UNIVERSITY OF SÃO PAULO

SCHOOL OF PHAMACEUTICAL SCIENCES

Post-Graduation Program in Food Science

Area of Bromatology

Probiotic potential of fermented milk with *Lactobacillus paracasei* subsp. *paracasei* F19 and *Streptococcus thermophilus* TH-4: impact of BSG supplementation and influence on the vitamin D receptor *in vivo*.

Carolina Battistini

Thesis presented for the degree of

Doctor in Sciences

Advisor:

Full Prof. Susana Marta Isay Saad

São Paulo

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São Paulo, _____, 2020.

DEDICATION

To my parents, Marcia and Sergio, and my brother, Marcos, for having faith and supporting me unconditionally.

To all scientists, who dedicate their lives for the benefit of humanity, especially to those that have been working in the frontline of the COVID-19 pandemic.

To all my Friends and all those who believe in love, kindness, and altruism, contributing to building a much better world for everyone!

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Firstly, I would like to express my gratitude to my parents and my brother, who supported me unconditionally and always believed in me! You are my strength and I could never get here without your love, care, patience, comprehension, and encouragement.

I also would like to thank all my Friends and family. You are so special, and I am so grateful that you are all with me on this journey!

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Life without love, is no life at all.

- Leonardo da Vinci

I don't trust a theologian who dismisses the beauty of science or a scientist who doesn't believe in the power of mystery.

- Brené Brown

RESUMO

BATTISTINI, C. **Potencial probiótico de leite fermentado com *Lactobacillus paracasei* subsp. *paracasei* F19 e *Streptococcus thermophilus* TH-4: impacto da suplementação com bagaço de malte e influência sobre o receptor de vitamina D *in vivo*.** 2020. 126 p. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2020.

Dentre os diversos benefícios à saúde relacionados ao consumo de probióticos, destacam-se a melhora da função de barreira intestinal, produção de ácidos graxos de cadeia curta, redução da resposta pró-inflamatória, modulação da microbiota intestinal, entre outros. No entanto, estes efeitos dependem da cepa empregada, da matriz de administração e da genética do hospedeiro, fatores importantes que devem ser levados em consideração na prescrição ou indicação de tratamentos. Alguns estudos sugeriram que o potencial anti-inflamatório dos probióticos parece ser regulado pelo receptor da vitamina D (VDR), que, além de mediar as funções da vitamina D, também atua como fator de transcrição associado à autofagia, função de barreira intestinal e respostas imunológicas. Entretanto, este é um tópico ainda pouco explorado e nenhum estudo avaliou a administração de probióticos em uma matriz alimentar na resposta anti-inflamatória relacionada ao VDR. Sendo assim, o presente trabalho avaliou o impacto da suplementação de leite fermentado (FM – *fermented milk*) com bagaço de malte de cevada (BSG) na sobrevivência de cepas potencialmente probióticas após a exposição a condições gastrointestinais (GI) simuladas *in vitro*. Adicionalmente, o efeito do FM probiótico na resposta inflamatória relacionada às funções do VDR foi avaliado por meio de um ensaio *in vivo*, empregando um modelo de colite induzida por DSS (dextrano sulfato de sódio). Este trabalho foi dividido em três etapas: I) Seleção de uma co-cultura composta por uma cepa probiótica e uma cepa *starter* para aplicação em FM probiótico; II) Avaliação da viabilidade dos microrganismos no FM ao longo do armazenamento e sua resistência às condições GI simuladas *in vitro*; III) Estudo do impacto do FM probiótico na expressão do VDR e em biomarcadores inflamatórios *in vivo*. Foram avaliadas 10 cepas probióticas (*Lactobacillus* (*L.*) *acidophilus* LA 5, *L. fermentum* PCC, *L. reuteri* RC-14, *L. paracasei* subsp. *paracasei* L. casei 431, *L. paracasei* subsp. *paracasei* F19, *L. rhamnosus* GR-1, *L. rhamnosus* LGG, *Bifidobacterium* (*B.*) *animalis* subsp. *lactis* BB-12, *B. longum* BB-46 e *B. longum* subsp. *infantis* BB-02) e 2 cepas *starter Streptococcus thermophilus* (TH-4 e STM-6). As culturas *L. paracasei* subsp. *paracasei* F19 e *S. thermophilus* TH-4 apresentaram os resultados mais promissores nos ensaios de fermentabilidade do BSG e, portanto, foram selecionadas para a produção do FM probiótico. No total, foram avaliadas quatro formulações: FM1 (TH-4); FM2 (TH-4 + BSG); FM3 (TH-4 + F19); FM4 (TH-4 + F19 + BSG). Em relação a viabilidade dos microrganismos, todas as formulações apresentaram populações acima de 10 log UFC por porção diária de 200 mL de FM durante os 28 dias de armazenamento a 4 °C. Além disso, estimamos que cerca de 10 log UFC de TH-4 e 8 log UFC de F19 poderiam chegar viáveis ao colón e possivelmente conferir benefícios à saúde. A co-cultura com F19 e/ou a adição de BSG aumentou a resistência do TH-4 ao estresse GI simulado *in vitro*, mostrando um potencial promissor do TH-4 como cultura *starter* e probiótica. Os experimentos com animais foram realizados com camundongos C57BL/7 *wild-type* (WT) e VDR *knockout*, em um modelo de colite induzida por DSS. PBS, leite ou FM foram administrados diariamente por 7 dias via gavagem oral e o tratamento com DSS (5% na água do bebedouro) foi iniciado 24 h após a primeira dose. O FM probiótico foi capaz de aumentar a expressão do VDR nos camundongos WT, enquanto reduziu o nível de IL-6. Por outro lado, em camundongos VDRKO, o FM probiótico agravou a inflamação, aumentando o nível dos marcadores inflamatórios IL-6 e lipocalina-2. Os resultados obtidos corroboram com a hipótese de que o efeito anti-inflamatório dos probióticos são regulados pelo VDR, contribuindo para o entendimento dos mecanismos pelos quais os probióticos exercem seus benefícios à saúde, fornecendo ferramentas para uma recomendação de tratamento mais assertiva e segura.

Palavras-chave: BSG; inflamação; leite fermentado; probiótico; receptor nuclear; VDR; vitamina D.

ABSTRACT

BATTISTINI, C. **Probiotic potential of fermented milk with *Lactobacillus paracasei* subsp. *paracasei* F19 and *Streptococcus thermophilus* TH-4: impact of BSG supplementation and influence on the vitamin D receptor *in vivo*. 2020.** 126 p. Thesis (PhD) – School of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2020.

Several health benefits are related to probiotic consumption, such as improvement of the gut barrier function, production of short-chain fatty acids, reduction of pro-inflammatory response, gut microbiota modulation, among others. However, these effects depend on the strain employed, the matrix of administration, and host genetics, which are important factors that should be taken into consideration when prescribing or indicating probiotic treatments. Previous studies suggested that the anti-inflammatory potential of probiotics seemed to be regulated by the vitamin D receptor (VDR), which, in addition to vitamin D functions, is a transcription factor associated with autophagy, gut barrier function, and immune responses. Nevertheless, this is a new subject that has hardly been explored and, to the best of our knowledge, the influence of the food matrix on the VDR-related anti-inflammatory potential of probiotics has not been studied yet. Therefore, the present study evaluated the impact of the supplementation of probiotic fermented milk (FM) with brewer's spent grain (BSG) on the survival of potentially probiotic strains after the exposure to *in vitro*-simulated gastrointestinal (GI) conditions. In addition, the effect of probiotic FM on the VDR-related inflammatory response was studied, employing an *in vivo* DSS (dextran sulfate sodium) colitis model. This study was divided into three steps: I) Selection of a co-culture of one probiotic and one starter strains for the application in probiotic FM; II) Evaluation of the viability of the microorganisms in the FM throughout storage, and their resistance to *in vitro*-simulated GI conditions; III) Study of the impact of probiotic FM on the VDR expression and inflammation biomarkers *in vivo*. Ten probiotic strains (*Lactobacillus* (*L.*) *acidophilus* LA-5, *L. fermentum* PCC, *L. reuteri* RC-14, *L. paracasei* subsp. *paracasei* L. casei 431, *L. paracasei* subsp. *paracasei* F19, *L. rhamnosus* GR-1, *L. rhamnosus* LGG, *Bifidobacterium* (*B.*) *animalis* subsp. *lactis* BB-12, *B. longum* BB-46, and *B. longum* subsp. *infantis* BB-02) and two starter strains *Streptococcus thermophilus* (TH-4 and STM-6) were evaluated. The cultures *L. paracasei* subsp. *paracasei* F19 and *S. thermophilus* TH-4 showed the most promising results regarding the fermentability of BSG. Thus, they were selected for the probiotic FM production. In total, four FM formulations were evaluated: FM1 (TH-4); FM2 (TH-4 + BSG); FM3 (TH-4 + F19); FM4 TH-4 + F19 + BSG). All formulations showed populations above 10 log CFU per daily portion of 200 mL of FM up to 28 days of storage at 4 °C. Moreover, we could estimate that around 10 log CFU of TH-4 and 8 log CFU of F19 may reach the colon viable, and possibly confer health benefits. The co-culture with F19 and/or the addition of BSG improved the resistance of TH-4 to *in vitro* GI stress, showing a promising potential of TH-4 to be employed both as a starter and as a probiotic culture. The animal experiments were performed with wild-type (WT) and VDR knockout C57BL/7 mice, employing a DSS colitis model. PBS, milk, or FM were administrated daily for 7 days by oral gavage and the DSS treatment (5% in drinking water) started 24 h after the first dose. The probiotic FM promoted an increase in the VDR expression in WT mice, while reduced the IL-6 level. On the other hand, in VDRKO mice, the probiotic FM worsened the inflammatory response, increasing the levels of the inflammation markers IL-6 and fecal lipocalin-2. These results corroborate with the hypothesis that the anti-inflammatory effects of probiotics are regulated by the VDR, contributing to the elucidation of the mechanisms by which probiotics exert their health benefits, providing tools to a more assertive and safe treatment recommendation.

Key words: BSG; fermented milk; inflammation; nuclear receptor; probiotic; VDR; vitamin D.

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ABBREVIATIONS

1,25-$(OH)_2$D - 1,25-dihydroxyvitamin D or calcitriol

25(OH)D – 25-OH-cholecalciferol or 25-hydroxyvitamin D

AMPs – antimicrobial peptides

ANOVA – Analysis of Variance

ATG16L1 - autophagy related 16 like 1

AXOS - arabinoxylooligosaccharides

AZA - Azathioprine

BMI – body mass index

BSG – Brewer's spent grain

CD - Crohn's Disease

CFU – colony forming units

C_t – threshold cycle

CYP27B1 - cytochrome P450 family 27 subfamily B member 1

CYP2R1 - cytochrome P450 family 2 subfamily R member 1

DOK2 - docking protein 2

DSS – dextran sulfate sodium

ESR - erythrocyte sedimentation rate

FAM10A4 - ST13 Hsp70 interacting protein (ST13)

FANCC - FA complementation group C

FHIT - fragile histidine triad

FM – fermented milk

FOS - fructooligosaccharides

GA - gastric antrum

GBGT1 - globoside alpha-1,3-N-acetylgalactosaminyltransferase 1 (FORS blood group)

GC - gastric corpus

GDP – gross domestic product

GI - gastrointestinal

GIT - gastrointestinal tract

GOS – galacto-oligosaccharides

GWAS - genome-wide association studies

HAT - histone acetyltransferase

HBI - Harvey-Bradshaw Index

HDAC - histone deacetylase

HDM - histone demethylase

HE - high sunlight exposure

HFD - high fat diet

HMO – human milk oligosaccharides

HMT - histone methyltransferase

HOMA-IR - homeostasis model of assessment-estimated insulin resistance

hs-CRP - high-sensitivity C-reactive protein

IBD - inflammatory bowel disease

IBS - irritable bowel syndrome

IFITM1 - interferon induced transmembrane protein 1

IFN-γ - interferon gamma

Ig A – Immunoglobulin A

IGFBP4 - insulin like growth factor binding protein 4

IL - interleukin

IOM - Institute of Medicine

IRGM - immunity related GTPase M

IU - International Units

JAK2 - Tyrosine-Protein Kinase JAK2

KO - knockout

LCACs - long chain acylcarnitines

LCN2 – lipocalin-2

LE - low sunlight exposure

lncRNAs - long ncRNAs

LPS - lipopolysaccharides

MAMPs - microbe-associated molecular patterns

MAPK – mitogen-activated protein kinase

MDP - muramyl dipeptide

miRNAs – microRNA

MP – mercaptopurine

mpr-MRS – modified phenol red MRS broth

mRNA – messenger RNA

MTX – methotrexate

MUC – mucin

NAFLD - non-alcoholic fatty liver disease

NASH - Non-Alcoholic Steatohepatitis

ncRNAs - noncoding RNAs

NF-κB – nuclear factor κB

NLR - NOD-like receptor

NOD2 - nucleotide binding oligomerization domain containing 2

PBS – phosphate buffered saline

piRNAs - PIWI-interacting RNAs

PMA – propidium monoazide

PUFA – polyunsaturated fatty acids

QoL - quality of life

qPCR – quantitative PCR

RTC - randomized controlled trial

RXR - retinoic acid receptor

SCFA - short-chain fatty acids

SD – standard deviation

siRNAs - short interfering RNAs

SNPs - single nucleotide polymorphisms

TAK1 - Transforming growth factor beta activated kinase 1

TE – Tris EDTA

Th1 - T helper 1

THRAP2 - Mediator Complex Subunit 13L

TLR - Toll like receptor receptor

TNFSF4 - Tumor Necrosis Factor Ligand superfamily member 4

TOS - *trans*-galactooligosaccharides

Tregs - regulatory T-cells

UC - Ulcerative colitis

UVB – ultraviolet B

VDR - vitamin D receptor

VDRE - vitamin D-response element

VDRKO – VDR knockout

VitD – vitamin D

WT – wild-type

ZO-1 - tight junction protein ZO-1

SUMMARY

PRESENTATION

This thesis is presented in the form of scientific articles (published, submitted, or to be submitted for publication), and is divided into the following chapters

Chapter 1: Probiotic, Vitamin D, and Vitamin D Receptor in Health and Disease

This chapter discusses the critical role of the vitamin D receptor in regulating the health benefits of probiotic consumption, and resulted in the following published book chapter: **Battistini, C.**; Nassani, N.; Saad, S. M. I.; Sun, J. (2020). **Probiotics, vitamin D, and vitamin D receptor in health and disease**. In: Albuquerque, M. A. C.; LeBlanc, A. M.; LeBlanc, J. G.; Bedani, R. (Eds), Lactic Acid Bacteria: A Functional Approach (pp. 93-105). Boca Raton: CRC Press. https://doi.org/10.1201/9780429422591.

Chapter 2: The potential use of vitamin D as an alternative approach for gut microbiota modulation in inflammatory bowel disease

This chapter delves into vitamin D deficiency and gut microbiota dysbiosis associated with Inflammatory Bowel Disease and the potential use of vitamin D as adjuvant therapy. The content resulted in the following review article under submission: **Battistini, C.**; Ballan, R.; Herkenhoff, M. E.; Sun, J.; Saad, S. M. I. (2020). The potential use of vitamin D as an alternative approach for gut microbiota modulation in inflammatory bowel disease. **To be submitted**.

Chapter 3: Brewer's spent grain enhanced the recovery of potentially probiotic strains in fermented milk after exposure to *in vitro*-simulated gastrointestinal conditions

This chapter aimed to evaluate the impact of BSG on the survival of potentially probiotic strains in fermented milk after the exposure to gastrointestinal conditions simulated *in vitro*. The following scientific article resulted from this chapter, which is under submission: **Battistini, C.**; Herkenhoff, M. E.; Leite, M. S.; Vieira, A. D. S.; Bedani, R.; Saad, S. M. I. (2020). Brewer's spent grain enhanced the recovery of potentially probiotic strains in fermented milk after exposure to *in vitro*-simulated gastrointestinal conditions. **To be submitted**.

Chapter 4: Probiotic fermented milk may worsen inflammation in mice lacking vitamin D receptor

This chapter evaluated the impact of probiotic fermented milk on the VDR functions and inflammation biomarkers employing an *in vivo* DSS colitis model. This study was conducted at the University of Illinois at Chicago, in Chicago – USA, under the supervision of Prof. Dr. Jun Sun ("Sandwich PhD" – PDSE CAPES Foundation Program). This content resulted in a scientific article that will be submitted: **Battistini, C.**; Zhang, Y. G.; Chatterjee, I.; Lu, R.; Zhang, J.; Saad, S. M. I.; Sun, J. (2020). Probiotic fermented milk may worsen inflammation in mice lacking vitamin D receptor. **To be submitted.**

JUSTIFICATION

The consumption of probiotics is associated with several health benefits, such as improvement of gut barrier function, gut microbiota modulation, and regulation of immune responses (SANDERS et al., 2019). Nevertheless, each individual responds to probiotic treatment differently and the clinical outcomes are still inconsistent. These facts make it difficult to state one unique treatment that fits all cases (OUWEHAND et al., 2017; ZMORA et al., 2018). Meanwhile, it has been suggested that the anti-inflammatory effect of probiotics may be regulated by the vitamin D receptor (VDR). Probiotics have the potential to improve the VDR signaling and reduce the inflammatory response. On the other hand, the lack of VDR may induce an exacerbated inflammatory response to probiotic treatment (WU et al., 2015). Studies exploring this critical role of VDR on probiotic health benefits are scarce but of great relevance to elucidate the mechanisms of action of probiotics, supporting the relevance of the present study.

This thesis was conducted in the context of the Research Project FAPESP 2018/21584-4 “Characterization of probiotic fermented milk supplemented with brewer's spent grain and *in vitro* and *in vivo* evaluation of potential health benefits”.

References

OUWEHAND, A. C. A review of dose-response of probiotics in human studies. **Beneficial Microbes**, v. 8, n. 2, p. 143-151, 2017.

SANDERS, M. E.; MERENSTEIN, D. J.; REID, G.; GIBSON, G. R.; RASTALL, R. A. Probiotics and prebiotics in intestinal health and disease: from biology to the clinic**. Nature Reviews Gastroenterology & Hepatology**, v. 16, p. 605-6016, 2019.

ZMORA, N.; ZILBERMAN-SCHAPIRA, G.; SUEZ J.; MOR, U.; DORI-BACHASH, M.; BASHIARDES, S.; KOTLER, E.; ZUR, M.; REGEV-LEHAVI, D.; BRIK, R. B. Z.; FEDERICI, S.; COHEN, Y.; LINEVSKY, R.; ROTHSCHILD, D.; MOOR, A. E.; BEN-MOSHE, S.; HARMELIN, A.; ITZKOVITZ, S.; MAHARSHAK, N.; SHIBOLET, O.; SHAPIRO, H.; PEVSNER-FISCHER, M.; SHARON, I.; HALPERN, Z.; SEGAL, E.; ELINAV, E. Personalized Gut Mucosal Colonization Resistance to Empiric Probiotics Is Associated with Unique Host and Microbiome Features. **Cell**, v. 174, n. 6, p. 1388-1405, 2018.

WU, S.; YOON, S.; ZHANG, Y. G.; LU, R.; XIA, Y.; WAN, J.; PETROF, E. O.; CLAUD, E. C.; CHEN, D.; SUN, J. Vitamin D receptor pathway is required for probiotic protection in colitis. **American Journal of Physiology - Gastrointestinal and Liver Physiology**, v. 309, n. 5, p. G341-G349, 2015.

OBJECTIVES

General

Evaluate the impact of potentially probiotic fermented milk on the vitamin D receptor (VDR) expression *in vivo*.

Specifics

- Evaluate the fermentability of brewer's spent grain (BSG) by starter and probiotic cultures and to select a suitable combination of one probiotic and one starter strain for the application in probiotic fermented milk (FM);
- Produce a probiotic fermented milk supplemented with BSG and evaluate the viability of the microorganisms in the FM throughout the storage period and their resistance to gastrointestinal stress simulated *in vitro*;
- Investigate the impact of probiotic fermented milk on the VDR expression *in vivo* and evaluate the VDR-related inflammatory response.

CHAPTER 1

Probiotic, vitamin D, and vitamin D receptor in health and disease

Abstract

Probiotic microorganisms are able to colonize the gastrointestinal tract, and among other health beneficial effects, help in the reduction of inflammatory conditions and promote the normalization of the intestinal microbiota. Meanwhile, vitamin D has its biological functions mainly through the mediation of the vitamin D receptor (VDR), acting on the antibacterial mechanism of the innate immune system. Vitamin D/VDR deficiency may be associated with the development or aggravation of certain illnesses, including inflammatory bowel diseases. The VDR is encountered in diverse sites of the body, which may explain this connection. Besides that, it has been found that probiotics may have a protective effect against induced colitis depending on the VDR status. The administration of butyrate, a microbial metabolite, raises the VDR expression, suggesting a direct relation between the potential health benefits of probiotics with the VDR expression. Furthermore, vitamin D and VDR expression are linked to the composition of the intestinal microbiota, as well as the susceptibility to the development of autoimmune diseases. Thus, this chapter aims to discuss the interaction of probiotics, vitamin D, and VDR regarding the intestinal microbiota and the possible immunomodulatory effects

Key words: Vitamin D, Vitamin D Receptor, VDR, Probiotic, Prebiotic, IBD, Microbiome

1 INTRODUCTION

The gut microbiome, “a newly discovered organ” of the body, plays a critical role in immunity and metabolism in the host’s health and disease states. Microbiome products released in the gut, e.g. short chain fatty acids, may reach and influence the function of organs and systems beyond the intestinal tract. Environmental factors, lifestyle, age, and sex influence the profile and function of the microbiome. Its imbalance, termed ‘dysbiosis’ is directly related to the development of various human diseases (COSTEA et al., 2018; SALVUCCI, 2019; WANG et al., 2016;).

Vitamin D (VitD) and the vitamin D receptor (VDR) are involved in many functions of the body, including but not limited to calcium absorption, immunity, glucose, and liver metabolism, in addition to gut microbiota modulation. For example, VitD deficiency and the lack or low expression of the VDR are directly related to inflammatory bowel diseases (IBD), and the treatment with VitD supplements might be effective in some cases (CELIBERTO et al., 2018; HAUSSLER et al., 2013; NATIONAL INSTITUTE OF HEALTH, 2016; OOI et al., 2013; WU et al., 2015a,b). Therefore, VDR could be used as a predictive biomarker for dysbiosis, and the development of strategies that boost its functions are of utmost importance for the gut microbiome restoration.

Dietary interventions with pre-, pro-, and/or synbiotic foods or supplements have shown to be effective in restoring the gut microbiome to a healthier pre-disease state pattern and are interesting and less harmful alternatives when compared to antibiotic therapies (COSTEA et al., 2018; SALVUCCI 2019). The benefits of probiotics are strain dependent and may comprise improvement of intestinal epithelial cells turnover, competition for nutrients and adhesion sites, production of short chain fatty acids, vitamins, bacteriocins, and anti-inflammatory compounds, regulation of the intestinal transit, modulation of the intestinal microbiota, and enhancement of immunity. The most common microorganisms known as having probiotic properties are bacteria from the *Lactobacillus, Streptococcus, Enterococcus,* and *Bifidobacterium* genera. (GIBSON, 2004; HILL et al., 2014; HOLZAPFEL & SCHILINGER, 2002; WILLIAMS, 2010).

Probiotics are usually consumed through food products, supplements or nutraceutical capsules. The food vehicle used, the presence of other bioactive substances, such as fibers, for example, and the consumption frequency are factors that influence probiotics efficacy (RANADHEERA; BAINES; ADAMS, 2010; SANDERS & MARCO, 2010). Besides, the

microorganisms' survival through the gastrointestinal tract passage (resistance to acids, bile, and enzymes) and the antibiotics resistance should also be taken into consideration (GIBSON, 2004; RANADHEERA; BAINES; ADAMS, 2010).

Additionally, another way to increase the population of beneficial bacteria in the intestinal microbiota is through the intake of prebiotic compounds, which are defined as "substrates that are selectively utilized by host microorganisms conferring a health benefit" (GIBSON et al., 2017). Some fibers might have prebiotic potential, once they are not digested in the small intestine and reach the colon intact, serving as substrates for the local microbiota (GIBSON et al., 2010; MARTINEZ; BEDANI; SAAD, 2015; PUUPPONEN-PIMIÄ et al., 2002;). The combination of prebiotic ingredients and probiotic microorganisms results in synbiotic foods or supplements and may confer a competitive advantage for the probiotics over the intestinal commensal microbiota or pathogenic bacteria (MARTINEZ; BEDANI; SAAD, 2015; PUUPPONEN-PIMIÄ et al., 2002). Food research should focus on the development of synbiotic products that target the increase of VDR expression, and consequently improve the anti-inflammatory responses and modulate the gut microbiota, which could be applied in a preventive approach for IBD and other diseases.

The clinical trials outcomes of probiotic treatments are still inconsistent and controversial. In this chapter, we will discuss the potential role of VDR in regulating probiotic functions, and whether they can be used as a therapeutic strategy to increase the VDR expression, and consequently, to promote the healthy microbiota.

2 VITAMIN D /VITAMIN D RECEPTOR

2.1 Chemical Structure and Functions

VitD (vitamin D) is a fat-soluble vitamin synthetized by the exposure of the skin to sunlight or consumed through nutraceutical supplements, fatty fishes, egg yolks, or fortified foods, such as dairy products. Commonly called pre-VitD, it is found in two chemical arrangements: vitamin D2, or ergocalciferol, and vitamin D3, or cholecalciferol. The main differences among these two forms are a double bond between carbons 22 and 23, and a methyl group bonded to carbon 24 in ergocalciferol (Figure 1). Nonetheless, these compounds are biologically inactive and should run through two hydroxylation before they have biological functions in the body (FDA, 2016; HEALTH CANADA, 2012; NATIONAL INSTITUTE OF HEALTH, 2016; ROSS et al., 2011b).

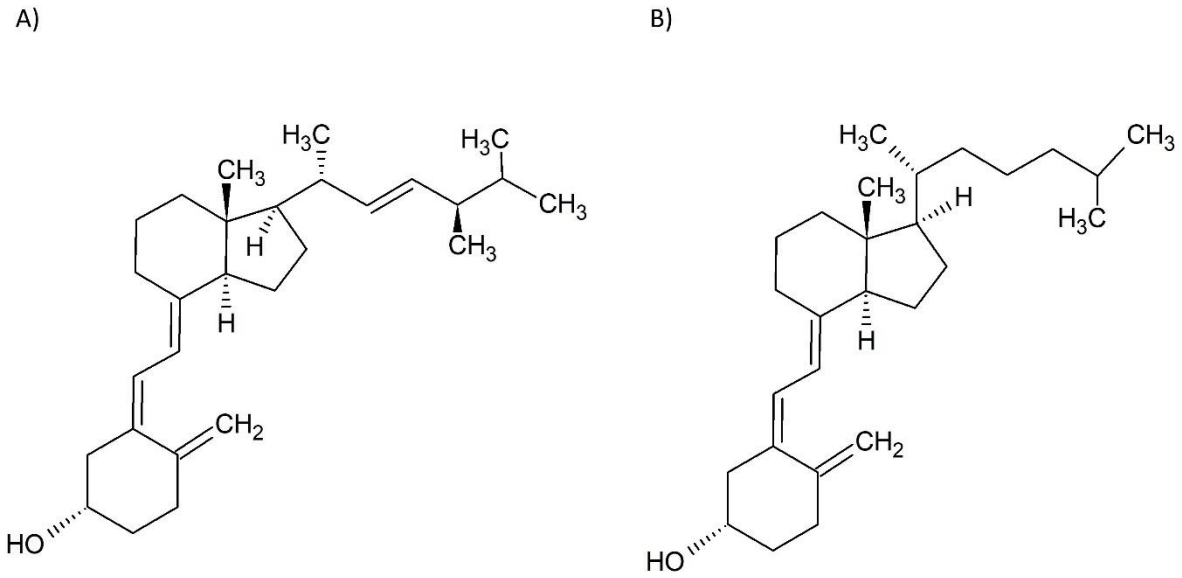

Figure 2: Chemical structure of ergocalciferol (A) and cholecalciferol (B).

In blood circulation, pre-VitD is bounded to the VitD binding protein, and then transported to the liver where the first hydroxylation occurs through the action of 25-hydroxylase, an enzyme encoded by the gene CYP2R1, generating the 25-OH-cholecalciferol (25(OH)D). This compound is then released in the bloodstream and is the major circulating form and the main marker of VitD status. Thereafter, the second hydroxylation may occur in the kidneys, brain, lung, prostate, placenta, or in the immune cells, resulting in VitD itself, or 1,25-$(OH)_2$D (BIVONA et al., 2018; GENETIC HOME REFERENCE, 2017; HOLICK, 2011a, b).

A great number of biological functions are associated with VitD through the mediation of the VitD receptor (VDR). Macrophages, when in contact with pathogens, induce the enzymatic activation of VitD in the cytoplasm, through the action of Toll-like receptors (TLR). When 1,25-$(OH)_2$D binds to VDR, both migrate to the cell nucleus and antibacterial compounds are produced, such as β-defensin 2 and cathelicidin, and the Th1 immune response is activated with the production of interferon gamma (IFN-γ). This whole process is regulated automatically. When 1,25-$(OH)_2$D is accumulated, the Th1 profile is inhibited and the Th2 immune response is activated. Hence, the production of IFN-γ is reduced, while the production of interleukin 4 (IL-4) increases (GATTI et al., 2016).

The VDR is found in several organs and tissues of the body, especially in the parathyroid gland, small intestines, and colon. In addition, it is also a receptor for the secondary bile acid lithocholic acid, and a transcriptional factor associated with immunomodulation, proliferation, intestinal barrier function, and autophagy, and shows a

similar sequence with the steroid and thyroid hormone receptor. When bound to the retinoid X receptor (RXR), they form a heterodimer that is involved in physiological effects, not only of VitD but, also, of microbial and dietary metabolites, like secondary bile acids and fatty acids. Furthermore, evidences indicate that VDR is genetically associated with the gut microbiota profile, IBD, liver diseases, cancer, and blood sugar regulation (BAKKE et al., 2018; WANG et al., 2016).

In summary, VitD and VDR influence body defenses and inflammatory responses. Thus, it is crucial to understand their mechanisms of action and establish potential therapies targeting the improvement of their status and functions for health maintenance.

2.2 Vitamin D Requirements

2.2.1 Recommended Circulating Level and Dietary Reference Intake

VitD status is defined by the serum concentration of 25(OH)D. However, there is no consensus worldwide about the adequate level. The Institute of Medicine (IOM) from the USA considers 20 ng/mL (50 nmol/L) of 25(OH)D sufficient (ROSS et al., 2011a,b), while other medical societies adopted a level of 30 ng/mL based on a possible reduction on the risk of falls and fractures in the elderly (AMERICAN GERIATRICS SOCIETY, 2014; HOLICK et al., 2011). In fact, there are a lot of controversial results from clinical trials regarding the effectiveness of VitD supplementation in different diseases and, for this reason, it is difficult to establish a suitable acceptance range of 25(OH)D level (BOLLAND et al., 2018; NEED et al., 2008; SANDERS et al., 2010; TRIVEDI et al., 2003).

According to the IOM, assuming minimal sunlight exposure, the daily recommended dietary allowance of VitD is 400 IU (10 mcg) for infants up to 12 months, 800 IU (20 mcg) for elderly (>70 years of age), and 600 IU (15 mcg) for other age groups (ROSS et al. 2011a,b). However, these values can vary depending on the geographic location, season of the year, skin pigmentation, and use of sun blocking agents (HOLICK, 1994).

An effective method to increase the levels of VitD is the exposure of the skin to sunlight. However, this is not often indicated due to the risk of skin cancer development. Alternatively, supplements intake or food fortification might be useful strategies against hypovitaminosis (PILZ et al., 2018). In addition to ergocalciferol and cholecalciferol, VitD metabolites may be administered under specific circumstances. Calcidiol, due to its hydrophilic properties and ability to bypass the hepatic 25-hydroxylation, is useful for individuals with fat malabsorption or liver disease, while calcitriol, which bypasses the one

alpha hydroxylation and activation phase, is indicated for patients with chronic kidney disease or type 1 VitD-dependent rickets.

2.2.2 Hypovitaminosis and Toxicity

VitD deficiency or resistance can be triggered by several factors, e.g. low availability of the vitamin, resulting from low dietary intake, lack of sunlight exposure, or low absorption; defects on the hydroxylation stage in the liver or activation in the kidneys, which is common in chronic renal disease and VitD-dependent rickets type 1; increased catabolism by the liver; resistance at the end organ level, like in hereditary VitD-resistant rickets type 2.

Of particular concern is the fact that low levels of VitD lead to malabsorption of calcium and phosphorus in the intestines, and when during a long period, this may cause rickets in children and osteomalacia in adults. Even though these diseases are less common in developed countries due to food fortification practices, subclinical VitD deficiency is common and may be associated with osteoporosis and increased risk of falls and fractures. In addition, patients with malabsorptive issues have an increased risk of VitD hypovitaminosis, such as patients suffering celiac disease, inflammatory bowel disease, and a history of gastrectomy.

The prescription of vitamin supplements is a good strategy to increase the circulating VitD, but clinicians should be cautious. High VitD doses, that have been associated with errors in the formulation and excessively fortified dairy products intake, can be toxic and lead to hypercalciuria. In this condition, the excretion of calcium through the urinary tract is increased, inducing kidney stones in some cases, or in worse scenarios, hypercalcemia is developed, when the concentration of calcium in the blood stream is high, and the patients usually report symptoms like fatigue, muscle weakness, weight loss, nausea, vomiting, soft tissue calcification, and tachycardia (HOLICK, 2003; JACOBUS et al., 1992; PILZ et al., 2018). In addition, increased levels of 25(OH)D seems to be related to an increased risk of falls and fractures, pancreatic and prostate cancer, and mortality (SANDERS et al., 2010; WORTSMAN et al., 2000).

Hence, medical societies do not have a current consensus about VitD adequacy, and their recommendations are based only on studies about bone health. On the other hand, a lot of other disorders are related to VitD deficiency and might need to be taken into consideration to determine the prescription of supplements. Thus, there is a gap and more studies are needed in the field in order to regulate the adequate levels of VitD, mostly for anti-inflammatory effects.

3 PROBIOTICS, VITAMIN D, AND VDR ON HEALTH

3.1 Probiotics, VDR, and the Gut Microbiota

The gut microbiota comprises all the microorganisms present in the gut, including bacteria, viruses, archeae, fungi, and yeasts. Its composition reaches maturity at the fourth year of life approximately, and it is influenced by non-genetic factors, such as age, sex, body mass index (BMI), smoking status, and dietary patterns, and at the genetic level by the VDR gene (CANI, 2018; CANI et al., 2019; FOUHY et al., 2019). A healthy intestinal environment is associated with the presence of beneficial microorganisms like *Bifidobacterium* spp., *Faecalibacterium prausnitzii*, and *Lactobacillus* spp., higher amounts of butyrate and anti-inflammatory cytokines, a thicker mucus layer, and improved barrier function (CELIBERTO et al., 2018; COSTEA et al., 2018).

The VDR is expressed in intestinal epithelial and immune cells, and regulates the transcription of barrier proteins, like claudin 2 (ZHANG et al., 2015); the expression of antimicrobial peptides (AMPs), like cathelicidin and β-defensins, promoting mucosal homeostasis and barrier function, protection against inflammation, and preventing epithelial apoptosis. Notably, paneth cells might play a role in preventing dysbiosis as they are responsible for autophagy and for the production of AMPs (ADOLPH, et al., 2013; WU et al., 2015b). In addition, both VitD and VDR, promote a tolerogenic and anti-inflammatory response in Crohn's disease patients, favoring regulator T cells (Tregs cells) and increasing the proportions of the Actinobacteria and the Firmicutes phyla (SCHÄFFLER et al., 2018).

The VDR status is implicated in inflammation, signal transduction, infections, amino acid and carbohydrate metabolism, and neoplasm (BAKKE & SUN, 2018; BAKKE et al., 2018; SUN, 2018). In addition, polymorphisms in VDR gene were associated with susceptibility to IBD. Studies with mice showed that VDR knockout mice presented a significant shift in their microbiota at a phylogenetic level and were more prone to autoimmune diseases (KONGSBAK et al., 2013). Moreover, animals incapable of producing the active form of VitD or VDR knockout developed a more severe colitis induced by dextran sulfate sodium (DSS) (AZAD et al., 2012; JIN et al., 2015; KONGSBAK et al., 2013; LEE & SONG, 2012; OOI et al., 2013; SIMMONS et al., 2000; WANG et al., 2016; WU et al., 2015b).

In a recent study, *Lactobacillus casei rhamnosus* (Lcr35®) was effective in increasing the populations of *Lactobacillus* and *Bifidobacterium* in the feces of hospitalized children with acute diarrhea. Besides, the probiotic treatment ameliorated the abdominal

discomforts and diarrhea, improving the patients' appetite and food intake (LAI et al., 2019). Similarly, *Bifidobacterium animalis* subsp. *lactis* (txid302911) was able to shape the gut microbiota of low birth weight infants to a more diverse and complex profile. The strain increased the counts of the *Bifidobacterium* and *Lactobacillus* genera in their faeces, whereas the control group presented a predominant presence of opportunistic pathogens associated with the risk of necrotizing enterocolitis (CHI et al., 2019).

Interestingly, VitD supplementation was associated with lower counts of *Clostridium difficile* in breast fed infants while increased *Lachnobacterium* and decreased *Lactococcus* in the gut microbiome of infants, age 3-6 months, were linked to cord blood VitD levels (SORDILLO et al., 2017).

In conclusion, the VDR status has an important role on the gut microbiota profile and with the development of IBD; dietary interventions with VitD and probiotics could be explored further, aiming at improving the VDR expression and restoration of the gut microbiome.

