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## Basic Study

**Lactobacillus bulgaricus** inhibits colitis-associated cancer via a negative regulation of intestinal inflammation in azoxymethane/dextran sodium sulfate model

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**Abstract****BACKGROUND**

Colitis-associated cancer (CAC) accounts for 2%-3% of colorectal cancer (CRC) cases preceded by inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis. Intestinal microbiota has been reported to play a central role in the pathogenesis of IBD and CAC. Recently, numerous prebiotics and probiotics have being investigated as antitumor agents due to their capacity to modulate inflammatory responses. Previous studies have indicated that lactic acid bacteria could be successfully used in managing sporadic CRC, however little is known about their role in CAC.

**AIM**

To investigate the effect of the probiotic *Lactobacillus bulgaricus* (*L. bulgaricus*) during the development of an experimental model of colitis associated colon

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**statement:** All authors declare that the Institutional Review Board approval was not applicable for this manuscript, once this study does not involve human beings.

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cancer (CAC).

## METHODS

C57BL/6 mice received an intraperitoneal injection of azoxymethane (10 mg/kg), followed by three cycles of sodium dextran sulphate diluted in water (5% w/v). Probiotic group received daily *L. bulgaricus*. Intestinal inflammation was determined by scoring clinical signs. Cytokines levels were determined from colon and/or tumor samples by ELISA BD OptEIA™ kits. The level of significance was set at  $P < 0.05$ . Graphs were generated and statistical analysis performed using the software GraphPad Prism 6.0.

## RESULTS

*L. bulgaricus* treatment inhibited of total tumor volume and mean size of tumors. In addition, the probiotic also attenuated the clinical signs of intestinal inflammation inducing a decrease in intestinal and tumor levels of IL-6, TNF- $\alpha$ , IL-17, IL-23 and IL-1 $\beta$ .

## CONCLUSION

Our results suggest a potential chemopreventive effect of probiotic on CAC. *L. bulgaricus* regulates the inflammatory response and preventing CAC.

**Key Words:** *Lactobacillus bulgaricus*; Colitis-associated cancer; Colorectal cancer; Carcinogenesis; Probiotics; Inflammation

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**Core Tip:** Recent studies suggested that consideration of the intestinal microbiota has an essential role in carcinogenesis. Probiotic supplementation is an alternative means of favourably modulating the intestinal microbiota. In this study, we investigate the effect of *Lactobacillus bulgaricus* (*L. bulgaricus*) during the development of an experimental model of colitis-associated colon cancer. Our results evidence an anti-inflammatory role and consequent antitumor effect of *L. bulgaricus* on colitis-associated cancer that may be used as a promising tool for the prevention and treatment of colitis-associated cancer.

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## INTRODUCTION

Colorectal cancer (CRC) remains one of the most incident type of cancer worldwide, being the third and second most frequently cancer diagnosed in men and women, respectively<sup>[1]</sup>. Colitis-associated cancer (CAC) specifically accounts for 2%-3% of CRC cases preceded by inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis<sup>[2]</sup>. The link between inflammation and cancer was firstly recognized in 1863 and has been recently exemplified by CAC. Patients with IBD have a higher risk for developing CRC and are affected by the disease earlier than patients with sporadic CRC<sup>[3,4]</sup>.

Currently, it has become increasingly evident that intestinal microbiota plays a crucial role in the pathogenesis of IBD and CRC. Changes in intestinal microbiota have been reported in patients with colon cancer, supporting this idea<sup>[5]</sup>. Among the gut microorganisms, probiotic bacteria may be defined as live microbial food supplements that confer benefits to the health of the consumer (WHO), including reduction of pathogen colonization by competition<sup>[6]</sup>, improvement in vitamin synthesis and nutrients absorption, stimulation of epithelial cell proliferation and differentiation,

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fortification of intestinal barrier and optimization of intestinal transit<sup>[7]</sup>.

In addition to the direct benefit of probiotics on the improvement of the host gut microbiota, probiotics have received considerable attention due to their anti-carcinogenic activities, mainly in CRC<sup>[8]</sup>. The underlying mechanisms for their anti-tumor effects are versatile and include: Modulation of host immune responses, such as proliferation of regulatory T cells, activation of macrophages and dendritic cells, and production of immunoglobulins and cytokines<sup>[9]</sup>; alteration of intestinal microbiota metabolism<sup>[10]</sup>; regulation of cell death, apoptosis, cell cycle, proliferation, invasion and metastasis<sup>[11]</sup>; competition with pathogenic bacteria<sup>[11]</sup>; and inactivation of carcinogenic compounds<sup>[12]</sup>.

