# Abstract

# 2.1 Introduction.

The human gut is home to a complex ecosystem of more than 100 trillion symbiotic microorganisms, which far exceeds the number of host cells. (Dekaboruah et al., 2020). The intestinal tract is the body's largest immune system, interacting with antigens and mechanisms of both the immune system and the central nervous system (CNS) **(Pelaseyed et al., 2014)**. It is estimated that around 70% of the immune system is generated in the gut **(Hashemi et al., 2023; Hou et al., 2022)**. This ecosystem, known as the gut microbiota, plays a fundamental role in human health, participating in essential biological processes such as nutrient extraction, metabolism, vitamin synthesis, and regulation of the immune system (Bouskra et al., 2008; Hou et al., 2022). Under balanced conditions, the gut microbiota contributes to stability, resilience, and beneficial symbiosis for the host, acting as an additional organ, preventing uncontrolled absorption of toxic or pathogenic compounds, as well as in the regulation of the immune response **(Hashemi et al., 2023; Hou et al., 2022)**. However, dysbiosis, or 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, modulation of gut microbiota using probiotics has emerged as a promising strategy for preventing and treating different pathologies. (Cremon et al., 2018; Sanders et al., 2019). Probiotics, defined as live microorganisms administered in adequate amounts that confer health benefits, have demonstrated their ability to protect against pathogens, inhibit colonization by 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), which are considered GRAS (Generally Recognised 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 subespecie thermophilus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Lactococcus* y *Escherichia coli Nissle* 1917, among others (Kumari et al., 2020).

Consequently, modification of gut microbial communities, whether by including or excluding specific microorganisms, can potentially prevent the development of diverse diseases. (Cani and 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. (Cerdó et al., 2019; Trejo & Sanz, 2013). These receptors, in addition, modulate the function of intestinal immune cells, thereby influencing immune homeostasis and inflammatory response. (Lee & Kim, 2007; Zmora et al., 2019). Therefore, the identification and characterization of the effect of a specific probiotic on the expression and modulation of genes associated with human pathologies are essential to understanding the underlying mechanisms of these diseases and developing more precise therapeutic interventions.

In this study, transcriptomic analysis was conducted on the cell lines Caco-2 and HT-29 treated with the probiotics *Bacillus subtilis* CW14 and *Propionibacterium freudenreichii* ITG P9, respectively. The objective of this analysis was to identify differentially expressed genes (DEGs) and to 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 integrated approach will advance the understanding of how probiotics can modulate gene networks associated with human diseases, opening new avenues for the development of gut microbiota-based therapies.

# 2.2. Materials and methods.

## 2.2.1. Differential expression analysis and data collection.

A comprehensive search of the Gene Expression Omnibus database was performed to obtain differential gene expression data related to probiotics' effect on colon cells. (GEO) (Clough & Barrett, 2016). A series of keywords, detailed in the [supplementary material S1](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/7_Selection%20criteria%20and%20search/README.md), were used to filter for relevant studies. From the results obtained, two studies were selected that met the following inclusion criteria: 1) colorectal adenocarcinoma cells as an experimental model, 2) evaluation of effect on the colon, and 3) probiotic-based treatments. The selected data includes 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).

Principal Component Analysis (PCA) was conducted using the DESeq2 (version 1.38.1) (Love et al., 2014) package to distinguish between the different groups and their respective controls. Differential expression analysis was performed with DESeq2 (version 1.38.1) (Love et al., 2014), to normalize the expression counts for each experiment. A base 2 logarithmic transformation of the fold change (log2FC (FC) ≥ 2 and an FDR value ≤ 0.05) was performed to interpret the results to account for differentially expressed genes. For better integration of the results, the names of the DEGs were converted to Entrez IDs using the Ensemble database. (Harrison et al., 2024) and **UniProt** (Consortium et al., 2025) through specific APIs. These identifiers facilitated querying and cross-annotation across different databases.

## 2.2.2. Functional enrichment and pathway analysis.

Gene ontology (GO) functional analysis was performed using Enrichr (E. Y. Chen et al., 2013), focusing on three main categories: GO Biological Process 2023 (Carbon et al., 2019). The identification of biological pathways associated with DEGs was performed using the following databases: KEGG 2021 Human (Ogata et al., 1999)and Elsevier Pathway Collection (Nesterova et al., 2019). These databases enabled the mapping of DEGs into metabolic pathways and signaling processes. As a result, a comprehensive biological context of probiotic-induced alterations in colon cells was established. It is important to note that the threshold for enrichment analyses was set at Padj ≤ 0.05.

## 2.2.3. Association of DEGs with human diseases.

Annotations were made to associate DEGs with human diseases, using specialized databases., such as DisGeNet (Piñero et al., 2020), GeDiNet 2023 (Kundu et al., 2023), Jensen DISEASES (Pletscher-Frankild et al., 2015), Virus-Host PPI P-HIPSTer 2020 (Lasso et al., 2019), Orphanet Augmented (Orphanet, 2025). This analysis enabled the identification of potential associations between affected genes and human diseases. The thresholds established for the enrichment analyses were Padj ≤ 0.05.

**2.2.4. Protein-Protein Interactions (PPIs) and Visualization of Results.**

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

# 2.3. Results.

## 2.3.1. HT-29 and Caco-2 cell lines treated with various probiotics show differentially expressed mRNA.

PCA analysis revealed marked transcriptional responses in both probiotics. (**Fig.1)**. For *P. freudenreichii* ITG P9 showed exceptionally high variance, with a PC1 of 94%, reflecting a clear separation between treatments and controls. While PC1 of *B. subtilis* CW14 explained 68% of the variance, suggesting that this axis contributes significantly to the separation of the groups. These results suggest that the treatment effect varies by probiotic, with a more pronounced response by *P. freudenreichii* *ITG P9* and indicating high mRNA heterogeneity as observed in the spatial distribution of treatments, which is evidence of intra-group variability.

Following filtration of transcripts with a log2FC ≥ 2 and an FDR ≤ 0.05, a total of 2,337 genes were obtained from the *P. freudenreichii* treatment, of which 1457 (62.34 %) were positively regulated and 880 (37.66 %) were negatively regulated. This suggests a broad and robust transcriptional response. In the case of *B. subtilis* CW14, 198 genes were obtained, 136 (68.69%) were positively regulated and 62 (31.31%) were negatively regulated. In both cases, a trend towards 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.

## 2.3.2. Functional enrichment analysis of biological processes and pathways.

Analysis of *B. subtilis* CW14-treated Caco-2 cells and *P. freudenreichii* ITG P9-treated HT-29 cells revealed significant modulation of DEGs through the implementation of expression change thresholds log2FC ≥ 2, an FDR value ≤ 0.05 and a Padj ≤ 0.05. These criteria allowed the selection of a set of genes associated with important biological processes, including cell cycle, immunity, adhesion, inflammation, and transport. In addition, key metabolic and signaling pathways were identified. All results obtained for gene ontology terms (Enrichr), metabolic and signaling pathways (KEGG and Elsevier Pathway Collection) can be viewed respectively in **Tables** [**S1**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S1_GO_Biological_Process_terms_sorted_with_FC.xlsx)**,** [**S2,**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S2_Elsevier_Pathway_Collection_terms_sorted_with_all.xlsx) and [**S3**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S3_KEGG_2021_Human_terms_sorted_with_all.xlsx).

