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Tese de Doutorado

Caracterização e Avaliação das Propriedades Probióticas de *Lactococcus lactis* NCDO 2118

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"Caracterização e avaliação das propriedades probióticas de *Lactococcus lactis* NCDO2118"

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"Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos".

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Capítulo1: Human gastrointestinal tract microbiota in health and disease

Capítulo 2: Anti-inflammatory effects of *Lactococcus lactis* NCDO 2118 during the remission period of chemically induced colitis

RESUMO

A prevalência das doenças inflamatórias intestinais (IBD, sigla do inglês *inflammatory bowel diseases*) tem aumentado em países que adotam um estilo de vida ocidental, incluindo o Brasil. As duas principais formas da IBD são a Colite Ulcerativa (UC, Ulcerative Colitis) e Doenças de Crohn (CD, Crohn's Disease). Descobertas há quase 100 anos atrás, a etiologia dessas doenças ainda não é bem compreendida, entretanto, existem crescentes evidências que as IBD resultam de uma resposta imune anormal à microbiota do intestino em indivíduos com predisposição genética, resultando em uma inflamação crônica do trato gastrointestinal. Os tratamentos, atualmente disponíveis, acarretam sérios efeitos colaterais. Estudos recentes tem demonstrado o potencial terapêutico de bactérias probióticas no tratamento destas doenças apresentando resultados promissores. Grande parte dos probióticos pertence ao grupo das Bactérias Lácticas (BL), que fazem parte de uma microbiota saudável. Entretanto pouco se sabe sobre os efeitos de bactérias presentes em produtos lácteos que fazem parte da nossa dieta, incluindo *Lactococcus lactis*. Neste estudo objetivou-se avaliar o potencial imunomodulador de três linhagens de *L. lactis* e analisar o seu potencial probiótico na prevenção de colite em modelo murino. Em primeiro lugar, analisamos o potencial de modulação imunológica de três lihagens de *L. lactis* (*L. lactis* subsp. *cremoris* MG1363, *L. lactis* subsp. *lactis* IL1403 e *L. lactis* subsp. *lactis* NCDO 2118) *in vitro*. O sobrenadante e as células de *L. lactis* foram co-cultivadas com as células epiteliais do intestino (células Caco-2) na presença da citocina pró-inflamatória IL-1β. Apenas uma linhagem, *L. lactis* NCDO 2118, foi capaz de reduzir os níveis de secreção de IL-8 induzida por IL-1β em células Caco-2, sugerindo um potencial efeito anti-inflamatório para esta linhagem. *In vivo*, esta linhagem foi administrada durante 4 dias em camundongos C57BL/6 durante um período de remissão entre um primeiro e um segundo curso de colite induzida por sulfato de sódio dextrano. O tratamento com *L. lactis* NCDO 2118 resultou em uma forma mais branda da colite recorrente do que a observada em camundongos que receberam apenas meio de cultura durante este mesmo período. Administração de *L. lactis* foi associada com o aumento precoce da citocina IL-6 e pela manutenção da citocina IL-10 no tecido do cólon. Camundongos que receberam *L. lactis* NCDO 2118 tiveram aumento no número de células T reguladoras que apresentam na superfície TGF-β sob a forma de peptídeo associado à latência ($CD4^+LAP^+$) em linfonodos mesentéricos e no baço bem como de células dendrídicas tolerogênicas. Os resultados deste estudo permitiram identificar uma nova linhagem anti-inflamatória, que, no futuro, poderá representar uma alternativa ao tratamento de IBD.

ABSTRACT

The prevalence of inflammatory bowel diseases (IBD) has increased in countries that adopt Western lifestyle, including Brazil. The two main IBD associated diseases are Ulcerative Colitis and Crohn's Disease. Discovered nearly 100 years ago, the etiology of these diseases is not yet fully understood; however, there is growing evidence that IBD results from abnormal immune responses to the gut microbiota in individuals with genetic predisposition, resulting in a chronic inflammation in the gastrointestinal tract. The treatments currently available are accompanied by serious side effects. Recent studies have demonstrated the potential therapeutic use of probiotic bacteria in the treatment of these diseases, and many probiotics, have generat promising results. Most of them belong to the Lactic Acid Bacteria (LAB) group, which makes part of our healthy microbiota. However, little is known about the effects of transiting dairy bacteria that make part of our diet, including *Lactococcus lactis*. In this study we aimed to evaluate the immunomodulatory potential of three strains of *L. lactis* and analyzed their probiotic potential in the prevention of colitis in murine model. Firstly, we analyzed the potential immune modulatory effects of three *L. lactis* strains (*L. lactis* subsp. *cremoris* MG1363, *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *lactis* NCDO 2118) *in vitro*. The supernatant and the *L. lactis* cells were co-cultured with intestinal epithelial cells (Caco-2 cells) in the presence of the proinflammatory cytokine IL1-β. Only one strain, *L. lactis* NCDO 2118, was able to reduce the IL1-β-induced IL-8 secretion in Caco-2 cells, suggesting a potential anti-inflammatory effect for this strain. *In vivo*, this strain was administered for 4 days to C57BL/6 mice during a remission period between a first and second course of colitis induced by dextran sulfate sodium. *L. lactis* NCDO 2118 treatment resulted in a milder form of recurrent colitis than observed in mice administered medium during this same period. Administration was associated with early increase in IL-6 production and maintenance of IL-10 in colonic tissue. Mice fed with *L. lactis* NCDO 2118 had an increased number of regulatory T cells bearing surface TGF-β in the form of latency-associated peptide ($CD4^+LAP^+$) in mesenteric lymph node and spleen as well as the number of tolerogenic dendritic cells. The results of this study allowed us to identify a new probiotic strain which may represent an alternative for IBD treatment.

INTRODUÇÃO GERAL

As interações bactéria-hospedeiro tem gerado crescente interesse junto ao meio científico devido a sua importância para a saúde humana. Bactérias residentes no trato gastrointestinal (TGI) podem estabelecer uma relação simbiótica com o hospedeiro, contribuindo com o desenvolvimento da arquitetura da mucosa intestinal, metabolismo de componentes alimentares não absorvidos, produção de vitaminas, antagonismo à patógenos e, modulando a barreira epitelial de mucosa e o sistema imunológico, favorecendo, assim, a homeostase intestinal. Em condições fisiológicas normais, a microbiota mutualista irá induzir um estado de tolerância imunológica (Faria e Weiner, 2006; Untersmayr e Jensen-Jarolim, 2006), sendo que a perturbação dessa tolerância poderá desencadear uma variedade de doenças tanto alérgicas quanto inflamatórias do TGI.

As doenças inflamatórias intestinais (IBD, do inglês, *Inflammatory Bowel Disease*) vêm apresentando uma incidência aumentada no mundo inteiro, sendo considerado um importante problema de saúde pública global (Gismera e Aladren, 2008). As IBD referem-se a desordens inflamatórias crônicas que afetam severamente o TGI, podendo levar, em longo prazo, ao comprometimento de sua estrutura e função de modo irreversível (Nikolaus *et al*., 1998; Shanahan, 2002).

As IBD são caracterizadas por etapas alternadas de recaída e remissão clínica, e têm sido associadas com o aumento do risco de câncer intestinal (Geremia, *et al*., 2013). Apresentam-se sob duas formas clínicas: em um processo inflamatório multifocal e transmural que pode afetar grande parte do trato digestivo (Doença de Crohn, CD, do inglês, *Crohn's Disease*), ou em uma inflamação contínua e superficial limitada ao intestino grosso ou cólon (Colite Ulcerativa, UC, do inglês, *Ulcerative Colitis*) (Nell, *et al*., 2010; Maloy, e

Powrie, 2011). Ambas as formas da doença apresentam como principais sintomas clínicos dor abdominal, diarréia, sangramento retal, mal-estar e perda de peso (Kaser, *et al*., 2010).

As IBD são doenças complexas cuja etiologia exata não está bem esclarecida, no entanto, evidências demonstram que indivíduos acometidos com IBD apresentam perda da tolerância à microbiota do TGI gerando inflamação (Kaser, *et al*., 2010). Acredita-se que o desenvolvimento da IBD está relacionado a uma combinação de fatores como a susceptibilidade genética, composição da microbiota, resposta imune e fatores ambientais (Figura 1) (Sartor, 2006).

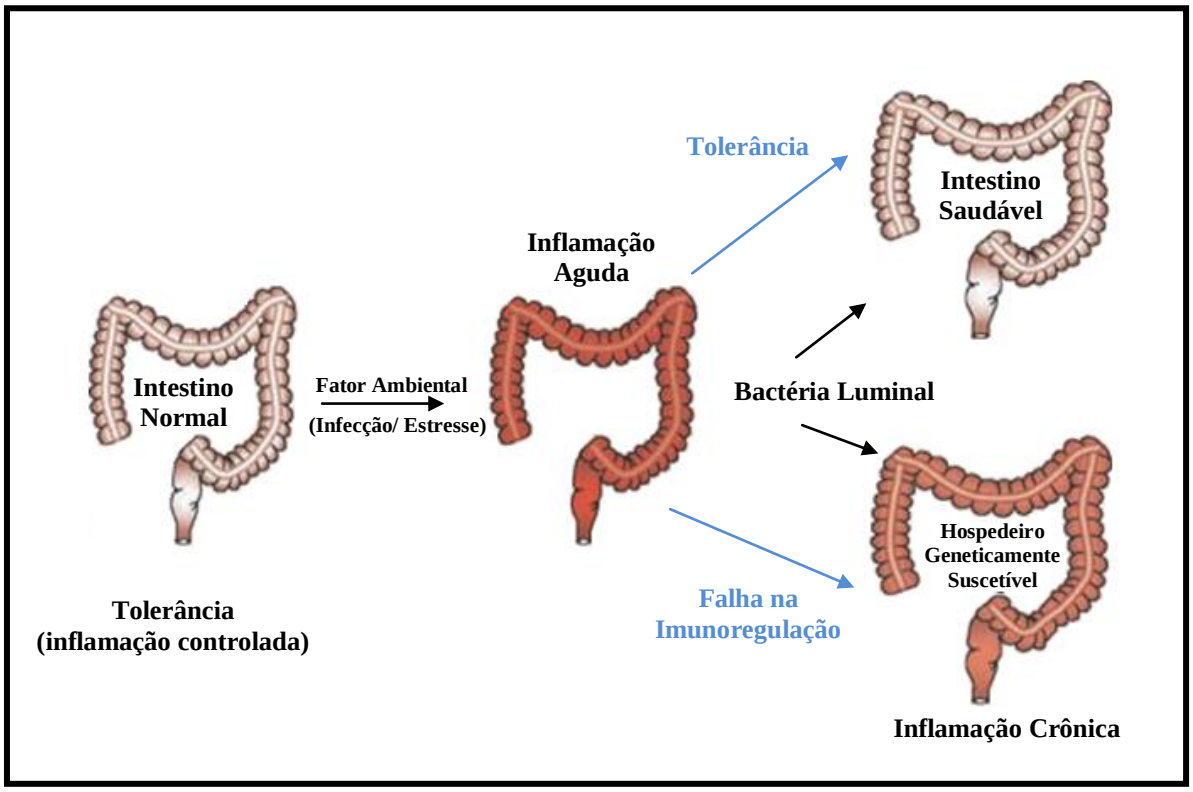

Figura 1. Resposta à agressão intestinal em hospedeiros geneticamente suscetíveis e resistentes a IBD. Adaptado de Sartor, 2006.

As opções de tratamento atualmente disponíveis contra as IBD incluem os corticosteróides e imunossupressores. Tais drogas, apesar de melhorarem o processo inflamatório, apresentam uma eficácia moderada e seu uso tem sido associado com sérios

efeitos colaterais. Neste contexto, em busca de novas terapias, mais seguras, eficazes e duradouras, o uso de probióticos tem-se mostrado uma alternativa promissora.

Probióticos são definidos como "microrganismos vivos que quando administrados em quantidades adequadas conferem benefício à saúde do hospedeiro" (FAO/WHO, 2002). A administração de probióticos tem sido extensivamente estudada como ferramenta para restaurar a tolerância à microbiota comensal, e, vários estudos, tanto em modelos animais quanto em ensaios clínicos em humanos, vem gerando resultados eficazes na prevenção/tratamento das IBD. Apesar dos mecanismos de ação implicada nos efeitos probióticos ser ainda pouco caracterizados, estes, podem ser agrupados em três principais categorias: (i) alteração da diversidade microbiana, (ii) o aumento da função de barreira epitelial, e (iii) a modulação da resposta imune (Servin e Coconnier, 2003; Lebeer, *et al*., 2008).

Grande parte dos probióticos estudados hoje em dia pertence ao grupo das bactérias lácticas (BL). As BL compreendem um grupo heterogêneo de bactérias Gram-positivas, não esporuladas e anaeróbias facultativas que tem como característica em comum a produção de ácido láctico como principal produto do metabolismo de carboidratos. *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Oenococcus* e *Lactococcus* compreendem os principais gêneros das BL (Ljungh, *et al*., 2006).

Dentre as BL, alguns membros podem ser classificados como verdadeiros residentes ou comensais, os quais colonizam o TGI formando uma comunidade estável e mantendo uma associação com um habitat em particular. Outras bactérias estão constantemente em trânsito através da alimentação e não possuem a habilidade de colonizar o TGI. Linhagens comensais de *Lactobacillus* são os probióticos mais bem caracterizados. O gênero *Lactococcus*, por sua vez, tem recebido pouca atenção sobre seu potencial probiótico, principalmente por não ser considerado um habitante natural do TGI. No entanto, sabe-se que não apenas bactérias

comensais apresentam ação probiótica, mas também bactérias que estão em trânsito pelo TGI através da ingestão de produtos fermentados (Santos Rocha, *et al*., 2012). Diante disso, surgiu nosso interesse em estudar o potencial probiótico do gênero *Lactococcus,* gênero este que inclui várias espécies utilizadas na produção de queijo há milhares de anos.

Lactococcus lactis é a espécie de BL mais bem caracterizada e figura como organismo modelo no estudo das mesmas; não só pela sua importância econômica, mas também devido ao fato de: (i) ser um microrganismo de fácil manipulação; (ii) ser "GRAS" (Generally Recognized As *S*afe); (iii) ter sido a primeira BL cujo genoma foi seqüenciado e (iv) possuir um grande número de ferramentas genéticas já desenvolvidas (Poquet, *et al.*, 1998; Duwat, *et al.*, 2000; Ravn, *et al.*, 2000; Bolotin, *et al.*, 2001; Nouaille, *et al.*, 2003; Mills, *et al*., 2006).

Existem duas subespécies de *L. lactis*: *L. lactis* subsp. *lactis* e *L. lactis* subsp. *cremoris* (Schleifer, 1987), sendo a linhagem *L. lactis* subsp. *lactis* IL1403 a primeira BL seqüenciada e extensivamente utilizada para a produção de diferentes produtos metabólicos, como vitamina B, diacetil e alanina, além da produção de proteínas recombinantes (Bolotin, *et al*., 2001; Kleerebezem, *et al*., 2002). Já a linhagem *L. lactis* subsp. *cremoris* MG1363 é a mais utilizada na pesquisa genética e fisiológica em todo mundo, sendo utilizada em várias aplicações biotecnológicas, como por exemplo, carreando vacinas orais ou peptídeos bioativos ao TGI (Hanniffy, *et al*., 2007).

L. lactis subsp. *lactis* NCDO 2118, nossa linhagem de interesse, é um isolado de ervilha congelada, que é utilizada rotineiramente em nosso laboratório para a produção de proteínas heterólogas. Recentemente, esta linhagem foi descrita como produtora de ácido gama-aminobutírico (GABA) (Mazzoli, *et al*., 2010). O GABA é produto da descarboxilação do L-glutamato mediado pela enzima glutamato descarboxilase (GAD, EC 4.1.1.15) e é conhecido por ter efeitos positivos sobre a saúde humana, sendo o neurotransmissor mais amplamente distribuído no sistema nervoso central de vertebrados. Este neurotransmissor é

capaz de diminuir a pressão sangüínea de pacientes levemente hipertensos (Inoue, *et al*., 2003), induzir efeito tranquilizante e diurético (Jakobs, *et al*., 1993; Wong, *et al*., 2003), prevenir diabetes (Hagiwara, *et al*., 2004), além de reduzir os níveis de resposta inflamatória de artrite reumatóide em modelo murino (Tian, *et al*., 2011).

OBJETIVO

Esta tese objetivou caracterizar e avaliar as propriedades probióticas de *L. lactis* subsp. *lactis* NCDO 2118, mais especificamente, estudar seu efeito imunomodulatório no contexto das IBD.

DELINEAMENTO DA TESE

Esta tese encontra-se dividida em dois capítulos. Antes de cada capítulo encontram-se resumos em português. Questões específicas abordadas em cada um destes encontram-se detalhadas abaixo:

Capítulo 1- Podemos considerar esta revisão como uma introdução aprofundada que revisa o conhecimento gerado ao longo dos anos sobre o trato gastrointestinal humano, na saúde e na doença, com destaque para as interações probiótico-hospedeiro no contexto das doenças inflamatórias intestinais. A revisão está em processo de submissão para a revista "FEMS Microbiology".

Capítulo 2- Demonstra a avaliação *in vitro* e *in vivo* do potencial imunomodulatório da linhagem *Lactococcus lactis* subsp. *lactis* NCDO 2118 em células epiteliais intestinais (IECs) e em um modelo murino de colite, respectivamente. O artigo está em processo de submissão para a revista "Plos One".

Ao final do Capítulo 2, apresentamos uma discussão geral, bem como as conclusões obtidas a partir deste trabalho.

CAPÍTULO 1

Microbiota do trato gastrointestinal humano na saúde e no contexto das IBD

Resumo

O trato gastrointestinal humano é colonizado por um número dez vezes maior de células bacterianas (microbiota) do que o número de células corporais, sendo que esta microbiota é capaz de impactar a saúde e o bem estar do hopedeiro. O advento de técnicas de biologia molecular, como o sequenciamento em larga escala de DNA de genomas bacterianos e de amostras de comunidades complexas do trato gastrointestinal (metagenoma) tem permitido conhecer melhor essas populações bacterianas, incluindo aquelas de difícil cultivo em laboratório. As bactérias residentes no trato gastrointestinal podem impactar seu hospedeiro tanto de forma positiva (simbiose), contribuindo com o metabolismo de produtos inacessíveis, na maturação e modulação do sistema imune, como de forma negativa (disbiose), em que a microbiota contribui com a patogênese de algumas doenças, como as doenças inflamatórias intestinais. Algumas bactérias isoladas do trato gastroitestinal humano, bem como aquelas que estão em constante trânsito pelo trato gastrointestinal através da alimentação, podem exercer efeitos benéficos ao hospedeiro. Essas bactérias, hoje em dia, são conhecidas como probióticos. Estudos têm demonstrado que uso de probióticos pode alterar a diversidade microbiana, aumentar a função da barreira epitelial e modular as respostas imunes do hospedeiro.

Este capítulo apresenta uma revisão bibliográfica sobre o impacto da microbiota intestinal para a saúde do hospedeiro, demonstrando a importância dos probióticos no tratamento e prevenção das doenças inflamatórias intestinais.

Human gastrointestinal tract microbiota in health and IBD context

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Abbreviations:

ATCC, American Type Culture Collection; CD, Crohn's disease; CpG-DNA, cytosine–phosphate–guanosine DNA; DC, dendritic cell; DNA, deoxyribonucleic acid; dsRNA, double stranded RNA; DSS, dextran sodium sulfate; EPS, exopolysaccharides; GALT, gut associated-lymphoid tissue; GF, germ-free; GI, gastrointestinal; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocytes; IκB, inhibitor of NF-κB; LAB, lactic acid bacteria; LPS, lipopolysaccharides; LTA, lipoteichoic acid; MAMPs, microbial-associated molecular patterns; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; NLRs, Nod-like receptors; NOD, nucleotide oligomerization domain; PGN, peptidoglycan; PRRs, pattern recognition receptors; RNA, ribonucleic acid; rRNA, ribosomal ribonucleic acid; SCFA, short chain fatty acid; SED, subepithelial dome; STAT, signal transducer and activator of transcription; SOC, suppressor of cytokine signaling; ssRNA, single-stranded RNA; TLR, toll-like receptor; TNBS, tri-nitro-benzene-sulfonic acid; TNF, tumor necrosis factor; Treg, regulatory T cells; UC, ulcerative colitis.

ABSTRACT

The human gastrointestinal tract is colonized by ten times more bacterial cells than human cells in the body and this microbial community can impact the health and the well-being of their host. Recent application of molecular biology techniques, including the high throughput sequencing of bacterial genomes, allowed a more representative description of the gastrointestinal tract microbiota members. The microbiota can influence their hosts in a positive way (symbiosis) contribuiting to the metabolism of undigested compounds and for the modulation of the immune system, and also in a negative way (dysbiosis), in which they can influence the pathogenesis os several deseases, including inflammatory bowel disesases. Some of the microbiota members isolated from the gastrointestinal tract, as well as transiting food bacteria, can exert beneficial effects in their hosts. They are now recognized as probiotics and several studies have demonstrated that they are able to alter the microbiota diversity, increase the epithelial barrier function and modulate immune responses. Here, we present a review of the present knowledge on the impact of the gut microbiota to the human health and the use of probiotics in the treatment of inflammatory bowel diseases.

INTRODUCTION

The study of bacteria - host interactions and their importance for human health is an area of growing interest. While much progress has been made in the understanding of interactions between pathogenic bacteria and the host, there is an increasing interest for commensal bacteria that reside in the human gastrointestinal tract (GIT), which constitute the densest bacterial population known to date. A growing number of studies indicate that the intestinal microbiota influences host energy balance and immune responses, and contributes to gut homeostasis (Bouma and Strober, 2003; Sartor, 2006, 2008). More recently, several studies indicate correlations between disturbed microbiota composition (dysbiosis) and diseases such as inflammatory bowel diseases (IBDs) (Kalliomaki and Walker, 2005; Winkler, *et al*., 2007; Artis, 2008; Hill and Artis, 2010; Santos Rocha, *et al*., 2012), obesity (Ley, *et al*., 2006; Neish, 2009), diabetes (Gill, *et al*., 2006; Rajilic-Stojanovic, *et al*., 2007), cancer (Mackie, *et al*., 1999; Huurre, *et al*., 2008) and several extra intestinal diseases (Mandar and Mikelsaar, 1996; Wang, *et al*., 2005; Guarner, 2006; O'Hara and Shanahan, 2006; Zoetendal, *et al*., 2006).

