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The probiotic-induced dysregulation of immune-related genes in colon cells and relation with colorectal cancer

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ABSTRACT

Supplemental probiotics available without a doctor's prescription have become a booming global market in the past few years. Medical research has shown that probiotics may benefit both healthy people and cancer patients by improving their immune systems and digestive health. Even though they seldom produce serious side effects, it's important to note that they are generally safe to use. But further investigation into the role of probiotics and gut microbes in the etiology of colorectal cancer is required. Here we used computational methods to identify the transcriptome alterations induced by the probiotic treatment of colon cells. The impacts of genes with substantially altered expression were assessed in relation to the progression of colorectal cancer. Following probiotic treatment, substantial and high-level changes in the expression of genes were determined. BATF2, XCL2/XCL1, RCVRN and, FAM46B were up-regulated while IL13RA2, CEMIP, CUL9, Cand XCL6, PTCH2 were down-regulated in probiotic-treated colonic tissue and tumor samples. Also, immune-related pathways were determined that contribute to colorectal cancer formation and progression, as well as genes with opposing roles. This suggests that the length and dosage of probiotic use, in addition to the specific bacterial strain, maybe the most important determinant in the association between probiotics and colorectal cancer.

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Introduction

Probiotics are beneficial living bacteria that may improve health when taken in therapeutic doses. It is known that probiotics have immunomodulatory effects, the ability to improve the endogenous defense barrier in the human intestine, and the ability to reduce mucosal permeability (1, 2). Probiotics stimulate mucus production by adhering to the intestinal wall, thereby interacting with host intestinal epithelial cells, and strengthening the intestinal barrier. Through such interaction, probiotics compete with pathogenic bacteria for nutrition and energy, preventing the growth and proliferation of pathogenic bacteria in the gut (3, 4).

Probiotics' capacity to improve health and prevent or aid in the treatment of a variety of ailments, including certain forms of cancer, has led to a dramatic growth in their popularity in recent years. Fermented milk products often utilize lactic acid bacteria strains as probiotics, notably *Lactobacillus* and *Bifidobacterium* species. The potential therapeutic benefits of probiotics on cancer prevention and therapy were shown in a review by Yu and Li, in comparison to the overall impact on enhancing the host gut flora. Anti-inflammatory and antipathogenic activities may prevent tumor growth and metastasis via immune regulation, reduced bacterial translocation, altered microbiota, and improved intestinal barrier function (5, 6).

Probiotics' therapeutic effects are often species-specific or even strain-specific, which means that although they may be useful against one form of cancer, they may not be successful against others. Multiple probiotic strains may have more efficacy than just one, and symbiotic (probiotic and prebiotic) combinations have also been proposed (7).

Consequently, we hypothesized that the co-culturing of colon cells with probiotics may influence the gene expression of colon cells and the progression of colorectal cancer. With the recent increase in the use of probiotics, especially as food additives or adjunctive therapy, this study aimed to illuminate how the 3 widely used probiotics (*Lactobacillus paracasei* BL23, *Lactobacillus plantarum* 299v, and the mutant of *Lactobacillus plantarum* 299v-), affect the effective genes and pathways in cancer development or progression in colon cells using in-silico approaches. Among bacterial strains, Lactobacillus paracasei BL23 and Lactobacillus plantarum 299v are highly adhere to extracellular matrix proteins and intestinal tissue while L. plantarum 299v−a, a mutant strain of L. plantarum 229v, is known to have less adherence to intestinal epithelial cells.

Materials and Methods

Microarray gene expression data

The data on gene expression was retrieved from the Gene Expression Omnibus (GEO) database (GSE23630). This dataset contains gene expression data from six patients with neoplasia whose colonic tissue was removed

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during surgery and was macroscopically normal. Phorbol 12-myristate 13-acetate (PMA)/ionomycin (IO) was added to the new culture medium to produce inflammation, and the old medium was discarded (Control sample). Culture media with 1×10^6 cfu/ml of *L. paracasei* BL23 (iBL23), *L. plantarum* 299v (iLP), or the mutant of *L. plantarum* 299v (iLP(A$^-$) was added to the samples, and were incubated for 24 hours.

Processing and normalization of data

Raw GSE23630 data were RMA normalized using the TAC software for transcriptome analysis. Approximately 45,045 unique genes/54,613 probe sets were used to generate the normalized transcription profile data. The data contains 18 samples in total; 3 control explants without any treatment, 3 directly frozen tissue, 3 explants treated successively with PMA/IO and BL23, 3 explants treated successively with PMA/IO and LP299v, 3 explants treated successively with PMA/IO and LP299v (A-) and 3 explants treated with PMA/IO.