Transcriptomic analysis of *B. subtilis* CW14-treated Caco-2 cells revealed a coordinated and simultaneous response of immune signaling pathways and defense mechanisms. Overexpression of chemokines and immunostimulatory 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-TNFSF3* (+3.07)and *PLAU* (+3.36). This observation suggests the activation of the *NF-κB* pathway, which likely facilitates the recruitment of T lymphocytes, neutrophils, and the differentiation of macrophages and granulocytes. This, in turn, promotes both the elimination of pathogens and the promotion of reparative processes in the intestinal epithelium (Anderson, 2023a; Peng et al., 2019; Upadhyay & Fu, 2013; Yamazaki et al., 2022). Furthermore, the increased expression of chemokines such as *CXCL8* (+4.65)*, CXCL10* (+4.34)*, CXCL11* (+2.82)*,* and *CX3CL2* (+3.01)indicates the activation of pathways that promote neutrophil chemotaxis and migration and mast cell activation, contributing to a coordinated immune response to microbial pathogens (Kochumon et al., 2020). Similarly, the increased expression of *CCL5* (+4.16) together 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 towards a reparative phenotype, which could contribute to mitigating epithelial damage under inflammatory conditions. Other genes such as *TNFAPI3* (+2.31) and *TNFSF14* (+2.95) could contribute to the control of intestinal inflammation, either by blocking *NF-κB* signaling or by apoptosis (Kolodziej et al., 2011; Krause et al., 2014).

Concurrently, effects on genes associated with stress response and metabolic activity were identified. Overexpression of the *CYP1B1* (+2.61) gene suggests the activation of detoxification pathways involved in the neutralization of xenobiotic compounds (such as mycotoxins), while the upregulation of *BIRC3* (+2.51) and downregulation of *RGS2* (-2.11) indicate the involvement of anti-apoptotic mechanisms that favor the survival of epithelial cells against oxidative stress (Pauletto et al., 2020). In contrast, the reduction in *HSPA6* (-2.72) expression may indicate an adaptation of the cellular system to stress conditions by optimizing resources in the face of a gastrointestinal environment that demands immune and repair responses (L. Chen et al., 2021; Neurath, 2014). Representative DEGs with a log2FC (LFC) doubling ≥ 2 for *B. subtilis* CW14 are shown in **Table 1**. All results obtained can be seen in **Tables** [**S1**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S1_GO_Biological_Process_terms_sorted_with_FC.xlsx)**,** [**S2,**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S2_Elsevier_Pathway_Collection_terms_sorted_with_all.xlsx) and [**S3**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S3_KEGG_2021_Human_terms_sorted_with_all.xlsx).

|  |  |  |  |
| --- | --- | --- | --- |
| Genes | Term | Adjusted P-value | Log2FoldChange |
| 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.

Transcriptomic analysis of HT-29 cells treated with *P. freudenreichii* ITG P9 revealed a coordinated response of *CDKN1A* (+4.21), *CDKN2B* (+2.84), and *CDKN1C* (+2.43) genes, which are associated with cell cycle regulation suggesting mechanisms associated with cell arrest through the G1/S transition phase and thus in reducing the proliferation of damaged cells (Abbas & Dutta, 2009). The BRSK2 (+2.64) and NES (+2.02) genes, which are involved in cycle transitions (G2/M), were also identified, suggesting the possibility of modulating the cell cycle under stress conditions (Cousin et al., 2016; Y. Wang et al., 2012). All results obtained for *P. freudenreichii* ITG P9 can be found in **Tables** [**S1**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S1_GO_Biological_Process_terms_sorted_with_FC.xlsx)**,** [**S2,**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S2_Elsevier_Pathway_Collection_terms_sorted_with_all.xlsx) and [**S3**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S3_KEGG_2021_Human_terms_sorted_with_all.xlsx)**.**

In a unified manner, changes in gene expression indicate that *B. subtilis* CW14 exerts a dual impact on intestinal cells. Firstly, it activates a controlled proinflammatory response by modulating chemokines and factors that promote the recruitment and activation of immune cells. Secondly, it modulates protective and detoxification mechanisms that contribute to the protection of epithelial integrity. In contrast, *P. freudenreichii* ITG P9 instigates cell cycle reprogramming to arrest cells in critical phases, such as G1/S and G2/M, whilst concurrently promoting defense mechanisms against stress.

## 2.3.3. Differentially expressed genes (DEGs) modulated by probiotics are repositioned as modulators in different pathologies having pluri-employment annotations.

**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**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S4_Genes_dysbiosis_diseases_with_FC_sorted_all.xlsx)**)**, neurological disorders **(Table** [**S5**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S5_Genes_neurological_diseases_with_FC_sorted_all.xlsx)**)**, and rare or orphan syndromes **(Table** [**S6**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S6_Genes_rare_diseases_with_FC_sorted_all.xlsx)**)** 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**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S1_GO_Biological_Process_terms_sorted_with_FC.xlsx)**)**. 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**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S4_Genes_dysbiosis_diseases_with_FC_sorted_all.xlsx)**,** [**S5**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S5_Genes_neurological_diseases_with_FC_sorted_all.xlsx)**,** [**S6**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S6_Genes_rare_diseases_with_FC_sorted_all.xlsx)**),** suggesting a dual role in the activation of innate immunity and pleiotropic mechanisms beyond the gut environment. In the context of the gut-brain axis, these findings become pertinent, as previous studies have demonstrated that probiotic strains, such as *B. subtilis*, can modulate the gut immune response. This, in turn, may have the capacity to influence neuroinflammatory processes and central nervous system (CNS) (Vida et al., 2024). Concurrently, modulation of CSF2 and CSF3, regulators of immune cell proliferation and differentiation, suggests that *B. subtilis* strain CW14 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**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S7_diseases_rare_dysbiosis_neurological.xlsx)**)**, 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**.

Gráfico, Gráfico de barras

El contenido generado por IA puede ser incorrecto.**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.

