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Traditional dairy-derived *Lactobacillus pentosus* enhances the anticancer activity of 5-fluorouracil against breast cancer cells

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ABSTRACT

Objective(s): Breast cancer remains a significant global health challenge and a leading cause of cancer-related mortality. Probiotics, key regulators of microbial and immune homeostasis, have emerged as promising candidates for cancer prevention and therapy. This study investigated the anticancer effects of Lactobacillus-derived cell-free supernatants (CFS) in *in vitro* and *in vivo* breast cancer models.

Materials and Methods: *Lactobacillus* strains were characterized for growth, viability, and antimicrobial activity. Standardized *Lactobacillus* cultures were used to prepare CFS by centrifugation and membrane filtration. The effects of CFS, alone or in combination with 5-fluorouracil (5-FU; 5 mg/kg every other day), on 4T1 breast cancer cell proliferation, migration, apoptosis, and inflammation were evaluated using MTT, flow cytometry, qPCR, and ELISA. An *in vivo* 4T1 breast cancer mouse model was established to assess the effects of CFS on tumor growth and associated molecular and histopathological changes. Statistical analyses were performed using Student's t-test or one-way ANOVA with Tukey's post hoc test; $P<0.05$ was considered significant.

Results: Co-treatment with CFS and 5-FU inhibits cell proliferation and cell-cycle progression by modulating p53 and cyclin D1 expression ($P<0.05$). CFS decreases tumor growth and promotes apoptosis by regulating BCL-2 and Bax ($P<0.05$). Moreover, co-treatment enhanced the inhibitory effect of 5-FU on cancer cell migration by modulating *E-cadherin*, *MMP2*, and *MMP9* expression ($P<0.05$).

Conclusion: Lactobacillus-derived CFS may enhance the antiproliferative and pro-apoptotic effects of 5-FU in breast cancer models. These findings provide a preclinical basis for further investigation into their therapeutic potential.

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Introduction

Breast cancer is the most common malignancy in women, with an incidence of approximately 2.3 million new cases and about 670000 deaths per year (1). Breast cancer tumorigenesis is a multifactorial process involving genetic, hormonal, immunological, nutritional, and environmental factors. Current therapeutic approaches for breast cancer include surgical intervention, radiotherapy, and chemotherapy (2). Although chemotherapy remains a cornerstone of cancer treatment, recent findings indicate that it can disrupt the composition of the gut microbiota, leading to a range of gastrointestinal adverse effects that markedly impair patient quality of life (3). Accumulating evidence suggests a strong association between gut microbiota dysbiosis and both tumor progression and poor clinical outcomes in breast cancer (4).

The gut microbiota can play a crucial role in preventing various human malignancies by producing several anti-mutagenic or anti-tumorigenic compounds. Moreover, the intestinal microbiota critically affects estrogen metabolism and systemic immune responses, and microbiota dysbiosis is associated with cancer growth and progression (5, 6). Therefore, probiotic supplementation has emerged as a potential microbiome-modulating intervention, as it can support intestinal microbial equilibrium and potentially attenuate microbiota alterations associated with cancer development and progression.

The World Health Organization defines probiotics as live microorganisms that, when administered in sufficient quantities, can improve host health. Emerging evidence indicates that probiotics confer several protective and therapeutic effects by increasing the secretion of antioxidant and anti-inflammatory metabolites, regulating tissue fibrosis, and enhancing immune activity (7). To further investigate the anti-tumor mechanisms of probiotics in human cancers, studies have found that probiotics inactivate potential carcinogens, inhibit cell cycle progression and

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proliferation, and induce apoptosis in gastrointestinal, breast, and gynecological cancers. Additionally, probiotic administration has been shown to improve chemotherapy-related gastrointestinal complications in cancer patients (8-10). Further *in vitro* studies have demonstrated that probiotics can positively influence the gut microbiota, promote the breakdown of potential carcinogens, prevent DNA damage in intestinal cells, and improve immune and inflammatory responses (11).

Lactobacillus is one of the most common intestinal probiotic genera and appears in the colon after consuming probiotic-containing meals. Recent preclinical *in vitro* studies suggest that *Lactobacillus* probiotics may have anti-cancer effects by inhibiting cell proliferation, producing anti-mutagenic metabolites, and enhancing immune responses (8, 12). Further supporting the immunomodulatory mechanism of probiotics, treatment with *Lactobacillus* probiotics has been shown to stimulate the activity of natural killer (NK) cells and cytotoxic T cells while modulating inflammatory cytokines, including IFN-γ and TGF-β, in a breast cancer animal model (13-15).

Despite these promising findings, the potential effects of probiotic *Lactobacillus* strains combined with conventional anticancer agents require further investigation. Therefore, this study aimed to evaluate the anticancer effects of selected *Lactobacillus*-derived CFS, alone and in combination with 5-FU, using cellular and animal breast cancer models. We specifically assessed whether probiotic-derived supernatant treatment could enhance the anticancer effects of 5-FU and explored its potential biological effects in these experimental models. The findings may provide further preclinical evidence regarding the potential use of selected probiotic bacteria as supportive agents alongside conventional breast cancer therapy.

Materials and Methods

Preparation of Lactic acid bacterium

A strain of lactic acid bacterium, originally isolated from a traditional local yogurt sample, was obtained from the Microbiology Laboratory of Islamic Azad University of Damghan. This bacterium was grown in De Man–Rogosa–Sharpe (MRS) broth (Merck, Germany) and then plated onto MRS agar. First, the probiotic potential of the bacteria was determined using conventional biochemical tests, and then their molecular identification was performed.

Carbohydrate fermentation profiling

Carbohydrate utilization patterns of lactic acid isolate were evaluated using a phenol red–based fermentation system optimized for lactobacilli (16). Sterile carbohydrate solutions, including glucose, fructose, sucrose, lactose, maltose, mannose, mannitol, arabinose, galactose, xylose, and trehalose, were incorporated individually into a deproteinized MRS derived basal medium at a final concentration of 2% (w/v). The medium also contained phenol red and inverted Durham tubes to monitor metabolic activity. Standardized cultures (OD_{600}=0.5) were inoculated at 1% (v/v) and incubated at 37 °C for 24 hr under anaerobic conditions. Acid production was quantified by the indicator shift from red to yellow, while CO_2 release was assessed by gas accumulation in Durham tubes. This assay allowed for the precise characterization of the metabolic profile specific to the single lactic acid bacterial strain. The carbohydrate fermentation profiles obtained from these assays are summarized in Table 1.

Acid tolerance evaluation

The capacity of the single lactic acid bacterium to withstand acidic conditions was assessed by measuring their viability at low pH (17). Active cultures, routinely maintained under anaerobic conditions in MRS broth at 37 °C for 24 hr, were standardized to a 0.5 McFarland turbidity standard. A 1% (v/v) inoculum (100 µl) was then transferred into 10 ml of modified MRS broth, which had been adjusted to pH 2.5 and 4.5 using 1 M HCl. MRS broth adjusted to pH 6.5 was utilized as the experimental control. Following incubation for 3 hr at 37 °C, viable cell counts were determined using the colony-forming unit (CFU) method. Bacterium strain maintaining a survival rate equal to or exceeding 106 CFU/ml were considered acid-tolerant.

