In Silico Identification of Alternative Molecular Targets from Probiotic Strains for Potential Therapeutic Repositioning in Gut Microbiome-Associated Diseases

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Master's Thesis

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Chapter 1

1.1. Abstract.

Human gut hosts over 100 trillion symbiotic bacteria, far exceeding the number of host cells. These microorganisms collectively form what is known as the gut microbiota. This microbiota performs a wide range of functions crucial for the human body, including protection against pathogens, nutrient extraction, metabolism, and immunity, which, under healthy conditions, result in stability, resilience, and beneficial symbiotic interactions. A probiotic microorganism is defined as a live microorganism that confers a health benefit to the host when administered in adequate amounts. Consequently, in recent years, the number of studies linking probiotic strains to prevent and treat several diseases, such as autoimmune diseases, atopy, metabolic syndrome, metabolic disorders, cancer, and certain behavioral disorders, has increased significantly. Similarly, more research is emerging that employs omics sciences, which involves obtaining comprehensive data that includes genomic, proteomic, metabolic, and other omics information, aiming to assess this data before and after probiotic treatment administration.

In this context, and thanks to open access data and cooperative Omics bioinformatic tools, this project proposes an *in-silico* approach to analyze the effects of probiotic strains on human cells, focusing on differentially expressed genes and their protein-protein interactions. The results highlight the ability of probiotics, such as *Propionibacterium freudenreichii* ITG P9 and *Bacillus subtilis* CW14, to modulate human cellular responses, particularly in pathways related to immunity and the cell cycle. This study emphasizes the role of probiotics in regulating genes associated with metabolic, neurological, and autoimmune diseases, revealing potential neuroprotective and antitumoral properties.

1.2. Background.

The human body is home to diverse microbiotas, which play critical roles in health and disease **(Hou et al., 2022)**. The oral microbiota, composed of complex microbial communities, supports both oral and systemic health, with its imbalance being linked to conditions such as periodontitis and cardiovascular diseases **(Santacroce et al., 2023)**. The urogenital microbiota, particularly in women, is dominated by Lactobacillus species, which regulate vaginal pH and protect against infections; disruptions are key factors in bacterial vaginosis and urinary tract infections **(Chee et al., 2020)**. The skin microbiota is located on the skin's surface, serves as a barrier against pathogens, and modulates immune responses; its imbalance is associated with conditions like atopic dermatitis and acne **(Wu & Yao, 2024)**. Lastly, the gut microbiota, one of the densest and most dynamic microbial communities **(Ley et al., 2006)**, plays a central role in digestion, immune regulation, and the synthesis of essential metabolites, significantly impacting overall health **(Yang & Cong, 2021)**. Despite their distinct anatomical locations and functional characteristics, these microbiotas are interconnected, underscoring the significance of maintaining their balance in the context of disease prevention and overall well-being **(Martínez et al., 2021)**.

1.3. The Gut Microbiota.

The human body hosts a variety of microorganisms that can be classified as commensal, mutualistic, or pathogenic. Commensal bacteria are microorganisms that live on or within the human body without causing harm, often contributing to physiological processes such as digestion and immune modulation **(Tarapatzi et al., 2022)**. The balanced relationship among these microorganisms gives rise to the concept currently defined as microbiota **(del Campo-Moreno et al., 2018)**. The gut microbiota is primarily divided into three groups: probiotics, a live microorganisms that, when administered in adequate amounts, confer a health benefit on the host **(Hill et al., 2014)**, such as *Bifidobacterium* and *Lactobacillus*; pathogenic bacteria, which can cause various diseases when their growth becomes uncontrolled, such as

Fusobacterium nucleatum, *Helicobacter pylori* **(Varela-Trinidad et al., 2022)**, *Vibrionaceae* **(Pérez-Reytor et al., 2018)** *Enterobacterales* **(González, 2022)** and *Clostridium perfringens* **(Kiu & Hall, 2018)**; and finally, opportunistic pathogens (mainly gram-positive bacteria), which are typically beneficial to health but can cause disease under certain conditions. In this context, one of the most complex and densely populated microbial communities in the human body is the microbiota found in the gastrointestinal mucosa, which plays a fundamental role in human health **(Holscher, 2017)**.

1.4. The impact of intestinal microbiota on human health.

The gut microbiota is a complex ecosystem comprising trillions of bacteria, fungi, viruses, and archaea **(Ruigrok et al., 2023)**. Collectively, the gut microbiota may contain approximately 150 times more genes than the entire human genome. Some of these genes may possess a greater degree of versatility than those found in humans. These genes can provide the body with unique enzymes and biochemical pathways from which the body can benefit. For example, these genes can increase the body's extraction of energy from food and optimize the acquisition of nutrients, such as some non-digestible carbohydrates **(Wang et al., 2017)**. Furthermore, the intestinal microbiota functions as a physical barrier against pathogens by producing antimicrobial substances and through a process of competitive exclusion **(Afzaal et al., 2022)**. It has been established that the intestinal microbiota plays a fundamental role in human health, the development of the intestinal mucosa and the immune system. In the context of healthy conditions, the intestinal microbiota fosters stability, resilience, and beneficial interactions for humans **(Clemente et al., 2012)**.

In recent years, there has been a notable increase in the number of studies addressing the role of these intestinal communities in various physiological processes, including lipid and protein homeostasis, vitamin synthesis, protection against pathogens, and immunity **(Belkaid & Hand, 2014)**. In this sense, the intestinal microbiota is responsible for the production of

short-chain fatty acids, including butyric, propionic, and acetic acids. These acids, upon absorption by the colon, have been shown to play a crucial role in regulating intestinal motility, inflammation, glucose homeostasis, and energy production **(Koh et al., 2016)**. Evidence has demonstrated that the intestinal microbiota plays a pivotal role in supplying humans with essential vitamins, including folates, vitamin K, biotin, riboflavin, cobalamin, and other B complex vitamins **(Wang et al., 2017)**. However, an imbalance or alteration in the composition of the human microbiota, known as microbial dysbiosis, has been demonstrated to be a causative agent for the development of several pathologies **(Petersen & Round, 2014)**. This microbial dysbiosis can be caused by a variety of factors, including alterations in diet, the use of antibiotics, stress, and specific disease conditions, among others **(Petersen & Round, 2014)**. The following list contains some examples of diseases associated with microbial dysbiosis: autoimmune diseases, cancer, obesity, inflammatory bowel disease, and metabolic and mental disorders **(Degruttola et al., 2016)**; reinforcing the close relationship between gut microbiota and human health **(Clemente et al., 2012)**.

1.5. Probiotics as co-adjuvants.

Probiotics are defined as a culture of bacteria that, when administered to humans or animals in specific doses, have a beneficial effect on the intestinal microbiota **(Hill et al., 2014)**. The human intestine harbors over 100 billion symbiotic bacteria, surpassing the number of host cells and forming the intestinal flora **(Lu et al., 2021)**. These probiotics provide protection against pathogens, stimulate the immune system, and modulate gastrointestinal hormones within the gut-brain axis **(Hemarajata & Versalovic, 2013; Ancona et al., 2021)**. They also regulate acute and chronic inflammation in intestinal mucosal tissue **(George Kerry et al., 2018)**.

Recent research highlights the ability of probiotics to modify microbiota composition, either by introducing or excluding microorganisms, to prevent and treat conditions such as irritable

bowel syndrome, colorectal cancer, eczema, and diabetes **(Sanders et al., 2019; Cani & Delzenne, 2009)**. Probiotics can stabilize the intestinal epithelial barrier and inhibit pathogens, while promoting microbial diversity and beneficial flora composition **(Lu et al., 2021)**. Specific mechanisms include immune modulation through pathways like nuclear factor kappa B (*NF-κB*), mitogen-activated protein kinases (*MAPK*), and interferon regulatory factors (IRF), triggered by probiotic interactions with innate pattern recognition receptors (PRRs) **(M. S. Lee & Kim, 2007;Trejo & Sanz, 2013)**.

Additionally, bacterial metabolites, such as butyrate produced by specific Firmicutes, play protective roles by modulating inflammation, epithelial proliferation, and apoptosis **(P. V Chang et al., 2014)**. Studies in animal models have demonstrated the potential of specific strains like Lactobacillus acidophilus and Lactobacillus plantarum in preventing colorectal cancer **(J.-H. Chang et al., 2012; H. A. Lee et al., 2015)**. Gram-positive bacteria, such as lactobacilli, have been shown to increase *TLR2*-positive cells and enhance immune responses, whereas Gram-negative bacteria induce *TLR4*-positive cells and cytokine production, demonstrating strain-specific immune interactions **(Grangette et al., 2005)**. This growing body of evidence emphasizes the need to study probiotic intake alongside dietary substrates to understand their impact on microbiota, metabolic pathways, and metabolite production, which collectively contribute to disease prevention and health promotion **(Kumari et al., 2021).**

1.6. Cell lines.

1.6.1. Caco-2.

Caco-2 cells, which are derived from human colon adenocarcinoma, play a critical role in the production and uptake of cytokines and chemokines. These molecules regulate epithelial activity by influencing various cellular processes, including cell proliferation, migration, and survival **(Francescone et al., 2015)**. Intestinal endothelial cells have also been shown to

contribute to the immune response. *In vitro*, these cells have been observed to differentiate into M-like cells that are capable of absorbing, transporting, and presenting antigens to subepithelial lymphoid cells. Additionally, these cells express Major Histocompatibility Complex II (MHC-II) **(Jung et al., 1995; Simon-Assmann et al., 2007)**.