Treatment of HT-29 cells with *P. freudenreichii* ITG P9 resulted in evidence of dual modulation of gene expression, characterized by both positive and negative regulation of specific genes **(Table** [**S1**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S1_GO_Biological_Process_terms_sorted_with_FC.xlsx)**)**. Enrichment analyses indicate that the *SH2D3C* (+6.80) and *CORO1A* (+3.79) both show an association with the polyhydramnios-megalencephaly-epilepsy symptomatic syndrome. Similarly, *KIFC2* (+2.14) has been linked to diseases such as Charcot-Marie-Tooth disease type 2P, type 4B3, and adult-onset dystonia-parkinsonism. The *KIAA0513* (+4.52) gene has been associated with intellectual disability-obesity-brain malformations-facial deformity syndrome and AD. This group of genes has been linked to synaptic plasticity, suggesting a potential role in maintaining the structure of the nervous system and thus a neuroprotective effect. This effect could be mediated by the regulation of genes involved in stabilizing the neuronal cytoskeleton, thereby strengthening the hypothesis of a connection between gut microbiota modulation and neurological pathways (Biggs et al., 2025; Gerik-Celebi et al., 2023; Herbin et al., 2016; Shimojima et al., 2017; Zheng et al., 2022; M. Zhu et al., 2020). In contrast, down-regulation of *KIF20A* (-2,19), a gene enriched in citrullinemia type II, a primary immunodeficiency with natural killer cell deficiency and adrenal insufficiency, was observed. Research has indicated a correlation between this gene and the suppression of glioblastoma cell invasion and proliferation, suggesting a potential tumor suppressor mechanism (Saadh et al., 2025). These findings are pertinent in the context of the gut-brain axis, as they suggest the modulation of an intestinal immune response, which influences neuroinflammatory processes and central nervous system homeostasis (Kim et al., 2024). All the results obtained can be seen in **Tables** [**S4**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S4_Genes_dysbiosis_diseases_with_FC_sorted_all.xlsx)**,** [**S5**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S5_Genes_neurological_diseases_with_FC_sorted_all.xlsx)**,** and [**S6**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S6_Genes_rare_diseases_with_FC_sorted_all.xlsx). A summary of the genes associated with different pathologies can be seen in **Fig. 3**.

Gráfico, Gráfico de líneas

Descripción generada automáticamente**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 differentially expressed genes (DEGs) with multi-association profiles to various pathologies **(Table** [**S7**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S7_diseases_rare_dysbiosis_neurological.xlsx)**)**. Genes with pleiotropic roles were highlighted, simultaneously linked to dysbiosis, cancer, neurological diseases, bacterial infections, and rare and viral diseases **(Figure 4)**. In this regard, the genes *CCL4*, *CSF2*, *CSF3*, *CXCL8*, *CXCL10,* and *CCL5* emerged as central nodes, showing a significant positive Log2FC and being associated with the pathological categories analyzed **(Table** [**S7**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S7_diseases_rare_dysbiosis_neurological.xlsx)**)**. 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.

Gráfico de dispersión

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**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.

## 2.3.4. Interacciones Proteína-Proteína (PPI)

As mentioned above, from the analysis of DEGs for cells treated with *B. subtilis* CW14 and 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 regard, the analysis of protein-protein interactions (PPIs) reveals the complex network of connections that the studied DEGs establish with other proteins and signaling pathways involved in the innate and adaptive immune response (see [Fig. S1](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Fig_S1_Bacillus_subtilis_CW14_CCL4_First_Neighbors.pdf) CCL4 network in the supplementary material). For example, the CXCL8 (IL-8) and CXCL10 genes and their association with multiple autoimmune and inflammatory diseases, support their role as potent chemokines involved in the migration and activation of leukocytes to the intestinal epithelium. Moreover, the presence of *CCL4* (MIP-1β) in the network underlines its potential role in the regulation of inflammatory responses and its link with chronic inflammatory processes, indicating its involvement in the regulation of immune cell recruitment in various pathological contexts. In addition, terms were identified that emphasize the importance of e.g. the ERK1/2 pathway in cell signaling, linking inflammation to processes of proliferation, differentiation, and cytokine response (Chandiok, 2024). Similarly, *CSF2* (GM-CSF) and *CSF3* (G-CSF) and their association with diseases in which the immune microenvironment and hematopoiesis are altered suggest that modulation of these two components may influence gut homeostasis and thus prevent systemic complications. The network also highlights the convergence of inflammatory and signaling pathways mediated by these DEGs with other pathways also linked to the adaptive immune response involving cytokines such as *IL-1, IL-4, IL-10,* and *IL-13*. This association suggests that *B. subtilis* CW14 not only affects the local epithelial response but may also have systemic effects by modulating other 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).

Diagrama

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**Figure 5. PPI networks of KIF20A and OASL in HT-29 cells after treatment with *P. freudenreichii* ITG P9.** The image displays 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.

# 2.4. Discussion.

The modulation of the intestinal cellular response through the implementation of probiotics has attracted the attention of researchers in recent years, especially for its potential to influence intestinal epithelial homeostasis, activation of immune pathways, reprogramming of the cell cycle by modulating critical phase arrests in damaged cells and the prevention of inflammatory, neoplastic and other human pathologies (Q. Wang et al., 2021). Previous studies have shown that the interaction between probiotics and intestinal epithelium promotes the modulation of local responses, which can lead to the improvement of the intestinal barrier, the inhibition of pathogens, the modulation and maturation of the immune system, and the reduction of inflammation and carcinogenic processes (Do Carmo et al., 2017; Foligné et al., 2010; Peng et al., 2019).

The results obtained show significant differences in the differential mRNA expression 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 observed through the overexpression of chemokines and inflammatory factors, meanwhile, in the second case, 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, in addition, related to molecular mechanisms regulating epithelial homeostasis, inflammation, cell adhesion, stress response, and cell cycle arrest.

## 2.4.1. General response of Caco-2 cells to *Bacillus subtilis* CW14 treatment.

Transcriptomic analysis revealed overexpression of chemokines and immunostimulatory factors **(Table** [**S1**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S1_GO_Biological_Process_terms_sorted_with_FC.xlsx)**)**. These genes are involved in the recruitment of T lymphocytes, neutrophils, and macrophage differentiation, suggesting activation of the *NF-κB* signaling pathway, a central axis in the inflammatory response and defense against pathogens (Peng et al., 2019). In this context, the chemokine *CCL4* is known to attract immune cells expressing the *CCR5* receptor, and its overexpression has been associated with inflammatory states in the gut and immune regulation (Chen et al., 2022). In addition, colony-stimulating factors such as *CSF2* (GM-CSF), and *CSF3* (G-CSF) modulate the proliferation, differentiation, survival, maturation, and functional activation of hematopoietic cells, including granulocytes and macrophages, enhancing the innate response through 4 distinct signaling pathways *PI3K/Akt, ERK1/2, JAK2/STAT5* y *NF-kB* (Bhattacharya et al., 2015). The hypothesis is supported by the fact that the over-expression of *NFKBIZ*, a regulator required for *NF-κB*-mediated modulation of transcription, is associated with cytokine release and amplification of the inflammatory response (Yamazaki et al., 2022). Furthermore, the over-expression of chemokines such as *CXCL8, CXCL10, CXCL11*, and *CX3CL2* indicates the activation of pathways that promote chemotaxis, resulting in the migration of neutrophils and *NK* cells to sites of infection or tissue damage. CXCL8 is one of the most important pro-inflammatory factors released by intestinal epithelial cells, playing a key role in various inflammatory diseases, through multiple signaling pathways, including *PI3k/Akt, MAPK,* and *NF-κB* (Y. Zhu et al., 2021).

