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In-Vitro Characterization of the Anti-Cancer Activity of the Probiotic Bacterium *Lactobacillus Fermentum* NCIMB 5221 and Potential against Colorectal Cancer Cells

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Abstract

Objective: Lactic acid bacteria (LAB), *Lactobacillus fermentum* for instance, have been shown to increase the levels of fecal short chain fatty acids (SCFAs). SCFAs are known for their beneficial role in colonic health and their production of anti-carcinogenic compounds, suggesting a potential in colorectal cancer (CRC) prevention. The aim of this study is to characterize the metabolic and anti-cancer features of *L. fermentum* NCIMB 5221 compared with two other *Lactobacillus* species.

Methods: A free fatty acid (FFA) profile was generated, and the anti-proliferative, and apoptotic effects of bacterial cell-free extracts were investigated. The effect on the growth of CRC cells compared with non-neoplastic colon cells was determined. The production of different SCFAs by the probiotic bacteria and the efficacy of their composition were analyzed.

Results: The FFA profile of *L. fermentum* was distinctive (~ 368 MAE, 16 h, $p < 0.01$) when compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. *L. fermentum* extracts significantly inhibited cancer cell growth up to ~ 40% and induced apoptosis up to ~ 30% in SW-480 CRC cells (24 h, $p < 0.05$) when compared with untreated cells. Although *L. fermentum* did not inhibit CRL-1831 non-neoplastic colon cell growth, it still had a significant anti-proliferative effect against Caco-2 cancer cells (~ 60%, 72 h, $p < 0.001$) compared with untreated cells. This was related to the higher levels of SCFAs produced (~ 377 mg/L). Similar concentrations of SCFA formulations (corresponding to those produced by *L. fermentum*) have shown the same inhibitory effect on Caco-2 cells with no effects against CRL-1381.

Conclusion: *L. fermentum* NCIMB 5221 was more potent in suppressing CRC cells and promoting normal epithelial colon cell growth through the production of SCFAs. Consequently, it could be considered as a biotherapeutic agent for the support of colonic health and the prevention of CRC.

Introduction

Colorectal cancer (CRC) is a leading cause of mortality worldwide [1]. However, it is a type of cancer for which chemoprevention is considered a therapeutic and preventive strategy [2]. Probiotics have been used as biotherapeutics that reduce cancer recurrence and side effects in CRC patients [3-5]. When orally administered, probiotic along with intestinal microbial-produced metabolites (e.g. organic acids, peptides), that interact with cellular proliferation, differentiation, and intestinal inflammation, reduce the risk of CRC [6]. In the large intestine, short chain fatty acids (SCFAs) produced by bacterial fermentation have shown to exert anti-inflammatory [7] and anti-carcinogenic actions. Studies have shown that fatty acids can mutually interact and protect against CRC. Nevertheless, the incorporation of fatty acids into CRC chemotherapies is still premature. The oral administration of specified probiotic bacteria remains the dominant method to increase the bio-production of these anti-tumorigenic compounds in the colon [8,9].

While the selection criteria of probiotic bacteria originating from the gut or from traditionally fermented products are fairly empirical, an emphasis on the importance of well-established *in vitro* and *in-*

vivo studies to select good candidates exists. Few studies have been found to establish a rigorous selection for new probiotic strains. Anti-cancer attributes, proper controls, and extensive evaluation of their anti-proliferative effect against cancer cells should be analyzed and compared with other established probiotic products. While studies have shown the ability of certain probiotics to affect SCFA levels, more evidence that the probiotic anti-cancer effect is solely due to the direct production of SCFAs [10-12] is needed. In this study the profile of *L. fermentum* NCIMB 5221, identified as a producer of ferulic acid (FA), was characterized. The latter is an anti-oxidant and anti-tumorigenic

compound [13], with the ability to generate a stronger free fatty acid (FFA). This ability was compared with that of *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 51303, both characterized in previous studies for their activity against tumor growth [14-17]. Notably, the total proliferative effect of this bacterial strain was investigated based on the effects of two types of probiotic cell-free extracts on the growth and apoptosis of CRC cells. A prior preliminary comparative study found that this particular strain exhibited more potent attributes associated with anti-cancer effects and survival when screened with other *L. fermentum* strains. To verify the non-cytotoxic effect of the probiotic extracts, the same assay was performed with non-cancerous colon cells. Validation of a correlation between the levels of SCFAs produced and the anti-cancer potency of the bacterium was performed. Quantification of *L. fermentum* NCIMB 5221 SCFAs was used to identify the extent to which SCFAs (acetic, propionic, and butyric acids) are responsible for the potential anti-cancer effect against CRC cells. *in vitro*. To confirm the level of efficacy of naturally produced SCFAs, the assay was performed using only a pure mixture of synthetic SCFAs with similar composition to the probiotic SCFAs.

Material and Methods

Materials

Agar and De Man, Rogosa, Sharpe (MRS) broth was bought from Fisher Scientific (Ottawa, ON, Canada). Dulbecco's modified Eagle's medium (DMEM) and Eagle's Minimum Essential Medium (EMEM), phosphate-buffered saline (PBS), Roswell Park Memorial Institute medium (RPMI-1640), and fetal bovine serum (FBS) were purchased from Invitrogen. Water was purified with two systems: EasyPure reverse osmosis and NanoPure Diamond Life Science (UV/UF) ultrapure water (Barnstead Dubuque, IA, USA). Sodium L-Lactic, propionic, acetic, and butyric acids were obtained from Sigma (St. Louis, MO, USA).

Bacterial cultures

L. fermentum NCIMB 5221 was purchased from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland, UK). Bacterial strains of *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 were obtained from Cedarlane Laboratories (Burlington, ON, Canada) and used as controls. Bacterial cultures were maintained by continuous subculturing in MRS broth at 1% (v/v) while bacterial growth was monitored with both OD at a wavelength of 620 nm (Perkin Elmer 1420 Multilabel Counter, USA) and by colony counting on agar plates.

Mammalian cultures

SW-480 colorectal cancer and Caco-2 epithelial colorectal cancer adenocarcinoma cells, as well as CRL-1831 normal epithelial colon cell line, were purchased from ATCC American Type Cell Collection (ATCC, Manassas, VA). Caco-2 cells were maintained in (EMEM) supplemented with 20% FBS. SW-480 cells were maintained in RPMI-1640 supplemented with 10% FBS, and CRL-1831 was maintained in complete DMEM (10% FBS, 37°C, 5% CO_2). Caco-2 cells were incubated in a CO_2 incubator at 37°C in air supplemented with 5% CO_2 for a maximum of two weeks for complete differentiation. For proliferation/viability assays, all cells were left to attach in 96-well plates until 50 - 60% confluence (24 - 48 h) before experimentation. The cell medium was then replaced by probiotic cell-free extracts mixed with serum/antibiotic-free DMEM.

Free fatty acid (FFA) analysis

In this analysis, the free fatty acids (FFAs) in the bacterial

supernatant were converted to their CoA derivatives and then oxidized. This resulted in the formation of a color measured at 570 nm (Figure 1). The assay was performed based on the manufacturer's instructions (Cell Biolabs Inc., CA, USA). For the induction of Acyl-CoA synthesis, a reaction mix was prepared. A 2 µl of ACS Reagent was added to all the standards (palmetic acid. sample wells were mixed and left to incubate (37°C, 30 min). The reaction mix (50 µl), f atty acid probe (2 µl), enzyme mix (2 µl), assay buffer (Igepal, 44 µl), and enhancer (N-ethylmaleimide, 2 µl) were mixed and vortexed briefly. Then, 50 µl of this reaction mixture was added to each well (standard or sample) and incubated (30 min, 37°C, away from light). Absorbance was measured at 570 nm for colorimetric assay in a microplate reader (Perkin Elmer, Victor 3, multi-label micro-plate reader, MA, USA).

