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

Imen Kahouli1,2, Meenakshi Malhotra1, Catherine Tomaro-Duchesneau1, Laëtitia Sonia Rodes1, Moulay A. Alaoui-Jamali2,3 and Satya Prakash1,2*

1Biomedical Technology and Cell Therapy Research Laboratory-Departments of Biomedical Engineering, Physiology, and Artificial Cells and Organs Research Center, Faculty of Medicine, McGill University, Canada
2Department of Experimental Medicine, Faculty of Medicine, McGill University, Canada
3Departments of Medicine and Oncology, Faculty of Medicine, McGill University, Canada
4Lady Davis Institute for Medical Research and Segal Cancer Centre, Sir Mortimer B. Davis-Jewish General Hospital, Canada

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. plantarum [21].

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

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 CO2 incubator (37°C, 5% CO2) 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% CO2), 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% CO2) to reach a late exponential phase (~16 h). The bacterial cells were collected from the culture broth by centrifugation (1000 x g, 15 min, 4°C) and washed with PBS. This bacterial pellet (1 x 10^6 cells/mL) was incubated in DMEM for 2 hours (37°C, 5% CO2). The medium was also centrifuged (1000 x 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), pancreatein (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 Zorbax Rx-180 (8%) column (150 mm x 7.80 mm, Phenomenex, Torrance, CA, USA) equipped with a UV-vis detector and diode array detector (DAD, 210 ± 5 nm). Growth and viability of bacterial cells were determined according to the manufacturer’s protocol [34]. After 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 would determine 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 OD530nm (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 CO2 incubator (37°C, 5% CO2) 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% CO2), before experiments. During assays, cell culture medium was substituted by probiotic conditioned medium (CM) mixed with serum and antibiotic-free media (DMEM + 10% FBS). 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 x 10^3 cells/well onto 96-well culture plates and left to attach for 24 - 48 h for the formation of an epithelial-like monolayer (37°C, 5% CO2). Caco-2 cells were incubated with the probiotic treatments for 24, 48 and 72 h, (37°C, 5% CO2, 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 (OD600nm) and viable bacterial cell count in SIF. The bacterial supernatant was collected by centrifugation (1000 × g, 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% CO2, 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 2797 and -8829. All L. fermentum strains produced significantly less lactic acid than L. acidophilus ATCC 314 (1900.0 ± 2100.0 mg/L, 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 acids.
(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, **p < 0.01 and ***p < 0.001, compared with L. acidophilus ATCC 314).

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 5a). 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 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_ NCIBM -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 8 and 16 h, _L. fermentum_ NCIBM -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_ NCIBM 2797 (70.11 ± 3.2% minimum) and _L. fermentum_ NCIBM 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
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 the production of lactic 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 acetic acid, 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 acetic acid 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 synthetic 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
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-cancer-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 < 0.005, 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<sup>min/+</sup> mouse 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 4](image-url)

**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.
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 | 39 | 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 SCFAs concentrations produced by L. fermentum NCIMB 8829.

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 corresponding SCFA formulation. The data produced indicates that the anti-proliferative effect...
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 \((p < 0.05, \text{ Figure 6b})\). 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 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.
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. J Bioanal Biomed 7: 104-115. doi:10.4172/1948-593X.1000132

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.

Acknowledgements

The authors would like to acknowledge a Canadian Institute of Health Research (CIHR) grant (MPO 64308) and grants from Micropharma Limited to Dr. Satya Prakash, a Fonds de Recherche du Québec–Sainte (FRSQ) Doctoral Awards and a Faculty of Medicine George G. Harris Fellowship to Imen Kohouli and Meneakshi Mahotra. We, also, thank the Analytical Laboratory Technicians of the Department of Chemical Engineering (McGill University), Andrew Golstajn and Ranjan Roy for their help with the analysis.

Conflicts of interest

The authors have no conflicts of interest to disclose.

