Screening and In-Vitro Analysis of Lactobacillus reuteri Strains for Short Chain Fatty Acids Production, Stability and Therapeutic Potentials in Colorectal Cancer

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Abstract

The use of probiotics as preventive agents in colorectal cancer is widely reported in the literature. However, the bioactivity of specific bacterial strains is only partially understood. Here, we identified Lactobacillus reuteri strains with anti-proliferative activity against colorectal cancer cells. We investigated the bioavailability and the efficacy of short chain fatty acids secreted by distinct Lactobacillus reuteri strains on the inhibition of colorectal cancer cells growth. Five L. reuteri strains were screened based on the short chain fatty acids bio-production and anti-proliferative effects on Caco-2 colon cancer cells. The composition of probiotic short chain fatty acids in cell culture conditioned medium was used to prepare short chain fatty acid synthetic formulations that were compared with L. reuteri cell culture conditioned medium. Later, the bio-stability of the bacteria in a simulated intestinal fluid was determined. Results showed that the production of short chain fatty acids was strain-dependent. L. reuteri NCIMB -11951, -701359 and -702656 were the most potent in producing total short chain fatty acids (402.2 ± 23.5, 3, p < 0.05 compared with the rest of strains) and inhibiting Caco-2 (by 56.7 ± 1.6 % compared to untreated cells at 72 h, p < 0.001). Comparing the inhibitory effect of the probiotic cell culture conditioned medium and the corresponding short chain fatty acid synthetic formulation showed that the role and relevance of short chain fatty acid production in colorectal cancer growth suppression was strain-dependent. L. reuteri NCIMB -702656 and -701359 showed resistance in simulated intestinal fluid (104.6 ± 0.6 % and 105.7 ± 4.1 % of viability at 4 h, respectively) and produced high amounts of total short chain fatty acids (1245.49 ± 0.49 - 1391.58 ± 4.84 mg/L at 24 h, respectively). Depending partly on short chain fatty acid bio-production, specific L. reuteri strains demonstrated growth inhibitory activity and may be considered as a potential chemopreventive agent against colorectal cancer.

Keywords: Biotherapeutic; Probiotics; Colorectal cancer; Short chain fatty acids, Cell proliferation, Intestinal fluid

Abbreviations: CRC: colorectal cancer; SCFA: short chain fatty acid; GI: gastrointestinal; L: Lactobacillus; LAB: Lactic acid bacteria; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; SIF: Simulated intestinal fluid; LA: Lactic acid; AA: Acetic acid; PA: Propionic acid; BA: Butyric acid; CFU: Colony-forming unit; OD: Optical density; CM: Conditioned cell culture medium; TNF: Tumor necrosis factor; L. reuteri-CM: Conditioned cell culture medium of L. reuteri bacteria. L. a 314: L. acidophilus ATCC 314; L r 11951: L. reuteri NCIMB 11951; L. r 701089: L. reuteri NCIMB 701089; L. r 701359: L. reuteri NCIMB 701359; L. r 702655: L. reuteri NCIMB 702655; L. r 702656: L. reuteri NCIMB 702656

Introduction

Colorectal cancer (CRC) is among the leading causes of cancer mortality worldwide, yet dietary intervention represents a valuable approach to prevent CRC development, particularly in susceptible human populations [1,2]. Several chemopreventive and biotherapeutic approaches have been reported for the prevention of CRC and other gut conditions [3,4]. Individuals with inherent gene defects that predispose to CRC, inflammatory bowel disease (IBD) and ulcerative colitis (UC) have been suggested to benefit from the consumption of probiotics. Millions of healthy people and patients with such conditions, who are at high risk of CRC, consumes probiotics as neurectheatical products [5,6]. Probiotics, defined as beneficial bacteria, have been proposed to balance disturbed gastrointestinal (GI) microflora and dysfunctions of the human GI tract [7]. Lactic acid bacteria (LAB) are predominantly reported to excrete components with protective properties against colon cancer causing-factors. They can release anti-carcinogenic compounds and, promote balanced bacterial growth in the colon to produce greater quantities of short chain fatty acids (SCFAs) such as acetate, propionate and butyrate, all which have anti-cancer properties [8]. Although several studies have reported the anti-proliferative or pro-apoptotic effect of probiotic bacteria on colon carcinoma cells [9-11], no systemic studies have been reported that screen or characterize...
certain LAB as potential candidates for CRC biotherapies such as *L. reuteri* bacteria, investigated for anti-pathogenic activity and capacity to produce conjugated linoleic acid [12-15]. *L. reuteri* is prominent among the *Lactobacillus* population in the GI ecosystem [16], and has been widely reported to be beneficial for some GI conditions such as UC, which is a strong risk indicator for CRC [17]. Other cases included constipation [18], diarrhea [19], maintaining mucosal barrier [20] and colon motility [21]. The biological activity of *L. reuteri* has been shown to be mediated in part by the production lactic acid and bacteriocins, which potentially influence the commensal micro-organisms [22, 23] and reduce intestinal absorption of endogenous and exogenous carcinogens [15]. According to the most current probiotic selection criteria, these bacteria have to stay active, withstand the intestinal environment and exert beneficial effects, once reaching the intestines.

