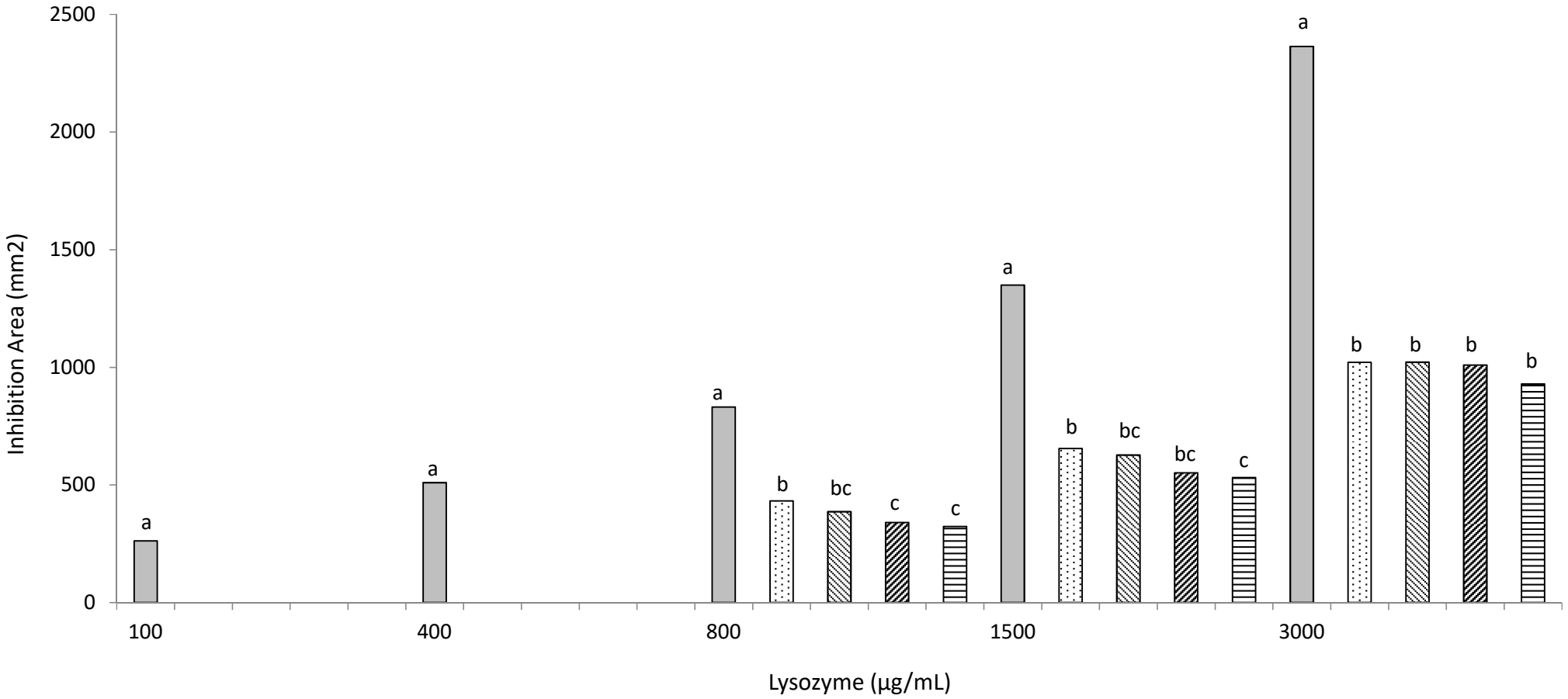

Figure 4.1 - Susceptibility to different lysozyme concentrations observed with strains of lactobacilli and the indicator microorganism. *Lactobacillus rhamnosus* DTA 79 and *Lactobacillus paracasei* DTA 83 and *Micrococcus luteus* (ATCC 4698; INCQS 356) to egg white lysozyme. Different letters in the same concentration of lysozyme indicates significant difference by Dunnett's test to 0.95 confidence.

Minami et al. (2016) researched different probiotic strains from human milk for tolerance to either egg white or human lysozyme. Among the infant-type human-resistant bacteria strains, all strains of *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium breve* grew well in breast milk, but the growth characteristics of *B. longum* subsp. *longum* and *B. bifidum* were strain-dependent. In contrast, the tested strains of adult-type human-resistant bacteria and non-human-resistant bacteria generally failed to grow and died after incubation in breast milk. Most infant-type strains were tolerant to high concentrations of lysozyme, while adult-type strains possessed intermediate tolerance to lysozyme, and non-human-resistant bacteria strains were susceptible to lysozymes of egg white or human origin. These data suggest that breast milk lysozyme content plays a leading role in the exclusion of non-human-resistant bacteria.

3.1.2 Biofilm inhibition

L. rhamnosus DTA 79 and *L. paracasei* DTA 83 reduced biofilm formation by *E. coli*, *C. albicans,* and *S.* Typhimurium by competition and exclusion mechanisms before and after GIC (Figures 4.2 and 4.3). *L. paracasei* lost the ability to inhibition *C. albicans* biofilm by exclusion mechanism (Figure 4.3).

When *L. rhamnosus* and *L. paracasei* were challenged to remove the attachment cells by displacement mechanism, it only removed *E. coli* biofilm. Controversially, stress due to GIC increase the ability of *L. rhamnosus* and *L. paracasei* to remove *E. coli* biofilm (Figures 4.2 and 4.3). On the other hand, *L. paracasei* was able to remove *C. albican*s biofilm by displacement mechanism only before GIC.

Neither lactobacilli displaced the biofilm raised by *S.* Typhimurium showing the strong attachment of the pathogenic bacterium. These results suggest that after any medical drug intake inducing negative impact on gut microbiome, it is advised to take probiotics, so it can adhere on the gut villi epithelium before the pathogen could do it. Moreover, gut infection leaded by pathogenic microorganism can only be avoided by regular intake of probiotics.