The Caco-2 cell line, which has been the focus of extensive research and characterization, exhibits characteristics of mature enterocytes, including the functionality of an intestinal barrier **(Lehto & Salminen, 1997)**. These cells are fundamental to probiotic research, as they facilitate the study of antimicrobial activity and the adhesion mechanisms of non-pathogenic microorganisms **(Jacobsen et al., 1999)**. Furthermore, Caco-2 cells exhibit responsiveness to a variety of stimulus, manifesting the expression of pro-inflammatory cytokines, including *TNF-α*, *IL-1*, *IL-6*, and *IL-8* **(Morita et al., 2002)**, and anti-inflammatory cytokines like transforming growth factor β (*TGF-β*) **(Jung et al., 1995)**. They also produce complement proteins, including C3, C4, and factor B **(Jung et al., 1995)**. These characteristics make Caco-2 cells a powerful model for investigating the molecular and cellular mechanisms underlying immune regulation in the intestinal epithelium.

1.6.2. HT-29.

The HT29 cell line, which was derived from a human colon adenocarcinoma, was first isolated in 1964 from a tumor in a 44-year-old Caucasian female patient **(Fogh & Trempe, 1975)** The HT29 cell line was initially utilized for the study of cancer biology. These cells have been observed to exhibit traits of mature intestinal cells, including enterocytes and mucus-producing cells **(Fogh & Trempe, 1975)**.

As an in vitro intestinal model, HT29 cells have been shown to offer certain advantages and face certain limitations. In their differentiated state, these cells bear a resemblance to small intestine enterocytes, exhibiting brush border hydrolases and a differentiation process

analogous to that observed in the intestinal tract **(Zweibaum et al., 1991).** It has been demonstrated that differentiated HT29 cells express receptors for peptides, including vasoactive intestinal peptides and insulin, as well as non-peptidic substances. Most of these receptors are analogous to those found in normal intestinal cells; however, the neurotensin receptor, which is present in HT29 cells but absent in normal colon epithelium, constitutes an exception to this rule **(Kitabgi et al., 1980)**. Additionally, opioid, serotonin, muscarinic, and *PPARβ/δ* receptors have been identified **(Ataee et al., 2010; Belo et al., 2011; Foreman et al., 2011; Zoghbi et al., 2006)**. For a comprehensive review, see **Zweibaum et al. (2011)**.

1.7. Using omics data to improve probiotic therapies.

Research on the response to probiotic treatment using omics approaches entails the acquisition of comprehensive data, encompassing genomic, transcriptomic, proteomic, metabolomic, and/or other omics information. The objective of these approaches is to evaluate the data obtained prior to and following probiotic administration. Most of these data are obtained through the application of transcriptomics, a field of study focused on the analysis of gene expression. Specifically, transcriptomics utilizes messenger RNA (mRNA) sequencing, a technique that detects alterations in gene expression that arise because of probiotic treatment **(Conesa et al., 2016)**. Furthermore, these studies can be complemented with analyses of the microbiome communities by metagenomics or metataxonomics to assess changes in the composition of the intestinal microbiota.

Next-generation sequencing has been influential in reducing the costs associated with sequencing and has helped scientists obtain whole genome sequences **(Rubio et al., 2020)**. This approach becomes particularly relevant in the context of probiotics, where precision strain identification contributes directly to understanding their impact on health **(Binda et al., 2020)**. In recent years, there has been a significant increase in the production of data through omics technologies, with both biomedical and industrial applications **(Vitorino,**

2024). The substantial volume of study data, accessible in publicly available databases, has prompted the adoption of the term "omics data mining," as it facilitates the acquisition of additional information beyond that of the original study. In data processing, there are multiple methods to achieve the expected results, and the choice of each step will have a considerable influence on the approach and validity of the results obtained. This range of influence extends from the selection of the type of library and the handling of the raw data to the use of specific programs for the identification of response variables of interest in these data sets, such as differentially expressed genes in these studies with probiotic treatments **(Eduardo et al., 2014)**.

1.8. The repositioning of probiotic strains is a scientific endeavor aimed at elucidating their functionality in a variety of health conditions.

Drug repositioning (DR) is defined as the identification of new therapeutic targets from already known molecules or active ingredients **(Luo et al., 2021)**. In this context, the employment of probiotic strains has emerged as a promising strategy to address current health challenges, leveraging the omics information available in databases. This approach entails the strategic repositioning of probiotic strains, which may result in the identification of novel therapeutic applications for probiotic strains or their metabolites. These substances possess well-documented health benefits and established safety profiles, which have the potential to reduce treatment costs and expedite their implementation in novel therapeutic interventions **(Zmora et al., 2019)**.

The primary challenge in implementing probiotic strains is ensuring their safety and efficacy. Probiotic strains are often associated with applications, and it is essential to comprehensively understand their safety and effectiveness profiles in diverse health contexts **(Sanders et al., 2019)**. The utilization of extensive omics datasets and *in silico* computational methodologies has become imperative in the identification and selection of probiotic strains that demonstrate

therapeutic potential **(Rodríguez Gómez, 2017)**. The employment of transcriptomic data derived from cell lines originating from conditions that have been treated with probiotic strains enables the employment of computational identification algorithms. These algorithms facilitate the detection of genes that exhibit altered expression levels in response to the presence of and interaction with these probiotic strains. The identification of these genes has the potential to reveal novel therapeutic avenues for a range of health conditions **(Dayama et al., 2020)**.

1.9. A methodical search of data sets (datasets).

1.9.1. Selection criteria and search strategy.

A comprehensive collection of studies was meticulously sourced from the Scopus and PubMed databases. This was achieved by employing bespoke scripts, meticulously crafted in the Python programming language. These scripts are available in the following repository and adhere strictly to the PRISMA methodology guidelines. This facilitated the identification of several studies deemed suitable for the execution of the proposal. Figure 1 presents a flow chart outlining the strategy employed for selecting pertinent articles.

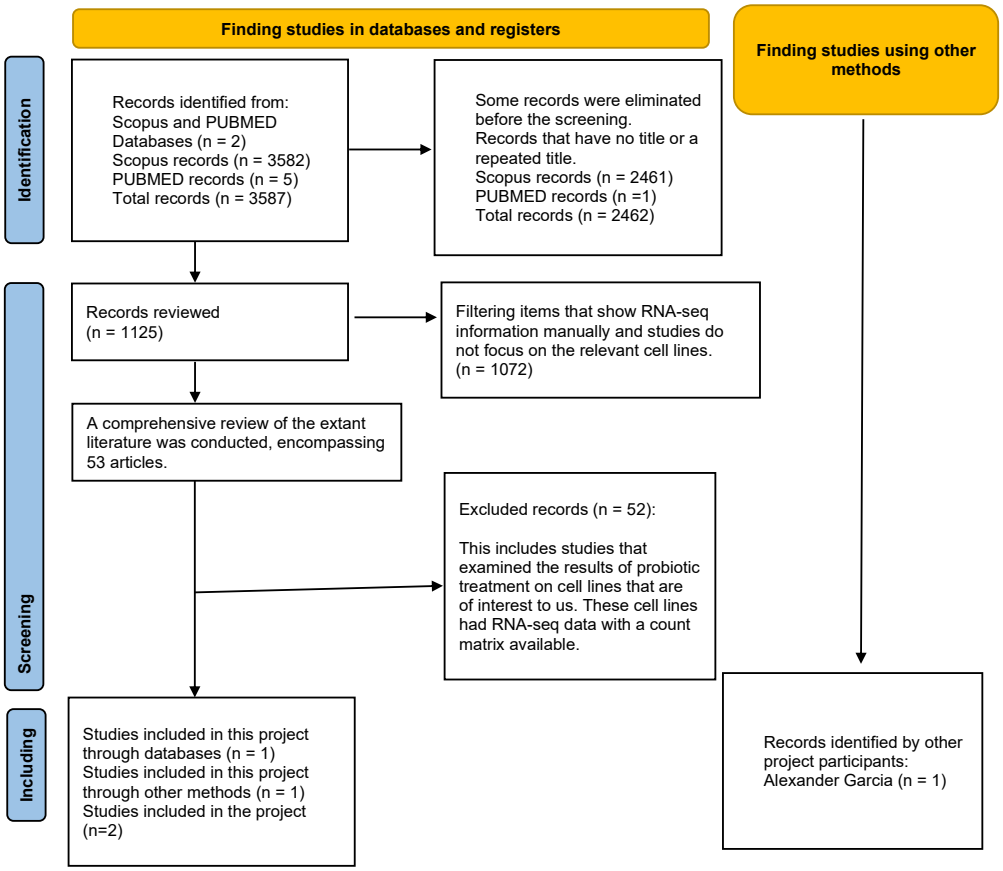

Figure 1. Flow chart showing the process of abstracting the studies of interest. A comprehensive literature search yielded a total of 3,587 references. After removing duplicates and records deemed null, 2,462 records were eliminated. The subsequent screening stage entailed the filtration of records that did not pertain to the cell lines of interest or that lacked ribonucleic acid sequencing (RNA-seq) data, resulting in the retention of 53 records. In the subsequent analysis, 52 articles were excluded from consideration due to their failure to analyze results before and after probiotic treatment of the pertinent cell lines, as indicated by the reporting of their respective RNA-seq data with the count matrix available. Subsequently, the results of the database search were integrated with findings from other sources that were consulted. Finally, Professor Alexander Garcia, who is also part of the project as an advisor, included the fourth article **(Sl. No. 2 in Table 1)**.

