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Original Research Article

# Invitro studies on colon cancer cell inhibition by cell free extracts of natural probiotics and *Bacillus clausii*

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## Abstract

**Background:** Colorectal cancer (CRC) is an increasingly concerning health issue, which signifies in searching for new alternatives to minimize the side effects associated with chemotherapy and radiation therapy. Probiotic organisms belonging to genus *Lactobacilllus* and other genera and their derivatives are gaining attention now a days for their potential role in this area.
**Materials and Methods:** In this study, cell-free extracts from four probiotic isolates-LC-8, LC-20, LC-27, LC-30 and *Bacillus clausii* whose sporulating suspension for maintaining gut microbiota were tested against the HCT-116 and COLO-205 cell lines. MTT assay was done to determine IC-50 values. Wound healing and colony formation assays at half and double IC-50 values were done in order to study cell signalling and colony formation in time and dose dependent manner.
**Results:** Two isolates LC-20 and LC-30 showed promising activities against HCT-116 cells as compared to COLO-205 cells. These crude cell free extracts showed significant antiproliferative effects when compared to the standard drug Adriamycin (ADR). Further investigation to inhibit the cell signalling and wound healing activities of cancer cells with the help of these extracts, tested at half and double IC-50 values, showed that the extracts effectively inhibited cell signalling and migration.
Conclusion: The findings suggest that cell-free extracts from probiotic organisms possess potent antiproliferative properties, making these postbiotics as a promising alternative or combination with chemotherapy for CRC treatment.

**Keywords:** CRC, HCT-116, COLO-205, Cell migration, Clonogenic assay, Chemotherapy

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## 1. Introduction

Colorectal cancer (CRC) is the type of gastric cancer occurring in humans. It is one of the most common cancers in the world which is related to death causes in humans worldwide.[1] Many reasons of occurrence are associated with CRC, which mainly includes less physical activity, obesity, high cholesterol food, low-fibre diets, and alcohol consumption. Recently the use of anti-inflammatory drugs has also contributed significantly in increasing the numbers of CRC.[2]

Probiotic organisms are known to have various health benefits, along with this their cell free extracts which are now called as postbiotics have shown very significant effects on human health, including antibacterial activity against pathogens occurring in intestine, reducing occurrence of colitis and regulating the immune system, and prevention of colon cancer.[3] Probiotics are generally defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[4] Most probiotics are found in the natural intestinal flora playing a significant role. *Lactobacillus spp* and *Bifidobacterium spp* are the most common LAB in human intestine inhabiting and providing numerous health benefits. Many recent studies have focused on the fact that probiotics and their cell free extracts can treat a range of gastrointestinal discomforts like acute diarrhoea, antibiotic-associated diarrhoea, and inflammatory intestinal disorders, by altering the intestinal microflora. It helps in

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establishing equilibrium and regulation of the intestinal epithelial systems. Moreover, probiotics and their cell free extracts can affect the quality and quantitative composition of intestinal ecosystems thereby maintaining normal intestinal ecosystem.[4,5]

Current trends in research have shown that anti-tumour effects of probiotics are particularly remarkable and helps in reducing cell migration of cancer cells. There has been increasing evidence in research of immunotherapy as a promising strategy in combating and treatment of solid tumours.[6] One report showed that the appropriate regulation of intestinal microbiota through the intake of probiotics and their derivatives may help in preventing tumour formation.[7] *L. plantarum* has been shown inhibitory effect of colon carcinoma cells in mice, mostly by altering the tumour microenvironment and promoting the migration of CD8 T cells and natural killer (NK) cells to the tumour tissue.[8] Another study indicates that oral administration of *L. casei* induce helper cells of immune response and cytotoxic T cell infiltration in the tumour tissue of tumour-bearing mice, resulting in tumour growth inhibition,[9] However use of cell free extracts obtained from probiotic organisms as potent anticancer agents and their in vitro effects in induction of various apoptotic pathways is summarized in **Table 1**.

## 2. Aim

Our study focuses on the inhibitory effects of cell free extracts obtained from probiotic organisms on COLO-205 and HCT 116 cells in *vitro*, and their probable role in cell signalling and induction of apoptotic pathways.

