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Original article

In *silico* analysis of the potential of *Bacillus subtilis* CW14 and *Propionibacterium freudenreichii* ITG P9 probiotics as modulators in the microbiota-gut-brain axis pathologies

Análisis in silico del potencial de los probióticos *Bacillus subtilis* CW14 y *Propionibacterium freudenreichii* ITG P9 como moduladores en las patologías del eje microbiota-intestino-cerebro

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Abstract

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

Keywords: Functional enrichment; Probiotics; Microbiota; Bioinformatics; Transcriptome.

Resumen

La microbiota intestinal comprende más de 100 billones de microorganismos y desempeña funciones críticas en la inmunidad, el metabolismo y la homeostasis. Su desequilibrio (disbiosis) se ha asociado con trastornos gastrointestinales, metabólicos, autoinmunes y neurológicos. Por otra parte, los probióticos, microorganismos vivos con efectos beneficiosos, han surgido como una estrategia terapéutica prometedora. Analizamos aquí el impacto de los probióticos *Bacillus subtilis* CW14 y *Propionibacterium freudenreichii* ITG P9 en células epiteliales intestinales de las líneas celulares Caco-2 y HT-29, respectivamente. Mediante un enfoque integrador basado en herramientas bioinformáticas, se examinaron genes expresados diferencialmente e interacciones proteína-proteína (IPP) para establecer el impacto de estos probióticos en la modulación génica y su relación con diversas patologías humanas. Los resultados mostraron efectos específicos para cada probiótico: *B. subtilis* CW14 moduló principalmente una respuesta inmunitaria coordinada y controlada que involucró quimiocinas y factores inflamatorios, en tanto que *P. freudenreichii* ITG P9 provocó una

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respuesta transcripcional caracterizada por la modulación de genes asociados al control del ciclo celular y el estrés. Se estableció el efecto pleiotrópico de ambos probióticos en genes vinculados a enfermedades metabólicas, neurológicas y autoinmunes, regulando en muchos casos genes con propiedades inmunomoduladoras, neuroprotectoras o antitumorales. De los resultados emergieron, además, mecanismos moleculares claves relacionados con la inmunomodulación, incluidos receptores innatos como el TLR y el NOD, y vías de señalización como la NF-κB y la MAPK, lo que respalda la relevancia de la conexión del eje intestino-cerebro a partir de los datos transcriptómicos como marco de futuras investigaciones.

Palabras clave: Enriquecimiento funcional; Probióticos; Microbiota; Bioinformática; Transcriptoma.

Introduction

The human gut harbors a complex microbial ecosystem comprising over 100 trillion symbiotic microorganisms that outnumber host cells (**Dekaboruah *et al***., 2020). This system, known as the gut microbiota, interacts dynamically with the immune system, 70% of which originates in the gut, regulating critical functions such as digestion, vitamin synthesis, metabolism, and defense against pathogens (**Hou *et al***., 2022). Under homeostatic conditions, the microbiota acts as a functional organ, promoting equilibrium through the epithelial barrier, competitive exclusion of pathogens, and the modulation of immune responses (**Hashemi *et al.***, 2023; **Hou *et al***., 2022). Its imbalance (dysbiosis), however, is associated with gastrointestinal, metabolic (e.g., diabetes, obesity), autoimmune (e.g., inflammatory bowel disease), and even neurological disorders (**Richard & Sokol,** 2019). In this context, probiotics, live microorganisms with beneficial effects, have emerged as promising therapeutic tools to restore microbial symbiosis (**Cremon *et al.***, 2018). These organisms not only compete with pathogens and reinforce the intestinal barrier but also modulate innate and adaptive immunity through diverse interactions (**Zmora *et al.***, 2019). Among the most studied strains are lactic acid bacteria (LAB) such as *Lactobacillus*, *Bifidobacterium*, and *Propionibacterium freudenreichii*, which are generally recognized as safe (GRAS) and are known for their immunomodulatory properties (**McFarland *et al***., 2018). Their ability to alter microbial composition and regulate disease-associated gene expression underscores their translational potential (**Trejo & Sanz,** 2013).

The exact mechanisms by which probiotics influence specific gene networks remain an active area of investigation. Here, we employed an *in-silico* approach to analyze the impact of two probiotic strains, *Bacillus subtilis* CW14 and *Propionibacterium freudenreichii* ITG P9, on intestinal epithelial cells (Caco-2 and HT-29), using transcriptomic data from the Gene Expression Omnibus (GEO) repository (**Clough & Barrett,** 2016). Through differentially expressed gene (DEG) analysis and protein–protein interaction (PPI) networks, two axes were explored: 1) the relationship of DEGs with proteins implicated in human diseases, and 2) gene-modulation mechanisms linked to immune and physiological responses. This integrative approach aims to elucidate how these strains regulate genes associated with metabolic, autoimmune, and neurological pathologies, identifying key molecular targets (e.g., chemokines, cell cycle factors) and shared signaling pathways.

Materials and methods

Data acquisition and differential expression analysis

We conducted a comprehensive search for differential gene expression data in the GEO database (**Clough & Barrett,** 2016). We used a series of keywords (https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos) to filter the studies. We selected two datasets meeting the following inclusion criteria: 1) use of colon adenocarcinoma cell lines as the experimental model, 2) evaluation of effects on the colon, and 3) probiotic-based treatments. The chosen datasets comprised 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).

For GSE115081, we obtained raw RNA-seq counts from 12 samples, six of which were selected for analysis: three untreated controls (GSM3164863, GSM3164864, GSM3164865) and three treated with *B. subtilis* CW14 (GSM3164866, GSM3164867, GSM3164868). For GSE67033, we obtained microarray data from 24 samples; the analysis focused on four untreated controls (NT replicates 1–4) and four treated with *P. freudenreichii* ITG P9 (SN replicates 1–4). In both datasets, three or four biological replicates per condition were available, ensuring adequate statistical power.

