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**RESEARCH ARTICLE**

# EXOPOLYSACCHARIDE (EPS) ISOLATED FROM *ENTEROCOCCUS FAECIUM* D36 SHOWS ANTI-CANCER AND ANTI-INVASIVE ACTIVITY POTENTIAL VIA DOWN-REGULATION OF MUC5AC GENE ON HUMAN COLORECTAL ADENOCARCINOMA (CACO-2) CELLS

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## ABSTRACT

To investigate the antiproliferative properties of exopolysaccharides (EPS) on human colorectal adenocarcinoma cell line (Caco-2) and the regulation of MUC5AC gene expression, the antiproliferative effect of EPS isolated from D36 strain was determined by MTT test and the regulation of MUC5AC gene expression was examined using Real-Time PCR. *Enterococcus faecium* D36 (*E. faecium* D36) were characterized by Ribotyping analysis. Some biochemical methodologies were preliminarily used to characterize the probiotic potential of *E. faecium* D36, including morphological, cultural, and physiological characteristics. EPS isolated from *E. faecium* D36 strain has an antiproliferative effect on Caco-2 cell line, and mucin gene (MUC5AC) expression levels decreased. These results suggest that EPS isolated from *E. faecium* D36 strain might be a source for a novel anticancer agent. Based on our results, it is believed that EPS obtained from *E. faecium* D36 can be used as a protective and therapeutic substance during the early stages of cancer, especially colon cancer. EPS affects colon cancer by reducing the invasion ability of cancer by decreasing MUC5AC expression. These findings are thought to shed light on future *in vivo* studies.

**Keywords:** Colorectal cancer, Lactic acid bacteria, *Enterococcus faecium*, Exopolysaccharide, MUC5AC

## 1. INTRODUCTION

Probiotics are described as beneficial microorganisms. Lactic acid bacteria (LAB) are one of these. LABs benefit human health and have become necessary due to their efficiency in secreting exopolysaccharides (EPS). EPS are long-chain and water-soluble polysaccharides that consist of branched and repeating units of sugars or sugar derivatives [1,2]. EPSs obtained from LAB have antitumor, antioxidant, antibiofilm, immune system support, and cholesterol-reducing properties [3,4]. Probiotics also exhibit anticancer activities against different carcinoma cells, such as the colon, bladder, and breast. Most studies have focused on the anticancer effect of probiotics on colon carcinoma cells [5]. Because of these probiotics characteristics, the food industry has expressed interest in them and their daily growth rate. Of the many probiotic microorganisms available, LABs are widely known and used.

Cancer is one of the leading causes of death. Colorectal cancer is one of the most seen types, and overall morbidity is high worldwide due to its high metastatic capacity. Humans consume a diet rich in proteins and carbohydrates but lack fibre, which results in various gastrointestinal problems, diseases, and cancer, especially colorectal cancer, related to these problems [6]. The high incidence of such problems

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leads humans to search for natural ways to overcome them. Foods containing probiotics are one of these ways [7].

The members of the genus Enterococcus are among the LABs found in many food products, the human gut, and the urogenital tract [8]. Several genes of Enterococcus are also used as probiotics in some countries [9,10,11]. Previous studies have shown the anticancer effects of LABs [12]. LAB strains produce microbicidal substances that act against gastric and bowel pathogens and other microbes or compete with them to bind to the cell surface and mucin-binding domains [13]. Changes have been shown in the location of mucin expression and expression levels, especially in cancer cells, as well as in the glycosylation of mucin glycoprotein. Some cancer cells developed invasion abilities because of these changes. For example, changing mucin expression and glycosylation changes in colon cancer cells increased the invasion ability of the cells. As a result, they gained the ability to metastasis [14,15,16]. Serine threonine O-glycosylation sites are abundant in glycoproteins bound to the cell surface or that secrete mucin. Mucins, highly glycosylated proteins, are the significant components of mucus. MUC5AC, MUC5B, and MUC8 are representative secretory mucins. However, O-glycans are widely found around cells that have been depolarized. This results in the loss of regular cell organization [17, 18, 19]. MUC5AC, a member of the group of mucins including MUC2, MUC5B, and MUC6, is located within the 11p15 chromosomal locus [20]. MUC5AC is not synthesized in normal colon epithelia but is *de novo* by colonic cancer cells [21, 22]. Pothuraju et al. (2020) reported that Overexpression of MUC5AC is observed in CRC (Colorectal cancer) patient tissues and cell lines. MUC5AC expression enhanced cell invasion and migration and decreased apoptosis of CRC cells [23]. This study investigated the effects of EPS isolated from LABs on cell proliferation of Caco-2 cell line and regulation of MUC5AC gene expression.