Bile salt tolerance assessment

The bile salt tolerance of the single lactic acid bacterial strain was evaluated to assess their potential survival during gastrointestinal transit (18). Initially, the lactic acid strains were revived by inoculating them into MRS broth and incubating them anaerobically at 37 °C for 24 hr. An overnight active culture sample (1 mL) was then inoculated into 9 ml of fresh MRS broth supplemented with 0.3% (w/v) Ox bile to serve as the treatment group. In contrast, an identical setup using MRS broth without bile salts was prepared simultaneously as the control. Both the treatment and control groups were subsequently incubated under anaerobic conditions at 37 °C for 8 hr. To monitor growth kinetics, the optical density (OD) of the cultures was measured hourly for 8 hr at 620 nm using an ELISA microplate reader. The bacterium Isolate demonstrating an inhibition coefficient equal to or less than 0.4 were considered to be bile-tolerant strains.

Thermal tolerance determination

The thermal growth characteristics of the single lactic acid bacterial strain included in this study were evaluated using the agar streaking method. Briefly, fresh cultures grown in MRS broth were transferred onto solid MRS agar plates using standard aseptic streaking techniques. To assess their ability to proliferate at extreme growth temperatures, the inoculated plates were incubated anaerobically at 15 °C and 45 °C for 24 hr. Microbial growth was subsequently monitored, and the strains were characterized by their viability at each temperature (19).

Hemolysis assay

The lactic acid bacterium was cultured in MRS medium at 37 ˚C for 18–24 hr. The streak plate method was performed on 5% sheep blood agar plates to analyze hemolytic activity (20).

Modified double-layer method

A modified agar overlay assay was used to determine the strain's antimicrobial activity (21). After 24 hr of anaerobic growth at 37 °C on MRS agar, the isolate was covered with a layer of 0.8% nutrient agar containing the target pathogens (*Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (PTCC 1338), *Pseudomonas aeruginosa* (ATCC 9027), and *Enterococcus faecalis* (ATCC 29212). Following an additional incubation phase (24 hr at 37 °C), the zones of inhibition surrounding the streaks were measured to quantify the strain's antimicrobial efficacy.

Preparation of cell-free supernatant

The lactic acid bacterium was cultured in MRS broth under anaerobic conditions at 37 ˚C for 24 hr. The overnight bacterial culture was centrifuged at 10,000 rpm for 10 min

at 4 °C. The collected supernatant was then passed through a 0.2 µm Millipore membrane filter for sterilization. The sterile CFS was aliquoted into microcentrifuge tubes and stored at -20 °C for further use, or combined with cell culture medium containing fetal bovine serum (FBS) as required for subsequent experiments (22).

Agar well diffusion assay

The antagonistic activity of the lactic acid-derived CFS against the selected pathogenic strains (*E. coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus*) was evaluated using the agar well diffusion method (23). Initially, the indicator pathogens were cultured in BHI broth until reaching a turbidity equivalent to a 0.5 McFarland standard (1.5×10^8 CFU/ml). Next, 100 µl of each bacterial suspension was uniformly spread onto Mueller-Hinton Agar (MHA) plates to create a confluent lawn of growth. Using a sterile cork borer, we punched 6-mm wells into the inoculated agar medium. Each well was then filled with 100 µl of the filtered CFS. To allow metabolites to pre-diffuse into the agar matrix, the plates were kept at 4 °C for 2 hr, then incubated for 24 hr at 37 °C under aerobic conditions. Sterile MRS broth and standard antibiotic discs were employed as negative and positive controls, respectively. The antibacterial efficacy was determined by measuring the diameter of the clear zones of inhibition (ZOI) around each well in millimeters using a digital caliper. All experiments were performed in triplicate to ensure statistical reliability.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The antibacterial activity of the lactic acid-derived CFS was evaluated against *E. coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus* using the broth microdilution method based on a 2-fold serial dilution protocol (24). The lactic acid-derived CFS was serially diluted twofold in Mueller-Hinton broth (MHB) and used as the stock solution (100% v/v). A series of 2-fold dilutions was prepared across the microtiter plate wells to achieve varying volume-to-volume (v/v) percentages, starting with the undiluted supernatant in the first well (100%), followed by sequential dilutions of 50% (1:2), 25% (1:4), 12.5% (1:8), 6.25% (1:16), and 3.125% (1:32) in the subsequent wells. The MBC was defined as the lowest concentration resulting in no colony formation. All assays were performed in triplicate across three independent sessions to ensure reproducibility.

Molecular characterization and phylogenetic analysis

To confirm the identity of the probiotic isolate, genomic DNA was isolated from overnight cultures in MRS broth utilizing a commercial extraction kit (Puyagene Azma, Iran) according to the manufacturer's protocol. The 16S rRNA gene was targeted for molecular identification through polymerase chain reaction (PCR) using the universal primers detailed in Table 2. Each 25 µl reaction mixture contained universal primers, Taq DNA polymerase, the DNA template, and a standardized PCR buffer. Thermal cycling was conducted under the following parameters: an initial denaturation phase at 95 °C for 5 min; 35 cycles of denaturation (95 °C for 30 sec), primer annealing (60 °C for 30 sec), and extension (72 °C for 30 sec); followed by a terminal extension at 72 °C for 5 min. Amplicons were separated on a 1.5% (w/v) agarose gel, stained with ethidium bromide, and documented using a UV-based GelDoc imaging system.

Taxonomic identification was performed through bidirectional sequencing of 16S rRNA amplicons, conducted by Puyagene Azma (Iran). Sequence alignment and homology assessments were executed using the Ez-BioCloud database. To illustrate evolutionary lineage, a phylogenetic tree was generated via the Maximum Likelihood algorithm in MEGA software (25).

Cell culture

The breast cancer cell lines, 4T-1 and MCF7, were cultured in RPMI 1640 medium with 10% FBS and 1% streptomycin/penicillin and maintained at 37 °C in a 5% CO_2 atmosphere.

Cell viability assay (MTT)

The cytotoxic effects of *Lactobacillus*-derived CFS on breast cancer cell lines were evaluated using the MTT assay. Briefly, the *Lactobacillus* probiotic was cultured at predetermined bacterial concentrations ranging from 10^5 to 10^{10} CFU/ml. Following centrifugation and membrane filtration to remove bacterial cells, the resulting CFS preparations were collected, and equal volumes of each preparation were added to 4T1, MCF-7, and 3T3 cell cultures. Cell viability was subsequently assessed using a microplate reader, and the half-maximal inhibitory concentration (IC_{50}) was determined based on the initial bacterial concentration used to generate the corresponding CFS.

Migration assay

To perform the scratch assay, breast cancer cells were cultured and treated with *Lactobacillus*-derived CFS. When the cells achieved 70% confluency, a scratch was created at the center of each well, and non-adherent cells were then removed by rinsing with PBS. The migratory activity of cells was investigated at multiple time points (between 0 and 72 hr) using a ZEISS microscope (Germany) and analyzed using ImageJ software (26).

Cell cycle analysis

The regulatory effects of bacterial-derived supernatants on cell cycle progression were investigated by cell cycle analysis (27). Briefly, 4T-1 cells were exposed to *Lactobacillus*-derived CFS and incubated with RNase enzyme. Subsequently, treated cells were stained with propidium iodide (PI), and cell cycle distribution was analyzed by a FACSCalibur (BD Biosciences) flow cytometer.

