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Chapter 9

Probiotics and Intestinal Microbiota: Implications in Colon Cancer Prevention

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1. Introduction

Colon cancer (CC) is one of the commonest causes of death among all types of cancers [1]. The development of cancer is a multifactorial process influenced by genetic, physiological, and environmental factors [2,3]. Regarding environmental factors, the lifestyle, particularly dietary intake, may affect the risk of CC developing [1,4]. Western diet, rich in animal fat and poor in fiber, is generally associated with an increased risk of colon cancer [5,6,7]. Thus, it has been hypothesized that the connection between the diet and CC, may be the influence that the diet has on the colon microbiota and bacterial metabolism, making both relevant factors in the etiology of the disease [8,9]. Additionally, it has been clearly demonstrated that the gut microbiota may be modulated by many factors including diet [10].

Several studies have indicated that the intestinal microbiota is an important determinant for general health of the human body [1]. Therefore, a beneficial modulation of the composition and metabolic activity of the gut microbiota might represent an interesting approach to improve health, reducing the risk of CC development. This modulation may be though about probiotic consumption.

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [11]. Among the best known probiotic microorganisms are strains belonging to the *Lactobacillus* and *Bifidobacterium* genera. However, other microorganisms, such as *Enterococcus* spp., *Streptococcus* spp., *Escherichia coli* Nissle 1917, some bacilli, and *Saccharomyces cerevisiae* subsp. *boulardii* have also been considered for use as probiotics [12].

Even though the mechanisms by which probiotics may inhibit colon cancer are not fully elucidated, certain potential mechanisms have been disclosed, such as the alteration of the

composition and the metabolic activities of the intestinal microbiota, the changing physicochemical conditions in the colon, the binding of dietary carcinogens, the production of short chain fatty acids (SCFA), the protection of the colonic mucosa and enhancement the immune system [1,3].

The anticarcinogenic effects of probiotic microorganisms *in vitro* and in animal studies are well documented [3]. In clinical trials, the probiotics are thought to play a protective role in the initial process of carcinogenesis. Nevertheless, it is important to determine whether the long-term administration of these microorganisms might result in changes in the incidence of CC in humans [13]. Additionally, there are several challenges for the development of probiotics, including the selection of the appropriate microorganisms, control of dietary intake, time and frequency of probiotic dosing and the use of accepted biomarkers for raised cancer risk that might be monitored during clinical trials [4,13]. Further experimental models are needed to understand the exact mechanisms involved in the influence of probiotics on colon cancer development.

Therefore, this chapter will discuss the effects of probiotics in colon cancer prevention and the possible mechanisms of action these microorganisms. Additionally, this chapter will also show the results of original work, carried out by our research group, about the effects of probiotic *Enterococcus faecium* CRL 183 (strain isolated from Tafí cheese, a homemade traditional highlands cheese the province of Tucumán, Argentina) on intestinal microbiota and colon cancer prevention.

2. Colon cancer

Social and economic transformations related to urbanization and industrialization in Brazil resulted in changes in the morbimortality profile of the population. While, in the first half of the 20th century infectious disease event were the most frequent, from the 1960 metabolic diseases and noncommunicable grievances occupied the first place, contributing to the process of demographic transition, which favours the spread of cardiovascular and respirator disease, cancer and diabetes, as does the nutritional transition, with a marked reduction of malnutrition and large growth in the number of overweight people [14].

Known for many centuries, cancer was widely regarded as a disease of developed countries with large financial resources. However, for approximately four decades, this situation has undergone transformation, and most of the global burden of cancer can be observed in developing countries, especially those with low to medium resources [15].

Cancer has become a global public health problem of course, since the World Health Organization (WHO) estimates that in the year 2030 there will be 27 mil new cases of cancer, 17 million deaths and 75 million people living with the disease [15].

Cancer of the colon and rectum is the third commonest type of cancer among men and the commonest in women. It is estimated that in 2011, in Brazil, 14,180 new cases of colon cancer and rectum, occurred in men and women. These values correspond to a perceived risk 15 new cases per 100 thousand men and 16 per 100 thousand women [15].

This neoplasia is considered to have a good prognosis when diagnosed in the early stages. Colon cancer like others forms of cancer develops as a result of interaction between endogenous and environmental factors. Among the factors that may affect the risk of developing this disease are age, eating habits, physical activity, alcohol consumption, smoking, nutritional status, presence of polyps, cancer history of self and family, cases of ulcerative enterocolitis and chronic constipation [15,16].

Most cases of CC occur sporadically, being the most common type of adenocarcinoma, which develops from glandular cells that cover the wall of the intestine [17]. Adenocarcinomas grow from normal epithelium through an accumulation of mutations that result in malignant transformation [19].

Genomic instability is fundamental to this process and is related to the rearrangement of genes, or loss of DNA fragments, aneuploidy and loss of heterozygosis [19]. In addition, inactivation of tumor suppressor genes, such as APC, DCC, DPC4 and p53, along with the activation of oncogenes, of which the family of *ras* genes are the best well described, play important parts in the appearance of malignancy [17].

Generally, the colon tumor is detected for the first time as a polyp (mass of cells growing out of the wall of the colon), although nowadays it is possible to detect small lesions affecting the crypts, called aberrant crypts foci (ACF) [18]. ACF are not only morphologically but also genetically distinct lesions and are precursors of adenoma and cancer. Tumors can appear anywhere in the colon, although most sporadic rectal colon cancers are located on the left side of the distal colon (including the rectum and sigmoid colon) [19].

Epidemiological studies have pointed to the high consumption of red meat, fat and low fiber intake, typical of the Western diet as risk factors in the etiology of this type of cancer [20].

One of the possible effects of a Western diet on colon cancer is related to increased excretion of bile acids [21]. In addition, the increased ammonia production in rats consuming a diet rich in protein has also been linked to an increased risk of cancer [22]. However, high consumption of fruits, cereals, fish and calcium may reduce the risk of developing colon cancer [23].

