

Lactiplantibacillus plantarum exerts anticancer effects and increase the chemosensitivity of 5-fluorouracil against oral cancer cells *in vitro*

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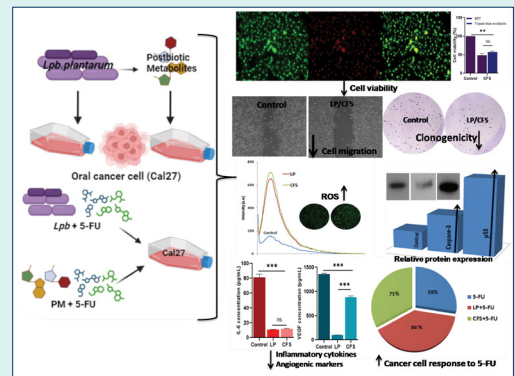
Abstract

Introduction: Probiotics are used to provide health benefits and can improve the immune response. They can also target cancer cells directly with anticancer effects through various mechanisms. In this study, *Lactiplantibacillus plantarum* (basonym: *Lactobacillus plantarum*) strain MCC 3016 and its postbiotic metabolites/cell free supernatant (CFS) were used against Cal27 oral cancer cells *in vitro*.

Methods: Standard assays were employed to investigate the effect of *Lpb. plantarum* on cell viability, proliferation, migration, and clonogenicity of Cal27 cells. The mechanism of action was assessed by measuring the levels of reactive oxygen species (ROS), interleukins (IL)-6 and IL-8, tumor necrosis factor- α (TNF- α), as well as the expression of Ki67, vascular endothelial growth factor (VEGF), p53 and caspase-3. Further, the effect of *Lpb. plantarum* and its CFS on the cytotoxicity of chemotherapy drug 5-fluorouracil (5-FU) was evaluated using cell viability assays.

Results: Cal27 cells treated with *Lpb. plantarum* and its CFS showed a significant decrease ($P < 0.01$) in cell viability, proliferation, migration, and clonogenicity, along with increased levels of ROS and induced apoptosis. It significantly reduced IL-6, IL-8, TNF- α , and VEGF levels and upregulated p53 and caspase-3 expression. The postbiotic metabolites also showed similar effects on Cal27 cells. Furthermore, the cytotoxic effect of 5-FU on Cal27 cells was enhanced by *Lpb. plantarum* and its CFS treatment.

Conclusion: *Lpb. plantarum* MCC 3016 and its postbiotic metabolites exhibited promising anticancer effects on oral cancer cells and improved drug efficacy, demonstrating their potential therapeutic value in oral cancer therapy.



Introduction

Oral cancer is one of the most common cancers globally, especially in Asian countries with mortality rate of 75.2% according to GLOBOCAN 2022.¹ The major risk factors for oral cancer include the consumption of alcohol, tobacco, poor oral hygiene, chronic ulceration, chronic inflammation, and microbial infections.² Like all other cancers, the clinical management of oral cancer involves surgery, chemotherapy, radiotherapy, immunotherapy or a combination of them.³ However, these therapies are reported to have several side effects that could result in patient morbidity and mortality. For example, radiotherapy can cause undesirable changes in the adjacent tissues of

the tumor site, and induce oral mucositis, dental caries, hyposalivation, and osteoradionecrosis.⁴ Oral mucositis limits the ability of the patient to withstand chemotherapy drugs such as 5-fluorouracil (5-FU) which can result in treatment failure.⁵ Due to these complications, new treatment strategies and therapeutic alternatives are gaining interest in the field of cancer research. Evidence from *in vitro* and *in vivo* studies has demonstrated the antitumor activities of probiotics as well as their crucial role in immunomodulation.^{6,7} Therefore, probiotics are considered as adjuvant and complementary treatments that can enhance the effectiveness of immunotherapy and chemotherapy.⁸⁻¹⁰ Probiotics are microorganisms with



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the ability to modify the immune response of the host, antagonize or compete with pathogens, and are therefore vastly applied in healthcare.

The understanding of the association between microbiota changes and diseases has contributed to the exploration of beneficial microbes in the treatment of various diseases.¹¹ Probiotics influence human health in several ways, such as enhancing systemic immune and anti-inflammatory activities, and reducing the risk of multiple chronic diseases including cancer.^{12,13} The *Lactobacillus* species are important members of the healthy human microbiome and are also found in various ecological niches.¹⁴ They have gained widespread attention as probiotics and can colonize the oral cavity, gastrointestinal tract, and genitourinary tract.¹⁵ The presence of *Lactobacillus spp.* can attract the colonization of other beneficial microbes, thereby displacing harmful pathogenic microbes and fostering a healthy microbiota.¹⁶ The probiotic properties of *Lactobacillus* have also been shown to be beneficial against different cancers.¹⁷⁻²⁰ In addition to the whole organism, the postbiotic metabolites produced by probiotic bacteria also known to offer similar beneficial effects.^{21,22} Treatment with postbiotic metabolite components, such as cell free supernatant (CFS), cytoplasmic extracts, and cell wall extracts of *Lactobacillus gasseri* and *L. crispatus*, induce anti-proliferative and apoptotic responses in cervical cancer cells.²³

Due to taxonomic changes in the genus *Lactobacillus*, *L. plantarum* is reclassified as *Lactiplantibacillus plantarum* (hereinafter *Lpb. plantarum*). Studies have shown that *Lpb. plantarum* treatment can reduce cell proliferation and induce apoptosis in colon cancer cell lines.^{24,25} The postbiotic metabolites produced by six *Lpb. plantarum* strains have shown anticancer effects against breast, colorectal, cervical, liver, and leukemia cancer cell lines.²³ The probiotic strains of *Lpb. plantarum* tend to improve the gut microbiota composition and immune system, regulate lipid metabolism, and reduce the risk of some cancers.^{26,27} Various mechanisms, such as increased production of reactive oxygen species (ROS), decreased inflammation, and regulation of proliferative and apoptotic pathways, have been associated with the action of probiotics on the cancer cells.¹⁹ In oral cancer, the pro-inflammatory cytokines and interleukins (IL) such as IL-6, IL-8, tumor necrosis factor- α (TNF- α), and vascular endothelial growth factor (VEGF) are upregulated.²⁸ Although reduced inflammation is one of the mechanisms by which probiotics inhibit cancer, detailed studies on the levels of inflammatory cytokines resulting from probiotic treatment of oral cancer cells are lacking.

Hence, in this study, we investigated the effects of a probiotic strain *Lpb. plantarum* MCC 3016 and its postbiotic metabolite against oral cancer cells *in vitro* to understand the mechanism of their action on oral

cancer cells. The probiotic strain used in the study was originally isolated from a healthy adult human gut, and the whole genome data and characterization studies have shown its potential probiotic properties.²⁹ The major hypothesis of our study was to investigate whether *Lpb. plantarum* and its postbiotic metabolites could induce an inhibitory effect on oral cancer cells by regulating inflammatory and apoptotic mediators. Additionally, we aimed to determine if *Lpb. plantarum* treatment could enhance the chemosensitivity of 5-FU on oral cancer cells for potentially improving the efficacy of oral cancer treatment.

Materials and Methods

Bacterial strain, cancer cell line, and culture conditions

Lpb. plantarum strain MCC 3016 was used for this study. It was cultured in a *Lactobacillus* MRS (Man Rogosa Sharpe) medium (HiMedia, India) at 37 °C under shaking (110 rpm) in a bacteriological incubator. The oral cancer Cal27 cell line (ATCC-CRL-2095) was cultured in a Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) and antibiotics (Penicillin-Streptomycin, Gibco, USA). The cells were maintained at 37 °C in a 5% CO₂ atmosphere (C170, CellXpert, Eppendorf, Germany). For the experiments involving live probiotic treatment, DMEM without antibiotics was used.

