RESEARCH PAPER

Bioactive Substances Derived From Probiotic Lactobacillus acidophilus Reduce Motility And Viability In Cervical Cancer Cells.

Yatakona Supriya[1], Sathasivam Sivamalar[1]*, Jamparapu Pranuthi Roy[1] ,Kasirajan Sudharsan[2]

[1]*Department of Research, Meenakshi Academy of Higher Education and Research (MAHER), Chennai-600078, Tamil Nadu, India*

Email : ysupriya230@gmail.com ,sv.biotech@gmail.com ,iampranuthi@gmail.com

[2]*Department of Microbiology, Vels Institute of Science and Technology & Advanced Studies (VISTAS) Pallavaram, Chennai, Tamil Nadu, 600117, India*

Email :ksudharsan.sls@velsuniv.ac.in

ABSTRACT

relatively higher incidence in developing countries. Chronic infection with high-risk human papillomavirus (HPV), particularly types 16 and 18, has been recognized as the principal causative factor. Although conventional treatments are highly effective, the limitations of these treatments, including toxicity, resistance, and recurrence, underscore the need for non-toxic complementary therapies. In this context, vaginal Lactobacillus species and their derivatives have been recognized as potential candidates for the prevention of cervical cancer. The anticancer potential of Lactobacillus acidophilus cell-free supernatant (LACFS) was examined in this investigation against human cervical cancer cell lines, SiHa (HPV Positive) and C33A (HPV Negative). L. acidophilus (ATCC 4356) was cultured anaerobically, and sterile cell-free supernatant was obtained for in vitro experiments.

Cytotoxicity was measured by MTT assay, morphological changes by phase contrast microscopy, migratory ability by scratch wound healing assay, and apoptosis induction by Annexin V-FITC/Propidium iodide staining and flow cytometry. Following treatment with LACFS, there was a significant concentration-dependent decrease in cell viability in both cell lines, with SiHa cells being more sensitive. Morphological analysis showed characteristic features of apoptosis such as cell shrinkage, membrane blebbing, and loss of attachment to the substrate. Scratch wound healing assay showed a significant decrease in wound closure, indicating a defect in migratory ability. Flow cytometry analysis further supported a significant increase in both early and late apoptotic cells, with very little necrosis being observed.These findings cumulatively and definitively demonstrate that the metabolites of L. acidophilus have strong cytotoxic, anti-migratory, and apoptosis-inducing effects, and therefore define their potential as a safe and cheap adjunct therapy for cervical cancer.

Keywords: Cervical cancer, Lactobacillus acidophilus, Cell-free supernatant, Apoptosis.

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INTRODUCTION

Cancer is presently considered among the top contributing factors to morbidity and mortality rates across the globe; it poses a significant risk to health worldwide with rising incidence and mortality rates across the population[1]. Cancer is considered a diverse category of diseases due to factors such as uncontrolled cell growth in the body, invasion of the surrounding tissue in addition to metastatic potential at a distance from the site of origin of the disease. Amongst various types of cancers prevalent across different parts of the world, cervical cancer is considered the fourth most common type of cancer in females; it results in the death of approximately 342,000 women every year due to 604,000 new incidences identified across the world in the year 2020; it is identified to predominantly affect low and middle-income countries across the world[2]. Cervical cancer is preventable; it is identified due to the process of human papillomavirus (HPV) testing and further HPV vaccination; however, deficiencies in the area of preventive practices have continued in perpetuating the presence of this type of cancer amongst the population in resource-limited countries across the world[3].

The major etiological factor for cervical carcinogenesis is persistent infection due to high-risk HPV types, especially HPV-16 and HPV-18, implicated in over 70% of cases[4]. HPV executes its oncogenic effect through viral proteins E6 and E7, which inactivate critical tumor suppressors such as p53 and retinoblastoma protein. The subsequent result of this leads to impaired apoptosis, uncontrolled cell cycle progression, genomic instability, and hence malignant transformation. Co-factors that contribute to the further aggravation of this process of progression from low-grade lesions to invasive carcinoma include smoking, immunosuppression, the use of oral contraceptives for a prolonged period of time, and co-infections[5].

