PRECLINICAL INVESTIGATIONS AND FUNCTIONAL STUDIES OF A NOVEL ACTIVE *LACTOBACILLUS* FORMULATION FOR POTENTIAL USE IN THE PREVENTION OF COLORECTAL CANCER.

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To my wonderful parents, my husband, my daughter, and all the rest of my family and friends, for their compassion and continuous support they provided during years of my training.

To my father who invested in my future far more than he ever could
To my mother who taught me of passion and perseverance
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To my husband whose love, patience and encouragements kept me going when I was at my lowest
To my daughter who taught me to be ever selfless and loving. May she follow the path of knowledge
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To my Tunisia, to its dunes, hills, and Mediterranean wonders
To my Canada the land where I chose to reborn, the land that welcomed me with open hands, the land where I was challenged and thrived beyond boundaries, the land that has become and will always be my home
I dedicate this modest work.

"Educationis radices amarae, fructus dulcis. Uno modo, certa, manifesta ratio practica; finem, in finem. Secundo, tui necessarii sunt ad fine; sapientiam pecuniae materia et modos. Tertio, adjust omne quod est ad finem. Nos quid debeamus facere saepius. Excellentia ergo non est actus, sed habitus"

"The roots of education are bitter, but the fruit is sweet.
First, have a definite, clear practical ideal; a goal, an objective. Second, have the necessary means to achieve your ends; wisdom, money, materials, and methods. Third, adjust all your means to that end. We are what we repeatedly do. Excellence, then, is not an act, but a habit".
Aristotle.

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PREFACE

In accordance with the McGill University Thesis Preparation Guidelines, as an alternative to the traditional thesis format, I have elected to take the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more accepted or published papers. This collection of articles, of which I am the first author along with other co-authors, are presented in Chapters 3, 4, 5, 6, 7, and 8, each divided into sections consisting of an abstract, introduction, materials and methods, results, discussion, and table and figures. An abstract, introduction, literature review, a final summary of results, conclusions, and a bibliography are included according to McGill guidelines.

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LIST OF ABBREVIATIONS

1H NMR: ^{1}H-nuclear magnetic resonance spectroscopy

APC: Adenomatous polyposis coli

ATCC: American Type Culture Collection

AUC: Area under the curve

CD3: Cluster of differentiation 3

CFU: Colony forming unit

CRC: Colorectal cancer

CRP: C-reactive protein

DAD: Diode array detector

DI/LC-MS/MS: direct injection liquid chromatography-tandem mass spectrometry

DMEM: Dulbecco's Modified Eagle Medium

FAO: Food and Agriculture Organization of the United Nations

FBS: Fetal bovine serum

FDA: American Food and Drug Administration

FFA: Free fatty acid

GALT: Gut-associated lymphoid tissue

GLM: General linear model

GIT: Gastrointestinal tract

GRAS: Generally recognized as safe

HPLC: High-pressure liquid chromatography

IBA-1: Ionized calcium binding adaptor molecule 1

IL: Interleukin

KC/GRO: Keratinocyte chemoattractant/human growth-regulated oncogene

LAB: Lactic acid bacteria

MALT: Mucosa-associated lymphoid tissue

Min: Multiple intestinal neoplasia

MRS: de Man, Rogosa and Sharpe

NCIMB: National Collection of Industrial, Food and Marine Bacteria

NIH: National Institutes of Health

NSAID: Non-steroidal anti-inflammatory drugs

PBS: Phosphate buffered saline

PCA: principal components analysis

PLS: Partial least squares discriminant analysis

PPAR-γ: Proliferator-activated receptor-γ

ROS: Reactive oxygen species

SEM: Standard error of the mean

SIF: Simulated intestinal fluid

AIJ: Artificial intestinal juice

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor alpha

IFNγ: Interferon gamma

VIP: Variable importance projection

WHO: World Health Organization

ZDF: Zucker diabetic fatty

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ABSTRACT

Colorectal cancer (CRC) is one of the leading causes of cancer-related death worldwide. The incidence of CRC influenced by several host and acquired factors, including dietary habits. An emphasis on the role of commensal bacteria revealed the potential effectiveness of probiotics in CRC prevention. Probiotics can modulate gut microbial populations toward a healthy profile and reduce the incidence and possibly recurrence of CRC in patients. There is still, however, a lack of research regarding the identification of potent probiotic bacteria and need to fully understand the potential mechanisms of action in reducing CRC risk, delaying intestinal neoplastic transformations, as well as halting CRC-associated inflammation. The purpose of this project is to identify potent probiotic bacterial strains and design a novel active probiotic *Lactobacillus* formulation as a CRC biotherapeutic. Specifically, in this thesis, many *Lactobacillus* strains, from *L. reuteri* or *L. fermentum* species, were screened for their anti-proliferative activity and fatty acid production, *in vitro*. This screening led to the identification of the most active candidate that was further validated *in vivo* for its anti-proliferative activity, in a genetically induced mouse model of CRC. Results indicated that the designed *Lactobacillus* formulation successfully reduced intestinal cellular and tumor proliferation and multiplicity in the intestines of *Apc* $^{Min/+}$ mice. The *Lactobacillus* biotherapy induced variations in the levels of exogenous (ingested) and endogenous metabolites both systemically and in the gut through the modulation of diverse metabolic pathways. The introduction of probiotic induced a different metabotype in the CRC animal model and showed the attenuation of systemic (INF-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α) and local (CD3 and IBA-1) inflammation in the intestines of *Apc* $^{Min/+}$ mice. This novel set of metabolomic findings indicated the potential of the *Lactobacillus*-based probiotic formulation as a biotherapeutic for CRC treatment and prevention. Further molecular studies are necessary for the full demonstration of *Lactobacillus* probiotic mechanisms and its potency in the management of CRC and possibly inflammation-associated health conditions.

RÉSUMÉ

Le cancer colorectal (CCR) est l'une des principales causes de décès liés au cancer dans le monde entier. L'incidence du CCR est influencée par plusieurs facteurs hôte et acquis, comme les habitudes alimentaires et la nutrition. L'accent mis sur le rôle des bactéries commensales a révélé l'efficacité potentielle des probiotiques dans la prévention du CCR. Les probiotiques peuvent moduler les populations microbiennes intestinales vers un profil sain et réduire le risque et éventuellement la récurrence du CCR chez les patients. Il reste, cependant, un manque de recherche concernant l'identification des souches de bactéries probiotiques efficaces et un besoin de comprendre les mécanismes d'action potentiels pour inhiber le risque du CCR, ce qui retarde les transformations néoplasiques intestinales, ainsi que la diminution de l'inflammation associée au CCR. Dans ce projet, l'objective c'est d'identifier les souches bactériennes probiotiques puissantes et concevoir une nouvelle formulation de probiotique *Lactobacillus* actives en tant que biothérapeutique de CCR. Plus précisément, dans cette thèse, de nombreuses souches de *Lactobacillus*, des espèces *L. reuteri* ou *L. fermentum*, ont été criblées pour leur activité de production d'acide gras et leur effet anti-prolifération *in vitro*. Ce processus de sélection a permis l'identification du candidat le plus actif qui a en outre été validée *in vivo* pour son activité anti-prolifération dans un modèle du CCR de souris génétiquement modifiées. Les résultats ont indiqué que la formulation *Lactobacillus* a réussi à réduire la prolifération cellulaire et la multiplicité des tumeurs dans les intestins des souris $Apc^{Min/+}$. La biothérapie de *Lactobacillus* a induit des variations dans la concentration des métabolites exogènes et endogènes à la fois par voie systémique et dans les intestins, par l'intermédiaire de la modulation de diverses voies métaboliques. L'influence du probiotique était induite par l'apparition d'un metabotype différent dans le modèle animal CCR. Le traitement probiotique a montré l'atténuation systémique (INF-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, et TNF-α) et locale (CD3 et IBA-1) de l'inflammation dans les intestins des souris $Apc^{Min/+}$. L'ensemble de ces observations métabolomiques et immunitaires a indiqué le potentiel de la formule probiotique à base de *Lactobacillus* en tant que biothérapeutique pour la prévention du CCR. D'autres études moléculaires sont nécessaires pour la pleine démonstration des mécanismes probiotiques de la formule *Lactobacillus* et sa puissance dans la gestion de CCR et peut-être aussi dans les conditions de santé associées à l'inflammation chronique.

CHAPTER 1. INTRODUCTION, RESEARCH HYPOTHESIS, THESIS OBJECTIVES AND OUTLINE

1.1. General introduction

Hippocrates said, “Let food be thy medicine and medicine thy food” [1]. Hippocrates, Galen and Avicenna believed “the stomach was the home of illness and diet was the main medicine.” Avicenna had made it the law of medicine, which is no longer the case in modern medical practices [2]. Still, in the last few decades, investigators started compiling evidence on the relevance of microorganisms residing the digestive system, the balance of which was associated with digestive, anti-cancer, anti-inflammatory, and neurological functions in the host [3, 4]. CRC and other CRC-associated GI conditions (e.g., IBD, IBS) appear to be increasing with our adaptation to a modern lifestyle. This includes consumption of processed foods and abandoning traditional ways of healthier dietary habits adopted for centuries, such as proper preparation of food and the use of fermentation to diminish anti-nutrients and enhance nutritional value. With changes in lifestyle in industrialized countries, the use of chemicals and processed foods has increased, while the use of fermented foods has diminished. Certain fermented foods like Natto, Kefir, Sauerkraut, Kombucha, and Yoghurt, etc., contain a complex and a significant population of beneficial bacteria, which play an essential bioactive role in improving the host metabolism, leading towards a healthier lifestyle and longevity of life [5-7].

CRC and CRC–associated GI conditions are emerging as one of the growing issues of public health concern with a global mortality of million deaths worldwide [8-10]. In 2014 the incidence of CRC cases reported was estimated to be 71,830 men and 65,000 women, with a mortality of 26,270 men and 24,040 women. Even though the lifetime probability of a CRC diagnosis is 4.7% in women and 5.0% in men, these subjects are likely to suffer other forms of GI conditions, such as CD and UC, which eventually develop towards CRC at later stages. In the last decade, CRC rates have increased from 2001 to 2010 among adults (< 50 years). CRC survival rate was reduced to 70.4% to 12.5% for patients with regional and distant-stage disease, respectively [11]. Only 40% of patients with CRC were diagnosed when the disease was localized had a maximum survival of 5-years [12]. Furthermore, in CRC major symptoms include rectal bleeding, abdominal pain, and change in bowel habits, anemia, occult bleeding, nausea, vomiting, and fatigue [13]. Patients with CRC, usually suffer from a poor intestinal epithelial barrier function, also known as “leaky gut”. A strong association between increased permeability (lack/poorly developed tight junctions) of the colon epithelium and tumor development has been proposed [14]. This relationship has led to systemic and intestinal

inflammation with a shift of microbiome towards a pathological state. CRC is associated with microflora imbalance, caused due to the reduction of *Lactobacillus* population and a nincrease of *Bacteroides/Prevotella* in the gut [15-18]. While, conventional CRC treatments lack efficacy, have long follow-up routines, and possess toxic side-effects with a risk of disease recurrence in a few years, the use of chemopreventive methods and biotherapeutics was suggested as the most promising therapeutic strategy [19].

SCFAs and antioxidants, such as conjugated linoleic acid (CLA), ferulic acid (FA), and butyrate are considered as anti-inflammatory and anti-carcinogenic bioactive molecules, which are available from dietary intake of probiotic rich food or are generated from the human microflora in the gut. These SCFAs present energy source for colonocytes and beneficial gut bacteria. At the cellular level, SCFAs have demonstrated the ability to induce apoptosis in tumor cells, affect DNA methylation and cell cycle. When orally administrated, these anti-oncogenic compounds were readily absorbed in the gut, before reaching the large intestines, without affecting the composition of gut microflora [3, 20, 21]. Probiotic bacteria digested by the host present a continuous delivery of active molecules (SCFAs) that improves the intestinal epithelium and the growth of other commensal bacteria in the lumen. For instance, some *L. reuteri, L. fermentum, L. acidophilus* and *L. rhamnosus* possess the ability to release higher levels of SCFAs, FA, and CLA by direct conversion of substrates (e.g. linoleic acid, carbohydrates) or by providing needed substrates (e.g. lactic acid) for gut flora to metabolize. This metabolic activity leads to increased metabolite concentrations in the intestinal lumen and beneficial modulation of gut bacteria. Several *Lactobacillus* strains showed features associated with decreased risk of CRC, metabolic syndrome, and IBD [20-25]. Thus, the selection of suitable candidates from screening several probiotic strains, and comparing them with previously researched bacteria is a crucial step for developing a probiotic formulation that can have a potential anticancer/anti-inflammatory effect on CRC.

1.2. Research hypothesis

A novel mechanism-based active probiotic (*Lactobacillus*) formulation can be screened characterized and used as a biotherapeutic for the treatment/prevention of CRC, based on its potent anticancer/anti-inflammation activity.

1.3. Research objectives

The main aim of this project are to identify and develop a novel active *Lactobacillus* formulation with potential for the treatment and/or prevention of CRC, with the following objectives:

1. To screen and study probiotic *Lactobacillus* bacteria (*L. reuteri* or *L. fermentum*) based on *in vitro* SCFA production, anti-proliferative action, and survival with exposure to challenging intestinal conditions.
2. To characterize the selected *Lactobacillus* (*L. reuteri* or *L. fermentum*) for metabolic activity, anti-proliferative, and pro-apoptotic effect on different CRC cells, without affecting the healthy colon cells and specifically identify the role of SCFAs compared to established probiotics.
3. To analyze and determine the synergistic effect of probiotic formulations, concerning anti-cancer effect in different colon cancer cell lines, while identifying the nature of bacterial bioactive compounds involved in inhibiting the proliferation of cancer cells.
4. To examine and validate the efficacy of the final *Lactobacillus* probiotic formulation *in vivo,* in inhibiting the intestinal tumor development and cellular proliferation markers in the intestine of genetically modified CRC $Apc^{Min/+}$ mice.
5. To demonstrate the effect of orally administered active *Lactobacillus* formulation on systemic and local inflammatory markers in a genetically modified CRC $Apc^{Min/+}$mice.
6. To explore the anti-tumorigenic mechanisms of the *Lactobacillus* biotherapy, by evaluating the probiotic effect on fecal and plasma metabolomes and identifying the most affected metabolic pathways in probiotic-treated $Apc^{Min/+}$mice.

1.4. Thesis outline

This existing body of work contains 11 chapters. A general introduction, along with details of the primary objectives are explained in **Chapter 1**. An in-depth literature review of the project is mentioned in **Chapter 2** (published). **Chapters 3-8** present the experimental, methodology, reasoning and analysis of the data, pertaining to each objective, as outlined in the thesis. These six chapters are original research manuscripts (three published, one submitted, and two to be submitted), for publication in peer-reviewed journals. **Chapter 9** provides a summary of the unique findings as presented in the thesis. **Chapter 10** concludes the thesis and underline the original contributions made to the existing knowledge. **Chapter 11** proposes future recommendations, aligned with the current research work.

CHAPTER 2. LITERATURE REVIEW

2.1. Colorectal cancer and probiotics

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in western countries [26]. According to the Canadian Cancer Society and the National Cancer Institute, in 2012, an estimated 23,300 Canadians and 1,43,460 Americans were diagnosed with CRC and 9,200 and 51,690, respectively, died of it. CRC develops by the accumulation of mutations, originating in stem cells at the base of the crypts [27] as non-cancerous polyps [28]. CRC incidence can be associated with a number of genetic factors such as germline mutations in the mismatch repair genes [29] and adenomatous polyposis coli (APC) gene [30]. In addition, genetic predispositions, environmental factors such as lifestyle and diet play an important role that can lead to CRC [31]. Researchers agree that a diet rich in red meat and processed food with lower consumption of fruits and vegetables, increases the risk of CRC incidence [32, 33]. This type of lifestyle and diet leads to disturbances in the intestinal environment, including the luminal content and microbiota [34]. The microbiota plays an important role in generating biochemical and physiological conditions that may increase the number of colonic pre-neoplastic lesions [35, 36]. Interestingly, consumption of beneficial bacteria can modulate the microorganisms of the gastrointestinal (GI) system [37]. Modulation of the imbalanced gut microbiota can provide a therapeutic and/or preventive effect by downgrading the carcinogenic stimulating events in the colon [38].

Probiotics are 'live microorganisms which, when administered in an adequate amount, confer a beneficial heath effect to the host' [39, 40]. Although probiotics have been used to manage a number of GI disorders such as diarrhea, infection and inflammation [41], their role in preventing and treating CRC is still under extensive investigation. In this context, probiotic bacteria should have potential features relevant to the development of CRC biotherapeutics. For example, Lactic acid bacteria (LAB) have shown protective effects against CRC by reinforcing and modulating the host's natural defence mechanisms [42]. LAB may also modify luminal secretions, reinforce the mucosal barrier [43], affect the epithelial cell proliferation [44] and reduce the exposure to toxic and carcinogenic compounds in the colon [45].

2.2. Probiotics and their role in modulating CRC-associated intestinal microbiota and gut integrity

2.2.1. Unbalanced gut microbiota in CRC

The normal human GI tract usually maintains a delicate balance of the microbiota with about 10^{12} bacteria per gram of luminal content and over 1000 species [46]. The gut microbiota is responsible for metabolizing nutrients, producing vitamins, endogenous hormones and toxic products (e.g. carcinogens), especially in the large intestine [47]. The microbiota is responsible for degrading organic compounds including food additives, bile salts and cholesterol [48, 49]. In CRC, the gut microbiota has been shown to be compromised and unbalanced [50]. Studies comparing human stool samples of healthy and CRC patients found a significant difference in bacterial genera [51]. Several *Lactobacillus* species from the intestinal flora were present in lower counts [52], while *Fusobacterium* [53, 54], *Bacteroides, Eubacterium, Proteobacteria* and *Prevotella* [51, 55], some *Salmonella* [56] and *Clostridium* species [57] were in higher counts in CRC patients.

Colon microbial carcinogenesis is a process that involves increased counts of CRC-causing bacteria such as *Bacteroides fragilis* that have been shown to induce colon tumor formation in multiple intestinal neoplasia (Min) mice [58]. It has therefore been suggested that a colon microbial imbalance may increase the proliferation of carcinogenic bacteria that enhance the production of carcinogenic compounds, secondary bile acids and cholesterol metabolites, driving oncogenic transformations in the epithelium and CRC pathogenesis [59]. However, further investigations are needed to establish this hypothesis.

2.2.2. Transient modulation of gut microbiota by probiotic bacteria

An unbalanced microbial composition can provide favorable conditions for colonic carcinogenesis [60]. It has been reported that a daily consumption of specific probiotic strains can improve human health, restore the microbiota balance [61] and inhibit intestinal colonization by pathogenic microorganisms (Fig.1). In a study using 1,2-dimethylhydrazine (DMH)-induced CRC rats, *Lactobacillus rhamnosus GG* administration reduced the number of coliforms and significantly elevated the count of lactobacilli [62]. According to a recent trial on goats, a mixture of *Lactobacillus reuteri* DDL 19, *Lactobacillus alimentarius* DDL 48, *Enterococcus faecium* DDE 39 and *Bifidobacterium bifidum* DDBA, significantly modified the microbiota by reducing enterobacteria and increasing bifidobacteria and LAB counts [63].

In a clinical trial with CRC patients, the oral administration of probiotic treatment increased the counts of *Bifidobacterium, Lactobacillus, and Enterococcus* and decreased the counts of *Escherichia coli* and *Staphylococcus aureus* [64, 65]. In addition, formulations of *Lactobacillus* and/or

Bifidobacterium strains such as *Lactobacillus gasseri* OLL2716: LG21 [66] and *Bifidobacterium lactis* Bb12 [67] have increased *Bifidobacterium* and *Lactobacillus* in the flora and decreased pathogen counts, including *Clostridium perfringens.*

For a better understanding of the action of probiotics on oncogenic/pathogenic bacteria further investigations are required. It was found that probiotic bacteria, delivered to the gut, rely on their antimicrobial, competitive, adhesive and anti-invasive properties to act on other microorganisms and regulate gut microbial activity [37, 68]. In addition, probiotics can provide intestinal and epithelial homeostasis, specifically improving epithelial barrier integrity [69]. Probiotics were found to produce antimicrobial compounds, such as bacteriocins, lactic acid, reuterin, hydrogen peroxide and deconjugated bile acids, which are essential substrates, required for the inhibition of pathogenic and carcinogenic microbes [70]. Some probiotic bacteria can bind or compete with pathogens for nutrients/molecules, adhere to epithelial cells and block the adherence of pathogens (competitive exclusion) [71, 72] and outcompete pathogens by forming biofilms [73].

2.2.3. Effects on the gut epithelial barrier

In the intestinal lumen, the epithelial cells form an impermeable barrier [74] and are covered with alayer of mucus[75]. This barrier protects the intestinal wall from physical and chemical damage, as well as from pathogens [76]. If pathogenic bacteria penetrate the intestinal epithelium, an inflammatory response is initiated at the site and in the adjacent intestinal mucosa causing damage to this epithelial barrier, increasing a risk of CRC [77]. It has been found that probiotic consumption can reinforce the epithelial barrier by preventing tight junction protein rearrangement [67], increasing the production of defensins and mucus by goblet cells [78], as well as reducing the leakage of harmful solutes, microorganisms and antigens (Fig.1) [75, 76]. A recent study indicated that components of *E. coli* strain *Nissle* 1917 decreased the permeability of 14C-mannitol, by restoring a disrupted epithelial barrier [79]. Preparations of *L. rhamnosus* GG and *B. lactis* Bb12, tested on CRC patients, have shown significant improvement of epithelial integrity in the intestinal lumen [80, 81]. Probiotics prevented epithelial barrier damage by inducing the production of cytoprotective heat-shock-proteins in stressed epithelial cells to maintain homeostasis [82] and promote cell survival [83, 84]. Interestingly, the epithelial cell signaling implicated is not only stimulated by bacterial metabolites but also by whole bacteria formulations [85].

2.2.4. Effects on the gut physicochemical conditions

Physicochemical properties of digesta in the colon such as bulking, water retention, pH, viscosity and levels of bile acids have been reported to be disrupted in CRC subjects [86-88]. This environment can be altered by probiotics to increase its resistance towards carcinogenesis. As demonstrated by Lan et al, upon exposure to probiotic propionibacteria short chain fatty acids (SCFAs, propionate and acetate), an acidic extracellular pH shifts cancer cell death from apoptosis to necrosis [89]. Moreover, a slight change in pH conditions (a lower pH in the feces) can block harmful enzymatic activity of the commensal bacteria and its binding to the surrounding epithelial cell wall and molecules [90]. The toxicity of fecal water content [91, 92] and the degree of water absorption by the colon, are one the first signs of irritation of the colonic mucosa [93]. Rats consuming *Bifidobacterium adolescentis* SPM1207 had less fecal water content than did control rats, decreasing colon toxicity, due to reduced exposure to soluble toxic compounds [61, 90]. A clinical trial on the daily consumption of *L. gasseri* OLL2716: LG21 for 12 weeks in CRC patients demonstrated a decrease in alkalosis in stool and fecal product synthesis (oxidized products from incomplete fermentation) such as putrescine, a cancer marker [63, 66]. Thus, mounting evidence suggests that the improvement of colonic environment by probiotic bacteria is strongly linked to a decrease in colonic irritation and lesions that cause inflammation and abnormal cell growth.

2.3. Effect of probiotics on metabolic and carcinogenic compounds

2.3.1. Activity of bacterial enzymes in CRC

An imbalanced gut microbiota may favour the secretion of bacterial enzymes such as β-glucuronidase, β-glucosidase, azoreductase [94] and nitroreductase, that produce carcinogens [95-97]. These harmful enzymes generate toxic metabolites such as aromatic amines [94, 98], transformed secondary bile salts [99], hydrogen sulphide [100], aglycones [101], acetaldehydes [102] and reactive oxygen species (ROS) [103]. β-Glucosidase, for example, can hydrolyse the detoxifying compound glucuronide, and produce other carcinogens. Bacterial β-glucuronidase produced by *Clostridium perfringens* [104] increases the genotoxicity of food mutagens, such as 2-amino-3-methylimidazo [4,5-f] quinolin (IQ) in the colon [105]. The bacterial enzymes azoreductase and nitroreductase, produced by bacteria such as *Bacteroides, Clostridium, Enterococcus, Salmonella,* and *Staphylococcus* [106] metabolize colourants, drugs, and aromatic nitro compounds to generate toxic aromatic amines [94]. *Enterobacter, Enterococcus, Streptococcus, Citrobacter* and *Escherichia* increase alcohol dehydrogenase activity and the production of acetaldehyde, a carcinogen [18].

2.3.2. Inhibition of harmful enzymatic activity

In CRC patients, bile acids and cholesterol are converted to microbial products faster in the colon leading to a disrupted enzymatic activity of the fecal flora and the generation of harmful enzymes [107]. These are reduced by the administration of probiotic formulations. Interestingly, several studies showed that *Bifidobacterium* or *Lactobacillus* consumption may limit the formation of toxic metabolites by decreasing the dehydroxylation of primary bile acids and reducing fecal deoxycholic acid concentrations [108]. *L. rhamnosus* GG has significantly shown to decrease the activity of β-glucuronidase [62]. Indeed, the activity of harmful bacterial enzymes can be reduced by certain LAB, as observed with *Butyrivibrio fibrisolvens* supplementation in a mouse CRC model [109] and with *Lactobacillus plantarum* given to rats with DMH-induced CRC [62]. Furthermore, *B. adolescentis* SPM1207 [61] and *B. adolescentis* SPM0212 [110] have shown to reduce the intestinal β-glucosidase, and β-glucuronidase [111], as well as tryptophanase and urease, which are producers of putrefactive products (indoles and ammonia) that are linked to higher incidence of CRC [112, 113].

2.3.3. Removal of carcinogenic products by probiotics

1.1.1.1 Carcinogenic compounds in the gut.

In patients with CRC, high oxidative and genotoxic levels have been observed in the gut [114, 115]. In fact, high levels of bile acids in the aqueous phase of feces were detected. Bile acids can exert cytotoxic effects on the colonic epithelium and increase malignant cell proliferation [116]. Bile acids (e.g., deoxycholic acid and lithocholic acid) are potential carcinogens and are negatively correlated with the levels of antineoplastic products in the colon, such as SCFAs [117]. The colonic mucosa is exposed to cancer-causing compounds [118, 119] that are mutagens and pro-mutagens such as N-methyl-N9-nitro-N-nitrosoguanidine (MNNG), IQ, benzo(α)pyrene and sodium azide [120-122]. Moreover, a high level of food-borne compounds [123] such as aflatoxin B1 (AFLB1) and 3-amino-I,4-dimethy-5H-pyrido (4,3-b) indole (TrpP-1), a fungal dietary contaminant, can also increase gut genotoxicity [118, 119]. Carcinogens such as *N*-nitroso compounds and indoles, generated from the intestinal metabolism of proteins, may increase fecal mutagenicity and increase CRC risk [124, 125]. Recent studies have demonstrated that probiotic bacteria can reduce carcinogen levels by deactivation or mechanical sequestration, reducing their impact on epithelial cells (**Figure 1)** [126].

1.1.1.2 Binding of carcinogens

L. rhamnosus GG and *L. rhamnosus* LC-705 were shown to bind carcinogens such as indole and AFLB1 and excrete them in the fecal matter [127, 128]. It was also demonstrated that

Bifidobacterium longum, *Lactobacillus acidophilus* and *Streptococcus salivarius* strains could bind heterocyclic amines and mutagens such as 2-amino-3,4-dimethylimidazo [4,5-f] quinoline (MeIQ), 2-amino-3-methyl-3H-imidazo [4,5-f] quinoline (MHIQ), and 5-phenyl-2-amino-l-methylimidazo [4,5-f] pyridine (PhMIP) and cause them to release in feces [129]. The administration of *L. reuteri* DDL 19, *L. alimentarius* DDL 48, *Enterococcus faecium* DDE 39 and *B. bifidum* DDBA to animals, and *L. gasseri*, to CRC patients have shown decreased mutagen fecal concentrations such as putrescine [130], cadaverine, and tryptamine (toxic amines) [66]. Better methodology for the investigation of binding capacity of probiotic bacteria as well as their effects on mutagens is still required.

1.1.1.3 Inactivation of carcinogens

LAB can decrease the activity of carcinogens such as MNNG and DMH by scavenging reactive intermediates and producing carcinogen-deactivating and antioxidative enzymes such as glutathione-S-transferase (GST), glutathione, glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase [131]. Remarkably, the treatment of colon cells with a supernatant from bacterial fermentation increased GST activity, an enzyme considered as having chemopreventive potential [132]. The probiotic suppression of DMH-induced rat CRC can be related to the detoxifying effect of antioxidant enzymes [133].

2.4. Anti-carcinogenic and antioxidant metabolites produced by probiotics prevent CRC.

Probiotics enhance the fermentation of dietary fibers [134] and increase the levels of antitumor compounds such as SCFAs, conjugated linoleic acids (CLAs) or phenols, with potential therapeutic effects against CRC [90, 135]. SCFAs are an energy source for colonocytes [136] and promote acidosis and apoptosis of CRC cells [137]. *B. lactis* increased the production of SCFAs promoting an acidic environment that fights the production of high levels of secondary bile acids [138] thus, lowering the incidence and multiplicity of colonic neoplasms [135]. A number of probiotic bacteria produce phenols with antioxidant capacity from lactic fermentation [139]. These bacteria also produce bioactive fatty acids such as CLA (Mladenova, Daniel *et al*. 2011), a group of isomers of linoleic acid, that possess anti-inflammatory and anti-carcinogenic properties [62]. During growth, *Pediococcus pentosaceus* 16:1, *L. plantarum* 2592 and *Lactobacillus paracasei* F19 produce antioxidants, which is equivalent to almost 100 mg of vitamin C [140]. This antioxidant capacity has shown to inhibit peroxidation and scavenge free radicals, preventing tumor formation [133]. On the

other hand, Watson has stated in his recent review that the antioxidant nutritional supplements may cause more cancers than they prevent [141]. It is clear that further research is needed in this field.

Several anti-carcinogenic and antioxidant probiotic products, potentially repress and prevent colon neoplastic growth [137] by the acceleration of apoptosis [134] and the inhibition of cancer cell proliferation. In addition, probiotic bacteria and their metabolites were found to promote cell differentiation [142, 143]; and reduce DNA damage in the colonic epithelium (**Table 2.2**) [144, 145].

2.5. Probiotics favorably modulate the host immune response to reduce CRC risk

Probiotics can both suppress and enhance the intestinal and systemic immune response, offering therapeutic and preventive options against inflammatory diseases and CRC [146, 147]. Probiotics affect immunological and cellular responses by enhancing the epithelial barrier and stimulating the production of anti-inflammatory, antioxidant and anti-carcinogenic compounds. Increasing evidence suggests that probiotics, interacting via Toll-like receptors (TLRs), induce anti-inflammatory cytokine production, initiate TNF production in epithelial cells, inhibit NF-κB in macrophages and influence the production of IL-8 needed for the recruitment of neutrophils [148]. Some strains of Lactobacilli can also promote regulatory T cell activity, stimulate bactericidal phagocytic activities of neutrophils in peripheral blood and natural killer (NK) cell activity involved in the suppression of tumorigenesis [86].

Lactobacillus and *Bifidobacterium* have been shown to decrease the expression of TLR-4, IL-8 secretion, and NF-κB activation [44], potentially caused by the release of bacterial products such as proteins, flagellin and LPS, etc. and to decrease the expression of peroxisome proliferator-activated receptors (PPAR) γ, a ligand for CLAs [149-151]. SCFAs have immunomodulatory functions that affect the inflammatory response, in some cases through interactions with G-protein-coupled receptors in the gut [152]. Recent animal and human studies have discussed the cellular and immunological effects of bacterial cells and products of recent probiotic formulations.

2.5.1. Animal studies

Lactobacillus fermentum FERM P-13857 and *Lactobacillus casei shirota* elicited IL-12 production in bone marrow cell-derived dendritic cells (DCs) in mice [146], which stimulates DCs and activates NK cells, involved in tumor-immune surveillance [146]. Also, *L. rhamnosus* GG and *B. adolescentis* bacterial extracts, given to rats, induced macrophage activation and significantly increased the production of TNF-α [62] and nitric oxide (NO) by macrophages [153], which can be cytotoxic or cytostatic to tumor cells [154]. Potential immunomodulatory and anti-tumorigenic properties of

924 microencapsulated *L. acidophilus* [155] and *Saccharomyces boulardii* [156] in a yogurt formulation
925 administered to $Apc^{Min/+}$ mice was demonstrated. This study reported a correlation between the
926 reduction of intestinal tumor growth, dysplasia and inflammation with the oral administration of
927 probiotics [155]. The mechanisms involved were related to the downregulation of extracellular-
928 signal-regulated kinases (Erk)1/2 activities through the inactivation of growth receptors such as
929 EGFR (epidermal growth factor receptor) EGFR and EGFR-Erk pathway [156].

930 **2.5.2. Human studies**

931 In a recent animal study, *L. gasseri* OLL2716: LG21 increased IL-1β, a cytokine that plays a central
932 role in the regulation of immune responses, and enhanced NK cell activity in the blood [66]. The
933 daily ingestion of fermented milk containing *L. casei shirota* for 3 weeks restored NK cell activity in
934 healthy subjects. Peripheral blood mononuclear cells (PBMCs) from healthy humans were cultured
935 in the presence of heat-killed *L. casei shirota*, which increased the activity of NK cells [157] that
936 plays a role in tumor-immune surveillance [158]. *L. rhamnosus* GG, *B. lactis* Bb12 and/or inulin
937 enriched with oligofructose demonstrated immune stimulatory effects by inducing the maturation of
938 DC [159], reinforcing the immune response against tumor cells [147]. This formulation has shown
939 anti-inflammatory effects by the activation of IL-10-secreting cells linked to the induction of
940 apoptosis in colon cancer and suppressing pro-carcinogenic factors [34, 149].

941 **2.6. Application of probiotics as a supplement to advanced-CRC treatments**

942 Based on their anticancer properties, probiotics can be used in combination with conventional CRC
943 therapies such as, surgery and chemotherapy [160]. Data obtained, although based on a limited
944 number of patients and samples, suggest an effective approach for achieving clinical benefits in
945 immune-compromised hosts by improving their intestinal environments [161]. The administration of
946 probiotics along with CRC treatment may alleviate the secondary effects related to chemotherapy
947 [162]. Moreover, clinical reports show that probiotics can improve the integrity of the gut mucosal
948 barrier and decrease infectious complications in surgical CRC patients [163]. Some of the recent
949 applications of probiotic strains in CRC are summarized in **Table 2.3**.

950 **2.6.1. With chemotherapy**

951 Recent studies showed the ability of LAB to enhance the apoptosis-induction capacity of 5-
952 fluorouracil (5-FU), a chemotherapeutic agent [160]. According to Osterlund et al., *L. rhamnosus*
953 GG supplementation reduced several undesirable effects of 5-FU-based chemotherapies such as the
954 frequency of severe diarrhea and abdominal discomfort [162]. Patients receiving *L. rhamnosus* GG

along with 5-FU-based regimens needed less hospital care, had less bowel toxicity, received fewer chemotherapy doses and suffered less from abdominal pain and diarrhea than patients with no probiotic administration [162]. Nagata et al., concluded from their study that the enteral administration of *Bifidobacterium breve Yakult* to cancer patients on chemotherapy was shown to prevent infections and particularly improve the fecal microbiota. The frequency of fever and the use of intravenous antibiotics were also reduced [161].

2.6.2. Effects on complications related to surgery

In patients with CRC, supplementation with viable probiotics, before surgery, can improve bacterial dysbiosis [164]. *L. casei Shirota* was given to patients whose colonic polyps were surgically removed in order to suppress the recurrence of CRC [157]. Infection following abdominal operation, considered as a factor affecting the morbidity of patients, was reduced using preoperative administration of probiotics. Patients who received daily encapsulated treatment containing *B. longum* BL-88, *L. acidophilus* La-11 and *L. plantarum* CGMCC No. 1258, before and after their operation, had better recovery of peristalsis, lower incidence of diarrhea [163] and reduced infection-related complications [163]. Likewise, Zhang and colleagues found that the preoperative use of viable *Bifidobacterium* stabilized the immune status and prognosis of patients undergoing CRC resection and diminished postoperative septic complications [164]. Probiotic mixtures supported the intestinal barrier function following CRC surgery, which may have prevented cancer recurrence [165]. Polypectomized patients and CRC patients who have undergone curative resection while receiving *B. lactis* and *L. rhamnosus*, had greater PBMCs producing IFN-γ and IL-2, both cytotoxic to cancer cells [159].

2.6.3. Effects on inflammation.

Lactobacillus johnsonii La1, given orally pre- and post-operatively, adhered to the colonic mucosa, reducing the counts of potentially pathogenic bacteria in the stool (enterobacteriaceae and enterococci). Gianotti and colleagues used *L. johnsonii* La1 in a formulation with *B. longum* BB536 and demonstrated the increased expression of naive and memory lymphocyte subsets, while reducing dendritic phenotypes dampening an over inflammatory response at the intestinal and distant sites in case of surgery [166]. In addition to alleviating several undesirable complications associated with CRC treatments, the administration of probiotics to patients may prevent cancer recurrence and improve their quality of life [165]. On the other hand, a mixture of probiotic bacteria: *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *L. paracasei* subsp. *paracasei* and *L. plantarum*, with

bioactive plant fibers β-glucans, inulin, pectin, resistant starch, post-operatively elevated the levels of anti-inflammatory cytokine IL-6 and prevented mild wound infection with fecal secretion. In this case, the synbiotic formulation did not have an anti-inflammatory effect, probably due to the absence of bowel cleaning [167]. As described, specific probiotic strains administered in different ways (mixture, period, and dose) were effective to a certain extent in bringing clinical benefits to CRC patients. However, more investigations are needed to improve probiotic formulations for better efficacy.

2.7. Significance and future directions of probiotic formulations in CRC

Very few reports demonstrate any limitations and negative aspects of probiotic oral supplementation. Some studies suggest that an increased bacterial translocation was related to mortality after supplementation with *Lactobacillus delbrueckii* UFV-H2b20 and *B. lactis* Bb12 in mice with DMH-induced injuries. These findings alert us to the potentially severe side effects associated with the use of probiotics under stressful situations, such as change in environmental and experimental conditions [168]. The variability observed in the documented benefits of probiotics in humans was shown to be dependent on the concomitant therapies and the health baseline status of the patient, the dosing and the addition of prebiotics or many strains into the formulation. Many reports brought to attention another important player minimizing the efficacy of orally administrated probiotics which is the loss in the viability of probiotics reaching the large intestine [169] Subsequently, microencapsulation, defined as the entrapment of viable cells in a polymer matrix, has been suggested to improve cell viability during GI transit [37, 170, 171]. Microencapsulation of probiotics can confer a significant resistance to gastric juice, thus protecting the bacterial cells during gastric and duodenal transit [172, 173]. Indeed, the use of artificial cell microcapsules allows for a '*pH controlled delivery'* of the probiotic bacteria through the gut. Concurrently, it allows the diffusion of oxygen, nutrients and metabolites while preventing white lymphocytes, antibodies, and cytokines from accessing the microcapsule [173-175]. As supported by previous research, this technology may assume a lot of importance in the near future for the development of active probiotic bacterial preparations in treating many diseases, including CRC.

Concurrently, recent research continues to support the idea that probiotic consumption may reduce tumor growth, modulate the host immune response and re-establish healthy gut conditions in CRC subjects. Recent studies continue to provide evidence that probiotic formulations have the potential to protect the gut and colon epithelial cells from toxic substances (digested or produced within the

intestine), from reactive metabolites and from pathogens or endogenous commensal bacteria [176, 177]. Several studies have shown the immunomodulatory impact of probiotics on the inhibition of tumor growth, as these probiotics modulate the cytokine production and signaling pathways that may initiate cancer or uncontrolled epithelial cell growth [18, 34, 178]. Research in this field still has to progress towards a solid understanding of the molecular interactions of the microorganisms with both healthy and compromised hosts [179]. The current treatments for CRC include invasive procedures and toxic drugs that not only attack cancer cells but also affect the normal cells [180]. In such a scenario, it appears challenging to portray probiotics as a therapy that can replace these treatments, but, the emerging outcomes of probiotic applications in CRC or other diseases (e.g. IBS, diabetes, allergies) [181] suggest the consideration and potential of probiotics for therapeutic and prophylactic purposes. Probiotics have shown clinical latency as a supplement for CRC patients especially when administrated prior/post-surgery or during prolonged hospitalization to manage symptoms related to the severity of the disease, side-effects and other complications related to the existing clinical treatments. However, further human studies are required to guide and to ascertain the decision of their establishment as a complementary treatment for CRC condition.

2.8. Acknowledgements

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2.9. Conflicts of interest

The authors have no conflicts of interest to disclose.

2.10. Figures and Tables

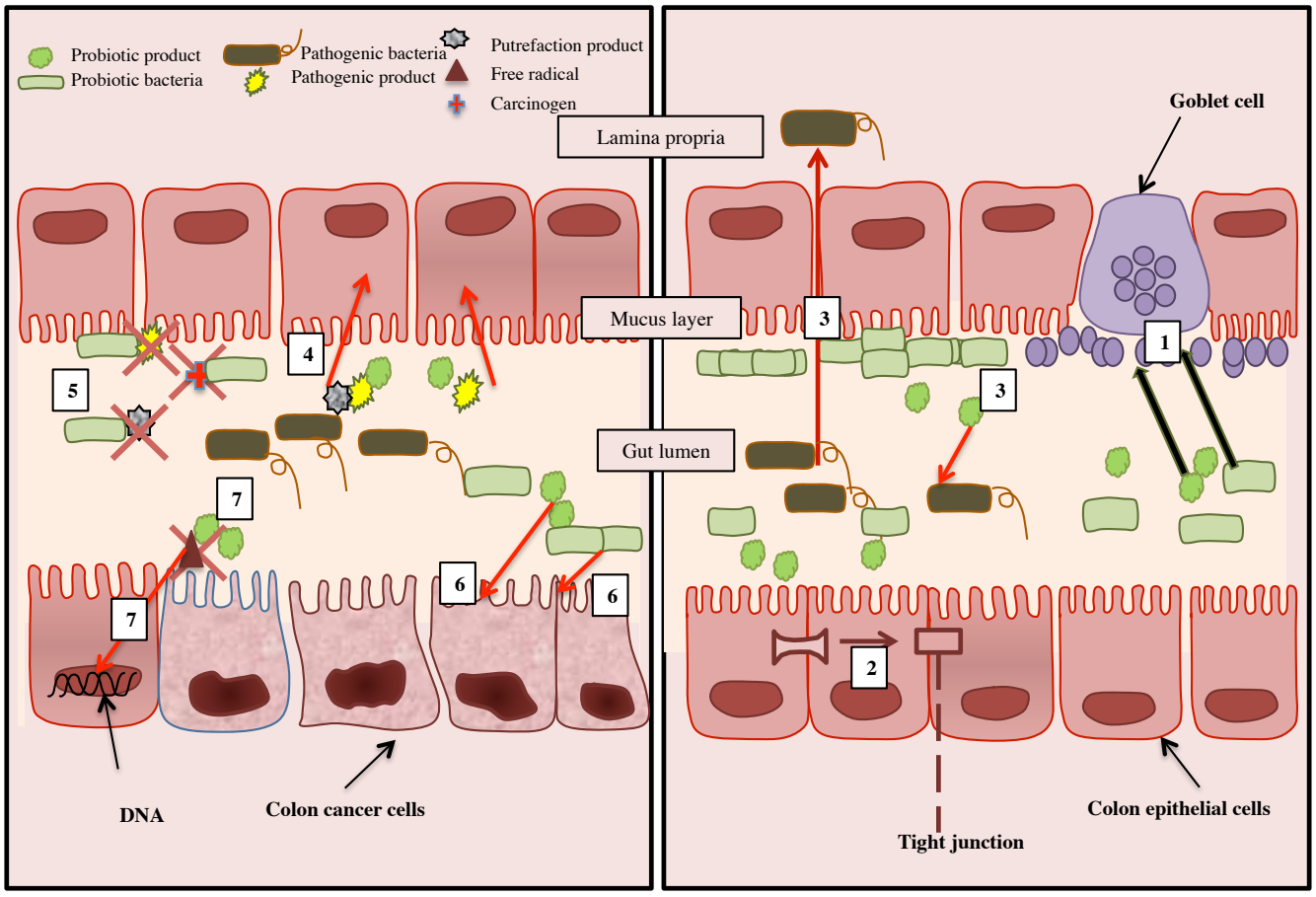

Figure 2.1: Potential mechanisms of action of probiotic bacteria in the improvement of the physicochemical conditions and the microbiota balance in the colon while producing beneficial metabolites and reducing toxic compounds.

(**1**) Enhancing mucus production from goblet cell. (**2**) Reinforcing intercellular integrity by increasing the integrity of apical tight junctions and produce beneficial metabolites that improve the growth of epithelial cells. (**3**) Antimicrobial activity by blocking pathogen entry into the epithelial cells and also by producing antimicrobial compounds. (**4**) Reduce carcinogens production by inhibiting the activity of harmful enzymes that generate potential carcinogens from bile salts, food and other products. (**5**) Detoxification of toxic compounds by decreasing fecal putrefaction, degrading and binding certain molecules. (**6**) Inhibiting cancer cell proliferation by producing anti-carcinogenic metabolites that suppress malignant growth and induce apoptosis in cancer cells. (**7**) Decreasing oxidative stress and genotoxicity by producing antioxidants that scavenge free radicals, such as reactive oxygen species (ROS), and reduce DNA damage in colon cells.

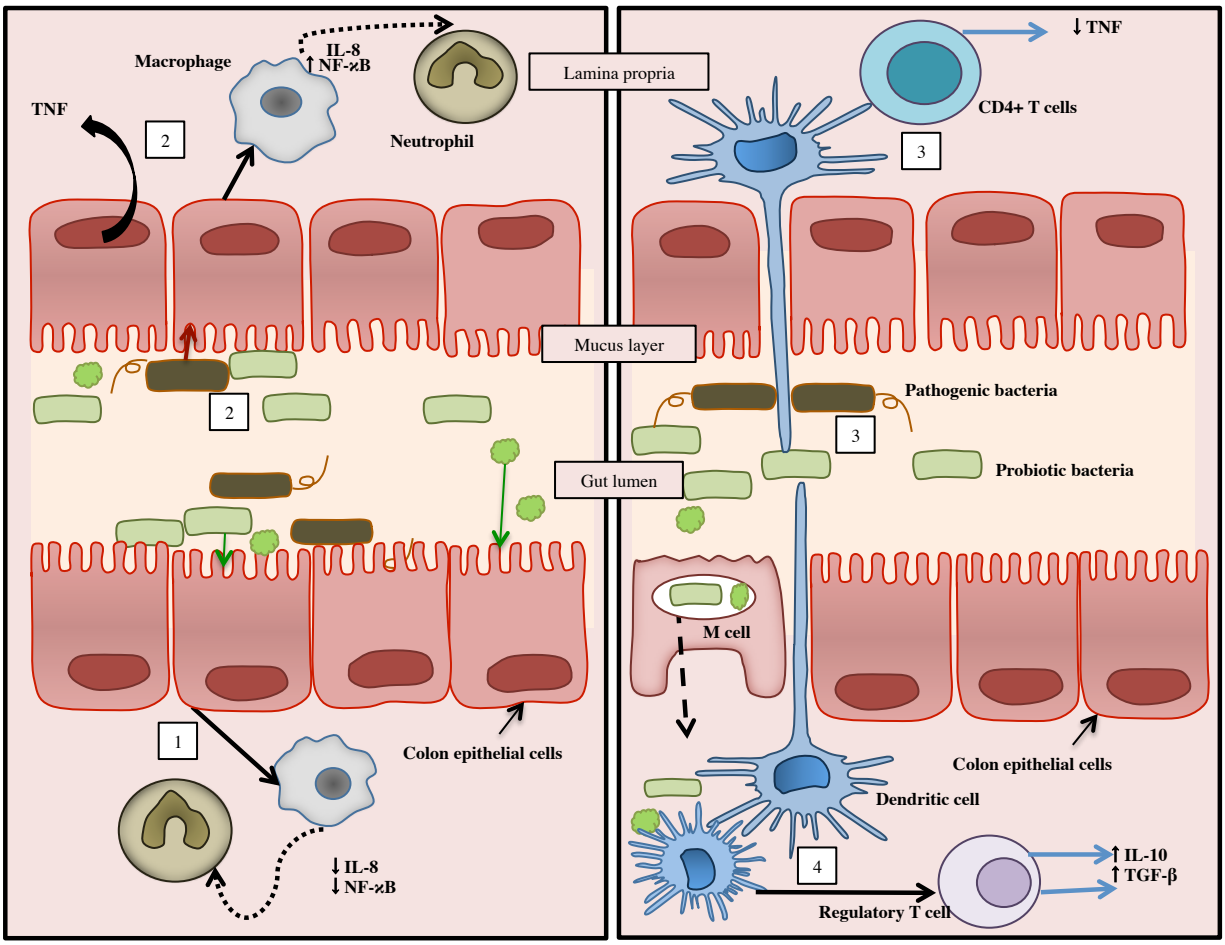

Figure 2.2: The mechanisms of action of probiotic bacteria in the induction of the immune and the anti-inflammatory response in the gut: involvement of macrophages and dendritic cells.

(**1**) Preventing the inflammatory response against pathogens: Probiotic bacteria may prevent the immune response against pathogens by inhibiting NF-κB in macrophages thus decreasing IL-8 (prevent the recruitment of neutrophils). (**2**) Enhancing the inflammatory response through macrophages: Probiotic bacteria activate the immune response by inducing epithelial cells to produce TNF, activation of NF-κB in macrophages thus IL-8 production. (**3**) Damping the inflammatory response through DCs: probiotic bacteria reduce the communication between DCs and pathogenic bacteria and prevent activation of CD4+ T-cells and TNF production as an inflammatory response to pathogens. (4) Inducing the anti-inflammatory response through DCs: probiotic bacteria can stimulate the innate immune system by signalling DCs which activates T-regulatory cells and induces the production of anti-inflammatory cytokines, mainly IL-10 and TGF-ß.

Table 2.1: Probiotic potential mode(s) of action in mitigating the factors responsible for CRC.

Factors linked to CRC	Potential mode(s) of action of probiotics in mitigating factors of CRC
-Unbalanced gut microbiota: ↑*Bacteroides, Eubacterium, Fusobacterium, Proteobacteria, Salmonella* and *Prevotella.*	-Modulation of gut microbiota: ↑*Bifidobacterium, Lactobacillus,* ↓*Escherichia, Staphylococcus.*
-Disrupted colonic physicochemical conditions: Alkalosis, Water absorption in the colon, Incomplete fermentation, Genotoxic fecal water content.	-Improvement of colonic physicochemical conditions: ↓pH, Improve fermentation ↓Putrefactive products: Putrescine, cadaverine, and tryptamine.
-Damaged epithelial barrier: Normal epithelial cell death, ↑Permeability, Tight junction protein rearrangement, Pathogen translocation,	-Reinforce gut epithelial barrier: ↑Defensins and mucus production by goblet cells, ↑Cytoprotective heat-shock-proteins, ↑Normal epithelial cell survival.
-↑Harmful bacterial enzymes: β-glucuronidase, β-glucosidase, azoreductase, nitroreductase, alcohol dehydrogenase.	-↓Bacteria producing harmful enzymes: *Bacteroides, Clostridium, Enterococcus, Salmonella, Enterobacter, Streptococcus, Citrobacter, Escherichia* and *Staphylococcus*.
-↑Carcinogenic products: IQ, tryptophanase, urease, acetaldehyde, MNNG, AFLB1, TrpP-1, *N*-nitroso compounds, aromatic amines, sodium azide, benzo(α)pyrene, transformed secondary bile salts, aglycones hydrogen sulphide and indoles.	-Binding, deactivation of carcinogens. -↑Detoxifying enzymatic antioxidants: GTS, glutathione, glutathione reductase, Glutathione peroxidase, superoxide dismutase and catalase.
-↑DNA damage -↑Abnormal cell growth: Dysplasia, tumor formation	-↑Anti-carcinogenic metabolites: SCFA, CLA, phenols. -↑Apoptosis, -↑Differentiation in cancer cells.
-Intestinal inflammation: ↑NF-κB, IL-8, IL6. -↓Immune response against tumor cells.	-↓Intestinal inflammation: ↓TLR-4, ↑IL-10, IL-8 secretion, NF-κB activation, -Immune response against tumor cells:

1073 Table 2.2: Inhibition of cancer cell proliferation and prevention of malignant transformation-
1074 Effects and mechanisms of probiotics.

Probiotics	**CRC model and treatment**	**Effects**	**Potential mechanisms**	**References**
***Enterococcus faecium* RM11 and *L. fermentum* RM2.**	Caco-2 cells; Live probiotic cells and supernatant.	↓ Cell viability.	↑Adherence. ↑Apoptosis. ↑Pan-caspases.	[182]
Saccharomyces boulardii.	HT-29, SW-480 or HCT-116; Probiotic cells. Apc (Min/+) mice; Oral administration of probiotic cells.	↓Colony formation and induction of apoptosis. ↓Intestinal tumor growth and dysplasia.	↓EGFR-Erk and EGFR-Akt pathways. ↓EGFR and receptor tyrosine kinase signaling. ↓HER-2, HER-3, and insulin-like growth factor-1 receptor.	[156]
***L. delbrueckii* CU/22.**	HT-29 cells; Probiotic supernatant.	↑Apoptosis and necrosis.	↑Bacterial hydrogen peroxide and superoxide radicals.	[183]
***L. acidophilus* 606 EPS.**	HT-29 cells; Isolated cell-bound exopolysaccharides (cb-EPS).	↑Tumor cell death via autophagy. Alteration of cell morphology.	↑Beclin-1 and GRP78. ↓Bcl-2 and Bak regulation.	[145]
***L. rhamnosus* GG and *B. lactis* Bb12: (aleurone(+)).**	HT29 and LT97 cells; Fermentation supernatant.	↓ Cell growth.	↑Cell cycle arrest in G(0)/G(1) and alkaline phosphatase activity. ↑Apoptosis and p21 and WNT2B.	[134]
B. lactis* and *L. rhamnosus.	Caco-2 cancer cell line; Live probiotic bacteria.	↑Apoptosis.	BAX translocation, cytochrome c release, and caspase-9 and -3 cleavage.	[184]
Bacillus polyfermenticus.	Colon, breast, cervical and lung cancers and AOM-treated NCM-460 colonocytes; Bacterial cell free supernatant.	↓Colony formation on soft agar. ↓Carcinogen-induced colony formation by normal colonocytes. ↓Tumor growth.	↓ErbB receptor-dependent pathway. ↓ErbB2 and ErbB3 protein and mRNA expression. ↓E2F-1-dependent transcriptional regulation of cyclin D1.	[185]

	Tumors implanted in the skin of nude mice; Injection of bacterial cell free supernatant.			
***L. paracasei subp. Paracasei* M5, X12, *L. fermentum* K11, K14 and *L. casei strain* X11.**	HT-29 cells; Cell walls and cytoplasm extracts.	↓Cell proliferation.	↑Apoptosis. S-phase accumulation.	[186]
***S. thermophilus* 14,085 and *B. infantis* 14,603.**	HT-29 and Caco-2 cells; Extracts from fermented soymilk with organic solvants.	↓Cell proliferation.	Antitumor bioactive compounds from bacterial fermentation,	[139]
***L. plantarum* AS1.**	CRC induced by DMH in rats; pre- and post-treatment with 1 ml containing 10^9 CFU of *L. plantarum* AS1 in saline/day.	↓Mean tumor volume diameter and total number of tumors.	Altering lipid peroxidation and antioxidant enzyme activities in the colon and in the plasma.	[133]
Propionibacterium freudenreichii.	HGT-1 cells; Fermented milk supernatant.	↑Apoptosis ↑cytotoxicity of camptothecin, a drug used in chemotherapy.	↑Chromatin condensation and formation of apoptotic bodies. ↑DNA laddering and cell cycle arrest. ↑ ROS. ↑Caspase activation and cytochrome c release.	[187]

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1077 **Table 2.3: Clinical applications of probiotic formulations in CRC patients.**

Probiotics	Treatment	Trial design and CRC conditions	Clinical study outcomes	References
***L. rhamnosus GG* LGG and *B. lactis* Bb12.**	10^{10} CFU of *L. rhamnosus GG* LGG and *B. lactis* Bb12 +10 g of oligofructose-enriched inulin. In a capsugel. Orally. Daily for 12 weeks	Randomized, double-blinded, placebo-controlled trial. For 12 wks. 37 CRC and 43 polypectomized patients.	↑Fecal *Bifidobacterium* and *Lactobacillus*. ↓ *Clostridium perfringens*. ↓CR proliferation. ↓Fecal water-induced necrosis in cancer cells. ↓Exposure to genotoxins. ↓Secretion of IL- 2. ↑Production of IFN-γ.	[188]
***L. rhamnosus* GG and *B. lactis* Bb12.**	10^{10} CFU of *L. rhamnosus* GG and 10^{10} CFU f *B. lactis* Bb12 +10 g of inulin enriched with oligofructose. Encapsulated. Orally. Daily for 12 weeks	Randomized double-blinded, placebo-controlled trial. 34 CRC patients with curative resection and 40 polypectomised patients.	↑IL-2 secretion by activated PBMCs. ↑Capacity of PBMC to produce IFN-γ. Minor stimulatory effects on the systemic immune system.	[159]
***B longum* BB536 and *L. johnsonii* La1.**	10^{7} or 10^{9} CFU of a mixture of *B longum* BB536 and *L. johnsonii* La1. Orally. 2 daily doses for 3 days before and 5 days postoperatively.	Randomized, double-blinded. 31 Subjects with elective resection for CRC.	Probiotic adherence to the colonic mucosa. ↓pathogens. ↓ Dendritic phenotypes CD83-123, CD83-HLADR. CD83-11c.	[166]
***Pediococcus pentosaceus, Leuconostoc mesenteroides, L. paracasei* 19 and *L. plantarum* 2362.**	10^{10} CFU of each Lactobacilli + 10 g fibre. Orally. Every 8 hours 2 days before operation and at day 2 postoperatively till day 4.	Prospective double-blinded randomized placebo-controlled trial. 68 Patients having mechanical bowel cleaning prior to the operation.	↑IL-6 after 72 h.	[167]

***L. plantarum* CGMCC No. 1258, *L. acidophilus* La-11 and *B. longum* BL-88.**	2 x 10^{11}CFU *L. plantarum* CGMCC No. 1258**,** 1x10^{10} CFU of *L. acidophilus* La-11 and 5 x 10^{10} CFU of *B. longum* BL-88. Daily. Encapsulated formulation 6 days preoperatively and 10 days post-operatively.	100 Patients with CR carcinoma.	↓Bacterial translocation. ↑Transepithelial resistance. ↓Transmucosal permeation of horseradish peroxidase and lactulose/mannitol ratio. ↓Ileal-bile acid binding protein. Positive rate of blood bacterial DNA. ↑Mucosal tight junction protein expression. ↓Blood enteropathogenic bacteria. Post-operative recovery of peristalsis. Improved infectious-related complications. ↓Incidence of diarrhea.	[163]
***L. rhamnosus* LGG.**	2 x 10^{10} CFU of *L. rhamnosus* LGG. Daily for 24 weeks on cycle days 7–14, for 8 days/month.	150 Patients having 5-FU-based regimens.	↓Frequency of severe diarrhoea and abdominal discomfort. ↓Chemotherapy dose. ↓Abdominal discomfort and diarrhoea.	[162].
B. breve Yakult.	Enteral.	42 CRC patients on chemotherapy.	↓Risk infection. Improved fecal micro flora and intestinal environments. ↓Frequency of fever. ↓Intravenous antibiotics use.	[161]
L. casei Shirota.	After surgery	Patients with surgically removed colonic polyps were	↓ Recurrence of CRC with moderate/severe atypia.	[157]
***L. johnsonii* La1 and *B. longum* BB536.**	2 x 10^{7} *L. johnsonii* La1 and 2 x 10^{9} CFU/d *B. longum* BB536, Orally for 3 days pre- and 6 days post-operative.	21 CRC patients.	↓Oathogens. ↑Expression of naive and memory lymphocyte subsets. ↓Expression of dendritic phenotypes.	[166]

Bifidobacterium	Administration of viable bacteria with routine enteral nutrition.	60 Patients undergoing CRC resection.	↓Postoperative *Bifidobacterium*/*E.coli* (B/E) ratio as compared to the preoperative. ↑Both preoperative and postoperative B/E ratios. ↑Stool SIgA, while↓ serum IgG, IgM, IgA, IL-6, CRP. ↓Postoperative septic complications.	[164]
n/a.	One-day bowel preparation with probiotics fro 3 days	60 patients with colonic surgery.	Maintain the intestinal barrier function after surgery CRC.	[165]
***Enterococcus. faecium* M-74.**	For three months.	60 CRC patients with colonic adenoma.	↑B iopsies with intracellular bacteria in adenoma and carcinoma group. ↑Intraepithelial bacteria in patients with large bowel adenoma and carcinoma	[189]

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2.11. Summary of the Literature and Thesis Research Goals

Reviewing the direction of current research towards understanding the role of microbiota in the CRC risk, it has become imperative to understand and adopt the methodologies that may lead towards discoveries of new biotherapeutics in order to establish a novel intervention that incorporatesprobiotic bacteria as a potential CRC therapeutic agents. Limitations are not only associated to the current CRC treatments, but also to the selection, understanding and investigation of potential probiotic candidates. Available strains with established health effect still need further characterizations. Thus, this project aims to answer questions about which strains have more potential in CRC, according to which features, and what are the mechanisms of action involved depending on the strain. This work aim to screen, identify and select new LAB based on their growth kinetics, metabolic activity and ability actively produce anti-cancer factors in different conditions, *in vitro* or *in vivo*. Later, this study objective is to validate the efficacy of selected strains in a genetically modified rodent CRC model and explain the effect of this probiotic formulation on intestinal inflammation, immune modulation, and metabolic fluctuations.

PREFACE TO CHAPTERS 3 - 8

To answer the fundamental question of this thesis, this project investigated different hypothesis in evolving methodological steps, six chapters were structured and contained the research performed to explore each of the objectives of this project. As stated below, each chapter reports, describes, and discusses key findings studied in this project.

Chapter 3: In the context of developing novel biotherapeutics to manage and prevent CRC. *L. reuteri* bacteria were screened, and the most potent candidate was identified based on bacterial survival, the effect on colon cells, and SCFA bio-production.

Chapter 4: Following the selection of a potent *L. reuteri* strain, based on **Chapter 3**, the bacterium was compared to other known *Lactobacilli*, in terms of FFA profile, SCFA bio-production, inhibition of colon cancer cells, and effect on non-neoplastic colon cells.

Chapter 5: For the identification of more potent strains than *L. reuteri*, ferulic acid-producing probiotic bacteria *L. fermentum* possess important antioxidant and anti-inflammatory features relevant for CRC. The goal of this chapter was to select a *L. fermentum* that is more potent to produce higher SCFAs, survive simulated intestinal environment, and suppress colon cancer cells.

Chapter 6: Following the selection of the most potent *L. fermentum* strain, in **Chapter 5**, there was a need to evaluate their anti-cancer effect compared with other known *Lactobacilli*. The FFAs profile, SCFA production and efficiency, the effect on normal colon cells was determined compared to *L. acidophilus* and *L. rhamnosus* strains.

Chapter 7: *L. fermentum* showed higher anti-CRC potential than *L. acidophilus in vitro.* Thus, the combination of both *L. fermentum* and *L. acidophilus* was tested and compared as mixed and pure cultures. We verified, *in vitro,* that the formulation of both strains possesses synergetic actions in term of growth, resistance, anti-proliferative effect, and beneficial role in normal colorectal cell growth. The natures of active bacterial factors were estimated based on the cell inhibition and apoptosis induced in CRC cells. *L. fermentum-L. acidophilus* bacteriotherapy was then validated in a genetically modified CRC mice model. The efficacy of this bio-treatment was evaluated in term of reducing tumor count, and cellular proliferation markers in the intestine of $Apc^{Min/+}$ mice. With the significant reduction in tumor burden, Ki-67 and ß-catenin expressions were significantly downregulated in the normal mucosa or in intestinal tumors of $Apc^{Min/+}$ mice.

Chapter 8: As we demonstrated that *L. fermentum-L. acidophilus* formulation attenuated intestinal tumor proliferation *in vivo* in **Chapter 7***,* the goal of the research presented in this chapter was to investigate the metabolomics and inflammatory mechanisms associated with the probiotic anti-tumorigenic effect. Together NMR fecal and DI/LC-MS/MS plasma analysis demonstrated that *L. fermentum-L. acidophilus* biotherapy in $Apc^{Min/+}$ mice reduced the production of toxic/pro-cancer compounds and enriched the production of factors related to an anti-oncogenic metabolism. As well, when several fecal and plasma anti-inflammatory compounds increased, many of pro-inflammatory cytokines were hindered and IBA-1 and CD 3 inflammatory cell infiltration was limited in intestinal tumors or normal mucosal tissues of $Apc^{Min/+}$ mice

Chapter 9: This chapter provides a summary of the findings described in the thesis.

Chapter 10: This chapter details the claims of the original contributions to knowledge and conclusions.

Chapter 11: This chapter provides relevant recommendations and perspectives for future research.

During the course of this research project, I contributed to 17 original research articles and reviews, as the first author, in seven of them, and the rest, ten are currently published, in press or in progress. I also contributed to 29 research presentations, in 12 of which I was a presenting author. In this thesis, seven articles (in press, published or in progress) in which I am the first author were included.

Original research articles included in the thesis:

1. **Kahouli I.** et al. (2013) Probiotics with colorectal cancer (CRC) with emphasis on mechanisms of action and current perspectives. Journal of Medical Microbiology. 62, 1107–1123. **Article Published**

2. **Kahouli I.** et al. (2015). Screening and *in vitro* analysis of *Lactobacillus reuteri* strains for short chain fatty acids production, stability and therapeutic potentials in colorectal cancer. Journal of Bioequivalence and Bioavailability. Volume 7(1): 039-050. **(Article Published)**

3. **Kahouli I.** et al. (2016) Characterization of *Lactobacillus 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. **(Article submitted to Journal of Medical Microbiology).**

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2. **Kahouli I.** et al. (2015). *In vitro* study on the screening of *Lactobacillus fermentum* strains for potential against colorectal cancer by characterizing short chain fatty acids production, anti-proliferative activity and survival intestinal fluid. The 12th Annual Meeting of the NHPRS of Canada: Convergence: Multi-sector Integration for Advancing Transdisciplinary NHP R&D. August 15th -17th, 2015- London (ON), Canada. *(Poster)*

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CONTRIBUTIONS OF CO-AUTHORS

As the first author of all the research articles included in my present thesis, I was responsible for conducting literature review, for the preparation, design, and implementation of all experimental protocol and studies, to formulate specific research objectives and methodologies for my thesis research project. I was responsible, as the first author on all original articles as presented in this thesis, for the collection data and analysis. The entire study was conducted under the supervision and guidance of Dr. Satya Prakash ad Dr. Moulay Aloui-Jamali. Dr. Prakash is the corresponding author in all manuscripts that originate from this thesis and Dr. Aloui-Jamali, co-supervised the entire study. The contributions of other co-authors who served on the original research articles are listed below.

Chapter 3: CTD, LR and MM collectively helped with the cell culture experiments, and troubleshooting at the various stages of the experiments. They also helped with manuscript writing, data analysis and results/discussion.

Chapter 4: MM provided assistance with the cell culture experiments, data interpretation and proofreading of the manuscript.

Chapter 5: CTD, LR and MM provided assistance with cell culture experiments, troubleshooting at the various stages of the experiments, data analysis and manuscript proof reading

Chapter 6: MM provided intellectual and technical assistance with all of the *in vitro* experiments, data interpretation, and proofreading of the manuscript.

Chapter 7: NH and AR assisted with planning *in vitro* and in-vivo experiments and manuscript preparation. They also provided guidance and troubleshooting throughout the various stages of the animal work, data collection, and analysis. MM along with others contributed to troubleshooting and proofreading of the manuscript.

Chapter 8: NH and AR assisted with planning the animal studies and manuscript preparation. They also provided guidance and troubleshooting throughout the different stages of the animal work. MM along with other authors helped with proofreading of the manuscript.

Original Research Article 1

CHAPTER 3. 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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Preface: Probiotic treatment can be a key component in a patient's recovery with CRC patient recovery or to prevent the condition in patients with high-risk of developing CRC. Therefore, the identification of new probiotic strains is required for the formulation of more efficient biotherapeutics. *L. reuteri* known for their anti-pathogenic activity needs to be investigated for their potential therapeutic role in CRC. The goal of this chapter is to screen and identify the most potent *L. reuteri* strains, and correlate their therapeutic effect based on their SCFA production. Thus, in this study, *in vitro*, *L. reuteri* strains were screened for SCFA production and their anti-proliferative effect was analyzed in CRC cells. The best screened strains were further investigated for their resistance in artificial intestinal conditions.

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3.1. Abstract

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

3.2. Introduction

Colorectal cancer (CRC) is among the leading causes of cancer mortality worldwide, dietary intervention represents a valuable approach to preventing CRC development, particularly in susceptible human populations [190, 191]. Several chemopreventive and biotherapeutic approaches have been reported for the prevention of CRC and other gut conditions [192, 193]. Individuals with inherent gene defects that predispose them to CRC, inflammatory bowel disease (IBD), or 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 developing CRC, consume probiotics as neutraceutical products [194, 195]. Probiotics, defined as beneficial bacteria, have been proposed to balance disturbed gastrointestinal (GI) microflora and dysfunctions of the human GI tract [196]. 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 SCFAs, such as acetate, propionate, and butyrate, all which have anti-cancer properties [197]. Although several studies have reported the anti-proliferative or pro-apoptotic effect of probiotic bacteria on colon carcinoma cells [182, 198, 199], no systemic studies have been reported that screen or characterize certain LAB as potential candidates for CRC biotherapies. Potential LAB include *L. reuteri* bacteria, which have previously been investigated for anti-pathogenic activity and ability to produce conjugated linoleic acid [200-203]. *L. reuteri* is prominent among the *Lactobacillus* population in the GI ecosystem [204], and has been widely reported to be beneficial for some GI conditions, such as UC, which is one of the strong risk indicators of CRC [205]. Other cases include constipation [206], diarrhea [207], maintaining the mucosal barrier [208] and colon motility [209]. The biological activity of *L. reuteri* has been shown to be mediated in part by the production of lactic acid and bacteriocins, which potentially influence the commensal microorganisms [210, 211] and reduce intestinal absorption of endogenous and exogenous carcinogens [203]. 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 the production of SCFAs and to assess the anti-proliferative effects of these SCFAs on colon cancer cells. 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. In this study it was determined why *L. reuteri* bacteria suppressed colon cancer cells *in vitro*. The role of SCFAs was also 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 an SCFA synthetic formulation was found to inhibit cancer cells less than the corresponding *L. reuteri* CM, then this would show that the SCFAs may not be the only anti-cancer compounds produced by the bacteria and there are as well other bacterial molecules excreted in the CM, which have anti-proliferative activity against colon cancer cells. However, if the SCFAs 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/solely responsible for the anti-proliferative effect.

3.4. Materials and Methods

3.4.1. 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/UF) ultrapure water system from Barnstead (Dubuque, IA, USA). Sodium L-Lactate, propionate, acetate, and butyrate were purchased from Sigma (St. Louis, MO, USA).

3.4.2. Bacterial cells

The bacterial strain of *L. acidophilus* ATCC 314 was purchased from Cedarlane Laboratories (Burlington, ON, Canada) and was used as a positive control 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 subculturing in MRS media 1% (v/v) and growth was monitored by measuring OD at a wavelength of 620 nm (Perkin Elmer 1420 Multilabel Counter, USA) and by colony counting.

3.4.3. Mammalian cells

Human epithelial CRC adenocarcinoma cell line Caco-2 (HTB-37) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 20% of fetal bovine serum (FBS). Cells were incubated in a CO_2 incubator at 37°C in air supplemented with 5% CO_2 for up to two weeks until fully differentiated. For proliferation assays, cells were incubated to adhere on 96-well plates for 24 - 48 h in complete growth media. At this point, cell medium was replaced by probiotic conditioned medium (CM) diluted with serum/antibiotic-free DMEM.

3.4.4. Preparation of probiotic treatments

The probiotic CM, a bacterial cell-free extract, was prepared with slight modifications from protocols adapted from Grabig *et al*. [212] and Kim *et al*., [213]. First, *L. reuteri* and *L. acidophilus* bacteria were incubated in *Lactobacillus* MRS broth at 37 °C in air supplemented with 5% CO_2 for 24 h and sub-cultured 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 pelleted out from the

MRS medium by centrifugation (1000 x g, 15 min, 4°C) and washed twice with PBS. Finally, the probiotic conditioned media (CM) was produced by incubating washed probiotic cells (10^7-10^9 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 2 M NaOH and 2 M HCl.

3.4.5. Quantification of lactic acid and SCFAs

SCFAs produced by the *L. reuteri* strains were measured after the preparation of the corresponding probiotic CM and during the growth of bacteria in SIF. SCFAs were separated using a HPLC method adapted from Dubey and Mistry, with modifications [214, 215]. 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 mm x 7.80 mm, Phenomenex, Torrance, CA, USA) fitted with an ion-exclusion microguard refill cartridge was used. Data was acquired using ChemStation supported with LC3D software Rev A.03.02 (Agilent Technologies, CO, USA). The mobile phase (A) 0.05 M H_2SO_4 (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 at 35°C. Lactic, acetic, propionic, and butyric acids were used to prepare a standard solution at concentrations of 1, 10, 100, 500, and 1000 ppm (in triplicate) to generate the standard curve. The amounts of SCFAs were calculated using the linear regression equations ($R^2 \geq$ 0.99) from the corresponding standard curves.

3.4.6. Assessment of cancer cell proliferation

The proliferation and viability of colon cancer cells treated with the probiotic treatments was evaluated using an ATP bioluminescence assay (CellTiter-Glo Luminescent Cell Viability Assay, Promega, USA). Caco-2 cells were seeded into 96-well culture plates at 5 x 10^3 cells per well and stabilized for 24 h (37°C, 5% CO_2). After exposure to *L. reuteri,* probiotic treatments (for 24, 48, and 72 h), cell viability was determined following the guidelines from the manufacturer [216]. After incubating the cells with the probiotic treatment, the plate and its contents were equilibrated at room temperature (RT) for approximately 30 min. Following which, 100 ul of luminescent reagent was added to 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, MAA, USA).

3.4.7. 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.*[217], 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_2PO_4, 3.3 g/L) dissolved in deionized water. The pH was adjusted to 6.8 using 2 M NaOH and 2M HCL. The solution was autoclaved at 120ºC for 15 min and cooled at RT before use.

3.4.8. Determination of Lactobacilli stability in SIF

The bacterial pellet was separated from a 16 - 24 h MRS-bacterial culture by centrifugation (1000 x g, 10 - 15 min, 4ºC) and washed twice with a NaCl solution of 0.85% (w/v). Then 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, 4, 8, 12, 16, and 24 h), the bacterial density (OD_{620nm}) was measured, bacterial viability (colony counting on agar plates) was estimated, a supernatant was filtered (5 mL of bacterial culture centrifuged and 0.22 µM filtered) and stored at -80ºC until further use.

3.4.9. Efficacy and role of SCFA

This method was used to determine the effect of different levels of SCFAs, naturally produced from *L. reuteri*- on cells. The SCFAs produced by *L. reuteri* bacteria were compared with synthetic SCFAs 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 *L. reuteri* bacteria in different media was measured. The concentrations of SCFAs were determined for each *Lactobacillus* CM. Then formulations containing the same compositions of 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.

3.4.10. Statistical analysis

Data are presented as means ± Standard Error of the Mean (SEM), presented in triplicates. Correlations were determined using Pearson's 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 post-hoc test and student's t-test. The SPSS statistics software package was used (version 20.0, IBM Corporation, New York, NY, USA). *P*-values of $p < 0.05$ were considered significant.

3.5. Results

3.5.1. *L. reuteri* produces lactic acid in a conditioned cell culture media (CM)

This experiment was designed to screen *L. reuteri* strains as LAB for their ability to produce lactic acid in DMEM media challenged with bacterial cells. As observed in, lactic acid produced by five *L. reuteri* strains was quantified to show 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 to other strains ($p < 0.001$), followed by *L. reuteri* NCIMB 1195, which produced 369.1 ± 15.1 mg/L of lactic acid. It was observed that *L. reuteri* NCIMB 701089 (208.3 ± 2.8 mg/L) and *L. reuteri* NCIMB 702655 (233.4 ± 7.3 mg/L) produced significantly less lactic acid ($p < 0.001$).

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

The SCFA (acetate, butyrate and propionate) bio-production ability of *L. reuteri* bacteria in CM was determined and quantified. For acetic acid production (**Figure 3.2a**), the *L. reuteri* NCIMB 701089 (43.4 ± 3.3 mg/L) produced the least amount of acetic acid, whereas, *L. reuteri* NCIMB 702656 (182.1 ± 15.4 mg/L) produced the highest amount ($p < 0.01$) followed by *L. reuteri* NCIMB 11951 (131.2 ± 4.8 mg/L, $p < 0.05$) and then *L. reuteri* NCIMB 701359 (116.0 ± 4 mg/L).

For the production of propionic acid (**Figure 3.2b**), *L. reuteri* NCIMB 701089 (38.7 ± 1.4 mg/L) and *L. reuteri* NCIMB 702655 (45.5 ± 6.4 mg/L) produced the least amount among the *L. reuteri* strains ($p < 0.05$). The highest amount of propionic acid was produced by *L. reuteri* NCIMB 702656 (161.4 ± 3 mg/L, $p < 0.01$) followed by *L. reuteri* NCIMB 11951 (111.2 ± 5.5 mg/L, $p < 0.05$) and *L. reuteri* NCIMB 701359 (78.5 ± 10.9 mg/L, $p < 0.05$). For butyric acid production (**Figure 3.2c**), *L. reuteri* NCIMB 702656 (58.75 ± 9.1 mg/L) was significantly better than all other bacteria ($p <$

0.001). Moreover, *L. reuteri* NCIMB 11951 (28.6 ± 4 mg/L) and *L. reuteri* NCIMB 701359 (27.62 ± 4.2 mg/L) produced significantly more butyrate compared with *L. reuteri* NCIMB 701089 (no butyrate detected, $p = 0.001$). Finally, *L. reuteri* NCIMB 702656 (402.21 ± 40.7 mg/L), *L. reuteri* NCIMB 701359 (222.07 ± 27, 04 mg/L), *and L. reuteri* NCIMB 11951 (271.03 ± 5.2 mg/L) produced significantly more total probiotic SCFAs than *L. reuteri* NCIMB 702655 (160.87 ± 20.4 mg/L, $p < 0.001$, $p = 0.006$, and $p = 0.002$, respectively) and *L. reuteri* NCIMB 701089 (82.12 ± 5.4 mg/L, $p < 0.001$, **Figure 3.2c**).

3.5.3. Identification of *L. reuteri* strains that suppressed colon cancer cell growth

Screening *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 (**Figure 3.3**). At 24, 48, and 72 h, the luminescence-based cell viability was determined. At 24 h (**Figure 3.3a**), *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 (**Figure 3.3c**), *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 (**Figure 3.2c**), the inhibition of colon cancer cells was best achieved with the CM of *L. reuteri* NCIMB 702656 (56.68 ± 1.61 %, $p < 0.001$) and *L. reuteri* NCIMB 701359 (55.58 ± 2.18 %, $p < 0.001$) compared with *L. reuteri* NCIMB 11951 (42.9 ±3.6 %, $p = 0.002$, $p = 0.01$, respectively), and all were significantly higher than *L. reuteri* NCIMB 702655 ($p < 0.001$) and *L. reuteri* NCIMB 701089 (6.43 ± 0.7 %, $p < 0.001$).

3.5.4. SCFAs produced by *L. reuteri* is partially responsible for their inhibitory effect

To verify whether the inhibitory effect of *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 3.1**, and tested on Caco-2 cells for 72 h (**Figure 3.5)**. No significant differences were observed in the anti-proliferative effects of *L. acidophilus* ATCC 314 and *L. reuteri* NCIMB 11951 or their SSFs. For *L. reuteri* NCIMB 701089 and *L. reuteri* 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 ($p < 0.05$). 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 % inhibition) significantly reduced cancer cell growth compared with SSF-r13+LA (9.07 ± 0.99 % 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.

3.5.5. Resistance of *L. reuteri* bacteria to SIF

This experiment was designed to monitor the density and viability of *L. reuteri* bacteria inoculated in the simulated intestinal media, to predict their ability to survive the harsh colonic and intestinal environment. As described in **Figure 3.6** and **Figure 3.7**, the viability and concentrations of *L. reuteri* NCIMB -701359, -11951, and -702656, identified in this study for their higher anti-proliferative activity, were evaluated in SIF at different periods. The data showed that our bacteria of interest demonstrated resistance to this environment, in comparison with other strains, for up to 4 h in SIF. No significant difference was observed between *L. reuteri* NCIMB 11951 (**Figure 3.7b**), *L. reuteri* NCIMB 701359 (**Figure 3.7d**), *L. reuteri* NCIMB 702656 (**Figure 3.7c**), and the control *L. acidophilus* ATCC 314 (94.9 ± 0.6 %, **Figure 3.7a**). 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.