Sl. No.	Title	Cellular Line	Reference	Probiotic or Microbial Treatment
1	The probiotic *Propionibacterium freudenreichii* as a new adjuvant for TRAIL-based therapy in colorectal cancer.	HT29	**(Cousin et al., 2016)**	*Propionibacterium freudenreichii* ITG P9
2	Probiotic *Bacillus subtilis CW14* reduces disruption of the epithelial barrier and toxicity of ochratoxin A to Caco-2 cells	Caco-2	**(Peng et al., 2019)**	*Bacillus subtilis* CW14

Table 1. Selected articles for the probiotic repositioning study. Considering the points mentioned earlier, the objective of this project is to implement an in-silico approach for the repositioning of probiotic strains, to identify molecular targets associated with various health conditions. The objective is to broaden the spectrum of available therapies based on probiotic cells or their derived metabolites.

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Chapter 2

2.1. Abstract.

The gut microbiota comprises more than 100 trillion microorganisms and plays critical roles in immunity, metabolism, and homeostasis. Its imbalance (dysbiosis) has been associated with gastrointestinal, metabolic, autoimmune, and neurological disorders. On the other hand, probiotics are live microorganisms with beneficial effects that have emerged as a promising therapeutic strategy. In this study, the impact of the probiotics Bacillus subtilis CW14 and Propionibacterium freudenreichii ITG P9 on intestinal epithelial cells from the Caco-2 and HT-29 cell lines, respectively, was analyzed. Using an integrative approach based on bioinformatics tools, differentially expressed genes and protein-protein interactions (PPIs) were examined to establish the impact of these probiotics on gene modulation and their relationship with various human pathologies. The results showed specific effects for each probiotic. It was observed that B. subtilis CW14 primarily modulates a coordinated and controlled immune response involving chemokines and inflammatory factors, while P. freudenreichii ITG P9 elicited a transcriptional response characterized by the modulation of genes associated with cell cycle control and stress. The pleiotropic effect of both probiotics on genes linked to metabolic, neurological, and autoimmune diseases was established, with many cases involving the regulation of genes with immunomodulatory, neuroprotective, or antitumor properties. Furthermore, key molecular mechanisms related to immunomodulation emerged from the results, including innate receptors such as TLR and NOD, and signaling pathways like NF-κB and MAPK, reinforcing the importance of the gut-brain axis connection.

2.2. Introduction.

The human gut harbors a complex ecosystem of more than 100 trillion symbiotic microorganisms—a figure that far exceeds the number of host cells **(Dekaboruah et al., 2020)**. The intestinal tract comprises the largest immune system in the body, interacting with antigens and mechanisms of both the immune system and the central nervous system (CNS) **(Pelaseyed et al., 2014)**. Approximately 70% of the immune system is generated in the gut **(Ygberg & Nilsson, 2012)**. This ecosystem, known as the gut microbiota, plays a fundamental role in human health by participating in essential biological processes such as nutrient extraction, metabolism, vitamin synthesis, and immune system regulation **(Bouskra et al., 2008; Hou et al., 2022)**. Under balanced conditions, the gut microbiota contributes to stability, resilience, and a beneficial symbiosis for the host by acting as an additional organ, preventing the uncontrolled absorption of toxic compounds or pathogens, as well as regulating the immune response **(Hou et al., 2022; Hashemi et al., 2023)**. However, dysbiosis, or the disruption of this balance, has been associated with a wide range of diseases, from gastrointestinal disorders to metabolic, autoimmune, and neurological conditions **(Richard & Sokol, 2019).**

In this context, the modulation of the gut microbiota using probiotics has emerged as a promising strategy for the prevention and treatment of various pathologies **(Cremon et al., 2018; Sanders et al., 2019)**. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits and have been shown to protect against pathogens, inhibit the colonization of harmful bacteria, strengthen the intestinal barrier, and modulate the immune response **(Ley et al., 2006; Richard & Sokol, 2019)**. Among the most widely used probiotics are lactic acid bacteria (LAB), considered GRAS (Generally Recognized as Safe) due to their well-established safety profile **(McFarland et al., 2018)**. Some of the most studied strains include *Propionibacterium freudenreichii*, *Lactobacillus subtilis*, *Lactobacillus acidophilus*, *Lacticaseibacillus casei*,

Limosilactobacillus reuteri, *Lactiplantibacillus plantarum*, *Bifidobacterium brevis*, *Streptococcus salivaris subsp*. *thermophilus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Lactococcus*, and *Escherichia coli Nissle* 1917, among others **(McFarland et al., 2018)**.

Thus, modifying intestinal microbial communities—either through the inclusion or exclusion of specific microorganisms—can help prevent the development of various diseases (**Cani & Delzenne, 2009)**. This phenomenon is closely related to the colonization of the intestinal tract by the microbiota, which can trigger immune responses mediated by the recognition of microbial signals through innate receptors **(Trejo & Sanz, 2013; Cerdó et al., 2019)**. These receptors, in turn, modulate the function of intestinal immune cells, influencing immune homeostasis and the inflammatory response **(Lee & Kim, 2007; Zmora et al., 2019)**. Therefore, identifying and characterizing the effect of a specific probiotic on the expression and modulation of genes associated with different pathologies is essential for understanding the underlying mechanisms common to these diseases, in order to develop more precise therapeutic interventions.

In this study, a bioinformatic analysis was conducted based on transcriptomic data obtained from the Gene Expression Omnibus (GEO) database **(Clough & Barrett, 2016)** from Caco-2 cell lines treated with the probiotic *Bacillus subtilis* CW14 and HT-29 cells treated with *Propionibacterium freudenreichii* ITG P9. The aim was to identify differentially expressed genes (DEGs) and explore their protein-protein interactions (PPIs). These interactions were analyzed in a relevant biological context, focusing on two main axes: **1)** the relationship with proteins associated with human diseases, and **2)** the identification of possible gene modulation mechanisms linked to immune and physiological responses. This comprehensive approach will advance our understanding of how probiotics can modulate gene networks associated with human diseases, opening new avenues for the development of gut microbiota-based therapies.

2.3. Materials and methods.

2.3.1. Data acquisition and differential expression analysis.

To obtain differential gene expression data in the context of the effects of probiotics on colon cells, an exhaustive search was performed in the GEO database **(Clough & Barrett, 2016)**. A series of keywords detailed in the supplementary material, was used to filter the pertinent studies. From the obtained results, two studies meeting the following inclusion criteria were selected: **1)** the use of colon adenocarcinoma cells as the experimental model, **2)** evaluation of the effect in the colon, and **3)** probiotic-based treatments. The selected data include: Caco-2 cells treated with *B. subtilis* CW14 (GSE115081) **(Peng et al., 2019)** and HT-29 cells treated with *P. freudenreichii* ITG P9 (GSE67033) **(Cousin et al., 2016)**.

A principal component analysis (PCA) was conducted to discriminate between the different groups and their respective controls using the package from **Love et al. (2014)**. Differential expression analyses were performed using the DESeq2 package (version 1.38.1) **(Love et al., 2014)** to normalize the expression counts for each experiment. A log base 2 transformation of the fold change (log2FC, with LFC ≥ 2 and an FDR value ≤ 0.05) was applied to interpret the results, thereby allowing for the identification of differentially expressed genes (DEGs). For better integration of the results, the names of the DEGs were converted to Entrez IDs using the Ensembl **(Harrison et al., 2024)** and UniProt **(Consortium et al., 2025)** databases via specific APIs. These identifiers facilitated querying and cross-referencing across various databases.

2.3.2. Functional enrichment and pathway analysis.

The functional analysis of gene ontology (GO) was performed using Enrichr **(Chen et al., 2013)**, focusing on the GO Biological Process 2023. The identification of biological pathways associated with the DEGs was carried out using the KEGG 2021 Human **(Ogata**

et al., 1999) and Elsevier Pathway Collection databases **(Nesterova et al., 2019)**. These resources enabled the mapping of DEGs to metabolic and signaling pathways, providing a broad biological context for the alterations induced by the probiotics in colon cells. The thresholds established for the enrichment analyses were Padj ≤ 0.05.

2.3.3. Association of differentially expressed genes (DEGs) with human diseases.

Annotations were performed to associate the DEGs with human diseases using specialized databases such as DisGeNet **(Piñero et al., 2020)**, GeDiNet 2023 **(Kundu et al., 2023)**, Virus-Host PPI P-HIPSTer 2020 **(Lasso et al., 2019)**, and Orphanet Augmented **(Orphanet, 2025)**. This analysis enabled the identification of potential links between modulated genes and human diseases. The thresholds established for these enrichment analyses were Padj ≤ 0.05.

2.3.4. Protein-Protein Interactions (PPI) and visualization of results.

The exploration of protein-protein interactions (PPIs) among the DEGs, as well as interactions with proteins associated with human diseases, was carried out using the STRING **(Szklarczyk et al., 2023)**, BioGRID **(Oughtred et al., 2021)**, and IntAct **(del Toro et al., 2022)** databases. The thresholds set for the PPI analyses were a combined score, quantitative score, and confidence value ≥ 0.9, respectively. These networks were visualized in Cytoscape v. 3.10.2 **(Shannon et al., 2003)** with a Padj ≤ 0.05.

