# 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). 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., 2022a). Under balanced conditions, the gut microbiota contributes to stability, resilience, and beneficial symbiosis for the host, acting as an additional metabolic organ (Hou et al., 2022b). 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 the inclusion or exclusion of specific microorganisms, has the potential to 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, a transcriptomic analysis of HT-29 and Caco-2 cell lines treated with probiotic strains was conducted. The probiotic strains used in this study were *Propionibacterium freudenreichii* and *Bacillus subtilis* CW14. The objective of the study 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 three main axes: 1) interaction with viral proteins, 2) relationship with human disease-associated proteins, and 3) identification of possible gene modulation mechanisms related to immune and physiological response. This comprehensive approach will advance our understanding of how probiotics can modulate gene networks associated with human disease, 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](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., 2019a), and HT-29 cells treated with ***P. freudenreichii* ITG P9** (GSE67033) (Cousin, Jouan-Lanhouet, Théret, Brenner, Jouan, Moigne-Muller, Dimanche-Boitrel, & Jan, 2016).

Principal Component Analysis (PCA) was conducted using the DESeq2 (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 Collectio (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 pathogens and genetic diseases.

Annotations were performed to associate DEGs with human pathogens and genetic 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 or a score ≥ 0.85.

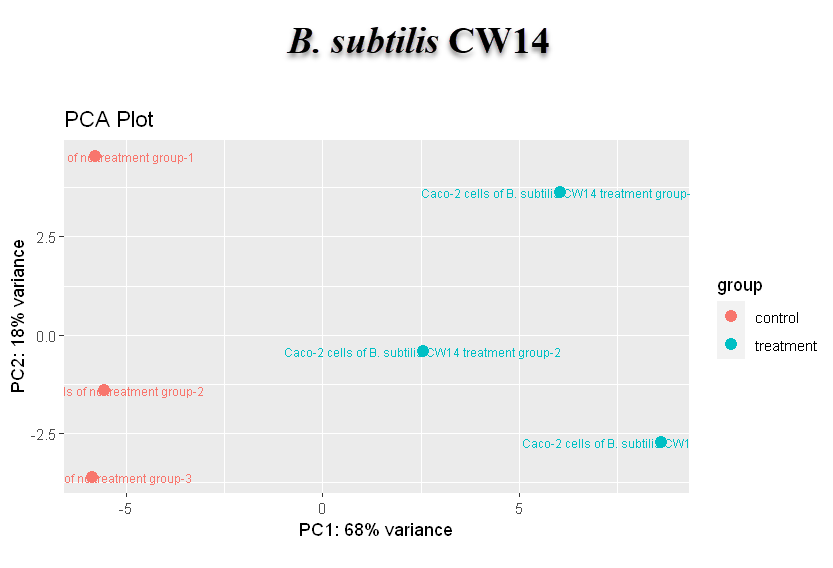
**2.2.4. Protein-Protein Interactions (PPIs) and visualization of Results.**

The exploration of protein-protein interactions (PPIs) of differentially expressed genes (DEGs), as well as their interaction with proteins associated with human diseases, viruses, and bacteria, 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 and biological pathways were generated using Cytoscape v. 3.10.2 (Shannon et al., 2003), the DEGs are represented by green, the interacting proteins by blue, and the biological terms associated with GO Biological Process with a Padj ≤ 0.05 by purple. These networks provide a clear and comprehensive representation of the molecular interactions and pathways affected by DEGs in response to probiotic treatments.

# 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.2)**. 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.

Tabla

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**Figure 2. PCA analysis showing transcriptional responses in *P. freudenreichii* ITG P9 and *B. subtilis* CW14 probiotics.**

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.

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

The present study analyzed the data obtained from *B. subtilis* CW14-treated Caco-2 cells and *P. freudenreichii* ITG P9-treated HT-29 cells, revealing significant modulation of DEGs through the implementation of expression change thresholds (log2FC ≥ 2), an FDR value ≤ 0.05 and a Padj ≤ 0.05. The implementation of these criteria enabled the selection of a set of genes associated with critical biological processes, including cell cycle, immunity, adhesion, inflammation, and transport. Furthermore, pathway analysis identified key metabolic and signaling pathways (KEGG and Elsevier Pathway Collection). The results obtained can be viewed in tables [ST1](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST1_Elsevier_Pathway_Collection_terms_sorted_with_all.xlsx) and [ST2](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST2_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, 2023; Peng et al., 2019b; Upadhyay & Fu, 2013; Yamazaki et al., 2022a). 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, 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 expression (-2.72) 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 (Z. Chen et al., 2025a; Neurath, 2014).