IBDs, including ulcerative colitis (UC) and Crohn's disease (CD), are characterized by a spontaneous and chronic inflammation of the GIT. Although the causes for IBD remain obscure, there is growing evidence that IBD results from abnormal immune responses to the gut microbiota in genetically predisposed individuals. Patients with IBD present an abnormal luminal microbiota and it has been suggested that a lack of bacteria with anti-inflammatory properties could be a key factor in the persistence of inflammation. The administration of probiotic bacteria may restore the balance and several probiotics have been proposed for IBD treatment, showing a protective effect in animal models of experimental colitis and for some of them also in human clinical trials. However, the mechanisms underlying the probiotic

effects are still poorly understood. Here we present a review of the present knowledge on bacteria - host interactions in the GIT with a special focus on IBDs. Mice deficient in IL-10 spontaneously develop enterocolitis from 3 months of age in a manner similar to human Crohn's disease (Kuhn *et al.,* 1993), however, when these mice are maintained in germ free conditions do not develop colitis, revealing the role of microbial antigens in triggering the disease (Elson and Cong, 2002).

THE GIT MICROBIOTA

The human GIT is colonized by up to 10^{14} bacteria, ten times more than the number of cells in the human body (Whitman, *et al*., 1998; Walter, 2008). Under normal conditions, the intestinal microbiota lives in a symbiotic relationship with the host (Eckburg, *et al*., 2005), where the host provides a nutrient-rich habitat, while the bacteria can supply essential functions and important benefits to the host. The study of commensal bacteria - host interactions is an area of growing interest and we are only beginning to appreciate the diversity and function of the gut microbiota. This knowledge is essential for a better understanding of the impact of the microbiota on our health and well-being, and to optimize the role that food or probiotic bacteria may play.

In humans, our knowledge on the composition of the GIT microbiota has long been based on microscopic observations and culturing of bacteria present in fecal samples. In this way some hundred species associated with the GIT have been distinguished (Hattori and Taylor, 2009). However, an estimated 70% of the GIT bacteria remain uncultivable and only the recent application of molecular biology techniques, including the sequencing of 16S rRNA genes and high throughput metagenomic sequencing, allowed a more representative description of the GIT microbiota. Today several research programs worldwide, such as MetaHIT (METAgenomics of the Human Intestinal Tract, http://www.metahit.eu) and the

NIH Human Microbiome project (http://commonfund.nih.gov/hmp/), aim to characterize the human GIT microbiota through large-scale sequencing of fecal DNA samples and individual bacterial genomes, thus providing an impression of the metagenome of the human GIT environment and the functions encoded therein (Turroni, *et al*., 2008). In the following paragraphs we will present an overview of the composition of the human gut microbiota based on these data.

The GIT is the most heavily colonized human organ and the colon alone is estimated to contain over 70% of all the microbes in the human body. Most of these microbes are bacteria and Archea, while fungi appear to be rare (Riesenfeld, *et al*., 2004; von Mering, *et al*., 2007). The composition and density of bacterial populations of healthy adults vary along the length of the GI tract (Reuter, 2001; Marteau and Shanahan, 2003). Relatively low numbers (up to 10^3) of bacteria are present in the upper GI tract (stomach, duodenum, jejunum and proximal ileum) where the presence of acid, bile and pancreatic secretions hamper bacterial colonization (Swidsinski, *et al*., 2005; Kang, *et al*., 2008). This part of the GI tract is populated by acid-tolerant bacteria such as streptococci and lactobacilli (Marteau and Shanahan, 2003; Borody, *et al*., 2004). Much higher numbers of bacteria reside in the lower compartments of the GI tract, where bacterial populations reach 10^{11} - 10^{12} per gram, the highest density for any microbial habitat known to date (Ott, *et al*., 2004) (Figure 1).

16S rRNA sequencing classified the majority of bacterial species in the human gut into three major phyla: the Gram-negative *Bacteroidetes* (23 to 48%) and the Gram-positive *Firmicutes* (48 to 76%) and *Actinobacteria* (0.2 to 38%) (Strober, *et al*., 2007). Most of the *Bacteroidetes* sequences are classified as *Bacteroides vulgatus* (31 % of Bacteroidetes), *Prevotellaceae* (23%), *Bacteroides thetaiotaomicron* (13%), *Bacteroides caccae*, *Bacteroides fragilis*, and *Bacteroides putredinis* phylotypes (Strober, *et al*., 2007).

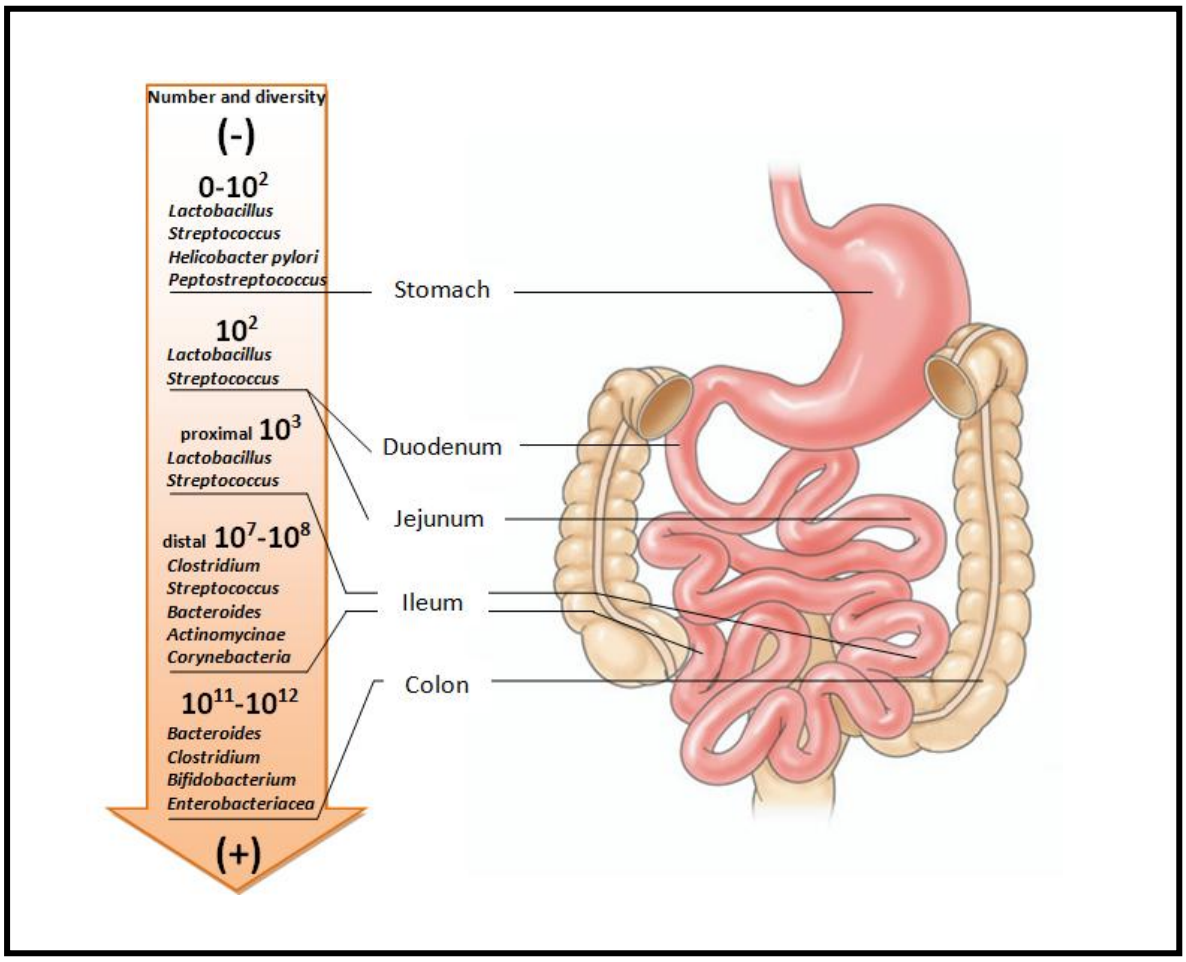

Figure 1. Distribution of predominant bacterial genera in the GI tract. Numbers indicate bacteria/gram (Figure adapted from (Sartor, 2008)).

Among the *Firmicutes*, *Clostridia* represent up to 82 % of the bacterial sequences, while 2 to 5 % and 0.2 to 11 % are members of the *Mollicutes* and *Bacilli* classes, respectively (Riesenfeld, *et al*., 2004; Strober, *et al*., 2007; Hattori and Taylor, 2009). The majority of Clostridia sequences belong to the genera *Eubacterium*, *Ruminococcus*, *Dorea*, *Butyrivibrio*, *Coprococcus* and *Faecalibacterium* (Strober, *et al*., 2007). Among the *Bacilli*, the genera *Streptococcus*, *Gemella*, and *Lactobacillus* are the most abundant. *Lactobacilli* constitute about 1 % of the fecal bacteria samples (Riesenfeld, *et al*., 2004; Borody, *et al*., 2004; Strober, *et al*., 2007). Contradictory results have been reported for the *Actinobacteria*, with frequencies varying from 0.2 to almost 38 %, depending on the age of the person (Marteau

and Shanahan, 2003; Riesenfeld, *et al*., 2004; Strober, *et al*., 2007; Sokol, *et al*., 2008). Irrespective of this variation, it appears that the majority of GI tract associated *Actinobacteria* correspond to the genera *Actinomyces*, *Corynebacterium, Rothia* and *Bifidobacterium* (Riesenfeld, *et al*., 2004; Strober, *et al*., 2007; Sokol, *et al*., 2009).

Metagenome sequencing represents a powerful alternative to rRNA sequencing for the analysis of complex microbial communities. The MetaHIT consortium recently published a metagenome sequence analysis of the human gut microbiota from fecal samples of 124 adult individuals (von Mering, *et al*., 2007). The data were used to establish a catalogue of non-redundant human intestinal microbial genes containing 3.3 million microbial genes, 150-fold more than the human gene complement. Essentially all (99,1%) of the genes identified are from bacterial origin, the remainder being mostly archaeal, with only 0.1% of eukaryotic and viral origins, thus confirming the previous results found by other metagenomic study (Riesenfeld, *et al*., 2004). The authors showed that almost 40% of the genes from each individual are shared with at least half of the other individuals analyzed. They also identified 75 species common to >50% of individuals and 57 species common to >90%, suggesting that this large number of shared genes and species supports the view that the prevalent human microbiota is of a finite and not overly large size.

The analysis also revealed a functional core, functions conserved in each individual of the cohort, which reflects the minimal human gut metagenome, encoded across many species and probably required for the proper functioning of the gut ecosystem. It includes functions known to be important for the host, such as degradation of complex polysaccharides, synthesis of short chain fatty acids (SCFA), amino acids and vitamins. Moreover, they identified functions that they attribute to a minimal gut bacterial genome, likely to be required by any bacterium to survive and thrive in the gut. Besides general housekeeping functions, the minimal meta-genome encompasses many genes of unknown function, rare in

sequenced genomes and possibly specifically required for the functioning of the gut ecosystem. These putative gut-specific functions include those involved in adhesion to the host proteins (collagen, fibrinogen, fibronectin).

Autochthonous and transiting bacteria

Within a given intestinal habitat, some microbial members can be classified as true residents or autochthonous species (Berg, 1996). These microorganisms have a long-term association with the particular habitat and form a stable community. Other bacteria transit with the food and under normal conditions do not colonize the GI tract (Berg, 1996). Fermented food products like yogurt and cheese are important sources of transiting bacteria, typically delivering 10^9 to 10^{10} live bacteria per serving. Studies aiming to reveal true resident and transient species are still emerging, and recent reports focus on the group of lactic acid bacteria (LAB) that are widely used in food fermentation (Berg, 1996; Reuter, 2001). Within this group, species such as *Lactobacillus plantarum*, *L. casei*, *L. paracasei*, *L. buchneri*, *L. brevis*, *L. rhamnosus*, *L. fermentum* and the thermophilic dairy lactobacilli *Lactobacillus delbrueckii* and *L. helveticus* appear not to form stable communities and can be classified as transit or allochthonous species. Some controversial results have been reported for *Bifidobacterium* species. The presence and persistence of *B. adolescentis*, *B. longum* and *B. bifidum*, largely vary between individuals (Reuter, 2001), making it difficult to draw more definite conclusions about which species are autochthonous or in transit. The survival of allochthonous bacteria in the GI tract depends on several factors among which their resistance to gastric acid, bile salts and pancreatic juice (Marteau and Shanahan, 2003).

Allochthonous bacteria can have important effects on their hosts, both in a negative and in a positive way. Transiting bacteria include several of the enteric food-borne pathogens which, in contrast to commensal microorganisms, may invade and disrupt the intestinal

epithelial barrier (Reuter, 2001; Neish, 2009). On the other hand, transiting bacteria also include species that are now recognized as probiotics. Probiotics and their beneficial effects for the human health will be discussed later.

GIT MICROBIOTA AND HUMAN HEALTH

Contributions to host metabolism

Gut bacteria aid their hosts in extracting maximal nutritional value from the diet through the breakage of undigested polysaccharides such as cellulose, xylan, resistant starch and host derived glycans. In the colon, several members of the microbiota are able to digest these polymers, releasing monosaccharides that are subsequently fermented by many other bacteria (Hooper, *et al*., 2002; Flint, *et al*., 2008; Hooper and Macpherson, 2010). The major end products of bacterial fermentation in the gut are short chain fatty acids (SCFA). Several tissues in the human body are able to oxidize SCFA for energy generation, and the bacterial formation of SCFA thus enables the host to salvage energy that would be lost otherwise. The three major SCFA produced in the gut are acetate, propionate and butyrate, the main fuel for enterocytes (Hooper, *et al.*, 2002; Blaut and Clavel, 2007; Louis, *et al.*, 2007).

In addition to their nutritional value, SCFA have important effects on other aspects of gut physiology. For example, SCFA are the predominant anions in the colon, and the absorption of water is coupled with sodium chloride as well as SCFA transport (Hooper, *et al.*, 2002). Interestingly, butyrate has been reported to play a role in the prevention of colitis and colorectal cancer (Hooper, *et al.*, 2002; Blaut and Clavel, 2007; Louis, *et al.*, 2007).

The ability of gut bacteria to synthesize vitamins was recognized many years ago. These vitamins are produced by several genera of gut bacteria, including *Bacteroides*, *Eubacterium*, *Propionibacterium*, and *Fusobacterium* (Albert, *et al.*, 1980). Minerals and

essential elements are crucial components of enzymes, structural proteins and redox transport chains. Cobalt, zinc, copper and iron are elements that can be exchanged in the gut between bacteria and their hosts thus increasing the availability of these elements for our utilization (Resta, 2009).

The results of recent studies also indicate that intestinal microorganisms directly affect the energy storage in adipose tissue (Bäckhed, *et al.*, 2004; Bäckhed, *et al.*, 2005). In a series of experiments with mice, Bäckhed *et al*. (2004) found that conventionally reared animals had a 40 % higher body fat content and 47 % higher gonadal fat content than germ free (GF) mice, even though they consumed less food than their GF counterparts. When the distal gut microbiota from the normal mice was transplanted into the GF mice, this resulted in a 60 % increase in body fat without any increase in food consumption or obvious differences in energy expenditure. The authors revealed that the microbiota promoted the absorption of monosaccharides derived from polysaccharides in the gut, and induced hepatic lipogenesis in the host. During lipogenesis, simple sugars derived from complex carbohydrates are converted to fatty acids, which are subsequently esterified with glycerol to form triacylglycerols, the main constituent of animal fats. The transplanted microbiota promoted the storage of triglycerides in adipocytes through the suppression of intestinal *Fiaf* (fasting induced adipose factor, also known as angiopoietin-like protein 4) in differentiated villus epithelial cells, resulting in an increase of lipoprotein lipase (LPL) activity, an increase in the absorption of triglycerides, and an increased storage of triglycerides in the adipocytes.

Maturation and modulation of the immune system

The GI tract of mammals is sterile at birth, and is subsequently colonized by the intestinal microbiota. This colonization plays an important role in the maturation and shaping

of both the mucosal and systemic immune systems. GF animals provide important insights into how the microbiota affects the host immune system.

The gut-associated lymphoid tissue (GALT) corresponds to the first line of defense for the intestinal mucosa and its development is defective in GF mice. They present fewer and smaller Peyer's patches, fewer and smaller mesenteric lymph nodes and isolated lymphoid follicles, and a less developed lamina propia of the small intestine relative to the conventional animals (Pollard and Sharon, 1970; Glaister, 1973; Hoshi, *et al*., 1992; Falk, *et al*., 1998; Macpherson and Harris, 2004). Besides, GF mice exhibit a reduced expression of Toll-like receptors (TLR) and the class II major histocompatibility complex (MHC II) which are involved in microbial sensing and antigen presentation, respectively, in the intestinal epithelial cells (IECs) (Matsumoto, *et al*., 1992; Lundin, *et al*., 2008). The number of their IgA-producing cells is reduced, as are the levels of secreted immunoglobulins (IgA and IgG) (Macpherson and Harris, 2004). They also exhibit irregularities in cytokine levels and profiles and are impaired in the generation of oral tolerance (Ishikawa, *et al*., 2008; Neish, 2009).

Intraepithelial lymphocytes (IELs) are interspersed with enterocytes, thus having direct contact with foreign antigens derived from the gut lumen, and play a key role in the immune responses toward these antigens and in pathogenesis of a variety of diseases (Brandtzaeg and Pabst, 2004; Bharhani, *et al.*, 2007). IELs from germ-free mice are reduced in number and their cytotoxicity is compromised (Umesaki, *et al.*, 1993; Imaoka, *et al.*, 1996). GF mice also have reduced numbers of $CD4^+$ T cells in the lamina propria and in the spleen (Bouskra, *et al.*, 2008; Niess, *et al.*, 2008) and the development of lymphoid follicles (specialized intestinal structures composed of dendritic cells (DCs) and B cell aggregates) is also disturbed (Bouskra, *et al.*, 2008).

Beyond the development and maturation, the microbiota also influences functional aspects of our immune system. It was demonstrated that GF mice are more susceptible to infectious agents, such as *Shigella flexneri* and *Leishmania* (Oliveira, *et al*., 2005; Smith, *et al.*, 2007). Therefore, the contribution of the microbiota to the development and function of the immune system appears to be crucial.

Several reports indicated that the conventionalization, or even the monocolonization with specific bacterial species are able to correct several defects found in the immune system of GF mice. Umesaki *et al*. (1999) showed that colonization of GF mice with segmented filamentous bacteria (SFB) increased the total number of IELs in the small intestine, the expression of MHC II molecules in IECs and also the number of IgA-producing cells in the small intestine. Mazmanian *et al*. (2005) demonstrated that the monocolonization of GF mice with *B. fragilis* was sufficient to correct several immunologic defects found in the absence of a bacterial microbiota. The authors focused on systemic T cell development, and in particularly $CD4^+$ T cells that are important for proper immune function. These cells are of two general subtypes, T helper 1 (T_H1) and T helper 2 (T_H2), each carrying out distinct and opposing activities (Neurath, *et al.*, 2002). A proper T_H1/T_H2 balance is critical for human and animal health, and over- or underproduction of either response is associated with immunologic disorders (Neurath, *et al.*, 2002; Mazmanian, *et al.*, 2005). A reduced proportion of $CD4^+$ T cells, which are skewed towards T_H2, are observed in newborn GF mice (Mazmanian, *et al.*, 2005). The authors showed that colonization with *B. fragilis* restores the proportions of $CD4^+$ T cells in the spleen and T_H1/T_H2 balance to the levels that are observed in conventional mice (Mazmanian, *et al.*, 2005).

Many cell types are influenced by our microbiota and, several reports indicate that commensals play an important role in $CD4^+$ T cell differentiation. Induction of each lymphocyte subset may be regulated by a distinct component of the microbiota. For instance,

SFB strongly induce T_H17 cells, which play a role in host resistance against intestinal pathogens and promote systemic autoimmunity (Gaboriau-Routhiau, *et al.*, 2009; Ivanov, *et al.*, 2009; Wu, *et al.*, 2010). Naïve $CD4^+$ T cells can also adopt a regulatory phenotype (Treg cells). Again, the microbiota may be critically involved in the differentiation of some gut Treg subsets. Induction of Tregs is an important target for treating both auto-immune and atopic disorders as these cells can suppress the activity of effector cells by inducing IL-10 production. Several gut bacteria, such as *Bifidobacteria longum* subsp. *infantis* and *F. prausnitzii*, have been shown to induce Tregs and IL-10 production in the gut (O'Mahony, *et al.*, 2008; Sokol, *et al.*, 2008).

Numerous other aspects of the immune system are influenced by gut microbiota. For instance, several reports indicate that commensal bacteria can interfere with important signaling pathways of their host, thus stimulating the production of anti-inflammatory mediators, while at the same time inhibiting the secretion of pro-inflammatory cytokines.

Disturbed microbiota composition and disease

As already described, the human body lives in symbiosis with a complex bacterial community. A growing number of reports indicate correlations between disturbed microbiota composition (dysbiosis) and diseases such as IBDs, obesity, diabetes, cancer and also several extra-intestinal diseases.

Evidence for the role of the microbiota in the pathogenesis of IBD is provided through studies demonstrating that antibiotic use can reduce or prevent inflammation both in patients and in murine models of IBD (Swidsinski, *et al*., 2005; Kang, *et al*., 2008). Besides, IBD patients presenting ulcerative colitis (UC) inoculated with stool collected from healthy donors exhibited disease remission within a week after the fecal transplantation, with a complete recovery after 4 months (Borody, *et al*., 2004).

IBDs have been associated with decreased microbial diversity, decreased numbers of Firmicutes and Bacteroidetes, and increased numbers of Proteobacteria and Actinobacteria (Ott, *et al*., 2004). In addition, biopsies from IBD patients contain higher amounts of bacteria associated with the mucus layer and the epithelial surface when compared with tissues obtained from healthy subjects (Strober, *et al*., 2007) but these studies do not support the presence of a specific, IBD causing, pathogenic microorganism (Strober, *et al*., 2007; Sartor, 2008).

Recent investigations propose that the absence of specific (groups of) bacteria may be important (Sokol, *et al*., 2008; Sokol, *et al*., 2009). Specially, Clostridial clusters IV and XIV exhibit relatively lower abundance in patients with IBD compared to healthy controls. Particular interest has been placed on members of this group since they are known producers of SCFAs with potent anti-inflammatory properties (Barcenilla, *et al*., 2000; Segain, *et al*., 2000; Ott, *et al*., 2004). For example, the specie *Faecalibacterium prausnitzii* have been reported to be underrepresented in ileal Crohn's disease (CD) patients (Sokol, *et al*., 2009). Besides, *F. prausnitzii* shows anti-inflammatory effects *in vitro* and *in vivo* (Sokol, *et al*., 2008). The studies described above indicate correlations between dysbiosis and IBDs. However, it remains to be seen whether these alterations in microbial communities are the cause or the consequence of such disease.

