UNIVERSITY OF SÃO PAULO
SCHOOL OF PHARMACEUTICAL SCIENCES
Post-Graduation Program in Pharmaceutical and Biochemical Technology
Food Technology

Characteristics of the gut microbiota and potential effects of probiotic supplements in individuals with type 2 diabetes *mellitus*

Rafael Ballan Maluhy

Dissertation presented for the Degree of Master of Science
Advisor: Full Prof. Susana Marta Isay Saad

São Paulo
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Rafael Ballan Maluhy

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Profa. Dra. Susana Marta Isay Saad
Advisor/President

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1st Examiner

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2nd Examiner

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3rd Examiner

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4th Examiner

São Paulo, _____, 2021.

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RESUMO

MALUHY, R.B. **Características da microbiota intestinal e potenciais efeitos de suplementos probióticos em indivíduos com diabetes *mellitus* tipo 2.** 2021. 95 p Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2021.

Com o avanço das pesquisas relacionadas ao uso dos probióticos e prebióticos na saúde humana nas últimas décadas, tem-se buscado avaliar seu impacto na microbiota intestinal e seu efeito terapêutico nas mais variadas doenças. A composição da microbiota intestinal varia entre indivíduos e é influenciada por fatores extrínsecos (estilo de vida, padrão alimentar, localização geográfica) e intrínsecos (idade, genética) e sua mudança tem sido associada com doenças metabólicas, doenças inflamatórias intestinais, doenças neurológicas, entre outras. Dentre essas doenças, destaca-se o diabetes *mellitus* tipo 2 (T2DM), que possui uma etiologia complexa e prevalência crescente em todo o mundo. A literatura atual indica que um desbalanço na composição microbiana intestinal (disbiose) está associada a um aumento de risco para T2DM. A microbiota intestinal de indivíduos com T2DM é caracterizada pela baixa diversidade, redução de bactérias produtoras de ácidos graxos de cadeia curta e bactérias produtoras de metabólitos de triptofano, além de um aumento na abundância de patógenos oportunistas, bactérias sintetizadoras de aminoácidos de cadeia ramificada (BCAA) e metabolizadoras de sulfato. Em conjunto, a produção de metabólitos e componentes bacterianos como trimetilamina, BCAAs, propionato de imidazol e lipopolissacarídeos desencadeiam respostas inflamatórias que contribuem para um aumento da resistência à insulina nessa população. A utilização de probióticos visa modular essas alterações, aumentando a diversidade microbiana, reduzindo a produção de metabólitos microbianos nocivos e reduzindo processos inflamatórios, podendo resultar em melhora de parâmetros metabólicos e antropométricos no T2DM. Apesar do crescimento expressivo de publicações neste tópico, existem controvérsias relevantes a respeito da eficácia, segurança e mecanismos de ação dos probióticos. Esse trabalho teve como objetivo revisar a bibliografia publicada, realizar uma análise crítica da literatura e, dentro das limitações dos estudos publicados, elucidar e consolidar o conhecimento científico disponível até o momento.

Palavras-chaves: diabetes *mellitus* tipo 2, microbiota intestinal, metabólitos microbianos, disbiose, probióticos.

ABSTRACT

MALUHY, R.B. **Characteristics of the gut microbiota and potential effects of probiotic supplements in individuals with type 2 diabetes *mellitus*.** 2021. 95 p Dissertation (Master of Science) – School of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2021.

With the advance of research related to the use of probiotics and prebiotics in human health in recent decades, their impact on the gut microbiota and their therapeutic effect on a variety of diseases has been explored. The composition of the gut microbiota varies between individuals and might be influenced by extrinsic (lifestyle, dietary pattern, geographic location) and intrinsic (age, genetics) factors, and its changes have been associated with metabolic diseases, inflammatory bowel diseases, neurological diseases, and other disorders. Among these diseases, type 2 diabetes mellitus (T2DM) stands out since it has a complex etiology and increasing prevalence worldwide. Current literature indicates that an imbalance in the intestinal microbial composition (dysbiosis) is associated with an increased risk for T2DM. The intestinal microbiota in individuals with T2DM is characterized by low diversity, reduction of short-chain fatty acid-producing bacteria, as well as in tryptophan metabolite-producing bacteria, in addition to an increase in the abundance of opportunistic pathogens, branched-chain amino acid-synthesizing bacteria (BCAA), and sulfate-metabolizing bacteria. Together, microbial metabolite production and bacterial components such as trimethylamine, BCAAs, imidazole propionate, and lipopolysaccharides trigger inflammatory responses that contribute to an increase in insulin resistance in this population. The use of probiotics aims to modulate the gut microbiota, increasing microbial diversity, reducing the production of harmful microbial metabolites, and reducing inflammatory processes, which may result in an improvement in metabolic and anthropometric parameters in T2DM. Despite the significant growth of publications on this topic, there are important controversies regarding the efficacy, safety, and mechanisms of action of probiotics. This dissertation aims to review the published bibliography, carry out a critical analysis of the literature, and, within the limitations of the published studies, elucidate and consolidate the scientific knowledge available so far.

Keywords: diabetes *mellitus* type 2, gut microbiota, microbial metabolites, dysbiosis, probiotics.

TABLES AND FIGURES LIST

ABBREVIATIONS

AKT – protein kinase b

ANVISA – Agência Nacional de Vigilância Sanitária

BA – bile acids

BCAA – branched-chain amino acids

BMI – body mass index

BSH – bile salt hydrolase

CRP – C-reactive protein

DCA – deoxycholic acid

DM – diabetes *mellitus*

DMG – gestational diabetes m*ellitus*

FBG – fasting blood glucose

FMO3 – flavin monooxygenase 3

FXR – farnesoid X receptor

GLP-1 – glucagon-like peptide 1

GM – gut microbiota

GPR – G-protein coupled receptor

HbA1c – hemoglobin A1c

HD – high dose

HDL – high density lipoprotein

HOMA-IR – Homeostatic Model Assessment for Insulin Resistance

IA – indoleacrylic acid

IAA – indoleacetic acid

IAId – indolealdehyde

IgA – immunoglobulin A

IL-1β – interleukin 1 beta

IL-6 – interleukin 6

ILA – indolelactic acid

ImP – imidazole propionate

IPA – indolepropionic acid

IR – insulin resistance

IRS-1 – insulin receptor substrate 1

ISAPP – International Scientific Association for Probiotics and Prebiotics

ISI – insulin sensitivity index

LCA – lithocholic acid

LD – low dose

LPS – lipopolysaccharides

mTORC1 – mechanistic target of rapamycin complex 1

N.s. – not significant

p62 – ubiquitin-binding protein p62

PAMPs – pathogen associated molecular patterns

PXR – pregnane X receptor

PYY – peptide YY

S6K1 – S6 kinase 1

SBP – systolic blood pressure

SCFA – short-chain fatty acids

SIRT1 – sirtuin 1

Skatole – 3-methyilindole

T2DM – type 2 diabetes *mellitus*

TC – total cholesterol

TG – triglycerides

TGR5 – G-protein-coupled bile acid receptor

TLR – toll-like receptors

TMA – trimethylamine

TMAO – trimethylamine N-oxide

TNF-α – tumor necrosis factor alpha

VDR – vitamin D3 receptor

WC – waist circunference

WHR – waist-to-hip ratio

Y – years

SUMMARY

PRESENTATION

In this Master of Science Dissertation, initially, an overview of the central topic of the project is presented, based on the scientific literature and current legislation, together with the justification of the project. This introduction is followed by an integrative review entitled: **"Characteristics of the gut microbiota and potential effects of probiotic supplements in individuals with type 2 diabetes *mellitus*".**

This review focuses on the potential effects of probiotic supplements on metabolic diseases, in this case, type 2 diabetes *mellitus*, aiming to identify the strains described in the scientific literature with effects on improving glucose metabolism, and the role of gut microbiota in the development of the disease.

Other scientific productions are attached and described below (attachment 3 and attachment 4).

Attachments:

1) Student record;
2) Curriculum Vitae Lattes;
3) First page of the article published, in 2021, in the scientific journal **International Journal of Molecular Sciences** (impact factor JCR 2019 = 4,556).

   - BATTISTINI, C.; **BALLAN, R.**; HERKENHOFF, M.E.; SAAD, S.M.I.; SUN, J. Vitamin D modulates intestinal microbiota in inflammatory bowel diseases. **International Journal of Molecular Sciences**, v.12, n.1, p.362-384, **2021**. [doi: 10.3390/ijms22010362; **ISSN:** 1422-0067]. Available at: https://www.mdpi.com/1422-0067/22/1/362

     ABSTRACT: Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract (GIT), including Crohn's disease (CD) and ulcerative colitis (UC), which differ in the location and lesion extensions. Both diseases are associated with microbiota dysbiosis, with a reduced population of butyrate-producing species, abnormal inflammatory response, and micronutrient deficiency (e.g., vitamin D hypovitaminosis). Vitamin D (VitD) is involved in immune cell differentiation, gut microbiota modulation, gene transcription, and barrier integrity. Vitamin D receptor (VDR) regulates the biological actions of the active VitD (1α,25-dihydroxyvitamin D3), and is involved in the genetic, environmental, immune, and microbial aspects of IBD. VitD deficiency is correlated with disease activity, and its administration

targeting a concentration of 30 ng/mL may have the potential to reduce disease activity. Moreover, VDR regulates functions of T cells and Paneth cells and modulates release of antimicrobial peptides in gut microbiota-host interactions. Meanwhile, beneficial microbial metabolites, e.g., butyrate, upregulate the VDR signaling. In this review, we summarize the clinical progress and mechanism studies on VitD/VDR related to gut microbiota modulation in IBD. We also discuss epigenetics in IBD and the probiotic regulation of VDR. Furthermore, we discuss the existing challenges and future directions. There is a lack of well-designed clinical trials exploring the appropriate dose and the influence of gender, age, ethnicity, genetics, microbiome, and metabolic disorders in IBD subtypes. To move forward, we need well-designed therapeutic studies to examine whether enhanced vitamin D will restore functions of VDR and microbiome in inhibiting chronic inflammation.

4) Chapter published, in 2020, of the book Jun Sun, ed. The microbiome in health and disease, from Elsevier.

- **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 science** (PMBTS)**.** Oxford: Elsevier, **2020**. v.171, p.265-300**.** Chap.9. Doi: 10.1016/bs.pmbts.2020.03.008; Available at: https://www.sciencedirect.com/science/article/pii/S1877117320300430

  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 potential increase in intestinal permeability leading to a systemic inflammation triggered by higher levels of circulating lipopolysaccharides and changes in the immune response caused by an overgrowth of a specific genus or of pathogens. These mechanisms may increase symptoms in gastrointestinal disorders or reduce glucose tolerance in metabolic diseases. Diet also has a significant impact on GM, and functional foods, namely prebiotics and probiotics, are a novel approach to reestablishing the indigenous microbiota. Prebiotics, like inulin and polyphenols, are selectively utilized by GM, releasing short-chain fatty acids (SCFA) and other metabolites which may reduce the intestinal lumen pH,

inhibit growth of pathogens, and enhance mineral and vitamin bioavailability. Probiotic microorganisms may increase the microbial diversity of GM and improve the integrity of the intestinal barrier, leading to an improvement of baseline and pathologic inflammation. In this chapter, we will discuss the potential roles of prebiotics and probiotics in health and diseases throughout and individual's lifetime and proposed mechanisms of action.

DISSERTATION PRESENTATION AND JUSTIFICATION

The term “microbiota” refers to the entire population of microorganisms that inhabit a given location, including bacteria, archaea, protozoa, fungi, and algae, whether it is an organ of the human body, food or the soil of a region (BERG *et al*., 2020). In the human body, the gut microbiota comprises the largest amount of bacterial genes, covering more than 2000 species (ALMEIDA *et al*., 2019). The composition of the gut microbiota is influenced by several factors, such as age, mode of birth and breast feeding, geographic location, and diet (DAVID *et al*., 2014; WANG *et al*., 2016).

Approximately 4 x 10^{13} bacteria comprise the human microbiota, which consists of a complex and dynamic ecosystem (SENDER; FUCHS; MILO, 2016). Due to the dynamism of microbial ecology, many authors have investigated how each factor (age, genetics, diet, diseases) influences the composition of the microbiota. It is known that, although bacterial genera are preserved over time, the relative abundance between them changes considerably, and even the use of antibiotics and the adoption of specific diets has a limited impact on the composition of the microbiota (RAJILIC-STOJANOVIC *et al*., 2012).

In the early 2000s, the gut microbiota was proposed to be an important environmental factor involved in the control of body mass and energy homeostasis (LEY *et al*., 2005; TURNBAUGH *et al*., 2006). The symbiosis relationship between microorganisms and host is based on the sharing of nutrients, such as the microbial processing of dietary components and the deposition of extracted energy in the host's adipose tissue (MORAN; SHANAHAN, 2014).

More recently, several authors have begun to investigate changes in the gut microbiota in various diseases. Some bacterial species have been associated with obesity, such as *Lactobacillus* and *Bifidobacterium* species, Prevotellaceae and *Blautia coccoides*, in addition to a high ratio between Firmicutes and Bacteroidetes (ALVAREZ-MERCADO *et al*., 2019). *Akkermansia muciniphila*, a bacteria capable of degrading mucus, has gained the attention of researchers as it is inversely associated with obesity, diabetes, cardiovascular disease, and low-grade inflammation (DAO; EVERARD *et al*., 2016).

These changes have intrigued researchers, who have sought to develop interventions that alleviate dysbiosis, characterized by microbial imbalance, and result in the improvement of metabolic parameters associated with these diseases.

Among these diseases, diabetes *mellitus* type 2 (T2DM) stands out, characterized by a persistent increase in serum glucose levels (AMERICAN DIABETES ASSOCIATION, 2014). Individuals with T2DM are at increased risk of hypertension and nephropathies, as a result of vascular damage caused by persistently high levels of glucose in the bloodstream. This results in a higher cost to the health system and a reduction in the quality of life of affected individuals.

According to the International Diabetes Federation, in 2019, Brazil had about 16.8 million people with diabetes, fifth in the world ranking. It is estimated that, in 2045, the number of individuals with T2DM will reach 26.0 million. Worldwide, the number of adults with this condition is 463 million (9.3% of adults), with Central-East and North Africa (MENA) being the regions with the highest prevalence of diabetic adults, at 12.2%. (WILLIAMS *et al*., 2020).

Due to the complications resulting from this condition, in addition to changes to healthier habits, adjuvant therapies, such as the use of probiotic supplements, can be a potential alternative to maintain adequate blood glucose levels (BOCK *et al*., 2021).

According to the last panel conducted by the International Scientific Association for Probiotics and Prebiotics (ISAPP), probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (HILL *et al*., 2014). Thus, its potential applications in various diseases must be studied, with the aim of improving the quality of life of these individuals.

According to the legislation published by the Agência Nacional de Vigilância Sanitária (ANVISA), in July 2018, minimum quantities are not established for the administration of probiotic supplement. For a food supplement containing probiotics to have a health benefit claim, the strain and its respective dose must be tested through randomized controlled trials that demonstrate causality between the consumption of the probiotic strain and the claimed effect (BRASIL, 2018). Therefore, studies with adequate scientific rigor must be carried out to support the use of probiotics in various health conditions.

In April 2020, Zheng *et al*. (2020) published a taxonomic reclassification covering species of the *Lactobacillaceae* family. Genetic techniques and identity markers (average nucleotide identity, average amino acid identity, central genomic phylogeny, among others) were used, resulting in 23 novel bacterial genera. These changes allow for a better ecological and functional understanding, helping the industry in the formulation of new products. Thus, the new nomenclatures were used in this dissertation, keeping it updated and disseminating

the knowledge published by the authors to the scientific community (ZHENG *et al*., 2020).

Several clinical trials with commercial products or isolated strains have been carried out to verify the effectiveness of probiotics in the most varied diseases. Inflammatory diseases, such as metabolic and inflammatory bowel diseases, are the target of this study. Although many of the studies described in the scientific literature indicate promising results, they are still in the experimental phase and often do not support the use of probiotics in clinical practice by health professionals. These effects were critically reviewed to elucidate whether there is sufficient evidence to support their use in reducing risk or improving metabolic parameters associated with type 2 diabetes *mellitus*.

REVIEW

Characteristics of the gut microbiota and potential effects of probiotic supplements in individuals with type 2 diabetes *mellitus*

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ABSTRACT

The increasing prevalence of type 2 diabetes *mellitus* (T2DM) worldwide has become a burden to healthcare systems. It is estimated that, in 2019, about 463 million adults lived with diabetes *mellitus*, and T2DM accounted for 90 to 95% of cases. The relationship between the gut microbiota and T2DM has been explored with the advent of metagenomic techniques. Genome-wide association studies evaluating the microbiota of these individuals have pointed to taxonomic, functional, and microbial metabolites imbalances and represent a potential intervention in the T2DM management. Several microbial metabolites and components, such as imidazole propionate, trimethylamine, and lipopolysaccharides, appear to impair insulin signaling, while short-chain fatty acids, secondary bile acids, and tryptophan metabolites may improve it. In addition, the use of probiotics with the aim of transiently restoring the microbial balance or reducing the effects of microbial metabolites that impair insulin sensitivity has been explored. Herein, we critically review the available literature on changes in the gut microbiota in T2DM, together with potential adjuvant therapies that may improve the health status of this population.

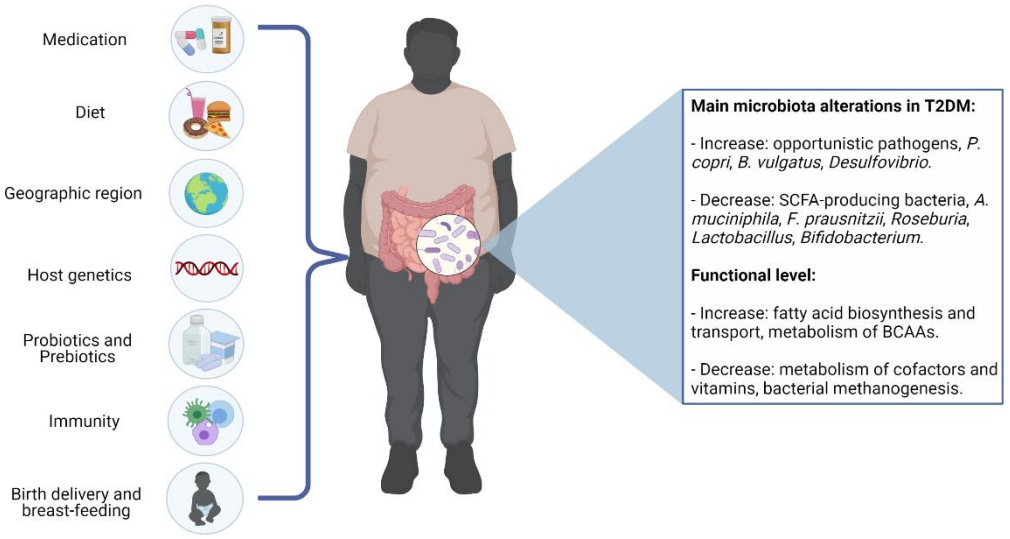
1 INTRODUCTION

Diabetes *mellitus* (DM) describes a group of metabolic disorders characterized mainly by chronic hyperglycemia, resulting from impaired insulin secretion or impaired insulin action or both mechanisms together, causing long-term complications (KERNER; BRUCKEL, 2014). Persistent hyperglycemia is associated with chronic micro and macrovascular complications. People with diabetes are at an increased risk of developing numerous health problems which may be life threatening, such as vascular damage that affects the heart, eyes, kidneys, and nerves (AMERICAN DIABETES ASSOCIATION, 2014). Clinical presentation and progression of type 1 and type 2 diabetes usually vary considerably due to the distinct pathophysiology of the diseases. An early accurate classification is important for determining therapy although sometimes this is not possible. (AMERICAN DIABETES ASSOCIATION, 2020).

