Pancreatitis-associated protein (pap) produced by different lactic acid bacteria can protect mice in an acute colitis model after oral delivery.

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NNT : 2018SACLS307

Thèse de doctorat

PANCREATITIS-ASSOCIATED PROTEIN (PAP) PRODUCED BY DIFFERENT LACTIC ACID BACTERIA CAN PROTECT MICE IN AN ACUTE COLITIS MODEL AFTER ORAL DELIVERY.

Thèse de doctorat de l'Université Paris-Saclay et de Universidade Federal de Minas Gerais
préparée à l'Úniversité Paris-Sud

École doctorale n°581 : agriculture, alimentation, biologie, environnement et santé (ABIES)
Spécialité de doctorat : sciences de la vie et de la santé

Thèse présentée et soutenue à Ville Jouy-en-Josas, le 14 Septembre 2018, par

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UNIVERSIDADE FEDERAL DE MINAS GERAIS

INSTITUTO DE CIÊNCIAS BIOLÓGICAS

DEPARTAMENTO DE BIOLOGIA GERAL

PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA

PhD Thesis

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Thesis presented as partial requirement for the degree of Doctor in Microbiology, to the Department of General Biology at the Institute of Biological Sciences, Federal University of Minas Gerais and the degree of Doctor in “Sciences du Vivant” to the University of Paris-Saclay.

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I dedicate this work to my niece Sophia, the light of my eyes and the joy of my days.

ACKNOWLEDGEMENTS

I would first like to thank my supervisors and co-supervisors for the opportunity to develop this work, for all the support and teachings.

To all my colleagues from LGCM, INRA and LIB for all the help in the experiments and for all the knowledge that was shared.

To all my family, especially my parents and brothers, for all unconditional love, for being the basis of my perseverance and for always having taught me that I am able to overcome all adversities.

To Leandro for having encouraged me to start this PhD and never to have let me give up, even in times of greater difficulties.

To all my friends who have always listened to me, advised me, wiped away my tears and smiled at me when I needed them most.

“If you can’t fly then run, if you can’t run then walk, if you can’t walk then crawl, but whatever you do you have to keep moving forward”

Martin Luther King, Jr.

ABSTRACT

Inflammatory bowel disease (IBD) is a group of chronic, complex and relapsing inflammatory conditions of GIT that has been a global health problem, with an increasing incidence. IBD is a group of closely related but heterogeneous disease processes. It includes two main forms, Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by alternating phases of clinical relapse and remission. One of the molecules that has been studied by our research group in the treatment of IBD is the Pancreatitis Associated Protein I (PAP). PAP is part of the proteins encoded by the regenerating islet-derived (REG) gene family, that many of them are associated with epithelial inflammation. PAP is expressed in the gastrointestinal, with their expression focused in the crypt base spreading from Paneth cells of jejunum and ileum and by the goblet cells and enterocytes in the colon, and is up-regulated in patients with inflammatory bowel disease. PAP has a variety of activities, which includes anti-apoptotic, anti-inflammatory, antibacterial effects and proliferative, maintaining host-bacterial homeostasis in the mammalian gut. Several new strategies using lactic acid bacteria (LAB) for the expression or ability to metabolize molecules capable of reducing inflammation in inflammatory bowel diseases have been studied in recent years. Some strains of LABs, such as *Lactobacillus casei Shirota* and *Bacillus bifidus communis*, have been considered as probiotics, which means “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”. Here, we first sought to determine whether PAP delivered at intestinal membrane by recombinant *Lactococcus lactis strain*, LL-PAP, is able to modulate the microbiota community and reduce the chemically induced intestinal inflammation. After a DiNitro-BenzeneSulfonic-acid (DNBS) challenge, mice treated with LL-PAP showed a decrease in the colitis severity compared to those treated with the control *L. lactis* strain. This effect was characterized by: protection against weight loss; lower macroscopical and histological scores; and down-regulation of pro-inflammatory cytokines secreted by lymphocytes in Mesenteric Lymph Node (MLN). Moreover after 5 days of treatment LL-PAP was able to increase the diversity of the microbiota and relative abundance of *Eubacterium plexicaudatum*, a butyrate producer. Based on our findings, we hypothesize that a treatment with LL-PAP shift the microbiota preventing thus the

severity of colon inflammation in acute colitis model through increase of *Eubacterium plexicaudatum*, butyrate-producing bacterium, which the mechanism is still elusive. Then, two important representants of LABs group, *Lactococcus lactis* and *Lactobacillus casei*, were used to express PAP under the control of the NICE (Nisin Controlled Gene Expression) system and tested in the treatment of acute colitis induced by DNBS. Beyond the comparison between both strains it was also compared two different protocols of administration, every day or every 3 days, considering the persistence time. The analysis of weight loss, macroscopic score and cytokines showed us that *Lactococcus lactis* should be administered every day to confer protection, while *Lactobacillus casei* should be administered every 3 days to show a tendency to protect mice. Our data showed the importance of the vector and the timing of the treatment, independent for which molecule is going to be tested in the treatment of induced-colitis. For that kind of approach, is clear the importance of a previous test to define the scheme of bacterium administration. We also performed the evaluation of the protection induced by a *L. lactis* strain delivering a plasmid for PAP expression by epithelial cells, LL-PAP cDNA, compared with LL-PAP in a murine model of DNBS acute colitis. Our results showed that both groups of recombinant *L. lactis* showed the same protective effect compared with LL empty group. Moreover, PAP-cDNA was able to induce the production of anti-inflammatory cytokines and this result may suggest an activation of Treg cells differentiation. Taken altogether, we can infer that the location of PAP delivery may influence its anti-inflammatory properties but showed the same effect regarding weight loss and macroscopic scores. These results confirmed the choice of the mechanism used to deliver the molecule is as important as the choice of the molecule per se.

RÉSUMÉ

Les Maladies Inflammatoires Chroniques Intestinales (MICI) sont un groupe de maladies inflammatoires chroniques, complexes et récidivantes du Tractus Gastro-Intestinal (TGI). Elles sont un problème de santé mondial ayant une incidence croissante. Les MICI présentent des processus pathologiques étroitement apparentés mais hétérogènes. Elles comprennent deux formes principales, la Maladie de Crohn (MC) et la Rectocolite Hémorragique (RH), caractérisées par des phases alternées de rechute clinique et de rémission. L'une des molécules qui a été étudiée par notre groupe de recherche dans le traitement des MICI est la Pancreatitis-Associated Protein I (PAP). La PAP fait partie des protéines de la famille des Regenerating islet-derived (REG), dont beaucoup sont associées à l'inflammation épithéliale. La PAP est exprimée dans le TGI, son expression provient principalement des cellules de Paneth du jéjunum et de l'iléon et des cellules caliciformes et des entérocytes du côlon. Son expression est régulée à la hausse chez les patients atteints de MICI. La PAP a différentes activités, qui comprennent des effets anti-apoptotiques, anti-inflammatoires, antibactériens et prolifératifs Elle participe au maintien de l'homéostasie intestinale chez les mammifères. Plusieurs nouvelles stratégies utilisant des bactéries lactiques (BL) pour l'expression de molécules capables de réduire l'inflammation intestinale ont été étudiées ces dernières années dans notre laboratoire. Certaines souches de BL, telles que *Lactobacillus casei Shirota* ou *Bacillus bifidus communis*, sont considérées comme des probiotiques, ce qui signifie "des microorganismes vivants qui, lorsqu'ils sont administrés en quantités adéquates, confèrent un bénéfice santé à l'hôte". Ici, nous avons d'abord cherché à déterminer si la PAP délivrée au niveau de la membrane intestinale par une souche recombinante de *L. lactis*, LL-PAP, est capable de moduler la composition du microbiote et de réduire l'inflammation intestinale. Après une inflammation provoquée par l'injection de de Di-Nitro-Benzène-Sulfonique (DNBS), les souris traitées avec la LL-PAP montrent une diminution de la sévérité de la colite par rapport à celles traitées avec la souche témoin *L. lactis*. Cet effet est caractérisé par: une protection contre la perte de poids; scores macroscopique et histologique plus faibles; et la régulation à la baisse des cytokines pro-inflammatoires sécrétées par les lymphocytes dans le ganglion

mésentérique lymphatique. Après 5 jours de traitement, la LL-PAP augmente la diversité du microbiote et l'abondance relative d'*Eubacterium plexicaudatum*, une bactérie productrice d'une molécule anti-inflammatoire, le butyrate. Cette modification du microbiote pourrait participer à l'effet anti-inflammatoire de LL-PAP. Ensuite, nous avons comparé LL-PAP avec une souche recombinante *Lactobacillus casei*, LC-PAP, exprimant la PAP dans le traitement de la colite aiguë induite par le DNBS. Au-delà de la comparaison entre les deux souches, nous avons également comparé deux protocoles d'administration différents : i) une administration journalière ; ou ii) une administration tous les 3 jours. L'analyse de la perte de poids, du score macroscopique et des cytokines nous a montré que *Lactococcus lactis* doit être administrée tous les jours pour conférer une protection, tandis que *Lactobacillus casei* doit être administrée tous les 3 jours pour montrer une tendance à protéger les souris. Nos données ont donc montré l'importance du vecteur et du timing du traitement. Nous avons également comparé la protection induite par une souche de *L. lactis* délivrant un plasmide codant pour l'expression de PAP dans les cellules épithéliales, LL-PAP cDNA, avec LL-PAP dans un modèle murin de colite aiguë au DNBS. Nous avons fait l'hypothèse que l'utilisation des deux différentes souches entraine l'augmentation de PAP à deux endroits différents. L'administration de LL-PAP va augmenter la PAP dans la lumière intestinale alors que celle de LL-PAP cDNA l'augmente dans les cellules épithéliales. Nos résultats ont montré que les deux groupes de *L. lactis* recombinants présentaient le même effet protecteur comparé au groupe LL vide. Néanmoins LL-PAP cDNA est capable d'induire la production de cytokines anti-inflammatoires et ce résultat suggére une activation de la différenciation des cellules Treg. Pris dans leur ensemble, nous pouvons déduire que l'emplacement de l'administration de PAP peut influencer ses propriétés anti-inflammatoires, mais pas les effets sur la perte de poids et les scores macroscopiques. Ces résultats confirment que le choix de la stratégie utilisée pour délivrer la molécule est aussi importante que le choix de la molécule proprement dite.

Abbreviations

CAPES	*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*
cDNA	complementary Deoxyribonucleic Acid
CFU	Colony Forming Unit
CM	Chloramphenicol
CNPq	*Conselho Nacional de Desenvolvimento Científico e Tecnológico*
DNBS	Dinitrobenzene Sulfonic Acid
ERY	Erythromycin
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	Fetal Calf Serum
GM17	M17 medium containing 0.5% glucose
IELs	Intestinal Epithelial Cells
IFNγ	Interferon *gamma*
IL	Interleukin
ILCs	Innate Lymphoid Cells
INRA	*Institut National de la Recherche Agronomique*
LGCM	Laboratory of Molecular and Cellular Genetics
MLN	Mesenteric Lymph Nodes
MRS	De Man, Rogosa and Sharpe culture medium
NICE	Nisin Controlled gene Expression
O.D.	Optical Density
PAP	Pancreatitis-Associated Protein
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute culture medium
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
TE	Tris and EDTA buffer
TGFβ	Transforming Growth Factor Beta
UFMG	*Federal University of Minas Gerais*

I THESIS PRESENTATION

I.1 Collaborators

This work was performed on the Laboratories of Molecular and Cellular Genetics (LGCM), at Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil and the Commensals and Probiotics-Host Interactions Laboratory, at Micalis Institute, Institut National de la Recherche Agronomique (INRA), Jouy-en-Josas, France.

The work was supported by: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

I.2 Thesis Outline

This manuscript begins with a general introduction review, which consists of a review of the literature on lactic acid bacteria, their use as probiotics and in the treatment of inflammatory bowel diseases. This introduction also addresses the PAP molecule and its relationship to inflammatory processes.

The first chapter of this work shows the protection induced by PAP by the induction of Treg in a model of DNBS when expressed by *Lactococcus lactis*. The second chapter presents the study of two different vectors for PAP presentation in a DNBS model: *Lactococcus lactis* and *Lactobacillus casei*. These two bacteria were tested in two different administration protocols: every day and every 3 days. The third chapter deals with the presentation of PAP to mice by expression of the protein by *Lactococcus lactis* or the bacterium serving as the delivery vector for a eukaryotic expression plasmid for the production of PAP by intestinal cells in a DNBS model.

After these chapters, we have the following sessions: general conclusions about the three chapters previously presented and directions for future work involving the use of PAP, *Lactococcus lactis* and *Lactobacillus casei* in the treatment of inflammatory bowel diseases. We also have the appendix session with the presentation of the main publications generated during the doctoral training. Finally, we present the bibliographic references used as basis for the elaboration of this work.

II GENERAL INTRODUCTION

1 GASTROINTESTINAL TRACT

The gastrointestinal tract (GIT) of mammals is a highly complex biological system whose main function is the digestion of food. The GIT is the largest surface that the body exposes to the outer world and because of its role in the digestion, it has a really large surface with around 400m^2 of area[1,2]. The system is composed by different regions with distinction in the anatomy and functionality, presenting a high diversity of cell types. These cell types include a diversity of specialized epithelial cells, the largest population of immune system cells in the body and the most complex system and largest number of neurons outside the central nervous system. The interaction between the different types of cells with the microbiota in the gut plays an important role in the homeostasis of the gut[3].

1.1 Structure of gastro-intestinal tract

The gastrointestinal tract goes from the mouth until the anus, including several organs specialized in the digestive process and associated with other organs including liver, pancreas and gall bladder[2,3]. The GIT has an anatomical division: (I) upper GIT that includes mouth, pharynx, esophagus and stomach. The chemical digestion starts in the mouth and continuous through all upper GIT while the food is transported. This chemical digestion has a key role to enable the degradation and absorption of the nutrients by the small intestine. (II) lower GIT, which comprises the small intestine (separated into duodenum, jejunum and ileum) and large intestine (divided into cecum, colon, rectum and anus). Here, the process of digestion has continuation. The most part of the absorption happens at the jejunum and has the conclusion the large intestine, where the excess of nutrients and water are removed and transported to the bloodstream. By the end of the process, at the colon, is formed a solid substance named stool[4–6].

The coordinate action of smooth muscle cells, intrinsic neurons and epithelial cells of several distinct types is responsible for the gut functions, like digestion, absorption, movement of gut contents and defecation. Nevertheless, other cells present within the gut wall play key roles in the gut functions, as specialized

interstitial cells, enteric glial cells and specially the cells involved in the mucosal immune system, that play a crucial role in the host defense[3].

1.2 Intestinal Immune System

The mucosa is the part of the intestine where the most part of the immunological processes occur. The mucosa is consisted by the epithelium, the underlying lamina propria and a thin muscle layer below the lamina propria (the muscularis mucosa) (FIG 1). The epithelial barrier is composed by a monolayer of enterocytes (or intestinal epithelial cells - IEC) that are strongly connected by tight junctions, among those cells there are other specialized cells, such as Paneth cells, M cells, Goblet cells, and enteroendocrine cells, as well as intestinal stem cells (undifferentiated cells). This barrier is responsible to separate the lamina propria from the lumen content. The lamina propria contains a large quantity of cells of the innate and adaptive immune systems, but some as the lymphocytes can be found in the epithelium. The lamina propria presents T cells, B cells, eosinophils, macrophages, mast cells and other cells from innate immune system, while the epithelium mainly contains T cells[7–11].

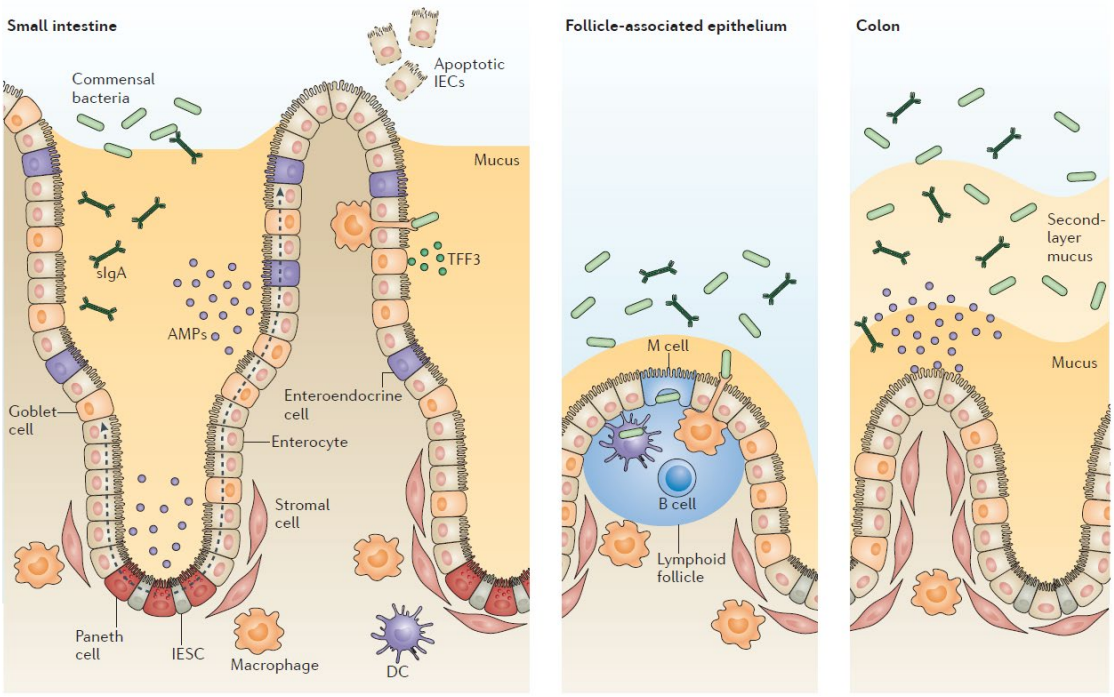

Fig 1. Anatomy of the intestinal mucosa and its immune apparatus[12].

The microvillus are apical protrusions in the IEC. This microvillus expands the area of the mucosa to something around 400m^2 and are responsible to enhance the absorptive and secretory functions of the intestinal epithelium. This surface has the cells replacement happening every two/three days, maintaining the integrity of the epithelium[13].

The Paneth cells are found in the small intestine and are present on the base of the crypts. They are responsible to the production of antimicrobial peptides such as defensins, regenerating islet-derived protein (Reg), and lysozyme[7,10,14]. These antimicrobial peptides have bactericidal activity against Gram-negative and/or Gram-positive bacteria using different strategies. One important strategy is the capacity to connect to the bacterial membrane to form a pore on it, promoting the disruption of the membrane[14,15]. Dysregulations in the Paneth cells functions led to a susceptibility to develop Crohn's Disease in a microbiota-dependent way[7].

The Goblet cells are 25% of all intestinal cells in the colon and 10% at small intestine and are responsible for the mucus production. At colon, the mucus is divided into two different forms: the inner, which is a dense layer directly in contact with the epithelial cells, and the outer, which is a loose layer with similar characteristics with the mucus layer found in the small intestine. In the small intestine the mucus layer is relatively permeable to bacteria and nutrients, while in the colon the two strata avoid that[7,16,17]. The mucus acts like a physical barrier very resistant to bacterial penetration, minimizing the contact of the lumen content with the epithelial surface and with antimicrobial properties because of the presence of mucin glycoproteins in its composition, which is toxic to several bacteria[7,12,18,19]. Those mucin-type glycoproteins have distinct functions. For example, MUC2, MUC5AC, MUC5B, and MUC6 are responsible to form the gel-like structure and MUC1, MUC3 and MUC17 are implicated in the maintenance of the integrity of the epithelial layer. Alterations in the mucus layer led to an increased penetration of bacteria, with a higher contact of them with the epithelial cells and promoting susceptibility to colitis and colon cancer[7,20–22].

Pattern recognition receptors (PRRs) are expressed by epithelial cells and can recruit and activate immune systems cells when activated. These PRRs can

recognize microbe-associated molecular patterns (MAMPs) that are microbial components, like lipopolysaccharide (LPS), lipoproteins, flagellin, lipotheic acids, peptidoglycan and nucleic acids. Innate immune responses are activated by the recognition of these MAMP[12,23–25]. After the recognition of the MAMP by the PRRs, a pro- or anti-inflammatory response can be elicited, even for the recognition of a microbiota compound. Some commensal bacteria that can elicit a pro-inflammatory answer are considered as pathobionts, it means with a potential to be pathogenic. As example of pathobionts we have *Escherichia coli*, *Clostridium difficile* and *Enterococcus faecalis*. The symbionts are the bacteria able to induce an anti-inflammatory response, like *Faecalibacterium prausnitzii*, *Bifidobacterium* sp. and *Propionibacterium* sp.[26–29]. The expression of PRRs present variations along the intestine, for example, TLR4 and CD14 are more often in the colon when compared with the small intestine, while TLR2 has an increased expression in the proximal colon than distal colon. This differences in the expression of PRRs seems to be related with the microbiota[7].

The gut-associated lymphoid tissue (GALT) is composed by subepithelial lymphoid aggregates located in the mucosa and submucosa. The GALT is composed by three distinct components: (I) Microfold cells (M cells) epithelial-type cells that are important component of the GALT, located between enterocytes. M cells are responsible to uptake and transport antigens (for example, bacteria) from the lumen to resident dendritic cell and then be presented to the adaptive immune system[7,12]. (II) The Peyer's Patches (PP) are in the small intestine, mainly in the distal ileum. They are formed by several B cells lymphoid follicles associated with smaller T cells areas, Antigen-Presenting Cells (APC), such as dendritic cells (DC) and macrophages. They are covered by M cells and are responsible for the production of immunoglobulin A[7,18] (FIG 2). (III) The intraepithelial lymphocytes (IELs) are located between enterocytes and present an extensive variety of effector and regulatory activities. The arrangement and concentration of the IEL differ between species and is influenced by antigen exposure and age. IEL also varies along the length of the intestine, with higher concentration in the proximal than distal small intestine and with a more accentuated decrease in the colon[7]. They are mainly composed by CD8+ cytotoxic T cells accumulated on wounded areas and induce lysis or apoptosis of

the cells by the secretion of cytotoxins in the moment of a infection[30]. Taking together, GALT plays a key role in the induction of immune responses against pathogens controlling the balance between tolerance and active immunity[18].

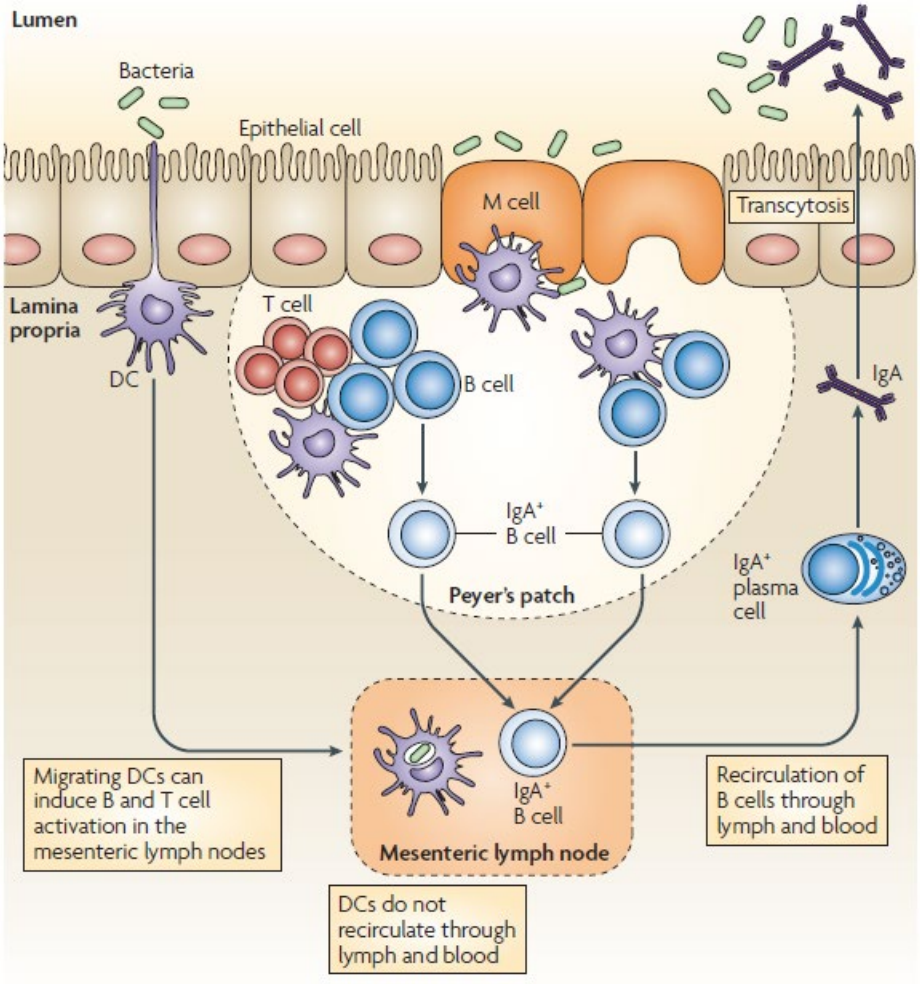

Fig 2. Production of IgA directed against intestinal bacteria[18].

The lymph nodes present in the intestine are in the highest quantity in the entire body and that reflects the continuous exposure of our GIT to environmental elements. Different regions in the intestine are drained by distinct lymph nodes, presenting different constitutions with specialized immunological characteristics[7,31–33].

In the lamina propria, the CD4+ T cells are two times more concentrated than CD8+ T cells and both display an effector memory phenotype. Treg cells increases from the duodenum to the colon, while Th17 has a lowest concentration

in the colon. IL-10-producing CD4+ T cells presents high concentration in the intestinal mucosa, mainly in the colon. By contrast, the frequencies of Th1 cells and Th2 cells do not seem to vary significantly along the human intestine[7,34–37].

Differently from others healthy tissues, the GIT presents a huge quantity of plasma cell in the lamina propria. Seventy five percent of the plasma cells from the duodenum are producer of IgA and increasing through the intestine until reaching 90% in the colon while the rest are IgM producer. Almost completely secretory IgA (SIgA) is microbiota-dependent[7].

The homeostasis of the GIT is responsibility to the macrophages, which are and the most abundant leukocytes in the lamina propria. They have a key role in the epithelial cell renewal by the production of important mediators and are responsible for the phagocytosis and degradation of dead cells and microorganisms. The IL-10 produced in large amount by the macrophages are responsible for the maintenance of life and functions of the Treg cells in the mucosa and to maintain the local homeostasis[7,38–40]. Those macrophages are constantly replaced from blood monocytes and differentiated locally by mucosal stimulus. They a present in a bigger number in the colon than in the small intestine, but both with high expression of MHC class II[7,41].

Despite their typical association with protection against worms and involved in allergic processes, mast cells and eosinophils are found in a high quantity in the intestinal mucosa of different mammals species[7,42–44]. Mast cells produce mediators involved in important processes like peristalsis, epithelial barrier integrity, permeability, vascular tone and detection of microorganisms trough TLRs[7,42]. Eosinophils are associated with tissue repair in large and small intestine, IgA class-switching in Peyer's patches, maintenance of IgA+ plasma cells, DCs and FOXP3^{+} Treg cells and IgE production.[7,45].

Therefore, the intestinal immune system is a complex of cells highly dependent of the microbiota and the environment. To confirm this, several works showed germ-free mice do not have a functional immune system, due to the absence of

microbiota. Once one or a group of bacteria colonize the intestine, these mice recover some immune functions[46–50].

1.3 Intestinal Microbiota

1.3.1 General Composition

The GIT is populated by a complex community of microorganisms, classified as being transient or indigenous. From the birth until elderly, our microbiota changes. Firstly, after birth the intestinal lumen receives the first microorganisms coming from the milk breast and from the environment[51,52]. These microorganisms can be considered as transient, in other words, they are not capable to survive in the difficult conditions find in the GIT for more than a few days, so do not colonize. However, those microorganisms are often found in the GIT by the fact that are present in the food consumed daily by humans, as breast milk or formula in early life, or as yogurts, cheeses and other fermented food in the other stages of the life. Otherwise, the indigenous microorganisms are adapted to the conditions found in the GIT and can survive, consequently they colonize the host[24,53,54].

The food and nutrients that we intake everyday transit through our GIT to be absorbed by our body and are associated with the presence in high concentrations of bacteria and other microbes. Part of theses microbes has the capacity to live associated with the host as a community and is called microbiota. This colonization starts at the birth and continues during our entire life, as mention before, culminating in a vast and diverse microbial ecosystem of 10^{14}–10^{15} microorganisms. The number of microorganisms present in our microbiota is around 10 times higher than the quantity of cells in our body. The size of human genome is 150 times smaller than the metagenome of microbiota. The microbiota composition is 94% bacteria, 3.6% of eukaryotic cells such as yeasts, 1.5% of archaebacteria and 0.61% of virus or phages[55–58].

The composition of the microbiota in the GIT varies greatly between species and inside each species. Mammalian GIT is formed by approximately 500-1000 species[7] that are mainly classified into three phyla: Gram-positive Firmicutes (48

to 76%), Gram-negative Bacteroidetes (23 to 48%) and Gram-positive Actinobacteria (0.2 to 38%)[54,59,60]. The Firmicutes includes the *Faecalibacterium prausnitzii*, which represents 3.5% of the microbiota in GIT, being the most abundant specie[61,62] and presents anti-inflammatory properties[63–66]. The Bacteroidetes comprises the *Bacteroides* genus, which has the capacity to degrade bile salt. The Actinobacteria embraces the Bifidobacteria, which are known for probiotic properties and are vastly present in child microbiota[67].