The effect of diet on carcinogenesis can be modulated by changes in metabolic activity and composition of the intestinal microbiota [23]. Several studies have trial to establish relationships between bacteria and colon cancer. We know that various bacterial metabolites are carcinogenic, examples being, the nitrosamines, phenol, indole, ammonia and amines [13].

There is multiple evidence that bacteria play a key role in the emergence of chronic inflammatory bowel diseases. Experimental studies demonstrate the impossibility of developing this inflammation in the absence of bacteria and researches have tried for many years trying to identify a possible causative agent of inflammatory bowel diseases. Studies suggest that chronic inflammatory intestinal activity seems, paradoxically to be triggered by bacteria belonging to the normal commensal which take on microbiota in situations as yet unknown, a pathological role that can activate the local immune apparatus [24].

There are many types of intestinal bacteria that produce a variety of metabolites that modulate the normal development and functioning of the host. On the other hand, the metabolic activity of intestinal microbiota can generate compounds that are harmful such as reactive oxygen intermediates. These molecules, which include superoxide, hydrogen peroxide, hypochlorous acid, singlet oxygen and hydroxyl radical, can cause oxidative damage to cellular DNA and increase the risk of colon cancer [25]. Studies have shown that *Enterococcus faecalis* can produce superoxide and hydrogen peroxide, causing damage to DNA in skin cells, in both *in vitro* and *in vivo* tests [26].

Given the role of intestinal microbiota in colon carcinogenesis, it is suggested that factors that modulate beneficially the composition and/or activity of the microbiota could inhibit the development of CC.

3. Evidences for relationship among intestinal microbiota, probiotics and colon cancer

3.1. The intestinal microbiota

The gastrontestinal (GI) microbiota undergoes changes in quantity and quality, depending on the location of colonization in the GI. Traditional culture-based characterization may take into account no more than 30% or so of the microorganisms that can be seen and enumerated by microscopic observation. The worldwide species diversity of commensal intestinal bacteria is immense. In that respect, the use of molecular tools has indicated that the majority of the dominant bacterial species observed in the faecal microbiota of an individual (approximately 80%) are specific to this individual [27]. Also, these species are not distributed homogeneously along the length of the GI, so the bacterial activities are considerably variable in different parts of the intestine [28].

The stomach and the small intestine contain few species, whereas the colon contains a complex and dynamic microbial ecosystem, with a great concentration of bacteria. Among these are the bifidobacteria and lactobacilli, considered non-pathogenic or beneficial bacteria [29]. The bacterial population in the large intestine is very large and reaches a maximum count of 10^{12} CFU.g^{-1}. In the small intestine, bacterial contents are considerably smaller from 10^4 to 10^7 CFU.g^{-1}, while in the stomach only 10^1 at 10^2 CFU.g^{-1} are found in function of low pH on this site. In total, the number of intestinal bacteria is approximately ten times the number of cells that make up the human body [30].

On the basis of rRNA sequencing 40,000 strains of intestinal bacteria can be indentified, including non-cultivable bacteria [31]. It was noted that 99% of intestinal bacteria consist of four phyla, Proteobacteria, Actinobacteria, and two main phyla Bacteroidetes and Firmicutes [32]. While the species in the phylum Bacteroidetes show a great variety between individuals, a large number of species in the phylum Firmicutes belong to clusters of clostridial butyrate producers [33].

With advances in molecular biology, it is known that the intestinal microbiome, contains 100 times more genes than the whole human genome [34]. Thus, a close relationship is evolving between the human gut microbiota. The human intestine exhibits to a symbiotic relationship that plays a key role in human homeostasis, including metabolism, growth and immunity [35].

One of the primary functions of the intestinal microbiota is the harnessing of energy from elements of the diet that could be lost through excretion [36]. The polysaccharides are not absorbed in the colon, but metabolized by resident microorganisms to short chain fatty acids (SCFA), such as propionate and butyrate, which are absorbed by passive diffusion [37]. SCFA production is dependent on the available fermentation of substrate, such as, starch or other polysaccharides, results butyrate, acetate and propionate [37]. SCFA concentrations are higher on the right side of the colon than on the left and this is probably due to the greater availability of carbohydrates [29]. The SCFA have an important role in the maintenance of the epithelial layer. Studies show that epithelial cells acquire about 70% of their butyrate oxidation [29]. The butyrate also acts as a trophic factor for cells in intact tissues [38]. In addition, it has been proposed that butyrate lowers the risk of colon cancer by its ability to inhibit the genotoxic activity of nitrosamines and hydrogen peroxide, as well as to induce various levels of apoptosis, differentiation and the cell cycle stop colon cancer in animal models [39].

Other researchers also cite the effect of butyrate on mediators of inflammation, it has been proved that this SCFA is able to inhibit the expression of some cytokines (TNF, IL-6, IL-1) and to inhibit the activation of nuclear factor κB (NF- κB) [40]. Other functions of the gastrointestinal microbiota include digestion of poorly digested nutrients, modification of bile acids, and nutritional supplementation by auxotrophic of mutants additional compounds that cannot be acquired by food consumption, such as folic acid and biotin [41].

The non-pathogenic commensal microbiota has a profound impact on the normal physiology of the GI tract. It ensures the efficiency of bowel motility, intestinal growth and immunity, as well as digestion, nutrient absorption and fortification of the mucus barrier [42].

Researchers have made advances in the characterization of GI microbiota defining the responses that may contribute to the development of inflammatory bowel diseases, such as, colon cancer [43]. Given the importance of a better understanding of intestinal microbiota, the TGI has been often studied. In recent decades, various intestinal simulators have been and are being developed, to facilitate the study of the intestinal microbial ecosystem and its interactions [44, 45].