The goal of this study was to screen five strains of *L. reuteri*, for Human epithelial CRC production of SCFAs and to assess their anti-proliferative effects of these SCFAs on colon cancer cells. Then, for the most potent candidates, the stability of the probiotic bacteria and the bio-production of lactic, acetic, propionic and butyric acids in a simulated intestinal fluid (SIF) were investigated. Within this study, to determine why *L. reuteri* bacteria suppressed colon cancer cells *in-vitro*, the role of SCFAs was simulated and a correlation between colon cancer cell growth inhibition and the concentrations of naturally produced SCFAs was established. Later, concentrations of SCFAs similar to the ones produced by *L. reuteri* bacteria were tested separately in formulations on colon cancer cells. For each *L. reuteri* strains, the SCFAs produced were quantified and those numbers were used to prepare similar SCFA synthetic formulations. If a SCFA synthetic formulation was found to inhibit cancer cells less than the corresponding *L. reuteri* CM, then this will show that the SCFAs may not be the only anti-cancer compounds produced by the bacteria and that there are probably other bacterial molecules excreted with anti-proliferative activity against colon cancer cells. However, if the SCFA synthetic formulation suppressed colon cancer cells equally or more than the *L. reuteri* bacteria then this would suggest that the levels of SCFAs of *L. reuteri* bacteria were mostly responsible for the anti-proliferative effect.

**Materials and Methods**

**Materials**

De Man, Rogosa, Sharpe (MRS) broth and agar were obtained from Fisher Scientific (Ottawa, ON, Canada). Eagle’s Minimum Essential Medium (EMEM), Dulbecco’s Modified Eagle’s Medium (DMEM), phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Invitrogen. Water was purified with an EasyPure reverse osmosis system and a NanoPure Diamond Life Science (UV/UO) ultrapure water system from Barnstead (Dubuque, IA, USA). Sodium L-Lactate, propionate, acetate, and butyrate were purchased from Sigma (St. Louis, MO, USA).

**Bacterial cells:** The bacterial strain of *L. acidophilus* ATCC 314 was purchased from Cedarlane Laboratories (Burlington, ON, Canada) and used as a positive control and for comparative purposes. Five *L. reuteri* strains: (*L. reuteri* NCIMB -11951, -701359, -701089, -702655, and -702656) were purchased from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland, UK). Bacterial cultures were maintained by continuous in MRS media at 1% (v/v) and growth was monitored by both OD at a wavelength of 620 nm (Perkin Elmer 1420 Multilabel Counter, USA) and colony counting.

**Mammalian cells:** Human epithelial colorectal cancer Mammalian cells: Human epithelial colorectal cancer: Human epithelial colorectal adenocarcinoma cell line Caco-2 (HTB-37) was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Eagle’s Minimum Essential Medium (EMEM) supplemented with 20% of fetal bovine serum (FBS). Cells were incubated in a CO₂ incubator at 37°C in air supplemented with 5% CO₂ for about two weeks till fully differentiated. For proliferation assays, all cells were left to attach for 24-48 h in 96-well plates before experiments, till they reached 50-60 % confluence. At this point, cell medium was replaced by probiotic conditioned medium (CM) diluted with serum/antibiotic-free DMEM.

**Preparation of probiotic treatments:** The probiotic conditioned medium (CM), a bacterial cell free extract, was prepared with slight modifications from protocols adapted from Grabig et al. [24] and Kim et al., [25]. First, *L. reuteri* and *L. acidophilus* bacteria were incubated in *Lactobacillus* MRS broth at 37°C in air supplemented with 5% CO₂ for 24 h and subcultured three times at 1% (v/v). At the third passage, the bacteria were incubated at 37°C to reach late exponential phase (14-16 h). Second, the bacteria were precipitated from the MRS medium by centrifugation (1000 x g, 15 min, 4°C) and washed twice with PBS. Finally, the probiotic conditioned cell culture medium (CM) was produced by incubating washed probiotic cells (10⁻¹⁰⁹ cfu/ml) in DMEM cell culture medium at 37°C for 2 h. The medium was centrifuged twice (1000 x g, 15 min, 4°C) and then sterile-filtered (0.2 μM-pore-size filter, Millipore). Prior to the treatment on the colon cancer cells, the probiotic CM of each bacterium was mixed with fresh DMEM at a ratio of 1:2 and the pH was adjusted to 7 using 2M NaOH and 2M HCl.

