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Characterization of *Limosilactobacillus fermentum* for anti-cancer activity using HCT 116 from Egyptian dairy products

Reham A. Madian[1], Sarah A. Aggag[1*], Mohamed A. Yacout[1], Sameh A. Awad[2] and Amel A. Ibrahim[2]

Abstract

Background Colon cancer (CRC) is one of the most significant health problems worldwide. Using Exopolysaccharides (EPSs)-produced probiotics as alternative colon cancer therapies depends on an anti-tumor effect and influences the immune system. This study isolated different probiotic EPS lactic acid bacteria (LAB) strain producers from traditional Egyptian fermented dairy products to evaluate their antiproliferative and anti-tumor effects on the HCT-116 colon cancer cell line.

Results EPS LAB were studied for their probiotic and antioxidant activity. The cytotoxicity effects on HCT-116 cells were analyzed. Two isolates *Limosilactobacillus fermentum* RE 245 (Accession No. PQ215810), and *Limosilactobacillus fermentum* RE 280 (Accession No. PQ215848) showed resistance against gastrointestinal conditions: low pH (> 40%), bile salt-resistant (57.36% and 76.21%, respectively), more than 90% when exposed to simulated gastric juice conditions. Isolates RE245 and RE 280 had the strongest inhibitory effect on HCT-116 cells reaching 86% and 70%, respectively, with an increase in the ratio of apoptosis induction. The induction of apoptosis was achieved via the up-regulation of IL-2 and the downregulation of BCL-2, PARK, TARC, LIF, IL-4, IL-6, CD1A, and CD1B genes in HCT-116 cells.

Conclusion From the EPS LAB isolates' results, they might be an excellent candidate for functional food production and as a potential alternative treatment to treat colon cancer.

Keywords Colon cancer, Exopolysaccharides, Lactic acid bacteria, Probiotic properties, Cytotoxicity, Apoptosis

Background

Colorectal cancer (CRC) is one of the significant health problems that lead to high mortality rates worldwide. Colorectal cancer incidence was ranked as the second leading cause of mortality prevalence in males and the third in females (Fortin et al. 2018). The current therapies for CRC include surgery, immunotherapy, chemotherapy, and radiotherapy. Most of the anti-cancer drugs used cause immunotoxicity and slow down the healing process, so they not only destroy cancer cell growth but also inhibit the growth of normal cells. As researchers study alternative anticancer treatments and prevention

*Correspondence:
Sarah A. Aggag
sarah.aggag@alexu.edu.eg
[1]Department of Genetics, Faculty of Agriculture, Alexandria University, Alexandria, Egypt
[2]Dairy Microorganisms and Cheese Research Laboratory (DMCR), Department of Dairy Science and Technology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt

approaches, evidence grows that alternative drug derived from natural products, such as microorganisms and plants, have generated extensive awareness and attention worldwide due to their efficacy, safety, and fewer side effects compared to chemotherapy (Ahmad et al. 2017).

According to the Food and Agricultural Organization (FAO) and the World Health Organization (WHO), probiotics are well-known for promoting gut health. Probiotics are defined as live microorganisms that, once consumed in adequate amounts (in food or as a dietary supplement), confer a health benefit on the host. The most common types of probiotic bacteria are *Lactococcus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus* species, and *Lactobacillus* (Isolauri et al. 2001). Scientists suggested that Lactobacilli have protective effects against cancers through apoptosis induction (Riaz Rajoka et al. 2017) by binding genotoxic carcinogens and stimulating the production of immune factors (Tuo et al. 2011). Therefore, in recent years, *Lactobacillus* has exerted potential anticancer activity and appears as a promising alternative to either replace or reduce the use of chemotherapy.

Recent studies have shown that exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) probiotics are supplemented in the treatment of human disorders due to their various biological activities and multiple health benefits, such as inflammatory bowel diseases, autoimmune diseases, colon cancer, gastric ulcers, cardiovascular diseases, and obesity (Yan et al. 2019). They are also used for their anti-tumor effects (Khalil et al. 2018), and anti-oxidative effects on the lungs, gut, and liver (Vasconcelos et al. 2019). EPS natural compounds are primary or secondary metabolites produced by microorganisms as prebiotics, usually synthesized during fermentation and then released in the fermentation medium (El-Deeb et al. 2018), which change the gastrointestinal microbiota and provide health benefits (Caggianiello et al. 2016). Many studies have reported that the structure and composition of EPSs tend to be strain-dependent (Li et al. 2014). EPS generally exists in two forms: a cell-bound exopolysaccharide (c-EPS) that firmly binds to the bacterial surface and a released EPS from LAB, which might, therefore, be a promising safe substitute for synthetic agents. Among microbial polysaccharides, exopolysaccharides produced by probiotic *Lactobacillus* exerted anticancer activity through different mechanisms, such as prevention of tumorigenesis formation, suppression of the growth of cancer cells by apoptosis induction, and improvement of immunity (El Ghany et al. 2015).

One of the best ways to discover new probiotic isolates is to study traditional fermented foods. Traditional fermented dairy products, made from raw milk without industrial methods, may have different and unique probiotic isolates than industrial products (Gupta et al. 2021). Studying these traditional dairy products could lead to discovering of novel probiotic isolates with various properties (Adikari et al. 2021). New probiotic isolates surviving food processing and GIT conditions are crucial for human health, as is increasing the diversity of probiotics in foods and supplements, which is important for maintaining a healthy gut microbiome (Faintuch and Faintuch 2019).

The objective of this study was isolation, screening probiotic exopolysaccharides LAB isolates from traditional dairy products and evaluate their possible anticancer activities, especially the apoptosis-inducing capacity of the newly selected isolates on different colon cancer cell lines. The occurrence of apoptosis induction was analyzed using the MTT assay, microscopic fluorescent analysis using Hoechst 33,342 staining, and flow cytometry. Changes in apoptotic markers and immune gene expression were revealed through RT-qPCR analysis.

Materials and methods

Samples collection

Twenty traditional Egyptian fermented dairy product samples (Laban Rayeb, Karish cheese, and yogurt) were collected from September until December 2021 aseptically from different locations of Alexandria, El-Behera, and Kafr El-Sheikh governments. The samples were collected, transported to the laboratory in the ice box, and stored at 4 °C until isolation.

Isolation of lactic acid bacteria

Three grams of each sample were cultured aseptically into 30 mL of sterilized reconstituted skimmed milk and incubated until coagulation at 30° C,37° C, and 42° C. Coagulated samples were then streaked on M17 (Biolife Italy) (for lactococci) and *Streptococcus thermophilus* (ST) agar media (for *Streptococcus thermophilus*). These samples streaked on de Man, Rogosa, and Sharpe (MRS) agar (for lactobacilli) were incubated under anaerobic conditions using the gas pack system (GENER box anae indicator Biomérieux) at 30° C, 37° C and 42° C for 48 h. The cultures were streaked on suitable media for their purification. The purified isolates were stored at -20° C in sterile reconstituted skimmed milk (12.5% W/V) supplemented with 15% glycerol. Their phenotypic evaluation was studied according to Kandler and Weiss 1986.