The number of bacteria also depend of the location inside the GIT. This quantity generally increases going down the gastrointestinal tract: 10^2–10^3 per ml in the highly acidic environment of the stomach, to 10^5 per ml in the upper small intestine and up to 10^{12} per ml in the colon. However, the terminal ileum might contain higher numbers of bacteria than in the colon. The distal colon is the site of the lowest diversity and the caecum with the highest one. While the small intestine is prevalent populated by aerobic species, there is a dominance of anaerobic species in the colon, consistent with the offer of oxygen on those sites (FIG 3)[7].

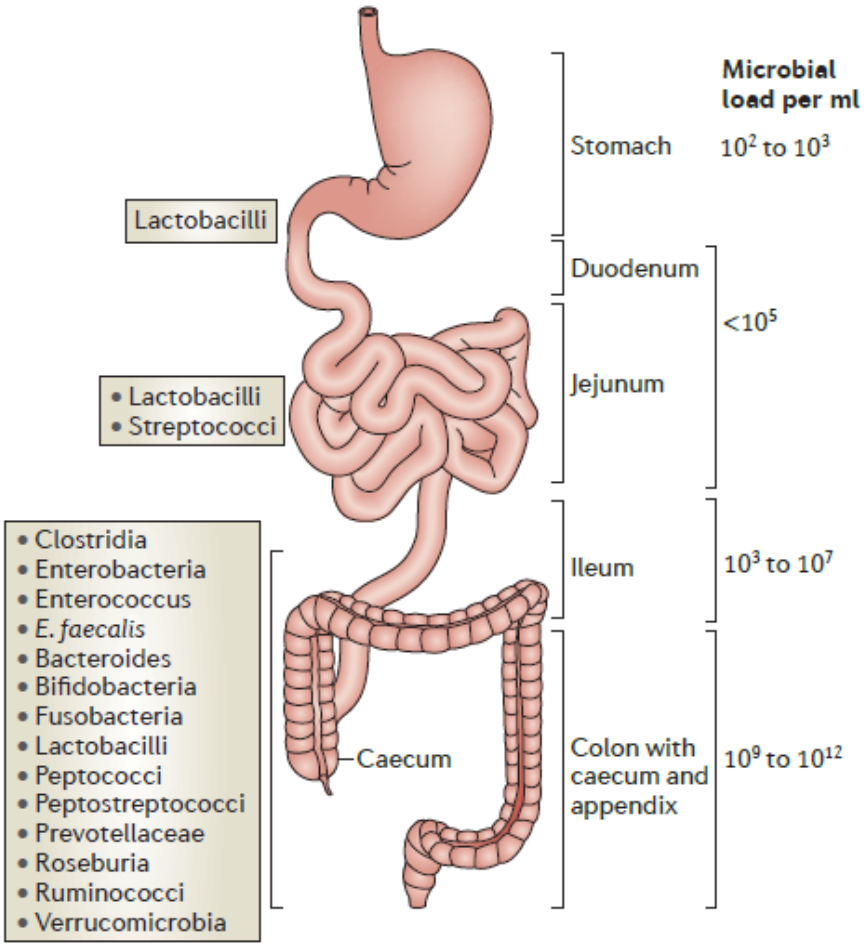

Fig 3. Distribution of environmental factors along the length of the intestine[7] (with modification).

The evolution of the microbiota arises in the beginning of life, from different sources, especially from gestational conditions, model of delivery, breast or use of formula feeding and from searching environment with mouth. The microbiota of a human has a dramatically change between the birth and 3-years old and then until reach the diversity and complexity of an adult, with large influence of age, diet, health status, stress and other conditions (Fig 4). The part of inheritance of microbiota remains unclear and studies are contradictory. It is a challenge to quantify which part of the microbiota is issued from the mother, function of host genes or dependent of the environment[68–71].

The postnatal period is especially important for the development of microbiota composition, immune cell maturation, homeostasis and host–microbe interactions. The life-long microbiota composition can be influenced by

regulations in the early neonatal period and the beneficial microbiota in the adult host is shaped during early infancy. Besides that, immune homeostasis and health in adulthood can be affected by a disturb in the establishment of the microbiota during early life caused by environmental factors during early life[72]. Modifications in the microbiota are related with augmented occurrence of autoimmune and allergic disorders. During early life, appropriate stimuli from intestinal microbiota are critical for inducing an immunoregulatory network at mucosal sites[73].

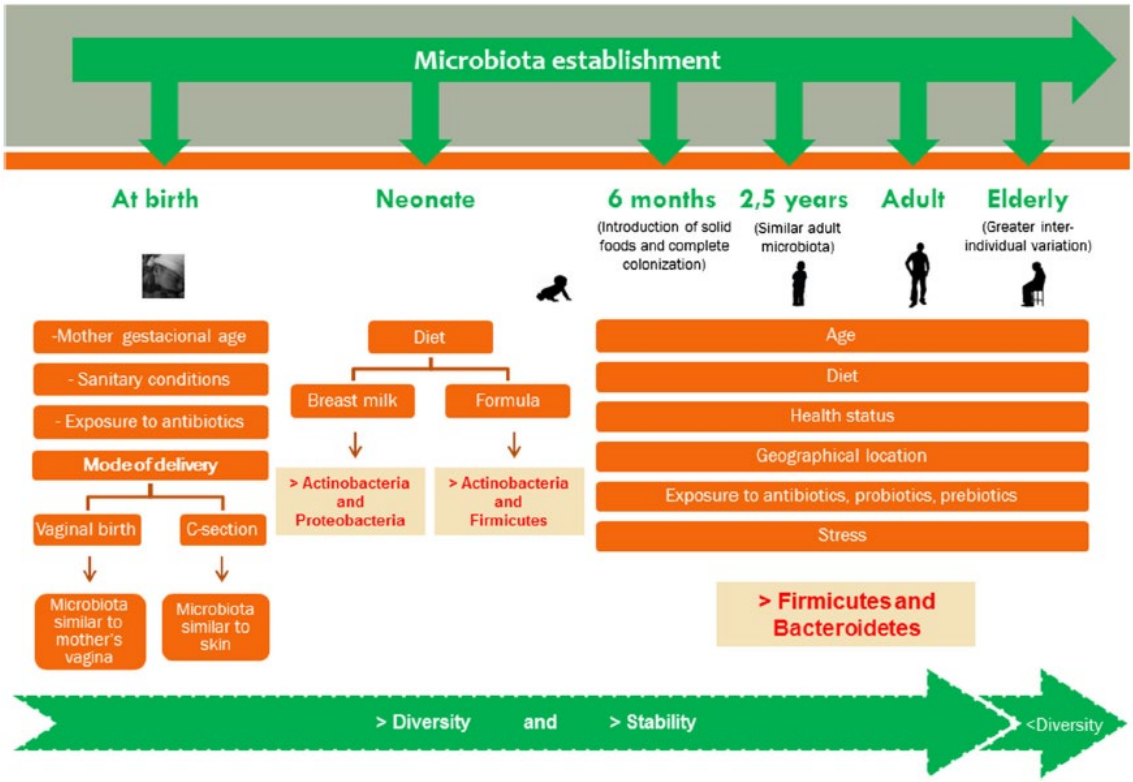

Fig 4. Factors involved in microbiota establishment from newborn to adult[71]

1.3.2 Relationships between host and microbiota

The extremely divergent arrangement of the gut microbiota between individuals is known. However, it has been described that the functional gene profiles are comparable, suggesting that the knowledge about of the metabolic activity of microbiota components could be more pertinent than its taxonomical composition. The key roles of the microbiota can be generally divided into three groups: (I) metabolic, including metabolism of lipids and cholesterol, cleavage of

some polysaccharides, as well as dietary fibers from plants into compounds as butyrate [anti-inflammatory properties]; metabolism of polyphenols [antioxidant and beneficial actions], and synthesis of amino acids and vitamins. (II) protection of the host against colonization by exogenous pathogens and potentially harmful indigenous microorganisms by competition, modulation of the immune system, production of antimicrobial peptides, and bacteriocins. Finally, (III) trophic by modulation of the proliferation, differentiation, maturation reduction of apoptosis of colonic epithelial cells[54,71,74–76].

Complex diseases as autism, Inflammatory Bowel Diseases (IBD) or obesity can be associated with the composition of the microbiota. In autism, it has been described a protective effect of *Bacteroides fragilis* in the development of the disease[77]. *Bacteroides fragilis* and *Faecalibacterium prausnitzii* are associated with healthy intestinal microbiota, and are reduced in patients with IBD. Action of *Bacteroides fragilis* is mediated by polysaccharide A, which has protective effect on colitis[78]. In obesity, the transfer of the microbiota from obese mice to germ free mice led to a higher weight intake[79]. It has also been shown that lean patients present a different microbiota composition when compared with obese patients. In the microbiota of obese patients was identified a reduction of Bacteroidetes and an increase of Firmicutes and Proteobacteria[69]. *Akkermansia muciniphila* has been associated with healthy patients, showing that an increase of *A. muciniphila* is promoted by as ingestion of oligofructose, leading to a reduction of weight[80].

1.4 Inflammatory Bowel Diseases

Inflammatory bowel disease (IBD) is a group of illnesses typified by a chronic bowel inflammatory disorders. The two main integrants of this group are Ulcerative Colitis (UC) and Crohn's Disease (CD), presenting distinct characteristics. UC is limited to the colon and is characterized by mucosal inflammation in a superficial way. CD typically causes transmural inflammation, affecting all the layers of the intestinal wall and can affect any region of the gastrointestinal tract in a discontinuous way. CD is normally related with the presence of strictures, abscesses and fistulas as complications. Beside these

differences, both diseases present similar symptoms like diarrhea, abdominal pain, rectal bleeding and weight loss. These symptoms on relapse can continue for days, weeks or even months[81–84]. Others chronic inflammatory disorders are highly associated with IBD, like osteoarthritis and psoriasis, and also complications such as colorectal cancer or blindness[85–87].

1.4.1 *Epidemiology*

IBD is considered as a global public health problem with a variation of the incidence across countries. More than five million people around the world is affected by UC and CD with 3 million in Europe and 1.4 million only in the US. Countries with low incidence are found in Asia, South America and southern and eastern Europe. The number of cases around the world is augmented year after year in both pediatric and adult patients [88–93] (FIG 5).

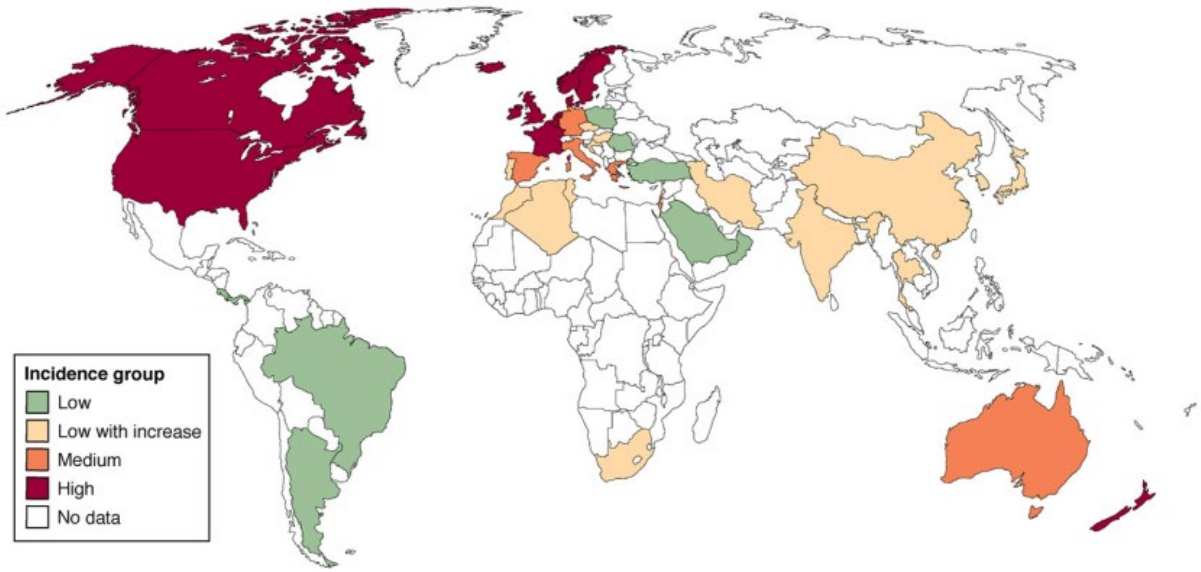

FIG 5. The global map of inflammatory bowel disease: red refers to annual incidence greater than 10/105, orange to incidence of 5–10/105, green to incidence less than 4/105, yellow to low incidence that is continuously increasing. Absence of color indicates absence of data[93].

1.4.2 IBD Pathogenesis

The IBD etiology is not really clear, but there are evidences of the influence of distinct aspects in the development of the diseases, like immune response, intestinal microbiota, genetic susceptibility and external environment[94].

1.4.2.1 Immune responses

The patients with IBD present alterations in the expression and function of PRRs such as toll-like receptors (TLRs) on the cell surface and NOD-like receptors in the cytoplasm, leading, for example, to the reduced production of antibacterial agents and increase of pathogenic microbial invasion[94–99]. IL-23 is a cytokine involved in the initial response against microorganisms and has been associated with UC and CD, promoting the chronic inflammation in the intestine. It also induces the production of Th17 cytokines by innate lymphoid cells (ILCs)[94,100], signature of the intestinal inflammation. The gene ATG16L1 is involved in autophagy processes, an important apparatus to the homeostasis maintenance, and a mutation in the gene is associated with an increase of the risk to develop CD[94,101]. Defects on antimicrobial peptides expression, damage epithelial barrier and augmented intestinal permeability have been detected in patients with IBD[94,102].

The mucosal immunity, particularly the T cell response, has been studied in the IBD pathogenesis. The Th1 and Th17 responses have been associated with CD, characterized by the production of IL-12, IL-23, IL-27 and IFN-γ, while a non-conventional Th2 response has been considered in UC with an overexpression of IL-4 and IL-13 [103,104]. High levels of IL-17A have been detected in the mucosa of patients with CD and UC, but its activity on IBD seems to be contradictory. IL-17A presents a pro-inflammatory activity by activating TNF-α or IL-1β and acting on neutrophils, fibroblasts, monocytes, macrophages and epithelium. In a mice experiment with colitis induced by TriNitroBenzene Sulfonic Acid (TNBS), is was observed that the absence of the receptor to IL-17A was associated with protection of the mice against the inflammatory process[105–108]. In opposition, the absence of IL-17A aggravates the inflammation in a model of colitis induced by Dextran sulfate sodium (DSS) while IL-17F (also Th17 profile cytokine) increased the colitis damages[109].

1.4.2.2 Intestinal microbiota and dysbiosis

The first time it was made an association between IBD and microbiota was after to see that patients submitted to diversion of fecal stream presented alleviation of IBD symptoms. Another observation was the fact that patients submitted to antibiotics therapy presented positive effect on IBD[110,111].

Several studies have been done to analyze the gut microbiota, they observed a reduced diversity associating with IBD. Analysis of fecal samples from UC and CD patients detected a significant reduction in the biodiversity compared with healthy controls. In those differences, we can highlight the lower quantity of *Faecalibacterium prausnitzii* and Lactobacilli. In patients with UC, it is detected an increase of *Escherichia coli* and a decrease in *Clostridium* spp., while in CD patients it is reported a significant reduction in the Firmicutes and Bacteroidetes and an increase of enterobacteria[59,63,112–117].

F. prausnitzii is a Gram-positive bacillus, representant of Firmicutes phylum. It is extremely sensitive to oxygen being difficult to culture and to study because of the necessity of special anaerobic equipment. *F. prausnitzii* has a remarkable abundance in the gut with more than 3.5% of the total fecal microbiota of healthy patients and this number can reach 15% in some persons[118].

In IBD, especially patients with CD, it is observed a higher quantity of bacteria associated with the colon mucus layer. *E. coli* was found to be strongly associated with the mucosa in both colon and ileum, and present inside granulomas in CD. Also in CD, it was described a phenotype of adherent and invasive *E. coli* (AIEC) that has a higher capacity to invade into epithelial cells and to replicate inside macrophages[94,119–123].

Patients with IBD present a reduction in the quantity of bacterial species responsible to produce Short Chain Fatty Acids (SCFA), such as species from Clostridium groups, mainly *F. prausnitzii*. SCFA are the primary end-products of fermentation of non-digestible carbohydrates that become available to the gut microbiota[124]. The genes involved in the metabolism of SCFA, like butyrate (that play a key role on the maturation of regulatory T cells) is reduced in patients with

IBD[29,63,125,126]. Reduction of certain species in the microbiota, as *F. prausnitzii*, could be used as reliable clinical marker in IBD because this reduction is commonly observed in patients during period of active disease or remission. Species from *Lactobacillus* and *Bifidobacteria* genus are find to be reduced in IBD with an important impact in the patients, since they are important to reduce the expression of pro-inflammatory cytokines in the GIT [127,128].

In opposition, species like *Clostridium difficile*, *Mycobacterium aviumparatuberculosis*, *Ruminococcus gnavus* and enterobacteria are considered pathobionts and find augmented in IBD[60,129–132]. Some species of pathobionts are able to reduce disulfide bonds that structure the mucus barrier in the GIT, allowing the contact of toxins and pathogenic bacteria with the epithelial cells of the host. Considering that, those sulphate-reducing pathobionts play an important role to start and to maintain the inflammation process in the IBD. In this condition, the patients are more susceptible to be colonized by facultative pathogens like invasive *E. coli* that is a specie able to adhere and invade the epithelial barrier in the gut[133–135]. The IBD clinical signs can be deteriorate by the establishment of pathogenic species like *Listeria monocytogenes*, *M. paratuberculosis* and Helicobacter species because those bacteria are able to induce pro-inflammatory responses in the host[27,136].

1.4.2.3 Genetic susceptibility

In the last decades, advances in the DNA sequencing and the analysis of these data allowed the support of the genetic contributions to IBD. The Single Nucleotide Polymorphisms (SNPs) identified through many Genome-Wide Association Studies (GWAS) making possible the identification of genes associated with UC and CD. Recent analysis has identified 163 gene loci associated with both IBD diseases, which 23 specific to UC, 30 specific to CD and 110 for both diseases. Those last ones might be the key to find the mutual pathogenesis between UC and CD. Most part of these genes are associated with cytokine receptor signaling, barrier function or T cell activation and are involved in IBD susceptibility in around 5% of world population [104,137–140].

The first gene discovered to be associated with the susceptibility for CD was NOD2 (nucleotide-binding oligomerization domain containing 2). The NOD2 gene codes for an intracellular receptor recognizing the muramyl dipeptide (MDP), a conserved motif present in peptidoglycan from both Gram-negative and Gram-positive bacteria. Activation of this receptor is associated with autophagy, control of the replication of bacteria, antigen presentation, and modulation of immune responses (innate and adaptative), including regulation of T-cell response. NOD2 mutations are still related with a deficient expression of α-defensins by Paneth cells in patients with IBD. Therefore, NOD2 deficient animals are good models in IBD investigation. This NOD2 alterations can be identified in 17 to 25% of CD patients [141–149]. TLR2 and TLR4 are PRRs implicated in recognition of luminal bacteria[150–152]. Under homeostasis condition, intestinal epithelial cells show low expression of TLR2 and TLR4 and are therefore unresponsive to TLR stimuli. However, under inflammation conditions or dysbiosis TLR expression is increased, and studies revealed the augmented expression of TLR2 and TLR4 is associated with inflammatory bowel disease[153].

The autophagy plays a key role in the immune responses in IBD and two genes have been described to be involved on it: ATG16L1 and IRGM. Autophagy is involved in the removal of intracellular microorganisms, resistance against infection and degradation and recycling of cytosolic contents, organelles, and dysfunctional cells, contributing to homeostasis. Mutations on these genes can lead to disorder in immune answer and homeostasis, so they are associated with an increased risk of CD[154–157].

The pathways of Th17 and IL-23 have been proved to be associated with the development of IBD, and the loci IL23R, IL12B, JAK2, and STAT3 recognized in the susceptibility to UC and CD. IL-23R gene encodes one subunit of the receptor for IL-23, a pro-inflammatory cytokine engaged in the generation of Th17 cells. IL-12B gene encodes the p40 subunit of IL-23 and IL-12[94,158,159].

Genetic polymorphisms related with transcription factor FoxP3, involved in the stimulation of Treg cells, are also associated with the susceptibility in IBD. In a study using IL-10 knockout specific pathogens free mice, the animals developed

colitis in a spontaneous way, showing that IL-10 is very important to induce tolerance to commensal microorganisms. In opposition, the colitis wasn't developed when IL-10 knockout germ-free mice were used, demonstrating the importance of the microbiota in the colitis progress[160–162].

Mutations in genes associated with protein folding can also be involved in IBD development. The problems associated with unfolded proteins can induce oxidative stress because of low disulfide binding and protease activity to degrade unviable protein[163]. Studies have shown the association between IBD and susceptibility gene loci, demonstrating the influence of the genetics in the pathogenesis of those diseases, but only 20-25% of these heritability susceptibilities can be explained until now. This phenomenon has been called "the mystery of missing heritability of common traits" or "genetic vacuum". There is the possibility that is not a case of missing genes to explain the association between genetics and diseases, but the key of explanation should be in the interactions between those genes and their products. Regarding these, future studies focused on the gene-gene interactions, gene-pathway interactions, and gene-environment interactions will give us more information about IBD pathogenesis than try to find new not so common genes associated with those diseases[94,164].

Studies of genome-wide association have identified risk variants of five epithelial-associated loci in ulcerative colitis. These are: ECM-1 (encodes an extracellular matrix protein), HNF4α (hepatocyte nuclear factor 4 alpha, an epithelial-specific transcriptional regulator), CDH1 locus (encodes the E-cadherin gene), GNA12 (a guanine nucleotide-binding protein) and LAMB1 (encodes laminin)[165]. C1ORF106 is a cell junction protein regulating epithelial junction formation and permeability. A reduction in its expression is associated with increased IBD risk[166]. Patients with CD presents a decrease in the expression of claudin-3, -5 and -8, besides that, presents an increased regulation of pore-forming claudin-2. In UC patients, there is a down-regulation of occludin, claudin-1 and -4 and an up-regulation of the pore-forming claudin-2[167].

1.4.2.4 External environment

There is a large number of evidences about the importance of the environment in the pathogenesis of IBD. Environmental factors such as diet, smoking, drugs, social stress, geography and psychological elements can be considered as risk factors for IBD. One of the most studied risk factors is the smoking, that is associated with a higher risk to CD, increasing twice the chance of smokers to develop the disease. In opposition, it showed a protective effect on the development of UC, including a low rate of relapse, but this protection is not seen in former smokers. It has been proved that cannabis exert an alleviating effect in IBD[168–175].

The little consumption of fiber and large ingestion of fat have been associated with an elevated risk of IBD. Some changes on diet seems to alleviates the symptoms[176,177]. Vitamin D has a large known role in bone health and calcium metabolism, but now has been growing numbers of studies focused on the immunologic properties of this vitamin. Vitamin D is obtained from sun exposure, food and diet complements. The low sun exposure is related to an increased incidence of IBD[178–181]. These patients has been commonly diagnosed with deficiency of vitamin D[178–181].

Despite the acknowledge about the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) and aspirin in the GIT, there is no strong evidences related to their ability to trigger the development or to induce a relapse of IBD. A study performed by Ananthakrishnan and collaborators showed in high doses, long-term and frequent uses of NSAIDs has direct effect in the risk to develop UC and CD, but the same result wasn't showed for aspirin[182]. The mechanism is still elusive, but we can assume that NSAIDs are able to disturb the epithelial barrier or dysbalance the immune response, increasing the chance to develop the diseases or the get a relapse. Regarding the antibiotics, another study has shown that their use has important influence in the risk to IBD because of their effect in the microbiota[183]. The inappropriate use of antibiotics during childhood is even more relevant because the microbiota is not really stablished yet. In this cases, there is a strong evidence associated with the use of antibiotics and the impact on the

intestinal microbiota and consequently to the development of IBD and other intestinal inflammations[29,90,104,184].

Some pathogenic infection has been observed previously the development of IBD, for example, patients shows predisposition to IBD after an infection with *Yersinia*[185,186]. This characteristic is due to a niche competition and subsequent dysbiosis. Another example, several strains of *E. coli* are innocuous, but they can turn in pathogenic because of the presence of mobile genetic elements. Adherent Invasive *E. coli* (AIEC) have the capacity to survive and multiply in phagosome after binding to the epithelium. Furthermore, they are phagocyted or enter directly to the cells and, finally, triggers Th1 answer. This pathogenic mechanism and immune response can provoke the process of IBD development. *E. coli* has also been observed in granulomas of over of 80% of CD patients[187,188].

Stress is another factor associated with the pathogenesis of UC and CD. Patients with anxiety and depression might be strongly affected in IBD, while individuals with low level of stress present reduced risk to develop the disease[94,189–194]. The industrialization promoted the increase of air pollution and in parallel raised evidences that it might contribute to the risk of UC and CD because of the elevated levels of NO_2, SO_2 and other particles. One study suggested that the air pollution might influence UC and CD by the association between the emission of total pollutant and the level of hospitalizations for both diseases[94,195–197].

1.4.3 Treatment

Nowadays the treatment accessible for IBD is based on the administration of immunosuppressive and/or anti-inflammatory drugs, different classes of antibiotics or even surgery. The corticoids are the immunosuppressives the most used, but at long time they can induce several side effects because they are derived from cortisol, a hormone implicated in numerous metabolic functions in the host. Those side effects include mood changes, headache, hyperglycemia, vomiting and weight gain. Besides that, those drugs turn the patient more vulnerable to get infections by the fact that they endanger the immune system of the host [195,198,199].

Aminosalicylates are extensively used for IBD, particularly for CD. Those drugs are able to suppress the production of pro-inflammatory chemokines and reduce the process of inflammation through the remission. However, aminossalicyates are involved in side effects as well, such as abdominal pain, headache, anemia, pancreatitis and hepatitis, they also disturb the absorption of folic acid[198,200].

Antibiotics can be used in the treatment for some complications in IBD, like fistulas, abscesses and infections by intestinal pathogens. Ciprofloxacin and metronidazole are broad spectrum antibiotics against Gram-negative and Gram-positive bacteria that are commonly used in the treatment of IBD. Besides their use in the clinical practice, there is controversies about the efficiency to reduce the general symptoms and eradicate dysbiosis because some weeks after the end of the treatment there is a return of the IBD signs[131,201,202].

In the worst case, a chirurgical intervention is necessary to remove part of the colon and rectum. However, even after the surgery it is necessary to continue with the drugs to avoid the return of symptoms. Nevertheless, a high number of patients present a relapse even after surgery and drugs treatment[63].

The anti-TNF monoclonal antibodies (infliximab, adalimumab and certolizumab) are effective mediators for the treatment of immune-driven disorders, such as inflammatory bowel disease (IBD). Nevertheless, there is the occurrence of failures and the needless prolongation of anti-TNF affects patients' quality of life and enforce adverse effects' risk without clinical justification[203].

Regarding the fact that the recent treatments have no complete effectiveness and present several critical side effects, it is essential to find new approaches to treat the patients with more safety and strong results[195,204,205].

As formerly pronounced, there is a loss of microbiota diversity in patients with IBD. Considering that, in order to reverse this microbiota issue, an innovative therapy used has been the fecal material transplant, which consists in the implantation of the microbiota from a healthy patient to an IBD patient. This

approach presented success against infection by *Chlostridium difficile*. For IBD, the new technique has shown promising results with decrease of symptoms, disease remission and allowing patients to stop the medication. Nevertheless, studies in large scale must be done to prove the causality of cure after fecal material transplant[206–208].

2 PROBIOTICS

The definition of probiotics is “live microorganisms that when administered in adequate amounts confer a health benefit on the host”[115,209–211]. Nevertheless, some studies has shown that dead microorganisms and bacterial DNA can also demonstrate positive effect on health[209]. The first association between probiotics and human health was made by Élie Metchnikoff in 1907, whom observed that people from Bulgarian villages had health and longevity improved by the ingestion of fermented dairy products, such as yogurt[115].

“Probiotic” derivates from *pro bios*, that in Greek means “for life”. In antique, people were already conscious about the beneficial effect provided by the consumption of fermented foods. At those times, illness such as atherosclerosis, gastrointestinal disorders, and liver diseases were treated with fermented dairy products, being considered as an exceptional medication in those cases[209,212].

In 1954, Ferdinand Vergina wrote a study showing the hostile effect in the intestinal microbiota of the use of antimicrobial preparations, such as antibiotics, and a positive effect of the use of some bacteria, described in the paper as “probiotika”[209].

The main benefits of the probiotics are the upgrade of host defense and modulation of host immunity. But to be considered as probiotic, a microorganism has to follow some criteria: I) to be able to survive the transit through the GIT; II) to be nonpathogenic; III) to have a real beneficial effect on the host[115,213].

The dose of probiotic that should be administered to confer real beneficial effect on the host is strain dependent and is influenced by the type of the product. Overall, a minimum quantity of viable cells should be available in the dose and the efficacy should be proved by clinical trials. In general, the dose should have something between 10^6 and 10^8 colony-forming units per gram (CFU/g). In Brazil this number is considered from 10^8 up to 10^9 CFU/daily. In Canada and Italy the dose is 10^9 CFU/daily[211,214].

Other important factor to be considered is the protocol of probiotic administration. For this, some aspects should be analyzed: I) the daily frequency, for example, 1, 2, 3 of 4 times per day. II) The time of administration, for example before, after or even during a meal. III) The period of administration for example, days, weeks or months. IV) The choice of vehicle of delivery, for example, capsule, powder, food, drink, and so on. And, finally V) The stability and viability of the probiotic strain[211].