Preparation of probiotic cell-free supernatants

Each probiotic bacterial colony was grown anaerobically in MRS broth for 12 - 16 h. The conditioned medium (CM) and probiotic supernatant (PS) were prepared with slight modifications of protocols adapted from Grabig et al. [14] and Kim et al [15]. For the preparation of the CM, bacterial cultures (16 h, 37 °C, 5% CO_2) were used to collect bacterial pellets, by centrifugation (4000 rpm, 15 - 20 min, 4°C), to be washed (PBS) and resuspended in DMEM. The bacterial pellets in DMEM were maintained and incubated in a shaker incubator for two hours (37°C, 5% CO_2, 100 rpm). After incubation, the culture medium was centrifuged (1000 x g, 15 min) and sterile-filtered (filter pore size 0.2 µM-pore-size filter). Prior to treating the cells, the CM of each *Lactobacillus* bacteria was combined with DMEM at a ratio of 1:2. For the preparation of the PS, the bacterial pellet was removed by centrifugation (4000 rpm, 15 min, 4°C), then the recovered supernatant was sterile-filtered (0.22 µm) and stored at −80 °C, until use.
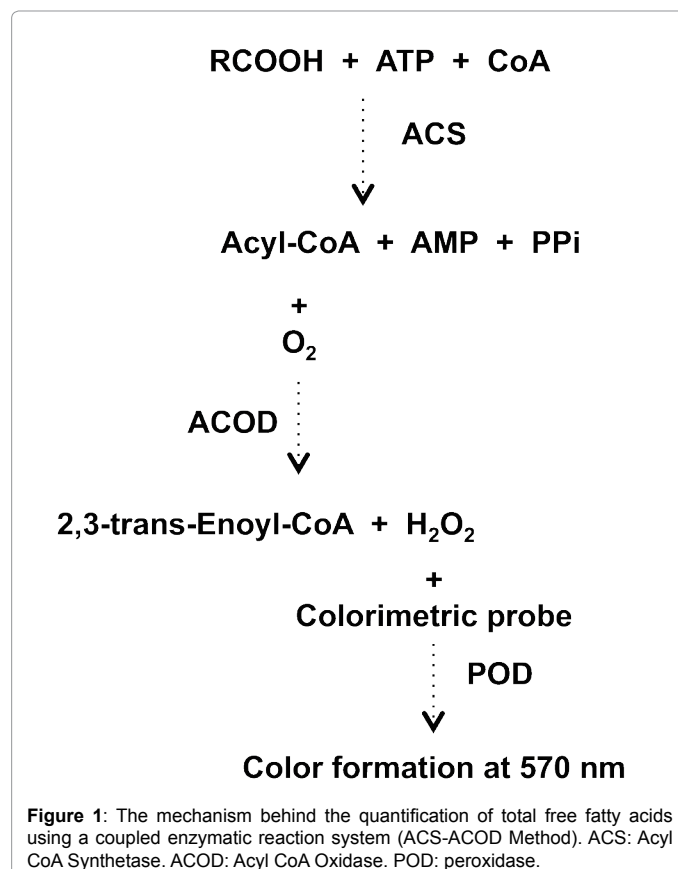

Figure 1: The mechanism behind the quantification of total free fatty acids using a coupled enzymatic reaction system (ACS-ACOD Method). ACS: Acyl CoA Synthetase. ACOD: Acyl CoA Oxidase. POD: peroxidase.

Cell viability assay

Cell viability was determined using ATP bioluminescence assay (CellTiter-Glo Luminescent Cell Viability Assay, Promega), following the manufacturer's protocol [16]. Colon cells (normal and cancer) were seeded onto 96-well culture plates (5 - 6×10³ cells/well, 100 μL/well) and stabilized for 24 - 48 h (37°C, 5% CO_2) for cell attachment. After incubating the cells with the probiotic treatments (24, 48, and 72 h), the 96-well plate was left at room temperature (RT, 30 min). A 100 μL of luminescent reagent was added to each well, followed by shaking (2 min, 200 rpm) and incubation at RT (10 min) to stabilize the luminescent signal. The signal was recorded using a spectrophotometer (Perkin Elmer, Victor 3, multi-label microplate reader, MA, USA).

Apoptosis assay

Apoptosis was determined by the assessment of caspase -3 and -7 using Caspase-Glo® 3/7 assay (Promega, USA). First, the buffer and the lyophilized substrate were equilibrated to RT before use. Both were mixed to dissolve fully the substrate. The blank reaction (DMEM without cells), negative control (untreated cells in DMEM) and the assays (treated cells in CA+DMEM) were all reactions prepared to detect caspase-3 and caspase-7 activity in cell cultures in 96-well white opaque plates. After incubation with the treatment, the plate was removed from the incubator and allowed to equilibrate at RT. A 100 μL of the luminescent reagent was added to each well with pre-filled 100 μL of blank, negative control or treated cells in DMEM. The plate was gently mixed on a plate shaker (300 - 500 rpm, 30 sec), followed by incubation at RT for 3 hours. Finally, the luminescence of each sample was measured in a plate-reading luminometer following the manufactures' instructions.

Probiotic effect on CRC cells vs. non-neoplastic colon cells

This assay was performed to demonstrate an anti-CRC effect of *L. fermentum* NCIMB 5221 by inhibiting CRC cell proliferation without affecting non-neoplastic colon cells. Caco-2 and CRL-1831 (4 - 5×10³ cells/ well) were seeded into 96-well culture plates (37°C, 5% CO_2) for 1, 2, 3, and 7 days. The cells in both populations were treated with probiotic CM. At each time point of incubation with probiotic treatments, cell proliferation was determined using an ATP bioluminescence assay.

Quantification of lactic acid and SCFAs

SCFAs produced by *L. reuteri* strains were measured during the growth of bacteria in SIF and after the preparation of corresponding CM. SCFAs were separated using a slightly modified HPLC method [17,18]. The Model 1050 of UV HPLC system (Hewlett-Packard HP1050 series, Agilent Technologies, USA), equipped with a UV-vis detector and diode array detector (DAD) set at 210 ± 5 nm was used. A 100 μl of sample was injected through an autosampler. A prepacked Rezex ROA-organic acid H+ (8%) (150mm × 7.80 mm, Phenomenex, Torrance, CA, USA) fitted with an ion-exclusion microguard refill cartridge was used. Data was acquired using ChemStation supported with LC3D software Rev A.03.02 (Agilent Technologies, CO, USA). The mobile phase (A) of H_2SO_4 (0.05 M) and the mobile phase (B) of acetonitrile (2%) pumped isocratically at a flow rate of 0.8 - 0.7 mL/min, through a column heated to 35°C. Lactic, acetic, propionic, and butyric acids were used to prepare a standard solution at concentrations of 1, 10, 100, 500, and 1000 ppm (in triplicate). The concentrations of SCFAs were estimated using the linear regression equations ($R^2 \geq 0.99$) generated from respective standard curves.

Product	Strain	*L. fermentum* NCIMB 5221
	Controls	*L. acidophilus* ATCC 314
		L. rhamnosus NCIMB 53103
	Characterization	Growth in MRS
		Fatty acids levels in bacterial culture
	Probiotic cell free extracts	PS: probiotic supernatant (culture based)
		Bacterial culture supernatant
		CM: conditioned medium (bacterial cell based)
		Cell media DMEM treated with bacterial cells
Effect	**Anti-proliferative effect**	Colon cancer cells + PS or CM (12h , 24 h, and 7 days)
		Proliferation and apoptosis
	Cancer cells vs. normal epithelial cells	CM+ colon cancer cell
		CM + non-epithelial colon cells
		(1, 2, 3 and 7 days)
Mechanism	**Role of SCFAs produced in CM**	Quantification (lactate, acetate, propionate and butyrate)
		Preparation of synthetic SCFA mixtures
	Testing different mixture of SCFAs	Decreasing doses of LA, AA, PA and BA and their mixtures

Table 1: Outline of the study on characterizing the anti-carcinogenic potential of *L. fermentum* NCIMB 5221 and the role of probiotic and synthetic SCFAs. LA: lactic acid; AA: acetic acid; PA: propionic acid; BA: butyric acid.

Role and efficacy of SCFAs: SCFA synthetic formulations vs. probiotic CM

This test intends to demonstrate the role and the relevance of naturally produced SCFAs by probiotic cells in their cell-free extracts (CM). SCFA synthetic formulations (SSF) with same composition of the naturally produced SCFAs in the probiotic CM were prepared (Table 1). Caco-2 cells (4 - 5 × 10³ cells/well), seeded onto 96-well plates (37°C, 5% CO_2, 72 h) were used to determine the inhibitory actions of these compounds and compare it to the probiotic CM. SCFA synthetic mixtures with the same effect as the bacterial extract would suggest that the inhibitory effect against CRC cell is due to the concentration of SCFAs produced by *L. fermentum*. Another set of mixtures (SSF+LA) was used after the addition of lactic acid (similar to that produced by the bacteria) to investigate the effect of another bacterial component on the action of SCFAs.

