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Characterization of *L. reuteri* NCIMB 701359 Probiotic Features for Potential Use as a Colorectal Cancer Biotherapeutic by Identifying Fatty Acid Profile and Anti-Proliferative Action against Colorectal Cancer Cells

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Abstract

Colorectal cancer (CRC), the third cause of cancer deaths worldwide, presents a condition with preventable aspects related to diet, lifestyle, and, in particular host gut microflora. Probiotic regimens have been proposed to diminish CRC risk and complications. Among *L. reuteri* strains previously screened some are still in need for further characterization to understand the connection between the probiotic metabolic activity and the potential anti-cancer features. Here, *L. reuteri* NCIMB 701359 was characterized for growth and fatty acid profile. The apoptotic and anti-proliferative capacities of the bacterial extracts (supernatant and conditioned medium) against cCRC cells have been assessed. To investigate a potential anti-cancer activity, the effect of *L. reuteri* on the proliferation of Caco-2 CRC cells compared with CRL-1831 normal coloretcal cells was analyzed. Later, short chain fatty acids that *L. reuteri* produced, were measured and the inhibitory action of short chain fatty acids against Caco-2 cells was investigated using short chain fatty acid synthetic formulations. Results revealed a significantly higher fatty acid production for *L. reuteri* during growth compared with other Lactobacilli. Also, both *L. reuteri* extracts, especially the conditioned cell culture medium, exhibited significant inhibitory effects against SW-480 cancerous cells and induced apoptosis. *L. reuteri* suppressed Caco-2 (cancer) but not CRL-1831 (non-neoplastic). Caco-2 inhibition strongly correlated with the concentration of bacterial short chain fatty acids and was confirmed to be partially but not totally because of short chain fatty acid production. This suggests the potential of *L. reuteri* NCIMB 701359 in suppressing colorectal cancer cell growth and survival, which may assist in the formulation of probiotic-based interventions to limit colorectal cancer development.

Keywords: Probiotic; *L. reuteri* NCIMB 701359; Colorectal cancer; Short chain fatty acids; Proliferation; Apoptosis

Introduction

Colorectal cancer is considered a leading cause of cancer mortality, the third most common cancer in men but the second in women, worldwide, with a high 5 year recurrence rates for patients [1]. Since developments in therapeutic strategies still have limitations in improving the survival rate of colorectal cancer patients [2], CRC is considered one of these types of cancers for which the mortality regressions comes significantly as a results of earlier detection and prevention making chemoprevention an attractive strategy for this disease [3]. Contrary to common chemo-preventive agents which long administration brings unknown risk factors and possible toxicity [4], probiotics have been shown more safety, reduced cancer recurrence and toxicity in CRC patients. Some of these probiotic formulations contained lactic acid bacteria (LAB) of which many were used in biopharmaceutical supplements for CRC patients [5-7]. As in the different stages of carcinogenesis, apoptosis and cell proliferation come as key factors, studies showed that many Lactobacilli extracts induced cell death, cell differentiation and apoptosis in cancer cells, in some cases, by the production of anti-carcinogenic products such as short chain fatty acids (SCFAs) and conjugated linoleic acid (CLA).

In fact, *L. acidophilus* [8-10] and *L. rhamnosus* [11-13] have showed a potential effect in colon cancer suppression, while for some species such as *L. reuteri,* only recently shown to possess probiotic efficacy, are less investigated. Most of the studies on *L. reuteri* bacteria focused on their ability to affect the production in intestinal environment [14,15], while very few showed production of SCFAs [16]. *L. reuteri* was connected to CRC by its ability to precipitate the deconjugated bile salts and a physically bind bile salts by the bacterium, thereby making the harmful bile salts less bioavailable [17]. It was found that administrating *L. reuteri* to mice colitis model have reduced colonic mucosal adherent and translocated bacteria and prevented the disease, some attribute that may indicate these bacteria to have potential to prevent CRC risk. Other finding demonstrated *L. reuteri* ATCC PTA 647 to secrete components that trigger cell death in myeloid leukemia-derived cells a feature that can associate to a potential CRC preventive effect [18].

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This report characterized, in general, the general bacterial metabolic activity of *L. reuteri* NCIMB 701359, using free fatty acid (FFA) profile and cell growth, then, the anti-proliferative activity of the bacterium cell free extracts, using the growth and apoptosis induction of colorectal cancer cells. To confirm that this effect is due to an anti-cancer effect for the bacterium and not to a cytotoxic effect, the same assays were performed on non-neoplastic colon cells. Subsequently, the probiotic the conditioned cell culture medium (CM) composition in SCFAs: acetic, propionic, and butyric acids was determined, leading to this question: Is this anti-cancer effect mainly related to the production of bacterial SCFAs other than other bacterial products? For this, the bacterial SCFAs were quantified; those amounts were used to formulate SCFA synthetic mixtures. When the SCFA synthetic formulation inhibits cancer cells less than its analogous *L. reuteri* CM, SCFAs, then, are not considered to be the single anti-cancer factor produced by the bacteria, *L. reuteri* NCIMB 701359 may have excreted other compounds with anti-proliferative activity. Meanwhile, if the SCFA synthetic formulation suppressed colon cancer cell growth at least evenly compared with the bacterial CM, we can speculate the levels of SCFAs produced by *L. reuteri* to be in total the only active probiotic component against CRC cells.

Material and Methods

Materials

De Man Rogosa Sharpe (MRS) broth and agar are 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 from Invitrogen. Purified water was generated by an Easy Pure reverse osmosis system. NanoPure Diamond Life Science (UV/UF) ultrapure water system from Barnstead (Dubuque, IA, USA) was used. Sodium L-Lactate and SCFAs were obtained from Sigma (St. Louis, MO, USA).

Bacterial and mammalian cells

L. reuteri NCIMB 701359 was acquired from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland, UK). *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 were procured from Cedarlane Laboratories (Burlington, ON, Canada). The growth of each culture, sustained in MRS broth (1% (v/v), 37°C, 5% CO_2) was characterized by $OD_{620\ nm}$ (Perkin Elmer 1420 Multilabel Counter, USA), then by viable cell count on agar plates.

SW-480 and Caco-2 colorectal cancer cell lines and CRL-1831 normal epithelial colon cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Caco-2 cells were growing in EMEM supplemented with 20 % FBS for about two weeks till fully differentiated. SW-480 was maintained in RPMI-1640 supplemented with 10% FBS, while CRL-1831 proliferated in complete DMEM (10% FBS, 37°C, 5% CO_2). In most assays, when mammalian cells have reached at 50-60% confluence (24-48 h), cell medium were replaced by probiotic cell free extracts and serum/antibiotic-free DMEM.

Free fatty acid (FFA) analysis

For the determination of the free fatty acid generated by the probiotic cells, bacterial cultures were maintained in MRS broth (12-16 h, 37°C, 5% CO_2) before extraction the supernatant. The latter was separated by the removal of the bacterial cells by centrifugation (1000 x g, 15-25 min, 4°C) and filtration with a 0.2 µM-pore-size filter, (Millipore). This assay was performed according to the manufacturer's protocol. Briefly, ACS Reagent (2 µl) was added to the standard (palmetic acid, 1 nmol/ul) and the sample in each well for Acyl-CoA synthesis (Figure 1), before incubation (37°C, 30 min). The reaction mix (50 µl) and an assay buffer (Igepal, 44 µl) were mixed. Then, fatty acid probe (2 µl) and an enzyme mix (2 µl) were added with an enhancer (N-ethylmaleimide, 2 µl) to be briefly vortexed. Am amount of50 µl of the reaction mix was added to either standard or test samples and all were incubated (30 min, 37°C)