BACTERIA-HOST INTERACTIONS IN GIT

While pathogenic bacteria and their interaction with the human host traditionally receive much attention, the interest for the beneficial role that non-pathogenic bacteria play at body surfaces and in the GI tract in particular is a more recent development. Therefore, still relatively little is known about the bacterial effectors and the mechanisms involved in health

beneficial bacteria - host interactions. In the next paragraphs we will review the main actors identified so far in this complex and important crosstalk between bacteria and the host.

The GIT epithelium

In addition to a diverse set of physiological functions including the absorption of nutrients, the gut epithelium creates a physical barrier between the external and internal environment and is responsible for the immune surveillance of commensal and (potentially) pathogenic bacteria. A key element of the mammalian strategy for maintaining homeostasis in the gut environment is to minimize the contact between luminal microorganisms and the intestinal epithelial cells (IEC) surface (Hill and Artis, 2010; Maloy and Powrie, 2011): specialized IECs, goblet cells, produce heavily glycosylated mucin-rich secretions that create a relatively impermeable, apically adhering glycocalyx (Hill and Artis, 2010). The colon has a mucus layer that presents itself in the dense portion next to the epithelial tissue and gradually less dense toward its outer portion. In this less dense portion are commensal bacteria (Atuma, *et al*., 2001; Johansson, *et al*., 2008; Johansson, *et al*., 2011; Ouwerkerk *et al*., 2013).

Mice lacking the mucin glycoprotein MUC2, the predominantly mucin type in the intestine, suffer from spontaneous intestinal inflammation, demonstrating the importance of the mucus barrier in maintaining a symbiotic relationship with the microbiota (Van der Sluis, *et al.*, 2006; Johansson, *et al.*, 2008; Hooper and Macpherson, 2010).

Tight junctions between IECs prevent paracellular traffic, and actin-rich microvillar extensions create an apical brush border that impedes microbial attachment and invasion. IECs also secrete a broad range of antimicrobial peptides, including defensins, cathelicidins and calprotectins (Sansonetti, 2004; Magalhaes, *et al.*, 2007). Together, all these adaptations provide a physical and biochemical shield and prevent the invasion of microorganisms into

IECs. It is believed that properties that permit to gain access to the apical surface of the intestinal epithelium and to rupture the physical barrier are the main characteristics that distinguish pathogenic from commensal microorganisms (Sansonetti, 2004).

IECs express different types of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), the best characterized class of PRR, Nucleotide oligomerization domain, the, Nod-like receptors (NLRs), peptidoglycan receptor proteins (PGRPs), C-type lectins, with lectin-like carbohydrate recognition domains (CRDs) in their extracellular carboxy-terminal domains (such as Dectin-1 and -2) that recognize microbial components (MAMPs, microbe associated molecular patterns) and modulate cellular responses (Hill and Artis, 2010). MAMPs are present too in fungi, protozoa and viruses. Furthermore a cross-talk between PRRs has been shown in immune cells. This interaction can be at the level of the cell surface, or at the level of intracellular pathways like NOD2/TLRs and Dectin-1/TLR2 or TLR4, that show a synergistic effect on cytokine production, leading to a specific innate immune response (Netea *et al*., 2005; Jin *et al*., 2007; Dennehy *et al*., 2008; Ferwerda *et al*., 2009). Table 1 shows the main PRRs and their ligands (Dziarski, 2004; Kim *et al*., 2004; Steiner, 2004; Martinon and Tschopp, 2005; Le Bourhis *et al*., 2007; Fitzner *et al*., 2008; Saijo and Iwakura, 2011).

Signal transduction

The binding of microbial MAMPs to their TLRs lead to the recruitment of single, or a specific combination of adaptor proteins (e.g. Myd88, TIRAP, TRIF or TRAM) and activation of specific downstream responses. Myd88 is utilized by almost all TLRs, with the exception of TLR3, and transmits signals culminating in NF-κB and mitogen-activated protein kinases (MAPKs) activation, leading to the induction of inflammatory cytokines. In contrast, TRIF is used by TLR3 and TLR4 and induces alternative pathways that lead to activation of the transcription factors IRF3 and NF-κB and the consequent induction of type I interferon and inflammatory cytokines. TRAM and TIRAP function as sorting adaptors that recruit TRIF to TLR4 and Myd88 to TLR2 and TLR4, respectively. Thus TLR signaling pathways are classified as either Myd88-dependent pathways, which drive the induction of

inflammatory cytokines, or TRIF-dependent pathways, which are responsible for the induction of type I interferon as well as inflammatory cytokines. TLR4 recruits four adaptor proteins and activates both the Myd88- and TRIF-dependent pathways (Kawai and Akira, 2011). TLR4 initially recruits TIRAP and subsequently facilitates the recruitment of Myd88 to trigger the initial activation of NF-κB and MAPK. TLR4 is subsequently trafficked to the endosome, where it forms a signaling complex with TRAM and TRIF, to initiate the TRIF-dependent pathway that leads to IFR3 activation as well as the late-phase activation of NF-κB and MAPK.

In summary, TLRs 1/2/6, 5, 7, 8 and 9 activate the Myd88-dependent pathway leading to NF-κB and AP-1 (MAPK) activation, and TLR3 and 4 activate Myd88-dependent and TRIF-dependent pathways. Activation of the cytosolic receptors NOD1 and NOD2 directly leads to the activation of NF-κB and subsequent transcription of pro inflammatory genes (Figure 2).

NF-κB pathway (Myd88-dependent pathway)

One of the central transcription factors mediating inflammatory responses is nuclear factor κB (NF-κB). NF-κB is required for the transcriptional activation of a number of inflammatory effectors, including IL-8, TNF-α, IL-6, Cox2, iNOS and many others. In most cells, NF-κB primarily resides in the cytoplasm, in an inactive complex with a member of the family of inhibitory IκB proteins. After engagement of TLRs by their cognate MAMPs, Myd88 recruits the IL-1 receptor-associated kinases (IRAK4, IRAK1). IRAK activation results in an interaction with TRAF6 which leads to the activation of MAPKs, TAK1 and MEKKs. These kinases activate the IKK complex which phosphorylates IκB and targets it for proteosomal degradation (Figure 3).

Table 1: PRR expression patterns and ligands

PRR	Ligands and their origins
TLR1	Forms heterodimers with TLR2, PGN, Lipoproteins
TLR2	LTA, PGN of gram-positive bacteria, Lipoproteins, lipopeptides, *Aspergillus fumigatus* GPI-anchored proteins, *Trypanosoma cruzei*, *Listeria monocytogenes* LAM, Mycobacteriae MALP-2, synthetic Manuronic acid polymers, *Pseudomonas aeroginosa* Membrane-associated proteins, *Neisserria meningitidis* Outer surface proteins A and B, *Borrelia burgdorferi* Pam3Cys, synthetic Phenol-soluble modulin, *Staphylococcus epidermis* Schistosome egg, *Schistosama mansoni* Soluble tuberculosis factor (STF), *Mycobacterium tubercolosis*, Zymosan, yeast
TLR3	Viral dsRNA, synthetic Poly(I:C)
TLR4	LPS of Gram-negative bacteria, ß-defensin 2, F Protein, RSV Heat-sensitive cell-associated mycobacterial factor, *Mycobacterium tuberculosis* Hsp60, Hsp70, host GP96, *Cryptococcus neoformans* Manuronic acid polymers, *Pseudomonas aeroginosa* Oligosaccharides of hyaluronan, host
TLR5	Flagellin of Gram-negative and Gram-positive bacteria
TLR6	Forms hetrodimers with TLR2
TLR7	ssRNA, virus Imiquimod, R-848, loxoribine, bropirimine
TLR8	ssRNA, HIV-1 R-848
TLR9	Unmethylated CpG DNA/ODN of bacteria and viruses
TLR10	Unknown
TLR11	Uropathogenic bacteria
NOD1	γ-d-Glu-DAP (iEDAP), d-lactyl-l-Ala-γ-Glu-meso-DAP-Gly, heptanolyl-γ-Glu-meso-DAP-Ala
NOD2	muramyl dipeptide (MDP), MurNAc-l-Ala-g-d-Glu-l-Lys

Abbreviations: CpG-DNA, cytosine–phosphate–guanosine DNA; dsRNA, double-stranded RNA; GPI, glycosylphosphatidylinositol; HSP, heat shock protein; HIV, human immunodeficiency virus; LAM, lipoarabinomannan; LTA, lipoteichoic acid; MALP, macrophage-activating lipopeptide 2; ODN, oligodeoxynucleotide; PGN, peptidoglycan; poly(I:C), polyinosinic–polycytidylic acid; RSV, respiratory syncytial virus; ssRNA, single-stranded RNA; TLR, Toll-like receptor; From (Winkler, *et al.*, 2007).

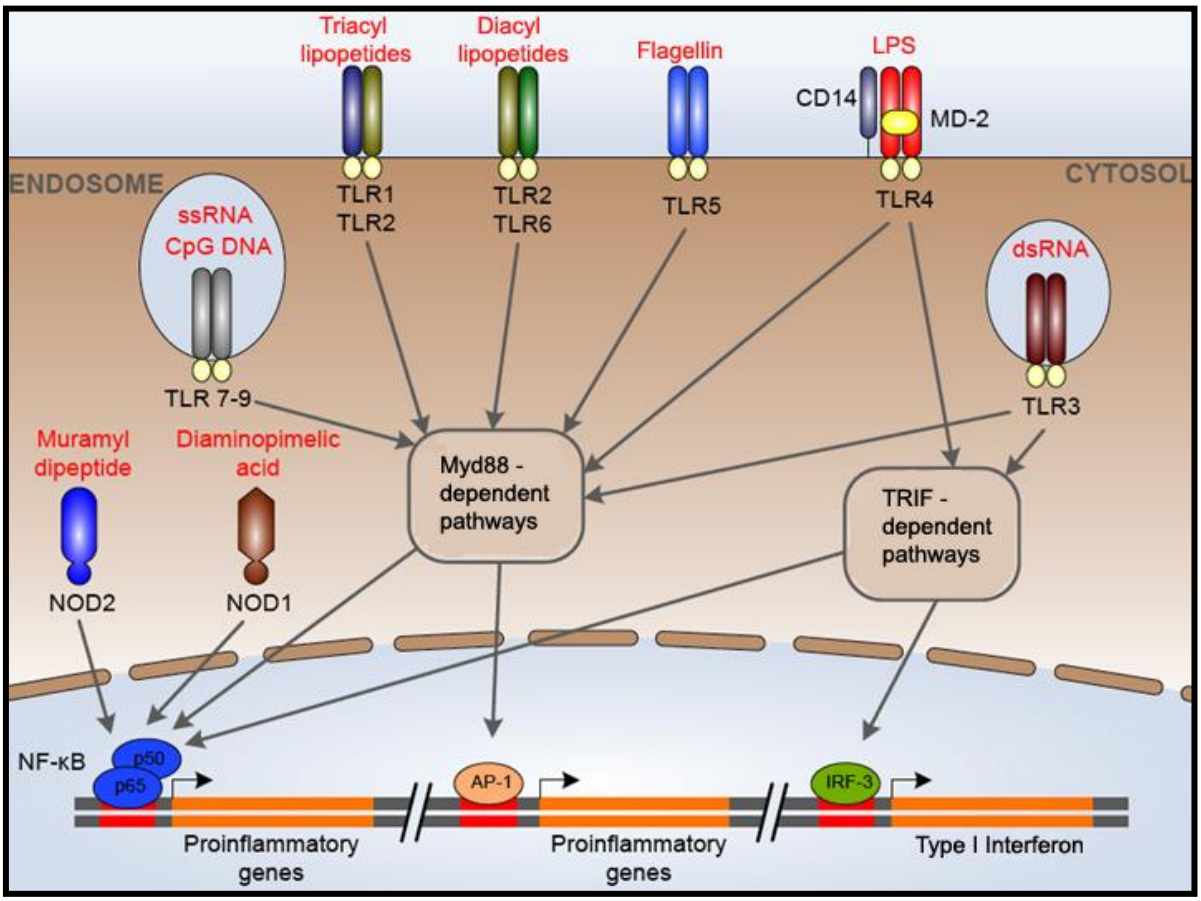

Figure 2. **Pattern recognition receptors and their typical ligands.** TLRs 1/2/6, 4 and 5 are located on the surface of most cells, whereas TLRs 3, 7-9 are located in endosomes in the cytosol. Signals through TLRs 1/2/6, 5, 7-9 are mediated exclusively by MyD88 leading to NF-κB and AP-1 activation. TLRs 3 and 4 also utilize MyD88-independent signalling leading to NF-κB and IRF-3 activation. NOD1 and NOD2 are located in the cytoplasmand activate NF-κB. Adapted from (Kalliomaki and Walker, 2005).

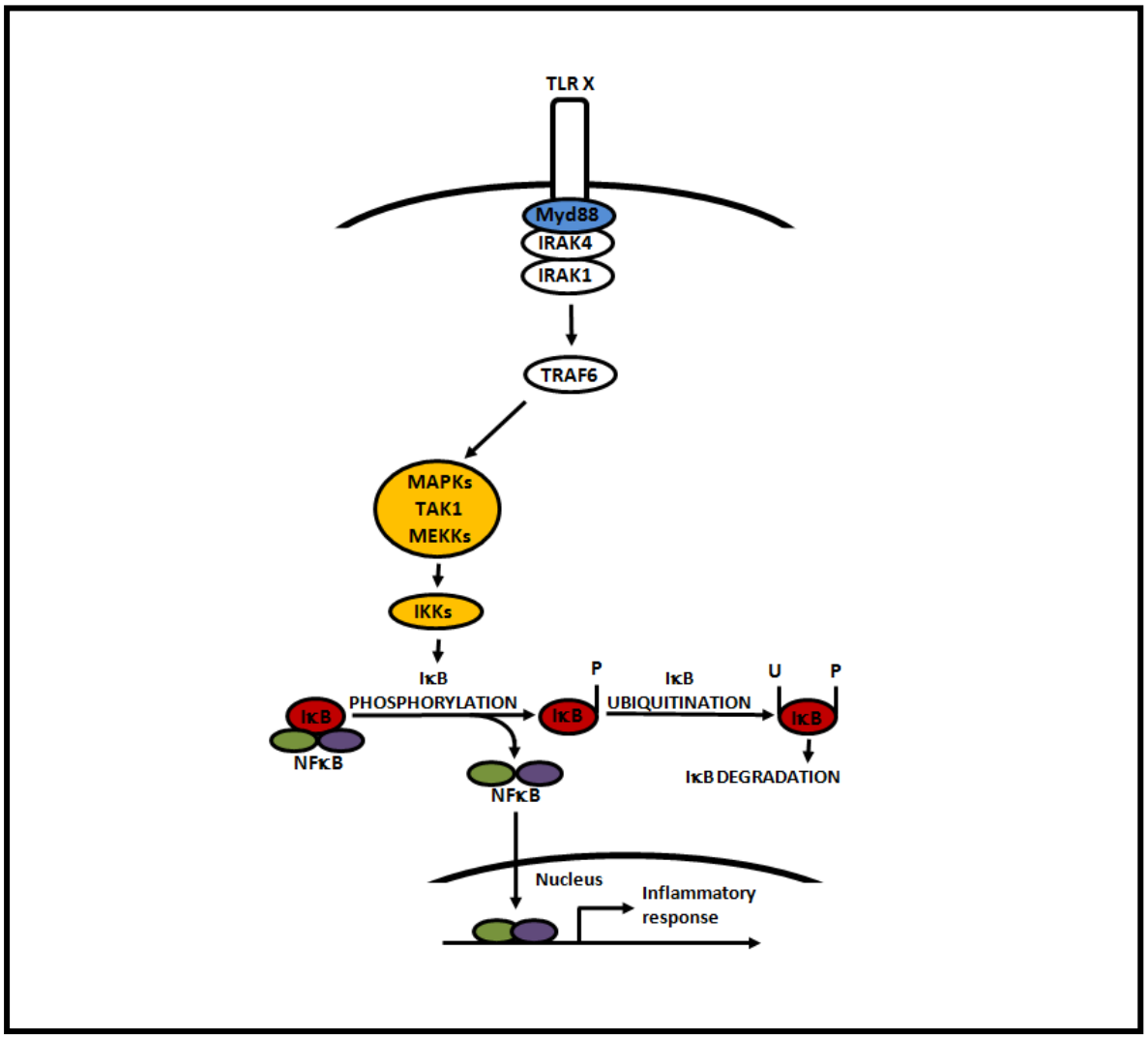

Figure 3. **Myd88-dependent NF-κB pathway.** After engagement of TLRs by their congnate MAMPs, Myd88 recruits IRAK4 and IRAK1. IRAK activation results in an interaction with TRAF6 which leads to the activation of MAPKs, TAK1 and MEKKs. These kinases activate the IKK complex which phosphorylates IκB and targets it for proteosomal degradation. Free NF-κB migrates to the nucleus where it will activate the transcription of several inflammatory mediators. Figure adapted from (Santos Rocha, *et al.*, 2012).

Multiple lines of evidence suggest that NF-κB activation has a dual role (detrimental and beneficial) in the intestine. NF-κB is reported to contribute to the development and maintenance of intestinal inflammation and was found to be overactivated in mucosal cells of IBD patients. Pharmacological inhibition of NF-κB activity ameliorates intestinal inflammation in mouse models of colitis (Schreiber, *et al.*, 1998). Therefore, these studies suggest that excessive NF-κB activation contributes to intestinal inflammation and that NF-

κB inhibition could have therapeutic effects in IBD. On the other hand, mice NF-κB deficient results in apoptosis of IECs mediated by TNF, reduction of microbial peptide production and compromising of the epithelial integrity allowing bacterial translocation (Nenci, *et al.*, 2007).

Since the GI tract epithelium is in close contact with commensals, there must be robust mechanisms to control excessive NF-κB activation and maintain its steady state activation. Thus, TLR signaling and subsequent events must be tightly regulated by inhibitors of TLR signaling to maintain immune balance. In fact, several inhibitors of TLR signaling pathways have been characterized, including the Peroxisome Proliferator-Activated Receptor γ (PPARγ) (Shibolet and Podolsky, 2007).

Most PPARγ are members of a nuclear receptor family and has been proposed as a therapeutic target in IBD (Dubuquoy, *et al.*, 2006). PPARγ's effects on inflammation may be mediated by TLRs. Signals from TLR4 and luminal bacteria may regulate PPARγ expression leading to the inhibition of NF-κB (Dubuquoy, *et al.*, 2003). Ogawa *et al*. (2005) showed that PPARγ and other nuclear receptors repress overlapping but distinct subsets of TLR inflammatory response genes, while Kelly *et al*. (2004) demonstrated that PPARγ directly associates with the RelA subunit of NF-κB and is responsible for its nuclear export limiting the duration of NF-κB's action. Interestingly, in this same work, they showed that the anti-inflammatory effect of the commensal *B. thetaiotaomicron* was correlated with its ability to increase PPARγ expression in a Caco-2 epithelial cell line.

Intestinal immunity

The intestinal epithelium not only functions as a physical barrier but also makes part of the digestive tract's immune system which involves IECs and the gut associated-lymphoid tissue (GALT), the largest immune organ in the human body (Sansonetti, 2004; Neish, 2009). The lymphoid tissue comprises Peyer's patches in the small intestine, and isolated lymphoid

follicles embedded in the lamina propria all along the intestinal tract (Rescigno and Di Sabatino, 2009). These sites of the GALT play a fundamental role in the induction of immune responses against pathogens that invade the epithelium and contribute to the maintenance of the balance between immunity and tolerance at the mucosal surface (Sansonetti, 2004; Artis, 2008; Neish, 2009). IECs known as M (microfold) cells that overlie Peyer's patches participate in the sampling of luminal content and microorganisms, and deliver these to the subepithelial dome (SED), an area that is populated by immune cells including professional antigen-presenting cells and a number of dendritic cell (DC) subsets (Sansonetti, 2004; Artis, 2008). In addition, specialized intestinal DCs located in the lamina propria of the small intestine express tight-junction proteins that allow for direct luminal sampling through the extension of dendrites between IECs, while keeping the IEC barrier intact (Rescigno, *et al.*, 2001). DCs in the mesenteric lymph nodes (MLN) promote differentiation of regulatory and effector T lymphocytes, as well as class switching of B lymphocytes, which then exit through the efferent lymph into the systemic circulation. Some of these cells home back to the intestine where they exert their effector functions.

DCs are key modulators of the adaptive immune system and provide a link between innate and adaptive immunity. Particularly in the gut, a subset of DCs has been shown to preferentially drive the development of Tregs, the main effectors of tolerance (Coombes and Powrie, 2008).

$CD4^+$ T cells are subdivided into T helper cells (T_H1, T_H2 and T_H17 cells) and Tregs. T_H1 cells are inflammatory cells that release IFN-γ and are involved in immunity against intracellular pathogens, whereas T_H2 cells are primarily involved in B cell help as they release B cell growth factors, like IL-4. T_H17 cells play a critical role in host defense against a variety of bacteria and fungi (Dubin and Kolls, 2008), but under pathological conditions such as autoimmunity, T_H17 cells exacerbate inflammation (Steinman, 2008). Tregs suppress

the function of effector T cells and are thus essential to counteract inflammatory responses (Powrie, *et al.*, 2003).

Intestinal DCs regulate local T cell response in part by production of IL-12 and IL-23. IL-12 is a regulatory cytokine that induces T_H1 cell differentiation (Trinchieri, 2003) while IL-23 drives inflammatory T_H17 responses (Ahern, *et al.*, 2008). They are tolerogenic compared with systemically circulating DCs, thus contributing to the generation of oral tolerance (Chirdo, *et al.*, 2005; Iliev, *et al.*, 2009). The mechanism by which intestinal DCs may present a tolerogenic phenotype is not fully understood, but includes reduced TLR expression and negative regulation of the NF-κB pathway via NOD2, for example. (Watanabe, *et al.*, 2004; Watanabe, *et al.*, 2006; Yang, *et al.*, 2007; Monteleone, *et al.*, 2008; Zeuthen, *et al.*, 2008). In conclusion, intestinal DCs are important to maintain mucosal homeostasis; however, the role of gut bacteria in promoting the tolerogenic profile of mucosal DCs remains to be studied.

In summary, healthy individuals have a tolerogenic response against the gut microbiota, where we found a balance between effector and regulatory T cells (Figure 4). When this balance is disrupted (Figure 4), either by an increase in the population of effector T cells, or by a decrease in the population of Tregs, the homeostasis is broken leading to mucosal inflammation. The loss of tolerance to the normal gut microbiota is observed in patients with IBD (Strober, *et al.*, 2007).

Several drugs are used for treating IBD such as aminosalicylates, corticosteroids, TNF-α antibodies, immunosuppressants, and antimicrobials. In some cases, patients are submitted to surgery. Drug treatments are rather effective but not curative. They are used to obtain or maintain remission, but are accompained by multiple side effects, such as fever, chills, pruritis, urticaria, allergic reactions, and liver problems. (Marteau, *et al*., 2006; Nielsen and Munck, 2007). In this context, probiotics appear as a promising alternative in the

treatment of IBD. Probiotics and their mechanisms of action will be discussed in the next section.

