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Identification of *Lactobacillus Fermentum* Strains with Potential against Colorectal Cancer by Characterizing Short Chain Fatty Acids Production, Anti-Proliferative Activity and Survival in an Intestinal Fluid: *In Vitro* Analysis

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Abstract

The use of probiotics as preventive agents in colorectal cancer (CRC), as widely suggested in many clinical and pre-clinical studies, was often linked to the potency of short chain fatty acids (SCFAs) in the gut. However, there remains an incomplete understanding of the fatty-acid-producing activity of certain probiotics and their cancer preventive potential. In the current study, *L. fermentum* strains were investigated for their potential use with CRC treatments. Using cell-free extracts, *L. fermentum* NCIMB -5221, - 2797, and -8829 were first compared based on their SCFAs production and anti-proliferative activity against Caco-2 colon cancer cells. The corresponding SCFAs synthetic formulations, similar to the ones produced by the bacteria, were prepared and compared with the latter to determine the role and efficacy of naturally produced SCFAs in inhibiting the proliferation of colon cancer cells. Subsequently, the bioactivity and stability of *L. fermentum* bacterial strains in a simulated intestinal fluid (SIF) was determined. Results showed that *L. fermentum* NCIMB -5221 and -8829 were the most potent in producing SCFAs, in particular, acetic (192.3 ± 4 mg/L minimum), propionic (69.2 ± 1.6 mg/L minimum), and butyric (35.4 ± 2.9 mg/L minimum) acids. They were also found to inhibit the growth of Caco-2 cells (53.4 ± 1.6%, 72 h, $p = 0.021$) in comparison with *L. acidophilus* ATCC 314. Additionally, they showed resistance to SIF (16.3 ± 1.9% minimum, 72 h, $p = 0.006$) and produced SCFAs in SIF at concentrations high enough to significantly inhibit Caco-2 proliferation (74.73 ± 2.1%, 72 h). Based on characteristics related to bacterial cell survival, SCFA production, and anti-proliferative activity, *L. fermentum* NCIMB -5221 and - 2797 could potentially be considered as biotherapeutic agents against CRC.

Keywords: *L. fermentum*; Probiotics; Colorectal cancer; Short chain fatty acids; Cell proliferation; Intestinal fluid

Introduction

The diagnosis and primary prevention strategies employed for colorectal cancer (CRC) have shown this disease to be a common public health problem especially in developing countries [1,2]. CRC accounts for 8.0 - 9.7% of all cancer cases and cancer-related deaths [3] and is considered not only a common type of cancer but also a complex and multifactorial disease [4,5] Despite the appreciable understanding of the disease's pathogenesis, as the environment is considered to play a vital role in its progression, the identification of reliable markers for primary preventive measures for CRC is still deficient [6]. Nevertheless, reports have shown that CRC incidence was reduced to a large extent (up to 80%) by a healthy lifestyle and environmental factors, with diet being a major controlling factor [7]. Dietary interventions have recently attracted increased attention from researchers and clinicians for the prevention and management of CRC [8]. Within this domain of dietary supplements, probiotics have emerged as attractive biotherapeutic agents with nutritional and health benefits. Probiotics, comprised of live microbial food supplements capable of beneficially affecting the gut microbiome, have long been known to augment a variety of immunological and metabolic parameters through diverse mechanisms [8]. A prominent class of probiotics, found to confer health-promoting attributes to the host are lactic acid-producing microorganisms. The *Lactobacillus* spp. is commonly found in fermented foods as well as in the gastrointestinal (GI) ecosystem. Several probiotic formulations containing *L. fermentum,* typically those surviving in both GI [9,10] and genital environments [11], were found to reduce infection [12] and overgrowth of harmful bacteria [13]. Also, they retained their beneficial metabolic activities when exposed to intestinal conditions, suggesting their potential for targeted colon delivery and increased colon bioproduction of anti-carcinogenic compounds [14]. *L. fermentum* have also shown to attribute potential beneficial GI health including anti-inflammatory [15,16] and anti-tumorigenic [17,18] activities. Some *L. fermentum* strains have shown greater or comparable effects than other probiotic bacteria, such as *L. reuteri* [19], *Bifidobacterium longum* [20] and *L. plantrum* [21].

Several bacterial products were found responsible for the mechanisms associated with these appreciable effects. Among them,

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short chain fatty acids (SCFAs) produced by the gut microflora are known for their ability to induce cancer cell death and provide a source of energy for colonocytes [22]. The SCFAs resulting from the microbial metabolism of non-digestible carbohydrates in the gut, play a central role in the intestinal homeostasis [23]. They also have shown certain effects, such as; anti-cancer cell-apoptotic effect, promotion of cancer cell cycle arrest, inhibition of cancer cell invasion, and inflammation in the colon [24]. A recent *in vitro* study showed the adherence property of *L. fermentum* to cancer cells and the associated anti-proliferative effect through the bioproduction of SCFAs [25]. However, comparative studies investigating the anti-proliferative effect of these bacteria *in vitro* against CRC cells and their activity in intestinal conditions are infrequent or inconclusive [14,26,27]. Thus, the current study screened a number of *L. fermentum* bacterial strains (NCIMB -5221, -2797, and -8829) in order to evaluate their biotherapeutic potential against CRC. These strains were previously investigated for the production of certain anti-inflammatory acids [28], cholesterol assimilation [14] in relation to targeted colon delivery [29], and for use in metabolic syndrome (MS) [30]. The aim of this study is to provide insight into SCFA production and anti-proliferative effects against colon cancer cells as well as the bacterial stability in intestinal conditions for *L. fermentum* bacteria NCIMB -5221, -2797, and -8829.

Materials and Methods

Materials

Cell culture media including Dulbecco's modified Eagle's medium (DMEM), Eagle's Minimum Essential Medium (EMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from Invitrogen. Bacterial culture broth De Man Rogosa Sharpe (MRS) and agar used for plating and growth were obtained from Fisher Scientific (Ottawa, ON, Canada). Water was purified with two systems from Barnstead (Dubuque, IA, USA): an EasyPure reverse osmosis system then a NanoPure Diamond Life Science (UV/UF) ultrapure water system. Reagents and acids such as propionate, acetate, and butyrate, and sodium L-Lactate, were obtained from Sigma (St. Louis, MO, USA).

Bacterial cultures

L. fermentum NCIMB -5221, -8829, and -2797 were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland, UK). *L. acidophilus* ATCC 314 was purchased from Cederlane Laboratories (Burlington, ON, Canada). To maintain the bacterial cultures, they were inoculated daily in new MRS broth at 1% (v/v). Growth and viability of bacterial cells were determined at OD_{620nm} (Perkin Elmer 1420 Multilabel Counter, USA) and colony counting using agar plates.

Mammalian cultures

Caco-2 human epithelial CRC adenocarcinoma cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in EMEM + 20% FBS and incubated in a CO_2 incubator (37°C, 5% CO_2) for up to two weeks for full differentiation. Caco-2 colon cancer cells were left to attach for up to 24 h to reach a confluence of 50-60% in 96-well plates in DMEM + 10% FBS (37°C, 5% CO_2), before experiments. During assays, cell culture medium was substituted by probiotic conditioned medium (CM) mixed with serum and antibiotic-free media (DMEM + 10% FBS).

