

Long-term exposure of *Lactobacillus rhamnosus* to quercetin enhances its anti-angiogenic and anti-colorectal cancer efficacy

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Abstract

Colorectal cancer (CRC) is the second leading cause of cancer death worldwide, with rising incidence projected over the coming decades. Here we show that prolonged exposure of *Lactobacillus rhamnosus* PTCC 1637 to quercetin (up to 200 µg/mL) improves its probiotic traits and markedly boosts the anticancer activity of its cell-free supernatant (que-CFS). Acid tolerance increased, and antibacterial zones against *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli* grew by 2–13 % using que-CFS compared with untreated CFS. In SW-480 colorectal adenocarcinoma cells, que-CFS lowered the 48-h IC₅₀ to 28.36 % (v/v) versus 65.7 % for CFS. Flow cytometry revealed 87.4 % apoptosis with que-CFS; DAPI staining confirmed greater nuclear fragmentation and a 57.9 % reduction in nuclear area. Que-CFS suppressed wound closure by 26.5 % at 48 h, reduced 3-D spheroid volume by 27 % at 96 h, and inhibited angiogenesis in the chick chorioallantoic membrane by 36.3 % versus 23.1 % for CFS. qRT-PCR showed stronger upregulation of Caspase-8, BAX and TP53. Proteinase K abolished antibacterial activity, confirming proteinaceous mediators. These data reveal that quercetin reprograms *L. rhamnosus* metabolism to yield metabolites with superior cytotoxic, anti-migratory, anti-spheroid, and anti-angiogenic effects, opening a dietary route to potentiate probiotic anticancer therapy.

Introduction

Cancer remains the second leading cause of mortality worldwide, surpassed only by cardiovascular diseases, with its incidence projected to double by 2070. Colorectal cancer (CRC), affecting both the colon and rectum, represents a major global health burden, accounting for nearly 1.9 million new cases and approximately 900,000 deaths in 2020. With population growth and aging, CRC incidence is expected to exceed 3.2 million cases by 2040 (Xi & Xu, 2021).

Conventional therapies, including surgical resection, chemotherapy, radiotherapy, immunotherapy, and targeted drugs, are largely stage-dependent. Despite their effectiveness, these approaches are limited by drug resistance, systemic toxicity, immune evasion, and high financial cost, often reducing therapeutic outcomes (Zafar et al., 2025). Consequently, there is growing interest in complementary and alternative approaches such as gene therapy (Hu et al., 2023), photodynamic therapy (Guidolin et al., 2022), dietary modulation (Zhong et al., 2025), and bacteriotherapy (Mueller et al., 2022). Evidence suggests that at least 50% of cancer cases are associated with specific compounds present in dietary patterns (Ross, 2010). Consequently, nutritional components, particularly probiotics, have attracted considerable research interest due to their potential impact on human health (Maftei et al., 2024). Probiotics are non-pathogenic microorganisms that confer health benefits to the host and have been suggested to possess anti-tumor properties. Administration of these beneficial bacteria in adequate amounts can modulate the composition and activity of the gut microbiota (Latif et al., 2023). While probiotics are widely employed to manage various gastrointestinal disorders, including diarrhea, infections, and inflammation, their potential role in the prevention and treatment of colorectal cancer remains under active investigation. Considering the influence of gut microbiota on colon cancer suppression, it is essential to develop novel interventions that can inhibit tumor cell growth while sparing healthy cells (Zhao et al., 2023). In this

context, probiotics emerge as a promising strategy. Probiotic bacteria may serve as effective chemopreventive agents against colon cancer by reducing cancer risk through multiple mechanisms, including modulation of the immune system, alteration of gut microbiota composition, enhancement of antioxidant activity, regulation of the cell cycle, and degradation of potential carcinogens such as mutagenic compounds and secondary bile acids produced by intestinal microbes (Ahmad et al., 2025). In addition, probiotics can strengthen the intestinal barrier, inhibit chronic inflammation, produce short-chain fatty acids with anti-proliferative effects, and modulate signaling pathways involved in tumor development and apoptosis (Neurath et al., 2025). Common probiotic strains include species from *Lactobacillus* and *Bifidobacterium* (Matera, 2024). *Lactobacillus* is a genus of Gram-positive, non-spore-forming bacteria commonly found in the human gut and fermented foods such as yogurt and kefir. These probiotics help maintain gut microbial balance by inhibiting pathogenic bacteria through the production of lactic acid and other antimicrobial compounds (Shah et al., 2024). They enhance intestinal barrier function, modulate the immune system by activating macrophages, dendritic cells, and regulatory T cells, and some strains exhibit potential anticancer effects by reducing inflammation, enhancing antioxidant activity, and influencing tumor-related signaling pathways (Mazziotta et al., 2023). *Lactobacillus* also contributes to overall gut health, aids in lactose digestion, and may provide protection against certain infections (Huang et al., 2022). *Lactobacillus rhamnosus* modulates the host immune system through regulation of cytokines and molecular signaling pathways, contributing to its anti-inflammatory and anti-cancer effects also increases anti-inflammatory cytokines such as IL-10 and TGF- β while reducing pro-inflammatory cytokines, including TNF- α , IL-6, IL-1 β , and IFN- γ . At the molecular level, LGG inhibits NF- κ B activation, interacts with TLR2 and TLR4, modulates MAPK, PI3K/Akt, and Wnt/ β -catenin pathways, and reduces STAT3 activation, collectively leading to decreased chronic inflammation, enhanced apoptosis in cancer cells, and improved gut microbial balance (Leser & Baker, 2024; Plaza-Diaz et al., 2014; Segers & Lebeer, 2014). Quercetin is a naturally occurring flavonoid found in fruits, vegetables, and plants such as apples, onions, grapes, broccoli, and green tea, and has attracted considerable attention due to its significant anticancer properties (Aghababaei & Hadidi, 2023). This compound can induce cell cycle arrest and promote apoptosis in cancer cells. Quercetin modulates tumor-related signaling pathways, including NF- κ B, MAPK, PI3K/Akt, and Wnt/ β -catenin, leading to the inhibition of cancer cell proliferation, reduction of metastasis, and suppression of tumor angiogenesis in various cancers, including colon, breast, prostate, and lung cancers (Reyes-Farias & Carrasco-Pozo, 2019). Furthermore, its antioxidant and anti-inflammatory activities help reduce oxidative stress and chronic inflammation, creating an unfavorable microenvironment for tumor progression and thereby contributing to cancer prevention (Lin et al., 2019). Emerging evidence suggests that quercetin may influence the activity of probiotics by modulating gut microbiota and enhancing their beneficial effects, particularly through antioxidant and anti-inflammatory mechanisms (D'Arrigo et al., 2024). Although direct studies on the synergistic effects of quercetin and probiotics are limited, these observations indicate a promising strategy for colorectal cancer prevention, warranting further investigation (Sreenesh et al., 2025). Dietary patterns likely shape the functional traits of probiotics within the host gastrointestinal environment by altering their metabolic output, stress resistance, and production of bioactive compounds that influence cancer-related cellular processes. In this context, this study

investigates the influence of quercetin on the anticancer activity of *L. rhamnosus* metabolites derived from quercetin-treated and untreated bacteria were evaluated against the SW-480 cell line. Cell viability was initially evaluated using the MTT assay to assess the cytotoxic effects of bacterial metabolites. Apoptotic and necrotic responses were subsequently examined through flow cytometry (Annexin V/PI) and supported by 4',6-diamidino-2-phenylindole (DAPI) staining to detect nuclear condensation and fragmentation. Additionally, a wound healing assay was performed to evaluate cell migration and determine the influence of different treatments on SW-480 cell motility. determine the inhibitory activity of CFSs, while a three-dimensional spheroid model was applied to examine its cytotoxic effects on tumoroids within a 3D microenvironment. The anti-angiogenic potential of CFS and que-CFS was confirmed using the chorioallantoic membrane (CAM) assay, and finally, the expression levels of selected genes were quantified using the quantitative real-time PCR (qRT-PCR) technique.

Experimental

Materials

The standard strains of *L. rhamnosus* PTCC 1637, *Staphylococcus aureus* PTCC 1917, *Escherichia coli* PTCC 1276, and *Salmonella typhi* PTCC 1639 were bought from the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The SW-480 human colorectal adenocarcinoma cell line was sourced from the Pasteur Institute, Tehran, Iran. de Man, Rogosa, and Sharpe (MRS) agar broth (catalogue No: 1.0661.0500), MRS agar (catalogue No: 1.0660.0500), Mueller-Hinton Agar (catalogue No: 1.05437.0500), Nutrient Broth (catalogue No: 1.05443.0500), and Yeast extract (catalogue No: 1.03753.0500) were provided by Merck (Darmstadt, Germany). Diphenyltetrazolium bromide (MTT) (catalogue No: 475989) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) (catalogue No: 16000044), RPMI 1640 medium (catalogue No: 11875093), and penicillin-streptomycin (catalogue No: 15140122) were obtained from Gibco Life Technologies (Waltham, MA, USA). Trypsin-EDTA (catalogue No: T4049), pipettes, and cell culture plates of 6, 24, and 96 wells were purchased from SPL Life Sciences (Gyeonggi-do, South Korea). Diethyl pyrocarbonate (DEPC), Triton X-100, and Proteinase K were purchased from Sigma-Aldrich (St. Louis, MO, USA). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany). Quercetin (catalogue NO Q4951) was provided by Sigma Aldrich. Apoptosis was assessed using the FITC Annexin V Apoptosis Detection Kit (purchased from Immunostep, Salamanca, Spain) according to the manufacturer's instructions.