3.2. Methods for *in vitro* evaluation of effects of probiotics on intestinal microbiota

The FAO/WHO refers to probiotics as live microorganisms that administered in adequate doses, benefit the health of the host [11]. The beneficial effects of ingesting probiotics

enhanced relief of the symptoms of lactose intolerance, treatment for diarrhea, reduction of serum cholesterol, enhanced immune response and anticarcinogenic effects [46].

The rising consumption of probiotic products by Europeans is mainly is in the form of dairy products containing generally *Lactobacillus* spp. and *Bifidobacterium* spp. However there are products in which the microorganisms used are strains of *Enterococcus* spp. or yeasts such as *Saccharomyces boulardii* [47]. Foods for human consumption containing lactic acid bacteria (LAB) include fermented milk, fruit juices, wine and sausages. Simple cultures or mixed microorganisms are used in probiotic preparations [48].

Several experimental observations have pointed to the potential protective effect of LAB against the development of tumors in the colon [49]. Within the intestinal microbiota, the LAB complex constitutes part of those bacteria able to promote a beneficial effect. They have an important role in retarding colon carcinogenesis by possibly of influencing metabolic, protective and immunological functions in the intestine [39]. The effect of intake of probiotics on intestinal native microbiota can be assessed through *in vivo* or *in vitro* models. *In vivo* models may involve healthy human volunteers, hospitalized patients or an animal model, but these models have some limitations such as high cost, delay in obtaining results and the type of food or drugs administered [50], whereas, *in vitro* models enable you to simplify the system and study separately the metabolism of native and added microbiota, in the presence of specific substrates [50].

In vitro fermentation models range from a simple batch system to more complex systems of continuous flow and multi-stage. *In vitro* gut fermentation models enable the stable cultivation of a complete intestinal microbiota for a defined and model-specific period of time. Selection of the appropriate model requires careful evaluation of the study objectives given the advantages and limitations exhibited by each type of system. Some existing systems are included in the batch, continuous culture, multi-stage continuous culture, continuous artificial digestive system and stationary systems [51].

Batch fermentation is the growth of a pure or mixed bacterial suspension in a carefully selected medium without the further addition of nutrients. These models are generally closed systems is sealed bottles or reactors containing suspensions of fecal material which are maintained under anaerobic conditions. Several studies have already been carried out, using this type of model in research on the prebiotic potential of fructans. This template is particularly useful to investigate metabolic profiles of SCFAs arising from active metabolism of dietary compounds by intestinal microbiota [50].

Continuous culture fermentation models exist as either single- or multistage systems and are necessary to perform long-term studies, as substrate replenishment and toxic product removal are facilitated. Single-stage continuous fermentation models are often used to elucidate proximal colon function and metabolic activity as the mixing of digest from both the caecum and ascending colon is well simulated in these models [52].

These models have several advantages, such as: the ease of use of the system, the possibility of using radioactive substances and the low operation cost [28].

A major advance for *in vitro* fermentation systems was the development of continuous multi-stage models, which allow the simulation of horizontal processes. This type of system makes it easy to study the nutritional and physicochemical properties of intestinal microbiota, through the combination of three reactors connected in series, simulating the proximal, distal and transversal colon (see Figure 1). Later, Molly et al. [44] developed the human microbial ecosystem simulator (SHIME ®), which consists of a succession of five connected reactors, which represent the different parts of the human gastrointestinal tract with their respective values of pH, residence time and volumetric capacity (Figure 1). The five reactors are continually agitated and kept at a temperature of 37 ºC by means of a thermostat. The medium is kept in the anaerobic state, by daily injection of N_2. The appropriate pH for each portion of the GI tract is controlled automatically by adding 1N NaOH or concentrated HCl [44, 45].

Figure 1. Computer controlled simulation of human microbial ecosystem (SHIME ®) housed in the Probiotics Research Laboratory of FCF/UNESP-Brazil. Sivieri et al.[53]

The adaptation, survival and proliferation of a human intestinal microbiota in continuous fermentation *in vitro* models are depended on environmental parameters such as pH, retention time, temperature, flow rate and oxygen depletion. The rigorous control of these factors allows steady established state in conditions the microbial composition and metabolic activity, creating a reproducible system.

The continuous cultivation model has been used in research on the metabolism and ecology of intestinal microbiota, with an emphasis on the use of probiotics [51, 54], prebiotics [55, 56] and the formation of fermentation products [57]. The *in vitro* modeling of host digestive functions in vitro coupled with multistage continuous fermentation, represents the most advanced attempt thus far at simulating interdependent physiological functions within the human gut, stomach lumen and small intestine. Human digestive functions that are

reproduced in the TIM-1 small intestine model include bile secretion, motility, pH and absorption capacity of the upper intestine. Proximal colon simulator models such as TIM-2 include other host functions such as peristaltic mixing and water and metabolite absorption. The combination of TIM-1 and TIM-2 models led to the creation of an artificial digestive system which has been used to investigate pharmaceutical drug delivery and advanced nutritional studies [58, 59].

The use of a multidisciplinary biological systems approach, in combination with '-omics' platforms as outlined will facilitate the most advanced system for unraveling the complex microbial and host factors governing human gut microbiota functionality [60].

In vitro fermentation models are an innovative technological platform where the greatest advantages are exhibited by the virtually limitless experimental capacity as experimentation is not restricted by ethical concerns. Host intestinal function is only partially simulated in some model designs (e.g. TIM-1 and TIM-2) and together with microbial population balancing remains a major challenge of in vitro gut fermentation modeling.

3.3. Inhibition on colon cancer by probiotics and the possible action mechanisms of these microorganisms

The evidence pointing to the beneficial effects of probiotics on colon cancer comes from *in vitro* tests, experiments with animals and clinical trials. Additionally, these has been much discussed on which step in the process of carcinogenesis might the effect by probiotics. It is likely that different probiotic strains act on different stages of carcinogenesis [20].