Exopolysaccharides production of LAB isolates

The screening of EPS production was limited to the isolates showing their mucoid or ropy appearance. For slime production, isolates were streaked on MRS media and incubated at the optimum temperature for 24 h. Formed colonies ropiness of colonies on agar surfaces was tested

using a metal loop to observe the formation of slimy filaments (Knoshaug et al. 2000).

Overnight cultures were streaked on the surface of plates containing ruthenium red milk (10% w/v, skim milk powder, 1% w/v, sucrose, and 0.08 g/L ruthenium red, 1.5% w/v agar), Ruthenium red stain tints the bacterial cell wall, so white colonies as positive EPS while red colonies as negative EPS was carried out according to the method described by Dabour et al. (2006).

The positive isolates were also tested for capsule production. The bacterial culture was mixed with India ink, spread on a clean slide, covered with a glass cover, and examined with phase contrast microscopy (ZEISS Microscope, West Germany).

Determination of the antioxidative activity of LAB isolates

LAB bacterial cells were harvested by centrifugation in 2010 xg for 15 min of the overnight grown culture at 37 °C. During preparation, the cell pellet was washed with PBS three times and resuspended in PBS. The cell numbers were adjusted to 10^9 CFU/mL. The cell-free supernatant (CFS) was sterilized with a 0.22 µm nylon membrane filter (Lin and Yen 1999).

The antioxidant activity of CFS from the selected LAB isolates was evaluated using DPPH radical scavenging activity and Ferric ion reducing power according to the method as described by Lin and Yen (1999) respectively. Ascorbic acid (45 µg/mL) was also tested at the same time as the control of standard antioxidants. The absorbance of the solution was measured at 517 and 700 nm in DPPH and Ferric ion-reducing power methods respectively by using a spectrophotometer (Pg T80+, England). The results were expressed as the following Eq. 1:

$$\text{Radical scavenging activity}\% = \frac{(\text{A control} - \text{A sample})}{\text{Acontrol}} \times 100 \quad (1)$$

Tolerance to simulated human GI tract of LAB isolates

Resistance to low pH, Pepsin, pancreatin, and tolerance to bile salts

The selected isolates were tested for tolerance to low pH was determined according to the method of Argyri et al. 2013. Briefly, overnight cultured bacteria were centrifuged at 5000 xg at 4 °C for 10 min, washed twice with PBS buffer (pH 7.2), and resuspended in PBS to approximately 1×10^8 CFU/mL. 0.1 mL of LAB suspension was transferred into 10 mL of different adjusted pH including 2.0, and 4.0. After 3 h incubation, Resistance to low pH was measured by counting viable colonies on MRS agar plates. For resistance of the lactobacilli to pepsin and pancreatin, the simulated gastric juice was freshly prepared consisting of pepsin (1:10000, sigma, USA) suspended in sterile PBS (pH 3) to a final concentration of 3 g/L. overnight medium containing cultured bacteria was centrifuged at 5000 xg at 4°c for 10 min, cells were washed twice with PBS buffer (pH 7.2), and then adjusted the cell number to 10^9 CFU/mL into simulated gastric juice (pH 3). The solution was incubated at 37 °C for 0 and 3 h. After incubation, 1 mL of the bacterial culture was transferred to 9 mL of simulated intestinal juice (pH 8) and incubated at 37 °C for 0 and 4 h. The viable cell populations were made using the pour plate method with MRS. The plates were incubated at 37 °C for 48 h (Li et al. 2018). All experiments were performed in triplicates and the survival rate (SR) was calculated according to the following Eq. 2:

$$\text{Survival rate}\% = \log \text{CFU } N_1 / \log \text{CFU } N_0 \times 100. \quad (2)$$

N_0: the total viable count of LAB strains before treatment(control), N_1: the total viable count of LAB strains after treatment.

Finally, to evaluate the effect of bile salts on the growth rate according to Guo et al. (2012), overnight cultured bacteria were inoculated into MRS broth with and without 0.3% bile salts (Sigma, USA), respectively. The mixtures were incubated at 37 °C, and absorbance was measured every hour at 600 nm by using a spectrophotometer (Pg T80+, England). The absorbance of growth was followed for 24 h or until a monitored difference of 0.3% of the bile salt unit was reached.

Determination of safety properties of LAB isolates

Hemolytic activity

LAB isolates were determined by streaking onto blood agar plates (Oxoid) containing 7% (v/v) sheep blood and incubated at 37 °C for 48 h. After incubation, the plates were detected by 3 categories: α -hemolysis, β -hemolysis, and γ-hemolysis (Abedi et al. 2018).

Antibiotic susceptibility

0.1 mL of each LAB isolate was cultured on MRS agar medium, and ten types of antibiotic discs (Penicillin-G (10 units); Streptomycin(10mcg); Ampicillin(10mcg); Vancomycin(30mcg); Tetracycline(30mcg); Erythromycin(15mcg); Kanamycin(30mcg); Gentamycin(10mcg); Chloramphenicol(30mcg); and Clindamycin(2mcg), HIMedia®, USA) were manually placed on plates and incubated at 37 °C for 24 h. The antibiotic resistance of the LAB isolates was determined by the disk diffusion method, Data were analyzed and classified according to disk producer guidelines (Haghshenas et al. 2014).

Identification of LAB isolates

Selected isolates were identified by using MALDI-TOF MS (Schwenninger et al. 2016) and 16 S rRNA. 16 S rRNA Gene sequence amplification and sequencing were carried out by ABI 3730xl DNA sequencer using the BLAST (N) program at the NCBI database (http.www.ncbi.nlm.nih.gov) for identification of bacterial isolates at species levels.

Cell culture

HCT 116 (ATCC® CCL-247) cell line was obtained from the colon of an adult male with colon cancer. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/L) and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin 100 µg/mL (Cegrogen Biotech, Germany) incubated under standard conditions in a humidified 37 °C incubator with 5% CO_2.

Assessment cell viability

MTT assay 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide was used with lactobacilli EPSs on the proliferation of HCT 116 cells. Cell-free supernatant (CFS) of EPSs was dissolved in antibiotic-free high glucose DMEM and filtered through a 0.22 µm nylon membrane syringe filter before analysis. HCT 116 cell lines ($\sim 5 \times 10^3$ /well) were seeded in triplicate in a 96-well plate incubated. After 24 h cells were treated with different concentrations of selected EPS isolates 500 µL/well with (25×10^3 CFU/mL) of a Lactobacillus Cell-Free Supernatant (LCFS), 5-fluorouracil (5-FU) used as a positive control, and untreated HCT-116 cells used as a negative control; incubated for 48 h, and 72 h at 37˚C in 5% CO_2. 200 µL of dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) was added to each well to solubilize formazan crystals. Finally, cell viability was observed at 490 nm and was measured using an ELISA microplate reader (Benchmark Microplate Reader, Bio-Rad Laboratories Inc., USA) (Parisa et al. 2020). The following formula is used to calculate the percentage of cell viability inside each well Eq. 3:

$$\text{Cell Viability\%} = (\text{Absorbance Sample}/\text{Absorbance Control}) \times 100. \quad (3)$$

Annexin-V/fluorescein isothiocyanate (FITC) and Annexin-V/propidium iodide (PI) staining assay

HCT cells ($\sim 2 \times 10^5$/ well) were incubated for 72 h at 37˚C in 5% CO_2 followed by staining with FITC-Annexin V Apoptosis Detection Kit (BD Bioscience, Heidelberg, Germany) according to the manufacturer´s protocol (Eray et al. 2001). Cells were analyzed with FITC-conjugated Annexin V and PI by flow cytometry using FACS Calibur (BD Bioscience, Heidelberg, Germany) and the Cell Quest Pro software (BD Bioscience).