Recent research has emphasized the role of the vaginal microbiota in the regulation of the persistence of HPV infection and the development of cervical cancer.Vaginal microbiota overrepresented by *Lactobacillus* species has been shown to be a favorable factor in decreasing the persistence of HPV infection, increasing mucosal

**Author for Correspondence: sv.biotech@gmail.com*

immunity, and preventing the development of cervical cancer[6]. However, altered microbiota characterized by reduced *Lactobacillus* levels and increased anaerobes promotes chronic inflammation and a tumor microenvironment[7]. These observations have led to increased interest in using the vaginal microbiota as a strategy in HPV infection and therapy[8].

Although there have been major progresses made in current conventional therapies like surgery, radiation therapy, and chemotherapy, these therapies are also faced with major drawbacks. The risks of infection, hemorrhage, and impact on fertility are commonly posed in surgical therapies; myelosuppression, neuropathy, gastrointestinal symptoms, andalopecia are induced in patients undergoing chemotherapy; while radiation therapy poses risks of tissue damage even when enhanced image-guiding is employed[9]. Furthermore, the issues of tumor recurrence, resistance, and survival of cancer stem cells also pose major health concerns in cancer therapy. Although innovative therapies like immunotherapy, oncolytic virus therapy, nanomedicine-based drug delivery systems, and gene editing have been shown to be very promising in cancer management, the major drawback is their high cost, susceptibility to immunogenic responses, and unknown long-term toxicities[10,11].

However, the limitations associated with these traditional therapies have brought forth advances in research and development of complementary and alternative modalities that are safer and better tolerated. Probiotics, which are live microbiological fractions which, when administered in adequate amounts, have health benefits, have been proposed to function as novel modulators of host microbiota, immune system, and cellular signaling pathways, showcasing evident therapeutic capability[12]. Of these, *Lactobacillus acidophilus* has been optimally investigated for its pro-capacity to secrete antimicrobial peptides, organic acids, and other metabolites that show apparent inhibition of microbe proliferation, anti-inflammatory properties, and intervention into cellular signaling processes[13]. From experimental proof, *L. acidophilus* metabolites have been shown to induce apoptosis, inhibit proliferation, and suppress migratory activity in multiple cancers, including human papillomavirus-positive cervical cancers, through pathways encompassing intrinsic and extrinsic apoptosis, epithelial to mesenchymal transition, and inhibition of matrix metalloproteinase[14].Consequently, these data underscore the proposed use of *Lactobacillus acidophilus* as a safe and less toxic auxiliary or alternative therapy for cervical cancers, thereby necessitating further exploration into its mechanistic pathways.

Materials and Methods

Collection and Maintenance of *Lactobacillus acidophilus* Culture

The probiotic strain *Lactobacillus acidophilus* (ATCC 4356) was procured from American Type Culture Collection, USA. The revival of the micro-organism was done by culturing in MRS Agar (Hi Media Laboratories Pvt. Ltd., India) under aseptic conditions with a class II biosafety cabinet facility. Well-isolated pure culture was inoculated in MRS broth and incubated under strict anaerobic conditions, temperature (37 °C), in a candle jar method for a period of 24 hours. After receiving growth, it was used for further experiments.

Gram-Stain Identification

The purity and identity of *Lactobacillus acidophilus* cultures were verified through Gram staining. The culture was Gram stained by taking a loopful of the overnight culture and placing it on a clear glass slide. The culture was then heat-fixed. The staining steps involved staining the culture with crystal violet stain, subsequent staining with iodine solution, decolorizing with alcohol decolorizer solution, and finally staining the cultures with safranin counterstain (Hi Media Laboratories Pvt. Ltd., India) and observed under binocular microscope[15].

Isolation and Preparation *of Lactobacillus acidophilus* Cell-Free Supernatant (LACFS)

Lactobacillus acidophilus was cultured using de Man, Rogosa, and Sharpe (MRS) medium under anaerobic conditions for 24 hours at 37°C for optimum cell growth and metabolism. A 24 h subculture with a turbidity equivalent to that of the 2.0 McFarland standardized value (containing 6.0 × 10^8 CFU/ml) was prepared, and subsequently, 2 ml of this suspension was transferred into fresh MRS broth. The MRS broth was incubated under anaerobic conditions at 37°C for 24 hours. The bacteria were then centrifuged at 6000 rpm for 15 minutes[16]. The cell-free supernatant was then sterilized by passing this solution through a sterile 0.22 µm pore size filter membrane, thereby removing any bacteria or bacteria cell residue, before making it ready for MTT cytotoxic assays, as well as the wound healing assay (Figure 1).