In general, the probiotics do not colonize the human gut, but some strains are can permanently colonize the indigenous microbiota [61].

The mechanisms by which probiotics may inhibit colon cancer are not yet fully characterized. However, several explanations have been suggested including: alteration of the metabolic activities of the intestinal microbiota; quantitative and qualitative changes in the intestinal microbial compositin; alteration of physicochemical conditions in the colon; binding and/or degradation of potential carcinogens; SCFA production; production of anti-tumorigenic or anti-mutagenic compounds; modulation of hosts's immune response, and/or physiology [3,62, 63].

Probiotics may modulate the metabolic activities of the intestinal microbiota by three possible mechanisms: competing with and displacing other components of the microbiota; producing antibacterial substances, including bateriocins, to control the growth of other members of the microbiota; producing lactic and other organic acids, which might lower the luminal pH and thus modulate enzyme activity [20,64].

Several investigations have shown that probiotics can influence bacterial enzymes activity related to the production of carcinogenic compounds, such as beta-glucuronidase, nitroreductase and azoreductase [65, 66, 67].

Bacterial glucuronidase appears to have an important role in the initiation of colon cancer, due to its ability to hydrolyze several glucuronides and carcinogenic aglycones in the intestinal lumen [65,68]. The nitroreductase and azoreductase take past in to the formation of aromatic amines harmful to the body [69].

Both harmful and beneficial bacteria are commonly found in the intestines and differ in their enzymatic activity [70]. In general, bacteria from the genera *Bifidobacterium* and *Lactobacillus* produced a very little activity of enzymes that convert pro-carcinogens into carcinogens, compared with bacteria from the genera *Bacteroides* and *Clostridium* [71]. Therefore, the activities of these enzymes in the lumen might be correlated with the number of lactic acid bacteria (LAB) in the intestine [72]. This suggests that increasing the proportion of LAB in the gut could diminish the levels of xenobiotic metabolizing enzymes [71]. Thus, the effect of probiotic microorganisms on fecal enzyme activities might be explained by this mechanism.

In a preliminary study, on feces of small animal, the animal supplementation of a high cholesterol diet with a mixture of probiotic strains of *L. johnsonii* and *L. reuteri* for 5 weeks significantly decreased the activity of fecal-glucuronidase and azoreductase [67].

Gorbach and Goldin [65] studied, in humans, the effect of ingestion of *L. acidophilus* NCFM strains about the activity of-glucuronidase, nitroreductase and azoreductase. Both strains had a similar effect and caused a significant decline in the activity of these three enzymes. A reverse effect was found 10 to 30 days after the end of the intake of these bacteria, suggesting that continuous consumption of *L. acidophilus* is necessary for maintaining.

Benno and Mitsuoka [73] and Spanhaak et al. [66] also found in humans, a significant reduction in the activity glucuronidase after intake of *Bifidobacterium longum* and *L casei* Shirota, respectively. On the other hand, Marteau et al. [74] verified in healthy volunteers that the regular consumption of a fermented dairy product (100 g three times per day) containing *L. acidophilus*, *B. bifidum*, *Streptococcus thermophilus* and *S. cremoris* for 3 weeks decreased the feces nitroreductase activity from baseline but not that of β-glucuronidase or azoreductase.

Feces metabolites are also indicators of bacterial activity. Changes in enzyme activities and the concentration of ammonia, phenol and cresol have been detected in volunteers who consumed Lactobacilli [65]. Other metabolites with possible adverse effects are N-nitroso compounds, diacylglycerol and secondary bile acids [49].

A wide variety of microrganisms can produce ammonia, for example, enterobacteria, bacteroides and clostridia. Ammonia is considered a potential promoter of tumor in the colon and it can increase the rate of neoplastic transformation in the intestine. According to Benno and Mitsuoka [73], reducing the proportion of clostridia and bacteroides could explain the decrease in the concentration of ammonia in individuals who consumed fecal *B. longum.*

Epidemiological studies indicate an association between the risk of developing colon cancer and the consumption of high fat diets [7,75, 76]. For the digestion of fats, bile acids

conjugated to glycine or taurine molecules are released into the small intestine and reabsorbed in the same location. It is believed that the deoxycholic acids may be cytotoxic to the epithelial cells, which could lead to the development of colon cancer [71]. Probiotic modulation of the intestinal microbiota may affect the activity of one of the enzymes (7a-dehydroxylase) forming these toxic products, but probiotics may also reduce the toxicity of bile salts that bind to them [77]. Lidbeck et al. [68] found that administering *L. acidophilus* to colon cancer patients for 6 weeks resulted in reduction in the concentration of soluble bile acids in the stool.

The consumption of fermented milk containing *L. acidophilus* may reduce the population of harmful bacteria, such as coliforms, and increased levels of lactobacilli in the intestine [78], suggesting that supplementation with this microorganism can have a beneficial effect since it inhibits the growth of bacteria that harmful are possibly involved in the production of tumor promoters and pro-carcinogens. Savard et al. [79] assessed the impact of four week's consumption of commercial yoghurt with *Bifidobacterium animalis* subsp. lactis (BB-12) and *Lactobacillus acidophilus* (LA-5) on fecal bacterial counts in healthy adults. The yoghurt had a positive effect on the bacterial population in that a the increase in beneficial bacteria and the reduction of potentially pathogenic bacteria was observed.

Not all studies show a correlation between the administration of probiotics and the activity of intestinal microbiota. Bartram et al. [80] argued that the fecal microbiota is relatively stable and generally unaffected by the administration of probiotics. In an intervention study, 12 individuals consumed yogurt (500 mL) enriched with *B. longum*. No significant difference was found in fecal weight, pH, concentration of fecal short chain fatty acids, bile acids and neutral sterols after 3 weeks of intervention. Despite the rise in the fecal concentration of *B. longum*, the results suggested litlle or no modulation of resident microbiota.

