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THESIS

Evaluation of recombinant *Lactococcus lactis* strain producing human Pancreatitis-associated Protein I in the treatment of DNBS-induced colitis and 5-Fluoracil-induced mucositis in mice models

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BELO HORIZONTE

Julho – 2016

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Thesis presented to the Post-Graduate program in Genetics as a partial requirement for receiving a Ph.D. in microbial genetics at the Institute of Biological Sciences, Federal University of Minas Gerais.

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SUMMARY

Abstract

Inflammatory Bowel Diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD) are complex intestinal disorders characterized by chronic inflammation of the gastrointestinal tract (GIT). IBD are caused by a deregulation of the mucosal immune system toward the native intestinal microbiota in genetically predisposed individuals, leading to excessive pro-inflammatory immune responses in the GIT. The incidence of both diseases is increasing in developed countries turning CD and UC a main gastroenterological problem as current treatment drugs are associated with serious side effects. Thus, recent research is focusing on the development of new strategies for the treatment of IBD. Probiotic bacteria, especially the ones belonging to the lactic acid bacteria (LAB) group, were shown to be capable of prevent and treat IBD by restoring the balance of disrupted microbiota and suppressing pro-inflammatory immune responses. In order to increase LAB probiotic effect, molecular cloning and expression of anti-inflammatory molecules are being carried out and LAB recombinant strains are also being evaluated as an alternative treatment for IBD. As the use of these strains, especially the model *Lactococcus lactis*, showed to be very effective in fighting intestinal inflammation, its administration as a therapy for treating other human GIT inflammatory diseases, such as mucositis, are also being evaluated. This disorder is a common side effect of patients undergoing radiotherapy or chemotherapy that strongly affects their quality of life. Like IBD, treatment for mucositis is very limited with few medicaments and procedures described to contain inflammation. Therefore, given the need to develop alternative treatments for both IBD and mucositis, this study aimed to test the efficacy of either dairy *L. lactis* NZ9000 or recombinant *L. lactis* strain expressing Pancreatitis Associated Protein I (PAP) to fight inflammation in mouse models of IBD and mucositis. PAP has been reported as a protein with antimicrobial properties that plays important roles to keep intestinal homeostasis. Firstly, we constructed and confirmed the expression of human PAP by recombinant *L. Lactis*. Afterwards, we evaluated the therapeutic effect of this strain in a mice model of dinitrobenzenosulfonic acid (DNBS)-induced colitis. We demonstrated that DNBS injection was able to cause severe acute inflammation in the colon of mice. Moreover, PAP delivery by lactococci protected animals from weight loss, intestinal permeability, and tissue damage. In addition, *L. lactis*-PAP treatment decreased Th1 (IFNγ), Th2 (IL-4, IL-5) and Th17 (IL-17) type-immune responses. It was also observed a higher expression of regulatory TGF-β cytokine and increased amount of T regulatory cells in treated mice. The anti-inflammatory effects of both *L. lactis*-PAP and dairy *L. lactis* NZ9000 strains were also measured in 5-fluoracil mucositis model. We showed that this model was successfully reproduced in BALB/c mice with an induction of acute inflammation in the small bowel of animals. Administration of *L. lactis* NZ9000 harboring pSEC vector without the cDNA of PAP was able to prevent histological damage, reduce eosinophils infiltrate and IgA secretion in the ileum of mice. On the other hand, *L. lactis* expressing PAP preserved mucosal architecture and improved Paneth cells activity. Taking together, our results demonstrate that *L. lactis*, expressing PAP peptide is a promising strategy to treat IBD. Moreover, *L. lactis* NZ9000 strain, derived from dairy *L. lactis* MG1363 used extensively for cheese production, surprisingly presented anti-inflammatory effects in mice injected with 5-FU.

Résumé

Les maladies inflammatoires chroniques de l'intestin (MICI), regroupent la colite ulcéreuse et la maladie de Crohn qui sont des troubles intestinaux complexes caractérisés par une inflammation chronique du tractus gastro-intestinal. Les MICI sont provoquées par un dysfonctionnement du système immunitaire de la muqueuse vers le microbiote intestinal chez les individus génétiquement prédisposés, menant à des réponses immunitaires pro-inflammatoires excessives dans le tractus gastro-intestinal. L'incidence de ces maladies augmente dans les pays développés, deviennent la colite ulcéreuse et la maladie de Crohn comme un grand problème gastroentérologie en tant qu'actuels médicaments de traitement qui sont associés à des effets collatéraux indésirables graves. Ainsi, les dernières recherches se concentrent sur le développement de nouvelles stratégies pour le traitement des maladies inflammatoires intestinales. Les bactéries probiotiques, en particulier ceux qui appartiennent au groupe des bactéries lactiques (BL), se montrent capables de prévenir et de traiter les MICI, en rétablissant l'équilibre du microbiote perturbé et en supprimant les réponses immunitaires pro-inflammatoires. Afin d'augmenter l'effet probiotique sur des BL, le clonage moléculaire et l'expression des molécules anti-inflammatoires ont été réalisées et les souches recombinantes des BL ont été évaluées comme un traitement alternatif pour les MICI. L'utilisation de ces souches, en particulier le modèle *Lactococcus lactis*, a montréson efficacitédans la lutte contre l'inflammation intestinale, son administration comme une thérapie pour traiter d'autres maladies inflammatoires du tractus gastro-intestinal humaine, tels que la mucite, ont été également évalués. Ce trouble est un effet secondaire fréquent sur les patients subissant une radiothérapie ou une chimiothérapie qui affecte fortement leur qualité de vie. Comme les MICI, le traitement de la mucite est assez limité avec quelques médicaments et procédures décrites pour contenir des ulcérations et l'inflammation. Par conséquent, il faut développer des traitements alternatifs pour les MICI et la mucite, cette étude avait pour but tester l'efficacité des produits laitiers *L. lactis* NZ9000 ou de la souche de *L. lactis* recombinant exprimant la protéine associée à la pancréatite I (PAP) pour lutter contre l'inflammation dans des modèles de souris des MICI et mucite. La PAP a été rapportée comme une protéine avec des propriétés antimicrobiennes qui joue un rôle important pour maintenir l'homéostasie intestinale. Tout d'abord, nous avons construit et confirmé l'expression de la PAP humaine par recombinant *L. lactis*. Ensuite, nous avons évalué l'effet thérapeutique de cette souche dans un modèle de souris de Dinitrobenzene acide sulfonique (DNBS) pour induire la colite. Nous avons démontré que l'injection DNBS a pu provoquer une inflammation aiguë sévère dans le côlon de souris. En outre, la livraison de PAP par lactocoques protégé les animaux de perte de poids, de la perméabilité intestinale, et des lésions tissulaires. En plus, le traitement *L. Lactis*-PAP a diminué Th1 (IFN-y), Th2 (IL-4, IL-5) et Th17 (IL-17) de type-réponses immunitaires. On a également observé une expression élevée de régulation de cytokines TGF-β et la quantité de cellules T régulatrices augmenté chez les souris traitées. Les effets anti-inflammatoires des *L. Lactis*-PAP et des souches des produits laitiers *L. lactis* NZ9000 ont également été mesurées dans le modèle d'inflammation des muqueuses 5-Fluorouracil (5-FU). Nous avons montré que ce modèle a été reproduit avec succès dans le modèle de souris BALB/c avec une induction de l'inflammation aiguë dans le petit intestin des animaux. L'administration de *L. lactis* NZ9000 hébergeant pSEC vecteur sans l'ADNc de PAP a été en mesure de prévenir les dommages histologiques,

réduire les éosinophiles infiltrent et la sécrétion d'IgA dans l'iléon de souris. D'autre part, *L. lactis* exprimant la PAP conservé l'architecture muqueuse et l'amélioration de l'activité des cellules de Paneth. En même temps, nos résultats démontrent que *L. lactis*, exprimant PAP peptide est une stratégie prometteuse pour traiter les MICI. En outre, la souche *L. lactis* NZ9000, provenant de produits laitiers de *L. lactis* MG1363 largement utilisé pour la production de fromage, a présenté de manière surprenante des effets anti-inflammatoires chez les souris injectées avec du 5-FU.

Abbreviations

5-FU – 5-Fluoracil

APC – Antigen presenting cell

CD - Crohn's disease

CFU – Colony forming units

DAMP - Damage associated molecular pattern

DTH – Delayed Type Hypersensitivity

DC – Dendritic cell

DNBS – Dinitrobenzenosulfonic acid

DSS - Dextran sulfate sodium

GI – Gastrointestinal

GIT – Gastrointestinal Tract

GALT – Gastrointestinal associated-lymphoid tissue

GRAS – Generally Regarded as Safe

IBD – Inflammatory Bowel Diseases

IEC - Intestinal epithelial cells

IEL – Intraepithelial lymphocytes

IFNγ – Interferon gama

IL - Interleukin

LP – Lamina Propria

LPS – Lipopolyssacharide

M - Microfold cells

MetaHIT – Metagenomics of the Human Intestinal Tract

MAMP – Microbe-associated molecular patterns

MLN – Mesenteric Limph nodes

MPO – Myeloperoxidase

NICE- Nisin Controlled Gene Expression

NF-kB – Nuclear factor kappa B

NLR – NOD-like receptor

PAP – Pancreatitis-associated Protein I

PP – Peyer's patches

PRR – Pattern recognition receptors

ROS – Reactive oxigen species

SCFA - Short chain fatty acids

sIgA – Secretory IgA

SOD – Superoxide dismutase

STAT3 - Signal transducer and activator of transcription 3

Treg- Regulatory T cells

TGF-β – Transforming growth factor β

Th – T helper

TLR – Toll-like receptor

TNBS – Trinitrobenzenesulfonic acid

TNF-α –Tumor Necrosis Fator α

UC – Ulcerative colitis

Usp45 – Unknown secreted protein of 45 KDa

ZO-1 – Zônula occludens 1

THESIS PRESENTATION

I. Collaborations

This work was developed under the collaboration between Laboratory of cellular and molecular genetics (LGCM), Laboratory of Immunobiology (LIB), both from the Institute of Biological Sciences (ICB), Federal University of Minas Gerais (UFMG) – Brazil, and the National Institute of Agronomic Research (INRA), located in Jouy-en-Josas – Ile de France – France. This collaboration was established due to the mutual interest of the mentioned groups to develop new biotechnological and therapeutic applications for Lactic Acid Bacteria, a group of microorganisms conventionally used in food industry to ferment foods for at least 4000 years. This study composes an International Joint thesis between UFMG and the University of Paris-Sud (Saclay - Ile de France - France) as an application to obtain a dual PhD degree in both universities. The thesis was conducted at UFMG under the supervision of Prof. Dr. Vasco Ariston de Carvalho Azevedo and co-supervision of Dr. Marcela Santiago Pacheco de Azevedo (LGCM-UFMG) and at the University of Paris-Sud under supervision of Dr. Luis Bermudez-Humaran. It received financial support from the “Coordination for the Improvement of Higher Education Personnel” (CAPES).

II. Thesis outline

This manuscript presents a general introduction of the thesis. Following, in its first chapter is presented a review describing the use of Lactic Acid Bacteria, especially recombinant *Lactococcus lactis* as an alternative therapy for inflammatory diseases of the gastrointestinal tract such as idiopathic Inflammatory Bowel Diseases (IBD) and mucositis for which conventional treatments are unsatisfactory. Furthermore, the literature review also discusses immunological and anatomical aspects of the intestinal mucosa and its relationship with the microbiota in the context of intestinal homeostasis or disease.

The second chapter presents the justification for this work. The third describes the general and specific goals of the thesis. The results obtained as well as the methodology that was used are presented and discussed in chapters IV and V. The first part (Chapter IV) describes the use of recombinant *Lactococcus lactis* expressing Pancreatitis Associated Protein I (PAP) strain as an alternative strategy for the treatment of Crohn's disease, a type of IBD, in the dinitrobenzenosulfonic acid (DNBS)-induced murine model of colitis. Chapter V presents the work aimed to determine

whether the same strain could alleviate symptoms of mucositis, an inflammatory condition of the gastrointestinal tract of patients under chemotherapy, in a murine model of 5-fluoracil-induced mucositis. Chapter VI and VII presents general conclusion and future directions of the thesis, respectively. This manuscript also presents an appendix section, showing at first the valorization of work (appendix A), containing a list of the main scientific publications obtained during the doctoral studies. A book chapter presented in the section B of the appendix describes a similar subject as the one in the introduction part; however, it shows another focus on the use of LAB as a tool to alleviate intestinal inflammation and as a vaccine to deliver recombinant antigens to mucosal surfaces. In addition to probiotic applications for LAB, the book chapter also discusses other biotechnological and therapeutic applications, including the use of *L. lactis* as a vehicle to deliver DNA vaccines to fight infectious diseases. Finally, references are presented.

GENERAL INTRODUCTION

General Introduction

The Gastrointestinal Tract (GIT) is colonized by a complex community of microorganisms, mainly bacteria classified as being indigenous or transient, known as the intestinal microbiota. Symbiont bacteria, for example short chain fatty acids (SCFA)-producing species from Lactobacillales order and *Faecalibacterium prausnitzii,* contribute to host metabolism and immune system, while occupying a protected environment rich in nutrients (Hooper & Macpherson, 2010; Vos & Vos, 2012, Chang and Lin, 2016). On the other hand, some pathobionts of the GIT, composed mainly by Proteobacteria members, such as *Escherichia coli* and *Clostridium dificille*, might present potential risk of disrupting the integrity of tissues if, for instance, they grow in number (Lebeer et al., 2010; Vangay et al, 2015).

Therefore, the host contains several biological structures that are essential to control bacterial overgrowth and invasion. In this context, the mucous layer protecting the Intestinal epithelial cells (IEC) plays an important role by restricting contact of harmful bacteria with host cells (Johansson et al., 2013; Peterson & Artis, 2014). In addition, specialized IEC, such as Paneth cells, secrete several antimicrobial peptides in order to eliminate microbes that eventually penetrate into the mucus (Salzman *et al*., 2007; Carlsson *et al*., 2013). When pathobionts translocate into the intestinal epithelium, the host immunity is activated in order to eliminate them by producing pro-inflammatory mediators. However, the over production of these compounds represent a risk, as they can inflame the tissue causing intestinal barrier disruption and mucosal dysfunctions to the host (Hidalgo-Cantabrana et al, 2014; Kashyap et al., 2014). Therefore, to keep intestinal homeostasis, specialized immunological structures, known as Gut-associated Lymphoid Tissue (GALT), must be able to specifically recognize and eliminate the pathogenic species, however, tolerate the commensals (Izcue et al, 2009; Carlsson et al., 2013).

Under normal conditions, GALT generates tolerance to commensals mainly through the action of regulatory T (Treg) cells. When the dynamic balance between Treg and activated effector T cells is broken, homeostasis is compromised and may lead to the development of mucosal inflammation in the gut (Strober et al., 2007). In addition to microbiota composition impairment, known as dysbiosis, other factors can influence the proper functioning of the GIT immune system, including individual genetic susceptibility, diet, use of drugs and environmental stress (Ananthakrishnan, 2015). The intersection of these factors may generate an exaggerated pro-inflammatory reaction against the microbiota causing Inflammatory Bowel Diseases (IBD), a group of idiopathic and chronic inflammatory conditions of the GIT, which primarily includes

ulcerative colitis (CD), and Crohn's disease (UC) (Vangay et al., 2015; Velasquez-Manoff, 2015). On the other hand, other factors such as the use of some medicaments can also contribute to the breakdown of this immunological tolerance against commensals commonly observed under normal conditions. It has been described that chemotherapeutic agents, as 5-Fluoracil, widely used in the treatment of advanced solid tumors, may also lead to the development of another inflammatory condition of the GIT known as mucositis. This painful inflammation and ulceration of the mucosal membranes arises as an adverse effect of chemotherapy and thus have great medical importance (Soares et al., 2013; Pedroso et al., 2015).

CD and UC are associated with severe intestinal inflammation, and patients report Gastrointestinal (GI) symptoms as abdominal pain, diarrhea, rectal bleeding and weight loss (Lennard-Jones, 1989; Stepaniuk et al., 2015). IBD represent a global health issue as incidence has increased in several countries and conventional therapy is not sufficient for immediate relief of the symptoms (Molodecky et al., 2012; Ananthakrishnan, 2015). Moreover, drugs adverse side effects present great risks to patients by affecting host immune system and thus increasing susceptibility to infections. In relation to 5-FU-induced mucositis, it has a great clinical impact as well, as cancer therapy is usually discontinued affecting patient's chances of survival (Generoso et al., 2015; Antunes et al., 2015). Up to date, mucositis treatment is also very limited relying mostly on mucosal coatings, cryotherapy, antibiotics and analgesics, which has not been sufficient to alleviate the disease. Hence, scientific community has sought novel therapeutic alternatives to fight both IBD and mucositis. As dysbiosis play a key role in the pathogenesis of both diseases, restoration of the patient's original microbiota via the administration of probiotic bacteria has been proposed, instead of using medicaments. Probiotics, a term defined by the World Health Organization as “live microorganisms administered in adequate amounts that confer a beneficial health effect on the host” (WHO, 2002), are live bacteria and yeasts; however, the majority of strains are gram-positive bacteria belonging to *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Lactococcus* genera. These genera are included in a diverse group of microorganisms known as Lactic Acid Bacteria (LAB), as they are able to convert sugars into lactic acid (Stiles & Holzapfel, 1997; Carr et al., 2002).

Recent data demonstrates promising results on the use of LAB strains to treat both UC and CD in pre-clinical tests, by restoring impaired microbiota and suppressing pro-inflammatory immune responses (Ljungh and Wadström, 2006; Santos Rocha et al., 2014; Quinto et al., 2014; Luerce et al., 2014; Thomas, 2016). As dysbiosis has been recently implicated in the overall development of intestinal mucositis, probiotic approach, such as LAB, especially *Lactobacillus* genus, are being used to treat the

disease in animal models, which have shown interesting results (Whitford et al 2009; Tooley et al, 2011; Prisciandaro et al, 2012; Wang et al., 2013).

In order to boost LAB probiotic characteristics, some strains have been modified using genetic engineering techniques to express heterologous proteins of medical interest, especially those with anti-inflammatory properties. Among LAB species, *Lactococcus lactis* is the most studied considering its metabolic pathways, physiology and genetics. Therefore, several tools for both cloning and heterologous expression became available for this bacterium and are allowing the construction of different recombinant strains capable of expressing anti-inflammatory molecules (Moreno et al, 2015; Wang et al., 2016).

Among all heterologous expression systems available for *L. lactis*, Nisin-Controlled Gene Expression system (NICE) is the most used. In brief, it is based on three genes involved in the production and regulation of a peptide named nisin in a genetically engineered *L. lactis* strain carrying an expression vector containing the nisin inducible promoter. NICE system has been successfully used to express and address a variety of heterologous proteins of medical and biotechnological interest, and, thus, is ranked as one of the best genetic tool already developed for gene cloning and expression in *L. lactis* (Nouaille *et al*., 2003; Le Loir *et al*., 2005).

In recent years, several research groups have obtained satisfactory results using recombinant *L. lactis* as the strains have been shown to reduce inflammation after oral administration in pre-clinical trials (Steidler et al., 2000; LeBlanc et al., 2011; Steidler et al., 2003; Del Carmen et al., 2014; Strukelj et al., 2014). In fact, a phase II clinical trial based on a genetically engineered *L. lactis* strain was carried out in IBD patients, suggesting the feasibility of mucosal therapy using recombinant lactococci (Braat et al., 2006). This trial opened the door for testing genetically modified *L. lactis* to treat other intestinal inflammatory conditions for which conventional treatment has been unsatisfactory, such as mucositis. Moreover, the use of genetically modified strains of *L. lactis* to produce proteins with anti-inflammatory properties has been promising to treat mucositis in phase II clinical trial as well (Caluwaerts et al., 2010).

Taken together, *L. lactis* proved to be a safe vehicle for delivering novel molecules with anti-inflammatory properties to treat GI disorders (IBD and mucositis). Key elements in the host-microbiota relationship, such as antimicrobial peptides, are considered potential candidates to be cloned in *L. lactis* as the oral delivery of such peptides could prevent microbe-driven inflammation and promote intestinal homeostasis. Antimicrobial peptides that are involved in maintaining the epithelial barrier could represent an interesting candidate to prevent microbiota-driven inflammatory signaling. Various antimicrobial peptides, such as defensins, cathelicidins

and histatins, that are naturally produced by Paneth cells, seems to play a critical role in intestinal homeostasis and their biological activity has been reported to be compromised in IBD patients (Clevers and Bevins, 2013; Peterson & Artis, 2014). Different research groups are investigating whether the administration of these peptides could have a protective effect against intestinal inflammation (Seo et al., 2012; Wong et al., 2012).

Among the antimicrobial peptides, Reg3A, also known as pancreatitis associated protein I (PAP), has been extensively studied due to its protective effect in several organs of human and animals during the inflammatory process (Abe et al., 2000; Graf et al., 2006; Zhang et al, 2004). In addition, human PAP expression has been shown to increase in IBD patients with CD or UC, a fact that has prompted this peptide as an object of study (Medveczky et al., 2009; Maranduba et al., 2015). Several studies have recently pointed out that PAP apparently exert bactericidal activity against Gram-positive bacteria (Cash et al., 2006b; Mukherjee et al., 2008; Mukherjee et al., 2014). Thus, it appears that activation of PAP expression in the intestinal mucosa is required in order to generate a protective response against intestinal microbiota during bacteria-driven inflammatory events.

As PAP has been able to reestablish homeostasis in inflamed organs such as the skin, pancreas and lung, and, moreover, is involved in the intestinal barrier function, its use to alleviate GIT inflammatory disorders such IBD and mucositis is very attractive. In this context, the use of *L. lactis* as a vehicle to deliver human PAP to intestinal mucosal surfaces represents a suitable strategy to evaluate its protective effects. Hence, this study was carried out to construct and evaluate the prophylactic effect of recombinant *L. lactis* expressing the antimicrobial peptide PAP in the treatment of IBD and mucositis using murine models of inflammation and *in vitro* assays.

Chapter I

Review: Use of wild type or recombinant Lactic Acid Bacteria as an alternative treatment for gastrointestinal inflammatory diseases: A focus on Inflammatory Bowel Diseases and mucositis

Chapter I - Review: Use of wild type or recombinant Lactic Acid Bacteria as an alternative treatment for gastrointestinal inflammatory diseases: A focus on Inflammatory Bowel Diseases and mucositis

This chapter presents a review on the use of wild type or recombinant Lactic Acid Bacteria (LAB), especially *Lactococcus lactis*, as an alternative therapy for inflammatory disorders affecting the gastrointestinal tract (GIT), as idiopathic Inflammatory Bowel Diseases (IBD) and mucositis for which conventional treatments are unsatisfactory. The literature review includes descriptive topics on several immunological aspects of the host-microbiota relationship in the context of intestinal homeostasis or disease. Moreover, it describes current animal models used to explore new alternative treatments for GIT tract inflammation; major outcomes obtained from recent studies on wild type and recombinant LAB, especially recombinant *L. lactis* strains expressing anti-inflammatory molecules to treat both IBD and mucositis. At last, the role of Pancreatitis-associated Protein I (PAP) in intestinal homeostasis is discussed and the use of recombinant *L. lactis* expressing PAP as a treatment for GIT inflammation is glimpsed. This manuscript will be submitted as a review article in Microbial Cell Factories).

Use of wild type or recombinant Lactic Acid Bacteria as an alternative treatment for gastrointestinal inflammatory diseases: A focus on Inflammatory Bowel Diseases and mucositis

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Abstract

The human gastrointestinal tract (GIT) is highly colonized by bacterial communities, which lives in a symbiotic relationship with the host in normal conditions. It has been shown that a dysfunctional interaction between the intestinal microbiota and the host immune system, known as dysbiosis or dysbacteriosis, is a very important factor responsible for the development of different inflammatory conditions of the GIT, such as the idiopathic inflammatory bowel diseases (IBD), a complex and multifactorial disorder of the GIT. Dysbiosis has also been implicated in the pathogenesis of other GIT inflammatory diseases such as mucositis usually caused as an adverse effect of chemotherapy. Both diseases have become a great clinical problem, as current treatment drugs are associated with serious side effects and the therapeutic effect commonly observed is relatively limited. Therefore, many research groups have been focusing on developing new strategies for the treatment of IBD and mucositis. In this review, we show that Lactic Acid Bacteria (LAB) have been capable at preventing and treating both disorders demonstrating to be safer and more effective when compared to conventional treatment. In addition, we present the most current studies on the use of wild type or genetically engineered LAB strains designed to express anti-inflammatory proteins as a promising strategy in the treatment of IBD and mucositis.

Keywords: Inflammatory bowel diseases, mucositis, Lactic Acid Bacteria, *Lactococcus lactis*, PAP, RegIII.

Introduction

The gastrointestinal tract (GIT) of mammals is a highly complex biological system whose main function is the digestion of food. This system is divided anatomically into (i) upper GIT, which comprises the mouth, pharynx, esophagus and stomach and (ii) lower GIT, which includes the small bowel (divided into duodenum, jejunum and ileum) and large bowel (separated into cecum, colon, rectum and anus). The upper GIT is responsible for continuing the chemical digestion process started in the mouth and for transporting food through the GIT, thus facilitating degradation and absorption of nutrients in the small intestine. The digestive process continues throughout the lower GIT, where the majority of nutrients are absorbed in the jejunum. The final stages of digestion occur in the large bowel where water and nutrients in excess are removed from the intestinal waste and transported to bloodstream. The remaining material moves along the colon, drying slowly and forming a solid substance called stool. As the lower GIT is rich in nutrients, particularly the ileum and colon, there is a dynamic community of microorganisms, known as intestinal microbiota, which plays a role in intestinal physiology and regulation (Gill et al, 2006; Sartor, 2008; Kumar et al, 2011).

Intestinal microbiota

The GIT is colonized by a complex community of microorganisms, mainly bacteria that are classified as being indigenous or transient. The first ones are well adapted to the intestinal luminal environment, such as extreme pH changes along the GIT and can colonize different parts of the GIT. In turn, the transient microorganisms coming from external environments, usually ingested through eating and drinking, are not able to survive more than a few days within this conditions and does not colonizes the GIT. Although being transient, some species are often found in the intestine because they are present in some foods daily ingested by human beings (i.g yogurts, cheeses, fermented milk). The intestinal microbiota comprises 500 to 1,000 species of bacteria that is estimated to exceed the total number of mammalian cells by a factor of 10 or more (Ley et al, 2006; Artis, 2008; Velasquez-Manof, 2015) However, a recent work demonstrated that the ratio of bacteria to human cells is actually 1:1 (Sender et al., 2016).

The composition of the intestinal microbiota in humans has been studied by microscopic observation or through culture of intestinal biopsy specimens and stool culture. This approach, however, has presented to be limited as only 30% of the bacteria found in the GIT are cultivable on artificial media. In this context, techniques of

molecular biology as the rRNA 16S gene sequence analysis and metagenomic sequencing has allowed a major breakthrough in the identification of the GIT bacterial species (Eckburg et al., 2005; Hattori & Taylor, 2009; Arumugam et al., 2011; Mayo Muñoz, 2015).

Many academic and industrial consortiums, such as the MetaHIT (Metagenomics of the Human Intestinal Tract), have attempted to characterize the microbiota associated with the human GIT through genomic sequencing, thus giving a more detailed description of the human intestinal microbiota composition and its function (Thompson et al, 2014;. Uyeno et al, 2015). The MetaHIT consortium published in 2010 a complete illumina-based metagenomic sequencing from faecal samples of 124 European individuals. It was characterized 3.3 million nonredundant microbial genes. The authors also showed that around 40% of the genes of each individual are shared with more than half of individuals. Essentially 99.1% of the genes identified are of bacterial origin, the remaining of archaea and only 0.1% of eukaryotic or viral origin. Furthermore, 75 species were identified in common with 50% of the subjects, and 57 species in common with 90% of the subjects (Qin et al., 2010). Nowadays, it is known that most species found in mammalian GIT can be classified into three phyla: gram-negative Bacteroidetes (23 to 48%), gram-positive Firmicutes (48 to 76%) and gram-positive Actinobacteria (0.2 to 38%) (Eckburg et al., 2005; Qin et al, 2010; Velasquez-Manof, 2015).

The bacterial colonization of human GIT starts soon after birth, when children are exposed to a complex microbial population during passage through the vaginal canal. Some studies indicate that children born in natural delivery acquire a microbiota resembling their own mother's vaginal microbiota, with the prevalence of *Lactobacillus*, *Prevotella* and *Sneathia* spp species (Bernstein, 2014; Geuking et al, 2014). Children born by caesarean section (C-section) show a very different microbiota composition because they are not exposed to bacteria present in the birth canals of their mothers. Thus, the very first bacterial colonization of these newborns occurs from other sources, such as the skin during breast-feeding. It has been described that species from *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* genus are more frequent in children born by C-section (Bernstein, 2014; Geuking et al, 2014).

During the first years of life, the microbiota composition is relatively simple and varies greatly among individuals. It has been reported that *Escherichia coli* and streptococci are the most common organisms isolated from the upper GIT shortly after birth (Velasquez-Manof, 2015; Vangay et al, 2015). These species are responsible to create a favorable environment promoting the colonization of anaerobic bacteria, in which *Bifidobacterium* and Bacteroidetes are the most prevalent group during the first

days after birth. In the course of the first two years of life, it has been reported an increase in lactobacilli, clostridia and *Fusobacterium* bacteria groups. Across time it was demonstrated by phylogenetic analysis that microbiota composition becomes more stable in older subjects (Dominguez-Bello et al, 2010; Bernstein, 2014). Gradually, it evolves becoming similar to the microbiota of adults, dominated by anaerobic bacteria, such as Bacteroides, as their capability to digest complex sugars, mantaining ancient symbiotic relationship with the host and central position in the gut microbiome might help them to sustain abundance over time (Rajilic-Stojanovic et al., 2007, Neu & Rushing, 2011; Bernstein, 2014; Rehman et al., 2015). However, although microbiota is stable in older persons, it can be altered in short term duration by dietary intervention. Dietary intake shifts, especially of nondigestible carbohydrates, may change the composition of some species of the gut microbiota by increasing the number of *Bifidum bacterium* spp and *Faecalibacterium prausnitzii*, although species does not respond in the same way in all subjects (Ramirez-Farias et al., 2009; Martínez et al., 2010; Flint et al., 2012).

The diversity and abundance of bacterial populations in healthy adults varies along the GIT (Wang et al., 2005; Zoetendal et al, 2006; Sartor, 2008; Velasquez-Manof, 2015). In the stomach and duodenum can be found a small number of microorganisms, up to 10^3 bacterial cells per gram of intestinal content, which are adhered to the mucosal surface or in transit through the GIT (Guarner, 2006; O'Hara & Shanahan, 2006; Lennon et al, 2014). Streptococci and lactobacilli are among of the most common group of bacteria found in this part of the intestine (Wang et al., 2005; Walter, 2008; Prakash et al, 2011). The bacterial population increases gradually along the jejunum and the ileum, reaching numbers around 10^4-10^7 per gram of intestinal content. However, it is in the lower GIT (colon) that the highest density of bacteria population is encountered, reaching a number of 10^{11}-10^{12} per gram, turning this area one of the most complex microbial ecosystems known to date on Earth (Sartor, 2008; Prakash et al, 2011).

Intestinal microbiota keeps a symbiotic relationship with the host, making fundamental contributions to host metabolism, while occupying a protected environment rich in nutrients (Hooper & Macpherson, 2010; Vos & Vos, 2012). In this context, the intestinal microbiota plays four major roles: (i) nutrition functions, such as vitamin production, digestion and absorption of nutrients, calcium and iron; (II) development and modulation of the host immune system; (III) trophic functions on the proliferation and differentiation of the intestinal epithelium; and (iv) prevention of pathogen adhesion, colonization and infection through the intestinal epithelial barrier

(Guarner, 2006; Flint et al., 2012; Moreno-Indias et al., 2014; Goldsmith and Sartor, 2014; Velasquez-Manoff, 2015).

The intestinal epithelial barrier and the associated microbiota

The mucosal surfaces lining the GIT separate the host from the external environment by a delicate epithelial barrier, composed mainly by a single layer of intestinal epithelial cells (IEC) (or enterocytes) connected together by tight junctions. It forms the first physical barrier between the lamina propria (LP) and the contents of the intestinal lumen restricting the trans-epithelial movement of particles and hydrophilic molecules of up to ~ 2 kDa between extra and intracorporeal regions (Gewirtz & Madara, 2001; Izcue et al, 2009; Carlsson et al., 2013; Trujillo et al, 2014).

The IEC show apical protrusions named microvillus that significantly increases the absorptive and secretory surface of the intestinal epithelium expanding the area to 400 m^2. Moreover, it has a polarized arrangement; where the apical zone is faced to the lumen while the basal area is faced to the LP, where blood vessels can be found. The integrity of this epithelium is maintained by the fact that this surface is replaced every two/three days (Geukin et al., 2014).

Mucosal surfaces are considered the main sites of interaction between the external environment and the host tissues. Microfold cells (M cells) are epithelial-type cells located between enterocytes able to capture, by endocytosis or phagocytosis, some proteins or bacteria present in the luminal content and transport them to the sub-epithelial dome region, an area populated by immune cells (Peterson & Artis, 2014). However, these surfaces also represent the largest portal for the entry of many potential pathogenic microorganisms. Therefore, the epithelial barrier is believed to be the body's first line of defense that keeping most potentially pathogenic species out of the body (Izcue et al, 2009; Peterson & Artis, 2014).

Besides acting as a physical barrier, preventing the invasion of pathogenic microorganisms, the epithelial barrier minimizes the contact between luminal microorganisms and the surface of IEC through the production of a mucus very resistant to bacterial penetration by specialized IEC called goblet cells (Hooper & Macpherson, 2010; Kim and Ho, 2010; Peterson & Artis, 2014). The number of goblet cells among absorptive epithelial cell types increases gradually from proximal small intestine (4%) to distal colon (16%), similar to the proportion of microbial organisms numbers distribution along the lower GIT (Kim and Ho, 2010). The mucus is formed mainly by mucin-type glycoproteins, for example MUC2, MUC5AC, MUC5B, and MUC6 that contribute to the formation of gel-like structure, while MUC1, MUC3 and MUC17

imply a bioactive role in maintaining the integrity of the surface epithelial layer (Hollingsworth et al., 2004; Andrianifahanana et al., 2006; Johansson et al., 2013).

In the small bowel, a thin layer of mucus relatively permeable to bacteria and nutrients can be observed (Johansson et al, 2011; Vaishnava et al, 2011). In contrast, in the large bowel there is a thick layer of mucus which is divided into two strata: an inner layer adhered to the IEC, that has high density and is virtually devoid of bacteria and an outer layer, which is less compact and inhabited by microorganisms. Thus, to control bacteria that could possibly penetrate the thinner mucus layer, mammalian beings have developed another type of specialized IEC, known as Paneth cells which are located in the crypts of the small intestine, capable of secreting antimicrobial peptides (Salzman *et al*., 2007; Carlsson *et al*., 2013). These peptides, also named defensins, are broad-spectrum antimicrobial molecules that confer bactericidal activity against either gram-positive or gram-negative bacteria, through different biological mechanisms. For instance, these peptides can interact and bind to phospholipids groups from the bacterial membrane creating a pore that can cause membrane disruption (Cash et al, 2006a; Salzman et al., 2007; Clevers & Bevins, 2013). It has been shown that the presence of commensal bacteria is crucial in stimulating Goblet and Paneth cells to secrete the mucus and produce defensins, respectively (Kim and Ho, 2010; Johansson et al., 2013).

Therefore, in order to have an impact on body, microbiota needs to be recognized by mammalian cells. Actually, commensal bacteria and their host have co-evolved diverse biological mechanisms turning possible this cross talk. For example, it was demonstrated that pattern recognition receptors (PRR), including Toll-like receptors (TLR), expressed by IEC are able to recognize microbe-associated molecular patterns (MAMP) of the commensal microbiota. These MAMP are microbial components, such as lipoproteins, nucleic acids (RNA and unmethylated CpG dinucleotides) lipopotheic acids, lipopolysaccharide (LPS), flagellin and peptidoglycan (Akira & Takeda, 2004; Artis, 2008; Winkler, 2007; Peterson & Artis, 2014). The recognition of a MAMP transduce signals that subsequently activates innate immune responses (Winkler, 2007; Peterson & Artis, 2014). Thus, depending from the origin of a MAMP, either pro or anti-inflammatory immune responses can be elicited. Certain bacterial species belonging to the commensal flora elicits a more pro-inflammatory immune response after binding to TLR while others induces a more anti-inflammatory profile (Lakatos et al., 2006; Lebeer et al., 2010; Royet et al., 2011). Generally, the first ones are considered to be potentially pathogenic species, known as pathobionts, composed mainly by Proteobacteria members, such as *Escherichia coli*, and species from the phylum Firmicutes, as *Clostridium dificille* and *Enterococcus faecalis*. The

second ones are the symbionts, for example species from Lactobacillales order and the phylum Actinobacteria (*Bifidobacterium* sp. and *Propionibacterium* sp.) (Lebeer et al., 2010; Vangay et al, 2015).

