Analysis of gene expression regulation of colon adenocarcinoma genes treated with three probiotic species.

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COVER LETTER

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To: Taylor & Francis Group

Editors-in-Chief

Gut Microbes

From: Jerson Alexander Garcia-Zea, Laura Sierra-Zapata, Alejandra Velez-García*.

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Subject: submission of manuscript "**Analysis of gene expression regulation in colon adenocarcinoma treated with three probiotic species**" to the editorial board of the journal Gut Microbes.

Dear Editors-in-chief and members of the editorial board,

We hereby present on behalf of ourselves, as well as the rest of authors, the original research article entitled "**Analysis of gene expression regulation in colon adenocarcinoma treated with three probiotic species**", which we would like to submit for consideration for the prestigious Journal that you preside. We confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

Our study investigates the impact of three probiotic species, with a particular focus on the probiotics *P. freudenreichii* SN and *B. subtilis* CW14, on gene expression in colon adenocarcinoma cells. We are pleased to inform you that our findings highlight the crucial role of these probiotics in various key aspects, including immune response, antiviral defense, autophagy, inflammatory response, cellular stress, and metabolism. Most notably, our results suggest a potential beneficial effect on intestinal health and possibly in preventing the development of colon cancer associated with the use of these probiotics. The complexity of gene responses observed to different probiotics is also underscored in our study, revealing a multifaceted range of impacts through the activation and inhibition of various pathways.

We firmly believe that our findings are highly relevant to the Gut Microbes audience, as they contribute to the understanding of the molecular mechanisms underlying the interaction between the intestinal microbiota and human health, as well as the therapeutic potential of probiotics in the context of colorectal cancer.

We have no conflicts of interest to disclose. Thank you for your consideration of this manuscript.

Sincerely,

Jerson Alexander Garcia-Zea, Laura Sierra-Zapata, Alejandra Velez-García*

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Chapter 1

Abstract

The human intestine is home to more than 100 billion symbiotic bacteria, which far exceeds the number of host cells. These microorganisms together constitute the so-called gut microbiota or intestinal flora. This microbiota performs a wide range of functions that are important to the human body, including protection against pathogens, nutrient extraction, metabolism and immunity that, under healthy conditions, result in stability, resilience and beneficial symbiotic interactions. Therefore, in recent years, the number of studies associating the use of probiotic strains for the prevention and treatment of different diseases, including cancer, has increased significantly. In this sense, several studies have shown that the microbiota can affect the origin and development of cancer and therefore can be used for cancer prevention and/or as an adjunctive therapy during chemotherapy against this pathology. Thus, the modification of the communities present in the microbiota, either by including or excluding microorganisms, can help prevent the development of different diseases. This is related to the colonization of the intestinal tract by the microbiota, which can trigger immune responses involved with the recognition of microbial signals and stimuli by innate receptors that can modulate the function of intestinal immune cells. Therefore, to understand the mechanisms associated with this disease, is essential to identify and thoroughly characterize the effect of a specific probiotic on the expression and modulation of genes associated with colon adenocarcinoma cells.

In the present study, we conducted a series of *in-silico* analyses through the integration of open-access data to evaluate the possible effects of various probiotic strains on two specific cell lines, Caco-2 and HT29, to understand the effects of these on the modulation of human genes linked to colon adenocarcinoma and thus establish the impact on key aspects such as immune response, antiviral defense, autophagy, cellular stress and metabolism.

1. Background

1.1. Colon Cancer

Cancer is a disease of uncontrolled proliferation of transformed cells that are subject to evolution by natural selection (**Brown *et al*., 2021**). In this regard, cancer is one of the terms used to call a large group of diseases that can affect any part of the human body and one of its defining characteristics is the rapid formation of abnormal cells that grow beyond their usual limits and can spread to other adjacent organs; this latter process is known as metastasis (**Liu *et al*., 2019**). This disease is one of the leading causes of death worldwide, accounting for nearly 10 million deaths in 2020 (**Ferlay et al., 2020**), with lung cancer being the leading cause of death from this condition, resulting in 1.8 million deaths, followed by colon cancer with 916,000 deaths, according to data reported by WHO https://www.who.int/es/news-room/fact-sheets/detail/cancer.

Cancer arises from the transformation of normal cells into tumor cells through a multi-stage process that typically progresses from a precancerous lesion to a malignant tumor (**Brown et al., 2021**). The changes that can occur in normal cells are the result of the interaction between genetic factors and external agents, including physical carcinogens such as ultraviolet and ionizing radiation; chemical carcinogens like asbestos, tobacco smoke components, alcohol, aflatoxins, and arsenic; and biological carcinogens, such as infections by certain viruses, bacteria, or parasites (NCCN 2023). In addition, there are risk factors that can influence the onset and development of cancer. These include advanced age, which likely reflects an accumulation of risks leading to specific cancers. Other notable risk factors are alcohol and tobacco use, unbalanced diets, lack of exercise, air pollution, and exposure to certain viruses such as human papillomavirus and hepatitis (NCCN 2023).

Colon cancer (CRC) is the third leading cause of cancer-related deaths in male and female worldwide (**Xi & Xu, 2021; Morgan *et al*., 2023**). In 2020, CRC accounted for 10% of global cancer incidence and 9.4% of cancer deaths; the number of new cases is expected to reach 3.2 million by 2040 (**Xi & Xu, 2021**). According to GLOBOCAN estimates for age-standardized mortality rates (ASMR), suggest a higher ASMR for CRC in men (11.0) than

in women (7.2) (**Roshandel *et al.*, 2024**). In men, it is the second most diagnosed tumor (8.6% of cases) behind prostate cancer, whereas in women, it is the third most common type of tumor (8.8% of cases), surpassed only by breast cancer and thyroid cancer (**Ferlay *et al.*, 2020**). There are several types of colon cancer, depending on the origin of the tumor cell from embryonic development; the most common is adenocarcinoma, which develops in the cells of the large intestine lining. However, there are other less common types such as primary colorectal lymphomas, gastrointestinal stromal tumors, leiomyosarcomas, carcinoid tumors, and melanomas (**Angell *et al.*, 2020**). Diet is one of the most significant exogenous factors identified to date, according to reports from the World Cancer Research Fund and the American Institute for Cancer Research (https://www.wcrf.org/diet-activity-and-cancer/), that have concluded that colorectal cancer can primarily be prevented with proper diets and the control of factors associated with the development of this pathology. Other factors that can induce the disease include tobacco use, alcohol consumption, an increase in intestinal aerobic flora, and bacteremia caused by *Streptococcus bovis* (**WHO, 2005**). The symptoms of colon cancer are nonspecific, ranging from changes in bowel habits, general abdominal discomfort, unexplained weight loss, and constant fatigue to intermittent abdominal pain, nausea or vomiting, bleeding, obstruction, and perforation. A palpable mass is common with right-sided colon cancer. Bleeding may be acute and most commonly appears as red blood mixed with stool. Chronic loss of occult blood with iron deficiency anemia occurs frequently. Such patients may present with weakness and high-output congestive heart failure (**WHO 2005**).

1.2. Colon cancer treatments

According to the stage of the disease, different treatments are applied. For earlier stages of cancer where there is no metastasis, colectomies can be performed to remove the cancerous cells. In cases where there is metastasis or the tumor is medically inoperable, chemotherapy or radiotherapy are used as treatments (NCCN 2023). Additionally, other treatments such as probiotics are used as adjuvants in the treatment of the disease. Each of these treatments is detailed below.

1.2.1 Chemotherapy

According to the National Comprehensive Cancer Network (NCCN), the clinical treatment of metastatic or advanced colon cancer should be carried out by means of chemotherapy using drugs such as FOLFOX, CapeOX or FOLFIRI. Table 1 details the mechanism of action of each of the drug compounds.

Drug/Brand name	Components	Mechanism of action	Reference
Folfox	Folinic acid, 5-Fluorouracil, Oxaliplatin	Folinic Acid: This acid works as an adjuvant in the treatment of colon cancer, acting as a biochemical modulator of 5-FU by enhancing its antineoplastic activity. 5-FU: It indirectly interacts with the folate cycle, inhibiting the enzyme thymidylate synthase, and thus the formation of deoxythymidine monophosphate (dTMP) and the production of thymidine, which can cause a breakdown of the DNA chain. Oxaliplatin: Contains a platinum (Pt) atom that interacts with the nitrogen 7 of the DNA's guanine and causes the formation of covalent adducts between two guanines, disrupting DNA replication and transcription.	**Schneiders *et al.*, 2010; Martinez-Balibrea *et al.*, 2015, Kim, 2016; Hussain, 2020**
CapeOX	Capecitabine,oxaliplatin.	It is an orally administered, tumor-activated anticancer agent that is rapidly absorbed through the intestine and converted into 5-FU by the enzyme thymidine phosphorylase (TP). It is used both as a standalone therapy and in combination in metastatic cancer. This drug is often used in	**Pouya *et al.*, 2021**

		conjunction with others, such as oxaliplatin, where it has been shown to inhibit certain signaling pathways, increase non-cancerous cell survival, reduce tumor growth, and decrease the side effects of capecitabine.	
Folfiri	Irinotecan, Folinic acid, Fluorouracil.	Irinotecan: A prodrug that produces the metabolite SN-38, which inhibits the enzyme topoisomerase I. It causes DNA supercoiling and inhibits cell proliferation in processes such as translation, transcription, and mitosis.	**Fujita *et al.*, 2015**

Table 1. Drugs used for colon cancer chemotherapy.

1.2.2 Biological drugs and immunotherapy

The spread of colon cancer can be controlled or mitigated using drugs that intervene in the mechanisms of angiogenesis, proliferative signaling and immune evasion. The mechanisms of cancer spread, and the drugs used for treatment are described below (Table 2).

- Angiogenesis: Angiogenesis is a normal process in the body promoted by different biomolecules. In this process, local or systemic endogenous chemical signals are produced, and coordinate the functions of endothelial cells and smooth muscle cells to repair damaged blood vessels (**Rajabi & Mousa, 2017**). As in angiogenesis the formation of vesicles occurs for the formation of new blood vessels from existing vessels, this process in some cases can promote tumor progression (**Zhao & Adjei, 2015**). To control this process, angiogenesis inhibitors can systematically alter blood vessel formation or eliminate existing vessels, these inhibitors work by acting on proteins that have been identified as angiogenic activators, including vascular endothelial growth factor (VEGF) which has a significant role in cell survival and motility **(Mousa & Davis, 2017**). Bevacizumab and

Panitumumab (**Table 2**) are the main drugs used to cause a blockade in angiogenic activators.

- Proliferative Signaling: Proliferation is one of the most critical factors in the development and progression of cancer, making it one of the most studied mechanisms. Development and progression are affected by the altered expression and activity of proteins related to the cell cycle and by oncogenes that encode proteins for proliferation, survival, and cell growth with one or more mutations (**Feitelson *et al.*, 2015**).

  Moreover, there are other processes that can positively affect cell proliferation, such as carcinogenesis, which involves changes in tissue architecture and the formation of preneoplastic nodules that precede the onset of cancer; hypoxia, which promotes the survival and growth of tissue stem cells (**Harris, 2002**); and angiogenesis (**Fang *et al.*, 2008**). Currently, Cetuximab (**Table 2**) is used as a treatment to reduce cell proliferation, which inhibits mitosis in adenocarcinomas and prevents cell growth, so the cell cannot survive (**Fornasier *et al.*, 2018**).

- Immune system evasion: According to several studies immune evasion by tumors can be divided into two broad categories: **1)** induction of tolerance by the developing tumor and **2)** resistance to destruction by activated immune effector cells (**Drake *et al.*, 2006**). There are multiple mechanisms by which immune evasion can occur as in the case of tolerance induction, the immune system "ignores" the tumor or the tumor may actively induce anergy among tumor-specific T cells and can even reduce regulatory T cells or lead to the elimination of tumor-specific T cells (**Drake *et al.*, 2006**).

  To prevent tumors from evading the immune system, immunotherapy has been used in recent years as a treatment, where substances that stimulate the immune system are used to fight cancer, infections and other diseases **(Sáez-López et al., 2017)**. One of the most used drugs in immunotherapy is Pembrolizumab (**Table 2**) which increases the efficacy of the T-cell response and the reactivation of antitumor immunity, which reduces tumor

growth and prolongs survival (**Mcdermott & Atkins, 2013; Deeks, 2016**).

Drug/Brand name	**Components**	**Mechanism of action**	**Reference**
Avastin (Bevacizumab)	Recombinant humanized monoclonal antibody against vascular endothelial growth factor (VEGF).	Inhibits the signaling of vascular endothelial growth factor (VEGF) that can affect tumor growth and progression through various mechanisms, including: 1) inhibiting the growth of new vessels, 2) regression of newly formed vasculature, 3) altering vascular function and tumor blood flow, 4) direct effects on tumor cells.	**Hicklin & Ellis, 2005; Wey *et al.*, 2005; Ellis, 2006; McCormack & Keam, 2008**
Panitumumab	Recombinant, fully human, anti-epidermal growth factor receptor (EGFR) IgG2 monoclonal antibody.	The mechanisms of Panitumumab's antitumor activity include negative regulation of EGFR, induction of apoptosis through inhibition of EGFR signaling pathways, cell cycle arrest, autophagy, and inhibition of angiogenesis.	**Foon *et al.*, 2004; Keating 2010, Tol & Punt, 2010**
Cetuximab	Recombinant chimeric IgG1 monoclonal antibody and epidermal growth factor receptor (EGFR) inhibitor.	It inhibits epidermal growth factor (EGF) and other ligands, which causes blocking of phosphorylation and activation of receptor-associated kinases (MAPK and PI3K/Akt), resulting in inhibition of cell growth, induction of apoptosis and decreased cancer invasion and metastasis. Additionally, it inhibits the development of new blood vessels by decreasing the production of vascular endothelial growth factor and activates the human anti-chimeric antibody (HACA).	**Lenz, 2007; Vincenzi *et al.*, 2008; Fornasier *et al.*, 2018**

Pembrolizumab	Humanized monoclonal antibody used against programmed death receptor 1 (PD-1). (PD-1).	Pembrolizumab acts by blocking PD1 receptor binding to PD-L1 and PD-L2 ligands, preventing PD1-mediated inhibition, allowing the normal T-cell immune response to tumor cells to occur. PD-1 blockade can increase natural killer cell activity and antibody production by B cells.	**Ascierto *et al.*, 2013; Tang & Heng, 2013; Mamalis *et al.*, 2014; Poole, 2014 Deeks, 2016**

Table 2. Drugs used for colon cancer immunotherapy.

1.2.3 Probiotics as co-adjuvants

There are more than 100 billion symbiotic bacteria in the human intestine, a number that far exceeds the number of host cells, which together constitute the intestinal flora (**Lu *et al.*, 2021**). In recent years, multiple studies have been conducted on the use of probiotics for the prevention and treatment of different diseases (**Sanders *et al.*, 2019**). Regarding this, probiotics play an essential role in the intestinal microbiota as they can exert a variety of beneficial effects on the host, including on metabolism, endocrinology, improvement of the intestinal barrier, in addition to the regulation of the immune system (**Ley *et al.*, 2006**). Therefore, by modifying the communities present in the microbiota, either by including or excluding microorganisms, it is possible to prevent or treat various intestinal diseases such as irritable bowel syndrome, cancer, and systemic disorders such as eczema, allergies, and diabetes (**Cani & Delzenne, 2009**).

The effects associated with gut flora can be categorized into non-specific and specific physiological effects. In the first case, probiotics can contribute to maintaining a healthy balance of the intestinal flora, as they can increase the bacterial strain count and thus promote beneficial changes in the diversity and composition of the intestinal flora, resulting in stabilization of the intestinal epithelial cell barrier and inhibition of pathogens (**Lu *et al.*, 2021**). And secondly, specific effects have been associated with the regulation of humoral immunity,

innate immunity, and cellular immunity through different mechanisms (**Cerdó *et al.*, 2019**).

Colonization of the intestinal tract by the microbiota triggers a mucosal immune response that comprises the recognition of microbial signals and stimuli by innate pattern recognition receptors (PRR), which modulate the function of mucosal immune cells (**Trejo & Sanz, 2013**). When probiotics bind to some of these receptors, three main signaling pathways in the immune system are activated: 1) nuclear factor kappa B (*NF-κB*), 2) mitogen-activated protein kinases (*MAPK*), and 3) interferon regulatory factors (IRF) (**Lee & Kim, 2007**). This activates a gene expression program for inflammatory genes encoding cytokines, immunoregulatory proteins, adhesion molecules, stress-associated proteins, and other mediators, as well as the recruitment of other immune cells (T cells, basophils, neutrophils, dendritic cells, and natural killers). The collective expression of these genes can lead to the elimination of pathogens and tumor cells.

Evidence suggests that bacterial metabolites have a protective effect. Butyrate, for example, produced solely by specific members of the *Firmicutes* phylum through the fermentation of dietary fiber and resistant starches, can modulate inflammation, epithelial proliferation, and apoptosis (**Chang *et al.*, 2014**). **Ganapathy *et al.* (2013**) demonstrated that butyrate was recognized by host colonic receptors *GPR109* and *GPR43* in mice, where a higher susceptibility to tumors was observed in mice lacking *GPR109*. On the other hand, **Belcheva *et al*. (2014)** showed that butyrate can, alternatively, promote epithelial proliferation leading to tumorigenesis in the case of genetic modifications.

Other studies have highlighted the specific role of lactobacilli in the prevention of colon cancer in animal models (**Chang *et al.*, 2012; Zhu *et al.*, 2014**). For example, protective effects of *Lactobacillus acidophilus* and *Lactobacillus salivarius* have been unveiled in the development of precancerous growths and colorectal carcinogenesis in rat models, respectively. **Lee *et al*. (2015)**

demonstrated how a specific strain of *Lactobacillus plantarum* inhibited colon cancer in a mouse model after chemical induction of cancer.