Some researchers have suggested that a high intestinal pH may be related to increased risk of colon cancer, whereas acidification of the colon could prevent the formation of carcinogens. Benno and Mitsuoka [73] found a significant reduction of faecal pH in health men who ingested *B. longum* for 5 weeks.

Evidence indicates that a high concentration of short chain fatty acids (acetate, propionate and butyrate) can assist in maintaining an appropriate pH in the lumen of the colon for the expression of many bacterial enzymes that probably metabolize carcinogens in the gut [81]. The activity of some dietary carcinogens, such as nitrosamines (resulting from commensal bacterial metabolic activity in individuals who consume a diet rich in proteins) can be neutralized by butyric acid produced by some probiotics [82]. Furthermore, production of ammonia, nitrosamines and secondary bile acids in the intestinal environment can be reduced by lowering the pH [83].

Butyrate, particularly, has received much attention as a potential chemopreventive agent [1,84]. While acting as an energy source for untransformed cells, butyrate possibly reduces survival of tumor cells by inducing apoptosis and differentiation, as well as by inhibiting

proliferation. These mechanisms may play an important role in the reduction and/or inhibition of promotion and progression of cancer [1, 85].

Studies show that the LAB may be involved in the detoxification of various carcinogens such as polycyclic aromatic hydrocarbons and heterocyclic aromatic amines [86]. The mechanisms of action of these bacteria are poorly known, but it is possible that the LAB bind directly to the carcinogen and catalyze detoxification reactions [62]. It is worth noting that the protective effects conferred by LAB only appear when these are at a high density and when there is a regular intake [87].

Evidence is accumulating that heterocyclic aromatic amines (HCAs), which are derived from amino acids in meat during cooking, might be involved in the etiology of human cancer [88]. Zsivkovits et al. [89] showed that *L. bulgaricus* 291, *S. thermophilus* F4, *S. thermophilus* V3 and *B. longum* BB536 are highly protective against the genotoxic effects of HCAs in rats. Additionally, the inhibition of HCAs induced DNA damage was dose dependent and significant when 1 x 10^7 cells/animal were administered. Other authors showed that *L. casei* DN 114001 may metabolize or adsorb HCAs and reduce their genotoxicity *in vitro* [89].

In vivo evidence that probiotics bond the carcinogens are still not conclusive. Hayatsu Hayatsu (1993) demonstrated the marked suppressive effect of orally administered *L. casei Shirota* (LcS) on the urinary mutagenicity arising from ingestion of fried ground beef by humans. In another clinical trial, the consumption of *L. acidophilus* decreased the urinary and fecal excretion of mutagens [68]. In view of the *in vitro* results, it is possible that the LAB supplements are influencing excretion of mutagens by simply binding them in the intestine [62]. Even though the binding of carcinogens is a possible mechanism for the inhibition of genotoxicity and mutagenicity by LAB in vitro, some researchers have reported that it does not appear to have any influence *in vivo* [90]. Additionally, the extent of the binding depends on the mutagen and bacterial strain used [71].

Several studies have also reported the effect of probiotics on the promotion phase of carcinogenesis. Rowland et al. [91] found that administration of *B. longum* (6 x 10^9 CFU/day) inhibited the formation of aberrant crypt foci (ACF) in rats that received an induced of carcinogenesis (azomethane). As the probiotic treatment began 1 week after exposure to the carcinogen, these results indicate an effect on the early promotional phase of carcinogenesis [71].

Goldin et al. [92] observed a lower incidence of colonic tumors in rats who consumed *Lactobacillu*s GG before, during and after chemical induction with dimethylhydrazine (DMH) than in animals that were fed the probiotic after receiving carcinogen. The researchers concluded that probiotics acted by inhibiting the initiation stage of carcinogenesis.

Kumar et al. [93] tested the efficacy of *L. plantarum* AS1 in the suppression of colorectal cancer induced by DMH in rats and formed that AS1 was capable of diminishing colon

tumor through its antioxidant activity. However, long-term administration of this strain was necessary to achieve the maximum inhibitory effect.

On the other hand, not all studies have shown significant effects of probiotic on carcinogen-induced ACF. Gallanger et al. [94] using na ACF promotion protocol together with *B. longum* and *L. acidophilus*, obtained inconsistent results, which they attributed to differences in the ages of rats when DMH was administered.

Several studies have correlated the effect of probiotic on colon cancer with the modulation of the immune system. There is evidences that probiotics may contribute to the development of the mucosal immune system by influencing the innate inflammatory response and reducing mucosal inflammation. Additionally, probiotics also act on dendritic and epithelial cells and native T cells in the lamina propria of the gut and can thus influence adaptive immunity [13, 95].

Probiotics may influence the immune system by the action of products, such as metabolites, cell-wall components and DNA. Thus, immune modulatory effects might even be achieved by dead probiotic microorganisms or just probiotic derived components such as peptidoglycan fragments or DNA. Probiotic products are recognized by host cells sensitive to them these because they are equipped with recognition receptors adhesion. The main target cells in this context are therefore gut epithelial and gut-associated immune cells. The adhesion of probiotics to epithelial cells might itself might already trigger a signaling cascade leading to immune modulation [96].

Recent advances in the understanding of the immunomodulatory activity of probiotics have resulted from the discovery of Toll-like pattern recognition receptors (TLRs). These are transmembrane proteins present on the surface of cells such as macrophages, monocytes, dendritic cells and epithelial cells [97].