Fluorescence microscopy and images

The fluorescent dye Hoechst 33,342 (Life Technologies, USA) was used to carry out the visual symptoms of apoptosis followed by the method described by Paolillo et al. (2009). The slides were examined using a confocal microscope, the Leica DMi8 (Leica, Wetzlar, Germany). The images were captured and analyzed using ImageJ software. The Hoechst 33,342 dye was excited at 350 nm and its dual-wavelength fluorescence was analyzed (blue, 460 nm).

Quantitative RT-PCR assay

Total cell RNA extraction was performed using an RNA extraction kit (GENEzol™ TriRNA Pure Kit, Taiwan) according to the manufacturer's protocol (cDNA) was synthesized from 3 µL of total RNA with 0.3 µL reverse transcriptase (TOPscript™ Reverse Transcriptase 200 unit/µl, Enzynomics, Korea, qRT-PCR) was carried out using TOPreal ™ qPCR 2X PreMIX (SYBR Green with low ROX) (Enzynomics, Korea) in an ABI step-1 plus instrument (Applied Biosystem, USA). Thermal cycler conditions were obtained as follows: initial incubation at 95˚C for 5–15 min, then 30–45 cycles alternating in turn with 95˚C for 15–30 s, 60˚C for 30–60 s, and 72˚C for 10–60 s. The primers for apoptosis-associated genes are listed in Table 1. All reactions were normalized relative to GAPDH and performed in triplicate. The expression level was evaluated using the $2^{-\Delta\Delta Ct}$ equation as a fold of relative intensity (Pfaffl 2001).

Statistical analysis

All experiments were repeated at least three times. Statistical analysis of data was carried out using Statistical Package for Social Studies software (SAS) software, version 9.2. (2009) Statistical Analysis System Institute. North Carolina USA v.9.2. Data is expressed as averages ± standard error. The student's t-test was used for statistical analysis by comparing treatment groups versus the control group. Data with a P-value of < 0.05 were considered to have statistically significant differences.

Results

Isolation of lactic acid bacteria

Ninety-one Gram-positive, catalase-negative LAB isolates were obtained from traditional Egyptian fermented dairy products including Laban Rayeb, Karish cheese, and yogurt. The LAB isolates were classified into 59 and 32 bacilli and cocci isolates, respectively.

Table 1 Sequence of primer pairs used in quantitative Real-Time PCR reactions

Gene	Forward primer	Reverse primer	Ref.
IL-2	GGA AAC ACA GGA ACA ACT GGA	TTC AAT TCT GTG ACC TTC TTG G	Ana and Rivera (2010)
IL-4	AGA GCT CGG TGA CCT CAG AC	CCT GCA TGG CGG GTC TTT AG	
IL-6	GAA AAC ACC AGG GTC AGC AT	CAG CCA CTG GTT TTT CTG CT	
TARC	GGC TGA CAA GGT GGT ACA AGA CTT C	CAG ATG GAC TTG CCT TGG ACA G	
LIF	TGA AGT GCA GCC CAT AAT GA	TTC CAG TGC AGA ACC AAC AG	
CD-A	ATG CTG TTC TGT TTC TTC CA	CCA GTG GAG GAT AAT GTC TTG	Aggag et al. (2022)
CD-B	ATG CTGC/ TTT CTG CCA/ GTTTC	TCAT/CA/GGATA/ GAT/ CCTGATATGA	
BCL2	GGT GGG GTC ATG TGT GTG G	CGG TTC AGG TAC TCA GTC ATC C	Riaz Rajoka et al. (2017)
PARK	ACC CAC CTA CAA CAG CTT TTT C	CAG CAA GAT GGG CCC TGG	Pallotta et al. (2017)
GAPDH	CAT TGC CCT CAA TGA CCA CT	TCC TTG GAG GCC ATG TAG AC	-

Exopolysaccharides production of LAB isolates

The ability of LAB isolates screened for EPS production is illustrated in Table 2. The results revealed that among these 91 isolates only *Limosilactobacillus fermentum* RE 245, *Limosilactobacillus fermentum* RE 280, and *Lactobacillus fermentum* RE 281 gave mucoid (or ropy) phenotypes, white colonies, and were recorded as capable of producing EPs (Table 2; Fig. 1).

Determination of the antioxidative activity of LAB isolates

DPPH free radical scavenging assay

The radical scavenging ability of the LAB isolates was compared with the standard antioxidants' ascorbic acid. The scavenging effects of DPPH radicals on three LAB isolates RE245, RE280, and RE281 were selected for their exopolysaccharide production, as their DPPH radical % was 62.43, 19.66 and 28.84%, respectively. As can be seen from the results, both DPPH radicals can be scavenged by these tested isolates, which shows the significant free radical degradation activity of these isolates.

Ferric ion-reducing activity

As shown in Table 2, the two tested isolates showed varying degrees of reducing activity, RE 245 recorded (30.08%) the highest ferric ion-reducing activity values, which was significantly different ($P<0.05$) than that of RE 281 (1.23%) and RE 280, had the lowest reducing activity (0.54%). These obtained results by Ferric ion-reducing activity confirmed the scavenging effects of DPPH radicals. This means that the antioxidant effect of LAB isolates was the same as examined with different methods.

Tolerance to simulated human GI tract of LAB isolates

Resistance to low pH, Pepsin, pancreatin, and tolerance to bile salts

The results obtained in Table 3 showed the acid, pepsin, pancreatin, and bile tolerance rates of RE 245, and RE 280, expressed a good survival rate for 1 h at pH 2 of 62.3%, and 41.31%, respectively, while RE 281 was observed as moderate survival rate 36.09%. Also, all of them showed very good resistance to pH 4 as presented in Table 3. Therefore, the result indicates a significant difference ($P<0.05$) in the survival rate of two isolates (RE 245, and RE 280) that exhibited the best scores in pepsin enzyme after incubation for 3 h 98.87% and 96.23%, respectively. Their survival rate in pancreatin juice after 4 h of incubation was about 100%, and the lowest survival rate was observed as a progressive reduction in survival rate to 0% for RE 281 isolates. Concerning tolerance to bile salts, viability rates were 57.36, 76.21, and 27.77% for RE245, RE280, and RE281 isolates. The outcome showed that these two isolates RE 245, and RE 280 displayed probiotic potential.