The most common microorganisms used as probiotics comprise a group of bacteria named lactic acid bacteria (LAB) that produces lactic acid as the primary metabolite of sugar metabolism, such as *Lactobacillus* and *Bifidobacterium*<sup>[13,14]</sup>. Although previous studies have indicated that LAB could be successfully used in managing food allergies, diarrhea, IBDs and sporadic CRC<sup>[15-17]</sup>, little is known about its role in CAC. In this study, we sought to investigate the effects of the probiotic *Lactobacillus bulgaricus* (*L. bulgaricus*) in colitis-associated carcinogenesis.

## MATERIALS AND METHODS

### *Mice and treatment protocol*

In the present study, we used male C57BL/6 wild type (WT) mice, between 4-6 wk old and weighing between 20-25 g. The animals were purchased from the Animal Facility of the University of São Paulo (USP) and housed at the facility of Ribeirão Preto College of Nursing - EERP/USP (Ribeirão Preto, SP, Brazil) under controlled temperature conditions (25 ± 2 °C) with 12/12 photoperiod hours. Water and food were available ad libitum. All experiments were handled in accordance with institutional ethical guidelines, and the study was approved by the Ethics Committee on Animal Research from the University of São Paulo (CEUA PUSP-RP: No. 14.1.418.53.1).

### *L. bulgaricus and treatment*

*Lactobacillus delbrueckii ssp bulgaricus*, LOT No. FK0201, identification LB-G040, Chinese origin, was purchased from Liane Drugstore, Ribeirão Preto, SP, Brazil and stored in at 4 °C. For mice treatment, 1 × 10<sup>9</sup> CFU were diluted in 200 µL of PBS and orally given to each mouse, 3 times a week during all experimental period. Prior to tumor induction, mice were randomly distributed in 2 groups (*n* = 10) and treated with PBS (control group) or *L. bulgaricus* (Lb group) by gavage (0.2 mL/mouse) for one week.

### *CAC induction*

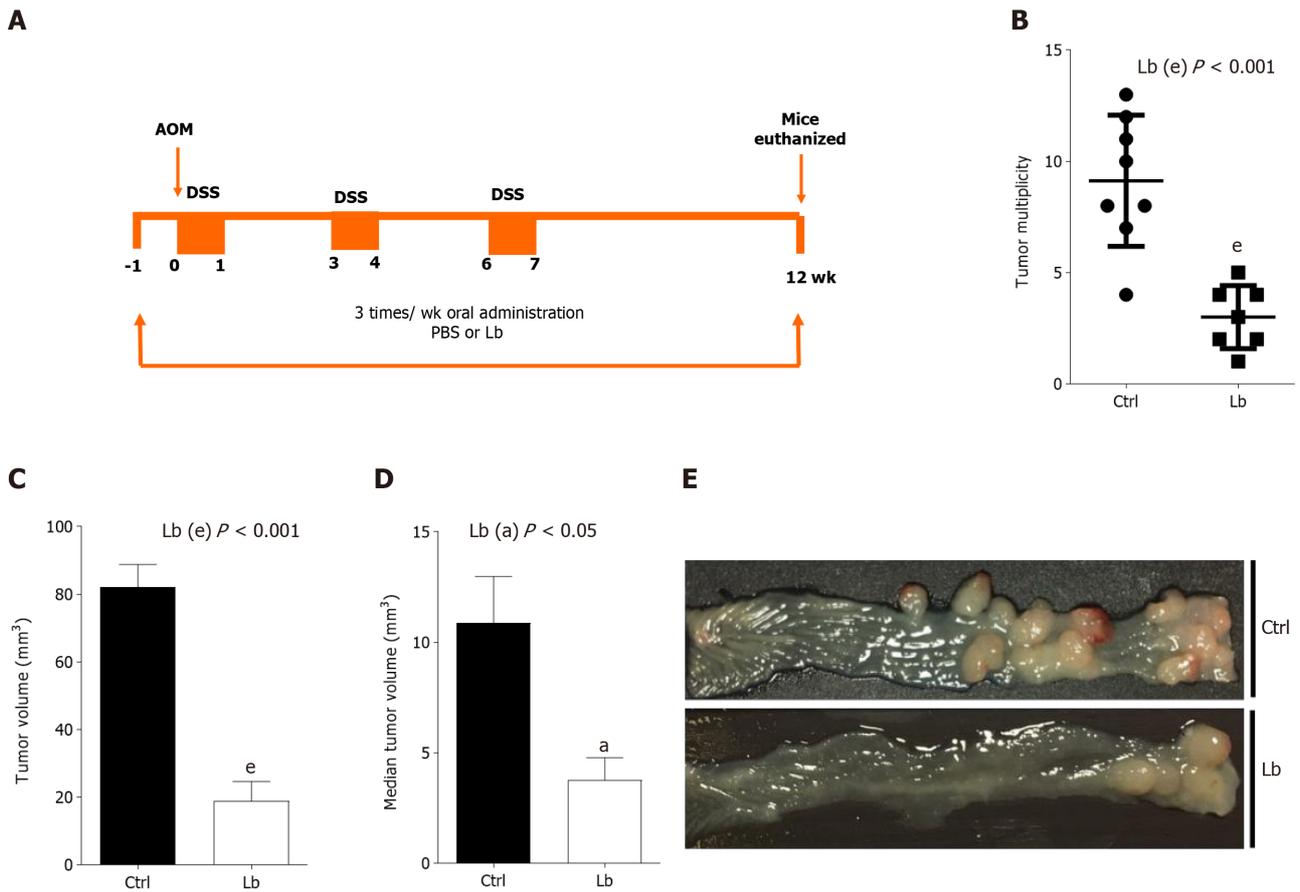
For CAC induction, mice were intraperitoneally (i.p.) injected with a single dose (10 mg/kg in 300 µL solution) of azoxymethene (AOM, Sigma-Aldrich), followed by 3 cycles of one week of 2.5% dextran sulfate sodium (DSS) diluted in drinking water intercalated for 2 wk of normal water<sup>[18]</sup>. Mice were euthanized 12th week after CAC induction (Figure 1A).