Lactic Acid Bacteria (LAB) group are the biggest group of probiotics, followed by other species, such as *Bifidobacterium* sp., the yeast *Saccharomyces boulardii* and one strain of the Gram-negative bacterium *Escherichia coli,* Nissle 1917[115,215]. The identification of probiotics strains is necessary to perform screening experiments to recognize those ones that have immunomodulatory properties[115,216]. A clinical trial with severe acute pancreatitis patients treated with a multispecies probiotic preparation showed an increased risk of mortality[217]. This kind of results show us the importance to choose not just a probiotic but also to select the right protocol of administration and the right quantity of probiotics to be administered to the patient[218,219].

Different kinds of diseases are associated with dysbiosis. Regarding this, a good strategy to reestablish the health and/or to avoid a healthy individual to develop dysbiosis could be the use of beneficial microorganisms to bring back the normal ecosystem. The use of probiotics as an efficient therapy has been demonstrated in irritable bowel syndrome (IBS), peptic ulcers, traveler's diarrhea, allergy and autoimmune disorders[220–225].

Intestinal disorders, like IBS, have benefited of the use of probiotics. IBS is characterized by bloating, discomfort, alteration of bowel habits and abdominal pain. *Lactobacillus* spp., *Bifidobacterium* and *Streptococcus* presented optimistic results in human patients with IBS in many studies[115,226]. *Bifidobacterium infantis* 35,624 strain was found to reduce of 20% the symptoms of IBS when compared with placebo group[227].

A study performed in Lebanon and France demonstrated that children with acute diarrhea were beneficiated with the supplementation of the milk with *Saccharomyces boulardii*. The children presented the restoration of the weight and a decrease in the diarrhea duration when compared with those who received the regular milk[209]. The addition of *Bifdobacterium lactis* and *Streptococcus thermophilus* in the powder milk was shown, by some authors, able to reduce the risk of infection by rotavirus and reduction of nosocomial diarrhea frequency[228].

VSL#3 is a probiotic preparation with 8 microorganisms: *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delrueckii subs. bulgaricus*, *Lactobacillus plantarum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis* and *Streptococcus salivarius subs. Thermophilus*. This preparation was tested in pediatric patients with UC and it was detected a great reduction in the symptoms recurrence when compared with the placebo group. While in adults, it was able to induce the remission in almost half of the patients, while in control group it happened only in 16% of them. In children, the induction of remission was even more expressive, reaching 92,8% of the patients[229,230].

E. coli Nissle 1917 (EcN1917) was isolated during an epidemy of *Shigella* infection in the First World War from the feces of a soldier that did not develop diarrhea[231]. The strain demonstrated positive results in clinical trials for UC treatment. The strain has the efficacy compared with mesalazine, considered the standard anti-inflammatory drug against the disease, with protection around 65% for both strategies after a year of treatment[209,232]. EcN1917 was tested in IBS patients and ameliorated the symptoms in 20% of the cases when administered for a long-term, compared with placebo group[233].

3 LACTIC ACID BACTERIA (LAB)

Louis Pasteur, in 1857, showed the presence of microorganisms capable to ferment milk. In 1873, Joseph Lister, following the antiseptic strategies published by Pasteur was able to isolate a pure culture of lactic acid bacteria, *Bacterium lactis*. Those bacteria can produce lactic acid after the fermentation of saccharides and are resistant to low pH and to a large range of temperatures. They can be found in the mouth, GIT and genital tract of animals, including humans [234–236]. LAB are living cells, prokaryote, Gram-positive bacteria, rods or cocci, acid-tolerant, non-sporulating and require complex organic molecules as an energy source[237].

Most part of the LAB is component of the phylum Firmicutes, a complex group of bacteria with low G+C content in its genomes. It includes the genera: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. There is a controversy around the genus *Bifidobacterium*. Many authors considerer it inside the LAB group mainly because the genus is also able to produce lactic acid as a product of the fermentation process. Nevertheless, the genus is part of the phylum Actinobacteria, which has high G+C content in its genomes and the process of carbohydrate fermentation is distinct from the phylum Firmicutes[211].

LAB are largely used in industrial process such as preservation and production of fermented food. Those bacteria have GRAS status (Generally Recognized As Safe) granted by the FDA (Food and Drug Administration), being thus considered safe for human consumption. They also present the status of Qualified Presumption of Safety (QPS) according to the European Food Safety Authority (EFSA). This safety has been proved by history of consumption and scientific evidences. The risk of infection with those bacteria is insignificant, but even with this proved safety it is necessary to use them with caution in immunocompromised patients, preterm infants and patients critically ill in intensive care[115,209,211,238]. Some LAB are also considered as opportunist

pathogens, such as *Streptococcus mutans*, an important agent on dental carries formation[239,240]. LAB can also be observed in mammalian microbiota. Lactobacilli and streptococci can be largely found in human ileum and jejunum[241].

Lactobacillus rhamnosus GG (LGG) shows positive effect on atopic eczema in children after perinatal administration and probably because of its anti-inflammatory properties. LGG has also shown the ability to induce the increase of IL-10 production in children with atopic dermatitis[115,242–246]. In a study with 500 children of age between 3 months and 3 years old, using different probiotics, LGG showed a reduction of the diarrhea period[247]. However, LGG wasn't able to show positive results in patients with CD in a clinical trial. On year after surgery, CD patients received the probiotic and the recurrence of the symptoms were 6% higher in the treated group than in the placebo group. In the endoscopic recurrence, it was 25% higher in the probiotic group compared with placebo group[248]. In a pediatric study, CD patients received LGG and presented remission for 9.8 months and relapse in 31% patients. In placebo group, the remission was 11 months and recurrence in 17% of the patients[249]. LGG was also tested for UC treatment and its efficacy was compared with mesalazine effect during 6 and 12 months. It was evaluated the capacity to maintain the remission. LGG demonstrated better effect than mesalazine in both period of treatment. The combination of both treatments presented better results in 6 months when compared with isolated ones. With 12 months the combination of both presented similar results to treatment with only LGG and superior to only mesalazine[250,251].

Lactobacillus johnsonii was tested in CD patients submitted to surgery during 6 months after the procedure and presented 51% of remission against 36% in the placebo group[249]. *Lactobacillus plantarum* 299v (Lp 299 v) was administered every day for four weeks in patients with IBS and was able to reduce the symptoms in a significant way[209].

Lacteol (Lacteol Fort, Rameda, Egypt) is a probiotic composed by two distinct species of *Lactobacillus* (*L. delbrueckii* and *L. fermentum*). In study with daily administration of Lacteol together with sulfasalazine during 8 weeks in patients with UC it was observed the alleviation of the inflammation and symptoms[250].

When administered to mice, *Lactobacillus casei* Shirota strain showed an inhibition of IgE production and a study with children with eczema treated with *Lactobacillus reuteri* ATCC 55730 strain also presented a reduction of IgE[252,253]. In a murine model of acute colitis induced by DSS it was showed an anti-inflammatory activity of *L. casei* BL23 strain and another using *L. reuteri*[50,221,232,254].

L. reuteri ATCC 55730 was tested in pediatric patients with UC. After intra-rectal administration for 8 weeks concomitant with mesalazine treatment, 100% presented positive clinical results against 53% on placebo group. Remission was observed in 31% of the children in *L. reuteri* group and none in placebo group. In the *L. reuteri* group it was observed a decrease in the expression of IL-1β, TNF-α, and IL-8 and an increase of IL-10[255].

A study evaluated the effect of *L. casei* DG in UC patients after oral and/or rectal administration for 8 weeks concomitant with oral 5-ASA. It was found a decrease in the scores of histological disease severity for both rectal and oral administration when compared with the group that only received mesalazine. The rectal administration induced the increased of *Lactobacillus* and decrease of Enterobacteriaceae cultured from biopsy. The same alterations weren't observed in the oral administration group. The rectal administration also induced the mucosal increase of IL-10 and decrease of IL-1β[256].

So far, we described several works proving the anti-inflammatory properties and the wide range of probiotics application. Some mechanisms of action of these probiotics have been reported, and others are still not clear. Further studies are necessary to contribute and improve these missing information.

4 ANTIMICROBIAL PEPTIDES

In higher organisms, antimicrobial peptides are part of the first line of defense against pathogens, while in microorganisms they are used in competition for nutrient resources[257]. They are an efficient mechanism of immune defense to quickly inactivate or kill microorganisms. In higher organisms, tissues such as skin, respiratory tract and intestine are the most important producers of AMPs because those epithelial surfaces have constantly contact with the environment and frequently meets microorganisms that can be source of illness. Besides that, the large quantity of microorganisms in the mammals' intestinal microbiota is also constantly risk to the integrity of the tissue barrier. Therefore, the large production of AMPs is important to fight against the invasion of potential pathogens and for the maintenance of homeostasis in the tissues[258].

Antimicrobial peptides can be arranged into different groups based on their length, sequence or structure. The rising number of identified antimicrobial peptides exceeds 2700[257]. AMPs can present different secondary structures and contain a substantial fraction of hydrophobic residues[259]. Those peptides have selective properties causing disruption in pathogens membrane, such as bacteria, but limited injury to the membranes of human cells. This selectivity is based in the difference of the composition of cell membranes (bacteria *versus* mammalian) contributing to the peptide binding and membrane destabilization[259–263].

The general mechanism of action of the AMPs is based on the attack against the bacteria cell wall, which is composed by membrane, peptidoglycan layer and the outer membrane in Gram-negative bacteria. This mechanism reduces the probability of the bacteria to create alternatives to avoid the AMPs action, since modifications in the cell wall structures directly affects the global fitness of the bacteria[258]. Regarding this, AMPs have being considered in the treatment for infections by microorganisms resistant to antibiotics[264].

The three main types of AMPs (defensins, lectins, and cathelicidins) are able to bind to bacterial membrane and then use of different strategies to disturb the membrane integrity (Fig 6). α-defesin is expressed as an inactive molecule. This pro-peptide needs to be activated by trypsin in humans system and then the active molecule forms an dimer pore stabilized by electrostatic interactions between defensin and the bacterial membrane. The C-type lectins of the REG3 family are also synthetized as a pro-peptide which needs to be activated by the proteolytic action of trypsin. After to bind to the peptidoglycan of Gram-positive bacteria, the active molecule forms an hexameric pore in the bacterial membrane and is also stabilized by electrostatic interactions between REG3 and membrane. The cathelicidins, like LL-37, is synthetized as a disordered peptide, which gets an α-helical structure after bind to the lipids of the membrane via electrostatic interactions. First the α-helix structure binds in a parallel position to the membrane and then gets inside the lipid bilayer to form the linear pore[258].

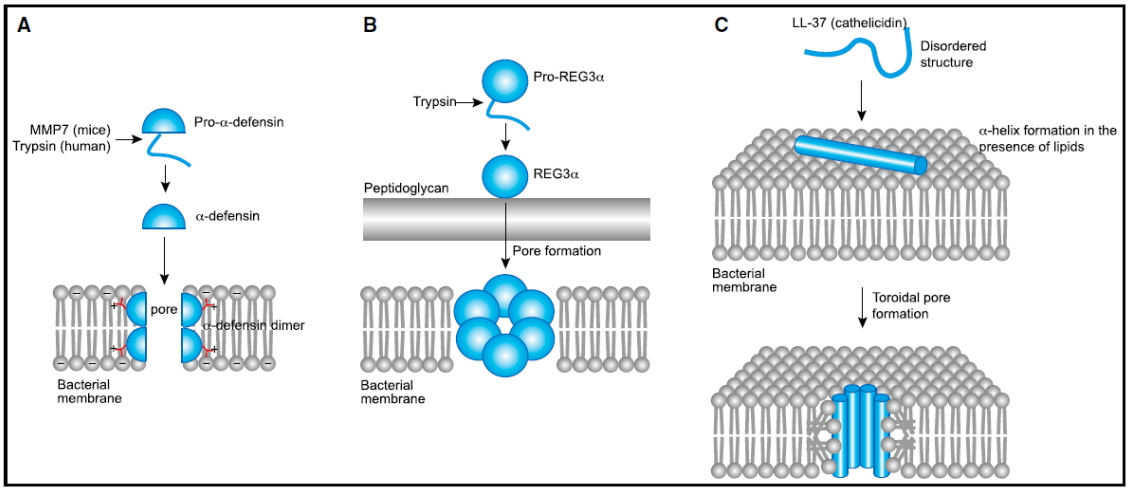

Fig 6. Models of Bacterial Membrane Permeabilization by Key Intestinal AMPs[258].

There are evidences concerning the intracellular targets of the antimicrobial peptides. They can act by the inhibition of DNA, RNA and protein synthesis, inhibition of enzymatic activity, inhibition of cell-wall synthesis, activation of autolysin, etc. (Fig 7)[259].

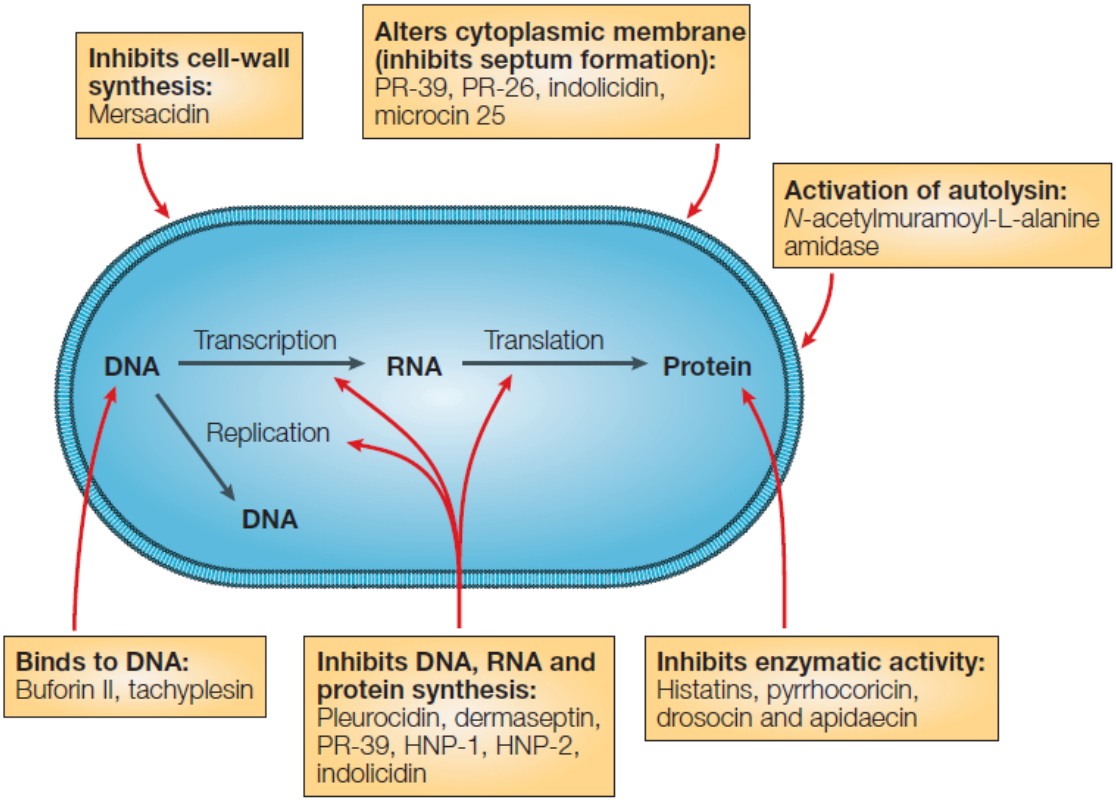

Fig 7. Mode of action for intracellular antimicrobial peptide activity[259].

The contact of the host mucosa with the enteric microbiota may induce the colitis development and this contact can be reduced by the production of AMPs, since those peptides are involved in the maintenance of intestinal barrier. AMPs are not only involved on the inhibition of pathogenic bacteria but evenly on immune responses activation. Another important role of AMPs is its application as a biomarker for some illnesses since their expression can be increased or decreased in some inflammatory processes and infections[264].

Regarding Reg gene family encodes a diverse group of proteins called C-type lectins with carbohydrate recognition domain (CRD). Those peptides present an average weight of 16 kDa, a N-terminal secretion signal and are divided into four subgroups (I, II, III and IV). The members of this group are mainly expressed in the small intestine. Mouse regenerating islet-derived protein 3γ (RegIIIγ) and human hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP) are two important homologous AMPs representatives of the C-type lectin family. RegIIIγ was found to be increased in conventional mice when

compared with germ-free mice and an inflammation caused by a mucosal damage can also increase this expression[265].

RegIIIγ was first isolated in rat pancreatic juice in the acute phase of pancreatitis and represented up to 5% of total protein. The human ortholog, RegIIIα (or PAP), was identified from the pancreatic juice of diabetic patients and reached up to 7.5% of the total secretory protein[266–268]. Despite their initial association with pancreas, most Reg proteins are expressed in multiple organs such as liver, lung and intestines, and are detected under normal and pathological conditions[268–270]. This peptide plays a protective effect, such as anti-inflammatory properties able to reduce the severity of colitis, preserving gut barrier and epithelial inflammation. PAP is mainly synthesized by goblet cells and enterocytes in the colon and in the small intestine by metaplasic Paneth cells located in the crypt[265,271–273] and secreted into the intestinal lumen where it will limit the contact between intestinal bacteria, resident microbes, and mucosal surface[258]. Moreover, intraepithelial lymphocytes (γδ IEL) have been evocated due to the important contribution on PAP expression and its participation on mucosal healing[274]. Several works demonstrated the expression of RegIIIγ in the intestine correlated with the richness of microbiota composition. They observed low expression of RegIIIγ in germ-free mice, but markedly increases after bacterial colonization[258,272,274]. PAP expressed by intraepithelial lymphocytes (IEL) and epithelial cells (IEC) also requires cytokine signals from Innate Lymphocyte Cells (ILC) subsets. One of them, the ILC3, produces IL22, which binds to IL22R (receptor) on epithelial cells and modulates epithelial function and AMP production, such as RegIIIγ, warranting the intestinal epithelial homeostasis[50,272]. This AMP is up-regulated in patients with inflammatory bowel disease[266,268,269,275,276]. PAP has a variety of activities, which includes anti-apoptotic, anti-inflammatory, antibacterial effects and proliferative, maintaining host-bacterial homeostasis in the mammalian gut.[267,275]. Regarding the intestinal homeostasis and PAP, recent work has showed the transgenic mice expressing PAP in pancreas were more resistant to develop colitis. Those mice presented microbiota diversity able to drive an anti-inflammatory environment ensuring the epithelial integrity and function[277]. Moreover, the bactericidal effect of PAP is contradictory, even many studies exert a direct bactericidal effect as a result of the capacity to bind to the

peptidoglycan layer of Gram positive bacteria even at low micromolar concentrations[267,275,276].

III AIMS OF THE STUDY

IV. 1 Main aim of the study

The main objective of this work is to study the PAP molecule in the treatment of intestinal inflammatory diseases through a model of acute colitis induced by DNBS.

IV. 2 Specific aims of the study

1. To evaluate if PAP expressed by *Lactococcus lactis* is able to protect mice against inflammation in DNBS-induced colitis model
2. To evaluate if PAP expressed by *Lactococcus lactis* is able to modulate the composition of the microbiota
3. To establish an efficient protocol to extract proteins from the pellet of *Lactobacillus casei* culture.
4. To establish an efficient protocol to induce the expression of PAP by the strain of *Lactobacillus casei* under the control of the NICE system.
5. To induce a DNBS-induced colitis model in mice and evaluate the protection against the inflammation when mice received daily treatment with *Lactococcus lactis* expressing or not PAP and *Lactobacillus casei* expressing or not PAP.
6. To induce a DNBS-induced colitis model in mice and evaluate the protection against the inflammation when mice received every 3 days treatment with *Lactococcus lactis* expressing or not PAP and *Lactobacillus casei* expressing or not PAP.
7. To induce a DNBS-induced colitis model and to perform the treatment of the mice with daily oral administration of *Lactococcus lactis* harboring a plasmid for eukaryotic expression of PAP;
8. To evaluate if the strain of *Lactococcus latics* harboring PAP cDNA was able to reduce the weight loss and the macroscopic score 4 days after the induction of inflammation;
9. To evaluate the immune response profile after the administration of *Lactococcus latics* harboring PAP cDNA 4 days after the induction of inflammation.

V CHAPTER 1

Oral delivery of Pancreatitis-Associated Protein (PAP) by *Lactococcus lactis* displays protective effects in DNBS-induced colitis model and is able to modulate the composition of the microbiota

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1 GENERAL BACKGROUND AND STORY OF THE PROJECT

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) belongs to Regenerating islet-derived III (RegIII) proteins family, is a C-type (Ca^{+2} dependent) lectin which binds selectively to specific carbohydrate structure of bacteria. PAP protein plays a protective effect presenting anti-inflammatory properties able to reduce the severity of colitis, preserving gut barrier and epithelial inflammation. Here, we sought to determine whether PAP delivered at intestinal membrane by recombinant *Lactococcus lactis* strain (LL-PAP) is able to reduce the severity of colitis chemically-induced. After construction and characterization of our

recombinant strains we tested their effects in DiNitro-BenzeneSulfonic-acid (DNBS) and Dextran Sulfate Sodium (DSS) colitis model. After DNBS challenge, mice treated with LL-PAP presented less severe colitis compared to PBS and LL-treated mice groups. Those mice showed protection against weight loss, lower epithelial damage (macroscopical and histological scores), down-regulation of pro-inflammatory cytokines secreted by lymphocytes in Mesenteric Lymph Node and colon and increase of butyrate producers members in microbiota. After DSS challenge no protective effects of our strain could be detected. We determined that after 5 days of administration LL-PAP increase butyrate producers bacteria especially *Eubacterium plexicaudatum*. Based on our findings, we hypothesize that a treatment with LL-PAP shifts the microbiota preventing the severity of colon inflammation in acute colitis model through intestinal microbiota modulation.

2 ACTORS IMPLIED IN THE PROJECT

We thank CAPES-Cofecub, CNPq for funding (NMB, PBVB, RDC). We thanks the members of Animal Facility (INRA, Jouy en Josas, France): Jérôme Pottier, Charline Pontlevoy, Marlène Héry ; Mathilde Bauducel and Andre Tiffoche. NMB and JMC designed the paper. NMB and PBVB performed the animal experiments, analyzed them and wrote the manuscript. We are grateful for the important help gave to us from MSA, MLM concerning immunology experiments and GF concerning to sequencing data analysis. We also thank C. Aubry, R. Martin, B. Lamas, G. da Costa, M.L. Richard, E. Jacouton, C. Michon, C. Severiano, D. Rama, T. Alain, J. Natividad, and S. Le Guin for fruitful discussion and technical help. We thank HS to microscopic score analyzes. We should mention the important help on the development of this manuscript from RDC, LGB, PL, HS, FC, TR, AM, VAA and PL. Funding was provided by Probihôte team (JMC and PL), Capes Cofecub (Brazil), Science without borders (Ciências sem Fronteiras), CNPq-Brazil.

3 GOALS

3.1 General goals

To study the efficiency of a recombinant strain of *Lactococcus lactis* expressing PAP in a colitis model induced by DNBS to protect the mice when compared with the controls groups.

3.2 Specific goals

a) To evaluate if PAP expressed by *Lactococcus lactis* is able to protect mice against inflammation in DNBS-induced colitis model
b) To evaluate if PAP expressed by *Lactococcus lactis* is able to modulate the composition of the microbiota

4 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 remit to condition of long-term morbidity. At the present day, 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 new tools are still required [2-5].

Therapeutic proteins are gaining increased popularity, owing to drug-drug interactions high activity and specificity, low toxicity and minimal nonspecific [6]. Antimicrobial peptides (AMPs) secreted by intestinal immune, epithelial cells, and lymphocytes are an important target [7-9]. They belong to the important effectors of innate immunity compartment, serving as a first line of the defense against pathogens. AMPs are key regulators in the host-microbiota relationships by restricting contact between commensal bacteria and epithelial surface. Consequently, they maintain the balance of the commensal bacteria community [9, 10].

Pancreatitis-associated protein (PAP) belongs to the REG gene family. PAP was first found in regenerating pancreatitis islets in rat encoding a small group of proteins involved in the control of epithelial cell proliferation and wound healing in various organs, included pancreas and intestine [7, 11-14]. This protein is characterized as C-type lectin able to bind selectively to carbohydrate structure, often in a Ca^{+2} dependent manners [10]. PAP kills bacteria through non-enzymatic mechanism of cell-wall attack being able to disrupt bacterial membranes charged negatively [15]. Inactive pro-RegIIIα/γ is converted to active form by trypsin-dependent proteolytic processing. RegIIIα kills gram-positive bacteria by first binding to peptidoglycan, then oligomerizing to form a hexameric membrane-penetrating pore that is stabilized by electrostatic interactions between RegIIIα cationic residues and the anionic phospholipids of the bacterial membrane [15, 16]. Therefore, PAP may be able to alter microbiota community.

PAP is mainly synthesized by goblet cells and enterocytes in the colon and in the small intestine by metaplasic Paneth cells located in the crypt [9, 10, 17, 18] and secreted into the intestinal lumen where it will limit the contact between intestinal bacteria, resident microbes, and mucosal surface [19]. Moreover, intraepithelial lymphocytes (γδ IEL) have been evocated due to the important contribution on PAP expression and its participation on mucosal healing [8]. Several works demonstrated the expression of RegIIIγ in the intestine correlated with the richness of microbiota composition. They observed low expression of RegIIIγ in germ-free mice, but markedly increases after bacterial colonization [8-10, 19]. PAP expressed by intraepithelial lymphocytes (IEL) and epithelial cells (IEC) also

requires cytokine signals from Innate Lymphocyte Cells (ILC) subsets. One of them, the ILC3, produces IL22, which binds to IL22R (receptor) on epithelial cells and modulates epithelial function and AMP production, such as RegIIIγ, warranting the intestinal epithelial homeostasis [9, 20].

Regarding the intestinal homeostasis and PAP, recent work has showed the transgenic mice expressing PAP in pancreas were more resistant to develop colitis. Those mice presented microbiota diversity able to drive an anti-inflammatory environment ensuring the epithelial integrity and function [21]. In counterpart, several works showed the use of living genetically engineered strains of the food-grade bacterium *Lactococcus lactis* delivering therapeutic molecules *in situ* as being promising to treat different human diseases as allergy [22, 23], cancer [24], obesity [25] or IBD [26-28]. Therefore, we hypothesized that exogenous PAP delivered by recombinant *L. lactis* might shape the intestinal microbiota and thus act against inflammatory process taking place in IBD. It may be useful as intervention approach to maintain the intestinal homeostasis or prevent the intestinal dysbiosis caused by genetic predisposition to IBD.

5 MATERIALS AND METHODS

5.1 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 [50] 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 [51] 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 [52] to obtain the strain LL-PAP. pSEC:PAP was also introduced into NZ9000htrA- [53]. 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.

5.2 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 [51, 54].

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.

5.3 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 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.

5.4 Induction of acute colitis and bacteria administration

The protocol of DNBS-induced acute colitis is detailed in Fig 3A. 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 11 days. Weight loss was monitored daily to assess the severity of colitis. Inflammation was monitored 4 days after DNBS administration by cytokine productions.

The protocol of DSS-induced acute colitis is detailed in Fig 4A. Briefly, at D0 colitis was induced by adding 2.5 % (w/v) of Dextran Sulfate Sodium Salt (DSS) at a molecular weight of 36,000–50,000 (MPBio) to the drinking water for 7 days. The mice were sacrificed at D12 (DSS recovery) after the DSS induction. For the recovery phase, DSS colitis induction was followed by 5 days of recovery with normal drinking water. As a control, mice have been fed during 12 days without DSS induction. Mice were monitored daily for weight loss (Fig.4C), fecal occult blood (Hemoccult, Beckman Coulter), and stool consistence. Disease Activity Index (DAI – Fig. 4B) has been calculated according to the protocol established by Cooper et al, 1993. Mice have been sacrificed by cervical dislocation and mesenteric lymphatic node (MLN) as well as colon have been harvested for colon washes, protein extraction and histological assessment.

5.5 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 [29, 30]. Briefly, the macroscopic criteria (assessed on a scale from 0 to 5) 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.

5.6 Histological assessment

For histological assessment, a colon sample was fixed in 4 % paraformaldehyde acid (sigma) and embedded in paraffin. Four micrometer sections were stained with hematoxylin/eosin and examined blindly [55].

5.7 Cytokine assays

Mesenteric Lymph Nodes (MLN) cells and spleen cells were isolated from mice and cultured in RPMI culture medium (Lonza) with 100 Unit of Streptomycin, Penicillin (PAA Laboratories) and 10% SVF (Lonza) at $2x10^6$ cells per well. Cells were re-activated with 4μg/μL pre-coated anti-mouse antibody CD3e and CD28 (eBioscience). Concentrations of cytokines IL-12, IL-17, IL-4, TSLP, and INF-γ (Mabtech) and TGF-β (R&D), in medium were assessed by ELISA after 48h of incubation.

One centimeter of colonic tissue was weighing and mashed by Gentle MaxTM (Miltenyl Biotec) in 1mL of PBS plus anti-protease (Roche). The lysate was centrifuged and the supernatantas used to measure cytokine level by ELISA. The cytokines tested were IFNγ, IL12, IL4, IL17, TSLP (Mabtech), and TGF-β (R&D systems) and the concentration was normalized by mg of tissue.

5.8 *Lamina propria* isolation

Black C57BL/6 mice were administered with 109 CFU of LL , LL-PAP or PBS for seven days before DNBS challenge. After 4 days since the challenge, animals were euthanized, colon were recovered to perform lamina propria extraction. After cleaning the tissue, digestion using DNAse and Liberase (Roche) was performed during 30 minutes at 37°C with constant shaking. The digested tissue was mashed in a cell stainer (100µm) and collected in complete medium (RPMI sigma). After centrifugation, cells resuspended in Percoll 40% were underlaid on 3 mL of Percoll 80%, tubes were centrifuged during 20min, 600g (without break), and the ring formed in the middle of the two phases was collected into another tube. Cells were centrifuged, washed with complete RPMI medium and counted using a flow cytometer.