Preparation of cell-free supernatant (CFS) from untreated and quercetin-treated *L. rhamnosus* (que-CFS)

The bacterial strain *L. rhamnosus* was incubated in MRS broth at 37°C for 24 h, and the culture density was adjusted to an OD₆₀₀ of 0.7–0.8 as determined by a UV–visible spectrophotometer (Cambridge, UK). Bacterial density was standardized to ~ 10⁹–10¹¹ CFU/mL, reflecting intestinal levels. Bacteria cultured with quercetin became resistant to higher doses through gradual exposure to increasing concentrations

(20,40,60,80,100,120,140,160,180 and 200 µg/mL). Bacteria were treated with each concentration of quercetin until the cultures reached a density of $\sim 10^9$ – 10^{11} CFU/mL. Post-incubation, bacterial cultures exhibiting resistance to the highest quercetin concentration (200 µg/mL), along with untreated controls, were centrifuged at $3500 \times g$ for 10 min at 4°C to separate supernatants from cell pellets. For the bacterial study, a bacterial cell pellet was used in the acid resistance assay. In the antibiogram test, the filtered cell-free supernatant from both quercetin-treated and untreated bacteria, adjusted to pH 7.4, was employed. For the in vitro study, bacterial cell pellets were rinsed twice with PBS, and RPMI was added as the culture medium 24 h prior to supernatant collection. After incubation, the supernatants were subjected to double filtration through 0.22 µm filters to ensure sterility before use. The supernatants from the untreated and quercetin-treated cultures were collected and referred to as CFS and que-CFS, respectively, and their potential anti-cancer effects against the SW-480 human colorectal cancer cell line were investigated.

Resistance to low pH environments

To simulate gastric acidic conditions (Naissinger da Silva et al., 2021), the acid tolerance of *L. rhamnosus* treated with quercetin was evaluated in phosphate buffer (0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4) adjusted to pH 3.0. Following 24 hours of incubation in MRS broth, both treated and untreated bacterial cultures were centrifuged ($3500 \times g$, 4°C, 10 min) and rinsed twice with PBS at pH 7.4. Bacterial pellets were resuspended in phosphate buffer (pH 3.0) and incubated for 0.5, 1, 2, and 3 hours. At each time point, using the pour plate technique, bacterial suspensions were inoculated onto MRS agar and incubated at 37°C for 48 hours to promote colony formation. The number of colonies was counted and compared between quercetin-treated and untreated groups at each time point to evaluate whether quercetin exposure had a positive, negative, or neutral effect on acid resistance and probiotic potential of *L. rhamnosus*.

Enzymatic digestion assay for identifying protein-dependent antibacterial metabolites

To evaluate whether the antibacterial metabolites produced by untreated and quercetin-treated *L. rhamnosus* are protein-dependent, the cell-free supernatants (CFS and que-CFS) were treated with Proteinase K (1 µL/mL) at 37°C for 55 minutes to digest potential protein components. The enzyme was then inactivated by incubating the samples at 70°C for 20 minutes. Subsequently, 150 µL of each digested supernatant was applied to sterile paper discs and placed on agar plates inoculated with pathogenic bacteria. After 24 hours of incubation at 37°C, antibacterial activity was assessed using the disc diffusion method. A reduction or loss of the inhibition zone indicated that the active metabolites were likely protein-dependent.

Antibacterial activity assessment by zone of inhibition test

The disk diffusion method was utilized to determine the growth inhibition zones were assessed to determine the antibacterial efficacy of CFSs against clinically isolated pathogenic bacteria (Abdelhamid et al., 2018; Tsega et al., 2023). (Gram-negative bacteria *Escherichia coli* and *Salmonella typhi* were used, and the gram-positive bacteria included *Staphylococcus aureus*). For this purpose, a fresh overnight culture from treated and untreated *L. rhamnosus* was prepared. In this study, MRS broth was used for the culture of *L. rhamnosus*, and Muller-Hinton Broth culture medium was used for the culture of pathogenic strains. After that, CFSs from treated and untreated *L. rhamnosus* were prepared and sterilized by filtration through a 0.22 µm filter. Following this, every pathogenic strain investigated in the study was cultured in separate plates. To assess antibacterial activity, sterile disks (5 mm) containing CFS or que-CFS (150 µL) were placed on agar plates, with PBS-soaked disks (pH 7.4) serving as negative controls and standard antibiotics as positive controls. Plates were incubated at 37°C for 24 h to facilitate the diffusion of compounds into Mueller-Hinton agar. The diameters of inhibition zones were determined using an electronic digital caliper, and the measurements were recorded for further analysis. For the antibacterial test, an Azithromycin (30 µg) disc was used as the sensitive antibiotic and an ampicillin (30 µg) disc as the resistant antibiotic for *Escherichia coli* and *Salmonella typhi*. For *Staphylococcus aureus*, Vancomycin (30 µg) was applied as the sensitive antibiotic and cefazolin (30 µg) as the resistant one.

Cell culture

The SW-480 colorectal cancer cell line was utilized in this study. Cryopreserved cells were thawed and cultured according to the standard protocol provided by the supplier. For experimental procedures, cells in the logarithmic growth phase with a confluency of approximately 60–80% were selected to ensure optimal viability and responsiveness to treatments. SW-480 human colorectal adenocarcinoma cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at 37°C in a humidified atmosphere containing 5% CO₂.

In-vitro MTT cell viability assay

The MTT assay is a colorimetric method used to assess cell viability. Metabolically active cells reduce the yellow MTT reagent to purple formazan crystals via mitochondrial enzymes. The crystals are then dissolved in a solvent such as DMSO, and absorbance is measured at 570 nm, providing a quantitative estimate of viable cells and cytotoxic effects of treatments (van Meerloo et al., 2011). In this research, the MTT assay was employed to evaluate the cytotoxic effects of CFS and que-CFS on SW-480 cells cultured in 96-well plates. Cells were seeded at a density of 1×10^4 cells per well and incubated for 24 hours before being treated with varying concentrations of CFS and que-CFS prepared in RPMI medium. Negative control consisted of cells treated with RPMI, and positive controls contained 5% (v/v) DMSO in the RPMI. After 24, 48, and 72 hours after treatment with CFSs, MTT solution (2 mg/mL) was added to each well and incubated for 4 hours. Formazan crystals formed by metabolically active cells were then dissolved in DMS (Absorbance was measured at 570 nm using a RayBiotech Stat Fax 4200 ELISA reader

(Norcross, GA, USA)). The optical density is proportional to the number of viable cells, allowing quantitative assessment of cell viability and cytotoxic effects of the tested compounds.

Assessment of nuclear morphology by 4',6-diamidino-2-phenylindole (DAPI) staining

To investigate nuclear morphological alterations indicative of apoptosis, DAPI staining was employed (Eidet et al., 2014). Sterile glass coverslips were positioned in 6-well culture plates, and 4×10^5 SW-480 cells were seeded into each well. After 24 h of incubation, the culture medium was replaced with treatment media comprising complete RPMI (negative control), RPMI supplemented with 5% (v/v) DMSO (positive control), and RPMI containing CFS or que-CFS at the 48-h IC_{50} concentration of que-CFS (28.36% v/v). Following 48 hours of the culture media were removed, and the cells were fixed with 4.5% paraformaldehyde for 40 minutes at room temperature. Cells were then washed with PBS and permeabilized with 0.5% (v/v) Triton X-100 for 5 minutes. Following additional PBS washes, nuclei were stained with DAPI (20 ng/mL) for 20 minutes in the dark. Microscopic images of the samples were captured using a fluorescence microscope (Olympus IX81). Images were captured in bright-field, fluorescence, and merged formats. For quantitative analysis, the ImageJ software was utilized to assess the nuclear area. Micrographs were converted to 8-bit grayscale, and threshold values were adjusted to ensure accurate nuclear segmentation. Non-nuclear artifacts were excluded, and overlapping nuclei were separated using the “Watershed” algorithm. The nuclear area was quantified using the “Analyze Particles” function. For each condition, 100 nuclei were selected for analysis. Results were expressed as normalized nuclear area relative to the negative control.

Migration assay

The scratch assay is commonly employed to evaluate cell migration and proliferation abilities (Gnerucci et al., 2020). In this assay, a confluent cell monolayer was established. Approximately 1×10^5 cells were seeded into each well of a 24-well plate, which was subsequently incubated under standard conditions. Upon reaching confluence (approximately 90–100%), a straight-line scratch was made across the cell monolayer using a pipette tip. Following the scratch, the cells were gently washed with PBS to remove any detached cells. The wells were then replenished with the appropriate concentrations of CFSs. Negative and positive controls consisted of RPMI medium and RPMI medium containing DMSO 5% (v/v), respectively. Cell migration images were captured using an inverted light microscope CKX41 (Olympus Corp., Tokyo, Japan) and Cell^AA software. The width of the cell-free gap was monitored over a period of three days, and the resulting data were analyzed using one-way ANOVA followed by Tukey's assay.