In addition to the activation of immune pathways, modulation of chemokines and immune factors that could promote defense and repair mechanisms of the intestinal epithelium were also observed. Overexpression of *CCL5* in association with *CCL22* and *CCL2* suggests the attraction of monocytes and Tregs, which would contribute to the polarization of macrophages towards a reparative and anti-inflammatory phenotype. This combination of effector and regulatory cell attraction is crucial for repairing epithelial damage and preventing inflammatory processes that can trigger gut barrier disruptions(Peng et al., 2019). Thus, the activation of different pathways such as *NF-κB*, mediated by probiotics, through the overexpression of different genes, not only promotes the inflammatory response but can also trigger the production of growth and tissue repair factors. Indeed, the *NF-κB* pathway has been associated with the activation of repair mechanisms, which facilitates the reduction of inflammation and restoration of epithelial integrity (Z. Liu et al., 2022).

Concurrently, alterations in genes linked to metabolic activity and stress were detected. Overexpression of *CYP1B1*(+2,60), an enzyme categorized within the cytochrome P450 family, was observed to be implicated in the detoxification of xenobiotics, in addition to lipid and steroid hormone metabolism (Shah et al., 2019). In this context, *CYP1B1* induction could be indicative of an adaptive response aimed at maintaining gut balance, thereby suggesting a mechanism of detoxification or metabolism of beneficial compounds derived from probiotics or microbiota. This contrasts with a promotion of proinflammatory signals, which would be consistent with a role in modulating pathways such as Ahr (aryl hydrocarbon receptor), which is key in the response to microbial metabolites (Schiering et al., 2017). For instance, the *AhR/HIF1α* pathway is activated by microbiota-derived ligands (e.g. tryptophan), which in turn induce *CYP1A1/CPYP1B1*, thereby facilitating the metabolism of these ligands and prevent immune overstimulation. This suggests that CPYP1B1 may function as a feedback regulator for immune homeostasis (Schiering et al., 2017).

Conversely, elevated *BIRC3* expression and the negative regulation of *RGS2* suggest the activation of anti-apoptotic mechanisms that promote epithelial cell survival under stress conditions (Pauletto et al., 2020). In this regard, *BIRC3* (+2,51), a member of the inhibitor of the apoptosis (IAP) family, through inactivation of CASP cascades, exerts this function, but in addition, it plays a crucial role in neuronal function through its NPD1-mediated positive regulation promoting neuronal cell survival (Martin-Gallausiaux et al., 2022). Furthermore, *BIRC3* has been reported to be associated with crypt regeneration and, consequently, in the renewal and function of the intestinal epithelial barrier (Martin-Gallausiaux et al., 2022). Conversely, the study conducted by Hu & Shao, 2022 revealed that positive regulation of *BIRC3*, mediated by the probiotic *Lactobacillus pentosus*, contributed to the inactivation of the *NLRC4* inflammasome, which suppressed neuronal pyroptosis, suggesting it as an alternative strategy for the treatment of neurodegenerative diseases.

Concerning *RGS2* (-2,11), it is a regulator of the G protein-coupled receptor (GPCR) signaling pathway, which belongs to the RGS superfamily of proteins. The expression of RGS2 is subject to epigenetic, transcriptional, and post-translational mechanisms (Pauletto et al., 2020). The negative regulation of *RGS2* suggests a possible influence of this probiotic on the modulation of GPCR-mediated signaling in the intestinal epithelium; since *RGS2* is involved in the regulation of T-cell immunity and antioxidant response, its downregulation could be related to a reduction of oxidative stress or inflammation in these cells. These findings are consistent with those reported by (Li et al., 2023), who have proposed that negative regulation of *RGS2* contributes indirectly to gastrointestinal homeostasis.

Finally, the finding of reduced expression of *HSPA6* (-2,72) is of interest, given that this gene encodes a chaperone of the heat shock protein (HSP) group. The induction of this gene is often observed in response to stress stimuli, such as exposure to toxins, oxidative stress, or heat conditions that compromise cellular integrity (Kim et al., 2024; B. Song et al., 2022). Its functions include protein quality control, including correct protein folding, refolding, and control of subsequent protein degradation (B. Song et al., 2022). Modulation of heat shock proteins such as *HSPA6* is closely associated with epithelial barrier function (Ohkawara et al., 2006). In this regard, the observed state of relaxation in the *HSPA6* gene may be a consequence of the modulation exerted by the probiotic, as, in the absence of stressors, cells do not require its activity, thereby maintaining intestinal cell homeostasis.

## 2.4.2. General response of HT-29 cells treated with *P. freudenreichii* ITG P9.

Treatment of HT-29 cells with *P. freudenreichii* ITG P9 resulted in a transcriptional response focused on cell cycle modulation. Positive overexpression of genes such as *CDKN1A, CDKN2B*, and *CDKN1C* was observed **(Table** [**S1**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S1_GO_Biological_Process_terms_sorted_with_FC.xlsx)**)**. These genes encode cyclin-dependent kinase inhibitors (*CKIs*) that play a critical role in cell cycle arrest at the G1/S transition (Cousin et al., 2016; Y. Wang et al., 2012). Their activation is crucial to prevent the proliferation of damaged and potentially cancerous cells, suggesting that *P. freudenreichii* may promote a protective arrest state that could prevent the spread of damaged cells or oxidative stress (Cousin et al., 2012; Cousin et al., 2016). In this sense, the induction of cell arrest at critical stages of the cell cycle is interpreted as a protective mechanism against stress that would allow the cell to initiate repair processes or drive the cell toward apoptosis in the event of irreparable damage. Previous studies have shown that the induction of *CDKN1A* (p21) by compounds that alter histone acetylation, such as short-chain fatty acids, is a mechanism in the prevention of colon carcinogenesis. Indeed, down-regulation of these three genes has been associated with aberrant proliferation and thus the potential to trigger tumor transformation, as it has been suggested, for example, that CDKN1A may play a key role in inhibiting epithelial-mesenchymal transition (EMT), migration, and invasion (Bueno-Fortes et al., 2022; Tu et al., 2017; Yang et al., 2023). This finding highlights the importance of positive and controlled regulation of *CDKN1A*, *CDKN2B,* and *CDKN1C*.