5.9 Treg cells population

Staining was performed according to manufacturer recommendations: around 106 cells/well were inserted in an opaque, white 96-Well Plate V format (Grener), centrifuged at 37°C for 2 minutes/2000 rpm. Supernatant was discarded, cells were washed with PBS before being incubated with anti-mouse CD3e APC-efluor 780, CD4 PE-Cy5 conjugated L3T4 and CD16/CD32 antibodies (diluted in PBS1x containing 2% FBS – PBS1XFluo). All antibodies are used at final concentration of 1µg/mL. Plate was incubated 20 minutes at 4°C protected from light and next centrifuged at 37°C for 2 minutes/2000 rpm. Supernatant was then discarded and incubated with PBS1XFluo, washed and resuspended with fixation/permeabilization solution. Cells were kept for 30 minutes at 4°C protected light, centrifuged, and incubated with anti-mouse/Rat Foxp3-FITC antibody for 30 minutes (prepared in permeabilization buffer). Cells were washed and resuspended with PBS for reading in the flow cytometer. Leukocytes were gated using forward scatter (FSC) and side scatter (SSC), and within the leukocyte gates, leucocytes were identified as Th cells ($CD3^+$, $CD4^+$) and the population of Treg was identified as $CD3^+CD4^+FoxP3^+$. CD16/CD32 are expressed in B cells, monocytes/macrophages, NK cells, granulocytes, mast cells, and dendritic cells and used as control.

5.10 Statistical Analysis

GraphPad software (GraphPad Sofware, 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.

5.11 Bioinformatics analysis

The assembled sequences were dereplicated and singletons were removed using the Vsearch tool using the “derep_fulllength” command. The dereplicated sequences were clustered into 99% identity groups to constitute the OTUs through the “cluster_fast” command. The initial reads were mapped to the constructed OTUs to quantify each Taxonomic Unit using the “usearch_global” Vsearch tool [10.7717/peerj.2584]. The taxonomic assignment was performed by the TAG.ME [10.1101/263293] R package using 515F-806R model.

Statistical analysis – The differential abundant OTUs were identified using the Deseq2 [10.1186/s13059-014-0550-8] R package with an adjusted pvalue threshold of 0.05. The Beta-Diversity visualization was performed through the Principal Coordinates Analysis using the Jensen-Shannon distance matrix.

6 RESULTS

6.1 Characterization of human PAP production by *Lactococcus lactis*.

PAP cDNA was inserted in pSEC or pCYT vectors, obtaining thus pSEC-PAP and pCYT-PAP (Table 1), in order to produce PAP secreted or cytoplasmic. Then pSEC-PAP and pCYT PAP were introduced in *L.lactis* strain NZ9000 where PAP expression was induced by nisin. We used then ELISA to test the ability of our

recombinant strains to produce and secrete human PAP. Highest PAP production was obtained with strains transformed with pSEC:PAP (Fig1). 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).

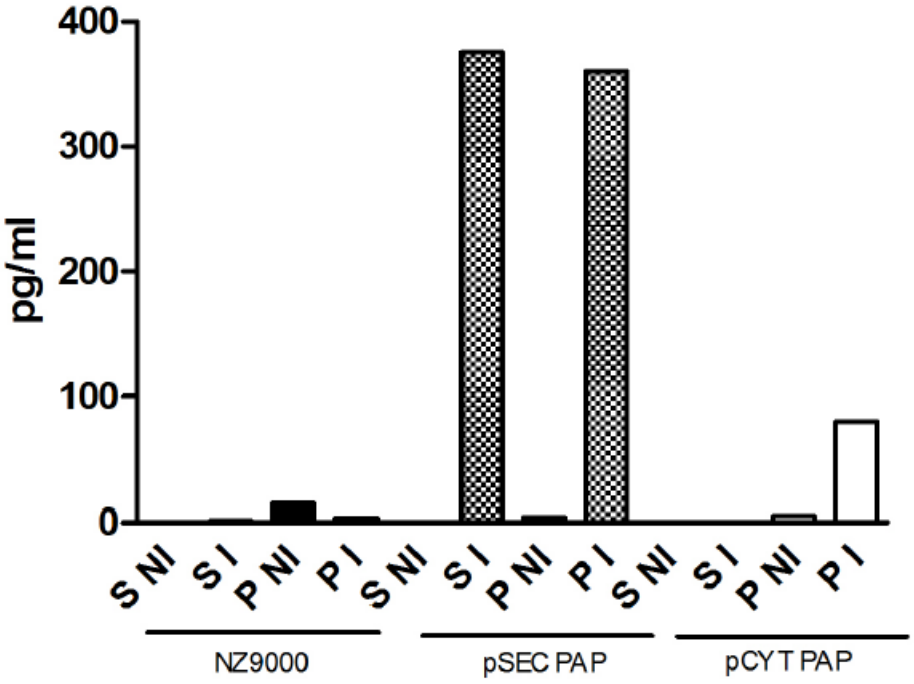

Fig 1. Characterization of human PAP production by *Lactococcus lactis*. PAP was identified in the pellet and supernatant of nisin-induced recombinant *L. lactis* PAP culture by ELISA. S NI = Supernatant from Non-Induced culture; S I = Supernatant from Induced culture; P NI = Pellet from Non-Induced culture; and P I = Pellet from Induced culture. NZ9000 : *L. lactis* control strain, containing the plasmid pNIS empty; pSECPAP : *L. lactis* strain secreting PAP; and pCYTPAP : *L. lactis* strain expressing PAP into the cytoplasm.

6.2 PAP shaped the intestinal microbiota after oral gavage

To assess the impact of LLPAP on gut microbiota, C57BL/6 mice were treated with LL-PAP during 7 days by oral gavage and LL-treated mice were used as a control. Fresh fecal samples were collected from each mouse on the 7th day and after DNBS challenge, and sent for 16S rRNA sequencing. The bar-coded sequencing provided 237,945 usable reads (6,012 operational taxonomic units [OTUs] with 99% identity threshold) from 10 fecal samples. Oral administration of

LL-PAP for 7 days remarkably shifted the overall structure of gut microbiota *in vivo*. The differential abundance test shows that 8 OTUs are different between LL and LL-PAP-treated (Fig 2A). The relative abundance of OTUs belonging to the families Lachnospiraceae and Ruminococcaceae, to the Ruminoclostridium genus, and specie *Eubacterium plexicaudatum* is highly increased in mice LL-PAP-treated compared to LL. In other hand, bacteria from the Genus *Clostridium* strict senso 1 and one OTU from the Lachnospiraceae NK4A136 group are moderately decreased in those mice (Fig2A). The boxplots showed an increasing of alpha-diversity into the LL-PAP-treated mice microbiota population compared to LL-treated mice (Fig2B).

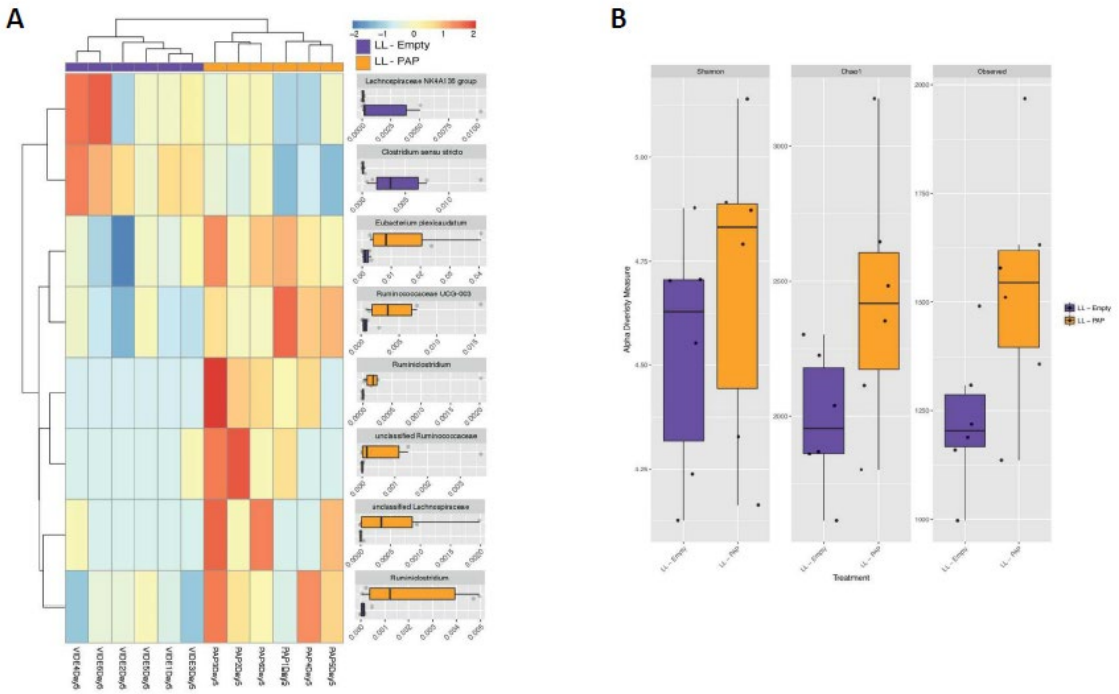

Fig 2. Intestinal microbiota after oral gavage with LL-PAP. (A) Comparison of the relative abundance of OTUs in LL and LL-PAP groups. (B) The α-diversity into the LL-PAP treated mice microbiota population compared to LL treated mice.

6.3 LL-PAP treatment reduces the severity of DNBS-induced acute colitis, but does not prevent damages in DSS-induced colitis

To validate the anti-inflammatory effects of LL-PAP *in vivo*, we used a well-established DNBS-induced colitis model [29-33]. The protocol used to develop

the murine model of DNBS-inflammation is detailed in Fig 3A. Briefly, conventional C57BL/6JRj mice, males with 6-week-old mice were orally administered with LL or LL-PAP during 7 days before and 4 days after intra-rectal injection of DNBS. Mice were sacrificed 4 days after DNBS injection. Animals administered with LL-PAP lost less weight than PBS- or LL-administered mice (Fig 3B). LL treated mice did not start to regain weight at D4 after DNBS even if the difference with the PBS group is not statistically significant. Permeability to FITC was significantly reduced when mice were treated with LL-PAP compared with LL (Fig 3C), showing an improvement of the intestinal permeability after DNBS in LL-PAP treated mice. Other parameters such as macroscopic and microscopic scores were reduced by ~75 and ~50 % respectively in LL-PAP group compared to PBS or LL group (Fig 3D, E). All parameters analyzed here showed LL-PAP mice developed a less severe colitis compared to LL- and PBS-treated mice.

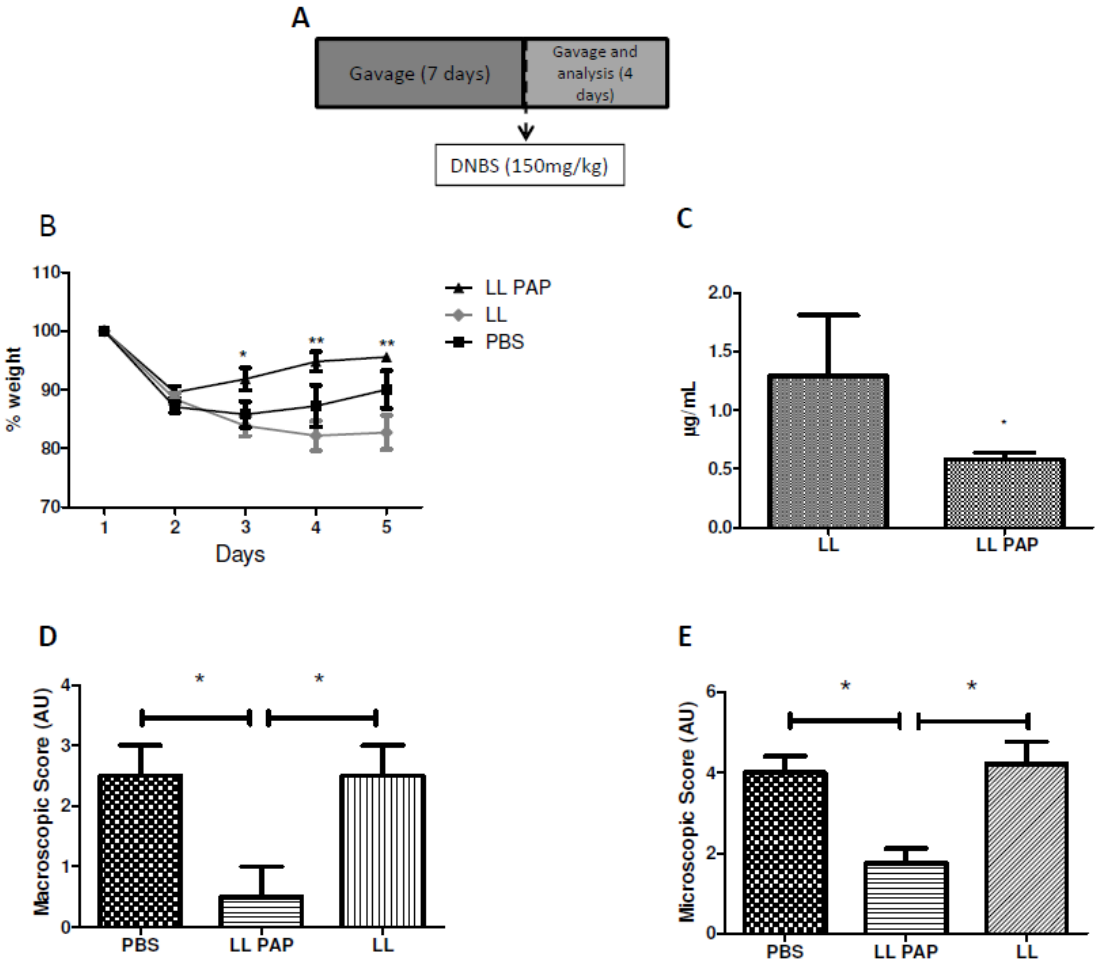

Fig 3. Effect of LL-PAP on DNBS-induced colitis. Mice were orally administered with LL or LL-PAP during 7 days before and 4 days after intra-rectal injection of DNBS. Mice were sacrificed 4 days after DNBS injection. (A) Experimental design. (B) Percentage of weight loss among the groups. (C) Intestinal permeability measured by the concentration of FITC present in the blood 4h after FITC oral administration. (D) Macroscopic score. (E) Microscopic score.

The protocol used to develop DSS-induced colitis model is detailed in Fig 4A. Shortly, mice were orally administered with LL or LL-PAP during all experiment long. After seven days, they received 2.5% DSS solution diluted in drink water *ad libitum*. The solution was changed each 3 days. After 7 days of DSS, mice were sacrificed. There is no difference in the weight loss, neither in the other parameters measured, such as consistence and presence of blood in the feces. All parameters analyzed here showed LL-PAP did not affect the severity of DSS colitis.

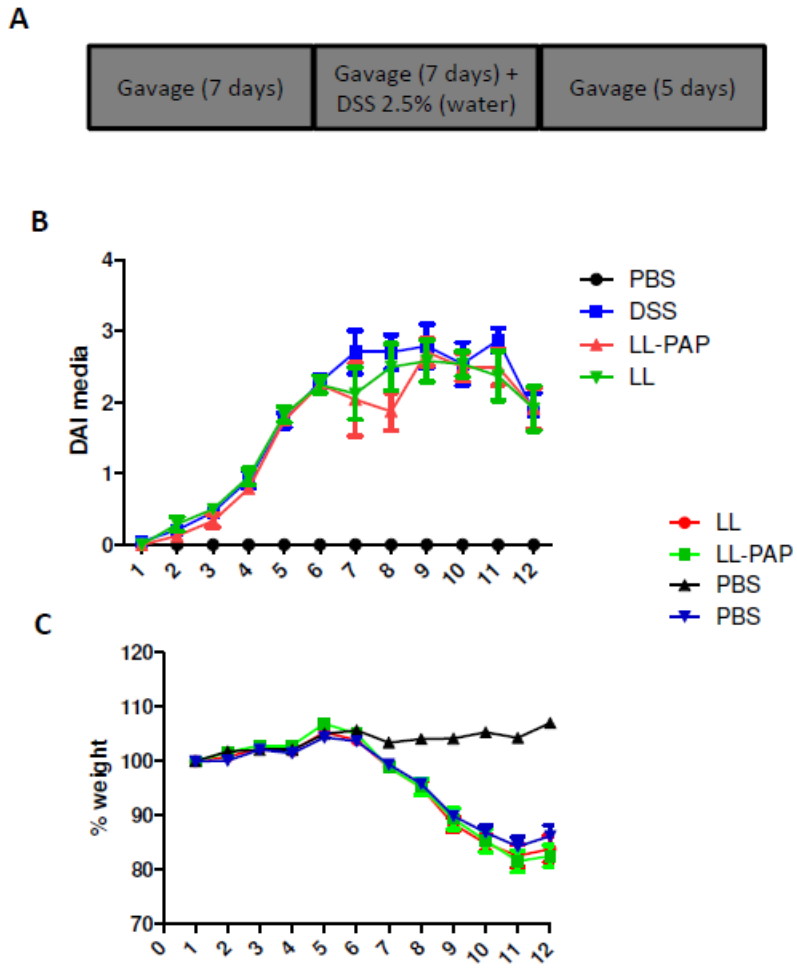

Fig 4. Effect of LL-PAP on DSS-induced colitis. Mice were orally administered with LL or LL-PAP during 7 days before and 7 days after DSS administration. Mice were sacrificed 5 days after DSS administration. (A) Experimental design. (B) Disease Activity Index. (C) Percentage of weight loss among the groups.

6.4 LL-PAP treatment is able to decrease the inflammatory immune response and increase TGF-β.

In order to know the effects of PAP delivered by *L. lactis* in mice inflamed with DNSB, amount of cytokines IFNγ, IL12p70, IL4 , TSLP, IL17 and TGF-□ were assayed in supernatant of activated lymphocytes isolated from MLN and protein extracts from colon tissue of those mice.

In MLN supernatant, Th1 cytokines (IL12 and IFN-γ) were decreased in LL-PAP-treated mice compared to LL-treated mice (Fig 5). While the anti-inflammatory cytokines, such as TGF-β and TSLP were expressed at higher level in LL-PAP treated mice. IL17 was reduced in LL-PAP treated mice compared to LL group. PBS has lower production, as expected. IL4 concentration was not different between groups (Fig.5).

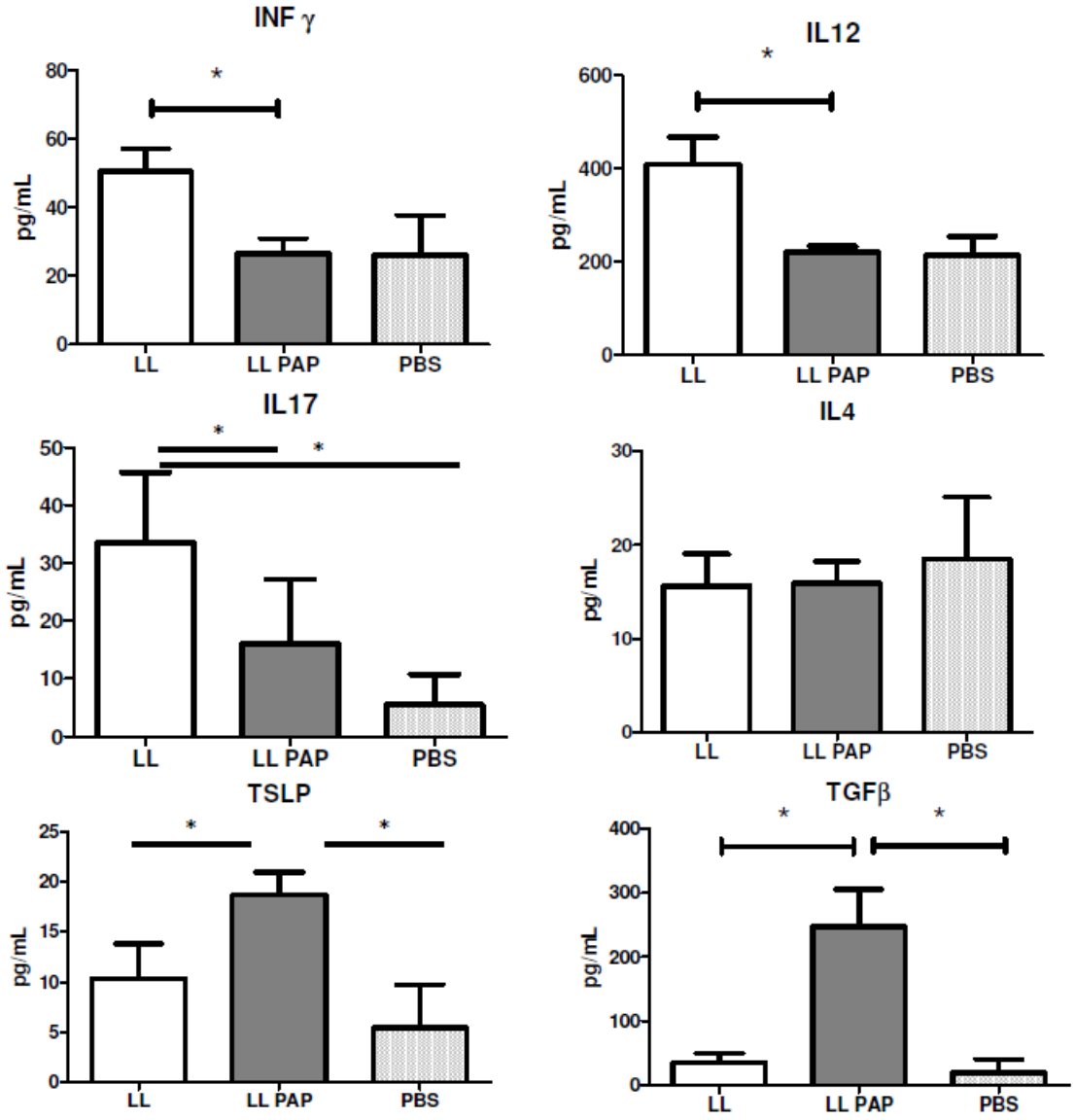

Fig 5. Cytokine production in mesenteric lymph nodes. Mice were orally administered with LL or LL-PAP during 7 days before and 4 days after intra-rectal injection of DNBS. Mice were sacrificed 4 days after DNBS injection. Cells were isolated from MLN and re-stimulated *in vitro* by anti-CD3 and anti-CD28 during 48h. Supernatants were recovered and cytokine measured using ELISA Kits.

In colon extracts, we observed a decrease of IL17 between LL- and LL-PAP-treated mice. Moreover, TSLP was increased in LL-PAP group compared to LL- and PBS-treated mice (Fig 6). No other differences could be described.

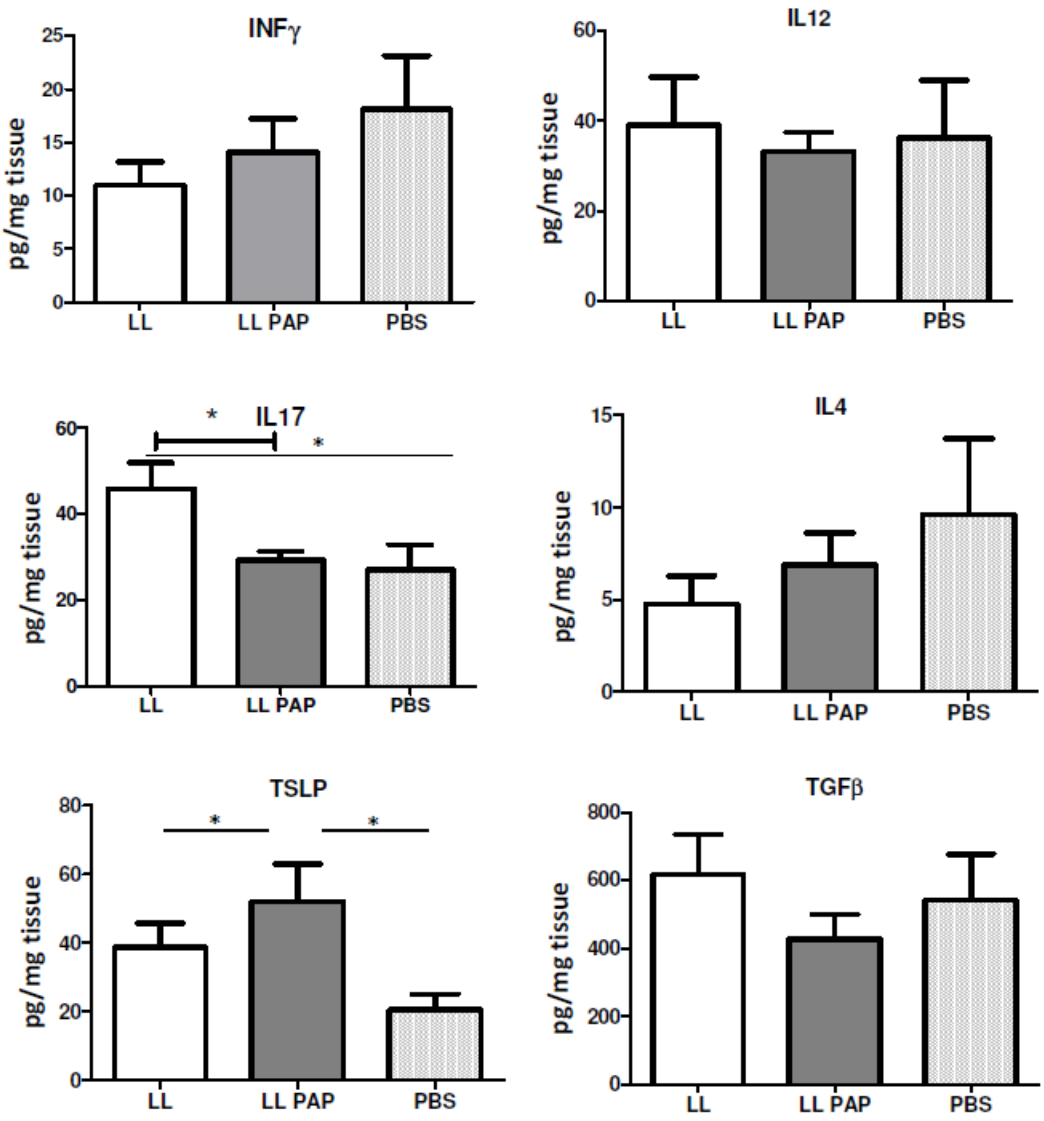

Fig 6. Cytokine production in colon. Mice were orally administered with LL or LL-PAP during 7 days before and 4 days after intra-rectal injection of DNBS. Mice were sacrificed 4 days after DNBS injection. Colon from each mouse was mashed in 1mL of PBS using Gentle Max and cytokines were measured using ELISA kits.

6.5 *L. lactis* restore Treg population in the intestinal *Lamina propria* in a PAP-independent way.

To access the mechanisms by which the inflammation is reduced in LL-PAP treated mice, we isolated cells from intestinal *lamina propria* from those mice (LL, LL-PAP, non-inflamed and inflamed controls) to measure the Treg cells population by flow cytometer. LL-PAP and LL-treated mice presented the same percentage of $CD4^{+}FoxP3^{+}$ cells. Moreover, the same percentage of these cells is found in non-inflamed mice (PBS), showing that *L. lactis* was able to increase

the Treg population in DNBS-challenged mice somehow, and independent of PAP expression (Fig. 7).

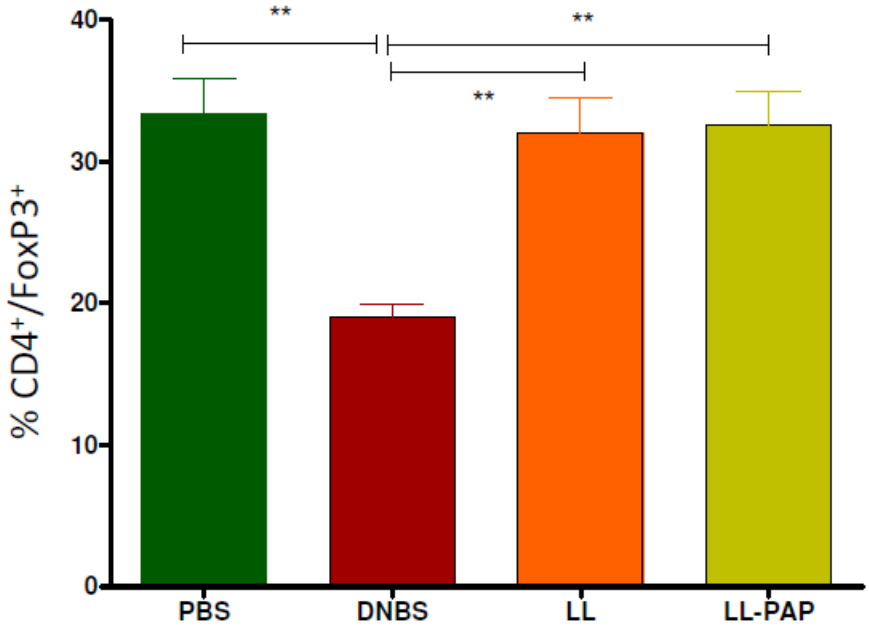

Fig 7. Percentage of FoxP3⁺ cells population from intestinal lamina propria. Mice were orally administered with LL or LL-PAP during 7 days before and 4 days after intra-rectal injection of DNBS. Mice were sacrificed 4 days after DNBS injection. Cells isolated from intestinal lamina propria from PBS, DNBS, LL and LL-PAP treated mice were stained with anti-CD4⁺ and anti-FoxP3⁺ and analyzed by Flow cytometer. The percentage of cells obtained is represented in the graph.

7 DISCUSSION

Our goal was to describe the anti-inflammatory properties of PAP and its impact in the intestinal homeostasis using recombinant lactococci. Previous works had shown that an overexpression, or oral and rectal administration, of antimicrobians led to significant changes in gut microbiota composition; however, the underlying mechanisms and health benefits provided by these changes remain to be demonstrated [21, 34, 35].