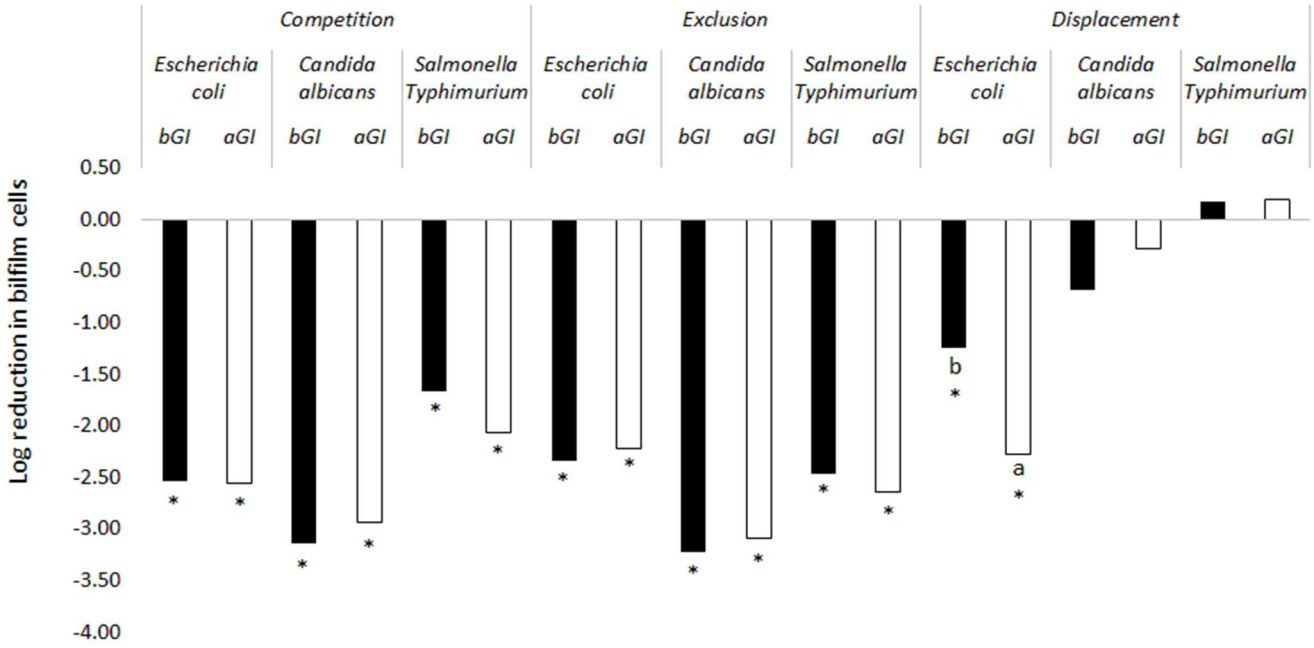

Figure 4.2 – Reduction of *Salmonella* Typhimurium, *Candida albicans*, and *Escherichia coli* biofilm formation by *Lactobacillus rhamnosus* DTA79 by the mechanisms of competition, exclusion and displacement either before (■) and after (□) gastrointestinal conditions. Biofilm cells were estimated subtracting the biofilm cell counts in cocultures from the monoculture pathogenic controls after incubation. Presence of asterisk below the column indicates significative difference when biofilm cells count was compared to the control by Dunnett's test ($P > 0.05$). Different lower-case letters to the same microorganism and to the same inhibition mechanism, show significative difference between biofilm cells count bGI and aGI by Fischer's test ($P > 0.05$). b (before) a (after) gastrointestinal conditions (GI).