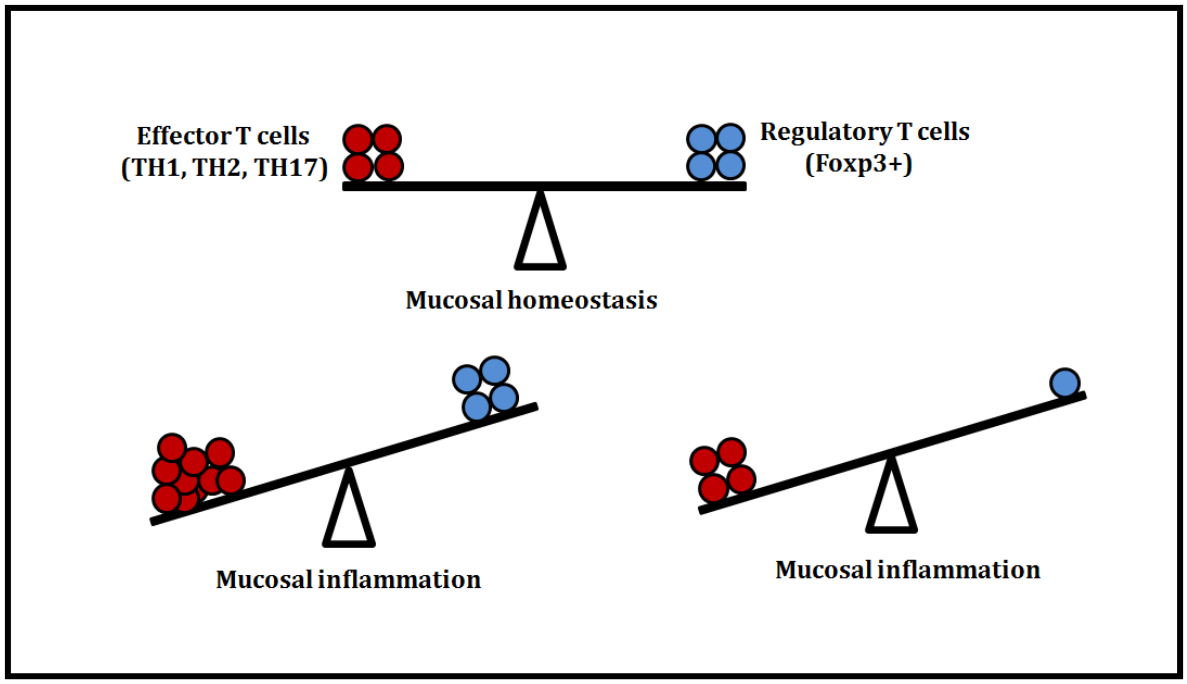

Figure 4. **Breakdown of mucosal homeostasis.** In healthy conditions there is a balance between effector and regulatory T cells. This balance can be disrupted either by an increase in the population of effector T cells, or by a decrease in the population of Tregs, leading to mucosal inflammation. Adapted from (Bouma and Strober, 2003).

PROBIOTICS

In the first decade of the 20th century, the scientist Ilya Metchnikoff suggested that certain bacteria present in fermented food products might have health beneficial effects. Metchnikoff hypothesized that living bacteria present in fermented milk products such as yogurt were responsible for the longevity of its consumers (Metchnikoff, 1908). Colonization of the intestine with what he called "Bulgarian Bacillus" (nowadays known as *Lactobacillus delbrueckii* ssp. *bulgaricus*) was thought to normalize bowel movements and combat intestinal disease (Metchnikoff, 1908). Several health beneficial effects have been attributed to a number of bacteria by recent experimental studies, and the hypothesis of Metchnikoff

paved the way for the present concept of probiotics, defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002).

The effect of probiotic bacteria are mainly evaluated after their oral ingestion. Thus, these bacteria must retain beneficial functional characteristics after its passage through the GIT. Therefore, it is interesting to evaluate the ability of bacteria to survive after their contact with the stomach and intestine environments. Also, their ability of adhesion to intestinal tissue, the inhibition of pathogenic microorganisms, their safety and their immunomodulatory effects (FAO/WHO, 2002). The experimental (both *in vitro* and in animal models) health effects should be clinically validated and, finally, they should be stable during processing and storage (FAO/WHO, 2002; Borchers, *et al.*, 2009). Today, they are used to prevent or treat a number of disease, including allergic diseases, gastrointestinal infections and IBDs (Borchers *et al*., 2009).

Lactobacillus and *Bifidobacterium* comprise the most used probiotic bacteria. They are present in GIT of animals and humans and have the Qualified Presumption of Safety (QPS) status due to the long history of safe use (Gaggìa *et al*., 2010). However, probiotic properties are strain specific and cannot be extrapolated to other strains (Servin and Coconnier, 2003; Foligne, *et al.*, 2007; Douglas and Sanders, 2008).

Besides identifying strains with probiotic characteristics, many works have been developed for better understanding the mechanism of action of these microorganisms. Being the three main categories of action: (i) alteration of microbial diversity; (ii) increasing epithelial barrier function; and (iii) modulation of the immune responses (Servin and Coconnier, 2003; Lebeer *et al*., 2008).

Alteration of microbial diversity

Healthy people have a mucus layer able to protect them against the endogenous microbiota and pathogens, whereas individuals suffering from IBD have their protection ability compromised, which favors the increased association of luminal bacteria to their mucus layer (Schultsz, *et al*., 1999). Analysis of the intestinal mucosa of IBD patients showed variation in the concentration of microorganisms associated, with an increased population of *E. coli* and reduction of *Bifidobacterium* species (Kleessen, *et al*., 2002; Swidsinski, *et al*., 2002).

Several studies have shown that probiotics can induce or inhibit changes in gut microbial species composition and diversity**.** VSL#3, a mix of four strains of lactobacilli (*L.plantarum*, *L.casei*, *L.acidophilus* and *L.delbrueckii* subsp. *bulgaricus*), three strains of bifidobacteria (*B.infantis*, *B.longum* and *B.breve*) and one strain of *Streptococcus* (*S.salivarius* subsp. *thermophilus*), induced an increase in bacterial (mainly *Lactobacilli* and *Bifidobacteria***)** and reduction in fungal diversity when compared to placebo-treated individuals. The increase in bacterial diversity was not caused by colonization with bacterial strains contained in VSL#3. However, it was not conclusive whether the anti-inflammatory effects of probiotics are primary or secondary to induction of changes in the diversity of the mucosal microbiota.

A mix of *Lactobacillus* (*L. rhamnosus* GG, *L. plantarum* CIP102021, *L. casei* CIP107868 and *L. delbrueckii* subsp. *lactis* CIP101028) and *Bifidobacterium* strains (*B. bifidum* CIP56.7, *B. infantis* CIP64.67, *B. lactis* CIP105256 and *B. adolescentis* CIP64.59) was found to ameliorate DSS-induced colitis in mice, however, the mechanism whereby probiotic administration attenuates colitis remains uncertain. This study showed that levels of *Bifidobacterium, Bacteroides* and *Lactobacillus acidophilus* significantly decreased in mice with DSS colitis, compared with controls or probiotic co-treated with DSS group.

Interestingly, although the probiotic mix used did not contain any organism of the *Bacteroides-Prevotella-Porphyromonas* group, animals given the probiotic mix showed normalization of levels of the bacteria of this group. The authors suggest that the maintenance of bacterial levels in the colon by probiotics may have induced changes in luminal metabolism leading to an anti-inflammatory effect (Nanda Kumar, *et al.*, 2008).

In order to study the impact of administration of exogenous strains of *Lactobacillus* normally used as probiotics upon endogenous microbial population, Fuentes *et al*. (2008) analyzed the impact in gut microbial diversity after feeding mice with *L. casei* and *L. plantarum* isolated from commercially available dairy products. The authors reported an increase in the diversity of gut lactobacilli (others than *L.casei* and *L.plantarum*) in the feces as well as in intestinal samples after treatment of mice with the two strains administered.

Derrien *et al.* (2011) proposed that *Akkermansia muciniphila* a Gram-negative commensal bacteria act in pathways related to immune tolerance and homeostasis of basal metabolism of the commensal microbiota. Mice treated with *A. muciniphila* had their microbial composition preserved even against the high-fat diet, contribution to the barrier function of the colonic mucosa during obesity. Furthermore *A. muciniphila* regulates antimicrobial peptides such as RegIIIγ, which has bactericidal activity against Gram-positive bacteria (Everard *et al*., 2013; Derrien *et al*., 2011).

Probiotics can also inhibit growth of pathogens by producing antimicrobial compounds or reduce their impact through competitive exclusion by occupying binding sites at the mucosal surface (Ljungh and Wadstrom, 2006). For example *L. johnsonii* strain La1 competes with several enteropathogens by using the same binding sites in the intestine, and competition for mucosal surface binding sites could also be the mechanism by which *L. casei* Shirota and *L. rhamnosus* GG displace enterovirulent *E. coli* and *S. enterica* from Caco-2 cells and human intestinal mucus in *in vitro* experiments (Ljungh and Wadstrom, 2006).

Increasing epithelial barrier function

Probiotics can increase mucosal barrier function leading to a decrease in the ability of harmful bacteria to reach the gut mucosa and a decrease in translocation of bacteria. These effects on barrier function are therefore considered as an important mode of action in the prevention and treatment of IBD**.** Numerous studies have shown that probiotics have the potential to modulate many of the processes involved in mucosal barrier formation and are able to upregulate expression of defensins, mucins or proteins associated with tight junctions such as claudins and occludins (Reiff and Kelly, 2010).

In mice with immune-mediated IBD, tumor necrosis factor receptor 2 (TNFR2) signaling increases long myosin light chain kinase isoform (MLCK) expression, resulting in tight junction dysregulation, barrier loss and induction of colitis (Su, *et al*., 2013).

Treatment of IL-10-knockout mice, a model of colitis, with VSL#3 resulted in normalization of colonic physiologic function and barrier integrity in conjunction with a reduction in mucosal secretion of IFN-γ and TNF-α and an improvement in the histologic parameters of the disease. The authors also demonstrated that VLS#3 prevents invasion of *Salmonella dublin* in T84 monolayer cells. This was attributed to either improvement in barrier integrity or binding of probiotic bacteria to surface receptors to block *S. dublin* invasion (Madsen, *et al*., 2001).

Zyrek *et al*. (2007) showed that the probiotic *E. coli* Nissle 1917 counteracts the disruptive effects of enterophatic *E. coli* EPEC on T84 cell monolayers by altering protein kinase C signaling and causing the redistribution and increased expression of zonulaoccludens-2 (ZO-2), an important factor in maintaining epithelial tight junction function.

Modulation of the immune responses

As mentioned, IECs are the first line of defense against pathogens and they communicate extensively with commensals and probiotics. Probiotics can affect IECs in multiple ways, some of which are enhancing barrier function (discussed above) and modulating inflammatory signaling pathways. Several probiotics are reported to modulate the NF-κB response and influence downstream cytokine secretion. Zhang *et al*. (2005) showed that both viable and heat-killed *L. rhamnosus* GG decrease IκB degradation and subsequent NF-κB translocation into the nucleus in Caco-2 cells. This resulted in decreased production of the inflammatory cytokines IL-8 and TNF-α (Zhang, *et al.*, 2005).

It is known that TNF-α-stimulated IL-8 secretion by IECs is mediated by extracellular signal-regulated kinase (ERK) and NF-κB (Jijon, *et al.*, 2002). Some Lactobacilli have shown inhibitory activity of TNF-alpha induced secretion of IL-8 (Ma, *et al.*, 2004).

Recent studies demonstrated that the anti-inflammatory effects of some bacteria involve inhibition of IκB degradation by targeting the different steps involved in this process (phosphorylation, ubiquitination or proteasome degradation) (Figure 5). *L. delbrueckii* can reduce NF-κB activation by reducing IκB phosphorylation (Santos Rocha, *et al.*, 2012). Similar results were reported for *Bifidobacterium brevis* (Heuvelin, *et al.*, 2009). *L. rhamnosus* GG ATCC 53103 modulates ubiquitin-mediated degradation of IκB through the generation of reactive oxygen species (ROS). Increased quantities of ROS inactivate the Ubc12 enzyme, which is responsible for neddylation of the cullin-1 subunit of the E3-$SCF^{\beta TrCP}$. In the absence of cullin-1 neddylation, the E3 ligase cannot contribute to polyubiquitination of IκB and NF-κB is not released (Kumar, *et al.*, 2007; Lin, *et al.*, 2009). VSL#3 produces soluble factors that inhibit the chymotrypsin-like activity of the proteasome in gut epithelial cells (Petrof, *et al.*, 2004).

PPARγ constitutes another target for probiotic modulation. As mentioned earlier, PPARγ diminishes colitis by inhibiting NF-κB activation. *L. crispatus* M247 uses hydrogen peroxide as a signal-transducing molecule to increase PPARγ activation and transcriptional activity (Voltan, *et al.*, 2008). PPARγ protein expression is reduced in colonic epithelial cells of patients with UC (Dubuquoy, *et al.*, 2003). Probiotic treatment that increases the expression of PPARγ can help ameliorate UC-associated inflammation. Other probiotics known to increase PPARγ expression include *B.thetaiomicron* (Kelly, *et al.*, 2004), *L.casei* (Eun, *et al.*, 2007) and VSL#3 (Ewaschuk, *et al.*, 2006) (Figure 5).

Riedel *et al*. (2006) tested the effect of eight *Bifidobacterium* strains on NF-κB modulation in HT-29 cells. None of the strains tested induced NF-κB. However, six out of eight strains inhibited LPS-induced NF-κB activation in a dose- and strain-dependent manner. In contrast, none of the eight strains affected TNF-α-induced NF-κB activation, indicating that the inhibitory effect observed is specific for LPS-induced inflammation. Inhibition of LPS-induced NF-κB was accompanied by a dose-dependent decrease of IL-8 and by lower mRNA levels for IL-8, TNF-α, cyclooxygenase 2 (Cox-2) and intercellular adhesion molecule 1 (ICAM-2).

Not all probiotic bacteria inhibit NF-κB activation. Some may stimulate NF-κB with consequent increased of transient cytokine secretion in the initial contact with host during microbial establishment. But, in this case, only the activation of NF-κB did not result in beneficial effects in TLR 2 dependent being signaling pathway (Ruiz, *et al.*, 2005). The activation of the NF-κB pathway is potentially important in IBD because it can mediate the production of Human beta-defensin-2 (HBD2), an antimicrobial peptide that improves of the epithelial barrier function, an effect observed with *E. coli* Nissle 1917 (Wehkamp, *et al*., 2004; Aldhous, *et al*., 2009). NF-κB-mediated IEC gene expression may have a protective role during acute intestinal inflammation. This is in line with the fact that TLR9 signaling by

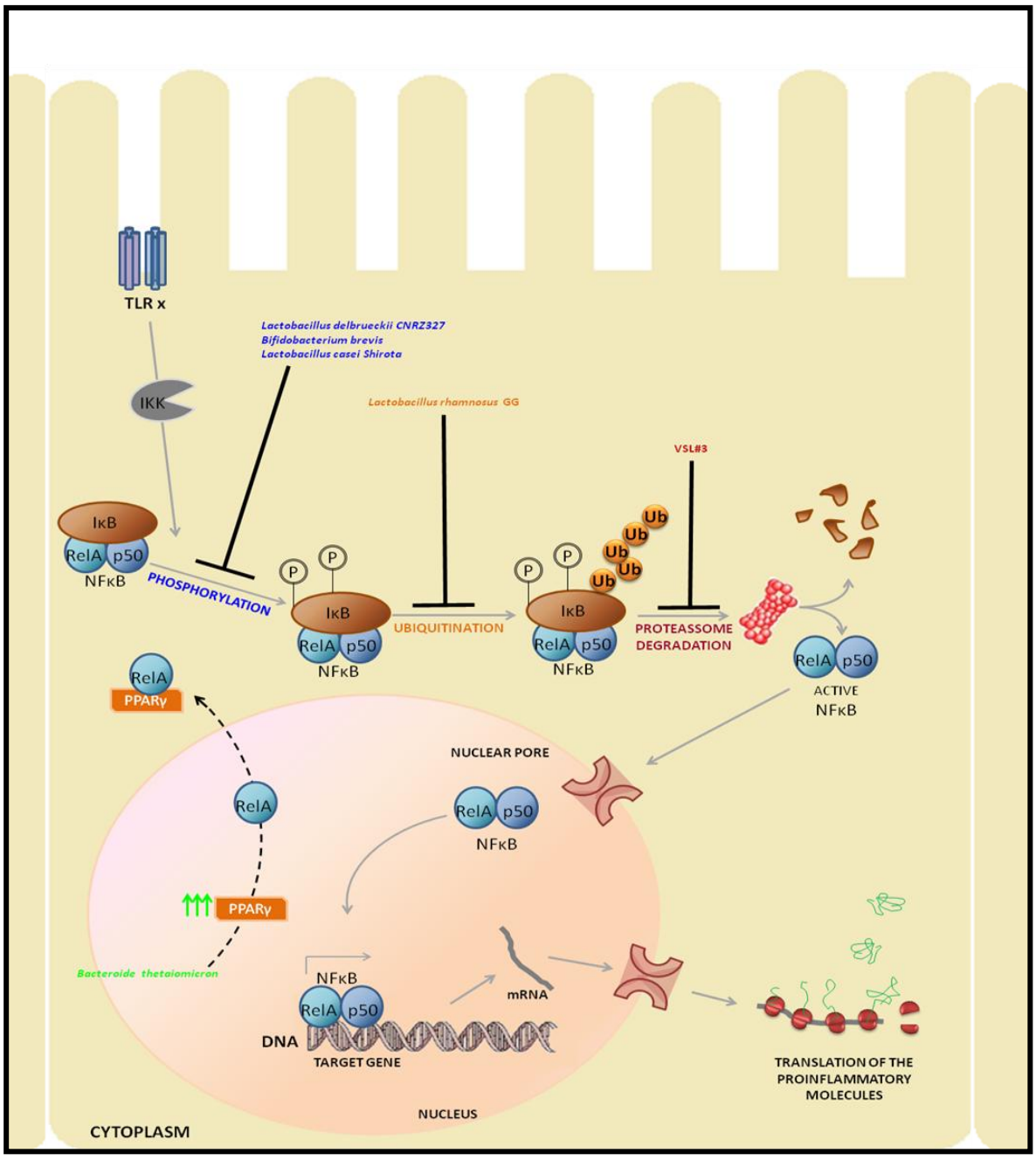

Figure 5. Diferrent mechanisms of NF-κB pathway inhibition by probiotics. In yellow, blue and red, bacterial strains which target the phosphorylation, ubiqutination and proteasome degradation process, respectively. In purple, Bacteroides thetaiomicron increases the expression of PPARγ leading to the nuclear export of the p65 subunit of NF-κB and, consequently, the inhibition of the pathway.

unmethylated CpG bacterial DNA decreases DSS- and hapten-induced colitis (Rachmilewitz, *et al.*, 2002). Some studies *in vitro* showed that NF-κB blockade of the specific expression of the IκBαAA super-repressor exacerbates DSS-induced experimental colitis (Weaver, *et al.*,

2001), suggesting that in contrast to previous observations of the protective effect of NF-κB blockade in TNBS (trinitrobenzene sulfonic acid) induced chronic colitis (Neurath, *et al.*, 1996). Thus, NF-κB activation may have a beneficial role mainly in the early onset of inflammation, but at the same time may be detrimental if activation persists. Some authors suggest that low levels of NF-κB activity induced by nonpathogenic commensal bacteria play a role in the maintenance of intestinal homeostasis (Haller, *et al.*, 2002).

The MAPK signaling pathway can also be affected by probiotics. Resta-Lenert *et al*. (2006) treated IECs with IFN-γ either after pretreatment or simultaneously with a combination of the probiotics *S. thermophilus* ATCC 19258 and *L. acidophilus* ATCC 4356, or with the commensal *B. thetaiotaomicron* ATCC 29184. Both the combination of probiotics and the commensal bacteria induced changes in MAPK signaling cascades, including sustained activation of ERK1/2 and increased p38 phosphorylation, which prevented IFN-γ-induced changes in ion transport (Resta-Lenert and Barrett, 2006).

Certain probiotics can also regulate apoptosis in IECs. *L. rhamnosus* GG ATCC 53103 was shown to activate anti-apoptotic Akt/protein kinase B and inhibited pro-apoptotic p38 MAPK in TNF-, IL-1α, or IFN-γ stimulated in human colon cells (Yan and Polk, 2002).

Some *in vivo* studies demonstrated that probiotics can affect a multitude of genes in their host's. Van Baarlen *et al*. (2009) analyzed the gene expression profiles of healthy human dudodenal samples 6 hours post oral ingestion of *L. plantarum* WCFS1. The strain was administered at different growth phases, including midlog and stationary phase, as well as dead bacteria. Stationary-phase and dead *L. plantarum* induced the upregulation of NF-κB-, JUN- and TNF-dependent pathways. In contrast, midlog-phase *L. plantar*um induced MYC- and cyclin D1-dependent pathways, positive regulators of proliferation (van Baarlen, *et al.*, 2009). This study demonstrated that proper selection of probiotics for a specific application

includes the appropriate species and strains as well as their physiological state (growth phase).

Although the intestinal epithelium acts a barrier to prevent the penetration of bacteria beyond the epithelial cells, commensals can breach this barrier, thus entering in direct contact with innate immune cells. Commensal bacteria that breach the IEC barrier typically succumb to rapid phagocytosis and elimination by lamina propria macrophages which are present in high numbers along the mammalian GI tract and are frequently in close contact with the epithelium (Lee, *et al.*, 1985). These cells kill the ingested organism through mechanisms that include antimicrobial proteins and reactive oxygen species (Smythies, *et al.*, 2005). Macrophages from many tissues, including lamina propria, secrete pro-inflammatory mediators that recruit neutrophils and activate T cells.

As described above for IECs, probiotics can also modulate various signaling pathways in macrophages. Treatment of RAW 267.4 mouse macrophages cell line with *E. coli* M17 led to a decrease of LPS-induced pro-inflammatory cytokines TNF-α, IL1-β and IL-6. The same strain also inhibited TNF-α-induced NF-κB. *In vivo* studies of *E. co*li M17 in a DSS-induced mice model of colitis revealed reduced secretion of colonic IL-12, IL-6, IL1-β and IFN-γ due to an inhibitory effect on NF-κB signaling (Fitzpatrick, *et al.*, 2008).