## 2. MATERIAL AND METHODS

### 2.1. Bacterial Culture and Identification

*Enterococcus faecium* was isolated from Turkish milk samples cultured on de Man, Rogosa, and Sharpe (MRS) Agar (Sigma-Aldrich, Steinheim, Germany), as well as on M-17 Agar (Sigma-Aldrich, Steinheim, Germany) plates for isolation of LAB. The plates were anaerobically incubated at 37 °C for 48 h, and isolates were examined by microscope. Cell morphology and Gram-staining reactions were determined. Next, isolates were tested for oxidase and catalase activities. Then, sugar fermentation patterns of the isolates were determined using API 20 STREP organisms according to manufacturer guidelines (BioMerieux, France). Growth at different temperatures (4, 15, and 45 °C) and pH values (3.9 and 9.6) was observed. Ammonia production from arginine was then studied. Next, growth was examined at different NaCl concentrations (6, 7.5 & 10%) [24, 25]. Finally, isolates were identified using an EcoRI automated RiboPrinter® microbial characterisation system according to manufacturer instructions. Cultures were maintained at -80 C in 20% glycerol.

### 2.2. Production of EPS

MRS Agar was inoculated into the broth and incubated for 24-48 hours under optimal conditions. At the end of the incubation period, the bacteria were centrifuged at 6000 rpm for 20 min at +4 °C. The supernatant that formed after was then transferred to another tube. Twenty % trichloroacetic acid was added to the tube and left overnight at +4 °C. Next, the samples were centrifuged at 10.000 rpm for 30 min at +4 °C. Then, the resultant supernatant was transferred to another tube, and an equal volume of chilled ethanol was added to the tube and left overnight at -20 °C. Finally, samples were centrifuged at 10.000 rpm for 30 min at +4 °C. The resultant supernatant was discarded. The hot distilled water was poured onto the pellet that had formed at the bottom of the tube, after which the pellet dissolved. The ensuing EPS solution was used.

### 2.3. Analysis of Partially Purified EPS Contents

Analyse the calibration standards, calibration verification standard CVS, and samples by HPLC using a Biorad Aminex HPX-87H column. 0.005 M sulfuric acid was used as mobile phase, which is 0.2 µm filtered and degassed. Flow rate and column temperature were adjusted to 0.6 mL/minute and 60 °C, respectively. Refractive Index Detector (RID) was used [26]. After pH adjustment, the sample was filtered by a 0,45µm syringe filter. Contents of the samples were calculated from areas of the obtained peaks.

### 2.4. Cells and Cell Culture

Caco-2 cell lines (HÜKÜK No: 98052301) were obtained from Şap Enstitüsü (Ankara, Turkey). DAPI staining was performed to check mycoplasma presence or not. Cells were cultured in minimum essential medium (MEM) (Sigma-Aldrich, Steinheim, Germany) containing 10% fetal bovine serum (Sigma-Aldrich, Steinheim, Germany), 1% nonessential amino acids (Sigma-Aldrich, Steinheim, Germany) and 1% penicillin/streptomycin (Sigma-Aldrich, Steinheim, Germany). Cells were maintained at 37 °C in a humidified incubator (Thermo Scientific Heracell, USA) containing 5% $CO_2$.

### 2.5. Cell viability assay

Two strategies were used to investigate EPS's effect on cell viability and protection.
Strategy 1: The aim was to study EPS's protective effect during early diagnosis. An experiment was designed that reduced the cell number to $5x10^3$, and the concentration-dependent effects of EPS at 24 and 48 h were subsequently analysed.
Strategy 2: Colon cancer cells form a tumour density by proliferation. To obtain an *in vitro* model, 20.000 and 30.000 cells were seeded into each well of a 96-well plate. A 24-h experiment was designed, and the appropriate concentration of EPS was studied.

The effect of EPS on cell viability was studied using the MTT protocol, an assay based on the reduction of the yellow dye, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide, to purple formazan crystals by mitochondrial dehydrogenase activity [27]. Briefly, as part of the continuous treatment procedure, 5000-20.000-30.000 cells (Caco-2) were seeded in each well a 96-well microplate at a final volume of 100 µL. After 48 h of seeding, cells were treated with 4-8-10-12.5-15-18-20 (mg/mL) EPS for 24 and 48 hours. Eight replicate wells per concentration were used, and the experiments were repeated in triplicate at different intervals. Untreated medium controls (blank) and solvent controls (ultra-pure water) were also assayed in parallel. After treatment with various concentrations of test samples for 24 and 48 h, the liquid media containing the relevant samples from each well was replaced with 100 µL fresh medium containing 0.5 mg/mL MTT dissolved in phosphate buffer saline (PBS). Samples were added to culture wells and incubated for 2 h at 37 °C. The supernatant solution was removed, 100 µL/well DMSO (dimethyl sulfoxide) (Sigma-Aldrich, Steinheim, Germany) was added, and samples were shaken for 5 min. Absorbance was measured at 570 nm with a Bio-Tek ELx808 microplate reader (http://www.biotek.com/).