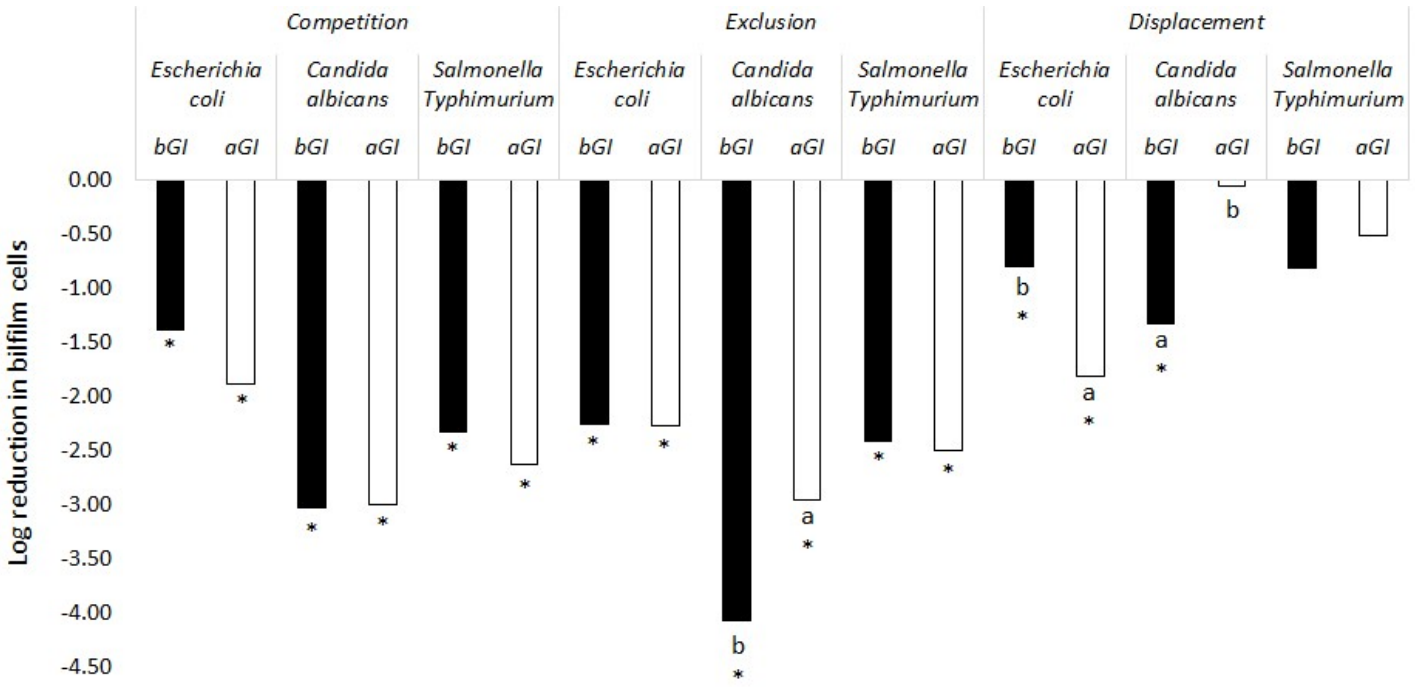

Figure 4.3 – Reduction of *Salmonella* Typhimurium, *Candida albicans*, and *Escherichia coli* biofilm formation by *Lactobacillus paracasei* DTA 83 by the mechanisms of competition, exclusion and displacement either before (■) and after (□) gastrointestinal conditions. Biofilm cells were estimated subtracting the biofilm cell counts in cocultures from the monoculture pathogenic controls after incubation. Presence of asterisk below the column indicates significative difference when biofilm cells count was compared to the control by Dunnett's test ($P > 0.05$). Different lower-case letters to the same microorganism and to the same inhibition mechanism, show significative difference between biofilm cells count bGI and aGI by Fischer's test ($P > 0.05$). b (before) a (after) gastrointestinal conditions (GI).

Candidiasis is a fungal infection caused by yeasts that belong to the genus *Candida*. When it affects the mouth, it is commonly called thrush. Signs and symptoms include white patches on the tongue or other areas of the mouth and throat. Elderly denture users are often affected by candidiasis infection due to injuries on mouth mucosa. Both *L. rhamnosus* and *L. paracasei* have showed inhibitory effect against *C. albicans* biofilm settlement. Pathogen biofilm inhibition assay was performed in polycarbonate microtiter plate, however a surface quite different from oral mucosa or gut villi epithelium. Nevertheless, these findings are an indication of antimicrobial activity and might be repeated using animal cells.

Daliri and Lee (2015) reported correlations between changes in composition and activity of the gut microbiota and common disorders such as cancer, hypertension, hypercholesterolemia, inflammatory bowel diseases, obesity, *etc.* These diseases may be avoided by probiotic, but they need to be in enough number.

3.1.3 Hemolysis on blood agar

L. paracasei DTA 83 and *L. rhamnosus* DTA 79 did not present alpha or gamma-hemolysis before or after CGI (Figure 4.4). According to Owusu-kwarteng et al. (2015) general absence of hemolysis or poor hemolysis activities expressed by lactic acid bacteria, it is indicative of their safety applications in food. In fact, it was not found reports in literature indicating some lactobacilli strain with hemolytic activity, either commercial or indigenous strains.

Figure 4.4– Absence of lactobacilli alfa or gamma-hemolysis on MRS agar added with sheep blood (5 %)

3.1.4 Antibiotic susceptibility assay

Susceptibility to seven antibiotics chosen among those for human use was tested (Table 4.5). *L. paracasei* was sensitive to chloramphenicol (CLO), tetracycline (TET), amoxicillin + clavulanic acid (AMC), and ampicillin (AMP). It showed intermediate sensitivity to cephalexin (CFX), and ciprofloxacin (CIP). *L. rhamnosus* was sensitive to chloramphenicol (CLO), tetracycline (TET), amoxicillin + clavulanic acid (AMC), and ampicillin (AMP). It presented intermediate sensitivity to cephalexin (CFX), and ciprofloxacin (CIP). As expected, both lactobacilli were resistant to nalidixic acid (NAL). The sensitivity was evaluated after *in vitro* simulated gastrointestinal conditions. Although in most of the cases a variation in the halo size was registered, it was not enough to cause significant changes in the sensitivity of the lactobacilli to the antibiotics studied after in vitro intestinal conditions.

Table 4.5 - Resistance of *Lactobacillus paracasei* and *Lactobacillus rhamnosus* to antibiotics.

Antibiotic	Gastrointestinal Condition	Mean ± Standard Deviation	
		Lactobacillus paracasei	*Lactobacillus rhamnosus*
CLO	Before	43.96 ± 2.50 (S)	29.71 ± 1.41 (S)
	After	42.10 ± 1.50 (S)	30.74 ± 0.42 (S)
NAL	Before	** **	** **
	After	** **	** **
TET	Before	35.55 ± 0.99 (S)	25.88 ± 0.69 (S)
	After	31.63 ± 1.02 (S)	24.06 ± 1.59 (S)
AMC	Before	35.51 ± 0.99 (S)	24.19 ± 1.05 (S)
	After	35.14 ± 0.25 (S)	25.99 ± 0.56 (S)
AMP	Before	34.92 ± 0.44 (S)	28.35 ± 0.67 (S)
	Afetr	34.33 ± 1.05 (S)	27.36 ± 0.75 (S)
CFX	Before	27.52 ± 2.62 (S)	16.52 ± 1.05 (I)
	After	28.33 ± 2.55 (S)	17.03 ± 0.52 (I)
CIP	Before	23.64 ± 0.31 (S)	18.65 ± 1.06 (I)
	After	25.50 ± 1.00 (S)	16.84 ± 1.65 (I)