A number of epithelial AMPs kill bacteria through non-enzymatic mechanisms of cell-wall attack; these include PAP, C-type lectins belonging to RegIII family. PAP has a net positive charge and thus interacts with the bacterial membrane through

electrostactic interactions [15]. Some studies have shown that RegIIIγ presents bactericidal activity and is selective for Gram-positive bacteria because peptidoglycan is generally accessible on the outer surfaces of Gram-positive bacteria but is shielded by the outer membrane in Gram-negative bacteria [10]. Moreover, the RegIII recognition of peptidoglycan involves a unique mechanism that allows high-affinity binding to extended carbohydrate chains. This selective binding carbohydrate chain length-dependent avoid competitive inhibition by shorter peptidoglycan chains shed by bacteria and are thus abundant in the intestinal environment [19].

In order to understand how PAP improves the health status after DNBS challenge, we should figure out the severity of colitis through cytokine profile and macroscopic and microscopic parameters from these mice. As described by Wallace and colleagues, DNBS is an alternative to induce severe colitis in rodents[36]. The ethanol in which DNBS is dissolved causes colonic mucosal barrier disruption allowing thus penetration of DNBS into the *lamina propria*. DNBS haptenize the colonic and gut microbial proteins becoming immunogenic and activating the host immune response [33]. In our study, we treated mice with LL and LL-PAP before and after DNBS challenge. We hypothesized that once delivered into the intestinal lumen PAP played an important anti-inflammatory role. Our results confirm LL-PAP mice recovered weight faster than the other groups and present low severity lesion markers, such as lower intestinal permeability and better preservation of the intestinal architecture in according with the endpoints established by [33, 36-38]. We observed a reduction of IL-17, IFNγ and IL12 production in LL PAP-treated mice compared to LL-treated mice. To note, those pro-inflammatory cytokines are involved in the progression of IBD [33, 37, 38]. Moreover, this treatment also showed an increase of TGF-β and TSLP. TGF-β is involved in the Treg cell differentiation and anti-inflammatory status [39, 40]. The relation between IL17 and TGF-β is very important. TGF-β is a pleiotropic cytokine required for the differentiation of Treg and Th17 cells. However, TGF-β is non-redundantly required to the development of Treg cells, but dispensable for the differentiation of Th17. In the last case, TGF-β can be replaced by IL1-β [41]. However, it is curious that one cytokine can drive different cells with opposite functions. The explanation is based on the concentration. Low

concentration of TGF-β and the synergy with IL6 induce T cells to differentiate to Th17. On the other hand, high concentration of TGF-β favors Foxp3^{+}Treg cells [41-43]. Our results showed an increase in production of TGF-β and TSLP while a reduction in IFNγ and IL12 production, in MLN, when we compare LL-PAP with LL treated mice. As we mentioned before, IL17 production, in MLN, was decreased in LL-PAP and PBS groups, suggesting, this way, TGF-β may be involved in Treg differentiation. Moreover, in colon TGF-β production was increased in LL treated mice compared to LL-PAP and PBS groups. Taken all together, MLN and colon results concerning TGF-β, LL-PAP, LL and PBS (control group) have a balanced TGF-β production. However, this feature was not enough to avoid the severity of colitis in LL treated mice. Meanwhile, TSLP was increased only in LL-PAP treated mice, in this case we can consider that PAP was responsible to improve TSLP and overcome the colitis, once TSLP is described as anti-inflammatory cytokine [38]. These results confirmed our hypothesis PAP has an anti-inflammatory effect on DNBS-induced colitis mice.

The TGF-β production among LL, LL-PAP and PBS groups incite us to verify the percentage of Treg cells population in those groups. Treg cells could be the key element in the maintenance of the intestinal integrity, preventing all inflammation markers, such as intestinal permeability, macroscopic and microscopic scores, and cytokine profile. In order to know how LL-PAP improved the mice health status, we isolated T cells from *lamina propria* from all treated groups (PBS, DNBS, LL, and LL-PAP) to compare the Treg cells population. The percentage of Treg cells present in *lamina propria* of LL and LL-PAP treated mice were the same, moreover both restore the Treg cells population after DNBS challenge at the same level to the non-inflamed group (PBS). Moreover, these cells are in a 150% higher level than in the inflamed group (DNBS). This result is according to TGF-β production. So far, we may conclude *L. lactis* was able to improve Treg cells population by balancing TGF-β production at MLN and colon in an adverse environment, such as colitis, PAP-independent. However, PAP did not affect this feature. However, the raised number of Treg was not enough to avoid the intestinal damage and neither to promote the weight recovering in LL treated mice. Since, although LL improves Treg cells independently of PAP, LL-PAP was able to modulate cytokine profile, ameliorating weight gain and intestinal barrier

integrity more. In order to explain this protective effect of PAP, we hypothesized the microbiota shaping as a potential mechanism by which PAP prevents the mucosal barrier damage.

The composition of a host's intestinal microbiota drives the type of mucosal and systemic immune response by affecting the proportion and number of functionally distinct T cells subsets. In particular, the microbiota affects the differentiation of intestinal T cells, which play crucial role in maintaining mucosal barrier of functions, besides controlling immunological homeostasis [44, 45]. Our results showed that the microbiota composition was different in both groups (LL and LL-PAP treated mice) before DNBS challenge. Treatment by LL-PAP increased the α-diversity or richness. Diversity is known now to be very important to resist against various pathologies. Moreover β-diversity analysis through PCA confirmed that the two groups (LL and LL-PAP) have different microbiota. At the genus level, we could see that LL-PAP treatment increased *Ruminoclostridium*, *Ruminococcaceae* and *Lachnospiraceae*. These genus belong to Firmicutes phylum, which are mainly butyrate producers. Butyrate has a protective role against colitis by improving gut barrier function, increasing antimicrobial peptides production, interacting with the immune system to drive to an anti-inflammatory profile, and reducing oxidative stress [46]. Remarkably, we noticed an increase of the specie *Eubacterium plexicaudatum*. Compared to the other bacteria modified by LL-PAP treatment *E. plexicaudatum* is abundant, 1-5% of total. *E. plexicaudatum* is a member of the altered Schaedler flora [47] and described as a butyrate producer (Wilkins T.D. *et al.,* 1974). We can suppose that this bacterium, butyrate producer, was able to prevent inflammatory signals, and consequently able to inhibit Th17 and Th1 differentiation. This explanation fits with our findings and confirms that somehow this bacterium was able to improve the intestinal health in LL-PAP treated mice after DNBS challenge. Butyrate-producing bacteria are reported to be decreased in HFD-fed animals and in some human's diseases such as IBD and obesity [48].

Recently, Darnaud et al. described that enteric delivery of PAP modifies the intestinal microbiota composition and controls inflammation. Similarly to our results, they showed that expression of PAP shift the composition of intestinal

microbiota toward enrichment in clostridiales (Rumincoccaceae, Lachnospiraceae)[21]. Nevertheless in contrary to our results they have a strong protective effect in DSS-induced colitis model of PAP expression. They used transgenic mice (TG) overexpressing PAP in liver delivering thus the AMP in the lower part of the intestinal tract. The delivery of PAP using recombinant LAB strategy has more chance to occur in the upper part of the intestine than in the lower part. Indeed, lactococci are highly sensitive to low pH and generally to the biochemical and physical-chemical conditions of the intestinal tract. They don't colonize and after entering the intestine they don't survive more than few hours[49]. Thus they deliver their load rapidly in the small intestine. It has to be noted too that they describe a mild protective effect of a 100 µg intrarectal injection of recombinant PAP. In our case with administer daily a quantity of PAP estimated around few hundreds of picograms which is far from what Darnaud et al. have injected.

Taken altogether, our results allow us to conclude LL-PAP was able to shift the microbiota through an enriched butyrate-producers microbiota which could be able to prevent intestinal epithelial damage, weight loss, and inflammatory status after DNBS challenge. More studies should be performed to demonstrate the role of the microbiota but we can propose *E. plexicaudatum* as a potential probiotic used to prevent intestinal inflammation damages.

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VI CHAPTER 2

Comparison of the efficiency of *Lactococcus lactis* and *Lactobacillus casei* strains expressing pap in the protection of mice in an acute colitis model.

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1 GENERAL BACKGROUND AND STORY OF THE PROJECT

Lactic acid bacteria (LAB) are known for their role in the food industry and have been widely used as probiotics for both humans and animals, ensuring homeostasis of health in various organs and systems[1–4]. One of the reasons for the wide use of LABs is given by the fact that they have the status of "GRAS", that means Generally Recognized As Safe. They also present the status of Qualified Presumption of Safety (QPS) according to the European Food Safety Authority (EFSA). For example, bacteria of the genera *Bifidobacterium* spp. and *Lactobacillus* spp. has a long history of safe consumption without any harmful effects on health. A good example of the use of these bacteria is VSL # 3, a cocktail of 8 probiotic microorganisms (4 strains of *Lactobacillus*, 3 strains of *Bifidobacterium* and 1 strain of *Streptococcus*) that presented results when used in human trials in patients with Crohn's disease, ulcerative colitis, pouchitis, and Irritable Bowel Syndrome[5–10]. In addition, these bacteria have been studied as a vector for the delivery of proteins and molecules for the treatment of many diseases, presenting as a safe, comfortable and effective way of administration in patients[1–4]. Inflammatory bowel diseases (IBD) have been one of the targets

of the use of LABs and recombinant LABs, since these diseases present treatments that are often not effective and with important side effects[1,4,7–9,11].

This project aimed to test two different LAB strains capable of producing the same molecule (pancreatitis associated protein I - PAP) under the control of the NICE (Nisin Controlled Gene Expression) system for the treatment of mice in a DNBS-induced colitis model. The PAP molecule has been studied in our research group on IBD models for its ability to shape the microbiota and thus protect animals against inflammatory processes. Thus, we choose two different vectors to produce the same molecule, *Lactococcus lactis* and *Lactobacillus casei*. However, before initiating the animal experiments, it was necessary to stablish protocols for extracting protein from the culture pellet and inducing the promoter for the expression of PAP in the *Lactobacillus casei* strain, as there were no efficient protocols for these. For *Lactococcus lactis* these protocols have already been tested and used in previous studies. In addition to the choice of two different vectors, the persistence times of each in the gastrointestinal tract of the animals were considered, leading us to test two protocols with different times of administration of the bacteria (every day and every 3 days).

2 ACTORS IMPLIED IN THE PROJECT

This project has been fully executed by me, from testing protocols for extracting pellet proteins and inducing PAP expression to the animal experiment, with all subsequent analyzes. All steps were taken at the Micalis Insitute at the INRA in Jouy-en-Josas. This work was carried out under the direct supervision of Jean-Marc Chatel and Vasco Ariston de Carvalho Azevedo and co-supervision of Natália Martins Breyner.

3 GOALS

3.1 General goals

To study the efficiency of two recombinant strains of *Lactococcus lactis* and *Lactobacillus casei* expressing PAP in a colitis model induced by DNBS to protect the mice when compared with the controls groups.

3.2 Specific goals

a) Establishing an efficient protocol to extract proteins from the pellet of *Lactobacillus casei* culture.
b) Establishing an efficient protocol to induce the expression of PAP by the strain of *Lactobacillus casei* under the control of the NICE system.
c) Inducing a DNBS-induced colitis model in mice and evaluate the protection against the inflammation when mice received daily treatment with *Lactococcus lactis* expressing or not PAP and *Lactobacillus casei* expressing or not PAP.
d) Inducing a DNBS-induced colitis model in mice and evaluate the protection against the inflammation when mice received every 3 days treatment with *Lactococcus lactis* expressing or not PAP and *Lactobacillus casei* expressing or not PAP.

4 INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic, complex and relapsing inflammatory conditions of GIT that has been a global health problem, with an increasing incidence[12,13]. IBD is a group of closely related but heterogeneous disease processes. It includes two main forms, Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by alternating phases of clinical relapse and remission[12,14,15]. CD can cause transmural inflammation and affect any part of the gastrointestinal tract (most commonly the perianal region or the

terminal ileum) in a non-continuous type. Classically presented with fatigue, fever, weight loss, prolonged diarrhea with or without severe bleeding and abdominal pain, commonly associated with complications such as fistulas, abscesses and stenosis. In contrast, UC is typified by mucosal inflammation and limited to the colon (involving the rectum) and exhibits symptoms that generally include rectal bleeding, frequent stools, rectal mucus secretion, tenesmus, and low abdominal pain[12,14]. IBD affects about 1.5 million Americans, 2.2 million people in Europe and a prevalence rate of 396 per hundred thousand individuals worldwide[13,14]. The exact etiology of IBD is still unknown, but recent research indicates that it involves the individual's genetic susceptibility, an uncontrolled immune-mediated inflammatory response, microbiome, and external environment[12–15].

One of the molecules that has been studied by our research group in the treatment of IBD is the Pancreatitis Associated Protein I (PAP). PAP is part of the proteins encoded by the regenerating islet-derived (REG) gene family, that many of them are associated with epithelial inflammation[16]. PAP was first isolated in rat pancreatic juice in the acute phase of pancreatitis and represented up to 5% of total protein. The human ortholog was identified from the pancreatic juice of diabetic patients and reached up to 7.5% of the total secretory protein[17–19]. Despite their initial association with pancreas, most Reg proteins are expressed in multiple organs and are detected under normal and pathological conditions[20]. PAP is expressed in the gastrointestinal, with their expression focused in the crypt base spreading from Paneth cells of jejunum and ileum and by the goblet cells and enterocytes in the colon, and is up-regulated in patients with inflammatory bowel disease[16,17,19–21]. PAP has a variety of activities, which includes anti-apoptotic, anti-inflammatory, antibacterial effects and proliferative, maintaining host-bacterial homeostasis in the mammalian gut.[18,21]. PAP exert a direct bactericidal effect as a result of the capacity to bind to the peptidoglycan layer of Gram positive bacteria even at low micromolar concentrations[16,18,21]. The anti-inflammatory effect of PAP has been shown in a number of studies, in different models of inflammation[22–24].

Several new strategies using lactic acid bacteria (LAB) for the expression or ability to metabolize molecules capable of reducing inflammation in inflammatory bowel diseases have been studied in recent years[7,8,25–29]. Some strains of LABs, such as *Lactobacillus casei Shirota* and *Bacillus bifidus communis*, have been considered as probiotics, which means “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”[6–9]. Two important representants of this group are *Lactococcus lactis* and *Lactobacillus casei* that were chosen to perform this study. In here, both strains were used to express PAP under the control of the NICE (Nisin Controlled Gene Expression) system and tested in the treatment of acute colitis induced by DNBS. Beyond the comparison between both strains it was also compared two different protocols of administration, every day or every 3 days, considering the persistence time. So far, no work has compared the efficiency of these two strains with different protocols of administration to see how this could reflect on the protection of the mice in a situation of acute inflammation.

5 MATERIALS AND METHODS

5.1 Bacterial strains and growth conditions

Lactococcus lactis strains were grown at 30°C in M17 medium without shaking containing 0.5% glucose (GM17). The antibiotics were added to the medium at the ideal concentrations: erythromycin (Ery) 5µg/mL or chloramphenicol (Cm) 10µg/mL.

Lactobacillus casei strains were grown at 37°C in MRS medium without shaking. When necessary, the antibiotics were added to the medium at the ideal concentrations: erythromycin (Ery) 5µg/mL or chloramphenicol (Cm) 10µg/mL.

5.2 Tests of protocols for extracting proteins from the pellet of *Lactobacillus casei* cultures.

Seven different protein extraction protocols were tested on the pellets of *L. casei* culture.

5.2.1 Protocol A

Pellets resuspended in TE buffer + 1x antiprotease were submitted to sonication with 10 pulses of 30 seconds, with intermittent cooling and interval of 30 seconds between the pulses. Samples were centrifuged at 10.000 RPM for 8 min at 4°C. The supernatants were collected and frozen at -80°C until use in the next steps.

5.2.2 Protocol B

Pellets resuspended in TE buffer + 1x antiprotease were submitted to lysis with 0.1mm diameter zirconium beads using the Precellys machine (3 cycles of 30 seconds at 4.500RPM and temperature at 4°C, with a 30 seconds interval between the cycles). Samples were centrifuged at 10.000 RPM for 8 min at 4°C. The supernatants were collected and frozen at -80°C until use in the next steps.

5.2.3 Protocol C

Pellets resuspended in TE buffer + 1x antiprotease were submitted to lysis with an enzymatic cocktail (200µL of the pellet + 60µL of the enzymatic solution containing lysozyme: 50mg/ml, mutanolysin: 233U/ml, lysostaphin: 13.3U/ml). The mix was incubated at 37°C for 30 minutes. The samples were then sonicated with 6 pulses of 10 seconds, with intermittent cooling and interval of 30 seconds between the pulses. Samples were centrifuged at 10.000 RPM for 8 min at 4°C. The supernatants were collected and frozen at -80°C until use in the next steps.

5.2.4 Protocol D

Pellets resuspended in TE buffer + 1x antiprotease were submitted to lysis with an enzymatic cocktail (200µL of the pellet + 60µL of the enzymatic solution containing lysozyme: 50mg/ml, mutanolysin: 233U/ml, lysostaphin: 13.3U/ml).

The mix was incubated at 37°C for 2 hours. Samples were centrifuged at 10.000 RPM for 8 min at 4°C. The supernatants were collected and frozen at -80°C until use in the next steps.

5.2.5 Protocol E

Pellets resuspended in TE buffer + 1x antiprotease were submitted to lysis with 0.1mm diameter zirconium beads using the Precellys machine (6 cycles of 30 seconds at 4.500RPM and temperature at 4°C, with a 30 seconds interval between the cycles). Samples were centrifuged at 10.000 RPM for 8 min at 4°C. The supernatants were collected and frozen at -80°C until use in the next steps.

5.2.6 Protocol F

Pellets resuspended in TE buffer + 1x antiprotease were submitted to sonication with 10 pulses of 30 seconds, with intermittent cooling and interval of 30 seconds between the pulses. The samples were then submitted to lysis with 0.1mm diameter zirconium beads using the Precellys machine (3 cycles of 30 seconds at 4.500RPM and temperature at 4°C, with a 30 seconds interval between the cycles). Samples were centrifuged at 10.000 RPM for 8 min at 4°C. The supernatants were collected and frozen at -80°C until use in the next steps.

5.2.7 Protocol G

Pellets resuspended in TE buffer + 1x antiprotease were submitted to lysis with an enzymatic cocktail (200µL of the pellet + 60µL of the enzymatic solution containing lysozyme: 50mg/ml, mutanolysin: 233U/ml, lysostaphin: 13.3U/ml). The mix was incubated at 37°C for 30 minutes. The samples were then submitted to lysis with 0.1mm diameter zirconium beads using the Precellys machine 3 cycles of 30 seconds at 4.500RPM and temperature at 4°C, with a 30 seconds interval between the cycles). Samples were centrifuged at 10.000 RPM for 8 min at 4°C. The supernatants were collected and frozen at -80°C until use in the next steps.

5.3 Evaluation of the proteins migration profile by the SDS-PAGE technique.

Proteins extracted from the pellet and precipitated from the supernatant of the different nisin-induced *L. casei* cultures were analyzed by the SDS-PAGE technique using 10% polyacrylamide gel and protein denaturation at 95°C for 5 minutes. The gels were stained with Coomassie Brilliant Blue to evaluate the migration profile of the proteins.

5.4 Tests of protocols for induction by nisin using NICE system in *Lactobacillus casei*.

Four different induction protocols were tested and different concentrations of nisin were tested in all protocols.

5.4.1 Protocol A

Lactobacillus casei strains were grown at 37°C without shaking in MRS medium + 5µg/mL erythromycin overnight. Further, we diluted 1/20 in MRS medium + 5µg/mL erythromycin until it reached an $O.D_{600nm}$ approximately around 0.15. The culture was maintained at 37°C without shaking until reaching an $O.D_{600nm}$ between 0.4 and 0.6. Later, the nisin was added at 3 different concentrations: 10 ng/mL, 25 ng/mL and 50 ng/mL and the culture was incubated at 37°C without shaking for 2 hours. Afterwards, cultures were centrifuged at 10.000 RPM for 8 minutes at 4°C. Pellets were resuspended in TE buffer + 1x antiprotease; and antiprotease (final concentration of 1x) was added into the supernatants. Pellets and supernatants were stored at -80°C until be used in the next steps.

5.4.2 Protocol B

Lactobacillus casei strains were grown at 37°C without shaking in MRS medium + 5µg/mL erythromycin overnight. Further, we diluted 1/20 in MRS medium + 5µg/mL erythromycin until it reached an $O.D_{600nm}$ approximately around 0.15. The culture was maintained at 37°C without shaking until reaching an $O.D_{600nm}$ approximately around 0.3. Later, the nisin was added at 3 different

concentrations: 10 ng/mL, 25 ng/mL and 50 ng/mL and the culture was incubated at 37°C without shaking for 3 hours. Afterwards, cultures were centrifuged at 10.000 RPM for 8 minutes at 4°C. Pellets were resuspended in TE buffer + 1x antiprotease; and antiprotease (final concentration of 1x) was added into the supernatants. Pellets and supernatants were stored at -80°C until be used in the next steps.

5.4.3 Protocol C

Lactobacillus casei strains were grown at 37°C without shaking in MRS medium + 5µg/mL erythromycin overnight. Further, we diluted in MRS medium + 5µg/mL erythromycin until it reached an $O.D_{600nm}$ approximately around 0.35. The culture was maintained at 37°C without shaking for 1 hour and 30 minutes. Later, the nisin was added at 3 different concentrations: 10 ng/mL, 25 ng/mL and 50 ng/mL and the culture was incubated at 37°C without shaking for 4 hours and 30 minutes. Afterwards, cultures were centrifuged at 10.000 RPM for 8 minutes at 4°C. Pellets were resuspended in TE buffer + 1x antiprotease; and antiprotease (final concentration of 1x) was added into the supernatants. Pellets and supernatants were stored at -80°C until be used in the next steps.

5.4.4 Protocol D

Lactobacillus casei strains were grown at 37°C without shaking in MRS medium + 5µg/mL erythromycin overnight. The overnight culture was then centrifuged, the supernatant was withdrawn and the pellet resuspended in MRS medium + 5µg/mL erythromycin until it reached an $O.D_{600nm}$ approximately around 2.5. The culture was maintained at 37°C without shaking for 1 hour and 30 minutes. Later, the nisin was added at 3 different concentrations: 10 ng/mL, 25 ng/mL and 50 ng/mL and the culture was incubated at 37°C without shaking for 2 hours. Afterwards, cultures were centrifuged at 10.000 RPM for 8 minutes at 4°C. Pellets were resuspended in TE buffer + 1x antiprotease; and antiprotease (final concentration of 1x) was added into the supernatants. Pellets and supernatants were stored at -80°C until be used in the next steps.

5.5 ELISA for PAP protein detection

The expression levels of the PAP protein after the induction protocols were defined by measuring PAP in the supernatant and in the pellet of each culture using the ELISA PancrePAP assay kit (Dynabio). The procedures were performed according to the instructions of the provider.

5.6 Mice Experiment

Conventional C57BL/6JRj mice, males with 6-week-old were purchased for Janvier Labs and hosted in INRA (Jouy-en-Josas, France) animal care facilities and acclimatized for 1 week prior to immunization, in accordance with current standards in the Unité d'Expérimentation Animale (Jouy-en Josas, France). Ten groups were used, each one with 8 mice. Four of these groups received $5x10^9$ (CFU) of the strains daily, intragastrically: *L. lactis* EMPTY (*L. lactis* + empty plasmid), *L. lactis* PAP (*L. lactis* expressing PAP), *L. casei* EMPTY (*L. casei* + empty plasmid) and *L. casei* PAP (*L. casei* expressing PAP). Four other groups received $5x10^9$ (CFU) of the same strains every 3 days, also intragastrically. Two control groups were used, one negative control group (Naïve) and another positive control group for inflammation (DNBS), both receiving only PBS intragastrically, daily. On the fifth day of bacterial administration, induction of inflammation was performed by DNBS intra-rectal administration at the rate of 150 mg/Kg of the animal. 50µl of DNBS solution diluted in 30% ethanol + PBS was administered. The negative control group received only 50µL of 30% ethanol + PBS. On the ninth day of bacterial administration the animals were sacrificed (Fig 1).

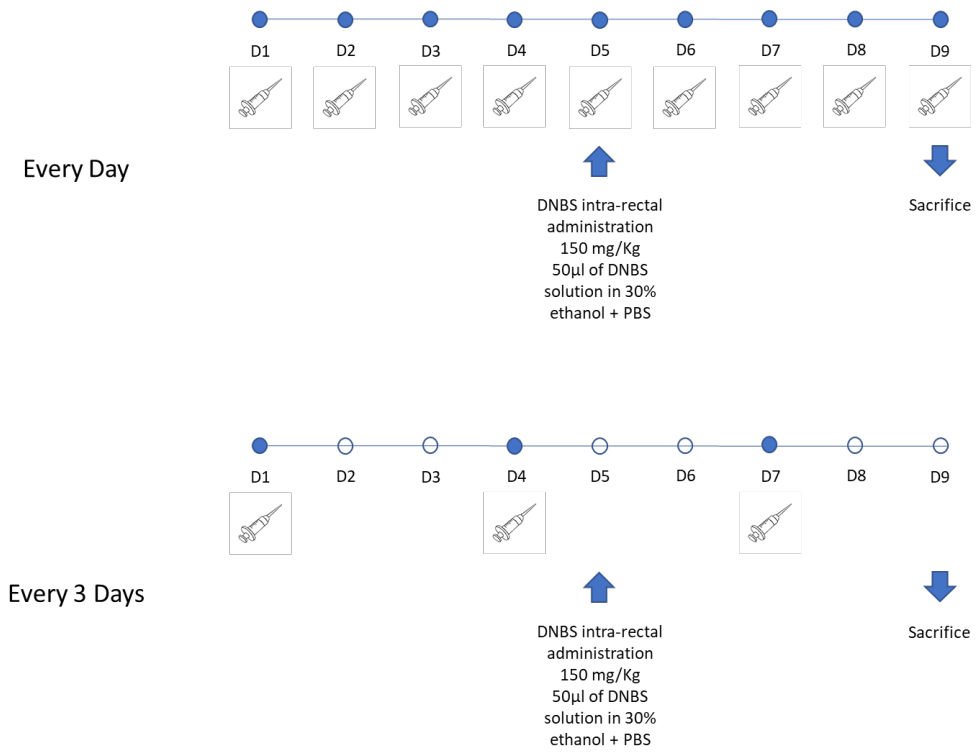

Fig 1. Mice Experiment. Protocol of bacterial administration, DNBS-induced colitis and sacrifice.

5.7 Analysis of weight recovery after inflammation induction

The mice weight was considered 100% on the day of DNBS administration. The mice were monitored for 4 days after inflammation induction and graphics were performed for loss and recovery of weight during that period.

5.8 Macroscopic evaluation of compromised colon

The macroscopic evaluation was performed during the sacrifice of the animals, assessed on a scale of 0–6,5, observing the following aspects: thickness of the tissue (equal or lower than the negative control = 0; higher than negative control = 1), presence of diarrhea (no = 0; yes = 1), hyperemia (no = 0; yes = 1), adhesions (no = 0; yes = 1) or ulcers (no = 0, one smaller than 2mm = 1, one with bigger than 2mm = 1,5; more than one smaller than 2mm = 2; more than one bigger than 2mm = 2,5).

5.9 Protein Extraction in colon and small intestine and measure of cytokines by ELISA

The proteins present in the tissues were extracted using zirconium beads with 1.4mm diameter in PBS + 1x antiprotease. The samples were submitted to the Precellys machine with 3 cycles of 30 seconds at 4.500 RPM. Afterwards, the samples were then centrifuged at 5000g for 1 minute, the supernatants were collected and frozen at -80°C in 500µL aliquots in deep well plates for subsequent cytokine dosage by the ELISA technique. Commercial kits were used and procedures were performed according to the manufacturer's instructions. The cytokines tested were Th1-related cytokine (IFNγ and IL12); Th2-related cytokines (IL4 and IL5); Th17-related cytokine (IL17) and Treg–related cytokines (IL10 and TGFβ), Th22-related cytokine (IL22).

5.10 Interleukin Secretion by Stimulated Lymphocytes and measure of cytokines by ELISA

Mesenteric Lymph Nodes (MLN) and spleen were isolated from mice during the sacrifice and then smashed and filtered using 70 µm filter. Lymphocytes were counted by flow cytometry and $2{,}5x10^6$ cells/mL were placed per well in 24 wells plate in RPMI with 10% Fetal Calf Serum (FCS) and 100 Unit of Streptomycin and Penicillin. The plates were pre-incubated with anti-CD3 and anti-CD28 antibodies, 4µg/mL of each antibody in PBS. Plates were incubated 48h at 37°C, 5% of CO_2. After this period, supernatants were collected and frozen at -80°C in 500µL aliquots in deep well plates for subsequent cytokine dosage by the ELISA technique. Commercial kits were used and procedures were performed according to the manufacturer's instructions. The cytokines tested were Th1-related cytokine (IFNg and IL12); Th2-related cytokines (IL4 and IL5); Th17-related cytokine (IL17) and Treg–related cytokines (IL10 and TGFb), Th22-related cytokine (IL22).

5.11 Statistical Analysis

All statistics and graphics have been performed on Prism-GraphPad®. Results represent means ± s.e.m.. Statistical significance was determined by the Mann-Whitney test. It has been considered that $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

6 RESULTS

6.1 Establishment of protocols for protein extraction from culture pellet and PAP secretion.

The proteins extracted from the pellet of *L. casei* culture were analyzed by the SDS-PAGE technique and it was found that the most efficient protocol was Protocol E, where the pellets were resuspended in TE buffer + 1x antiprotease and submitted to lysis with zirconium beads (0.1mm of diameter) using the Precellys apparatus with 6 cycles of 30 seconds at 4.500RPM and temperature of 4°C, with a 30 second interval between cycles (Fig 2).