Assessment of cell death mechanism by Annexin V-FITC/PI staining

Apoptosis, including early and late stages as well as necrosis, was quantitatively evaluated using the Annexin V-FITC/PI apoptosis detection kit (Immunostep). Annexin V, a calcium-dependent phospholipid-

binding protein, binds specifically to phosphatidylserine (PS), which is translocated to the outer plasma membrane during early apoptosis. Propidium iodide (PI), a membrane-impermeable dye, stains DNA in cells with compromised membranes, thereby identifying late apoptotic or necrotic cells (Kari et al., 2022). SW-480 cells were seeded in 6-well culture plates at a density of 4×10^5 cells/well. After 24 hours, the culture medium was replaced with treatment media: complete RPMI (negative control), RPMI containing DMSO 5% (v/v) (positive control), and RPMI supplemented with CFS and que-CFS at a final concentration of 28.36% (v/v), corresponding to the IC_{50} value of que-CFS determined by MTT assay. Following 48 hours of treatment, cells were trypsinized, collected, and centrifuged at 300 g for 6 minutes at 10°C. The cell pellets were washed with PBS and resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). To stain the cells, 5 μ L each of Annexin V-FITC and PI was added to 500 μ L of cell suspension and incubated at room temperature for 15 minutes in the dark. Flow cytometric analysis was subsequently carried out on a BD FACSCalibur™ flow cytometer (FACSQuant; Miltenyi, Germany), recording 10,000 events per sample.

Evaluation of CFS- and que-CFS–Induced Modulation of Pro-Apoptotic Genes Using Real-Time qPCR

The expression levels of the pro-apoptotic genes Caspase-8, Bax, and TP53 following treatment with CFS and que-CFS were quantified using real-time PCR (qPCR). GAPDH served as the housekeeping gene for normalization. Forward and reverse primers for each target gene were designed using Oligo 7 software, synthesized by Sinacolon (Tehran, Iran), and are listed in Table 1.

SW-480 human colorectal adenocarcinoma cells were seeded in 6-well plates and cultured under standard conditions prior to treatment. Cells were then exposed to the respective IC_{50} concentrations of the CFSs and incubated for 48 hours. Following treatment, total RNA was extracted and reverse-transcribed into cDNA.

qPCR reactions were performed using a Bio system instrument with 2X SYBR® Green Applied Master Mix in a 20 μ L reaction volume comprising 10 μ L master mix, 1 μ L cDNA, 0.5 μ L of each primer, and nuclease-free water. The thermal cycling protocol included an initial denaturation at 94°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, gene-specific annealing at 60–62°C for 30 seconds, and extension at 72°C for 25 seconds.

Relative gene expression levels were calculated using the Pfaffl method, with CT values normalized to GAPDH. Negative controls were included in each run, and all reactions were performed in triplicate.

Table 1
Primers utilized for quantitative RT-PCR.

Gene name	Primer	Primer sequence (5'→3')	Length of product	Genbank No
BAX	Forward	GATGCGTCCACCAAGAAG	163	NR_027882
	Reverse	AGTTGAAGTTGCCGTCAG		
Caspase-8	Forward	GGTCTGAAGGCTGGTTGTTC	237	NM_001228.4
	Reverse	AATCTCAATATTCCCAAGGTTCAAG		
TP-53	Forward	AGATAGCGATGGTCTGGC	147	NM_000546.6
	Reverse	ATGGTGGTACAGTCAGAGC		
GAPDH	Forward	AAGCTCATTTCCTGGTATGACAACG	126	NM_002046.3
	Reverse	TCTTCCTCTTGCTCTTGCTGG		

Evaluation of the effects of CFS and que-CFS on the three-dimensional (3D) model of SW-480

To evaluate the cytotoxic effects of the treatments, a 3D spheroid model of SW-480 cells was utilized, as it better mimics the *in vivo* tumor environment compared to conventional 2D cultures. This model is widely used to study cell proliferation, viability, and drug responses in cancer research (Mangani et al., 2025). Spheroids were generated in 96-well round-bottom plates. Initially, 40 μ L of agarose (1% w/v) solution was added to the bottom of the well to prevent cell attachment to the surface. After the agarose solidified, 1×10^4 cells were seeded into each well, and the plates were centrifuged at $160 \times g$ for 15 minutes to facilitate compact spheroid formation. The plates were then incubated for 6–7 days to allow spheroid development. Following spheroid maturation, the culture medium was replaced with treatment media, including CFS, que-CFS, negative control, and positive control with DMSO 5% (v/v). Spheroid images were captured using an inverted light microscope CKX41 (Olympus Corp., Tokyo, Japan) and Cell A software. Spheroid size was measured up to 96 hours post-treatment, and the data were analyzed as the percentage of size reduction normalized to the negative control to evaluate the effects of treatments on spheroid growth. The morphological groups of spheroids, including negative control and the spheroids treated with CFS and que-CFS, were investigated using a Field-Emission Scanning Electron Microscopy (FE-SEM) (MV2300, Brno, Czech Republic). Groups were fixed in 2.5% (v/v) glutaraldehyde at 4°C for 4 h, dehydrated using a graded ethanol series (50%–90%, 30 min per step), and stored in absolute

ethanol. Following air-drying, spheroids were mounted on SEM stubs and sputter-coated with a thin gold layer using a direct current sputter coater (Emitech K450X, UK) via physical vapor deposition (PVD). Scanning electron microscopy (SEM) was then employed to acquire micrographs.

Chick chorioallantoic membrane (CAM) angiogenesis assay

The chick chorioallantoic membrane (CAM) assay was employed to assess the anti-angiogenic effects of the treatments. The CAM model is widely used for angiogenesis research due to its dense vascularization and accessibility, which allow direct in vivo observation of vessel formation (Kennedy et al., 2021). Fertilized ROSS 308 chicken eggs were incubated at 37.5°C with 60% relative humidity in an egg incubator. On day 11 of incubation, a small window was carefully created over an area with a well-developed vascular network, and sterile discs containing the respective treatments were gently placed onto the chorioallantoic membrane. Following 24 hours of incubation, the CAMs were examined for angiogenic responses. The experimental groups included a negative control (PBS), a positive control (bevacizumab (Gholipour et al., 2023), 25 mg/mL), CFS, and que-CFS. Images of the CAM vasculature were captured and analyzed using ImageJ software. Each image was converted to 8-bit format, and the threshold was adjusted to enhance vessel visibility. The vascularized area was quantified and normalized to the negative control group to determine the relative inhibition of angiogenesis.

Statistical analysis

All experiments were independently repeated three times, and data were expressed as mean $\pm$ standard deviation (Mean $\pm$ SD). Differences among experimental groups were analyzed using one-way ANOVA followed by Tukey's post hoc test, conducted in GraphPad Prism (version 10). Statistical significance was defined as $p < 0.05$.

Results

Progressive quercetin exposure resulted in full adaptation and resistance in *L. rhamnosus*

L. rhamnosus bacteria were cultured in MRS broth and exposed to progressively increasing concentrations of quercetin in a stepwise manner to promote adaptation and enhance resistance to high quercetin levels. Quercetin was dissolved in MRS broth and applied at concentrations ranging from 20 to 200 $\mu\text{g/mL}$. **Figure 1(A)** presents the growth profiles of *L. rhamnosus* across these concentrations, normalized to the growth of untreated controls.

As shown in **Figure 1(A)**, the initial introduction of quercetin resulted in a pronounced reduction in bacterial growth. However, repeated subculturing at the same concentration led to a recovery of growth to levels comparable to, or exceeding, those of untreated bacteria. In many cases, growth performance improved beyond baseline. The results also demonstrate that while early growth patterns differed among the various quercetin concentrations, continuous subculturing in the presence of the compound gradually produced more uniform growth trajectories. These observations indicate successful bacterial

adaptation, culminating in the ability of *L. rhamnosus* to withstand and grow in 200 µg/mL quercetin. This outcome confirms the development of enhanced tolerance and stable resistance to quercetin.

Once optimal and stable growth was achieved at the final quercetin concentration, cell-free supernatants from both quercetin-treated (que-CFS) and untreated (CFS) cultures were obtained by centrifugation at 3000×g. These supernatants were subsequently used in further experiments to compare the antibacterial and cytotoxic activities of metabolites produced by quercetin-adapted versus untreated *L. rhamnosus* through a series of antibacterial and cellular assays.

Quercetin-adapted *L. rhamnosus* demonstrated enhanced survival under acidic conditions

The acid resistance of quercetin-treated *L. rhamnosus* was evaluated using a phosphate-buffered solution adjusted to pH 3.0, simulating the acidic environment of the gastrointestinal tract. As illustrated in **Figures 1(Aa), (Ab), and (Ac)**, quercetin-adapted bacteria showed consistently higher colony counts than untreated bacteria when exposed to acidic conditions (pH 3.0) across multiple time points (0.5, 1, 2, and 3 hours). These results indicate that the adaptation of *L. rhamnosus* to quercetin not only preserved its inherent acid tolerance but further enhanced its ability to withstand and survive under low-pH conditions.

Quercetin-adapted *L. rhamnosus* displayed increased antibacterial activity across all tested pathogens

As presented in **Table 2** and **Figure 1(Aa) and (Ab)**, both CFS and que-CFS derived from *L. rhamnosus* demonstrated antibacterial activity against all assessed pathogenic bacteria. However, que-CFS consistently produced larger inhibition zones than CFS, measuring 11.8 vs. 11.5 mm for *S. aureus*, 10.7 vs. 10.2 mm for *S. typhi*, and 11.6 vs. 10.4 mm for *E. coli*. Although the magnitude of these differences was modest, the uniform increase observed across all pathogens indicates that quercetin exposure enhanced the antibacterial capacity of the probiotic. The reproducible improvement suggests that adaptation to quercetin may promote elevated production or enhanced functionality of antimicrobial metabolites, thereby strengthening the inhibitory performance of *L. rhamnosus*.