3.2 Probiotics, Vitamin D, and VDR in IBD and Metabolic Disorders

3.2.1 Inflammatory Bowel Diseases

Inflammatory bowel disease is a chronic inflammation of the gastrointestinal tract (GIT), caused by abnormal immune responses. The main types of IBD are ulcerative colitis (UC), which affects the large intestine, and Crohn's disease (CD), which may affect the whole intestinal tract (FEUERSTEIN et al., 2017), although more recent studies suggest that they may be part of the same spectrum.

VitD deficiency is recurrent in patients with IBD. However, it is unclear whether it is a cause or a consequence. Actually, it has been reported that the consumption of products with lactose may worsen the abdominal discomforts in IBD, which is in completely accordance with the lower concentrations of VitD because dairy products are the main fortified foods with VitD and calcium, and IBD patients stop or decrease consumption of these products to avoid abdominal discomforts. In addition, studies have shown that VitD supplementation is a promising tool to improve the clinical outcome and quality of life of IBD patients, with the potential to inhibit the activity of CD and maintain remission (KABBANI et al., 2016; MIHELLER et al., 2009; SCOTTI et al., 2019; WILLIAMS et al., 2018; YANG et al., 2013).

The expression of VDR is decreased in IBD. In fact, investigations with mice have shown that VDR knockout animals are more prone to develop severe colitis while transgenic

overexpression of VDR may confer a protective effect (BELIZÁRIO & NAPOLITANO, 2015; KONG et al., 2008; LIU et al., 2013; OOI et al., 2013; WU et al., 2015b). This anti-inflammatory response seems to be directly related to NF-κB pathway, given that 42 Single Nucleotide Polymorphisms (SNPs) linked with immune disorders are binding sites for both VDR and NF-κB. VDR is also involved in pro-inflammatory response to bacterial endotoxin LPS and with autophagy regulation (SINGH et al., 2017).

Interestingly, VDR expression is crucial for probiotic anti-inflammatory effects. In a *Salmonella* infection model, *Lactobacillus plantarum* showed physiological and histological protection only for wild-type mice, whereas no effect was observed in VDR knockout mice (WU et al., 2015). Similarly, our research group found that the administration of probiotic fermented milk with *Lactobacillus paracasei* subsp. *paracasei* F19 showed a promising increase in the VDR expression at the mRNA level in wild-type mice, whereas VDR knockout mice presented an exacerbated inflammation induced by DSS when compared to wild-type mice (unpublished data).

3.2.2 Metabolic Disorders

Metabolic syndrome, insulin resistance, obesity, and coronary disease have also been related to low levels of VitD and defects on VDR signaling (CHANG & KIM, 2017; MORENO-SANTOS et al., 2017; OH et al., 2015). Nonetheless, there is a lack of studies involving VitD and both type 1 and type 2 diabetes. A few reports have shown controversial, but promising results (COOPER et al., 2011; DONG et al., 2013; KRUL-POEL et al., 2017; SONG et al., 2013).

Interestingly, experiments with VDR knockout mice showed that lean animals resist weight gain induced by high-fat diet (PEIRCE et al., 2014), while interventional studies reported that VitD supplementation improved insulin signaling (JIN et al., 2015; VRIEZE et al., 2012). Meanwhile, VitD supplementation may improve lipid profile and inflammatory markers in non-alcoholic fatty liver disease (NAFLD). In a study with a Non-Alcoholic Steatohepatitis (NASH) mice model, VitD significantly decreased steatosis and NAFLD activity (GIBSON et al., 2018; ZHU et al., 2013).

Probiotics are alternatives to enhance metabolic disease status. The consumption of the probiotic strain *Lactobacillus reuteri* NCIMB 30242 improved VitD status of hypercholesterolemic adults by 22.4% when compared with the placebo group (JONES et al., 2013). In the same trend, obese adults that consumed synbiotic fermented milk produced with *Bifidobacterium lactis* Bb-12 and inulin showed a significant reduction in the serum insulin

and blood triglycerides levels, and at the same time, improved their VitD status and insulin sensitivity (MOHAMMADI-SARTANG et al., 2018).

Moreover, individuals with metabolic syndrome who consumed probiotic fermented milk (containing *Bifidobacterium lactis* Bb-12 and *Lactobacillus acidophilus* La5) for eight weeks presented lower levels of blood glucose and vascular cell adhesion molecules, whereas no significant changes were observed for insulin level, homeostasis model of assessment-estimated insulin resistance (HOMA-IR), and for other metabolic and cardiovascular markers (REZAZADEH et al., 2019).

The co-supplementation of VitD and probiotics seems to have synergic effects, and more studies should be conducted in order to investigate these observations. Nevertheless, in a clinical trial, the combination of VitD with a pool of probiotics improved the 25(OH)D levels, whereas it significantly reduced serum insulin levels, HOMA-IR, and high-sensitive C-reactive protein in patients with type 2 diabetes and coronary heart disease (RAYGAN et al., 2018).

Probiotic anti-inflammatory properties are dependent on VDR expression. Meanwhile, both VitD and probiotics influence the markers for intestinal microbiome and metabolic disease. Their synergic effect is not well explored and more *in vivo* and clinical trials should be carried out to answer this unexplored path.

4 CONCLUSIONS AND FUTURE DIRECTION

This chapter discussed how the VDR status influences the gut microbiome and its critical impact on the potential benefit of probiotics against inflammation and infections. Overall, VitD and probiotics have great potential to modulate the gut microbiota, improving the anti-inflammatory response and metabolic markers. Nonetheless, VDR is an important factor for homeostasis and is directly involved in the probiotics' mechanisms of action. It is a double way path, since VDR expression is crucial for probiotics anti-inflammatory potential, while the administration of probiotic may enhance the VDR expression.

Nevertheless, there is promising evidence of synergic effects of co-supplementation with VitD and probiotics. However, more *in vivo* studies and clinical trials combining probiotics and VitD supplementation are of utmost importance for the comprehension of their roles. Therefore, it is possible to establish novel biomarkers and develop alternative treatments for the prevention of inflammation and other diseases.

To the best of our knowledge, only *Lactobacillus* strains were investigated regarding the potential of probiotics to improve VDR expression, and future studies should include other genera like *Bifidobacterium.* Furthermore, the impact of prebiotic compounds and food processing technologies, such as microencapsulation, could be used as strategies to increase the probiotic survival after passage through the gastrointestinal tract, and possibly boost their potential health benefits. IBD patients avoid dairy products due to lactose content (SCOTTI et al., 2019). Thus, other food matrices should be explored as vehicles for probiotics, such as soy, rice, coconut, almond, among other vegetable beverages. This knowledge can be exploited to develop novel strategies by restoring healthy VDR functions and normal host-microbe interactions.

REFERENCES

ADOLPH, T. E.; TOMCZAK, M. F.; NIEDERREITER, L.; KO, H.-J.; BÖCK, J.; MARTINEZ-NAVE, E.; GLICKMAN, J. N.; TSCHURTSCHENTHALER, M.; HARTWIG, J.; HOSOMI, S.; FLAK, M. B.; . CUSICK, J. L; KOHNO, K.; IWAWAKI, T.; BILLMANN-BORN, S.; RAINE, T.; BHARTI, R.; LUCIUS, R.; KWEON, M.-N.; MARCINIAK, S. J .; CHOI, A.; HAGEN, S. J.; SCHREIBER, S.; ROSENSTIEL, P.; KASER, A.; BLUMBERG, R. S. Paneth cells as a site of origin for intestinal inflammation. **Nature**, v. 503, p. 272‑276, 2013.

AMERICAN GERIATRICS SOCIETY WORKGROUP ON VITAMIN D SUPPLEMENTATION FOR OLDER ADULTS. Recommendations abstracted from the American Geriatrics Society Consensus Statement on vitamin D for Prevention of Falls and Their Consequences. **Journal of the American Geriatrics Society**, v. 62, n. 1, p. 147-152, 2014.

AZAD, A. K.; SADEE, W.; SCHLESINGER, L. S. Innate immune gene polymorphisms in tuberculosis. **Infection and Immunity**, v. 80, n. 10, p. 3343-3359, 2012.

BAKKE, D.; CHARTTERJEE, I.; AGRAWAL, A.; DAI, Y.; SUN, J. Regulation of Microbiota by Vitamin D Receptor: A Nuclear Weapon in Metabolic Diseases. **Nuclear Receptor Research**, v. 5, p. 101377, 2018.

BAKKE, D.; SUN, J. Ancient nuclear receptor VDR With New functions: Microbiome and inflammation. **Inflammatory Bowel Disease**, v. 24, n. 6, p. 1149‑1154, 2018.

BELIZÁRIO, J. E.; NAPOLITANO, M. Human microbiomes and their roles in dysbiosis, common diseases, and novel therapeutic approaches. **Frontiers in Microbiology**, v. 6, p. 1050, 2015.

BIVONA, G.; AGNELLO, L.; CIACCIO, M. The immunological implication of the new vitamin D metabolism. **Central-European Journal of Immunology**, v. 43, n. 3, p. 331-334, 2018.

BOLLAND, M. J.; GREY, A.; AVENELL, A. Effects of vitamin D supplementation on musculoskeletal health: a systematic review, meta-analysis, and trial sequential analysis. **Lancet Diabetes and Endocrinology**, v. 6, n. 11, p. 847-858, 2018.

CANI, P. D. Human gut microbiome: hopes, threats and promises. **Gut**, v. 67, n. 9, p. 1716-1725, 2018

CANI, P. D.; HUL, M. V.; LEFORT, C.; DEPOMMIER, C.; RASTELLI, M.; EVERARD, A. Microbial regulation of organismal energy homeostasis. **Nature Metabolism**, v. 1, p. 34-46, 2019.

CELIBERTO, L. S., GRAEF, F. A.; HEALEY, G. R.; BOSMAN, E. S.; JACOBSON, K.; SLY, L. M.; VALLANCE, B. A. Inflammatory bowel disease and immunomodulation: novel therapeutic approaches through modulation of diet and the gut microbiome. **Immunology**, v. 155, n. 1, p. 36-52, 2018

CHANG, E.; KIM, Y. Vitamin D Insufficiency Exacerbates Adipose Tissue Macrophage Infiltration and Decreases AMPK/SIRT1 Activity in Obese Rats. **Nutrients**, v. 9, n. 4, p. 338, 2017.

CHI, C.; XUE, Y.; LIU, R.;WANG, Y.; LV, N.; ZENG, H.; BUYS, N.; ZHU, B.; SUN, J.; YIN, C. Effects of a formula with a probiotic *Bifidobacterium lactis* Supplement on the gut microbiota of low birth weight infants. **European Journal of Nutrition**, v. 59, p. 1493-1503, 2020.

COOPER, J. D.; SMYTH, D. J.; WALKER, N. M.; STEVENS, H.; BURREN, O. S.; WALLACE, C.; GREISSL, C.; RAMOS-LOPEZ, E.; HYPPÖNEN, E.; DUNGER, D. B.; SPECTOR, T. D.; OUWEHAND, W. H.; WANG, T. J.; BADENHOOP, K.; TODD, J. A. Inherited variation in vitamin D genes is associated with predisposition to autoimmune disease type 1 diabetes. **Diabetes**, v.60, n. 5, p. 1624-1631, 2011.

COSTEA, P. I.; HILDEBRAND, F.; ARUMUGAN, M.; BÄCKHED, F.; BLASER, M. J.; BUSHMAN, F. D.; DE VOS, W. M.; EHRLICH, S. D.; FRASER, C. M.; HATTORI, M.; HUTTENHOWER, C.; JEFFERY, I. B.; KNIGHTS, D.; LEWIS, J. D.; LEY, R. E.; OCHMAN, H.; O'TOOLE, P. W.; QUINCE, C.; RELMAN, D. A.; SHANAHAN, F.; SUNAGAWA, S.; WANG, J.; WEINSTOCK, G. M.; WU, G. D.; ZELLER, G.; ZHAO, L; RAES, J.; KNIGHT, R.; BORK, P. Enterotypes in the landscape of gut microbial community composition. **Nature Microbiology**, v. 3, p. 8-16, 2018.

DONG, J. Y.; ZHANG, W. G.; CHEN, J. J.; ZHANG, Z. L.; HAN, S. F.; QIN, L. Q. Vitamin D intake and risk of type 1 diabetes: a meta-analysis of observational studies. **Nutrients**, v. 5, n. 9, p. 3551-3562, 2013.

FDA – Food and Drug Administration. **FDA Approves an Increase to the Amount of Vitamin D for Milk and Milk Alternatives**. 2016. Available at: https://www.fda.gov/Food/NewsEvents/ConstituentUpdates/ucm510556.htm. Accessed: Sep 2018.

FEUERSTEIN, J. D.; NGUYEN, G. C.; KUPFER, S. S.; FALCK-YTTER, Y.; SINGH, S. American Gastroenterological Association Institute Guideline on Therapeutic Drug Monitoring in Inflammatory Bowel Disease. **Gastroenterology**, v. 153, n. 3, p. 827‑834, 2017.

FOUHY, F.; WATKINS, C.; HILL, C. J.; O'SHEA, C. A.; NAGLE, B.; DEMPSEY, E. M.; O'TOOLE, P. W.; ROSS, R. P.; RYAN, C. A.; STANTON, C. Perinatal factors affect the gut microbiota up to four years after birth. **Nature Communications**, v. 10, p. 1517, 2019.

GATTI, D.; IDOLAZZI, L.; FASSIO, A. Vitamin D: not just bone, but also immunity. **Minerva Medica**, v. 107, n. 6, p. 452-460, 2016.

GENETIC HOME REFERENCE. **CYP2R1 gene**. 2017. Available at: https://ghr.nlm.nih.gov/gene/CYP2R1. Accessed: Jan 2019.

GIBSON, G. R. Fibre and effects on probiotics (the prebiotic concept). Clinical Nutrition Supplements, v. 1, n. 2, p. 25-31, 2004.

GIBSON, G. R.; HUTKINS, R.; SANDERS, M. E.; PRESCOTT, S. L.; REIMER, R. A.; SALMINEN, S. J.; SCOTT, K.; STANTON, C.; SWANSON, K. S.; CANI, P. D.; VERBEKE, K.; REID, G. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. **Nature Reviews Gastroenterology & Hepatology**, v. 14, p. 491-502, 2017.

GIBSON, G. R.; SCOTT, K. P.; RASTALL, R. A.; TUOHY, K. M.; HOTCHKISS, A.; DUBERT-FERRANDON, A.; GAREAU, M.; MURPHY, E. F.; SAULNIER, D.; LOH, G.; MACFARLANE, S.; DELZENNE, N.; RINGEL, Y.; KOZIANOWSKI, G.; DICKMANN, R.; LENOIR-WIJNKOOP, I.; WALKER, C.; BUDDINGTON, R. Dietary prebiotics: current status and new definition. **Food Science & Technology Bulletin: Functional Foods**, v. 7, n. 1, p. 1-19, 2010.

HAUSSLER, M. R.; WHITFIELD, G. K.; KANEKO, I.; HAUSSLER, C. A.; HSIEH, D.; HSIEH, J. C.; JURUTKA, P. W. Molecular mechanisms of vitamin D action. **Calcified Tissue International**, v. 92, n. 2, p. 77-98, 2013.

HEALTH CANADA. **Vitamin D**. 2012. Available at: < https://www.canada.ca/en/health-canada/services/food-nutrition/food-nutrition-surveillance/health-nutrition-surveys/canadian-health-measures-survey/vitamin-nutrition-biomarkers-cycle-1-canadian-health-measures-survey-food-nutrition-surveillance-health-canada-1.html >. Accessed: Sep 2018.

HILL, C.; GUARNER, F.; REID, G.; GIBSON, G. R.; MERENSTEIN, D. J.; POT, B.; MORELLI, L.; CANANI, R. B.; FLINT, H. J.; SALMINEN, S.; CALDER, P. C.; SANDERS, M. E. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. **Nature Reviews Gastroenterology & Hepatology**, v. 11, p. 506-514, 2014.

HOLICK, M. F. McCollum Award Lecture, 1994: vitamin D--new horizons for the 21st century. **The American Journal of Clinical Nutrition**, v. 60, n. 4, p. 619-630, 1994.

HOLICK, M. F. Vitamin D: A millenium perspective. **Journal of Cellular Biochemistry**, v. 88, n. 2, p. 296-307, 2003.

HOLICK, M. F. Vitamin D: a d-lightful solution for health. **Journal of Investigative Medicine: The Official Publication of The American Federation for Clinical Research**, v. 59, n. 6, p. 872–880, 2011a.

HOLICK, M. F. Vitamin D: evolutionary, physiological and health perspectives. **Current Drug Targets**, v. 12, n. 1, p. 4–18, 2011b.

HOLZAPFEL, W. H.; SCHILLINGER, U. Introduction to pre- and probiotics. **Food Research International**, v. 35, n. 2-3, p. 109–116, 2002.

JACOBUS, C. H.; HOLICK, M. F.; SHAO, Q.; CHEN, T. C.; HOLM, I. A.; KOLODNY, J. M.; FULEIHAN, G. E.; SEELY, E. W. Hypervitaminosis D associated with drinking milk. **The New England Journal of Medicine**, v. 326, n. 18, p. 1173-1177, 1992.

JIN, D.; WU, S.; ZHANG, Y. G.; LU, R.; XIA, Y.; DONG, H.; SUN, J. Lack of Vitamin D Receptor Causes Dysbiosis and Changes the Functions of the Murine Intestinal Microbiome. **Clinical Therapeutics**, v. 37, n. 5, p. 996–1009.e7, 2015.

JONES, M. L.; MARTONI, C. J.; PRAKASH, S. Oral Supplementation With Probiotic *L. reuteri* NCIMB 30242 Increases Mean Circulating 25-Hydroxyvitamin D: A Post Hoc Analysis of a Randomized Controlled Trial. **The Journal of Clinical Endocrinology and Metabolism**, v. 98, n.7, p. 2944-2951, 2013.

KABBANI, T. A.; KOUTROUBAKIS, I. E.; SCHOEN, R. E.; RAMOS-RIVERS, C.; SHAH, N.; SWOGER, J.; REGUEIRO, M.; BARRIE, A.; SCHWARTZ, M.; HASHASH, J. G.; BAIDOO, L.; DUNN, M. A.; BINION, D. G. Association of Vitamin D Level With Clinical Status in Inflammatory Bowel Disease: A 5-Year Longitudinal Study. **American Journal of Gastroenterology**, v. 111, n. 5, p. 712–719, 2016.

KONG, J.; ZHANG, Z.; MUSH, M. W.; NING, G.; SUN, J.; HART, J.; BISSONNETTE, M.; LI, Y. C. Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. **American Journal of Physiology - Gastrointestinal and Liver Physiology**, v. 294, n. 1, p. G208-G216, 2008.

KONGSBAK, M.; LEVRING, T. B.; GEISLER, C.; VON ESSEN, M. R. The vitamin d receptor and T cell function. **Frontiers in Immunology**, v. 4, p. 148, 2013.

KRUL-POEL, Y. H.; TER WEE, M. M.; LIPS, P.; SIMSEK, S. MANAGEMENT OF ENDOCRINE DISEASE: The effect of vitamin D supplementation on glycaemic control in patients with type 2 diabetes mellitus: a systematic review and meta-analysis. **European Journal of Endocrinology**, v. 176, n. 1, p. R1-R14, 2017.

LAI, H. H.; CHIU, C. H.; KONG, M. S.; CHANG, C. J.; CHEN, C. C. Probiotic *Lactobacillus casei*: Effective for Managing Childhood Diarrhea by Altering Gut Microbiota and Attenuating Fecal Inflammatory Markers. **Nutrients**, v. 11, n. 5, p. 1150, 2019.

LEE, Y. H.; SONG, G. G. Pathway analysis of a genome-wide association study of ileal Crohn's disease. **DNA and Cell Biology**, v. 31, n. 10, p. 1549-1554, 2012.

LIU, W.; CHEN, Y.; GOLAN, M. A.; ANNUNZIATA, M. L.; DU, J.; DOUGHERTY, U.; KONG, J.; MUSH, M.; HUANG, Y.; PEKOW, J.; ZHENG, C.; BISSONNETTE, M.; HANAUER, S. B.; LI, Y. C. Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. **The Journal of Clinical Investigation**, v. 123, n. 9, p. 3983–3996, 2013.

MARTINEZ, R. C. R.; BEDANI, R.; SAAD, S. M. I. Scientific Evidence for Health Effects Attributed to the Consumption of Probiotics and Prebiotics: An Update for Current Perspectives and Future Challenges. **The British Journal of Nutrition**, v. 114, n. 12, p. 1993-2015, 2015.

MIHELLER, P.; MUZES, G.; HRITZ, I.; LAKATOS, G.; PREGUN, I.; LAKATOS, P. L.; HERSZÉNYI, L.; TULASSAY, Z. Comparison of the effects of 1,25 dihydroxyvitamin D and 25 hydroxyvitamin D on bone pathology and disease activity in Crohn's disease patients. **Inflammatory Bowel Diseases**, v. 15, n. 11, p. 1656-1662, 2009.

MOHAMMADI-SARTANG, M.; BELLISSIMO, N.; ZEPETNEK, J. O. T.; BRETT, N. R.; MAZLOOMI, S. M.; FARAROUIE, M.; BEDELTAVANA, A.; FAMOURI, M.; MAZLOOM, Z. The effect of daily fortified yogurt consumption on weight loss in adults with metabolic syndrome: A 10-week randomized controlled trial. **Nutrition, Metabolism, and Cardiovascular Diseases**, v. 28, n. 6, p. 565-574, 2018.

MORENO-SANTOS, I.; CASTELLANO-CASTILLO, D.; LARA, M. F.; FERNANDEZ-GARCIA, J. C.; TINAHONES, F. J.; MACIAS-GONZALES, M. M. IGFBP-3 Interacts with the Vitamin D Receptor in Insulin Signaling Associated with Obesity in Visceral Adipose Tissue. **International Journal of Molecular Cciences**, v. 18, n. 11, p. 2349, 2017.

NATIONAL INSTITUTE OF HEALTH. **Vitamin D Fact Sheet for Consumers**. 2016. Available at: https://ods.od.nih.gov/factsheets/VitaminD-Consumer/. Accessed: Jul 2019.

NEED, A. G.; O'LOUGHLIN, P. D.; MORRIS, H. A.; COATES, P. S.; HOROWITZ, M.; NORDIN, B. E. Vitamin D metabolites and calcium absorption in severe vitamin D deficiency. **Journal of Bone and Mineral Research: The Official Journal of The American Society for Bone and Mineral Research**, v. 23, n. 11, p. 1859-1863, 2008.

OH, J.; RIEK, A. E.; DARWECH, I.; FUNAI, K.; SHAO, J.; CHIN, K.; SIERRA, O. L.; CARMELIET, G.; OSTLUND, R. E.; BERNAL-MIZRACHI, C. Deletion of macrophage Vitamin D receptor promotes insulin resistance and monocyte cholesterol transport to accelerate atherosclerosis in mice. **Cell Reports**, v. 10, n. 11, p. 1872-1886, 2015.

OOI, J. H.; LI, Y.; ROGERS, C. J.; CANTORNA, M. T. Vitamin D Regulates the Gut Microbiome and Protects Mice from Dextran Sodium Sulfate – Induced Colitis. **The Journal of Nutrition**, v. 143, p. 1679–1686, 2013.

PEIRCE, V.; CAROBBIO, S.; VIDAL-PUIG, A. The different shades of fat. **Nature**, v. 510, p. 76-83, 2014.

PILZ, S.; MÄRZ, W.; CASHMAN, K. D.; KIELY, M. E.; WHITING, S. J.; HOLICK, M. F.; GRANT, W. B.; PLUDOWSKI, P.; HILIGSMANN, M.; TRUMMER, C.; SCHWETZ, V.; LERCHBAUM, E.; PANDIS, M.; TOMASCHITZ, A.; GRÜBLER, M. R.; GAKSCH, M.; VERHEYEN, N.; HOLLIS, B. W.; REJNMARK, L.; KARRAS, S. N.; HAHN, A.; BISCHOFF-FERRARI, H. A.; REICHRATH, J.; JORDE, R.; ELMADFA, I.; VIETH, R.; SCRAGG, R.; CALVO, M. S.; VAN SCHOOR, N. M.; BOUILLON, R.; LIPS, P.; ITKONEN, S. T.; MARTINEAU, A. R.; LAMBERG-ALLARDT, C.; ZITTERMANN, A. Rationale and Plan for Vitamin D Food Fortification: A Review and Guidance Paper. **Frontiers in Endocrinology**, v. 9, p. 373, 2018

PUUPPONEN-PIMIÄ, R.; AURA, A. M.; OKSMAN-CALDENTEY, K. M.; MYLLÄRINEN, P.; SAARELA, M.; MATTILA-SANDHOLM, T.; POUTANEN, K. Development of functional ingredients for gut health. **Trends in Food Science and Technology**, v. 13, n. 1, p. 3–11, 2002.

RANADHEERA, R. D. C. S.; BAINES, S. K.; ADAMS, M. C. Importance of food in probiotic efficacy. **Food Research International**, v. 43, n. 1, p. 1–7, 2010.

RAYGAN, F.; VAHIDREZA, O.; BAHMANI, F.; ASEMI, Z. The effects of vitamin D and probiotic co-supplementation on mental health parameters and metabolic status in type 2 diabetic patients with coronary heart disease: A randomized, double-blind, placebo-controlled trial. **Progress in Neuro-Psychopharmacology and Biological Psychiatry**, v. 84, Part A, p. 50-55, 2018

REZAZADEH, L.; GARGARI, B. P.; JAFARABADI, M. A.; ALIPOUR, B. Effects of probiotic yogurt on glycemic indexes and endothelial dysfunction markers in patients with metabolic syndrome. **Nutrition**, v. 62, p. 162-168, 2019.

ROSS, A. C.; MANSON, J. E.; ABRAMS, S. A.; ALOIA, J. F.; BRANNON, P. M.; CLINTON, S. K.; DURAZO-ARVIZU, R. A.; GALLAGHER, J. C.; GALLO, R. L.; JONES, G.; KOVACS, C. S.; MAYNE, S. T.; ROSEN, C. J.; SHAPSES. S. A. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. **The Journal of Clinical Endocrinology and Metabolism**, v. 96, n. 1, p. 53-58, 2011a.

ROSS, A. C.; TAYLOR, C. L.; YAKTINE, A. L.; DEL VALLE. H. B. **Dietary Reference Intakes for Calcium and Vitamin D**. Washington, DC: National Academies Press, 2011b.

SALVUCCI, E. The human-microbiome superorganism and its modulation to restore health. **International Journal of Food Science and Nutrition**, v. 70, n. 7, p. 781-795, 2019.

SANDERS, K. M.; STUART, A. L.; WILLIAMSON, E. J.; SIMPSON, J. A.; KOTOWICZ, M. A.; YOUNG, D.; NICHOLSON, G. D. Annual high-dose oral vitamin D and falls and fractures in older women: a randomized controlled trial. **JAMA**, v. 303, n. 18, p. 1815-1822, 2010.

SANDERS, M. E.; MARCO, M. L. Food formats for effective delivery of probiotics. **Annual Review of Food Science and Technology**, v. 1, n. 1, p. 65–85, 2010.

SCHÄFFLER, H.; HERLEMANN, D. P.; KLINITZKE, P.; BERLIN, P.; KREIKEMEYER, B.; JASTER, R.; LAMPRECHT, G. Vitamin D administration leads to a shift of the intestinal bacterial composition in Crohn's disease patients, but not in healthy controls. **Journal of Digestive Diseases**, v. 19, n. 4, p. 225-234, 2018.

SCOTTI, G. B.; AFFERRI, M. T.; DE CAROLIS, A.; VAIARELLO, V.; FASSINO, V.; FERRONE, F.; MINISOLA, S.; NIEDDU, L.; VERNIA, P. Factors affecting vitamin D deficiency in active inflammatory bowel diseases. **Digestive and Liver Disease: Official Journal of The Italian Society of Gastroenterology and the Italian Association for the Study of the Liver**, v. 51, n. 5, p. 657-662, 2019.

SIMMONS, J. D.; MULLIGHAN, C.; WELSH, K. I.; JEWELL, D. P. Vitamin D receptor gene polymorphism: association with Crohn's disease susceptibility. **Gut**, v. 47, n. 2, p. 211-214, 2000.

SINGH, P. K.; VAN DEN BERG, P. R.; LONG, M. D.; VREUGDENHIL, A.; GRIESHOBER, L.; OCHS-BALCOM, H. M.; WANG, J.; DELCAMBRE, S.; HEIKKINEN, S.; CARLBERG, C.; CAMPBELL, M. J.; SUCHESTON-CAMPBELL, L. E. Integration of VDR genome wide binding and GWAS genetic variation data reveals co-occurrence of VDR and NF-κB binding that is linked to immune phenotypes. **BMC Genomics**, v. 18, n. 1, p. 132, 2017.

SONG, Y.; WANG, L.; PITTAS, A. G.; DEL GOBBO, L. C.; ZHANG, C.; MANSON, J. E.; HU, F. B. Blood 25-hydroxy vitamin D levels and incident type 2 diabetes: a meta-analysis of prospective studies. **Diabetes Care**, v. 36, n. 5, p. 1422-1428, 2013.

SORDILLO, J. E.; ZHOU, Y.; MCGEACHIE, M. J.; ZINITI, J.; LANGE, N.; LARANJO, N.; SAVAGE, J. R.; CAREY, V.; O'CONNOR, G.; SANDEL, M.; STRUNK, R.; BACHARIER, L.; ZEIGE, R.; WEISS, S. T.; WEINSTOCK, G.; GOLD, D. R.; LITONJUA, A. A. Factors influencing the infant gut microbiome at age 3-6 months: Findings from the ethnically diverse Vitamin D Antenatal Asthma Reduction Trial (VDAART). **The Journal of Allergy and Clinical Immunology**, v. 139, n. 2, p. 482-491, 2017.

SUN, J. Dietary vitamin D, vitamin D receptor, and microbiome. **Current Opinion in Clinical Nutrition and Metabolic Care**, v. 21, n. 6, p. 471‑474, 2018.

TRIVEDI, D. P.; DOLL, R.; KHAW, K. T. Effect of four monthly oral vitamin D3 (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: randomised double blind controlled trial. **BMJ**, v. 326, n. 7387, p. 469, 2003.

VRIEZE, A.; VAN NOOD, E.; HOLLEMAN, F.; SALOJÄRVI, J.; KOOTTE, R. S.; BARTELSMAN, J. F. W. M.; DALLINGA-THIE, G M.; ACKERMANS, M. T.; SERLIE, M. J.; OOZEER, R.; DERRIEN, M.; DRUESNE, A.; VAN HYLCKAMA VLIEG, J. E. T.; BLOKS, V. W.; GROEN, A. K.; HEILIG, H. G. H. J.; ZOETENDAL, E. G.; STROES, E. S.; DE VOS, W. M.; HOEKSTRA, J. B. L.; NIEUWDORP, M. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. **Gastroenterology**, v. 143, n. 4, p. 913-916, 2012.

WANG, J.; THINGHOLM, L. B.; SKIECEVIČIENĖ, J.; RAUSCH, P.; KUMMEN, M.; HOV, J. R.; DEGENHARDT, F.; HEINSEN, F. A.; RÜHLEMANN, M. C.; SZYMCZAK, S.; HOLM, K.; ESKO, T.; SUN, J.; PRICOP-JECKSTADT, M.; AL-DURY, S.; BOHOV, P.; BETHUNE, J.; SOMMER, F.; ELLINGHAUS, D.; BERGE, R. K.; HÜBENTHAL, M.; KOCH, M.; SCHWARZ, K.; RIMBACH, G.; HÜBBE, P.; PAN, W. H.; SHEIBANI-TEZERJI, R.; HÄSLER, R.; ROSENSTIEL, P.; D'AMATO, M.; CLOPPENBORG-SCHMIDT, K.; KÜNZEL, S.; LAUDES, M.; MARSCHALL, H. U.; LIEB, W.; NÖTHLINGS, U.; KARLSEN, T. H.; BAINES, J. F.; FRANKE, A. Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. **Nature Genetics**, v. 48, p. 1396-1406, 2016.

WILLIAMS, C. E.; WILLIAMS, E. A.; CORFE, B. M. Vitamin D status in irritable bowel syndrome and the impact of supplementation on symptoms: what do we know and what do we need to know? **European Journal of Clinical Nutrition**, v. 72, n. 10, p. 1358-1363, 2018.

WILLIAMS, N. T. Probiotics. **American Journal of Health-System Pharmacy**, v. 67, n. 6, p. 449–458, 2010.

WORTSMAN, J.; MATSUOKA, L. Y.; CHEN, T. C.; LU, Z.; HOLICK, M. F. Decreased bioavailability of vitamin D in obesity. **The American Journal of Clinical Nutrition**, v. 72, n. 3, p. 690-693, 2000.

WU, S.; YOON, S.; ZHANG, Y. G.; LU, R.; XIA, Y.; WAN, J.; PETROF, E. O.; CLAUD, E. C.; CHEN, D.; SUN, J. Vitamin D receptor pathway is required for probiotic protection in colitis. **American Journal of Physiology - Gastrointestinal and Liver Physiology**, v. 309, n. 5, p. G341-G349, 2015a.

WU, S.; ZHANG, Y.; LU, R.; XIA, Y.; ZHOU, D.; PETROF, E. O.; CLAUD, E. C.; CHEN, D.; CHANG, E. B.; CARMELIET, G.; SUN, J. Intestinal epithelial vitamin D receptor deletion leads to defective autophagy in colitis. **Gut**, v.64, n. 7, p. 1082–1094, 2015b.

YANG, L.; WEAVER, V.; SMITH, J. P.; BINGAMAN, S.; HARTMAN, T. J.; CANTORNA, M. T. Therapeutic effect of vitamin d supplementation in a pilot study of Crohn's patients. **Clinical and Translational Gastroenterology**, v. 4, n. 4, p. e33, 2013

ZHANG, Y. G.; WU, S.; LU, R.; ZHOU, D.; ZHOU, J.; CARMELIET, G.; PETROF, E.; CLAUD, E. C.; SUN, J. Tight junction CLDN2 gene is a direct target of the vitamin D receptor. **Scientific Reports**, v. 5, p.10642, 2015.

ZHU, L.; BAKER, S. S.; GILL, C.; LIU, W.; ALKHOURI, R.; BAKER, R. D.; GILL, S. R. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. **Hepatology**, v. 57, n. 2, p. 601-609, 2013.

CHAPTER 2

The potential use of vitamin D as an alternative approach for gut microbiota modulation in inflammatory bowel disease

Abstract

Inflammatory bowel disease (IBD) is a chronic disease in the gastrointestinal tract (GIT). The two major types of IBD are ulcerative colitis (UC), which generally affects only the large intestine mucosa and submucosa, and Crohn's disease (CD), which may affect any part of the GIT by transmural inflammation. Both UC and CD are associated with an imbalance of the gut microbiota composition and injuries in the intestinal mucosa. In fact, the intestinal dysbiosis is related to a reduction in butyrate-producing species, impairing the anti-inflammatory response of the immune system, and is commonly associated with micronutrients deficiency, e.g. vitamin D hypovitaminosis. Vitamin D, in addition to calcium homeostasis and bone metabolism, is involved in several critical functions, including immune cell differentiation, regulation of microbiota, gene transcription, and barrier integrity. Vitamin D supplementation in IBD patients showed promising results in reducing the disease activity, gut microbiota modulation, and thus, resulting in improvement of the health status. Therefore, in this review, we will discuss the potential use of vitamin D supplementation as adjuvant therapy to restore gut microbiota balance, promote beneficial metabolites, and inhibit inflammation status in patients with IBD.

Key words: Vitamin D, VDR, inflammation, microbiome, metabolites, nuclear receptor, probiotics, tight junctions

1 INTRODUCTION

Inflammatory bowel disease (IBD) is defined as a chronic inflammation of the gastrointestinal tract (GIT) that affects more than 6 million people worldwide (AGA, 2017; ALATAB et al., 2017). The most common types are Crohn's disease (CD) and ulcerative colitis (UC), which will differ in the location and extension of the lesions throughout the GIT (AGA, 2017). CD is a segmental, asymmetrical, and transmural inflammation that may affect the whole GIT, but is more frequently observed in the ileum and colon. On the other hand, UC is more related to mucosal inflammation from the rectum to the proximal colon (AGA, 2017; TORRES et al., 2017; UNGARO et al., 2017). In fact, IBD has a great impact on the physical, psychological, and social aspects of life, and depression and anxiety are usually increased in these patients. Thus, the management of these diseases is of utmost importance for the quality of life of the patients (ALATAB et al., 2017).

Studies have suggested that IBD may be triggered by an abnormal immune response to gut commensal bacteria in genetically predisposed individuals and is associated with an impaired intestinal barrier function and a less diverse gut microbiota composition (LEVINE; SIGALL BONEH; WINE, 2018; RYAN et al., 2020; SCHIRMER et al., 2019; STANGE; SCHROEDER, 2019). Several factors are associated with the risk of IBD development, such as country development degree, smoking, sex, age, use of antibiotics or oral contraceptives, lower serum levels of vitamin D, and diet (ALATAB et al., 2017; PIOVANI et al., 2019).

The gut microbiota is comprised of more than 2000 species of bacteria distributed throughout the GIT. The population density increases from the stomach to the colon, reaching 10^{10}-10^{12} CFU/mL at the end of the large intestine. Innumerous functions are attributed to the gut microbiota, like metabolism of nutrients from the diet, fiber fermentation, short-chain fatty acids (SCFA) production, vitamin production, barrier function and tight junctions regulation, antimicrobial compounds secretion, immune regulatory, among others (ADAK; KHAN, 2019; ALMEIDA et al., 2019). Microbial metabolites released by the gut microbiota circulate and may affect the proper function of other organs and systems of the body. Therefore, strategies that address the gut microbiota modulation, improvement of the gut barrier function, and decrease in the intestinal mucosa inflammation are of the greatest significance for IBD treatment (SALVUCCI, 2019; STANGE; SCHROEDER, 2019).