S – sensitive (Ø < 17 mm), I – Intermediate (17 ≤ Ø ≥ 23mm), R – Resistant (Ø > 23 mm)
** Disc diameter = 6.00 mm

3.2 Evaluating of Technological Lactobacilli Probiotic Properties

3.2.1 Acidification potential and coagulation properties

L. paracasei DTA 83 and *L. rhamnosus* DTA 79 did not interfere with dairy process nor with milk coagulation kinetics. Table 4.6 shows the descriptive statistics and variance analysis of milk acidification (pH), lactobacilli total counts, traditional coagulation properties and modelling according to Bittante and Cecchinato (2013). The great variability observed was due to both the effect of time interval (the Figure 4.5 is an average of 12 hours) and the type of treatment. In fact, as expected, both were significant factors influencing the acidity level. In most cases, at the beginning the pH was 6.69, while the lowest value (pH 5.91) was registered after 12 hours of incubation. The same was found with the control milk (C), not inoculated with Lactobacilli.

Table 4.6 Descriptive statistics (mean and SD) and ANOVA results (Fischer's test) for the acidification milk (pH), total count of lactobacilli, traditional coagulation properties and CFt equation parameters

Variable	Mean	SD	Treatment (T)	Time interval (I)	T*I	Pendle	R^2, %	RMSE
pH	6.49	0.21	7.0^{***}	190.6^{***}	-	-	95.6	0.05
Total count of lactobacilli:[1]								
TBC_{MRS}	5.79	2.97	7099.2^{***}	1780.2^{***}	53.0^{***}	-	99.9	0.13
Traditional coagulation property:[2]								
RCT, min	9.68	4.87	352.2^{***}	2466.7^{***}	20.7^{***}	7.9^{***}	99.7	0.41
k_{20}, min	1.97	0.59	21.7^{***}	114.5^{***}	1.9^{***}	4.8^{***}	94.8	0.21
a_{30}, mm	65.5	9.2	36.1^{***}	171.7^{***}	2.8^{***}	9.2^{***}	96.5	2.64
a_{45}, mm	71.1	5.5	11.1^{***}	40.3^{***}	1.3	12.7^{***}	91.3	2.50
a_{60}, mm	73.1	4.6	4.9^{***}	10.8^{***}	0.8	11.5^{***}	85.3	2.74
Parameter CF_t :[3]								
RCT_{eq}, min	9.24	5.07	371.9^{***}	2565.2^{***}	20.8^{***}	9.0^{***}	99.7	0.42
k_{CF}, %×min^{-1}	8.57	1.76	25.8^{***}	441.9^{***}	5.3^{***}	4.8^{***}	98.3	0.35
k_{SR}, %×min^{-1}	0.67	0.03	4.7^{***}	43.7^{***}	1.7^{**}	4.8^{***}	89.2	0.02
CF_P, mm	98.1	6.1	5.1^{***}	14.1^{***}	0.9	13.0^{***}	87.0	3.39
CF_{max}, mm	73.2	4.6	5.1^{***}	14.1^{***}	0.9	13.0^{***}	87.0	2.53
t_{max}, min	54.5	7.9	2.0^{*}	15.1^{***}	1.2	2.0^{*}	77.4	5.80

SD = Standard Deviation; [1]TBC_{MRS} = $\log_{10}$ cfu/ml of the total bacterial count of *Lactobacillus paracasei* and *Lactobacillus rhamnosus*; [2]RCT = coagulation time measured; k_{20} = period between the beginning of gelation and achieve the consistency of the curd 20mm; a_{30} (a_{45}, a_{60}) = consistency of the curd 30 (45, 60) min after rennet; [3]RCT_{eq} = RCT estimated by modelling on the basis of the curd texture changes over time (CF_t); k_{CF} = constant rate of curd firmness; CF_P = potential asymptotic value of the consistency of the curd; k_{SR} = constant rate of syneresis; CF_{max} = maximum firmness of the clot obtained in 45 min; t_{max} = time is reached CF_{max}. *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

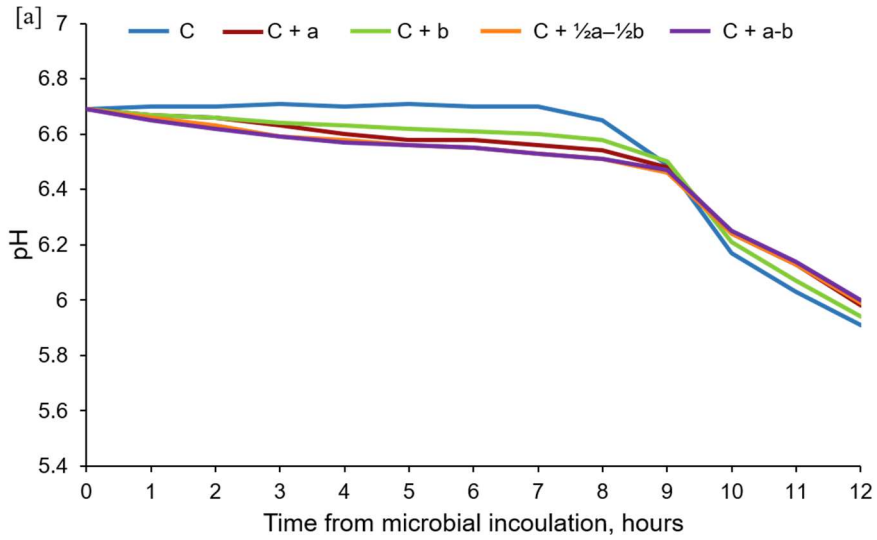

Figure 4.5 - Acidity of milk (pH) calculated with the least squares method, according to the interaction between inoculation time interval and the microbial treatment (C = control; C + a = control + *L. paracasei*; C = control + b + *L. rhamnosus*; C + ab = control + *L. paracasei* and *L. rhamnosus*; C + ½A-½b = control + ½ ½ *L. paracasei* and *L. rhamnosus.*

Figure 4.6 shows the control group and 4 experimental group the pattern of the milk samples consistency of the coagulum after adding rennet (modeling of CFt), according to the interaction between time interval from microbial inoculation (I, 0 to 11 uM) and treatments.