Proteinase K treatment eliminated the antibacterial activity of both CFS and que-CFS, confirming the protein-based nature of their active metabolites

Probiotics are well known for their ability to inhibit pathogenic microorganisms. In this study, the protein-based characteristics of antimicrobial metabolites produced by untreated and quercetin-treated *L. rhamnosus* (CFS and que-CFS, respectively) were evaluated. As shown in **Table 2** and **Figure 1(Cb)**,

pretreatment of both supernatants with proteinase K completely abolished their antibacterial activity, demonstrated by the disappearance of inhibition zones against all tested pathogens, including *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli*. The loss of inhibitory activity following enzymatic digestion confirms that the active antibacterial compounds present in both CFS and que-CFS are protein-derived, likely representing bacteriocins or antimicrobial peptides. This observation is consistent with the established behavior of probiotic-associated antimicrobial agents, which are characteristically heat-labile and sensitive to proteolytic degradation.

Table 2. Inhibition zone diameters of CFS and que-CFS against pathogenic bacteria *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli*.

Strain	Supernatant type	Dimme
<i>Staphylococcus aureus</i>	CFS	11.5 ±1.2
	CFS+ PK	0.0 ±0.0
	que-CFS	11.8 ±0.4
	que-CFS+PK	0.0 ±0.0
	Positive Control (Vancomycin)	0.0 ±0.0
	Negative Control (Cefazolin)	0.0 ±0.0
	PBS	0.0 ±0.0
<i>Salmonella typhi</i>	CFS	10.2 ±0.4
	CFS+ PK	0.0 ±0.0
	que-CFS	10.7 ±0.4
	que-CFS+PK	0.0 ±0.0
	Positive Control (Azitromycin)	23.3 ±0.3
	Negative Control (Ampicilin)	0.0 ±0.0
	PBS	0.0 ±0.0
<i>Escherichia. coli</i>	CFS	10.4±0.1
	CFS+ PK	0.0 ±0.0
	que-CFS	11.6 ±0.1
	que-CFS+PK	0.0 ±0.0
	Positive Control (Azitromycin)	21.1 ±0.9
	Negative Control (Ampicilin)	0.0 ±0.0
	PBS	0.0 ±0.0

Que-CFS exhibited significantly enhanced cytotoxicity against SW-480 colorectal cancer cells

The cytotoxic effects of CFS and que-CFS were evaluated using the SW-480 human colorectal cancer cell line. As shown in **Figure 2(A)** and **(B)**, exposure to increasing concentrations of both CFS and que-CFS (35%, 50%, 75%, 85%, and 100% v/v) resulted in a dose-dependent reduction in cell viability. Notably, que-CFS consistently produced higher levels of cytotoxicity compared to CFS at all tested concentrations. MTT assay analysis further demonstrated that the IC₅₀ value for CFS at 48 hours was 65.7% (v/v),

whereas que-CFS exhibited a markedly lower IC₅₀ of 28.36% (v/v), as shown in **Figure 2(C)**. This reduction was statistically significant ($P < 0.0001$), indicating a substantial enhancement in the cytotoxic potential of the metabolites produced by quercetin-adapted *L. rhamnosus*.

Que-CFS induced pronounced apoptotic nuclear changes in SW-480 cells

DAPI staining revealed distinct nuclear morphological alterations associated with apoptosis across different treatment groups. As shown in **Figure 3(A)**, bright-field, fluorescence, and merged microscopy images demonstrated that untreated control cells maintained elongated morphology with uniformly stained, healthy nuclei. In contrast, DMSO-treated positive control cells exhibited characteristic apoptotic features, including nuclear condensation and fragmentation. Cells treated with CFS displayed mild apoptotic changes, with a few condensed and intensely stained nuclei. Notably, que-CFS-treated cells exhibited extensive nuclear fragmentation, indicative of late-stage apoptosis.

Quantitative analysis performed using ImageJ and GraphPad Prism Version 10 confirmed a significant reduction in nuclear area: 15.9% in the CFS group and 57.86% in the que-CFS group relative to the negative control (**Figure 3(B)**). Consistent with these observations, Annexin V/PI flow cytometry of SW-480 cells revealed that que-CFS induced a significantly higher rate of apoptosis compared to both CFS-treated and control cells. These findings collectively indicate that quercetin adaptation markedly enhances the pro-apoptotic potential of *L. rhamnosus* metabolites.

Que-CFS significantly inhibited Sw-480 cell migration

Cell migration, a critical process in cancer metastasis (Castaneda et al., 2022), was assessed in SW-480 cells using a scratch assay to evaluate the effects of CFS and que-CFS. Cells were treated with the respective supernatants for 24 and 48 hours, and wound closure was quantified at each time point. At 24 hours, que-CFS-treated cells exhibited a 15.7% reduction in wound closure, whereas CFS-treated cells showed a 9.3% reduction compared to untreated controls. By 48 hours, que-CFS inhibited migration by 26.5%, in contrast to an 18.2% reduction observed in the CFS group (**Fig. 3(C)** and **(D)**).

These findings indicate that que-CFS more effectively suppresses SW-480 cell migration than CFS, suggesting that metabolites produced by quercetin-adapted *L. rhamnosus* may contribute to the inhibition of metastatic potential in colorectal cancer cells.

Que-CFS enhanced pro-apoptotic gene expression in SW-480 cells

Quantitative PCR (qPCR) was employed to assess changes in gene expression and to validate findings from the preceding functional assays. As shown in **Fig. 4(C)**, treatment with que-CFS significantly upregulated the pro-apoptotic gene Bax compared to both CFS-treated and control cells. Expression of

Caspase-8 was also markedly elevated, with significant increases observed in the CFS group ($***P < 0.01$) and que-CFS group ($****P < 0.0001$) relative to the control, and higher levels in que-CFS-treated cells compared to CFS-treated cells. Although TP53 expression was not significantly altered, both CFS and que-CFS groups displayed a noticeable increase compared with controls ($**P < 0.1$).

These results confirm that que-CFS enhances apoptosis in SW-480 cells through the upregulation of key pro-apoptotic genes, including Bax, Caspase-8, and TP53, supporting the mechanistic basis for the increased cytotoxicity and apoptosis observed in earlier assays.

Que-CFS exerts enhanced cytotoxic effects in 3D tumor spheroids

Three-dimensional (3D) spheroid models closely recapitulate the native tumor microenvironment by providing physiologically relevant cell–cell and cell–matrix interactions, realistic nutrient and oxygen gradients, and more predictive responses to therapeutic agents compared to conventional two-dimensional (2D) cultures (Dave et al., 2025). The cytotoxic effects of CFS and que-CFS on SW-480 3D spheroids were evaluated over 96 hours, as shown in **Figure 5(A)** and **(C)**. Both treatments reduced spheroid size, with que-CFS demonstrating a more pronounced inhibitory effect. At 24 hours, spheroid size decreased by 7.23% in the CFS group and 8.69% in the que-CFS group, representing a 16.8% greater inhibition by que-CFS, although this difference was not statistically significant. By 48 hours, the inhibitory effect became more distinct, with CFS reducing spheroid size by 10.25% and que-CFS causing a 16.43% reduction, reflecting a significant 37.6% greater inhibition. At 72 hours, que-CFS inhibited 18.01% of spheroid growth compared to 13.58% for CFS (25% greater inhibition). By 96 hours, que-CFS achieved a 21.08% reduction in spheroid size, whereas CFS caused a 15.33% reduction, corresponding to a significant 27% greater inhibition by que-CFS. These results indicate that que-CFS exerts a more potent inhibitory effect on spheroid growth and structural integrity.

Morphological assessment using scanning electron microscopy (SEM) further supported these findings (**Figure 5(B)**). Representative SEM micrographs revealed that control spheroids maintained a well-organized architecture, with smooth surfaces, strong intercellular adhesion, and an intact cellular network indicative of healthy morphology. CFS-treated spheroids exhibited structural alterations, including surface irregularities, partial loss of cell-to-cell cohesion, and disruption of the cellular network. Notably, que-CFS-treated spheroids displayed pore formation and cellular shrinkage, indicating significant structural damage and compromised cellular integrity. These observations demonstrate that quercetin adaptation enhances the antitumor efficacy of *L. rhamnosus* metabolites in a physiologically relevant 3D colorectal cancer model.

Que-CFS more effectively inhibited angiogenesis

While angiogenesis is necessary for normal tissue development, its dysregulation plays a key role in tumor progression and metastasis (Asprițoiu et al., 2021). Targeting angiogenesis has become a

promising therapeutic approach in cancer management. Owing to its rich vascular network, the chorioallantoic membrane (CAM) assay serves as a robust, cost-effective, and versatile *in vivo* model for assessing the angiogenic or anti-angiogenic potential of therapeutic agents (Dhara et al., 2018). Probiotics can modulate angiogenesis by regulating inflammatory pathways and vascular growth factors, thereby potentially inhibiting the formation of new blood vessels in tumor tissues (Dhara et al., 2018). The CAM assay was performed using the CFS Que-CFS bevacizumab (an anti-VEGF agent) was used as the positive control, and PBS served as the negative control. As shown in **Fig. 6(B)**, the CFS inhibited angiogenesis by 23.13% and the que-CFS by 36.24% after 24 hours compared to the negative control. Moreover, the vessel diameter in the que-CFS was smaller compared to that observed in the CFS.

