

# Exploring Anticancer Potential of *Lactobacillus* Strains: Insights into Cytotoxicity and Apoptotic Mechanisms on HCT 115 Cancer Cells

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**Introduction:** This study aims to systematically assess the anticancer potential of distinct *Lactobacillus* strains on Human Colorectal Tumor (HCT) 115 cancer cells, with a primary focus on the apoptotic mechanisms involved. *Lactobacillus* strains were isolated from sheep milk and underwent a meticulous microbial isolation process. Previous research indicates that certain probiotic bacteria, including *Lactobacillus* species, may exhibit anticancer properties through mechanisms such as apoptosis induction. However, there is limited understanding of how different *Lactobacillus* strains exert these effects on cancer cells and the underlying molecular pathways involved.

**Methods:** Cytotoxicity was evaluated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays and exposure durations of *Lactobacillus* cell-free lyophilized filtrates. Additional apoptotic features were characterized using 4,6-diamidino-2-phenylindole (DAPI) analysis for nuclear fragmentation and Annexin V/PI analysis for apoptosis quantification. Genetic analysis explored the modulation of apoptotic proteins (Bax and Bcl<sub>2</sub>) in response to *Lactobacillus* treatment. Whole-genome sequencing (WGS) was performed to understand the genetic makeup of the *Lactobacillus* strains used in the study.

**Results:** The study demonstrated a significant reduction in HCT 115 cell viability, particularly with *L. plantarum*, as evidenced by Sulforhodamine B (SRB) and MTT assays. DAPI analysis revealed nuclear fragmentation, emphasizing an apoptotic cell death mechanism. Annexin V/PI analysis supported this, showing a higher percentage of early and late apoptosis in *L. plantarum*-treated cells. Genetic analysis uncovered up-regulation of pro-apoptotic protein Bax and down-regulation of anti-apoptotic protein Bcl<sub>2</sub> in response to *Lactobacillus* treatment. WGS study revealed a strain reported to NCBI PRJNA439183.

**Discussion:** *L. plantarum* emerged as a potent antiproliferative agent against HCT 115 cancer cells, inducing apoptosis through intricate molecular mechanisms. This study underscores the scientific basis for *L. plantarum*'s potential role in cancer therapeutics, highlighting its impact on antiproliferation, adhesion, and gene-protein regulation. Further research is warranted to elucidate the specific molecular pathways involved and to evaluate the therapeutic potential of *L. plantarum* in preclinical and clinical settings.

**Keywords:** sheep milk, *Lactobacillus plantarum*, cytotoxicity, apoptosis, human colorectal tumor 115, cancer therapeutics

## Introduction

Colorectal cancer (CRC) continues to impose a significant global health burden, necessitating ongoing exploration of innovative strategies for prevention and treatment.<sup>1</sup> These are the third most commonly diagnosed cancer and according

to recent data, CRC accounts for approximately 10% of all new cancer cases and 9% of all cancer-related deaths globally.<sup>2</sup> Presently utilized therapeutic approaches, which encompass surgery, radiotherapy, and chemotherapy, have the potential to induce numerous undesirable side effects.<sup>3</sup> These may include but are not limited to clinical conditions. Furthermore, the drugs employed in these treatments face challenges related to their lack of selectivity and the development of chemoresistance.<sup>4</sup> These issues pose significant hurdles to the safety and efficacy of cancer therapy, impacting its overall management. Nutrition seems to play a pivotal role in preventing, initiating, and progressing cancer, complementing diverse and effective treatment approaches.<sup>5</sup> The digestive system forms an intricate ecosystem hosting a variety of both advantageous and detrimental microorganisms.<sup>6</sup> Probiotics, particularly various strains of *Lactobacillus*, have emerged as potential agents with diverse health benefits, including anticancer properties as elimination of carcinogens, modification of colon physicochemical conditions, metabolic activities in the intestinal microflora, production of anti-mutagenic or anti-tumorigenic compounds, and stimulation of the immune system.<sup>7,8</sup> Few lactic acid bacteria (LABs), such as *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus acidophilus*, control over oncological markers in human CRC and preventive effects on carcinogen-induced colon malignancies in experimental animal models.<sup>9</sup> LABs impede the proliferation of cells by interacting with them.<sup>10</sup> This study focuses on a comparative assessment of the cytotoxic effects of novel *Lactobacillus* strains – *L. plantarum*, *L. rhamnosus*, and *L. acidophilus* – specifically targeting the HCT 115 colon cancer cell line. The findings of Gholipour et al suggested that the metabolomics of LABs directly induce toxicity against cancer cells. The primary objective is to unravel and understand the apoptotic mechanisms induced by these probiotic strains, shedding light on their potential as therapeutic interventions against colorectal cancer. The distinct characteristics of *L. plantarum*, *L. rhamnosus*, and *L. acidophilus* warrant investigation, particularly regarding their potential impacts on colon cancer cells. This study aims to provide valuable insights that could have implications for their application as adjuvants in colorectal cancer therapy. The isolation of *Lactobacillus* strains from sheep milk forms the foundation of our study. This study aims to contribute novel insights into the comparative cytotoxicity of distinct *Lactobacillus* strains on colon cancer cells. The elucidation of apoptotic mechanisms not only advances our understanding of probiotics' anticancer potential but also provides a foundation for potential therapeutic applications. The findings hold promise for the evolving field of probiotics in oncology, particularly within the context of colorectal cancer research.