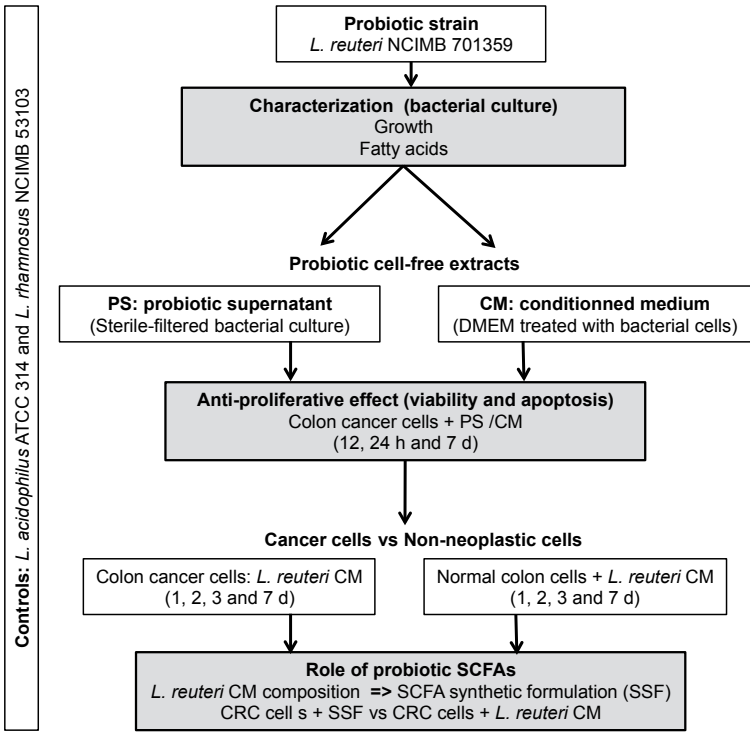

Figure 1: Experimental outline for investigating the anti-cancer characteristics of *L. reuteri* NCIMB 701359 and the role performed by probiotic compared with synthetic SCFAs.

while protected from light. At this point, the fatty acids present are converted to CoA derivatives which are oxidized forming a color to be measured at 570 nm.

Preparation of probiotic cell-free extracts

Pure cultures of probiotic bacteria were maintained in MRS broth for 12-16 h (37°C, 5% CO_2). Cell culture conditioned medium (CM) and probiotic supernatant (PS) of *L. reuteri* NCIMB 701359 were prepared similarly to Grabig et al. [19], and Kim et al. [20]. First, probiotic bacteria were left to grow for 24 h (37°C, 5% CO_2) and were passaged at 1%. At the 3rd-4th culture, the bacteria were incubated for 14-16 h (37°C, 5% CO_2). Then, bacterial cell precipitation was performed by centrifugation (1000 x g, 15-25 min, 4°C) and the collected pellet was washed with PBS. In the following step, washed probiotic cells (10^7-10^9 cfu/mL) were transferred in DMEM, were shacked at 37°C for 2 h (50 rpm). The suspension was centrifuged twice (1000 x g, 15 min, 4°C) and sterile-filtered (0.2 µm-pore-size filter) to obtained the probiotic cell culture conditioned medium (CM). For the preparation of bacterial PS, sterile-filtered (supernatant was obtained after removal of the bacterial pellet (1000 x g, 15-25 min, 4°C). Before incubation with colorectal cells, CM and PS were each mixed with DMEM (ratio 1:2, pH 7) and the pH was adjusted with 2 M NaOH and 2 M HCl solutions.

Determination of CRC cell proliferation

To determine the proliferation of probiotic-treated CRC cells CellTiter-Glo Luminescent Cell Viability Assay (Promega, USA), based on ATP bioluminescence, was used. Caco-2 cells were distributed into 96-well culture plates (5×10^3 cells/well) and left to attach for 24 h (37°C, 5% CO_2) and, later, were incubated with probiotic supernatants (CM or PS) For 12 h, 24 h, and 7 d, viability was determined based on the manufacturer instructions [21]. At each time point, each 96-well plate was left at room temperature (RT, 30 min) before addition of a 100 µL of luminescent reagent in each well. To induce cell lysis in each well, the 96-well plate was shacked on an orbital shaker (2 min, 200 rpm). Next, the plate was left to incubate at RT for 10 min before recording the luminescent signal with a spectrophotometer (Perkin Elmer, Victor 3, multi-label microplate reader, MA, USA). Cancer cell inhibition was determined by evaluating the proliferation of treated cells compared with untreated cells at each time point.

Apoptosis Assay

To determine if the suppression of CRC cell growth is related to inducing cell death, apoptosis was evaluated by assessing caspace-3 and -7 using Caspase-Glo® 3/7 assay (Promega, USA). Briefly, the lyophilized substrate, provided with the kit, was dissolved with a buffer at RT. Luminometer-compatible white-walled 96-well plates were filled with samples (100 µL) of negative control, treated cells, and blank. After probiotic treatment, the plate was left to equilibrate at RT. An amount of the luminescent reagent (100 µL) was added to each well. Then, the plate was covered with a plate lid and was placed on a plate shaker (300-500 rpm, 30 s). Later, incubation at RT for 30 min to 3 h, will take place. The luminescence in each well was captured by a plate-reading luminometer as pointed by the manufacturer.

Determination of Probiotic Action on Neoplastic and Non-Neoplastic Colon Cells

In order to verify that the suppressive effect of probiotic cell free extract is due to an anti-cancer activity and not cytotoxic, the treatments were tested on both normal (CRL-1831) and cancer (Caco-2) colon cell proliferation. Cells were seeded into 96-well culture plates (5×10^3 cells/well) before incubation (37°C, 5% CO_2, 24 h). Colon cells were treated with *L. reuteri* NCIMB 701359 cell-free extracts for at 24 h and 48 h before assessing viability, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 as positive controls.

Lactic Acid and SCFA Analysis

SCFAs produced by *L. reuteri* NCIMB 701359, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103, were measured with HPLC [22,23] after the preparation of all CM. The HPLC system (Hewlett-Packard HP1050 series, Model 1050 UV, Agilent Technologies, USA) used was equipped with a UV-Vis detector and diode array detector (DAD, 210 ± 5 nm). Samples (100 µl) were injected through a pre-packed column (Rezex ROA-organic acid H+ (8%), 150 × 7.80 mm, Phenomenex, CA, USA) attached to an ion-exclusion microguard refill cartridge. Chromatographs were generated using ChemStation LC3D software (Rev A.03.02, Agilent Technologies, CO, USA). The two mobile phases H_2SO_4 (0.05 M) and acetonitrile (2%) were pumped through the column with an isocratic gradient (0.8-0.7 mL/min, 35°C). Lactic, acetic, propionic, and butyric acids were used to prepare a standard solution at different concentrations (1, 10, 100, 500 and 1000 ppm). The concentrations of SCFAs were calculated from the generated standard curves and their respective linear regression equations ($R^2 \geq 0.99$).

Efficacy and Role of SCFAs Produced by *L. reuteri* NCIMB 701359

This method was used to determine the role of the naturally produced SCFAs in the inhibition of colorectal cancer cells, probiotic SCFAs produced by *L. reuteri* bacteria were compared with SCFA synthetic formulations, which are reproduced at the same concentrations. The concentrations of SCFAs were determined for each cell free extract CM, and then formulations containing the same composition in SCFAs were prepared. The anti-proliferative effect was evaluated by treating colon cancer cells with SCFAs synthetic formulations at a pH=7, a ratio of 1:2 for 72 h. An ATP bioluminescence assay was used for the analysis to determine the inhibitory effects of SCFAs synthetic formulation on colon cancer growth in comparison with *L. reuteri* NCIMB 701359-CM.

Statistical Analysis

Data were presented as means ± Standard Error of the Mean (SEM). Statistical significance was obtained for the treated groups compared with each other. One-way analysis of variances (ANOVA) coupled with Tukey's comparison test was run by SPSS statistics software (version 20.0, IBM corporation, NY, USA). *P*-value of $p<0.05$ was admitted as significant. Regressions were determined based on Pearson correlation method.