Numerous studies have shown that the expression of receptors such as toll-like types differs between commensal and pathogenic bacteria, as well as between Gram-positive and Gram-negative bacteria. It has been reported that Gram-positive bacteria increase the number of *TLR2*-positive cells (**Grangette *et al.*, 2005**), while for Gram-negative bacteria, the number of *TLR4*-positive cells increases in parallel with the induction of different cytokines (interleukin *IL-10* and *IL-12*). Thus, the use of different probiotics can trigger various immune responses, granting different or common outcomes depending on the case.

1.3 Cell lines

1.3.1 Caco-2

Caco-2 cells, derived from human colon adenocarcinoma, play a crucial role as producers and receptors of cytokines and chemokines. These signals are key in modulating epithelial activity, influencing proliferation, migration, and cell survival (**Francescone *et al.*, 2016**). Intestinal endothelial cells also contribute to the immune response by differentiating *in vitro* into M-like cells, responsible for absorbing, transporting, and presenting antigens to the lymphoid cells of the sub epithelium (**Simon-Assmann *et al.*, 2007**), expressing Major Histocompatibility Complex II (**Jung *et al.*, 1995**).

The Caco-2 cell line has been extensively implemented and characterized; it originated from human colon adenocarcinoma cells and exhibits characteristics of a mature enterocyte (**Lehto & Salminen, 1997**). These cells are a fundamental tool in probiotic microorganisms research, as they allow for the exploration of aspects such as antimicrobial activity (**Jacobsen *et al.*, 1999**) and the adhesion mechanisms of non-pathogenic microorganisms. Additionally, Caco-2 cells are susceptible to both the expression and induction of pro-inflammatory and anti-inflammatory cytokines in response to various stimuli (**Jung *et al.*, 1995**).

Among the pro-inflammatory cytokines released by these cells are *TNF-α, IL-1, IL-8, and IL-6* (**Morita *et al.*, 2002**), as well as complement proteins (C3, C4, and factor B) (**Jung *et al.*, 1995**). On the other hand, Caco-2 cells express anti-inflammatory cytokines, such as transforming growth factor β (TGF-β) (**Jung *et al.*, 1995**). These characteristics make Caco-2 cells a valuable tool for exploring the intricate cellular and molecular mechanisms that regulate the immune response in the context of the intestinal epithelium.

1.3.2 HT29

The HT29 cell line, a human colon adenocarcinoma, was originally isolated in 1964 from a primary tumor of a 44-year-old Caucasian woman by **Fogh & Trempe (1975).** Since its discovery, many cell lines derived from human colon cancers have been developed. At the start, the HT29 line was used to study various aspects of the biology of human cancers, particularly for its ability to express characteristics of mature intestinal cells, such as enterocytes and mucus-producing cells (**Fogh & Trempe, 1975**).

The use of the HT29 cell line as an *in vitro* model of intestinal cells presents advantages and limitations that have been summarized by **Zweibaum *et al.* (2011)**. In their differentiated phenotype, these cells resemble enterocytes of the small intestine in terms of structure, presence of brush border-associated hydrolases, and the temporal course of the differentiation process, comparable to the one found in the small intestine.

Regarding the expression of cell surface receptors, differentiated HT29 cells have been reported to express receptors for peptides such as vasoactive intestinal peptide or insulin, as well as receptors for non-peptidic substances. Although most receptors are equivalent to those of normal intestinal cells, the neurotensin receptor, characterized in HT29 cells (**Kitabgi *et al.*, 1980**), but not detected in normal human colon epithelium, is noteworthy. Also, the presence of opioid, serotonin, muscarinic, and *PPARβ/δ* receptors has been observed in this cell line in recent studies (**Zoghbi *et al.*, 2006; Ataee *et al.*, 2010; Belo *et al.*, 2011; Foreman *et al.*, 2011**). More detailed information about receptor studies in this cell line can be found in **Zweibaum *et al.* (2011).**

In this context, this work aims to identify the effect of various probiotics on the expression and modulation of genes associated with colon adenocarcinoma cells through the study of different cell lines.

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Chapter 2

2. Materials and methods

2.1. Data collection and differential expression analysis

For data collection, a search for secondary information was conducted in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), using the keyword "*Probiotic*" as a filter. This search yielded 47 article results, of which, after reading the abstracts, three were selected that met the following criteria: 1) the sample or organ used for the research was in colon adenocarcinoma cells (Caco-2 and/or HT29), 2) the effect to be evaluated was in the colon, and 3) the treatment used was a probiotic.

For instance, RNA data was collected from **Peng *et al.*, (2019)** who used the probiotic strains *Bacillus subtilis* CW14, **Lépine *et al.*, (2018)** where *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W36, and *Lactobacillus casei* W56 were used, and **Cousin *et al.*, (2016)** with *Propionibacterium freudenreichii* ITG P9. The accession numbers are as follows: GSE115081, GSE115022, GSE67033, from the Gene Expression Omnibus (GEO) database (**Clough & Barrett, 2016**) respectively, which belong to the NCBI database (**Sayers *et al.*, 2022**). Using Caco-2 cells (validation dataset) and HT29 cells (validation dataset) with 6-hour treatment for all probiotics, the RNAseq data was analyzed from FASTQ files, which were filtered excluding low-quality sequences and adapter sequences were removed using Trimmomatic software (V0.32) (**Bolger *et al.*, 2014**). Sequences were mapped against version 38 of the human genome (GRCh38) using TopHat2 (**Kim *et al.*, 2013**). Counts for each gene were obtained using the Feature Counts program (**Anders *et al.*, 2015**). DESeq2 (version 1.38.1) (**Love *et al.*, 2014**) was used to normalize the expression counts. mRNAs were considered differentially expressed if their |fold change| (FC) ≥ 2, and FDR ≤ 0.05

2.2. Gene enrichment analysis

Common genes differentially expressed in the 3 probiotics were retrieved from comparison of remarks derived from enrichment analyses.

2.3. Common genes retrieval

For the analysis of common gene enrichment, Enrichr was used (Ontologies: GO Biological Process 2023 and Pathway: Elsevier Pathway Collection) (**Chen *et al*., 2013**) along with the David database (Biological process and KEGG pathway) (**Sherman *et al*., 2021**). The results from both databases were cross-referenced to get a better understanding of the biological processes and pathways involved. For a specific approach to links with colon cancer (COAD), a second assessment involved analyzing the common genes identified, using the GSCA platform (Gene Set Cancer Analysis) (**Liu *et al*., 2023**). This analysis aimed to determine the potential effects of mRNA from differentially expressed common genes on pathway activity (activation or inhibition), in which case GSCA includes 10 pathways linked to cancer. The pathways the following: Tuberous Sclerosis Complex/mammalian Target of Rapamycin (TSC/mTOR), related to cell growth regulation and protein synthesis; Receptor Tyrosine Kinase (RTK), which participates in cellular signal transduction; Rat Sarcoma/Mitogen-Activated Protein Kinase (RAS/MAPK), an intracellular signaling pathway associated with regulation of cell growth and differentiation; Phosphoinositide 3-Kinase/Protein Kinase B (PI3K/AKT), involved in cell survival, growth, and proliferation; estrogen receptors (Hormone ER), involved in the response to female sex hormones; androgen receptors (Hormone AR), androgen receptors that respond to male sex hormones; Epithelial-Mesenchymal Transition (EMT), referring to the biological process related to cell mobility and invasion; DNA Damage Response, response to DNA damage, which includes mechanisms of repair and cellular regulation; Cell Cycle, indicating the cell cycle and including phases of cell division and growth; and Apoptosis pathways, referring to the process of programmed cell death, essential for maintaining cellular balance and eliminating damaged or unnecessary cells.

2.4. Transcription factor motif analysis

Homer software (**Heinz *et al.*, 2010**) was used to detect transcription factor motifs overrepresented in the promoter of the expressed genes. Promoters were considered significant if they were located between nucleotides -300 and +50 relative to the start of the transcription site. Binding sites were considered significantly overrepresented if they had a $p < 0.01$.

2.5. Interaction network

Based on the differentially expressed genes, protein-protein interaction networks were mapped using STRING (**Snel *et al.*, 2000; Szklarczyk *et al.*, 201**7), which was also used to identify functional interactions among the corresponding genes. The network visualization was plotted using the open-source software platform Cytoscape v. 3.6.13 (**Shannon *et al.*, 2003**).

3. Results:

3.1 Colon adenocarcinoma cells (Caco-2 and HT29) treated with different probiotics show differentially expressed mRNA.

Principal Component Analysis (PCA) was performed to distinguish between different groups of probiotics and the control Caco-2 and HT29 cells (**Fig. 1A**). The PCA for each platform demonstrated high mRNA heterogeneity as observed in the spatial distribution of treatments, indicating intragroup variability. For the treatment with *B. subtilis* CW14, approximately 20,510 transcripts were obtained, for *P. freudenreichii* SN 58,718, and for *L. acidophilus* W37, *L. brevis* W63, and *L. casei* W56, 33,298 mRNA transcripts were filtered with a log2 fold change ≥ 1 and log2 fold change ≤ -1 and a *p-value* ≤ 0.05. These analyses explained that the different treatments significantly affected gene expression. In the case of *B. subtilis* CW14, in total 786 genes were affected, of which 712 (39.86%) were positively regulated and 1,074 (60.13%) negatively regulated. For the treatment with *P. freudenreichii* SN, a total of 1,782 genes were affected, of which 1,093 (61.33%) were positively regulated and 689 (38.66%) negatively, whereas for the treatments with *L. acidophilus* W37, *L. brevis* W63,

and *L. casei* W56, 3,224, a total of 295, and 258 genes were affected respectively, where 1,459, 166, 121 (45.25%, 56.27%, 46.89%) were positively regulated and 1,765, 129, and 137 (54.74%, 43.72%, 53.10%) were negatively regulated (**Fig. S1**). These results show that the probiotics used modulate the expression of genes associated with the maintenance of the intestinal barrier.

Furthermore, changes in differentially expressed genes showed that, for the treatment with *B. subtilis* CW14, 20% of the positively regulated genes had an FC exceeding the threshold of at least 5-fold, for the treatment with *P. freudenreichii* SN this was observed in 40%, whilst for the treatments with *L. acidophilus* W37, *L. brevis* W63, and *L. casei* W56 this limit was not exceeded and lower FC values were observed, where 80% of the genes did not exceed the 1.5-fold limit (**Fig. 1B**). Comparable results were observed for negatively regulated genes as for positively regulated genes (**Fig. 1C**). The heatmaps shows the different gene expression profiles for each of the treatments (**Fig. 1D**).

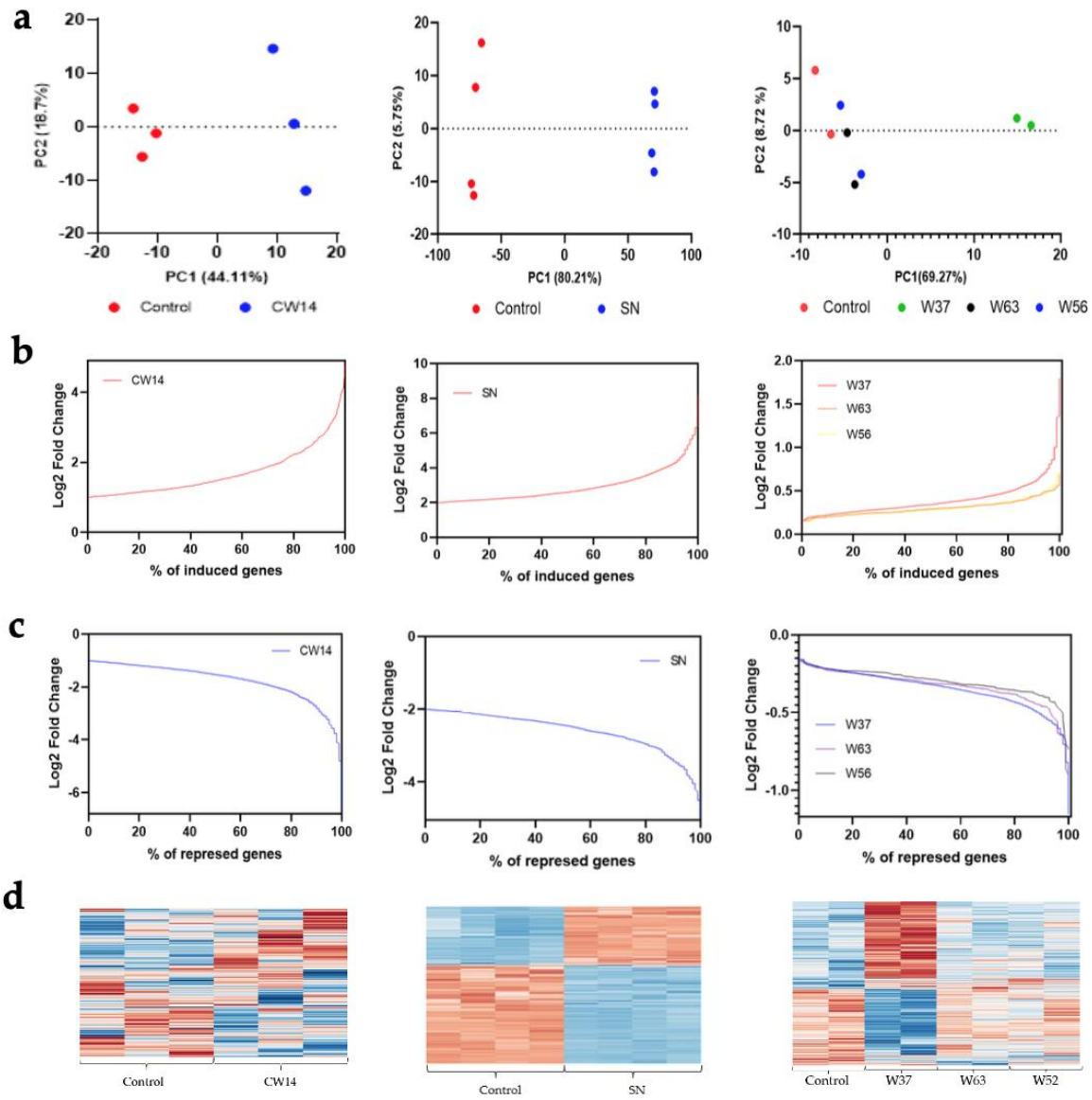

Fig. 1. Transcriptome characterization of intestinal epithelial cells in contact with various probiotic treatments (*B. subtilis* CW14, *P. freudenreichii* SN, *L. acidophilus* W37, *L. brevis* W63, and *L. casei* W56), using Illumina, Agilent, and Affymetrix as technologies for gene expression quantification. (A) Principal Component Analysis of mRNA data, each principal component (PC1, PC2) displays the percentage of variance. **(B and C)** Cumulative frequency distribution indicated as a percentage (%, x-axis), of differentially expressed genes (red line) upregulated and (blue line) downregulated (log2-fold change, y-axis). **(D)** Heat map of differentially expressed genes, normalized with a Z-score. Negatively and positively regulated genes with absolute fold-change values > 1 and *p-value* <= 0.05 are shown in red and blue, respectively.

3.2 Probiotics activate different gene expression programs in colon adenocarcinoma cells (Caco-2 and HT29).

To understand the biological processes affecting the gene expression programs in colon adenocarcinoma cells activated by probiotics, we peformed a gene ontology (GO) analysis. We hypothesized that various probiotics promote or suppress the expression of common genes associated with colon adenocarcinoma cells, and that these are modulated independently of the probiotic stimulus.

3.2.1 Analysis of common genes using David and Enrichr.

After analyzing the differential expression data of genes in Caco-2 and HT29 cells, the next step was to characterize the common genes, resulting in 69 genes (**Table S1, Fig. S2**). This analysis provided information on the common general response of both cell lines regarding perhaps fundamental processes impacted regardless of the probiotic stimulus. To understand the biological processes associated with these genes, we analyzed the enrichment results. Thus, the analysis with David and Enrichr led to 40 (62.5%) genes associated with biological processes (**Table S2, S3, S4**), showing 36 gene ontology groups clustered into different related modules, such as transcription regulation, apoptosis, immunity, autophagy, virus-host interactions, cell cycle, DNA repair, DNA damage, cell adhesion, transport, among other processes (**Table S3**).

We examined the modulation of differentially expressed genes linked to immune response, autophagy, inflammation, cellular stress, and metabolism (**Fig. 2**). There were six immune response genes, three related to the innate immune response (N4BP3, NEURL3, PLA2G2F) and one to the adaptive immune response (DBNL); the remaining two genes were classified as immune response (ULBP1, ULBP2). Particularly, all were expressed positively and significantly (FC > 2) under treatment with the probiotic *P. freudenreichii* SN. However, for the probiotics *B. subtilis* CW14 and *L. acidophilus* W37, the expression was variable. In terms of virus-host interactions, we observed that some of these genes also participate in this process (NEURL3, ULBP, ULBP2), along with the genes PIM1 and PRMT6, in which case the regulation of their expression varied with the different treatments. Likewise, we identified

a specific gene involved in antiviral defense (DDIT4), that showed positive regulation mediated by the probiotic *P. freudenreichii* SN. Also, the genes associated with autophagy are noteworthy, since they are key to maintaining intestinal homeostasis and cell survival (**Lapaquette *et al*., 2021**). Thus, three genes characterized for this process were identified. Two of them, C9ORF72 and MAPILC3B, showed positive modulation by the probiotic *P. freudenreichii* SN, whereas ATG10 was negatively regulated. In terms of inflammatory response and stress response, the identified genes were *CCL20* and *DUPS1*. Both genes were positively regulated by the probiotic *P. freudenreichii* SN. Regarding metabolism, we observed that the gene GSTM2, an important detoxifier of electrophilic compounds including carcinogens or environmental toxins **(Sauer *et al.,* 2007**), was positively modulated by the probiotics *P. freudenreichii* SN and *B. subtilis* CW14. Another interesting finding was related to the negative regulation of the gene EPC1 by the three probiotics. Such regulation has been associated with an increase in apoptosis power through the mediation of the E2F1 gene (**Wang *et al*., 2016; Denechaud *et al.*, 2017**). All results can be seen in **Table S1**.