3.5.6. *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 **Figure 3.8.** 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 (**Figure 3.9a**), the *L. reuteri* NCIMB 702656 (650 ± 0.02 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 (**Figure 3.9b**), *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 production (**Figure 3.9c**), although *L. reuteri* NCIMB 701359 (1300.3 ± 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, $p < 0.001$).

3.6. 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 [218, 219]. Hence, 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 this 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 incidence of CRC [197]. Since probiotic SCFAs, (mainly acetate, propionate and butyrate) are recognized for their anti-cancer activity on colon cancer, several studies have demonstrated that this effect acts through arrested growth, and apoptosis [220]. In particular, some studies have shown restored GPR43 expression coupled with propionate treatment that induced an upregulation of p21, a decrease in the levels of cyclin D3, and cyclin-dependent kinases (CDKs) 1 and 2. After propionate/butyrate treatment, G0/G1 cell cycle arrest and activated caspases were induced, leading to increased apoptotic cell death [221]. Importantly, administration of *L. reuteri* strains was shown to alter the levels of fecal SCFAs in animals [218, 219] and in fermentation systems [198]. In addition, *L. reuteri* bacteria have been shown to affect the colonic fermentation of fibers and to stimulate the production profile of SCFAs [222] in simulated intestinal conditions [219]. Thus, the first objective was to screen a number of *L. reuteri* strains: *L. reuteri* NCIMB -11951, -701089, -

701359, -702655, and -702656, for the concentrations of SCFAs in their CM, as well as lactic acid as the primary characteristic of these LAB. In this study, we reported that the *L. reuteri* bacteria were bioactive and produced detectable amounts of lactic acid in the cell culture conditioned media (CM) using DMEM (**Figure 3.1**). Later, SCFAs were quantified in the CM and the levels of acetic, propionic, and butyric acids produced have shown to be strain-dependent (**Figure 3.2**). Moreover, we showed that *L. reuteri* NCIMB -11951, -701359, and -702656 produced higher concentrations of SCFAs and lactic acid compared with other *L. reuteri* strains. It was noted that the concentrations of acetic and propionic acids measured in this study were half the optimal doses used in the literature to induce anti-proliferative effect on Caco-2 cells [223], which predicts a possible inhibitory effect of the probiotic treatment on colon cancer cells.

Very few studies have investigated the effect of *L. reuteri* bacteria on colon cancer cells. In one case study, *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) [224]. Occasionally, the identification of the anti-proliferative effect of probiotics in-vitro can be measured, by the effect of bacterial extracts on colon cancer cell colony formation and have shown similar results to the proliferation assay in a number of studies [225, 226]. We determined that *L. reuteri* NCIMB -11951, -701359, and -702656 exhibited the greatest inhibition of colon cancer cell proliferation (72 h, **Figure 3.3**), respectively, compared with untreated cells. These observations, shown for the first time, are consistent with the findings that LAB and, more specifically *L. reuteri,* may have anti-cancer activity induced by SCFA production in the colon, which may decrease tumor growth, inhibit colon cancer cell growth, and promote apoptosis [225, 226]. As described in **Figure 3.4**, 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, with and without lactic acid, were prepared as different mixtures and tested on colon cancer cells (**Figure 3.5**). It is important to note, that the *L. reuteri*-CM, comprised of DMEM media modified by the incubation of probiotic bacterial

cells, which had changed the composition of the cell culture media, thereby, producing SCFAs and other bacterial products. This makes the addition of SCFAs to standard cell media, in the case of SCFA synthetic formulations, only an approximation of the effect of SCFAs alone and may affect the evaluation of cell anti-proliferation activity versus the effect of bacterial SCFAs production. This assay showed differential levels of inhibition between the natural probiotic treatments "*L. reuteri*-CM" and the SCFA synthetic doses, which argues for the responsibility of the naturally produced SCFAs in the CM to kill cancer cell deaths. Surprisingly, the SCFA formulations, corresponding to *L. reuteri* strains, that inhibited colon cancer cell proliferation the most were SSF-r1, SSF-r13, and SSF-r56, which correspond, to *L. reuteri* NCIMB -11951, -701359, and -702656; respectively. These strains produced the highest levels of SCFAs and inhibited the cancer cells the most (**Figure 3.5**). A correlation was shown between the effect of SCFA synthetic formulations and *L. reuteri*-CM on colon cancer cell proliferation ($r = 0.84$, $p = 0.001$). This suggests that the anti-proliferative effect of the CM is due in part, to the concentration of bacterial SCFAs but the effect is not only related to the presence of SCFAs. This is demonstrated in the case of *L. reuteri* NCIMB 701359 (**Figure 3.5**), where the corresponding SCFA synthetic formulation had a significantly less anti-proliferative effect than the *L. reuteri*-CM. This implies the presence of an additional acting bacterial factor produced in the *L. reuteri*-CM. This is supported by the fact that some microbial components, such as CpG DNA, flagellin, and lipopolysaccharide (LPS) inhibited tumor growth by activating pattern recognition receptors in colon cancer epithelial cells [227]. This indicates that the presence of other bacterial products may complement and enhance the anti-proliferative and the anti-carcinogenic activities 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 was found to produce different components (organic acids, bacteriocins, peptides, etc.) that interact with the 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 bioactive SCFAs: 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 [228]. Conversely, for *L. reuteri* NCIMB -701089, -702655, and -702656, the respective SCFA synthetic formulations had a significantly higher anti-proliferative effect than the probiotic extracts CM, suggesting that *L.reuteri* bacteria had also produced other factors that may have diminished the action of SCFAs. A positive correlation was found between the inhibition of cancer cells and the concentration of lactic acid ($r = 0.92$, $p < 0.001$) produced by *L. reuteri* bacteria. This could entail that the more *L. reuteri* produced lactic acid the more it suppressed CRC proliferation. Nevertheless, when lactic acid was added to the synthetic SCFA formulations, the inhibitory effect drastically decreased ($p < 0.001$, SSF+LA compared with CM, and SSF compared with SSF+LA, **Figure 3.5**), showing that lactic acid itself has no significant direct effect on the inhibition of colon cancer cell proliferation, but it could be strongly implicated in the action of SCFAs. It is possible that probiotic bacteria have altered colon cancer cell metabolism by the production of SCFAs and lactic acid. Few reports have related the effect of probiotic SCFAs to the production of lactate in cancer cells [228].Yet, some studies showed that butyrate analogs, such as propionate and L-lactate, significantly inhibit the uptake of butyrate in cancer cells [228].

In addition, they have the potential to decrease glycolysis and lactate secretion, thus killing the cancer cells [228]. These facts denoted that the lactic acid, added later to the SSFs, could have suppressed the ability of cancer cell to uptake SCFAs resulting in the lower efficacy of SSF+LA.

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 *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 *in-vitro* anti-cancer activity, *L. reuteri* NCIMB -701359, -11951, and -702656, were selected for the characterization of their bacterial cultures (**Figure 3.6**) and survival in SIF (**Figure 3.7**). 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 [229]. In an animal-based study, probiotic bacteria were administrated at

10^{10} cfu and reached the intestine at 6 - 8 log, similarly to our strains, and persisted for days [230]. Another study that screened 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 between 35% - 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 [231]. In our case, both *L. reuteri* NCIMB -702656 (**Figure 3.7c**) and -701359 (**Figure 3.7d**), showed similar resistance to SIF in early incubation in comparison with *L. reuteri* NCIMB 11951 (**Figure 3.7b**) and *L. acidophilus* ATCC 314 (**Figure 3.7a**) and 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 [232].

Although 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 significantly 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, **Figure 3.8**). However, in terms of bacterial acetate and propionate production in SIF, no significant difference among these three strains was observed. Remarkably, the levels of total SCFAs produced by *L. reuteri* NCIMB 11951 ($p = 0.331$), the strains with significantly higher survival in the SIF ($p < 0.001$), were not with *L. reuteri*, NCIMB 702656 ($p = 0.07$) and *L. reuteri* NCIMB 701359 ($p = 0.332$). 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 (**Figure 3.9**). The results also indicated that *L. reuteri* NCIMB -701359 and -702656 have the potential to produce effectively more SCFAs in the colonic environment than *L. reuteri* NCIMB 11951. In comparison with other studies using different media, this study found propionate produced in SIF to be double the amount produced by different *L. reuteri* isolates, whereas acetic and lactic acids levels were relatively low [233]. This study confirmed similar research which demonstrated *L. reuteri* to have the ability to increase SCFAs production and

fermentation in human simulated digestive fluids [203]. It is possible 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 CRC prevention.

3.7. Conclusion

In this study, we determined whether *L. reuteri* bacteria might produce SCFA that significantly 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 were shown to produce SCFAs in CM or SIF and suppress colon cancer cell growth. This study was the first to screen 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 the ability to produce anti-carcinogenic active compounds, thus indicating a potential biotherapeutic effect in CRC that could be investigate further.

3.8. Acknowledgements

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3.9. Conflicts of interest

The authors have no conflicts of interest to disclose.

3.10. Tables and Figures

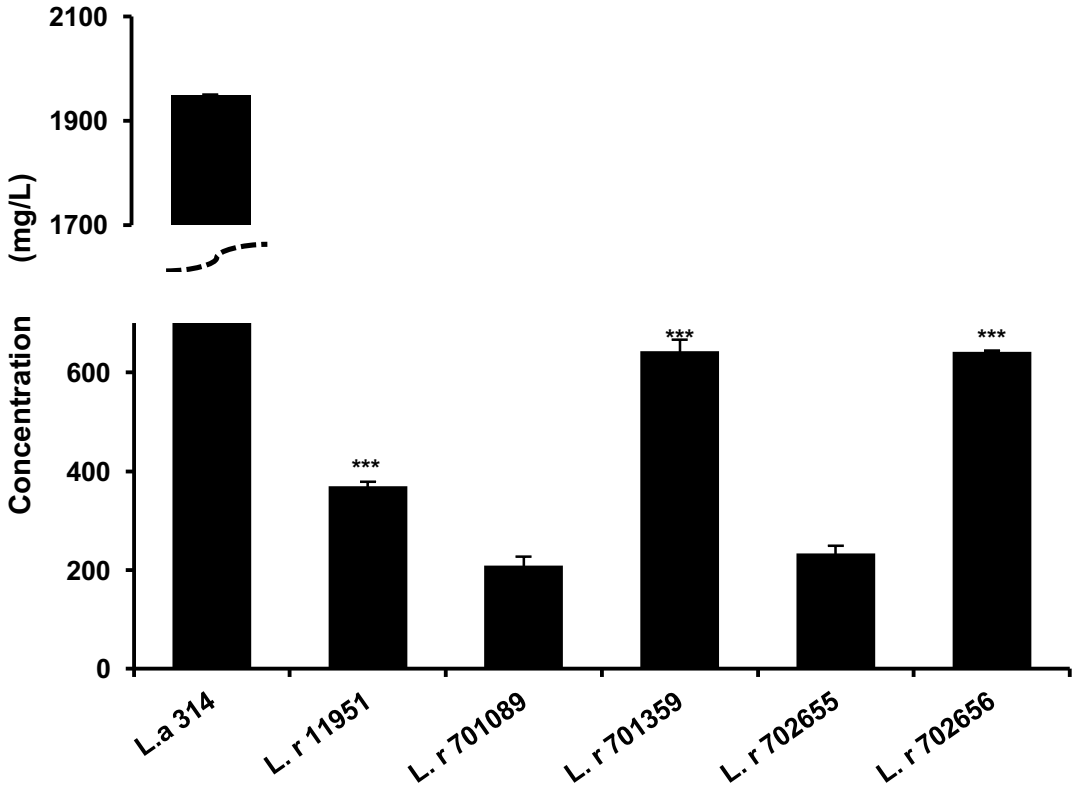


Figure 3.1: Illustrating the ability of *L. reuteri* strains to produce lactic acid in the cell culture conditioned medium (CM).

Lactic acid was produced after incubating bacterial cells of *L. reuteri* NCIMB -11951, -701089, -701359, -702655, or -702656 in DMEM cell media (2 h, 37^{o}C, 5% CO_2). *L. acidophilus* ATCC 314 is used as a control. Data are presented as mean ± SEM (n = 3). ***$p < 0.001$ compared with *L. reuteri* NCIMB 701089.

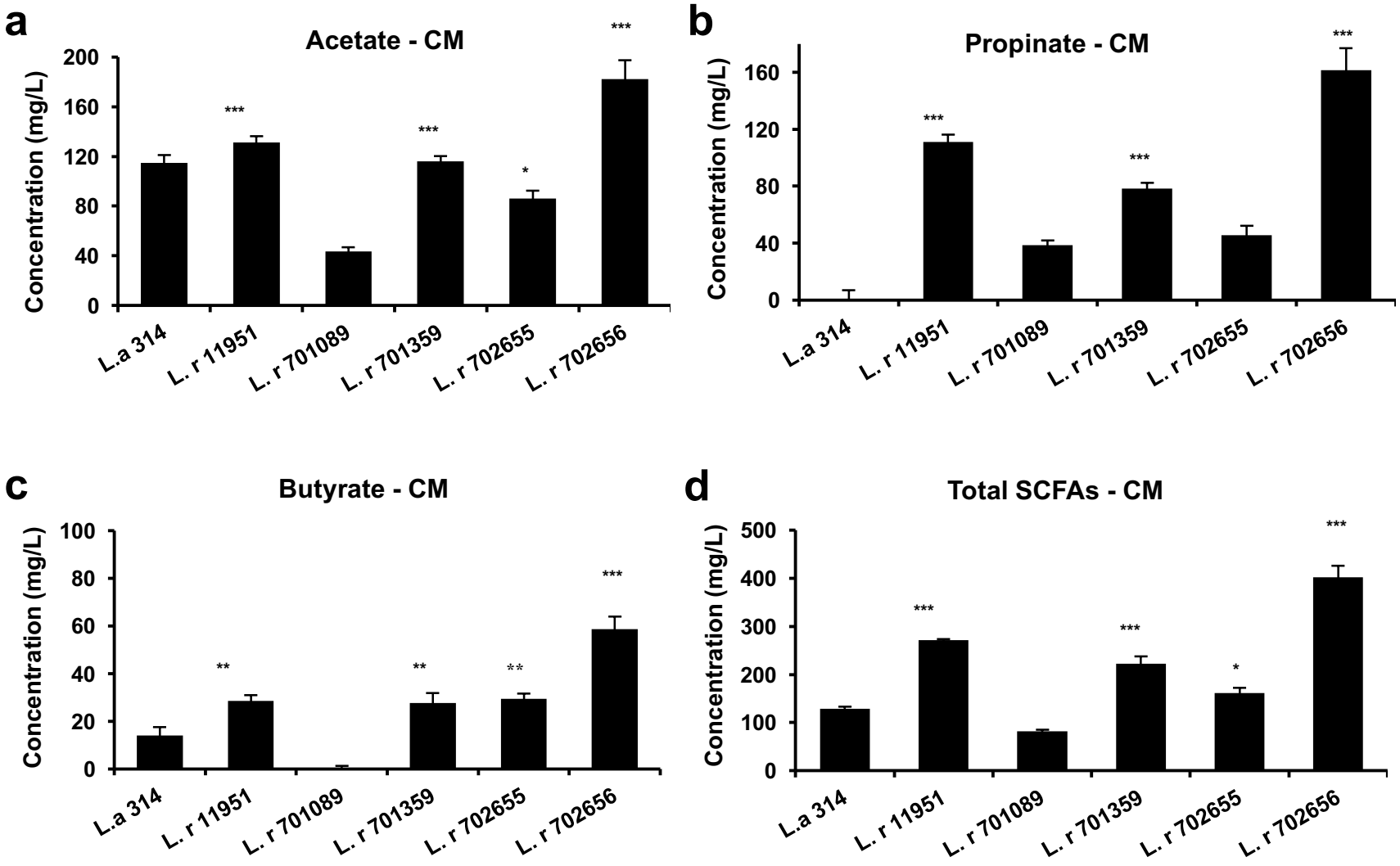
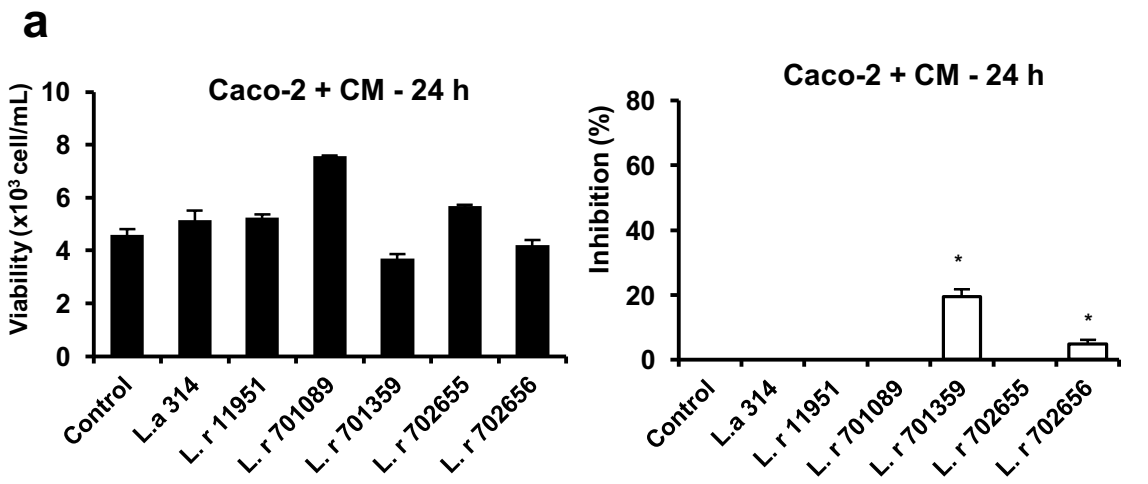
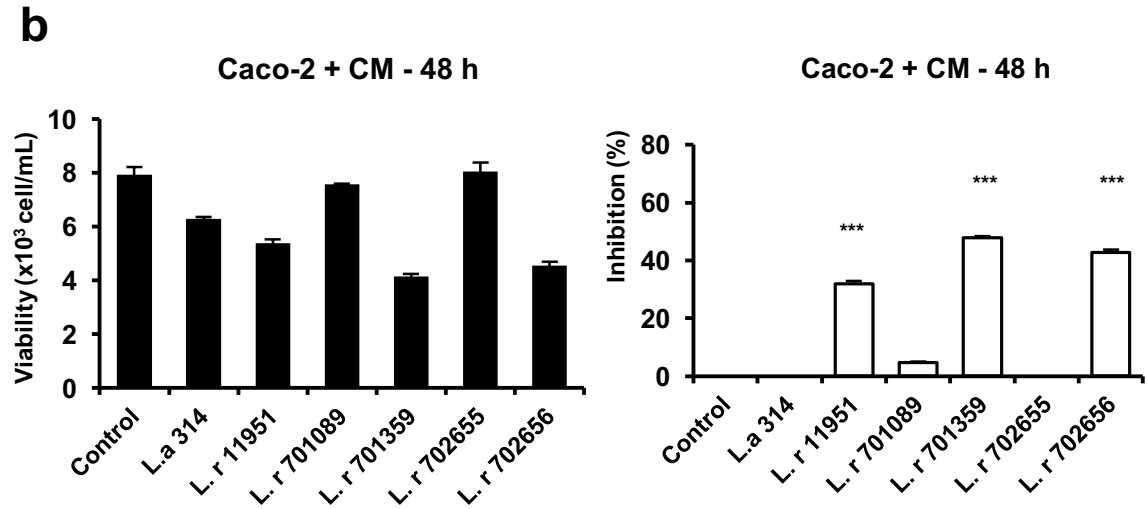

a
Acetate - CM
Concentration (mg/L)
200
160
120
80
40
0
***
***
*
***
L.a 314
L. r 11951
L. r 701089
L. r 701359
L. r 702655
L. r 702656
b
Propinate - CM
Concentration (mg/L)
160
120
80
40
0
***
***
***
L.a 314
L. r 11951
L. r 701089
L. r 701359
L. r 702655
L. r 702656
c
Butyrate - CM
Concentration (mg/L)
100
80
60
40
20
0
**
**
**
***
L.a 314
L. r 11951
L. r 701089
L. r 701359
L. r 702655
L. r 702656
d
Total SCFAs - CM
Concentration (mg/L)
500
400
300
200
100
0
***
***
*
***
L.a 314
L. r 11951
L. r 701089
L. r 701359
L. r 702655
L. r 702656

Figure 3.2: Study of the bio-production of SCFAs by *L. reuteri* strains in cell culture conditioned medium (CM).

In order to establish if *L. reuteri* NCIMB -11951, -701089, -701359, -702655, and -702656, produces SCFAs known as active anti-cancer compounds in-vitro conditions; the bacterial cells were incubated in DMEM (2 h, 37^{o}C, 5% CO_2). Then, the acetic, propionic, and butyric acids were separated and quantified by HPLC method. *L. acidophilus* ATCC 314 is used as control. Data are presented as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001, compared with *L. reuteri* NCIMB 701089.


a
Caco-2 + CM - 24 h
Viability (x10³ cell/mL)
10
8
6
4
2
0
Control
L.a 314
L. r 11951
L. r 701089
L. r 701359
L. r 702655
L. r 702656
Caco-2 + CM - 24 h
Inhibition (%)
80
60
40
20
0
*
*
Control
L.a 314
L. r 11951
L. r 701089
L. r 701359
L. r 702655
L. r 702656

33


b
Caco-2 + CM - 48 h
Viability (x10³ cell/mL)
10
8
6
4
2
0
Control
L.a 314
L. r 11951
L. r 701089
L. r 701359
L. r 702655
L. r 702656
Caco-2 + CM - 48 h
Inhibition (%)
80
60
40
20
0
***
***
***
Control
L.a 314
L. r 11951
L. r 701089
L. r 701359
L. r 702655
L. r 702656

34

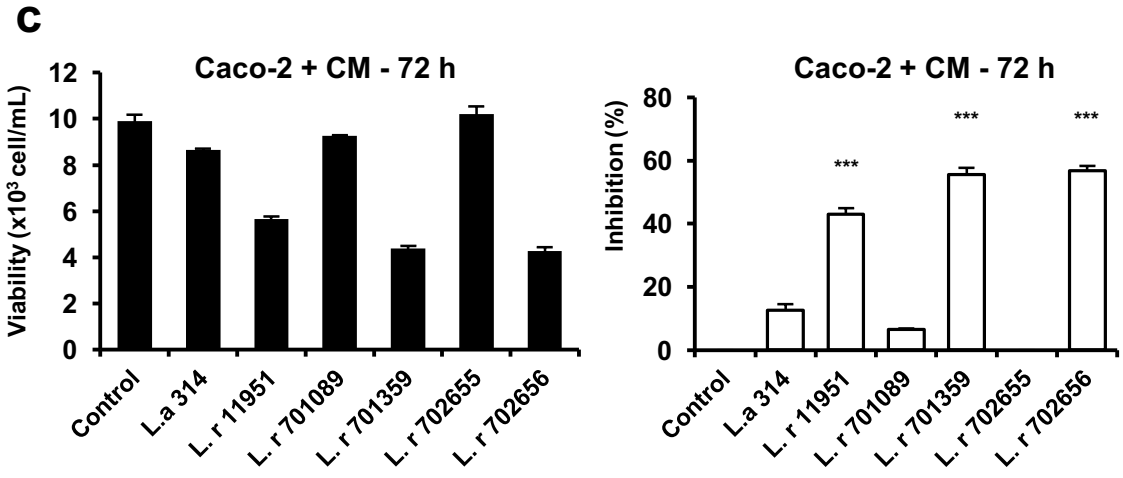


Figure 3.3: Screening of *L. reuteri* strains for a potential anti-proliferative effect against colon cancer cells.

To investigate the anti-proliferative effect of probiotic *L. reuteri* bacteria, the cell culture conditioned medium (CM) of *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 *L. reuteri*-CM was measured after incubation with probiotic treatments for (a) 24 h, (b) 48 h and (c) 72 h, using ATP bioluminescence. *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 *L. reuteri* NCIMB 702655.

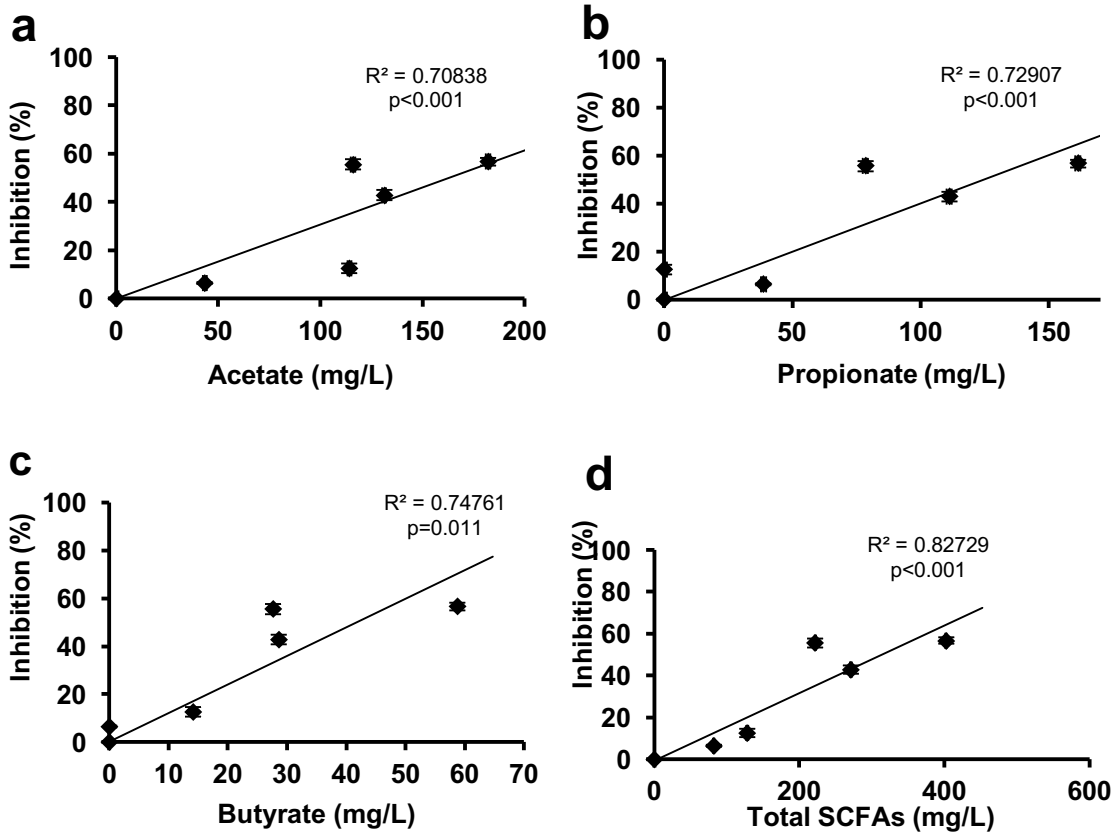


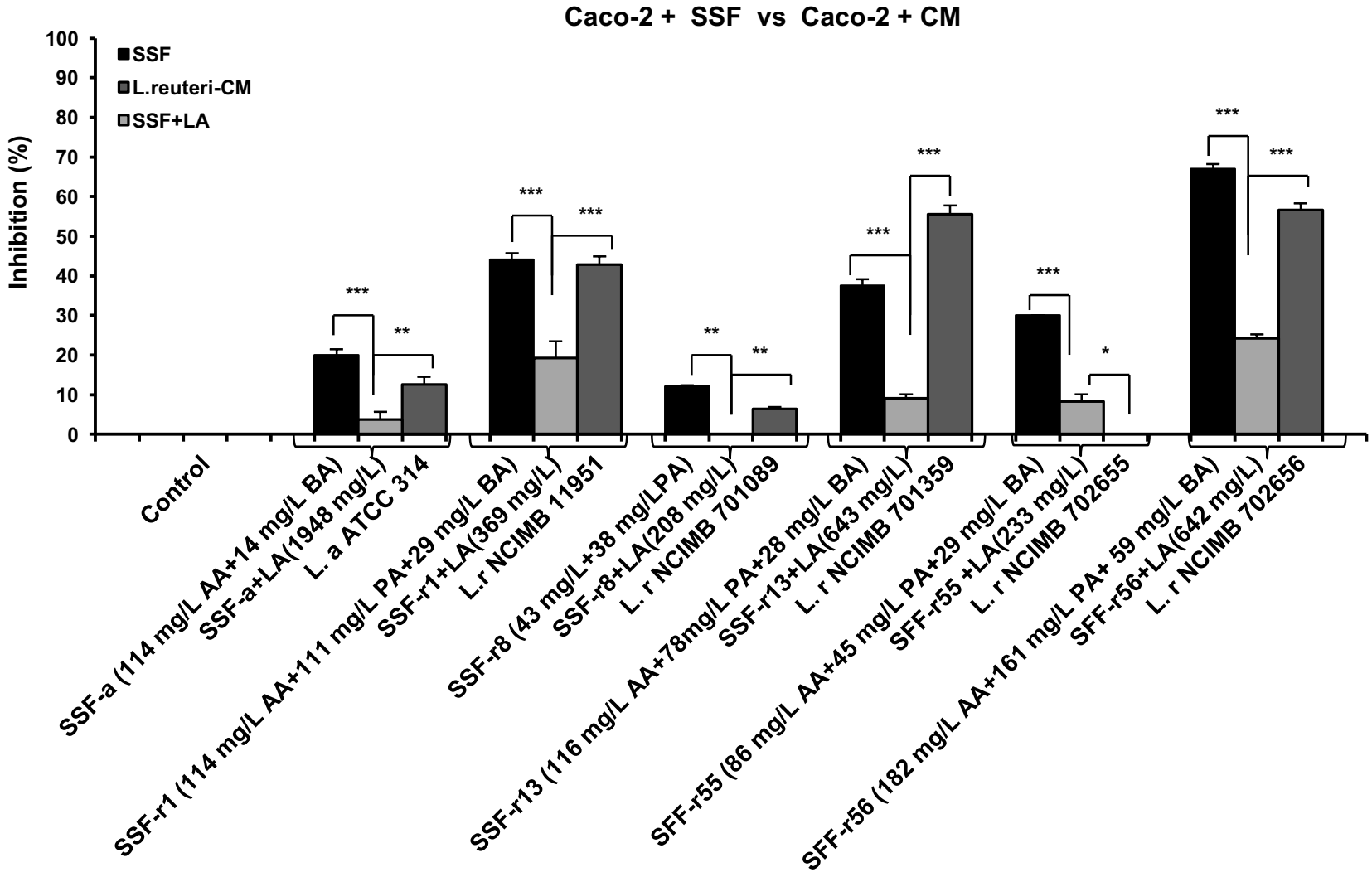
Figure 3.4: Correlation analysis between the production of probiotic SCFAs and the inhibition of colon cancer cell by probiotic bacteria *L. reuteri*.

The correlation was determined between the concentrations of **(a)** acetate, **(b)** propionate, **(c)** butyrate and **(d)** total SCFAs in *L. reuteri*-CM and the inhibition of colon cancer cell growth by *L. reuteri*-CM. This test was performed to examine if *L. reuteri*-CM suppressed colon cancer cell growth due, in part, to the presence of SCFAs produced by the probiotic bacteria. The inhibition of Caco-2 proliferation by *L. reuteri*-CM was measured after 72 h of treatment. SCFAs were measured in the *L. reuteri*-CM used to treat Caco-2 cell. Plots represent the data of cell growth inhibition described in Figure 3.2. The lines were obtained by linear regression analysis.

Table 3.1: 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 SSF was prepared by the addition of respective concentrations of lactate same as the one produced by *L. reuteri*-CM. SSF: SCFA synthetic formulation.

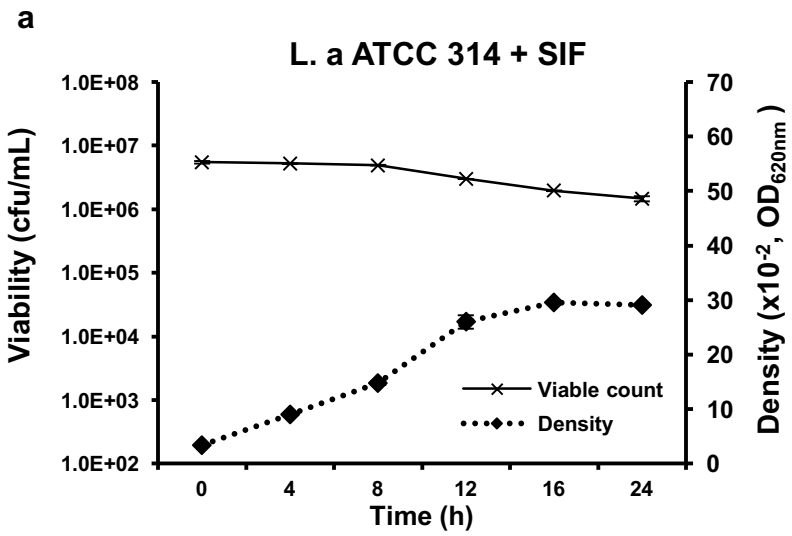
Corresponding CM	SSF	Composition (mg/L)			SSF+LA	Composition (mg/L)			
		Acetate	Propionate	Butyrate		Lactate	Acetate	Propionate	Butyrate
L. a 314	SSF-a	114	0	14	SSF-a+LA	1948	114	0	14
L. r 11951	SSF-r1	131	111	29	SSF-r1+LA	369	131	111	29
L. r 701089	SSF-r8	43	38	0	SSF-r8+LA	208	43	38	0
L. r 701359	SSF-r13	116	78	28	SSF-r13+LA	643	116	78	28
L. r 702655	SSF-r55	86	45	29	SSF-r55+LA	233	86	45	29
L. r 702656	SSF-r56	182	161	59	SSF-r56+LA	642	182	161	59

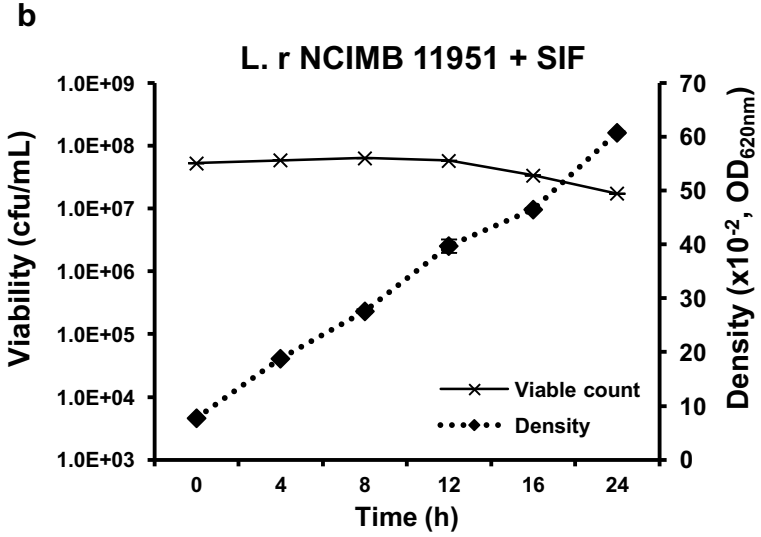

Caco-2 + SSF vs Caco-2 + CM
SSF
L.reuteri-CM
SSF+LA
Inhibition (%)
100
90
80
70
60
50
40
30
20
10
0
***
**
***
***
**
**
***
***
***
*
***
***
Control
SSF-a (114 mg/L AA+14 mg/L BA)
SSF-a+LA(1948 mg/L)
L. a ATCC 314
SSF-r1 (114 mg/L AA+111 mg/L PA+29 mg/L BA)
SSF-r1+LA(369 mg/L)
L.r NCIMB 11951
SSF-r8 (43 mg/L+38 mg/LPA)
SSF-r8+LA(208 mg/L)
L. r NCIMB 701089
SSF-r13 (116 mg/L AA+78mg/L PA+28 mg/L BA)
SSF-r13+LA(643 mg/L)
L. r NCIMB 701359
SFF-r55 (86 mg/L AA+45 mg/L PA+29 mg/L BA)
SFF-r55 +LA(233 mg/L)
L. r NCIMB 702655
SFF-r56 (182 mg/L AA+161 mg/L PA+ 59 mg/L BA)
SFF-r56+LA(642 mg/L)
L. r NCIMB 702656

89

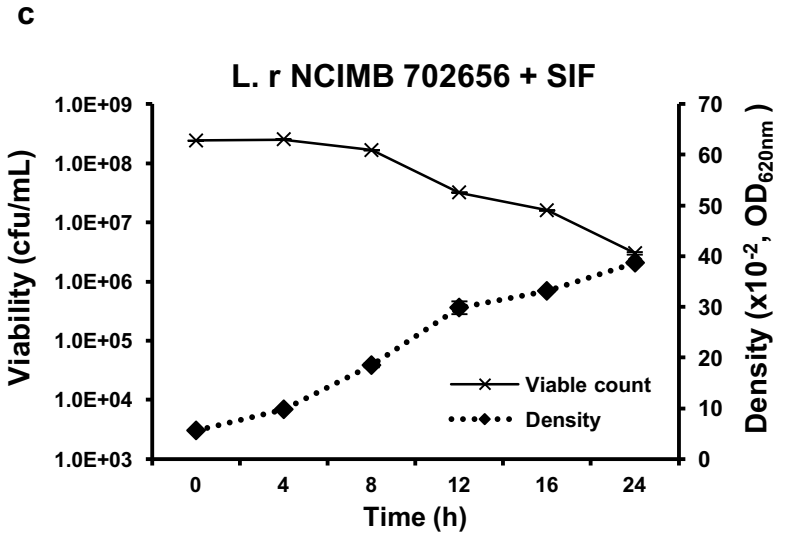
Figure 3.5: Comparison of the anti-proliferative effect of SCFA synthetic formulations (SSFs) with the anti-proliferative effect of *L. reuteri*-CM.

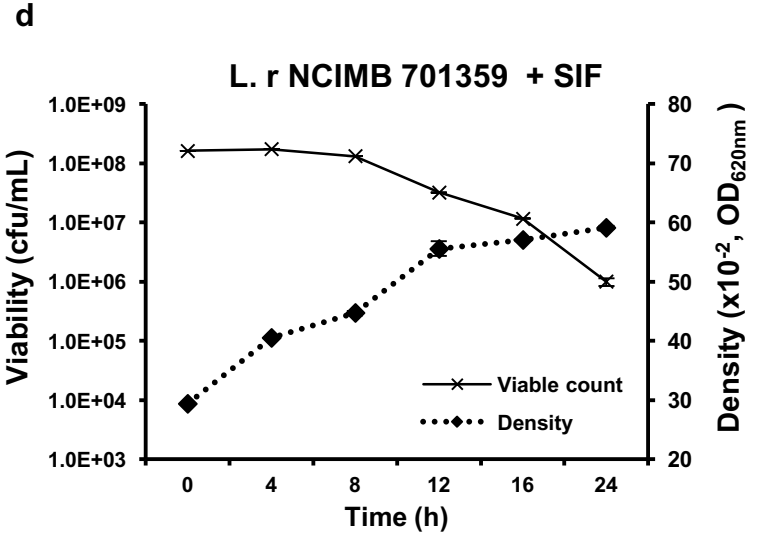
This evaluation was performed to study if *L. reuteri*–CM owe in part, their effect in suppressing colon cancer cells to the levels of naturally produced SCFAs they produced? The quantities of chemical compounds in the synthetic formulations are the same as naturally produced by *L. reuteri* bacteria (*L. reuteri* NCIMB -11951, -701089, -701359, -702655, and 702656) in CM, as presented in Table 1. *L. acidophilus* ATCC 314 is used as a positive control and for comparative purposes. Data are represented as mean ± SEM ($n = 5$). *$p < 0.05$ and ***$p < 0.001$, compared with untreated groups.


a
L. a ATCC 314 + SIF
Viability (cfu/mL)
Density (x10-2, OD620nm)
Time (h)
Viable count
Density


b
L. r NCIMB 11951 + SIF
Viability (cfu/mL)
Density (x10-2, OD620nm)
Time (h)
Viable count
Density

109

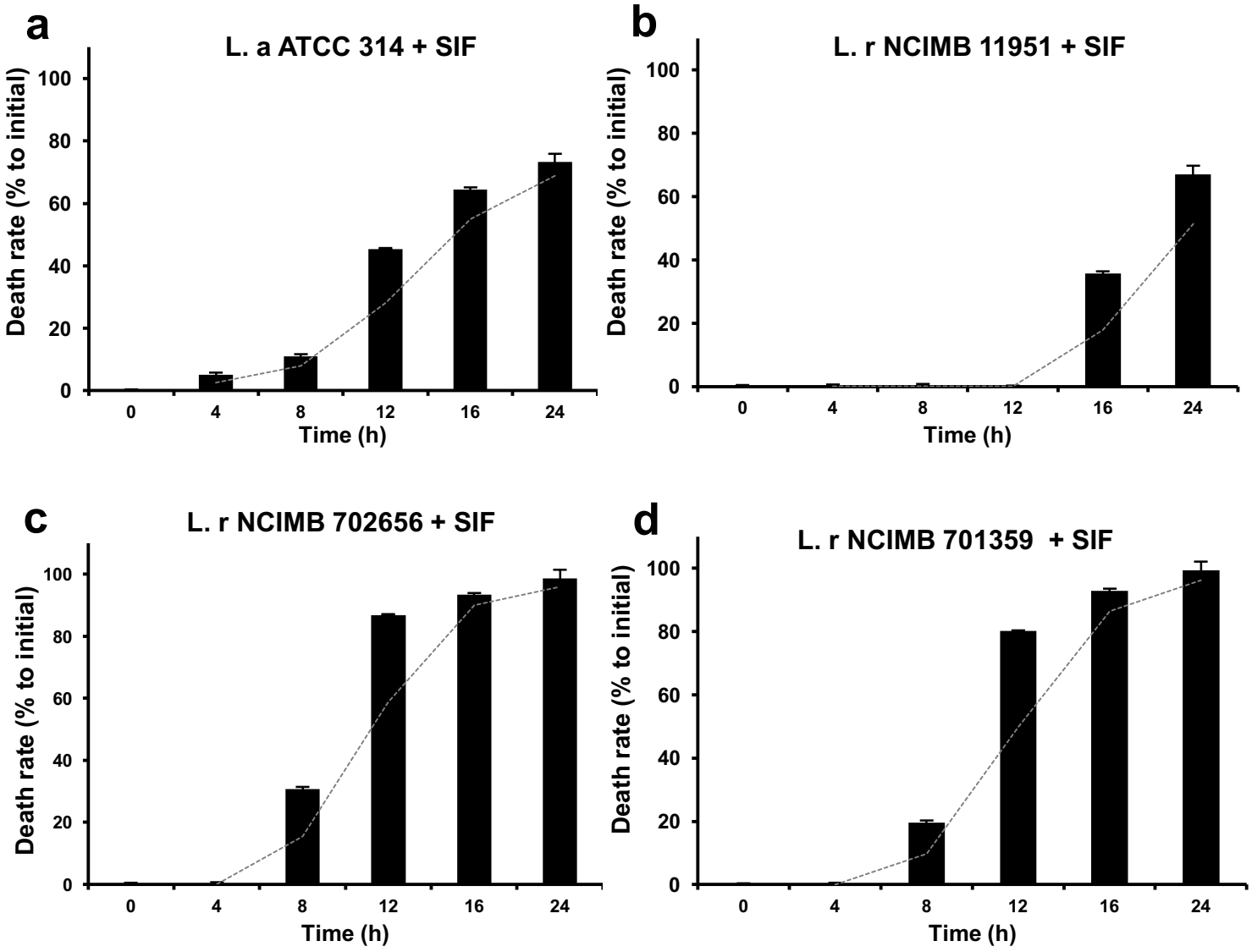

c
L. r NCIMB 702656 + SIF
Viability (cfu/mL)
Density (x10-2, OD620nm)
Time (h)
Viable count
Density


d
L. r NCIMB 701359 + SIF
Viability (cfu/mL)
Density (x10-2, OD620nm)
Time (h)
Viable count
Density

110

Figure 3.6: Bacterial cell culture characterization for *L reuteri* strains in a simulated intestinal fluid (SIF) (pH = 6.8, 24 h).

The variation in bacterial viable cell count and cell culture absorbance of **(b)** L. reuteri NCIMB -11951, **(c)** -701359, and **(d)** -702656, in addition to **(a)** *L. acidophilus* ATCC 314, was determined in micro-anaerobic conditions. The SIF used contained glucose (5.5g/L), yeast extract (3.5g/L), pancreatin (2g/L), oxgall (1.5g/L), pectin (2g/L), inulin (0.54g/L) fructooligosaccharides (0.85g/L), starch (3g/L), and monobasic potassium phosphate (KH2PO4, 3.3g/L). The data is presented by the mean ± SEM (n = 3).

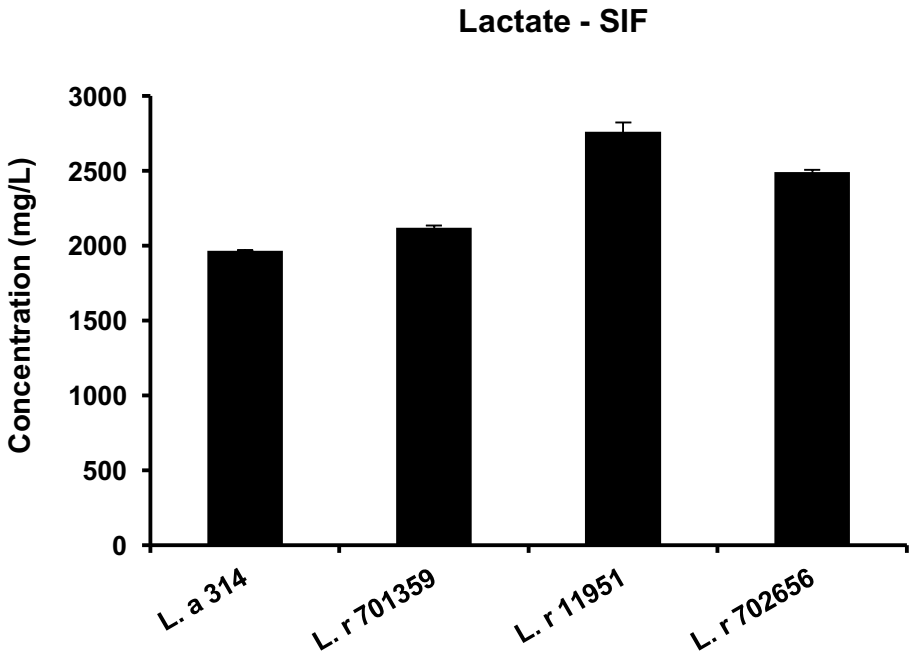

a
L. a ATCC 314 + SIF
Death rate (% to initial)
Time (h)
b
L. r NCIMB 11951 + SIF
Death rate (% to initial)
Time (h)
c
L. r NCIMB 702656 + SIF
Death rate (% to initial)
Time (h)
d
L. r NCIMB 701359 + SIF
Death rate (% to initial)
Time (h)

129

130

Figure 3.7: Death rate of *L reuteri* bacteria in a simulated intestinal fluid (SIF) (pH = 6.8, 24 h. SIF was a mixture of glucose (5.5g/L), yeast extract (3.5g/L), pancreatin (2g/L), oxgall (1.5g/L), pectin (2g/L), inulin (0.54g/L) fructooligosaccharides (0.85g/L), starch (3g/L), and monobasic potassium phosphate (KH_2PO_4, 3.3g/L).

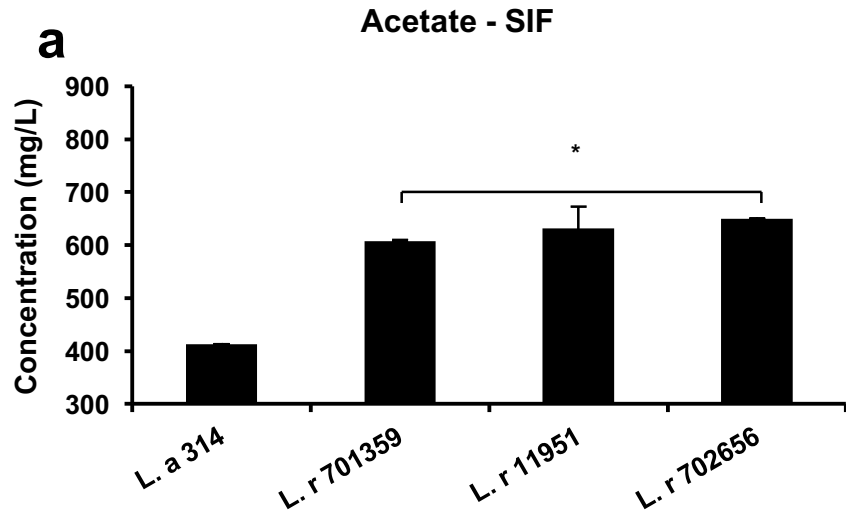
The percentage of dead bacterial cell compared to initial count of (**b**) *L. reuteri* NCIMB -11951, (**c**) -701359, and (**d**) -702656 in addition to (**a**) *L. acidophilus* ATCC 314, was determined in micro-anaerobic conditions. The data is presented by the mean ± SEM (n = 3).



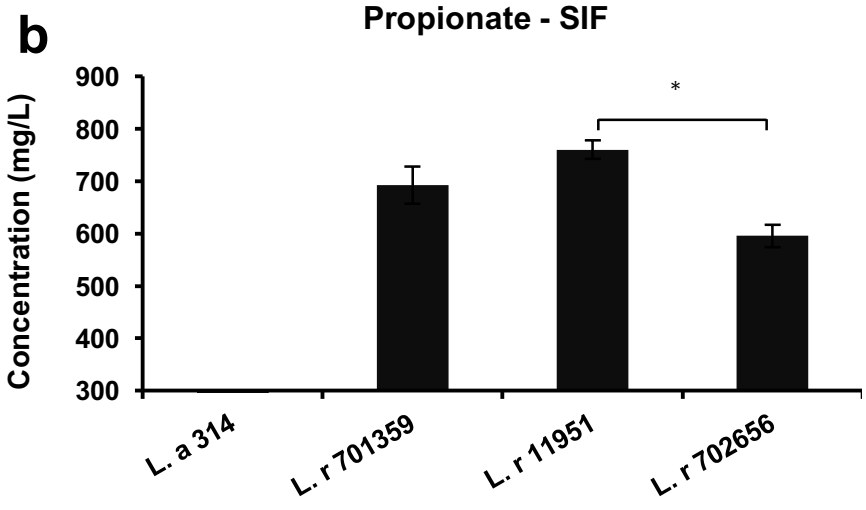
148

149 **Figure 3.8: Ability of *L. reuteri* to produce lactic acid in simulated intestinal conditions.**

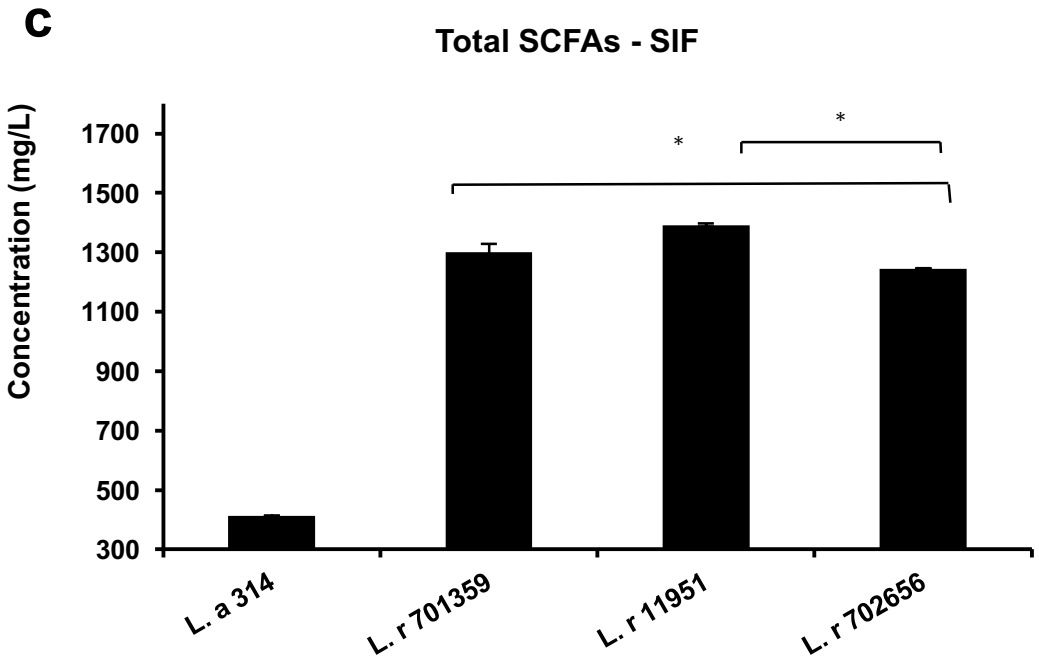
150 The concentrations of lactic acid produced by *L. reuteri* NCIMB -11951, -701359 and -702656 in
151 SIF (pH = 6.8, 24 h, 37^{o}C, 5% CO_2) were identified and compared to *L. acidophilus* ATCC 314.
152 Data are presented as mean ± SEM (n = 3).


a
Acetate - SIF
Concentration (mg/L)
900
800
700
600
500
400
300
*
L. a 314
L. r 701359
L. r 11951
L. r 702656

153


b
Propionate - SIF
Concentration (mg/L)
900
800
700
600
500
400
300
*
L. a 314
L. r 701359
L. r 11951
L. r 702656

154



155

156 **Figure 3.9: Quantification of the SCFAs produced by in simulated intestinal conditions.**

157 This experiment was perfomed to verify if *L. reuteri* NCIMB -11951, -701359 and -702656 are
158 still able to produce significant amounts SCFAs, in an intestinal environment, and induce potential
159 inhibitory effect against colon cancer cells in the gut. The concentrations of (a) acetate, (b)
160 propionate, butyrate (ND) and (c) total SCFAs produced by *L. reuteri* NCIMB -11951, -701359,
161 and -702656 in SIF (pH = 6.8, 24 h, 37oC, 5% CO2) were identified by HPLC. *L. acidophilus*
162 ATCC 314 is used as a control. Data are presented as mean ± SEM (n = 3) at *$p < 0.05$.

Original Research Article 2

CHAPTER 4. CHARACTERIZATION OF *LACTOBACILLUS 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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[5]

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Preface: This chapter contains further characterizations for the previously selected *L. reuteri* strain. The potency of *L. reuteri* was investigated when compared with two established *Lactobacillus bacteria: L. acidophilus and L. rhamnosus.* Metabolic activity of *L. reuteri* bacterium was studied based on FFAs and SCFAs production. The anti-proliferative effect was defined when testing two different extracts on two CRC cell lines. The anti-CRC potential was confirmed when verifying the bacterial extracts have not negatively impacted the normal colon cells. The concentrations of SCFAs produced by *L. reuteri* bacterium were tested for their efficacy in inhibiting the CRC cell growth.

Submitted to the Journal of Medical Microbiology

4.1. Abstract

Colorectal cancer (CRC), although a major cause of death 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 CRC cells have been assessed. To investigate a potential anti-cancer activity, the effect of L. reuteri on the proliferation of Caco-2 CRC cells as compared with CRL-1831 normal colorectal cells was analyzed. Later, short chain fatty acids (SCFAs) as produced by *L. reuteri* were measured and the inhibitory action of SCFAs against Caco-2 cells was investigated using SCFAs synthetic formulations. Results revealed a significantly higher fatty acid production for L. *reuteri* during growth compared with two other *Lactobacilli* used as controls*: L. acidophilus* and *L. rhamnosus.* Also, both *L. reuteri* extracts, mostly the conditioned cell culture medium (CM), exhibited significant inhibitory effects against SW-480 cancerous cells and induced apoptosis. *L. reuteri* suppressed Caco-2 (cancer) but not CRL-1831 (non-neoplastic) cells. Caco-2 inhibition correlated with the concentration of bacterial SCFAs and was confirmed to be partially but not totally due to SCFAs bio-production. This suggests the potential of *L. reuteri* NCIMB 701359 in suppressing CRC risk.

4.2. Introduction

Colorectal cancer (CRC) is considered a leading cause of cancer mortality, the third most common cancer in men and the second most common in women, worldwide, with a high 5-year recurrence rates for patients [234]. Developments in therapeutic strategies for CRC still have limitations in improving the survival rate of patients [235]. CRC is considered a form of cancer for which the mortality regression comes significantly as a result of earlier detection and making chemoprevention an attractive strategy for this disease [236]. Contrary to common chemopreventive agents for which long administration brings unknown risk factors and possible toxicity [237], probiotics have been shown to more safely reduce cancer recurrence and toxicity in CRC patients [3]. Some of these probiotic formulations contained lactic acid bacteria (LAB) which

have been used in many biopharmaceutical supplements for CRC patients [191, 238, 239]. Studies have shown that many *Lactobacilli* extracts induced 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).

For instance, *L. acidophilus* [155, 240, 241] and *L. rhamnosus* [242-244] have shown a potential effect in colon cancer suppression. Nonetheless, *L. reuteri* bacteria have recently shown to possess probiotic efficacy, however, lacks adequate investigation. Most studies on *L. reuteri* bacteria have focused on their ability to affect the production of certain anti-microbial metabolites in variable intestinal environments [200, 202] and very few have shown significant production of SCFAs [245]. Some *L. reuteri* were connected to CRC by its ability to precipitate the deconjugated bile salts and physically bind bile salts, thereby making the harmful bile salts less bioavailable [246]. It was found that administrating *L. reuteri* to mice colitis model reduced colonic mucosal adherent, translocated bacteria and prevented the disease, some attributes which indicate these bacteria may have potential to prevent CRC risk. Other findings demonstrated *L. reuteri* ATCC PTA 647 secretion of components that trigger death in myeloid leukemia-derived cells which can be associated to a potential CRC preventive effect [247].

This report characterized the general bacterial metabolic activity of *L. reuteri* NCIMB 701359, using free fatty acid (FFA) profile and cell growth. The anti-proliferative activity of the bacterium cell free extracts was also characterized, using the growth and apoptosis induction in CRC cells. To confirm that this effect is due to an anti-cancer effect of the bacterium and not to a cytotoxic effect, the same assay was performed on non-neoplastic colon cells. Subsequently, the probiotic conditioned cell culture medium (CM) composition in SCFAs (acetic, propionic, and butyric acids) was determined. It was necessary to determine if the anti-cancer effect was mainly due to the production of bacterial SCFAs rather than other bacterial products. To investigate this, as the bacterial SCFAs were quantified; those amounts were used to formulate SCFA synthetic mixtures. If the SCFA synthetic formulation inhibits cancer cells less than its analogous *L. reuteri*-CM, SCFAs would not be considered 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 that the levels of SCFAs produced by *L. reuteri* are in total the only active probiotic component produced against CRC cells.

4.3. Materials and Methods

4.3.1. 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 EasyPure 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).

4.3.2. Bacterial and mammalian cells

L. reuteri NCIMB 701359 was acquired from the National Collection of Industrial Marine and Food 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 ^{o}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 CRC 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 ^{o}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 (CM) and serum/antibiotic-free DMEM.

4.3.3. 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 extracting 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. 50 µl of the reaction mix was added to either standard or test samples and all were incubated for 30 min at 37 °C) in dark. At this point, the fatty acids present are converted to CoA derivatives which gets oxidized and give a color, which can be measured at 570 nm.

4.3.4. 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, [212] and Kim et al., [213]. First, probiotic bacteria were left to grow for 24 h (37 °C, 5 % CO_2) and were passaged at 1 %. For the 3^{rd} - 4^{th} 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 culture medium and were incubated at 37 °C for 2 h, under shaking (50 rpm). The suspension was centrifuged twice (1000 x g, 15 min, 4°C) and sterile-filtered (0.2 µm-pore-size filter) to obtain the probiotic CM. For the preparation of bacterial PS, sterile-filtered supernatant was obtained after centrifuging out the bacterial pellet (1000 x g, 15 - 25 min, 4 °C). Before incubation with colorectal cells (cancerous or normal), 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.

4.3.5. 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 x 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's instructions [216]. 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 agitated on an orbital shaker (2 min, 200 rpm). Following this, the plate was left to incubate at RT for 10 min before recording the luminescent signal on a spectrophotometer (Perkin Elmer, Victor 3, multi-label

315 microplate reader, MA, USA). Cancer cell inhibition was determined by evaluating the
316 proliferation of treated cells compared with untreated ones at each time point.

317 **4.3.6. Apoptosis assay**

318 To determine if the suppression of CRC cell growth is related to cell death upregulation, apoptosis
319 was evaluated by assessing caspace -3 and -7 using Caspase-Glo® 3/7 assay (Promega, USA).
320 Briefly, the lyophilized substrate, provided with the kit, was dissolved with a buffer at RT.
321 Luminometer-compatible white-walled 96-well plates were filled with samples (100 µL) of
322 negative control, treated cells, and blank. After probiotic treatment, the plate was left to equilibrate
323 at RT.100 µL of the luminescent reagent was added to each well, covered with a lid and was placed
324 on plate shaker (300 - 500 rpm, 30 sec).This was followed by incubation at RT for 30 minutes to
325 3 hours. The luminescence in each well was captured using a multi-plate reader spectrophotometer.

326 **4.3.7. Determination of *L. reuteri* probiotic action on neoplastic and non-neoplastic colon**
327 **cells**

328 In order to verify that the suppressive effect of probiotic cell-free extract is due to an anti-cancer
329 activity and not cytotoxic, the treatments were tested on both normal (CRL-1831) and cancer
330 (Caco-2) colon cells. Cells were seeded into 96-well culture plates (5 x 10^3 cells/well) before
331 incubation (37°C, 5% CO_2, 24 h). Colon cells were treated with *L. reuteri* NCIMB 701359 cell-
332 free extracts for at 24 h and 48 h before assessing viability, compared with *L. acidophilus* ATCC
333 314 and *L. rhamnosus* ATCC 53103 as positive controls.

334 **4.3.8. Lactic acid and SCFA analysis**

335 After the preparation of all CM, SCFAs produced by *L. reuteri* NCIMB 701359, in comparison
336 with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103, were measured using HPLC [214,
337 215]. The HPLC system (Hewlett-Packard HP1050 series, Model 1050 UV, Agilent Technologies,
338 USA) used was equipped with a UV-vis detector and diode array detector (DAD, 210 ± 5 nm).
339 Samples (100 µl) were injected through a prepacked column (Rezex ROA-organic acid H+ (8 %),
340 150 x 7.80 mm, Phenomenex, CA, USA) attached to an ion-exclusion microguard refill cartridge.
341 Chromatographs were generated using ChemStation LC3D software (Rev A.03.02, Agilent
342 Technologies, CO, USA). The two mobile phases H_2SO_4 (0.05 M) and acetonitrile (2%) were
343 pumped through the column with an isocratic gradient (0.8 - 0.7 mL/min, 35 °C). Lactic, acetic,
344 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 ($R^2 \geq 0.99$).

4.3.9. Efficacy and role of SCFAs produced by *L. reuteri* NCIMB 701359

This method was used to determine the role of the naturally produced SCFAs by *L. reuteri* bacteria in the inhibition of CRC cells. The probiotic SCFAs produced were compared with their analogue SCFA synthetic formulations. Specifically, 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, at a ratio of 1:2, for 72 h. An ATP bioluminescence assay, as described above, was used for the analysis to determine the inhibitory effect of SCFAs synthetic formulation on colon cancer cells in comparison with the CM of *L. reuteri* NCIMB 701359-CM.

4.3.10. Statistical analysis

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

4.4. Results

4.4.1. *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, the FFA content of the bacterial culture supernatant was characterized during the different growth phases (0 - 72 h) (**Figure 4.1 and 4.2**). During the lag and the stationary phases, *L. acidophilus* ATCC 314 (**Figure 4.2a**) and *L. rhamnosus* ATCC 53103 (**Figure 4.2b**), did not increase the levels of total FFAs in the culture, while *L. reuteri* NCIMB 701359 did increase the FFA levels (**Figure 4.2c**). At the beginning of the stationary phase, *L. reuteri* NCIMB 701359 (**Figure 4.2f**, $p < 0.05$) had significantly higher FFA levels compared with *L. acidophilus* ATCC 314 (**Figure 4.2d**) and *L. rhamnosus* ATCC 53103 (**Figure 4.2e**). However, during the death phase, *L. reuteri* NCIMB 701359 (**Figure 4.2i**, $p < 0.01$) released significantly less FFAs than *L. acidophilus* ATCC 314 (**Figure 4.2g**) and *L. rhamnosus* ATCC 53103 (**Figure 4.2h**). Using the viable bacterial cell number and total bacterial weight for further normalizations,

the concentrations of FFA/bacterial cell (**Figure 4.2j**) and FFA/g of bacterial pellet (**Figure 4.2k**) were significantly superior to *L. reuteri* NCIMB 701359 ($p < 0.001$). A minor exception was found during the death phase, where only *L. rhamnosus* ATCC 53103 was capable of significantly increasing the levels of FFAs up to 109.5 ± 1.8 μM PAE per viable bacterial cell, compared with *L. reuteri* NCIMB 701359 ($p < 0.001$).

4.4.2. *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 4.3**). For PS, cancer cell proliferation was significantly less than untreated cells. In the case of PS of *L. acidophilus* ATCC 314, *L. reuteri* NCIMB 701359 and *L. rhamnosus* ATCC 53103 (**Figure 4.3a)**, the 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 4.3c**), respectively, compared with the control. Conversely, when probiotic CM was tested, results showed that the CM of *L. reuteri* NCIMB 701359 have inhibited at 24 h (**Figure 4.3e),** 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 4.3f**), 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.

4.4.3. *L. reuteri* NCIMB 701359 cell-free extracts induced apoptosis in CRC cells

Both types of bacterial extracts were tested for their ability to induce cell death through apoptosis induction in cancer cells (**Figure 4.4**). In case of probiotic PS, after 12 h **(Figure 4.4a**), *L. reuteri* NCIMB 701359 (21.7 ± 12.4 %) induced less apoptosis when compared with *L. rhamnosus* ATCC 53103 (26 ± 13.9 %, $p = 0.04$) and induced higher apoptosis when compared with *L. acidophilus* ATCC 314 at 18.9 ± 8.6 %. For probiotic CM, data collected at 24 h (**Figure 4.4d**) showed that *L. reuteri* NCIMB 701359 (27.2 ± 9.4 %) significantly induced cell death, when compared with *L. acidophilus* ATCC 314 ($p = 0.031$).

4.4.4. *L. reuteri* NCIMB 701359 suppressed CRC cells but not normal colon cells

The purpose of this experiment was to verify the anti-cancer activity of the probiotic bacteria and determine that the inhibitory effect was specific to cancer cells and not to normal colon epithelial cells. Thus, the effect of *L. reuteri* NCIMB 701359 on the viability of both Caco-2 and non-cancerous CRL-1831 were determined using CM, compared with *L. acidophilus* ATCC 314, *L. rhamnosus* ATCC 53103, and untreated cells (**Figure 4.5**). Results showed that for *L. reuteri* NCIMB 701359 (**Figure 4.5c)** and *L. rhamnosus* ATCC 53103 (**Figure 4.5b**), cancer cell growth was inhibited, at 24 h, by 22.41 ± 2.14 % and 6.33 ± 1.04 % ($p < 0.01$) respectively, when compared with the untreated cells. However, at 48 h, cancer cell viability was reduced by 42.68 ± 4.44 % (**Figure 4.5c**), and 11.42 ± 1.75 % (**Figure 4.5d**) respectively, compared with untreated group ($p < 0.01$). 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 4.5c**), 23.95 ± 2.49 % (**Figure 4.5b**), and 12.59 ± 1.92 % (**Figure 4.5a**), respectively, compared with untreated cells ($p < 0.05$). Moreover, at 7 days of probiotic treatment, Caco-2 cell growth was inhibited 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 viability was observed in CRL-1831 treated with probiotic extracts. The results specified that *L. acidophilus* ATCC 314 (**Figure 4.5d**), *L. reuteri* NCIMB 701359 (**Figure 4.5f**), and *L. rhamnosus* ATCC 53103 (**Figure 4.5e**), had stimulated CRL-1831 epithelial normal colon cells significantly, after 24, 48, 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 4.5b, 4.5e, and 4.5f)**).

4.4.5. *L. reuteri* NCIMB 701359 secreted higher concentrations of SCFAs

We hypothesized that the significant anti-cancer effect by *L. reuteri* NCIMB 701359 is due to the production of SCFAs. Therefore, the quantification of lactic acid and SCFAs produced by this bacterium was analyzed in order to confirm their presence in the cell-free extract (**Figure 4.6**). The results showed that *L. reuteri* NCIMB 701359 produced the highest amount of acetate (**Figure 4.6c**) and propionate **(Figure 4.6d**), compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 ($p < 0.05$). *L. reuteri* NCIMB 701359 was observed to produce significantly higher levels of butyrate (**Figure 4.6d**) and higher levels of total SCFAs ($p < 0.001$, **Figure 4.6e**). In

addition, *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 did not significantly inhibit colon cancer growth and did not generate detectable amounts of propionic and acetic acids (**Figures 4.6b and 4.6c**), but produced higher amounts of lactic acid i.e. 1970.6 ± 9.56 and 3239.8 ± 9.9 mg/L, respectively, compared with *L. reuteri* NCIMB 701359 **(Figure 4.6a**).

4.4.6. 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 against colon cancer cells (**Figure 4.7**). First, concentrations equal to the acetate, propionate, and butyrate amounts produced were tested separately, and it was observed that their growth inhibitory effect did not exceed 35 % (**Figure 4.7a**) compared to bacterial CM. In fact, the SCFA synthetic formulation corresponding to *L. reuteri* NCIMB 701359 was significantly more effective than the bacterial CM itself ($p < 0.001$) or the SCFA synthetic formulation following lactate addition ($p < 0.01$, **Figure 4.7a**).

4.5. Discussion

Despite CRC wide symptoms and treatment side-effects, this disease can be regulated through modifications of diet. There is a need to identify dietary components such as probiotics, as health supplements that have shown potential to restore intestinal metabolism and beneficially alter CRC biomarkers. In the last decade, many papers have suggested that probiotic *lactobacilli* have positive effect on colon health and can impact in the reduction of 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 to many *in vitro* studies aiming to determine the possibility of their use as novel bio-preventive treatments. However, there is a void in the evaluation of novel bacteria, establishing their degree of efficacy compared with other stains of *lactobacilli,* defining specific metabolic effects, and *in vitro* action on both CRC cells and healthy colon cells.

The current study, for the first time, shows the characterization of *L. reuteri* NCIMB 701359 activity in producing potential anti-cancer compounds. This feature was determined by measuring their general ability to affect FFA 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 extract 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 than both *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 with excretion of more FFAs (two fold) in the growth media. As known, FFAs have diverse potent biological activity against potential pathogenic or opportunistic microorganisms, in addition to being cytotoxic to some mammalian cells. Some reports stated that LAB bacteria could produce FFAs which are considered beneficial functional lipids [248], predominantly butyric and linoleic (LNA) acids that are converted to CLA. 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 benefits to products supplemented by this bacterium [249]. 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 have attracted interest as being a novel type of beneficial functional lipids [250] or other phytochemicals [251].

In this study, we have studied the effect of both PS and CM, prepared from the bacterial culture of *L. reuteri* NCIMB 701359, against SW-480 colon cancer cells. The results suggested that both types of probiotic bacterial cell-free extracts contained bacterial metabolites with anti-proliferative (**Figure 4.3**) and apoptotic activities (**Figure 4.4**). Results showed no significant ability of PS to induce apoptosis in cancer cells at 12 h, 24 h, and 7 days of treatment in comparison to the positive controls. Meanwhile, the CM of *L. reuteri* NCIMB 701359 had a 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 the least growth when compared with untreated cells, an effect that was observed until 7 days of incubation ($p < 0.001$). For both types of probiotic extracts, the bacteria appeared to secrete inhibitory and apoptotic actions against colon cancer cell in the CM.

Very few studies have demonstrated the effect of probiotic extracts on normal colon cells. However, in our study, we have shown that *L. reuteri* NCIMB 701359 had no cytotoxic effect on non-neoplastic epithelial colon cells. We found that not only had *L. reuteri* NCIMB 701359

suppressed the most cancer cells, but it also promoted the best healthy proliferation of normal colon cells CRL-1831 (**Figure 4.5**). Hence, we can hypothesize that this mechanism of action involves compounds that can be a source of energy for normal cells and in addition inhibit the growth of cancer cells. It is established that SCFAs, especially propionate, an inhibitor of histone acetylases (HDACI), affects androgen receptor (AR) co-regulators expression and transcription activity in cancer cells, while exhibiting minimal effect on normal prostate cells [252]. This would explain why ineffective L. *acidophilus* produced less propionate or none, whle *L. reuteri* with the highest anti-cancer effect exhibited no cytotoxic effect on normal 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 on their composition of SCFAs and lactic acid (**Figure 4.6**), 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 latter 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 the *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, possesses significant immunoregulatory functions and cancer prophylactic potential. He demonstrated that, similar to other SCFAs, propionic bacterial supernatant or propionate upregulated the expression of the NKG2D ligands on cancer cells and activated T lymphocytes [253].

A strong correlation ($r^2 = 0.9401$, $p = 0.024$) was observed between the inhibitory effect of probiotic CM and the concentrations of total SCFAs, AA, PA and PA+BA (**Figure 4.6**). Our bacterium *L. reuteri* NCIMB 701359 secreted significantly higher concentrations of propionate than both other tested bacteria (*L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103). This confirmed that the observed beneficial probiotic effect is, probably, dependent of the production of propionate [254, 255]. Till now, there has been more emphasis on the physiological and pathological role of butyrate and SCFA in combination along with an undervaluation of the potential effects of propionic acid. Researchers have mainly investigated propionate in the context of ruminant physiology, particularly, liver physiology and metabolism. Though, it was demonstrated as the primary precursor for glucose production [256], as some of the evidence have

suggested the role of propionic acid in human physiology. Moreover, propionate was shown to exert anti-inflammatory and anti-microbial activity against pathogenic bacteria in the gastrointestinal (GI) tract [257].

One of the primary objectives of the current study was to confirm that the mechanism of action of *L. reuteri* NCIMB 701359, in suppressing the growth of cancer cells, is by the secretion of SCFAs/propionate. The following steps were taken to examine this objective: (i) Examination of the effect of pure SCFAs at the same concentration produced by *L. reuteri* 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 naturally were tested and this demonstrated that SCFA is not 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 for their anti-proliferative activity, since *L. rhamnosus* ATCC 53103 did not secrete any SCFAs. Thus, the SCFA synthetic formulation corresponding to *L. reuteri* NCIMB 701359 was observed to significantly inhibit the CRC cell growth, however, less than the probiotic cell-free extract CM (**Figure 4.7b**), 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 4.7b**) was hindered. This could be explained by the potential presence of bacterial products with a complementary effect or whose activity is necessary to the action of SCFAs against cancer cells.

Some studies have identified other components secreted in probiotic CM and PS that demonstrated bacterial regulation of colon cellular responses through the production of active molecules by different bacterial strains. In recent studies, the cell-free extracts of *L. rhamnosus* GG induced cellular effects by the secretion of multiple low-molecular-weight compounds regulating epithelial cellular responses, such as p75 and p40. p75 and p40 were 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 [258, 259]. With all this in mind, we can speculate that a LAB producing a higher level of fatty acids, is considered an energy source for non-neoplastic epithelial colon cells [260]. Consequently, they may also have potential

552 for superior anti-tumorigenic activity and the ability to reduce colonic lesions, maintain a healthy
553 gut cell lining, preventing tumor development and reducing cancer risk**.**

554 **4.6. Conclusion**

555 *L. reuteri* NCIMB 701359 presents a relevant potential biotherapeutic candidate that can be
556 considered in probiotic formulations for CRC, when compared *in vitro* with *L. acidophilus* ATCC
557 314 and *L. rhamnosus* ATCC 53103. Findings described the characteristic FFA profile of *L. reuteri*
558 NCIMB 701359 and showed that it produced higher amounts of SCFAs. *L. reuteri* NCIMB 701359
559 had a significant anti-proliferative effect that correlated with the levels SCFAs secreted in CM.
560 This bacterium had alos promoted healthy growth of normal colon cells suggesting its ability to
561 enhance the devolpment of a healthy colon mucosa which can be relevant ofr future investigations.
562 This suggests that a mechanism of action by which the fermentation of non-digestible compounds
563 is associated with the production of other active compounds (e.g. phenolic fatty acids derivatives
564 and biopeptides) [261]. These conclusions emphasized concerns regarding the use of *L. reuteri*
565 NCIMB 701359 as cancer-causing lesions preventer that possesses comparable anti-cancer activity
566 to other LAB. It also has a potential to be an effective component of a functional food strategy for
567 tumor growth inhibition and cancer prevention and as a biotherapeutic agent in CRC.

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577 preparation of the manuscript.

578 **4.8. Conflicts of Interest**

579 No author has conflicts of interest to disclose.

4.9. Figures and Tables

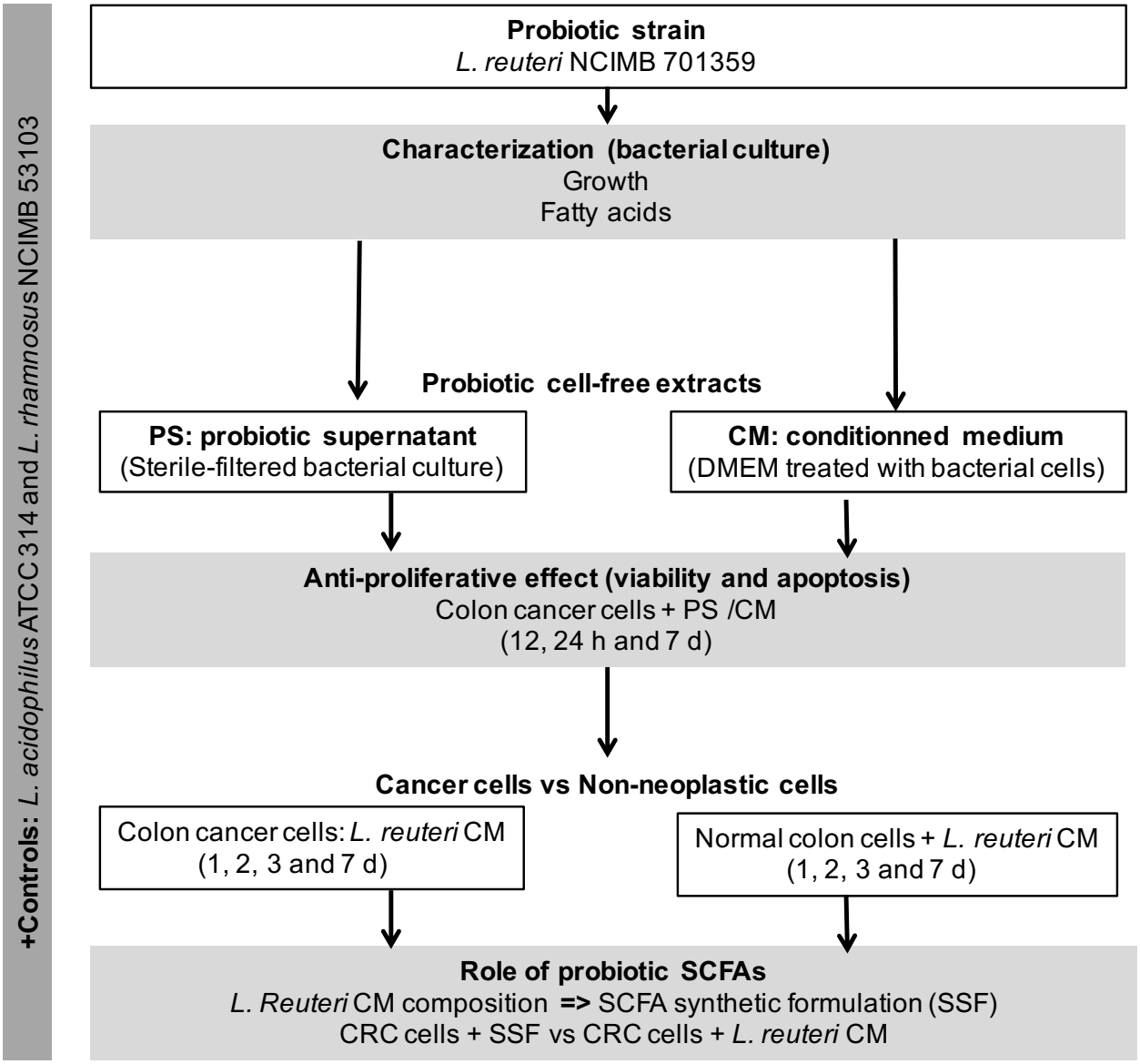
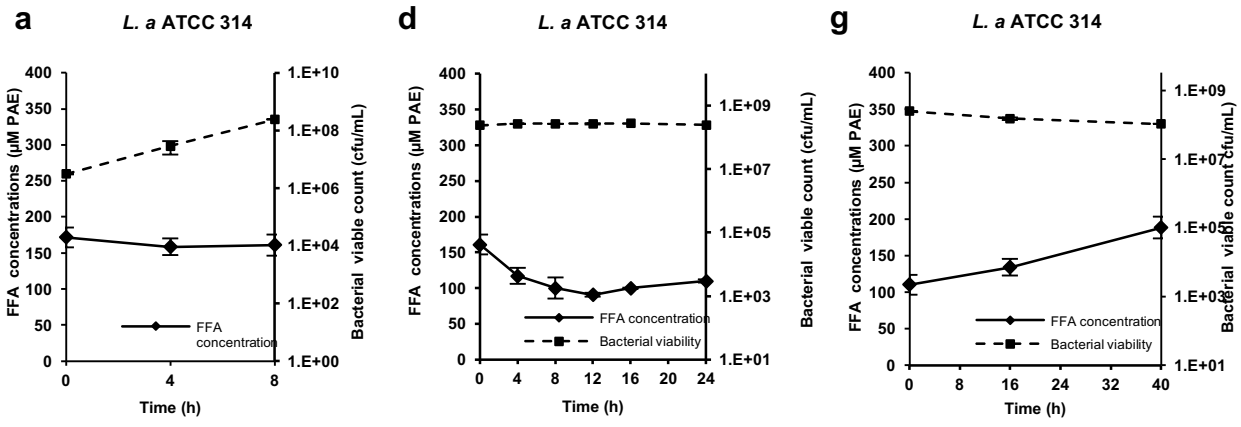


Figure 4.1: Experimental outline for investigating the anti-cancer characteristics of *L. reuteri* NCIMB 701359 and the role performed by probiotic compared with synthetic SCFAs.