2.4. Results.

2.4.1. Caco-2 and HT-29 cell lines treated with B. subtilis CW14 and P. freudenreichii ITG P9 exhibit differentially expressed mRNA.

Principal component analysis (PCA) revealed marked transcriptional responses for both probiotics **(Fig. 1)**. P. *freudenreichii* ITG P9 displayed exceptionally high variance, with PC1 accounting for 94%, reflecting a clear separation between treatments and controls. In

contrast, the PC1 of *B. subtilis CW14* explained 68% of the variance, suggesting that this axis significantly contributes to the separation between groups. These results indicate that the treatment effect varies according to the probiotic, with a more pronounced response from *P. freudenreichii* ITG P9 and highlights the high heterogeneity of mRNA as observed in the spatial distribution of treatments, evidencing intragroup variability.

When transcripts were filtered with a $\log_2FC \geq 2$ and an FDR ≤ 0.05, the treatment with *P. freudenreichii* yielded 2,337 genes, of which 1,457 (62.34%) were upregulated and 880 (37.66%) downregulated. This suggests a broad and robust transcriptional response. For *B. subtilis* CW14, 198 genes were identified, with 136 (68.69%) upregulated, and 62 (31.31%) downregulated. In both cases, a trend toward gene activation was observed **(Fig. 1)**.

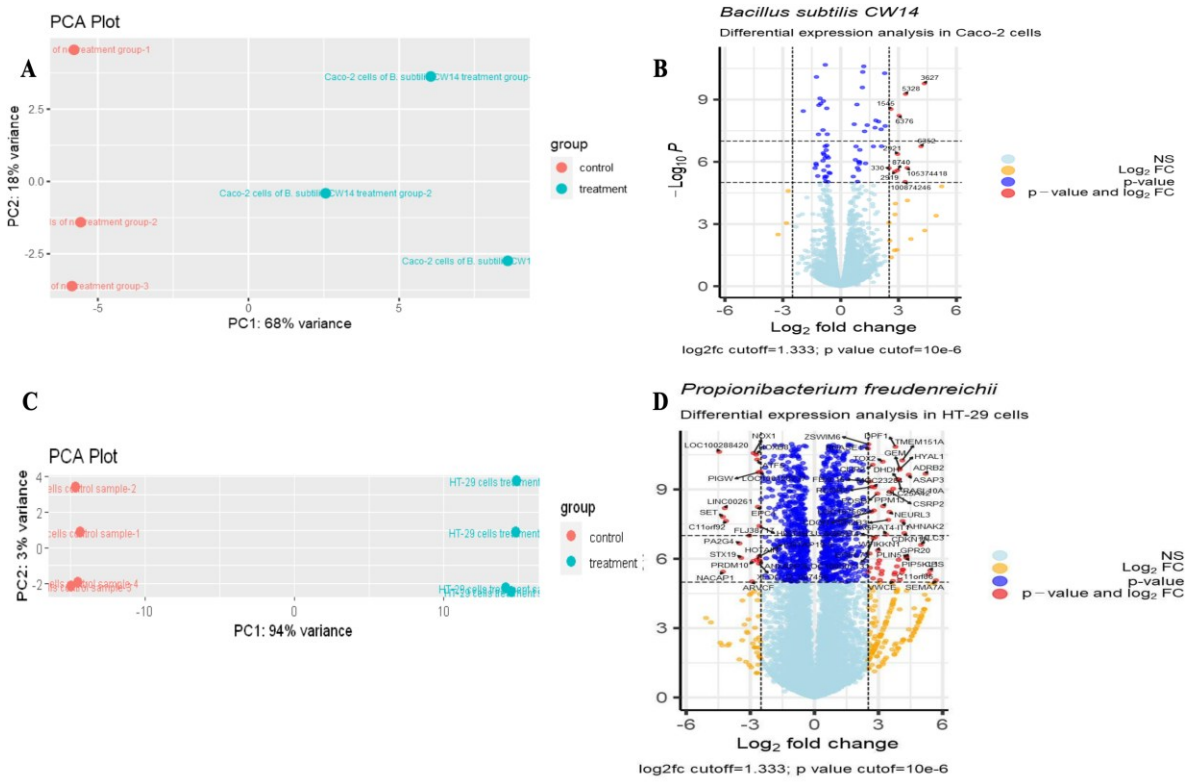

Figure 1. PCA and Volcano Plot Analysis of Genomic Data in Probiotic-Treated Caco-2 and HT-29 Cells. (A and C) Principal Component Analysis (PCA) of Genomic Data. In panel A (for *B. subtilis* CW14), PC1 accounts for 68% of the variance and PC2 for 18%. The points represent control samples (red) and treatment samples (blue) from Caco-2 cells. In panel C (for *P. freudenreichii* ITG P9), PC1 explains 94% of the variance and PC2 3%, with control samples depicted in red and treatment samples in blue from HT-29 cells. **(B and D) Volcano Plots**. In panel B (for *B. subtilis* CW14), the x-axis shows the logarithmic change in gene expression, and the y-axis displays the -log10 of the p-value. The red points represent genes with both significant log2FC and significant p-value; the blue points represent genes with only a significant p-value; the yellow points represent genes with only a significant log2FC; and the grey points represent non-significant genes. A cutoff of a log2FC of 1,333 and a p-value threshold of 10e-6 is applied for graphics. Panel D (for *P. freudenreichii* ITG P9) follows the same criteria.

2.4.2. Functional enrichment analysis of biological processes and pathways.

The analysis of Caco-2 cells treated with *B. subtilis* CW14 and HT-29 cells treated with *P. freudenreichii* c revealed significant modulation of DEGs by applying thresholds of log_2FC

≥ 2, FDR ≤ 0.05, and Padj ≤ 0.05. These criteria allowed the selection of a set of genes associated with important biological processes, including the cell cycle, immunity, adhesion, inflammation, and transport. Moreover, they enabled the identification of key metabolic and signaling pathways. All results obtained for gene ontology terms (Enrichr), metabolic pathways, and signaling pathways (KEGG and Elsevier Pathway Collection) are shown in **Tables S1, S2,** and **S3**, respectively.

The transcriptomic analysis performed on Caco-2 cells treated with *B. subtilis* CW14 revealed a coordinated and simultaneous response involving immune signaling pathways and defense mechanisms. Notably, an overexpression of chemokines and immune system stimulatory factors was observed, as reflected by increased expression of genes such as *CCL4* (+5.24), *CSF2* (+5.03), *CSF3* (+4.95), *NFKBIZ* (+2.28), *LTB* (+3.07), and *PLAU* (+3.36). This overexpression suggests activation of the *NF-κB* pathway, which likely promotes the recruitment of T lymphocytes, neutrophils, and the differentiation of macrophages and granulocytes, thereby facilitating both the elimination of pathogens and the promotion of reparative processes in the intestinal epithelium **(Upadhyay et al., 2013; Peng et al., 2019; Yamazaki et al., 2022; Anderson, 2023)**.

Similarly, the overexpression of *CXCL8* (+4.65), *CXCL10* (+4.34), *CXCL11* (+2.82), and *CX3CL2* (+3.01) indicates the activation of pathways that promote the chemotaxis and migration of neutrophils, as well as mast cell activation, contributing to a coordinated immune response against microbial pathogens **(Kochumon et al., 2020)**. Furthermore, the increased expression of *CCL5* (+4.16), along with the modulation of *CCL22* (+2.51) and *CCL2* (+2.55), points to the attraction of monocytes, regulatory T cells (Tregs), and the polarization of macrophages toward a reparative phenotype, which could help mitigate epithelial damage under inflammatory conditions. Other genes, such as *TNFAPI3* (+2.31)

and *TNFSF14* (+2.95), may contribute to the control of intestinal inflammation either through the blockade of *NF-κB* signals or via apoptosis **(Kolodziej et al., 2011; Krause et al., 2014)**. Simultaneously, effects were identified in genes related to stress response and metabolic activity. The overexpression of the *CYP1B1* gene (+2.61) suggests the activation of detoxification pathways involved in neutralizing xenobiotic compounds, while the increased expression of *BIRC3* (+2.51) and the downregulation of *RGS2* (-2.11) indicate the involvement of anti-apoptotic mechanisms that favor the survival of epithelial cells in the face of oxidative stress **(Pauletto et al., 2020)**. In contrast, the reduction in *HSPA6* expression (-2.72) may suggest an adaptation of the cellular system to stress conditions by optimizing resources in a gastrointestinal environment that simultaneously demands immune and repair responses **(Chen et al., 2021; Neurath, 2014)**. **Table 1** shows representative DEGs with a $\log_2$FC (LFC) ≥ 2 for *B. subtilis* CW14. All results obtained can be seen in **Tables S1, S2,** and **S3**.