Micronutrient deficiencies are often observed in IBD patients, and mostly low levels of vitamin D and zinc, even during disease remission (MACMASTER et al., 2020).

Observational studies have reported that low levels of vitamin D are directly associated with increased disease activity, mucosal inflammation, clinical relapse, and quality of life. Thus, vitamin D deficiency might be both, the cause, and a consequence of IBD (GUBATAN et al., 2019; MACMASTER et al., 2020). In fact, chronic diarrhea, nutrients malabsorption, low exposure to sunlight, and reduced consumption of vitamin D-fortified foods, like dairy products, are frequent in IBD patients, which may lead to vitamin D deficiency (MYINT; SAUK; LIMKETKAI, 2020).

Additionally, several studies about epigenetic factors associated with IBD have been conducted. Indeed, epigenetics may explain how environment and genetics might be involved in the development, progression, pathogenicity, and response to treatments. Also, epigenetic markers related to immunoregulation, intestinal epithelial barrier, and autophagy are differently expressed among IBD and healthy controls, and between UC and CD patients as well, and miRNAs (microRNA) may be used as biomarkers for disease assessment in the future, as they are more convenient than endoscopy and biopsies, mainly for patients with active disease (ZENG; MUKHERJEE; ZHANG, 2019).

In this review, we will explore vitamin D deficiency and gut microbiota dysbiosis associated with IBD, and the potential use of vitamin D in the management of the disease. In addition, epigenetic factors involved in IBD and vitamin D mechanisms will also be discussed.

2 PATHOGENESIS OF INFLAMMATORY BOWEL DISEASES

2.1 Genetics

In the last decades, the understanding of the pathophysiology of IBD has markedly evolved. In addition to environmental, genetic, and microbial factors, the pathogenesis of IBD also involves the function of cells related to the inflammatory process, such as adipose, epithelial, and endothelial cells, together with regulatory RNAs and inflammasome. For a better elucidation of the disease, a broader approach of all these factors must be performed to clarify the underlying mechanisms that results in the abnormal immune response associated to these diseases (DE SOUZA; FIOCCHI, 2016). Here we will focus on the main mechanisms related to genetic factors and intestinal microbiota that affect the immune response.

It is known that there is an important genetic component that predisposes the development of both UC and CD, and many of these variants are shared in these diseases, thus the mechanistic pathways may be similar. A meta-analysis regarding genome-wide

association studies (GWAS) showed that, although 110 variants are shared in IBD, there are 23 specifics for UC and 30 for CD. The identified loci are enriched for primary immunodeficiencies, reduced circulating T- cell levels, and mycobacterial diseases (JOSTINS et al., 2012).

The strongest genetic risk associated with IBD is NOD2 (RADFORD-SMITH; PANDEYA, 2006). The receptor belongs to the NOD-like receptor (NLR) family and encodes the primary receptor for muramyl dipeptide (MDP) present in all Gram-positive and negative bacteria. NOD2 is expressed in macrophages, Paneth cells, and lamina propria lymphocytes and is pivotal for bacterial recognition. Therefore, it acts in the innate immune response and regulation of commensal microbiota (SALZMAN et al., 2010). After binding to MDP, the NOD2 oligomer activates TAK1, which leads to activation of NF-κB and MAPK, resulting in the production of inflammatory cytokines (MEINZER; HUGOT, 2005). Changes in the microbiome with an abnormal NOD2 response can result in an exacerbated immune response and inflammation, which is usually present in CD. Still, NOD2 variants can reduce the transcription of IL-10 anti-inflammatory cytokine (NI et al., 2017; STROBER; WATANABE, 2011).

There are other genetic variants associated with autophagy identified by GWAS and related to CD, such as ATG16L1 and IRGM. Activation of NOD-2 by bacterial MDP in epithelial cells leads to activation of autophagy and increases bacterial killing, a process that is impaired in individuals with CD associated with NOD-2 variants (HOEFKENS et al., 2013). This further compromises the secretion of antimicrobial peptides, such as α-defensins and other cryptdins (STROBER; WATANABE, 2011). Cryptdins are antimicrobial peptides that are produced by Paneth cells, and their antimicrobial activity is important in reducing infection by pathogenic bacteria such as *Listeria monocytogenes* (NI et al., 2017; OUELLETTE et al., 2000; STROBER; WATANABE, 2011).

2.2 Microbiota and Immune response

The human intestinal microbiota holds approximately 10^{14} bacterial cells and about 100-fold the number of human genes (microbiome), and the most representative phyla are Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (BÄCKHED et al., 2015; THURSBY; JUGE, 2017). Microbiota is shaped since before birth and is influenced by the mode of birth, the surrounding environment, breastfeeding, availability of nutrients, and other factors (AAGAARD et al., 2014; DOMINGUEZ-BELLO et al., 2010). After the introduction of food, the infant's microbiota becomes more similar to that of an adult, and its maturity

occurs around 3 years of age (BÄCKHED et al., 2015). Early colonization is essential for the development and maturation of the immune system. Children born by cesarean have delayed colonization and present lower diversity and reduced Th1 response (JAKOBSSON et al., 2014).

Immune receptors, such as Toll like receptor receiver (TLR) and NLR, recognize microbe-associated molecular patterns (MAMPs) and play a chief role in intestinal homeostasis (HOOPER; LITTMAN; MACPHERSON, 2012). The microbial composition is conditioned by the products of the immune and epithelial cells, such as IgA, mucus, and defensins. Regarding the mucosal immunity, it is regulated by the microbiota. *Bacteroides fragilis* promotes the differentiation of T helper 1 (Th1) and Clostridia of T helper Reg (Treg), for example, in a symbiotic relation (IMAM et al., 2018).

Increased intestinal permeability is frequent in CD and UC (COUFAL et al., 2019). A defect in the intestinal barrier could be a primary cause of immunopathogenesis in IBD since increased permeability facilitates the absorption of food and microbial products able to induce an exacerbated immune response and lead to inflammation (TURNER, 2009; WYATT et al., 1993). This is possibly due to a change in the mucus layer in the intestinal lumen. In patients with CD, a reduction in the expression of MUC3, MUC4, and MUC5B mRNA in the ileal mucosa and MUC1 mRNA in the inflamed ileum has already been observed (BUISINE et al., 1999; DOROFEYEV et al., 2013). Also, the colon mucus of animals that develop UC spontaneously and patients with active UC has been shown to allow bacteria to penetrate and reach the intestinal epithelium (JOHANSSON et al., 2014). The variants of the NOD2, JAK2, MUC1, and MUC13 genes are associated with impaired intestinal barrier function and may predispose to infectious and inflammatory diseases and handle an abnormal immune response to luminal antigens (BUHNER et al., 2006; PRAGER et al., 2012; SHENG et al., 2013).

In an experimental colitis model, it was demonstrated that mice with the gut epithelium vitamin D receptor (VDR) deletion developed a more severe clinical colitis and worsened epithelial cell apoptosis, leading to an increased intestinal mucosa permeability, and promoted the Th1 and Th17 mucosal response (HE et al., 2018). This suggests that the downregulation of the colonic VDR observed in patients with IBD may be related to an increased intestinal mucosa permeability.

The immunological profile of IBD patients is a combination of Th1 and Th1/Th17 in CD and atypical Th2 in UC (ROSEN et al., 2017). An increase in the pro-inflammatory cytokine IL-17 is observed in the intestinal mucosa and blood, especially in patients with CD. Since Th17 cells also produce IL-22 and IL-21, it promotes IFN-γ production and Th1

response. In UC, an immune response Th-2 is characterized by the production of IL-5, IL-13, and IFN-y. There is still disagreement regarding the pattern of cytokines secreted in different diseases and studies showed that the cytokine profile does not always match the type of immune response (TATIYA-APHIRADEE et al., 2019).

In IBD, there is an increased immune response against microbial antigens. This is noted by the circulating levels of antibodies against microbial antigens and glycans. Several studies have pointed out differences in the composition of the microbiota between the IBDs. The patients present an imbalance related to microbial diversity and relative abundance of specific bacteria, namely dysbiosis (CHASSAING; DARFEUILLEMICHAUD, 2011).

In comparison to healthy individuals, individuals with IBD show an increase in bacteria of the Proteobacteria phylum, such as Enterobacteriaceae and *Escherichia coli* (ANDOH et al., 2011). Patients with CD usually present a reduction in the phylum Firmicutes, especially *Faecalibacterium prausnitzii,* which is reduced in relative abundance in the stool, and increased abundances of Bacteroidetes and Proteobacteria. In UC, the gut microbiota is characterized by the low abundance of butyrate-producing bacteria, and a high ratio of *B.fragilis*/*F. prausnitzii* is associated with a weaken anti-inflammatory response (CHASSAING; DARFEUILLEMICHAUD, 2011; MIQUEL et al., 2013; SITKIN; POKROTNIEKS, 2019).

2.3 Therapies and adverse effects

Although IBDs are characterized by a chronic inflammatory disorder, there are several degrees in the severity of symptoms. Some patients may have the disease controlled with aminosalicylates and glucocorticoids treatments, while others require biological therapy and immunosuppressants (QUEZADA; MCLEAN; CROSS, 2018).

Aminosalicylates have a long history of safe use. Their adverse effects are similar to placebo controls and are usually controlled with a dose reduction (ROGLER, 2010).

The most common immunomodulators are azathioprine (AZA), methotrexate (MTX), and mercaptopurine (MP), which are usually efficient in controlling symptoms without the need for corticosteroids. The most frequent adverse effects related to these medications are liver toxicity, nausea, vomiting, diarrhea, and fatigue. Anti-TNF-α is the first class of biological therapy created to treat moderate to severe IBD. They comprise a series of monoclonal antibodies that are effective for both maintenance and remission of CD and UC. Nonetheless, these therapies may increase the risk for autoimmunity, demyelinating disease, and opportunistic infections, and about a third of patients can develop infections during the

first year of using the medication. Thus, some precautions are recommended before starting therapy, such as screening for tuberculosis and hepatitis B virus (QUEZADA; MCLEAN; CROSS, 2018).

Additionally, there are the anti-integrin and anti-interleukin 12/23 therapies. Despite the short history of use, they have shown to be a promising and effective treatment due to their role in different molecules of the pro-inflammatory cascade (QUEZADA; MCLEAN; CROSS, 2018).

The main reason for malnutrition in IBD is the reduced oral food intake due to symptoms of the disease, such as nausea and vomiting, fasting during hospitalization, or prolonged restrictive diets. Despite this, the use of medications may affect the absorption and use of micronutrients. Sulfasalazine, for example, is a folic acid antagonist, which may lead to anemia when used for a long period. On the other hand, glucocorticoids decrease the absorption and use of calcium, zinc, and phosphorus and impair vitamin D metabolism (SCALDAFERRI et al., 2017).

2.4 Diet and Quality of Life in IBD

IBD patients often report reduced quality of life (QoL) and have elevated levels of anxiety and depression when compared to healthy individuals. This may be due to the associated symptoms, disruption to usual life activities, employability, stigma, or disability (KNOWLES et al., 2018a). In a recent systematic review, Knowles et al. (2018b) verified the impacts of the disease on QoL when compared to healthy individuals. There is a significant reduction in both mental and physical health, similar to other medical conditions. QoL was also lower when the disease was active, and worse for those with CD compared to UC (KNOWLES et al., 2018a). It has also been shown that the QoL of patients with IBD improves over time, which means that there is an adaptive process to the disease (KNOWLES et al., 2018a,b).

It is common for patients with IBD to self-impose dietary restrictions, which is generally associated with insufficient macro and micronutrients in the diet (LIM; KIM; HONG, 2018). One study compared patients with inactive or average CD with healthy controls. An inadequate nutrient intake due to the exclusion of food groups, such as milk, vegetables, and grains in CD group was observed (SOUSA GUERREIRO et al., 2007). More than a third of the individuals with IBD had BMI above 25, showing malnutrition accompanied by obesity, which may be due to physical inactivity or treatment with corticosteroids. The main micronutrient deficiencies observed in patients with IBD are zinc,

iron, vitamin B12, and vitamin D, contributing to a critical condition and influencing on well-being (MASSIRONI et al., 2013; ROCHA et al., 2019).

In the meta-analysis conducted by Gubatan et al. (2019), the relationship between low levels of vitamin D and the risks of clinically active disease, mucosal inflammation, clinical relapse, and low quality of life scores among 8316 IBD patients from observational studies was evaluated. Low levels of 25(OH)D were significantly associated with an increase in the clinically active disease [UC (pooled OR 1.47, 95% CI 1.03-2.09, P = .03, I2 = 0%); CD (pooled OR 1.66, 95% CI 1.36-2.02, P < .00001, I2 = 0%)] and clinical relapse [UC (pooled OR 1.20, 95% CI 1.01-1.43, P = .04, I2 = 0%); CD (pooled OR 1.35, 95% CI 1.14-1.59, P = .0004, I2 = 0%)]. Meanwhile, low vitamin D levels were associated with increased mucosal inflammation and low quality of life scores only in CD patients. In fact, mucosal inflammation may lead to malabsorption of vitamin D in CD, thus low levels of vitamin D could be considered as an inflammation biomarker for CD (GUBATAN et al., 2019). Accordingly, MacMaster et al. (2020) observed that around 30% of 93 IBD patients in remission presented vitamin D deficiency (MACMASTER et al., 2020).

Hospitalization of IBD patients is associated with disease complications, surgical procedures, and a lack of specialized follow-up. Thus, the maintenance of UC and CD remission is of utmost importance. In fact, according to an integrative review conducted by Rocha et al. (2019), malnutrition is related to hospitalization of patients affected by the disease. Moreover, nutritional status may influence hospitalization in IBD, although no comparison with adequate nutritional status was evaluated (ROCHA et al., 2019). Low or insufficient levels of vitamin D have already been linked to an increased need for hospitalization and surgery in IBD, when compared to normal serum levels (ANANTHAKRISHNAN et al., 2013; KABBANI et al., 2016). This highlights the importance of maintaining levels considered as adequate for vitamin D, since its anti-inflammatory effect is very well studied, and these patients can benefit and improve their well-being.

3 VITAMIN D AS AN ALTERNATIVE APPROACH IN IBD

3.1 Mechanisms of action of Vitamin D

Vitamin D is a fat-soluble vitamin that can be found in two different chemical structures: cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2). It can be absorbed either by exposure of the skin to UVB rays from the sun, when the 7-dehydrocholesterol,

present in the skin, is converted to cholecalciferol, or by the consumption of some fatty fishes, sun-exposed mushrooms, fortified foods - mostly dairy products, or even by supplements. Vitamin D is transported to the liver by the circulation and transformed into 25(OH)D (25-hydroxyvitamin D), the main circulation form and vitamin D status marker, by the enzyme 25-hydroxylase (CYP2R1). Nonetheless, the 25-hydroxyvitamin D should suffer another hydroxylation in the kidneys by the enzyme 1-α-hydroxylase (CYP27B1), where it is converted to calcitriol or 1,25-$(OH)_2$D (1,25-dihydroxyvitamin D), the active form of the vitamin (HOLICK, 2017).

The functions of calcitriol in the body are mediated by the VDR. The VDR bounded to 1,25-$(OH)_2$D forms an heterodimer with the retinoic acid receptor (RXR), which migrates to the cell nucleus and binds to the vitamin D-response element (VDRE) in the promoter regions of target genes, acting as a nuclear transcription regulator (BAKKE et al., 2018; HAUSSLER et al., 2011; HOLICK, 2017). In addition, the VDR is expressed in various tissues (e.g. parathyroid gland, small intestines, and colon). The VDRE is found in several genes, explaining the mechanisms associated with vitamin D, like autophagy (WU et al., 2015), cell proliferation (JIN et al., 2017), intestinal barrier function (ZHANG et al., 2015; ZHANG et al., 2019), gut microbiota modulation (WANG et al., 2016; WU et al., 2015; ZHANG et al., 2020), and immune functions (BASHIR et al., 2016; VELDMAN; CANTORNA; DELUCA, 2000), besides the most well-known mechanism, regarding calcium homeostasis and bone health (BAKKE et al., 2018; HAUSSLER et al., 2011; WANG et al., 2016).

Vitamin D immunomodulatory effects are directly related to antigen presenter cells (e.g. macrophages and dendritic cells) and T-cells functions. It seems that 1,25-$(OH)_2$D modulates the T-cell differentiation, shifting from a pro-inflammatory Th1 immune response to an anti-inflammatory Th2 immune response, increasing the secretion of IL-4 while decreasing the secretion of IL-2 and IFN-γ. Moreover, 1,25-$(OH)_2$D may inhibit dendritic cell differentiation and IL-12 production while increasing IL-10. Also, the lack of 1,25-$(OH)_2$D harms regulatory T-cells (Tregs) differentiation and weakens its functions, which may trigger autoimmune diseases (CANTORNA, 2016; LIM; HANAUER; LI, 2005; SZODORAY et al., 2008).

There is no consensus about the ideal circulating level of vitamin D. According to the Institute of Medicine (IOM), for the majority of the population, a minimum 25(OH)D serum level of 20 ng/mL (50 nmol/L) is considered enough, in case of a minimum sun exposure. Meanwhile, the risk of vitamin D deficiency is considered when the 25(OH)D

serum level is below 12 ng/mL (30 nmol/L) (INSTITUTE OF MEDICINE, 2011). Nevertheless, the Clinical Practice Guideline from the Endocrine Society defined vitamin D deficiency as serum level of 25(OH)D below 20 ng/mL (50 nmol/L) and values between 21-29 ng/mL (525-725 nmol/L) are considered as vitamin D insufficiency (HOLICK et al., 2011). These thresholds of vitamin D serum levels were established for bone health. However, it is known that vitamin D deficiency may also be related to certain types of cancer, cardiovascular diseases and hypertension, type 2 diabetes and metabolic syndrome, autoimmune diseases (e.g. type 1 diabetes, rheumatoid arthritis, IBD, CD, systemic lupus erythematosus, and multiple sclerosis), and infectious diseases (e.g. tuberculosis and upper respiratory infections), autism, depression, among others (HOLICK, 2017; HOLICK et al., 2011; INSTITUTE OF MEDICINE, 2011; SZODORAY et al., 2008). Furthermore, it is important to point out that the exposure to sunlight is the most effective natural source of vitamin D. However, people usually avoid sunlight exposure or use sunscreen due to skin cancer risk and it is difficult to reach the minimum required through the diet, thus supplementation is often necessary (HOLICK, 2017; HOLICK et al., 2011; INSTITUTE OF MEDICINE, 2011).

3.2 Vitamin D as an alternative treatment for gut microbiota modulation and improvement of inflammation in IBD

It has been suggested that low levels of circulating vitamin D are related to increased IBD disease activity and relapses, in addition to gut microbiota dysbiosis. In fact, IBD is characterized by an abnormal immune response to gut commensal bacteria in genetically predisposed individuals. There are few studies with humans exploring the impact of vitamin D in IBD management and gut microbiota modulation, which will be discussed hereafter and are summarized in table1.

Table 1: Summary of Vitamin D studies in Inflammatory Bowel Disease

Group	Type of IBD	Treatment/Condition	Duration of study	Outcome	Ref.
Adults N = 90	UC	Single intramuscular injection: Vitamin D_3: 300,000 IU	Follow up after 3 months	↑ 25(OH)D ↓ TNF-α, IFN-γ, IL-12p70, hs-CRP, ESR ↓ Th1 immune response	SHARIFI et al., 2016; SHARIFI et al., 2019
Children and adolescents N= 61	UC and CD	Oral liquid preparation: Arm A - Vitamin D_2: 2,000 IU daily Arm B - Vitamin D_3: 2,000 IU daily Arm C - Vitamin D_2: 50,000 IU weekly	Follow up after 6 weeks	↑ 25(OH)D	PAPPA et al., 2012
Adolescents N= 40	UC and CD	Oral pills: • Vitamin D_3: 5,000 IU/10 kg of body weight (máx. 25,000 IU weekly) or 10,000 IU/10 kg or body weight (máx. 50,000 IU weekly)	6 weeks (follow up after 2, 8, and 12 weeks)	↑ 25(OH)D	SIMEK et al., 2016
Adults N= 10	UC and CD	Oral liquid preparation: Vitamin D_3: 5,000-10,000 IU daily	12 weeks (follow up at week 16)	↑ 25(OH)D ↓ CD clinical disease activity (HBI)	GARG et al., 2018b
Adults N= 25	UC	Oral pills: Vitamin D_3: 4,000 IU weekly	8 weeks	↑ 25(OH)D ↓ clinical disease activity ↓ fecal calprotectin ↓ inflammation in active UC Trend in reducing mucolytic species in fecal microbiota	GARG et al., 2018a

CD = Crohn's Disease; ESR = erythrocyte sedimentation rate; GIT = gastrointestinal tract; HBI = Harvey-Bradshaw Index; HE = high sunlight exposure; hs-CRP = high-sensitivity C-reactive protein; IBD = inflammatory bowel disease; IU = International Units; LE = low sunlight exposure; UC = Ulcerative colitis.

Table 1: continued

Group	Type of IBD	Treatment/Condition	Duration of study	Outcome	Ref.
Adults N= 17	CD in clinical remission	Oral: Vitamin D_3: Day 1 – 3: 20,000 IU Day 4 – 28 (alternated): 20,000 IU	4 weeks	↑ 25(OH)D ↑ week 1: *Alistipes*, *Barnesiella*, *Roseburia*, *Anaerotruncus*, *Subdoligranulum* ↑ week 2: *Faecalibacterium*, *Veillonella*, *Blautia*, *Fusicatenibacter*, *Intestinibacter* ↑ week 4: *Lactobacillus*, *Megasphera* ↓ reduced diversity	SCHÄFFLER et al., 2018
Adults N= 16	Healthy volunteers	Oral drops: Vitamin D_3: First 4 weeks: 980 IU/ kg of body weight weekly (max. 68,600 IU weekly) Last 4 weeks: 490 IU/ kg of body weight weekly (max. 34,300 IU weekly)	8 weeks	↑ 25(OH)D Upper GIT: ↓ Gammaproteobacteria – Pseudomonas spp., Escherichia/Shiguela spp. ↑ bacterial richness Terminal ileum: ↑CD8+ T cell	BASHIR et al., 2016
Adults N= 87	CD and UC active or in remission	Comparison between Seasonal 25-(OH)D circulating levels (supplemented or not)	Summer/autumm (HE) vs winter/spring (LE)	25(OH)D levels were correlated with changes in microbiome ↓ 25(OH)D → balanced microbiome composition	SOLTYS et al., 2020

CD = Crohn's Disease; ESR = erythrocyte sedimentation rate; GIT = gastrointestinal tract; HBI = Harvey-Bradshaw Index; HE = high sunlight exposure; hs-CRP = high-sensitivity C-reactive protein; IBD = inflammatory bowel disease; IU = International Units; LE = low sunlight exposure; UC = Ulcerative colitis.

3.2.1 Vitamin D and gut microbiota modulation

Schäffler et al. (2018) reported that vitamin D_3 supplementation altered the gut microbiota composition only in remission CD patients, and no changes were noted in the healthy controls. Throughout 4 weeks, an increase in the abundance of beneficial bacteria like *Alistipes*, *Parabacteroides*, *Roseburia*, and *Faecalibacterium* was observed, even though it was transient. The authors suggested that 4 weeks might have been a too short intervention period to detect a greater change. However, these results suggest that vitamin D administration has potential as an adjuvant therapy for CD patients (SCHÄFFLER, et al., 2018). It is noteworthy that the reduced abundance of the *Faecalibacterium* genus is commonly associated with both diseases, UC and CD. Its characteristic of producing butyrate has already been shown to be a way to reduce inflammation and promote a balance between Th17 and Treg (ZHOU et al., 2018).

In an interesting cohort study, the possible connection between the seasonality of serum vitamin D levels and changes in the microbiome was evaluated. The target population was composed by adults (n = 87) with IBD (CD or UC), who lived in regions far from the equator, and both the intestinal mucosa and the fecal samples microbiome were evaluated. After confirming the differences in the concentrations of 25 (OH) D, which were higher in periods with higher sun exposure (summer / autumn), some changes in the microbial composition were also observed. In the summer / autumn period, an increase in the abundance of *Pediococcus* spp., *Clostridium* spp., and *Escherichia* / *Shigella* spp. was observed. In contrast, inflammation-related bacterial genera such as *Eggerthella lenta*, *Fusobacterium* spp., *Helicobacter* spp., and *Faecalibacterium prausnitzii* showed lower relative abundance. Unlike other studies, low levels of vitamin D were associated with a more balanced composition of the microbiome. It should be noted that it was not a randomized controlled trial (RTC), but vitamin D levels were correlated with changes in the microbiome in individuals with IBD (SOLTYS et al., 2020).

Vitamin D supplementation in healthy individuals has also been shown to alter the microbiome. In a study conducted in healthy adults (n = 16) supplemented with vitamin D_3 (first 4 weeks: 980 IU / kg of body weight; last 4 weeks: 490 IU / kg of body weight) for 8 weeks, the supplemented group had the upper GIT (gastric corpus, antrum, and duodenum) microbiome composition changed (BASHIR et al., 2016). The abundance of Proteobacteria was reduced in the gastric corpus (GC) and antrum (GA), along with an increase in Bacteroidetes in the GC and the descending part of the duodenum, while no changes were

observed in the microbiome of the lower GIT and fecal samples. The supplemented group also showed an increase in bacterial richness and significant changes in Gammaproteobacteria in the upper GIT, such as a reduction in *Pseudomonas spp*. and *Escherichia* / *Shigella*. The authors suggested that the increase in the phylotype richness and the microbial changes in the upper GIT, mainly due to the reduction in typically opportunistic pathogens, supports the beneficial effects of vitamin D on the human gut microbiome, especially in the upper GIT (BASHIR et al., 2016). Still, similarly to what had been previously observed by Veldman et al. (2000), a trend in the increase of CD8 + T cell, the immune cell with the highest expression of VDR, was observed in almost all regions of the gastrointestinal tract evaluated (BASHIR et al., 2016; VELDMAN et al., 2000).

In another interventional study conducted with healthy individuals, higher circulating levels of 25(OH)D (above 20 ng/mL) were related to a higher abundance of the beneficial bacteria *Akkermansia muciniphila* and a reduced abundance of the pathogen *Porphyromonas* spp. Moreover, after supplementation with vitamin D_3 for 8 weeks (600, 4,000, or 10,000 IU/day), an increase in the relative abundances of *Bacteroides* spp. and *Parabacteroides* spp. was observed. This fact is usually associated with an improvement in the IBD activity, and a decrease in the Firmicutes:Bacteroidetes ratio (CHAROENNGAM et al., 2020). Accordingly, Luthold et al. (2017) reported that 25(OH)D circulating levels were inversely correlated with the fecal abundances of the Gram-negative genera *Haemophilus* and *Veillonella*, in addition to higher LPS levels, suggesting the vitamin D role in the intestinal homeostasis and inflammation (LUTHOLD et al., 2017).

3.2.2 Anti-inflammatory potential of vitamin D in IBD

Supplementation of vitamin D in IBD patients is challenging due to nutrients malabsorption issues, and higher doses are often necessary to achieve the recommended circulating level (above 20 ng/L, according to IOM). A meta-analysis conducted by Guzman-Prado et al. (2020) indicated that the administration of vitamin D to IBD patients might improve the vitamin status while reducing the disease activity index and the levels of hs-CRP (high-sensitivity C-reactive protein) (GUZMAN-PRADO et al., 2020). Nevertheless, these benefits seemed to be more pronounced in CD cases when compared to UC (GUBATAN et al., 2019; GUZMAN-PRADO et al., 2020).

Supplementation of vitamin D_2 (2,000 IU daily or 50,000 IU weekly) or vitamin D_3 (2,000 IU daily) were able to increase vitamin D level in children and adolescents with IBD and vitamin D insufficiency. However, the higher vitamin D_2 dose (50,000 IU weekly) was

the most successful treatment, achieving a serum level of 25(OH) above 32 ng/mL in 75% of the patients enrolled in this group (PAPPA et al., 2012). Likewise, adolescents with IBD and vitamin D deficiency that received oral vitamin D_3 supplementation during 6 weeks (5,000 IU or 10,000 IU per 10 g of body weight) showed an improvement in the vitamin D status up to 12 weeks of follow up (SIMEK et al., 2016). Nonetheless, none of these clinical trials evaluated inflammatory markers or reported the impact on the disease status.

An observational study with 206 IBD patients showed that vitamin D deficiency and insufficiency were observed in both CD and UC patients, but were more frequent in CD patients. In addition, moderate and severe clinical disease activities reported were significantly associated with vitamin D deficiency in CD (MENTELLA et al., 2019). Similarly, Mechie et al. (2019) reported that the majority of IBD patients had vitamin D deficiency and the serum levels of 25(OH)D may be considered an important marker for IBD disease activity, in addition to hs-CRP and fecal calprotectin (MECHIE et al., 2019).

In a RCT with 90 adults with UC, a single dose injection of 300,000 IU of vitamin D_3 significantly increased the serum levels of 25(OH)D while it decreased inflammatory markers hs-CRP and ESR (erythrocyte sedimentation rate) and suppressed the Th1 immune response in UC patients (SHARIFI et al., 2016; SHARIFI et al., 2019). Similarly, in the study conducted by Garg et al. (2018b), UC and CD patients that received 5,000-10,000 IU of vitamin D_3 daily for 12 weeks showed a significant increase in the 25(OH)D serum levels (GARG et al., 2018b). At the same time, CD patients reported a decrease in the clinical disease activity index, even though the biomarkers did not confirm this anti-inflammatory effect (GARG et al., 2018a).

In summary, vitamin D plays a critical role in the proper immune responses, and its status should be monitored in individuals from risk groups, such as IBD patients. Moreover, a limited number of interventional studies evaluating the impact of vitamin D in the inflammation pathways and in the gut microbiota modulation in IBD were conducted. However, the outcomes are hopeful, and future studies are encouraged.

4 EPIGENETICS AND IBD, VITAMIN D/VDR

Genetics is popularly known as the study of heredity, evaluating the changes in nucleic acids and their performance in organisms. On the other hand, epigenetics is defined as changes in gene expression or reversible hereditary changes without altering the DNA sequence (FEINBERG, 2007). A central goal of epigenomics is to understand the gene

expression alteration by dietary molecules (CARLBERG; ULVEN; MOLNÁR, 2016), and it makes a joint focus with nutrigenomics and epigenomics (CARLBERG, 2019).

Epigenetics changes occur in the following ways: DNA methylation, histone modifications, chromatin remodeling, and noncoding RNAs regulation (CAVALLI; HEARD, 2019).

The most well-known epigenetic modification is DNA methylation, which is characterized by the addition of a methyl group covalently to the C5 carbon of a cytosine (BIRD, 2002). The degree or number of methylations defines the expression of a gene. High degree of methylations (hypermetilation) silences the promoter of tumor suppressor genes, while under-methylated DNA (hypomethylation) induces proto-oncogenes, for example (HERCEG, 2007).

The second group of epigenetic changes are the histone modifications, which either activate or repress gene expression. Based on post-translational modifications in the histone tail at lysine, arginine, and serine residues (KOUZARIDES, 2007), such as acetylation, methylation, and phosphorylation (CARLBERG; ULVEN; MOLNÁR, 2016).

Chromatin remodeling are the third group of epigenetics modification. Chromatin is modulated by a group of enzymes that catalyze changes in histone residues, such as the addition or removal of acetyl or methyl groups. Acetylation (addition of acetyl) is generally associated with transcriptional activation. These reactions are performed by two classes of enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC), while for histone methylation there are two classes of enzymes with opposite functions, histone methyltransferase (HMT) and histone demethylase (HDM) (CARLBERG; ULVEN; MOLNÁR, 2016).

There are several classes of RNA, including the noncoding RNAs (ncRNAs). This class of molecules are grouped in the fourth group of epigenetics modification. NcRNA is a group of RNA that has several other smaller groups, and neither their production nor their functions can be generalized. Many of them regulate post-transcriptional processes, and others are involved in transcriptional regulation. They can be divided in long ncRNAs (lncRNAs), with up to more than 100 kilobases, and short ncRNAs, with less than 30 nucleotides, such as microRNAs (miRNAs), short interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs) (CAVALLI; HEARD, 2019).

4.1 Epigenetics and IBD

For a long time, a genetic susceptibility in IBD pathogenesis was suggested, and the technological progress in DNA/RNA sequencing allowed many GWAS, and thus, the single nucleotide polymorphisms (SNPs) identification (ANNESE, 2020; GAYA et al., 2006; ZHANG; LI, 2014).

The identification of markers for the diagnosis of IBD is of utmost importance, and DNA methylation and miRNAs are special biomarkers for diagnosis at the molecular level. Indeed, studies have shown a strong sensitivity, specificity, and precision of these markers in the diagnosis of IBD (ZENG; MUKHERJEE; ZHANG, 2019).

Compared with a healthy control group, patients with IBD showed different changes in the mucosa methylation of the THRAP2, FANCC, GBGT1, DOK2, and TNFSF4 markers. Differences were also observed between patients with CD and UC. CD patients had hypermethylated GBGT1, IGFBP4, and FAM10A4 and hypomethylated IFITM1 when compared to UC patients. Thus, enabling them as markers for differentiating CD and UC (COOKE et al., 2012). Recently, Kim et al. (2020) identified that the fragile histidine triad (FHIT) gene was hypermethylated in patients with CD, therefore a possible biomarker for this disease (KIM et al., 2020).

4.2 Vitamin D/VDR epigenetic role in IBD

It is common to associate vitamin D with skeletal homeostasis (LAMBERG-ALLARDT, 2006). However, VDR is linking at hundreds of sites in the genome (CARLBERG; CAMPBELL, 2013) and is associated with the regulation of more than 60 genes (ALI; VAIDYA, 2007; THIBAULT; CANCEL-TASSIN; CUSSENOT, 2006). VDR regulates the opening of ion channels, as well as the activity of various enzymes such as kinases, phosphatases, and phospholipases (HAUSSLER et al., 2011).

The role of vitamin D/VDR in the secretion of intestinal mucus may be regulated by the expression of CYP27B1 (ZHU et al., 2019). In both UC and CD, VDR expression is down-regulated while CYP27B1 is up-regulated (CHEN et al., 2014; DU et al., 2017; LIU et al., 2013). This reduced VDR expression can also be attributed to the miRNA-346 (CHEN et al., 2014), one of the post-transcriptional mechanisms explained further.

Another way to prevent intestinal inflammation is by regulation of junctional proteins (CAMPBELL; MAIERS; DEMALI, 2017). Although there is a variety of functions and routes that vitamin D / VDR plays a role, there are few studies exploring gene regulation of junctional proteins (FAKHOURY et al., 2020). Liu et al. (2017) showed that VDR binds to

histone inhibiting transcription of ZO-1, claudin-5, and occludin genes (LIU et al., 2017). On the other hand, Zhang et al. (2015) have identified that VDR increases the leaky protein *claudin-2* as a direct target of the VDR signaling pathway (ZHANG et al., 2015). Inflammatory cytokines could also increase the expression of Claudin-2 and enhance intestinal permeability. Thus, lacking intestinal VDR regulation in IBD leads to hyperfunction of Claudin-2 in the intestine and exaggerates the inflammatory responses (ZHANG et al., 2017).

Besides the junctional proteins, other studies pointed out the influence of other proteins related to IBD on the modulation of miRNAs or modulated by them. MiRNA are a class of small non-coding RNA (17-22 nucleotides) that regulate gene expression post-transcriptionally. Liu et al. (2018) performed a meta-analysis exploring the association of SNPs from miRNAs and IBD. They reported three polymorphisms (rs11614913, rs2910146, and rs3746444) in miRNA-196a2, miRNA-146a, and miRNA-499 in patients with IBD (LIU et al., 2018). In addition, other miRNAs expression profiles changed during IBD. Among them, are the following: miRNA-21, miRNA-122a, miRNA-155 or miRNA-150, which have been associated to intestinal epithelial permeability (BIAN et al., 2011; SUN et al., 2013; TIAN et al., 2013; YE et al., 2011); and miRNA-126, miRNA-146a or miRNA-155, which are linked to innate and adaptive immune response in intestinal inflammation (FENG et al., 2012; GHORPADE et al., 2013; SINGH et al., 2014). Moreover, miRNA-146a and miRNA-155- are directly related to the communication between the GIT microbiome and the brain, and miRNA-155 acts on 3 proteins, for which down-regulation is related to Alzheimer's Disease (ALEXANDROV et al., 2019).

4.3 Probiotic role on epigenetic changes by Vitamin D/VDR

The potential use of probiotics for gut microbiota modulation and IBD management have been studied (BIANCHI et al., 2019; LEE et al., 2018; SARTOR, 2011). Ryan et al. (2020) reported that inflamed and non-inflamed colonic segments in CD and UC differ in microbiota composition and epigenetic profiles (RYAN et al., 2020). Moreover, it has been suggested that probiotics may modulate the expression of miRNAs (CURRÒ et al., 2017).

Importantly, the proper function of VDR is crucial for probiotic anti-inflammatory effects, while probiotic consumption may improve the VDR status as well (BATTISTINI et al., 2020). Recently, Lu et al. (2020) reported that the anti-inflammatory and anti-infectious activity of lactobacilli strains isolated from Korean kimchi depends on the VDR expression (LU et al., 2020). Yet, in an earlier study, Lu et al. (2019) investigated the tissue-specific role

of intestinal epithelial VDR apoptosis and autophagy. The authors concluded that VDR loss impairs autophagy and enhances cell death through apoptosis. They suggested that this mechanism is mediated by the action of vitamin D in ATG16L1 and Beclin-1, which promotes cell survival and thus an anti-inflammatory role in the intestine (LU et al., 2019). Likewise, *Saccharomyces boulardii* revealed to be a promising probiotic specie for the management of IBD, increasing the expression of miRNA-155 and miRNA-223, while decreasing the expression miRNA-143 and miRNA-375 (RODRÍGUEZ-NOGALES et al., 2018).