584 LAG AND EXP. P. STATIONARY P. DEATH P.



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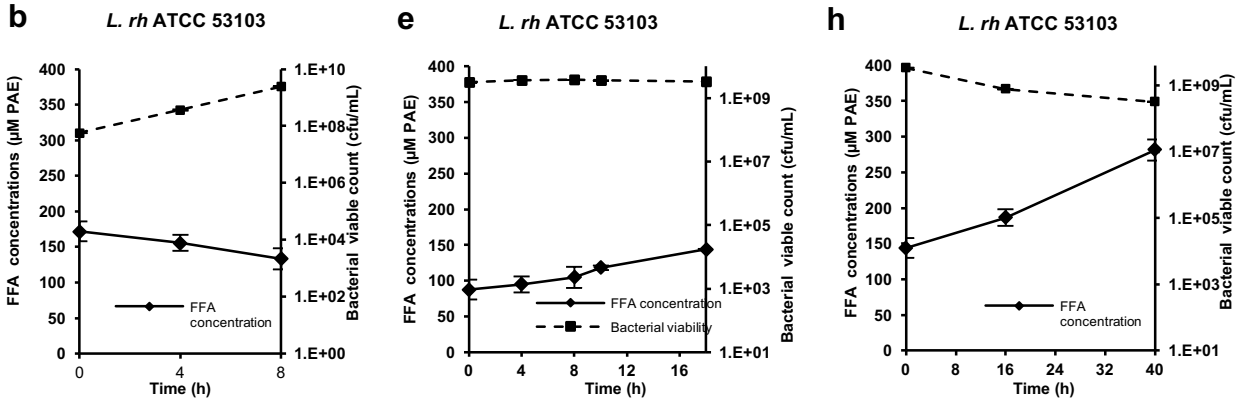

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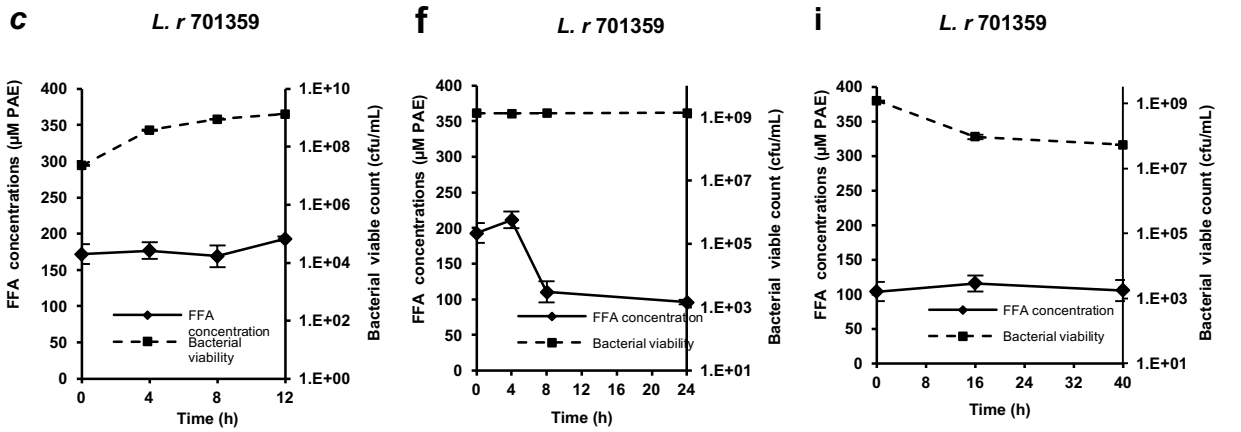

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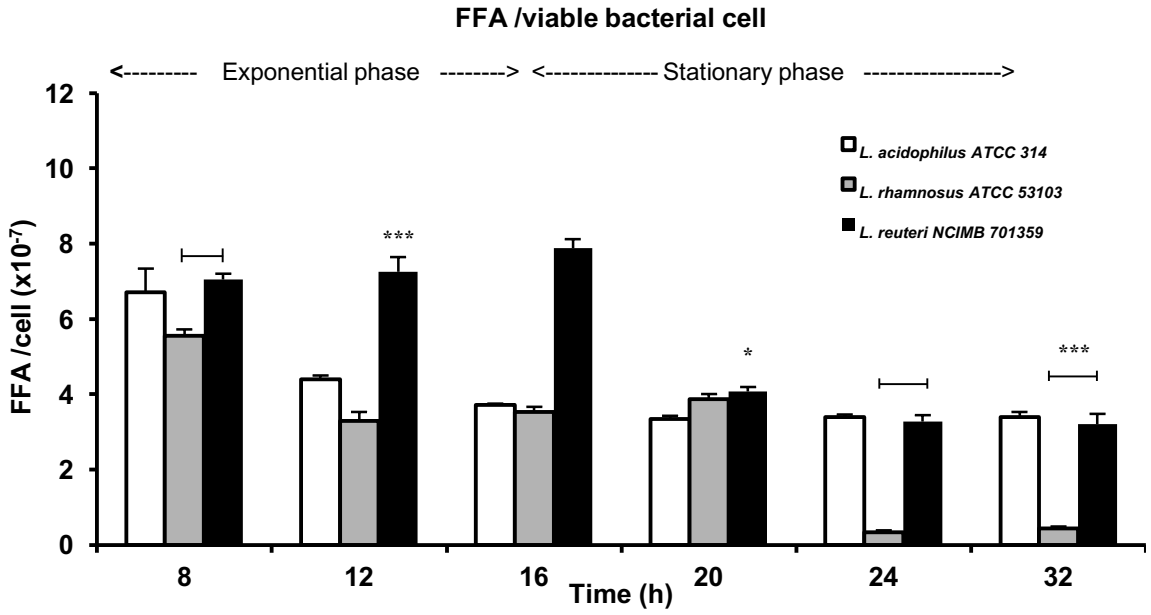
FFA /viable bacterial cell
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FFA /cell (x10-7)
Time (h)
L. acidophilus ATCC 314
L. rhamnosus ATCC 53103
L. reuteri NCIMB 701359
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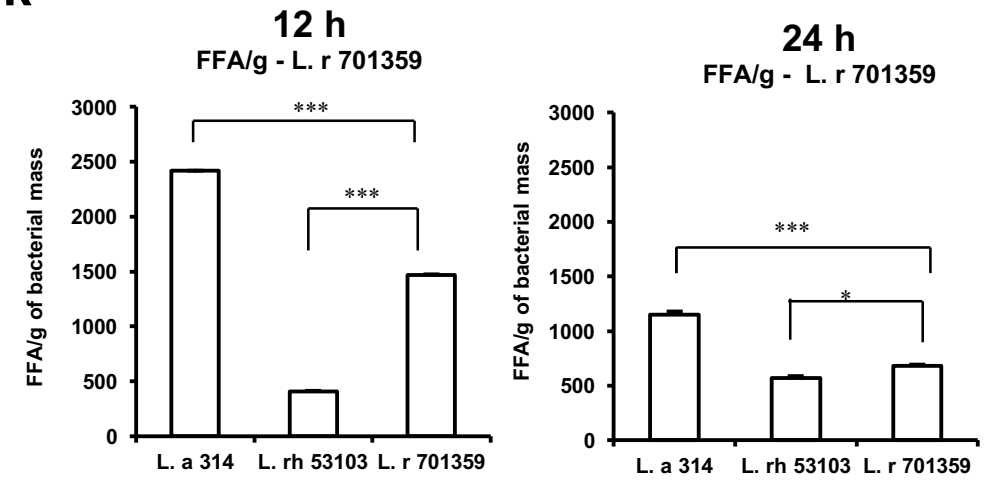
12 h
FFA/g - L. r 701359
24 h
FFA/g - L. r 701359
FFA/g of bacterial mass
L. a 314
L. rh 53103
L. r 701359
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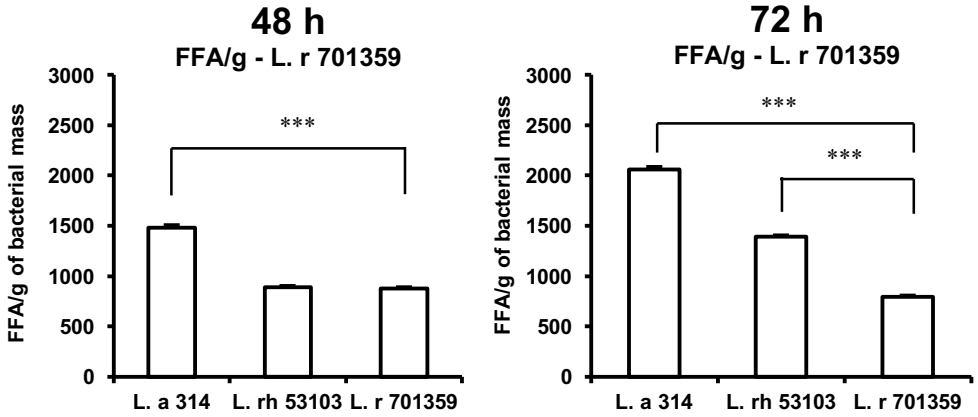
589



590

591 **Figure 4.2: Description of the metabolic activity of *L. reuteri* NCIMB 701359 based on bacterial growth**
592 **and free fatty acid (FFA) profiles.**

593 The content of total FFAs in the bacterial culture of *L. reuteri* NCIMB 701359 (**c, e and h**) were determined
594 for different phases for bacterial growth, in comparison with two of *Lactobacillus* bacteria (positive
595 controls): (**a, b and c**) *L. acidophilus* ATCC 314 and (**d, f and i**) *L. rhamnosus* ATCC 53103. (**j**) Description
596 of the variation of FFA levels per viable bacterial cell (exponential and stationary phases). (**k**) Illustration of
597 the levels of FFA/g of bacterial cells at (12, 24, 48 and 72 h). *L. reuteri* NCIMB 701359, *L. acidophilus*
598 ATCC 314 and *L. rhamnosus* ATCC 53103 were growing in each culture during 72 h in MRS (37^{o}C, 5 %
599 CO_2). *$p < 0.05$, **$p < 0.005$, and ***$p < 0.001$, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus*
600 ATCC 53103. Data represent the mean ± SEM ($n = 3$). P.: phase; EXP: exponential; PAE: Palmetic acid
601 equivalents. *L.a* 314. *L. rh* 53103: *L. rhamnosus* 53103, *L. a* 314: *L. acidophilus* ATCC 314, *L. r* 701359: *L.*
602 *reuteri* NCIMB 701359.

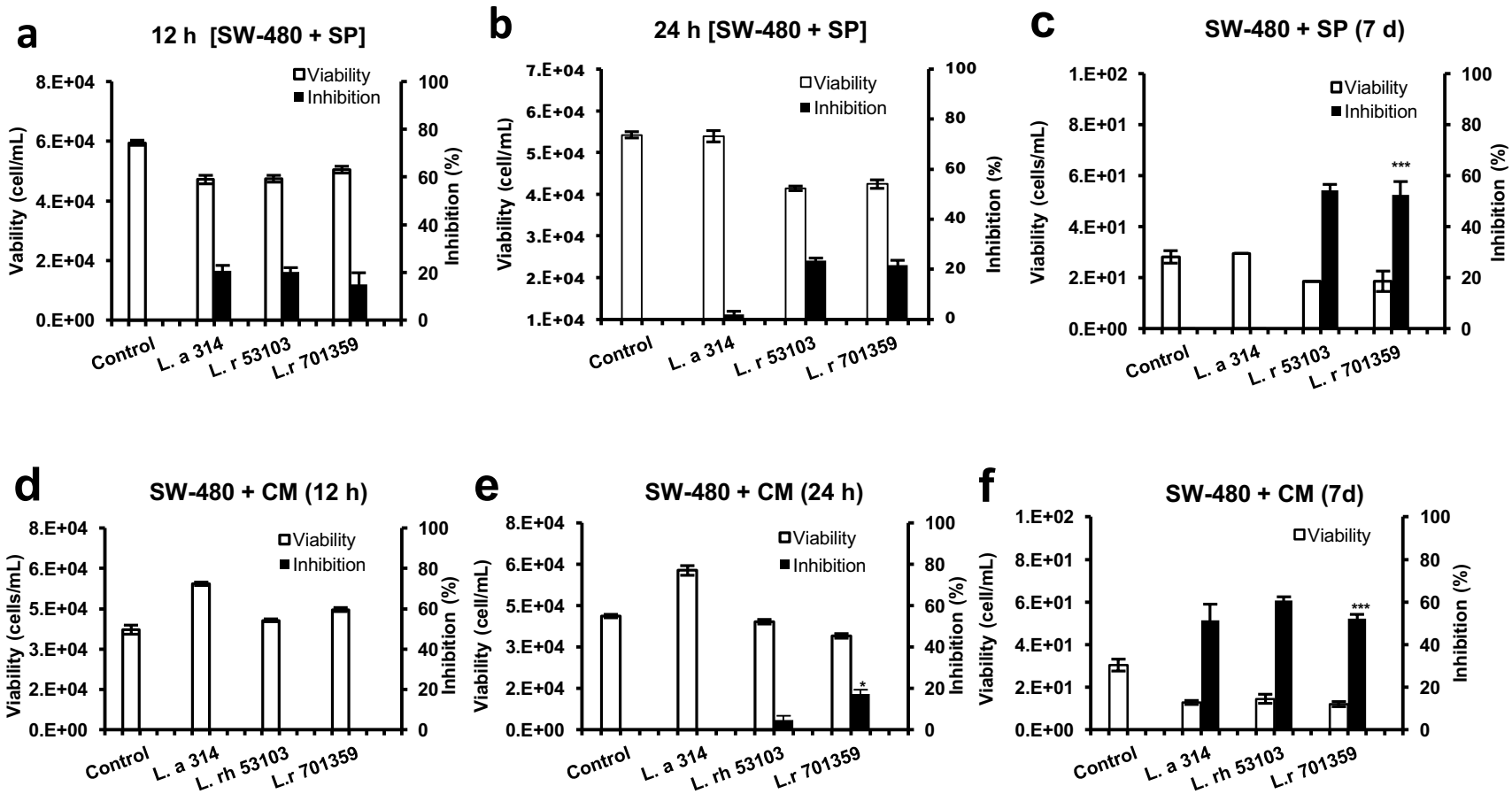


Figure 4.3: Investigation of cancer cell inhibition by different bacterial cell-free extracts of *L. reuteri* NCIMB 701359.

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. a* ATCC 314, *L. rh* 53103, and negative control. Data are presented as mean ± SEM ($n = 4$). *L. rh* 53103: *L. rhamnosus* 53103, *L. a* 314: *L. acidophilus* ATCC 314, *L. r* 701359: *L. reuteri* NCIMB 701359.

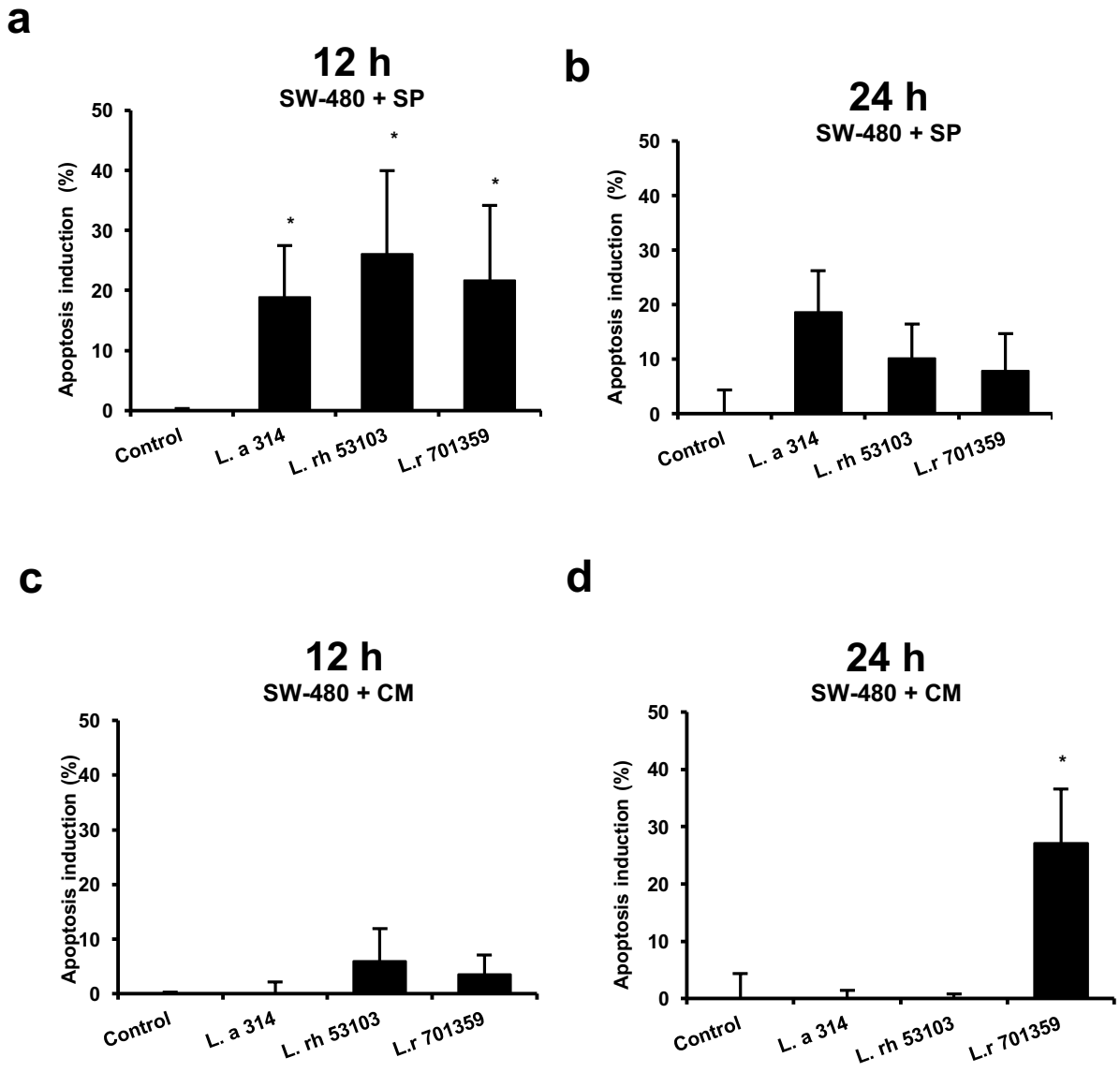
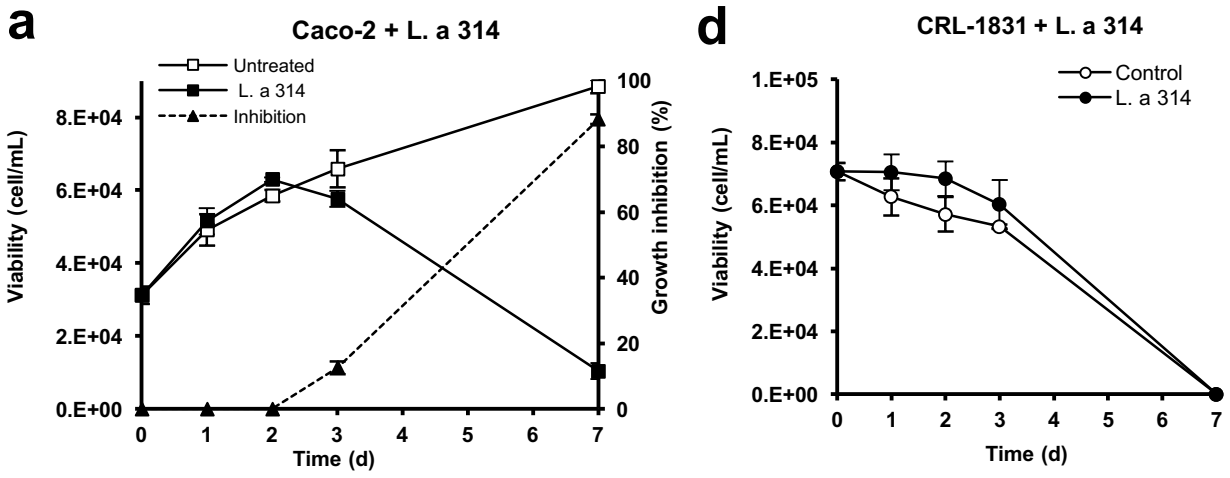
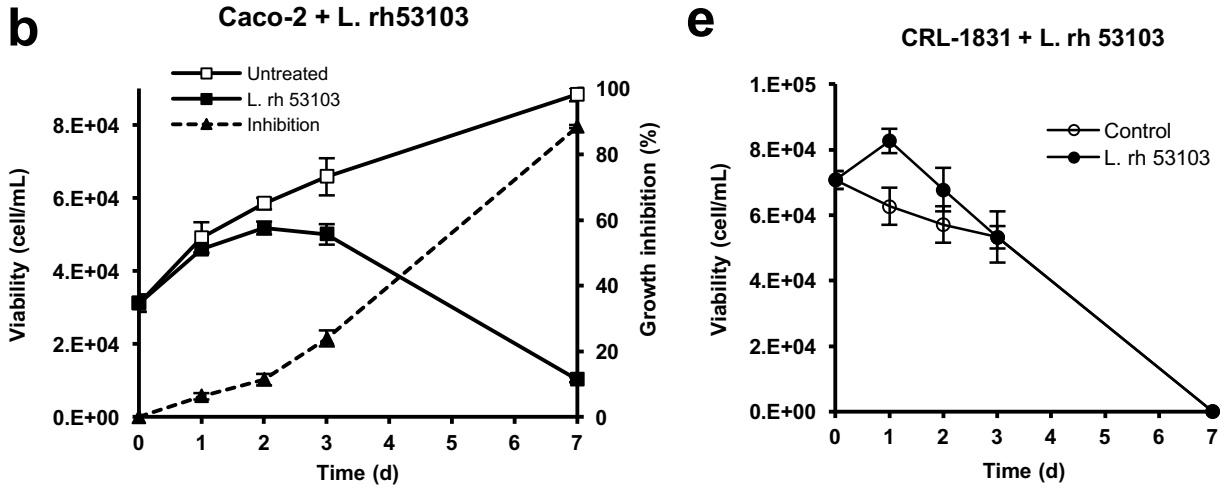


Figure 4.4: Determination of CRC cell apoptosis induced by *L. reuteri* NCIMB 701359.

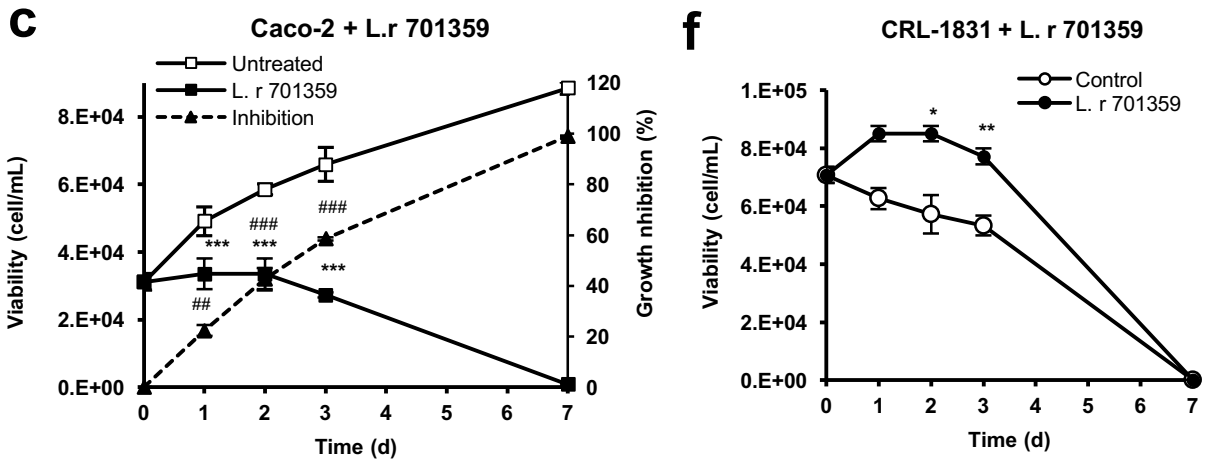
The bacterial cell-free extracts (PS and CM) of *L reuteri* NCIMB 701359 increased cell death in SW-480, when treated for (**a, c**) 12 h and (**b, d**) 24 h. The data values represent the mean ± SEM (n = 4). *$p < 0.05$, compared with control. # $p < 0.05$, compared with *L. acidophilus* ATCC 314 and *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.


a
Caco-2 + L. a 314
Untreated
L. a 314
Inhibition
Viability (cell/mL)
Growth inhibition (%)
Time (d)
d
CRL-1831 + L. a 314
Control
L. a 314
Viability (cell/mL)
Time (d)

618


b
Caco-2 + L. rh53103
Untreated
L. rh 53103
Inhibition
Viability (cell/mL)
Growth inhibition (%)
Time (d)
e
CRL-1831 + L. rh 53103
Control
L. rh 53103
Viability (cell/mL)
Time (d)

619


c
Caco-2 + L.r 701359
Untreated
L. r 701359
Inhibition
Viability (cell/mL)
Growth nhibition (%)
Time (d)
f
CRL-1831 + L. r 701359
Control
L. r 701359
Viability (cell/mL)
Time (d)

620

Figure 4.5: 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 cancer 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 ($n = 4$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, compared with untreated cells. #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. *L. rh* 53103: *L. rhamnosus* ATCC 53103, *L. a* 314: *L. acidophilus* ATCC 314, L. r 701359: *L. reuteri* NCIMB 701359.

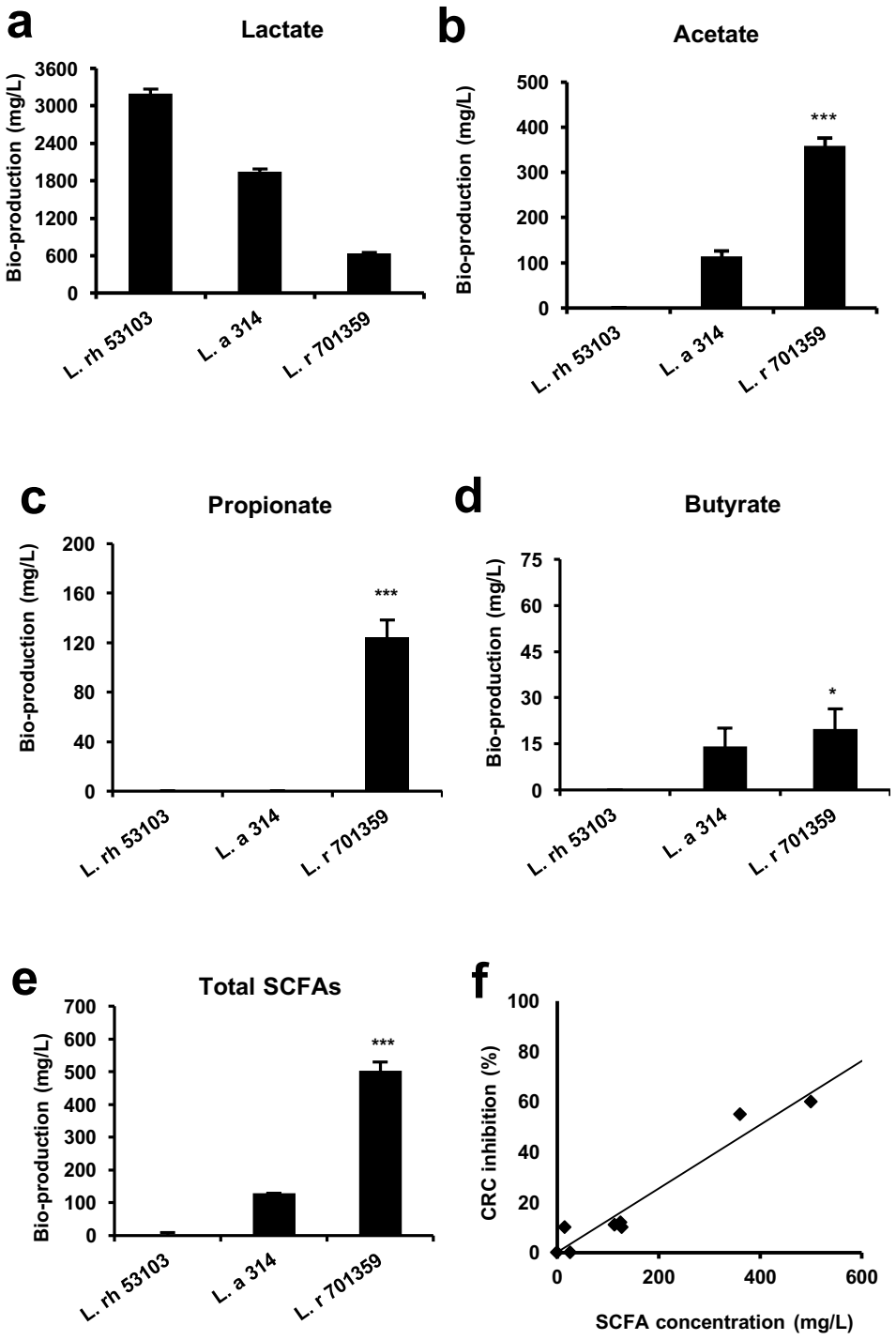


Figure 4.6: Bio-production of lactic acid and SCFAs by *L. reuteri* NCIMB 701359.

(**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 4.5** were used to run linear regression analysis. The CM was prepared by incubating probiotic bacterial pellet, in DMEM for 2 h (37^{o}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).

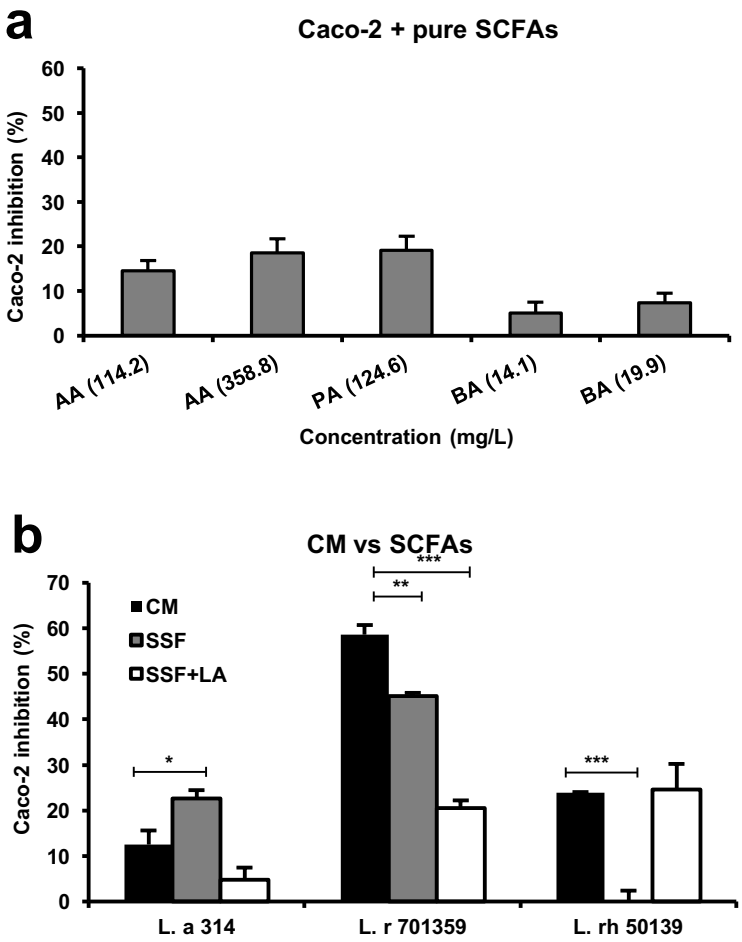


Figure 4.7: Confirmation of the involvement of SCFAs produced by *L. reuteri* NCIMB 701359.

(**a**) The anti-proliferative effect of SCFAs doses at similar concentrations than what was produced by *L. reuteri* NCIMB 701359 (**Figure 4.5**). (**b**) The anti-proliferative activity of SSF and SFF+LA corresponding to *L. reuteri* NCIMB 701357, against CRC cells, compared with *L. reuteri*-CM (72 h). Positive controls used 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 4.6 (n = 6). *p < 0.05, **p < 0.01, and ***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.

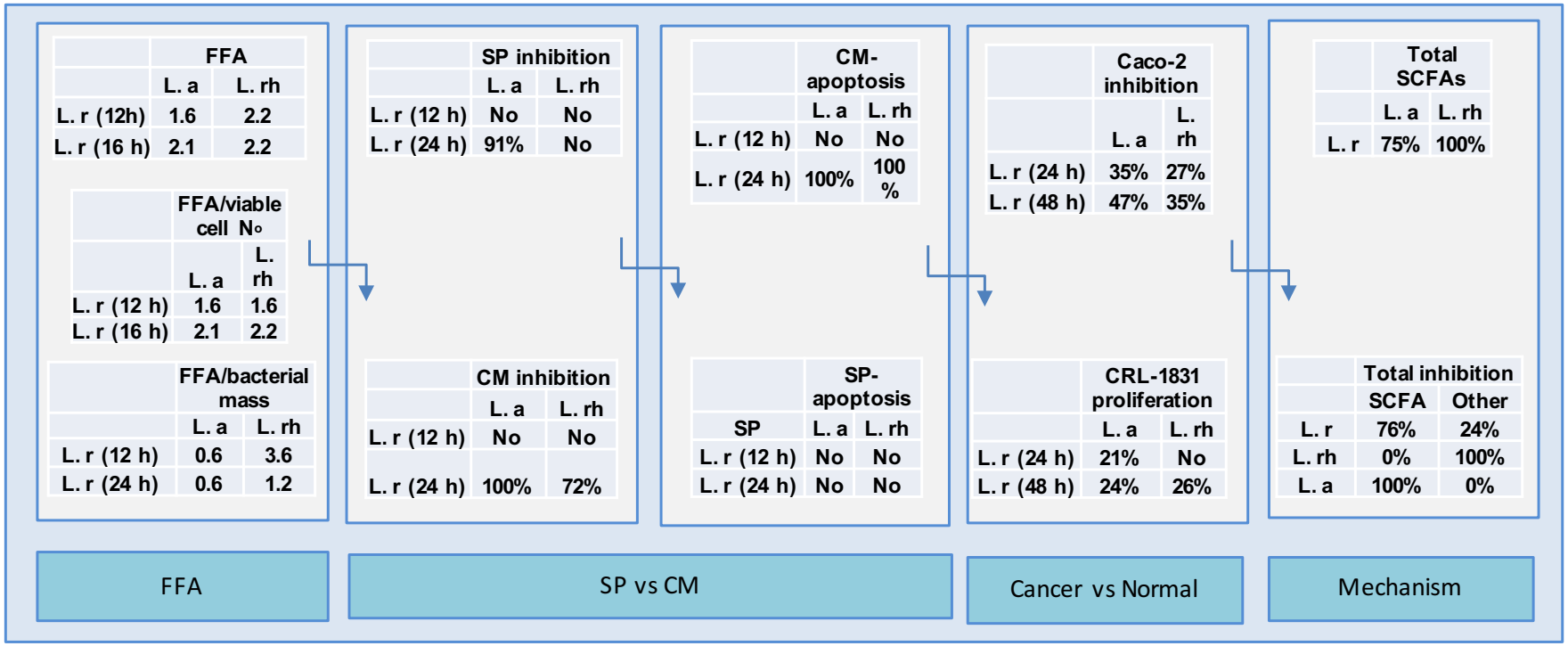


Figure 4.8: Schematic overview of the main features and potential mechanisms of action of *L. reuteri* NCIMB 701359 based on the current *in vitro* study compared with other *Lactobacilli*.

L. reuteri NCIMB 70135 was compared with *L. rhamnosus* ATCC 53103 and *L. acidophilus* ATCC 314, in percent or in fold. Only significant differences were presented ($p < 0.05$). No: no significant difference. L. rh: *L. rhamnosus* ATCC 53103, L. a: *L. acidophilus* ATCC 314, L. r: *L. reuteri* NCIMB 70135.

Original Research Article 3

CHAPTER 5. IDENTIFICATION OF *LACTOBACILLUS FERMENTUM* STRAINS WITH POTENTIAL AGAINST COLORECTAL CANCER BY CHARACTERIZING SHORT CHAIN FATTY ACIDS PRODUCTION, ANTI-PROLIFERATIVE ACTIVITY AND SURVIVAL IN AN INTESTINAL FLUID: IN VITRO ANALYSIS

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Preface: Since the previously selected *L. reuteri* bacteria showed a relative potential in inhibiting CRC, the focus of this chapter is to consider other possible *Lactobacillus* bacteria that were recently found to produce anti-inflammatory and antioxidant molecules. For this purpose, we considered screening of ferulic-acid-producing *L. fermentum* bacterial strains. The screening was performed based on the similar criteria's established for the selection of *L. reuteri* in the previous chapters. Within the screened strains, *L. fermentum* were able to produce higher SCFAs, inhibited the growth of colon cancer cells and more significantly showed survival in challenging conditions. Some of the *L. fermentum* bacteria were able to produce amounts of SCFAs, in simulated intestinal fluid. Such conditions are more relevant and indicate a close relation and prediction of the effects of probiotic strains, as biotherapeutics and to inhibit the growth of colorectal cancer cells

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http://dx.doi.org/10.4172/1948-593X.1000132

5.1. 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.

5.2. 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 [262, 263]. CRC accounts for 8.0 - 9.7% of all cancer cases and cancer-related deaths [264] and is considered not only a common type of cancer but also a complex and multifactorial disease [265, 266]. 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 [267]. 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 [236]. Dietary interventions have recently attracted increased attention from researchers and clinicians for the prevention and management of CRC [268]. 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 [268]. 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 [269, 270] and genital environments [271], were found to reduce infection [272] and overgrowth of harmful bacteria [273]. 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 [175]. *L. fermentum* have also shown to attribute potential beneficial GI health including anti-inflammatory [274, 275] and anti-tumorigenic [182, 276] activities. Some *L. fermentum* strains have shown greater or comparable effects than other probiotic bacteria, such as *L. reuteri* [277], *Bifidobacterium longum* [278] and *L. plantrum* [279].

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 [280]. The SCFAs resulting from the microbial metabolism of non-digestible carbohydrates in the gut, play a central role in the intestinal homeostasis [281]. 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 [282]. 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 [283]. 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 [175, 284, 285]. Thus, the current study screened a number of *L. fermentum* bacterial strains (NCIMB -5221, -2797, and -8829) in order to evaluate their biotherapeutic potential against CRC. These strains were previously investigated for the production of certain anti-inflammatory acids [286], cholesterol assimilation [175] in relation to targeted colon delivery [287], and for use in metabolic syndrome (MS) [21]. 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.

5.3. Materials and Methods

5.3.1. 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).

5.3.2. Bacterial cultures

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

5.3.3. Mammalian cultures

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

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

5.3.5. 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. [217]. The SIF solution contained; glucose (5.5 g/L), yeast extract (3.5 g/L), pancreatin (2 g/L), oxgall (1.5 g/L), pectin (2 g/L), inulin (0.54 g/L), fructooligosaccharides (0.85 g/L), starch (3 g/L), and monobasic potassium phosphate (KH2PO4, 3.3 g/L) dissolved in deionized water. The pH was adjusted to 6.8 using 2 M NaOH and 2 M HCl, followed by autoclaving at 120°C for 15 min and cooled at room temperature (RT) before use.

5.3.6. 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) [214, 215] (described below in detail).

5.3.7. Analysis of lactic acid and SCFAs

Lactic acid and SCFAs were separated using a slightly modified HPLC method [214, 215]. The HPLC system used (Model 1050 UV, Hewlett- Packard HP1050 series, Agilent Technologies, USA) was equipped with a UV-vis detector and diode array detector (DAD, 210 ± 5 nm). The column used was a prepacked Rezex ROA -organic acid H+ (8%) column (150 mm x 7.80 mm, Phenomenex, Torrance, CA, USA) attached to an ion- exclusion microguard refill cartridge and heated to 35°C. Data were obtained using ChemStation equipped with LC3D software (Rev A.03.02, Agilent Technologies, CO, USA). The mobile phases (0.05 M H_2SO_4 and 2% of acetonitrile) were pumped at an isocratic gradient with a 0.7 - 0.8 mL/min flow rate. A 100 µl of sample was injected through an autosampler. Lactic, acetic, propionic, and butyric acids were used to prepare standard solutions at concentrations of 1, 10, 100, 500, and 1000 ppm. The concentrations of samples were calculated using the linear regression equations ($R^2 \geq 0.99$) from each standard curve.

5.3.8. Cancer cell proliferation assay

The growth of colon cancer cells was determined using an ATP bioluminescence-based assay (CellTiter-Glo® Luminescent Cell Viability Assay, Promega). Caco-2 cells were seeded at 5×10^3 cells/ well onto 96-well culture plates and left to attach for 24 - 48 h for the formation of an epithelium-like monolayer (37°C, 5% CO_2). Caco-2 cells were incubated with the probiotic treatments for 24, 48 and 72 h, (37°C, 5% CO_2, pH 7). Cell growth inhibition and viability were determined according to the manufacturer's protocol [216]. 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).

5.3.9. Determination of bacterial stability in SIF

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

5.3.10. Relevance of SCFAs produced by *L. fermentum* strains

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

5.3.11. 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.

5.4. Results

5.4.1. *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 5.1**). Data showed that *L. fermentum* NCIMB 5221 (455.3 ± 9.3 mg/L, $p < 0.001$) produced the highest amounts of lactic acid when compared with *L. fermentum* NCIMB -2979 and -8829. All *L. fermentum* strains produced significantly less lactic acid than *L. acidophilus* ATCC 314 (1947.7 ± 23.3, $p < 0.0001$).

5.4.2. *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 5.2a**). 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 5.2b**). 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 5.2d**).

5.4.3. *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 5.3**). At 24 h (**Figure 5.3a**), 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 5.3b**), 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 5.3c** 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).

5.4.4. 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 5.4**). 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 5.4d**). Cancer cell inhibition correlated with the production of butyric ($r = 0.89$, $p < 0.001$) and acetic ($r = 0.0771$, $p < 0.001$) acids (**Figures 5.4c and 5.4b**). 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 5.4f**).

5.4.5. The action of probiotic SCFAs is strain-dependent

Establishing a correlation between *L. fermentum* bacteria SCFA production and their anti-proliferative effect against CRC cells is not sufficient to demonstrate that the inhibition of CRC cell growth is due to SCFAs. Therefore, an additional approach was taken using synthetic SCFAs. Initially, pure SCFAs corresponding to the concentrations produced by the bacteria were tested separately, and the resulting concentrations of acetic, propionic, and butyric acids showed significantly less inhibition (maximum of 20.3 ± 2.5%) than *L. fermentum*-CM (31.2 ± 1.5% minimum, $p < 0.05$, **Figure 5.5a**).

Secondly, SCFA synthetic formulations corresponding to the concentrations of SCFAs produced by the bacteria and containing acetic, propionic, and butyric acids were prepared (as described in **Table 5.1**). SCFA synthetic formulations were then tested on Caco-2 cells and compared with *L. fermentum*-CM (**Figure 5.5b**). These findings showed that the above mentioned mixtures had variable effects on the alteration of cell viability compared with *L. fermentum*-CM treated cancer cells. For *L. acidophilus* ATCC 314, the CM (12.6 ± 1.9%) had significantly less efficacy than its corresponding SCFA synthetic formulation (SSF-a, 22.9 ± 1.0%, $p < 0.05$). For *L. fermentum* NCIMB 5221, there was no significant difference ($p = 0.094$) between the SSF (58.9 ± 1.8%) and CM (57.9 ± 0.7%). However, for *L. fermentum* NCIMB 2797 (53.4 ± 1.6%) and *L. fermentum* NCIMB 8829 (31.2 ± 1.5%), *L. fermentum*-CM was significantly more effective than SCFA synthetic formulations (SSF-f2, 43.8 ± 2.2%, $p = 0.026$) and SSF-f8 (19.12 ± 1.6%, $p = 0.015$, **Figure 5.5b**).

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 5.5b**), indicating a loss of SCFA efficacy against cancer cells.

5.4.6. *L. fermentum* bacteria demonstrated resistance in SIF

The growth and viability of *L. fermentum* bacteria were strain-dependent. For *L. fermentum* NCIMB -2797 and -5221, the bacterial culture density (0.38 ± 0.001 minimum) was significantly higher compared with *L. acidophilus* ATCC 314 (0.29 ± 0.003%, $p < 0.001$, **Figure 5.6a**). Between 4 and 8 h, *L. fermentum* NCIMB -2797 (16.3 ± 1.9%) and -5221 (28.4 ± 2.4%) showed a significant increase in bacterial growth compared with the initial count. This was not the case with *L. acidophilus* ATCC 314 (**Figure 5.6a**).

In terms of decrease in viable bacterial cells, compared with initial count, a significant difference was determined (12 - 16 h), where *L. fermentum* NCIMB 2797 (70.11 ± 3.2% minimum) and *L. fermentum* NCIMB 5221 (94.02 ± 0.4% minimum) had higher death rate than *L. acidophilus* ATCC 314 (64.5 ± 0.7% maximum, $p < 0.01$, **Figure 5.6b**).

5.4.7. *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 5.7**). Results indicate that both *L. fermentum* strains produced significantly higher concentrations of lactic, acetic, and propionic acids (**Figures 5.6a – 5.6c**, respectively) than *L. acidophilus* ATCC 314 in SIF. *L. fermentum* strains also showed higher production of total SCFAs in SIF, as represented in **Figure 5.6d**. *L. acidophilus* ATCC 314 produced 1968.5 ± 0.3 mg/L and 413.1 ± 0.1 mg/L of total SCFAs, respectively. *L. fermentum* NCIMB 2797 produced 2491.9 ± 11.4 mg/L of lactate, 689.4 ± 2.1 mg/L of acetate, and 686.3 ± 35.7 mg/L of propionate. Also, *L. fermentum* NCIMB 5221 produced 2407.3 ± 42.3 mg/L of lactate, 637.99 ± 5.7 mg/L of acetate and 648.8 ± 17.8 mg/L of propionate. When considering the concentration of total SCFAs produced depending on bacterial culture density, both *L. fermentum* NCIMB -2797 and -5221 were significantly more potent than *L. acidophilus* ATCC 314 ($p < 0.0001$, **Figure 5.7e**).

5.4.8. 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 5.8a**). 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 5.2**. 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 5.8a**). For the inhibition of Caco-2 epithelium-like monolayer,

L. fermentum synthetic formulation SSF-SIF-f was significantly more efficient than the *L. acidophilus* systhetic formulation SSF-SIF-a (**Figure 5.8b**, $p = 0.0381$).

5.5. Discussion

CRC is a leading cause of death and an economic burden with a therapeutic market worth billions of dollars worldwide [288]. However, thanks to the preventive potential of this disease [289] it was found that a lifestyle and dietary measures, supplemented with digestive enzymes and probiotics, can substantially decrease CRC incidence [290]. 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 [291]. This study investigated three *L. fermentum* strains that have demonstrated antioxidant and anti-inflammatory potential by the production of ferulic acid [292, 293]. *L. fermentum* NCIMB -2797, -8829 and -5221 were investigated for anti-cancer-associated features, such as the production of SCFAs and anti-colon-cancer-cell-proliferative effects *in vitro*. For this, the cell culture conditioned medium (CM) of each bacterium was used as a probiotic extract treatment for the *in vitro* study. The metabolic activity of these LAB, when incubated in the CM was verified by the concentrations of lactic acid produced. It was observed that *L. fermentum* NCIMB 5221 produced significantly high levels of lactic acid as represented in **Figure 5.1**. Lactic acid is used by lactate-utilizing butyrate-producing bacteria in the gut [294] and is considered an anti-inflammatory component [295], which has the ability to increase anti-tumor immunoreactivity [296]. 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 [297]. 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 [298]. Thus, SCFAs secreted by *L. fermentum,* were quantified and produced at significantly different concentrations (**Figure 5.2**). *L. fermentum* NCIMB -2797, -8829, and -5221 produced significantly higher amounts of total SCFAs in their CM, compared with *L. acidophilus* ATCC 314 ($p < 005$, **Figure 5.2d**), but significantly lower amounts of lactate in their respective CM ($p < 0.001$, **Figure 5.1**). This result suggests that *L. fermentum* may act as an anti-colon cancer agent due to the production of higher quantities of SCFAs distinctively from *L. acidophilus* ATCC

314. Consequently, *L. fermentum* may produce anti-tumorigenic and anti-inflammatory activities as shown in a CRC $Apc^{Min/+}$ mice model [191]. 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 [299]. 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 [223], 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 these studies associated the potential anti-cancer activity of probiotic bacteria with the production of SCFAs; however, few have validated this theory [300]. 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 5.3**).

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 5.4e and 5.4f**). 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 5.4a** 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 5.4)**. Overall, the synthetically prepared mixtures of SCFAs showed a closer effect to *L. fermentum*-CM (**Figure 5.5b**). More specifically, *L. fermentum* NCIMB 5221 had the same effect as its corresponding SCFA formulation. The *L. fermentum* NCIMB -2797 and -8829 significantly inhibited colon cancer cell growth less than the corresponding SCFAs synthetic formulations ($p < 0.05$), indicating that the bacteria have potentially secreted additional anti-cancer products. Nonetheless, *L. acidophilus* ATCC 314 was significantly less effective than its SCFA synthetic cocktail. This indicates the presence of other bacterial factors, produced in the CM, which hindered

the effect of the naturally produced probiotic SCFAs. The data produced indicates that the anti-proliferative effect 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 5.1**, 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 [301] and adhesive [302] proteins that bind to the intestinal and gastric mucus as DNA fragments, or lipopolysaccharides [227]. 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 5.6a**) and resistance to the bile exposure for 4 h, which was significantly higher than for *L. acidophilus* ATCC 3 ($p < 0.05$, **Figure 5.6b**). 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 [229]. Between 12 h and 16 h, *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 5.7a)**, acetate (**Figure 5.7b**), propionate (**Figure 5.7c**), and total SCFAs (**Figure 5.7d**) than *L. acidophilus* ATCC 314 (**Figure 5.7**, $p < 0.01$). 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 5.7e**). 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 5.7**) [303] 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 5.3**. Notably, the only SCFA *L. acidophilus* ATCC 314 that did not produce detectable levels was propionate (**Figure 5.2b)**. 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 5.8a**), suggesting that propionate production is a major mechanism for colon cancer inhibition by *L. fermentum* in the intestinal environment.

5.6. 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 5.9**). 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.

5.7. Acknowledgements

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5.8. Conflicts of interest

The authors have no conflicts of interest to disclose.

5.9. Figures and Tables

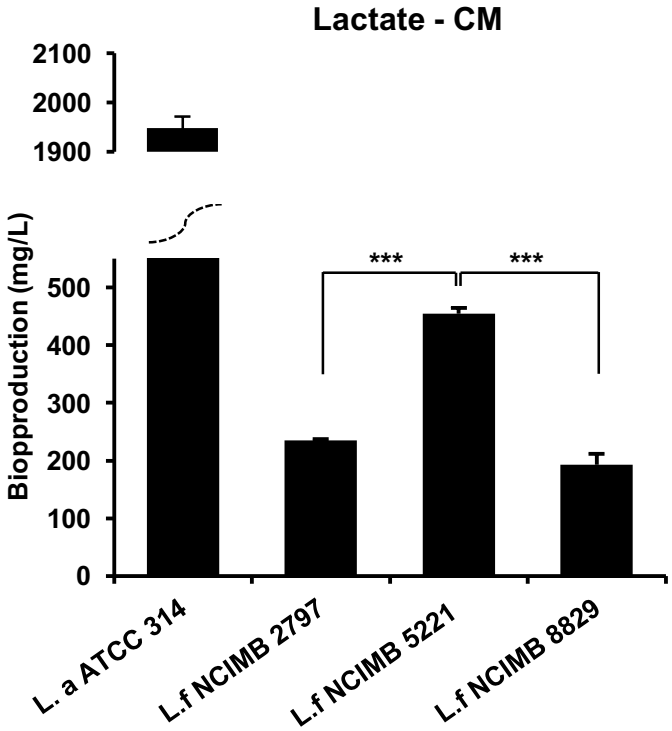


Figure 5.1: Determination control for of the ability of *L. fermentum* strains to produce lactic acid in conditioned cell culture medium (CM).

L. fermentum NCIMB -2797, -5221 and, -8829 were active enough to produce different concentrations of lactic acid when incubated in DMEM (2 h, 37°C, 5% CO2). *L. acidophilus* ATCC 314 is used as a control. Data are presented as mean ± SEM (n = 3), ***$p < 0.005$.

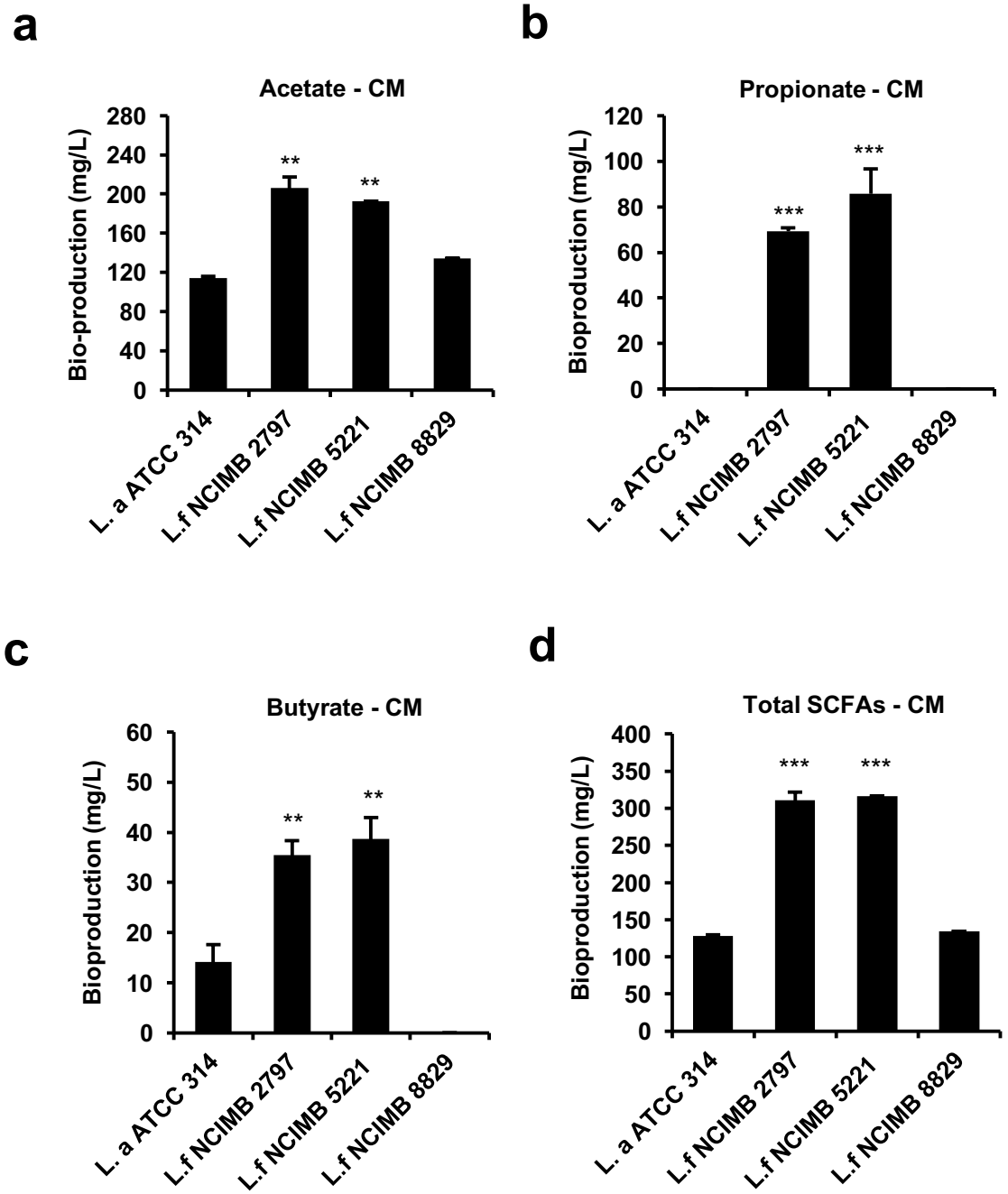
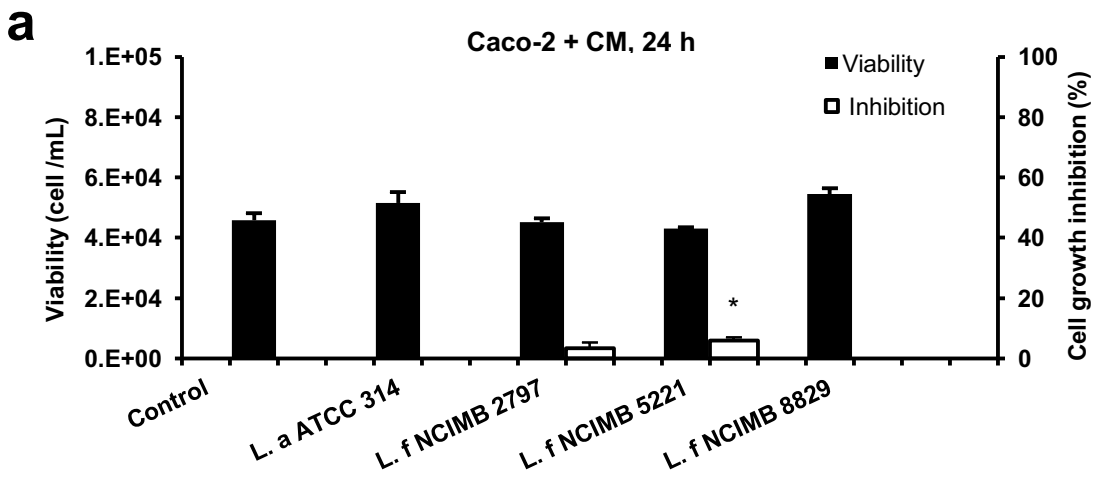
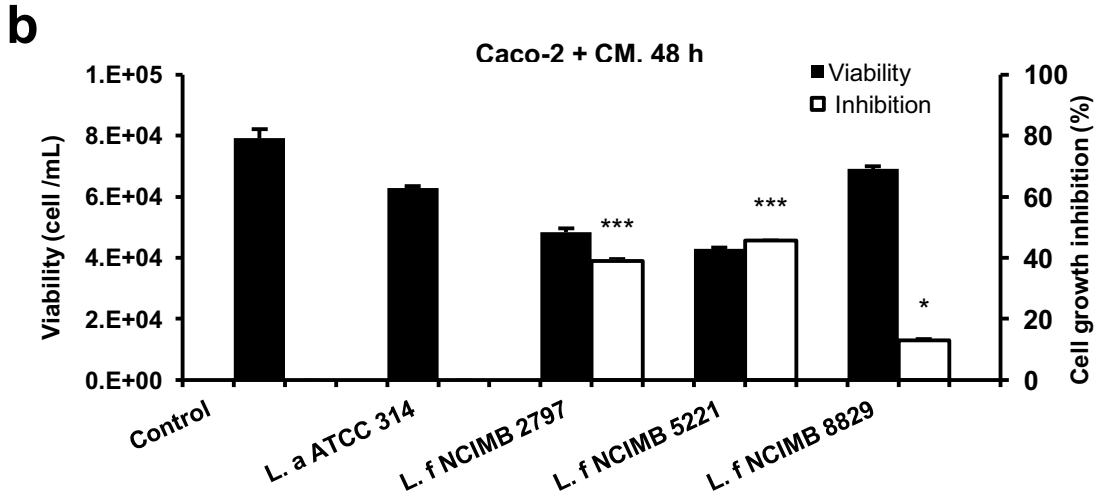


Figure 5.2: Analysis of the bio-production of SCFAs by *L. fermentum* strains in the conditioned cell culture medium (CM).

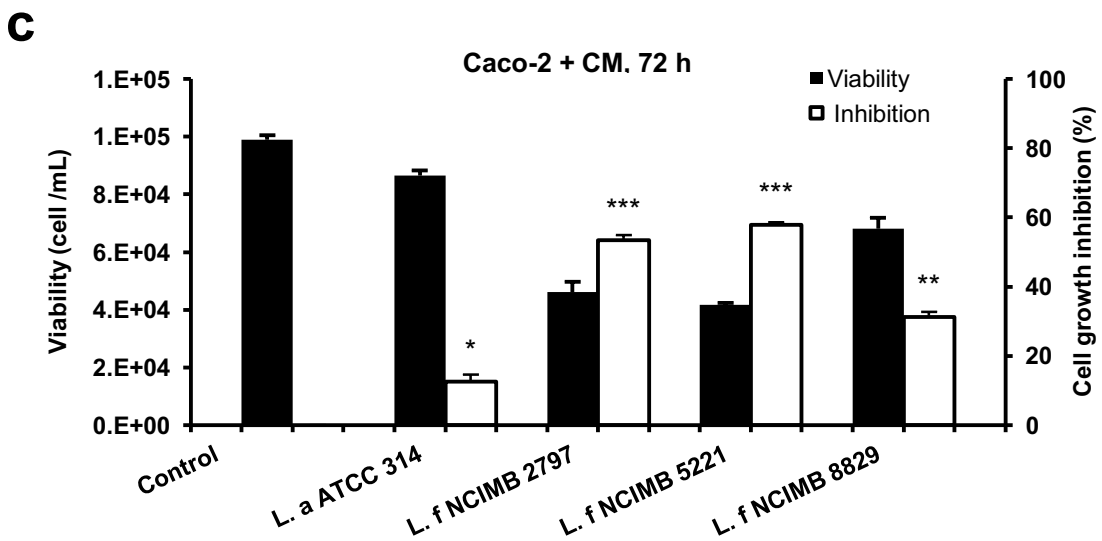
L. fermentum strains produced variable levels of SCFAs in a strain-dependent manner. The levels (**a**) acetic, (**b**) propionic, (**c**) butyric acids, and (**d**) total SCFAs, produced by *L. fermentum* NCIMB -2797, -5221, and -8829 were quantified in CM and compared with each other, while *L. acidophilus*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


a
Caco-2 + CM, 24 h
Viability
Inhibition
Viability (cell /mL)
Cell growth inhibition (%)
1.E+05
8.E+04
6.E+04
4.E+04
2.E+04
0.E+00
100
80
60
40
20
0
*
Control
L. a ATCC 314
L. f NCIMB 2797
L. f NCIMB 5221
L. f NCIMB 8829

389


b
Caco-2 + CM. 48 h
Viability
Inhibition
Viability (cell /mL)
Cell growth inhibition (%)
1.E+05
8.E+04
6.E+04
4.E+04
2.E+04
0.E+00
100
80
60
40
20
0
***
***
*
Control
L. a ATCC 314
L. f NCIMB 2797
L. f NCIMB 5221
L. f NCIMB 8829

390


c
Caco-2 + CM. 72 h
Viability
Inhibition
Viability (cell /mL)
Cell growth inhibition (%)
1.E+05
1.E+05
8.E+04
6.E+04
4.E+04
2.E+04
0.E+00
100
80
60
40
20
0
*
***
***
**
Control
L. a ATCC 314
L. f NCIMB 2797
L. f NCIMB 5221
L. f NCIMB 8829

391

Figure 5.3: Screening of *L. fermentum* strains for a proliferation inhibitory effect against CRC cells. To investigate the anti-proliferative effect of the different *L. fermentum* strains, the cell culture conditioned cell culture media (CM) of *L. fermentum* NCIMB -2797, -5221, and -8829 were incubated with Caco-2 cancer cells.

The viability and growth inhibition rate of Caco-2 cells for **(a)** 24 h, **(b)** 48 h, and **(c)** 72 h of incubation showed a time-dependent effect. *L. acidophilus* ATCC 314 is used as a positive control. $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$, compared with *L. acidophilus* ATCC 314. Data are presented as mean ± SEM ($n = 4$).

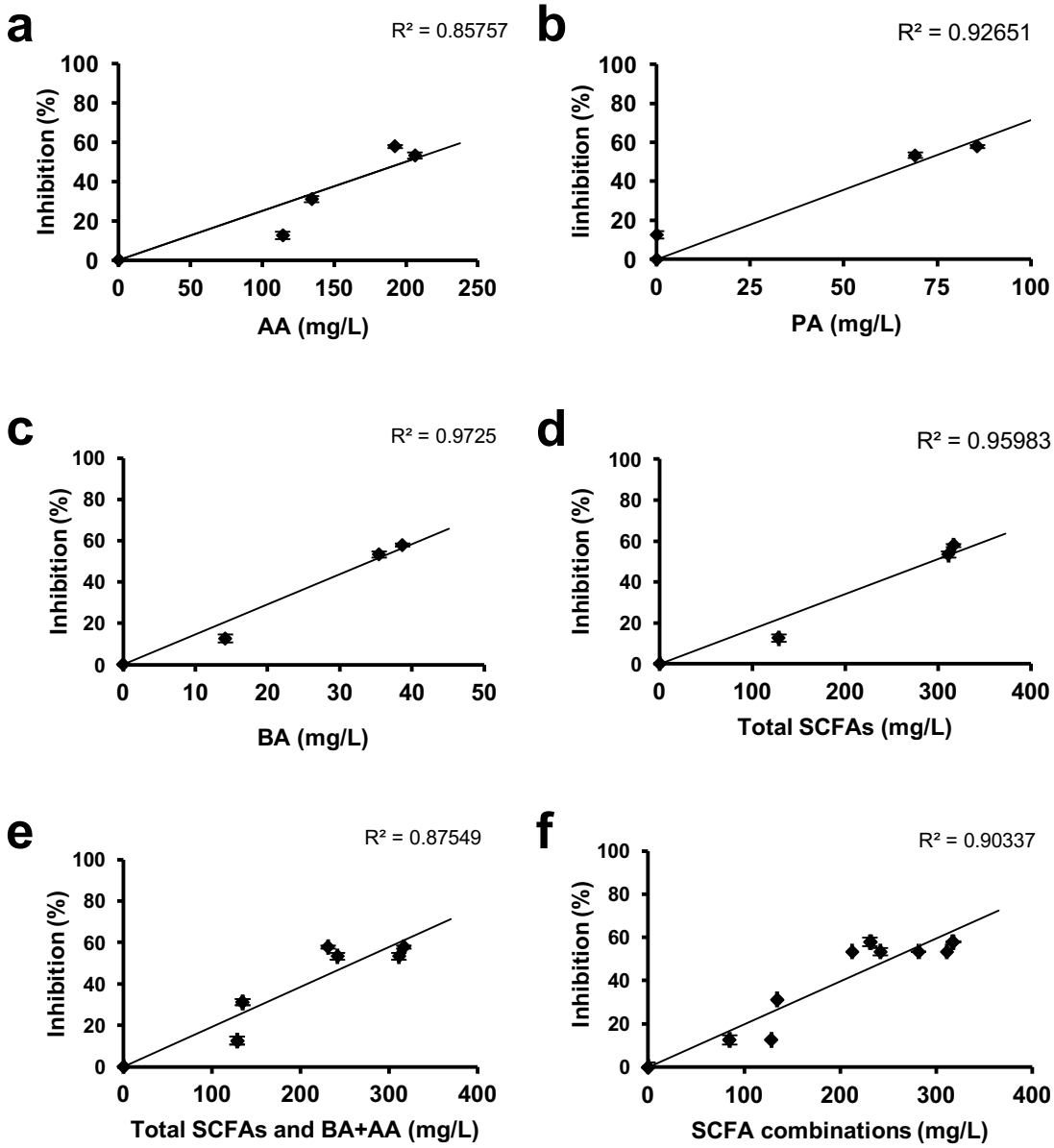


Figure 5.4: Investigation of the correlation between cell growth inhibition and the different concentrations of naturally produced SCFAs in probiotic CM.

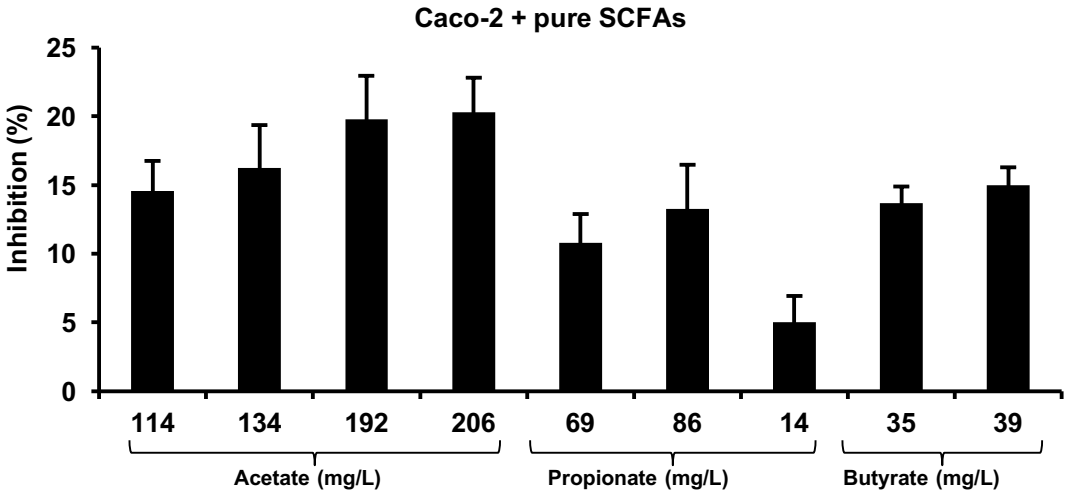
The dependent variables used are the values for: (**a**) acetate, (**b**) propionate, (**c**) butyrate, (**d**) total SCFAs, (**e**) total SCFAs and BA+AA, and (**f**) SCFA combinations: 7x BA and PA+[7xBA], Plots represent the data of cell growth inhibition at 72 h (presented in **Figure 5.2c**). The lines were obtained by linear regression analysis. LA: lactic acid; AA: acetic acid; PA: propionic acid.

Table 5.1: 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 SSFs containing lactic acid was prepared by the addition of the respective concentrations of lactic acid at the same concentrations produced by *L. fermentum*-CM. SSF-a: SCFA synthetic formulation corresponding to *L. acidophilus* ATCC 314; SSF-f7: SCFA synthetic formulation corresponding to SCFA concentrations produced by *L. fermentum* NCIMB 2797; SSF-f5: SCFA synthetic formulation corresponding to SCFAs concentrations produced by *L. fermentum* NCIMB 5221; and SSF-f8: SCFA synthetic formulation corresponding to SCFA concentrations produced by *L. fermentum* NCIMB 8829.

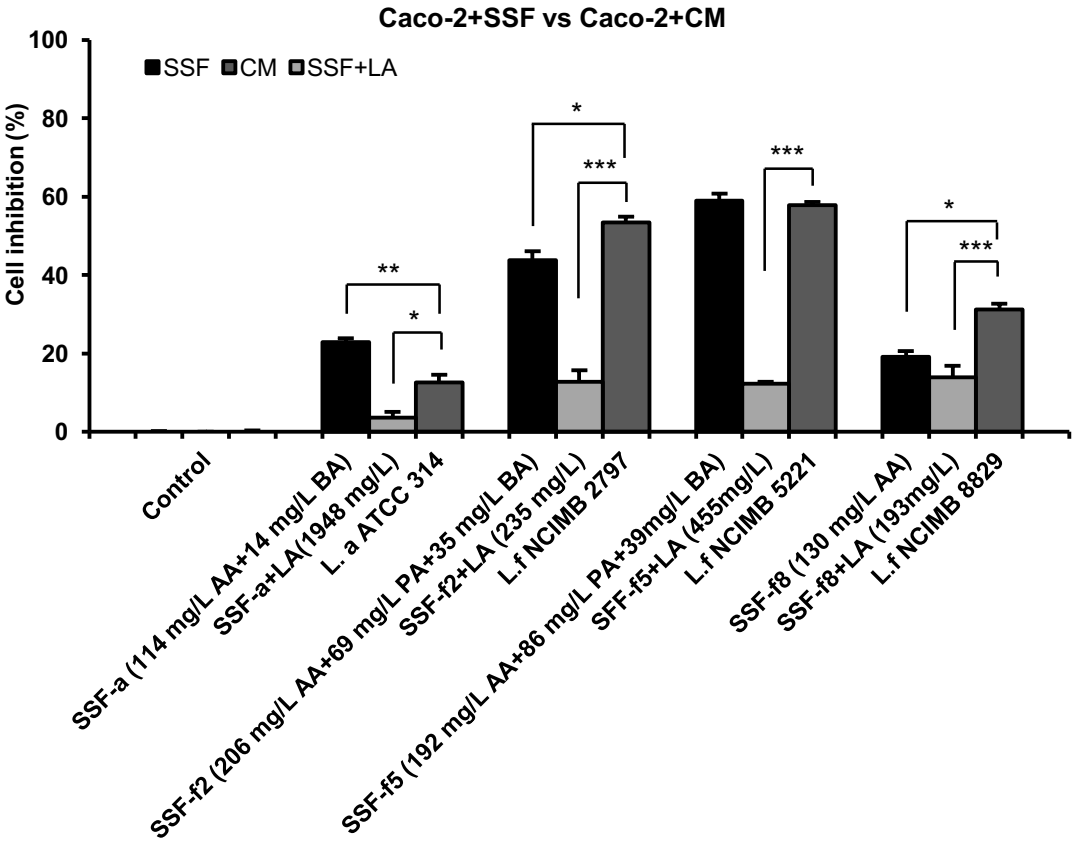
	SSF	Composition (mg/L)			SSF+LA	Composition (mg/L)			
		Acetate	Propionate	Butyrate		Lactate	Acetate	Propionate	Butyrate
L. a 314	SSF-a	114	0	14	SSF-a+LA	1948	114	0	14
L. f 2797	SSF-f2	206	69	35	SSF-f2+LA	235	206	69	35
L. f 5221	SSF-f5	192	86	39	SSF-f5+LA	455	192	86	39
L. r 8829	SSF-f8	130	0	0	SSF-f8+LA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

a


Caco-2 + pure SCFAs
Inhibition (%)
25
20
15
10
5
0
114
134
192
206
69
86
14
35
39
Acetate (mg/L)
Propionate (mg/L)
Butyrate (mg/L)

424

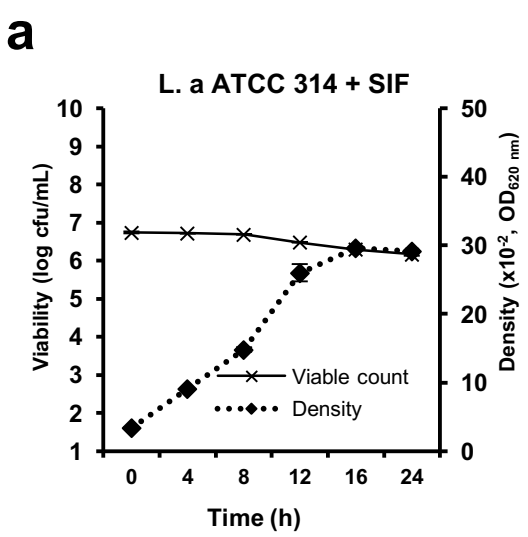
b

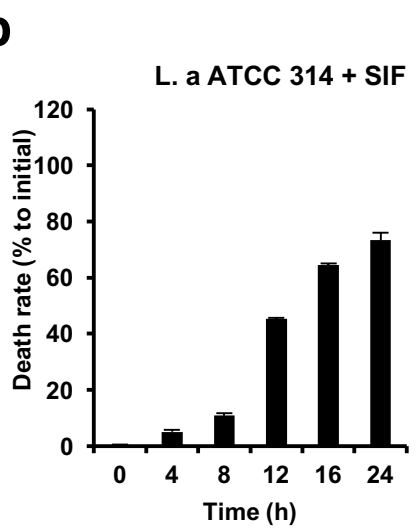

Caco-2+SSF vs Caco-2+CM
SSF
CM
SSF+LA
Cell inhibition (%)
100
80
60
40
20
0
**
*
*
***
***
*
***
Control
SSF-a (114 mg/L AA+14 mg/L BA)
SSF-a+LA(1948 mg/L)
L. a ATCC 314
SSF-f2 (206 mg/L AA+69 mg/L PA+35 mg/L BA)
SSF-f2+LA (235 mg/L)
L.f NCIMB 2797
SSF-f5 (192 mg/L AA+86 mg/L PA+39mg/L BA)
SFF-f5+LA (455mg/L)
L.f NCIMB 5221
SSF-f8 (130 mg/L AA)
SSF-f8+LA (193mg/L)
L.f NCIMB 8829

425

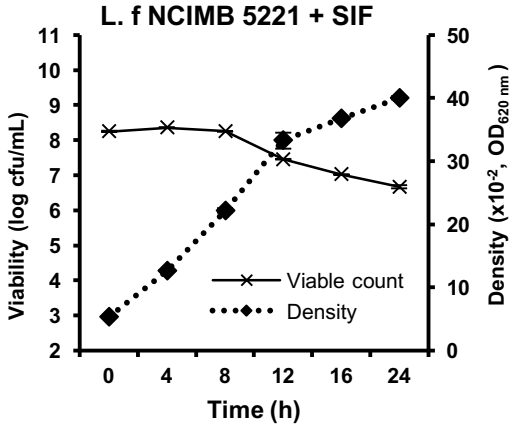
Figure 5.5: Investigation of the role and effectiveness of SCFAs produced by *L. fermentum* bacteria.