Genes	Term	Adjusted P-value	Log2Fold Change
CCL4	Cytokine-cytokine receptor interaction, Viral protein interaction with cytokine and cytokine receptor.	1,86e-17, 3,08e-16	5,23
CSF2	TNF signaling pathway, Rheumatoid arthritis, Cytokine-cytokine receptor interaction.	7,38e-21, 5,01e-18, 1,86e-17	5,02
CSF3	Cytokine-cytokine receptor interaction, IL-17 signaling pathway, Malaria, Coronavirus disease.	1,86e-17, 2,86e-15, 4,92e-07	4,95
CXCL8	Rheumatoid arthritis, Cytokine-cytokine receptor interaction, Viral protein interaction with cytokine and cytokine receptor, IL-17 signaling pathway, NF-kappa B signaling pathway.	5,01e-18, 1,86e-17, 3,08e-16, 2,86e-15, 1,15e-14	4,64
CXCL10	TNF signaling pathway, Cytokine-cytokine receptor interaction, Viral protein interaction with cytokine and cytokine receptor, IL-17 signaling pathway, Chemokine signaling pathway, Toll-like receptor signaling pathway.	7,38e-21, 1,86e-17, 3,08e-16, 2,86e-15, 1,02e-09, 3,49e-08	4,34

Table 1. Functional enrichment of positively regulated genes in Caco-2 cells treated with *B. subtilis* CW14. This table summarizes the key genes identified in the transcriptomic analysis, their associated enriched biological pathways, the adjusted p-values for these enrichments, and the corresponding log2FC in gene expression, highlighting their potential roles in immune signaling pathways and inflammatory responses.

The transcriptomic analysis of HT-29 cells treated with *P. freudenreichii* ITG P9 revealed a coordinated response involving the genes *CDKN1A* (+4.21), *CDKN2B* (+2.84), and *CDKN1C* (+2.43), which are associated with cell cycle regulation. This suggests mechanisms related to cell cycle arrest at the G1/S transition phase, thereby reducing the proliferation of damaged cells **(Abbas & Dutta, 2009)**. Additionally, the genes *BRSK2* (+2.64) and *NES*

(+2.02), implicated in cell cycle transitions (G2/M), were identified, suggesting the possibility of modulating the cell cycle under stress conditions **(Wang et al., 2012; Cousin et al., 2016)**. All results obtained for *P. freudenreichii* ITG P9 can be seen in **Tables S1, S2,** and **S3.**

Overall, the changes in gene expression indicate that *B. subtilis* CW14 exerts a dual impact on intestinal cells; on one hand, it activates a controlled pro-inflammatory response through the modulation of chemokines and factors that promote the recruitment and activation of immune cells, and on the other, it modulates protective and detoxification mechanisms that contribute to preserving epithelial integrity. In contrast, *P. freudenreichii* ITG P9 induces a reprogramming of the cell cycle aimed at promoting arrest at critical phases such as G1/S and G2/M, in addition to enhancing defense mechanisms against stress.

2.4.3. The differentially expressed genes (DEGs) modulated by the probiotics are repositioned as regulators in various pathologies, exhibiting multifunctional annotations.

2.4.3.1. Modulation of *B. subtilis* CW14 in Caco-2 cells and *P. freudenreichii* ITG P9 in HT-29 cells on genes associated with diseases, neurological, dysbiosis, and rare syndromes.

The analysis of DEGs in intestinal cells following probiotic treatment has revealed a complex gene regulatory network associated with dysbiosis **(Table S4)**, neurological disorders **(Table S5)**, and rare or orphan syndromes **(Table S6)** through log2FC (LFC) ≥ 2, an FDR value ≤ 0,05 and Padj filter ≤ 0,05. The results underline the critical role of the microbiota in modulating cross-cutting pathophysiological pathways, mediated by the regulation of chemokines, cytokines, and growth factors.

In Caco-2 cells treated with *B. subtilis* CW14, positive regulation of several genes associated with proinflammatory and immunomodulatory pathways was observed, with log2FC values ranging from +2,10 to +5.23 **(Table S1)**. Among these, *CCL4* (+5,23), *CSF2* (+5,02), *CSF3* (+4,95), *CXCL8* (+4,64), and *CXCL10* (+4,34) genes stood out for their enrichment. These genes, according to enrichment analyses (Table 2), are associated not only with local inflammatory processes but also with neurological diseases (Epilepsy, Parkinson's Disease (PD), Alzheimer's Disease (AD)), rare disorders (amyloidosis, antibody-mediated glomerulonephritis) and gut dysbiosis, linked to metabolic disorders such as obesity, inflammatory bowel disease (IBD) and diabetes mellitus **(Table S4, S5, S6),** This suggests a dual role in the activation of innate immunity and in pleiotropic mechanisms that extend beyond the intestinal environment.

These findings acquire even greater relevance in the context of the gut-brain axis, where previous studies have demonstrated that probiotic strains such as *B. subtilis* modulate the intestinal immune response, thereby influencing neuroinflammatory processes and CNS homeostasis **(Sarkar et al., 2016; Bravo et al., 2011)**. For example, the overexpression of *CXCL10* (+4.34) and *CCL4* (+5.23), both associated with autoimmune and neurodegenerative diseases, supports the hypothesis that the gut microbiota may serve as an intermediary in neuroprotection and in maintaining the integrity of the blood-brain barrier through the controlled regulation of these genes **(Vida et al., 2025)**. In parallel, the modulation of *CSF2* (+5.02) and *CSF3* (+4.95), regulators of immune cell proliferation and differentiation, indicates that the *B. subtilis* CW14 strain could promote a controlled inflammatory response in diseases such as multiple sclerosis (MS) or AD **(Mayer et al., 2014)**.

Concerning the connection between dysbiosis and neuroinflammation, the results are reinforced when considering the role of genes such as *CXCL8* (+4,64), which is associated

with the intestinal inflammatory response. Its regulation by *B. subtilis* CW14 points to a mechanism by which this probiotic could regulate intestinal homeostasis, mitigating systemic inflammation and its impact on neurological disorders **(Mayer et al., 2014)**. This finding is consistent with the evidence linking dysbiosis to alterations in the gut-brain axis, increasing susceptibility to metabolic and neurodegenerative pathologies **(Bercik et al., 2011; Cryan et al., 2019)**. Taken together, the results emphasize the potential of *B. subtilis* CW14 as a modulator of gut immunity and its cross-cutting effect on neurological and metabolic diseases **(Table S7)**, reinforcing the concept that the gut microbiota acts as a connector between the immune and nervous systems **(Sarkar et al., 2016)**. A summary of the genes associated with different pathologies can be seen in **Fig. 2**.

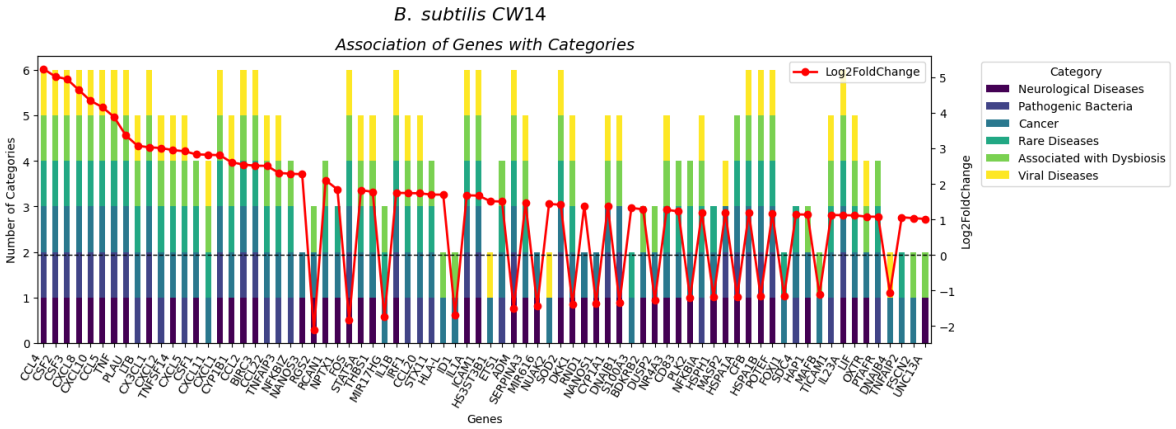

Figure 2. Association of genes with categories and Log2FoldChange in *B. subtilis CW14*. This graph illustrates the association between regulated genes and various disease categories following treatment with *B. subtilis* CW14. On the x-axis, the analyzed genes are displayed, while the left y-axis indicates the number of disease categories linked to each gene. The stacked bars break down these counts by type: neurological diseases, pathogenic bacteria, cancer, rare diseases, dysbiosis, and viral diseases. Superimposed on the bars, a red line represents the logarithmic change in gene expression (Log2FoldChange), with its values shown on the right y-axis. A dotted horizontal line marks a reference value of Log2FoldChange = 0.

In HT-29 cells treated with *P. freudenreichii* ITG P9, a dual modulation of gene expression was observed, characterized by both positive and negative regulation of specific genes **(Table S1)**. According to the enrichment analyses, the genes *SH2D3C* (+6.80) and *CORO1A* (+3.79) are both associated with symptomatic polyhydramnios-megalencephaly-epilepsy syndrome. Likewise, *KIFC2* (+2.14) was linked to diseases such as Charcot-Marie-Tooth disease type 2P and type 4B3, as well as adult-onset dystonia-parkinsonism. The gene *KIAA0513* (+4.52) was associated with the syndrome of intellectual disability-obesity-cerebral malformations-facial dysmorphism and with AD. This group of genes has been linked to synaptic plasticity, suggesting a potential role in maintaining neuroarchitecture and, consequently, a neuroprotective effect. This effect could be mediated by the regulation of genes involved in stabilizing the neuronal cytoskeleton, reinforcing the hypothesis of a connection between the modulation of the gut microbiota and neurological pathways **(Herbin et al., 2016; Shimojima et al., 2017; Zhu et al., 2020; Zheng et al., 2022; Gerik-Celebi et al., 2023; Biggs et al., 2025)**. In contrast, a downregulation of *KIF20A* (-2.19) was observed, a gene enriched in type II citrullinemia, primary immunodeficiency with natural killer cell deficiency, and adrenal insufficiency. This gene has been associated with reduced invasion and proliferation of glioblastoma cells, suggesting a potential tumor-suppressive mechanism **(Saadh et al., 2025)**. These results are relevant in the context of the gut-brain axis, as they suggest modulation of an intestinal immune response that influences neuroinflammatory processes and CNS homeostasis **(Kim et al., 2023)**. All the results obtained can be seen in **Tables S4, S5,** and S6. A summary of the genes associated with different pathologies can be seen in **Fig. 3**.