Discussion

The modulation of the human gut microbiota has recently emerged as a promising strategy for both the prevention and management of colorectal cancer (Dehghani et al., 2021). CRC is a multifactorial disease. Among known risk factors are diet, lifestyle, and genetics. Studies indicate that the gut microbiome significantly influences CRC development. As most of the body's microbiota resides in the colon, disruptions in this microbial balance can impair metabolism and immunity, contributing to gastrointestinal disorders and cancer progression (Ghorbani et al., 2022). The gut microbiome contributes to the biosynthesis of metabolites that act as key mediators in oncogenic signaling pathways, thereby playing an important role in both the prevention and regulation of cancer development (Jaye et al., 2022). Probiotics, as beneficial members of the intestinal microbiota, are live microorganisms that promote host health (Ağagündüz et al., 2023). Their protective effects are attributed to strengthening the intestinal barrier, inhibiting pathogenic bacteria, and producing antioxidant and anticarcinogenic metabolites such as short-chain fatty acids and vitamins (Ferrer et al., 2024). Probiotics can bind and eliminate carcinogenic compounds through interactions with their cell wall components. They also reduce chronic intestinal inflammation, a key factor in colorectal cancer, by modulating immune responses, enhancing mucin and IgA production, and promoting apoptosis and cell differentiation to inhibit tumor growth (Takeuchi & Ohno, 2022). *L. rhamnosus* is a highly resilient probiotic with several proven benefits. It can survive acidic gastric conditions, strongly adhere to the intestinal mucosa, and form protective biofilms that support epithelial integrity. *L. rhamnosus* also inhibits pathogenic bacteria, protects healthy intestinal cells from physical and chemical stress, and modulates immune responses by enhancing Th1 activity, reducing inflammation, and promoting anti-tumor immunity. Clinically, it is widely used to manage diarrhea, mucositis, leaky gut, and gut inflammation linked to infections, antibiotics, and cancer therapies (Huang et al., 2019). Long-term dietary patterns play a crucial role in modulating the composition and metabolic activity of the gut microbiota, primarily by influencing the production of microbial-derived metabolites (Kuru-Yaşar & Üstün-Aytekin, 2024). Quercetin is a naturally occurring flavonoid with well-documented anti-inflammatory, antioxidant, and anticancer properties. It exerts its effects by scavenging free radicals and modulating key signaling pathways such as PI3K/Akt and NF-κB, thereby promoting apoptosis and inhibiting cancer

cell proliferation and metastasis (Neamtu et al., 2022). In a previous study, it was reported that quercetin exerts anticancer effects in human colon cancer cells (CACO-2 and SW-620) by inducing apoptosis. The underlying mechanisms involved inhibition of the NF- κ B signaling pathway, Bcl-2, and upregulation of Bax, supporting the potential clinical application of quercetin in colon cancer management (Zhang et al., 2015). Motivated by the potential chemopreventive and anticancer properties of quercetin, as well as its ability to modulate cellular signaling pathways involved in apoptosis and oxidative stress, the present study aimed to investigate the effects of quercetin on the metabolic profile of the probiotic *L. rhamnosus* and its subsequent impact on colorectal cancer cell proliferation and apoptosis. This approach provides insight into the synergistic interactions between dietary flavonoids and probiotic-derived metabolites in the context of colorectal cancer prevention and therapy. Similarly, our prior work revealed that High doses of curcumin and crocin, natural food antioxidants, were found to alter the metabolomics of *L. plantarum* and *L. lactis*, conferring enhanced anticancer properties (Gholipour et al., 2023; Gholipour et al., 2024). Furthermore, we have shown that elevated levels of quercetin, a natural dietary antioxidant, can reprogram the metabolomic activity of *L. rhamnosus*, thereby enhancing its anticancer potential. MTT assay results demonstrated that both que-CFS and CFS inhibited the proliferation of SW-480 colorectal cancer cells in a dose-dependent manner, with IC₅₀ values of 28.36% (v/v) and 65.7% (v/v) after 48 hours, respectively. A widely recognized strategy for cancer therapy involves the induction of apoptosis in tumor cells. Recent studies have demonstrated that the CFS of *L. rhamnosus* can effectively promote apoptotic cell death in colorectal cancer cell lines, highlighting the potential of probiotic-derived metabolites as adjunctive agents in anticancer interventions (Dehghani et al., 2021). In line with these data, flow cytometry analysis indicated that cell death occurred primarily through apoptosis, as evidenced by Annexin V/FITC staining and DAPI imaging. Nuclear fragmentation and apoptotic body formation, along with the induction of late apoptosis, were observed in que-CFS-treated cells, whereas CFS predominantly induced early apoptosis. Thoda et al (2023) demonstrated that CFS derived from *L. rhamnosus* GG inhibited CRC cell invasion by reducing matrix metalloproteinase-9 (MMP-9) expression and increasing tight junction proteins such as zona occludens-1 (ZO-1) and tissue inhibitor of metalloproteinase (TIMP) levels (Thoda & Touraki, 2023). In this study, we evaluated the anti-migratory potential of que-CFS. Consistent with previous findings, our scratch assay demonstrated that que-CFS more effectively inhibited cell migration compared to CFS, showing 19% and 30% greater suppression at 24 and 48 hours, respectively. A study in 2025 reported that the supernatant of *L. rhamnosus* DS3316 reduced spheroid aggregation and inhibited cell growth in HCT116 3D models (Lee et al., 2025). This is in line with our findings, a consistent reduction in spheroid size was observed throughout all time points of the analysis and structural integrity following treatment, highlighting that probiotic metabolites can substantially modulate cancer cell behavior in physiologically relevant 3D contexts. Collectively, these data indicate that bacterial metabolites not only affect cell proliferation but also influence spheroid structure, compactness, and morphological characteristics. Recent evidence also suggests that *L. rhamnosus* exerts anti-angiogenic effects in various experimental models. For instance, a 2022 study demonstrated that the cell-free supernatant of *L. rhamnosus* GG modulated inflammatory signaling and inhibited angiogenesis by regulating formyl peptide receptor 1 (FPR1) activity in gastric cancer cells (Liotti et al., 2022). Similarly, a 2024 investigation reported that extracellular vesicles released from *L.*

rhamnosus GG (LGG-EVs) modulated endothelial cell behavior, influencing tube formation and vascular remodeling processes. These findings highlight the ability of *L. rhamnosus* and its metabolites to interfere with angiogenic pathways, further supporting the notion that probiotic-derived factors can modulate tumor microenvironmental dynamics and potentially contribute to the suppression of tumor vascularization (Wang et al., 2024). We further assessed the potential anti-angiogenic properties of CFS and que-CFS using the CAM assay. CFS and que-CFS both inhibited angiogenesis, with que-CFS showing an approximately 13% stronger effect compared to CFS. These results suggest that que adaptation strengthens the anti-angiogenic potential of *L. rhamnosus*-derived metabolites. Quantitative PCR analysis showed that both que-CFS and CFS upregulated pro-apoptotic genes Bax, Caspase-8, and TP53. These findings align with previous studies investigating the cytotoxic and apoptotic effects of *L. rhamnosus*, *L. plantrum* and *L. lactis* supernatants on HT-29 and HCT-116 cells, where treatments with PG, TBHQ, curcumin and crocin similarly inhibited cell growth in a dose- and time-dependent manner and induced apoptosis, as confirmed by flow cytometry and gene expression analyses (Gholipour et al., 2023; Gholipour et al., 2024; Salmanzadeh et al., 2018). Our results corroborate these earlier observations, particularly in the context of *L. rhamnosus* treated with quercetin. In the present study, quantitative PCR analysis revealed that que-CFS treatment markedly modulated the expression of key apoptosis-related genes. As shown in **Figure. 4(C)**, the expression level of Bax was significantly upregulated in cells treated with que-CFS compared with both the CFS and control groups. Bax, a pro-apoptotic member of the Bcl-2 family, plays a crucial role in mitochondrial outer membrane permeabilization and the release of cytochrome c into the cytosol, thereby initiating the intrinsic apoptotic cascade. Thus, the observed increase in Bax transcription suggests that que-CFS may potentiate the induction of programmed cell death through the mitochondrial apoptotic pathway (Qian et al., 2022). Furthermore, caspase-8 expression was significantly elevated in both the CFS (* $P < 0.01$) and que-CFS (*** $P < 0.0001$) groups compared with the control, with a more pronounced increase in the que-CFS-treated cells. Caspase-8 is a key initiator caspase in the extrinsic apoptosis pathway, mediating death receptor signaling and, through the cleavage of BID to tBID, linking the extrinsic and intrinsic apoptotic pathways. Therefore, the strong induction of caspase-8 by que-CFS indicates that this treatment may simultaneously activate both the extrinsic and intrinsic apoptotic cascades, resulting in a more robust apoptotic response (Orning & Lien, 2021). Although the change in TP53 expression was not statistically significant between the CFS and que-CFS groups, both treatments showed a trend toward increased TP53 mRNA levels relative to the control ($P < 0.1$). TP53 encodes the tumor suppressor protein p53, a central regulator of cellular stress responses and a transcriptional activator of several pro-apoptotic genes, including Bax, NOXA, and PUMA. Even a modest elevation in TP53 expression may therefore reflect the initiation of p53-mediated transcriptional programs in response to cellular stress induced by que-CFS. Previous studies have reported that the activation of p53 contributes to the upregulation of pro-apoptotic members of the Bcl-2 family and enhances cellular sensitivity to apoptosis (Wang et al., 2021). The present study provides compelling evidence that que-CFS exerts its cytotoxic effects on SW-480 cells through the induction of apoptosis. Mechanistically, que-CFS enhances apoptotic signaling by upregulating the caspase-8 pathway and leading to BAX activation in a TP53-independent manner. Overall, the findings of this study

demonstrate that quercetin treatment alters the metabolomic profile of the probiotic *L. rhamnosus*, leading to an enhancement of its anticancer and anti-angiogenic activities.