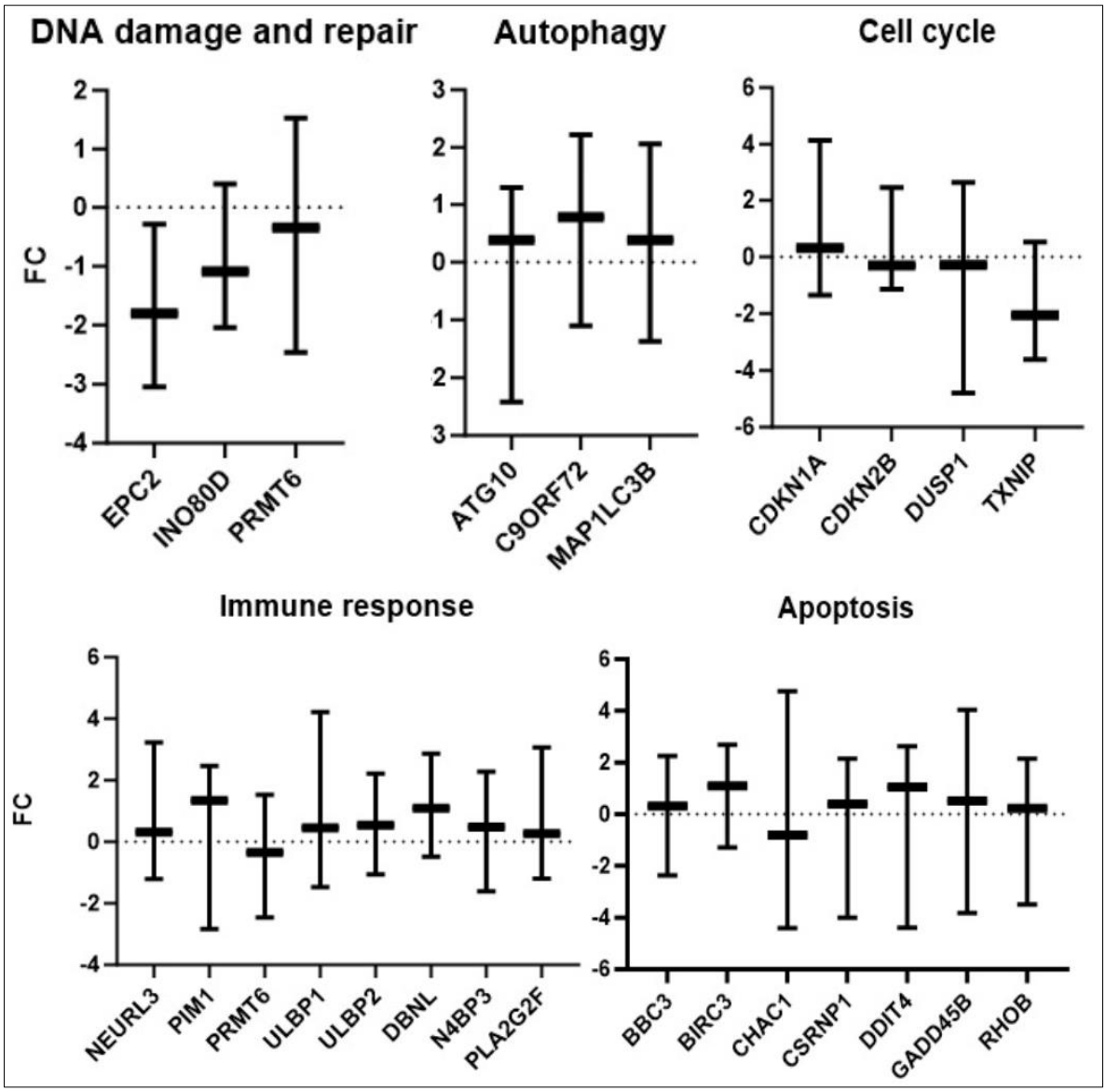

Figure 2. Analysis of common genes by David and Enrich. Different gene ontology groups are shown grouped in five related modules: DNA damage and repair, autophagy, cell cycle, immune response, and apoptosis. On the "Y" axis the FC values obtained by the probiotics used (*P. freudenreichii* SN, *B. subtilis* CW14 and *L. acidophilus* W37) and on the "X" axis the genes associated with the biological processes.

3.2.2 Analysis of common genes through Gene Set Cancer Analysis (GSCA)

Through this analysis, we associated the genes to 9 out of the 10 pathways included in GSCA, excluding the hormone AR pathway. Out of the 69 common genes, 41 (59.4%) were involved in some of the pathways. Of these, 30 (73%) were associated with the activation of one of the pathways and 31 (76%) to the inhibition (**Fig. 2 and Tables S5 to S8**). Among the genes,

we highlight *DENND3* and *EGR2*, which engaged in the activation of the most pathways (6), while *DENND3, TXNIP*, and *ZSWIM6* participated in the inhibition of the most pathways (3) (**Fig. 3 and Tables S5, S6)**.

From the analysis of specific genes that were differentially and positively expressed, we highlight those with a |fold change| (FC) ≥ 3 that activate any of the previously mentioned pathways. The most meaningful results were mainly linked to the treatment with the probiotic *P. freudenreichii* SN. In this regard, the genes *CHAC1* linked to the cell cycle, *DUSP5*, *GADD45B, GEM,* and *SERPINE* associated with the EMT pathway, and *TNFSF9* involved in apoptosis were observed. For specific genes negatively regulated and involved in inhibition, the most significant results were associated with the probiotic *B. subtilis* CW14, where the associated genes were *CHAC1* to the cell cycle, *CSRNP1* with the RTK and TSCm/TOR pathways, *DUSP1* associated with RTK, *FOS* and *FOSB* linked to the RAS/MAPK, RTK, and TSCm/TOR pathways, *GADD45B* and *GEM* with the EMT pathway, and TXNIP linked to RAS/MAPK (**Tables S5 to S8**). Suggesting that the inhibition or activation of a pathway varies depending on the probiotic stimulus and that a gene can be involved in the modulation of one or several pathways.

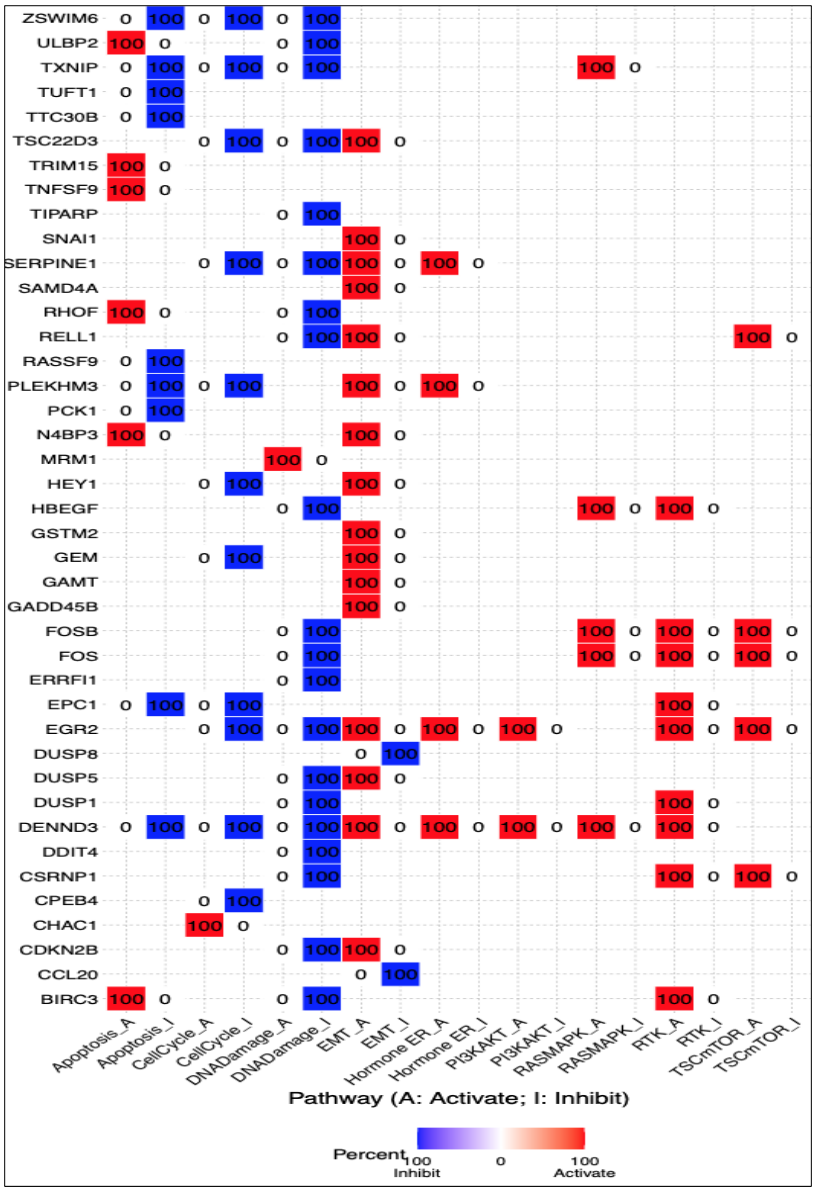

Figure 3. Analysis of common genes through GSCA (Gene Set Cancer Analysis). Summary of the percentage of colon cancer cases in which the mRNA expression of a specific gene has a potential effect on pathway activity. Red indicates activation and blue indicates inhibition of any of the pathways. The number in each cell indicates the percentage in which a gene showed a significant association for colon cancer. On the "X" axis the pathways included in GSCA for the analysis of association with pathways related to colon cancer. Apoptosis: genes involved in the process of programmed cell death; Cell Cycle: genes related to the cell cycle involved in different phases of cell division and growth; DNA Damage Response: genes linked to the response to DNA damage, including repair and cellular regulation mechanisms; Epithelial-Mesenchymal Transition (EMT): biological processes related to cell mobility and invasion; (Hormone ER): genes involved in the response to female sex hormones and androgen receptors; Phosphoinositide 3-Kinase/Protein Kinase B (PI3K/AKT): genes associated with cell survival, growth, and proliferation; Rat Sarcoma/Mitogen-Activated Protein Kinase (RAS/MAPK): genes linked to intracellular signaling associated with the regulation of growth and cell differentiation; Receptor Tyrosine Kinase (RTK): receptors that participate in cellular signal transduction; Tuberous Sclerosis Complex/mammalian Target of Rapamycin (TSC/mTOR): related to the regulation of cell growth and protein synthesis. On the "Y" axis the common genes and their role in activating (red) or inactivating (blue) the pathways associated with colon cancer.

3.3 Transcriptional Regulation of Genes in Caco-2 Cells

Given the results, we hypothesize that the changes induced by the probiotic *B. subtilis* CW14 in gene expression in Caco-2 cells may be associated with transcriptional control. To address this, we performed an analysis of transcription factors (TFs). Out of 100 expressed TFs, *B. subtilis* CW14 affected the expression of 2 TFs, resulting in changes of more than 1.5-fold. The associated genes were negatively regulated (*TNFAIP3* and *ATXN7*). It has been shown that the deletion of *TNFAIP3* in intestinal epithelial cells increases susceptibility to experimental colitis, in addition to playing a significant role in preventing autoimmune inflammation by regulating the activation and antigen-presenting functions of dendritic cells (**Kelly & Mulder 2012; Murphy *et al*. 2014**). On the other hand, *ATXN7* plays an important role in transcriptional regulation, and it has been observed that mRNA expression of *ATXN7* was significantly reduced in renal cell carcinoma (RCC), associating it with a lower survival rate (**Han *et al.*, 2016**).

Furthermore, we analyzed the enrichment of transcriptional motifs in the promoter sequences of differentially expressed genes. DNA binding motifs associated with *RBM5* and *bHLH122* were found in promoter regions of specific genes (**Fig. 4**). *RBM5* had an association with the *STON2* gene, while *bHLH122* was linked to the *TNFAIP3* gene. Both genes exhibited negative regulation. The negative regulation of these genes suggests that these TFs play a significant role in modulating biological processes associated with these genes. In this regard, it has been suggested that *STON2* is related to the progression and prospects of ovarian carcinoma, and it has been demonstrated that the overexpression of this gene is involved with intestinal metastasis (**Sun *et al.*, 2017**), making it a potential therapeutic target for colon adenocarcinoma.

Down-Regulated Promoters			
Logo Motif	Fg. %	Bg. %	Match
	3.58	1.63	RBM5
	1.92	0.83	bHLH

Figure 4. Transcription factors mediating gene expression in *B. subtilis* CW14-treated Caco-2 cells. The novo motif analysis was performed at promoters (-300 and +50 relative to the transcription start site, TSS) of up- and down-regulated genes. Motifs were compared using the HOMER transcription factor database to determine the closest matches. Percentage (%) represents a fraction of foreground (Fg) and background (Bg) sequences containing at least one motif occurrence.

3.4 Relationship between common genes and their expression in response to each of the probiotics used.

The network displayed various interactions among genes, highlighting certain gene groups involved in biological processes (**Fig. 5, Fig. S3**). Among the most notable interactions, we observed a relationship between the genes *ATG10* and *MAP1LC3B*, associated with autophagy processes (**Fig. 5c**). The genes *EPC2*, *INO80D*, and *PRMT6*, associated with DNA damage and repair processes (**Fig. 5a**), are also interconnected in the network, as are the genes *CDKN1A* and *CDKN2B*, which relate to cell cycle regulation (**Fig. 5c**). The analyses also indicate certain patterns; when filling the network circles based on FC, it was identified that for the treatment with *P. freudenreichii* SN, the network assumes pinkish hues, indicating positive gene expression (**Fig. S3b**), while for the treatment with *B. subtilis* CW14, the network predominantly turns green, indicating negative gene regulation (**Fig. S3a**). For the treatment with *L. acidophilus* W37, the circles are mostly white, light pink, and light green, indicating that the gene expression with this treatment is not overrepresented (**Fig. 3c**).

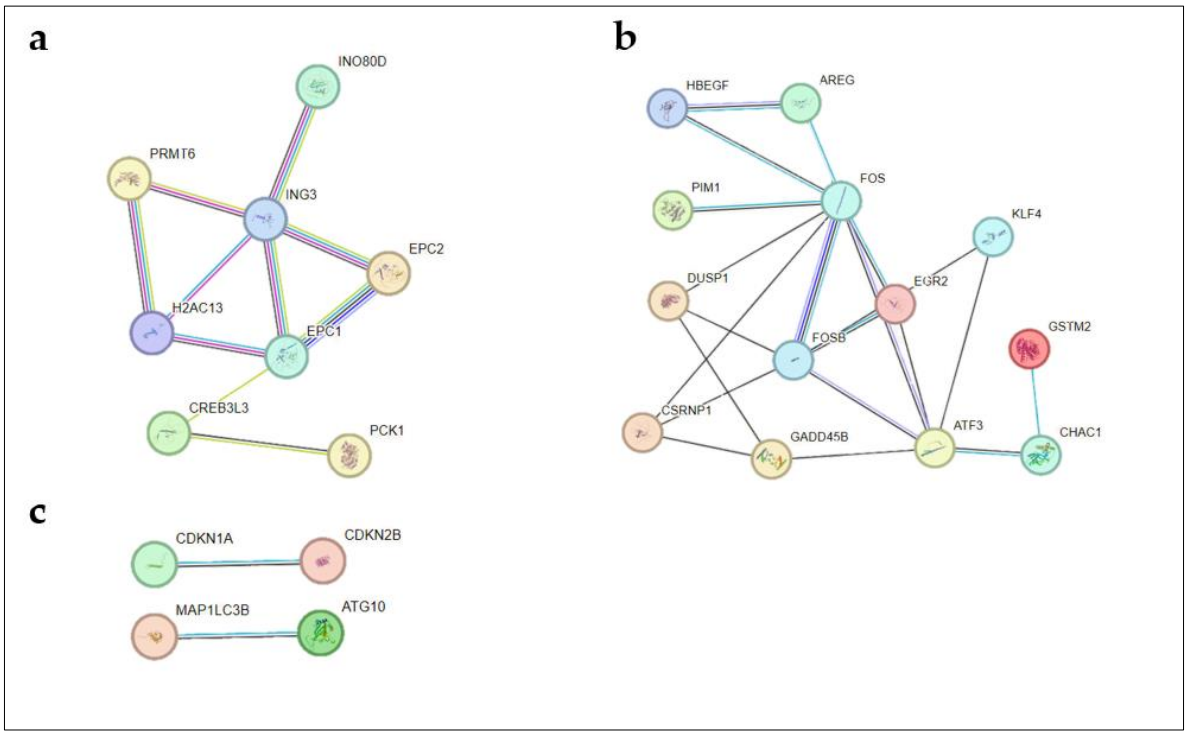

Figure 5. Interaction network designed of the 69 common genes identified in the previous results using STRING v 12.0. The main relationships are shown, where the curated database (light blue line), co-occurrence (dark blue line), co-expression (black line), closeness (green line) and fusion (red line) between genes are used as interaction criteria. Two main interaction clusters (**a,b**) and two pairs of genes with interactions relevant to this study **(c)** were identified for their role in regulating cancer metabolism

4. Discussion:

The impact of a probiotic is linked to the specific strain; hence it is crucial to identify and characterize exhaustively (**Butel, 2014**). The findings of this study highlight the heterogeneity in modulation induced by different probiotics, emphasizing the importance of both phenotypic and genotypic identification of these microorganisms. Thus, in this work, we evaluated the effects of various bacterial strains (*P. freudenreichii* SN, *B. subtilis* CW14, *L. acidophilus* W37) on Caco-2 and HT29 cell lines. The most meaningful results were associated with the probiotics *P. freudenreichii* SN and *B. subtilis* CW14, demonstrating their capacity to modulate the expression (positive or negative) of various genes related to colon cancer pathways.