**Quantification of lactic acid and SCFAs:** SCFAs produced by the *L. reuteri* strains were measured during the growth of bacteria in SIF and after the preparation of the corresponding probiotic CM. SCFAs were separated using a HPLC method adapted from Dubey and Mistry, with modifications [26,27]. A Model 1050 UV HPLC system (Hewlett-Packard HP1050 series, Agilent Technologies, USA), equipped with a UV-vis detector and diode array detector (DAD) set at 210 ± 5 nm, was used. 100 μl of sample was injected through an autosampler. A prepacked Rezex ROA-organic acid H⁺ (8%) (150 x 7.80 mm, Phenomenex, Torrance, CA, USA) fitted with an ion exclusion microguard refill cartridge was used. Data were acquired using ChemStation supported with LC3D software Rev A.03.02 (Agilent Technologies, CO, USA). The mobile phase (A) 0.05 M H₂SO₄ (very polar) and the mobile phase (B) of acetonitrile (2%) were used with an isocratic gradient pumped at a flow rate of 0.7-0.8 mL/min, through a column heated to 35°C. Lactic, acetic, propionic, and butyric acids were used to prepare a standard solution at concentrations of 1, 10, 100, 500, 1000 ppm (in triplicate) to generate the standard curve. The amounts of SCFAs were calculated using the linear regression equations (R²>0.99) from the corresponding standard curves.

**Assessment of cancer cell proliferation:** The proliferation of colon cancer cells treated with the probiotic treatments was evaluated using an A595 bioluminescence assay (CellTitre-Glo Luminescent Cell Viability Assay, Promega, USA). Caco-2 cells were seeded into 96-well culture plates at 5 x 10⁴ cells per well and stabilized for 24 h (37°C, 5% CO₂). After exposure to *L. reuteri* probiotic treatments for 24 h, 48 h, and 72 h, cell viability was determined following the guidelines from the manufacturer [28]. After incubating the cells with the probiotic treatment, the plate and its contents were equilibrated at room temperature (RT) for approximately 30 min. 100 μl of luminescent reagent was added to the an equal volume of the cell culture medium present in each well. The contents of the 96-well plate
were mixed for 2 minutes on an orbital shaker (200 rpm) to induce cell lysis. Afterwards, the plate was allowed to incubate at RT for 10 min to stabilize the luminescent signal, and the data was recorded using a spectrophotometer (Perkin Elmer, Victor 3, multi-label microplate reader, MA, USA).

**Preparation of SIF:** To determine the potential of *L. reuteri* bacteria in surviving intestinal conditions, a simulated intestinal fluid (SIF) was prepared as described previously by Qian Zhao et al. [29], with some modifications. In brief, the solution of SIF 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$_2$PO$_4$, 3.3 g/L) dissolved in deionized water. The pH was adjusted to 6.8 using 2M NaOH and 2M HCL, the solution was autoclaved at 120ºC for 15 min and cooled at RT before use.

**Determination of Lactobacilli stability in SIF:** The bacterial pellet was separated from a 16-24 h MRS-bacterial culture by centrifugation (1000xg, /10-15 min, 4ºC) and washed twice with a NaCl solution of 0.85% (w/v). 3% of the bacterial suspension was used to inoculate 15 mL of SIF solution, which was sealed and incubated micro-anaerobically. *Lactobacillus* cultures were incubated for 24 h in triplicate. At each time point (0 h, 4 h, 8 h, 12 h, 16 h and 24 h), the bacterial density (OD$_{620}$ nm) was measured, bacterial viability (colony counting on agar plates) was estimated, a supernatant was filtered (5 µm), and stored at -80ºC until further use.

**Efficacy and role of SCFAs:** This method was used to determine the role of the levels of naturally produced SCFAs in the inhibition of colorectal cancer cells, probiotic SCFAs produced by *L. reuteri* bacteria were compared with SCFA synthetic formulations, made at the same concentrations. Thus, the anti-proliferative effect of bioactive compounds, such as lactic acid and the SCFAs (acetic, propionic and butyric acids), produced by *Lactobacillus* bacteria in different media was measured. The concentrations of SCFAs were determined for each *Lactobacillus* CM, then formulations containing the same composition in lactic acid and the SCFAs were prepared and added to the culture media of colon cancer cells for 72 h. Viability of the colon cancer cells was determined using an ATP bioluminescence assay. This analysis determined the inhibitory effects of SCFAs on colon cancer growth in comparison with the *Lactobacillus* cell free extracts.

**Statistical analysis:** Data are presented as means ± Standard Error of the Mean (SEM) of replicates. Correlations were determined using Pearson correlation method. Statistical significance was generated for the treated groups as compared with each other by means of the one-way analysis of variances (ANOVA) with the Tukey’s comparison test and student’s t-test. SPSS statistics software package was used (version 20.0, IBM corporation, New York, NY, USA). *P*-value of *p* < 0.05 were considered significant.

**Results**

**L. reuteri does produce lactic acid in a conditioned cell culture media (CM)**

This experiment was designed to screen these LAB for their ability to produce lactic acid in DMEM media challenged with bacterial cells. As observed in Fig. 1, lactic acid produced by five *L. reuteri* strains was quantified and the data shows that *L. reuteri* NCIMB 702656 (642.5 ± 9.3 mg/L) and *L. reuteri* NCIMB 701359 (643.1 ± 9.3 mg/L) produced significantly higher amounts of lactic acid compared with all strains (*p* < 0.001), followed by *L. reuteri* NCIMB 1195, which produced 369.1 ± 9.3 mg/L of lactic acid. It was observed that *L. reuteri* NCIMB 701359 (643.1 ± 9.3 mg/L) and *L. reuteri* NCIMB 702656 (642.5 ± 9.3 mg/L) produced significantly less lactic acid (*p* < 0.001).