Results

L. reuteri NCIMB 701359 highly affected the levels of FFAs

To distinguish the metabolic activity of *L. reuteri* NCIMB 701359 and its potential in producing a high amount of beneficial metabolites, compared with other LAB bacteria, the FFA content of the bacterial culture supernatant was characterized during the different growth phases (0 h to 72 h) (Figures 1 and 2). During the lag and the stationary phases, *L. acidophilus* ATCC 314 (Figure 2a) and *L. rhamnosus* ATCC 53103 (Figure 2b), did not increase the levels of total FFAs in the culture, while *L. reuteri* NCIMB 701359 slightly did (Figure 2c). At the start of stationary phase, *L. reuteri* NCIMB 701359 (Figure 2f, $p<0.05$)

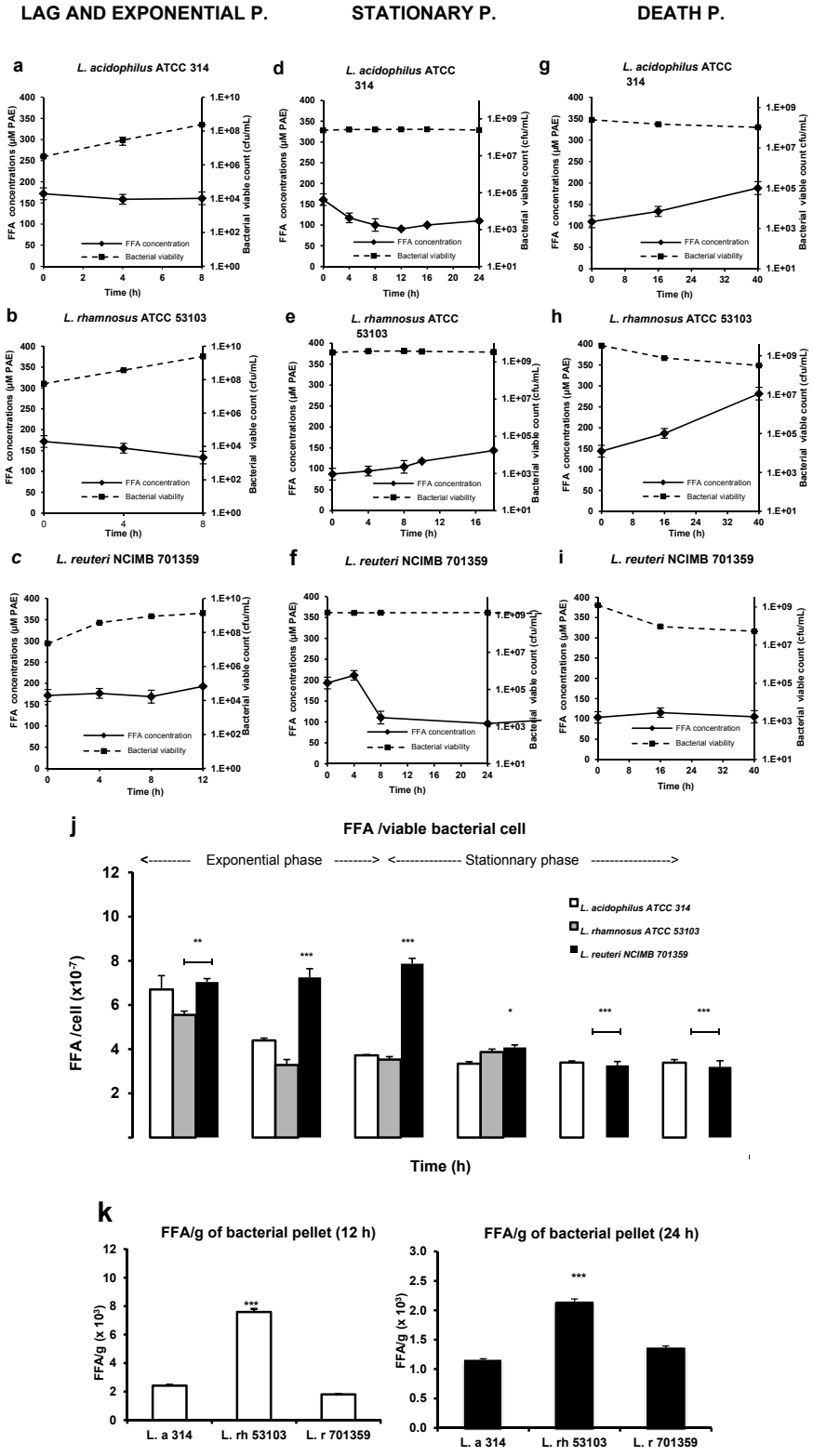

Figure 2: Description of the metabolic activity of *L. reuteri* NCIMB 701359 based on bacterial growth and free fatty acid (FFA) profiles. The content of total FFAs in the bacterial culture of *L. reuteri* NCIMB 701359 **(c, e, h)** were determined for different phases for bacterial growth, in comparison with two of *Lactobacillus* bacteria (positive controls): **(a, b, c)** *L. acidophilus* ATCC 314 and **(d, f, i)** *L. rhamnosus* ATCC 53103. **(j)** Description of the variation of FFA levels per viable bacterial cell (exponential and stationary phases). **(k)** Illustration of the levels of FFA/g of bacterial cells (12 h and 14 h). *L. reuteri* NCIMB 701359, *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 were growing in each culture during 72 h in MRS (37°C, 5% CO_2). *p<0.05, **p < 0.005 and ***p < 0.001, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103.. Data represent the mean ± SEM (n=3). P: phase; PAE: Palmetic acid equivalents. *L.a* 314. *L. rh* 53103: *L. rhamnosus* 53103, *L. a* 314: *L. acidophilus* ATCC 314, *L. r* 701359: *L. reuteri* NCIMB 701359.

had significantly higher FFA levels compared with *L. acidophilus* ATCC 314 (Figure 2d) and *L. rhamnosus* ATCC 53103 (Figure 2e). However, during the death phase, *L. reuteri* NCIMB 701359 (Figure 2i, $p<0.01$) did significantly release less FFAs than *L. acidophilus* ATCC 314 (Figure 2g) and *L. rhamnosus* ATCC 53103 (Figure 2h). In the other hand, using the viable bacterial cell number and total bacterial weight, the concentrations of FFA/bacterial cell (Figure 2j) and FFA/g of bacterial pellet (Figure 2k) were significantly superior to *L. reuteri* NCIMB 701359 ($p<0.001$). An exception was shown during the death phase, where only *L. rhamnosus* ATCC 53103 was capable to significantly increase the levels of FFAs to 109.5 ± 1.8 µM PAE per viable bacterial cell compared with *L. reuteri* NCIMB 701359 ($p<0.001$).

L. reuteri NCIMB 701359 cell-free extracts inhibited colon cancer cells

To study the anti-cancer action of *L. reuteri* NCIMB 701359 against CRC cell *in vitro*, different bacterial cell-free extracts were tested on colon cancer cells SW-480: A probiotic supernatant (PS), prepared for the MRS bacterial culture and a cell culture conditioned medium (CM), that was pre-enriched with bacterial cells (Figure 3). For PS, cancer cells proliferation was significantly less than untreated cells. In the case of PS of *L. acidophilus* ATCC 314, *L. reuteri* NCIMB 701359