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. This suggests mechanisms associated with cell arrest through the G1/S transition phase thus 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; Wang et al., 2012).

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 with the objective of arresting 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, metabolic diseases (obesity, diabetes), neurological disorders, cancer, and rare syndromes through log2FC (LFC) ≥ 2, an FDR value ≤ 0.05 and Padj filter ≤ 0.05 (Table [ST3](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST3_Genes_dysbiosis_diseases_with_FC_sorted_all.xlsx), [ST4](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST4_Genes_neurological_diseases_with_FC_sorted_all.xlsx), [ST5](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST5_Genes_rare_diseases_with_FC_sorted_all.xlsx)). The results obtained 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 [ST6](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST6_KEGG_2021_Human_terms_sorted_with_all.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, Alzheimer's), rare disorders (amyloidosis, antibody-mediated glomerulonephritis) and gut dysbiosis, linked to metabolic disorders such as obesity, inflammatory bowel disease (IBD) and diabetes mellitus (Table [ST7](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST7_Genes_neurological_diseases_with_FC_sorted_Bacilus.xlsx), [ST8](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST8_Genes_rare_diseases_with_FC_sorted_Bacilus.xlsx), [ST9](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST9_Genes_dysbiosis_diseases_with_FC_sorted_Bacilus.xlsx)), suggesting a dual role in the activation of innate immunity and in 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 homeostasis (Vida et al., 2024). For instance, the over-expression of genes such as CXCL10 and CCL4, which have been linked to autoimmune and neurodegenerative diseases, supports the hypothesis that the gut microbiota may play a role in mediating neuroprotection and maintaining blood-brain barrier integrity by regulating these genes in a controlled manner (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 or Alzheimer's disease (Mayer et al., 2014).

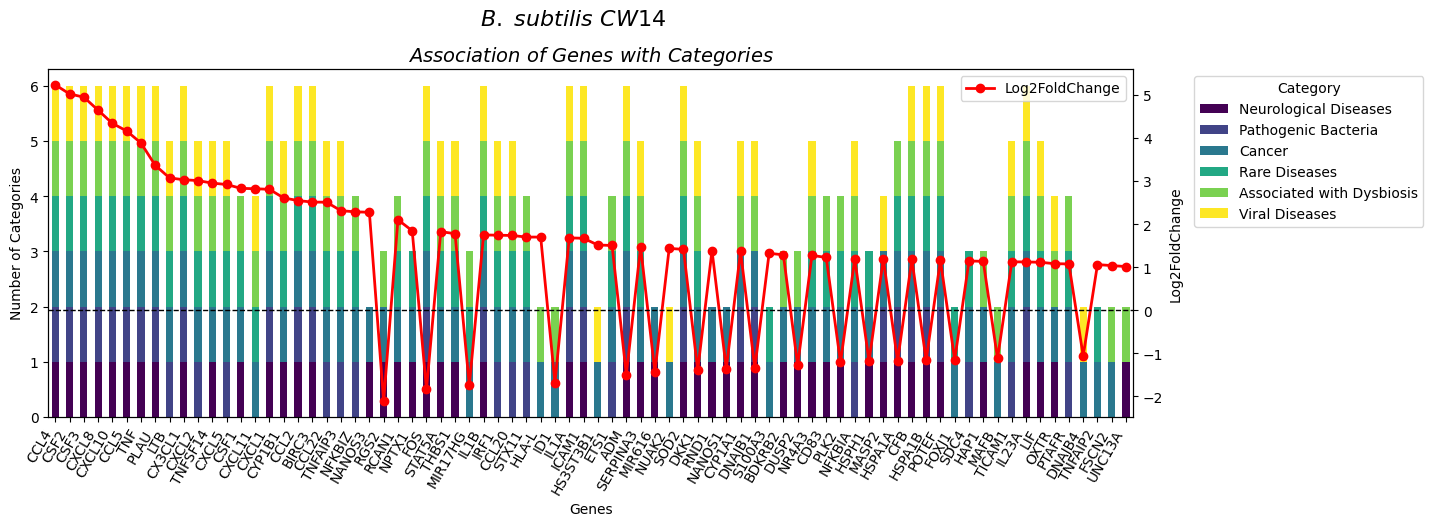
|  |  |  |  |
| --- | --- | --- | --- |
| **Genes** | **Term** | **Adjusted P-value** | **Log2FoldChange** |
| CCL4 | Cytokine-cytokine receptor interaction, Viral protein interaction with cytokine and cytokine receptor. | 1.86e-17, 3.08e-16 | 5.23 |
| CSF2 | TNF signaling pathway, Rheumatoid arthritis, 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 2. Functional enrichment of positively regulated genes in Caco-2 cells treated with *B. subtilis* CW14.**