Identification of differentially expressed genes as a result of probiotic treatment

In the first step, differentially expressed genes were determined using GEO2R analysis in the frozen control group compared to the control group. In the same way, the PMA group was compared with the control group. The purpose of this analysis was to identify genes whose expression changes were not related to probiotics. The identified genes were excluded from the list of differentially expressed genes.

The groups treated with probiotics (BL23, 299V and 299V-) were compared with the control group by GEO2R analysis, and statistically differentially expressed genes were determined (p.val <0.05). Then, genes with a log fold change value greater than 1.5 were determined.

Hierarchical clustering

Common genes which were determined as statistically differentially expressed in all probiotic-treated groups versus the control group were hierarchically clustered with mean standardized gene expression values with Cluster 3.0 program. After performing cluster analysis, we applied our standards to the resulting data and analyzed them in Treeview. Genes and arrays were clustered using the full linkage approach and Euclidean distance to create a hierarchical cluster.

Functional pathway analysis

Database for Annotation, Visualization, and Integrated Discovery" (DAVID) was used to analyze the functional connections between these genes. Our genes' related pathways have been identified accordingly.

Gene set enrichment analysis (GSEA)

Following the GSEA method guidelines, we performed a gene set enrichment analysis (GSEA)(http://software.broadinstitute.org/gsea/docGSEAUserGuideFrame.html). Analyses were carried out using data obtained from the GSE23630 data. There are a total of 54,613 probe sets included in this data (45,046 different genes). In order to understand the pattern of probiotics' effects, we compared the untreated control group with the treated groups that received probiotics. The primary objective of this research is to establish which gene is substantially enriched in which GSEA gene set and which gene set is considerably enriched in which groups.

Enrichment score (ES), normalized enrichment score (NES), nominal *P* value (NOM *P* value), false discovery rate *q* value (FDR *q* value), and familywise error rate *P* value (FWER) are all computed using GSEA. It is possible to identify upregulated genes with the use of the ES value, which represents the highest departure of a gene from other gene sets. Expression differences and similarities across genes are reflected in the NES value. A larger NES number indicates an increase in permutations. So, gene sets with a higher NES value are more significant. The NOM P value is an additional measure of the significance of the ES computation, complementing the ES and NES values. As a result, the ES and NES values were both exactly proportional to the NOM P value. The crucial impact of ES is shown by the rise in the NOM P value. Decreased FWER P values are directly and strongly connected with the accuracy of NES computation, as they imply a lower risk of false positives in the NES. The most important value of this study is the FDR q value. The enrichment of gene sets is more significant as this value falls below 0.25, while it is still significant even at lower levels.

Network Analysis

Network analysis was performed using GeneMANIA software to better demonstrate the relationship of differentially expressed genes between the two groups.

Determination protein expressed genes in different types of cancers

In order to determine the protein expression of determined genes in colorectal cancer compared to other cancer types, we used the human protein atlas online tool. With the use of a combination of omics technologies, such as antibody-based imaging, transcriptomics, and systems biology, the Human Protein Atlas initiative aims to map every human protein present in cells, tissues, and organs.

Compromising between differentially expressed genes expression in colorectal cancer and normal tissue

Gene Expression Profiling Interactive Analysis (GEPIA) was used to compare differentially expressed genes between colon cancer and normal tissues after treatment with probiotics. A newly designed online web server GEPIA has been created in order to analyze the RNA sequencing data of 9.736 tumors and 8.587 normal samples from the TCGA and the GTEx projects (log 2 fold change = 1, p-val < 0.01)

Differential expression comparison between tumors and healthy tissues, cancer type or stage profiling, the study of patient survival, identification of orthologous genes, correlation analysis, and dimensionality reduction analysis are only a few of the adaptable features offered by GEPIA.

Results

Identification of differentially expressed genes in probiotic-treated groups

Comparing the whole genome expression values of the groups treated with BL23, 299V and 299V- bacterial

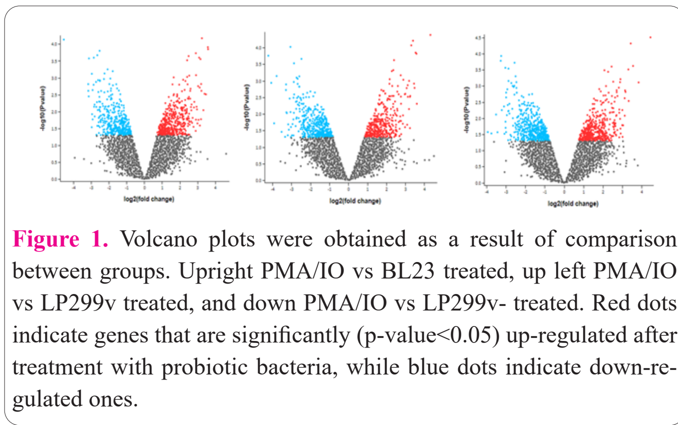

Figure 1. Volcano plots were obtained as a result of comparison between groups. Upright PMA/IO vs BL23 treated, up left PMA/IO vs LP299v treated, and down PMA/IO vs LP299v- treated. Red dots indicate genes that are significantly (p-value<0.05) up-regulated after treatment with probiotic bacteria, while blue dots indicate down-regulated ones.