Moreover, the regulation of genes involved in the G2/M transition, such as *BRSK2* and *NES*, suggests that they may not only act in the G1/S phase but also modulate cycle progression in later phases. The coordination of these mechanisms may be crucial to arrest cell proliferation in response to a hostile environment, such as that generated in inflammatory conditions or the presence of oxidative stress, thus preventing the accumulation of mutations and eventual malignant transformation (Cousin et al., 2016; Y. Wang et al., 2012). In this context, *BRSK2* is known to be a serine/threonine protein kinase, a member of the *AMPK* family, which plays roles in apoptosis and cell polarity that are crucial for normal physiology (Z. Chen et al., 2025; Y. Wang et al., 2012). In the context of pancreatic cancer, the study by (Saiyin et al., 2017) showed that pancreatic ductal adenocarcinoma (PDAC) neoplastic cells respond to nutrient deprivation by inducing *BRSK2*. This repression removes the feedback that mTORC1 normally exerts on *Akt* signaling, allowing Akt activity to increase, and giving cancer cells a survival advantage in an energetically unfavorable environment. However, *BRSK2* is positively modulated after treatment with *P. freudenreichii* ITG P9. This finding suggests that metabolites or signals released by this probiotic could induce a state of metabolic stress like that of nutrient deprivation. Consequently, the activation of *BRSK2* in intestinal cells (HT-29) could represent an adaptive response comparable to that observed in PDAC, with implications for the regulation of *Akt/mTOR* pathways. This is particularly relevant given that *P. freudenreichii* ITG P9 is known to produce short-chain fatty acids (e.g. propionate and acetate), compounds that can alter cellular metabolism and activate energy stress sensors such as *AMP* kinases (*AMPKs*) and their related members, including *BRSK2*. Thus, the positive modulation of *BRSK2* in HT-29 cells following exposure to *P. freudenreichii* ITG P9 may be part of a cellular response aimed at coping with an environment of energy deprivation or metabolic stress-induced, for example, by bacterial metabolites. In this sense, the increase in *BRSK2* modulated by this probiotic could contribute to a better management of metabolic stress, protecting cellular integrity. Furthermore, the positive modulation of *BRSK2* is directly related to the *Akt/mTOR* pathway, which could favor the coordination of this pathway, allowing cells to adapt to conditions of low energy availability without compromising crucial functions.

In the case of *NES*, a cytoskeletal protein expressed in a variety of neural stem cells, it has been linked to the maintenance of the balance between neuronal apoptosis and neurogenesis (Lombardi & Dicks, 2022). Studies in transgenic mice suggest that NES expression is a signature of neuronal plasticity in the adult ENS (enteric nervous system) (De Vadder et al., 2018). A study showed that adult enteric neurogenesis is an active process that is balanced by apoptosis (Kulkarni et al., 2017). In contrast, another study showed that *NES* is essential for tumor cells such as hepatocellular carcinoma cells to progress through the G2/M phase, as it participates in the formation of the mitotic spindle (Wang et al., 2021). In this context, the increase in *NES* suggests that HT-29 cells activate mechanisms that favor mitotic spindle assembly and G2 phase transition. This could be interpreted as the activation of regenerative pathways that help intestinal cells to efficiently progress through the G2/M phase. This would be beneficial in situations of stress or damage, where rapid and orderly cell turnover is crucial for tissue repair. In addition, *NES* contributes to the stabilization of *βII-tubulin* and the correct assembly of the spindle (Wang et al., 2021), its expression in intestinal cells could promote orderly mitotic division. This mechanism would ensure that, even under adverse conditions, cells can complete their cell cycle without errors that compromise tissue integrity, which may ultimately contribute to the maintenance of intestinal epithelial cell integrity and function. However, the mechanisms by which gut bacteria or probiotics impact ENS organization remain unknown. Therefore, it is likely that nitrergic and serotonergic pathways interact with the microbiota to establish a functional and stable ENS (De Vadder et al., 2018).

## 2.4.3. Pleiotropic effects of probiotics *B. subtilis* CW14 and *P. freudenreichii* ITG P9.

Over the past decades, it has been recognized that the gut microbiota plays an essential role in the regulation of immune and metabolic homeostasis (Belnap et al., 2024; Zhao et al., 2023), but more interesting is the growing number of studies linking the microbiota-gut-brain axis in the modulation of critical molecular pathways that influence not only systemic homeostasis but also the regulation of complex diseases, including neurological, metabolic disorders and rare syndromes that may have shared pathophysiological complexities (Belnap et al., 2024; Zhao et al., 2023). The intestinal tract is the largest immune system in the body and interacts with antigens and immune system 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 sustaining the homeostasis of the immune system and preventing the uncontrolled absorption of toxic or pathogenic compounds (Hashemi et al., 2023). Intestinal micro-organisms are also known to have a significant effect on metabolism, cell population balance, immune response, and the neurological system (Bienenstock et al., 2015). Thus, the microbiota may influence the onset and development of both intestinal and other microbial diseases (Plaza-Diaz et al., 2014; Sarkar et al., 2016). In this regard, accumulating evidence suggests that probiotic interventions not only restore the balance of the microbiota but also modulate gene expression in intestinal epithelial cells by affecting the secretion of chemokines, cytokines, and growth factors that affect inflammatory and neuroimmunological responses (Plaza-Diaz et al., 2014; Sarkar et al., 2016). Studies have shown that administration of certain probiotic strains induces changes in gene expression, which may have therapeutic effects in conditions as diverse as AD, PD, metabolic disorders, and rare syndromes (Hashemi et al., 2023). The results obtained in this study reinforce the idea that probiotics play a crucial role in the intestinal epithelium, as well as being closely linked to the balance in immune and brain modulation.

## 2.4.4. Pleiotropic effects *B. subtilis* CW14.

Enrichment analyses for *B. subtilis* CW14-treated Caco-2 cells showed a diversity of pleiotropic effects for the different disease categories **(Table S4 to S7, Fig. 2)**. When analyzing the modulated genes, a clear overlap between genes associated with inflammatory bowel, metabolic and neurological diseases was observed. This suggests that this probiotic may play a key role in the regulation of systemic inflammatory processes. The most obvious pattern is the observed interconnection between gut dysbiosis and neurodegenerative diseases **(Table** [**S7**](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Table_S7_diseases_rare_dysbiosis_neurological.xlsx)**)**.

First, there are chemokines, a class of proteins that can attract and activate leukocytes. These are classified into two groups according to their functions: inflammatory chemokines and homeostatic chemokines (Wang et al., 2024). Some inflammatory chemokines exhibit homogeneous functions, while others can be positively regulated under certain extraordinary conditions (Vergunst & Tak, 2005; C. Wang et al., 2024). Inflammatory chemokines are crucial elements in many pathological processes, including autoimmune diseases (C. Wang et al., 2024). In the CNS, these inflammatory chemokines also play roles beyond their chemotactic function (Nombela-Cabrera et al., 2023). In this sense changes in the expression of inflammatory chemokines together with their respective receptors are involved in the pathogenesis of neurological diseases (e.g. AD, PD) (C. Wang et al., 2024). Functions include neuromodulation, regulation of the neuroendocrine axis, control of permeability, regulation of neurogenesis, neuroprotection, neurotoxicity and regulation of axonal germination and elongation (Semple et al., 2010).

In this context, this study showed that exposure of Caco 2 cells to the probiotic *B. subtilis* CW14 promotes a positive modulation in the expression of a set of chemokines (*CCL2, CCL4, CCL5, CCL22, CXCL1, CXCL2, CX3CL1* and *CXCL8, CXCL10*), which is of particular interest when considering the relevance of these molecules in the regulation of inflammatory processes and their involvement in several neurodegenerative and cognitive diseases, as mentioned above. For example, *CCL2* elevation is recognized for its dual role; while under homeostatic conditions its function in attracting and activating monocytes and microglia may favor the elimination of protein aggregates, its overexpression in pathological contexts is associated with exacerbation of neuroinflammation and blood-brain barrier dysfunction, contributing to the development and progression of diseases such as AD and PD (Wang et al., 2024). Additionally, the positive modulation of *CCL4* and *CCL5* supports the concept of increased immune cell recruitment, which may lead to a chronic inflammatory response. In the context of the gut-brain axis, this response could contribute to neurodegeneration (Semple et al., 2010).