We performed a differential expression analysis using the DESeq2 package (**Love *et al.***, 2014) for RNA-seq data (GSE115081) and the limma package (**Ritchie *et al.***, 2015) for microarray data (GSE67033). In both analyses, the experimental design was defined as a single factor with two levels (control vs. treatment), and the condition variable was set with the control as the reference level. No batch effects were identified in the original studies, as samples were processed simultaneously under identical conditions, and, therefore, batch correction was not required.

We performed a principal component analysis (PCA) on variance-stabilized transformed (VST) counts for RNA-seq data and on normalized $\log_2$-transformed expression values for microarray data to visualize group separation and assess overall variability between treatment groups and controls (**Love *et al***., 2014).

Before differential expression testing, low-expression genes were filtered to reduce noise and multiple-testing burden. For the RNA-seq dataset, genes with a total read count of less than 10 across all samples were excluded from the analysis. For the microarray dataset, probes with signal intensity below background in all samples were removed based on the manufacturer's detection thresholds.

Differentially expressed genes (DEGs) were defined using an adjusted p-value (FDR) ≤ 0.05 and an absolute $\log_2$ fold change ($\log_2$FC) ≥ 2.0 for RNA-seq data. For microarray data, DEGs were selected with an adjusted p-value ≤ 0.05 and an absolute $\log_2$FC ≥ 1.5 due to their lower dynamic range. All p-values were adjusted for multiple testing using the Benjamini–Hochberg (BH) procedure to control the false discovery rate.

For integrative purposes, DEG gene symbols were converted to Entrez IDs via the Ensembl (**Yates *et al***., 2016) and UniProt (**Bateman *et al***., 2025) databases, facilitating cross-database queries and annotations. To avoid redundancies and statistical inflation during multi-database integration, only unique gene symbols were retained after conversion, and duplicate entries from overlapping databases were manually curated and consolidated. Enrichment and disease-association analyses were performed on the final non-redundant gene sets.

Functional and pathway enrichment analysis

Gene Ontology (GO) functional enrichment was performed using Enrichr (**Chen *et al***., 2013), focusing on the GO Biological Process 2023 terms. Biological pathway identification for DEGs was carried out against the KEGG 2021 Human (**Ogata *et al.***, 1999) and Elsevier Pathway Collection (**Elsevier,** 2020) databases. Enrichment analyses were considered significant at an adjusted p-value (Padj) ≤ 0.05.

Association of differentially expressed genes with human diseases

DEGs were annotated for association with human diseases using DisGeNET (**Piñero *et al.***, 2019), GeDiNet 2023 (**Qumsiyeh *et al.***, 2022), Virus-Host PPI P-HIPSTer 2020 (**Lasso *et al.***, 2019), and Orphanet Augmented (**Rath *et al***., 2012) databases. Those associations with an adjusted p-value ≤ 0.05 were deemed significant. It is important to note that these databases compile reported associations between genes and diseases from the literature, including genetic, text-mining, and curated sources; such associations do not imply direct biological causality but rather indicate prior documented links. To minimize redundancy and potential inflation of associations, results from multiple databases were merged by gene symbol and disease concept, retaining unique entries. The associations with an adjusted p-value ≤ 0.05 were deemed significant.

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Protein–protein interaction (PPI) networks and visualization

PPI analysis of DEGs and disease-associated proteins was performed using STRING (**Szklarczyk *et al.***, 2023), BioGRID (**Oughtred *et al.***, 2021), and IntAct (**del Toro *et al.***, 2022) databases. Interaction thresholds were set at a combined score, quantitative score, and confidence value ≥ 0.9, respectively. Interactions from the three databases were merged, and non-redundant high-confidence interactions were retained for network construction. Resulting networks were visualized in Cytoscape (**Shannon *et al.***, 2003), applying Padj ≤ 0.05 for network filtering.

The entire protocol can be replicated by following these steps: https://github.com/nicolasbroldan/Protocolo_Reposicionamiento_Probioticos.git

Results

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

The principal component analysis (PCA) revealed pronounced transcriptional responses to both probiotics (**Figure 1**). *P. freudenreichii* ITG P9 displayed exceptionally high variance, with PC1 accounting for 94%, clearly separating treated samples from controls. In contrast, PC1 for *B. subtilis* CW14 explained 68% of the variance, indicating a substantial contribution of this axis to group separation. These findings suggest that treatment effects differ by probiotic strain, with a more pronounced response elicited by *P. freudenreichii* ITG P9, and highlight high mRNA heterogeneity as evidenced by the spatial distribution and intra-group variability of treated samples. Treatment with *P. freudenreichii* ITG P9 yielded 2,337 DEGs, 1,457 (62.34%) of which were up-regulated and 880 (37.66%) down-regulated. For *B. subtilis* CW14, 198 DEGs were identified: 136 (68.69%) up-regulated and 62 (31.31%) down-regulated. In both cases, there was a clear trend toward gene activation (**Figure 1**).