The best-characterized PRR are the TLR expressed in the surface of IEC or in the membrane of endosomes vesicles inside of the cell. There are nine types of TLR described in humans; all contains conserved leucine-rich repeats and cysteine-rich repeats in the N-terminal portion of the protein, followed by both transmembrane and an intracellular domain at the C-terminal end, that activates signaling pathways. TLR can bind to either exogenous or endogenous ligands, such as modified lipids or other soluble mediators after its activation. For example, LPS is recognized by TLR4 and flagellin by TLR5, both important virulence factors involved in bacterial adhesion and invasion of the epithelial barrier. TLR3, TLR7, TLR8 and TLR9 are expressed in endosomes and recognize nucleic acids: TLR3 interact with double-stranded RNA, TLR7 and TLR8 recognize single-stranded RNA, and TLR9 binds to unmethylated CpG dinucleotides, quite abundant in bacterial genomes (Cario, 2010; O'Neill et al, 2013). In order to extend the spectrum of ligands, these receptors are able to form heterodimers; for instance TLR2-TLR1 heterodimer attach to lipopeptides of gram-negative bacteria while TLR2-TLR6 recognizes lipopeptides of gram-positive bacteria (O'Neill et al., 2013). Other PRR include NOD-like receptors (NLR), which are located in the cytoplasm of epithelial cells and are also capable of interacting with intracellular ligands (Artis, 2008; Iwasaki, 2007; Winkler, 2007; O'Neill et al, 2013). RIG-like receptors (RLR) and C-type lectins receptors are other examples of PRR. It has been shown that upon activation all these receptors are able to induce innate immune responses against bacteria. Therefore, their expression and proper functioning is extremely important to link innate and adaptive immunity, protecting the body against invading pathogens, or to promote immune tolerance towards commensals. In fact, the expression of PRR and their interaction with the microbiota is very important for the healthy development of the host immune system (Wells et al., 2010; Royet et al., 2011; Sommer and Bäckhed, 2013; Kashyap et al., 2014).

GIT immune system

In order to peacefully co-exist with the complex microbiota and in the same time generate protection against pathogenic species, the intestinal tract contains gut associated lymphoid tissue (GALT) and other immunological structures coupled to the intestinal mucosa necessary to assist this balance (Neish, 2009; Sansonetti, 2004; Hidalgo-Cantabrana et al, 2014). The GALT can be divided into two parts (i) the Peyer's patches (PP) located in the small bowel, which are connected to the

mesenteric lymph nodes (MLN), and (ii) the intraepithelial lymphocytes (IEL), distributed throughout the intestinal tract (Bienenstock & Befus, 1980; Izcue et al, 2009; Mowat, 2003; Hooper & Macpherson, 2010). The PP are covered by M cells and formed mainly by aggregates of B and T lymphocytes as well as Antigen-Presenting Cells (APC), such as dendritic cells (DC) and macrophages, located in the mucosal sub-epithelial layer. The major specialized sites designed for antigen presentation are the PP and MLN, where the link between innate and adaptive defense occurs. After antigen presentation by APC, naïve T helper (Th) cells are activated giving rise to a clone of effector cells which are CD4+. Basically, these cells are divided into three sub-types based on their repertory of cytokine production: T helper type 1 (Th1), type 2 (Th2) and type 17 (Th17). In the other hand, naïve Th cells can also differentiate into regulatory T cells (Treg) after being activated by certain APC, such as DC103+ cells. Cytokines produced by effector Th cells or Treg are able to activate B-lymphocytes, which differentiate into plasma cells that secrete large quantities of immunoglobulins, also known as antibody (classes: IgM, IgD, IgG, IgE, or IgA) (Baker et al., 2010; Harrison and Powrie, 2013).

Interleukin-12 (IL-12) secreted by DC subsets in the intestinal LP drive mucosal T helper 1 cell differentiation while IL-23 production drive the differentiation into Th17 cells and IL-4 secretion induces the proliferation of Th2 lymphocytes (Trinchieri, 2003; Zaph et al, 2008). Usually, the proliferation of Th1 cells is observed when the body needs to fight against intracellular pathogens (viruses or bacteria), as these cells produces more pro-inflammatory cytokines (IL-1, tumor necrosis factor α -TNF-α-, IL-12 and interferon- γ -IFN-γ) (Martinez et al, 2008; Gordon and Martinez, 2010). Immune response mediated by Th2 cells is important to combat infection with helminthes (Spencer et al., 2011). Th17 responses, in turn, operate primarily in intestinal infections by extracellular bacteria and fungi through the secretion of IL-17 and IL-22 cytokines. It was observed that IL-17 induces inflammatory reactions, while IL-22 plays a pleiotropic activity depending on the tissue and the condition in which it was produced (Blaschitz and Raffatellu, 2010). Some studies has appointed its involvement in the maintenance of epithelial cell integrity, especially in stimulating tissue repair and secretion of antimicrobial peptides by Paneth cells (Ouyang & Zheng, 2008; Ivanov et al, 2009; Littman & Rudensky, 2010). Finally, Treg cells promote tolerance, preventing inflammatory reactions against the commensal microbiota by producing immunosuppressive cytokines, such as IL-10 and TGF-β (Harrison and Powrie, 2013; Chistiakov et al., 2015)

The MLN serve as a crossroad between the peripheral and mucosal recirculation pathways. Antigens from the lymph are brought by APC to the PP where

they are presented to naïve lymphocytes stimulating adaptive immune responses (Th1, Th2, Th17 or Treg). Intestinal IEL in humans are primarily composed of CD8+ cytotoxic T cells that accumulate on injured sites where they secrete cytotoxins (perforin, granzyme and granulysin) causing lysis or apoptosis of the cell in the case of an infection (Boschetti et al., 2016). Another IEL that can be found are the ones expressing the gamma delta (γδ) receptor that has been shown to promote repair of the intestinal barrier (Witherden and Havran, 2013). Taking together, GALT plays a key role in the induction of immune responses against pathogens controlling the balance between tolerance and active immunity (Abreu-Martin & Targan, 1996; Hooper & Macpherson, 2010).

Crosstalk between the microbiota and the host

Although commensal microorganisms show beneficial effects on the host, some pathobionts of the GIT might present potential risk of disrupting the integrity of tissues if, for instance, they grow in number. As described above, the host epithelium contains several biological structures that are essential to control bacterial overgrowth and invasion. However, some species have different mechanisms of evasion of host protective responses and, thus, are capable of ensuring their propagation. When pathobionts translocate the intestinal epithelium, the host immunity is activated and usually enough to eliminate them. Nonetheless, the over production of pro-inflammatory cytokines necessary to eliminate this type of microorganisms would represent a risk once inflammation may also be problematic causing cell disruption and infection to the host. Therefore, to reach intestinal homeostasis, GALT must be able to recognize and eliminate specifically these pathogens that are usually numerically rare compared to the number of commensal microorganisms that inhabit the GIT (Izcue et al, 2009; Carlsson et al., 2013).

Detection of virulence factors from pathogenic species is mediated by PRR (i.g toll-like receptors) located on the apical surface of IEC. TLR signaling results in the activation of nuclear factor-kappa B (NF-kB) pathway, allowing the transcription of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and chemokines involved in the immune responses against intracellular pathogens, as mentioned above, generating tissue damage (Neish, 2009; Winkler 2007 O'Neill et al, 2013; Peterson & Artis, 2014). Differently, it has been demonstrated that commensal microbes are able to strengthen the epithelial barrier through PRR activation (Lakatos et al., 2006; Lebeer et al., 2010). Moreover, they are capable of stimulating both defensins and mucins production fortifying tight junctions barrier, instead of triggering inflammatory processes (Salzman *et al*., 2007; Carlsson *et al*., 2013; Johansson et al., 2013). Furthermore, the

commensals were reported to induce signals of immunological tolerance, such as secretion of TGF-β cytokine that inhibit NF-κB signaling pathway inside epithelial cells. It has been shown that activation of TLR by commensal bacteria also promote the development of CD103 dendritic cells, which are responsible to drive the development of Treg cells (Uematsu et al. 2008 ; Kinnebrew et al., 2012; de Kivit et al., 2014). It is known that Treg cells suppress effector T cell responses mainly through the production of IL-10 and TGF-β. IL-10 inhibits IL-12 production by macrophages and also inhibits the activity of pro-inflammatory DC. TGF-β produced by Treg cells was demonstrated to prevent the recruitment of granulocytes, suppressing the activation of macrophages, neutrophils and endothelial cells. In addition, Treg cells expressing TGF-β and IL-10 drive B cells to undergo antibody class switching to produce IgA antibody, the major humoral defense of mucosal surfaces. Secretory IgA (sIgA) contributes to mucosal homeostasis through a process known as immune exclusion. sIgA is able to bind to opportunistic pathogens avoiding its dissemination throughout the body (Brandtzaeg, 1998; Suzuki & Fagarasan 2008 ; Brown et al., 2013).

Finally, the GIT provides a highly effective defense to infectious agents while inducing tolerance to commensal bacteria (Kutzler and Weiner, 2008; Artis & Peterson, 2014). Intestinal barrier dysfunction generates an imbalance between immune responses observed for protective and harmful intestinal bacteria and thus contributes for the onset of numerous inflammatory conditions of the GIT (Artis, 2008; O'Neill et al, 2013).

Intestinal homeostasis breakdown rising inflammatory conditions of the GI tract

GIT is constantly being challenged by antigens from the intestinal microbiota. Under normal conditions the intestinal mucosa generates tolerance to commensals mainly through the action of Treg cells. When the dynamic balance between Treg and activated effector T cells is broken, homeostasis is compromised and may lead to the development of mucosal inflammation in process known as dysbiosis (Strober et al., 2007). Multiple factors can influence the proper functioning of the GIT immune system, including individual genetic composition, diet, use of drugs and environmental stress. Other studies has also provided some evidence that the reduction of certain bacterial populations which naturally induces immunological mechanisms of tolerance within the body, such as species belonging to *Bifidobacterium* and *Lactobacilli* genera, is associated with the development of mucosal inflammation. The intersection of these factors generates an exaggerated pro-inflammatory reaction against the microbiota causing Inflammatory Bowel Diseases (IBD), a group of idiopathic and chronic

inflammatory conditions of the GIT, which primarily includes ulcerative colitis (CD), and Crohn's disease (UC) (Vangay et al., 2015; Velasquez-Manoff, 2015).

Other factors such as the use of some medicaments can also contribute to the breakdown of this immunological tolerance against commensals commonly observed under normal conditions. It has been described that chemotherapeutic agents, as 5-Fluoracil, widely used in the treatment of advanced solid tumors, may also lead to the development of another inflammatory condition of the GIT known as mucositis. This painful inflammation and ulceration of the mucous membranes arises as an adverse effect of chemotherapy and thus have great medical importance (Soares et al., 2013; Pedroso et al., 2015).

Inflammatory Bowel Diseases

IBD is a group of chronic, complex and relapsing inflammatory conditions of the GIT. The exact etiology is still not completely known, however, some factors such as environmental factors, emotional distress, genetic background, diet, smocking are implicated in the development of this conditions (Ko and Auyeung, 2014; Ananthakrishnan, 2015). Ulcerative colitis (CD) and Crohn's disease (UC) are the main types. Clinical symptoms of both are similary found in patients, such as abdominal pain, diarrhea, rectal bleeding and weight loss (Lennard-Jones, 1989; Stepaniuk et al., 2015). Relapse symptoms can last days, weeks, or even months (Falvey et al., 2015). CD is characteristically discontinuous, with inflamed areas that can be found in all the layers of the intestinal wall while UC is characterized as a continuous and superficial inflammation limited to the colon (Cho, 2008; Tontini, 2015). The incidence of these diseases varies widely across countries, however, in recent years, it has increased considerably worldwide being considered a global public health problem. This increase has been associated with the modern lifestyle that includes the ingestion of processed foods usually high in fats and sugar and low in fiber (Shivananda et al., 1996; Molodecky et al., 2012; Ananthakrishnan, 2015). It was also reported that Caesarean delivery and the inappropriate use of antibiotics, especially during childhood when the microbiota has not yet been established, are factors that can contribute to the development of intestinal inflammation (Nell et al, 2010; Koren et al., 2013; Ananthakrishnan, 2015; Vangay et al., 2015). Both CD and UC have different immunological aspects when it comes to innate and adaptive immunity (Duchmann et al., 1995; Mow et al., 2004). Pro-inflammatory cytokines and chemokines are overexpressed in IBD patients; however, the predominant set of cytokines observed in CD patients is the ones secreted by Th1 and Th17 cells (IL-12, IL-23, IL-27, IFN-γ)

whereas in UC patients a Th2 immune response, characterized by the production of IL-4 and IL-13, appears to be predominant (Sartor, 2006; Koren et al, 2013).

Dysbiosis in IBD

It has been reported that patients with IBD have a reduced diversity of GIT microbiota, characterized by the depletion of commensal species belonging to the phylum Firmicutes and Bacteroidetes (Walker et al., 2011; Kamada, 2016).

Metagenomic studies has pointed out a decreasing in Short chain fatty acids (SCFA) metabolism genes such as butyrate, which play an inportant role on the maturation of regulatory T cells, in the gut microbiota of IBD patiens (Chang and Lin, 2016). Moreover, it was observed that patients have a reduction in the amount of many SCFA-producing species from Clostridium groups, remarkably *F. prausnitzii* (Sokol et al., 2008; Vangay et al, 2015; Takahashi et al., 2016). Microbiota patterns such as the reduction of *F. prausnitzii* has been consistently associated to CD patients, during period of active disease or remission, from different geographical regions including Europe and South Asia and may thus serve as reliable clinical marker (Manichanh et al., 2012; Rehman et al., 2015). The reduction of especies from Lactobacillus and Bifidobacteria genus have an important role in IBD pathogenesis as well, as these bacteria can down-regulate the expression of key proinflammatory cytokines and chemokines in the gut (Manichanh et al., 2012). In the other hand, other species considered as pathobionts, such as *Mycobacterium aviumparatuberculosis*, *Clostridium difficile*, *Ruminococcus gnavus* and enterobacteria are increased (Peterson et al., 2008; Clayton et al, 2009; Qin et al, 2010; Sartor, 2010; Schwiertz et al, 2010). Sulphate-reducing pathobionts are considered a key factor in the initiation and maintenance of IBD as these bacteria reduce disulfide bonds composing the mucus barrier, thereby allowing exposure of the host cells to pathogenic bacteria and toxins (Ijssennagger et al., 2016). Therefore, these patients develop a certain predisposition for the colonization of facultative pathogens such as invasive *E. coli,* that express several virulence factors involved in adhesion and invasion of the epithelial barrier (Lodes et al., 2004; Sitaraman et al, 2005). Moreover, colonization by pathogenic species, such as *M. paratuberculosis*, *Listeria monocytogenes*, or *Helicobacter species* can worsen the symptoms of IBD as these bacteria can activate proinflammatory-signaling cascades into the host (Sartor, 1997; Lebeer et al., 2010).

Genetic background in IBD

Up to date, more than 160 genes, previously known to be involved either in barrier function, T cell activation or cytokine receptor signalling, have been associated with susceptibility to IBD, in average 5% of world population (Knights et al., 2013; Huang et al., 2015; Liu and Stappenbeck, 2016; Ward et al., 2016). Accumulating evidence suggest that genetic polymorphisms associated with factors involved in the stimulation of Treg cells, such as the transcription factor FoxP3, may increase susceptibility to IBD (Sarmento et al., 2016). It was shown in a very interesting study that IL-10 Knockout mice spontaneously develop colitis upon exposure to a convencional microbiota, demonstrating the importance of this cytokine to keep immunological tolerance to commensals (Balish and Warner, 2002; Scheinin et al., 2003). Intriguing, the disease was not observed in IL-10 knockout germ-free mice, highlighting the role of the microbiota in the development of colitis (Kühn et al., 1993). Other polymorphisms are related to the expression of PRR genes. For example, in IBD patients it was described low expression of α-defensins by Paneth cells associated with NOD2 mutations (Hugot et al., 2001; Ogura *et al*., 2001). it has been reported that 17 to 25% of CD patients have mutations in the NOD2/CARD15 gene, which regulates host responses to bacteria (Hampe et al., 2001). Others PRR genes involved in recognition of luminal bacteria, such as TLR2 and TLR4, have also been associated to excessive immune response in the gastrointestinal tract, caused by mutation which lead to their up-regulation in IBD patients (Cario and Podolsky, 2000; Hausmann et al., 2002; Cheng et al., 2015).

IBD available treatment

Treatment available for IBD is based on the administration of anti-inflammatory and/or immunosuppressive drugs, different classes of antibiotics or even surgery. The current immunosuppressives used are corticoids. Because they are derived from cortisol, a hormone involved in various host metabolic functions, their prolonged use can generate serious side effects, including vomiting, mood changes, weight gain, hyperglycemia and headache (Bernstein, 2015). Moreover, it was demonstrated that this medicaments might compromise the host immune system, turning the patient more susceptible to infections (Thia et al., 2008, Orlicka et al., 2013). Aminosalicylates are also widely employed in the treatment of IBD, especially CD, as they can suppress inflammatory processes during remission period being capable of inhibit the synthesis of pro-inflammatory chemokines (Bernstein, 2015). Nevertheless, the use of aminossalicyates was also associated with many side effects observed in patients, as

headache, abdominal pain, hepatitis, pancreatitis and anemia. Moreover, this drug demonstrated to affect folic acid absorption (Habr- Gama et al., 2011).

Antibiotics most widely used in clinical practice for the treatment of IBD are metronidazole and ciprofloxacin, both have broad spectrum activity against both gram-positive and gram-negative bacteria (*Bacteroides fragilis*, *Fusobacterium sp*, *Clostridium sp*, *Eubacterium sp*, *E. coli*, *Shigella sp*, *Salmonella sp*, *Klebsiella sp*, *Enterobacter spp*, *Proteus sp, Yersinia* sp; *Aeromonas sp*, *Plesiomonas sp*, *Pasteurella sp*, *Listeria sp*, *Corynebacterium sp)* (Sartor, 2010). It was observed that antibiotics are effective to treat certain conditions generated by intestinal inflammation, such as abscesses, fistulas and some infections with intestinal pathogens. Nonetheless, their efficacy to alleviate global symptoms of IBD and eliminate dysbiosis has been considered controversial. Some studies have reported good results with metronidazole administration in patients with IBD. However, few weeks after treatment ends, patients are diagnosed again with dysbiosis and nausea, diarrhea and abdominal pain returns (Rahimi et al., 2007, Khan et al, 2011).

In some cases, surgery to remove the entire colon and rectum may be recommended. After surgery, the patient is usually treated with the mentioned immunosuppressive drugs and antibiotics to prevent reappearance of symptoms. However, even with the drug treatment, a high percentage of symptoms recurrence is reported in patients that underwent surgery (Lakatos & Lakatos., 2007; Sokol et al, 2008). Thus, considering that standard treatment cause serious side effects and is not completely effective, the development of new therapeutic strategies with better and more durable responses in favor of patients are necessary (Thia et al., 2008; Marteau, 2006; Nielsen & Munck, 2007).

Mucositis

Inflammation of the GIT associated with the use of chemotherapy drugs is known as mucositis. These medicaments, as 5-fluoracil (5-FU), doxorubicin and irinotecan (CPT-11) are commonly used in oncology practice and widely prescribed in the treatment of head, neck and gastrointestinal cancer (Prisciandaro et al., 2011; Herbers et al., 2014; Koohi-Hosseinabadi et al., 2015). They are effective in cancer treatment because they inhibit cell proliferation. 5-FU, for example, causes cytotoxic effect through competitive inhibition of the enzyme thymidylate synthase, preventing methylation of uracil to thymine and therefore inhibiting DNA replication. Moreover this medicament can also be incorporated into RNA molecules interfering with their processing and function. Depletion of DNA and RNA molecules caused by the

administration of 5-FU is quite effective in cells with a high mitotic index such as malignant cells. However, as an adverse consequence, the medicament shows also an effect in normal cells that has a higher turnover rate, such as GIT enterocytes (McCarthy et al., 1998). In this context the use of 5-FU is associated with a number of side effects like myelosuppression, dermatitis, cardiac toxicity and mucositis. Among them, gastrointestinal mucositis is being regarded as a major risk, occurring in 80% of patients receiving 5-FU (Sonis, 2004; Chang et al., 2012).

Patients with mucositis develop symptoms like odynophagia (pain in swallowing), vomiting, abdominal pain and diarrhea, which make eating difficult. Therefore, weight loss and malnutrition are also reported and quality of patient`s life is greatly affected (Soares et al., 2013). The risk of systemic sepsis in mucositis has been reported, however, it has been shown that the disease usually does not threaten the patient's life. Nevertheless, it causes great discomfort leading to treatment discontinuation thus compromising cancer therapeutic benefit (Generoso et al., 2015; Antunes et al., 2015).

Pathophysiology

Gastrointestinal mucositis is characterized by morphological alterations in the mucosal architecture, as villous atrophy, increased crypts apoptosis, that exposes the mucosa to intestinal pathogens, which are able to translocate across intestinal epithelial cells leading to inflammatory responses (Antunes et al., 2015).

Recently been appreciated, the pathophysiology process of mucositis is thought to be very complex and divided into four phases: (I) Initiation, (II) Primary damage response, (III) Ulceration and (IV) Resolution (Duncan and Grant, 2003). The first stage occurs when chemotherapy and/or radiotherapy starts. As a consequence of the treatment, both normal epithelial cells and connective tissue cells are affected by damaging their DNA. Reactive oxygen species (ROS) are formed inducing the release of endogenous damage-associated molecular pattern (DAMP) molecules. In phase II, DAMP activates NF-kB pathway, which induces the expression of more than 200 genes, including pro-apoptotic enzymes such as caspases, pro inflammatory cytokines, such as TNF-α, IL1-β and IL-6, and chemokines involved in the recruitment of neutrophils and eosinophils. In stage III, there is loss of mucosal integrity and endothelial damage characterized by ulceration, increased intestinal permeability, and sepsis. In phase IV, healing process occurs, where macrophages are transported to the damaged epithelium, inducing tissue repair, cell division and differentiation to restore mucosal integrity (Sonis, 2004; Chang et al., 2012).

Dysbiosis in Mucositis

The gastrointestinal microbiota and its influence on mucositis has become recently a remarkable field of research. Previous studies demonstrated that microbiota might be involved in the development of mucositis induced by 5-FU or irinotecan (Stringer et al., 2009; Bültzingslöwen et al., 2006; Savva-Bordalo et al., 2010; Stringer and Logan, 2015). It has been shown that commensals have a very important role in promoting many clinical aspects involved in the pathogenesis of mucositis (Vliet et al., 2010). It was demonstrated that germ free mice are more resistant to mucositis induction, suggesting microbiota as a key factor for mucositis development (Brandi et al., 2006; Pedroso et al., 2015). Moreover, commensals, such as *Entercocccus faecalis* can alter intestinal permeability during anticancer treatments and promote disruption of the epithelial mucus layer (Vliet et al., 2010). These effects may contribute to bacterial translocation during mucositis and causes more serious infection, such as sepsis. Translocation of commensals across IEC exacerbates inflammatory responses and amplifies the damage to the intestinal mucosa (Brandi et al., 2006).

Other studies attempt to demonstrate the impact of chemotherapy drugs as well as antibiotics in microbiota composition and their role in the development of dysbiosis (Touchefeu et al., 2014). Stringer and colleagues (2009) demonstrated that 5-FU injection was associated to a decrease in *Clostridium* spp., *Lactobacillus* spp. and *Streptococcus* spp., species that are more prevalent in the GIT of healthy individuals, and an increase of enteropathogenic species, as *Escherichia* spp.

Other chemotherapy drugs, like irinotecan, were also reported to alter microbiota composition. It was shown that concomitant treatment with 5-FU and irinotecan in rats was associated with a drastic change in microbiota composition, causing an increase of *Escherichia spp*., *Clostridium spp*. and *Enterococcus spp* (Lin et al., 2014). Although many studies have pointed out that changes in microbiota composition might be caused by the administration of chemotherapy drugs that allows the proliferation of opportunistic bacteria, the direct role of the microbiota in the development of mucositis have yet to be fully elucidated.

Mucositis available treatment

Currently, no intervention that is completely successful at preventing mucositis has been described (Ribeiro Júnior et al., 2010). Usually, treatment is based on local anesthetics, analgesics and/or antibiotics. The use of local anesthetics to control the symptoms of the mucositis is questioned, as their effect are short, affects taste and reduces the flow of saliva affecting the ingestion of food as well (Herbers et al., 2014).

Applying ice at the oral cavity minutes before starting the chemotherapy cycle (topical cryotherapy) may ameliorate oral mucositis as it reduces the exposure of the epithelium to 5-FU. Another common procedure is applying laser to the oral mucosa in order to minimize the risk of infection. These measures are considered safe and effective in 80% of patients, however, are inadequate for controlling GI mucositis (Köstler et al., 2001; Vokurka et al., 2012).

Broad-spectrum antibiotics (i.g chlorhexidine) are also administrated to patients in favor of preventing infection of the inflamed tissue. However, their use has been associated with side effects such as abdominal pain, altered taste and abnormal dental pigmentation (Ata-Ali et al., 2015). In addition, they may also lead to GI dysbiosis altering microbiota composition. Thus, the prolonged use of antibiotics is not indicated (Touchefeu et al., 2014).

Current animal models used to explore new alternative treatments for GI tract inflammation

As treatments available for either IBD or mucositis disorders are not enough to contain both diseases, scientific community is investing in the development of several experimental animal models of intestinal inflammation to verify the efficacy of new alternative therapies, mentioned later in this review. Many animal species are being explored as a model to study both diseases, like dogs, mice, rabbits, rats, pigs as well as horses. These models are undoubtedly providing new insights into the biological mechanisms operating for the development of both IBD and mucositis (Kiesler et al., 2015). However, the most often used models are the ones based on mice strains, as they are biologically similar to humans, can be genetically manipulated, are easy to handle and transport and, thus, are an efficient research tool (Low et al., 2013).

Mouse models of inflammatory bowel diseases

Mouse models of IBD can be divided into four categories: (i) animals that spontaneously develop colitis as a result of natural genetic abnormalities (ii) genetically engineered animals via the insertion or deletion of genes involved in immune responses, (iii) inflammation induced by the transference of T cells to immunodeficient mice (iv) animal models of inflammation induced by chemical agents (MacDonald *et al.*, 2005).

Mouse models of spontaneous mucosal inflammation can be used to define which genetic factors are causing GIT inflammation (Blumberg *et al.*, 1999). These include SAMP1/ Yit and C3H-HeJBir mice, which develop colitis at three weeks of age.

On the other hand, genetically modified mice are commonly used to understand differential cytokines gene expression profiles during inflammatory processes (Blumberg *et al.*, 1999; Bouma & Strober, 2003). The most frequently used knockout mouse models are for IL-2, IL-7, IL-10 cytokines genes, for major histocompatibility complex (MHC) class II molecules and T-cell receptor (TCR) gene (Strober *et al*., 2007). In the T cell transfer model, colitis is induced by the selective transfer of naïve $CD45RB_{high}$, to lymphopenic mice, unable to produce Treg, such as Severe Combined Immunodeficiency mice (SCID), *Recombinant Activating Gene* and TGF-β (- / -). Therefore, naïve T cells received from the animal donor can be differentiated into pro-inflammatory subtypes, such as Th1 and Th17, as they get contact with the host antigens, but not into T regulatory cells, ,developing inflammation within 5-8 weeks after cell transfer (Blumberg *et al.*, 1999). The last category includes the use of chemical agents that cause intestinal inflammation, which most commonly used to study GIT inflammation as it is relatively simple and resembles important clinical, immunological and histopathological aspects of human IBD (Wirtz *et al.*, 2007**;** Oh et al., 2014; Taghipour et al., 2016).

Chemical-induced inflammatory bowel diseases models in mice

Several chemicals have been reported to induce colitis in mice, such as the dextran sulfate sodium (DSS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,4-Dinitrobenzenesulfonic Acid (DNBS), acetic acid, indomethacin and others (Okayasu *et al*., 1990; Morampudi et al., 2014).

One commonly employed model to study ulcerative colitis (UC), is DSS-induced colitis model as it provokes an acute bowel inflammation that resembles human UC. DSS diluted in the drinking water 2% (w/v) concentration. Oral administration of DSS during seven days leads to acute colitis characterized by epithelial disruption, infiltration of neutrophils and macrophages in the damaged segments (Wirtz et al., 2007). This model has been reported to be particularly useful for studying the contribution of innate immune responses in the development of colitis. The damage induced by DSS preferentially affects the distal colon and caecum, produces mucosal erosion, ulceration and infiltration of leukocytes. Moreover, administration of DSS generates various macroscopic alterations, including rectal bleeding, weight loss and diarrhea (Geier *et al*., 2005; Huang *et al*., 2010). The mechanism by which DSS promotes UC remains unclear, nonetheless, it has been suggested that the toxic effects observed in the epithelium after DSS administration might be due to disruption in mucin composition (Kitajima *et al*., 2001, Taghipour et al., 2016).

Another well-established model that has been widely used is the haptenizing TNBS-induced colitis. This chemical agent is diluted in 30-50% ethanol solution and must be administered intra-rectally to induce colitis in mice (Neurath & Strober, 2000; Brenna et al., 2013). The intestinal inflammation observed after TNBS injection is a result of covalent binding of the haptenizing agent to autologous host proteins, generally stimulating an antigen-specific Th1 immune response (Fiorucci *et al*., 2002). There are three types of TNBS-induced colitis models described: one that generates an acute inflammation in which a nonspecific inflammatory response is observed; a second where an established colitis is detected and a more specific immune response can be obtained; lastly, the chronic TNBS-induced colitis that demonstrates a Crohn's disease-like cytokine profile (Kiesler et al., 2015).

DNBS-induced colitis is very similar to TNBS model, however DNBS compound presented to be safer for human being. As it is less toxic, it can be handled more safely in a ventilated room with basic equipment such as gloves, laboratory apron and goggles. Furthermore, DNBS showed to be capable of reproducing an inflammatory response similar to TNBS. DNBS lesions include (i) infiltration of macrophages, T cells and neutrophils, (ii) strong induction of myeloperoxidase (MPO) enzyme produced neutrophils (Neurath *et al*., 1995; Morampudi et al., 2014), accompanied by clinical features such as progressive weight loss, bloody diarrhea, ulceration and bowel wall thickening (Neurath & Strober, 2000; Scheiffele and Fuss, 2002). There are three DNBS-induced colitis models described as well: (i) acute colitis, (ii) Delayed type IV hypersensitivity (DTH) and (iii) chronic colitis. To reproduce the acute colitis, a single dose of DNBS is employed, which generate a nonspecific acute inflammation condition in mice GIT, leading to a disruption of the epithelial barrier and formation of reaction oxygen species (ROS) (Scheiffele and Fuss, 2002). In the second model two doses of the haptenizing compound is administered within a period of time generating a prolonged Th1 immune response against autologous antigens bound to DNBS. Subsequently, cytotoxic T CD8+ lymphocytes are activated causing direct damage to the tissue, characterizing the DTH (D'Amato and Rioux, 2013). Finally, chronic colitis is obtained with several alternative cycles of DTH in order to induce a long-lasting Th1 response (Te Velde *et al*., 2006; Brenna et al., 2013). In contrast to the intestinal inflammation generated by DSS compound, injection of DNBS results in a more substantial adaptive inflammation by stimulating Th1 mediators, including IFNγ and IL-12 cytokines. DNBS-induced colitis also demonstrated to differentiate T cells into Th17 subsets, an immune profile encountered in CD patients (Neurath *et al*., 1995; Oh et al., 2014; Morampudi et al., 2014).

Mouse models of mucositis

Radiation therapy was used massively in the early twentieth century until now to reduce tumors and kill cancer cells of patients. Mucositis has been reported as a side effect from X-ray irradiation treatment and thus, it has been used to induce mucositis in animal models. However, as new cancer treatments are arising, like chemotherapy, including the use of methotrexate, irinotecan and 5-FU medicaments, new animal models have emerged as mucositis appears as an adverse reaction caused by these therapies as well (Logan *et al*., 2007; Pereira et al., 2015). Current models are divided into two categories: the ones mimicking oral mucositis and others resembling gastrointestinal mucositis. Intraperitoneal injection of the drug irinotecan may be used to induce intestinal mucositis in mice, although this medicament needs to be prepared in sorbitol latic acid buffer (pH3.4) in order to be activated prior to administration. A single dose of 225 mg/kg injection in mice can cause severe myelosuppression, diarrhea and damage to the small bowel mucosa (Bowen et al., 2007; Bastos et al., 2016). Methotrexate have been used preferentially to induce intestinal mucositis rats by sub-cutaneous injection (single dose of 2mg/kg), as these animals are more susceptible to this drug effect (Tooley et al., 2006; Southcott et al., 2008; Shi et al., 2016), although methotrexate-induced mucositis have been adapted in mice models (single dose of 100 mg/kg) (Koning et al., 2006). Therefore both models show mucosal damage characterized by apoptosis of intestinal crypts, villous shortening and reducing mucus secretion (Logan 2008, Shi et al., 2016).

Generally, the majority of the studies are based on models that use 5-FU to generate a severe mucositis, as it is the most commonly used drug in clinical oncology (Rubenstein, et al., 2004; Chang et al., 2012). Mice injected with 5-FU doses of 300mg/kg generally develop an acute inflammation in the small bowel while humans present both intestinal and oral mucositis. 5-FU model has proved to generate many important clinical aspects of the disease as well, such as diarrhea, increased intestinal permeability, sepsis, villous atrophy, acute inflammation characterized by the infiltration of leukocytes, especially neutrophils and eosinophils in the GIT (Logan et al, 2008; Soares et al., 2013; Maioli et al., 2014; Kwon, 2016).

Use of probiotic Lactic Acid Bacteria in the treatment of gastrointestinal inflammation

Scientific community has been mobilized to search effective alternative treatments for IBD and, more recently, to mucositis. As dysbiosis is observed in both conditions, administration of microorganisms that induces immunological tolerance and are capable to occupy niches at GIT have been suggested, replacing drug administration. Actually, Elie Metchnikoff over a century ago was the first to propose the rationale for using host-friendly bacteria found in yogurt to manipulate the intestinal microbiome. He also predicted the existence of bacterial translocation and described theories associating the microbiota with intestinal inflammation and other diseases (Mackowiak, 2013). At present, several research groups confirmed his hypothesis demonstrating that the administration of certain bacterial species tested in several animal models actually provides health benefits to alleviate inflammation, such as containment of inflammatory mediators, stimulation of the immune system and microbiota restoration by competitive exclusion with potentially pathogenic species (Ljungh and Wadström, 2006; Santos Rocha et al., 2014 ; Quinto et al., 2014; Luerce et al., 2014; Thomas, 2016). These microorganisms are considered to be probiotics, a term defined by the World Health Organization as “live microorganisms administered in adequate amounts that confer a beneficial health effect on the host” (WHO, 2002).

Probiotics are live bacteria and yeasts; however, the majority of strains are gram-positive bacteria belonging to *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Lactococcus* genera. These genera are included in a diverse group of microorganisms entitled Lactic Acid Bacteria (LAB), as they are able to convert sugars into lactic acid (Stiles & Holzapfel, 1997; Carr et al., 2002). Regarding Gram-negative bacteria, some strains of *Escherichia coli* are considered to promote health as well, for instance *Escherichia coli* Nissle 1917 (EcN1917), originally isolated from faeces of a soldier during the First World War that did not develop infectious diarrhea during an outbreak of contagious *Shigella* (Westendorf et al. 2005; Henker et al. 2007).

Although Metchnikoff introduced the concept of probiotic only in 1907, some of these microorganisms are used in food industry for centuries to prepare yogurt, sourdough breads, sauerkraut, cucumber pickles and olives, as they are able to produce lactic acid, as mentioned before (Mackowiak, 2013; Vikhanski, 2016). In the latter half of the 20th century, probiotics have gained visibility as there has been an increasing interest in applying them in other areas, such as in pharmaceutical industry. Thus, the selection of new probiotic strains, development of new food products based on probiotics and freeze-dried probiotic pharmaceutical formulations has gained much

importance. There are many studies being conducted focusing on the development of probiotic-based pharmaceutical formulations which can be applied to either gastrointestinal, nasal, or vaginal mucosa, as well as to the skin of the patient (Vicariotto et al., 2012; Guglielmetti et al., 2010; Iannitti and Palmieri, 2010).