Preparation of probiotic treatments

For the probiotic treatment used on colon cancer (Caco-2) cells, a conditioned cell culture medium (CM) was prepared according to Grabbing et al. [24] and Kim et al. [25] with slight modifications. Bacterial cultures of *L. fermentum* and *L. acidophilus* were passaged for 72 h (37°C, 5% CO_2) to reach a late exponential phase (~16 h). The bacterial cells were collected from the culture broth by centrifugation (1000 × g, 15 min, 4°C) and washed with PBS. This bacterial pellet (10^7-10^9 cru/mL) was incubated in DMEM for 2 hours (37°C, 5% CO_2). The medium was also centrifuged (1000 × g, 15 min, 4°C) to remove the bacteria, then sterile-filtered (0.2 µm-pore-size filter, Millipore). The pH was adjusted to 7 using 2 M NaOH and 2 M HCl. Before use, the CM of each bacterium was diluted twice with DMEM.

Preparation of simulated intestinal fluid (SIF)

To determine the ability of *L. fermentum* bacteria to survive in intestinal conditions, a simulated intestinal fluid (SIF) was prepared, with some modification, as described previously by Qian Zhao et al. [31]. The SIF solution contained; glucose (5.5 g/L), yeast extract (3.5 g/L), pancreatin (2 g/L), oxgall (1.5 g/L), pectin (2 g/L), inulin (0.54 g/L), fructooligosaccharides (0.85 g/L), starch (3 g/L), and monobasic potassium phosphate (KH2PO4, 3.3 g/L) dissolved in deionized water. The pH was adjusted to 6.8 using 2 M NaOH and 2 M HCl, followed by autoclaving at 120°C for 15 min and cooled at room temperature (RT) before use.

Bioactivity of *L. fermentum* bacteria

It was necessary to determine if *L. fermentum* bacteria were metabolically active in CM or SIF. Since all bacteria are lactic acid bacteria, the concentrations of lactic acid, potentially produced by bacterial cells, were separated and measured by HPLC method, adapted from Dubey and Mistry (1996) [32,33] (described below in detail).

Analysis of lactic acid and SCFAs

Lactic acid and SCFAs were separated using a slightly modified HPLC method [32,33]. The HPLC system used (Model 1050 UV, Hewlett- Packard HP1050 series, Agilent Technologies, USA) was equipped with a UV-vis detector and diode array detector (DAD, 210 ± 5 nm). The column used was a prepacked Rezex ROA -organic acid H+ (8%) column (150 mm x 7.80 mm, Phenomenex, Torrance, CA, USA) attached to an ion- exclusion microguard refill cartridge and heated to 35°C. Data were obtained using ChemStation equipped with LC3D software (Rev A.03.02, Agilent Technologies, CO, USA). The mobile phases (0.05 M H_2SO_4 and 2% of acetonitrile) were pumped at an isocratic gradient with a 0.7 - 0.8 mL/min flow rate. A 100 µl of sample was injected through an autosampler. Lactic, acetic, propionic, and butyric acids were used to prepare standard solutions at concentrations of 1, 10, 100, 500, and 1000 ppm. The concentrations of samples were calculated using the linear regression equations ($R^2 \geq 0.99$) from each standard curve.

Cancer cell proliferation assay

The growth of colon cancer cells was determined using an ATP bioluminescence-based assay (CellTiter-Glo® Luminescent Cell Viability Assay, Promega). Caco-2 cells were seeded at 5 × 10^3 cells/well onto 96-well culture plates and left to attach for 24 - 48 h for the formation of an epithelium-like monolayer (37°C, 5% CO_2). Caco-2 cells were incubated with the probiotic treatments for 24, 48 and 72 h, (37°C, 5% CO_2, pH 7). Cell growth inhibition and viability were determined according to the manufacturer's protocol [34]. After

incubation, the plate was equilibrated at RT (30 min) and the media was replaced with 100 µL of luminescent reagent and 100 µL of DMEM. The plate was agitated on an orbital shaker (200 rpm, 3 min), followed by incubation at RT for 10 min. Signals were recorded using a multi-label microplate reader (Perkin Elmer, Victor 3, MA, USA).

Determination of bacterial stability in SIF

Each bacterial culture in MRS broth passaged for 72 h was used to inoculate 15 ml of SIF at 3% (v/v), sealed and incubated micro-anaerobically. At 0, 4, 8, 12, 16, and 24 h, samples were taken to determine the density ($OD_{620\ nm}$) and viable bacterial cell count in SIF. The bacterial supernatant was collected by centrifugation (1000 × g, 30 min, 4°C), using 5 ml of bacterial culture, filtered (0.22 µm sterile filters), then stored at -80°C until use.

Relevance of SCFAs produced by *L. fermentum* strains

To determine whether the concentrations of SCFAs present within the bacterial cell-free extract were the active factors behind suppressing CRC cell growth, the anti-proliferative effect of SCFAs alone was determined. First, lactic, acetic, propionic, and butyric acids produced by each *L. fermentum* strain were quantified in CM. Mixtures containing the same composition were formulated in DMEM, then added to the colon cancer cells (37°C, 5% CO_2, pH 7, 72 h). Cell viability was determined using an ATP bioluminescence assay, as described above.

Statistical analysis

Results were presented as means ± standard error of the mean (SEM). Statistical significance was calculated using one-way analysis of variances (ANOVA) with the Tukey's comparison test and Student's t-test. Pearson's correlation method was followed to determine correlation between variables. SPSS statistics software package (version 20.0, IBM Corporation, NY, USA) was used. *P*-values of $p < 0.05$ were considered significant.

Results

L. fermentum bacteria produce lactate in the conditioned medium (CM)

Before using the CM of *L. fermentum* bacteria as a probiotic treatment *in vitro*, the activity of the bacterial cells incubated in the CM was established by quantifying the level of lactic acid produced. All bacterial strains were active in CM and produced variable amounts of lactic acid (Figure 1). Data showed that *L. fermentum* NCIMB 5221 (455.3 ± 9.3 mg/L, $p < 0.001$) produced the highest amounts of lactic acid when compared with *L. fermentum* NCIMB -2979 and -8829. All *L. fermentum* strains produced significantly less lactic acid than *L. acidophilus* ATCC 314 (1947.7 ± 23.3, $p < 0.0001$).

L. fermentum strains produced variable amounts of SCFAs

To confirm that *L. fermentum* bacteria may produce anti-carcinogenic active compounds in the cell-free extract, three SCFAs were quantified in the conditioned cell CM acetic, propionic, and butyric acids. The results described the quantities of naturally produced SCFAs by the bacteria. For the bioproduction of acetic acid, *L. fermentum* NCIMB 2797 (206.3 ± 8.7 mg/L, $p < 0.01$) and *L. fermentum* NCIMB 5221 (192.3 ± 4 mg/L, $p < 0.01$) produced significantly more than either *L. acidophilus* ATCC 314 (114.2 ± 11.9 mg/L, $p < 0.01$) or *L. fermentum* NCIMB 8829 (134.3 ± 5.7 mg/L, (Figure 2a). Again, *L. fermentum* NCIMB 2797 (69.2 ± 1.6 mg/L, $p < 0.001$) and *L. fermentum* NCIMB 5221 (85.7 ± 10.9 mg/L, $p < 0.001$) were the only bacteria to produce propionic acid, but not *L. acidophilus* ATCC 314 or *L. fermentum* NCIMB 8829 (**Figure 2b**). Similarly, *L. fermentum* NCIMB 2797 (35.4 ± 2.9 mg/L) and *L. fermentum* NCIMB 5221 (38.7 ± 4.2 mg/L, $p < 0.05$) produced significantly higher amount of butyric acid than *L. fermentum* NCIMB 8829 (butyrate not detected) and *L. acidophilus* ATCC 314. In terms of total SCFA production, *L. fermentum* NCIMB 2797 (35.4 ± 2.9 mg/L) and *L. fermentum* NCIMB 5221 (38.7 ± 4.2 mg/L) had significantly higher production compared with *L. acidophilus* ATCC 314 (14.1 ± 5.9, $p < 0.01$) or *L. fermentum* NCIMB 8829 (Not detectable, $p < 0.0001$, Figure 2d).