Apoptosis assay

To examine the effect of bacterial-derived CFS on breast cancer cell apoptosis, an Annexin V (FITC-conjugated)-Propidium iodide (PI) flow cytometric method was used as previously described. Briefly, breast cancer cells were treated with *Lactobacillus*-derived CFS for 24 hr. Treated cells were resuspended in Annexin binding buffer and then stained with Annexin V-FITC/PI solution. Finally, the percentages of live, apoptotic, and necrotic cells were determined by a BD FACSCalibur (BD Biosciences) flow cytometer at excitation/emission wavelengths of 488/525 nm for Annexin V and 488/675 nm for PI. Data analysis was carried out using FlowJo V10_CL software (27).

***In vivo* experiments**

Animal experiment

All animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals approved by the Islamic Azad University-Damghan Branch

Research Ethics Committee. The study Approval ID was (IR.IAU.DAMGHAN.REC.1403.011). Eight-week-old inbred BALB/C female mice were purchased from the Bu Ali Institute (Mashhad, Iran). 4T-1 cells (1×10^6 cells) were subcutaneously implanted into the right flank of BALB/c mice. When tumors reached a volume of 80-100 mm^3, animals were randomly categorized into four groups: 1) No treatment (Negative control), 2) 5-FU-treated (5 mg/kg every other day, intraperitoneal injection), 3) treated with 0.2 ml of *Lactobacillus*-derived CFS, harvested from a bacterial culture that had reached a standardized cell density of 1×10^8 CFU/ml before centrifugation and filtration, for 3 weeks, 4) Combination group treated with 5-FU and probiotic-derived supernatants for 3 weeks. To monitor oncogenic development, tumor volumes were measured every two days with digital calipers. Following a 16-day experimental period, the animals were sacrificed, and the mammary tumors were excised. The collected specimens were then processed for histological evaluation using both Masson's trichrome and H&E staining protocols to assess tissue morphology (28).

Real-time PCR analysis

Real-time PCR was performed following published protocols. Briefly, total RNA was extracted from tumor tissues and reverse-transcribed into cDNA using a reverse transcription kit according to the manufacturer's protocol (TaKaRa Bio, Shiga). Real-time PCR was performed on an ABI-PRISM StepOne instrument (Applied Biosystems, Foster City, CA) using specific primers for target genes (Table 3).

Enzyme-linked immunosorbent assay (ELISA)

Tumor tissues were homogenized, and protein concentrations were measured using a BCA quantification kit. ELISA assays were performed to quantify the levels of interleukin-6 (IL-6), transforming growth factor-beta (TGF-β), and tumor necrosis factor-alpha (TNF-α) in the samples.

Statistical analysis

All experiments were conducted in triplicate, and data are presented as mean±standard deviation (SD). The Kolmogorov–Smirnov test was performed to investigate the normal distribution of continuous data. Statistical comparisons were made using Student's t-test and one-way analysis of variance (ANOVA). Differences were considered statistically significant at *P-value*<0.05.

Results

Biochemical tests

Biochemical and molecular tests showed that the probiotic strain is catalase-negative, oxidase-negative, indole-negative, and Gram-positive (Table 1). According to the results presented in Table 1, the probiotic isolate demonstrated a comprehensive fermentative profile. It successfully utilized all eleven tested carbohydrates, ranging from simple hexoses like glucose and mannose to complex disaccharides such as trehalose and sugar alcohols like mannitol.

Acid and bile salt resistance assay

The acid and bile stability of the probiotic strain was assessed through qualitative monitoring of growth patterns (Table 1). Samples were deemed positive when discernible colonies emerged, while the absence of cellular expansion was interpreted as negative, reflecting the strain's specific adaptive responses to these chemical stressors.

Thermal tolerance

The thermal tolerance of the isolated strain was evaluated to assess its viability under varying environmental conditions. As shown in Table 1, the lactic acid bacterial isolate grew at 15 °C but did not grow at 45 °C. These results indicate that while the strain maintains metabolic activity at lower temperatures, it lacks heat tolerance in the thermophilic range.

Hemolysis assay

Hemolysis assay results showed that the bacterial strain had no adverse effects on erythrocyte integrity. Consequently, the lack of hemolysis supports the safety

Table 1. Phenotypic and functional characterization of the bacterial isolate

Category	Test	Results
Morphology	Gram staining	Positive (Purple)
	Cell morphology	Small bacilli, single or in chains
Biochemical Profiles	Oxidase test	Negative
	Catalase test	Negative
	Indole test	Negative
	Hemolytic activity	Non-hemolytic
	Motility	Non-motile
Carbohydrate Fermentation	Glucose, Fructose, Sucrose, Lactose, Maltose	Positive
	Mannose, Mannitol, Fructose, Arabinose	Positive
	Galactose, Xylose, Trehalose	Positive
Physiological Tolerance	Growth at 15°C	Positive
	Growth at 45°C	Negative
	Acid tolerance (pH 2.5, 4.5, 6.5) after 3 h	Tolerant
	Bile tolerance (0.3% bile salt) after 3 h	Tolerant
Antimicrobial Potential	Antibacterial activity against *Escherichia coli*	Sensitive
	Antibacterial activity against *Staphylococcus aureus*	Sensitive
	Antibacterial activity against *Enterococcus faecalis*	Sensitive
	Antibacterial activity against *Pseudomonas aeruginosa*	Sensitive

The bacterial isolate was characterized based on its morphological and biochemical properties, carbohydrate fermentation profile, tolerance to different temperatures, acidic and bile salt conditions, and antibacterial activity against selected bacterial pathogens.

Table 2. Characteristics and nucleotide sequences of the universal primers used for 16S rRNA gene amplification

Primer name	Sequence (5'-3')	TM	GC%
16S - F	AGAGTTTGATCCTGGCTCAG	57.3	50
16S - R	CGGTTACCTTGTTACGACTT	55.25	45

profile of this probiotic candidate, as detailed in Table 1.

Modified double-layer method

To assess the antibacterial potential of *Lactobacillus* probiotics, the modified double-layer method was employed against a diverse panel of pathogenic strains. The resulting antagonistic profiles, detailed in Table 1, revealed that the probiotic exhibited broad-spectrum antibacterial activity, successfully restricting the growth of all tested microorganisms. The magnitude of growth inhibition varied across the different pathogens. The most pronounced antibacterial effects were directed against *E. coli* and *S. aureus*, which displayed clearance zones of 26.5 mm and 23 mm, respectively. Strong inhibitory activity was likewise observed against *E. faecalis* (21 mm). Conversely, *P. aeruginosa* was the least susceptible organism in the panel, though it was still notably inhibited, with an inhibition zone of 18 mm.

Well diffusion assay

The agar well diffusion method was used to evaluate the probiotic isolate's antagonistic activity against four pathogenic bacteria. As illustrated in Table 1, the probiotic exhibited clear zones of inhibition against all tested pathogens. The largest inhibition zones were recorded for *E. coli* (17.5 mm) and *S. aureus* (15 mm), followed by *E. faecalis* (13.5 mm) and *P. aeruginosa* (10 mm). Although the isolate's inhibition zones were smaller than those produced by the positive control, Gentamicin (GM, 10 µg), the results confirm the isolate's significant antimicrobial potential against both Gram-positive and Gram-negative indicators.