Lactic Acid Bacteria group

LAB group includes heterogeneous group of ubiquitous microorganisms that obtains energy through the conversion of sugars into lactic acid. Morphologically LAB bacteria can resemble cocci, rods or bacilli. They are gram-positive microorganisms that present low GC content (54%) in their genome, facultative anaerobic, non-spore-forming, immovable and do not produce catalase (Stiles & Holzapfel, 1997; Carr *et al*., 2002). The species of this group can be naturally found in different environments that are rich in nutrients, such as decomposing vegetables and fruits, and even in the oral, urogenital or intestinal tract of mammals and other animals. They can also be found in several kinds of dairy foods, as some strains are used to produce them (Holzapfel et al., 1998; Liu et al., 2014). LAB species found in the human GIT can be autochthonous as indigenous GI microflora, especially those belonging to *Lactobacillus* and *Streptococcus* genera, or allochthonous, transient in the GIT, such as *Lactococcus sp* and some strains of *Lactobacillus* used to produce yogurts. Some species, especially the ones belonging to *Streptococcus* genera are pathogenic, however, the vast majority of LAB strains have a positive impact on human health being granted as Generally Regarded as Safe (GRAS) in United States by Food and Drug Administration Agency (FDA) (Felis & Dellaglio, 2007).

After the pioneering work of Elie Metchnikoff who first suggested that the ingestion of dairy foods produced by LAB fermentation could prevent intestinal infections and promote both health and human longevity, the scientific community is continuously exploring in more details the positive effects promoted by these bacteria (Johnson & Klaenhammer, 2014; Vikhanski, 2016). Among all LAB species described exerting probiotic effects, *Lactobacillus* spp, *Streptococcus* spp and *Lactococcus* sp stand out for therapeutic application for both treatment and prevention of various intestinal disorders (Majamaa *et al*., 1997; Ouwehand *et al*., 2002; Prescott & Bjorksten, 2007; Ohland & MacNaughton, 2010; Santos *et al*., 2014; Luerce *et al*., 2014). This topic has been widely studied and certain immunological aspects of LAB anti-inflammatory properties have been described.

Effects of probiotic lactic acid bacteria in animal models of gastrointestinal inflammation

Accumulating evidence reveals that probiotic LAB is able to protect the host against potentially pathogenic species that inhabit the GIT of animals, including man. It seems that Lactobacilli strains, such as *L. acidophilus* LA1 can prevent the colonization of the intestine with pathogenic bacteria, such as *Staphylococcus aureus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* by competitive exclusion (Bernet-Camard et al., 1997; Adolfsson et al., 2004). Apparently, these LAB compete for nutrients and adhesion sites in the intestinal epithelium with other potentially pathogenic bacteria present in the GIT that are in consequence eliminated from the host organism. The secretion of lactic acid and bacterocins (natural antibiotics) by probiotics species has been also implicated in the cause of the pathogens elimination (Ogawa et al., 2001; Moal et al., 2007).

Another manner by which LAB strains may protect the host from pathogen invasion is boosting the intestinal mucosal barrier. Some strains are capable of interacting with epithelial PRR, mainly TLR2, TLR4 and NOD-like receptors, through the activation of protein kinases. This activation induces several protective mechanisms restoring tissue damage, such as modulation of tight junctions stability (Lebeer et al., 2010; Ohland & MacNaughton, 2010; Villena and Kitazawa, 2014; Bajaj et al., 2015). Species like *B. infantis, L. plantarum* and *L. casei* have been shown to increase the expression of proteins involved in tight junction barrier functioning, as ocludins and zonula occludens-1 (ZO-1) (Ewaschuk et al., 2008 ; Anderson et al., 2010; Eun et al., 2011).

Another example is some *Lactobacillus* strains that are capable of increasing the production of other proteins involved in the maintenance of epithelial barrier homeostasis, as mucin-2 (MUC2), the most abundant glycoprotein of the mucus. *In vitro* studies showed that increased MUC2 expression by intestinal epithelial Caco-2 cells demonstrated to block the adhesion of pathogenic *E. coli* (Mattar et al., 2002; Mack et al, 2003). Furthermore, an *in vivo* study has demonstrated that mice treatment with VSL#3 mixture, consisting of *S. thermophilus* four strains of *Lactobacillus* (*L. delbruekii, L. casei, L. acidophilus and L. plantarum*) and three species of *Bifidobacterium* (*B. longum, B. infantis and B. breve*) during seven days increased about 60-fold the production of MUC2 in treated animals (Gaudier et al., 2005).

Other studies suggested that some LAB strains are able to induce the secretion of defensins by enterocytes, related to biological control of potentially pathogenic species in the lumen. Administration of certain species of *Lactobacillus* or the VSL#3 mixture in mice demonstrated to increase the production of β-defensin-2, which has

microbicidal activity against important opportunistic pathogens, as *P. aeruginosa, E. coli* and *Candida albicans* (Harder et al., 2004; Schlee, et al., 2008).

Another well-established probiotic effect is the stimulation of the host immune system and suppression of pro-inflammatory responses. One of the major mechanisms is the stimulation of immunological tolerance to GIT microbiota, with an increase in IL-10 secretion and significant reduction in IFNγ and IL-12 expression. This probiotic effect is caused due to the interaction of "good" bacteria with intestinal DC that drives the development of Treg cells and IgA-producing B cells (Fedorak, 2000; Ng et al., 2009). Administration of *B. lactis B. bifidum* and B*. infantis* in mice previously infected with rotavirus or pathogenic *E. coli* (EHEC) has been shown to increase the titers of specific IgA (Shu and Gill, 2001; Qiao et al., 2002). Santos and collaborators (2014) showed for instance that the probiotic effect of *L. delbrueckii* strain CNRZ327 was related to an expansion of Treg cells and an increase of total IgA in Dextran sulfate sodium (DSS)-induced colitis in mice. This effect showed to be enough to treat them from inflammation (Santos-Rocha et al., 2014). Recently, it was reported that *Lactococcus lactis* ssp. *lactis* NCDO2118 strain prevented DSS-induced colitis in mice and the protective effect was related to IL-10 increased levels in the colon and Treg cells induction in the mesenteric lymphnodes (Luerce et al., 2014). In another study using a similar colitis model, *L. lactis* FC ssp. *cremoris* demonstrated a protective role in preventing inflammation in mice, by inhibiting NF-kB pathway and decreasing IL-8 expression in epithelial cells (Nishitani et al, 2009).

LAB has also been studied and generating promising results either *in vitro* or *in vivo* in other models of intestinal inflammation, such as preclinical mucositis models (Tooley et al, 2006; Bowen et al., 2007; Smith et al, 2008; Southcott et al, 2008; Whitford et al 2009; Tooley et al, 2011; Prisciandaro et al, 2012). *In vitro* it was observed that intestinal epithelial cells previously treated with 5-FU when co-cultured with *L. rhamnosus* presented reduced levels of cytotoxicity and apoptosis through the inhibition of caspase-3 and caspase-7 (Prisciandaro et al., 2012). *In vivo*, *Lactobacillus fermentum* BR11 administered in mice once injected with 5-FU reduced levels of intestinal inflammation and myeloperoxidase enzyme activity, a marker of eosinophilic inflammation (Smith et al., 2008). In another study, VSL#3 was used in the treatment of mucositis induced in rats through the injection of chemotherapy drug known as Irinotecan. The probiotic administration has shown to improve weight loss and reduce diarrhea in Irinotecan-treated animals. These findings were associated with significant improvement in the integrity of crypts in the jejunum and reduction in apoptosis levels in both small and large intestines (Bowen et al., 2007). Whitford and colleagues in 2009 compared the efficiency of live *S. thermophilus* TH-4 strain, dead TH-4 and TH-4

bacterial culture supernatant in rats treated with 5-FU. He showed that live TH-4 significantly reduced disease severity scores as well as crypt fission index. Still, Tooley and colleagues (2011) ascertained the effects of live TH-4 on small intestinal damage, generated by the injection of methotrexate (MTX), a chemotherapy drug that induces mucositis, and tumor progression in tumor-bearing rats. It was verified that although TH-4 did not protect animals completely from chemotherapy-induced mucositis, progression of mammary adenocarcinoma was unaffected (Tooley et al., 2011).

The efficacy of cow's milk yogurt containing *L. johnsonii*, sheep's milk yogurt containing *Lactobacillus bulgaricus* and *S. thermophilus* were assed in MTX-induced mucositis in rats. It was showed that all types of yogurts reduced intestinal permeability, revealing to be useful to restore intestinal barrier function (Southcott et al, 2008).

As they were shown to be capable of acting in many diverse biological processes within the host, probiotics have been remarkably experimented as an alternative therapy against GIT inflammatory disorders. In order to enhance probiotic properties, research are focusing on the development of genetically modified bacterial strains expressing heterologous proteins of medical interest, such as anti-inflammatory molecules. Recently, the use of recombinant LAB strains with natural probiotic activity have shown promising results in pre-clinical studies as an alternative therapy to fight against, cancer, obesity, especially GI tract inflammation (Bermúdez-Humarán et al., 2007; Cortes-Perez et al., 2007; Bahey-El-Din et al., 2010; Bermudez-Humaran et al, 2013; Wang et al., 2016).

The use of recombinant Lactic Acid Bacteria for the treatment of GIT inflammatory diseases

Since 1960 molecular biologists have developed several sophisticated techniques to identify, isolate, and manipulate genetic components of the bacterial cell. This knowledge enabled the construction of different LAB recombinant strains with boosted anti-inflammatory properties. A well-reported example was the construction of *L. casei*, *L. plantarum*, *S. thermophilus* and *L. lactis* strains capable of expressing either superoxide dismutase (SOD) enzyme or catalase, which increased both anti-inflammatory and antioxidant potential of the above mentioned strains (Han et al., 2006; LeBlanc et al., 2011; del Carmen et al, 2014). Thus, several studies focusing on the use of recombinant anti-inflammatory LAB could be an interesting alternative treatment for GIT inflammatory diseases (Moreno et al, 2015; Wang et al., 2016).

LAB has proven to successfully express proteins of interest in different cell compartments (in the cytoplasm, anchored to the cell membrane or secreted to the extracellular medium) (Miyoshi et al., 2010; Pontes et al., 2011; Pereira et al., 2014). It has been shown that LAB can be administrated at the oral route, thus facilitating treatment as needles or clean syringes is not required in this type of immunization. Actually, the World Health Organization (WHO) recommends immunization or treatment through the oral route due to economic, logistical and security reasons. Furthermore, this route offers important advantages over systemic administration, such as reducing side effects, as molecules are administered locally and has the ability to stimulate the GALT immune responses (Levine & Dougan, 1998; Neutra & Kozlowski, 2006; Bermudez-Humaran et al, 2011).

The majority of works found in literature describes the genetic engineering of *L. lactis*, because it is the best-characterized member of the LAB group, both physiologically and genetically, with a large number of genetic tools available for genetic manipulation. Moreover, other features that turns *L. lactis* one of the most extensively studied bacterium is related to its economic importance in cheese production, is easy to grown and to manipulate and was the first LAB to have its genome completely sequenced (Bolotin et al., 1999; de Vos, 1999; Felis & Dellagio, 2007; Wells & Mercenier, 2008; Bermudez-Humaran et al, 2011). In addition, it does not produce endotoxins such as Lipopolysaccharide (LPS) and secretes few proteins facilitating purification processes of heterologous proteins. Actually, only the Unknown Secreted Protein of 45 kDa (Usp45) is detectable after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Brilliant Blue (van Asseldonk et al., 1990; Bahey El-Din & Gahan, 2010).

Lactococcus lactis, the model Lactic Acid Bacteria for the expression of anti-inflammatory molecules

Properties of Lactococcus lactis

L. lactis is a mesophilic facultative heterofermentative bacterium, with an optimum growth temperature around 30°C, considered to be very important in dairy industry, especially for cheese production. There are two subspecies (ssp) reported to *L. lactis,* ssp. *lactis* and ssp. *cremoris*. Both of them can be found naturally in plants, especially grass. As they are used in the food industry for milk fermentation, both species can also be encountered in dairy products such as cheeses, yogurts, some breads and wines as well (Carr et al., 2002). *L. lactis* subsp. *cremoris* MG1363 is the most commonly used in dairy industry, as it has no plasmids and does not produce any

extracellular proteases. In addition, this strain was cataloged by the FDA and the European Food Safety Authority (EFSA) as a safe microorganism (GRAS), non-invasive and non-pathogenic, reinforcing its use as a factory for the production of anti-inflammatory molecules. Even though being considered GRAS, another strain of *L. lactis* spp. lactis was reported to cause infection in two individuals, who had been diagnosed with cardiac abnormalities. Afterwards, they were treated with antibiotics and infection was cleared. Both patients did not develop any further infection with *L. lactis* (Mercenier et al., 2000; Bermudez et al, 2011). As *L. lactis* does not colonizes human GIT, most works have been focusing in studying beneficial effects of LAB strains included into *Lactobacillus* and *Bifidobacteria* genus, which are allochthones. However, recent works demonstrated some Lactococci strains have anti-inflammatory properties, such as *L. lactis* NCDO2118 sub. lactis and *L*. *lactis* FC sub. cremoris, when administered to animals receiving chemical agent DSS (Nishitani et al, 2009;. Luerce et al, 2014).

As mentioned before, there are several expression systems for heterologous proteins production available for use in *L. lactis* (Miyoshi et al., 2010). Therefore, it has allowed the cloning and the great expression of different heterologous anti-inflammatory proteins by the use of both cloning and expression vectors designed for *L. lactis* (Langella & Le Loir., 1999; Le loir et al., 2005; Bermudez-humaran et al., 2011).

Heterologous protein Expression Systems in L. lactis

Among all expression systems available for *L. lactis*, Nisin-Controlled Gene Expression system (NICE) is the most used (Figure 1). In brief, it is based on (i) three within eleven genes involved in the production and regulation of a peptide named nisin, naturally secreted by various strains of *L. lactis,* (ii) in a genetically engineered *L. lactis* strain and, at last, (iii) in an expression vector harboring nisin inducible promoter*.* Because of its antimicrobial properties, nisin is widely used as a natural food preservative. The eleven genes (*nisABTCIPRKFEG*) mentioned are responsible for the production, modification and secretion of nisin peptide across *L. lactis* strains. *NisA* gene encodes the nisin precursor peptide that contains 57 amino acid residues; *nisBC* are involved in its post-translational modifications; *nisT* gene is required for nisin transport across the cytoplasmic membrane while *nisP* is associated with signal peptide cleavage. *NisI* along with *nisFEG* genes encodes a lipoprotein that confers to the strain nisin resistance. Finally, *nisR* and *nisK* encodes two-component regulatory system (NisRK), which controls the expression of these 11 genes through the activation of signal transduction pathways (Kuipers *et al.,* 1993). The strains used in

this system is a genetically modified version of *L. lactis* MG1363 strain, called *L. lactis* NZ9000, in which both *nisR* and *nisK* regulatory genes were inserted into its chromosome. The expression vector contains the nisin promoter P*nisA*, followed by multiple cloning sites (MCS) for the insertion of heterologous genes coding for anti-inflammatory molecules or antigens (Kuipers et al., 1993; Mireau & Kleerebezem, 2005). Because expression vectors of NICE systems exist in different versions, heterologous proteins are addressed to different cellular compartments. Besides cytoplasm, recombinant protein can be anchored to the bacterial cell wall due to the presence of the cell wall anchor (CWA) peptide, composed of 30 amino acids located in the carboxy-terminal portion (C-terminus) of the protein. CWA is recognized by the cell anchoring machinery and is usually covalently attached to peptidoglycan from the cell membrane. Furthermore, recombinant proteins may be coupled with a short (5-30 amino acids long) peptide present at the N-terminus region of the heterologous protein, allowing its translocation across the cell membrane and secretion to the extracellular medium (Le Loir *et al*., 1994; Piard *et al*., 1997).

NICE system has been successfully used to express and address a variety of heterologous proteins of medical and biotechnological interest, and thus, is ranked as one of the best genetic tool already developed for gene cloning and expression in *L. lactis* (Nouaille *et al*., 2003; Le Loir *et al*., 2005).

Therapeutic interventions using recombinant Lactococcus lactis strains to contain GI intestinal inflammation

As *L. lactis* can be genetically modified to efficiently produce and secrete different anti-inflammatory proteins, recombinant strains of *L. lactis* has been tested in pre-clinical and clinical experimental trials to treat or prevent various human diseases, including intestinal inflammation (Steidler et al., 2000; LeBlanc et al., 2011; Rochat et al., 2007, Bermúdez-Humaran, 2013; Del Carmen et al, 2014). The oral administration of *L. lactis* expressing anti-inflammatory proteins is very interesting strategy to fight GIT inflammation, as this specie is non-invasive and allochthonous, as commented earlier, being unable to colonize GIT, thus, reducing the potential to elicit adverse effects on host microbiota related to its long-term administration (Nouaille et al., 2003).

It has been shown that the oral administration of recombinant *L. lactis* strain expressing SOD enzyme, naturally produced by *Bacillus subtilis*, reduced inflammation scores in animals treated with TNBS. This therapeutic effect was implicated to the antioxidant properties of the recombinant SOD (Rochat et al., 2005). Later, the same strain was able to prevent the development of colorectal cancer cells in mice.

Another strategy has been the use of *L. lactis* to secrete either regulatory cytokines involved in the regulation of inflammation processes or antibodies that neutralizes pro-inflammatory cytokines. *L. lactis* strains able to secrete anti-TNFα antibodies that bind to TNF-α, one of the most important mediator of inflammation, were described (Yoshida et al., 2008; Strukelj et al., 2014). It was demonstrated that the oral administration of *L. lactis* expressing murine anti-TNFα employed in DSS-induced colitis mouse model showed positive results in reducing inflammation, as well as the work described by Bermudez-Humaran and collaborators in which a recombinant *L. lactis* strain expressing TGF-β cytokine was able to ameliorate symptoms in the same DSS model of intestinal inflammation (Yoshida et al., 2008; Bermudez-Humaran et al., 2015). Another strain that is presenting very good results in pre-clinical trials is the one expressing IL-10, an anti-inflammatory cytokine capable of suppressing many inflammatory pathways. The effect of recombinant IL-10 producing *L. lactis* has been tested in several IBD animal models, such as IL-10 knockout mice, TNBS or DSS models (Schotte et al., 2000; Steidler et al., 2000 ; Steidler et al., 2003; Braat et al., 2006; Del Carmen et al., 2014). Production of IL-10 by recombinant *L. lactis* had demonstrated promising results in pre-clinical as well as in clinical trials. Indeed, a very big clinical trial using recombinant *L. lactis* secreting the human IL-10 was conducted in patients with Crohn's disease (CD) ten years ago. Its use in human was allowed by regulatory agencies, as the Genetically Modified Organisms (GMO) European Commission, because of a biological-containment strategy that was developed. The essential gene coding for thymidylate synthase (ThyA) located in *L. lactis* chromosome was exchanged for human IL-10 gene. Therefore, strain was only able to survive in the presence of thymine or thymidine artificially provided into the medium culture, turning *L. lactis*-IL-10 critically dependent on this compound. Inside human body the strain could survive and deliver IL-10 as thymine or thymidine is available. Nonetheless, outside the body GMO strain showed to be unable to survive, avoiding to spread into the environment (Steidler et al., 2003). Unfortunately, clinical results showed no significant improvement between patients receiving IL-10 producing *L. lactis* strain with those who received placebo (Braat et al., 2006).

Few studies regarding the treatment of mucositis using recombinant *L. lactis* strains expressing therapeutic molecules are reported. Most pre-clinical studies found in literature describes the use of purified anti-inflammatory compounds intended to eliminate disease. An example is the systemic administration of either IL-11 or TGF-β regulatory cytokines in patients. Sadly, authors noted that this alternative treatment was not able to contain oral mucositis. Possible causes of this failure were linked to an inadequate dosage, route of administration and drug stability (Antin et al., 2002; De

Koning et al, 2007). Other clinical studies have tested growth factors that stimulate cell proliferation thereby maintaining epithelial barrier integrity, such as the granulocyte-macrophage factor colony stimulating (GM-CSF) and the epidermal growth factor (EGF). However, their use was associated with increased risk and progression of tumor (Hong et al, 2009).

Few studies using recombinant *L. lactis* to treat mucositis are described. In 2010, Caluwearts and colleagues evaluated the effect of *L. lactis* secreting the trefoil factor I (TFF-1), naturally involved in the repair of the epithelial barrier, administered in hamsters with oral mucositis. It was noticed that recombinant *L. lactis* was able to reduce mucosal inflammation (Caluwaerts et al., 2010). Moreover, as undesired reactions were not detected in pre-clinical trials, another genetically modified *L. lactis* strain (AG013) capable of secreting human TFF1 was engineered based on the ThyA biological confinement system. A phase 1 clinical trial was performed in patients with oral mucositis who tolerated the treatment well. Moreover, AG013 strain administration showed to be safe (Limaye et al., 2013).

Several molecules with anti-inflammatory properties has been sought to be cloned and expressed in *L. lactis*, which proved to be a safe vehicle for the treatment of GI intestinal disorders. Anti-inflammatory cytokines, anti-oxidant enzymes, epithelial growth factor and especially antimicrobial peptides produced by *L. lactis* are being the focus of future research efforts for the development of a possible treatment for GI tract inflammation.

Human antimicrobial peptides produced by Lactococcus lactis as possible treatment for intestinal inflammation

Antimicrobial peptides that are involved in maintenance of the epithelial barrier could represent an interesting candidate to prevent microbiota-driven inflammatory signaling. Various antimicrobial peptides, such as defensins, cathelicidins and histatins, that are produced by Paneth cells, seems to play a critical role in intestinal homeostasis and their biological activity has been reported to be compromised in IBD patients (Clevers and Bevins, 2013; Peterson & Artis, 2014). Different research groups are investigating whether the administration of these peptides could have a protective effect against intestinal inflammation. A study conducted by Seo and coleagues (2012) demonstrated the *in vitro* co-incubation of α-defensin (HD5) and human β-defensin 2 (HBD2), expressed and purified from the probiotic *E. coli* Nissle 1917, with pathogenic *E. coli*, *S. typhimurium* and *Listeria monocytogenes* were able to inhibit bacterial growth (Seo et al., 2012).

Another antimicrobial peptide called Cathelicidin was expressed in *L. lactis*, and recombinant strain efficacy in decreasing intestinal inflammation was evaluated in a DSS murine model. Authors observed a reduced number of bacteria in the feces from animals that received *L. lactis*-Cathelicidin, showing an anti-microbial effect of the strain as well as reduced tissue damage and MPO activity (Wong et al., 2012).

Among the antimicrobial peptides, the C-type lectin, Reg3A has been extensively studied due to its protective effect in several organs of human and animals during the inflammatory process. This peptide belongs to Reg family that is located in tandem repeats on chromosome 2 in humans, and encodes a diverse group of proteins called secreted C-type lectins with carbohydrate recognition domain (CRD).The proteins of this family have an average weight of 16 kDa, containing the CRD domain plus a signal peptide at the N-terminal portion and are classified into sub-groups I, II, III and IV. The RegIII belonging to the group are produced predominantly in the small intestine of mammals mainly by Paneth cells, including RegIIIγ also found in mice (Christa et al., 1996).

The RegIIIγ has been the focus of several studies aimed at identifying its role in the intestinal mucosa (Cash et al., 2006a). In the study of Vaishnava and coleagues (2011) RegIIIγ (- / -) knockout mice were developed. The authors found that there is an increase of the intestinal microbiota adherence to the epithelium and animals showed to develop a stronger adaptive response against microbiota, suggesting that RegIIIγ is essential for the maintenance of intestinal microflora growth. Later in the work of Ogawa et al (2013) in which the expression of RegIIIγ mRNA was assessed in the mouse intestine, it could be observed higher expression levels in the distal ileum where the density of microorganisms is higher, while in the intestines of germ-free mice RegIIIγ expression is relatively smaller, showing that its production in mice could be stimulated in response to intestinal bacteria. Another study confirmed that RegIIIγ could be stimulated in animals after *B. breve* monocolonization (Natividad et al., 2013). In this study they used both conventional and knockout for Myeloid Differentiation factor 88 (MyD88), an important signaling component of TLR pathway of the innate immune response. The authors observed a reduction in the expression of the RegIIIγ in MyD88 knockout animals indicating that the expression of this peptide is regulated by bacterial activation of TLR. Moreover, Zheng and coleagues (2008) had previously suggested that the expression of RegIIIγ is possibly induced by IL-22 through STAT3 pathway in the intestinal adaptive immune response. The IL-22/ STAT3 pathway is involved in several epithelial barrier protective mechanisms, including the induction of mucin in the goblets cells and defensins by Paneth cells. The human orthologue Reg3A, also known as pancreatitis associated protein I (PAP) which has been first identified in pancreatic

cells taken from patients with pancreatitis, has 66% amino acid identity with the murine RegIIIγ. Initially Reg3A was associated with pancreatitis acute phase, which in fact resulted in its clinical use as a molecular marker of acute pancreatitis, and only later other studies identified its production in other organs such as liver, lung and intestines, but still generally associated with inflammatory processes (Abe et al., 2000; Graf et al., 2006; Zhang et al, 2004). In addition, human PAP expression has also been shown to increase in IBD patients with CD or UC (Parikh et al., 2009; Granlund et al., 2013), a fact that has prompted this peptide as an object of study (Medveczky et al., 2009; Maranduba et al., 2015). Several studies have recently pointed out that PAP apparently exerts a similar activity to its murine orthologue, RegIIIγ, in the small intestine, as both are mainly produced by Paneth cells in the crypts of the small bowel (Cash et al., 2006a; Granlund et al., 2013), and exert bactericidal activity against Gram-positive bacteria. In the latter study, both RegIIIγ as PAP were able to inhibit the growth of Gram-positive bacteria in vitro, including *L. monocytogenes* and *E. faecalis*, whereas none of these peptides was able to inhibit Gram-negative bacteria such as *E. coli* and *Salmonella* sp (Cash et al., 2006b; Mukherjee et al., 2008; Medveczky et al., 2009). Mukherjee and coleagues (2014) have suggested by crystallographic analysis and computational modeling, a bactericidal mechanism in which PAP promotes the formation of pores in the membrane of Gram-positive bacteria (Figure 2). Thus, it appears activation of PAP expression in intestinal mucosa would be required to generate a protective response against intestinal microbiota during bacteria-driven inflammatory events. In other organs some work has demonstrated that PAP is associated with inhibition of apoptotic processes induced by oxidative stress and TNF-α in epithelial tissue of lung and pancreas (Christa et al., 1999;. Malka et al., 2000), and more recently Lai and colleagues (2012) showed that PAP is involved in proliferation of keratinocytes in the epidermis regeneration process after injury events.

Conclusion

The efficiency of probiotic LAB for treating IBD has been demonstrated by many studies in the last decades, and more recently in mucositis animal models. The use of genetically engineered *L. lactis*, able to deliver anti-inflammatory proteins *in situ*, has also been considered as an alternative tool to treat GIT inflammation in several models, despite of the few studies involving their use in intestinal mucositis models, maybe because dysbiosis has been shown recently to be implicated on the physiopathology of mucositis. In this context, the use of probiotic LAB, highlighting *L. lactis* recombinant strains could represent a promising strategy to fight mucositis once they are very effective to prevent microbiota-driven inflammation in IBD. However, PAP protective effects in intestinal proinflammatory immune responses still need to be investigated. Since PAP has been able to reestablish homeostasis in inflammed organs such as the skin, pancreas and lung, but also is involved in the intestinal barrier function, its use to ameliorate GIT inflammatory disorders such IBD and mucositis is very attractive. In this context, as showed above in this review, the use of *L. lactis* expression systems have been very effective to produce recombinant proteins and deliver them to the intestinal mucosal surface, and therefore could represent a suitable strategy to evaluate PAP protective effects.

Figure legends

Figure 1. Inducible Heterologous protein expression through NICE system in Lactococcus lactis

Figure 2. Reg3A protein binding and pore formation in Gram-positive bacteria.

Figure 1

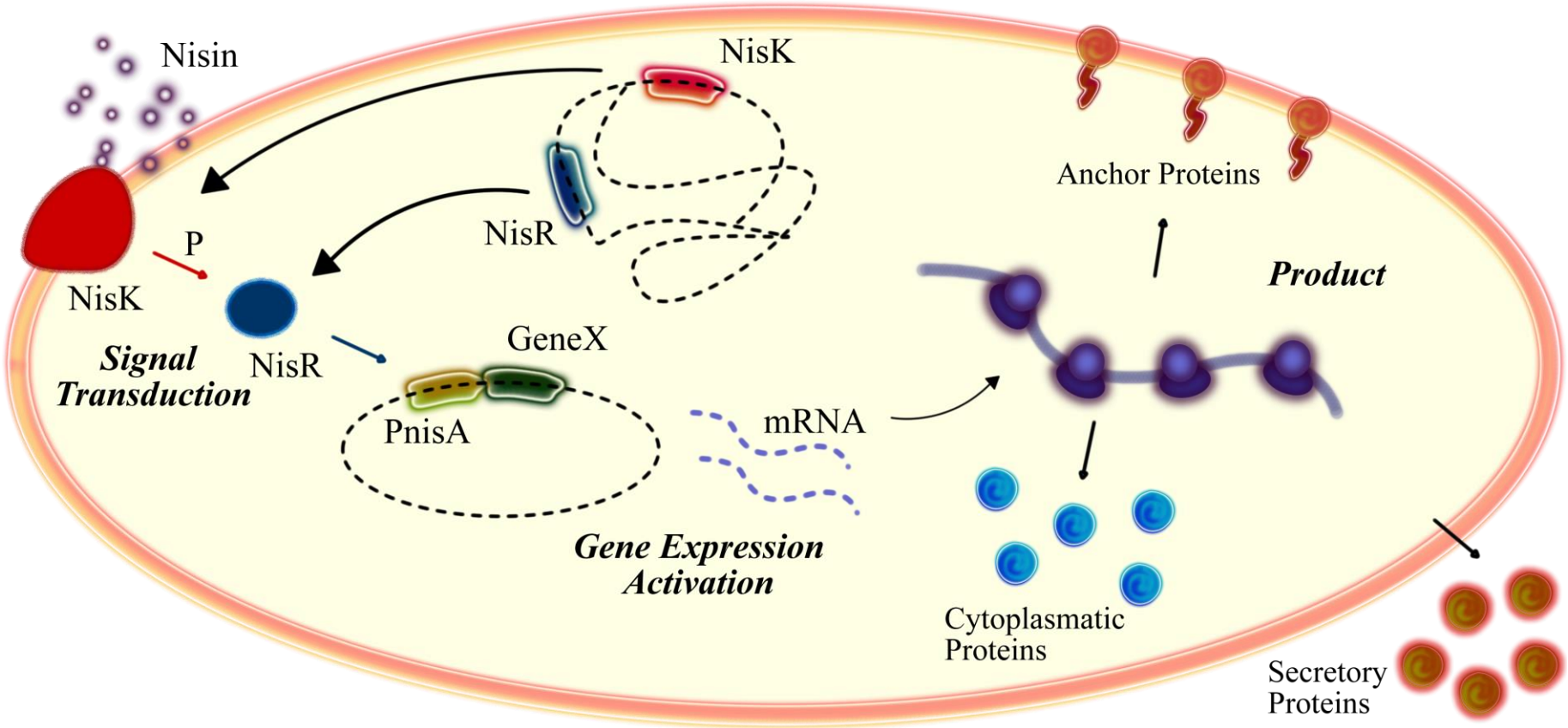
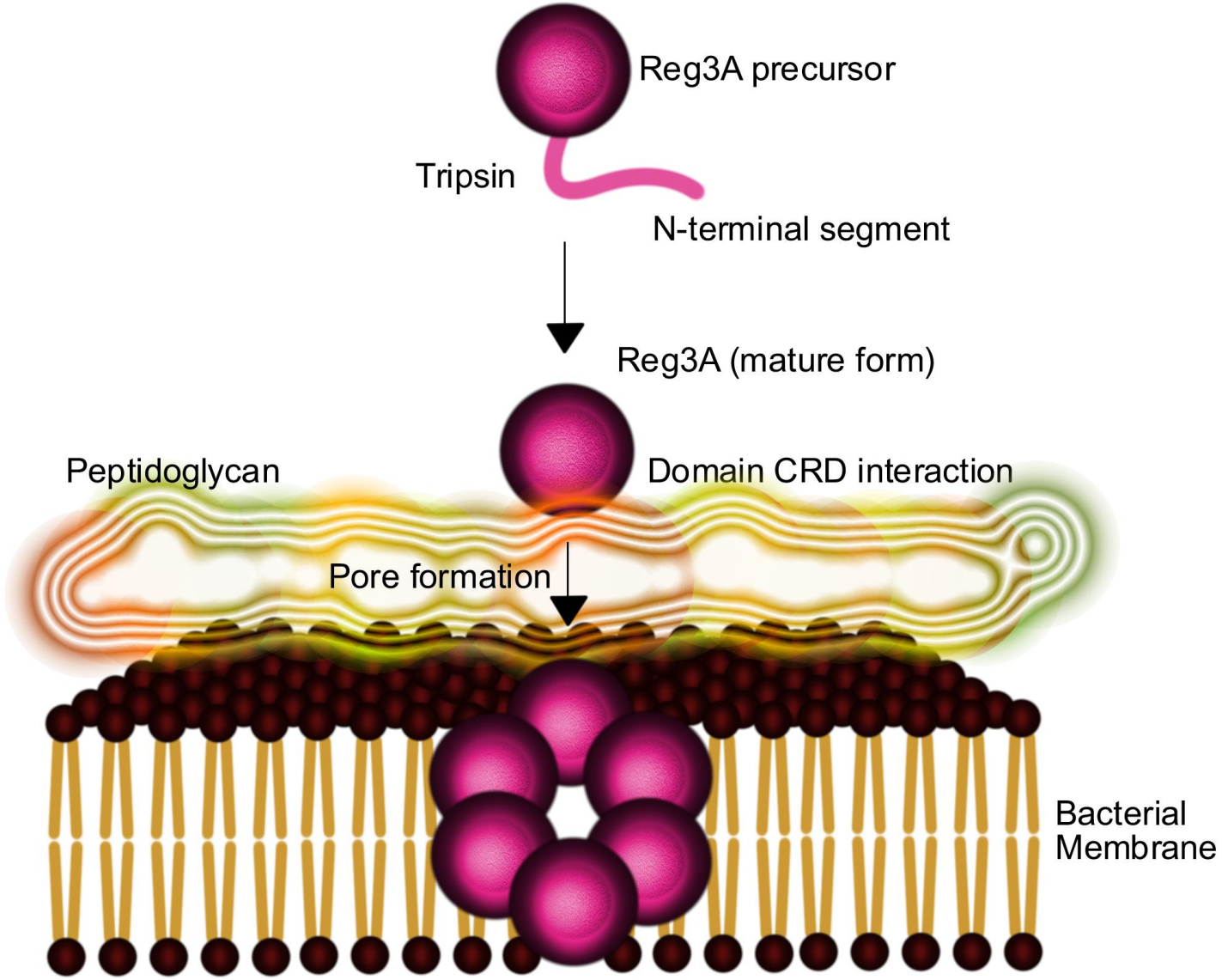

Nisin
NisK
NisR
P
NisK
Signal Transduction
NisR
GeneX
PnisA
mRNA
Gene Expression Activation
Anchor Proteins
Product
Cytoplasmatic Proteins
Secretory Proteins

Figure 2


Reg3A precursor
Tripsin
N-terminal segment
Reg3A (mature form)
Peptidoglycan
Domain CRD interaction
Pore formation
Bacterial
Membrane

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Chapter II

Justification for the study

2. Justification for the study

Inflammatory Bowel Diseases (IBD) are chronic diseases triggered by a number of factors, such as genetic predisposition, dysbiosis and several environmental conditions. In summary, these disorders can be described as microbiota-driven pro-inflammatory responses in the human gastrointestinal (GIT). Clinical common forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Both are associated with severe intestinal inflammation, and patients report GI symptoms as abdominal pain, diarrhea, rectal bleeding and weight loss. IBD represent a global health issue since incidence has increased in several countries and conventional therapy is not sufficient for immediate relief of the symptoms. Moreover, drugs adverse side effects present great risks to patients by affecting the host immune system and thus increasing susceptibility to infections. Hence, scientific community has sought novel therapeutic alternatives to fight IBD. As dysbiosis play a key role in the pathogenesis of the disease, restoration of the patient's original microbiota via the administration of probiotic bacteria has been proposed, instead of using medicaments, especially those belonging to the lactic acid bacteria group (LAB). Recent data demonstrates that some LAB strains are probiotic, presenting several positive effects on human health, for instance restoring impaired microbiota and suppressing pro-inflammatory immune responses. Up to date, there have been more than a hundred studies showing promising results on the use of probiotics to treat both UC and CD in pre-clinical tests. Probiotic interventions in clinical trials conducted in patients who have been diagnosed with IBD have also shown promising outcomes for both diseases as well. In addition, LAB is considered safe for human ingestion as they are used in food industry for centuries and few side effects have been observed from long-term consumption.