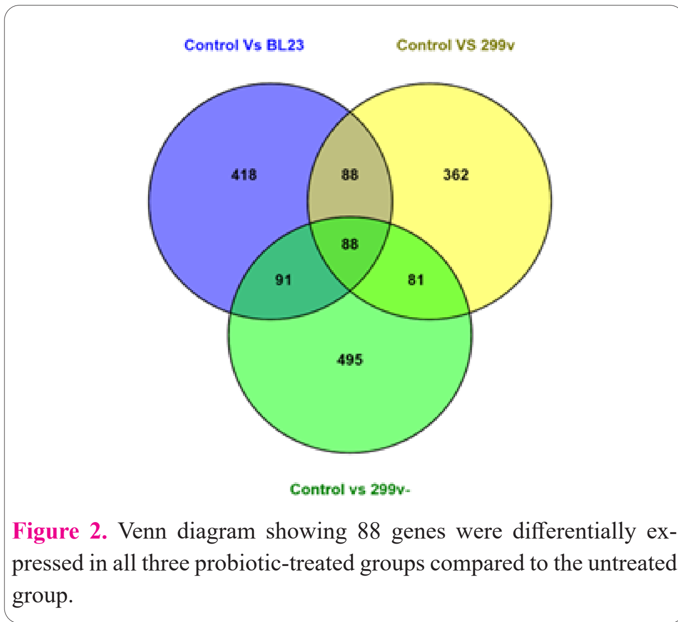

Figure 2. Venn diagram showing 88 genes were differentially expressed in all three probiotic-treated groups compared to the untreated group.

strains with the control group, we found that 685, 619 and 755 genes were expressed differently respectively (Figure 1).

Among these 88 genes were common in all groups (Figure 2).

The list of these genes with the p-value and log fold change of each gene are given in Supplementary Data 1.

Genes whose expression change resulted from PMA/IO treatment or freezing protocol, and genes whose expression change was in different directions in different groups were excluded. Nine genes (*PTCH2, BATF2, IL13RA2, XCL2///XCL1, RCVRN, FAM46B, CEMIP, CUL9, CXCL6*) expression was discovered statistically significant and at high levels altered after treatment with probiotics (Table 1).