Type 2 diabetes (T2DM), which accounts for 90 to 95% of all DM cases, is the most common metabolic disorder and is characterized by insulin resistance and pancreatic β-cell dysfunction as a consequence of unstable hyperglycemia (AKBARI; HASSAN-ZADEH, 2020; HAMEED *et al*., 2015). It has a complex and multifactorial etiology, involving genetic and environmental components and usually affects individuals from the fourth decade of life, although there has been an increase of the incidence of diabetes in children and young people. The risk factors for T2DM are genetic susceptibility, age, obesity, physical inactivity, previous diagnosis of pre-diabetes or gestational diabetes (DMG), inadequate diet, and stress (AMERICAN DIABETES ASSOCIATION, 2014; KOLB; MARTIN, 2017).

According to the Diabetes Atlas of the International Diabetes Federation 2019 (INTERNATIONAL DIABETES FEDERATION, 2019), approximately 463 million adults (20-79 years) in the world, corresponding to 9.3% of the world population, are living with diabetes; it is estimated that this number will increase to 700 million in 2045. In 2019, 374 million people were at risk of developing T2DM, and this proportion has increased in many countries. The largest number of people with diabetes are between 40 and 59 years old. For every two people with diabetes, one did not know they had the disease, or 263 million. In Latin America, it is estimated that 40% of the people with diabetes do not know that they have the disease. Brazil ranks 4th among the countries with the highest number of people who are unaware of their diagnosis of diabetes *mellitus* (SAEEDI *et al*., 2019; OLIVEIRA, MONTENEGRO, VENCIO, 2017).

The most probable explanations for the increased prevalence of diabetes are social and economic changes, including changes towards a sedentary lifestyle, an unbalanced diet leading to unfavorable nutritional changes, increased frequency of overweight, and growing urbanization. On the other hand, improved health care has increased the life expectancy of people with diabetes. Another explanation is the availability of more recent data reporting increasing cases of diabetes (CHO *et al*., 2018). Figure 1 summarizes the main gut microbiota microbial and functional alterations in T2DM, as well as the main factors which lead to the changes involved.



Created with Biorender.com

Figure 1. Factors affecting the profile of the host gut microbiota. Gut microbial composition is initially affected by the type of birth delivery and breast-feeding, along with the host's genetics, geographic region, medications, and immunity status. Probiotics and prebiotics are potential tools to restore the gut microbial balance when altered (dysbiosis). The main microbial shifts observed in T2DM patients are an increase in opportunistic pathogens, *Prevotella copri*, *Bacteroides vulgatus*, and *Desulfovibrio* species, and a decrease in SCFA-producing bacteria, *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Roseburia*, *Lactobacillus*, and *Bifidobacterium* species. At the functional level, an increase in BCAA metabolism, and fatty acid biosynthesis and transport is observed while there is a decrease in the metabolism of cofactors and vitamins, and bacterial methanogenesis pathways. Abbreviations: T2DM: type 2 diabetes *mellitus*; SCFA: short-chain fatty acids; BCCA: branched-chain amino acids.

Both genetic and epigenetic factors have been implicated in the development of inflammation associated with T2DM. The dysregulation of epigenetic control mechanisms that control the expression of a great amount of genes has been linked to the pathogenesis of several disorders related to the immune system, including T2DM. It is well established that the presence of a pro-inflammatory phenotype is strongly associated with the development of insulin

resistance, β cells, and vascular complications in a patient with DM (ESSER *et al*., 2014). Hyperglycemia and dyslipidemia cause abnormal epigenetic changes that promote the activation of the main inflammatory pathways and contribute to the development of a state of low-grade chronic inflammation in T2DM. This state of chronic inflammation impairs insulin secretion and sensitivity, leading to the development of T2DM and its comorbidities (AKBARI; HASSAN-ZADEH, 2020). The development and perpetuation of hyperglycemia occur concomitantly with hyperglucagonemia, resistance of peripheral tissues to the action of insulin, increased hepatic glucose production, incretin dysfunction, increased lipolysis, and consequent increase in free circulating fatty acids, increased renal reabsorption of glucose, and varying degrees of deficiency in the synthesis and insulin secretion by the pancreatic β cell. T2DM may be controlled through measures such as changes in lifestyle, adoption of healthier diets in association with medications, if necessary (KOLB; MARTIN, 2017).

Drug treatment used to treat this condition involves several classes of drugs, which act to reduce glucose production and absorption, increase insulin production and response, or increase urine glucose excretion. The standard drugs used to treat T2DM are metformin and glucagon-like peptide 1 (GLP-1) receptor agonists (AMERICAN DIABETES ASSOCIATION, 2020). However, there are limitations in the treatment with medications, as some patients are allergic to medication or have serious adverse effects such as diarrhea and lactic acidosis (BICSAK; WALSH; FINEMAN, 2017; DUJIC *et al*., 2016). Metformin use has already been linked to an increase in the abundance of *Escherichia*, *Intestinibacter*, *B. adolescentis*, and *Akkermansia muciniphila*. An increase in short chain fatty acids (SCFA) production was also observed, suggesting that modulation of the gut microbiota mediates some of the antidiabetic effects of metformin (WU *et al*., 2017).

The gut microbiota plays an important metabolic role, either through the fermentation of non-digestible carbohydrates and the synthesis of micronutrients or through its interaction with the immune system (ROWLAND *et al*., 2018). The term microbiota refers to the assemblage of living microorganisms including bacteria, archaea, protozoa, fungi, and algae present in a defined environment (BERG *et al*., 2020). Recently, changes in the human intestinal microbiota have been associated with pathological conditions such as obesity and other metabolic disorders such as type 2 diabetes mellitus, metabolic syndrome, and insulin resistance (MARCHESI *et al*., 2016; MUNOZ-GARACH; DIAZ-PERDIGONES; TINAHONES, 2016). Among the mechanisms that associate the intestinal microbiota with diabetes and insulin resistance, there is an increase in the permeability of the intestinal barrier, with consequent

metabolic endotoxemia. An increased production of branched-chain amino acids (BCAA), imidazole propionate, and of trimethylamine N-oxide (TMAO), as well as interaction with bile acids, changes in fatty acid metabolism, and intestinal hormones. These changes may lead to increased levels of adiposity and impaired insulin signaling (GURUNG *et al*., 2020; HEIANZA *et al*., 2019; MUNOZ-GARACH; DIAZ-PERDIGONES; TINAHONES, 2016).

Since T2DM represents the variation of the disease that affects most diabetic patients and is frequently associated with obesity and cardiovascular diseases, efforts have been made to develop new therapies to control and prevent the disease (YARIBEYGI; SATHYAPALAN; SAHEBKAR, 2019).

With the development of the metagenomics techniques, the whole-genome sequencing of all the DNA contained in a sample, a taxonomic investigation at the level of species and strains became possible, also providing a functional profile of metabolic pathways present in a community. With these features, a better understanding of the relationship between the gut microbiota and T2DM should lead to advances in therapeutic approaches and the development of new therapies, such as the use of probiotics.

Herein, we critically summarize recent findings on the role of microbiota in T2DM, as well as the use of probiotic supplements in the metabolic parameters of individuals with T2DM.

2 OBJECTIVES

2.1 General Objective:

Perform an integrative review of the available scientific literature to synthesize and elucidate current knowledge of the influence of the intestinal microbiota, the microbial metabolites, and the effects of probiotic supplementation on the health of patients with type 2 diabetes mellitus and its practical applicability.

2.2 Specific objectives:

- Identify which strains have proven efficacy, based on clinical trials which employed appropriate methods.
- Elucidate the role of microbial metabolites in type 2 diabetes *mellitus*.
- Explore changes in the gut microbiota associated with type 2 diabetes *mellitus*.

3 MATERIAL AND METHODS

Search method. The integrative review was performed using the following terms: type 2 diabetes mellitus AND (microbiota OR microbiome OR probiotics OR *Lactobacillus* OR *Bifidobacterium* OR *Akkermansia*). Trials were identified by searching MEDLINE (via Pubmed), Scopus, and Web of Science databases between 2011 and March 2021. We included human studies published in English, Spanish, or Portuguese. Studies evaluating whether an intervention with probiotic supplement compared with placebo has any effect in at least one parameter related to the glucose profile (e.g., hemoglobin A1C, fasting plasma glucose or insulin levels) were included. To explore the association between gut microbiota and type 2 diabetes mellitus, studies evaluating gut microbiota or functional changes in type 2 diabetes mellitus were included.

Eligibility criteria and study selection. Duplicates were removed manually. For the association between gut microbiota and type 2 diabetes mellitus, cohorts or case-control studies evaluating microbial composition by genetic sequencing (16S rRNA or metagenomic sequencing) were included. For the assessment of supplementation with probiotics, randomized controlled trials in which probiotics in the form of any pharmaceutical formulation administered to adult patients with T2DM were included after title and abstract screening. Combination therapy (e.g., minerals, prebiotics, fatty acids or phytosterols) or associated diseases were exclusion criteria. Subsequently, full texts of the articles were reviewed for inclusion of eligible studies.

Figure 2 outlines the steps followed for the selection of studies included in this review.

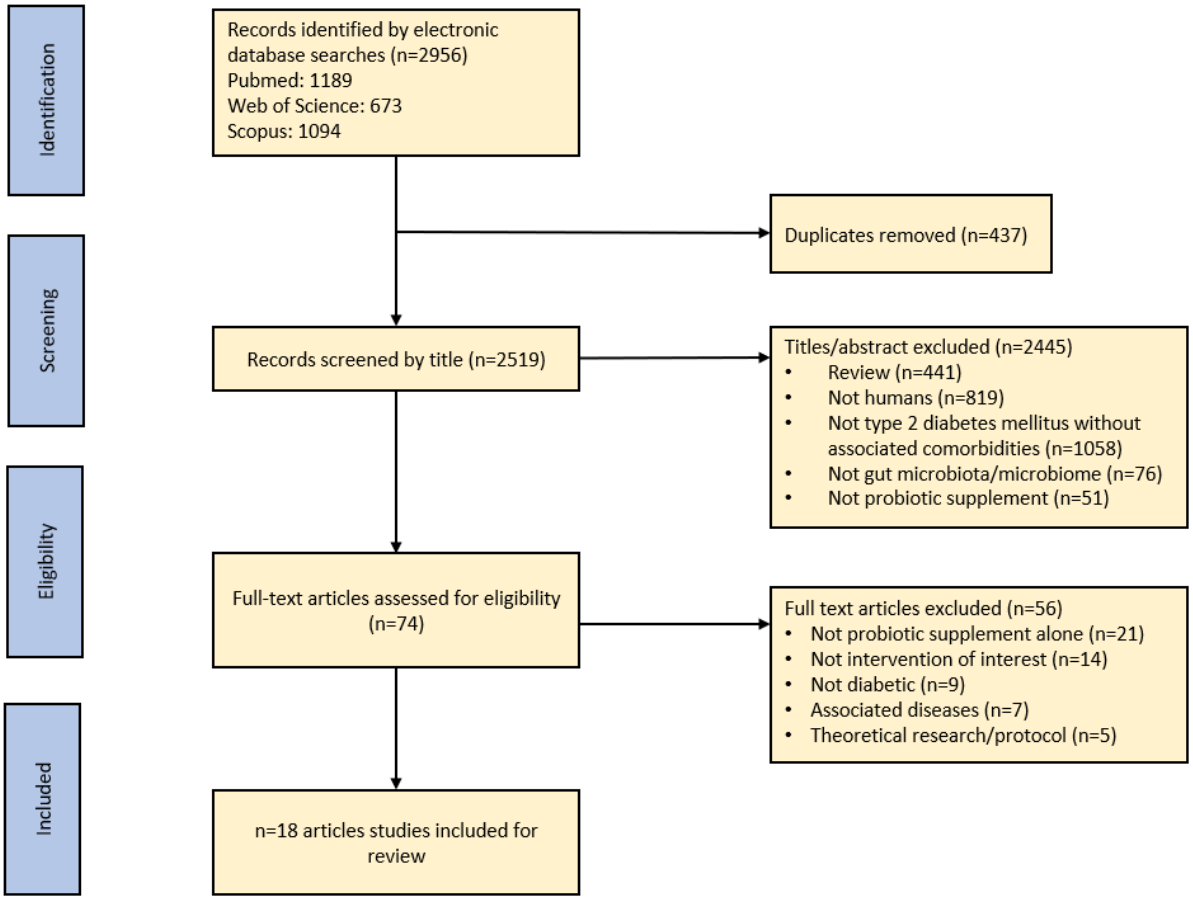


Figure 2. A flow diagram detailing the process followed for the selection of studies for the integrative review.

4 GUT MICROBIOTA AND ASSOCIATION WITH T2DM

Several studies suggest that the susceptibility, development, and progression of T2DM is influenced by the gut microbiota (DOUMATEY *et al*., 2020; QIN *et al*., 2012; WU; TREMAROLI *et al*., 2020). This is due to a reduction in diversity and a microbial imbalance, leading to an impact on the immune system and the emergence and growth of pathogens. Dysbiosis is also associated with obesity, insulin resistance, and low-grade inflammation, which reflects a possible causality linking these pathologies (SIRCANA *et al*., 2018).

Several human studies have reported bacterial genera or species that are reduced or increased in T2DM patients compared to healthy controls. A summary of recent studies evaluating changes in the gut microbiota found in T2DM is shown in **Table 1**.

Table 1 – Summary of studies evaluating microbial and functional changes in individuals with T2DM (continue).

Sample size	Age (y)	Sex	Technique	Microbiota modifications	Functional modifications	Study
183 T2D 185 Controls (Chinese)	13-86	Women (153) Men (209)	Metagenomic sequencing	Increased in T2D: *Akkermansia muciniphila*, *Bacteroides caccae*, *Bacteroides Intestinalis*, *Clostridium hathewayi*, *Clostridium ramosum*, *Clostridium symbiosum*, *Desulfovibrio sp.*, *Eggerthella lenta*, and *Escherichia coli* Decreased in T2D: *Clostridiales sp. SS3/4*, *Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, and *Roseburia inulinivorans.*	Increased in T2D: membrane transport of sugars, branched-chain amino acid (BCAA) transport, methane metabolism, xenobiotics degradation and metabolism, and sulphate reduction Increased in control: bacterial chemotaxis, flagellar assembly, butyrate biosynthesis, and metabolism of cofactors and vitamins.	Qin *et al*., 2012
53 T2D 49 IGT 43 Controls (Swedish)	69-72	Women (145)	Metagenomic sequencing	Increased in T2D: *Clostridium clostridioforme*, *Lactobacillus gasseri*, and *Streptococcus mutans* Decreased in T2D: *Roseburia*, *Clostridium spp*., *Eubacterium eligens*, *Coriobacteriaceae* and *Bacteroides intestinalis*.	Increased in T2D: starch and glucose metabolism, fructose and mannose metabolism, ABC transporters for amino acids, ions and simple sugars, fatty acid biosynthesis and, cysteine and methionine metabolism Increased in control: flagellar assembly, and riboflavin metabolism.	Karlsson *et al*., 2013
13 T2D 64 Prediabetes 44 Controls (Chinese)	52-55	Not available	16S rRNA V3-V5 region	Increased in T2D: *Clostridiales*, *Dorea*, *Prevotella*, *Collinsella*, and *Ruminococcus* Decreased in T2D: *Bacteroides*, *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Haemophilus parainfluenzae*, and *Roseburia*	-	Zhang *et al*., 2013

Table 1 – Summary of studies evaluating microbial and functional changes in individuals with T2DM (continuation).

Sample size	Age (y)	Sex	Technique	Microbiota modifications	Functional modifications	Study
75 T2M 291 Controls (Danish)	50-66	Women (187) Men (179)	Metagenomic sequencing	Increased in T2D: *Prevotella copri* and *Bacteroides vulgatus* Decreased in T2D: *Roseburia*, *Bifidobacterium*, *Faecalibacterium*, *Oscillibacter*, *Coprococcus*, and *Butyrivibrio*	Increased in T2D: lipopolysaccharide and BCAA biosynthesis Decreased in T2D: BCAA transport into bacterial cells, methanogenesis and pyruvate oxidation.	Pedersen *et al*., 2016
22 T1D 23 T2D 23 Controls (Polish)	20-65	Women (40) Men (28)	16S rRNA	Increased in T2D: *Ruminococcus*, *Enterobacteriaceae*, *Verrucomicrobia* Decreased in T2D: *Bacteroides*, *Roseburia*, *Faecalibacterium* (n.s.)	-	Salamon *et al*., 2018
20 T2D 40 Controls (Chinese)	20-60	Women (42) Men (18)	16S rRNA V4-V5 region	Increased in T2D: *Dorea*, *Fusobacterium*, and *Faecalibacterium prausnitzii* Decreased in T2D: *Parabacteroides*, *Akkermansia*, *Bifidobacterium*, and *Streptococcus*	Increased in T2D: : butyrate production via transferase, methanol conversion and pentose phosphate pathway Decreased in T2D: tyrosine degradation, leucine degradation, and anaerobic fatty acid beta-oxidation	Li *et al*., 2020

Table 1 – Summary of studies evaluating microbial and functional changes in individuals with T2DM (end).