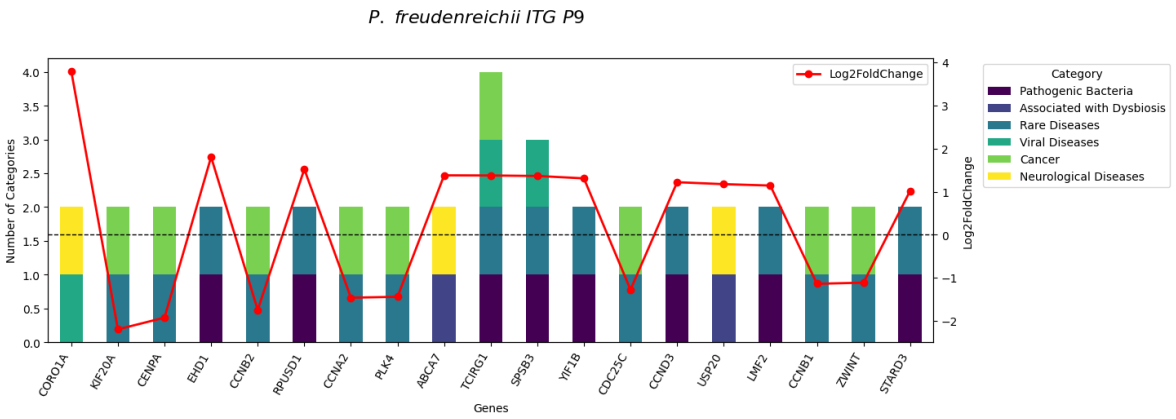

Figure 3. Association of genes with categories and Log2FoldChange in *P. freudenreichii* ITG P9. This graph displays the association between regulated genes and various disease categories following treatment with *P. freudenreichii* ITG P9. It follows the same structure as the previous graph, with the x-axis showing the analyzed genes and the left y-axis indicating the number of disease categories linked to each gene. A red line overlays the bars to show the Log2FoldChange in gene expression (values on the right y-axis), while a dotted horizontal line marks a reference level of Log2FoldChange = 0.

Transcriptomic analyses also revealed a set of DEGs with multi-association profiles to various pathologies **(Table S7)**. Among these, genes with pleiotropic roles—linked simultaneously to dysbiosis, cancer, neurological disorders, bacterial infections, rare diseases, and viral conditions—stood out **(Fig. 4)**. In this regard, the genes *CCL4*, *CSF2*, *CSF3*, *CXCL8*, and CXCL10 emerged as central nodes, displaying a significant positive $\log_2$FC and associating with the pathological categories analyzed **(Table S7)**. It is noteworthy that this group of genes exhibits a Log2FC that is twice the established (Log2FC ≥ 2), which may indicate a role as cross-cutting biomarkers in multiple pathophysiological pathways. Furthermore, *CCL4*, *TNF*, and *CSF2* genes also function as central nodes, connecting dysbiosis to neurological diseases including AD, PD, and epilepsy. For instance, *CSF2* (+5,03), associated with microglial activation in AD, and *TNF* (+3,88), implicated in neuroinflammation in amyotrophic lateral sclerosis (ALS), underscore the existence of shared mechanisms between gut inflammation and neuronal degeneration.

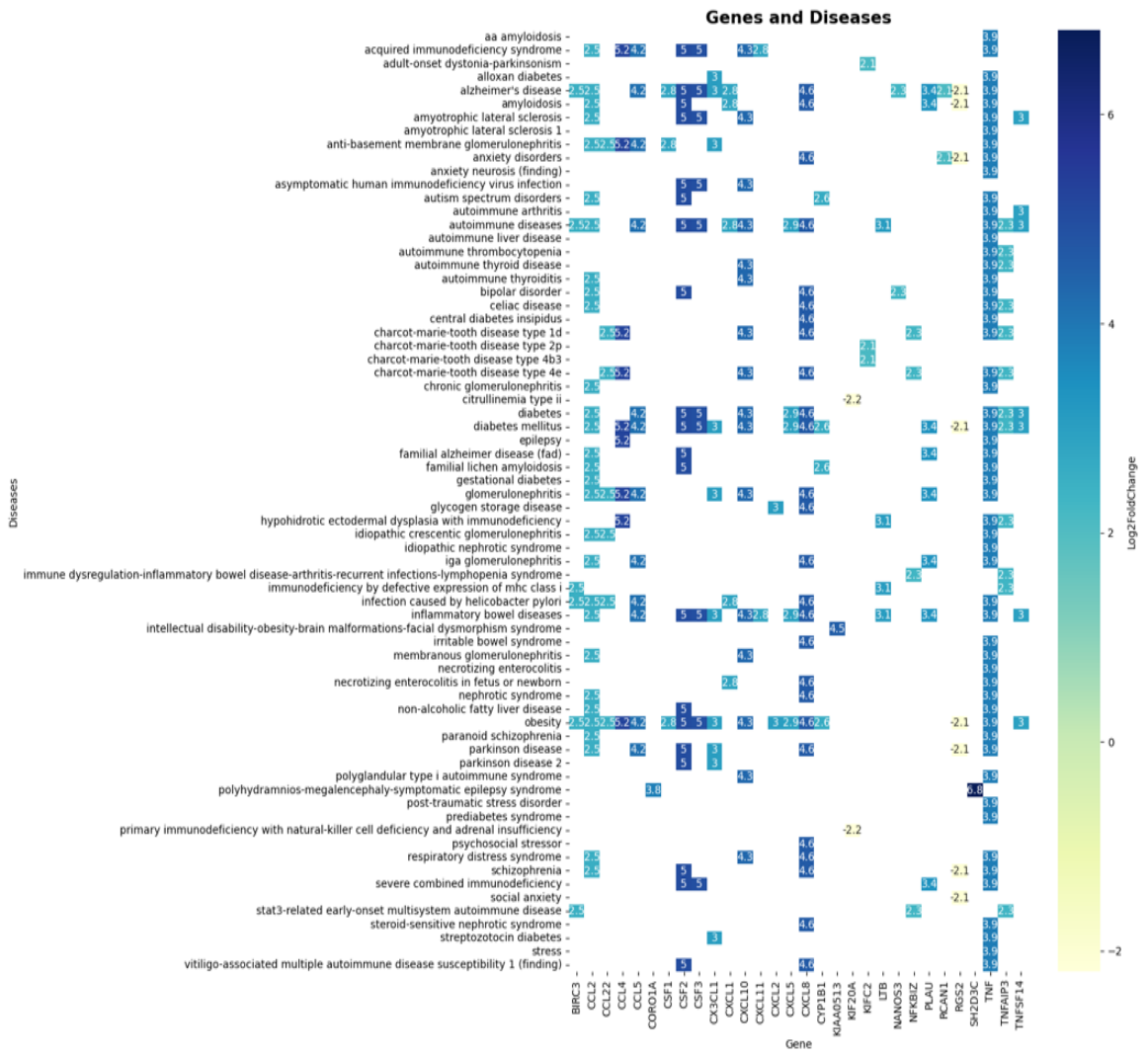

Figure 4. Heatmap of Log2FC in gene expression across diseases. This heatmap illustrates the Log2FC in gene expression for a range of genes across several diseases. The vertical axis lists the diseases associated, while the horizontal axis displays the genes analyzed. Each cell's color intensity represents the magnitude of the Log2FC, deeper blues indicate strong upregulation, whereas yellows reflect downregulation. Numerical values are overlaid on each cell for precise quantification.

2.4.4. Protein-Protein Interactions (PPI).

As mentioned previously, from the analysis of DEGs for cells treated with *B. subtilis* CW14, when mapped against various pathologies (dysbiosis, neurological diseases, and rare diseases), genes or core nodes with pleiotropic roles emerged that were simultaneously linked to these pathologies (*CCL4, CSF2, CSF3, CXCL8,* and *CXCL10*). In this context, the PPI analysis highlights the complex network of connections that the studied DEGs establish with other proteins and signaling pathways involved in both the innate and adaptive immune responses **(see Fig. S1 for the CCL4 network in the supplementary material)**. For example, the genes *CXCL8* (IL-8) and *CXCL10*, and their association with multiple autoimmune and inflammatory diseases, support their function as potent chemokines involved in the migration and activation of leukocytes to the intestinal epithelium. On the other hand, the presence of *CCL4* (*MIP-1β*) in the network underscores its potential role in regulating the inflammatory response and its link to chronic inflammation processes, indicating its involvement in modulating immune cell recruitment in various pathological contexts.