## Methods

### Sample Collection and *Lactobacillus* Strains Isolation

A total of 180 sheep milk samples (Indian breed) were collected from local places in the Kolhapur, Sangli, and Admapur areas of Maharashtra, India. For bacterial enumeration, milk samples (1 mL) were kept at  $-80^{\circ}\text{C}$  in 15% glycerol before use. Isolation and purification were performed on de Man-Rogosa-Sharpe (MRS) broth and agar medium.<sup>11</sup> The samples were inoculated on MRS agar and incubated for a period of 48 hrs under microaerophilic conditions. After incubation, the individual colonies formed were transferred into a sterile MRS broth medium to enrich. The purified cultures were identified using 16sRNA PCR assay and sequencing. The universal primers pair F-5'-CTTGTCACACCGCCCGTCA-3' and reverse R-5'-CTCAAACACTAAACAAAGTTTC-3' were used. The PCR protocol was adopted from our laboratory.<sup>12</sup>

### Genome Drafting of *Lactobacillus* Strains

The identified strains were subjected to DNA extraction. Total DNA was extracted using a PureLink genomic DNA extraction kit (Life Technologies), following the manufacturer's instructions. The sequencing libraries were prepared using a Nextera XT library kit, and whole-genome sequencing of *L. plantarum*, *L. rhamnosus*, and *L. acidophilus* strain denoted as JRARSH was performed on the Illumina NextSeq 500 sequencing platform (150-bp paired-end reads) with a shotgun strategy.<sup>13</sup> The Illumina-generated sequence reads were quality filtered by FastQC v.0.10.1 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and low-quality reads were removed before assembly. The quality-filtered reads were assembled de novo using SPAdes v.3.9,<sup>14,15</sup> producing 31 contigs. The gene prediction was performed using the Rapid Annotations using Subsystems Technology (RAST) server v.2.0 ([rast.nmpdr.org/](http://rast.nmpdr.org/)), with default parameters.<sup>16–18</sup> Predicted genes were annotated using the Prokaryotic Genome Annotation Pipeline (PGAP) v.4.9<sup>19</sup>

and BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Default parameters were used for all software unless otherwise specified.

## HCT 115 Cell Culture and Cell-Free Lyophilized Filtrate Preparation

The human colon cancer cell line HCT 115 is developed as monolayers by maintaining it in RPMI (Roswell Park Memorial Institution medium). The procurement of these cells was carried out from NCCS Pune, India. These media are augmented with 100 U/mL streptomycin-penicillin and 11% temperature maintained heated killed fetal bovine serum (FBS). The cells were maintained in a humidified atmospheric condition of 4.6% CO<sub>2</sub> at around 36°C (approximately). After 80–85% confluence, cells were treated with the cell-free lyophilized filtrate of the bacterial samples. The *L. plantarum*, *La* and *L. rhamnosus* cultures of *Lactobacillus* were grown in MRS broth at 37°C overnight with a concentration of  $1 \times 10^{7-8}$  cfu count/mL. Later, these cultures were centrifuged at 5100 x g for 5.5 min at 4°C to get pellets. The formed cell pellets were washed with PBS before ultrasonic disruption in a cooling ice bath (4°C) for 15 min at an interval of 28 sec. The formed supernatant is passed through the 0.22 µm pore microfilter (Himedia, USA). Further for cell-free lyophilized filtrates, centrifugation was used to extract the bacterial cells at 5000 rpm for 15 minutes at 4°C when the cultures attained the required OD600. The bacterial pellet was carefully separated from the supernatant, which included the released metabolites and bacterial by-products. A 0.22 µm sterile membrane filter was used to filter the supernatant in order to make sure there were no remaining cellular contamination. Using a freeze dryer, the resultant filtrate—which was devoid of bacterial cells—was lyophilised. After the material was lyophilised, it was reconstituted in sterile phosphate-buffered saline (PBS) to create a concentrated stock solution. This solution was then kept at –80°C until it was needed again. In the tests that followed, cytotoxicity and apoptosis were examined using this cell-free lyophilised filtrate (CFLF). The sterilized filtrate of supernatant was freeze-dried and various concentration was used for cell viability assays.<sup>20</sup>

## Sulforhodamine B (SRB) Assay of *L. plantarum*, *L. rhamnosus*, and *L. acidophilus*

The cellular protein content is measured in SRB assay by determination of cell density. In brief, the cell in the concentration of  $1 \times 10^{5-6}$  cells/mL was incubated for 24 hrs using a 96-well microtitre plate. After incubation, the cells were treated with the cell-free lyophilized filtrate of the *L. plantarum*, *L. rhamnosus* and *L. acidophilus* in concentrations of 10, 20, 30, 40 and 50 (µg/mL). Then, the total medium was incubated at 38°C in 5% CO<sub>2</sub> atmospheric conditions for 24 hrs, 48 hrs, and 72 hrs. After the incubation period, the formed cell monolayers were fixed with 9.9% (w/v) trichloroacetic acid. Further, it was stained for 30 min with SRB and for removing the excess dye, 1% (v/v) acetic acid was used. The OD was calculated at 500 nm by dissolving the protein-bound dye in 9.9 mm Tris base solution using a microplate reader for determination of the cell viability. The percentage of cell viability was calculated by comparing with the values of the control well without any LABs samples and using Equation 1. *L. acidophilus* sample was used as a reference control group.<sup>21</sup>

$$\% \text{ Cell viability} = \text{Absorbance the cell under treatment} / \text{Control cell absorbance.} \quad (1)$$