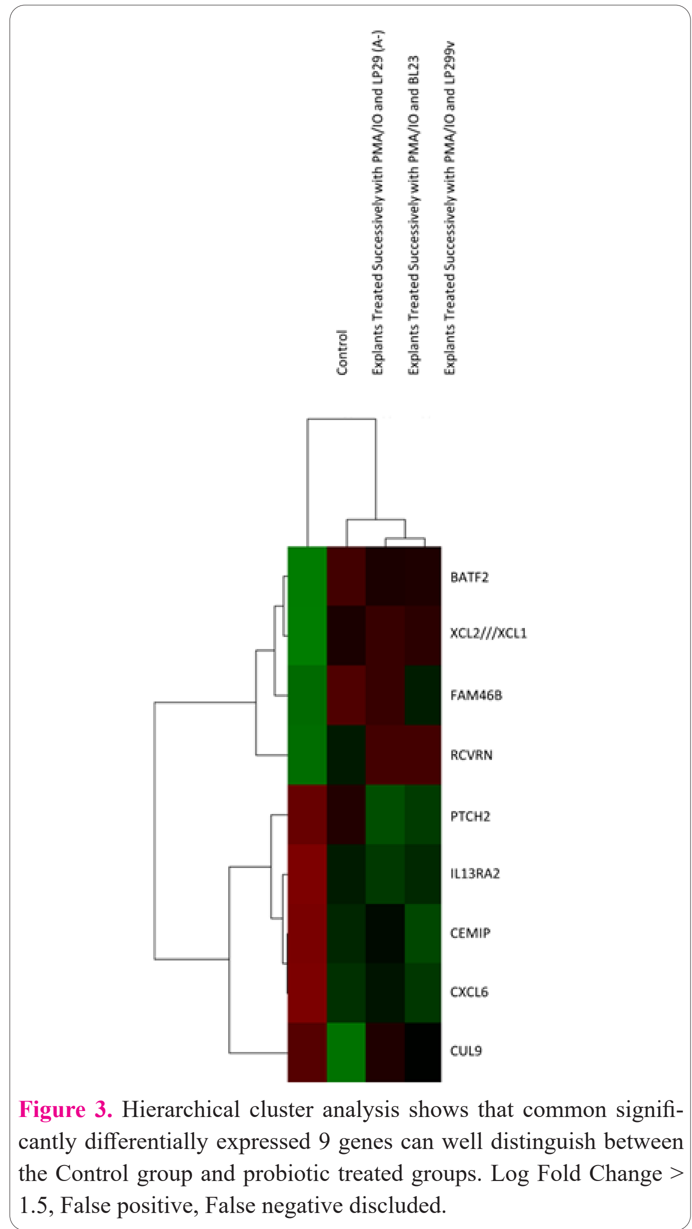

Figure 3. Hierarchical cluster analysis shows that common significantly differentially expressed 9 genes can well distinguish between the Control group and probiotic treated groups. Log Fold Change > 1.5, False positive, False negative discluded.

Hierarchical clustering analysis shows that these nine genes (p-value <0.05, Log Fold change>1.5) can distinctly distinguish cells treated with probiotic bacterial strains from the control group (Figure 3).

Table 1. Log Fold change and p-values of 9 genes which most significantly differentially expressed between probiotics-treated and untreated groups.

Gene Symbol	p value			Log FC vs Control		
	BL23	299v	299v-a	BL23	299v	299v-a
PTCH2	0.001741	0.001629	0.000237	3.02536	3.06	3.55
BATF2	0.002503	0.002266	0.001132	-2.07833	-2.18	-2.47
IL13RA2	0.004098	0.027276	0.01653	2.57796	2.24	2
XCL2///XCL1	0.006976	0.010788	0.010787	-2.19428	-2.25	-1.92
RCVRN	0.008601	0.015884	0.016253	-2.54805	-2.33	-2.48
FAM46B	0.010431	0.016781	0.006721	-1.81627	-1.61	-2.07
CEMIP	0.019412	0.022317	0.004681	1.864884	1.84	2.46
CUL9	0.022613	0.029946	0.035539	2.458151	2.18	2.12
CXCL6	0.041789	0.01017	0.023988	1.558165	1.72	1.94

FC, Fold Change. Probiotic bacteria; *Lactobacillus paracasei* BL23, *Lactobacillus plantarum* 299v, mutant of *Lactobacillus plantarum* 299v-a. p-values ≤ 0.05 and fold change cutoff of ≥1.5.

Table 2. Pathway analysis presents most of the nine common genes are involved in immune-related pathways.

Category	Term	Count	%	P-Value	Benjamini
UP_KW_CELLULAR_COMPONENT	Secreted	4	44,4	6,2E-2	5,0E-1
UP_KW_PTM	Disulfide bond	5	55,6	9,0E-2	6,3E-1
UP_KW_MOLECULAR_FUNCTION	Cytokine	3	33,3	5,5E-3	5,5E-2

Category: Original database/resource where the terms orient, Term: Enriched terms associated with our gene list, Count: Number of genes involved. In the term, %: involved genes/total genes, Benjamini: Modified Fisher Exact P-value, EASE Score.The smaller, the more enriched.

Four genes out of these nine genes were up-regulated (*BATF2, XCL2/XCL1, RCVRN, FAM46B*)(Figure 4A) in probiotic-treated colonic tissue while five genes were down-regulated (*IL13RA2, CEMIP, CUL9, CXCL6, PTCH2*)(Figure 4B).

Pathway analysis of differentially expressed genes

By performing pathway functional annotation clustering analyses, we determined three immune-related clusters with significant Benjamini-adjusted p-value, in which these most significant common genes enrolled. Table 2 shows the enrichment score and p-value for each cluster (Table 2).

Determination of enriched genesets in the probiotic-treated group

Gene sets enriched in control and bacterial-treated groups were determined by GSEA. Significant enrichment at FDR 25% was observed in gene sets associated with T helper and T cytotoxic lymphocyte immune response in probiotic-treated samples, including CD8+ alpha-beta T cell activation, regulation of CD4+ alpha-beta T cell differentiation, regulation of CD8+ alpha-beta T cell activation, and regulation of CD4+ alpha-beta T cell activation, while in the control group, 103 gene sets were substantially enriched at a false discovery rate (FDR) of 25% (Table 3 and Supplementary Data 2).

Comparison of differentially expressed genes in colorectal cancer samples with probiotic-treated colon cells

GEPIA analysis was performed to show the expression change of these most significant nine genes in tumor samples when compared with normal samples. In this analysis, 275 tumor samples were compared to 349 normal samples. With regard to the analysis results *BATF2, CEMIP, IL13RA2, CXCL6,* and *XCL2* were found to be up-regulated in tumor samples (Figure 5A) while *RCVRN, FAM46B, CUL9, PTCH2* genes were down-regulated (Figure 5B). Figure 6 shows a strong network relationship between differentially expressed genes. Together with the results of pathway and gene set enrichment analyses, this result is an expected result (Figure 6).