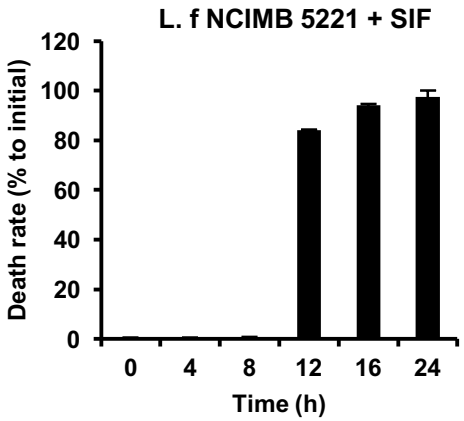
(**a**) The anti-proliferative effect of pure SCFAs at the same concentrations as what was produced by probiotic bacteria in *L. fermentum*-CM (as described in **Figure 5.4**). The inhibitory effect of SCFAs on Caco-2 cells (72 h) increased with higher doses. (**b**) Comparison of the anti-proliferative effect of SCFA synthetic formulations (SSFs) with the anti-proliferative effect of *L. fermentum*-CM. The SSFs are reconstituted mixtures of acetic, propionic, and butyric acids (**Table 5.1**) with or without lactic acid, at concentrations similar to the naturally produced ones by *L. fermentum* bacteria. These formulations, used to treat Caco-2 cells for 72 h, were compared with their corresponding *L. fermentum*-CM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Data are represented as mean ± SEM ($n = 5$).


a
L. a ATCC 314 + SIF
Viability (log cfu/mL)
Density (x10-2, OD620 nm)
Viable count
Density
Time (h)

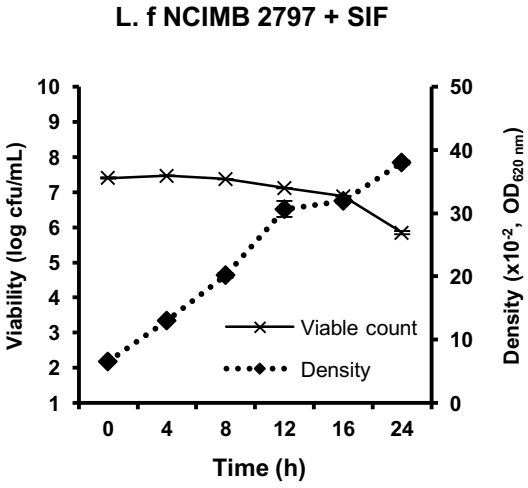

b
L. a ATCC 314 + SIF
Death rate (% to initial)
Time (h)

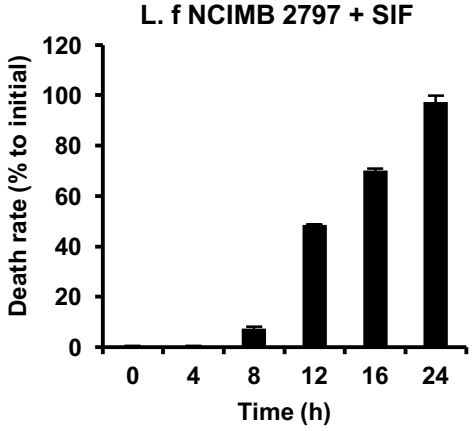
437


L. f NCIMB 5221 + SIF
Viability (log cfu/mL)
Density (x10-2, OD620 nm)
Viable count
Density
Time (h)


L. f NCIMB 5221 + SIF
Death rate (% to initial)
Time (h)

438

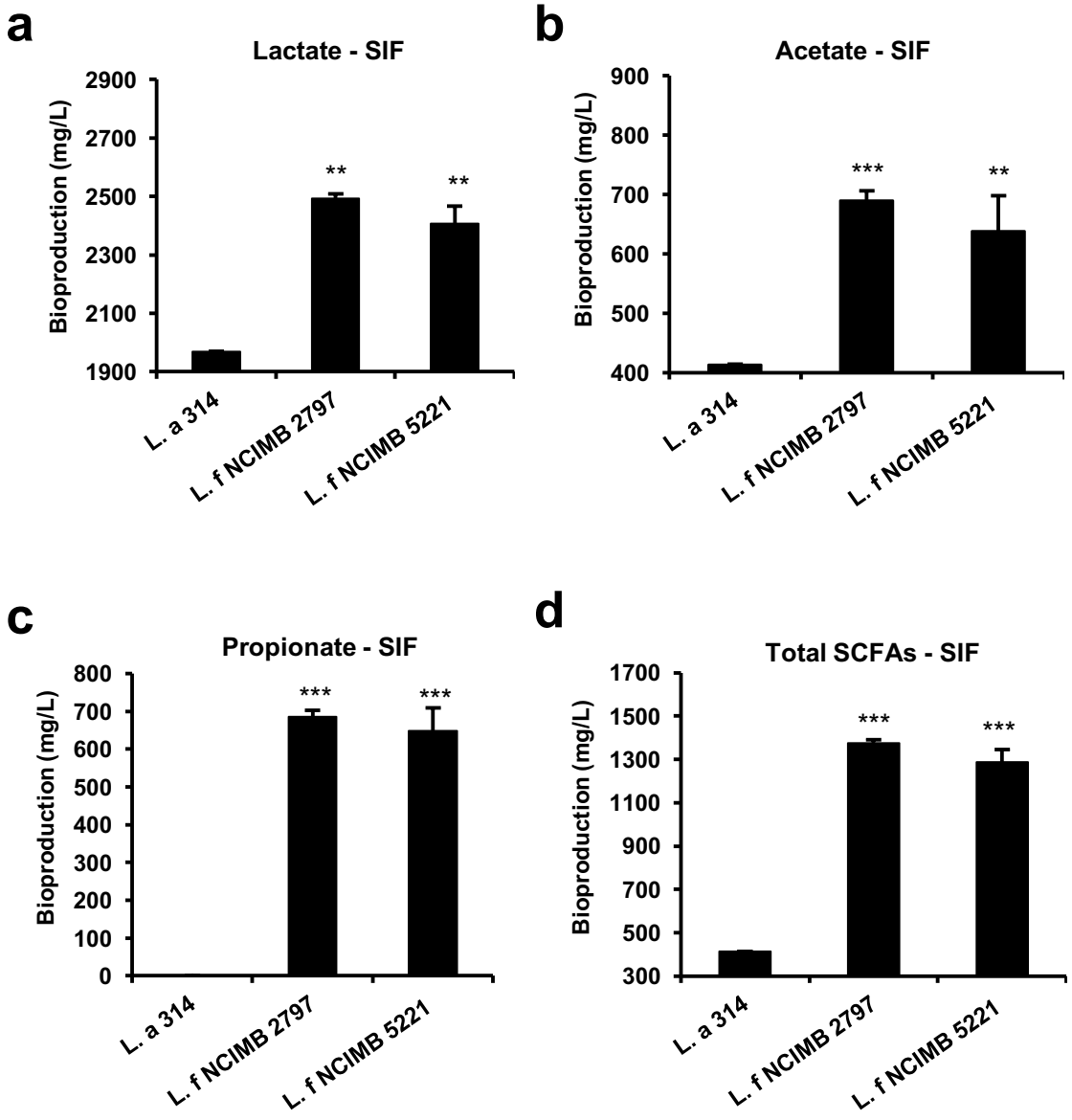

L. f NCIMB 2797 + SIF
Viability (log cfu/mL)
Density (x10-2, OD620 nm)
Viable count
Density
Time (h)


L. f NCIMB 2797 + SIF
Death rate (% to initial)
Time (h)

439

Figure 5.6: Characterization of *L. fermentum* bacterial cell resistance in a simulated intestinal fluid (SIF).

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


a
Lactate - SIF
Bioproduction (mg/L)
2900
2700
2500
2300
2100
1900
**
**
L. a 314
L. f NCIMB 2797
L. f NCIMB 5221
b
Acetate - SIF
Bioproduction (mg/L)
900
800
700
600
500
400
***
**
L. a 314
L. f NCIMB 2797
L. f NCIMB 5221
c
Propionate - SIF
Bioproduction (mg/L)
800
700
600
500
400
300
200
100
0
***
***
L. a 314
L. f NCIMB 2797
L. f NCIMB 5221
d
Total SCFAs - SIF
Bioproduction (mg/L)
1700
1500
1300
1100
900
700
500
300
***
***
L. a 314
L. f NCIMB 2797
L. f NCIMB 5221

450

451

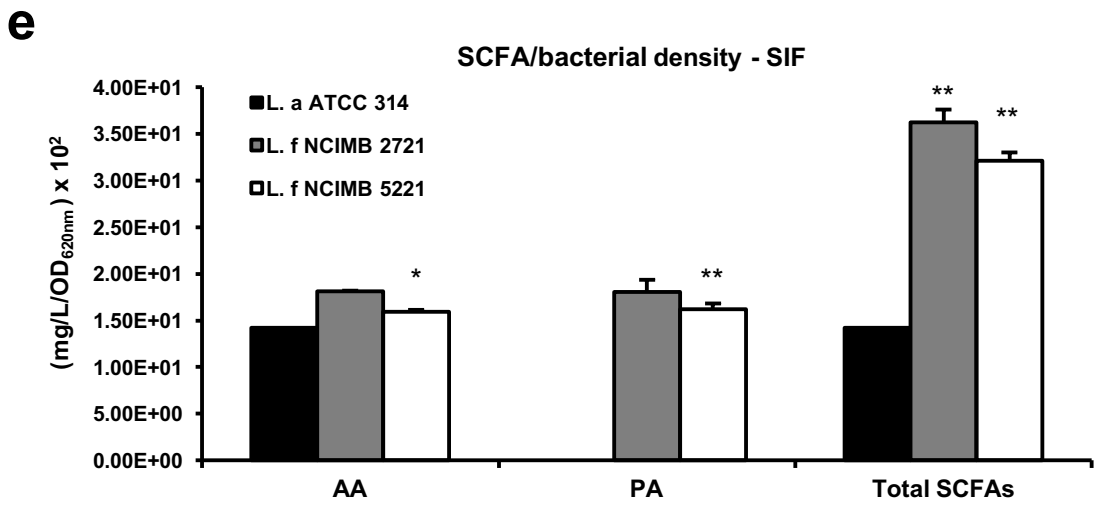


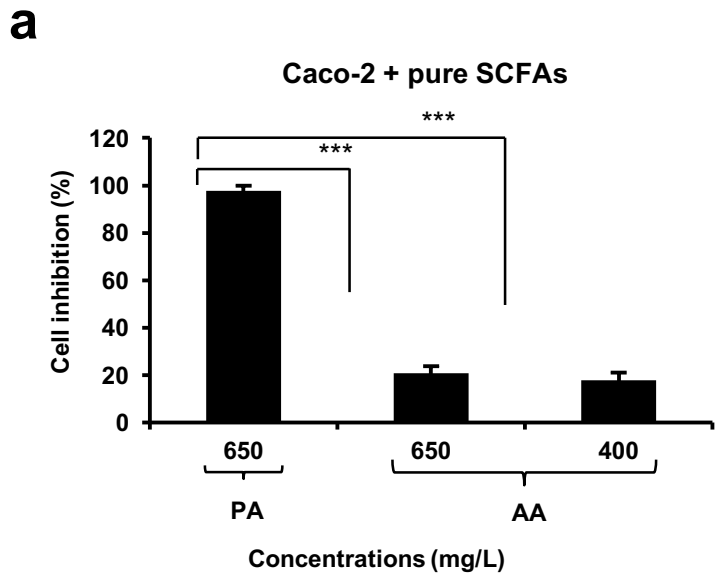
Figure 5.7: Quantification of the lactic acid/SCFAs produced by *L. fermentum* strains in SIF.

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

Table 5.2: Levels of lactic, acetic, propionic, and butyric acids produced by *L. acidophilus* ATCC 314, *L. fermentum* NCIMB -2797 and -5221 after 24 h incubation in SIF, with *L. acidophilus* ATCC 314 as a control.

The data is presented by the mean ± SEM (n = 3).

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



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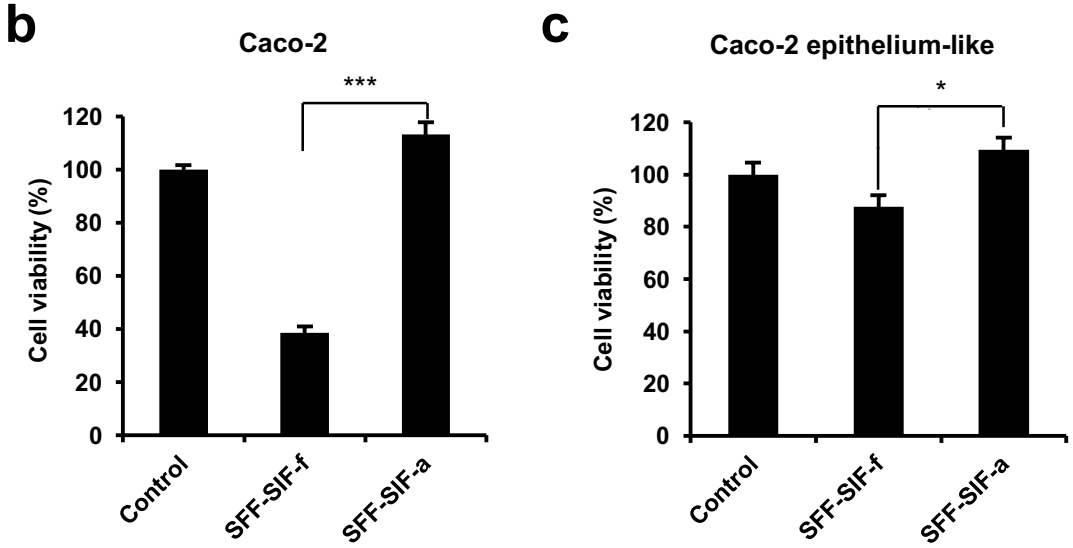

470

471 **Figure 5.8: Confirmation of the efficacy of SCFAs produced in SIF, by *L. fermentum*.**

472 (**a**) The inhibitory effect of propionic and acetic acids produced by *L. fermentum* in SIF was
473 described. The effect of the SCFA synthetic formulations (SSF-SIF-a and SFF-SIF-f) against CRC
474 cells (**b**) cell culture, and (**c**) epithelium-like cell culture. SSF-SIF-a and SFF-SIF-f represented
475 synthetic mixtures of SCFAs that have the same composition as the probiotic SCFAs naturally
476 produced in SIF by *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB -5221 and -2797,
477 respectively (**Table 5.2**). Data are presented as mean ± SEM (n = 5). *$p < 0.05$ and ***$p < 0.01$,
478 compared with control or *L. acidophilus* ATCC 314. SSF-SIF-f: formulation of SCFAs produced
479 in SIF corresponding to both *L. fermentum* bacteria (NCIMB -5221 and -2797); SSF-SIF-a: SCFA
480 formulation of SCFAs produced in SIF by *L. acidophilus* ATCC 314.

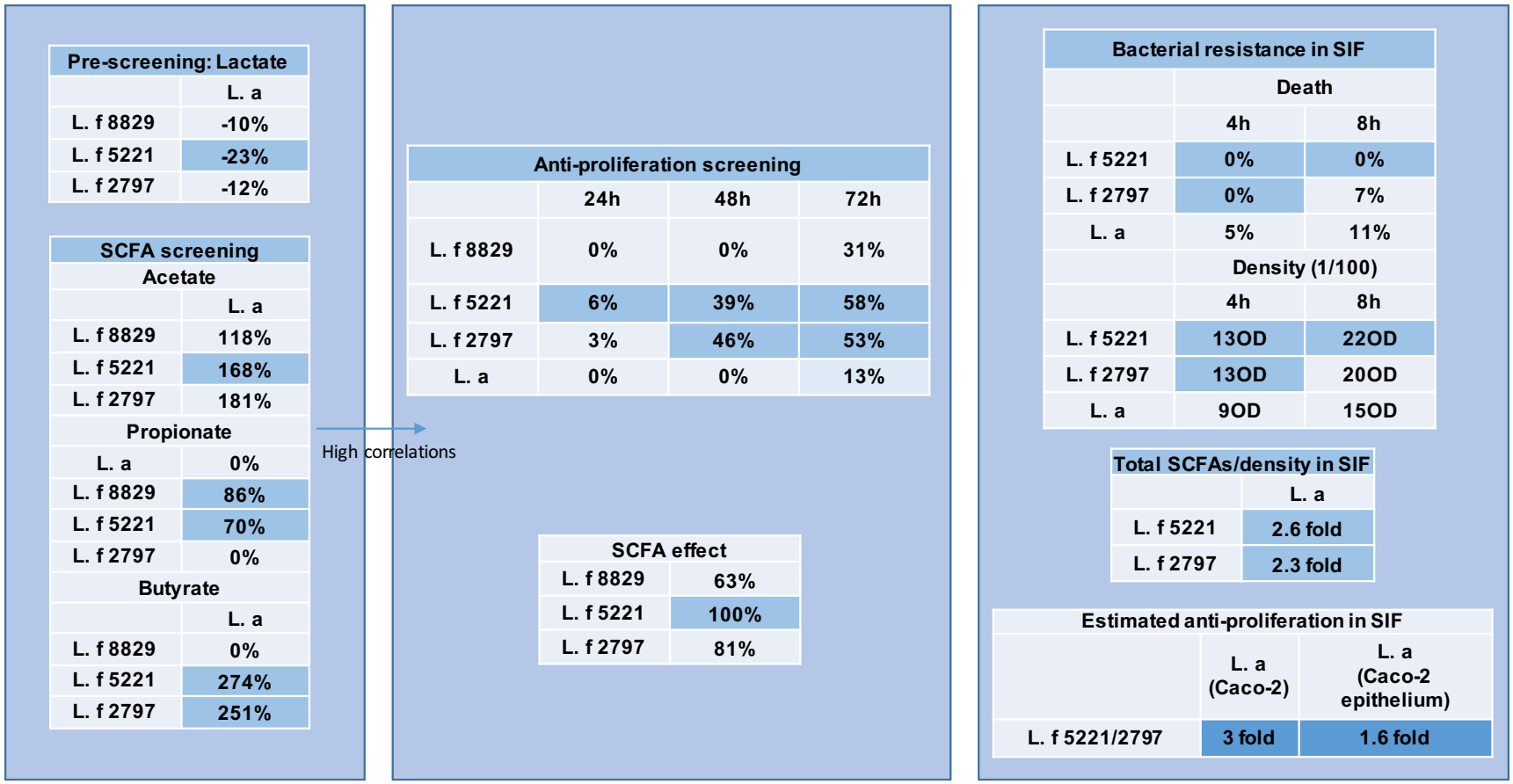

Figure 5.9. Overview of *L. fermentum* strain screening and relevance depending on growth, metabolic, and anti-CRC proliferative criteria.

Original Research Article 4

CHAPTER 6. *IN VITRO* CHARACERIZATION OF THE ANTI-CANCER ACTIVITY OF THE PROBIOTIC BACTERIUM *LACTOBACILLUS FERMENTUM* NCIMB 5221 AND POTENTIAL AGAINST COLORECTAL CANCER CELLS

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Preface: After the selection of a most relevant *L. fermentum* strain, further investigations were undertaken to characterize its FFA and SCFAs production, anti-proliferative effect through different extracts with different CRC cells, and in comparison with normal colon cells. Evaluating the metabolic and anti-cancer efficacy of this bacterium against CRC cells was evaluated based on two previously investigated *Lactobacillus* bacteria (*L. acidophilus* and *L. rhamnosus*). We demonstrate that SCFAs produced by *L. fermentum exeret a significant and selective antiproliferative and anti-survival activities* against CRC cells compared to non-transformed normal colon epithelial cells.

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6.1. Abstract

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

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

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

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

6.2. Introduction

Colorectal cancer (CRC) is a leading cause of mortality worldwide [234]. However, it is a type of cancer for which chemoprevention is considered a therapeutic and preventive strategy [236]. Probiotics have been used as biotherapeutics that reduce cancer recurrence and side effects in CRC patients [191, 238, 239]. When orally administered, probiotic along with intestinal microbial-produced metabolites (e.g. organic acids, peptides), that interact with cellular proliferation, differentiation, and intestinal inflammation, reduce the risk of CRC [304]. In the large intestine, short chain fatty acids (SCFAs) produced by bacterial fermentation have shown to exert anti-inflammatory [257] and anti-carcinogenic actions. Studies have shown that fatty acids can mutually interact and protect against CRC. Nevertheless, the incorporation of fatty acids into CRC chemotherapies is still premature. The oral administration of specified probiotic bacteria remains the dominant method to increase the bio-production of these anti-tumorigenic compounds in the colon [305, 306].

While the selection criteria of probiotic bacteria originating from the gut or from traditionally fermented products are fairly empirical, an emphasis on the importance of well-established *in vitro* and *in-vivo* studies to select good candidates exists. Few studies have been found to establish a rigorous selection for new probiotic strains. Anti-cancer attributes, proper controls, and extensive evaluation of their anti-proliferative effect against cancer cells should be analyzed and compared with other established probiotic products. While studies have shown the ability of certain probiotics to affect SCFA levels, more evidence that the probiotic anti-cancer effect is solely due to the direct production of SCFAs [283, 307, 308] is needed. In this study the profile of *L. fermentum* NCIMB 5221, identified as a producer of ferulic acid (FA), was characterized. The latter is an anti-oxidant and anti-tumorigenic compound [21], with the ability to generate a stronger free fatty acid (FFA). This ability was compared with that of *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 51303, both characterized in previous studies for their activity against tumor growth [14-17]. Notably, the total proliferative effect of this bacterial strain was investigated based on the effects of two types of probiotic cell-free extracts on the growth and apoptosis of CRC cells. A prior preliminary comparative study found that this particular strain exhibited more potent attributes associated with anti-cancer effects and survival when screened with other *L. fermentum* strains. To verify the non-cytotoxic effect of the probiotic extracts, the same assay was performed

with non-cancerous colon cells. Validation of a correlation between the levels of SCFAs produced and the anti-cancer potency of the bacterium was performed. Quantification of *L. fermentum* NCIMB 5221 SCFAs was used to identify the extent to which SCFAs (acetic, propionic, and butyric acids) are responsible for the potential anti-cancer effect against CRC cells. *in vitro*. To confirm the level of efficacy of naturally produced SCFAs, the assay was performed using only a pure mixture of synthetic SCFAs with similar composition to the probiotic SCFAs.

6.3. Material and Methods

6.3.1. Materials

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

6.3.2. Bacterial cultures

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

6.3.3. Mammalian cultures

SW-480 colorectal cancer and Caco-2 epithelial colorectal cancer adenocarcinoma cells, as well as CRL-1831 normal epithelial colon cell line, were purchased from ATCC American Type Cell Collection (ATCC, Manassas, VA). Caco-2 cells were maintained in (EMEM) supplemented with 20% FBS. SW-480 cells were maintained in RPMI-1640 supplemented with 10% FBS, and CRL-1831 was maintained in complete DMEM (10% FBS, 37°C, 5% CO_2). Caco-2 cells were incubated in a CO_2 incubator at 37°C in air supplemented with 5% CO_2 for a maximum of two weeks for complete differentiation. For proliferation/viability assays, all cells were left to attach in 96-well

plates until 50 - 60% confluence (24 - 48 h) before experimentation. The cell medium was then replaced by probiotic cell-free extracts mixed with serum/antibiotic-free DMEM.

6.3.4. Free fatty acid (FFA) analysis

In this analysis, the free fatty acids (FFAs) in the bacterial supernatant were converted to their CoA derivatives and then oxidized. This resulted in the formation of a color measured at 570 nm (**Figure 6.1**). The assay was performed based on the manufacturer's instructions (Cell Biolabs Inc., CA, USA). For the induction of Acyl-CoA synthesis, a reaction mix was prepared. A 2 µl of ACS Reagent was added to all the standards (palmetic acid. sample wells were mixed and left to incubate (37°C, 30 min). The reaction mix (50 µl), f atty acid probe (2 µl), enzyme mix (2 µl), assay buffer (Igepal, 44 µl), and enhancer (N-ethylmaleimide, 2 µl) were mixed and vortexed briefly. Then, 50 µl of this reaction mixture was added to each well (standard or sample) and incubated (30 min, 37°C, away from light). Absorbance was measured at 570 nm for colorimetric assay in a microplate reader (Perkin Elmer, Victor 3, multi-label micro-plate reader, MA, USA).

6.3.5. Preparation of Probiotic cell-free supernatants

Each probiotic bacterial colony was grown anaerobically in MRS broth for 12 - 16 h. The conditioned medium (CM) and probiotic supernatant (PS) were prepared with slight modifications of protocols adapted from Grabig et al. [212] and Kim et al [213]. For the preparation of the CM, bacterial cultures (16 h, 37 °C, 5% CO_2) were used to collect bacterial pellets, by centrifugation (4000 rpm, 15 - 20 min, 4°C), to be washed (PBS) and resuspended in DMEM. The bacterial pellets in DMEM were maintained and incubated in a shaker incubator for two hours (37°C, 5% CO_2, 100 rpm). After incubation, the culture medium was centrifuged (1000 x g, 15 min) and sterile-filtered (filter pore size 0.2 µM-pore-size filter). Prior to treating the cells, the CM of each *Lactobacillus* bacteria was combined with DMEM at a ratio of 1:2. For the preparation of the PS, the bacterial pellet was removed by centrifugation (4000 rpm, 15 min, 4°C), then the recovered supernatant was sterile-filtered (0.22 µm) and stored at −80 °C, until use.

6.3.6. Cell viability assay

Cell viability was determined using ATP bioluminescence assay (CellTiter-Glo Luminescent Cell Viability Assay, Promega), following the manufacturer's protocol [216]. Colon cells (normal and cancer) were seeded onto 96-well culture plates (5 - 6×10^3 cells/well, 100 µL/well) and stabilized for 24 - 48 h (37°C, 5% CO_2) for cell attachment. After incubating the cells with the probiotic

631 treatments (24, 48, and 72 h), the 96-well plate was left at room temperature (RT, 30 min). A 100
632 µL of luminescent reagent was added to each well, followed by shaking (2 min, 200 rpm) and
633 incubation at RT (10 min) to stabilize the luminescent signal. The signal was recorded using a
634 spectrophotometer (Perkin Elmer, Victor 3, multi-label microplate reader, MA, USA).

635 **6.3.7. Apoptosis assay**

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

647 **6.3.8. Probiotic effect on CRC cells vs. non-neoplastic colon cells**

648 This assay was performed to demonstrate an anti-CRC effect of *L. fermentum* NCIMB 5221 by
649 inhibiting CRC cell proliferation without affecting non-neoplastic colon cells. Caco-2 and CRL-
650 1831 (4 - 5×10^3 cells/ well) were seeded into 96-well culture plates (37°C, 5% CO_2) for 1, 2, 3,
651 and 7 days. The cells in both populations were treated with probiotic CM. At each time point of
652 incubation with probiotic treatments, cell proliferation was determined using an ATP
653 bioluminescence assay.

654 **6.3.9. Quantification of lactic acid and SCFAs**

655 SCFAs produced by *L. fermentum* strains were measured during the growth of bacteria in SIF and
656 after the preparation of corresponding CM. SCFAs were separated using a slightly modified HPLC
657 method [214, 215]. The Model 1050 of UV HPLC system (Hewlett-Packard HP1050 series,
658 Agilent Technologies, USA), equipped with a UV-vis detector and diode array detector (DAD) set
659 at 210 ± 5 nm was used. A 100 µl of sample was injected through an autosampler. A prepacked
660 Rezex ROA-organic acid H+ (8%) (150mm × 7.80 mm, Phenomenex, Torrance, CA, USA) fitted

with an ion-exclusion microguard refill cartridge was used. Data was acquired using ChemStation supported with LC3D software Rev A.03.02 (Agilent Technologies, CO, USA). The mobile phase (A) of H_2SO_4 (0.05 M) and the mobile phase (B) of acetonitrile (2%) pumped isocratically at a flow rate of 0.8 - 0.7 mL/min, through a column heated to 35°C. Lactic, acetic, propionic, and butyric acids were used to prepare a standard solution at concentrations of 1, 10, 100, 500, and 1000 ppm (in triplicate). The concentrations of SCFAs were estimated using the linear regression equations ($R^2 \geq 0.99$) generated from respective standard curves.

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

This test intends to demonstrate the role and the relevance of naturally produced SCFAs by probiotic cells in their cell-free extracts (CM). SCFA synthetic formulations (SSF) with same composition of the naturally produced SCFAs in the probiotic CM were prepared (**Table 6.1**). Caco-2 cells (4 - 5 × 10^3 cells/well), seeded onto 96-well plates (37°C, 5% CO_2, 72 h) were used to determine the inhibitory actions of these compounds and compare it to the probiotic CM. SCFA synthetic mixtures with the same effect as the bacterial extract would suggest that the inhibitory effect against CRC cell is due to the concentration of SCFAs produced by *L. fermentum.* Another set of mixtures (SSF+LA) was used after the addition of lactic acid (similar to that produced by the bacteria) to investigate the effect of another bacterial component on the action of SCFAs.

6.3.11. Effect of SCFAs on CRC cell compared with normal cells

The objective of this step was to investigate the dose-dependent effect of SCFAs, pure or in a mixture, and the nature of their potential synergistic effect on both normal and cancerous colon cells. Different concentrations of lactate, acetate, propionate and butyrate were prepared (**Table 6.2**) and tested on Caco-2 and normal non-neoplastic colon cells CRL-1831 cells. Cells were seeded onto 96-well culture plates (4 - 5 × 10^3 cells/ well, 37°C, 5% CO_2), left to stabilize and attach (24 - 48 h), followed by incubation with treatment samples (37°C, 5% CO_2, 72 h). SCFA treatments included increasing concentrations of lactate (0, 325, 650, and 1300 mg/L), acetate (0, 325, 650 and 1300 mg/L), propionate (0, 100, 200, and 400 mg/L) and butyrate (0, 75, 150, and 300 mg/L). The concentration of each SCFA was mixed to prepare the following four compositions; 1) SC4 (325 mg/L of lactate, 325 mg/L of acetate, 100 mg/L of propionate and 75 mg/L of butyrate), 2) SC3 (325 mg/L of lactate, 650 mg/L of acetate, 200 mg/L of propionate, 150 mg/L of butyrate), 3) SC2 (650 mg/L of lactate, 1300 mg/L of acetate, 400 mg/L of propionate

acid and 300 mg/L of butyrate acid), and 4) SC1 (1300 mg/L of lactate, 1300 mg/L of acetate, 400 mg/L of propionate and 300 mg/L of butyrate).

6.3.12. Statistical analysis

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

6.4. Results

6.4.1. *L. fermentum* NCIMB 5221 a higher producer of FFAs

L. fermentum NCIMB 5221 was characterized for its growth and production of FFAs in bacterial cultures. Data sets describing the total FFA concentration (μM PAE) in the bacterial supernatant (**Figure 6.2a, b, and c**) included FFA concentration per viable bacterial cell (**Figure 6.2d**) and FFA concentration per gram of bacterial pellet. Results showed that *L. fermentum* NCIMB 5221 growth significantly increased FFA concentrations (367.8 ± 10.5 μM PAE) compared with *L. acidophilus* ATCC 314 (117.1 ± 3 μM PAE, $p < 0.001$) and *L. rhamnosus* ATCC 53103 (87.4 ± 0.1 μM PAE, $p < 0.001$, **Figure 6.2b**). The high FFA concentrations were maintained at 367.8 ± 10.5 μM PAE and 366.7 ± 6.6 μM PAE between 12 and 16 h of growth and then started dropping at the beginning of the stationary phase. Even at the end of the death phase (**Figure 6.2c**), *L. fermentum* NCIMB 5221 induced a significantly higher level of FFAs (320.8 ± 12.6 μM PAE) compared with *L. acidophilus* ATCC 314 (188.2 ± 6.9 μM PAE, $p = 0.0161$) and *L. rhamnosus* ATCC 53103 (281.32 ± 1.77 μM PAE, $p = 0.0487$). Values of FFAs per viable bacterial cell measured during both log phase and stationary phase (**Figure 6.2d**), remained significantly higher for *L. fermentum* NCIMB 5221 ($6.7 \pm 0.2\times10^{-7}$ - $13.6 \pm 0.2\times10^{-7}$ μM PAE/cell, $p < 0.05$, **Figure 6.2**) compared with *L. acidophilus* ATCC 314 ($3.3 \pm 0.1\times10^{-7}$ – $4{,}4 \pm 0.2\times10^{-7}$ μM PAE/cell) and *L. rhamnosus* ATCC 53103 ($3.5 \pm 0.1\times10^{-7}$ – $5.8 \pm 0.1 \times10^{-7}$ μM PAE/cell). Regarding the bacterial mass, FFA generated per gram of the bacterial mass was the highest for *L. fermentum* NCIMB 5221 at 12 h and 24 h ($p < 0.001$, **Figure 6.2e**).

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

The anti-proliferative and apoptotic effect of *L. fermentum* NCIMB 5221 against SW-480 CRC cells was compared with controls: *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103, using two types of probiotic cell -free extracts, PS and CM. At 12 h, SW-480 cells treated with *Lactobacilli* PS showed no difference between groups in terms of proliferation and apoptosis (**Figure 6.3a**), whereas for *Lactobacilli* CM, *L. fermentum* NCIMB 5221 showed significant inhibition of CRC cell proliferation (12.9 ± 1.8%) compared with controls (p = 0.034, **Figure 6.3d**). At 24 h, *Lactobacilli* SP inhibited cancer cell proliferation with no significant difference between treatments (p = 0.0754, **Figure 6.3b**). Whereas for *Lactobacilli* CM, *L. fermentum* NCIMB 5221 significantly killed CRC cells with 38.1 ± 1.9% of inhibition (**Figure 6.3e**), and 29.8 ± 10.4% of apoptosis (**Figure 6.3e**) compared with other treatments (p = 0.0004, p = 0.0471, respectively). Interestingly, at 7 days, the SP of *L. fermentum* NCIMB 5221 significantly reduced cell growth by 42.6 ± 5.1% ($p < 0.05$), compared with control and *L. acidophilus* ATCC 314 (**Figure 6.3c**). The CM of *L. fermentum* NCIMB 5221 significantly suppressed CRC cell growth by 67.7 ± 2% compared with untreated cells ($p < 0.001$, **Figure 6.3f**). For the induction of cell death in SW-480 cancer cells by SP treatments, there was no significant difference between the treated groups (**Figures 6.4a and 6.4b**). However, for *L. fermentum* NCIMB 5221, the effect of CM in inducing apoptosis in CRC cells at 12 h (23.6 ± 7.5%, $p < 0.05$, **Figure 6.4c**) and 24 h (29.9 ± 10.4%, $p < 0.05$, **Figure 6.4d**) was shown to be significantly higher than controls. This result suggests that cancer cells might not be affected by bacteria-cell contact but by soluble bacterial factors or other microbial associated molecular patterns (MAMPs)

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

It was necessary to elucidate and determine the mechanism by which bacterial symbionts affect cell growth in the epithelium in a tumor environment. To do so, CM prepared from probiotic cells of *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 and *L. rhamnosus* ATCC 53103, was evaluated on the growth of both cancer (Caco-2) and non-cancerous (CRL-1831) colon cells (**Figure 6.5**). Results show that at 24 h of incubation with CM of *L. fermentum* NCIMB 5221 and *L. rhamnosus* ATCC 53103, cancer cell growth was inhibited by 28.6 ± 3.7% and 6.3 ± 1% ($p <$ 0.01, **Figure 6.5b and 6.5c**), respectively, compared with untreated cells. At 48 h of incubation,

cancer cell viability was reduced by 42.2 ± 2.2% and 11.4 ± 1.7% ($p < 0.01$), respectively, compared with untreated cells. At 72 h of incubation, *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 and *L. rhamnosus* ATCC 53103, inhibited cancer cell proliferation by 12.6 ± 1.9%, 59.4 ± 4.2% and 23.9 ± 2.5%, respectively, compared with untreated cells. Moreover, after 7 days, Caco-2 cell growth was reduced by *L. fermentum* NCIMB 5221 to 99.5 ± 0.1% ($p < 0.05$) compared with the control treatments (Figures 5a and 5b). Interestingly, the data indicates that *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 and *L. rhamnosus* ATCC 53103 promoted the growth of CRL-1831 epithelial normal colon cells by 12.5 ± 5.3%, 11.9 ± 1% 32 ± 3.4%, respectively, compared with untreated cells. After 48 h of treatment by CM of *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 increased CRL-1831 growth by 13 ± 8.4%, and 43.2 ± 3% ($p < 0.05$), respectively. At 72 h, *L. acidophilus* ATCC 314 (**Figure 6.5d**) showed no significant anti-proliferative effect, whereas *L. fermentum* NCIMB 5221 reduced cell growth by 59.4 ± 9.8.2% ($p < 0.05$), compared with untreated cells (**Figure 6.5f**).

6.4.4. *L. fermentum* NCIMB 5221 produced higher levels of SCFAs

The effect of anti-proliferative activity induced by the cells incubated in CM used to treat both cancer and normal cell lines, was characterized by SCFA composition, especially lactic, acetic, propionic, and butyric acids. The results as displayed in **Figure 6.5** show levels of SCFAs produced by different strains of *Lactobacillus* bacteria in the media. *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 did neither inhibit CRC growth nor produce detectable amounts of propionate in the media but had higher amounts of lactate, i.e. 1970.6 ± 9.6 and 3239.8 ± 9.9 mg/l, respectively, compared with *L. fermentum* NCIMB 5221 (480.6 ± 13.3 mg/l, **Figure 6.6a**). *L. fermentum* NCIMB 5221 produced the highest amount of acetate and butyrate, i.e. 224.2 ± 8.8 and 81.17 ± mg/L, respectively, (**Figure 6.6d**) compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 ($p < 0.05$, **Figure 6.6b, 6.6d**). *L. fermentum* NCIMB 5221 was the only probiotic bacterium to produce propionate (76.7 ± 7.9 mg/L, **Figure 6.6c**) compared with controls.

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

To determine if the anti-proliferative effect of *L. fermentum* NCIMB 5221 is the result of a specific SCFA, separate concentrations of SCFAs produced by the bacteria were tested. This revealed that acetic, propionic, and butyric acid concentration, quantified in **Figure 6.6** have significantly less

effect than the bacterial extract CM (**Figure 6.7a**). Only *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC314, not *L. rhamnosus* ATCC 53103, produced SCFAs (**Figure 6.6**). Thus, only their corresponding synthetic SCFA formulations were used for this experiment to verify the role of bacterial SCFAs. The results demonstrated that the synthetic SCFA formulation, corresponding to *L. fermentum* NCIMB 5221, significantly decreased Caco-2 viability by 67.8 ± 7.2% compared with synthetic SCFA formulation corresponding to *L. acidophilus* ATCC 314 (22.6 ± 2.6%, p = 0.018). Thus, for *L. fermentum* NCIMB 5221, the synthetic SCFA formulation showed no significant difference with the probiotic CM (**Figure 6.7b**), whereas after addition of lactic acid to the synthetic mixture, the SSF + LA corresponding to *L. fermentum* NCIMB 5221 decreased Caco-2 viability only by 21.1 ± 2.9%.

6.4.6. Doses of SCFAs have differential effects on normal and cancer cells

To investigate and differentiate the effects of pure SCFAs and their mixtures, with or without lactic acid, on colon normal and cancer cells, increasing doses (**Table 6.1**) of acetic, propionic, and butyric acids were tested on Caco-2 and CRL-1831 cells (**Figure 6.8**). Increasing concentrations of acetic acid to 1300 mg/L did not exceed more than 26% inhibition of cancer cells (**Figure 6.8g**), with no significant effect on normal cells (**Figure 6.8b**). For propionic acid, the inhibition was dose dependent, and 400 mg/L of propionate (**Figure 6.8h**) showed 43% inhibition with no inhibition on normal cells (**Figure 6.8c**). In the case of butyric acid, the inhibitory effect on CRC cells was dose-dependent and 300 mg/L of butyrate inhibited cell proliferation with a maximum inhibition of 93% (**Figure 6.8i**) with no significant effect on CRL 1831 normal colon cells (**Figure 6.8d**). Later, increasing doses of SCFAs were mixed to formulate synthetic SCFA mixtures: SSM1, SSM2, SSM3, and SSM4 (**Table 6.1**). The effect of each SCFA mixture was significantly higher (**Figure 6.8j**, $p < 0.05$) than the total effect of separate doses of SCFAs, with no significant effect observed on CRL-1831 (**Figure 6.8e**). However, when the different concentrations of lactic acid were added to each mixture (+LA), the anti-proliferative effect was significantly reduced (**Figure 6.8j**, p <0.001). When the doses of lactic acid were tested, they had no significant effect on the proliferation of both normal and cancer cells (**Figure 5.8a and 6.8f**).

6.5. Discussion

This study demonstrated, for the first time, that *L. fermentum* NCIMB 5221 has a higher anti-proliferative effect against CRC cells related to a higher metabolic activity, than to other LAB

bacteria (*L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103) [155, 309-311]. As a general characterization of this strain, *L. fermentum* NCIMB 5221 significantly ($p < 0.01$) affected the level of FFAs during most of the growth phases and surpassed both controls *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 for the following parameters: concentration of FFA in the bacterial supernatant (**Figures 6.2a, 6.2b, and 6.2c**), FFA/viable bacterial cell (**Figure 6.2d**) and FFA/g of bacterial pellet (**Figure 6.2e**). This reflected a significantly higher metabolic activity of this bacterium, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103, additionally to its ability to produce more fatty acids, a feature that relates to the production of anti-cancer fatty acid compounds. For instance, SCFAs, linolenic acid [312] or conjugated linoleic acid (CLA) [313] are considered to be locally produced in the colon to target immune cell function and suppress the disease/inflammation [314, 315]. Furthermore, fatty acids, classified as short-chain (SCFA), medium-chain (MCFA) or long-chain (LCFA) fatty acids, demonstrated potential as chemotherapeutic agents for the treatment of colorectal cancer. For instance, lauric acid holds promise for preferential antineoplastic properties by higher induction of apoptosis in cancer cells [316].

Investigating the anti-cancer effect of *L. fermentum* NCIMB 5221 was performed with a verification of the probable effect of its bacterial cell-free extract on colon cancer cell proliferation (**Figure 6.3**) and cell death (**Figure 6.4**). The suppression of cancer cell growth and induction of apoptosis reflect a significant effect of those extracts against colon cancer cells. Each extract seemed to contain bacterial compounds with anti-proliferative effect expressed in different time points. Compared with SP, the CM bacterial extract was most effective in inhibiting cancer cell proliferation after 24 h and 7 days of treatment ($p < 0.05$, **Figure 6.3e and 3f**) and in inducing apoptosis at 24 h ($p < 0.05$, **Figure 6.4e**). As the supernatant (PS) contains sodium acetate (found in MRS broth), which may interfere with the efficacy of the test, more interest was focused on the conditioned medium (CM). Previous studies have shown that some *L. fermentum* strains have greater potency compared with other *Lactobacilli,* in terms of soluble factors produced in the supernatant and not to the bacterial pellet itself. This aligns with other studies where probiotic CM have shown effects similar to living bacteria [318] and supported the potential of probiotics against CRC [220]. To provide relevant evidence of the potential beneficial effect of probiotic bacteria *L. fermentum* NCIMB 5221 against colon cancer, the probiotic was tested on both Caco-2 cancer

cells and CRL-1831 normal cells *in vitro*. *L. fermentum* NCIMB 5221 was shown to reduce CRC cell viability in a time- dependent manner (**Figure 6.5e**) compared with controls (**Figures 6.5a and 6.5b**). It also supported constant non-neoplastic cell growth in a serum-free media compared with untreated cells (**Figure 6.5f**). In fact, among all tested probiotic bacteria, the probiotics that inhibited the most cancer cells also showed the greatest proliferation of non-cancerous colon cell growth. In order of potency, the tested probiotics were *L. fermentum* NCIMB 5221, *L. rhamnosus* ATCC 53103, and *L. acidophilus* ATCC 314. Those observations support the finding that an optimal anti-cancer drug would be one that destroys neoplastic cells but not healthy cells.

Interestingly, *L. fermentum* NCIMB 5221 produced the most SCFAs ($p < 0.001$, **Figure 6.6**) compared with *L. rhamnosus* ATCC 53103 and *L. acidophilus* ATCC 314. This observation agrees with some if the reported actions of probiotic bacteria in producing factors that prevented tumor-initiating events in the colon while promoting a healthy epithelium. The considerable increase in the production of butyrate observed during the administration of *L. fermentum* was of importance in relation to colonic cancer [319]. SCFAs are defined as products of the anaerobic metabolism of mal-absorbed or non-absorbed dietary carbohydrates by luminal bacteria and identified as the dominant ion species in the aqueous phase of feces (190 mM) [320]. In fact, it has been thusly proposed that to alter intestinal epithelial cell function, including colonic SCFA utilization (mainly butyrate [218]), luminal bacteria can be the first target. Approximately, SCFA concentrations in the lumen are in the range of 70 - 130 mM, with molar ratios of acetate: propionate: butyrate varying from 75:15:10 to 40:40:20. It has been estimated that SCFAs can contribute with about 10% of the total caloric requirements in humans. Luminal SCFAs, especially butyrate, serve as the major energy source for human colonocytes, especially in the distal colon [321]. In addition to its role as a fuel, butyrate is notable for its function as an inhibitor of histone deacetylases (HDACs), leading to hyperacetylation of chromatin, thereby influencing gene expression. During the concentration-dependent absorption of SCFAs, bicarbonate, salt, and water transport improves, maintaining a neutral or alkaline colonic pH [322]. In animals, they accelerated the restoration of colonic anastomoses and experimental colitis [323, 324] and resulted in increased regional blood flow and oxygen uptake [325]. *L. fermentum* NCIMB 5221 is considered a fitter candidate based on its higher production of propionate and butyrate. Nonetheless, *L. rhamnosus* ATCC 53103 and *L. acidophilus* ATCC 314 may also have a beneficial effect through their

elevated production of lactate (up 3200 mg/L, **Figure 6.6a**, $p < 0.001$). Here, lactate is a substrate for luminal lactate-utilizing bacteria that produce acetate and butyrate, as well as some propionate [326] and a regulator of epithelial proliferation in the gut through the repression of cyclin E1/D1 gene transcription [318].

Other features that could support the prophylactic potential of *L. fermentum* NCIMB 5221 in colorectal health could be the transformation of LA to CLA and other compounds with antioxidant and anti-inflammatory properties [327]. When orally administered *L. fermentum* NCIMB 5221 was used to alleviate markers of metabolic syndrome in ZDF rats hypothetically through the release of FA, a phenolic acid found in foods [21].

Further analysis confirmed that *L. fermentum* NCIMB 5221 activity was not due to one of the SCFAs alone (**Figure 6.7a**). Since there was no significant difference between the bacterial extract and the SCFAs synthetic formulation (**Figure 6.7b**), *L. fermentum* NCIMB 5221 may owe its anti-cancer effect to SCFA release in total. After addition of lactic acid to the SCFA mixture, the effect of SSF+LA was significantly less than SSF or the CM. This implies that lactate may have repressed SCFA metabolism/intake in CRC cells and other non-anti-cancer factors produced by *L. fermentum* NCIMB 5221 that support the activity of secreted SCFAs in suppressing cancer cells. The transport of butyrate into cells is greatly inhibited by the presence of its analog, lactate, a monocarboxylic acid transported into cells via monocarboxylated transporter (MCT), or propionate that is found in the colonic lumen and structurally similar to butyrate [328]. Similarly, it was demonstrated that the uptake of 500 μM butyrate in Caco–2 cells was reduced by 49.6% in the presence of propionate and by 57.2% in the presence of 10 mM L-lactate [329]. Under in vivo conditions, where butyrate and propionate are present at >10 mM in the colon, the transporter plays only a minor role in the entry of these compounds into colon cells. When SCFAs are at low concentrations, there is involvement of SLC5A8 as transporters of butyrate and propionate with a Michaelis constant of ~0.05 mM. However, at high concentrations, SCFAs diffuse into cells bypassing the transporter [330].

Importantly, the absorption and action of SCFAs within the extract of *L. fermentum* NCIMB 5221 on cancer cells could have involved other mechanisms that were repressed with the addition of lactate. *L. fermentum* NCIMB 5221 extract may have contained molecules playing a role in

assuring the action of SCFAs involved with cell transporters. These include the monocarboxylated transporter 1 (MCT-1) and sodium-coupled monocarboxylate transporter (SMCT-1) receptor found on colonocytes. Their function is to transport SCFAs or SCFA receptors GPR41/ free fatty acid receptor 3 (FFAR3) and GPR43/ free fatty acid receptor 2 (FFAR2), expressed in a subpopulation of ghrelin and gastrin cells [331]. Recent studies have identified the plasma membrane transporter SLC5A8 and the cell-surface receptors GPR109A and GPR43 as essential for the biologic effects of SCFAs in the colon [332]. Gpr109a was found crucial for butyrate-mediated induction of IL-18 in colonic epithelium. It was actively involved in promoting anti-inflammatory properties in colonic macrophages and dendritic cells and enabling them to induce differentiation of Treg cells and IL-10-producing T cells [333]. Several bacterial effectors may alter the action of soluble factors that probiotic bacteria have produced. For example, a probiotic-derived polyphosphate have shown to inhibit progression of CRC, inactivate the ERK pathway and induce cancer cell apoptosis [334] or cell-bound exopolysaccharides (c-EPS) with anti-tumor activity [333]. In certain cases, it was revealed that the pro-SCFA compound might be a macromolecule such as a protein, nucleic acid, or a polysaccharide [333].

Validation tests on the SCFAs' effect on non-neoplastic cells and cancer cells were used to confirm the fact that different concentrations and mixtures of pure/synthetic SCFAs have significant suppressive effect on cancer cells but not against normal epithelial cells. The test also verified the effect of the addition of lactate with SCFAs on cancer cell proliferation (**Figure 6.8**). First, lactic acid did not affect cancer cell proliferation when tested at different doses (up to 1300 mg/L, **Figure 6.8f**); however, when added to SCFAs, they lost a significant part of the cancer-suppressing activity**.** This confirms that lactic acid could inhibit SCFA metabolism/uptake in cancer cells as described in some studies [335] and also concluded with SCFA synthetic formulations, as presented in this study (**Figure 6.7b**), Thus, this emphasize that the presence of another bacterial factor promoted the role of SCFAs to suppress cell growth. If we assume, as concluded above, that lactate and propionate inhibited the uptake of butyrate (by 31% for *L. fermentum* NCIMB5221), then in the presence of lactate, only acetate (18. 6 ± 3.1%) will be responsible for the inhibitory effect that was closer to SSF + LA (21.1 ± 2.9%, **Figure 6.7**). In the case of non-neoplastic colon cells, no significant effect was observed on CRL-1831 cell growth when treated with SCFAs and/or lactate when compared with cancer cells. Whereas with *L. fermentum* NCIMB 5221, there

was a promotion of cell growth (**Figure 6.5f**), implying that, in addition to SCFAs, other soluble or non-soluble bacterial compounds could have a beneficial action on normal cells. For example, lipoteichoic acid (LTA) was shown to induce signaling in colon epithelial cells through Toll-like receptor 2 (TLR2)-CD14 and/or TLR2-TLR6 heterodimers. It activates extracellular-signal-regulated kinases (ERKs), NF involved protein kinase C (PKC)- and mitogen-activated protein kinase (MAPK)-dependent pathways, and inhibits cytokine-induced epithelial cell apoptosis and damage through a phosphoinositide 3-kinase-AKT-dependent pathway [336]. Those proteins were demonstrated to present resistance against apoptosis and induce epithelial barrier fortification in intestinal epithelial cells by activating the p38 and ERK signaling pathways [337].

6.6. Conclusion

In this study, *L. fermentum* NCIMB 5221 showed the same CRC cell inhibitory effect as the SCFAs by themselves. This would suggest the use of these bacteria as preventive vehicles is not limited to SCFA-producing ability, as was suggested in some studies [316, 338-340]. Notably, the use of the bacteria as a delivery mechanism for active compounds such as the SCFAs could be a better option, especially since *L. fermentum* NCIMB 5221 can produce antioxidant, anti-inflammatory, and anti-carcinogenic effects in soluble and non-soluble components within the gut. Here, *L. fermentum* NCIMB 5221 was identified with an increased anti-proliferative effect against CRC cells in comparison with some other LAB (*L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103) characterized in previous studies for their potential anti-cancer effect [155, 309-311]. Interestingly, this bacterium exhibited a reverse effect on normal colon cells suggesting that this bacterium is harmful to cancer cells but beneficial to normal cells. These effects were strongly related in this work, showing the significant ability of *L. fermentum* to produce more FFAs and significantly more acetic, propionic, and butyric acids compared with other probiotics. *L. fermentum* NCIMB 5221 has also been shown to produce antioxidant and anti-cancer compounds that make it more suitable as an alternative bioprophylactic and biotherapeutic agent for CRC treatment.

6.7. Conflicts of Interest

The authors have no conflicts of interest to disclose.

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6.8. Figures and Tables

Table 6.1: Outline of the study on characterizing the anti-carcinogenic potential of *L. fermentum* NCIMB 5221 and the role of probiotic and synthetic SCFAs. LA: lactic acid; AA: acetic acid; PA: propionic acid; BA: butyric acid.

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

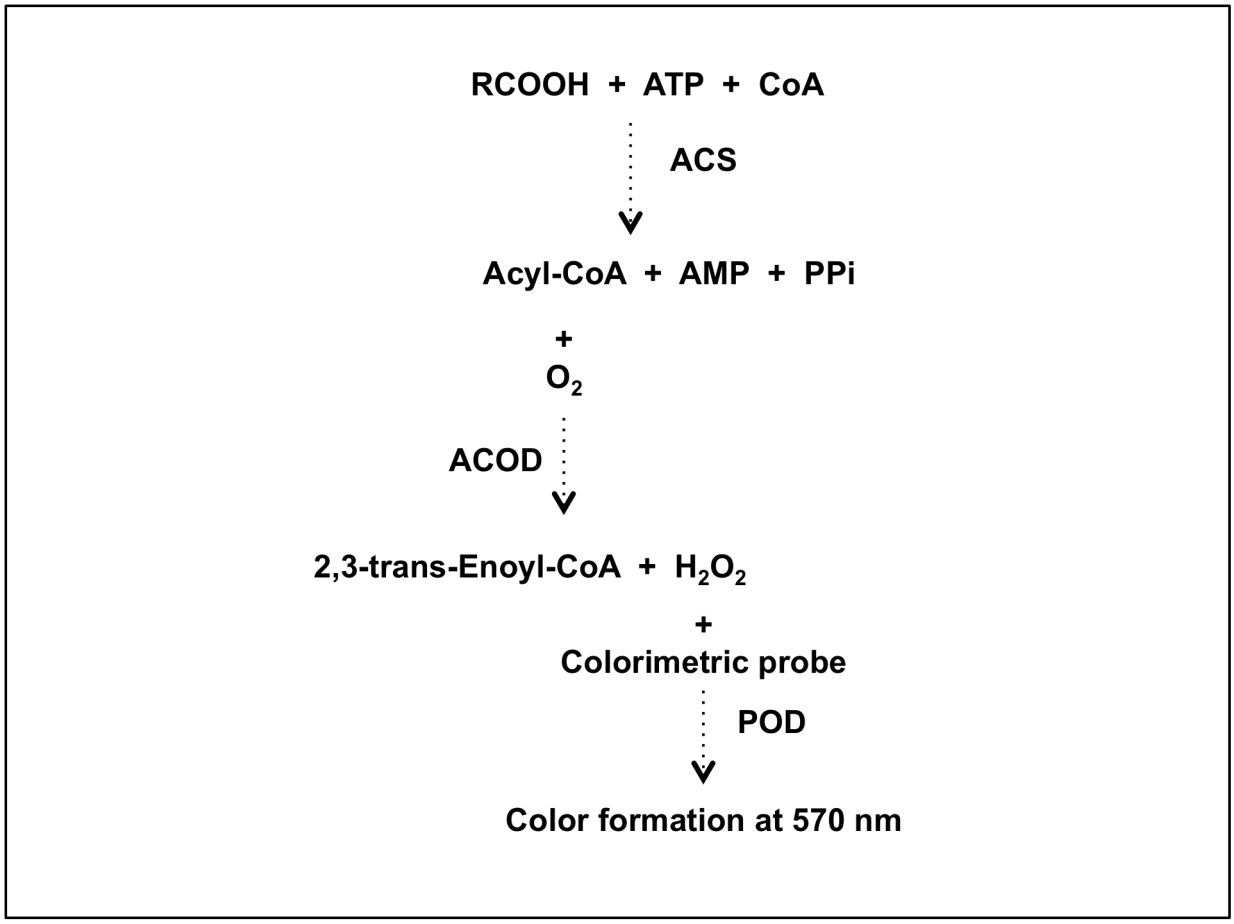
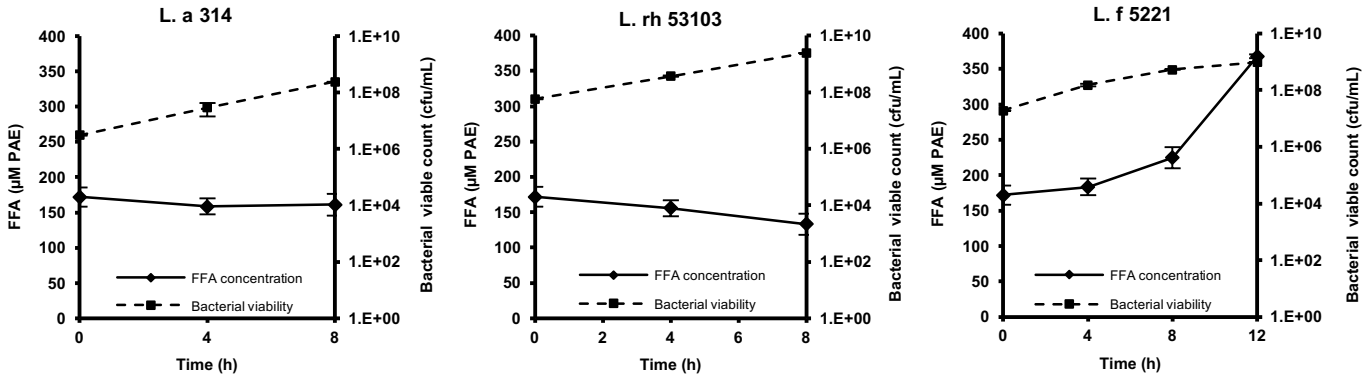


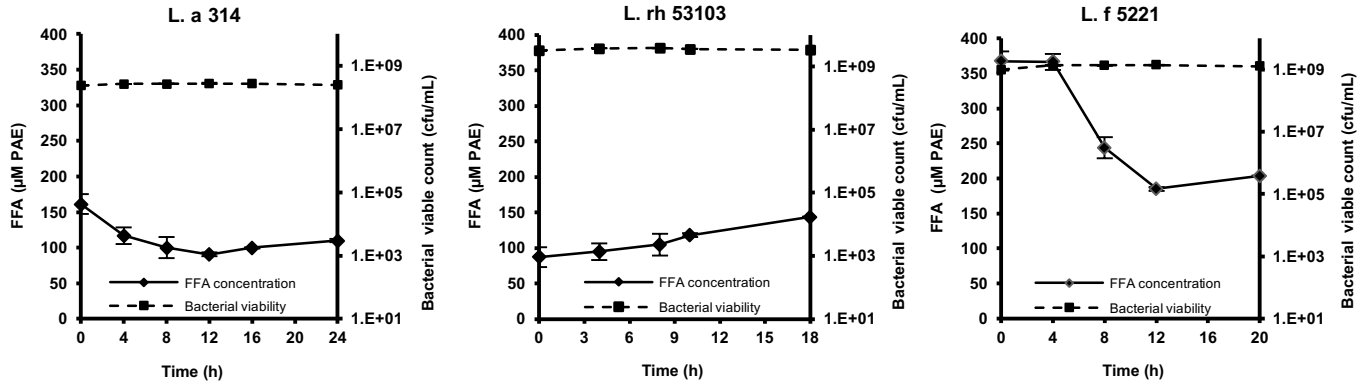
Figure 6.1: The mechanism behind the quantification of total free fatty acids using a coupled enzymatic reaction system (ACS-ACOD Method).

ACS: Acyl-CoA Synthase. ACOD: Acyl CoA Oxidase. POD: peroxidase.

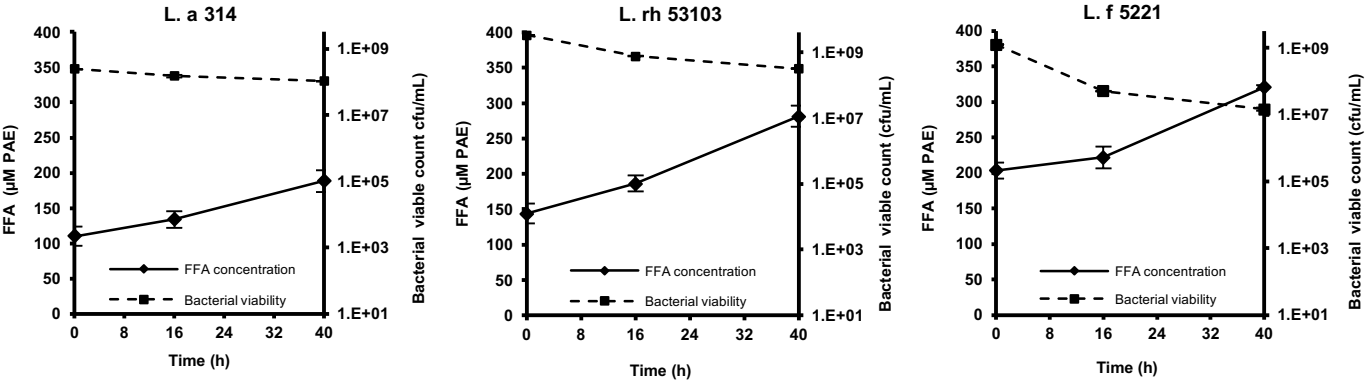
a


LAG AND EXPONENTIAL PHASE
L. a 314
L. rh 53103
L. f 5221
FFA (µM PAE)
Bacterial viable count (cfu/mL)
FFA concentration
Bacterial viability
Time (h)

b


STATIONARY PHASE
L. a 314
L. rh 53103
L. f 5221
FFA (µM PAE)
Bacterial viable count (cfu/mL)
FFA concentration
Bacterial viability
Time (h)

c


DEATH PHASE
L. a 314
L. rh 53103
L. f 5221
FFA (µM PAE)
Bacterial viable count cfu/mL
Bacterial viable count (cfu/mL)
FFA concentration
Bacterial viability
Time (h)

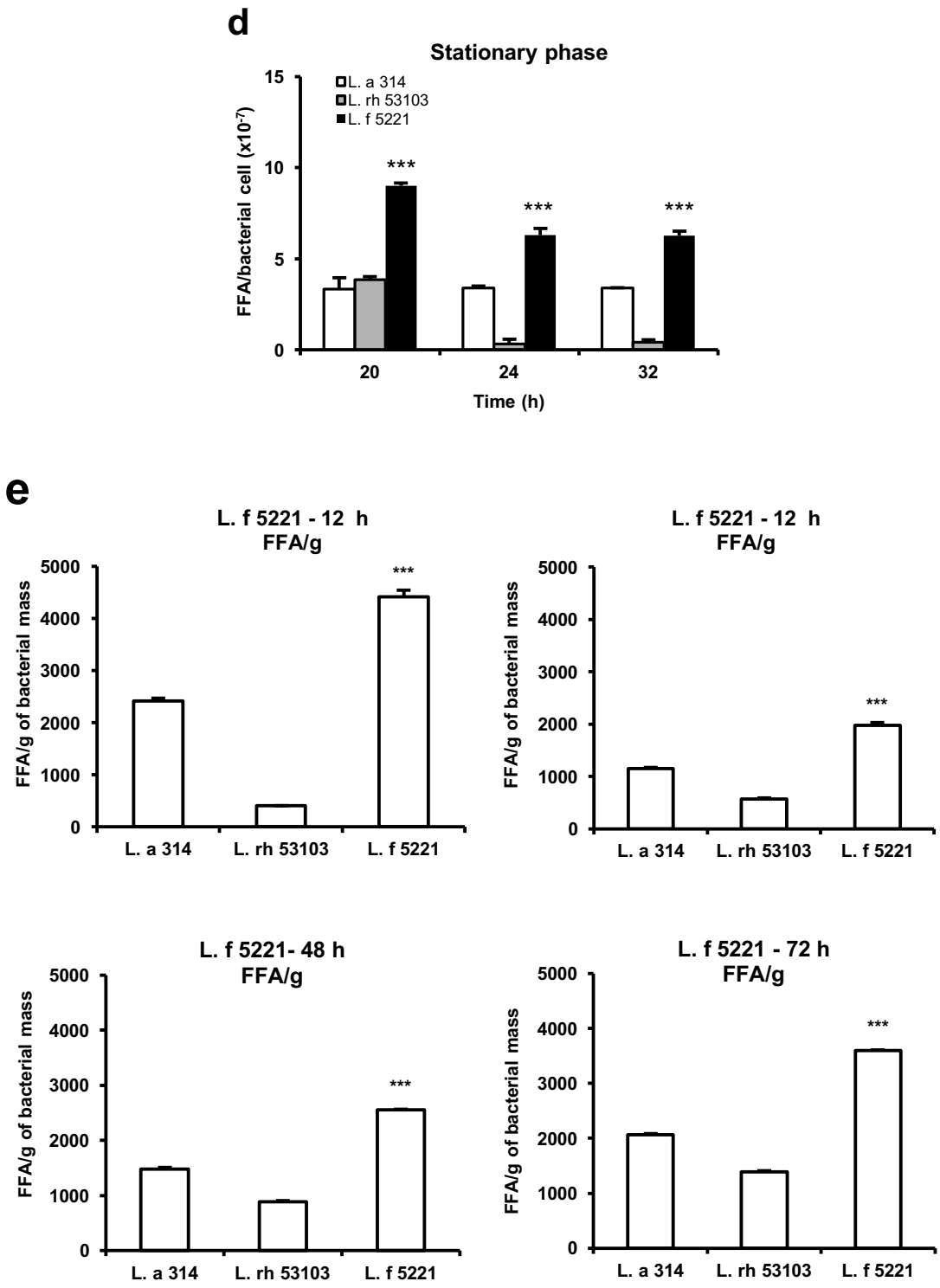

d
Stationary phase
L. a 314
L. rh 53103
L. f 5221
FFA/bacterial cell (x10-7)
15
10
5
0
***
***
***
20
24
32
Time (h)
e
L. f 5221 - 12 h
FFA/g
FFA/g of bacterial mass
5000
4000
3000
2000
1000
0
***
L. a 314
L. rh 53103
L. f 5221
L. f 5221 - 12 h
FFA/g
FFA/g of bacterial mass
***
L. a 314
L. rh 53103
L. f 5221
L. f 5221- 48 h
FFA/g
FFA/g of bacterial mass
***
L. a 314
L. rh 53103
L. f 5221
L. f 5221 - 72 h
FFA/g
FFA/g of bacterial mass
***
L. a 314
L. rh 53103
L. f 5221

Figure 6.2: Study of the metabolic activity of *L. fermentum* NCIMB 5221: Determination of growth pattern and total free fatty acid (FFA) profile.

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

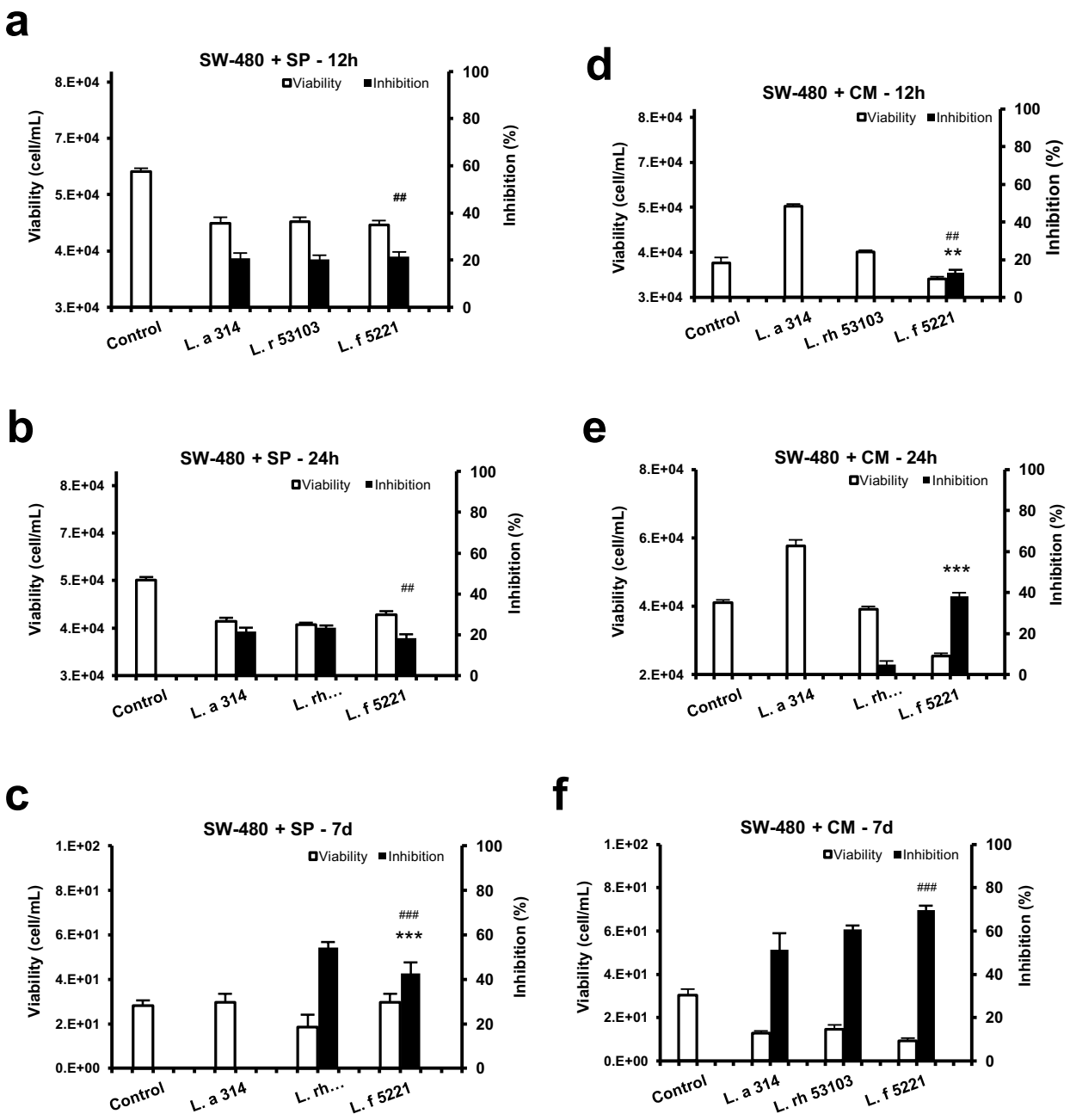


Figure 6.3: Analysis of human colon cell growth inhibition by *L. fermentum* NCIMB 5221 using different bacterial cell-free extracts.

The effect of (**a, b, c**) the probiotic supernatants (PS), and (**d, e, f**) the conditioned cell culture medium (CM) of *L. fermentum* NCIMB 5221 on the viability of CRC cells (SW-480) for 12 h, 24 h, and 7 days. $^{**}p < 0.01$ and $^{***}p < 0.001$, compared with *L. acidophilus* ATCC 314 or *L. rhamnosus* ATCC 53103. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with untreated cells. Data are presented as mean ± SEM ($n = 4$). *L. a* 314: *L. acidophilus* ATCC 314; *L. rh* 53103: *L. rhamnosus* ATCC 53103; *L. f 5221*: *L. fermentum* NCIMB 5221.

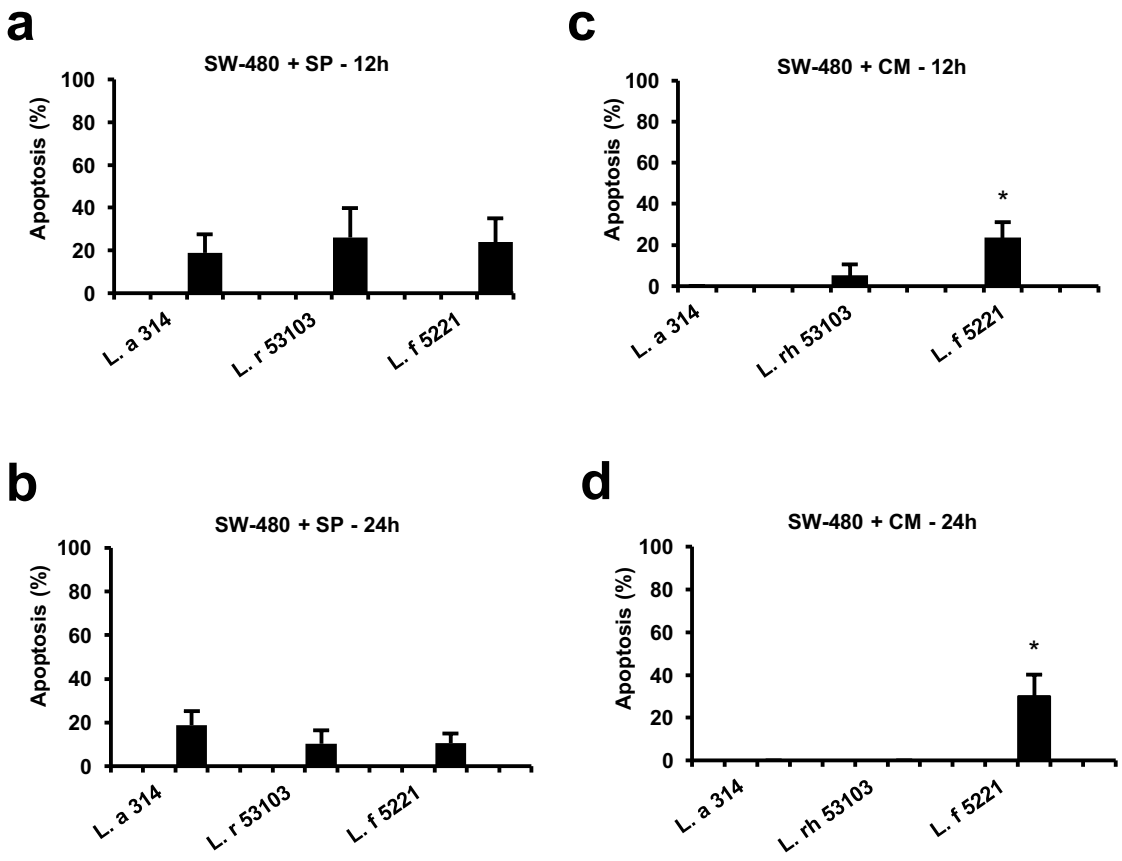


Figure 6.4: Assessment of apoptosis induction in CRC cells after treatment with *L. fermentum* NCIMB 5221 extracts.

Both probiotic supernatant (PS) and conditioned cell culture medium (CM) of *L. fermentum* NCIMB 5221 had induced apoptosis in SW-480 cells, when treated for (**a, c**) 12 h and (**b, d**) 24 h. The data values represent the mean ± SEM ($n = 4$). *$p < 0.05$, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. *L. a* 314: *L. acidophilus* ATCC 314; *L. rh* 53103: *L. rhamnosus* ATCC 53103; *L. f* 5221: *L. fermentum* NCIMB 5221

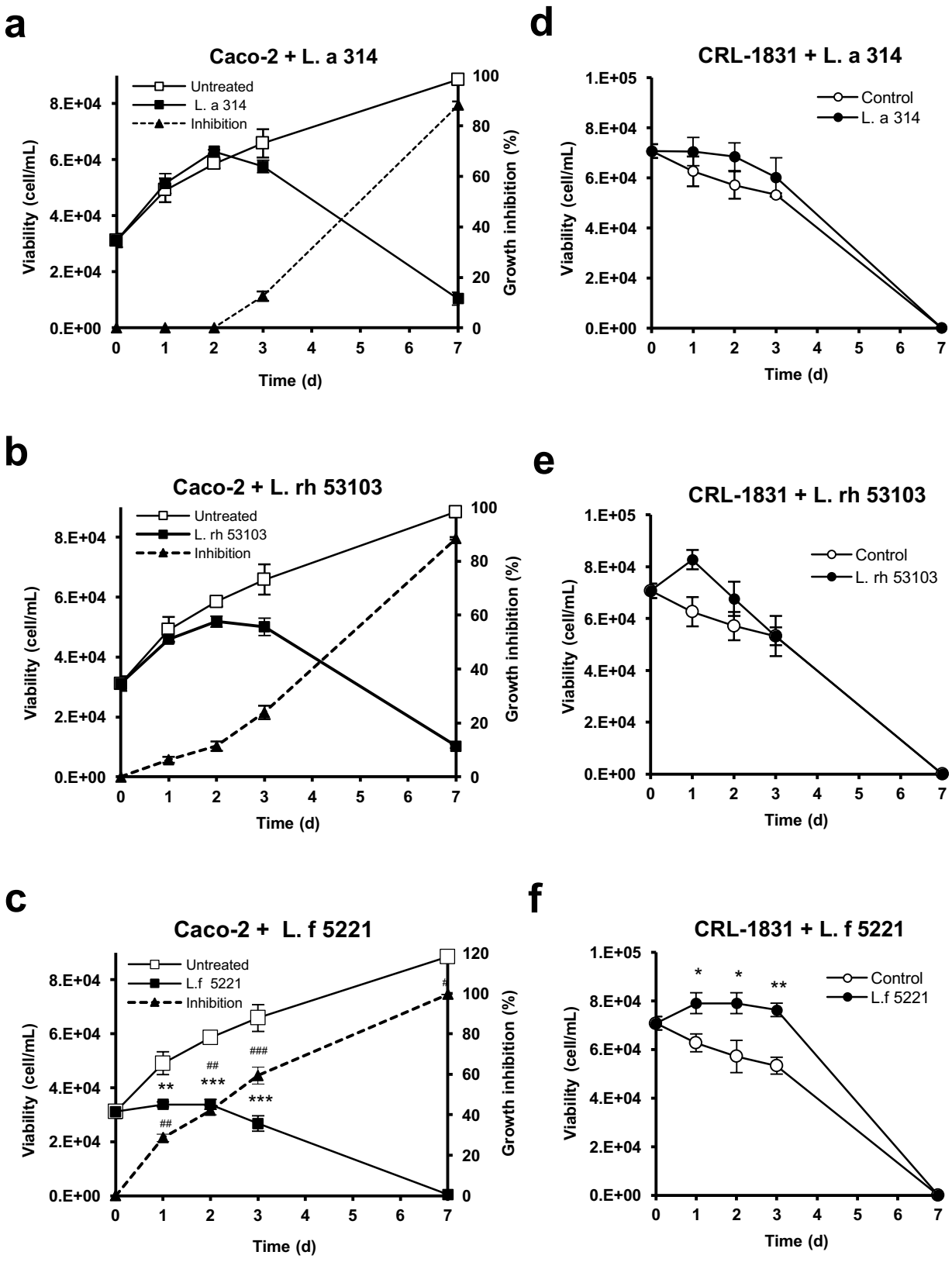

a
Caco-2 + L. a 314
Untreated
L. a 314
Inhibition
Viability (cell/mL)
Growth inhibition (%)
Time (d)
d
CRL-1831 + L. a 314
Control
L. a 314
Viability (cell/mL)
Time (d)
b
Caco-2 + L. rh 53103
Untreated
L. rh 53103
Inhibition
Viability (cell/mL)
Growth inhibition (%)
Time (d)
e
CRL-1831 + L. rh 53103
Control
L. rh 53103
Viability (cell/mL)
Time (d)
c
Caco-2 + L. f 5221
Untreated
L.f 5221
Inhibition
Viability (cell/mL)
Growth inhibition (%)
Time (d)
f
CRL-1831 + L. f 5221
Control
L.f 5221
Viability (cell/mL)
Time (d)

Figure 6.5: Investigation of the anti-colon-cancer proliferative and the non-cytotoxic effects of *L. fermentum* NCIMB 5221 using non-neoplastic and CRC cells.

This assay was performed by comparing the viability of Caco-2 colon carcinoma cells and CRL-1831 normal epithelial colon cells, incubated with the CM of *L. fermentum* NCIMB 5221, during 1, 2, and 3 days. The data values represent the mean ± SEM (n = 4). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, compared with untreated cells. #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. *L. a* 314: *L. acidophilus* ATCC 314; *L. rh* 53103: *L. rhamnosus* ATCC 53103; L. f 5221: *L. fermentum* NCIMB 5221.

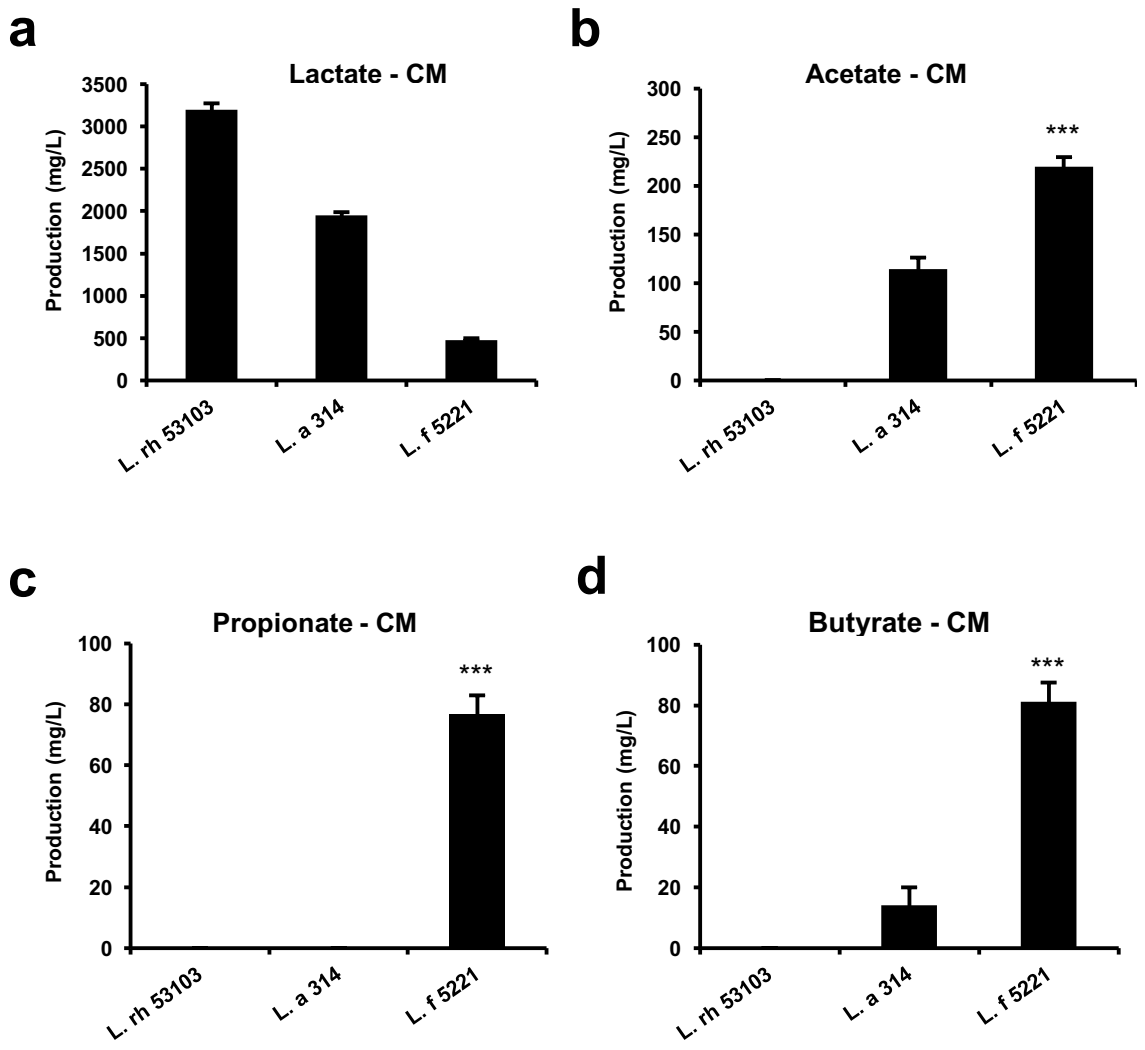


Figure 6.6: Production of SCFAs and lactic acid by *L. fermentum* NCIMB 5221.