Particularly, *P. freudenreichii* SN is a bacteria that has recently shown promising probiotic potential, since it is capable of surviving the digestive stress caused by oral ingestion. Analysis of this probiotic revealed high FC values, suggesting a strong effect on genes associated with colon cancer. **Jan *et al*. (2002)** have demonstrated in vitro assays that this probiotic induces genes involved in apoptosis processes via the intrinsic death pathway and dimethylhydrazine (DMH)-induced carcinogenesis (**Lan *et al.,* 2008**). Also, **Cousin *et al*. (2016)** specifically demonstrated that metabolites produced by this bacteria such as propionate and acetate can induce intrinsic apoptosis in CRC cells. Moreover, recent studies indicate that butyrate-producing bacteria such as *P. freudenreichii*, which belong to the next-generation probiotics (NGP), may have beneficial applicability in cancer therapy (**Kvakova *et al*., 2022).** Among the genes that were overexpressed by *P. freudenreichii* SN, the ones related to cell differentiation and the immune system were identified (**Table S3**), which may indicate that it can have a protective effect on the intestinal epithelium and be beneficial in preventing the appearance of cancer cells, as it has been observed that *P. freudenreichii* SN can reduce inflammation in human intestinal epithelial cells (HIEC) (**Illikoud *et al*., 2023**). Between these genes we find *CDKN1A*, known as the cyclin-dependent kinase inhibitor p21, a negative regulator of both cell cycle progression and gene expression; where uncontrolled crossing of cells through the G1/S checkpoint by negative regulation or loss of p21 could induce aberrant proliferation (**Bueno-Fortes *et al*. 2021**), therefore the positive regulation of genes by this probiotic suggests inhibitory effects on abnormal cell proliferation or tumor progression. We also recorded a positive regulation of *Creb3l3/CREB-H*, a transcription factor that is essential for regulating glucose, lipid, and lipoprotein metabolism (**Wade *et al*., 2023**), in addition to other complex biological processes such as endoplasmic reticulum stress, mitochondrial oxidative stress, atherosclerosis, cell growth, inflammation, and autophagy (**Yuxiong *et al*., 2023).** For example, in hepatocellular carcinoma (HCC), *Creb3l3* shows high expression in normal hepatic cells and low in HCC cells, although the precise mechanism of *Creb3l3* in HCC is unclear, and its effects are not fully understood. Therefore, this gene may be an interesting target in the study of colon cancer.

On the other hand, *B. subtilis* has been used in recent years in trials against the prevention and growth of colon adenocarcinoma (**Peng *et al.,* 2019**). This strain has been investigated

for its antimicrobial, antiviral, and anticancer effects (**Lee *et al.,* 2019**). It is proven that *B. subtilis* can inhibit the adhesion of *Salmonella enteriditis*, *Listeria monocytogenes*, and *E.coli*, for example, in addition to exhibiting antiviral activity against the influenza virus, herpes virus, and encephalomyelitis virus (**Lee *et al.,* 2019**). The results of this study showed elevated levels of overexpression in genes involved in cancer pathways. Some of the genes that were overexpressed or under expressed by this probiotic are related to the protection of the intestinal barrier, as observed in previous research, by improving the expression of zonula occludens-1 (ZO-1) (**Gu *et al.,* 2014**), and intestinal homeostasis, through the membrane of a host carrier cell (*OCTN2*) (**Fujiya *et al.,* 2007**). We also observed a positive regulation of the *DBNL* gene (drebin), which has been reported to play an important role in duodenal inflammation through the immunological synapse by acting as a common effector of antigen receptor signaling pathways in leukocytes and also as a key component of the immunological synapse for the regulation of T-cell activation (**Kaarbø *et al.,* 2023**). We also noted the positive expression of the *GSTM2* gene, which belongs to a large superfamily of genes that encode glutathione S-transferase (**Sharma *et al.,* 2004**), known to effectively detoxify O-quinones (e.g., aminochrome), oxidation products of catecholamines that may be associated in the development of Parkinson's disease (**Pool-Zobel *et al.,* 2005**). This finding is significant, as *GSTM2* is part of a dynamic and interactive defense mechanism that protects against cytotoxic electrophilic chemicals and facilitates adaptation to oxidative stress (**Martinez *et al.,* 2023**).

In L. acidophilus, the expression levels were not as high, but we observed expression in some genes that support the function of the intestinal immune barrier (**Esvaran & Conway, 2012**), and the host-virus interaction, which could indicate an improvement in pathogen resistance, as previously reported (**Weiss *et al.,* 2010**).

As mentioned in the results, the analyses indicated the presence of six immune response genes, associated with the development and progression of colon cancer. Similarly, it has been shown that this depends on the complex tumor microenvironment (TME) in which it develops (**Angell *et al.,* 2020**). **Galon *et al.* (2006) and Galon *et al.* (2007)**, have reported that the infiltration of adaptive immune cells generates prognoses superior to classic criteria

of tumor invasion, including the degree, stage, and metastatic status. Therefore, tumor progression and patient survival reflect the complex cellular and molecular interactions of the tumor with the host's immune system (**Galon *et al.*, 2013**), making the use of these genes as molecular markers or modulating them to enhance the immune response potentially very helpful for the management and evolution of colon cancer.

Additional processes identified included autophagy, apoptosis, and inflammatory response. In this context, it has been observed that autophagy frequently occurs during tumorigenesis and chemotherapy and that it can protect cancer cells from apoptosis during chemotherapy, leading to drug resistance and refractory cancers **(Li *et al.*, 2017**). However, other studies have shown that it can cause cell death, inhibit cell growth, or have no effect at all; this depends on the type of tissue, the stage of tumor development, and the degree of autophagy activity (**Kaluzki *et al.*, 2019**). Alternatively, the apoptosis that leads to cell death, is unidirectional (**Hoeppner *et al.*, 2001; D'Amelio *et al.*, 2010**). Studies indicate that cancer overexpresses anti-apoptotic proteins to resist apoptosis (**Korsmeyer *et al.*, 1993**), which helps cancer cells survive, proliferate, and resist drugs (**Fulda, 2009**). Regarding the inflammatory and stress responses, these are processes that can help in the development of colon cancer, as it has been observed that treatment with anti-inflammatory drugs can prevent or delay the development of this cancer in both hereditary and sporadic cases (**Friis *et al.*, 2015**), and it is known that inflammation is a major driver of tumorigenesis in colon cancer (**Long *et al.*, 2017**). Therefore, autophagy, apoptosis, and inflammation can be effective, yet counterproductive in tumors, and modulation of genes related, so these processes must be carefully analyzed so that gene therapy can function properly.

The analysis of common genes through GSCA and the comparison of the effects of different probiotics on the modulation of common genes revealed significant diversity in the induced genetic responses. For example, we observed a positive regulation of the *CHAC1* gene (related to the cell cycle pathway) by *P. freudenreichii* SN, while *B. subtilis* CW14 exerted negative regulation. So, it has been reported that positive regulation of *CHAC1* enhances apoptosis in endothelial cells in response to oxidized phospholipids (**Mungrue *et al.*, 2009**). Moreover, it has been suggested that this same gene could be an important target of the *ATF4*-

ATF3-CHOP signaling pathway, which also promotes apoptosis, as *CHAC1* could be directly regulated by these genes (**Mungrue *et al.*, 2009**).

We also observed the positive expression of the *TNFSF9* gene (involved in the apoptosis pathway) by *P. freudenreichii* SN, in contrast to its negative regulation by *B. subtilis* CW14. This gene is part of the tumor necrosis factor (TNF) family, and its positive expression has been associated with several types of human tumors, including colorectal cancer (CRC) (**Wu *et al.*, 2021**). Furthermore, it has been linked to the inhibition of cancer cell proliferation and the induction of apoptosis (**Wu *et al.*, 2021**). Additionally, *TNFSF9* is expressed not only in immune cells but also in non-immune cells like endothelial cells, fibroblasts, and epithelial cells, suggesting that further study of this gene could yield promising results regarding protection against colon cancer (**Kwon, 2009**).

The results also showed positive regulation of some genes related to the epithelial-mesenchymal transition (EMT) pathway, such as *DUSP5*, *GADD45B*, *GEM*, and *SERPINE1*. These findings suggest that the positive expression of DUSP5 by *P. freudenreichii* SN may be associated with tumor suppression, as indicated by **Yan *et al.* (2016)**, who suggest that high expression of *DUSP5* is related to tumor suppression through the mitogen-activated protein kinase (MAPK) signaling pathway, besides being associated with a better prognosis for the progression of CRC patients and their response to chemotherapy (**Yan *et al.*, 2016**). Even though *B. subtilis* CW14 also positively regulated this gene, its effect was not as significant as that observed with *P. freudenreichii* SN.

In addition, the *GADD45B* gene, a member of the DNA damage-inducible gene family, stops cell growth and plays critical roles in DNA repair, cell growth, and p53-mediated apoptosis (**Wang *et al.*, 2012**). Here, *P. freudenreichii* SN showed positive regulation. Overexpression of *GADD45B* is associated with a worse prognosis for CRC patients (**Martinez-Romero *et al.*, 2018**). In contrast, *B. subtilis* CW14 showed negative regulation, suggesting a beneficial effect of this probiotic in modulating *GADD45B.* However, it is important to highlight that there is still controversy regarding the expression and function of *GADD45B* in various tumors (**Wang *et al.*, 2012**).

Besides, we observed positive regulation of the SERPINE1 gene by *P. freudenreichii* SN, but negative and insignificant regulation by *B. subtilis* CW14. This gene influences the onset and progression of colon cancer and the poor prognosis for this pathology (**Wang *et al.*, 2023).** It has also been reported to promote peripheral angiogenesis, regulate endothelial homeostasis, and interact with inflammatory factors, which could suggest a relationship with the tumor microenvironment (**Chen *et al.*, 2022**).

We also report positive modulation of the *GEM* gene by *P. freudenreichii* SN and negative by *B. subtilis* CW14. This gene was originally identified by its overexpression in mitogen-stimulated T-lymphocytes (**Maguire *et al.*, 1994**). Its role is linked to the rearrangement of the cytoskeleton, but more importantly, to cell adhesion, cellular morphology, and cytokinesis, and especially, to migration (**Akakura *et al.*, 2012**). It's under expression has been reported in some epithelial and hematopoietic cancer cell lines, however, the expression of this gene and its prognostic value have not been determined in the different types of cancer (**Huang *et al.*, 2014**).

Concerning transcriptional control, while the data indicate genes related to immunity such as *TNFPAIP3,* and genes associated with the prognosis and evolution of cancer such as *STON2* and *ATXN7*, our *in-silico* approach is limited by the lack of experiments to quantify the specific DNA-binding activity of the transcription sequence. Nonetheless, the results from the motif analysis for negatively regulated genes identified the enrichment of 2 transcription factors (*RBM5* and *bHLH122*). Evidence suggests that *RBM5* is associated with the ability to modulate apoptosis, positively regulating the proapoptotic protein *BAX* and negatively regulating the antiapoptotic proteins *BCL*-2 and *BCL-XL* (**Sutherland *et al.*, 2010**). As for *bHLH122,* it is part of a large superfamily of transcriptional regulators involved in various processes including sex determination, cell cycle, cell lineage, and tumorigenesis (**Jones, 2004**). Although the role of *bHLH122* in humans is unknown, in plants, it is an activator of immunity against pathogens through the MAPK cascade (**Wang *et a*l., 2020**).

In the interaction network, two main clusters were identified where interesting interactions were established, such as the case with the genes *EPC1*, *EPC2*, and *INO80D* (**Fig. 5a**) which

show a co-expression relationship. It has been observed that these genes encode a protein that is a member of the Polycomb group (PcG) family, being a component of the *NuA4* histone acetyltransferase complex and can function as both transcriptional activator and repressor (**Attwooll *et al.*, 2005).** This protein has been associated with apoptosis and DNA repair (**Zhang *et al.*, 2009**). Within the same cluster, a co-expression relationship between the genes *ING3* and *INO80D* was also identified; these genes have been involved in the transcriptional activation of genes through the acetylation of nucleosomal histones and transcriptional programs associated with the induction of growth facilitated by oncogenes and proto-oncogenes, the arrest of growth facilitated by tumor suppressors, and replication (**Zhang *et al.*, 2017; Cheng *et al.*, 2024**).

Lastly, we highlighted the co-expression relationship between the genes *HBEGF*, *FOS*, and *FOSB* (**Fig. 5b),** as they play a key role in cell differentiation and proliferation, and in some cases have been associated with cell death by apoptosis (**Malsy *et al.* 2023; Yonemitsu *et al.* 2021**). In the case of the relationship between the genes *CDKN1A* and *CDKN2B*, and *MAP1LC3B* and *ATG10* (**Fig. 5c**), co-expression relationships were identified, and it was found that they are involved in the control of the cell cycle, especially the genes *MAP1LC3B* and *ATG10*, which are associated with ubiquitination and deubiquitinating processes in the regulation of cancer metabolism, which is of great importance for post-translational modifications in metabolic reprogramming (**Sun *et al.*, 2020**). These functions and relationships are of significant importance for the development of new therapeutic approaches in the treatment of colon cancer, therefore it is necessary to continue deepening these interactions to be able to develop more effective treatments.

5. Conclusions

Overall, the findings highlight an especially important role for the probiotics *P. freudenreichii* SN and *B. subtilis* CW14, which demonstrated significant impacts on key aspects such as immune response, antiviral defense, autophagy, inflammatory response, cellular stress, and metabolism. This suggests their potential benefits for intestinal health and probably in the prevention of colon cancer development. The results also highlight the

complexity of genetic responses to different probiotics through the activation and inhibition of various pathways, suggesting a multifaceted impact. Future research could deepen into the specific mechanisms of action of the probiotics analyzed here, thus offering new therapeutic approaches and molecular insights related to colon cancer.

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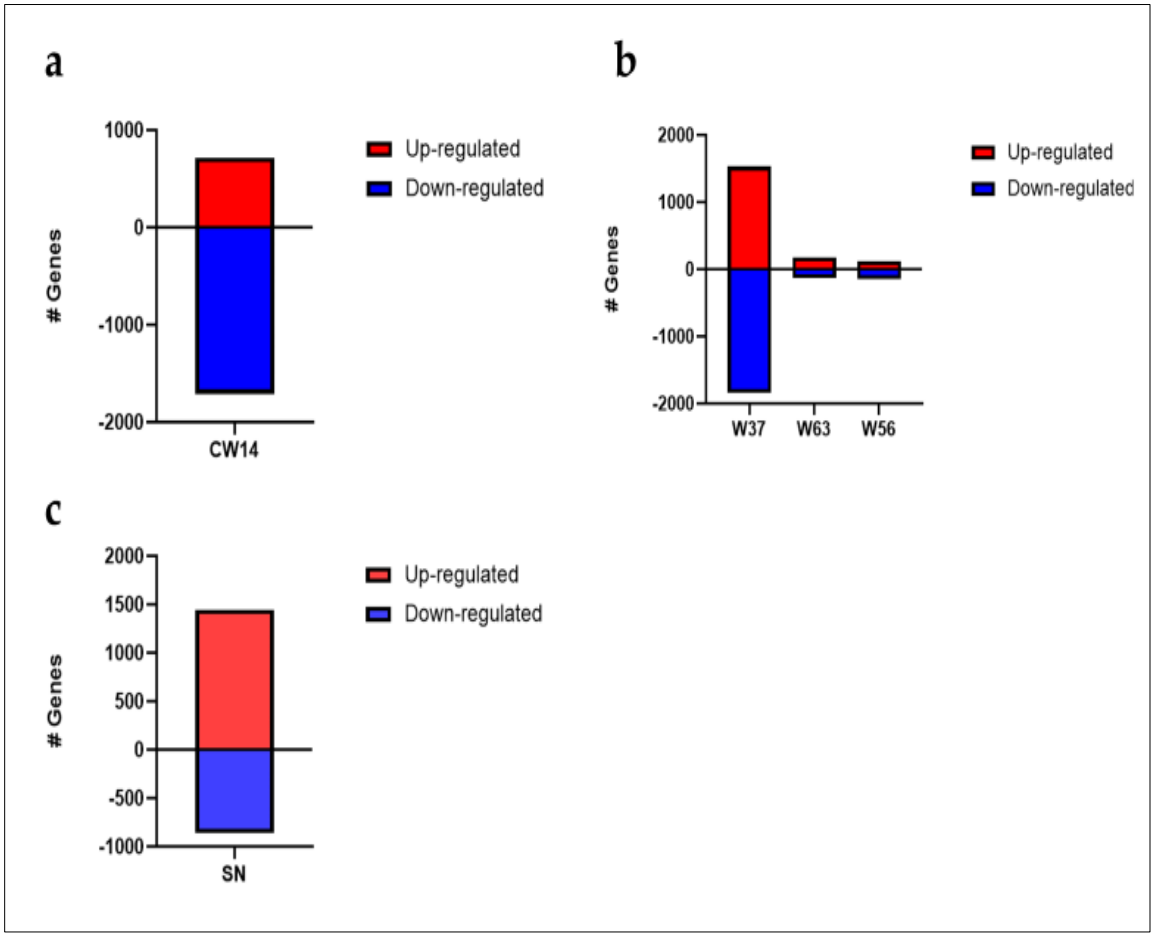
Additional information