Furthermore, in a study conducted by Chatterjee et al. (2020), it became clear that the VDR signaling affects both the microbiome and the metabolomics profile. Indeed, impaired VDR together with a high fat diet (HFD), promoted a significant impact on bile acid metabolism, which was more pronounced in female mice. In addition to microbiome regulation, long chain acylcarnitines (LCACs), tocopherol, and equol metabolisms were also influenced by VDR function and HFD. Thus, it can be concluded that both, diet and VDR status, play a role in metabolic diseases, inflammation, risk of colon cancer, and epigenetic pathways (CHATTERJEE et al., 2020).

In summary, emerging studies have pointed out the role of vitamin D/VDR in regulating proteins that are related to IBD, especially promoting transcription factors, such as miRNAs. There is also evidence that probiotics play a role in these modulations. Our recent study has shown that VDR promotes healthy microbial metabolites and microbiome in a tissue specific and gender specific manner (CHATTERJEE et al., 2020). However, more studies are necessary to elucidate this influence and all the metabolic steps associated.

5 CONCLUSIONS AND FUTURE DIRECTIONS

In this review, we addressed the immunomodulatory effects of vitamin D. The knowledge about the particularities of IBD has advanced substantially in recent years, and the anti-inflammatory and modulating effect of vitamin D and VDR expression has been widely studied and publicized. However, despite the fact that the chronic inflammation profile in IBD is the key role that the microbiota plays in this relationship, studies in humans are still scarce.

Vitamin D immunomodulatory effects seem to be related to the suppression of the pro-inflammatory Th1 immune response, while lower levels of 1,25-$(OH)_2$D impair Tregs, triggering autoimmune diseases. Indeed, the supplementation of vitamin D improved the

hs-CRP and fecal calprotectin status, while reduced the disease activity index and relapses, predominantly in CD patients.

So far, it is possible to observe that vitamin D supplementation contributes to the reduction of inflammation in individuals with IBD and can promote changes in the human microbiota. However, the studies reported have several limitations, such as the small sample, the unmatched methodology, or even the lack of definition of what would be the composition of a healthy microbiota. Surely, VDR is a crucial factor for gut microbiota homeostasis, having a great impact on the metabolome profile as well. In addition, its proper functions influence several genes associated with inflammation, barrier function, cancer, autophagy, among others. Therefore, more studies to assess the microbiota at the metabolic level are needed, which would be more appropriate than in the taxonomic level, although alterations in genera and species have already been associated with the disease.

Finally, a different profile of miRNA is expressed in CD, UC, or healthy control individuals and epigenetics markers revealed to be a highly sensitive, specific, and precise tool for IBD diagnosis, therefore a promising and less invasive alternative when compared with endoscopy and biopsies, which are employed nowadays. Moreover, vitamin D also plays a role in IBD regulating transcription factors associated with barrier function and immune responses. The exact mechanisms are not well understood and more studies are encouraged to explore the therapeutic potential of vitamin D in the gut microbiota modulation and anti-inflammatory effects in IBD in the metabolic, immunological, and epigenetic levels.

REFERENCES

AAGAARD, K.; MA, J.; ANTONY, K. M.; GANU, R.; PETROSINO, J.; VERSALOVIC, J. The placenta harbors a unique microbiome. **Science Translational Medicine**, v. 6, n. 237, p. 237ra65, 2014.

ADAK, A.; KHAN, M. R. An insight into gut microbiota and its functionalities. **Cellular and Molecular Life Sciences**, v. 76, n. 3, p. 473-493, 2019.

AGA Patient Information Section. Inflammatory Bowel Disease. **Clinical Gastroenterology and Hepatology**, v. 15, n. 7, p. A21, 2017.

ALATAB, S.; SEPANLOU, S. G.; IKUTA, K.; VAHEDI, H.; BISIGNANO, C.; SAFIRI, S.; SADEGHI, A.; NIXON, M. R.; ABDOLI, A.; ABOLHASSANI, H.; ALIPOUR, V.; ALMADI, M. A. H.; ALMASI-HASHIANI, A.; ANUSHIRAVANI, A.; ARABLOO, J.; ATIQUE, S.; AWASTHI, A.; BADAWI, A.; BAIG, A. A.A.; BHALA, N.; BIJANI, A.; BIONDI, A.; BORZÌ, A. M.; BURKE, K. E.; CARVALHO, F.; DARYANI, A.; DUBEY, M.; EFTEKHARI, A.; FERNANDES, E.; FERNANDES, J. C.; FISCHER, F.; HAJ-MIRZAIAN, A.; HAJ-MIRZAIAN, A.; HASANZADEH, A.; HASHEMIAN, M.; HAY, S. I.; HOANG, C. L.; HOUSEH, M.; ILESANMI, O. S.; BALALAMI, N. J.; JAMES, S. L.; KENGNE, A. P.; MALEKZADEH, M. M.; MERAT, S.; MERETOJA, T. J.; MESTROVIC, T.; MIRRAKHIMOV, E. M.; MIRZAEI, H.; MOHAMMAD, K. A.; MOKDAD, A. H.; MONASTA, L.; NEGOI, I.; NGUYEN, T. H.; NGUYEN, C. T.; POURSHAMS, A.; POUSTCHI, H.; RABIEE, M.; RABIEE, N.; RAMEZANZADEH, K.; RAWAF, D. L.; RAWAF, S.; REZAEI, N.; ROBINSON, S. R.; RONFANI, L.; SAXENA, S.; SEPEHRIMANESH, M.; SHAIKH, M. A.; SHARAFI, Z.; SHARIF, M.; SIABANI, S.; SIMA, A. R.; SINGH, J. A.; SOHEILI, A.; SOTOUDEHMANESH, R.; SULERIA, H. A. R.; TESFAY, B. E.; TRAN, B.; TSOI, D.; VACANTE, M.; WONDMIENEH, A. B.; ZARGHI, A.; ZHANG, Z. J.; DIRAC, M.; MALEKZADEH, R.; NAGHAVI, M. The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. **The Lancet Gastroenterology & Hepatology**, v. 5, n. 1, p. 17-30, 2020.

ALEXANDROV, P. N.; ZHAO, Y.; LI, W.; LUKIW, W. J. Lipopolysaccharide-stimulated, NF-kB-, miRNA-146a- and miRNA-155-mediated molecular-genetic communication between the human gastrointestinal tract microbiome and the brain. **Folia Neuropathologica**, v. 57, n. 3, p. 211-219, 2019.

ALI, M. M.; VAIDYA, V. Vitamin D and cancer. **Journal of Cancer Research and Therapeutics**, v. 3, n. 4, p. 225-230, 2007.

ALMEIDA, A; MITCHELL, A. L.; BOLAND, M.; FORSTER, S. C.; GLOOR, G. B.; TARKOWSKA, A.; LAWLEY, T. D.; FINN, R. D. A new genomic blueprint of the human gut microbiota. **Nature**, v. 568, p. 499-504, 2019.

ANANTHAKRISHNAN, A. N.; CAGAN, A.; GAINER, V. S.; CAI, T.; CHENG, S. C.; SAVOVA, G.; CHEN, P.; SZOLOVITS, P.; XIA, Z.; DE JAGER, P. L.; SHAW, S. Y.; CHURCHILL, S.; KARLSON, E. W.; KOHANE, I.; PLENGE, R. M.; MURPHY, S. N.; LIAO, K. P. Normalization of plasma 25-hydroxy vitamin D is associated with reduced risk of surgery in Crohn's disease. **Inflammatory Bowel Diseases**, v. 19, n. 9, p. 1921-1927, 2013.

ANDOH, A.; IMAEDA, H.; AOMATSU, T.; INATOMI, O.; BAMBA, S.; SASAKI, M.; SAITO, Y.; TSUJIKAWA, T.; FUJIYAMA, Y. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. **Journal of Gastroenterology**, v. 46, p. 479-489, 2011.

ANNESE, V. Genetics and epigenetics of IBD. **Pharmacological Research**, v. 159, p. 104892, 2020.

BÄCKHED, F.; ROSWALL, J.; PENG, Y.; FENG, Q.; JIA, H.; KOVATCHEVA-DATCHARY, P.; LI, Y.; XIA, Y.; XIE, H.; ZHONG, H.; KHAN, M. T.; ZHANG, J.; LI, J.; XIAO, L.; AL-AAMA, J.; ZHANG, D.; LEE, Y. S.; KOTOWSKA, D.; COLDING, C.; TREMAROLI, V.; YIN, Y.; BERGMAN, S.; XU, X.; MADSEN, L.; KRISTIANSEN, K.; DAHLGREN, J.; WANG, J. Dynamics and stabilization of the human gut microbiome during the first year of life. **Cell Host & Microbe**, v. 17, n. 5, p. 690-703, 2015.

BAKKE, D.; CHARTTERJEE, I.; AGRAWAL, A.; DAI, Y.; SUN, J. Regulation of Microbiota by Vitamin D Receptor: A Nuclear Weapon in Metabolic Diseases. **Nuclear Receptor Research**, v. 5, p. 101377, 2018.

BASHIR, M.; PRIETL, B.; TAUSCHMANN, M.; MAUTNER, S. I.; KUMP, P. K.; TREIBER, G.; WURM, P.; GORKIEWICZ, G.; HÖGENAUER, C.; PIEBER, T. R. Effects of high doses of vitamin D3 on mucosa-associated gut microbiome vary between regions of the human gastrointestinal tract. **European Journal of Nutrition**, v. 55, n. 4, p. 1479-1489, 2016.

BATTISTINI, C.; NASSANI, N.; SAAD, S. M. I.; SUN, JUN. **Probiotics, Vitamin D, and Vitamin D Receptor in Health and Disease**. In: ALBUQUERQUE, M. A. C.; LEBLANC, A. M.; LEBLANC, J. G.; BEDANI, R. (eds.), Lactic acid bacteria: a functional approach. CRC Press, Boca Raton, p. 93–105, 2020.

BIAN, Z.; LI, L.; CUI, J.; ZHANG, H.; LIU, Y.; ZHANG, C. Y.; ZEN, K. Role of miR-150-targeting c-Myb in colonic epithelial disruption during dextran sulphate sodium-induced murine experimental colitis and human ulcerative colitis. **The Journal of Pathology**, v. 225, n. 4, p. 544-553, 2011.

BIANCHI, F.; DUQUE, A. L. R. F.; SAAD, S. M. I.; SIVIERI, K. Gut microbiome approaches to treat obesity in humans. **Applied Microbiology and Biotechnology**, v. 103, p. 1081-1094, 2019.

BIRD, A. DNA methylation patterns and epigenetic memory. **Genes & Development**, v. 16, n. 1, p. 6-21, 2002.

BUHNER, S.; BUNING, C.; GENSCHEL, J.; KLING, K.; HERRMANN, D.; DIGNASS, A.; KUECHLER, I.; KRUEQER, S.; SCHMIDT, H. H. J.; LOCHS, H. Genetic basis for increased intestinal permeability in families with Crohn's disease: Role of CARD15 3020insC mutation? **Gut**, v. 55, n. 3, p. 342-347, 2006.

BUISINE, M. P.; DESREUMAUX, P.; DEBAILLEUL, V.; GAMBIEZ, L.; GEBOES, K.; ECTORS, N.; DELESCAUT, M. P.; DEGAND, P.; AUBERT, J. P.; COLOMBEL, J. F.; PORCHET, N. Abnormalities in mucin gene expression in Crohn's disease. **Inflammatory Bowel Diseases**, v. 5, n. 1, p. 24-32, 1999.

CAMPBELL, H. K.; MAIERS, J. L.; DEMALI, K. A. Interplay between tight junctions & adherens junctions. **Experimental Cell Research**, v. 358, n. 1, p. 39-44, 2017.

CANTORNA, M. T. IBD: Vitamin D and IBD: Moving towards clinical trials. **Nature Reviews Gastroenterology & Hepatology**, v. 13, p. 322-323, 2016.

CARLBERG, C. Nutrigenomics of Vitamin D. **Nutrients**, v. 11, n. 3, p. 676, 2019.

CARLBERG, C.; CAMPBELL, M. J. Vitamin D receptor signaling mechanisms: Integrated actions of a well-defined transcription factor. **Steroids**, v. 78, n. 2, p. 127-136, 2013.

CARLBERG, C.; ULVEN, S. M.; MOLNÁR, F. **Nutrigenomics**. 1st ed. Springer International Publishing, 2016.

CAVALLI, G., HEARD, E. Advances in epigenetics link genetics to the environment and disease. **Nature**, v. 571, p. 489–499, 2019.

CHAROENNGAM, N.; SHIRVANI, A.; KALAJIAN, T. A.; SONG, A.; HOLICK, M. F. The effect of various doses of oral Vitamin D3 supplementation on gut microbiota in healthy adults: A Randomized, Double-blinded, Dose-response Study. **Anticancer Research**, v. 40, n. 1, p. 551-556, 2020.

CHASSAING, B.; DARFEUILLEMICHAUD, A. The commensal microbiota and enteropathogens in the pathogenesis of inflammatory bowel diseases. **Gastroenterology**, v, 140, n. 6, p. 1720-1728, 2011.

CHATTERJEE, I.; LU, R.; ZHANG, Y. G.; ZHANG, J.; DAI, Y.; XIA, Y.; SUN, J. Vitamin D receptor promotes healthy microbial metabolites and microbiome. **Scientific Reports**, v. 10, p. 7340, 2020.

CHEN, Y.; DU, J.; ZHANG, Z.; LIU, T.; SHI, Y.; GE, X.; LI, Y. C. MicroRNA-346 mediates tumor necrosis factor α-induced downregulation of gut epithelial vitamin D receptor in inflammatory bowel diseases. **Inflammatory Bowel Diseases**, v. 20, n. 11, p. 1910-1918, 2014.

COOKE, J.; ZHANG, H.; GREGER, L.; SILVA, A. L.; MASSEY, D.; DAWSON, C.; METZ, A.; IBRAHIM, A.; PARKES, M. Mucosal genome-wide methylation changes in inflammatory bowel disease. **Inflammatory Bowel Diseases**, v. 18, n. 11, p., 2128-2137, 2012.

COUFAL, S.; GALANOVA, N.; BAJER, L.; GAJDAROVA, Z.; SCHIEROVA, D.; JIRASKOVA ZAKOSTELSKA, Z.; KOSTOVCIKOVA, K.; JACKOVA, Z.; STEHLIKOVA, Z.; DRASTICH, P.;TLASKALOVA-HOGENOVA, H.; KVERKA, M. Inflammatory Bowel Disease Types Differ in Markers of Inflammation, Gut Barrier and in Specific Anti-Bacterial Response. **Cells**, v. 8. n. 7, p. 719, 2019.

CURRÒ, D.; IANIRO, G.; PECERE, S.; BIBBÒ, S.; CAMMAROTA G. Probiotics, fibre and herbal medicinal products for functional and inflammatory bowel disorders. **British Journal of Pharmacology**, v. 174, n. 11, 1426-1449, 2017.

DE SOUZA, H. S. P.; FIOCCHI, C. Immunopathogenesis of IBD: Current state of the art. **Nature Reviews Gastroenterology & Hepatology**, v. 13, p. 13-27, 2016.

DOMINGUEZ-BELLO, M. G.; COSTELLO, E. K.; CONTRERAS, M.; MAGRIS, M.; HIDALGO, G.; FIERER, N.; KNIGHT, R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. **Proceedings of the National Academy of Sciences of the United States**, v. 107, n. 26, p. 11971-11975, 2010.

DOROFEYEV, A. E.; VASILENKO, I. V.; RASSOKHINA, O. A.; KONDRATIUK, R. B. Mucosal barrier in ulcerative colitis and Crohn's disease. **Gastroenterology Research and Practice**, v. 2013, p. 431231, 2013.

DU, J.; WEI, X.; GE, X.; CHEN, Y.; LI, Y. C. Microbiota-dependent induction of colonic CYP27B1 is associated with colonic inflammation: Implications of locally produced 1,25-dihydroxyvitamin D3 in inflammatory regulation in the colon. **Endocrinology**, v. 158, n. 11, p. 4064-4075, 2017.

FAKHOURY, H. M. A.; KVIETYS, P. R.; ALKATTAN, W.; AL ANOUTI, F.; ELAHI, M. A.; KARRAS, S. N.; GRANT, W. B. Vitamin D and intestinal homeostasis: Barrier, microbiota, and immune modulation. **The Journal of Steroid Biochemistry and Molecular Biology**, v. 200, p. 105663, 2020.

FEINBERG, A. P. Phenotypic plasticity and the epigenetics of human disease. **Nature**, v. 447, p. 433-440, 2007.

FENG, X.; WANG, H.; YE, S.; GUAN, J.; TAN, W.; CHENG, S.; WEI, G.; WU, W.; WU, F.; ZHOU, Y. Up-Regulation of microRNA-126 May Contribute to Pathogenesis of Ulcerative Colitis via Regulating NF-kappaB Inhibitor IkBa. **PLoS One**, v. 7, n. 12, p. e52782, 2012.

GARG, M.; HENDY, P.; DING, J. N.; SHAW, S.; HOLD, G.; HART, A. The effect of Vitamin D on intestinal inflammation and faecal microbiota in patients with ulcerative colitis. **Journal of Crohn's & Colitis**, v. 12, n. 8, p. 963-972, 2018a.

GARG, M.; ROSELLA, O.; ROSELLA, G.; WU, Y.; LUBEL J. S.; GIBSON, P. R. Evaluation of a 12-week targeted vitamin D supplementation regimen in patients with active inflammatory bowel disease. **Clinical Nutrition**, v. 37, n. 4, p. 1375-1382, 2018b.

GAYA, D. R.; RUSSELL, R. K.; NIMMO, E. R.; SATSANGI, J. New genes in inflammatory bowel disease: lessons for complex diseases? **The Lancet**, v. 367, n. 9518, p. 1271-1284, 2006.

GHORPADE, D. S.; SINHA, A. Y.; HOLLA, S.; SINGH, V.; BALAJI, K. N. NOD2-nitric oxide-responsive microRNA-146a activates Sonic hedgehog signaling to orchestrate inflammatory responses in murine model of inflammatory bowel disease. **Journal of Biological Chemistry**, v. 288, n. 46, p. 33037-33048, 2013.

GUBATAN, J.; CHOU, N. D.; NIELSEN, O. H.; MOSS, A. C. Systematic review with meta-analysis: association of vitamin D status with clinical outcomes in adult patients with inflammatory bowel disease. **Alimentary Pharmacology & Therapeutics**, v. 50, p. 1146-1158, 2019.

GUZMAN-PRADO, Y.; SAMSON, O.; SEGAL, J. P.; LIMDI, J. K.; HAYEE, B. Vitamin D Therapy in Adults With Inflammatory Bowel Disease: A Systematic Review and Meta-Analysis. **Inflammatory Bowel Diseases**, 2020. In press. doi: 10.1093/ibd/izaa087.

HAUSSLER, M. R.; JURUTKA, P. W.; MIZWICKI, M.; NORMAN, A. W. Vitamin D receptor (VDR)-mediated actions of $1,25(OH)_2$vitamin D_3: Genomic and non-genomic mechanisms. **Best Practice & Research Clinical Endocrinology & Metabolism**, v. 25, n. 4, p. 543-559, 2011.

HE, L.; LIU, T.; SHI, Y.; TIAN, F.; HU, H.; DEB, D. K.; CHEN, Y.; BISSONNETTE, M.; LI, Y. C. Gut epithelial Vitamin D receptor regulates microbiota-dependent mucosal inflammation by suppressing intestinal epithelial cell apoptosis. **Endocrinology**, v. 159, n. 2, p. 967, 979, 2018.

HERCEG, Z. Epigenetics and cancer: Towards an evaluation of the impact of environmental and dietary factors. **Mutagenesis**, v. 22, n. 2, p. 91-103, 2007.

HOEFKENS, E.; NYS, K.; JOHN, J. M.; VAN STEEN, K.; ARIJS, I.; VAN DER GOTEN, J.; VAN ASSCHE, G.; AGOSTINIS, P.; RUTGEERTS, P.; VERMEIRE, S.; CLEYNEN, I. Genetic association and functional role of Crohn disease risk alleles involved in microbial sensing, autophagy, and endoplasmic reticulum (ER) stress. **Autophagy**, v. 9, n. 12, P. 2046-2055, 2013.

HOLICK, M. F. The vitamin D deficiency pandemic: Approaches for diagnosis, treatment and prevention. **Reviews in Endocrine and Metabolic Disorders**, v. 18, p. 153-165, 2017.

HOLICK, M. F.; BINKLEY, N. C.; BISCHOFF-FERRARI, H. A.; GORDON, C. M.; HANLEY, D. A.; HEANEY, R. P.; MURAD, M. H.; WEAVER, C. M. Evaluation, treatment, and prevention of vitamin D deficiency: An endocrine society clinical practice guideline. **The Journal of Clinical Endocrinology & Metabolism**, v. 96, n. 7, p. 1911-1930, 2011.

HOOPER, L. V.; LITTMAN, D. R.; Macpherson, A. J. Interactions between the microbiota and the immune system. **Science**, v. 336, n. 6086, p. 1268-1273, 2012.

IMAM, T.; PARK, S.; KAPLAN, M. H.; OLSON, M. R. Effector T helper cell subsets in inflammatory bowel diseases. **Frontiers in Immunology**, v. 9, p. 1212, 2018.

INSTITUTE OF MEDICINE. **Dietary Reference Intakes for Calcium and Vitamin D**. Washington DC: National Academies Press, 2011.

JAKOBSSON, H. E.; ABRAHAMSSON, T. R.; JENMALM, M. C.; HARRIS, K.; QUINCE, C.; JERNBERG, C.; BJÖRKSTÉN, B.; ENGSTRAND, L.; ANDERSSON, A. F. Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by Caesarean section. **Gut**, v. 63, n. 4, p. 559-566, 2014.

JIN D.; ZHANG, Y. G.; WU, S.; LU, R.; LIN, Z.; ZHENG, Y.; CHEN, H.; CS-SZABO, G.; SUN, J. Vitamin D receptor is a novel transcriptional regulator for Axin1. **The Journal of Steroid Biochemistry and Molecular Biology**, v. 165, p. 430-437, 2017.

JOHANSSON, M. E. V.; GUSTAFSSON, J. K.; HOLMEN-LARSSON, J.; JABBAR, K. S.; XIA, L.; XU, H.; GHISHAN, F. K.; CARVALHO, F. A.; GEWIRTZ, A. T.; SJOVALL, H.; HANSSON, G. C. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. **Gut**, v. 63, n. 2, p. 281-291, 2014.

JOSTINS, L.; RIPKE, S.; WEERSMA, R. K.; DUERR, R. H.; MCGOVERN, D. P.; HUI, K. Y.; LEE, J. C.; PHILIP SCHUMM, L.; SHARMA, Y.; ANDERSON, C. A.; ESSERS, J.; MITROVIC, M.; NING, K.; CLEYNENM I.; THEATRE, E.; SPAIN, S. L.; RAYCHAUDHURI, S.; GOYETTE, P.; WEI, Z.; ABRAHAM, C.; ACHKAR, J. P.; AHMAD, T.; AMININEJAD, L.; ANANTHAKRISHNAN, A. N.; ANDERSEN, V.; ANDREWS, J. M.; BAIDOO, L.; BALSCHUN, T.; BAMPTON, P. A.; BITTON, A.; BOUCHER, G.; BRAND, S.; BÜNING, C.; COHAIN, A.; CICHON, S.; D'AMATO, M.; DE JONG, D.; DEVANEY, K. L.; DUBINSKY, M.; EDWARDS, C.; ELLINGHAUS, D.; FERGUSON, L. R.; FRANCHIMONT, D.; FRANSEN, K.; GEARRY, R.; GEORGES, M.; GIEGER, C.; GLAS, J.; HARITUNIANS, T.; HART, A.; HAWKEY, C.; HEDL, M.; HU, X.; KARLSEN, T. H.; KUPCINSKAS, L.; KUGATHASAN, S.; LATIANO, A.; LAUKENS, D.; LAWRANCE, I. C.; LEES, C. W.; LOUIS, E.; MAHY, G.; MANSFIELD, J.; MORGAN, A. R.; MOWAT, C.; NEWMAN, W.; PALMIERI, O.; PONSIOEN, C. Y.; POTOCNIK, U.; PRESCOTT, N. J.; REGUEIRO, M.; ROTTER, J. I.; RUSSELL, R. K.; SANDERSON, J. D.; SANS, M.; SATSANGI, J.; SCHREIBER, S.; SIMMS, L. A.; SVENTORAITYTE, J.; TARGAN, S. R.; TAYLOR, K. D.; TREMELLING, M.; VERSPAGET, H. W.; DE VOS, M.; WIJMENGA, C.; WILSON, D. C.; WINKELMANN, J.; XAVIER, R. J.; ZEISSIG, S.; ZHANG, B.; ZHANG, C. K.; ZHAO, H.; INTERNATIONAL IBD GENETICS CONSORTIUM (IIBDGC); SILVERBERG, M. S.; ANNESE, V.; HAKONARSON, H.; BRANT, S. R.; RADFORD-SMITH, G.; MATHEW, C. G.; RIOUX, J. D.; SCHADT, E. E.; DALY, M. J.; FRANKE, A.; PARKES, M.; VERMEIRE, S.; BARRETT, J. C.; CHO, J. H. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. **Nature**, v. 491, n. 7422, p. 119-124, 2012.

KABBANI, T. A.; KOUTROUBAKIS, I. E.; SCHOEN, R. E.; RAMOS-RIVERS, C.; SHAH, N.; SWOGER, J.; REGUEIRO, M.; BARRIE, A.; SCHWARTZ, M.; HASHASH, J. G.; BAIDOO, L.; DUNN, M. A.; BINION, D. G. Association of Vitamin D Level With Clinical Status in Inflammatory Bowel Disease: A 5-Year Longitudinal Study. **American journal of gastroenterology**, v. 111, n. 5, p. 712–719, 2016.

KIM, T. O.; PARK, D. I.; HAN, Y. K.; KANG, K.; PARK, S. G.; PARK, H. R.; YI, J. M. Genome-Wide Analysis of the DNA Methylation Profile Identifies the Fragile Histidine Triad (FHIT) Gene as a New Promising Biomarker of Crohn's Disease. **Journal of Clinical Medicine**, v. 9, n. 5, p. 1338, 2020.

KNOWLES, S. R.; GRAFF, L. A.; WILDING, H.; HEWITT, C.; KEEFER, L.; MIKOCKA-WALUS, A. Quality of Life in Inflammatory Bowel Disease: A Systematic Review and Meta-analyses-Part I. **Inflammatory Bowel Diseases**, v. 24, n. 4, p. 742-751, 2018a.

KNOWLES, S. R.; KEEFER, L.; WILDING, H.; HEWITT, C.; GRAFF, L. A.; MIKOCKA-WALUS, A. Quality of Life in Inflammatory Bowel Disease: A Systematic Review and Meta-analyses-Part II. **Inflammatory Bowel Diseases**, v. 24, n. 5, p. 966-976, 2018b.

KOUZARIDES, T. Chromatin Modifications and Their Function. **Cell**, v.128, n. 4, p. 693-705, 2007.

LAMBERG-ALLARDT, C. Vitamin D in foods and as supplements. **Progress in Biophysics and Molecular Biology**, v. 92, n. 1, p. 33-38, 2006.

LEE, E. S.; SONG, E. J.; NAM, Y. D.; LEE, S. Y. Probiotics in human health and disease: from nutribiotics to pharmabiotics. **Journal of Microbiology**, v. 56, p. 773-782, 2018.

LEVINE, A.; SIGALL BONEH, R.; WINE, E. Evolving role of diet in the pathogenesis and treatment of inflammatory bowel diseases. **Gut**, v. 67, n. 9, p. 1726-1738, 2018.

LIM, H. S.; KIM, S. K.; HONG, S. J. Food Elimination Diet and Nutritional Deficiency in Patients with Inflammatory Bowel Disease. **Clinical Nutrition Research**, v. 7, n. 1, p. 48-55, 2018.

LIM, W. C.; HANAUER, S. B.; LI, Y. C. Mechanisms of disease: Vitamin D and inflammatory bowel disease. **Nature Clinical Practice Gastroenterology & Hepatology**, v. 2, p. 308-315, 2005.

LIU, F. H.; Li, S. S.; Li, X. X.; WANG, S.; LI, M. G.; GUAN, L.; LUAN, T. G.; LIU, Z. G.; LIU, Z. J.; YANG, P. C. Vitamin D3 induces vitamin D receptor and HDAC11 binding to relieve the promoter of the tight junction proteins. **Oncotarget**, v. 8, n. 35, p. 58781-58789, 2017.

LIU, W.; CHEN, Y.; GOLAN, M. A.; ANNUNZIATA, M. L.; DU, J.; DOUGHERTY, U.; KONG, J.; MUSH, M.; HUANG, Y.; PEKOW, J.; ZHENG, C.; BISSONNETTE, M.; HANAUER, S. B.; LI, Y. C. Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. **The Journal of clinical investigation**, v. 123, n. 9, p. 3983–3996, 2013.

LIU, Y.; XIONG, L.; ZHOU, Y.; ZHENG, B.; LIU, T.; XIE, W. Association of Three Polymorphisms rs11614913, rs2910146, and rs3746444 in miRNA-196a2, miRNA-146a, and miRNA-499 with Inflammatory Bowel Disease: A systematic review and meta-analysis. **Gastroenterology Research and Practice**, v. 2018, p. 7295131, 2018.

LU, R.; SHANG, M.; ZHANG, Y. G.; JIAO, Y.; XIA, Y.; GARRETT, S.; BAKKE, D.; BÄUERL, C.; MARTINEZ, G. P.; KIM, C. H.; KANG, S. M.; SUN, J. Lactic Acid Bacteria Isolated From Korean Kimchi Activate the Vitamin D Receptor-autophagy Signaling Pathways. **Inflammatory Bowel Diseases**, v. 26, n. 8, p. 1199-1211, 2020.

LU, R.; ZHANG, Y. G.; XIA, Y.; SUN, J. Imbalance of autophagy and apoptosis in intestinal epithelium lacking the vitamin D receptor. **The FASEB Journal**, v. 33, n. 11, p. 11845-11856, 2019.

LUTHOLD, R. V.; FERNANDES, G. R.; FRANCO-DE-MORAES, A. C.; FOLCHETTI, L. G. D.; FERREIRA, S. R. G. Gut microbiota interactions with the immunomodulatory role of vitamin D in normal individuals. **Metabolism**, v. 69, p. 76-86, 2017.

MACMASTER, M. J.; DAMIANOPOULOU, S.; THOMSON, C.; TALWAR, D.; STEFANOWICZ, F.; CATCHPOLE, A.; GERASIMIDIS, K.; GAYA, D. R. A prospective analysis of micronutrient status in quiescent inflammatory bowel disease. **Clinical Nutrition**, 2020. In press. doi: 10.1016/j.clnu.2020.05.010.

MASSIRONI, S.; ROSSI, R. E.; CAVALCOLI, F. A.; DELLA VALLE, S.; FRAQUELLI, M.; CONTE, D. Nutritional deficiencies in inflammatory bowel disease: Therapeutic approaches. **Clinical Nutrition**, v. 32, n. 6, p. 904-910, 2013.

MECHIE, N. C.; MAVROPOULOU, E.; ELLENRIEDER, V.; PETZOLD, G.; KUNSCH, S.; NEESSE, A.; AMANZADA, A. Serum vitamin D but not zinc levels are associated with different disease activity status in patients with inflammatory bowel disease. **Medicine**, v. 98, n. 15, p. e15172, 2019.

MEINZER, U.; HUGOT, J. P. Nod2 and Crohn's disease: Many connected highways. **The Lancet**, v. 368, n. 9473, p. 1752-1754, 2005.

MENTELLA, M. C.; SCALDAFERRI, F.; PIZZOFERRATO, M.; GASBARRINI, A.; MIGGIANO, G. A. D. The association of disease activity, BMI and phase angle with vitamin D deficiency in patients with IBD. **Nutrients**, v. 11, n. 11, p. 2583, 2019.

MIQUEL, S.; MARTÍN, R.; ROSSI, O.; BERMÚDEZ-HUMARÁN, L. G.; CHATEL, J. M.; SOKOL, H.; THOMAS, M.; WELLS, J. M.; LANGELLA, P. *Faecalibacterium prausnitzii* and human intestinal health. **Current Opinion in Microbiology**, v. 16, n. 3, p. 255-261, 2013.

MYINT, A.; SAUK, J. S.; LIMKETKAI, B. N. The role of vitamin D in inflammatory bowel disease: a guide for clinical practice. **Expert Review of Gastroenterology & Hepatology**, v. 14, n. 7, p. 539-552, 2020.

NI, J.; WU, G. D.; ALBENBERG, L.; TOMOV, V. T. Gut microbiota and IBD: Causation or correlation? **Nature Reviews Gastroenterology & Hepatology**, v. 14, p. 573-584, 2017.

OUELLETTE, A. J.; SATCHELL, D. P.; HSIEH, M. M.; HAGEN, S. J.; SELSTED, M. E. Characterization of luminal paneth cell alpha-defensins in mouse small intestine. Attenuated antimicrobial activities of peptides with truncated amino termini. **The Journal of Biolical Chemistry**, v. 275, n. 43, p. 33969-33973, 2000.

PAPPA, H. M.; MITCHELL, P. D.; JIANG, H.; KASSIFF, S.; FILIP-DHIMA, R.; DIFABIO, D.; QUINN, N.; LAWTON, R. C.; VARVARIS, M.; VAN STRAATEN, S.; GORDON, C. M. Treatment of vitamin D insufficiency in children and adolescents with inflammatory bowel disease: A randomized clinical trial comparing three regimens. **The Journal of Clinical Endocrinology and Metabolism**, v. 97, n. 6, p. 2134-2142, 2012.

PIOVANI, D.; DANESE, S.; PEYRIN-BIROULET, L.; NIKOLOPOULOS, G. K.; LYTRAS, T.; BONOVAS, S. Environmental Risk Factors for Inflammatory Bowel Diseases: An Umbrella Review of Meta-analyses. **Gastroenterology**, v. 157, n. 3, p. 647-659, 2019.

PRAGER, M.; BÜTTNER, J.; HAAS, V.; BAUMGART, D. C.; STURM, A.; ZEITZ, M.; BÜNING, C. The JAK2 variant rs10758669 in Crohn's disease: Altering the intestinal barrier as one mechanism of action. **International Journal of Colorectal Disease**, v. 27, n. 5, p. 565-573, 2012.

QUEZADA, S. M.; MCLEAN, L. P.; CROSS, R. K. Adverse events in IBD therapy: the 2018 update. **Expert Review of Gastroenterology & Hepatology**, v. 12, n. 12, p. 1183-1191, 2018.

RADFORD-SMITH, G.; PANDEYA, N. Associations between NOD2/CARD15 genotype and phenotype in Crohn's disease-Are we there yet? **World Journal of Gastroenterology**, v. 12, n. 44, p. 7097-7103, 2006.

ROCHA, R.; SOUSA, U. H.; REIS, T. L. M.; SANTANA, G. O. Nutritional status as a predictor of hospitalization in inflammatory bowel disease: A review. **World Journal of Gastrointestinal Pharmacology and Therapeutics**, v. 10, n. 2, p. 50-56, 2019.

RODRÍGUEZ-NOGALES, A.; ALGIERI, F.; GARRIDO-MESA, J.; VEZZA, T.; UTRILLA, M. P.; CHUECA, N.; GARCÍA, F.; RODRÍGUEZ-CABEZAS, M. E.; GÁLVEZ, J. Intestinal anti-inflammatory effect of the probiotic *Saccharomyces boulardii* in DSS-induced colitis in mice: Impact on microRNAs expression and gut microbiota composition. **The Journal of Nutritional Biochemistry**, v. 61, p. 129-139, 2018.

ROGLER, G. Gastrointestinal and liver adverse effects of drugs used for treating IBD. **Best Practice & Research Clinical Gastroenterology**, v. 24, n. 2, p. 157-165, 2010.

ROSEN, M. J.; KARNS, R.; VALLANCE, J. E.; BEZOLD, R.; WADDELL, A.; COLLINS, M. H.; HABERMAN, Y.; MINAR, P.; BALDASSANO, R. N.; HYAMS, J. S.; BAKER, S. S.; KELLERMAYER, R.; NOE, J. D.; GRIFFITHS, A. M.; ROSH, J. R.; CRANDALL, W. V.; HEYMAN, M. B.; MACK, D. R.; KAPPELMAN, M. D.; MARKOWITZ, J.; MOULTON, D. E.; LELEIKO, N. S.; WALTERS, T. D.; KUGATHASAN, S.; WILSON, K. T.; HOGAN, S. P.; DENSON, L. A. Mucosal Expression of Type 2 and Type 17 Immune Response Genes Distinguishes Ulcerative Colitis From Colon-Only Crohn's Disease in Treatment-Naive Pediatric Patients. **Gastroenterology**, v. 152, n. 6, p. 1345-1357, 2017.

RYAN, F. J.; AHERN, A. M.; FITZGERALD, R. S.; LASERNA-MENDIETA, E. J.; POWER, E. M.; CLOONEY, A. G.; O'DONOGHUE, K. W.; MCMURDIE, P. J.; IWAI, S.; CRITS-CHRISTOPH, A.; SHEEHAN, D.; MORAN, C.; FLEMER, B.; ZOMER, A. L.; FANNING, A.; O'CALLAGHAN, J.; WALTON, J.; TEMKO, A.; STACK, W.; JACKSON, L.; JOYCE, S. A.; MELGAR, S.; DESANTIS, T. Z.; BELL, J. T.; SHANAHAN, F.; CLAESSON, M. J. Colonic microbiota is associated with inflammation and host epigenomic alterations in inflammatory bowel disease. **Nature Communications**, v. 11, p. 1512, 2020.