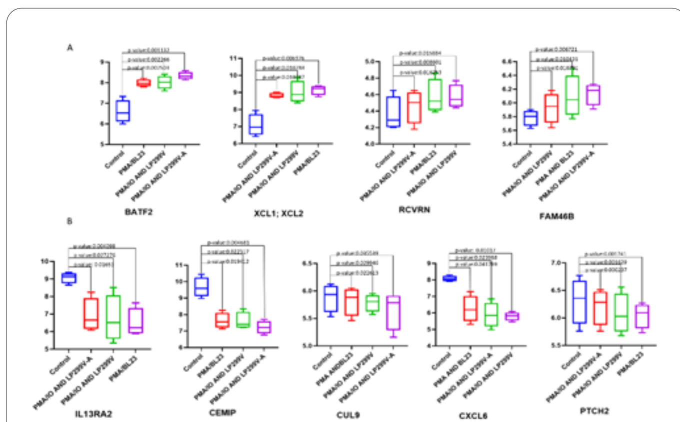

Figure 4. Four genes (*BATF2, XCL2, RCVRN,* and *FAM46B*) were significantly up-regulated after treatment of colon cells with probiotics (A) while, five genes (*IL13RA2, CEMIP, CUL9, CXCL6,* and *PTCH2*) were found to be down-regulated (B).

Discussion

Recently, there has been an increase in the use of pro-

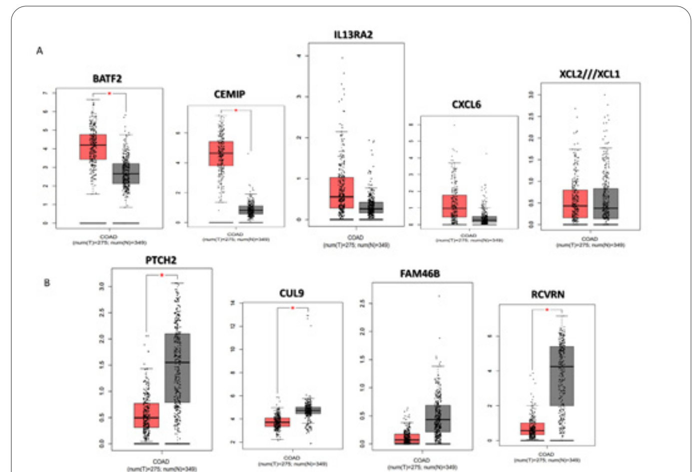

Figure 5. GEPIA analysis shows two out of nine common genes (*BATF2* and *CEMIP*)(A) are up-regulated significantly in tumor samples, while three (*PTCH2, CUL9*, and *FAM46B*)(B) are down-regulated significantly.

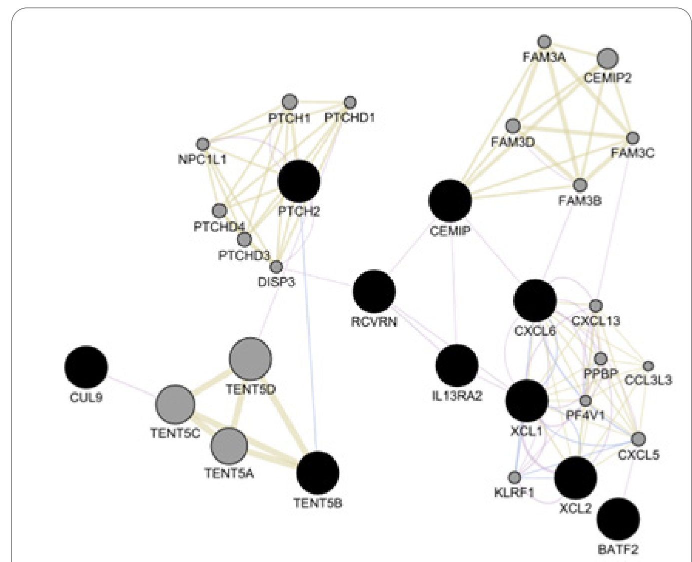

Figure 6. Network analysis of statistically differentially expressed genes. There is a strong network of co-expression and genetic interaction between these genes. Gray dots are other genes associated with these 10 genes identified by GeneMANIA. Dot size correlates with close network.

Table 3. Gene sets that were significantly enriched at FDR < 25% in the probiotic-treated groups compared to the control group.