## 3. Materials and Methods

### *3.1. Preparation of cell free extracts*

Four Lactobacillus spp. isolates were obtained and characterized according to the Bergey's Manual of Systemic Bacteriology Volume II (Edition III). LC-8, LC-20, LC-27 and LC-30 were inoculated in MRS broth and incubated for 48h at 37 ± 1 °C, Bacillus clausii was inoculated in Luria broth for 48 h at 37 ± 1 °C. Following incubation, the cells were harvested from the broth through centrifugation at 10,000 rpm for 10 minutes at 4°C, to counteract the effects of lactic acid and other organic acids produced by these organisms, the pH of the supernatant (s) was adjusted to 5.5 by adding 12% ammonium hydroxide. Proteins from the supernatant(s) were precipitated by using fractional precipitation method by gradually adding increasing concentrations of ammonium sulfate (30%, 60%, and 90%) followed by dialysis. Estimation of protein was done using Folin-Lowry method.[16,17]

### *3.2 Antiproliferative activity by MTT assay*

Anti-proliferative activities of the cell free extracts were determined by MTT assay.

### *3.3. Cell culture and growing of cells*

Cell Culture HCT-116 (Human Colorectal Carcinoma) and COLO-205 (Human colon adenocarcinoma) cell lines were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in DMEM (Dulbecco Modified Eagle Medium) (Hi-media) supplemented with 10% (v/v) fetal bovine serum FBS, 1% L-glutamine, 1% NEAA, 1% penicillin, and 1% streptomycin. All cells were maintained in a humidified 5% $CO_2$ incubator at 37 $^0$C.

### *3.4. Cell viability by MTT assay (antiproliferative activity)*

Cell lines, HCT-116 and COLO-205 were cultured, and $1\times10^3$ cells were inoculated in 96 well plates following treatment with eight concentrations of cell free extract 3.91, 7.81,15.63, 31.25, 62.5, 125, 250 and 500 µg/ml, where in serial dilution dilution method was used. The assay was carried out in triplicates with incubation period of 48 h. After 48 h media was removed and cells were incubated with 5mg/ml MTT solution for 4 h. MTT formazan crystals were dissolved in tissue culture grade Dimethyl sulfoxide (DMSO) and absorbance was recorded at 570 nm using Read well Touch Automatic Elisa Plate Reader (Robotnik India Private Limited) after incubation of 30 minutes. The percentage viability of the cells treated with cell free extracts and standard drug ADR was compared with the viability of untreated control cells, considering it as 100% viable. Percent cell viability was calculated by following formula

% Cell viability = (Abs of sample − Abs of blank) (Abs of control − Abs of blank) x 100

The IC50 values were calculated by GraphPad prism software 9.0.0. An inverted microscope (Zeiss Axio Observer. Z1) with 10 X, to 100 X air objective (NA 0.8) was used for image acquisition. Zeiss image analysis software used for Initial image processing.[18-21]

### *3.5. Cytotoxicity against peripheral blood mononuclear cells (PBMCs) by cell viability assay*

**Isolation of PBMCs:** Isolation of peripheral blood mononuclear cells (PBMCs) was done using Ficoll-Hypaque according to standard method. PBMCs were isolated by density gradient centrifugation. To isolate PBMCs, whole blood, diluted with PBS, was gently layered over an equal volume of Ficoll in a Falcon tube and centrifuged for 30-40 minutes at 400-500 g without break. PBMCs were removed from white, cloudy layer with the help of Pasteur pipette and added into warm medium or PBS to wash off any remaining platelets. The pelleted cells were counted in Neubauer chamber under inverted microscope with the help of Trypan blue staining.[22,23]

**Table 1:** In vitro effects of postbiotics on CRC cells.

| Probiotic Bacteria | Cell Line used | Probable Effect | Reference |
|---|---|---|---|
| *Bacillus coagulans* | COLO-205 | Cytotoxic effect on cells, progression towards apoptosis by activating various apoptosis inducing genes like Bax Puma and BCL-2 | [10] |
| *Faecalibacterium prausnitzii* | HCT 116 | Cytotoxic activity induced in dose- time dependent manner | [11] |
| *L. rhamnosus GG* | HCT-116, Caco-2, HT-29 | Antiproliferative activity, cell cycle arrest | [12] |
| *Lactobacillus spp.* | HT-29, Caco-2 | Cytotoxic activity induction and upregulating of apoptotic genes | [13] |
| *Propionibacterium freudenreichii* | HCT-116 | Cytotoxic activity with respect to dose kinetics | [11] |
| *L. plantarum* | Caco-2, HT-29 | Antiproliferative effect | [14] |
| *Lactiplantibacillus plantarum* | HCT 116, HT-29 | Dose-dependent cell toxicity | [15] |