Functional enrichment analysis of biological processes and pathways

DEG sets from Caco-2 cells treated with *B. subtilis* CW14 and HT-29 cells treated with *P. freudenreichii* ITG P9 were filtered using thresholds of $\log_2FC \geq 2$, FDR ≤ 0.05, and Padj

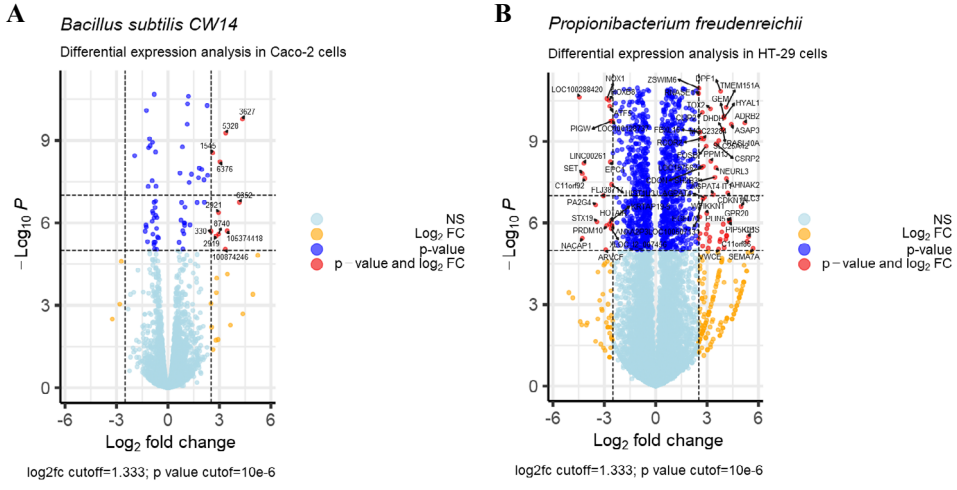

Figure 1. Volcano plot analysis for Caco-2 and HT-29 cells treated with probiotics. In panel **A** (for *B. subtilis* CW14), the x-axis shows the logarithmic change in gene expression ($\log_2FC$), and the y-axis shows the $-\log_{10}$ of the p-value. Red points represent genes with both significant $\log_2$ FC and significant p-value; blue points represent genes with a significant p-value; yellow points represent genes with a significant $\log_2$ FC, and grey points represent non-significant genes. A cutoff of 1,333 for $\log_2FC$ and a p-value threshold of 10^{-6} were applied for the plots. **Panel B** (for *P. freudenreichii* ITG P9) follows the same criteria.

≤ 0.05. This selection highlighted genes involved in key biological processes, including cell cycle regulation, immunity, adhesion, inflammation, and transport, as well as critical metabolic and signaling pathways. Full enrichment results for GO terms (Enrichr), KEGG pathways, and Elsevier Pathway Collection are provided in **Tables S1,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5340, **S2,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5341 **and S3,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5342, respectively.

In Caco-2 cells exposed to *B. subtilis* CW14, a coordinated up-regulation of immune signaling and defense mechanisms was observed. Notably, chemokines and immune-stimulating factors were overexpressed: CCL4 (+5.24), CSF2 (+5.03), CSF3 (+4.95), NFKBIZ (+2.28), LTB (+3.07), and PLAU (+3.36). This profile suggests activation of the NF-κB pathway, likely promoting T-cell and neutrophil recruitment, as well as macrophage and granulocyte differentiation, processes that facilitate pathogen clearance and epithelial repair (**Anderson,** 2023; **Peng *et al.***, 2019). Elevated expression of CXCL8 (+4.65), CXCL10 (+4.34), CXCL11 (+2.82), and CX3CL2 (+3.01) further indicate enhanced neutrophil chemotaxis, mast cell activation, and a coordinated antimicrobial response. Up-regulation of CCL5 (+4.16), together with modulation of CCL22 (+2.51) and CCL2 (+2.55), points to recruitment of monocytes and regulatory T cells (Tregs) and polarization of macrophages toward a reparative phenotype, potentially mitigating epithelial damage during inflammation. Additional regulators such as TNFAIP3 (+2.31) and TNFSF14 (+2.95) may help control intestinal inflammation via NF-κB inhibition or induction of apoptosis (**Krause *et al.***, 2014).

Simultaneously, genes related to stress response and metabolic activity were modulated. Overexpression of CYP1B1 (+2.61) suggests induction of detoxification pathways for xenobiotic neutralization, while up-regulation of BIRC3 (+2.51) and down-regulation of RGS2 (-2.11) imply engagement of anti-apoptotic mechanisms that enhance epithelial cell survival under oxidative stress (**Pauletto *et al.***, 2020). Conversely, decreased HSPA6 expression (-2.72) may reflect cellular adaptation to stress by reallocating resources in a demanding gastrointestinal environment that requires both immune activation and repair (**Chen *et al.***, 2022; **Neurath,** 2014). Representative DEGs for *B. subtilis* CW14 ($log_2FC \geq 2$) are listed in **Table 1**; comprehensive results appear in **Tables S1-S3**.

Table 1. Functional enrichment of positively regulated genes in Caco-2 cells treated with *B. subtilis CW14*

Genes	Term	Adjusted P-value	Log2 fold change
CCL4	Cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor	1.86e-17, 3.08e-16	5.23
CSF2	TNF signaling pathway, rheumatoid arthritis, cytokine-cytokine receptor interaction	7.38e-21, 5.01e-18, 1.86e-17	5.02
CSF3	Cytokine-cytokine receptor interaction, IL-17 signaling pathway, malaria, coronavirus disease	1.86e-17, 2.86e-15, 4.92e-07	4.95
CXCL8	Rheumatoid arthritis, cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, IL-17 signaling pathway, NF-kappa B signaling pathway	5.01e-18, 1.86e-17, 3.08e-16, 2.86e-15, 1.15e-14	4.64
CXCL10	TNF signaling pathway, cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, IL-17 signaling pathway, chemokine signaling pathway, toll-like receptor signaling pathway	7.38e-21, 1.86e-17, 3.08e-16, 2.86e-15, 1.02e-09, 3.49e-08	4.34

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Table 1 summarizes the key genes identified in the transcriptomic analysis, their associated enriched biological pathways, the adjusted p-values for these enrichments, and the corresponding log_2FC in gene expression, highlighting their potential roles in immune signaling pathways and inflammatory responses.