Concerning the connection between dysbiosis and neuroinflammation, the results are reinforced when considering the role of genes such as CXCL8, 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, reinforcing the concept that the gut microbiota acts as a connector between the immune and nervous systems (Sarkar et al., 2016).

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 [S10](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST10_Genes_multiple_categories_with_FC_sorted_Propionibacterium.xlsx)). Enrichment analyses indicate that the SH2D3C (+6.80) and CORO1A (+3.79) genes are associated with symptomatic polyhydramnios-megalencephalic-epilepsy 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 gene (+4.52) has been associated with intellectual disability-obesity-brain malformations-facial deformity syndrome and Alzheimer's disease. 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 up-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., 2024a).

Transcriptomic analyses also revealed a set of differentially expressed genes (DEGs) with multi-association profiles to various pathologies (Table [S11](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST11.xlsx)). Among these, genes with pleiotropic roles were highlighted, simultaneously linked to dysbiosis, cancer, neurological diseases, bacterial infections, and rare and viral diseases (Figure 3). In this regard, the genes *CCL4*, *CSF2*, *CSF3*, *CXCL8*, *CXCL10, and CCL5* emerged as central nodes, showing a significant positive LFC and being associated with the five pathological categories analyzed (Table [S11](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST11.xlsx)). It is noteworthy that this group of genes exhibits an LFC that is twice the established (LFC ≥ 2) **(Figure 3)**, which may indicate a role as cross-cutting biomarkers in multiple pathophysiological pathways. Furthermore, genes such as *CCL4*, *TNF,* and *CSF2* also function as central nodes, connecting dysbiosis to neurological diseases including Alzheimer's, Parkinson's, and epilepsy. For instance, *CSF2* (LFC +5,03), associated with microglial activation in Alzheimer's disease, and *TNF* (LFC +3,88), implicated in neuroinflammation in amyotrophic lateral sclerosis (ALS), underscore the existence of shared mechanisms between gut inflammation and neuronal degeneration.

 Gráfico, Gráfico de líneas

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**Figure 3. Association of genes with categories and Log2FoldChange in *B. subtilis* CW14 and *P. freudenreichii* ITG P9.**

## 2.3.4. Interacciones Proteína-Proteína (PPI)

En células Caco-2 tratadas con *B. subtilis* CW14, se destacó un grupo de genes con roles pleiotrópicos, descritos anteriormente. En la red, los genes CCL4, CSF2, CSF3, CXCL8, CXCL10 y CCL5 emergen como nodos centrales (véase [red CCL4](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/Cytoscape_network_ppi_go_biological_process.cys) en el material suplementario), se identificaron nuevas conexiones entre IL1B, IL1A y CCL20 mediante el análisis de PPI. La red resalta términos GO enriquecidos vinculados a la inflamación y a la quimiotaxis de leucocitos (linfocitos, monocitos, neutrófilos y eosinófilos). En este contexto, CCL4 desempeña un papel esencial en el reclutamiento inmunitario hacia sitios de infección o inflamación.

Asimismo, se identificaron términos que enfatizan la importancia de la vía ERK1/2 en la señalización celular, conectando la inflamación con procesos de proliferación, diferenciación y respuesta a citocinas (Chandiok, 2024). Adicionalmente, rutas mediadas por citoquinas y quimiocinas, que involucran a IL1A, IL1B, CXCLs, CCL20 y CCL5, indican respuestas a infecciones bacterianas, fúngicas y parasitarias. Finalmente, el nodo “Negative Regulation by Host of Viral Transcription” sugiere un componente antiviral, ampliando la función de CCL4 a mecanismos de defensa frente a infecciones virales.

