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REVIEW

Unlocking the Potential of Probiotics: A Comprehensive Review on Research, Production, and Regulation of Probiotics

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Abstract
This review provides a comprehensive overview of the current state of probiotic research, covering a wide range of topics, including strain identification, functional characterization, preclinical and clinical evaluations, mechanisms of action, therapeutic applications, manufacturing considerations, and future directions. The screening process for potential probiotics involves phenotypic and genomic analysis to identify strains with health-promoting properties while excluding those with any factor that could be harmful to the host. In vitro assays for evaluating probiotic traits such as acid tolerance, bile metabolism, adhesion properties, and antimicrobial effects are described. The review highlights promising findings from in vivo studies on probiotic mitigation of inflammatory bowel diseases, chemotherapy-induced mucositis, dysbiosis, obesity, diabetes, and bone health, primarily through immunomodulation and modulation of the local microbiota in human and animal models. Clinical studies demonstrating beneficial modulation of metabolic diseases and human central nervous system function are also presented. Manufacturing processes significantly impact the growth, viability, and properties of probiotics, and the composition of the product matrix and supplementation with prebiotics or other strains can modify their effects. The lack of regulatory oversight raises concerns about the quality, safety, and labeling accuracy of commercial probiotics, particularly for vulnerable populations. Advancements in multi-omics approaches, especially probiogenomics, will provide a deeper understanding of the mechanisms behind probiotic functionality, allowing for personalized and targeted probiotic therapies. However, it is crucial to simultaneously focus on improving manufacturing practices, implementing quality control standards, and establishing regulatory oversight to ensure the safety and efficacy of probiotic products in the face of increasing therapeutic applications.

Keywords Probiotics · Probiogenomics · Probiotic regulations · Functional characterization · Human and animal models

Background

For many years, health-promoting bacteria, i.e., probiotic bacteria, have been widely added as live components to many food preparations. However, the precise impact of probiotic bacteria on the functioning of the human gastrointestinal tract (GIT) is not fully understood. The elucidation of the precise mechanisms by which probiotics influence human health and guaranteed biosafety is essential for developing novel and effective probiotic products. This must be considered when discovering and developing the next generation of probiotic bacteria [1].

Every bacterial strain must possess specific properties to be considered a potential probiotic. The Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) [2] guide the evaluation of probiotics in food, emphasizing the importance of accurately identifying potential probiotic strains and conducting various in vitro tests to assess their functional characteristics. This is because probiotic properties are unique to each

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strain, influenced by specific conditions and dosage, making it unlikely for two strains of the same species to possess identical probiotic attributes [3]. Numerous strain-specific criteria have been developed as in vitro assays for the initial selection of probiotic strains. Subsequently, in vivo studies are conducted to investigate the potential probiotic properties of the selected strains in healthy individuals [3].

To select new probiotic strains, microbial cultures obtained from unconventional ecosystems, i.e., anywhere not food-related, such as plants, soil, and the gut of mammals, must undergo a comprehensive evaluation process, which includes in silico characterization, in vitro experiments, animal testing, and clinical trials [4]. For identification, the FAO/WHO guidelines recommend the utilization of the most current and valid methodologies, employing a combination of phenotypic and genotypic methods for the speciation of probiotic candidate strains [2]. Using molecular methods for strain identification is highly recommended, as phenotypic identifications alone are deemed insufficiently reliable [5]. Regarding in vitro assays, the FAO/WHO guidelines list commonly employed tests for screening and characterizing potential probiotic strains. These tests evaluate resistance to gastric acidity, bile salt hydrolase (BSH) activity and resistance to bile salt, adherence to mucus and/or human epithelial cells, and antimicrobial and antagonistic activity against potential pathogenic bacteria. For instance, models simulating intestinal or systemic inflammation are employed to investigate the pathogenesis of inflammation-related conditions [6]. Additionally, diverse animal models have been developed to study metabolic disorders [7], cholesterol reduction ability [8], antioxidant activity [9], or cytotoxic effects against cancer cells [10]. Furthermore, clinical trials that recruit healthy volunteers and patients are conducted to establish correlations between clinical outcomes and specific molecular changes induced by probiotic supplementation [11]. Collectively, these approaches contribute to advancing our understanding of probiotics and their therapeutic potential [12]. Nevertheless, it is worth mentioning that it is not mandatory for a strain to fulfill all these selection criteria to be considered a probiotic strain [13].

Although there are many guidelines worldwide regarding the characterization of probiotic strains, the function of each test and how these would apply to the strain's beneficial effect are unclear. In this review, we aim to present the steps taken to characterize a newly discovered strain until it becomes a commercial probiotic product, focusing on the status of research on probiotics and the existing problems, and propose solutions for those. This paper will be divided into sections, as described in Fig. 1. The first section will cover the most common places for isolating probiotic strains. In contrast, the second section will describe the techniques used to identify the probiotic candidate to the strain level and evaluate potential pathogenic traits, survivability, adhesion, and antagonistic activity of the strain used in silico and in vitro tests. The third section brings the probiotic candidate to the test, evaluating its safety in vivo and the effects of the strain in different disease models, going further to clinical trials in humans. This section brings results in models of intestinal inflammatory diseases, metabolic disorders, bone health, and central nervous system (CNS) disorders. Finally, the fourth section brings to light the creation of the commercial probiotic product, the regulations, and the evaluation of viability and safety.

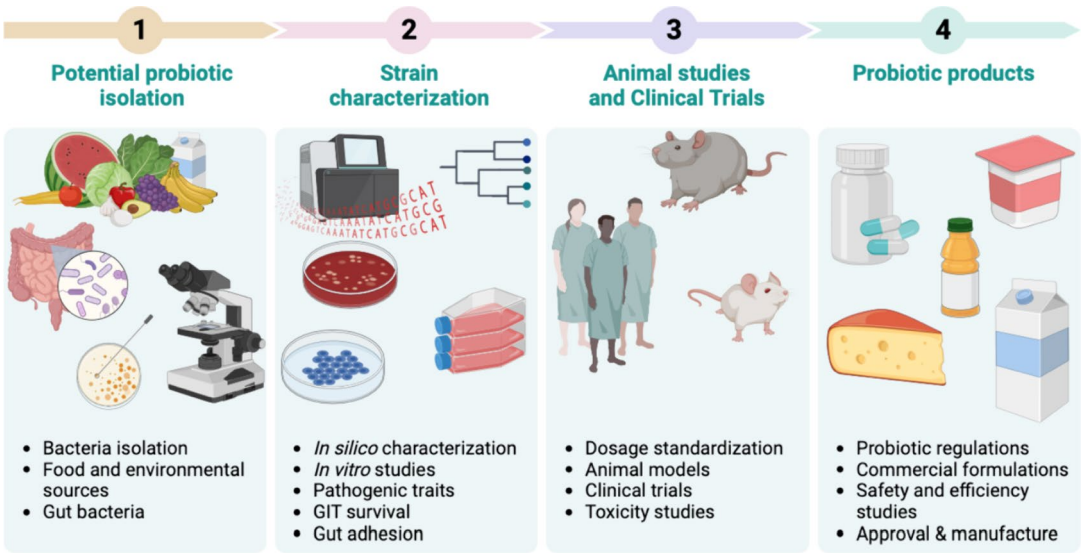

Fig. 1 Main approaches used in the discovery, characterization, and production of a probiotic

Potential Probiotic Isolation

Potential probiotic strains can originate from various sources, such as humans, animals, plants, and the environment. Nonetheless, it is recommended that when selecting bacterial strains as probiotics for animals, they should be sourced from the animals' intestinal microbiota. This approach facilitates smoother intestinal colonization and ensures a more targeted and effective application.

The GIT and breast milk are viable sources for identifying potential probiotic strains for human use [14]. Extensive research confirms that human milk plays a critical role in establishing the microbiota in the sterile intestinal environment of newborns. Given the acknowledged health-promoting advantages of bacteria present in breast milk, researchers have directed attention toward isolating potential probiotic strains from this source [15–18]. Historically, the isolation of such strains has primarily encompassed traditional bacterial species from the families bifidobacteria and lactobacilli, owing to their well-established safety and efficacy in the domains of nutrition and health [19]. However, ongoing research exploring unconventional bacterial species may uncover new possibilities for these organisms to function as probiotics, enhancing overall gut health. Consequently, it has been proposed that human milk harbors bacterial strains with the potential to serve as probiotic agents [20, 21]. Furthermore, research indicates that the fecal samples of adults, children, and infants also contain abundant probiotic bacteria [22]. Animal-derived food sources such as unprocessed milk [23–25] or fermented products [26, 27], as well as plant-based fermented foods [28, 29], offer ample opportunities for identifying potential probiotic strains. Numerous research studies have demonstrated that probiotic strains obtained from fermented foods can serve as a basis for creating starter cultures essential for the industrial production of fermented probiotic foods [30–32].

Independent of the potential probiotic strain source, the following steps involve identifying strain levels, genomic and phenotypic characterization, and in vivo studies. These steps are described in the sections that follow.

Strain Characterization

Identification of Bacterial Species and Strains

Accurate identification is crucial in probiotic research, requiring the correct classification of each potential probiotic strain through the utilization of both phenotypic and genotypic methods. This approach emphasizes the genotype and employs molecular techniques to characterize microorganisms at both the species and strain levels [2]. While phenotypic methods, such as morphological evaluation and gram staining, are still employed for initial screening, computer-assisted commercial phenotypic identification systems have become outdated due to their limitations. These methods can be challenging and time-consuming, particularly for slow-growing and fastidious organisms. Additionally, identifying novel isolates using computerized numerical taxonomy does not always provide satisfactory species identification due to the reliance on characteristics observed in reference strains under optimal growth conditions, which can vary under stress [33]. Examples of such systems include the Biolog microbial identification system (Biolog Inc., Hayward, CA, USA) for carbon source utilization, which identifies bacteria, molds, and filamentous fungi [34], as well as the API system (bioMérieux, Marcy l'Etoile, France) that identifies bacteria based on their carbohydrate fermentation patterns [35].

In addition to phenotypic methods, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a rapid, dependable, and high-capacity diagnostic tool tailored for the swift identification of microorganisms. This advanced technology enables the identification of bacterial species within minutes. Employing MALDI-TOF MS for identification has consistently surpassed conventional methods [36]. In a specific study involving the prospective analysis of 980 routine clinical isolates through MALDI-TOF MS, 92% of isolates were accurately identified down to the species level, as opposed to 83.1% of isolates identified using conventional identification methods. Notably, traditional methods exhibited a higher frequency of incorrect genus-level identifications (1.6%), in contrast to the lower rate of 0.1% observed with MALDI-TOF MS [37]. The proficiency of MALDI-TOF MS remains generally resilient to factors such as culture media, cultivation conditions, or incubation durations. This inherent resilience contributes significantly to bacterial identification's consistent and reproducible nature via MALDI-TOF MS [38, 39]. This technique, however, does not show high specificity at strain levels, especially for newly discovered strains, which is essential for correct probiotic identification [40].

In contrast, genotypic identification methods have gained prominence for species identification and differentiation of microbial strains. Various molecular methods, such as pulse field gel electrophoresis, sequencing of rRNA genes, protein profiling, ribotyping, conventional polymerase chain reaction (PCR), random amplification of polymorphic DNA, and repetitive element palindromic PCR, can be employed [41]. Among the various genotypic identification methods, 16S rRNA gene sequence analysis is commonly favored for microbial identification due to its high accuracy and ability to determine taxonomical relationships among microbial strains

[42]. This method is preferred over others, such as multilocus sequence typing (MLST) [43] and metagenome-assembled genomes (MAGs) [44], because of 16S rRNA sequencing widespread use, the availability of reference databases, and its capability to provide reliable species and strain-level identification [45]. Other identification methods may be time-consuming, require extensive resources not readily available in many laboratories, or necessitate a substantial collection of reference strains [2]. Therefore, 16S rRNA gene sequence analysis has emerged as a frequently employed and reliable method for microbial identification in probiotic research.

Using long-read sequencing technology, other methods have been developed to identify bacteria at the strain level from a microbial mixture [46]. Although promising, limitations include focusing only on long reads, the limited reference database, and the abundance dependency on mixture samples. This highlights the importance of choosing the right tool for identifying and characterizing a potential probiotic strain, considering its environment and DNA extraction method. A well-identified strain is a requirement for a probiotic to be approved by the major regulatory agencies. To this date, whole-genome sequencing (WGS) is considered the golden standard for bacteria identification at the strain level. With this technique, all DNA is sequenced (chromosome and plasmids), allowing the precise characterization of known and newly discovered strains, with very large databases, such as the NCBI's Genbank, available for comparison. WGS also allows an in-depth analysis of the genome composition and the prediction of phenotypes.

Genomic Characterization of Probiotics

Probiotics, as defined by the International Scientific Association for Probiotics and Prebiotics consensus statement, are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [47]. These organisms can be divided into two classes: classic probiotics, which comprise *Lactococcus* spp., the *Lactobacillaceae* family., *Bifidobacterium* spp., *Bacillus* spp., *Enterococcus* spp., *Weissella* spp., *Escherichia coli* Nissle 1917, and *Saccharomyces* spp., and next-generation probiotics (NGP), referring to a group of microbial organisms that meet the conventional criteria for probiotics but have not been historically utilized for health enhancement [48]. These microorganisms also align with the definition provided by the US Food and Drug Administration (FDA) for live biotherapeutic products (LBPs), which entails being composed of live organisms, such as bacteria, intended for preventing, treating, or curing human diseases or conditions, excluding vaccines [49]. Several microbial commensals have undergone assessment as NGPs. *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Anaerobutyricum hallii*, *Prevotella copri*, and *Bacteroides thetaiotaomicron* have shown significant promise. It is worth noting that NGPs are genetically distant from lactic acid bacteria (LAB), which belong to the *Firmicutes* (Bacilli class) or *Actinobacteria* phyla. These NGP taxa, such as *Prevotella*, *Bacteroides*, and *Akkermansia*, originate from different phyla, namely, *Bacteroidetes* and *Verrucomicrobia*. On the other hand, taxa like *Faecalibacterium*, *Roseburia*, and *Eubacterium* are part of the *Firmicutes* phylum but belong to a different class, namely, *Clostridia*. This diversity in taxonomic classification highlights the wide range of potential candidates for NGPs, expanding the possibilities for health promotion through probiotic interventions [48]. Figure 2 shows a schematic representation of probiotic bacteria classified into the five phyla of bacteria. Furthermore, it becomes clear that there are numerous uncharted territories to investigate regarding the variety of

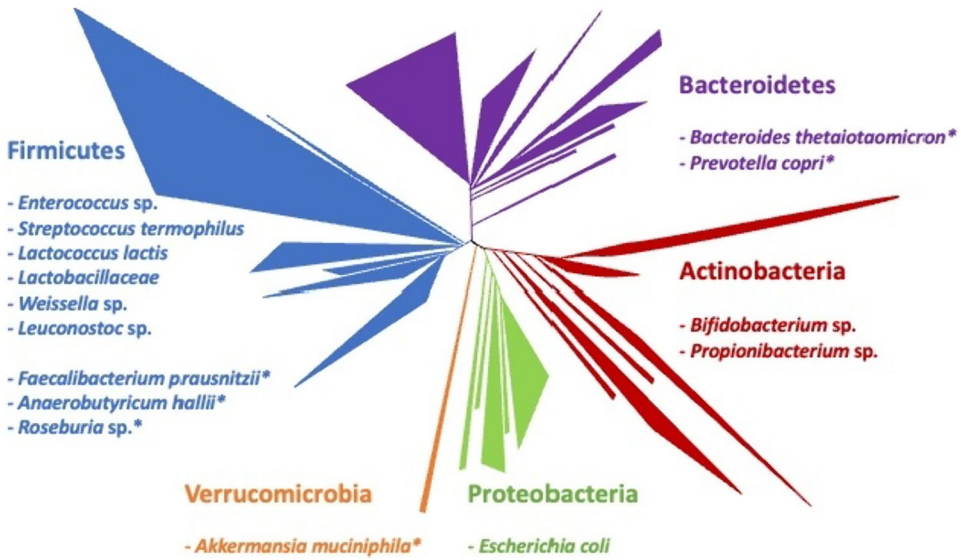

Fig. 2 Schematic representation of probiotic bacteria by phylum. Bacterial species belong to different phyla that can be ordered on a phylogenetic tree. The two major phyla are Firmicutes and Bacteroidetes. Actinobacteria, Proteobacteria, and Verrucomicrobia are in the minority but are not less essential. NGPs are highlighted with an asterisk (*)

bacterial strains that exhibit unique and previously undiscovered beneficial characteristics, and the probiogenomic is a powerful tool to tackle this issue.