### 2.6. mRNA isolation and Real-Time PCR

Total RNA was isolated using a RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Total RNA quantity was measured by NanoVue™ Plus Spectrophotometer (GE Healthcare, Buckinghamshire, UK). For quantitative RT-PCR, mRNA was converted to cDNA using a QuantiTect Reverse Transcription cDNA kit (QIAGEN, Valencia, CA, USA) and amplified on a Palm Cycler using a Taqman® Universal PCR Master Mix (QIAGEN) following manufacturer instructions.

DD-Hu-600 MUC5AC gene (Primerdesign, Ltd, Southampton, UK) primer and double-dye assay (Taqman-style) was used as target gene. HK-DD-Hu-18S rRNA (Primerdesign, Ltd, Southampton, UK) primer and double-dye assay were used as a housekeeping gene, and fluorescent signals generated during PCR amplifications were monitored by a QIAGEN Corbett Rotor-Gene® PCR (Corbett Life Science, USA). To determine the efficiency of each Taqman-style gene expression assay, standard curves were generated by dilution of cDNA, and quantitative evaluations of target and housekeeping gene levels were analysed by Rotor-Gene 6000 software.

***Statistical analysis***

The SPSS® Statistical Package for Social Sciences software was used to analyse all data. Data were evaluated using one-way ANOVA (Analysis of Variance) followed by Tukey's test. A value of $p < 0.05$ was considered significant. All data were expressed as means and standard deviations of triplicate measures determined in 3 independent experiments.

## 3. RESULTS

### 3.1. Properties of EPS

The results of different temperatures and salt concentrations on cell morphology are shown in Table 1. The isolates were Gram-positive as well as catalase- and oxidase-negative cocci. Sugar fermentation of the strains was performed using the API ID 32 STREP system. According to phenotypic tests, the strains were identified as E. faecium. Similarly, the automated EcoRI ribotyping results (Fig. 1) showed that phenotypic characterisation was confirmed for E. faecium. Partially purified EPS contents were analysed by HPLC (Table 2).

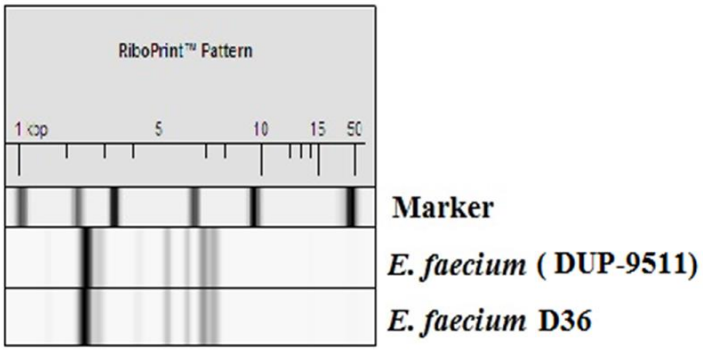


**Figure. 1.** Ribotyping profiles of *E. faecium* D36 and standards DUP-9511

**Table 1** Morphological, cultural and physiological characteristics of the *E. faecium* D36

| Gram reaction | Morphology | Catalase | Oxidase | +4 °C | 20°C | 45°C | %6 NaCl | %7.5 NaCl |
|---|---|---|---|---|---|---|---|---|
| + | coc | - | - | + | + | + | + | + |
| | | | | | | | | |
| %10 NaCl | $NH_3$ - Arginine | pH 3.9 | pH 9.6 | Glucose | Lactose | Sucrose | Fructose | |
| - | + | + | + | + | +++ | + | + | |

**Table 2** EPS Contents

| Glucose (mg/mL) | Xylose (mg/mL) | Ethanol (mg/mL) |
|---|---|---|
| 0.276 | 0.051 | 9.121 |

### 3.2. MTT Assay

According to MTT results, there was a decrease in cell viability of Caco-2 cells after EPS application in a concentration and time-dependent manner.

Strategy 1: The EPS's protective effect for early diagnosis was studied. An experiment was designed to reduce cell number to 5000. The concentration-dependent effect of EPS at 24 and 48 h were studied. It was shown that EPS isolated from *Enterococcus faecium* D36 strain decreased cancer cells by 25% at 24 h at a 20 mg/mL concentration and by 35% at 48 h at a 12.5 mg/mL concentration (Figure 2).