In order to boost LAB probiotic characteristics, some strains have been modified using genetic engineering techniques to express heterologous proteins of medical interest, especially those with anti-inflammatory properties. Among LAB species, *Lactococcus lactis* is the most studied considering its metabolic pathways, physiology and genetics. Therefore, several tools for both cloning and heterologous expression became available for this bacterium and are allowing the construction of different recombinant strains capable of expressing anti-inflammatory molecules. Thus, recombinant *L. lactis* strains have been tested as an alternative strategy to treat IBDs as well as wild type LAB strains. In recent years, several research groups have obtained satisfactory results using recombinant *L. lactis* as the strains have been shown to reduce inflammation after oral administration in pre-clinical trials. In fact, a phase II clinical trial based on a genetically engineered *L. lactis* strain was carried out in IBD patients, suggesting the feasibility of mucosal therapy using recombinant

lactococci. This trial opened the door for testing genetically modified *L. lactis* to treat other intestinal inflammatory conditions for which conventional treatment has been unsatisfactory, such as mucositis. This disorder may result from cancer treatment with the use of chemotherapy drugs, such as 5-Fluoracil (5-FU), which is vastly prescribed by oncologists to contain several kinds of cancers. 5-FU induced mucositis main symptoms are odynophagia, vomiting, abdominal pain, ulcers throughout the mouth and diarrhea, altering quality of life in patients. Furthermore, mucositis has a great clinical impact, as cancer therapy is usually discontinued affecting patient's chances of survival. Up to date, mucositis treatment relies mostly on mucosal coatings, cryotherapy, antibiotics and analgesics, which has not been sufficient to alleviate the disease. Hence, as dysbiosis has been recently implicated in the overall development of intestinal mucositis, probiotic approach, such as LAB, are being used to treat the disease in animal models, which have shown interesting results. Moreover, the use of genetically modified strains of *L. lactis* to produce proteins with anti-inflammatory properties has been promising to treat mucositis in phase II clinical trial as well.

Thus, *L. lactis* represents a safe vehicle for delivering novel molecules with anti-inflammatory properties to treat GIT disorders. Key elements in the host-microbiota relationship, such as antimicrobial peptides, are considered potential candidates to be cloned in *L. lactis* as the oral delivery of such peptides could prevent microbe-driven inflammation and promote intestinal homeostasis. It has been reported by current studies that the antimicrobial Pancreatitis-associated protein I (PAP) is secreted by specialized enterocytes, such as Paneth cells in response to microbe invasion and inflammatory damage in the intestinal mucosa. Indeed, cDNA encoding PAP protein delivered by an adenoviral vector to rats has been capable to prevent inflammation elicited by dextran sodium sulfate (DSS) administration.

As conventional treatment for both mucositis and IBD are limited and elicits strong adverse effects, scientific community is experimenting other alternative therapies, as the oral administration of either wild type or recombinant LAB, especially *L. lactis*, designed to express anti-inflammatory molecules. Therefore, in this work we decided to construct and evaluate the therapeutic effect of recombinant *L. lactis* expressing the antimicrobial peptide PAP using either dinitrobenzene sulfonic acid (DNBS)-induced murine model of colitis or 5-FU induced small bowel mucositis in a mouse model as well.

Chapter III

Aims of the study

3. Aims of the study

3.1 Main aim of the study

The main aim of the study was to evaluate the therapeutic effect of recombinant *L. lactis* strain expressing human pancreatitis associated protein I (PAP) using a murine experimental model of colitis induced by dinitrobenzene sulfonic acid (DNBS) or in a mouse model of 5-Fluorouracil-induced small intestinal mucositis

3.2 Specific aims of the study

-Induce the expression of recombinant human PAP previously cloned into *Lactococcus lactis* NZ9000 strain using the lactococcal bacteriocin nisin;

-Measure PAP expression in different cells compartments from induced and noninduced *L. lactis* cultures by enzyme-linked immunosorbent assay (ELISA);

-Induce acute colitis in specific pathogen-free C57BL/6 mice through DNBS administration;

-Immunize both inflamed and non-inflammed mice by intragastric gavage with recombinant and non-recombinant lactococci;

-Evaluate if PAP-expressing *L. lactis* strain reduces either macroscopic or microscopic scores of inflammation in C57BL /6 DNBS-treated mice;

-Assess immune responses elicited in non-treated and DNBS-treated mice after therapy with recombinant lactococci;

-Verify the inhibitory activity of cultures supernatants from *L. lactis* NZ9000 and *L. lactis* expressing PAP strains against pathogenic *Listeria monocytogenes;*

-Induce mucositis in conventional BALB/c mice through 5-Fluoracil administration;

-Immunize both inflamed and non-inflammed mice by continuous feeding with recombinant and non-recombinant lactococci producing PAP;

-Assess Immunoglobulin A secretion in non-treated and 5-Fluoracil-treated mice after therapy with recombinant lactococci;

-Evaluate the therapeutic effect of *L. lactis* NZ9000 and *L. lactis* expressing PAP in the treatment of 5-FU-induced mucositis in convetional BALB/c mice.

Chapter IV

Oral delivery of Pancreatitis-Associated Protein (PAP) by the food-grade bacterium *Lactococcus lactis* displays protective effects in mouse acute colitis model through Treg cells induction

Chapter IV- Oral delivery of Pancreatitis-Associated Protein (PAP) by the food-grade bacterium *Lactococcus lactis* displays protective effects in mouse acute colitis model through Treg cells induction

This chapter presents a brief description of the study of the use of recombinant *Lactococcus lactis* strains expressing PAP as an alternative strategy for treatment of Crohn's disease. The manuscript of the study, which will be submmited as a research article, is presented below. This study was conducted in the Institut national de la recherche agronomique (INRA-France) in collaboration with the Laboratory of cellular and molecular genetics (LGCM) from Federal University of Minas Gerais (UFMG-Brazil). It has contributed to the development of an international joint supervision PhD thesis between the University of Paris-sud (France) and UFMG. Therefore, experiments were carried out at INRA in order to construct recombinant *L. lactis* strains expressing human PAP; verify its expression by the strains through ELISA and western blotting. Particularly, I was involved in the *in vivo* evaluation of *L. lactis*-PAP protective effects to prevent acute colitis in C57BL/6 mice.

4.1 Introduction

Over the last two decades, IBD have been considered a worldwide problem, especially in industrial-urbanized societies. Current treatment drugs used for alleviating the symptoms are associated with serious side effects, which can last for months and cause great discomfort to the patients (Nell *et al*., 2010; M'Koma, 2013; Koren *et al*., 2013; Ananthakrishnan, 2015). Probiotic bacteria have been proposed and tested as an alternative therapy for IBD because they are very effective to treat dysbiosis, a key factor for the development of gastrointestinal (GIT) inflammation. Most bacterial probiotics species belong to lactic acid bacteria (LAB) group, in which many species are considered generally regarded as safe, such as *Lactococcus lactis.* Recently, the use of genetically modified strains of *L. lactis* to produce proteins with anti-inflammatory properties has been promising to treat IBD as well (Del Carmen *et al*., 2014; Zurita-Turk *et al*., 2014; Moreno *et al*., 2015). Thus, key elements in the host-microbiota relationship are considered potential candidates to be cloned in *L. lactis*. Current studies have shown that the antimicrobial pancreatitis-associated protein I (PAP) could present anti-inflammatory properties as it is over-expressed in IBD patients. Actually, it was demonstrated that PAP have antimicrobial function and anti-inflammatory properties (Seo *et al*., 2012; Wong *et al*., 2012). In this context, this study aimed to construct a recombinant *L. lactis* strain expressing human PAP and evaluate whether the same strain could prevent inflammation in a murine model of colitis induced by dinitrobenzene sulfonic acid (DNBS).

4.2 Materials and Methods

In order to construct recombinant *L. lactis* strains, the gene *reg3A* encoding mature human PAP was inserted in pSEC or in pCYT vector, both harboring the nisin-inducible promoter, for obtainment of secreted and cytoplasmic PAP, respectively. Afterwards, either pSEC:PAP or pCYT:PAP were transformed into *L. lactis* NZ9000. To increase the yield of secreted PAP, pSEC:PAP plasmid was transformed in *L. lactis* NZ9000htrA- strain, which is deficient in HtrA protease. Concentration of cytoplasmic and secreted PAP was measured in the culture medium of all strains by enzyme-linked immunosorbent assay (ELISA). Therefore, C57BL/6 mice were administered with *L. lactis* harboring pSEC empty vector (LL) or the same bacterium containing pSEC:PAP (LL-pSEC:PAP) plasmid for 7 days by gavage and then injected or not with DNBS. During this time, animals were monitored daily to assess the severity of colitis. Mice were euthanized; colon, mesenteric lymph nodes (MLN) was removed for clinical, histological and immunological analysis. One Way ANOVA and "Bonferroni" post-test was used to determine statistical significance across groups.

4.3 Results and discussion

L. lactis NZ9000 transformed with pSEC:PAP was able to express the highest amount of recombinant PAP, encountered in the supernatant from induced cultures of LL-pSEC:PAP. Thus, this data confirmed the ability of *L. lactis* to efficiently express PAP using the NICE system. The use of *L. lactis* lacking the extracellular protease HtrA did not improve PAP secretion. *L. lactis* NZ9000 containing pSEC:PAP was chosen for further *in vivo* experiments. DNBS was used to simulate immunological and histological aspects from human IBD in C57BL/6 mice (Barnett & Fraser, 2011). In order to investigate the protective effect of LL-pSEC:PAP in DNBS-induced colitis, body weight was measured because it reflects the severity of gut inflammation (Ananthakrishnan, 2015). As expected, animals administered with DNBS presented a substantial weight loss while group treated with LL-pSEC:PAP recovered weight after DNBS challenge. Moreover, DNBS-treated mice from the control group showed increased macroscopic clinical index, demonstrating that an acute colitis was successfully reproduced in the animals. On the other hand, LL-pSEC:PAP strain significantly decreased disease index and protected the mucosa against damage induced by DNBS. These results are in agreement with a previous study in which a cDNA encoding PAP protein delivered by an adenoviral vector to rats suppressed inflammation and ulceration elicited by dextran sodium sulfate (DSS) administration (Lv et al., 2012). Furthermore, LL-pSEC:PAP administration decreased intestinal mucosal permeability when compared to control group, demonstrating that PAP produced by *L. lactis* has a role in maintaining barrier function. It was also demonstrated a decrease in the expression of Th1 pro-inflammatory cytokines, as IL-12 and IFNγ, Th2 cytokines, as IL-4 and IL-5, and Th17 cytokines (IL-17) in mice MLN. TGF-β expression was also quantified, as this cytokine plays key role in the induction of T regulatory immune responses. It was observed increased TGF-β concentrations after LL-pSEC:PAP treatment in mice MLN as well as Foxp3 regulatory T cells in intestinal *lamina propria.* The expansion of these cells were related to the down-regulation of pro-inflammatory cytokines and amelioration of colitis in treated mice. Nevertheless, PAP exact mechanism of action to be investigated.

4.4 Conclusions

The study demonstrated the successful cloning and expression of human PAP expression by *L. lactis* NZ9000 harboring either pSEC:PAP or pCYT:PAP. The use of the strain lacking HtrA extracellular protease did not improve PAP secretion. Lastly, mice injected with DNBS and treated with *L. lactis* carrying pSEC:PAP vector showed reduced scores of inflammation.

Oral delivery of Pancreatitis-Associated Protein (PAP) by the food-grade bacterium *Lactococcus lactis* displays protective effects in mouse acute colitis model through Treg induction.

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Abstract

Antimicrobial peptides secreted by intestinal immune and epithelial cells are important effectors of innate immunity. They play an essential role in the maintenance of intestinal homeostasis by limiting microbial epithelium interactions and preventing unnecessary microbe-driven inflammation. Pancreatitis-associated protein (PAP), member of the Regenerating islet-derived III (RegIII) proteins family, is a C-type (Ca+2 dependent) lectin which binds selectively to specific carbohydrate structure of bacteria establishing thus a balance between pathogens and symbionts. Different epithelial cells secrete PAP and its expression is increased in the mucosa of patients with inflammatory bowel diseases (IBD). Afterwards, emerging evidence suggests that this protein may represent an anti-inflammatory agent, once it can inhibits NF-κβ activation and down-regulates cytokine production and adhesion molecule expression in inflamed tissue. Here, we sought to determine whether PAP delivered at intestinal membrane by recombinant *Lactococcus lactis* strain, LL-PAP, is able to reduce the intestinal inflammation in chemically-induced colitis model. We showed, using acute colitis model induced by a DiNitro-BenzeneSulfonic-acid (DNBS) challenge, a decrease in colitis severity in mice when treated with LL-PAP compared to those treated with the control *L. lactis* strain characterized by: i) protection against weight loss; ii) lower macroscopical and histological scores; iv) down-regulation of pro-inflammatory cytokines; v) up-regulation of TGF-β and Treg cells. Based on these findings, we hypothesize that a treatment with LL-PAP is able to reduce colon inflammation and enhance barrier integrity in acute colitis model through Treg cells induction.

Keywords: RegIII, PAP, IBD, *Lactococcus lactis*, colitis model.

Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders located in the large and/or small intestine, including ulcerative colitis and Crohn's disease. These diseases are multi-factorial driven mainly by an inappropriate immune response to gut microbes in a genetically predisposed host [1, 2]. This group of diseases has a substantial socioeconomic impact worldwide, being a significant health problem in Western societies. Indeed, these diseases affect millions of patients, which may have relapse and also remit to condition of long-term morbidity. Moreover, there is no permanent drug cure; therefore their treatment represents a medical challenge [1-3]. Some of the existing treatments for IBD include anti-inflammatory and immunosuppressive drugs presenting severe side-effects. In later years there has been a landmark of discoveries and advancements for the therapeutic intervention of IBD but nevertheless new tools are required [2-5].

Antimicrobial peptides secreted by intestinal immune and epithelial cells are important effectors of innate immunity. They are key elements in the host-microbiota relationships by restricting contact between commensal bacteria and epithelial surface [6, 7]. PAP is a member of the type III subclass of the REG gene family discovered in regenerating pancreatitis islets in rat encoding a small group of proteins involved in the control of epithelial cell proliferation in various organs, included pancreas and intestine [8-12]. This protein is characterized as C-type lectin able to bind selectively to carbohydrate structure, often in a Ca^{+2} dependent manners [6]. In the colon, PAP is the main protein synthesized by goblet cells and in the small intestine by Paneth cells, located in the crypt. Moreover, in *lamina propria*, PAP is secreted by intraepithelial lymphocytes. Clinical observations showed that PAP is over-expressed in colonic tissue of active IBD patient and could have anti-inflammatory properties [9, 13, 14]. Some

studies showed that PAP have a protective effect against inflammatory damage by preventing TNFα to induce NFkB activation in macrophage, down-regulating inflammatory cytokines production and adhesion molecule expression in inflamed tissue [9, 11]. PAP and its murine counterpart RegIIIα are thus antimicrobial peptides showing anti-inflammatory properties [6].

The use of living genetically engineered strains of the food-grade bacterium *Lactococcus lactis* delivering therapeutic molecules *in situ* has been promising to treat different human diseases as allergy [15, 16], cancer [17], obesity [18] or IBD [19-21]. We hypothesized that exogenous PAP delivery by recombinant *L. lactis*-PAP might act against inflammatory process taking place in IBD. An increase of PAP delivered locally at mucosal level may enhance the epithelial barrier integrity and protect from inflammation.

Methods

Cloning of the human Pancreatitis-Associated Protein (PAP) gene in *L. lactis*

A 478-bp DNA fragment encoding for mature human PAP (i.e., without the signal peptide) was PCR amplified from the pSPORT1:PAP vector [22] using primers *Nsi*I-PAP (5'-CC A***ATGCAT***CAGAAGAACCCCAGAGGGAACTG-3') and *Eco*RI-PAP (5'-GG***GAATTC***A CTCAGTCCCTAGTCAGTGAACTTGCAGACA-3'). The resulting fragment was directly digested with *Nsi*I and *Eco*RI enzymes (restriction sites on the primers are indicated in bold and italics) and cloned into purified backbone isolated from the *Nsi*I-*Eco*RI-cut pSEC-E7 vector [23] resulting in pSEC:PAP or *Nsi*I-*Eco*RI-cut pCYT-E7 vector resulting in pCYT:PAP. Both plasmids were introduced into *L. lactis* strain NZ9000 carrying the regulatory genes nisR and nisK [24] to obtain the strain LL-PAP. pSEC:PAP was also introduced into NZ9000htrA- [25]. As a negative control, NZ9000 was transformed with a pSEC empty vector to generate strain LL. Recombinant *L. lactis* clones were selected by the addition of 10 µg/ml chloramphenicol.

Inducible expression of PAP

For the induction of PAP expression from the nisin promoter, strains were grown in M17 medium (Difco) supplemented with 1% glucose (GM17) at 30°C without agitation until an optical density at 600 nm of 0.6. Recombinants *L. lactis* were selected by the addition of 10 µg/ml chloramphenicol. Afterwards, the strains were induced with 10 ng of nisin (Sigma) per ml for 2 h. *L. lactis* culture extraction and immunoblotting assays were performed as follows, using a polyclonal serum specific from Human Reg3A (R&D Systems). Protein samples were prepared from 2 ml of induced culture at a DO_{600} = 1. After centrifugation (5 min, 10,000 rpm), the cell pellet and supernatant were treated separately. The supernatants were treated with 100 µl of 100% trichloroacetic

acid (TCA) to precipitate proteins. Samples were incubated for 1 h on ice, and proteins were recovered from the pellets after centrifugation at 4°C for 30 min at 13,000 rpm. The cell fractions were resuspended in PBS supplemented with anti-protease and sonicated (6 cycles of 10 seconds sonicating and 10 second rest) on ice. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, Western blotting, and immunodetection were performed as previously described [23, 26].

The concentrations of PAP secreted in the medium and retained in cell fractions were assessed by an enzyme-linked immunosorbent assay (ELISA) kit (Dynabio) too. Human commercial PAP (BioVendor) was used as a control in Western blotting and ELISA.

Animals

Specific pathogen-free C57BL/6 mice (6-8 weeks old; Janvier, France) were maintained under normal husbandry conditions in the animal facilities of the National Institute of Agricultural Research (UEIERP, INRA, Jouy-en-Josas, France). All animal experiments began after the animals were allowed 1 week of acclimation and were performed according to European Community rules of animal care and with authorization 78-149 of the French Veterinary Services.

Induction of acute colitis and bacteria administration

The protocol of DNBS-induced acute colitis is detailed in Fig 2. Briefly, mice of approximately 20 g were fully anesthetized by intraperitoneal (*i.p.*) injection of 150 µl of 0.1% ketamine (Imalgene 1000, Merial, France) and 0.06% xylazine (Rompun) and a 3.5 catheter (French catheter, Solomon Scientific) attached to a tuberculin syringe was inserted into the colon. A dose of 150 mg/kg of DNBS solution (ICN, Biomedical Inc.) in 30% ethanol (EtOH) was then injected intra-rectally (*i.r.*) to induce colitis. Control mice (without colitis) received only 30% EtOH. Mice were gavaged with $5x10^9$ CFU in 200 µl of either LL or LL-PAP in PBS, or PBS alone daily for 12 days. Weight loss was

monitored daily to assess the severity of colitis. Inflammation was monitored 4 days after DNBS administration by cytokine productions, macro and microscopy scores.

Macroscopic damage scores

Mice were sacrificed by cervical dislocation and the abdominal cavity was opened, the colon was removed and opened longitudinally and damage was immediately assessed macroscopically. Macroscopic scores were recorded using a previously described system [27, 28]. Briefly, the macroscopic criteria (assessed on a scale from 0 to 9) include macroscopic mucosal damages such as ulcers, thickening of the colon wall, the presence of adhesions between the colon and other intra-abdominal organs, the consistency of fecal material (as an indicator of diarrhea) and the presence of hyperemia.

Cytokine assays

One centimeter samples of distal colon were recovered and homogenized in an appropriate volume of PBS buffer containing protease inhibitors (Sigma-Aldrich) in a Tissue Lyser (Qiagen). The samples were centrifuged for 20 min and the supernatants collected and frozen at -80ºC until analysis. After Mesenteric Lymph Nodes (MLN) isolation from mice, lymphocytes were counted by and resuspended in RPMI culture medium (Lonza) with 100 Unit of Streptomicin, Penicilin (PAA Laboratories) and 10% SVF (Lonza) and $2x10^6$ cells were activated with 4μg/μL coated anti-mouse antibody CD3 and CD28 (eBioscience). Concentrations of cytokines IL-12, IL-17, IL-4, IL-5, and INF-γ (Mabtech) and TGF-β (R&D), in medium were assessed by ELISA after 48h of incubation.

Treg cells counting

Colon samples from treated and non-treated mice were fixed in 4 % paraformaldehyde acid (Sigma) and embedded in paraffin. Four micrometer sections were stained with

H&E and then with anti-FoxP3 ab54501 (10μg/ml - Abcam) staining FOXP3 (red) in paraffin embedded mice colon sections. In order to count FOXP3 stained cells we selected only epithelial surface and sort an area randomly. In the graph, cells are expressed in number of cells / pm2.

Statistical Analysis

GraphPad software (GraphPad Software, La Jolla) was used for statistical analysis. Results are presented as bar graphs or dot plots with means +/- SEM. Most comparisons involved one-way analysis of variance followed by the Bonferroni multiple comparison post hoc analysis. For data sets that were non-Gaussian or based on a score or on a percentage, the non-parametric Mann Whitney test was used. A *p* value of less than 0.05 was considered significant.

Results

Characterization of human PAP production by *Lactococcus lactis*.

PAP cDNA was inserted in pSEC or pCYT vectors, to obtain pSEC-PAP and pCYT-PAP (table 1), in order to produce PAP secreted or in cytoplasm. Then pSEC-PAP was introduced in two different strains NZ9000 or NZHtrA- and pCYT-PAP only in NZ9000. PAP expression was induced by nisin (10ng/ml) as described in Methods and tested for immunodetection in supernatant or bacterial pellet. The ability of LL-PAP to produce and secrete human PAP was then tested by ELISA. Highest PAP production was obtained with strains transformed with pSEC: PAP (Fig1). The use of NZhtrA- didn't increase the secretion of PAP. Recombinant strain NZ9000 containing pSEC:PAP (LL-PAP) was used in further experiments. A band of ~19 kDa in cytoplasm was detected in nisin-induced cultures of the LL-PAP by western-blot (data not shown).

LL-PAP treatment reduces the severity of DNBS-induced acute colitis

To validate the anti-inflammatory effects of LL-PAP *in vivo,* we used a well-established DNBS-induced colitis model [27-30]. The protocol used to develop the murine models of DNBS-inflammation is detailed in Fig 2A. Briefly mice were orally administered with LL or LL-PAP during 7 days until intra-rectal injection of DNBS. Mice were sacrificed 4 days after DNBS injection. Animals administered with LL-PAP lost less weight than control and even, after a 10% loss, they start to regain it at D2 (Fig 2B). Permeability to FITC was significantly decreased when mice were treated with LL-PAP than with LL (Fig 2C). Other parameters such as macroscopic (including ulcers, hyperemia, thickening of the colon wall, the presence of adhesions between the colon and other intra-abdominal organs and the consistency of fecal material) and microscopic scores (including architectural derangements, goblet cell depletion,

edema/ulceration, and degree of inflammatory cell infiltrate) were reduced by ~75 and ~50 % respectively (Fig 2D, E).

LL-PAP treatment is able to decrease the pro-inflammatory immune response.

Amount of main pro-inflammatory cytokines IFN-γ, IL-12p70, IL-4, IL-5 and IL-17 detected in supernatant of activated lymphocytes isolated from MLN of DNBS-mice were lower in LL-PAP-treated mice than in control mice (Fig 3). While the anti-inflammatory TGF-β cytokine was expressed at higher levels in LL-PAP administered mice.

LL-PAP treatment increase the number of Treg cells.

Considering the increase of TGF-β in LL-PAP treated mice we looked for Treg cells in the lamina propria. Treg were detected using an anti-FoxP3 antibody and counted. Treg number decrease slightly in mice treated by DNBS compared to control one (Fig 4). Mice orally administered with LL-PAP showed a 2 fold increase of Treg number compared to LL or PBS group (Fig 4).

DISCUSSION

The central question of this work was to study the protective properties of PAP. To achieve this goal we used recombinant *L.lactis* strains expressing PAP. And so PAP delivered by recombinant *L. lactis* in the lumen of the digestive tract might have a protective role in DNBS-induced acute colitis model.

Anti-microbial activity of PAP is controversial. It has been described that PAP is bactericidal specifically to gram-positive bacteria [6] but other authors report that PAP show no anti-microbial activity [31, 32]. To be active PAP needs to be processed. Thus a flexible N-terminal prosegment is removed *in vivo* by trypsin [33]. For Lactococci being gram-positive bacteria, we have produced PAP with its prosegment avoiding thus any potential anti-microbial activity *in vitro*. We hypothesize that recombinant *L.lactis* produce and secrete *in vivo* a non-active PAP which will be processed and activated by endogenous trypsin in the digestive tract.

Anti-inflammatory properties of PAP have been first shown in mice in pancreatitis model. Administration of antibodies specific from PAP in a pancreatitis murine model increases creases the inflammation in the pancreas [34]. Similar results have been obtained using an antisense strategy [35]. But PAP has also anti-inflammatory properties in the gut. In IBD patients PAP serum levels is increased and correlated with clinical symptoms. Moreover, co-incubation of purified recombinant PAP with explants from active Crohn's disease patients reduces the secretion of pro-inflammatory cytokines through the inhibition of NF-kB pathway [9]. Here we confirm the anti-inflammatory activities of PAP by a new mechanism of action. Indeed, we showed that oral administration of PAP is able to have protective effect on weight loss, intestinal permeability, macroscopic score and tissue damage. Moreover oral PAP administration

decrease the Th1 (IL-12, IFNγ), but also Th2 (IL-4, IL-5) and Th17 (IL-17) immune response in MLN. Both Th1 (IFNγ and IL12) and Th17 (IL17) are implicated on the development of colitis and linked to severity colitis. In the opposite we could detect an increase of TGF-β. TGF-β is a major player in the development of the Treg immune response. Considering these results we decided to look for Treg proliferation in the lamina propria and were able to detect an increase of Treg cells in LL-PAP treated mice compared to LL-group. To our knowledge it's the first time that PAP has been described to play a role in Treg development through TGF-β stimulation.

PAP protective effect on DSS-induced colitis model has been shown in rats [36]. Authors used adenovirus strategy to deliver PAP cDNA in host cells and thus increase the expression of PAP. We haven't been able to show any protective effects of PAP delivered by *L.lactis* in DSS (data not shown). Oral administration of LL-PAP didn't improve any criteria (weight loss, integrity of the epithelial membrane, immune response) in our DSS-induced colitis model. In these two strategies the main difference could be that the PAP is not produced/delivered in the same area. PAP production after adenovirus delivery will be local at the level of the mucus layer. Usually PAP is secreted by goblet cells to reduce bacterial concentration in the mucus layer. The mucus layer of RegIII KO mice is invaded by bacteria which elicit a low grade inflammation [37]. Oral administration of recombinant LL-PAP should increase the concentration of PAP in the lumen acting thus in a different place and possibly targeting another population of bacteria.

In conclusion, all our results confirm the potential use of the *L. lactis* to deliver human PAP aiming reduce or prevent intestinal inflammation. However although this study is a proof of concept on the used modified microorganisms to treat a subset of IBD, more

studies should be performed before considering this type of treatment as a feasible therapy to be tested in humans.

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Figure 1

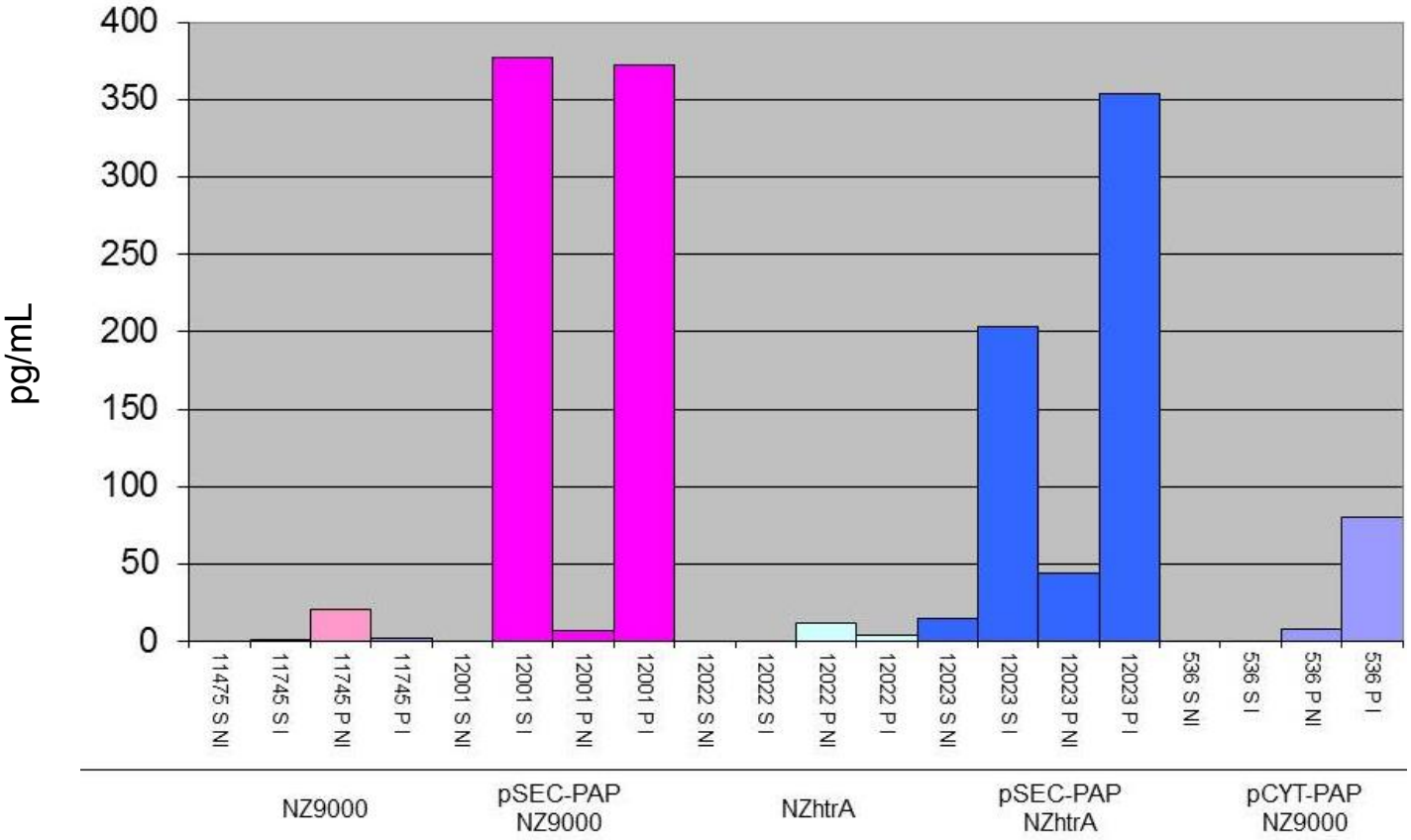
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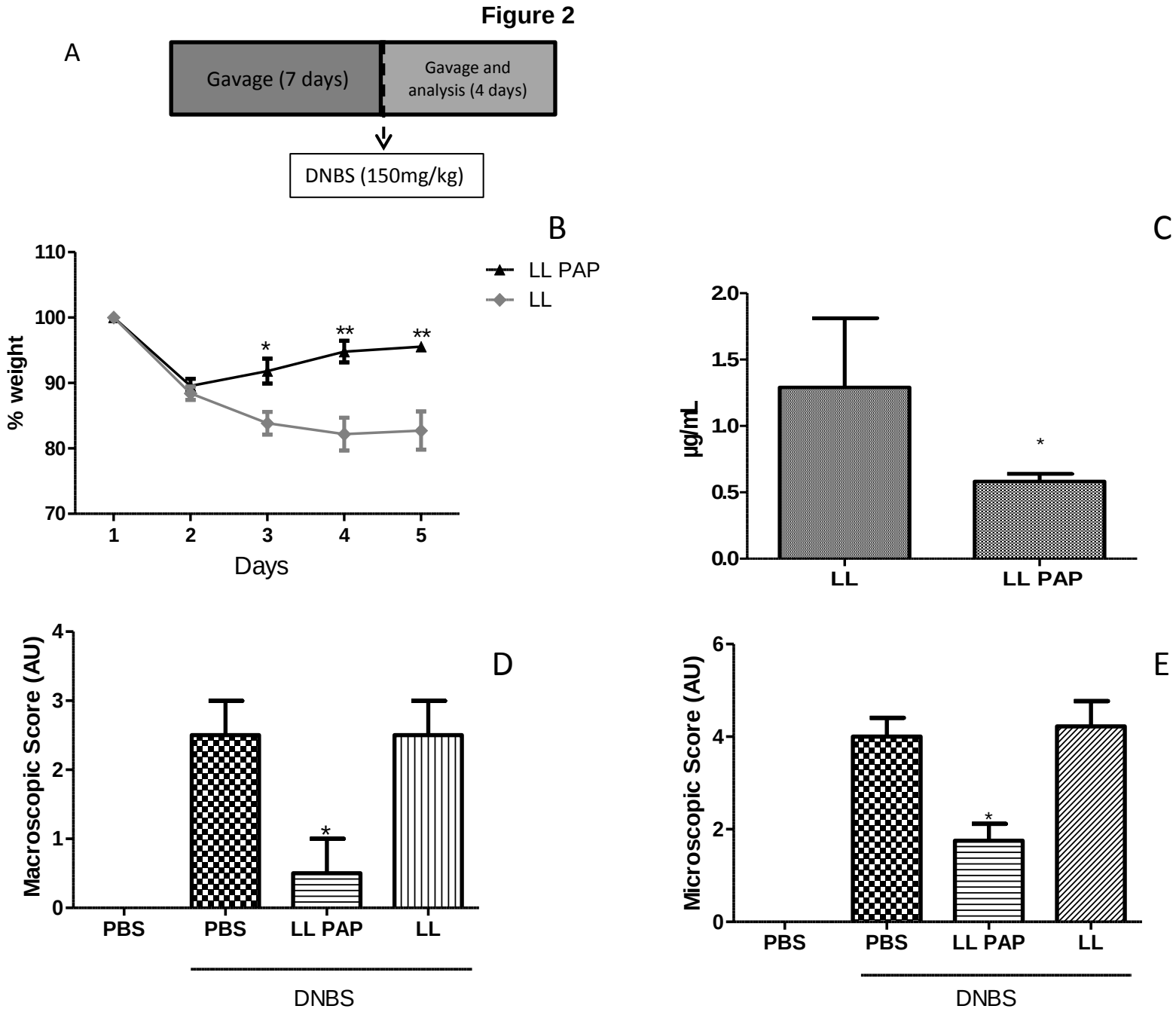
Figure 2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Gavage and analysis (4 days)
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LL
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Figure 3