The chemokine *CCL22* and its receptor *CCR4* play a crucial role in homeostasis and inflammatory responses (Scheu et al., 2017). Microbial products, such as lipopolysaccharides, and cytokines, such as *IL-1β*, tumor necrosis factor (*TNF*) and *CD40* ligand, induce *CCL22* production in dendritic cells (Yamashita & Kuroda, 2002). *CCL22* is believed to be involved in various pathologies, ranging from allergic reactions, autoimmunity such as MS and metabolic syndromes (type I diabetes) (Montane et al., 2011; Scheu et al., 2017; Yamashita & Kuroda, 2002). Interestingly, the immunomodulatory properties of *CCL22* in autoimmunity involve Treg cell responses (Scheu et al., 2017). In this regard, overexpression of *CCL22* in pancreatic *β-cells* in type I diabetes model mice prevented autoimmune attack by recruiting Treg cells, ultimately providing protection against the progression of type I diabetes (Montane et al., 2011).

On the other hand, chemokines such as *CXCL1* and *CXCL2* are crucial in attracting neutrophils and enhancing local inflammatory responses; in the intestinal model, their upregulation may reflect an immune surveillance mechanism that, via distant signaling, contributes to the regulation of the neuroimmune microenvironment (C. Wang et al., 2024). *CX3CL1* also plays an interesting role, since, under equilibrium conditions, it promotes communication between neurons and microglia and, in pathological situations, it can participate in the deregulated activation of microglia, generating inflammation (H. Liu et al., 2020). Finally, the chemokine *CXCL8* (*IL-8*) is known both for its ability to recruit neutrophils and for its modulatory effects on the release of proinflammatory mediators; its overexpression has been linked to alterations in the integrity of the blood-brain barrier and the induction of neurotoxic processes in disease models (C. Wang et al., 2024). However, some experiments suggest that *CXCL8* may be neuroprotective, as it can inhibit *Aβ* peptide-induced neuronal apoptosis and increase neuronal production of brain-derived neurotrophic factor (John et al., 2008). Consequently, *CXCL8* may play a protective role in the pathogenesis of AD for example.

Another pro-inflammatory chemokine is *CXCL5*, which is associated with tissue remodeling (Hummitzsch et al., 2021). *CXCL5* has been shown to function as a promoter of angiogenesis (Ma et al., 2024). The biological effects of *CXCL5* in brain inflammation and neurodegenerative diseases have been achieved by interacting with *CXCR2* receptors and activating the p38 MAPK kinase signaling pathway (Yu et al., 2021). This underlines the importance of a coordinated modulation of *CXCL5*.

*CXCL10* is another chemokine expressed by cells of the immune system that influences the migration/relocation of macrophages, dendritic cells, NK cells and activated T-cell subsets in areas of inflammation (Skinner et al., 2019). Initially described as potentially important in attracting T-cells to psoriatic plaques (Gottlieb et al., 1988). *CXCL10* was subsequently shown to be expressed in numerous human inflammatory diseases including autoimmune diseases and cancer (Karin & Razon, 2018). *CXCL10* is also expressed in response to microbial infections and is important in attracting specific *CXCR3*-positive leukocytes to sites of infection that help control/eliminate the invading pathogen (Van Raemdonck et al., 2015). *CXCL11* has also been shown to have a higher affinity for *CXCR3* (Karin, 2020) and its function is aimed at directing the development of the regulatory T cell lineage-1 (Tr1) (Karin et al., 2016), it has therefore been suggested that these chemokines play an opposing role in the direction of T cell polarization, and as chemokine 11 (*CXCL11*) has a higher affinity for *CXCR3*, it is likely to dominate immune regulation.

In the same line of findings, the *RGS2* gene has been reported to be associated with different mental illnesses such as major depressive disorder (MDD), AD, schizophrenia, bipolar disorder (BD), autism spectrum disorder (ASD), post-traumatic stress disorder (PTSD) and Huntington's disease (HD) (Bhuvaneshwar & Gusev, 2024; Hadar et al., 2016). Negative regulation of this gene, as also evidenced in this study (-2,11), has been found to act as a neuroprotective factor (Hadar et al., 2016). A notable aspect of *RGS2* is its unique ability to inhibit the translation of the *eIF2Bε* protein mRNA (ε subunit of eukaryotic initiation factor 2B). This process is crucial for proper protein folding, a process that is impaired in neurodegenerative disorders such as HD, AD, prion diseases, and mutations that cause infantile (Bazan, 2014; Hu & Shao, 2022). The observation that *RGS2* is negatively regulated by *B. subtilis* CW14 is significant, as its reduction could assist in maintaining or restoring the translation efficiency of *eIF2Bε* mRNA, a critical component for proper protein folding. This is particularly relevant in HD, AD, and other conditions where protein misfolding contributes to pathology.

Positive regulation of *BIRC3* (+2,51) also has neuroprotective effects (Bazan, 2014; Hu & Shao, 2022). This gene plays crucial roles in neuronal function, as well as being involved in the steps preceding the switch between *NF-κB* pathway activation, apoptosis, and necrosis (Calandria et al., 2015). In line with the above, the study by Hu & Shao et al. (2022) showed that overexpression of *BIRC3* promoted by the probiotic *L. pentosus* suppressed the inflammatory response and neuronal pyroptosis, suggesting a neuroprotective role also in neurodegenerative diseases by this probiotic, which could be extrapolated to the case of *B. subtilis* CW14.

*RCAN1* (+2,10) is another gene associated with AD and other neurodegenerative diseases, in addition to Down syndrome when its elevation is prolonged (Ermak & Davies, 2013). Although the neurodegeneration-associated pathways associated with *RCAN1* are not entirely clear, it has been proposed that it may inhibit calcineurin and increase *GSK-3β* activity leading to neurodegeneration (Ermak & Davies, 2013). *RCAN1* may also reduce *ADP/ATP* exchange and *ATP* levels and cause increased mitochondrial production of superoxide and its dismutation product, hydrogen peroxide, leading to cell damage (Balaban et al., 2005). Furthermore, *RCAN1* can induce the opening of mitochondrial permeability transition pores (*mtPTP*) leading to necrosis, and it has been shown that *RCAN1* can facilitate neuronal apoptosis in cell culture (Sun et al., 2011). In this context, the positive modulation of this gene by B. subtilis CW14 suggests that it may enhance the extension of adaptive protective cellular responses to stress through the control of these pathways.