Preparation of probiotic bacterial cells and postbiotic metabolites

The *Lpb. plantarum* cells were harvested from the MRS broth cultures incubated for 24 h by centrifugation (6,000 rpm at 4 °C for 5 min) and washed with sterile phosphate-buffered saline (PBS). The cell pellet was re-suspended in sterile PBS, serially diluted, and cultured on agar plates to count the cells as colony-forming units (CFU). Based on the CFU counts, the multiplicity of infection (MOI) was calculated for cancer cell culture experiments.

For the preparation of postbiotic metabolites, the bacterial culture was incubated for 24 h, harvested by centrifugation (8000 rpm at 4 °C for 10 min), and the CFS was collected. The pH of each CFS was adjusted to physiological pH of 7.2–7.4 using 0.1 mM sodium hydroxide and filtered through 0.22 μ m syringe filter prior to the assay.²³

Treatment of Cal27 cells with live Lpb. plantarum and its CFS

The Cal27 cells were seeded into a 96-well microplate (Nunc, Roskilde, Denmark) at a cell density of 5000 cells per well. The cells were allowed to attach overnight, then treated with *Lpb. plantarum* at MOI 10 and MOI 100, and incubated in a CO₂ incubator at 37 °C (5% CO₂) for 6 h. *Streptococcus mutans* ATCC 25175 was used as the negative control. To study the effect of CFS, a separate 96-

well microplate seeded with cells was also similarly used. The cells were treated with different concentrations of CFS (5-25% v/v) separately for 6 h and 24 h. Untreated cells and MRS broth were used as the control and negative control respectively.

Cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

The effect of the probiotic and CFS treatments on Cal27 cell viability was assessed using the MTT assay.³⁰ After the incubation period, the supernatant media from the treated wells were removed, and the cells were washed using PBS. MTT reagent (1 mg/mL) was added to cells, and the contents were incubated at 37 °C in the dark for 4 h. After the incubation, the cells were washed using PBS, and the formazan crystals formed were dissolved using dimethyl sulfoxide (DMSO). The absorbance of the solubilized formazan was quantified as OD₅₄₀ using a multimode microplate reader (FLUOstar Omega, BMG Labtech, Germany). The percentage of cell viability in comparison to the control group was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{OD}_{540} \text{ of test}}{\text{OD}_{540} \text{ of control}} \times 100$$

Trypan blue dye exclusion assay

To determine the number of viable cells after the probiotic and CFS treatment, trypan blue dye exclusion assay was used.³¹ In this assay, dead cells are stained, while live cells exclude trypan blue dye due to their intact cell membranes. For this experiment, Cal27 cells were seeded in a 24-well plate at a density of 20,000 cells per well and then treated with live *Lpb. plantarum* for 6 h and with CFS for 24 h separately. After the incubation, the cells were trypsinized and collected by centrifugation (1500 rpm for 5 min). The cell pellet was resuspended in fresh DMEM and stained using an equal volume of the trypan blue dye (0.4%). The cells were counted using a hemocytometer, and based on the dye uptake, the percentage of viable cells were calculated relative to the untreated control group using the formula:

$$\text{Viable cell (\%)} = \frac{\text{Total no. of viable cells}}{\text{Total no. of cells seeded}} \times 100$$

Live-dead assay

To investigate whether *Lpb. plantarum* and its CFS treatments have apoptotic effects, the live-dead staining method was used as described elsewhere.³² Briefly, Cal27 cells were seeded in 24-well plates at a density of 20,000 cells per well, incubated overnight for cell attachment, and then treated with *Lpb. plantarum* for 6 h and with CFS for 24 h separately. After incubation, the medium was removed, and the cells were gently washed with sterile PBS. Subsequently, cells were incubated with acridine-orange and ethidium bromide (AO-EB) (1 µg/mL each)

in the dark for 20 min at room temperature. The stained cells were observed using a fluorescent imager (Zoe Bio-Rad, California, USA), and images were captured with green, red, and merged filters.

Cell migration assay

The scratch assay was used to test the effect of probiotic on the cell migration of Cal27 cells.³³ Approximately 55,000 cells were seeded in a 6-well plate and incubated until confluence was reached. A linear scratch was created using a p200 microtip, and the dead and floating cells were removed. The cells were then treated with *Lpb. plantarum* (MOI 100) or CFS and then incubated in a CO₂ incubator at 37 °C. The cell migration across the scratch was monitored at regular intervals of time and observed using an inverted microscope (Primo Vert-Zeiss, Germany). The area under the scratch was measured from the photographs using ImageJ software to calculate the percentage of cell migration in the treatment groups compared to the untreated controls.

Clonogenic assay

A clonogenic assay was performed to evaluate the effect of *Lpb. plantarum* and its CFS treatments on the ability of a single cell to grow into a colony.³⁴ Cal27 cells were seeded in 6-well plates, allowed to adhere, and treated with *Lpb. plantarum* or CFS separately. Following the treatment period, fresh medium was added, and cells were incubated for 10–14 days to allow the cells to grow into colonies. The colonies were stained with crystal violet, and colonies containing 50 or more cells were manually counted. The results were normalized to the plating efficiency (PE) of the control. The survival fraction was calculated based on the number of colonies formed in the treated cells relative to those in the untreated control.

Immunofluorescence assay for the proliferation marker Ki67

The commonly used cell proliferation marker Ki67 was employed to study the impact of probiotic and its CFS on cancer cell proliferation using an immunofluorescence assay.³⁵ Cal27 cells were cultured in 8-chambered slides (Eppendorf, Germany) at a seeding density of 5,000 cells per well in DMEM and treated with *Lpb. plantarum* (MOI 100) for 6 h. Similarly, in another set, the cells were treated with CFS for 24 h. After incubation, the cells were washed with PBS, fixed using 4% formaldehyde, and permeabilized with Triton-X-100. The cells were incubated with Ki67 primary antibody (Cell Signaling Technology, USA) overnight at 4 °C, followed by an incubation with anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technology, USA) for 1 h. Subsequently, the cells were washed with PBS, mounted using 4', 6-diamidino-2-phenylindole (DAPI), and observed using a fluorescent imager (Zoe, Bio-Rad,

California, USA). The Ki67-stained cells were scored and compared with untreated control cells.

Estimation of ROS levels in the treated cells

The total cellular ROS produced by Cal27 cells treated with *Lpb. plantarum* and its CFS was determined using the 2',7'-dichlorofluorescein diacetate (DCFDA) method.³⁶ In a 24-well plate, Cal27 cells (10,000 cells per well) were seeded and treated with probiotic bacteria or with CFS. After incubation, the cells were washed, and DCFDA solution (100 μ M) was added, and kept for 30 min at 37 °C. Fluorescent images were captured in the green channel using a fluorescent imager (Zoe, Bio-Rad, California, USA). After imaging, the cells were lysed using radio-immunoprecipitation assay (RIPA) buffer, incubated on ice, and centrifuged (5,000 rpm at 4 °C for 10 min). To quantify the activity, the fluorescent intensity of the supernatant was measured at 522 nm using a fluorescence spectrophotometer (F -2700, Hitachi, Tokyo, Japan).

Quantification of IL-6, IL-8, TNF- α and VEGF by ELISA

The levels of inflammatory cytokines, namely, IL-6, IL-8, TNF- α , and VEGF, were measured using the ELISA method. For this experiment, Cal27 cells were cultured in 6-well plates (55,000 cells per well) until confluence and then treated with *Lpb. plantarum* for 6 h or with CFS for 24 h. Following the incubation, the supernatant was collected, and ELISA was performed to detect IL-6, IL-8, TNF- α , and VEGF using the respective GenLISA™ kits (Krishgen Biosystems, India) following the manufacturer's instructions. The concentrations of the secreted cytokines were quantified using a standard curve and compared with the control group.