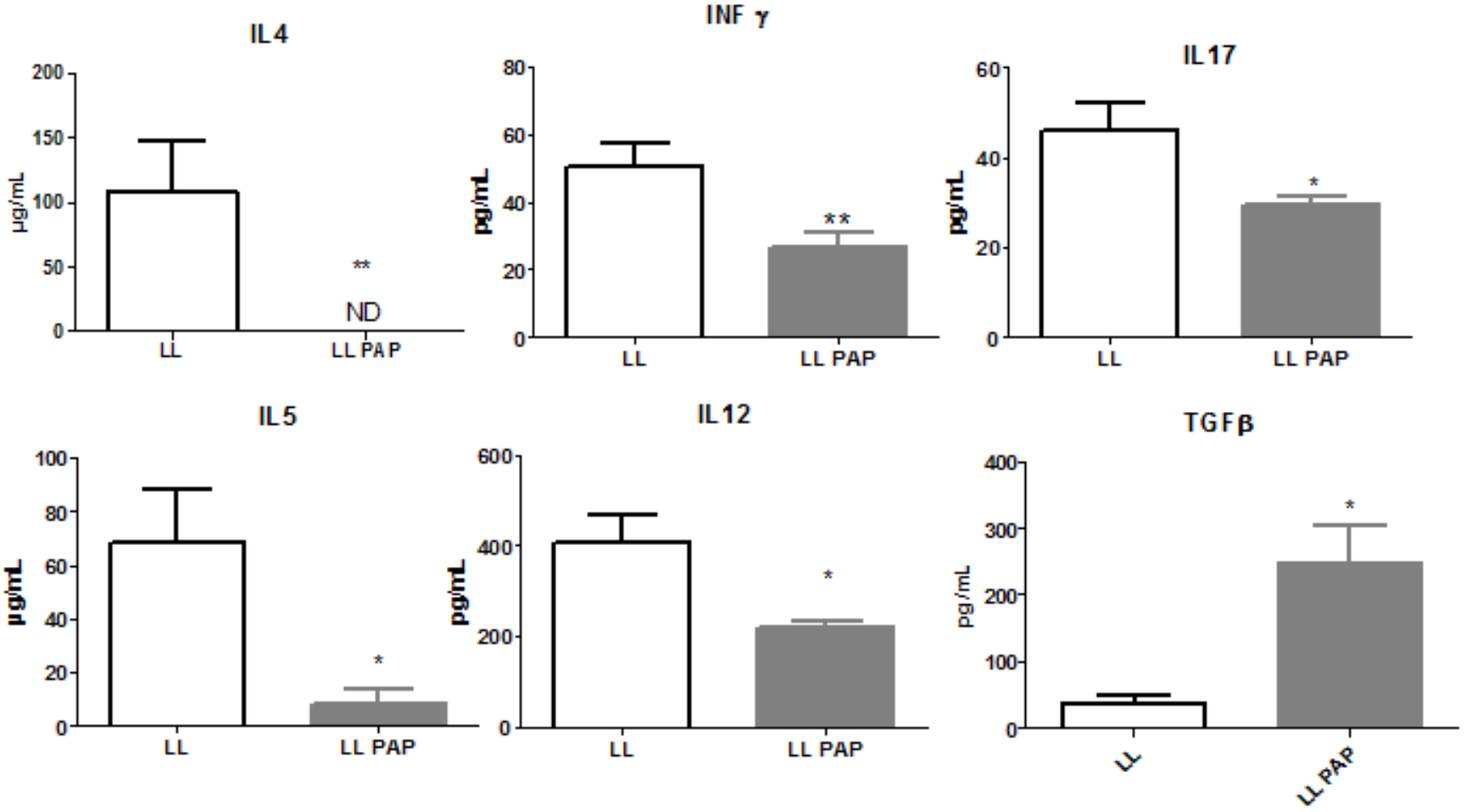
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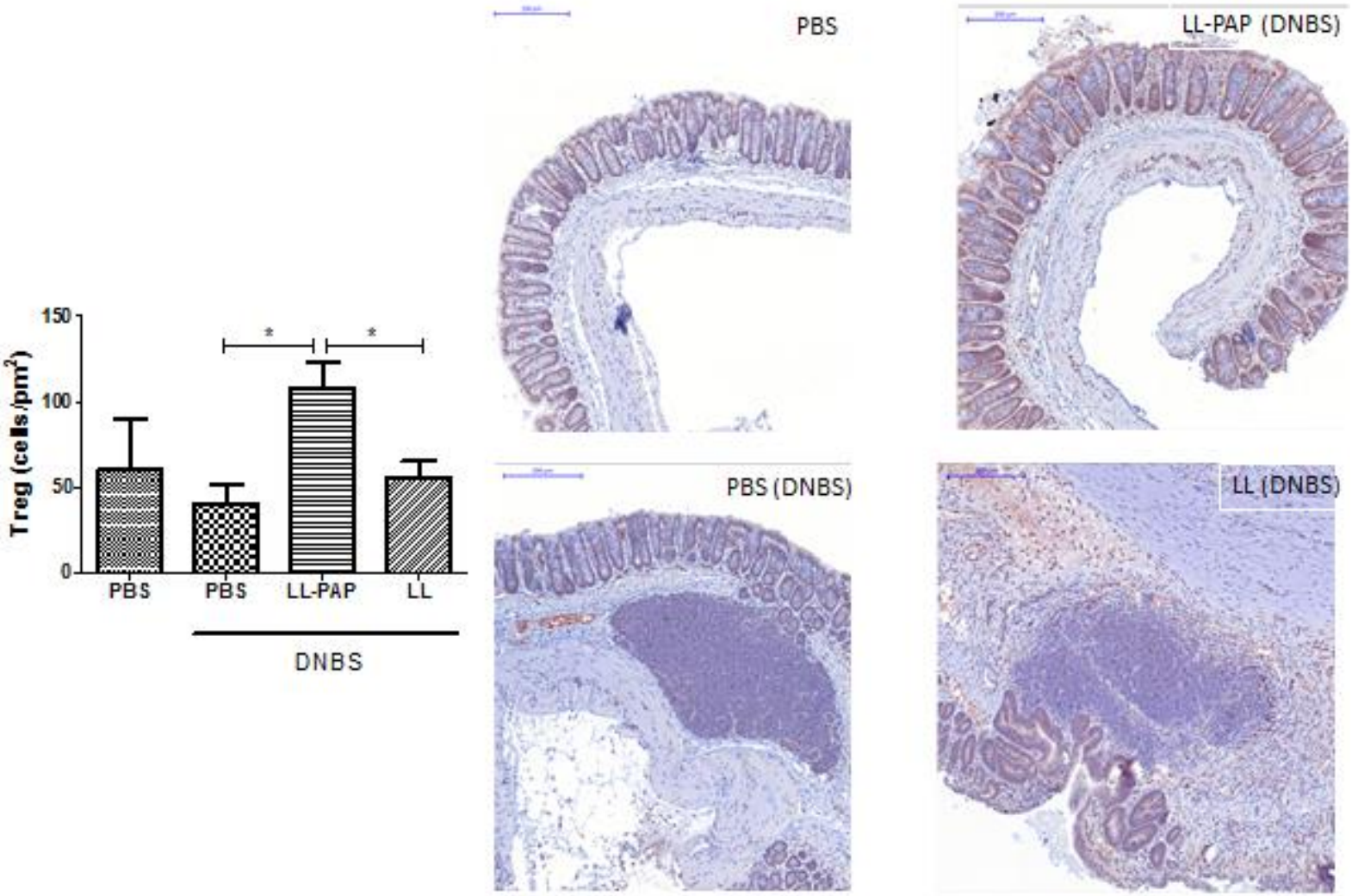
Figure 4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Chapter V

Secretion of biologically active pancreatitis-associated protein I (PAP) by dairy genetically modified *Lactococcus lactis* NZ9000 in the prevention of intestinal mucositis

Chapter V - Secretion of biologically active pancreatitis-associated protein I (PAP) by dairy genetically modified Lactococcus lactis NZ9000 in the prevention of intestinal mucositis

This chapter presents a brief description of the study that was designed to evaluate *L. lactis* expressing PAP as an alternative strategy for the treatment of mucositis. In the next content, the manuscript, which will be submitted as a research article to the journal Microbial cell Factories is presented. This study was conducted in UFMG (Brazil), as part of an international joint supervision thesis with the University of Paris-Sud (France). We have contributed to the present study overall development through the investigation of PAP protective role in the prevention of mucositis. For this purpose *in vivo* experiments were carried out in order to induce mucositis in conventional BALB/c mice; immunize mice using PAP-expressing *L. lactis* strains; assess immune responses elicited in non- 5-FU-treated mice. The French institute INRA has collaborated with this work by providing the recombinant and wild type Lactococci strains.

5.1 Introduction

Mucositis is a gastrointestinal (GI) inflammatory disorder affecting the quality of life of patients that make use of chemotherapy drugs, such as 5-Fluoracil (5-FU) (Sonis et al., 2004; Pedroso, 2015). As conventional treatments presents limitations in alleviating the disease, scientific community has been encouraged to investigate alternative strategies (Plevová, 1999; Sonis, 2009). The oral administration of recombinant *L. lactis* strains, designed to express anti-inflammatory molecules has been shown capable to prevent others GI inflammatory disorders such as the Inflammatory Bowel Diseases (IBD) and thus represent a promising strategy to treat mucositis as well (Del Carmen *et al*., 2014 ; Zurita-Turk *et al*., 2014; de Moreno *et al*., 2015). It has been reported that the antimicrobial Pancreatitis-associated Protein I (PAP) may play a key role on the host-microbiota relationship by restricting contact of gram-positive bacteria to epithelial cells, inhibiting elicitation of pro-inflamatory responses the intestines (Vaishnava et al., 2011; Lv et al., 2012; Mukherjee et al., 2014). Recently, our research group has shown that the use of *L. lactis* expressing PAP could prevent colitis in a chemically induced murine model of IBD. Hence, the present study aimed to evaluate the therapeutic effect of *L. lactis* expressing PAP in a murine model of mucositis induced by 5-FU.

5.2 Materials and Methods

L. lactis NZ9000 harboring pSEC:PAP (LL-PAP) was previously obtained (unpublished). The inhibitory activity of PAP in *L. lactis* supernatant or the same strain harboring empty pSEC vector (LL) was assessed against *L. monocytogenes.* Therefore, BALB/c mice were administered with LL or LL-PAP for 14 days by continuous feeding and then injected intraperitoneally with 5-FU. During the experiment, mice weight was monitored daily. Following euthanasia, mice ileum were removed and reserved for histological analysis and determination of EPO activity. The remaining segment of small bowel was used for assessing secretory IgA (sIgA) levels. One Way ANOVA and “Bonferroni” post-test was used to determine statistical significance across groups.

5.3 Results and discussion

The growth of *L. monocytogenes* decreased after 4h of incubation in LL supernatant, while incubation in LL-PAP supernatant caused no significant reduction. We suppose the presence of lactic acid or others metabolites produced by *L. lactis* NZ9000 could have an antagonistic activity against this pathogen whereas the inhibitory effect of PAP could not be detected due to its low concentration in the supernatant. Next, 5-FU was used to induce mucositis in BALB/c mice and, as expected, our data has showed an intense inflammatory process affecting the ileum mucosa of mice, which is consistent with several previous studies

which also used this medicament to induce mucositis (Soares *et al*., 2013; Maioli *et al*., 2014; Antunes *et al*., 2015; Generoso et al., 2015). LL culture treatment in mice was capable to prevent histological damage caused, while PAP delivery improved only mucosal architecture preservation. However, the treatment with both strains could not restore the weight of the animals. Then, morphometric analysis was used to determine epithelial integrity. LL-PAP treatment provided improved villus height and villus/crypt ratio, and in addition, improved Paneth cells activity by increasing granular density. These results could be related to PAP proliferative function in epithelial cells, which has been reported by previous studies (Lai *et al*., 2012; Lv et al., 2012). To estimate eosinophil influx into in *lamina propria*, we sought to determinate EPO activity in mice ileum. Our results demonstrate that the administration of LL in mice caused reduced eosinophil infiltration in the mucosa, whereas no further reduction was observed in the LL-PAP treated mice. Lastly, we investigated IgA secretion in the intestinal fluid of mice. LL culture administration decreased IgA secretion, suggesting this strain might prevent microbe-associated damage in the small bowel. In the other hand, PAP delivery did not caused a significant reduction. Finally, we investigated IgA secretion in the intestinal fluid of mice. LL culture administration reduced IgA concentration, suggesting that this strain was able to prevent microbe-driven inflammatory damage in the small bowel. Moreover, PAP delivery did not show a significative reduction.

5.4 Conclusions

The study demonstrated that PAP expressed in *L. lactis* harboring pSEC:PAP did not have inhibitory activity against *L. monocytogenes.* 5-FU-induced mucositis could be successfully reproduced in the animals. Pre-treatment with recombinant PAP-expressing *L. lactis* strain did not prevented mucositis in mice, although positive effects were observed in the animals such as increased villi preservation and Paneth cells activity.

Secretion of biologically active pancreatitis-associated protein I (PAP) by dairy genetically modified Lactococcus lactis NZ9000 in the prevention of intestinal mucositis

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Abstract

Mucositis is one of the most relevant gastrointestinal inflammatory conditions in humans, generated by the use of chemotherapy drugs, such as 5-Fluoracil (5-FU). 5-FU-induced mucositis affects 80% of patients undergoing oncological treatment causing mucosal gut dysfunctions and great discomfort. As current therapy drugs presents limitations in alleviating mucositis symptoms, alternative strategies are being pursued. Recent studies have shown that the antimicrobial pancreatitis-associated protein I (PAP) has a protective role in intestinal inflammatory processes. Indeed, it was demonstrated that a recombinant strain of *Lactococcus lactis* expressing human PAP (LL-PAP) could prevent and improve murine DNBS-induced colitis, an inflammatory bowel disease (IBD) that causes severe inflammation of the colon. Hence, in this study we sought to evaluate the protective effects of LL-PAP on 5-FU-induced experimental mucositis in BALB/c mice as a novel approach to treat the disease. Our results show that non-recombinant *L. lactis* NZ9000 have antagonistic activity against the enteroinvasive gastrointestinal pathogen *L. monocytogenes in vitro.* Moreover, *L. lactis* was able to prevent histological damage, reduce both eosinophil infiltration and secretory Imumoglobulin-A in mice injected with 5-FU. Recombinant lactococci carrying antimicrobial PAP did not improve those markers of inflammation, although its expression was associated with villous architecture preservation and increased secretory granules density inside Paneth cells in response to 5-FU inflammation. Hence, *L. lactis* NZ9000 strain, derived from dairy *L. lactis* MG1363 used extensively for cheese production, reveals to be a promising alternative to treat mucositis.

Keywords: Pancreatitis-associated protein, mucositis, *Lactococcus lactis*, 5-Fluoracil.

Introduction

Mucositis is a gastrointestinal inflammatory disorder caused by radiotherapy or chemotherapeutic agents in oncology patients [1-3]. This enfeebling condition has been reported in 80% of patients undergoing clinical treatment with 5-Fluorouracil (5-FU), a drug commonly prescribed for treating several types of cancer, including gastrointestinal, breast, pancreas, head and neck [2, 4]. As 5-FU present nonspecific cytotoxicity to cells with high replication rate, it inhibits the proliferation of both cancer and normal cells, such as the enterocytes lining the *digestive* tract [5,6]. Hence, this process often leads to mucosal alterations, characterized by leukocyte infiltration, ulcers, villus shortening and decreased villus/crypt ratio, favoring systemic translocation of harmful bacteria colonizing the gut, increasing thus the possibility of infections and fatal consequences [7-11]. Moreover, 5-FU-induced mucositis has been associated with mucosal gut dysfunctions, affecting patients' food consumption, provoking vomiting, abdominal pain, and diarrhea which may cause dehydration [1,3].,

Up to date, mucositis treatment relies mostly on mucosal coatings, cryotherapy, antibiotics and analgesics administration. However, no current therapy has been efficient to alleviate the disease [12,13]. Thus, alternative strategies are currently being investigated. Contemporary studies have described promising achievements with the administration of lactic acid bacterium (LAB), instead of drugs [14-18]. These bacteria are considered to be probiotics, a term defined as “live microorganisms administered in adequate amounts that confer a beneficial health effect on the host” [19]

Species like *Streptococcus thermophilus* and several *Lactobacilli* have been shown to reduce intestinal inflammation caused by chemotherapy drugs injection, such as 5-FU, Irinotecan or methotrexate, in rats [14-16]. Moreover, administration of yogurts containing *L.*

johnsonii or *L. bulgaricus* and *S. thermophilus*, seems to be useful to restore intestinal barrier function in these animals [17].

The use of recombinant LAB strains, such as the model *Lactococus lactis* for delivering biologically active molecules with anti-inflammatory properties has also been explored as an alternative therapy for the treatment of mucositis and other gastrointestinal inflammatory disorders, as Inflammatory Bowel Diseases (IBD) [20-23]. In 2006, a biological confinement strategy to contain the dissemination of genetically engineered *L. lactis* strain expressing IL-10 was carried out in phase I clinical trial with IBD patients, suggesting the feasibility of mucosal therapy using recombinant lactococci [24]. This trial provided novel possibilities for testing genetically modified *L. lactis* to treat similar intestinal disorders, such as mucositis. In fact, recent studies have designed recombinant strains of *L. lactis* to produce anti-inflammatory proteins, involved in the maintenance of epithelial barrier integrity, such as Trefoil factor 1 (TFF-1), which has been promising to treat mucositis in clinical trial as well [25-26]. Therefore, other key elements in the host-microbiota relationship are being glimpsed as potential candidates to be cloned in *L. lactis.*

Delivery of antimicrobial peptides (AMPs) that protects the host by killing harmful bacteria has been shown to prevent inflammation in colitis mice models [27-28]. This positive effect was associated with host epithelial cell surface protection against pro-inflammatory bacteria in the intestinal mucosa [27-28]. As AMPs have been recently considered to be a very effective approach to fight inflammation, various types are being explored in basic research such as Reg3A, also known as Pancreatitis associated Protein (PAP). It has been extensively studied due to its protective effect in the intestinal inflammatory process [29-34]. This protein belongs to the RegIII gene sub-family, which encodes proteins involved in the regulation of epithelial cell proliferation and antimicrobial activity in several organs, including intestines [29-33]. In the small intestine, PAP is mainly produced by Paneth cells

that are located in mucosal crypts and exerts bactericidal activity against gram-positive bacteria species that might pose risk of infection to the host [33,34]. Recently, our research group, constructed and confirmed the expression of human PAP by recombinant *L. Lactis* NZ9000 using the inducible Nisin Control Expression System (NICE) [35]. In the same work, we evaluated the therapeutic effect of this strain in mice model of dinitrobenzenosulfonic acid (DNBS)-induced colitis. We found out that PAP delivered by lactococci revealed to be anti-inflammatory [35]. As this strategy have shown to be useful in the treatment of IBD, we sought to investigate LL-PAP protective role in another important inflammatory gastrointestinal disorder for which conventional therapy is not enough, as mucositis, using the 5-FU intestinal mucositis experimental mouse model.

Methods

Bacterial strains and growth conditions

Lactococcus lactis NZ9000 strain harboring pSEC:PAP vector (LL-PAP) and *L. lactis* NZ9000 strain carrying pSEC vector without the open reading frame of PAP (LL) [unpublished data], were grown in M17 medium (Difco) supplemented with 0.5% glucose (GM17) at 30°C without shaking. Recombinant strains were selected by the addition of chloramphenicol (Cm, 10 μg/mL). For nisin-induced PAP expression, LL-PAP was cultivated until the optical density at 600 nm reached 0.6. Afterwards, 10 ng/mL of nisin (Sigma) were added to the medium and cultures were maintained at 30°C for 2 h before experimentation. For *in vitro* antagonistic assays, *L. lactis* strains were grown in Brain-Heart Infusion (BHI) medium containing Cm (10 μg/mL) at 30°C, as well as *Listeria monocytogenes* ATCC 15313 strain, which were grown in BHI containing or not Cm (10 μg/mL) at 37°C without shaking.

Antagonistic activity assay against pathogenic *Listeria monocytogenes*

Antimicrobial activity of PAP secreted by LL-PAP strain was assessed against the food-borne pathogen *L. monocytogenes* using a previously described method [36]. Briefly, LL and LL-PAP were grown separately in BHI medium containing Cm (10 µg/mL) and, after OD600 reached 0.6, 10 ng/mL of nisin were added. Cultures were then centrifuged in an OD600 of 1.0, at 4,000*g* for 10 min and supernatants were sterilized using 0.20 μm millipore filters (Sarstedt, Nümbrecht, Germany). *L. monocytogenes* was then inoculated into filter-sterilized lactococcal supernatant or in BHI medium containing Cm (10 µg/mL) and nisin (10 ng/mL) at an initial OD600 of 0.1. Cultures were incubated at 37°C and after 2 and 4 hours, serial dilutions were seeded in BHI agar plates that were maintained at 37°C for 24h. Thereafter, number of viable *L. monocytogenes* was estimated through counting of bacterial colony forming units (CFUs).

Animals, bacterial administration and experimental groups

Conventional female BALB/c mice between 6 and 8 weeks of age were obtained at Federal University of Minas Gerais (UFMG – Belo Horizonte, Brazil) and the study was approved by the Brazilian Ethics Committee on Animal Use (CEUA). Mice were kept in a temperature-controlled room with *ad libitum* access to water and standard chow diet 24h prior to experiments. Animals were fed daily with 50 mL of water; or with 50 mL of M17 medium supplemented with Cm (10 µg/mL) and nisin (10 ng/mL) (M17/Cm/Nisin); or with M17/Cm/Nisin containing $2{,}5x10^{10}$ CFU/mL of either LL or LL-PAP strains for 13 days. In order to induce mucositis, mice received a single intraperitoneal injection of 5-Fluoracil (300 mg/kg) on day 10 and were euthanized on day 14. An injection of saline (NaCl 0,9%) was used as a control. For experimentation, BALB/c mice were divided into eight groups, each containing six to nine animals. Animals from group 1 to 4 were injected with 0.9% saline on

day 10 (noninflamed groups); group 1 received water, group 2 were fed with M17/Cm/Nisin medium; group 3 were administered with LL culture, group 4 received *L. lactis* expressing PAP. Mouse from group 5 to 8 were injected with 5-FU on day 10 (inflamed groups); group 5 received water; group 6 received M17/Cm/Nisin medium; group 7 were administered with LL culture and finally group 8 received LL-PAP strain.

Intestinal histology and morphology

After euthanasia, the distal portion of the small bowel (ileum) from the animals was collected, and, after washing, rolls were prepared for histomorphological analysis. Rolls were fixed with 10% buffered formaldehyde. Material was then embedded in paraffin, and a 4μm section of each sample was placed on a glass slide and stained with hematoxylin and eosin (HE). Slides of each experimental group were photographed using a digital camera (Moticam 2500, China) coupled to an optical microscope (Olympus Optical Co., Japan). The histological score was determined using a previously described method [10], which measures the intensity of both mononuclear and polymorphonuclear cells infiltrate in the *lamina propria*, changes in mucosal architecture and presence of ulceration. For each parameter it was used the ranking values: absent (0), mild (1), moderate (2) and severe (3). For morphological analysis, ten images from the ileum of each animal were randomly captured and analyzed through ImageJ software. Granular density inside Paneth cells was determined by measuring the intracellular area occupied by secretory granules. Villus height and crypt depth was measured vertically from the tip of a villus to the base of the adjacent crypt. Villus height/crypt height ratio from the intestinal epithelium was also obtained.

Determination of intestinal eosinophil peroxidase activity

The extent of tissue eosinophil infiltration was assessed by measurement of eosinophil peroxidase (EPO) activity, as previously described [37]. Briefly, 100 mg of intestine were weighted, homogenized with 1.9 mL of PBS and centrifuged at 12,000g for 10 minutes. Supernatant was discarded, and pellet was resuspended in 1.9 mL of 0.5% hexadecyltrimethyl ammonium bromide diluted in PBS. After being frozen three times in liquid nitrogen, samples were centrifuged at 4°C, 12,000 g during 10 minutes. To test EPO activity, the obtained supernatant was mixture with a substrate (1:1) containing 1.5 mmol/L of o-phenylenediamine, 6.6 mmol/L of H_2O_2 and 0.075 mmol/L of Tris-HCl (pH 8). Reaction was stopped with 50 µL of 1M H_2SO_4, and absorbance was measured at 492 nm.

Secretory IgA

Levels of secretory IgA were determined by enzyme linked immunosorbent assay (ELISA) in small bowel intestinal fluids. Microtitre plates (Nunc-Immuno Plates, MaxiSorp) were coated with goat anti-mouse antibody (Southern Biotechnology, Birmingham, AL, USA) in carbonate-bicarbonate buffer (0,1M Na2CO3/NaHCO3 - pH 9.6) for 18h at 4°C. Wells were washed with washing solution (saline 0.9% plus 0.05% tween 20) and blocked with 200µL of 0.05% casein in PBS for 1h at room temperature. Intestinal fluids previously centrifuged at 432*g* for 20 min were added to the plate and diluted in PBS-0.25% casein (two times until dilution 1:80). After incubation of 1 hour at room temperature, plate was washed and biotin conjugated anti-mouse IgA antibody (Southern Biotechnology) in PBS-0.25% casein (1:10.000) was added to the wells. After incubation of one hour at 37°C, peroxidase-streptavidin goat anti-mouse IgA (Southern Biotechnology, Birmingham, AL, USA) was added; plate was incubated for one hour more and, then, coated with 100µL/well of orthophenylenediamine (OPD) (1 mg/mL) (Sigma, St. Louis, MO, USA) and 0.04% H_2O_2

substrates. Color was developed at room temperature and reaction was stopped by the addition of 20µL/well of 2N H_2SO_4. Absorbance was measured at 492 nm using a Bio-Rad Model 450 Microplate Reader. Results were expressed as concentration (µg/mL), according to the standard curve.

Statistical Analysis

Differences between groups were statistically evaluated by one way analysis of variance (ANOVA). Bonferroni test was applied to calculate statistical significance across groups. Non-parametric Mann Whitney test was used for data sets based on scores or percentages. All data was processed using GraphPad Prism 5.0 software. P values under 0,05 were considered significant.

Results

L. lactis* supernatant inhibits *in vitro* growth of *L. monocytogenes

The antagonistic activity of LL or LL-PAP culture supernatants was assessed against the food-borne pathogen *L. monocytogenes.* After two hours, it was not observed any statistical differences in CFU counts of *L. monocytogenes* inoculated in BHI+Cm+Nisin medium or in the supernatant of LL and LL-PAP, both containing Cm and Nisin (Fig 1A). However, after 4 hours, it was observed a 2-fold decrease in CFU counts of *L. monocytogenes* grown either in LL+Cm+Nisin or in LL-PAP+Cm+Nisin culture supernatants when compared to counts obtained for the pathogen inoculated in BHI+Cm+Nisin medium. Presence of PAP in the supernatant did not reduce *L. monocytogenes* counts (Fig1B).

***L. Lactis* administration prevents 5-FU-induced mucosal inflammation in the ileum**

Mice injected with 5-FU showed significant decreases in body weight (10% and 17% loss) from groups fed with water or M17+Cm+Nisin medium as was expected (Fig 2). Treatment

with *L. lactis* strains did not improve mouse weight as groups receiving LL or LL-PAP cultures lost approximately 10% of their initial body weight after 5-FU injection (Figure 2B). Histological analysis revealed mucosal pattern within normal limits in all groups injected with 0.9% saline (Fig 3A). 5-FU injection in mice receiving water or M17+Cm+Nisin medium caused lesions in the small intestine characterized by an inflammatory cellular infiltrate in lamina propria, as well as in the submucosa and muscular layers, with increased number of histopathological parameters (Fig 3B). Mice immunized with LL prevented 5-FU-induced mucosal inflammation in the ileum, showing reduced infiltration by polymorphonuclear neutrophils, ulceration and reduced alterations of intestinal mucosal architecture. Treatment with LL-PAP strain did not improve the histological score (Fig 3B).

Delivery of human PAP by *L. lactis* improves villous architecture preservation and Paneth cells activity in ileum

Morphometric analysis was carried out to evaluate epithelial integrity. A decrease was observed in villus height (Fig 4A), villus/crypt ratio (Fig 4C) and granular density inside Paneth cells (Fig 5) after 5-FU injection in mice receiving water or M17+Cm+Nisin medium. No statistical differences in crypt depth were observed across groups (Fig 4B). Inflamed mice treated with LL-PAP showed increased villus height (Fig 4A), vilus/crypt ratio (Fig 4B) and granular density within Paneth cells (5A, B) when compared to mice treated with LL.

***L. Lactis* treatment reduces eosinophilic infiltration in the ileum**

To evaluate whether treatment with LL or LL-PAP strain would have an effect in reducing polymorphonuclear cells infiltration in the small bowel of mice, EPO activity was measured in cell lysates of the ileum. As expected, 5-FU administration increased intestinal EPO activity in mice administered with water or M17+Cm+Nisin medium (Fig 6). Treatment with

LL culture demonstrated to reduce eosinophilic infiltration when compared to mice that were given M17+Cm+Nisin medium. Expression of PAP by *L. lactis* did not reduce EPO activity (Fig 6).

L. Lactis reduces IgA secretion in the small bowel

Secretory IgA (sIgA) response was also measured in the small bowel of mice as it plays an important role in mucosal protection. sIgA secretion increased in mice injected with 5-FU receiving water or M17+Cm+Nisin medium, as expected (Fig 7). Group of animals immunized with LL or LL-PAP strain demonstrated reduced levels of IgA when compared to M17-treated group (Fig 7). However, LL-PAP administration did not caused a significant reduction of sIgA when compared to LL-treated mice (Fig 7).

Discussion

PAP anti-microbial role against gram-positive pathogenic bacteria has been demonstrated in previous studies [29-34]. Hence, we decided to investigate whether recombinant PAP expressed by *Lactococcus lactis* would have an inhibitory *in vitro* effect against the gram-positive foodborne pathogen *Listeria monocytogenes*. Interestingly, we found that the bacterial supernatant from either *L. lactis* NZ9000 or *L. lactis* expressing PAP cultures could statistically inhibit *L. monocytogenes* growth. However, expression of PAP did not seem to enhance this inhibitory effect. In our work we did not add trypsin, which is necessary for PAP N-terminal cleavage and activation [32-34], to the medium as a recent work demonstrated that intact unprocessed PAP protein was capable of binding and killing gram-positive bacteria [29-31]. Therefore, we hypothesized that the lacking of PAP inhibitory activity against *L. monocytogenes* could be due to its low concentration within culture supernatant, as a non-concentrated supernatant from LL-PAP strain was used for the assay. Thus, growth inhibition

of *L. monocytogenes* could be caused by expression of some molecules as lactic acid, bacteriocins or metabolites naturally produced by *L. lactis*. Actually, Charlier and colleagues [38] had tested the inhibitory effect of seventy-five strains of *L. lactis,* including *L. lactis* NZ9000 strain, against gram-positive pathogenic *Staphylococcus aureus.* They demonstrated that medium acidification was not involved in the inhibition of *S. aureus* in early growth phases, suggesting that further experimentation is required to characterize the molecular bases of *L. lactis* NZ9000 antagonistic activity against this pathogen [38].

Besides its antimicrobial activity, PAP has been reported to be involved in maintaining intestinal homeostasis [33]. Indeed, a recent report from our research group showed that *L. lactis* expressing PAP was capable to efficiently prevent colitis in mice [35]. Therefore, we supposed that this strain could also display protective effects in other inflammatory disorders of the gastrointestinal tract, as mucositis in which dysbiosis have been implicated as key factor for inducing intestinal inflammation [38-43]. The medicament 5-FU commonly used in oncology generate as an adverse effect mucositis, causing weight loss to patients as around 80% of their epithelial cells population are destroyed by apoptosis thus leading to lower absorption of nutrients [44]. In our study, pretreatment with both *L. lactis* NZ9000 and *L. lactis*-PAP strains during 13 days had no influence on weight recovery of inflamed mice. Differently, Bowen and colleagues have shown that VSL#3 probiotic mixture could reduce weight loss in mice with mucositis after a long period of administration (28 days) [15]. Therefore, we believe that longer treatment duration is required to recover weight of mice injected with 5-FU.

A common feature of mucositis patients receiving 5-FU is the presence of inflammatory cells infiltrating into the *lamina propria* with increased NF-kappa-B transcription factor activity that play roles in inflammatory responses [2,4]. Another characteristic of mucositis disorder is altered intestinal morphology, such as villus shortening and decreased villus/crypt ratio, with

loss of the mucosal barrier integrity [7-11]. Thus, all this parameters were evaluated in this work in order to test the anti-inflammatory activity of both LL and LL-PAP strains. Histological analysis revealed that the administration of *L. lactis* NZ9000 in inflamed mice decreased the histopathological scores, with less polymorphonuclear infiltration and ulceration observed in samples. Surprisingly, PAP expression by *L. lactis* did not improve these parameters. Despite LL-PAP did not improve histopathological scores, morphometric analysis showed that it was able to better preserve intestinal villous architecture. Moreover, LL-PAP was able to preserve and improve Paneth cells activity, thus augmenting antimicrobial gastrointestinal function, as these cells are associated with the secretion of antimicrobial peptides. This was a very interesting result because PAP has also been reported to stimulate the proliferation of epithelial cells in the colon, including Paneth cells [46]. Recent works demonstrated that the increased influx of eosinophils is an important event for the pathogenesis of mucositis [2, 43]. Thus, we measured the activity of EPO enzyme, naturally produced by eosinophils, to estimate eosinophil influx into intestinal *lamina propria* from non-inflamed and inflamed mice receiving water, medium or LL and LL-PAP strains. We showed that *L. lactis* NZ9000 strain was able to reduce eosinophil infiltration while the administration of *L. lactis* expressing PAP did not promote this effect.

Levels of secretory IgA were also determined as it is crucial to prevent pathogens to penetrate the epithelial barrier and, thus, to contain inflammatory processes [45]. We found that animals with mucositis that did not receive lactococci treatment had increased sIgA levels in the small bowel. This effect was expected, as the amount of this immunoglobulin increases during intestinal inflammatory process as a defense mechanism of the host [45,47]. Interestingly, *L. lactis* NZ9000 administration could decrease IgA secretion in mouse intestinal lumen. Again, no improvements were obtained with the use of *L. lactis* secreting PAP strain.

Altogether, our results have shown that *L. lactis* NZ9000 strain carrying pSEC vector without the cDNA of PAP was able to prevent 5-FU-induced intestinal inflammation in BALB/c mice. This was a very surprising and intriguing result because *L. lactis* NZ9000 strain is a derivative of *L. lactis* MG1363 used as starter cultures for cheddar cheese production [48,49], and has not been reported as improving host health. Actually, the only report regarding beneficial effects of the species L. lactis was demonstrated by two studies, in which the strains NCDO2118 sub. lactis and FC sub. cremoris have shown anti-inflammatory properties when administered to mice receiving chemical agent Dextran Sulphate Sodium (DSS) [47, 50].

Furthermore, PAP expression by *L. lactis* subtly alleviate mucositis damage, as it did not show to decrease markers of inflammation such as, ulceration, pro-inflammatory cells infiltrate and IgA levels, but preserved architecture and increased secretory granules density inside Paneth cells in response to 5-FU inflammation.

Conclusion

In conclusion, we have demonstrated that the allochthonous bacterium, *L. lactis* NZ9000, derived from dairy *L. lactis* MG1363 reveals to be a promising tool to prevent chemotherapy drug 5-Fluoracil-induced mucositis. Moreover, we opened the doors for future studies investigating possible factors involved in *L. lactis* NZ9000 anti-inflammatory effects. As a slight beneficial effect has been demonstrated by the recombinant LL-PAP strain, further studies should be considered, such as biological confinement strategies preventing its dissemination into nature, in order to make it a safe approach to be tested in humans.

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Figure legends

Figure 1. Inhibitory activity of bacterial supernantants from *L. lactis* and *L. lactis*-PAP against pathogenic *L. monocytogenes*, after 2 (A) and 4 hours (B) incubation. *, p>0,05.

Figure 2. Time-course of body weight for mice injected with saline or 5-fluoracil receiving water, M17+Cm+Nisin medium or *L. lactis* and *L. lactis*-PAP strains (A). Weight loss observed after 5-FU injection and differences across groups (B).*, *p*<0,05; ***, *p*<0,0001.

Figure 3. Representative images from mucosal histopathology (A) and histopathological scores obtained for experimental groups (B). ***, p<0,0001; **, p<0,003;

Figure 4. Morphometric analysis of villus height (A), crypts depth (B) and villus height/crypt depth ratio (C). ***, p<0,0001; **, p<0,003; *, p<0,005.

Figure 5. Representative images from Paneth cells morphology (A) and microscopic morphometric analysis of Paneth cell secretory granules (B) *, p>0,05.

Figure 6. Differences across experimental groups in eosinophil peroxidase activity from cell lysates of the ileum **, p<0,003; *, p>0,05.