(**a**) Lactic, (**b**) acetic, (**c**) propionic, and (**d**) butyric acids, and (**e**) total SCFAs have been produced by probiotic bacteria in the conditioned cell culture medium (CM). The data values represent the mean ± SEM (n = 3). ***$p < 0.001$, compared with *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. (f) Analysis of the correlation between cell growth inhibition by probiotic CM and the concentrations of SCFAs secreted in the CM. Plots represent the data of cell growth inhibition showed in Figure 6.5. The lines were obtained by linear regression analysis. The data values represent the mean ± SEM (n = 3).

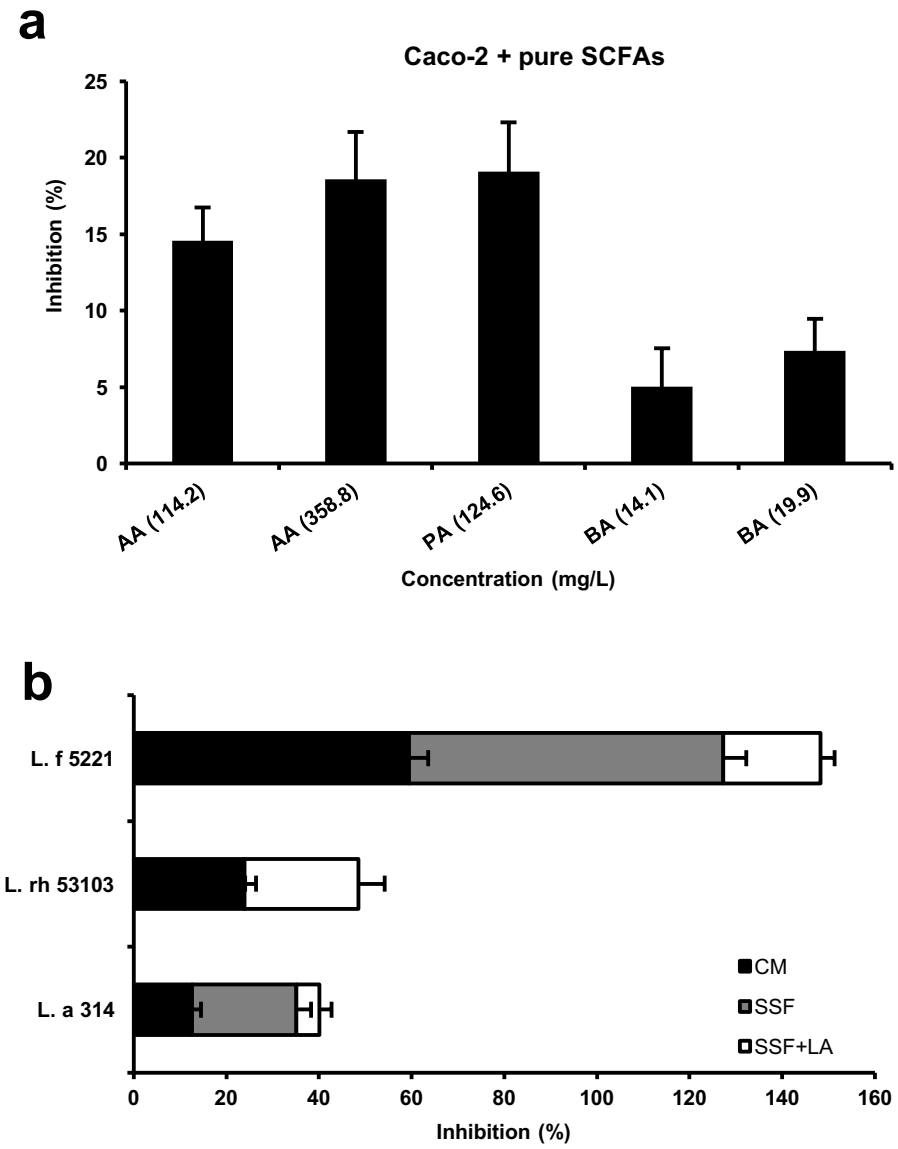


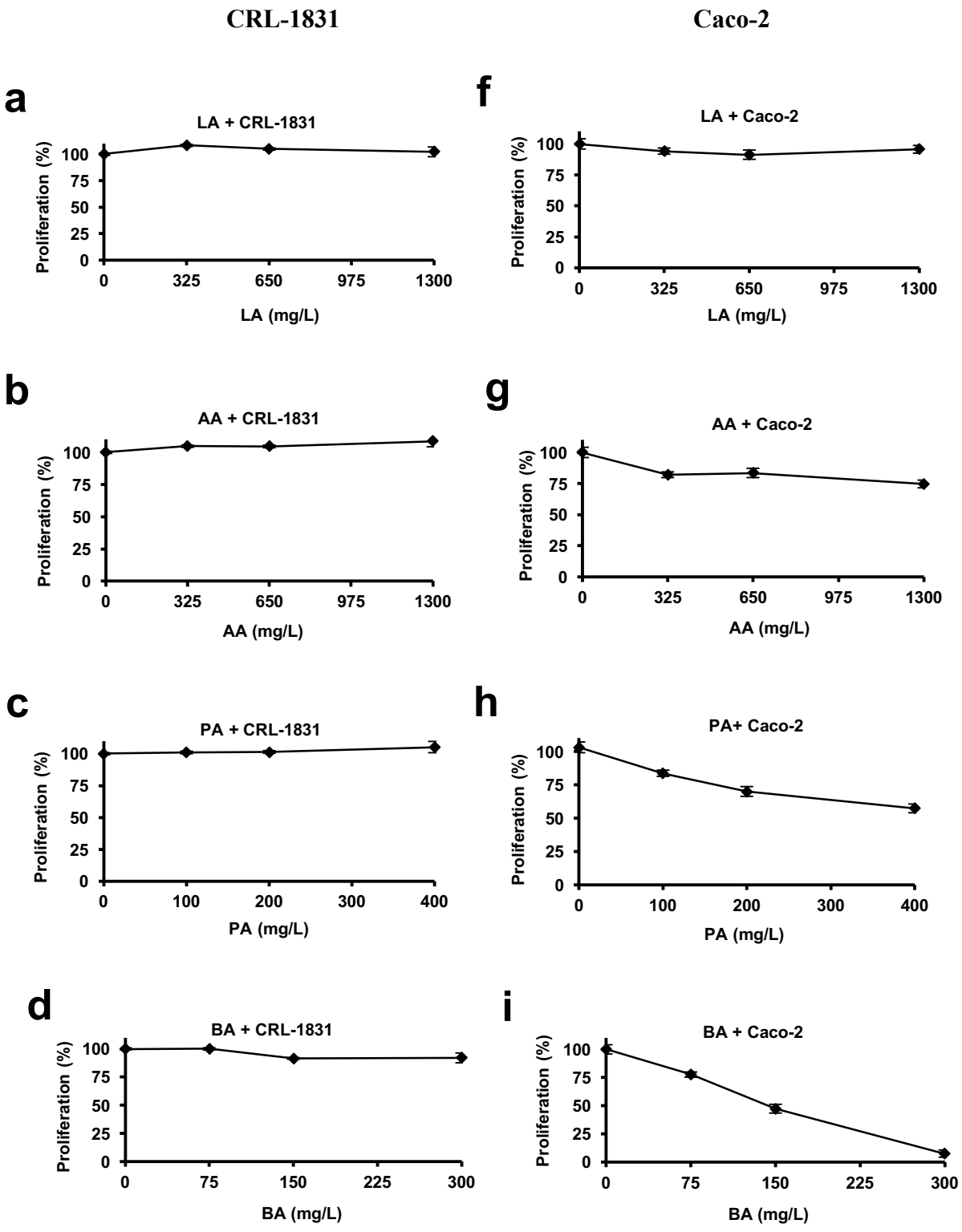
Figure 6.7: Verification of the implication of SCFAs produced by *L. fermentum* NCIMB 5221 in suppressing CRC cell growth.

Anti-proliferative effect of SCFAs doses at similar concentrations as what was produced by *L. fermentum* NCIMB 5221 as described in Figure 6.6. (**b**) Inhibitory effect of the SSFs and SFF+LA (formulations corresponding to *L. fermentum* NCIMB 5221) on the proliferation of CRC cells for 72 h, compared with the bacterial conditioned cell culture medium (CM) containing naturally produced probiotic SCFAs. *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 were positive controls. SSF+LA mixtures were prepared with the addition of different concentrations of lactic acid and pure SCFAs to DMEM media (**Figure 6.6**). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, compared with controls. The data values represent the mean ± SEM (n = 6). L. a. 314: *L. acidophilus* ATCC 314; L. rh 53103: *L. rhamnosus* 53103, *L. f* 5221: *L. fermentum* NCIMB 5221; SSF: SCFA synthetic formulation.

Table 6.2: Doses of single SCFAs and the composition of SCFA mixtures to be tested on Caco-2 and CRL-1831 colon cells.*

	Doses (mg/L)			
LA	0	325	650	1300
AA	0	325	650	1300
PA	0	100	200	400
BA	0	75	150	300
	Composition (mg/L)			
	LA	AA	PA	BA
Control	0	0	0	0
SSM1	1300	1300	400	300
SSM2	650	1300	400	300
SSM3	325	650	200	150
SSM4	325	325	100	75

* LA: lactic acid; AA: acetic acid; PA: propionic acid; BA: butyric acid.


CRL-1831
Caco-2
a
LA + CRL-1831
Proliferation (%)
100
75
50
25
0
0
325
650
975
1300
LA (mg/L)
f
LA + Caco-2
Proliferation (%)
100
75
50
25
0
0
325
650
975
1300
LA (mg/L)
b
AA + CRL-1831
Proliferation (%)
100
75
50
25
0
0
325
650
975
1300
AA (mg/L)
g
AA + Caco-2
Proliferation (%)
100
75
50
25
0
0
325
650
975
1300
AA (mg/L)
c
PA + CRL-1831
Proliferation (%)
100
75
50
25
0
0
100
200
300
400
PA (mg/L)
h
PA+ Caco-2
Proliferation (%)
100
75
50
25
0
0
100
200
300
400
PA (mg/L)
d
BA + CRL-1831
Proliferation (%)
100
75
50
25
0
0
75
150
225
300
BA (mg/L)
i
BA + Caco-2
Proliferation (%)
100
75
50
25
0
0
75
150
225
300
BA (mg/L)

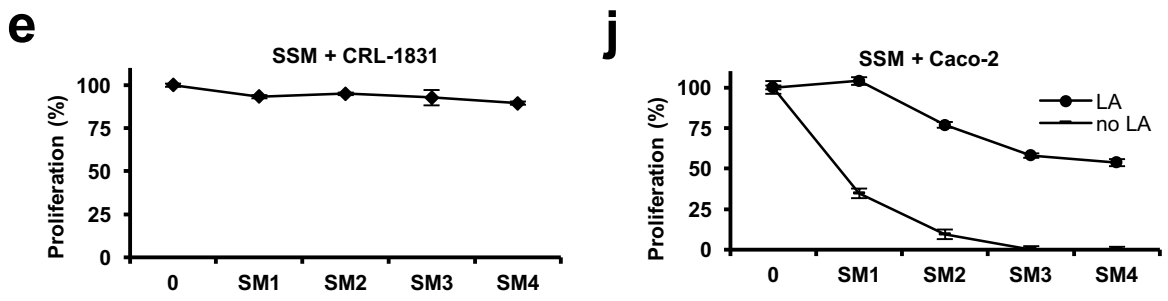


Figure 6.8: Study of the effect of synthetic/pure SCFAs (separately or mixed) on CRC and non-neoplastic colon cells.

Confirmation of the non-cytotoxic effect of (**a**) lactic, (**b**) acetic, (**c**) propionic, (**d**) and butyric acids, and (**e**) their mixtures on normal epithelial colon cells (CRL-1831). Determination of the anti-proliferative activity of (**f**) lactic, (**g**) acetic, (**h**) propionic, (**i**) and butyric acids, and (**j**) their mixtures on CRL-1831 normal cells anti-proliferative activity (**f, g, h, i, and j**, respectively) on Caco-2 cancer cells. The data values represent the mean ± SEM (n = 5). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with control. LA: lactic, AC: acetic, PA: propionic, BA: butyric acid.

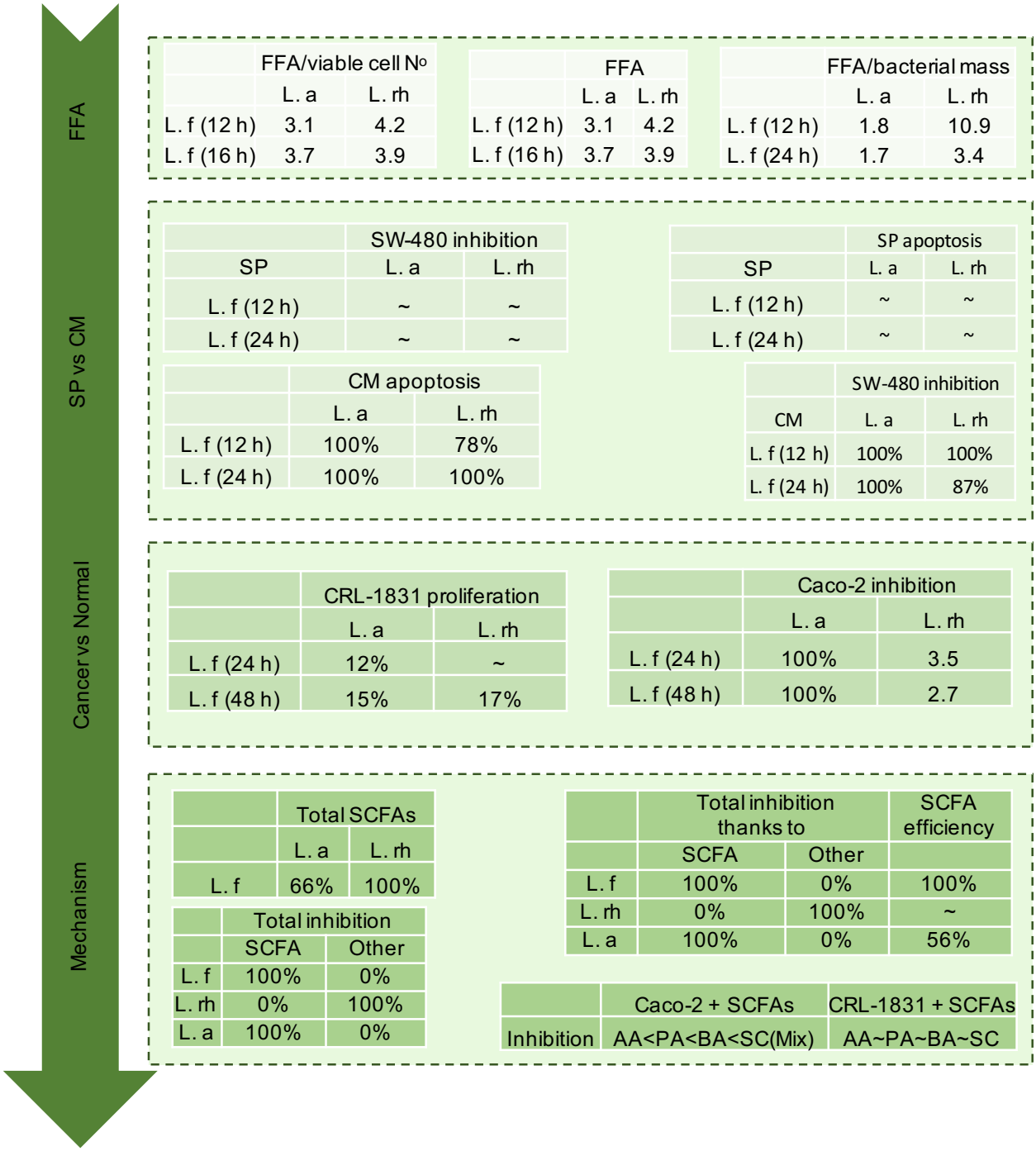


Figure 6.9: A descriptive overview of the CRC potent features and potential mechanisms of actions of *L. fermentum* NCIMB 5221.

The latter was evaluated based on *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. Comparative values were estimated in percent or fold change. ~: No significant effect ($p > 0.05$). L. rh: *L. rhamnosus* ATCC 53103. L. a: *L. acidophilus* ATCC 314 L. f: *L. fermentum* NCIMB 5221.

Original Research Article 5

CHAPTER 7. DESIGN AND VALIDATION OF AN ORALLY ADMINISTRATED ACTIVE *LACTOBACILLUS FERMENTUM-LACTOBACILLUS ACIDOPHILUS* PROBIOTIC FORMULATION USING COLORECTAL CANCER *APC*$^{MIN/+}$ MOUSE MODEL

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Preface: On comparing the anti-cancer potential of *L. fermentum* NCIMB 5221 with *L. acidophilus* ATCC 314 in the previous chapter, the anticancer effect of *L. fermentum* NCIMB 5221 was found significantly higher and more efficient than the previously selected *L. reuteri* NCIMB 701359. As *L. acidophilus* ATCC 314 have already shown anti-tumorigenic effect in CRC animal model, this bacterial strain was selected and tested in combination with *L. fermentum* NCIMB 5221, to determine if this combination can have a greater effect. Results in this chapter indicate that this combination had a significantly higher effect than using each bacterium separately, in terms of anti-proliferative effect, and positive effect on colon cells treated with or without a carcinogen. Finally, to validate these findings the mixed probiotic formulation of active live cells was orally administered to a genetically modified CRC mouse model. The efficacy of the probiotic treatment was determined based on intestinal tumor enumeration and assessment of cellular proliferation markers in both intestinal tumors and normal mucosa.

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7.1. Abstract

Probiotics have been shown to have beneficial properties in attenuating the risk of colorectal cancer (CRC) development. However, functional evidence to support such effects for some probiotic bacteria are relatively unknown. Here we document a significant antioxidant, anti-proliferative and pro-apoptotic activities of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 on CRC cells., particularly when used in combination (La-Lf). Furthermore, a superior synergistic activity on the inhibition of tumor growth and modulation of cell proliferation and epithelial markers in the $Apc^{Min/+}$ CRC mouse model was explored, based on the expression levels of Ki-67, E-cadherin, β-catenin, and cleaved caspase-3 (CC3) proteins. The anti-cancer activity of La-Lf co-culture was significantly enhanced *in vitro* with significant reduced proliferation (38.8 ± 6.9 %, $P = 0.009$) and increased apoptosis (413 RUL, $P < 0.001$) towards cancer cells, as well as significant protection of normal colon cell growth from toxic treatment (18.6 ± 9.8%, $P = 0.001$). La-Lf formulation (10^{10}cfu/animal/day) altered aspects of intestinal tumorigenesis by significantly reducing intestinal tumor multiplicity (1.7 fold, $P = 0.016$) and down-regulating cellular proliferation markers, including β-catenin ($P = 0.041$) and Ki-67 ($P = 0.008$). In conclusion, La-Lf showed greater protection against intestinal tumorigenesis supporting a potential use as a biotherapeutic for the prevention of CRC.

Keywords: *L. acidophilus*, *L. fermentum*, probiotics, colorectal cancer, apoptosis, proliferation.

7.2. Introduction

Active research is being conducted to unravel the effectiveness of probiotic biotherapeutics for some of the gastrointestinal (GI) disorders, in particular, one of the most common malignancies worldwide is the colorectal cancer (CRC) (Ambalam, Raman, Purama,Doble 2016; Tjalsma, Boleij, Marchesi,Dutilh 2012). Development of CRC is a multistage and multifactorial process that is associated with chronic inflammation, increased mutation to cell exposed to oncogenes and highly proliferating dysplastic lesions. Treated patients are suffering side effect and are left with high risk of risk of cancer recurrence (Levin, Rozen, Spann,Young 2005). Although some studies did not confirm such beneficial effects (Roessler, Forssten, Glei, Ouwehand,Jahreis 2012), others have reported that individuals consuming fermented milk or yogurt have a lower incidence of CRC (L Madsen 2012) or a lower propensity to develop large adenocarcinomas (Ohara, Yoshino,Kitajima 2009). Epidemiologically and clinically, it is suggested that CRC risk, recurrence, and health related issues (diarrhea, treatment toxicity, etc.) could be attenuated and managed by the consumption of certain functional foods. The identification of dietary constituents, such as probiotic bacteria, that prevent CRC, is a growing area of research in health, biotechnology and applied microbiology. A clear conclusion on which probiotic bacteria are superior candidates is not fully defined (Kuppusamy, Yusoff, Maniam, Ichwan, Soundharrajan,Govindan 2014). To evaluate the therapeutic efficacy of certain probiotic bacteria in CRC, several *Lactobacillus* strains have been tested. The bacteria were used, as potential active oral supplements or extracts, against cancer cells, tumor formation, and CRC recurrence in animals and humans (Kahouli, Malhotra, Alaoui-Jamali,Prakash 2015; Kahouli, Malhotra, Tomaro-Duchesneau, Rodes, Aloui-Jamali,Prakash 2015b; Kahouli, Malhotra, Tomaro-Duchesneau, Saha, Marinescu, Rodes, Alaoui-Jamali,Prakash 2015). Despite promising findings from experiments on animals, clinical trials, and epidemiological studies, there are a few clear-cut results to select best suited strains use in CRC (Kahouli, Tomaro-Duchesneau,Prakash 2013b; Raman, Ambalam, Kondepudi, Pithva, Kothari, Patel, Purama, Dave,Vyas 2013). Clearly, further studies are needed before the beneficial effects of certain probiotics in the prevention of human CRC can be confirmed. To establish a particular probiotic formulation as a biotherapeutic for CRC,

testing and characterization of relevant probiotic strains are crucial steps to prove efficacy and understand the mechanisms of action.

L. acidophilus ATCC 314 and *L. fermentum* NCIMB 5221 had shown features that directly and indirectly relate to GI health (Kahouli, Tomaro-Duchesneau,Prakash 2013a; Tomaro-Duchesneau, Saha, Malhotra, Jones, Labbé, Rodes, Kahouli,Prakash 2014). For instance, the oral administration of *L. acidophilus* ATCC 314 showed anti-inflammatory (Amdekar, Roy, Singh, Kumar, Singh,Sharma 2012) and anti-tumorigenic effect in CRC mouse model. *L. acidophilus* ATCC 314 was added to a yogurt formulation and was shown to protect $Apc^{Min/+}$mice from increased intestinal tumors, without being tested, however, as a pure free bacterial cells (Urbanska, Bhathena, Martoni,Prakash 2009). Previously, *L. fermentum* NCIMB 5221 was established as a superior ferulic acid-producing bacteria and shown a positive outcome in a metabolic syndrome rat model (Jones, Kahouli, Labbé, Prakash, Tomaro-Duchesneau, Rodes, Saha,Malhotra). Recent studies had demonstrated *L. fermentum* NCIMB 5221 had a greater *in vitro* anti-cancer potential than many *L. fermentum* strains, *L. fermentum* NCIMB 5221 (Kahouli, Malhotra, Alaoui-Jamali,Prakash 2015; Kahouli, Malhotra, Tomaro-Duchesneau, Rodes, Aloui-Jamali,Prakash 2015a) . Here, we hypothesize that the combination of these bacteria could potentially secrete synergistic metabolic activity and boost the biotherapeutic potential.

The present study was designed to study possible synergistic bacterial properties of mixed *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 and establish their effect on both cancer and normal colon cells. Then, the final step was an assessment to validate the effect and underlying mechanisms by which the probiotic formulation inhibits intestinal tumorigenesis in a recognized mouse model for human intestinal cancer, $Apc^{Min/+}$ mice. Before oral administration in animals, the survival and antioxidant capacity of live active bacterial cells of *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314, used in combination in simulated intestinal fluids were depicted. First, modified and unmodified bacterial cell-free extracts of *L. fermentum* NCIMB 5221, *L. acidophilus* ATCC 314, or their bacterial co-culture were tested on cancerous (Caco-2) and non-cancerous colorectal (CRL-1831) cell lines, in order to depict the nature of the active bacterial compounds. The *in vivo* effectiveness of daily administration of the potent probiotic formulation was investigated in an $Apc^{Min/+}$ mouse CRC model. This genetic animal model mimics human

intestinal colorectal carcinogenesis and has been used extensively in chemopreventive studies (Corpet and Pierre 2003). Tumor load was identified using haematoxylin and eosin (H&E) staining. These mice carry a mutation of the human adenomatous polyposis coli (*APC*) that is a negative regulator of ß-catenin, essential for epithelial cell, and hemostatic when it constitutes the adherence junction together with E-cadherin (Conacci-Sorrell, Zhurinsky,Ben-Ze'ev 2002; Rubinfeld, Souza, Albert, Muller, Chamberlain, Masiarz, Munemitsu,Polakis 1993). Thus, both ß-catenin, E-cadherin expressions were assessed and additional cellular proliferation markers (Ki-67, cleaved caspase 3 (CC3) were identified by Immunohistochemistry.

7.3. Materials and Methods

7.3.1. Reagents

Agar and De Man, Rogosa, Sharpe (MRS) broth (Fisher Scientific, Ottawa, ON, Canada) was used for *Lactobacillus* bacterial cultures. Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and Eagle's Minimum Essential Medium (EMEM, Invitrogen) were used for mammalian cell culture. Phosphate-buffered saline (PBS), Roswell Park Memorial Institute medium (RPMI-1640), fetal calf serum (FCS), and fetal bovine serum (FBS) were purchased from Invitrogen. Water was purified with two systems: EasyPure Reverse Osmosis and NanoPure Diamond Life Science (UV/UF) Ultrapure Water (Barnstead, Dubuque, IA, USA). Phosphate buffered formalin (PBF), Trypan Blue dye, acetic and propionic acids were obtained from Sigma (St. Louis, MO, USA). All chemicals and reagents were of HPLC grade, purchased from Sigma.

7.3.2. Bacterial cultures

L. fermentum NCIMB 5221 and *L. acidophilus* ATCC 314 were procured from the National Collection of Industrial, Marine and Food Bacteria (NCIMB, Aberdeen, Scotland, UK) and Cedarlane Laboratories (Burlington, ON, Canada), respectively. Bacterial cultures were maintained by continuous subculturing in MRS broth at 1% (v/v) while bacterial growth was monitored with both optical density (Perkin Elmer 1420 Multilabel Counter, USA) and colony counting. Three bacterial cultures were maintained: one inoculated only with *L. fermentum* NCIMB 5221, another inoculated only with *L. acidophilus* ATCC 314, and lastly a co-culture inoculated with both *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314 simultaneously.

7.3.3. Mammalian cultures

Caco-2 epithelial CRC adenocarcinoma cells were purchased from ATCC (Manassas, VA) and maintained in EMEM supplemented with 20 % FBS. CRL-1831 normal epithelial colon cell line, obtained from ATCC (Manassas, VA), was grown in complete DMEM (10 % FBS, 37^{o}C, 5% CO_2). Cells were left to differentiate in a humidified incubator at 37°C, supplemented with 5% CO_2 for up to two weeks. For experiments assessing viability and apoptosis, cells were left to attach for 24 - 48 h in 96-well plates, until they are at 50 - 60 % confluence level. Then, cell culture medium was replaced by probiotic cell-free extracts diluted twice with DMEM (serum/antibiotic-free).

7.3.4. Resistance in simulated intestinal fluids

Before using *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314 formulations *in vivo*, their corresponding bacterial cultures were characterized and tested in a simulated intestinal environment *in vitro*. Three bacterial cultures, as described in **Figure 7.1,** *L. fermentum* NCIMB 5221 (culture 1), *L. acidophilus* ATCC 314 (culture 2) and co-culture of both *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314 (culture 3), were prepared in MRS broth and simulated intestinal fluid (SIF). Bacterial cell resistance, in terms of viability and cell density, was monitored in each culture for 12 h. SIF used was prepared as described by Qian Zhao et al. (Zhao, Mutukumira, Lee, Maddox,Shu 2012), and artificial intestinal juice (AIJ) as used by Ganan et al., with some modifications (Ganan, Carrascosa, de Pascual-Teresa,Martinez-Rodriguez 2012). The solution of SIF contained glucose (10 g/L), yeast extract (5 g/L), pancreatin (5 g/L), oxgall (1 g/L), starch (10 g/L), and monobasic potassium phosphate (KH_2PO_4, 3 g/L) dissolved in deionized water. Artificial intestinal juice (AIJ) was prepared by adding 1 g/L of pancreatin and 0.3 g/L oxgall to MRS broth (pH = 6.8).

7.3.5. Total antioxidant capacity (TAC)

To determine the antioxidant capacity of *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314, before dose administration to animals, the three bacterial cultures: culture 1, 2, and 3 in MRS and AIJ, were used (**Figure 7.1**). In each probiotic culture, cell density and total antioxidant production (TAC) in each probiotic culture were assessed for 12 h. TAC was measured using a QuantiChrom^{TM} Antioxidant Assay Kit. A standard curve was generated for Trolox at concentrations of 0, 300, 600, and 1000 µM plotted against A_{570nm} ($R^2 \geq 0.99$). Probiotic bacteria were subcultured from MRS broth at 1 % (v /v) in MRS and AIJ. Non-inoculate media were used as negative controls, and TAC was described in µM Trolox equivalent (µMTE).

7.3.6. Preparation of probiotic cell-free extracts

To identify the most potent probiotic formulation, three different probiotic treatments were compared in this study: *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 and their co-culture (**Figure 7.1**). For the preparation of cell-free extracts, specifically, cell culture conditioned media (CM), with three different bacterial cultures were incubated separately in MRS broth (8 - 12 h, 37°C)**.** Bacterial pellets were harvested from each culture

by centrifugation (1000 x g, 15 min) then washed twice with PBS. Each bacterial pellet was re-suspended in an equal volume of DMEM and incubated for 2 hours (37°C, 5% CO_2). The culture medium was centrifuged (1000 x g, 15 min) and sterile-filtered (0.2 μM-pore-size filter). To identify the physiochemical nature of the active compound in these probiotic formulations, the probiotic CM was subjected to different treatments, that are heat inactivation (100^o C, 45 min), filtration using an ultra-centrifugal filter of 30 MWCO, centrifuged at 1000 g for 20 min and treatment with proteinase K (10, 50, and 100 ug/mL, 37^o C, 1h). The CM of each strain was diluted twice with the culture medium, DMEM before incubation with mammalian cells.

7.3.7. Trypan Blue dye - Exclusion assay

CRC cells (normal or cancer) were seeded at 5 x 10^6 cells/well cell suspension added into 12-well plates incubated at 37°C for 24 - 48 h (**Figure 7.1**). Then, 1 ml of CM was added to each well and incubated further for 27 h. The cell survival was examined using Trypan Blue Exclusion staining with a Neubauer haemocytometer. The analysis was conducted in triplicates, and cell viability was expressed in cell number per mL or percent cell viability [% viability = (live cell count/total cell count) x 100].

7.3.8. Cell viability assay

Assessment of cell viability was based on an ATP bioluminescence assay (CellTiter-Glo® Luminescent Cell Viability Assay, Promega, WI, USA) following the manufacturer's protocol (Andreotti, Cree, Kurbacher, Hartmann, Linder, Harel, Gleiberman, Caruso, Ricks, Untch,et al. 1995; Ganan, Carrascosa, de Pascual-Teresa,Martinez-Rodriguez 2012). Colon cells (normal or cancer) were seeded into 96-well culture plates (5 - 6 x 10^3 cells per well, 100 μL per well) and stabilized for 24 - 48 h (37°C, 5% CO_2) for cell attachment. After incubating the cells with the probiotic supernatants (24, 48, and 72 h), the plates were incubated at room temperature (RT, 30 min) before adding the luminescent reagent (100 μL). After which, the plate was placed on a plate-shaker and was agitated (2 min, 200 rpm) and incubated at RT (10 min) and the luminescent signal was recorded using a spectrophotometer (Perkin Elmer, Victor 3, multi-label microplate reader, MA, USA).

7.3.9. Apoptosis assay

Apoptosis was determined using caspases -3 and -7 using Caspase-Glo® 3/7 assay (Promega, USA). CRC cells were seeded into 96-well culture plates (5 - 6 x 10^3 cells per

well, 100 µL per well) and stabilized for 24 - 48 h (37°C, 5% CO_2) before exposure to probiotic supernatants. Before use, the buffer and lyophilized substrate were equilibrated to RT were dissolved. Three treatment groups were prepared: the blank group (DMEM without cells), negative control (untreated cells in DMEM) and the treatments (treated cells) to detect caspase-3 and -7 activities in cell cultures using a luminometer-compatible white walled 96-well plates. After incubation with the treatment, the plate was removed from the incubator (37°C, 5% CO_2) and allowed to equilibrate at RT (10 min). A volume of 100 µL luminescent reagent was added to each well of a 96-well plate previously filled with 100 µL of blank, negative control cells, or treated cells in culture medium. Then, the plate was covered and the content was gently mixed using a plate shaker (300 - 500 rpm, 30 s). Later, after incubation at RT (30 min - 3 h) the luminescence of each sample was measured using a plate-reading luminometer as instructed by the manufacturer.

7.3.10. Assessment of the probiotic anti-carcinogenic effect

To verify the action of probiotic treatments as described above in protecting normal cells against a carcinogen exposure, (**Fig 1**) CRL-1831 cells were grown in a media supplemented with probiotic CM and seeded (5 x 10^6 cell per well) into a 12-well platesand left to adhere for 24 – 48 h. First, the CM prepared - CM of *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 and their co-culture - were used to treat normal colon cells CRL-1831 for 72 h to identify cell viability. Then, the plates were treated with azoxymethane (AOM, 10^{-6} M) for seven days. Treated cells were subjected to F-actin-staining using, fluorescein isothiocyanate (FITC)-phalloidin actin staining and image analysis. The organization of the actin network was evaluated by a laser-scanning confocal system (Rainbow Radiance 2100), attached to an inverted microscope (Nikon, Bio-Rad-Zeiss). CRL-1831 cell monolayer visualization was performed by F-actin localization and nuclear staining after fixing the cells in 2% PBF solution (10 min, RT), washed in PBS, and permeabilized with 0.05% saponin and 10% FCS (30 min, RT), and incubated (30 min, RT, in the dark) in a 0.1 µM FITC-phalloidin solution (0.05% saponin and 10% FCS). Also, a double-labelling of the nuclei was performed by incubating the filters for 2 min in 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 300 nM) with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The wells were then mounted in fluorescent mounting medium (Dako), and stored in the dark at 4°C until microscopic examination. Preparations were mounted using

antifade reagent and analyzed with an inverted laser-scanning confocal microscope. Serial optical sections were processed with ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/, 1997-2011).

7.3.11. Preparation of the probiotic oral supplement

For the preparation of La-Lf probiotic supplement, pure cultures of probiotic bacteria were grown micro-anaerobically in MRS broth for 16 h (37 °C, 5% CO_2, **Figure 7.1**). The bacterial cells were grown for few passages before being harvested as pellets (8 – 10 h growth) by centrifugation (1000 x g, 15 min, 4°C), then washed twice with PBS. Probiotic pellets were diluted with saline 0.85 % (w /v) NaCl (~ pH 7) to obtain the desired concentration for a final daily dose of 1 x 10^{10} cfu [La-Lf = 0.5 x 10^{10} cfu of *L. acidophilus* ATCC 314 and 0.5 x 10^{10} cfu of *L. fermentum* NCIMB 5221]. This dose ensures efficient colonization of the colon and terminal ileum and is considered biologically relevant for an adult human (70 kg) (Dai, Zheng, Meng, Zhou, Sang,Jiang 2013; Zavisic, Petricevic, Radulovic, Begovic, Golic, Topisirovic,Strahinic 2012).

7.3.12. $Apc^{Min/+}$ mice - Experimental protocol

Adult male wild-type C57BL/6J-Apc^{Min}/J four week old mice were purchased from Charles River Laboratories (Wilmington, MA, USA). The mice were fed Constant Nutrition® formula basal rodent diet (Formulab Labdiet® 5008, ON, Canada) since admission. All mice were maintained in a temperature controlled and light-controlled facility where they consume water and food *ad libitum*. Animals were housed at 22°C temperature and humidity with a controlled 12-h light/dark cycle. Experimentation was executed with the approval of the Animal Care Committee at McGill University.

After a week of acclimatization, mice were randomized into two groups (n = 5), where the control group was administered 0.2 mL of saline, and the treatment group was given La-Lf formulation for a period 12 weeks, intragastrically by gavage. Food consumption, water intake, and animal mass were monitored weekly. At the end the experiment, the $Apc^{Min/+}$ mice were euthanized, intestines were collected, fixed and stained for further assessments (**Figure 7.6**).

7.3.13. Histopathological analysis

For tumor assessment, mice were sacrificed at 17 weeks of age by asphyxiation (CO_2). The small intestine and colon were dissected and washed with ice-cold 0.9 % NaCl solution.

The small intestine was divided while the cecum and colon were kept together. Upon removal, the intestines (~ 10 cm) were infused with 10 % phosphate-buffered formalin (PBF), then were rolled into cassettes and immersed in 10% PBF as a fixative. All membranes were dehydrated through graded alcohols and xylene, followed by embedding in paraffin. Tissue blocks were sectioned at 4 μm and stained with H&E for histological evaluation. A veterinary pathologist who was blinded to the treatments examined the sections for the presence of tumors and other lesions. Using Aperio-Imagescope image analysis software (Leica Biosystems, Concond, ON, Canada), all proliferative lesions were manually circled and the software provided the areas for each lesion surface. Other organs, including stomach, liver, lung, spleen and pancreas, were collected for detection of any abnormalities between groups.

7.3.14. Immunohistochemistry and imaging analysis

Immunohistochemical procedures were performed using automated immunostainer Intellipath FLX, from Biocare Medical (Concord, CA, USA) and monoclonal and polyclonal antibodies for E-cadherin (dilution 1:150), β-catenin (dilution 1:150), Ki-67 (dilution 1:150), and CC3 (dilution 1:150). After blocking for 15 min in dual enzyme blocking solution and 15 min in a protein blocking solution (Dako, Carpinteria, CA, USA), sections were incubated with primary antibodies for 1 hour at RT, followed by incubation with horseradish peroxidase (HRP)-bound secondary goat anti-rabbit antibody for 30 min at RT. Tissue membranes were incubated with 3,3'-diaminobenzidine (DAB, Dako) for 10 min and counterstained with hematoxylin (Carpinteria, CA, USA) for 1 min.

Slides were digitally scanned with Aperio ScanScope slide automated scanner and Aperio ScanScope Console (version 10.2.0.0) at 20 X magnification. Quantification of staining was performed using digitized images and the Aperio-Imagescope (version 6.25 software). Selected areas were analyzed for brown staining using the Aperio Imagescope software's color deconvolution algorithm, adapted to the color characteristics of the DAB and hematoxylin used. The color threshold tool in the Aperio Imagescope was used to identify DAB signal. The number of positive cells and the total number of tumor cells were counted using the Aperio Imagescope software. The index was expressed as the percentage of the number of positive cells in total and the data was plotted using GraphPad Prism 5.0 (GraphPad Inc., CA)

7.3.15. Statistical analysis

Data is presented as means ± Standard Error of the Mean (SEM). Statistical significance was generated for the treated groups as compared to each other using the one-way analysis of variances (ANOVA) with the Tukey's comparison test using SPSS statistics software package (version 20.0, IBM corporation, NY, US). P values of $P < 0.05$ were considered significant.

7.4. Results

7.4.1. Probiotic bacterial characterization - Resistance in simulated intestinal fluid

This assay was performed to verify whether the growth-related properties of the co-culture of *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314 was better than either of the individual cultures. The bacterial density in the co-culture was significantly higher ($P < 0.001$, 8 - 12 h) than each of the pure cultures of L. f 5221 and L. a 314, in MRS (1.3 ± 0.01 maximum, **Figure 7.2a**) or in SIF (0.5 ± 0.01 maximum, **Figure 7.c,** $P < 0.001$**)**. For viability in MRS, the count of L. a 314 and L. f 5221, at 12 h in the co-culture, was at 2.4 x 10^8 ± 2.8 x 10^7 cfu/mL and 5.12 x 10^8 ± 1.3 x 10^7 cfu/mL (**Figure 7.2b),** respectively. In SIF, however, the viability of L. a 314 and L. f 5221 was the highest, at 8 h in the co-culture, and was at 2.5 x 10^7 ± 4.4 x 10^6 cfu/mL and 8.3 x 10^7 ± 1.5 x 10^7 cfu/mL, **(**$P < 0.05$, **Figure 7.2d),** respectively. Overall, the probiotic co-culture La-Lf showed higher growth than separate bacteria (10h -12h, $P < 0.001$, **Figure 7.2e**).

7.4.2. Probiotic bacteria characterization - Antioxidant activity

This test verified whether *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314 as a co-culture, could be more active with no bacterial competition and have higher or equal antioxidant capacity when mixed. For culture density, in AIJ, the bacterial co-culture La-Lf was gave absorbance values between 0.4 - 0.5 compared with 0.02 - 1.3 in MRS (**Figure 7.2f**). For TAC, in standard growth conditions, bacterial co-culture (304.9 ± 35.7 µMTE, $P < 0.05$) had significantly higher TAC than pure cultures, while in AIJ, it preserved the same levels of TAC (112 ± 3.1 µMTE) as L. f 5221 and remained significantly higher than La- 314. ($P < 0.001$, **Figure 7.2g**).

7.4.3. Probiotic bacteria inhibit CRC but not normal colon cells growth and survival

To determine the effect of probiotic treatments L. a 314 and L. f 5221 and La-Lf (co culture of L. a 314 and L. f 5221), on the viability and death of CRC cells, the viable cells and dead cells were counted by Trypan Blue assay. The treatments have all negatively affected CRC proliferation at different levels (**Figure 7.3**).

Caco-2 cells treated with the CM of L. a 314 (70.7 ± 5.5 %, $P = 0.126$) had no significant growth inhibition compared with the control. Cancer cells treated with L. f5221 (38.02 ± 5.8 %, $P = 0.004$) or La-Lf (38.8 ± 6.9 %, $P = 0.009$) exhibited significant diminution in cell proliferation (**Figure 7.3a**). The effect of each treatment was evaluated by the number

of dead Caco-2 cells observed under miscroscope. Although for L. f 5221 and La-Lf treatments dead cells seemed to be higher than L. a 314 and untreated cells, data showed no significant difference between treatments (**Figure 7.3b**).

To confirm that the probiotic treatments have anti-proliferative effect against cancer cells and not normal cells, when, specifically, tested on CRL-1831 cells. The probiotic treatments L. f 5221 ($1.9 \times 10^4 \pm 8.8 \times 10^2$ cells, $P = 0.002$) and La-Lf ($1.9 \times 10^4 \pm 1.01 \times 10^3$ cells, $P = 0.001$) significantly promoted CRL-1831 cell proliferation. While L. a 314 alone ($1.00 \times 10^4 \pm 5.8 \times 10^2$ cells, $P = 0.025$) significantly altered normal colon cell growth compared with untreated cells ($1.3 \times 10^4 \pm 8.8 \times 10^2$, **Figure 7.3c).**

7.4.4. Effect of heat inactivated-probiotic treatments on CRC cells

In order to characterize the heat-resistant ability of the probiotic bacterial effectors that altered cell proliferation, the conditioned media: L. a 314 and L. f 5221 and La-Lf were heat-inactivated and compared with unmodified treatments (**Figure 7.4**).

Unmodified CM of each probiotic formulation have significantly reduced Caco-2 to 70.7 ± 5.5 % ($P < 0.05$) for L. a 314, to 38. 03 ± 5.8 % ($P < 0.001$) for L. f 5221, and to 38.8 ± 6.8 % ($P < 0.001$) for La-Lf, respectively, compared with untreated cells. Both L. f 5221 and La-Lf were significantly more efficient than L. a 314 ($P < 0.01$).

However, heat-inactivated extracts showed significantly less effect ($P < 0.001$) than unmodified extracts. Caco-2 cells treated by heat-inactivated L. a 314 (26 ± 2.9 %, $P < 0.01$) and heat-inactivated La-Lf (19.3 ± 2.4 %, $P < 0.01$) had significantly proliferated more than heat-inactivated control (4.7 ± 2.1 %) and heat-inactivated L. f 5221 (0.6 ± 0.04 %, **Figure 7.4a).**

To determine if growth inhibition was linked to induction of cell death, cell apoptosis induced by probiotic treatment was assessed in Caco-2 cells. For non-modified treatments, the CM of L. a 314 (194.8 ± 38.8 RUL, $P < 0.05$), L. f 5221 (194.8 ± 38.8 RUL, $P < 0.001$) and La-Lf (412.5 ± 41.5 RUL, $P < 0.001$) induced significantly more apoptosis than the control. The CM containing *L. fermentum* NCIMB 5221 extract: L. f 5221 ($P < 0.05$) and La-Lf ($P < 0.001$) were more effective in inducing apoptosis compared with L. a 314.

Cancer cells treated with heat-inactivated *L. a 314*-containing CM: L. a 314 (566.6 ± 16.7 RUL) and La-Lf (611.5 ± 27.5 RUL) have undergone less apoptosis than the control (803.3 ± 12.6 and Lf 5221 (611.5 ± 27.5 RUL, $P < 0.001$, **Figure 7.4b**).

7.4.5. Effect of proteinase K-treated probiotic extracts against CRC cells

For each probiotic treatment, the protein content in the CM was inhibited by 10, 50, and 100 μg/mL of proteinase K before incubation with Caco-2 cells. When treated with 50 – 100 ng/mL of proteinase K, the growth of Caco-2 cells was inhibited almost completely in all groups, which indicates that the proteins essential for the maintenance of cell growth were removed (**Figure 7.4c and 7.4d**). Thus, this high concentration was not used to analyze the effect of the treatments. Interestingly, only in the case of the proteinase K treatment of 10 ng/mL, the control was significantly different from treatments (**Figure 7.4c**). The CM containing 10 ng/mL of proteinase K-treated L. a 314, L. f 5221 and La-Lf significantly reduced cell viability to 7.4 ± 1.4 %, 2.3 ± 0.4 %, and 3.2 ± 0.8 % ($P < 0.001$), respectively, compared with control (10 ng/mL of proteinase K-treated DMEM) or compared with their corresponding unmodified probiotic treatments. For cell death, the same treatments 10 ng/mL of proteinase K-treated L. a 314 (769.4 ± 9.7 RUL), L. f 5221 (804.5 ± 3.2 RUL), and La-Lf (796.5 ± 4.9 RUL) had significantly induced more apoptosis than the 10 ng/mL of proteinase K-treated control (191.01 ± 67.3 RUL, $P < 0.001$). Moreover, they had significantly limited proliferation and induced cell death in Caco-2 cancer cells more efficiently than unmodified probiotic treatments ($P < 0.001$, **Figure 7.4d**).

7.4.6. Effect of filtered probiotic extracts against CRC cells

To characterize the active component in bacterial extract in size, the probiotic treatments: L. a 314 and L. f 5221 and La-Lf were subject to filtration using a 30 MWCO centrifugal filters. The filtrate obtained after centrifuging the probiotic treatments were evaluated for their anti-proliferative and apoptotic effects on Caco-2 cells (**Figure 7.4e and 4f**).

The CM of L. a 314, L. f 5221 and La-Lf (after 30 MWCO filtration) significantly reduced the cell viability to 23.1 ± 2.8 %, 24.5 ± 2.2 % and 20.6 ± 2.3 %, respectively, compared with control (DMEM with 30 MWCO, 66.8 ± 14.7 %, $P < 0.001$). For cell death, the 30 MWCO treatment of L. a 314 (910.3 ± 45.8 RUL), L. f 5221 (910.3 ± 45.8 RUL) and La-Lf (1038.5 ± 35.1 RUL) significantly increased apoptosis compared with control ($P < 0.001$). After 30 MWCO, all probiotic CM significantly limited proliferation and enhanced cell death in Caco-2 cells compared with unmodified treatments ($P < 0.001$, **Figure 7.4f**).

7.4.7. Probiotic extracts protected normal colon cells from azoxymethane

After treatment with L. a 314 and L. f 5221 and La-Lf, CRL-1831 cells were exposed to the carcinogen AOM. Following F-actin and nuclei staining, cell images were analyzed by software to investigate whether prior incubation with probiotic treatments provided protection from CRL-1831 cells from carcinogen alteration **(Figure 7.5b).** After evaluation of cell size, count and area on images (**Figure 7.5a)**, data showed that there was no significant change in the overall area of all cells. While for cell count, La-Lf was the only formulation that significantly increased digital cell count compared with probiotic-untreated cells ($P < 0.01$). Both La-Lf (78.5 ± 10.1 cells, $P < 0.01$) and L. f 5221 (67 ± 9.6 cells, $P < 0.05$) significantly increased cell count compared with L. a 314 (38.2 ± 9.5 cells, **Figure 7.5a**). Similarly, for the percentage of cell area, La-Lf was the only formulation that significantly increased the percentage of cell area per image surface compared with probiotic-untreated cells ($P < 0.05$). After AOM exposure, CRL-1831 normal cells treated with La-Lf (0.8 ± 0.1 %, $P < 0.01$) and L. f 5221 (0.7± 0.2 %, $P < 0.05$) had significantly higher total cell area compared with L. a 314 (0.4 ± 0.2 ± %, **Figure 7.5a**).

7.4.8. Animal weight, food and water intake after probiotic supplementation

For the effect of oral administration of the selected La-Lf probiotic formulation on body mass and dietary intake, food and water consumption and animal weight were monitored throughout the 12 weeks' period. (**Figure 7.6**). In both groups, the animals gained body weight steadily (**Figure 7.7**). At the end of the treatment period, there was no significant difference in body mass between the saline and the probiotic group. However, in the last the six weeks, a significant difference in food (**Figure 7.7c,** $P < 0.05$) and water (**Figure 7.7d,** $P < 0.05$) consumption between the treated and the control groups was observed, where La-Lf-fed mice significantly consumed more food and water on average. However, none of the animals fed with La-Lf produced any gross changes in collected organs (pancreas, liver, lung, and kidney).

7.4.9. La-Lf formulation may reduce tumor burden

Oral administration of an active formulation containing both *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 live bacterial cells to $Apc^{Min/+}$ mice suppressed intestinal polyp formation close to 40% (24.8 ± 6.1 tumors per mouse, $P = 0.016$, **Figure 7.8b**) when compared with the control saline group (43.0 ± 4.7 tumors/mouse). This difference

accounted for about 1.7-fold reduction in the intestinal polyps formation. For total tumor area (P = 0.152, **Figure 7.8a**) and mean tumor area, there was no significant difference observed (P = 0.982, **Figure 7.8c**). For $Apc^{Min/+}$ mice treated with La-Lf, total tumor area (23.9 ± 4.4 mm^2, P = 0.152) was slightly smaller than saline group (32.6 ± 7.7 mm^2), but no significance was detected (**Figure 7.8a**).

7.4.10. Effect of La-Lf oral administration on β-catenin and E-cadherin protein levels in ApcMin/+ mice normal and tumor intestinal tissues

As aberrant β-catenin signaling is a key molecular event in the development of tumors in $Apc^{Min/+}$ mice, the first target of our investigation was β-catenin expression in both normal and tumorous tissue of the intestines, using immunohistochemistry (**Figure 7.9**).

As illustrated in **Figure 7.9f**, it appeared that some cells at the base of crypts displayed cytoplasmic and sporadic nuclear stain, while the staining of normal epithelia in the upper crypts showed a distinctive membranous staining of β-catenin. In the saline group, the adenomas had enhanced nuclear and cytoplasmic staining, but reduced membranous β-catenin staining. Whereas in the normal crypt from mice treated with La-Lf (46.2 ± 3.4 %, P = 0.041), nuclear staining of β-catenin significantly was reduced compared with saline group (54.7 ± 0.9 %, **Figure 7.9d)**. In normal crypt from the treated group, the intensity of cytoplasmic staining was restored**.** ß-catenin expression was less in tumors, but no significance was observed (P = 0.728, **Figure 7.9e).** These data suggest that the aberrant β-catenin signaling in the tumors was suppressed by La-Lf administration. Although, E-cadherin regulation is a possible upstream event for β-catenin signaling (Wijnhoven, Dinjens,Pignatelli 2000), for E-cadherin protein levels (**Figure 7.9c**), the treatment with La-Lf downregulated the nuclear staining intensity or positive-staining cells in the normal crypt (**Figure 7.9a**) or tumor (P = 0.225, **Figure 7.9b**), but not significantly.

To determine the effect of the oral administration of live probiotic cells La-Lf to $Apc^{Min/+}$ mice on cell proliferation and apoptosis, antibodies against Ki-67 and CC3 were used. Strong Ki-67 expression was located mostly in the lower part of crypts (**Figure 7.9l**). In the La-Lf treated group, the Ki-67 staining was decreased in tumors and normal crypts (**Figure 7.9j and 7.9k**). In the normal crypt, the proliferation index of La-Lf group (11.1 ± 1.3 %, P = 0.008) was significantly lower as compared with the proliferation index from the saline group (16.6 ± 0.8 %). La-Lf probiotic treatment (27.8 ± 2.5 %, P = 0.012) has

significantly reduced Ki-67 expression in intestinal tumors, compared with animal receiving saline only (38.4 ± 2.2 %, **Figure 7.9k**). Nevertheless, apoptotic cells, Caspase-3 positive, were observed in tumors, while no appreciable change was detected in normal mucosa (P = 0.022, **Figure 7.9g**). The number of apoptotic cells in tumor cells between La-Lf treated mice and saline treated mice had no significant difference (P = 0.392, **Figure 7.9h**).

7.5. Discussion

Supplementation with live probiotic bacteria is one of the strategies to improve the balance of gut flora and provide the host with an additional wall of defense against the risk of colorectal neoplastic development. Oral digestion of certain *Lactobacilli* could shift the bacterial gut populations, the most abundant body of microbes in the human GI tract, towards a healthier composition (Kahouli, Malhotra, Alaoui-Jamali,Prakash 2015; Kahouli, Malhotra, Tomaro-Duchesneau, Rodes, Aloui-Jamali,Prakash 2015a; Kahouli, Tomaro-Duchesneau,Prakash 2013a; Macfarlane and Macfarlane 2012). The health beneficial effect of intestinal *lactobacilli* in the host microflora (Maghsoudi, Zakeri,Lockshin 2012) and its importance for animal and human health (Fuller 1992) have been reported. Epidemiological and experimental studies have suggested that *Lactobacilli* may exert substantial health-promoting effects to the host and reduce the risk and may play an important role in CRC prevention (Rafter 2003).

To our knowledge, this is the first study investigating the *in vitro* **(Figure 7.1)** and *in vivo* (**Figure 7.6**) effect of probiotic combinations using live bacterial cells of *L. acidophilus* ATCC 314 mixed with *L. fermentum* NCIMB 5221. The bacterial treatments were screened and characterized for their anti-proliferative, apoptotic activity against CRC cells, beneficial effect on normal colon cells, as well as, anti-tumorigenic effect in a genetically modified CRC mouse model. This type of study might be important for a preliminary selection, formulation and characterization of a potential probiotic mixture in an animal model of CRC.

Our data suggest that a combination of both *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 have more potent anti-cancer potential than individually. Prepared *in vitro* as cell-free extract, this formulation showed the ability to inhibit cancer cells but not normal colon cells, and may have protected them from damaging effect of a carcinogen. The active compound in the extracts appeared to be heat sensitive, with a size below 30 kD. Later, administered as live free cells, this formulation reduced tumor enumeration in the intestines of $Apc^{Min/+}$ mice, which seemed to be correlated with a reduced expression of cellular proliferation markers Ki-67 and β-catenin.

For *in vitro* screening and characterization, probiotic CM were prepared from L. a 314, L. f 5221 bacterial cultures and the third from a co-culture of both L. a 314 and L. f 5221 (La-

Lf, **Figure 7.1**). La-Lf co-culture have shown higher density (**Figure 7.2a and 7.2c**) and viability (**Figure 7.2b and 7.2d**) than pure cultures in SIF or AIJ. In addition, La-Lf co-culture has shown higher or similar density (**Figure 7.2a**) and antioxidant capacity (**Figure 7.2b**) as the pure cultures, in artificial juices. This will allow characterizing the antioxidant capacity of the probiotic formulation before being administered to animals as was done in other studies (Tomaro-Duchesneau, Saha, Malhotra, Jones, Labbé, Rodes, Kahouli,Prakash 2014). Results suggest that the combination of both probiotic bacteria might produce a more active culture with higher resistance and superior metabolic activity once digested through the gut, than individual bacteria, as summarized in **Table 7.1S**. Add analysis for **Figure 7.2f and 7.2g**

Only the formulations containing *L. fermentum* NCIMB 5221 (L. f 5221 and La-Lf) reduced viable Caco-2 cancer cell count and increased viable count of CRL-1831 normal cells (**Figure 7.3**). L. a 314 had no effect on cancer cells and reduced viable cell count of normal colon cells. Some studies showed the ability of probiotic extract or butyrate to kill cancer cells but not non-neoplastic colon cells (Scheppach, Luehrs, Melcher, Gostner, Schauber, Kudlich, Weiler,Menzel 2004). While, others demonstrated that some probiotic strains have inhibited the proliferation of both cancer and normal cells, *in vitro,* which makes it non-specific to the anti-cancer action but rather emphasizes on the cytotoxic effect (Sadeghi-Aliabadi, Mohammadi, Fazeli,Mirlohi 2014). Interestingly, L. a 314 did not show any significant anti-cancer effect, however, in a formulation with L. f 5221, the total extract seemed to be more effective, possibly due to a possible synergistic effect ($P < 0.05$).

Next, each of the above formulations was modified/treated, then tested on cancer cells for viability and apoptosis to identify the nature of the active components acting against cancer cell proliferation, as similarly done by Ma et al, (Ma, Choi, Choi, Pothoulakis, Rhee,Im 2010). Before heat-inactivation, the treatments containing *L. fermentum* NCIMB 5221 (L. f 5221 and La-Lf) were the superior at killing and inhibiting cancer cells, while L. a 314 was less potent (**Table 7.2S**). Conversely, after heat-inactivation, extracts containing *L. acidophilus* ATCC 314 (L. a 314 and La-Lf) promoted cancer cell proliferation and minimized apoptosis, suggesting that *L. acidophilus* ATCC 314 produced heat-resistant factors that enhance cell growth in general (**Figure 7.4a and 4b**). The effective anti-cancer factor produced, however, is not heat stable and is possibly a DNA bacterial product. Some

studies have explained that heating probiotic products, containing immunostimulatory DNA as active component, failed to have the same effect as untreated ones. Other published works have provided evidence showing that bacterial DNA could be behind the probiotic beneficial effects (Laudanno, Vasconcelos, Catalana,Cesolari 2006; Rachmilewitz, Katakura, Karmeli, Hayashi, Reinus, Rudensky, Akira, Takeda, Lee, Takabayashi,Raz 2004).

When treated with 10 ng/mL of Proteinase-K, cancer cells were able to proliferate in the modified controlled environment, similarly to the unmodified one, and cancer cells with probiotic CM were inhibited. Thus, after the inhibition of a certain amount of proteins, the anti-cancer compounds were observed to be more effective. This effect is possibly due to the enhanced absorption of anti-cancer compounds by cancer cells (**Figure 7.4 b and 4c**). When protein of more than 30 kD in size were removed by filtration, the effectiveness of L. f 5221 and La-Lf was unchanged (**Figure 7.4e and 4f**), which demonstrates that the major active anti-cancer components were at a similar size than 30 kD in size. The sum of these observations (**Table 7.2S**) shows that the active factors responsible for cancer cell death in the bacterial supernatant could be described as soluble, heat sensitive effectors such as fatty acids or other proteins. These factors were able to induce cell death mostly through induction of apoptosis and they can be identified as TLR ligands or metabolic products, such as SCFAs, that affect cell signaling and interferes with cell cycle and proliferation (Sartor 2004). On top of SCFAs (Isono, Katsuno, Sato, Nakagawa, Kato, Sato, Seo, Suzuki,Saito 2007) and bacteriocins (Lewus and Montville 1991), studies have reported probiotic extracts to contain phospholipids (Frick, Schenk, Quitadamo, Kahl, Koberle, Bohn, Aepfelbacher,Autenrieth 2007) and proteins, such as p40 and p75, that induced increased Akt activation, inhibition of cytokine-induced epithelial cell apoptosis, and growth promotion (Yan, Cao, Cover, Whitehead, Washington,Polk 2007). Similarly to this assay, probiotic bacteria was found to act through TRAIL apoptotic action and reduce colon cancer cell proliferation (Cousin, Jouan-Lanhouet, Théret, Brenner, Jouan, Le Moigne-Muller, Dimanche-Boitrel,Jan 2016).

It was left to determine which one of these probiotic treatments could better protect against carcinogens exposure and the progression of neoplastic growth in the colonic epithelium. In the previous assay, normal epithelial colon cells CRL-1831 treated with La-Lf had

proliferated significantly healthier than other treatments ($P < 0.05$, **Figure 7.5**). This demonstrates that probiotic CM prepared from a co-culture of both *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314 produced more effective active compounds (quantitatively or/and qualitatively) that support the proliferation of non-neoplastic colorectal cells, as previously suggested (Belcheva, Irrazabal,Martin 2015). In addition, La-Lf may have been contracting the toxic effect of AOM by potentially preventing the rearrangement of actin cytoskeletton (**Figure 7.5b**) and the degradation of tight junction proteins as explained by Lindfors et *al*., (Lindfors, Blomqvist, Juuti-Uusitalo, Stenman, Venäläinen, Mäki,Kaukinen 2008). Overall, La-Lf demonstrate a better alternative with its greater protective effect on normal colon cells and inhibitory activity against cancer cells.

The *APC* tumor suppressor is the most commonly altered gene in CRC, and the genetic and epigenetic alterations may be associated with dietary and lifestyle risk factors for CRC (Gay, Mitrou, Keen, Bowman, Naguib, Cooke, Kuhnle, Burns, Luben,Lentjes 2012), making the $Apc^{Min/+}$ mouse CRC model a suitable model to evaluate La-Lf probiotic active formulation (Chu, Esworthy, Chu, Longmate, Huycke, Wilczynski,Doroshow 2004; Kettunen, Kettunen,Rautonen 2003). After *in vitro* characterization of *L.* f 5221 and *L.* a 314 synergistic action, the potential of this probiotic mixture was investigated in the context of an animal trial (**Figure 7.6).** We have found that the animals fed with this formulation exhibited a fewer occurrences of intestinal adenocarcinomas ($p < 0.005$, **Figure 7.8**) through the alteration of a number of cellular proliferation biomarkers. Although no significant differences were found in tumor area and total tumor area between groups, we observed an interesting trend where both parameters were lower in La-Lf treated animals.

The $Apc^{Min/+}$ mice demonstrate a predisposition to multiple intestinal neoplasias (Min). They carry a mutation in the tumor suppressor gene, *APC* that leads to aberrant activation of the Wnt/β -catenin signaling pathway in the colonic epithelium (Gregorieff and Clevers 2005). A mutation which is recognized as the initiating event leading to tumor formation, β-catenin has been shown to participate in many key processes maintaining normal cell function and microarchitecture of the epithelia in the intestine. In a mouse model of colonic hyperplasia, increased cytosolic and nuclear expression of β-catenin was associated with hyperproliferation, as well as with increased steady-state levels of c-myc and cyclin D1 (Sellin, Umar, Xiao,Morris 2001; Sparks, Morin, Vogelstein,Kinzler 1998).

Overexpression of β-catenin is known to be an oncogenic event in the intestinal tract leading to dysplasia and adenomatous polyps in the small and large intestines (Harada, Tamai, Ishikawa, Sauer, Takaku, Oshima,Taketo 1999; Romagnolo, Berrebi, Saadi-Keddoucci, Porteu, Pichard, Peuchmaur, Vandewalle, Kahn,Perret 1999). Interestingly, La-Lf probiotic treatment significantly reduced β-catenin expression ($P < 0.05$, **Figure 7.9d-f**), which would affect the E-cadherin/β-catenin complex, and thus reinforce the barrier function of GI epithelial cells (Hummel, Veltman, Cichon, Sonnenborn,Schmidt 2012). Thus, La-Lf oral administration could have reduced tumor burden by the suppression of transcriptional activation of β-catenin (Kettunen, Kettunen,Rautonen 2003), promotion of cell differentiation, and restoring cell polarity in CRC cells (Mariadason, Bordonaro, Aslam, Shi, Kuraguchi, Velcich,Augenlicht 2001; Naishiro, Yamada, Takaoka, Hayashi, Hasegawa, Imai,Hirohashi 2001). These findings make the prevention of β-catenin cellular accumulation a possible mechanism for La-Lf in CRC prevention (Pajari, Rajakangas, Päivärinta, Kosma, Rafter,Mutanen 2003). Similar to findings of other studies, the addition of probiotics to the diet induced a significant reduction in the incidence of colon tumors with altered β-catenin expression in both tumors and "normal-appearing" crypts (de Moura, Caetano, Sivieri, Urbano, Cabello, Rodrigues,Barbisan 2012).

Furthermore, the probiotic formulation La-Lf had significantly attenuated Ki-67, an antigen not expressed in quiescent or resting cells in the G_0-phase and an excellent operational marker for determining cell proliferation and the aggressiveness of tumors (Xiao, Zhao, Zhao, Zheng, Gou, Takano,Zheng 2013). Our data revealed that Ki-67 was disturbed in both intestinal adenomas and non-neoplastic tissues after La-Lf probiotic intervention ($P < 0.05$, **Figure 7.9 (j-l)**). This suggest that, in addition to inhibiting tumor cells from further proliferation, La-Lf acted by preventing normal healthy intestinal tissues from undergoing neoplastic transformation that begins with cell overgrowth. In agreement with this result, other studies have related probiotic anti-carcinogenic activity to an inhibition of Ki-67 and β-catenin expressions. In contrast, expression of an important biomarker of the Wnt signaling pathway (Hummel, Veltman, Cichon, Sonnenborn,Schmidt 2012), E-cadherin was unaffected by La-Lf treatment **(Figure 7.9 (d-f)**). On the other hand, CC3, a protein that is activated directly by caspase-8, -9 and -10 in apoptotic cells to initiate apoptosis (Xiao, Zhao, Zhao, Zheng, Gou, Takano,Zheng 2013), was reduced but not significantly

changed in tumors **(Figure 7.9 (g-i))**. Nonetheless, it seemed to be slightly inhibited (P = 0.022), in normal-appearing tissues of $Apc^{Min/+}$ mice, suggesting La-Lf involvement in preserving the integrity of intestinal epithelial barrier **(Figure 7.9 (g-i))**. In consonance with these findings, previous studies confirmed that some probiotics such as *L. rhamnosus* LGG (Lin, Nasr, Berardinelli, Kumar,Neish 2008) and VSL#3 (Mennigen, Nolte, Rijcken, Utech, Loeffler, Senninger,Bruewer 2009) suppress intestinal epithelial apoptosis in cultured cells and reduced chemically induced epithelial apoptosis *ex vivo*, when orally administered to live animals.

Some of the particular differences in the administration of oral probiotic treatments may present a relevant factor in the efficacy of the treatment and the mechanism of action involved. Several assays were conducted to examine and improve *L. acidophilus* ATCC 314 or *L. fermentum* NCIMB 5221 survival in GI conditions/fluids by microencapsulation which is considered a targeted delivery of these cells to the colon that is heavily inhabited with gut flora compared to the stomach and small intestines. However, the administration of free probiotic cells is, in reality, a flooding of the upper intestines, the least inhabited with commensal bacteria, with probiotic bacteria assuming a surviving number of resistant cells will reach the colon and induce an effect. Studies differentiating both probiotic-delivery strategies are lacking, based on which it would be possible to suggest studies with both microencapsulated and probiotic free cells for an enhanced biotherapeutic effect that acts within different part of the gut.

In this light, formulating and characterizing the combination of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221, *in vitro,* suggested they may exert a significant synergistic beneficial effect against CRC initiation and progression by releasing enhanced levels of anti-neoplastic compounds, inducing cancer cell death, and maintaining a healthy mucosa. It is noteworthy, that this study demonstrated that biotherapeutic supplementation using live active *L. acidophilus* ATCC 314 mixed with *L. fermentum* NCIMB 5221 have prevented the formation of more spontaneous intestinal polyposis in $Apc^{Min/+}$mice through the downregulation of important tumor proliferation markers. Our findings may represent a first insight and a preliminary ground to future investigations in the exact mechanisms of La-Lf oral formulation. Nonetheless, we can still speculate that the mechanisms of action (**Figure 7.10**), in this study, could include increase in the production of beneficial

675 postbiotics (Kliegman and Willoughby 2005), as well as the intensity and the complexity
676 of probiotic-epithelial interactions. Like this, the oral administration of La-Lf formulation
677 can be envisaged as a potential nutraceutical towards the prevention or treatment of CRC.
678 Further trials are warranted to understand more of the mechanisms by which this probiotic
679 therapy exerts its biotherapeutic effect.

7.6. Acknowledgements

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7.7. Conflicts of interest

The authors have no conflicts of interest to disclose.

7.8. Figures

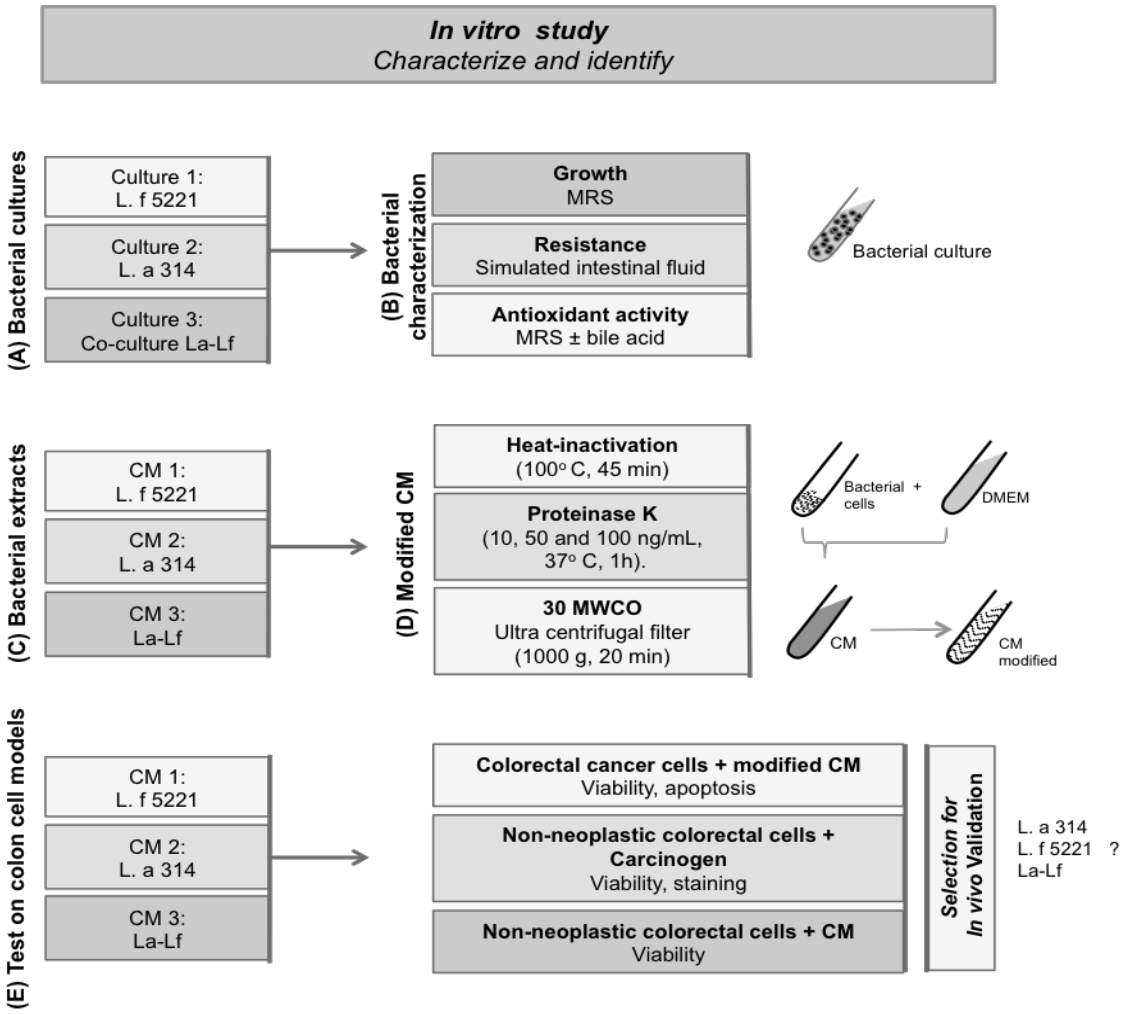
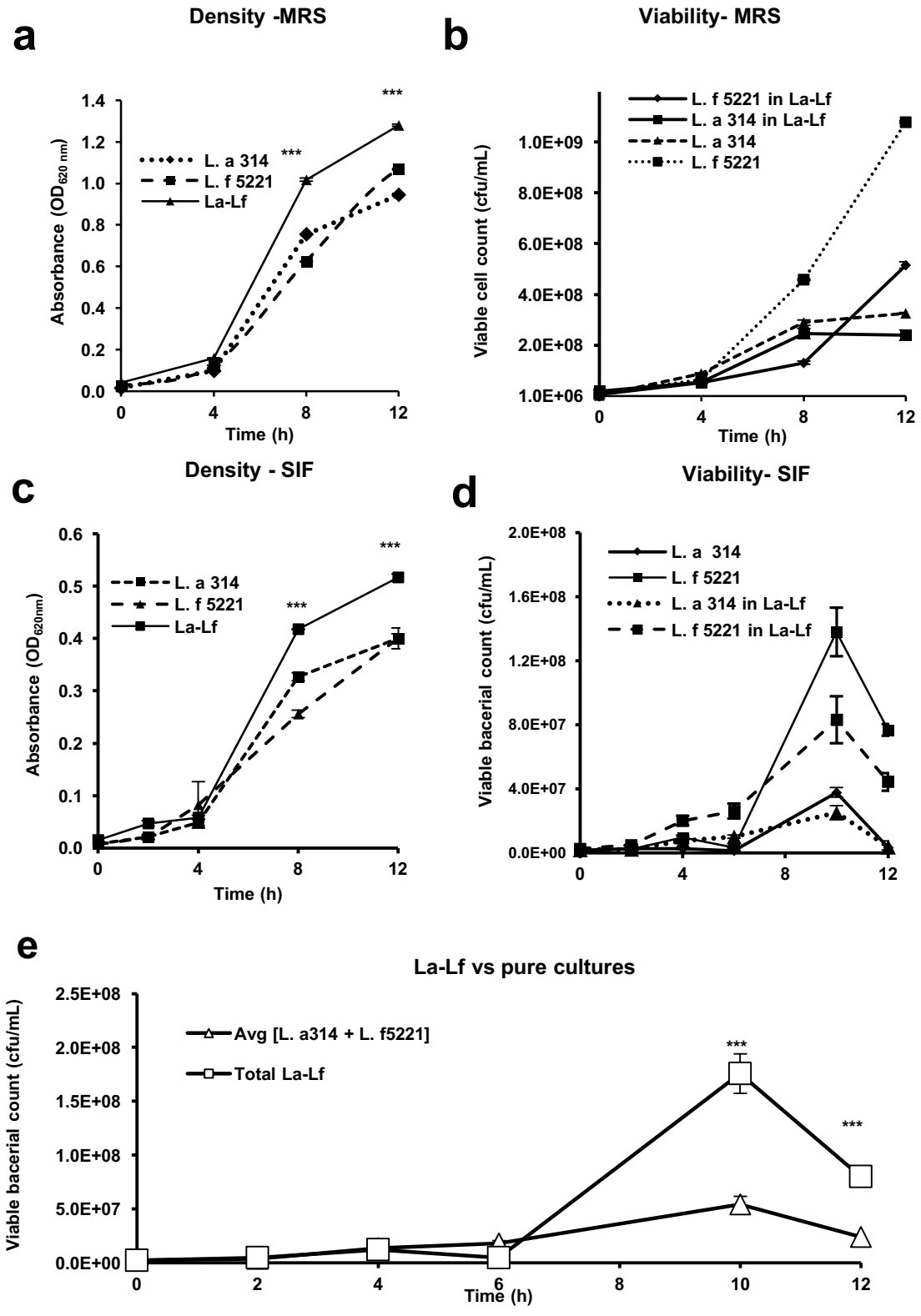


Figure 7.1: Outline and details of the *in vitro* experimentations - Characterization of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 formulations, using chemical and physical modifications*.

The main interest is to determine if the combination of both *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 have a greater (**A and B**) metabolic bacterial activity growth kinetic, antioxidant activity, and resistance) and higher (**E**) anti-CRC action (anti-proliferative and pro-apoptotic). This will allow later for validation of anti-tumorigenic effect *in vivo* (detailed in **Figure 7.9)**. These tests were designed to identify the features of the bacterial cultures (1, 2, and 3) and their extracts in different settings, including resistance in intestinal conditions, total antioxidant capacity, and finally the nature of active metabolites in the cell culture conditioned medium (CM). *(**C and D**) The chemical and physical modifications, represented by heat inactivation, proteinase K treatment, and 30 MWCO, were performed on all probiotic CM and tested on CRC cell proliferation.


a
Density -MRS
Absorbance (OD620 nm)
L. a 314
L. f 5221
La-Lf
***
Time (h)
b
Viability- MRS
Viable cell count (cfu/mL)
L. f 5221 in La-Lf
L. a 314 in La-Lf
L. a 314
L. f 5221
Time (h)
c
Density - SIF
Absorbance (OD620nm)
L. a 314
L. f 5221
La-Lf
***
Time (h)
d
Viability- SIF
Viable bacerial count (cfu/mL)
L. a 314
L. f 5221
L. a 314 in La-Lf
L. f 5221 in La-Lf
e
La-Lf vs pure cultures
Viable bacerial count (cfu/mL)
Avg [L. a314 + L. f5221]
Total La-Lf
***
Time (h)

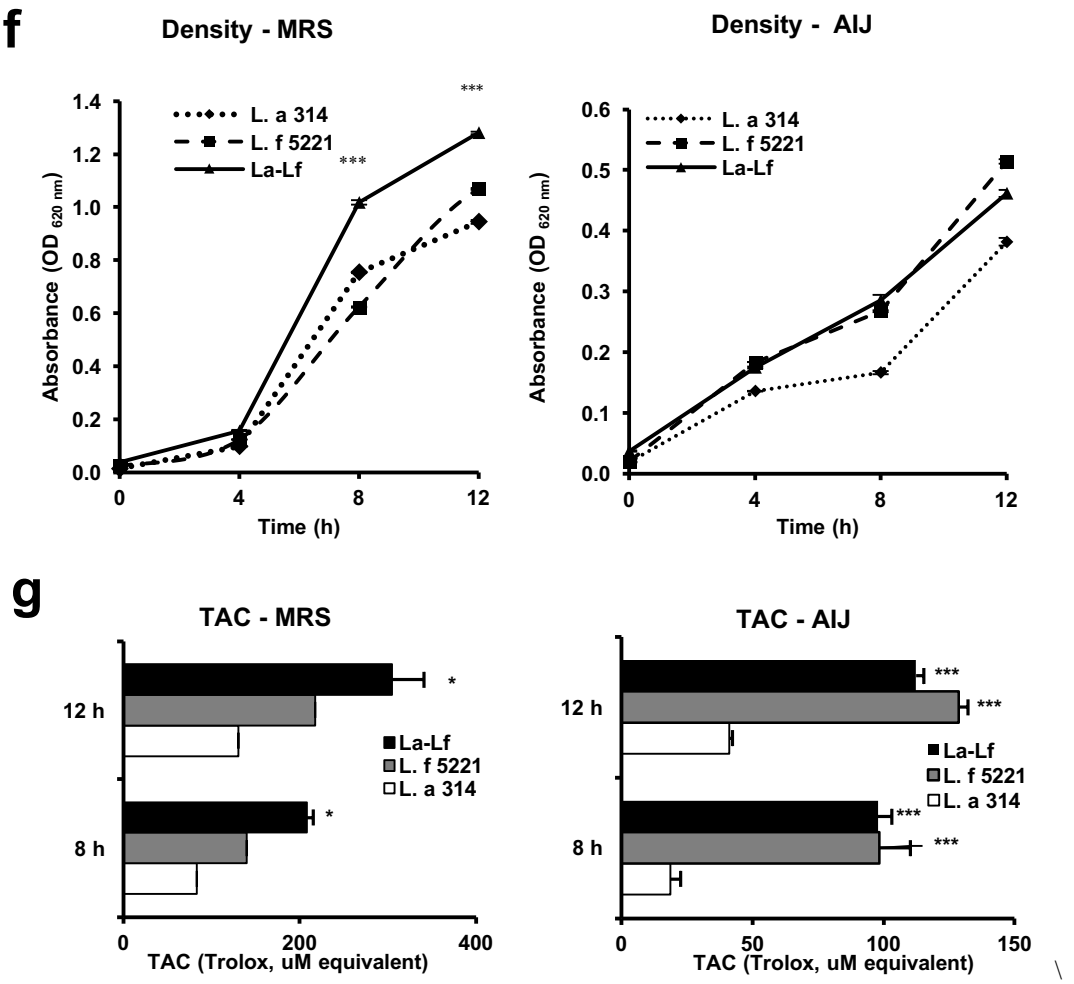


Figure 7.2: Total antioxidant capacity (TAC) and bacterial growth and survival profiles of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221, separate or mixed, in simulated intestinal juices.