MIC and MBC results

Antibacterial activity tests against various pathogenic bacteria showed activity against the listed pathogens (Table 1). The tested bacterial strains varied substantially in susceptibility to the crude supernatant across species. The supernatant showed potent antibacterial activity against S. aureus, with inhibitory and bactericidal effects up to a 1:8 dilution; MIC and MBC were 12.5% (v/v), indicating high susceptibility to the treatment. In contrast, growth inhibition was observed at a 1:4 dilution (MIC=25% v/v) for *E. coli*. Complete eradication required a higher concentration at a 1:2 dilution (MBC=50% v/v), suggesting a predominantly bacteriostatic effect at lower concentrations that requires a 2-fold increase to achieve a bactericidal outcome. Furthermore, *P. aeruginosa* showed the highest resistance among the tested pathogens, with susceptibility only at the 1:2 dilution and identical MIC and MBC values of 50% (v/v), meaning the supernatant must constitute at least half of the total well volume to inhibit or actively kill the bacteria. Overall, the supernatant demonstrated a significantly higher antibacterial efficacy against Gram-positive bacteria, particularly *S. aureus*. Because the MIC and MBC values for most strains were either identical or within a single 2-fold dilution factor, the bioactive supernatant can be classified as primarily bactericidal rather than bacteriostatic.

Molecular identification

To determine the taxonomic and phylogenetic position of the isolate under study, we partially sequenced the 16S rRNA gene. We compared the resulting nucleotide sequence with reference sequences in the EZBioCloud database. Genotypic identification based on the partial 16S rRNA gene sequence identified the isolate as *Lactobacillus pentosus*. The 16S rRNA gene sequence was 1,304 bp long and was deposited in the NCBI GenBank database under accession number PX495930, where it was registered as *L. pentosus* strain TNFM. To further investigate the phylogenetic relationship

Table 3. Sequences of primers used for quantitative real-time PCR analysis of target genes in mouse tissues

Gene	Source	Primer	Sequence
GAPDH	Mouse	Forward	CAACGACCCCTTCATTGACC
		Reverse	CTTCCCATTCTCGGCCTTGA
Cyclin D1	Mouse	Forward	GCGTACCCTGACACCAATCTC
		Reverse	ACTTGAAGTAAGATACGGAGGGC
MMP-2	Mouse	Forward	AACTGTTGCTTTTGTATGCCCT
		Reverse	CGATGTCAGACAACCCGAGT
MMP-9	Mouse	Forward	GCGTCGTGATCCCCACTTAC
		Reverse	CAGGCCGAATAGGAGCGTC
p53	Mouse	Forward	GGACAGCTTTGAGGTTCGTG
		Reverse	TCATTCAGCTCCCGGAACAT
Bax	Mouse	Forward	AGACAGGGGCCTTTTTGCTAC
		Reverse	AATTCGCCGGAGACACTCG
BCL-2	Mouse	Forward	GAGAGGAGAACGCAGGTAGT
		Reverse	TGGCCACCTAAAGCTCCG
E-cad	Mouse	Forward	GTCTACCAAAGTGACGCTGA
		Reverse	GGGAAACATGAGCAGCTCTG
Col3a1	Mouse	Forward	CTGTAACATGGAAACTGGGGAAA
		Reverse	CCATAGCTGAACTGAAAACCACC
Col1a2	Mouse	Forward	GTTCTCAGGGTAGCCAAGGT
		Reverse	CCTTCAAAACCAAAGTCATAGCC

Note: GAPDH was used as the reference gene. Primer sequences are presented in the 5′–3′ direction

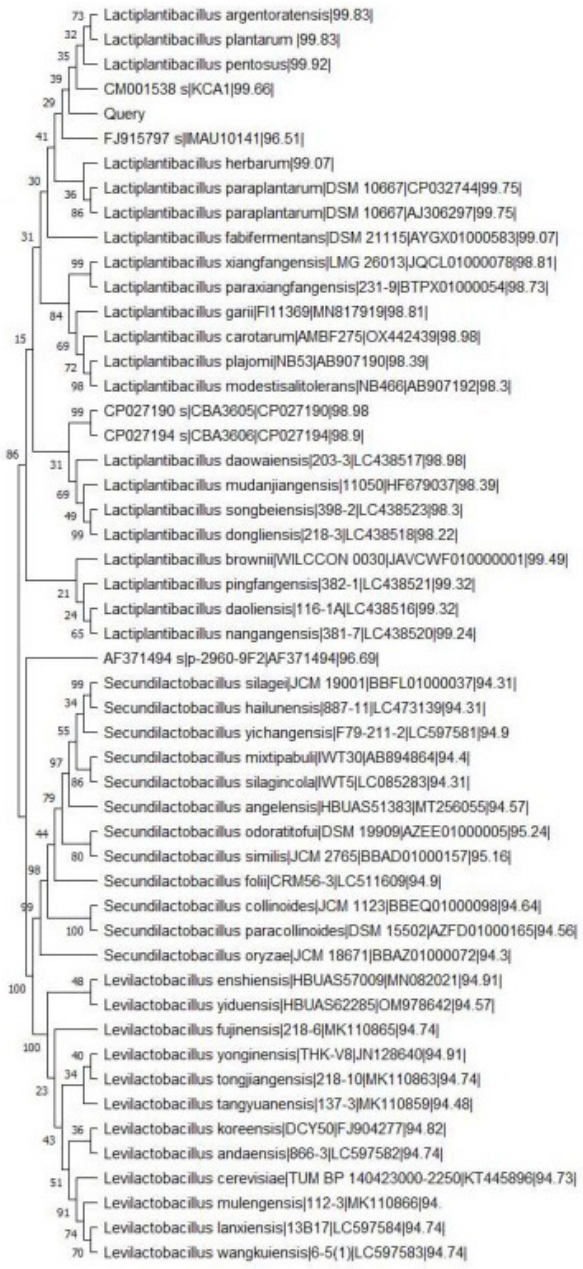

Figure 1. Phylogenetic analysis of the isolate based on partial 16S rRNA gene sequences
The tree was constructed using the Maximum Likelihood method in MEGA with 1,000 bootstrap replicates. Bootstrap values are shown at the nodes, and the isolate is indicated as "Query." Percentages represent sequence similarity.

of the isolate with closely related taxa, a phylogenetic tree was constructed using the Maximum Likelihood (ML) method implemented in MEGA, with 1,000 bootstrap replicates. The results showed that the isolate under study (Query) was positioned within the *Lactiplantibacillus* lineage and clustered with closely related members of the *Lactiplantibacillus* genus, including *L. pentosus*, *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, and *Lactobacillus fabifermentans*. This phylogenetic placement was consistent with identifying the isolate as *L. pentosus* based on 16S rRNA sequence comparison and its corresponding GenBank record.

As shown in Figure 1, the phylogenetic tree comprised three major lineages. The first lineage included species in the genus *Lactiplantibacillus*, including *L. pentosus*, *L. plantarum*, *L. paraplantarum*, and *L. fabifermentans*, within which the isolate under study was positioned. The second lineage included species of the genus *Secundilactobacillus*, such as *S. silagincola* and *S. angelensis*, whereas the third lineage comprised species of the genus *Levilactobacillus*, including *L. brevis* and *L. koreensis*. The isolate's position within the *Lactiplantibacillus* lineage and its close association with *L. pentosus* and other members of the *L. plantarum* group support its placement within this phylogenetic group.

Bootstrap support values varied among the nodes of the phylogenetic tree, with some nodes showing relatively low support and others showing moderate to high support. Thus, the bootstrap values indicate different levels of statistical support for the inferred phylogenetic relationships and should not be interpreted as uniformly high support across the tree. The clustering pattern in Figure 1, along with the 16S rRNA sequence comparison and the GenBank record, supports identification of the isolate as *L. pentosus* strain TNFM. Overall, partial 16S rRNA gene sequencing, sequence comparison using the EZBioCloud database, phylogenetic analysis, and GenBank deposition support identification of the isolate as *L. pentosus* strain TNFM (GenBank accession number PX495930).