Conclusion

Quercetin adaptation of *L. rhamnosus* PTCC 1637 generated a cell-free supernatant (que-CFS) that outperformed untreated CFS across every measured anticancer endpoint in SW-480 colorectal adenocarcinoma cells. The 2.3-fold lower IC₅₀, 4% higher apoptosis, 58% smaller nuclear area, 27% greater spheroid inhibition, 26.5% stronger migration block, and 36.3% angiogenesis suppression—all statistically robust—demonstrate that a simple dietary flavonoid can reprogram probiotic metabolism to amplify multiple tumor-suppressive mechanisms. Proteinase K sensitivity proves the active principles are peptides or bacteriocin-like proteins whose production or activity quercetin enhances. These findings establish a proof-of-concept for nutritional preconditioning of probiotics to raise their therapeutic index against CRC.

The quercetin-induced metabolic shift in *L. rhamnosus* invites targeted metabolomic and proteomic profiling to pinpoint the upregulated peptides and secondary metabolites responsible for the observed gains. In vivo CRC models—orthotopic xenografts or APC^{min/+} mice—should quantify tumor burden, metastasis, and survival after oral que-CFS administration, alongside faecal microbiome sequencing to confirm persistence of the adapted strain. Clinical translation will require GMP-scale fermentation of quercetin-adapted *L. rhamnosus*, stability testing of lyophilised que-CFS, and phase-I safety trials in CRC patients. Combining que-CFS with checkpoint inhibitors or anti-angiogenic drugs may yield synergistic regimens, while extension to other flavonoids (*e.g.*, rutin, kaempferol) could broaden the approach to other cancers.

Declarations

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Ethics declaration

The chorioallantoic membrane (CAM) angiogenesis assay was performed in compliance with the ARRIVE guidelines. The study protocol was approved by the Iran National Committee for Ethics in Biomedical Research (IR.TBZMED.VCR.REC.1402.267).

AI Declaration

During the preparation of this work, the authors utilized Microsoft Copilot to enhance the readability and clarity of the text. Final decisions regarding content, structure, and interpretation were made by the authors, who accept full responsibility for the content of the published material.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

PK: Investigation, Writing-original draft. **NA:** Investigation. **HD:** Investigation, Formal analyses. **SV:** Data Curation, Validation. **ME:** Conceptualization, Supervision, Project administration, Writing - Review & Editing. **AM:** Supervision, Writing - Review & Editing. **HMS:** Supervision, Writing - Review & Editing.

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Figures

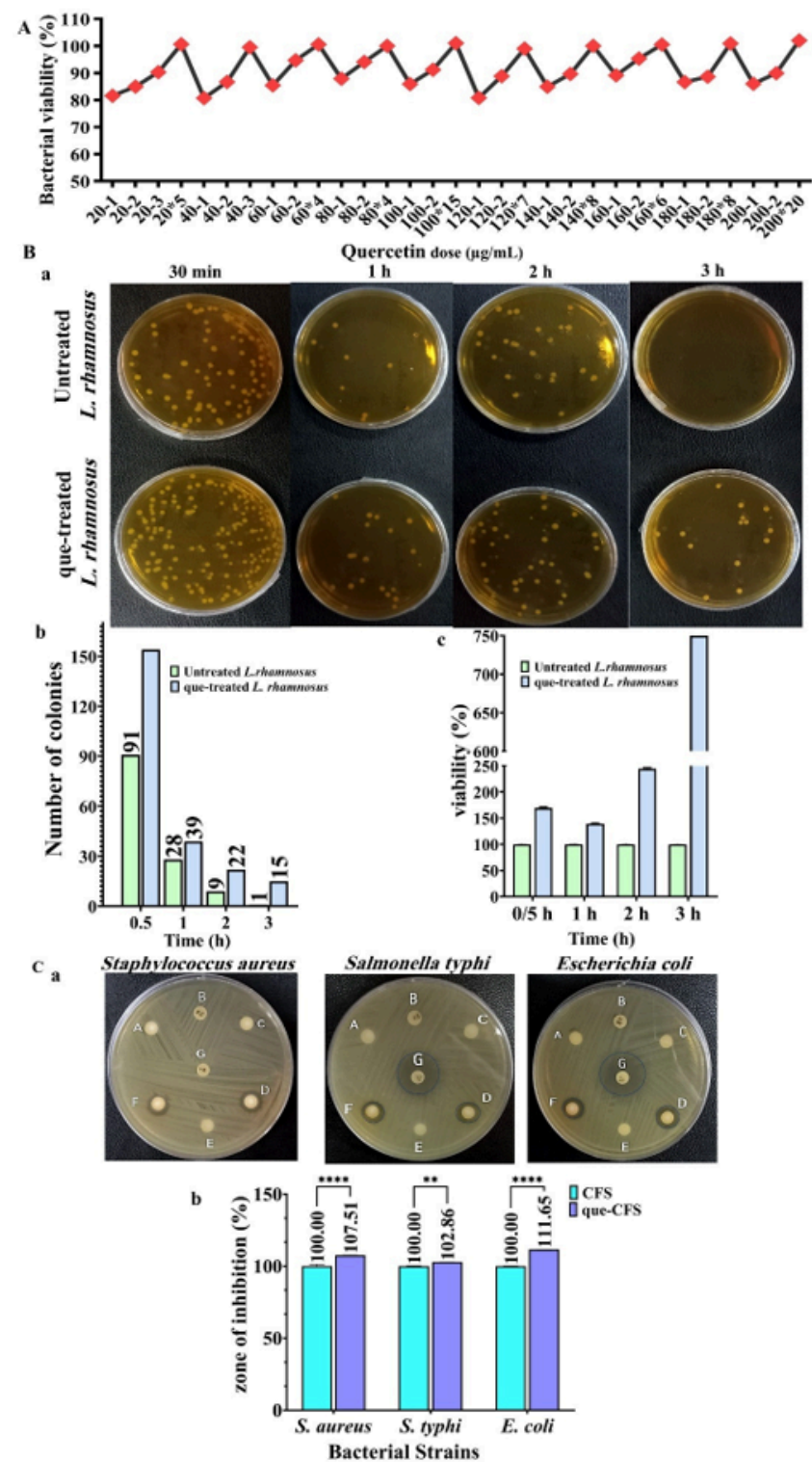


Figure 1

(A) Adaptation of *L. rhamnosus* to quercetin. (Ba) Growth of untreated and quercetin-treated *L. rhamnosus* in pH 3.0 at 0.5, 1, 2 and 3 h that were grown on MRS agar at 37°C for 24 h. (Bb) The number of visible colonies present on an agar plate of untreated *L. rhamnosus* and quercetin-treated *L. rhamnosus* at different time intervals (0.5, 1, 2, 3 h) at pH 3.0. (Bc) Survival rate of *L. rhamnosus* treated with the antioxidant quercetin under acidic gastric conditions (pH 3.0) at different time intervals (0.5, 1, 2, and 3 h), compared with the acid resistance of untreated bacteria under the same conditions and time points.) (Ca) In vitro antibacterial test results of zone of inhibition caused by metabolites in CFS and que-CFS against *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli*. (A) CFS + Protease K, (B) a, b, and c Vancomycin and Azithromycin, respectively, (C) que-CFS + Protease K, que-CFS, (E) PBS, (F) CFS, and (G) a, b, and c Cefazolin and Ampicillin, respectively. (Cb) Quantitative comparison of inhibition zone diameters. Que-CFS exerted remarkably greater antimicrobial activity than CFS against all tested pathogens (**** $P \leq 0.0001$).

