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Influence of *Enterococcus faecium*, a probiotic component, on ion channels in colon cancer

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ABSTRACT

Aims: This study aimed to investigate the effect of probiotic bacterium *Enterococcus faecium (E. faecium)* on the gene expression of ion channels in colon cancer cells.

Methods: The cytotoxic effect on SW480 colon cancer cell line was analyzed by MTT cell proliferation test using *E. faecium* bacterial cell culture free supernatants. The effect of *E. faecium* on ion channel genes in SW480 cells was determined by qRT-PCR analysis. STRING analysis was used to reveal protein-protein interactions of ion channel proteins. Bioinformatic analysis of healthy and colon cancer patient data on ion channels was revealed with GEPIA platform.

Results: The SW480 cell line's viability was enhanced by *E. fecieum* bacterial cell culture free supernatants dosages of 0.5 and 1.09 mg/ml, but it dramatically declined between 2.187 and 17.5 mg/ml doses, according the MTT study. Analysis of gene expression revealed that the TRPV2 and TRPM8 genes had significantly increased. The examined ion channel proteins were discovered to be substantially linked to one another based on STRING analysis. The TRPM2 gene we looked at revealed a notable rise in colon cancer patients based on data from both healthy and colon cancer patients on the GEPIA platform.

Conclusion: *E. faecium* has been shown to have many beneficial effects on health. Our study has shown that it also has an effect on ion channels in cancer cells and that ion channels are of great importance for cell survival and death.

Keywords: Colon cancer, ion channels, microbiota, *E. faecium*

INTRODUCTION

Colorectal cancer (CRC) is the second most frequent disease in women after breast cancer and the third most common cancer in males after prostate cancer.[1] The aging of the population and the rise in known risk factors such obesity, sedentary lifestyles, smoking, and chronic inflammatory diseases are contributing to an overall increase in the incidence and prevalence of colorectal cancer.[2,3]

Reactive oxygen species (ROS), reactive nitrogen species (RNS), and other electrophiles are examples of oxidative stress mediators that TRP channels react well to. Hydrogen peroxide (H_2O_2) activates the transient receptor potential melastatin-2 (TRPM2) channel, the first identified ROS-sensitive channel that mediates a variety of cellular responses, such as cell death and chemokine synthesis.[4] TRPM7, also known as transient receptor potential melastatin 7, functions as an ion-channel protein for the transportation of calcium and magnesium.[5] The TRPM7 channel controls cell survival and death physiologically by maintaining calcium and magnesium balance. When cancer cells exhibit malignant characteristics, TRPM7 expression is elevated in many malignancies, and its absence inhibits the proliferation of cancer cells.[6] TRPM8 is often expressed at low levels in epithelial cells, however it is expressed at significantly higher levels in tumor cells.[7] While TRPM8 is significantly downregulated in androgen-independent prostate cancer metastases, it is highly upregulated in a number of malignancies, including those of the prostate, breast, pancreatic, and skin.[8,9]

The genus *Enterococcus*, part of the family Enterococcaceae, comprises gram-positive, facultatively anaerobic bacteria commonly found in the gastrointestinal tracts of humans and animals. These resilient organisms can endure extreme environments, including high salt concentrations, varying pH levels, and temperatures ranging from 10°C to 45°C.[10] This adaptability not only underscores their ecological significance but also their potential applications in various fields, including food microbiology and probiotics. Among the species within this genus, *Enterococcus faecium (E. faecium)* is particularly recognized for its probiotic properties that contribute to gut health. As a member of the lactic acid bacteria (LAB) group, *E. faecium* thrives in the gastrointestinal environment,

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enhancing its effectiveness as a probiotic. It plays essential roles in supporting gut barrier integrity, modulating the immune response, and producing bacteriocins that inhibit pathogenic bacteria. Additionally, certain strains of *E. faecium* have demonstrated cholesterol-lowering properties and antimicrobial activity, further emphasizing its importance in maintaining gut microbiome balance and overall health.[11] These characteristics make *E. faecium* a valuable candidate for probiotic formulations and health intervention.