Supplementary Figures



Supplementary Figure 1. Genes expressed positively and negatively by the different probiotics used. **A)** Genes expressed by *Bacillus. subtilis* CW14. **B)** Genes expressed by *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W36 and *Lactobacillus casei* W56. **C)** Genes expressed by *Propionibacterium freudenreichii* SN

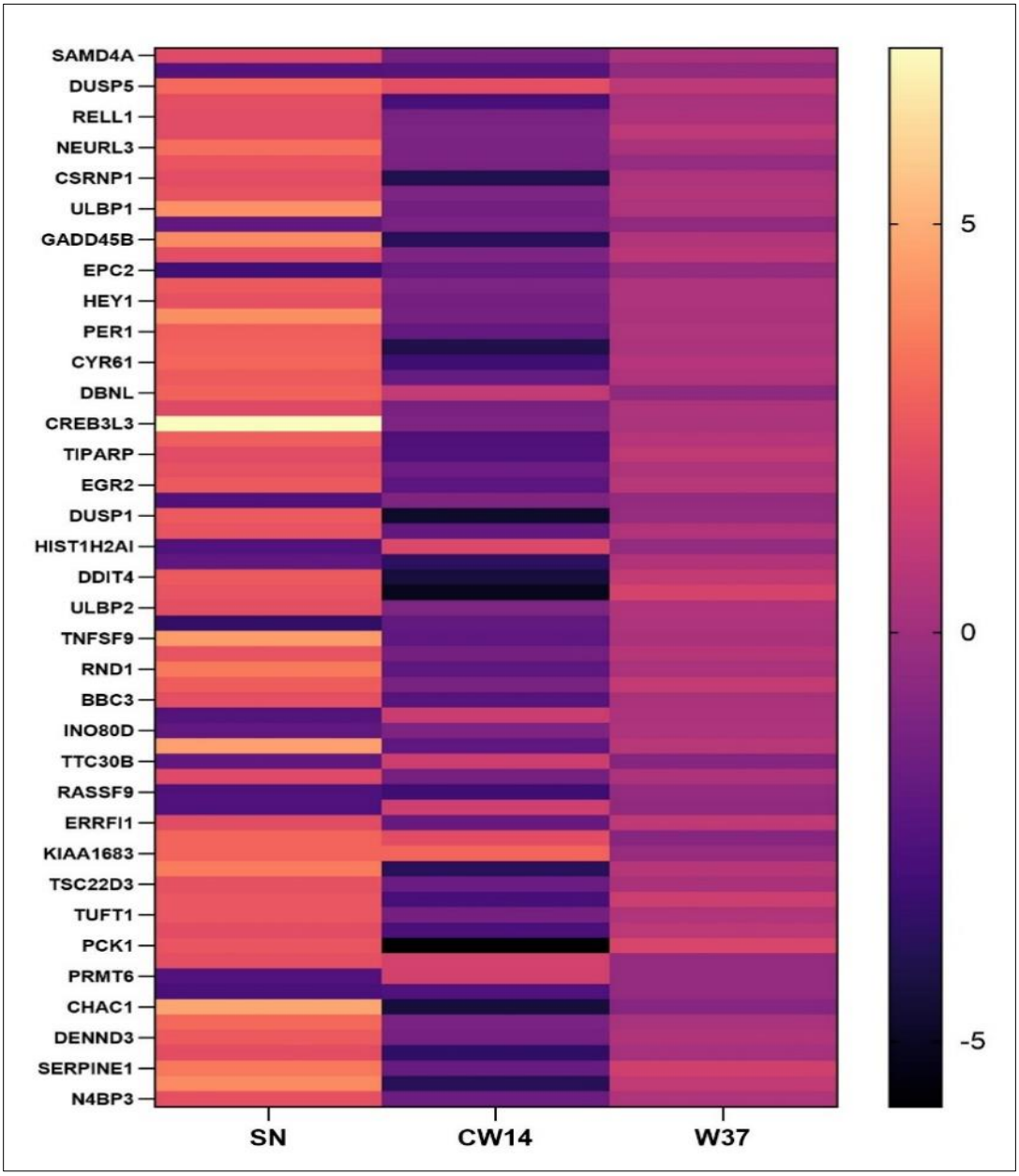
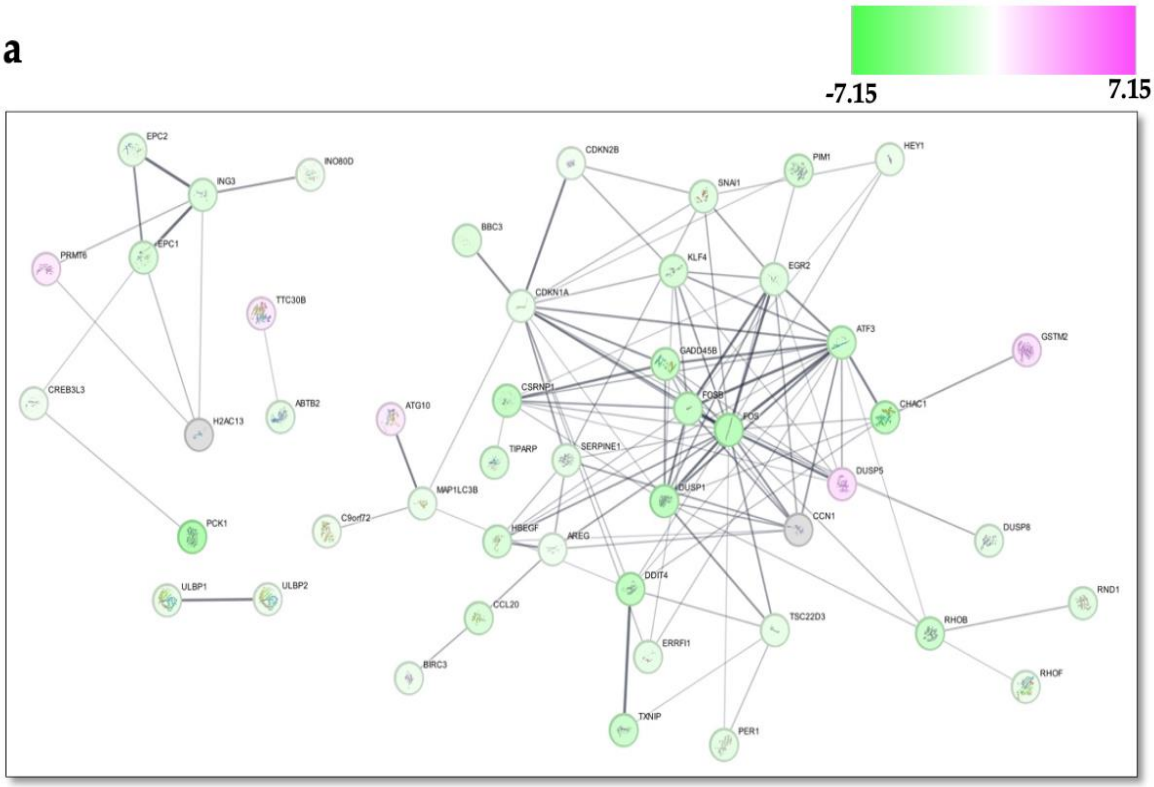


Figura suplementaria 2. Heatmap for common genes identified between treatments: *Propionibacterium freudenreichii* (SN), *Bacillus. subtilis* (CW14), *Lactobacillus acidophilus* (W37).

a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ULBP2
CDKN2B
SNAI1
PIM1
HEY1
BBC3
KLF4
EGR2
CDKN1A
ATF3
GSTM2
GADD45B
CSRNP1
FOSB
FOS
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TIPARP
SERPINE1
DUSP5
MAP1LC3B
DUSP1
C9orf72
CCN1
DUSP8
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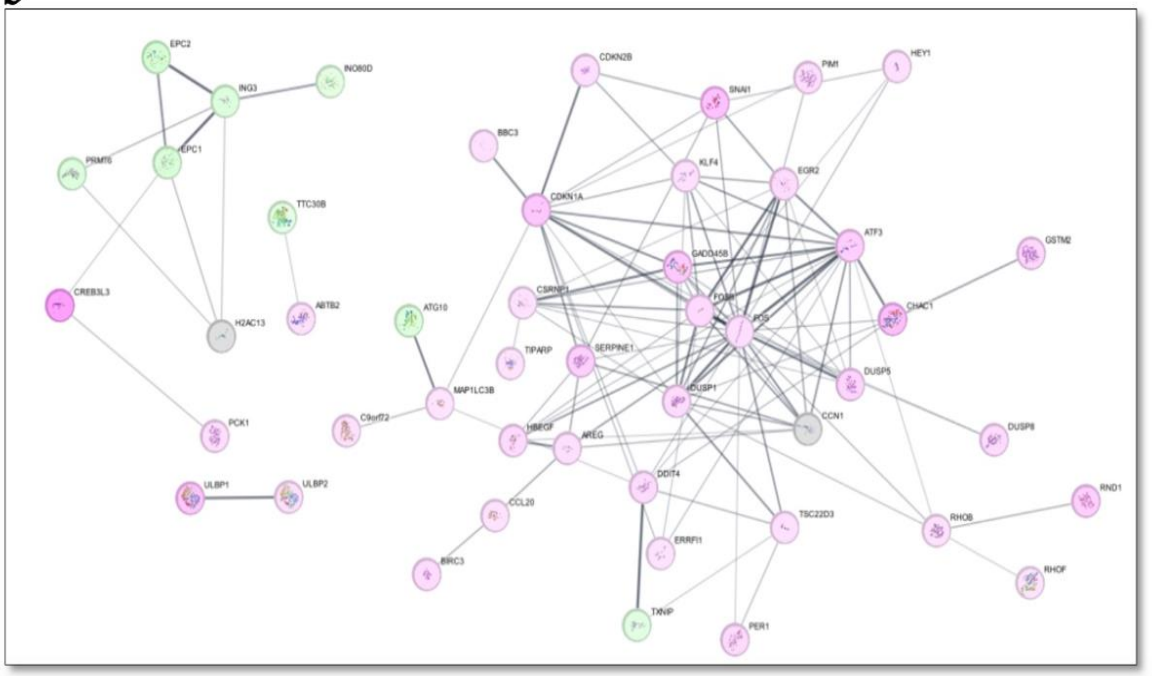
b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CDKN2B
SNAI1
PIM1
HEY1
BBC3
KLF4
EGR2
CDKN1A
ATF3
GSTM2
GADD45B
CSRNP1
FOSB
FOS
CHAC1
ATG10
TIPARP
SERPINE1
DUSP5
MAP1LC3B
DUSP1
C9orf72
CCN1
DUSP8
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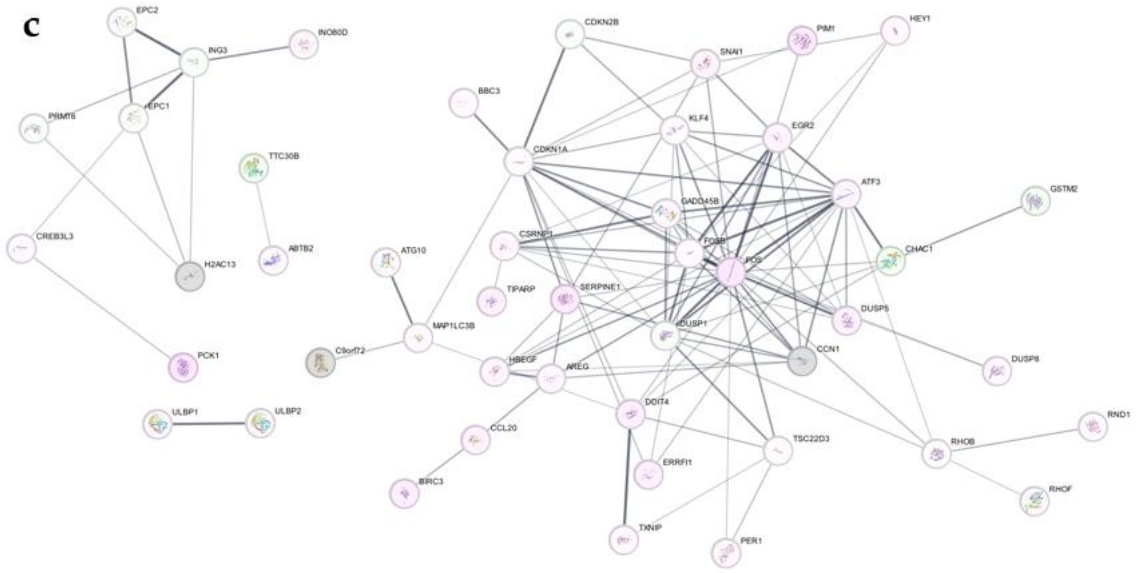

Supplementary Figure 3. General interaction network designed for the common genes. The colors of the circles indicate the FC value obtained for two of the treatments that obtained the most representative values. **A)** Interaction network obtained for the FC values of *B. subtilis* CW14. **B)** Interaction network obtained for the FC values of *Propionibacterium freudenreichii*. **C)** Interaction network obtained for the FC values of *Lactobacillus acidophilus W37*

Supplementary Tables

Gene	*P. freudenreichii* SN	*B. subtilis* CW14	*L. acidophilus W37*
SAMD4A	2.060218802	-1.260431252	0.286324193
ING3	-2.474594971	-2.326271411	-0.394236602
DUSP5	3.113308959	2.244688175	0.854372738
KLF4	2.222904981	-2.804629879	0.290705032
RELL1	2.116161159	-1.205909242	0.377321768
PLEKHM3	2.093706316	-1.085288545	0.858883126
NEURL3	3.231713979	-1.204574492	0.322916606
CDKN2B	2.442793778	-1.137334783	-0.292432603
CSRNP1	2.144744996	-4.001301109	0.399630454
AREG	2.347701182	-1.092913387	0.556996301
ULBP1	4.224916852	-1.467987523	0.458110466
C9ORF156	-2.039032465	-1.205871536	-0.465707448
GADD45B	4.033562113	-3.820862308	0.519060708
C9ORF72	2.223505399	-1.100004974	0.783621858
EPC2	-3.047191125	-1.804674602	-0.288225698
CPEB4	2.667168725	-1.085460896	0.397006936
HEY1	2.301619953	-1.446907274	0.39985353
CDKN1A	4.145435818	-1.344943088	0.316979512
PER1	2.751279578	-1.894040306	0.500666372
FOSB	2.846832732	-4.095951395	0.346130448
CYR61	3.001909269	-3.13623667	0.648583174
DUSP8	2.64960148	-1.872106926	0.53666191
DBNL	2.860324381	1.096649535	-0.477856217
RHOF	2.000712992	-1.249829298	0.431601432
CREB3L3	7.154331421	-1.090938051	0.349592234
HBEGF	2.764099767	-2.63784825	0.640226003
TIPARP	2.078272891	-2.566224816	0.997868476
ABTB2	2.309892747	-1.658054439	0.46367195
EGR2	2.647349721	-2.195299208	0.766066769
MEPCE	-2.482861458	-1.026678503	-0.338706785
DUSP1	2.638649242	-4.790882954	-0.280932976
ZSWIM6	2.40776305	-1.978324752	0.545869885
HIST1H2AI	-2.485070121	1.925087606	-0.395482824
TXNIP	-2.051271484	-3.618691936	0.536240057
DDIT4	2.63279181	-4.397904657	1.052758659
FOS	2.419779429	-5.130458519	1.650062643
ULBP2	2.209782335	-1.062194767	0.533616404
RTP4	-3.476417582	-2.009486348	0.400657998
TNFSF9	4.50259234	-2.075551523	0.341600625
TRIM15	2.382312553	-1.339581244	0.694597568
RND1	3.540203164	-2.049091371	0.361813609
BIRC3	2.684212055	-1.283924182	1.108931976
BBC3	2.250657209	-2.369575721	0.323245606

ATG10	-2.423769218	1.293660346	0.381474341
INO80D	-2.040593726	-1.087376815	0.395546362
SNAI1	4.593224568	-2.075996799	0.752354426
TTC30B	-2.028140226	1.451921574	-0.819182137
MAP1LC3B	2.062576815	-1.374699314	0.388434274
RASSF9	-2.60209124	-3.082365328	-0.319943314
MRM1	-2.598332679	1.473989165	-0.456662107
ERRFI1	2.110601407	-1.854467017	0.999331395
GSTM2	2.977679777	2.135280632	-0.800252128
KIAA1683	2.950676596	2.939413782	-0.287868548
ATF3	3.652939264	-3.731890868	0.719818784
TSC22D3	2.276822852	-1.713002909	0.303133377
PIM1	2.467655567	-2.835260123	1.343479032
TUFT1	2.477456232	-1.37876813	0.488500227
CCL20	2.12636019	-2.704863364	0.886127745
PCK1	2.430492606	-5.804802007	1.797024908
GAMT	2.265594949	1.56647045	-0.337347274
PRMT6	-2.460123309	1.531618592	-0.347878649
EPC1	-2.74234178	-2.600158869	-0.327081866
CHAC1	4.751713003	-4.403959352	-0.804060767
PLA2G2F	3.066483269	-1.197770857	0.262960372
DENND3	2.622734546	-1.33643839	0.536050663
RHOB	2.154911955	-3.487420099	0.235407508
SERPINE1	3.512026896	-1.770117774	1.463536901
GEM	3.994523375	-3.807691716	1.067264569
N4BP3	2.281034064	-1.601316917	0.481406369

Supplementary Table 1. Genes comunes. Probióticos: *Propionibacterium freudenreichii* ITG P9 SN, *Bacillus subtilis* CW14, *Lactobacillus acidophilus* W37.