Determination of safety properties of LAB isolates

Hemolytic activity

The hemolysis test done for safety reasons for selected probiotic exopolysaccharides Lactobacilli, showed gamma hemolysis activity as represented in Table 4.

Table 2 Antioxidant activity and exopolysaccharide production of selected LAB isolates

Isolates Code	Antioxidant activity %		Exopolysaccharide's production	Identification of LAB isolates by MALDI-TOF MS	Identification by 16 S rRNA	Accession Number
	DPPH %	FRAP %				
RE245	$62.43^{a} \pm 2.48$*	$30.08^{a} \pm 1.03$	+	*Lactobacillus fermentum*	*Limosilactobacillus fermentum*	PQ215810
RE280	$19.66^{b} \pm 0.72$	$0.54^{b} \pm 0.23$	+	*Limosilactobacillus fermentum*	*Limosilactobacillus fermentum*	PQ215848
RE281	$28.84^{b} \pm 1.63$	$1.23^{b} \pm 0.41$	+	*Lactobacillus fermentum*	-	-

*Values are expressed as mean ± SD; means in the same column followed by different letters are statistically significantly different ($p<0.05$) (–) no EPS production; (+) EPS production

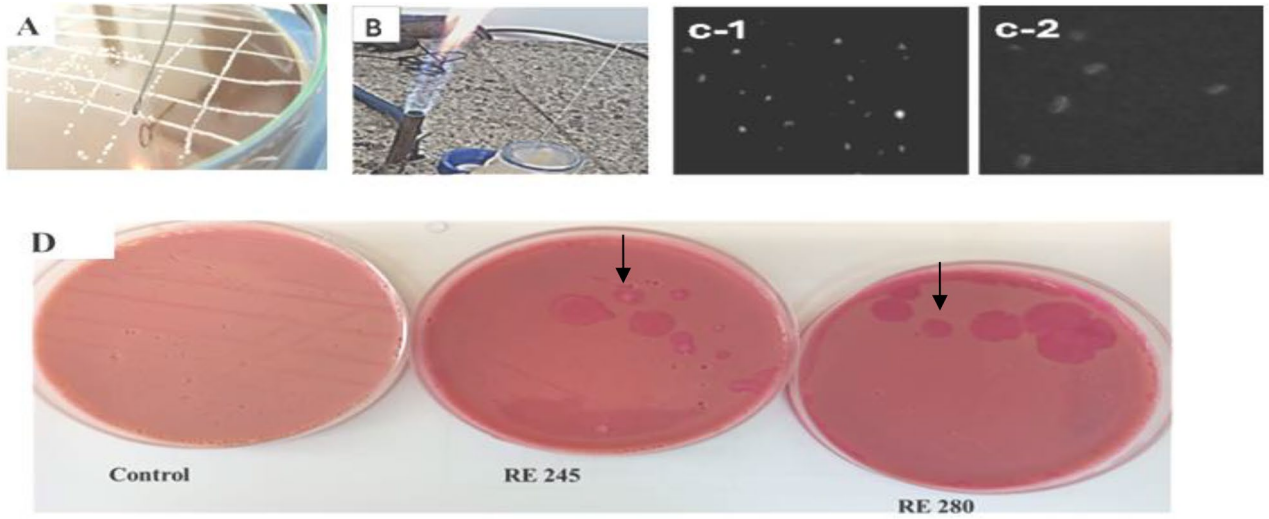

Fig. 1 (**A**) Mucoid colonies phenotype; (**B**) visually examined for coagulation and ropy EPS expression. (**C**) Phase contrast microscopy of India ink-stained cells showing the presence of the capsule-like EPS, (C1) *Limosilactobacillus fermentum* RE 245 (Accession No.215810), and (C2) *Limosilactobacillus fermentum* RE 280(Accession No.215848); (**D**) Ruthenium red stain tints the bacterial cell wall, so white colonies (+) EPS while red colonies (-) EPS

Table 3 Surviving percentage of LAB isolates in 0.3% bile salt, low pH 2 and 4, and in the artificial gastric juices

Isolates	Survival rate %				
	Bile Salt Tolerance (0.3% Ox gall)	Low pH		Gastric Acid Tolerance	
	24 h	pH 2	pH 4	Pepsin	Trypsin
RE245	$57.36^{b} \pm 0.12$*	$60.93^{a} \pm 0.08$	$93.76^{b} \pm 0.16$	$98.87^{a} \pm 0.02$	$100^{a} \pm 0$
RE280	$76.21^{a} \pm 0.19$	$41.31^{b} \pm 0.28$	$98.12^{a} \pm 0.11$	$96.23^{b} \pm 0.002$	$99.99^{a} \pm 0.01$
RE281	$27.77^{c} \pm 0.07$	$36.09^{c} \pm 0.06$	$87.32^{c} \pm 0.06$	$0^{c} \pm 0$	$0^{b} \pm 0$

*Viable counts are presented as the mean ± SD, and the values with different lowercase letters are significantly different ($P < 0.05$)

Table 4 Susceptibility of LAB isolates against some antibiotics and hemolysis activities of different isolates

Isolates	Antibiotic susceptibility (inhibition zone in cm)										Blood hemolysis
	P	S	Amp	Va	Te	E	K	Gm	C	CC	
RE 245	R (0)	S (2.4)	S (4)	I (1.6)	S (3.2)	S (4.6)	I (1.6)	I (1.9)	S (4)	S (4)	γ
RE 280	R (0)	R (1.1)	S (4.4)	R (0)	S (3.4)	S (4)	R (1)	S (2)	S (4)	S (4.3)	γ

Antibiotic Susceptibility expressed as R (resistant), I (intermediate susceptibility), or S (susceptible). P, penicillin-G (10 units); S, Streptomycin(10mcg); Amp, Ampicillin(10mcg); Va, vancomycin(30mcg); Te, tetracycline(30mcg); E, erythromycin(15mcg); K, Kanamycin(30mcg); Gm, gentamycin(10mcg); C, chloramphenicol(30mcg); and CC, clindamycin(2mcg)

Antibiotic susceptibility

The antibiotic susceptibility profile of *L. fermentum* RE 245, as detailed in Table 4, revealed resistance to penicillin, intermediate susceptibility to vancomycin, kanamycin, and gentamicin, and susceptibility to the remaining tested antibiotics. The impact of antibiotics on *Li. fermentum* RE 280 was sensitive to all of them except penicillin, streptomycin, vancomycin, and kanamycin was resistant to them.

Identification of LAB isolates

Two lactic acid bacteria isolates were selected from ninety-one isolates based on their exopolysaccharide's functional properties. The two isolates were identified by MALDI-TOF MS (Matrix Supported Laser Desorption/Ionization) to be *Limosilactobacillus fermentum*, and *Limosilactobacillus fermentum*, respectively as shown in Table 2. The identification of RE 245 and RE 280 were confirmed by the 16 S rRNA gene. It was found that RE 245 (PQ215810) showed 99.88% similarity with *Limosilactobacillus fermentum MJM60807* (OQ073800.1), and RE 280 (PQ215848) shared 100% similarity with *Limosilactobacillus fermentum AD62* (OM807266.1).