Lactobacillus-derived CFS inhibits cell growth and cell cycle progression in breast cancer

The growth-inhibitory effects of *Lactobacillus*-derived CFS on two breast cancer cell lines were evaluated using the MTT assay. Investigation of cytotoxic effects of *L. pentosus*-derived CFS on MCF-7 and 4T-1 cells indicated that the probiotic-derived CFS inhibited breast cancer cells in a dose-dependent manner. As shown in Figure 2A, treatment with CFS prepared from a 1010 CFU/ml *L. pentosus* bacterial culture reduced 4T-1 cell proliferation and viability to 58.5±7% compared with untreated cells. Similarly, CFS prepared from the same bacterial concentration decreased MCF-7 cell viability to 54±6% compared with the control group. Moreover, the MTT assay showed that the bacterial supernatant had no toxic effects on normal 3T3 fibroblast cells.

To further investigate the anti-proliferative effects of *Lactobacillus* CFS on breast cancer, we treated 4T-1 cells with *L. pentosus*-derived CFS and 5-FU and analyzed cell cycle distribution by flow cytometry. Our results showed that probiotic-derived CFS, alone or in combination with 5-FU, significantly altered cell cycle progression in 4T-1 cells. Specifically, single treatments with 5-FU or CFS led to a significant increase in the Sub-G1 population compared to the untreated Control group ($P<0.05$). Notably, the combination treatment (CFS+5-FU) exhibited a potent regulatory effect, resulting in a more than 4-fold increase in the Sub-G1 fraction relative to the control baseline ($P<0.01$ vs Control; $P<0.05$ vs single treatments), confirming a substantial accumulation of cells in the Sub-G1 phase (Figure 2B).

Taken together, these results indicate that *L. pentosus*-derived CFS may effectively reduce tumor cell proliferation by repressing cell cycle progression in breast cancer cells.

Lactobacillus -derived supernatant inhibits tumor growth in a mouse model of breast cancer

The suppressive impact of *L. pentosus*-derived CFS on tumor progression was assessed in a murine model of breast carcinoma. Histopathological analysis further indicates that exposure to the probiotic-derived CFS induced marked

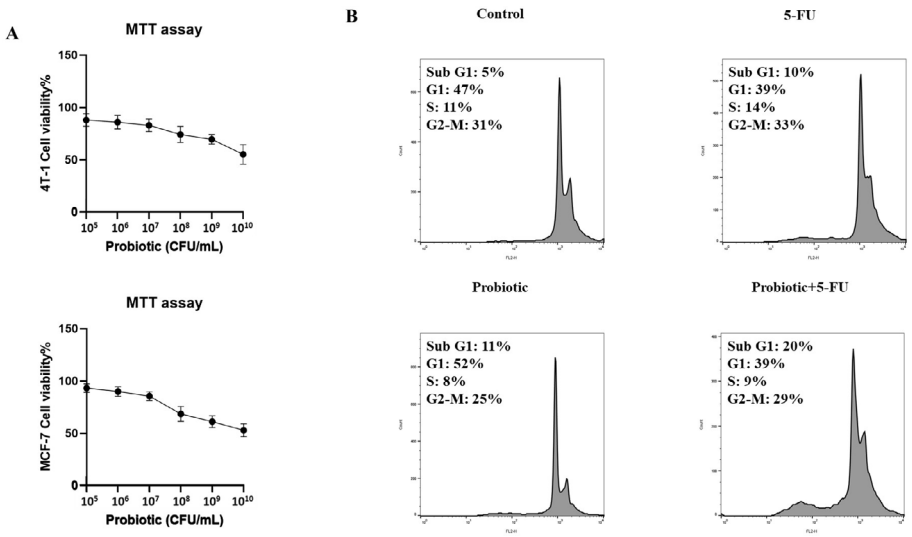

Figure 2. *Lactobacillus* cell-free supernatant (CFS) inhibits cell proliferation and induces cell cycle arrest in breast cancer cells
(A) 4T-1, MCF-7, and 3T3 cells were treated with the *Lactobacillus pentosus*-derived CFS for 24 hr, and cell viability was determined by MTT assay. (B) The cell cycle analysis by PI staining is shown. 4T-1 cells were treated with Lactobacillus-derived CFS for 24 hr, then stained with propidium iodide (PI) and analyzed by flow cytometry. Flow cytometry histograms are representative of three independent biological experiments (n=3). The accompanying bar chart presents the quantitative analysis of the Sub-G1 phase population expressed as Mean±SD. All experiments were performed in triplicate (n=3), and statistical significance was assessed using one-way ANOVA followed by Tukey's multiple-comparisons test (*P<0.05, **P<0.01, ***P<0.001).

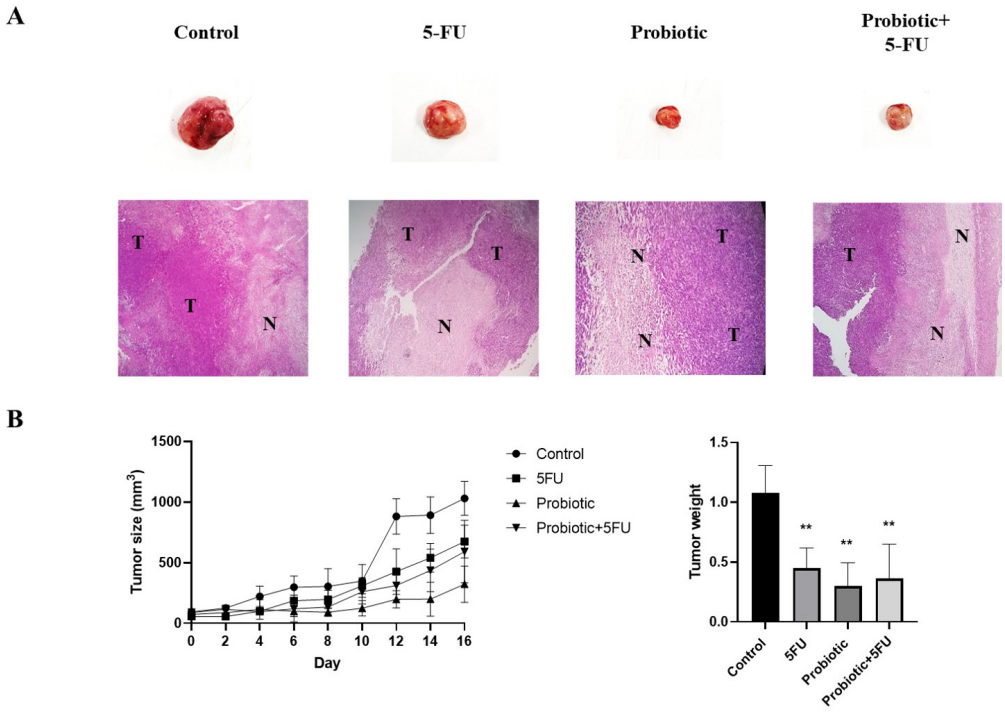

Figure 3. *Lactobacillus* cell-free supernatant (CFS) reduces tumor growth in the breast cancer mouse model (n=6 in each group)
(A) Histological staining of tumor tissues by hematoxylin and eosin (H&E) for evaluating aggregation of tumor cells (T) and necrotic area (N). (B) Tumor size and weight change in a breast cancer mouse model treated with CSF, 5-FU, and their combination. Data are presented as mean±SEM; statistical significance was assessed using one-way ANOVA followed by Tukey's multiple-comparisons test (*P<0.05, **P<0.01, ***P<0.001).

tissue necrosis in the breast cancer model (Figure 3A). Consistent with the *in vitro* results, oral administration of the *L. pentosus*-derived CFS significantly reduced tumor size and weight (P<0.01) (n=6 in each group) (Figure 3B). Notably, co-treatment with the probiotic-derived CFS and 5-FU enhanced the chemotherapeutic efficacy of 5-FU (Figure 3A and B) (P<0.01).