Figure 7. Evaluation of IgA secretion in the small bowel from inflamed and noninflamed mice receveing water, M17 medium or *L. lactis* and *L. lactis*-PAP strains ***, p<0,0001; **, p<0,003

Figure 1A

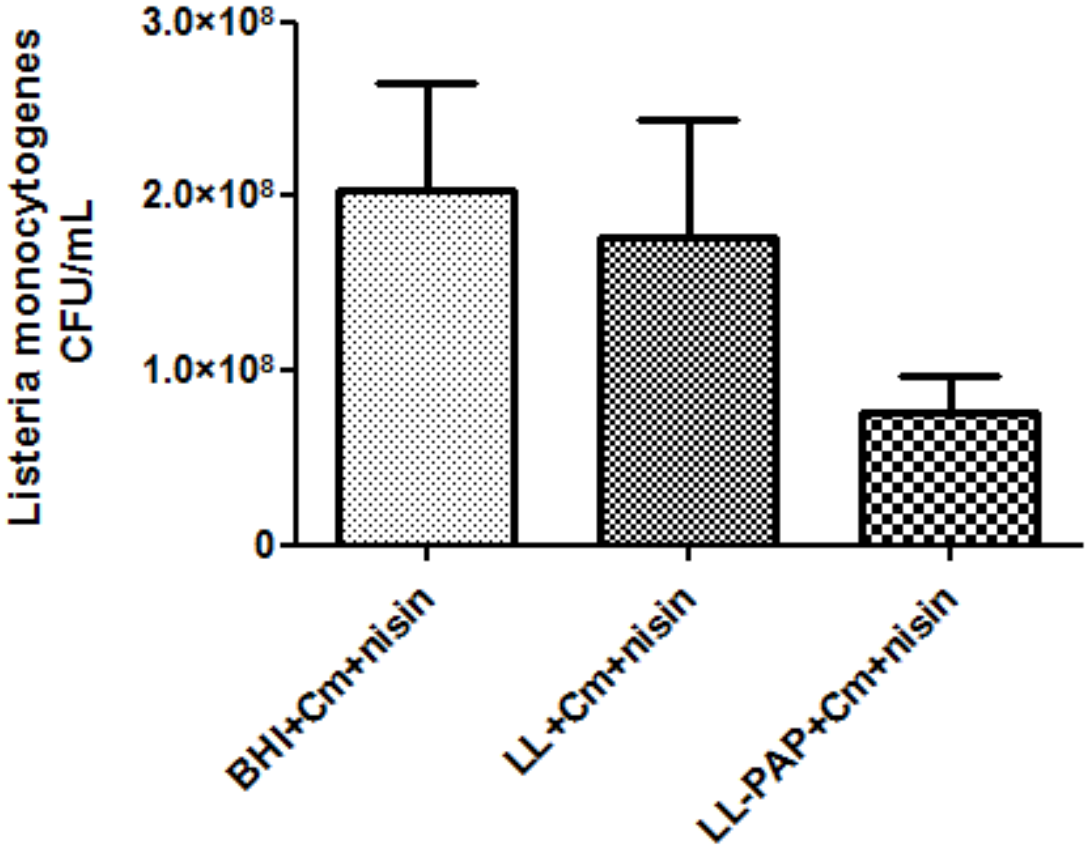

Listeria monocytogenes CFU/mL
3.0×10⁸
2.0×10⁸
1.0×10⁸
0
BHI+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin

Figure 1B

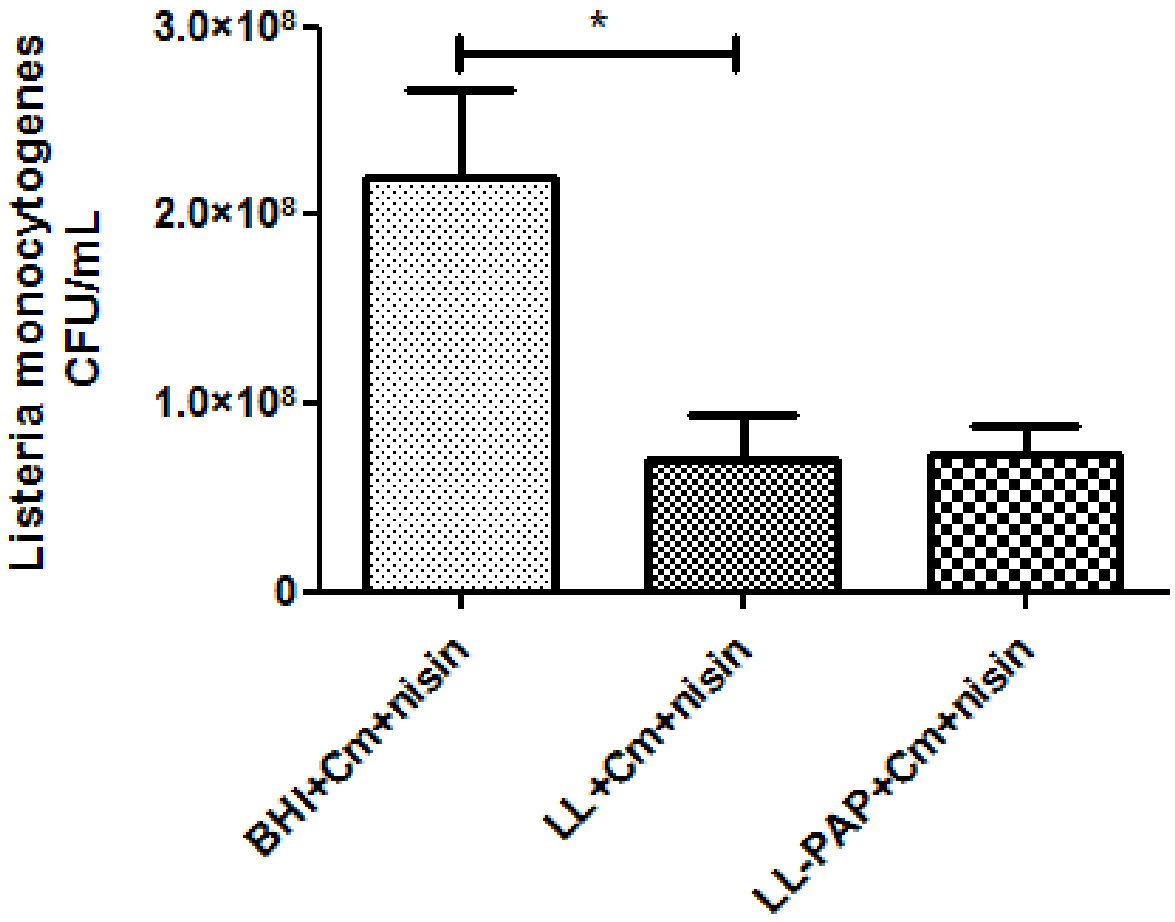

Listeria monocytogenes CFU/mL
3.0×10⁸
2.0×10⁸
1.0×10⁸
0
*
BHI+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin

Figure 2A

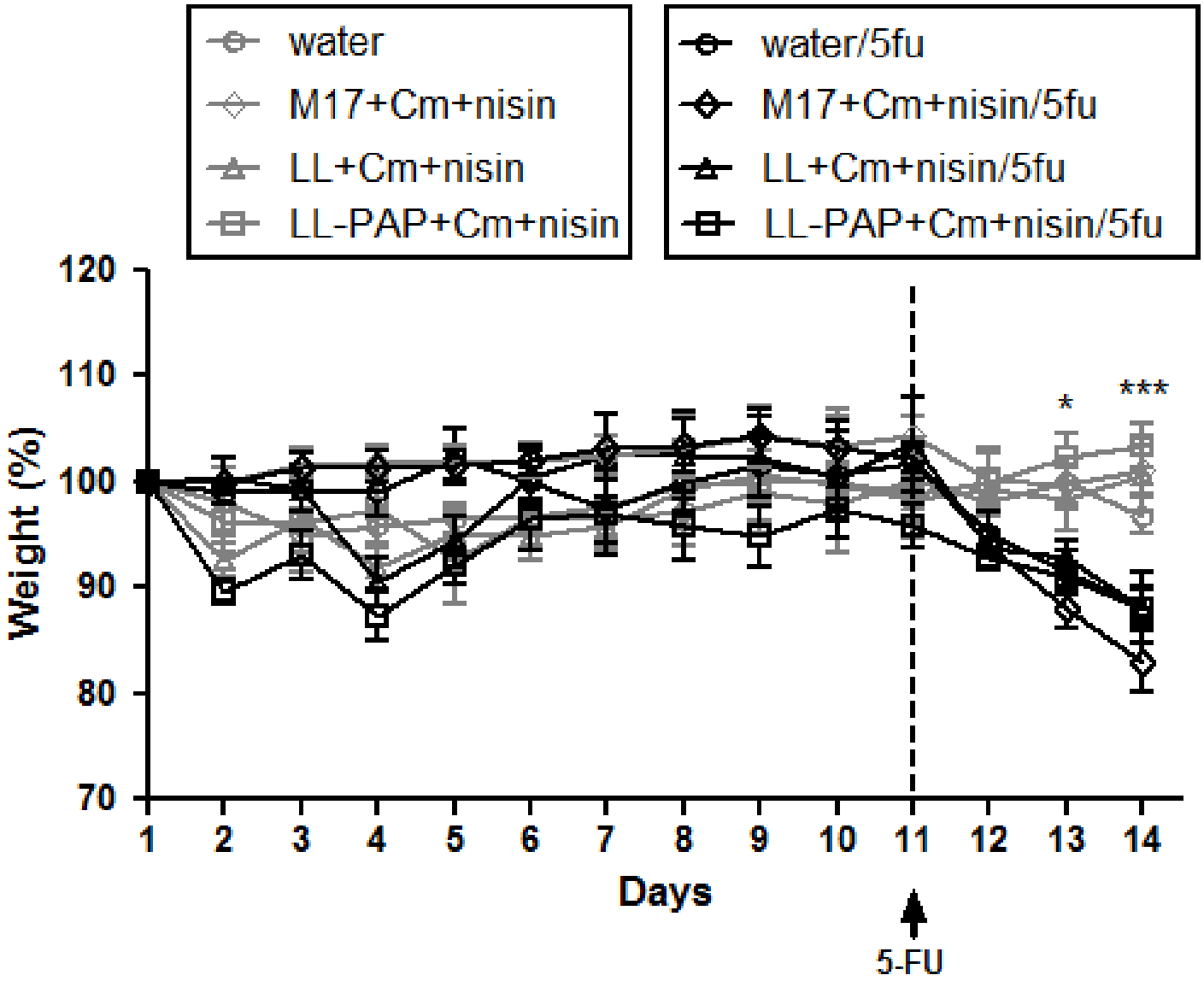

water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
water/5fu
M17+Cm+nisin/5fu
LL+Cm+nisin/5fu
LL-PAP+Cm+nisin/5fu
Weight (%)
120
110
100
90
80
70
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days
*
***
5-FU

Figure 2B

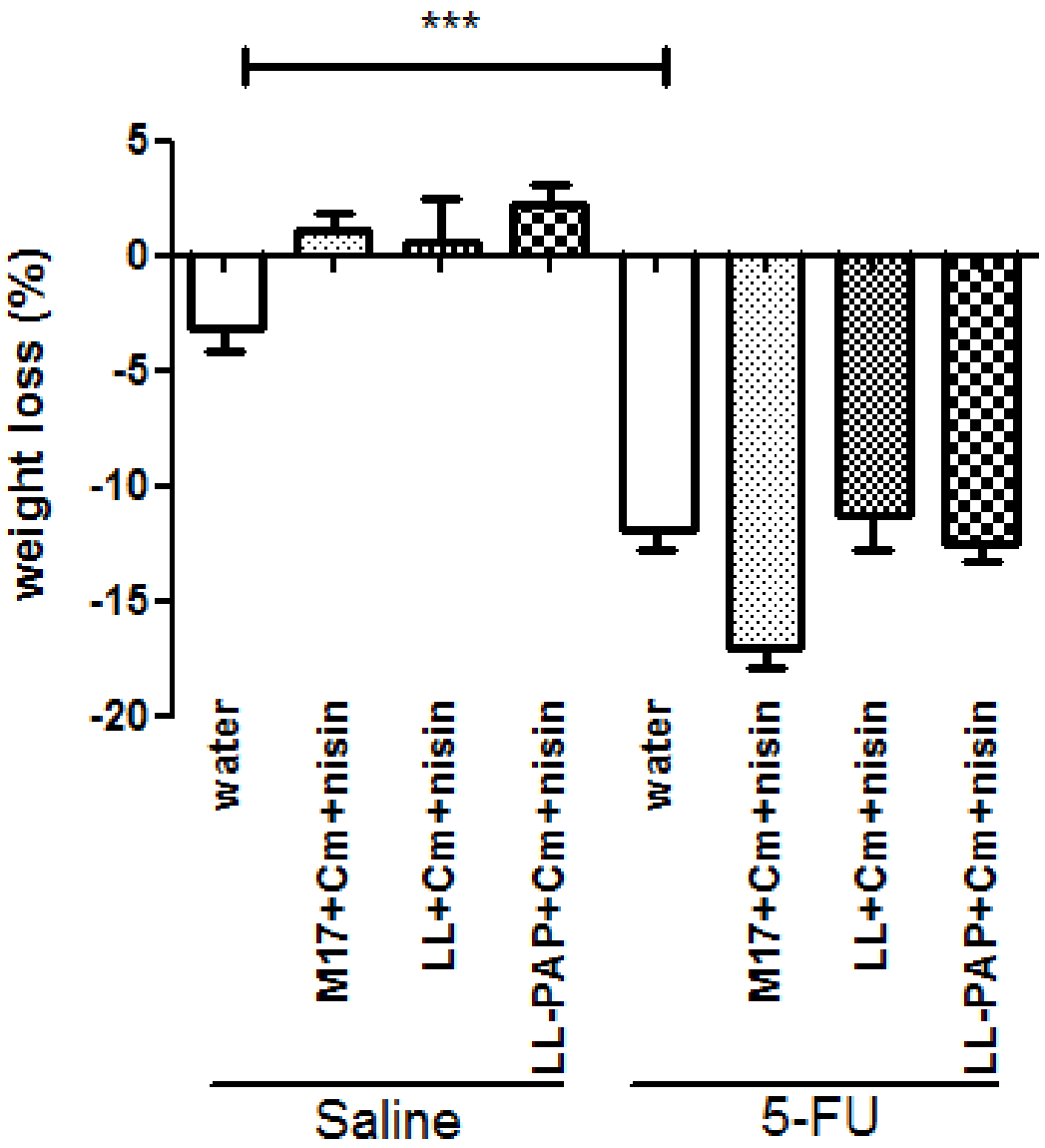

***
5
0
-5
-10
-15
-20
weight loss (%)
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
Saline
5-FU

Figure 3A

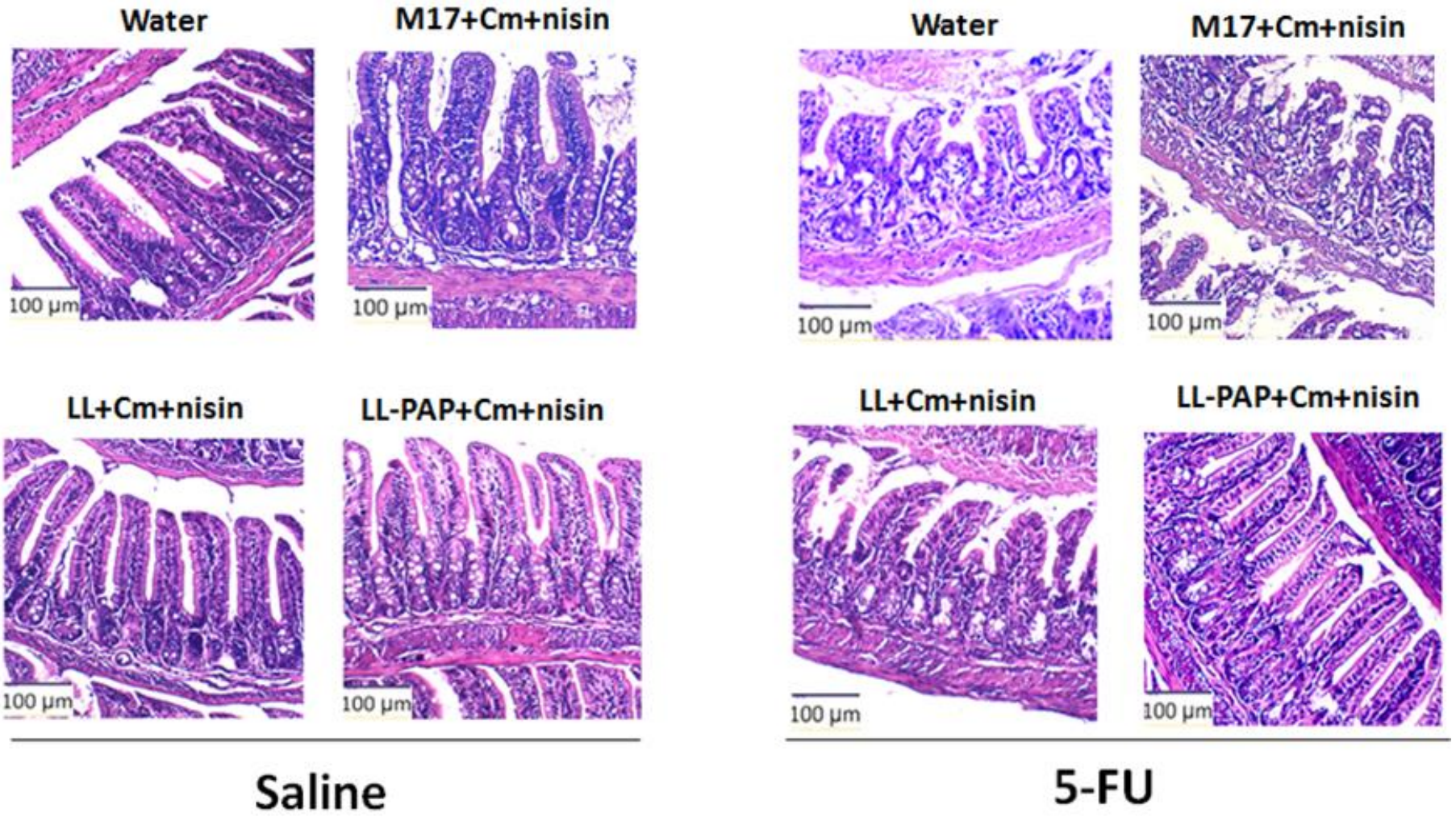

Water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
100 µm
Saline
5-FU

Figure 3B

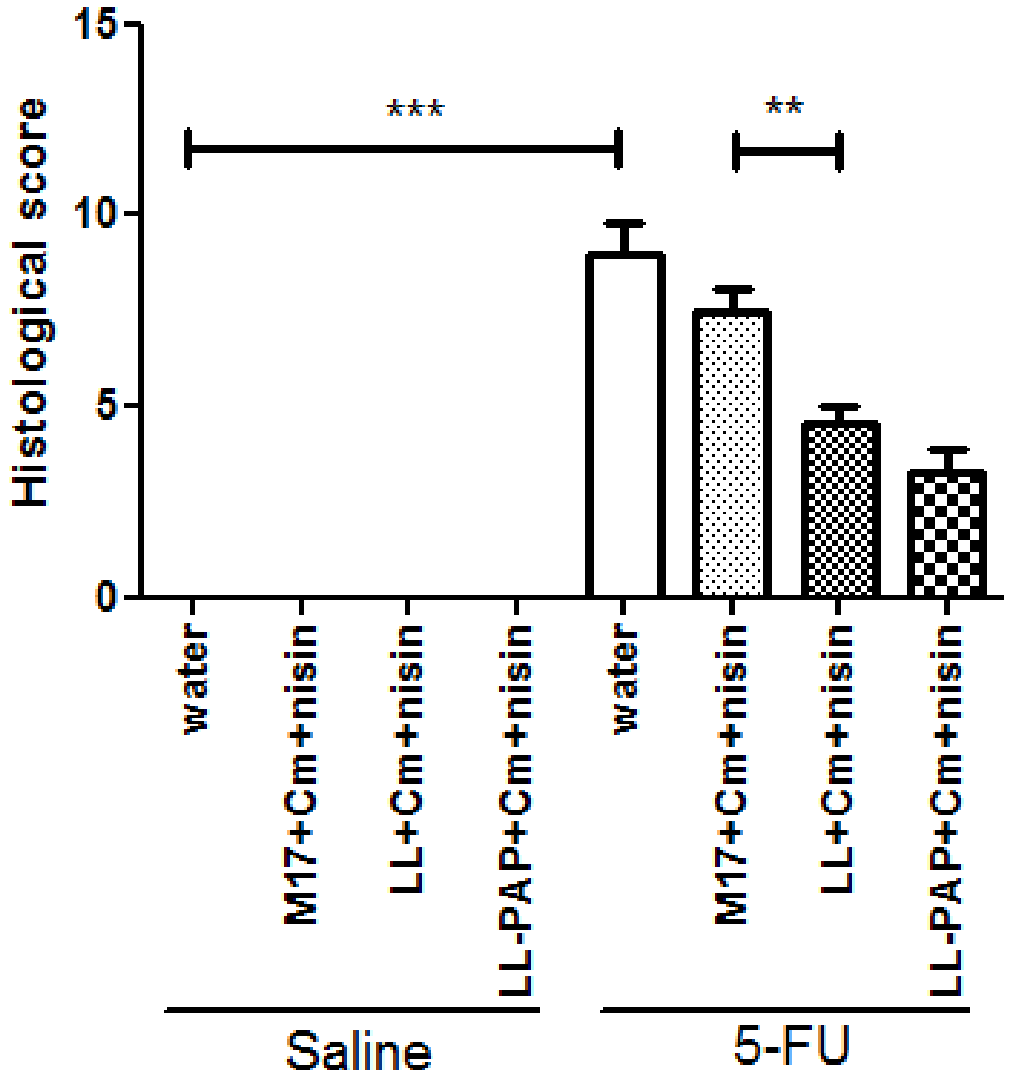

Histological score
15
10
5
0
***
**
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
Saline
5-FU

Figure 4A

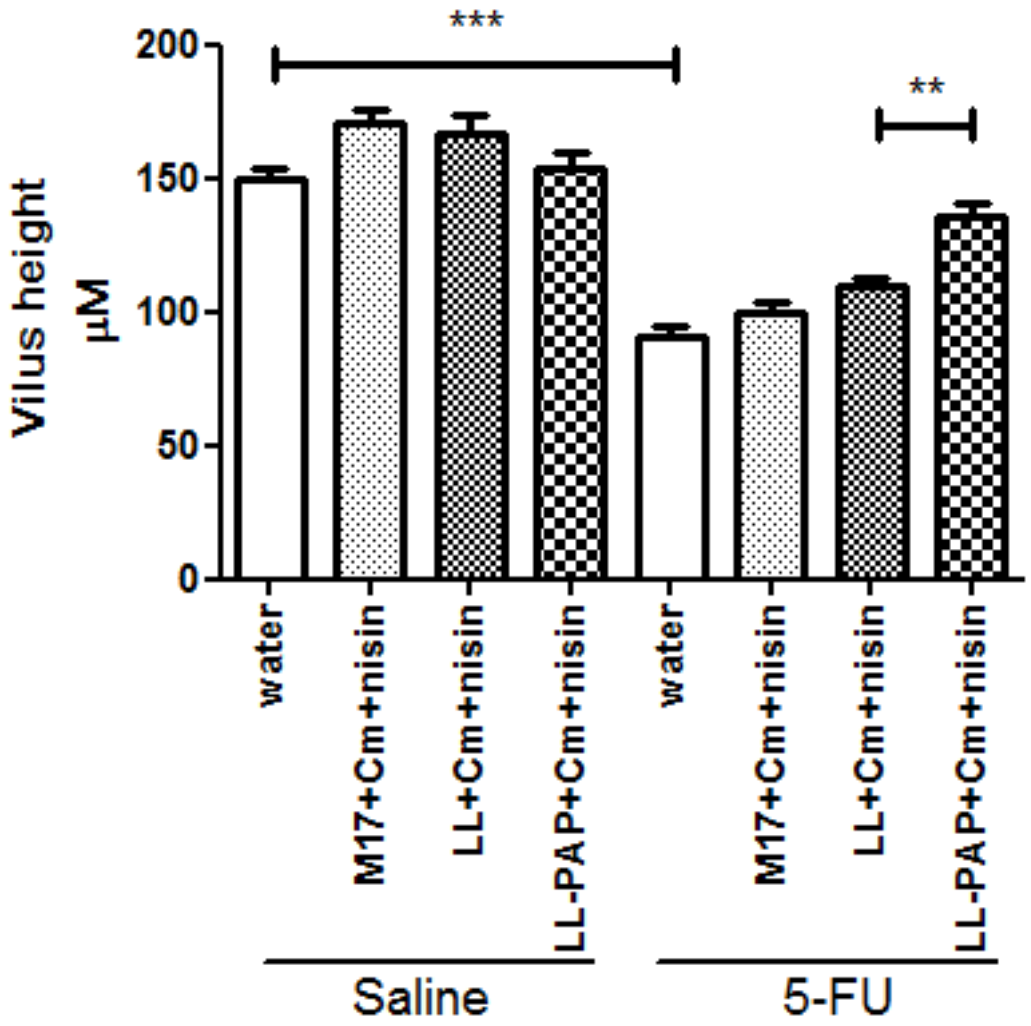

***
**
200
150
100
50
0
Vilus height
µM
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
Saline
5-FU

Figure 4B

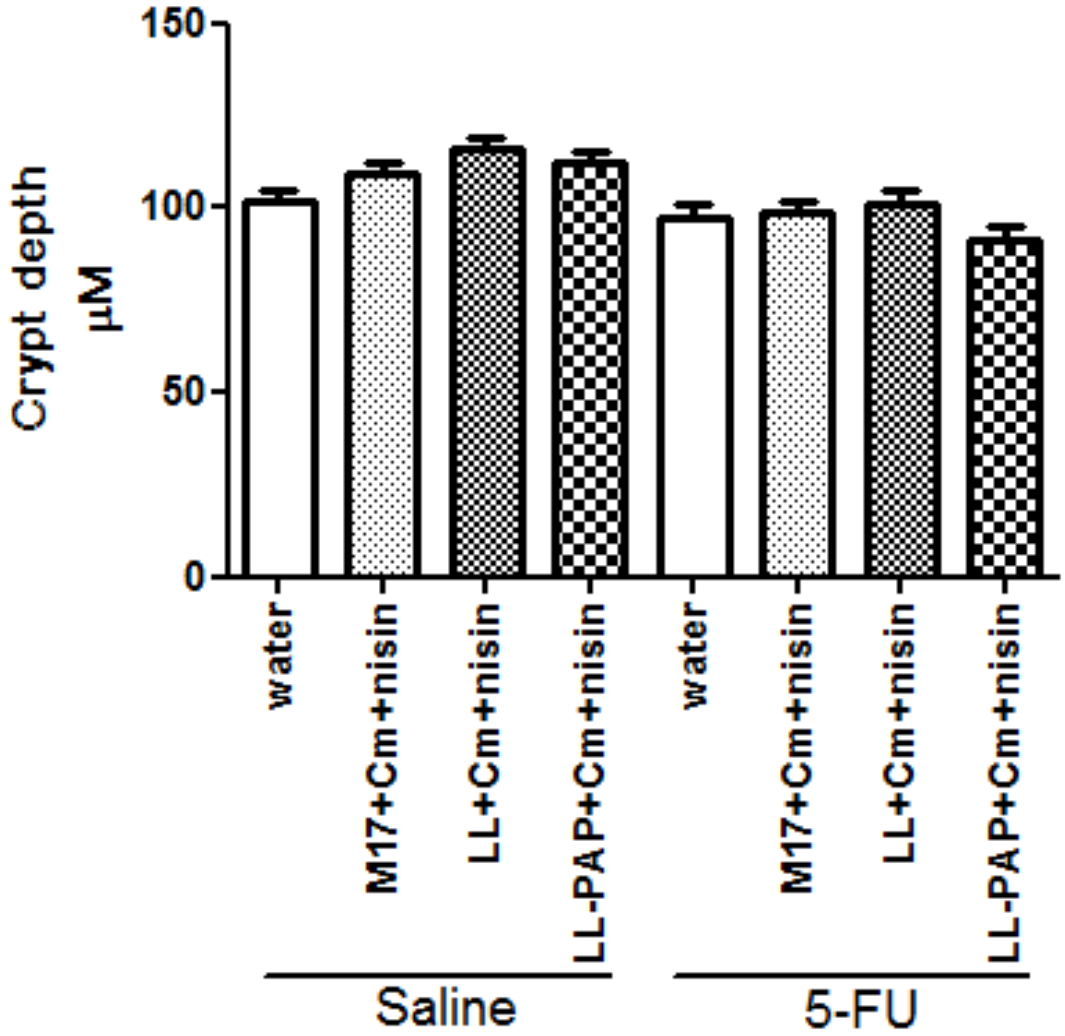

Crypt depth
µM
150
100
50
0
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
Saline
5-FU

Figure 4C

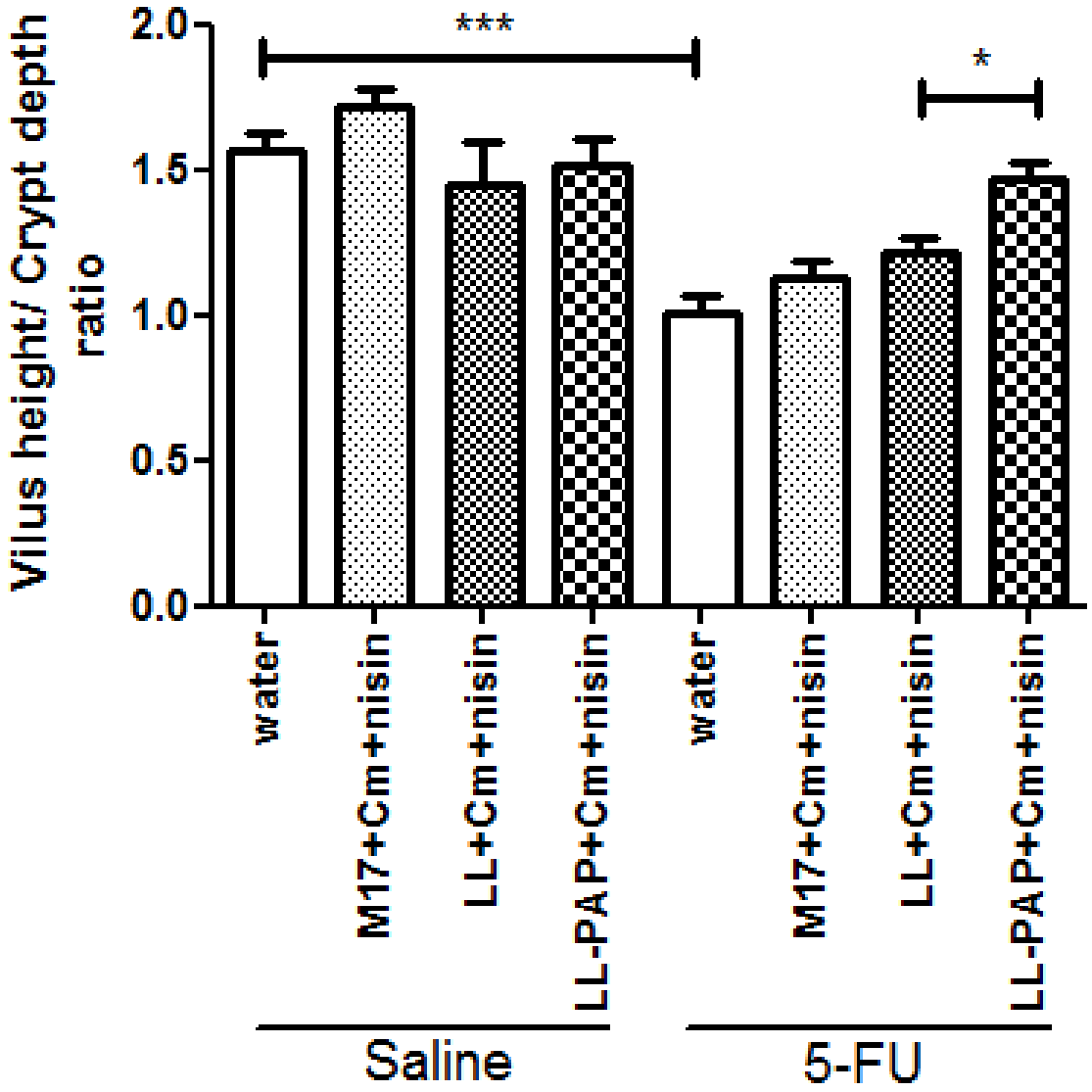

Vilus height/ Crypt depth ratio
2.0
1.5
1.0
0.5
0.0
***
*
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
Saline
5-FU

Figure 5A

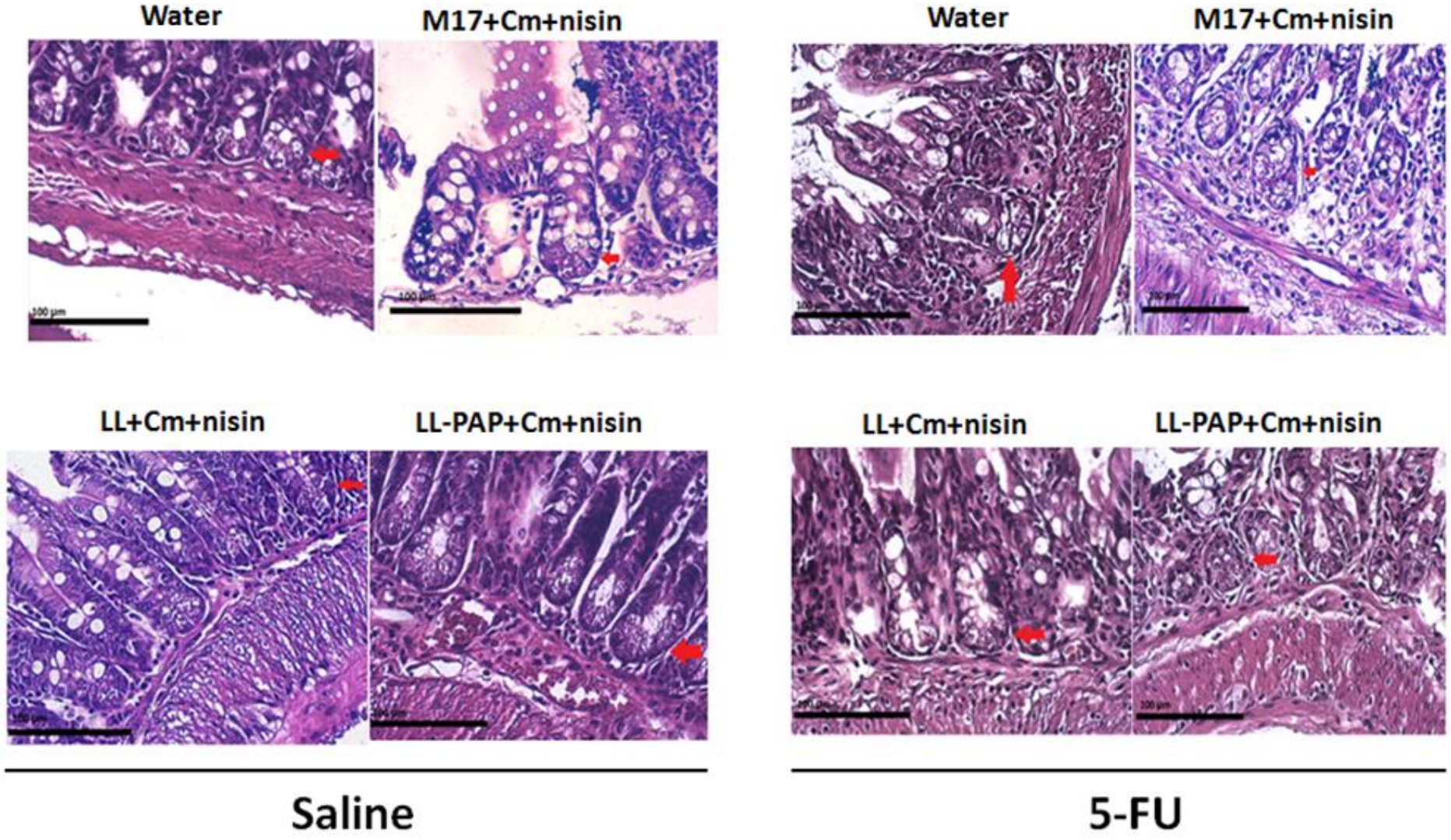

Water
M17+Cm+nisin
Water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
Saline
5-FU