MTT antiproliferative effect of RE 245 and RE 280 on HCT-116 cancerous cells

The cultures of HCT-116 cells were treated with bacteria after cell growth and differentiation. The MTT assay was performed at 48 and 72 h of simultaneous high doses of the bacteria. The highest cytotoxic effect of bacterial

metabolites on the HCT 116 cell line was observed at 86% in *Li. fermentum* RE 245 and 70% in *Li. fermentum* RE 280 at 72 h. As depicted in Fig. 2, the cell viability of the treated cancer cells showed a significant difference ($P < 0.05$) between 5-FU and bacteria. Both isolates exhibited a decrease in HCT-116 growth.

Apoptosis assessment using Annexin V assay and Hoechst 33,342 staining assay

To determine the induction of apoptosis in the HCT-116 colon cancer cell line treated with *Li. fermentum* RE 245 and *Li. fermentum* RE 280, flow cytometry analysis was performed. According to the bacterial treatment results, the highest percentage of induced apoptosis belonged to *Li. fermentum* RE 280, displaying 81.19% total apoptosis (5.24% early and 75.95% late apoptosis), while *Li. fermentum* RE 245 induced 68.58% total apoptosis (3.58% early and 65% late apoptosis). Moreover, the apoptosis induction by 5-FU was 31.54 (12.38% early and 19.07% late apoptosis) (Fig. 3).

Compared with the HCT-116 cells, the nuclear and chromatin morphology of cell changes visualized by Hoechst 33,342 staining, with white staining showing numerous fragmented micronuclei, yellow staining showing apoptotic body formation, red staining showing apoptotic cells with membrane blebbing, and green staining showing secondary necrotic cells (Fig. 3).

Quantitative Real-time PCR

The effect of EPS CFCs *Li. fermentum* RE 245 and *Li. fermentum* RE 280 as anticancer effects on the HCT-116 colon cancer cell line was measured using the expression of genes related to immunomodulatory cytokines, namely IL-2, IL-4, IL-6, CD1A, and CD1B. The results in Fig. 4 demonstrated that there was no significant difference in the amounts of increased expression of the IL-2 gene in RE 245, RE 280, and 5-FU. The results showed that IL-4, IL-6, CD1A, and CD1B genes were downregulated in all treated cells. The IL-4 gene expression data revealed a substantial increase in expression for RE 245 ($P < 0.00006$) and RE 280 ($P < 0.0004$) compared to 5-FU. Colon cancer cells HCT-116 treated with the two bacterial isolates (RE 245, RE 280) and the 5-FU drug showed downregulation in the gene expression of BCL-2, PARK, TARC, and LIF (Fig. 5). Cells treated with RE 245 showed a significant difference in the expression ratios of BCL-2 and TARC ($p < 0.03$ and $p < 0.02$, respectively). PARK gene expression levels in RE 245 increased significantly compared to RE 280 ($p < 0.01$ and $p < 0.04$, respectively). However, the gene expression of LIF in RE 245 and RE 280 showed an extremely substantial decrease ($p < 0.008$ and $p < 0.006$).

Discussion

In recent years, clinical studies have increased interest in LAB as a cancer preventive and for treating bacteria that exert their anti-cancer properties through different factors and proposed mechanisms, including cell surface components and metabolites, to impact human health (Lebeer et al. 2010). This offers safer and fewer side effects as an alternative therapy compared to the current anticancer therapies, such as chemotherapy and radiotherapy, given to cancer patients who are limited by the toxicities associated with the latter therapies. In the current study, significant effort has been made to isolate lactic acid bacteria from traditional dairy products as a good source to find probiotic lactic acid bacteria, as demonstrated by Hassanzadazar et al. (2012). Our screening protocols were focused on potential probiotic characterization, antioxidant production, and exopolysaccharide production. This study aimed to select and identify new promising isolates that display the most promising probiotic and exopolysaccharide characteristics, as well as determine their anti-proliferative, immunomodulation, and anticancer bioactivities against HCT-116 colon cancer cells to use as a functional supplement and as an adjunctive treatment for cancer.

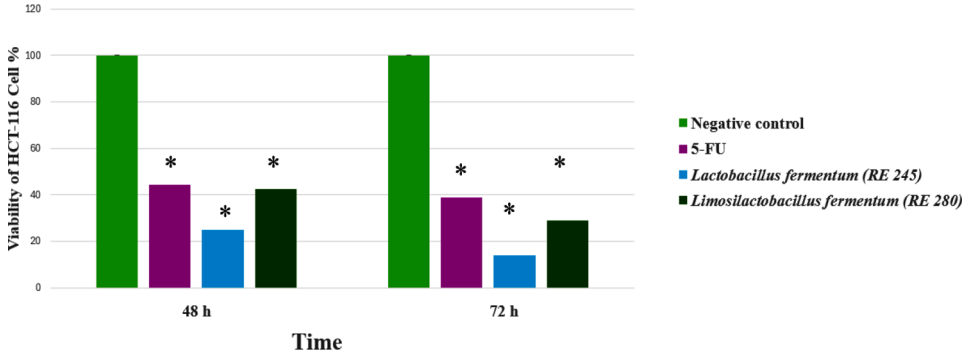

Fig. 2 The time- and dose-dependent anti-proliferative effect of *Limosilactobacillus fermentum* RE 245 (Accession No.215810), *Limosilactobacillus fermentum* RE 280 (Accession No.215848), and 5-FU on HCT-116 cells was evaluated by MTT assay for 48 and 72 h. control: untreated HCT-116 cells (human colon cancer cells); 5-FU: used as a positive control. The p-values determined using Student's t-test were $*p < 0.05$, statistically significant