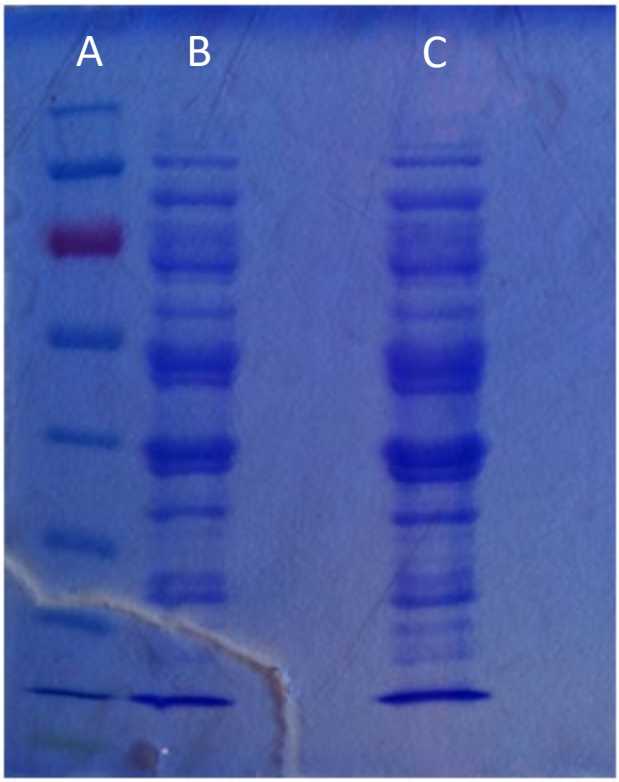

Fig 2. Evaluation of the protein migration profile by SDS-PAGE with 10% polyacrylamide gel after protein extraction from pellet of the *Lactobacillus casei* culture using Protocol E. A= Protein Ladder. B= 5x concentrated pellet. C= 10x concentrated pellet.

Expression levels of PAP protein were determined in the supernatant and pellet from cultures of *L. casei* by ELISA. Protocol B (nisin added in the culture with OD around 0.3 for 3 hours) using 25ng/mL was determined the best results (Fig 3).

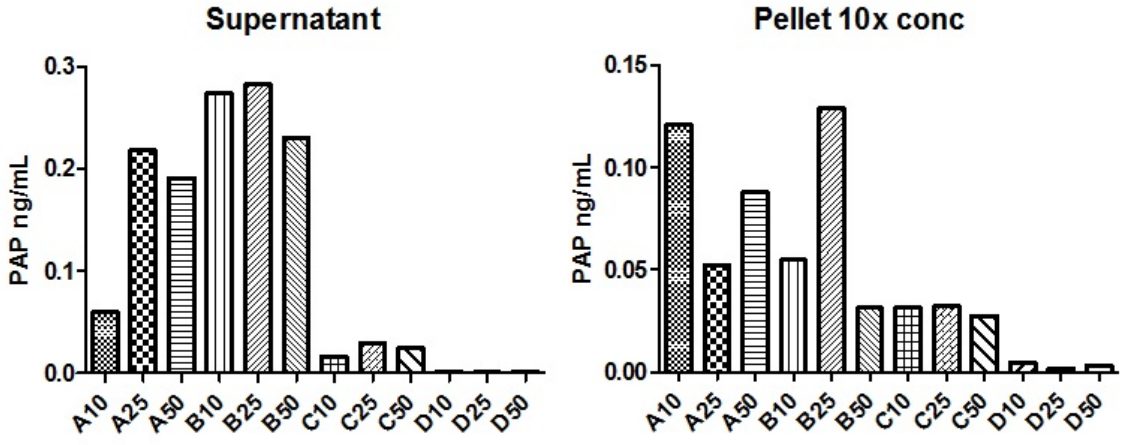

Fig 3. Evaluation of PAP expression levels by *Lactobacillus casei* after different nisin induction protocols. PAP was measured in the supernatants and pellets of the cultures using the PancrePAP assay kit ELISA (Dynabio).

6.2 Effect of daily administration of *Lactococcus lactis* or *Lactobacillus casei* expressing PAP on weight loss in acute colitis model.

The first aspect to be analyzed was the recovery of weight of the animals after the induction of inflammation. Animals from the *L. lactis* PAP group showed better weight recovery 4 days after induction of colitis by DNBS, compared to the other groups (Fig 4).

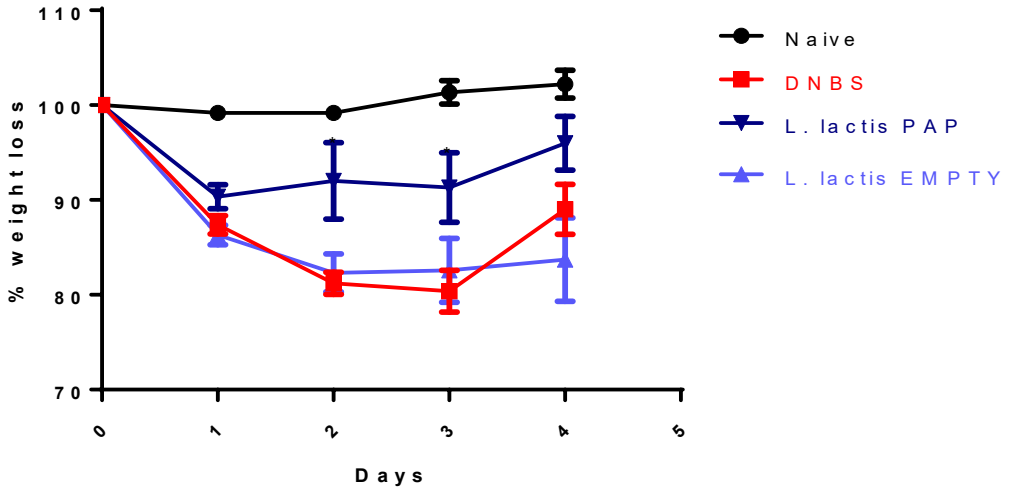

Fig 4. Evaluation of weight recovery of the mice after induction of DNBS inflammation. Animals received daily treatment with *Lactococcus lactis* expressing PAP (*L. lactis* PAP) or not (*L. lactis* EMPTY). *p<0.05

The animals of the *L. casei* PAP group presented a recovery similar to DNBS group, but better than the group treated with empty *Lactobacillus casei* at D4 (Fig 5).

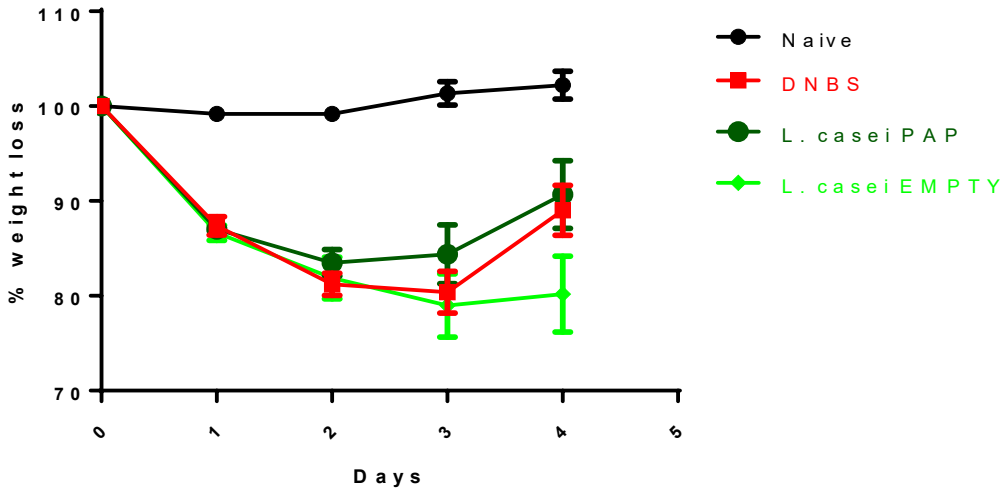

Fig 5. Evaluation of weight recovery of the mice after induction of DNBS. Animals received daily treatment with *Lactobacillus casei* expressing PAP (*L. casei* PAP) or not (*L. casei* EMPTY).

6.3 Effect of every 3 days administration of Lactococcus lactis or Lactobacillus casei expressing PAP on weight loss in acute colitis model.

Animals from the *L. lactis* PAP group showed no weight recovery after induction of colitis by DNBS when treatment was performed every 3 days. The same result was observed with the group treated with *L. lactis* EMPTY (Fig 6).

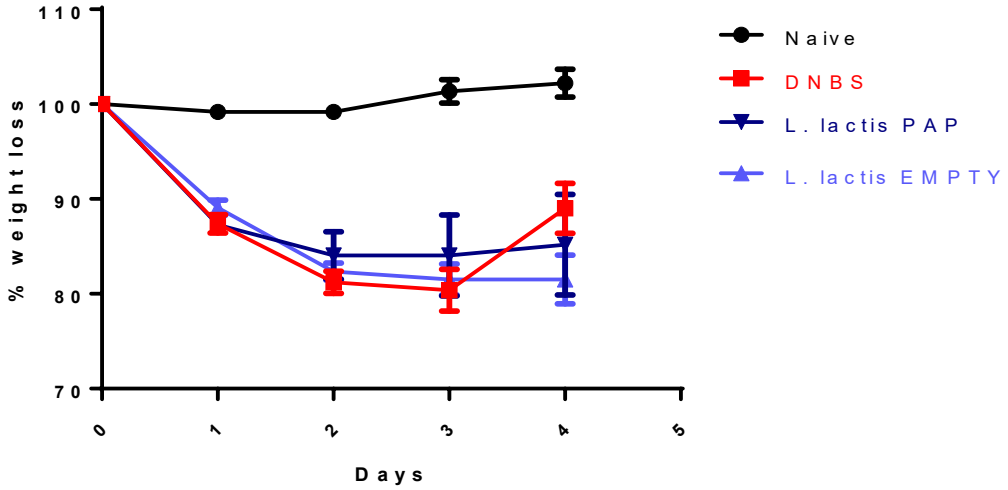

Fig 6. Evaluation of weight recovery of the mice after administration of DNBS. Animals received treatment with *Lactococcus lactis* expressing or not PAP every 3 days.

The animals of the *L. casei* PAP group or *L. casei* EMPTY showed a slightly better weight recovery when compared to DNBS group (Fig 7).

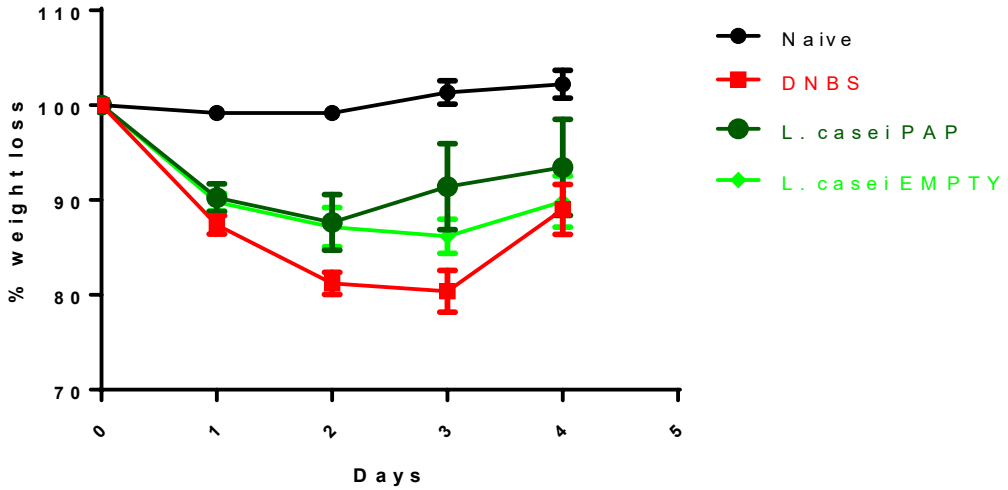

Fig 7. Evaluation of weight recovery of the mice 4 days after administration of DNBS. Animals received treatment with *Lactobacillus casei* expressing or not PAP every 3 days.

6.4 Effect of daily administration of *Lactococcus lactis* or *Lactobacillus casei* expressing PAP on macroscopic score in acute colitis model.

The second aspect to be analyzed was the macroscopic evaluation of the colon 4 days after induction of inflammation.

When the treatment was performed every day, the group that received *L. lactis* PAP presented a lower macroscopic score when compared with DNBS group and *L. lactis* EMPTY (Fig 8).

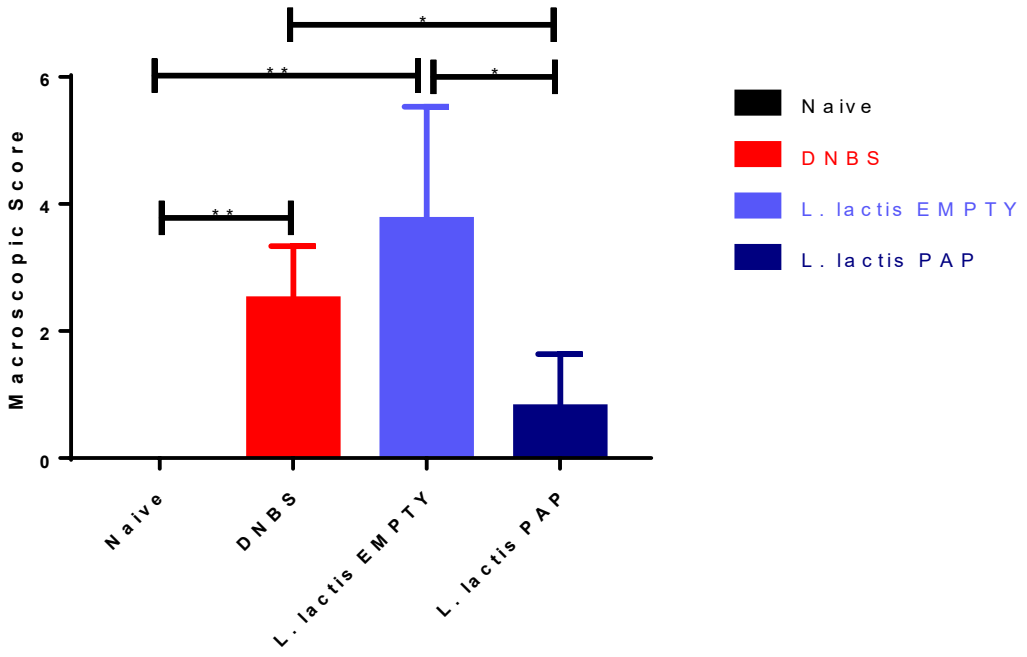

Fig 8. Macroscopic evaluation of the colon 4 days after the induction of inflammation by DNBS. Animals were treated daily with *Lactococcus lactis* expressing or not PAP. ∗P < 0.05, ∗∗P < 0.01

When the treatment was performed using *L. casei,* the group expressing PAP presented a reduction of macroscopic score when compared with group treated with empty *L. casei*, but not significant, and no difference either with DNBS group (Fig 9).

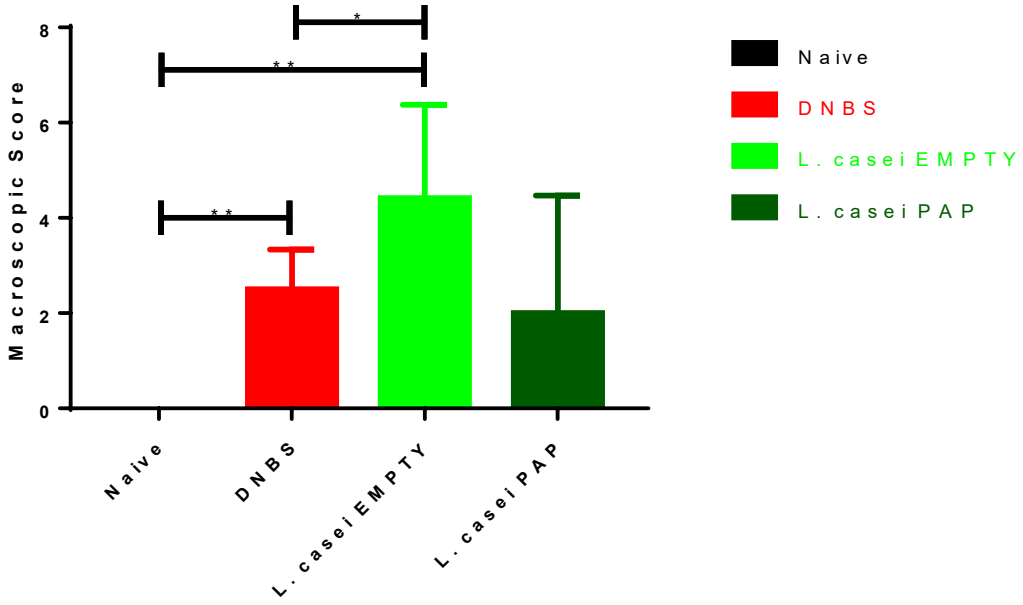

Fig 9. Macroscopic evaluation of the colon 4 days after the induction of inflammation by DNBS. Mice were treated daily with *Lactobacillus casei* expressing or not PAP. ∗P < 0.05, ∗∗P < 0.01

6.5 Effect of every 3 days administration of *Lactococcus lactis* or *Lactobacillus casei* expressing PAP on macroscopic score in acute colitis model.

When the treatment was performed every 3 days, both groups receiving *L. lactis* (expressing or not PAP) were not able to reduce the macroscopic score when compared with DNBS group (Fig 10).

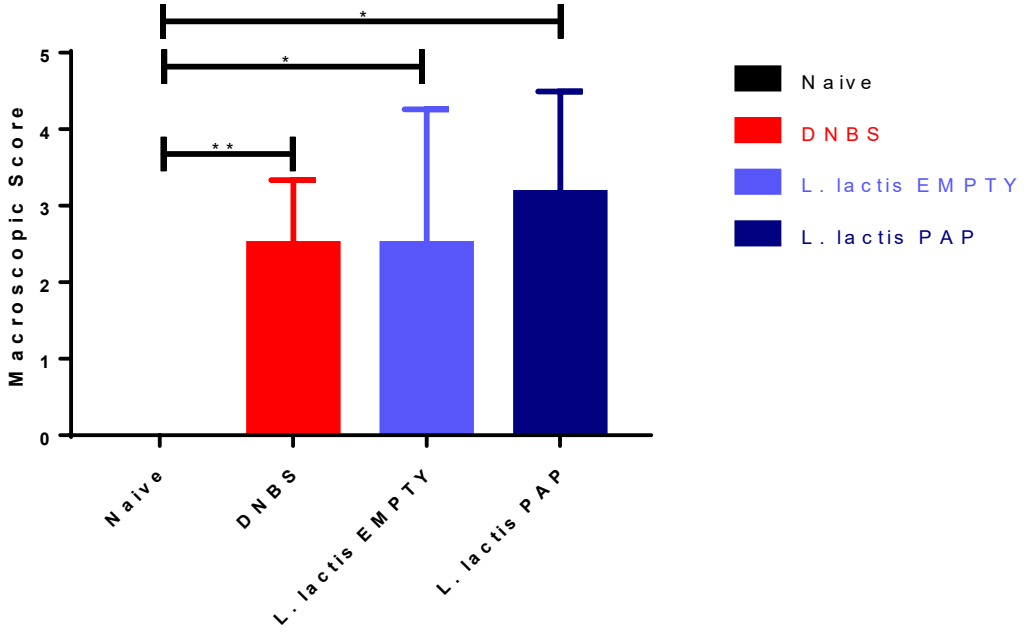

Fig 10. Macroscopic evaluation of the colon 4 days after the induction of inflammation by DNBS. Animals were treated every 3 days with *L. lactis* expressing or not PAP. ∗P < 0.05, ∗∗P < 0.01

When the treatment was performed every 3 days using *L. casei*, the empty group presented a reduction of macroscopic score when compared with DNBS group, but not significant. The group treated with *Lactobacillus casei* expressing PAP was able to reduce the macroscopic score in a discrete way, but also not significant (Fig 11).

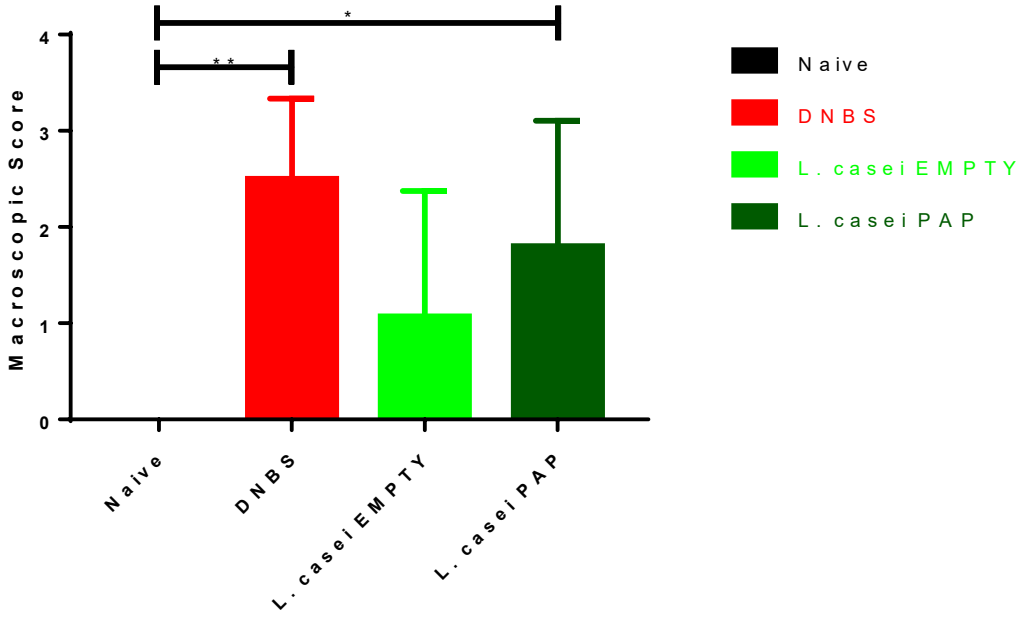

Fig 11. Macroscopic evaluation of the colon 4 days after the induction of inflammation by DNBS. Mice were treated every 3 days with *Lactobacillus casei* expressing or not PAP. ∗P < 0.05, ∗∗P < 0.01.

6.6 Effect of daily administration of *Lactococcus lactis* or *Lactobacillus casei* expressing PAP on immune system in acute colitis model.

The third aspect to be analyzed was the immune response. We monitored the concentrations of pro (Th1, Th17) and anti-inflammatory cytokines (Th2) in the colon, ileum and secreted by lymphocytes from MLN and spleen 4 days after induction of inflammation by DNBS. Measurements of IL-4, IL-5, IL-10, IL-12, IFN-γ, IL-17, IL-22 and TGF-β were performed.

When *Lactococcus lactis* expressing or not PAP were administered every day, no significant changes in the cytokines profile where observed on MLN or colon samples. A significant decrease in pro-inflammatory cytokine IL17 was found in supernatant of splenocytes in *L. lactis* expressing PAP group when compared with DNBS group. The group *L. lactis* EMPTY group also showed a decrease in IL17 levels, but not significant (Fig 12).

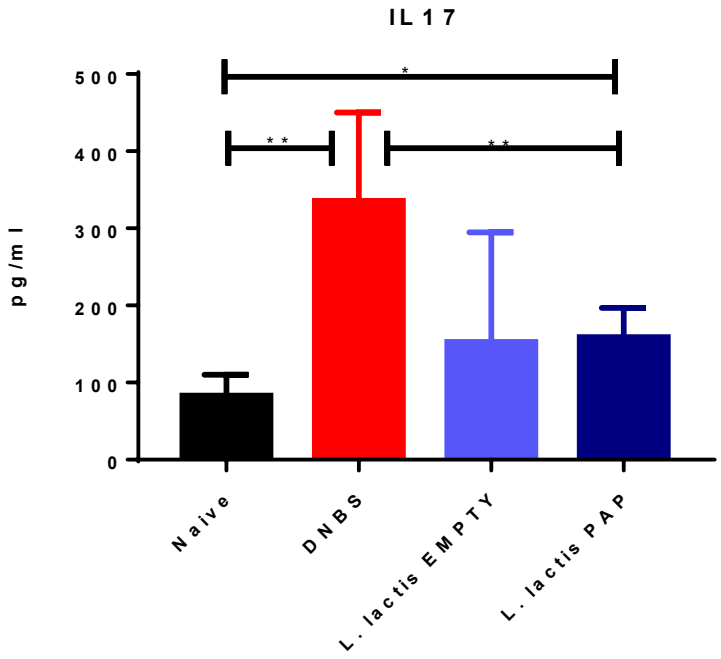

Fig 12. IL17 concentration in medium of splenocytes 4 days after induction of inflammation by DNBS. Mice received treatment every day with *Lactococcus lactis* expressing or not PAP. ∗P < 0.05, ∗∗P < 0.01

When *Lactobacillus casei* expressing or not PAP were administered every day, no significant changes in the cytokines profile where found in MLN, ileum or colon samples. A significant increase in IL4 and IL5, both Th2 anti-inflammatory cytokines, was found in supernatant of spleenocytes in *L. casei* expressing PAP group when compared with DNBS group. The group *L. casei* EMPTY group also showed an increase in IL5 levels, but not significant. A significant decrease in IFNγ (Th1 pro-inflammatory) was also found in supernatant of lymphocytes from spleen in *L. casei* expressing PAP group when compared with DNBS group (Fig 13).

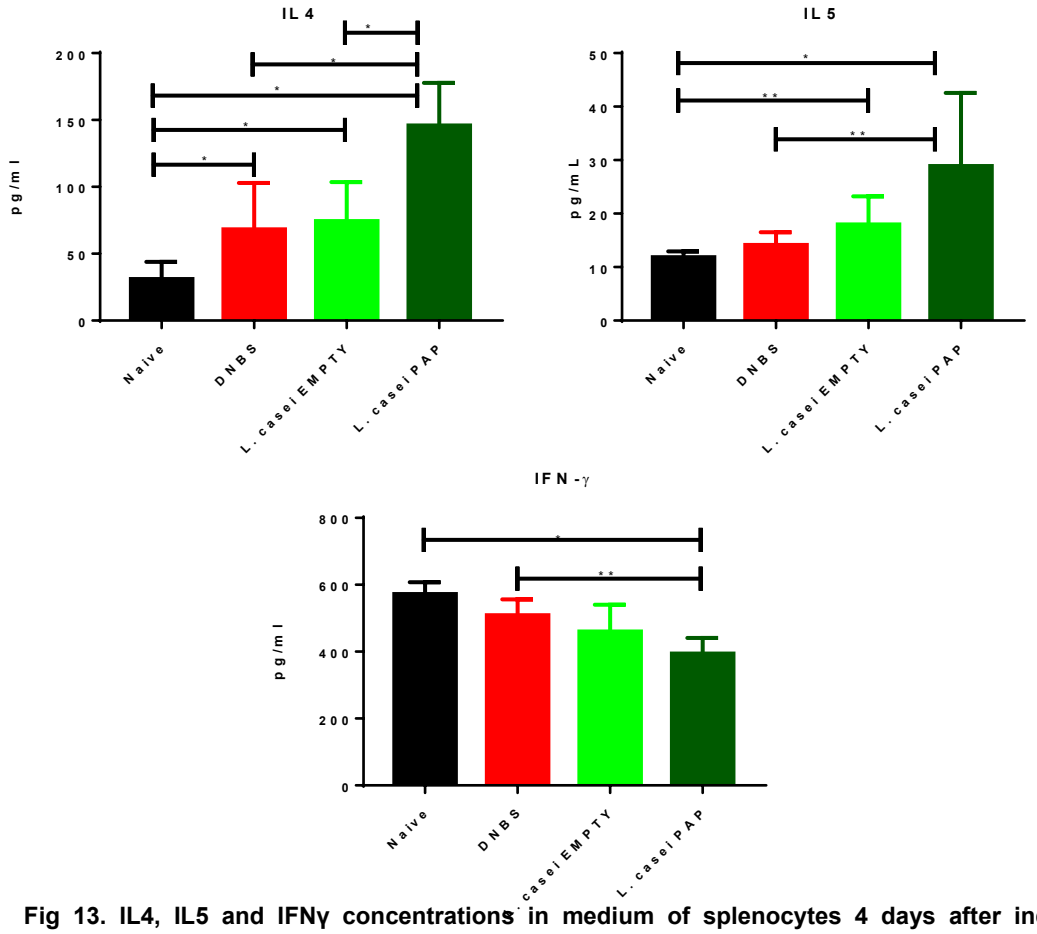

Fig 13. IL4, IL5 and IFNγ concentrations in medium of splenocytes 4 days after induction of inflammation by DNBS. Mice received treatment every day with *Lactobacillus casei* expressing or not PAP. ∗P < 0.05, ∗∗P < 0.01.

6.7 Effect of every 3 days administration of *Lactococcus lactis* or *Lactobacillus casei* expressing PAP on immune system in acute colitis model.

When *Lactococcus lactis* expressing or not PAP were administered every 3 days, no significant changes in the cytokines profile where found on MLN, spleen or colon. A significant increase in IL4, IL5, IL12, IL17 and IFNγ was found in the proteins extracted from ileum in *L. lactis* expressing PAP group when compared with DNBS group. For IL12 and IL17 this difference was also significant when compared with *L. lactis* EMPTY group (Fig 14).