L. fermentum inhibits colon cancer cell proliferation

In this experiment, the ability of *L. fermentum* bacteria to inhibit colon cancer cell growth was investigated. Caco-2 cancer cells were incubated with bacterial CM for 24 h, 48 h, and 72 h. The results showed a time-dependent effect of the probiotic extracts on the viability of Caco-2 cells (Figure 3). At 24 h (Figure 3a), only *L. fermentum* NCIMB 5221 (6.02 ± 1.04%, $p < 0.05$) inhibited cancer cell growth when compared with remaining treated and untreated cells. After 48 h of probiotic treatment (Figure 3b), results showed that *L. fermentum* NCIMB 2797 (39.00 ± 1.56%) and *L. fermentum* NCIMB 5221 (45.77 ± 0.37%) were significantly better in reducing CRC cell proliferation ($p < 0.001$). Data presented in Figure 3c shows that *L. fermentum* NCIMB 2797 (53.4 ± 1.6%), and *L. fermentum* NCIMB 5221 (57.9 ± 0.7%) significantly induced greater inhibition of colon cancer proliferation compared to all other treatments tested ($p < 0.001$, 72 h). Moreover, *L. fermentum* NCIMB 5221 significantly inhibited more cancer cell proliferation than *L. fermentum* NCIMB 2797 ($p = 0.033$, 72 h).

The inhibition of colon cancer cells correlates with SCFAs production

To relate the action of *L. fermentum* bacteria in suppressing CRC cell growth with respect to the production of SCFAs, a correlation analysis was conducted (Figure 4). Regression analysis showed that the suppression of colon cancer cell proliferation by *L. fermentum*-CM significantly correlated with the levels of total SCFAs produced by the bacteria in the CM ($r = 0.87$, $p < 0.001$, Figure 4d). Cancer cell inhibition correlated with the production of butyric ($r = 0.89$, $p < 0.001$) and acetic

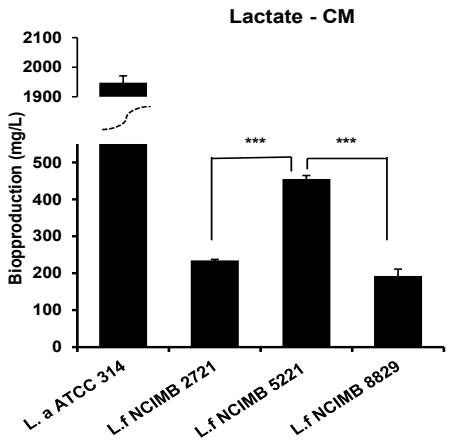

Figure 1: Determination control for of the ability of *L. fermentum* strains to produce lactic acid in conditioned cell culture medium (CM). *L. fermentum* NCIMB -2797, -5221 and, -8829 were active enough to produce different concentrations of lactic acid when incubated in DMEM (2 h, 37°C, 5% CO_2). *L. acidophilus* ATCC 314 is used as a control/for comparative purpose. Data are presented as mean ± SEM ($n = 3$). ***$p < 0.005$.

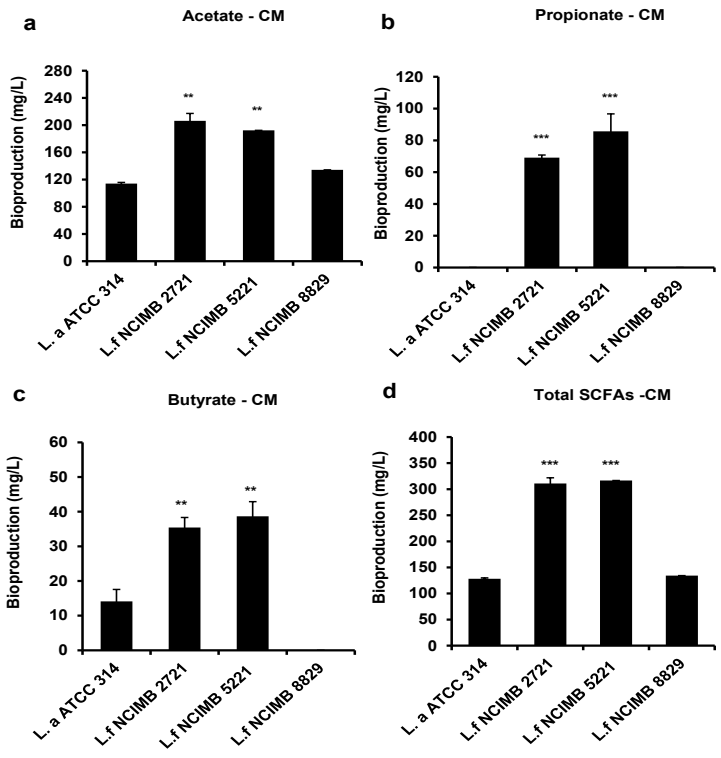

Figure 2: Analysis of the bioproduction of SCFAs by *L. fermentum* strains in the conditioned cell culture medium (CM). *L. fermentum* strains produced variable levels of SCFAs in a strain-dependent manner. The levels **(a)** acetic, **(b)** propionic, **(c)** butyric acids, and **(d)** total SCFAs, produced by *L. fermentum* NCIMB -2797, -5221, and -8829 were quantified in the conditioned medium cell culture and compared with each other while *L. acidophilus* ATCC 314 was used as a control. Data are presented as mean ± SEM (n = 3). *p < 0.05, **p < 0.01 and ***p < 0.001, compared with *L. acidophilus* ATCC 314.

(r = 0.0771, p < 0.001) acids (Figures 4c and 4b). The highest correlation was with propionic acid concentrations (r = 0.89, p < 0.001) and with different combinations of SCFAs (butyrate and propionate) (r = 0.95, p < 0.001, Figure 4f).

The action of probiotic SCFAs is strain-dependent

Establishing a correlation between *L. fermentum* bacteria SCFA production and their anti-proliferative effect against CRC cells is not sufficient to demonstrate that the inhibition of CRC cell growth is due to SCFAs. Therefore, an additional approach was taken using synthetic SCFAs.

Initially, pure SCFAs corresponding to the concentrations produced by the bacteria were tested separately, and the resulting concentrations of acetic, propionic, and butyric acids showed significantly less inhibition (maximum of 20.3 ± 2.5%) than *L. fermentum*-CM (31.2 ± 1.5% minimum, p < 0.05, Figure 5a).