The innate immune system recognizes a large number of molecular structures from bacteria, such as, lipopolysaccharides and lipoteichoic acid, and is able to distinguish whether a particular microorganism is part of its microbiota or not. Different structures can activate different TLRs [98]. For example, TLR-2 recognizes the peptidoglycan, lipoteichoic acid, which is a component of the wall of Gram-positive bacteria such as lactobacilli and bifidobacteria [99], whereas TLR-4 is the most important receptor for lipopolysaccharide, the main component of the wall of Gram-negative bacteria [100].

Rachmilewitz et al. [101] using a probiotic mixture of 8 strains of freeze-dried lactic acid bacteria (*Bifidobacterium longum, B. infantis, B. breve, Lactobacillus acidophilus, L. casei, L. delbrueckii subsp. bulgaricus, L. plantarum, Streptococcus salivaris subsp. thermophilus*), reported that the chromosomal DNA of this mixture was responsible, via TLR-9 receptors for an anti-inflammatory effect observed in mice with colitis.

The connection of components of microorganisms to these receptors can lead to a cascade of inflammatory reactions via the activation of nuclear factor-kB (NF-kB), with subsequent release of cytokines, epitope chemokines and lipid mediators of reactive oxygen and

nitrogen species [102]. Studies have shown that probiotics can activate elements responsible for the formation of cytokines and epitope chemokine's, although that response was weaker for *L. rhamnosus* if than for a Gram-positive pathogen (*Streptococcus pyogenes*) [103]. Some authors have suggested that a possible mechanism of action of probiotics would be the inhibition of NF-kB activation by reducing intestinal inflammation [104]. However, the possible mechanisms of probiotics against carcinogenesis, regarding the modulation of the immune system, are complex and still need to be better further elucidated.

An inflammatory immune response produces monocytes and macrophages, activated by cytokines that release cytotoxic molecules capable of the lyzing tumor cells *in vitro* [105]. The cytokines IL-1 and inflammatory TNF (tumor necrosis factor) exert cytotoxic and cytostatic effects on neoplastic cells *in-vitro* [106]. Natural-killer cells (NK) are effective against tumor cells and low activity of this cell type has been linked to a risk of cancer [107]. Matsuzaki and Chin [108] found that in mice, NK cell activity and inflammatory responses increased with the administration of probiotic strains.

Several studies in humans have shown an increase of NK cells in response to the consumption of probiotics [109, 110], and the same has been in animal models. When Takagi et al. [111] administered the strain *L. casei* Shirota, in order to inhibit tumor development induced by methylcholantracene in mice, there were high levels of NK cells in the group treated with the probiotic, which slowed the early development of the tumor, compared to the control group.

On the other hand, Berman et al. [112] did not observed any increasing in NK cells in healthy subjects who consumed during 8 weeks a formulation containing 4 species of probiotics (*L. rhamnosus*, *L. plantarum*, *L. salivarus* and *B. bifidum*). However, the researchers did note an increase in phagocytosis by neutrophils and monocytes.

Evidence has shown that the probiotic *Lactobacillus casei* Shirota has anti-tumor effects and antineoplastic action in rodents (biologically or chemically induced). Intrapleural administration of the strain in mice with tumor induced the production of various cytokines, such as interferon IL-1 and TNF in the thoracic cavity, which resulted in tumor inhibition and increased survival [113]. A study on *B. longum* and *B. animalis* showed that these bacteria induce the production of inflammatory cytokines (IL-6 and TNF-) [114].

In a clinical trial, the effect of *L. casei* Shirota on NK cell activity in humans was investigated. The activity of NK was increased as a likely consequence of *L. casei* Shirota-induced IL-12 production which was detected in *in vitro* assays [115].

According to the results of the various studies mention here, the probiotic microorganisms are capable of modulating the immune system in a strain-specific manner [116]. Therefore, different strains may induce different immune responses that might lead to the inhibition of carcinogenesis.

4. Effects of *Enterococcus faecium* CRL 183 on intestinal microbiota and colon cancer

Enterococcus spp. are Gram-positive, non-sporulating, catalase and oxidase negative facultative anaerobes [72]. Species of this genus are natural constituents of the intestinal microbiota of humans and comprise the third-largest genus of lactic acid bacteria (LAB), after *Lactobacillus* spp. and *Streptococcus* spp. [117].

It is hard to determine the exact number of enterococci species, but from a microbiological and functional point of view, *Enterococcus faecalis* and *Enterococcus faecium* are considered the most important [117, 118].

Some strains of *Enterococcus* spp. exhibit antibiotic resistance, possess virulence factors (adhesions, invasins, pili and haemolysin) and may cause bacteremia, endocarditis and other infections [117]. However, commercial pharmaceutical preparations of enterococci include *Enterococcus faecium* SF68® (NCIMB 10415, produced by Cerbios-Pharma SA, Barbengo, Switzerland) and *Enterococcus faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany), are on the market without reported health problems. Since 2008, *Enterococcus faecium* has been authorized for use in food and recognized as a probiotic microorganism in Brazil [119].

Currently, several strains of *Enterococcus faecium* are considered safe for human consumption, being used as starter cultures in cheese making and other fermented products and recognized as probiotic microorganisms [120]. The use of *Enterococcus faecium* as a starter culture in various fermented foods can be explained by its resistance to high concentrations of NaCl and low pH, and its ability to produce different aromas.

The strain of *Enterococcus faecium* CRL 183 was isolated by researchers from at the Reference Center for Lactobacillus (Cerela-Argentina), from cheese samples of Tafí – a traditional homemade cheese from the highland province of Tucuman, Argentina [121]. *In vitro* and *in vivo* studies showed that *Enterococcus faecium* CRL 183 is able to adhere to the intestinal cells, resists the gastrointestinal environment and colonizes the large intestine of rats, thus satisfying the requirement for a probiotic microorganism [122, 123]. Furthermore, this strain has no antibiotic resistance and no virulence factors, ensuring its safe use as a starter culture [121].