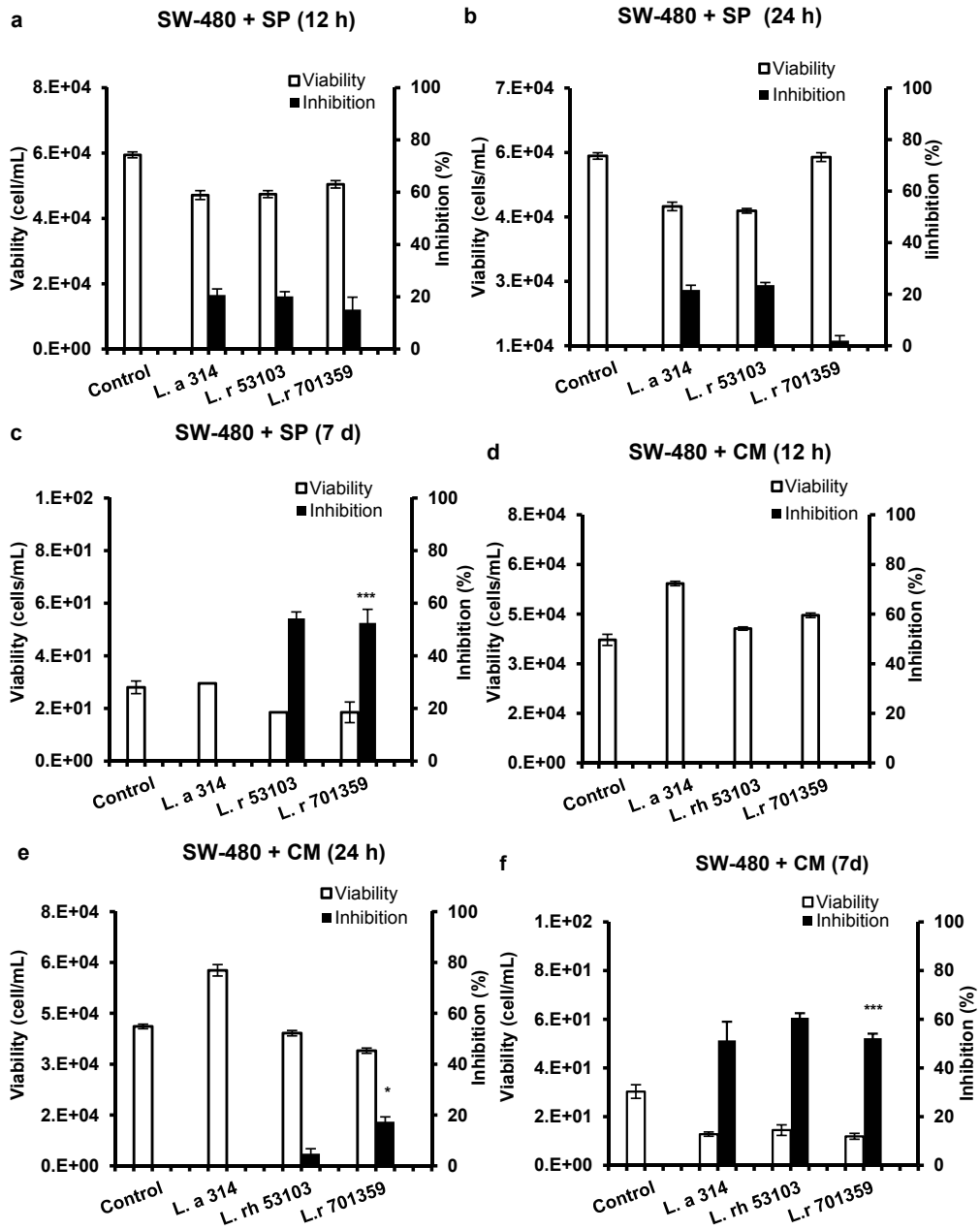

Figure 3: Investigation of cancer cell inhibition by different bacterial cell free extracts of *L. reuteri* NCIMB 701359. Viability of human colon cancer cells (SW-480) at 12 h, 24 h, and 7 days, after exposure to **(a, b, c)** probiotic supernatant (PS) and **(d, e, f)** conditioned cell culture medium (CM) of *L. reuteri* NCIMB 701359, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. *$p<0.05$ and ***$p<0.001$, compared with *L. acidophilus* ATCC 314, *L. rhamnosus* ATCC 53103, and negative control. Data are presented as mean ± SEM ($n=4$). *L.a* 314. *L. rh* 53103: *L. rhamnosus* 53103, *L. a* 314: *L. acidophilus* ATCC 314, *L. r* 701359: *L. reuteri* NCIMB 701359.

and *L. rhamnosus* ATCC 53103 (Figure 3a), cancer cell proliferation was inhibited by 20.71 ± 2.3%, 15.16 ± 4.73% and 20.21 ± 1.8%, for 12 h, respectively. Interestingly, after 7 days, only PS of *L. reuteri* NCIMB 701359 and *L. rhamnosus* ATCC 53103 has inhibited CRC cell growth by 52.55 ± 3.86%, 54.26 ± 2.43% (p<0.05 Figure 3c), respectively, compared with the control. In the other hand, when probiotic CM was tested, results showed that the CM of *L. reuteri* NCIMB 701359 have inhibited, at 24 h (Figure 3e), cell proliferation by 17.4 ± 2.3%, while *L. rhamnosus* ATCC 3103 inhibited cells by 4.82 ± 1.935 compared with untreated cells. Interestingly, after 7 days of treatment (Figure 3f), there was significant cell inhibition by the CM of *L. reuteri* NCIMB 701359 (60.66 ± 5.31%), *L. acidophilus* ATCC, 314 (51.35 ± 7.7%) and *L. rhamnosus* ATCC 53103 52.25 ± 8.26% (p<0.05), respectively, compared with untreated cells.

L. reuteri NCIMB 701359 cell-free extracts induced apoptosis in colon cancer cells

Both types of bacterial extracts were tested if they induce apoptosis in cancer cells (Figure 4). In case of probiotic PS, after 12 h (Figure 4a), *L. reuteri* NCIMB 701359 (21.7 ± 12.4%) induced less apoptosis than *L. rhamnosus* ATCC 53103 (26 ± 13.9 %, p=0.04) and more than *L. acidophilus* ATCC 314 at 18.9 ± 8.6%. For probiotic CM, data collected at 24 h (Figure 4d) showed that *L. reuteri* NCIMB 701359 (27.2 ± 9.4%) significantly induced cell death compared to *L. acidophilus* ATCC 314 (p=0.031).

L. reuteri NCIMB 701359 suppressed cancer colon cells but not normal colon cells

The purpose of this experiment is to verify the anti-cancer activity of the probiotic bacteria and determine that the inhibitory effect was specific to cancer cells and not normal colon cells. Thus, the effect of *L. acidophilus* ATCC 314, *L. rhamnosus* ATCC 53103 and *L. reuteri* NCIMB 701359 on the viability of both Caco-2 and non-cancerous CRL-1831 were determined using CM (Figure 5). Results showed that for *L. reuteri* NCIMB 701359 (Figure 5c) and *L. rhamnosus* ATCC 53103 (Figure 5b), cancer cell growth was inhibited, at 24 h, by 22.41 ± 2.14% and 6.33 ± 1.04% (p<0.01) compared with untreated cells, respectively. However, at 48 h, cancer cell viability was reduced by 42.68 ± 4.44% (Figure 5c), and 11.42 ± 1.75% (Figure 5d) respectively, compared with untreated group (p<0.01). Then, at 72 h, *L. reuteri* NCIMB 701359 and *L. rhamnosus* ATCC and *L. acidophilus* ATCC 314 have inhibited cancer cell proliferation by 58.57 ± 0.66% (Figure 5c), 23.95 ± 2.49% (Figure 5b), and 12.59 ± 1.92% (Figure 5a), respectively, compared with untreated cells (p<0.05). Moreover, at 7 days of probiotic treatment, Caco-2 cell growth has been reduced by of 88.23 ± 1.47% and 88.41 ± 0.45%, 99 ± 0.26%, respectively, compared with the untreated group (p<0.05).

By contrast, no significant decrease in cell viability was observed in CRL-1831 treated with probiotic extracts. The results specifies that *L. acidophilus* ATCC 314 (Figure 5d), *L. reuteri* NCIMB 701359 (Figure 5f), and *L. rhamnosus* ATCC 53103 (Figure 5e), has stimulated CRL-1831 epithelial normal colon cells significantly, after 24 h, 48 h and 72 h of treatment, compared with untreated cells (p<0.05). Even at 7 days, all probiotic treatments showed no significant inhibitory effect on the growth of CRL-1831 (Figures 5b, 5e and 5f).