Association of probiotic-modulated DEGs with human pathologies

The annotation of probiotic-modulated DEGs revealed their involvement in dysbiosis (**Table S4,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5343), neurological disorders (**Table S5,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5344), and rare/orphan syndromes (**Table S6,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5345) using criteria of $log_2FC \geq 2$, FDR ≤ 0.05, and Padj ≤ 0.05. In Caco-2 cells treated with *B. subtilis* CW14, several pro-inflammatory and immunomodulatory genes were up-regulated (log_2FC +2.10 to +5.23; **Table S1,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5340), notably CCL4 (+5.23), CSF2 (+5.02), CSF3 (+4.95), CXCL8 (+4.64), and CXCL10 (+4.34). These DEGs are linked to neurological diseases (e.g., epilepsy, Parkinson's disease, Alzheimer's disease), rare disorders (e.g., amyloidosis, antibody-mediated glomerulonephritis), and metabolic dysbiosis (obesity, IBD, diabetes mellitus) (**Tables S4-S6**), which suggests a dual role in innate immunity activation and pleiotropic mechanisms extending beyond the gut, a hypothesis that requires *in vivo* validation.

Our findings gain significance in the gut-brain axis context, where probiotic strains like *B. subtilis* CW 14 have been shown to modulate intestinal immune responses and could potentially influence neuroinflammatory processes and central nervous system homeostasis (**Sarkar *et al.***, 2016). For instance, overexpression of CXCL10 (+4.34) and CCL4 (+5.23), both associated with autoimmune and neurodegenerative conditions in database annotations, elicits the hypothesis that the gut microbiota might contribute to neuroprotection and blood-brain barrier integrity through controlled regulation of these genes, although it has to be tested in appropriate models (**Vida *et al.***, 2025). In this context, the observed up-regulation of these chemokines contrasts with reports linking their sustained elevation to neuroinflammation; thus, the net effect may depend on the timing, magnitude, and cellular context of the modulation. Similarly, up-regulation of CSF2 (+5.02) and CSF3 (+4.95), which govern immune cell proliferation and differentiation, indicates that *B. subtilis* CW14 might foster a balanced inflammatory response in diseases such as multiple sclerosis or Alzheimer's disease (**Mayer *et al***., 2014), although this remains speculative without *in vivo* validation.

Regarding dysbiosis neuroinflammation links, CXCL8 regulation (+4.64) by *B. subtilis* CW14 suggests a potential mechanism whereby this probiotic could contribute to maintaining intestinal homeostasis, which, in turn, might influence systemic inflammation and its impact on neurological disorders (**Mayer *et al.***, 2014). This aligns with evidence connecting dysbiosis to alterations in the gut–brain axis, increasing susceptibility to metabolic and neurodegenerative diseases (**Cryan *et al.***, 2019). These results underscore the *in silico*-derived potential of *B. subtilis CW14* as an intestinal immune modulator with cross-pathology effects (**Table S7,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5346), highlighting the concept of the gut microbiota as a bridge between the immune and nervous systems, which warrants further investigation (**Sarkar *et al.***, 2016). A summary of pathology-associated genes is presented in **Figure 2**.

In HT-29 cells treated with *P. freudenreichii* ITG P9, we observed a dual modulation of gene expression, characterized by both up- and down-regulation of specific genes **(Table S1,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5340). For example, SH2D3C (+6.80) and CORO1A (+3.79) are associated with the polyhydramnios–megalencephaly–symptomatic epilepsy syndrome. Likewise, KIFC2 (+2.14) has been linked to Charcot–Marie–Tooth disease types 2P and 4B3, as well as adult-onset dystonia–parkinsonism. The gene KIAA0513 (+4.52) is implicated in the intellectual disability–obesity–brain malformation–facial dysmorphism syndrome and Alzheimer's disease.

These genes have also been associated with synaptic plasticity, suggesting a potential role in maintaining neuroarchitecture and, consequently, a neuroprotective effect (**Biggs *et al.***, 2025). In contrast, KIF20A, a gene enriched in disorders such as citrullinemia type II and primary immunodeficiency with natural killer cell deficiency and adrenal insufficiency, was down-regulated (-2.19). KIF20A down-regulation has been linked to reduced glioblastoma cell invasion and proliferation, suggesting a possible tumor-suppressive mechanism. These findings are relevant to the gut-brain axis, as they indicate a modulation of the intestinal immune response that could, in theory, influence neuroinflammatory processes and central nervous system homeostasis *in vivo*, a possibility that requires experimental validation (**Kim *et al***., 2024). Full results are available in **Tables S4, S5, and S6**, and a summary of pathology-associated genes is shown in **Figure 3**.

The differential expression analysis also revealed a set of pleiotropic genes simultaneously associated with dysbiosis, cancer, neurological diseases, infections, rare disorders, and viral diseases (**Figure 4**) (**Table S7,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/534). In this context, CCL4, CSF2, CSF3, CXCL8, and CXCL10 emerged as central nodes, exhibiting significant positive $\log_2$FC values and linking with the pathological categories analyzed (**Table S7,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/534). Notably, these genes exceeded the $\log_2$FC threshold (≥ 2) by 2, suggesting they are potential candidates for further investigation. Furthermore, CCL4, TNF, and CSF2 could serve as central nodes in neurological conditions such as Alzheimer's disease, Parkinson's disease, and epilepsy. Overall, these results underscore the critical role of gut microbiota in modulating convergent pathophysiological pathways through the regulation of chemokines, cytokines, and growth factors.