Lipoteichoic acid (LTA) from selected probiotics has been shown to have a potent anti-inflammatory effect, in contrast to LTA from pathogens. LTA isolated from *L. plantarum* K8 inhibits *Staphylococcus aureus* LTA-induced TNF-α production by preventing signal transduction through both NF-κB and MAPK pathways in THP-1 (human monocytic cell line) cells (Kim, *et al.*, 2008a). Further experiments performed by Kim *et al*. (2008b) demonstrated that the expression of many PRRs, such as TLR4, NOD1 and NOD2, in THP-1 cells is decreased following pretreatment with LTA from *L. plantarum*. Besides, IRAK-M, a negative regulator of TLRs, was induced while LITAF, a molecule involved in LPS-induced

TNF-α expression, is decreased, resulting in an overall reduction in TNF-α (Kim, *et al.*, 2008b). In contrast to these studies of anti-inflammatory effects, other studies have demonstranted that *Lactobacillus* LTA has a proinflammatory effect. LTAs from *L. casei* YIT9029 and *L. fermentum* YIT0159 stimulate TNF-α production and activation of NF-κB in RAW 264.7 macrophage cells (Matsuguchi, *et al.*, 2003). Interestingly, D-alanylation of LTA has been reported to affect the anti-inflammatory properties of the probiotic *L. plantarum* strain NCIMB8826. A mutant which incorporated much less D-Ala in its LTA than the *wt* strain caused a significantly higher production of the anti-inflammatory cytokine IL-10 *in vivo* and *in vitro* (Grangette, *et al.*, 2005).

Treg cells differentiation and expansion is promoted by tolerogenic DC actuation. DC in the gut are generally hyporesponsive and can induce anergy in antigen-specific T cells with tolerogenic function (Coombes and Powrie, 2008; Rutella and Locatelli, 2011).

Several probiotics have been reported to exert anti-inflammatory effects by the modulation of innate and adaptive immune responses. It has been shown that they can modulate the balance between Th1, Th2, Th17 and Treg cells, down-regulate the production of pro-inflammatory cytokines and stimulate anti-inflammatory cytokine production (Round and Mazmanian, 2010; Di Giacinto, *et al.*, 2005).

Recent reports indicate that probiotics can induce Tregs and tolerogenic DCs. VSL#3 ameliorates TNBS-induced colitis in mice by inducing IL-10-dependent TGF-β-bearing regulatory T cells (Di Giacinto, *et al.*, 2005). The same probiotic mix, administered after surgery in patients undergoing ileal pouch anal anastomosis for UC, reduced the pouchitis disease activity index and increased the number of mucosal Tregs (Pronio, *et al.*, 2008). Foligne *et al*. (2007) showed that DCs treated with LAB strains such as *L. salivarius* Ls33, *L. rhamnosus* Lr32 or *L. acidophilus* NCFM ameliorate TNBS-induced colitis in mice. The protection was associated with a reduction of inflammation scores and colonic expression of

pro-inflammatory genes, while a high local expression of the immunoregulatory enzyme indolamine 2,3 dioxygenase (IDO) was observed. The preventive effect of probiotic-pulsed DCs required not only MyD88-, TLR2- and NOD2-dependent signaling, but also the induction of IL-10 independent $CD4^+$ $CD25^+$ regulatory cells. Although the mechanism is not yet clear, this response may have been generated by peptidoglycan of tolerogenic probiotic that could impact on the TLR2 signaling cascade through NOD2 interaction, or by potential role of DNA mediated by TLR9 signaling in the effect of probiotics on DC function (Foligne, *et al.*, 2007). These examples illustrate that probiotics have the potential to promote gut tolerance via instruction of DCs to generate immune-regulatory T cells. Further investigations are needed to identify specific mechanisms of action.

The immunomodulatory effects of probiotics discussed in this chapter are summarized in the tables 2 (*in vitro* effects) and 3 (experimental colitis models). Some other examples were also added to the tables.

Bacterial-derived molecules underlying probiotics effects

Bacterial effectors of different nature have been implicated in probiotic effects. These include peptidoglycan, lipoproteins and lipoteichoic acids, lipopolysaccharides, flagelin and CpG motifs in DNA, all of which bind to eukaryotic PRRs inducing distinct patterns of gene expression in the host cell that guide the activation of innate immunity and initiate the development of antigen-specific acquired immunity (Akira and Takeda, 2004). Bacterial surface proteins have also been implicated in the binding of bacteria to epithelial host cells, mucus or fibronectin (Sanchez, *et al.*, 2008). The picture of these interactions is far from complete, however.

Table 2: *In vitro* immunomodulatory effects of probiotics

Probiotic species	Model system	Probiotic effect(s)	Mechanisms involved	Reference(s)
Lactobacillus rhamnosus GG	IECs	Inhibits NF-κB	Decreases IκB degradation	(Zhang, *et al.*, 2005)
Lactobacillus reuteri	IECs	Inhibits NF-κB	Decreases IκB degradation	(Ma, *et al.*, 2004)
Bifidobacterium longum	IECs	Inhibits NF-κB	-	(Bai, *et al.*, 2004)
Lactobacillus bulgaricus	IECs	Inhibits NF-κB	-	(Bai, *et al.*, 2004)
Lactobaacillus plantarum ATCC 8040	IECs	Inhibits NF-κB and ERK	Decreases IκB degradation	(Ko, *et al.*, 2007)
Bifidobacterium lactis NCC362	IECs	Inhibits NF-κB	-	(Riedel, *et al.*, 2006)
Lactobacillus delbrueckii	IECs	Inhibits NF-κB	Decreases IκB phosphorylation	(Santos Rocha, *et al.*, 2012)
Bifidobacterium brevis	IECs	Inhibits NF-κB	Decreases IκB phosphorylation	(Heuvelin, *et al.*, 2009)
Lactobacillus rhamnosus GG	IECs	Inhibits NF-κB	Decreases IκB ubiquitination	(Kumar, *et al.*, 2007; Lin, *et al.*, 2009)
VSL#3	IECs	Inhibits NF-κB	Decreases IκB proteasome degradation	(Petrof, *et al.*, 2004)
Bifidobacterium lactis BB12	IECs	Induces MAPKs	Increases p38 phosphorylation	(Ruiz, *et al.*, 2005)
Bacteroides vulgatus	IECs	Induces NF-κB	Induces IκB phosphorylation	(Haller, *et al.*, 2002)
Lactobacillus crispatus M247	IECs	Inhibits NF-κB	Increases PPARγ expression-	(Voltan, *et al.*, 2008)
VSL#3	IECs	Inhibits NF-κB	Increases PPARγ expression-	(Ewaschuk, *et al.*, 2006)
Bacteroides thetaiomicron	IECs	Inhibits NF-κB	Increases PPARγ expression-	(Kelly, *et al.*, 2004)
Escherichia coli M17	Macrophages	Inhibits NF-κB	-	(Fitzpatrick, *et al.*, 2008)
Lactobacillus casei Shirota	Macrophages	Inhibits NF-κB	Decreases IκB phosphorylation	(Watanabe, *et al.*, 2009)

Probiotic species	Model system	Probiotic effect(s)	Mechanisms involved	Reference(s)
Lactobacillus rhamnosus GG	Macrophages	Induces IL-10 production	Stimulates SOCS3	(Latvala, *et al.*, 2011)
Lactobacillus plantarum K8	Macrophages	Inhibits NF-κB and MAPKs	Decreases TLR4, NOD1 and NOD2 expression	(Kim, *et al.*, 2008a)
Lactobacillus casei YIT9029	Macrophages	Induces NF-κB	-	(Matsuguchi, *et al.*, 2003)
Lactobacillus crispatus	Macrophages	Induces NF-κB	-	(Klebanoff, *et al.*, 1999)
Lactobacillus rhamnosus GG	Macrophages	Induces NF-κB and STATs	-	(Miettinen, *et al.*, 2000)

Table 3: Immunomodulatory effects of probiotics in experimental colitis model

Probiotic species	Model system	Probiotic effect(s)	Mechanisms involved	Reference(s)
Escherichia coli M17	DSS	Attenuates colitis	Inhibits NF-κB, decreases colonic IL-12, IL-6, IL1-β and IFN-γ	(Fitzpatrick, *et al.*, 2008)
Lactobacillus casei	TLR4 KO and DSS	Attenuates colitis	Reduces pro-inflammatory cytokines secretion and neutrophil recruitment	(Chung, *et al.*, 2008)
Faecalibacterium prausnitzii	TNBS	Attenuates colitis	Increases colonic IL10 and decreases colonic IL12. Tends to correct the dysbiosis associated with TNBS colitis	(Sokol, *et al.*, 2008)
Mix of four lactobacillus or four Bifidobacterium species	DSS	Attenuates colitis	Reduces colonic pro-inflammatory cytokines	(Nanda Kumar, *et al.*, 2008)
VSL#3	TNBS	Attenuates colitis	Increases production of IL-10 and Tregs	(Di Giacinto, *et al.*, 2005)
Lactobacillus salivarius Ls33	TNBS	Attenuates colitis	Increases IL-10 production and Tregs	(Macho Fernandez, *et al.*, 2011)
Lactobacillus plantarum DSM 15313, *Lactobacillus fermentum* 35D	DSS	Attenuates colitis	Reduces bacterial translocation	(Osman, *et al.*, 2008)
Bacteroides fragilis	TNBS	Attenuates colitis	Increases production of IL-10 and Tregs	(Round and Mazmanian, 2010)
Lactobacillus salivarius 433118, *Bifidobacterium infantis*	IL-10 KO	Attenuates colitis	Reduces inflammatory cytokines	(McCarthy, *et al.*, 2003)
Lactobacillus casei Shirota	DSS	Attenuates colitis	Reduces IL-6 production by LPMC	(Matsumoto, *et al.*, 2005)

Abbreviations: ERK, extracellular signal-regulated kinases; IEC, intestinal epithelial cell; IκB, inhibitor of NF-κB; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappaB; PPARγ, peroxisome proliferator activated receptor-gamma; SOCS, suppressor of cytokine signaling; LPMC, lamina propria mononuclear cells; STAT, signal transducer and activator of transcription; DSS, Dextran sulfate sodium; TNBS, tri-nitro-benzene-sulfonic acid.

Cell surface structures. A number of studies demonstrated that direct contact between bacteria and IECs was required for certain effects observed, suggesting that cell surface factors are involved in those effects. However, most of the studies did not identify the probiotic elicitors.

L. reuteri was reported to inhibit IL-8 secretion by T84 and HT29 cells and to block the translocation of NF-κB to the nuclei of HeLa cells. The authors showed that *L.reuteri* must be preincubated with IECs and be adherent and alive to induce its inhibitory effect. The effect was not reproduced by conditioned media, bacterial lysates or heat-killed or gamma-irradiated bacteria (Ma, *et al.*, 2004). *L. acidophilus* ATCC 4536 was able to mediate anti-inflammatory and anti-apoptotic effects only in direct contact with epithelial cells, by the activation of MAPK and the prevention of NF-κB activation (Resta-Lenert and Barrett, 2003; Resta-Lenert and Barrett, 2006; Yan, *et al.*, 2007). Some important cell surface factors from lactobacilli involved in immune modulation effects have been identified. For instance, LTA from *L. johnsonii* La1 and *L. acidophilus* La10 inhibited LPS induced IL-8 release by HT29 cells (Vidal, *et al.*, 2002). PSA from *B. fragilis* is protective against colitis induced by *Helicobacter hepaticus* (Mazmanian, *et al.*, 2008). PGN which makes up the body of the bacterial cell wall is among the main surface components of Gram positive bacteria recognized by the innate immune system (Guan and Mariuzza, 2007). While most research has highlighted the role of PGN in the pathogenesis of various bacteria (Boneca, 2005), relatively few papers reported beneficial effects of this bacterial compound. Shida *et al*. (2009) showed that PGN from *L .johnsonii* JCM 2012 and *L. plantarum* ATCC 14917 inhibited IL-12 production by macrophages. Recently, Macho Fernandez *et al*. (2011) showed that PGN purified from *L. salivarius* Ls33 rescued mice from TNBS-induced colitis in an IL-10-dependent manner and favored the developments of $CD103^+$ DCs and

$CD4^+Foxp3^+$ Tregs. Bleau *et al*. (2010) reported that exopolysaccharides (EPS) from *L. rhamnosus* RW-9595M increase IL-10 production by macrophages.

Surface proteins can also interact with host cells, mainly by TLR pathways, as has been demonstrated mainly for pathogenic bacteria (Bulut, *et al.*, 2005; Yarovinsky, *et al.*, 2005; Schröder, *et al.*, 2008).

Surface proteins of *L. delbrueckii* are involved in its anti-inflammatory effects in HT-29 cells (Santos Rocha, *et al.*, 2012). Hoermannsperger *et al*. (2009) showed that cell surface proteins of the *L. casei* strain present in the probiotic mix VSL#3 were responsible for the inhibition of TNF-α-induced secretion of T-cell chemokine interferon-inducible protein (IP-10) in Mode-K cells, demonstrating that cell surface proteins of *L. casei* are able to elicit anti-inflammatory effects in IEC.

Secreted factors. Several studies reported probiotic effects for which direct cell contact was not required. Soluble peptides of the probiotic mixture VSL#3 were shown to inhibit the degradation of IκB and to induce heat shock proteins through proteasome inhibition (Petrof, *et al.*, 2004). VSL#3 was also reported to stabilize tight junctions and induce mucins in IECs by a large (>50 kDa) but unidentified proteinaceous soluble factor (Otte and Podolsky, 2004). Secreted proteins of *L. rhamnosus* GG (p40 and p75) stimulated activation of Akt, promoted epithelial cell growth and inhibited TNF-α-induced epithelial cell apoptosis. These two proteins show similarity with putative cell wall-associated hydrolases or cell wall-modifying enzymes and are abundantly present in *L. rhamnosus* GG culture supernatants (Yan and Polk, 2002; Yan, *et al.*, 2007). Recently, Kaci *et al*. (2011) showed that small soluble factors (<3 kDa) of *Streptococcus salivarius* were able to inhibit TNF-α-induced NF-κB in IECs.

Unmethylated CpG DNA.

CpG DNA from the probiotic VSL#3 was shown to inhibit IL-8 secretion, reduce p38 MAPK and NF-κB activation in IECs (Jijon, *et al.*, 2004). TLR9 signaling has been shown to mediate the anti-inflammatory effects of VSL#3 DNA in a DSS-induced model of colitis (Rachmilewitz, *et al.*, 2004).

The examples describe above illustrate that multiple cell surface and secreted factors as well as DNA of probiotics seem to exert protective effects *in vitro* and in animal models of colitis. However, the effector-receptor interaction still needs to be studied in most cases. The identification of other bioactive probiotics molecules will provide new insights for the development of probiotic-based drug treatment for IBD.

Conclusion

The microbiota consists of a complex community of microorganisms that have been better understood as their interactions due to the advent of molecular biology techniques. Capable of extend the spectrum of the host metabolism; contribute to the maturation and modulation of the immune system, and even its diversity directly related to the health of hospededeiro. The reestablishment of a healthy microflora has been attempted as an alternative treatment for diseases that correlate with disturbed microbiota, as in the case of people suffering from inflammatory bowel diseases. For this, several preclinical studies and clinical trials have been conducted to evaluate potential probiotic being observed in many cases satisfactory results, although their mechanism of action remain to be elucidated further. Thus, in addition to contributing to intestinal health condition when acquired naturally, beneficial bacteria, may favor the return of the conditions homeostásicas gut when eaten after being rationally selected.

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CAPÍTULO 2

Efeito anti-inflamatório de *Lactococcus lactis* NCDO 2118 durante o período de remissão da colite induzida quimicamente

Resumo

Muitas bactérias probióticas são descritas como uma ferramenta promissora no tratamento de doenças inflamatórias intestinais (IBD). A maioria destes pertence ao grupo das bactérias lácticas (LAB), que fazem parte da nossa microbiota saudável. No entanto, pouco se sabe sobre os efeitos das bactérias que estão constantemente presentes em nossa dieta, incluindo *Lactococcus lactis*. No presente estudo, foram analisados os efeitos de modulação do sistema imunológico de três linhagens de *L. lactis in vitro* utilizando células epiteliais do intestino, sendo a linhagem com melhor desempenho, avaliada em um modelo murino de colite ulcerativa. Apenas uma linhagem de *L. lactis*, NCDO 2118, foi capaz de reduzir a secreção de IL-8, induzida por IL1-β em células Caco-2, sugerindo um potencial efeito anti-inflamatório desta lihagem. *In vivo*, *L. lactis* NCDO 2118 foi administrada durante 4 dias em camundongos C57BL/6 durante o período de remissão entre um primeiro e um segundo curso de colite induzida por sulfato de sódio dextrano. O tratamento com *L. lactis* NCDO 2118 resultou em uma forma mais branda da colite recorrente do que o observado em camundongos que receberam apenas meio de cultura durante este mesmo período. Este efeito protetor não foi atribuível às mudanças na secreção de IgA, contudo, a administração de NCDO 2118 foi relacionado com o aumento precoce na produção de IL-6 e pela manutenção da IL-10 no tecido do cólon. Camundongos tratados com *L. lactis* NCDO 2118 tiveram aumento no número de células T reguladoras $CD4^+$ que apresentam na superfície TGF-β sob a forma de peptídeos associados à latência (LAP^+ células T) em linfonodos mesentéricos e no baço. Os resultados deste estudo permitiu identificar uma nova linhagem probiótica que poderá ser utilizada no tratamento da IBD, bem como alguns dos mecanismos envolvidos no seu efeito anti-inflamatório.

Neste capítulo encontra-se o resultado da avaliação probiótica de *L. lactis* NCDO 2118.

Anti-inflammatory effects of *Lactococcus lactis* NCDO 2118 during the remission period of chemically-induced colitis

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Abbreviations:

Caco-2 cells, human colon adenocarcinoma cell line; CD, Crohn´s Disease; CFU, unit colony forming units; DSS, dextran sulphate sodium; IBD, Inflammatory Bowel Diseases; IECs, intestinal epithelial cells; IL1-β, Interleukin-1 beta; IFN-γ, interferon gamma; GIT, gastrointestinal tract; LAB, Lactic Acid Bacteria; LAP, latency-associated peptide; MOI, multiplicity of infection; NF-κB, nuclear factor kappa B; sIgA, Secretory Immunoglobulin A; TGF-β, Transforming growth factor-beta; TNBS, tri-nitro-benzene-sulfonic acid; Treg, regulatory T cells; UC, ulcerative colitis.

ABSTRACT

Many probiotic bacteria have been described as promising tools in the therapy and prevention of Inflammatory Bowel Diseases (IBD). Most of them belong to the Lactic Acid Bacteria group, which is an important part healthy human microbiota. However, little is known about the effects of transient bacteria present in normal diets, including *Lactococcus lactis*. In the present study, we analyzed the immune modulation effects of three *L. lactis* strains *in vitro* using intestinal epithelial cells. Only one strain, *L. lactis* NCDO 2118, was able to reduce the IL1-β-induced IL-8 secretion in Caco-2 cells, suggesting a potential anti-inflammatory effect. *In vivo*, *L. lactis* NCDO 2118 was administered for 4 days to C57BL/6 mice during a remission period of colitis induced by dextran sulfate sodium, resulting in a milder form of recurrent colitis than observed in control mice. This protective effect was not attributable to changes in secretory IgA, however NCDO 2118 administration was associated with early increase in IL-6 production and by maintaining IL-10 concentrations in colonic tissue. Mice fed with *L. lactis* NCDO 2118 increased the number of regulatory $CD4^+$ T cells bearing surface TGF-β in the form of latency-associated peptide in mesenteric lymph nodes and spleen. The results of this study allowed us to identify a new probiotic strain that could be used in the treatment of IBD as well as some of the mechanism involved in its anti-inflammatory effect.

INTRODUCTION

Inflammatory Bowel Diseases (IBD), such as Ulcerative Colitis (UC) and Crohn´s Disease (CD), have very complex causes, including genetic, environmental and geographic factors (Khor, *et al*., 2011). It is now thought to result from inappropriate and ongoing activation of the mucosal immune system driven by the presence of an abnormal gut microbiota resulting in chronic inflammation of the gastrointestinal tract (GIT) (LeBlanc, *et la*., 2013). It has been shown that infiltrating T lymphocytes responsive to the gut microbiota might be associated with a loss of tolerance in the intestinal milieu (Podolsky, 2002).

Current IBD treatments include the use of anti-inflammatory drugs, which induce or maintain remission, but these are not curative. Moreover, their use is accompanied by several side effects such as allergic reactions, pruritis, chills, fever, urticaria and liver problems (Nielsen and Munck, 2007; Marteau, *et al*., 2006). In this context, biologic agents, like probiotics with anti-inflammatory properties have been proposed as tools in the prevention or treatment of IBD (de Moreno de Leblanc *et al*., 2011).

Most of the probiotics used and studied today belong to Lactic Acid Bacteria (LAB) group, mainly lactobacilli, which have been isolated from the human GIT, but alsot include some *Bifidobacterium* (Cronin, *et al*., 2011) and *Streptococcus* strains (Wescombe, *et al*., 2009). Members of the *Lactobacillus* genus have shown therapeutic properties, with improvement of the normal microbiota (Kuhbacher, *et al*., 2006; Nanda Kumar *et al*., 2008), prevention of infectious diseases and food allergies (Chai, *et al*., 2013; Castillo, *et al*., 2013; Isolauri, *et al*., 2012), stabilization of the gut mucosal barrier (Reiff and Kelly, 2010; Madsen, *et al*., 2001) and modulation of the innate and adaptive immune responses (Zhang, *et al*.,

2005; Jijon, *et al*., 2002; Riedel, *et al*., 2006; Di Giacinto, *et al*., 2005; Santos Rocha, *et al*., 2012).

The *Lactococcus* genus, in turn, has received little attention on its probiotics activities, mainly because they are not traditionally considered as commensal bacteria (Kimoto, *et al*., 1999). However, *Lactococcus lactis* strains are in constantly transient through the GIT after ingestion of fermented dairy and vegetable products, and only a few studies have shown that they can exertbeneficial effects (Santos Rocha, *et al*., 2012). Among these, Nishitani *et al.* (2009) demonstrated that *L. lactis* subsp. c*remoris* FC possess a potent anti-inflammatory activity. Oral administration of *L. lactis* FC reduced inflammatory cytokines production as well as inducible oxide nitric expression in DSS (dextran sulphate sodium)-induced colitis in mice, suggesting that orally administered *L. lactis* FC may have good implications for human IBD treatment (Nishitani, *et al*., 2009). Thus, the objective of this study was to evaluate the potential mechanisms involved in the anti-inflammatory effects of *L. lactis* strains which are still poorly understood.

MATERIAL AND METHODS

***Bacterial strains and growth conditions*.** Three *L. lactis* strains were used in this study: *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *lactis* NCDO 2118 and *L. lactis* subsp. *cremoris* MG1363 (Table 1). They were grown at 30^{o}C in M17 medium (Difco), containing 0.5% glucose (GM17), without agitation or in the same medium solidified with 1.5% agar during 18 hours.