ID	Gene Name	BIOLOGICAL_PROCESS
ATF3	activating transcription factor 3(ATF3)	KW-0804~Transcription,KW-0805~Transcription regulation,
ATG10	autophagy related 10(ATG10)	KW-0072~Autophagy,KW-0653~Protein transport,KW-0813~Transport,KW-0833~Ubl conjugation pathway,
BBC3	BCL2 binding component 3(BBC3)	KW-0053~Apoptosis,
BIRC3	baculoviral IAP repeat containing 3(BIRC3)	KW-0053~Apoptosis,KW-0833~Ubl conjugation pathway,
C9ORF72	C9orf72-SMCR8 complex subunit(C9orf72)	KW-0072~Autophagy,
CCL20	C-C motif chemokine ligand 20(CCL20)	KW-0145~Chemotaxis,KW-0395~Inflammatory response,
CDKN1A	cyclin dependent kinase inhibitor 1A(CDKN1A)	KW-0131~Cell cycle,
CDKN2B	cyclin dependent kinase inhibitor 2B(CDKN2B)	KW-0131~Cell cycle,
CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1(CHAC1)	KW-0053~Apoptosis,KW-0524~Neurogenesis,KW-0834~Unfolded protein response,KW-0914~Notch signaling pathway,
CREB3L3	cAMP responsive element binding protein 3 like 3(CREB3L3)	KW-0804~Transcription,KW-0805~Transcription regulation,KW-0834~Unfolded protein response,
CSRNP1	cysteine and serine rich nuclear protein 1(CSRNP1)	KW-0053~Apoptosis,KW-0804~Transcription,KW-0805~Transcription regulation,
DBNL	drebrin like(DBNL)	KW-0254~Endocytosis,KW-0391~Immunity,KW-0813~Transport,KW-1064~Adaptive immunity,
DDIT4	DNA damage inducible transcript 4(DDIT4)	KW-0051~Antiviral defense,KW-0053~Apoptosis,

DUSP1	dual specificity phosphatase 1(DUSP1)	KW-0131~Cell cycle,KW-0346~Stress response,
EGR2	early growth response 2(EGR2)	KW-0804~Transcription,KW-0805~Transcription regulation,KW-0833~Ubl conjugation pathway,
EPC1	enhancer of polycomb homolog 1(EPC1)	KW-0221~Differentiation,KW-0341~Growth regulation,KW-0744~Spermatogenesis,KW-0804~Transcription,KW-0805~Transcription regulation,
EPC2	enhancer of polycomb homolog 2(EPC2)	KW-0227~DNA damage,KW-0234~DNA repair,KW-0804~Transcription,KW-0805~Transcription regulation,
GADD45B	growth arrest and DNA damage inducible beta(GADD45B)	KW-0053~Apoptosis,KW-0221~Differentiation,
GSTM2	glutathione S-transferase mu 2(GSTM2)	KW-0443~Lipid metabolism,
HEY1	hes related family bHLH transcription factor with YRPW motif 1(HEY1)	KW-0804~Transcription,KW-0805~Transcription regulation,KW-0914~Notch signaling pathway,
ING3	inhibitor of growth family member 3(ING3)	KW-0341~Growth regulation,KW-0804~Transcription,KW-0805~Transcription regulation,
INO80D	INO80 complex subunit D(INO80D)	KW-0227~DNA damage,KW-0233~DNA recombination,KW-0234~DNA repair,KW-0804~Transcription,KW-0805~Transcription regulation,
KLF4	KLF transcription factor 4(KLF4)	KW-0804~Transcription,KW-0805~Transcription regulation,
MAP1LC3B	microtubule associated protein 1 light chain 3 beta(MAP1LC3B)	KW-0072~Autophagy,KW-0833~Ubl conjugation pathway,
MRM1	mitochondrial rRNA methyltransferase 1(MRM1)	KW-0698~rRNA processing,
N4BP3	NEDD4 binding protein 3(N4BP3)	KW-0391~Immunity,KW-0399~Innate immunity,KW-0524~Neurogenesis,

NEURL3	neuralized E3 ubiquitin protein ligase 3(NEURL3)	KW-0391~Immunity,KW-0399~Innate immunity,KW-0833~Ubl conjugation pathway,KW-0945~Host-virus interaction,
PCK1	phosphoenolpyruvate carboxykinase 1(PCK1)	KW-0312~Gluconeogenesis,
PER1	period circadian regulator 1(PER1)	KW-0090~Biological rhythms,KW-0804~Transcription,KW-0805~Transcription regulation,
PIM1	Pim-1 proto-oncogene, serine/threonine kinase(PIM1)	KW-0053~Apoptosis,KW-0131~Cell cycle,KW-0945~Host-virus interaction,
PLA2G2F	phospholipase A2 group IIF(PLA2G2F)	KW-0391~Immunity,KW-0399~Innate immunity,KW-0442~Lipid degradation,KW-0443~Lipid metabolism,
PRMT6	protein arginine methyltransferase 6(PRMT6)	KW-0227~DNA damage,KW-0234~DNA repair,KW-0804~Transcription,KW-0805~Transcription regulation,KW-0945~Host-virus interaction,
RHOB	ras homolog family member B(RHOB)	KW-0037~Angiogenesis,KW-0053~Apoptosis,KW-0130~Cell adhesion,KW-0221~Differentiation,KW-0653~Protein transport,KW-0813~Transport,
SAMD4A	sterile alpha motif domain containing 4A(SAMD4A)	KW-0810~Translation regulation,
TRIM15	tripartite motif containing 15(TRIM15)	KW-0833~Ubl conjugation pathway,
TUFT1	tuftelin 1(TUFT1)	KW-0091~Biomineralization,
TXNIP	thioredoxin interacting protein(TXNIP)	KW-0131~Cell cycle,KW-0804~Transcription,KW-0805~Transcription regulation,
ULBP1	UL16 binding protein 1(ULBP1)	KW-0391~Immunity,KW-0945~Host-virus interaction,
ULBP2	UL16 binding protein 2(ULBP2)	KW-0391~Immunity,KW-0945~Host-virus interaction,
ZSWIM6	zinc finger SWIM-type containing 6(ZSWIM6)	KW-0524~Neurogenesis,

Supplementary Table 2. Summary of the biological processes associated with common differentially expressed genes across David.

ID Biological process	process	genes	count
KW-0145	Chemotaxis	CCL20	1
KW-0395	Inflammatory response	CCL20	1
KW-0254	Endocytosis	DBNL	1
KW-0399	Innate immunity	PLA2G2F	1
KW-0051	Antiviral defense	DDIT4	1
KW-0346	Stress response	DUSP1	1
KW-0744	Spermatogenesis	EPC1	1
KW-0233	DNA recombination	INO80D	1
KW-0698	rRNA processing	MRM1	1
KW-0312	Gluconeogenesis	PCK1	1
KW-0090	Biological rhythms	PER1	1
KW-0037	Angiogenesis	RHOB	1
KW-0130	Cell adhesion	RHOB	1

KW-0091	Biomineralization	TUFT1	1
KW-0442	Lipid degradation	PLA2G2F	1
KW-0810	Translation regulation	SAMD4A	1
KW-0653	Protein transport	ATG10, RHOB	2
KW-0524	Unfolded protein response	CHAC1, CREB3L3	2
KW-0834	Notch signaling pathway	CHAC1, HEY1	2
KW-0221	Differentiation	EPC1, RHOB	2
KW-0341	Growth regulation	EPC1, ING3	2
KW-0443	Lipid metabolism	GSTM2, PLA2G2F	2
KW-0072	Autophagy	ATG10, C9ORF72, MAP1LC3B	3
KW-0813	Transport	ATG10, DBNL, RHOB	3
KW-0524	Neurogenesis	CHAC1, N4BP3, ZSWIM6	3
KW-1064	Adaptive immunity	DBNL, N4BP3, NEURL3	3
KW-0227	DNA damage	EPC2, INO80D, PRMT6	3
KW-0234	DNA repair	EPC2, INO80D, PRMT6	3
KW-0131	Cell cycle	CDKN1A, CDKN2B, DUSP1, TXNIP	4
KW-0945	Host-virus interaction	NEURL3, PIM1, PRMT6, ULBP1, ULBP2	5
KW-0833	Ubl conjugation pathway	ATG10, BIRC3, EGR2, MAP1LC3B, NEURL3, TRIM15	6
KW-0391	Immunity	DBNL, N4BP3, NEURL3, PLA2G2F, ULBP1, ULBP2	6
KW-0053	Apoptosis	BBC3, BIRC3, CHAC1, CSRNP1, DDIT4, GADD45B, PIM1, RHOB	8
KW-0804	trancription	ATF3, CREB3L3, CSRNP1, EGR2, EPC2, HEY1, ING3, INO80D, KLF4, PER1, PRMT6, TXNIP	12
KW-0805	transcription regulation	ATF3, CREB3L3, CSRNP1, EGR2, EPC2, HEY1, ING3, INO80D, KLF4, PER1, PRMT6, TXNIP	12

Supplementary Table 3. Summary of biological processes by category and associated gene by gene through David.

ID	Gene Name	KEGG_PATHWAY
AREG	amphiregulin(AREG)	hsa04010:MAPK signaling pathway,hsa04012:ErbB signaling pathway,hsa04151:PI3K-Akt signaling pathway,hsa04390:Hippo signaling pathway,hsa05210:Colorectal cancer,
ATG10	autophagy related 10(ATG10)	hsa04136:Autophagy - other,hsa04140:Autophagy - animal,
BBC3	BCL2 binding component 3(BBC3)	hsa01524:Platinum drug resistance,hsa04115:p53 signaling pathway,hsa04210:Apoptosis,hsa04215:Apoptosis - multiple species,hsa04390:Hippo signaling pathway,hsa05016:Huntington disease,hsa05162:Measles,hsa05200:Pathways in cancer,hsa05210:Colorectal cancer,
BIRC3	baculoviral IAP repeat containing 3(BIRC3)	hsa01524:Platinum drug resistance,hsa04064:NF-kappa B signaling pathway,hsa04120:Ubiquitin mediated proteolysis,hsa04210:Apoptosis,hsa04215:Apoptosis - multiple species,hsa04217:Necroptosis,hsa04390:Hippo signaling pathway,hsa04510:Focal adhesion,hsa04621:NOD-like receptor signaling pathway,hsa04668:TNF signaling pathway,hsa05132:Salmonella infection,hsa05145:Toxoplasmosis,hsa05168:Herpes simplex virus 1 infection,hsa05200:Pathways in cancer,hsa05202:Transcriptional misregulation in cancer,hsa05222:Small cell lung cancer,

C9ORF72	C9orf72-SMCR8 complex subunit(C9orf72)	hsa04140:Autophagy - animal,hsa05014:Amyotrophic lateral sclerosis,hsa05022:Pathways of neurodegeneration - multiple diseases,
CCL20	C-C motif chemokine ligand 20(CCL20)	hsa04060:Cytokine-cytokine receptor interaction,hsa04061:Viral protein interaction with cytokine and cytokine receptor,hsa04062:Chemokine signaling pathway,hsa04657:IL-17 signaling pathway,hsa04668:TNF signaling pathway,hsa05323:Rheumatoid arthritis,
CDKN1A	cyclin dependent kinase inhibitor 1A(CDKN1A)	hsa01522:Endocrine resistance,hsa01524:Platinum drug resistance,hsa04012:ErbB signaling pathway,hsa04066:HIF-1 signaling pathway,hsa04068:FoxO signaling pathway,hsa04110:Cell cycle,hsa04115:p53 signaling pathway,hsa04151:PI3K-Akt signaling pathway,hsa04218:Cellular senescence,hsa04630:JAK-STAT signaling pathway,hsa04921:Oxytocin signaling pathway,hsa04928:Parathyroid hormone synthesis, secretion and action,hsa04934:Cushing syndrome,hsa05160:Hepatitis C,hsa05161:Hepatitis B,hsa05163:Human cytomegalovirus infection,hsa05165:Human papillomavirus infection,hsa05166:Human T-cell leukemia virus 1 infection,hsa05167:Kaposi sarcoma-associated herpesvirus infection,hsa05169:Epstein-Barr virus infection,hsa05200:Pathways in cancer,hsa05202:Transcriptional misregulation in cancer,hsa05203:Viral carcinogenesis,hsa05205:Proteoglycans in cancer,hsa05206:MicroRNAs in cancer,hsa05210:Colorectal cancer,hsa05211:Renal cell carcinoma,hsa05212:Pancreatic cancer,hsa05213:Endometrial cancer,hsa05214:Glioma,hsa05215:Prostate cancer,hsa05216:Thyroid cancer,hsa05217:Basal cell carcinoma,hsa05218:Melanoma,hsa05219:Bladder cancer,hsa05220:Chronic myeloid leukemia,hsa05222:Small cell lung cancer,hsa05223:Non-small cell lung cancer,hsa05224:Breast cancer,hsa05225:Hepatocellular carcinoma,hsa05226:Gastric cancer,
CDKN2B	cyclin dependent kinase inhibitor 2B(CDKN2B)	hsa04068:FoxO signaling pathway,hsa04110:Cell cycle,hsa04218:Cellular senescence,hsa04350:TGF-beta signaling pathway,hsa04934:Cushing syndrome,hsa05166:Human T-cell leukemia virus 1 infection,hsa05200:Pathways in cancer,hsa05203:Viral carcinogenesis,hsa05222:Small cell lung cancer,hsa05226:Gastric cancer,

CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1(CHAC1)	hsa00480:Glutathione metabolism,hsa01100:Metabolic pathways,
CPEB4	cytoplasmic polyadenylation element binding protein 4(CPEB4)	hsa04114:Oocyte meiosis,hsa04914:Progesterone-mediated oocyte maturation,
CREB3L3	cAMP responsive element binding protein 3 like 3(CREB3L3)	hsa04022:cGMP-PKG signaling pathway,hsa04024:cAMP signaling pathway,hsa04151:PI3K-Akt signaling pathway,hsa04152:AMPK signaling pathway,hsa04211:Longevity regulating pathway,hsa04261:Adrenergic signaling in cardiomyocytes,hsa04668:TNF signaling pathway,hsa04714:Thermogenesis,hsa04725:Cholinergic synapse,hsa04728:Dopaminergic synapse,hsa04911:Insulin secretion,hsa04915:Estrogen signaling pathway,hsa04916:Melanogenesis,hsa04918:Thyroid hormone synthesis,hsa04922:Glucagon signaling pathway,hsa04925:Aldosterone synthesis and secretion,hsa04926:Relaxin signaling pathway,hsa04927:Cortisol synthesis and secretion,hsa04928:Parathyroid hormone synthesis, secretion and action,hsa04931:Insulin resistance,hsa04934:Cushing syndrome,hsa04935:Growth hormone synthesis, secretion and action,hsa04962:Vasopressin-regulated water reabsorption,hsa05016:Huntington disease,hsa05020:Prion disease,hsa05030:Cocaine addiction,hsa05031:Amphetamine addiction,hsa05034:Alcoholism,hsa05161:Hepatitis B,hsa05163:Human cytomegalovirus infection,hsa05165:Human papillomavirus infection,hsa05166:Human T-cell leukemia virus 1 infection,hsa05203:Viral carcinogenesis,hsa05207:Chemical carcinogenesis - receptor activation,hsa05215:Prostate cancer,
DDIT4	DNA damage inducible transcript 4(DDIT4)	hsa04140:Autophagy - animal,hsa04150:mTOR signaling pathway,hsa04151:PI3K-Akt signaling pathway,hsa05206:MicroRNAs in cancer,