Secondly, SCFA synthetic formulations corresponding to the concentrations of SCFAs produced by the bacteria and containing acetic, propionic, and butyric acids were prepared (as described in Table 1). SCFA synthetic formulations were then tested on Caco-2 cells and compared with *L. fermentum*-CM (Figure 5b). These findings showed that the above mentioned mixtures had variable effects on the alteration of cell viability compared with *L. fermentum*-CM treated cancer cells. For *L. acidophilus* ATCC 314, the CM (12.6 ± 1.9%) had significantly less efficacy than its corresponding SCFA synthetic formulation (SSF-a, 22.9 ± 1.0%, p < 0.05). For *L. fermentum* NCIMB 5221, there was no significant difference (p = 0.094) between the SSF (58.9 ± 1.8%) and CM (57.9 ± 0.7%). However, for *L. fermentum* NCIMB 2797 (53.4 ± 1.6%) and *L. fermentum* NCIMB 8829 (31.2 ± 1.5%), *L. fermentum*-CM was significantly more effective than SCFA synthetic formulations (SSF-f2, 43.8 ± 2.2%, p = 0.026) and SSF-f8 (19.12 ± 1.6%, p = 0.015, Figure 5b).

After addition of lactic acid to each formulation, the inhibitory effect of "SSF+LA" was up to 50%, lower than either *L. fermentum*–CM or SSFs (p < 0.001, Figure 5b), indicating a loss of SCFA efficacy against cancer cells.

L. fermentum bacteria demonstrated resistance in SIF

The growth and viability of *L. fermentum* bacteria were strain-dependent. For *L. fermentum* NCIMB -2797 and -5221, the bacterial culture density (0.38 ± 0.001 minimum) was significantly higher compared with *L. acidophilus* ATCC 314 (0.29 ± 0.003%, p < 0.001, Figure 6a). Between 4 and 8 h, *L. fermentum* NCIMB -2797 (16.3 ± 1.9%) and -5221 (28.4 ± 2.4%) showed a significant increase in bacterial growth compared with the initial count. This was not the case with *L. acidophilus* ATCC 314 (Figure 6a).

In terms of decrease in viable bacterial cells, compared with initial count, a significant difference was determined (12 - 16 h), where *L. fermentum* NCIMB 2797 (70.11 ± 3.2% minimum) and *L. fermentum* NCIMB 5221 (94.02 ± 0.4% minimum) had higher death rate than *L. acidophilus* ATCC 314 (64.5 ± 0.7% maximum, p < 0.01, Figure 6b).

L. fermentum strains produced SCFAs in SIF

Despite the decrease in the viability of *L. fermentum* bacteria in SIF, the bacteria were still able to produce an anti-colon-cancer–proliferative effect in a simulated intestinal fluid environment. To

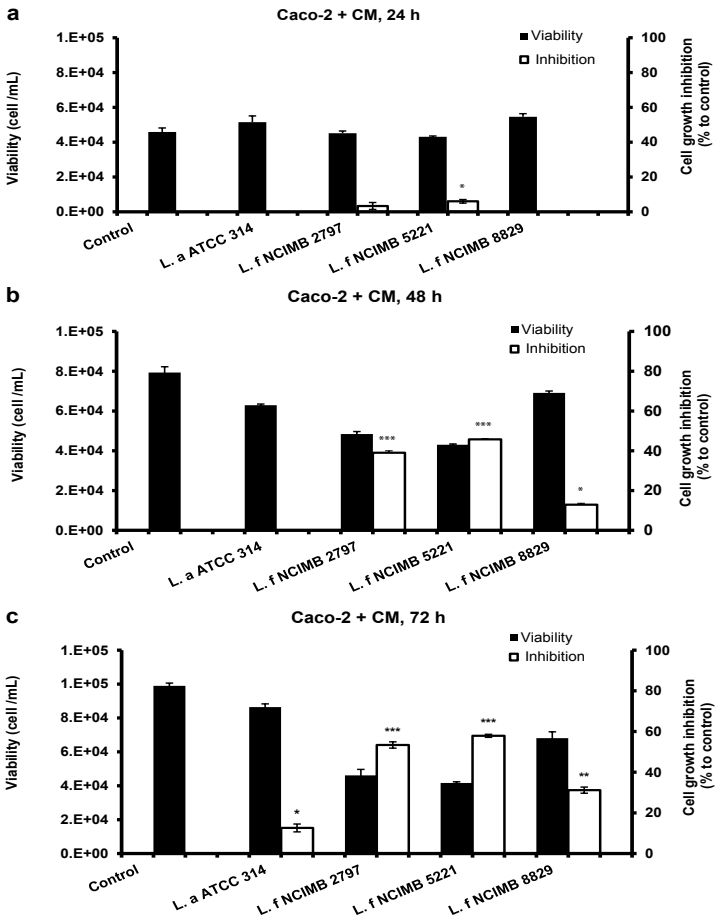

Figure 3: Screening of *L. fermentum* strains for a proliferation inhibitory effect against colorectal cancer cells. To investigate the anti-proliferative effect of the different *L. fermentum* strains, the cell culture conditioned cell culture media (CM) of *L. fermentum* NCIMB -2797, -5221, and -8829 were incubated with Caco-2 cancer cells. The viability and growth inhibition rate of Caco-2 cells for **(a)** 24 h, **(b)** 48 h and **(c)** 72 h of incubation showed a time-dependent effect. *L. acidophilus* ATCC 314 is used as a positive control. *p < 0.05, **p < 0.01 and ***p < 0.005 compared to *L. acidophilus* ATCC 314. Data are presented as mean ± SEM (n = 4).

confirm this, the production of lactic acid and SCFAs was determined in SIF after 24 h of incubation (Figure 7). Results indicate that both *L. fermentum* strains produced significantly higher concentrations of lactic, acetic, and propionic acids (Figures 6a-6c, respectively) than *L. acidophilus* ATCC 314 in SIF. *L. fermentum* strains also showed higher production of total SCFAs in SIF, as represented in Figure 6d. *L. acidophilus* ATCC 314 produced 1968.5 ± 0.3 mg/L and 413.1 ± 0.1 mg/L of total SCFAs, respectively. *L. fermentum* NCIMB 2797 produced 2491.9 ± 11.4 mg/L of lactate, 689.4 ± 2.1 mg/L of acetate, and 686.3 ± 35.7 mg/L of propionate. Also, *L. fermentum* NCIMB 5221 produced 2407.3 ± 42.3 mg/L of lactate, 637.99 ± 5.7 mg/L of acetate and 648.8 ± 17.8 mg/L of propionate. When considering the concentration of total SCFAs produced depending on bacterial culture density, both *L. fermentum* NCIMB -2797 and -5221 were significantly more potent than *L. acidophilus* ATCC 314 (p < 0.0001, Figure 7e).

Efficacy of the levels of SCFAs produced in SIF

To verify that *L. fermentum* bacteria could produce an anti-proliferative activity against colon cancer in an intestinal environment, the same concentrations of bacterial SCFAs as produced in the SIF were tested on CRC cells. SCFA synthetic formulations corresponding to the levels of SCFAs produced by the *L. fermentum* (NCIMB -2797 and -5221) in SIF (SSF-SIF-f) were reconstituted. Additionally, separate concentrations of propionic and acetic acids at the same levels as produced in SIF were tested.

Propionic acid doses used were significantly more efficient in inhibiting colon cancer cell growth than acetic acid (p < 0.001, Figure 8a). For SCFA synthetic formulations representing the concentrations of SCFAs naturally produced by *L. fermentum* bacteria in SIF (SSF-SIF-f), two formulations were prepared, as described in Table 2. SSF-SIF-f significantly reduced Caco-2 proliferation by 74.73 ± 2.1% when compared with SSF-SIF-a (38.51 ± 2.46%, p = 0.0012) and untreated cells (p = 0.0018, Figure 8a). For the inhibition of Caco-2 epithelium-like monolayer, *L. fermentum* synthetic formulation SSF-SIF-f was significantly more efficient than the *L. acidophilus* systhetic formulation SSF-SIF-a (Figure 8b, p = 0.0381).