**The production of SCFAs by L. reuteri is strain-dependent**

To determine the SCFA bio-production ability of *L. reuteri* bacteria in CM, acetate, propionate, and butyrate produced by the bacteria were
quantified. For acetic acid production (Fig. 2 (a)), the \textit{L. reuteri} NCIMB 701089 (43.4 ± 3.3 mg/L) produced the least amount of acetic acid, whereas, \textit{L. reuteri} NCIMB 702656 (182.1 ± 15.4 mg/L) produced the highest amount (p < 0.01) followed by \textit{L. reuteri} NCIMB 11951 (131.2 ± 4.8 mg/L, p < 0.05) and then \textit{L. reuteri} NCIMB 701359 (116.0 ± 4 mg/L). For the production of propionic acid (Fig. 2 (b)), \textit{L. reuteri} NCIMB 701089 (38.7 ± 4 mg/L) and \textit{L. reuteri} NCIMB 702655 (45.5 ± 6.4 mg/L) produced the least amount among the \textit{L. reuteri} strains (p < 0.05). The highest amount of propionic acid was produced by \textit{L. reuteri} NCIMB 702656 (161.4 ± 3 mg/L, p < 0.01) followed by \textit{L. reuteri} NCIMB 11951 (111.2 ± 5.5 mg/L, p < 0.05) and \textit{L. reuteri} NCIMB 701359 (78.5 ± 10.9 mg/L, p < 0.05). For butyric acid production (Fig. 2 (c)), \textit{L. reuteri} NCIMB 702656 (58.7 ± 9.1 mg/L) was significantly better than all other bacteria (p < 0.001). Moreover, \textit{L. reuteri} NCIMB 11951 (28.6 ± 4 mg/L) and \textit{L. reuteri} NCIMB 701359 (27.6 ± 4.2 mg/L) significantly produced more butyrate compared with \textit{L. reuteri} NCIMB 701089 (no butyrate detected, p = 0.001). Finally, \textit{L. reuteri} NCIMB 702656 (402.21 ± 40.7 mg/L), \textit{L. reuteri} NCIMB 701359 (222.07 ± 27.04 mg/L), and \textit{L. reuteri} NCIMB 11951 (271.03 ± 5.2 mg/L) produced significantly more total probiotic SCFAs than \textit{L. reuteri} NCIMB 702655 (160.87 ± 20.4 mg/L, p < 0.001, p = 0.0064 and p = 0.002, respectively) and \textit{L. reuteri} NCIMB 701089 (82.12 ± 5.4 mg/L, p < 0.001, Fig. 2(c)).

Identification of \textit{L. reuteri} strains that suppressed colon cancer cell growth

Screening \textit{L. reuteri} strains based on the inhibitory effect on Caco-2 colon cancer cells was performed using the corresponding probiotic CM at a ratio of 1:2 at different time points (Fig. 3). At 24 h, 48 h and 72 h, the luminescence-based cell viability was determined. At 24 h (Fig. 3 (a)), \textit{L. reuteri} NCIMB -701359 and -702656 inhibited cancer cell growth by 19.5 ± 2.22 % and 4.78 ± 1.3 %, compared with the untreated cells, respectively. For 48 h of treatment (Fig. 3 (c)), \textit{L. reuteri} NCIMB -11951, -701089, -701359, and -702656 inhibited colon cancer growth by 32.02 ± 0.97 % (p < 0.001), 4.71 ± 0.3 % (p < 0.05), 47.76 ± 0.69 %, 42.78 ± 1.08 % (p < 0.001), respectively, compared with untreated cells. As observed, at 72 h post treatment (Fig. 2 (c)), the inhibition of colon cancer cells was best achieved with the CM of \textit{L. reuteri} NCIMB 702656 (56.68 ± 1.61 %), \textit{L. reuteri} NCIMB 701359 (55.58 ± 2.18 %, p < 0.001) and \textit{L. reuteri} NCIMB 701089 (6.43 ± 0.7 %, p < 0.001).

SCFAs produced by \textit{L. reuteri} may be responsible of their inhibitory effect

To verify whether the inhibitory effect of \textit{L. reuteri} bacteria is due to the production of probiotic SCFAs, SCFA synthetic formulations (SSFs) containing acetic, propionic and butyric acids were prepared, as described in Table 1, and tested on Caco-2 cells for 72 h (Fig. 5). The results showed no significant difference observed in the anti-proliferative effects of \textit{L. acidophilus} ATCC 314 and \textit{L. reuteri} NCIMB 11951 and their SSFs. For \textit{L. reuteri} NCIMB 701089 and \textit{L. reuteri} NCIMB 702656 (182.1 ± 15.4 mg/L, p < 0.001), 4.71 ± 0.3 %, 47.76 ± 0.69 %, 42.78 ± 1.08 % (p < 0.001), respectively, and all were significantly higher than \textit{L. reuteri} NCIMB 702656 (p < 0.001) and \textit{L. reuteri} NCIMB 701089 (6.43 ± 0.7 %, p < 0.001).