References

1. Bishehsari F, Mahdavinia M, Vacca M, Malekzadeh R, Mariani-Costantini R (2014) Epidemiological transition of colorectal cancer in developing countries: environmental factors, molecular pathways, and opportunities for prevention. World J Gastroenterol 20: 6055-6072.
2. Altobelli E, Lattanzi A, Paduano R, Varassi G, di Orio F (2014) Colorectal cancer prevention in Europe: burden of disease and status of screening programs. Prev Med 62: 132-141.
3. Theodoratou E, Farrington SM, Tenesa A, Cetnarskyj R, et al. (2014) Associations between dietary and lifestyle risk factors and colorectal cancer in the Scottish population. Eur J Cancer Prev 23: 8-17.
4. Young PE, Womeldorf CM, Johnson EK, Maykel JA, Brucher B, et al. (2014) Early detection of colorectal cancer recurrence in patients undergoing surgery with curative intent: current status and challenges. J Cancer 5: 262-271.
5. Siegel R, Ma J, Zou Z, Jemal A (2014) Cancer statistics, 2014. CA Cancer J Clin 64: 9-29.
6. Chapman CG, Rubin DT (2014) The Potential for Medical Therapy to Reduce the Risk of Colorectal Cancer and Optimize Surveillance in inflammatory bowel disease. Gastrointest Endosc Clin N Am 24: 353-365.
7. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA Cancer J Clin 63: 1-30.
8. Birt DF, Phillips GJ (2014) Diet, genes, and microbes: complexities of colon cancer prevention. Toxicol Pathol 42: 182-188.
9. Gardiner GE, Heinemann C, Baroja ML, Bruce AW, Beuerman D, et al. (2002) Oral administration of the probiotic combination Lactobacillus rhamnosus GR-1 and *L. fermentum* RC-14 for human intestinal applications. International Dairy Journal 11: 191-196.
10. Son Chu-Ky, Thi-Khanh Bui, Tien-Long Nguyen, Phu-Ha Ho (2014) Acid adaptation to improve viability and *X-prolyl dipeptidyl aminopeptidase* activity of the probiotic bacterium *Lactobacillus fermentum* HN6 exposed to simulated gastrointestinal tract conditions. International Journal of Food Science & Technology 49: 565-570.
11. Gardiner GE, Heinemann C, Baroja ML, Bruce AW, Beuerman D (2002) Persistence of *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 but not *L. rhamnosus* GG in the human vagina as demonstrated by randomly amplified polymorphic DNA. Clin Diag Lab Immunol 9: 92-96.
12. Maldonado J, Cañafate F, Sempere L, Vela F, Sánchez AR, et al. (2012) Human milk probiotic *Lactobacillus fermentum* CECT5716 reduces the incidence of gastrointestinal and upper respiratory tract infections in infants. J Pediatr Gastroenterol Nutr 54: 55-61.
13. Stolzer PO, Blomberg L, Conway PL, Henriksson A, Abrahamsson H (1996) Probiotic treatment of small intestinal bacterial overgrowth by *Lactobacillus fermentum* KLD. Scand J Infect Dis 28: 615-619.
14. Tomaro-Duchesneau C, Saha S, Mahotra M, Coussa-Charley M, Kohouli I, et al. (2012) Probiotic ferulic acid esterase active *Lactobacillus fermentum* NCIMB 5221 APA macropapules for oral delivery: preparation and in vitro characterization. Pharmaceuticals 5: 236-248.
15. Peran L, Camuesso D, Comalada M, Nieto A, Concha A, et al. (2006) *Lactobacillus fermentum*, a probiotic capable to release glutathione, prevents colonic inflammation in the TNBS model of rat colitis. Int J Colorectal Dis 21: 737-746.
16. Geier MS, Butler RN, Gifford PM, Howarth GS (2007) *Lactobacillus fermentum* BR1, a potential new probiotic, alleviates symptoms of colitis induced by dextran sulfate sodium (DSS) in rats. Int J Food Microbiol 114: 267-274.