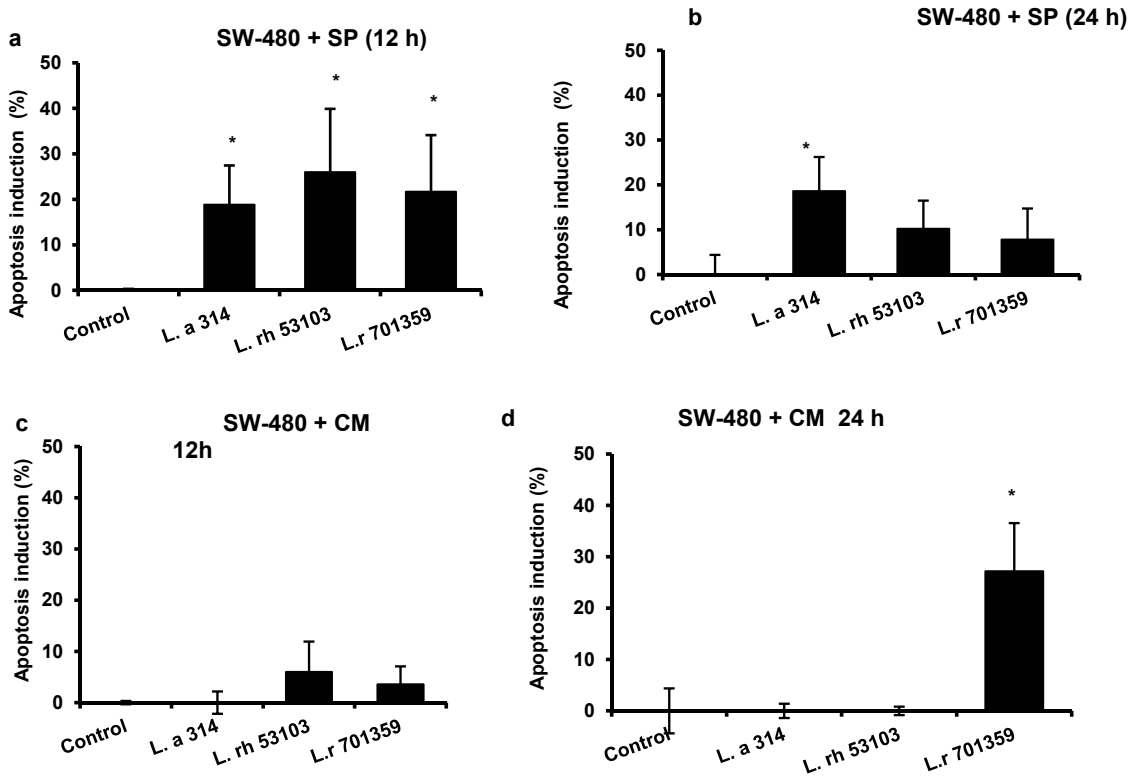

Figure 4: Determination of CRC cell apoptosis induced by *L. reuteri* NCIMB 701359. The bacterial cell-free extracts (PS and CM)- of *L reuteri* NCIMB 701359 increased cell death in SW-480 , when treated for **(a, c)** 12 h and **(b, d)** 24 h. The data values represent the mean ± SEM (n=4). *p<0.05, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. *L.a.* 314. *L. rh* 53103: *L. rhamnosus* 53103, *L. a* 314: *L. acidophilus* ATCC 314, L. r 701359: *L. reuteri* NCIMB 701359.

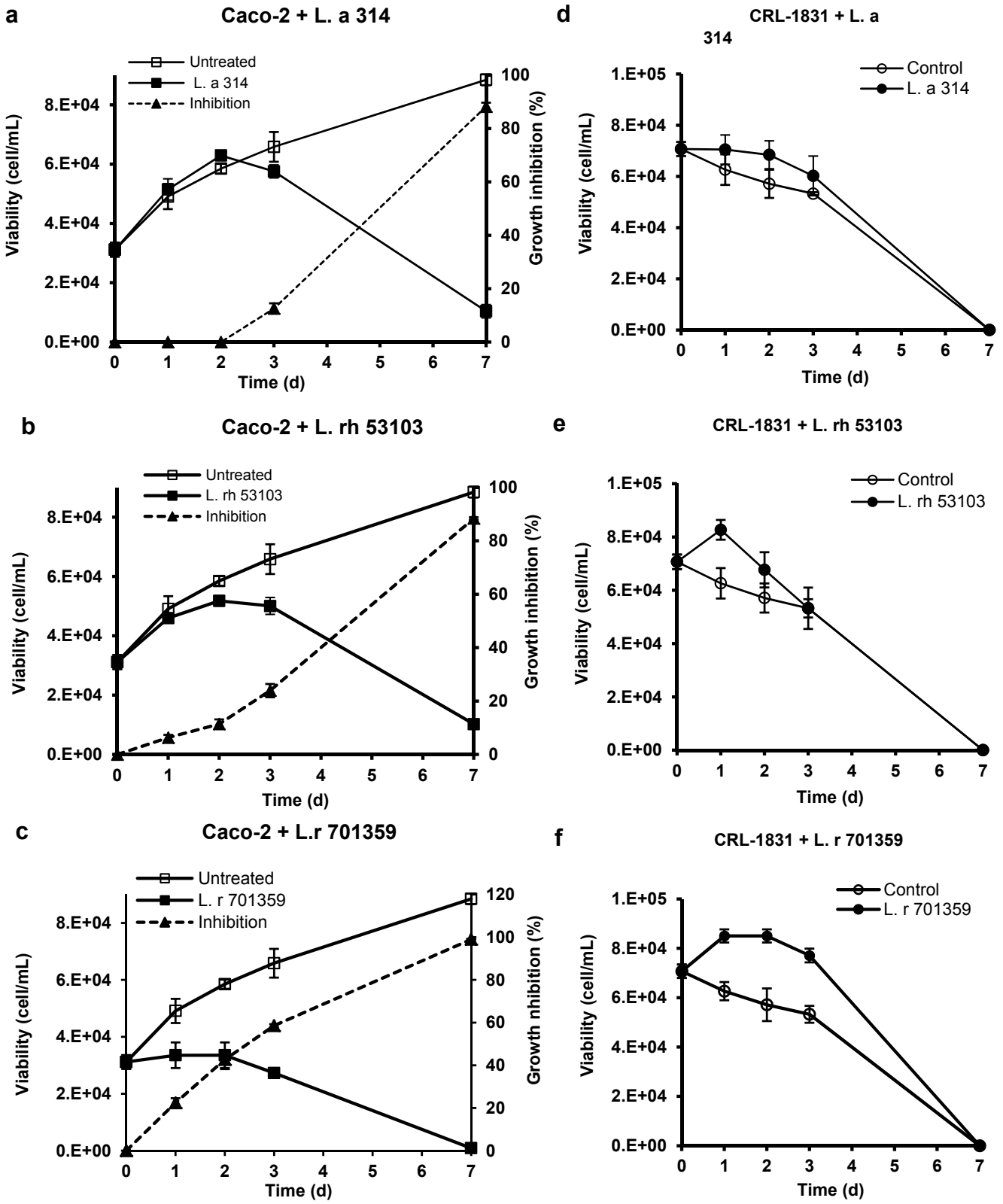

Figure 5: Comparison of the anti- proliferative and the non-cytotoxic effect of *L. reuteri* NCIMB 701359 using cancer and non-neoplastic colorectal cells. The viability of Caco-2 colon carcinoma cells and CRL-1831 normal epithelial colon cells incubated with *L. reuteri*-CM for three days was differentially evaluated. The values represent the mean ± SEM (*n*=4). *L. rh* 53103: *L. rhamnosus* ATCC 53103, *L. a* 314: *L. acidophilus* ATCC 314, L. r 701359: *L. reuteri* NCIMB 701359.