Discussion

CRC is a leading cause of death and an economic burden with a therapeutic market worth billions of dollars worldwide [35]. However, thanks to the preventive potential of this disease [36] it was found that a lifestyle and dietary measures, supplemented with digestive enzymes and probiotics, can substantially decrease CRC incidence [37]. It is

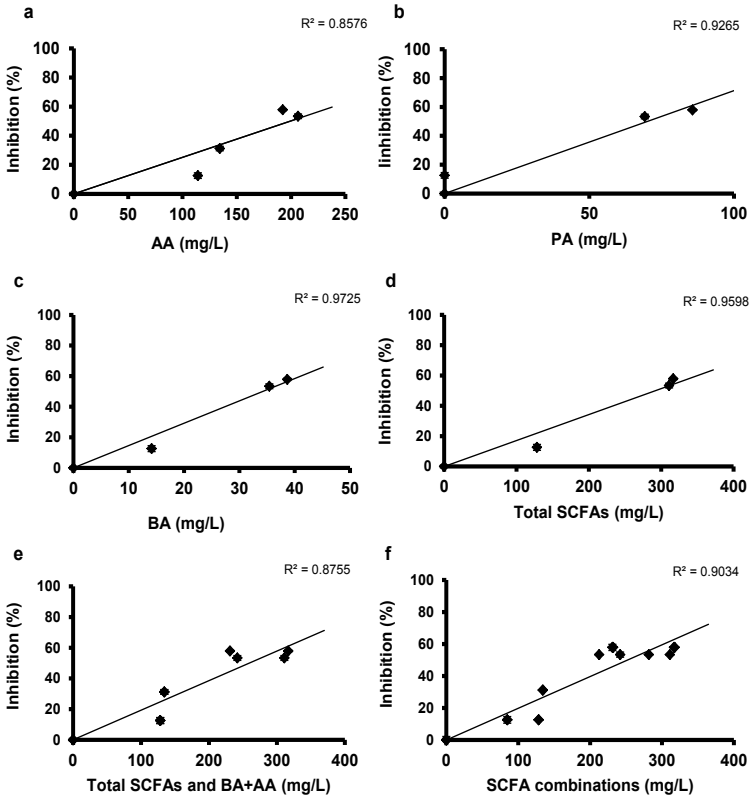

Figure 4: Investigation of the correlation between cell growth inhibition and the different concentrations of naturally produced SCFAs in probiotic CM. The dependent variables used are the values for: **(a)** acetic acid, **(b)** propionic acid, **(c)** butyric acid, **(d)** total SCFAs, **(e)** total SCFAs and BA+AA and **(f)** SCFA combinations: 7x BA and PA+[7xBA], Plots represent the data of cell growth inhibition at 72 h (Figure 2c). The lines were obtained by linear regression analysis. LA: lactic acid; AA: acetic acid; PA: propionic acid.

proposed, that increasing the rate of SCFA production through higher gut bacterial carbohydrate fermentation is essential for the maintenance of a healthy colon, with reduction of intestinal injuries, and abnormal cell growth in the lining of the intestines. However, a limited number of probiotic bacteria have been investigated as novel candidates against CRC [38]. This study investigated three *L. fermentum* strains that have demonstrated antioxidant and anti-inflammatory potential by the production of ferulic acid [39,40]. *L. fermentum* NCIMB -2797, -8829 and -5221 were investigated for anti-cancer-associated features, such as the production of SCFAs and anti-colon-cancer-cell-proliferative effects *in vitro*. For this, the cell culture conditioned medium (CM) of each bacterium was used as a probiotic extract treatment for the *in vitro* study. The metabolic activity of these LAB, when incubated in the CM was verified by the concentrations of lactic acid produced. It was observed that *L. fermentum* NCIMB 5221 produced significantly high levels of lactic acid as represented in Figure 1. Lactic acid is used by lactate-utilizing butyrate-producing bacteria in the gut [41] and is considered an anti-inflammatory component [42], which has the ability to increase anti-tumor immunoreactivity [43]. SCFAs secreted by gut bacteria induce apoptosis in CRC cells and may, therefore, be relevant for the prevention and therapy of CRC. For example, microbial-derived butyrate was found to promote the stabilization of transcription factors related to epithelial barrier protection [44]. Butyrate and propionate inhibited the activity of histone deacetylases (HDACs) in colonocytes and immune cells and induced anti-inflammatory effects via the differentiation of regulatory T-cells [45]. Thus, SCFAs secreted by *L. fermentum,* were quantified and produced at significantly different concentrations (Figure 2). *L. fermentum* NCIMB -2797, -8829, and -5221 produced significantly higher amounts of total SCFAs in their CM, compared with *L. acidophilus* ATCC 314 ($p < 005$, Figure 2d), but significantly lower amounts of lactate in their respective CM ($p < 0.001$, Figure 1). This result suggests that *L. fermentum* may act as an anti-colon cancer agent due to the production of higher quantities of SCFAs distinctively from *L. acidophilus* ATCC 314. Consequently, *L. fermentum* may produce anti-tumorigenic and anti-inflammatory activities as shown in a CRC $Apc^{Min/+}$ mice model [46]. The higher levels of lactate produced may provide more substrate for anti-oncogenic bacteria in the gut. Therefore, *L. fermentum* bacteria may play a vital role in CRC prevention through SCFAs production rather than by modulating the gut microbiota. This effect may also provide growth support for other beneficial microbiota, or inhibition of CRC-associated bacteria due to the production of lactic acid [47]. This study also showed that the concentrations of acetic acid and propionic acid measured are about half of the optimal doses suggested in the literature to induce inhibitory effects on Caco-2 cells [48], which predicts a more efficient cancer-suppressive effect of the probiotic treatment by the *L. fermentum* bacteria.

The role of microbial SCFAs in colon carcinogenesis is debatable and poorly understood. Several reports have provided evidence on the effect of probiotic bacterial supernatants or separately tested pure SCFAs in the mechanism of cancer cell inhibition. Many of

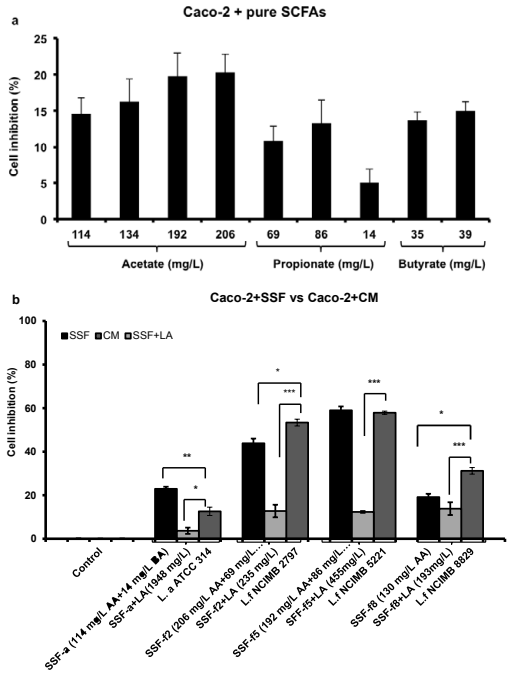

Figure 5: Investigation of the role and effectiveness of SCFAs produced by *L. fermentum* bacteria. **(a)** The anti-proliferative effect of pure SCFAs at the same concentrations as what was produced by probiotic bacteria in *L. fermentum*-CM (as described in Figure 4). The inhibitory effect of SCFAs on Caco-2 cells (72 h) increased with higher doses. **(b)** Comparison of the anti-proliferative effect of SCFA synthetic formulations (SSFs) with the anti-proliferative effect of *L. reuteri*-CM. The SCFA synthetic formulations are reconstituted mixtures of acetic, propionic, and butyric acids (Table 1) with or without lactic acid, at concentrations similar to the naturally produced ones by *L. fermentum* bacteria. These formulations, used to treat Caco-2 cells for 72 h, were compared with their corresponding *L. fermentum*-CM. *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$. Data are represented as mean ± SEM ($n = 5$).