Sample size	Age (y)	Sex	Technique	Microbiota modifications	Functional modifications	Study
98 T2D 193 Controls (Africans)	41-70	Not available	16S rRNA V4 region	Increased in T2D: *Desulfovibrio piger*, *Prevotella*, *Eubacterium*, and *Peptostreptococcus* Decreased in T2D: *Collinsella*, *Ruminococcus lactaris*, *Anaerostipes*, *Epulopiscium*, and *Clostridium*	Increased in T2D: proteasome pathway Decreased in T2D: none	Doumatey *et al*., 2020
134 T2D 37 Controls (Chinese)	45-67	Women (92) Men (79)	16S rRNA V3-V4 region	Increased in T2D: *Prevotella*, *Dialister*, *and Sutterella* Decreased in T2D: *Bacteroides*, *Bifidobacterium*, *Clostridium XIVa*, *Parabacteroides*, *Staphylococcus*, *Granulicatella*, *Porphyromonas*, *Clostridium XI*, *Blautia*, *Anaerostipes*, *Clostridium XVIII*, *Fusicatenibacter*, *Enterococcus*, *Clostridium IV*, *Eggerthella*, and *Flavonifractor*.	-	Wang *et al*., 2020
46 T2D 75 CGI 178 IGT 189 IFG 523 Controls (Swedish)	57-61	Women (568) Men (443)	Metagenomic sequencing	Increased in T2D: *Coprococcus eutactus*, *Clostridiales bacterium*, and *Lachnospiraceae bacterium* Decreased in T2D: *Clostridium sp.*, *Clostridium hathewayi*, *Clostridium bolteae*, *Clostridium symbiosum*, and *Roseburia faecis*	Increased in T2D: two-component systems, phosphotransferase systems, fructose and mannose metabolism, pentose phosphate pathway, bacterial biosynthesis of branched-chain amino acids, and metabolism of the B-group vitamins biotin and thiamine. Decreased in T2D: bacterial methanogenesis, glycolysis, peptidoglycan biosynthesis, vancomycin resistance, and DNA replication and transcription.	Wu *et al*., 2020

Abbreviations: y: years

The idea that the microbiota might contribute to the development of diseases such as obesity comes from animal studies carried out in the early 2000s. Among the main findings are the increased Firmicutes/Bacteroidetes ratio in obese patients and the association of microbiota with obesity (LEY *et al*., 2005; TURNBAUGH *et al*., 2006). By transferring the gut microbiota from obese to germ-free mice, their energy harvest capacity and adiposity were increased, establishing a possible causal relationship.

In the following years, several other studies reported enrichment or depletion of bacterial genera or species in the gut microbiota, indicating a connection with adiposity, insulin resistance, and T2DM. The first study to describe differences in the microbial composition between healthy individuals and individuals with T2DM dates from 2010 (LARSEN *et al*., 2010). Thirty-six stool samples were evaluated using 16S rRNA amplicon sequencing between individuals with T2DM and healthy controls. T2DM was associated with dysbiosis at the phylum level, with a reduction in the proportion of Firmicutes and an increase in *Bacteroidetes* and *Proteobacteria*, while overall diversity of the gut microbiota was positively correlated with plasma glucose levels in T2DM patients (LARSEN *et al*., 2010). On the other hand, these results were not observed in two large-scale metagenome-wide association studies performed in China and Europe (KARLSSON *et al*., 2013; QIN *et al*., 2012).

Controversial results also occurred for the species *Akkermansia muciniphila*. In a study conducted with 368 Chinese subjects, *A. muciniphila* was found to be increased in T2DM, while in other two studies with Chinese subjects, a reduction in abundance was found in T2DM. Animal studies systematically report that *A. muciniphila* abundance is inversely correlated with body weight, fat mass, insulin resistance, glucose intolerance and inflammation (EVERARD *et al*., 2013; EVERARD *et al*., 2014; PLOVIER *et al*., 2017; SCHNEEBERGER *et al*., 2015). In a randomized, double-blind, placebo-controlled clinical trial conducted with overweight or obese insulin-resistant patients, supplementation of pasteurized *A. muciniphila* resulted in weight loss, improved insulin resistance, reduced insulinemia, and plasma total cholesterol. However, it is important to emphasize that this was only a pilot study to verify the safety and tolerance of its supplementation (n=32) (DEPOMMIER *et al*., 2019). A potential mechanism suggested for these positive effects is an interaction between thermostable outer membrane protein Amuc 1100 found in pasteurized *A. muciniphila* and with Toll-like receptor 2 (PLOVIER *et al*., 2017).

Overall, patients with TDM2 have a reduced abundance of SCFA producing species (*Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Roseburia inulinivorans*, *Roseburia*

faecis, *Akkermansia muciniphila*, *Bifidobacterium* spp., and *Eubacterium* rectale) and tryptophan metabolite producing bacteria (*Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, Bacteroides, and Clostridium). On the other hand, these patients have an increased abundance of opportunistic pathogens (*Clostridium hathewayi*, *Clostridium ramosum*, *Bacteroides caccae*, *Escherichia coli*, and *Eggerthella lenta*), sulfate-reducing bacteria (*Desulfovibrio* spp.), and branched-chain amino acid producing bacteria (*Prevotella copri*, and *Bacteroides vulgatus*), in comparison with healthy controls (DOUMATEY *et al*., 2020; KARLSSON *et al*., 2013; LI *et al*., 2020; PEDERSEN *et al*., 2016; QIN *et al*., 2012; SALAMON *et al*., 2018; WANG *et al*., 2020; WU *et al*., 2020; ZHANG *et al*., 2013).

At the functional level, the main pathways enriched in T2DM were the metabolism of BCAAs and the fatty acid biosynthesis and transport while bacterial methanogenesis and metabolism of cofactors and vitamins were depleted (KARLSSON *et al*., 2013; LI *et al*., 2020; PEDERSENN *et al*., 2016; QIN *et al*., 2012; WU; TREMAROLI *et al*., 2020). BCAA concentrations are known to correlate positively with insulin resistance, and *Prevotella copri* was found to be the major drive species between microbial biosynthesis in the gut and insulin resistance, suggesting a potential causal relation that deserves better investigation (PEDERSEN *et al*., 2016). A Chinese and a Swedish study also found increased expression of the microbial genes involved in oxidative stress, suggesting that the gut microbiota in T2DM stimulates bacterial defense mechanisms against the oxidative stress characteristic of the disease (KARLSSON *et al*., 2013; QIN *et al*., 2012).

Despite the similarities found in these studies, some of the conflicting results must be explained by differences in geographic locations, genetics, drug treatment, and sequencing techniques. The development of novel technologies is highly desirable to understand whether these associations of gut microbiota and T2DM are causal or consequences of the development of the disease.

5 MICROBIAL METABOLITES AND COMPONENTS LINKED TO T2DM

In the last years, low-grade inflammation has been hypothesized to be the link between the microbiome and the risk for developing T2DM, due to mechanisms related to microbial metabolites such as bacterial toxins, short-chain fatty acids, bile acids, TMAO, tryptophan metabolites, and BCAA metabolites. Microbial metabolites allow us to better understand the underlying mechanisms by which bacterial taxa contributes to host health and disease. The main microbial metabolites related to T2DM are shown in **Table 2**.

Table 2 – Main microbial metabolites related to T2DM (continue).

Metabolite	Metabolite-producing bacteria (genus or species)	Mechanism of T2DM risk	Reference
SCFA (acetate, propionate, and butyrate)	SCFA: *Akkermansia*, *Ruminococcus*, *Faecalibacterium prausnitzii*, *Eubacterium*, *Roseburia*, *Blautia*, *Coprococcus*, *Anaerostipes*, and others	- Increases epithelial barrier function by regulation of TJP - Reduces the passage of LPS improving inflammation - Stimulates the secretion of PYY and GLP-1 from L-cells in a GPR41 and GPR43 dependent manner - Reduces appetite, insulin secretion, plasma glucose levels, and slow gastric emptying through stimulation of GLP-1 and GLP-2 secretion.	Canfora *et al*., 2015 Morrison *et al*., 2016 Markowiak-Kopeć *et al*., 2020
Imidazole propionate	Imidazole propionate: *Eggerthella lenta*, *Streptococcus mutans*, *Aerococcus urinae*, *Brevibacillus laterosporus*, and others	- Impairs glucose tolerance and insulin signaling by activating the p38γ-p62-mTORC1 pathway.	Koh *et al*., 2018
TMAO	TMA: *Desulfovibrio desulfuricans*, *Providencia*, *E. coli*, *Klebsiella pneumoniae*, *Sporosarcina*, and others.	- Exacerbates blockage of the insulin signaling pathway and promotes inflammation in adipose tissue.	Zeisel *et al*., 2017

Table 2 – Main microbial metabolites related to T2DM (end).

Metabolite	Metabolite-producing bacteria (genus or species)	Mechanism of T2DM risk	Reference
Branched-chain amino acids	BCAA: *Lactobacillus*, *Weissella*, *Leuconostoc*, *P. copri*, *and B. vulgatus*	- Promotes insulin resistance through serine phosphorylation of IRS-1 by persistent activation of mTORC1/S6K1.	Pedersen *et al*., 2016 Mutaguchi *et al*., 2018 Tett *et al*., 2019
Bile acids	Secondary bile acids: *Ruminococcus*, *Bifidobacterium*, *Bacteroides*, *Clostridium*, *Lactobacillus*, *Eubacterium*, *Listeria*, and others.	- Ligands of nuclear receptors, such as VDR, PXR, and FXR, which induce TGR5 expression and regulates insulin and glucose sensitivity.	Jia *et al*., 2018 Chiang *et al*., 2019
Tryptophan metabolites	Tryptophan metabolites and tryptamine: *Clostridium bartlettii*, *Clostridium sporogenes*, *Ruminococcus gnavus*, Bacteroides ovatus, *Lactobaccilus acidophilus*, *Lactobacillus reuteri*, , *Bifidobacterium fragilis*, *Bifidobacterium bifidum*, and others.	- Improves intestinal epithelial barrier function by activation of PXR - Stimulates insulin secretion, supress appetite, and slow gastric emptying by stimulating GLP-1 secretion - Promotes gastrointestinal motility by stimulating serotonin release - Anti-inflammatory and anti-oxidative effects in the systemic circulation.	Roager *et al*., 2018

Abbreviations: SCFA: short chain fatty acids; TJP: tight junction proteins; LPS: lipopolysaccharides; PYY: peptide YY; GLP-1: glucagon-like peptide; GPR: G-protein coupled receptor; mTORC1: mechanistic target of rapamycin complex 1; TMAO: trimethylamine-N-oxide; TMA: trimethylamine; BCAA: branched-chain amino acids; IRS-1: insulin receptor substrate 1; VDR: vitamin D3 receptor; PXR: pregnane X receptor; FXR: farnesoid X receptor; TGR5: G-protein-coupled bile acid receptor.

5.1 Low-grade inflammation

Metabolic diseases, such as obesity and T2DM, share a characteristic, the chronic state of low-grade inflammation. The mechanism proposed in the literature for this inflammation is the activation of toll-like receptors (TLR) by the lipopolysaccharides (LPS) present in the cell wall of the Gram-negative bacteria. LPS is an endotoxin, which leads to a chronic systemic inflammatory response, when consistently increased in serum levels. This situation occurs in T2DM, due to exacerbated bacterial translocation (MEDZHITOV, 2008; YU *et al*., 2019).

TLRs, on the other hand, comprise a large family of cellular membrane proteins, which play a crucial role in the innate immune response, providing the first line of defense against host pathogens. Through the recognition of Pathogen Associated Molecular Patterns (PAMPs), TLRs activate cascades signaled by inflammatory cytokines in target tissues of insulin. This, in turn, leads to the activation of the phosphorylation of kinases, c-Jun n-Terminal and IκB, increasing the inflammatory response (KUMAR; KAWAI; AKIRA, 2009). The result of this sequence of molecular events is the inhibition of the insulin transducer signal, via phosphorylation of insulin receptor substrate 1 (IRS1) in serine, leading to insulin resistance in liver, adipose, and muscle tissues. This mechanism inhibits the signaling of the insulin receptor tyrosine kinase and protein kinase b (AKT), contributing to the degradation of IRS-1 and towards insulin resistance (SALTIEL; KAHN, 2001; TANIMURA *et al*., 2008).

5.2 Short-Chain Fatty Acids

T2DM patients are known to have reduced abundance of SCFA-producing bacteria such as *Roseburia*, *Eubacterium*, *Faecalibacterium* and *Ruminococcus* species (DOUMATEY *et al*., 2020; QIN *et al*., 2012). This leads to a reduction in the production of the SCFAs acetate, butyrate, and propionate in the host colon, derived from the fermentation of non-digestible carbohydrates. Among their functions, SCFAs play a role in the cell growth and differentiation, maintenance of intestinal epithelial integrity, immunomodulatory and anti-inflammatory effects (CANFORA; JOCKEN; BLAAK, 2015; MORRISON; PRESTON, 2016). By binding to G-protein coupled receptors 41 and 43, SCFAs stimulate the production of GLP-1 peptides from colonic enteroendocrine L cells and peptide YY (PYY) (CANFORA; JOCKEN; BLAAK, 2015). These peptides reduce gastric emptying, control the appetite, stimulate insulin secretion, and inhibit glucagon secretion. In a clinical trial, the increase in SCFA levels promoted by dietary fiber intake was associated with lower levels of HbA1c, partially due to the increase in

GLP-1. The authors concluded that recovering active SCFA-producing bacteria may alleviate T2DM phenotypes and represent a new way to manipulate the microbiota in T2DM and other dysbiosis-related diseases (ZHAO *et al*., 2018) .

5.3 Trimethylamine N-Oxide

Trimethylamine (TMA) is an organic compound synthesized by microbial metabolism of dietary phosphatidylcholine, choline, and carnitine. After being absorbed, it is transported to the liver through the portal vein. TMA is converted in the liver by flavin monooxygenase 3 (FMO3) to form TMAO (TAESUWAN *et al*., 2017).

Several strains of bacteria are potentially TMA/TMAO producers *in vivo*, including Firmicutes, Proteobacteria, *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *C. hathewayi*, *C. sporogenes*, *Escherichia fergusonii*, *Proteus penneri*, *Providencia rettgeri*, *Edwardsiella tarda*, and *Desulfuricanibrio desulfuricans* (QI *et al*., 2018; ZEISEL; WARRIER, 2017).

T2DM patients have increased TMAO serum levels. Higher TMAO plasma levels are associated with an increased risk of T2DM, cardiac events, and mortality (TANG *et al*., 2017). In animals, TMAO intake has been associated with worsening impaired glucose tolerance and insulin resistance induced by a high fat diet (HFD). This is also mediated by the insulin signaling pathway in the liver, increasing the production of inflammatory cytokines in the adipose tissue (GAO *et al*., 2014).

In contrast, a recent Mendelian randomization found that T2DM elevates TMAO levels, suggesting reverse causality. Therefore, relationship between T2DM and TMAO requires more investigation to elucidate the issue (JIA *et al*., 2019).

5.4 Imidazole propionate

Imidazole propionate (ImP) is a microbially produced histidine-derived metabolite. Bacteria usually produce ImP from its precursor urocanate. Potentially ImP producers are *Eggerthella lenta*, *Streptococcus mutans*, *Aerococcus urinae*, *Brevibacillus laterosporus*, and others. Patients with T2DM show an increase in Imp-producing bacteria, in addition to having increased levels in the portal vein and peripheral blood (KOH *et al*., 2018; MOLINARO *et al*., 2020). Administration of ImP in mice demonstrated that it impairs the glucose tolerance. Consistent with the findings in mice, in the human liver, phosphorilation of p62 and S6K1 was

higher than in healthy controls, indicating a role for imidazole propionate in impairing insulin signaling through the p62 and mTORC1 pathway (KOH *et al*., 2018).

ImP represents an important target for the development of approaches that modify its production from bacteria, resulting in an improvement in insulin resistance.

5.5 Branched-chain amino acids

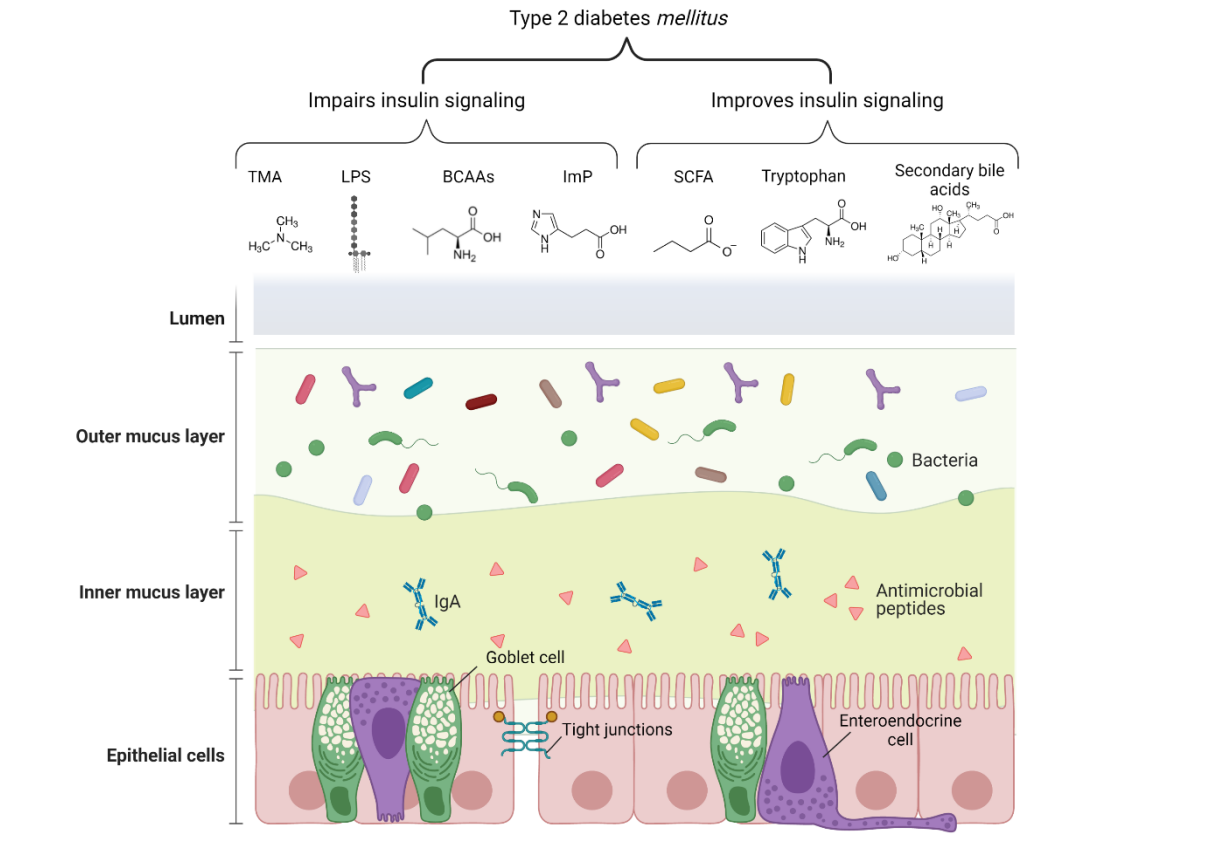
Branched-chain amino acids (BCAAs), which include leucine, isoleucine, and valine, are essential amino acids. BCAA-rich diets are usually associated with positive effects on the regulation of body weight, muscle protein synthesis, and glucose homeostasis. *Lactobacillus*, *Weissella* and *Leuconostoc* are among the genera capable of producing BCAAs. In addition, *Prevotella copri* and *Bacteroides vulgatus* are known as the main species driving the association between biosynthesis of BCAAs and insulin resistance (TETT *et al*., 2019). Despite this, several studies highlight that the metabolism of microbial amino acids may play a role in the development of insulin resistance. Human studies have shown that the increased intake of BCAAs is associated with an increased risk of insulin resistance and T2DM (ASGHARI *et al*., 2018). This may be due to the insulinogenic activity of these amino acids. Constant high levels of BCAAs persistently activate mTORC1 signaling pathway, leading to IR with serine phosphorylation of insulin receptor substrate 1 (IRS-1), which occurs in response to persistent aminoacidemia or hyperinsulinemia (LYNCH; ADAMS, 2014; NEWGARD *et al*., 2009). Among BCAAs, leucine seems to be more important in this process, as it has a greater effect on the mediation of mTORC1 activity (LYNCH; ADAMS, 2014). In contrast, BCAAs were associated with a lower risk of developing T2DM in Japanese women (NAGATA *et al*., 2013). The increase in insulin resistance caused by increased plasma levels of BCAAs appears to be context-dependent and needs further investigation.