Table 1: Strains used in this work

Strain	*Relevant feature*	*Reference*
Lactococcus lactis subsp. *lactis* NCDO 2118 [NCDO2118]	Plasmid-free, isolated from frozen peas (test group)	Miyoshi, *et al*., 2004
Lactococcus lactis subsp. *lactis* IL1403 [IL1403]	Plasmid-free, is derived from the *L. lactis* IL594 strain, an isolated from starter culture of cheese (control group)	Chopin, *et al*., 1984
Lactococcus lactis subsp. *cremoris* MG1363 [MG1363]	Plasmid-free, from the curing of the *L. lactis* NCDO 712 strain, is the prototype of lactic acid bacterium (control group)	Gasson, 1983

***Epithelial cell culture*.** Caco-2 cells, a human colon adenocarcinoma cell line, were cultured in RPMI medium (Sigma) supplemented with 10 % (v/v) of fetal bovine serum (FBS) (Gibco), 2 mM of L-glutamine, 0.1 mM of non-essential amino acids, 1 mM sodium pyruvate solution, $5x10^{-5}$ M 2-ME (Sigma-Aldrich) and 25 ng/mL of gentamicin in an atmosphere containing 5% CO_2 at 37°C.

***Epithelial cell treatments*.** Caco-2 cells were seeded at $3x10^5$ cells/well in 24-wells plates and incubated at 37°C, 5% CO_2 for 24 hours before treatment. Secretion of the pro-inflammatory cytokine IL-8 by the cells was induced by the addition of human recombinant IL-1β (BD Bioscience) to a final concentration of 10 ng/mL. *L. lactis* cultures at stationary phase of growth were centrifuged for fractions separation (supernatant and cells), and each fraction was co-incubated with Caco-2 cells. Bacterial cells washed 2 times with PBS (137 mM/L NaCl, 2.7 mM/M KCl , 10 mM/L $Na_2HPO4 \bullet 2 H_2O$, 2 mM/L KH2PO4) and added at a *multiplicity* of *infection* [MOI] of 5 and filtered supernatant at a final concentration of 10% (v/v). Caco-2 cells without IL-1β treatment was used as control. After 6 hours of co-incubation, the supernatant of cell cultures was collected and stored at -80ºC until analysis.

IL-8 levels were measured using Human IL-8 ELISA set (BD Biosciences), following the manufacturer's instructions. Data were analyzed from three independent experiments.

Animals. Conventional inbred female C57BL/6 mice, 10 to 12 weeks of age, were obtained from Universidade Federal de Minas Gerais (UFMG), Brazil. Mice were maintained in an environmentally controlled room with 12h light-dark cycle. All procedures were approved by the local ethical committee for animal research (protocol number 114/2012).

DSS-induced colitis. Chemically colitis was induced by replacing the drinking water of mice with a 2 % (w/v) dextran sodium sulphate; (DSS , MP Biomedicals) aqueous solution during 7 consecutive days. After this time, mice received orally GM17 medium (DSS group) or the oral treatment with *L. lactis* NCDO 2118 (DSS + NCDO2118 group), during four consecutive days. Fresh NCDO2118 total culture (bacteria plus supernatant at stationary phase of growth) were prepared daily before being offered to the mice. Since each mouse drank about 5 mL of culture per day (data not shown), the total dose of bacteria per mouse was estimated to be $5x10^9$ bacteria/day. Mice were killed at day 14 (just after the oral treatment) or upon a second DSS cycle (at day 21). The control group of mice received water during the 21 days of experiment. Throughout the experimental period, all mice had unlimited access to food. Figure 2A shows the schematic representation of the experimental procedure.

Macroscopic and microscopic assessment of colitis. The macroscopic score of DSS induced colitis was derived by scoring three major clinical signs, which were weight loss, diarrhea, and rectal bleeding, 7 days after DSS administration as described by Cooper *et al*. (1993). Loss of body weight was calculated as the difference between the initial and actual weight. Diarrhea was shown as mucus/fecal material adherent to anal fur. The presence or absence of diarrhea was confirmed by examination of the colon following completion of the experiment. Mice were killed and the colons and spleens were excised from the animals.

Diarrhea was defined by the absence of fecal pellet formation in the colon and the presence of continuous fluid fecal material in the colon. Rectal bleeding was defined as diarrhea containing visible blood and gross rectal bleeding and scored as described for diarrhea. The three major clinical signs (weight loss, diarrhea, and occult/gross bleeding) were scored separately. The macroscopic score was calculated from the score of the clinical signs using the following formula: (weight loss score) + (diarrhea score) + (rectal bleeding score). Colon samples were fixed in formalin and processed for histological analysis. Hematoxylin-eosin stained sections were blindly scored based on a semi-quantitative scoring system previously described (McCafferty, *et al*., 2000) where the following features were graded: extent of destruction of normal mucosal architecture (0, normal; 1, 2 and 3, mild, moderate and extensive damage, respectively), presence and degree of cellular infiltration (0, normal; 1, 2 and 3, mild, moderate and transmural infiltration, respectively), extent of muscle thickening (0, normal; 1, 2 and 3, mild, moderate and extensive thickening, respectively), presence or absence of crypt abscesses (0, absent; 1, present) and the presence or absence of goblet cell depletion (0, absent; 1, present). Scores for each feature were summed up to a maximum possible score of 11.

***Colon tissue preparation and cytokine assay*.** Colon samples were weighed and homogenized in PBS containing 0.05 % (v/v) Tween-20, 0.1mM phenylmethylsulphonyl fluoride, 0.1mM benzetho-nium chloride, 10mM EDTA and 20 KIU Aprotinin A using a tissue homogenizer (100 mg tissue/ml buffer) (Gomes-Santos *et al*., 2012). Suspensions were centrifuged at 12.000g for 20 min at 4^{o}C and the supernatants collected for cytokine assay. Plates were coated with purified monoclonal antibodies reactive for the cytokines IL-6, IL-12, IFN-gamma, IL-17, IL-10 and TGF-beta (BD-Pharmingen) overnight at 4^{o}C. In the following day, wells were washed, supernatants were added and plate was incubated overnight at 4^{o}C. In the third day, biotinylated monoclonal antibodies against cytokines were

added and plates were incubated for 2 hour at room temperature. Color reaction was developed at room temperature with 100µl/well of orthophenylenediamine (1mg/ml), 0.04 % (v/v) H_2O_2 substrate in sodium citrate buffer. Reaction was interrupted by the addition of 20 µl/well of 2N H_2SO_4. Absorbance was measured at 492 nm using a microplate reader (BIO-RAD).

***Secretory IgA (sIgA) assay*.** Levels of sIgA were determined by ELISA. Briefly, 96-well plates (NUNC) were coated with Ig goat anti-mouse UNLB antibody, in coating buffer pH 9.6 overnight at 4ºC. Wells were washed and blocked with 200µl of PBS contain 0.25% casein for 1h at room temperature. Samples were added to the plate and incubated for 1h at 37ºC and washed, then peroxidase- streptavidin goat anti-mouse IgA- HRP (Southern Biotechnology) 1:10000 was added, and plates were incubated for 1 h at 37°C. Color reaction was developed at room temperature with 100µl/well of orthophenylenediamine (1mg/ml) (SIGMA), 0.04% H_2O_2 substrate in sodium citrate buffer. Reaction was interrupted by the addition of 20 µl/well of 2N H_2SO_4. Absorbance was measured at 492nm by an ELISA microplate reader (Bio-Rad).

***Antibodies and FACS analysis*.** Fluorescein isothiocyanate-conjugated (FITC) (mAbs) to CD69, CD25 and CD11c; phycoerithrin (PE)-conjugated mAbs to CD45RB and CD11b and biotinylated antibodies to CD103; streptavidin-Cy5-Chrome were purchased from BD Biosciences. The biotinylated anti-human LAP (TGF-β) antibody was purchased from R&D System. Surface staining was performed according to standard procedures at density of 0,5-1x10^6 cells per well. Samples were analyzed in a FACSCan instrument (BD) and analyzed in FlowJo Software (Tree Star Inc).

Statistical analysis. Results were expressed as the mean ± standard error of the mean (SEM). Normal distribution of samples was confirmed by the Kolmogorov–Smirnov test. Significance of differences among groups was determined by Student's t-test or analysis of

variance (ANOVA) (Tukey's post test). Means were considered statistically different when $P < 0.05$.

RESULTS

L. lactis shows a strain-dependent anti-inflammatory effect on IECs

None of the strains tested induced IL-8 secretion above background levels, indicating that these *L. lactis* strains do not induce inflammatory events in IECs (Figure 1A and 1B). To investigate whether *L. lactis* posseses an anti-inflammatory activity in IECs, the ability of the strains to block the secretion of IL-8 induced by IL-1β was analyzed. Caco-2 cells secrete a baseline level of IL-8, which increases after stimulation with IL-1β. None of the live cell fractions was able to reduce IL-1β-induced IL-8 secretion (Figure 1A); however, the culture supernatant of the strain NCDO2118 reduced the production of IL-8 by 45% (Figure 1B), whereas the other 2 supernants did not show similar effects, demonstrating a strain-dependent anti-inflammatory role of *L. lactis*.

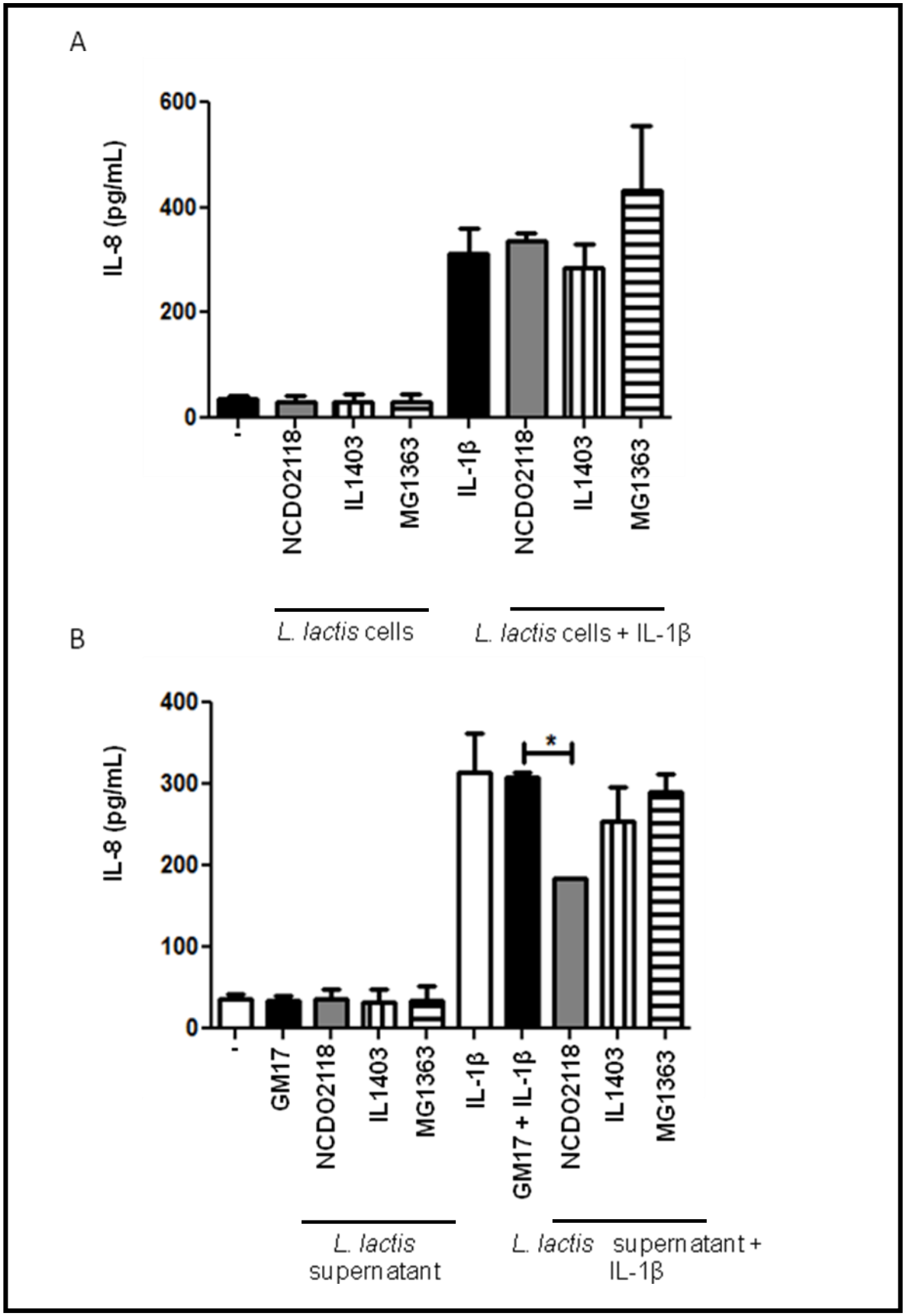

Figure 1. IL-8 levels after co-incubation of *L. lactis* strains with Caco-2 cells stimulated or not with IL1-β. **(A)** *L. lactis* cells. **(B)** *L. lactis* supernatant. Dash, without addition of IL-1β or bacteria; IL-1β, only IL-1β was added; GM17, only the medium was added. Bars represent the mean and the MSE of three independent experiment. *., $p<0.05$.

L. lactis NCDO 2118 oral administration alleviates colitis symptoms

Based on our *in vitro* results, NCDO2118 was then chosen to be tested *in vivo*. The effect of oral administration of this strain was tested in a murine model of chemically-induced colitis during the remission period and after a second colitis cycle. This experimental protocol mimics the remission and active periods of IBD. As shown in figure 2B, body weight significantly decreased during DSS treatment compared to water-treated mice (control group). After DSS withdrawal, mouse body recovered gradually in all experimental groups. However, at day 11, the oral treatment with NCDO2118 gently slows weight gain but in the second colitis cycle the body weight in NCDO2118 group remained higher than in the DSS group (Figure 2B). A reduction in colon length at day 14 in DSS and DSS-treated NCDO2118 group was also observed (Figure 2C). Nevertheless, at the end of the experiment at day 21, the oral treatment with *L. lactis* led to the reestablishment of this parameter (Figure 2C). Mice consuming *L. lactis* exhibited significantly reduced clinical symptoms (macroscopic inflammatory score) in the recovery phase and upon colitis induction (Figure 2D), despite the severity of inflammation after the second colitis cycle. These findings suggest that *L. lactis* NCDO2118 administered *in vivo* has an anti-inflammatory role.

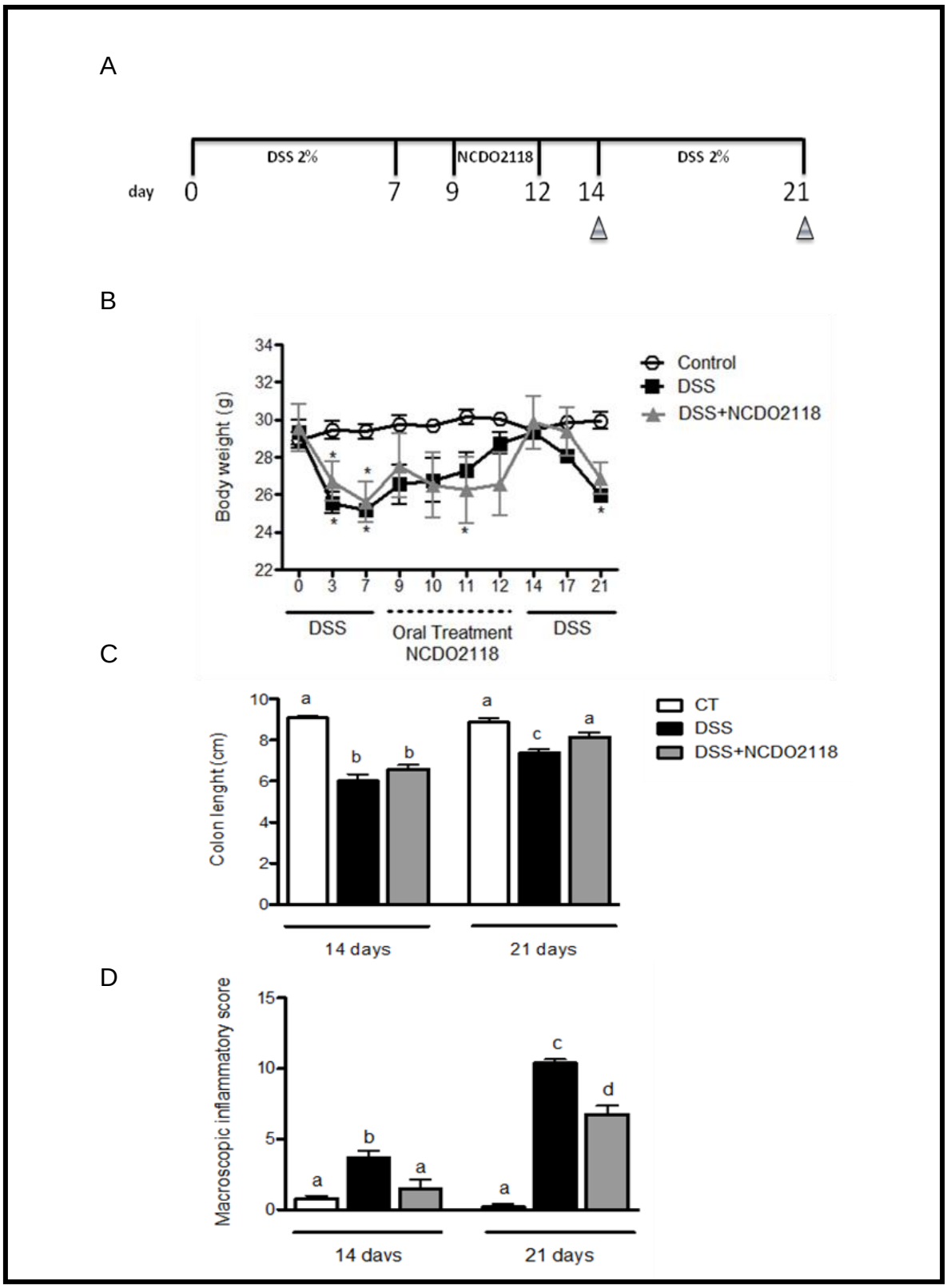

Figure 2. Oral administration of *L. lactis* NCDO 2118 improved colon shortening and macroscopic score of colitis. **(A)** Experimental protocol. C57BL/6 mice received DSS 2% during 7 days. *L. lactis* NCDO 2118 was continually administrated for 4 consecutive days during the remission period of colitis (arrows) between a first and second course of colitis. Control group received medium. Mice were killed at days 14 and 21 (arrows head). **(B)** Body weight from day 0 to day 21. **(C)** Colon length measured in cm. **(D)** Macroscopic score of colitis including body weight, diarrhea and rectal bleeding. Bars are the mean of 6 mice/group and data are representative of three independent experiments; ANOVA, post-test Tukey. Treatments followed by letters are sygificantly different ($p < 0.05$).

L. lactis NCDO 2118 prevents intestinal inflammation

The ability of NCDO2118 to prevent DSS-induced colonic damage was evaluated at the histological level. Control mouse colon sections, presented intact epithelium, well defined crypt length, and no neutrophil infiltration in mucosa and submucosa layers (Figure 3A). In contrast, colon tissue from DSS treated mice showed severe inflammatory lesion extensively throughout the mucosa and submucosa (Figure 3B). The oral administration of NCDO2118 was able to prevent histological damage in the second colitis cycle, but did not immediately improve the inflammatory status at the gut mucosa on day 14 (Figure 3C, D).

L. lactis NCDO 2118 did not alter the secretory IgA

The sIgA was evaluated in mouse feces at day 14 and 21, and levels of IgA were raised up only after the second colitis cycle. The oral administration of *L. lactis* NCDO 2118 maintains the sIgA in intermediated levels (Figure 4A). To verify if *L. lactis* was able to modify the sIgA levels in a physiological scenario, we measured the sIgA levels after 2, 3 or 4 days of *L. lactis* administration and control mice groups. NCDO2118 did not changes the sIgA levels in feces (Figure 4B), discarding the possibility of IgA modulation as a regulatory *L. lactis* mechanism.

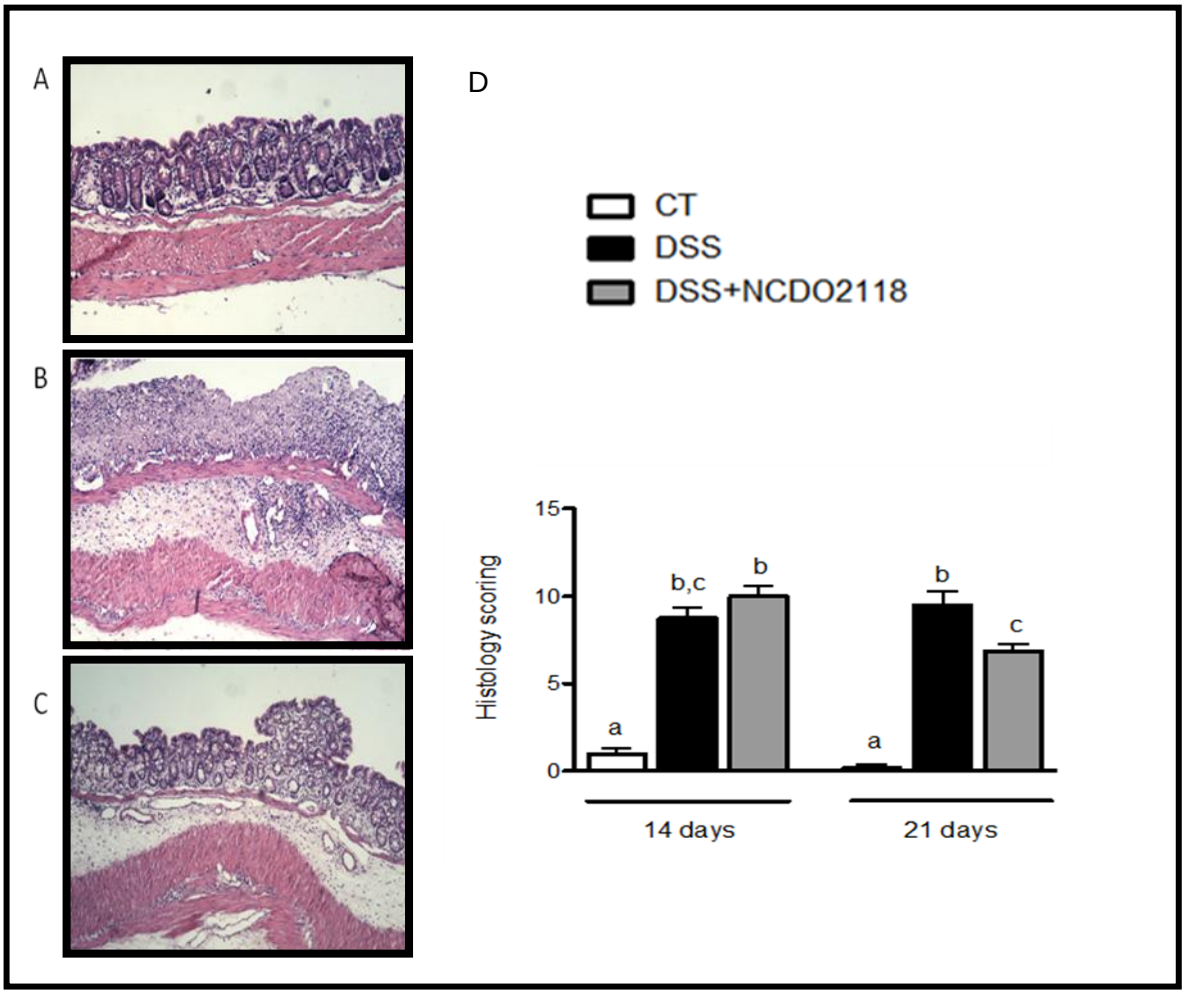

Figure 3. Oral administration of *L. lactis* NCDO 2118 prevented histological damage of colitis. Photograph (X100) of HE-stained paraffin sections of a representative colon from control **(A)**, DSS **(B)** and DSS + NCDO2118 **(C)** groups at day 21. **(D)** Histological scores of colon sections of DSS-colitis mice with or without oral administration of *L. lactis*. Values represent the means ± MSE (n=6). Treatments followed by letters are sygificantly different ($p < 0.05$).