Protein–protein interaction (PPI) networks

DEGs PPI analysis in *B. subtilis* CW14-treated cells identified pleiotropic hub genes (CCL4, CSF2, CSF3, CXCL8, and CXCL10). Network mapping revealed complex interactions with proteins and pathways involved in innate and adaptive immune responses (see **Figure S1,** https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5339, for the CCL4). For instance, CXCL8 (IL-8) and CXCL10, associated with multiple autoimmune and inflammatory diseases, function as potent chemokines mediating

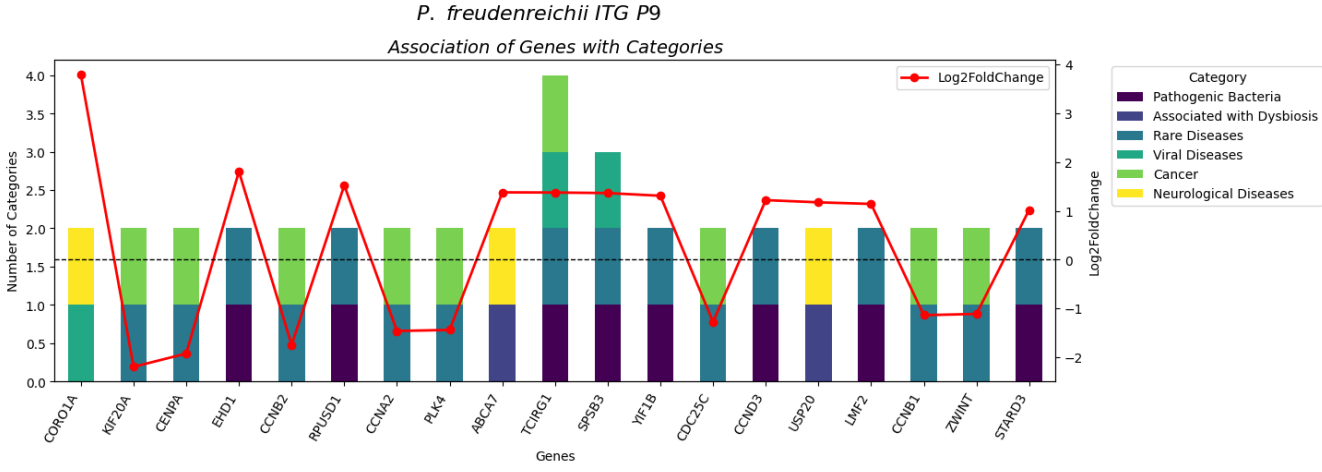

Figure 2. Association of genes with disease categories and $\log_2$ fold change in *B. subtilis* **CW14**. The graph shows the number of disease categories (left yaxis) associated with each differentially expressed gene following treatment with *B. subtilis* CW14 based on annotations from DisGeNET, Orphanet, and GeDiNet databases. Categories include neurological diseases, pathogenic bacteria, cancer, rare diseases, dysbiosis, and viral diseases. The stacked bars represent the count of associations per category for each gene. The red line indicates the $\log_2$ fold change ($\log_2$FC) of gene expression (right yaxis), with a dotted horizontal line marking $\log_2$FC = 0. The associations reflect database annotations and do not imply direct biological causality or demonstrated therapeutic effects.

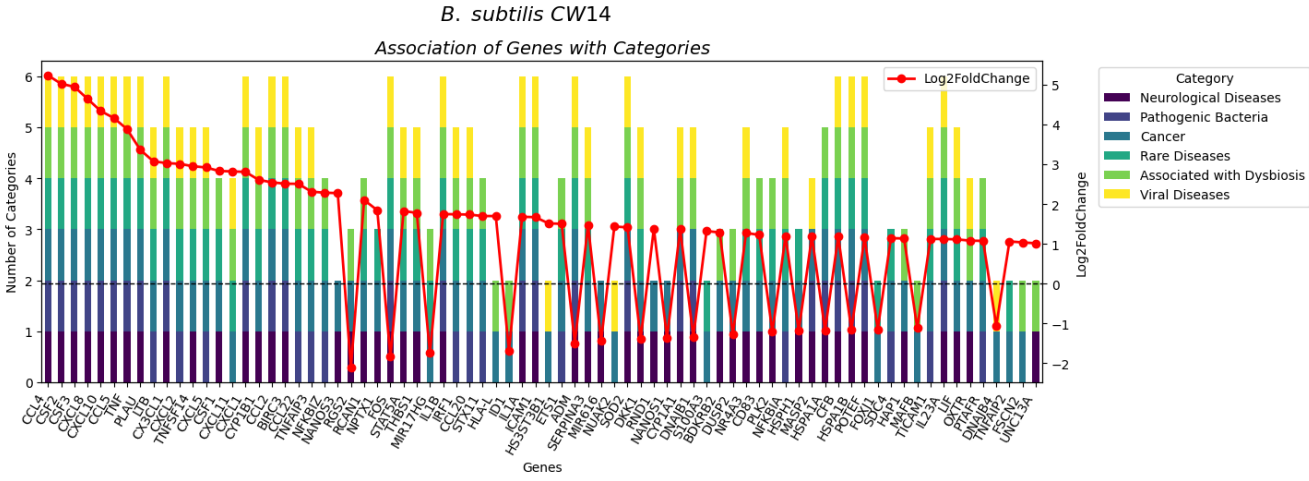

Figure 3. Association of genes with disease categories and log₂ fold change in *P. freudenreichii* **ITG P9**. The graph follows the same structure as **Figure 2**, displaying the number of disease categories (left yaxis) associated with each differentially expressed gene after treatment with *P. freudenreichii* ITG P9 based on annotations from DisGeNET, Orphanet, and GeDiNet databases. The stacked bars indicate the count of associations per category for each gene. The red line represents the log₂ fold change (log₂FC) in gene expression (right yaxis), with a dotted horizontal line at log₂FC = 0. Here, again, the reported associations are derived from curated databases and should be interpreted as hypothesisgenerating rather than evidence of direct biological or therapeutic effects.