### *Disease score evaluation*

Intestinal inflammation *in vivo*, or disease score, was determined by scoring clinical signs as previously described<sup>[19]</sup>. Briefly, we used a scoring system in which one point (1.0) was attributed to each signal presented by the mouse, including: Weight loss ≥ 5% and < 10% of body weight compared to the previous day; presence of humid perianal region; presence of diarrhea; blood in the stool or perianal region; hyporactivity and piloerection. When weight loss was ≥ 10% 2 points were attributed to the "weight loss" signal. Finally, the final sum of these points determined the clinical score of the disease.

### *Determination of colon length*

The severity of intestinal inflammation was also assessed by measuring the length of the entire large intestine. After euthanasia, colons were collected, carefully placed on a clean surface and photographed. The images were calibrated by the presence of a graduated ruler that served as a scale for the analyzes. Subsequently, images of the large intestine were evaluated using ImageJ software.



**Figure 1** *Lactobacillus bulgaricus* inhibits tumor progression in azoxymethane/dextran sulfate sodium-exposed mice. A: Prior to tumor induction, mice were treated 3 times with PBS or the probiotic by gavage (0.2 mL/mouse) for one week. After that, colitis-associated cancer was induced by intraperitoneal injection of a single dose of azoxymethane (AOM), followed by 3 cycles of 2, 5% dextran sulfate sodium (DSS) in drinking water for one week and normal drinking water for 2 wk; B: After euthanasia at 12th week, colons were longitudinally opened, washed and examined for multiplicity; C: Total tumor volume; D: Mean tumor volume; E: The dimensions of colorectal tumors were measured with pachymeter and the volumes calculated as:  $(\text{Width})^2 \times \text{length} / 2$ . Illustrative and endoscopic images of AOM/DSS-induced tumors.  $^aP < 0.05$ ;  $^eP < 0.001$  vs controls. AOM: Azoxymethane; Ctrl: Controls; DSS: Dextran sulfate sodium; Lb: *Lactobacillus bulgaricus*; PBS: Phosphate-buffered saline.

### Determination of tumor volume and multiplicity

After euthanasia, the colons were longitudinally opened, washed and examined with regards to presence of tumors. The multiplicity of tumors was verified for each animal in the experimental groups. The dimensions of the colorectal tumors were measured with pachymeter and the volumes were calculated by the formula:  $(\text{Width})^2 \times \text{length} / 2$ <sup>[20]</sup>. Total tumor volume, indicates the sum of the volumes of all tumors found in each colon. Mean tumor volume refers to the mean tumor size, i.e., total tumor volume divided by the number of tumors of each colon.