Western blot analysis for the expression of p53 and caspase-3

The expression of p53 and caspase-3 was studied by western blot analysis using cell lysate of the cells treated with *Lpb. plantarum* and its CFS. Total protein was isolated from the cell lysates and quantified using the Bradford method. For western blotting, approximately 30 μ g/mL of protein was loaded and subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to a nitrocellulose membrane (HiMedia, India), the separated proteins were blocked using 5% skimmed milk in PBS with Tween 20 (PBST) (pH 7.4). The blots were incubated overnight with the primary antibodies of p53 (R&D Systems, USA) and caspase-3 (Cell Signaling Technology, USA) at 4 °C, followed by incubation with an HRP-linked secondary antibody (Cell Signaling Technology, USA) for 1 h. After incubating with enhanced chemiluminescence (ECL) reagent (BioRad, USA), the protein bands were visualized using a gel documentation system (Vilber, Fusion Solo S, France).

Effect of *Lpb. plantarum* treatment on the chemosensitivity of 5-FU against Cal27 cells

5-FU is a basic chemotherapeutic drug used to treat various cancers such as breast, stomach, pancreatic, and colorectal cancers.³⁷ The anticancer drug 5-FU was used to determine if *Lpb. plantarum* and its CFS treatment can enhance chemotherapy effect. For this, Cal27 cells were initially treated with *Lpb. plantarum* (MOI 100) or CFS (13.5% v/v) for 6 h, followed by treatment with different concentrations (0-100 μ M) of 5-FU for 24 h. The cells treated with 5-FU without probiotic treatment were taken as the control. Cytotoxicity of the drug on the probiotic-treated cancer cells was assessed using MTT assay. Additionally, a live-dead assay was performed to observe apoptosis using AO-EB staining, as explained in the previous section. For this, the cells treated with *Lpb. plantarum* (MOI 100) or CFS (13.5% v/v) for 6 h were subsequently challenged with 5-FU at its IC₅₀ concentration for 24 h and then subjected to the staining procedure.

Statistical analysis

All the experiments were performed in both technical and biological triplicates. From these replicates, the mean and standard deviation were calculated. Comparisons between treatments were carried out using the Student's t-test. To compare the multiple groups, a one-way analysis of variance (ANOVA) coupled with Tukey's multiple comparisons test was performed, and a statistical difference of $P < 0.05$ was considered significant, unless specified otherwise.

Results

***Lpb. plantarum* MCC 3016 and its CFS target the Cal27 cell viability and proliferation**

The MTT assay results were compared to determine the direct effect of *Lpb. plantarum* MCC 3016 treatment against cancer cell viability and proliferation inhibition. The probiotic treatment significantly reduced cell viability and proliferation compared to the control group ($P < 0.001$) (Fig. 1a). Both at MOI 10 and 100, the cell viability was reduced significantly, and a significant difference in the cell viability was observed between the treatments at MOI 10 and 100 ($P < 0.05$). The results of the trypan blue assay were also consistent with the MTT assay results (Fig. 1b). The live-dead staining revealed early apoptotic Cal27 cells treated with live *Lpb. plantarum* (Fig. 1c). The micrographs clearly showed the distorted morphology in the probiotic-treated cells compared to the intact cell morphology of the control. No significant effect on Cal27 cells was observed for the *S. mutans* treatment, which was used as a negative control (Supplementary file 1, Fig. S1).

The CFS of *Lpb. plantarum* was used at different concentrations to treat Cal27 cells, and the results showed

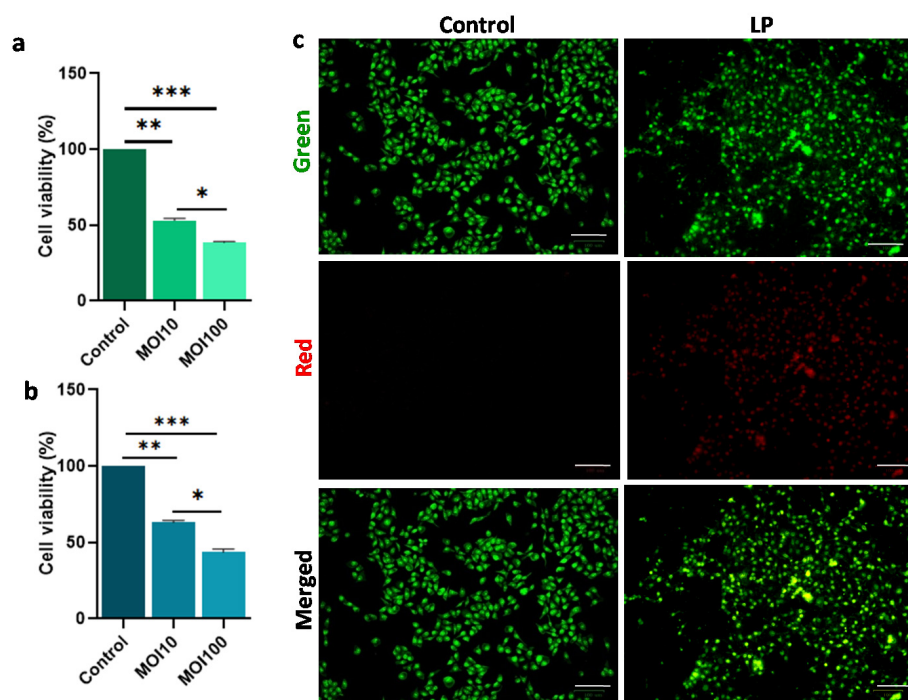


Fig. 1. *Lpb. plantarum* (LP) MCC 3016 treatment decrease the Cal27 viability. Results of (a) MTT and (b) Trypan blue dye exclusion assay showing significantly decreased cell viability of Cal27 cells treated with *Lpb. plantarum* at MOI 10 and MOI 100 for 6 h, compared to untreated control cells, (c) fluorescent images showing apoptotic Cal27 cells treated with *Lpb. plantarum* at MOI 100 for 6 h. The cells were stained using acridine orange and ethidium bromide (Scale bar = 100 μ m). Results are expressed as mean \pm SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