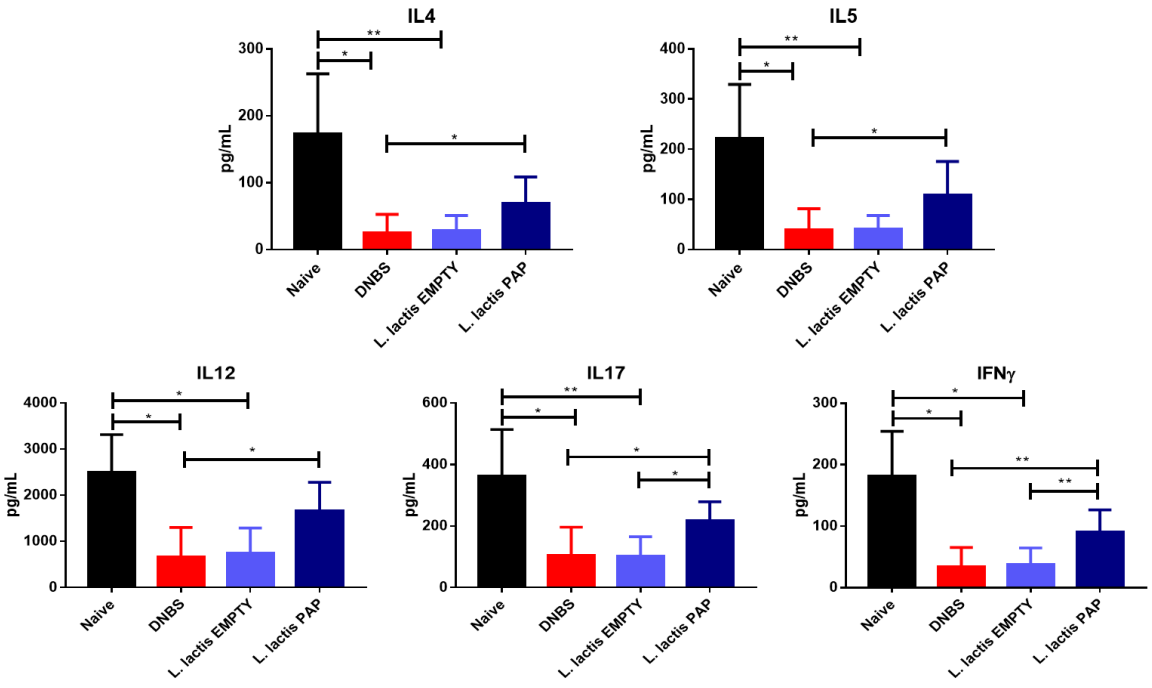

Fig 14. IL4, IL5, IL12, IL17 and IFNγ concentrations in protein extraction from ileum 4 days after induction of inflammation by DNBS. Mice received treatment every 3 days with *Lactococcus lactis* expressing or not PAP. ∗P < 0.05, ∗∗P < 0.01.

When *Lactobacillus casei* expressing or not PAP was administered every 3 days, no significant changes in the cytokines profile where found on ileum or colon. A significant increase in IL4, IL10 and IL22 was found in supernatant of lymphocytes from MLN in *L. casei* EMPTY and *L. casei* expressing PAP groups when compared with DNBS group (Fig 15).

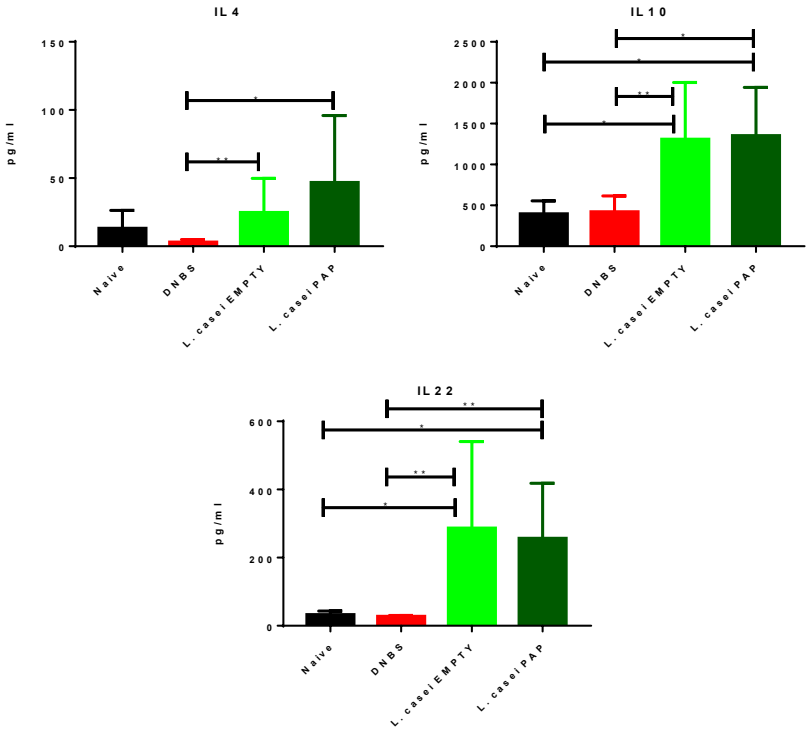

Fig 15. IL4, IL10 and IL22 concentrations in medium of lymphocytes culture from MLN 4 days after induction of inflammation by DNBS. Mice received treatment every 3 days with *Lactobacillus casei* expressing or not PAP. ∗P < 0.05, ∗∗P < 0.01.

A significant increase in IL10 was found in supernatant of splenocytes in *L. casei* EMPTY when compared with DNBS group. *L. casei* expressing PAP also showed an increase in IL10, but this difference was not significant when compared with the other groups (Fig 16).

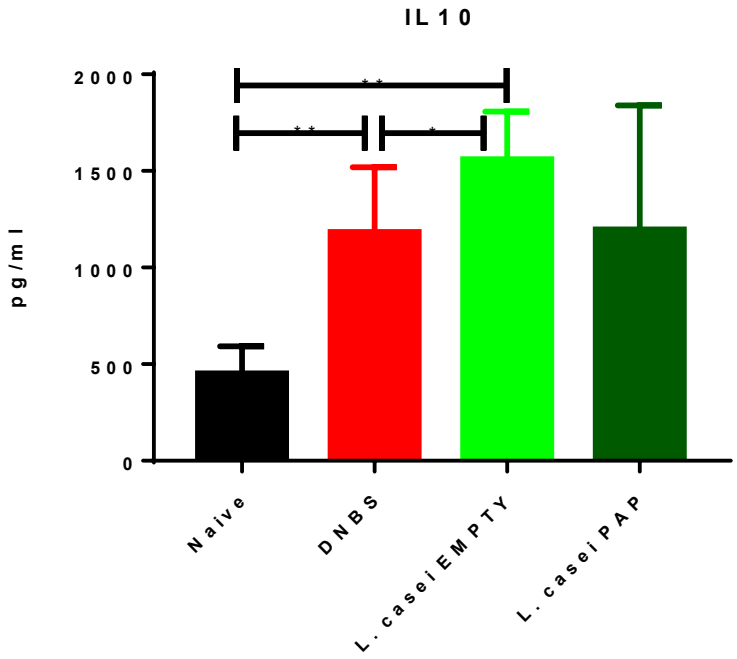

Fig 16. IL10 concentration in medium of lymphocytes culture from spleen 4 days after induction of inflammation by DNBS. Mice received treatment every 3 days with *Lactobacillus casei* expressing or not PAP. ∗P < 0.05, ∗∗P < 0.01.

7 DISCUSSION

The present work was based on two species of lactic acid bacteria, *Lactococcus lactis* and *Lactobacillus casei*, and their actions in a murine model of intestinal inflammation induced by the intra rectal administration of DNBS. For the two bacterial species, we used recombinant strains expressing PAP (pancreatitis associated protein) under the control of the NICE (Nisin Controlled gene Expression) system, where nisin is used as an inductor molecule for the activation of the plasmid promoter.

Pancreatitis-Associated Protein (PAP) has been studied in different models of inflammatory processes in the gastrointestinal tract and has shown potential anti-inflammatory properties when daily administered in *L. lactis*[24]. Usually, PAP is

produced by intestinal epithelial cells and show bactericidal and anti-inflammatory activity[30].

The aim objective of this study is to show that independent of the protein used for the treatment, the vector could have a key role in the response to the inflammation and the mechanisms to it should be studied. Here, we evaluated the variation of the weight after the intrarectal administration of DNBS, macroscopic score (length of the colon, thickness of the tissue, presence of diarrhea, hyperemia or ulcers) at the day of the mice sacrifice and the cytokines detected in the colon, ileum, lymphocytes from MLN and splenocytes [25,31–35].

Extraction of proteins from the culture pellet of Lactic Acid Bacteria could be an issue. The extraction protocol using sonication works effectively for the strain of *Lactococcus lactis*, however, for *Lactobacillus casei* there was no determination of a protocol capable of extracting the proteins efficiently and without degradation. In order to obtain a high level of protein with a low level of degradation, different protocols were tested with adaptation of those found in the literature[36–38]. The tested protocols used enzymatic mixes, zirconium beads, sonication or a combination of more than one of these strategies. After analyzing the products of the extractions by SDS-PAGE, most part of the protocols showed a low level of extraction or a high level of degradation (data not show). The best result was observed when a mechanical extraction was performed, using zirconium beads and agitation. Moreover, this protocol was able to show an effective extraction with a low level of degradation. Therefore, the further analysis and *in vivo* experiments were performed using this protocol for *L. casei*.

The second step is the protein induction. The nisin induction protocol in the NICE system for *L. lactis* is already well established, presenting good levels of expression by our strain with the protocol used [24,39–41]. However, we should test different protocols to find the most appropriate for induction in *L. casei*. Whereof, 4 different induction protocols were tested, and in all protocols different concentrations of nisin were also used [36–38,41]. These protocols vary in initial $O.D_{600nm}$., time of nisin addition and for how long the culture is going to be exposure to the nisin. The best protocols were Protocols A and B for all nisin concentrations and for pellet and supernatant, when compared with Protocols C

and D. The similarity between the best protocols was the low $O.D_{600nm}$ in the beginning of the culture (0,15 for both protocols) and the point of adding the nisin (between 0,4 and 0,6 for protocol A and 0,3 for protocol B). With that, our results allow us to infer that the metabolism of the bacterium when the nisin is added is crucial for the activation of the promoter showing better results when the bacterium is at the exponential phase. Another interesting observation is the fact that the highest concentration of nisin (50ng/mL) didn't give the best result, probably because nisin show a bacteriostatic effect when added to the culture and this concentration may affect the bacterial metabolism[42–47].

L. lactis and *L. casei* are both LAB but their physiology and immunological properties are different. *L. lactis* is mainly considered as a neutral or pro-inflammatory vehicle whereas *L. casei* has been described as anti-inflammatory. *L. lactis* doesn't colonize the GIT whereas after oral administration *L. casei* can persist during 2-3. That's why *L. casei* is often considered as a better vehicle than *L. lactis*. In order to determine which is the best bacterial vector (*L. lactis* x *L. casei*) we used two different protocols schemes. In the first, we performed daily gavages for the two bacteria, in the second, the gavages were performed every 3 days, in the total of 3 administrations during the experiment. These two protocols schedules with different time of intervals between bacterial administrations are related to the time of each strain remains in the gastrointestinal tract of the animals[48–53].

The protocol using every day administration demonstrated *Lactococcus lactis* EMPTY does not show protection against the weight loss during the entire experiment. Otherwise, *L. lactis* expressing PAP was able to protect mice against the weight loss since the beginning of the experiment. The animals lost only 10% of the original weight. The weight loss results was significant in days 2 and 3, when compared with DNBS group and *L. lactis* EMPTY group. Those animals lost around 20%. This result showed that the PAP molecule was crucial to confer protection in mice against the weight loss when delivered by *L. lactis* daily. This was confirmed by the effect on macroscopic score.

When *L. lactis* expressing or not PAP is administered every 3 days no protective effect in mice against the weight loss or macroscopic score was obtained. Thus the same bacterial vector exerts different responses depending on the time of administration. In this case, we may infer the importance of the bacteria remains in the gastrointestinal tract of the animals. *L. lactis* secreting PAP should be administered every day to confer protection against the weight loss.

On the opposite *Lactobacillus casei* expressing or not PAP was not able to protect mice against the weight loss when administered every day. Taken altogether, we may propose no longer *L. casei* remains for more time than *L. lactis* in the gastrointestinal tract of the animals. With the daily administration, the overload of bacteria and/or protein (PAP) could be not efficient for the gastrointestinal tract homeostasis.

Meanwhile, *Lactobacillus casei* expressing or not PAP presented a tendency to protect mice against the weight loss when administered every 3 days, especially at days 2 and 3 when compared with the DNBS group. In this case, we may infer the time that the bacteria remain in the gastrointestinal tract of the animals is crucial to determinate the bacterial strain administration. Moreover, the results obtained using *L. casei* demonstrated that this bacterium should not be administered every day to confer protection against the weight loss.

Otherwise, when *L. casei* expressing or not PAP was administered every day, there was no effect on the macroscopic score for both strains compared with DNBS group. There is a tendency to reduce the score when the same bacteria were administered every 3 days, especially *L. casei* EMPTY, but those differences are not significant when compared with DNBS group. Once again, these results corroborate with the results obtained in the weight loss and the fact that *Lactobacillus casei* should not be administered every day to confer protection against inflammation induced by DNBS.

Cytokines are proteins/hormones secreted by immune cells. They are important to orchestrate the immune response, facilitate communication between cells, control the development, growth, activation and function of innate and adaptive

immune cells, and mediate local and systemic inflammation[54,55]. The analysis of cytokines in MLN and colon was performed according to the literature showing that an effective treatment of colitis is capable to decrease pro-inflammatory and to increase anti-inflammatory cytokines in that organs in different models of colitis[25,26,28,31,33,56]. There is no significant difference in the protocol where both strains were administrated every day. Separately, regarding *L. lactis,* secreting or not PAP, was able to decrease IL-17 in the spleen, only. This result may suggest a reduction of Th17 cells activity that are responsible to recruit neutrophils to the sites of active inflammation[14,57–59]. However, in the colon and MLN we did not found any evidences in terms of this cytokine.

With respect to the other strain, *L. casei* secreting PAP induce an increasing of IL4 and IL5 compared with DNBS group, however, only IL4 is increased compared with *L. casei* EMPTY in the spleen. These results showed a Th2 response compatible with the profile of the inflammation on colitis, principally in UC[14,57–61]. Moreover, *L. casei* secreting PAP reduce the level of IFN-γ in the same organ comparing with DNBS group. IFN-γ is a pro-inflammatory cytokine, secreted by Innate Lymphoid Cells (ILCs), Mucosal T-cells, Intestinal Epithelial Cells (IELs) and so on. This cytokine is responsible for the induction of TNF-α production by activated tissue macrophages, which causes epithelial cell apoptosis and affects the tight junction activity. It also triggers the production of others inflammatory cytokines like IL-1, IL-6, IL-8, IL-12, and IL-18, contributing to the maintenance of the inflammation on colitis, principally CD[14,60,62,63]. These results may suggest a protective effect of PAP secreted by *L. casei* when administrated every day. However, this protective effect was also not shown on colon and MLN, as far as may explain the macroscopic score and weight loss results.

When bacteria were administered every 3 days, no significant results were found on cytokines concentration in MLN, colon and spleen from mice that received *L. lactis* expressing or not PAP. In the ileum, the level of IL-4, IL-5, IL-12, IL-17 and IFN-γ were increased. These results may suggest an inflammatory effect with a mixed immune response profile: Th1 (IL-12 and IFN-γ), Th2 (IL-4 and IL-5) and Th17 (IL-17). Despite the ileum is not the main affected part on this model, we

can clearly see the compromise of the tissue by the inflammatory response. The mice group treated every 3 days with *L. lactis* did not show weight loss recovering. Differently, *L. casei*, expressing or not PAP, induced an increase of IL-10 in MLN and spleen (just the empty one). These results may indicate a protective effect through Treg activation[63–65] once IL10 is involved in Treg differetiantion. In according with this observation, the mice treated with *L. casei* PAP and *L. casei* EMPTY presented reduced level of weight loss compared with the DNBS group. However, the macroscopic score is conflicting. Therefore, both *L. casei* strains, expressing or not PAP, were also able to induce the production of IL-22 in MLN. IL22 plays a key role on gut homeostasis, wound healing, epithelial regeneration, production of antimicrobial peptides and might be involved in mucus production. As well, IL22 is responsible to induce the secretion of PAP by Paneth cells and epithelial cells[63,64,66,67]. Here, we may suggest *L. casei per si* overwhelming the inflammation caused by DNBS. Previous work showed *L. casei* as an anti-inflammatory bacterium[68–71]. Further analysis should be done to evaluate whether this potential is related to the shaping of the microbiota or AhR activation[72]. Moreover, if confirmed this protective effect of *L. casei*, new strategies can be designed to increase the interest of the food industry in our studies and products. However, PAP expressed in *L. casei* does not seem to be relevant to protect mice against the inflammation caused by DNBS [14,58,73].

8 CONCLUSIONS

The main goal of this work was to compare two strains of lactic acid bacteria able to produce a recombinant protein with anti-inflammatory properties in a model of acute colitis. One of the strains, *Lactococcus lactis* expressing PAP, already had stablished protocols for extract the protein from the culture pellet and to induce the production of the recombinant protein in the presence of the nisin, the promoter inductor. Regarding *Lactobacillus casei* expressing PAP, new protocols were necessary to be developed. The protocol selected to extract the protein from the pellet was using zirconium beads and agitation, showing a great level of

extraction with low levels of degradation. The protocol selected to induce the production of the recombinant protein used a low $O.D_{600nm}$ in the beginning of the culture and at the point of adding the nisin, presenting good levels of expression at the pellet and at the supernatant of the culture.

The next step was to perform the animal experiment. The mice had the colitis induced by the intrarectal administration of DNBS and received oral treatment by gavage, starting 4 days before the induction of inflammation. The gavage was performed every day or every 3 days for all bacteria (expressing or not PAP). The analysis of weight loss, macroscopic score and cytokines showed us that *Lactococcus lactis* should be administered every day to confer protection, while *Lactobacillus casei* should be administered every 3 days to show a tendency to protect mice.

Taken altogether, our data showed for the first time a comparison between two different recombinant lactic acid bacteria strains and the importance of the vector and the timing of the treatment, independent for which molecule is going to be tested in the treatment of induced-colitis. For that kind of approach, is clear the importance of a previous test to define the scheme of bacterium administration.

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VII CHAPTER 3

Comparison between *Lactococcus lactis* strain delivering an eukaryotic expression plasmid for PAP expression by intestinal cells and *L. lactis* strain delivering PAP as a protein.

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1 GENERAL BACKGROUND AND STORY OF THE PROJECT

The technique of DNA vaccines is based on the transference to the host cells of a plasmid harboring a cDNA under the control of an eukaryotic promoter. This approach induces immune responses similar with those ones induced by attenuated pathogens[1]. The cDNA can be injected intramuscularly, but this route does not provide stability to this nucleic acid[2]. Therefore, an alternative route is the mucosal delivery, which is able to induce local and systemic immune responses. However, as in the gut has different physico-chemical conditions, what can damage the DNA, it is very important to protect this molecule. Regarding this protection, studies suggest bacteria as an efficient vector[3]. Lactic Acid Bacteria is a large group of bacteria, most of them display probiotics properties, as well as they are well know and widely used in fermented food, due to their status of GRAS (Generally Recognized as Safe) by the World Health Organization. These properties warrant them as potential vector[4–8]. This strategy based on the DNA vaccines to reduce inflammatory processes has been applied in several studies[3,9–14]. For example, Inflammatory Bowel Diseases (IBD) can be

favored by this approach. IBD is characterized by a chronic bowel inflammatory disorders, which two mains integrant of this group are Ulcerative Colitis (UC) and Crohn's Disease (CD), presenting distinct characteristics but with similar symptoms like diarrhea, abdominal pain, rectal bleeding and weight loss[15–18].

Our study aimed to test a *Lactococcus lactis* strain delivering a plasmid for Pancreatitis Associated Protein (PAP) expression by epithelial cells. This approach was used to focus on the treatment of mice in a DNBS-induced colitis model. The PAP molecule has been studied in our research group on IBD models. PAP presented the ability to shape the microbiota and thus protect animals against inflammatory processes. In order to confirm the efficiency of this strategy, we compared it with a *Lactococcus lactis* strain capable of producing PAP under the control of the NICE (Nisin Controlled Gene Expression) system, as used in our previous studies. For this, we induced the colitis with the intrarectal administration of DNBS and treated the mice with both strains. The parameters evaluated were the weight loss, macroscopic score and measured TGF-β and IL-10 in the supernatant of MLN culture.

2 ACTORS IMPLIED IN THE PROJECT

This project has been fully executed by me, from the animal experiment to the subsequent analyzes. All steps were taken at the Micalis Insitute at the INRA in Jouy-en-Josas. This work was carried out under the direct supervision of Jean-Marc Chatel and Vasco Ariston de Carvalho Azevedo and co-supervision of Natália Martins Breyner.

3 GOALS

3.1 General goals

To evaluate the protection induced by a *Lactococcus lactis* strain delivering a plasmid for PAP expression by epithelial cells in a model of acute colitis induced by DNBS.

3.2 Specific goals

a) To induce acute colitis by intrarectal administration of DNBS in C57BL/6 female mice;
b) To perform the treatment of the mice with daily oral administration of *Lactococcus lactis* harboring a plasmid for eukaryotic expression of PAP;
c) To determine the efficacy of *Lactococcus latics* harboring PAP cDNA to reduce the weight loss and the macroscopic score 4 days after the induction of inflammation;
d) To evaluate the immune response profile after the administration of *Lactococcus latics* harboring PAP cDNA 4 days after the induction of inflammation.

4 INTRODUCTION

The strategy of DNA vaccination has been widely studied for its capacity to induce cell-mediated and humoral immune responses. Since the plasmid DNA is administrated via intramuscular, a production of antibodies and T helper cells and cytotoxic cells responses is established. This approach has shown promising results regarding the ability of the host to fight against infections caused by virus and bacteria [19,20]. However, the injection of naked DNA, albeit is safe, it has low immunogenicity, one it has no capacity to replication. Therefore, due to a small amount of antigens produced, new strategies to improve the potency of DNA vaccines should be developed[21]. Bacteria used as vehicle for DNA delivery to eukaryotic cells presents an interesting approach to solve this problem. The advantages in this strategy include: (i) possibility of easy oral administration, (II) induction of mucosal and systemic immune responses, (III) work as immune

adjuvant, (IV) protect the plasmid against degradation, (V) can carry large-sized plasmids and (VI) does not require a large quantity of purified plasmid DNA[22–24]. Among the large amount of bacteria, Lactic Acid Bacteria (LAB) presents as an excellent choice for this kind of approach. LAB is a group of bacteria largely used in industrial process such as preservation and production of fermented food. Those bacteria have GRAS status (Generally Recognized As Safe) granted by the FDA (Food and Drug Administration), being thus considered safe for human consumption. They also present the status of Qualified Presumption of Safety (QPS) according to the European Food Safety Authority (EFSA). This safety has been proved by history of consumption and scientific evidences[5,6,8,12]. Several studies have been done using strategies based in the DNA vaccines to reduce inflammatory processes[3,9–14]. One important group of illness that can be favored by this approach is the Inflammatory Bowel Diseases (IBD), typified by a chronic bowel inflammatory disorders. The two main representative of this group are Ulcerative Colitis (UC) and Crohn's Disease (CD). They present distinct characteristics, such as UC is limited to the colon and is characterized by mucosal inflammation in a superficial way. Although, CD typically causes transmural inflammation, affecting all the layers of the intestinal wall and can affect any region of the gastrointestinal tract in a discontinuous way. Moreover, CD is normally related with the presence of strictures, abscesses and fistulas as complications. Beside these differences, both diseases present similar symptoms like diarrhea, abdominal pain, rectal bleeding and weight loss. These symptoms on relapse can continue for days, weeks or even months[15–18]. For the moment, there is no cure for these diseases. In addition, the available treatments present strong side-effects, such as immunosuppression, abdominal pain, nausea, and so on. Taken altogether, it is important to develop new strategies to improve the immune response aiming combat these diseases[25–27].

5 MATERIALS AND METHODS

5.1 Culture conditions

Lactococcus lactis strains were grown at 30°C in M17 medium without shaking containing 0.5% glucose (GM17). When necessary, chloramphenicol (Cm) was added to the medium at the ideal concentrations of 10µg/mL.

5.2 DNBS-Induced Colitis

Conventional C57BL/6JRj mice, males with 6-week-old were purchased for Janvier Laboratory, France and settled in animal care facilities and acclimatized for 1 week prior to immunization, in accordance with current standards in the Unité d'Expérimentation Animale (Jouy-en Josas, France). Five groups were used, each one with 10 mice. Three of these groups received $5x10^9$ (CFU) of the strains daily, intragastrically: EMPTY (*L. lactis* + empty plasmid), PAP-PROT (*L. lactis* expressing PAP) and PAP-cDNA (*L. lactis* harboring PAP cDNA for eukaryotic expression). Two control groups were used, one negative control group (Naïve) and another positive control group for inflammation (DNBS), both receiving only PBS intragastrically, daily. On the fifth day of bacterial administration, induction of inflammation was performed by DNBS intra-rectal instillation at the rate of 150mg/Kg of the animal weight. 50µl of DNBS solution diluted in 30% ethanol + PBS was administered. The negative control group received only 50µL of 30% ethanol + PBS. On the ninth day of bacterial administration the animals were sacrificed.

5.3 Evaluation of weight loss and recovery

The mice weight was considered 100% on the day of DNBS administration. The mice were monitored for 4 days after inflammation induction and graphics were performed for loss and recovery of weight during that period.

5.4 Colon analysis for macroscopic score

The macroscopic evaluation was performed during the sacrifice of the animals, observing the following aspects: length of the colon, thickness of the tissue, presence of diarrhea, hyperemia or ulcers.

5.5 Interleukin Secretion by Stimulated Lymphocytes

Mesenteric Lymph Nodes (MLN) were isolated from mice during the sacrifice and then smashed and filtered using 70 µm filter. Lymphocytes were counted by flow cytometry and $2.5x10^6$ cells/mL were placed per well in 24 wells plate in RPMI with 10% Fetal Calf Serum (FCS) and 100 Unit of Streptomycin and Penicillin. The plates were pre-coated with anti-CD3 and anti-CD28 antibodies, 4µg/mL of each antibody in PBS. These cells were incubated 48h at 37°C, 5% of CO_2. After this period, supernatants were collected and frozen at -80°C in 500µL aliquots in deep well plates for subsequent cytokine dosage by the ELISA technique. Commercial kits were used and procedures were performed according to the manufacturer's instructions. The cytokines tested were Th1-related cytokine (IFNγ); Th17-related cytokine (IL17) and Treg–related cytokines (IL10 and TGF-β).

5.6 Statistical Analysis

All statistics and graphics have been performed on Prism-GraphPad®. Results represent means ± s.e.m. Statistical significance was determined by the Mann-Whitney test. It has been considered that $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

6 RESULTS

PAP-cDNA strain administration protects on weight loss in a DNBS-induced acute colitis model as well as PAP-PROT strain.

The first parameter analyzed was the weight loss of the mice during four days after the induction of inflammation by intrarectal administration of DNBS. The control group for the inflammation presented equivalent results when compared with the groups that received *L. lactis* expressing PAP (PAP-PROT) and *L. lactis* harboring PAP cDNA (PAP-cDNA) during the entire experiment, not showing significant difference between them. In contrast, *L. lactis* EMPTY presented the highest level of weight loss, showing significant results when compared with PAP PROT (day 4) and PAP cDNA (days 2, 3 and 4) (Fig 1).

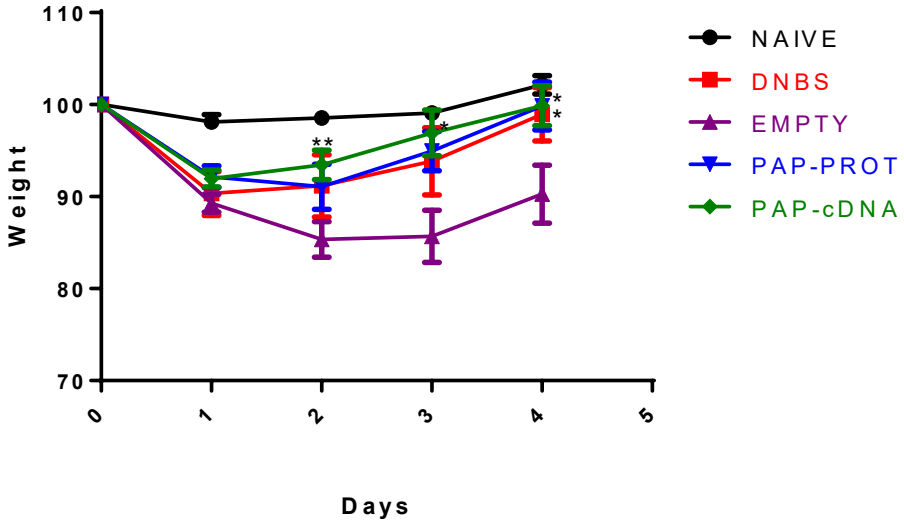

Fig 1. Effect of the strains EMPTY, PAP-PROT and PAP-cDNA on the weight loss during 4 days after DNBS-induced colitis. Mice were orally administered with EMPTY, PAP-PROT or PAP-cDNA strains ($5x10^9$ CFU) during 5 days before and during 4 days after colitis induction. Weight was monitored daily. ∗P < 0.05, ∗∗P < 0.01.

PAPcDNA strain administration protects on macroscopic score in a DNBS-induced acute colitis model as well as PAP-PROT strain.