L. reuteri NCIMB 701359 secreted higher concentrations of SCFAs

We hypothesized that *L. reuteri* NCIMB 701359 has excreted its effect due to the production of SCFAs. Therefore, the quantification of lactic acid and SCFAs produced by this bacterium intent to confirm their presence in the cell free extract (Figure 6). The results showed that *L. reuteri* NCIMB 701359 produced the highest amount of acetate (Figure 6c) and propionate (Figure 6d) compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 ($p<0.05$). *L. reuteri* NCIMB 701359

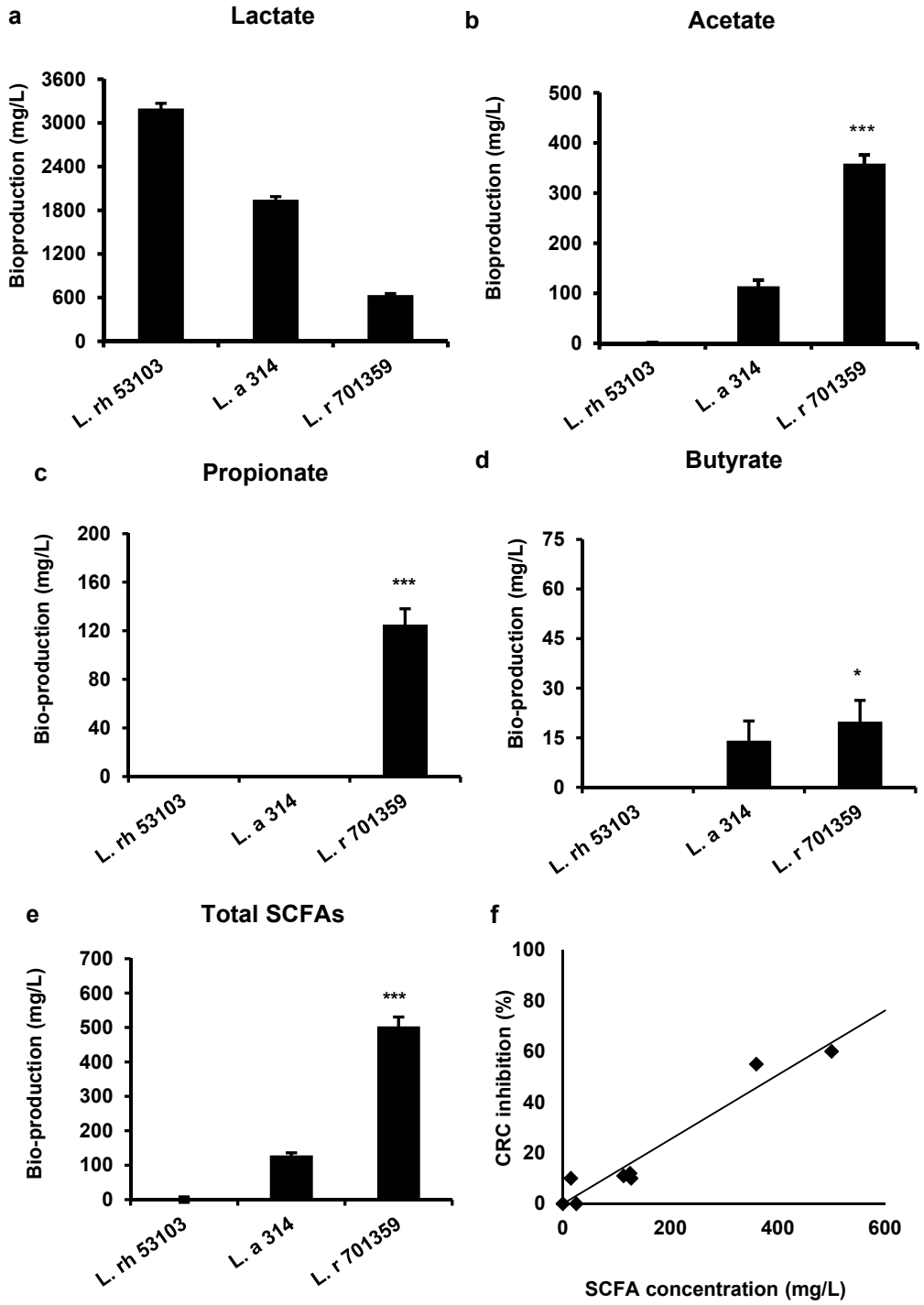

Figure 6: Bioproduction of lactic acid and SCFAsby *L. reuteri* NCIMB 701359. **(a)** The concentrations of lactic, **(b)** acetic, **(c)** propionic, and **(d)** butyric acids, and **(e)** total SCFAs produced by *L. reuteri* NCIMB 701359 and bacterial controls in their CM. The data values represent the mean ± SEM ($n=3$). ***$p<0.001$ compared to *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. **(f)** Examination of the correlation between inhibited CRC cell growth by probiotic CM and concentrations of SCFAs produced in CM. The data of cell growth inhibition in Figure 5 were used to run linear regression analysis. The CM was prepared by incubating probiotic bacterial pellet, in DMEM for 2 h (37°C, 5% CO_2), and then it was incubated at a ratio of 1:2 with colon cells. The data values represent the mean ± SEM ($n=3$).

produced significantly higher levels of butyrate (Figure 6d). *L. reuteri* NCIMB 701359 produced significantly higher levels of total SCFAs that both controls (p<0.001, Figure 6e). In addition, *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103did not significantly inhibit colon cancer growth, did not generate detectable amount of propionic and acetic acid (Figures 6b and 6c)) but produced higher amount of lactic acid, i.e., 1970.6 ± 9.56 and 3239.8 ± 9.9 mg/L, respectively, compared with *L. reuteri* NCIMB 701359 (Figure 6a).

SCFAs produced by *L. reuteri* could be the main inhibitory compounds

We noticed that the bacteria with the highest levels of SCFAs had the best suppressive effect against CRC cell growth. Therefore, to investigate the anti-CRC-cell-proliferative activity of *L. reuteri* NCIMB 701359 in relation to the secreted concentrations of SCFAs, pure and mixed synthetic doses of SCFAs were tested on colon cancer cells (Figure 7). First, concentrations equal to acetate, propionate and butyrate amounts produced were tested separately and their growth inhibitory effect did not exceed 35% (Figure 7a). In fact, The SCFA synthetic formulation of *L. reuteri* NCIMB 701359 was significantly more effective than the CM (p<0.001) or following lactate addition (p<0.01, Figure 7a).

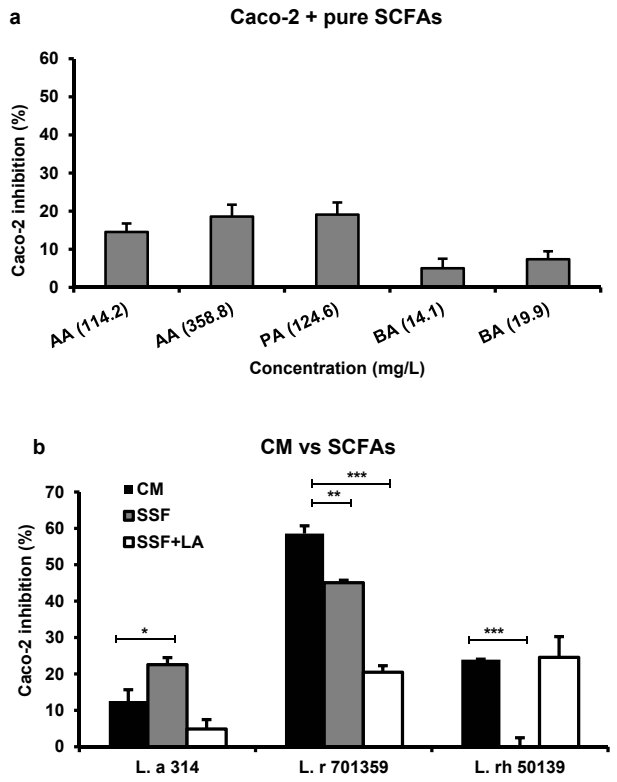

Figure 7: Confirmation of the involvement of SCFAs produced by *L. reuteri* NCIMB 701359. **(a)** The anti-proliferative effect of SCFAs doses at similar concentrations than what was produced by *L. reuteri* NCIMB 701359 (Figure 5). **(b)** The anti-proliferative activity of SSF and SFF+LAcorresponding to *L. reuteri* NCIMB 701357, against CRC cells, compared with *L. reuteri*-CM (72 h). Positive controls are *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. SSFs were prepared by mixing different doses of SCFAs to DMEM media based on probiotic SCFAs concentrations measured in CM as shown in Figure 6 (n=6,). *p<0.05, **p<0.01, ***p<0.001, compared with control. The data values represent the mean ± SEM (n=6). *L. a.* 314: *L. acidophilus* ATCC 314, *L. r* 701359: *L. reuteri* NCIMB 701359. SSF: SCFA synthetic formulation.