Effect of SCFAs on CRC cell compared with normal cells

The objective of this step was to investigate the dose-dependent effect of SCFAs, pure or in a mixture, and the nature of their potential synergistic effect on both normal and cancerous colon cells. Different concentrations of lactate, acetate, propionate and butyrate were prepared (Table 2) and tested on Caco-2 and normal non-neoplastic colon cells CRL-1831 cells. Cells were seeded onto 96-well culture plates (4 - 5 × 10³ cells/ well, 37°C, 5% CO_2), left to stabilize and attach (24 - 48 h), followed by incubation with treatment samples (37°C, 5% CO_2, 72 h). SCFA treatments included increasing concentrations of lactate (0, 325, 650, and 1300 mg/L), acetate (0, 325, 650 and 1300 mg/L), propionate (0, 100, 200, and 400 mg/L) and butyrate (0, 75, 150, and 300 mg/L). The concentration of each SCFA was mixed to prepare the following four compositions; 1) SC4 (325 mg/L of lactate, 325 mg/L of acetate, 100 mg/L of propionate and 75 mg/L of butyrate), 2) SC3 (325 mg/L of lactate, 650 mg/L of acetate, 200 mg/L of propionate, 150 mg/L of butyrate), 3) SC2 (650 mg/L of lactate, 1300 mg/L of acetate, 400 mg/L of propionate acid and 300 mg/L of butyrate acid), and 4) SC1

(1300 mg/L of lactate, 1300 mg/L of acetate, 400 mg/L of propionate and 300 mg/L of butyrate).

Statistical analysis

Data are presented as means ± Standard Error of the Mean (SEM) of replicates. Correlations were determined using Pearson's correlation. Statistical significance was generated for the treated groups as compared with each other using of the one-way analysis of variances (ANOVA), with Tukey's post hoc test using SPSS statistics software package (v. 20.0, IBM Corporation, New York, NY, USA). Values of $p < 0.05$ were considered significant.

Results

L. fermentum NCIMB 5221 a higher producer FFAs

L. fermentum NCIMB 5221 was characterized for its growth and production of FFAs in bacterial cultures. Data sets describing the total FFA concentration (μM PAE) in the bacterial supernatant (Figure 2a, 2b, and 2c) included FFA concentration per viable bacterial cell (Figure 2d) and FFA concentration per gram of bacterial pellet. Results showed that *L. fermentum* NCIMB 5221 growth significantly increased FFA concentrations (367.8 ± 10.5 μM PAE) compared with *L. acidophilus* ATCC 314 (117.1 ± 3 μM PAE, $p < 0.001$) and *L. rhamnosus* ATCC 53103 (87.4 ± 0.1 μM PAE, $p < 0.001$, Figure 2b). The high FFA concentrations were maintained at 367.8 ± 10.5 μM PAE and 366.7 ± 6.6 μM PAE between 12 and 16 h of growth and then started dropping at the beginning of the stationary phase. Even at the end of the death phase (Figure 2c), *L. fermentum* NCIMB 5221 induced a significantly higher level of FFAs (320.8 ± 12.6 μM PAE) compared with *L. acidophilus* ATCC 314 (188.2 ± 6.9 μM PAE, $p = 0.0161$) and *L. rhamnosus* ATCC 53103 (281.32 ± 1.77 μM PAE, $p = 0.0487$). Values of FFAs per viable bacterial cell measured during both log phase and stationary phase (Figure 2d), remained significantly higher for *L. fermentum* NCIMB 5221 ($6.7 ± 0.2×10^{-7}$ - $13.6 ± 0.2×10^{-7}$ μM PAE/cell, $p < 0.05$, Figure 2) compared with *L. acidophilus* ATCC 314 ($3.3 ± 0.1×10^{-7}$ – $4,4 ± 0.2×10^{-7}$ μM PAE/cell) and *L. rhamnosus* ATCC 53103 ($3.5 ± 0.1×10^{-7}$ – $5.8 ± 0.1 x10^{-7}$ μM PAE/cell). Regarding the bacterial mass, FFA generated per gram of the bacterial mass was the highest for *L. fermentum* NCIMB 5221 at 12 h and 24 h ($p < 0.001$, Figure 2e).

L. fermentum NCIMB 5221 displays anti-proliferative activity against CRC cells in a time-dependent manner

The anti-proliferative and apoptotic effect of *L. fermentum* 5221 against SW-480 CRC cells was compared with controls: *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103, using two types
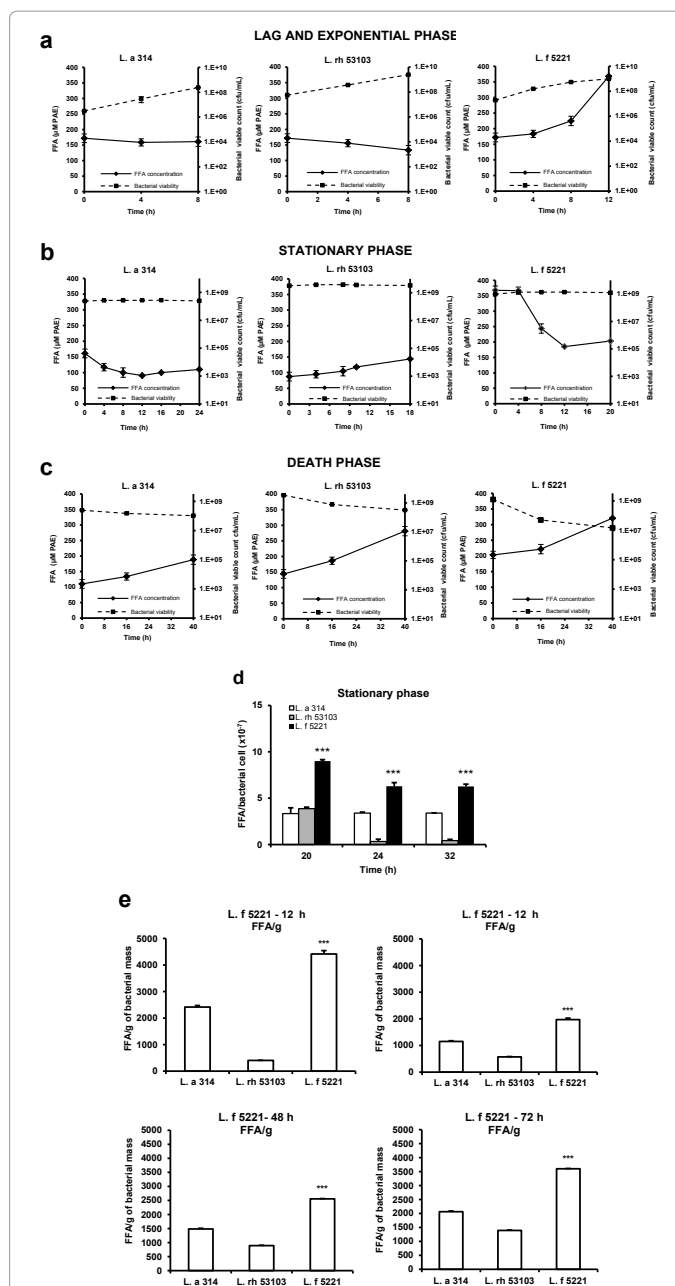

Figure 2: Study of the metabolic activity of *L. fermentum* NCIMB 5221: Determination of growth pattern and total free fatty acid (FFA) profile. The concentrations of total FFAs (μM PAE) in the probiotic bacterial culture of *L. fermentum* NCIMB 5221 was determined during the (a) lag and exponential, (b) stationary, and (c) death phases of bacterial growth. *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 were used as controls. (d) Variation of total FFAs levels per viable bacterial cell for the exponential and stationary phases. (e) Description of the levels of total FFA per gram of bacterial pellet at 12 h and 14 h of growth. All these features of *L. fermentum* NCIMB 5221 were compared to *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 at the same conditions, for 32 h in MRS broth (37°C, 5 % CO_2) *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. Data represent the mean ± SEM (*n* = 3). PAE: Palmetic acid equivalents. *L. a 314*: *L. acidophilus* ATCC 314; *L. rh 53103*: *L. rhamnosus* ATCC 53103; *L. f 5221*: *L. fermentum* NCIMB 5221.