5.6 Tryptophan metabolites

Tryptophan is one of the nine essential amino acids, and, because it is not synthesized by the human body, it needs to be supplied by the diet. Tryptophan is absorbed in the small intestine, but the fraction that reaches the colon can be catabolized by the gut bacteria in several indole-derivatives. Many bacterial species are able to catabolize tryptophan. They belong to the genera *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Lactobacillus*, *Anaerostipes*, beside others, and produce the metabolites Indole, 3-methylindole (Skatole), Indoleacetic acid (IAA), Indoleacrylic acid (IA), Indolealdehyde (IAId), Indolelactic acid (ILA), Indolepropionic acid

(IPA), and Tryptamine (ROAGER; LICHT, 2018).

Figure 3 illustrates the main microbial components and metabolites affecting insulin signaling.



Adapted from "Structure of Mucosal Barrier", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

Figure 3. Main microbial components/metabolites affecting insulin signaling in type 2 diabetes *mellitus*. Abbreviations: TMA: trimethylamine; LPS: lipopolysaccharide; BCAAs: branched-chain amino acids; ImP: imidazole propionate; SCFA: short chain fatty acids; IgA: immunoglobulin A.

In mice, IPA was able to regulate the intestinal barrier function by acting as a pregnane X receptor (PXR) ligand, and to reduce intestinal permeability in mice fed with HFD (JENNIS *et al*., 2018; VENKATESH *et al*., 2014). IA was also able to promote intestinal epithelial barrier function and reduce inflammatory response in mice by mucus production and promoting goblet cell differentiation (WLODARSKA *et al*., 2017). Higher serum IPA levels were associated with a reduced risk of developing T2DM and better insulin secretion, possibly through preservation of β-cell function (DE MELLO *et al*., 2017).

Also, Indole was able to induce GLP-1 secretion in mouse colonic enteroendocrine L cells, suggesting that indoles might play an important role in the glucose metabolism

(CHIMEREL *et al*., 2014). The increase in GLP-1 levels stimulates insulin production, reduces appetite, and delays gastric emptying, which could benefit T2DM patients. Human studies endorsing these effects are desirable and should be performed in the future (HOLST, 2007).

5.7 Bile acids

Bile acids are produced and secreted by the liver and then released into the intestine. Secreted bile acids are reabsorbed in the intestine, mostly in the ileum. In the ileum and colon, gut bacterial bile salt hydrolase (BSH) converts primary conjugated bile salts into deconjugated bile acids (BA) that are subsequently converted into secondary BA (CHIANG; FERRELL, 2019). BSH activity is high in bacteria belonging to the genera *Clostridium*, *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* (JIA; XIE; JIA, 2018). Gut bacteria regulates bile acid composition and pool size to modulate the intestinal farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5) signaling. While primary bile acids activate FXR, secondary bile acids bind to the G protein-coupled TGR5 receptor, which results in GLP-1 secretion in enteroendocrine L cells (CHIANG; FERRELL, 2019). This mechanism has been demonstrated in obese diabetic mice, where intestinal FXR activation altered bile acid metabolism by increasing lithocholic acid (LCA)-producing bacteria in the gut (PATHAK *et al*., 2018). Higher LCA levels activated intestinal TGR5, which then stimulates GLP-1 to improve hepatic glucose and lipid metabolism. This mechanism represents a potential therapeutic target for the treatment of T2DM and other metabolic diseases.

6 EFFECTS OF PROBIOTICS SUPPLEMENTS IN T2DM

Probiotics are defined as live microorganisms that, when administered in an adequate amounts, confer a health benefit on the host (HILL *et al*., 2014). Controversies about the effects of probiotics have generated repercussions among researchers, and some studies warn about the potential risks of their use (SUEZ *et al*., 2019). However, several benefits of taking probiotics have been reported, including immunomodulation, SCFAs production, antagonism with pathogens, improvement of the barrier function, gut microbiota modulation, production of enzymes, and production of small molecules with systemic effects (SANDERS *et al*., 2019).

Currently, most clinical trials mainly focus on probiotics of the *Lactobacillus* and *Bifidobacterium* genera. But with the advances in microbiota research, single bacterial strains associated with the improvement of inflammation-related diseases are screened and isolated. Some of these strains are expected to emerge as next generation probiotics (CHANG *et al*., 2019).

In the search for new approaches to control T2DM, the number of experiments using probiotics to improve the glycemic and lipid profile in T2DM patients is rising, but the number is still low. However, it is necessary to exercise caution when evaluating these studies as it is known that the effect of probiotics depends on the strain chosen, the characteristics of the group being studied, the pathophysiology of the disease, the food matrix or pharmaceutical form, whether it is single or multi-strain, the intervention period, and a sufficient dose (OUWEHAND, 2017).

In this review, we have focused on studies exclusively using probiotic supplements, without other compounds or substances, such as fatty acids, vitamins, and minerals, to avoid confounding factors. A summary of the clinical trials is shown in **Table 3**.

Table 3 – Summary of clinical trials evaluating the effect of probiotic supplementation alone in individuals with T2DM (continue).

Sample size	Design	Duration	Intervention	Metabolic outcomes	Microbiota modifications	Functional modifications	Study
Placebo (18) Intervention (16)	Single-blind clinical trial	6 weeks	Probiotic: 3000mg/ day of *L. acidophilus*, *L. bulgaricus*, *L. bifidum* and *L. casei*	Probiotic: n.s.	-	-	Mazloom *et al.*, 2013
Placebo (53) Intervention (48)	Randomized, double-blind, parallel-group, controlled clinical trial	12 weeks	Probiotic: 3 x 10^{10} CFU/day of *L. acidophilus*, *L. casei*, *L. lactis*, *B. bifidum*, *B. longum* and *B. infantis*	Probiotic: ↓HbA1c, FI, HOMA-IR	Probiotic: ↑ *Bifidobacterium* spp., *Lactobacillus* spp.	-	Firouzi *et al.*, 2017
Placebo (39) Intervention (39)	Randomized, single-centre, double-blind, placebo-controlled	12 weeks	Probiotic: 10^{10} CFU/day of *B. bifidum* W23, *B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63, *L. casei* W56, *L. salivarius* W24, *Lactococcus lactis* W19 and *Lactococcus lactis* W58	Probiotic: ↓HOMA-IR, FBG, Insulin, C-peptide, TG, LDL-c, WHR	-	-	Sabico *et al.*, 2017
Placebo (15) Intervention (29)	Randomized, double-blind, placebo-controlled trial	12 weeks	Probiotic: *L. reuteri* DSM 17938 LD: 10^{8} CFU/day HD: 10^{10} CFU/day	Probiotic: HD: ↑ ISI, DCA LD: ↑ unconjugated bile acids	Probiotic: ↑ *L. reuteri*	-	Mobini *et al.*, 2017
Placebo (22) Intervention (46)	Randomized, double-blind, placebo-controlled trial	9 months (6 month intervention)	Group 1: 4 x 10^{9} CFU/day of probiotic *L. reuteri* ADR-1 Group 2: 2 x 10^{10} CFU/day heat-killed *L. reuteri* ADR-3	Group 1: ↓HbA1c, TC Group 2: ↓ SBP, IL-1β	Group 1: ↑ *L. reuteri* Group 2: ↑ *Bifidobacterium*	-	Hsieh *et al.*, 2018

Table 3 – Summary of clinical trials evaluating the effect of probiotic supplementation alone in individuals with T2DM (continuation).

Sample size	Design	Duration	Intervention	Metabolic outcomes	Microbiota modifications	Functional modifications	Study
Placebo (22) Intervention (31)	Randomized, double-blind, single-centre, clinical trial	8 weeks	Probiotic: 1 sachet (10g)/day of 14 probiotic strains of *Lactobacillus* + *Lactococcus* (6×10^{10} CFU/g), *Bifidobacterium* (1×10^{10}/g), *Propionibacterium* (3×10^{10}/g), *Acetobacter* (1×10^{6}/g) genera	Probiotic: ↓HOMA-IR, TNF-alpha, IL-1β, WC	-	-	Kobyliak *et al*., 2018
Placebo (30) Intervention (31)	Randomized, single-centre, double-blind, placebo-controlled clinical trial	6 months	Probiotic: 10^{10} CFU/day of *B. bifidum* W23, *B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63, *L. casei* W56, *L. salivarius* W24, *Lactococcus lactis* W19 and *Lactococcus lactis* W58	Probiotic: ↓HOMA-IR, FBG, Insulin, C-peptide, TG, TC, TC/HDL, CRP, TNF-α, IL-6, resistin, endotoxin ↑ adiponectin	-	-	Sabico *et al*., 2019
Placebo (20) Intervention (20)	Randomized, parallel-group, placebo-controlled trial	8 weeks	Probiotic: 10^{8} CFU/day of *L. casei*	Probiotic: ↓FBG, HOMA-IR, Insulin, fetuin-A, weight, BMI, WC ↑ SIRT1	-	-	Khalili *et al*., 2019

Table 3 – Summary of clinical trials evaluating the effect of probiotic supplementation alone in individuals with T2DM (end).

Sample size	Design	Duration	Intervention	Metabolic outcomes	Microbiota modifications	Functional modifications	Study
Placebo (30) Intervention (30)	Randomized, double-blind, single-centre, placebo-controlled pilot trial	12 weeks	Probiotic: 2 x 10^{11} CFU/day of *L. plantarum* Lp-115, *L. bulgaricus* Lb-64, *L. gasseri* Lg-36, *B. breve* Bb-03, *B. animalis* sbsp. *lactis* Bi-07, *B. bifidum* Bb-06, *Streptococcus thermophilus* St-21 and *Saccharomyces boulardii* DBVPG 6763	Probiotic: ↑ plasma butyrate Subgroup (metformin): ↓ FBG, HbA1c, insulin resistance, and zonulin ↑ plasma butyrate	Probiotic: n.s. beta diversity ↓ *Prevotella copri*, *Flavonifractor plautii* ↑ *B. breve*, *Bacteroides caccae*, *Bacteroidales bacterium* ph8, *Akkermansia muciniphila*, *Clostridium hathewayi* Subgroup (metformin): ↓ *Bactoides uniformis* ↑ *B. breve*, *Bacteroides caccae*, *Anaerotruncus colihominis*	Subgroup (metformin): pyruvate fermentation to butanoate, and *Bifidobacterium* shunt pathways	Palacios *et al*., 2020

Abbreviations: N.s.: not significant; HbA1C: haemoglobin A1c; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance; FBG: fasting blood glucose; IR: insulin resistance; TG: triglycerides; WHR: waist-to-hip ratio; LD: low dose; HD: high dose: ISI: insulin sensitivity index ; DCA: deoxycholic acid; SBP: systolic blood pressure; IL-1β: interleukin 1 beta; TNF-α: tumor necrosis factor alpha; WC: waist circunference; TC: total cholesterol; HDL: high density lipoprotein; CRP: C-reactive protein; BMI: body mass index; SIRT1: sirtuin 1.

Sabico *et al*. (2017) evaluated the use of 10^{10} CFU/day of a multi-strain probiotic preparation containing *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W19, and *Lactobacillus lactis* W58) regarding metabolic endotoxemia levels and cardiometabolic parameters in adult patients recently diagnosed with T2DM for 12 weeks. A clinically significant improvement in HOMA-IR (Homeostatic Model Assessment-Insulin Resistance) and a reduction in waist-hip ratio between groups were observed. Within-group comparisons in the probiotic group resulted in lower levels of fasting blood glucose (FBG), insulin, insulin resistance, C-peptide, triglycerides, and LDL-c. No significant changes in endotoxin levels were observed (SABICO *et al*., 2017). In another study, the effects of the same commercial probiotic preparation, which included strains of *Lactobacillus*, *Lactococcus*, and *Bifidobacterium*, in the same amount, and considering the same parameters as the previous study, were evaluated for a period of 6 months. Again, a clinically significant difference was observed in HOMA-IR, and a borderline significant improvement in insulin levels in the probiotic group was also seen. Within-group comparisons showed a reduction in the inflammatory markers (TNF-α, IL-6, C-reactive protein) and an improvement in the endotoxin and adiponectin levels. The results obtained were satisfactory, corroborating the idea that the probiotic supplementation time is also essential for its action (SABICO *et al*., 2019). Despite these results, the same product was shown to be able to improve the intestinal epithelial barrier function *in vitro* (HEMERT; ORMEL, 2014).

Integrity of the intestinal barrier has been one of the main focuses of improvement with the use of probiotics. A reduction in low-grade inflammation and improvement in the insulin signaling cascade are expected by reducing the passage of LPS. In this context, Karczewski *et al*. (2010) elegantly evaluated the impact of probiotic *Lactobacillus plantarum* strain WCFS1 injected directly into the duodenum of a group of individuals, followed by tissue biopsy 6 hours after the intervention. The authors observed an increased translocation of *zonula occludens*-1 and occludin close to the tight junctions, promoting intestinal epithelium integrity in a TLR2-dependent manner (KARCZEWSKI *et al*., 2010). Similar results were obtained in cell culture for several strains of the *Lactobacillus* genus (HUMMEL *et al*., 2012).

Hsieh *et al*. (2018), in a 9-month double-blinded, randomized, placebo-controlled study, observed a decrease in serum levels of HbA1C and cholesterol in patients with T2DM who received capsules containing probiotic *Lactobacillus reuteri* ADR-1 (HSIEH *et al*., 2018). The reduction in HbA1C remained even after three months of follow-up without probiotic intake.

Microbiota analysis revealed increased levels of *L. reuteri* in the probiotic group and that changes in HbA1C were negatively correlated with upregulation of total *L. reuteri* and positively correlated with the *Bacteroidetes* or *Bacteroidetes*/*Firmicutes* ratio. Together, these results indicated that the degree of HbA1C reduction is affected by the level of upregulation of *L. reuteri* in T2DM after *L. reuteri* ADR-1 consumption (HSIEH *et al*., 2018).

Similarly, an RCT conducted by Mobini *et al.* (2017) evaluated the intake of *L. reuteri* DSM 17938 at different concentrations (low dose: 10^8 CFU/day vs high dose: 10^{10} CFU/day) for 12 weeks but did not observe a reduction in HbA1C in T2DM patients. An improvement in the insulin sensitivity index (ISI) was observed in the high dose group even though it was not significant. However, post-hoc analysis based on ISI improvement showed a significant reduction in HbA1C and secondary bile acids after *L. reuteri* intake and evidenced that responders had higher microbiota diversity at baseline (MOBINI *et al*., 2017).

A 12-week intervention with a probiotic multi-strain (3 x 10^{10} CFU/day of *L. acidophilus*, *L. casei*, *L. lactis*, *B. bifidum*, *B. longum* and *B. infantis*) with 101 T2DM adults was able to reduce HbA1C levels, fasting insulin, and insulin resistance, together with an increase in *Bifidobacterium* and *Lactobacillus* species in the gut microbiota of the probiotic group (FIROUZI *et al*., 2017).

An RCT conducted by Palacios *et al*. (2020) evaluated the effect of a probiotic multi-strain (2 x 10^{11} CFU/day, containing *L. plantarum* Lp-115, *L. bulgaricus* Lb-64, *L. gasseri* Lg-36, *B. breve* Bb-03, B. *animalis* sbsp. *lactis* Bi-07, *B. bifidum* Bb-06, *Streptococcus thermophilus* St-21, and *Saccharomyces boulardii* DBVPG 6763) in patients with prediabetes and T2DM (PALACIOS *et al*., 2020). Only an increase in the butyrate plasma levels was found between the intervention and the placebo groups. Interestingly, the sub-group analysis in participants taking metformin and probiotic showed reductions in levels of FBG, HbA1c, insulin resistance, and zonulin, a marker and modulator of the intestinal permeability. The authors hypothesize that probiotics and metformin altered zonulin levels, encouraging the microbiome to increase luminal butyrate production. Microbiota analysis revealed that either the probiotic alone or in combination with metformin was able to increase SCFA-producing bacteria after 12 weeks of intervention (PALACIOS *et al*., 2020).

Among the nine clinical trials here selected, three of them were of short duration (8 weeks or less). Mazloom *et al*. (2013) did not find significant differences in anthropometric and metabolic parameters with the use of probiotics for 6 weeks in T2DM (MAZLOOM; YOUSEFINEJAD; DABBAGHMANESH, 2013). A reduction in FBG, insulin concentration,

and insulin resistance along with an increase in SIRT1 and a decrease of fetuin-A was observed by Khalili *et al*. (2017), using a single-strain probiotic (10^8 CFU/day of *L. casei*) for 8 weeks (KHALILI *et al*., 2019). Also, Kobyliak *et al*., (2018) found a reduction in HOMA-IR and inflammatory markers (TNF-α, IL-1β), when administering a multi-strain probiotic (14 probiotic bacteria genera *Bifidobacterium, Lactobacillus, Lactococcus, Propionibacterium*) for 8 weeks (KOBYLIAK *et al*., 2018).

Searching for probiotic effects under altered parameters in individuals with T2DM has been a challenge, due to the scarcity of literature, inappropriate experimental designs, and high heterogeneity in form of administration. In a recent meta-analysis that included only randomized clinical trials conducted with T2DM, Kocsis *et al*. (2020) found that the probiotics were able to improve fasting blood glucose (−16.52 mg/dL, 95% CI − 23.28; −9.76, $p < 0.001$), HbA1c (− 0.33%, 95% CI − 0.53; − 0.13, $p = 0.001$), and fasting insulin (1.40 μIU/mL, 95% CI − 2.52, − 0.27, $p = 0.015$) (KOCSIS *et al*., 2020). However, probiotics in foods and therapies combined with other substances were considered. Additionally, in the subgroup analysis, no significant improvement was observed in long-term interventions, higher doses, or the use of multi-strain probiotics, although other studies have found an improvement (OUWEHAND, 2017; SUN; BUYS, 2016; ZHANG; WU; FEI, 2016).

We must consider that the application of probiotics is strain-specific and disease-specific, and grouping results obtained with different strains for different outcomes may lead to inaccurate conclusions. Often, only the genus or genus and species are reported in studies, making comparison difficult. Here, only two studies used the same probiotic formulation as an intervention and obtained positive results mainly for insulin resistance (SABICO *et al*., 2019; SABICO *et al*., 2017). Two species of the *Lactobacillus* genus were evaluated, also resulting in benefits in glucose metabolism in T2DM patients (HSIEH *et al*., 2018; KHALILI *et al*., 2019; MOBINI *et al*., 2017). Two other RCTs included identified that the gut microbiota composition seems to be decisive in obtaining a response with the intervention of probiotics (HSIEH *et al*., 2018; MOBINI *et al*., 2017). Only one study evaluated the microbiota at the functional level with results that do not resemble those observed in the cohorts dedicated to evaluating these alterations (PALACIOS *et al*., 2020).