The characterization of probiotic bacteria was based on the density, on the viable cell count, and on the survival of bacteria in each culture. Monitoring of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 pure cultures and co-culture in **(a)** MRS in **(b)** SIF. **(e)** To compare growth kinetics of La-Lf co-culture to both pure cultures, the total number of viable cells in La-Lf was compared to the average number of cell in L. a 314 culture and L. f 5221 culture. Data are presented as mean ± SEM (n = 4). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared L. a 314 or L. f 5221. **(f)** Bacterial cell density and **(g)** TAC produced by *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221, in pure or co-culture, in MRS and AIJ. Data are presented as mean ± SEM (n = 4). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with unmodified treatments. L. a 314: *L. acidophilus* ATCC 314; L. f 5221: *L. fermentum* NCIMB 5221; La-Lf: the co-culture of L. a 314 and L. f 5221; TAC: Total antioxidant capacity

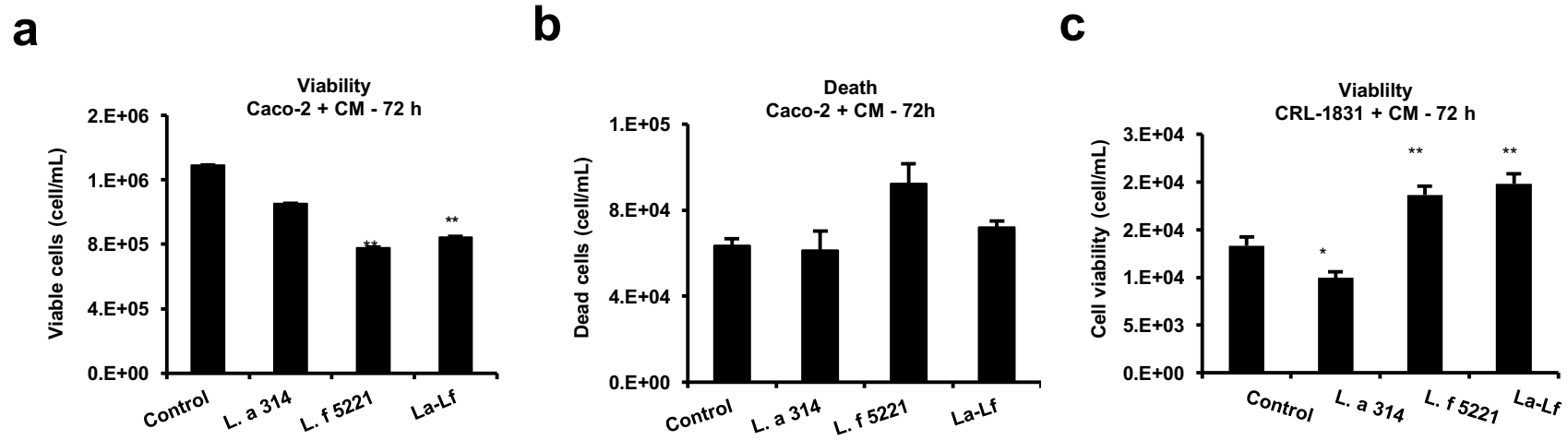
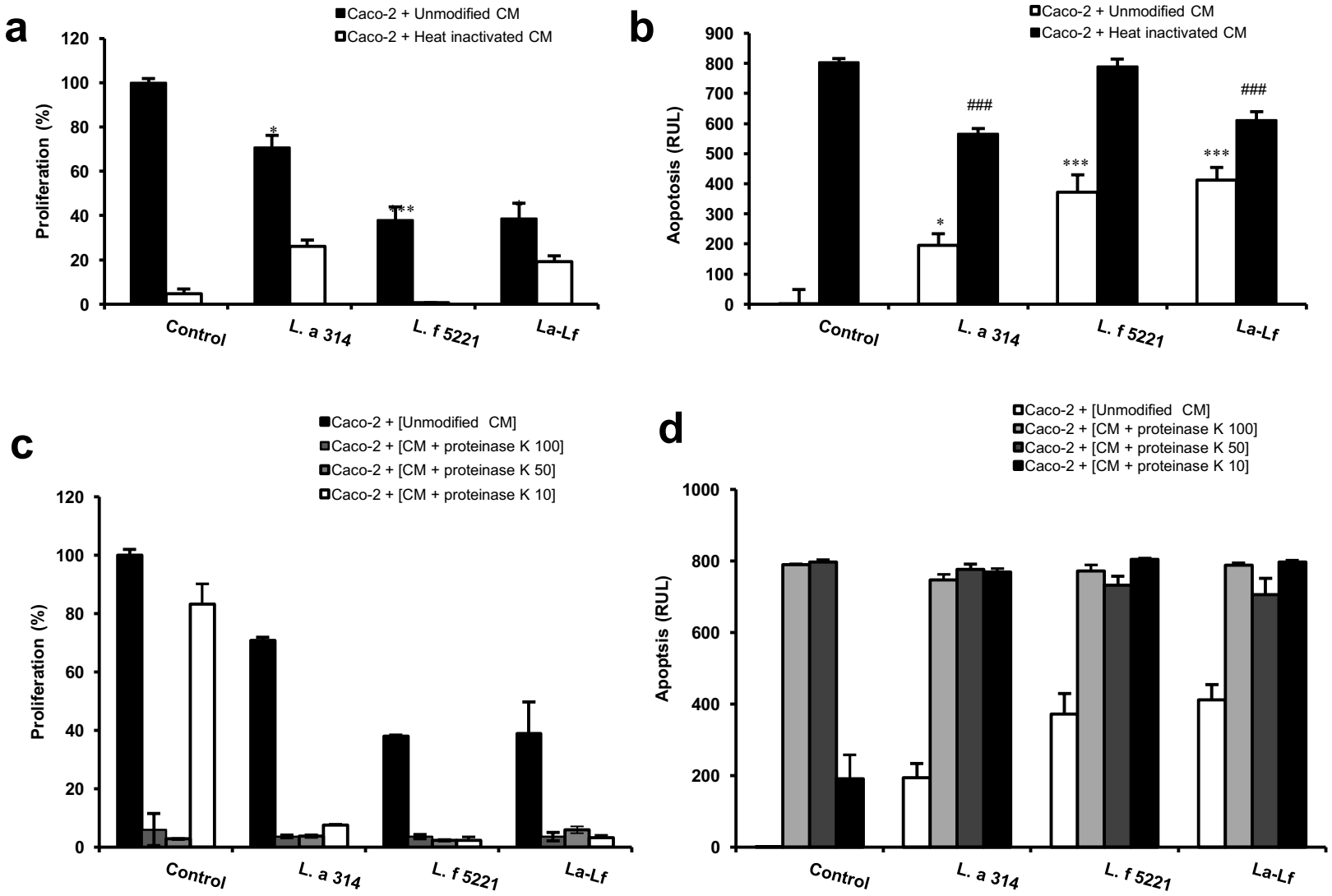


Figure 7.3: Study of the anti-proliferative activity of *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 and their co-culture against colon cancer and normal colon cells.

The count of **(a)** live and **(b)** dead Caco-2 CRC cells and **(c)** viable CRL-1831 colon normal cells treated with the conditioned cell culture medium (CM) of *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 and their co-culture, for 72 h. Data are presented as mean ± SEM (n = 4). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with control. L. a 314: *L. acidophilus* ATCC 314; L. f 5221: *L. fermentum* NCIMB 5221; La-Lf: co-culture of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221


a
Caco-2 + Unmodified CM
Caco-2 + Heat inactivated CM
Proliferation (%)
120
100
80
60
40
20
0
*
**
Control
L. a 314
L. f 5221
La-Lf
b
Caco-2 + Unmodified CM
Caco-2 + Heat inactivated CM
Aopotosis (RUL)
900
800
700
600
500
400
300
200
100
0
*
###
***
***
###
Control
L. a 314
L. f 5221
La-Lf
c
Caco-2 + [Unmodified CM]
Caco-2 + [CM + proteinase K 100]
Caco-2 + [CM + proteinase K 50]
Caco-2 + [CM + proteinase K 10]
Proliferation (%)
120
100
80
60
40
20
0
Control
L. a 314
L. f 5221
La-Lf
d
Caco-2 + [Unmodified CM]
Caco-2 + [CM + proteinase K 100]
Caco-2 + [CM + proteinase K 50]
Caco-2 + [CM + proteinase K 10]
Apoptsis (RUL)
1000
800
600
400
200
0
Control
L. a 314
L. f 5221
La-Lf

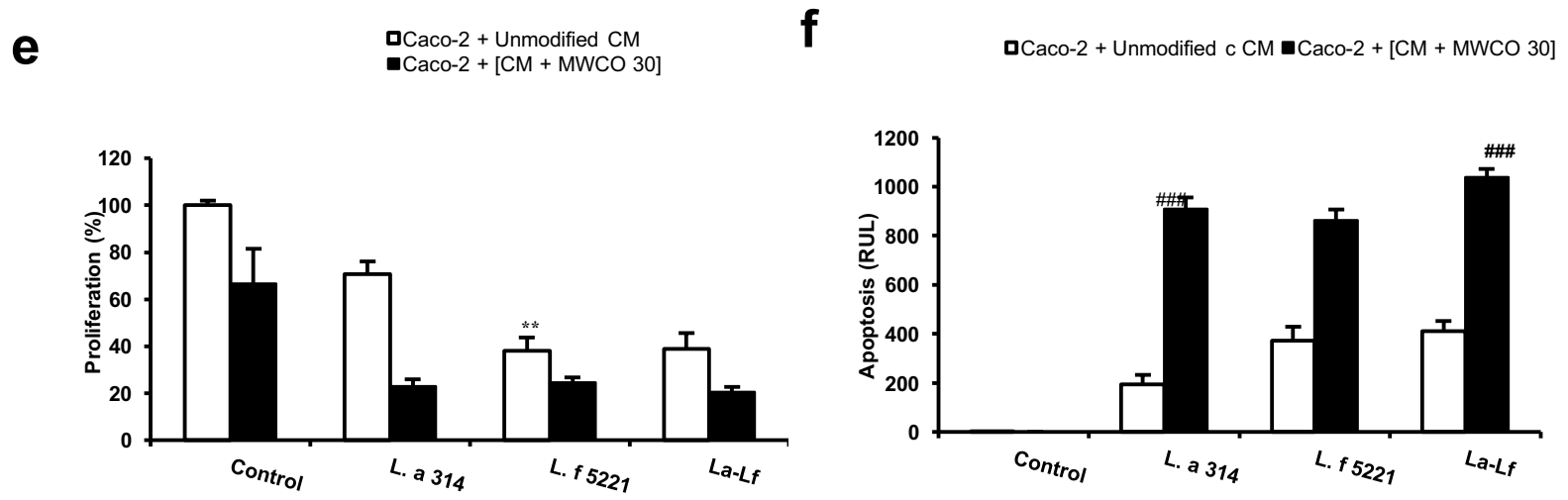


Figure 7.4: The efficacy of different probiotic formulations containing L. acidophilus ATCC 314 and/or L. fermentum NCIMB 5221 before and after physical and chemical modifications.

The conditioned cell culture media (CM) of each probiotic formulation for *L. acidophilus* ATCC 314 and/or *L. fermentum* NCIMB 5221 were subject to a heat inactivation treatment, proteinase K treatments, or a molecular weight cut-off of 30 kD (MWCO 30). The anti-proliferative effect **(a)** and apoptosis induction **(b)** of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 formulations against CRC cells (Caco-2) were evaluated compared with untreated cells and cells treated with unmodified probiotic CM. **(c)** The proliferation and **(d)** apoptosis of Caco-2 cells at 72 h of treatment with proteinase K modified CM. Probiotic CM was treated with different concentration of proteinase K: 100, 50 and 10 ng/mL for 1 h at 37^{o}C. The probiotic CM of *L. acidophilus* ATCC 314 and/or *L. fermentum* NCIMB 5221 had a MWCO 30, then were incubated for 72 h with Caco-2 CRC cells. (**e**) After treatment with those modified CM, cell proliferation and (**f**) apoptosis in cancer cells were identified. Data are presented as mean ± SEM (n = 4). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with unmodified treatments. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$, compared with heat inactivated treatments. L. a 314: *L. acidophilus* ATCC 314; L. f 5221: *L. fermentum* NCIMB 5221; La-Lf: the co-culture of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221

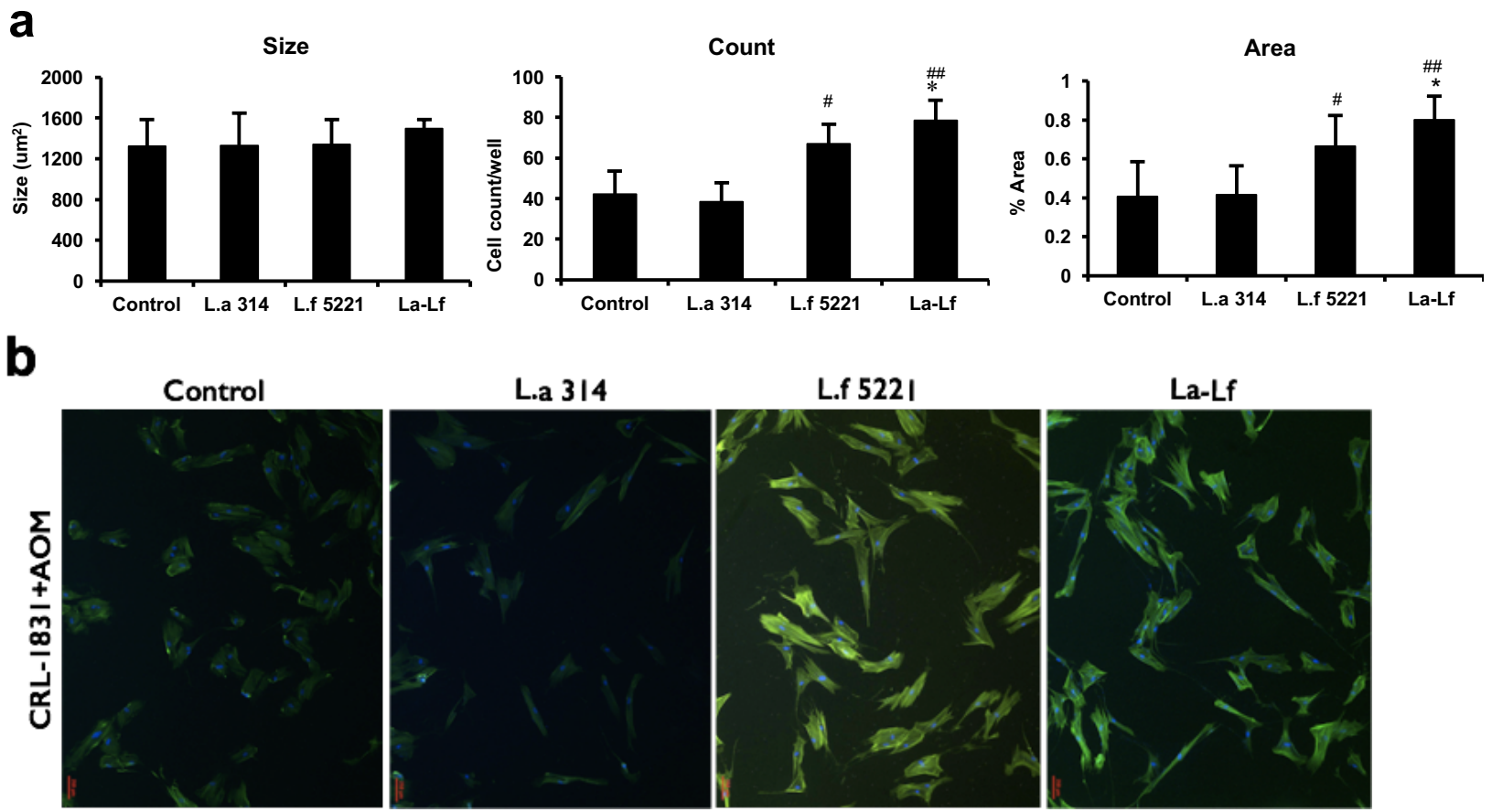


Figure 7.5: Estimation of a potential protective effect of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 formulations on non-neoplastic epithelial colon cells treated with a carcinogen.

CRL-1831 normal epithelial colon cells were first incubated with a probiotic conditioned cell culture medium (CM) of *L. acidophilus* ATCC 314, *L. fermentum* NCIMB 5221 or their co-culture for 72 h, then treated with a carcinogen: azoxymethane (AOM, 10^{-6} M) for 7 days. **(a)** Image quantifications (size, cell count, and area) of CRL-1831 cells after probiotic and carcinogenic treatments. scale bar (in

red) = 250 µm. Data are presented as mean ± SEM (n = 4). **(b)** After F-actin staining, the surface of attached CRL-1831 cells for each treatment was analyzed twice on 3 captures taken from random wells, using an imaging software. Data are presented as mean ± SEM (n = 4). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with control. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with L. a 314. L. a 314: *L. acidophilus* ATCC 314; L. f 5221: *L. fermentum* NCIMB 5221; La-Lf: the co-culture of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221.

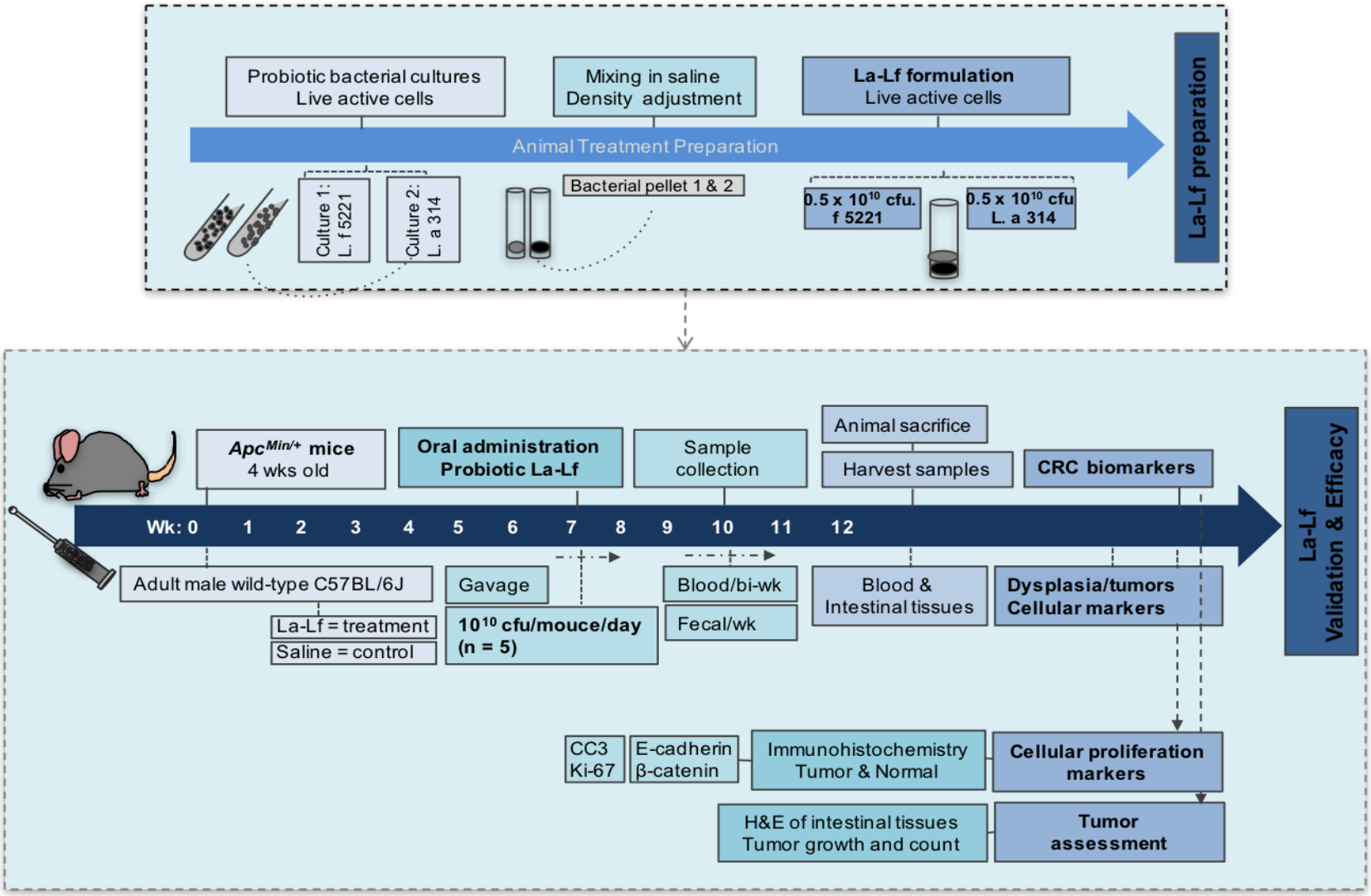

Probiotic bacterial cultures
Live active cells
Mixing in saline
Density adjustment
La-Lf formulation
Live active cells
Animal Treatment Preparation
La-Lf preparation
Culture 1:
L. f 5221
Culture 2:
L. a 314
Bacterial pellet 1 & 2
0.5 x 10^10 cfu.
f 5221
0.5 x 10^10 cfu
L. a 314
Apc^Min/+ mice
4 wks old
Oral administration
Probiotic La-Lf
Sample
collection
Animal sacrifice
Harvest samples
CRC biomarkers
La-Lf
Validation & Efficacy
Wk: 0 1 2 3 4 5 6 7 8 9 10 11 12
Adult male wild-type C57BL/6J
La-Lf = treatment
Saline = control
Gavage
10^10 cfu/mouce/day
(n = 5)
Blood/bi-wk
Fecal/wk
Blood &
Intestinal tissues
Dysplasia/tumors
Cellular markers
CC3
Ki-67
E-cadherin
β-catenin
Immunohistochemistry
Tumor & Normal
Cellular proliferation
markers
H&E of intestinal tissues
Tumor growth and count
Tumor
assessment

Figure 7.6: . The timeline of the animal experiment.

Oral interventional administration of active live probiotic La-Lf formulation using a CRC *Apc*$^{Min/+}$ mouse model. Bacterial masses of each *L. acidophilus* and *L. fermentum* were collected and prepared at a density of 0.5 x 10^{10} cfu for each bacterium in saline. A total of ~10^{10} cfu bacteria was ingested by each mouse daily (n = 5). After 12 weeks of treatment, adult male wild-type C57BL/6 *Apc*$^{Min/+}$ mice were sacrificed, and tissues/blood were harvested for assessment of cellular markers and tumorigenesis. Intestinal sections were H&E stained for tumor enumeration. Then, more sections were immunostained against E-cadherin, ß-catenin, CC3, and Ki-67 antibodies.

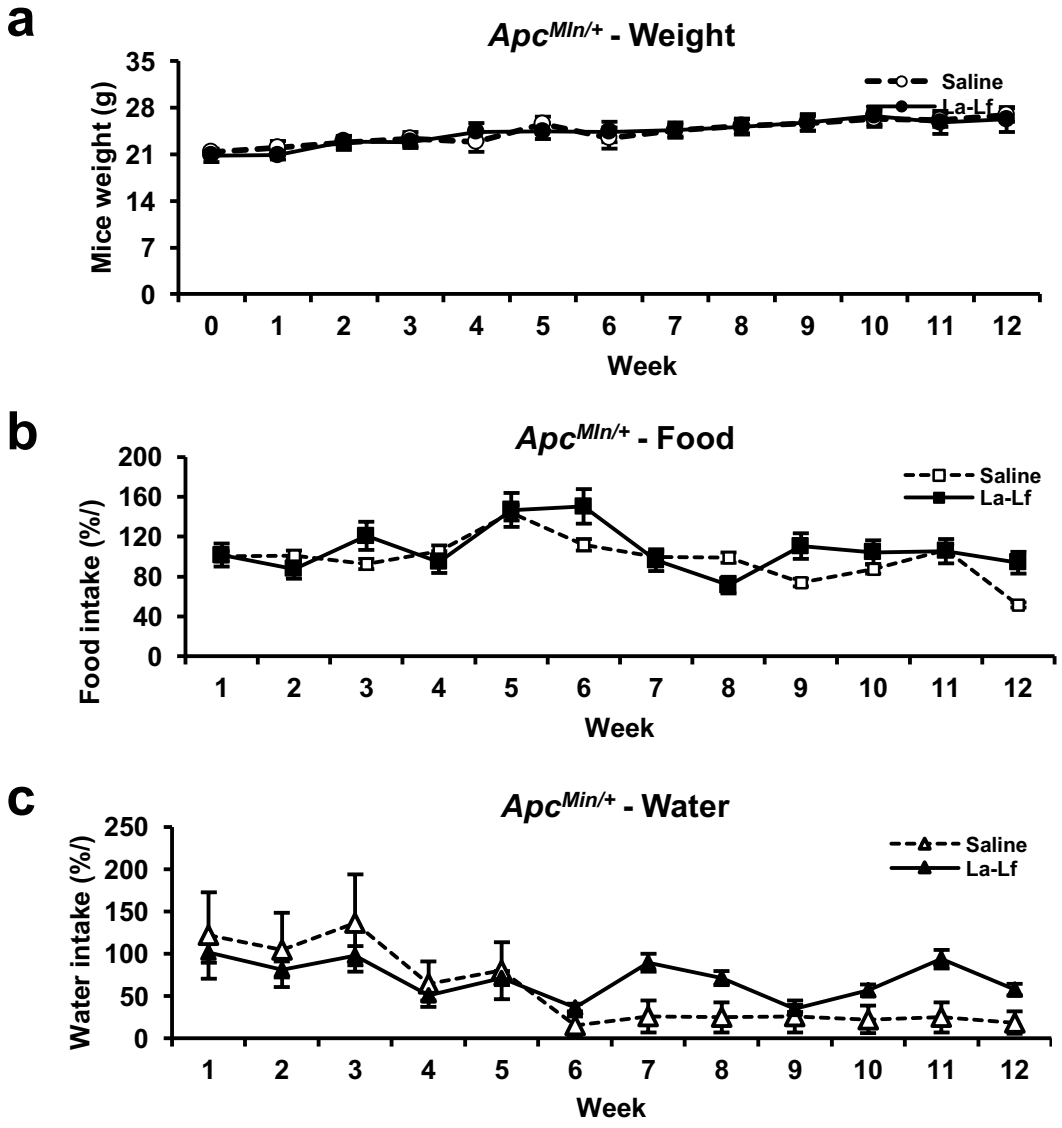


Figure 7.7: Animal weight, food and water intake after oral intake of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 formulation.

(a) Weight change, **(b)** food intake, and **(c)** water consumption among La-Lf-supplemented and un-supplemented *Apc*$^{Min/+}$ mice during 12 weeks. The gavage of the probiotic formulation started at four weeks old daily with a dose of La-Lf (10 cfu/d/mouse). La-Lf= [0.5 x 10^{10} cfu/d of *L. acidophilus* ATCC 314 and 0.5 x 10^{10} cfu/d of *L. fermentum* NCIMB 5221]. Saline is given to control group. Normalization/adjustments of bacterial density, in saline (0.2 mL), stabilized at RT before administration. Data are presented as mean ± SEM (n = 5)

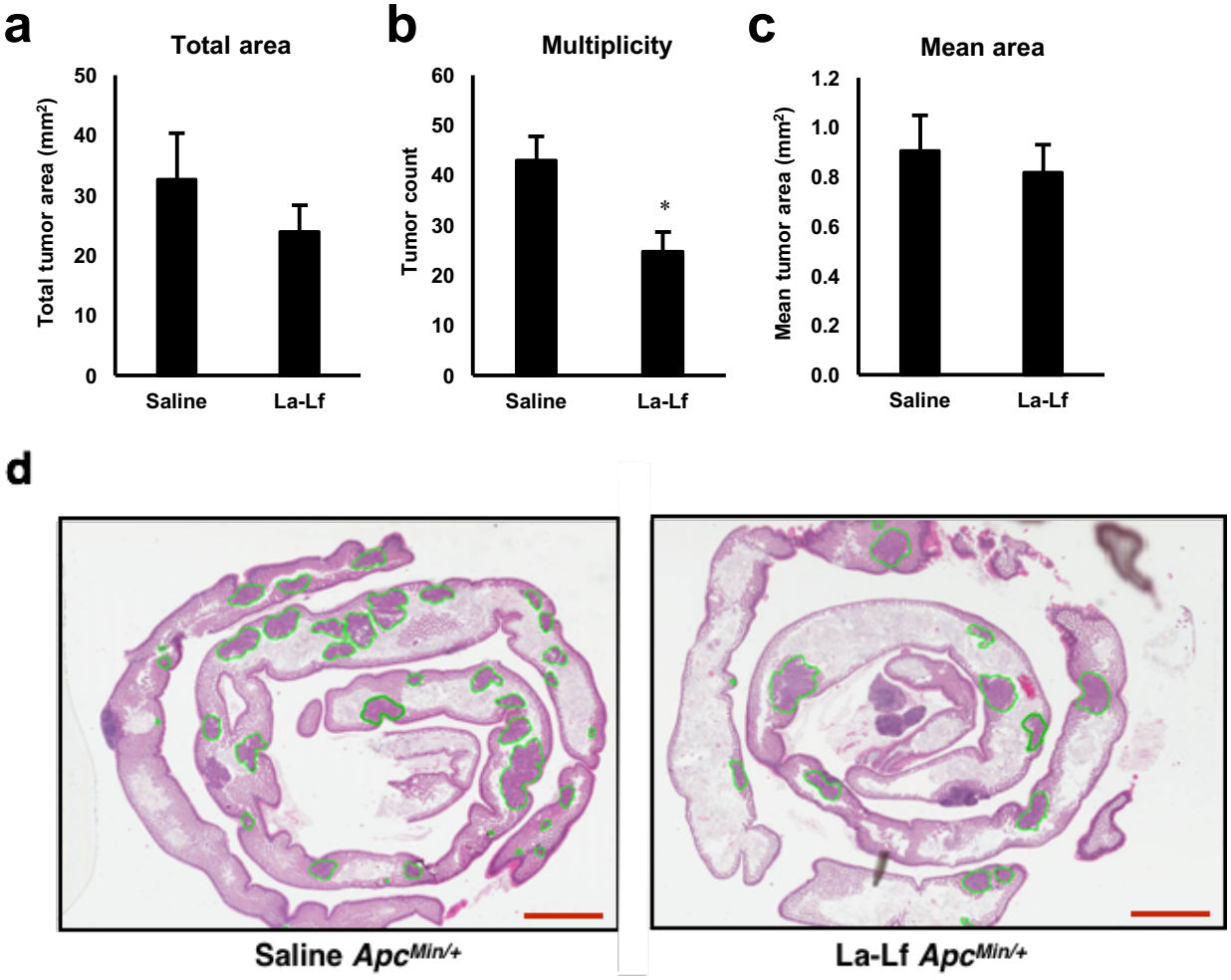
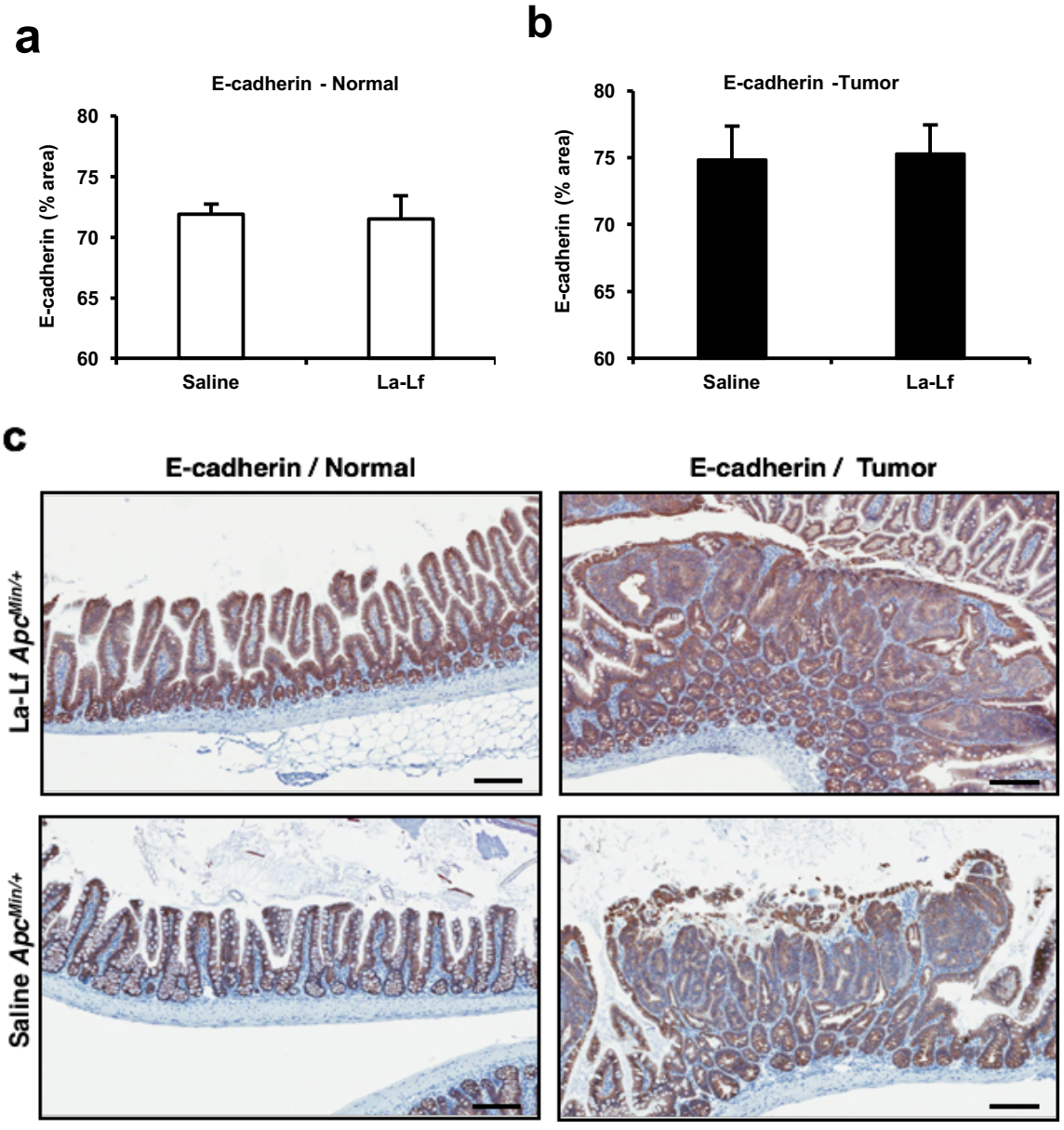
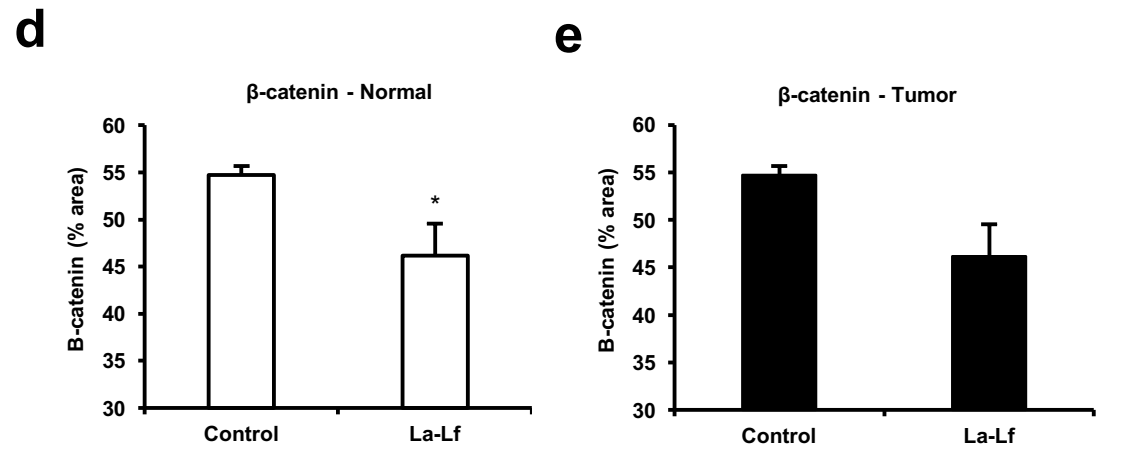


Figure 7.8: The efficacy of the oral administration of active probiotic formulation (La-Lf) in a CRC *Apc*$^{Min/+}$mouse model.

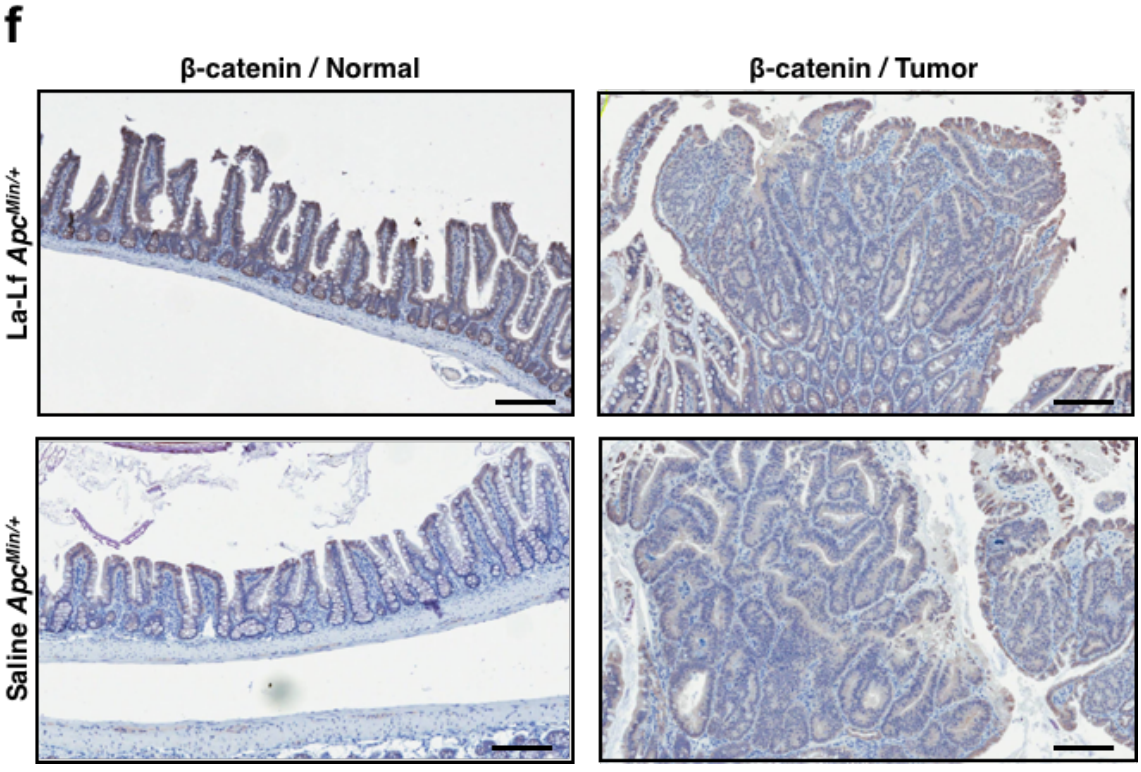
(a) Tumor enumeration, **(b)** tumor area, and **(c)** mean tumor area assessments for the probiotic treatments administrated to *Apc*$^{Min/+}$mice, compared with saline. Probiotic doses were administered orally on a daily basis. After 12 weeks of La-Lf feeding, *Apc*$^{Min/+}$mice were sacrificed, and the 10 cm section of the distal small intestine was harvested, from which the number and size of intestinal adenoma were measured. Tumor number is reduced in *Apc*$^{Min/+}$mice treated with La-Lf compared with control untreated *Apc*$^{Min/+}$mice. Tumor size is also reduced in the treated group, as well as total tumor surface area. Data represent the mean SEM per group (n = 5). **(d)** Representative H&E stained images for histopathology identification of intestinal adenomas. The proximal sections of the intestines were H&E stained. Representative images illustrate the contrast between La-Lf-treated *Apc*$^{Min/+}$mice

and control. Scale bar (in red) = 5mm. * $P < 0.05$, compared with untreated mice, using independent samples t-test.

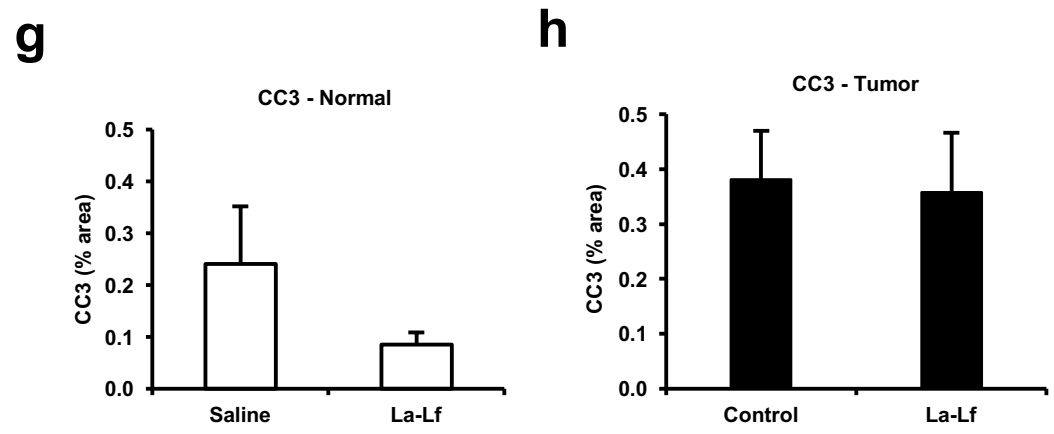



d
β-catenin - Normal
B-catenin (% area)
60
55
50
45
40
35
30
*
Control
La-Lf
e
β-catenin - Tumor
B-catenin (% area)
60
55
50
45
40
35
30
Control
La-Lf

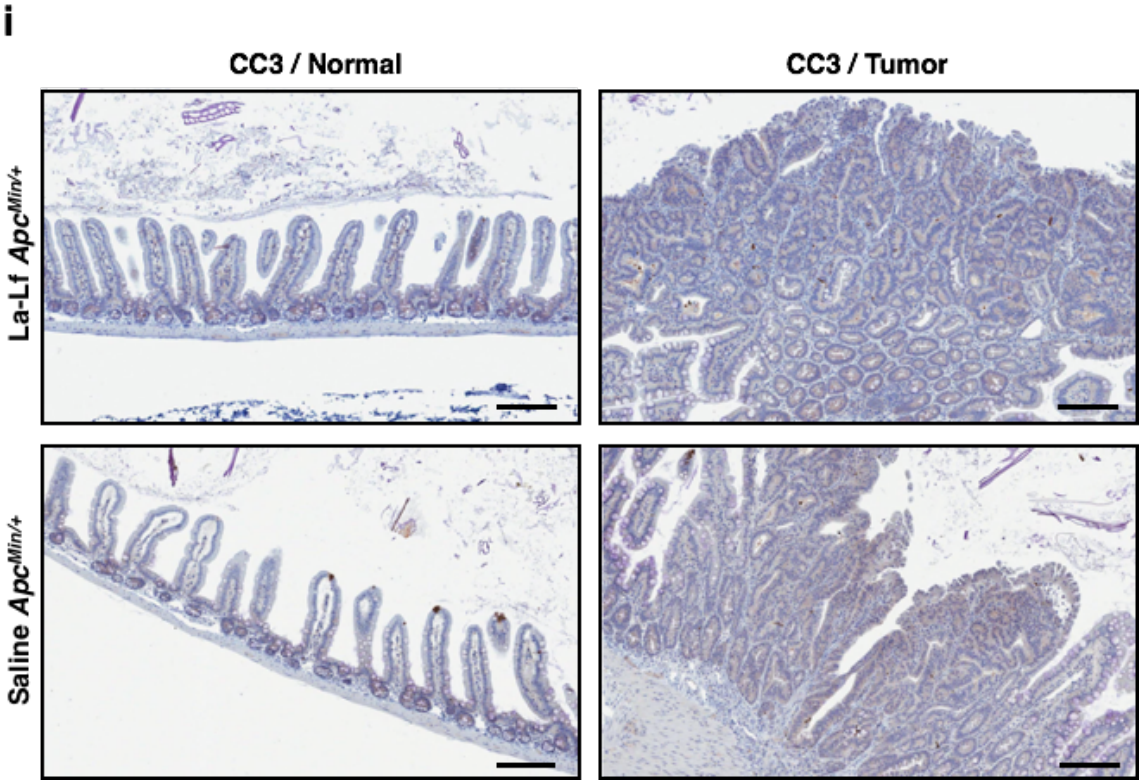
33


f
β-catenin / Normal
β-catenin / Tumor
La-Lf ApcMin/+
Saline ApcMin/+

34


g
CC3 - Normal
CC3 (% area)
0.5
0.4
0.3
0.2
0.1
0.0
Saline
La-Lf
h
CC3 - Tumor
CC3 (% area)
0.5
0.4
0.3
0.2
0.1
0.0
Control
La-Lf

35


i
CC3 / Normal
CC3 / Tumor
La-Lf ApcMin/+
Saline ApcMin/+

36
37

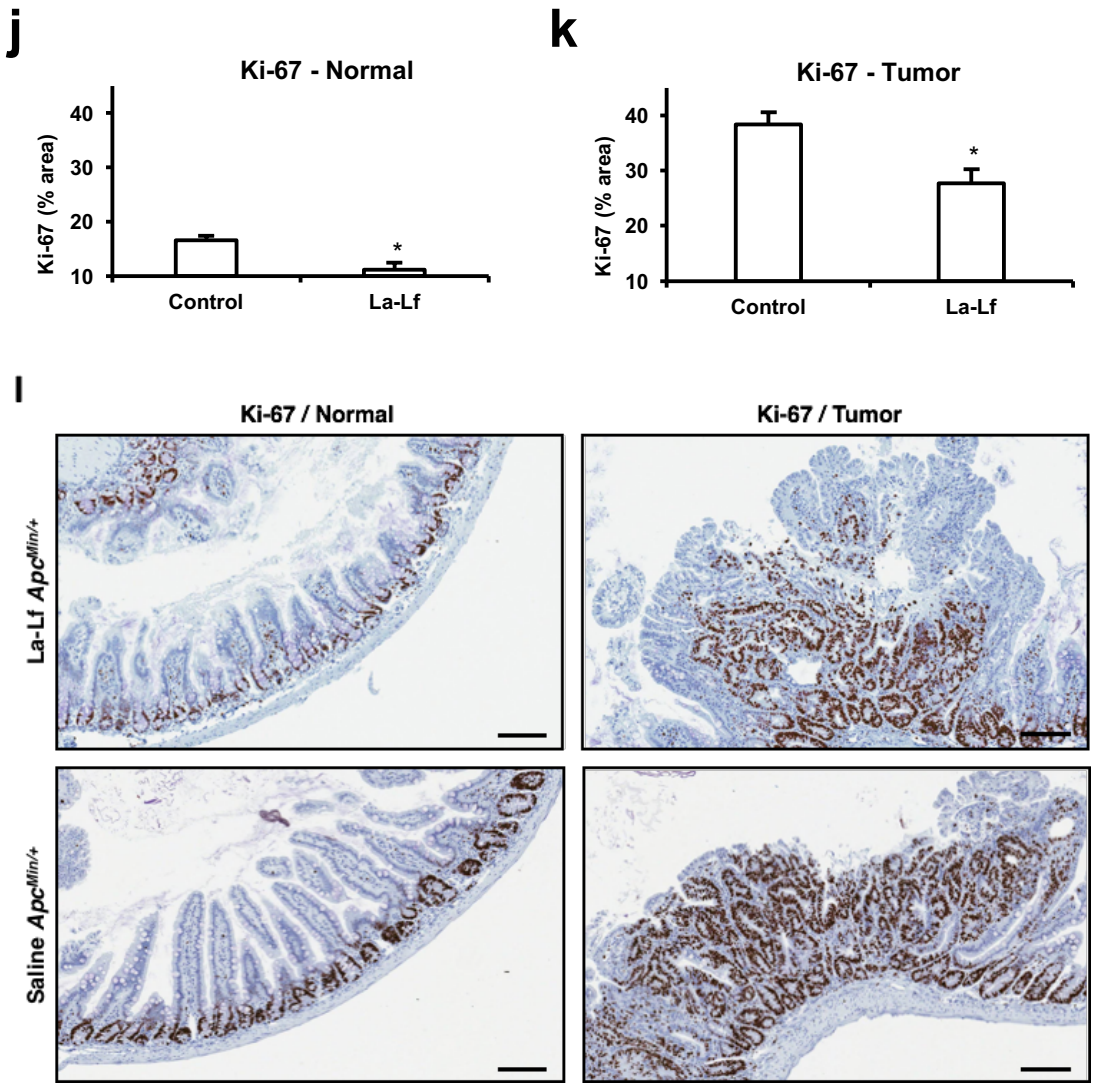


Figure 7.9: Orally administrated *Lactobacillus* active formulation containing *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 demonstrated potential in attenuating molecular neoplastic markers of cell proliferation in intestinal tissues in a CRC $Apc^{Min/+}$ mouse model.

The parentage of stained cells in intestinal sections for different markers: **(a, b)** E-cadherin, **(d, e)** β-catenin, **(g, h)** CC3, and **(j, k)** Ki-67, respectively, in "normal appearing" and tumor intestinal tissues. **(l)** Immunohistochemical staining showing the effect of La-Lf probiotic treatment on β-catenin and E-cadherin on cell proliferation in the intestinal crypts of $Apc^{Min/+}$ mice. The intestines were stained using antibodies and counterstained with hematoxylin. Dark colored nuclear staining indicates positive

cells. Cells with blue nuclei are negative. Representative images are shown to compare the different staining intensities against each antibody. Scale bar (in black) = 200 μm. Data are presented as mean ± SEM (n = 5). * $P < 0.05$, compared with untreated animals.

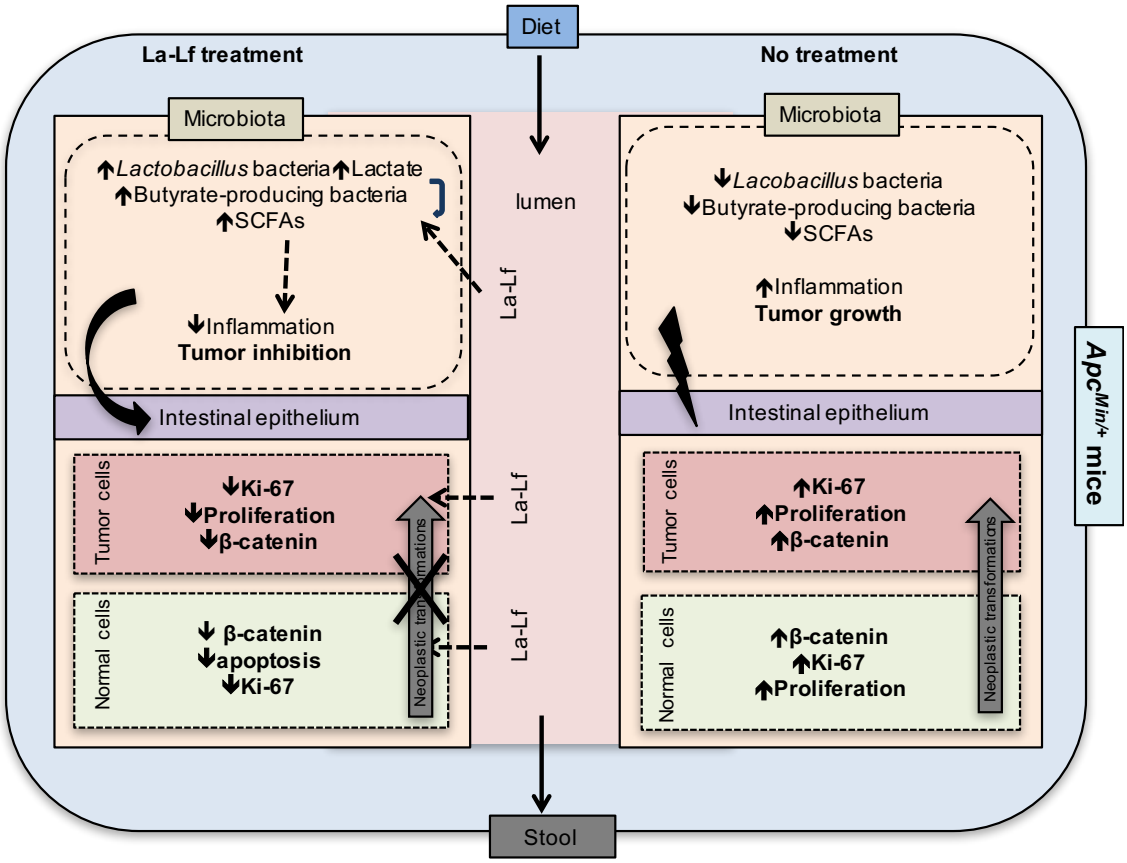


Figure 7.10: Schematic hypothetical illustration of how La-Lf oral administration might inhibit CRC tumor growth based on the speculations of the present study.

In summary, with the introduction of the bio-therapeutic factor in a CRC *Apc*$^{Min/+}$ mouse model, tumorigenic processes are attenuated through indirect modulation of microbiota or direct action on the intestinal epithelium. As unbalanced microbiota aggravates GI health, La-Lf modifies this negative status. All those events aim to attenuate the levels of inflammation, cellular proliferation in tumors and slowdown ongoing normal cell transformation in intestinal epithelium.

7.9. Supplementary materials

Table 7.1S. Summary and comparison of bacterial culture growth kinetics. Data are recapitulated from results obtained in **Figs. 2 and 3**.

	L. a 314	L. f 5221	La-Lf
Viability MRS	+	+	+
Viability SIF	+	+	+
Density MRS	+	++	+++
Density SIF	++	+	+++
Density AIJ	+	++	+++
Antioxidant MRS	+	++	+++
Antioxidant AIJ	+	++	++
Caco-2 viable count	+	+++	+++
Caco-2 dead count	~	+	~
CRL-1831 viable count	-	++	++

Table 7.2S. Comparative table summarizing the anti-proliferative and pro-apoptotic activity of modified and non-modified probiotic extracts on CRC cells.

	CRC cell inhibition			
	None	Heat-inactivation	Proteinase K	30 MWCO
L. a 314	+	−−	−−−	+++
L. f 5221	+++	+	−−−	+++
La-Lf	++	−−	−−−	+++

	CRC cell apoptosis			
	None	Heat-inactivation	Proteinase K	30 MWCO
L. a 314	+	−−−	+++	+++++
L. f 5221	+++	0	+++	+++++
La-Lf	+++	−−−	+++	+++++

Original Research Article 6

CHAPTER 8. ORALLY ADMINISTRATED ACTIVE *LACTOBACILLUS FERMENTUM-LACTOBACILLUS ACIDOPHILUS* PROBIOTIC FORMULATION MODULATES INFLAMMATORY AND METABOLOMIC MARKERS IN THE COLORECTAL CANCER *APC*$^{MIN/+}$ MOUSE MODEL

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Preface: As validated in the previous chapter, the anti-tumorigenic effect of *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314 *in vivo* was validated, thus further investigations on the metabolic and inflammatory mechanism of actions were needed. Using *Apc*$^{Min/+}$ mice CRC model, NMR and DI/LC-MS/MS methods generated plasma and fecal metabolomes. Coupled with PCA and PLS-DA analysis, it was possible to identify the set of major metabolites that distinguish the probiotic formulation from the untreated group. This probiotic bacteriotherapy induced significant simultaneous reduction or elevation in the concentrations of major metabolites with effect in developing CRC risk and microbiota modulation. To study the anti-inflammatory effect of *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314 oral intake, the plasma profile of pro-inflammatory cytokines was determined. The expression of inflammatory cells in normal-appearing and tumor mucosa was studied in *Apc*$^{Min/+}$ mice after the biotherapy.

Submitted to Cancer Research

8.1. Abstract

Oral probiotic supplementation with active *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 formulation (La-Lf) in genetically modified CRC mice has previously shown potential at inhibiting intestinal tumor growth. Thus, the purpose of this current study was to discover specific biomarkers and mechanisms of actions associated with the La-Lf probiotic bio-intervention. This study evaluated the metabolic and anti-inflammatory impact of this active probiotic formulation in a CRC $Apc^{Min/+}$ mouse model by assessing local and systemic inflammatory markers (immune cells and pro-inflammatory cytokines) and analyzing plasma and fecal metabolomes (^{1}H NMR, DI/LC-MS/MS). Supervised partial least squares discriminant analysis (PLS-DA) showed that the plasma and fecal profiles contained discriminant metabolites between treated and control groups. The La-Lf supplementation modified the fecal metabolic profile toward higher levels of tyrosine, leucine, proline, pyruvate, aspartate, sarcosine, succinate, lactate, glycerol, acetate, glucose, phenylacetate, choline, and methanol, with lower concentrations of fecal ethanol in treated mice compared with the control. However, in the plasma, the La-Lf treatment resulted in higher levels of 12 glycerophospholipids, with lower symmetric dimethylarginine (DMA). Moreover, in La-Lf-fed mice, plasma INF-γ, IL-1β, KC/GRO, TNF-α, and IL-10 were significantly diminished ($p < 0.05$), the expression of IBA-1 and CD3 were significantly downregulated in intestinal tissues compared with the control ($p < 0.05$), making La-Lf relevant for inflammation-driven CRC as well. The results showed that La-Lf treatment-associated inflammatory, fecal, and plasma profiles in CRC $Apc^{Min/}$ mice may indicate treatment efficacy and provide grounds for the anti-tumorigenic activity of the La-Lf probiotics in CRC.

8.1. Introduction

Gastrointestinal (GI) disease, particularly colorectal cancer (CRC) is a major health concern that has increased awareness regarding the role of intestinal microbial ecosystems in host hemostasis. In recent times, we have arrived at an understanding that healthy gut flora play a vital role in human health by combating pathogens and infections, supporting the integrity of intestinal membrane barrier, strengthening the immune system, and excreting essential and bioactive metabolites (1,2). However, the population balance of microorganisms is susceptible to intense fluctuations in response to diet and lifestyle (3). An altered intestinal microbiota has been associated with variable conditions, including CRC (4,5), inflammatory bowel diseases (IBD), and irritable bowel syndrome (IBS) (6) as well as obesity and Type 2 diabetes (7). Dietary manipulation of the gut microbiota through oral probiotic supplementation is described as a potential alternative to improve or restore a disturbed microbial community and render the host potentially less prone to intestinal lesions, inflammation, and epigenetic alterations. Despite the advances in investigating the benefits of probiotics and gut microflora in host metabolism and immunity, key metabolic and inflammatory activities of several lactic acid bacteria (LAB) are not completely understood (8,9). Few metabolomics studies were able to reveal that certain *Lactobacillus* strains can modulate numerous host metabolic pathways such as inducing beneficial changes in lipid profiles, gluconeogenesis, and amino acid metabolism (10,11).

Recently, *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 (La-Lf) were found to exhibit an anti-tumorigenic effect in $Apc^{Min/+}$ mice, which is anticipated to result from fine-tuning the balance between different inflammatory and metabolic functions using active probiotic manipulation of the gut microflora. This requires further substantiation of the effects of probiotics on the functional levels of gut−microbiota homoeostasis. The current study was established with an aim to extend the previous study (12) and verify how the oral administration of an active mixture of Lactobacilli significantly affects the intensities of specific cellular and bacterial

168 metabolic markers. For this purpose, two methods were used: direct injection liquid
169 chromatography-tandem mass spectrometry (DI/LC-MS/MS) for plasma profiling,
170 and ^{1}H-nuclear magnetic resonance spectroscopy (^{1}H-NMR) for fecal extracts. In
171 addition, this pre-clinical trial explored components in the inflammatory and immune
172 profiles modified by La-Lf intervention in *Apc*$^{Min/+}$ mice. Therefore, pro-
173 inflammatory cytokines in plasma, including interferon (INF)-γ, interleukin (IL)-1β,
174 IL-2, IL-4, IL-5, IL-6, keratinocyte chemoattractant (KC)/human growth-regulated
175 oncogene (GRO), IL-10, IL-12p70, and tumor necrosis factor (TNF)-α were analyzed.
176 In this study, the expression and infiltration of two markers IBA-1 (macrophage) and
177 CD3 (T lymphocyte) were applied in tumors or normal crypts of *Apc*$^{Min/+}$ mice
178 intestines to demonstrate the ability of La-Lf in modulating specific mucosal immune
179 responses and, thereby, ameliorate colonic carcinogenesis regression.

8.2. Materials and methods*

8.2.1. Experimental procedure for La-Lf bio-intervention in *Apc*$^{Min/+}$ mice

The *Apc*$^{Min/+}$ animal trial took place to assess metabolic and immune markers after La-Lf probiotic intervention as illustrated in **Figure 8.1**. Adult male C57BL/6J-*Apc*^{Min}/J four-week-old mice were purchased from Charles River Laboratories (Wilmington, MA, USA). The mice were fed Constant Nutrition® formula basal Purina rodent diet (Formulab Labdiet® 5008, ON, Canada, **Table 8.1S**) during the entire trial and were maintained at constant temperature (~22 °C) and humidity in a light-controlled (12 h light:12 h dark) facility while allowed consumption of water and food *ad libitum*. All animal experiments were approved by the Animal Care Committee at McGill University and the Canadian Council on Animal Care guidelines (Protocol Number 5189).

After one week of acclimatization, mice were distributed randomly into two groups (n = 5), where one was orogastrically supplemented with La-Lf formulation and the other group received the same volume of saline solution during a 12-week treatment period. Probiotic pellets were suspended in a saline solution to obtain a dose of La-Lf containing 0.5 x 10^{10} cfu of *L. acidophilus* ATCC 314 mixed with 0.5 x 10^{10} cfu of *L. fermentum* NCIMB 5221. The probiotic doses were prepared freshly on a daily basis. The stool was massed on a weekly basis and was stored at −80 ^{o}C until analysis. Lateral saphenous vein blood samples were collected bi-weekly into microtainer dipotassium ethylenediaminetetraacetic acid-coated tubes (EDTA-Microtainer®, Becton Dickinson (BD) 365974, BD Biosciences NJ, USA) were used for immediate separation of plasma as previously described (13). Tubes were repetitively inverted to mix blood with anti-coagulant before centrifugation (3500 x g, 15 min, 4 ^{o}C); then, plasma was aspired carefully and inspected for turbidity before storing in cryovials at −80 °C for later analysis. At the completion of the trial, the *Apc*$^{Min/+}$ mice were sacrificed by CO_2 asphyxiation and feces and blood/plasma (by cardiac puncture) were collected. All samples were stored as described above for subsequent metabolomic and

inflammatory analysis. Additionally, the intestines were collected, fixed, and stained for immunohistochemistry evaluations of immune markers as detailed below.

8.2.2. ^{1}H NMR spectroscopic fecal analysis

For ^{1}H NMR analysis of *Apc*$^{Min/+}$ mice fecal samples, the extraction of fecal water was performed after La-Lf intervention as described by Costabile *et al.,* with minor modifications (14). Chenomx NMR Suite Professional software package version 7.6 (Chenomx Inc., Edmonton, ALB, Canada) allowed the processing and analysis of all ^{1}H NMR spectra and ^{1}H NMR signal assignment. Assigned metabolites were mainly identified based on the Human Metabolome Database (15).

8.2.3. DI/LC-MS/MS spectroscopic plasma analysis

For metabolomic plasma analysis of *Apc*$^{Min/+}$ mice, after La-Lf bio-therapy, preserved samples were thawed and analyzed using the Absolute*IDQ*™ kit (Biocrates Life Sciences AG, Austria). This assay allows identification of about 162 metabolites including 76 phosphatidylcholines, 15 lysophosphatidylcholines, 41 acylcarnitines, 19 biogenic amines, 15 sphingolipids, and 14 amino acids. The use of this kit is based on a sample preparation workflow integrated with direct flow injection mass spectrometry (DI/LC-MS/MS) as well as Met*IQ*^{TM} software (Biocrates Life Sciences AG, Austria), similarly to what was conducted by Shultz *et al*. (16).

8.2.4. Plasma pro-inflammatory cytokines quantification using an enzyme-linked immunosorbent assay

Plasma samples from mice were collected in EDTA-coated blood collection tubes (described above) and stored at −80 °C for bulk analysis. Cytokine measurements were performed using electrochemiluminescent multiplex assays with the Multi-Spot® Assay System (Meso Scale Discovery®, MD, USA). The plasma levels of ten cytokines (INF-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α) were determined simultaneously, with an intra-assay correlation ≤ 10% and inter-assay variance ≤ 10%. Cytokine concentrations were determined using the MSD Workbench 3.0 software (Meso Scale Discovery®, MD, USA) based on curve fit models ($\log-\log$ or four-parameter $\log-$logistic). Following the manufacturer's

instructions, specific cytokine concentrations were computed using the curve fit models of Softmax® Pro (Version 4.6 software) for each reaction.

8.2.5. Histopathological analysis

To examine intestinal tissues for the presence of intestinal tumors and confirmation of tumor total decline in the La-Lf-fed *Apc*$^{Min/+}$ mice compared with the saline group, animals were sacrificed at 17 weeks of age by CO_2 asphyxiation. The small intestines and colon were divided and gently rinsed with ice-cold NaCl solution (0.9% w/v). The intestines were infused with 10% phosphate buffered formalin (PBF) and rolled into cassettes immersed in 10% PBF. Tissue dehydration was performed using graded alcohols and xylene, before embedding in paraffin. Tissues paraffin blocks were sectioned (4 μm) and stained with hematoxylin and eosin (H&E) stain for histological evaluation. A veterinary pathologist, blinded to the treatments, re-examined the stained sections for proliferative lesions and immune cell infiltration using Aperio-Imagescope image analysis software (Leica Biosystems, ON, Canada).

8.2.6. Immunohistochemistry and imaging analysis

This set of analyses was conducted to reveal the role of probiotic La-Lf treatment in modulating intestinal immune responses. In particular, the intensity of CD3 (T cell marker, anti-CD3, dilution 1:200, Abcam, Toronto, ON, Canada) and IBA-1 (macrophage marker, anti-IBA-1, dilution 1:200, Wako Chemicals, Richmond, VA, USA) expressions and infiltration in tumors and non-tumor tissues were assessed by immunohistochemical staining using automated immunostainer Intellipath FLX, from Biocare Medical (Concord, CA, USA).

8.3. Data and statistical analysis

After the concentration values were determined, MetaboAnalyst 3.0 (Canada) (accessible at http://www.metaboanalyst.ca) was used to perform statistical analysis as suggested by Bijlsma *et al.* (17). For immune and inflammatory-related assays, data are presented as means ± Standard Error of the Mean (SEM). Statistical significance was generated by student's t-test. The statistics software package used was SPSS

(version 20.0, IBM Corporation, New York, NY, USA). *P*-values of $p < 0.05$ were considered significant.

*See Supplementary material and methods for more details.

8.4. Results

8.4.1. NMR fecal metabolites and analysis using a volcano plot

The ^{1}H NMR spectra of fecal water samples revealed a broad range of metabolites. Thirty-nine of the identified metabolites were assigned and integrated using Chenomx NMR Suite Professional software based on the Human Metabolome Database (15). The obtained ^{1}H NMR spectra contained resonances primarily from short chain fatty acids (SCFAs), branched chain fatty acids, biogenic amines, bile acids, organic acids, and amino acids (**Supplementary Figure 8.1S**). A pronounced effect of La-Lf probiotic administration was observed in the NMR profile. Visual inspection of spectra showed that samples collected after La-Lf intervention had a markedly different fecal metabolite profile than saline control samples. To study the difference between La-Lf treated and untreated $Apc^{Min/+}$ mice, a volcano assay was performed to show the most significantly affected metabolites by the treatment based on independent t-test and fold change (FC) (**Figure 8.2A**). La-Lf treatment significantly affected the 16 metabolites whose *p*-values and FCs are described in **Table 8.2S**.

8.4.2. Pattern recognition analysis to translate La-Lf impact on bacterial metabolism

To extract the La-Lf-treatment-induced modifications in the fecal profile and to identify coordinated affected pathways, the NMR spectra recorded on the fecal samples of $Apc^{Min/+}$ mice were subjected to multivariate data analysis. Constructed PCA score plots were visualized and demonstrated no significant outliers in the dataset; however, there was an observable separation between both groups (**Figure 8.2B**). The next step was a supervised analysis by constructing a PLS-DA model using the NMR $Apc^{Min/+}$ mice fecal dataset (*x*-matrix) and treatment group parameters (*y*-matrix). The resulted PLS-DA score provided a complete discrimination between the saline and the probiotic groups, with a clear separation of clustering between groups and the optimal complexity being two components explaining 31.5% and 24.9% of *x*-variance, respectively (**Figure 8.2C**). The predictability and accuracy of the PLS-DA plot was assessed by values presented in **Figure 8.2D**, indicating acceptable predictive

power and accuracy. To evaluate the robustness of this model, a permutation test was conducted with 2,000 permutations, which demonstrated that the observed separation was not due to chance ($p < 0.005$) or random overfitting of the data. Moreover, the analysis of variance was applied in the PLS-DA model to abstract the statistical significance of the difference between the two groups, which was highly significant with a score of $p = 3.4 \times 10^{-8}$.

8.4.3. La-Lf-treated *Apc*$^{Min/+}$ mice possess a distinguished set of bacterial co-metabolites

The present NMR-based metabolomic study sought to assess the anti-tumorigenic effect of La-Lf on the metabolic fecal profile in tumor-bearing mice. Therefore, heatmaps of data clustering (**Figure 8.2F**) and correlations (**Figure 8.3G**) between metabolites within samples were illustrated. The heatmaps showed metabolite variations and correlations as color-coded loading plots according to their absolute correlation coefficient value.

Metabolites were ranked according to their power to segregate the La-Lf effect from untreated mice. Variable importance in projection (VIP) values were generated as described in the methods section. The VIP scores calculate the influence of each metabolite on cluster formation among groups in the PLS model. Higher VIP scores indicate greater importance of the selected metabolite in predicting the efficacy of La-Lf bio-treatment. Inspection of the VIP scores, together with cross-model validation, showed that 15 fecal metabolites were most relevant for the discrimination between control and treated *Apc*$^{Min/+}$ mice (**Figure 8.3A**). PLS-DA results showed that the variation in the fecal metabolites could be readily differentiated. To identify which compounds were responsible for this distinct fecal metabolic signature, the following parameters were used as criteria: VIP > 1 and $p < 0.05$. Among the fifteen metabolites, the following discriminant metabolites were enriched: tyrosine, leucine, proline, pyruvate, aspartate, sarcosine, succinate, lactate, ethanol, glycerol, acetate, glucose, phenylacetate, choline, and methanol **(Table 3).** Nonetheless, ethanol was reduced, in the La-Lf treatment group when compared with the saline group. The VIP plot, which

was used to rank the importance of the variables, showed methanol and choline as the most influential variables.

8.4.4. La-Lf modulates bacterial metabolic pathways in *Apc*$^{Min/+}$ mice

Based on the fecal metabolic profile, the variations of metabolites were translated to provide the biological interpretation behind the probiotic effect. All significantly affected pathways were ranked and their fold change was estimated (**Figure 8.3B** and **Table 8.4S**).

8.4.5. Volcano plot for DI/LC-MS/MS plasma metabolites

To extract the significant differences between plasma metabolites of La-Lf-treated and untreated *Apc*$^{Min/+}$mice (**Supplementary Figure 8.1S)**, the volcano test was applied and generated the most significantly affected metabolites based on independent t-test and their fold change (FC) (**Figure 8.4A**), namely, symmetric DMA ($p = 0.0116$), PC aa C36:1 ($p = 0.0353$), and PC aa C32:1 ($p = 0.0413$) (**Table 8.5S**).

8.4.6. La-Lf produces a distinct systemic set of metabolites *Apc*$^{Min/+}$ mice

The DI/LC-MS/MS recorded data on La-Lf and control plasma samples were processed with multivariate analysis to extract sets of metabolic pathways changed after probiotic administration to *Apc*$^{Min/+}$ mice. The PCA and PLS-DA score plots were composed with an *X*-matrix for DI/LC-MS/MS and a *Y*-matrix for group labels (**Figure 8.4**). The score plot depicts two distinct clusters along PC1 **(Figures 8.4B and 8.4C)**. The PLS-DA resulted in a clear separation between groups. The plasma metabolomic profile of La-Lf-treated mice contained significant elevation of certain metabolites (listed below). The predictability and accuracy of the PLS-DA plot were assessed by characteristic values as presented in **Figures 8.4D and 8.4E**, indicating acceptable predictive power and accuracy. The permutation analysis with 2,000 resamplings was performed and confirmed that the observed separation was unlikely to be caused by chance ($p < 0.005$) or random data overfitting. The analysis of variance was applied in the PLS-DA model and resulted in a score of $p = 31.6 \times 10^{-5}$.

After robust PCA and PLS-DA scoring and evaluation of VIP values, the principal metabolic groups identified belonged to the groups of glycerophospholipids, biogenic

amines, and acethylcarnitines. The list of the most relevant metabolites with their VIP values starting from the most important are as following: symmetric DMA, PC aa C40:1, PC ae C38:2, PC ae C34:1, PC ae C40:3, PC ae C32:1, PC aa C40:2, PC aa C32:1, PC aa C42:2, PC aa C40:3, PC ae C36:1, PC ae C38:1, PC aa C34:1, PC aa C36:1, and methionine sulfoxide (**Figure 8.5A)**. All these metabolites were elevated in the treated group, except for symmetric DMA, which was significantly ($p < 0.05$) reduced in $Apc^{Min/+}$ mice plasma (**Table 8.6S)**.

8.4.7. La-Lf biotherapy modulates $Apc^{Min/+}$ mice metabolism

In terms of systemic impact of La-Lf treatment, the most enriched pathways are ranked in **Figure 8.5B**. The most significantly disturbed pathways were the following: tryptophan metabolism; valine, leucine, and isoleucine degradation; propanoate, betaine, and methionine metabolism; glycine, serine, and threonine metabolism; protein metabolism; and glutathione metabolism (**Table 8.7S**).

8.4.8. La-Lf decreases systemic inflammation in $Apc^{Min/+}$ mice

After oral administration of La-Lf to $Apc^{Min/+}$ mice, a number of plasma pro-inflammatory cytokines were found significantly negatively affected, whereas none were increased. Only IL-2, IL-4, and IL-12p70 (below detection range (BDR), **Figures 8.6C, 8.6D, and 8.6I**) were undetectable for both groups. La-Lf treatment lowered IL-1β (BDR, **Figure 8.6B**) to an undetectable level compared with saline (0.3 ± 0.1 pg/mL). In La-Lf-treated animals, plasma INF-γ (0.2 ± 0.1 pg/mL) was significantly lower compared with control (1.5 ± 0.5 pg/mL, $p = 0.039$, **Figure 8.6A).** La-Lf significantly lowered KC/GRO levels (47.7 ± 8.9 pg/mL) compared with saline (118.9 ± 21.7 pg/mL, $p = 0.016$). Similarly, TNF-α (7.3 ± 0.5 pg/mL) was lowered by La-Lf compared with control (13.5 ± 2.6 pg/mL, $p = 0.045$, **Figure 8.6J**).

8.4.9. La-Lf treatment suppresses the expression of IBA-1 and CD3

In tumor tissues, a significant effect of the La-Lf treatment in lowering the expression of CD3 (0.36 ± 0.01, (**Figure 8.7E**) was observed compared with the saline group (0.58 ± 0.02%, $p < 0.001$, **Figure 8.7A**). In normal-appearing mucosa (**Figure 8.7B**), no significant effect between saline and La-Lf groups ($p = 0.177$) was observed. When

intestinal tumor tissues were analyzed for IBA-1-positive cell expression (**Figure 8.7F**), no significant difference was noted (p = 0.2, **Figure 8.7C**) between the saline and La-Lf groups. However, in normal-appearing mucosa, La-Lf (6.02 ± 0.6%) significantly reduced IBA-1 expression compared with saline (7.69 ± 0.9%, p = 0.014, **Figure 8.7D**).

8.5. Discussion

Extensive probiotic selection protocols were identified before the trial was undertaken. This included extensive screening and in vitro characterization of formulations and validation of their anti-tumorigenic effect in a CRC mouse model (18,19). We have established, heretofore, that oral administration of La-Lf probiotic formulation, a mixture L. acidophilus ATCC 314 and L. fermentum NCIMB 5221 in mice harboring adenomatous polyposis coli (APC) gene mutation-induced in a CRC animal model (ApcMin/+ mice), significantly attenuated intestinal tumorigenesis and downregulated cell proliferation in the epithelial cells. To unravel the mechanisms by which the La-Lf probiotic intervention provides its benefits, we focused on elucidating for the first time the effect of La-Lf-induced fluctuations in different biological compartments (plasma and stool) for different types of biomarkers: metabolic, inflammatory, and immunological. We identified a total of 190 fecal and plasma metabolites in $Apc^{Min/+}$ mice using a combination of two metabolomic methods (^{1}H NMR and DI/LC-MS/MS, **Figure 8.1**) and we looked at changes in plasma cytokines and intestinal tissue immune cell markers. Further, this study established that the probiotic-associated pro-inflammatory cytokine profile and several plasma and fecal biochemical pathways are expressed differently in probiotic-treated and untreated mice. To our knowledge, this is the first report investigating a probiotic treatment containing live active *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 in a genetically modified CRC model with $Apc^{Min/+}$ mice and indicating distinct CRC-related metabolic profiles induced by the probiotic formulation

A robust PLS-DA model based on these identified metabolites was able to distinguish all La-Lf-treated mice from controls, where 35 metabolites were differentially

expressed in La-Lf $Apc^{Min/+}$ mice. Compared with previous CRC anti-tumorigenic studies and different metabolomic findings (20-24), several key metabolic pathways in association with CRC, including , amino acid, glutathione, fatty acid metabolism, and gut microbial metabolism, were consistently disparate.

The PLS-DA models and heatmaps between treated and control mice revealed diverse metabolic signatures induced by the probiotic La-Lf treatment in feces of $Apc^{Min/+}$ mice (**Figure 8.2**), mainly involving the methyl group metabolism of the transmethylation process as indicated by changes in the related metabolites, namely, choline, methionine, methylamine, and methanol in the feces. Among the discriminant fecal metabolites of the La-Lf bacterial effect, the PLS-DA models displayed methanol with the highest discriminant power (**Table 8.1S**). Methanol, described as an important bacterially produced metabolite derived from gut microflora, was found elevated and considered to distinguish IBD patients from healthy subjects. Methanol was found to decline in the urine (25,26) and feces (27) of IBD patients compared with healthy individuals. Furthermore, methanol is used by fecal Methanobacteriales and Methanobrevibacterium to produce methane and all correlated with CRC development (28). Interestingly, in our study, fecal methanol levels were reconstituted after La-Lf biotherapy in $Apc^{Min/+}$ mice, corroborating a major mechanism of action robustly associated with microflora modulation and a potential decrease in methanogenic bacteria.

Unexpectedly, we found that lactate was enriched in the colon, a bacterial SCFA through which La-Lf could reduce colonic pH, decrease pathogen populations (29), and yield higher production of butyrate (30). Also, it was found that microbiota-derived lactate accelerates colon epithelial cell turnover in mice (31) and inhibit Helicobacter pylori NCTC 11637 (32).

Fecal succinate, a citric acid cycle intermediate and energy metabolite, on the other hand, was increased by La-Lf treatment, which is otherwise found at decreased levels in CRC (33) or IBD patients (34).

In line with these observations, La-Lf seemed to significantly upregulate the levels of fecal SCFA acetate. Acetate makes up around 60%−75% of the total SFCAs released in the lumen and absorbed by colonocytes as a primary energy source (35). Our data also support the hypothesis of a molecular mechanism where microbial fermentation of fibers (~3%) present in the diet used in this study yields SCFAs. These serve as the preferred energy source of normal colonocytes and supports homeostasis. Nonetheless, these also accumulate in cancerous colonocytes (36) due to the Warburg effect and functions as an HDAC inhibitor to deter proliferation and induce apoptosis. This may render tumor cells less resistant to apoptosis, and contribute to the probiotic microbe-induced anti-neoplastic effect (36). Acetate, the production of which was enhanced by La-Lf treatment, is known to cause mitochondrial apoptotic death in CRC cells (37). The role of acetate in the intestine maintained the proliferation of normal crypt cell while reducing muscle contractions in colonic smooth muscle (38), ileal motility, and colonic blood flow (39).

While some studies reported insignificant variations with some metabolites associated with bacterial metabolism, including glycerol and glucose, others did not show any significant correlation with colorectal cancer (40). Two important metabolites in our study, glycerol and glucose, were found at higher levels, but have previously received little attention as fecal metabolites. In addition, it was found that human faecal microbiota display variable patterns of glycerol metabolism that are related to tumor and inflammation (41).

Related reports explain that an increase in glycerol metabolite, associated in this study to upregulated glycolipid metabolism (42) in the case of an optimal ratio with glucose (43), not only affects the microbial community composition, but also increases the rate of conversion of substrates to beneficial levels of acetate and produces a significant shift in the Lactobacillus–Enterococcus population (44). In addition, it was found that human faecal microbiota display variable patterns of glycerol metabolism that are related to tumor and inflammation (41).

In the same context of glucose as a discriminatory metabolite, QEA shows that some of the most affected pathways were glycolysis metabolism, gluconeogenesis (45-47), and the insulin signaling glucose−alanine cycle (occur in the mitochondria (48)); all were found inter-linked and dysregulated in cancer patients. Glucose metabolism was found in general to connect gut microbiota with the endocannabinoid system as well (49).

Other reports show as well that glucose in gluconeogenesis was found depleted in the serum of the patients with metastatic CRC as well as lactate, pyruvate, alanine, and glutamine (50).