Discussion

Despite CRC aggressive treatments and symptoms, this disease can be regulated through diet highlights. There is a need to identify dietary components such as probiotics, emerging as health supplements that have shown potential to restore intestinal metabolism and beneficially alter CRC biomarkers. In the last decade, many papers suggested that probiotic *lactobacilli* have positive effect on colon health and an impact in reducing CRC incidence. The search for potent LAB with anti-cancer attributes and the characterization of their features as biotherapeutic agents in CRC has been subject of many *in vitro* studies aiming to determine the possibility of their use as novel preventive treatments. However, there is a lack in the evaluation of novel bacteria, establishing their degree of efficacy compared with other stains of Lactobacilli and defining metabolic effects and action on CRC cells, *in vitro* or/and non-cancerous/healthy colon cells.

For the first time, we characterized, in this study, the activity of *L. reuteri* NCIMB 701359 in producing potential anti-cancer compounds by measuring their general ability to affect FFAs levels in their growth media compared with other known LAB (*L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103). Later, the activity of *L. reuteri* NCIMB 701359 CM was tested on the proliferation of both colon normal and colon cancer cells, and the presence of SCFAs was determined.

We reported that *L. reuteri* NCIMB 701359 possess a higher metabolic and lipolytical activity rather than both *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 with excretion of more FFAs (two times) in the growth media. As known, FFAs have diverse potent biological activity against potential pathogenic or opportunistic microorganisms, in addition to be cytotoxic to some cells. Some reports stated that LAB bacteria could produce FFAs, predominantly butyric acid and linoleic acid (LNA) that is converted to CLA, all considered beneficial functional lipids [24]. These observations suggested that this bacterium, possessed higher activity and could be effective in producing beneficial metabolites if administered to the colon, or may confer nutritional and therapeutic values to products supplemented by this bacterium [25]. Several studies have reported that the addition of LAB to nutritional products may contribute to the production of FFAs, such as CLA and LNA, which attracted interest as being a novel type of beneficial functional lipids [26] or other phytochemicals [27].

We studied further the effect of both PS and CM, prepared from the bacterial culture of *L. reuteri* NCIMB 701359, against SW-480 cancer cells. The results suggested that both types of probiotic bacterial cell-free extracts contained bacterial metabolites with anti-proliferative (Figure 3) and apoptotic activities (Figure 4). Results showed no significant ability of PS to induce apoptosis in cancer cells at 12 h, 24 h and 7 days of treatment, compared with the positive controls. Meanwhile, the CM of *L. reuteri* NCIMB 701359 had more significant anti-proliferative and apoptotic effect, at 24 h, in comparison with both control groups. When PS was used as a treatment, all probiotic bacteria inhibited and killed SW-480 cells for 12 h and for a longer period of 7 days. However, for SW0-480 cells treated with CM, the inhibitory effect and cell death were observed at 24 h. When treated with *L. reuteri* NCIMB 701359, cancer cells had significantly the least growth compared untreated cell an effect that was observed till 7 days of incubation (p<0.001). For both types of probiotic extracts, the bacteria appeared to excrete inhibitory and apoptotic actions against colon cancer cell, however CM was used of the rest of the study, due to the homogeneity of the extract and the relevance of the effect it generated.

Very few studies demonstrate the effect of probiotic extracts on

normal colon cells. However in our study, we showed that it has no cytotoxic effect on non-neoplastic epithelial colon cells while inducing cancer cell death. We found that not only had *L. reuteri* NCIMB 701359 suppressed the most cancer cells, but it also promoted the best the healthy proliferation of normal colon cells CRL-1831 (Figure 5). Hence, we can hypothesize that this mechanism of action involves compounds that can be a source of energy for normal cell but also kill cancer cells. It is established that SCFAs, especially propionate, an inhibitor of histone acetylases (HDACI), affects AR co-regulators expression and transcription activity in cancer cells. But, it has minimal effect on normal prostate cells.

To validate this hypothesis, the SCFA profile of *L. reuteri* NCIMB 701359 and controls was analyzed and compared in relation to their effect on the proliferation of both normal and cancer colon cells.

Based on CM analysis for their composition in SCFAs and lactic acid (Figure 6), we have noticed that *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 produced significantly higher levels of lactic acid than *L. reuteri* NCIMB 701359, while the latest secreted the highest amount of total SCFAs ($p<0.001$). Since bacterial propionate and acetate were identified as the major cytotoxic components secreted, this implies that this *L. reuteri* strain could constitute a probiotic efficient in CRC prophylaxis by producing apoptosis-inducing SCFAs. More interestingly, Anderesen et al. suggested that propionic acid; possess significant immunoregulatory functions and cancer prophylactic potential. He demonstrated that, same as other SCFAs, propionibacteria supernatant or propionate unregulated expression of the NKG2D ligands on cancer cells, as well as activated T lymphocytes [28].

There is a strong correlation ($r^2=0.9401$, $p=0.024$) observed between the inhibitory effect of probiotic CM and the concentrations of total SCFAs, AA, PA and PA+BA (Figure 6). Our bacterium secreted significantly higher concentrations of propionate than both other tested bacteria. This confirms the fact that the observed beneficial probiotic effect is, probably, dependent of the production of this acid [29,30]. To note, there has been more emphasis on the physiological and pathological role of butyrate and SCFA combinations versus an undervaluation of the potential effects of propionic acid. Despite the fact that researchers have mainly investigated propionate in the context of ruminant physiology, particularly liver physiology and metabolism, it was though demonstrated it as the primary precursor for glucose production [31], there is evidence that propionic acid is an important factor in human physiology as well. Moreover, propionic acid was showed to exerted anti-inflammatory and anti-microbial activity against pathogenic bacteria in the gastrointestinal (GI) tract [32].

One of the main points was to confirm that the mechanism of action of *L. reuteri* NCIMB 701359 in killing cancer cells and suppressing their growth is by the secretion of SCFAs/propionate. The following steps were taken: (i) Examination of the effect of pure SCFAs at the same concentration produced by NCIMB 701359; (ii) reproduce the probiotic CM composition of SCFAs and test this synthetic mixture on cancer cells. For the first approach (i) separate concentrations of different SCFA similar to the one produced were tested and this demonstrated that not a SCFA is solely responsible of the anti-proliferative effect. Second (ii) only the SCFA formulations of *L. acidophilus* ATCC 314 and *L. reuteri* NCIMB 701359 were considered, since *L. rhamnosus* ATCC 53103 did not excrete SCFAs. The SCFA synthetic formulation corresponding to *L. reuteri* NCIMB 701359 significantly inhibited CRC cell growth less than the probiotic cell-free extract CM (Figure 7b). Suggesting that 13% of the activity of *L. reuteri* NCIMB 701359 is due to the presence of different bacterial compounds. When LA was added to SCFA mixture the inhibitory effect of SFF+LA (Figure 7b) was hindered. This could be explained by the potential; presence of bacterial products with a complementary effect or whom activity is necessary to the action of SCFAs against cancer cells. Some studies have identified other components secreted in probiotic CM and PS and demonstrated bacterial regulation of colon cellular responses through the production of active molecules in different bacterial strains. In recent studies, the PS and the CM of *L. rhamnosus* GG induced cellular effects by the secretion of multiple low-molecular-weight compounds regulating epithelial cellular responses, such as the case of two proteins p75 and p40, the first probiotic bacterial proteins found to affect apoptosis and epithelial cell proliferation. By promoting intestinal epithelial homeostasis through specific signaling pathways these probiotic bacterial components were suggested to be useful for preventing cytokine- mediated GI diseases [33,34].