	Doses (mg/L)			
LA	0	325	650	1300
AA	0	325	650	1300
PA	0	100	200	400
BA	0	75	150	300
	Composition (mg/L)			
	LA	AA	PA	BA
Control	0	0	0	0
SSM1	1300	1300	400	300
SSM2	650	1300	400	300
SSM3	325	650	200	150
SSM4	325	325	100	75
* LA: lactic acid; AA: acetic acid; PA: propionic acid; BA: butyric acid				

Table 2: Doses of single SCFAs and the composition of SCFA mixtures to be tested on Caco-2 and CRL-1831 colon cells.

of probiotic cell-free extracts, PS and CM. At 12 h, SW-480 cells treated with *Lactobacilli* PS showed no difference between groups in terms of proliferation and apoptosis (Figure 3a), whereas for *Lactobacilli* CM,
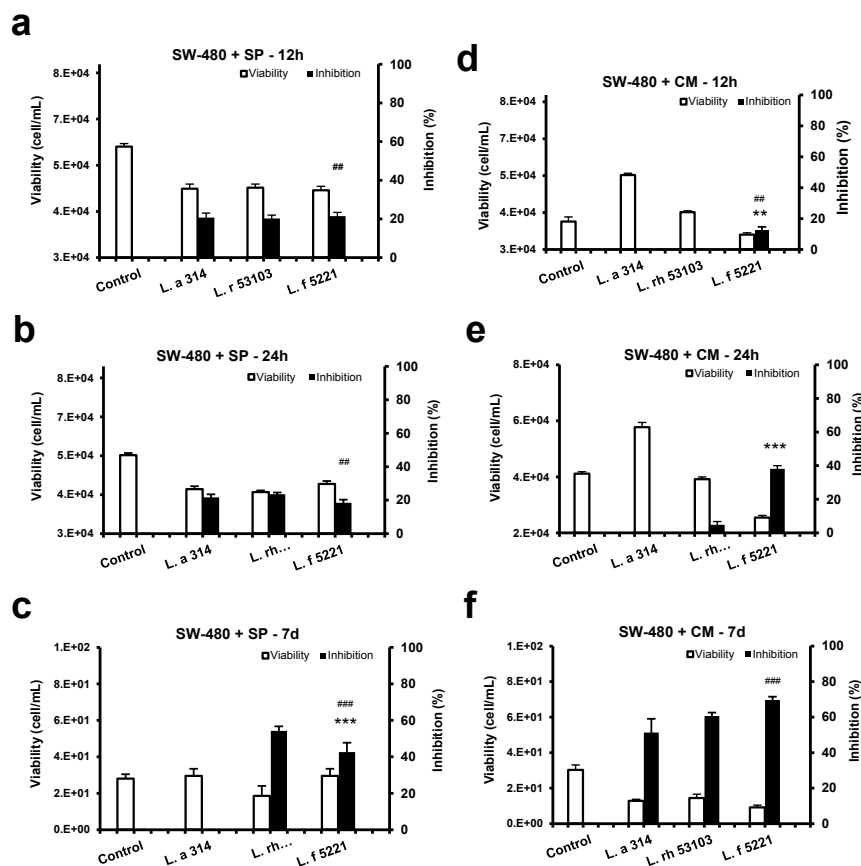
Figure 3: Analysis of human colon cell growth inhibition by *L. fermentum* NCIMB 5221 using different bacterial cell free extracts. The effect of (a, b, c) the probiotic supernatants (PS) and (d, e, f) the conditioned cell culture medium (CM) of *L. fermentum* NCIMB 5221 on the viability of CRC cells (SW-480) for 12 h, 24 h, and 7 days. **$p < 0.01$ and ***$p < 0.001$ compared with *L. acidophilus* ATCC 314 or *L. rhamnosus* ATCC 53103. ##$p < 0.01$ and ###$p < 0.001$ compared to untreated cells. Data are presented as mean ± SEM ($n = 4$). *L. a* 314: *L. acidophilus* ATCC 314; *L. rh* 53103: *L. rhamnosus* ATCC 53103; *L. f* 5221: *L. fermentum* NCIMB 5221.

L. fermentum NCIMB 5221 showed significant inhibition of CRC cell proliferation (12.9 ± 1.8%) compared with controls ($p = 0.034$, Figure 3d). At 24 h, *Lactobacilli* SP inhibited cancer cell proliferation with no significant difference between treatments ($p = 0.0754$, Figure 3b). Whereas for *Lactobacilli* CM, *L. fermentum* NCIMB 5221 significantly killed CRC cells with 38.1 ± 1.9% of inhibition (Figure 3e), and 29.8 ± 10.4% of apoptosis (Figure 3e) compared with other treatments ($p = 0.0004$, $p = 0.0471$, respectively). Interestingly, at 7 days, the SP of *L. fermentum* NCIMB 5221 significantly reduced cell growth by 42.6 ± 5.1% ($p < 0.05$), compared with control and *L. acidophilus* ATCC 314 (Figure 3c). The CM of *L. fermentum* NCIMB 5221 significantly suppressed CRC cell growth by 67.7 ± 2% compared with untreated cells ($p < 0.001$, Figure 3f). For the induction of cell death in SW-480 cancer cells by SP treatments, there was no significant difference between the treated groups (Figures 4a and 4b). However, for *L. fermentum* NCIMB 5221, the effect of CM in inducing apoptosis in CRC cells at 12 h (23.6 ± 7.5%, $p < 0.05$, Figure 4c) and 24 h (29.9 ± 10.4%, $p < 0.05$, Figure 4d) was shown to be significantly higher than controls. This result suggests that cancer cells might not be affected by bacteria-cell contact but by soluble bacterial factors or other microbial associated molecular patterns (MAMPs)

L. fermentum NCIMB 5221 inhibits CRC cells but not normal cells

It was necessary to elucidate and determine the mechanism by which bacterial symbionts affect cell growth in the epithelium in a tumor environment. To do so, CM prepared from probiotic cells of *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 and *L. rhamnosus* ATCC 53103, was evaluated on the growth of both cancer (Caco-2) and non-cancerous (CRL-1831) colon cells (Figure 5). Results show that at 24 h of incubation with CM of *L. fermentum* NCIMB 5221 and *L. rhamnosus* ATCC 53103, cancer cell growth was inhibited by 28.6 ± 3.7% and 6.3 ± 1% ($p < 0.01$, Figure 5b and 6.5c), respectively, compared with untreated cells. At 48 h of incubation, cancer cell viability was reduced by 42.2 ± 2.2% and 11.4 ± 1.7% ($p < 0.01$), respectively, compared with untreated cells. At 72 h of incubation, *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 and *L. rhamnosus* ATCC 53103, inhibited cancer cell proliferation by 12.6 ± 1.9%, 59.4 ± 4.2% and 23.9 ± 2.5%, respectively, compared with untreated cells. Moreover, after 7 days, Caco-2 cell growth was reduced by *L. fermentum* NCIMB 5221 to 99.5 ± 0.1% ($p < 0.05$) compared with the control treatments (Figures 5a and 5b). Interestingly, the data indicates that *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 and *L. rhamnosus* ATCC 53103 promoted the growth of CRL-1831 epithelial normal colon cells by 12.5 ± 5.3%, 11.9 ± 1% 32 ± 3.4%, respectively, compared with untreated cells. After 48 h of treatment by CM of *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 increased CRL-1831 growth by 13 ± 8.4%, and 43.2 ± 3% ($p < 0.05$), respectively. At 72 h, *L. acidophilus* ATCC 314 (Figure 5d) showed no significant anti-proliferative effect, whereas *L. fermentum* NCIMB 5221 reduced cell growth by 59.4 ± 9.8.2% ($p < 0.05$), compared with untreated cells (Figure 5f).
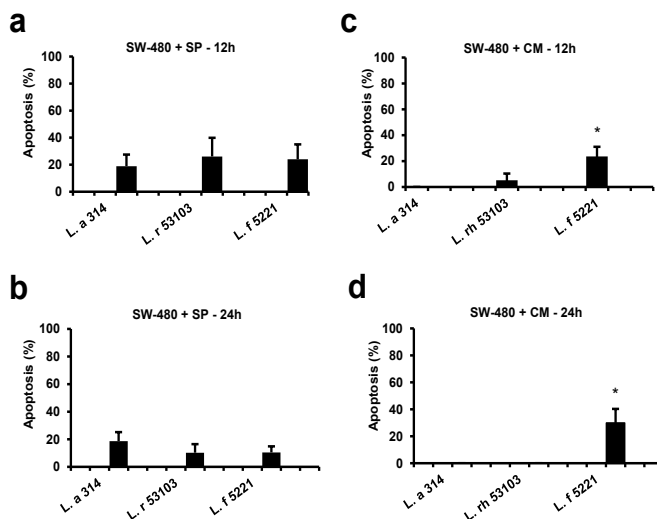
Figure 4: Assessment of apoptosis induction in CRC cells after treatment with *L. fermentum* NCIMB 5221 extracts. Both probiotic supernatant (PS) and conditioned cell culture medium (CM) of *L. fermentum* NCIMB 5221 had induced apoptosis in SW-480 cells, when treated for (a, c) 12 h and (b, d) 24 h. The data values represent the mean ± SEM ($n = 4$). *$p < 0.05$ compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. *L. a* 314: *L. acidophilus* ATCC 314; *L. rh* 53103: *L. rhamnosus* ATCC 53103; *L. f* 5221: *L. fermentum* NCIMB 5221.