SALVUCCI, E. The human-microbiome superorganism and its modulation to restore health. **International Journal of Food Science and Nutrition**, v. 70, n. 7, p. 781-795, 2019.

SALZMAN, N. H.; HUNG, K.; HARIBHAI, D.; CHU, H.; KARLSSON-SJÖBERG, J.; AMIR, E.; TEGGATZ, P.; BARMAN, M.; HAYWARD, M.; EASTWOOD, D.; STOEL, M.; ZHOU, Y.; SODERGREN, E.; WEINSTOCK, G. M.; BEVINS, C. L.; WILLIAMS, C. B.; BOS, N. A. Enteric defensins are essential regulators of intestinal microbial ecology. **Nature Immunology**, v. 11, p. 76-82, 2010.

SARTOR, R. B. Efficacy of probiotics for the management of inflammatory bowel disease. **Gastroenterology & Hepatology**, v. 7, n. 9, p. 606-608, 2011.

SCALDAFERRI, F.; PIZZOFERRATO, M.; LOPETUSO, L. R.; MUSCA, T.; INGRAVALLE, F.; SICIGNANO, L. L.; MENTELLA, M.; MIGGIANO, G.; MELE, M.C.; GAETANI, E.; GRAZIANI, C.; PETITO, V.; CAMMAROTA, G.; MARZETTI, E.; MARTONE, A.; LANDI, F.; GASBARRINI, A. Nutrition and IBD: Malnutrition and/or Sarcopenia? A Practical Guide. **Gastroenterology Research and Practice**, v. 2017, p. 8646495, 2017.

SCHÄFFLER, H.; HERLEMANN, D. P.; KLINITZKE, P.; BERLIN, P.; KREIKEMEYER, B.; JASTER, R.; LAMPRECHT, G. Vitamin D administration leads to a shift of the intestinal bacterial composition in Crohn's disease patients, but not in healthy controls. **Journal of digestive diseases**, v. 19, n. 4, p. 225-234, 2018.

SCHIRMER, M.; GARNER, A.; VLAMAKIS, H.; XAVIER, R. J. Microbial genes and pathways in inflammatory bowel disease. **Nature Reviews Microbiology**, v. 17, p. 497-511, 2019.

SHARIFI, A.; HOSSEINZADEH-ATTAR, M. J.; VAHEDI, H.; NEDJAT, S. A randomized controlled trial on the effect of vitamin D3 on inflammation and cathelicidin gene expression in ulcerative colitis patients. **Saudi Journal of Gastroenterology: Official Journal of The Saudi Gastroenterology Association**, v. 22, n. 4, p. 316-323, 2016.

SHARIFI, A.; VAHEDI, H.; NEDJAT, S.; RAFIEI, H.; HOSSEINZADEH-ATTAR, M. J. Effect of single-dose injection of vitamin D on immune cytokines in ulcerative colitis patients: a randomized placebo-controlled trial. **APMIS**, v. 127, n. 10, p. 681-687, 2019. 2019;127(10):681–7. doi: 10.1111/apm.12982.

SHENG, Y. H.; TRIYANA, S.; WANG, R.; DAS, I.; GERLOFF, K.; FLORIN, T. H.; SUTTON, P.; MCGUCKIN, M. A. MUC1 and MUC13 differentially regulate epithelial inflammation in response to inflammatory and infectious stimuli. **Mucosal Immunology**, v. 6, n. 3, p. 557-568, 2013.

SIMEK, R. Z.; PRINCE, J.; SYED, S.; SAUER, C. G.; MARTINEAU, B.; HOFMEKLER, T.; FREEMAN, A. J.; KUMAR, A.; MCELHANON, B. O.; SCHOEN, B, T.; TENJARLA, G.; MCCRACKEN, C.; ZIEGLER, T. R.; TANGPRICHA, V.; KUGATHASAN, S. Pilot study evaluating efficacy of 2 regimens for hypovitaminosis D repletion in pediatric inflammatory bowel disease. **Journal of Pediatric Gastroenterology and Nutrition**, v. 62, n. 2, p. 252-258, 2016.

SINGH, U. P.; MURPHY, A. E.; ENOS, R. T.; SHAMRAN, H. A.; SINGH, N. P.; GUAN, H.; HEGDE, V. L.; FAN, D.; PRICE, R. L.; TAUB, D. D.; MISHRA, M. K.; NAGARKATTI, M.; NAGARKATTI, P.S. miR-155 deficiency protects mice from experimental colitis by reducing T helper type 1/type 17 responses. **Immunology**, v. 143, n. 3, p. 478-489, 2014.

SITKIN, S.; POKROTNIEKS, J. Clinical Potential of Anti-inflammatory Effects of *Faecalibacterium prausnitzii* and Butyrate in Inflammatory Bowel Disease. **Inflammatory Bowel Diseases**, v. 25, n. 4, p. e40-41, 2019.

SOLTYS, K.; STUCHLIKOVA, M.; HLAVATY, T.; GAALOVA, B.; BUDIS, J.; GAZDARICA, J.; KRAJCOVICOVA, A.; ZELINKOVA, Z.; SZEMES, T.; KUBA, D.; DRAHOVSKA, H.; TURNA, J.; STUCHLIK, S. Seasonal changes of circulating 25-hydroxyvitamin D correlate with the lower gut microbiome composition in inflammatory bowel disease patients. **Scientific Reports**, v. 10, p. 6024, 2020.

SOUSA GUERREIRO, C.; CRAVO, M.; COSTA, A. R.; MIRANDA, A.; TAVARES, L.; MOURA-SANTOS, P.; MARQUES VIDAL, P.; NOBRE LEITÃO, C. A comprehensive approach to evaluate nutritional status in Crohn's patients in the era of biologic therapy: A case-control study. **The American Journal of Gastroenterology**, v. 102, p. 2551-2556, 2007.

STANGE, E. F.; SCHROEDER, B. O. Microbiota and mucosal defense in IBD: an update. **Expert Review of Gastroenterology & Hepatology**, v. 13, n. 10, p. 963-976, 2019.

STROBER, W.; WATANABE, T. NOD2, an intracellular innate immune sensor involved in host defense and Crohn's disease. **Mucosal Immunology**, v.4, n. 5, p. 484-495, 2011.

SUN, K.; XIE, C.; XU, D.; YANG, X.; TANG, J.; JI, X. *Lactobacillus* isolates from healthy volunteers exert immunomodulatory effects on activated peripheral blood mononuclear cells. **Journal of Biomedical Research**, v. 27, n. 2, p. 116-126, 2013.

SZODORAY, P.; NAKKEN, B.; GAAL, J.; JONSSON, R.; SZEGEDI, A.; ZOLD, E.; SZEGEDI, G.; BRUN, J. G.; GESZTELYI, R.; ZEHER, M.; BODOLAY, E. The complex role of vitamin D in autoimmune diseases. **Scandinavian Journal of Immunology**, v. 68, n. 3, p. 261-269, 2008.

TATIYA-APHIRADEE, N.; CHATUPHONPRASERT, W.; JARUKAMJORN, K. Immune response and inflammatory pathway of ulcerative colitis. **Journal of Basic and Clinical Physiology and Pharmacolgy**, v. 30, n. 1, p. 1-10, 2019.

THIBAULT, F.; CANCEL-TASSIN, G.; CUSSENOT, O. Low penetrance genetic susceptibility to kidney cancer. **BJU International**, v. 98, n. 4, p. 735-738, 2006.

THURSBY, E.; JUGE, N. Introduction to the human gut microbiota. **Biochemical Journal**, v. 474, n. 11, p. 1823-1836, 2017.

TIAN, R.; WANG, R. L.; XIE, H.; JIN, W.; YU, K. L. Overexpressed miRNA-155 dysregulates intestinal epithelial apical junctional complex in severe acute pancreatitis. **World Journal of Gastroenterology**, v. 19, n. 45, p. 8282-8291, 2013.

TORRES, J.; MEHANDRU, S.; COLOMBEL, J. F.; PEYRIN-BIROULET, L. Crohn's disease. **The Lancet**, v. 389, n. 10080, p. 1741-1755, 2017.

TURNER, J. R. Intestinal mucosal barrier function in health and disease. **Nature Reviews Immunology**, v. 9, p. 799-809, 2009.

UNGARO, R.; MEHANDRU, S.; ALLEN, P. B.; PEYRIN-BIROULET, L.; COLOMBEL, J. F. Ulcerative colitis. **The Lancet**, v. 389, n. 10080, p. 1756-1770, 2017.

VELDMAN, C. M.; CANTORNA, M. T.; DELUCA, H. F. Expression of 1,25-dihydroxyvitamin D3 receptor in the immune system. **Archives of Biochemistry and Biophysics**, v. 374, n. 2, p. 334-338, 2000.

WANG, J.; THINGHOLM, L. B.; SKIECEVIČIENĖ, J.; RAUSCH, P.; KUMMEN, M.; HOV, J. R.; DEGENHARDT, F.; HEINSEN, F. A.; RÜHLEMANN, M. C.; SZYMCZAK, S.; HOLM, K.; ESKO, T.; SUN, J.; PRICOP-JECKSTADT, M.; AL-DURY, S.; BOHOV, P.; BETHUNE, J.; SOMMER, F.; ELLINGHAUS, D.; BERGE, R. K.; HÜBENTHAL, M.; KOCH, M.; SCHWARZ, K.; RIMBACH, G.; HÜBBE, P.; PAN, W. H.; SHEIBANI-TEZERJI, R.; HÄSLER, R.; ROSENSTIEL, P.; D'AMATO, M.; CLOPPENBORG-SCHMIDT, K.; KÜNZEL, S.; LAUDES, M.; MARSCHALL, H. U.; LIEB, W.; NÖTHLINGS, U.; KARLSEN, T. H.; BAINES, J. F.; FRANKE, A. Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. **Nature Genetics**, v. 48, p. 1396-1406, 2016.

WU, S.; ZHANG, Y.; LU, R.; XIA, Y.; ZHOU, D.; PETROF, E. O.; CLAUD, E. C.; CHEN, D.; CHANG, E. B.; CARMELIET, G.; SUN, J. Intestinal epithelial vitamin D receptor deletion leads to defective autophagy in colitis. **Gut**, v.64, n. 7, p. 1082–1094, 2015.

WYATT, J.; VOGELSANG, H.; HÜBL, W.; WALDHOER, T.; LOCHS, H. Intestinal permeability and the prediction of relapse in Crohri's disease. **The Lancet**, v. 341, n. 8858, p. 1437-1439, 1993.

YE, D.; GUO, S.; AL-SADI, R.; MA, T. Y. MicroRNA regulation of intestinal epithelial tight junction permeability. **Gastroenterology**, v. 141, n. 4, p. 1323-1333, 2011.

ZENG, Z.; MUKHERJEE, A.; ZHANG, H. From genetics to epigenetics, roles of epigenetics in inflammatory bowel disease. **Frontiers in Genetics**, v. 10, p. 1017, 2019.

ZHANG, Y. G.; LU, R.; WU, S.; CHATTERJEE, I.; ZHOU, D.; XIA, Y.; SUN, J. Vitamin D Receptor Protects Against Dysbiosis and Tumorigenesis via the JAK/STAT Pathway in Intestine. **Cellular and Molecular Gastroenterology and Hepatology**, v. 10, n. 4, p. 729-746, 2020.

ZHANG, Y. G.; LU, R.; XIA, Y.; ZHOU, D.; PETROF, E.; CLAUD, E. C.; SUN, J. Lack of Vitamin D receptor leads to hyperfunction of claudin-2 in intestinal inflammatory responses. **Inflammatory Bowel Diseases**, v. 25, n. 1, p. 97-110, 2019.

ZHANG, Y. G.; LU, R.; XIA, Y.; ZHOU, Z. PETROF, E. O.; CLAUD, E. C.; SUN, J. Lack of Intestinal VDR Regulation Leads to Dysfunction of Claudin-2 in Inflammatory Responses. **Gastroenterology**, v. 152, n. 5, p. S413, 2017.

ZHANG, Y. G.; WU, S.; LU, R.; ZHOU, D.; ZHOU, J.; CARMELIET, G.; PETROF, E.; CLAUD, E. C.; SUN, J. Tight junction CLDN2 gene is a direct target of the vitamin D receptor. **Scientific Reports**, v. 5, p.10642, 2015.

ZHANG, Y. Z.; LI, Y. Y. Inflammatory bowel disease: Pathogenesis. World Journal of Gastroenterology, v. 20, n. 1, p. 91-99, 2014.

ZHOU, L.; ZHANG, M.; WANG, Y.; DORFMAN, R. G.; LIU, H.; YU, T.; CHEN, X.; TANG, D.; XU, L.; YIN, Y.; PAN, Y.; ZHOU, Q.; ZHOU, Y.; YU, C. *Faecalibacterium prausnitzii* Produces Butyrate to Maintain Th17/Treg Balance and to Ameliorate Colorectal Colitis by Inhibiting Histone Deacetylase 1. Inflammatory Bowel Diseases, v. 24, n. 9, p. 1926-1940, 2018.

ZHU, W.; YAN, J.; ZHI, C.; ZHOU, Q.; YUAN, X. 1,25$(OH)_2D_3$ deficiency-induced gut microbial dysbiosis degrades the colonic mucus barrier in *Cyp27b1* knockout mouse model. Gut Pathogens, v. 11, p. 8, 2019.

CHAPTER 3

Brewer's spent grain enhanced the recovery of potentially probiotic strains in fermented milk after exposure to in vitro-simulated gastrointestinal conditions

Abstract

In beer manufacturing, the by-product brewer's spent grain (BSG) is generated, which is rich in fiber and other compounds with interesting nutritional and functional properties. The fermentability of BSG by ten probiotics and two starter cultures was evaluated to select a suitable combination of one probiotic and one starter strain for the application in fermented milk (FM). The co-culture chosen for the development of the probiotic FM was composed of *Lactobacillus paracasei* subsp. *paracasei* F19 and *Streptococcus thermophilus* TH-4. Four formulations were studied, using *Streptococcus thermophilus* TH-4 as starter culture: FM1 (control; without probiotic culture and BSG); FM2 (only with BSG); FM3 (with probiotic culture and without BSG); FM4 (with probiotic culture and BSG). The viability of the microorganisms in the FM throughout 28 days and their resistance to *in vitro*-simulated gastrointestinal (GI) stress was monitored. The BSG did not influence the fermentation kinetics or the populations of both microorganisms in the FM during storage. Nevertheless, the addition of BSG and/or the co-culture with F19 improved the survival of TH-4 against GI stress simulated *in vitro*. All the formulations evaluated have potential as probiotic fermented beverages, since total probiotic populations were always above 10^{10} CFU in a daily portion of 200 mL of FM, and a minimum of 10^{10} and 10^{8} CFU equivalent of, respectively, *St. thermophilus* TH-4 and *Lb. paracasei* subsp. *paracasei* F19 was recovered after the GI stress. Therefore, *St. thermophilus* TH-4 has potential as a probiotic strain in addition to its starter feature, while BSG may be employed as a possible prebiotic ingredient for food application. Nonetheless, *in vivo* and clinical trials to confirm the health benefits of the products developed herein are needed.

Key words: probiotic; BSG; fermented milk; *in vitro* gastrointestinal resistance; prebiotic; synbiotic.

1 INTRODUCTION

Brazil is the third-largest beer producer worldwide, and this market is responsible for 2% of the GDP (gross domestic product) and 14% of the national industry, generating around 2.7 million of jobs and contributing to the economy with R$ 25 billion per year in taxes (CERVBRASIL, 2014; SINDICERV, 2020).

The beer production involves some processes, which comprise the malting step when the enzymatic content of the barley grains is increased, and these enzymes are activated. Next, the grains are dried, generating flavor components. Afterward, in the mashing step, the malted barley is milled and mixed with water, and the temperature is slowly increased, converting the starch in fermentable sugars, which will serve as a substrate for yeast fermentation and alcohol production in beer. Finally, the wort obtained is filtered, generating the by-product brewer's spent grain (BSG) (FĂRCAŞ et al., 2015; MUSSATO; DRAGONE; ROBERTO, 2006; STOJCESKA, 2019).

Studies have shown that 100 liters of beer produced generate approximately 20 kg of BSG. In 2016, more than 13 billion liters of beer were produced in Brazil, thus a great amount of by-product was generated, which might be an environmental problem (CERVBRASIL, 2014; SINDICERV, 2020). Moreover, the BSG has around 70% of fibers, mainly arabinoxylans, lignin, and cellulose, is rich in protein, contains linoleic, palmitic, and oleic fatty acids, and phenolic compounds like flavonoids, having antioxidant activity. Thus, BSG may have potential as a prebiotic ingredient, and novel applications of this by-product are of utmost interest for the development of functional foods with more added value (FĂRCAŞ et al., 2015; GIBSON et al., 2017; MUSSATO; DRAGONE; ROBERTO, 2006; ROBERTSON et al., 2010; SAJIB et al., 2018; STOJCESKA, 2019).

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (HILL et al., 2014). Among the various mechanisms of action attributed to probiotic microorganisms, stands out the capacity of gut microbiota modulation, barrier function, competition for nutrients and for adhesion sites, production of short-chain fatty acids (SCFA), organic acids, certain vitamins, bacteriocins, and anti-inflammatory compounds, and improvement of the immune response (HILL et al., 2014; SANDERS et al., 2019). In addition, the survival of probiotics during the food production and storage processes and, also, through the gastrointestinal tract (GIT) stress

(resistance to acid environment, bile, and enzymes) are extremely important factors for action and efficacy of probiotics (GIBSON, 2004; RANADHEERA; BAINES; ADAMS, 2010).

Besides the direct administration of probiotics, another way to raise the population of beneficial bacteria in the intestinal microbiota is through the intake of prebiotics. Since 2017, prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (GIBSON et al., 2017). The prebiotic ingredients with so far approved claims were lactulose, inulin, fructooligosaccharides (FOS), and *trans*-galactooligosaccharides (TOS). However, with the new definition, other compounds, such as insoluble fibers, polyphenols, and polyunsaturated fatty acids, may be considered as prebiotics if the beneficial effect to host health is proven (GIBSON et al., 2010; GIBSON et al., 2017; MARTINEZ; BEDANI; SAAD, 2015; SANDERS et al., 2019).

Furthermore, a synbiotic is defined as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (SWANSON et al., 2020). The combination of prebiotic and probiotic results in the "complementary synbiotic", which may confer a better adaptation of the microorganism in the food matrix, besides a possible competitive advantage of the probiotics over the intestinal microbiota (MARTINEZ; BEDANI; SAAD, 2015, SANDERS et al., 2019, SWANSON et al., 2020). In addition, dairy products have shown to be excellent food matrixes for the development of probiotic foods, from the technological point of view, as well as the consumers' acceptability (SANDERS & MARCO, 2010). Therefore, the present study aimed to evaluate the potential of BSG to improve the recovery of potentially probiotic strains in fermented milk after the exposure to *in vitro*-simulated gastrointestinal (GI) conditions.

2 MATERIAL AND METHODS

2.1 Production of BSG flour

For the BSG flour manufacturing, the barley beer by-product provided by the local craft brewery *Cervejaria Nacional* (São Paulo, SP, Brazil), was blanched in boiling water for 12 minutes, and then oven-dried (FABBE, São Paulo, Brazil) at 60 °C for 24 hours. Afterward, the dried BSG was milled and sieved to obtain a flour with particle size ≤ 0.42 mm (BEDANI; ROSSI; SAAD, 2013; SANTOS et al., 2003). The BSG flour was stored at -18 °C.

2.2 Evaluation of the fermentability of BSG flour by probiotic and starter cultures

An *in vitro* fermentation assay was carried out to evaluate the effect of BSG flour on the growth of probiotic and starter strains from Christian Hansen (Table 1) in culture media and in UHT fat-free milk.

Table 1: Probiotic and starter strains tested, and culture media and incubation conditions (37 °C) employed for each culture.

Group	Strain	Code	Agar	Incubation condition
Probiotic	*Bifidobacterium animalis* subsp. *lactis* BB-12	BB-12	LP-MRS[1]	Anaerobic[6]
	Bifidobacterium longum BB-46	BB-46		
	Bifidobacterium longum subsp. *infantis* BB-02	BB-02		
	Lactobacillus acidophilus LA-5	LA-5	mMRS with maltose[2]	Aerobic
	Lactobacillus fermentum PCC	PCC	MRS[3]	
	Lactobacillus reuteri RC-14	RC-14		
	Lactobacillus paracasei subsp. *paracasei L.casei* 431	431	acidified MRS (pH 5.4)[4]	Anaerobic[6]
	Lactobacillus paracasei subsp. *paracasei* F19	F19		
	Lactobacillus rhamnosus GR-1	GR-1		Aerobic
	Lactobacillus rhamnosus LGG	LGG		
Starter	*Streptococcus thermophilus* TH-4	TH-4	M17[5]	Aerobic
	Streptococcus thermophilus ST-M6	STM		

1 –MRS agar (Oxoid) containing sodium propionate (3 g/L, Sigma-Aldrich, St. Louis, USA) and lithium chloride (2 g/L, Merck, Darmstadt, Germany) (VINDEROLA et al., 2000); 2 – Modified MRS agar, formulated with the replacement of glucose for maltose (IDF, 1995); 3 – MRS agar (Oxoid); 4 – MRS agar (Oxoid) acidified with a 3 M acetic acid solution until pH 5.4 (BURITI; CARDARELLI; SAAD, 2007); 5 - M17 agar with lactose (Oxoid) (RICHTER & VEDAMUTHU, 2001); 6 –AnaeroGen™ Anaerobic System (Oxoid).

Previously, the BSG flour was irradiated at the Nuclear and Energy Research Institute (IPEN, São Paulo, Brazil) with a dose of 25 kGy in the equipment Gammacell 220 (Atomic Energy of Canada Ltd., Ottawa, Canada). The irradiated BSG flour was tested to confirm the absence of any possible contaminating microorganism (ALBUQUERQUE et al., 2016).

Modified phenol red MRS broth (mpr-MRS), prepared according to Buriti et al. (2014), and UHT skimmed milk (MOLICO, Nestlé Brasil Ltda.) were supplemented with 1 g/100 mL of BSG flour, inoculated with each strain separately, and then incubated at 37 °C (ALBUQUERQUE et al., 2016; BURITI et al., 2014). The counts of each microorganism were determined before (0 h) and after 24 h of fermentation using selectively agar and incubation conditions appropriated for each microorganism as described in Table 1. Negative controls were also evaluated for each strain, in which the mpr-MRS and UHT skimmed milk

were inoculated and fermented without supplementation with BSG flour. The experiments were performed in triplicates.

2.3 Production of fermented milk with probiotic co-culture

According to the results from the fermentability assay (item 2.2), one probiotic and one starter strains were selected to produce the probiotic FM based on their ability to ferment the BSG in UHT skimmed milk.

The FM were produced according to Figure 1 and four formulations were evaluated, using *Streptococcus thermophilus* TH-4 as starter culture: FM1 (control; without probiotic culture and BSG); FM2 (with BSG only and no probiotic culture); FM3 (with probiotic culture and without BSG); FM4 (with probiotic culture and BSG).

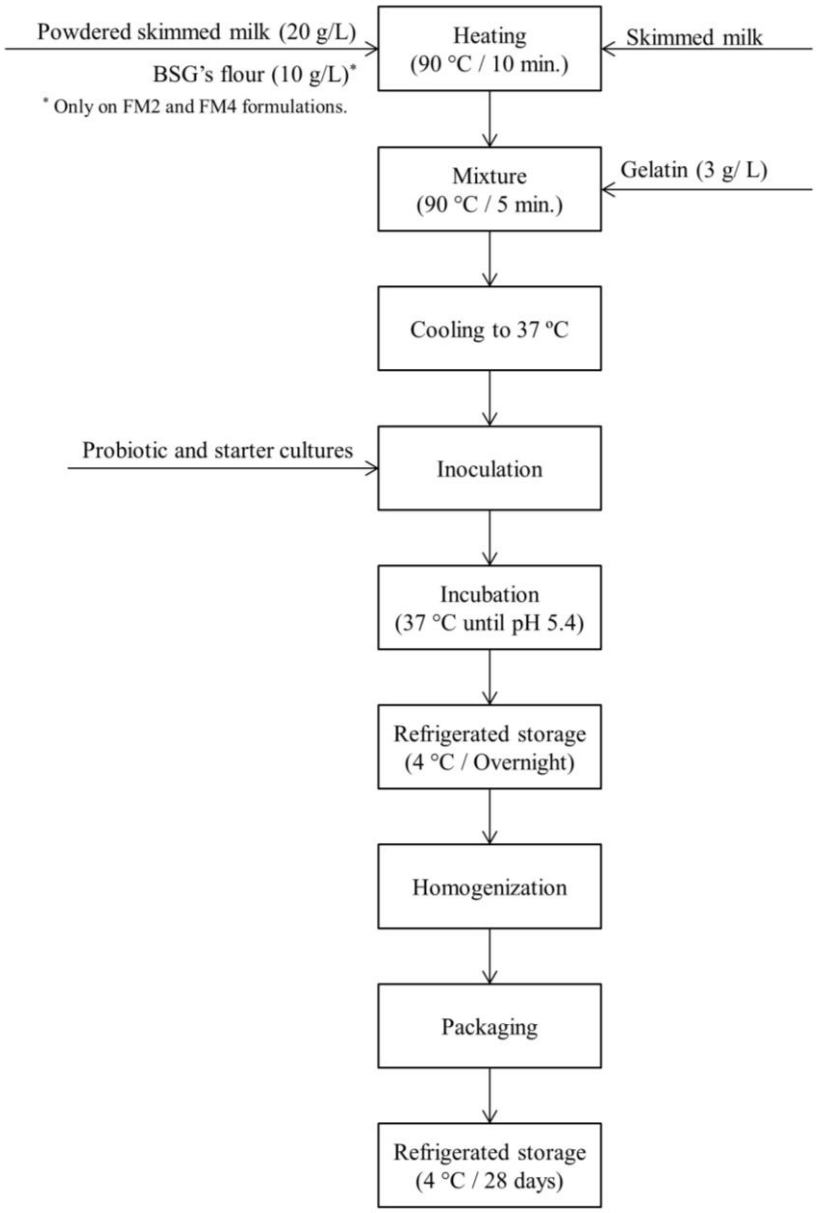

Figure 1: Schematic representation of the fermented milks production steps employed.

In accordance with the Codex *Alimentarius* (JOINT FAO/WHO CODEX ALIMENTARIUS COMMISSION, 2010), FM is a product obtained through the fermentation of milk, when the pH is decreased, but not necessarily reaching the isoelectric point of the casein (pH 4.6 at 25 ºC). For this reason, the fermentation was stopped when the pH reached 5.4, therefore not following the common sense of usually fermenting milk until the pH reaches 4.6. This fermentation endpoint was chosen since the reduction in the pH continues during the cooling and storage processes, which might affect the survival of the microorganisms.

2.4 Characterization of the probiotic fermented milk and BSG

The pH was monitored through an Orion Three Stars equipment (Thermofisher Scientific), using a penetration electrode, model 2A04-GF (Analyser).

The chemical composition and fiber content were determined by the following methods: moisture (BSG flour) and total solid contents (FM) by oven-drying at 70 °C, under vacuum; ashes by incineration in a muffle at 550 °C; protein by micro-Kjeldahl, using the conversion factors 6.38 and 5.83 for the FM and BSG flour, respectively (INSTITUTO ADOLFO LUTZ, 2008); lipids by the Bligh-Dyer method (BLIGH & DYER, 1959); total fiber content of BSG by the enzymatic gravimetric method 991.43 from AOAC (A.O.A.C., 2011), and carbohydrates determined by difference to reach 100% of the chemical composition.

2.5 Viability of *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19 in fermented milk

The viability of the microorganisms in FM was determined after 1, 7, 14, 21, and 28 days of storage at 4 °C.

Samples of the FM were submitted to successive serial decimal dilutions in sterile saline solution (NaCl, 0.85 g/100 mL). For the enumeration of *St. thermophilus* TH-4, aliquots of each dilution were pour plated in M17 agar (Oxoid), and the plates incubated in aerobic conditions during 48 h at 37 °C (RICHTER & VEDAMUTHU, 2001). For the enumeration of *Lb. paracasei* subsp. *paracasei* F19, aliquots of each dilution were pour plated in acidified MRS agar (Oxoid), and the plates incubated in anaerobic conditions (AnaeroGenTM Anaerobic System, Oxoid) during 48 h at 37 °C (BURITI; CARDARELLI; SAAD, 2007).

2.6 Submission of fermented milk to gastrointestinal conditions simulated *in vitro*

The FM were submitted to an *in vitro* assay simulating the passage through the GIT in order to estimate the viable population of probiotic bacteria that may reach the colon and, thus, resulting in the possible health benefits of these microorganisms.

To mimic the GIT conditions, the assay was divided into gastric, enteric I, and enteric II phases, as described previously by Bedani, Rossi & Saad (2013). In each step, appropriated enzymes were added, and the pH adjusted with hydrochloric acid or alkaline solutions, as illustrated in Figure 2 (BEDANI; ROSSI; SAAD, 2013). At the end of all stages, samples were collected to evaluate the viable cell number of the microorganisms employed in the FM by Real-Time quantitative PCR (PADILHA et al., 2016).

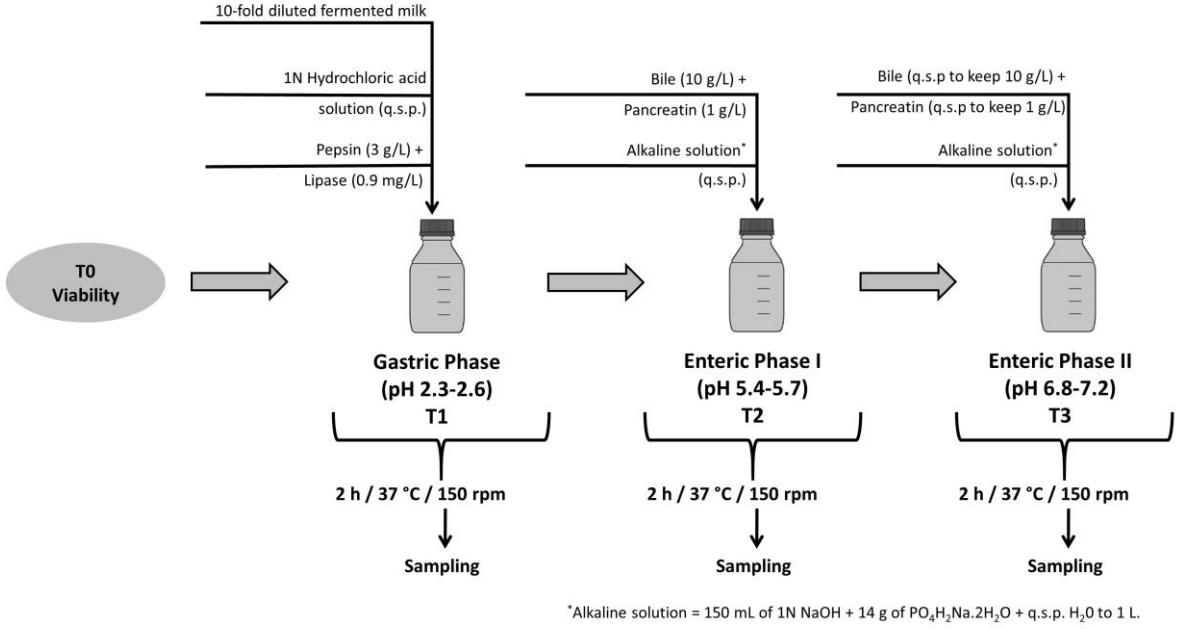

Figure 2: Schematic representation of the *in vitro*-simulated gastrointestinal conditions assay.

2.7 Survival of *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19 under gastrointestinal conditions simulated *in vitro*

After each phase of the GI *in vitro* assay, aliquots of 3 mL were mixed with 27 mL of 2% (w/v) trisodium citrate dihydrate solution and incubated at 45 °C for 30 min. Next, the cell pellets were harvested by centrifugation (9000 *g* / 10 min / 4 °C), washed twice with Tris EDTA (TE) buffer (10mM Tris-HCL, 1 mM EDTA, pH 8), and resuspended in 499 µL of PBS buffer prior to PMA (propidium monoazide) treatment (PADILHA et al., 2016; VILLARREAL, 2013).

To avoid the amplification of the DNA from dead cells, the PMA treatment was performed. The cell suspensions were transferred to a 2 mL light-transparent microcentrifuge tube and homogenized with 1.25 µL of PMA (Biotium) solution (final concentration of 50 mM, as indicated by the manufacturer). After 5 min of dark incubation at 22 °C, the tubes were light-exposed for 15 min using the Glo-Plate™ Blue LED Illuminator (Biotium). The cells pellet were harvested by centrifugation (10000 *g* / 10 min / 4 °C), washed with PBS buffer, resuspended in 500 µL of TE buffer, and transferred to a 2 mL screw-cap tube containing 0.3 g of 0.1 mm zirconia beads and 150 µL of buffer-saturated phenol (Invitrogen) (PADILHA et al., 2016; VILLARREAL, 2013).

The cells were mechanically lysed using the bead beating system FastPrep-24™ Classic (MP Biomedicals) for 1 min at 5.0 m/s, and cooled down on ice for 1 min. This step was repeated one more time. Thereafter, the DNA was isolated by successive extraction with phenol-chloroform: isoamyl alcohol followed by precipitation with ethanol, as described by Padilha et al. (2016). The DNA was collected by centrifugation (15700 *g* / 5 min / 4 °C), resuspended in 100 µL of TE buffer (PADILHA et al., 2016; VILLARREAL, 2013), and the quality and concentration determined in a NanoPhotometer® N60 (IMPLEN).

For enumeration of viable cells of *St. thermophilus* TH-4 and *Lb. paracasei* subsp. *paracasei* F19 quantitative PCR (qPCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems™). The amplification reactions were composed of 12.5 µL of 2X Power SYBR® Green PCR Master Mix (Applied Biosystems™), 5 µL of the DNA sample, each primer at the appropriate concentration, and q.s.p. DNase/RNase-Free distilled water (UltraPure™, Invitrogen ®) water to complete 25 µL. The primer sequences and concentrations and respective amplification programs are detailed in Table 2.

The standard curves were built with 10-fold dilution series of the genomic DNA isolated from the pure cultures of *St. thermophilus* TH-4 (genome size: 2102268 bp; NCBI RefSeq: NZ_CP038020.1) and *Lb. paracasei* subsp. *paracasei* F19 (genome size: 3063698 bp; GenBank: CP016355.1) from 100 to 5×10^8 genome copies per amplification reaction. The viable cell number were determined by the comparison of the threshold cycle (C_t) of each sample with the standard curves (PADILHA et al., 2016).

Table 2: Primers and cycling parameters applied to determine the viable cells numbers of *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19 by quantitative PCR (qPCR).

Streptococcus thermophilus TH-4			
Primers	Sthermo-F (forward)[1] (5' - GTTCACACTGTGACGGTAGCTT – 3')		500 nM
	Sthermo-R (reverse)[1] (5' – GAGCCACAGCCTTTAACTTCAGA – 3')		500 nM
Cycling		1×	50 °C – 2 min. 95 °C – 5 min.
		40×	95 °C – 30 s 60 °C – 30 s 72 °C – 1 min
Lactobacillus paracasei subsp. *paracasei* F19			
Primers	CRISPR2F (forward)[2] (5'- CGTGTGCCGATATAATGGGAACG-3')		100 nM
	CRISPR2R (reverse)[2] (5'-CCAAAGATCATCAAGCGTGCCAT-3')		100 nM
Cycling		1×	50 °C – 2 min. 95 °C – 10 min.
		40×	95 °C – 15 s 60 °C – 1 min.

[1] FALENTIN et al., 2012; [2] SIEUWERTS & HÅKANSSON, 2016.

2.8 Statistical analysis

The results were expressed as mean ± standard deviation (SD). To quantify the viable cell number, the Ct values were automatically converted to CFU equivalent/mL by the 7500 Real-Time PCR System Software (Applied Biosystems™). The homogeneity and normal distribution of the collected data were evaluated. Once a normal distribution was found, the Student's t-test was applied to compare two samples and for three or more samples the Analysis of Variance (ANOVA) followed by Tuckey test was applied. Results were considered as significantly different when $p \leq 0.05$ (significance level of 95%). Minitab® Statistical Software (Minitab, LLC), version 19, was used for the statistical analysis.

3 RESULTS

3.1 Evaluation of fermentability of BSG by probiotic and starter cultures

In general, all strains tested, except for *St. thermophilus* TH-4, presented a good growth in mpr-MRS with or without BSG flour supplementation (Table 3). Regarding the probiotic strains, *Bifidobacterium* spp. showed higher population in the absence of the BSG ($p < 0.05$) after 24 h of fermentation, while PCC, 431, GR-1, and LA-5 counts were significantly higher with the addition of BSG. On the other hand, the strains RC-14, F19, and LGG showed similar growth with or without the BSG supplementation. Regarding the

St. thermophilus strains evaluated, ST-M6 counts were higher when fermented with BGS, while those of TH-4 decreased in both conditions after 24 h of fermentation. Even so, the reduction in TH-4 populations was attenuated in the presence of BSG ($p < 0.05$).

Most of the strains grew in the negative control. Thus, fermentability of the strains in milk was also evaluated, since this was the food matrix chosen for the development of the probiotic fermented food product.