Lactobacillus-derived CFS inhibits fibrosis and inflammatory responses in breast cancer tissues

Fibrosis arises from dysregulation of the normal wound-healing process and is characterized by excessive collagen accumulation in the extracellular matrix (ECM). To evaluate the effect of *L. pentosus*-derived CFS on collagen accumulation and fibrosis, tumor tissues from each experimental group were subjected to Masson's trichrome staining. The histological analysis demonstrated that exposure to the *Lactobacillus*-derived CFS markedly

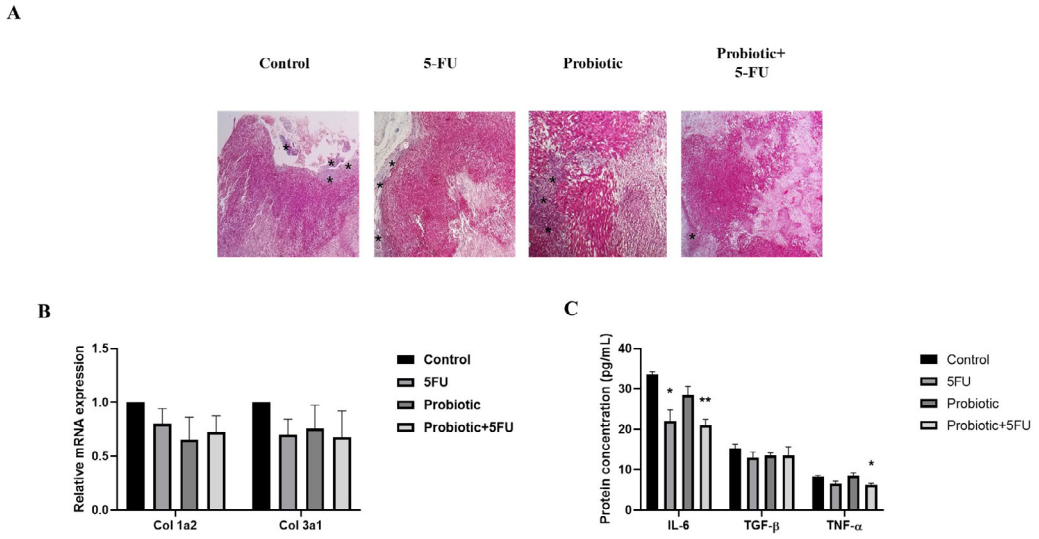

Figure 4. Regulatory mechanism of *Lactobacillus* cell-free supernatant (CFS) on fibrosis and inflammatory cytokines
(A) Trichrome staining for investigating the deposition of collagen fibers in breast cancer tissues (n=6 in each group). (B) Tumor extracts were subjected to q-PCR analysis to evaluate *Col 1a2* and *Col 3a1* expression. qPCR data were normalized to the reference gene *GAPDH* using the $2^{-\Delta\Delta Ct}$ method. (C) Breast tumor tissues were subjected to ELISA to assess inflammatory cytokine expression. Data are presented as mean±SD; all experiments were performed in triplicate (n=3), and statistical significance was assessed using one-way ANOVA followed by Tukey's multiple-comparisons test (*P<0.05, **P<0.01, ***P<0.001).

reduced collagen deposition in tumor tissues, confirming their anti-fibrotic potential against breast cancer (Figure 4A). Consistently, our results indicate that treatment with *L. pentosus*-derived CFS reduces the expression of fibrosis-related genes, including *Col1a2* and *Col3a1,* in tumor tissues (Figure 4B). To investigate further their anti-inflammatory activity, protein analysis in the breast cancer model revealed that co-administration of *L. pentosus*-derived CFS with 5-FU significantly decreased the protein levels of TNF-α (P<0.05) and IL-6 (P<0.01) in breast cancer tissues (Figure 4C). No significant change in TGF-β protein levels was observed following treatment (Figure 4C).

Lactobacillus-derived CFS induces apoptosis in breast cancer cells

We next evaluated the regulatory role of the *Lactobacillus*-derived CFS on breast cancer cell apoptosis. Our results indicated that co-administration of the bacterial-derived supernatants with 5-FU further enhanced its apoptotic activity in 4T-1 cells relative to the control group (Figure 5A). Consistently, bacterial supernatant treatment modulated the expression of key apoptosis regulators, including *p53*, *Bax*, and *BCL-2*, in breast cancer tissues

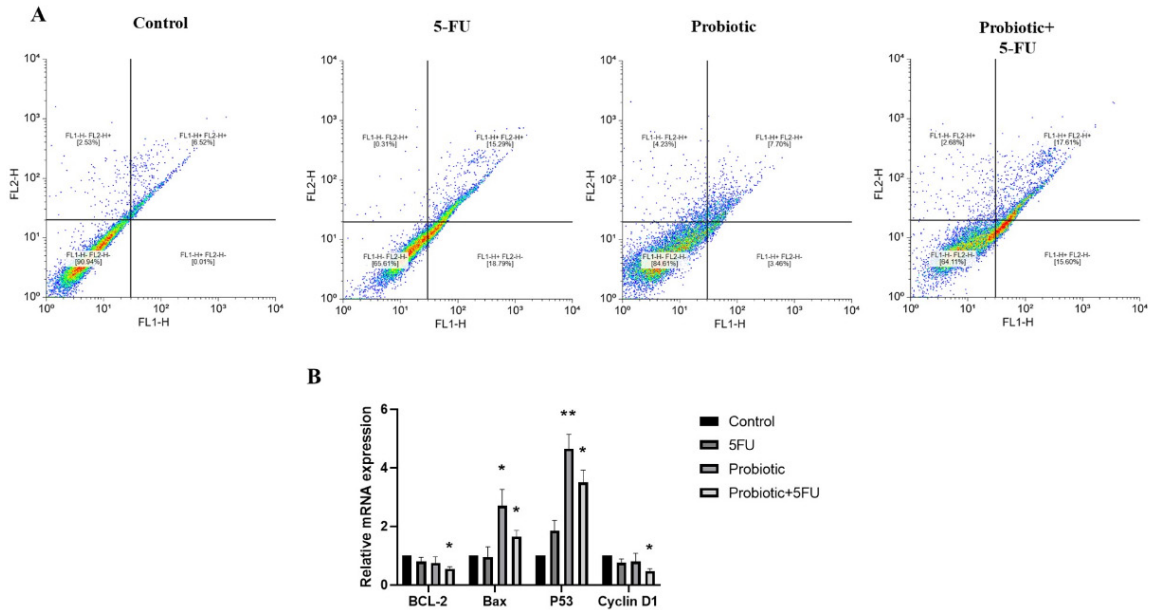

Figure 5. *Lactobacillus* cell-free supernatant (CFS) promotes cell apoptosis in breast cancer cells
(A) 4T-1 cells were treated with *Lactobacillus pentosus*-derived CFS for 24 hr, and apoptosis was explored by flow cytometry using annexin V/PI staining. The lower-right and upper-right quadrants indicate the percentage of cells in early and late apoptosis, respectively. (B) Breast tumor tissues were subjected to q-PCR analysis to evaluate the mRNA expression of tumor suppressor and apoptosis-related genes. Data are presented as mean±SD; all experiments were performed in triplicate (n=3), qPCR data were normalized to the reference gene *GAPDH* using the $2^{-\Delta\Delta Ct}$ method, and statistical significance was assessed using one-way ANOVA followed by Tukey's multiple-comparisons test (*P<0.05, **P<0.01, ***P<0.001).