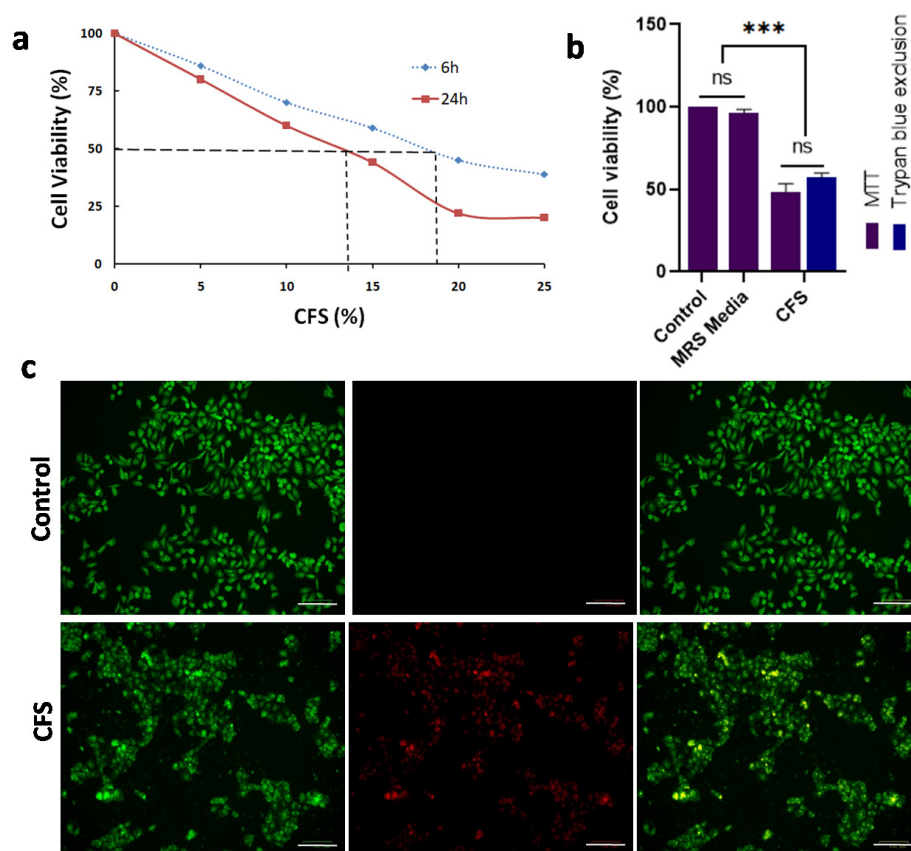


Fig. 2. *Lpb. plantarum* CFS treatment targets Cal27 cell viability. (a) Decrease in the Cal27 cell viability with increasing concentration of CFS treatment for 6 h and 24 h. Untreated cells were used as control. (b) Percentage inhibition of the cell viability with CFS treatment [13.5% (v/v)] for 24 h calculated from MTT and trypan blue dye exclusion assays. Untreated cells and cells treated with MRS broth were used as control and negative control respectively. (c) Photomicrographs showing apoptosis in the Cal27 cells treated with CFS for 24 h (Scale bar = 100 μ m). The cells were stained using acridine orange and ethidium bromide. Results are expressed as mean \pm SD. ns represent non-significant, $P > 0.05$, and *** $P < 0.001$.

a dose-dependent reduction in cell viability (Fig. 2a). At all the tested concentrations, a significant ($P < 0.001$) decrease in cell viability compared to the control and negative control (MRS broth) groups was observed. At the highest concentration of CFS (25% v/v), the residual cell viability was less than 20% (at 24 h). A significant difference was observed in the cell viability between CFS treatments of 6 h and 24 h. From this data the volume of CFS required for a 50% reduction in cell viability of Cal27 cells at 6 h and 24 h was estimated to be 18.5% and 13.5% (v/v) respectively. Further, the trypan blue assay performed with 13.5% CFS treatment for 24 h showed ~50% cytotoxicity, confirming the cell-killing results (Fig. 2b). The microscopic evaluation of the fluorescent stained CFS-treated Cal27 cells showed apoptosis in most of the cells, with a loss of cell morphology (Fig. 2c).

Lpb. plantarum and its CFS inhibit Cal27 cell migration

Cancer cell migration is an important parameter; therefore, the impact of *Lpb. plantarum* and its CFS treatment on the migratory activity of cancer cells was assessed using a scratch assay. Untreated Cal27 cells exhibited progressive cell migration during the incubation period, with a significantly higher rate of scratch closure between 8 h and 18 h. Conversely, the cells treated with

Lpb. plantarum showed a significantly lower rate of cell migration compared to the control ($P < 0.001$). At 18 h, the control group showed 80% closure of the scratch, whereas the probiotic and CFS-treated groups showed less than 50% closure (Fig. 3a and 3b). Notably, there was no significant difference in the cell migration rates between the groups treated with live bacteria and those treated with CFS ($P > 0.05$).

Expression levels of Ki67

The expression of the nuclear protein Ki67 is associated with cancer cell proliferation and growth. In the control group, Cal27 cells showed higher Ki67 expression than the cells treated with *Lpb. plantarum*, which did not express the proliferative marker Ki67, indicating the arrest of cell proliferation (Fig. 4a and 4b). Similarly, there was no expression of Ki67 in the cancer cells treated with CFS for 24 h (Fig. 4c and 4d).

Lpb. plantarum treatment reduced colony formation and increased the production of ROS

The clonogenic assay was conducted to evaluate the cell proliferation ability of the Cal27 cells treated with the probiotic and its CFS. Prominent colonies were observed in the control group on the 14th day, while the probiotic-

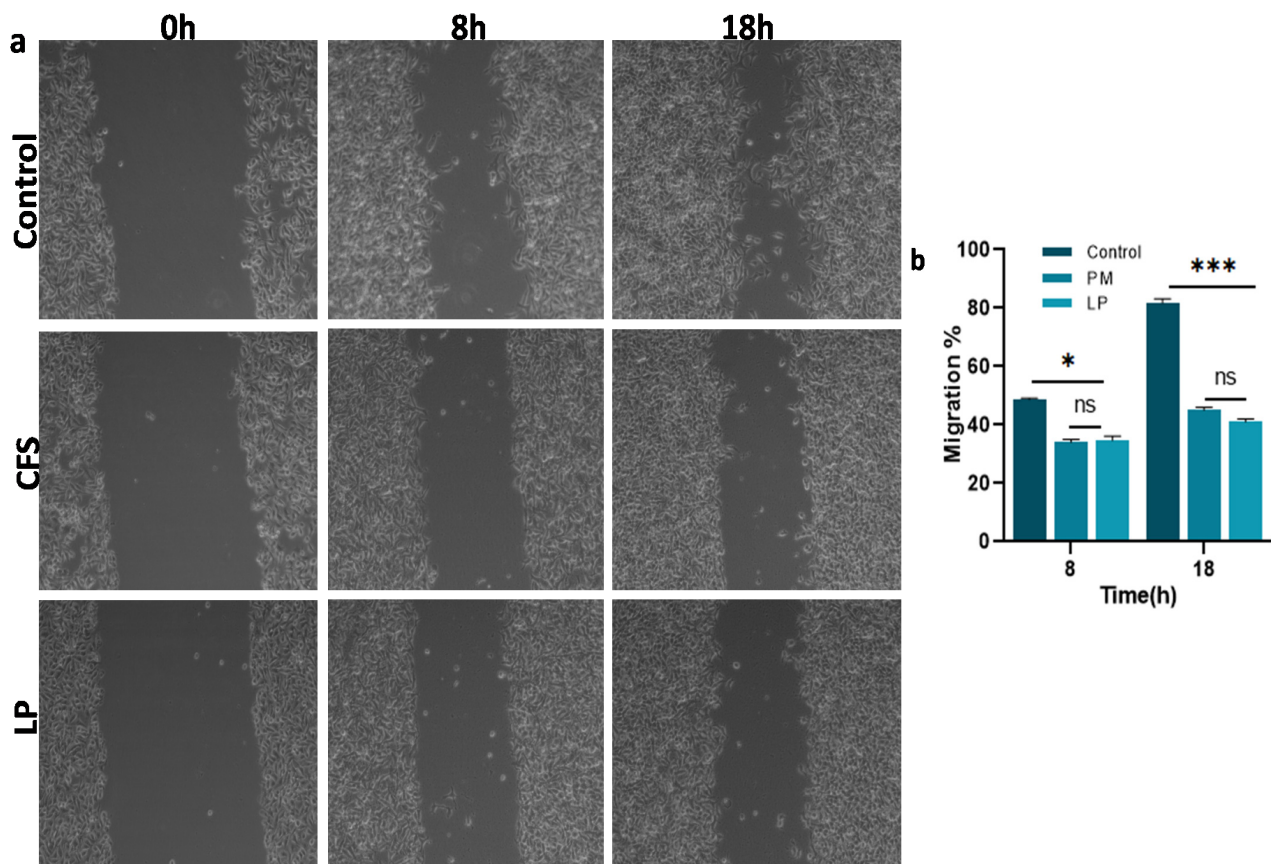


Fig. 3. Cal27 cell migration on *Lpb. plantarum* (LP) and CFS treatment. (a) Micrographs taken at 0, 8 and 18 h post-treatment with LP and CFS from the scratch assay, (b) graphical representation showing the migration rate of the Cal27 cells in treated and control groups at different time points. Results are presented as the mean \pm standard deviation. ns represent non-significant $P > 0.05$, * $P < 0.05$ and *** $P < 0.001$.