17. Thirabunyanon M, Boonprasop S, Niarnsup P (2009) Probiotic potential of lactic acid bacteria isolated from fermented dairy milks on antiproliferation of colon cancer cells. Biotechnol Lett 31: 571-576.
18. Iida N, Dzutsue A, Stewart CA, Smith L, Bouladoux N, et al. (2013) Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. Science 342: 967-970.
19. Morita H, Toh H, Fukuda S, Horikawa H, Osakada K, et al. (2008) Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cohabalan production. DNA Res 15: 151-161.
20. Likotraifi E, Tuohy KM, Gibson GR, Rastall RA (2014) An in vitro study of the effects of probiotics, prebiotics and symbiotics on the elderly faecal microbiota. Anaerobe 27: 50-55.
21. Asha A, Gayathri D (2012) Synergistic impact of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *corynebacterium* on 2-Dimethylhydrazine-induced colorectal carcinogenesis in mice. Exp Ther Med 3: 1049-1054.
22. Zeng H, Lazarova DL, Bordonaro M (2014) Mechanisms linking dietary fiber, gut microbiota and colon cancer prevention. World J Gastroenterol Oncol 6: 41-51.
23. Puertollano E, Kolidis S, Yaqoob P (2014) Biological significance of short-chain fatty acid metabolism by the intestinal microbiome. Curr Opin Clin Nutr Metab Care 17: 139-144.
24. Casanova M (2015) Development of an improved propionibacterium for potential use as a nutraceutical towards the prevention-treatment of colorectal cancer.
25. Thirabunyanon M, Hongwittayakorn P (2013) Potential probiotic lactic acid bacteria of human origin induce antiproliferation of colon cancer cells via synergic actions in adhesion to cancer cells and short-chain fatty acid bioproduction. Appl Biochem Biotechnol 169: 511-525.
26. Saikali J, Picard C, Freitas M, Holt P (2004) Fermented milks, probiotic cultures, and colon cancer. Nutr Cancer 49: 14-24.
27. Kohouli I, Tomaro-Duchesneau C, Prakash S (2013) Probiotics in colorectal cancer (CRC) with emphasis on mechanisms of action and current perspectives. J Med Microbiol 62: 1107-1123.
28. Tomaro-Duchesneau C, Saha S, Mahotra M, Coussa-Charley M, Al-Salamy H, et al. (2012b) *Lactobacillus fermentum* NCIMB 5221 has a greater ferulic acid production compared to other ferulic acid esterase producing *Lactobacillus*. International Journal of Probiotics & Prebiotics 7: 23-32.
29. Tomaro-Duchesneau C, Jones ML, Shah D, Jain P, Saha S, et al. (2014) Cholesterol assimilation by *Lactobacillus* probiotic bacteria: an in vitro investigation. Biomed Res Int 2014: 380316.
30. Tomaro-Duchesneau C, Saha S, Mahotra M, Jones ML, Labbé A, et al. (2014) Effect of orally administered *L. fermentum* NCIMB 5221 on markers of metabolic syndrome: an in vivo analysis using ZDF rats. Appl Microbiol Biotechnol 98: 115-126.
31. Zhao Q, Mutukumira A, Lee SJ, Maddox I, Shu Q (2012) Functional properties of free and encapsulated *Lactobacillus reuteri* DPC16 during and after passage through a simulated gastrointestinal tract. World J Microbiol Biotechnol 28: 61-70.
32. Liong MT, Shah NP (2005) Optimization of cholesterol removal, growth and fermentation patterns of Lactobacillus acidophilus ATCC 4962 in the presence of mannitol, fructo-oligosaccharide and inulin: a response surface methodology approach. J Appl Microbiol 98: 1115-1126.