GS	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val	RANK AT MAX
GOBP_CD8_POSITIVE_ALPHA_BETA_T_CELL_ACTIVATION	24	-0.77	-2.28	0	0	0	1493
GOBP_REGULATION_OF_CD4_POSITIVE_ALPHA_BETA_T_CELL_DIFFERENTIATION	48	-0.62	-2.18	0	0.003	0.007	2398
GOBP_REGULATION_OF_CD8_POSITIVE_ALPHA_BETA_T_CELL_ACTIVATION	17	-0.77	-2.13	0	0.009	0.035	1493
GOBP_CENTROMERE_COMPLEX_ASSEMBLY	26	-0.69	-2.13	0	0.008	0.038	1449
OBP_REGULATION_OF_ALPHA_BETA_T_CELL_ACTIVATION	96	-0.54	-2.12	0	0.007	0.042	2958
GOBP_REGULATION_OF_CD4_POSITIVE_ALPHA_BETA_T_CELL_ACTIVATION	58	-0.58	-2.08	0	0.014	0.1	2958
GOBP_KINETOCHORE_ORGANIZATION	21	-0.72	-2.06	0	0.019	0.155	1162
GOBP_POSITIVE_REGULATION_OF_CD4_POSITIVE_ALPHA_BETA_T_CELL_ DIFFERENTIATION	31	-0.65	-2.02	0.002	0.027	0.234	1784
GOBP_REGULATION_OF_ALPHA_BETA_T_CELL_DIFFERENTIATION	65	-0.54	-2	0	0.038	0.349	2398
GOBP_REGULATION_OF_MITOTIC_SISTER_CHROMATID_SEGREGATION	46	-0.57	-1.98	0	0.043	0.42	1162
GOBP_NEGATIVE_REGULATION_OF_INTERLEUKIN_1_BETA_PRODUCTION	29	-0.63	-1.97	0	0.049	0.507	5761
GOBP_CYTOLYSIS	24	-0.66	-1.96	0	0.05	0.536	2905
GOBP_REGULATION_OF_T_HELPER_CELL_DIFFERENTIATION	37	-0.58	-1.95	0	0.057	0.607	2398
GOBP_POSITIVE_REGULATION_OF_CELL_CYCLE_G2_M_PHASE_TRANSITION	31	-0.6	-1.94	0	0.059	0.645	2022
GOBP_GAMMA_DELTA_T_CELL_ACTIVATION	18	-0.7	-1.93	0	0.063	0.703	3034
GOBP_REGULATION_OF_DNA_DEPENDENT_DNA_REPLICATION	49	-0.54	-1.93	0	0.061	0.716	4608
GOBP_RESPONSE_TO_PROTOZOAN	23	-0.64	-1.93	0.003	0.061	0.74	2041
HP_EPISPADIAS	26	-0.63	-1.92	0.002	0.06	0.753	3266
GOBP_REGULATORY_T_CELL_DIFFERENTIATION	33	-0.59	-1.91	0	0.066	0.796	1494
GOBP_POSITIVE_REGULATION_OF_CD4_POSITIVE_ALPHA_BETA_T_CELL_ ACTIVATION	37	-0.57	-1.9	0	0.079	0.862	1784

GS, Gene Sets; ES, Enrichment Score; NES, Normalized Enrichment Score; NOM, Nominal; FDR, False Discovery Rate; FWER, Familywise Error Rate.

biotic-containing drug-like, supplements and food additive products other than food products (8). In the long term, it is necessary to investigate in more detail how the use of probiotics will affect the pathological conditions in humans, especially different types of cancer (9, 10). This study is a transcriptomic study evaluating the risk of colon cancer development and progress after exposure of normal colon tissues to probiotics. With regard to our results, four genes become upregulated after treatment of normal colon tissue samples with probiotics. Our results mostly support our hypothesis and according to our results, it has been shown that some of the genes differentially expressed in colon cells after treatment with probiotics contribute to the development of colorectal cancer risk.

BATF2, a novel IFN-stimulated gene, showed that inhibits colorectal cancer tumor cell proliferation, invasion, and migration. The expression of *BATF2* was discovered to be inversely linked with the expression of MET in cancer cells (11). By downregulating the activity of the hypoxia-inducible factor 1 alpha (HIF-1)/(VEGF) axis, it was discovered that inducing *BATF2* in colorectal cancer cells inhibited the HGF/MET signaling pathway and suppressed angiogenesis and tumor development. Inhibition of growth and induction of apoptosis are only two of the many cellular activities that are controlled by *BATF2* (12).