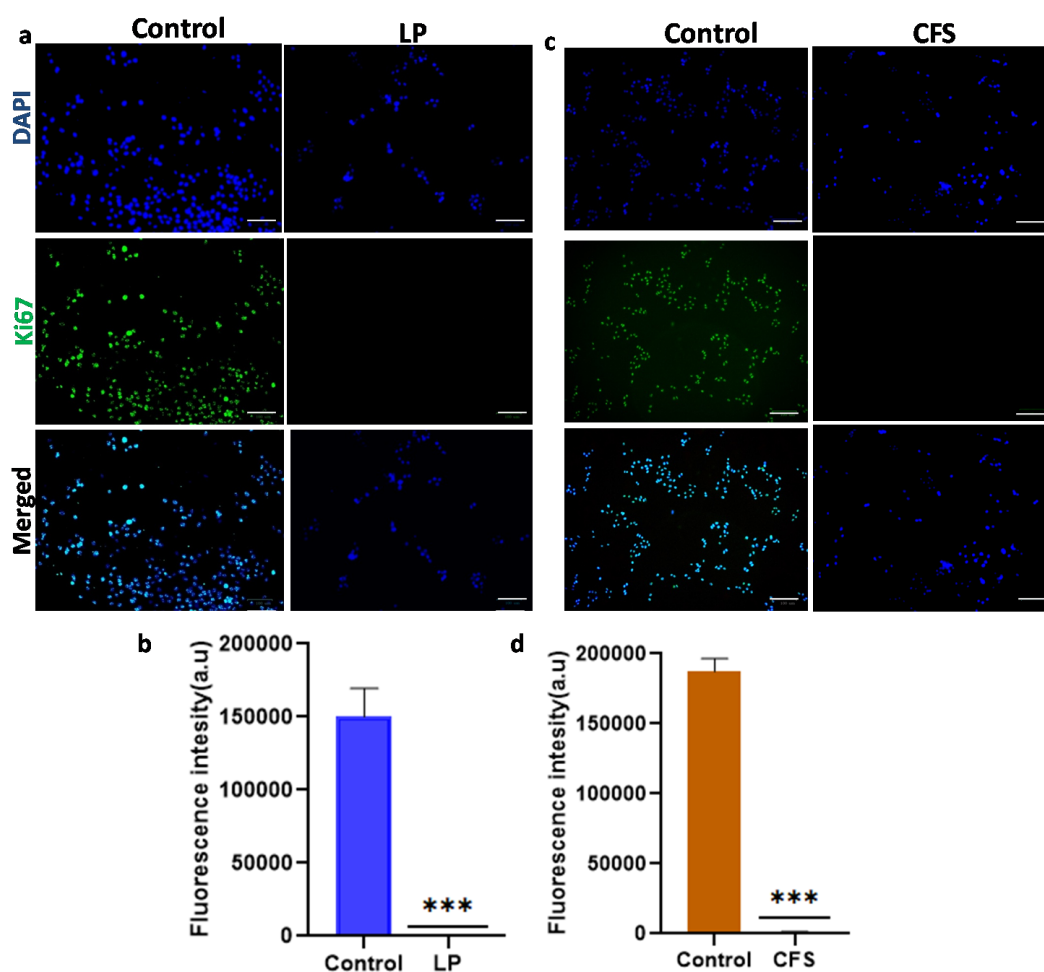


Fig. 4. Immunofluorescence showing the expression profile of Ki67 in Cal27 cells treated with *Lpb. plantarum* (LP) and its CFS compared to control. Fluorescence microscopic image and fluorescence intensity of Cal27 cells treated with (a and b) LP for 6 h and (c and d) CFS for 24 h. Green color indicates cells expressing Ki67 and blue color indicates nuclear staining using DAPI (Scale bar = 100 μ m). LP was used at MOI 100 and CFS at a volume required for 50% cell viability reduction. Results are expressed as mean \pm SD. *** P < 0.001.

treated cells formed a significantly lower number of colonies (P < 0.001) (Fig. 5a and 5b). Additionally, the sizes of the colonies in the treated groups were smaller than those in the control group. The survival fractions in the *Lpb. plantarum* and CFS-treated groups were found to be 50% and 60%, respectively (Fig. 5c). A significant difference was observed in the number of colonies and survival fraction between the live and CFS-treated groups (P < 0.05).

Elevated level of ROS is associated with signaling pathways that lead to apoptosis and is a central target for cancer therapy. Using the DCFDA method, ROS levels in the Cal27 cells treated with probiotic and its CFS were measured (Fig. 5d and e). The results showed significantly higher ROS levels in the *Lpb. plantarum* and CFS-treated groups compared to the control group (P < 0.001) (Fig. 5f). No significant difference in ROS levels was observed between live *Lpb. plantarum* and CFS treatments (P > 0.05).

Up-regulation of p53 and caspase-3

The p53 gene is a tumor suppressor gene, and tumor cells accumulate the p53 protein. The *Lpb. plantarum* treatment significantly upregulated the expression of p53 and caspase-3 in Cal27 cells compared to the control group (P < 0.001) (Fig. 6a). Similarly, overexpression of both proteins was observed in the CFS-treated groups as well (Fig. 6b). No significant difference in the expression levels of p53 was observed among the treatment groups (P > 0.05) (Fig. 6c). However, the expression level of caspase-3 was significantly lower in the CFS group compared to the live *Lpb. plantarum* treatment (P < 0.001) (Fig. 6d).

IL-6, IL-8, TNF- α , and VEGF levels

The culture supernatants of the probiotic and CFS-treated Cal27 cells were used to measure IL-6, IL-8, TNF- α , and VEGF levels. The ELISA results showed significantly lower levels of the IL-6, IL-8, TNF- α , and VEGF in the culture