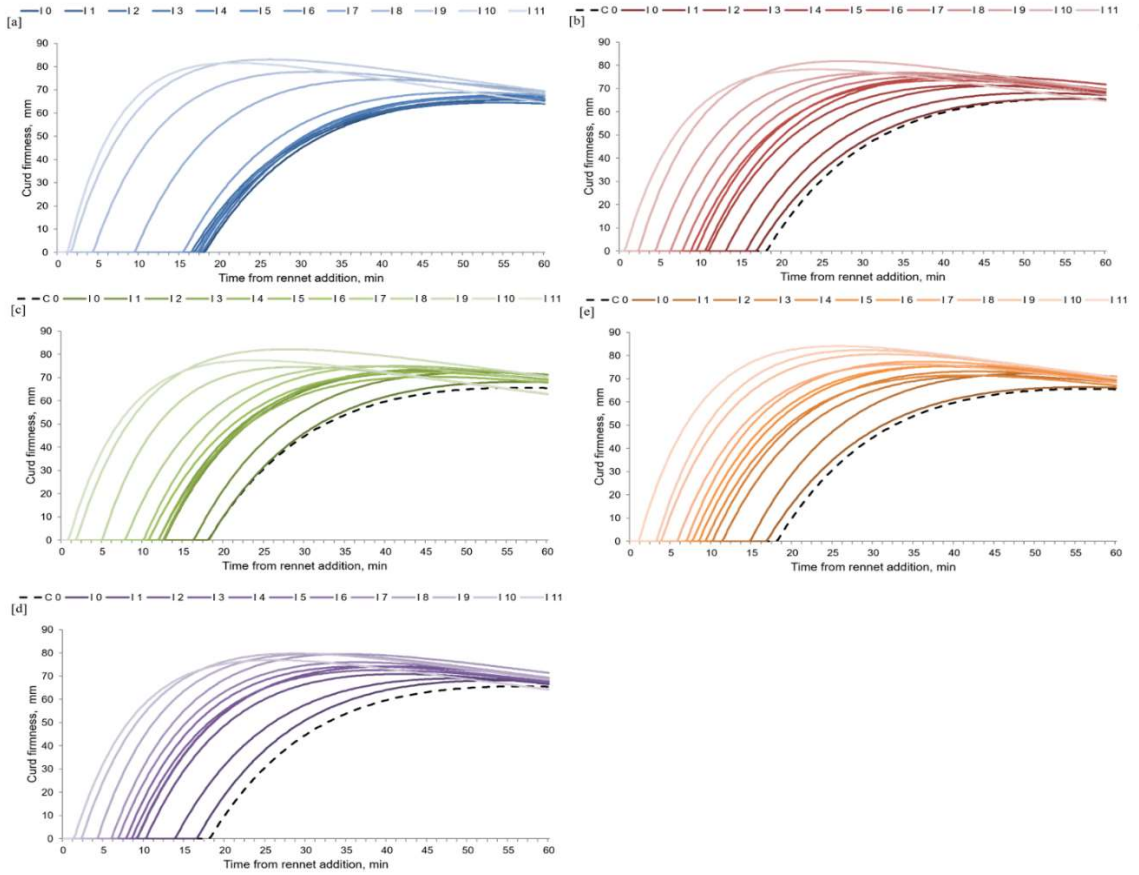

Figure 4.6 – Pattern of clot consistency. [a] control. [b] control + *Lactobacillus paracasei* DTA 83. [c] control + *Lactobacillus rhamnosus* DTA 79. [d] control + *Lactobacillus paracasei* DTA 83 and Lactobacillus rhamnosus DTA 79. [e] control + ½ *Lactobacillus paracasei* DTA 83 and ½ *Lactobacillus rhamnosus* DTA 79.

The Figure 4.6a shows a trend of control milk coagulation almost unchanged in the first 6 hours of modelled clotting parameters, with a rennet coagulation time estimated from the equation (RCTeq) which was maintained at above 17 minutes. From the seventh hour, the clotting time began to reduce significantly (-10.5% compared to I6). The pH drop of the milk was probably the cause of this phenomenon (Figure 4.5), at the first 6 hours the pH value was basically unchanged (pH 6.7). From the sixth hour, it began a downward trend more or less pushed until it reaches a pH of 6.3 to I-11. Most drastic changes in terms of RCTeq have occurred after the seventh hour, passing by a RCTeq of 15.46 min (I-7) at 1.60 min (I-11). Optimal milk (type A) should present an RCT = 13 min, while sub-optimal are milks with RCT of 16 min (type B), 11 min (type C) and 9.5 min (type D) (BITTANTE et al., 2012). Following this classification only rounds I-7 and I-8 may be defined as sub-optimal, while the rounds I-9 and I-10 fall within the definition of milks defective, more precisely DD type (RCT <5 min; k20 <3 min). As expected the rate of clot firmness is increased progressively with decreasing pH, passing from a minimum of 6.42% × min^{-1} (I-0) to a maximum of 14.7% × min^{-1} (I-11). Micelar aggregation is favoured at pH 6.0-6.3 then lowering the clotting time favouring formation of a firmer clot (LÓPEZ et al., 2017). The highest value of the potential asymptotic value of the consistency of the curd; (CFP) has been reached to interval 10 (104.28 mm). Rate of syneresis 9-10-11 obtained in intervals is very thorough, respectively 0,694%, 0.675%, and 0.742% × min^{-1}. This is most likely due to the fact that pH <6.0 the excessive dissolution of the colloidal calcium phosphate (CCP) tends to push a higher bleeding rate of the curds (Ibid).