Enterococcus faecium CRL 183 has been investigated by our research group for about 20 years, with the objective of defining its functional properties in the free form or associated with food products [122,123,124,125,126, 127, 128, 129, 130].

The best functional effects of *Enterococcus faecium* CRL 183 were obtained when this microorganism was used as a starter culture of a yogurt-like fermented soy product (soy yogurt) [129]. This product has sensorial and technological properties similar to fermented-milk yogurt drinks and has exhibited functional properties in animal tests and clinical trials. Among the beneficial effects of the soy product fermented with *Enterococcus faecium* CRL 183, the following deserve special attention: improved of lipid profile, modulation of the

immune system, positive changes in the intestinal microbiota, and reduction of colon cancer development [123, 125,128,130] .

Sivieri et al [123] studied the effect of daily ingestion of *Enterococcus faecium* CRL 183 (8 log CFU/mL) on the incidence of colorectal tumors induced by 1,2 dimethylhydrazine (DMH) in rats (20 mg/kg body weight, in a weekly dose, for 14 weeks). The experiment was conducted over 42 weeks and the rats were allocated to three groups: G1 - Control (not induced); G2 – Induced with DMH; G3 – Induced with DMH + *E. faecium* CRL 183. Thioglycollate-elicited peritoneal exudate cells (PECs) were harvested from animals in PBS and the adherent cells were obtained after incubation with LPS or RPMI-1640 (CO2 - 95:5, v/v). The cytokine levels (IL-4, IFN- γ and TNF- α) were determined in the supernatant of of the cell culture by ELISA. After euthanasia, colons were removed for histological analysis. The animals with induced colorectal cancer and that received the suspension of *Enterococcus faecium* CRL 183 (G3) showed a 50% reduction in average number of tumors compared to G2 ($P < 0.001$) (Figures 2 and 3). The total number of aberrant crypt foci (ACF), the total ACF/mm^2, the number of crypts per ACF and the adenocarcinoma were also reduced in G3. In addition, G3 exhibited increased production of IL-4, IFN- γ and TNF- α by PECs compared to G2.

Anti-tumor activities of probiotic acid lactic bacteria have been attributed to an enhanced immune response [132]. The induction of TNF- α by probiotic bacteria would be necessary to initiate cross-talk between the immune cells associated with the lamina propria and the intestinal epithelial cells. IFN- γ is involved in the maturation of immune cells (dendritic cells), controls their cellular proliferation at the intestinal level and induce other cytokines, especially IL-4, IL-5 and IL-10. Because of its role in mediating macrophage and NK cell activation, IFN- γ is important in the host defense against intracellular pathogens, viruses and tumors [133]. According to Perdigón et al. [134] IL-4 exerts control over the inflammatory response induced by the carcinogen. In that study, the antitumor activity of *Enterococcus faecium* CRL 183 was attributed to its ability to modulate the immune response.

It has been suggested that increasing the consumption of red meat and animal fat lead to an increased risk of developing cancer colon, in comparison with a vegetarian diet [23]. Several studies have demonstrated that the microbiota of the colon is involved in the etiology of the colon cancer and that the some strains of probiotic microorganism can have beneficial effects on the composition of the intestinal microbiota, stimulates the production of short chain fatty acids (SCFA) and inhibit the activity of enzymes that convert pro-carcinogens into carcinogens [39,49, 135, 136].

Based in these evidence, a study was carried out to determine if consumption of a soy product fermented with *E. faecium* CRL 183 was able to modify the fecal microbiota of rats fed a diet containing red meat [122]. The experiment was conducted during over days and the animals were randomly divided into six groups: GI - standard casein-based rodent feed; GII to GVI - beef-based feed. From the 30th day, G III–VI also ingested the following products: G III, *E. faecium*-fermented soy product; G IV, pure suspension of *E. faecium*; G V, sterilized fermented soy product; and G VI, unfermented soy product (3 mL kg^{-1} BW day^{-1}). The feces of each animal were collected at the start (T0) and on the 30th (T30) and 60th (T60)

days of the experiment, to determine the viable cell counts of total aerobic and anaerobic bacteria, *Enterococcus* spp., Enterobacteria, *Lactobacillus* spp., *Clostridium* spp., *Bacteroides* spp. and *Bifidobacterium* spp.

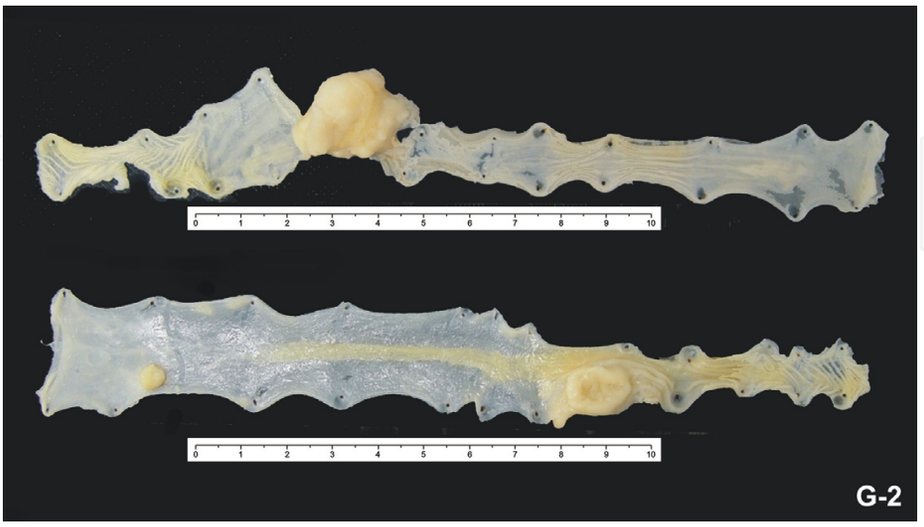

Figure 2. Topographic view of macroscopic growths by G2 – Induced with DMH. Sivieri et al [123]