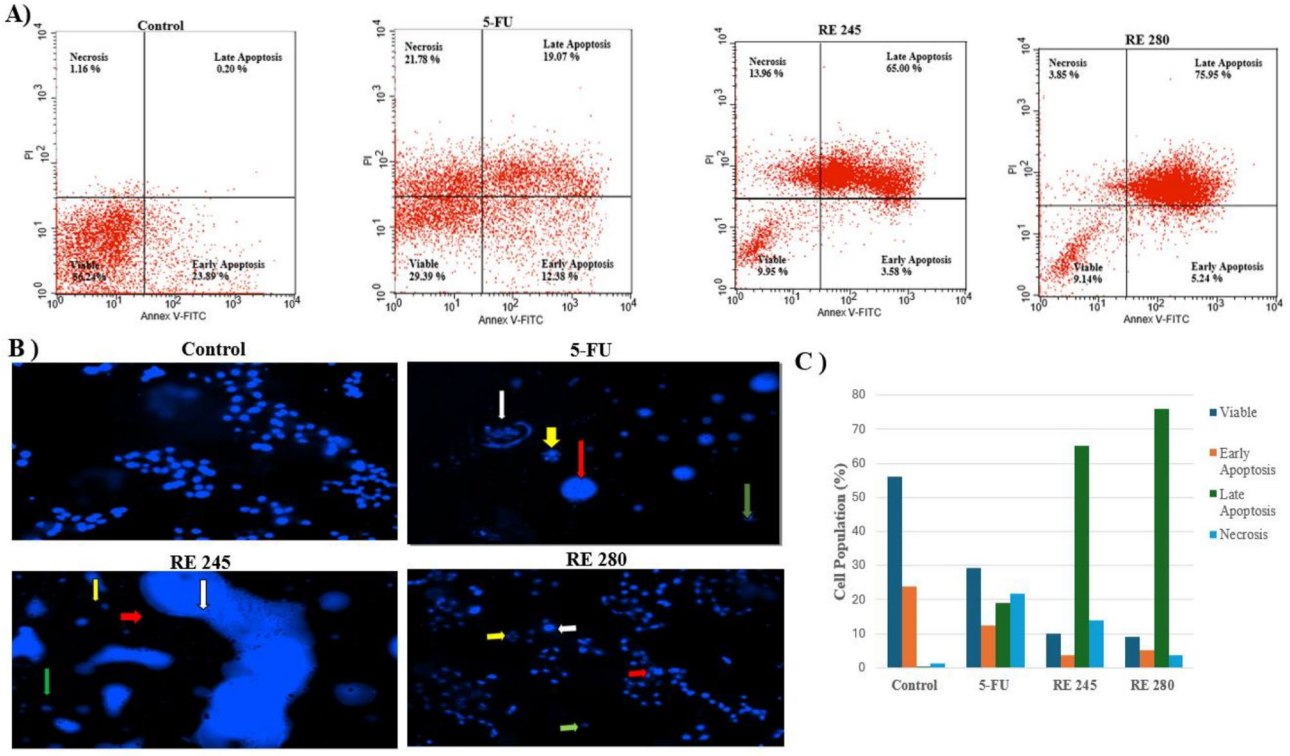

Fig. 3 Determination effect of *Limosilactobacillus fermentum* RE 245 (Accession No.215810), *Limosilactobacillus fermentum* RE 280 (Accession No.215848), and 5-FU on HCT-116 cells. (**A**) represent flow cytometric analysis results of untreated control and treated HCT-116 cells, Lower left column: annexin VI^-/PI^- (viable cells), lower right column: annexin Vi^+/PI^- (early apoptotic cells), upper right column: annexin Vi^+/PI^+ (late apoptotic cells) and upper left column annexin VI^-/PI^+ (necrotic cells). (**B**) Fluorescent microscopy of Hoechst 33,342 staining of untreated HCT-116 cells, treated HCT-116 by 5-FU, RE245, and RE280, respectively. Cell Morphological changes and induction of apoptosis were indicated by arrows with numerous fragmented micronuclei (white), apoptotic body formation (yellow), apoptotic cells with membrane blebbing (red), and secondary necrotic cells (green). (**C**) Bar plots demonstrate quantitative alterations (viable, necrosis, and early/late apoptosis) of treated/untreated HCT-116 cells from flow cytometry analysis

According to Osuntoki and Korie (2010), EPS derived from LAB are promising candidates for immunotherapeutic agents against cancer since they typically have fewer side effects and are less cytotoxic. Also, enhanced cell-mediated immune responses such as natural killer cell tumoricidal activity, T-lymphocyte proliferation, and mononuclear cell phagocytic capacity have been reported (LeBlanc et al. 2002) and may provide physiological benefits that include immunomodulation, antitumor activity, antioxidant activities, and cholesterol-lowering ability (Adebayo-Tayo and Popoola 2017).

In the current experiment, we investigated the production of EPSs by only three *Lactobacillus* isolates: *Li. fermentum* (RE 245), *Li. fermentum* (RE280), and *L. fermentum* (RE 281) out of ninety-one LAB isolates as observed by the presence of ropy white mucus on a medium containing ruthenium red, in addition to the slimy filaments of isolates observed when touching colonies using a metal loop (Fig. 1A). These findings agreed with Van den Berg et al.'s (1993) results, who reported that 30 out of 607 LAB isolates tested could be able to produce exopolysaccharides. Recently, *L. fermentum* isolates with functional characteristics have demonstrated their capacity to generate EPS (Ale et al. 2020). Wang et al. (2019) observed the EPS obtained from *L. fermentum* S1 was a heteropolysaccharide mainly composed of mannose, rhamnose, glucose, and galactose. Also, Wei et al. (2019), characterized an EPS from *L. fermentum* YL-11 as mainly composed of galactose, glucose, mannose, and arabinose. Our data are agreed with previous studies, isolates RE 245, RE 280, and RE 281 exhibited exopolysaccharide producers belonging to *Li. fermentum* and *L. fermentum.*

The antioxidant activities of LAB isolates could be associated with the production of cell surface compounds such as extracellular polysaccharides such as peptidoglycan, lipoteichoic acid, and proteins produced by isolates (Li et al. 2012). As is clear from isolate RE 245, which produces exopolysaccharides and has high antioxidant activity (62.43% for DPPH radical scavenging activity), RE 280 and RE 281 were also able to produce exopolysaccharides, but they had low antioxidant activity compared to the RE 245 isolate (19.66% and 28.84% respectively, for DPPH radical scavenging activity). There are many reports on EPS from LAB with potential antioxidant activity that were found to participate in the removal of