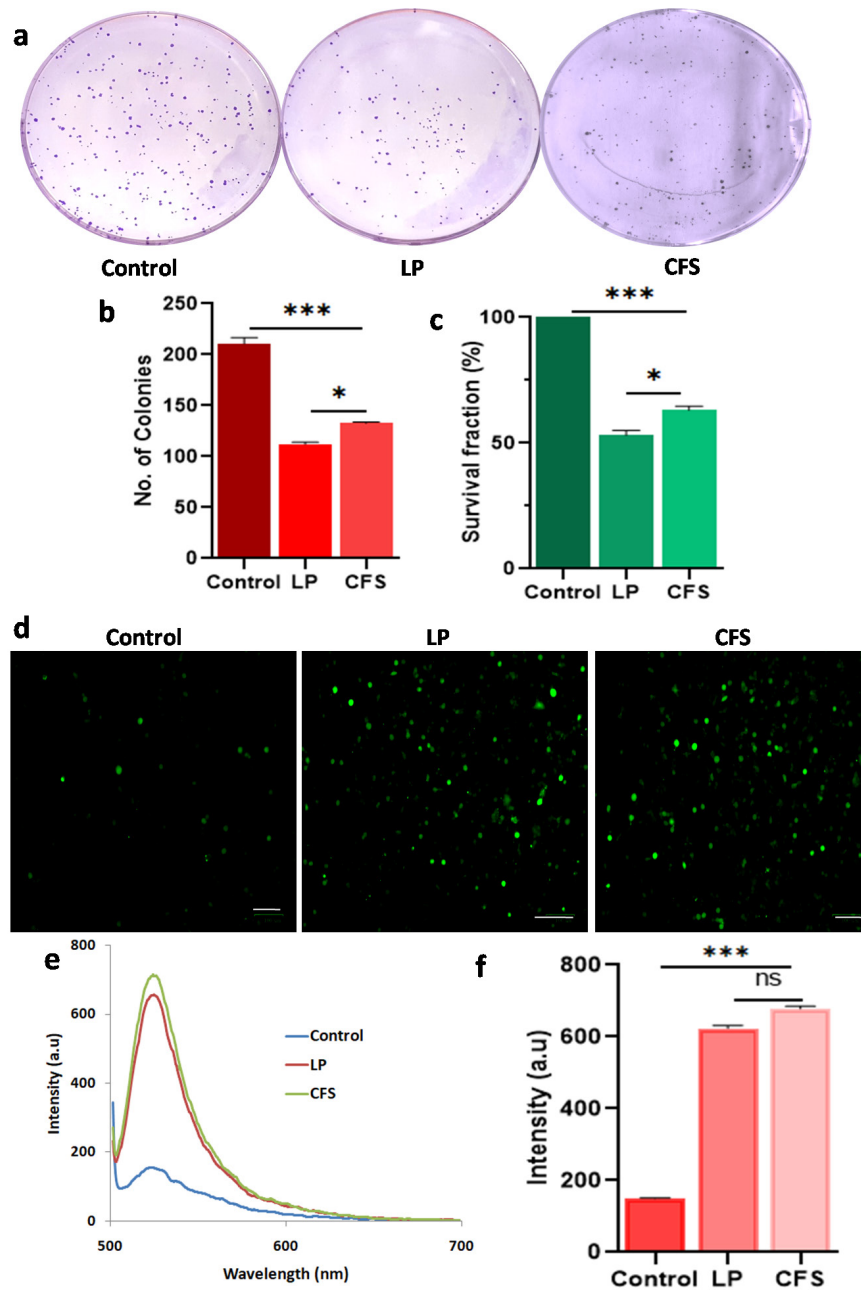


Fig. 5. Colony formation and induction of ROS in the Cal27 cells treated with *Lpb. plantarum* (LP) and its CFS. (a) Photomicrographs of the crystal violet stained colonies (b) number of colonies formed in the different treatment groups and (c) survival fraction of LP (MOI 100) and CFS treatments compared to control. (d) Cal27 cells stained with DCFDA showing ROS production, (e and f) fluorescent spectra and quantification of ROS based on fluorescent intensity with LP and CFS treatments. Results are expressed as mean \pm SD. ns represent non-significant $P > 0.05$, $*P < 0.05$ and $***P < 0.001$.

medium of Cal27 cells treated with both *Lpb. plantarum* and CFS than the control group ($P < 0.01$). Between *Lpb. plantarum* and CFS treatments, no significant differences were observed in IL-6 and TNF- α levels (Fig. 7a and b). However, a significant difference was observed in the levels of IL-8 ($P < 0.05$) and VEGF ($P < 0.001$) between *Lpb. plantarum* and CFS treatments (Fig. 7c and 7d).

***Lpb. plantarum* and its CFS enhance the cytotoxicity of 5-FU against Cal27 cells**

The cytotoxicity of 5-FU on *Lpb. plantarum* and CFS-

treated Cal27 cells were analyzed using the MTT assay. The 5-FU treatment, along with *Lpb. plantarum* and its CFS, showed increased cytotoxicity compared to 5-FU alone (Fig. 8a). The IC_{50} of 5-FU was calculated and found to be 7.2 μ M, and in combination with live probiotic and its CFS, it was reduced to 3.8 and 4.1 μ M respectively. The apoptotic activity of 5-FU on *Lpb. plantarum* treated Cal27 cells was higher than the control (Fig. 8b). Further, the co-treatment of 5-FU with *Lpb. plantarum* resulted in increased chemosensitivity of the drug leading to better inhibition of oral cancer Cal27 cells.

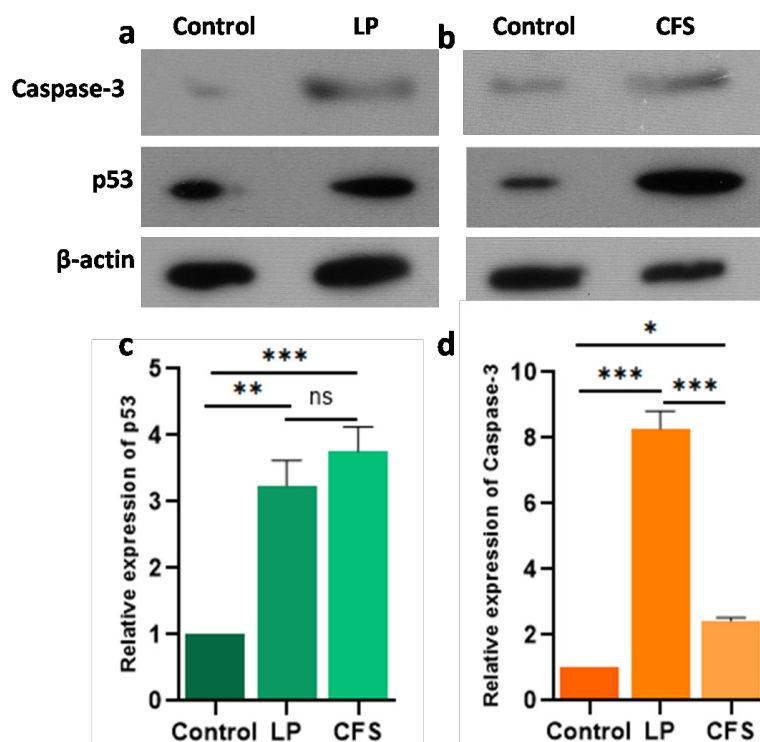


Fig. 6. Relative expression of p53 and caspase-3 in Cal27 cells treated with *Lpb. plantarum* (LP) and its CFS. Representative western blot showing expression of p53 and caspase-3 protein in Cal27 cells treated with (a) LP (MOI 100) and (b) CFS (13.5% v/v). The histogram shows the relative levels of (c) p53 and (d) caspase-3 proteins in the treatments compared to the control. Western blot analysis was performed using proteins isolated from the cell lysates of Cal27 cells treated with LP for 6 h and CFS for 24 h against p53 and caspase-3 primary antibodies. Beta actin was used as loading control to normalize the expression levels. Results are expressed as mean \pm SD. ns represents non-significant $P > 0.05$, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

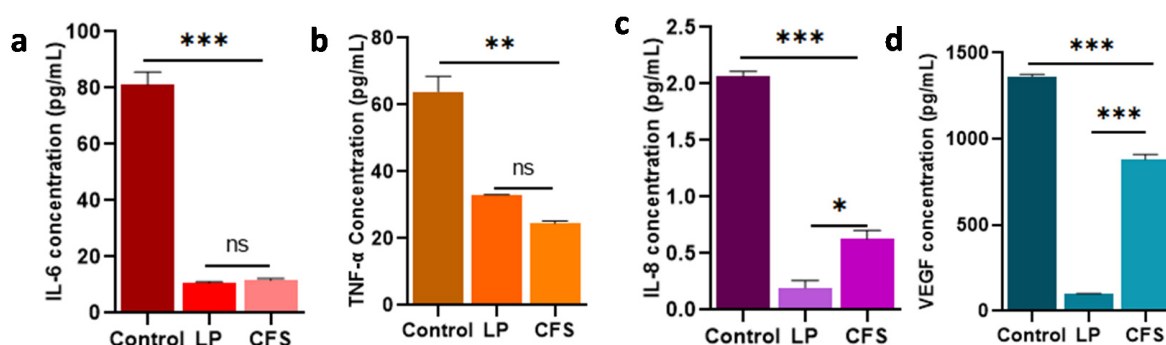


Fig. 7. Effect of *Lpb. plantarum* (LP) and its CFS on the expression levels of inflammatory cytokines and angiogenic mediator in Cal27 cells. (a) IL-6, (b) TNF- α , (c) IL-8, and (d) VEGF levels in the supernatants of LP and CFS-treated Cal27 cells. ELISA method was employed to quantify the protein levels from the CFS of Cal27 cells treated with LP at MOI 100 and its CFS at the IC_{50} dose for 6 h and 24 h respectively. Results are expressed as mean \pm SD. ns represent non-significant $P > 0.05$, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Discussion