Our study aimed to investigate the relationship between cancer-related ion channels and *E. faecium*, exploring how this probiotic may influence cancer pathways and potentially contribute to therapeutic strategies. We seek to understand the mechanisms by which *E. faecium* interacts with these ion channels and its implications for cancer treatment outcomes.

METHODS

Ethics

This is cell line culture study. This study was exempt from ethical approval because no direct human or animal samples were used.

Cell Line Culture

The colon cancer cell line SW480 (ATCC® CCL-228TM) was cultured in high glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 50 µg/ml L-glutamine, and 1% penicillin-streptomycin. The culture was kept growing until it reached an adequate amount in an incubator with 5% CO_2 and 95% humidity.

Bacterial Cell Culture And Cell-Free Culture Supernatant (CFCS) Preparing

The *E. faecium* strain used in this study was isolated from locally sourced kefir. To confirm its identity, we performed 16S rRNA gene amplification and sequencing (**Supp. Data 1**), which verified that the strain belongs to *E. faecium*. *E. faecium* was inoculated in De Man, Rogosa, and Sharpe (MRS) broth and incubated at 37°C overnight. Following incubation, the culture was centrifuged at 10.000 rpm for 10 minutes to pellet the cells. The supernatant was carefully collected using a sterile syringe with a needle and then filtered through a sterile polyethersulfone (PES) membrane filter with a pore size of 0.45 µm. The freshly prepared cell-free culture supernatant (CFCS) was checked for contamination, while the remaining portions were stored at -20°C for further studies.

Supp. Data 1. The primers used to confirm 16S rRNA gene amplification and sequencing

	Primer sequences	Product lenght
Forward primer (008F)	5' -AGAGTTTGATCMTGGC-3'	1387 bp
Reverse primer (1387r)	5'-GGGCGGWGTGTACAAGRC-3'	

MTT Assay for Cell Viability

The viability of SW480 cells was assessed using the MTT assay. Briefly, SW480 cells were seeded at a density of 5.000 cells per well in a 96-well plate and allowed to adhere overnight. Following treatment with different doses of CFCS, the cells were incubated at 37°C with 5% CO_2 for 24 hours. After the treatment, 10 µL of MTT solution (5 mg/ml in PBS) was added to each well, and the plate was incubated for an additional 4 hours at 37°C. Following the incubation, the medium was carefully removed, and 100 µL of DMSO was added to each well to dissolve the formazan crystals. The absorbance of the solution was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells. Each experiment was conducted in triplicate, and results were presented as mean±standard deviation (SD).

RNA Isolaton and cDNA Syntesis

Following the manufacturer's directions, RNA was isolated from cancer cells that were suitably removed at the conclusion of the intended times and applications in the indirect culture system (Invitrogen™ TRIzol™ Reagent, USA). Following the manufacturer's instructions, cDNA synthesis was carried out from quality-controlled RNA samples using a kit (Bio-Rad iScript cDNA Synthesis, USA).

Real-Time qPCR Analysis

Target gene primer designs were created with primer quest (http://eu.idtdna.com/home/home.aspx). Using cDNAs containing target gene primers, RT-qPCR analysis was performed using the BioRAD CFX Connect tool to determine variations in gene expression of SW480 (**Table**).

Table. Used primers in this study

Gene	Primers	Amplicon size (bp)
TRPM2	F:5-TCGGACCCAACCACACGCTGTA-3 R:5-CGTCATTCTGGTCCTGGAAGTG-3	339
TRPM7	5-CTTATGAAGAGGCAGGTCATGG-3 5-CATCTTGTCTGAAGGACTG-3	214
TRPM8	F:5-TGAACTCTTCTCCAACCACTTC-3 R:5-CGTGAGGAGGGCATCATTATAG-3	85
TRPV2	F:5-GACCCTTGACATCTCCATCTG-3 R:5-CATCTTCTTGGCCTCCATCTAA-3	127
GAPDH	F:5-TGAACGGGAAGCTCACTGG-3 R:5-TCCACCACCCTGTTGCTGTA-3	307

RT-qPCR was performed with BrightGreen qPCR MasterMixR, using 0.5 pmol of each primer, which was designed with the IDT PrimerQuest™ Tool. The qPCR protocol began with an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The relative expression levels of the target genes were calculated and GAPDH served as the reference gene for normalization.