Furthermore, terms emphasizing the importance of the *ERK1/2* pathway in cellular signaling were identified, connecting inflammation with processes of proliferation, differentiation, and cytokine responses **(Chandiok, 2024)**. Similarly, *CSF2* (*GM-CSF*) and *CSF3* (*G-CSF*), and their relation to diseases in which the immune microenvironment and hematopoiesis are altered, suggest that modulation of these two components may influence intestinal homeostasis and, consequently, prevent systemic complications. The network also highlights the convergence of inflammatory and signaling pathways mediated by these DEGs with other routes linked to the adaptive immune response, involving cytokines such as *IL-1*, *IL-4*, *IL-10*, and *IL-13*. This interconnection indicates that *B. subtilis* CW14 not only impacts the local epithelial response but could also have systemic repercussions through the modulation of additional inflammatory pathways.

In the case of *P. freudenreichii* ITG P9, two major networks were identified, centered on two genes: *KIF20A* and *OASL* **(Fig. 5)**. PPI analysis revealed that both networks are related to processes such as cell cycle and antiviral response. *KIF20A* is positioned as a central node in the first network. This gene interacts with cyclins (*CCNA2, CCNB1, CCNB2*), kinases (*AURKA, PLK1, TTK*), and centromere components (*CENPA, INCENP, NCAPG, NUF2*), suggesting an essential role in cytokinesis, cell cycle progression, and chromosomal stability. On the other hand, *OASL* emerged as a central regulator of the antiviral immune response. This gene interacts with genes such as *IRF7, IFI44, IFIT3, ISG15*, and *RNASEL*. The direct relationship between *OASL* and *RNASEL* suggests a mechanism for degrading viral RNA and inhibiting replication, as well as modulating antiviral gene expression through *IRF7* **(Jung-Rodriguez et al., 2024; Lee et al., 2013)**. Upregulation of *OASL* by *P. freudenreichii* ITG P9 may enhance the innate immune response, limit viral spread, and protect epithelial cells, thereby strengthening the intestinal immune barrier. **(Weiss, 2020)**.

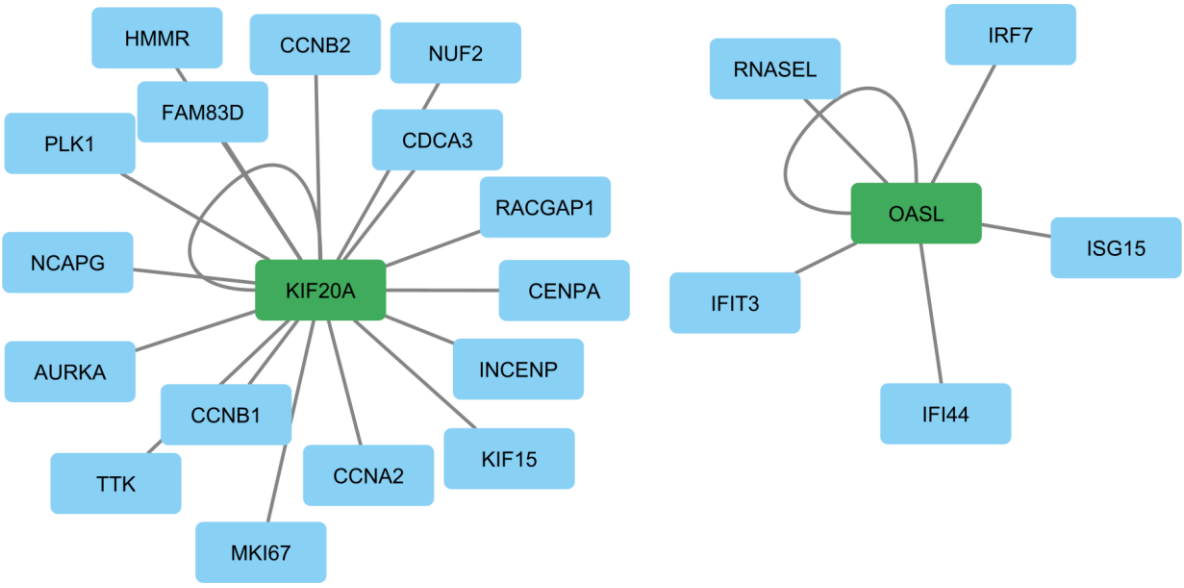

Figure 5. PPI networks of KIF20A and OASL in HT-29 cells after treatment with *P. freudenreichii* ITG P9. The image illustrates two protein-protein interaction (PPI) networks derived from the analysis of *P. freudenreichii* ITG P9. Network 1, centered on *KIF20A* (in green), connects with genes including *CCNA2, CCNB1, CCNB2, AURKA, PLK1, TTK, CENPA, INCENP, NCAPG*, and *NUF2*, suggesting a role in cytokinesis, cell cycle progression, and chromosomal stability. Network 2, centered on *OASL* (in green), interacts with *IRF7, IFI44, IFIT3, ISG15,* and *RNASEL*, indicating its central role in the antiviral response through viral RNA degradation and the induction of antiviral genes.

2.5. Discussion.

The intestinal tract is the largest immune system in the body and interacts with antigens and immune mechanisms **(Pelaseyed et al., 2014)**. Approximately 70% of the immune system is generated in the gut **(Ygberg & Nilsson, 2012)**. As a result, the intestinal epithelium is crucial for maintaining immune homeostasis and preventing the uncontrolled absorption of toxic compounds or pathogens **(Hashemi et al., 2023)**, which can lead to improvements in the intestinal barrier, pathogen inhibition, modulation and maturation of the immune system, and a reduction in inflammatory and carcinogenic processes **(Foligné et al., 2010; Bienenstock et al., 2015; do Carmo et al., 2017; Peng et al., 2019)**.

The modulation of the intestinal cellular response through the use of probiotics has captured the attention of researchers in recent years, particularly due to its potential to influence the homeostasis of the intestinal epithelium, activate immune pathways (via the secretion of chemokines, cytokines, and growth factors that impact inflammatory and neuroimmune responses), reprogram the cell cycle by modulating arrest in critical phases in damaged cells, and prevent inflammatory, neoplastic, and other human pathologies **(Wang et al., 2021; Liu et al., 2022)**. Even more interesting is the growing number of studies associating the gut-microbiota-brain axis with the modulation of critical molecular pathways, which not only influence systemic homeostasis **(Plaza-Diaz et al., 2014; Sarkar et al., 2016)** but also regulate complex diseases, including neurological disorders, metabolic alterations, and rare syndromes that may share pathophysiological complexities such as those seen in AD and PD **(Kirby & Ochoa-Repáraz, 2018; Zhao et al., 2023; Hashemi et al., 2023; Belnap et al., 2024)**.

In this work, significant differences were observed in the DEGs of Caco-2 cells treated with *B. subtilis* CW14 and HT-29 cells treated with *P. freudenreichii* ITG P9. In the first case, a coordinated immune response was noted through the overexpression of chemokines and inflammatory factors, whereas in the second, a transcriptional response characterized by the modulation of genes related to cell cycle control and stress was observed. The observed diversity suggests that the effects of probiotics on intestinal cells may be strain-specific and related to molecular mechanisms that regulate epithelial homeostasis, inflammation, cell adhesion, stress response, and cell cycle arrest.

2.5.1. Pleiotropic effects and integrated molecular mechanisms.

2.5.1.1. Immune modulation and tissue repair in the intestinal epithelium.

In Caco-2 cells treated with *B. subtilis* CW14, a coordinated activation of genes related to the inflammatory response and epithelial repair was observed. The overexpression of chemokines such as *CXCL1*, *CXCL2*, *CCL2*, *CCL4*, *CCL5*, and *CCL22* suggests that this strain is capable of recruiting and activating various immune cell types, including T lymphocytes, neutrophils, macrophages, and NK cells **(Scheu et al., 2017; Chen et al., 2022)**. This modulation is twofold: on one hand, it creates a microenvironment that favors local defense against pathogens; on the other, it promotes the activation of the *NF-κB* pathway—reinforced by the overexpression of *NFKBIZ*—which not only promotes the inflammatory response but also facilitates tissue repair processes and the regeneration of intestinal crypts **(Zhang et al., 2019; Liu et al., 2022; Xiong et al., 2021; Yamazaki et al., 2022; Feng et al., 2023)**.

The ability to induce the expression of immunological mediators is not only crucial for maintaining the integrity of the intestinal barrier but also has systemic repercussions. For example, the regulation of *CCL2*—which under homeostatic conditions contributes to the clearance of protein aggregates—gains therapeutic importance in neurological diseases such as AD and PD, where the accumulation of aberrant proteins and neuroinflammation are central factors **(Semple et al., 2010; Wang et al., 2024)**. Similarly, the modulation of chemokines such as *CXCL8*, *CXCL10*, *CXCL11*, and *CX3CL2* may influence the permeability of the blood-brain barrier and the activation of microglia, suggesting a role that extends beyond the intestinal domain to affect neuroimmunomodulation **(John et al., 2008; Karin et al., 2016; Karin & Razon, 2018; Karin, 2020; Zhu et al., 2021; Wang et al., 2024)**.

2.5.1.2. Metabolic response and detoxification.

The overexpression of *CYP1B1* in Caco-2 cells after exposure to *B. subtilis* CW14 highlights another pleiotropic aspect: the activation of detoxification mechanisms and xenobiotic metabolism. This effect is mediated, in part, by the aryl hydrocarbon receptor (*AhR*) pathway, which is activated in response to microbially derived metabolites, for example, from tryptophan **(Schiering et al., 2017; Shah et al., 2019)**. This adaptive response not only contributes to the neutralization of potentially harmful compounds but also adjusts the metabolic balance of the intestinal epithelium, which could have implications in metabolic diseases such as diabetes and metabolic syndrome **(Schiering et al., 2017)**, where imbalances in detoxification pathways and the metabolism of lipids and steroid hormones are common **(Shah et al., 2019)**.