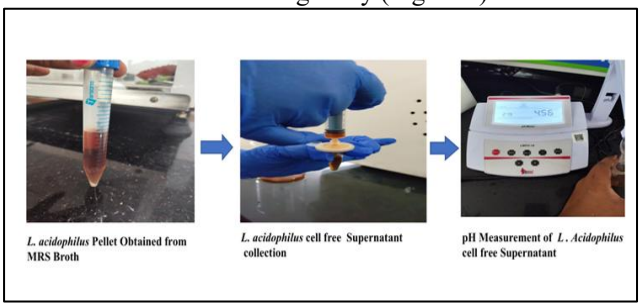

Figure1: Preparation of Probiotic Cell-Free Supernatant

Cell line maintenance

Human Cervical carcinoma cell lines (SiHa and C33A) ware obtained from NCCS, Pune. The cells were maintained in T25 culture flasks in DMEM media supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin). The cells were maintained at 37ºC in a humid atmosphere of 5% CO2. The cells were trypsinized and subcultured upon confluency[17].

Cell viability (MTT) assay

The experiment examined the effect of cell-free supernatant of *Lactobacillus acidophilus* (LACFS) on the survival of human cervical cancer cell lines SiHa and C33A as

determined by the MTT assay (Cayman chemical). SiHa and C33A cells were seeded at a concentration of 5×10^3 cells/well in a 96-well plate and allowed to attach overnight at 37°C in a humidified incubator with 5% CO_2. After overnight attachment, the wells were washed twice with serum-free DMEM and then serum-starved for 3 hours[18]. The cells were then exposed to varying concentrations of LACFS (5-80 µL/mL) for 24 hours, with untreated serum-free cells as controls. After treatment, 100 µL of MTT solution (0.5 mg/mL in DMEM) was added and incubated for 4 hours at 37°C to allow the formation of formazan crystals. The medium was aspirated, and the crystals were dissolved in 100 µL of DMSO, followed by a 1-hour dark incubation with gentle shaking[19]. The absorbance at 570 nm was measured using a Micro ELISA plate reader, all experiments were performed in triplicates, Cell viability can be determined by the following formula:

$$\text{Cell Viability (\%)} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of control} - \text{Absorbance of blank}} \times 100$$

Microscopic study of Cellular Morphology

Morphological analysis was initiated by plating SiHa and C33A cervical cancer cells at a concentration of 2×10^5 cells per well in 6-well plates and allowing them to attach overnight. The following day, cells were treated with the *Lactobacillus acidophilus* cell-free supernatant at their IC50 concentrations (SiHa, 29.56 ± 6.21 µL/mL; C33A, 33.44 ± 0.80 µL/mL) for 24 hours. Following treatment, the medium was aspirated, and cells were gently washed with PBS (pH 7.4). Morphological changes in both treated and untreated samples were then analysed using a phase-contrast microscope[20]. The IC50 values used in these experiments were determined from the MTT assay and used in the wound healing and apoptosis assays as described.

Scratch wound healing assay

In vitro wound healing process involved seeding of 2×10^5 cells per well onto six-well culture plates. 200µl tip was employed to inflict a wound on the cell monolayer, following which PBS was used to clean the wound and take a photograph with an inverted microscope[21] . The same microscope was employed to take the picture of the injured area following a 24-hour treatment cycle of Probiotic supernant (SiHa, 29.56 ± 6.21 µL/mL; C33A, 33.44 ± 0.80 µL/mL) and control cells treated with serum-free growth medium.

Apoptosis Analysis by Annexin V–FITC/PI Staining

SiHa and C33A cervical cancer cells were plated in 6-well plates and allowed to adhere overnight. The cells were treated with *Lactobacillus acidophilus* cell-free supernatant (LCFS) at IC_{50} concentrations for 24 hours, while the untreated cells served as controls. After treatment, both adherent and floating cells were harvested, washed with phosphate-buffered saline (PBS), and stained with Annexin V-FITC and propidium iodide (PI) purchased from BD bioscience company.The samples were left in the dark for 15 minutes at room temperature and immediately analyzed by a flow cytometer. The data obtained was used for early apoptotic, late apoptic, and necrotic cells, which enabled the evaluation of the pro-apoptotic activity of *L. acidophilus* CFS[22].

Statistical analysis

All the information collected were examined with one way ANOVA and then Students-t-test by using GraphPad Prism software version 10, and the results are expressed as mean ± SD of triplicate experiments, with a significance level of $p < 0.05$.