Probiotics are increasingly explored for the management of cancer therapy as adjuvants and as protection against recurrence. Our observations demonstrated that the probiotic strain used in the study could inhibit the growth and survival of oral cancer cells. The direct treatment of viable cells of *Lpb. plantarum* MCC 3016 could significantly decrease the cancer cell viability by 48% at MOI 10 and by 60% at MOI of 100 within 6 h of treatment. These results indicate the direct cell killing effect of live probiotic bacteria against oral cancer cells.

Earlier studies have reported a 15% to 27% inhibition of gastric and colon cancer cell viability when treated with live *L. paracasei* and *L. rhamnosus*.¹⁸ However, inhibition of cell viability was lower (3 to 15%) against HT-29 colon cancer cells treated with 15 different probiotic strains of *L. acidophilus* compared to the results of our study.³⁸ The ratio of number of bacteria to the cancer cells also has an effect on the efficacy. In the present study the MOI 100 showed higher inhibition than MOI 10. In an earlier study, *L. acidophilus* at MOI of one could not show inhibitory effect while, MOI 100 was able to bring about 41% inhibition of HT29 cells at 48 h treatment.³⁸ We

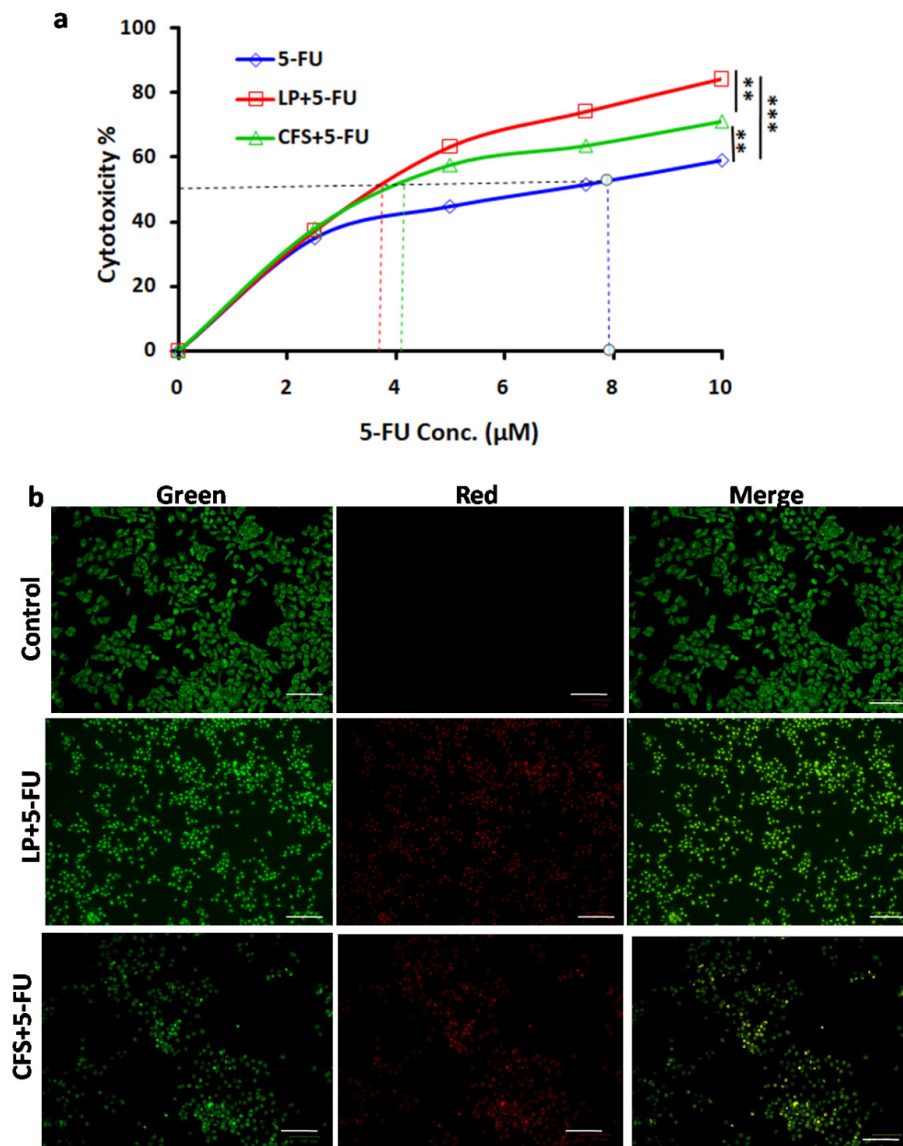


Fig. 8. Effect of 5-FU on *Lpb. plantarum* (LP) and CFS-treated Cal27 cells. (a) Increased cytotoxic effect of 5-FU with LP (MOI 100) and CFS [13% (v/v)] treatment on Cal27 cells was observed in MTT assay. Results are expressed mean \pm SD. ** $P < 0.01$ and *** $P < 0.001$. (b) Micrographs showing the apoptotic activity of 5-FU on Cal27 cells treated with *Lpb. plantarum* and CFS. The cells were treated with 5-FU at its IC_{50} for 24 h post LP treatment and AO-EB staining was carried out (Scale bar = 100 μ m)

observed 15 to 75% inhibition in the cell viability of Cal27 cells treated with the CFS of *Lpb. plantarum* MCC 3016 at concentrations from 5-25% (v/v) for 24 h. However, treating Cal27 cells for 6 h resulted in a maximum 50% reduction at the CFS concentration of 25% (v/v). The CFS derived from *L. rhamnosus* GG, used at concentrations ranging from 10-90% (v/v) to treat gastric and colon cancer cells for 48 h showed 50 to 80% inhibition in cell viability, with IC_{50} values ranging from 74% to 92% across four different cell lines.³⁹ Notably, the IC_{50} calculated for the CFS in our study was 13.5% (v/v) for 24 h treatment, indicating a better inhibitory effect.

Cancer cell migration and proliferation are crucial processes in tumor development, facilitating invasion and leads to metastasis. Inhibiting these processes is essential for controlling cancer growth and metastasis.