In order to evaluate the level of injury at the colon, we established a macroscopic score based on following parameters: length of the colon, thickness of the tissue, presence of diarrhea, hyperemia or ulcers. Similar the weight loss, there's no significant difference between DNBS when compared with PAP-PROT, PAP-cDNA or when compared with EMPTY group. But, we have found statistical differences when we compare the EMPTY group with PAP-PROT and with PAP-

cDNA. It's important to notice that there is no difference between NAÏVE and PAP-PROT (Fig 2).

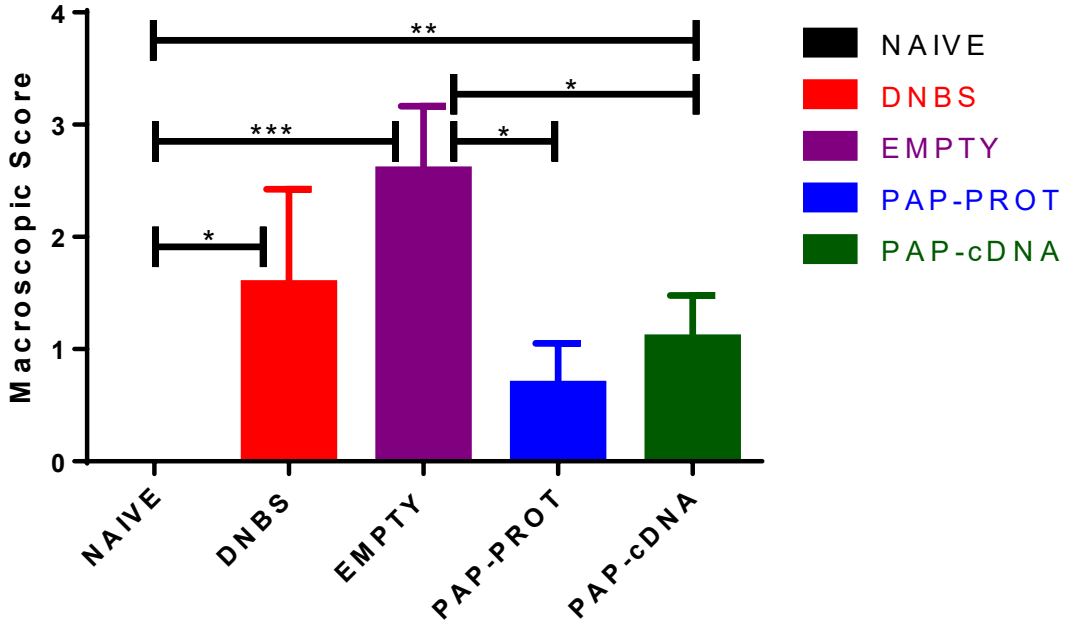

Fig 2. Effect of the strains EMPTY, PAP-PROT and PAP-cDNA on macroscopic scores in a DNBS-induced colitis. Mice were orally administered with EMPTY, PAP-PROT or PAP-cDNA during 5 days before and during 4 days after colitis induction. The macroscopic score was performed at the day of sacrifice. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001.

PAP-cDNA strains administration showed a better protective effect on immune response in a DNBS-induced acute colitis model than PAP-PROT.

The last parameter analyzed was the dosage of pro and anti-inflammatory cytokine secreted by lymphocytes from MLN 4 days after inflammation induction by DNBS. Measurements of IL-10, TGF-β, IL-17 and IFN-γ were performed. IL17 and IFN-γ showed no significant difference among the groups. For TGF-β, the group PAP-cDNA presented the highest level of secretion when compared with all other groups (Fig 3). For IL-10, it was found an increase of the levels in the group PAP-cDNA when compared with the positive control group. The groups EMPTY and PAP-PROT also presented an increase in the IL-10 production but not in a significant way.

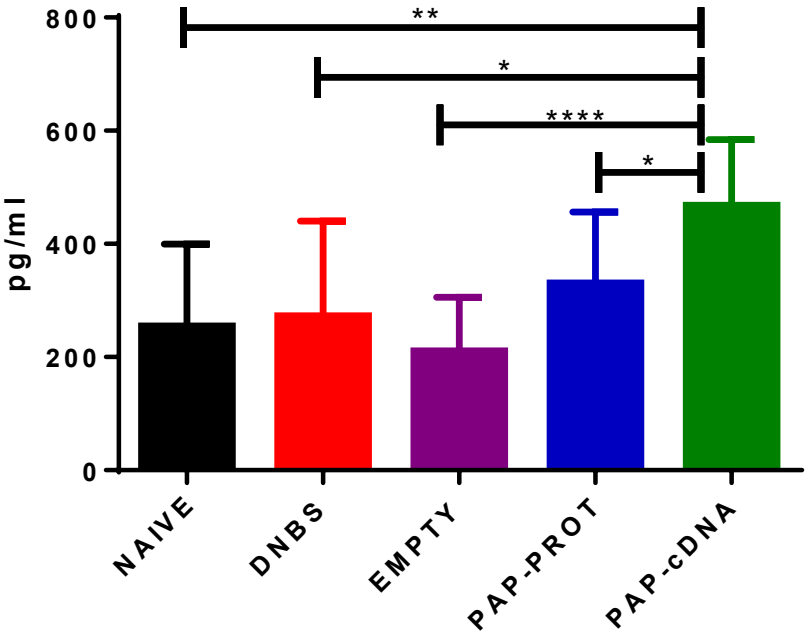

Fig 3. TGF-β secreted by reactivated lymphocytes from MLN in DNBS-induced colitis model. Mice were orally administered with EMPTY, PAP-PROT or PAP-cDNA during 5 days before and during 4 days after colitis induction. The MLN was recovered at the day of sacrifice. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001.

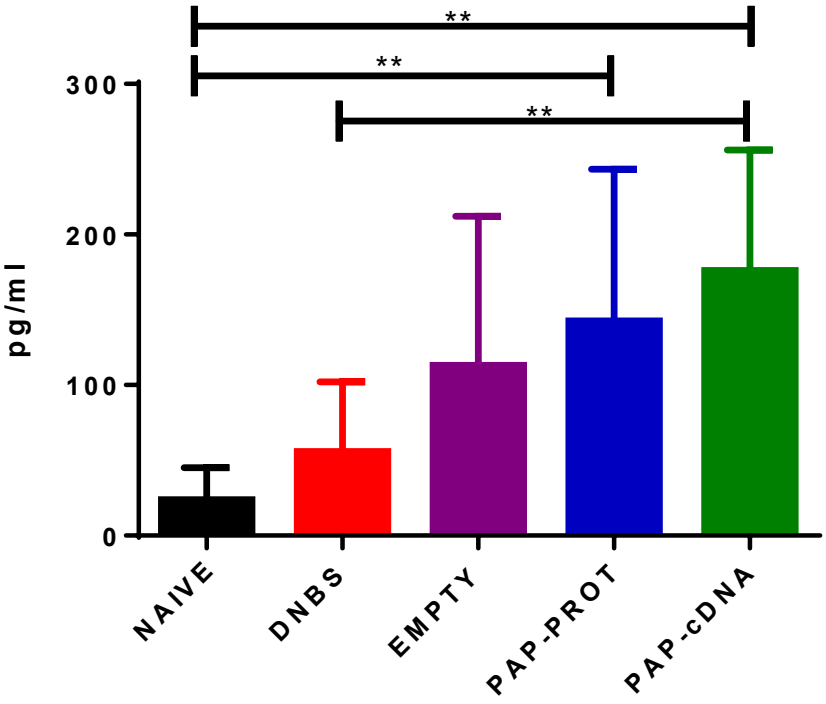

Fig 4. IL-10 secreted by reactivated lymphocytes from MLN in DNBS-induced colitis model. Mice were orally administered with EMPTY, PAP-PROT or PAP-cDNA during 5 days before and during 4 days after colitis induction. The MLN was recovered at the day of sacrifice. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001.

7 DISCUSSION

The present work had as main objective to compare the protection induced by a *Lactococcus lactis* strain delivering a plasmid for PAP expression by epithelial cells in DNBS-induced colitis with *L. lactis* secreting PAP. Preliminary studies have showed the potential of the cDNA delivery to treat inflammatory processes[3,9–14]. In this strategy the anti-inflammatory molecule of interest is produced by the eukaryotic cells of the host. So, in this approach, the molecule is produced directly at the site of interest[14,28–33]. Considering that, we hypothesized the delivery of PAP cDNA could enhanced the satisfactory results already showed by the protein delivery with PAP produced by *Lactococcus lactis*, thus we decided to compare both strategies.

According to the analysis of the weight loss 4 days after the induction of inflammation by DNBS, there is no difference between the groups DNBS (positive control), PAP-PROT and PAP-cDNA. This result is also seen at the macroscopic score, where there is a slight reduction in the groups PAP-PROT and PAP-cDNA when compared with DNBS group, but this difference is not significant. A probable reason for this close results could be the fact that the level of the inflammation induced by the DNBS in this experiment was lowest than usual. In general, it is expected a weight loss between 10 and 20% and a macroscopic score between 3 and 5[34–36] for the groups not treated (DNBS). Here, the highest weight loss for DNBS group was around 10% at day 1. Another possibility to explain that could be the high heterogeneity inside the groups, especially in DNBS groups, regarding the weight loss and macroscopic score. Otherwise, we can see a significant difference between PAP-PROT and EMPTY at day 4 and between PAP-cDNA and EMPTY at days 2, 3 and 4, allowing us to infer an early protective effect of PAPc-DNA on the weight loss compared to PAP-PROT. At the macroscopic score, we observe a significant reduction of the score in the groups PAP-PROT and PAP-cDNA compared with EMPTY group. These results agreeing with our previous data presented in the paper Scientific Reports (Breyner and Vilas Boas et al 2018 submitted) and with Darnaud et al 2018[37].

Taken altogether, we confirm the anti-inflammatory effect of PAP independent of the expression way (secreted or expressed by intestinal cells).

Despite the fact that PAP-PROT deliver PAP into the lumen and PAP-cDNA induces an over expression of PAP in epithelial cells, PAP-PROT or PAP-cDNA showed a protective effect in acute model of colitis induced by DNBS regarding weight loss and macroscopic score. Those results lead us to suggest that independent of the mechanism used to express PAP, when we use *L. lactis* as vector with daily gavage is possible to observe PAP anti-inflammatory properties[38–40]. Meanwhile, PAP-cDNA and PAP-PROT in these experiments didn't show significant effect on macroscopic score and weight loss when compared to DNBS group. However, there is a significant difference between EMPTY and PAPcDNA from the day 2 after DNBS-induced colitis, and between EMPTY and PAP-Prot from the day 4. Our results lead us to infer that *L. lactis* empty has a pro–inflammatory effect. Other authors have shown that the type of immune response elicited by LAB is strain dependent as they may favour a Th1 response, a Th2 humoral or tolerogenic, or only an inflammatory response[41–44]. Moreover, the PAP presence (being by protein secretion or cell over-expression) was able to overcome that effect and improve the animals' health status.

Analyzing the secretion of cytokines by reactivated lymphocytes from MLN, we can see an increase of TGF-β and IL10 by PAP-cDNA compared with DNBS, EMPTY and PAP-PROT. Both cytokines production are related to decreasing of inflammation status, since they are involved with Treg cells differentiation and activity. In according to previous studies, which showed this same profile, such as an increase of anti-inflammatory and a decrease of pro-inflammatory cytokines in MLN when an effective treatment of colitis is performed in different models of colitis[9,10,33,45–47]. Both cytokines increased by PAP-cDNA at this study, TGF-β and IL-10, are the primary mediators of local immune suppression. These cytokines have as the principal action the suppression of proinflammatory cytokines which play a critical role in the immune response of IBD. In other words, TGF-β and IL-10 are implicated in regulatory T cell function, preventing the activation and the effector function of T cells and mucosal macrophages that have escaped from other mechanisms of tolerance, contributing to the maintenance of homeostasis

in gut inflammation. These results may indicate a protective effect through Treg activation[48–52].

8 CONCLUSIONS

The main goal of this work was to compare two strains of *Lactococcus lactis* in a model of acute colitis. One of these strains was able to produce a recombinant protein with anti-inflammatory properties, PAP. The other one was able to deliver a cDNA to the production of the same protein by the intestinal cells of the mice. Both strains were tested in a model of acute colitis induced by intrarectal administration of DNBS and analyzed the protection induced by those strains.

The first aspect analyzed was the weight loss, which none of the strains was able to protect the mice when compared with DNBS group but showed a significant protection effect when compared with the group that received the treatment with the EMPTY bacterium. The same kind of result was seen on the macroscopic score, the second aspect analyzed. The last aspect observed was the production of cytokines by reactivated lymphocytes from MLN, where were detected the increased of TGF-β and IL-10 on PAP-cDNA group. Both, IL10 and TGF-β, are anti-inflammatory cytokines responsible to differentiation of Treg cells.

In resume, our results showed that both groups of *L. lactis* PAP do not show difference on end-clinical aspects, considering weight loss and macroscopic score, comparing with DNBS group. However, PAP-cDNA was able to protect against weight loss earlier than PAP-PROT. Moreover, PAP-cDNA induces the production of anti-inflammatory cytokines and this result may suggest an activation of Treg cells differentiation. Taken altogether, we can propose that the location of PAP delivery may influence its anti-inflammatory properties. These results confirmed the choice of the mechanism used to deliver a molecule is so important as the choice of the molecule *per si*. Further studies should be done to confirm the PAP-cDNA mechanism of protection.

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VIII GENERAL CONCLUSIONS

Our studies aimed to point out the anti-inflammatory properties of PAP. PAP is an antimicrobial peptide, belonging to enteric innate immune molecules, which plays an important role in gut barrier function and gut microbiota homeostasis[1]. PAP or RegIIIγ, as previously described, had showed widely related to the intestinal homeostasis. Several strategies to deliver PAP were tested and mechanisms to explain the beneficial effects on IBD were still elusive. Here, we presented three therapeutic approaches consisting of increasing the intra-luminal concentration of PAP aiming to preserve host-microbiota homeostasis and thus prevent intestinal inflammation and we propose a mechanism through PAP overcome the inflammation caused by DNBS.

Firstly, we tested a strain of *Lactococcus lactis* carrying the plasmid containing PAP attached to a secretion promoter. We confirmed the ability of this strain to secret PAP and we followed the test with this strain in both DNBS and DSS-induced colitis model. For the murine model of DNBS-inflammation, LL-PAP presented protective effects, considering weight loss, permeability to FITC, macroscopic and microscopic scores when compared to PBS or LL groups. However, in the protocol used to develop DSS-induced colitis, no difference in weight loss and DAI was observed in LL-PAP compared to PBS or LL groups. Our first results encouraged us to follow a deep analysis addressing to the mechanism of protection displayed by PAP secreted by *L. lactis*. Our landmark result at this point was *L. lactis* secreting PAP was able to modulate the immune response, increasing TGF-β production and decreasing pro-inflammatory cytokines. TGF-β is involved in Treg cells differentiation and these cells are implicated in control of immune response. Thus, we supposed PAP was able to improve the Treg cells population.

Farther, in order to confirm this hypothesis, cells were isolated from inflamed mice treated with *L. lactis* and *L. lactis* PAP, and healthy mice. Cells from these mice were stained with anti-CD4 to confirm those T cells, and anti-FoxP3 to quantify the Treg cell population. We observed that inflamed mice treated with *L. lactis* or *L. lactis* secreting PAP presented the same population of Treg, and more, they were at the same level of healthy mice. This result lead us to confirm that *L. lactis* was able to restore Treg cells, in a PAP-independent way. Moreover, we may

suggest *L. lactis* strain (with or without PAP) improved the Treg cell population; however, this feature was not enough to avoid the inflammation during the firsts 4 days after DNBS instillation. However, the question for which mechanism can PAP protect mice during the DNBS-induced inflammation was still unresponsive. Observing the Darnaud and colleagues' results, in which they showed an alteration in the microbiota ecology in mice when PAP was present, and consequently those animals were less sensitive to DSS-induced colitis[1]. Thus, we decided to investigate the microbiota of mice before receive DNBS, but previously treated daily with *L. lactis* and *L. lactis* secreting PAP. Could PAP secreted by *L. lactis* modulate microbiota to favor a better settlement of damage caused by DNBS? Therefore, it did. Mice treated only with *L. lactis* were prone to develop severe inflammation after DNBS instillation. However, mice treated with *L. lactis* secreting PAP presented an increase of an anaerobic strain, *Eubacterium plexicaudatum*, known as a butyrate producing, which is able to protect the integrity of intestinal epithelium and exert anti-inflammatory effects[2]. Consequently, those mice were resistant to the inflammation after DNBS instillation.

Then, in order to choose the better vehicle to deliver PAP into the lumen, we tested different vectors. In our work, we constructed for the first time a *Lactobacillus casei* carrying the plasmid containing PAP attached to a secretion promoter. Further, we confirmed the ability of this strain to secret PAP and we followed the test to compare *L. lactis* x *L. casei*, both secreting PAP, in a DNBS-induced colitis model. According to the capacity of these strains to remain in the intestinal tract, we tested different protocols concerning the time of administration, such as every day or each 3 days. Our results demonstrated the better vehicle to deliver PAP was *L. lactis* administrated daily. Mice treated with this protocol better recovered the weight, had lower macroscopic score, and finally presented an immunomodulation circumvented the inflammation caused by DNBS. As previous demonstrate in the literature, we confirmed the probiotic effect of *L. casei*, regarding its ability to circumvent the inflammation, however PAP did not interfere in this effect, neither improving nor worsen[3–6].

Previous studies have shown *the L. lactis* holding an eukaryote expression vector carrying cytokine gene, such as IL4 and IL10, protected mice against TNBS-induced and DSS-induced inflammations[7–9]. In order to improve these anti-inflammatory effects of PAP we decide to compare PAP-protein secreted by *L. lactis* directly into the lumen with PAP-cDNA delivered by *L. lactis*, able to integrate the cell host machinery, inducing PAP production by mammalian cells. Our results showed that cDNA strategy prompt the organism to fight early than PAP-protein. The protective mechanism should be further analyzed in terms of a microbiota modulation and the consequently resistance against DNBS-induced inflammation.

All results considered here, lead us to confirm the anti-inflammatory properties of PAP in a DNBS-induced colitis model by modulating the microbiota, increasing the secretion of anti-inflammatory cytokines and reducing the pro-inflammatory ones. In addition, mice treated with *L. lactis* secreting PAP presented less macroscopic damages, such as mucosal damage like ulcers and hyperemia; as well as less microscopic damages, for example villus and crypt length and architecture. Our work highlighted yet the importance of the vehicle to deliver the molecule of the interest and to achieve their anti-inflammatory effects.

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IX DIRECTIONS FOR FUTURE WORKS

As perspective of this work, concerning the luminal bacteria and their ability to limit the inflammation in the DNBS model, as we showed here, it is important to perform a fecal microbiota transfer. This approach will confirm the ability of the different microbiota modulated by PAP to increase the resistance to develop colitis after DNBS. Moreover, using this proposition we can confirm whether altered microbiota will be able to circumvent the problem, or PAP should be present to modulate the environment.

In order to confirm the protective effect of *L. lactis* containing PAP (cDNA) we should test in a DSS-induced colitis model. Albeit, in this present work, we have already shown that *L. lactis* PAP (protein) was not able to protect mice in the DSS model, Darnaud and colleagues[1] showed mice hepatocytes overproducing PAP were able to circumvent the inflammation caused by DSS by altered microbiota. In Darnaud's paper, they use transgene mice overexpressing PAP (RegIIIγ). Moreover, in our studies, we observed an earlier protective effect in DNBS model when mice were treated with LL-PAPcDNA. Another interesting point that justify both tests, fecal transplant and DSS model, is the ability of the mice overexpressing PAP in Darnaud's paper to alter the microbiota and reduce ROS, preventing the inflammation. In addition, they confirmed that through fecal transplantation, receptor mice were protected against DSS inflammation. To note, DSS model is widely used for the screening of potential therapeutic agents[2,3]. In addition, DSS does not require T or B cell response, but the luminal bacteria may play a role in the development of this type of colitis[2,4].

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APENDIX

PUBLICATIONS DURING THE PhD

BREYNER, NATALIA M. ; MICHON, CRISTOPHE ; DE SOUSA, CASSIANA S. ; **VILAS BOAS, PRISCILLA B.** ; CHAIN, FLORIAN ; AZEVEDO, VASCO A. ; LANGELLA, PHILIPPE ; CHATEL, JEAN M. . Microbial Anti-Inflammatory Molecule (MAM) from Faecalibacterium prausnitzii Shows a Protective Effect on DNBS and DSS-Induced Colitis Model in Mice through Inhibition of NF-κB Pathway. Frontiers in Microbiology (Online), v. 8, p. 114, 2017.

ALMEIDA, SINTIA ; DORNELES, ELAINE M. S. ; DINIZ, CARLOS ; ABREU, VINÍCIUS ; SOUSA, CASSIANA ; ALVES, JORIANNE ; CARNEIRO, ADRIANA ; **BAGANO, PRISCILLA** ; SPIER, SHARON ; BARH, DEBMALYA ; LAGE, ANDREY P. ; FIGUEIREDO, HENRIQUE ; AZEVEDO, VASCO . Quadruplex PCR assay for identification of Corynebacterium pseudotuberculosis differentiating biovar Ovis and Equi. BMC Veterinary Research, v. 13, p. 1, 2017.

ALMEIDA, JULIANA F. ; BREYNER, NATALIA M. ; MAHI, MILOUD ; AHMED, BENSOLTANE ; BENBOUZIANE, BOUASRIA ; **BOAS, PRISCILLA C.B. VILAS** ; MIYOSHI, ANDERSON ; AZEVEDO, VASCO ; LANGELLA, PHILIPPE ; BERMÚDEZ-HUMARÁN, LUIS G. ; CHATEL, JEAN-MARC . Expression of fibronectin binding protein A (FnBPA) from Staphylococcus aureus at the cell surface of Lactococcus lactis improves its immunomodulatory properties when used as protein delivery vector. Vaccine (Guildford), v. x, p. 1, 2016.

Title: Pancreatitis-associated protein (PAP) produced by different lactic acid bacteria can protect mice in an acute colitis model after oral delivery.

Keywords: Inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC), Pancreatitis Associated Protein I (PAP)

Abstract: Inflammatory bowel disease (IBD) is a group of chronic, complex and relapsing inflammatory conditions of GIT that has been a global health problem, with an increasing incidence. IBD is a group of closely related but heterogeneous disease processes. It includes two main forms, Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by alternating phases of clinical relapse and remission. One of the molecules that has been studied by our research group in the treatment of IBD is the Pancreatitis Associated Protein I (PAP). PAP is expressed in the gastrointestinal, with their expression focused in the crypt base spreading from Paneth cells of jejunum and ileum and by the goblet cells and enterocytes in the colon, and is up-regulated in patients with inflammatory bowel disease. PAP has a variety of activities, which includes anti-apoptotic, anti-inflammatory, antibacterial effects and proliferative, maintaining host-bacterial homeostasis in the mammalian gut. Several new strategies using lactic acid bacteria (LAB) for the expression or ability to metabolize molecules capable of reducing inflammation in inflammatory bowel diseases have been studied in recent years. Some strains of LABs have been considered as probiotics, which means “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”. Here, we first sought to determine whether PAP delivered at intestinal membrane by recombinant *Lactococcus lactis strain*, LL-PAP, is able to modulate the microbiota community and reduce the chemically induced intestinal inflammation. After a DiNitro-BenzeneSulfonic-acid (DNBS) challenge, mice treated with LL-PAP showed a decrease in the colitis severity compared to those treated with the control *L. lactis* strain. This effect was characterized by:

protection against weight loss; lower macroscopical and histological scores; and down-regulation of pro-inflammatory cytokines secreted by lymphocytes in Mesenteric Lymph Node (MLN). Moreover after 5 days of treatment LL-PAP was able to increase the diversity of the microbiota and relative abundance of *Eubacterium plexicaudatum*, a butyrate producer. Based on our findings, we hypothesize that a treatment with LL-PAP shift the microbiota preventing thus the severity of colon inflammation in acute colitis model through increase of *Eubacterium plexicaudatum*, butyrate-producing bacterium, which the mechanism is still elusive. Then, two important representants of LABs group, *Lactococcus lactis* and *Lactobacillus casei*, were used to express PAP and tested in the treatment of acute colitis induced by DNBS. Beyond the comparison between both strains it was also compared two different protocols of administration, every day or every 3 days, considering the persistence time. The analysis of weight loss, macroscopic score and cytokines showed us that *L. lactis* should be administered every day to confer protection, while *L. casei* should be administered every 3 days to show a tendency to protect mice. Our data showed the importance of the vector and the timing of the treatment, independent for which molecule is going to be tested in the treatment of induced-colitis. For that kind of approach, is clear the importance of a previous test to define the scheme of bacterium administration. We also performed the evaluation of the protection induced by a *L. lactis* strain delivering a plasmid for PAP expression by epithelial cells, LL-PAP cDNA, compared with LL-PAP in a murine model of DNBS acute colitis. Our results showed that both groups of recombinant *L. lactis* showed the same protective effect compared with LL empty group. Moreover, PAP-cDNA was able to induce the production of anti-inflammatory cytokines. Taken altogether, we can infer that the location of PAP delivery may influence its anti-inflammatory properties but showed the same effect regarding weight loss and macroscopic scores. These results confirmed the choice of the mechanism used to deliver the

molecule is as important as the choice of the molecule per se.

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Titre: Étude des effets protecteurs de la protéine PAP et de ses mécanismes d'action dans des modèles de colite aiguë

Mots clés: maladies inflammatoires chroniques de l'intestin (MICI), maladie de Crohn (CD), rectocolite hémmoragique (UC), pancreatitis associated protein (PAP)

Résumé: Les maladies inflammatoires chroniques de l'intestin (MICI) sont un groupe d'affections inflammatoires chroniques complexes et récurrentes du tractus gastro-intestinal (TGI) qui constitue un problème de santé mondial à incidence croissante. Les MICI ont des processus pathologiques étroitement liés mais hétérogènes. Elles comprennent deux formes principales, la maladie de Crohn (CD) et la rectocolite hémmoragique (UC), caractérisées par des phases alternées de rechute clinique et de rémission. L'une des molécules étudiées par notre groupe de recherche dans le traitement des MICI est la pancreatitis associated protein (PAP). La PAP est exprimée dans le système gastro-intestinal, son expression étant centrée dans les cryptes à partir des cellules de Paneth du jéjunum et de l'iléon, ainsi que dans les cellules caliciformes et les entérocytes dans le côlon. Elle est régulée positivement chez les patients atteints de MICI. La PAP a une variété d'activités, notamment des effets anti-apoptotiques, anti-inflammatoires, antibactériens, antiprolifératifs et globalement le maintien de l'homéostasie hôte-bactérienne dans les intestins des mammifères. Plusieurs nouvelles stratégies utilisant des bactéries lactiques (LAB) pour l'expression ou la capacité à métaboliser des molécules capables de réduire l'inflammation dans les maladies inflammatoires de l'intestin ont été étudiées ces dernières années. Certaines souches de LAB sont considérées comme des probiotiques, ce qui signifie "des

micro-organismes vivants qui, lorsqu'ils sont administrés en quantités suffisantes, confèrent un bénéfice pour la santé de l'hôte". Ici, nous avons d'abord cherché à déterminer si la PAP délivrée au niveau de la membrane intestinale par la souche recombinante de *Lactococcus lactis*, LL-PAP, était capable de moduler la communauté du microbiote et de réduire l'inflammation intestinale induite chimiquement. Après une administration d'acide DiNitro-benzène sulfonique (DNBS), les souris traitées avec la LL-PAP ont montré une diminution de la sévérité de la colite par rapport à celles traitées avec la souche contrôle *L. lactis*. Cet effet est caractérisé par: une protection contre la perte de poids; des scores macroscopiques et histologiques inférieurs; et la régulation à la baisse des cytokines pro-inflammatoires sécrétées par les lymphocytes dans les ganglions mésentériques (MLN). De plus, après 5 jours de traitement, la LL-PAP augmente la diversité du microbiote et l'abondance relative *d'Eubacterium plexicaudatum,* une bactérie productrice de butyrate. Sur la base de nos résultats, nous émettons l'hypothèse que le traitement par LL-PAP modifie le microbiote, empêchant ainsi la gravité de l'inflammation du côlon dans le modèle de colite aiguë en augmentant *Eubacterium plexicaudatum*. Ensuite, deux représentants importants du groupe LAB, *Lactococcus lactis* et *Lactobacillus casei*, ont été utilisés pour exprimer la PAP et testés dans le traitement de la colite induite par la DNBS. Au-delà de la comparaison entre les deux souches, il a également été comparé deux protocoles d'administration différents, tous les jours ou tous les 3 jours. L'analyse de la perte de poids, du score macroscopique et des cytokines ont montré que *L. lactis* devait être administré tous les jours pour conférer une protection, tandis que *L. casei* devait être administré tous les 3 jours afin de montrer une tendance à la protection des souris. Nos données ont montré l'importance du vecteur et le moment du traitement, quelle que soit la molécule à tester dans le traitement de la colite induite. Pour ce type d'approche, il est clair qu'un test préalable permet de définir le schéma d'administration de bactéries. Nous

avons également effectué l'évaluation de la protection induite par la souche de *L. lactis* délivrant un plasmide pour l'expression de PAP par les cellules épithéliales, contenant l'ADNc de LL-PAP, par rapport à LL-PAP dans un modèle murin de colite aiguë au DNBS. Nos résultats ont montré que les deux groupes de *L. lactis* recombinant présentaient le même effet protecteur par rapport au groupe vide LL. De plus, l'ADNc de PAP était capable d'induire la production de cytokines anti-inflammatoires. Dans l'ensemble, nous pouvons en déduire que la localisation de la délivrance de PAP peut influer sur ses propriétés anti-inflammatoires, mais qu'elle a eu le même effet sur la perte de poids et les scores macroscopiques. Ces résultats confirment l'importance du choix du mécanisme utilisé pour délivrer la molécule et l'importance de la molécule en soi.