Figure 5: Investigation of the anti-colon-cancer proliferative and the non-cytotoxic effects of *L. fermentum* NCIMB 5221 using non-neoplastic and CRC cells. This assay was performed by comparing the viability of Caco-2 colon carcinoma cells and CRL-1831 normal epithelial colon cells, incubated with the CM of *L. fermentum* NCIMB 5221 during 1, 2, and 3 days. The data values represent the mean ± SEM ($n = 4$). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, compared with untreated cells. #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. *L. a* 314: *L. acidophilus* ATCC 314; *L. rh* 53103: *L. rhamnosus* ATCC 53103; L. f 5221: *L. fermentum* NCIMB 5221.
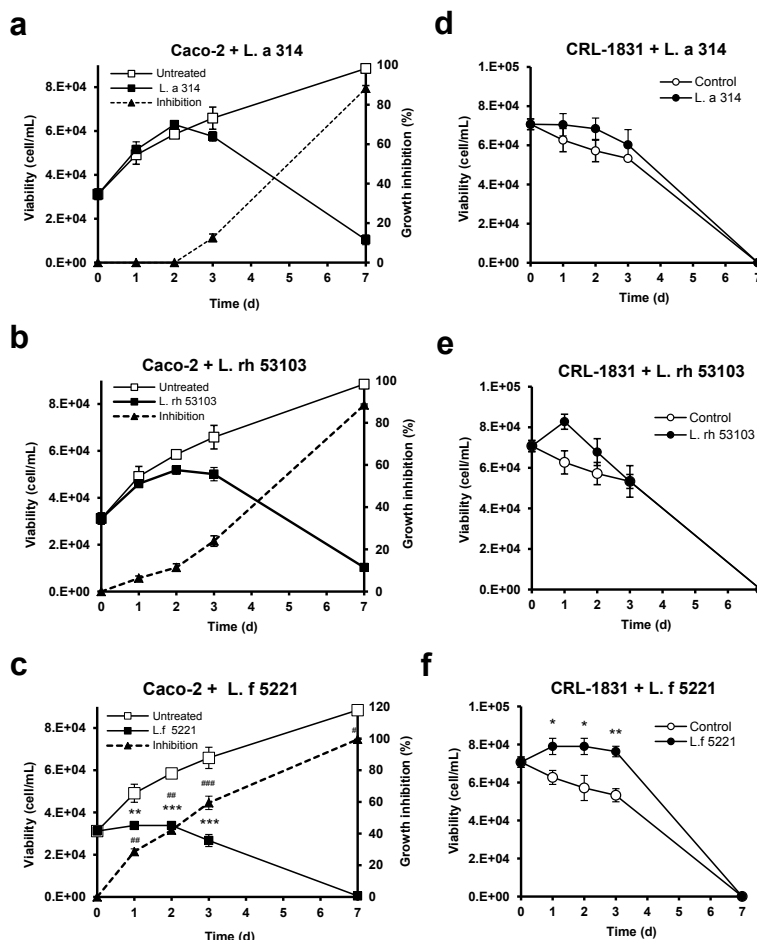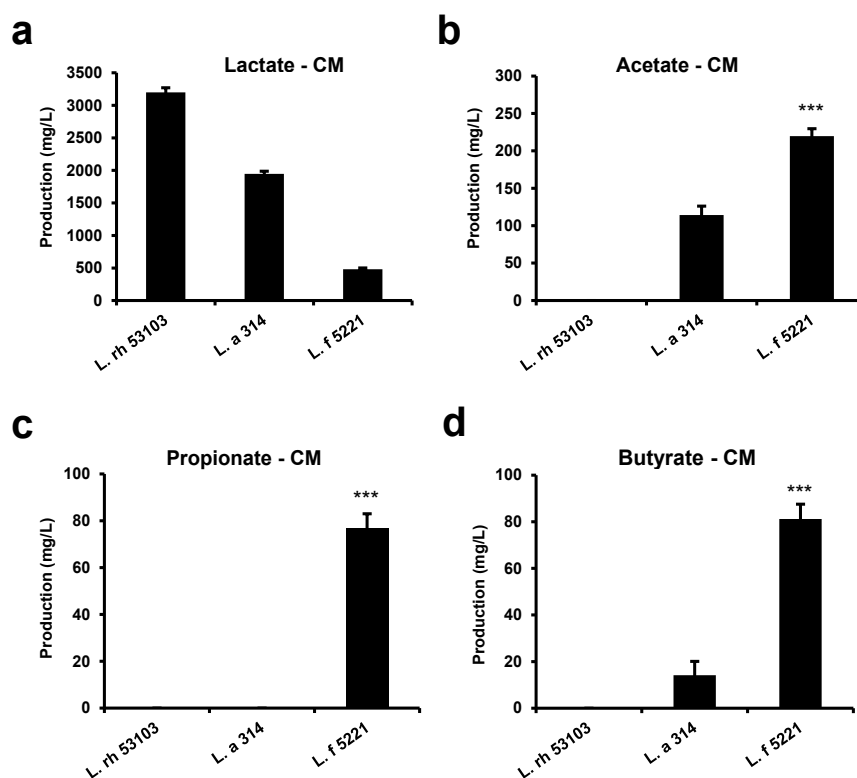
Figure 6: Production of SCFAs and lactic acid by *L. fermentum* NCIMB 5221. (a) Lactic, (b) acetic, (c) propionic, and (d) butyric acids, and (e) total SCFAs have been produced by probiotic bacteria in the conditioned cell culture medium (CM). The data values represent the mean ± SEM ($n = 3$). ***$p < 0.001$ compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. (f) Analysis of the correlation between cell growth inhibition by probiotic CM and the concentrations of SCFAs secreted in the CM. Plots represent the data of cell growth inhibition showed in Figure 5. The lines were obtained by linear regression analysis. The data values represent the mean ± SEM ($n = 3$).

L. fermentum NCIMB 5221 produced higher levels of SCFAs

The effect of anti-proliferative activity induced by the cells incubated in CM used to treat both cancer and normal cell lines, was characterized by SCFA composition, especially lactic, acetic, propionic, and butyric acids. The results as displayed in Figure 5 show levels of SCFAs produced by different strains of *Lactobacillus* bacteria in the media. *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 did neither inhibit CRC growth nor produce detectable amounts of propionate in the media but had higher amounts of lactate, i.e. 1970.6 ± 9.6 and 3239.8 ± 9.9 mg/l, respectively, compared with *L. fermentum* NCIMB 5221 (480.6 ± 13.3 mg/l, Figure 6a). *L. fermentum* NCIMB 5221 produced the highest amount of acetate and butyrate, i.e. 224.2 ± 8.8 and 81.17 ± mg/L, respectively, (Figure 6d) compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 ($p < 0.05$, Figure 6b, 6.6d). *L. fermentum* NCIMB 5221 was the only probiotic bacterium to produce propionate (76.7 ± 7.9 mg/L, Figure 6c) compared with controls.

SCFAs produced by *L. fermentum* NCIMB 5221 are responsible for the inhibitory effect

To determine if the anti-proliferative effect of *L. fermentum* NCIMB 5221 is the result of a specific SCFA, separate concentrations of SCFAs produced by the bacteria were tested. This revealed that acetic, propionic, and butyric acid concentration, quantified in Figure 6 have significantly less effect than the bacterial extract CM (Figure 7a). Only *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC314,

not *L. rhamnosus* ATCC 53103, produced SCFAs (Figure 6). Thus, only their corresponding synthetic SCFA formulations were used for this experiment to verify the role of bacterial SCFAs. The results demonstrated that the synthetic SCFA formulation, corresponding to *L. fermentum* NCIMB 5221, significantly decreased Caco-2 viability by 67.8 ± 7.2% compared with synthetic SCFA formulation corresponding to *L. acidophilus* ATCC 314 (22.6 ± 2.6%, $p = 0.018$). Thus, for *L. fermentum* NCIMB 5221, the synthetic SCFA formulation showed no significant difference with the probiotic CM (Figure 7b), whereas after addition of lactic acid to the synthetic mixture, the SSF + LA corresponding to *L. fermentum* NCIMB 5221 decreased Caco-2 viability only by 21.1 ± 2.9%.