Both the live and the CFS of *Lpb. plantarum* MCC 3016 demonstrated a significant reduction in cell migration, as evidenced by the results of scratch assay. Compared to control, a 36-38% decrease in the migration rate of Cal27 cells was observed with live *Lpb. plantarum* MCC 3016 and its CFS treatment. Previously, the CFS from *Lpb. plantarum* OC01 exhibited inhibitory effect on the cell migration against HCT116 and HT29 cells in both 2D and 3D cultures at 72 h.⁴⁰ These observations indicate the potential role of probiotic and its metabolites against cancer cell migration. Tumor cell proliferation is commonly assessed by Ki67 expression, which serves as a prognostic indicator in cancer.⁴¹ Cancer cells overexpress Ki67 due to the active proliferation. In this study, Cal27 cells in the control group showed profused expression of Ki67. However, cells treated with *Lpb. plantarum* MCC

3016 did not express Ki67, suggesting that the proliferation of the tumor cells was arrested by the probiotic treatment. Further, we also observed the downregulation of Ki67 expression with CFS treatment and similar observations were reported earlier on human melanoma cells (A375P and A375SM) treated with CFS from *Lpb. plantarum* L-14.⁴²

Colony formation is considered one of the characteristics of cancer cells and is used to assess the cellular growth and the cytotoxic effects of various anticancer drugs and radiation treatments on cancer cell.^{34,43} The results from the clonogenic assay revealed that treatment with *Lpb. plantarum* MCC 3016 and its CFS reduced the ability of Cal27 cells to form the colonies. A probiotic mixture containing *Bifidobacterium longum*, *B. bifidum*, *L. acidophilus*, and *Lpb. plantarum* significantly reduced colony formation in colon cancer CT-26 cells, which is consistent with our results.⁴⁴ In an earlier study, the CFS of *Lpb. plantarum* 125 and *L. rhamnosus* GG treated HT29 cells formed significantly fewer colonies than control.⁴⁵ Interestingly, in our study, live *Lpb. plantarum* MCC 3016 showed significantly higher inhibition of clonogenicity compared to its CFS.

Probiotics are known to target the key pathways involved in cancer cell proliferation and survival by inducing oxidative stress.⁴⁶ In this study, increased ROS production in Cal27 cells treated with both probiotic and its CFS was observed. ROS stress can induce cellular apoptosis by damaging cellular components and modulating apoptosis pathways.⁴⁷ Yue et al found that treating colon cancer HT-29 cells with *L. acidophilus* (MOI 100) resulted in excessive ROS production, which was associated with apoptosis.³⁸ In our study, the cellular apoptosis can also be attributed to significantly higher expressions of p53 and caspase-3, which may be mediated by the ROS. The nuclear transcription factor p53 is well known for its pro-apoptotic activities, and p53-induced apoptosis depends on increased ROS levels. Caspase-3 is an important protease involved in apoptosis and regulates cancer cell migration, invasion, and metastasis.⁴⁸ Similar observations of the induction of apoptosis in colon cancer cells treated with heat killed and CFS of probiotic bacteria by upregulation of both p53 and caspase-3 are reported.^{49,50}

Further, the levels of inflammatory cytokines IL-6, IL-8, and TNF- α , which were explored in the study, were reduced in oral cancer cells treated with *Lpb. plantarum* and its CFS. IL-6 is an important mediator of inflammation found in tumor microenvironment and tends to promote pro-tumorigenic activities like survival, invasion, growth, and angiogenesis, and plays a crucial role in inflammation-driven oral carcinogenesis.⁵¹

The cell migration of HT-29 and HCT116 colon cancer cells, stimulated by IL-6, was inhibited by treatment with the CFS of *L. plantarum* OC01 indicating the potential

of probiotics in the cancer treatment.⁴⁰ Similarly, IL-8 and TNF- α are also associated with increased cancer cell proliferation, migration and invasion.^{52,53} Probiotic treatment can bring the levels of IL-8 and TNF- α down due to their role in increasing nuclear factor kappa B (NF- κ B) transcriptional activity, an important molecule linking chronic inflammation to cancer.^{54,55} Both IL-6 and IL-8 can activate the janus kinase/signal transducers and activators of transcription (JAK2/STAT3) and extracellular signal-regulated kinases and microtubule associated protein kinases (ERK/MAPK) pathway, involved in cancer cell survival, growth and proliferation.^{56,57} Neoangiogenesis supports the tumor growth and its key contributor, VEGF, is downregulated by *Lpb. plantarum* MCC 3016 and its CFS treatment in this study. A previous study on colon cancer cells treated with the CFS of *L. plantarum* YYC-3 showed downregulation of VEGF/MMPs signaling pathway.⁵⁸ Additionally, VEGF initiates several downstream signaling pathways vital for the growth, survival, and angiogenesis, including phosphoinositide 3 kinase Akt/mammalian target of rapamycin (PI3K-AKT/Mtor), p38 mitogen-activated protein kinases (p38 MAPK), Ras/Raf/mitogen-activated protein kinase/ERK (Ras/Raf/MEK/ERK) pathways.⁵⁹ Clinical evidences on the probiotic supplementation via oral administration in post-surgical colorectal cancer patients suggests the beneficial effects of *Lactobacillus* and *Bifidobacterium*, leading to reduced levels of pro-inflammatory cytokines, including TNF- α , IL-6, IL-10, IL-12, IL-17A, IL-17C, and IL-22.⁶⁰

As probiotic treatments have been hypothesized to improve therapeutic outcomes, findings from our investigation show the effect of probiotic treatment on the drug sensitivity. We found that *Lpb. plantarum* MCC 3016 and its CFS increased the efficacy of 5-FU against Cal27 cells in a dose-dependent manner. Here, we have observed approximately 25% more cytotoxicity in treatment of 5-FU with *Lpb. plantarum* (MOI 100) compared to 5-FU alone (10 μ M). The ability of live *Lpb. plantarum* to enhance 5-FU sensitivity on Cal27 cells was found to be higher than that of its CFS. The combination of the probiotic with 5-FU, however, was not able to bring about a synergistic effect. Nevertheless, the significant increase in cancer cell apoptosis can have a positive influence during the treatment of oral cancer. Previous studies have shown that a combination of live *L. acidophilus* and *L. casei* with 5-FU increases apoptosis in colorectal cancer cells by activating caspase-3 and downregulating p21.²⁰ Live *Lpb. plantarum* and its metabolites also enhanced 5-FU sensitivity in colorectal cancer by downregulating claudin-1 and through the PDK2-mediated glucose metabolic pathway.^{61,62} Therefore, using probiotics or their postbiotic metabolites to enhance the efficacy of chemotherapy in oral cancer can reduce the economic burden and improve the treatment outcomes favorably.

Research Highlights

What is the current knowledge?

- Probiotic bacteria such as *Lactiplantibacillus plantarum* have beneficial effect against cancer cells and induce apoptosis.
- The probiotics and their metabolites increase chemosensitivity of 5-FU in colorectal cancer.

What is new here?

- *Lactiplantibacillus plantarum* MCC 3016 and its postbiotic metabolites decreased oral cancer Cal27 cell viability, migration, proliferation and clonogenicity.
- Increased levels of reactive oxygen species and reduced expression of Ki67 result in apoptotic and antiproliferative activity.
- Upregulation of p53, caspase-3, and downregulation of IL-6, IL-8, TNF- α , and VEGF contribute to anticancer activity.
- *Lactiplantibacillus plantarum* increased cytotoxic effect of chemodrug 5-fluorouracil on Cal27 cells.
- *Lactiplantibacillus plantarum* and its postbiotic metabolites could be used as a possible therapeutic strategy in oral cancer.

Probiotics can also be prescribed for the high-risk group as preventive measures after carefully studying the long-term health benefits through clinical trials. Further studies can explore the major metabolites of the probiotic bacteria that are responsible for their activity and can be developed into drug molecules.

Conclusion

The findings of the study underscore the potential of the probiotic strain *Lpb. plantarum* MCC 3016 and its postbiotic metabolites in oral cancer treatment due to their direct and combined effects with the chemotherapy drugs. The ability of *Lpb. plantarum* to adhere to cancer cells and colonize them offers promise as a preventive measure during the pre-cancer stages and against cancer recurrence, as well as to improve drug treatment efficacy. Additionally, it can be used to restructure the oral microbiome as a preventive measure in high-risk patients. Further research is needed to elucidate the precise mechanisms underlying the interaction between probiotic bacteria and cancer cell.

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Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author

Ethical Statement

This article does not contain any studies with human participants or animals performed by the authors

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