Figure 5B

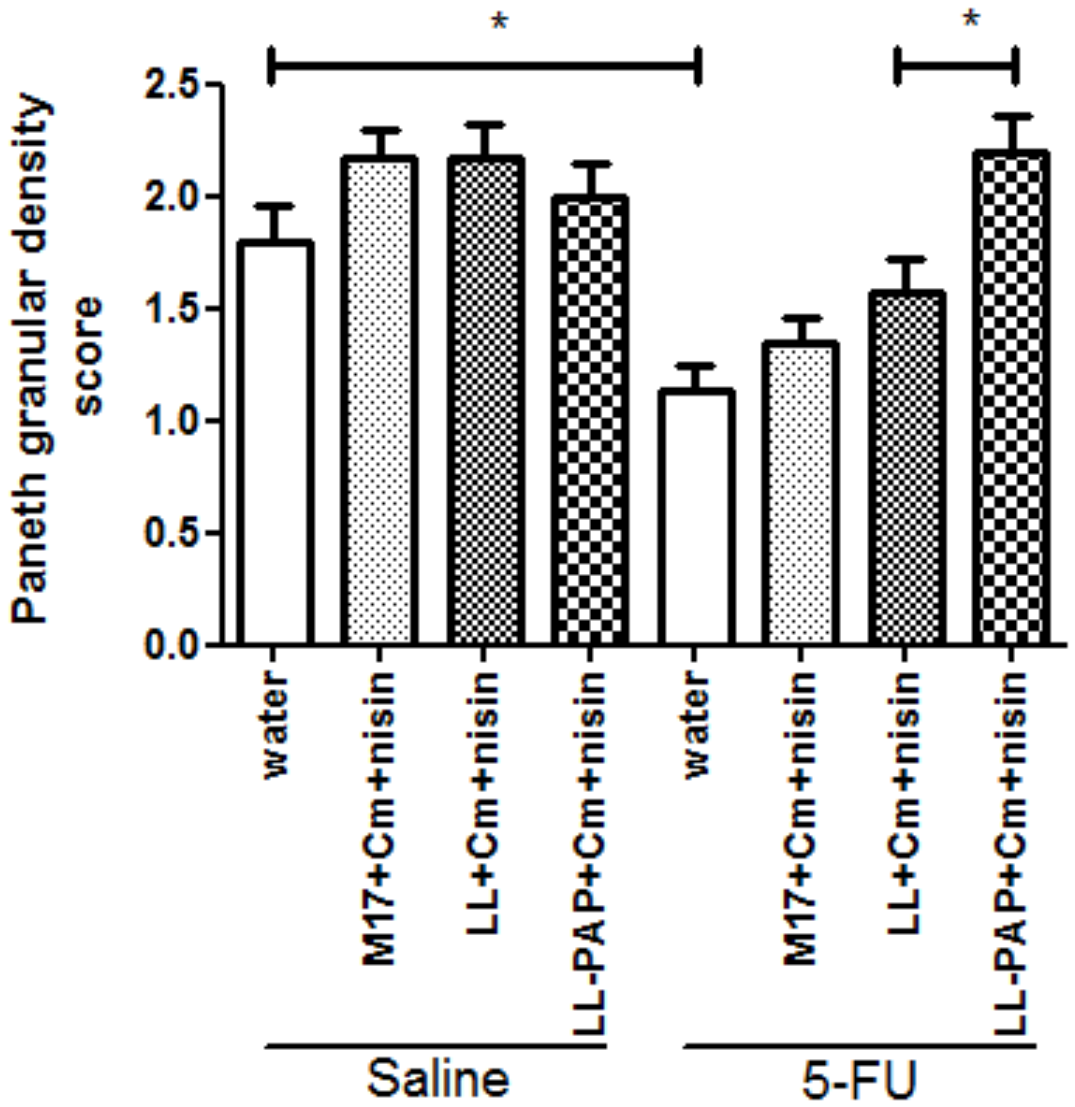

*
*
Paneth granular density score
2.5
2.0
1.5
1.0
0.5
0.0
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
Saline
5-FU

Figure 6

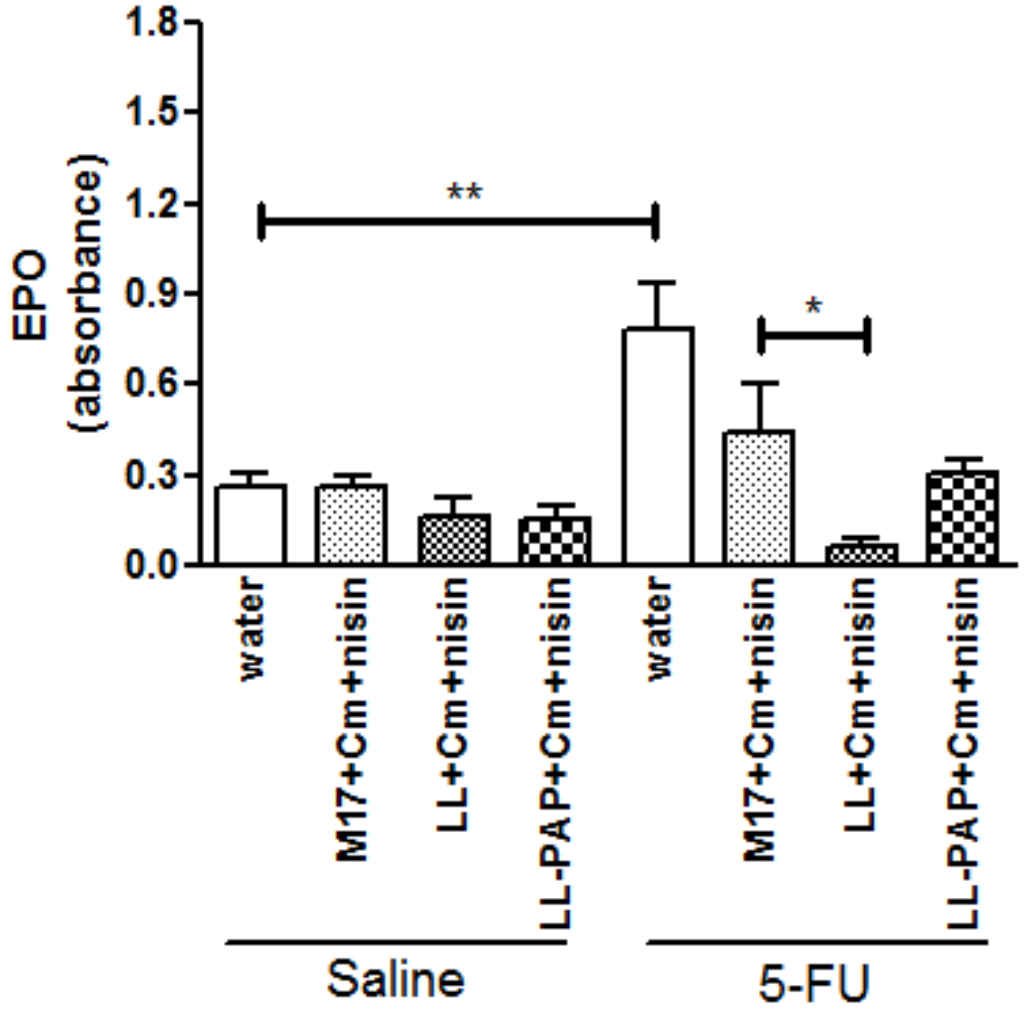
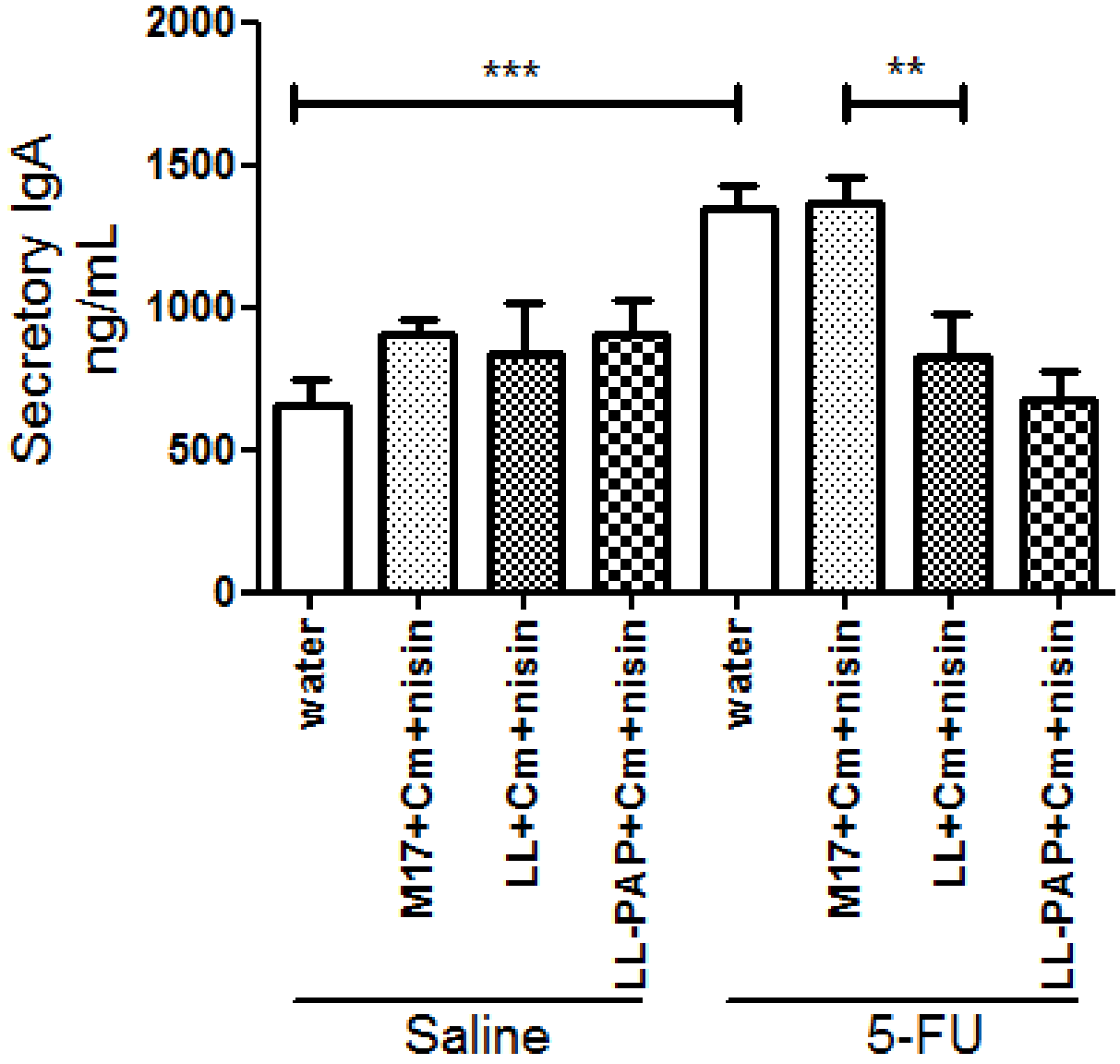

EPO
(absorbance)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
**
*
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
Saline
5-FU

Figure 7


Secretory IgA
ng/mL
2000
1500
1000
500
0
***
**
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
water
M17+Cm+nisin
LL+Cm+nisin
LL-PAP+Cm+nisin
Saline
5-FU

Chapter VI – General conclusion

6. General Conclusion

This Thesis was developed in a Joint supervision between the University of Paris-Sud (France) and Universidade Federal de Minas Gerais (UFMG – Brazil). The first part was conducted in France at the Institut National de La Recherche Agronomique (INRA). We have constructed and confirmed the expression of human PAP by recombinant *Lactococcus lactis* strains. Hereafter, we evaluated its anti-inflammatory effects in a mice model of DNBS-induced colitis. We demonstrated that DNBS injection was able to cause severe acute inflammation in the colon of mice. Furthermore, PAP delivery by lactococci protected animals from weight loss, intestinal permeability and tissue damage. In addition, *L. lactis*-PAP treatment decreased Th1 (IFNγ), Th2 (IL-4, IL-5) and Th17 (IL-17) type-immune responses. Furthermore, it was observed a higher expression of regulatory TGF-β cytokine and increased amount of T regulatory cells in treated mice.

As PAP-producing *L. lactis* revealed itself a very promising tool to prevent colitis, we sought to investigate its therapeutic effect in 5-Fluoracil (5-FU)-induced mucositis, which is another important clinical gastrointestinal (GI) inflammatory disorder. This study, carried out in Brazil at UFMG, showed that the 5-FU model was successfully reproduced in BALB/c mice inducing an acute inflammation in the small bowel of the animals. Administration of *L. lactis* NZ9000 harboring pSEC vector without the cDNA of PAP was able to prevent histological damage, reduce eosinophils infiltrate and IgA secretion in the ileum of mice. On the other hand, *L. lactis* expressing PAP preserved mucosal architecture and improved Paneth cells activity.

Taking together, our results demonstrate that *L. lactis* NZ9000 strain, derived from dairy *L. lactis* MG1363 used extensively for cheese production, surprisingly was able to prevent intestinal inflammation in the 5-FU mucositis model. Expression of PAP peptide by this bacterium was very succesfull to treat experimental IBD and boosted *L. lactis* antiinflamamtory properties in the mucositis model used by 5-FU administration.

Chapter VII – Directions for future work

7. Directions for future work

Our research group in Federal University of Minas Gerais (UFMG-Brazil) will explore the use of *Lactococcus lactis* expressing PAP as an alternative strategy for the treatment of mucositis in near future. In parallel, further investigation on the use of *L. lactis*-PAP for the treatment of DNBS-induced colitis will be performed by our colleagues in France (INRA). For this reason, this chapter presents only the perspectives related to chapter V that will be performed in Brazil.

7.1 Directions for future work (Chapter V)

- Evaluate the effect of both *L. lactis* NZ9000 and *L. lactis* expressing PAP in the microbiota composition from conventional BALB/c and 5-FU-treated mice, through 16S sequencing in stool samples.

- Evaluate the microbiota composition from inflamed and non-inflamed mice through Real time PCR using oligonucleotides targeting specific clusters of microorganisms.

- Confirm the *in vitro* inhibitory effect of both *L. lactis* NZ9000 and *L. lactis* expressing PAP in the growth of representative species from each cluster of microorganisms defined after sequencing and real time PCR analysis.

- Evaluate the therapeutic effect of either *L. lactis* NZ9000 or *L. lactis* expressing PAP in the treatment of 5-FU-induced mucositis in tumor-bearing mice.

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APPENDIX

Appendix A – Valorisation of the work

Before being engaged with Lactic Acid Bacteria studies, at the beginning of my Ph. D. I was involved in another line of reaserch leaded by Prof. Vasco Azevedo, focused on the study of genetic regulation of the pathogen *Corynebacterium pseudotuberculosis* in the adaptive response to stress. Therefore, during my first year as a Ph.D. student, I have contributed to the development of some works as a collaborator within this theme. During the second year until the end of the doctorate, I was involved in the studies aimed to test the anti-inflammatory activities of recombinant *Lactococcus lactis* expressing PAP for the treatment of both colitis and mucositis. As this thesis was conducted in a co-tutelle format, I spent one year and a half at INRA where I was involved with the experiments to evaluate the antiinflammatory effects of *L. lactis*-PAP using the DNBS mouse model. After returning to Brazil, I continued my work using the same lactococci strain, however, testing it in another mouse model of intestinal inflammation, 5-FU, under the supervision of Prof. Dr. Vasco Azevedo and Dr[a]. Marcela Santiago. This work rendered one research paper and one review article.

I-Research articles

Rodrigo D. Carvalho, Natalia Breyner, Zelia Menezes, Nubia M. Rodrigues, Luisa Lemos, Tatiane U. Maioli, Danielle Da G. Souza, Denise Carmona, Ana M. C. De Faria, Philippe Langella, Jean-Marc Chatel, Luis G. Bermúdez-Humarán, Henrique C. P. Figueiredo, Vasco A. Azevedo[1*] And Marcela S. De Azevedo[1*]. Secretion of biologically active pancreatitis-associated protein I (PAP) by dairy genetically modified Lactococcus lactis NZ9000 in the prevention of intestinal mucositis. Microbial Cell Factories (Submited 2016).

Natalia Breyner, **Rodrigo D De Carvalho**, Tatiana Rochat, Priscilla Bagano Vilas Boas, Daniela Pontes, Florian Chain, Harry Sokol, Marcela Azevedo, Anderson Myioshi, Vasco A. Azevedo, Philippe Langella, Luis G. Bermúdez-Humarán, Jean-Marc Chatel. Oral delivery of Pancreatitis-Associated Protein (PAP) by the food-grade bacterium *Lactococcus lactis* displays protective effects in mouse acute colitis model through Treg induction. (Manuscript in preparation – 2016).

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II- Review articles

Rodrigo D. Carvalho, Alberto Junior, Philippe Langella, Jean-Marc Chatel, Luis G. Bermúdez-Humarán, Vasco Azevedo[1]* And Marcela S. De Azevedo[1]*. Use of wild type or recombinant Lactic Acid Bacteria as an alternative treatment for gastrointestinal inflammatory diseases: A focus on Inflammatory Bowel Diseases and mucositis. Microbial Cell Factories. (Submitted 2016).

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III-Book chapters

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Appendix B - Book Chapter: Novel Biotechnological and Therapeutic Applications for Wild Type And Genetically Engineered Lactic Acid Bacteria

This section of the manuscript presents a book chapter accepted in Taylor Francis group in the Book Fermented Foods of Latin America. The book chapter describes general biotechnological and therapeutic applications of Lactic Acid Bacteria, Including probiotic use, heterologous protein production for developing new DNA vaccines and therapy for gastro intestinal disorders.

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Novel Biotechnological and Therapeutic Applications for Wild type and Genetically Engineered Lactic Acid Bacteria

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Abstract

The human gastrointestinal (GI) tract is colonized by a complex and dense microbial community that can be divided into three major phyla—Bacteroidetes, Firmicutes and Actinobacteria, which, under normal conditions, live in a symbiotic relationship with the host. However, it has been shown that a dysfunctional interaction between the microbiota and the host can lead to several intestinal disorders, thus being considered a field of growing interest by the scientific community. In this context, some studies have been carried out to elucidate functions of true resident bacteria, while other research has attempted to assess transient bacteria. In addition, some studies have focused on the group of lactic acid bacteria (LAB) that are widely used as starter cultures in food fermentation of a large variety of fermented foods. It has being reported that allochthonous LAB bacteria may have positive effects on the host when administrated in adequate amounts, thereby allowing them to be classified as probiotics microorganisms. Our research group recently investigated the mechanisms underlying the protective effects of dairy *Lactobacillus delbrueckii* Lb CNRZ327 *in vitro* and *in vivo*assays and have deposited the complete genome of *Lactococcuslactis* NCDO 2118, which will enable a greater understanding of its intrinsic characteristics. Beyond the classical employment of LAB, our group gathered research works using genetically engineered LABs, more specifically lactococci and lactobacilli as mucosal delivery vectors for therapeutic proteins and DNA

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vaccines. In this context, several studies have been conducted to develop new strains and efficient expression systems to use LAB as "cell factories" for the production of anti-inflammatory proteins, where we provide the recombinant *L. lactis* strains efficiency in the prevention of the intestinal damage associated with inflammatory bowel disease in murine models. Moreover, the use of LAB as cell carriers for the production and presentation of antigens has contributed significantly to the development of new vaccines. A growing number of publications on biotechnological or therapeutic employment of LAB has emerged showing their effectiveness against disease but also its safety and immune efficiency, the fact that there are varied ongoing studies with tests at different stages of clinical phase, strengthens our belief that their use will soon benefit the population against most diseases whose treatment and cure is difficult or non-existent.

Introduction

The human gastrointestinal (GI) tract is colonized by up to 10^{14} bacteria, ten times higher than the number of cells in the human body (Artis 2008; Ley et al. 2006). Most of these microorganisms are bacteria and fungi appear to be rare (Gill et al. 2006). The composition and density of bacterial populations vary along the gastrointestinal tract (GI) of healthy adults (Wang et al. 2005; Zoetendal et al. 2006). Low numbers (10^3) of bacteria, mainly belonging to the streptococci and lactobacilli group, are present in the upper GI tract, while in contrast, much higher numbers reside in the lower compartments, where bacterial populations reach 10^{11}–10^{12} (Whitman et al. 1998). Bacterial species from both upper and lower GI are classified into three phyla—Bacteroidetes, Firmicutes and Actinobacteria (Eckburg et al. 2005).

Under normal conditions, the intestinal microbiota lives in a symbiotic relationship with the host and this interaction has become a field of growing interest for the scientific community, which is beginning to understand the diversity and function of this microbiota that plays an important role in human health. Metagenomic sequencing studies are currently being developed with the intent to (i) identify the different species that lives in the GI tract and (ii) understand their specific function that are thought to be essential for the proper functioning of the gut ecosystem. This includes functions known to be important for the host, such as degradation of complex polysaccharides, metabolism of mineral, carbohydrates, and lipids, synthesis of short chain fatty acids (SCFA), amino acids and vitamins, activation of bioactive food components, maturation and modulation of the immune system, as well as protection against potentially pathogenic species (Arumugam et al. 2011; Qin et al. 2010). It has been shown that a dysfunctional interaction between the microbiota of the gut and the mucosal immune system of the host can lead to inflammatory intestinal diseases known as inflammatory bowel diseases (IBDs) in genetically disposed individuals (Sartor 2006).

Some microbial members can be classified as true residents, indigenous or autochthonous species, which have a long-term association with the intestinal habitat forming a stable community. On the other hand, there are microbial species that under normal conditions do not colonize the intestine but they do occur in the GI, at least temporarily, as they are present in the food intake and disappear a few days after (Berg 1996). These transiting or allochthonous bacterial species are usually present in fermented food products like yogurt and cheeses. Studies aimed to assess

differences among true resident and transient bacteria are still incipient, and recent reports focus on the group of lactic acid bacteria (LAB) that are widely used as starter cultures in food fermentation of a large variety of fermented foods (Reuter 2001). Examples that illustrate this class of bacteria are *Lactobacillus plantarum*, *L. casei*, *L. paracasei*, *L. buchneri*, *L. brevis*, *L. rhamnosus*, *L. fermentum* and the thermophilic dairy lactobacilli *Lactobacillus delbrueckii* and *L. helveticus* (Marteau and Shanahan 2003).

Probiotic Lactic Acid Bacteria

It has being reported that LAB allochthonous bacteria may have positive effect on their host. Actually, several health beneficial effects have been attributed to this group of bacteria, and the hypothesis of Metchnikoff that claimed that certain bacteria present in fermented food products might have positive effects on the consumers, improving their life expectancy proved to be correct (Metchnikoff 1907). Thus, the World Health Organization (WHO) in 2001 defined this group of microorganisms as probiotics–live microorganisms when administered in adequate amounts confer a health benefit on the host (FAO/WHO 2002). Most of the probiotics used and exploited today are lactobacilli, especially *L. acidophilus*, *L. gasseri* and *L. johsonii*, most of which have been isolated from the human GI tract. Other representatives include *L. casei*, *L. plantarum*, *L. reuteri, L. fermentum, L. salivarius, L. paracasei and L. rhamnosus*(Borchers et al. 2009; FAO/WHO 2002; Ventura et al. 2009; Walter 2008). A number of other bacteria like *Escherichia coli* strain Nissle, *Enterococcus faecium*, *E. faecalis* and some species from the *Bifidobacterium* genus are considered as probiotics as well (Borchers et al. 2009; Ventura et al. 2009).

Many research projects have shown that probiotics can induce changes in the gut microbial species composition and diversity, suggesting that an increase in bacterial diversity may have a therapeutic role to attenuate intestinal inflammation. Probiotics can also inhibit growth of pathogens by producing antimicrobial compounds reducing their population at mucosal surfaces through competitive exclusion (Ljungh and Wadström 2006). Moreover, they are able to increase mucosal barrier function turning gut mucosa resistant to pathogens that in other conditions would be capable to translocate the epithelium and cause disease. Another characteristics of probiotics are that they can modulate inflammatory signaling pathways (for example NF-kB pathway) in macrophages, dendritic cells (DCs) and intestinal epithelial cells (IECs) decreasing the secretion of pro-inflammatory cytokines, such as IL-8, TNF-α and INF-y (Fitzpatrick et al. 2008; Haller et al. 2002). Several studies have indicated that probiotics can induce the proliferation of regulatory DCs and T lymphocytes (Treg) establishing an anti-inflammatory environment with the predominance of TGF-B and IL-10 cytokines (Foligne et al. 2007; Di Giacinto et al. 2005; Pronio et al. 2008). Another very interesting feature is that certain probiotics were shown to be able to regulate apoptosis in IECs (Yan and Polk 2002).

Bacterial factors implied in the probiotic effect from different bacterial strains of LAB still remain to be identified. These molecules might be factors secreted by the bacterium, as the induction of probiotic effects usually does not require direct cell contact. For instance, Lipoteichoic acid (LTA), a secreted factor from selected probiotics, has been shown to have a potent anti-inflammatory effect *in vitro* (Kim et al. 2008). In 2008, Mazmanian and collaborators demonstrated that the anti-inflammatory

effect of *Bacteroides fragilis* was due to a single microbial molecule, polysaccharide A, PSA (Mazmanian et al. 2008). Another example is the soluble peptides from the probiotic mixture VSL#3 that were shown to block NF-kB pathway decreasing the secretion of pro-inflammatory cytokines by the host (Petrof et al. 2004).

As strains of probiotics are capable of reverting an inflammatory to an anti-inflammatory environment, some are being tested as a therapeutic tool to fight against inflammatory intestinal diseases, such as IBD (Jurjus et al. 2004). With this purpose, several models of experimental colitis have been described in order to understand the pathogenesis and exploit probiotics as treatment for IBD (Jurjus et al. 2004; Prantera et al. 2002).

Lactobacillus delbrueckii

Our research group recently screened a collection of dairy *L. delbrueckii*and tested its immune modulation effect *in vitro* through the quantification of nuclear factor kappa B (NF-κB) activation in a human intestinal epithelial cell line. All strains showed anti-inflammatory effects that varied from strong to light and this effect was due to bacterial surface exposed proteins. One strain (Lb CNZ327) that exhibitedan extraordinarily anti-inflammatory function in the *in vitro* assays and was able to significantly reduce macroscopic and microscopic symptoms of dextran sulfate sodium (DSS) induced colitis in mice (Santos Rocha et al. 2012). In order to investigate the mechanisms underlying the protective effects of Lb CNRZ327 *in vivo*, mice were administrated with DSS and many immunological parameters were measured. It was observed that Lb CNRZ327 strain modulated the production of TGF-β, IL-6, and IL-12 in the colonic tissue and of TGF-β and IL-6 in the spleen causing the expansion of CD4+Foxp3+ regulatory T cells in the cecal lymph nodes, modulating not only mucosal but also systemic immune responses (Santos Rocha et al. 2014). Despite positive results from some pre-clinical or clinical trials using probiotics as treatment for intestinal inflammatory diseases, like IBD, our knowledge on the use of this group of bacteria is still preliminary. Due to the variability activity of different probiotic strains, well-designed studies and research projects are required to employ probiotics in medical practice.

Lactococcus lactis

Most beneficial effects of probiotics comprising the group of LAB have often been attributed to bacterial strains included into *Lactocbacillus* and *Bifidobacteria* genus. However, little is known about the effects of bacteria that are constantly present in our diet, such as *Lactococcus lactis*. This species is a facultative heterofermentative and mesophilic bacteria (optimum growth temperature around 30°C) whose participation in the dairy industry is very relevant, especially for cheese production. There are two *L. lactis* subspecies reported to date: lactis and cremoris, both are found naturally in plants, especially grass. But they are also artificially found in the fermented foods as yoghurt, bread and in some types of wines, once they are used as starter cultures (Carr et al. 2002).

In order to understand the beneficial effect of these bacteria and their mechanism of action some strains of *L. lactis* were selected for assessment of their immunomodulatory potential *in vitro*. For this purpose three strains were chosen: (i) *L.*

lactis subsp. *lactis* IL1403, the first LAB sequenced and extensively used for the production of various metabolic products such as vitamin B, diacetyl and alanine, as well for the production of recombinant proteins (Bolotin et al. 2001; Kleerebezem et al. 2002); (ii) the *L. lactis* subsp. cremoris MG1363 strain, is most widely used in genetic and physiological research throughout the world and is employed in several biotechnological applications, such as oral vaccines or delivery of bioactive peptides to mucosal GI (Hanniffy et al. 2007); and (iii) the *L. lactis* subsp. *lactis* NCDO 2118 (LLNCDO2118) strain, isolated from frozen pea, it has been routinely used in our laboratory for cloning and expressing proteins. Recently, this strain was described as a gamma-aminobutyric acid (GABA) producer (Mazzoli et al. 2010). GABA, the most widely distributed neurotransmitter in the central nervous system of vertebrates, is the product of L-glutamate decarboxylation mediated by the enzyme glutamate decarboxylase (GAD, EC 4.1.1.15) and is known to have positive effects on human health. This neurotransmitter is able to lower blood pressure in mildly hypertensive patients (Inoue et al. 2003), induce tranquilizer and diuretic effect (Jakobs et al. 1993; Wong et al. 2003), prevent diabetes (Hagiwara et al. 2004) and reduce the levels of inflammatory response in rheumatoid arthritis murine model (Tian et al. 2011).

The initial evaluation of *L. lactis* properties was performed in *in vitro* inflammation model, using intestinal epithelial Caco-2 cell line which, in culture, exhibit enterocytes characteristics (Pinto 1983). When these cells are stimulated with the proinflammatory cytokine IL-1β, transcriptional factor NF-kB is activated and consequently, the production of inflammatory mediators, including IL-8, TNF-α, IL-6, Cox2, iNOS. Our results demonstrated that LLNCDO2118 does not induce proinflammatory events, and the culture supernatant decreased the secretion of IL-8 levels by 45%, a cytokine which is overproduced in mucosal cells of IBD patients. The ability to inhibit IL-8 secretion or its pathway suggestsan immunomodulatory effect of LLNCDO2118, and shows its potential use for IBD treatment, since its inhibition can result in improvement of symptoms of these intestinal diseases (Neurath et al. 1996) .

Later, LLNCDO2118 was evaluated *in vivo* for their potential in the prevention of ulcerative colitis (UC) chemically induced by DSS in mice. As UC is a chronic inflammation characterized by remission and recurrence periods, a protocol that mimics this behavior was employed (Travis et al. 2011). Thus, the animals were subjected to an initial 7-day cycle DSS ingestion followed by 7 days of rest (without DSS ingestion), allowing for a regression of symptoms as well as in the period of remission in UC, in which the treatment (*ad libitum*) with LLNCDO2118 strain was carried out. A second cycle of DSS was used to simulate the disease recurrence. This new DSS cycle started on 14th day and finished on the 21st, which were the days chosen to evaluate the effectiveness of the strain. On the 14th, the group treated with LLNCDO2118 showed improvement in clinical signs of colitis, particularly diarrhea, suggesting that this strain has a local effect, contributing to epithelial cells protection. The analysis carried out on day 21 showed that animals treated with LLNCDO2118 had a reestablishment of the colon size, as well as microscopic scores improvement. This beneficial effect was not due to the production of sIgA levels, an important factor to prevent bacterial translocation (Malin et al. 1996; O'Sullivan 2001), since they were unchanged. However, the cytokine profile of the LLNCDO2118 treated group was able to maintain intermediate levels of anti-inflammatory cytokine IL-10 in colon tissue, while animals that did not receive the strain showed reduced IL-10 levels. Furthermore, LLNCDO2118 administration was associated with an early

increase in IL-6 production in the same tissue. IL-6 is a cytokine which can present both a pro-inflammatory and anti-inflammatory effect. In this case we suggest that IL-6 is related to increased mucosal repair by epithelial restitution (Chalaris et al. 2010; Dann et al. 2008; Grivennikov et al. 2009; Podolsky 1999; Scheller et al. 2011). As the DSS induced colitis is caused by the loss of immunological tolerance against the commensal microbiota antigens, being tolerance maintained primarily by Treg cells (and its relative ratio to activated T cells), the regulatory cells levels could then be associated with *L. lactis* anti-inflammatory mechanism. Thus, after colitis induction, the activated T cells ($CD69^+$) were quantified, which only animals fed with LLNCDO2118 showed higher levels in the spleen, suggesting that some *L. lactis* product could be able to activate T cells.The $CD4^+CD25^+CD45RB^{low}$ and $CD4^+CD25^+LAP^+$ cells was also analyzed in the mesenteric lymph node and spleen of animals treated with LLNCDO2118, since these specialized T-cell response counterbalance pro-inflammatory ones (Bouma and Strober 2003; Strober et al. 2007). Although the anti-inflammatory activity of LLNCDO2118 did not increase $CD4^+CD25^+CD45RB^{low}$ Treg, there was, however, induction of Treg cells characterized by the surface expression of peptides associated with latency (LAP) both in the mesenteric lymph nodes and spleen of animals treated with this strain. Similar results were observed following treatment with VSL # 3 probiotic which was also administered during the remission period of colitis induced by TNBS (trinitrobenzenesulfonic acid), which has been shown to increase $CD4^+LAP^+$ cells, which is essential for the VSL # 3 probiotic effect (Di Giacinto et al. 2005).

As the differentiation of effector T cells (activated) and regulatory are modulated by DCs (Chen 2006), the profile of DCs expressing $CD103^+$ surface markerwas investigated. These tolerogenic DCs are related to the differentiation of naïve $CD4^+$ T cells into Tregs (Coombes and Powrie 2008). In the inflamed group, which received only DSS, there was an increase in the population of $CD11c^+CD103^+$ cells compared to the control group (non-inflamed). LLNCDO2118 administration was able to further increase the amount of these cells, suggesting the expansion of Tregs, such as $CD4^+LAP^+$. So it was proposed that a second effect of LLNCDO2118 was observed on 21st day of experiment after the second DSS cycle, which no longer had the presence of the strain in the intestines of animals. In this second stage it was observed that the immunomodulatory capacity of LLNCDO2118 clearly depended on the expansion / recruitment of regulatory cells and their products, resulting in a milder form of UC. Similarly, Nishitani et al. observed that the *L. lactis* subsp. *cremoris* FC strain when co-cultured with Caco-2 stimulated cells, was able to significantly reduce expression of IL-8 mRNA, and also inhibit nuclear translocation of NF-kB using RAW264.7 cells *in vitro* model (Nishitani et al. 2009). Now, LLNCDO2118 immunomodulation effect on Treg cells is considered the best characterized mechanisms of action. Recently our research group has deposited the complete genome of this probiotic strainalso, which will enable a greater understanding of their intrinsic characteristics (Oliveira et al. 2014).

Heterologous Protein Production

Genetic engineering strategies in LAB have been employed to improve carbohydrate fermentation, metabolite production, enzymatic activities, or conferring them the capacity to produce beneficial compounds such as bacteriocins, and

exopolysaccharides, vitamins, antioxidant enzymes and anti-inflammatory molecules (LeBlanc et al. 2013). In this context, several studies has been conducted to develop new strains and efficient expression systems to use LAB as "cell factories" for the production of proteins (de Moreno et al. 2011).

L. lactis is the best characterized LAB group member, being regarded as a model organism for the production of heterologous proteins for (i) being an easy to handle microorganism, (ii) being safe for human use and (iii) was the first LAB to have its genome fully sequenced (Bolotin et al. 1999) thus, it has currently a large number of genetic tools for cloning and expressing (Guimarães et al. 2009; de Vos 1999).

Gene expression regulation L. lactis

Transcription

Bacteria gene transcription starts when the sigma subunit (σ) of RNA polymerase recognizes a specific region located on the DNA. This region, called the "promoter" is located on an upstream sequence of gene or operon characterized by the presence of 2 consensus motives, –35 (TTGACA) and –10 (TATAAT) base pairs from the transcription start site. After recognition of these hexanucleotides, the transcription process is carried out (Bolotin et al. 1999). In *L. lactis* a number of promoters have been described through comparative and functional analysis of already identified genes (Kuipers et al. 1993). They feature sequences –35 and –10 similar to those found in *E. coli* and *B. subtilis* and also a "TG" (thymine-guanine) motif located on the first upstream base pair of the –10 sequence. The primary sigma factor in *L. lactis* is encoded by rpoD gene (Araya-Kojima et al. 1995; Bolotin et al. 2001) and shows homology to the σ^{70} and σ^{A} factors genes of *E. coli* and *B. subtilis,* respectively. The transcription stops in the 3' portion of genes and operons, where a palindromic sequence of nucleotides rich in guanine and cytosine and thymine, called "transcriptional terminators", signals the end of the process. Most genes and operons *L. lactis* have such sequences.

Translation

Once transcription has occurred, the translation process initiates. In *L. lactis* translation start signals are also similar to those described in *E. coli* and *B. subtilis*. The ribosome attachment site or "RBS" is located in the 5 'portion of mRNA to be translated, and is complementary to the sequence 3' to the 16S rRNA (3'CUUUCCUCC 5') of *L. lactis* (Chiaruttini and Milet 1993). Although most of the initiation codons are AUG, other codons, such as GUG were also observed (van de Guchte et al. 1992).