Results and Discussion

Probiotic–Microscopic Examination

Gram staining of the Probiotic *Lactobacillus acidophilus* isolate showed the dominance of Gram-positive bacilli, which were distinguished by the retention of the crystal violet stain and appeared as deep purple, slender rods, occurring singly, in pairs, and short chains, without spore formation or branching, as described in the classical morphological definition of this species[23].The smear showed a dense growth of uniformly stained cells, indicating prolific growth and optimal culture conditions. It is pertinent to note that the absence of Gram-negative or morphologically distinct contaminants indicated the purity of the isolate, ensuring that the biological effects could be attributed solely to probiotic metabolites. The purity and uniform morphology of the *L. acidophilus* culture shown in this study ensure that the biological effects of its cell-free supernatant on SiHa and C33A cells can be attributed specifically to probiotic-derived metabolites, providing a sound basis for the subsequent cytotoxicity, apoptosis, and migration experiments.

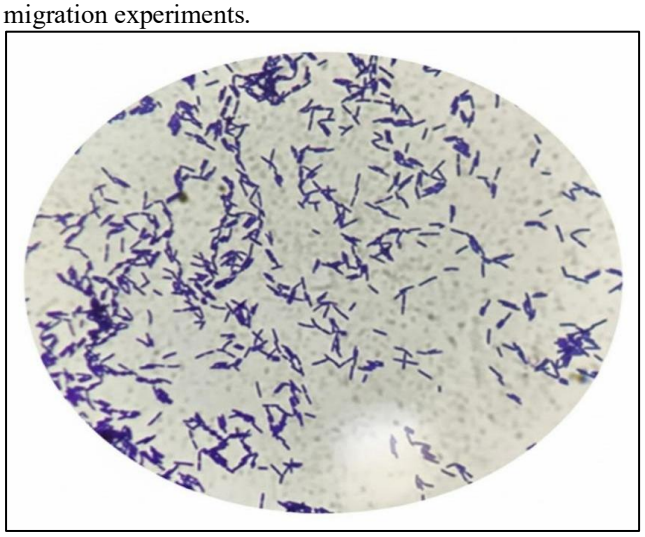

Figure 2: Purple-Stained Gram-Positive Bacilli

Cytotoxic activity of Probiotic *L. acidophilus* supernatant on Cervical cells

The cytotoxic activity of Probiotic *Lactobacillus acidophilus* cell-free supernatant (CFS) against SiHa and C33A cervical cancer cells was assessed using the MTT assay, and the results showed a concentration-dependent decrease in cell viability from 5 to 80 µL/mL. Lower concentrations of 10-20 µL/mL caused mild cytotoxicity (>70% viability), whereas intermediate concentrations of 40-60 µL/mL and higher concentrations of 80 µL/mL caused a significant reduction in viability to approximately

45-40% compared with controls. The IC_{50} value was lower in HPV-16 positive SiHa cells (29.56 ± 6.21 µL/mL) than in HPV-negative C33A cells (33.44 ± 0.8 µL/mL), indicating greater sensitivity in HPV-positive cells. This antiproliferative effect is in line with the earlier study that demonstrated the antiproliferative effect of the metabolites of probiotics, such as organic acids, bacteriocins, and antimicrobial peptides, which could potentially inhibit mitochondrial function, induce intracellular pH alteration, and induce oxidative stress in cancer cells[24]. The higher sensitivity of SiHa cells may be explained by the presence of oncogenes E6 and E7 of the HPV virus, which cause irregularities in cell cycle and apoptosis, making these cells more sensitive to cytotoxic stress[25]. The statistically significant reduction in cell viability at higher concentrations ($P < 0.05$) further supports the specificity and biological significance of *L. acidophilus* CFS as an anticancer agent for both HPV-positive and HPV-negative cervical cancer cells

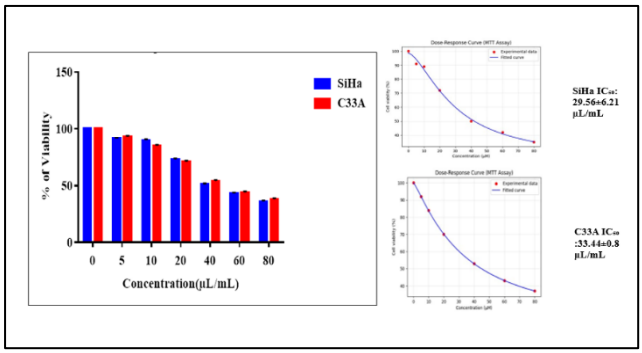

Figure 3: The cytotoxic effects of probiotic supernatant on SiHa and C33A cell lines