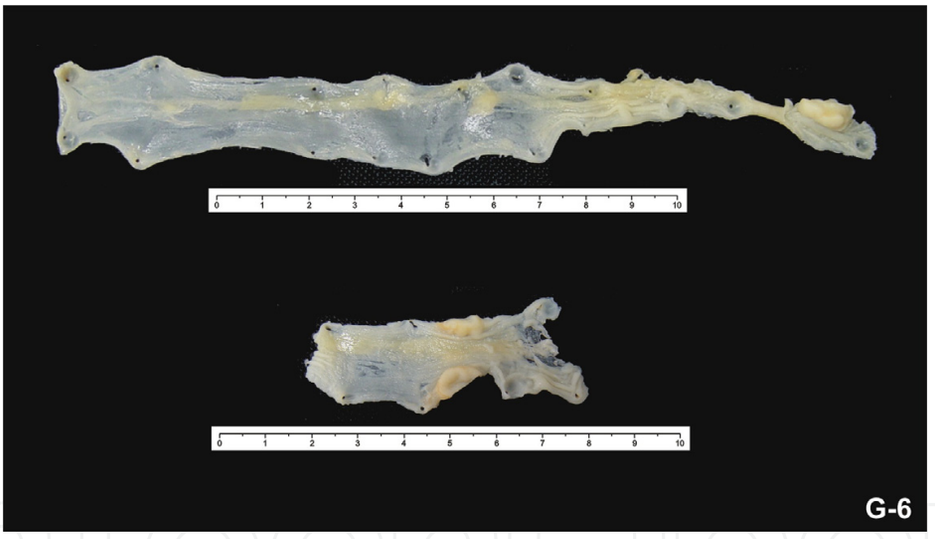

Figure 3. Topographic view of macroscopic growths by G3 – Induced with DMH + *E. faecium* CRL 183. Sivieri et al [123]

By day T30 days of experiment, rats on a red meat-based diet exhibited an increase in the population of total anaerobes, enterobacteria and enterococci and a decrease in the numbers of lactobacilli and bifidobacteria. From T30 to T60, the obtained results showed that fermented soy product and pure *Enterococcus faecium* CRL 183 suspension promoted an increase in the numbers of lactobacilli (0.45 log CFU g^{-1} and 1.83 log CFU g^{-1}, respectively). During the same period, only the animals treated with pure *Enterococcus faecium* CRL 183 suspension showed a rise in the fecal bifidobacterium population. The fermented soy product promoted a slight fall in the *Bacteroides* spp. population (2.80 ± 0.20 to 2.34 ± 0.07 log CFU g−1), but the counts of *clostridia*. and enterobacteria were unchanged.

Another study, using New Zealand rabbits with induced hypercholesterolemia as an animal model, was conducted to investigate the possible correlation between fecal microbiota, serum lipid parameters and atherosclerotic lesion development. It was show that, after 60 days of the experiment, intake of the probiotic soy product (with or without isoflavones) was correlated with significant increases ($P<0.05$) in *Lactobacillus* spp., *Bifidobacterium* spp. and *Enterococcus* spp. and a decrease in the enterobacteria population (Cavallini et al., 2011).

The studies conducted by Bedani et al (2010) and Cavallini et al. (2011) suggest that daily ingestion of the soy product fermented with *Enterococcus faecium* CRL 183, or the pure culture of this probiotic microorganism, may contribute to a beneficial balance of the fecal microbiota.

Currently, other studies, using animal models and an *in vitro* simulator of human intestinal microbial ecosystem (SHIME), are being conducted by our research group in order to elucidate the possible mechanisms involved in the protective effect of *Enterococcus faecium* CRL 183 against colon cancer and the importance of the modulation of fecal microbiota and stimulation of the immune system in the disease pathogenesis [53].

5. Conclusions

From the above discussion, it is evident that probiotics have the capacity to modulate the intestinal microbiota and the immune system, to the benefit of the host organism, reducing the risk of many chronic degenerative diseases, among them colon cancer. It appears also that the actions performed by probiotics are species-strain-dependent, so that several effects or actions can occur with the same bacterial genus. However, the results of several experiments reported in the literature, highlight a degree of controversy concerning the effects observed, especially regarding the various types of cancers and it is difficult to compare these studies. Such controversies are due mainly to large variations in the time of the experiment - usually prevailing those of short duration the experimental models, bacterial strains and the doses and frequencies of administration of probiotics. In this sense, it is important that further studies be done to define and standardize these variables mentioned, and especially to elucidate the mechanisms involved in each of the observed effects.

It showed also be mentioned that, according there is also in the literature, that probiotics studied are taken almost exclusively in milk as can be observed in the products available on the market. This condition often makes them inappropriate for certain lactose intolerant population groups on those and allergic to milk proteins. Thus, alternative vehicles for probiotics, free of lactose and of β-lactoglobulin, such as the aqueous extract of soybeans, for example, deserve special attention from researchers seeking to develop products with a good nutritional profile and suitable to transport the probiotic specified for the purpose desire. It is expected that in the near future, as results of the interaction of various fields of study such as food science and technology, nutrition, microbiology, genetic engineering and molecular biology the market can offer consumers products that are more accessible and effective, reducing the risk of certain diseases, particularly certain types of cancer, and acting as adjuvants in specific treatments for existing diseases.

Finally, from the results obtained by our research group in the studies of probiotics in relation to colon cancer, and even other diseases, it appears that these was always variability between individuals, either in clinical trials or in studies with animal models, suggesting a possible specificity of these individuals in relation to consumption of given probiotics. This leads us to wonder, if today nutrigenomics is already a reality, is it not the moment to propose studies on something like "probiogenomics" or even about self-probiotics? Certainly the future will provide an answer to that question

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