Activation of *PLAU* (+3,36) has been associated with inflammatory bowel processes, including diseases such as IBD. In this context, *PLAU*-mediated proteolysis could promote mucosal remodeling and resolution of chronic inflammation through the *NF-κB* pathway that exerts as a *PLAU* regulator (Anderson, 2023). *PLAU* has been reported to be involved in the degradation of beta-amyloid aggregates in AD, suggesting that its modulation may influence neuroinflammation and amyloid plaque accumulation, and its modulation by the *NF-κB* pathway also contributes to the interaction between *PLAU* and the mitochondrial melatonergic path, which may be related to diseases such as AD, glioblastoma, breast cancer, ALS and type 1 diabetes (Anderson, 2023a, 2023b). Positive upregulation of *PLAU* by *B. subtilis* CW14 could be associated with a protective mechanism favoring tissue repair and restoration of the intestinal barrier. Furthermore, its interaction with the fibrinolytic system suggests a role in the regulation of the intestinal inflammatory microenvironment, limiting fibrin accumulation and promoting a state of homeostasis (Liang et al., 2020). In the context of the gut-brain axis, a healthy gut microbiota, regulated by probiotics such as *B. subtilis* CW14, could contribute to the modulation of *PLAU* and thus reduce neuroinflammation and possible protection against neurodegeneration.

Genes related to autoimmunity also emerged from the enrichment analysis. In this regard, the gene κBζ (encoded by *NFKBIZ* (+2,28)), an inflammatory factor belonging to the IkappaB family of proteins, plays a central role in cytokine expression and the regulation of functions of the mononuclear phagocytic system, NK cells, T cells, and B cells (Feng et al., 2023). An increasing number of publications show that *IκBζ* is related to autoimmune diseases by regulating the release of proinflammatory or anti-inflammatory factors through the *NF-κB* pathway to cause autoimmune diseases and inhibit or promote tumors, as well as being related to inflammatory diseases (Crohn's disease and ulcerative colitis) (Kerami et al., 2014; Nanki et al., 2019), autoimmune diseases (Psoriasis) (Feng et al., 2023; Zhang et al., 2019) and neurological conditions (neuronal ischemia) (Xiong et al., 2021). In this regard, psoriasis is one of the autoimmune diseases most closely related to *NFKBIZ*. *IκBζ* acts as a transcriptional activator of proinflammatory genes in keratinocytes, and its positive regulation has been associated with disease progression (Feng et al., 2023). In this context, positive modulation of *NFKBIZ* by *B. subtilis* CW14 could influence skin inflammatory response and immune homeostasis by regulating *NFKBIZ* expression levels. Likewise, chronic antibody-mediated rejection has been observed in transplantation, indicating a key role in modulating the adaptive immune response (Feng et al., 2023; Zhang et al., 2019). *NFKBIZ* has also been found to be involved in neuroinflammatory processes. In models of neuronal ischemia, positive regulation of *NFKBIZ* has been shown to attenuate apoptosis and promote neuronal activity, suggesting a neuroprotective role in hypoxic-ischemic encephalopathy (Xiong et al., 2021). The fact that *B. subtilis* CW14 induced *NFKBIZ* expression in Caco-2 cells reinforces the idea of the importance of the gut-skin axis as well as the gut-brain axis and their roles in the homeostasis of both axes.

Another gene was *TNFAIP3* (+2.31), which encodes the A20 protein, a negative regulator of inflammation (Ciccacci et al., 2019; Musone et al., 2011). It modulates *TNF* signaling, Toll-like receptors (TLR), nucleotide-binding oligomerization domain (*NOD*), and *NF-κB* pathways (Ciccacci et al., 2019). Its role is essential to avoid excessive activation of the immune response and to prevent chronic inflammation and programmed cell death (Musone et al., 2011). *TNFAIP3* deficiency in immune cells has been linked to inflammatory processes and autoimmunity, leading to increased expression of the cytokine *IL-17* which is implicated in these diseases (Zhou et al., 2015). In this regard, dysregulation of *TNFAIP3* has been associated with multiple inflammatory bowel diseases, including Crohn's disease and ulcerative colitis (Burton et al., 2007; L. Wang et al., 2010). In these disorders, a decrease in A20 activity may lead to an exacerbated inflammatory response, while its positive upregulation may contribute to the resolution of intestinal inflammation (Musone et al., 2011). In autoimmune diseases such as rheumatoid arthritis (RA) (Dieguez-Gonzalez et al., 2009), primary Sjögren's syndrome (Ciccacci et al., 2019), loss of A20 function may lead to hyperactivation of the *NF-κB* pathway, promoting inflammation and tissue damage. In this line of findings, positive modulation of *TNFAIP3* by *B. subtilis* CW14 could favor epithelial protection by reducing inflammation in the gut, in addition to reducing the hyperinflammation characteristic of autoimmune diseases.

*TNFSF14* (*LIGTH*) (+2.95) is a member of the complex *TNF* family system (Ware et al., 2022). It is mainly expressed in inflammatory effector cells, including dendritic cells, natural killer (*NK*) cells, macrophages, neutrophils, innate lymphoid cells, T cells, and activated effector and memory *CD4* and *CD8* T cells (Croft et al., 2012). *TNFSF14* acts as a ligand for receptors such as *HVEM* (*TNFRSF14*) and *LTβR* (Ware et al., 2022). Activation of these pathways promotes the activation of the transcription factor *NF-κB*, triggering the expression of genes associated with inflammatory responses, cell differentiation, proliferation, and survival (Kou et al., 2019). These processes are essential for the coordination of the innate and adaptive immune response, and in appropriate physiological contexts, contribute to an efficient defense against different pathologies (Ware et al., 2022). Several studies have associated altered *TNFSF14* levels with the pathogenesis of *IBD*, asthma, autoimmune diseases (atopic dermatitis, scleroderma, MS), idiopathic pulmonary fibrosis (IPF), and metabolic diseases (type 2 diabetes, non-alcoholic fatty liver disease) (Ware et al., 2022). Since *TNFSF14* signaling by *HVEM* and *LTβR* activates proinflammatory gene pathways via the NF κB pathway and modulation of cytokines and chemokines, leading to recruitment and activation of T lymphocytes and other effector cells, a *B. subtilis* CW14 mediated modulation could be seen as a mechanism that controls and coordinates cellular communication between the epithelium and the immune system, resulting in homeostasis of the intestinal barrier and immune response.

Taken together, these results suggest that the action of *B. subtilis* CW14 on the intestinal epithelium may have beneficial effects in modulating systemic inflammatory responses, promoting a controlled modulation of the gut-brain axis. Moreover, the signaling pathways associated with these chemokines, involving G protein-coupled receptors (such as *CCR2*, *CCR4, CCR5, CXCR1,* and *CXCR2*, among others) and cascades such as *MAPK* and *NF-κB* activation, are closely linked to the pathological mechanisms underlying neurodegenerative, cognitive and autoimmune disorders. In this sense, the ability of this probiotic to induce the controlled expression of these chemokines could, depending on the context and magnitude of the response, favor repair mechanisms and immune surveillance.

## 2.4.5. Pleiotropic effects *P. freudenreichii* ITG P9

In the case of HT-29 cells treated with *P. freudenreichii* ITG P9, the enrichment analyses for pleiotropic effects for different diseases were not as diverse as those shown for the probiotic *B. subtilis CW14*, and no general pattern was observed interconnecting the enrichment analyses for the different disease categories studied **(Table S4 to S7, Fig. 3)**.