Doses of SCFAs have differential effect on normal and cancer cells

To investigate and differentiate the effects of pure SCFAs and their mixtures, with or without lactic acid, on colon normal and cancer cells, increasing doses (Table 1) of acetic, propionic, and butyric acids were tested on Caco-2 and CRL-1831 cells (Figure 8). Increasing concentrations of acetic acid to 1300 mg/L did not exceed more than 26% inhibition of cancer cells (Figure 8g), with no significant effect on normal cells (Figure 8b). For propionic acid, the inhibition was dose dependent, and 400 mg/L of propionate (Figure 8h) showed 43% inhibition with no inhibition on normal cells (Figure 8c). In the case of butyric acid, the inhibitory effect on CRC cells was dose-dependent and 300 mg/L of butyrate inhibited cell proliferation with a maximum inhibition of 93% (Figure 8i) with no significant effect on CRL 1831

normal colon cells (Figure 8d). Later, increasing doses of SCFAs were mixed to formulate synthetic SCFA mixtures: SSM1, SSM2, SSM3, and SSM4 (Table 1). The effect of each SCFA mixture was significantly higher (Figure 8j, $p < 0.05$) than the total effect of separate doses of SCFAs, with no significant effect observed on CRL-1831 (Figure 8e). However, when the different concentrations of lactic acid were added to each mixture (+LA), the anti-proliferative effect was significantly reduced (Figure 8j, $p < 0.001$). When the doses of lactic acid were tested, they had no significant effect on the proliferation of both normal and cancer cells (Figure 5.8a and 6.8f).

Discussion

This study demonstrated, for the first time, that *L. fermentum* NCIMB 5221 has a higher anti-proliferative effect against CRC cells related to a higher metabolic activity, than to other LAB bacteria (*L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103) [19-22]. As a general characterization of this strain, *L. fermentum* NCIMB 5221 significantly ($p < 0.01$) affected the level of FFAs during most of the growth phases and surpassed both controls *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 for the following parameters:

Figure 7: Verification of the implication of SCFAs produced by *L. fermentum* NCIMB 5221 in suppressing CRC cell growth. (a) Anti-proliferative effect of SCFAs doses at similar concentrations as what was produced by *L. fermentum* NCIMB 5221 as described in Figure 6. (b) Inhibitory effect of the SSFs and SFF+LA (formulations corresponding to *L. fermentum* NCIMB 5221) on the proliferation of CRC cells for 72 h, compared with the bacterial conditioned cell culture medium (CM) containing naturally produced probiotic SCFAs. *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 were positive controls. SSF+LA mixtures were prepared with the addition of different concentrations of lactic acid and pure SCFAs to DMEM media (Figure 6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with controls. The data values represent the mean ± SEM (n = 6). *L. a.* 314: *L. acidophilus* ATCC 314, *L. r* 701359: *L. reuteri* NCIMB 701359. SSF: SCFA synthetic formulation. LA: lactic acid.

concentration of FFA in the bacterial supernatant (Figures 2a, 2b, and 2c), FFA/viable bacterial cell (Figure 2d) and FFA/g of bacterial pellet (Figure 2e). This reflected a significantly higher metabolic activity of this bacterium, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103, additionally to its ability to produce more fatty acids, a feature that relates to the production of anti-cancer fatty acid compounds. For instance, SCFAs, linolenic acid [23] or conjugated linoleic acid (CLA) [24] are considered to be locally produced in the colon to target immune cell function and suppress the disease/inflammation [25,26]. Furthermore, fatty acids, classified as short-chain (SCFA), medium-chain (MCFA) or long-chain (LCFA) fatty acids, demonstrated potential as chemotherapeutic agents for the treatment of colorectal cancer. For instance, lauric acid holds promise for preferential antineoplastic properties by higher induction of apoptosis in cancer cells [27].

Investigating the anti-cancer effect of *L. fermentum* NCIMB 5221 was performed with a verification of the probable effect of its bacterial cell-free extract on colon cancer cell proliferation (Figure 3) and cell death (Figure 4). The suppression of cancer cell growth and induction of apoptosis reflect a significant effect of those extracts against colon cancer cells. Each extract seemed to contain bacterial compounds with anti-proliferative effect expressed in different time points. Compared with SP, the CM bacterial extract was most effective in inhibiting cancer cell proliferation after 24 h and 7 days of treatment ($p < 0.05$, Figure 3e and 3f) and in inducing apoptosis at 24 h ($p < 0.05$, Figure 4e). As the supernatant (PS) contains sodium acetate (found in MRS broth), which may interfere with the efficacy of the test, more interest was focused on the conditioned medium (CM). Previous studies have shown that some *L. fermentum* strains have greater potency compared with other *Lactobacilli,* in terms of soluble factors produced in the supernatant and not to the bacterial pellet itself [28]. This aligns with other studies where probiotic CM have shown effects similar to living bacteria [29] and supported the potential of probiotics against CRC [30]. To provide relevant evidence of the potential beneficial effect of probiotic bacteria *L. fermentum* NCIMB 5221 against colon cancer, the probiotic was tested on both Caco-2 cancer cells and CRL-1831 normal cells *in vitro*. *L. fermentum* NCIMB 5221 was shown to reduce CRC cell viability in a time-dependent manner (Figure 5e) compared with controls (Figures 5a and 5b). It also supported constant non-neoplastic cell growth in a serum-free media compared with untreated cells (Figure 5f). In fact, among all tested probiotic bacteria, the probiotics that inhibited the most cancer cells also showed the greatest proliferation of non-cancerous colon cell growth. In order of potency, the tested probiotics were *L. fermentum* NCIMB 5221, *L. rhamnosus* ATCC 53103, and *L. acidophilus* ATCC 314. Those observations support the finding that an optimal anti-cancer drug would be one that destroys neoplastic cells but not healthy cells.