Colon cancer cell lines: Caco-2, HT-29, COLO-205

**Cell Viability of PBMCs by MTT Assay:** Cell viability assay of PBMCs was performed by MTT assay and calculated by formula,

% cell viability = (Abs of sample − Abs of blank) (Abs of control − Abs of blank) x 100

*3.6. Clonogenic Assay (Colony formation assay)*

Clonogenic assay was performed on HCT-116 cell line according to protocol described in literature with some modifications. Cell free extract showing significant IC -50 values as compared to standard ADR, LC- 20, LC-30 and *Bacillus clausii* were used. HCT-116 cells were plated at a seeding density of 500 cells per well in a 6-well cell culture grade plate. After 24h, the culture medium was changed, new medium was added and the cells were treated with half and double IC-50 concentrations to study dose dependent kinetics. The treated cells were incubated for 24 h in 5% CO2. Then, the treated cells were exposed to fresh media and kept in the media for 7 days for colony formation with media change for 2days. The obtained colonies were fixed with ethanol and were stained with 0.5% crystal violet solution.[24,25]

*3.7. Wound healing assay*

Wound healing assay was performed on HCT-116 cell line according to protocol described in literatures with some modifications. HCT-116 cells at a density of $1 \times 10^4$ cells/well were incubated for 24 h at 37 $^0$C and 5% CO2 to allow cell adhesion and the formation of a confluent monolayer. After 24 hours of incubation media was removed, and the cell monolayer was scratched in a straight line with a sterile pipette tip to leave a scratch width of approximately 0.5 mm Cells were gently washed three times with phosphate-buffered saline (PBS) to remove the debris and replaced with a fresh serum supplemented culture medium. Then, the cells were immediately treated with cell free extracts showing significant IC-50 values selection of half and double IC-50 values were done to understand dose and time dependent kinetics. The cell migration ability was recorded after different time interval. Migrated cells were observed under the inverted microscope. The photographs of the scratch were taken at the 0, 12, and 24 h time interval.[26,27]

*3.8. Cell cycle analysis of cell free extracts*

Flow cytometry with Propidium Iodide (PI) staining was done to investigate the effect of cell free extracts and standard *Bacillus clausii* on various stages of the cell cycle. HCT-116 cells seeded at a density of $1 \times 10^4$ cells/well were incubated at 37 $^0$C for 24 hrs with 5% $CO_2$ to allow cell adhesion and the formation of a confluent monolayer. Adhered cells were then gently washed for three times with sterile phosphate-buffered saline (PBS) to remove the cell debris and replaced with a fresh serum supplemented DMEM culture medium. The confluent cells were immediately treated with cell free extracts showing significant IC-50 values, where half and double IC-50 dose values were selected to understand dose and time dependent kinetics on the cell cycle progression. The cells were stained with propidium iodide before acquiring. A total of 10,000 cells were acquired in each tube.[21]

## 4. Results

*4.1. Protein estimation*

**Table 2:** Protein estimation by Folin Lowry method

| Isolate | Protein Concentration in mg/ml |
|---|---|
| LC-8 | 6.9 |
| LC-20 | 6.2 |
| LC-27 | 5.9 |
| LC-30 | 1.3 |
| *Bacillus clausii* | 3.6 |

*4.2 Anti-proliferative activity*

The anti-proliferative activities of the crude extracts, extracted from isolates were tested against human colon

cancer cell lines namely HCT-116, and COLO-205 through MTT assay. All the experiments were performed in triplicates. The results for each crude extract are expressed as the percent cell viability at different concentrations in ug/ml. ADR (Doxorubucin), was used as standard. The susceptibility of cells to the crude extract was assessed by $IC_{50}$ and depicted in **Table 3**.