| Figure 2: Study of the bio-production of SCFAs by \textit{L. reuteri} strains in cell culture conditioned medium (CM): | \textbf{Acetate - CM} | \textbf{Propionate - CM} | \textbf{Butyrate - CM} | \textbf{Total SCFAs - CM} |
|---|---|---|---|---|
| L. r. 11951 | L. r. 701089 | L. r. 701359 | L. r. 702655 |
| L. r. 11951 | L. r. 701089 | L. r. 701359 | L. r. 702655 |
| L. r. 11951 | L. r. 701089 | L. r. 701359 | L. r. 702655 |
| L. r. 11951 | L. r. 701089 | L. r. 701359 | L. r. 702655 |

| Table 1: \textbf{Comparison of inhibitory effect on \textit{Caco-2} cell viability} by \textit{L. reuteri} strains and \textit{L. acidophilus} ATCC 314

| \textbf{L. reuteri} strains | \textbf{Inhibitory effect on cell viability} |
|---|---|
| NCIMB 11951 | 42.78 ± 1.08 % (p < 0.001) |
| NCIMB 701089 | 4.71 ± 0.3 % (p < 0.05) |
| NCIMB 701359 | 47.76 ± 0.69 % (p < 0.001) |
| NCIMB 702655 | 42.02 ± 0.97 % (p < 0.001) |

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NCIMB 702656, the SSFs were significantly more effective than the L. reuteri-CM (p < 0.05). For L. reuteri NCIMB 702655, the L. reuteri-CM had no anti-proliferative effect while the SSF did. However, for L. reuteri NCIMB 701359, SSFs showed significantly less effect that L. reuteri-CM. After addition of lactic acid to each SSFs, SSF-r56+LA (24.15 ± 1.03 % of cell inhibition) and, SSF-r1+LA (19.23 ± 4.3 % of cell inhibition) significantly reduced cancer cell growth compared with SSF-r13+LA (9.07 ± 0.99 % of cell inhibition, p = 0.001, p = 0.26, respectively) and SSF-r55+LA (8.32 ± 1.78 % of cell inhibition, p < 0.001, p = 0.016). SSF-r8+LA had no effect compared with the treated or untreated cells (p > 0.05).

### Resistance of L. reuteri bacteria to SIF

One of the criteria of probiotic selection is their ability to resist the harsh colonic and intestinal environment. Therefore, this experiment was designed to monitor the density and the viability of bacteria inoculated in the simulated intestinal media. As described in Fig. 6 and 7, the viability and concentration of L. reuteri NCIMB -701359, -11951, and -702656, identified in this study for their higher anti-proliferative activity, was evaluated in SIF at different time points. The data showed that our bacteria of interest demonstrated showed resistance to this environment in comparison with the other strains up to 4 h in SIF. No significant difference was observed between L. reuteri NCIMB 11951 (Fig. 7 (b)), L. reuteri NCIMB 701359 (Fig. 7 (d)), L. reuteri NCIMB 702656 (Fig. 7 (c)), and the control L. acidophilus ATCC 314 (94.9 ± 0.6 %, Fig. 7 (a)). It was only after 8 h of incubation that the viability of each L. reuteri strain decreased, depending on its resistance to the SIF. At 8 h of incubation in SIF, L. reuteri NCIMB 11951 had higher viability (121 ± 6.4 %, p < 0.001) than all other bacteria: L. acidophilus ATCC 314 (89.1 ± 0.8 %); L. reuteri NCIMB 701359 (80.5 ± 2.2 %); and L. reuteri NCIMB 702656 (69.2 ± 1.4 %). However, the viability of L. reuteri NCIMB 701359 was not significantly different from L. reuteri NCIMB 702656 after 12 h of incubation.

### L. reuteri produces lactate and SCFAs in SIF

Following the investigation of bacterial resistance to simulated intestinal conditions, and to understand the in-vitro fermentation of probiotic bacteria in a SIF, the production of SCFAs and lactic acid was determined for L. reuteri NCIMB -701359, -11951 and -702656, as shown in Fig. 8. Results showed that lactic, acetic and propionic acids were produced at different levels in the SIF. For lactic acid production, L. reuteri NCIMB 11951 (2762.9 ± 106.6 mg/L) and L. reuteri NCIMB 702656 (2491.8 ± 17.2 mg/L) were significantly higher compared with L. acidophilus ATCC 314 (p < 0.001 and p = 0.003, respectively) and L. reuteri NCIMB 701359 (2121.3 ± 17.3 mg/L, p = 0.001 and p = 0.016, respectively). For acetic acid bio-production in SIF (Fig. 9 (a)), the L. reuteri NCIMB 702656 (650 ± 0.2 mg/L) was significantly higher, followed by L. reuteri NCIMB 11951 (631.2 ± 58 mg/L) and L. reuteri NCIMB 701359 (608 ± 3.2 mg/L, p = 0.016 and p = 0.01, respectively) in comparison with L. acidophilus ATCC 314 (p = 0.003, p = 0.005 and p = 0.009, respectively). However, for the production of propionic acid in SIF (Fig. 9 (b)), L. reuteri NCIMB 11951 (760.4 ± 44.5 mg/L) produced significantly higher amounts of propionic acid, followed by L. reuteri NCIMB 701359 (692.3 ± 21.5 mg/L) and L. reuteri NCIMB 702656 (595.5 ± 0.3 mg/L), when compared with L. acidophilus ATCC 314 (413.1 ± 0.1 mg/L, p < 0.001). In terms of total SCFAs acid production ((Fig. 9 (c)), while L. reuteri NCIMB 701359 (1303.0 ± 27.2 mg/L) was not significantly different from L. reuteri NCIMB 11951 (1391.6 ± 4.8 mg/L, p = 0.332), the latter produced significantly higher amounts of total SCFAs, compared with L. reuteri NCIMB 702656 (1245.5 ± 0.5 mg/L, p = 0.07) and L. acidophilus ATCC 314 (413.1 ± 0.1 mg/L, p < 0.001).