Corresponding CM	**SSF**	Composition (mg/L)			**SSF+LA**	Composition (mg/L)			
		Acetate	Propionate	Butyrate		Lactate	Acetate	Propionate	Butyrate
L. a 314	**SSF-a**	114	0	14	**SSF-a+LA**	1948	114	0	14
L. f 2797	**SSF-f2**	206	69	35	**SSF-f2+LA**	235	206	69	35
L. f 5221	**SSF-f5**	192	86	39	**SSF-f5+LA**	455	192	86	39
L. f 8829	**SSF-f8**	130	0	0	**SSF-f8+LA**	193	130	0	0

Table 1: Composition of SCFA synthetic formulations (SSFs) containing different concentrations of acetate, propionate, and butyrate (no bacteria was used), designed at the respective concentrations of naturally produced SCFs in the cell culture conditioned media (CM) of *L. fermentum* NCIMB -5221, -2797, and -8829. A second set of SSF, containing lactic acid was prepared by the addition of the respective concentrations of lactic acid at the same concentrations produced by *L. fermentum*-CM. SSF-a: SCFA synthetic formulation corresponding to *L. acidophilus* ATCC 314; SSF-f7: SCFA synthetic formulation corresponding to SCFA concentrations produced by *L. fermentum* NCIMB 2797; SSF-f5: SCFA synthetic formulation corresponding to SCFAs concentrations produced by *L. fermentum* NCIMB 5221; and SSF-f8: SCFA synthetic formulation corresponding to SCFA concentrations produced by *L. fermentum* NCIMB 8829.

	LA and SCFAs in SIF (mg/L)		
	LA	**AA**	**PA**
***L. acidophilus* ATCC 314**	2000	400	0
***L. fermentum* NCIMB -2797 or -5221**	2500	650	650

Table 2: Levels of lactic, acetic, propionic and butyric acid produced by *L. acidophilus* ATCC 314, *L. fermentum* NCIMB -2797 and -5221 after 24 h incubation in SIF with *L. acidophilus* ATCC 314 as a control. The data is presented by the mean ± SEM (n=3).

these studies associated the potential anti-cancer activity of probiotic bacteria with the production of SCFAs; however, few have validated this theory [49]. In this study, *L. fermentum*-CM significantly inhibited CRC cell proliferation, in a time-dependent manner, compared with untreated cells and cells treated with *L. acidophilus* ATCC 314 ($p < 0.05$, Figure 3).

Linear regression analysis was applied to the percentage of Caco-2 cells inhibited by *L. fermentum*–CM and the concentrations of SCFAs produced by *L. fermentum* bacteria highlighting a strong correlation between them (Figures 4e and 4f). To identify potential factors other than SCFAs involved in this activity, concentrations of synthetic SCFAs prepared as a mixture were tested on CRC cells. Figure 4a demonstrates that artificially prepared doses of pure SCFAs have significantly less effect when compared with the probiotic bacterial extracts CM ($p < 0.01$). This fact supports the ability of a particular naturally produced SCFA to induce inhibitory effects (Figure 4). Overall, the synthetically prepared mixtures of SCFAs showed a closer effect to *L. fermentum*-CM (Figure 5b). More specifically, *L. fermentum* NCIMB 5221 had the same effect as its corresponding SCFA formulation. The *L. fermentum* NCIMB -2797 and -8829 significantly inhibited colon cancer cell growth less than the corresponding SCFAs synthetic formulations ($p < 0.05$), indicating that the bacteria have potentially secreted additional anti-cancer products. Nonetheless, *L. acidophilus* ATCC 314 was significantly less effective than its SCFA synthetic cocktail. This indicates the presence of other bacterial factors, produced in the CM, which hindered the effect of the naturally produced probiotic SCFAs. The data produced indicates that the anti-proliferative effect

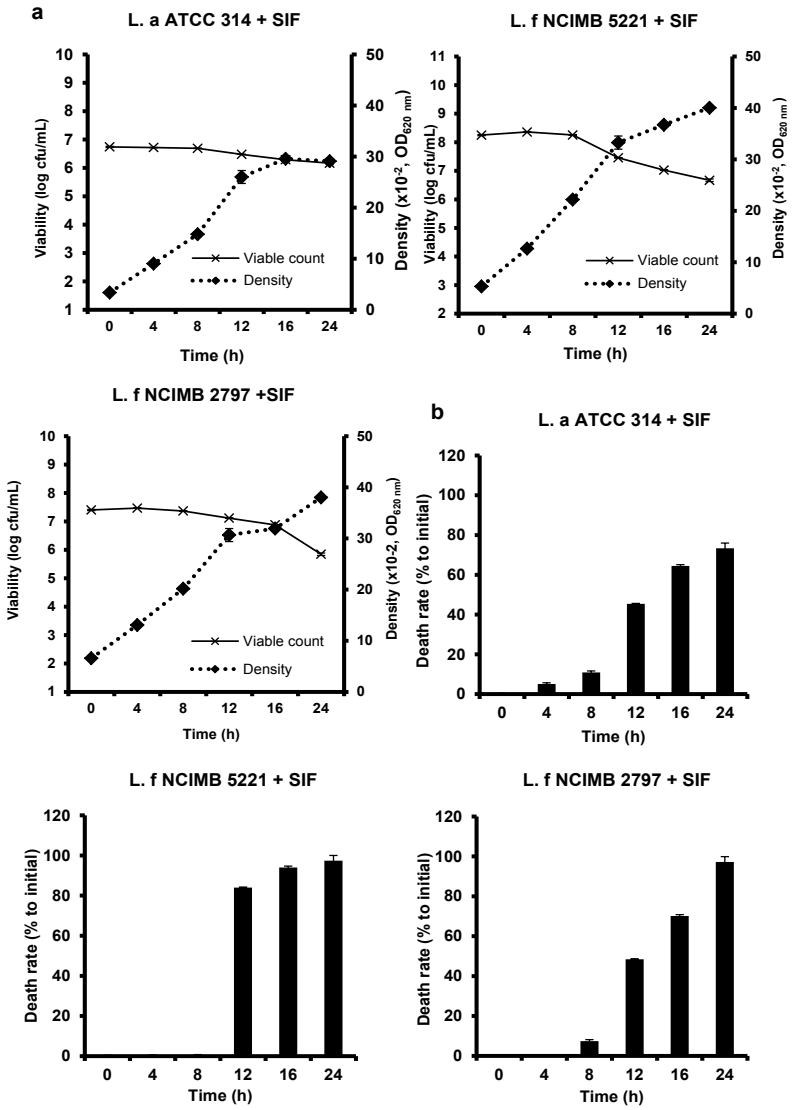

Figure 6: Characterization of *L. fermentum* bacterial cell resistance in SIF. **(a)** Bacterial cell culture characterization for *L. fermentum* strains in a simulated intestinal fluid (SIF), (pH = 6.8, 24 h). It was determined by bacterial viable cell count and cell culture absorbance of *L. fermentum* NCIMB -5221, -2797, and -8829, in addition to *L. acidophilus* ATCC 314 used as a control. **(b)** Death rate of *L. fermentum* bacteria in a SIF (pH = 6.8, 24 h). The death rate in all bacteria showed a transition at 8 h. The SIF used contained glucose (5.5 g/L), yeast extract (3.5 g/L), pancreatin (2 g/L), oxgall (1.5 g/L), pectin (2 g/L), inulin (0.54 g/L), fructooligosaccharides (0.85 g/L), starch (3 g/L), and monobasic potassium phosphate (KH_2PO_4, 3.3 g/L). Data are presented as the mean ± SEM (n = 3).