**Table 3:** *In vitro* anti-proliferative activity of crude extracts against HCT-116 and COLO-205 after 48-hour-exposure.

| Crude extract | $IC_{50}$ values (ug/ml) | |
|---|---|---|
| | HCT116 | COLO-205 |
| LC8 | > 100 | 214.3 ± 4.28 |
| LC20 | 5.07 ± 0.67 | 207.4 ± 1.09 |
| LC27 | 204.5 ± 2.40 | 280.7 ± 1.65 |
| LC30 | 19.84 ± 0.03 | 223.4 ± 3.14 |
| *Bacillus clausii* | 24.8 ± 0.15 | 236.8 |
| ADR | 27.8 ±0.54 | 32.6 ±1.50 |

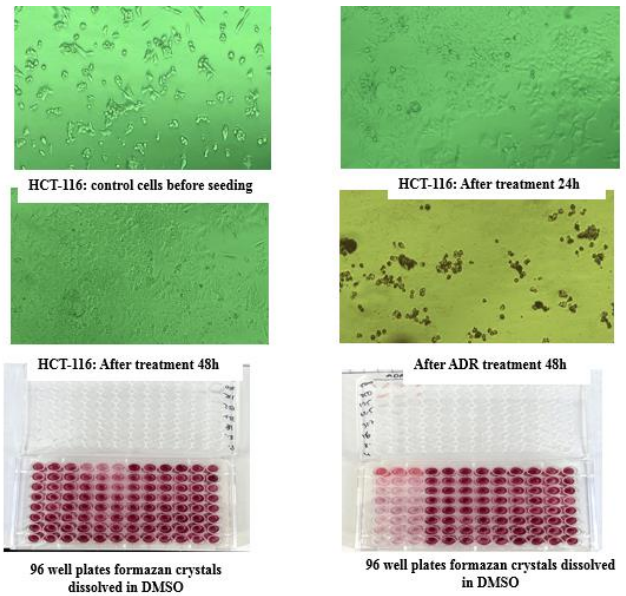


**Figure 1:** Anti-proliferative activity measured after 48 hours exposure.Experiment was done in triplicates. Adriamycin (ADR) was used as control.

The MTT assay results are summarized in **Table 3**. Significant effects were seen on HCT-116 cells by cell free extracts of LC-20, LC-30 and *Bacillus clausii* as compared to the standard ADR whereas no significant effects were seen in COLO-205 cell line. Based on these IC-50 values of cell free extracts of LC-20, LC-30 and *Bacillus clausii* were used for further experiments on HCT-116 cell line.

*4.3 Anti-proliferative activity against HCT-116*

Cell free extracts of LC-20 and LC-30 displayed potent inhibition with IC50 values of 5.07 and 19.84 µg against HCT-116 cancer cells as compared to standard drug ADR that shows IC50 27.8 µg. LC-8 showed IC50 more than 100 µg which was not significant as compared to standard control. LC-27 showed moderate inhibition activity against HCT-116 cells whereas standard probiotic crude extract obtained from *Bacillus clausii* showed activity with IC-50 24.8 µg. Thus, these experiments indicated that the crude extracts exhibit moderate to well anti-proliferative activity against HCT-116 cells.

*4.4. Anti-proliferative activity against COLO-205*

All cell free extract of LC-8, LC-20, LC-27, LC-30, and *Bacillus clausii* displayed low inhibition with IC-50 values of 214.3,207.4,280.7,223.4 236.8 µg against COLO- 205 cancer cells as compared to standard drug ADR that shows IC-50 32.6 µg. Thus, these experiments indicated that the crude extracts show low anti-proliferative activity against COLO-205 cells.

*4.5. Cytotoxicity against peripheral blood mononucleocytes (PBMC)*

**Table 4:** Cytotoxicity effect on PBMC

| Crude extract | IC-50 (µg /ml) |
|---|---|
| LC-8 | 340.9± 1.09 |
| LC-20 | 294.1±2.63 |
| LC-27 | 387.3±3.15 |
| LC-30 | 302.9±1.86 |
| *Bacillus clausii* | 359.2±2.45 |

There was no cytotoxicity effect against PBMCs enabling the use of cell free extracts as potent antiproliferative agent.

*4.6. Colony formation assay*

To further evaluate the cytotoxic effects of LC-20 and LC-30, colony formation assay (clonogenic assay) was performed using HCT-116 cells with reference to results and selectivity of cell line obtained in MTT assay. To study dose dependent kinetics in cell signalling, HCT-116 cells were treated with half and double IC-50 values of crude cell free extract for 24 hours followed by incubation for seven days with change in media. The crystal violet staining of nuclei revealed that double IC-50 dose concentration considerably reduced the clonogenic ability of HCT-116 cells. These results confirmed that the cell signalling was inhibited by double IC-50 values. The colony formation assay with HCT-116 cell line showed that the proliferation rate and colony numbers were significantly decreased after the treatment of double IC-50 value, in comparison with the control cells (**Table 2**).