In probiotic research, it is necessary to focus on understanding the strain-specific mechanisms of action by exploring their unique genetic and metabolic characteristics. Relying solely on traditional tests to determine probiotic safety and efficacy is not always reliable, making it challenging to predict their functionality. Besides having no universally essential attributes for all probiotics, they can exhibit multiple mechanisms linked to a specific clinical benefit [50, 51]. These gaps in knowledge add complexity to the task of understanding and forecasting the safety and functionality of probiotics. To tackle these challenges, the concept of "probiogenomics" has risen as a growing field of research interest [52]. It aims to explore the diversity and evolution of commensal and probiotic bacteria while uncovering the molecular basis for their beneficial activities in promoting health [53]. Probiogenomics encompasses advanced techniques like genomics, transcriptomics, proteomics, and metabolomics, which provide valuable resources for identifying uncharacterized strains and developing predictive models for the rational selection of new probiotics [52, 54]. The public availability of complete genome sequences has significantly enriched our understanding of these microorganisms' biology, providing vast information on their metabolic capabilities, genetics, and phylogeny [52]. Sequencing of probiotic strains has become more affordable and accessible. Moreover, many bioinformatics tools are readily accessible to assist with genome assembly, annotation, and phylogenetic analysis [55].

The emergence of probiogenomics as a distinct field within genomics encompasses significant progress in probiotic research [1]. The advancement of high-throughput sequencing technology and improvements in bioinformatics have introduced new and powerful tools for conducting in-depth analyses of the evolutionary patterns of bacterial strains that are of interest [12]. These advancements have facilitated in-depth investigations into the biology of probiotics, their impact on cellular responses, and the validation of their health-promoting properties, playing a crucial role in identifying probiotic traits, such as bacteriocin production or predicting acid tolerance [56], while metagenomic studies have shed light on probiotic-microbiota interactions [57]. Transcriptomic, proteomic, and metabolomic platforms have enabled the examination of host-microbe crosstalk and provided a comprehensive understanding of the holistic effects of probiotic supplementation [58]. Additionally, recent comparative studies have strongly supported the notion that probiotic actions are species-, disease-, sex-, and host-specific, underscoring the importance of targeted interventions and personalized medicine [59].

By utilizing the genome sequences of probiotic bacteria, researchers are uncovering the mechanisms and interactions involved in their activity within the host GIT by integrating functional genomic techniques. In this context, four critical areas of probiotic action are being emphasized: (i) the ability to survive the passage through the GIT and adhere to the epithelial cells of the intestines, (ii) engage in competitive exclusion and display antimicrobial activity, (iii) modulate the immune system of the host GIT, and (iv) interact and modulate the host intestinal microbiota (especially by NGPs, which are well-known resident of the human microbiota) [60]. Understanding microbe-host interaction is crucial for rationalizing potential probiotics targeting specific health objectives. Currently, the selection of probiotic organisms is primarily based on ecological considerations and phenotypic traits that ensure safety, robust manufacturing, storage stability, and survival throughout the GIT. Empirical studies focusing on colonization and host modulation functions have contributed to a growing confidence in the effectiveness of probiotics [61]. However, the intricate nature of biological interactions involving probiotics, the intestinal ecosystem, and the host has made it challenging to definitively identify specific probiotic effector molecules and their functions. Thus far, the probiotic effector molecules that have been identified are mainly located on the bacterial surface or are secreted [62]. Also, there is an increasing body of evidence demonstrating specific and measurable physiological effects on the host following the consumption of probiotics [63].

Comparative genomics has significantly contributed to predicting probiotic traits and attributes [64], being a valuable approach used to explore the extent of phenotypic variation among strains and identify strain-specific genes within the pangenome (the complete gene set of all strains within a monophyletic group) while highlighting shared characteristics [65]. It helps uncover conserved sequences in probiotics' genomes that encode essential cellular functions and regulatory elements governing their expression. Analysis of clusters of orthologous genes allows for categorizing these findings into various functional categories, such as transcription, metabolism, cell motility, and signal transduction, among others [66]. Furthermore, comparing the genomes of newly discovered isolates with those of well-characterized probiotics can reveal functional properties like adhesion to epithelial cells, auto-aggregation, stress response mechanisms, defense mechanisms (including virulence factors), and antibiotic resistance [67].

In Silico Identification of Potential Pathogenic Traits in Probiotic Strains

The main theoretical risks associated with probiotics include the possibility of infection, adverse effects caused by toxins produced by the probiotic strains or contaminants, and

immunological effects [68]. Safety assessments should consider factors such as the nature of the probiotic microorganism, method of administration, level of exposure, health status of the recipients, and the intended physiological functions of the microbes [68]. Although a universal international standard for the safety evaluation of probiotics is currently lacking, the FDA in the USA designates which bacteria receive the Generally Recognized as Safe (GRAS) for human consumption status, in a manner that probiotic blends or supplements must exclusively include strains that have achieved the GRAS status [69, 70]. Furthermore, the European Food Safety Authority (EFSA) has proposed the concept of "Qualified Presumption of Safety" (QPS), which may apply to specific groups of microorganisms [71]. The determination of QPS status is based on four key considerations: (i) taxonomy: the taxonomic level or grouping for which QPS is being sought; (ii) familiarity: the extent of knowledge available regarding the proposed group of organisms to enable a decision on their safety; (iii) pathogenicity: whether the group considered for QPS contains known pathogens, and if so, whether sufficient information exists about their virulence determinants or toxigenic potential to exclude pathogenic strains; and (iv) end use: whether viable organisms from the group enter the food chain or if they are utilized in the production of other products [71].

Probiotic strains, including members of the Lactobacilli family, *Lactococcus* genus, *Bifidobacterium* genus, and the strain *Streptococcus thermophilus*, have a long-standing record of safe use and commonly receive the GRAS status [72]. Evidence suggests that the consumption of probiotic lactobacilli does not pose a greater risk of infection than commensal strains, and it is doubtful to associate lactobacilli consumption with a risk of death [14]. Infection caused by lactobacilli and bifidobacteria is rare and estimated to account for only 0.05% to 0.4% of infective endocarditis and bacteremia [22]. As for NGP, comprehensive and extensive research on safety and tolerability must be conducted through animal and human trials. Currently, human trials are lacking for most NGP candidates. When performed, they are primarily exploratory, featuring small sample sizes and excluding sensitive populations such as frailty subjects, older people, or children [48].

While most species and genera of LAB are considered safe, certain strains may present concerns, such as those known to produce biogenic amines (BAs) from protein sources. Additionally, the transmission of antibiotic resistance genes among different bacterial strains is a health concern, and the FAO/WHO guidelines recommend assessing the antibiotic resistance/susceptibility pattern of each probiotic strain [2]. Some studies have attempted to identify virulence factors for lactobacilli. Nevertheless, such approaches are more applicable to known pathogens and may be inherently flawed when applied to normal commensals such as lactobacilli or bifidobacteria [14].

Before considering their potential application, selected strain candidates must be characterized based on various safety traits in multiple published works, reports, and recommendations provided by diverse committees, organizations, and expert/advisory groups [73, 74]. One crucial aspect of evaluating probiotic safety involves thoroughly examining intrinsic properties, such as pathogenicity/virulence, toxin production, and antibiotic resistance. This assessment has become obligatory because several studies suggest that probiotics may adversely affect the host's health, primarily resulting in allergic and infectious diseases, harmful metabolic activities, and infectivity [75]. Therefore, ensuring that probiotic safety can be achieved through individual or integrated probiogenomics approaches.

Virulence Factors

Virulence factors play a crucial role in microbial pathogenesis and encompass a variety of components, such as enzymes, toxins, secreted effectors, and cell-associated products. A comprehensive safety assessment of probiotics must include an evaluation of their potential to express virulence or toxin genes that could lead to disease. This assessment typically involves two main aspects: determining whether the probiotic strain belongs to a species known for virulence or toxigenicity and investigating the presence of virulence or toxin genes within the microbe's genome.

Defining the virulence of a microbe at the molecular genetic level is challenging, as it greatly depends on the dynamic host-microbe relationship. Generally, factors contributing to colonization, invasion, and evasion of host immune-related elements are considered critical genetic foundations of virulence [51]. Other factors may enable a microbe to thrive in a specific host environment and contribute to virulence by complementing the harmful effects of toxins and other directly acting agents associated with virulence [76].

It is important to note that individual "virulence" factors do not act in isolation. Instead, their coordinated expression and underlying genetic foundation collectively contribute to disease potential [51]. A prime example is the species of *E. coli*, which includes both commensal strains and pathogenic strains causing disease. This example highlights the complexity of host-microbe interactions and the role of designated "virulence factors" in pathogenicity. Determining pathogenicity requires a systems biology approach and a deeper understanding of the mechanisms underlying the beneficial relationship between probiotics and the host [77].

High-resolution information on core genome relationships and accessory genome elements can further enhance our understanding of the genetic content of concern. Recent developments in this field have prompted the EFSA to update its QPS list of microorganisms to address these changes [78].

Abriouel et al. [79] conducted an in silico evaluation of the safety of *Weissella confusa* LBAE C39-2 and *Weissella cibaria* KACC 11862 strains by analyzing their whole-genome sequences. The analysis revealed that *W. confusa* LBAE C39-2 possesses four virulence factors associated with genes encoding collagen adhesion, hemolysin, and mucus-binding proteins. On the other hand, *W. cibaria* KACC 11862 was found to contain two virulence factors coding for hemolysins. Although these factors have been linked to invasion and infectivity events, their specific role in the virulence of *Weissella* species remains unknown, thus requiring further studies to confirm their expression.

Similarly, Li et al. [80] assessed the safety of the potentially probiotic strain *Enterococcus durans* KLDS6.0930, which was isolated from a traditional fermented cream. WGS and analysis were employed to examine virulence-related genes, and 45 putative virulence factors were identified, predominantly related to cell surface molecules involving host or surface adhesion and promoting biofilm formation. These factors have been recognized as essential elements in the initiation of infections. However, it is worth noting that an additional analysis using PathogenFinder [81], a web-based server that predicts bacterial pathogenicity through the analysis of user-provided proteome, genome, or raw reads, predicted *E. durans* KLDS6.0930 as a non-pathogenic bacterium.

In a related study, the genome sequences of *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI, obtained from fecal samples of healthy breast-fed infants, were compared with the genome sequences of four significant human pathogens: *E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus* using VirulenceFinder tool [82], a part of a freely accessible web-based platform for analyzing WGS data. No virulence-associated genes related to offensive traits, such as Shiga toxin, exoenzymes, or genes involved in immune evasion or alteration, were detected. Consequently, the authors proposed that these strains could have the GRAS status [83]. This analysis is mainly helpful to confirm the avirulent status of strain coming from a GRAS species. However, it could significantly help to identify if the presence of genes generally recognized as virulence factors, such as adhesins, biofilm formation, and mucus-biding, is a determinant factor for safety as they could be found on many probiotic strains and help these strains to survive/compete on the host.

Prophages and Integrases

Unwanted genetic traits, such as those related to disease-causing factors and resistance to antimicrobial agents, are frequently connected to mobile genetic elements (MGEs) that can be acquired during adaptive evolution. Therefore, it is essential to examine the mobilome of a potential probiotic strain, which includes components like phages, plasmids, genomic islands (GEI), transposons, and insertion sequences (IS). This examination is crucial for determining whether its health-promoting benefits are innate characteristics or acquired attributes [84, 85]. Prophages are segments of bacteriophage genomes inserted and integrated into bacterial chromosomes. They are commonly found in probiotic genomes; however, their presence requires further investigation due to their association with a higher likelihood of encoding virulence factors and promoting genetic variability [86].

The complete genome of *Lactiplantibacillus plantarum* 5–2, isolated from fermented soybeans, was sequenced to explore the potential presence of prophage elements [87]. The analysis revealed that the genome of strain 5–2 harbored four prophage regions, three of which were intact and one was incomplete. Within the three intact prophage elements, three integrases were identified. These integrases are known to be associated with phage morphogenesis, including the packaging, head, tail gene clusters, and lysis cassette. Furthermore, the presence of intact prophage regions suggests their recent acquisition into the bacterial genome, while the incomplete prophage region implies strong selection by the bacteria, leading to prophage inactivation [88]. Sequences belonging to prophages are recognized for their lack of stability in maintaining the genome's integrity. Complete and functional prophage regions indicated a high risk of gene transfer from the strain to others present in the host, needing further investigation on the content of these sequences. Similarly, Abriouel et al. [89] conducted a comprehensive analysis of the complete genome sequence of *Lactiplantibacillus pentosus* MP-10, a bacterium isolated from the brine of naturally fermented olives, to identify prophage DNA elements. The genome of MP-10 was found to harbor five temperate phage regions: two regions were determined to be intact (region 2 and region 5), two regions were uncertain (region 1 and region 4), and one region was incomplete (region 3). Each prophage region contained integrases, which are responsible for encoding proteins. Based on these findings, the authors recommended further investigations into the application of *Lp. pentosus* MP-10.

More recently, Tarrah et al. [90] conducted a study to explore potential safety characteristics, particularly the presence of prophages, in *Lacticaseibacillus paracasei* DTA93, which was isolated from fecal samples of healthy infants. The analysis involved complete genome sequencing, and the data revealed the presence of only three incomplete prophage regions in the DTA93 genome. The authors suggested that these prophage remnants did not contain sufficient genes to be considered complete functional phages.

Pei et al. [91] evaluated 1472 lactobacilli strain genomes from 16 species, presenting various prophages. The referred prophage distribution is uneven, with various genome characteristics and distinct clusters linked to host species. This allows us better to understand the genetic diversity of

prophages in lactobacilli. Additionally, antibiotic resistance genes (ARGs) were found in prophages from 10% of the studied strains. The study also showed that most of the intact identified prophages could be induced to produce temperate phages successfully [92]. The transfer of the genes and, consequently, the phenotype related to the gene were not tested. The findings of this study could be of interest to a variety of biotechnological and clinical fields that require a more comprehensive safety assessment and functional understanding of lactobacilli species.

In general, results show that prophage's genetic content must be extensively characterized to guarantee the absence of any pathogenic genes that could lead to a pathogenic trait, avoiding the transfer to commensal or even already pathogenic strains. As a result, absence of intact prophages is preferable to uphold genome stability and guarantee the appropriateness of probiotic bacteria for use in industrial contexts [88].