### Discussion

There is a need to systematically evaluate the potential use of novel probiotic bacteria in CRC therapies. Until now, L. reuteri bacteria exhibited few features related to colon health by altering the levels of fecal SCFAs [30,31]. In this study, we screened and characterized five strains of L. reuteri bacteria according to their effects on colon cancer cell inhibition and SCFAs production. The main purpose of the study was to distinguish a strain-dependent effect of a number of L. reuteri bacteria in suppressing colon cancer cell growth and to depict the role of bacterial SCFAs as a mechanism, either generally or strain-dependently.

Reduced colonic SCFAs levels have been reported in human populations with high CRC incidents [8]. As probiotic SCFAs, mainly acetate, propionate and butyrate, were recognized for their anti-cancer activity on colon cancer, several studies have shown that this effect acts through arrested growth, and apoptosis [32]. In particular, some studies showed restored GPR43 expression, coupled with propionate treatment that, induced an upregulation of p21 and a decrease in the levels of cyclin D3 and cyclin-dependent kinases (CDKs) 1 and 2. After propionate/butyrate treatment, and G0/G1 cell cycle arrest and activated caspases were induced, leading to increased apoptotic cell death [33]. Importantly, administration of L. reuteri strains was shown to alter the levels of fecal SCFAs in animals [30, 31] and in fermentation systems [10]. In addition, L. reuteri bacteria has been shown to affect the colonic fermentation of fibers and to stimulate the production profile of SCFAs [34] in simulated intestinal conditions [31]. This supports our hypothesis that L. reuteri produces SCFAs which kills cancer cells.

Thus, the first objective was to screen a number of L. reuteri strains: L. reuteri NCIMB -11951, -701089, -701359, -702655, and -702656. Another set of SSFs was prepared by the addition of respective concentrations of lactate, same as the one produced by L. reuteri-CM.

| Corresponding CM | SSF Composition (mg/L) | SSF+LA Composition (mg/L) |
|------------------|------------------------|---------------------------|
| L. a 314         | SSF-a                  | Lactate                   |
| L. r 11951       | SSF-r1                 | 114                       | 0                        | 14                      |
| L. r 701089      | SSF-r8                 | 131                       | 111                      | 29                      |
| L. r 701359      | SSF-r13                | 116                       | 78                       | 28                      |
| L. r 702655      | SSF-r55                | 86                        | 45                       | 29                      |
| L. r 702656      | SSF-r56                | 182                       | 161                      | 59                      |

Table 1: Composition of SCFA synthetic formulations (SSFs) containing different concentrations of acetate, propionate and butyrate (no bacteria were used), designed at the respective concentrations of naturally produced SCFAs in the CM of L. reuteri NCIMB -11951, -701089, -701359, -702655, and -702656. Another set of SSFs was prepared by the addition of respective concentrations of lactate, same as the one produced by L. reuteri-CM.
DMEM (Fig. 1). Later, SCFAs were quantified in the CM and the levels of acetic, propionic, and butyric acids produced shown to be strain-dependent (Fig. 2). Moreover, we showed that \textit{L. reuteri} NCIMB -11951, -701359, and -702656 produced higher concentrations of SCFAs and lactic acid compared with the other \textit{L. reuteri} strains. It was noted that the concentrations of acetic and propionic acids measured in this study were about half the optimal doses used in the literature to induce anti-proliferative effect on Caco-2 cells [35], which predict a possible inhibitory effect of the probiotic treatments on colon cancer cells.