Protein-Protein Interaction Network Analysis

To better illustrate the functional link among transient receptor potential cation channel subfamily members, protein-protein interaction network of target genes were constructed using STRING (https://string-db.org/) database. This database utilizes textmining, databases, coexpression, experimentally verified and neigborhood interactions to build an interaction network among multiple proteins. The K means algorithm was used for clustering of the constructed network to identify functional modules. K-means algorithm is a widely-applied clustering approach for anomaly-based

intrusion detection. It attemps to classify a provided set of data into k (a previously defined number) categories.[12,13]

Gene Expression Analysis in Colon Adenocarcinoma and Normal Tissues

The Gene Expression Profiling Interactive Analysis (GEPIA) bioinformatics tool (http://gepia2.cancer-pku.cn/) is utilized to analyze differences between gene expression levels of tumor and normal samples.[14] The transcript levels in tumor tissues are retrieved from The Cancer Genome Atlas (TCGA) dataset while the expression levels in healthy samples are sourced from the Genotype Tissue Expression (GTEx) project. The boxplot diagrams are automatically produced via GEPIA platform. The cut-off values for p-value and log2 fold change are set to 0.05 and 1, respectively.

Statistical Analysis

The Ct values of the genes of investigation in the study were normalized in relation to the reference gene using the $2^{-\Delta\Delta Ct}$ method. The "Multiple t test" was used in GraphPad®Prism version 5 to evaluate the gene expression levels of the groups. If $p<0.05$, the statistics were considered significant.

RESULTS

E. faecium CFCS Reduced the Viability of Colon Cancer Cells

The Figure 1 demonstrates that *E. faecium* CFCS significantly reduces the viability of colon cancer cells across various doses. The control group establishes a baseline viability rate, while the lowest dose (0.5 mg/ml) shows a higher viability rate, indicating a potential proliferative effect at lower concentrations. However, as the lysate dose increases to 2.187, 4.375, 8.75, and 17.5 mg/mL, there is a marked decline in cell viability, with statistical significance represented by asterisks. This trend underscores the effectiveness of higher concentrations of *E. faecium* CFCS in inhibiting the growth of colon cancer cells, suggesting its potential role as a therapeutic agent in cancer management.

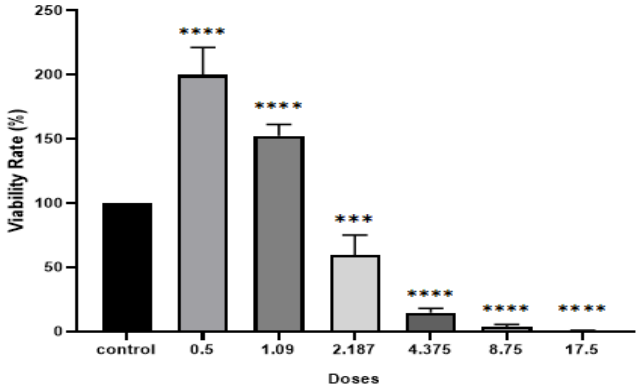

Figure 1. The *E. faecium* cell-free culture supernatant significantly reduces the viability of colon cancer cells

E. faecium Lysates Altered the Expression of Ion Channel-Related Genes in Colon Cancer Cells

The Figure 2 illustrates the expression levels of various TRP channels, highlighting significant differences in fold change between the CFCS-treatment and control groups. Specifically, TRPV2 and TRPM8 exhibited statistically significant increases in expression, while TRPM2 and TRPM7 showed no significant changes (ns). The most notable change was observed in TRPM8, where expression increased over 15-fold, suggesting a potential regulatory role in the response to CFCS-treatment. These findings emphasize the differential modulation of TRP channels by the treatment, pointing to specific targets for further investigation in the context of therapeutic strategies and cellular mechanisms involved in gut health and cancer biology.