## MTT Assay of *L. plantarum*, *L. rhamnosus*, and *L. acidophilus*

The anchorage-dependent viability in the form of the cell growth was determined by the colorimetric assay using [3-(4-5-dimethylthiazol-2yl)-2, 5diphenyl tetrazolium bromide] MTT reagent. In the MTT assay, the purple formazan colour of the reduced MTT dye is found directly proportional to the viable cell number quantitated by the spectrophotometric study. In brief, 9000–10,500 cells of HCT 115 were seeded (triplicates) in the 96-well flat-bottomed tissue-culture plates for 24 hrs. After 24 hrs, the cells were treated with the cell-free lyophilized filtrate of the bacterial samples of *L. plantarum*, *L. rhamnosus* and *L. acidophilus* in a concentration of 10, 20, 30, 40 and 50 (µg/ mL). The negative control group kept for the assay consisted of a non-inoculated RPMI medium. The cells washed with the PBS solution after 24 hrs post-treatment were inoculated with 5 mg/mL solution of the MTT in PBS. Further, the cells were incubated for 4 hrs at 37°C for the mitochondrial dehydrogenase activity which was later visualized by the purple formazan crystals formation. Finally, in each well 120 µL of DMSO (dimethyl sulfoxide) was added and incubated for 28 min at 37°C. By

the spectrophotometric method using a microplate reader, the colour intensity was measured at 570 nm. All the experiments were executed in triplicates (n=3), and the data were represented as percent viability compared to the control.<sup>22,23</sup>

## Nuclear Staining with DAPI of *L. plantarum*, *L. rhamnosus*, and *L. acidophilus*

The cell-specific apoptotic process is characterized by DAPI assay. In HCT 115 cells, the analysis of nuclear fragmentation along with the chromatin condensation was done by DAPI staining using fluorescence microscopy. After treatment of 70–80% confluent HCT 115 cells with cell-free lyophilised filtrate of *L. plantarum*, *L. rhamnosus* and *L. acidophilus* (50 µg/mL in RPMI) for 24 hrs the cells were quickly washed with ice-cold PBS and fixed in ice-chilled acetone:methanol (1:1) mixture for 10 min at 4°C in the dark. The cells after washing with ice-cold PBS were incubated for 20 min with the DNA-specific fluorochrome, DAPI. The ice-cold PBS wash was carried out to remove the excess DAPI. Finally, the cells were observed using a fluorescence microscope at 20–40X resolution. *L. acidophilus* sample was used as a reference control group.<sup>24</sup>

## Annexin-V and Propidium Iodide (PI) Assay of *L. plantarum*, *L. rhamnosus*, and *L. acidophilus*

HCT 115 cells were cultured in a dish of 60 mm and further treated with the cell-free filtrate of *L. plantarum*, *L. rhamnosus* and *L. acidophilus* (500 µL). After a 24-hr incubation period, the harvested cells were washed with cold PBS. A 1% Annexin binding buffer solution was prepared. 5 µL of the 1 mg/mL PI stock solution was used to prepare a diluted 100 µg/mL working solution of PI. The final solution along with the inoculated cell was centrifuged and the cells were suspended in 1X Annexin-binding buffer after discarding the supernatant solution. The final volume of 100 µL of cells per assay of buffer was prepared by diluting the 1X Annexin-binding buffer to  $\sim 1 \times 10^5$ – $6$  cells/mL. 1 µL 100 µg/mL PI working solution and 6 µL of Alexa Fluor® 488 Annexin V were added to form 100 µL of cell suspension. Later, the cells were kept at room temperature for about 12 min for the incubation. Finally, the cells were inoculated with the 390–410 µL of 1X Annexin-binding buffer and were placed in an ice bath. The stained cells were evaluated by measuring the fluorescence emission at 520 nm using flow cytometry.<sup>20</sup>

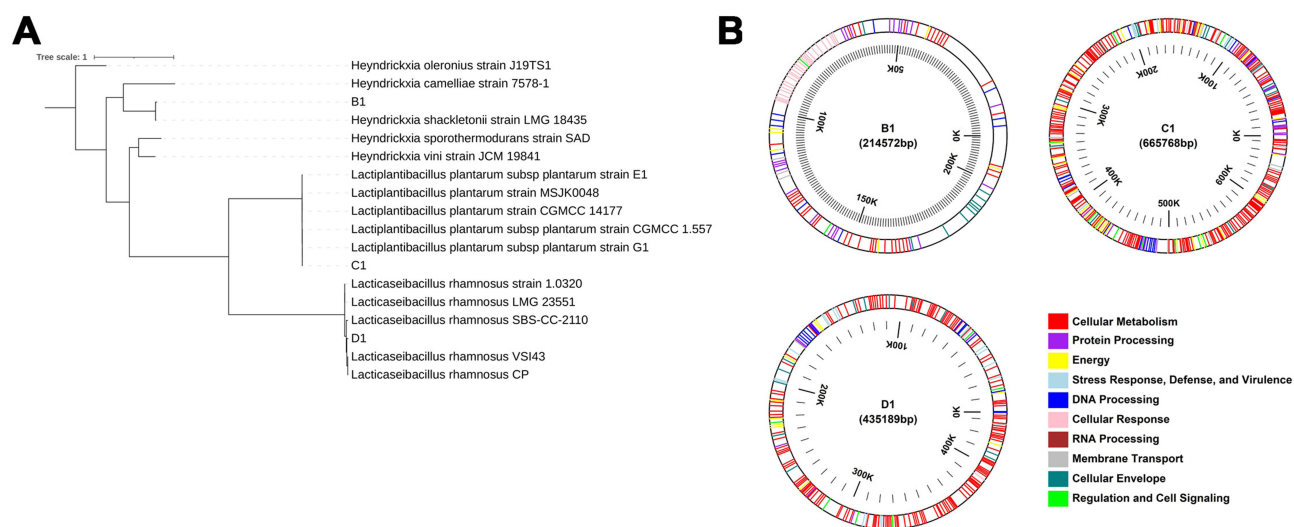
## Statistical Analysis

One way analysis of variance (ANOVA) with Tukey's multiple comparison tests was used to compare the results of the probiotic and control groups using GraphPad Prism 5.01 software, USA. Standard deviations and significant differences were presented as \*p<0.05, \*\*p<0.01.