Antibiotic Resistance

The global health concern of antibiotic resistance is rapidly spreading. As mentioned above, genes culminating in antibiotic resistance are commonly found inside prophage and other MGE. Probiotic bacteria with inherent antibiotic resistance are generally considered safe, as they pose minimal risk of transferring drug-resistant genes to more harmful species. However, in probiotic strains where ARGs are acquired, primarily carried on MGEs, like plasmids, transposons, prophages, and integrons, there is a significant potential for horizontal transmission, presenting a serious safety concern [93].

Certain probiotics may serve as reservoirs for ARG, so it is reasonable to speculate that genetic transfer of antibiotic resistance to opportunistic bacteria could occur. Therefore, it is essential to investigate resistance mechanisms, the genetic nature of resistance traits, and the elements contributing to resistance dissemination [75]. Evaluating antibiotic resistance in novel probiotic strains involves phenotypic assessment through determining minimal inhibitory concentrations [96] and genotypic analysis using PCR-based techniques and sequencing [94, 95]. DNA microarrays and WGS platforms can also identify and locate previously unidentified ARG [95, 96].

The EFSA has established a protocol for evaluating antibiotic resistance, including assessing phenotypic resistance based on MIC (minimum inhibitory concentration) values above pre-established thresholds and searching the genome for ARG [97, 98]. According to EFSA's guidance, any functional antibiotic resistance above the threshold should be further characterized as intrinsic or transmissible, and the genetic basis of resistance should be identified and explained. The updated EFSA guidance from 2018 emphasizes the need for phenotypic (MIC) and genotypic analysis of antimicrobial resistance without limiting the analysis to resistance above a specific threshold or cutoff value [98]. This aligns with the safety requirements of several countries worldwide.

Understanding the genetic nature of antibiotic resistance is crucial for assessing the risk of potential spread, given the transferability of resistance genes among bacteria. It is well known that live microbes can harbor ARGs, and horizontal gene transfer in the human gut has been demonstrated [99]. Strains with intrinsic resistance to antibiotics, commonly found within strains from the same species, pose a low risk of horizontal spread and are considered safe to use by regulatory agencies [98]. Acquired resistance is not typically present in a species and is considered a higher concern. However, chromosomal mutations leading to acquired resistance are associated with a low potential for horizontal spread [97]. Strains carrying transmissible resistance genes flanked by genetic elements known to mediate horizontal transfer must be addressed and explained, as per EFSA guidance, and strains for which the nature of resistance cannot be explained should be avoided [97, 98].

Biogenic Amine Production

BAs are organic nitrogen compounds formed mainly by decarboxylation of amino acids by microorganisms. They are associated with toxicity concerns, particularly their microbial production in certain fermented foods like cheeses [100]. They are considered undesirable metabolic products of bacteria due to their adverse effects and potential toxicological risks. While BAs are essential for various biological functions, such as neurotransmitters, excessive levels can lead to toxic effects [101]. Some commonly studied BAs include monoamines (e.g., histamine, tyramine), diamines (e.g., cadaverine, putrescine), and polyamines (e.g., spermidine, spermine). Histamine and tyramine are particularly concerning due to their potential toxicities. Therefore, probiogenomics studies can provide insights into whether probiotic strains harbor genes or operons responsible for the biosynthesis of BA. The presence of genes encoding BA production can be determined using WGS or targeted PCR amplification to assess the potential for additional BA production by administered probiotic strains. Phenotypic confirmation of biogenic amine production by bacterial species can be analyzed using decarboxylase screening media and high-performance liquid chromatography [102].

Li et al. [103] investigated the production of BAs by *Lactobacillus helveticus* KLDS1.8701, isolated from Chinese traditional fermented dairy products, using WGS and LC/MS–MS analysis. The genome of *Lb. helveticus* KLDS1.8701 contained pseudogenes related to the conversion of arginine into putrescine and the conversion of ornithine into putrescine. Additionally, the strain harbored a cluster

for spermidine/putrescine ABC transporter. Despite the presence of these genes, LC/MS–MS analysis revealed the absence of BA production by *Lb. helveticus* KLDS1.8701, indicating that the pathway may be incomplete and, therefore, non-functional.

The potential production of BAs by *Bifidobacterium animalis* subsp. *lactis* AD011, isolated from an infant fecal sample, was recently investigated using a metabolomics approach through LC/MS–MS analysis [104, 105]. Since the presence of BA can serve as a quality indicator for fermented probiotic products, the authors focused on identifying four main BAs, namely, putrescine, cadaverine, histamine, and tyramine, in two different growth media (MRS and whole milk) after 15 h of bacterial cultivation. The results demonstrated the absence of detectable BA production in both supernatants, suggesting that, regarding the production of BA, AD011 is likely safe and acceptable for human consumption as a probiotic. Other pathogenicity traits were still to be evaluated to confirm strain safety fully.

Mucin Degradation

Mucin, a protein found in mucus, plays a crucial role in the structure of mucosal surfaces in the digestive tract and is a protective barrier against microbial and chemical invasion [106]. More than 40 bacterial genes have been identified in mucin metabolism [107]. WGS can be utilized to identify bacterial genes associated with mucin degradation, and the activity of these genes can be assessed in vitro by evaluating the ability of bacterial strains to grow in the presence of mucin as the sole carbon source in liquid or agar media [108].

The gut microbiota naturally degrades mucus to utilize it as a carbon source. For example, *A. muciniphila* feeds on mucins and converts them into short-chain fatty acids (SCFA), an essential energy source for the cells lining the GIT. However, a disruption in the balance between mucin-degrading bacteria and other bacteria has been suggested to contribute to human disease and infection [109, 110]. It is important to note that mucin degradation is tightly regulated within gut microbes and influenced by the availability of dietary polysaccharides [111]. Assessing the mucin degradation capability of probiotic strains is part of a comprehensive safety analysis. An excessive increase in mucin-degrading bacteria would destroy the intestinal protective barrier, allowing pathogenic and opportunistic bacteria to cause infections. However, determining the associated risk requires a systems biology approach and a deeper understanding of the mechanisms underlying the beneficial relationship between probiotics and the host. Therefore, the number of mucin-degrading genes present in the genome of a probiotic species should be considered as part of a holistic assessment of the strain's safety rather than being solely regarded as a virulence characteristic.

In Vitro Characterization of Probiotic Candidates

Numerous health advantages associated with consuming these microorganisms have been elucidated through clinical investigations. These benefits encompass a range of outcomes, such as the mitigation of diarrhea's duration and occurrence, relief from lactose intolerance symptoms, decreased likelihood of pathogenic infections, immune system stimulation, and regulation of the inflammatory response [112]. The GIT serves as a primary site where probiotic microorganisms exert their effects. The underlying concept behind incorporating probiotics into food and supplements to harness their potential health benefits relies on the microorganisms' capacity to withstand the transit through the GIT, ensuring that their adequate quantity of live probiotic bacteria reaches either the small or large intestine and interact with, adhere to, and colonize the host. However, probiotic microorganisms encounter various challenges during their passage through the GIT, including the stomach's highly acidic environment (pH 1.5–4.0), bile salts, and digestive enzymes [113].

FAO and WHO have established guidelines for probiotics in food. These guidelines propose several criteria for selecting probiotics, including resistance to adverse conditions in the human body, ability to adhere to epithelial cells, antimicrobial activity, and safety assessment [47]. Evaluating the stress tolerance characteristics of novel strains under gastrointestinal conditions is crucial to ensure their functionality and expand the range of microbial species and products available as probiotics.

Acid Tolerance and Survival in Artificial Gastric Juice

Bacterial strains must possess several crucial characteristics to be considered probiotics, including maintaining their viability and activity throughout production, product storage, and passage through the GIT [114]. The GIT poses a stressful environment for probiotics, beginning with the stomach. The transit time in the stomach can vary from less than 1 to 4 h, influenced by factors such as individual differences, diet, and other variables [115]. During this time, the pH in the stomach can reach deficient levels, around 1.5, while the concentration of bile in the upper intestinal tract can be unpredictable and vary [116]. Simulated stomach survival methods in vitro are commonly employed as initial steps in evaluating new probiotic strains. These methods involve subjecting the strains to incubation in an acidified medium (such as MRS broth/buffer/peptone; pH 2.0–3.0 for 1–4 h) or simulated gastric juices (pH 2.0–3.0 in the presence of pepsin) [117, 118]. Additionally, more complex gastrointestinal models are utilized to simulate various aspects of gastrointestinal transit and provide a more comprehensive evaluation of probiotic survival. In vitro tests assessing acid

and bile tolerance often serve as a predictive measure of a strain's ability to survive in the host's body environment. However, it should be noted that acid and bile tolerance primarily pertain to the oral administration of probiotics and may not be relevant for other applications [13].

The resistance to gastrointestinal conditions varies among different genera and species. Lactobacilli are generally more resistant, while bifidobacteria tend to be more sensitive to low pH, resulting in lower survival rates at pH 2 and pH 3 [4]. In a study on Serpa cheese, 116 LAB strains were isolated to investigate their probiotic properties. None of the LAB isolates survived at pH 2.5, but all survived at pH 3.0. Additionally, 20 isolates survived at an intermediate pH of 2.75 after a 2-h exposure [119].

Lee et al. [120] conducted a study to examine the acid tolerance of LAB, exposing them to a pH of 2.5 for 1 h. Only *Limosilactobacillus reuteri* and *Lactobacillus gasseri* exhibited tolerance to acidic conditions among the LAB tested. In a study by Song et al. [121], the functional properties of LAB isolated from various sources were screened. Except for *Lactobacillus acidophilus* M23, all the tested lactobacilli strains demonstrated tolerance to pH 2.5 for 2 h in the presence of pepsin. It has been reported that bifidobacteria are more susceptible to a pH of 2.0 in an HCl-acidified medium when compared to a medium acidified by a mixture of pepsin and HCl, which better simulates the composition of gastric juices. Furthermore, it has been suggested that pepsin may protect bifidobacterial cells during exposure to low pH by reducing their hyperpolarization, which is associated with H + -ATPase activity [122].

Finally, dynamic models of human digestion have been used to evaluate the survival of probiotic bacteria during transit and determine the impact of the food matrix, or the probiotic encapsulation, during simulated digestion. Rabah et al. [123] tested the survival of *Propionibacterium freudenreichii* CIRM-BIA 129 through the GIT using in vitro models of static [124] and dynamic [125] digestion with three different matrices, milk ultrafiltrate, milk, and a mono-strain "Swiss-type" cheese. During static digestion, all three matrices could be recovered at a viability of around 40% after the gastric phase; when later submitted to the intestinal phase, only the cheese matrix could maintain the same viability, while milk and milk ultrafiltrate reduced to 20%. When submitted to the dynamic digestion, the cheese matrix maintained viability up to 60%, while milk ultrafiltrate had CFU reduced by up to 3 logs after 2 h of gastric phase. After the intestinal phase, both matrices maintained their viability after the end of the gastric phase. In addition, this study [123] evaluated the integrity of the SlpB (surface-layer protein B), an anti-inflammatory protein found on the surface of some *P. freudenreichii* strains, during the digestion using both digestion models. It was found that SlpB was wholly degraded during the static gastric phase on milk and milk ultrafiltrate matrices, while the cheese matrix protected it. As for dynamic digestion, SlpB was found to be up to 80 min of digestion (mid gastric phase) on milk ultrafiltrate matrix and to 150 min (30 min at intestinal phase). This demonstrates the importance of the probiotic matrix not only on the probiotic viability but also on their immunomodulatory function, as we have seen here with the preservation of SlpB protein.

An *E. coli* probiotic candidate, *E. coli* CEC15, could survive through the GIT test and be compared with the probiotic *E. coli* Nissle 1917 [126]. Both strains were simultaneously submitted to an in vitro simulated digestion [127] consisting of 2 h incubation in simulated gastric juice (with pepsin) and 2 h in simulated intestinal fluid (with pancreatin and bile salts). Both strains demonstrated low survival to the gastric phase with low pH (pH3), with CEC15 presenting better results (6.3% for CEC15 against 0.91% for Nissle 1917). After the intestinal phase, CEC15 recovered its viability, restoring values to 57.85%, while the Nissle 1917 strains maintained the viability of 2.77%. These results show that even strains from the same bacteria species can present very different survivability rates, and each strain's genetic components could determine this.

Bile Tolerance and Metabolism

Passage of probiotics through the small intestine can pose challenges as they encounter bile acids, bile salts, and pancreatic enzymes, which can significantly reduce viability. Unlike the stomach, the small intestine's neutral pH range of 6.1 to 7.8 does not inhibit probiotic survival [127, 128]. Bile acids play a crucial role in lipid digestion in the small intestine and impact the microbial ecosystem in the small and large intestines [129]. The liver produces bile acids to aid lipid digestion, while bile salts are secreted into the duodenum [129]. These components comprise more than 50% of the organic composition of bile. Furthermore, bile acids act as digestive surfactants, facilitating the emulsification of lipids for easier absorption [130]. Bile acids have also been shown to possess antimicrobial properties against various bacterial species [131].

Conjugated bile salts, conjugated ionically with either taurine or glycine by hepatic enzymes, have been observed to inhibit the growth of both gram-negative and gram-positive bacteria (e.g., *E. coli* and *Klebsiella* and *Enterococcus* genera) [132]. However, gram-positive bacteria tend to be more susceptible to the inhibitory effects of bile salts when compared to gram-negative bacteria [133]. Some probiotic strains can produce BSH, which allows them to hydrolyze bile salts. This ability is believed to confer resistance to conjugated bile salts and is a defense mechanism against their toxic effects. The hydrolysis of glycine- and taurine-conjugated bile salts by BSH leads to the release of the corresponding amino acids and deconjugated bile acids

[134]. The BSH activity of probiotic bacteria has been considered a significant criterion in selecting potential probiotic strains due to its association with various mechanisms that contribute to reducing plasma cholesterol levels in the host [135]. Deconjugated bile salts, less soluble than their conjugated counterparts, are more likely to be excreted in the feces. Consequently, the synthesis of new bile salts from cholesterol in the liver is reduced, resulting in decreased plasma cholesterol levels. Furthermore, deconjugated bile salts can precipitate with cholesterol, reducing its solubility and promoting its excretion via feces. It should be noted, however, that while BSH activity contributes to cholesterol reduction, excessive deconjugation of bile salts may have negative implications in the human intestine. This is because other intestinal bacteria can convert primary bile acids into secondary bile acids, which have been shown to exhibit mutagenic and tumor-promoting properties in animal models. Thus, excessive deconjugation of bile salts may pose potential harm to the human host [136].

In a study by Song et al. [121], the bile salt tolerance of 10 *Lactobacillus* strains was assessed. The results revealed that *Lactobacillus sp*. JNU 8829, *Lacticaseibacillus casei* MB3, *Latilactobacillus sakei* MA9, *Lt. sakei* CH8, and *Lb. acidophilus* M23 exhibited a high tolerance to bile acid. The assessment was performed using MRS broth supplemented with 0.3% oxgall and incubated for 24 h at 37 °C. In a study conducted by Bin Masalam et al. [137], it was found that the most bile-tolerant strains were predominantly enterococci, including *Enterococcus faecium* ZiNb3, *E. faecium* Rashad3, and *E. faecium* SMBM3. Other species such as *Ls. casei* BgShn3, *Ls. casei* Dwan5, *Ls. casei* MSJ1, *Lp. plantarum* EyLan2, *Lb. acidophilus* Musallam2, *Ls. paracasei* NMBM1, *Streptococcus bovis* Salam7, *Lactococcus garvieae* Emad4, *L. garvieae* ZSJ5, and *W. confusa* SYary1 also demonstrated bile tolerance. The assessment was performed using MRS broth supplemented with 0.5% wt/vol bile and incubated for 4 h.