Interestingly, the only important metabolite that La-Lf biotherapy seemed to deplete was ethanol. Interestingly, ethanol is a substantial factor for the formation of colon carcinogenesis as its first oxidative metabolite is acetaldehyde, which is mutagenic and carcinogenic (51). Knowing that fecal bacteria are able to process ethanol to acetaldehyde (52), we can assume that La-Lf oral intake was able to limit this activity, thus lowering the risk of tumor formation.

The QEA detailed the biological effect of La-Lf and the pathways through which it affected the levels of metabolites. For the increase in phenylacetate by La-Lf, we can mention that this compound is also a drug that reduces glutamine availability in the blood, which explains the relevance of glutamate metabolism in the QEA (**Figure 8.6**). Phenylacetate inhibits the proliferation of tumor cells and promotes cell differentiation (53). In the case of CRC patients, the levels of several metabolites derived from microbial metabolism in the gut were altered, including phenylacetate in urine (54). Phenylacetate could arise from endogenous synthesis via β-oxidation of phenylbutyrate, phenylalanine metabolism (55), or through the intake of phenylaceticacid from plant-food sources (56). The gut microflora transforms phenylalanine to phenylacetate through endogenous enzymatic action. Phenylacetate is then conjugated with glycine to form phenylacetylglycine (57). This is an aromatic SCFA derivative and an HDAC inhibitor, detectable in human fecal water (58) from

498 the transformation of polyphenols in fruits and vegetables during GI passage (58,59).
499 In our study, rodent diet ingredients (**Table 8.1S**) were able to provide *Apc*$^{Min/+}$ mice
500 with levels of plant-source polyphenols (60). Factors alike may have assisted ingested
501 *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314-treated microflora to
502 restore phenylacetate levels in the feces, thus enhancing the transformation of dietary
503 polyphenols to phenylacetate.
504 Each of the fecal choline and sarcosine concentrations were found to be significantly
505 increased after La-Lf treatment (**Figure 8.5**). Sarcosine is known for its onco-
506 protective properties (61) and both factors are depleted in a choline-deficient diet (62).
507 Choline, increased by La-Lf supplementation (**Table 8.22**), is an essential nutrient
508 relevant in different metabolic processes (i.e., methyl group metabolism, cell
509 membrane structure and signaling, neurotransmitter synthesis, and lipid transport)
510 (63), which could be associated to the reason phospholipid biosynthesis was one of the
511 most affected pathways (**Figure 8.6**). Abnormal choline metabolism was associated
512 with proliferation, oncogenesis, tumor progression, and corresponding enzymes such
513 as choline kinase. A fecal concentration of choline depends on their conversion by gut
514 bacteria to methylamines and dietary intake as well as intestinal absorption (64).
515 Notwithstanding, choline was considered anti-inflammatory agent and was associated
516 with lower levels of inflammatory markers (65-67). In fact, we notice that
517 methylamine diminished in La-Lf mice stool (**Figure 8.2**) and negatively correlated
518 with choline. Adversely, dysbiosis-associated changes in bacterially produced
519 metabolites in IBD patients included lower levels of urinary (25) and higher levels of
520 fecal (27) methylamine (68). It has been stated that deficiencies in those transfer
521 factors, methionine and choline (69,70), induce colorectal tumors (71). Based on our
522 finding, this impaired status was reversed by La-Lf oral ingestion in *Apc*$^{Min/+}$ mice,
523 where choline and methionine levels increased with the decrease of methylamine
524 concentrations in the fecal matter.
525 It was established that administration of a diet deficient in methyl-donor nutrients, such
526 as folic acid, methionine, choline, and vitamin B12, are responsible for a 95%

reduction in tumor multiplicity in *Apc*$^{Min/+}$ mice (72). Here, betaine metabolism was one of the most affected by La-Lf since betaines (products of choline oxidation) are catalyzed to homocysteine, yielding DMG and methionine (**Table 8.4S**).

While probiotic La-Lf increased their levels, patients with CRC are known to have lower levels of leucine (50,73) and pyruvate (74). Here, pyruvate metabolism was one of the most affected pathways. Pyruvate, a highly promising therapeutic, was found to alleviate clinical symptoms of mitochondrial myopathy stroke-like episodes (MELAS) syndrome and other conditions (40). On the other hand, proline levels, increased by La-Lf digestion, have previously shown an anti-tumor effect in the CRC mice model (75). La-Lf not only affected proline, but had an impact on arginine and proline metabolism based on QEA analysis (**Figure 8.6**). Depleted proline in the serum of CRC patients had probably further induced arginine metabolism downregulation and reflected disturbed proline metabolism and degradation, which correlates with p53-dependent inhibition of apoptosis in CRC (76).

Conjointly, DI/LC-MS/MS identification of plasma metabolites showed an appreciable variation of systemic metabolites (**Table 8.6S** and **Figure 8.4**) after La-Lf treatment and affected different metabolic pathways (**Figure 8.5 and Table 8.7S).** Two hypermethylated metabolites of arginine, namely, *symmetric*-dimethylarginine (DMA) and *asymmetric*-DMA, were also progressively elevated in CRC. Conversely, the metabolic signature of lamina propria tissues in *Apc*$^{Min/+}$ was defined by a decrease in glycerophosphocholine and the gut-microbial co-metabolite DMA relative to wild type (23). For biogenic amines, methylarginines have been shown to interfere with nitric oxide (NO) formation by inhibiting nitric oxide synthase-asymmetric-DMA and cellular L-arginine uptake into the cell. The formation of methylarginines in mammalian cells is carried out by protein arginine methyltransferases (PRMTs), some of which are overexpressed in CRC (77), and for which somatic mutations were found in large intestine cancer (78).

It may not be considered coincidence that after La-Lf oral intake, plasma of *Apc*$^{Min/+}$ mice fluctuated in different glycerophospholipids (**Figure 8.4**). In fact, the

development of a lipogenic phenotype is one of the metabolic changes tumor cells undergo when progressing toward malignancy. Highly proliferating cancer cells display a status of deregulated lipogenesis when continually and robustly synthesizing fatty acids *de novo* to provide glycerophospholipids, particularly for membrane production (79). Neoplastic entities employ exogenous palmitate for the generation of structural and oncogenic glycerophospholipids, sphingolipids, and ether lipids (80). Other studies showed that oral and intravenous administration showed that some of those metabolites are effective in inhibiting the growth of tumors in Rag2M mice (81) However, several glycerophospholipids (82) have bioactive roles and were identified along with ether lipids (83,84) as being inversely associated with the aggressiveness of the cancer disease (80). Those facts showed the effectiveness of La-Lf in increasing those anti-CRC metabolites in plasma. For methionine sulfoxide, increased by La-Lf, it is a metabolite that was at a low level in rat renal failure (85). It was tested for anti-inflammatory activity as an oxidized product of methionine and was found to be the least active (86).

In view of La-Lf discriminant effects on the group of metabolites associated with immunoregulatory and anti-inflammatory responses, systemic or intestinal, a number of immune and inflammatory markers were reported. We hypothesized that La-Lf introduction would modify intestinal homeostasis and induce both gut microflora and the intestinal epithelium to produce an anti-inflammatory response. The potential of La-Lf to prevent the progression and development of CRC is also linked to its ability to modulate immune responses and reduce inflammation involved in tumor progression. In fact, commensal bacteria have emerged as cofactors in the development of ileocolitis and intestinal malignancies (87) as it can trigger inflammation in the intestinal tract (88). As chronic inflammation is increasingly recognized as a major contributor of CRC, probiotics can remodel inflammatory microbiota community for beneficial effects instead (89). In this assay, the most significantly affected inflammatory cytokines after La-Lf intervention were INF-γ, IL-1β, TNF-α, and KC/GRO ($p < 0.005$, **Figures 8.6A, 8.6B, 8.6G,** and **8.6J**). Similarly,

KC chemokines (mouse homologues of GRO) were found to be the principal chemokines induced by LPS and pro-inflammatory cytokines IL-1 and TNF-α via nuclear factor kappa B (NF-κB) signaling in ovarian surface epithelial cancer cells (90). Chemotactic cytokines are important in angiogenesis and attraction of immune cells and many GROs were overexpressed in CRC tissues (91). Moreover, although no statistical significant changes were concluded, the levels of IL-6 seemed to be slightly decreased. This suggested that La-Lf oral administration demonstrated significant anti-inflammatory effect through the gut. This conclusion is comparable with other research on VSL#3® probiotic treatment, where probiotics induced the downregulation of LPS-activated IL-8 production and the secretion of INF-γ, IL- 1β, and TNF-α, consistently with activation of PPARγ by the gut microflora (92). At the tissue level, CD3 T cells revealed a different degree of infiltration in the intestinal mucosa after La-Lf probiotic treatment ($p < 0.001$, **Figure 8.7A**) compared with the saline group. We observed IBA-a (marker of macrophages/microglia) in intestinal tumors ($p < 0.05$, **Figure 8.7D**). Probiotic La-Lf active formulation induced CD3 downregulation in tumors, whereas IBA-1 was less expressed in normal-appearing tissue. Hence, it would appear that oral administration of La-Lf affects immune responses in the intestinal epithelium, attenuates inflammation, and affects intestinal pathophysiology. Macrophages are involved in tumorigenesis with stimulating effects (93) associated with several factors, such as NO synthase, vascular endothelial growth factors, metalloproteinases, and other cytokines (e.g., TNF-α and IL-1α) (94,95). This may explain why PGE_2 enhances macrophage infiltration, leading to tumors when macrophages are activated by gut flora. La-Lf may have played a role in negatively regulating intestinal inflammation and inflammatory cells infiltration.

8.6. Conclusion

In summary, a panel of differentially expressed host−microbiota co-metabolites was identified in mice with CRC precursors and found to be related to several biomarkers validated in previous CRC metabolomics studies. This present study demonstrated that

plasma- and fecal-based metabolomics are able to discriminate probiotic-treated animals from untreated controls. We were able to advance our understanding of the immune and metabolic mechanisms underlying the cancer protective effect of probiotic supplementation. Similarly, we have identified the metabolic pathways where potential molecular or cellular targets can be recognized in the context of probiotic or different chemopreventive interventions. Our findings suggest that probiotic supplementation has a significant capacity to revert intestinal adenoma by regulating inflammatory cell functions, shifting intestinal microbiota functional status, and repressing tumor cell proliferation. This was similarly demonstrated in other metabolomic studies with different health conditions, where several metabolic pathways where affected by the functional state of gut microbes (96). This goes hand-in-hand with observations on the effect of *L. acidophilus* ATC 314 and *L. fermentum* NCIMB 5221 to attenuate chronic inflammation in rats and mice, delaying the pro-tumor inflammatory factors. Moreover, La-Lf could be considered beneficial for CRC-associated chronic intestinal inflammation in IBD with augmented mucosal permeability and relentless impairment along the gastrointestinal tract. La-Lf appears to exert its beneficial effects by multiple potential mechanisms of action and represents a promising strategy for novel and selective anti-cancer biotherapies that need to be explored and addressed in our future studies.

8.7. Acknowledgements

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8.8. Conflicts of interest

No authors have conflicts of interest to disclose.

8.9. Figures and tables

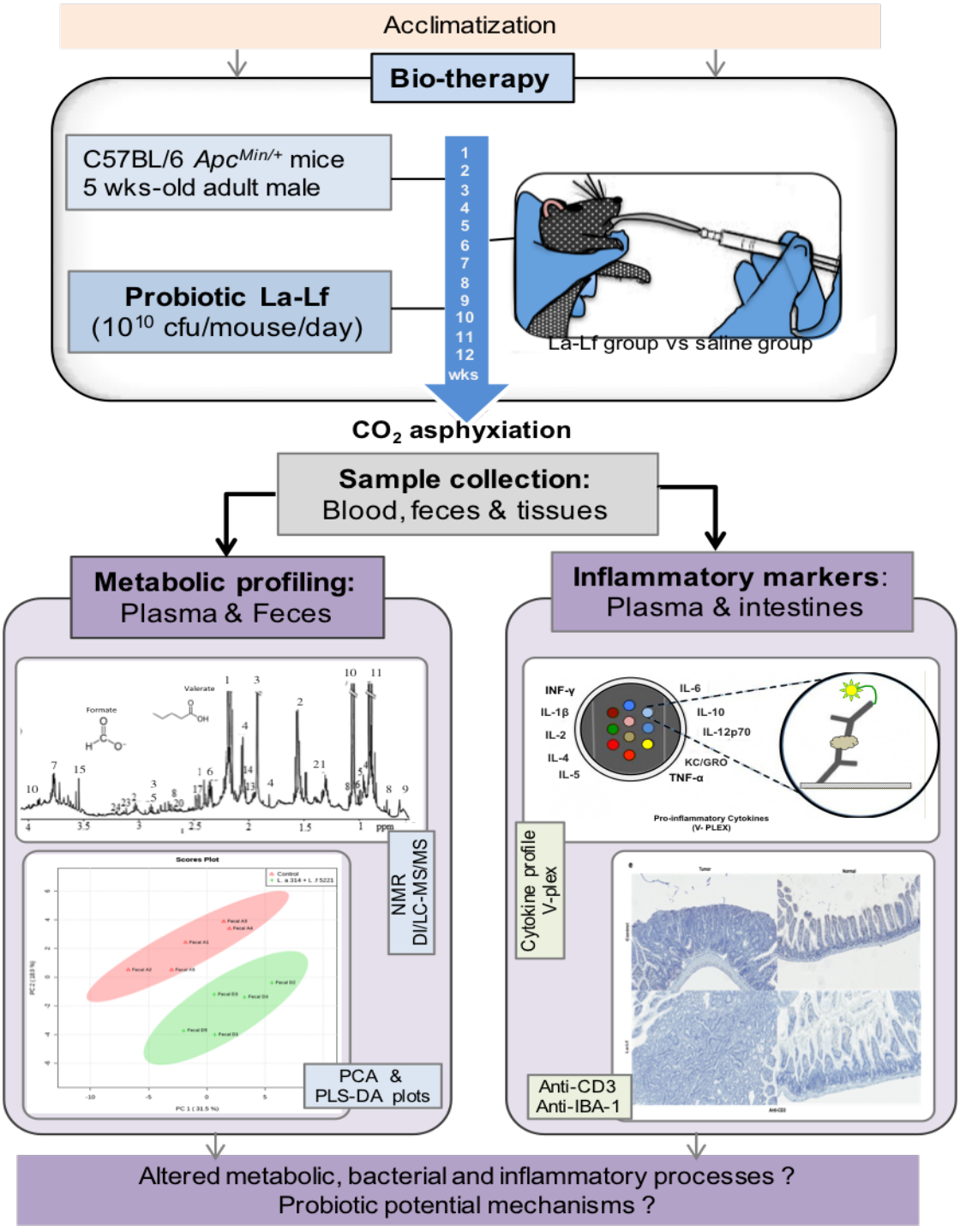
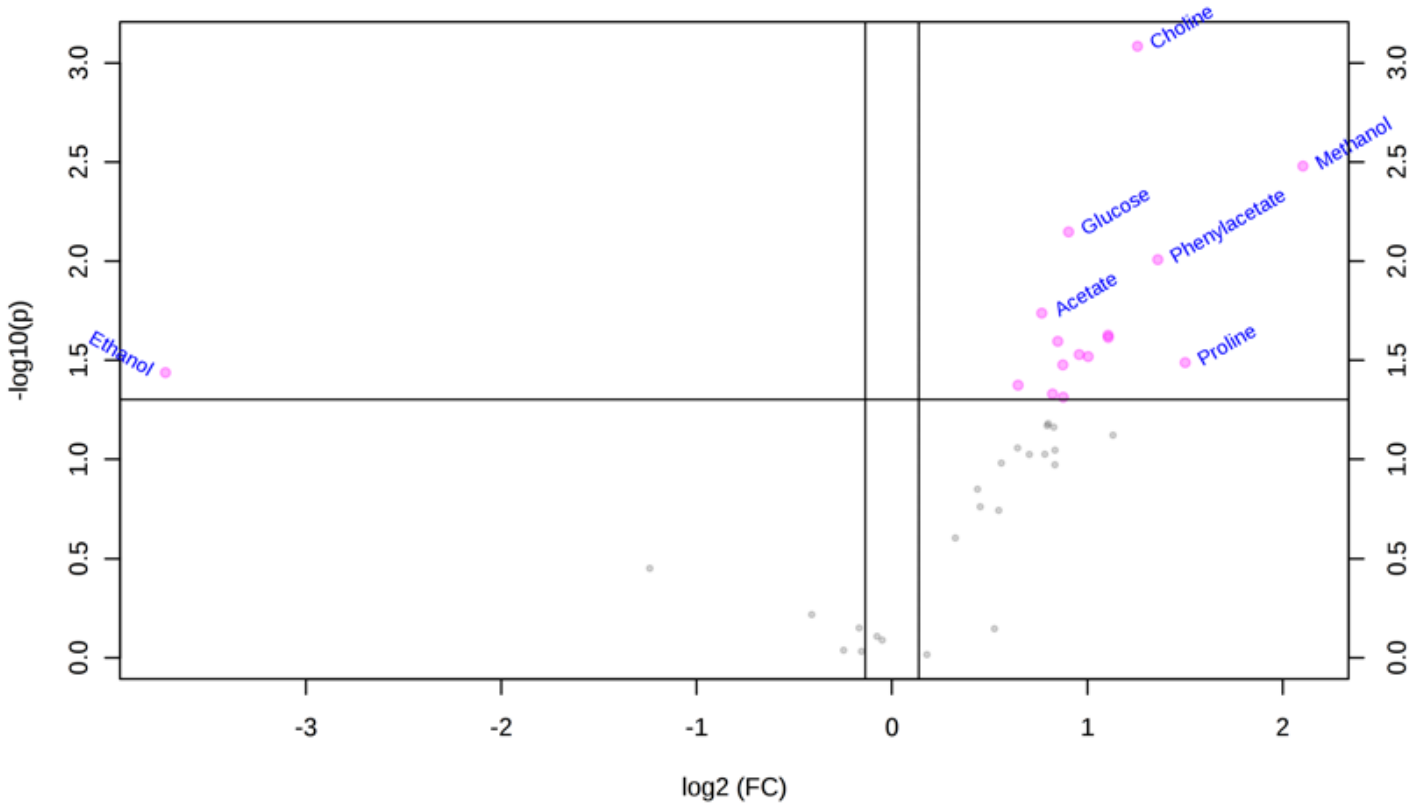


Figure 8.1 : Overview of the study design to research the metabolic and anti-inflammatory outcomes of La-Lf probiotic biotherapy in the CRC *Apc*$^{Min/+}$ mouse model.

Experimental workflow for immune and metabolomic data generation and analysis. La-Lf probiotic mixture (*L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314) was orally administrated to C57BL/6J *Apc*$^{Min/+}$ mice (10^{10} cfu/mouse/day, 12 weeks). Mice stool was profiled with ^{1}H NMR and plasma samples were analyzed by DI/LC-MS/MS. In addition, plasma was analyzed for pro-inflammatory cytokine profile and the intestinal tissues (tumor or non-neoplastic) were stained for the expression of immune cell inflammatory marker.

A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Methanol
Glucose
Phenylacetate
Acetate
Proline
Ethanol
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log2 (FC)
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1.5
2.0
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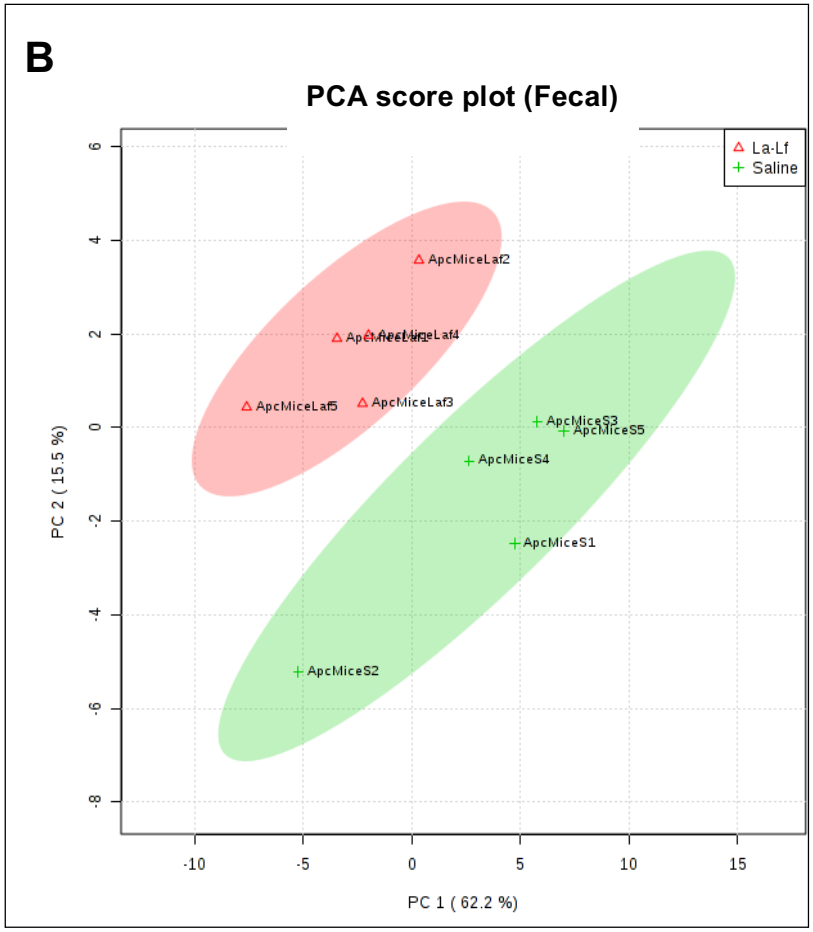
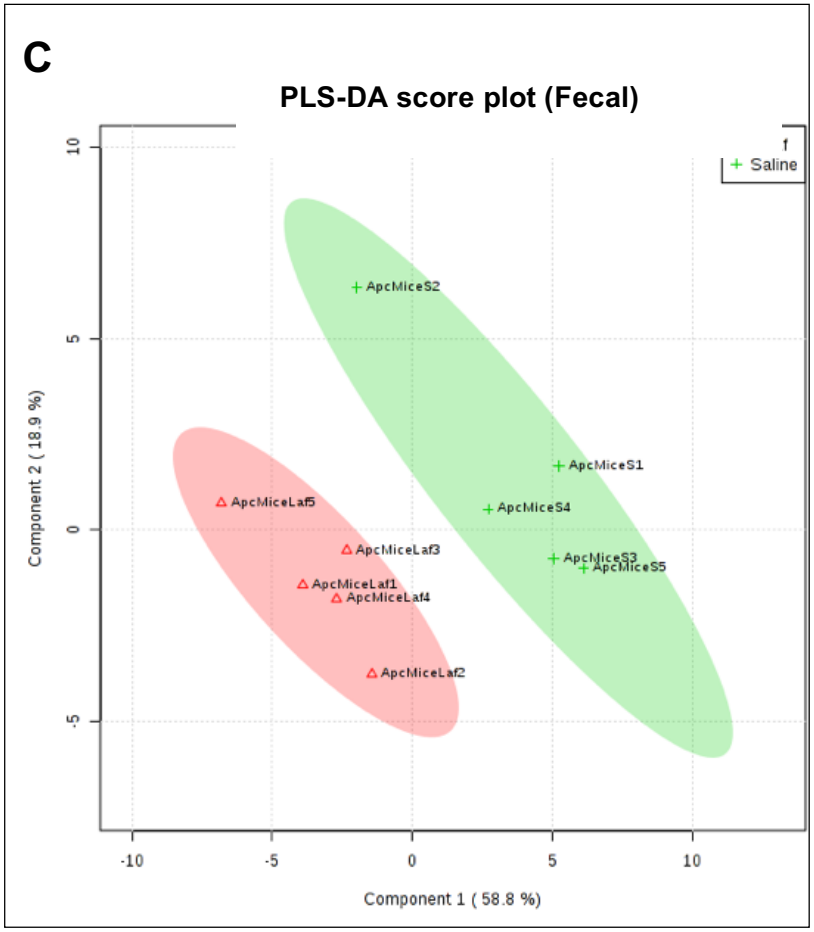
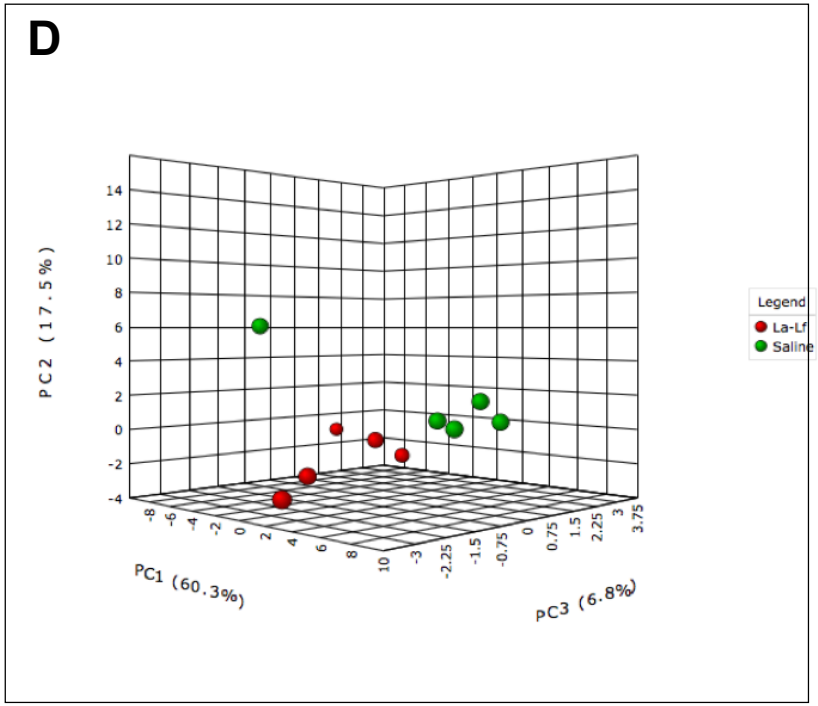
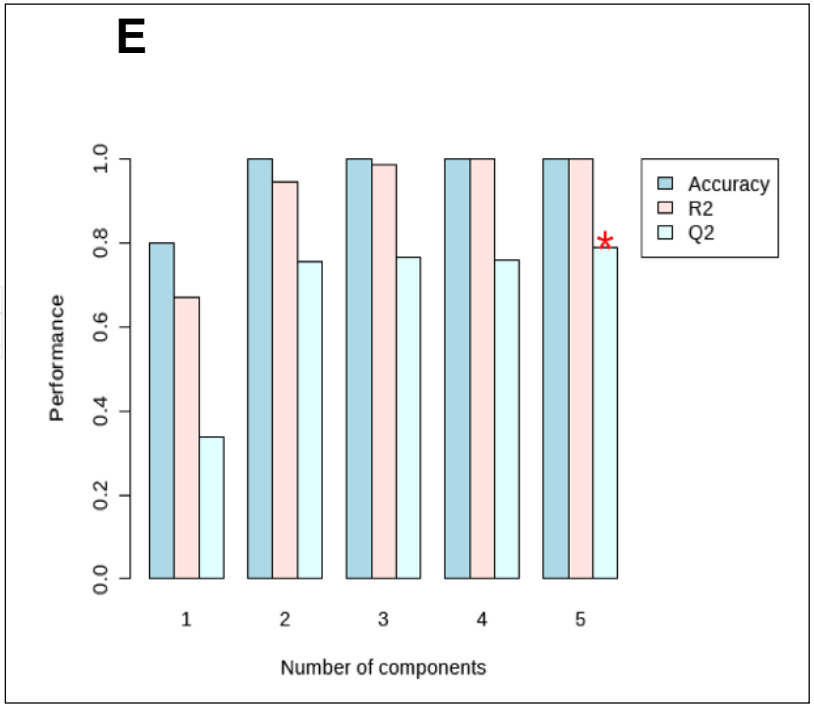
1
2


B
PCA score plot (Fecal)
La-Lf
Saline
ApcMiceLaf2
ApcMiceLaf4
ApcMiceLaf5
ApcMiceLaf3
ApcMiceS3
ApcMiceS5
ApcMiceS4
ApcMiceS1
ApcMiceS2
PC 2 (15.5 %)
PC 1 (62.2 %)


C
PLS-DA score plot (Fecal)
f
Saline
ApcMiceS2
ApcMiceS1
ApcMiceS4
ApcMiceLaf5
ApcMiceLaf3
ApcMiceS3
ApcMiceS5
ApcMiceLaf1
ApcMiceLaf4
ApcMiceLaf2
Component 2 (18.9 %)
Component 1 (58.8 %)


D
PC2 (17.5%)
PC1 (60.3%)
PC3 (6.8%)
Legend
La-Lf
Saline


E
Performance
Number of components
Accuracy
R2
Q2

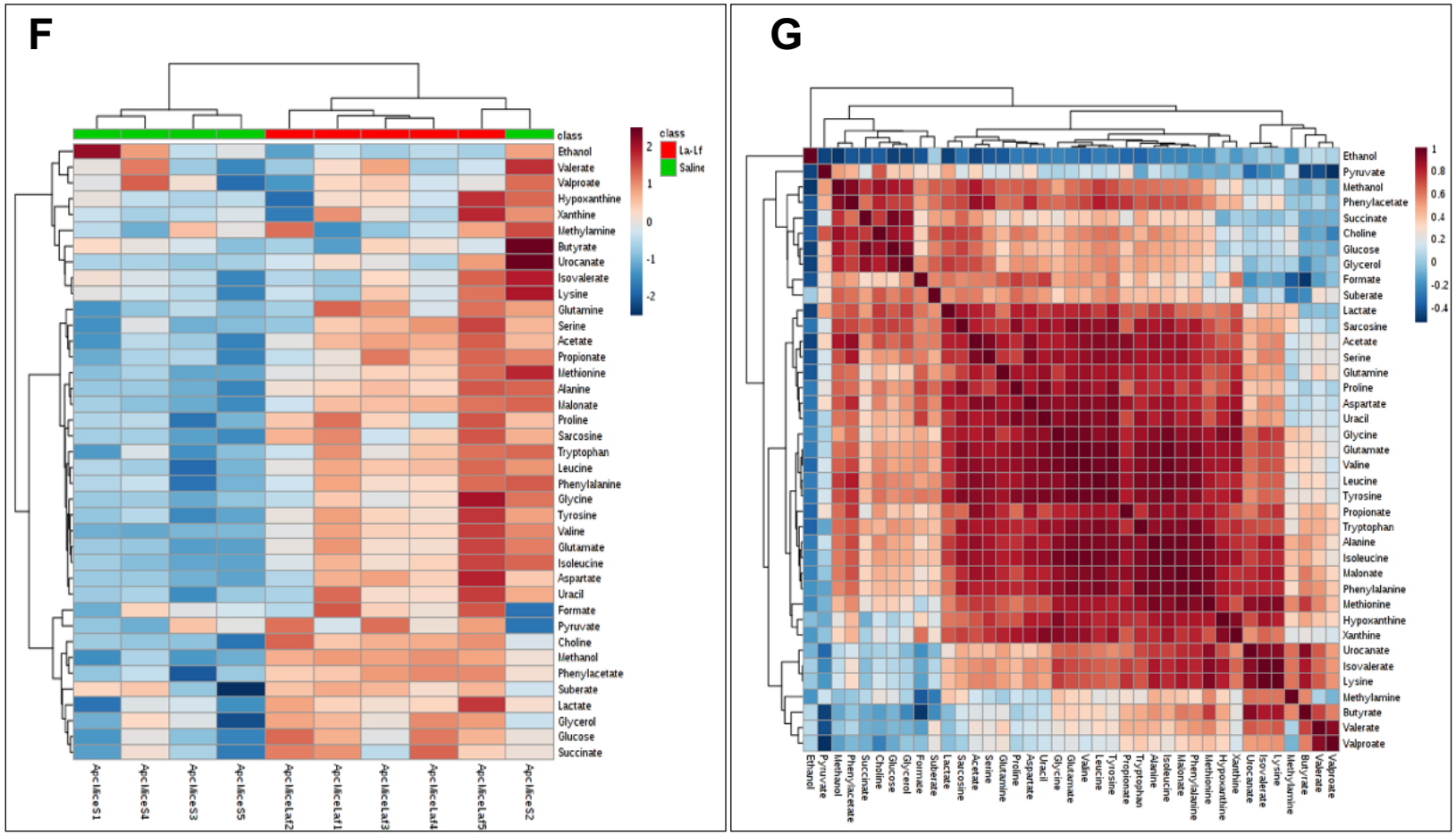


Figure 8.2. Univariate analysis and multivariate analysis on the concentrations of endogenous metabolites in La-Lf-treated mice fecal water compared with saline group. (A) After orally administrating La-Lf probiotic formulation to C57BL/6J-*Apc*$^{Min/+}$ CRC mice, fecal water was extracted and analyzed using ^{1}H NMR spectroscopy. Volcano plot was generated to analyze the significant variation in individual metabolites quantified. Data represent the mean SEM per group (n = 5). La-Lf: a mixture of *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314. Overview of differences in fecal metabolic profiles. Separation between probiotic-fed and saline mice describes the score plots along principal components (PCs) generated from **(B)** PCA and **(C)** PLS-DA of ^{1}H fecal water extracts from La-Lf (L. f 5221 + L. a 314) and saline mice, to compare the fecal

metabolome of from controls (red triangles) with La-Lf-treated *Apc*$^{Min/+}$ mice (green plus signs) (n = 5). "+": La-Lf = La 314 + Lf 5221. "Δ": Control (Saline). **(D)** 3D scores plot between the selected PCs. The explained variances are shown in brackets. (**E**) PLS-DA classification using different number of components. The red star indicates the best classifier. (**F**) Unsupervised hierarchical clustering plot as heatmap for control (saline) and probiotic La-Lf groups of *Apc*$^{Min/+}$ mice of the most important 25 fecal metabolites. A heatmap was constructed using the 25 most relevant metabolites. The metabolites and samples were hierarchically grouped by the Ward algorithm using Euclidian distance. Each column represents a unique feature with a characteristic concentration. As shown in the heatmap alignment, the two groups were clustered by an unsupervised algorithm, which confirms the presence of discriminating features between La-Lf and control groups. (**G**) Heatmap exemplification of the inter-fecal-metabolite correlations, in response to the probiotic La-Lf treatment in *Apc*$^{Min/+}$ mice. Correlations coefficients were calculated based on Pearson's correlation.

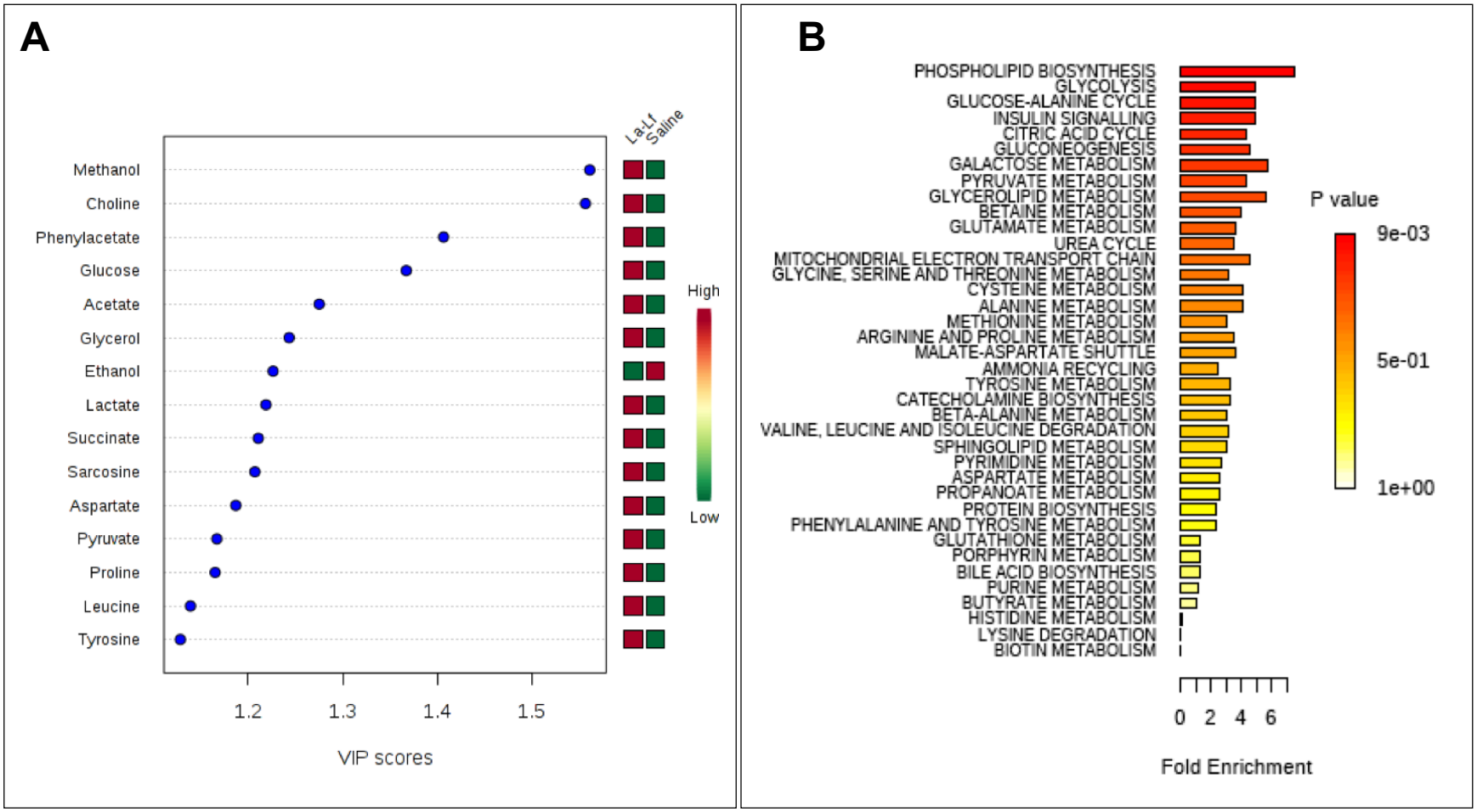
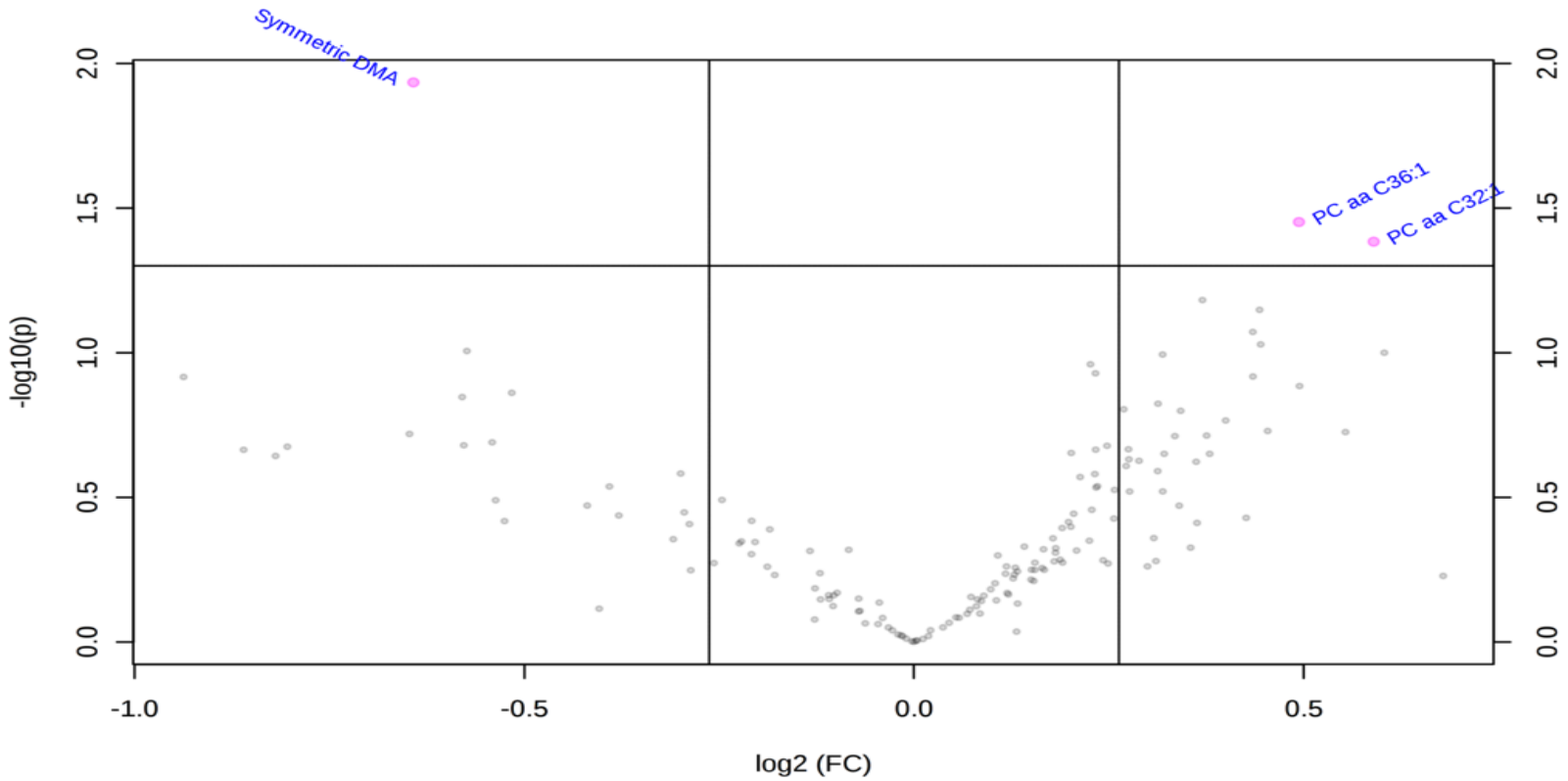


Figure 8.3: **(A) Determination of the most significant fecal metabolites that were affected by La-Lf oral administration to *Apc*$^{Min/+}$ mice. The VIP plot from PLS-DA modeling of DI/LC-MS/MS data is presented. (B) Summary plot of quantitative enrichment analysis (QEA), which ranks the most affected metabolic pathways depending on the fold change in metabolite concentrations and statistical significance.**

A


Symmetric DMA
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PC aa C32:1
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log2 (FC)
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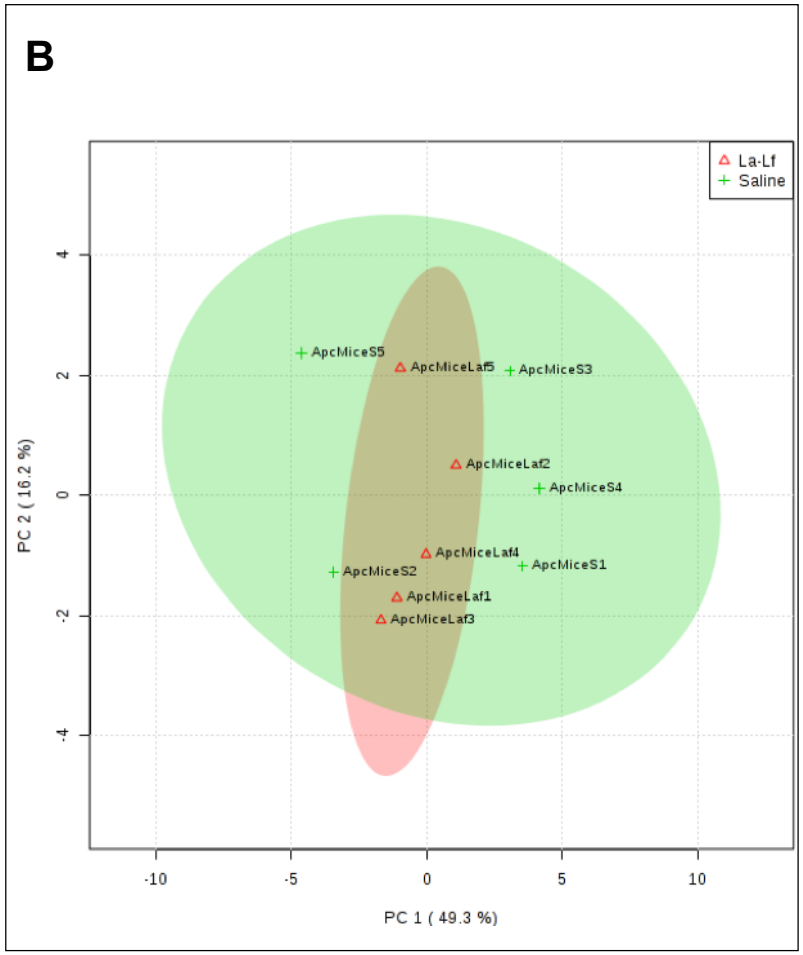
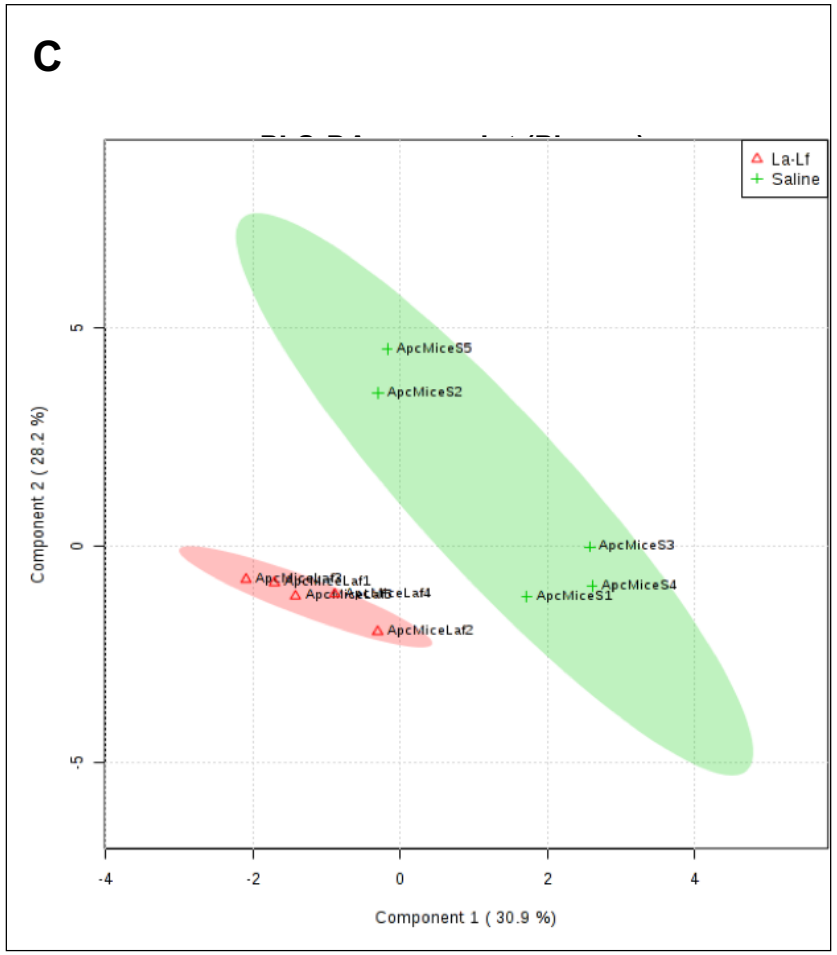
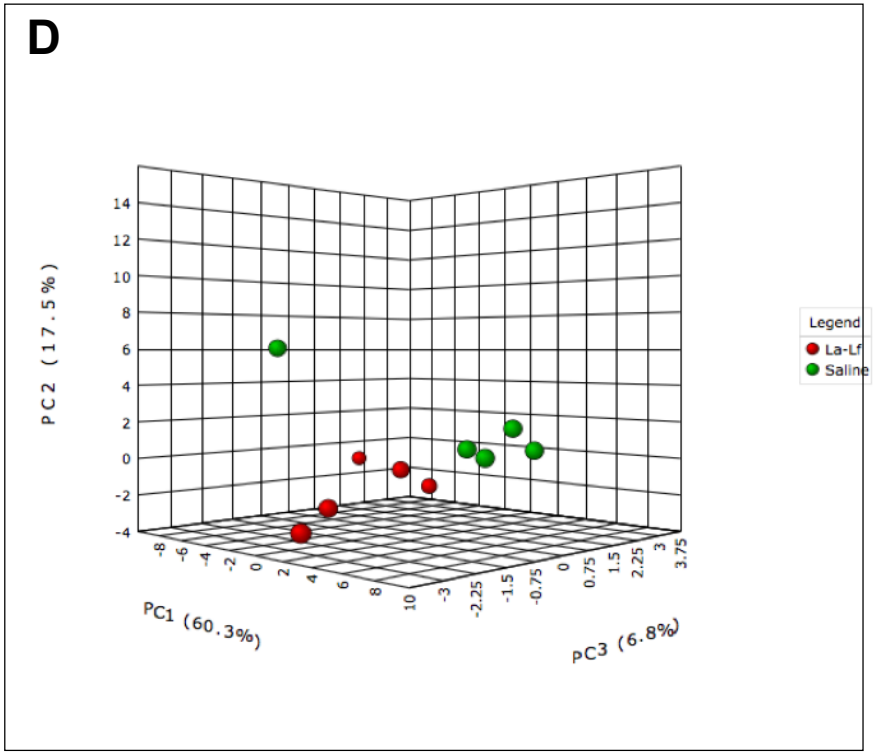
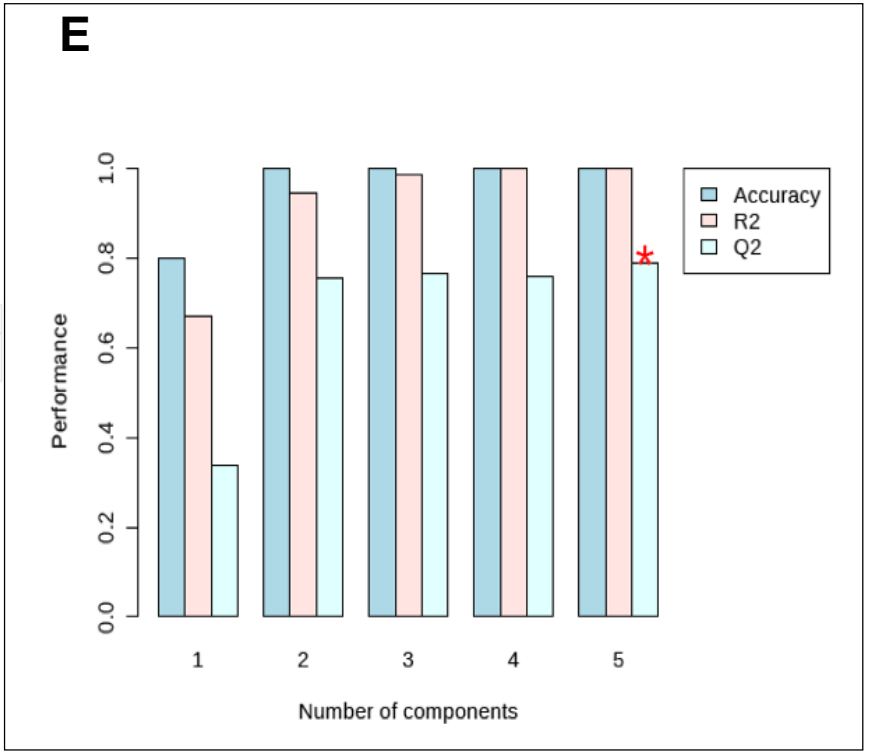
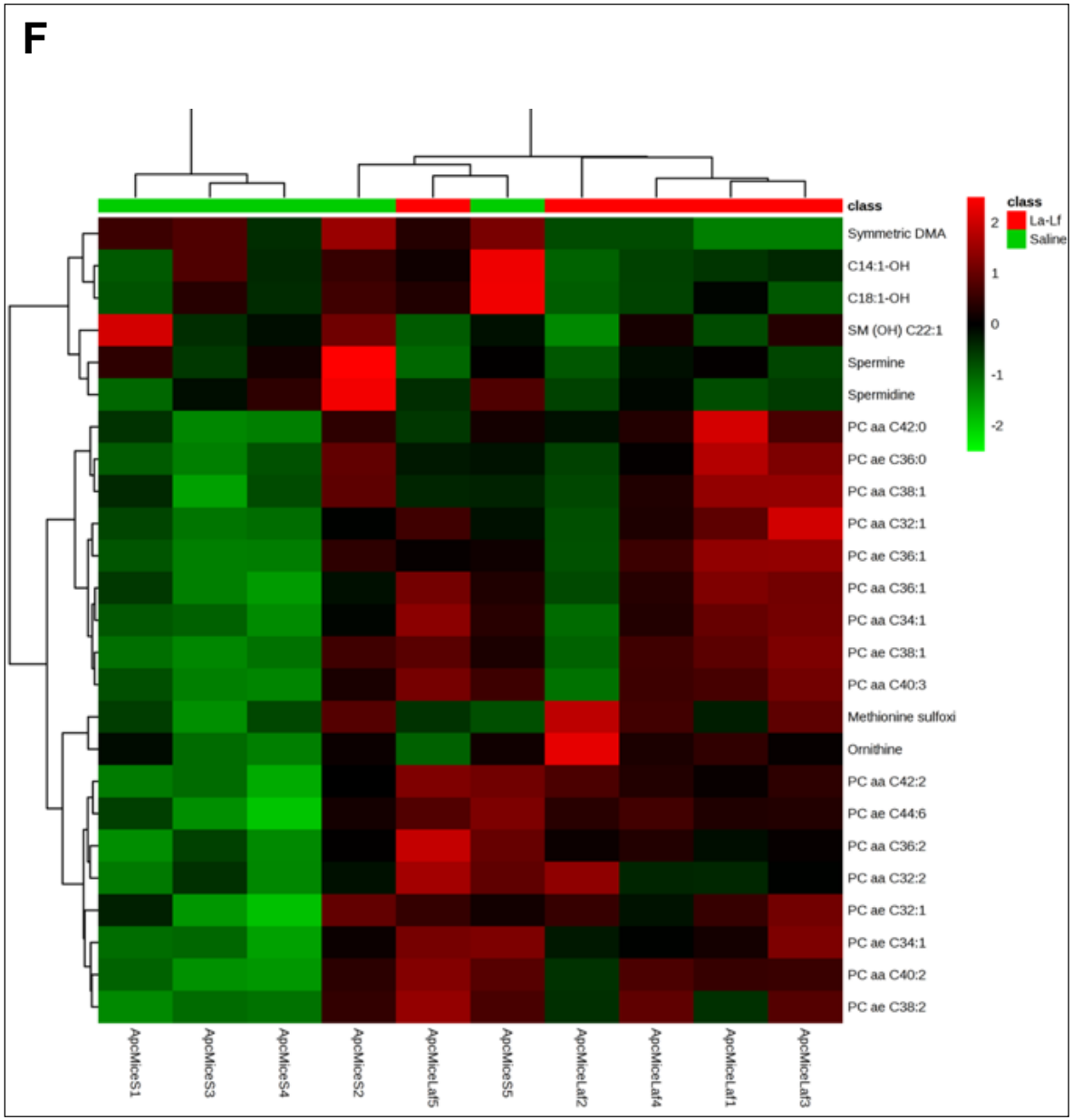
1.5
1.0
0.5
0.0
-1.0
-0.5
0.0
0.5


B
La-Lf
Saline
ApcMiceS5
ApcMiceLaf5
ApcMiceS3
ApcMiceLaf2
ApcMiceS4
ApcMiceLaf4
ApcMiceS2
ApcMiceS1
ApcMiceLaf1
ApcMiceLaf3
PC 2 (16.2 %)
PC 1 (49.3 %)
-10
-5
0
5
10
4
2
0
-2
-4


C
La-Lf
Saline
ApcMiceS5
ApcMiceS2
ApcMiceS3
ApcMiceS4
ApcMiceS1
ApcMiceLaf2
Component 2 (28.2 %)
Component 1 (30.9 %)
-4
-2
0
2
4
5
0
-5


D
PC2 (17.5%)
PC1 (60.3%)
PC3 (6.8%)
Legend
La-Lf
Saline


E
Performance
Number of components
Accuracy
R2
Q2
*


F
class
Symmetric DMA
C14:1-OH
C18:1-OH
SM (OH) C22:1
Spermine
Spermidine
PC aa C42:0
PC ae C36:0
PC aa C38:1
PC aa C32:1
PC ae C36:1
PC aa C36:1
PC aa C34:1
PC ae C38:1
PC aa C40:3
Methionine sulfoxi
Ornithine
PC aa C42:2
PC ae C44:6
PC aa C36:2
PC aa C32:2
PC ae C32:1
PC ae C34:1
PC aa C40:2
PC ae C38:2
ApcMiceS1
ApcMiceS3
ApcMiceS4
ApcMiceS2
ApcMiceLaf5
ApcMiceS5
ApcMiceLaf2
ApcMiceLaf4
ApcMiceLaf1
ApcMiceLaf3
class
La-Lf
Saline
2
1
0
-1
-2

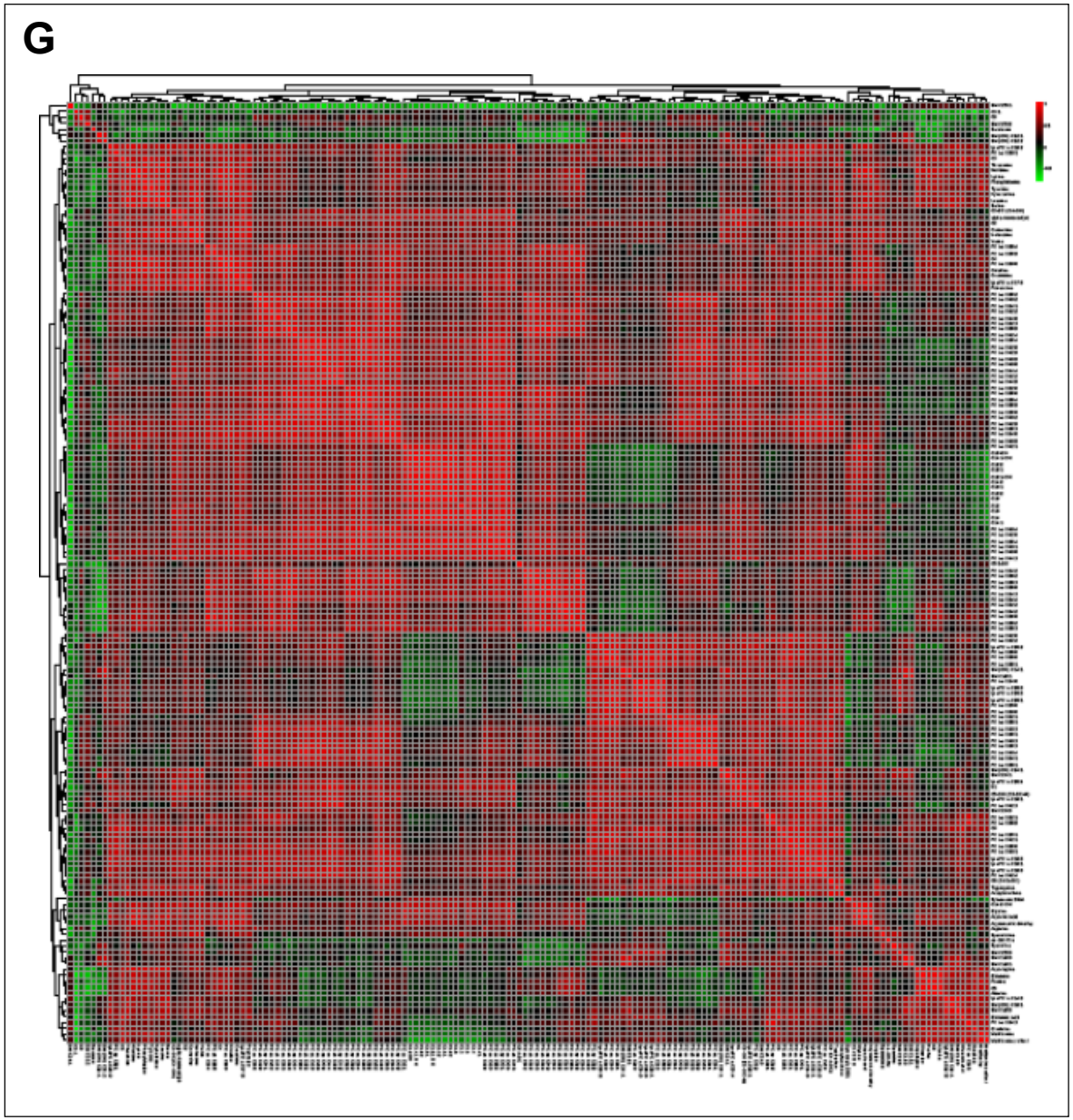

G

Figure 8.4: Univariate and multivariate analysis of metabolites composition in La-Lf *Apc*$^{Min/+}$ plasma.

(A) Volcano plot for plasma metabolite composition of La-Lf-treated mice plasma in a wide range of endogenous metabolites (acylcarnitines, glycerophospholipids, sphingolipids, amino acids, and biogenic amines). La-Lf: a mixture of *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314. After orally administrating La-Lf probiotic formulation to C57BL/6J *Apc*$^{Min/+}$ CRC mice for 12 weeks, plasma was collected and analyzed using DI/LC-MS/MS. Data represent the mean ± SEM per group (n = 5). Examination of dissimilarities in plasma metabolic profiles for the parting of probiotic-fed and saline mice. (**B**) and (**C**) Score plots along principal components (PCs) generated from PCA and PLS-DA of plasma metabolome from DI/LC-MS/MS spectra to compare controls (red triangles) with La-Lf-treated *Apc*$^{Min/+}$ mice (green plus signs) (n = 5). "+": La-Lf = La 314 + Lf 5221. "Δ": Control (saline). (**D**) PLS-DA classification using different number of components. The red star indicates the best classifier. (**E**) 3D scores plot between the selected PCs. The explained variances are shown in brackets. Hierarchical cluster analysis for plasma metabolome.(**F**) Unsupervised hierarchical clustering plot as heatmap for control (saline) and probiotic La-Lf groups of *Apc*$^{Min/+}$ mice of the most important 25 plasma metabolites. A heatmap was constructed using the 25 most relevant metabolites. The metabolites and samples were hierarchically clustered using Euclidian distance (Ward algorithm). (**G**) Heatmap exemplification of the inter-metabolite correlations in response to the probiotic La-Lf treatment in *Apc*$^{Min/+}$ mice, based on Pearson's correlation.

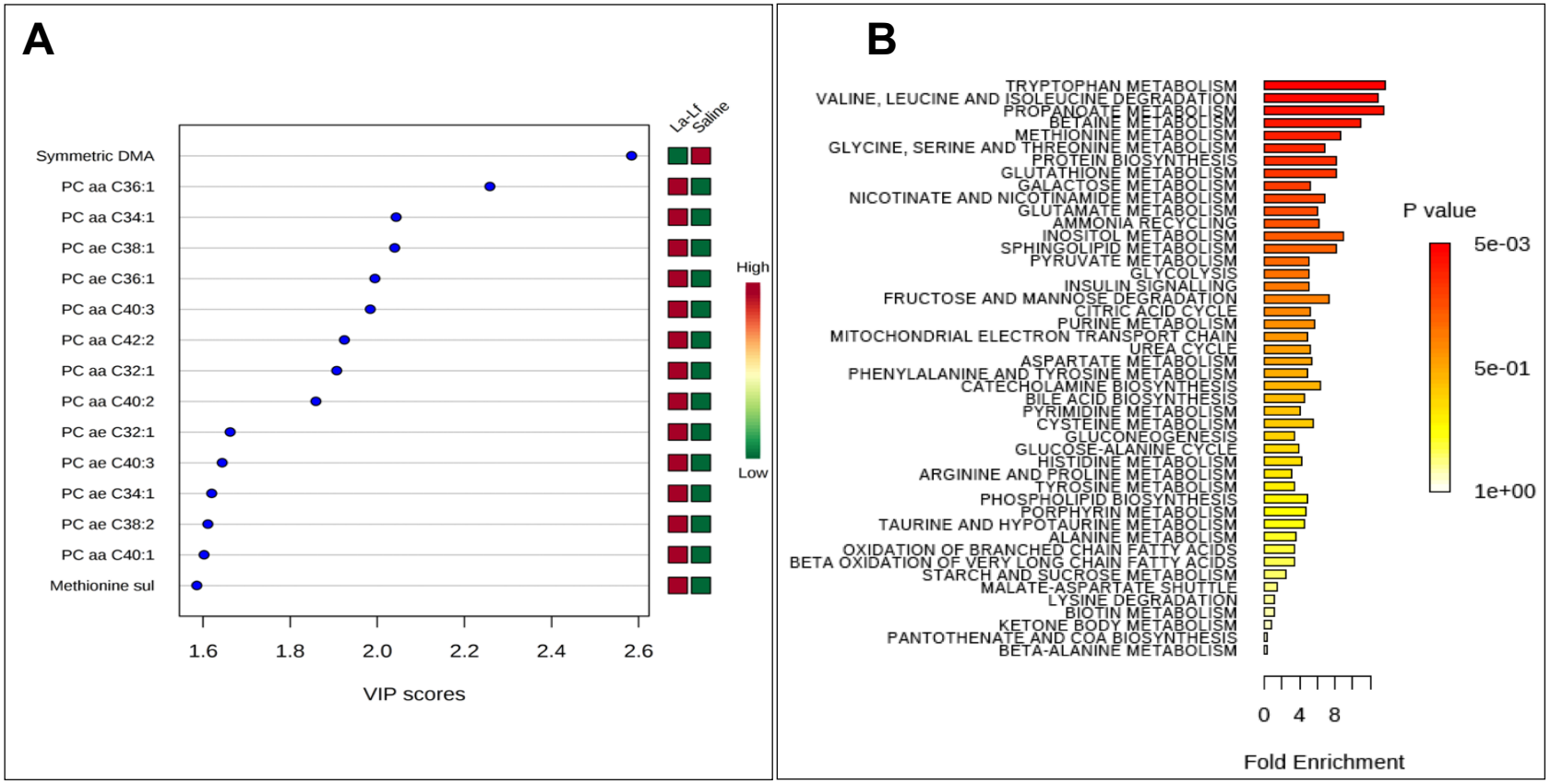


Figure 8.5: (A) Determination of the important metabolites in the plasma that were affected the most by the oral administration of La-Lf treatment to *Apc*$^{Min/+}$ mice. This presents the VIP plot from PLS-DA modeling of DI/LC-MS/MS data. The data present measurements of five replicates per group (n = 5). La-Lf: a mixture of *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314. La 314 + Lf 5221: La-Lf. **(B) Arrangement of the most enriched systemic metabolic pathways in *Apc*$^{Min/+}$ mice after La-Lf biotherapy.** Each enriched metabolism is rated based on its fold change and significance.

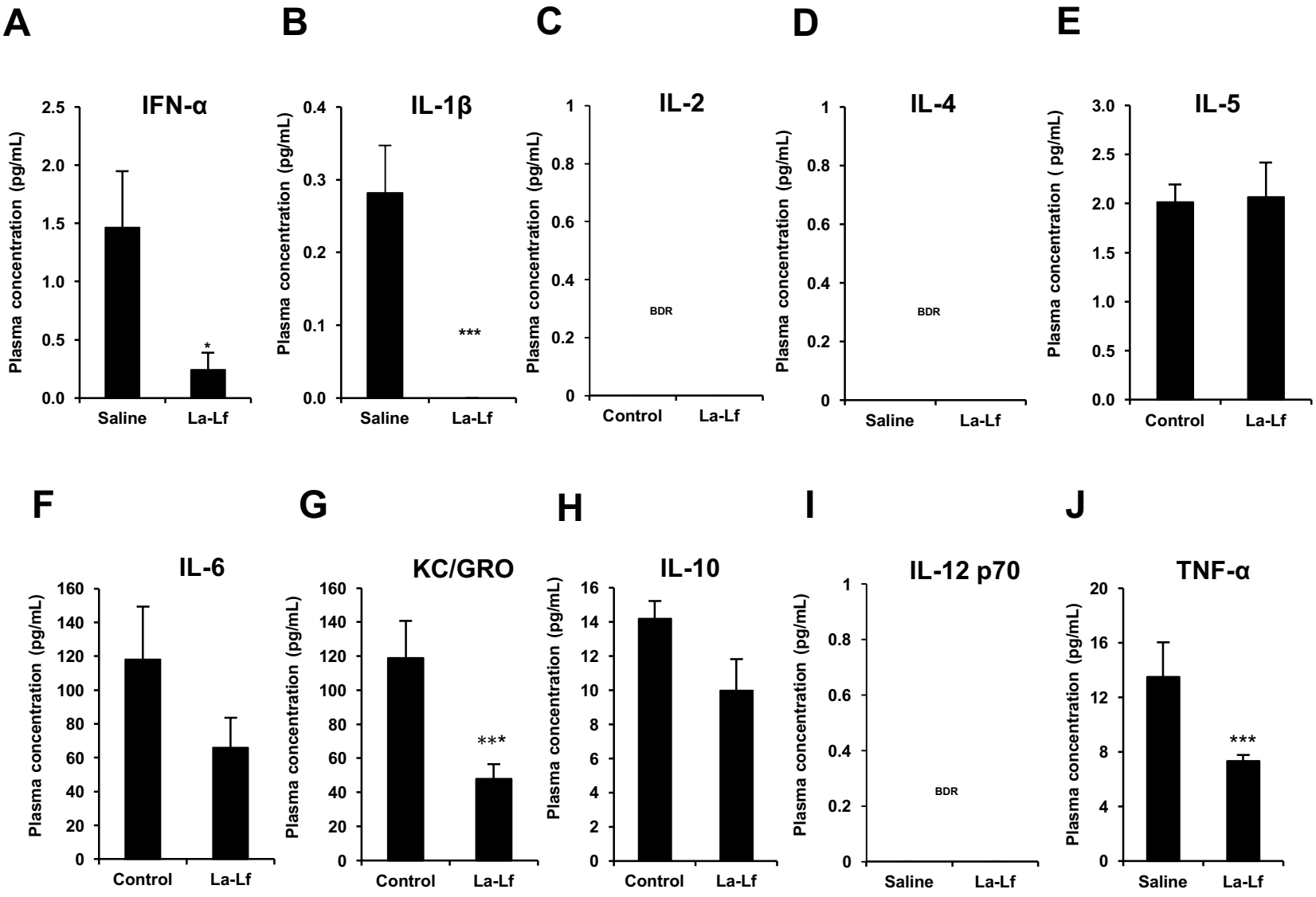
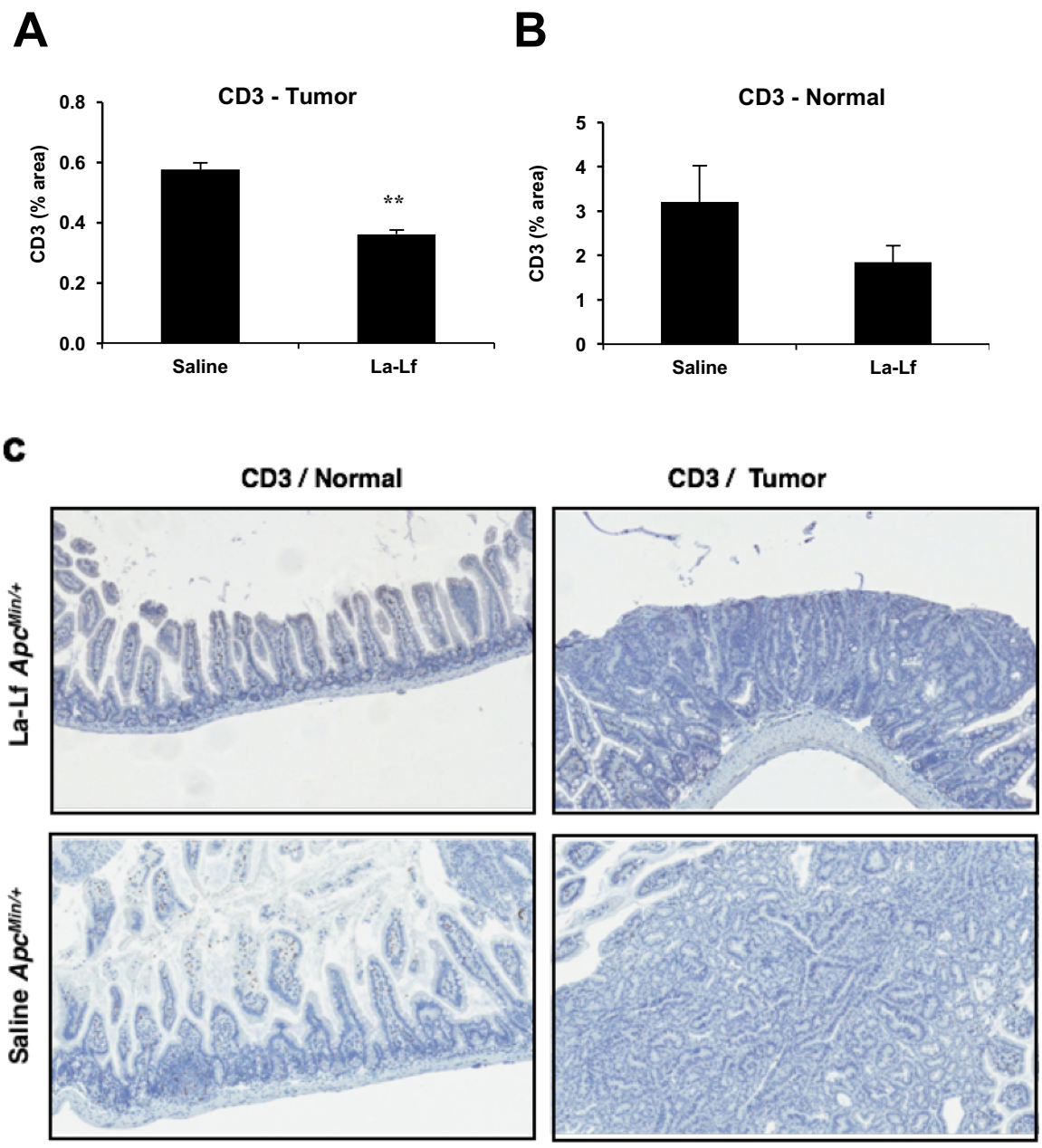

A
IFN-α
Plasma concentration (pg/mL)
2.5
2.0
1.5
1.0
0.5
0.0
*
Saline
La-Lf
B
IL-1β
Plasma concentration (pg/mL)
0.4
0.3
0.2
0.1
0.0
***
Saline
La-Lf
C
IL-2
Plasma concentration (pg/mL)
1
0.8
0.6
0.4
0.2
0
BDR
Control
La-Lf
D
IL-4
Plasma concentration (pg/mL)
1
0.8
0.6
0.4
0.2
0
BDR
Saline
La-Lf
E
IL-5
Plasma concentration (pg/mL)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
La-Lf
F
IL-6
Plasma concentration (pg/mL)
160
140
120
100
80
60
40
20
0
Control
La-Lf
G
KC/GRO
Plasma concentration (pg/mL)
160
140
120
100
80
60
40
20
0
***
Control
La-Lf
H
IL-10
Plasma concentration (pg/mL)
16
14
12
10
8
6
4
2
0
Control
La-Lf
I
IL-12 p70
Plasma concentration (pg/mL)
1
0.8
0.6
0.4
0.2
0
BDR
Saline
La-Lf
J
TNF-α
Plasma concentration (pg/mL)
20
16
12
8
4
0
***
Saline
La-Lf

Figure 8.6: Effect of La-Lf probiotic bio-intervention on the plasma pro-inflammatory cytokine profile of *Apc*$^{Min/+}$ mice.

The evaluation of the levels of pro-inflammatory cytokines in plasma of C57BL/6J-*Apc*$^{Min/+}$ mice after oral administration of *L. acidophilus* ATCC 314 mixed with *L. fermentum* NCIMB 5221 (total = 1 x 10^{10} cfu/mL) for 12 weeks. The cytokines assessed in the plasma were **(A)** INF-γ, **(B)** IL-1β, **(C)** IL-2, **(D)** IL-4, **(E)** IL-5, (**F**) IL-6, (**G**) KC/GRO, (**H**) IL-10, **(I**) IL-12p70, and (**J**) TNF-α. * $p < 0.05$. Data present measurements of five replicates per group ($n = 5$). (BDR: below detection range)


A
CD3 - Tumor
CD3 (% area)
0.8
0.6
0.4
0.2
0.0
**
Saline
La-Lf
B
CD3 - Normal
CD3 (% area)
5
4
3
2
1
0
Saline
La-Lf
C
CD3 / Normal
CD3 / Tumor
La-Lf Apc^Min/+
Saline Apc^Min/+

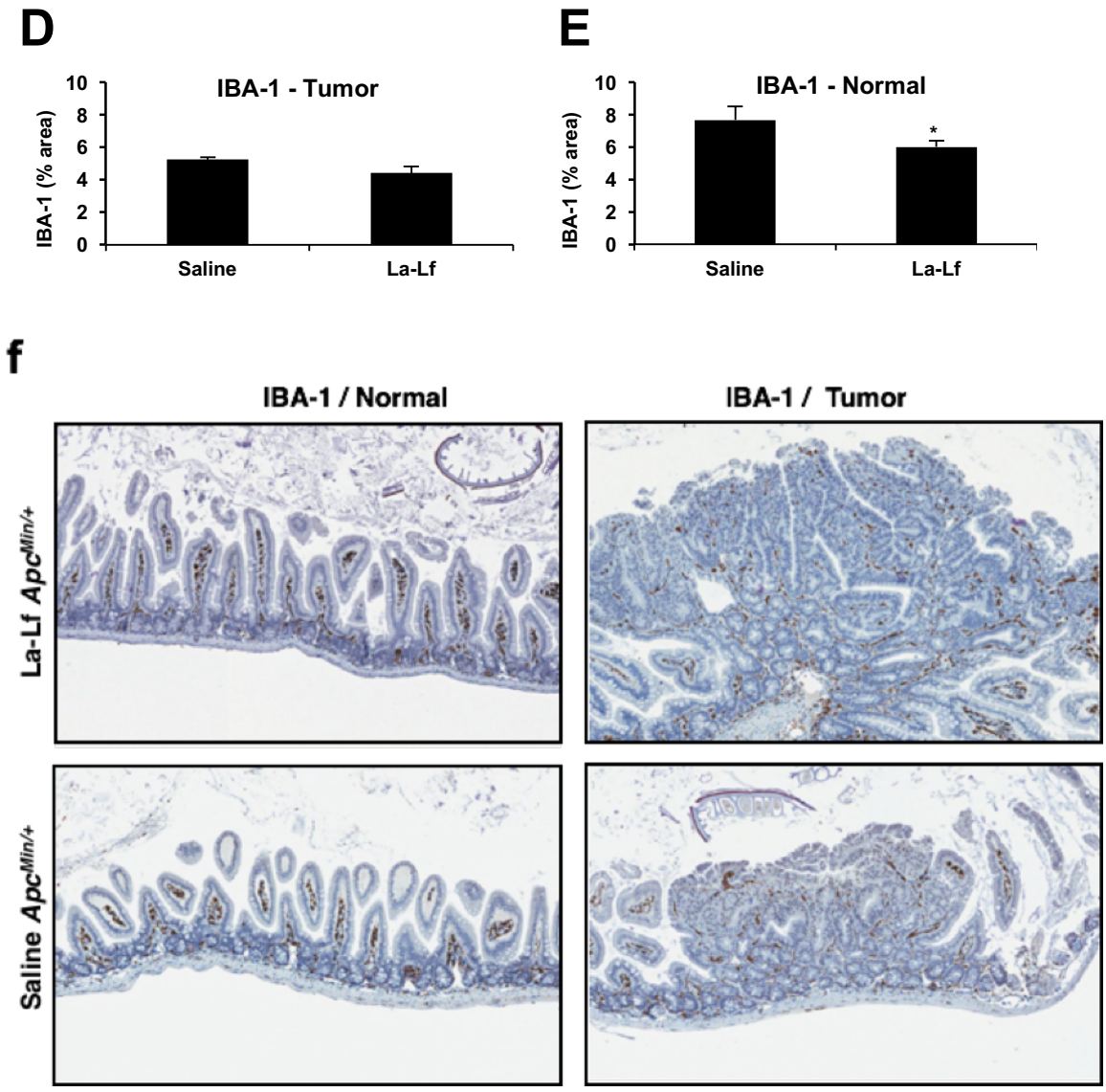


Figure 8.7: Orally administrated *Lactobacillus* active formulation of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 modulates the expression of IBA-1 and CD3 inflammatory markers in the intestines of $Apc^{Min/+}$ mice.

The percentage of stained cells in intestinal sections for: **(A)** and **(B)** anti-CD3, and **(C)** and **(D)** anti-IBA-1, respectively, in tumor and normal-appearing tissues. Section of villi from $Apc^{Min/+}$ mice with immunohistochemical staining of the intestine with macrophage-specific antibodies (IBA-1), demonstrating the effect of La-Lf probiotic treatment on the expression of **(E)** CD3 and **(F)** IBA-1 in $Apc^{Min/+}$ mice after oral treatment for 12 weeks. The intestines were stained using antibodies and counterstained with hematoxylin. Dark-colored nuclear staining indicates positive cells. Cells with blue nuclei are negative. Data are presented as mean ± SEM ($n = 5$). * $p < 0.05$ compared with untreated animals, using independent samples t-test. Images are at 100x magnification.