## Results

### Lactobacillus Strains and Genomic Study

The 16sRNA sequencing confirmed the three stains as *L. plantarum*, *L. rhamnosus*, and *L. acidophilus* and the phylogenetic tree was prepared (Figure 1A). The final assembly yielded a 3.20-Mb genome with a mean G+C content of 44.5%, an N50 value of 174,093 bp, an L50 of 6, and a total of 3128 genes, of which 2980 are protein-coding genes, 59 are tRNA genes, 5 are rRNA genes (35S rRNAs, 116S rRNA, and 123S rRNA), and 4 are noncoding RNA (ncRNA) genes. The genome also contained 80 pseudogenes. Within the total of 3128 genes, biological functions were defined for 2135 (68.25%). The predicted genes that were identified are associated with cellular metabolism (n =38), protein processing (n =323), energy (n = 276), stress response defence and virulence (n = 182), DNA processing (n = 181), cellular response (n = 163), RNA processing (91), membrane transport (n = 33), the cellular envelope (n = 28), and regulation and cell signalling (n = 18) (Figure 1B). Moreover, the genes were each assigned a putative function. Putative functions included cell adhesions, acid tolerance, bile toxicity, molybdenum cofactor biosynthesis, folate and pteridine biosynthesis, aromatic compound degradation, exopolysaccharide production, riboflavin and bacteriocin production, thiamine and methionine biosynthesis, phosphate metabolism, sulfur metabolism, dormancy and sporulation, and denitrification. This *L. plantarum* bacterial strain also proves to be an attractive candidate for the metabolic engineering



**Figure 1** (A) Phylogenetic Tree based on 16S rRNA Sequencing Confirms *L. plantarum* (B1), *L. rhamnosus* (C1), and *L. acidophilus* (D1). (B) Genome Annotation Overview of the Assembled *Lactobacillus* Genome. Different colors represent various functional categories. Cellular Metabolism (Red), Protein Processing (Purple), Energy (Yellow), Stress Response, Defense, and Virulence (Light Blue), DNA Processing (Dark Blue), Cellular Response (Pink), RNA Processing (Brown), Membrane Transport (Gray), Cellular Envelope (Teal), Regulation and Cell Signaling (Green).

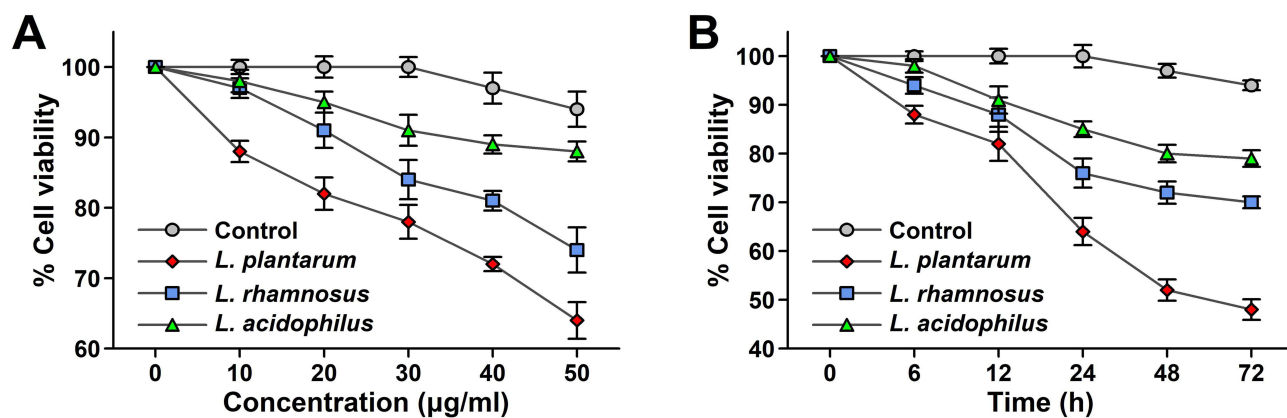
of lignocellulosic biomass to biofuels, owing to its characteristic natural abilities to metabolize the hexose sugars and to tolerate high ethanol and acid concentrations.