According to Table 3, in UHT skimmed milk supplemented with BSG, the populations of BB-46, PCC, RC-14, F19, and LGG increased significantly when compared to their respective negative control ($p < 0.05$). Regarding all the other strains, probiotic or starter, no significant differences were observed between the counts after fermentation with or without BSG. Additionally, *Lb. paracasei* subsp. *paracasei* F19 showed the highest population after 24 h of fermentation and seemed to be the strain that benefited most with the addition of BSG. Therefore, this strain was selected for the development of a probiotic FM supplemented with BSG in co-culture with *St. thermophilus* TH-4.

3.2 Production and characterization of probiotic fermented milk and submission to *in vitro*-simulated gastrointestinal conditions

The chemical composition of the FM formulations and BSG flour are presented in Table 4. Overall, no important differences were observed in the composition among the FM formulations, despite a little higher fat content in the FM with the addition of BSG (FM2 and FM4). Moreover, no soluble fiber was found in the BSG and around 90% of the carbohydrate content was comprised of insoluble fiber.

The co-culture of *Lb. paracasei* subsp. *paracasei* F19 (F19) and *St. thermophilus* TH-4 (TH-4) showed a trend to reduce the fermentation time when compared to the FM with only TH-4 (Table 5). However, no significant difference in the acidification rate was observed. Overall, the pH of all the FM decreased significantly between the 1st and 7th day of storage (data not shown), remaining stable until the end of storage. No difference was observed in the pH among the FM on day 1. However, on day 28, the FM formulations with F19 and TH-4, namely FM3 and FM4, presented significantly lower pH values ($p < 0.05$) when compared to FM1 and FM2 (Table 5).

Table 3: Variations in probiotic and starter populations in modified phenol red MRS broth (mpr-MRS) and in UHT skimmed milk, with or without BSG flour, from before (0 h) and after 24 h of fermentation for the different strains tested.

		Δ log CFU/mL 0-24 h*			
		Phenol red MRS broth		UHT skimmed milk	
Group	Strain	With BSG	Negative control**	With BSG	Negative control**
Probiotic	*Bifidobacterium animalis* subsp. *lactis* BB-12	2.45 ± 0.09^{DEb}	2.71 ± 0.07^{CDa}	3.35 ± 0.04^{Ca}	3.29 ± 0.19^{CDa}
	Bifidobacterium longum BB-46	2.31 ± 0.05^{Eb}	2.54 ± 0.09^{CDa}	4.03 ± 0.02^{ABa}	3.75 ± 0.07^{ABb}
	Bifidobacterium longum subsp. *infantis* BB-02	2.45 ± 0.10^{DEb}	2.79 ± 0.07^{Ca}	3.35 ± 0.07^{Ca}	3.54 ± 0.06^{BCa}
	Lactobacillus acidophilus LA-5	2.97 ± 0.11^{Ca}	2.48 ± 0.04^{Db}	4.04 ± 0.10^{ABa}	3.90 ± 0.02^{Aa}
	Lactobacillus fermentum PCC	2.56 ± 0.08^{DEa}	2.16 ± 0.05^{Eb}	1.96 ± 0.10^{Da}	1.14 ± 0.06^{Fb}
	Lactobacillus reuteri RC-14	3.56 ± 0.03^{Ba}	3.68 ± 0.09^{Aa}	2.18 ± 0.39^{Da}	$- 0.08 \pm 0.04^{Gb}$
	Lactobacillus paracasei subsp. *paracasei* L.casei 431	4.28 ± 0.04^{Aa}	3.37 ± 0.03^{Bb}	3.19 ± 0.06^{Ca}	3.31 ± 0.03^{CDa}
	Lactobacillus paracasei subsp. *paracasei* F19	2.56 ± 0.18^{DEa}	2.53 ± 0.15^{Da}	4.26 ± 0.05^{Aa}	3.18 ± 0.03^{Db}
	Lactobacillus rhamnosus GR-1	3.78 ± 0.13^{Ba}	2.69 ± 0.03^{CDb}	3.89 ± 0.08^{Ba}	3.51 ± 0.07^{BCa}
	Lactobacillus rhamnosus LGG	2.74 ± 0.21^{CDa}	2.73 ± 0.14^{CDa}	3.06 ± 0.09^{Ca}	$2,73 \pm 0.11^{Eb}$
Starter	*Streptococcus thermophilus* TH-4	-0.15 ± 0.04^{Ba}	-0.76 ± 0.15^{Bb}	4.29 ± 0.11^{Aa}	4.35 ± 0.07^{Aa}
	Streptococcus thermophilus ST-M6	2.88 ± 0.15^{Aa}	2.43 ± 0.05^{Ab}	4.05 ± 0.16^{Aa}	4.01 ± 0.07^{Ba}

* Δ log CFU/mL 0-24 h = Population T24 – Population T0 (log CFU/mL); T0 = initial population; T24 = population after 24 h of fermentation. ** Negative control = fermentation without the addition of BSG flour. [A,B] Means in the same column for the same group (probiotic or starter) with different capital letters are significantly different ($p < 0.05$). [a,b] Means in the same row for the same media (mpr-MRS or UHT skimmed milk) with different lower case letters are significantly different ($p < 0.05$).

Table 4: Chemical composition of the fermented milks (FM) and brewer's spent grain flour (BSG).

	BSG	FM1	FM2	FM3	FM4
Moisture (g/100 g)	2.48 ± 0.12	88.12 ± 0.19^{A}	88.31 ± 0.31^{A}	88.46 ± 0.14^{A}	87.19 ± 0.18^{B}
Protein (g/100 g)	19.20 ± 0.33	4.42 ± 0.22^{A}	4.51 ± 0.02^{A}	4.36 ± 0.13^{A}	4.55 ± 0.30^{A}
Fat (g/100 g)	10.93 ± 0.63	0.38 ± 0.02^{BC}	0.54 ± 0.05^{A}	0.37 ± 0.03^{C}	0.47 ± 0.03^{AB}
Ash (g/100 g)	3.79 ± 0.05	1.02 ± 0.02^{A}	1.02 ± 0.02^{A}	0.92 ± 0.01^{C}	0.97 ± 0.01^{B}
Carbohydrates (g/100 g)	63.60 ± 2	6.05 ± 0.10^{AB}	5.62 ± 0.47^{B}	5.89 ± 0.02^{AB}	6.81 ± 0.05^{A}
Total dietary fiber (g/100 g)	57.67 ± 0.14*	N/A	N/A	N/A	N/A

FM1 (control; without probiotic culture and BSG); FM2 (with BSG only and no probiotic culture); FM3 (with probiotic culture and without BSG); FM4 (with probiotic culture and BSG). ^{A,B} Means in the same row with different capital letters are significantly different ($p < 0.05$). * No soluble fiber was detected. N/A (not applicable).

Table 5: Fermentation time, acidification rate, and pH of fermented milks (FM) after 1 and 28 days of storage at 5 °C.

	Formulations			
	FM1	FM2	FM3	FM4
Time to reach pH 5.4 (h)	3.1 ± 0.1^{B}	3.4 ± 0.1^{A}	2.9 ± 0.1^{BC}	2.8 ± 0.0^{C}
Acidification rate (pH U/h)	0.43 ± 0.05^{A}	0.39 ± 0.03^{A}	0.45 ± 0.05^{A}	0.42 ± 0.02^{A}
pH (day 1)	5.1 ± 0.2^{A}	5.2 ± 0.2^{A}	4.9 ± 0.3^{A}	4.9 ± 0.4^{A}
pH (day 28)	4.7 ± 0.2^{A}	4.8 ± 0.2^{A}	4.2 ± 0.3^{B}	4.2 ± 0.2^{B}

FM1 (control; without probiotic culture and BSG); FM2 (with BSG only and no probiotic culture); FM3 (with probiotic culture and without BSG); FM4 (with probiotic culture and BSG). ^{A,B} Means in the same row with different capital letters are significantly different ($p < 0.05$).

The addition of BSG and/or the co-culture with F19 did not show any influence in the viability of TH-4. The counts were similar for all the FM formulations, remaining above 8.4 log CFU/mL throughout the storage time evaluated (**Erro! Fonte de referência não encontrada.** 6). Likewise, the addition of BSG did not influence the viability of F19 in the FM, which was maintained above 8.9 log CFU/mL during the 28 days of storage (Table 6).

As illustrated in Figures 3 and 4, the viability of TH-4 and F19 significantly decreased after the GIT stress simulated *in vitro* ($p < 0.05$). Nevertheless, the addition of BSG in the FM conferred a significant protection to TH-4 throughout the storage time ($p < 0.05$) and the co-culture with F19 did not show any negative effect. Meanwhile, the resistance of F19 was improved with the addition of BSG only at day 1 ($p > 0.05$).

Table 6: Viabilities of *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19 (log CFU/mL) in the fermented milks during storage at 4 ºC for up to 28 days.

Microorganism	Time (days)	Formulations			
		FM1	FM2	FM3	FM4
S. thermophilus TH-4	1	8.90 ± 0.00^{Aa}	8.83 ± 0.01^{Aa}	8.55 ± 0.06^{Aa}	8.61 ± 0.61^{Aa}
	7	8.81 ± 0.19^{Aa}	8.88 ± 0.11^{Aa}	8.50 ± 0.00^{Aa}	8.46 ± 0.57^{Aa}
	14	8.91 ± 0.08^{Aa}	8.89 ± 0.20^{Aa}	8.56 ± 0.01^{Aa}	8.60 ± 0.77^{Aa}
	21	8.91 ± 0.04^{Aa}	8.94 ± 0.03^{Aa}	8.54 ± 0.04^{Aa}	8.40 ± 0.69^{Aa}
	28	8.94 ± 0.03^{Aa}	8.94 ± 0.08^{Aa}	8.54 ± 0.00^{Aa}	8.39 ± 0.71^{Aa}
Lb. paracasei subsp. *paracasei* F19	1	N/A	N/A	8.91 ± 0.09^{Ab}	8.95 ± 0.13^{Ab}
	7	N/A	N/A	8.99 ± 0.08^{Aab}	9.16 ± 0.10^{Aab}
	14	N/A	N/A	9.16 ± 0.01^{Aa}	9.17 ± 0.00^{Aab}
	21	N/A	N/A	9.19 ± 0.07^{Aa}	9.26 ± 0.07^{Aa}
	28	N/A	N/A	9.15 ± 0.09^{Aa}	9.25 ± 0.07^{Aa}

^{A,B} Means in the same row with different capital letters are significantly different ($p < 0.05$). ^{a,b} Means in the same column, for a same microorganism (TH-4 or F19) with different lower case letters are significantly different ($p < 0.05$). FM1 (control; without probiotic culture and BSG); FM2 (with BSG only and no probiotic culture); FM3 (with probiotic culture and without BSG); FM4 (with probiotic culture and BSG); N/A (not applicable).

4 DISCUSSION

Several studies with barley are being conducted to explore the possible stimulation of probiotic growth and the gut microbiota modulation. These effects may be related to the barley's β-glucan and arabinoxylooligosaccharides (AXOS) contents, which are considered as having potential prebiotic effects (ARENA et al., 2014; GÓMEZ et al., 2015; SAJIB et al., 2018; SHEN et al., 2012). In the study conducted by Gómez et al. (2015), the fermentation of AXOS from BSG increased the population of bifidobacteria and lacobacilli in an *in vitro* assay with human feces, while clostridia populations decreased. In addition, more SCFA were produced when compared to the negative control group (GÓMEZ et al., 2015). Likewise, Shen et al. (2012) reported that the administration of barley and oat β-glucans to rats increased the fecal SCFA and the population of *Bifidobacterium* and *Lactobacillus* in the colon, while it decreased the population of *Enterobacteriaceae* (SHEN et al., 2012).

In the present study, we found that BSG flour from barley improved the fermentation of *Lactobacillus* spp. in a strain-dependent manner and *Lb. paracasei* subsp. *paracasei* F19 was the strain to most benefit with the addition of BSG flour in FM. In contrast, almost no

effect was observed for *Bifidobacterium* spp., which showed slightly increased populations in FM only for *Bifidobacterium longum* BB-46. Pallin et al. (2016) also observed that lactobacilli strains grew well in the presence of barley flour, with counts always above 7.30 log cfu/g for the heat-treated flour, and above 9.00 log cfu/g for the untreated flour. Likewise, Arena et al. (2014) reported that food matrices containing β-glucan could stimulate the growth of *Lactobacillus* spp. and increase the resistance of certain strains to the passage through the oral and GIT simulated *in vitro*. Moreover, β-glucan enriched carriers promoted a faster growth of all the lactobacilli strains tested by the authors after the GIT stresses; however, these effects were strain-dependent like herein observed (ARENA et al., 2014).

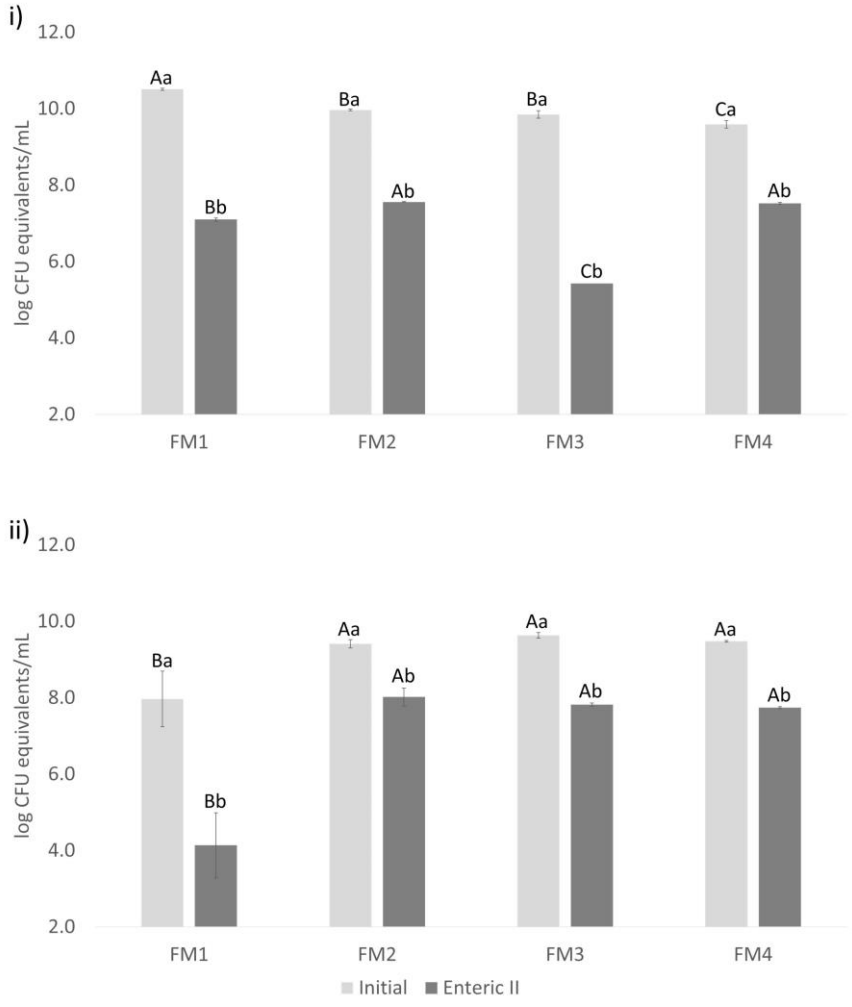

Figure 3: Population of *Streptococcus thermophilus* TH-4 in fermented milk formulations before (Initial) and after gastrointestinal stress simulated *in vitro* (Enteric II) after 1 (i) and 28 (ii) days of storage at 4 °C. [A,B] For each phase, different capital letters represent significant differences between FM formulations ($p < 0.05$). [a,b] For each FM formulation, different lower case letters represent significant differences between phases of the *in vitro* assay ($p < 0.05$). FM1 (control; without the addition of probiotic culture and BSG); FM2 (with BSG only and no probiotic culture); FM3 (with probiotic culture and without BSG); FM4 (with probiotic culture and BSG).

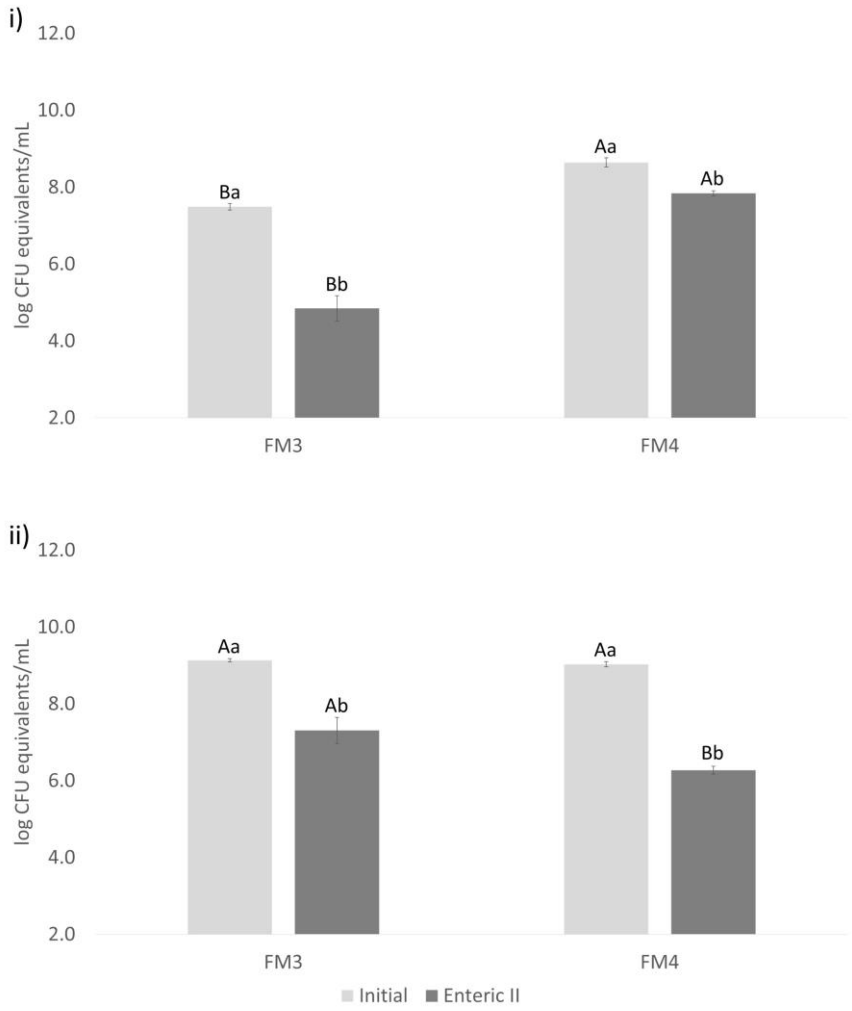

Figure 4: Population of *Lactobacillus paracasei* subsp. *paracasei* F19 in fermented milk (FM) formulations before (Initial) and after gastrointestinal stress simulated *in vitro* (Enteric II) after 1 (i) and 28 (ii) days of storage at 4 °C. ^{A,B} For each phase, different capital letters represent significant differences between FM formulations ($p < 0.05$). ^{a,b} For each FM formulation, different lower case letters represent significant differences between phases of the *in vitro* assay ($p < 0.05$). FM3 (with probiotic culture and without BSG); FM4 (with probiotic culture and BSG).

Regarding the starter strains, the growth media was the most important factor. *St. thermophilus* TH-4 did not ferment the mpr-MRS but showed great potential as a starter culture for FM production. No major differences were observed in the behavior of *St. thermophilus* ST-M6 in mpr-MRS or milk, with or without BSG supplementation. The ability of *St. thermophilus* to utilize different sugars and its proteolytic activities are important factors involving milk fermentation and is directly related to each strain genetic (CUI et al., 2016). Moreover, according to the manufacturer, TH-4 has potential as a probiotic, so it was

selected to study the possible application of this strain both as a starter and a probiotic culture in FM.

The composition of the BSG may differ between the breweries. On average, the results obtained herein was consistent with earlier reports with a slightly higher protein recovery (ROBERTSON et al., 2010; SAJIB et al., 2018; STOJCESKA, 2019). Meanwhile, the addition of BSG did not show any inhibitory effect in the kinetics of milk fermentation. In fact, the acidification rates were in agreement with previously observed for milk or soymilk fermentation added with commercial prebiotic ingredients FOS and/or inulin (BATTISTINI et al., 2018; OLIVEIRA et al., 2009).

Concerning the viability of the microorganisms in the FM throughout the storage time evaluated, no major effects were observed with the co-culture and/or BSG supplementation. The counts of *St. thermophilus* TH-4 and *Lb. paracasei* subsp. *paracasei* F19 were always above, respectively, 10^{10} and 10^{11} CFU per daily portion of 200 mL of FM for all the studied formulations, reaching the minimum usually associated with probiotic health benefits (HEALTH CANADA, 2019; HILL et al., 2014; OUWEHAND, 2017).

The resistance of TH-4 against GIT stress simulated *in vitro* was significantly improved by the addition of BSG and/or the co-culture with F19. Except for FM1 (without BSG or co-culture with F19), we could estimate that a daily portion of 200 mL of all other FM studied formulations could deliver around 10^{10} CFU equivalent viable cells of *St. thermophilus* TH-4 to the colon, corroborating the potential use of this strain as a starter and probiotic culture, if the health benefits are supported by clinical trials.

Previous studies have described interesting features of *Lb. paracasei* subsp. *paracasei*. These include genetic stability, resistance to the GIT stress, improvement of IBS (irritable bowel syndrome) symptoms, and gut microbiota modulation (BERTAZZONI et al., 2013; CRITTENDEN et al., 2002; DI CERBO & PALMIERI, 2013; LOMBARDO; VERNETTO; BLANCO, 2009; MÄTTÖ et al., 2006). In fact, the present study showed survival of 10^{8} and 10^{9} CFU equivalents after the exposure of the FM formulations, respectively, with and without BSG to *in vitro* GIT conditions, considering a daily portion of 200 mL of FM. This outcome is very promising when compared with the results of 10^{6} and 10^{9} CFU/g of F19 fecal recovery reported by Crittenden et al. (2002) and Mättö et al. (2006), respectively.

Ouwehand (2017) stated that the dose-response of probiotic health effects is not clear yet. Regarding probiotic fecal recovery, the dose administered was directly proportional to the

amount of the strains detected in the patient feces. Similar results were found for antibiotic-associated diarrhea, for which the risk is reduced with a dose above 10^{10} CFU. On the other hand, no dose-response could be found for *Clostridium difficile* associated diarrhea, as well as for immune modulation. (OUWEHAND, 2017).

It is important to highlight that molecular biology techniques, namely qPCR in combination with PMA treatment (PMA-qPCR), should be employed for the quantification of viable cells after the GIT conditions assay. In agreement with what was previously stated by Villarreal et al. (2013), the traditional plate count method is not reliable for the enumeration of probiotic microorganisms after stress, since they might be in a viable but not cultivable state. In fact, we did not detect any viable cells of TH-4 and significantly lower viable cells of F19 after GIT stress simulated *in vitro* whilst employing traditional plate-counting methods (data not shown).

5 CONCLUSION

The BSG revealed to be an interesting ingredient rich in fiber that may be employed in a synbiotic approach. The addition of BSG in FM, alone or in combination with *Lb. paracasei* subsp. *paracasei* F19, improved the resistance of *St. thermophilus* TH-4 to GIT conditions simulated *in vitro*, suggesting a prospective application of this strain both as starter and as probiotic culture. Nonetheless, the employment of PMA-qPCR was crucial for the assessment of viable cells of TH-4 and F19. Furthermore, all the evaluated FM formulations have potential as probiotic foods, since total probiotic populations were always above 10^{10} CFU in a daily portion of 200 mL of FM during the 28 days of storage, delivering to the colon an estimated viable cells above 10^{10} and 10^{8} CFU equivalent of, respectively, *St. thermophilus* TH-4 and *Lb. paracasei* subsp. *paracasei* F19,. Future *in vivo* and clinical trials to evaluate the possible health benefits conferred by the consumption of FM with TH-4 and F19 are encouraged.

REFERENCES

A.O.A.C. - Association of Official Analytical Chemists, 2011. **Official methods of analysis of AOAC international**, 18. ed. Maryland: AOAC International.

ALBUQUERQUE, M. A. C.; BEDANI, R.; VIEIRA, A. D. S.; LE BLANC, J. G.; SAAD, S. M. I. Supplementation with fruit and okara soybean by-products and amaranth flour increases the folate production by starter and probiotic cultures. **International Journal of Food Microbiology**, v. 236, p. 26-32, 2016.

ARENA, M. P.; CAGGIANIELLO, G.; FIOCCO, D.; RUSSO, P.; TORELLI, M.; SPANO, G.; CAPOZZI, V. Barley β-Glucans-Containing Food Enhances Probiotic Performances of Beneficial Bacteria. **International Journal of Molecular Sciences**, v. 15, p. 3025-3039, 2014.

BATTISTINI, C.; GULLÓN, B.; ICHIMURA, E. S.; GOMES, A. M. P.; RIBEIRO, E. P.; KUNIGK, L.; MOREIRA, J. U. V.; JURKIEWICZ, C. Development and characterization of an innovative synbiotic fermented beverage based on vegetable soybean. **Brazilian Journal of Microbiology**, v. 49, p. 303-309, 2018.

BEDANI, R.; ROSSI, E. A.; SAAD, S. M. I. Impact of inulin and okara on *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product and probiotic survival under in vitro simulated gastrointestinal conditions. **Food Microbiology**, v. 34, p. 382-389, 2013.

BERTAZZONI, E.; DONELLI, G.; MIDTVEDT, T.; NICOLI, J.; SANZ, Y. Probiotics and clinical effects: is the number what counts? **Journal of Chemotherapy**, v. 25, n. 4, p. 193-212, 2013

BLIGH, E. G.; DYER, W. J. A rapid method for total lipid extraction and purification. **Canadian Journal of Biochemistry and Physiology**, v. 37, p. 911–917, 1959.

BURITI, F. C. A.; CARDARELLI, H. R.; SAAD, S. M. I. Biopreservation by *Lactobacillus paracasei* in coculture with *Streptococcus thermophilus* in potentially probiotic and synbiotic fresh cream cheeses. **Journal of food protection**, v. 70, n. 1, p. 228–235, 2007.

BURITI, F. C. A.; DOS SANTOS, K. M. O.; SOMBRA, V. G.; MACIEL, J. S.; TEIXEIRA SÁ, D. M. A.; SALLES, H. O.; OLIVEIRA, G.; DE PAULA, R. C. M.; FEITOSA, J. P. A.; MONTEIRO MOREIRA, A. C. O.; MOREIRA, R. A.; EGITO, A. S. Characterisation of partially hydrolysed galactomannan from *Caesalpinia pulcherrima* seeds as a potential dietary fibre. **Food Hydrocolloids**, v. 35, p. 512–521, 2014.

CERVBRASIL - **Associação Brasileira da Indústria da Cerveja. Anuário 2014**. 2014. Available at: <http://www.cervbrasil.org.br/arquivos/anuariofinal2014.pdf>. Accessed: Aug 2015.

CRITTENDEN, R.; SAARELA, M.; MÄTTÖ, J.; OUWEHAND, A. C.; SALMINEN, S.; PELTO, L.; VAUGHAN, E. E.; DE VOS, W. M.; VON WRIGHT, A.; FONDÉN, R.; MATTILA-SANDHOLM, T. *Lactobacillus paracasei* subsp. *paracasei* F19: Surival, ecology and safety in the human intestinal tract – A survey of feeding studies within the PROBDEMO Project. **Microbiology Ecology in Health and Disease**, v. 14, n. 1, p. 22-26, 2002.

CUI, Y.; XU, T.; QU, X.; HU, T.; JIANG, X.; ZHAO, C. New insights into various production characteristics of *Streptococcus thermophilus* strains. **International Journal of Molecular Sciences**, v. 17, n. 10, p. 1701, 2016

DI CERBO, A.; PALMIERI, B. *Lactobacillus paracasei* subsp. *paracasei* F19; a farmacogenomic and clinical update. **Nutrición Hospitalaria**, v. 28, n. 6, p. 1842-1850, 2013.

FALENTIN, H.; HENAFF, N.; LE BIVIC, P.; DEUTSCH, S. M.; PARAYRE, S.; RICHOUX, R.; SOHIER, D.; THIERRY, A.; LORTAL, S.; POSTOLLEC, F. Reverse transcription quantitative PCR revealed persistency of thermophilic lactic acid bacteria

metabolic activity until the end of the ripening of Emmental cheese. **Food Microbiology**, v. 29, n. 1, p. 132-140, 2012.

FĂRCAŞ, A. C.; SOCACI, S. a.; DULF, F. V.; TOFANĂ, M.; MUDURA, E.; DIACONEASA, Z. Volatile profile, fatty acids composition and total phenolics content of brewers' spent grain by-product with potential use in the development of new functional foods. **Journal of Cereal Science**, v. 64, p. 34–42, 2015.

GIBSON, G. R. Fibre and effects on probiotics (the prebiotic concept). **Clinical Nutrition**, Supplement, v. 1, n. 2, p. 25–31, 2004.

GIBSON, G. R.; HUTKINS, R.; SANDERS, M. E.; PRESCOTT, S. L.; REIMER, R. A.; SALMINEN, S. J.; SCOTT, K.; STANTON, C.; SWANSON, K. S.; CANI, P. D.; VERBEKE, K.; REID, G. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. **Nature Reviews Gastroenterology & Hepatology**, v. 14,p. 491-502, 2017.

GIBSON, G. R.; SCOTT, K. P.; RASTALL, R. A.; TUOHY, K. M.; HOTCHKISS, A.; DUBERT-FERRANDON, A.; GAREAU, M.; MURPHY, E. F.; SAULNIER, D.; LOH, G.; MACFARLANE, S.; DELZENNE, N.; RINGEL, Y.; KOZIANOWSKI, G.; DICKMANN, R.; LENOIR-WIJNKOOK, I.; WALKER, C.; BUDDINGTON, R. Dietary prebiotics: current status and new definition. **Food Science and Technology Bulletin: Functional Foods**, v. 7, n. January, p. 1–19, 2010.

GÓMEZ, B.; MÍGUEZ, B.; VEIGA, A.; PARAJÓ, J. C.; ALONSO, J. L. Production, Purification and *in vitro* evaluation of the prebiotic potential of arabinoxylooligosaccharides from Brewer's Spent Grain. **Journal of Agriculture and Food Chemistry**, v. 63, n. 38, p. 8429-8438, 2015.

HEALTH CANADA. **Probiotic Claims**. 2019. Available at: < https://www.inspection.gc.ca/food-label-requirements/labelling/-f-for-industry/former-health-claims/eng/1514559099172/1514559100331?chap=9#s21c9>. Accessed: Jun 2020.

HILL, C.; GUARNER, F.; REID, G.; GIBSON, G. R.; MERENSTEIN, D. J.; POT, B.; MORELLI, L.; CANANI, R. B.; FLINT, H. J.; SALMINEN, S.; CALDER, P. C.; SANDERS, M. E. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. **Nature Reviews Gastroenterology & Hepatology,** v. 11, p. 506 – 514, 2014.

IDF - INTERNATIONAL DAIRY FEDERATION. Fermented and non-fermented milk products: detection and enumeration of *Lactobacillus acidophilus*. **Bulletin of the International Daity Federation**, v. 306, p. 23–33, 1995.

INSTIUTO ADOLFO LUTZ. **Métodos físico-químicos para análise de alimentos.** 4ª ed. Instituto Adolfo Lutz, São Paulo, SP, 2008.

JOINT FAO/WHO CODEX ALIMENTARIUS COMMISSION. **STANDARD FOR FERMENTED MILKS. 2010**. Available at: http://www.fao.org/fao-who-codexalimentarius/shproxy/en/?lnk=1&url=https%3A%2F%2Fworkspace.fao.org%2Fsites%2Fcodex%2FStandards%2FCODEX+STAN+243-2003%2FCXS_243e.pdf. Acessed: Apr 2017.

LOMBARDO, L.; VERNETTO, A.; BLANCO, I. Clinical evaluation of *Lactobacillus paracasei* subsp. *paracasei* F19 with gluco-oligosaccharides in the short-term treatment of irritable bowel syndrome. **Microbiology Ecology in Health and Disease**, v. 21, n. 1, p. 28-32, 2009.

MARTINEZ, R. C. R.; BEDANI, R.; SAAD, S. M. I. Scientific Evidence for Health Effects Attributed to the Consumption of Probiotics and Prebiotics: An Update for Current Perspectives and Future Challenges. **The British Journal of Nutrition**, p. 1–23, 7 out. 2015.

MÄTTÖ, J.; FONDÉN, R.; TOLVANEN, T.; VON WRIGHT, A.; VILPPONEN-SALMELA, T.; SATOKARI, R.; SAARELA, M. Intestinal survival and persistence of probiotic *Lactobacillus* and *Bifidobacterium* strains administered in triple-strain yoghurt. **International Dairy Journal**, v. 16, n. 10, p. 1174-1180, 2006.

MUSSATTO, S. I.; DRAGONE, G.; ROBERTO, I. C. Brewers' spent grain: Generation, characteristics and potential applications. **Journal of Cereal Science**, v. 43, n. 1, p. 1–14, 2006.

OLIVEIRA, R. P. S.; PEREGO, P.; CONVERTI, A.; OLIVEIRA, M. N. Growth and acidification performance of probiotics in pure culture and co-culture with *Streptococcus thermophilus*: The effect of inulin. **LWT - Food Science and Technology**, v. 42, n. 5, p. 1015-1021, 2009

OUWEHAND, A. C. A review of dose-response of probiotics in human studies. **Beneficial Microbes**, v. 8, p. 143-151, 2017.

PADILHA, M.; MORALES, M. L. V.; VIEIRA, A. D. S.; COSTA, M. G. M.; SAAD, S. M. I. A prebiotic mixture improved *Lactobacillus acidophilus* and *Bifidobacterium animalis* gastrointestinal *in vitro* resistance in *petit-suisse*. **Food & Function**, v. 7, p. 2312 – 2319, 2016

PALLIN, A.; AGBACK, P.; JONSSON, H.; ROOS, S. Evaluation of growth, metabolism and production of potentially bioactive components during fermentation of barley with *Lactobacillus reuteri*. **Food Microbiology**, v. 57, p. 159-171, 2016.

RANADHEERA, R. D. C. S.; BAINES, S. K.; ADAMS, M. C. Importance of food in probiotic efficacy. **Food Research International**, v. 43, n. 1, p. 1–7, 2010.

RICHTER, R. L.; VEDAMUTHU, E. R. Milk and Milk Products. In: DOWNES, F.P.; ITO, K., eds. **Compendium of methods for the microbiological examination of foods**. 4 ed. Washington: APHA, 2001. p. 483–495.

ROBERTSON, J. A.; I'ANSON, K. J. A.; TREIMO, J.; FAULDS, C. B.; BROCKLEHURST, T. F.; EIJSINK, V. G. H.; WALDRON, K. W. Profiling brewer's spent grain for composition and microbial ecology at the site of production. **LWT - Food Science and Technology**, v.43, n. 6, p. 890-896, 2010.

SAJIB, M.; FALCK, P.; SARDARI, R. R. R.; MATHEW, S.; GREY, C.; KARLSSON, E. N.; ADLERCREUTZ, P. Valorization of Brewer's spent grain to prebiotic oligosaccharide: Production, xylanase catalyzed hydrolysis, *in-vitro* evaluation with probiotic strains and in a batch human fecal fermentation model. **Journal of Biotechnology**, v. 268, p. 61-70, 2018.

SANDERS, M. E.; MARCO, M. L. Food formats for effective delivery of probiotics. **Annual Review of Food Science and Technology**, v. 1, n. 1, p. 65–85, 2010.

SANDERS, M. E.; MERENSTEIN, D. J.; REID, G.; GIBSON, G. R.; RASTALL, R. A. Probiotics and prebiotics in intestinal health and disease: from biology to the clinic**. Nature Reviews Gastroenterology & Hepatology**, v. 16, p. 605-6016, 2019.

SANTOS, M.; JIMÉNEZ, J. J.; BARTOLOMÉ, B.; GÓMEZ-CORDOVÉS, C.; DEL NOZAL, M. J. Variability of brewer's spent grain within a brewery. **Food Chemistry**, v. 80, n. 1, p. 17–21, 2003.

SHEN, R. L.; DANG, X. Y.; DONG, J. L.; HU, X. Z. Effects of oat β-glucan and barley β-glucan on fecal characteristics, intestinal microflora, and intestinal bacterial metabolites in rats. **Journal of Agriculture and Food Chemistry**, v. 60, n. 45, p. 11301-11308, 2012.

SIEUWERTS, A.; HÅKANSSON, J. Development of a standardized method for the quantification of *Lactobacillus paracasei* F19 in stoll samples of various ages. **EC Nutrition**, v. 3, n. 3, 633-642, 2016.

SINDICERV – **Sindicato Nacional Da Indústria Da Cerveja. O setor em números**. Available in: < https://www.sindicerv.com.br/o-setor-em-numeros/>. Accessed: 26 jun 2020.

STOJCESKA, V. **Brewer's spent grain from by-product to health: A rich source of functional ingredients.** In: Preedy, V. R., Watson, R. R. (Eds.), Flour and Breads and their Fortification in Health and Disease Prevention. Academic Press, p. 189-198, 2019.

SWANSON, K. S.; GIBSON, G. R.; HUTKINS, R.; REIMER, R. A.; REID, G.; VERBEKE, K.; SCOTT, K. P.; HOLSCHER, H. D.; AZAD, M. B.; DELZENNE, N. M.; SANDERS, M. E. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotic. **Nature Reviews Gastroenterology & Hepatology**. In press. 2020. https://doi.org/10.1038/s41575-020-0344-2

VILLARREAL, M. L. M.; PADILHA, M.; VIEIRA, A. D. S.; FRANCO, B. D. G. M.; MARTINEZ, R. C. R.; SAAD, S. M. I. Advantageous direct quantification of viable closely related probiotics in *petit-suisse* cheeses under *in vitro* gastrointestinal conditions by propidium monoazide – qPCR. **PLoS One**, v. 8, n. 12, p. e82102, 2013.