of the CM is possibly due, in a minor part, to the concentration of bacterial SCFAs; however the effect is not solely related to the presence of SCFAs. As described in Table 1, lactic acid was added to each SCFA synthetic formulation. These lactic acid-containing SCFA mixtures had significantly less effect than either SCFA synthetic formulation or probiotic CM ($p < 0.001$). This implies that the presence of lactic acid may have reduced the efficacy of SCFAs on the metabolism of cancer cells. This is supported by a study where L-lactate significantly inhibited uptake of butyrate in cancer cells [41], suppressing the anti-cancer effect of the latter. Hence, the lactate, added later to the SSFs, could have suppressed the ability of cancer cell to uptake SCFAs resulting in the decreased action of SSF containing lactate. Some of the bacterial products released by *L. fermentum* bacteria were indicated as surface [50] and adhesive [51] proteins that bind to the intestinal and gastric mucus as DNA fragments, or lipopolysaccharides [52]. As explained, the anti-proliferative effect of *L. fermentum* may not only be based on the activity of SCFAs but also on the release of other bacterial products that may have preserved or enhanced the effect of SCFAs.

Another feature related to probiotic strain selection was the loss of viability of *L. fermentum* bacteria in simulated human intestinal conditions as well as the ability to produce SCFAs. Interestingly, *L. fermentum* NCIMB -5221 and -8829, which exhibited higher anti-colon cancer potential, showed similar densities /absorbances (Figure 6a) and resistance to the bile exposure for 4 h, which was significantly higher than for *L. acidophilus* ATCC 3 ($p < 0.05$, Figure 6b). Some studies have shown that *L. fermentum* have resistance to gut conditions; however, this feature varied according to the glucose and other nutrient availability in the gut. *L. fermentum* tolerance to intestinal conditions was observed, mainly, for a maximum of 4 h, compared with other probiotic bacteria [53]. Between 12 h and 16 h,

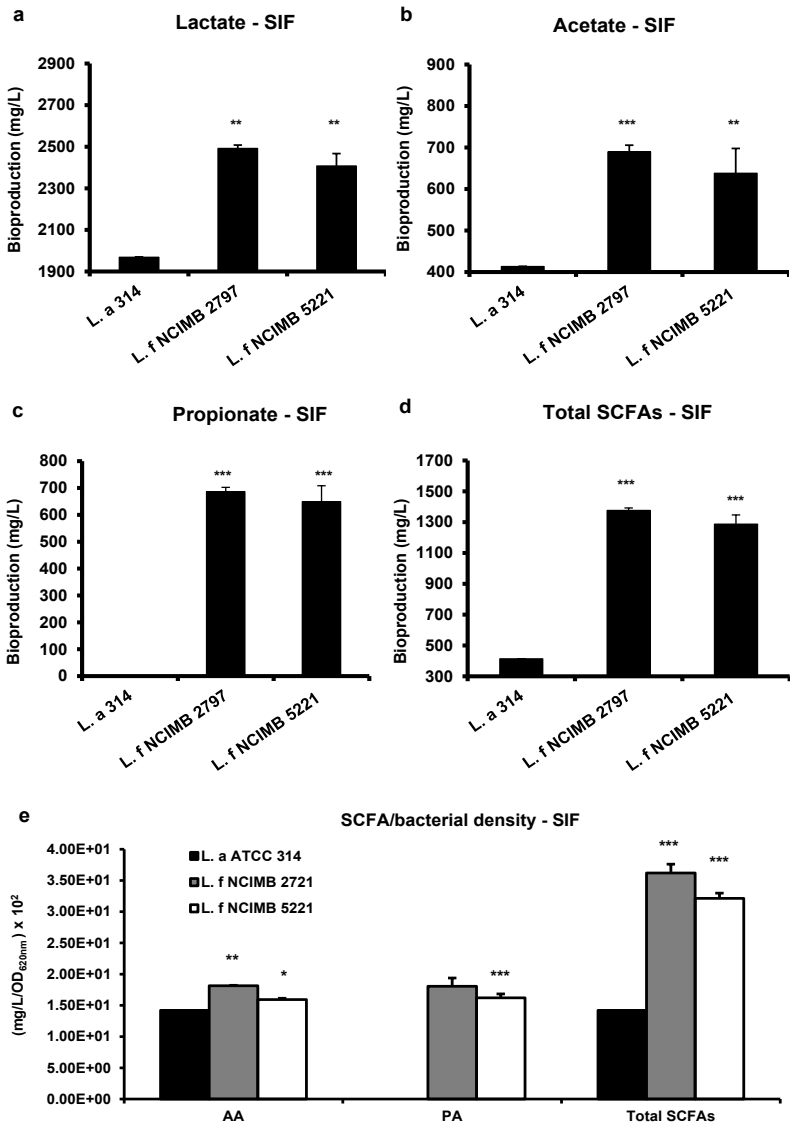

Figure 7: Quantification of the lactic acid/SCFAs produced by *L. fermentum* strains in SIF. **(a)** Lactic, **(b)** acetic, **(c)** propionic acids and **(d)** total SCFAs produced by *L. fermentum* NCIMB -2797 and -5221 were measured in a simulated intestinal fluid (SIF, 24 h, pH=8.6). **(e)** Comparison of SCFAs production in SIF depending on the bacterial culture density of *L. fermentum* NCIMB -2797 and -5221 with *L. acidophilus* ATCC 314 (mg/L/OD_{620nm} x 10^2). The SIF was prepared by mixing glucose (5.5 g/L), yeast extract (3.5 g/L), pancreatin (2 g/L), oxgall (1.5 g/L), pectin (2 g/L), inulin (0.54 g/L), fructooligosaccharides (0.85 g/L), starch (3 g/L), and monobasic potassium phosphate (KH_2PO_4, 3.3 g/L). *L. acidophilus* ATCC 314 is used as a positive control (n=3). *L. acidophilus* ATCC 314 is used as a control. Data are presented as mean ± SEM (n = 3). *p < 0.05, **p < 0.01 and ***p < 0.005 compared with *L. acidophilus* ATCC 314.