Very few studies showed the effect of \textit{L. reuteri} bacteria on colon cancer cells. In some one case study, \textit{L. reuteri} promoted TNF-induced apoptosis and suppressed cell proliferation and anti-apoptotic proteins by down-regulating nuclear factor-κB (NF-κB)-dependent gene products that mediate cell proliferation (Cox-2, cyclin D1) and cell survival (Bcl-2, Bcl-xL) [36]. In some cases, the identification of the anti-proliferative effect of probiotics in-vitro can, also, be measured by the effect of bacterial extracts on colon cancer cell colony formation and have shown similar results to the proliferation assay [37,38]. We determined that \textit{L. reuteri} NCIMB -11951, -701359, and -702656 exhibited the greatest inhibition of colon cancer cell proliferation (72 h, Fig. 3), respectively, compared to untreated cells. These observations, shown for the first time, are consistent with the findings that LAB and, more specifically, \textit{L. reuteri}, may have anti-cancer activity induced by SCFA production in the colon which may

**Figure 3: Screening of \textit{L. reuteri} strains for a potential anti-proliferative effect against colon cancer cells.**

To investigate the anti-proliferative effect of probiotic \textit{L. reuteri} bacteria, the cell culture conditioned medium (CM) of \textit{L. reuteri} NCIMB -11951, -701089, -701359, -702655, and -702656 was used. The viability and growth inhibition of human epithelial CRC adenocarcinoma cells (Caco-2) by the \textit{L. reuteri}-CM was measured after incubation with probiotic treatments for (a) 24 h, (b) 48 h and (c) 72 h, using ATP bioluminescence. \textit{L. acidophilus} ATCC 314 is used as a positive control for comparative purposes. Data are presented as mean ± SEM (n=4).

*\(p<0.05\), *\(p<0.01\) and ***\(p<0.001\), compared with \textit{L. reuteri} NCIMB 702655.
decrease tumor growth, inhibit colon cancer cell growth, and promote apoptosis [39]. It is possible that probiotic bacteria have altered colon cancer cell metabolism. Few reports have related the effect of probiotic SCFAs to the production of lactate in cancer cells [40]. Nevertheless, some studies showed that butyrate analogs such as propionate and L-lactate significantly inhibited uptake of butyrate in cancer cells [41], but they may still have a potential effect in decreasing glycolysis /lactate secretion and thus killing cancer cells [42].

As described in Fig. 4, the correlations, between the suppression of colon cancer cell growth by *L. reuteri* and the SCFAs, produced in probiotic CM, were analyzed. A positive correlation has been observed between the inhibition of cancer cells and the concentration of acetic acid (*r* = 0.78, *p* < 0.001), propionic acid (*r* = 0.79, *p* < 0.001), butyric acid (*r* = 0.66, *p* = 0.011) and total SCFAs (*r* = 0.77, *p* < 0.001) produced by *L. reuteri* bacteria.

To investigate this further, concentrations of pure SCFAs, both, with and without lactic acid, were prepared as different mixtures and tested on colon cancer cells (Fig. 5). These, however, showed different levels of inhibitions, which argues for the ability of naturally produced SCFAs in the CM to kill cancer cells. Surprisingly, the SCFA formulations, corresponding to the *L. reuteri* strains, that inhibited the most colon cancer cell proliferation the most were SSF-r1, SSF-r13 and *L. reuteri* NCIMB-11951, -701359, and -702656; that, as shown above, produced the highest levels of inhibitions, which argues for the ability of naturally-containing CM (*p* < 0.001, SCFA formulations vs CM, Fig. 5) is probably due to the presence of other microbial components such as CpG DNA, flagellin and lipopolysaccharide (LPS) which are, shown to inhibit tumor growth by activating pattern recognition receptors in colon epithelial cells [43]. This indicates also that the presence of other bacterial product may complement and enhance the anti-proliferative and thus the anti-carcinogenic activity of SCFAs in the bacterial extract. These findings extend our understanding of the complexity of the interactions between probiotic bacterial products and colon cells. In fact, gut microbiota were found to produce different components (organic acids, bacteriocins, peptides, etc.) that interacts with tumor microenvironment. SCFAs were the primary components related to tumor growth in the colon, since fibers and fermentable oligosaccharides gut microbial breakdown, could result primarily in the production of SCFAs bioactive molecules: acetate, propionate, and butyrate, with a general ratio of 60:25:15. Formate, valerate, caproate, and branched-chain fatty acids (isobutyrate, 2-methylvalerate, and isovalerate, etc) are produced in low quantities from the catabolism of some branched-chain amino acids. Interestingly, studies have shown that polyunsaturated fatty acids and SCFAs mutually interact and protect against colon cancer [44].

Several criteria can be evaluated for probiotic bacteria such as safety, growth, and survival and, in the case of oral administration, the tolerance...
of the bacterium to harsh intestinal conditions. Thus, this study also evaluated the loss of viability of \textit{L. reuteri} bacteria in simulated human intestinal conditions and the preservation of fermentative ability, as determined by the concentration of SCFAs produced in SIF. Of note, the best probiotic candidates, in terms of potential \textit{in-vitro} anti-cancer activity, \textit{L. reuteri} NCIMB -701359, -7011951, and -702656, were selected
for the characterization of their bacterial cultures (Fig. 6) and survival in SIF (Fig. 7). Interestingly, all strains showed similar resistance and survived bile exposure of 4 h. A number of studies have shown that L. reuteri have resistance to gut conditions; however, this feature varied according to the availability of glucose and other nutrients in the gut. L. reuteri tolerance to intestinal conditions was evaluated, mainly, for a maximum of 4 h of exposure while being compared with other probiotic bacteria [45]. In an animal-based study, probiotic bacteria was administrated at $10^9$ cfu, reached the intestine at 6-8 log, similarly to our strains, and persisted for days [46]. Another study, screening the resistance of L. reuteri bacteria to acidic conditions and bile acid, tested several strains for 2-3 h of exposure to different bile acid concentrations and showed a survival rate of between 35% and 70% after just 3 h, with a decrease of up to log 5. In addition, only 73% of the 35 screened L. reuteri strains were not able to survive up to 3 h [47]. In our case, both L. reuteri NCIMB -702656 (Fig. 7 (c)) and -701359 (Fig. 7 (d)), showed similar resistance to SIF in early incubation in comparison with L. reuteri NCIMB 11951 (Fig. 7 (b)) and L. acidophilus ATCC 314 (Fig. 7 (a)). This may answer the question: Are these bacteria suitable as an effective chemopreventive agent against CRC development, since they have the same survival as other bacteria for 4 h in SIF? Furthermore, even after 24 h, they are still viable at log 6, which strongly suggests they are viable in the intestinal environment [48].