Genetic tools for the production of heterologous proteins in L. lactis

Heterologous proteins expression systems in *L. lactis* were obtained by the progress of genetic knowledge, the development of molecular biology techniques and studies of regulatory elements of gene expression, such as constitutive or inductive promoters (Miyoshi et al. 2004). This combination has allowed a variety of proteins from different sources to be cloned and highly expressed in *L. lactis* through

several plasmidial vectors (Bermúdez-Humarán et al. 2011; Langella and Le Loir 1999; Mercenier et al. 2000).

Heterologous proteins expression systems and cell targeting

An early gene expression system for use in *L. lactis* was based on the promoter Plac and the regulatory gene *lacR* from the bacterial lactose operon. This operon is activated when the Plac promoter is induced in the presence of lactose and the transcription repressor gene (*lacR*) is suppressed in the same condition, allowing the target gene to be expressed (van Rooijen et al. 1992) .

Subsequently another system was developed, consisting of three vectors that matched the lac operon elements and 2 more elements from the bacteriophage T7 of *E. coli*, allowing a higher level of induction of the protein of interest (Wells et al. 1993). In this system, the gene coding RNA polymerase from phage T7 (T7 RNA pol) was placed under the control of Plac promoter in a first vector while in a second vector, the target protein is under control of the T7 promoter. Thus, this system works in a way that when lactose was added to the culture medium, the Plac induces the expression of T7 RNA pol, which activates expression of the gene of interest controlled by the T7 promoter. However, in order for the cell to be capable of metabolizing soluble lactose in the medium, a third vector containing the lac operon was necessary. Although this system allowed fine control of gene expression and higher levels of production, it became infeasible because it required three antibiotic resistance markers making it unsuitable for food and pharmaceutical industry employment (Wells et al. 1993).

Gene expression regulation studies on the common phages from the *Lactococcus* sp. were the basis for developing more simple expression systems like the "operator-repressor system" based on r1t, a *L. lactis* phage (Nauta et al. 1996). In the same vector, the gene coding of the protein of interest is placed under the control of P_{ORF5} phage promoter, which is repressed by the phage protein Rro. When added to the medium, the mutagen mitomycinC, causes the proteolytic breakdown of the repressor protein, Rro, and the consequent release of th epORF5 promoter. Free of repression, the promoter induces the expression of the gene under its control. This system was tested using the lacZ reporter gene rom *E. coli* and subsequently using the *acmA* gene (autolysin) from Lactococcus sp. However, the use of mitomycin C as inductor prevents the use of this system for protein production in fermenters as well in food products.

In another system, the genetic elements from phage φ31 were used to develop an expression system that matched the P_{15A10} promoter and the replication origin, ori31 (O'Sullivan 2001). Here, as in other systems, the gene of interest cloned under the control of P15A10, and ori31, are in the same vector. After the start of φ31 phage infection, ori31 becomes target of the phage replication machinery and the amount of vector copies within the cell is increased. Due to this increase and due to the strength of P_{15A10} promoter, the gene of interest expression level is also increased. After cell lysis caused by phage replication, the protein molecules in question are released into the environment. The major disadvantage of this system is the need to obtain cell infection induction; which leads to the destruction of the cell culture, thus impeding their industrial use, in fermenters.

In this context, many studies have been conducted in order to develop safer and more suitable vectors for food industry. One of the most powerful expression systems already developed for use in food industry are based on genes involved in biosynthesis and regulation of the antimicrobial nisin, a peptide naturally secreted by several strains of *L. lactis.* Because of its antimicrobial properties, it is widely used as a natural food preservative. The Nisin Controlled Gene Expression–NICE system was developed in *L. lactis* where the genes *nisR* and *nisK* were inserted into the chromosome in the MG1363 strain, and the P_{nisA} promoter in the expression vector followed by multiple cloning sites (MCS) for insertion of genes of interest (Kuipers et al. 1993; Mierau and Kleerebezem 2005). In this system, induction of expression of heterologous proteins can be achieved by adding nisin in the extracellular medium, in which the *nisK* gene functions as a membrane sensor that recognizes the extracellular presence of nisin, while the signal is transferred to NisR by a phosphorylation process, turning *NisR* capable of binding to P_{nisA} promoter and consequently activate the gene of interest transcription.

This system is also versatile, making the heterologous protein able to accomplish their desired biological activity by addressing them properly to its final cell destination: (i) cytoplasm (ii) membrane or (iii) the extracellular medium. In bacteria, the protein secretion is accomplished by the addressing of specific sequences which encode a hydrophobic negatively charged signal peptide (SP) located at their amino-terminal portion (N-terminal). This SP is recognized and cleaved by the secretion machinery allowing translocation of the protein across the cell membrane, and thereby released in the extracellular medium. Another signal sequence is Cell Wall Anchor (CWA) that encondes a peptide composed of 30 amino acids which is located in the carboxy-terminal portion (C-terminus) of the protein. The CWA has a conserved motif (LPXTG) which is recognized by anchoring machinery. Thereby, the protein containing this motif is covalently attached to peptidoglycan present in the cell membrane (Le Loir et al. 1994, Mierau and Kleerebezem 2005; Piard et al. 1997).

Xylose-Inducible Expression System

In 2004, Miyoshi et al. developed a new gene expression system for *L. lactis*. The system, called Xylose-Inducible Expression System (XIES) based on the xylose permease gene promoter, (PxylT) from *L. lactis* NCDO2118, fully described and functionally characterized (Jamet and Renault, 2001). In the presence of some sugars, as glucose, fructose and/or mannose, PxylT was shown to be repressed; Otherwise, PxylT is transcriptionally activated by xylose in *L. lactis* (Miyoshi et al. 2004). Thereby, this promoter could be successively turned on by adding xylose and off by washing the cells and growing them on glucose (Jamet and Renault 2001). Myoshi et al. (2004) developed a new lactococcal xylose-inducible expression system that also incorporates the ability to target heterologous proteins to cytoplasm or extracellular medium. This system contains two plasmids that are derived from two broad-host-range expression vectors, pCYT:Nuc and pSEC:Nuc that would send the protein to the cytoplasm or to the extracellular medium, respectively (Bermúdez-Humarán et al. 2003). The system combines the PxylT (Jamet 2001), the ribosome-binding site (RBS) and the signal peptide (SP) of the lactococcal secreted protein, Usp45 (van Asseldonk et al. 1990) and the *Staphylococcus aureus* nuclease gene (nuc) as the reporter (Le Loir et al. 1994; Shortle 1983) and was successfully applied to high-level

Nuc production and correct protein targeting and was tested in the *L. lactis* subsp. lactis strain NCDO2118. These systems have great advantages once they are considered less expensive and safer for laboratory use as compared to the other available expressions methods (Azevedo et al. 2012).

De Azevedo et al. (2012) constructed the recombinant *L. lactis* strains that were able to produce and properly send the *Mycobacterium leprae* 65-kDa HSP (Hsp65) to the cytoplasm or to the extracellular medium, using XIES. Heat shock proteins (HSPs) expression in host is induced by a wide variety of stresses (including high temperature, anoxia, and ethanol) (Lindquist and Craig 1988). Hsp65 are also known to play a major role in immune modulation, controlling autoimmune responses. Some authors showed that oral administration of a recombinant *L. lactis* strain that produces and releases LPS-free Hsp65 prevented the development of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice, reduced the incidence of type I diabetes in non-obese diabetic mice and attenuated atherosclerosis in low-density lipoprotein receptor-deficient mice (Jing et al. 2011; Ma et al. 2014; Rezende et al. 2013). Some *L. lactis* produced recombinant Hsp65 that could be used for biotechnological and therapeutic applications. rHsp65 was efficiently produced in both the cytoplasm and secreted forms to the extracellular medium, confirming the ability of the XIES system to produce and correctly address recombinant proteins (Azevedo et al. 2012).

Some works have used the XIES system to express anti-inflammatory molecules as an alternative therapy against IBDs, like use of a fermented dairy product containing IL-10-producing *L. lactis* for the prevention and/or treatment of IBD using a rodent model of CD (del Carmen et al. 2011). IL-10 has a central role in down-regulating inflammatory cascades, which makes it a good candidate for use in the therapeutic intervention in inflammatory processes (Marinho et al. 2010). The expression system XIES was effective in milk food matrix, as observed by significant increases of the cytokine in fermented milks with IL-10-producing strains (Cyt and Sec). Once, XIES is a LAB expression system that can be added in to the food matrix that is tightly regulated by xylose, which is rarely found in conventional foods, it can act perfectly as an inductor. In this way IL-10 expression can be up- or down regulated, which is especially useful for expression studies of the cytokines in media or milk. The use of an inducible expression system is very interesting from a genetics point of view, because genes in this expression system are only expressed when required by adding the inducer (in this case xylose). A constitutive expression system would continuously produce the IL-10 (or other genes under its control) when it might not be required, especially in the cases of bacterial strains that could persist in the gastrointestinal tract (del Carmen et al. 2011). Induction with xylose increased the cytokine levels production by IL-10 *L. lactis* producers (>500 pg/ml for the Cyt strain and >1,000 pg/ml for the Sec strain) (Marinho et al. 2010). There is some controversial data in the literature about the more effective IL-10 producer *L. lactis* strain. It was demonstrated by Marinho et al. (2010) that *L. lactis*- producing IL-10 in the cytoplasm showed a higher immunomodulatory potential in a murine lung inflammation model, hypothesizing that the recombinant IL-10 produced in the cytoplasmic form stored IL-10 for a longer period of time and is slowly released in the tissue when the bacterial host lysis occurs. However, Del Carmen et al. (2011) found that the secreting IL-10 strain showed a higher anti-inflammatory effect compared to the cytoplasmatic producing IL-10 strain. This could be due to the fact that this cytokine

and the *L. lactis* Sec strain are probably both protected by the food matrix (milk), resulting in a more efficient delivery of IL-10 in the gut. The IL-10 produced by these lactococci strains was able to induce an anti-inflammatory effect in our TNBS model and this effect was attributed to IL-10 producers while the Wt strains did not exert any effect. Prevention of intestinal damages (macroscopic and microscopic) was also observed in mice that received the milks fermented by both of these strains (Cyt and Sec producers) thereby proving the anti-inflammatory effect of these products. The promising results obtained in these studies showed that the employment of fermented milks as a new form of administration of IL-10-producing *L. lactis* could represent an alternative treatment for IBDs (del Carmen et al. 2011; Marinho et al. 2010).

In another study, researchers evaluated the production and the delivery of 15-lipoxygenase-1 (15-LOX-1) by *L. lactis* NCDO2118 containing XIES in a TNBS- induced colitis model in mice (Saraiva et al. 2015). 15-LOX-1 is found in endothelial/epithelial cells that plays a key role in the oxidative metabolism of arachidonic acid responsible for lipoxins production, lipid mediators with potent anti-inflammatory actions (Lee et al. 2011; Serhan 2005). They concluded that 15-LOX-1 producing *L. lactis* was effective in the prevention of the intestinal damage associated to inflammatory bowel disease in a murine model, proving the effectiveness and efficiency of XIES expression system (Saraiva et al. 2015).

Production of several staphylococcal proteins in *L. lactis* has been reported. However, these studies were not dedicated to the development of an antigen for oral vaccination, most of them were dedicated to expression-secretion systems development, such as staphylococcal nuclease to be used as a reporter protein, used latter as a reporter protein in XIES system (Le Loir et al. 1994); the characterization of staphylococcal virulence factors as ClfA and FnbA. (Que et al. 2001), ClfB (Clarke et al. 2009), IsdA (Innocentin et al. 2009): or to increase adhesion properties of recombinant *L. lactis* strains (Harro et al. 2012). Asensi et al. (2013) were the first to evaluate the production of a staphylococcal antigen in a recombinant LAB strain to be used for oral vaccination where two recombinant *L. lactis* strains allowed the production of rSEB (Staphylococcal enterotoxin type B), a potent superantigenic exotoxin, either cytoplasmatic or secreted in the intestinal mucosa of mice, using XIES expression system. Oral immunization with the recombinant strains induced a protective immune response against a lethal challenge with *S. aureus* ATCC 14458, an SEB producer strain, in murine model.

DNA Vaccines

Vaccination is one of the main tools to combat and eradicate diverse pathogenic and/or infectious agents widespread around the world.

DNA vaccines are the third generation vaccine, which utilizes genetically engineered DNA to produce an immunologic response. The first study about this platform started in the early 1990s when Wolff and collaborators observed that injection of a "naked" plasmid DNA encoding foreign antigens in mice made their muscle cells capable of expressing these same antigens (Wolff et al. 1992); since this first publication the use of DNA as a strategy for vaccination has progressed very quickly. The Norwegian Biotechnology Advisory Board defines this platform as "the intentional transfer of genetic material (DNA or RNA) to somatic cells for the purpose of

influencing the immune system" (The Norwegian Biotechnology Advisory Board 2003).

This platform has the propriety to induce humoral and cellular immune response against different kind of microorganisms such as parasites, bacteria and disease-producing viruses (Ulmer et al. 1993; Wolff et al. 1992). Moreover, it was also utilized on several tumor models (Cheng et al. 2005). The components of DNA vaccine are: (i) the plasmid backbone which contains a bacterial origin of replication needed for the vector's maintenance and propagation inside the bacteria; the "CpG motifs" (cytosine-phosphate-guanine unmethylated), called immunostimulatory sequences (ISS), these motifs could be a contribution to DNA immunogenicity. In the mammalian genome CpG have a low frequency and are mainly methylated, but bacterial DNA contains many unmethylatedCpG motifs allowing this motif to be recognized by mammals as a pathogen associated molecular pattern (PAMP). In this way,CpG motifs are the ones in charge of increasing the magnitude of the immune response because they can interact with Toll-like receptors (TLR), such as TLR9, adding adjuvant activity (Tudor et al. 2005); a resistance marker, required to permit selective growth of the bacteria that carries the plasmid; (ii) the transcriptional unit, essential for eukaryotic expression, which harbors a promoter/enhancer region, introns with functional splicing donor and acceptor sites, as well as the ORF (open reading frame) encoding the antigenic protein of interest, and the polyadenylation sequence (poly A), signal required for efficient and correct transcription termination of the ORF and transfer of the stable mRNA from the nucleus to the cytoplasm (Azevedo et al. 1999; Kowalczyk and Ertl 1999).

After an intramuscular or intradermal injection, the naked plasmid DNA will transfect somatic cells, such as myocytes and keratinocytes, and/or resident Antigen Presenting Cells (APCs) like DCs and macrophages located in the lamina propria (Kutzler and Weiner 2008; Liu 2011). Due to the fact that antigens are expressed intracellularly, both humoral and cell-mediated immunity can be activated to generate a broad immune protection. After the transcription and translation of the transgene the host-synthesized antigens become the target of immune surveillance in the context of both major histocompatibility complexes (MHC) class I and class II molecules of APCs. The APC cells have the propriety to move to the draining lymph nodes where they present the antigenic peptide-MHC complexes to stimulate naïve T cells. In the other hand, B cells are activated, beginning the antibody production cascades. Although plasmid DNA vaccines vectors can induce antibody and CD4+ T cell helper responses, they are particularly suited to induce CD8+ T cell responses (Anderson and Schneider 2007). CD8+ T-cells, the cytotoxic T lymphocytes, which are important in controlling infections (Leifert and Whitton 2000) induced by DNA vaccine can occur in two main pathways: (i) the direct DNA transfection of the APCs like as dendritic cells (DCs) and (ii) cross-presentation approach, when somatic cells such as myocytes are transfected with DNA and the expressed antigens are taken up by the APCs, or when the transfected apoptotic cells are phagocytosed by the APCs (Xu et al. 2014).

DNA vaccines have an extensive range of features that give them many advantages over other vaccination platforms like traditional vaccines developed against pathogens, including either killed or attenuated pathogenic agents. DNA vaccines

are relatively cheap and easy to produce, which is an important feature when considering an emerging pandemic threat (Liu 2011). An essential concern about vaccine products is safety. DNA vaccine are considered safe because they lack the risk of reversion to a disease causing state or secondary infection. Also the risk of integration of the plasmid into the host cell causing insertional mutagenesis, which may lead to the inactivation of tumor suppressor genes or activation of oncogenes, is found to be significantly lower than the spontaneous mutation rate (Nichols et al. 1995; Wang et al. 2004). No adverse effects have been reported ineither tolerance to the antigen or autoimmunity (Liu and Ulmer 2005), animal studies showed that there is no increase in anti-nuclear or anti-DNA antibodies after DNA vaccination. There has been no evidence that autoimmunity is associated with DNA vaccines (Le et al. 2000; Tavel et al. 2007).

Vaccine manufacturing is a simple and low cost method as it requires only the use of cloning techniques in order to clone the protein of interest. They are stable at room temperature, easy to store and transport, presents thermal stability and have a long shelf life (Grunwald and Ulbert 2015; Pereira et al. 2014). A systemic inflammation, which might conduce the increase of cardiovascular risk, is a rising concern about vaccination in general (Gherardi and Authier 2012; Ramakrishnan et al. 2012), although DNA vaccines are still contemplated as a relatively new approach to vaccination, and its potential to induce systemic inflammation must be overlooked (Xu et al. 2014). Many studies have shown that DNA vaccines are generally satisfactory with an acceptably good safety profile, and no systemic inflammation has been reported (Goepfert et al. 2011; Jaoko et al. 2008; Kalams et al. 2012, Ledgerwood et al. 2011) .

Therefore, DNA vaccines portraysas a smart tool due to its property to induce all three points of adaptive immunity: antibodies, helper T cells (TH) and cytotoxic T lymphocytes (CTLs), as well as being capable of stimulating innate immune responses (Li et al. 2012) with safety. However, among the disadvantages,the poor immunogenicity of naked-DNA platform when is administrated in large animals (Kim et al. 2010) can be highlighted along with the necessity of using adjuvants besides the gene encoding of the protein. These platform are limited to protein immunogens and are not useful for non-protein based antigens such as bacterial polysaccharides (Kuby et al. 2007).

To circumvent this problem a delivery vehicle is needed to protect the DNA vaccine against endonucleases degradation. Thus, pathogenic bacteria attenuated strains appear as an interesting delivery method as they have innate tropism for specific tissues of host, which makes them attractive to use as a vehicle delivery to DNA vaccine. The use of bacteria as a delivery vector has numerous benefits: they can maintain the plasmid in a high copy number, they are easy to manufacture, they are less laborious and the cost is low as there is no need to amplify and purify the plasmid before hand (Becker et al. 2008; Schoen et al. 2004), large-size plasmid can be housed inside the bacteria, permitting the insertion of multiple genes of interest (Hoebe et al. 2004; Seow and Wood 2009). Another important features is the possibility of these vectors being used for mucosal administration, without the use of a needle, thus having the ability to stimulate both mucosal and systemic immune responses (Srivastava and Liu 2003).

In the intestinal mucosal, the bacteria carrying a DNA vaccine are able to cross the intestinal barrier, mainly via specialized epithelial cells called Microfold cells (M cells). M cells overlying Peyer's patches (PPs) whose lymphoid follicles are isolated while draining gut mesenteric lymph nodesare considered more accessible to antigens and bacteria present in the luminal compartment. DCs located in the PPs, are another pathway that bacteria have to access the body. Immature DC are able to open the tight junctions between epithelial cells, extend their dendrites outside the epithelium and directly sample bacteria, thereby monitoring the contents of the intestinal lumen (Rescigno et al. 2001). The IECs lining mucosal surfaces, can be invaded by bacteria through bacterial proteins called invasins. This characteristic refers to the capacity of attenuated pathogenic vectors to deliver DNA vaccines as they are able to naturally produce invasins.

Once inside the cells, bacteria vector have the ability to escape from the phagolysosome vesicles by the secretion of a variety of phospholipases and pore-forming cytolysins and enter the cytoplasm of the host cells (Hoebe et al. 2004; Schoen et al. 2004). The microtubules net are used by the plasmid to reach the nucleus. In the nucleus, using the host cell's transcription machinery, the protein of interest carried by the plasmid can be encoded, translated, and secreted afterwards (Grillot-Courvalin et al. 1999; Schoen et al. 2004) by the cell or be presented on the surface of epithelial cell or DCs. The MHC class-II, from APCs, presents the exogenous proteins, turning naïve T cells activated into CD4+ T-cells. Furthermore, the exogenous protein may also be processed into small peptides, which are then presented on the surface of MHC class-I molecules to CD8+ T-cells, and stimulate them (Saha et al. 2011).

The pattern recognition receptors (Toll-like and Nod-like receptors) expressed by IECs, B-lymphocytes and DCs located in the sub epithelial lamina propria are the other components of immunity used by bacteria. The bacterial components known as microbe-associated molecular patterns (MAMPs) are recognized by pattern recognition receptors and trigger intracellular signaling pathways that lead to cytokine secretion and immune cell activation (Barbosa and Rescigno 2010; Steinhagen et al. 2011). The bacterial recognition by the immune system modulates the innate immune response, thereby supporting a vigorous and lasting adaptive response (Hoebe et al. 2004).

Enteropathogenic bacteria like *Salmonella typhi, Listeria monocytogenes, Shigellaflexneri, Yersinia enterocolitica* and *Escherichia coli* are the species that are most widely used as bacterial delivery systems into mammalian cells (Schoen et al. 2004) because of their natural tropism for macrophages as well as DCs in the lymphoid tissue of the intestinal mucosal surface (Becker et al. 2008).

The method that use senteropathogenic species as a bacterial carrier is being considered an advantage because of their capacity to infect human colonic mucosa after oral administration. However, they need to be attenuated or inactivated as they present the risk to revert to the virulent phenotype, there by compromising its safety. Therefore, World and Health Organization (WHO) does not recommend their use in children and immunocompromised individuals. Thus, to counteract this severe problem, the use of non-pathogenic bacteria, such as LAB as vectors for genetic immunization has been investigated (Wells and Mercenier 2008).

Lactic acid bacteria vehicles for DNA vaccine delivery

Regarding *L. lactis* as a vehicle to deliver DNA vaccines, many interesting features can be highlighted: (i) it was proved in different laboratories all over the world that they can carry recombinant plasmids and express antigens and therapeutic molecules at different cellular localizations (Le Loir et al. 2001; Wells et al. 1993); (ii) it was successfully demonstrated that *L. lactis* can deliver DNA into eukaryotic cells and in vivo to mice IECs (Chatel et al. 2008a; Guimarães et al. 2005a; Innocentin et al. 2009); (iii) they can induce both systemic and mucosal immunity when administrated at mucosa surfaces (Chang et al. 2003; Robinson et al. 1997); (iv) they can resist the acid environment of the stomach, are able to survive into the gastrointestinal tract, ensuring recombinant protein or plasmid delivery (Pereira et al. 2014). Regardingits extraordinary safety profile (Salminen et al. 1998), because*L. lactis* is not very immunogenic, it can be orally administrated several times (Guimarães et al. 2006). All these characteristics makes it a good option for being used in immunization programs (Macauley-Patrick et al. 2005).

Research that used wild-type (wt) *L. lactis*as a vector for genetic immunizationhave demonstrated both *in vitro* (Guimarães et al. 2006) and *in vivo* (Chatel et al. 2008a) that the percentage of gene transferred observed was low, as well as a low and transitory Th1-type immune response after immunization trials (Chatel et al. 2008a). To solve this problem scientist develop recombinant *L. lactis* expressing different invasins to improve bacterial interaction with IECs (Azevedo et al. 2012; Guimarães et al. 2005; Innocentin et al. 2009).

Regarding non-invasive LABs, Guimarães et al. (2006) carried out *in vitro* studies using non-invasin strains of *L. lactis* as DNA delivering vehicles. The pLIG:BLG plasmid, containing an eukaryotic expression cassette with the cDNA of the bovine -lactoglobulin (BLG) under the control of the human cytomegalovirus eukaryotic promoter (Pcmv) was used to transform *L. lactis* MG1363. Caco-2 human cells were co-incubated with purified pLIG:BLG, MG1363 (pLIG:BLG), MG1363 and a mix of MG1363(pLIG) and pLIG:BLG. Only the cells that were co-incubated with MG1363 (pLIG:BLG) exhibited the presence of BLG cDNA and the subsequent expression of BLG. This result indicated that there was the delivery of the BLG cDNA to the mammalian epithelial cells. The authors suggested that after the co-culture, some bacteria are internalized and lysed by the host phagolysosome and, consequently, the BLG cDNA was released in the cytosol.

After these *in vitro* results Chatel et al. (2008) described for the first time *in vivo* the transfer of functional genetic material from non-invasive food-grade transiting from bacteria to host. The delivery of a eukaryotic expression plasmid coding of the BLG to the epithelial cells of the intestinal membrane of mice using *L. lactis* is possible. This demonstrates the capacity of using these bacteria in the delivery of DNA vaccines. In this study, mice were submitted to intragastrically gavage. The BLG cDNA was detected in the epithelial membrane of the small intestine in 40% of the mice. Moreover, the BLG was produced by 53% of them. In addition, the BLG production was responsible for inducing a protective immune response when the mice were sensitized with cow's milk proteins. In this case, the induction of a Th1 immune response counteracting a Th2 response was observed. The delivery of a functional plasmid by *L. lactis* to the mice intestinal wall providesus with the understanding of the host-bacterium interaction and the modulation of host immune response due to the delivered DNA.

Regarding the use of invasive LABs, Guimarães et al. (2005) constructed a *L. lactis* strain capable of invading epithelial cells by cloning and expressing the internalin A gene (*inlA* gene) of *Listeria monocytogenes* under the control of a native promoter. Western Blot and immunofluorescence experiments showed that the cell wall anchored form of InlA was efficiently exhibited by the recombinant lactococci,that favored the internalization of *L. lactisinlA+* in Caco-2 cells. Invasivity test showed that *L. lactisinlA+* was 100 times more invasive than *L. lactis* wt. Moreover, *L. lactisinlA+* could deliver the eukaryotic expression plasmid coding the GFP gene to Caco-2 cells, as it was possible to detect the GFP in 1% of the invaded cells. Finally, *in vivo* studies using *L. lactisinlA+* for oral inoculation of guinea pigs revealed that *L. lactisinlA+* was able to penetrate intestinal cells.

With these invasive lactococci, DNA delivery by this bacterium can be measured. In order to achieve this, a new vector has been developed resulting from the co-integration of two replicons: one from *E. coli* and the other from *L. lactis,* named pValac (Vaccination using Lactic acid bacteria). The pValac is formed by the fusion of (i) cytomegalovirus promoter (CMV), that allows the expression of the antigen of interest in eukaryotic cells, (ii) polyadenylation sequences from the bovine Growth Hormone (BGH), essential to stabilize the RNA transcript, (iii) origins of replication that allow its propagation in both *E. coli* and *L. lactis* hosts, and (iv) a chloramphenicol resistance gene for selection of strains harboring the plasmid. The functionality of pValac was observed after transfecting plasmids harboring the *gfp* ORF into mammalian cells, PK15. PK15 cells were able to express GFP protein.

Although the interesting results obtained by the utilization of *L. lactisinlA+*, *in vivo* experimental studies are limited to guinea pigs or mutated mice, InlA cannot bind the murine E-cadherin. Thus, Innocentin et al. (2009) performed comparative studies using both *L. lactis* expressing the Fibronectin-Binding Protein A of *Staphylococcus aureus* (LL-FnBPA+) as a InlA. In this study, it was verified that LL-FnBPA+ or the truncated form coding only C and D domains of FnBPA (LL-CD+) were internalized by the Caco-2 intestinal epithelial cells as efficiently as *L. lactisinlA+*. Also in this study, it was evidenced for the first time that lactococci can be internalized in high levels and they as heterogeneously distributed in the cell monolayer. Finally, studies were performed using *L. lactis* InlA, *L. lactis* FnBPA and *L. lactis*CD carrying GFP and all of them were able to trigger GFP expression in Caco-2 cells.

Pontes et al. (2012) in *in vitro* and *in vivo* studies used invasive *L. lactis* expressing FnBPA of *Staphylococcus aureus* (LL-FnBPA+) and demonstrated that the production of FnBPA increased the plasmid transfer to Caco-2 cells (Pontes et al. 2012). When the invasiveness of Caco-2 cells by LL-FnBPA+ carrying the pValacBLG plasmid (LL-FnBPA+BLG) or not (LL-FnBPA+) was compared with the LL-wide type (LL-wt) and LL-BLG, it was observed that LL-FnBPA+BLG and LL-FnBPA+ were 10 times more invasive than LL-wt and LL-BLG. After the Caco-2 cells were co-incubated with LL-FnBPA+BLG and LL-BLG. It was found that the cells incubated with LL-FnBPA+BLG produced 30 times more BLG than the cells co-incubated with the non-invasive strain. Moreover, using BLG and GFP under the control of a eukaryotic promoter, the potential of LL-FnBPA+ as a DNA vaccine delivery vehicle was characterized *in* vivo. After the oral administration of LL-FnBPA+BLG and LL-BLGto mice, it was detected the plasmid transfer to enterocytes had no difference between both strains. The same result was observed when LL-FnBPA+GFP were used. Regarding the expression of BLG by mice, the oral administration of

LL-FnBPA+BLG led to an increase in the number of mice able to produce BLG, but there was no difference in the levels of the BLG produced. In other words, *L. lactis* increased the plasmid transfer but not the quantity of plasmid transferred.

As mentioned before, InlA cannot bind the murine E-cadherin. Moreover, FnBPA requires an adequate amount of fibronectin to be used by the integrins. Therefore, to bypass these problems and to better understand the steps of DNA transfer to mammalian cells, De Azevedo et al. (2012), engineered *L. lactis* to express a mutated form of InlA (mInLA+) which allowed the affinity to murine E-cadherin and, consequently, *in vivo* experiments using conventional mice. The results of the tests with Caco-2 cells demonstrated that LL-mInlA+ were 1000 times more invasive than LL. To analyze the role of this strain of *L. lactis* as a DNA delivery vector, a plasmid carrying the BLG cDNA (pValacBLG) was used and the transfer to intestinal epithelial cells (IECs) was measured. *In vitro* results showed that LL-mInlA+BLG were 10 times more invasive than LL-BLG. *In vivo*, after oral administration of LL-mInlA+BLG and LL-BLG, the number of mice producing BLG in isolated enterocytes was slightly higher in mice administered with LL-mInlA-BLG than with LL-BLG.

Our research group was very interested to know whether uptake of L. lactis DNA vaccines by DCs could also lead to antigen expression, as observed in IECs, as they are unique in their ability to induce antigen-specific T cell responses. We demonstrated that both non-invasive and invasive lactococci could transfect bone-marrow DCs (BMDCs), inducing the secretion of the pro-inflammatory cytokine IL-12. This plasmid transfer to BMDCs was also measured through a polarized monolayer of IECs, mimicking the situation found in the GI tract. Co-incubation of strains in this co-culture model showed that DCs incubated with LL-mInlA+ containing pValac:BLG could express significant levels of BLG, suggesting that DCs could sample bacteria containing the DNA vaccine across the epithelial barrier and express the antigen (de Azevedo et al. 2015).

With reference to the IBDs, del Carmem et al. (2013) used LL-FnBPA+ carrying pValac:il-10. Interleukin-10 (il-10) is an important anti-inflammatory cytokine involved in the intestinal immune system (del Carmen et al. 2014). Transfection and invasiveness assays using cell cultures showed the functionality of the plasmid and the invasive strain. Fluorescence microscopy using mice confirmed the *in vitro* results. After that, a trinitrobenzene sulfonic acid (TNBS) model for induction of intestinal inflammation in mouse was performed. Mice that received LL-FnBPA+ carrying pValac:il-10 plasmid exhibited lower damage scores by macroscopic and microscopic analysis of the large intestine, lower microbial translocation to liver and the anti-inflammatory/pro-inflammatory cytokine ratios were increased more than the mice that received *L. lactis* FnBPA+ without the pValac:*il-10* plasmid. These results suggest that this DNA delivery strategy was efficient in preventing inflammation in this colitis murine model.

Continuing in the IBDs research line, Zurita-Turk et al. (2014) used LL-FnBPA+pValac:Il-10, LL-FnBPA+, LL-pValac:Il-10 and LL-wt in a different colitis model, the dextran sodium sulphate (DSS) model for induction of intestinal inflammation (Zurita-Turk et al. 2014). The results showed that both LL-FnBPA+pValac:Il-10 and LL-pValac:Il-10 were able to diminish the intestinal inflammation. Therefore, both strains delivered the eukaryotic expression vector to host cells directly at the sites of inflammation and lead in situ IL-10 production and its anti-inflammatory properties.

Christophe et al. (2015), working with another LAB, *Lactobacillus plantarum*, and aiming to increase the DNA delivery by these bacteria, constructed a strain targeting DEC-205, a receptor located at the surface of dendritic cells (Christophe et al. 2015). The objective was to increase the bacterial uptake and, consequently, improve the delivery of the cDNA to immune cells. For that, anti-DEC-205 antibody (aDec) was displayed at the surface of *L. plantarum*using a covalent anchoring of aDec to the cell membrane, a covalent anchoring to the cell wall and a non-covalent anchoring to the cell wall. The results show that aDec was successfully expressed in the three strains, but surface location of the antibody could only be demonstrated for the strains with a covalent and a non-covalent anchoring to the cell wall. To verify the plasmid transfer, a plasmid for GFP expression under the control of a eukaryotic promoter was used to transform the three strains. GFP expression in DC cells was increased when using the strains producing cell-wall anchored aDec. However, *in vivo* tests using the mouse model exhibited a higher expression of GFP when the strain with a covalent anchoring to the cell membrane was used. It seems to be that the more embedded location of aDec in this strain is beneficial when cells are exposed to the gastrointestinal tract conditions.

For further reading on this topic, the following works can be of great value: Almeida et al. (2014) evaluated the invasiveness of recombinant strains *of L. lactis* expressing FnBPA under the control of its constitutive promoter or driven by the strong NICE system (Almeida et al. 2014; Pontes et al. 2014) compared immune responses elicited by DNA immunization using LL-FnBPA+BLG and LL-BLG and they verified that the immune response could be modified by production of invasins on the cell surface (Pontes et al. 2014). They showed that intranasal or oral DNA administration using invasive LL-FnBPA+BLG elicited a TH2 primary immune response whereas the LL-BLG elicited a classical TH1 immune response; Pontes et al. (2012) revised not only the expression of heterologous protein but also the delivery systems developed for *L. lactis*, and its use as an oral vaccine carrier (Pontes et al. 2012); Bermúdez-Humarán et al. (2011) gathered research works using LABs, more specifically lactococci and lactobacilli, as mucosal delivery vectors for therapeutic proteins and DNA vaccines (Bermúdez-Humarán et al. 2011).

Recently, our team has developed another vector called pExu, to be used in *L.lactis*. This vector will be also be used in genetic immunization like the pValac.

DNA vaccines in clinical trial phase and already licensed for use

Since the early 1990s, when studies of DNA vaccines were started, and till date, more than 18,000 scientific papers have been published on this subject. Of these, almost 500 were published in the first half of 2015's (PUBMED 2015). However, while research has advanced, currently, only four DNA vaccines are licensed and commercially available in the world, all of them for veterinary use.

The first two prophylactic vaccines based on recombinant DNA technology have been approved for use and licensed in 2005. The first one against horses West Nile Virus (West Nile- Innovation®) (Davidson et al. 2005; Davis et al. 2001) and the second one against salmonids Infectious hematopoietic necrosis virus (IHNV) (Apex-IHN®) (Anderson et al. 1996; Garver et al. 2005).

In 2008, a gene therapy based on the same technology has been licensed for pigs' treatment in Australia. Administration of a single dose of LifeTide® (plasmid containing the GHRH gene–growth hormone releasing hormone), in reproductive age females was able to reduce perinatal morbidity and mortality, thereby increasing productivity (Khan et al. 2010a,b).

The latter permit a DNA vaccine occurred in 2010. Once pt™ was developed to be used as immunotherapy for melanoma in dogs and its effectiveness is related to antibodies production that prevent the development and aggravation of the disease (Bergman et al. 2003; Liao et al. 2006).

While the number of studies with DNA-based vaccines are high, and currently there are already licensed treatment for veterinary use and any type of vaccine or treatment based on this technology are available for use in humans.

According to clinical trials (www.clinicaltrials.gov), currently there are 33 studies involving DNA vaccines in clinical trial phase worldwide. The vast majority of studies, more than 57%, are related to cancers. The testing HIV vaccines are also significant, accounting for over 27% of all ongoing studies. The other 21% are related to other diseases such as Ebola virus, HPV, hepatitis, etc.

As the licensing process for the commercialization of DNA vaccines in humans is long and meticulous, taking into account not only effectiveness against disease but also their safety and immune efficiency, the fact that there are already licensed DNA vaccines for veterinary use and various ongoing studies with tests at different stages of clinical phase, makes us believe that vaccinology based on recombinant DNA technology is a tool that will soon benefit the population against most diseases whose treatment and cure is difficult or non-existent.

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