BSH enzymes have been found in various bacterial genera, including *Enterococcus*, *Listeria*, lactobacilli, *Bifidobacterium*, *Clostridium*, and *Bacteroides* [138, 139]. Generally, most bifidobacteria and lactobacilli strains in the GIT possess BSH enzymes [138]. Lactobacilli strains originating from the gut have been observed to deconjugate glyco- and tauro-conjugated bile acids. In contrast, lactobacilli strains isolated from fermented milk products exhibit excellent capability to deconjugate glyco-conjugated bile acids [130]. BSH activity serves multiple purposes, including utilizing liberated amino acids as nutrients (carbon and nitrogen sources) and reducing bile salt toxicity. Additionally, it may play a role in incorporating cholesterol into the cell wall [139]. Some lactobacilli strains have been reported to reduce cholesterol levels through mechanisms such as cholesterol binding to their cells, potentially leading to lower serum cholesterol levels in vivo [140].

In a study by Saravanan et al. [141], 10 strains were isolated from traditional fermented foods belonging to the genera *Leuconostoc* spp., *Weissella* spp., *Pediococcus* spp., *Lactococcus* spp., and *Bacillus* spp. All exhibited strong BSH activity when tested on MRS agar containing 0.5% taurodeoxycholic acid (TDCA) sodium salt at 30 °C for 72 h. However, Kumari et al. [142], who tested 20 different lactobacilli strains isolated from fermented foods and beverages, found that none exhibited BSH activity. Awasti et al. [143] evaluated BSH activity in 12 bifidobacteria isolates obtained from human sources using an MRS medium supplemented with 0.5% sodium salts of taurocholic acid (TCA), tauroglycocholic acid (TGCA), and TDCA at 37 °C for 48 h. Out of the 12 isolates, 5 showed high levels of BSH activity, and 8 exhibited moderate activity, while 1 strain (NBIF-1) did not demonstrate BSH activity on TCA and TDCA. These findings highlight the variability in BSH activity among different bacterial species and even within strains of the same species, indicating the influence of strain-specific factors and environmental adaptation.

Adherence to the Host's Intestinal Wall

The capacity to adhere to intestinal epithelial cells is critical for successfully colonizing probiotic strains and is often regarded as a prerequisite for colonization. Consequently, it represents one of the primary criteria for selecting a probiotic microorganism [144]. The colonization of the human intestinal microbiota begins at birth and continues throughout life. However, the composition of the intestinal microbiota can change over the host's lifetime, and probiotics have the potential to influence these changes. Although probiotics do not permanently colonize the host's intestine, they can have a transient colonization effect [145]. The adhesion of probiotic strains is an essential factor in their colonization ability, and this adhesion can be associated with changes in the intestinal microbiota. For instance, the decline in bifidobacteria in elderly individuals may be attributed to reduced adhesion of bifidobacteria to intestinal mucus, which correlates with age [146].

Several mechanisms have been proposed to explain bacterial adhesion to the intestinal mucosa. One possible mechanism is hydrophobic interactions between the probiotic candidates and the intestine's surface [147]. The presence of mucin-binding proteins in the bacterial cell envelope enhances adhesion capability by binding to the mucin proteins of the mucus layer on the gastrointestinal epithelia [148]. Pili, which are hair-like appendages, can also contribute to bacterial attachment to the mucosal surface of the intestine. Certain bacteria, such as bifidobacteria, can employ pili to facilitate adhesion [149]. Fibronectin-binding and surface-layer proteins are additional surface proteins

embedded in the bacterial cell wall that promote bacterial adherence to the intestinal mucosa [150]. Producing extracellular polysaccharides by probiotic candidates has also been associated with adhesion to the intestinal surface [151].

The in vitro adhesion test is commonly used to evaluate the ability of a probiotic candidate to attach to human epithelial cell lines such as Caco-2, HT-29, and fetal I-407 [152]. This test examines the capability of the probiotic candidate to attach to epithelial cell lines (notably to mucin) [152, 153]. However, the in vitro adhesion test has several drawbacks. One primary concern is the reproducibility of the test. The conditions under which the adherence of the probiotic candidate is assessed in the epithelial cell lines deviate from the natural conditions in the host intestine. Essential factors such as other microorganisms, digestive enzymes like pancreatin, and physical contractions are absent in the in vitro test [152, 153].

Production of Antimicrobials and Pathogen Antagonists

The evaluation of antimicrobial activity against pathogens is a crucial factor in the selection of potential probiotic strains. While the production of antimicrobial compounds is a primary mechanism for this activity, additional mechanisms are involved. These include competition between probiotic and pathogenic strains for nutrients, the attachment of probiotics to epithelial cells, and the stimulation of the immune system. These combined mechanisms contribute to the overall antimicrobial efficacy exhibited by probiotic strains [154].

Antimicrobial substances produced by LAB can be classified into two main groups: non-bacteriocin antimicrobial substances and bacteriocins [155]. The non-bacteriocin antimicrobial metabolites encompass a variety of compounds such as organic acids (e.g., lactic acid, acetic acid), hydrogen peroxide, diacetyl, acetaldehyde, acetoin, carbon dioxide, reuterin, reutericyclin, antifungal cyclic dipeptides, phenyllactic acid, 4-hydroxyphenyllactic acid, and 3-hydroxy fatty acids. Among them, mainly lactic and acetic, organic acids are the most important and extensively studied. LAB plays a significant role in modulating the intestinal environment by producing organic acids, which leads to a decrease in pH and favors the colonization by beneficial microorganisms while reducing the population of pathogens [156]. Heterofermentative LAB species possess flavoprotein oxidase enzymes that catalyze oxygen reduction, producing hydrogen peroxide. The antimicrobial activity of hydrogen peroxide is attributed to its oxidative impact on bacterial cells, leading to the disruption of essential molecular structures of cell proteins [157].

The second group of antimicrobial compounds produced by LAB consists of bacteriocins, peptides, or proteins synthesized within the ribosomes of certain bacterial strains. Bacteriocins exhibit antimicrobial activity against other bacteria, while the cells producing them are immune to their bacteriocins [158]. To be classified as bacteriocins, these antimicrobial peptides must be modified or unmodified peptide antimicrobials produced by bacteria, accompanied by a dedicated immunity system that protects the producer cells [159]. The investigation of LAB for their bacteriocin-like inhibitory activity has gained significant attention in recent studies [22].

Probiotic Modulation of Gene Expression In Vitro

In vitro studies are also helpful in understanding how probiotics modulate different cell types in the host. Immortalized cells and freshly isolated cells have been widely used to evaluate the modulation of the expression of key genes related to barrier function, signaling, inflammation, differentiation, and chemoattraction of immune cells. This helps to predict the effect that could be obtained in a complex organism. As most probiotics aim to treat intestinal diseases, intestinal epithelial cells, such as Caco-2 and HT-29 (human colorectal adenocarcinoma), and PBMCs (peripheral blood mononuclear cells), such as lymphocytes, monocytes, natural killer (NK) cells, or dendritic cells (DC), are the most used cell lines in in vitro studies.

One of the well-recognized effects of probiotics is their ability to induce a shift from Th2 to Th1 cells, leading to a reduction in allergic reactions. When human peripheral blood lymphocytes and PBMCs are exposed to LAB, they exhibit an increase in the production of interferon-gamma (IFN-g) by T and NK cells [160, 161]. These findings align with in vitro experiments that demonstrate that lactobacilli present in fermented foods strongly stimulate the production of pro-IFN-g cytokines like IL-12 and IL-18 by both human and murine leukocytes [162, 163]. This capacity to steer the immune response toward a Th1 profile could prove beneficial in conditions characterized by Th2-driven inflammation, including atopic disorders and other Th2-associated inflammatory diseases.

Certain probiotic strains can exert varying effects on NK cells. For example, *Lacticaseibacillus rhamnosus* GG and *Lm. reuteri* DSM 17938 hinder the activation of T cells and NK cells, as well as the release of IFN-gamma from PBMCs stimulated with *S. aureus* [164]. The intricate interplay among probiotics, DC, and NK cells emphasizes how distinct strains can uniquely shape the immune system and inflammatory responses, potentially yielding advantageous outcomes by balancing NK and DC interactions [165]. An emerging strain, *Lm. reuteri* LMG P-27481, discovered and studied by Sagheddu et al. [166], demonstrates a remarkable ability to prompt significant secretion of IL-10 when exposed to immature human DCs. Compared to other *Lm. reuteri* strains, it manifests a more pronounced anti-inflammatory impact. In vitro co-culture experiments reveal that *Lm. reuteri* LMG P-27481 effectively curbs the growth of

E. coli, *Salmonella*, and rotavirus, with the unique ability to hinder *Clostridium difficile* growth. Notably, its genetic makeup enables it to metabolize lactose, proving especially valuable in diarrhea scenarios [166].

Luerce et al. [167], through a colitis-recurrence model conducted on Caco-2 cells, demonstrated the capability of *Lactococcus lactis* NCDO 2118 to reduce the secretion of IL-8 triggered by IL-1beta. Similarly, *B. animalis* subsp. *lactis* and *Lb. acidophilus* have also exhibited the ability to decrease IL-8 production, suppress the expressions of pro-inflammatory agents, and enhance TLR2 expression in an in vitro model. This anti-inflammatory effect influences the TLR2-mediated NF-kB and mitogen-activated protein kinase (MAPK) signaling pathways within inflamed intestinal epithelial cells [168].

Certain lactobacilli species have shown the ability to mitigate barrier disruptions by upregulating tight junction (TJ) proteins. For instance, *Lb. acidophilus* and *Lp. plantarum* have been demonstrated to increase the occludin protein expression in vivo and in vitro models, respectively [169, 170]. Moreover, *Lp. plantarum* triggers the relocalization of ZO-1 and occludin to the apical region of cells by stimulating Toll-like receptor 2 (TLR2) [171, 172]. It is important to note, however, that while coincubation of Caco-2 cells with *Lp. plantarum* leads to increased transcription of genes related to the disassembly of TJs and occludin degradation, the elevated occludin expression and apical localization might be a defensive response prompted by initial bacterial-induced degradation of the TJ structures, rather than a means of maintaining them [169].

In some instances, realizing the beneficial effects of probiotics hinges on the prior disruption of TJ homeostasis. *E. coli* Nissle 1917 incubation of T84 human intestinal epithelial cells does not significantly alter intestinal barrier function [173]. However, when T84 cells are co-incubated with enteropathogenic *E. coli* (EPEC), causing barrier disruptions, supplementing with *E. coli* Nissle 1917 restores barrier integrity to levels like those in control cells. Consequently, *E. coli* Nissle 1917's beneficial effects on the intestinal barrier might manifest post-infection without requiring preemptive supplementation, which can be challenging from a clinical standpoint. Additionally, alongside reduced barrier permeability, *E. coli* Nissle 1917 supplementation leads to heightened expression of ZO-2 and its robust relocalization to the TJ [173]. While these initial findings do not establish definitive causation, they suggest that the positive impact of *E. coli* Nissle 1917 on intestinal barrier function might be influenced by the bacterium's ability to regulate ZO-2 expression and localization.

Mucins play a crucial role in maintaining the protective function of the intestinal barrier. Probiotic bacteria exhibited diverse impacts on mucin gene and protein expression. Furthermore, the effects of probiotic treatments on mucin gene expression varied. When Caco-2:HT29–MTX (90:10) co-cultures were incubated with *Ls. rhamnosus* HN001, increased levels of all mucin mRNA were observed, with a significant increase in MUC5AC mRNA compared to untreated samples [174]. This contrasted with a study by Mack et al. [175], where *Lp. plantarum* 299v increased the expression of MUC2 and MUC3 genes in HT29 cells. This difference might arise from the co-culture conditions in this study versus the monoculture of mostly undifferentiated HT29 cells in the previous one.

Although important for identifying the mechanism of probiotics' actions and predicting the effects in the host, in vitro studies lack the complexity of a tissue, an organ, and an organism. To fulfill the gaps left by in vitro studies and to continue the tests on probiotic effects, in vivo studies involving animal models and, later, clinical trials in humans are widely used.

Animal Studies and Clinical Trials

The search for proof of efficacy in vivo while exploring the potential of probiotics has resulted in the development of various biological models with varying levels of complexity. These models encompass a wide range, from simple multicellular organisms, such as worms and invertebrates, to advanced knock-out (KO) models in rodents and even clinical trials involving different populations of humans [153]. Hence, although the ultimate evaluation of probiotic functionality should ideally be conducted directly in the target population, such as the general population or a specific subgroup with a particular condition [176], the initial selection of strains to be included in these costly clinical trials may require the use of suitable in vivo models. Rodent models, especially mice and rats, serve as cost-effective and publicly acceptable screening tools, but they still fall short of representing human physiology. Therefore, developing more relevant experimental models for evaluating probiotic functionality is necessary. These models should allow studying various dynamic states and address specific diseases with multifactorial origins. It is important to note that the accuracy of results obtained from animal models is not always the same as the results in humans and can sometimes present challenges. As recently stated, there can be inflammatory findings and difficulties in extrapolating results from one species to another [177, 178]. The use of animals to predict human response to drugs, chemicals, or foods (including probiotics) remains a contentious issue. While some advocate for a ban on animal experimentation due to a perceived lack of scientific evidence for human predictivity [179], the relevance of animal disease models, such as mice, for studying human conditions has been positively evaluated [180].

The effective utilization of rodent models for probiotic research will rely on rigorous standardization, including the microbiota composition. It is crucial to consider the relevance to the human situation since many bacterial species that are commensal in humans can be pathogenic in mice and vice versa [181, 182]. Despite these potential drawbacks, rats and mice will continue to be used as models to address numerous probiotic research questions. This includes evaluating immune and metabolic responsiveness, regulatory processes, and neuro-endocrinological and nutritional aspects, all playing essential roles in the complex relationships between the microbiota and the host. Additionally, small animals allow for mimicking specific diseases with genetically modified specimens (conditional and tissue-specific knock-in/KO mutants) or specific chemicals (e.g., TNBS to induce intestinal inflammation) and infectious challenges. Manipulating the microbiota also enables the investigation of the role of specific microorganisms in these models [153].

The regulations permit the use of animals for scientific purposes but require strict adherence to restrictive conditions. To enhance the welfare of animals in research, applying the 3Rs serves as an ethical guide. 3R means replacement (the use of alternative methods such as in vitro, in silico, ex vivo, and the use of less sentient species, i.e., invertebrates), reduction (to utilize the least number possible of animals without interfering with the statistics), and refinement (experimental protocols curation aiming to minimize stress and suffering on the animals)—proposed originally by Russel and Burch in 1959 [183, 184]. Implementing the 3Rs principle enhances the well-being of animals utilized in scientific research. It tackles multiple issues related to animal use in science, prioritizes the welfare of individual animals, incorporates new knowledge and insights, strikes a balance between scientific requirements and animal welfare, and fosters collaboration among diverse stakeholders concerned about animal welfare [185]. Numerous Replacement technologies offer notable benefits, including enhanced consistency and accuracy, rapid results, and lower costs when compared to using animals. Besides, specific studies are impractical to conduct in animal models due to limitations in throughput or the necessity of human-relevant tissues [183]. By employing stringent criteria in experimental design to ensure reproducibility, Reduction also leads to improved scientific outcomes, and diligently applying the principles of Refinement helps minimize stress as a scientific variable, guaranteeing a more refined and reliable research environment [183].