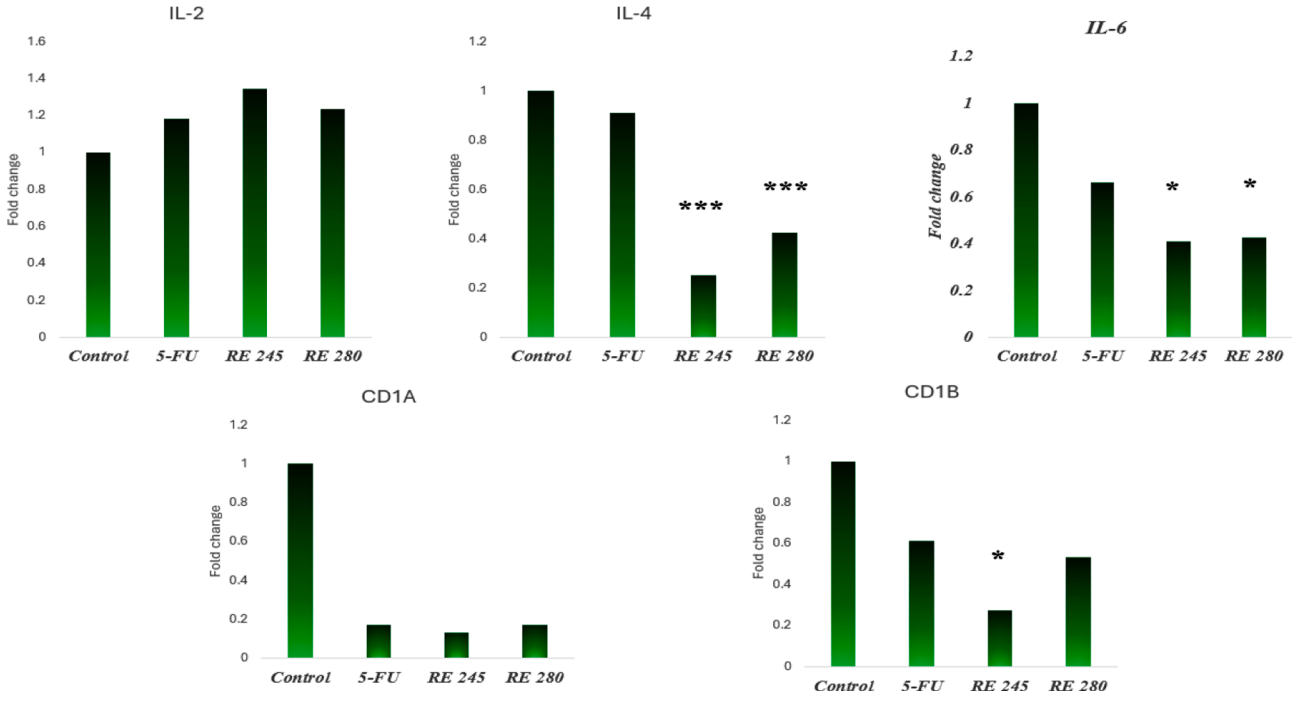

Fig. 4 RT-PCR of IL-2, IL-4, IL-6, CD1A, and CD1B of HCT-116 cells treated by RE 245(Accession No.215810), and RE 280 (Accession No.215848), (relative to 5-FU treated cells) the gene expression was normalized relative to GAPDH and performed in triplicate, For the quantification, the formula RQ = 2-ΔΔct was used. Statistical analysis was performed using T-Test. *$p < 0.05$, **$P < 0.01$, ***$P < 0.001$ statistically significant differences from the positive control. Untreated cells were used as control and HCT-116 cells treated by 5-FU were used as positive control

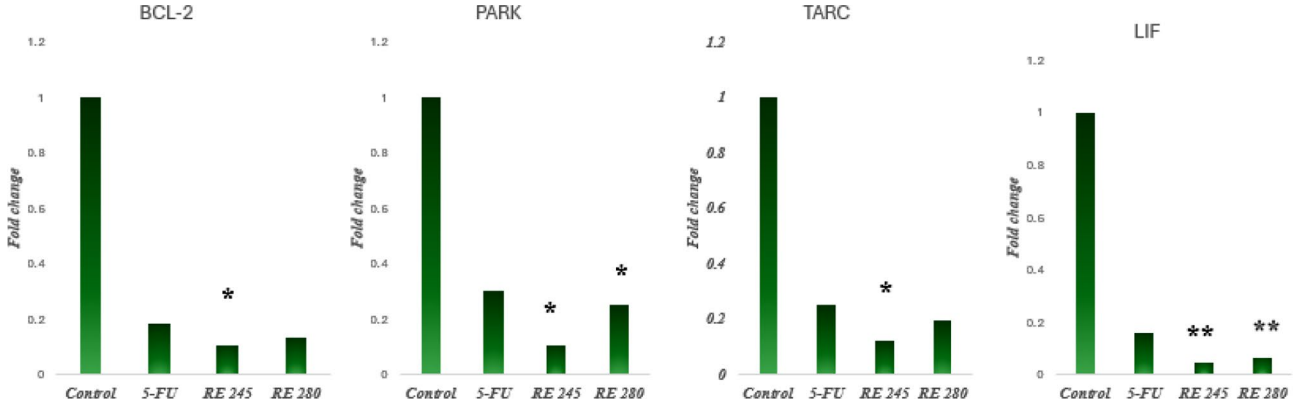

Fig. 5 RT-PCR of cancerous-related genes BCL-2, PARK, TARC, and LIF of HCT-116 cells treated by RE 245 (Accession No.215810), and RE 280 (Accession No.215848), (relative to 5-FU treated cells) the gene expression was normalized relative to GAPDH and performed in triplicate, For the quantification, the formula RQ = 2-ΔΔct was used. Statistical analysis was performed using T-test. *$p < 0.05$, **$P < 0.01$ statistically significant differences from the positive control. Untreated cells were used as control and HCT-116 cells treated by 5-FU were used as positive control

free radicals, degradation of superoxide anion and hydrogen peroxide, reduction of ROS, and metal chelating activity through inherent cellular antioxidant defense by secreting enzymes like superoxide dismutase and assists the production of the major non-enzymatic antioxidant and free radical scavenger glutathione (GSH) (Adesulu-Dahunsi et al. 2018; Ismail and Nampoothiri 2013). Many studies in this regard have shown that Good EPS antioxidant activity is related to the presence of hydroxyl groups and other functional groups, and various factors, such as monosaccharide composition, and molecular weight of the EPS that produce a more stable free radical (Min et al. 2019).

According to the above results, a low pH, gastric juice, and bile-salt tolerance study in vitro was designed to assess LAB use as an efficient probiotic as per the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) 2002, general guidelines as well as safety properties involving antibiotic resistance and hemolytic activity. Therefore, the probiotic bacteria

should exert these important characteristics that are required to confirm its ability to survive while it passes through the gastrointestinal tract to reach the large intestine (Nueno-Palop and Narbad 2011).

It appeared from our results that three identified *Lactobacillus* spp. had antioxidant activity and were exopolysaccharide producers. Only two isolates (RE 245 and RE 280) displayed good survival rates under low pH (> 40%), were highly bile salt-resistant (57.36% and 76.21%, respectively), and exhibited higher viability of more than 90% when exposed to simulated gastric juice conditions. These results are in contrast with RE 281, isolate which showed the lowest survival rate among them at 36.09% in low pH, 27.77% in bile salt survival rate, and can't survive in gastric juice conditions. According to Ben Salah et al. (2012), low pH tolerance is related to H-ATPase activity, and high tolerance capacity is associated with the bilayer membrane structure, which facilitates tolerance of inverse conditions. These results are in harmony with the study carried out by Melchior et al. 2020; who recorded that polysaccharides on the outer cell membrane may be the cause of LAB bile salt resistance.

Oh and Jung (2015) reported that the absence of antibiotic resistance and hemolytic activity are the basic requirements for the selection of a new, safe probiotic isolates. Also, according to the Clinical and Laboratory Standards Institute (2018) (CLSI) guidelines, these isolates were found to be safe for human health. Isolated isolates had a strong sensitivity to numerous tested antibiotics, including streptomycin, ampicillin, clindamycin, erythromycin, gentamycin, kanamycin, tetracycline, and chloramphenicol. Our results agreed with Prabhurajeshwar and Chandrakanth (2017), who found that most LAB isolates were sensitive to ampicillin, streptomycin, and gentamycin. All isolates have no hemolytic activity.