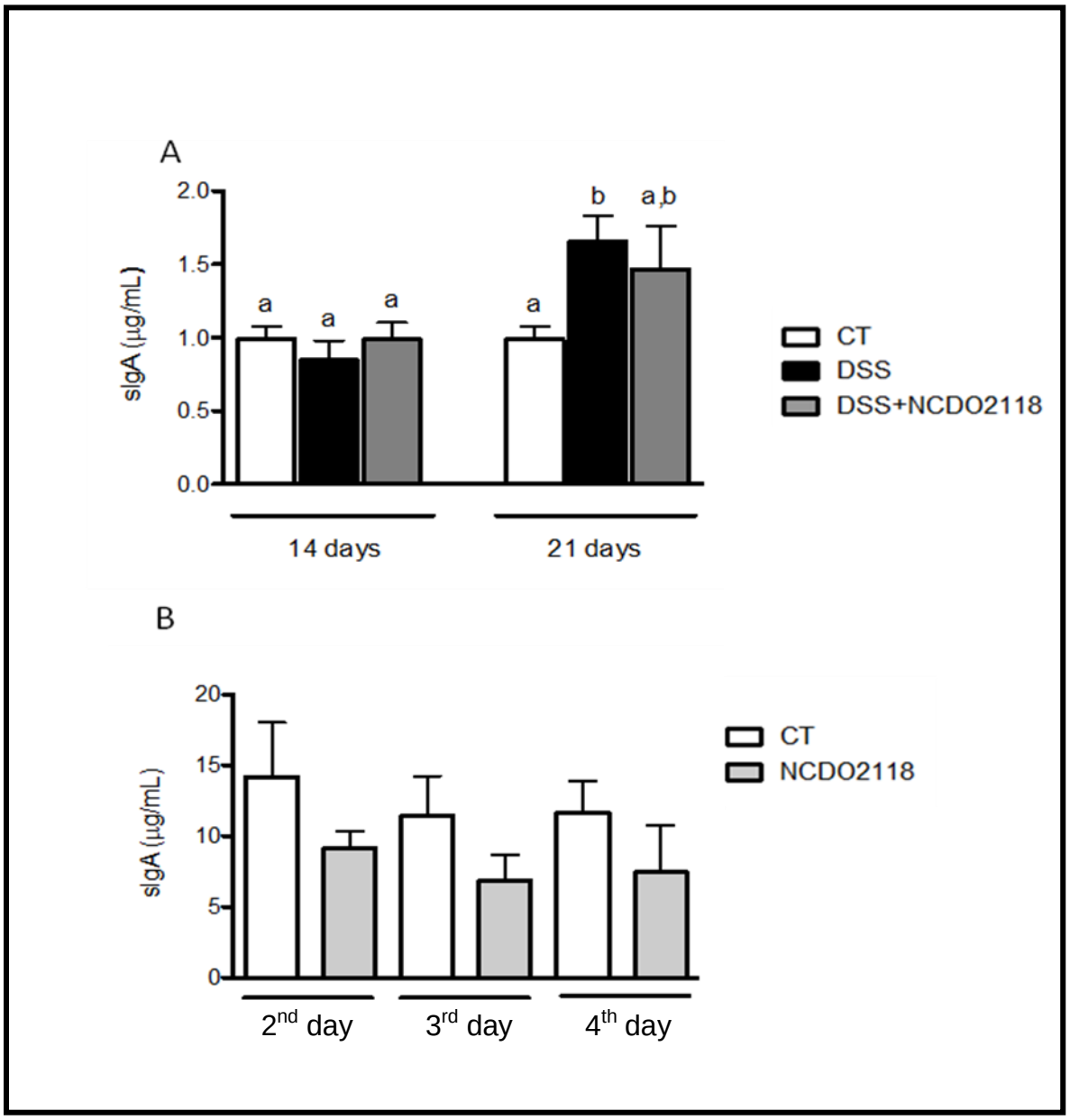

Figure 4. Oral administration of *L. lactis* NCDO 2118 did not change the secretory IgA production. (A) Intestinal feces were collected and total sIgA was measured by ELISA in mice from control, DSS and DSS + NCDO2118 groups. (**B**) intestinal feces from health mice were collected after 2, 3 or 4 days of *L. lactis* administration and total sIgA was measured by ELISA. Bars represent the mean ± MSE of 5 mice per group. Treatments followed by letters are sygificantly different ($p < 0.05$).

L. lactis NCDO 2118 modulates the production of cytokines in intestinal tissue

To further identify potential mechanisms by which *L. lactis* NCDO 2118 exerted its beneficial effect, the cytokine profiles in colonic tissue were evaluated at days 14 and 21. Oral administration of NCDO2118 significantly increased the levels of IL-6 at day 14, while the levels of this cytokine were higher at day 21 in both DSS and DSS-NCDO2118 treated groups (Figure 5A). The exposure of C57BL/6 to 2% of DSS led to increased IL-12 levels only at day 21, and *L. lactis* did not affect this behavior (Figure 5B). Despite this, the levels of IFN-γ did not change due to DSS or *L. lactis* treatment (Figure 5C). The levels of IL-17 were reduced at day 21 in both DSS and DSS-NCDO2118 (Figure 5D). TGF-β was not affected neither by DSS or *L. lactis* (Figure 5E). Finally, the anti-inflammatory cytokine IL-10 was significantly lowered in DSS-treated group at day 21, and NCDO2118 prevented this reduction (Figure 5F).

L. lactis NCDO 2118 enhanced regulatory T cells in mesenteric lymph node and spleen

Since the intestinal inflammation in DSS-induced colitis is triggered by microbial antigens, the induction of oral tolerance to microbiota could be one of the potential mechanisms by which *L. lactis* NCDO 2118 stimulates the immue system. Because oral tolerance is maintained mainly by Tregs cells (Faria and Weiner, 2005), we analyzed the changes in $CD4^{+}CD25^{+}CD45RB^{low}$ and $CD4^{+}CD25^{+}LAP^{+}$ T cells in mesenteric lymph node and spleen of the mice *L. lactis* NCDO 2118 did not change the numbers of activated T cells in mesenteric lymph node. However, this treatment enhanced the number of activated T cells in spleen (Figure 6A). This suggests that some *L. lactis* product is able to activated T cells *in vivo*. The population of $CD4^{+}CD25^{+}CD45RB^{low}$ regulatory T cells was not affected by DSS or DSS-NCDO2118 treatment (Figure 6B).

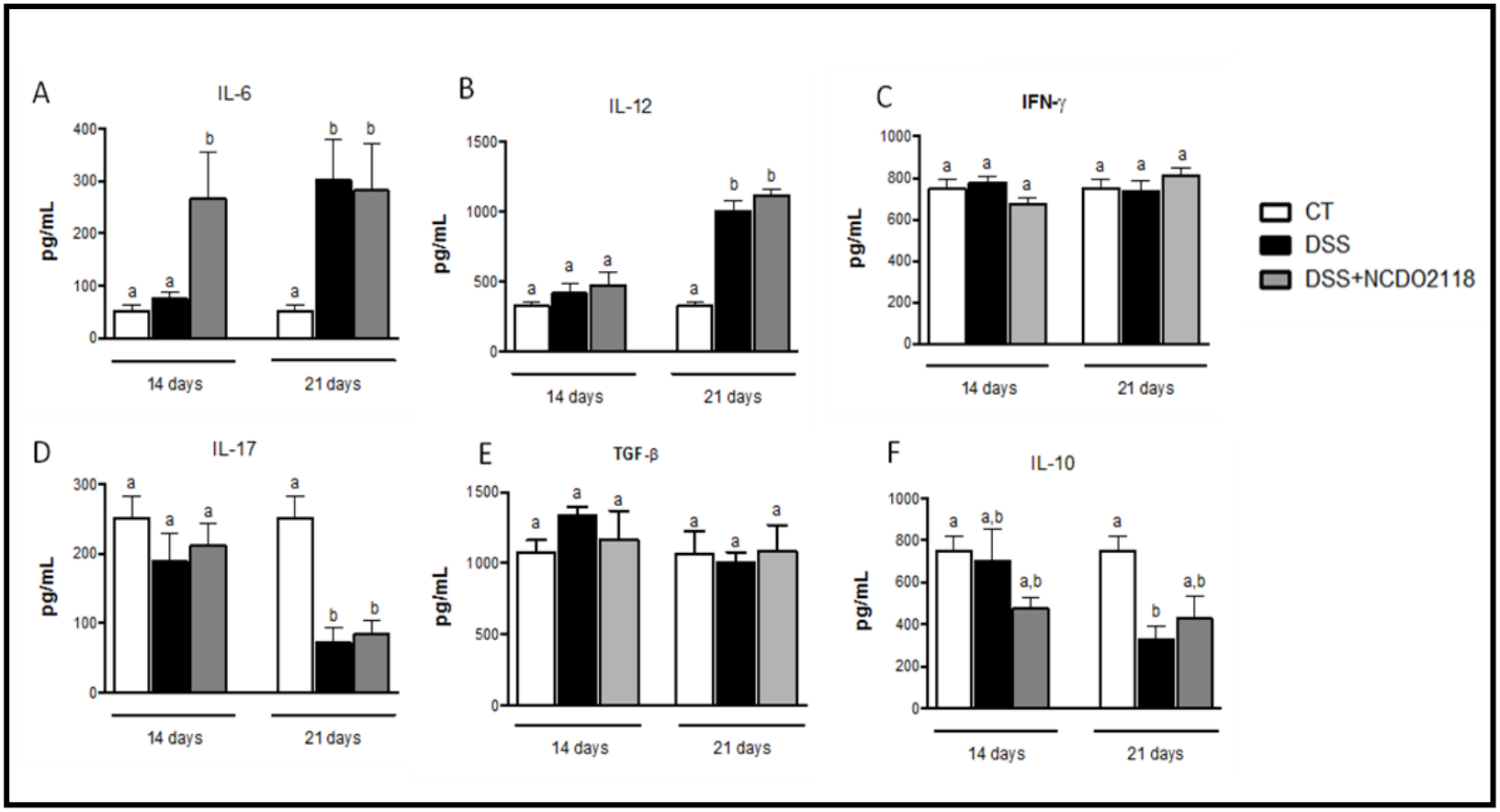

Figure 5. The effect of *L. lactis* NCDO 2118 in cytokine production in colonic tissue. Colonic IL-6 **(A),** IL-12 **(B),** IFN-γ **(C),** IL-17 **(D),** TGF-β **(E),** e IL-10 **(F).** were measured by ELISA in mice from control, DSS and DSS + NCDO218 groups. One representative result from two independent repetitions is shown. Bars represent the mean ± MSE of 5 mice per group. Treatments followed by letters are sygificantly different ($p < 0.05$).

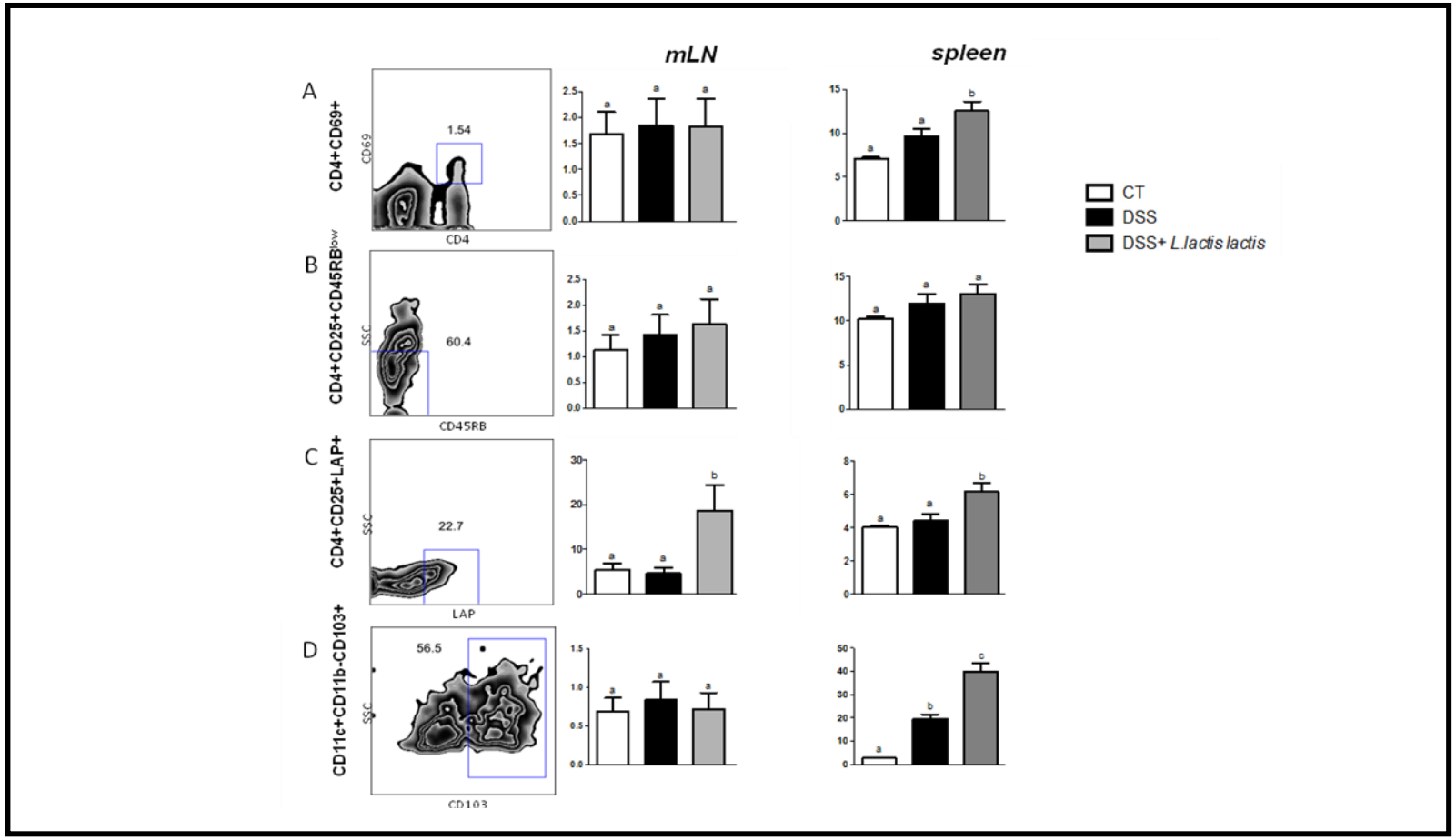

Figure 6. **The effect of *L. lactis* NCDO 2118 in the number of activated T cells, regulatory T cells and tolerogenic dendritic cells in chemically-induced colitis**. Cells from the mesenteric lymph nodes (mLN) and spleen were obtained and stained at day 21. **(A)** Number of CD4+CD69+ T cells. **(B)** CD4+CD25+CD45RB^{low} T cells. **(C)** CD4+CD25+LAP+ T cells. **(D)** CD11c+CD11b-CD103+ cells. Bars are the mean of 5 mice/group and data are representative of two independent experiments; ANOVA, post-test Tukey. Treatments followed by letters are sygificantly different ($p< 0.05$).

Nevertheless, the induced regulatory T cells $CD4^+CD25^+LAP^+$ were increased in the NCDO2118 treated mice in the mesenteric lymph node and spleen. Finally, we verified the dendritic cell population described as tolerogenic DCs expressing the integrin alfa-E beta 7. Although we did not find differences in $CD11c^+CD11b^-CD103^+$ cells in mesenteric lymph node, this population was increased during colitis (DSS group) but enhanced much more in DSS+*L. lactis* treated group.

DISCUSSION

Here we showed in an *in vitro* assay using IECs the immunomodulatory effect of *L. lactis* NCDO 2118 strain. This test is based on the fact that the IL-1β is associated with transcriptional activation of pro-inflammatory genes in cells of the intestinal epithelium. Thus, IL-1β activate transcription factors, including the nuclear factor κB (NF-κB), which induces an increased expression of pro-inflammatory mediators, such as IL-8, TNF-α, IL-6, Cox2 and many others (Wang, *et al*., 2009; Hoffmann, *et al*., 2002).

The expression of IL-8 is mainly regulated by NF-κB, and it has been shown that this transcriptional factor is overactivated in mucosal cells of IBD patients, while pharmacological inhibition of NF-κB activity modulates intestinal inflammation in mouse models of colitis (Neurath, *et al*., 1996). We showed that the culture supernatant of NCDO2118 was able to reduce the levels of IL-8 production in Caco-2 cells stimulated by IL-1β, suggesting that NCDO2118 is able to reduce NF-κB activation in these cells. Several probiotics, mainly commensals, are reported to modulate NF-κB response and influence downstream cytokine secretion in IEC, such as *Lactobacillus rhamnosus* GG (Ma, *et al*., 2004), *Lactobacillus reuteri* (Bai, *et al*., 2004), and *Bifidobacterium longum* (Bai, *et al*., 2004) while for *L. lactis*, little has been published. Co-cultures of *L. lactis* subsp. *cremoris* FC with Caco-2 cells resulted in significant down-regulation of IL-8 mRNA expression in Caco-2 cells and inhibition of NF-κB nuclear translocation in RAW264.7 cells (Nishitani, *et*

al., 2009). Interestingly, we found that IL-8 inhibition is dependent of the strain used. Similar results were reported by Santos Rocha *et al.* (2012). They also found a strain dependent NF-κB inhibition by the dairy bacteria *Lactobacillus delbrueckii* (Santos Rocha, *et al*., 2012).

Due to these interesting *in vitro* results, *L. lactis* NCDO 2118 appears to have potential to be used as a probiotic for IBD therapy. Thus, *in vivo* experiments were performed in order to evaluate the effectiveness of this strain in a murine colitis model, chemically induced by DSS.

We have shown that *L. lactis* NCDO 2118 was able to prevent a second colitis cycle induced by DSS. In our study, intestinal injury was assessed by a variety of methods including body weight, colon length and histology. By macroscopic and microscopic observations, *L. lactis* NCDO 2118 inhibited colonic injury. The experimental protocol used resembles the typical remission period of IBD. We opted to administrate the bacteria after the onset of colitis. This could have a clinical importance since it is not possible to predict when the disease onset or when it became active.

L. lactis NCDO 2118 also improves the macroscopic score of colitis after the oral administration (at day 14), especially the diarrhea. However, at this time point, the colon length and histology scoring were not ameliorated. The analyses of two time points (at day 14 and 21) allowed us to separate two different anti-inflammatory effects of *L. lactis*. The first one is a local effect of the bacteria possible based on the protection of intestinal epithelial cell and barrier function since the diarrhea diminished. The second is clearly an immunomodulatory effect and it seems to be dependent of expansion or recruitment of regulatory immune cells and their products. We opted to investigate the second effect since the improvement of colitis was more evident after the second colitis cycle.

Several studies showed that consumption of probiotics were associated with increased gut secretory IgA (sIgA) levels, which could promote the gut immunological barrier by

limiting the penetration of bacteria (commensals and pathogenics) into host tissues (O'Sullivan, 2001; Malin, *et al*., 1996) suggesting a regulatory mechanism of disease protection. This is particularly relevant in DSS model of colitis since DSS is directly toxic to gut epithelial cells and also enhanced the bacterial translocation (Okayasu, *et al*., 1990; Laroui *et al*., 2012). However, *L. lactis* did not alter the sIgA production after oral treatment.

The cytokine produced in gut mucosa greatly influence the resulting immunological outcome. The production of anti-inflammatory cytokines induces the mucosal tolerance and high levels of pro-inflammatory cytokines induce protective immune response and inflammation. The most intriguing aspect of probiotic modulation of immune response is through its effects on cytokine production. The effect of *L. lactis* on cytokine production was investigated in few works. Kimoto *et al*. (2004) showed that *L. lactis* G50 induces Th1 type immune responses *in vitro*. Pavan *et al*. (2003) observed a significantly increase in IFN-gamma production in the ilea of mice fed with *L. lactis* MG1363; however, in our study, *L. lactis* did not change the production of IFN-γ in colonic tissue.

Here, we found enhanced levels of IL-6 in the colon of mice fed with *L. lactis* after colitis induction. In acute situations, such as in chemically colitis induced by DSS, mice deficient for IL-6, showed higher levels of inflammation, than that observed in the control group. Where antigens breach the epithelial barrier, IL-6 enhances mucosal repair by epithelial restitution (Podolsky, 1999; Grivennikov, *et al*., 2009; Dann, *et al*., 2008; Chalaris, *et al*., 2010; Scheller, *et al*., 2011). Thus, we speculate that *L. lactis* could, via the production of IL-6, promote the restitution of epithelia and in consequence, reduced the diarrhea as discussed above.

IL-17 is generally thought to have a proinflammatory role in the intestine (Shen and Durum, 2010). However, the neutralization of IL-17 can aggravate acute DSS-induced colitis in mice, suggesting a protective role of IL-17 in colonic inflammation (Ogawa, *et al*., 2004).

In our study, the levels of IL-17 diminished in mice that received the second cycle of DSS but these levels were not affected by the oral administration of *L. lactis* NCDO 2118.

IL-10 is probably the most important cytokine in shaping immune responses at gut mucosa. IL-10 deficient mice spontaneously develop gut inflammation (Gomes-Santos, *et al*., 2012). In the present study, after the second colitis cycle, the levels of IL-10 were decreased in the colon of DSS-treated mice but *L. lactis* NCDO 2118 prevented this reduction. Thus, maintaining IL-10 levels seems to be responsible, at least in part, for the anti-inflammatory effect of *L. lactis* NCDO 2118.