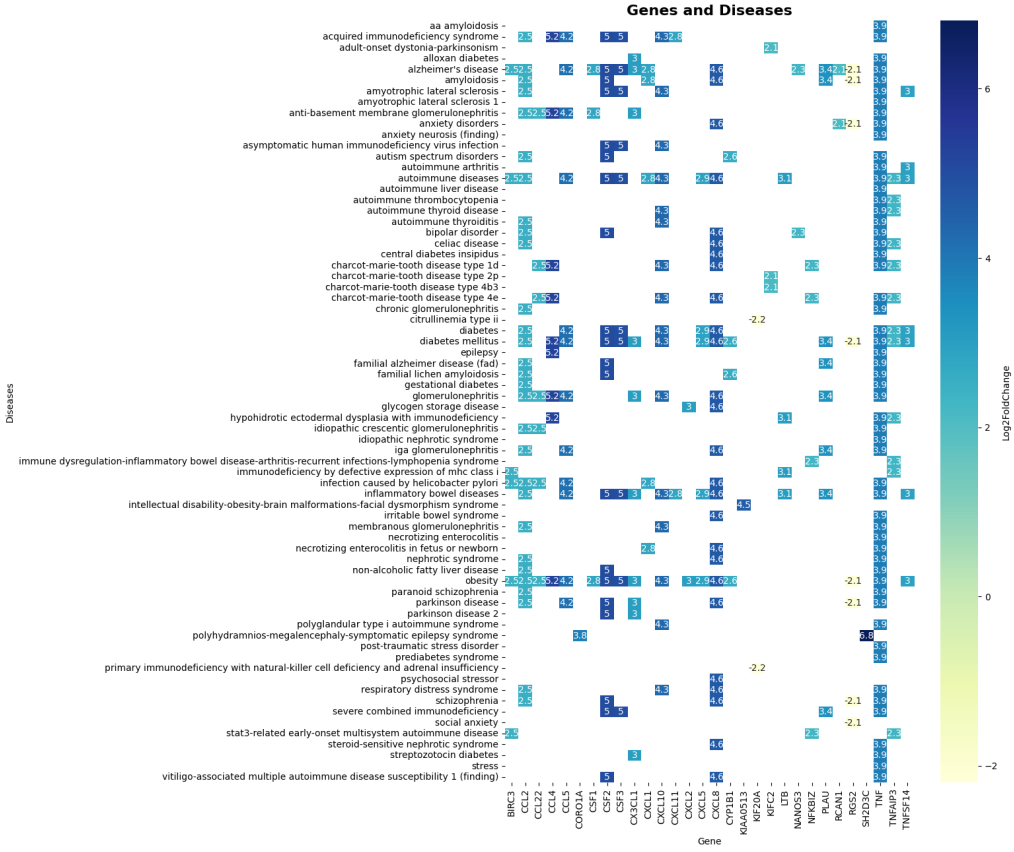

Figure 4. Heatmap of Log2FC in gene expression across diseases. The heatmap illustrates the log₂FC in gene expression for a range of genes across several diseases. The vertical axis lists the diseases associated, while the horizontal axis displays the genes analyzed. Each cell's color intensity represents the magnitude of the log₂FC: deeper blues indicate strong upregulation, whereas yellows reflect downregulation. Numerical values are overlaid on each cell for precise quantification.

leukocyte migration and activation in the intestinal epithelium. CCL4 (MIP-1β) has a role in regulating inflammatory responses and chronic inflammation by recruiting immune cells in diverse pathological contexts. Terms pointing to ERK1/2 pathway involvement further link inflammation to proliferation, differentiation, and cytokine responses (**Chandiok,** 2024). CSF2 (GM-CSF) and CSF3 (G-CSF) were connected to diseases characterized by altered immune microenvironments and hematopoiesis. Moreover, these inflammatory and signaling pathways intersect with adaptive immune routes involving IL-1, IL-4, IL-10, and IL-13, suggesting that *B. subtilis* CW14 may exert systemic effects beyond local epithelial responses.

In *P. freudenreichii* ITG P9-treated cells, we identified two principal networks centered on KIF20A and OASL (**Figure 5**), both involving cell cycle regulation and antiviral responses. KIF20A interacted with cyclins (CCNA2, CCNB1, CCNB2), kinases (AURKA, PLK1, TTK), and centromere components (CENPA, INCENP, NCAPG, NUF2), indicating a key role in cytokinesis, cell cycle progression, and chromosomal stability. OASL was linked to antiviral immunity, interacting with IRF7, IFI44, IFIT3, ISG15, and RNASEL. The direct OASL–RNASEL interaction suggests a mechanism for viral RNA degradation and replication inhibition, as well as modulation of antiviral gene expression via IRF7 (**Jung-Rodríguez *et al.***, 2024). The regulation of OASL by *P. freudenreichii* ITG P9 could enhance innate immune defenses, limit viral spread, and protect epithelial cells, thereby strengthening the intestinal immune barrier (**Weiss,** 2020).

Discussion

The intestinal tract is the largest immune organ in the body, interacting with antigens and immune mechanisms, as approximately 70% of the immune system is generated in the gut (**Ygberg & Nilsson,** 2012). Consequently, the intestinal epithelium is crucial for maintaining immune homeostasis and preventing uncontrolled uptake of toxic compounds or pathogens (**Richard & Sokol,** 2019), which can manifest as enhanced barrier integrity, pathogen inhibition, immune modulation and maturation, and reduced inflammatory and carcinogenic processes (**do Carmo *et al.***, 2017; **Peng *et al.***, 2019).