Coagulation curves profile from Figure 4.6b (control + *L. paracasei*, Figure 1.10b) we note that already at time 0 has been obtained by a shorter RCTeq compared to I-0 control, however, accompanied by a rate firming of the lower clot (6.42 vs 6.27 % × min^{-1}) and a syneresis rate higher (0.592 vs 0.616 % × min^{-1}). In addition, already one hour after the inoculation of the starter the pH is lowered (Figure 4.5) and RCTeq decreased from 16.854 min (I-0) to 15.56 min (I-1). Optimal clotting time has been reached already to I-2 (13.04 min), with KCF = 7.18 % × min-1 and KSR = 0649 % × min^{-1}. Even in this case CFP has reached the highest value at the tenth interval (103.32 mm) and then reduced to I-11 (97.88 mm). In the last three rounds the rate of the curds drain has shown tends to be lower than the KSR detected in the same shifts to the control.

Otherwise, *L. paracasei*, the *L. rhamnosus* (Figure 4.6b) apparently has no influence on RCTeq interval to 0, which is a result very close to RCTeq to I-0 of the control thesis. However, he reached a CFP comparatively higher (94.13 vs 90.03 mm), thus suggesting that there has been an influence on the strength of aggregation of caseins. The 2-3-4 intervals have very similar performance in terms of RCT, which KCF and KSR. I-5 is distinguished particularly with respect to the preceding and subsequent intervals due to lower CFP, while maintaining constant rates of curd firmness and very similar syneresis. Finally, the results reappointed as CFP tends to reach the highest value in tenth interval. We also observe how the thesis 3 has reached to I-10 a CFP very similar to that obtained in the same interval from the thesis 2 (103.34 vs. 103.32 mm), therefore hypothesize that after 10 hours of incubation the two probiotics have on the medium milk similar effects.

The Figure 4.6d shows how the probiotic bacteria have influenced the modeling parameters when inoculated together in a double dose. As in the argument we had 2 RCTeq to I-0 faster (16.50 min) compared to C-0 (18.09 min), reinforcing the hypothesis of the influence of *L. paracasei*. Until the beginning of the sixth hour RCTeq test is decreased faster than the test 5 (Figure 4.6e), probably due to higher concentration of inoculated probiotics, and KCF is maintained tend to be higher. For the thesis I-11 showed a higher RCTeq than the counterpart (1.34 vs 2.01 min). After the first hour of incubation was reached RCTeq of 13.82 min, a value that is close to that expected for an optimal type A milk (13 min) (LÓPEZ et al., 2017) also comparing the overall trend of clot consistency with graphs of the test 5 (Figure 4.6e) stands

out as there was a considerable difference in terms of CF. In the first case the highest value was obtained interval 11, with CF_{MAX} = 84 mm, while in this thesis was obtained a lower average CF_{MAX}, with the highest value reached to I-10 (79.7 mm), for a difference of 4.3 mm. Finally, the KSR values occurred again erratic.

When *L. paracasei* and *L. rhamnosus* were inoculated in pairs in a single dose (Figure 4.6b), there has been a decrease in the clotting time for I-0 with respect to the control thesis (C-0), similarly to what happened in the Figures 4.6c and 4.6d. The milk with RCT near sub-optimal values according with BITTANTE et al. (2012) was obtained already after the first hour of incubation (14.79 min). It can also be observed that the clotting time has been reduced more rapidly than the test in which it was inoculated a single probiotic, in fact were obtained tends to better performance in terms of RCTeq. Regarding the potential value of the asymptotic curd firmness, it was seen that it has reached the maximum value to I-9 (104.6 mm) and then declined in the following ranges, however, the highest value was obtained CF_{MAX} to I- 11 (84 mm). The curd firmness rate increased faster in this thesis with respect to both theses 2 and 3, until the eighth hour. The KSR has presented a variable and non-linear trend during all 11 hours, with values that ranged between 0.586% × min-1 (I-0) and 0.701% × min-1 (I-11).

Among the traditional coagulation parameters, results showed very low average RCT, of 9.68 ± 4.87 min, as well as very low the curd firming time (k20 = 1.97 min). In fact, according to a study conducted by DE MARCHI et al. (2007), the milk produced by cattle breeds commonly used in Italy (Holstein Friesian, Brown Swiss, Simmental, Alpine Gray) the average RCT was 15.5-18.4 min and k20 of 6.0-8.8 min, much higher values compared to that obtained in this study due to the effect of treatment and time interval. Although the consistency of the clot occurred very high from 30 to 60 minutes from the beginning of the analysis dynamographic milk. In fact, the average value of a_{30} found was of 65.5 ± 9.2 mm, when an optimal milk or suboptimal for the cheese making normally presents a curd firmness to 30 minutes between 29 and 54 mm (BITTANTE, 2011). The high variability for all parameters is due both to the time interval of 12 hours in which it is carried out the test, the effect of which is highly significant ($P < 0.001$), both to the treatment (T), which is also highly significant in all measured parameters. The interaction T * I is highly significant for RCT, k20 and a30 (Table 4.6).

3.2.2 Lactobacilli acidification potential

L. rhamnosus DTA 79, *L. paracasei* DTA 83, and *S. thermophilus* TH 895 were unable to acidify the milk to pH 5.2 in 9 nine hours at 37 °C when in axenic cultures. However, *S. thermophilus* TH 1435 acidified very well the milk. Association between *L rhamnosus* or *L. paracasei* with S. *thermophilus* TH 1435 also acidified the fermented milk below to pH 5.2 but this acidification probably is due fermentation potential from TH 1435 (Figures 4.7A and 4.7B).