Strategy 2: EPS concentrations were applied to different cell numbers. An *in vitro* density forming model was required. To create this *in vitro* model, 20.000 and 30.000 cells were seeded into each well of a 96-well plate, and a 24h experiment was designed. Then 4-8-10 mg/mL EPS concentrations were explored. There was no effect of 4-8-10 mg/mL EPS concentrations on Caco-2 cells, but increased cell viability (Figure 3).

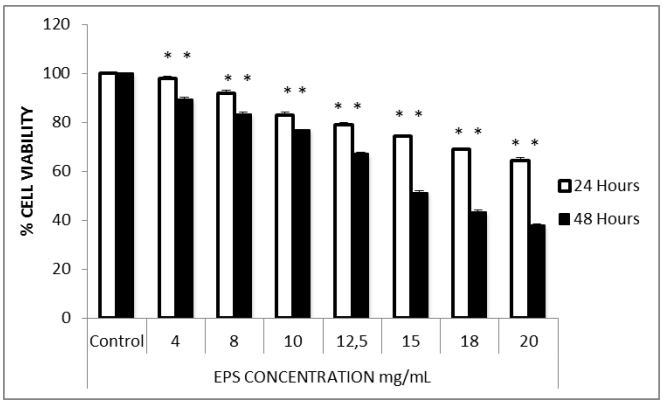


**Figure 2.** Cytotoxic activity of EPS obtained from *Enterococus faecium* D-36 strain isolated from milk on Caco-2 cell line as determined in the MTT assay. Concentration-response graph of MTT assay of the antiproliferative effect of EPS after 24 and 48 hours of treatment. Results are expressed as mean SD. *indicates a significant difference compared to the control group by the Tukey test ($p < 0.05$)

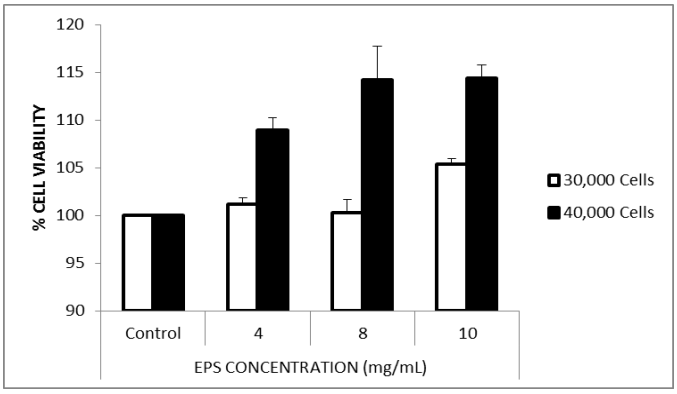


**Figure 3** Cytotoxic activity of EPS obtained from *Enterococus faecium* D-36 strain isolated from milk on Caco-2 cell line as determined in the MTT assay. Concentration-response curves of the anti-proliferative effect of EPS for MTT assays performed after 24 h treatment. The results are expressed as the mean_SD.

### 3.3. Gene Expression

For Real-Time PCR, 220.000 cells were seeded in a T25 flask. EPS obtained from *Enterococcus faecium* D36 strain isolated from milk was applied to Caco-2 cells at dosages of 15, 18, and 20 mg/mL, respectively. At the end of 24 h, exposure and its effect on the MUC5AC gene expression increased on Caco-2 cells. Expression levels was evaluated by Real-Time PCR. Compared to the control group, there was a concentration-dependent decrease in MUC5AC gene expression levels (Figure 4).

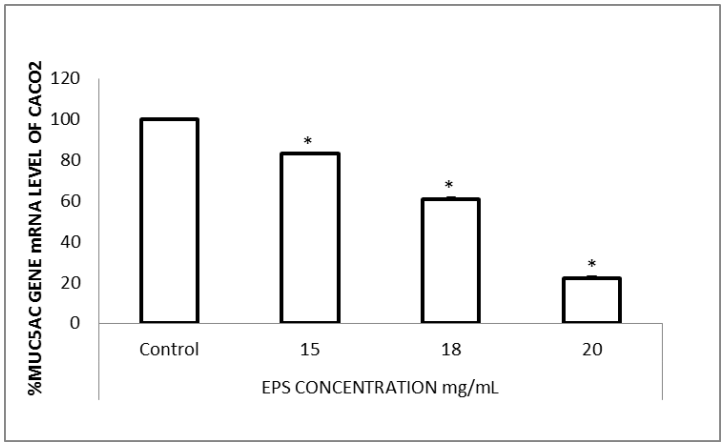


**Figure 4** Effect of EPS obtained from *Enterococus faecium* D-36 strain isolated from milk regulation of MUC5AC gene expression level on Caco-2 cell line as determined by Real Time PCR. Concentration-response curves of MUC5AC gene expression level of EPS for Real Time PCR performed after 24 h treatment. Results are expressed as mean SD. *indicates a significant difference compared to the control group by the Tukey test ($p < 0.05$)