We can speculate that the LAB producing a higher level of fatty acids, is considered as an energy source for non-neoplastic epithelial colon cells [35], will be the ones with superior anti-tumorigenic activity and the ability to reduce colonic lesions, preventing tumor development and reducing cancer risk**.**

Conclusion

Compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103, *L. reuteri* NCIMB 701359 presents a relevant potential biotherapeutic candidate that can be considered in probiotic formulations for CRC. Findings described the characteristic FFA profile of *L. reuteri* NCIMB 701359 that produced higher amounts of SCFAs. *L. reuteri* NCIMB 701359 had a significant anti-proliferative effect that correlated with the levels SCFAs secreted in CM. This suggests that a mechanism of action by which the fermentation of non-digestible compounds exert a beneficial effect by modulating apoptosis and proliferation in cancer cells to a greater extent and probably due to the participation of other compounds (e.g. phenolic fatty acids derivatives and biopetides). These conclusions emphasized concerns regarding the use of *L. reuteri* NCIMB 701359 as cancer-causing lesions preventer by exerting prebiotic-like effects. Therefore, we suggested *L. reuteri* NCIMB 701359 as a propionate-producer bacterium that process comparable anti-cancer activity in CRC with some other LAB and are able to make the intestinal microbiota a target for nutritional intervention and a factor influencing the biological activity of other food compounds acquired orally. It also has a potential to be an effective component of a functional food strategy for tumor growth inhibition and cancer prevention that could be introduced as a biotherapeutic agents in CRC.

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Conflicts of Interest

All authors have no actual or potential conflicts to disclose.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, et al. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917.

2. O'Connell MJ, Campbell ME, Goldberg RM, Grothey A, Seitz JF, et al. (2008) Survival following recurrence in stage II and III colon cancer: Findings from the ACCENT data set. J Clin Oncol 26: 2336-2341.

3. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA Cancer J Clin 63: 11-30.
4. Cerella C, Sobolewski C, Dicato M, Diederich M (2010) Targeting COX-2 expression by natural compounds: A promising alternative strategy to synthetic COX-2 inhibitors for cancer chemoprevention and therapy. Biochem Pharmacol 80: 1801-1815.
5. Gianotti L, Morelli L, Galbiati F, Rocchetti S, Coppola S, et al. (2010) A randomized double-blind trial on perioperative administration of probiotics in colorectal cancer patients. World J Gastroenterol 16: 167-175.
6. Kahouli 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.
7. Liu Z, Qin H, Yang Z, Xia Y, Liu W, et al. (2011) Randomised clinical trial: the effects of perioperative probiotic treatment on barrier function and post-operative infectious complications in colorectal cancer surgery–a double-blind study. Aliment Pharmacol Ther 33: 50-63.
8. Goldin BR, Gorbach SL (1980) Effect of Lactobacillus acidophilus dietary supplements on 1,2-dimethylhydrazine dihydrochloride-induced intestinal cancer in rats. J Natl Cancer Inst 64: 263-265.
9. Urbanska AM, Bhathena J, Martoni C, Prakash S (2009) Estimation of the potential antitumor activity of microencapsulated *Lactobacillus acidophilus* yogurt formulation in the attenuation of tumorigenesis in Apc(Min/+) mice. Dig Dis Sci 54: 264-273.
10. Verma A, Shukla G (2013) Probiotics *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* suppresses DMH-induced pro-carcinogenic fecal enzymes and pre-neoplastic aberrant crypt foci in early colon carcinogenesis in Sprague Dawley rats. Nutr Cancer 65: 84-91.
11. Escamilla J, Lane MA, Maitin V (2012) Cell-free supernatants from probiotic *Lactobacillus casei* and *Lactobacillus rhamnosus* GG decrease colon cancer cell invasion *in vitro*. Nutr Cancer 64: 871-878.
12. Orlando A, Refolo MG, Messa C, Amati L, Lavermicocca P, et al. (2012) Anti-proliferative and pro-apoptotic effects of viable or heat-killed *Lactobacillus paracasei* IMPC2.1 and *Lactobacillus rhamnosus* GG in HGC-27 gastric and DLD-1 colon cell lines. Nutr Cancer 64: 1103-1111.
13. Russo F, Orlando A, Linsalata M, Cavallini A, Messa C (2007) Effects of *Lactobacillus rhamnosus* GG on the cell growth and polyamine metabolism in HGC-27 human gastric cancer cells. Nutr Cancer 59: 106-114.
14. Coakley M, Ross RP, Nordgren M, Fitzgerald G, Devery R, et al. (2003) Conjugated linoleic acid biosynthesis by human-derived Bifidobacterium species. J Appl Microbiol 94: 138-145.
15. Sieber R, Collomb M, Aeschlimann A, Jelen P, Eyer H (2004) Impact of microbial cultures on conjugated linoleic acid in dairy products—a review. International Dairy Journal 14: 1-15.
16. Stewart ML, Savarino V, Slavin JL (2009) Assessment of dietary fiber fermentation: Effect of *Lactobacillus reuteri* and reproducibility of short-chain fatty acid concentrations. Mol Nutr Food Res. 53: S114-S120.
17. De Boever P, Wouters R, Verschaeve L, Berckmans P, Schoeters G, et al. (2000) Protective effect of the bile salt hydrolase-active *Lactobacillus reuteri* against bile salt cytotoxicity. Appl Microbiol Biotechnol 53: 709-714.
18. Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN (1999) Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology 116: 1107-1114.
19. Grabig A, Paclik D, Guzy C, Dankof A, Baumgart DC, et al. (2006) *Escherichia coli* strain Nissle 1917 ameliorates experimental colitis via toll-like receptor 2- and toll-like receptor 4-dependent pathways. Infect Immun 74: 4075-4082.
20. Kim Y, Whang JY, Whang KY, Oh S, Kim SH (2008) Characterization of the cholesterol-reducing activity in a cell-free supernatant of *Lactobacillus acidophilus* ATCC 43121. Biosci Biotechnol Biochem. 72: 1483-1490.
21. Andreotti PE, Cree IA, Kurbacher CM, Hartmann DM, Linder D, et al. (1995) Chemo-sensitivity testing of human tumors using a microplate adenosine triphosphate luminescence assay: Clinical correlation for cisplatin resistance of ovarian carcinoma. Cancer Res 55: 5276-5282.
22. Dubey UK, Mistry VV (1996) Effect of bifidogenic factors on growth characteristics of bifidobacteria in infant formulas. J Dairy Sci 79: 1156-1163.
23. 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.
24. Desbois AP, Smith VJ (2010) Antibacterial free fatty acids: Activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol 85: 1629-1642.
25. Bazzan AJ, Newberg AB, Cho WC, Monti DA (2013) Diet and nutrition in cancer survivorship and palliative care. Evid Based Complement Alternat Med 2013: 917647.
26. Soto C (2013) Effect of isomalto-oligosaccharide and gentio-oligosaccharide on the growth and fatty acid profile of *Lactobacillus plantarum*. Electronic Journal of Biotechnology 16: 8.
27. Laparra JM, Sanz Y (2010) Interactions of gut microbiota with functional food components and nutraceuticals. Pharmacol Res 61: 219-225.
28. Andresen L, Hansen KA, Jensen H, Pedersen SF, Stougaard P, et al. (2009) Propionic acid secreted from propionibacteria induces NKG2D ligand expression on human-activated T lymphocytes and cancer cells. J Immunol 183: 897-906.
29. Gamet L, Daviaud D, Denis-Pouxviel C, Remesy C, Murat JC (1992) Effects of short-chain fatty acids on growth and differentiation of the human colon-cancer cell line HT29. Int J Cancer 52: 286-289.
30. Hinnebusch BF, Meng S, Wu JT, Archer SY, Hodin RA (2002) The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. J Nutr 132: 1012-1017.
31. Bergman EN (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70: 567-590.
32. Al-Lahham SaH, Peppelenbosch MP, Roelofsen H, Vonk RJ, Venema K (2010) Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. Biochim Biophys Acta 1801: 1175-1183.
33. Yan F, Cao H, Cover TL, Whitehead R, Washington MK, et al. (2007) Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. Gastroenterology 132: 562-575.
34. Yan F, Liu L, Dempsey PJ, Tsai YH, Raines EW, et al. (2013) A *Lactobacillus rhamnosus* GG-derived soluble protein, p40, stimulates ligand release from intestinal epithelial cells to transactivate epidermal growth factor receptor. J Biol Chem 288: 30742-30751.
35. Prakash S, Tomaro-Duchesneau C, Saha S, Rodes L, Kahouli I, et al. (2014) Probiotics for the prevention and treatment of allergies, with an emphasis on mode of delivery and mechanism of action. Curr Pharm Des 20: 1025-1037.