However, despite the efforts to find alternative methods, the current state of knowledge does not yet allow these methods to comprehensively address all scientific questions in biology and medical research. Therefore, alternative methods often serve as complementary approaches to in vivo methods rather than complete replacements, as they may not fully substitute for the complexity of biological processes and physiological interactions observed in living organisms.

Animal Toxicity Studies

When the history of use for a particular probiotic strain is unknown or insufficient, authoritative guidance suggests conducting additional safety studies, including animal studies. However, there is limited specific guidance on the design and conduct of probiotic animal toxicity studies. Unlike chemicals, a standard non-clinical toxicology testing paradigm may not apply to probiotics due to their unique nature and may provide limited information [68, 186]. Therefore, specific requirements for probiotic testing should be carefully evaluated on a case-by-case basis.

Conducting tests in animal models for certain probiotic strains may be reasonable. For example, if insufficient historical use data is available for a particular strain or species or for "novel" or NGP, animal toxicity assessment may be necessary before testing in humans. Even when candidate probiotic strains are human commensal microbes, they are still considered "not-self" and cannot be assumed harmless.

Rousseau et al. [187] provided an overview of in vitro, ex vivo, and in vivo non-clinical models that they considered relevant for microbiome research on various products, including probiotics. While these models hold promise for microbiome research purposes, most are unsuitable for assessing standard toxicology endpoints.

There are indeed notable differences between rodents and humans that should be considered when evaluating potential probiotic strains. Factors such as differences in mucus growth rate and dietary patterns between rodents (herbivores) and humans (omnivores) can impact the relevance of animal models to human outcomes. In this respect, pigs constitute preclinical animal models with a higher similarity when compared to rodents in terms of physiology, digestive and associated metabolic processes, nutritional requirements, and intestinal microbiota [188]. It is essential to acknowledge that no animal model can fully represent humans [51].

However, when toxicity studies are deemed necessary for probiotics, relying on standard rodent models, such as rats or mice, is common. Rodents have a long history of use in toxicology studies and provide a wealth of data on toxicity findings that are specific to rodents and do not have a direct correlation to humans (lack clinical relevance). This historical data can be informative in assessing the safety of probiotics [73].

Once an appropriate rodent model is chosen, the next question concerns the duration of dosing required to support further safety testing in human clinical trials. Interestingly, a review of traditional oral repeated dose animal toxicity studies with probiotics, as reported in the scientific literature, has not revealed any adverse effects regardless of dosing duration. This includes acute (single dose), repeated dose (e.g., 14–28 days), sub-chronic (e.g., 28–90 days), and even chronic (12 + months) studies conducted at high doses of the probiotic under investigation [73, 189].

For example, studies have been conducted where rats were dosed with a proprietary preparation of *Bacillus coagulans* for 90 days at high doses with no observed toxicity. Similarly, a 12-month study in rats with *Clostridium butyricum* and a sub-chronic rodent study with a probiotic product containing various strains showed no toxicity [190].

The dosing duration in toxicity studies with probiotics is connected to considering relevant toxicity endpoints. When assessing the safety of live microorganisms, such as probiotics, specific endpoints related to in vivo administration should be evaluated. One important endpoint is translocation, which refers to the passage of live microbes from the GIT to other sites within the body. To assess translocation, various organs such as lymph nodes, spleen, liver, bloodstream, or other tissues are collected at necropsy, homogenized, and plated for enumeration of bacterial colonies [191]. Genetic methods can be used to confirm the presence of specific bacterial strains. The translocation of microbes to other organs is a concern because it could potentially lead to infection in the host, such as bacteremia or septicemia.

Therefore, when studying the translocation potential of probiotics, it is more relevant to use healthy animals in the research [192]. Several in vivo studies have assessed the translocation potential of various probiotic strains in healthy mice or rats, typically lasting 4 weeks. This timeframe is sufficient to observe potential translocation and infectivity [193].

Shorter-term studies, such as repeated dose studies, may provide sufficient assurance of safety to proceed with clinical studies in healthy humans. These initial human studies should be carefully designed to collect safety endpoint data and ensure appropriate monitoring of potential adverse effects [190]. It is important to note that the safety assessment of probiotics should be tailored to the characteristics of the strain and the intended use, and a case-by-case approach is recommended.

Besides toxicity studies, animal models and clinical trials have been extensively used to identify the effects of potential probiotics in disease models and healthy individuals. These effects attributed to probiotics found in these studies are summarized in Fig. 3 and described in more detail in the following sections.

Probiotic Modulation of Intestinal Diseases In Vivo

The commensal microbiota has constant interaction with the GIT, which maintains mucosal immune homeostasis under normal conditions and promotes benefits to the host [194–196]. However, when the intestinal microbial balance is disturbed, the intestinal microbiota might have a role in the establishment and/or development of chronic inflammatory diseases, such as inflammatory bowel diseases (IBDs) and intestinal mucositis [197, 198].

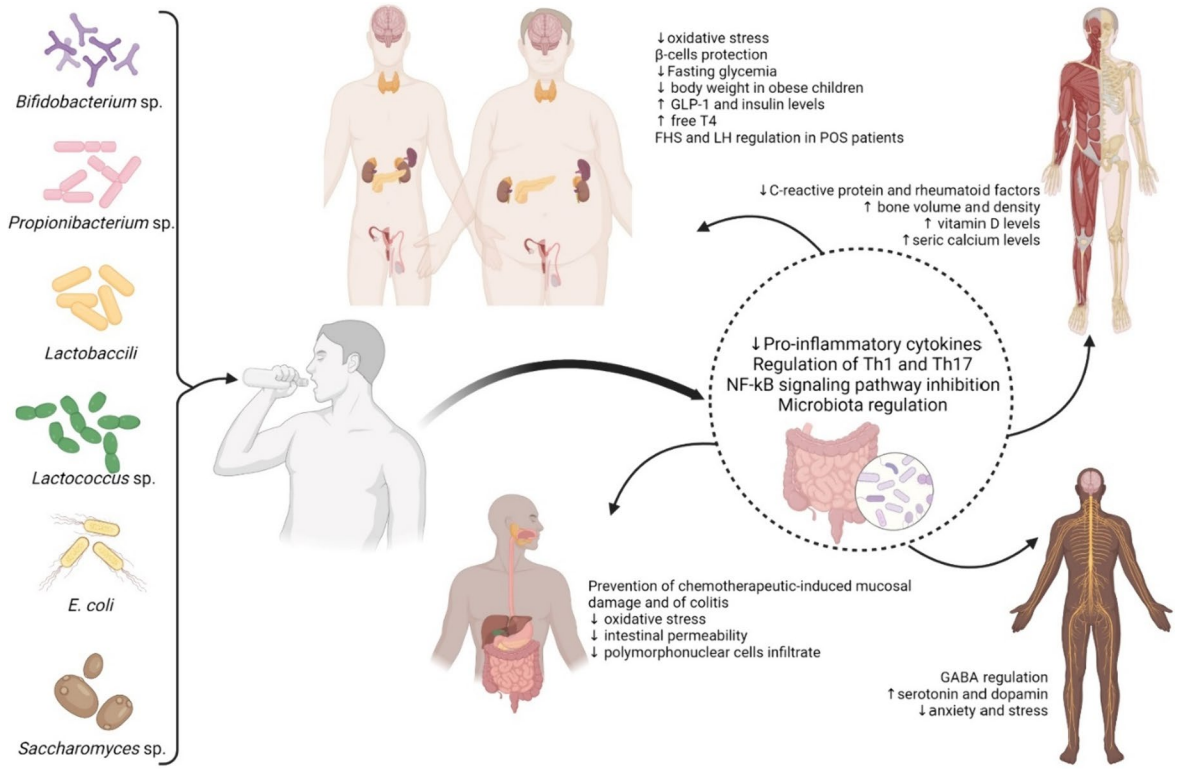

Fig. 3 Main effects of probiotics in the inflammatory process of diverse systems

The IBDs are described as multifactorial disorders that involve chronic inflammation of the GIT. IBDs include ulcerative colitis (UC), which affects mainly the mucosa and submucosa of the colon, as well as Crohn's disease (CD), which causes a multifocal transmural granulomatous inflammation along the lining of GIT [199]. The UC mainly affects adults aged 30–40, with no sex predominance. The CD has a bimodal distribution between ages 15 and 30 and 40 and 60. UC's highest incidence and prevalence are in Northern Europe and North America. In contrast, the CD is most seen in the Western developed world in North America, Northern Europe, and New Zealand [200–202]. The IBD-associated disorders are commonly characterized by blood in the stool, diarrhea, tenesmus, fatigue, fevers, increased frequency of bowel movements, abdominal pain, and weight loss. The diagnosis is based on clinical information, physical examination, and endoscopic and histological investigations [203, 204]. The main risk factors associated with these diseases' etiology are family history, genetic susceptibility, environmental factors (e.g., cigarette smoking, stress), intestinal dysbiosis, and altered immune response [200–202].

A loss of tolerance toward enteric commensal bacteria and an exacerbated Toll-like or NOD-like receptors' expression levels lead to an activated innate (macrophage, neutrophil) and acquired (T and B cells) immune response in IBD patients. These immune cells increase the production of different cytokines and chemokines involved in Th1, Th2, and Th17 responses, such as pro-inflammatory markers tumor necrosis factor-alpha (TNF-α), IFN-γ, interleukins (IL-1β, IL-18, IL-6, IL-17, IL-23), chemokine (c–c motif) ligand (CCL2, CCL4, CCL5), and chemokine (C-X-C ligand) motif (CXCL8, CXCL10) [205, 206]. All these immunological factors are associated with CD and UC development. In this way, to suppress the inflammatory responses, current treatments for IBDs have been done with corticosteroids, aminosalicylates, monoclonal antibodies anti-TNF-a (e.g., infliximab and adalimumab), antibiotics, and surgery. Moreover, the choice of the therapeutic approach is based on the extent and severity of the disease and inducing and maintaining clinical remission [204].

Probiotics as Alternative Therapeutic Approach for Treatment of IBD

Knowing that dysbiosis also plays a vital role in the pathogenesis of IBDs, the modulation of the patient microbiota via the administration of probiotic bacteria has been proposed as a promising therapeutic approach for the treatment of these diseases due to selected probiotic strains' anti-inflammatory effects on GIT and the microbiota restoration/regulation [207]. A strain-dependent positive effect of probiotic consumption in IBD treatment has been extensively proven in animal models and clinical trials. However, most studies are still conducted in animal models, especially colitis induced by DSS (dextran sodium sulfate) and TNBS (2,4,6-trinitrobenzene sulfonic acid) in rats and mice.

The positive effect of probiotic administration has been tested in clinical trials. The commercial probiotic preparation VSL#3 (including *Lp. plantarum*, *Lb. delbrueckii*, *Ls. casei*, *Lb. acidophilus*, *B. longum*, *B. breve*, *B. infantis*, and *Streptococcus salivarius*) was able to prevent CD recurrence after surgery by reducing mucosal inflammatory cytokine levels (IL-8 and IL-1β) and improving IBD questionnaire score [208]. In another study, this commercial probiotic formulation could also induce remission in patients with UC [209]. Meta-analysis of randomized controlled clinical trials confirmed the strong evidence of VSL#3 efficacy in IBD, with 8 conclusive clinical trials [210].

The beneficial effects of *Ls. rhamnosus* GG administration were also reported, improving the gut barrier function and clinical status in children with mildly to moderately active CD [211]. Promising results were also found for the *Lb. delbrueckii* and *Limosilactobacillus fermentum*, whose consumption was associated with intestinal inflammation reduction in patients with UC. This included decreased colonic concentration of IL-6, TNF-α, and NF-κB p65 expression, leukocyte recruitment, and colonic MPO (myeloperoxidase) activity [212].

A review analyzed 18 studies on the effectiveness of probiotics, prebiotics, and symbiotics in inducing or maintaining remission of UC in adults and children. It concluded that probiotics are beneficial in achieving remission in patients with UC [213]. One of the reviewed studies demonstrated changes in the composition of the intestinal microbiota, the more significant number of bifidobacteria on the mucosal surface of patients fed with a formula containing *B. longum* and the prebiotic Synergy1® (inulin and oligofructose) [213].

As for the studies conducted in animal models, preclinical trials showed that oral administration of *Lb. delbrueckii* subsp. *lactis* CNRZ327 (2.5×10^{10} colony-forming units (CFU)/mL) [214] or of *P. freudenreichii* CIRM-BIA 129 (2×10^{9} CFU/mL) [215] was able to attenuate DSS- and TNBS-induced colitis in mice, respectively. These probiotic bacteria showed anti-inflammatory, as evidenced by a reduction of oxidative stress markers (cyclooxygenase 2 (Cox-2) and heme oxygenase (Hmox)) and neutrophil inflammatory infiltrate (MPO assay) [215]. These probiotics modulated the balance between Th1, Th2, Th17, and Treg cells [214] and epithelial architecture damage [214, 215]. The same results were observed in rats for *P. freudenreichii* KCTC 1063 strain, according to Ma et al. [216]. They administrated this probiotic strain (10^{8} CFU/rat/day) for 22 days, and it showed an improvement of DSS (5%)-induced colitis (last 8 days) in rats by stimulating MUC2 protein expression and down-regulating the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β [216].

Jang et al. [217] showed that administration of *B. longum* LC67 (2×10^9 CFU/mL) reduced the severity of TNBS-induced colitis in mice by preventing MPO activity, inhibiting NF-κβ activation, restoring Th17/Treg balance and gut microbiota composition by restoring the Proteobacteria-to-Bacteroidetes ratio.

Finally, the VSL mentioned above #3 mix of 8 probiotic strains, which was effective against IBD in clinical trials, also afforded protection against DSS-induced colitis and TNBS-induced colitis in rats and mice. Li et al. [220] show that VSL#3 fosters anti-inflammatory characteristics in rats through a lowered disease activity index and reduced MPO activity. Furthermore, administering VSL#3 over 7 days led to a decline in iNOS, COX-2, NF-κB, TNF-α, IL-6, and p-Akt levels, accompanied by an elevation in IL-10 expression within the colonic tissue. Simultaneously, this reduced TNF-α and IL-6 levels, while IL-10 serum levels increased. As for TNBS-induced colitis, VSL#3 treatment prevented weight loss and mitigated colon shortening. Additionally, VSL#3 treatment demonstrated the ability to reduce damage to intestinal epithelial cells and the infiltration of inflammatory cells within the lamina propria and submucosa. Moreover, VSL#3 restored the levels of HMGB1, a pro-inflammatory mediator crucial in experimental colitis, as well as F4/80 + levels, a pan-marker indicating macrophages within the lamina propria mononuclear cells of mice with TNBS-induced colitis. The notable reduction in the expression of TJ proteins ZO-1, occludin, and claudin-1 in TNBS-exposed mice, caused by TNBS administration, was facilitated through VSL#3 treatment [218]. These results highlight the potential effects of certain probiotic strains on IBD, opening pathways for their future use as therapeutics for humans.