Overall, further well-controlled RCTs with strains or formulations that have proven effectivity should be performed to improve the level of evidence that is currently available. Furthermore, the administration of species that are inversely correlated with diabetes markers, such as *Akkermansia muciniphila*, should be carried out with the possibility of obtaining better

results. Finally, evaluating changes at the functional level of the gut microbiota is also desired to elucidate the way in which probiotics modify microbial metabolism and impact the host.

7 CONCLUSION AND FUTURE PERSPECTIVES

Despite the substantial increase in publications evaluating the effect of probiotics on T2DM, the methodologies are highly heterogeneous, usually lack adequate control, and often are not comparable. This review was dedicated towards evaluating the studies using only probiotic supplements, to avoid confounding effects caused by food matrices and other substances in association. It is known that the probiotics effects are strain-specific and disease-specific, and in the future clinical trials dedicated to evaluating the effect of the most promising strains should be carried out, thereby for allowing a better comparison between them.

Many of the bacterial species altered in the T2DM gut microbiota are not cultivable, which represents a challenge to investigate the effects of their supplementation. Despite this, a pilot clinical trial has already been developed to verify the safety and tolerance with *Akkermansia muciniphila*, a bacterium difficult to culture due to its sensitivity to oxygen. This fact leads to the possibility that new technological and cultivation techniques may allow for the cultivation of strictly anaerobic strains from the host gut microbiota. Together, SCFA-producing bacteria supplementation should be performed due to its low abundance in the T2DM gut microbiota.

The association of changes in the gut microbiota, both at the compositional and functional levels, and T2DM traits is well documented. Together with the microbial metabolites, they play a critical role to the etiology of T2DM. Future research aiming, not only at taxonomic changes in the microbiota, but also at altered metabolic pathways in T2DM, should be performed in human clinical trials with the possibility of obtaining effective and comparative results. Current literature indicates that some strains appear to have a beneficial effect in patients with T2DM, but further studies are needed to support the use of these probiotics as an adjuvant therapy in the management of T2DM.

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ATTACHMENTS

ATTACHMENT 1 – Student record

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
FICHA DO ALUNO

9133 - 11042043/2 - Rafael Ballan Maluhy

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBT5781-5/3	Culturas Probióticas: Aplicações Tecnológicas (1)	20/08/2018	10/09/2018	60	4	100	A	N	Concluída
FBT5788-1/4	Aplicação de Alimentos Probióticos na Modulação de Imunidade de Mucosas	09/09/2019	29/09/2019	60	4	100	A	N	Concluída
FBA5753-2/2	Nutrigenômica e Programação das Doenças Crônicas Não-Transmissíveis	30/09/2019	06/10/2019	30	2	100	A	N	Concluída
FBA5752-2/1	Probióticos em Alimentos e suas Implicações na Saúde Humana	15/10/2019	25/11/2019	60	4	100	A	N	Concluída
HNT5759-3/3	Genômica Nutricional no Contexto das Doenças Crônicas não Transmissíveis (Faculdade de Saúde Pública - Universidade de São Paulo)	05/11/2019	09/12/2019	60	4	93	A	N	Concluída
FBA5905-2/3	Planejamento Experimental e Análise Multivariada	18/02/2020	14/06/2020	60	4	100	A	N	Concluída
BMF5825-5/6	Seminários Gerais de Farmacologia (Instituto de Ciências Biomédicas - Universidade de São Paulo)	04/03/2020	16/06/2020	30	2	100	A	N	Concluída
FBT5773-8/5	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica	09/03/2020	17/05/2020	30	2	100	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito da dissertação	
Disciplinas:	0	25	26
Estágios:			
Total:	0	25	26

Créditos Atribuídos à Dissertação: 71

Observações:
1) Disciplina(s) cursada(s) isoladamente e aceita(s) pelo(a) orientador(a) do(a) candidato(a)

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 15/03/2021
Impresso em: 06/06/2021 15:17:35

Janus - Sistema Administrativo da Pós-Graduação

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Curso:	Mestrado
Programa:	Tecnologia Bioquímico-Farmacêutica
Área:	Tecnologia de Alimentos
Data de Matrícula:	10/04/2019
Início da Contagem de Prazo:	10/04/2019
Data Limite para o Depósito:	12/07/2021
Orientador:	Prof(a). Dr(a). Susana Marta Isay Saad - 10/04/2019 até o presente. Email: susaad@usp.br
Proficiência em Línguas:	Inglês, 10/04/2019
Data de Aprovação no Exame de Qualificação:	Aprovado em 04/11/2020
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 10/04/2019

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 15/03/2021
Impresso em: 06/06/2021 15:17:35

Janus - Sistema Administrativo da Pós-Graduação

ATTACHMENT 2 – Curriculum Vitae Lattes

Rafael Ballan Maluhy

Endereço para acessar este CV: http://lattes.cnpq.br/7748760484316319
ID Lattes: **7748760484316319**
Última atualização do currículo em 02/06/2021

Possui graduação em Nutrição pelo Centro Universitário São Camilo (2017). Mestrando em Tecnologia Bioquímico-Farmacêutica (2019). Tem experiência na área de Nutrição, com ênfase em Tecnologia de Alimentos. Atuando principalmente nos seguintes temas: Probióticos, Prebióticos e Doenças Metabólicas e Intestinais. **(Texto informado pelo autor)**

Identificação

Nome	Rafael Ballan Maluhy
Nome em citações bibliográficas	BALLAN, R.;Ballan, Rafael
Lattes iD	http://lattes.cnpq.br/7748760484316319

Endereço

Formação acadêmica/titulação

2019	Mestrado em andamento em Programa de Pós Graduação em Tecnologia Bioquímico Farmacêutica. Faculdade de Ciências Farmacêuticas - USP, FCF, Brasil. Título: Aplicação de probióticos visando a melhoria de tolerância à glicose em indivíduos obesos pré-diabéticos,Orientador: Susana Marta Isay Saad. Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.
2014 - 2017	Graduação em Nutrição. Centro Universitário São Camilo, USC, Brasil. Título: Disbiose Intestinal: Relação da Microbiota Intestinal com a Obesidade. Orientador: Roseli Espíndola Balchiunas.

Formação Complementar

Áreas de atuação

1.	Grande área: Ciências da Saúde / Área: Nutrição.

Idiomas

Português	Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Inglês	Compreende Bem, Fala Razoavelmente, Lê Bem, Escreve Razoavelmente.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica

1. BATTISTINI, CAROLINA ; **Ballan, Rafael** ; HERKENHOFF, MARCOS EDGAR ; Saad, Susana Marta Isay ; SUN, JUN . Vitamin D Modulates Intestinal Microbiota in Inflammatory Bowel Diseases. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES JCR, v. 22, p. 362, 2021.

   Citações: WEB OF SCIENCE ™ 1

Capítulos de livros publicados

1. **Ballan, Rafael**; Xavier-Santos, Douglas ; Saad, Susana Marta Isay . Interactions of probiotics and prebiotics with the gut microbiota. In: Jun Sun. (Org.). Progress in Molecular Biology and Translational Science. 9ed.: Elsevier, 2020, v. 171, p. 265-300.

Eventos

Participação em eventos, congressos, exposições e feiras

1. Espectrometria de massas aplicada à análise de metabólitos naturais- instrumentação analítica e metabolômica. 2020. (Outra).
2. 24º Congresso Brasileiro Multidisciplinar em Diabetes. 2019. (Congresso).
3. PCR em Tempo-Real: Além do positivo e negativo. 2019. (Outra).
4. Producing and delivering efficacious probiotics to the consumer: the practical side of probiotics. 2019. (Outra).
5. Curso online: Bioquímica e Metabolismo. 2018. (Outra).
6. SOCESP - Sarcopenia e doenças cardiovasculares: a ingestão proteica como uma peça do quebra-cabeça. 2018. (Outra).
7. 38º Congresso da SOCESP. 2017. (Congresso).
8. SIMPONUTRI. 2017. (Simpósio).
9. SOCESP - Café e Vinho & Coração: Aspectos clínicos. 2017. (Outra).
10. Curso: Bioquímica e Biologia Molecular aplicada à Nutrição. 2016. (Outra).
11. EXPONUTRITION CONFERENCE. 2016. (Congresso).
12. SIMPONUTRI. 2016. (Simpósio).
13. 1st Annual ISSN-Brasil Meeting - Módulo Nutrição Esportiva. 2015. (Encontro).
14. 3º Meeting Nutrir+ - Hipertrofia Muscular - Oferta de proteínas e aminoácidos sob a ótica científica. 2015. (Outra).
15. 3º Meeting Nutrir+ - Suplementos em atividade física aeróbia. 2015. (Outra).
16. Arnold Conference - Módulo de Nutrição Esportiva. 2015. (Congresso).
17. Circuito Nacional de Nutrição Esportiva. 2015. (Outra).
18. EXPONUTRITION CONFERENCE. 2015. (Congresso).
19. SIMPONUTRI. 2015. (Simpósio).
20. EXPONUTRITION CONFERENCE. 2014. (Congresso).
21. Seminário Internacional de Nutrição Esportiva. 2014. (Seminário).
22. Workshop de Nutrição Esportiva e Treinamento da Probiótica. 2014. (Outra).

ATTACHMENT 3 – First page of the scientific article published in *International Journal of Molecular Sciences*, entitled "Vitamin D Modulates Intestinal Microbiota in Inflammatory Bowel Diseases"

Open Access Review

Vitamin D Modulates Intestinal Microbiota in Inflammatory Bowel Diseases

by Carolina Battistini [1,2,†], Rafael Ballan [1,2,†], Marcos Edgar Herkenhoff [1,2], Susana Marta Isay Saad [1,2,*] and Jun Sun [3,4,*]

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† These authors contributed equally to this work.

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Abstract

Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract (GIT), including Crohn's disease (CD) and ulcerative colitis (UC), which differ in the location and lesion extensions. Both diseases are associated with microbiota dysbiosis, with a reduced population of butyrate-producing species, abnormal inflammatory response, and micronutrient deficiency (e.g., vitamin D hypovitaminosis). Vitamin D (VitD) is involved in immune cell differentiation, gut microbiota modulation, gene transcription, and barrier integrity. Vitamin D receptor (VDR) regulates the biological actions of the active VitD (1α,25-dihydroxyvitamin D3), and is involved in the genetic, environmental, immune, and microbial aspects of IBD. VitD deficiency is correlated with disease activity and its administration targeting a concentration of 30 ng/mL may have the potential to reduce disease activity. Moreover, VDR regulates functions of T cells and Paneth cells and modulates release of antimicrobial peptides in gut microbiota-host interactions. Meanwhile, beneficial microbial metabolites, e.g., butyrate, upregulate the VDR signaling. In this review, we summarize the clinical progress and mechanism studies on VitD/VDR related to gut microbiota modulation in IBD. We also discuss epigenetics in IBD and the probiotic regulation of VDR. Furthermore, we discuss the existing challenges and future directions. There is a lack of well-designed clinical trials exploring the appropriate dose and the influence of gender, age, ethnicity, genetics, microbiome, and metabolic disorders in IBD subtypes. To move forward, we need well-designed therapeutic studies to examine whether enhanced vitamin D will restore functions of VDR and microbiome in inhibiting chronic inflammation. View Full-Text

Keywords: antimicrobial peptides (AMP); Crohn's disease; dysbiosis; epigenetics; inflammation; metabolites; microbiome; micronutrient; nuclear receptor; probiotics; tight junctions; ulcerative colitis; vitamin D; VDR

▼ Show Figures

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,*]

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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

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potential increase in intestinal permeability leading to a systemic inflammation triggered by higher levels of circulating lipopolysaccharides and changes in the immune response caused by an overgrowth of a specific genus or of pathogens. These mechanisms may increase symptoms in gastrointestinal disorders or reduce glucose tolerance in metabolic diseases. Diet also has a significant impact on GM, and functional foods, namely prebiotics and probiotics, are a novel approach to reestablish the indigenous microbiota. Prebiotics, like inulin and polyphenols, are selectively utilized by GM, releasing short-chain fatty acids (SCFA) and other metabolites which may reduce the intestinal lumen pH, inhibit growth of pathogens, and enhance mineral and vitamin bioavailability. Probiotic microorganism may increase the microbial diversity of GM and improve the integrity of the intestinal barrier, leading to an improvement of baseline and pathologic inflammation. In this chapter, we will discuss the potential roles of prebiotics and probiotics in health and diseases throughout an individual's lifetime and proposed mechanisms of action.

1. Introduction

The term "microbiota" refers to the entire population of microorganisms in a given location. The largest population of microorganisms is the microbiota of the gut, which is comprised of more than 2000 bacterial species and is significantly influenced by intrinsic (genetics, age) and extrinsic (body mass index, smoking, physical activity, diet) factors, mainly by diet, which regulates microbial activity and gene expression (Fig. 1).[1–3] Studies have shown that several diseases are associated with an imbalance of the bacterial composition of the gut, which is referred to as a dysbiosis. Dietary interventions may be effective in restoring it to a healthier state.[4]

In this sense, functional foods, which in addition to their nutritional properties must confer a defined benefit to the consumer's health, seem to be interesting alternatives for dietary interventions targeting at improving the microbiota shape. The most known functional foods or ingredients are polyphenol, polyunsaturated fatty acids, prebiotics, and probiotics.[5,6]

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host."[7] While the definition of probiotics has been debated, an expert consensus from 2013 has agreed on this description.[8] The major mechanisms of action of probiotics on human health are multifold and include competition with pathogens for nutrients and adhesion sites, production of bacteriocin, vitamins, and short-chain fatty acids (SCFA), immunomodulation, improvement of the intestinal barrier, and production of neurotransmitters.[9]

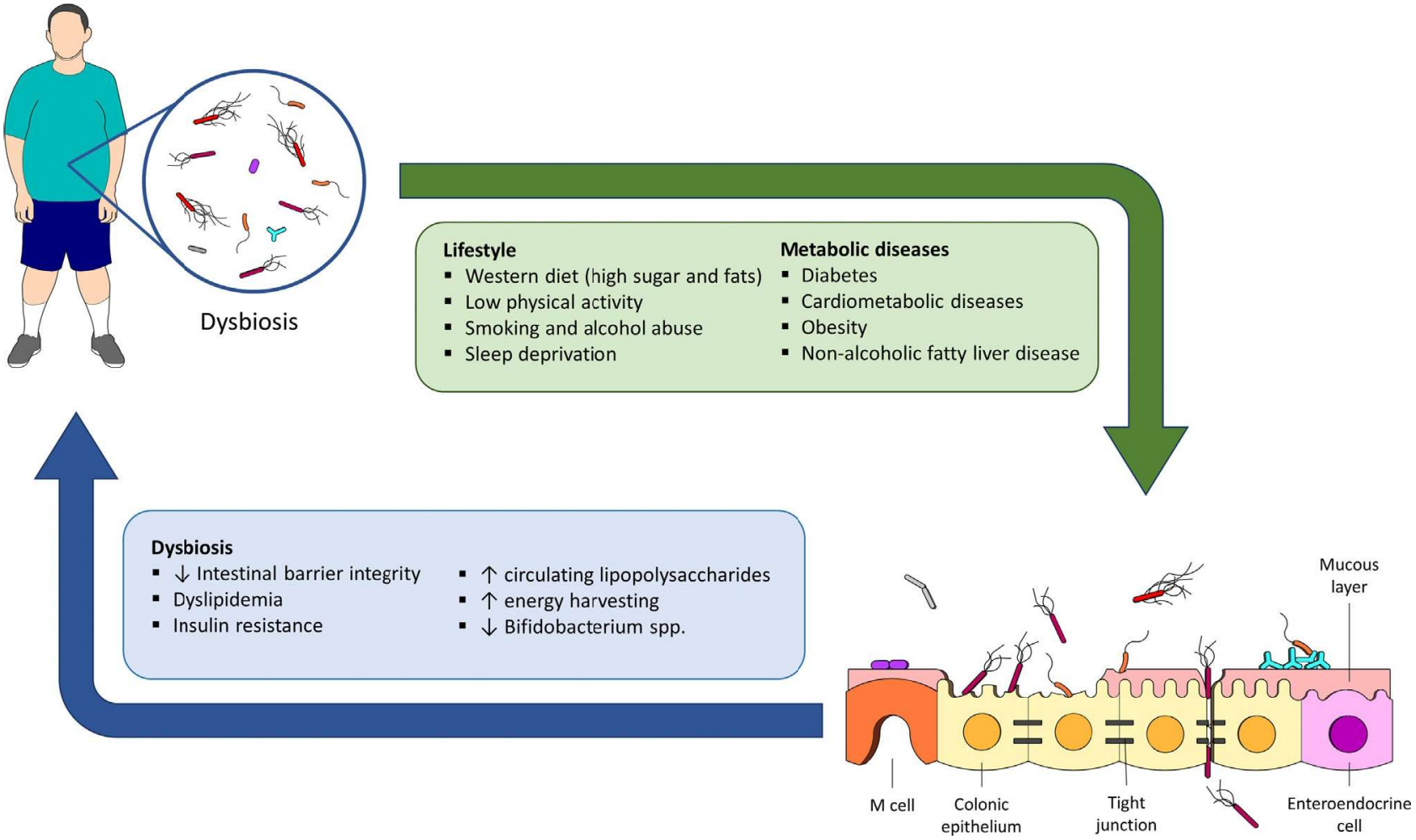

Fig. 1 Factors that influence the gut microbiota.

In the last years, studies related to probiotics and their effects on human health have increased exponentially. The vehicles or food matrices used to deliver probiotics affect the viability of the microorganisms and, therefore, have different impacts on the host microbiota. In addition, prebiotics and bioactive food compounds may interact with the commensal microorganisms that inhabit the human gut.

Several clinical trials have been conducted in order to evaluate the effect of probiotics, primarily those containing bacteria from the *Lactobacillus* and *Bifidobacterium* genera, on different food matrices and their impacts on the human health, leading to heterogeneous results. In this chapter, we will delve into different bioactive and probiotic foods and their effects on the human health.