## SRB Assay on HCT 115 Cell Line

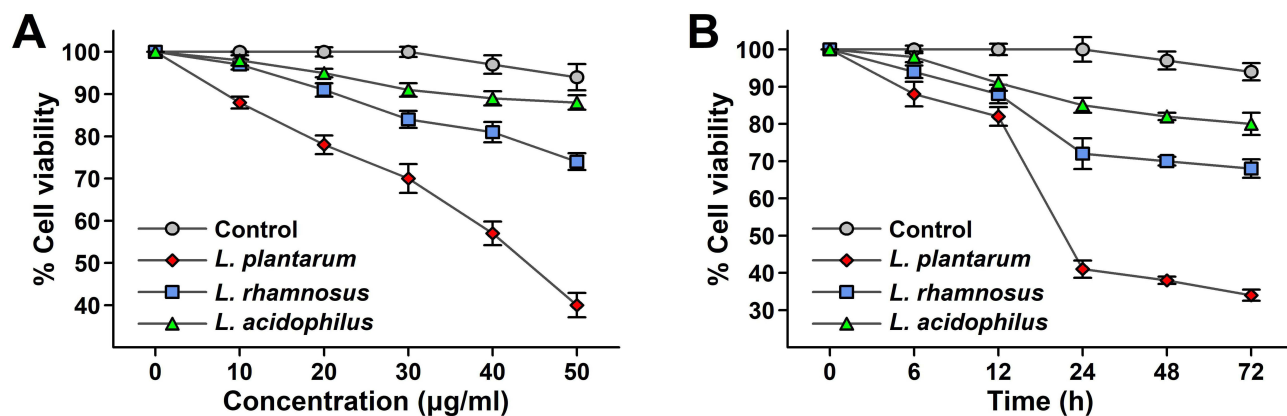
The viability study conducted by SRB assay on the HCT 115 cell line shows a decrease in percentage cell viability of approximately 64% in the case of *L. plantarum* for 50  $\mu\text{g/mL}$  concentration concerning the control group. Similarly, the cell viability results obtained in the case of *L. rhamnosus* and *L. acidophilus* show 74% and 88% viability of the HCT 115 cell line for 50  $\mu\text{g/mL}$  with respect to the control group. Time-dependent SRB assay was carried out for a period of 24 hrs, 48 hrs, and 72 hrs. The study reveals that the cell viability decreased with time. *L. plantarum* shows a decrease in viability approximately from 64% at 24 hrs, 52% at 48 hrs to 48% after 72 hrs as compared to the control group (Figure 2A). Similarly, the cell viability decreased with time in the case of *L. rhamnosus* with an approximate decrease in viability from 76% at 24 hrs to 72% at 48 hrs and 70% at 72 hrs (Figure 2B). A very negligible decrease in the viability of HCT 115 cell lines are observed in the case of *L. acidophilus*. Thus, both time-dependent and concentration-dependent decreases in the cancer cell line viability is observed significantly in cases of *L. plantarum* as compared to *L. rhamnosus* and *L. acidophilus* by SRB assay.

## MTT Assay

In this assay, the MTT reagent passes through the viable cells and enters into the mitochondria, producing the dark blue/purple insoluble compound called formazan. This is observed only in viable cells, as the yellow tetrazolium salt from the MTT reagent is reduced to coloured purple dye only in the living cells. These viable colored cells get homogenized and soluble because of the use of organic solvent like isopropanol resulting in the formation of the formazan. The dead cells never show these activities thus, this assay gives the count of the living cells in the form of the viability count and determines the number of metabolically active cells. The viability study conducted by MTT assay on HCT 115 cell lines shows a decrease in percentage cell viability, approximately around 40% in the case of the *L. plantarum* for its highest dose of 50  $\mu\text{g/mL}$  and further, showing the strongest antiproliferative activity as compared to the control group. In the case of *L. plantarum*, except for the dose of 10  $\mu\text{g/mL}$  the other doses from 20  $\mu\text{g/mL}$  to 50  $\mu\text{g/mL}$  show antiproliferative activity. Similarly, the cell viability results obtained in the case of *L. rhamnosus* and *L. acidophilus* are 74% and 88% on the HCT 115 cell line for its highest dose of 50  $\mu\text{g/mL}$  with respect to the control group. *L. acidophilus*, which is used as the reference control group, does not show any antiproliferative activity (Figure 3A). Time-dependent



**Figure 2 (A)** Effects of the cell-free lyophilized filtrate of LAB on the viability of HCT 115 cell line for various concentrations by SRB assay. Values are expressed as viability mean ratio  $\pm$  SD\*, n = 3. **(B)** Effects of the cell-free lyophilized filtrate of LAB on the viability of HCT 115 cell line for various incubation time by SRB assay. Values are expressed as viability mean ratio  $\pm$  SD\*, n = 3.

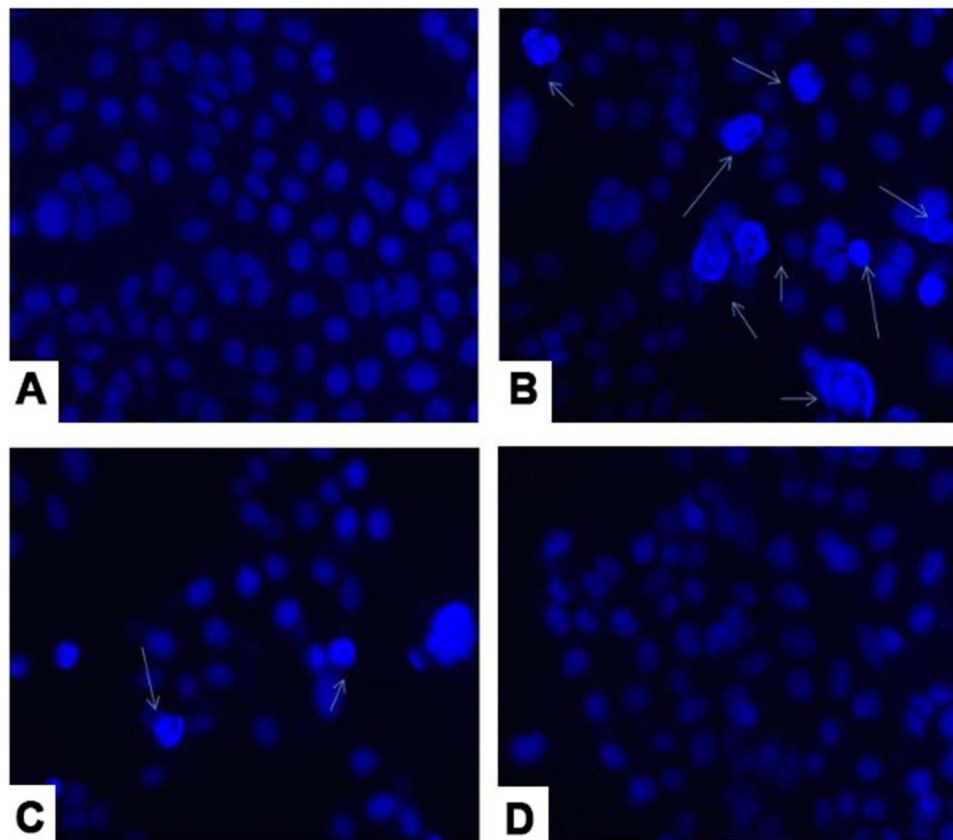


**Figure 3 (A)** Effects of the cell-free lyophilized filtrate of LAB on the viability of HCT 115 cell line for various concentrations by MTT assay. Values are expressed as viability mean ratio  $\pm$  SD\*, n = 3. **(B)** Effect of the cell-free lyophilized filtrate of LAB on the viability of HCT 115 cell line for various incubation time by MTT assay. Values are expressed as viability mean ratio  $\pm$  SD\*, n = 3.