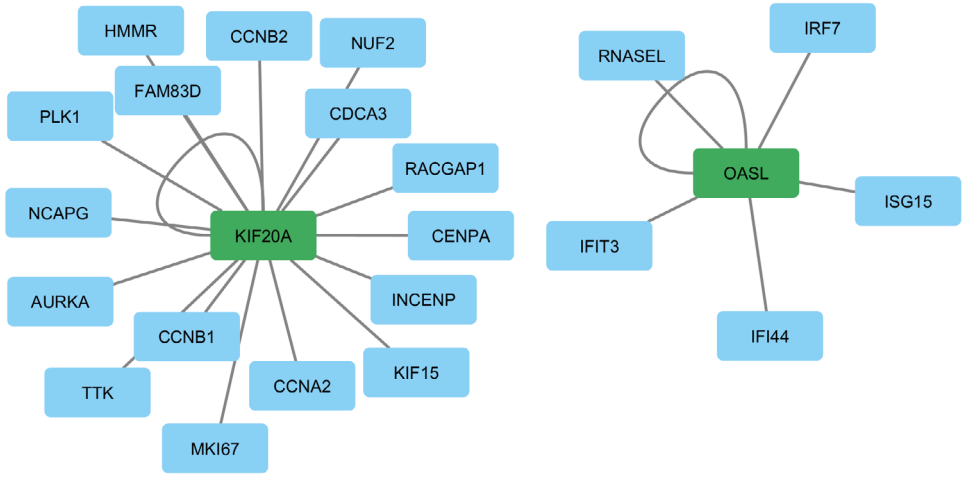

Figure 5. KIF20A and OASL PPI networks in HT-29 cells after treatment with *P. freudenreichii* ITG P9. The image illustrates two protein-protein interaction (PPI) networks derived from the analysis of *P. freudenreichii* ITG P9. Network 1, centered on KIF20A (in green), connects with genes including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

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Pleiotropic effects and integrated molecular mechanisms

Immune modulation and tissue repair in the intestinal epithelium in Caco-2 cells treated with *B. subtilis* CW14 entailed a coordinated activation of genes involved in inflammatory response and epithelial repair. Overexpression of chemokines such as CXCL1, CXCL2, CCL2, CCL4, CCL5, and CCL22 suggests that this strain recruits and activates multiple immune cell types, including T lymphocytes, neutrophils, macrophages, and NK cells (**R. Chen *et al.***, 2022). This dual modulation both enhances local defense against pathogens and promotes NF-κB pathway activation, reinforced by up-regulation of NFKBIZ, which, besides driving inflammation, facilitates tissue repair and regeneration of intestinal crypts (**Feng *et al***., 2023; **Liu *et al***., 2022; **Yamazaki *et al***., 2022; **Zhang *et al***., 2019).

The ability to induce immunological mediators is not only vital for barrier integrity but also has potential systemic repercussions to be explored in future studies. For instance, homeostatic regulation of CCL2 aids in the clearance of protein aggregates, marking it a potential therapeutic target in neurodegenerative diseases such as Alzheimer›s and Parkinson›s, where protein misfolding and neuroinflammation are central features (**Wang *et al.***, 2024). Likewise, the modulation of chemokines CXCL8, CXCL10, CXCL11, and CX3CL2 has been reported to influence blood-brain barrier permeability and microglial activation, implying a role that would extend beyond the gut to neuroimmunomodulation, although this was not evaluated in our *in-silico* model (**Wang *et al***., 2024; **Zhu *et al.,*** 2021).

Metabolic response and detoxification

CYP1B1 overexpression in Caco-2 cells highlights a pleiotropic effect on xenobiotic detoxification and metabolism. This is mediated in part by the aryl hydrocarbon receptor (AhR) pathway, which is activated by microbially derived metabolites such as tryptophan catabolites (**Shah *et al***., 2019). Such adaptive response not only neutralizes harmful compounds but also adjusts epithelial metabolic balance, with potential implications for metabolic diseases like diabetes and metabolic syndrome, conditions that are characterized by dysregulated detoxification and lipid/steroid hormone metabolism (**Shah *et al.***, 2019).

Regulation of apoptosis and cell survival

The modulation of BIRC3, a member of the inhibitor-of-apoptosis family, represents another key mechanism induced by *B. subtilis* CW14. Its overexpression protects the epithelium from apoptotic loss while supporting intestinal cell regeneration and renewal (**Hu & Shao,** 2022; **Pauletto *et al.,*** 2020). Conversely, down-regulation of RGS2 suggests attenuation of G-protein-coupled receptor (GPCR) signaling, potentially reducing oxidative stress and inflammation to foster a more stable, resilient cellular environment (**Bhuvaneshwar & Gusev,** 2024; **Pauletto *et al***., 2020). The reduced expression of HSPA6, a heat-shock protein induced by stress, indicates that, in the absence of external insults, cells can maintain proteostatic homeostasis. Balanced stress-response modulation is essential for preserving integrity in high-turnover tissues such as the intestinal epithelium (**Kim *et al.***, 2024).

Cell-cycle regulation and metabolic stress response in HT-29 cells treated with *P. freudenreichii* ITG P9 exhibited transcriptional signatures indicative of cell-cycle control. Up-regulation of cyclin-dependent kinase inhibitors CDKN1A, CDKN2B, and CDKN1C suggests a G_1/S checkpoint arrest, serving as a protective barrier against proliferation of damaged cells and neoplastic transformation (**Bueno-Fortes *et al.***, 2021; **Cousin *et al***., 2016; **Yang *et al***., 2023). This is particularly relevant to rare and neoplastic diseases, where cell-cycle dysregulation is etiologically significant. Modulation of genes such as BRSK2 and NES, implicated in G_2/M transition and mitotic spindle organization, further underscores *P. freudenreichii's* capacity to induce adaptive responses to metabolic stress (**Chen *et al.***, 2025). The induction of BRSK2, an AMPK-related kinase, suggests that bacterial metabolites (e.g., short-chain fatty acids) trigger a controlled stress state, prompting cells

to adjust metabolism via the Akt/mTOR pathway (**Saiyin *et al***., 2017). Such a mechanism may preserve cellular integrity under low-energy conditions and has implications for metabolic disorders and rare syndromes linked to aberrant energy-signaling (**Chen *et al***., 2025; **Saiyin *et al***., 2017). NES overexpression indicates the activation of regenerative mechanisms ensuring accurate cell division, contributing to tissue repair under damage, as seen in degenerative prevention (**Dicks,** 2022; **Wang *et al***., 2021).