Morphological Changes Caused by probiotic strain

Morphological analysis of SiHa and C33A cervical carcinoma cells treated with the cell-free supernatant (CFS) of Probiotic *Lactobacillus acidophilus* at their respective IC_{50} values showed significant morphological changes compared to untreated controls (Figure 4). The untreated cells were seen to have typical epithelial morphology with elongated or polygonal shapes, intact plasma membranes, strong cell-to-cell adhesion, and a dense monolayer, whereas the CFS-treated cells showed cytotoxic stress-induced changes such as cell rounding, shrinkage, membrane blebbing, loss of adhesion, monolayer disruption, and reduced cell density. SiHa cells treated with 29.56 ± 6.21 µL/mL CFS showed extensive loss of adhesion with many floating and broken cells, suggesting severe growth inhibition, whereas C33A cells treated with 33.44 ± 0.8 µL/mL CFS showed cytoplasmic condensation, irregular cell borders, and partial monolayer disruption. These morphological changes are typical of apoptosis and not necrosis and provide strong support to the cytotoxic effects seen in the MTT assay, suggesting a controlled and therapeutically desirable form of cell death[26]. The extent of damage in SiHa cells is consistent with the lower IC_{50} value and can be explained by the molecular changes induced by the high-risk HPV E6 and E7 oncoproteins, which regulate mitochondrial function and apoptosis[27]. The bioactive compounds in *L. acidophilus* CFS, such as organic acids and bacteriocins, have been shown to influence mitochondrial membrane potential and apoptosis, and provide strong morphological evidence for its apoptotic and anticancer properties on both HPV-positive and HPV-negative cervical cancer cells

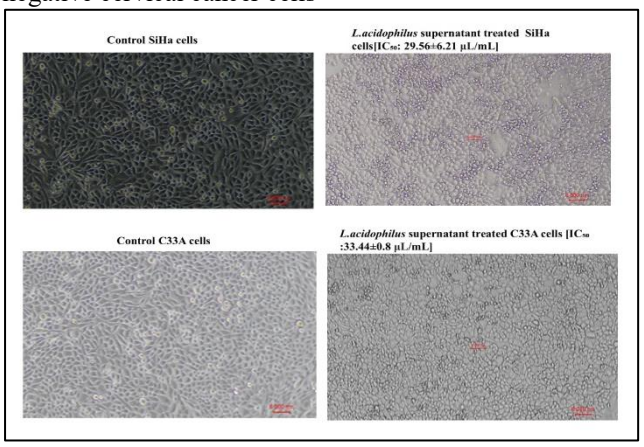

Figure 4: Morphological Alterations in SiHa and C33A Cell lines

Scratch Wound healing assay

The antimigratory effect of *Lactobacillus acidophilus* cell-free supernatant (LACFS) was assessed in SiHa and C33A cervical cancer cells by an in vitro scratch wound healing assay (Figure 5). At 0 h, all experimental groups showed an equal scratch, ensuring proper wound formation, whereas control cells demonstrated time-dependent migration with almost complete wound closure at 24 h. However, treatment of cells with LACFS at IC_{50} values (SiHa: 29.56 ± 6.21 µL/mL, C33A: 33.44 ± 0.8 µL/mL) significantly reduced cell migration, showing little wound closure and a visible wound gap at 24 h. The result was more evident in SiHa cells, suggesting greater sensitivity of HPV-16-positive cells to the antimigratory effect of LACFS. These results indicate that LACFS efficiently inhibits the migration of cervical cancer cells, which is a key process involved in the invasion and metastasis of cancer cells[28]. The decreased migratory ability could be attributed to the organic acids and bacteriocins produced by probiotics, which affect intracellular pH balance and actin filament dynamics, and the application of IC_{50} values verifies that the inhibition of migration is a direct effect, not a secondary effect of cytotoxicity[29]