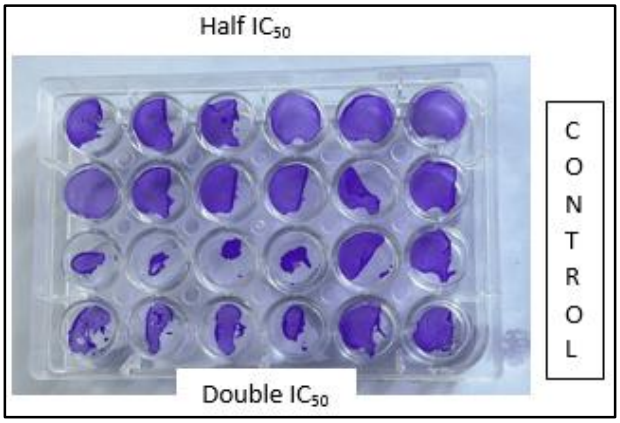


**Figure 2:** Clonogenic assay for half and double IC50 values

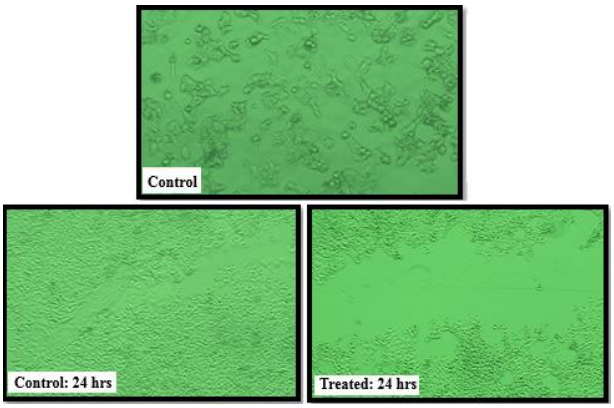


**Figure 3:** Effect on cell migration by in vitro wound healing assay

*4.7. Effect on cell migration by in vitro wound healing assay*

An important characteristic of cancer metastasis and inflammation is cell migration. Therefore, the cell free extracts of LC-20 and LC-30 were evaluated for its effect on the cell migration of HCT-116 cells by *in vitro* wound healing assay (scratch assay). The cells were treated with half and double IC-50 concentrations for 24 hrs. Positive control with no treatment were maintained simultaneously. After 24 hrs, a progressive inhibition in cell migration was seen as compared to the control of non-treated cells.(**Figure 3**)

*4.8. Cell cycle analysis of cell free extracts*

In a dose and time dependent manner, the cell free extracts of LC-20 and LC-30 that showed cytotoxicity against HCT-116 cell line were chosen for cell cycle analysis. Half the IC-50 and double IC-50 dose values of extracts of LC-20 and LC-30 and *Bacillus clausii* were used against HCT-116 cells for 12 hours and 24 hours, respectively. After 12 and 24 hours, half IC-50 and double IC-50 concentrations of both LC-20 and LC-30 showed noticeable changes in Sub G0 population as compared to the control and *Bacillus clausii*. There were small changes in the cell population of other phases of cell cycle as well. At half and double IC-50 concentration, after 12 and 24 hours, LC-20, LC-30 and *Bacillus clausii* caused changes in G0/G1population, as compared to the control (**Table 5**a -d)

**Table 5:** Half IC-50 12 hrs

| **a: Half IC-50 12 hrs** | | | | |
|---|---|---|---|---|
| **Isolate** | **G0/G1** | **S** | **G2/M** | **SUB G0** |
| **Control** | 48.6 | 27.1 | 19.9 | 4.1 |
| **LC-20** | 50.1 | 24 | 22 | 3.5 |
| **LC-30** | 51.9 | 25.5 | 17.6 | 4.5 |
| ***B.clausii*** | 47.6 | 26.1 | 20.8 | 3.1 |
| **b: Half IC-50 24 hrs** | | | | |
| Isolate | G0/G1 | S | G2/M | SUB G0 |
| **Control** | 61 | 22.8 | 12.1 | 5 |
| **LC-20** | 71.9 | 16.4 | 4.8 | 4.9 |
| **LC-30** | 68 | 19.9 | 3.2 | 8.9 |
| ***B. clausii*** | 60 | 21 | 13 | 6 |
| c: Double IC-50 12 hrs | | | | |
| **Isolate** | **G0/G1** | **S** | **G2/M** | **SUB G0** |
| **Control** | 50.6 | 26.1 | 20.9 | 4.1 |
| **LC-20** | 50.8 | 22.8 | 20.9 | 4.9 |
| **LC-30** | 68.4 | 22.9 | 5.6 | 5.1 |
| ***B.clausii*** | 48.2 | 21.9 | 6.8 | 4.2 |
| **d: Double IC-50 24 hrs** | | | | |
| **Isolate** | **G0/G1** | **S** | **G2/M** | **SUB G0** |
| **Control** | 60 | 21.8 | 11.1 | 7 |
| **LC-20** | 63.5 | 20 | 10.9 | 4.5 |
| **LC-30** | 74.2 | 17.7 | 1.8 | 6.2 |
| ***B.clausii*** | 59 | 20 | 12 | 8 |