Based on the results of this study, LAB isolates RE 245 and RE 280 were selected for application on HCT-116 colon cancer cells because they significantly produced exopolysaccharides and received the highest scores in the probiotic in vitro tests Tuo et al. (2010) claimed that LAB's anticancer action is achieved by a variety of mechanisms, including apoptosis induction, cancer cell line differentiation, binding of genotoxins and carcinogens, anti-proliferating activity, and immune system interaction. RE 245 had a highly inhibitory effect on the HCT 116 cell line after 72 h among the bacterial and 5-FU treatments. All EPS CFCS from LAB isolates could inhibit the proliferation of cancer cells in a time-dependent manner (Tukenmez et al. 2019). the outcomes matched Wang et al. (2015), who mentioned that the highest anti-proliferative impact of EPS from the *L. plantarum* strain against HT-29 cells was seen after 72 h.

Cell death in HCT-116 was induced by RE 245, RE 280, and 5-FU. RE 245 showed the most potent cytotoxic effect on HCT-116 cells. The apoptosis induction of EPS CFCS from LAB isolates showed an anti-proliferative impact. They induced both early and late apoptosis in HCT-116 cells. As suggested by Haghshenas et al. (2014), the main cytotoxic mechanism for the extracted metabolites of *L. lactis* subsp. *lactis* 44Lac was late apoptosis. Those results developed a new efficacious anti-cancer drug that leads cancer cells to apoptosis (Tukenmez et al. 2019).

Staining-treated cancer cell lines were analyzed through a fluorescent microscope to visualize the apoptosis symptoms in cancerous cells. It demonstrated morphological changes in the cell membrane, chromatin, and cytotoxicity expressions (Haghshenas et al. 2015). The fluorescent microscope results, according to Chuah et al. (2019), obtained shrinking cells with a condensed numerous nuclei or fragmented micronuclei, apoptotic body formation, apoptotic cells with membrane blebbing, and secondary necrotic cells. These variations of apoptotic bodies in terms of number, size, and composition were also reported by Elliott et al. (2007).

EPS CFCS bacterial isolates showed downregulation of cancerous gene expression, including BCL-2, PARK, TARC, and LIF, compared to 5-FU. Oh (2008) demonstrated the same result of decreasing the anti-apoptotic gene expression level of Bcl-2. Ahmed et al. (2018) observed a decrease in the expression level of Bcl-2 due to the effect of exopolysaccharide from Marine Bacillus velezensis MHM3 on MCF-7. Wu et al. (2018) showed a significant up-regulation of LIF expression in CRC patients. The overexpression of LIF is associated with a poor prognosis in CRC (Yu et al. 2014). HCT-116 cells were treated with EPS to down-regulate LIF expression. The TARC marker showed overexpression in patients with colorectal cancer and was related to tumor necrosis factor stimulation. The upregulation of TARC was observed in the colon- cancer cells with interferon (IFN)-g but not with interleukin IL-4 stimulation (Berin et al. 2001). In the present study, both EPS isolates decreased down-regulating TARC expression.

Previous studies have indicated that evaluation of immunomodulatory cytokine expression helps to detect ongoing activation of immune cells at a distance from the tumor. IL-2 expression is rarely detected in tumor tissues from numerous types of cancer, including CRC (Barth et al. 1996). IL-2 gene expression seems to be down-regulated in the tumor cells. Notably, this quantitative determination of cytokines elucidates the effects of the interactions between cytokines and the progression of disease prediction (CSISZÁR et al. 2004).

Our findings matched those of Walia et al. (2003), who found that the IL-6 gene was down-regulated when Caco-2 BBE cell lines were pretreated with TGFb. Rubie et al. (2007) explained that there were influences on

aspects of cancer progression and inflammatory reactions and concluded that IL-6 proinflammatory cytokines are linked to colorectal cancer development. IL-4 is a well-known characteristic of immune reactions, proinflammatory cytokines, and chemoattractant factors in leukocytes (O'Garra et al. 2000). CD1A and CD1B genes are members of the CD1 transmembrane glycoprotein family. They present as lipid antigens to T-cell receptors (Shahine 2018). Hayakawa et al. (2004) suggested that CD1 plays an important role in antitumor defense. and produces rapid anti-tumor cytotoxicity, which can promote tumor rejection. Previous reports have indicated that CD1B is not expressed in healthy livers but is detectable within tumor liver cells (Kenna et al. 2007). Furthermore, CD1 had potential targets for cancer immunotherapy (Nieda et al. 2004). Colon cancer cells treated by bacterial isolates showed downregulation in the CD1A and CD1B genes.

The current in vitro results, demonstrating the potent anti-proliferative and pro-apoptotic effects of RE 245 and RE 280 on the HCT-116 cancer cell line provide the basis for future in vivo studies. However, the in vivo experiments will provide a more physiologically relevant assessment of safety and tolerability that will ultimately inform the clinical potential of these isolates. Future in vitro studies will also investigate the effects of RE 245 and RE 280 on normal cell lines to complement the in vivo findings and further elucidate their safety profiles.

Conclusion

Li. fermentum RE 245 and *Li. fermentum* RE 280 could be potential new safe probiotics that exhibit desirable probiotic properties, including resistance to low pH and high bile salts, adaptation to gastric conditions, and susceptibility to some antibiotics. *L. fermentum* showed probiotic properties with a high antioxidant percentage and an exopolysaccharide producer. *Li. fermentum* shows probiotic properties and exopolysaccharide production. Therefore, the newly isolates identified *Li. fermentum* as exopolysaccharides exerted higher antitumor effects on the HCT-116 cancer cell line than the cytotoxicity displayed by 5-FU. Their immunomodulatory effects via apoptotic mechanisms stimulate the immune response. The effects of these EPS isolates could show promising future applications in colon cancer treatment, as they could be considered functional nutritional supplements. However, in vivo investigations and clinical trials are required to recommend EPS isolates for oral administration.

Abbreviations

CRC	Colon cancer
LAB	Lactic acid bacteria
MRS	deMan, Rogosa, and Sharpe
L. fermentum	Lactobacillus fermentum
EPS	Exopolysaccharides
PBS	Phosphate buffer saline
DPPH	2,2-Diphenyl-1-picrylhydrazyl
MALDI-TOF MS	Matrix-Supported Laser Desorption/Ionization
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
CFS	Cell-Free Supernatant
5-FU	5-fluorouracil
DMSO	Dimethyl sulfoxide
cDNA	Complementary DNA
qRT-PCR	Quantitative real-time PCR
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
FAO	Food and Agricultural Organization
WHO	World Health Organization

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Author contributions

Conceptualization, Reham Abd El-Gaber; Investigation, Amel Ibrahim, and Sarah Aggag; Methodology, Reham Abd El-Gaber; data curation, Reham Abd El-Gaber, writing—original draft preparation Reham Abd El-Gaber and Amel Ibrahim, Sarah Aggag writing—review and editing, Mohamed Yacout and Sameh Awad - review and editing. All authors have read and agreed to the published version of the manuscript.

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Data availability

All data supporting the findings of this study are available within the manuscript.

Declarations

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

No conflict of interest with any authors.

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