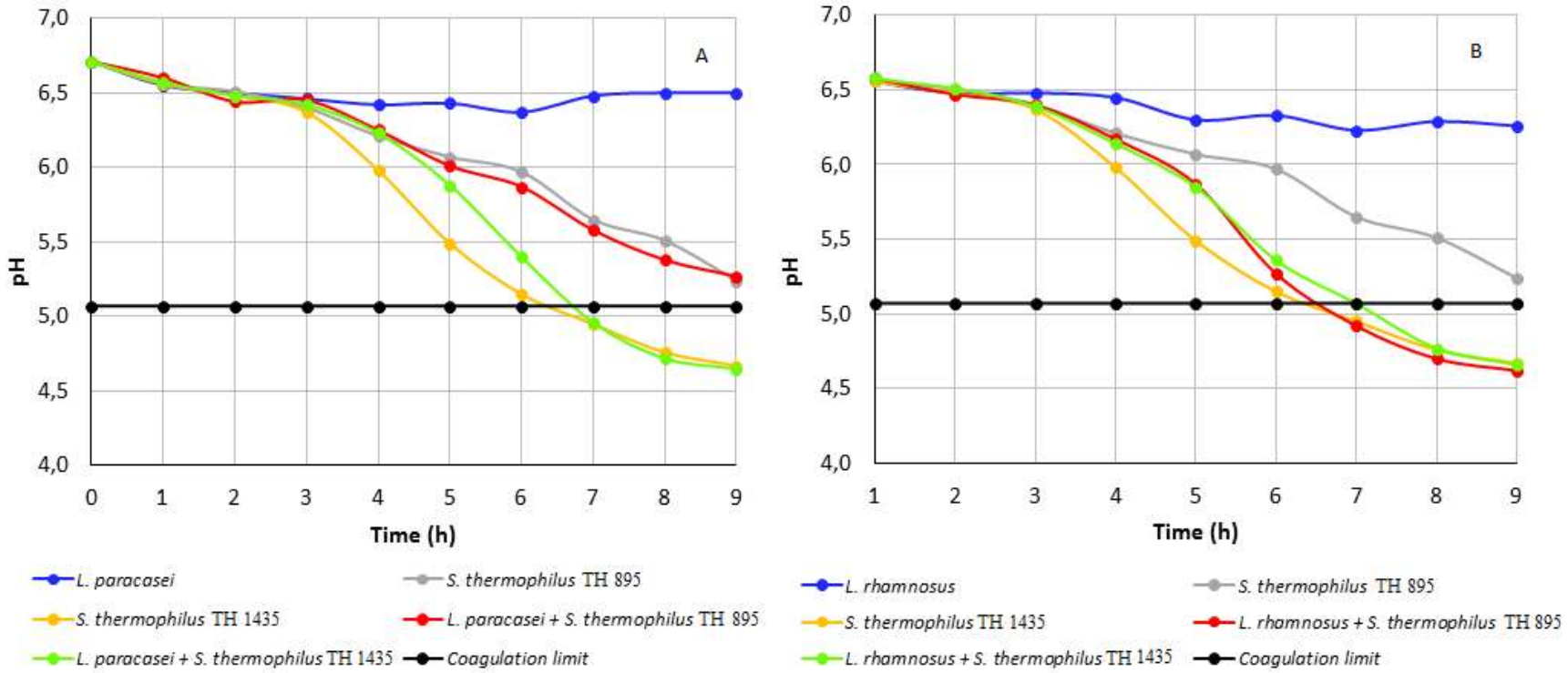

Figure 4.7 – Milk acidification profiles by *Lactobacillus paracasei* (A) and *Lactobacillus rhamnosus* (B) with *Streptococcus thermophilus* TH 895 or TH 1435 in axenic or co-cultured growth.

Figure 4.8 – Potential of milk acidification by lactobacilli. Partial skimmed milk (A), samples preparation (B), incubation step (C;D), Ph measurement (E).

Dairy producers search for lactic acid bacteria strains with a fast coagulation potential, and consequently, a fast adaptation (lag) phase and a high metabolic performance. Association between lactic acid culture are a suitable tool to improve the technological and sensorial characteristics of probiotic products. Mutualistic type of interaction was found with *L. rhamnosus* DTA 79 and *S. thermophilus* TH 985 strains speeding up the milk acidification to pH 5.2, showing potentiality for the starter-cultures development (Figure 4.7B). Differently, *S. thermophilus* TH 1435 does not interacts with other lactic acid bacteria. Furthermore, streptococci are not recognized as probiotic microorganisms yet, when it is co-cultured with probiotic strain the intention is to improve probiotic viability and technological properties of the products. These results show that the mutualistic association between lactobacilli and streptococci occurs between specific strains. However, bio-adjusted cultures should be previously studied for these properties prior to use together.

L. paracasei species are commonly used in probiotic dairy products, such as yogurt, cheese, fermented milk, and ice cream (STEFANOVIC et al., 2017). Dose levels of probiotics to reach the desired benefits should be based on levels found to be efficacious in human studies (GANGULY et al., 2011; AKALIN et al., 2017). In a more advanced approach, it shows that when probiotics are intended for gut benefits, they shall be resistant to the passage through the gastrointestinal tract. Foods are good probiotic matrices, since they are consumed daily by humans and animals (LEE et al., 2015; VILLALVA et al., 2017). The Figure 4.8 shows the potential of milk acidification performance.

3.2.3 Effect of honey on lactobacilli survivability

Honey did not have positive impact on *L. paracasei* DTA 83 survivability, but addition of honey (5% w/v) in fermented milk improved the survival of *L. rhamnosus* DTA 79 during CGI. In the presence of honey, the population of *L. rhamnosus* after CGI was more than one log cycle higher than control without honey (Figure 4.9).