MTT assay is carried out for a period of 24 hrs, 48 hrs, and 72 hrs. The study reveals that cell viability decreases with time. *L. plantarum* shows a decrease in viability count approximately from 41% at 24 hrs, 38% at 48 hrs to 34% after 72 hrs as compared to the control group. Similarly, the cell viabilities decreased with time in the case of *L. rhamnosus* with an approximate decrease in viability from 72% at 24 hrs, 70% at 48 hrs and 68% at 72 hrs (Figure 3B). While the negligible decrease in the viability in HCT 115 cell lines are observed in the case of the control group. Thus, both time-dependent and concentration-dependent decrease in the cancer cell line viability is observed significantly in the case of *L. plantarum* as compared to *L. rhamnosus* and *L. acidophilus* by MTT assay.

## DAPI Analysis

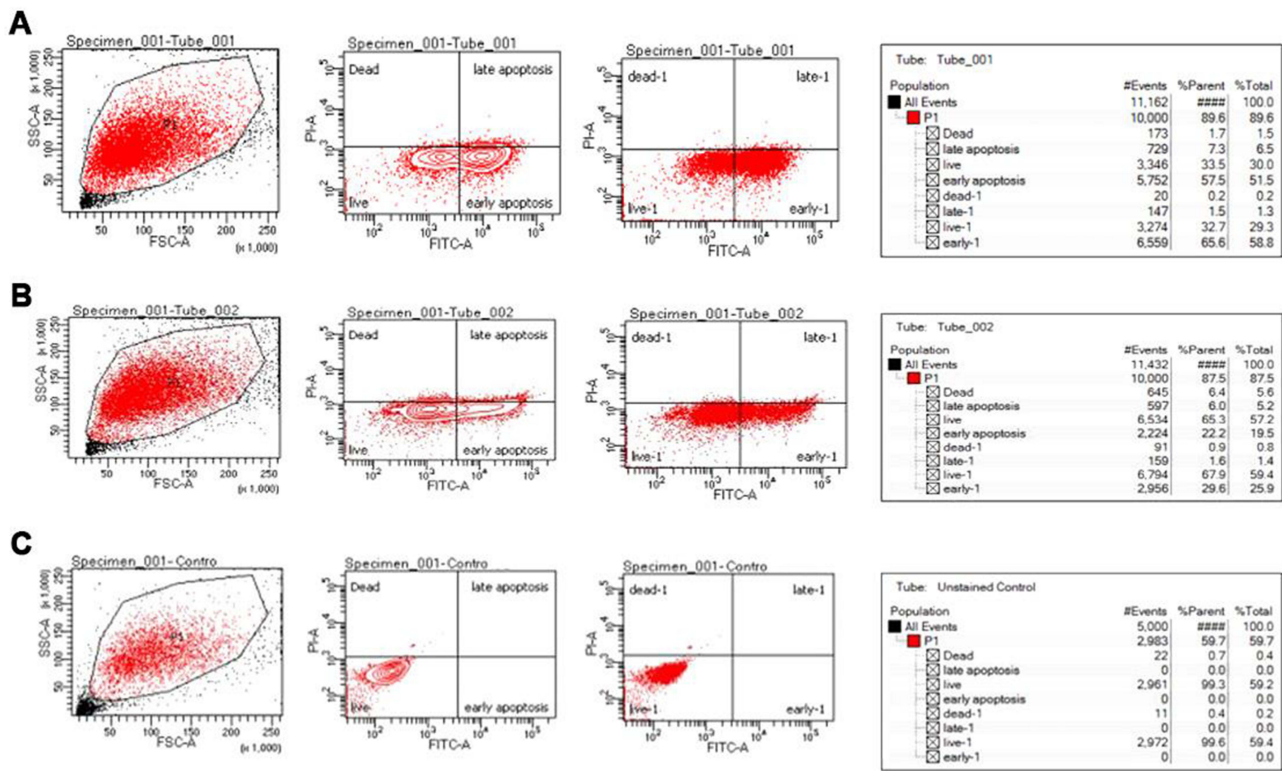
The cell-free lyophilized filtrate of all three strains was analyzed for 24 hrs on HCT 115 cell lines. Significant apoptotic signals were observed in the case of *L. plantarum* as compared to *L. rhamnosus*. Non-apoptotic signals are observed in the case of the *L. acidophilus* which is used as a reference control group. The observed apoptotic cells were with fragmented condensed nuclei as compared to the intact colour normal cells. After 24 hrs incubation, the *L. plantarum* treated HCT 115 cells show apoptotic shrunk cells with nuclear fragmentation pattern as compared to the *L. rhamnosus*. The other features observed in the case of the apoptotic cells are the formation of a micronucleus blebbing cell membrane with the shrinkage of the nucleus in *L. plantarum*. While *L. rhamnosus* shows a blebbing membrane with a micronucleus. Apoptosis is visualized by DAPI (Figure 4A–D).