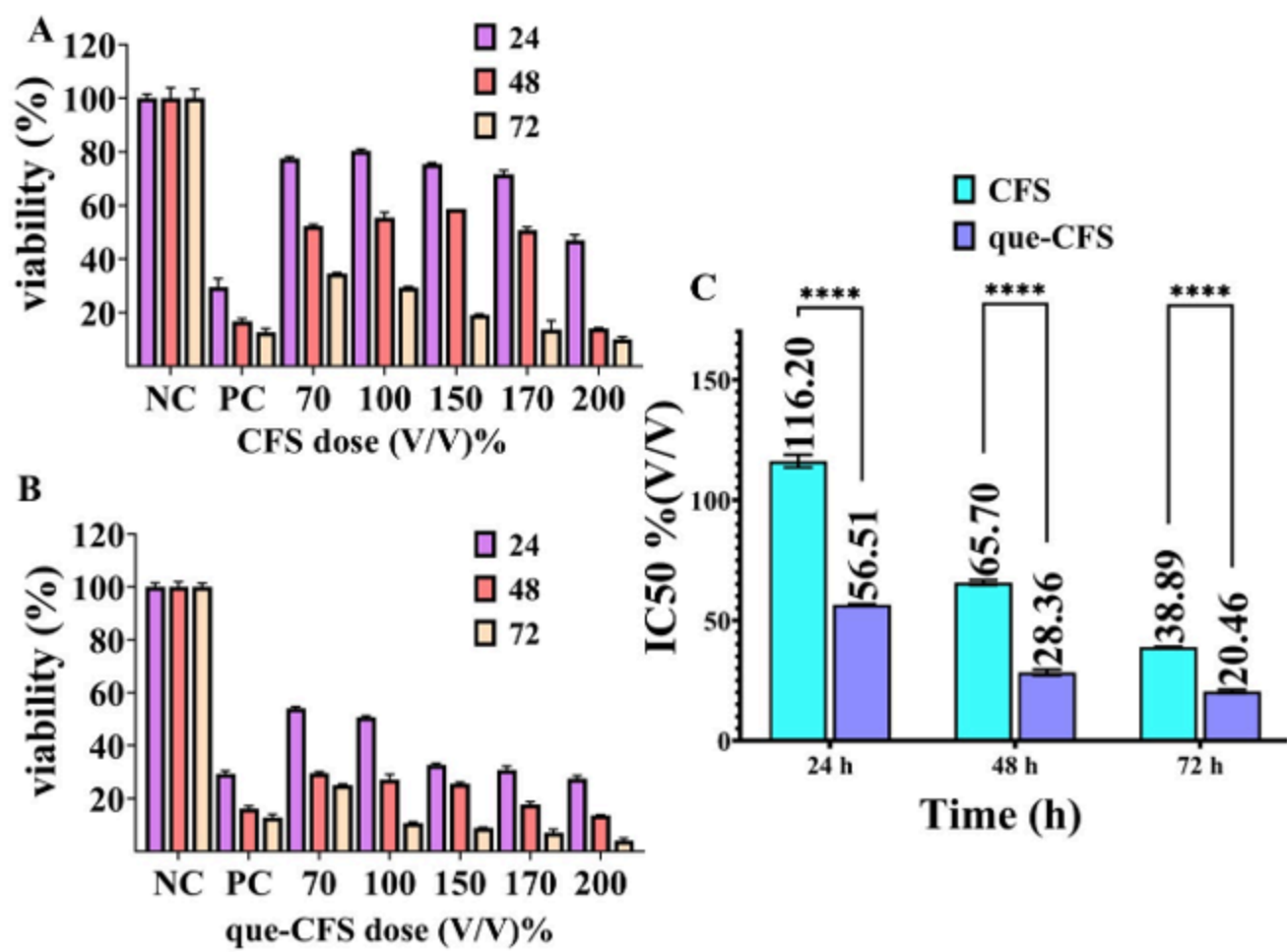


Figure 2

(A) (B) MTT assay results of SW-480 cells treated with varying concentrations of CFS and que-CFS at 24, 48, and 72 hours. (C) Indicates IC₅₀. Both CFS and que-CFS induced cytotoxicity in a dose- and time-dependent manner. At 48 hours, que-CFS exhibited significantly higher cytotoxic effects and a lower IC₅₀ compared to CFS.

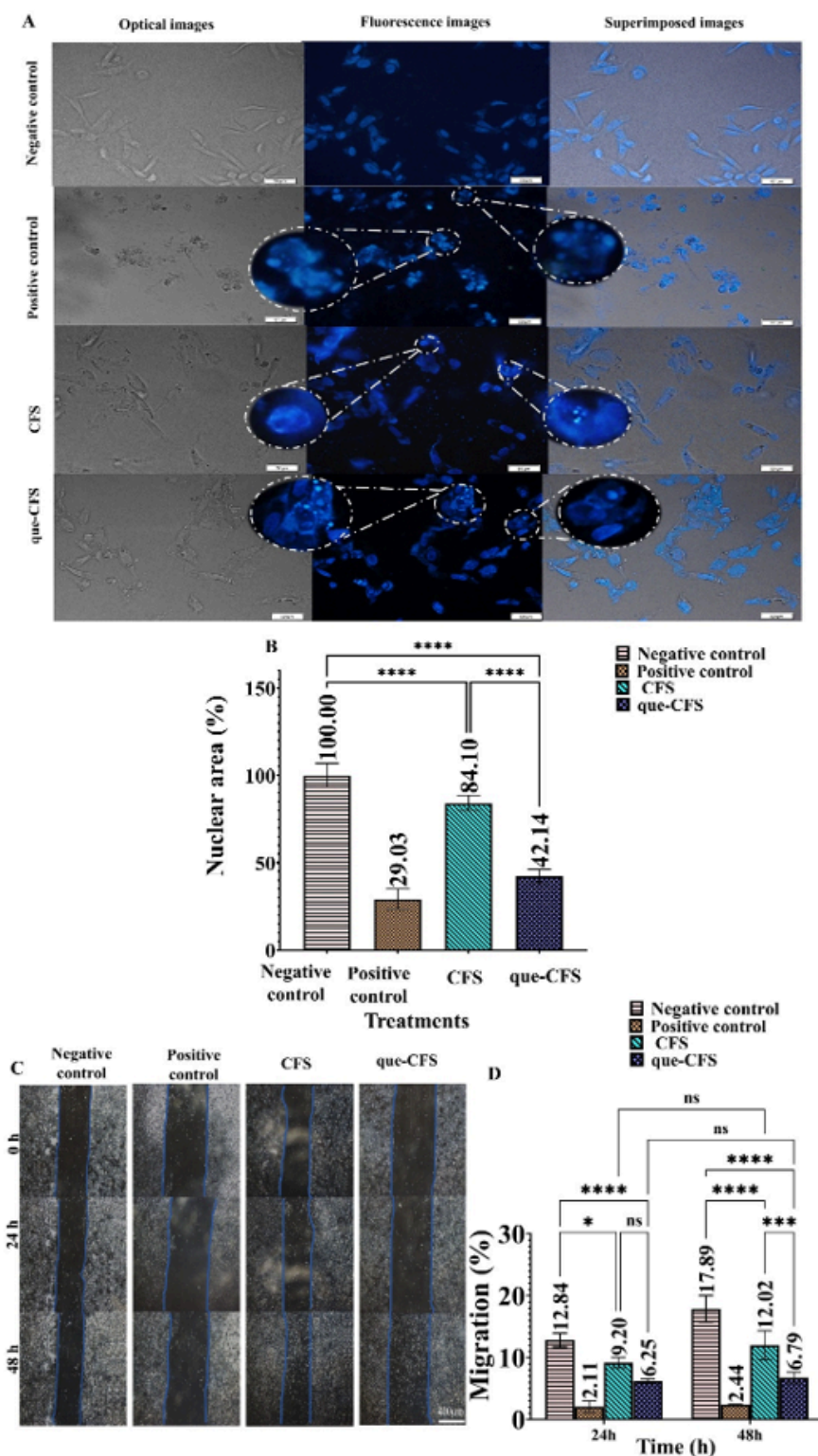


Figure 3

(A) Nuclear staining results using DAPI are presented as bright-field, fluorescence, and merged bright-field/fluorescence micrographs. (B) Comparison of nuclear area in treated cells showed that the average nuclear area in CFS-treated cells was significantly larger than in cells treated with que-CFS. Statistical significance is indicated as follows: ns: $P > 0.05$, **: $P \leq 0.01$, ****: $P \leq 0.0001$. (C) Micrographs of the cell migration assay at different time points: before treatment (0 h), 24 and 48 hours post-treatment. (D)

Comparative graph illustrating the percentage of cell migration under different treatment conditions at 24 and 48hours after treatment.

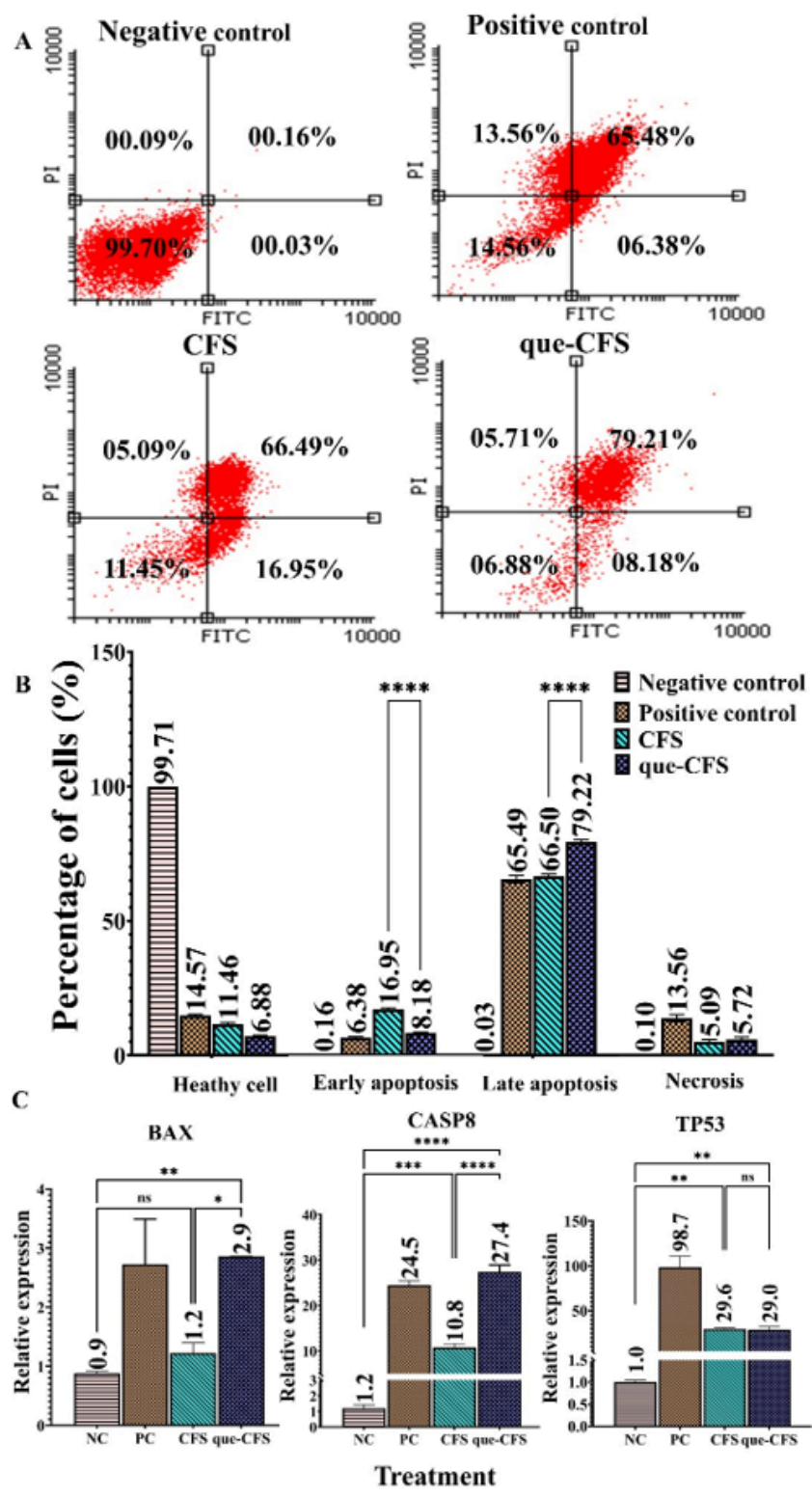


Figure 4

(A) The flow cytometry graphs result of apoptosis and necrosis. (B) Flow cytometry analysis of apoptosis and necrosis in SW-480 colon cancer cells treated with CFS and que-CFS, compared to unstained and positive controls. Apoptosis increased to 83.44% following CFS treatment and to 87.39%