VINDEROLA, C. G.; PROSELLO, W.; GHIBERTO, D.; REINHEIMER, J. A. Viability of probiotic (*Bifidobacterium*, *Lactobacillus acidophilus* and *Lactobacillus casei*) and nonprobiotic microflora in Argentinian fresco cheese. **Journal of Dairy Science**, v. 83, n.9, p. 1905–1911, 2000

CHAPTER 4

Probiotic fermented milk may worsen inflammation in mice lacking vitamin D receptor

Abstract

Functional foods are promising alternative approaches to alleviate the symptoms or prevent the development of certain illnesses, like inflammatory bowel diseases. Meanwhile, probiotics are known to improve inflammatory conditions, gut barrier function, and production of short-chain fatty acids. Nonetheless, these effects vary between genera or strains, and each health benefit depends on the host's genetics, route of administration, and matrix of delivery. In addition, studies have shown that the vitamin D receptor, in addition to vitamin D functions, is a critical factor associated with the health benefits of probiotics, acting directly on the intestinal permeability, inflammatory responses, and is a key genetic factor for shaping the gut microbiome. This study aimed to evaluate the impact of the administration of a probiotic fermented milk (FM) containing *Lactobacillus paracasei* subsp. *paracasei* F19 and *Streptococcus thermophilus* TH-4, a potentially probiotic food matrix, on the inflammatory responses and VDR expression using a DSS (dextran sulfate sodium)-induced colitis mouse model. The administration of probiotic FM showed an anti-inflammatory effect only for wild-type (WT) mice, reducing the serum level of IL-6, while increasing the VDR expression at the mRNA and protein levels. In contrast, VDR Knockout (KO) mice showed an aggravation in the inflammatory response with the probiotic treatment, presenting a more significant body weight loss and increased serum IL-6 and fecal lipocalin 2 concentrations. Therefore, the probiotic FM studied presented a promising anti-inflammatory potential against inflammation in a VDR-dependent manner.

Key words: fermented milk, functional foods, inflammation, nuclear receptor, probiotics, vitamin D receptor.

1 INTRODUCTION

Functional foods are those to confer a health benefit in addition to basic nutrition and should be offered as a “normal food pattern” and not as pills or capsules. Moreover, the recommended daily portion to provide the associated health claims must be well-suited for a normal diet (DOMÍNGUEZ DÍAZ; FERNÁNDEZ-RUIZ; CÁRMARA, 2020). An interesting approach would be to use functional foods as an adjuvant therapy to alleviate the symptoms or prevent the development of certain illnesses. The most well-known types of functional foods are those enriched with dietary fibers, bioactive compounds (e.g. polyphenols), omega-3 PUFA (polyunsaturated fatty acids), prebiotics (e.g. inulin, FOS, GOS, HMO), and probiotics (BALLAN et al., 2020).

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (HILL et al., 2014). The health benefits related to the consumption of probiotics include improvement of the barrier function, competition with pathogens for nutrients and adhesion sites, and production of organic acids, SCFA (short-chain fatty acids), neurochemicals, vitamins, antimicrobials, mucin, and enzymes. Furthermore, they may reduce the pro-inflammatory responses, interact with the gut microbiota by the cross-feeding mechanism, or metabolizing substrates that will interact with the host through the intestinal epithelium, and may enhance the immune functions by increasing the phagocytic activity and production of immunoglobulins (BALLAN et al., 2020; SANDERS et al., 2019).

It is noteworthy that the pathways by which probiotics interact with the host may differ at genus or strain levels. In this way, the efficacy of probiotics will depend on several factors, such as host genetics, route of administration (e.g. oral or topic), and matrix of delivery (e.g. fermented foods, capsules, or supplements, etc) (CHAMPAGNE; CRUZ; DAGA, 2018; SANDERS et al., 2018; ZMORA et al., 2018). Still, studies have shown that the incorporation of probiotics in like dairy products, may improve their resistance to the gastrointestinal (GIT) stress. However, this fact is not always associated with an improvement of beneficial effects. Therefore, the technological and functional points of view should be taken into consideration when developing and/or indicating the consumption of probiotics (BALLAN et al., 2020; CHAMPAGNE; CRUZ; DAGA, 2018; PÁPAI et al., 2020; SANDERS et al., 2018).

Among the genetic factors associated with the probiotic's mechanisms of action, stands out the vitamin D receptor (VDR). The VDR is a transcriptional factor which, in addition to its regulation of vitamin D functions, is involved in autophagy (WU et al., 2015b), proliferation (JIN et al., 2017; OGBU; BAKKE; SUN, 2020), anti-tumorigenesis (ZHANG et al., 2020a; ZHANG et al., 2020b), gut microbiota modulation (CHATTERJEE et al., 2020; OOI et al., 2013; WANG et al., 2016; WU et al., 2015b), intestinal barrier function (ZHANG et al., 2015; ZHANG et al., 2019; ZHANG et al., 2020b), anti-inflammatory effects (OOI et al., 2013; WU et al., 2015b), immune functions (OOI; CHEN; CANTORNA, 2012; VELDMAN; CANTORNA; DELUCA, 2000), and neurodevelopment disorders (OGBU; XIA; SUN, 2020) pathways.

Studies have shown that the proper function of VDR is crucial for the potential health benefits associated with the consumption of probiotics. Indeed, impaired VDR expression inhibited the protective effect of probiotic treatment against *Salmonella* infection *in vivo* or *in vitro*, whereas in normal conditions probiotics improved the VDR transcriptional activity and the expression of antimicrobial peptides (e.g. cathelicidin, defensins, and lysozyme) (WU et al., 2015a). Still, the direct administration of butyrate, a beneficial bacterial metabolite that is released by probiotics, directly or indirectly (cross-feeding), appeared to increase the VDR expression and exert an anti-inflammatory effect in wild-type (WT) mice (WU et al., 2015b). Meanwhile, the lack of VDR may trigger the predisposition to DSS (dextran sulfate sodium)-induced colitis and intestinal permeability, in addition to an aggravated inflammation and gut microbiota dysbiosis (OOI et al., 2013).

To the best of our knowledge, only the probiotic conditional media or pure cultures have been studied regarding their effects on VDR-related anti-inflammatory responses. Therefore, this study aimed to evaluate the influence of a food matrix in the potential of probiotics to improve VDR functions and inflammation biomarkers employing an *in vivo* DSS colitis mouse model.

2 MATERIAL E METHODS

2.1 Fermented milk production

The fermented milk (FM) formulations were produced as illustrated in Figure 1, using *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19, as starter and probiotic cultures, respectively, both from Chr. Hansen. Brewer's spent grain flour (BSG) (Cervejaria Nacional, São Paulo, Brazil) was added as fiber source ingredient.

Four formulations were evaluated: FM1 (TH-4); FM2 (TH-4 + BSG); FM3 (TH-4 + F19); FM4 (TH-4 + F19 + BSG).

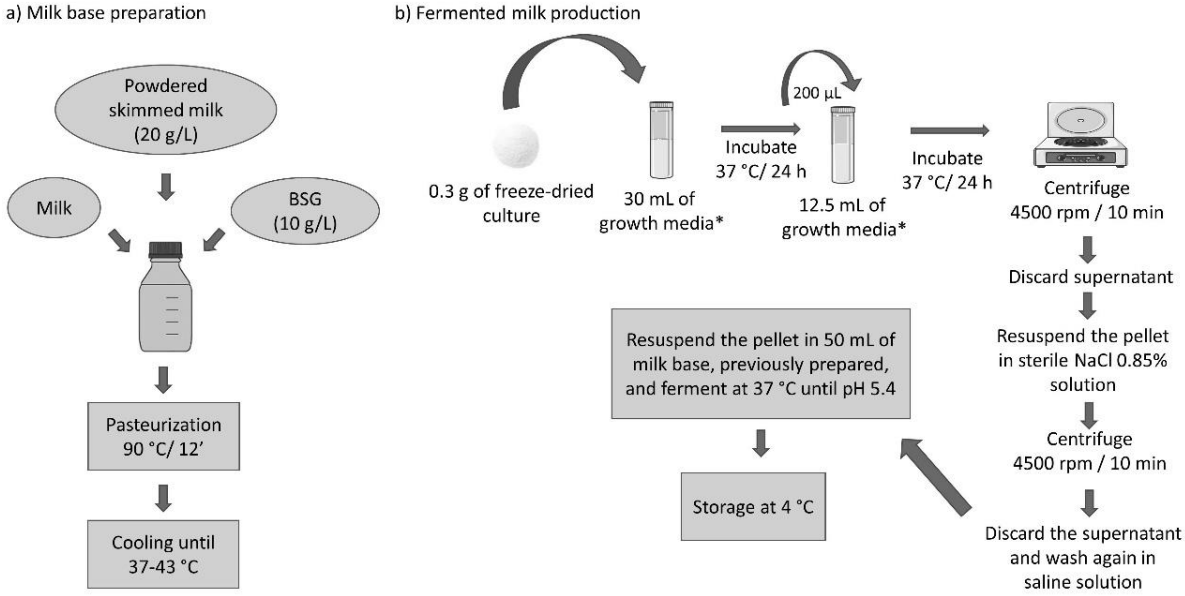

Figure 1: Schematic representation of the fermented milks production. BSG = Brewer's spent grain flour. * Growth media for *S. thermophilus* TH-4 was M17 broth (Sigma-Aldrich) and for *L. paracasei* subsp. *paracasei* F19 was MRS broth (Difco).

2.2 Animals

Animal experiments were performed with wild-type (WT) and VDR knockout (VDRKO) C57BL/6 mice with 2-3 months of age. Mice were provided with water and food *ad libitum* and maintained in a 12-hour dark-light cycle. The animal work was approved by the UIC Office of Animal Care (ACC Number: 17-218).

2.2.1 Pilot Study

A pilot study was conducted to select the fermented milk (FM) formulation with greater potential to improve VDR status for a subsequent interventional study. Six groups of 3 WT mice received 100 µL of PBS, milk, or FM by oral gavage, and were euthanized 24 h after treatment by anesthesia followed by cervical dislocation, as illustrated in figure 2a. Colon epithelial cells were collected for VDR protein expression assessment by Western blot as described by Zhang, Xia & Sun (2020).

The counts of starter and/or probiotic were always above 10^8 CFU/mL for all FM formulations. Thus, the administered dose was 10^7 CFU.

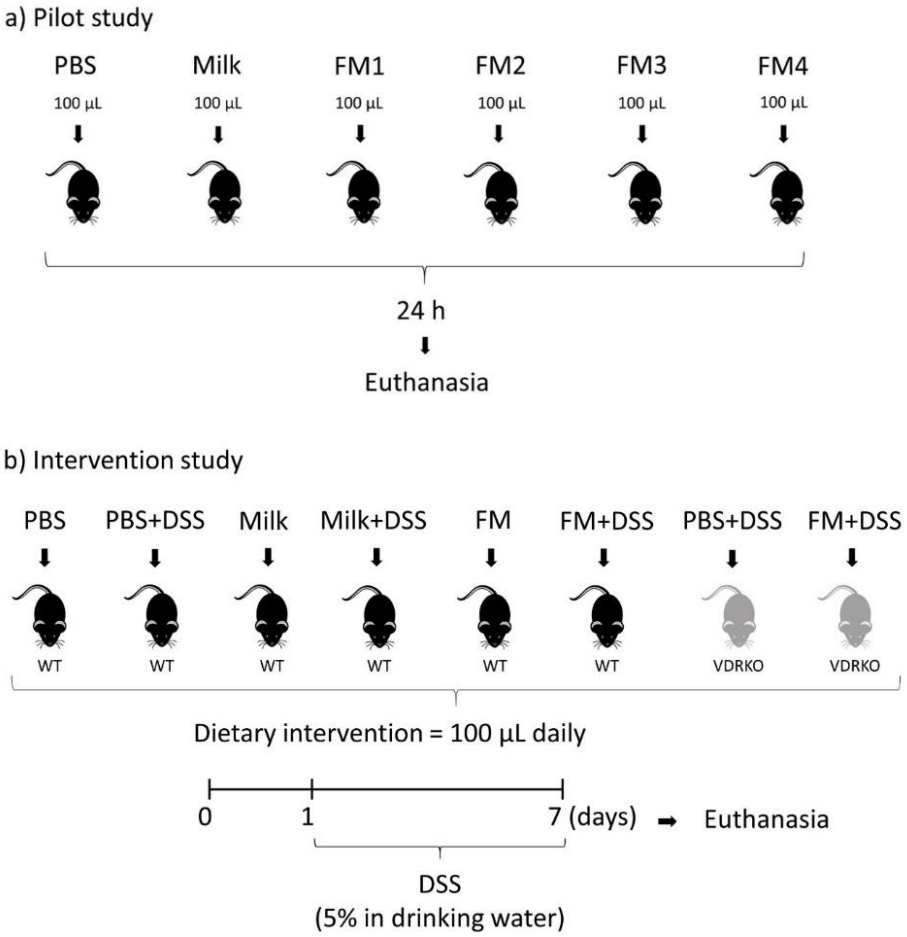

Figure 2: Schematic representation of the animal studies. A) Pilot Study. B) Intervention Study. PBS = Phosphate Buffered Saline; TH-4 = *Streptococcus thermophilus* TH-4; F19 = *Lactobacillus paracasei* subsp. *paracasei* F19; BSG = Brewer's spent grain; FM = Fermented milk; FM1 (TH-4); FM2 (TH-4 + BSG); FM3 (TH-4 + F19); FM4 (TH-4 + F19 + BSG); VDR = vitamin D receptor; WT = wild-type; VDRKO = VDR knockout; DSS = Dextran Sulfate Sodium.

2.2.2 Intervention Study

After the pilot study, one FM formulation was selected for an interventional study to evaluate its feasible anti-inflammatory effect in a DSS (dextran sulfate sodium) colitis model.

Six groups of WT mice (n=8) received, by oral gavage, 100 µL/day of FM (10^7 CFU), milk, or PBS. Additionally, two groups of VDRKO mice (n=3) received, by oral gavage, 100 µL/day of FM (10^7 CFU) or PBS. In the assigned groups, the DSS treatment started 24 h after the first dose, as detailed in figure 2b. Fecal samples were collected daily, as described by Zhang, Xia & Sun (2020), and the body weight assessed as well. The animals were euthanized on day 7 by anesthesia followed by cervical dislocation.

2.3 Vitamin D receptor expression

After the euthanasia, the colon lengths were determined, and tissues samples were collected to evaluate the colonic VDR expressions at protein and mRNA levels.

2.3.1 Western blot

The VDR expression at protein level was determined by western blot. Protein from colon samples were extracted by sonication in lysis buffer (1% Triton X-100, 150 mmol/L NaCL, 10 mmol/L Tris pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA pH 8.0, 0.2 mmol/L sodium ortho-vanadate, and protease inhibitor cocktail), separated by SDS-polyacrylamide gel electrophoresis, and finally detected by immunoblotting with primary antibodies to mouse VDR and β-actin (Santacruz, CA, USA). The ECL kit (Thermo Fisher Scientific) was used for visualization (CHATTERJEE et al., 2020; WU et al., 2015b; ZHANG; XIA; SUN, 2020).

2.3.2 Gene expression

The VDR expression at mRNA level was determined by qPCR. Total RNA from colon samples was extracted using TRIzol reagent (Invitrogen) according to manufacturer instructions, followed by RNA reverse transcription using the iScript cDNA synthesis kit (Bio-Rad). Next, qPCR was performed using Bio-Rad CFX 96 Real-time system and SYBR green supermix (Bio-Rad). All reactions were performed in triplicates and the results were normalized to β-actin level from the same sample. The $2^{-\Delta\Delta Ct}$ calculation was employed to determine the VDR relative gene expression, as previously described by Zhang, Xia & Sun (2020).

2.4 Inflammation

Blood was collected by cardiac puncture and transferred to tubes with EDTA (10 mg/mL). The IL-6 level was measured using an ELISA kit (IL6 ELISA kit, KE 1000-7, Mouse IL6, Proteintech) according to the manufacturer's instructions (WU et al., 2015a).

Fecal samples were prepared as described by Lu et al. (2019), and lipocalin 2 (LCN-2) level was estimated using the Duoset murine Lcn-2 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.5 Statistical analysis

The results were expressed as mean ± standard deviation (SD). The homogeneity and normal distribution of the collected data were evaluated. Once a normal distribution was

found, Analysis of Variance (ANOVA) followed by Tuckey test was applied. Results were considered significantly different when $p \leq 0.05$ (significance level of 95%). Minitab® Statistical Software (Minitab, LLC), version 19, was used for the statistical analysis

3 RESULTS

3.1 Pilot Study

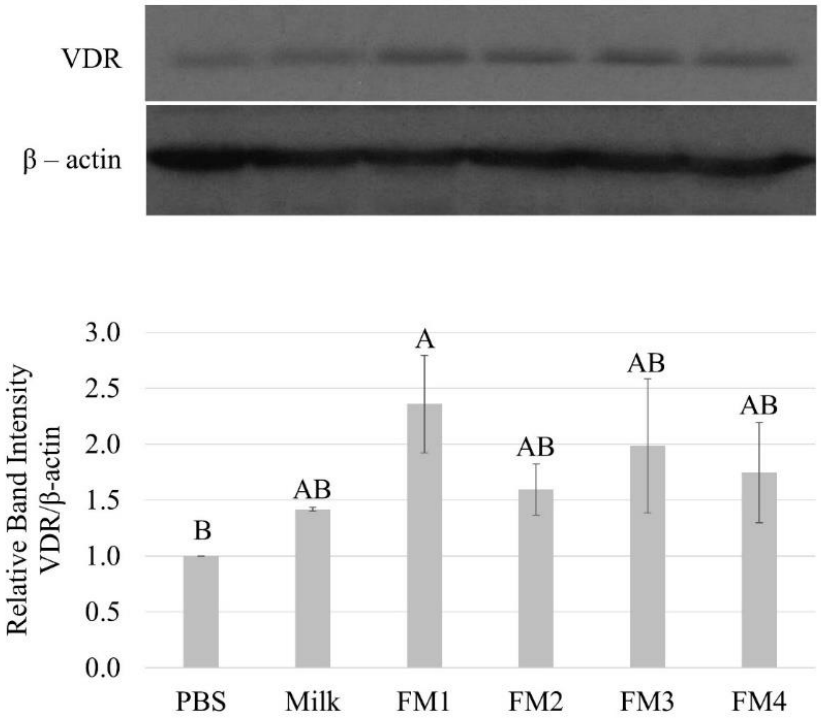

Figure 3: Protein Expression of VDR in colon samples from Pilot Study. VDR = vitamin D receptor; PBS = Phosphate Buffered Saline; TH-4 = *Streptococcus thermophilus* TH-4; F19 = *Lactobacillus paracasei* subsp. *paracasei* F19; BSG = Brewer's spent grain; FM = Fermented milk; FM1 (TH-4); FM2 (TH-4 + BSG); FM3 (TH-4 + F19); FM4 (TH-4 + F19 + BSG). A,B Different capital letters represent significant differences between treatments ($p < 0.05$).

The administration of FM showed a promising potential to increase the colonic VDR protein expression when compared to PBS, as shown in figure 3. Nonetheless, the addition of BSG as a fiber source did not show any positive effect to improve the VDR status. Still, our unpublished data have shown that *S. thermophilus* TH-4 has potential as a probiotic strain, in addition to its starter features, and the co-culture with *L. paracasei* subsp. *paracasei* F19 enhanced its survival after exposure to GIT stress simulated *in vitro*. Thus, the FM formulation selected for the intervention study was the FM3, with the co-culture of TH-4 and F19, without BSG, now on referred just as FM.

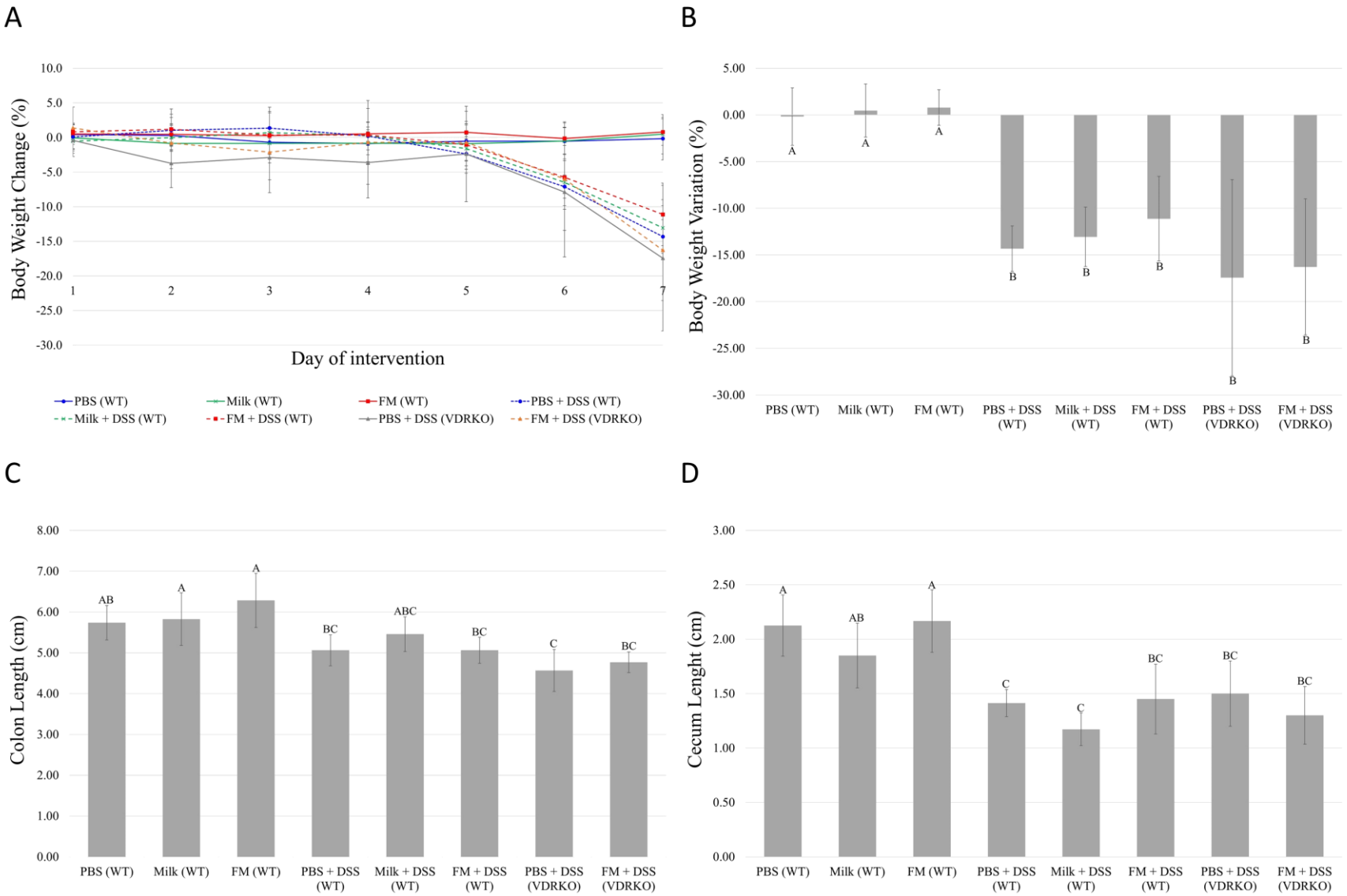

Figure 4: A) Body weight changes during the dietary intervention. B) Total body weight variation. C) Colon lengths. D) Cecum lengths. FM = fermented milk with the co-culture *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19, DSS = dextran sulfate sodium, WT = wild-type, VDRKO = vitamin D receptor knockout. A,B Different capital letters represent significant differences between treatments ($p < 0.05$).

3.2 Intervention Study

All the groups that were submitted to DSS treatment showed a significant body weight loss, as illustrated in figure 4B, which was more pronounced at days 6 and 7 (Figure 4A). Meanwhile, rectal bleeding and softer feces were observed from day 5 until the end of the study. In addition, all DSS groups showed shortened cecum lengths when compared to their respective controls (Figure 4D), and FM showed a trend to attenuate it in WT mice. Regardless of statistical differences, no major differences were observed in colon lengths (Figure 4C).

The administration of probiotic FM showed a promising trend to improve the VDR status in the colon at both mRNA and protein levels, as observed in figure 5A and B. Nonetheless, this positive effect was only observed in the groups that had not been submitted to DSS treatment.

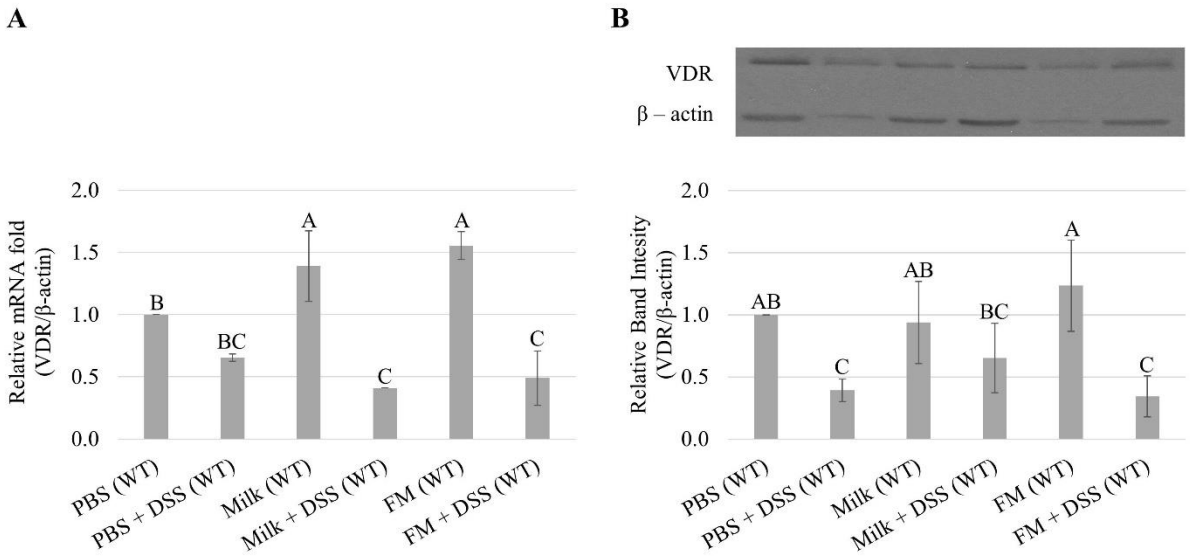

Figure 5: VDR expression in colon samples at mRNA (A) and protein (B) levels. VDR = vitamin D receptor, FM = fermented milk with the co-culture *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19, DSS = dextran sulfate sodium, WT = wild-type, VDRKO = vitamin D receptor knockout. A,B Different capital letters represent significant differences between treatments ($p < 0.05$).

Regarding the inflammation biomarkers, as shown in figure 6 A, the administration of milk or probiotic FM significantly reduced the serum level of IL-6 when compared to the PBS control ($p < 0.0.5$). In addition, between DSS groups, probiotic FM showed a trend towards a decrease in the IL-6 release, but this effect was dependent on VDR functions. In fact, the IL-6 concentration was extremely higher in VDRKO mice that received probiotic FM when compared to all other DSS groups. Furthermore, fecal lipocalin 2 was also significantly increased with the DSS treatment, and the probiotic FM showed a tendency to

worsen this inflammation marker when compared to the PBS control in VDRKO mice (Figure 6B).

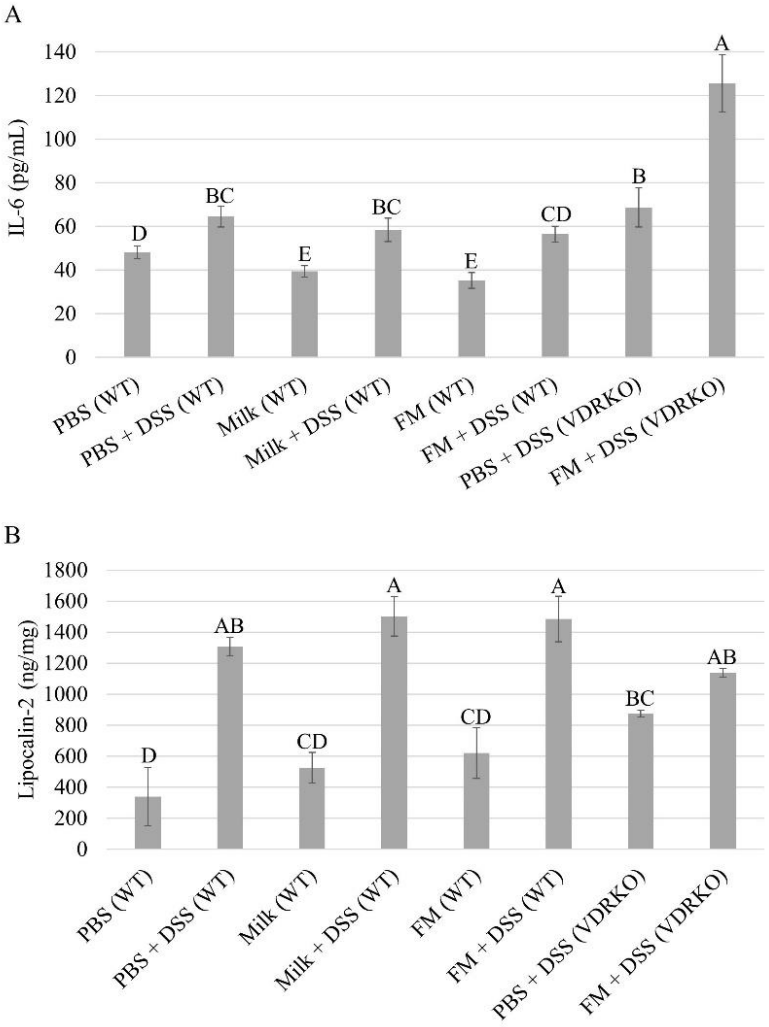

Figure 6: Serum of IL-6 (A) and fecal lipocalin-2 (B) levels. IL-6 = interleukin 6, FM = fermented milk with the co-culture *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19, DSS = dextran sulfate sodium, WT = wild-type, VDRKO = vitamin D receptor knockout. A,B Different capital letters represent significant differences between treatments ($p < 0.05$).

4 DISCUSSION

In the present study, we have shown that probiotic FM increased the VDR expression in colon at the mRNA and protein levels and a tendency in reducing the serum concentration of IL-6 in DSS-colitis in WT mice. On the other hand, in VDRKO mice, the treatment with probiotic FM triggered an abnormal inflammatory response to DSS-induced colitis.

The critical role of VDR in the anti-inflammatory outcomes of probiotic interventions has hardly been reported. In the study conducted by Lu et al. (2020),

conditional media from *Lactobacillus paracasei* DKL121, a lactic acid bacteria isolated from Korean kimchi, increased the *in vitro* VDR expression of human colon cancer cells (HCT116) at RNA and proteins level, while improved autophagic response and cathelicidin gene expression. Furthermore, pre-treatment with the probiotic showed a protective effect against inflammation (LU et al., 2020). Similarly, Wu et al. (2015a) reported that *Lactobacillus rhamnosus* LGG and *Lactobacillus plantarum* improved the VDR and cathelicidin signaling *in vitro*, while *Lactobacillus plantarum* protected WT mice against *Salmonella* infection and improved Paneth cells functions. However, in VDRKO mice probiotic treatment caused a more intense inflammatory response, corroborating with the principle that VDR signaling is essential for probiotics anti-inflammatory effects (WU et al., 2015a).

The impaired intestinal barrier may have triggered the abnormal inflammation of the VDRKO mice that received the probiotic FM treatment when compared to the other DSS treated groups. Several studies reported that the lack of VDR drastically increases the vulnerability to DSS-induced colitis and the proper functions of VDR are critical for tissue recovery. In addition to higher levels of pro-inflammatory cytokines, the junctional proteins seemed to be very compromised in DSS-treated VDRKO mice, increasing gut permeability, whereas a possible bacteria translocation from the gut to the circulation may occur (FROICU; CANTORNA, 2007; HE et al., 2018; KONG et al., 2008; OOI et al., 2013). In fact, claudin-2 is overexpressed in VDRKO mice under inflammation, which may explain the impaired gut barrier in association with a higher IL-6 level (ZHANG et al., 2019). The IL-6 is directly related to chronic inflammatory diseases and bacterial infection. Meanwhile, its overexpression may inhibit Treg (regulatory T cell) differentiation, impairing the immune response (KISHIMOTO, 2005; ROSE-JOHN, 2020; TANAKA; NARAZAKI; KISHIMOTO, 2012). Therefore, we hypothesized that the probiotic bacteria present in the FM could have been translocated, becoming a threat to VDRKO mice, aggravating the body weight loss and the inflammation biomarkers.

As expected, the fecal level of lipocalin 2 (LCN2) has substantially increased with DSS treatment as well. Fecal LCN2 has been shown to be a sensitive and noninvasive inflammation marker related to bacterial infection (CHASSAING et al., 2012; LU et al., 2019; STALLHOFER et al., 2015). In the present study, we observed that LCN2 concentration was slightly higher in WT that received milk or FM when compared those on PBS, with or without DSS treatment. Nevertheless, in VDRKO mice, the effect of probiotic in increasing LCN2 was more pronounced. Curiously, Chiang et al. (2012) reported that the

downregulation of VDR reduced the effect of vitamin D in suppressing the LCN2 expression and tumorigenesis in an *in vitro* cancer model, suggesting that LCN2 may be regulated by VDR.

An important mechanism of action of probiotics in the gut is the metabolization of dietary components into SCFA, such as butyrate, propionate, and acetate. These compounds may improve several functions related to the gut epithelium and anti-inflammatory responses (PEARCE et al., 2020; SANDERS et al., 2018; SANDERS et al., 2019). Therefore, an interesting alternative approach for VDR deficient target groups would be the administration of beneficial metabolites, such as butyrate, as a pre-treatment prior probiotics intervention, aiming to enhance the gut barrier integrity, and possibly avoid translocation and aggravated inflammation/infection. Indeed, the depletion of VDR exclusively in the intestinal epithelial cells leads to a shift in the gut microbiota, decreasing the population of butyrate-producer bacteria. On the other hand, the administration of butyrate may enhance the VDR expression while regulates autophagy, Paneth cells, and inflammation (WU et al., 2015b). In addition, butyrate may increase the expression of lysozyme and boost the barrier integrity (PEARCE et al., 2020).

It is noteworthy that milk itself showed an improvement in the VDR expression and IL-6 level in WT mice. Dairy products are the major vitamin D fortified food, and the administration of vitamin D appeared to improve immune functions and shape the gut microbiota, ameliorating Inflammatory bowel diseases (IBD) symptoms. Likewise, *in vivo* studies have reported that vitamin D may reduce the severity of induced colitis while enhancing gut barrier integrity (ZHU et al., 2015). Thus, we believed these facts could explain why the animals that received only milk showed these positive outcomes when compared to the PBS control group. On the other hand, intestinal inflammation is usually associated with nutrients malabsorption problems, and the vitamin D amount present in milk or FM was not sufficient to confer a protective effect against DSS colitis (CANTORNA, 2016).

The health outcomes regarding the administration of probiotics in IBD are still inconsistent and an official recommendation does not exist yet (WU et al., 2015a). VDR is usually downregulated in IBD patients. Pre-disposed individuals present an atypical immune reaction to commensal bacteria, in addition to increased gut permeability and gut microbiota dysbiosis (LEVINE; SIGALL BONEH; WINE, 2018; RYAN et al., 2020). Zhang, Xia & Sun (2020) showed that the VDR signaling may be assessed using fecal samples, which is a noninvasive exam and could offer a promising diagnostic tool. Therefore, we suggest that

VDR functions should be assessed prior to probiotic interventions, which will allow a more precise and safe treatment recommendation.

Hence, the present study reinforces that VDR plays a critical role in the potential anti-inflammatory effects of probiotics. To the best of our knowledge, this is the first study evaluating the influence of a food matrix as a probiotic vehicle in the VDR functions. Our study has some limitations that should be addressed in future studies, such as the need to increase the number of VDRKO animals, including the evaluation of probiotics in VDRKO mice without DSS treatment, histological studies, expression of tight junction proteins, and other pro-inflammatory and anti-inflammatory biomarkers. We encourage studies with different probiotic genus and strains and/or food matrices (e.g. plant-based products or milk without vitamin D fortification), which will certainly contribute to elucidate the probiotic health benefits in anti-inflammation.

5 CONCLUSIONS

The probiotic FM produced with the co-culture *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19 has a promising anti-inflammatory potential and may improve VDR signaling. Meanwhile, the lack of VDR may trigger an abnormal inflammatory response to probiotic FM treatment.

REFERENCES

BALLAN, R.; BATTISTINI, C.; XAVIER-SANTOS, D.; SAAD, S. M. I. **Interactions of probiotics and prebiotics with the gut microbiota**. In: Jun Sun (ed.), The microbiome in health and disease. Series title: Progress in molecular biology and translational sciences (PMBTS). Oxford: Elsevier, v. 171, p. 265-300, 2020.

CANTORNA, M. T. IBD: Vitamin D and IBD: Moving towards clinical trials. **Nature Reviews Gastroenterology & Hepatology**, v. 13, p. 322-323, 2016.

CHAMPAGNE, C. P.; CRUZ, A. G.; DAGA, M. Strategies to improve the functionality of probiotics in supplements and foods. **Current Opinion in Food Science**, v. 22, p. 160-166, 2018.

CHASSAING, B.; SRINIVASAN, G.; DELGADO, M. A.; YOUNG, A. N.; GEWIRTZ, A. T.; VIJAY-KUMAR, M. Fecal Lipocalin 2, a Sensitive and Broadly Dynamic Non-Invasive Biomarker for Intestinal Inflammation. **PLoS One**, v. 7, n. 9, p. e44328, 2012.