Interestingly, *L. fermentum* NCIMB 5221 produced the most SCFAs ($p < 0.001$, Figure 6) compared with *L. rhamnosus* ATCC 53103 and *L. acidophilus* ATCC 314. This observation agrees with some if the reported actions of probiotic bacteria in producing factors that prevented tumor-initiating events in the colon while promoting a healthy epithelium. The considerable increase in the production of butyrate observed during the administration of *L. fermentum* was of importance in relation to colonic cancer [31]. SCFAs are defined as products of the anaerobic metabolism of mal-absorbed or non-absorbed dietary carbohydrates by luminal bacteria and identified as the dominant ion species in the aqueous phase of feces (190 mM) [32]. In fact, it has been thusly proposed that to alter intestinal epithelial
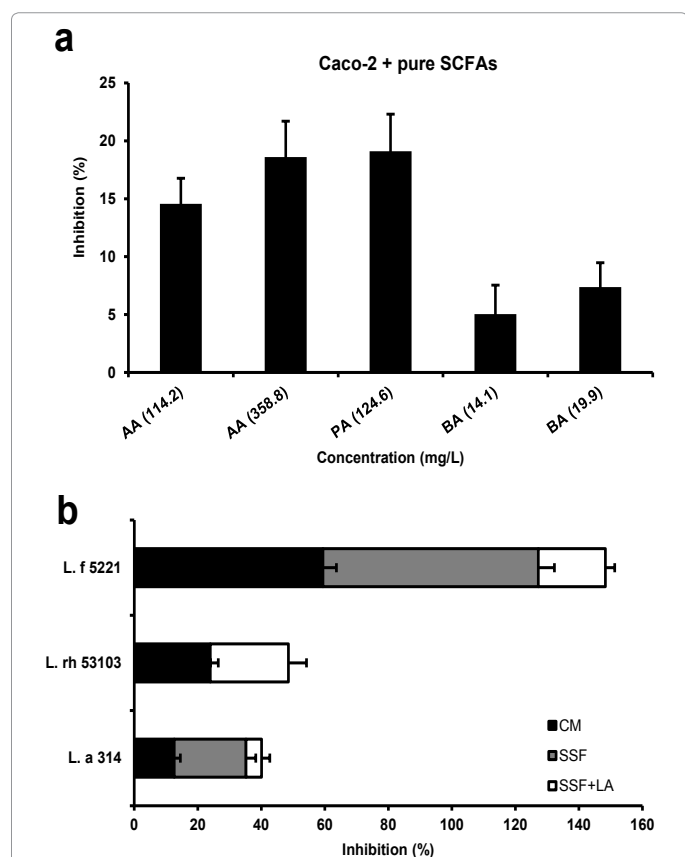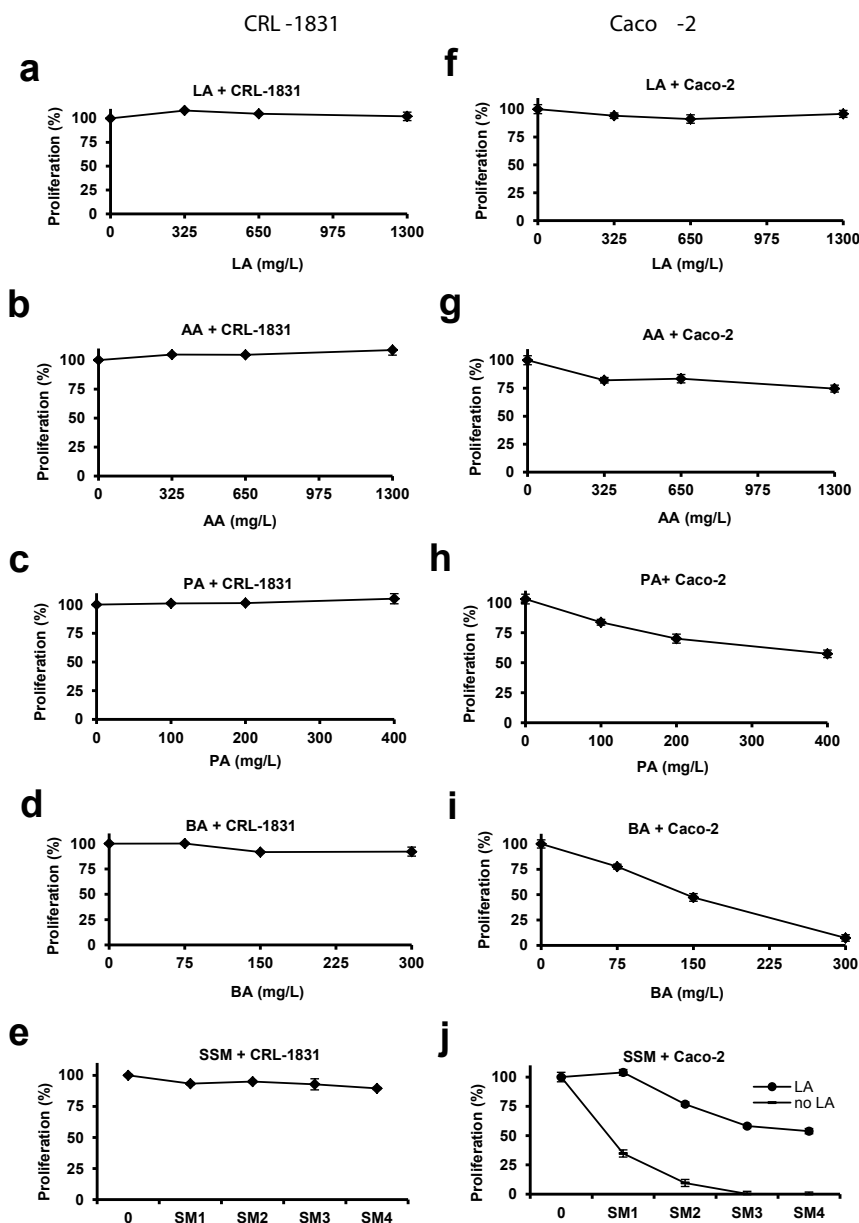
Figure 8: Study of the effect of synthetic/pure SCFAs (separately or mixed) on CRC and non-neoplastic colon cells. Confirmation of the non-cytotoxic effect of (a) lactic, (b) acetic, (c) propionic, (d) and butyric acids, and (e) their mixtures on normal epithelial colon cells (CRL-1831). Determination of the anti-proliferative activity of (f) lactic, (g) acetic, (h) propionic, (i) and butyric acids, and (j) their mixtures on CRL-1831 normal cells anti-proliferative activity (f, g, h, i, and j, respectively) on Caco-2 cancer cells. The data values represent the mean ± SEM (n = 5). *p < 0.05, **p < 0.01, and ***p < 0.001 compared with control. LA: lactic, AC: acetic, PA: propionic, BA: butyric acid.

cell function, including colonic SCFA utilization (mainly butyrate [33]), luminal bacteria can be the first target. Approximately, SCFA concentrations in the lumen are in the range of 70 - 130 mM, with molar ratios of acetate: propionate: butyrate varying from 75:15:10 to 40:40:20. It has been estimated that SCFAs can contribute with about 10% of the total caloric requirements in humans. Luminal SCFAs, especially butyrate, serve as the major energy source for human colonocytes, especially in the distal colon [34]. In addition to its role as a fuel, butyrate is notable for its function as an inhibitor of histone deacetylases (HDACs), leading to hyperacetylation of chromatin, thereby influencing gene expression. During the concentration-dependent absorption of SCFAs, bicarbonate, salt, and water transport improves, maintaining a neutral or alkaline colonic pH [35]. In animals, they accelerated the restoration of colonic anastomoses and experimental colitis [36,37] and resulted in increased regional blood flow and oxygen uptake [38]. *L. fermentum* NCIMB 5221 is considered a fitter candidate based on its higher production of propionate and butyrate. Nonetheless, *L. rhamnosus* ATCC 53103 and *L. acidophilus* ATCC 314 may also have a beneficial effect through their elevated production of lactate (up 3200 mg/L, Figure 6a, *p* < 0.001). Here, lactate is a substrate for luminal lactate-utilizing bacteria that produce acetate and butyrate, as well as some propionate [39] and a regulator of epithelial proliferation in the gut through the repression of cyclin E1/D1 gene transcription [29].
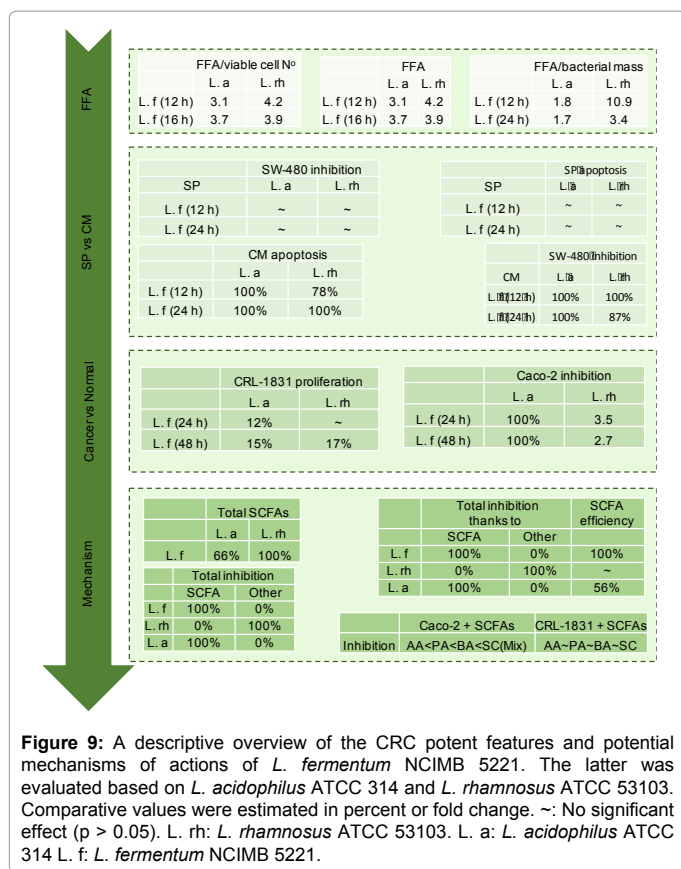
FFA