8.10. Supplementary Material and Methods

8.10.1. Probiotic oral treatment preparation

To prepare a probiotic dose that delivers enough active cells to the colon, a total of 1010 cfu per animal was prepared daily as suggested in previous studies (92). Each of L. acidophilus ATCC 314 (Cedarlane Laboratories, Burlington, ON, Canada) and L. fermentum NCIMB 5221 (NCIMB, Aberdeen, Scotland, UK) bacterial cultures were maintained in MRS broth for 16 h (37 °C, 5% CO2). After three passages, cultures were allowed to grow for eight hours in MRS broth (37°C, 5% CO2) and the bacterial pellets were harvested by centrifugation (1000 x g, 15 min, 4°C) and washed with PBS.

8.10.2. Preparation of fecal samples for ^{1}H NMR

For ^{1}H NMR analysis of *Apc*$^{Min/+}$ mice fecal samples, the extraction of fecal water was performed after La-Lf intervention as described by Costabile et *al.* (14), with minor modifications. Mice stool samples were finely powdered in liquid nitrogen and quickly transferred to an Eppendorf tube. Fecal water was extracted at 1:1 (w/v) in ice-cold PBS (1 M, pH 7.4). The use of this buffer during extraction allowed the reduction of chemical shift inconsistency between samples. Homogenization was performed in a stomacher bag (filtra-bag, VWR, Edmonton, AB, Canada, 5 min) followed by sonication (4°C, 20 min). The samples were further subjected to vortex shaking (250 rpm, 20 min) and ultracentrifugation (15000 x g, 1 h, 4°C, Beckman Optima L90K Ultracentrifuge; Beckman Limited, High Wycombe, U.K.) to ensure the precipitation of any particulate residual fecal matter and to obtain the pure extract as a supernatant. was transferred. The supernatants were carefully removed and stored in a 1.5 mL Eppendorf tubes at -20 °C, until analysis. To prepare sample for the NMR analysis, a 285 µL aliquot of mice fecal water was placed in a 1.5 mL Eppendorf tube and supplemented with 35 µL of D_2O and 30 µL of a standard buffer solution containing 585 mM NaHPO4, (pH 7.0)), 11.667 mM disodium-2,2-dimethyl-2-silapentane-5-sulphonate (DSS), and 0.47% NaN_3 in H_2O. The samples (350 µL) were then transferred to a Shigemi^{TM} NMR tube (Shigemi Co., Tokyo, Japan) and NMR spectral analysis was performed.

8.10.3. ^{1}H NMR spectroscopic fecal analysis

All ^{1}H NMR spectra were collected on a Varian 500 MHz Inova spectrometer (Inova 600, Varian Medical Systems, Palo Alto, CA, USA) equipped with a 5 mm HCN Z-gradient

pulsed-field gradient (PFG) cryogenic probe. ^{1}H NMR spectra were acquired at 25°C using the first transient of the Varian tnnoesy pulse sequence with a high degree of selective water suppression and quantitative accuracy of resonances, based on a standard one-dimensional NMR experiment with the Carr-Purcell-Meiboom-Gill technique. Water suppression pulses were calibrated to achieve a bandwidth of 80 Gauss. For each spectrum, 128 transients were collected into 48000 complex data points with a spectral width of 20 ppm at 24.8^{o}C with eight steady-state scans, a 4 second acquisition time and a one second relaxation delay. Before spectral analysis, free induction decays were multiplied by an exponential function equivalent to a 0.5 Hz line-broadening factor prior to Fourier Transformation and zero-filled to 64,000 data points. The methyl singlet, generated from a known concentration of DSS, was the internal standard (set to 0 ppm) used as a reference for chemical shift and quantification.

Using the Chenomx NMR Suite software, each NMR spectrum was qualitatively and quantitatively analyzed by manually fitting spectral intensities from an internal database. The heteronuclear single quantum correlation (HSQC) 2D NMR technique was applied to confirm the identity of metabolites. For the 2D experiment, a spectral width of 6127 Hz (^{1}H dimension) and 27164 Hz (^{13}C dimension) was acquired with a matrix (4096 x 1024), a 512 transients and a 2s relaxation delay. Major peaks were annotated with a compound name. About 90% of visible peaks and spectra areas were assigned and were routinely fit using the Chenomx spectral analysis software.

8.10.4. DI/LC-MS/MS spectroscopic plasma analysis

For metabolite quantification, isotope-labeled internal standards were integrated into the kit plate filter. First, 14 wells were used for a blank (one), zero samples (three), standards (seven), and quality control samples (three). Samples left to thaw on ice were vortexed and centrifuged at 13000 x g (4^{o}C, 15 min). A volume of 10 µL of supernatant for each sample was loaded on a filter paper of the plate and dried with nitrogen. Extraction of the metabolites was then achieved using methanol containing 5 mM ammonium acetate.

8.10.5. Immunohistochemistry and imaging analysis

The membranes were blocked for 15 min in dual enzyme and 15 min in a protein blocking solution (Dako, Carpinteria, CA, USA). Later, simultaneous incubation of the sections (1 h, RT) with primary antibodies: was conducted with anti-CD3 (1:200), and anti-IBA-1

(1:200) was performed. Following which the sections were incubated with secondary antibody (goat anti-rabbit IgG-horseradish peroxidase (HRP)) for 30 min at RT. Treated sections were then incubated with 3,3'-diaminobenzidine (DAB, Dako, 10 min) and counterstained 1 min with hematoxylin (Carpinteria, CA, USA).

The Aperio ScanScope slide automated scanner and Aperio ScanScope console (v.10.2.0.0) were used to scan digitally scan slides at 20 x magnification. The staining intensity was quantified from digitized images with the mean of Aperio-Imagescope software (v.6.25), where selected surface areas were analyzed for the corresponding staining using a color deconvolution algorithm, adapted to the color characteristics of the DAB and hematoxylin used. The marker index was expressed as the percentage of the number of positive cells in the total number of cells. A "% positive area" was calculated and plotted using GraphPad Prism 5.0 (GraphPad Inc., CA, USA).

8.10.6. Plasma pro-inflammatory cytokines - Enzyme-linked immunosorbent assay

Each well of the 96-well plate-based Multi-Spot® Array assays contained antibodies for each cytokine (INF-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α). When running the MSD assay, electrochemiluminescence was used as the basis for detection and calibration curves were generated based on assay diluent for mice plasma (40000 pg/ml - 1.2 pg/ml) specific to the cytokine. Standards were provided at a linear range of 0.2 to 10000 pg/mL. First, well reactions containing 25 µl/well of assay diluent, were shaken at room temperature (RT, 30 min). Samples and calibrators (25 µl) were added in duplicate to their corresponding wells for shaking (RT, 2 h), and subsequently washed with PBS + 0.05% Tween 20. The detection antibody reagent (25 µl) was added and the plate was agitated for 2 hrs (RT). The 96-well plate array was washed (PBS + 0.05% Tween 20) before adding the detection buffer and proceeding to reading the array values on the Sector® Imager 2400-253 (Meso Scale Discovery®, MD, USA).

8.11. Data and statistical analysis

Data normalization critical to creating a normal or Gaussian distribution of metabolite values was completed by autoscaling normalization, log transformation and/or range data scaling which allows conventional statistical tests to be performed and it simplifies data interpretation.

Univariate and Multivariate data analyses were performed to explore whether the La-Lf bio-intervention led to systematic metabolic changes, and to identify which metabolites differentiated the most La-Lf-treated subject from saline group. T-test and volcano plots were used to extract the most significant metabolites. The principal component analysis of the preprocessed NMR spectra was performed to visualize general grouping, trend, and outliers in the data. Subsequently, supervised partial least squares discriminant analysis (PLS-DA) was carried out to discriminate between the treated and control samples. Initially, principal components analysis (PCA) is an unsupervised classification technique that allows one to detect whether two or more sets of data using Pareto scaled data. PCA was performed to visualize general grouping, trend, and outliers in the data. PCA data were visualized by plotting the PCA scores (one sample) and the PCA loadings (one mass/retention time pair). The PCA analysis comprises computing correlation coefficients between sets of data to extricate Eigen values and Eigen vectors based on linear transformations. Thus, a set of "vectors" of different metabolites is generated and used to plot out the metabolite data on an X-Y cluster plot. The first and most significant vector is the 1^{st} principle component (x-axis) and the second is the 2^{nd} principle component (y-axis). In the case of separable data, two sets of clusters appear indicating that there are some significant metabolic/metabolite differences between the two sets of samples.

Subsequently, supervised PLS-DA was performed to discriminate between the treated and untreated subjects. PLS-DA analysis is a supervised classification technique, used as a last resort if no PCA separation is observable. From the loading plots PLS-DA, various metabolites could be identified as responsible for the separation between control and treated groups. The data is then processed similarly to PCA with the constraint to separate the two groups as best as it can. Although data plot separation between groups is achieved, this step can still be misleading which is a reason for a permutation test to confirm that group segregation has not happened by chance. This step consists of randomly re-labeling the metabolomic data and re-running the PLS-DA. This command is recurrently repeated (2000 times), with different random labeling. Subsequently, we can calculate the *p*-value, which is, in this context, the probability that the initial separation was a random event. The lists of metabolites with variable importance projection (VIP) values of more than 1.0 and

p-values of less than 0.05 (threshold) were selected as metabolites that could discriminate between the La-Lf-probiotic treated and untreated subjects.

Table 8.1S: The main composition of the diet fed to *Apc*$^{Min/+}$ mice.

As provided by the manufacturer (http://www.labdiet.com), the ingredients included in the rodent diet are ground corn, soybean meal, whole wheat, wheat middlings, animal fat, cane molasses, meat and bone meal, ground oats, wheat germ, brewer yeast, alfalfa meal, beet pulp, whey, calcium carbonate, salt, menadione dimethylpyrimidinol bisulfite, choline chloride, cholecalciferol, DL-methionine, vitamin A acetate, pyridoxine hydrochloride, dl-alpha tocopheryl acetate, folic acid, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, and cobalt carbonate.

Nutrients		***Minerals***	
Protein	**23.60%**	Ash	6.10%
Arginine	1.52%	Calcium	0.95%
Cystine	0.40%	Magnesium	0.20%
Glycine	1.26%	Phosphorus	0.70%
Histidine	0.60%	Phosphorus (non-	0.41%
Isoleucine	0.97%	Potassium	1.09%
Leucine	1.79%	Sulfur	0.28%
Lysine	1.36%	Sodium	0.28%
Methionine	0.43%	Chloride	0.49%
Phenylalanine	1.05%	Fluorine	15 ppm
Tyrosine	0.70%	Iron	210ppm
Threonine	0.89%	Zinc	87 ppm
Tryptophan	0.27%	Manganese	75 ppm
Valine	1.09%	Copper	14 ppm
Serine	1.15%	Cobalt	0.53 ppm
Aspartic Acid	2.56%		
Glutamic acid	4.84%	Iodine	0.97 ppm
Alanine	1.37%	Chromium	0.01 ppm
Proline	1.53%	Selenium	0.37 ppm
Taurine	0.03%		
Fat	**6.7 - 8.1%**	***Vitamins***	
Linoleic acid	1.39%	Carotene	0.8 ppm
Linolenic acid	0.10%	Vitamin K	3.2 ppm
Arachidonic acid.	0.02%	Thiamin	15 ppm
Omega-3 fatty	0.30%	Riboflavin	5.1 ppm
Total saturated	2.13%	Niacin	78 ppm
Total	2.38%	Choline chloride	2000 ppm
Cholesteral, ppm	238ppm	Folic acid	2.9 ppm
Fiber	**3.30%**	Pyridoxine	6.0 ppm
Neutral detergent	13.00%	Biotin	0.2 ppm
Acid detergent	4.10%	B_{12}	20 mcg/kg
Nitrogen-free	**50.30%**	Vitamin A	15 IU/gm
Starch	29.40%	Vitamin D_3	3.4 IU/gm
Glucose	0.21%	Vitamin E	62 IU/kg

Sucrose	2.79%	Pantothenic acid	15 ppm
Lactose	0.47%		
Total digestible	**79.30%**	***Calories***	
Gross energy	**4.36kcal/gm**	Protein	26.53%
Physiological	**3.56kcal/gm**	Fat	16.97%
Metabolizable	**3.23kcal/gm**	Carbohydrates	56.50%

8.12. Supplementary results.

Table 8.2S: Distribution criteria of individual fecal metabolites based on the Volcano plot. The main individual metabolites, from the volcano plot, presented in function of their fold change (FC) and *p*-values.

	FC	log^2(FC)	*p*-value
Choline	2.3894	1.2567	0.00082525
Methanol	4.2987	2.1039	0.0033167
Glucose	1.8713	0.90408	0.0071233
Phenylacetate	2.5685	1.3609	0.0098308
Acetate	1.7015	0.76681	0.018298
Succinate	2.153	1.1063	0.023634
Lactate	2.1543	1.1072	0.024287
Sarcosine	1.8009	0.84872	0.02539
Aspartate	1.9433	0.95851	0.0296
Glycerol	2.006	1.0043	0.0303
Proline	2.8305	1.501	0.032495
Pyruvate	1.8338	0.8748	0.033361
Ethanol	0.0759	-3.7191	0.036493
Tyrosine	1.5643	0.64556	0.042247
Valine	1.7679	0.822	0.046781
Leucine	1.8359	0.87651	0.048583

Table 8.3S: Description of the value of VIP score for each of the discriminative fecal metabolites.

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Fecal metabolites	VIP scores
Acetate	1.0434
Aspartate	1.0028
Choline	1.3765
Ethanol	1.2526
Glucose	1.1803
Glycerol	1.0926
Lactate	1.1342
Leucine	0.99011
Methanol	1.3238
Proline	0.96031
Pyruvate	1.4328
Sarcosine	1.0014
Succinate	1.0912
Tyrosine	0.96246

Table 8.4S: List of the top significantly enhanced fecal metabolic pathways based on the QEA. *P* values and total compounds involved were described.

	Total fecal compounds	*p-value*
Phospholipid biosynthesis	19	0.00022937
Glycolysis	21	0.00067502
Glucose-alanine cycle	12	0.00067502
Insulin signaling	19	0.00067502
Citric acid cycle	23	0.00078333
Gluconeogenesis	27	0.0014453
Galactose metabolism	25	0.0043017
Pyruvate metabolism	20	0.0046768
Glycerolipid metabolism	13	0.0060484
Betaine metabolism	10	0.0069761
Glutamate metabolism	18	0.0079246
Urea cycle	20	0.014133
Mitochondrial electron transport	15	0.020051
Glycine, serine and threonine	26	0.025249
Cysteine metabolism	8	0.032367
Alanine metabolism	6	0.032367
Methionine metabolism	24	0.036523
Arginine and proline metabolism	26	0.042445
Malate-aspartate shuttle	8	0.049509

Table 8.5S: Distribution criteria of individual plasma metabolites based on the Volcano plot. The main individual metabolites generated form the volcano analysis with description of g in their fold change (FC) and *p*-values.

Metabolites	FC	log^2(FC)	*p*-value
Svmmetric DMA	0.64068	-0.64231	0.011611
PC aa C36:1	1.4082	0.49384	0.035301
PC aa C32:1	1.505	0.58973	0.041279

Table 8.6S: Description of the value of VIP score for each of the discriminative plasma metabolites.

Plasma metabolites	VIP scores
Symmetric DMA	2.5839
PC aa C36:1	2.2585
PC aa C34:1	2.0432
PC ae C38:1	2.04
PC ae C36:1	1.9945
PC aa C40:3	1.9838
PC aa C42:2	1.9244
PC aa C32:1	1.9069
PC aa C40:2	1.859
PC ae C32:1	1.6622
PC ae C40:3	1.6439
PC ae C34:1	1.6198
PC ae C38:2	1.611
PC aa C40:1	1.6022
Methionine sulfoxide	1.2729

Table 8.7S:. List of the top significantly enhanced systemic metabolic pathways. *P* values and total compounds involved were described.

	Total	plasma	*p-value*
Tryptophan metabolism	34		0.00011851
Valine, leucine and isoleucine degradation	36		0.00011954
Propanoate metabolism	18		0.00013942
Betaine metabolism	10		0.00014515
Methionine metabolism	24		0.00017852
Glycine, serine and threonine metabolism	26		0.00024586
Protein biosynthesis	19		0.0004808
Glutathione metabolism	10		0.0011587
Galactose metabolism	25		0.001486
Nicotinate and nicotinamide metabolism	13		0.0014862
Glutamate metabolism	18		0.0017952
Ammonia recycling	18		0.0021191
Inositol metabolism	19		0.002215
Sphingolipid metabolism	15		0.0037584
Pyruvate metabolism	20		0.0042678
Glycolysis	21		0.0055285
Insulin signaling	19		0.0055285
Fructose and mannose degradation	18		0.0058535
Citric acid cycle	23		0.0074342
Purine metabolism	45		0.0076722
Mitochondrial electron transport chain	15		0.0088969
Urea cycle	20		0.0091251
Aspartate metabolism	12		0.0092578
Phenylalanine and tyrosine metabolism	13		0.0099556
Catecholamine biosynthesis	5		0.011289
Bile acid biosynthesis	49		0.012047
Pyrimidine metabolism	36		0.013383
Cysteine metabolism	8		0.017447
Gluconeogenesis	27		0.018221
Glucose-alanine cycle	12		0.018729
Histidine metabolism	11		0.01927
Arginine and proline metabolism	26		0.022785
Tyrosine metabolism	38		0.025431
Phospholipid biosynthesis	19		0.026571
Porphyrin metabolism	22		0.02814
Taurine and hypotaurine metabolism	7		0.03235

8.13. Supplementary Figures.

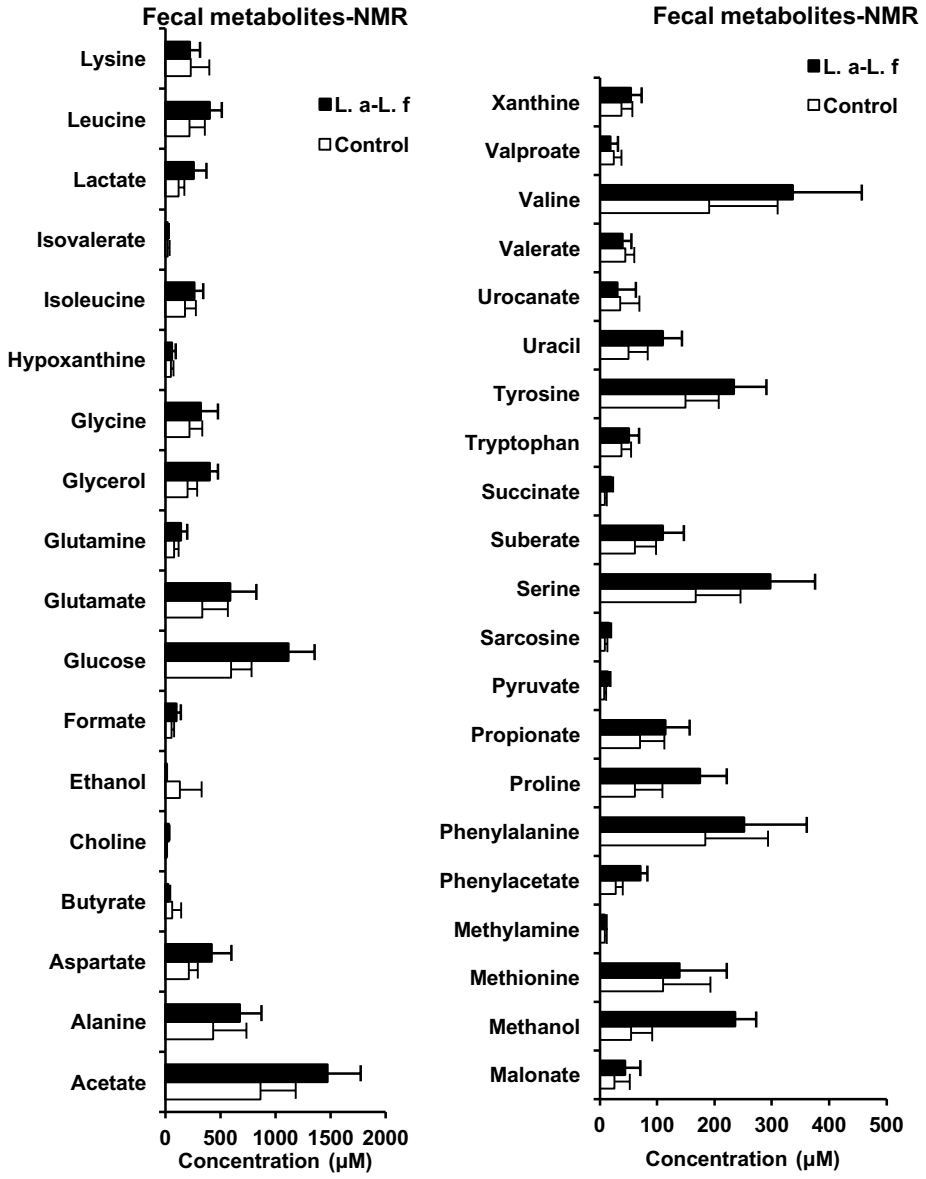
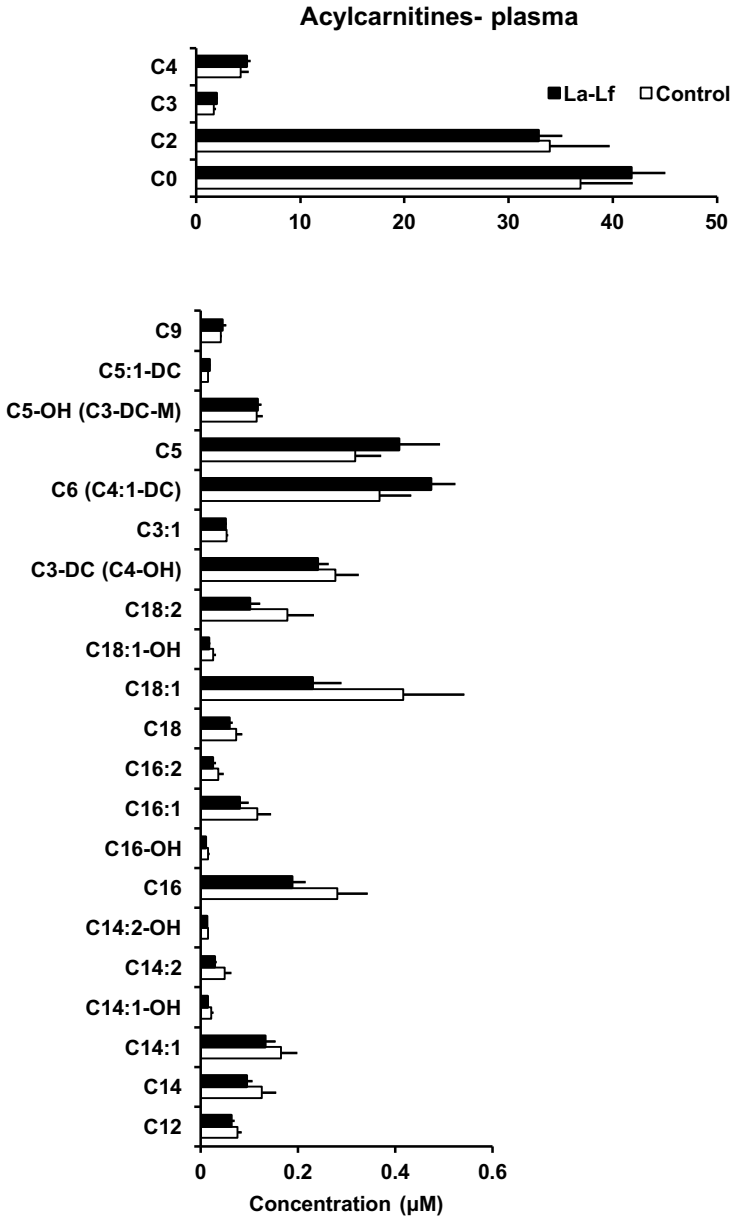


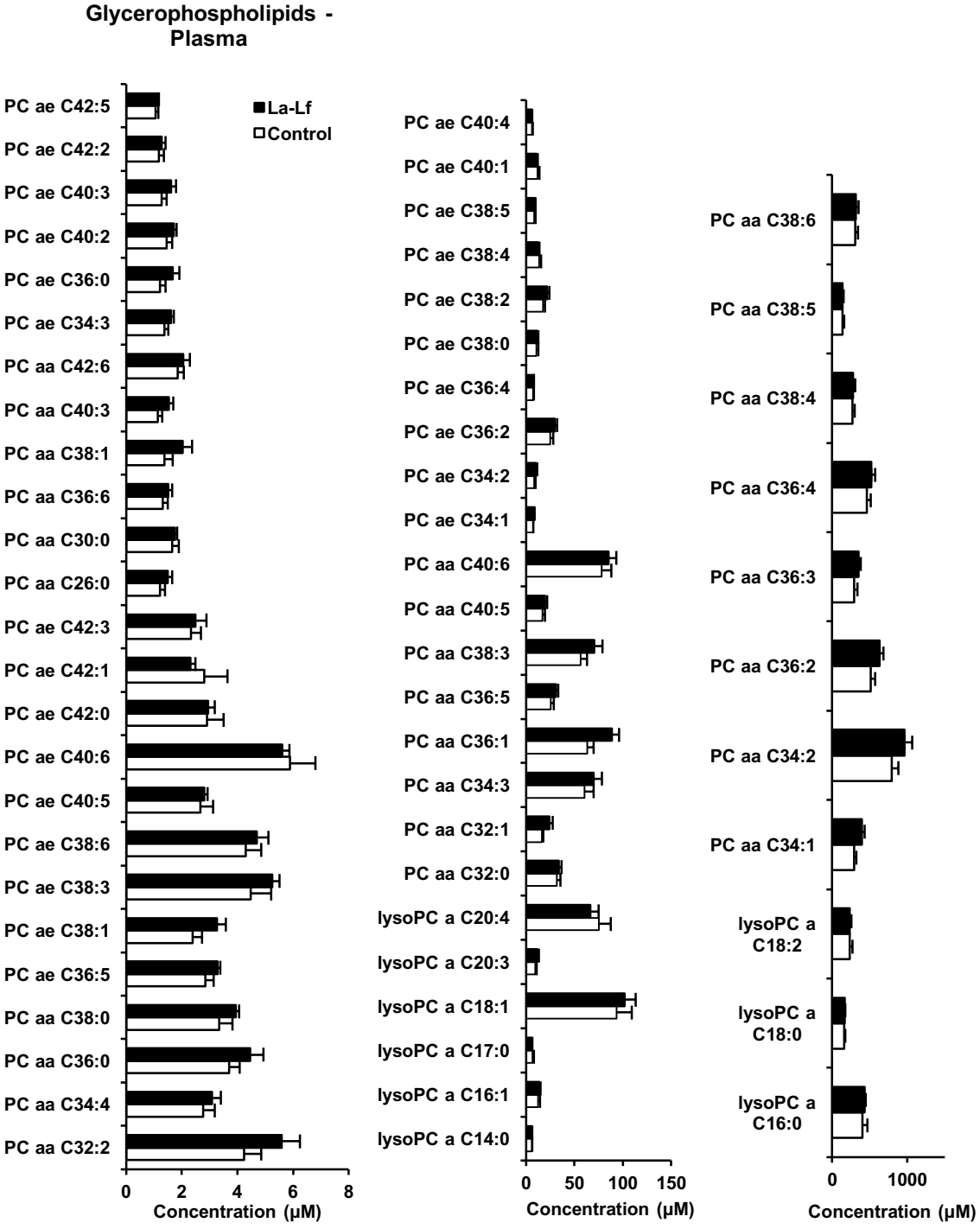
Figure 8.1S: Assessment of the composition of La-Lf-treated mice fecal water and concentrations of endogenous metabolites.

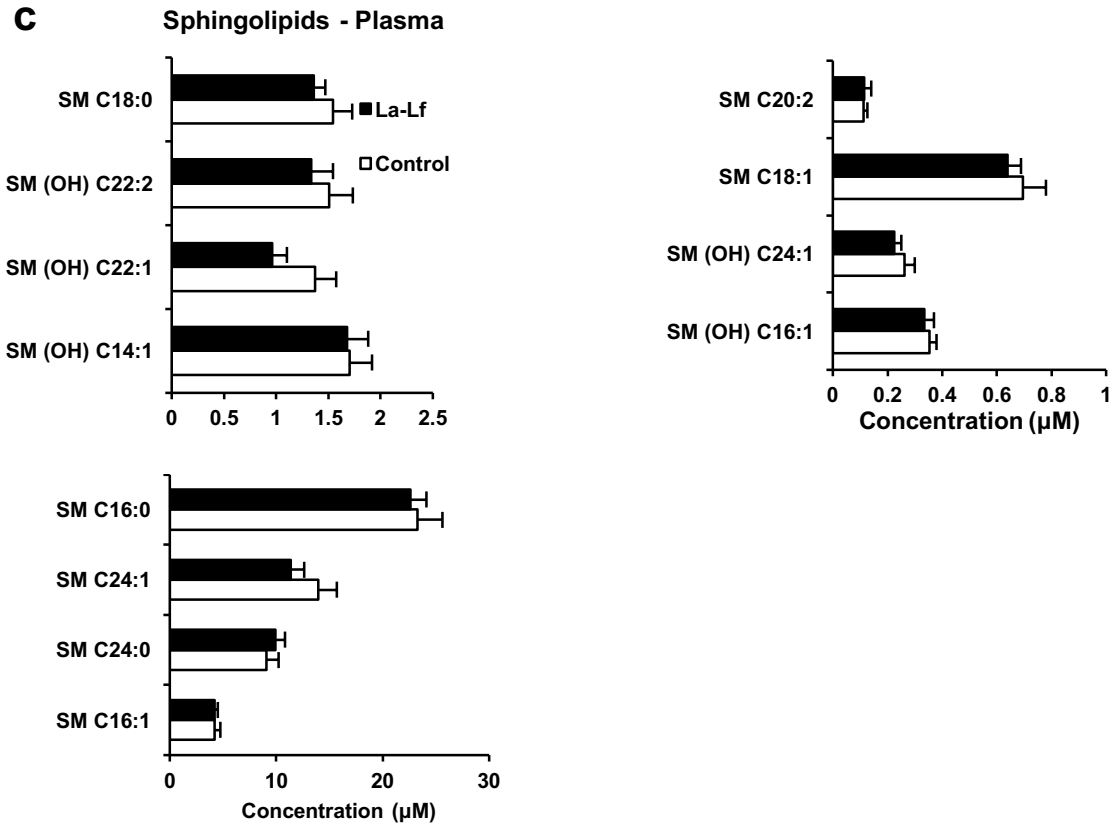
After orally administrating La-Lf probiotic formulation to C57BL/6J-*Apc*$^{Min/+}$ CRC mice, fecal water was extracted and analyzed for using ^{1}H NMR spectroscopy. Data represent the mean SEM per group (n = 5). La-Lf: a mixture of *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC **314.**

a

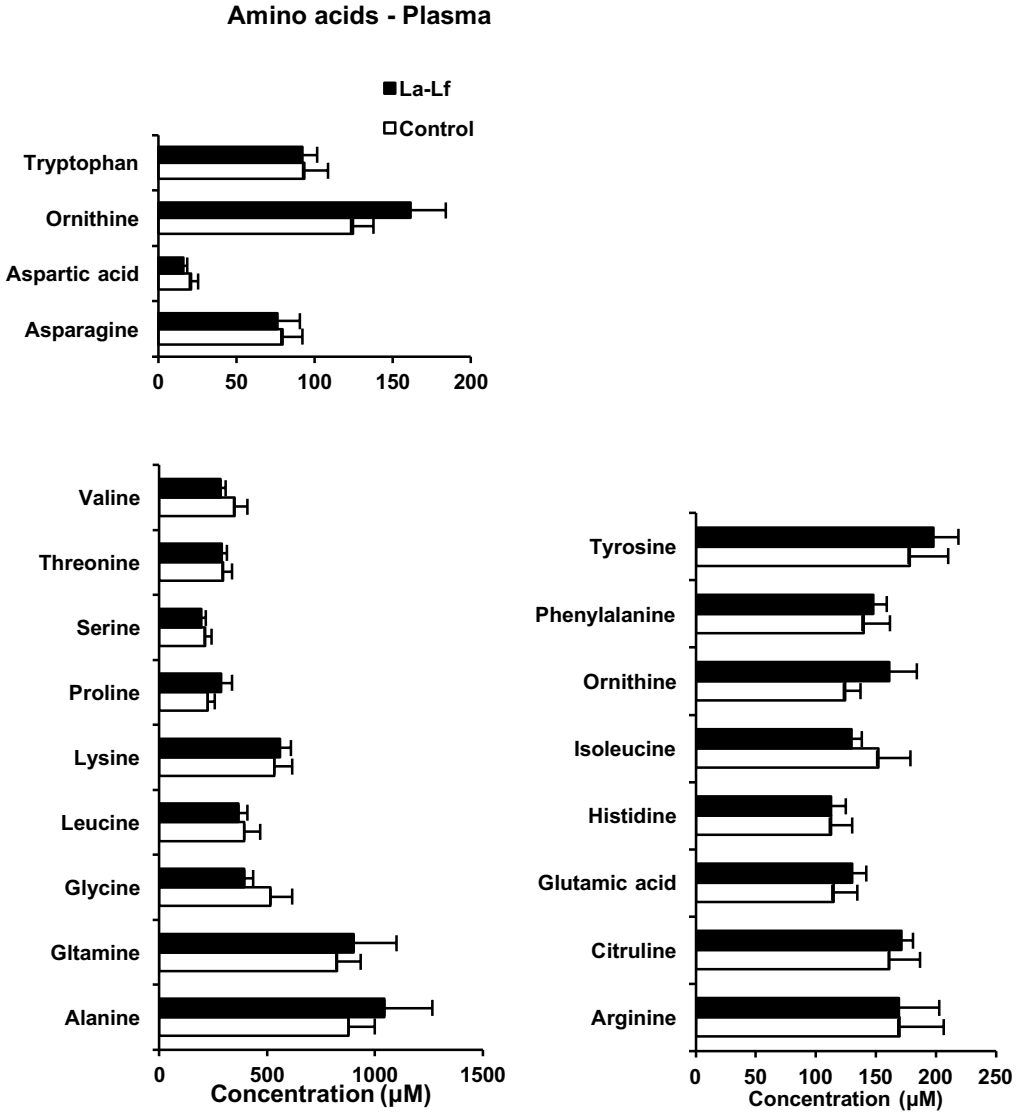

Acylcarnitines- plasma
La-Lf
Control
C4
C3
C2
C0
0
10
20
30
40
50
C9
C5:1-DC
C5-OH (C3-DC-M)
C5
C6 (C4:1-DC)
C3:1
C3-DC (C4-OH)
C18:2
C18:1-OH
C18:1
C18
C16:2
C16:1
C16-OH
C16
C14:2-OH
C14:2
C14:1-OH
C14:1
C14
C12
0
0.2
0.4
0.6
Concentration (μM)

b


Glycerophospholipids - Plasma
La-Lf
Control
PC ae C42:5
PC ae C42:2
PC ae C40:3
PC ae C40:2
PC ae C36:0
PC ae C34:3
PC aa C42:6
PC aa C40:3
PC aa C38:1
PC aa C36:6
PC aa C30:0
PC aa C26:0
PC ae C42:3
PC ae C42:1
PC ae C42:0
PC ae C40:6
PC ae C40:5
PC ae C38:6
PC ae C38:3
PC ae C38:1
PC ae C36:5
PC aa C38:0
PC aa C36:0
PC aa C34:4
PC aa C32:2
0 2 4 6 8
Concentration (µM)
PC ae C40:4
PC ae C40:1
PC ae C38:5
PC ae C38:4
PC ae C38:2
PC ae C38:0
PC ae C36:4
PC ae C36:2
PC ae C34:2
PC ae C34:1
PC aa C40:6
PC aa C40:5
PC aa C38:3
PC aa C36:5
PC aa C36:1
PC aa C34:3
PC aa C32:1
PC aa C32:0
lysoPC a C20:4
lysoPC a C20:3
lysoPC a C18:1
lysoPC a C17:0
lysoPC a C16:1
lysoPC a C14:0
0 50 100 150
Concentration (µM)
PC aa C38:6
PC aa C38:5
PC aa C38:4
PC aa C36:4
PC aa C36:3
PC aa C36:2
PC aa C34:2
PC aa C34:1
lysoPC a C18:2
lysoPC a C18:0
lysoPC a C16:0
0 1000
Concentration (µM)


c
Sphingolipids - Plasma
SM C18:0
SM (OH) C22:2
SM (OH) C22:1
SM (OH) C14:1
La-Lf
Control
0
0.5
1
1.5
2
2.5
SM C16:0
SM C24:1
SM C24:0
SM C16:1
0
10
20
30
Concentration (µM)
SM C20:2
SM C18:1
SM (OH) C24:1
SM (OH) C16:1
0
0.2
0.4
0.6
0.8
1
Concentration (µM)

d


Amino acids - Plasma
La-Lf
Control
Tryptophan
Ornithine
Aspartic acid
Asparagine
0
50
100
150
200
Valine
Threonine
Serine
Proline
Lysine
Leucine
Glycine
Gltamine
Alanine
0
500
1000
1500
Concentration (µM)
Tyrosine
Phenylalanine
Ornithine
Isoleucine
Histidine
Glutamic acid
Citruline
Arginine
0
50
100
150
200
250
Concentration (µM)

e

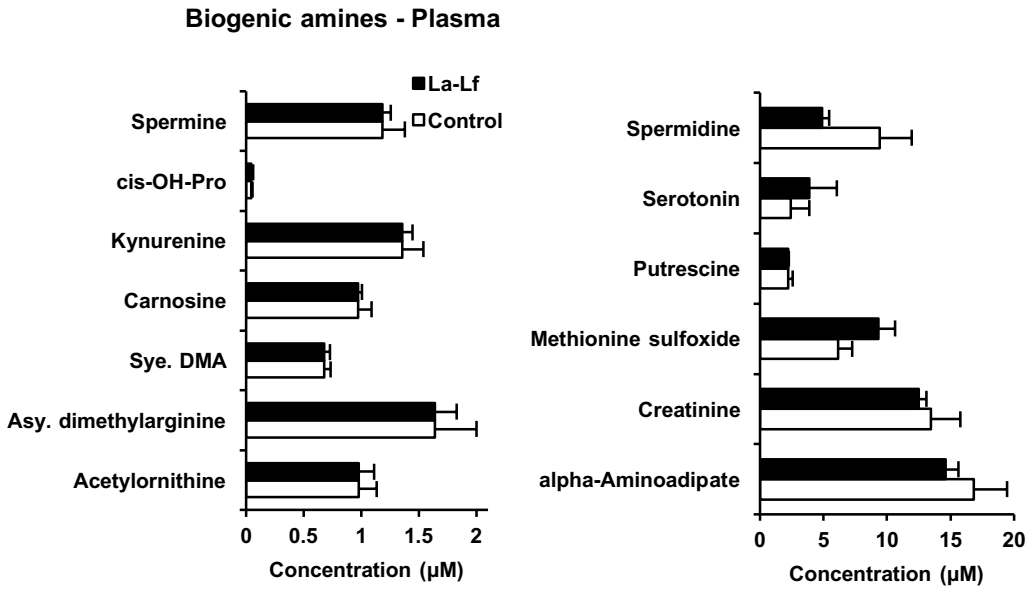


Figure 8.2S. The assessment of the composition of La-Lf-treated mice plasma in a wide range of endogenous metabolites: (a) acylcarnitines, (b) glycerophospholipids, (c) sphingolipids, (d) amino acids, and (d) biogenic amines.

After orally administrating La-Lf probiotic formulation to C57BL/6J *Apc*$^{Min/+}$ CRC mice for 12 weeks, plasma was collected and analyzed using DI/LC-MS/MS. Data represent the mean ± SEM per group (n = 5). La-Lf: a mixture of *L. fermentum* NCIMB 5221 and *L. acidophilus* ATCC 314.

CHAPTER 9. GENERAL DISCUSSION

Despite the development of advanced pharmaceuticals and surgical techniques, CRC morbidity and mortality remains high in the population worldwide. If primary CRC regimes seem to be effective, side effects, infections, quality of life, economic burden, and recurrence of the cancer are remaining greater concerns. Elevated risk of CRC is epidemiologically and geographically clustered in industrialized countries, and predisposed with individuals suffering from different GI conditions and chronic inflammation [3]. As gut microbiota is found to be severely altered in high-risk individuals, balancing back the microbiota flora to fight event-causing cancer in the gut is beginning to be an attractive strategy that may improve patient well–being, intestinal epithelial proliferation, gut permeability, immune responses locally and systematically (**Table 2.2)**. For instance, Lactic acid bacteria (LAB), in particular, *Lactobacilli* were found to be microbial markers between a healthy individual and CRC patients in which intestinal *Lactobacillus* population was diminished [260, 479].

Probiotics are 'live microorganisms which, when administered in an adequate amount, confer a beneficial health effect to the host' [39, 40]. Many reports demonstrated that probiotic bacteria possess potential features associated with lower risks of CRC. LAB, in particular, have shown protective effects against CRC by reinforcing and modulating the host's natural defense mechanisms [42]. LAB were shown to reinforce the mucosal barrier [43], reduce the exposure to toxic and carcinogenic compounds in the colon [45], and dimish CRC recurrence in treated patients [3].

Probiotic supplementation showed great promise for CRC patients during conventional treatments (chemotherapy or surgical) and improved several clinical aspects (e.g., lowering the recurrence of the disease). However, there is still a lack of definitive data about the preventive and therapeutic outcomes, as well as specific benefits of probiotic interventions [480]. There is a deficit in proper screening tools for *in vitro* selection of most potent

strains, as well as, need to use high-throughput pre-clinical and clinical analytical methods that reflect a clear map of the mechanisms of action exhibited [3, 22-24].

Considerable interest was focused on the major events that a healthy microbiota or potent probiotic bacteria produces, in particular, carbohydrate fermentation for the production of fatty acids. Most importantly, SCFAs, differentially produced in healthy and tumor-bearing intestines are readily absorbed by colon epithelial cell as an energy source, and considered anti-carcinogenic towards neoplastic epithelial colon cells. Direct ingestion of SCFAs could be beneficial but limited as they are absorbed before arriving at the large intestines [481]. Hence, intake of active *Lactobacillus* bacteria could present a delivery mechanism not only for SCFAs but a vast spectrum of antioxidant, anti-inflammatory, anti-oncogenic probiotic bacterial products as well. Many of these factors with total or relative benefits to the colon include CLA, secreted bacterial proteins, DNA fragments, ferulic acid, etc., [482]. We hypothesized, in the context of this research thesis, on the possibility to identify and design an active *Lactobacillus* probiotic oral supplement that can inhibit CRC cell proliferation, reduce tumor formation by lessening intestinal and systemic inflammation and enriching the availability of anti-cancer metabolites within the gut microbiota ecosystem. We went further to the identification of metabolic mechanisms of action of the probiotic treatment using metabolomic methods.

The first objective of this project included a preliminary screening of different *L. reuteri* strains, previously investigated in the context of GI health (**Chapter 3**). As SCFAs are microbial by-products of large concern to colon health, they were used as the first criteria for screening. SCFAs possess anti-cancer, pro-apoptotic and cell differentiation activity in CRC cells. They are considered anti-bacterial, antioxidant, and anti-inflammatory agents [483]. The second major criteria for screening was the ability of *L. reuteri* strains to reduce CRC cells growth. Later, the correlation between those two criteria showed a high association between CRC cell-inhibitory potential and SCFAs production by *L. reuteri*.

Another important measure to consider for the selection of probiotic bacteria is their resistance to intestinal conditions, which was evaluated by assessing bacterial survival and their ability to produce SCFAs in simulated intestinal juice. For this screening, *L. reuteri* NCIMB 701359 and *L. reuteri* NCIMB 11989 were most successful at producing the highest amounts of SCFAs based on their viability.

Consequently, the previous conclusions lead to the second objective of this project **(Chapter 4**). There, *L. reuteri* NCIMB 701359 was characterized further based on other investigated *Lactobacilli* from different species that possess apparent anti-tumorigenic effect [243, 484, 485]. General metabolic characterization was structured to evaluate the particularity of *L. reuteri* NCIMB 701359 general metabolic activity. Thus, FFA levels measured during growth phases showed partial superiority compared with different *Lactobacilli.* Diverse types of probiotic extracts can be used to study probiotic bacteria in mammalian cells. Here, two cell-free extracts SP and CM were tested against CRC cells based on proliferation and apoptosis to show different efficacies. For the rest of the study, we evaluated the probiotic effect of *L. reuteri* on both normal and cancer colon cell lines. Interestingly, *L. reuteri* modestly inhibited CRC cell proliferation and slightly improved cell growth for normal epithelial colon cells, a significant confirmation of the validity of the anti-cancer effect *in vitro*.

Furthermore, mounting levels of research showed that gut ecosystem modulation by oral probiotics increase SCFAs concentrations in the proximal colon, antioxidant levels, and improve gut integrity while reducing apoptosis and lesions in colonocytes and inducing cell death in tumor cells [486, 487]. Therefore, *L. reuteri* total SCFA bio-production was shown higher than both *L. rhamnosus* ATCC 53103 and *L. acidophilus* ATCC 314. Although butyrate, or propionate to a lesser extent, are more popular for their efficacy in the context of CRC, we demonstrated that a single concentration of a specific SCFA is not responsible for the overall anti-proliferative effect of *L. reuteri* NCIMB 701359. In fact, all SCFAs produced in their preserved ratio are behind the anti-cancer effect of this

bacterium. Nevertheless, *L. reuteri* NCIMB 701359 relative potential, compared with *L. rhamnosus* ATCC 53103 and *L. acidophilus* ATCC 314, was not superior for all criteria, and despite the detected anti-cancer activity *in vitro*, consideration for limited effect *in vivo* testing were to be taking in consideration if further pre-clinical trials are planned (**Figure 4.8)**.

In a previous study, *L. fermentum* were compared with *L. reuteri* in other GI disorders and showed more efficacy in attenuating many symptoms of the disease. The aim of **Chapter 5** is to continue the search for *Lactobacillus* bacterium with higher colorectal anti-cancer effect, than what previous *L. reuteri* demonstrated. Therefore, we screened ferulic acid-producing *L. fermentum* bacteria, with a verified anti-inflammatory effect in metabolic syndrome [488], as potential strains to consider for CRC.

Among the screened *L. fermentum* strains, *L. fermentum* NCIMB -5221 and -2797 showed significantly higher anti-proliferative activity against Caco-2 cancer cells and SCFA bio-production in their CM, making them attractive candidates for further characterization. They, also, showed resistance in SIF (up to 8 h) and produced a higher level of SCFAs at ratios that can inhibit CRC cells, in the artificial intestinal fluid. This fact was supported by additional testing of identified ratios of produced SIF-SCFAs in Caco-2 cell culture or epithelium-like culture. Observations collected in these assays strongly suggested the ability of *L. fermentum* NCIMB -5221 and -2797 to survive colon environment and actively produce SCFAs at relevant concentrations for the inhibition of malignant growth.

Based on the overall criteria summarized in **Figure 5.9** of the previous chapter**,** *L. fermentum* NCIMB 5221 was selected for further investigations, as detailed in **Chapter 6**. This *Lactobacillus* not only produced significantly larger amounts of FFAs and higher concentrations of total SCFAs than *L. rhamnosus* ATCC 53103 and *L. acidophilus* ATCC 314 but it was also more apoptotic and CRC cell anti-proliferative. Different cell-free extracts, SP and CM, of *L. fermentum* NCIMB 5221 were more proficient in inducing cancer cell death and anti-proliferative effect, as verified, totally based, not on the release

of specific SCFAs, but on the total composition of produced acetate, propionate, and butyrate altogether. It is taken that a highly effective drug for CRC or patients with high risk would ideally kill cancer cells, limit neoplastic transformation of colonocytes, without damage to normal colon cells. Therefore, simultaneous assays were designed to search *L. fermentum* grander features against the CRC initiation/development, by inhibiting CRC cells, not non-neoplastic cells if not providing a source of energy for healthy proliferation. Using synthetic SCFA formulations, the ratios of acetate, propionate, and butyrate, released by *L. fermentum* NCIMB 5221 were reproduced, and their efficacy was evaluated based on other *Lactobacilli* (*L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103). At this level, the particularity of our bacterium, *L. fermentum* NCIMB 5221, may reside on both the levels and the composition of SCFAs produced. In previously tested SCFA concentrations, some bacteria seemed to have less effect than the SCFAs they produced, i.e., the bacteria have released a product that lowered the anti-cancer capacity of the bacteria, which was not the case of *L. fermentum* NCIMB 5221. This being said, SCFAs to reduce Caco-2 viability at different concentrations with no significant adverse effect on CRL-1831 normal cells compared to cancer cells. The schematic overview of CRC-related properties in **Figure. 6.9** as investigated in this chapter, may assist in deciding the faith of *L. fermentum* NCIMB 5221 in further *in vivo* studies.

Chapters 5 and 6 provided compelling and novel findings on the anti-CRC ability of *L. fermentum* NCIMB 5221. When it comes to animal trial, additional parameters are to be taken into consideration, particularly, whether the bacteria could survive at realistic numbers while reaching the large intestines, produce other relevant metabolites, act differently though signaling with colonocytes, as it is delivered as a whole cell and not an isolated supernatant?

When reporting about *L. fermentum* NCIMB 5221, the bacterium *L. acidophilus* ATCC 314 used as a positive control, was first established as an anti-tumorigenic agent from Dr. Prakash group [155]. In this work, *L. acidophilus* ATCC 314 showed advanced survival

over 8 h in SIF with a significant release of lactate ($p < 0.001$), compared with *L. fermentum* NCIMB 5221. Even if *L. acidophilus* ATCC 314 *in vitro* direct action on CRC cells appeared restricted, this probiotic could act indirectly by providing therapeutic levels of SCFAs-production substrate (lactate) or normalize the lumen physiochemical characteristics for an optimal growth of anti-oncogenic gut-residing bacteria [413]. Thus, instead of using *L. fermentum* NCIMB 5221 as a single probiotic *in vivo*, both probiotics *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 were analyzed as pure cultures or as a mixed co-culture in **Chapter 7**. In previous studies several probiotic formulations containing more than one probiotic strain were investigated in different health conditions, such as VSL#3®, thanks to the synergistic effect of the various bacteria it contains [315, 489]. Hence, the fifth objective of this thesis is to identify if *L. fermentum* NCIMB 5221 combination with *L. acidophilus* ATCC 314 is more efficient in inhibiting CRC then pursue validation in a genetically modified CRC animal model. Accomplishing this objective revealed, initially, that the *Lactobacilli* co-culture had improved growth kinetics and total antioxidant capacity. Besides, the probiotic mixture had significantly more pro-apoptotic and inhibitory activities against Caco-2 cells, with a positive effect on the proliferation of normal colon cells, and protected them also from exposure to the carcinogen (AOM). This detailed study was designed to show the superiority of probiotics *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 combination over the use of separate bacteria. The different probiotic cell-free extracts were examined on Caco-2 proliferation before and after being physically or chemically modified and preserved the synergistic/enhanced effect. These observations brought some light on the nature of probiotic secreted active factors that were able to reduce cancer growth and induce apoptosis. Identification of the mechanisms of action through which *Lactobacillus* bacteria negatively affect CRC is strongly associated with the bacterial components secreted into the lumen or supernatant. Depending on the effect investigated, several studies went in the direction of identifying and isolating active probiotic molecules (e.g., LPS, bacterial protein or peptides) [315,

489]. Many investigations concluded that some probiotic extracts can prevent neoplastic transformation of normal cells [226, 490]**.** In our report, normal cells were exposed to AOM, a toxic chemical used to induce CRC in animal models. AOM was shown to possess toxic effect on cells at appropriate doses. Here again, the synergistic ability of La-Lf was demonstrated in protecting normal cells from the AOM cytotoxic action, indicating their La-Lf enhanced carcinogenic effect, in the case of a longer exposure [226]. As a conclusion, La-Lf was selected as a more efficient alternative to using in a pre-clinical study with $Apc^{Min/+}$ mice. The interest, here, is to validate La-Lf formulation and report investigated cellular anti-tumor mechanisms of action. Oral intake of this active probiotic mixture induced positive outcomes by reducing tumor number and attenuating upregulated cellular proliferation in both normal appearing and tumor crypts. The results of this report, as summarized in **Figure 7.14,** were successful at establishing *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 formulation as a therapeutic with potential in CRC and direct the research toward identifying particular probiotic mechanisms of action.

The focus of this work was mostly related to early stage CRC represented by the use of mice with Apc mutations. However, in the case of advanced CRC where patients have a higher chance of developing KRAS mutations [491, 492], probiotic administration have shown an effect in reducing chemotherapy doses and other side effects in patients undergoing conventional treatments. La-Lf could be tested in a model of KRAS mutation of CRC, and it would have potential, at higher doses this time, of increasing SCFAs in the gut and still inhibiting inflammation, if not slightly reducing tumor cells growth [3].

Straightaway that an anti-tumorigenic effect of La-Lf active bio-treatment was observed, the last objective of this thesis was to seek a wider understanding of specific mechanisms of action of the digested bacteria in tumor-bearing mice (**Chapter 8**). In the last decade, new advances in "omics" and system biology are becoming the basis of analytic clinical methods. Metabolomic profiling of fluids and biological matters, in host compartments, allowed the recognition of disease and intervention fingerprints and disclosed variations of

a vast number of metabolites in association to specific pathological conditions or therapeutic strategies [404]. To our knowledge, this is a novel investigation that studies the metabolomic outcomes of probiotics composed of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221. Initially, La-Lf, taken orally, produced a distinct metabolic signature detected in the fecal matter of $Apc^{Min/+}$ mice. The goal is to identify how La-Lf affects fecal metabolome and pathways. The metabolites that were mostly changed, reflected how La-Lf bacteria modulated the microflora toward the release of anti-inflammatory and anti-carcinogenic products. For instance, tyrosine, leucine, proline, pyruvate, aspartate, sarcosine, succinate, lactate, glycerol, acetate, glucose, phenylacetate, choline, and methanol were increased, while ethanol was depleted. As explained in previous metabolomics studies, the most affected fecal metabolites in this study originated from microflora and are linked to the reduction of inflammation and tumorigenic events [403, 409, 415, 427, 440, 457, 493]. After La-Lf intake, the pattern of variations in these metabolites was represented in a significant impact on many metabolic pathways including phospholipid biosynthesis, glycolysis, glucose-alanine cycle, citric acid cycle, gluconeogenesis, galactose, pyruvate, glycerolipid, betaine glutamate metabolism, urea cycle, mitochondrial electron transport chain, glycine, serine and threonine metabolism. Those observations revealed the mechanisms through which La-Lf oral intake affect the host fecal metabolic profile. Based on many fecal metabolomics studies, many of the pathways and metabolites that La-Lf modulated were established as biomarkers and found altered in the case of GI conditions and CRC [494, 495]**.**

Chronic inflammation plays a key role in CRC, as a symptom, and in inflammation-induced CRC, as a cause. Since individuals at risk of CRC may suffer from chronic low-grade inflammation, the additional important goal is to describe how La-Lf affected CRC mice systemically, which took place by lowering pro-cancer metabolites and pro-inflammatory cytokines, as revealed using DI/LC-MS-MS method and V-plex mesoscale analysis, respectively. For the plasma metabolomics analysis, symmetric DMA was reduced, while

some glycerophospholipids were elevated. The overall metabolic effect of La-Lf bacteriotherapy on different groups of systemic metabolites is translated on significant impact on cancer-related metabolic pathways. La-Lf formulation was able to significantly affect several pathways that play an important role in CRC, including tryptophan metabolism, valine, leucine and isoleucine degradation, propanoate, betaine, methionine, glycine, serine, and threonine metabolism, protein biosynthesis, glutathione, galactose, nicotinate and nicotinamide, glutamate metabolism, ammonia recycling, inositol, sphingolipid, and pyruvate metabolism [415].

Interestingly, La-Lf not only reduced inflammation at the systemic level (INF-γ, IL- 1β, TNF-α, and KC/GRO) but also acted locally by downregulating inflammatory immune cell markers (CD3 and IBA-1). In unhealthy subjects, inflammatory cells produce reactive oxygen and nitrogen species leading to an inflamed intestinal mucosa and shifting of biological pathways towards a carcinogenic state [496]. As described by Kahouli et *al.,* some of the mechanisms of action of orally digested probiotic bacteria to suppress CRC development are inducing colon epithelial cells and immune cells to diminsh the production of pro-inflammatory cytokines in the mucosa [3]. La-Lf bacteriotherapy may have interfered with the recurrent inflammation in $Apc^{Min/+}$ and enhanced the production of several anti-inflammatory and anti-tumor agents in the mucosa, such as choline [497], proline [498], pyruvate [499], succinate [483], phenylacetate [500], and many glycerophospholipids [501]. Here, we discover a stronger aspect of La-Lf anti-inflammatory potential, at local and systems levels, which goes in hand with the nature of both discriminative fecal and plasma metabolites sorted out in the metabolic profile in this study. More importantly, these “multi-metaboinflammatory” findings significantly linked La-Lf to major metabolic pathways and markers associated with CRC.

Our findings supported the dietary modulation of the composition and activity of the microbiota by the use of probiotics. The results revealed the potential of La-Lf probiotic bacteriotherapy to induce positive outcomes on the large intestine one of the most

metabolically active organs and the resident highly dense of living bacterial populations [502]. The health benefits of La-Lf are suggested to be part the modification of the host immune response and interference with the colonic microbial ecosystem, resulting in a modulation of the colonic bacterial metabolism and altered systemic profile [503]. Notably, La-Lf bio-treatment may have stimulated bacterial activity in the gut, through an increased uptake of nitrogen, amino acids, and other metabolites [504]. La-Lf probiotic activity may have affected saccharolytic and proteolytic processes in the intestines as an indirect effect on the fermentation of carbohydrate and protein. There is a noticeable increase in carbohydrate breakdown in the presence of probiotic bacteria through higher production of lactate and succinate. Similarly to La-Lf, previous metabolomic studies reported that probiotic treatments increased isobutyrate and isovalerate, and increased urinary excretion of phenolic and indolic. As demonstrated in **Chapter 8** for La-Lf**,** some probiotics supplementations were linked to specific amino acid patterns, lipid profiles, gluconeogenesis, and methylamine metabolism [505, 506]. Other possible factors could be taken in consideration for future studies such as the possible modulation of the bacterial enzyme activity in the colon. (e.g. -glucuronidase activity), fecal activities of carcinogen-metabolizing enzymes, as well as the metabolism secondary bile acid considered metabolism and carcinogenic [507].

Many of the cytokines and immune cells investigated in our study, after La-Lf supplementation, are involved in GI mucosal immunity associated to intestinal microbiota [508]. Previous probiotic supplementation has also shown effects on systemic inflammation, which was explained by the secretion/inhibition of variable immune-related metabolites (inflammatory and non-inflammatory) on the systemic and luminal levels.[509].

It is fundamental to note that those promising and significant findings, in this body of work, are in fact in greater need for further metabolomic studies that involve different biofluids. This is essential for a complete picture of the mechanisms of action of such biotherapy and

the potential side effects. Conversely, some studies stated that even though probiotics are considered beneficial and safe, their effect could be described as temporary and unclear, while, in some case, it can cause infections, allergic and autoimmune disturbances [510]. Besides, some probiotics can induce modifications in gene expression, activation of disease-related signaling pathways, and transfer of undesirable of genes [511-513]. This unfavorable view explains the need for establishing clear correlations between the metabolic and the microbial variations in the host and complete studies with multi-metabolomic profiles in animals/humans harboring (health and unhealthy) to extract any unfavorable outcomes and determine the safety of probiotic formulations. Some studies suggested the selection and development of natural metabiotics that could be analogs or forms of natural probiotic compounds produced, which could present a natural evolution of the probiotic concept [514, 515]. Nonetheless, this only can only be established after complete understanding of probiotic bacteria role in human metabolome, genome, and microbiota complexities and interactions. Usually, drug action is affected by drug interaction with gut microbiota that may enhance or reduce drug absorption and activity in the intestine [276, 516]. A question could be asked about drug-drug interactions between probiotic bacteria and chemical drugs. Interestingly, in the case of treated CRC patients, many clinical trials reported synergistic beneficial effects of oral probiotic supplementation before and after surgery, chemotherapy, and/or radiation. Patients needed fewer drug doses which mean the probiotic sensitized cancer cells and/or enhanced the effect of the drugs [517, 518].

Alternately, confirmation of probiotic challenged or improved metabolomes in more than one animal model could be done before moving to clinical trials, where the safety of La-Lf probiotic doses can be assessed. In clinical settings, the probiotic doses and administration should undergo adjustments and optimization dependent on the category of patients, their medication, and the stages of their disease. Additional research is still needed in the field of metabolomics for the identification of complete sets CRC biomarkers in animal and

humans, on which we can base the evaluation of probiotic interventions. Still, this strategy could be coupled with proteomic and genomic studies to link metabolic changes to gene expression and customize doses and treatments depending on patient responses. This will allow more functional analysis for omics integration, and it became easier to map genes, proteins, and metabolites together, or generate specifically mapped network with the available data generate behind the metabolic pathways established, displaying the metabolic difference between the La-Lf treatment and the control groups.

Finally, *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 formulation is suitable as a biotherapeutic for CRC and understanding its action is in progress. On a view of the nature of inflammatory and metabolic markers La-Lf regulated, this formulation could also be with an appropriate use in other medical conditions, such as GI or autoimmune inflammatory disorders.

CHAPTER 10. SUMMARY OF OBSERVATIONS AND CLAIMED ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

10.1. Symmary of observations

*The marked observations are claimed as original contributions to knowledge.

The research outcomes described in this thesis demonstrate that a novel active Lactobacillus formulation can be used as a biotherapeutic for CRC. The summary of the main observations is:

The exact novel discoveries are presented as following:

1. As a lactic acid bacterium, *L. reuteri* bacteria were active in the cell culture conditioned medium (CM) and were able to produce variable but significant amounts of lactic acid compared with each other ($p < 0.05$).

2. *As SCFAs were produced significantly by gut microbiota in healthy colon and are able to induce cell death and cell differentiation in CRC cells, probiotic *L. reuteri* bacteria were preliminarily screened for ability to produce SCFAs. *L. reuteri* bacteria produced significantly variable amounts of SCFAs: acetate, propionate, and butyrate (all $p < 0.05$).

3. *Probiotic *L. reuteri* bacteria were screened for anti-proliferative properties against CRC cells. The CM of each screened strain presents the excretory products released by the bacteria in the cancer cell medium. The effect of the bacteria depends on the active factors secreted that have potential activity against CRC cell. Probiotic *L. reuteri* strains inhibited CRC cells strain-dependently. *L. reuteri* NCIMB -701359 and -11989 were the best strains to suppress CRC cell proliferation in a time-dependant manner ($p < 0.05$).

4. Production of SCFAs is not a proof of the anti-cancer effect of probiotic bacteria. Evaluation of the role of SCFAs produced by each *L. reuteri* shows that the inhibitory effect of probiotic bacteria be partially responsible for their anti-proliferative activity against CRC cells, depending on the strain.

5. *The efficacy of probiotic treatment depends greatly on the delivery of resistant bacteria that will survive GI conditions and stay active in the colon. *L. reuteri* NCIMB -701359 and -11989 were characterized depending on their resistance to artificial intestinal conditions. *L. reuteri* viability and density in a simulated intestinal fluid indicated if the strain will survive best intestinal conditions and induce greater effect. The concentration of SCFAs released was also quantified confirming the release of potential anti-cancer factors produced. *L. reuteri* were found viable in the simulated intestinal fluid ($p < 0.05$, 8 h) and produced significant levels of total SCFAs acetate, and propionate ($p < 0.05$).

6. **L. reuteri* NCIMB 701359 was further characterized based on different established Lactobacilli: *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 in terms of growth kinetics and FFA production. Total FFA levels of *L. reuteri* NCIMB 701305 significantly exceeded other Lactobacilli, suggesting it may be more active in producing fatty acids with anti-cancer activity.

7. **L. reuteri* NCIMB 701305 was characterized for its anti-cancer effect *in vitro* based on different established Lactobacilli: *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. *L. reuteri* NCIMB 701305 cell-free extracts, the supernatant (SP, 15.16 ± 4.73 %) and cell culture conditioned medium (CM, 17.4 ± 2.3 %) showed inhibition of SW-480 cancer cell growth at different time points. Both extract induced apoptosis in SW-480 cells, while CM (27.2 ± 9.4%, $p = 0.031$), was significantly the highest.

8. *A beneficial probiotic strain would play a role inhibiting tumor formation and provide beneficial factors for healthy normal colon cells. *L. reuteri* NCIMB 701305 (42.68 ± 4.44 %, 48 h, $p < 0.01$) suppressed Caco-2 cancer cell proliferation, without inhibiting CRL-1831 normal cells.

9. *SCFAs were showed to be responsible in part for the anti-proliferative effect of *L. reuteri* NCIMB 701305. This bacterium was tested to confirm the levels of SCFAs produced compared to two Lactobacilli: *L. acidophilus* ATCC 314 and L. *rhamnosus* ATCC 53103, then, link those concentrations to the ability to inhibit cancer cells. Total

SCFAs ($p < 0.001$) produced by *L. reuteri* NCIMB 701305 were significantly higher that the positive controls and they were, in total, responsible for the anti-proliferative activity.

10. **L. fermentum* bacteria were screened for their ability to produce products with an anti-cancer activity, such as SCFAs. *L. ferm*entum NCIMB 2797 and *L. fermentum* NCIMB 5221 produced the highest amount of total SCFAs in their cell culture conditioned medium ($p < 0.001$).

11. *L fermentum* NCIMB 2797 and *L fermentum* NCIMB 5221 were the best at inhibiting Caco-2 cancer proliferation in a time-dependent manner.

12. For the screened *L fermentum* bacteria, there was a significant correlation between SCFAs produced and CRC cell inhibited.

13. For the screened *L fermentum* bacteria, the role of SCFAs concentrations produced was estimated. SCFAs they secreted were considered totally responsible for the inhibition of CRC cells.

14. *Survival profile (density and viability) of L. *fermentum* NCIMB 2797 and *L fermentum* NCIMB 5221 in artificial intestinal fluid demonstrated resistance to those challenging conditions. During 8 h of incubation in simulated intestinal fluid *L. fermentum* NCIMB 5221 exhibited the least cell death in the simulated intestinal fluid, followed by *L. fermentum* NCIMB 2797.

15. *L. fermentum* NCIMB 2797 and *L fermentum* NCIMB 5221 metabolic activity was tested in simulated intestinal fluid. Both strains were able to produce a significant amount of SCFAs.

16. *The composition of SCFAs produced by *L. fermentum* NCIMB 2797 and/or *L fermentum* NCIMB 5221 in the simulated intestinal fluid was tested on CRC cells. The levels of SCFAs produced the simulated intestinal fluid were able to significantly suppress Caco-2 cancer cell viability in free-cell or epithelium-like culture.

17. *The probiotic bacterium selected for the highest anti-proliferative activity and SCFA bio-production, *L. fermentum* NCIMB 5221, was compared with conventional strains from

different species: *L. acidophilus* ATCC 314 and *L. rhamnosus*. The latter are Lactobacilli that displayed anti-cancer effects in previous *in vitro* and/or *in vivo* studies, and they are used as positive controls for a better evaluation of *L. fermentum* NCIMB 5221 preventive anti-CRC potential.

18. *Many fatty acids produced by the gut bacteria and probiotic bacteria is considered beneficial for CRC prevention (e.g., CLA, ferulic acid, and SCFAs) hold anti-inflammatory and anti-carcinogenic properties. The probiotic FFA profile, describing FFA per bacterial mass or per viable bacterial count during growth and stationary phases of growth, reflected *L. ferme*ntum NCIMB 5221 to be more metabolically active, compared to each of *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103 ($p < 0.001$).

19. *Investigating the anti-cancer effect of a probiotic bacteria starts with testing a particular cell-free extract to be incubated with cancer cells in vitro. Two different bacterial extracts from *L. fermentum* NCIMB 5221 showed anti-proliferative effect ($p < 0.0001$) and induced apoptosis ($p < 0.0001$) in CRC cells. In these tests, *L. fermentum* NCIMB 5221 showed more efficacy than *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103.

20. After showing a negative effect on CRC cells in vitro, several studies omit to test the probiotic treatments on normal colonic cells which are supposed to verify that the probiotic effect in not only due to the cytotoxic effect of bacterial products against mammalian cells. Here, *L. fermentum* NCIMB 5221 inhibited Caco-2 cancer cells ($p < 0.0001$), but have no cytotoxic effect on normal colon cells, it has promoted healthy cell growth in nutritionally minimal conditions.

21. Bio-produced SCFAs process a role in the anti-proliferative activity *L. fermentum* bacteria that was compared to pre-established *L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 53103. The production of SCFAs by *L. fermentum* NCIMB 5221 correlated with its anti-cancer activity.

22. SCFAs play a dual role in the prevention of CRC, and they present an energy source for colonocytes while killing cancer cells. Increasing concentrations of SCFAs in different

mixtures are tested on both cancer and normal colon cells and showed a synergistic effect in cancer cells, while no significant cytotoxic effect on colon cells, *in vitro*.

23. **L. fermentum* NCIMB 5221 produced increasing levels of FFAs, generate higher concentrations of SCFAs, inhibited more CRC cells, suggesting the ability to beneficiate normal colon cells by probiotic SCFA production. The sum of those observation confirms the potential anti-cancer of this bacterium as an active agent against CRC.

24. *In previous studies, *L. acidophilus* ATCC 314 exhibited anti-inflammatory and anti-tumorigenic effect, while *L. fermentum* NCIMB 5221 showed potential against metabolic diseases and is an adequate producer of ferulic acid, known for anti-cancer and antioxidant properties. Both were combined and tested for potential synergistic effect in terms of bacterial properties. *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 co-culture showed higher density during exponential growth ($p < 0.001$) in different media. The co-culture had a higher antioxidant production 304.9 ± 35.7 μMTE ($p < 0.05$), and showed resistance in simulated intestinal fluids ($p < 0.05$) than separately. Those observations suggested that a combination of these two Lactobacillus produced a metabolically superior probiotic co-culture with elevated growth kinetics.

25. **L. acidophilus* ATCC 314 mixed with *L. fermentum* NCIMB 5221 significantly reduced viability (38.8 ± 6.9 %, $p = 0.009$) and induce apoptosis ($p < 0.001$) in Caco-2 cells. Similarly, it has enhanced CRL-1831 non-neoplastic cell growth ($p = 0.001$) more and protected them for the toxicity of AOM carcinogen. Thus, La-Lf probiotic formulation demonstrated the potential to kill cancer cells, promote healthy growth of normal colon cells while protecting them carcinogens.

26. **L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 mixture promoted normal colon cell growth and protected them from carcinogen toxicity more significantly than separately. Both La-Lf (78.5 ± 10.1 cells, $p < 0.01$) and L. f 5221 (67 ± 9.6 cells, $p < 0.05$) significantly increased cell count, compared with L. a 314. These results suggest the

suitability of those Lactobacilli to reduce colon lesions caused by ingested or generated carcinogenic/mutagenic products throughout the gut.

27. *Investigations with *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 formulation were performed to validate whether this mixture could influence tumor proliferation and CRC biomarkers in genetically induced CRC in an animal model. La-Lf oral intake, in $Apc^{Min/+}$ mice, was validated to successfully reduced tumor burden by about 50% ($p = 0.016$).

28. *Since *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 formulation significantly lowered tumor number in the intestines of genetically modified CRC $Apc^{Min/+}$ mice, some cellular proliferation biomarkers were assessed, including K-67, CC3, β-catenin and E-cadherin. Cellular index examinations were not only undertaken in tumors, but also in adjacent normal-appearing intestinal tissues since they are still prone to later tumor development. Results showed less Ki-67 expression in both normal-appearing ($p = 0.008$) or tumor tissues ($p = 0.012$), while β-catenin activation was loss intense in normal-appearing intestinal crypts ($p = 0.041$). The downregualtion of cellular proliferation markers in intestinal mucosa describes potential mechanisms of action of La-Lf probiotic formulation against of CRC

29. The *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 formulation demonstrated, *in vivo* and *in vitro*, the potential to lower the risk of CRC by suppressing the upregulation of cellular proliferation markers in the intestine and reducing intestinal tumor progression.

30. Tumor-bearing $Apc^{Min/+}$ mice housed higher intestinal inflammation rate. La-Lf may have reduced tumor formation through anti-inflammatory mechanisms, since La-Lf probiotics had already produced significant antioxidant capacity, *in vitro*, and resulted on higher acetate in the colon.

31. The anti-tumorigenic activity of orally ingested active bacterial cells of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 may have involved significant modulation of

the overall mammalian and bacterial metabolic homeostatic in the gut. Thereafter, to discover those mechanisms, novel extensive identification of La-Lf-treated *Apc*$^{Min/+}$ mice plasma and fecal metabotypes were generated from DI/LC-MS-MS and 1H NMR spectra, respectively.

32. *Oral feeding of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 to *Apc*$^{Min/+}$ mice significantly altered the fecal metabolic profile by increasing the levels choline (p = 0.00082525), methanol (p = 0.0033167), glucose (p = 0.0071233), phenylacetate (p = 0.0098308), acetate (p = 0.018298), succinate (p = 0.023634), lactate (p = 0.024287), sarcosine (p = 0.02539), aspartate (p = 0.0296), glycerol (p = 0.0303), proline (p = 0.032495), pyruvate (p = 0.033361), tyrosine (p = 0.042247), valine (p = 0.046781), leucine (p = 0.048583) and decreasing the levels of ethanol (p = 0.036493). The results suggested several mechanisms by which La-Lf biotherapy may have reduced CRC-related biomarkers in the colon.

33. *After La-Lf probiotic therapy, the most important fecal metabolites based on the VIP values are: tyrosine (VIP = 1.1282), leucine (VIP = 1.1389), proline (VIP = 1.165), pyruvate (VIP = 1.1669), aspartate (VIP = 1.1868), sarcosine (VIP = 1.2071), succinate (VIP = 1.2106), lactate (VIP = 1.2187), ethanol (VIP = 1.2263), glycerol (VIP = 1.2432), acetate (VIP = 1.275), glucose (VIP = 1.3671), phenylacetate (VIP = 1.4065), choline (VIP = 1.5565), and methanol (VIP = 1.5611).

34. *The efficacy of the probiotic intervention is evaluated depending on its effect on the metabolic fluctuation in *Apc*$^{Min/+}$ mice plasma. La-Lf treatment significantly changed three metabolites, namely symmetric DMA (p = 0.011611), PC aa C36:1 (p = 0.035301), and PC aa C32:1 (p = 0.041279). The oral ingestion of La-Lf formulation may reduced intestinal tumor growth by changing the levels of systemic metabolites.

35. *Based on fecal QEA, the most affected metabolic pathways after La-Lf probiotic therapy are phospholipid biosynthesis (p = 0.00022937), glycolysis (p = 0.00067502), glucose-alanine cycle (p = 0.00067502), insulin signaling (p = 0.00067502), citric acid

cycle (p = 0.00078333), gluconeogenesis (p = 0.0014453), galactose (p = 0.0043017), pyruvate (p = 0.0046768), glycerolipid (p = 0.0060484), betaine (p = 0.0069761), glutamate metabolism (p = 0.0079246), urea cycle (p = 0.014133), mitochondrial electron transport chain (p = 0.020051), glycine, serine and threonine metabolism (p = 0.025249). Thus, at the intestinal level, the La-Lf probiotic therapy may have reduced CRC risk by modulating several metabolic pathways.

36. *The most relevant plasma metabolites associated to La-Lf oral intake were extracted with their VIP values starting from the most important are as following: symmetric DMA (VIP = 2.5839), PC aa C40:1 (VIP = 1.6022), PC ae C38:2 (VIP = 1.611), PC ae C34:1 (VIP = 1.6198), PC ae C40:3 (VIP = 1.643), PC ae C32:1 (VIP = 1.6622), PC aa C40:2 (VIP = 1.859), PC aa C32:1 (VIP = 1.9069), PC aa C42:2 (VIP = 1.9244), PC aa C40:3 (VIP = 1.9838), PC ae C36:1 (VIP = 1.9945), PC ae C38:1 (VIP = 2.04), PC aa C34:1 (VIP = 2.0432), PC aa C36:1 (VIP = 2.2585), and methionine sulfoxide (VIP = 1.5856). By affecting the systemic levels of those metabolites, the probiotic treatment inhibited tumor-causing agents and increased anti-tumor substrates.

37. *Based on the effect of La-Lf treatment on plasma metabolic profile, the 15 most affected pathways are tryptophan metabolism (p = 0.00011851), valine, leucine and isoleucine degradation (p = 0.00011954), propanoate (p = 0.00013942), betaine (p = 0.00014515), methionine (p = 0.00017852), glycine, serine and threonine metabolism (p = 0.00024586), protein biosynthesis (p = 0.0004808), glutathione (p = 0.0011587), galactose (p = 0.001486), nicotinate and nicotinamide (p = 0.0014862), glutamate metabolism (p = 0.0017952), ammonia recycling (p = 0.0021191), inositol (p = 0.002215), sphingolipid (p = 0.0037584), and pyruvate metabolism (p = 0.0042678). La-Lf probiotic treatment succeeded to differentially modulate systemic pathways linked to oncogenic signalings and thereby suppress tumor development and CRC risk.

38. *Supplementation with active mixture of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 to $Apc^{Min/+}$ resulted in reduced plasma pro-inflammatory profile, where pro-

inflammatory cytokines: INF-γ ($p = 0.039$), KC/GRO ($p = 0.016$), and TNF-α ($p = 0.045$) were significantly downregulated. These results demonstrated the systemic anti-inflammatory activity of digested probiotic bacteria, by lowering inflammation and, thus, suppressing CRC development.

39. *The active mixture of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 reduced the expression of intestinal inflammatory markers in $Apc^{Min/+}$, such as IBA-1 ($p = 0.014$), in normal-appearing crypts, and CD3 ($p < 0.001$), in tumor tissues. These results described the ability of La-Lf bio-intervention in intestinal immune modulation, a mechanism involved in reducing CRC risk.

40. The oral administration of *L. acidophilus* ATCC 314 and *L. fermentum* NCIMB 5221 probiotic formulation demonstrated the potential, *in vitro* and *in vivo*, to affect key risk factors associated with CRC and many GI disorders by suppressing CRC cell growth, attenuating intestinal tumorigenesis, protecting non-neoplastic colon cells, reducing intestinal and systemic inflammation, as well as regulating different metabolic pathways.

10.2. Conclusions

In the recent years, scientific evidence giving isan increasing credit to the host microbiome in preventing or initiating many GI conditions leading directly or indirectly to CRC. The use of probiotic bacteria, *Lactobacillus* in particular, as a strategy to balance and heal the gut microflora is investigated as a tool to affect the host metabolic processes toward fighting cancer-causing events. CRC mortality, recurrence, and incidence considered significant economic and health concerns worldwide, were found limited in populations consuming beneficial bacteria. Some probiotic therapies enhanced conventional CRC treatments and reduced chemotherapy side effects [3]. Nonetheless, the selection and formulation of probiotic supplements are not clear, and the mechanism of actions are partially understood. Novel probiotic development and use of screening methods should be established actively. This multistep study allowed the design of a synergistic combination of *L. fermentum* 5221 with *L. acidophilus* ATCC 314 that is capable of the suppression of

tumor formation in the intestines of genetically modified mouse model *Apc*$^{Min/+}$ mice. It reduced inflammation and modulating intestinal immune responses, all key factors in the prevention and/or treatment CRC and other associated GI-conditions.

Sets of *L. reuteri* and *L. fermentum* strains were screened for identification of strains with higher potential as a CRC biotherapeutic. The most potent bacterium *L. fermentum* 5221 mixed with *L. acidophilus* ATCC 314 had a superior activity against CRC development, reduced tumor formation by suppressing CRC cell growth and protecting normal colon cells. This La-Lf formulation suppressed CRC cellular proliferation markers, induced higher production of SCFAs, attenuated intestinal inflammation systemically and locally as demonstrated after oral intake in *Apc*$^{Min/+}$ mice. This work was based on extensive investigations on the mechanism of actions of La-Lf at the metabolomic level. NMR and DI/LC-MS-MS metabolomics studies revealed the main metabolic pathways affected by the La-Lf active probiotic formulation. This study produced a novel formulation, brought new insight into its mechanism of action and confirmed the strong potential of *L. fermentum* 5221 and *L. acidophilus* ATCC 314 oral biotherapy for the treatment and/or prevention of CRC.

10.3. RECOMMENDATIONS

The project conducted within this thesis demonstrates the potential of an active probiotic formulation containing a mixture of *L. fermentum* 5221 and *L. acidophilus* ATCC 314 as an effective CRC biotherapeutic. The observations concluded from this study was able to fulfill the objectives and confirm the hypothesis. These findings bring more questions about observing the same benefits or more in different animal models and humans with additional methods.

First, dose optimization, stability, and safety could be undertaken for this formulation in GI model, animal, and humans, for the delivery of adequate doses. Optimizing the production and storage process of the oral formulation and characterizing this probiotic mixture regarding the production of SCFAs, CLA, and ferulic acid and other active anti-cancer compounds are all strong elements to consider.

Conducting metabolomics analysis allowed a strong view on the action of the probiotic therapy on the metabolic levels. There still a need for running metabolomic analysis with the same technique on the host biofluids to produce an overall profile of metabolic fluctuation in host components. As the gut is home to the primary immune system, immuno-scoring would be a necessary testing to plan for in future studies.

This study researched a wide number of criteria and features that makes of the selected bacteria of this formulation an appreciable candidate for CRC and other inflammatory conditions. However, the different assays conducted represent only a part of broad aspects (mechanisms or biomarkers) in need for more evaluation.

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