33. Dubey UK, Mistry VV (1996) Effect of bifidogenic factors on growth characteristics of bifidobacteria in infant formulas. J Dairy Sci 79: 1156-1163.

34. Andreotti PE, Cree IA, Kurbacher CM, Hartmann DM, Linder D, et al. (1995) Chemosensitivity testing of human tumors using a microplate adenosine triphosphate luminescence assay: clinical correlation for cisplatin resistance of ovarian carcinoma. Cancer Res 55: 5276-5292.

35. Ledford H (2010) ‘Biosimilar’ drugs poised to penetrate market. Nature 468: 58-10.

36. Odegard AO, Koh WP, Yuan JM (2013) Combined lifestyle factors and risk of incident colorectal cancer in a Chinese population. Cancer Prev Res (Phila) 6: 360-367.

37. Erdrich J, Zhang X, Giovannucci E, Willett W (2015) Proportion of colon cancer attributable to lifestyle in a cohort of US women. Cancer Causes Control.

38. Shmuely H, Domniz N, Cohen D (2013) Probiotics in the prevention of colorectal cancer. Curr Colorectal Cancer Reports 9: 31-36.

39. Tomaro-Duchesneau C, Saha S, Malhotra M, Coussa-Charley M, Al-Salami H, et al. (2012a) Lactobacillus fermentum NCIMB 5221 has a greater ferulic acid production compared to other ferulic acid esterase producing Lactobacillus. Int J probiotics prebiotics 7: 23-32.

40. Tomaro-Duchesneau C, Saha S, Malhotra M, Coussa-Charley M, Al-Salami H, et al. (2012c) Lactobacillus fermentum NCIMB 5221 has a greater potential for the production of ferulic acid when compared to other ferulic acid esterase active Lactobacillus. International Journal of Probiotics and Prebiotics 7: 23-32.

41. De Vuyst L, Leroy F (2011) Cross-feeding between bifidobacteria and butyrate-producing colon bacteria explains bifidobacterial competitiveness, butyrate production, and gas production. International journal of food microbiology 149: 73-80.

42. Yang G, Xu H, Goldsmith J, Kelly C, Chen X (2012) Identification of Lactic Acid From Probiotic Yeast as an Anti-Cancer and Anti-Inflammatory Component. P-188 YI. Inflammatory Bowel Diseases 18: S90-S91.

43. Ogawa M, Shimizu K, Nomoto K, Tanaka R, Hamabata T, et al. (2001) Inhibition of in vitro growth of Shiga toxin-producing Escherichia coli O157:H7 by probiotic Lactobacillus strains due to production of lactic acid. Int J Food Microbiol 68: 135-140.

44. Basson MD, Emenaker NJ, Hong F (1998) Differential modulation of human (Caco-2) colon cancer cell line phenotype by short chain fatty acids. Proc Soc Exp Biol Med 217: 476-483.

45. Ohigashi S, Sudo K, Kobayashi D, Takahashi D, Takahashi T, 2013. Changes of the Intestinal Microbiota, Short Chain Fatty Acids, and Fecal pH in Patients with Colorectal Cancer. Dig Dis Sci 58: 1717-1726.

46. Rojas M, Ascencio F, Conway PL (2002) Purification and characterization of a surface protein from Lactobacillus fermentum 104R that binds to porcine small intestinal mucus and gastric mucin. Appl Environ Microbiol 68: 2330-2336.

47. Henriksson A, Szewzyk R, Conway PL (1991) Characteristics of the adhesive determinants of Lactobacillus fermentum 104. Appl Environ Microbiol 57: 499-502.

48. Jin B, Sun T, Yu XH, Yang YX, Yeo AE (2012) The effects of TLR activation on T-cell development and differentiation. Clin Dev Immunol 2012: 836485.

49. Charalampopoulos D, Pandiella SS, Webb C (2003) Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. Int J Food Microbiol 80: 1-10.

50. Bultman SJ (2014) Molecular pathways: gene-environment interactions regulating dietary fiber induction of proliferation and apoptosis via butyrate for cancer prevention. Clin Cancer Res 20: 799-803.