DUSP1	dual specificity phosphatase 1(DUSP1)	hsa04010:MAPK signaling pathway,hsa04726:Serotonergic synapse,hsa05012:Parkinson disease,hsa05418:Fluid shear stress and atherosclerosis,
DUSP5	dual specificity phosphatase 5(DUSP5)	hsa04010:MAPK signaling pathway,
DUSP8	dual specificity phosphatase 8(DUSP8)	hsa04010:MAPK signaling pathway,
EGR2	early growth response 2(EGR2)	hsa04625:C-type lectin receptor signaling pathway,hsa05161:Hepatitis B,hsa05166:Human T-cell leukemia virus 1 infection,hsa05203:Viral carcinogenesis,
EPC1	enhancer of polycomb homolog 1(EPC1)	hsa03082:ATP-dependent chromatin remodeling,
EPC2	enhancer of polycomb homolog 2(EPC2)	hsa03082:ATP-dependent chromatin remodeling,
FOS	Fos proto-oncogene, AP-1 transcription factor subunit(FOS)	hsa01522:Endocrine resistance,hsa04010:MAPK signaling pathway,hsa04024:cAMP signaling pathway,hsa04210:Apoptosis,hsa04380:Osteoclast differentiation,hsa04620:Toll-like receptor signaling pathway,hsa04657:IL-17 signaling pathway,hsa04658:Th1 and Th2 cell differentiation,hsa04659:Th17 cell differentiation,hsa04660:T cell receptor signaling pathway,hsa04662:B cell receptor signaling pathway,hsa04668:TNF signaling pathway,hsa04713:Circadian entrainment,hsa04725:Cholinergic synapse,hsa04728:Dopaminergic synapse,hsa04915:Estrogen signaling pathway,hsa04917:Prolactin signaling pathway,hsa04921:Oxytocin signaling pathway,hsa04926:Relaxin signaling pathway,hsa04928:Parathyroid hormone synthesis, secretion and action,hsa04932:Non-alcoholic fatty liver disease,hsa04935:Growth hormone synthesis, secretion and action,hsa05031:Amphetamine addiction,hsa05130:Pathogenic Escherichia coli infection,hsa05132:Salmonella

		infection,hsa05133:Pertussis,hsa05135:Yersinia infection,hsa05140:Leishmaniasis,hsa05142:Chagas disease,hsa05161:Hepatitis B,hsa05162:Measles,hsa05166:Human T-cell leukemia virus 1 infection,hsa05167:Kaposi sarcoma-associated herpesvirus infection,hsa05170:Human immunodeficiency virus 1 infection,hsa05171:Coronavirus disease - COVID-19,hsa05200:Pathways in cancer,hsa05207:Chemical carcinogenesis - receptor activation,hsa05208:Chemical carcinogenesis - reactive oxygen species,hsa05210:Colorectal cancer,hsa05224:Breast cancer,hsa05231:Choline metabolism in cancer,hsa05235:PD-L1 expression and PD-1 checkpoint pathway in cancer,hsa05323:Rheumatoid arthritis,hsa05417:Lipid and atherosclerosis,hsa05418:Fluid shear stress and atherosclerosis,
FOSB	FosB proto-oncogene, AP-1 transcription factor subunit(FOSB)	hsa04380:Osteoclast differentiation,hsa04657:IL-17 signaling pathway,hsa05030:Cocaine addiction,hsa05031:Amphetamine addiction,hsa05034:Alcoholism,
GADD45B	growth arrest and DNA damage inducible beta(GADD45B)	hsa04010:MAPK signaling pathway,hsa04064:NF-kappa B signaling pathway,hsa04068:FoxO signaling pathway,hsa04110:Cell cycle,hsa04115:p53 signaling pathway,hsa04210:Apoptosis,hsa04218:Cellular senescence,hsa05169:Epstein-Barr virus infection,hsa05200:Pathways in cancer,hsa05202:Transcriptional misregulation in cancer,hsa05210:Colorectal cancer,hsa05212:Pancreatic cancer,hsa05213:Endometrial cancer,hsa05214:Glioma,hsa05216:Thyroid cancer,hsa05217:Basal cell carcinoma,hsa05218:Melanoma,hsa05220:Chronic myeloid leukemia,hsa05222:Small cell lung cancer,hsa05223:Non-small cell lung cancer,hsa05224:Breast cancer,hsa05225:Hepatocellular carcinoma,hsa05226:Gastric cancer,
GAMT	guanidinoacetate N-methyltransferase(GAMT)	hsa00260:Glycine, serine and threonine metabolism,hsa00330:Arginine and proline metabolism,hsa01100:Metabolic pathways,
GSTM2	glutathione S-transferase mu 2(GSTM2)	hsa00480:Glutathione metabolism,hsa00980:Metabolism of xenobiotics by cytochrome P450,hsa00982:Drug metabolism - cytochrome P450,hsa00983:Drug metabolism - other enzymes,hsa01100:Metabolic pathways,hsa01524:Platinum drug resistance,hsa05200:Pathways in cancer,hsa05204:Chemical carcinogenesis - DNA adducts,hsa05207:Chemical

		carcinogenesis - receptor activation,hsa05208:Chemical carcinogenesis - reactive oxygen species,hsa05225:Hepatocellular carcinoma,hsa05418:Fluid shear stress and atherosclerosis,
HBEGF	heparin binding EGF like growth factor(HBEGF)	hsa01522:Endocrine resistance,hsa04012:ErbB signaling pathway,hsa04912:GnRH signaling pathway,hsa04915:Estrogen signaling pathway,hsa04928:Parathyroid hormone synthesis, secretion and action,hsa05120:Epithelial cell signaling in Helicobacter pylori infection,hsa05171:Coronavirus disease - COVID-19,hsa05205:Proteoglycans in cancer,hsa05219:Bladder cancer,
HEY1	hes related family bHLH transcription factor with YRPW motif 1(HEY1)	hsa04330:Notch signaling pathway,hsa05165:Human papillomavirus infection,hsa05200:Pathways in cancer,hsa05224:Breast cancer,
ING3	inhibitor of growth family member 3(ING3)	hsa03082:ATP-dependent chromatin remodeling,
INO80D	INO80 complex subunit D(INO80D)	hsa03082:ATP-dependent chromatin remodeling,
KLF4	KLF transcription factor 4(KLF4)	hsa04550:Signaling pathways regulating pluripotency of stem cells,hsa05207:Chemical carcinogenesis - receptor activation,
MAP1LC3B	microtubule associated protein 1 light chain 3 beta(MAP1LC3B)	hsa04137:Mitophagy - animal,hsa04140:Autophagy - animal,hsa04216:Ferroptosis,hsa04371:Apelin signaling pathway,hsa04621:NOD-like receptor signaling pathway,hsa05014:Amyotrophic lateral sclerosis,hsa05022:Pathways of neurodegeneration - multiple diseases,hsa05131:Shigellosis,hsa05167:Kaposi sarcoma-associated herpesvirus infection,
PCK1	phosphoenolpyruvate	hsa00010:Glycolysis / Gluconeogenesis,hsa00020:Citrate cycle (TCA cycle),hsa00620:Pyruvate metabolism,hsa01100:Metabolic pathways,hsa03320:PPAR signaling pathway,hsa04068:FoxO signaling pathway,hsa04151:PI3K-Akt signaling

	carboxykinase 1(PCK1)	pathway,hsa04152:AMPK signaling pathway,hsa04910:Insulin signaling pathway,hsa04920:Adipocytokine signaling pathway,hsa04922:Glucagon signaling pathway,hsa04931:Insulin resistance,hsa04964:Proximal tubule bicarbonate reclamation,
PER1	period circadian regulator 1(PER1)	hsa04710:Circadian rhythm,hsa04713:Circadian entrainment,
PIM1	Pim-1 proto-oncogene, serine/threonine kinase(PIM1)	hsa04630:JAK-STAT signaling pathway,hsa04933:AGE-RAGE signaling pathway in diabetic complications,hsa05200:Pathways in cancer,hsa05206:MicroRNAs in cancer,hsa05221:Acute myeloid leukemia,
PLA2G2F	phospholipase A2 group IIF(PLA2G2F)	hsa00564:Glycerophospholipid metabolism,hsa00565:Ether lipid metabolism,hsa00590:Arachidonic acid metabolism,hsa00591:Linoleic acid metabolism,hsa00592:alpha-Linolenic acid metabolism,hsa01100:Metabolic pathways,hsa04014:Ras signaling pathway,hsa04270:Vascular smooth muscle contraction,hsa04972:Pancreatic secretion,hsa04975:Fat digestion and absorption,
RELL1	RELT like 1(RELL1)	hsa04060:Cytokine-cytokine receptor interaction,
RHOB	ras homolog family member B(RHOB)	hsa05132:Salmonella infection,
RND1	Rho family GTPase 1(RND1)	hsa04360:Axon guidance,
SERPINE1	serpin family E member 1(SERPINE1)	hsa04066:HIF-1 signaling pathway,hsa04115:p53 signaling pathway,hsa04218:Cellular senescence,hsa04371:Apelin signaling pathway,hsa04390:Hippo signaling pathway,hsa04610:Complement and coagulation cascades,hsa04933:AGE-RAGE signaling pathway in diabetic complications,hsa05142:Chagas disease,
SNAI1	snail family transcriptional repressor 1(SNAI1)	hsa04520:Adherens junction,

TNFSF9	TNF superfamily member 9(TNFSF9)	hsa04060:Cytokine-cytokine receptor interaction,
TXNIP	thioredoxin interacting protein(TXNIP)	hsa04621:NOD-like receptor signaling pathway,
ULBP1	UL16 binding protein 1(ULBP1)	hsa04650:Natural killer cell mediated cytotoxicity,
ULBP2	UL16 binding protein 2(ULBP2)	hsa04650:Natural killer cell mediated cytotoxicity,

Supplementary Table 4. Summary of the pathways associated with each gene through David.

Cancer type	symbol	pathway	fdr	class
COAD	BIRC3	Apoptosis	0,049768876	Activation
COAD	BIRC3	RTK	0,016490782	Activation
COAD	CDKN2B	EMT	0,003340515	Activation
COAD	CHAC1	CellCycle	0,01888459	Activation
COAD	CSRNP1	RTK	0,005519796	Activation
COAD	CSRNP1	TSCmTOR	0,012661895	Activation
COAD	DENND3	EMT	0,01514656	Activation
COAD	DENND3	Hormone ER	0,024575444	Activation
COAD	DENND3	PI3KAKT	0,012635615	Activation
COAD	DENND3	RASMAPK	0,034966052	Activation
COAD	DENND3	RTK	0,003751729	Activation
COAD	DUSP1	RTK	0,001548405	Activation
COAD	DUSP5	EMT	0,009071089	Activation
COAD	EGR2	EMT	3,6531E-06	Activation
COAD	EGR2	Hormone ER	0,021831074	Activation
COAD	EGR2	PI3KAKT	0,016733748	Activation
COAD	EGR2	RTK	0,016733748	Activation
COAD	EGR2	TSCmTOR	0,026478178	Activation
COAD	EPC1	RTK	0,027228535	Activation
COAD	FOS	RASMAPK	0,000214335	Activation
COAD	FOS	RTK	0,001121979	Activation
COAD	FOS	TSCmTOR	0,000027943	Activation
COAD	FOSB	RASMAPK	0,041883188	Activation
COAD	FOSB	RTK	0,024477014	Activation
COAD	FOSB	TSCmTOR	0,002630827	Activation
COAD	GADD45B	EMT	3,60158E-06	Activation
COAD	GAMT	EMT	0,00750831	Activation
COAD	GEM	EMT	0,000282005	Activation
COAD	GSTM2	EMT	0,018121749	Activation
COAD	HBEGF	RASMAPK	0,03103446	Activation
COAD	HBEGF	RTK	0,03103446	Activation
COAD	HEY1	EMT	0,004818676	Activation
COAD	MRM1	DNADamage	0,004678163	Activation
COAD	N4BP3	Apoptosis	0,0234712	Activation
COAD	N4BP3	EMT	0,028593803	Activation
COAD	PLEKHM3	EMT	0,034264783	Activation
COAD	PLEKHM3	Hormone ER	0,034264783	Activation
COAD	RELL1	EMT	0,000759434	Activation

COAD	RELL1	TSCmTOR	0,019932408	Activation
COAD	RHOF	Apoptosis	0,016064346	Activation
COAD	SAMD4A	EMT	6,4053E-08	Activation
COAD	SERPINE1	EMT	7,56592E-07	Activation
COAD	SERPINE1	Hormone ER	0,020507241	Activation
COAD	SNAI1	EMT	3,1575E-07	Activation
COAD	TNFSF9	Apoptosis	0,003575694	Activation
COAD	TRIM15	Apoptosis	0,020293551	Activation
COAD	TSC22D3	EMT	5,93596E-07	Activation
COAD	TXNIP	RASMAPK	0,009800588	Activation
COAD	ULBP2	Apoptosis	0,023298235	Activation

Supplementary Table 5. Colon Cancer Links (COAD) through the GSCA (Gene Set Cancer Analysis) platform by analyzing 10 pathways linked to cancer to determine the potential effects of the mRNA of common differentially expressed genes on the activity of a pathway (activation).

Cancer type	symbol	pathway	fdr	class
COAD	BIRC3	DNADamage	0,000453869	Inhibition
COAD	CCL20	EMT	0,035884534	Inhibition
COAD	CDKN2B	DNADamage	0,046324174	Inhibition
COAD	CPEB4	CellCycle	0,02434988	Inhibition
COAD	CSRNP1	DNADamage	0,005228962	Inhibition
COAD	DDIT4	DNADamage	0,006463797	Inhibition
COAD	DENND3	Apoptosis	0,013978403	Inhibition
COAD	DENND3	CellCycle	0,003751729	Inhibition
COAD	DENND3	DNADamage	0,005279217	Inhibition
COAD	DUSP1	DNADamage	0,00323488	Inhibition
COAD	DUSP5	DNADamage	0,02516704	Inhibition
COAD	DUSP8	EMT	0,011595109	Inhibition
COAD	EGR2	CellCycle	0,030471642	Inhibition
COAD	EGR2	DNADamage	0,016733748	Inhibition
COAD	EPC1	Apoptosis	0,006620921	Inhibition
COAD	EPC1	CellCycle	0,000169628	Inhibition
COAD	ERRFI1	DNADamage	0,018366472	Inhibition
COAD	FOS	DNADamage	0,001433017	Inhibition
COAD	FOSB	DNADamage	0,002630827	Inhibition
COAD	GEM	CellCycle	0,001760306	Inhibition
COAD	HBEGF	DNADamage	0,019289829	Inhibition
COAD	HEY1	CellCycle	0,004818676	Inhibition
COAD	PCK1	Apoptosis	0,041561542	Inhibition

COAD	PLEKHM3	Apoptosis	0,034264783	Inhibition
COAD	PLEKHM3	CellCycle	0,034264783	Inhibition
COAD	RASSF9	Apoptosis	0,005690819	Inhibition
COAD	RELL1	DNADamage	0,000507069	Inhibition
COAD	RHOF	DNADamage	0,013475737	Inhibition
COAD	SERPINE1	CellCycle	0,010414502	Inhibition
COAD	SERPINE1	DNADamage	0,010414502	Inhibition
COAD	TIPARP	DNADamage	0,001298889	Inhibition
COAD	TSC22D3	CellCycle	0,007137325	Inhibition
COAD	TSC22D3	DNADamage	0,002447762	Inhibition
COAD	TTC30B	Apoptosis	0,00006419	Inhibition
COAD	TUFT1	Apoptosis	0,000729808	Inhibition
COAD	TXNIP	Apoptosis	0,023159864	Inhibition
COAD	TXNIP	CellCycle	1,29862E-06	Inhibition
COAD	TXNIP	DNADamage	0,029855743	Inhibition
COAD	ULBP2	DNADamage	0,041771236	Inhibition
COAD	ZSWIM6	Apoptosis	0,017149298	Inhibition
COAD	ZSWIM6	CellCycle	0,029051819	Inhibition
COAD	ZSWIM6	DNADamage	0,029051819	Inhibition

Supplementary Table 6. Colon Cancer Links (COAD) through the GSCA (Gene Set Cancer Analysis) platform by analyzing 10 pathways linked to cancer to determine the potential effects of the mRNA of common differentially expressed genes on the activity of a pathway (inhibition).

GSCA			ENRICHR	KEGG_PATHWAY DAVID
Genes	**Uniques activation**	**Uniques inhibition**		
ABTB2				
AREG			AREG	hsa04010:MAPK signaling pathway,hsa04012:ErbB signaling pathway,hsa04151:PI3K-Akt signaling pathway,hsa04390:Hippo signaling pathway,hsa05210:Colorectal cancer,
ATF3			ATF3	
ATG10				hsa04136:Autophagy - other,hsa04140:Autophagy - animal,
BBC3	BIRC3	BIRC3	BBC3	hsa01524:Platinum drug resistance,hsa04115:p53 signaling pathway,hsa04210:Apoptosis,hsa04215:Apoptosis - multiple species,hsa04390:Hippo signaling pathway,hsa05016:Huntington disease,hsa05162:Measles,hsa05200:Pathways in cancer,hsa05210:Colorectal cancer,
BIRC3			BIRC3	hsa01524:Platinum drug resistance,hsa04064:NF-kappa B signaling pathway,hsa04120:Ubiquitin mediated proteolysis,hsa04210:Apoptosis,hsa04215:Apoptosis - multiple species,hsa04217:Necroptosis,hsa04390:Hippo signaling pathway,hsa04510:Focal adhesion,hsa04621:NOD-like receptor signaling pathway,hsa04668:TNF signaling pathway,hsa05132:Salmonella infection,hsa05145:Toxoplasmosis,hsa05168:Herpes simplex virus 1 infection,hsa05200:Pathways in

			cancer,hsa05202:Transcriptional misregulation in cancer,hsa05222:Small cell lung cancer,
C9orf72		C9orf72	
C9ORF72			hsa04140:Autophagy - animal,hsa05014:Amyotrophic lateral sclerosis,hsa05022:Pathways of neurodegeneration - multiple diseases,
C9ORF156			
CCL20	CCL20	CCL20	hsa04060:Cytokine-cytokine receptor interaction,hsa04061:Viral protein interaction with cytokine and cytokine receptor,hsa04062:Chemokine signaling pathway,hsa04657:IL-17 signaling pathway,hsa04668:TNF signaling pathway,hsa05323:Rheumatoid arthritis,
CDKN1A		CDKN1A	hsa01522:Endocrine resistance,hsa01524:Platinum drug resistance,hsa04012:ErbB signaling pathway,hsa04066:HIF-1 signaling pathway,hsa04068:FoxO signaling pathway,hsa04110:Cell cycle,hsa04115:p53 signaling pathway,hsa04151:PI3K-Akt signaling pathway,hsa04218:Cellular senescence,hsa04630:JAK-STAT signaling pathway,hsa04921:Oxytocin signaling pathway,hsa04928:Parathyroid hormone synthesis, secretion and action,hsa04934:Cushing syndrome,hsa05160:Hepatitis C,hsa05161:Hepatitis B,hsa05163:Human cytomegalovirus infection,hsa05165:Human papillomavirus infection,hsa05166:Human T-cell leukemia virus 1 infection,hsa05167:Kaposi sarcoma-associated herpesvirus infection,hsa05169:Epstein-Barr virus infection,hsa05200:Pathways in cancer,hsa05202:Transcriptional misregulation in

				cancer,hsa05203:Viral carcinogenesis,hsa05205:Proteoglycans in cancer,hsa05206:MicroRNAs in cancer,hsa05210:Colorectal cancer,hsa05211:Renal cell carcinoma,hsa05212:Pancreatic cancer,hsa05213:Endometrial cancer,hsa05214:Glioma,hsa05215:Prostate cancer,hsa05216:Thyroid cancer,hsa05217:Basal cell carcinoma,hsa05218:Melanoma,hsa05219:Bladder cancer,hsa05220:Chronic myeloid leukemia,hsa05222:Small cell lung cancer,hsa05223:Non-small cell lung cancer,hsa05224:Breast cancer,hsa05225:Hepatocellular carcinoma,hsa05226:Gastric cancer,
CDKN2B	CDKN2B	CDKN2B	CDKN2B	hsa04068:FoxO signaling pathway,hsa04110:Cell cycle,hsa04218:Cellular senescence,hsa04350:TGF-beta signaling pathway,hsa04934:Cushing syndrome,hsa05166:Human T-cell leukemia virus 1 infection,hsa05200:Pathways in cancer,hsa05203:Viral carcinogenesis,hsa05222:Small cell lung cancer,hsa05226:Gastric cancer,
CHAC1	CHAC1		CHAC1	hsa00480:Glutathione metabolism,hsa01100:Metabolic pathways,
CPEB4		CPEB4	CPEB4	hsa04114:Oocyte meiosis,hsa04914:Progesterone-mediated oocyte maturation,
CREB3L3			CREB3L3	hsa04022:cGMP-PKG signaling pathway,hsa04024:cAMP signaling pathway,hsa04151:PI3K-Akt signaling pathway,hsa04152:AMPK signaling pathway,hsa04211:Longevity regulating pathway,hsa04261:Adrenergic signaling in cardiomyocytes,hsa04668:TNF signaling

				pathway,hsa04714:Thermogenesis,hsa04725:Cholinergic synapse,hsa04728:Dopaminergic synapse,hsa04911:Insulin secretion,hsa04915:Estrogen signaling pathway,hsa04916:Melanogenesis,hsa04918:Thyroid hormone synthesis,hsa04922:Glucagon signaling pathway,hsa04925:Aldosterone synthesis and secretion,hsa04926:Relaxin signaling pathway,hsa04927:Cortisol synthesis and secretion,hsa04928:Parathyroid hormone synthesis, secretion and action,hsa04931:Insulin resistance,hsa04934:Cushing syndrome,hsa04935:Growth hormone synthesis, secretion and action,hsa04962:Vasopressin-regulated water reabsorption,hsa05016:Huntington disease,hsa05020:Prion disease,hsa05030:Cocaine addiction,hsa05031:Amphetamine addiction,hsa05034:Alcoholism,hsa05161:Hepatitis B,hsa05163:Human cytomegalovirus infection,hsa05165:Human papillomavirus infection,hsa05166:Human T-cell leukemia virus 1 infection,hsa05203:Viral carcinogenesis,hsa05207:Chemical carcinogenesis - receptor activation,hsa05215:Prostate cancer,
CSRNP1	CSRNP1	CSRNP1		
CYR61			CYR61	
DBNL			DBNL	
DDIT4		DDIT4	DDIT4	hsa04140:Autophagy - animal,hsa04150:mTOR signaling pathway,hsa04151:PI3K-Akt signaling pathway,hsa05206:MicroRNAs in cancer,
DENND3	DENND3	DENND3		