Even though both L. reuteri NCIMB -702656 and -701359 were less viable in comparison with L. reuteri NCIMB 11951 and L. acidophilus ATCC 314 at 24 h in SIF, they were able to produce the same concentrations of SCFAs, which shows they may have superior activity in the gut.

In fact, L. reuteri strains appeared to be active and resistant enough in the SIF to produce considerable amounts of lactic, acetic and propionic acids, which was higher than what was produced in the CM ($p < 0.001$). This suggests that a higher beneficial effect may be observed in the intestinal environment. At first, the data suggested that, in SIF, L. reuteri NCIMB and L. reuteri NCIMB 702656 produced significantly more lactic acid than L. reuteri NCIMB 701359 ($p = 0.01$ and $p = 0.016$, respectively, Fig. 8). However, in terms of bacterial acetic and propionic acid production in SIF, no significant difference among these three strains was observed. Remarkably, the levels of total SCFAs produced by L. reuteri NCIMB 11951 ($p = 0.031$) with significant higher survival in the SIF ($p < 0.001$), was non significant with L. reuteri, NCIMB 702656 ($p = 0.07$) and L. reuteri NCIMB 701359 ($p =
Figure 8: Ability of L. reuteri to produce lactic acid in simulated intestinal conditions. The concentrations of lactic acid produced by L. reuteri NCIMB -11951, -701359 and -702656 in SIF (pH=6.8, 24 h, 37ºC, 5% CO2) were identified and compared to L. acidophilus ATCC 314. Data are presented as mean ± SEM (n = 3) *p < 0.05 ND: not detected.

Figure 9: Quantification of the SCFAs produced by L. reuteri in simulated intestinal conditions. To show that L. reuteri NCIMB -11951, -701359 and -702656 are still able to produce significant amounts SCFAs, in an intestinal environment, and induce potential inhibitory effects against colon cancer cells in the gut, the concentrations of (a) acetate, (b) propionate, butyrate (ND) and (c) total SCFAs produced by L. reuteri NCIMB -11951, -701359, and -702656 in SIF (pH = 6.8, 24 h, 37ºC, 5% CO2) were identified by HPLC. L. acidophilus ATCC 314 is used as a control. Data are presented as mean ± SEM (n = 3) *p < 0.05 ND: not detected.

0.332). This result demonstrated that the L. reuteri NCIMB -702656 and -701359 are the best potential strains for the production of SCFAs in simulated intestinal conditions (Fig. 9). The results also indicated that L. reuteri NCIMB -701359 and -702656 have the potential to effectively produce more produce SCFAs in the colonic environment than L. reuteri NCIMB 11951. In comparison with other studies using different media, this study found propionic acid produced in SIF to be double the amount produced by different L. reuteri isolates, whereas acetic and lactic acids levels were relatively low [49]. This study confirmed similar research which demonstrated L. reuteri to have the ability to increase SCFAs production and fermentation in human simulated digestive fluids [15]. It is not unlikely that L. reuteri bacteria will favor cell death in tumor cells via local production of colonic SCFAs, making it an interesting candidate for biotherapeutic application in colon health and cancer prevention.

Conclusion

In this study, we determined whether L. reuteri bacteria may produce SCFAs to inhibit colon cancer cell proliferation. For this purpose, five strains of L. reuteri (L. reuteri NCIMB -11951, -701089, -701359, -702655, and -702656), were selected for their tolerance to intestinal stress, and shown to produce SCFAs in CM or SIF and suppress colon cancer cell growth. This study was the first to compare the anti-proliferative effect of L. reuteri probiotic bacterial strains in-vitro, while evaluating a potential connection with SCFAs.

Together, our findings identified a significant impact of L. reuteri NCIMB -701359 and -702656 in inhibiting colon cancer cell growth that was, related to the bacterial production of SCFAs. These strains also showed a significant efficiency in producing SCFAs in intestinal conditions. Undoubtedly, L. reuteri bacteria showed a potential anti-cancer effect and the ability to produce anti-carcinogenic active compounds, thus indicating its potential biotherapeutic effect in CRC.

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