Probiotics as Alternative Therapeutic Approach for Treatment of Intestinal Mucositis

Intestinal mucositis is a cytotoxic effect resulting from non-selective antineoplastic drugs (e.g., 5-fluorouracil (5-FU), oxaliplatin, methotrexate, and irinotecan) that, besides destroying neoplastic cells, promote damage to healthy cells [219]. Inflammation-related signaling pathways mainly characterize this inflammatory disorder. These include NF-κB and MAPK activation and, consequently, pro-inflammatory cytokine and chemokine production (e.g., TNF-α, IL-6, IL-22, CXCL1, CXCL5), loss of the intestinal epithelial barrier, damage to the crypts and villus shortening, mucus-producing goblet cells reduction, inflammatory cells infiltration in the lamina propria (e.g., macrophages, neutrophils, and eosinophils), microbiota composition alteration, TJ disruption, increasing intestinal permeability, and predisposition to infections by pathogenic microorganisms [220, 221].

Intestinal mucositis is a severe gastrointestinal condition in humans, and alteration of the intestinal microbiota has a relevant role in the progression of this inflammatory condition [198]. The modulation of the digestive microbiota through oral administration of probiotic bacteria has thus been proposed as a therapeutic alternative for intestinal mucositis treatment. In this context, several studies have demonstrated the strain-dependent beneficial effects of probiotic bacteria on the prevention of chemotherapy-induced intestinal mucositis [222–224].

Bifidobacterium infantis (10^9 CFU/day) ameliorated the mucosal damage induced in a synergic colorectal cancer (CRC) model in rats with 5-FU (75 mg/kg/3 days) and oxaliplatin (8 mg/kg/3 days). It improved intestinal mucositis by increasing the CD4 + , CD25 + , Foxp3 + , and Tregs cells and decreasing Th1 and Th17 cell response [225]. Positive effects were also reported for *B. bifidum* G9-1 (10^7–10^9 CFU), which reduced the intestinal damage induced by 5-FU (50 mg/kg/9 days) in mice via regulation of the intestinal microbiota (increasing Bacteroidetes and decreasing Firmicutes abundances) and reduction of inflammatory infiltrate and pro-inflammatory IL-1β and TNF-α cytokine levels [226].

Promising results were also reported using different lactobacilli strains. Epithelial damage induced by 5-FU (450 mg/kg) in mice was attenuated by the consumption of *Lb. acidophilus*, which improved the intestinal mucositis via inhibition of the NF-κB signaling pathway and reduced the levels of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1β, and CXCL-1 [227]. *Ls. casei* triggered a similar protective effect against intestinal damage caused by chemotherapy association with 5-FU (30 mg/kg), leucovorin (10 mg/kg), and oxaliplatin (1 mg/kg) for 5 days in a syngeneic CRC model. This was related to the downregulation of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β, IFN-γ) due to NF-kβ inhibition and microbiota regulation (decreasing Firmicutes and increasing Bacteroidetes abundance) [228].

Probiotic formulations containing different bacteria strains and fermented products have also effectively mitigated intestinal damage induced by 5-FU chemotherapy. A probiotic formulation containing *Lb. acidophilus*, *Ls. paracasei*, *Ls. rhamnosus*, and *Bifidobacterium lactis* prevented epithelial damage in mice induced by 5-FU (450 mg/kg). It increased the villus/crypt ratio, glutathione (GSH) levels, reduced neutrophil infiltrate, and malondialdehyde (MDA) and pro-inflammatory cytokine (TNF-α and IL-6) levels in the duodenum, jejunum, and ileum [229]. Another study also showed that administration of the commercial DM#1 (*Lb. acidophilus* DM8302, *Ls. casei* DM8121, *Bifidobacterium breve* DM8310, and *S. thermophilus* DM8309) ameliorated the intestinal damage of mice treated with 5-FU (30 mg/kg/5 days) by decreasing intestinal permeability, neutrophil

infiltration, and pro-inflammatory cytokines (TNF-α, IL-4, IL-6) [230]. Also, fermented milk formulations composed of *P. freudenreichii* CIRM-BIA138 and/or *Ls. paracasei* BL23 supplemented with whey protein (30%) [223] and *Lb. delbrueckii* CIDCA 133 (7.5×10^7 CFU) [231] were able to prevent intestinal mucosa damage induced by 5-FU (300 mg/kg). These studies observed the prevention of villus shortening, goblet cell degeneration, and polymorphonuclear cell infiltration reduction [223, 231].

Probiotic yeasts can also be highlighted in intestinal mucositis treatment. Post-treatment with *Saccharomyces cerevisiae* UFMG A-905 (10^9 CFU/mL) was able to protect mice against irinotecan (75 mg/kg)-induced intestinal mucositis, reducing the weight loss, villus shortening, intestinal permeability, oxidative stress, and prevented goblet cells degeneration [232]. Promising results were also reported for *Saccharomyces boulardii* (10^9 CFU/kg/3 days), which prevented 5-FU (450 mg/kg)-induced intestinal mucositis, controlling TLR2, TLR4, and MyD88 and reducing NF-κB, ERK1/2, phospho-p38, phospho-JNK, and pro-inflammatory markers TNF-α, IL-1β, and CXCL-1 [233]. Therefore, all these findings show that oral administration of probiotic microorganisms modulates the inflammatory response in chemotherapeutic agent–induced mucositis, revealing a promising therapeutic strategy for treating this intestinal inflammatory disorder.

Intestinal Dysbiosis Resolution and Microbiota Modulation by Probiotics

The human intestinal microbiota fulfills diverse functions in the host, including intestinal development, homeostasis, and protection against pathogenic bacteria. Moreover, various studies have indicated that intestinal microbiota dysbiosis contributes to the development of metabolic diseases, including obesity, diabetes, and intestinal diseases such as antibiotic-associated diarrhea (AAD), IBDs, and CRC [11, 234–236]. Modulating the microbiota by probiotic supplementation seems to be a promising way to treat and prevent diverse conditions.

In dysbiosis, probiotics can restore intestinal biodiversity, returning it to normal [237]. The overgrowth of the Proteobacteria and/or reduction of Bacteroidetes are observed conditions of dysbiosis related to unhealthy dietetic habitats. Abnormal increase in Proteobacteria levels can lead to energetic imbalance between different bacteria species and growing suppression of other bacteria species. The proliferation of some species of Proteobacteria also may cause illness development [238]. In celiac disease, studies suggested that patients with gastrointestinal symptoms present different microbiota compositions, a higher abundance of Proteobacteria phylum, and a lower abundance of Bacteroidetes and Firmicutes compared to control groups [235, 239]. These microbiota alterations may have pathogenic implications, promoting persistent gastrointestinal symptoms [239].

The mechanisms by which probiotics alter the intestinal microbiota are diverse. They may stimulate mucin production by intestinal goblet cells, induce antimicrobial peptides production, improve stability of cell junctions, increase the release of IgA by activated B cells, and inhibit the growth of pathogens or promote their elimination through the production of antimicrobial molecules, such as SCFA, bacteriocins, and microcin [240]. These mechanisms maintain the homeostasis of the microbiota, prevent the adhesion and proliferation of potentially pathogenic microorganisms, and promote health in the host.

Wu et al. [244] performed a clinical trial with 276 previously untreated patients infected with *Helicobacter pylori*, who were divided into two groups through random assignment. One group ($n = 140$) received a 14-day esomeprazole, bismuth, amoxicillin, and furazolidone, supplemented with probiotics (*Bifidobacterium* tetra vaccine tablets), while the other group ($n = 136$) received a placebo for 28 days. The occurrence of gastrointestinal adverse events was notably lower in the probiotic group compared to the placebo group (23.6% vs. 37.7%, $p = 0.016$). Immediate eradication of *H. pylori* led to considerable disruptions in the gut microbiota, with Proteobacteria replacing commensal Firmicutes and Bacteroidetes. However, this alteration gradually normalized after 2 weeks. Adding probiotics counteracted the reduction of gut Bacteroidetes induced by eradication drugs. The gastric microbiota fully restored itself as *H. pylori* decreased and other taxa flourished. Importantly, individuals treated with probiotics exhibited more stable fluctuations in gastric microbiota than those who received a placebo.

In another trial [241], fifty patients receiving hemodialysis were enrolled and randomized, receiving either probiotics (2.2×10^9 CFU *B. longum* NQ1501, 0.53×10^9 CFU *Lb. acidophilus* YIT2004, and 1.1×10^9 CFU *Enterococcus faecalis* YIT0072) or placebo for 6 months. Compared to the placebo group, the administration of probiotics did not significantly alter species diversity within the fecal microbiome. However, probiotics did play a role in restoring the community composition, and this effect was particularly notable in non-diabetic hemodialysis patients ($p = 0.007$). Specifically, based on the findings from linear discriminate analysis effect size, the introduction of probiotics led to an increase in the proportions of the Bacteroidaceae and Enterococcaceae families while reducing the presence of Ruminococcaceae, Halomonadaceae, Peptostreptococcaceae, Clostridiales Family XIII, Incertae Sedis, and Erysipelotrichaceae families in non-diabetic hemodialysis patients.

B. longum BB536 and *Ls. rhamnosus* HN001, combined with vitamin B6, were administered to 23 lactose-intolerant individuals who continued to experience symptoms despite adhering to a lactose-free diet [242]. This administration

took place over 30 days. Compared to the placebo, probiotics and vitamin B6 intake significantly reduced bloating ($p = 0.028$) and improved constipation ($p = 0.045$). The composition of the fecal microbiome varied between the treatment group and the placebo. The treatment led to the enrichment of several genera associated with lactose digestion, including bifidobacteria. Additionally, there were changes in the relative abundance of certain compounds, such as an increase in acetic acid, 2-methyl-propanoic acid, nonenal, and indolizine 3-methyl and a decrease in phenol.

These findings underscore the importance of specific probiotics, and sometimes adjuvants, in alleviating symptoms and addressing gut dysbiosis in individuals with dysbiosis and persistent functional gastrointestinal symptoms.

Probiotics in Metabolic Diseases

Metabolic syndrome refers to a set of metabolic disorders characterized by dyslipidemia, hyperglycemia, insulin resistance, oxidative stress, inflammation, hypertension, and neurodegeneration. These disorders are associated with various metabolic diseases like obesity, diabetes mellitus (DM), non-alcoholic fatty liver disease (NAFLD), and osteoarthritis [243]. It is common for multiple metabolic diseases to coexist, where obesity increases the risk of type 2 DM, and excessive body weight contributes to the development of NAFLD [244]. Recent studies have highlighted the significant role of imbalanced gut microbiota in metabolic diseases [245]. Therefore, modulating the gut microbiota has emerged as a promising approach to address this situation [246].

The approach to maintaining a healthy gut microbiota balance is using probiotics. Experimental studies and clinical trials have shown promising effects of probiotics in alleviating conditions such as obesity, type 2 DM, and other metabolic diseases in many cases [247]. Consumption of probiotic kefir was reported to improve serum apoloprotein A1 in metabolic syndrome patients [248]. Consumption of *Lp. plantarum* [249], *Lb. acidophilus*, and some *Bifidobacterium* species (*B. bifidum*, *B. lactis*, and *B. longum*) [250] led to a reduction in blood sugar and cholesterol. More precisely, consuming *Lp. plantarum* for 90 days reduced LDL cholesterol, blood glucose, and homocysteine levels in postmenopausal women [249].

Diabetes Mellitus

Experimental studies have suggested a possible beneficial effect of probiotics in preventing and treating DM. This metabolic syndrome results from the lack of insulin and/or the inability of insulin to adequately exert its effects, characterizing permanently high blood sugar levels (hyperglycemia) [251, 252]. Probiotics were shown to be able to attenuate hyperglycemia; improve the function of pancreatic β cells [253], insulin secretion [254], and insulin resistance [255]; regulate lipid and lipoprotein metabolism [256]; and modulate oxidative stress and inflammatory processes, improving the body weight [257] and preventing micro- and macrovascular complications [258], being considered an alternative for treatment and maintenance of DM.

Lactobacilli is the bacteria family most used in DM studies because it can improve hyperglycemia in the short and long term, reducing fasting and postprandial plasma glucose, HbA1c (glycated hemoglobin), serum insulin concentration, and insulin resistance [259]. *Lb. acidophilus* and *Ls. casei* reduced oxidative stress and exhibited an anti-diabetic effect in animals [260, 261]. Matsuzaki et al. [262] reported that ingesting *Ls. casei* by alloxan-treated Balb/c mice inhibited the reduction of pancreatic β-cells. In another study, researchers observed that autoimmune destruction of pancreatic β-cells was also inhibited by the oral administration of *Ls. casei* in non-obese diabetic mice. The administration of *Ls. rhamnosus* GG significantly delayed elevated glucose intolerance and hyperglycemia during the development of streptozotocin-induced diabetes in rats [263].

Oral administration of dahi (a fermented milk product from India containing *Lb. acidophilus* and *Ls. casei*) delayed the progression of streptozotocin-induced diabetes in rats. The results suggested that the supplementation of probiotic cultures increased effectiveness in suppressing chemically induced diabetes through insulin depletion. In addition, the product prevented diabetic dyslipidemia, inhibiting lipid peroxidation and nitrite formation [260].

Supplementation with *Lb. acidophilus* NCFM was evaluated in healthy or insulin-sensitive individuals. According to Andreasen et al. [264], after treatment, insulin sensitivity was preserved in the probiotic group and decreased in the placebo group, and the inflammatory markers and systemic inflammatory response (TNF, IL-6, IL1ra, and C-reactive protein) were not affected in either group.

Over 13 randomized clinical trials involving 840 subjects with type 2 DM have shown that probiotic administration can improve glucose metabolism with a potentially more significant effect when the duration of treatment is higher than 8 weeks [265]. These studies involved the administration of different probiotic strains, including *Lb. acidophilus*, *B. lactis*, *Ls. casei*, *Ls. rhamnosus*, *Lactobacillus bulgaricus*, *B. breve*, *B. longum*, *S. thermophilus*, *Lactobacillus bifidum*, *Lactobacillus sporogenes*, *Lp. plantarum*, *B. bifidum*, *B. animalis* subsp. *lactis*, *Lm. reuteri*, *Lm. fermentum*, and *Bacillus coagulans* which show the high variability of bacterial species that present anti-diabetic effects.

Lb. gasseri BNR17, from human breast milk, significantly reduced fasting and postprandial glycemia and HbA1c in a murine model of type 2 DM [266]. *Ls. rhamnosus* CCFM0528

and *Ls. casei* CCFM 0412 also reduced fasting glucose, postprandial glycemia, and HbA1c and increased serum insulin levels and hepatic glycogen after 13 weeks of intervention in diabetic rats, showing an improvement in glucose tolerance [267, 268]. Another study with diabetic rats using soy milk fermented with *Ls. rhamnosus* CRL 981 demonstrated a significant reduction in fasting glycemia [269].

In addition, the use of shubat (also known as chal), a Turkic beverage of fermented camel milk, sparkling white with a sour flavor, and a mixture of LAB strains (*Lp. plantarum*, *Lb. helveticus*, *Schleiferilactobacillus harbinensis*, *Lentilactobacillus hilgardii*, *Ls. rhamnosus*, *Limosilactobacillus mucosae*, *Ls. paracasei* subsp. tolerans, *Lp. pentosus*, and *L. lactis*) and yeasts (*Kluyveromyce smarxianus*, *Pichia membranifaciens*, *Candida ethanolica*, and *Issatchenkia orientalis*), promoted a reduction of fasting blood glucose and HbA1c and increased in serum levels of C-peptide and GLP-1 [270].

In studies with gestational DM, probiotics led to significant reductions in fasting glucose and insulin resistance and a tendency to increase insulin receptor sensitivity, as reported before [271].