**Figure 4** DAPI analysis of *Lactobacillus* strain (A) untreated cell line, (B) *L. plantarum* (C) *L. rhamnosus* and (D) *L. acidophilus*.

### Annexin V/PI Analysis of *L. plantarum*, *L. rhamnosus*, and *L. acidophilus*

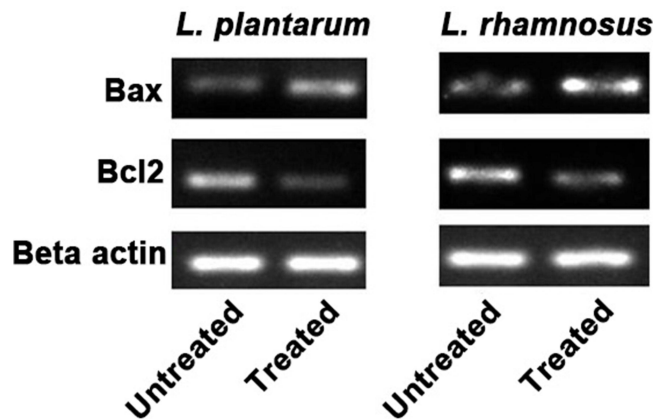
HCT 115 cells are treated with the supernatant of *L. plantarum*, *L. rhamnosus*, and *L. acidophilus* for 24 hrs. Thus, mixed results of the fluorescent dots (annexin V+/PI-) are observed in the lower right quadrant indicating the early apoptosis phenomenon in the case of *L. plantarum*, and *L. rhamnosus*, while no such dots are observed in control *L. acidophilus*. Similarly mixed results of the fluorescent dots (annexin V+/PI-) are observed in the upper right quadrant indicating the late apoptosis phenomenon in cases of *L. plantarum*, and *L. rhamnosus*, while no such fluorescent dots are observed in control. The results revealed that about 57.5% of the early and 7.3% late apoptosis are observed on the HCT 115 cell lines by treatment with the *L. plantarum* supernatant (Figure 5A). In the case of the *L. rhamnosus*, the early and late apoptosis observed on the HCT 115 cell line was about 22.2% and 6.0% respectively (Figure 5B). The results also revealed that 0.0% and 0.1% of the early and late apoptosis are observed on the HCT 115 cell lines by treatment with the La supernatant, which was used as the reference standard control group in the study (Figure 5C). The necrotic dead count observed in the upper left quadrant in the case of *L. plantarum*, *L. rhamnosus*, and *L. acidophilus* were 1.7%, 6.4% and 0.7% respectively which are not affecting the overall study which was conducted. The pro-inflammatory agents are released due to the death of the cells by the necrosis phenomenon, while the death of the cells by apoptosis is the cascade event. It concludes that the death in the cell lines is due to the phagocytosis phenomenon, induced by the macrophages and cytokines due to the complementary system activation. The PI inclusion in the assay assesses the membrane integrity during the apoptosis process. The staining with the FITC-conjugated annexin V differentiates the category of death as necrosis or apoptosis (early or late). The current study proves that the *L. plantarum* exhibits as early and late apoptosis as compared with the *L. rhamnosus* with negligible necrosis events. Earlier studies have proven that *L. plantarum* SBL with a concentration of about 50  $\mu\text{g/mL}$  after 24 hrs showed 20% and 51.5% early and late apoptosis on Hela cell lines.



**Figure 5** Annexin V/PI Analysis of *L. plantarum*, *L. rhamnosus*, and *L. acidophilus* on HCT 115 Cells, (A) *L. plantarum* Treatment, Panel (B) *L. rhamnosus* Treatment, Panel (C) *L. acidophilus* Treatment (Control).

### Up/Down-Regulation of the Apoptotic Protein Expression of Lactobacillus Strains

The apoptotic proteins were investigated by the up-regulation and downregulation of the Bax, Bcl2 and beta-actin along with its expression ratio. Bax acts as pro-apoptotic proteins, it shows a significant increase ( $p < 0.01$ ) in the Bax of *L. plantarum* (treated) compared with the (untreated) control group. Similarly, there is a significant increase ( $p < 0.05$ ) in the Bax of *L. rhamnosus* compared with the control group. Thus increase in Bax is found higher in *L. plantarum* as compared to *L. rhamnosus* (Figure 6). Similarly Bcl2 acts as anti-apoptotic proteins, it shows a significant decrease ( $p < 0.01$ ) in the Bcl2 of *L. plantarum* compared with the control group. Similarly, there is a significant decrease ( $p < 0.05$ ) in the Bcl2 of *L. rhamnosus* compared with the control group. Thus, a decrease in Bcl2 expression is seen higher in



**Figure 6** Up/Down-Regulation of Apoptotic Protein Expression of Lactobacillus Strains. Analysis of Bax (pro-apoptotic), Bcl2 (anti-apoptotic), and Beta-actin expression in treated and untreated groups.

*L. plantarum* as compared to *L. rhamnosus*. No significant changes are observed in the Beta-actin expression in case of *L. plantarum* and *L. rhamnosus* for both treated and untreated groups. This shows that *L. plantarum* shows significantly higher apoptotic protein expression properties as compared to *L. rhamnosus*. Apoptotic activities were reported by *L. reuteri* on HT 29 colon cancer cell lines by an increase in Bax and decrease in Bcl2. In a similar way, *L. plantarum* shows apoptotic activity by up-regulation of Bax and down-regulation of Bcl2.