XCL2 and its important paralog *XCL1* were also found to be upregulated in treated tissue samples. Inducible cytotoxic immunity is mediated by the XCL ligand and the receptor family of genes was shown to be drawn to the tumor microenvironment in breast cancer tissues by the chemokine *XCL1.* One allele of the rs1024176 gene, called rs1024176-A, is causally linked to increased *XCL1* expression, which in turn is associated with enhanced DC1 signatures and a more favorable disease course. The results of this investigation lend credence to the hypothesis that a genetic predisposition to poor prognosis in breast cancer might be explained by the recruitment of XCL1-induced DC1 in the tumor microenvironment (13).

However, data shows that *XCL1* secreted by MCT-derived squamous cell carcinomas might promote PD1/PD-L1 interaction and CD8+ T cell dysfunction in the tumor microenvironment. Expression of *XCL1* has been suggested as a potential new biomarker for identifying MCTs that have undergone a malignant change (14). Lung cancer has been shown to have considerably greater levels of *XCL2* and *CX3CL1* expression compared to normal lung tissue in comparative studies. In another study regarding to breast cancer microenvironment, it has been shown when comparing tumors with different numbers of metastatic lymph nodes, the expression level of these genes was shown to be considerably greater in those with more nodes involved. Both *XCL2* and *CX3CL1* chemokines are upregulated as tumor malignancy advances, suggesting they may be useful gene therapy targets for this disease (15). It seems that high expression of this gene by immune cells results in host benefit, while high expression by cancer cells adversely affects the cancer process.

The protein encoded by the *RCVRN* gene is a neuronal calcium sensor related to the family known as recoverin. However, recoverin expression is not directly related to cancers that are neuroendocrine in nature (16). It may be specifically located in the p13.1 region of chromosome 17, which is on the short arm of the chromosome. The tumor suppressor gene p53 is one of several cancer-related genes found in this area. For a cancer cell to progress to the malignant stage, it needs just one mutation that renders the p53 gene inactive throughout the tumor-forming stage.

In several malignancies, recoverin is highly expressed (17, 18). Cancers of the cervix, uterus, ovaries, and fallopian tubes are included in this category. Transfection of human recoverin cDNA into non-expressing lung cancer A549 cells resulted in a decrease in the rate of cell proliferation (18).

In this case, TENT5B might also be referred to as *FAM46B*. The protein-coding gene tnt5B has been hypothesized to function as a poly(A) RNA polymerase that is distinct from the classical poly(A) RNA polymerase (19). There is evidence that low levels of *FAM46B* expression in prostate tumor tissue predict shorter survival times in prostate cancer patients. Compared to a non-malignant prostate cell line, *FAM46B* shRNA increased MYC levels, while overexpression of *FAM46B* in cancer cells lowered glucose absorption, and LDH activity, and triggered death (20). Multiple forms of cancer, including hepatoma, have been shown to have their survival ensured by the actions of the compound norcantharidin (NCTD), and a member of the gene family known as *FAM46C* has been identified as being particularly important in this process (21). Not only that but *FAM46C* expression was also shown to be low in colorectal cancer tissues and cells. Results showed that colorectal cancer cells treated with NCTD had dramatically increased apoptosis and decreased glycolysis (22).

Tumors of many different types, including the pancreas, ovaries, breast, colon, and melanomas, show significant expression of the interleukin-13 receptor subunit alpha-2 (*IL13RA2*), a high-affinity membrane receptor of IL-13 (23, 24). Colorectal cancer patients whose tumors were more aggressive and whose *IL13R2* expression was higher had shorter overall survival (25).

Proliferation and Cell Division A gene called clathrin-coated endocytic membrane-associated protein (*CEMIP*) has been linked to the carcinogenesis of colorectal cancer because it facilitates the depolymerization of hyaluronic acid (HA) at the cell membrane. Subsequent research revealed that *CEMIP* shRNA inhibited nuclear-catenin and Snail levels, demonstrating suppression of Wnt/-catenin/Snail signaling transduction in CRC cells. *CEMIP* has been shown to have a role in the CRC cells' metastatic phenotype (26).

Posttranslational modification of p53 is a signaling mechanism that is activated in response to a broad variety of cellular stressors, including DNA damage. It is becoming clear that p53's cytoplasmic actions play a significant role in tumor suppression (27).

CXCL6 (GCP2) is a member of the CXC chemokine family that exhibits many of the same functions as *CXCL8* (31% amino acid sequence identity). Matrix metalloproteinase-9 (MMP-9) and other proteases are secreted by granulocytes, and this factor enhances this process. Chemotactic and angiogenic actions of *CXCL6* and *CXCL8* are mediated via binding to the CXCR1 and CXCR2 receptors (28). Malignant colorectal tissue has been shown to contain much greater amounts of the angiogenic factors *CXCL6* and VEGF than normal tissues (29). Due to its ability to trigger EMT, *CXCL8* aided in the development of colorectal cancer (30). The expression of EMT markers was decreased in colorectal cancer cells when *CXCL2*, another *CXCL*, was inhibited by short hairpin RNA (31).