2.5.1.3. Regulation of apoptosis and cell survival.

The modulation of genes such as *BIRC3*, which belongs to the inhibitor of apoptosis (IAP) family, constitutes another critical pleiotropic mechanism induced by *B. subtilis* CW14. The overexpression of *BIRC3* not only protects the epithelium from apoptotic processes but is also associated with the regeneration and renewal of intestinal cells **(Pauletto et al., 2020; Hu & Shao, 2022)**. Conversely, the reduction in *RGS2* suggests an attenuation of signaling mediated by G protein-coupled receptors (GPCR) **(Hadar et al., 2016; Pauletto et al., 2020; Bhuvaneshwar & Gusev, 2024)**, which could decrease oxidative stress and inflammation, thereby creating a more stable and resilient cellular environment. Similarly, the decrease in *HSPA6*, a heat shock protein induced in response to stress conditions, indicates that in the absence of external aggressors, cells can maintain a basal state of protein homeostasis. A balanced modulation of stress response is essential for preserving cellular integrity, especially in tissues with high turnover such as the intestinal epithelium **(Ohkawara et al., 2006; Song et al., 2022; Kim et al., 2024)**.

2.5.1.4. Regulation of the cell cycle and response to metabolic stress in HT-29 Cells.

Treatment of HT-29 cells with *P. freudenreichii* c exhibited a transcriptional profile primarily oriented toward cell cycle regulation. The overexpression of cyclin-dependent kinase inhibitors—*CDKN1A*, *CDKN2B*, and *CDKN1C*—suggests a mechanism of arrest at the G1/S transition, acting as a protective barrier against the proliferation of damaged cells and eventual neoplastic transformation **(Wang et al., 2012; Cousin et al., 2016; Cousin et al., 2018; Bueno-Fortes et al., 2021; Yang et al., 2023)**. This effect is particularly relevant in the context of rare and neoplastic diseases, where dysfunction in cell cycle control can be an etiological factor.

Furthermore, the modulation of genes such as *BRSK2* and *NES*—implicated in the G2/M transition and in the organization of the mitotic spindle—reinforces the capacity of *P. freudenreichii* to induce adaptive responses to metabolic stress **(Wang et al., 2012; Chen et al., 2025)**. The induction of *BRSK2*, a protein kinase related to the *AMPK* pathway, suggests that bacterial metabolites, such as short-chain fatty acids, stimulate a controlled stress state, prompting the cell to adjust its metabolism via the *Akt/mTOR* pathway **(Saiyin et al., 2017)**. This mechanism would not only preserve cellular integrity under conditions of low energy availability but could also have implications in metabolic diseases and in rare syndromes associated with alterations in cellular energy signaling **(Saiyin et al., 2017; Chen et al., 2025)**. In contrast, the overexpression of *NES* indicates the activation of regenerative mechanisms that ensure precise cell division, contributing to tissue repair even under damaging conditions **(Kulkarni et al., 2017; De Vadder et al., 2018; Wang et al., 2021; Dicks, 2022)**, which may be crucial in preventing degenerative processes.

2.5.2. Implications for neurological, rare, and metabolic diseases.

Neurological diseases: The modulation of chemokines such as *CCL2*, *CXCL5*, *CXCL8*, *CXCL10*, and *CXCL11* suggests a direct impact on the gut–brain axis. In conditions such as Alzheimer's and Parkinson's, dysfunction in immune signaling (for example, the p38 *MAPK* signaling pathway) **(Yu et al., 2021)** and alterations in the blood–brain barrier have been shown to contribute to neuroinflammation and neuronal deterioration **(Karin & Razon, 2018; Wang et al., 2024; Semple et al., 2010)**. Moreover, the regulation of genes such as *RGS2*, *RCAN1*, and *BIRC3* – the latter associated with *NPD1*-mediated neuronal protection – points to neuroprotective mechanisms that could be exploited in therapies for neurodegenerative diseases and neuronal ischemia **(Ermak & Davies, 2013; Van Raemdonck et al., 2015; Bazan, 2014; Hu & Shao et al., 2022)**. The ability to modulate the inflammatory response in the intestine, which in turn influences microglia and the permeability of the blood–brain barrier, opens the possibility of using these probiotic strains as part of therapeutic repositioning strategies in neurological disorders.

The integration of the results suggests that both probiotics can have positive effects in modulating systemic inflammatory responses, promoting a controlled regulation of the gut–brain axis. Additionally, signaling pathways associated with chemokines—which are linked to G protein-coupled receptors (such as *CCR2*, *CCR4*, *CCR5*, *CXCR1*, *CXCR2*, among others) and cascades like *MAPK* and *NF-κB* activation—are closely related to the pathological mechanisms underlying neurodegenerative and cognitive disorders. In this sense, the ability of these probiotics to induce controlled expression of these chemokines could, depending on the context and the magnitude of the response, favor mechanisms of repair and immunological surveillance.

Metabolic diseases: The activation of *CYP1B1* and the adaptive response mediated by the *AhR* receptor, together with the modulation of metabolic pathways related to *Akt/mTOR*

through *BRSK2* and the influence of *TNFSF14* as a ligand for receptors such as *HVEM* (*TNFRSF14*) and *LTβR* for the activation of the transcription factor *NF-κB* **(Kou et al., 2019; Ware et al., 2022; Wang et al., 2012; Chen et al., 2025)**, position these strains as candidates for intervention in metabolic diseases. Disorders such as type I and II diabetes, non-alcoholic fatty liver disease, and metabolic syndrome are characterized by imbalances in metabolic signaling and in the response to oxidative stress. The ability of these probiotics to rebalance these processes could translate into improvements in insulin sensitivity and a reduction in metabolic stress, opening new avenues for the complementary treatment of these conditions **(Schiering et al., 2017; Chen et al., 2025)**.

Rare diseases and autoimmune disorders: Dysfunction in cell cycle control and immune signaling is common in certain rare syndromes and autoimmune diseases, such as ulcerative colitis, Crohn's disease, rheumatoid arthritis, and systemic lupus erythematosus (SLE) **(Musone et al., 2011; Ciccacci et al., 2019)**. The induction of cell cycle inhibitors (*CDKN1A*, *CDKN2B*, *CDKN1C*) by *P. freudenreichii* ITG P9 not only prevents aberrant proliferation but may also help restore control mechanisms in pathologies where dysregulation of the cell cycle plays a central role. Additionally, the positive modulation of mediators such as *TNFAIP3* **(Ciccacci et al., 2019)** and *NFKBIZ* reinforces the ability of these probiotics to regulate the inflammatory response in a balanced manner, suggesting a potential use in managing autoimmune diseases and certain rare disorders where chronic inflammation is a determining factor **(Musone et al., 2011; Zhang et al., 2019; Feng et al., 2023)**.

2.5.3. Therapeutic Repositioning Potential.

The integration of these findings suggests that the pleiotropic effects of *B. subtilis* CW14 and *P. freudenreichii* ITG P9 could be harnessed for the therapeutic repositioning of these strains across a wide range of pathologies. In the context of neurological diseases, the ability to

modulate the gut–brain axis—through the regulation of chemokines and protection against neuroinflammation—could be key in developing adjuvant interventions for the treatment of Alzheimer's, Parkinson's, neuronal ischemia, and even other neurodegenerative disorders. Influencing mediators that control microglial activity and the integrity of the blood–brain barrier opens new perspectives for combating pathological processes that have traditionally been difficult to address with conventional therapies.

Regarding metabolic diseases, the capacity to induce detoxification mechanisms and regulate signaling pathways related to cellular metabolism (such as Akt/mTOR and the AhR pathway) position these strains as potential modulators of metabolism. Restoring metabolic balance, reducing oxidative stress, and improving insulin sensitivity are critical aspects for managing diabetes and metabolic syndrome, conditions that currently represent significant public health challenges.

Finally, in the realm of rare diseases and autoimmune disorders, the regulation of the cell cycle and the modulation of anti-inflammatory and autoimmune mediators—for example, *TNFAIP3* (which modulates signals from *Toll-like* receptors (*TLRs*), nucleotide-binding oligomerization domain (*NOD*) pathways, and the *NF-κB* pathway) and *NFKBIZ*—offer an intriguing strategy to slow the progression of pathologies in which dysfunction in these processes is a key etiological factor. The ability to induce cell cycle arrest at critical phases and promote tissue repair suggests that these strains could be useful in preventing neoplastic transformations and in stabilizing autoimmune conditions, where control of cell growth and immune response is essential.

2.6. Conclusions.

Overall, the results obtained through the implementation of an integrative methodology highlighted the positive effects of the probiotics *P. freudenreichii* ITG P9 and *B. subtilis* CW14 on intestinal cells, demonstrating that each can exert specific effects, particularly in pathways related to immunity and the cell cycle. Moreover, the pleiotropic effects of these probiotics on genes linked to metabolic, neurological, and autoimmune diseases are emphasized, as they often regulate genes with neuroprotective or antitumor effects, thereby reinforcing the importance of the gut–brain axis connection. Additionally, key molecular mechanisms related to immunomodulation emerged from the results—such as innate receptors (*TLR* and *NOD*) and signaling pathways (*NF-κB* and *MAPK*). Finally, the findings of this work reinforce the concept of the microbiota as a dynamic organ with systemic impact.

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