2. An overview into the human microbiota

The human microbiota consists of a complex ecosystem that holds approximately 10^{14} bacteria. Much has been discussed about the factors that may contribute to its shape in the prenatal period, since the mother's oral microbiota has recently been identified as being similar to that found in the placenta, comprising Firmicutes, Tenericutes, Proteobacteria, Bacteroides, and Fusobacteria phyla. One hypothesis is that these bacteria are transported from the oral cavity to the fetus through the bloodstream.[10]

Outside of any in utero exposures, the first contact of the newborn with various microorganisms occurs during birth, which is influenced by the mode of delivery.[11] In the vaginal delivery, the skin and mucous membranes of the baby are in contact with the local microbiota, which leads to colonization by *Lactobacillus* spp., reflecting the mother's vaginal microbiota. In contrast, when delivery occurs by cesarean section, the newborn's mouth, intestines, and skin are colonized by microorganisms present in the birth and skin environments, such as *Staphylococcus* and *Propionibacteria*.[12,13]

During the first months of life, the gut microbiota adapts to the conditions of the surrounding environment and are influenced by anaerobic conditions, nutrient availability, and microbial interactions during community succession.[13] Cesarean-born infants often have fewer important maternally-transmitted microorganisms, such as Bacteroides and Bifidobacteria. Infants delivered via c-section continue to exhibit a lower diversity and reduced Th1 response during the first two years of life, although this difference in colonization between delivery modes is gradually reduced over time.[12,13]

Breastfeeding is the other major contributor to early microbial colonization of infants. Breast milk may contain up to 10^7 bacterial cells in 800 mL, mainly from the *Lactobacillus*, *Streptococcus*, and *Staphylococcus* genera.[14] In addition, it contains oligosaccharides that stimulate the selective growth of *Bifidobacterium* and *Lactobacillus* spp. and through fermentation in the gut, SCFA are released, reducing the colonic pH. Initially, this reduces the diversity of the infant's intestinal microbial community, which is likely due to acidification of the intestinal pH. Meanwhile, acidification acts as a defense against pathogens that cannot survive under these conditions.[15] Furthermore, the presence of immunoglobulin A, defensins, and lactoferrin in human milk provide other benefits to the infant's health,[16] and exclusive breastfeeding in the first months of life has been associated with a reduction in several childhood diseases, such as obesity, infections, and atopic diseases.[17–19]

After food introduction and, consequently, increased nutrient pool ingestion, the infant's intestinal microbiota resembles an adult's microbiota. It is generally reached in the third year of life and will be influenced together with the environment in which the child is inserted. A diverse microbiota enhances the vitamins and amino acids biosynthesis and the ability to metabolize carbohydrates.[13,20]

In adulthood, the microbiota is composed mainly of Firmicutes and Bacteroidetes, whereas members of other phyla, like Actinobacteria, Proteobacteria, and Verrucomicrobia are present in lower populations. The gut microbiota plays a role in various functions of the body, such as energy storage, intestinal barrier integrity, immune system development, including tolerogenicity, and neurotransmitter production. The host's microbiota is dynamic and, until now, it is difficult to establish how different factors, such as lifestyle, illnesses, geographical location, and age, may impact its stability.[21,22]

Presently, there is a collective effort to identify what would be a healthy microbiota, also known as a "core microbiota." However, due to the highly heterogenicity among individuals, it is easier to compare with the same individual along his lifetime. In fact, a core microbiome exists at the level of metabolic functions.[23] Throughout life, the gut microbiota is resilient, and transient or punctual situations do not seem to significantly affect its composition, such as adherence to a specific diet, use of antibiotics, and exposure to different environments.[24]

Several clinical trials have demonstrated that a number of diseases are related to changes in the relative abundance of a phylum or genus. For example, obesity or high caloric diet are associated with a greater relative

abundance of Firmicutes over Bacteroidetes. This also occurs in populations that eat a typical western diet, which is rich in processed foods, carbohydrates, and sugars.[25,26] Other metabolic diseases, such as diabetes, cardiometabolic diseases, and low-grade inflammation associated with obesity are inversely associated with *Akkermansia muciniphila* abundance.[27]

In conclusion, mechanisms involved in shifts of the gut microbiota are still not well understood. However, dietary interventions with functional foods, like probiotics, are being studied extensively and have been proposed as a way of ameliorating various diseases and as a potential adjunctive therapy or preventative strategy, in spite of the fact that they are not able to promote permanent changes in the host's microbiota.

3. Functional foods

It is well known that functional foods may be good alternatives in preventing or ameliorating the *status* of certain diseases. Moreover, they can be used in combination with traditional therapies as adjuvants and, unlike antibiotics, will not have a detrimental effect on beneficial populations of bacteria. The major functional foods or ingredients explored are prebiotics, probiotics, the combinations of both in synbiotic foods, in addition to dietary fibers and bioactive compounds.

3.1 Prebiotics

A prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit."[5] The potential health benefits related to prebiotics include gut microbiota modulation along with beneficial microbial metabolites release (e.g., SCFA and tryptophan). However, these effects should be verified in the target host (animal or human).[5,28,29] Several prebiotics are commercially available, like fructo-oligosaccharides (FOS), inulin, β-galactooligosaccharides (GOS), lactulose, isomalto-oligosaccharides (IMO), and resistant starches (i.e., the fraction of starch that resists to digestion in the small intestine).[30]

Innumerous studies explored the potential health benefits of fiber consumption, with or without prebiotic effect. The main pathway associated with prebiotics involves selective fermentation by beneficial microorganism present in the gut, including *Lactobacillus* and *Bifidobacterium*, which produce acetate and lactate and in turn may stimulate production of butyrate by other bacteria. Moreover, the production of SCFA bestows diverse health benefits like improvement of mineral absorption and barrier function.[5,28,29]

In addition, prebiotics may be used to enhance fermentation processes or as encapsulating material. According to Oliveira et al., inulin, one of the most studied prebiotic compounds, may increase the acidification rate of milk by co-cultures of probiotic and starter strains.[31] Meanwhile, Santos et al. also reported higher resistance to GIT stress simulated in vitro by the probiotic strain *Lactobacillus acidophilus* La-5 microencapsulated with inulin when compared to free cells. Moreover, when incorporated in a food matrix, the survival rate was further increased.[32] Similarly, Rosolen et al. stated that the combination of whey and inulin as a coating material for *Lactococcus lactis* R7 promoted resistance to heat treatment and GIT stress simulated in vitro.[33]

3.2 Polyphenols

Polyphenols are a large group of compounds that are well recognized for their anti-inflammatory and antioxidant properties and their potential health benefits on cardiometabolic diseases and brain function. The most common phenolic compounds are flavonoids (e.g., flavonols, isoflavones, and anthocyanins), phenolic acids (e.g., ellagic and caffeic acids), lignans, and stilbenes (e.g., resveratrol), which can be consumed in black and green tea, red wine, apples, blueberries, dark chocolate, onions, almonds, soy, pomegranates, and coffee, among others.[34–36]

After ingestion, some polyphenols are absorbed in the small intestines, while a significant amount can reach the large intestines intact where they can be metabolized by the gut microbiota and release active metabolites, like phenolic acids. Moreover, studies have shown that some compounds may stimulate beneficial bacteria, like *Lactobacillus* spp., *Bifidobacterium* spp., *Akkermansia muciniphila*, and *Faecalibacterium prausnitzii*. Conversely, other compounds may inhibit pathogens, like *Helicobacter pylori*, *Staphylococcus aureus*, and *Listeria monocytogenes*.[34–36]

Hence, there is evidence that polyphenols have potential as prebiotics and an innovative option for gut microbiota modulation. Furthermore, fermentation by lactic acid bacteria may be an alternative to improve bioavailability of these compounds, which are generally low in unprocessed foods.[36–38]

3.3 Omega-3

Consumption of omega-3 polyunsaturated fatty acids has been associated with a reduction in cardiovascular diseases and improvement of cognition,

depression, behavioral disorders, and brain function. Besides gut microbiota modulation, anti-inflammatory effects, and increased SCFA release, showing direct influence in the gut-brain axis.[39–42]

The major bioactive forms of omega-3 are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which can be found in some fatty fishes or in nutraceutical supplements. Their precursors are α-linolenic acid (ALA), contained in nuts, flaxseed, and canola and soy oil.[39,40]

Studies have reported the potential of omega-3 fatty acids to modulate the gut microbiota and to act as prebiotic candidates. According to Prossomariti and colleagues, the consumption of EPA was able to improve the mucosal inflammation in ulcerative colitis (UC) patients, while decreasing *Clostridium* spp. in the feces in a short-term intervention.[41] In another study, a high omega-3 diet increased significantly *Bifidobacterium* and *Lactobacillus* population in mice throughout life. Meanwhile, it influenced positively brain function and behavior of the animals.[42]

3.4 Probiotics

Potential health benefits associated with probiotic consumption are widely known. Nonetheless, several factors should be taken into consideration when developing a food product or supplement with claims of health benefits. The microorganisms should be well recognized as GRAS (Generally Recognized as Safe), claims of health benefits may only be associated with select strains, not all genuses or species, or with the target population in that specific clinical trial (e.g., infants, chronic disease patients, elderly, etc.). In addition, a recommended daily dose of colony-forming unit (CFU) to achieve health benefits should be indicated and the microorganisms should be viable during the shelf-life of the products.[7,8,43]

The most common probiotics are species from the *Bifidobacterium* and *Lactobacillus* genera. However, other microorganisms may be classified as probiotics, such as *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Streptococcus thermophilus*, *Lactococcus lactis*, and *Saccharomyces bourlardii*.[8,44,45] Moreover, each strain has different multiplication and survival rates after passage through the gastrointestinal tract (GIT) depending on the food matrix (e.g., milk, soymilk, and fruit juice), pH, incorporated oxygen (e.g., stirred-yogurt), temperature of storage (e.g., room temperature, refrigerated or frozen), presence of prebiotics or any other food ingredient, and microencapsulation.[45–49]

In addition to health effects, probiotics can improve food matrices by reducing the level of undesirable compounds, like stachyose and raffinose in soymilk, or by producing compounds of interest, like vitamins.[46,47,50] Moreover, starter strains, such as *Streptococcus thermophilus*, are usually combined with probiotic cultures to reduce fermentation times, which when co-cultured with *Bifidobacterium* strains prevent off-flavors due to acetic acid release by species from this genus.[31,47,51]

Prebiotic compounds may be used in combination with probiotics, resulting in synbiotic foods, which have synergistic effects including reduction of fermentation time or enhancement of the survival of probiotics during passage through the GIT.[31,44] However, any changes in matrices may affect the behavior of strains differently. Bedani et al. reported that *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 in fermented soymilk had higher survival rates after GIT stress simulated in vitro when compared to their fresh cultures, without any food matrix.[52] On the other hand, the addition of tropical fruit pulps in probiotic fermented soymilk showed the opposite effect, despite an improvement in the consumers' acceptance of the product.[53]

Besides prebiotics or other ingredients, emerging food technologies, such as microencapsulation and ohmic heating, can be applied to improve fermentation processes or to increase the resistance to GIT stress.[31–33,47,51] Nevertheless, it has recently been suggested that microbial metabolites alone are enough to confer health benefits and viable cells in the gut are not necessarily essential. This concept might completely change the directions of probiotic food and supplements research.[47]

In conclusion, there are several factors that influence probiotic stability in different matrices and their survival through the GIT, and consequently, their health benefits. Nevertheless, there is a lot of heterogeneity in the intervention studies reported, like participants' age, sex, body mass index, metabolic diseases, daily dose, duration of the study, different prebiotic and/or probiotic cultures administered, and different food matrices. Therefore, it is difficult to compare outcomes and identify one unique answer to fit all cases and design of a tailored treatment is likely a more biologically plausible approach to probiotic use.

4. Effects on healthy individuals

A limited number of clinical trials have studied the impact of probiotics or bioactive food consumption on the gut microbiota, especially

in healthy individuals. Several studies evaluated the fecal recovery of probiotic strains in order to verify if the administered microorganisms survived through the GIT, in addition to checking the participants' adherence to the dietary intervention. Indeed, molecular biology techniques, like gene sequencing or real-time PCR quantification, are helpful in understanding the actual effects of probiotics on the gut microbiota. However, they are costly and are not always available to all researchers.

In a randomized double-blind placebo-controlled trial, Rampelli et al. administered a probiotic biscuit daily containing *Bifidobacterium longum* Bar33 and *Lactobacillus helveticus* Bar13, 10^9 CFUs per serving versus placebo to 32 elderly participants living in Italy. Fecal samples were collected at baseline (T0) and after 30 days (T30). When compared to healthy adults, elderly subjects had higher levels of pathobionts, such as *Clostridium difficile*, *C. perfringens*, *Bacillus cereus*, *Campylobacter*, and Enterobacteriaceae, whose influence on the host occurs indirectly via stimulation of the immune system. After treatment, the placebo group microbiota remained the same while the intervention group had decreased opportunistic pathogens. Furthermore, increased content of health-promoting bacteria such as *Akkermansia muciniphila*, *B. longum*, and Lactobacillaceae was observed in the treatment group. These results suggest that dietary intervention with probiotic food may improve age-related dysbiosis, promoting a transitory healthier gut microbiota.[54]

Fermented milk containing *Lactobacillus casei* strain *Shirota* (LCS) is known to promote immune system function in healthy individuals. Mechanisms, such as an increase in natural Killer (NK) cell function, activation markers on circulating T cells, and increased concentrations of IgA1 and IgA2 in the oral mucosa, are well described.[55,56] Nevertheless, little is known about the effects of its consumption on the intestinal microbiota. In a study conducted in 25 healthy Chinese adults, subjects consumed daily 100 mL of a probiotic drink containing 10^8 CFU/mL of viable LCS (Yakult). Fecal recovery showed that the strain survived the GIT and promoted the growth of beneficial bacteria, especially *Bifidobacterium* and *Anaerostipes*, an efficient lactate fermenter, suggesting a synergistic effect, once *Bifidobacterium* is a lactate producer. Furthermore, population of butyrate-producing bacteria, such as *Roseburia intestinalis* and *Clostridium*, declined. The strain demonstrated poor ability to persist in the GIT during follow-up (intervention, day 21: 10^7 CFU/g of feces; endpoint, day 42: 10^4 CFU/g of feces) and decreased ability to produce SCFA. This suggests that, despite modifying the microbiota composition of adults, regular consumption is necessary.[57,58]

Interestingly, Klein et al. conducted a cross-over study evaluating the impact of the consumption of a yogurt (300 g/day) containing probiotic strains *Lactobacillus acidophilus* 74-2 (9.3×10^8 CFU/g) and *Bifidobacterium lactis* 420 (3.0×10^6 CFU/g) on the host microbiota. After the run-in period (3 weeks), subjects consumed the probiotic or control yogurt for 5 weeks and then they have switched groups for another 5 weeks. Probiotics were significantly increased in fecal samples, mainly *B. lactis* 420 counts, but this was transient. The levels of cholesterol, LDL-c and HDL-c, and SCFA did not change, nor did other specific immune parameters.[59] It is noteworthy that adults were healthy, there was no washout period, and the probiotic doses administered were probably not sufficient to promote health benefits.[60]

The effects of the consumption of probiotic strains on healthy adults were recently reviewed by Khalesi et al. and found that current literature supports the use of probiotics to improve defection frequency, stool consistency, bowel movement, immune system responses, and vaginal lactobacilli concentration.[61] It seems that the use of probiotics in improving GI discomfort is most effective in healthy individuals.

The most well-known properties of prebiotics in healthy individuals is their ability to relieve intestinal discomfort or increase absorption of minerals. Improvement in stool consistency, defecation frequency, regulation of motility by SCFA-signaling hormones, flatulence and bloating, and increased calcium solubility by pH reduction are also described frequently.[62–64]

Supplementation of prebiotic GOS at 5 g/day increased calcium absorption, together with an increase in fecal bifidobacteria in young girls (9–13 years of age), suggesting that bifidobacteria may mediate this mechanism.[62] Similarly, the use of a mixture of inulin-type fructans (8 g/day) has been shown to be effective in increasing calcium absorption in adolescents and may be an interesting approach for improving or preventing bone mass loss.[65]

The effect of β-fructans, such as oligofructose and fructo-oligosaccharides, has been evaluated in healthy and ill subjects. A recent meta-analysis of human studies that tested β-fructans as a single supplementary ingredient and applied in a product or in the form of dietary supplementation demonstrated that short-chain and not long-chain β-fructans supplementation (degree of polymerization <10) contributed to an increased stool frequency (0.36 defecation ± 0.06 per day; $P < 0.001$). In addition, β-fructans improved stool consistency and stool wet weight. The effects promoted by β-fructans appear to improve these features up to a dose of 18 g/day, with no substantial effects above that. Furthermore, bifidobacteria growth

stimulation appears to be dose-related, between 2.5 and 10 g/day, and doses above this only lead to marginal increases in stool frequency. Thus, different types of prebiotics might be useful for those with chronic constipation and may promote well-being by regulating bowel function.[66]

There are divergent opinions about studies designed to investigate the ability of probiotics to colonize the gut. Many studies fail to perform a follow-up of study participants' microbiota. However, since lifestyle is one of the factors shaping the autochthonous microbiota and probiotics have poor adherence to the intestinal mucosa, it is to be expected that the consumption of probiotic foods on a daily basis is recommended to produce the benefits observed in clinical trials.

5. Effects on various diseases

5.1 Application on early life conditions and diseases

Gastrointestinal disturbances are common in the early years of life. Therefore, many clinical trials are dedicated to preventing and treating diseases that affect this population, including acute and antibiotic-associated diarrhea, infantile colic, prevention of *Clostridium difficile*-associated diarrhea, necrotizing enterocolitis, *H. pylori* infection and neonatal sepsis.[67]

It is common for infants not to receive exclusive breastmilk and to minimally receive its benefits.[68] Thus, some infant formulas have been supplemented with prebiotics, such as GOS and FOS, aiming to prevent diseases. Enrichment of infant formulas with prebiotics has been effective in reducing respiratory tract infections to comparable levels observed breast fed children.[69,70]

5.1.1 *Acute gastroenteritis, antibiotic associated diarrhea,* Clostridium difficile*-associated diarrhea*

The most well-established condition for probiotic supplementation in infants is acute diarrhea. The use of probiotics is strongly recommended by the European Society of Pediatric Infectious Diseases (ESPGHAN) guidelines as an adjunct with rehydration for reducing the length of hospitalization (1.12 days, 95% confidence interval [CI] −1.16 to 0.38) and disease symptoms. In addition, the best-established strains with high quality clinical trials conducted are *L. rhamnosus* GG and *S. boulardii* CNCM I-745.[71]

Antibiotic-associated diarrhea (AAD) is a major global health concern in children, especially in Asia-Pacific where antibiotics are overused.[72,73] The main causative agent is *C. difficile* and its complications are severe.