CXCL6 may influence the EMT process in cancer, much as *CXCL2* or *CXCL8* (32).

One of the functions of Protein Patched Homolog 2 (PTCH2) is to regulate cell proliferation. In addition, Creb and Src protein phosphorylation is induced in tandem with Gli activation through Ptch2-mediated Hedgehog (Hh) signaling, revealing a previously unrecognized Ptch2-specific signal pathway (33). Due to the classical WNT signaling pathway's negative control of hedgehog signaling, this route is seldom active in colorectal cancer (34). Ptch2 levels were likewise decreased in other tumor tissues, as suggested by the available data (34).

In addition to the nine individually highly differentially expressed genes, our data shows that six gene sets were considerably enriched in colorectal tissue samples exposed to probiotic bacteria.

It is believed that the cysteine endopeptidases Cathepsin B and L, which are mostly located in the lysosomes of healthy tissues, are involved in the protein turnover. Post-translational processing of hormones and growth factors may also include these cathepsins. Cysteine endopeptidases play a role in the breakdown of proteins inside cells, therefore they should be present in any tissue with lysosomes.

Cathepsin B and L activities were shown to be significantly higher in tumors than in normal mucosa and were found to be highest in Dukes' A and B stage cancers and lowest in Dukes' C and D stage cancers. Adenomas from patients with colorectal cancer, on the other hand, showed no abnormalities in cathepsin B expression, suggesting that an increase in cathepsin B expression may be a sensitive marker for the transition to the malignant state of colorectal cancer (35).

The steroid hormone has important functions in human physiology and pathology. Epidemiological studies have shown mixed results when looking at the possible role of endogenous sex steroid hormones in the development of colorectal cancer. Epidemiological research has shown that postmenopausal women whose hormone replacement treatment regimens contain E1-S and progestins had a lower chance of developing colorectal cancer. As a result, Gilligan et al. examined E1-S's effects on colorectal cancer model cell lines. E1-S is converted to active estrogens and activates GPER after being translocated, presumably through OATP4A1. Agonists of the GPER receptor, which normally inhibit it, paradoxically enhance steroid sulfatase activity (35). So, the risk of colorectal cancer (CRC) in women is linked to whether or not they have gone through menopause, and the use of estrogen to treat prostate cancer reduces the occurrence of gastric cancer (36, 37).

It is possible to detect amyloid-beta (A) in the brain, CSF, and blood since it is a natural byproduct of APP proteolysis by - or -secretase (37). Synaptic dysfunction, neuronal loss, cognitive impairment, and ultimately amyloid beta (A) deposition, when it occurs abnormally, are all linked to tau aggregation (38). As reported by Meng et al. amyloid beta protein precursor was discovered to have a role in the proliferation of human colon cancer cells (39).

Since the other three gene sets found significant in the probiotic-treated group are associated with specific pathological conditions additional *in vitro* and *in vivo* evidence is needed to fully elucidate the association of these gene sets with colorectal cancer progress.

As a result of the pathway analysis, it is not a big surprise that chemokines and their associated pathways are found in the first cluster. As a matter of fact, it is an expected situation that immune pathways are active in our cells exposed to bacteria. However, it has been emphasized that the pathways in the third cluster (polymorphism, splice variant, alternative splicing, and sequence variant) may be important in the development of colorectal cancer in different studies. When we look at the enriched gene sets in the untreated (control) group, we see that the gene sets associated with the normal functions of the cells are active, as expected, and especially inflammation-related gene sets are absent.

In parallel to our pathway analysis results our GSEA results also demonstrate an increase in enrichment, especially in gensets related to CD4+ and CD8+ T cell differentiation and activation, in colon cells after being treated with probiotics. However, additional in vitro and in vivo experiments are required to fully elucidate the causes and mechanisms of activation of genes specifically associated with T cells after exposure to probiotics in colon cells (39-41).

We investigated the actual expression values of these nine genes in tumor samples. GEPIA analysis showed that among the genes which we found up regulated in probiotic treated tissues *BATF2* is also overexpressed in tumor samples while *FAM46B* is down regulated. Regard to genes which found down regulated in probiotic treated tissues *CXCL6* and *PTCH2* are also down regulate in tumor samples while *CEMIP* is up regulated. Although regard to our analyzes, we detected some genes and pathways that have a role in the development and progression of colorectal cancer, on the other hand genes, and pathways with opposite functions were also identified. This gives the idea that might be the critical factors in relation between probiotic and colorectal cancer are duration and dose of consumption as well as bacterial strain.

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Conflict of interest
The authors declare no conflict of interest

Author’s contribution
Seyhan Turk: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision.
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