En células HT-29 tratadas con *P. freudenreichii* ITG P9 se identificaron dos redes principales, cada una centrada en un gen clave: KIF20A y OASL (Figura 4). Aunque no se encontraron interacciones con términos de GO, el análisis de PPI reveló que ambas redes se derivan de PPI, vinculando a KIF20A y OASL con procesos como el ciclo celular y la respuesta antiviral.

Diagrama

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**Figura 4. Redes PPI de KIF20A y OASL en células HT-29 tras tratamiento con *P. freudenreichii* ITG P9.**

KIF20A, con regulación negativa (-2,19), se posiciona como nodo central en la primera red. Este gen interactúa con ciclinas (CCNA2, CCNB1, CCNB2), quinasas (AURKA, PLK1, TTK) y componentes del centrómero (CENPA, INCENP, NCAPG, NUF2), lo que sugiere un rol esencial en la citocinesis, progresión del ciclo celular y estabilidad cromosómica. Su interacción con RACGAP1, clave para la citocinesis, indica que la regulación negativa inducida por *P. freudenreichii* ITG P9 puede provocar defectos en la división celular, inestabilidad genómica y apoptosis en células cancerosas, resaltando su potencial antitumoral (Eid et al., 2022).

Por otro lado, OASL (+2,03) emerge como un regulador central en la respuesta inmune antiviral. Este gen interactúa con IRF7, IFI44, IFIT3, ISG15 y RNASEL, lo que sugiere que activa RNASEL para degradar ARN viral e inhibir la replicación, además de modular la expresión de genes antivirales a través de IRF7 (Jung-Rodriguez et al., 2024; Lee et al., 2013). La regulación de OASL por *P. freudenreichii* ITG P9 podría potenciar la respuesta inmune innata, limitando la propagación viral y protegiendo las células epiteliales, fortaleciendo así la barrera inmune intestinal (Weiss, 2020).

# 3. 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., 2019a).

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 former, a coordinated immune response was observed through the overexpression of chemokines and inflammatory factors, whereas in the latter, a transcriptional response characterized by the modulation of genes related to cell cycle control and stress was observed. The diversity observed suggests that the effects of probiotics on intestinal cells may be strain-specific and may be related to molecular mechanisms regulating epithelial homeostasis, inflammation, cell adhesion, stress response, and cell cycle arrest.

## 3.1. Response of Caco-2 cells to Bacillus subtilis CW14 treatment.

Transcriptomic analysis revealed overexpression of chemokines and immunostimulatory factors (Table [ST2](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST2_KEGG_2021_Human_terms_sorted_with_all.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., 2019b). 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 (R. Chen et al., 2022). Asimismo, factores de estimulantes de colonias como CSF2 (GM-CSF) y CSF3 (G-CSF) modulan la proliferación, diferenciación, supervivencia, maduración y activación funcional de células hematopoyéticas, incluidos de granulocitos y macrófagos, reforzando la respuesta innata, y mediante 4 vías de señalización diferentes 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., 2022b). 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. In particular, the importance of the proinflammatory factor, IL-8, in various inflammatory diseases is well-documented, with its role being to act via 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., 2019b). 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 (Liu et al., 2022).

Concurrently, alterations in genes linked to metabolic activity and stress were detected. Overexpression of CYP1B1, 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 preventing 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 (Hu & Shao, 2022). In this regard, BIRC3, a member of the inhibitor of 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, 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 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., 2024b; Song et al., 2022). Its functions include protein quality control, including correct protein folding, refolding, and control of subsequent protein degradation (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.

## 3.2. 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 [S6](https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos/blob/main/8_Supplementary%20Material/ST6_KEGG_2021_Human_terms_sorted_with_all.xlsx)). These genes encode cyclin-dependent kinase inhibitors (CKIs) that play a critical role in cell cycle arrest at the G1/S transition (Cousin, Jouan-Lanhouet, Théret, Brenner, Jouan, Moigne-Muller, Dimanche-Boitrel, Jan, et al., 2016b; Y. Wang et al., 2012b). 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., 2016b). 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).

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 in the presence of oxidative stress, thus preventing the accumulation of mutations and eventual malignant transformation (Cousin et al., 2016b; Y. Wang et al., 2012b). 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., 2025b; Y. Wang et al., 2012b). 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.

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