According to current guidelines for the Asia-Pacific region, *L. rhamnosus* GG and *S. boulardii* CNCM I-745 may be considered in pediatric populations, since they are effective in the prevention of AAD and *C. difficile* infections.[74] In contrast, the ESPGHAN group strongly recommends the use of *L. rhamnosus* GG or *S. boulardii* for AAD prevention. The recommendation to use *S. boulardii* to prevent *C. difficile* associated diarrhea is not as strong and includes the caveat that physicians should be cautious and include evaluation of risk factors for these conditions before prescribing use of these probiotic strains.[75]

5.1.2 Necrotizing enterocolitis (NEC)

The incidence of NEC in preterm infants is high, mainly in those with very low birth weight (VLBW) <1500 g, increasing the interest in evaluating probiotic effects that improve intestinal barrier function, prevent bacterial translocation, promote mucosal IgA production, and inhibit potential pathogens.[76,77]

Several meta-analyses show that the combination of *B. infantis* + *Str. thermophilus* + *B. bifidus* and *L. acidophilus* + *B. infantis*, were the most effective in reducing risk of NEC and mortality in VLBW infants, but not for nosocomial sepsis.[78] Thus, Latin-American (LATAM) experts' consensus grades 1a (Systematic Review with homogeneity of RCTs) recommend use of the following probiotic strains: *B. brevis*, *L. rhamnosus* GG, *L. acidophilus*, and *L. reuteri* DSM 17938 and mixtures of *Bifidobacterium* and *Streptococcus*.[67]

Although several studies have demonstrated the efficacy of probiotics from the *Lactobacillus* and *Bifidobacterium* species in reducing the incidence of NEC and NEC-associated mortality, recently conducted high quality RCTs have not been able to demonstrate that BBG-001 strain *B. brevis* is effective in reducing the risk of NEC. However, the latest expert consensus for the Asia-Pacific region states that probiotics may be considered to prevent NEC due to evidence of their effectiveness in reducing risk and mortality in high-risk populations.[74]

5.1.3 Infant colic

Colic is perceived as a condition that requires intervention in infants. There is usually an increase in gas production and changes in bowel motility, resulting in abdominal pain. The *L. reuteri* DSM 17938 strain was effective and safe in many clinical trials for the prevention of colic episodes, reducing infant fussiness, regurgitation, and constipation. Therefore, Latin-American experts classify the recommendation of this strain as grade 1a (prevention)

and 1b (treatment), while for Asia-Pacific population the evidence is considered weak, but the use of *L. reuteri* DSM 17938 may be considered.[67,74]

5.1.4 Inflammatory bowel disease

The role of probiotics in inflammatory bowel diseases is mainly targeted toward UC and Crohn's disease (CD). Probiotics are thought to modulate the host mucosal immune response, resulting in anti-inflammatory effects in IBD patients. Particularly for UC, guidelines suggest that commercial probiotic VSL#3, a multi-strain product including Bifidobacteria, Lactobacilli and Streptococcal species, is effective in inducing and maintaining remission of mild to moderate UC.[79] Another strain well studied is *E. coli Nissle*, for which the guidelines indicate that it may be as effective as standard mesalazine treatment in maintaining remission in UC.[74]

Crohn's disease management seems to be less effective, although several studies have shown changes in the intestinal microbiota to be relevant in this condition. One possible reason is that CD may be sub-classified, due to the genetic component present.[80] Current guidelines do not support effective probiotic therapy in maintaining remission of CD. The last expert panel conducted by Cameron et al. for the Asian-Pacific region concluded that the evidence is weak to indicate the use of probiotics in IBD in children, and did not recommend it overall.[74] In contrast, LATAM expert consensus recommended the use of VSL#3 only for UC (Evidence 1b—Individual RCT, with narrow Confidence Interval).[67,81]

5.1.5 Helicobacter pylori infection

With the increase in antibiotic resistance by pathogenic bacteria, interest for probiotics has grown with the objective of using them as a primary and adjuvant therapy. Some *Lactobacillus* species studied were unable to eradicate *H. pylori*, even in combination with drugs, such as antibiotics and proton pump inhibitors and probiotics only moderately improved eradication rates.[82,83] The recommendation for their use in the Asia-Pacific region is classified as "very low quality," but stronger evidence exists for the use of *S. boulardii*. There are currently no recommendations for probiotic use for *H. pylori* eradication in other geographic regions.[67,74,75]

It is noticeable that the evaluation of probiotic effects in the first years of life is directed toward health outcomes and not necessarily their effects on the intestinal microbiota. There are many RCTs conducted in infants, since they are a high-risk population with high mortality rates, garnering interest to improve the health status of this vulnerable population.

Additionally, specific guidelines have been developed for different regions, recognizing that each bacterial strain has unique properties that are affected by geography.

5.2 Adults

5.2.1 Metabolic diseases

With increasing prevalence of obesity and metabolic diseases such as diabetes mellitus type 2 (T2DM) in developed and emerging countries, the contributing factors for these diseases have been widely studied.[84] The GM plays a key role in energy homeostasis and low-grade inflammation, and its unbalance is associated with the development of the diseases mentioned above, a fact that has aroused the attention of researchers. Some bacterial species have been associated with obesity, such as *Lactobacillus* and *Bifidobacterium* species, Prevotellaceae and *Blautia coccoides*, as well as a high ratio between Firmicutes and Bacteroidetes.[85] Interventions that restore bacterial homeostasis in the gut may represent an important target for the use of probiotics and prebiotics and several studies have been conducted with the purpose of testing this approach. Such studies and respective results are discussed below.

In one study conducted by Stenman and colleagues, the strain *B. animalis* ssp. *lactis* 420 (B420) was administered to 225 healthy overweight or obese volunteers during a six-month period. The studied population was divided into four groups—probiotic only (B420, 10^{10} CFU/day), prebiotic Litesse® Ultra polydextrose alone (LU, 12 g/day), probiotic + prebiotic (B420: 10^{10} CFU/day + LU: 12 g/day), and placebo. In this study, B420 with or without LU was found to be effective in reducing body fat mass, waist circumference, energy intake, and body weight compared to placebo. This finding suggests a synergistic effect between the probiotic and the prebiotic and a major role for the chosen strain, as the prebiotic group did not show any difference when compared to the placebo. Results also demonstrated that expression of zonulin, a potential marker of intestinal permeability, was significantly correlated with changes in subjects' truncal fat mass ($r = 0.349$, $P < 0.0001$) in the B420 + LU group. Thus, the authors hypothesized that serum zonulin may be involved with gut microbiota modulation and intestinal integrity.[86]

Afterwards, Hibbert and colleagues aimed to explore possible changes in the microbiota and metabolites in the same population. The authors reported that *Lactobacillus* and *Akkermansia* were more abundant in the B420 group at the end of the intervention. *Methanobrevibacter*, Christensenellaceae, and *Akkermansia* were increased in B420 + LU, and LU alone groups, across

the intervention period and had a negative correlation with the waist-area body fat mass at the end of the intervention.[87]

Akkermansia muciniphila, a mucin degrading bacteria, has gained attention since its abundance is known to be inversely associated with obesity, diabetes, cardiometabolic diseases, and low-grade inflammation.[88,89] Animal studies have shown that treatment with *A. muciniphila* reduces obesity and associated comorbidities, such as insulin resistance and hepatic steatosis.[90,91] Due to difficulties cultivating this bacteria, its effects after heat treatment were evaluated in animal studies and demonstrated an even greater impact on adiposity, glucose tolerance, and insulin resistance after *A. muciniphila* was heat-treated (HT). Thus, Depommier et al. developed a synthetic medium and conducted a clinical trial on overweight/obese humans as a proof-of-concept exploratory study.[92]

The study was conducted with 40 volunteers, and 32 completed the 3-month protocol with daily administration of *A. muciniphila* at a concentration of 10^{10} CFUs/day. Subjects who received the HT *A. muciniphila* had improved insulin sensitivity ($+28.62 \pm 7.02\%$, $P = 0.002$), reduced insulinemia ($-34.08 \pm 7.12\%$, $P = 0.006$), and decreased total plasma cholesterol levels ($-8.68 \pm 2.38\%$, $P = 0.02$), when compared with the placebo group. Together, a small reduction in body weight (-2.27 ± 0.92 kg, $P = 0.091$) was observed, compared with the placebo group. Fat mass (-1.37 ± 0.82 kg, $P = 0.092$) and hip circumference (-2.63 ± 1.14 cm, $P = 0.091$) were reduced compared to baseline. As this was the first human study with *A. muciniphila* administration, tolerability and safety were among the primary outcomes, while metabolic effects were the secondary. These interesting results will definitely arouse interest from other researchers to conduct other RCTs that may potentially replicate these results and validate beneficial effects on other metabolic disorders.[92] Despite the results obtained, supplementation with either HT or living cells of *A. muciniphila* did not affect the overall structure of the gut microbiome. In fact, it only increased the quantity of *A. muciniphila* recovered in the feces by 1.7 and 2.6 log, respectively, in the HT and living cells groups.[92]

In recent years, several clinical trials evaluating the effects of probiotics on obesity have been done, with positive results in weight reduction,[93–96] waist circumference,[93,97–100] and fat mass,[93,96,99,101] and mainly *Lactobacillus* and *Bifidobacterium* species were applied, besides mixtures of probiotic strains and synbiotics. Altogether, these studies demonstrated that the use of probiotics, especially if combined with a dietary intervention, were able to assist in weight loss and fat mass reduction in obesity, probably by modulating the intestinal microbiota.

The effects of probiotic and prebiotic food have also been investigated in other metabolic diseases such as T2DM and non-alcoholic fatty liver disease (NAFLD). Studies using the *Lactobacillus* La-5 and the *Bifidobacterium* Bb-12 strains as an intervention in individuals with T2DM showed positive results for fasting glucose[102] and hemoglobin fraction A1c (HbA1c),[103] as well as improved lipid profile.[102–104]

In a recent meta-analysis of RCTs, conducted by Zheng et al., the effects of different probiotics and synbiotics on inflammatory biomarkers and oxidative stress in diabetic individuals were evaluated. Despite the methodological limitations emphasized by the authors, the consumption of probiotics and synbiotics was able to reduce high-sensitivity C-reactive protein (standardized mean difference [SMD] = − 0.38; 95% confidence interval [CI] levels: −0.51, −0.24; $P=0.000$) and malondialdehyde (SMD = −0.61; 95% CI: −0.89, −0.32; $P=0.000$) levels, together with a total increase in the antioxidant capacity (SMD = 0.31; 95% CI: 0.09, 0.52; $P=0.006$), in nitric oxide (SMD, 0.62; 95% CI, 0.25–0.99; $P=0.001$), and glutathione (SMD = 0.41; 95% CI: 0.26, 0.55, $P=0.000$) levels, in diabetic patients compared to those in the placebo group. The authors highlighted the heterogeneity between strains and intervention time, as well as the low amount of available data. Nevertheless, the probiotic and synbiotic approach demonstrated efficacy in improving the evaluated biomarkers.[105]

Verifying the effects of probiotics on altered parameters in individuals with T2DM has been a challenge, due to the lack of literature, inappropriate experimental designs, and high heterogeneity in the administration of probiotics. In a meta-analysis that included only RCTs conducted with diabetic subjects or with associated risk factors, Sun et al. evaluated the following outcomes: fasting blood glucose, glycated hemoglobin (HbA1c), insulin, or HOMA-IR (Homeostatic Model Assessment-Insulin Resistance). The results of the 11 included studies showed that probiotics were able to reduce blood glucose (mean: −0.50 mmol/L; range: 0.09–1.29 mmol/L), HbA1c (mean: −0.48%; range: −0.3 to 1.21%), whereas they were not able to decrease insulin and HOMA-IR significantly.[106]

NAFLD is characterized by the accumulation of fat in the liver (over 5% of liver weight).[107] This classification involves simple steatosis to non-alcoholic steatohepatitis (NASH) and may lead to severe conditions such as fibrosis and cirrhosis.[108] Because obesity, inflammation, insulin resistance, and dyslipidemia are risk factors for the disease, researchers have been studying the potential effects of probiotics and prebiotics on the disease.[109]

Fatty liver improvement was reported by Ahn et al. in a study conducted with 34 obese and NAFLD individuals, using a mixture of *Lactobacillus* and

Bifidobacterium. Using 16S rRNA microbiome sequencing gene, an increase in *Ruminococcaceae-2*, *Lachnospiraceae-2*, *Coprococcus*, *Lachnospiraceae-1*, *Ruminococcus*, and *Dorea* was observed.[110] Oligofructose supplementation (8 g/day for 12 weeks followed by 16 g/day for 24 weeks) in adults with liver-biopsy-confirmed NASH was also able to modulate the intestinal microbiota, increasing *Bifidobacterium* and reducing *Clostridium* clusters XI and I, together with ameliorating liver steatosis histologically-confirmed in NASH patients, compared to placebo.[111]

There are studies supporting the use of probiotics and prebiotics in NAFLD. A recent review targeting the effect of synbiotics on NAFLD found evidence to suggest that synbiotics can reduce inflammation, insulin resistance, and anthropometric parameters in NAFLD patients. However, the effects on dyslipidemia and oxidative stress are inconsistent. Additional RCTs in larger study cohorts including more accurate methods for the evaluation of the severity of diseases, such as liver enzymes, are chief to improve our understanding of probiotic usage in NAFLD.[112]

5.2.2 Inflammatory bowel disease (IBD)

The two main forms of IBD are UC and CD. In these conditions, the intestinal immune system is imbalanced, leading to non-specific chronic inflammation of the intestinal tract.[113]

Studies assessing the intestinal microbiota of individuals with IBD found a dysbiosis that could contribute toward an intestinal barrier dysfunction. In CD it is common to observe a reduction in the complexity of the Firmicutes phyla, specifically a reduced abundance of *F. prausnitzii*, as well as an increase in enteropathogenic *E. coli*.[114,115] In UC, the intestinal microbiota is characterized by a high ratio of *B. fragilis*/*F. prausnitzii* and a low abundance of butyrate-producing bacteria.[116]

There is a lot of controversy in the literature about how effective and safe the use of probiotics and prebiotics in patients with IBD might be, due to the intestinal mucosal sensitivity and a potential worsening of the disease.

In a recent meta-analysis, Astó et al. proposed to evaluate the effects of prebiotics, probiotics, and synbiotics on UC. The meta-analysis included 12 placebo-controlled trials, involving a total of 886 individuals. At first, high heterogeneity between studies was found ($I^2 = 71\%$), but after a subanalysis using only the UCDAI (Ulcerative Colitis Disease Activity Index) and DAI (Disease Activity Index) indexes, FDA-recommended scales, the heterogeneity reduced dramatically ($I^2 = 29\%$). In this case, a statistically significant difference between the probiotic and the placebo groups was observed, for which relative risk (RR) was 1.55 (CI 95% 1.13–2.15,

P-value = 0.007), and the OR (odds ratio) was 2.12 (CI 95% 1.36–3.31, *P*-value = 0.000, $I^2 = 12\%$).[117,118] When a sub-analysis was performed for probiotics containing bifidobacteria, studies using this genus showed efficacy in inducing remission in patients with active UC (RR = 1.73 (CI 95% 1.23–2.43, *P*-value = 0.002, $I^2 = 35\%$)) and OR = 2.50 (CI 95% = 1.33–4.70, *P*-value = 0.005, $I^2 = 44\%$). For maintaining remission, no statistically significant differences were observed. The authors concluded that probiotics are a promising approach to achieve remission in patients with UC, especially bifidobacteria-containing products, but the results depend on the scale used in the studies (UCDAI and DAI).[117] The greater effect obtained with bifidobacteria-containing products seems to be related to a higher SCFA production in the intestinal lumen, which could reduce inflammation and maintain the gut barrier function.[119]

Although the administration of probiotics and prebiotics in CD appears to be a promising intervention, there is no current recommendation for how to use them in this disease. In fact, the European Crohn's and Colitis Organization recently published a review of complementary medicine in IBD. The organization maintained their position that evidence to support use of probiotics and prebiotics in CD for induction of remission, maintenance of remission, or even prevention of relapse in CD patients following surgically induced remission, is inconclusive. However, the organization recognizes that the use of *E. coli Nissle* 1917 or a multi-strain probiotic containing a combination of lactic acid bacteria, *Streptococcus* and *Bifidobacterium* may be effective in inducing and maintaining remission in UC.[120]

Here we discuss the main applications of probiotics and bioactive foods in various diseases explored more recently. With the new definition of prebiotics, it is likely that more RCTs, including phenolic compounds and omega-3 fatty acids, will be performed in the near future, as their beneficial health properties are recognized and well-studied. There are other applications for probiotics and bioactive foods which were not emphasized here but are worth mentioning including the use of galactooligosaccharides (GOS) and fructo-oligosaccharides (FOS) to reduce the incidence of atopic dermatitis[121] and prebiotic-supplemented formula to reduce the prevalence of allergies in the first years of life.[122]

6. Proposed mechanisms of action

There are several pathways associated with potential health benefits of prebiotic and probiotic consumption. Fig. 2 summarizes some of them that will be discussed hereafter.

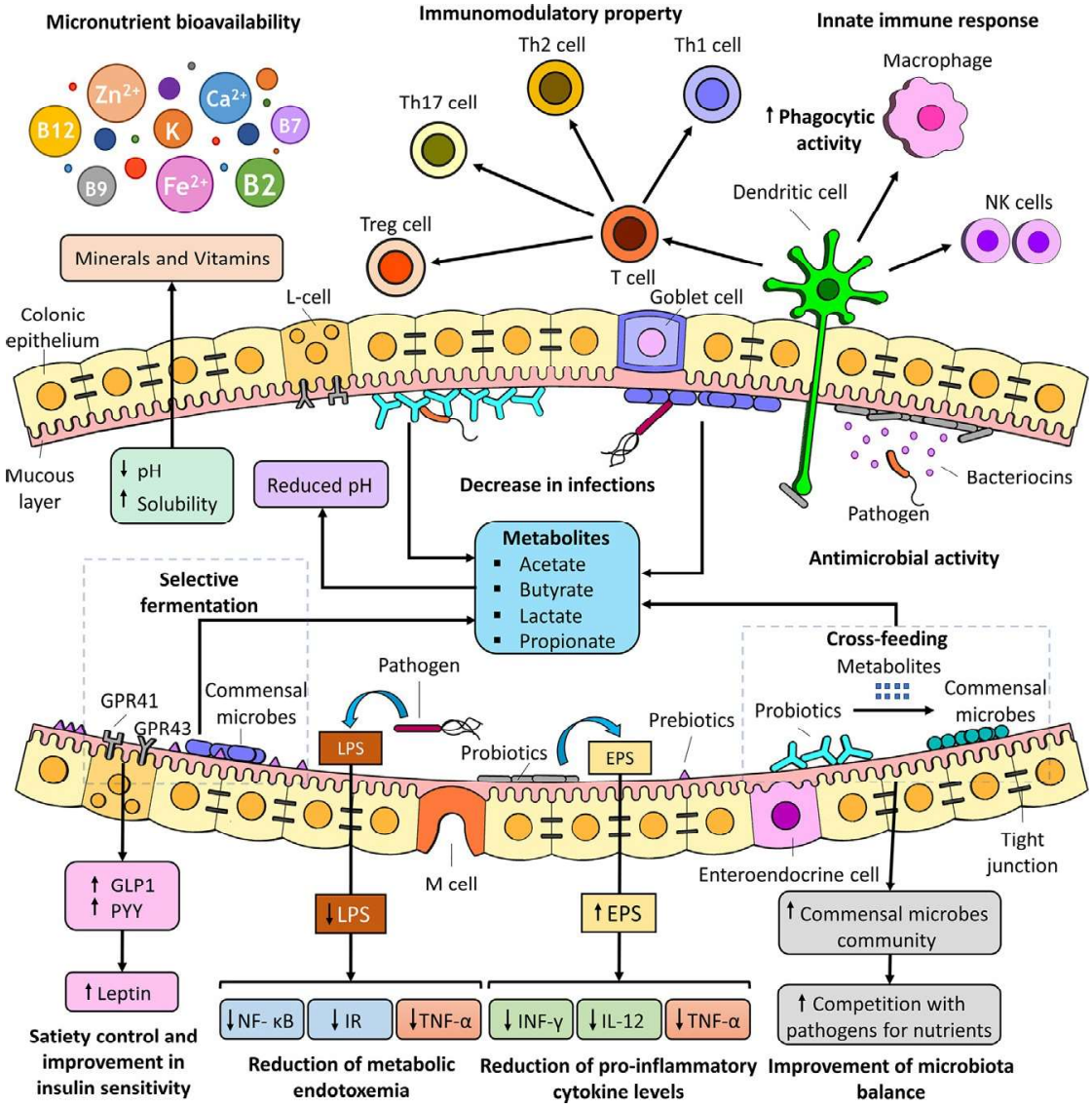

Fig. 2 Main mechanisms of action of prebiotics and probiotics. Prebiotics are selectively utilized by the commensal microbiota, releasing metabolites like short chain fatty acids (SCFA) and organic acids, reducing the lumen pH and thus increasing the absorption of minerals and inhibiting the growth of pathogens. Metabolic products from probiotics may stimulate butyrate producer bacteria by cross-feeding mechanism. Probiotics may also increase the phagocytic activity and modulate the production of immunoglobulins, improving the immune response, promoting the microbiota modulation by competition for nutrients and adhesion sites, in addition to bacteriocin release, reducing the pro-inflammatory response, and enhancing the barrier functions. L-cell, enteroendocrine L-cell; Th1 cell, type 1T helper cell; Th2 cell, type 2T helper cell; Th17 cell, type 17T helper cell; Treg cell, regulatory T cell; NK cells, natural killer cell; GPR 41, G protein-coupled 41; GPR 43, G protein-coupled 43; GLP1, glucagon like peptide 1; PYY, peptide YY; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; IR, insulin resistance; TNF-α, tumor necrosis factor-α; EPS, exopolysaccharide; INF-γ, interferon-γ; IL-12, interleukin 12; M cell, microfold cell.