## Discussion

Studying the cytotoxic effects of *Lactobacillus* strains on cancer cell lines is a major advancement in cancer research and treatment. This study aimed to investigate the the impact of three different *Lactobacillus* species (*Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus acidophilus*) on HCT 115 colon cancer cells. We conducted several tests and examinations to understand the mechanics behind their ability to kill cells and to evaluate their potential as new options for cancer treatment. We used the SRB assay as the main method to evaluate cell viability and determine the cytotoxic effects of the *Lactobacillus* strains. This test, based on detecting cellular protein levels, provides information on cell viability by measuring their ability to bind the dye.<sup>25</sup> We discovered that cell viability decreased in a dose-dependent manner after treatment with different *Lactobacillus* strains. Among them, *L. plantarum* showed the most antiproliferative effects compared to *L. rhamnosus* and *L. acidophilus*. This observation highlights the strong ability of *L. plantarum* to kill HCT 115 colon cancer cells, making it a good option for further investigation as a cancer treatment. This first report on the HCT15 cell line shows *L. plantarum* kills the colon cancer cells.

We used the SRB assay as the main method to evaluate cell viability and determine the cytotoxic effects of the lyophilized filtrates of *Lactobacillus* strains. The MTT assay measures the activity of mitochondrial enzymes in living cells to quantify cell viability by tracking the conversion of MTT into formazan crystals.<sup>26</sup> Consistent with the SRB assay findings, we discovered that cell viability decreased in a dose-dependent manner after treatment with different *Lactobacillus* filtrates, especially *L. plantarum*. A similar kind of time and concentration-dependent MTT study was carried out on the *L. plantarum* DGK-17 with a dose of 25 to 250 µg, which showed the effective decline in the colon cancer cell line after 24 hrs.<sup>27</sup> To the best of our knowledge, this is the first report on the HCT 115 cell line showing *L. plantarum* secretions' cytotoxic effects on colon cancer cells.

We conducted further investigations, such as DAPI staining and Annexin V/propidium iodide (PI) analysis, to understand the processes of cell death caused by *Lactobacillus* strains.<sup>28</sup> DAPI labelling allowed for the observation of nuclear structure, making it easier to identify characteristics of apoptosis such as nuclear condensation and disintegration. The results showed that cells treated with *L. plantarum* filtrates exhibited apoptotic features such as nuclear fragmentation and cell shrinkage, which are signs of programmed cell death. *L. rhamnosus*-treated cells showed reduced apoptotic signals, whereas La treatment did not trigger apoptosis, as indicated by the lack of apoptotic characteristics. *L. plantarum* mainly causes cell death by apoptosis, suggesting its promise as a focused treatment for cancer. An in-depth understanding of the apoptotic process was achieved by doing an Annexin V/PI analysis, which allows for the differentiation between the early and late stages of apoptosis. The study showed that *L. plantarum* and *L. rhamnosus* treatments induced both early and late apoptosis in HCT 115 cells, but *L. acidophilus* therapy resulted in modest apoptotic activity. This research offers additional proof of the particularity of *L. plantarum*-induced cell death and highlights its potential for use in cancer therapy. Furthermore, examination of apoptotic protein expression supported the apoptotic process responsible for the cytotoxic effects of *L. plantarum*.<sup>23–31</sup>

The pro-apoptotic protein Bax increased while the anti-apoptotic protein Bcl2 decreased in cells treated with *L. plantarum* filtrates, suggesting activation of the intrinsic apoptotic pathway. On the other hand, treatment with *L. rhamnosus* also resulted in higher Bax expression compared to *L. plantarum*, indicating distinct apoptotic signalling pathways amongst various *Lactobacillus* strains. Overall, our results highlight the strong inhibitory effects of *L. plantarum* filtrates on HCT 115 colon cancer cells by inducing apoptosis. The results provide important information on the molecular pathways responsible for the cytotoxic effects of *Lactobacillus* strains and highlight their potential as new therapeutic agents for treating cancer. Additional study is needed to clarify the precise signalling pathways involved in *L. plantarum*-induced apoptosis and to assess its effectiveness in both preclinical and clinical environments. Furthermore, investigating the possible synergistic impacts of *Lactobacillus* strains in combination with traditional

cancer treatments could improve treatment results and lead to personalised cancer therapy strategies. Our focus on specific *Lactobacillus* strains may not fully represent the diverse probiotic landscape. Mechanistic intricacies of *L. plantarum*-induced apoptosis, though partially elucidated, require further exploration. The clinical relevance of our findings necessitates extensive research for safety, efficacy, and optimal dosage before considering *L. plantarum* filtrates as a therapeutic agent in cancer treatment. The study's scope did not encompass the assessment of synergistic effects with traditional cancer treatments, suggesting avenues for future investigations into combination therapies for enhanced treatment outcomes.

## Conclusions

The present study deals with the comparative cytotoxicity studies of *Lactobacillus* against the cancerous cell lines. The study shows that *L. plantarum* acts as an effective antiproliferative agent against cancer cell lines as compared to the *L. rhamnosus* and *L. acidophilus*. *L. plantarum* shows cell death by the apoptosis due to nuclear fragmentations. The current study shows that the *L. plantarum* exhibit the cell death mechanism as early and late apoptosis as compared with the *L. rhamnosus* with negligible necrosis events. The apoptotic protein expression studies show that *L. plantarum* kills the cancerous cell by up-regulation of Bax and downregulation of Bcl2. Thus, the in-vitro study shows the cancer killing tendency of the *L. plantarum* is by the combination of antiproliferative, and gene-protein regulation mechanisms.

## Data Sharing Statement

The original contributions presented in the study are included in the article further inquiries can be directed to the corresponding author Sandip Patil (sandipatil1309@yahoo.com).

## Author Contributions

Luolin Wang and Zhenglei Xu contributed equally to this work and share the first authorship. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

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