Obesity

The etiology of obesity includes several genetic, metabolic, inflammatory mechanisms, and dysbiosis [272, 273]. Microbial changes in the human gut can be considered a factor involved in obesity development in humans [279], and the modulation of the bacterial strains in the digestive tract may help to reshape the metabolic profile in the obese human host [274, 275]. Evidence supports the connection between gut bacteria and obesity in infants and adults, where dysbiosis has been suggested to contribute to the development of obesity [281].

Lactobacilli species (i.e., *Ls. casei* strain Shirota, *Lb. gasseri*, *Ls. rhamnosus*, and *Lp. plantarum*) and *Bifidobacterium* species (i.e., *B. infantis*, *B. longum*, and *B. breve*) were used with success in well-established animal models of obesity due to their safety [276]. These treatments led to decreased weight gain and fat accumulation compared to the placebo group [277]. However, experimental studies differ in the duration of treatment (ranging from 4 weeks to 6 months) and the dosage of probiotics that were administered daily, leading to highly variable effects on body weight or fat mass [278]. Some studies using different species of lactobacilli and bifidobacteria have failed to demonstrate the beneficial effects of probiotic therapy in obese animals. Various strains of *Lp. plantarum*, *Lb. acidophilus* NCDC13, *Lb. gasseri* SBT2025, *Ls. casei* shirota, and *Loigolactobacillus coryniformis* CECT57 showed no significant effect on the weight of obese rats [278]. Bubnov et al. [279] reported that a combination of *B. animalis* VKB and *B. animalis* VKL did not have significant anti-obesity effects, although both probiotics administered alone reduced body weight in female BALB/C mice fed with a high-fat diet, suggesting potential interactions between food ingredients and certain probiotic strains [276].

Regarding the role of probiotics on newborn children from obese mothers, it was found that administration of *Ls. rhamnosus* GG 1 month before delivery to 6 months after led to less weight gain for the child up to 4 years post-partum [277]. Regarding the administration to subjects from different age groups, the supplementation with *Ligilactobacillus salivarius* ls-33 or with VSL#3®, in obese adolescents, was not able to reduce body weight, waist circumference, and visceral fat. However, VSL#3® showed a beneficial effect on body mass index (BMI), liver fat index, insulin resistance, and GLP-1 levels in obese children [280, 281]. In addition, Sanchis-Chordà et al. [282] showed that body weight was significantly reduced after administering *Bifidobacterium pseudocatenulatum CECT 7765* to obese children with insulin resistance.

Studies have shown that administration of *Lactobacillus curvatus* HY7601 and *Lp. plantarum* KY1032 [283], *Lb. acidophilus* LA-14, *Ls. casei* LC-11, *L. lactis* LL-23, *B. bifidum* BB-06, *B. lactis* BL-4 [284], and *Pediococcus pentosaceus* LP28 [285] strains led to a significant reduction in body weight, BMI, waist circumference, and fat mass in overweight human subjects. The administration of *Ls. rhamnosus* CGMCC1.3724 and a restricted-calorie diet caused significantly more significant weight loss in obese women than men [286]. The same results were not observed in a study with only obese women using supplementation of different doses of Ecologic® (*B. bifidum* W23, *B. lactis* W51, *B. lactis* W52, *Lb. acidophilus* W37, *Levilactobacillus brevis* W63, *Ls. casei* W56, *Lg. salivarius* W24, *L. lactis* W19, and *L. lactis* W58) [287]. These results have shown that probiotics are commonly used as adjuvants in treating diabetes and weight loss processes, besides their already-known effects on inflammation and immunomodulation. It highlights the importance of testing the effects of potential probiotics in different disease models.

Probiotics and Inflammation of the Bone-Muscular System

It is well known that the crosstalk between intestinal microbiota and host cells is critical in regulating many crucial biological processes. However, the link between allying intestinal microbiota and bone health remains elucidated. Current research suggests a complex relationship that demands further investigation to establish the exact mechanisms by which these microorganisms may modulate bone health [288]. In this context, the term "Osteomicrobiology" was introduced by [289] to refer to the research field on the role of microbiota in bone health and disease.

It has been shown that the imbalance in the communities of intestinal microorganisms (dysbiosis) directly contributes to the development of several bone inflammatory diseases and bone loss in general [290]. Sjögren et al. [291] showed that female germ-free mice presented an increase in bone mass associated with low osteoclasts in the trabecular bone. When colonizing germ-free females with intestinal microbiota of healthy animals, normalization in bone mass parameters, osteoclasts, and bone marrow immune status was observed, revealing intestinal microbiota's physiological importance to maintaining bone mass [291]. Thus, treatment with probiotic microorganisms can beneficially modulate the microbiota to improve general bone health, which several studies have corroborated.

The prominent bone inflammatory condition is osteoporosis, whose risk factors are highly associated with women's aging process and menopause [292]. This disease affects more than 200 million people worldwide. It is characterized by a reduction in bone mass, resulting in deterioration of bone microarchitecture due to a combination of causes: decreased absorption of calcium by the intestine, inactivation of vitamin D, osteoblast lifetime, and sex hormones [293]. Such factors resulted in increased bone fragility and a higher occurrence of fractures, a global concern due to the increasing aging population [293]. Current therapies for the prevention and treatment of osteoporosis comprise calcium and vitamin D supplementation. For high-risk patients, however, antiresorptive drugs are more often prescribed. Nevertheless, it has side effects, including gastrointestinal irritation, osteonecrosis of the jaw, and atypical subtrochanteric femoral fractures [294]. In this context, many studies revealed the role of probiotics as novel therapies in preventing and controlling postmenopausal osteoporosis models like the administration of *Lp. plantarum* A41 and *Lm. fermentum* SRK414 in ovariectomized rats [295], soymilk-honey fermented with *Ls. casei* subsp. *casei* R-68 and soymilk-honey fermented with *Lp. plantarum* 1 R 1.3.2 administered in menopausal women [296], *Ls. paracasei* DSM 13434, *Lp. plantarum* DSM 15312 and *Lp. plantarum* DSM 15313 in postmenopausal women [297], *Bacillus subtilis* C-3102 in postmenopausal women [298], *Lm. reuteri* 6475 in postmenopausal women [299], and GeriLact® (*Ls. casei*, *B. longum*, *Lb. acidophilus*, *Ls. rhamnosus*, *Lb. bulgaricus*, *B. breve*, and *S. thermophilus*) in postmenopausal women [300].

Probiotic *Bacillus clausii* (Enterogermina ®) was consumed orally as a suspension of 200 µL (10^9 CFU/mL daily) in drinking water for 6 weeks in female BALB/c mice after ovariectomy (which simulates postmenopausal osteoporosis conditions). In this study, treated animals showed a lower rate of bone resorption; increased bone volume, trabecular density, and bone mineral density (BMD); and reduced pro-inflammatory cytokines, proving *B. clausii* as an excellent therapeutic candidate [301]. Another report showed that *Lm. reuteri*, *Ls. casei*, and *B. coagulans* significantly increased serum vitamin D concentrations in ovariectomized rats [302]. Additionally, *Lb. acidophilus*, *Ls. casei*, and *Bifidobacterium* sp. significantly increased serum calcium compared to non-treated groups. *Lb. acidophilus* and *Ls. casei* indicated the most beneficial effects on BMD. Regarding bone marrow concentration and bone area, *Lb. acidophilus*, *Lm. reuteri*, and *Ls. casei* showed the most significant enhancement [303]. A recent meta-analysis study with 497 postmenopausal women showed that daily supplementation with probiotics for 24 weeks to 12 months was associated with decreased bone turnover marks compared to the placebo group. BMD loss at the lumbar spine was significantly lower in the probiotic group, while hips did not have a significant BMD difference [304].

Another clinically meaningful bone inflammatory condition is rheumatoid arthritis, a chronic autoimmune disease that damages bones and cartilage, leading to severe joint pain, disability, and premature death if not adequately treated [305]. The current therapies are focused on non-steroidal anti-inflammatory drugs, glucocorticoids, and disease-modifying antirheumatic drugs (methotrexate). However, even with improvement in arthritis treatment, the frequency and degree of responses are restricted, and some patients do not reach the treatment targets of clinical remission [306]. Due to these challenges, probiotic therapies are also pointed out as possible adjuvant or alternative therapies [307].

Ls. casei ATCC 334 was reported to promote an anti-inflammatory effect on collagen-induced arthritis in female Wistar rats due to Cox-2 and NF-κB downregulation [308]. Likewise, it was found to inhibit the increase of inflammatory markers like erythrocyte sedimentation rate, serum C-reactive protein, serum rheumatoid factor, and serum TNF-α [309]. Also, *Lp. plantarum* effectively exerted anti-arthritic activity in a model of complete Freund's adjuvant-induced arthritis in female Wistar rats.

A meta-analysis involving 361 patients showed that pro-inflammatory cytokine IL-6 was lower in those who received probiotics than placebo. However, there was no improvement in disease activity scores between probiotics and placebo groups [310]. Hence, there are signs of the beneficial effect of probiotics in treating human arthritis. However, more randomized controlled trials are still needed.

Regarding bone loss, studies showed that pretreatment with *Ls. casei* for 8 weeks before a surgical process for administration of CoCrMo to the cranium of C57BL/J6 mice (which promotes rapid osteolysis), followed by 2 more weeks of treatment, was able to reduce 40% in bone porosity and osteoclast formation, as well as activated M1 and M2 macrophages with an anti-inflammatory profile [311]. This same microorganism was tested in elderly patients who had suffered a fracture in the distal region of the radius. Those patients received daily supplementation with *Ls. casei*

Shirota for 6 months and were evaluated monthly according to pain level and limb function recovery. Results showed a significant reduction in pain level during the first 4 months of treatment and greater flexibility and strength in the wrist than in the placebo group, accelerating the fracture healing process [312]. In addition, *Lm. reuteri*, known for its immunomodulatory potential, improved bone health in female BALB/c mice submitted to a surgical incision on the back three times a week with probiotic administration. Probiotic consumption increases bone volume and trabecular density and reduces pro-inflammatory cytokines [313].

Several studies correlate improvement in bone condition and probiotic supplementation. However, mechanisms involving such benefits and signaling pathways in this complex interaction remain poorly studied. Therefore, metabolomics and proteomics studies and high-quality randomized controlled trials are essential for further clarifying this complex intestine-bone interaction.

Probiotics and the Central Nervous System

Psychobiotics refer to a specific group of probiotics that influence functions and behaviors of the CNS through the gut-brain axis (GBA). This communication occurs via various immune, humoral, neural, and metabolic pathways. The application of psychobiotics in both animal models and clinical trials improves gastrointestinal function and exhibits potential antidepressant and anxiolytic effects, opening a new frontier in neuroscience research [314].

Probiotics modulate the intestinal microbiota, increasing the diversity of microorganisms and the composition of beneficial bacteria, modulating the CNS via direct and indirect mechanisms [315]. Psychobiotics can modulate, in human and animal models, important neurotransmitters and proteins, such as gamma-aminobutyric acid (GABA), serotonin, glutamate, tryptophan metabolism, and brain-derived neurotrophic factor (BDNF) [316]. These substances control neural excitatory-inhibitory balance, mood, cognitive functions, learning, and memory processes [317–319]. These microbiologically synthesized neurotransmitters can cross the intestinal mucosa, acting indirectly on the enteric nervous system (ENS) [320, 321]. Much of the research on psychobiotics is conducted through animal studies, where stress is induced, and behavioral tests are performed on rodents to assess motivation, anxiety, and depression [320].

Certain species of lactobacilli and bifidobacteria, like *Lv. brevis*, *Bifidobacterium dentium*, and *Lp. plantarum*, have been found to produce GABA and serotonin, as well as *L. lactis* strains [322–325]. Specific lactobacilli species, such as *Lp. plantarum*, can also produce acetylcholine [326]. Recent studies have also demonstrated that microbes can regulate serotonin synthesis in the gut [314]. *Ls. rhamnosus* JB-1 has demonstrated the potential to reduce anxiety and depression. Its intake results in specific changes in GABA receptor expression within different brain regions and decreases plasma corticosterone levels [327]. According to Fasano et al. [334], *Lp. plantarum* PS128 activity in CNS functions in mice; increased locomotor activity; decreased anxiety, depression, and corticosteroid levels; and increased serotonin levels, with a dose of 10^9 CFU in 28-day treatment. Studies with *B. breve* and *Lm. fermentum* strains had an anxiolytic effect, reducing the anxiety behavior [328]. Similarly, the administration of a single strain, *B. longum* NCC3001, has shown effectiveness in treating anxiety. This strain also upregulates the expression of BDNF in the hippocampus [329]. Apart from promising results from animal studies, several research studies have also shown positive effects of probiotics on mental health in humans. In one study, healthy volunteers who received *B. longum* 1714 for 4 weeks experienced reduced stress levels and improved memory [330]. Furthermore, a randomized, double-blind, placebo-controlled trial investigated the effects of probiotic yogurt (containing *Lb. acidophilus* LA5 and *B. lactis* BB12) and probiotic capsules (comprising *Ls. casei*, *Lb. acidophilus*, *Ls. rhamnosus*, *Lb. bulgaricus*, *B. breve*, *B. longum*, and *S. thermophilus*) on petrochemical workers [331]. The participants who consumed both probiotic yogurt and probiotic capsules demonstrated improvements in mental health parameters, as assessed by the depression, anxiety, and stress scale (DASS) and the general health questionnaire [331].

Alzheimer's disease (AD) is a chronic neurodegenerative disorder that is characterized by cognitive and memory impairments. However, the evidence regarding the effects of probiotics in ameliorating cognitive disorders, including AD, is currently limited [314]. A study examined the impact of multiple probiotic strains, namely, *Lb. acidophilus*, *Lm. fermentum*, *B. lactis*, and *B. longum*, on an animal model of AD. After the probiotic intervention, there was an increase in the total counts of bifidobacteria and lactobacilli in the stool, while coliform counts decreased [332]. Moreover, the study found that the probiotic supplementation improved learning and memory deficits in AD rats compared to the control rats. Additionally, the Alzheimer-probiotic group showed reductions in the number of amyloid plaques. It decreased inflammation and oxidative stress, suggesting the potential therapeutic benefits of probiotics in mitigating certain aspects of AD [332]. In a study conducted by Mehrabadi and Sadr [333], it was demonstrated that treatment with probiotic strains *Lm. reuteri*, *Ls. rhamnosus*, and *B. infantis* at a dose of 10 billion CFU/day for 10 weeks showed beneficial effects in rat models of AD. The probiotic treatment was found to be effective in reducing inflammation and oxidative stress in these animal models of AD.

Parkinson's disease (PD) is a neuropsychiatric disorder that affects around 2% of the elderly population. Among the various nonmotor symptoms experienced by patients with

PD, constipation is a common issue [334]; in a randomized controlled study focusing on inflammation, insulin, and lipid-related genes in PBMCs from individuals with PD, a 12-week intervention with a probiotic supplement resulted in significant changes in gene expression. The subjects with PD who received the probiotic supplement showed downregulation of IL-1, IL-8, and TNF-a expression. At the same time, there was an upregulation of TGF-b and PPAR-g compared to the placebo control group [335]. In a study conducted by Hsieh et al. [336], it was reported that the consumption of a probiotic mixture containing *B. bifidum*, *B. longum*, *Ls. rhamnosus* GG, *L. lactis* subsp. *lactis*, and *Lp. plantarum* LP28 at a dose of 10 billion CFU/day for 16 weeks effectively protected dopamine-releasing neurons. This protection subsequently led to a reduction in the deterioration of motor dysfunctions in MitoPark PD mice. In another clinical study, the effects of fermented milk containing 6.5×10^9 of *Ls. casei* Shirota were assessed in PD patients over 5 weeks. The study reported that fermented milk consumption reduced bloating, decreased constipation, and reduced abdominal pain in PD patients [337].