DUSP1	DUSP1	DUSP1	DUSP1	hsa04010:MAPK signaling pathway,hsa04726:Serotonergic synapse,hsa05012:Parkinson disease,hsa05418:Fluid shear stress and atherosclerosis,
DUSP5	DUSP5	DUSP5		hsa04010:MAPK signaling pathway,
DUSP8		DUSP8		hsa04010:MAPK signaling pathway,
EGR2	EGR2	EGR2	EGR2	hsa04625:C-type lectin receptor signaling pathway,hsa05161:Hepatitis B,hsa05166:Human T-cell leukemia virus 1 infection,hsa05203:Viral carcinogenesis,
EPC1	EPC1	EPC1		hsa03082:ATP-dependent chromatin remodeling,
EPC2				hsa03082:ATP-dependent chromatin remodeling,
ERRFI1		ERRFI1	ERRFI1	
FOS	FOS	FOS	FOS	hsa01522:Endocrine resistance,hsa04010:MAPK signaling pathway,hsa04024:cAMP signaling pathway,hsa04210:Apoptosis,hsa04380:Osteoclast differentiation,hsa04620:Toll-like receptor signaling pathway,hsa04657:IL-17 signaling pathway,hsa04658:Th1 and Th2 cell differentiation,hsa04659:Th17 cell differentiation,hsa04660:T cell receptor signaling pathway,hsa04662:B cell receptor signaling pathway,hsa04668:TNF signaling pathway,hsa04713:Circadian entrainment,hsa04725:Cholinergic synapse,hsa04728:Dopaminergic synapse,hsa04915:Estrogen signaling pathway,hsa04917:Prolactin signaling pathway,hsa04921:Oxytocin signaling pathway,hsa04926:Relaxin signaling pathway,hsa04928:Parathyroid hormone synthesis,

				secretion and action,hsa04932:Non-alcoholic fatty liver disease,hsa04935:Growth hormone synthesis, secretion and action,hsa05031:Amphetamine addiction,hsa05130:Pathogenic Escherichia coli infection,hsa05132:Salmonella infection,hsa05133:Pertussis,hsa05135:Yersinia infection,hsa05140:Leishmaniasis,hsa05142:Chagas disease,hsa05161:Hepatitis B,hsa05162:Measles,hsa05166:Human T-cell leukemia virus 1 infection,hsa05167:Kaposi sarcoma-associated herpesvirus infection,hsa05170:Human immunodeficiency virus 1 infection,hsa05171:Coronavirus disease - COVID-19,hsa05200:Pathways in cancer,hsa05207:Chemical carcinogenesis - receptor activation,hsa05208:Chemical carcinogenesis - reactive oxygen species,hsa05210:Colorectal cancer,hsa05224:Breast cancer,hsa05231:Choline metabolism in cancer,hsa05235:PD-L1 expression and PD-1 checkpoint pathway in cancer,hsa05323:Rheumatoid arthritis,hsa05417:Lipid and atherosclerosis,hsa05418:Fluid shear stress and atherosclerosis,
FOSB	FOSB	FOSB	FOSB	hsa04380:Osteoclast differentiation,hsa04657:IL-17 signaling pathway,hsa05030:Cocaine addiction,hsa05031:Amphetamine addiction,hsa05034:Alcoholism,
GADD45B	GADD45B			hsa04010:MAPK signaling pathway,hsa04064:NF-kappa B signaling pathway,hsa04068:FoxO signaling pathway,hsa04110:Cell cycle,hsa04115:p53 signaling pathway,hsa04210:Apoptosis,hsa04218:Cellular senescence,hsa05169:Epstein-Barr virus

				infection,hsa05200:Pathways in cancer,hsa05202:Transcriptional misregulation in cancer,hsa05210:Colorectal cancer,hsa05212:Pancreatic cancer,hsa05213:Endometrial cancer,hsa05214:Glioma,hsa05216:Thyroid cancer,hsa05217:Basal cell carcinoma,hsa05218:Melanoma,hsa05220:Chronic myeloid leukemia,hsa05222:Small cell lung cancer,hsa05223:Non-small cell lung cancer,hsa05224:Breast cancer,hsa05225:Hepatocellular carcinoma,hsa05226:Gastric cancer,
GAMT	GAMT		GAMT	hsa00260:Glycine, serine and threonine metabolism,hsa00330:Arginine and proline metabolism,hsa01100:Metabolic pathways,
GEM	GEM	GEM	GEM	
GSTM2	GSTM2		GSTM2	hsa00480:Glutathione metabolism,hsa00980:Metabolism of xenobiotics by cytochrome P450,hsa00982:Drug metabolism - cytochrome P450,hsa00983:Drug metabolism - other enzymes,hsa01100:Metabolic pathways,hsa01524:Platinum drug resistance,hsa05200:Pathways in cancer,hsa05204:Chemical carcinogenesis - DNA adducts,hsa05207:Chemical carcinogenesis - receptor activation,hsa05208:Chemical carcinogenesis - reactive oxygen species,hsa05225:Hepatocellular carcinoma,hsa05418:Fluid shear stress and atherosclerosis,
HBEGF	HBEGF	HBEGF	HBEGF	hsa01522:Endocrine resistance,hsa04012:ErbB signaling pathway,hsa04912:GnRH signaling pathway,hsa04915:Estrogen signaling

				pathway,hsa04928:Parathyroid hormone synthesis, secretion and action,hsa05120:Epithelial cell signaling in Helicobacter pylori infection,hsa05171:Coronavirus disease - COVID-19,hsa05205:Proteoglycans in cancer,hsa05219:Bladder cancer,
HEY1	HEY1	HEY1		hsa04330:Notch signaling pathway,hsa05165:Human papillomavirus infection,hsa05200:Pathways in cancer,hsa05224:Breast cancer,
HIST1H2AI				
ING3				hsa03082:ATP-dependent chromatin remodeling,
INO80D				hsa03082:ATP-dependent chromatin remodeling,
KIAA1683				
KLF4			KLF4	hsa04550:Signaling pathways regulating pluripotency of stem cells,hsa05207:Chemical carcinogenesis - receptor activation,
MAP1LC3B			MAP1LC3B	hsa04137:Mitophagy - animal,hsa04140:Autophagy - animal,hsa04216:Ferroptosis,hsa04371:Apelin signaling pathway,hsa04621:NOD-like receptor signaling pathway,hsa05014:Amyotrophic lateral sclerosis,hsa05022:Pathways of neurodegeneration - multiple diseases,hsa05131:Shigellosis,hsa05167:Kaposi sarcoma-associated herpesvirus infection,
MEPCE				
MRM1	MRM1			
N4BP3	N4BP3			
NEURL3				
PCK1		PCK1	PCK1	hsa00010:Glycolysis / Gluconeogenesis,hsa00020:Citrate cycle (TCA cycle),hsa00620:Pyruvate

				metabolism,hsa01100:Metabolic pathways,hsa03320:PPAR signaling pathway,hsa04068:FoxO signaling pathway,hsa04151:PI3K-Akt signaling pathway,hsa04152:AMPK signaling pathway,hsa04910:Insulin signaling pathway,hsa04920:Adipocytokine signaling pathway,hsa04922:Glucagon signaling pathway,hsa04931:Insulin resistance,hsa04964:Proximal tubule bicarbonate reclamation,
PER1			PER1	hsa04710:Circadian rhythm,hsa04713:Circadian entrainment,
PIM1			PIM1	hsa04630:JAK-STAT signaling pathway,hsa04933:AGE-RAGE signaling pathway in diabetic complications,hsa05200:Pathways in cancer,hsa05206:MicroRNAs in cancer,hsa05221:Acute myeloid leukemia,
PLA2G2F			PLA2G2F	hsa00564:Glycerophospholipid metabolism,hsa00565:Ether lipid metabolism,hsa00590:Arachidonic acid metabolism,hsa00591:Linoleic acid metabolism,hsa00592:alpha-Linolenic acid metabolism,hsa01100:Metabolic pathways,hsa04014:Ras signaling pathway,hsa04270:Vascular smooth muscle contraction,hsa04972:Pancreatic secretion,hsa04975:Fat digestion and absorption,
PLEKHM3	PLEKHM3	PLEKHM3		
PRMT6			PRMT6	
RASSF9		RASSF9		

RELL1	RELL1	RELL1		hsa04060:Cytokine-cytokine receptor interaction,
RHOB			RHOB	hsa05132:Salmonella infection,
RHOF	RHOF	RHOF		
RND1				hsa04360:Axon guidance,
RTP4			RTP4	
SAMD4A	SAMD4A			
SERPINE1	SERPINE1	SERPINE1	SERPINE1	hsa04066:HIF-1 signaling pathway,hsa04115:p53 signaling pathway,hsa04218:Cellular senescence,hsa04371:Apelin signaling pathway,hsa04390:Hippo signaling pathway,hsa04610:Complement and coagulation cascades,hsa04933:AGE-RAGE signaling pathway in diabetic complications,hsa05142:Chagas disease,
SNAI1	SNAI1		SNAI1	hsa04520:Adherens junction,
TIPARP		TIPARP	TIPARP	
TNFSF9	TNFSF9		TNFSF9	hsa04060:Cytokine-cytokine receptor interaction,
TRIM15	TRIM15			
TSC22D3	TSC22D3	TSC22D3	TSC22D3	
TTC30B		TTC30B		
TUFT1		TUFT1		
TXNIP	TXNIP	TXNIP	TXNIP	hsa04621:NOD-like receptor signaling pathway,
ULBP1				hsa04650:Natural killer cell mediated cytotoxicity,
ULBP2	ULBP2	ULBP2		hsa04650:Natural killer cell mediated cytotoxicity,
ZSWIM6		ZSWIM6		

Supplementary Table 7. Summary of genes associated with colon cancer pathways through GSCA and annotated using Enrichr and David.

Genes	Uniques activation	Uniques inhibition	pathway activation	pathways count	pathway inhibition	pathways count	David
ABTB2							
AREG							
ATF3							KW-0804~Transcription,KW-0805~Transcription regulation,
ATG10							KW-0072~Autophagy,KW-0653~Protein transport,KW-0813~Transport,KW-0833~Ubl conjugation pathway,
BBC3							
BIRC3	BIRC3	BIRC3	Apoptosis	1	DNADamage	2	KW-0053~Apoptosis,KW-0833~Ubl conjugation pathway,
C9ORF156							
C9ORF72							KW-0072~Autophagy,
CCL20		CCL20			EMT	2	
CDKN1A							KW-0131~Cell cycle,
CDKN2B	CDKN2B	CDKN2B	EMT		DNADamage	1	
CHAC1	CHAC1		CellCycle	1			
CPEB4		CPEB4			CellCycle	1	
CREB3L3							KW-0804~Transcription,KW-0805~Transcription regulation,KW-

							0834~Unfolded protein response,
CSRNP1	CSRNP1	CSRNP1	RTK, TSCmTOR	3	DNADamage	1	
CYR61							
DBNL							KW-0254~Endocytosis,KW-0391~Immunity,KW-0813~Transport,KW-1064~Adaptive immunity,
DDIT4		DDIT4					
DENND3	DENND3	DENND3	EMT, Hormone ER, PI3KAKT, RASMAPK, RTK	6	Apoptosis, CellCycle, DNADamage	3	
DUSP1	DUSP1	DUSP1	RTK	1	DNADamage	1	
DUSP5	DUSP5	DUSP5	EMT	1	DNADamage	1	
DUSP8		DUSP8			EMT	1	
EGR2	EGR2	EGR2	EMT, Hormone ER, PI3KAKT, RTK, TSCmTOR	6	CellCycle, DNADamage	2	
EPC1	EPC1	EPC1	RTK	1	Apoptosis, cellCycle	2	
EPC2							KW-0227~DNA damage,KW-0234~DNA repair,KW-0804~Transcription,KW-

							0805~Transcription regulation,
ERRFI1		ERRFI1			DNADamage	1	
FOS	FOS	FOS	RASMAPK, RTK, TSCmTOR	3	DNADamage	1	
FOSB	FOSB	FOSB	RASMAPK, RTK, TSCmTOR	3	DNADamage	1	
GADD45B	GADD45B		EMT	1			
GAMT	GAMT		EMT	1			
GEM	GEM	GEM	EMT	1	CellCycle	1	
GSTM2	GSTM2		EMT	1			
HBEGF	HBEGF	HBEGF	RASMAPK, RTK	2	DNADamage	1	
HEY1	HEY1	HEY1	EMT	1	CellCycle	1	
HIST1H2AI							
ING3							KW-0341~Growth regulation,KW-0804~Transcription,KW-0805~Transcription regulation,
INO80D							KW-0227~DNA damage,KW-0233~DNA recombination,KW-0234~DNA repair,KW-0804~Transcription,KW-0805~Transcription regulation,
KIAA1683							

KLF4							KW-0804~Transcription,KW-0805~Transcription regulation,
MAP1LC3B							KW-0072~Autophagy,KW-0833~Ubl conjugation pathway,
MEPCE							
MRM1	MRM1		DNADamage	1			
N4BP3	N4BP3		Apoptosis, EMT	2			
NEURL3							KW-0391~Immunity,KW-0399~Innate immunity,KW-0833~Ubl conjugation pathway,KW-0945~Host-virus interaction,
PCK1		PCK1			Apoptosis	1	
PER1							KW-0090~Biological rhythms,KW-0804~Transcription,KW-0805~Transcription regulation,
PIM1							KW-0053~Apoptosis,KW-0131~Cell cycle,KW-0945~Host-virus interaction,
PLA2G2F							KW-0391~Immunity,KW-0399~Innate immunity,KW-0442~Lipid degradation,KW-0443~Lipid metabolism,

PLEKHM3	PLEKHM3	PLEKHM3	EMT, Hormone ER	2	Apoptosis, cellCycle	2	
PRMT6							KW-0227~DNA damage,KW-0234~DNA repair,KW-0804~Transcription,KW-0805~Transcription regulation,KW-0945~Host-virus interaction,
RASSF9		RASSF9			Apoptosis	1	
RELL1	RELL1	RELL1	EMT, TSCmTOR	2	DNADamage	1	
RHOB							KW-0037~Angiogenesis,KW-0053~Apoptosis,KW-0130~Cell adhesion,KW-0221~Differentiation,KW-0653~Protein transport,KW-0813~Transport,
RHOF	RHOF	RHOF	Apoptosis	1	DNADamage	1	
RND1							
RTP4							
SAMD4A	SAMD4A		EMT	1			
SERPINE1	SERPINE1	SERPINE1	EMT, Hormone ER	2	CellCycle, DNADamage	2	
SNAI1	SNAI1		EMT	1			
TIPARP		TIPARP			DNADamage	1	
TNFSF9	TNFSF9		Apoptosis	1			
TRIM15	TRIM15		Apoptosis	1			

TSC22D3	TSC22D3	TSC22D3	EMT	2	CellCycle, DNADamage	2	
TTC30B		TTC30B					
TUFT1	TUFT1						
TXNIP	TXNIP		RASMAPK	1	Apoptosis, CellCycle, DNADamage	2	
ULBP1							KW-0391~Immunity,KW-0945~Host-virus interaction,
ULBP2	ULBP2		Apoptosis	1	DNADamage	1	
ZSWIM6	ZSWIM6				Apoptosis, CellCycle, DNADamage	2	

Supplementary Table 8. Summary of common genes associated with 10 colon cancer pathways via GSCA and annotated using David to determine potential effects of differentially expressed mRNAs on the activity of a pathway (activation/inhibition).