Implications for neurological, rare, and metabolic diseases

Neurological disorders: The modulation of chemokines CCL2, CXCL5, CXCL8, CXCL10, and CXCL11 suggests a potential impact on the gut-brain axis that merits further investigation. While these genes have been previously associated with neurological conditions in curated databases, it is important to note that such associations do not establish causality; they simply indicate documented links that may involve either protective or pathogenic roles depending on the biological context. In Alzheimer›s and Parkinson›s diseases, immune-signaling dysfunction (e.g., p38 MAPK pathway) and blood-brain barrier compromise drive neuroinflammation and neuronal loss (**Karin & Razon,** 2018; **Yu *et al.***, 2021). The direction of transcriptional change observed in our study, for example, up-regulation of CXCL8 and CXCL10, must be interpreted with caution, as these chemokines are often associated with pro-inflammatory states in the central nervous system, yet their controlled modulation at the intestinal level might exert indirect homeostatic effects. The regulation of RGS2, RCAN1, and BIRC3 (the latter linked to neuroprotection via NPD1) raises the hypothesis of neuroprotective mechanisms that could be exploitable in neurodegenerative therapies, though this remains to be demonstrated (**Hu & Shao,** 2022). By calibrating intestinal inflammation, which might, in turn, influence microglia and barrier permeability, these probiotics are hypothesized to serve as adjuvant strategies in neurological disorders, warranting validation *in vivo.*

Metabolic diseases: The activation of CYP1B1 and AhR-mediated adaptive responses, combined with the regulation of Akt/mTOR via BRSK2 and TNFSF14 signaling through HVEM (TNFRSF14) and LTβR, suggests a basis for considering these strains as candidates for metabolic intervention in future studies. Dysregulation in diabetes, non-alcoholic fatty liver disease, and metabolic syndrome involves impaired metabolic signaling and oxidative stress. Based on our transcriptomic findings, probiotic-mediated rebalancing could hypothetically improve insulin sensitivity and reduce metabolic stress, pointing to complementary therapeutic avenues that require experimental confirmation (**Kou *et al.***, 2019).

Rare diseases and autoimmune disorders: Dysregulated cell-cycle control and immune signaling are hallmarks of certain rare syndromes and autoimmune conditions (e.g., ulcerative colitis, Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus) (**Ciccacci *et al***., 2019). *P. freudenreichii's* modulation of CDKN1A/B/C raises the possibility of preventing aberrant proliferation and restoring checkpoint mechanisms, although this is speculative at this stage. Concurrent up-regulation of anti-inflammatory mediators such as TNFAIP3 and NFKBIZ suggests its potential in managing chronic inflammatory and autoimmunological pathologies, which should be tested in appropriate models (**Ciccacci *et al***., 2019; **Feng *et al***., 2023; **Zhang *et al***., 2019).

Potential therapeutic implications and hypotheses derived from functional enrichment

These findings suggest the possibility of exploring pleiotropic effects of *B. subtilis* CW14 and *P. freudenreichii* ITG P9 in various pathologies, though they do not constitute evidence of therapeutic repositioning as defined in pharmacology. In neurological diseases, their gut-brain-immune modulation via controlled chemokine expression and barrier protection could, in theory, inform adjunctive interventions in Alzheimer's, Parkinson's, and related disorders, pending validation *in vivo*. In metabolic conditions, induction of detoxification pathways and regulation of central metabolic signaling suggest prospects for improving insulin sensitivity and mitigating oxidative stress that require further investigation.

Finally, in rare and autoimmune diseases, targeted cell-cycle arrest and anti-inflammatory signaling point to a potential strategy to stabilize cell proliferation and curb chronic inflammation, which remains to be experimentally tested.

Conclusions

The results obtained using an integrative methodology highlight the transcriptomic effects of the probiotics *P. freudenreichii* SN and *B. subtilis* CW14 on intestinal cells, suggesting that each strain may exert specific actions, particularly in pathways related to immunity and the cell cycle. Furthermore, they suggest a pleiotropic association of these probiotics with genes linked to metabolic, neurological, and autoimmune diseases, modulating genes previously described as having neuroprotective or antitumor functions in other contexts. Key molecular mechanisms of immunomodulation also emerged from the data, such as innate TLR and NOD receptors, and signaling pathways including NF-κB and MAPK. Finally, our findings provide a basis for further exploration of the concept of the microbiota as a dynamic organ with systemic influence and a crucial role in the gut-brain axis.

Supplementary information

Figure S1: https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5339
Table S1: https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5340
Table S2: https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5341
Table S3: https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5342
Table S4: https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5343
Table S5: https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5344
Table S6: https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5345
Table S7: https://www.raccefyn.co/index.php/raccefyn/article/view/4034/5346

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Author contribution

NBR: Writing of the original draft, visualization, validation, software, methodology, data curation, formal analysis, and investigation; **JAGZ:** Writing, review, and editing, supervision, project administration, methodology, and conceptualization; **LSZ:** Writing, review, editing, supervision, methodology.

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Conflicts of interest

The authors have no conflict of interest to declare.

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