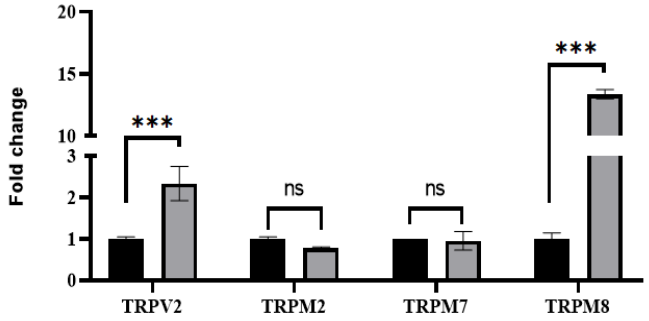

Figure 2. The *E. faecium* cell-free culture supernatant-treatment change the genes expression of transient receptor potential channels

Significant Connections between Ion Channel Proteins were Revealed using STRING Analysis

The functional impacts of target genes were also presented at system levels analysis by constructing protein-protein interaction (PPI) network. The constructed network consisted of 4 nodes (transient receptor potential cation channel subfamily members) and 4 edges, where the strength of interaction score was adjusted to greater than 0.4 (PPI enrichment p-value was 5.49E-09). Performed STRING PPI network analysis revealed significant functional link and close association among investigated genes. The black colored-line between all investigated extrinsic pathway genes confirmed the functional link among them (Figure 3).

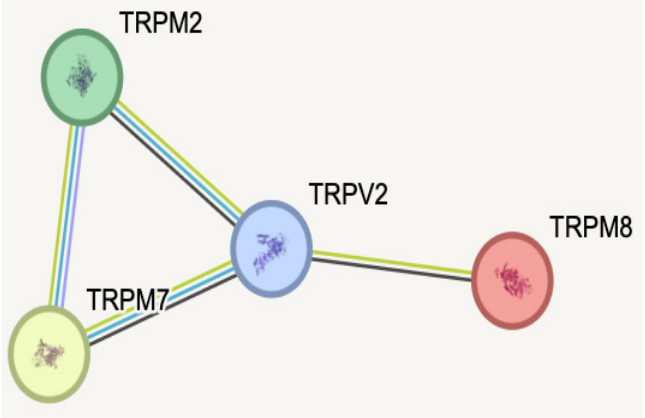

Figure 3. Protein-protein interaction network of TRPM2, TRPM7, TRPM8 and TRPV2 is drawn using STRING v12. The black lines denote confirmed co-expression thereby functional link among proteins while green-colored lines show the interactions based on textmining. Dark-blue lines predict interaction based on gene co-occurance whereas light-blue colored lines indicate protein homology. Moreover, cyan and purple-colored lines show known interactions from curated databases and experimentally-determined results, respectively. The calculated interaction score set on greater than 0.4.

GEPIA Data Showed that Patients with Colon Cancer Had Considerably Higher Levels of TRPM2 Expression

Changes in the transcript levels of genes are highly encountered between normal and tumor tissues. This fact urged us to check the differential expression profiles of target genes between colon adenocarcinoma (COAD) and normal tissues. The transcript levels of transient receptor potential cation channel subfamily members, namely TRPM7, TRPM8 and TRPV2 did not show any significant differences in COAD samples as compared to healthy colon tissues (**Figure 4B-D**, p-values>0.05). We observed a significantly higher expression of TRPM2 (**Figure 4A**, p-value<0.05) in colon cancer tissues than normal samples.

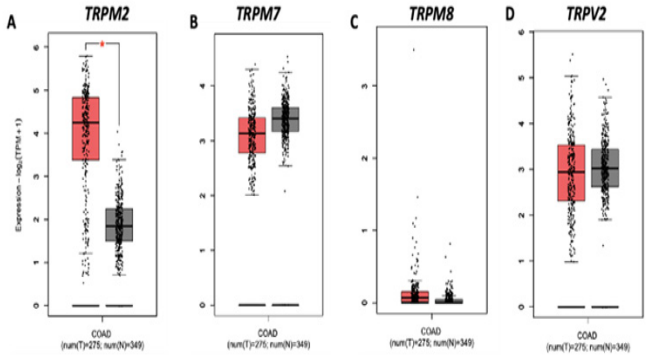

Figure 4. Expression analysis of transient receptor potential cation channel subfamily members performed via GEPIA2 in colon adenocarcinoma (COAD) samples (red, T=275) and normal tissue (grey, n=349) from TCGA and GTEx datasets, respectively. The (log2 (TPM+1)) transformed gene expression data are used in graphical representations for (**A**) TRPM2, (**B**) TRPM7, (**C**) TRPM8, and (**D**) TRPV2. p-values less than 0.05 are considered as statistically significant and represented with asterisk (*).