Figure 4.9 – Survival of *Lactobacillus rhamnosus* DTA 79 and *Lactobacillus paracasei* DTA 83 in fermented milk with or without honey bee and after gastric and intestinal condition. Asterisk indicate significant difference by Dunnett's test at 0.95 of confidence.

FAVARIN et al. (2015) found that suspending free cells of two *Bifidobacterium* strains in honey solutions resulted in a protective effect, equivalent to the plain microencapsulation with sodium alginate 3% and concluded that microencapsulation and the addition of honey improved the ability of *Bifidobacterium* to tolerate CGI.

3.2.4 Probiotic tablets with viable lactobacilli

L. paracasei DTA 83 and *L. rhamnosus* 79 remained viable in the tablets, at above 7 cfu/tablet up to 3 months at room temperature. Between the third and sixth month of storage, they reduced to 5 and 4 logs, respectively. The major finding of this study was related due to lactobacilli can inhibit *C. albicans* at least by competition and exclusion mechanisms. Moreover, they was able to keep viable in the probiotic tablets. Therefore, probiotic tablets have a potential to reduce oral candidiasis in elderly but *in vivo* study shall be performed to confirm the allegations.

3.2.5 Stress-induced by Processing and Storage of Ice Cream Bar or Ice-Lolly on *Lactobacillus paracasei* Survivability

There was no reduction on *L. paracasei*-DTA 83 viability in ice cream, but in ice-lolly (water based), there was approximately 2 logs of reduction (Figure 4.10).

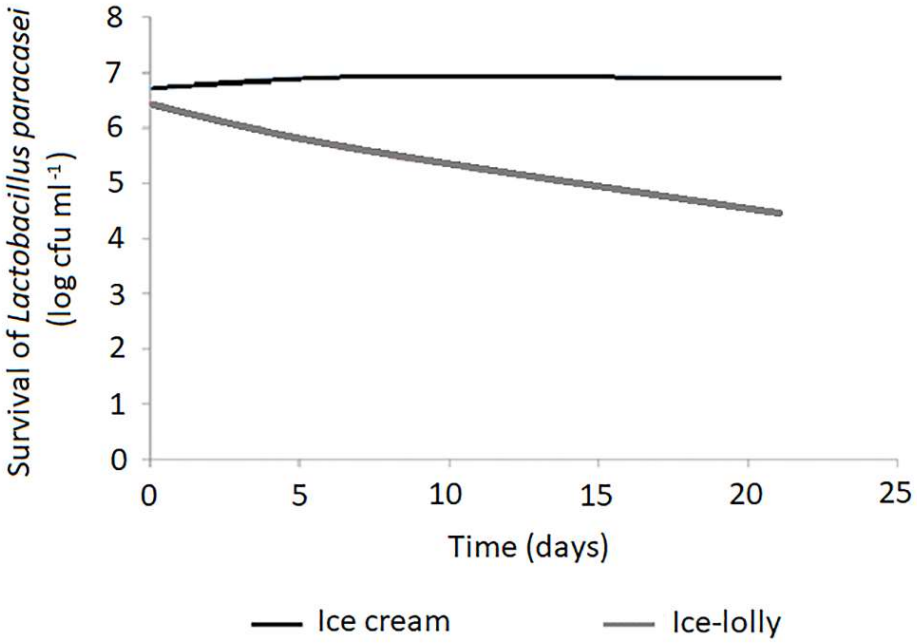

Figure 4.10 - Survival of *Lactobacillus paracasei* after gastrointestinal conditions simulation. ($p<0.05$) when compared with sample before simulation (A). Survival of *Lactobacillus paracasei* in ice cream bar (milk based), and in ice-lolly (water based) (B). (*) indicate significant difference by Dunnett's test to 0.95 of confidence.

In ice cream bar, there was no reduction on the viability of DTA 83, but when in ice-lolly (water based), there was up 2 logs of reduction (Figure 2C). Milk protects *Lactobacillus* during freezing/storage and acidity from the acerola. Probiotic ice cream in a milk based carrier is satisfactory to maintain viability of *L. paracasei* at dose levels above of 6 logs standardized by regulatory agencies (BRASIL, 2007).

Losses in the viability of probiotic bacteria in ice cream unavoidably occur during product formulation, processing, storage, and melting. During these steps, probiotic cells are subjected to different stresses related to pH, acidity, redox potential, freezing, oxygen (especially in overrun step), sugar concentration and osmotic effects, hydrogen peroxide, and mechanical shearing. It seems that the rate of loss of probiotic cells is greater during the freezing process than during storage (MOHAMMADI et al., 2011).

Acerola (*Malpighia emarginata*) is an acid fruit, with high content of vitamins (mainly vitamin C), anthocyanins, flavonoid, minerals such as magnesium, iron, and calcium (FERNANDES et al., 2011). During the crystallization process, the milk or water base can produce different structure at low temperature (PARK et al., 2015). *L. paracasei* survive better in ice cream bar, especially due to milk fat that protects the microorganism during crystallization at low temperature (HAMOSH et al., 1999).

4 CONSLUSIONS

- *L. rhamnosus* DTA 79 and *L. paracasei* DTA 83 are safe strains for human consumption
- There was a mutualistic type association between *L. rhamnonus* DTA 79 and *S. thermophilus* TH 895 speeding up the milk acidification to pH 5.2, showing potentiality for the starter-cultures development.
- *L. rhamnosus* DTA 79 and *L. paracasei* DTA 83 fermented milks resulted in a very high clot consistency and a very low syneresis rate, influencing the intensity of electrostatic protein interactions.
- Fermented milk and ice cream bar are a suitable matrix to delivery both lactobacilli strains ice-lolly is an unsuitable frozen-dessert to vehicle the *L. paracasei* DTA 83.
- Honey addition to fermented milk provide protection to lactobacilli strains increasing its survivability,
- Probiotic tablets can be a potential alternative to reduce oral candidiasis in elderly.

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