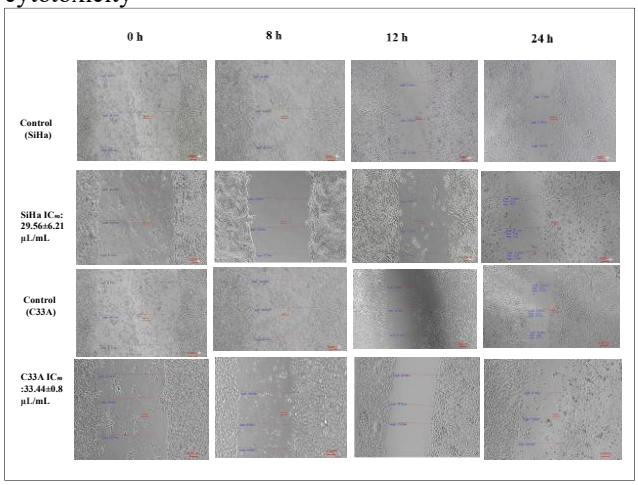

Figure 5: In vitro scratch wound healing assay

Apoptic assay

Apoptotic cell death induced by *Lactobacillus acidophilus* cell-free supernatant (LA-CFS) in SiHa and C33A cervical carcinoma cells was determined by Annexin V-FITC/PI staining (Figure 6). Untreated control cells of both lines were mainly distributed in the viable region (Annexin V^-/PI^-), suggesting high viability and low spontaneous apoptosis. Treatment with IC_{50} concentrations of LA-CFS resulted in a significant increase in early and late apoptotic areas, with a corresponding reduction in viable cells. SiHa cells exhibited greater apoptosis than C33A cells, indicating greater sensitivity of HPV-16-positive cells to LA-CFS-induced apoptosis. The low number of PI^+/Annexin V^- cells suggest low necrosis, confirming that LA-CFS-induced apoptosis is a regulated process rather than non-specific cytotoxicity[30]. These results are in agreement with previous studies that showed probiotic-derived metabolites induce apoptosis in cancer cells through mitochondrial dysfunction, oxidative stress, and caspase activation[31].

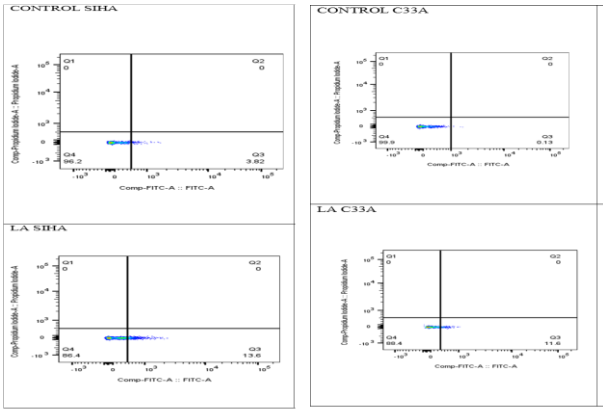

Figure 6. Apoptosis analysis of SiHa and C33A cells by Annexin V–FITC/PI staining.

Conclusion

The present study provides experimental evidence that the cell-free supernatant of *Lactobacillus acidophilus* (LACFS) has a significant inhibitory effect on cervical cancer in human cell lines SiHa and C33A. The results clearly show a dose-dependent decrease in cell viability with evident morphological alterations typical for apoptosis. In addition, the migratory capacity of cells treated with LACFS is significantly reduced, which underlines the potential of probiotic metabolites in interfering with the most important invasion and metastasis events. It is pertinent to note that the SiHa cells infected with HPV were more sensitive to LACFS than the HPV-uninfected C33A cells, which suggests that the probiotic metabolites may have different interactions with the oncogenic events induced by HPV. This difference in sensitivity emphasizes the potential of LACFS as a therapeutic agent in the development of cervical cancer caused by HPV.

Moreover, apoptosis was identified as the method of cell death via Annexin V-FITC/PI staining. Hence, the anticancer therapeutic strategy is focused on the results. Apoptosis-inducing agents have been the preferred agents for the treatment of cancer, as they induce less damage to normal cells. For example, the use of cell-free supernatant is a safer and more manageable procedure than the use of live probiotic. Therefore, the current research has highlighted the anticancer activity of *Lactobacillus acidophilus* metabolites, such as the capacity of *Lactobacillus acidophilus* to restrain the proliferation, survival, migration, and apoptosis of cervical cancer cell lines. The study lends credence to the emerging concept that modulating the vaginal microbiota may prove to be a useful approach in the prevention and treatment of cervical cancer. However, to better isolate the potential of LACFS as a treatment, further research along more mechanistic lines, as well as in vivo and clinical studies, are required. In conclusion, *L. acidophilus* clearly represents a promising candidate for the development of novel probiotic adjunctive therapies in cervical cancer management.

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CONFLICT OF INTEREST

The authors declare that none of their known competing financial interests or personal relationships could influence the findings of this study.

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DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable

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