In order to investigate the effect of *L. lactis* on T cell populations, we performed evaluated these cells in the mesenteric lymph node and spleen of mice treated or not with *L. lactis* NCDO 2118 during chronic colitis. IBD is generally believed to be driven by T cells and has been thought to be associated with increases in inflammatory cytokines, especially from Th1 and Th17 cells. Specialized regulatory T cells counterbalance these proinflammatory responses (Bouma and Strober, 2003; Strober, *et al*., 2007). The numbers of activated T cells expressing the earliest inducible cell surface glycoprotein acquired during lymphoid activation, CD69, were analyzed after the colitis induction. The numbers of $CD4^{+}CD69^{+}$ T cells enhanced only in the spleen of mice fed with *L. lactis* NCDO 2118. Therefore, some *L. lactis* products are able to activated T cells.

In mice, the transfer of $CD4^{+}CD25^{+}CD45RB^{low}$ T cells can prevent colitis induced by T cell transfer or innate immune activation (Veltkamp, *et al*., 2005). This population was further identified as a regulatory T cell. Despite the anti-inflammatory activity of NCDO2118, this strain did not enhance this regulatory T cell population. However, other type of peripherally induced Treg characterized by their surface expression of the latency-associated peptide (LAP), which is the N-terminal propeptide of TGF-beta precursor, were increased in the mesenteric lymph nodes and spleen of mice treated with NCDO2118.

Previously, Di Giacinto *et al*. (2005) showed that the probiotics VSL#3 administration during the remission period of TNBS induced colitis increases the numbers of regulatory $CD4^{+}LAP^{+}$ T cells and this is essential to the protective effect of probiotics.

Since dendritic cells modulate T cell differentiation into effector or regulatory T cells (Chen, 2006) the profile of dendritic cells (DCs) was evaluated. Previously it was shown that $CD103^{+}$ DCs have been shown to induce Tregs cells. In chemically colitis model, we found increased numbers of $CD11c^{+}CD103^{+}$ DCs in mice treated with DSS. However, the oral administration of NCDO2118 enhanced even more the numbers of $CD11c^{+}CD103^{+}$ DCs. Gyu Jeon *et al*. (2012) recently showed that intestinal $CD103^{+}$- dendritic cells mediated by *Bifidobacterium breves* induced the development of IL-10-producing T cells. Thus, *L. lactis* may induce a regulatory phenotype in DCs that drives the expansion of induced regulatory T cells such as $CD4^{+}LAP^{+}$. Thus we propose the following working model of *L. lactis* NCDO 2118 activity *in vivo* (Figure 7).

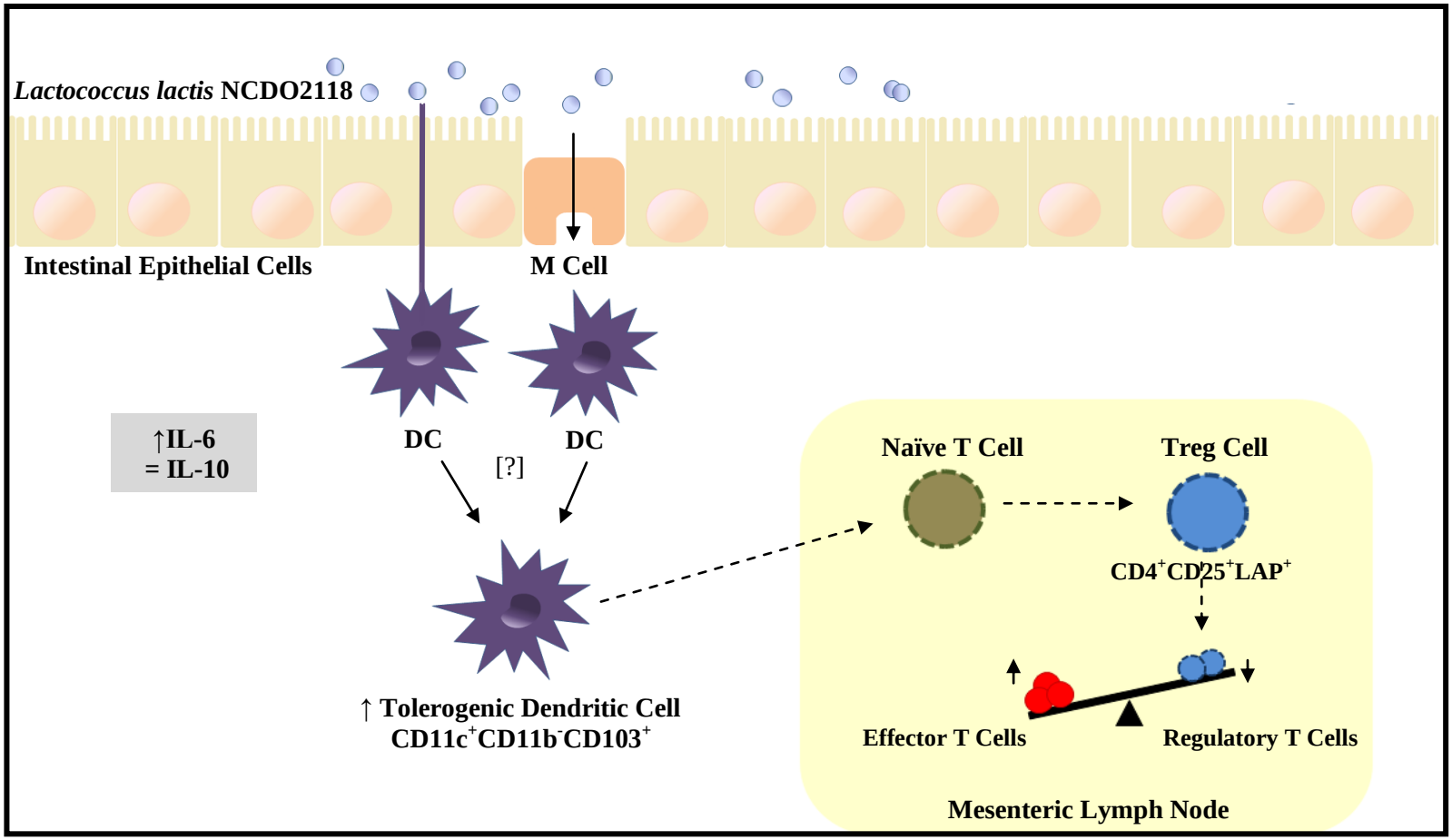

Figure 7. Schematic representation the immunomodulatory effect of *Lactococcus lactis* NCDO 2118 in dextran sulfate sodium (DSS)-induced ulcerative colitis murine model. Orally administered *Lactococcus lactis* NCDO 2118 is able to induce an early increase in IL-6 production and to sustain IL-10 secretion in colonic tissue. It also increases the number of local tolerogenic dendritic cells ($CD11c^{+}CD11b^{-}CD103^{+}$). These cells migrate to the mesenteric lymph nodes, stimulate the expansion of CD4+CD25+LAP+ cells, a regulatory type of T cell (Treg), leading ultimately to downmodulation of colitis.

In conclusion, we showed that *L. lactis* NCDO 2118 has anti-inflammatory activity in IEC and in chemical colitis model induced by DSS. Moreover, we showed the effectiveness of *in vitro* screenings in order to choose the proper strain to be tested *in vivo.* The mechanism involved in such anti-inflammatory effects include the modulation of colonic cytokines and the expansion of regulatory T cells and anti-inflammatory DCs. Taken together, our results suggest that not only commensal, but also dairy bacteria that form part of our diet can have probiotic effects. The nature of *L. lactis* NCDO 2118 components responsible for its anti-inflammatory effects is under investigation.

ACKNOWLEDGMENTS

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DISCUSSÃO GERAL E CONCLUSÕES

A linhagem *L. lactis* NCDO 2118 quando avaliada em modelo experimental de inflamação *in vitro*, demonstrou não induzir eventos pró-inflamatórios. Sendo, porém, capaz de apresentar efeito anti-anflamatório. O modelo de inflamação utilizado para a avaliação das propriedades de *L. lactis* NCDO 2118 consistiu no uso de células epiteliais intestinais da linhagem Caco-2, as quais, em cultura, apresentam características de enterócitos diferenciados (Pinto *et al*., 1983). Quando estas células são estimuladas com a citocina pró-inflamatória IL-1β, ocorre a ativação do fator transcricional NF-κB (*nuclear factor* κB) e consequente, produção de mediadores inflamatórios, que incluem IL-8, TNF-α, IL-6, Cox2, iNOS. Nossos resultados demontraram que o sobrenadante da cultura de *L. lactis* NCDO 2118, quando em contato com estas células estimuladas, foi capaz de reduzir os níveis de secreção da citocina pró-inflamatória IL-8, sugerindo assim, que NCDO2118 apresenta um efeito imunomodulador *in vitro*. E este efeito, provavelmente, esta relacionado à inibição da via NF-κB.

Considerando que o fator transcricional NF-κB encontra-se super expresso nas células mucosas de pacientes com IBD (Neurath, *et al*., 1996), e que sua inibição resulta em melhora dos sintomas destas doenças intestinais, *L. lactis* NCDO 2118 foi então avaliado *in vivo*, quanto ao seu potencial na prevenção da colite ulcerativa (UC) induzida quimicamente por DSS em modelo murino.

Como a UC é caracterizada por surtos e períodos de remissão (Travis and Dinesen, 2006), foi utilizado um protocolo que mimetiza tal comportamento. Assim, os animais passaram por um ciclo inicial de 7 dias ingerindo DSS juntamente com a água, para a indução da UC, que foi seguido por 7 dias de descanso (sem ingestão do DSS), permitindo uma regressão dos sintomas como ocorre no período de remissão. Sendo realizado o tratamento

com a linhagem NCDO2118 (*ad libitum*) dentro deste período. Um segundo ciclo de DSS foi utilizado para simular a reeincidência da doença. Este novo ciclo de DSS foi iniciado dia 14 e finalizado dia 21, sendo estes os dias escolhidos para avaliar a efetividade da linhagem.

No dia 14, o grupo tratado com *L. lactis* NCDO 2118 apresentou melhora nos sinais clínicos da colite ulcerativa, especialmente a diarréia. De modo que este grupo manteve o escore macroscópico equivalente ao grupo não inflamado, e ambos mostraram-se estatísticamente diferente do grupo inflamado, que apresentou um índice mais elevado. Assim, sugerimos que em um primeiro momento a linhagem *L. lactis* NCDO 2118 apresenta um efeito local, contribuindo para a proteção das células epiteliais e seu efeito barreira, principalmente devido à diminuição da diarréia.

Em contrapartida, as análises realizadas no dia 21 demonstraram que os animais tratados com NCDO2118 apresentaram um reestabelecimento do tamanho do colon, além de melhora no escore microscópico.

Este efeito benéfico de NCDO2118, não foi devido aos níveis de produção de sIgA, um importante fator para evitar a translocação bacteriana (O'Sullivan, 2001, Malin *et al*., 1996), uma vez que estes mantiveram-se inalterados. Porém quando observado o perfil de citocinas, o grupo tratado com NCDO2118 foi capaz de manter níveis intermediários da citocina anti-inflamatória IL-10 no tecido do cólon, enquanto que os animais que não receberam a linhagem apresentaram redução dos níveis. Além disso, a administração de NCDO2118 foi relacionada com o aumento precoce na produção de IL-6 neste mesmo tecido. IL-6 é uma citocina que pode apresentar tanto um efeito pró-inflamatório quanto anti-inflamatório. Neste caso propomos que IL-6 esta relacionado ao aumento do reparo da mucosa pela restituição do epitélio (Podolsky, 1999; Grivennikov, *et al*., 2009; Dann, *et al*., 2008; Chalaris, *et al*., 2010; Scheller, *et al*., 2011).

Como a colite induzida por DSS é desencandeada pela perda da tolerância imunológica contra antígenos da microbiota comensal, e a tolerância é mantida principalmente por células Tregs (e seu equilíbrio em relação às células T ativadas), a regulação dos níveis destas células poderia ser, então, o mecanismo de atuação de *L. lactis*. Assim, após a indução da colite, foram quantificadas as células T ativadas ($CD69^+$), que se mostraram elevadas apenas no baço dos animais que ingeriram NCDO2118, sugerindo que algum produto de *L. lactis* seja capaz de ativar células T. Também foram analisadas as células $CD4^+CD25^+CD45RB^{low}$ e $CD4^+CD25^+LAP^+$ do linfonodo mesentérico e baço dos animais tratados com *L. lactis* ou não, durante a fase crônica da UC, uma vez que essas células T especializadas contrabalançam uma resposta pró-inflamatória (Bouma e Strober, 2003; Strober e Mannon, 2007). Embora a atividade anti-inflamatória de *L. lactis* não tenha aumentado as células Tregs $CD4^+CD25^+CD45RB^{low}$, houve, no entanto, a indução de células Treg caracterizadas pela expressão de superfície de peptídeos associados à latência (LAP) nos linfonodos mesentéricos e baço dos animais tratados com *L. lactis*. Resultados similares foram observados após tratamento com o probiótico VSL#3, também administrado durante o período de remissão da colite induzida pelo TNBS (ácido trinitrobenzeno sulfônico), onde foi demonstrado que o aumento de células T $CD4^+LAP^+$ era essencial para o efeito probiótico (Di Giacinto *et al*., 2005).

Como a diferenciação de células T em efetoras (ativadas) e reguladoras são moduladas por células dendríticas (DCs) (Chen, 2006), investigamos o perfil de DCs expressando o marcador de superfície $CD103^+$. Essas DCs tolerogênicas estão relacionadas com a diferenciação de células T $CD4^+$ *naïve* em células Tregs (Coombes *et al*., 2007). No grupo inflamado, que recebeu apenas DSS, houve um aumento na população de células $CD11c^+CD103^+$ em relação ao grupo controle (não inflamado). Porém, a admnistração de NCDO2118 foi capaz de aumentar ainda mais a quantidade dessas células, sugerindo que este

aumento impulsionou a expansão das células Tregs, tais como $CD4^{+}LAP^{+}$.

Assim podemos propor um segundo efeito de *L. lactis* NCDO2118 observado aos 21 dias de experimento, após o segundo ciclo de DSS, quando não havia mais a presença da linhagem no intestino dos animais. Neste segundo momento foi evidenciada a capacidade imunomoduladora de *L. lactis* NCDO2118, claramente dependente da expansão / recrutamento de células reguladoras e seus produtos, o que resultou em uma forma mais branda da UC.

De modo semelhante, Nishitani *et al*. (2009) observaram que a linhagem de *L. lactis* subsp. *cremoris* FC, quando co-cultivada com células Caco-2 estimuladas, foi capaz de reduzir significativamente a expressão de mRNA IL-8, além de inibir a translocação nuclear de NF-κB em outro modelo de inflamação utilizando as células RAW264.7. Determinaram ainda, o perfil de citocinas estimuladas *in vivo* em modelo de murino para DSS quimicamente induzido, porém nenhum mecanismo relacionado a células reguladoras foi averiguado, fazendo com que, até o momento a linhagem de *L. lactis* NCDO 2118 seja a espécie probiótica, dentro do gênero *Lactococcus*, mais bem caracterizada quanto aos seus mecanismos de ação.

TRABALHO EM ANDAMENTO E PERSPECTIVAS

- Estudar de modo mais aprofundado o mecanismo de inibição de *L. lactis* NCDO 2118 sobre a via NFκ-B;
- Confirmar o tamanho do genoma de *L. lactis* pela técnica de Pulsed-field Gel Electrophoresis (PFGE);
- Realizar análise comparativa entre os genomas completos das linhagens de *L. lactis* depositadas em banco de dados;
- Confirmar o status GRAS da linhagem *L. lactis* NCDO 2118 através da análise do seu genoma completo e relacionar estas informações com dados microbiológicos;
- Caracterizar as proteínas exportadas do sobrenadante da cultura de *L. lactis* NCDO 2118 na fase estacionária de crescimeto atráves do sequenciamento dessas proteínas;
- Caracterizar as moléculas com potencial benéfico; Avaliar diferentes protocolos de administração da linhagem probiótica, para otimizar a quantidade de doses (2, 4 e 7 dias) e verificar a memória (15 e 21 dias após o término da administração). Bem como avaliar a presença de IgA no intestino delgado 2, 3 e 4 dias;
- Avaliar *in vitro,* a capacidade anti-inflamatória das células mortas de *L. lactis* NCDO 2118;
- Estudar a variação da microbiota causada pela administração de *L. lactis* NCDO 2118;
- Produzir e avaliar um leite fermentado com a linhagem *L. lactis* NCDO 2118 como um produto probiótico.

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APÊNDICE A - Sequenciamento do Genoma de *Lactococcus lactis* subsp. *lactis* NCDO 2118, uma linhagem produtora de GABA

Este apêndice exibe os dados obtidos através do sequenciamento do genoma de *Lactococcus lactis* subsp. *lactis* NCDO 2118 utilizando uma plataforma de nova geração. Este trabalho foi submetido na forma de artigo à revista Genome Announcements ASM.

Resumo

Lactococcus lactis subsp. *lactis* NCDO 2118 é uma bactéria láctica, fermentadora de xilose e produtora de ácido gama-aminobutírico (GABA), isolada a partir de ervilhas congeladas. Esta linhagem com potencial probiótico foi sequenciada por nosso grupo de pesquisa.

Genome sequence of *Lactococcus lactis* subsp. *lactis* NCDO 2118, a GABA-producing strain

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***Lactococcus lactis* subsp. *lactis* NCDO 2118 is a lactic acid bacterium, non-dairy, xylose fermenter and gamma-aminobutyric acid (GABA) producer isolated from frozen peas. Here, we report the partial genome sequence of the *L. lactis* NCDO 2118, a strain with probiotic potential activity.**

Lactic acid bacteria (LAB), in general, acquire energy from conversion of sugars into lactic acid (Carr, *et al*., 2002). Thus, usually, LAB are used for production of many fermented products, such as cheese, bread, acid milk, yogurt, butter, silage and wine. Food conservation is due to the medium acidification and production of molecules that inhibit the growth of undesirable microbiota which contributes for the development of desirable organoleptic properties in the final product (van de Guchte, *et al*., 2001). Moreover, some specific species of LAB strains, can also produce bioactive molecules such as the gamma-aminobutyric acid (GABA) (Zareian, *et al*., 2012), which is a product of glutamate decarboxylation by the glutamic acid decarboxylase (GAD) enzyme. Usually, GABA acts by modulating the central nervous system, contributing to smooth muscle relaxation and presenting a hypotensor activity (Inoue, *et al*., 2003). Also, it can immunomodulate the immune system (Jin, *et al*., 2013). Therefore, GABA-producing bacteria, generally, present probiotic properties (Mazzoli, *et al*., 2010).

Lactococcus lactis is the best-characterized member of the LAB group. *L. lactis* subsp. *lactis* NCDO 2118 is a non-dairy strain, xylose fermenter and gamma-aminobutyric acid (GABA) producer isolated from frozen peas. Some plant-associated strains usually present gene sets for degradation and uptake of subtracts inherent to the environment, such as xylose (Mazzoli, *et al*., 2010, Siezen, *et al*., 2008).

L. lactis NCDO 2118 genome was decoded with SOLiD 5500xl platform (Applied Biosystems) with mate-paired libraries that generated 85,550,956 reads of 60 bp in size (5,133,057,360 bp), which is equivalent to a genome sequence coverage of 2,053 times. The reads were subjected to quality filter Phred 20 using the Quality Assessment software (Ramos, *et al*., 2011). The genome assembly was performed with the CLC Genomics Workbench software, generating a total of 1,641 overlapping sequences. The redundant overlapping sequences were removed with the Simplifier software (Ramos, *et al*., 2012), ordered and oriented based on the reference *L. lactis* subsp. *lactis* KF147 genome sequence (a plant-associated strain; accession number CP001834). After, manual curation was performed using the Artemis software (Rutherford, *et al*., 2000); and, the SSPACE (Boetzer, *et al*., 2011) and Gapfiller (Nadalin, *et al*., 2012) were used to generate the scaffold and resolve gaps, respectively. At the end of curation and sequence assembly, it was obtained a total of 409 scaffolds (2,874,854 bp) with G+C content of 34.9 %.

***Nucleotide sequence accession number*.** The *Lactococcus lactis* NCDO 2118 genome has been deposited at DDBJ/EMBL/GenBank under the accession ASAE00000000. The version described in this paper is the first version, ASAE00000000.1.

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ANEXO A- Bactérias Lácticas Como Vacinas Vivas de Mucosa

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Resumo

As bactérias lácticas (BL), amplamente utilizadas na indústria alimentícia, estão presentes no intestino da maioria dos animais, incluindo os humanos. O uso potencial destas bactérias como veículos vivos para a produção e o endereçamento de proteínas heterólogas de interesse biotecnológico tem sido extensivamente investigado, assim como vacinas orais, e tem mostrado resultados inovadores. As aplicações profiláticas e terapêuticas destas bactérias e os benefícios à saúde a elas atribuídos podem contribuir para o desenvolvimento de novos produtos farmacêuticos e alimentícios para a sociedade. Além disto, a utilização das bactérias lácteas, em especial *Lactococcus lactis* como um veículo para a entrega de antígenos vacinais e citocinas diretamente à superfície de mucosas, o que constitui um novo passo rumo à validação da eficácia e efetividade de novas vacinas baseadas em bactérias lácticas, seja nativas ou geneticamente modificadas; o que também poderá fornecer informações valiosas para a pesquisa e o desenvolvimento de vacinas contra outros patógenos, com potencial para maior exploração em diversas áreas do conhecimento.

ANEXO B - Local and systemic immune mechanisms underlying the anti-colitis effects of the dairy bacterium *Lactobacillus delbrueckii*

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ABSTRACT

Several probiotic bacteria have been proposed for treatment or prevention of inflammatory bowel diseases (IBD), showing a protective effect in animal models of experimental colitis and for some also in human clinical trials. However, the mechanisms underlying the protective effects are still poorly understood. We recently reported that a *Lactobacillus* strain isolated from cheese, *L. delbrueckii* subsp. *lactis* CNRZ327, possess anti-inflammatory effects *in vitro* and *in vivo*, demonstrating that common dairy bacteria may be useful in the treatment or prevention of IBD. The mechanisms involved in the anti-inflammatory effect of Lb CNRZ327 were studied *in vivo,* in a mouse dextran sodium sulfate (DSS) colitis model. During colitis, Lb CNRZ327 modulated the production of TGF-β, IL-6, and IL-12 in colonic tissue and of TGF-β and IL-6 in the spleen, and expanded the frequency of $CD4^+$ $Foxp3^+$ regulatory T cells in the cecal lymph nodes. A strong tendency to $CD4^+$ $Foxp3^+$ expansion was also observed in the spleen. The results of this study show that orally administered dairy lactobacilli can not only modulate mucosal but also systemic immune responses.