FFA/viable cell Nº

	L. a	L. rh
L. f (12 h)	3.1	4.2
L. f (16 h)	3.7	3.9

FFA

	L. a	L. rh
L. f (12 h)	3.1	4.2
L. f (16 h)	3.7	3.9

FFA/bacterial mass

	L. a	L. rh
L. f (12 h)	1.8	10.9
L. f (24 h)	1.7	3.4

SP vs CM

SP — SW-480 inhibition

	L. a	L. rh
L. f (12 h)	~	~
L. f (24 h)	~	~

SP apoptosis

	L. a	L. rh
L. f (12 h)	~	~
L. f (24 h)	~	~

CM apoptosis

	L. a	L. rh
L. f (12 h)	100%	78%
L. f (24 h)	100%	100%

CM — SW-480 inhibition

	L. a	L. rh
L. f (12 h)	100%	100%
L. f (24 h)	100%	87%

Cancer vs Normal

CRL-1831 proliferation

	L. a	L. rh
L. f (24 h)	12%	~
L. f (48 h)	15%	17%

Caco-2 inhibition

	L. a	L. rh
L. f (24 h)	100%	3.5
L. f (48 h)	100%	2.7

Mechanism

Total SCFAs

	L. a	L. rh
L. f	66%	100%

Total inhibition

	SCFA	Other
L. f	100%	0%
L. rh	0%	100%
L. a	100%	0%

Total inhibition thanks to / SCFA efficiency

	SCFA	Other	SCFA efficiency
L. f	100%	0%	100%
L. rh	0%	100%	~
L. a	100%	0%	56%

	Caco-2 + SCFAs	CRL-1831 + SCFAs
Inhibition	AA<PA<BA<SC(Mix)	AA~PA~BA~SC

Figure 9: A descriptive overview of the CRC potent features and potential mechanisms of actions of *L. fermentum* NCIMB 5221. The latter was evaluated based on *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. Comparative values were estimated in percent or fold change. ~: No significant effect (p > 0.05). L. rh: *L. rhamnosus* ATCC 53103. L. a: *L. acidophilus* ATCC 314 L. f: *L. fermentum* NCIMB 5221.

Other features that could support the prophylactic potential of *L. fermentum* NCIMB 5221 in colorectal health could be the transformation of LA to CLA and other compounds with antioxidant and anti-inflammatory properties [40]. When orally administered *L. fermentum* NCIMB 5221 was used to alleviate markers of metabolic syndrome in ZDF rats_hypothetically through the release of FA, a phenolic acid found in foods [13].

Further analysis confirmed that *L. fermentum* NCIMB 5221 activity was not due to one of the SCFAs alone (Figure 7a). Since there was no significant difference between the bacterial extract and the SCFAs synthetic formulation (Figure 7b), *L. fermentum* NCIMB 5221 may owe its anti-cancer effect to SCFA release in total. After addition of lactic acid to the SCFA mixture, the effect of SSF+LA was significantly less than SSF or the CM. This implies that lactate may have repressed SCFA metabolism/intake in CRC cells and other non-anti-cancer factors produced by *L. fermentum* NCIMB 5221 that support the activity of secreted SCFAs in suppressing cancer cells. The transport of butyrate into cells is greatly inhibited by the presence of its analog, lactate, a monocarboxylic acid transported into cells via monocarboxylated transporter (MCT), or propionate that is found in the colonic lumen and structurally similar to butyrate [41]. Similarly, it was demonstrated that the uptake of 500 μM butyrate in Caco–2 cells was reduced by 49.6% in the presence of propionate and by 57.2% in the presence of 10 mM L-lactate [42]. Under in vivo conditions, where butyrate and propionate are present at >10 mM in the colon, the transporter plays only a minor role in the entry of these compounds into colon cells. When SCFAs are at low concentrations, there is involvement of SLC5A8 as transporters of butyrate and propionate with a Michaelis constant of ~0.05 mM. However, at high concentrations, SCFAs diffuse into cells bypassing the transporter [43].

Importantly, the absorption and action of SCFAs within the extract of *L. fermentum* NCIMB 5221 on cancer cells could have involved other mechanisms that were repressed with the addition of lactate. *L. fermentum* NCIMB 5221 extract may have contained molecules playing a role in assuring the action of SCFAs involved with cell transporters. These include the monocarboxylated transporter 1 (MCT-1) and sodium-coupled monocarboxylate transporter (SMCT-1) receptor found on colonocytes. Their function is to transport SCFAs or SCFA receptors GPR41/ free fatty acid receptor 3 (FFAR3) and GPR43/ free fatty acid receptor 2 (FFAR2), expressed in a subpopulation of ghrelin and gastrin cells [44]. Recent studies have identified the plasma membrane transporter SLC5A8 and the cell-surface receptors GPR109A and GPR43 as essential for the biologic effects of SCFAs in the colon [45]. Gpr109a was found crucial for butyrate-mediated induction of IL-18 in colonic epithelium. It was actively involved in promoting anti-inflammatory properties in colonic macrophages and dendritic cells and enabling them to induce differentiation of Treg cells and IL-10-producing T cells [46]. Several bacterial effectors may alter the action of soluble factors that probiotic bacteria have produced. For example, a probiotic-derived polyphosphate have shown to inhibit progression of CRC, inactivate the ERK pathway and induce cancer cell apoptosis [47] or cell-bound exopolysaccharides (c-EPS) with anti-tumor activity [46]. In certain cases, it was revealed that the pro-SCFA compound might be a macromolecule such as a protein, nucleic acid, or a polysaccharide [46].

Validation tests on the SCFAs' effect on non-neoplastic cells and cancer cells were used to confirm the fact that different concentrations and mixtures of pure/synthetic SCFAs have significant suppressive effect on cancer cells but not against normal epithelial cells. The test also verified the effect of the addition of lactate with SCFAs on cancer cell proliferation (Figure 8). First, lactic acid did not affect cancer cell proliferation when tested at different doses (up to 1300 mg/L, Figure 8f); however, when added to SCFAs, they lost a significant part of the cancer-suppressing activity. This confirms that lactic acid could inhibit SCFA metabolism/uptake in cancer cells as described in some studies [48] and also concluded with SCFA synthetic formulations, as presented in this study (Figure 7b), Thus, this emphasize that the presence of another bacterial factor promoted the role of SCFAs to suppress cell growth. If we assume, as concluded above, that lactate and propionate inhibited the uptake of butyrate (by 31% for *L. fermentum* NCIMB5221), then in the presence of lactate, only acetate (18. 6 ± 3.1%) will be responsible for the inhibitory effect that was closer to SSF + LA (21.1 ± 2.9%, Figure 7). In the case of non-neoplastic colon cells, no significant effect was observed on CRL-1831 cell growth when treated with SCFAs and/or lactate when compared with cancer cells. Whereas with *L. fermentum* NCIMB 5221, there was a promotion of cell growth (Figure 5f), implying that, in addition to SCFAs, other soluble or non-soluble bacterial compounds could have a beneficial action on normal cells. For example, lipoteichoic acid (LTA) was shown to induce signaling in colon epithelial cells through Toll-like receptor 2 (TLR2)-CD14 and/or TLR2-TLR6 heterodimers. It activates extracellular-signal-regulated kinases (ERKs), NF involved protein kinase C (PKC)- and mitogen-activated protein kinase (MAPK)-dependent pathways, and inhibits cytokine-induced epithelial cell apoptosis and damage through a phosphoinositide 3-kinase-AKT-dependent pathway [49]. Those proteins were demonstrated to present resistance against apoptosis and induce epithelial barrier fortification in intestinal epithelial cells by activating the p38 and ERK signaling pathways [50].

Conclusion

In this study, *L. fermentum* NCIMB 5221 showed the same CRC cell inhibitory effect as the SCFAs by themselves. This would suggest the use of these bacteria as preventive vehicles is not limited to SCFA-producing ability, as was suggested in some studies [27,51-53]. Notably, the use of the bacteria as a delivery mechanism for active compounds such as the SCFAs could be a better option, especially since *L. fermentum* NCIMB 5221 can produce antioxidant, anti-inflammatory, and anti-carcinogenic effects in soluble and non-soluble components within the gut.

Here, *L. fermentum* NCIMB 5221 was identified with an increased anti-proliferative effect against CRC cells in comparison with some other LAB (*l. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103) characterized in previous studies for their potential anti-cancer effect [19-22]. Interestingly, this bacterium exhibited a reverse effect on normal colon cells suggesting that this bacterium is harmful to cancer cells but beneficial to normal cells. These effects were strongly related in this work, showing the significant ability of *L. fermentum* to produce more FFAs and significantly more acetic, propionic, and butyric acids compared with other probiotics. *L. fermentum* NCIMB 5221 has also been shown to produce antioxidant and anti-cancer compounds that make it more suitable as an alternative bioprophylactic and biotherapeutic agent for CRC treatment.

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