The *KIAA0513* (+4.52) gene is involved in both synaptic and apoptotic signaling and is associated with the cytoskeleton through binding proteins (Lauriat et al., 2006; M. Zhu et al., 2020), is enriched in neurons of normal human brains, and is closely associated with the *KIBRA* gene, a cytoplasmic phosphoprotein that exerts neuroprotective effects in AD (Lauriat et al., 2006; L. Song et al., 2019). **Lauriat et al. (2006)** indicated that low *KIAA0513* expression could reflect a pathological condition of the brain suggesting it is a potential biomarker for early diagnosis such as AD. These findings suggest that this probiotic could be promoting an anti-inflammatory response or modulating cell signaling which, by increasing the expression of *KIAA0513,* would indicate a protective mechanism of action or adaptive responses to cellular stress, resulting in benefits against pathologies such as AD.

Another gene associated with AD was *SH2D3C* (+6,80), which is crucial for cell adhesion and migration, with evidence of its role in promoting the adhesion and movement not only of T cells but also of other cell types, which has even been linked to invasiveness in certain cancers, such as breast cancer (Gomi et al., 2018; Qiao & Li, 2023; L. Wang et al., 2010). The study by **Gomi et al. (2018)** reported that exposure to *amyloid-β* oligomers induces *SH2D3C* overexpression in cortical neurons, a finding that correlated with increased levels of this protein in murine models of AD compared to controls. This evidence raises the hypothesis that positive regulation of *SH2D3C* not only modulates cell migration and adhesion in the immune system and tumor invasion processes but in the neuronal context, its overexpression may trigger neurotoxic mechanisms that contribute to the pathogenesis of neurodegenerative diseases such as AD. Therefore, *SH2D3C* activation could reflect the activation of signaling pathways like those activated in neurons exposed to *β-amyloid*. Although in HT29 cells, these findings open the possibility that modulation of *SH2D3C* by probiotics influences the regulated control of gut-brain-axis communication, suggesting new perspectives in the investigation of therapeutic strategies in both inflammatory processes and neurodegenerative diseases.

The positive modulation of *CORO1A* (+3.79) suggests a relevant interaction between this probiotic and the regulation of key cellular functions. *Coro1A*, a protein linked to the actin cytoskeleton, is involved in crucial processes such as cell migration, phagocytosis, and intracellular signaling, both in the immune system and in the NCS (Martorella et al., 2017; Pick et al., 2017). Immunologically, the regulation could affect macrophage and neutrophil function by influencing adhesion, migration and phagocytic response. As CORO1A is a critical regulator of neutrophil trafficking and β2 integrin (CD11/CD18) adhesion (Jayachandran & Pieters, 2015; Pick et al., 2017), its modulation could enhance the innate immune response in the context of infection or dysbiosis. In addition, *CORO1A* plays a critical role in neuronal signaling through the *cAMP/PKA* pathway, suggesting that its regulation may have neurophysiological implications. Previous studies in animal models have shown that *CORO1A* deficiency alters synaptic connectivity and social behavior **(Martorella et al., 2017)**, raising the possibility that its modulation by probiotics may have beneficial effects on neuronal function and the gut-brain axis. Notably, *CORO1A* has also been implicated in autoimmune diseases and immunodeficiencies (Pieters et al., 2013). *CORO1A* deficiency is a factor associated with severe combined immunodeficiency (*SCID*), which is characterized by selective T-cell lymphopenia despite a normal thymus and normal levels of B cells and natural killer cells (Jayachandran & Pieters, 2015). Therefore, modulation of *CORO1A* by *P. freudenreichii* may have therapeutic implications in regulating the adaptive immune response and preventing autoimmune diseases.

The positive modulation of *KIFC2* (+2,14) suggests a relevant interaction between this probiotic and the regulation of intracellular transport and neuronal development. *KIFC2* is a neuronal kinesin involved in multivesicular vesicle transport (MVB) and dendrite formation, and its function is key in the maintenance of synaptic connectivity and neuronal homeostasis (Beatriz et al., 2021; Saito et al., 1997). Studies have shown that kinesins, including KIFC2, play an essential role in the intracellular transport of proteins and organelles, a process essential for neuronal communication and the maintenance of synaptic structure (Hirokawa & Takemura, 2003; Saito et al., 1997). Deficiencies in *KIFC2* have been associated with defects in dendritic development, which may affect neuronal plasticity and contribute to neurodegenerative diseases **(Beatriz et al, 2021)**. From a pathological point of view, kinesin dysfunction has been implicated in diseases such as Charcot-Marie-Tooth hereditary neuropathy type 2A, in which axonal transport of synaptic vesicle precursors is impaired **(Hirokawa & Takemura, 2003)**. In this sense, the positive regulation of *KIFC2* by *P. freudenreichii* could represent a potentially beneficial mechanism in the prevention or alleviation of dysfunctions associated with this type of disorder, by regulating synaptic vesicle trafficking, promoting the efficiency of neuronal communication and strengthening the adaptive response of the nervous system.

In the same direction as the kinesins, a down-regulation of *KIF20A* (-2,19) was found. This is essential for cytokinesis and mitotic spindle assembly (Wu et al., 2019). Overexpression of *KIF20A* has been reported in multiple cancer types, including colorectal cancer, where it promotes cell proliferation and invasion **(Wu et al., 2019)**. Its inhibition can induce cell cycle arrest in the G2/M phase and increase apoptosis(Yan et al., 2012), suggesting that negative modulation of *KIF20A* by *P. freudenreichii* may represent a potential anti-tumor mechanism. In addition to its role in cell proliferation, *KIF20A* has been implicated in neurological pathways, interacting with the *RGS3* protein, a regulator of G-protein signaling, at the intercellular bridges of neuronal progenitor cells and regulating the division of these cells in cortical neurogenesis (Geng et al., 2018). Its inactivation has been observed to affect cortical neurogenesis, leading to defects in neuronal progenitor cell division and alterations in central nervous system development **(Geng et al., 2018)**. Given that imbalances in neuronal proliferation and differentiation may be associated with neurodegenerative and developmental disorders, negative modulation of *KIF20A* by *P. freudenreichii* may have implications for neuronal homeostasis. This suggests a potential link between the effects of probiotics and the regulation of key processes in neurological function.

# Conclusions

The results of implementing an integrative methodology have shown the positive effects of probiotics P. freudenreichii ITG P9 and B. subtilis CW14 on intestinal cells. The results also show that each probiotic can have specific effects, especially on pathways related to immunity and the cell cycle. Furthermore, the comprehensive regulatory capabilities of probiotics on genes associated with metabolic, neurological, and autoimmune diseases have been emphasized, highlighting their potential to modulate genes with neuroprotective or antitumoral properties. The findings underscore the significance of the gut-brain axis connection and highlight the key molecular mechanisms involved in immunomodulation, such as innate *TLR* and *NOD* receptors, and signaling pathways such as NF-κB and MAPK. The findings of this work underscore the concept of the microbiota as a dynamic organ with systemic impact.

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