CHATTERJEE, I.; LU, R.; ZHANG, Y. G.; ZHANG, J.; DAI, Y.; XIA, Y.; SUN, J. Vitamin D receptor promotes healthy microbial metabolites and microbiome. **Scientific Reports**, v. 10, p. 7340, 2020.

CHIANG, K. C.; YEH, C. N.; LIN, K. J.; SU, L. J.; YEN, T. C.; PANG, J. H. S.; KITTAKA, A.; SUN, C. C.; CHEN, M. F.; JAN, Y. Y.; CHEN, T. C.; JUANG, H. H.; YEH, T. A. Chemopreventive and chemotherapeutic effects of dietary supplementation of vitamin D on cholangiocarcinoma in a Chemical-Induced animal model. **Oncotarget**, v. 5, p. 3849–3861, 2014.

DOMÍNGUEZ DÍAZ, L.; FERNÁNDEZ-RUIZ, V.; CÁMARA, M. The frontier between nutrition and pharma: The international regulatory framework of functional foods, food supplements and nutraceuticals. **Critical Reviews in Food Science and Nutrition**, v. 60, n. 10, p. 1738–1746, 2020.

FROICU, M.; CANTORNA, M. T. Vitamin D and the vitamin D receptor are critical for control of the innate immune response to colonic injury. BMC Immunology, v. 8, p. 5, 2007.

HE, L.; LIU, T.; SHI, Y.; TIAN, F.; HU, H.; DEB, D. K.; CHEN, Y.; BISSONNETTE, M.; LI, Y. C. Gut epithelial Vitamin D receptor regulates microbiota-dependent mucosal inflammation by suppressing intestinal epithelial cell apoptosis. **Endocrinology**, v. 159, n. 2, p. 967, 979, 2018.

HILL, C.; GUARNER, F.; REID, G.; GIBSON, G. R.; MERENSTEIN, D. J.; POT, B.; MORELLI, L.; CANANI, R. B.; FLINT, H. J.; SALMINEN, S.; CALDER, P. C.; SANDERS, M. E. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. **Nature Reviews Gastroenterology & Hepatology,** v. 11, p. 506 – 514, 2014.

JIN D.; ZHANG, Y. G.; WU, S.; LU, R.; LIN, Z.; ZHENG, Y.; CHEN, H.; CS-SZABO, G.; SUN, J. Vitamin D receptor is a novel transcriptional regulator for Axin1. **The Journal of Steroid Biochemistry and Molecular Biology**, v. 165, p. 430-437, 2017.

KISHIMOTO, T. INTERLEUKIN-6: From basic science to medicine - 40 Years in immunology. **Annual Review of Immunology**, v. 23, p. 1–21, 2005.

KONG, J.; ZHANG, Z.; MUSCH, M. W.; NING, G.; SUN, J.; HART, J.; BISSONNETTE, M.; LI, Y. C. Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. **American Journal of Physiology - Gastrointestinal and Liver Physiology**, v. 294, n. 1, p. G208-G216, 2008.

LEVINE, A.; SIGALL BONEH, R.; WINE, E. Evolving role of diet in the pathogenesis and treatment of inflammatory bowel diseases. **Gut**, v. 67, n. 9, p. 1726-1738, 2018.

LU, R.; SHANG, M.; ZHANG, Y. G.; JIAO, Y.; XIA, Y.; GARRETT, S.; BAKKE, D.; BÄUERL, C.; MARTINEZ, G. P.; KIM, C. H.; KANG, S. M.; SUN, J. Lactic Acid Bacteria Isolated From Korean Kimchi Activate the Vitamin D Receptor-autophagy Signaling Pathways. **Inflammatory Bowel Diseases**, v. 26, n. 8, p. 1199-1211, 2020.

LU, R.; ZHANG, Y. G.; XIA, Y.; SUN, J. Imbalance of autophagy and apoptosis in intestinal epithelium lacking the vitamin D receptor. **The FASEB Journal**, v. 33, n. 11, p. 11845-11856, 2019.

OGBU, D.; BAKKE, D.; SUN, J. Myeloid vitamin D receptor influences cell proliferation in small intestine. **The FASEB Journal**, v. 34, n. S1, 2020. doi: 10.1096/fasebj.2020.34.s1.09382.

OGBU, D.; XIA, E.; SUN, J. Gut instincts: vitamin D/vitamin D receptor and microbiome in neurodevelopment disorders. **Open Biology**, v. 10, p. 200063, 2020.

OOI, J. H.; CHEN, J.; CANTORNA, M. R. Vitamin D regulation of immune function in the gut: Why do T cells have vitamin D receptors? **Molecular Aspects of Medicine**, v. 33, n. 1, p. 77-82, 2012.

OOI, J. H.; LI, Y.; ROGERS, C. J.; CANTORNA, M. T. Vitamin D Regulates the Gut Microbiome and Protects Mice from Dextran Sodium Sulfate – Induced Colitis. **The Journal of Nutrition**, v. 143, p. 1679–1686, 2013.

PÁPAI, G.; TORRES-MARAVILLA, E.; CHAIN, F.; VARGA-VISI, E.; ANTAL, O.; NAÁR, Z.; BERMÚDEZ-HUMARÁN, L. G.; LANGELLA, P.; MARTÍN, R. The Administration Matrix Modifies the Beneficial Properties of a Probiotic Mix of *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus acidophilus* LA-5. Probiotics and Antimicrobial Proteins, In press. 2020. doi: 10.1007/s12602-020-09702-2

PEARCE, S. C.; WEBER, G. J.; VAN SAMBEEK, D. M.; SOARES, J. W.; RACICOT, K.; BREAULT, D. T. Intestinal enteroids recapitulate the effects of short-chain fatty acids on the intestinal epithelium. **PLoS One**, v. 15, n. 4 , p. e0230231, 2020.

ROSE-JOHN, S. Interleukin-6 signalling in health and disease. **F1000Research**, v. 9, p. 1013, 2020

RYAN, F. J.; AHERN, A. M.; FITZGERALD, R. S.; LASERNA-MENDIETA, E. J.; POWER, E. M.; CLOONEY, A. G.; O'DONOGHUE, K. W.; MCMURDIE, P. J.; IWAI, S.; CRITS-CHRISTOPH, A.; SHEEHAN, D.; MORAN, C.; FLEMER, B.; ZOMER, A. L.; FANNING, A.; O'CALLAGHAN, J.; WALTON, J.; TEMKO, A.; STACK, W.; JACKSON, L.; JOYCE, S. A.; MELGAR, S.; DESANTIS, T. Z.; BELL, J. T.; SHANAHAN, F.; CLAESSON, M. J. Colonic microbiota is associated with inflammation and host epigenomic alterations in inflammatory bowel disease. **Nature Communications**, v. 11, p. 1512, 2020.

SANDERS, M. E.; BENSON, A.; LEBEER, S.;, MERENSTEIN, D. J.; KLAENHAMMER, T. R. Shared mechanisms among probiotic taxa: implications for general probiotic claims. **Current Opinion in Biotechnology**, v. 49, p. 207-2016, 2018.

SANDERS, M. E.; MERENSTEIN, D. J.; REID, G.; GIBSON, G. R.; RASTALL, R. A. Probiotics and prebiotics in intestinal health and disease: from biology to the clinic**. Nature Reviews Gastroenterology & Hepatology**, v. 16, p. 605-6016, 2019.

STALLHOFER, J.; FRIEDRICH, M.; KONRAD-ZERNA, A.; WETZKE, M.; LOHSE, P.; GLAS, J.; TILLACK-SCHREIBER, C.; SCHNITZLER, F.; BEIGEL, F.; BRAND, S. Lipocalin-2 Is a Disease Activity Marker in Inflammatory Bowel Disease Regulated by IL-17A, IL-22, and TNF-α and Modulated by IL23R Genotype Status. **Inflammatory Bowel Disease**, v. 21, n. 10, p. 2327–2340, 2015

TANAKA, T.; NARAZAKI, M.; KISHIMOTO, T. Therapeutic targeting of the interleukin-6 receptor. **Annual Review of Pharmacology and Toxicology**, v. 52, p. 199–219, 2012.

VELDMAN, C. M.; CANTORNA, M. T.; DELUCA, H. F. Expression of 1,25-dihydroxyvitamin D3 receptor in the immune system. **Archives of Biochemistry and Biophysics**, v. 374, n. 2, p. 334-338, 2000.

WANG, J.; THINGHOLM, L. B.; SKIECEVIČIENĖ, J.; RAUSCH, P.; KUMMEN, M.; HOV, J. R.; DEGENHARDT, F.; HEINSEN, F. A.; RÜHLEMANN, M. C.; SZYMCZAK, S.; HOLM, K.; ESKO, T.; SUN, J.; PRICOP-JECKSTADT, M.; AL-DURY, S.; BOHOV, P.; BETHUNE, J.; SOMMER, F.; ELLINGHAUS, D.; BERGE, R. K.; HÜBENTHAL, M.; KOCH, M.; SCHWARZ, K.; RIMBACH, G.; HÜBBE, P.; PAN, W. H.; SHEIBANI-TEZERJI, R.; HÄSLER, R.; ROSENSTIEL, P.; D'AMATO, M.; CLOPPENBORG-SCHMIDT, K.; KÜNZEL, S.; LAUDES, M.; MARSCHALL, H. U.; LIEB, W.; NÖTHLINGS, U.; KARLSEN, T. H.; BAINES, J. F.; FRANKE, A. Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. **Nature Genetics**, v. 48, p. 1396-1406, 2016.

WU, S.; YOON, S.; ZHANG, Y. G.; LU, R.; XIA, Y.; WAN, J.; PETROF, E. O.; CLAUD, E. C.; CHEN, D.; SUN, J. Vitamin D receptor pathway is required for probiotic protection in colitis. **American Journal of Physiology - Gastrointestinal and Liver Physiology**, v. 309, n. 5, p. G341-G349, 2015a.

WU, S.; ZHANG, Y.; LU, R.; XIA, Y.; ZHOU, D.; PETROF, E. O.; CLAUD, E. C.; CHEN, D.; CHANG, E. B.; CARMELIET, G.; SUN, J. Intestinal epithelial vitamin D receptor deletion leads to defective autophagy in colitis. **Gut**, v.64, n. 7, p. 1082–1094, 2015b.

ZHANG, Y. G.; LU, R.; WU, S.; CHATTERJEE, I.; ZHOU, D.; XIA, Y.; SUN, J. Vitamin D Receptor Protects Against Dysbiosis and Tumorigenesis via the JAK/STAT Pathway in Intestine. **Cellular and Molecular Gastroenterology and Hepatology**, v. 10, n. 4, p. 729-746, 2020a.

ZHANG, Y. G.; LU, R.; XIA, Y.; ZHOU, D.; PETROF, E.; CLAUD, E. C.; SUN, J. Lack of Vitamin D receptor leads to hyperfunction of claudin-2 in intestinal inflammatory responses. **Inflammatory Bowel Diseases**, v. 25, n. 1, p. 97-110, 2019.

ZHANG, Y. G.; LU, R.; ZHANG, J.; GARRETT, S.; SUN, J.; XIA, Y. 1019 Vitamin D Receptor Protects Intestinal Barriers Against Tumorigenesis. **Gastroenterology**, v. 158, n. 6, Supplement 1, p. S-204, 2020b.

ZHANG, Y. G.; WU, S.; LU, R.; ZHOU, D.; ZHOU, J.; CARMELIET, G.; PETROF, E.; CLAUD, E. C.; SUN, J. Tight junction CLDN2 gene is a direct target of the vitamin D receptor. **Scientific Reports**, v. 5, p.10642, 2015.

ZHANG, Y. G.; XIA, Y.; SUN, J. A simple and sensitive method to detect vitamin D receptor expression in various disease models using stool samples. Genes & Diseases, In press. 2020. doi: 10.1016/j.gendis.2020.03.002.

ZHU, T.; LIU, T. J.; SHI, Y. Y.; ZHAO, Q. Vitamin D/VDR signaling pathway ameliorates 2,4,6-trinitrobenzene sulfonic acid-induced colitis by inhibiting intestinal epithelial apoptosis. **International Journal of Molecular Medicine**, v. 35, n. 5, p.1213–1218, 2015.

ZMORA, N.; ZILBERMAN-SCHAPIRA, G.; SUEZ J.; MOR, U.; DORI-BACHASH, M.; BASHIARDES, S.; KOTLER, E.; ZUR, M.; REGEV-LEHAVI, D.; BRIK, R. B. Z.; FEDERICI, S.; COHEN, Y.; LINEVSKY, R.; ROTHSCHILD, D.; MOOR, A. E.; BEN-MOSHE, S.; HARMELIN, A.; ITZKOVITZ, S.; MAHARSHAK, N.; SHIBOLET, O.; SHAPIRO, H.; PEVSNER-FISCHER, M.; SHARON, I.; HALPERN, Z.; SEGAL, E.; ELINAV, E. Personalized Gut Mucosal Colonization Resistance to Empiric Probiotics Is Associated with Unique Host and Microbiome Features. **Cell**, v. 174, n. 6, p. 1388-1405, 2018.

GENERAL CONCLUSIONS

In this study, we explored the application of brewer's spent grain (BSG), a beer industry by-product, as a potential prebiotic ingredient in fermented milk with probiotic strains. The BSG revealed to be an interesting ingredient rich in fiber that may improve the resistance of potentially probiotic strains in fermented milk to gastrointestinal (GI) stress simulated *in vitro*. Furthermore, we showed that *Streptococcus thermophilus* TH-4, in addition to its starter feature, has potential as probiotic strain when in combination with BSG and/or *Lactobacillus paracasei* subsp. *paracasei* F19. The fermented milk with *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19 has a promising anti-inflammatory potential against DSS-induced colitis and may improve the vitamin D (VDR) expression. Nevertheless, we observed that the lack of VDR aggravated the inflammation after probiotic treatment.

Our study has some limitations that should be addressed in future studies, such as increase the sample size of VDR knockout mice, perform histological studies, and explore the expression of tight junction proteins and other inflammation biomarkers. We encourage studies with different probiotic genus and/or food matrices (e.g. plant-based products or milk without vitamin D fortification), which will contribute to elucidate the probiotic health benefits related to the VDR.

ATTACHMENTS

Attachment 1. Proof of publication (book chapter): Probiotic, Vitamin D, and Vitamin D Receptor in Health and Disease

LACTIC ACID BACTERIA
A Functional Approach

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6

Probiotics, Vitamin D, and Vitamin D Receptor in Health and Disease

Carolina Battistini,[1,2] *Najib Nassani*,[3] *Susana MI Saad*[1,2] and *Jun Sun*[3,*]

Introduction

The gut microbiome, "a newly discovered organ" of the body, plays a critical role in immunity and metabolism in the host's healthy and diseased states. Microbiome products released in the gut, e.g., short chain fatty acids, may reach and influence the function of organs and systems beyond the intestinal tract. Environmental factors, lifestyle, age, and sex influence the profile and function of the microbiome. Its imbalance, termed 'dysbiosis', is directly related to the development of various human diseases (Wang et al. 2016, Costea et al. 2018, Salvucci 2019).

Vitamin D (VitD) and the vitamin D receptor (VDR) are involved in many functions of the body, including but not limited to calcium absorption, immunity, glucose and liver metabolism, in addition to gut microbiota modulation. For example, VitD deficiency and the lack or low expression of the VDR are directly related to inflammatory bowel diseases (IBD), and the treatment with VitD supplements might be effective in some cases (Haussler et al. 2013, Ooi et al. 2013, Wu et al. 2015a,b, National Institute of Health 2016, Celiberto et al. 2018). Therefore, VDR could be

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Attachment 2. Review article under submission: The Potential Use of Vitamin D as an Alternative Approach for Gut Microbiota Modulation in Inflammatory Bowel Disease

To be submitted

The Potential Use of Vitamin D as an Alternative Approach for Gut Microbiota Modulation in Inflammatory Bowel Disease

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Conflicts of interest: None of the authors have conflicts of interest.

Attachment 3. Scientific article under submission: Brewer's spent grain enhanced the recovery of potentially probiotic strains in fermented milk after exposure to *in vitro*-simulated gastrointestinal conditions

To be submitted to

Brewer's spent grain enhanced the recovery of potentially probiotic strains in fermented milk after exposure to *in vitro*-simulated gastrointestinal conditions

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Running Title: Brewer's spent grain increased probiotic resistance in fermented milk.

Attachment 4. Proof of Ethical Committee Approval for the animal experiments and certificates of related training

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

September 6, 2018

Jun Sun
Medicine/Gastroenterology and Hepatology
M/C 716

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

Dear Dr. Sun:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on ***09/05/2018***.

Title of Application: **How Vitamin D Receptor Influences Intestinal Barriers**

ACC Number: **17-218**

Modification Number: **06**

Nature of Modification: **Personnel Added: Carolina Battistini**

Condition of Initiation: ***New personnel added must complete facility orientation/zoonotic training and enter the UIC Occupational Health Program for Individuals with Animal Contact prior to initiation of any work with animals. For rodents, contact Dr. Jeanette Purcell (996-7051) for BRL, BBC, MBRB, COD, SES, and all satellites), Dr. Cynthia Adams for the mouse barrier in COMRB (996-9236), or Ms. Kelly Pavlik for BSB (996-7810). For large animal areas of the BRL and COMRB, contact Dr. Kelly Garcia (996-8619). For primates, contact Dr. Lisa Halliday (996-9453). Facility access will not be granted until this condition is completed.***

Protocol Approved: **2/5/2018**

Current Approval Period: **2/5/2018 to 12/19/2018.** ***Protocol is eligible for 2 additional years of renewal prior to expiration and resubmission.***

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*How Vitamin D Receptor Influences Intestinal Barriers (Institutional # 00364828)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 DK114126 (A1 v., yrs 1-5)	*Funded*		*UIC*	*Jun Sun*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

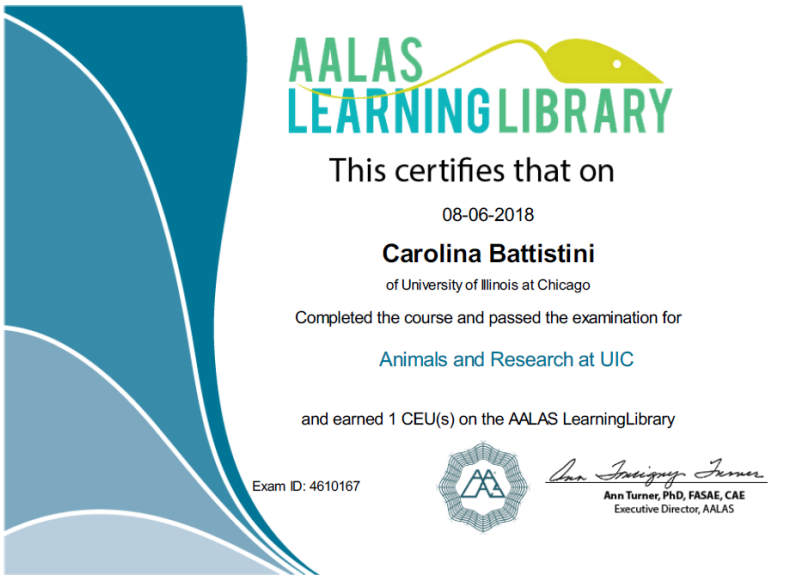
Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Mary B Bowman PhD

Mary B Bowman, PhD
Director, OACIB
mbb /ss
cc: BRL, ACC File, Yong-Guo Zhang, Rong Lu, Danika Bakke

Phone (312) 996-1972 • Fax (312) 996-9088



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This certifies that on

08-06-2018

Carolina Battistini

of University of Illinois at Chicago

Completed the course and passed the examination for

Animals and Research at UIC

and earned 1 CEU(s) on the AALAS LearningLibrary

Exam ID: 4610167

Ann Turner, PhD, FASAE, CAE
Executive Director, AALAS

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Annual Biohazard Training for Investigative Staff Using ABSL-2 Agents in Rodents

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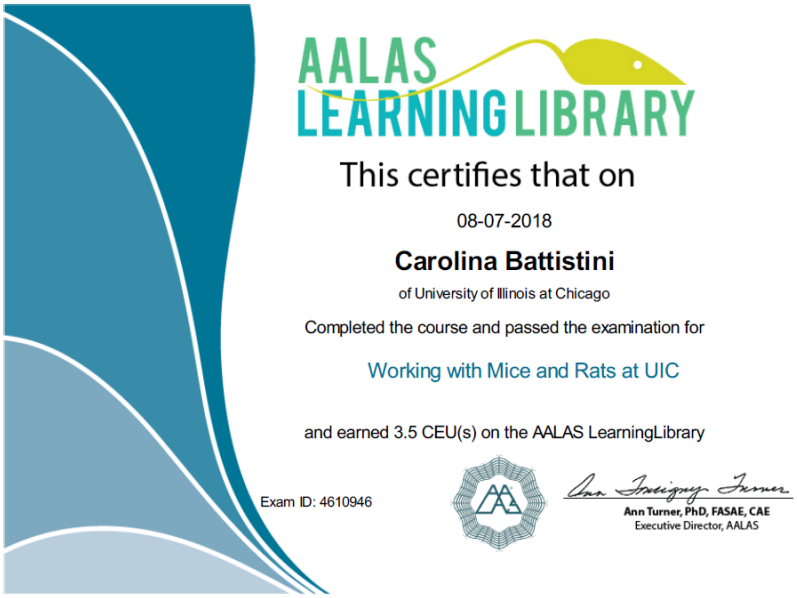
Ann Turner, PhD, FASAE, CAE
Executive Director, AALAS



AALAS LEARNING LIBRARY

This certifies that on

08-07-2018

Carolina Battistini

of University of Illinois at Chicago

Completed the course and passed the examination for

Working with Mice and Rats at UIC

and earned 3.5 CEU(s) on the AALAS LearningLibrary

Exam ID: 4610946

Ann Turner, PhD, FASAE, CAE
Executive Director, AALAS

Attachment 5. Proof of publication (book chapter related to the thesis): Interactions of probiotics and prebiotics with the gut microbiota

CHAPTER NINE

Interactions of probiotics and prebiotics with the gut microbiota

Rafael Ballan[a,b], Carolina Battistini[a,b], Douglas Xavier-Santos[a,b,c], Susana Marta Isay Saad[a,b,*]

[a]Department of Pharmaceutical and Biochemical Technology, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil
[b]Food Research Center, University of São Paulo, São Paulo, SP, Brazil
[c]Department of Civil, Chemical and Environmental Engineering, University of Genoa, Pole of Chemical Engineering, Genoa, Italy
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Contents

Abstract

The gut microbiota (GM) composition varies among individuals and is influenced by intrinsic (genetics, age) and extrinsic (environment, diet, lifestyle) factors. An imbalance or dysbiosis is directly associated with the development of several illnesses, due to the

Progress in Molecular Biology and Translational Science, Volume 171
ISSN 1877-1173
https://doi.org/10.1016/bs.pmbts.2020.03.008

Attachment 6. Abstracts published and presented in scientific meeting related to the thesis

V International Symposium on Lactic Acid Bacteria – SIBAL 2016

152 EVALUATION OF A BY-PRODUCT FROM BEER INDUSTRY AS SUBSTRATE FOR GROWTH OF PROBIOTIC AND STARTER BACTERIA

C. Battistini, R. Bedani, S. M. I. Saad

Faculdade de Ciências Farmacêuticas, Universidade de São Paulo. Av. Prof. Lineu Prestes, 580 05508-000 - São Paulo, SP, Brasil. E-mail:carolina.battistini@usp.br

In the beer manufacturing, after the malting and brewing processes, the slurry obtained is filtered. Afterwards, the liquid fraction is fermented for beer production, whilst the solid fraction is the main by-product generated by the beer industries, called brewer's spent grain (BSG). The BSG is rich in protein, fibre (mainly arabinoxylan, lignin, and cellulose), and phenolic compounds. When disposed, BSG may become an environmental problem; however, due to its relatively low cost and nutritive properties, this by-product has attracted the attention of researchers. Until now, to the best of our knowledge, BSG has only been used in animal feed and in bakery products. Nevertheless, BSG could be an inexpensive raw material with potential human health benefits when incorporated in different foods, including fermented products. Thus, the aim of this study was to evaluate the BSG flour as a substrate for growth of the probiotic strains *Bifidobacterium (B.) animalis* subsp. *lactis* BB-12, *B. longum* BB-46, *B. longum* subsp. *infantis* BB-02, *Lactobacillus (Lb.) acidophilus* LA5, *Lb. rhamnosus* GR1, *Lb. rhamnosus* LGG, *Lb. paracasei* subsp. *paracasei, L. casei* 431, *Lb. paracasei* subsp. *paracasei* F19, and *Streptococcus (St.) thermophilus* TH4, and the starter culture *St. thermophilus* STM6. Modified MRS broth containing phenol red (as indicator) and BSG flour (1 g·100 mL^{-1}) was inoculated with 45 log $CFU \cdot mL^{-1}$ of each strain individually, and then incubated at 37 °C. The counts of each microorganism were determined on selective agar before (0 h) and after 24 h and 48 h of aerobic incubation at 37°C. After 24 h, the strains LA5, *L. casei* 431 and GR1 showed the highest viable cells counts, 8.0 ± 0.2, 8.1 ± 0.1, and 8.3 ± 0.02 log $CFU \cdot mL^{-1}$, respectively. On the other hand, the populations of LGG, BB02, BB-12, BB-46, and F19 were approximately 7.4 log $CFU \cdot mL^{-1}$ for the same period. Regarding streptococci strains, STM6 showed higher population (7.9 ± 0.1 $CFU \cdot mL^{-1}$) than TH4 (4.7 ± 0.08 $CFU \cdot mL^{-1}$) after 24 h. Overall, the populations of probiotic and starter strains kept stable after 48 h. Therefore, BSG showed great potential as substrate for the growth of the probiotic and starter microorganisms tested. However, according to our results, the ability to ferment this type of substrate was strain-dependent.

This is to certify that Carolina Battistini has attended to the

V International Symposium on LACTIC ACID BACTERIA

Benefitting from Lactic Acid Bacteria
Progress in Health and Food

Dr. Fernanda Mozzi
President

BICENTENARIO de la Independencia Argentina 1816 - TUCUMÁN - 2016

San Miguel de Tucumán, Tucumán, Argentina. October 19 - 21, 2016

29° Congresso Brasileiro de Microbiologia

TITLE: SELECTION OF STARTER AND PROBIOTIC CULTURES FOR APPLICATION IN A BLACKBERRY PROBIOTIC FERMENTED MILK SUPPLEMENTED WITH BEER'S INDUSTRY BY-PRODUCT

AUTHORS: BATTISTINI, C.; BEDANI, R.; SAAD, S. M. I.

INSTITUTION: FACULDADE DE CIÊNCIAS FARMACÊUTICAS, UNIVERSIDADE DE SÃO PAULO (AV. PROF. LINEU PRESTES, 580, CEP 05508-000, SÃO PAULO - SP, BRAZIL). FOOD RESEARCH CENTER, UNIVERSITY OF SÃO PAULO, SÃO PAULO, BRAZIL.

ABSTRACT:

Probiotic microorganisms and fibers can beneficially modulate the intestinal microbiota, improving the host's health and consequently reducing the risk for the development of diseases. In the beer production, is generated the brewer's spent grain, which has around 70% of fibers. The addition of brewer's spent grain flour (BSG) in probiotic fermented milk (FM) might enhance the beneficial properties to health of this type of functional food. Thus, this study evaluated the fermentability of BSG by starter and probiotic cultures and selected a suitable strain combination for the application in blackberry probiotic FM supplemented with BSG. Probiotic cultures *Bifidobacterium (B.) animalis* (BB-12), *B. longum* (BB-46 and BB-02), *Lactobacillus (L.) acidophilus* (LA-5), *L. rhamnosus* (GR-1 and LGG), *L. fermentum* (PCC), *L. reuteri* (RC-14), *L. paracasei* (431 and F19), and the starter cultures *Streptococcus thermophilus* (TH-4 and STM-6) were evaluated regarding their ability to ferment the BSG. The fermentability assay consisted of supplementing UHT skimmed milk (MILK) with 1 g·100 mL^{-1} of BSG, followed by inoculation with each strain individually. The counts of each microorganism were determined on selective agar before (0 h) and after 24 h after incubation at 37 °C. The two cultures that showed the highest growth in the presence of BSG were selected for FM production. Four FM formulations with blackberry pulp were assessed: FM1 (control); FM2 (with BSG); FM3 (with probiotic culture); FM4 (with probiotic culture and BSG); the starter culture was added in all formulations. The products were stored at 4 °C for 28 days and the viability of the microorganisms was determined weekly, using selective agar. Regarding fermentability assay, the strains that significantly increased their populations after 24 h of fermentation with BSG were BB-46, PCC, RC-14, F19, and LGG ($p<0.05$). However, TH-4, STM-6, and F19 showed the highest population after 24 h, with counts around 9.1 CFU·mL^{-1}. Thus, the co-culture chosen for application in blackberry probiotic FM was composed by F19 and TH-4. In the FM formulations, both TH-4 and F19 remained stable, respectively, above 8.0 and above 8.5 CFU·mL^{-1} over 28 days, and the addition of BSG did not influence their counts. Therefore, all the FM formulations tested have potential as probiotic fermented milks, since the probiotic populations were always above 10^{10} CFU in a daily portion of 100 mL of the FM.

Keywords: blackberry, brewer's spent grain, fiber, functional food, probiotic

Financial support: FAPESP (Project #2013/50506-8) and CAPES

VITAMIN D RECEPTOR CONTRIBUTES TO THE HEALTH BENEFITS OF PROBIOTIC CONSUMPTION

Carolina Battistini [1] - Yong-guo Zhang [2] - Ishita Chatterjee [2] - Rong Lu [2] - Jilei Zhang [2] - Susana M I Saad [1] - Jun Sun [2]

University of São Paulo, School of Pharmaceutical Sciences / Department of Pharmaceutical and Biochemical Technology / FoRC - Food Research Center, São Paulo, Brazil [1] - University of Illinois at Chicago, Department of Medicine, Chicago, United States [2]

Objective

Inflammatory bowel disease affect millions worldwide. Probiotics are known to improve inflammatory conditions by modulating gut microbiota, however, the exact mechanisms involved are not well-understood. Meanwhile, vitamin D receptor (VDR), besides mediation of vitamin D functions, is involved in cell differentiation, growth, anti-inflammatory actions, and a key factor for shaping gut microbiome. This work aimed to evaluate the impact of probiotic fermented milk (FM) on the inflammatory responses and expression of VDR *in vivo*

Methods

Probiotic FM was produced with the co-culture *Streptococcus termophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19. Animal experiments were performed with wild-type (WT) and VDR knockout (VDRKO) C57BL/7 mice in a dextran sulfate sodium (DSS) colitis model (5% in drinking water 24h after first gavage). PBS or FM were gavaged (100 microliter) daily for 7 days.

Results

Probiotic FM showed an anti-inflammatory effect only for WT mice, worsening the inflammation in VDRKO mice. After DSS treatment, IL-6 level was significantly lower in the WT-FM+DSS group when compared with WT-PBS+DSS ($p<0.05$). In contrast, for VDRKO mice, the IL-6 levels were dramatically high in VDRKO-FM+DSS group when compared with VDRKO-PBS+DSS ($p<0.05$). Moreover, at mRNA level, FM increased the VDR relative expression in colon cells when compared with control groups.

Connclusions

The probiotic FM produced with the co-culture *Streptococcus thermophilus* TH-4 and *Lactobacillus paracasei* subsp. *paracasei* F19 presented a promising anti-inflammatory potential against DSS induced colitis in mice, but VDR expression is needed. Therefore, enhancing VDR levels may contribute to potential health benefits driven by probiotic consumption.

10TH PROBIOTICS, PREBIOTICS & NEW FOODS, NUTRACEUTICALS AND BOTANICALS for NUTRITION & HUMAN and MICROBIOTA HEALTH

ROME, SEPTEMBER 8-10 2019
UNIVERSITÀ URBANIANA

Rome, October 1, 2019

This is to certify that Carolina Battistini has attended to the 10th Probiotics, Prebiotics & New Foods and presented the work entitled "VITAMIN D RECEPTOR CONTRIBUTES TO THE HEALTH BENEFITS OF PROBIOTIC CONSUMPTION", accepted as oral communication

The authors are:
Carolina Battistini[1,2], Yong-guo Zhang[3], Ishita Chatterjee[3], Rong Lu[3], Jilei Zhang[3], Susana M I Saad[1,2], Jun Sun[3]

[1]Department of Pharmaceutical and Biochemical Technology, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil
[2]Food Research Center, University of São Paulo, São Paulo, SP, Brazil
[3]Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA

The Organizing Secretariat

ADDITIONAL FILES

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial

FICHA DO ALUNO

9131 - 8725570/2 - Carolina Battistini

Email:	carolina.battistini@usp.br
Data de Nascimento:	24/10/1987
Cédula de Identidade:	RG - 44.351.692-3 - SP
Local de Nascimento:	Estado de São Paulo
Nacionalidade:	Brasileira
Graduação:	Engenheiro - Escola de Engenharia Mauá - Instituto Mauá de Tecnologia - São Paulo - Brasil - 2012
Graduação:	Engenheiro - Escola de Engenharia Mauá - Centro Universitário do Instituto Mauá de Tecnologia - São Paulo - Brasil - 2012
Mestrado:	Mestre em Engenharia de Processos Químicos e Bioquímicos (1) - Centro Universitário do Instituto Mauá de Tecnologia - São Paulo - Brasil - 2015

Curso:	Doutorado
Programa:	Ciência dos Alimentos
Área:	Bromatologia
Data de Matrícula:	07/12/2015
Início da Contagem de Prazo:	07/12/2015
Data Limite para o Depósito:	02/10/2020
Orientador:	Prof(a). Dr(a). Susana Marta Isay Saad - 07/12/2015 até o presente. Email: susaad@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 04/12/2015
Prorrogação(ões):	120 dias Período de 04/06/2020 até 02/10/2020
Data de Aprovação no Exame de Qualificação:	Aprovado em 11/12/2017
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 07/12/2015 Prorrogação em 11/10/2019

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor de 20/04/2013 até 28/03/2018).
Última ocorrência: Matrícula de Acompanhamento em 27/07/2020
Impresso em: 24/09/2020 16:46:00

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial

FICHA DO ALUNO

9131 - 8725570/2 - Carolina Battistini

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBA5758-1/1	Fundamentos do Planejamento Experimental e Otimização Simplex	26/01/2016	01/02/2016	30	2	100	A	N	Concluída
FBT5788-1/1	Aplicação de Alimentos Probióticos na Modulação de Imunidade de Mucosas	28/03/2016	17/04/2016	60	4	100	A	N	Concluída
FBA5752-1/3	Probióticos em Alimentos e Suas Implicações na Saúde Humana	05/04/2016	16/05/2016	60	4	100	A	N	Concluída
FBA5728-4/2	Aprimoramento Pedagógico	31/05/2016	27/06/2016	60	4	88	A	N	Concluída
FBT5781-5/1	Culturas Probióticas: Aplicações Tecnológicas	09/08/2016	15/09/2016	60	4	100	A	N	Concluída
Atividade do Programa	Participou da Etapa de Estágio Supervisionado em Docência do Programa de Aperfeiçoamento de Ensino junto à Disciplina FBT0530 - Física Industrial do Departamento Tecnologia Bioquímico-Farmacêutica, ministrada aos alunos de graduação do curso de Farmácia e Bioquímica da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (2)	01/07/2017	30/11/2017	-	3	-	-	-	-
FBA5896-7/2	Tópicos em Ciência dos Alimentos e Nutrição II	17/11/2017	25/01/2018	30	2	100	A	N	Concluída
Atividade do Programa	Publicação do Capítulo "Probiotics, Vitamin D, and Vitamin D Receptor in Health and Disease",de autoria de Carolina Battistini, Najib Nassani, Susana M. I. Saad, Jun Sun, no livro "Lactic acid bacteria: a functional approach", da editora CRC Press (Taylor & Francis), lançado em fevereiro de 2020. (3)	03/02/2020	03/02/2020	-	2	-	-	-	-

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	20	25
Estágios:			
Total:	0	20	25

Créditos Atribuídos à Tese: 172

Observações:

1) Curso com validade nacional, de acordo com o disposto na Portaria MEC nº 1.077, de 31.08.2012..

2) Créditos atribuídos de acordo com o disposto na Portaria GR-3588 e GR-4391 - PAE, de 31.08.09 e aprovados pela Comissão de Pós-Graduação, em Sessão de 06/06/2018.

3) Créditos atribuídos de acordo com o Artigo 60 do Regimento de Pós-Graduação e aprovados pela Comissão de Pós-Graduação, em Sessão de 15/04/2020.

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 27/07/2020
Impresso em: 24/09/2020 16:46:00

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http://www.esalq.usp.br

AGRADECIMENTO

O Curso de Graduação em Ciências dos Alimentos da ESALQ / USP

agradece a

Mestre Carolina Battistini

a valiosa contribuição que seus conhecimentos e experiência trouxeram aos alunos da disciplina LAN0415 – Alimentos Funcionais ao proferir a palestra

"Probióticos, Prebióticos e Fibras"

no dia 28 de Maio de 2020, das 19h00 às 22h20.

Profa. Dra. Aline Silva Mello Cesar
Responsável pela disciplina

FoRC
FOOD RESEARCH CENTER

CERTIFICADO

Certificamos que **CAROLINA BATTISTINI**

Ministrou palestra na Fundação Salvador Arena com o título "Microorganismos probióticos: conceitos e aplicações", São Paulo - SP, em 05 de novembro de 2019.

São Paulo, 06 de maio de 2020.

Dra. Bernadette D.G.M.Franco
Diretora FORC