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by challenges in social communication and interactions in various settings. These difficulties are often accompanied by repetitive and restricted patterns of behaviors, interests, and activities [338]. In a study conducted by Shaaban et al. [339], the beneficial effects of probiotics on behavioral and gastrointestinal manifestations of ASD were reported. Autistic children were treated with probiotic strains containing *Lb. acidophilus*, *Ls. rhamnosus*, and *B. longum* for 3 months. The treatment resulted in an increase in the population of bifidobacteria and lactobacilli levels in the gut. Additionally, the children showed weight reduction and improvement in gastrointestinal symptoms, indicating potential positive impacts of probiotics in managing symptoms related to ASD.

The balance on the intestine-brain axis can be maintained by metabolites derived from probiotic bacteria, which aid in the production of neurotransmitters and the maturation of the nervous system. Probiotic bacteria produce digestion and fermentation metabolites of nutritional components that affect the brain process and immune responses [340]; therefore, probiotics are crucial to maintaining the balance on the brain-gut axis [341]. These findings highlight the potential of specific probiotic strains to positively influence mental health and brain function.

Probiotic Applications on Skin-Related Pathogenies

Probiotics have demonstrated significant potential in the management of vaginal- and skin-related diseases, owing to their ability to modulate the local microbiota and exert beneficial effects on host health. In the realm of vaginal health, probiotics have been extensively studied for their ability to prevent and treat conditions such as bacterial vaginosis, vulvovaginal candidiasis, and urinary tract infections. Research suggests that specific strains of lactobacilli, notably *Ls. rhamnosus* and *Lm. reuteri*, can restore the balance of vaginal flora by inhibiting the growth of pathogenic microorganisms and promoting the production of antimicrobial [342, 343]. Moreover, a randomized, double-blind, placebo-controlled trial involving 64 healthy women found that oral supplementation with *Ls. rhamnosus* GR-1 and *Lm. fermentum* RC-14 significantly altered vaginal flora, supporting their potential therapeutic role [342].

Similarly, in dermatology, probiotics have emerged as a promising therapeutic option for various skin conditions, including acne, atopic dermatitis, and wound healing. Probiotic formulations applied topically or taken orally have been shown to modulate the skin microbiota, reduce inflammation, and enhance the skin barrier function [344, 345]. For instance, a randomized, double-blind, placebo-controlled study demonstrated that supplementation with *Ls. paracasei* NCC 2461 normalized skin expression of genes implicated in insulin signaling and improved adult acne symptoms [345]. Additionally, a randomized controlled trial involving infants with atopic dermatitis found that the administration of *Ls. rhamnosus* GG resulted in significant improvements in disease severity and reduced the need for topical corticosteroids [346].

Overall, the application of probiotics in vaginal- and skin-related diseases represents a promising area of research, with potential implications for the development of novel therapeutic strategies. Further clinical trials are warranted to elucidate the specific mechanisms of action and optimal strains and dosages for different conditions.

Probiotic Products

Probiotics are utilized not only in managing various health conditions but are marketed to consumers to enhance or preserve health, largely fueled by media coverage. Bacteria with alleged probiotic properties are now readily available as dairy products, juices, capsules, drops, powders, and functional foods [347, 348]. Probiotics have also been dehydrated and formulated as food supplements to cater to consumer convenience, improving the bacterial strains' shelf life. Probiotic supplement products require refrigeration to preserve the viability of the bacteria, with a minimum count of 10^7 CFU/g [349]. Probiotic products can be presented as a single strain or a combination of multiple strains. These multi-strain products offer the advantage of providing a broader range of health benefits compared to products with only one strain [350]. Additionally, the term multispecies describes products that contain strains from multiple genera, indicating a broader range of bacterial diversity within the

product [351]. Ensuring the viability of probiotic species is crucial when selecting strains for use, as they need to survive in the food product or capsule and throughout the digestive system while maintaining their original health-promoting effects [352]. Recent technological advancements have led to probiotic products being more stable at room temperature, maintaining many viable cells, resisting acidic conditions, and demonstrating resilience against bile in the small intestinal tract. However, there is currently a lack of information on commercial probiotic supplements regarding the survival of the stain(s) when exposed to the GIT passage [353].

An important factor to consider in the manufacturing of probiotic products is the presence of dead bacteria. Dead bacteria are inevitable in probiotic products and can originate from various manufacturing stages, such as harvesting, lyophilization (freeze-drying), and degradation processes [354]. These dead bacterial bodies accompany the live bacteria from the early manufacturing stages and cannot be eliminated from the final product. To ensure that the advertised number of live bacteria is maintained, manufacturers often "overfill" each sachet or capsule with excess bacteria, considering the inevitable loss of viability during storage. This practice compensates for the product's expected number of dead bacteria [355]. Current regulations for labeling probiotic products require informing consumers about the number of live bacteria expressed as CFU per dose [354]. However, this information does not consider the number of dead bacteria. Consequently, the CFU information does not accurately inform consumers about the "total number" of bacteria they ingest [355]. As a result, health professionals are unaware of this "hidden content" and the true potency of the product they administer to patients. It could harm the balance between anti- and pro-inflammatory cytokines and other cellular functions [355–357]. A well-defined study on the properties and effects of the dead probiotic strain or its fragments could aid in predicting if the excess of bacteria (live and dead) is going to represent a risk to the patient.

Role of Probiotic Matrix on the Probiotic Effect

One additional factor that contributes to the variability observed in the results of clinical studies, apart from variations in study populations, selection criteria, and study design, is the utilization of different production conditions, growth media, drying conditions, or cryoprotectants for the same bacterial strain, as well as the combination of a successful probiotic with other bacteria or strains [358].

For instance, research has demonstrated that the adhesion properties of the *Ls. rhamnosus* GG strain (ATCC 53103) are contingent upon the composition of the growth media and the number of starter culture transfers [359]. Furthermore, when *Ls. rhamnosus* GG was combined with *Ls. rhamnosus* LC705, *B. breve* Bb99, and *P. freudenreichii* ssp. *shermanii*, no significant clinical or immunological effects were observed [360]. Likewise, as early as 1983, it was found that the clinical outcome of *Lb. acidophilus* varied depending on the specific production lot [361].

Various factors, including fermentation, matrix composition, cell harvesting, spray-drying, freeze-drying, and storage conditions such as temperature, humidity, and pH, play significant roles in determining the microorganisms' viability, growth, and survival. Ultimately, these factors can influence the outcomes of research studies and clinical trials involving probiotics [358, 362, 363].

Lb. delbrueckii CIDCA 133, for example, showed different results in an animal model of 5-FU-induced mucositis when administered with fermented milk [231] or fermented MRS medium [364]. *Lb. delbrueckii* CIDCA133 fermented milk at a dosage of 10^7 CFU/mL effectively protected the intestinal mucosa from damage caused by 5-FU, better than the fermented MRS administration. Fermented milk presented better results at reducing intestinal neutrophil infiltration, protecting against weight loss, and protecting the intestinal epithelial architecture, including preservation of villus and crypts, more effectively. Although both treatments reduced the effects promoted by the 5-FU administration, these experiments clarified the role of the matrix on probiotic activity.

De Filippis et al. [368] tested the effect of *P. freudenreichii* CIRM-BIA129 on a DSS-induced colitis model in mice with three different fermented matrices: milk ultrafiltration permeate, skim milk, and whole milk. The work showed that the increase in protein and fat in the fermented matrix positively influenced the anti-inflammatory effect of *P. freudenreichii*, with fermented whole milk obtaining the best results. This shows that the production of fermented functional foods should consider bacterial fermentation, the matrix's various components, and the food's structure. This highlights the importance of considering the interactions between the microorganisms, the food matrix, and the overall food structure to develop effective and beneficial fermented functional products.

Regulation and Safety of Probiotic Products

When using probiotics as a treatment, it is essential to consider safety due to the potential consumption of a substantial quantity of bacteria. Safety considerations encompass two aspects. Firstly, it involves determining the adverse effect profile of specific mono- and multi-strain preparations to assess the products' safety. Secondly, it ensures that marketed probiotic preparations adhere to rigorous quality standards. This ensures that the product's correct strains of bacteria are present and contamination-free [77]. These measures are in place to safeguard the well-being of individuals using probiotic treatments.

The relatively unregulated nature of the probiotic market allows for the transferal of claims from tested products to

others that may have notable differences in formulation or manufacturing processes. This practice gives rise to numerous problems and questions. Furthermore, when probiotic formulations are utilized to manage significant conditions like IBD or disorders associated with immunosuppression, such as human immunodeficiency virus, the lack of strict regulation can have severe consequences for patients [354].

Notably, most commercially available probiotics are derived from fermented foods with a long history of safe consumption or microbes that naturally colonize healthy individuals [365]. EFSA considers all common probiotic species safe for the general population [366]. However, this definition does not offer specific guidance for the increasing use of probiotics in individuals with medical conditions. It is important to mention that EFSA is cautious in accepting the term "probiotic," although health authorities tolerate it in certain countries like Italy. In the USA, the FDA evaluates and classifies probiotics individually, but many have been classified as safe for food products [367]. Regulations concerning NGPs remain inadequate and vary among different countries. In Europe, any microorganisms not used in foods before 1997 must undergo a thorough evaluation by the EFSA [49] before being approved for the market, whether they are intended as novel foods or drugs [368]. As mentioned, the FDA Center for Biologic Evaluation and Research (CBER) defines NGPs as LBPs. This category presents a promising opportunity for novel microorganisms extracted from the microbiota. However, they require meticulous characterization of any microorganism falling under this category, akin to the standards demanded for vaccines [369]. Although the pathway for human research on LBPs is well-defined, no known examples have completed this process, and the Investigational New Drug (IND) process must be followed [369]. It is worth noting that in the past, the FDA classified almost all probiotic research as drug research.

Most clinical trials investigating probiotics have not raised significant safety concerns [68]. However, several isolated cases of serious adverse effects have been documented independently of the formulation, dosage, and daily intake. These adverse effects include instances of bacterial sepsis associated with lactobacilli-containing probiotic supplements and the death of a preterm infant due to gastrointestinal mucormycosis, a severe and rare fungal infection resulting from a category of molds known as mucormycetes, which was linked to mold contamination in a probiotic supplement [77, 370]. Furthermore, in patients with predicted severe acute pancreatitis, treatment with a multispecies probiotic preparation was associated with an elevated risk of mortality [371]. Therefore, a thorough safety evaluation is necessary before the use of probiotics in vulnerable populations, including individuals with compromised intestinal mucosa or immune dysregulation, as seen in patients with IBD, liver diseases, HIV, and other conditions [68]. Safety concerns become even more crucial when dealing with a few products that contain high concentrations of probiotic bacteria, ranging from 450 to 900 billion bacteria per dose. The yeast *S. boulardii*, a natural yeast found in some probiotic formulations, has been associated with fungemia in critically ill patients and immunocompromised individuals [372, 373].

The accuracy of labeling for commercial probiotic products currently on the market is not always reliable. It has been observed that some microorganisms claimed to be present in these products may be absent, or their quantities may be lower than what is stated on the label. In a study conducted by Weese [374], deficiencies were identified in the labels of numerous Canadian commercial probiotics intended for oral consumption. Specifically, 43% of the analyzed products had improperly identified bacteria, and 25% of the products had misspelled content [374]. Similarly, Toscano et al. conducted a quality assessment of the leading probiotic products available in the Italian market in 2011 [375] and obtained results like those of the study by Weese [374]. The Italian research observed that 42% of the analyzed products did not contain the declared number of bacteria for at least one labeled strain. Additionally, 17% of the products showed no viable microorganisms, and 8% were contaminated with *E. faecium* [375]. The presence of an undisclosed microorganism, which may potentially possess pathogenic traits, poses a significant risk to the host's health. These studies underscore the necessity for specific legislation that mandates accurate identification and characterization of probiotic strains in commercial products and thorough testing of all available products.

Ensuring the stability of strain characteristics in the final product is essential to produce functional probiotic foods. This becomes particularly important when treating young infants with compromised gut barrier function, abnormal gut microbiota, and increased sensitivity to dietary substances [358]. It is increasingly recognized that the existing regulatory approach is insufficient and can give rise to issues related to quality, safety, and the validity of claims in commercial probiotic products used in medical contexts, including products used in vulnerable populations. A regulatory void must be addressed to ensure appropriate oversight and regulation of probiotic products.

Final Considerations and Future Perspectives

This review explores the current state of probiotic research and development, from the initial characterization of potential probiotic strains to clinical applications and commercial product considerations. Identifying and screening new probiotic candidates involves a combination of traditional phenotypic assays and advanced genomic analysis. Genomic analysis provides valuable insights into the genetic factors related to functionality and safety, including the absence of pathogenicity factors and

antibiotic resistance genes. Indeed, the main criteria for selecting probiotic microorganisms in many studies include their tolerance to acid and bile and their adhesion ability, among others. However, the variation in experimental conditions (in vitro), such as the types of bile, adhesion test methods, medium composition, pH, and duration of the tests, hinders the overall comparison of results. Standardization of testing methods and conditions and support from genomic data are essential for meaningful comparisons and reliable conclusions regarding probiotic characteristics. This allows for a better understanding of probiotic strains' potential benefits and functionality and facilitates the selection of appropriate candidates for further research and application. These tests serve as predictive measures of a strain's ability to survive the journey through the GIT and exert beneficial effects. While animal models are useful for preliminary screening, they have limitations in accurately replicating human physiology and clinical outcomes. Therefore, well-designed human trials are crucial to demonstrate the health benefits of probiotic strains in a disease-specific and often strain-specific manner. Probiotics have shown promising results in animal models and clinical trials in mitigating various conditions, including infectious diarrhea, IBD, mucositis, metabolic disorders, musculoskeletal inflammation, and psychiatric conditions.

However, translating probiotics from the laboratory to the market encounters several challenges. Manufacturing processes significantly impact probiotic products' bacterial growth, viability, and functional properties. The composition of the product matrix and supplementation with prebiotics or other bacterial strains can further modify the effects of the primary probiotic strains. Currently, there is a lack of regulatory oversight in the probiotic market, leading to product quality, safety, and label accuracy issues. Improvement in manufacturing practices, labeling requirements, and regulation is necessary to ensure consumer safety and confidence, particularly for vulnerable populations relying on probiotic products.

Advances in probiogenomics and multi-omics approaches will expand mechanistic knowledge and allow for predictive modeling to select novel probiotics for specific health goals rationally. Relevant in vitro and animal models that better represent human intestinal conditions will improve clinical predictability. Elucidating the bioactive molecules derived from probiotics and their impact on cellular signaling is critical to developing "postbiotic" therapies beyond live cells. With greater personalization on the horizon, combinations tailored to an individual's microbiome, genetics, and health status may provide more significant benefits than broad spectrum probiotic products currently dominating the market. Overall, exciting innovations in probiotic research and application are promising to revolutionize therapeutic approaches for diverse conditions and improve public health. However, these require parallel efforts to improve quality standards, manufacturing practices, and regulatory oversight of probiotic products to ensure safety and efficacy.

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Declarations

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