6.1 Immunomodulation of the host by humoral response and cell-mediated functions

The ability to increase phagocytosis, natural killer cell function, and dendritic cell activity has been attributed to some probiotic strains.[123–125] Probiotics interact with gut associated lymphoid tissue (GALT), composed mainly of Peyer's patches and lymphoid cells distributed along the gut mucosa, where antigen-presenting cells (APC), including macrophages and dendritic cells, first interact with antigens and initiate an immune response. Bacteria have a conserved structure known as microbial-associated molecular patterns (MAMPs), which are recognized by pattern-recognition-receptors (PRPs), such as Toll-like receptors, Nod-like receptors, and C-type lectin receptors. Recognition of MAMP by these receptors precipitates the maturation of APCs to define the type of immune response that will ensue—regulatory (Treg) or effector (Th1, Th2 or Th17). This immunomodulatory property of probiotic strains is the main target for allergies and inflammatory bowel disease (IBD).[126,127]

Together, probiotics demonstrate the ability to upregulate adaptive immune responses, improving vaccine response and defense against pathogens through modulation of IgA and IgM production.[128,129] There are many cell-surface components in probiotics regulating pro-inflammatory cytokine production, such as the exopolysaccharides from *Bifidobacterium breve*. These metabolites are able to reduce the expression of interferon-γ, TNF-α (tumor necrosis factor-α), and IL-12, and impair the persistence of the pathogen *Citrobacter rodentium*. *Lactobacillus rhamnosus* strain GG is capable of reducing cytokine-induced epithelial cell apoptosis and protecting against experimental colitis, due to activation of the epidermal growth factor receptor pathway and secretion of protein p40.[130,131] Bacterial strains in other probiotics express different cell-surface architecture, like fimbriae, flagella, secreted proteins, and cell wall associated polysaccharides.[123]

6.2 Reduced lumen pH by production of organic acids

The quantitatively and metabolically most important short chain fatty acids (SCFA) are acetate, butyrate, and propionate, occurring as end products of the human colon fermentation process.[132] The production of SCFA as primary end products of the carbohydrate metabolism is well known and described everywhere. Probiotic species from the *Bifidobacterium* and *Lactobacillus* genera produce lactic and acetic organic acids, which reduce the local pH, inhibiting the growth of pathogenic microorganisms and

provide a higher bioavailability of vitamins and minerals.[119,133] Although *Lactobacillus* and *Bifidobacterium* do not produce the main SCFA, butyrate, they promote the growth of commensal bacteria that do so, such as *Faecalibacterium prausnitzii* and *Roseburia intestinalis* by means of a cross-feeding mechanism.[134,135] It has been shown that SCFA can signal via cell-surface G protein-coupled receptors (GPCRs), such as GPR41 and GPR43, pathways related to the improvement in insulin sensitivity, regulation of energy intake through secretion of the hormones GLP-1 and PYY and satiety by increased levels of leptin. Increasing the levels of these organic acids in the intestinal lumen seems to be a promising approach against metabolic and intestinal diseases.[135,136]

6.3 Interactions with host gut microbiota

Probiotics may interact with the host microbiota in different ways: competition with pathogens for nutrients and epithelium adhesion, antagonism through the production of antimicrobial substances, cross-feeding other commensal bacteria, and inhibition of bacterial toxin production.[137,138] *Lactobacillus* spp. may produce antibacterial peptides like bacteriocins, including class II and III bacteriocins, which can be active in different mucosa inhibiting replication of pathogens.[139,140] The production of organic acids due to a saccharolytic property contributes to an acid environment that hinders the growth of pathogens.[141] As mentioned above, *F. prausnitzi* is able to use the *Bifidobacterium* fermentation end product acetate as substrate, characterizing the cross-feeding mechanism and consequently may improve the microbiota balance.[119] A probiotic *B. clausii* strain showed surprising results, inhibiting the cytotoxic effect of *Clostridium difficile* and *Bacillus cereus* through the secretion of an alkaline protease, elucidating a novel way to oppose enterotoxinogenic pathogens.[138]

6.4 Improvement in barrier function

The phenomenon known as "leaky gut" has been explored in the application of probiotics as a means of restoring intestinal barrier integrity in various diseases. Especially in metabolic diseases such as diabetes *mellitus* and obesity, probiotics have been associated with increased intestinal permeability, promoting the translocation of high amounts of LPS endotoxin from the cell wall of Gram-negative bacteria to the systemic circulation, causing what is called metabolic endotoxemia. When LPS is recognized by TLR-4 receptors in the intestinal mucosa, it promotes a pro-inflammatory response,

leading to the activation of NF-κB and production of cytokines such as TNF-α, which results in increased insulin resistance due to an IRS-1 phosphorylation in serine.[142,143] Interestingly, some scientists have attempted to clarify this mechanism and showed controversial results. *Bifidobacterium lactis* and the association of *Lactobacillus rhamnosus* 19070-2 and *L. reuteri* DSM 12246 have been shown to be effective in improving the integrity of the intestinal barrier. On the other hand, *L. rhamnosus* GG (LGG) and *L. plantarum* 299v did not affect or aggravate the intestinal integrity.[144–147] These results illustrate how the mechanism of each strain must be elucidated, as well as the pathophysiology of the disease in order to effectively use probiotics.

6.5 Production of enzymes and vitamins

Production of bile salt hydrolase (BSH) and β-galactosidase enzymes by some probiotic bacteria may improve cholesterol levels and lactose digestion. A large proportion of the world's population suffers from undesirable symptoms when consuming milk or dairy (lactose-containing products) in their diet. Several strains have β-galactosidase activity and in clinical trials have demonstrated symptom relief in individuals with lactose intolerance and malabsorption.[148,149] In one clinical trial, *Lactobacillus reuteri* NCIMB 30242 reduced cholesterol levels and increased BSH activity, showing that probiotic strains with high levels of BSH may be useful in the management of chronic diseases.[150] Besides, other microorganisms, such as yogurt starter cultures (i.e., *Streptococcus thermophilus* and *Lactobacillus bulgaricus*), may have a similar effect on lactose intolerance and malabsorption due to their capacity to produce β-galactosidase.[151–153]

It is well known and established that several food-related lactic acid bacteria (LAB) and commensal bacteria have the ability to produce B-group and K vitamins.[154,155] Some species of *Bifidobacterium* like *Bifidobacterium bifidum* and *Bifidobacterium longum* subsp. *infantis* can produce high levels of folate via de novo synthesis, and therefore, could help in the recovery of the nutritional status. Vitamins like riboflavin, cobalamin, niacin, and pyridoxine (the last two on a smaller scale) may also be produced by LAB, representing an interesting way to bio-enrich food products.[156] Since there are studies linking chronic fatigue syndrome with changes in the microbiota and the B-group vitamins are important cofactors for several enzymes that participate in the ATP generation in the citric acid cycle, these probiotics may provide an alternative to improve the fatigue status in these diseases.[157]

With the increased application of genome sequencing techniques allowing the selection and understanding of the mechanisms by which strains produce vitamins, it is likely that enrichment of fermented foods with B-producing vitamins will be stimulated, even within public health policies, in order to prevent nutritional deficiencies.[155]

6.6 Production of neurochemicals

Bidirectional interactions between the gut and the brain, referred to as the gut-brain-axis, have gained attention due to large amounts of neurochemicals produced directly or indirectly by the gut, which are now known to be modulated by the host microbiota.[158] Such neurochemicals include serotonin, dopamine, acetylcholine, and gamma-aminobutyric acid. Interactions between the gut microbiota and brain are complex and not yet well understood, but a dysbiosis is associated with neurological disorders such as anxiety, depression, and degenerative processes.[159] The use of probiotics has been shown to relieve part of the symptoms related to these diseases, such as, improving global scores and reducing cortisol levels.[160] In the next future, with studies advancing in this area, safer and more assertive approaches should emerge and benefit people under these conditions.

6.7 Microbiota modulation by prebiotics

Given that prebiotics stimulate selective growth of indigenous gut bacteria and that the gut is a complex ecosystem, the ingestion of different prebiotics may result in an increase of particular species depending on the individual metabotype (i.e., the metabolic responses of a group of individuals).[161,162] Just like probiotics, different prebiotics can modulate the gut microbiota through different ways, with effects primarily on the immune response, pathogen-defense mechanisms, bowel function and stool consistency, satiety-related hormone production, along with other secondary effects on metabolism. Although prebiotics are primarily known as carbohydrate-based, recent research has shown that bioactive compounds present in plants may also have the ability to shape the gut microbiota. Phenolic compounds and polyunsaturated fatty acids are two critical ones.

Reduction of Th2-type immune response seems to be the main mechanism related to the prebiotics' ability to modulate the immune function. Several clinical trials performed on infants have shown a reduction in both the incidence and the prevalence of allergic diseases such as atopic dermatitis.[163] In vitro studies have demonstrated that the inhibition of pathogens

occurs in the same manner as in the case of probiotics. So, the higher production of organic acids due to the increased proportion of beneficial bacteria leads to a reduction in the luminal pH, helping to maintain a stable microbiota in which commensal bacteria will reduce the availability nutrients to pathogens.[164]

Moreover, increasing SCFA concentrations due to prebiotic fermentation may result in changes in the intestinal motility and stool consistency, especially in infants.[63,165] However, it is important to emphasize that each SCFA may lead to different effects. As an example, higher propionate levels are related to delayed motility due to secretion of peptide YY (PYY), whereas higher butyrate levels increase motility, as shown in animal experiments.[64] Also, SCFA is capable of interacting with fatty acid receptors, GPR41 and GPR43, signaling the production of the anorexigenic hormones PYY and GLP-1, therefore suppressing appetite and improving insulin sensitivity. In addition, the administration of a synbiotic, containing L. *plantarum* ATCC 202195 + 150 mg of FOS and GOS alone, were able to prevent sepsis and decrease the intestinal permeability, showing that just as probiotics, prebiotics may have an influence on the expression of tight junction proteins, even though this mechanism has yet to be elucidated.[166,167] Together, these mechanisms suggest that the ingestion of prebiotics may be an adjuvant therapy in the prevention or management of infectious and inflammatory diseases.

Bioactive compounds have most recently been studied for their prebiotic-like effects on the gut microbiota. Among them, polyphenols and polyunsaturated fatty acids are the most frequently studied. Ingested polyphenols are metabolized by the microbiota, which may increase or decrease their bioavailability depending on the compound.[168] This biotransformation is highly dependent on the gut ecology and is heterogeneous inter-individual. Individuals can be stratified for their ability to produce specific polyphenol-derived gut microbiota metabolites (metabotypes), for example, is the low ability to produce equol from soy isoflavones in western populations (25–30%) compared to the eastern population (50%), who has the regular habit of consuming soy in their diet.[169–171] Thus, only the individuals producing higher amounts of equol benefit from the cardiometabolic effects of soy isoflavone consumption.[172]

The bioactive compounds of black and green tea, such as epigallocatechin, epicatechin, and catechin, are known potent inhibitors of pathogens including *Listeria monocytogenes*, *Salmonella typhimurium* DT104, and *Helicobacter pylori*. Other polyphenols, such as grape polyphenols and anthocyanidins, promote an increase in beneficial bacteria including

the genera *Bifidobacterium* and *Lactobacillus*, as well as *F. prausnitzii* and *Akkermansia muciniphila*.[173,174] These bioactive compounds might also be able to modify the relative abundances of specific phyla, reducing the ratio of Firmicutes to Bacteroidetes.[175] Although promising, some of these changes have yet to be tested in humans.

Omega-3 fatty acids also seem to impact the intestinal microbiota, although only a limited number of studies demonstrate effects on the microbial composition. An increase in the genus *Bifidobacterium* and a reduction in enterobacteria appear to promote a state of eubiosis, leading to increased SCFA production and suppression of metabolic endotoxemia, which results in the improvement of inflammation.[39]

7. Future perspectives

Throughout this chapter, we have discussed the various effects of probiotics and prebiotics and their implications on the human health (Fig. 3).

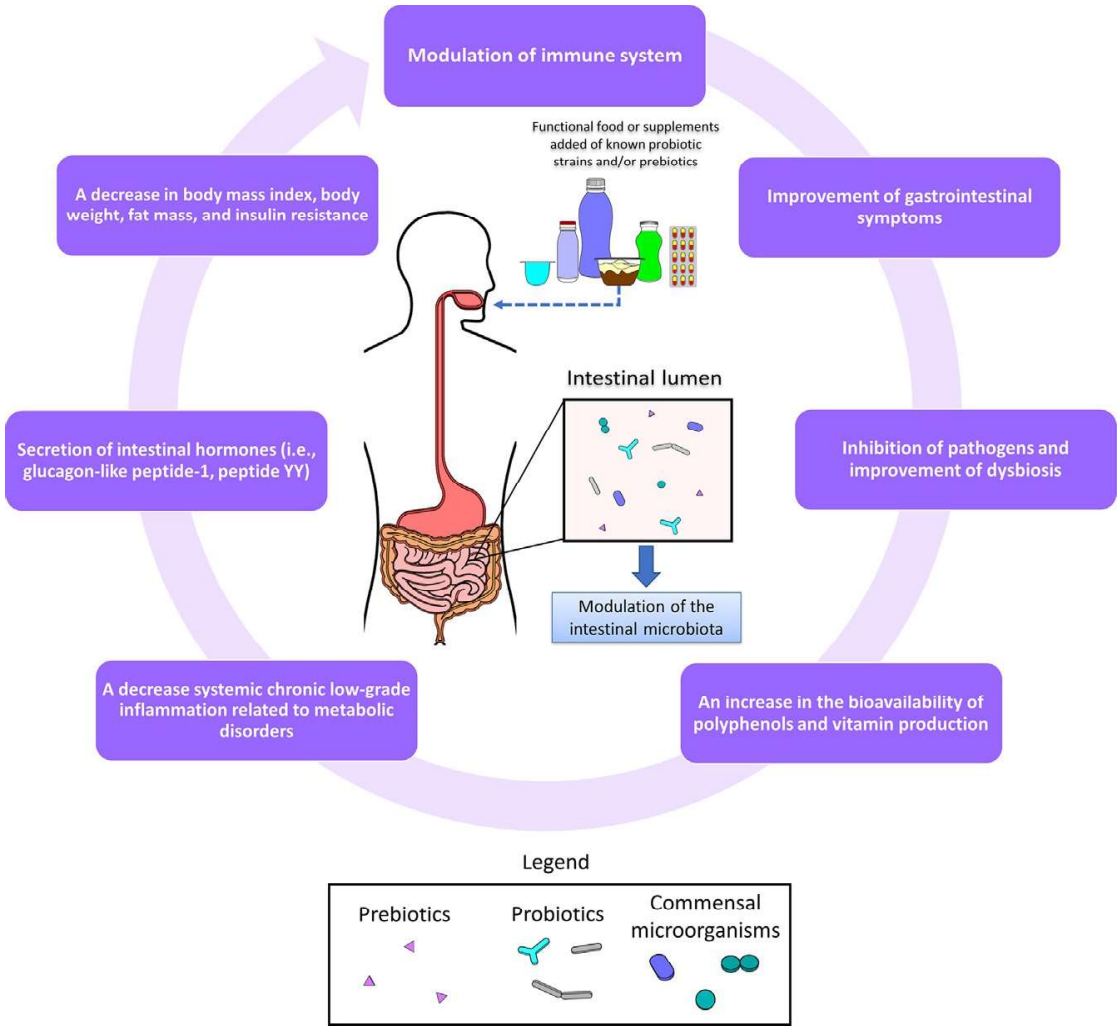

Fig. 3 Major health effects resulting from the consumption of prebiotics and probiotics.

However, it is noteworthy to mention that results in human studies are still heterogeneous and may not be applied universally to diseases, bacterial strains, and individuals from different geographic regions.

The key understanding when we talk about probiotics is that their effects are strain specific and are usually studied in a particular pathophysiological condition. Herewith, we have the differences among microbiotas of individuals, which are influenced by the type of birth, geographical location, individual genetics, lifestyle, and diet, besides other factors.

The availability of advanced genome sequencing techniques, as well as the large number of data available in libraries such as KEGG (Kyoto Encyclopedia of Genes and Genomes) and GenBank, are providing a better understand of the human microbiota, together with bioinformatics, which facilitates the processing of these large datasets.

As appropriate strains for certain health conditions are established, it will be possible to assign health claims and to make safe and effective recommendations for health professionals who are qualified to prescribe them. Effects observed with the use of bioactive and probiotic foods tend to be more significant in individuals with impaired health statuses, usually with an intestinal dysbiosis, allowing the bioactive/probiotic food to restore a health microbiota or at least improve this imbalance in microbes.

A smart use of food matrices, food technology, and different pharmaceutical forms should contribute by enabling adequate viability of probiotic strains and regular consumption, ensuring that the end consumer enjoys the benefit provided by these foods/supplements.

Lastly, the mechanism of action of each strain must be established in in vitro, in vivo, and proof-of-concept assays. With a good understanding of the microbiota of each individual or population, predictive models and algorithms may help in establishing which groups of individuals may benefit from the use of probiotics and prebiotics. Conducting better designed, multi-center clinical trials in larger populations with relevant and objective clinical outcomes could make the use of these supplements and functional foods a routine therapy and preventive health strategy for gastrointestinal well-being.

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