DISCUSSION

Regulation of ion channels has an effective role in cell survival and death. Numerous recent investigations have demonstrated that different kinds of cancer exhibit aberrant TRP channel expression. The effects of TRP channel subtypes on a wide variety of cancer cells, as well as the connection between TRP channel expression and surveillance in these malignancies, have been eloquently shown in a number of studies.[15] TRP channels have also been shown to play a significant part in the metastatic pathways and to be able to react to the physicochemical signals of metastatic cells in cancer cells.[16] It has been observed that tumor and immune system cells' migration and cell death are significantly impacted by TRPM2 channels.[17] Through bacterial peptide and cytokine activation, cell migration, and oxidative stress, the TRPM2 protein has been demonstrated to directly cause cell death.[18] It has been documented that adenocarcinomas of the head and neck, bladder, liver, and lung, especially breast cancer, exhibit heightened expression of TRPM2 channels.[19] In our study, *E. faecium* decreased the gene expression of TRPM2 ion channel in colon cancer cells, albeit insignificantly. TRPM2, which increased with tumor progression, was decreased when treated with *E. faecium*. As cancer cells exhibit malignant tendencies, TRPM7 expression is elevated in many malignancies, and its absence inhibits the proliferation of cancer cells.[20] In addition to having a detrimental impact on the prognosis of patients and the progressive tumor behavior of gastric cancer, overexpression of TRPM7 has been linked to a lower survival rate in breast cancer.[20,21] According to similar studies, TRPM7 expression was found to decrease in colon cancer cells treated with *E. faecium*, but it was not significant.

TRPM8 is now seen as a prospective target for cancer, especially prostate cancer, because it regulates cell proliferation and apoptosis.[22] Colorectal cancer tissues are among the primary tumors where TRPM8 mRNA has been found.[7] In line with the literature, TRPM8 expression was increased in colon cancer cells in our study, but *E. faecium* application did not change this increase. Therefore, it was determined that TRPM8, which was expected to be suppressed, was not suppressed.

TRPV2 activation causes apoptosis, lowers cell viability, and raises intracellular calcium levels.[23] Mizuno et al.[24] found that the murine MBT2 BC cell line had higher levels of TRPV2 expression than normal mouse urothelial cells; TRPV2 suppression in MBT-2 cells using RNA interference boosted cell proliferation, while TRPV2 activators had the reverse effect. Alptekin et al.[25] showed a strong correlation between TRPV2 overexpression and GBM patient survival, indicating for the first time that TRP channels, particularly TRPV2, play a role in the progression and survival of GBM patients. Studies have shown that overexpression of TRPV2 induces apoptosis. Our study has shown that *E. faecium* application significantly increases TRPV2 expression. Therefore, *E. faecium* has an anticancer effect in colon cancer cells.

CONCLUSION

E. faecium has many beneficial effects in the intestinal system as a probiotic. It plays a major role in regulating the intestinal system and strengthening the immune system. In our study, the cellular effect of *E. faecium* via ion channels was examined. According to the results obtained, results compatible with the literature were obtained in terms of TRPV2, TRPM2 and TRPM7.

However, the effect of TRPM8 observed following *E. faecium* application did not align with the anticipated outcomes. Consequently, further investigations are required to elucidate this relationship. Future studies should focus on exploring the interactions of additional ion channels and their associated cellular pathways to gain deeper insights into the underlying mechanisms.

ETHICAL DECLARATIONS

Ethics Committee Approval

This study was exempt from ethical approval because no direct human or animal samples were used.

Informed Consent

Written informed consent is not required in this study as no direct human or animal samples were used.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Financial Disclosure
The authors declared that this study has received no financial support.

Author Contributions
All the authors declare that they have all participated in the design, execution, and analysis of the study and that they have approved the final version.

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