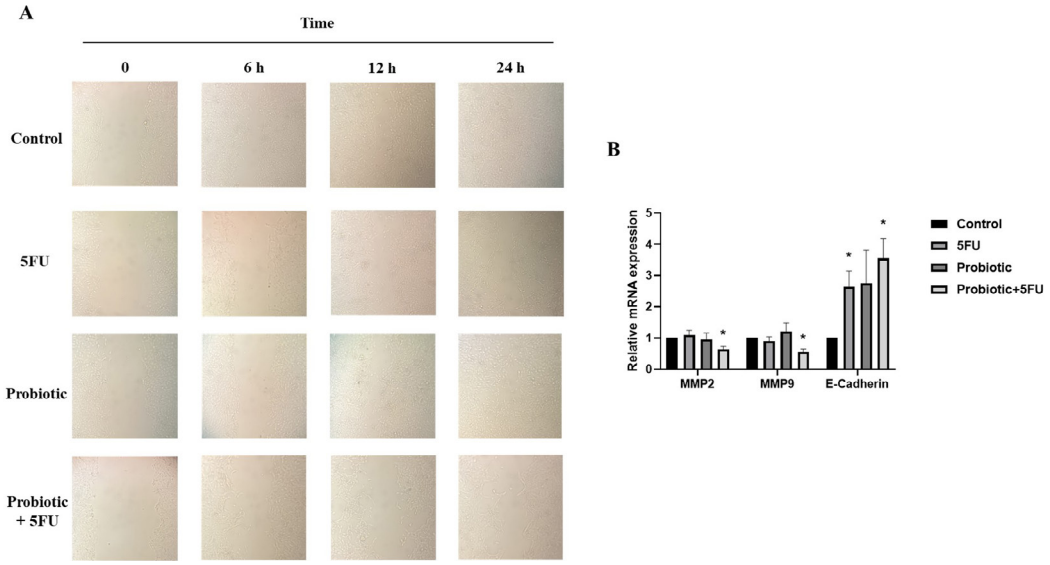

Figure 6. Regulatory effects of *Lactobacillus*-derived cell-free supernatant (CSF) on breast cancer cell migration
(A) 4T-1 cells were treated with *Lactobacillus pentosus*-derived CFS for 24 hr, and the resulting inhibitory effects on breast cancer cell migration were measured. (B) Breast tumor tissues were subjected to qPCR analysis to evaluate *E-cadherin*, matrix metalloproteinase (*MMP2*, and *MMP9*) gene expression. Data are presented as mean±SD; all experiments were performed in triplicate (n=3), qPCR data were normalized to the reference gene *GAPDH* using the $2^{-\Delta\Delta Ct}$ method, and statistical significance was assessed using one-way ANOVA followed by Tukey's multiple-comparisons test (*P<0.05, **P<0.01, ***P<0.001).

(Figure 5B). In agreement with the flow cytometry results, co-treatment of the *Lactobacillus*-derived CFS with 5-FU significantly induced the expression of *p53* and *Bax* in tumor tissues (P<0.05). Moreover, *cyclin D1* expression was investigated in breast tumor tissues. As shown in Figure 5B, cyclin D1 expression was significantly down-regulated in the combination group compared with the control group (P<0.05). These results suggest that *L. pentosus*-derived CFS induces 5-FU-mediated apoptosis by up-regulating pro-apoptotic genes in breast cancer cells and tumor tissues.

Lactobacillus-derived CFS prevents breast cancer cell migration

We further examined the anti-migratory potential of *Lactobacillus*-derived CFS on breast cancer cells. As shown in Figure 6A, *L. pentosus*-derived CFS treatment markedly impaired cellular migratory activity; meanwhile, combined treatment with 5-FU enhanced its inhibitory effects on cancer cell migration. Consistent with these observations, treatment with *Lactobacillus*-derived CFS down-regulated *MMP2* and *MMP9 mRNA* expression while up-regulating *E-cadherin* levels in breast cancer tissues (P<0.05) (Figure 6B). Taken together, these results demonstrate that the probiotic-derived CFS effectively attenuates the migratory behaviors of breast cancer cells.

Discussion

Probiotics have been investigated as natural immunomodulatory agents that can regulate immune responses, highlighting their therapeutic potential in managing diverse immune-related disorders like allergies, infections, and cancers (29, 30). In the present study, *Lactobacillus* strains were first cultured and then subjected to microbiological tests to confirm their identity and characteristics. Subsequently, the CFS was separated from the bacterial cells and used for subsequent experiments. The anticancer effects of *Lactobacillus*-derived CFS were then evaluated in breast cancer cell lines and a murine tumor model.

Our findings indicate that *L. pentosus*-derived CFS may modulate multiple cancer-related processes, including cell proliferation, apoptosis, inflammatory responses, fibrosis, and cellular migration. Furthermore, *Lactobacillus*-derived CFS may potentiate the antitumor activity of 5-FU, as shown by enhanced apoptosis and greater suppression of tumor growth following combination treatment. Collectively, these results expand upon previous evidence by supporting the potential anticancer activity of *Lactobacillus*-derived CFS in breast cancer and highlighting its potential as an adjunct to 5-FU therapy.

Probiotic survival and functional efficacy within the gastrointestinal tract depend heavily on tolerance to acidic and bile-rich environments. Our findings revealed that the isolated *Lactobacillus* strains exhibited robust acid tolerance across low pH levels and maintained an 80% survival rate in 0.3% bile salt conditions, supporting their potential as viable probiotics. To evaluate potential cytolytic or membranolytic off-target effects, a red blood cell hemolysis assay was conducted. The *Lactobacillus* strains did not compromise erythrocyte membrane integrity, suggesting that their cytotoxic effects on cancer cells are mediated through specific apoptotic or osmotic pathways rather than non-specific membrane lysis.

Recently, probiotics have attracted considerable attention for their capacity to inhibit cell cycle progression and cancer cell proliferation. Dehghani *et al.* demonstrated that administration of *Lactobacillus rhamnosus* induces cell cycle arrest at the G0/G1 phase and enhances apoptosis in HT-29 cancer cells (31). In line with this, we have recently studied the effect of *Lactobacillus* probiotics on cell cycle arrest and apoptosis in colorectal cancer (CRC) cells. Our previously published data show that *Lactobacillus* CFS administration induces apoptosis in CRC cells and tumor tissues by up-regulating the tumor suppressor p53 and modulating apoptosis-related genes, including Bax and BCL-2 (8).