## 4. DISCUSSION and CONCLUSION

EPSs are used in the food industry as stabilisers and anticoagulants. Also, medicinal biochemistry has shown that they have tumorigenesis inhibition properties, support the immune system, macrophage and lymphocyte activation, and lower cholesterol levels [19, 28, 29]. Enterococcus species are Gram-positive bacteria [30]. These bacteria have also been utilised as probiotics to improve the intestine's microbial balance and treat gastroenteritis in humans and animals [30, 31]. Name et al. (2014) showed that secreted metabolites of Enterococcus faecalis strain showed high anticancer activity against the cancer cell lines HeLa, AGS, MCF-7 and HT-29.

In this study, EPS isolated from *Enterococcus faecium* D36 strain isolated from milk which affected cell proliferation of Caco-2 cell line, a human colon cancer cell line and investigated expression levels of the MUC5AC gene that expresses mucin glycoprotein in Caco-2 cell line.

Two different approaches were used to study the effect of EPS's isolated from *Enterococcus faecium* D36 strain isolated from milk on cell viability of Caco-2 cell line. First, 5000 cells were used and exposed to EPS for 24 and 48 h. In the second approach, cells were exposed to EPS for 24 h using a density of 20.000-30.000 cells. Strategy 1: According to the results obtained, a decrease in cell viability was observed after 24 and 48 hours. Cell viability was particularly evident in the results of the 48-h experiment. While there was a decrease depending on the concentration, a 60% decrease was observed at the highest concentration.

Strategy 2: It was found that the changing concentration did not cause a decrease in cell viability, but an increase in cell viability was observed in cells exposed to EPSs for 24 hours.

In colon cancer, changes occur in mucin glycoprotein expression and glycosylation, similarly to many other proteins. This change in mucin glycosylation enables cells to develop invasive properties, which means cells move toward blood vessels and become metastatic [14, 32, 33]. As mucin gene expression levels increase, mucin protein increases and continue with faulty glycosylation [32]. An effective concentration of EPS obtained from *Enterococcus faecium* D36 isolated from Milk was determined by MTT analysis. Following this, the MUC5AC gene responsible for mucin expression in Caco-2 cell line was detected using Real-Time PCR.

This experiment has shown that MUC5AC gene expression was decreased compared to the control group in a concentration-dependent manner. EPS from *Enterococcus faecium* D36 reduced MUC5AC gene expression levels in a concentration-dependent manner. This observation suggests that this substance has the effect of decreasing mucin expression in colon cancer cells and that it can reduce invasion ability and metastatic ability.

Probiotics protect the gut against the formation of precancerous lesions by suppressing the activity of carcinogen enzymes such as azoreductase [34, 35]. It has been observed that EPS obtained from *Enterococcus faecium* D36 was not effective in high cell numbers but low cell numbers. This reminds us of the importance of early diagnosis since colon cancer can form a tumoral mass from a single cell [36, 37]. Based on these results, it is believed that EPS obtained from *Enterococcus faecium* D36 can be used as a protective and therapeutic substance during the early stages of cancer, especially colon cancer.

Cancer patients have a high demand for a diet containing natural products. Although probiotic foods are among these natural products, the microorganism content is a disadvantage. Since chemotherapy drugs and radiotherapies are taken during cancer treatment weaken the immune system, foods containing probiotic microorganisms cause a danger to cancer patients. It has been suggested that if the EPS (D36) obtained can be added to foods as a pure additive (eg EPS containing yogurt) and made into food, it can be used as a food supplement in patients receiving chemotherapy and radiotherapy who consume these foods, and can play a protective role in healthy individuals.

Finally, our findings showed that EPS (D36) might have protective and possible therapeutic effects on early-stage colon cancer and on reducing the invasion ability of cancer by decreasing mucin expression. However, further *in vivo* studies are required to support these findings

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## CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

## AUTHORSHIP CONTRIBUTIONS

**Burcugül Altuğ-Tasa:** Literature search, Writing – Original draft preparation, Analysis, Investigation, Visualization, Conceptualization. **Merih Kıvanç:** Supervision, Review and editing, **Ayşe Tansu Koparal;** Supervision**,** Review and editing.

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