in que-CFS-treated cells, significantly higher than in the control group (****: $P \leq 0.0001$). Furthermore, the apoptosis rate in the que-CFS-treated group was notably higher than that in the CFS-treated group. (C) Alteration in Bax, Caspase-8 and TP53 mRNA expression levels in SW-480 colon cancer cells upon treatment with CFS and que-CFS evaluated by qPCR technique. ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

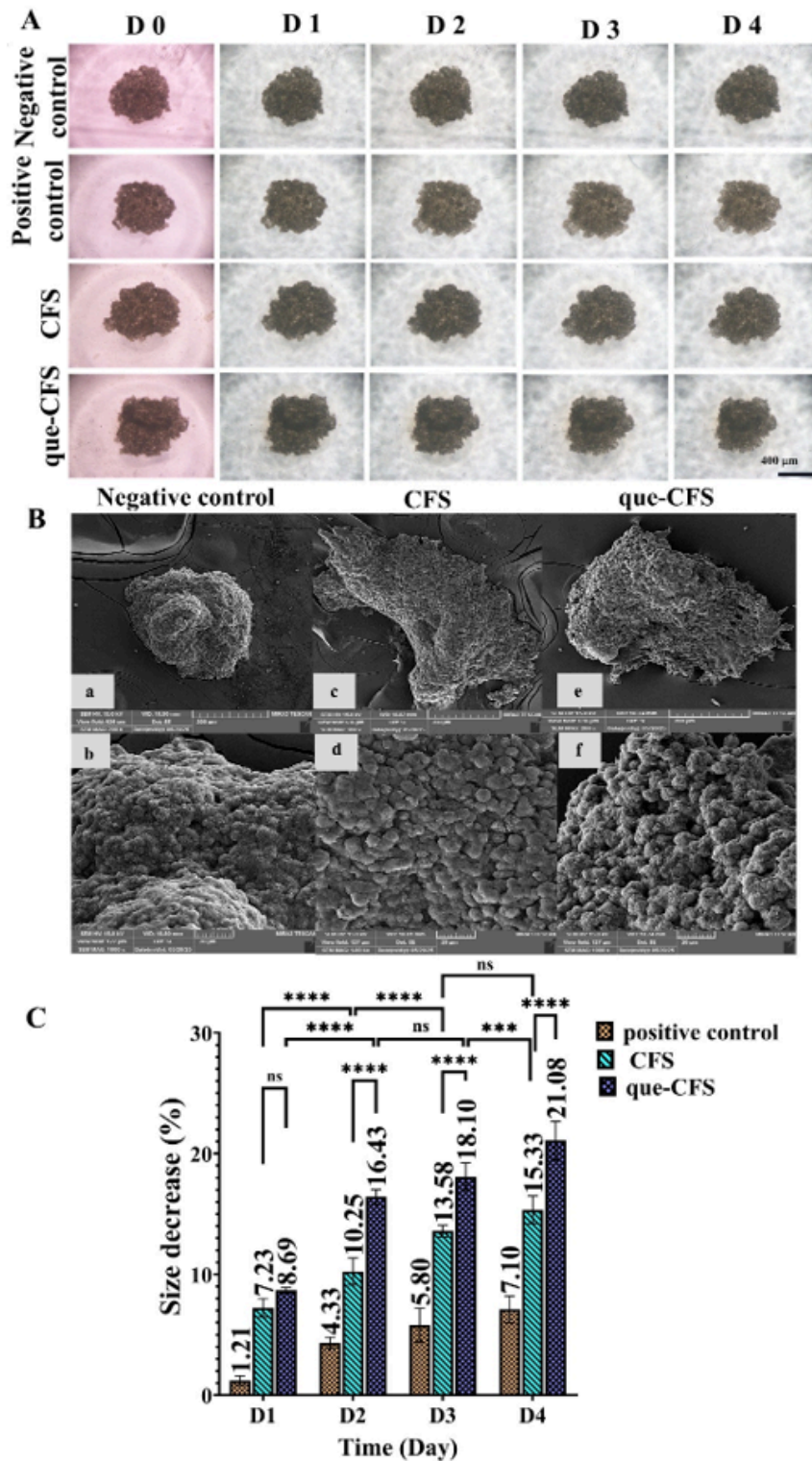


Figure 5

(A) Micrographs of the cell migration assay at different time points: before treatment (0 h), 24 and 48 hours post-treatment, and a Comparative graph illustrating the percentage of cell migration under different treatment conditions at 24 and 48hours after treatment. (B) FE-SEM graphs also show disrupted structure of SW-480 spheroids treated with CFS (c, d) and que-CFS (e, f); in contrast, intact cellular morphology was observed in the negative control (a, b).

(C) Effects of CFS and que-CSF treatments on spheroid size. Representative images of each treatment are shown over 4 days, and a Graph of spheroid size reduction at 24, 48, 72, and 96 hours post-treatment. (ns: $P > 0.05$, ***: $P \leq 0.001$, **: $P \leq 0.0001$).

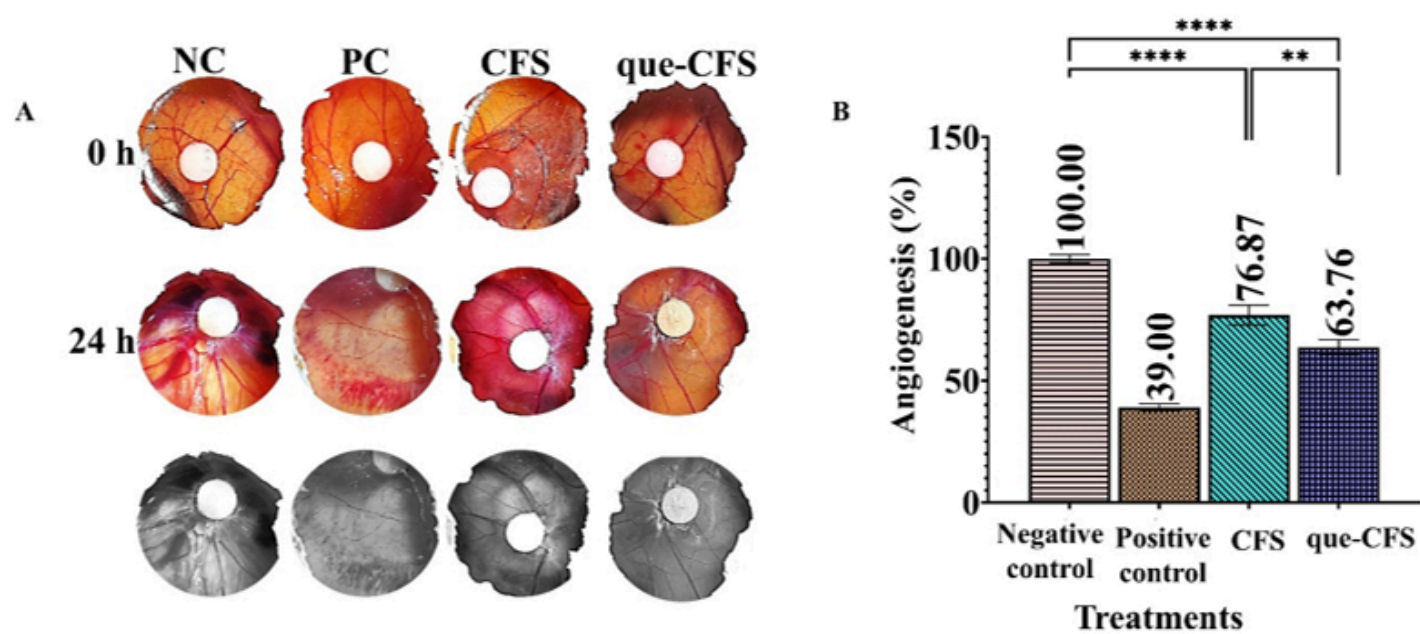


Figure 6

(A) Images of chick embryos show that blood vessels are reduced in the treatment group. The group treated with que-CFS exhibited a greater inhibition of angiogenesis compared to CFS. (B) Quantitative study showed significant angiogenesis inhibition compared to the control group.

Supplementary Files

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- [GA.jpg](#)