The tumor suppressor protein p53 is known for its ability to halt cell cycle progression and initiate apoptosis

by transcriptionally activating pro-apoptotic mediators such as *Bax*. In contrast, *Bcl-2* acts as an oncogenic factor that enhances cell survival, enabling malignant cells to circumvent programmed cell death. *Bax* antagonizes this effect by directly interacting with *Bcl-2* and neutralizing its anti-apoptotic function (32). In line with these mechanisms, the impact of *Lactobacillus*-derived CFS on cell cycle dynamics and apoptosis was investigated in breast cancer cells and mouse models. Our results suggest that *L. pentosus*-derived CFS treatment may induce apoptotic cell death, associated with suppression of Bcl-2 expression and concomitant up-regulation of *Bax* and *p53* in breast tumor cells. Interestingly, we showed that treatment with the bacterial-derived supernatant enhances the pro-apoptotic effects of 5-FU, as shown by increased apoptosis and up-regulation of *Bax* and *p53*. Taken together, our results suggest that administration of *L. pentosus*-derived CFS may enhance the antiproliferative potential of 5-FU by inducing cancer cell death in breast cancer cells and tumor tissues.

Accumulating data indicate the critical role of *Lactobacillus* probiotics in regulating systemic immune responses that inhibit tumor growth and dissemination (33, 34). Elevated pro-inflammatory cytokine levels play a pivotal role in host defense and wound healing; Nevertheless, a shift toward persistent inflammatory signaling often establishes a microenvironment that facilitates neoplastic development (35, 36). This sustained immune dysregulation is closely linked to accelerated tumor development and the disruption of cell cycle checkpoints (37). Given the role of inflammatory responses in tumor genesis and development, regulating inflammation is a promising approach to preventing and managing human cancers. Consistent with these findings, Yue *et al.* showed *that L. plantarum* YYC-3 suppresses CRC tumorigenesis by decreasing immune cell activity and repressing proinflammatory markers such as *IL-6* and *IL-22 (38)*. Further results indicate that treatment with *Lactobacillus* probiotics, *Lactobacillus paracasei, Lactobacillus casei,* and *L. plantarum* inhibits immune responses by repressing inflammatory cytokines, including *IL-6*, *IL-23*, *TNF-α*, and *IFN-γ* in CRC cells (39-41). Consistently, we show that treatment with *L. pentosus*-derived CFS reduces inflammatory responses in breast cancer cells by repressing *TNF-α* and *IL-6* protein levels in tumor tissues.

Chronic fibrosis plays a crucial role in tumor initiation and development. It is characterized by up-regulated inflammatory cytokines, extracellular matrix proteins, and growth factors that stimulate tumor cell growth and progression (42, 43). In a study, the anti-fibrotic effects of *Lactobacillus acidophilus* were investigated in a colitis mouse model, and the findings demonstrated that treatment with *Lactobacillus acidophilus* reduced inflammatory responses and intestinal fibrosis by down-regulating *Acta2* and type-I collagen (44). Further studies reported that probiotic administration inhibits fibrosis in liver, intestinal, and renal tissues (45, 46). To investigate the role of *Lactobacillus*-derived CFS on collagen deposition and fibrosis, we conducted Masson's trichrome staining, and our findings showed that treatment with these bacterial- derived CFS reduced collagen accumulation in breast tumor tissues (Figure 4A). Moreover, our molecular results indicate that the anti-fibrosis mechanism of *L. pentosus*-derived CFS can be mediated by down-regulation of profibrotic genes, including *Col1a2* and *Col3a1*.

To further support the inhibitory effect of *Lactobacillus*-derived CFS on breast cancer cell migration, we evaluated its influence on 4T-1 cell migratory behavior. As shown in Figure 6A, administration of *L. pentosus*-derived CFS inhibits cell migration. These findings show that the anti-migratory effect of *Lactobacillus* CFS on 4T-1 cells is not due to cytotoxicity.

Growing evidence indicates that the Wnt/β-catenin signaling pathway regulates cancer cell migration and invasion. β-catenin, the central effector of the Wnt signaling pathway, contributes to metastasis and cell adhesion by regulating MMP expression and E-cadherin levels. Down-regulation of E-cadherin in tumor cells is associated with epithelial-mesenchymal transition (EMT) and metastatic spread. Moreover, cyclin D1 and MMPs, as other β-catenin downstream targets, are reported to be up-regulated in aggressive breast cancers (47-49). Increased MMP expression promotes angiogenesis, cell migration, and metastasis, which are associated with adverse clinical outcomes. Accumulating data suggest that inhibiting MMPs suppresses tumor cell migration and metastasis, as these endopeptidases degrade the extracellular matrix (ECM) and promote cancer cell invasion (50, 51). To investigate the anti-migratory effects of *Lactobacillus*-derived CFS, we performed qPCR to examine the mRNA expression of *cyclin D1*, *MMP-2*, *MMP-9*, and *E-cadherin* in breast tumor tissues. We showed that *L. pentosus*-derived CFS administration down-regulates *cyclin D1* and *MMPs* and enhances *E-cadherin* expression. Taken together, our findings suggest that the anti-migratory effects of *L. pentosus* may be mediated by inhibiting enzymatic ECM degradation and promoting E-cadherin-mediated cell adhesion. However, because we did not directly assess β-catenin protein levels, subcellular localization, transcriptional activity, or Wnt pathway activation, our findings do not establish that *Lactobacillus*-derived CFS directly inhibits Wnt/β-catenin signaling. Thus, this pathway remains a plausible hypothesis that requires direct experimental validation.

The findings of this study should also be interpreted in light of several limitations. First, the investigation relied exclusively on *in vitro* cell lines (4T1 and MCF-7) and a murine tumor model and did not evaluate human clinical samples or clinical data, which limits the generalizability of the observed responses across diverse human breast cancer subtypes. Second, because the experiments evaluated CFS rather than viable *Lactobacillus* strains, these results cannot be interpreted as evidence for the therapeutic effects of live probiotic colonization or bacterial administration. Third, the specific bioactive metabolites and active soluble compounds within the CFS responsible for the observed antitumor activity remain unisolated and structurally uncharacterized. Fourth, although changes in inflammatory cytokines were observed, comprehensive characterization of tumor-infiltrating immune-cell populations was not performed. This is particularly relevant because the tumor microenvironment and immune response may substantially influence the effects of bacterial-derived products. Finally, while alterations in key downstream molecular markers were observed, direct pathway-specific mechanistic validations were not conducted. In particular, because β-catenin protein levels, subcellular localization, and transcriptional activity were not directly measured, the precise involvement of pathways such as Wnt/β-catenin remains a plausible hypothesis that requires experimental confirmation.

Conclusion

We have shown that *L. pentosus*-derived CFS exerts

potent anticancer effects by regulating inflammatory responses, metastasis, and apoptosis in breast tumor cells and tissues. Our findings suggest that *L. pentosus*-derived CFS could potentiate the anticancer effects of 5-FU against breast cancer. Nevertheless, translating these preliminary findings will require future studies to isolate specific bioactive metabolites, validate the underlying molecular signaling pathways, and confirm efficacy in clinically relevant translational models.

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Authors' Contributions

F R, M K, and N AS designed the experiments; T G performed the experiments and collected data; F R and MK discussed the results and strategy; F R supervised, directed, and managed the study; F R, M K, N AS, and T G approved the final version for publication.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We acknowledge the use of [ChatGPT (OpenAI)] for language editing and proofreading during the preparation of this manuscript. The AI-generated suggestions were carefully reviewed and modified by the authors, who take full responsibility for the final content.

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