L. fermentum NCIMB 2797 had a significantly lower death rate than *L. fermentum* NCIMB 5221. Furthermore, at 24 h, *L. fermentum* bacteria were still viable at log 6 - 7, strongly suggesting the ability to stay viable in an intestinal environment. Although *L. fermentum* NCIMB -5221 and -8829 displayed significantly less viability (24 h), compared with *L. acidophilus* ATCC 314 in SIF ($p < 0.05$), they were both able to produce significantly higher concentrations of lactate (Figure 7a), acetate (Figure 7b), propionate (Figure 7c), and total SCFAs (Figure 7d) than *L. acidophilus* ATCC 314 (Figure 7, $p < 0.01$). Moreover, SCFA concentrations per bacterial density were significantly higher for *L. fermentum* NCIMB -5221 and -8829 compared with *L. acidophilus* ATCC 314 ($p < 0.05$, Figure 7e). This data implied that *L. fermentum* bacterial cells are more active and have the potential to produce efficiently higher concentrations of anti-cancer bioactive compounds than *L. acidophilus* ATCC 314. Testing those concentrations separately on CRC cells (Figure 7) [54] confirms this finding. The levels of SCFAs produced by *L. fermentum* bacteria in SIF were shown to significantly reduce CRC cell proliferation, compared with *L. acidophilus* ATCC 314, in adherence with the superior inhibitory effect of the *L. fermentum* cell-free extract described in Figure 3. Notably, the only SCFA *L. acidophilus* ATCC 314 that did not produce detectable levels was propionate (Figure 2b). Nevertheless, the propionic acid concentration produced in the SIF seemed significantly more effective in decreasing the Caco-2 viability than acetic acid SIF concentrations ($p < 0.001$, Figure 8a), suggesting that propionate production is a major mechanism for colon cancer inhibition by *L. fermentum* in the intestinal environment.

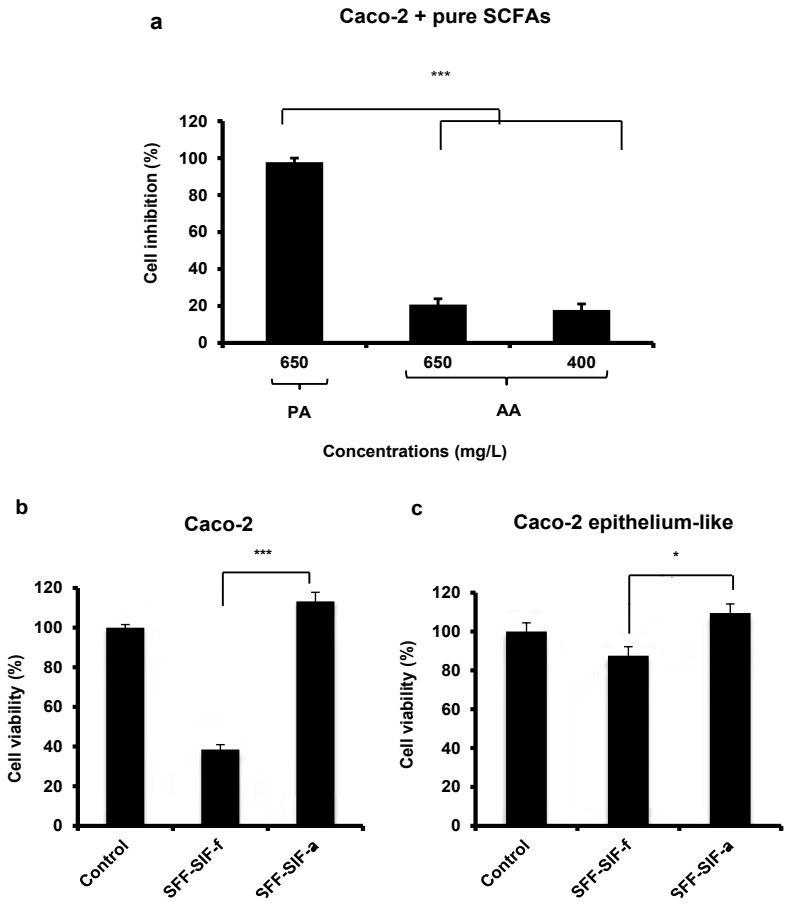

Figure 8: Confirmation of the efficacy of SCFAs produced in SIF, by *L. fermentum*. **(a)** The inhibitory effect of propionic and acetic acids produced by *L. fermentum* in SIF was described. The effect of the SCFA synthetic formulations (SSF-SIF-a and SFF-SIF-f) against CRC cells **(b)** cell culture, and **(c)** epithelium-like cell culture. SSF-SIF-a and SFF-SIF-f represented synthetic mixtures of SCFAs that have the same composition as the probiotic SCFAs naturally produced in SIF by *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB -5221 and -2797, respectively (Table 2). Data are presented as mean ± SEM (n = 5). *p < 0.05 and ***p < 0.005, compared with control or *L. acidophilus* ATCC 314. SSF-SIF-f: formulation of SCFAs produced in SIF corresponding to both L. fermentum bacteria (NCIMB -5221 and -2797); SSF-SIF-a: SCFA formulation of SCFAs produced in SIF by *L. acidophilus* ATCC 314.

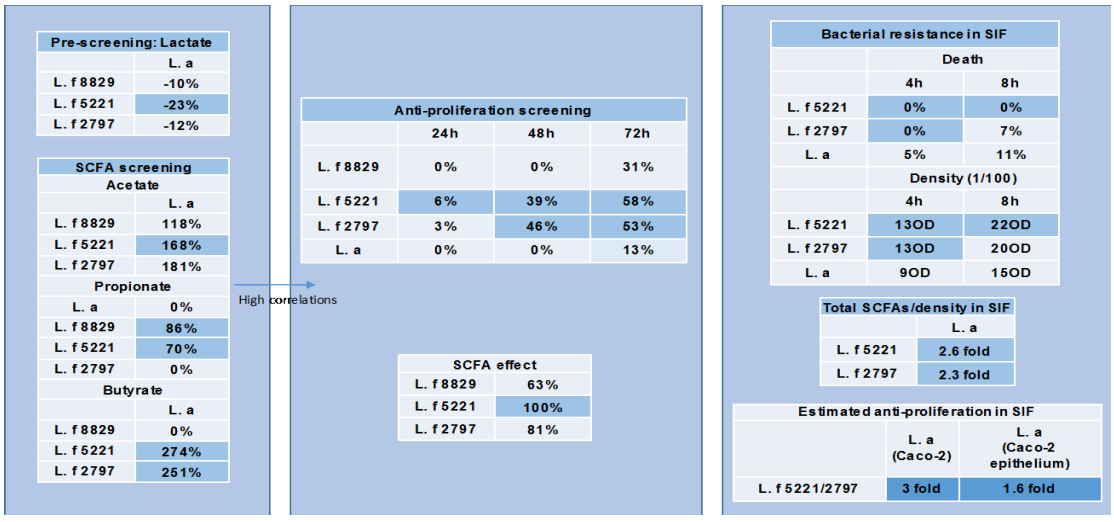

Figure 9: Overview of *L. fermentum* strain screening and relevance depending on growth, metabolic, and anti-CRC proliferative criteria.

Conclusion

This present study is the first to explore and compare the potential suitability of *L. fermentum* NCIMB -5221, -2797, and -8829 as CRC biotherapeutics *in vitro* (Figure 9). These strains were characterized for their production of active molecules relevant to CRC and their tolerance to intestinal stress. They also exhibit the production of SCFAs in different environments (supernatant CM or intestinal fluid SIF) and the suppression of CRC cell growth. We were able to compare the anti-proliferative effect of *L. fermentum* probiotic bacterial strains *in vitro* while evaluating the efficacy of SCFAs bioproduction as a mechanism. Our findings identified a significant effect of *L. fermentum* strains in inhibiting colon cancer cells which correlate with the ability of these bacteria to produce SCFAs. These strains also showed significant efficiency in producing SCFAs in intestinal conditions, suggesting an ability to generate an appreciable anti-carcinogenic effect in the colon.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

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