## 5. Discussion

Colorectal cancer (CRC) still remains a significant challenge as the third most common cancer worldwide and a leading cause of cancer-related deaths. The disease is strongly influenced by environmental and lifestyle factors, including poor dietary habits, sedentary behaviour, and limited physical activity. In the search for safer and more effective therapeutic alternatives to conventional treatments, which often involve chemotherapy and radiation with severe side effects, recent research has turned its focus towards the potential use of probiotics and their derivatives, known as post biotics.[17] Our findings indicate the anticancer properties of post biotics derived from specific probiotic strains, including four isolates of Lactobacillus spp. (LC-8, LC-20, LC-27, LC-30)[28] and *Bacillus clausii*. These cell-free extracts were tested against two colorectal cancer cell lines, HCT-116 and COLO-205, using a different in vitro assay. These findings revealed significant anticancer activity with LC-20, LC-30, and *Bacillus clausii* post biotics. They show strong effects in inhibiting cancer cell viability by changing some of the metabolic pathways, by inducing time- and dose-dependent cytotoxic activity, anti-proliferative mitotic arrest, and synergistic action.[11,12] These postbiotics were more effective as compared to the standard chemotherapeutic agent Adriamycin that was used in assays.

The MTT assay showed that the post biotics exhibited dose-dependent cytotoxicity, reducing the viability of cancer cells while no activity was seen on normal cells. Further, wound healing and clonogenic assays indicated that these postbiotics inhibited cancer cell migration and colony formation, respectively, thereby targeting key processes in cancer progression. After 12 and 24 hours, half IC-50 and double IC-50 concentrations of LC-20 and LC-30 showed noticeable changes in sub G0 population as compared to the control and *Bacillus clausii*. There were small changes in cell population in other phases of cell cycle as well. At half and double IC-50 concentration, cell free extracts of LC-20 and LC-30 showed more accumulation of cells in G0/G1 as compared to the control. These findings indicate activation of specific mechanistic pathways leading to apoptosis and the slowing of cell cycle progression. The ability to delay cell proliferation and migration highlights the therapeutic potential of these post biotics in combating colorectal cancer.[10] Overall, this research highlights the promising role of post biotics as a natural, less toxic alternative to conventional chemotherapy. The results pave the way for further investigation into the molecular mechanisms underlying their anticancer activity and their potential.

## 6. Conclusion

Probiotics have gained increasing medical significance due to the beneficial effect on the human body associated with the prevention and support for the treatment of many diseases without the absence of side effects. Our study shows that isolate LC -20, LC-30, *and Bacillus clausii* show significant anticancer activities against HCT-116 cell line. As an outcome, our study indicate the antitumor properties of probiotic organisms. These results confirm the effectiveness of probiotics and their cell free extracts as potential alternative only for prevention of cancer or as alternating treatment for chemotherapy. However, it would be essential to validate these in-vitro results through studies in living organisms, utilizing appropriate animal models of colorectal cancer. Such in-vivo investigation will be able to understand how the extracts will behave within a complex biological system and show their absorption, distribution, metabolism, and excretion, as well as their impact on tumour growth and spread. Positive outcomes in these preclinical models would be a crucial step towards considering future studies in human patients. Further research will explain specific pathways and targets within cancer cells that are affected by these extracts. Understanding these mechanisms will not only set the scientific basis for their use but could also help to identify which patients might benefit most. Furthermore, given the challenges of treating colorectal cancer, exploring the possibility of combining these extracts with existing chemotherapy regimens holds significant promise. Thus, in -vivo experiments along with clinical trials are needed to be done in order to confirm the potential of probiotic microorganisms and their products in this regard.

## 7. Conflict of Interest

The authors have no conflict of interest regarding this investigation.

## 8. Source of Funding

None.

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