### Histological analysis

Distal colon parts were fixed in 4% p-formaldehyde in phosphate-buffered formalin and unblocked in paraffin. Tissue sections (4.0  $\mu\text{m}$ ) were prepared from the paraffin-embedded tissue blocks, stained with hematoxylin and eosin and evaluated in a blinded fashion by an experienced pathologist (MOB). Normal colon, polyp without dysplasia, adenoma with low-grade dysplasia, adenoma with high-grade dysplasia, and invasive adenocarcinoma were identified in the different groups.

### Cytokine quantification

Cytokines levels were determined from colon and/or tumor samples. Tissues were collected, weighed and immediately homogenized in PBS in the presence of protease inhibitors (Roche) using a tissue homogenizer (Polytron System PT 1200E). The material was then centrifuged at 6000 rpm for 15 min at 4 °C and the supernatant collected, aliquoted and stored at -80 °C until the time of use. The concentrations of TNF- $\alpha$ , IL-6, IL-12 (p70), IL-17, IFN- $\gamma$ , IL-1  $\beta$ , IL-10, TGF- $\beta$ , IL-23 were determined by ELISA BD OptEIA™ kits (BD Biosciences Pharmingen) or DuoSet (R&D Systems).

The protocol was performed according to the manufacturers' instructions. Cytokine concentrations were determined with reference to the linear regression line obtained with the serial dilution data of each recombinant mouse cytokine.

### Statistical analysis

Data were analyzed using the statistical program GraphPad Prism version 6. Parametric and non-parametric samples were analyzed by one-way Analysis of Variance (ANOVA test) and Kruskal-Wallis test followed by Dunn's test, respectively. The probability was considered statistically significant if  $P < 0.05$ . Results were expressed as mean  $\pm$  SEM.

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## RESULTS

### *L. bulgaricus* treatment inhibited tumor progression in AOM/DSS-induced model of colon carcinogenesis

To investigate whether *L. bulgaricus* is able to inhibit the progression of CAC, we compared tumor development in AOM/DSS-induced mice treated or not with the probiotic (Figure 1A). As shown in Figure 1, animals from both groups developed tumors at the end of the experimental protocol, whereas those treated with the probiotic developed fewer and smaller tumors. Control mice developed between 4-13 colorectal tumors, whereas animals treated with *L. bulgaricus* developed only 1-5 (Figure 1B). *L. bulgaricus*-treated animals showed a total tumor volume (Figure 1C) and a mean tumor volume (Figure 1D and E) about 4.4-fold and 3-fold lower, respectively. However, no difference was observed in the incidence of tumors (data not shown).

### *L. bulgaricus* attenuated intestinal inflammation in AOM/DSS-induced model of colon carcinogenesis

Once inflammation plays a critical role in CAC carcinogenesis, we evaluated intestinal inflammation in AOM/DSS-induced mice treated with *L. bulgaricus* by three different parameters: Body weight, disease score and colon length. Although we did not observe differences in body weight loss between control and *L. bulgaricus*-treated (Figure 2A), we found differences in clinical signals in *L. bulgaricus*-treated mice, which showed a lower clinical score on the 13th and 15th days after tumor initiation (Figure 2B).

In addition to the attenuation of intestinal inflammation score, we observed that the treatment with *L. bulgaricus* reduced the DSS-induced shortening of the colon (Figure 2C and D) so that the control group had a shorter colon extension when compared to Lb group (Figure 2C and D). After histopathological evaluation of the tumor sections, we observed that, regardless of treatment, both groups of mice presented morphologically similar neoplastic lesions. In general, colorectal tumors were lesions of the polypoid adenoma type with variation between low and high degrees of dysplasia and mixed inflammation (Supplementary Figure 1).

### *L. bulgaricus* inhibits the production of proinflammatory cytokines in tumors and colons of AOM/DSS-induced mice

Once we observed that *L. bulgaricus* regulates gut inflammation, we also measured the intestinal concentration of inflammatory mediators involved in CAC pathogenesis. In segments of the large intestine that did not present tumors (inflamed colon) we observed a reduction of at least 2-fold in the levels of the cytokines TNF- $\alpha$ , IL-1  $\beta$ , IL-23 and IL-17 in *L. bulgaricus*-treated mice in comparison to controls (Figure 3). In contrast, increased concentrations of IFN- $\gamma$  were also observed in Lb group (Figure 3). We did not observe differences in IL-6 levels (Figure 3).

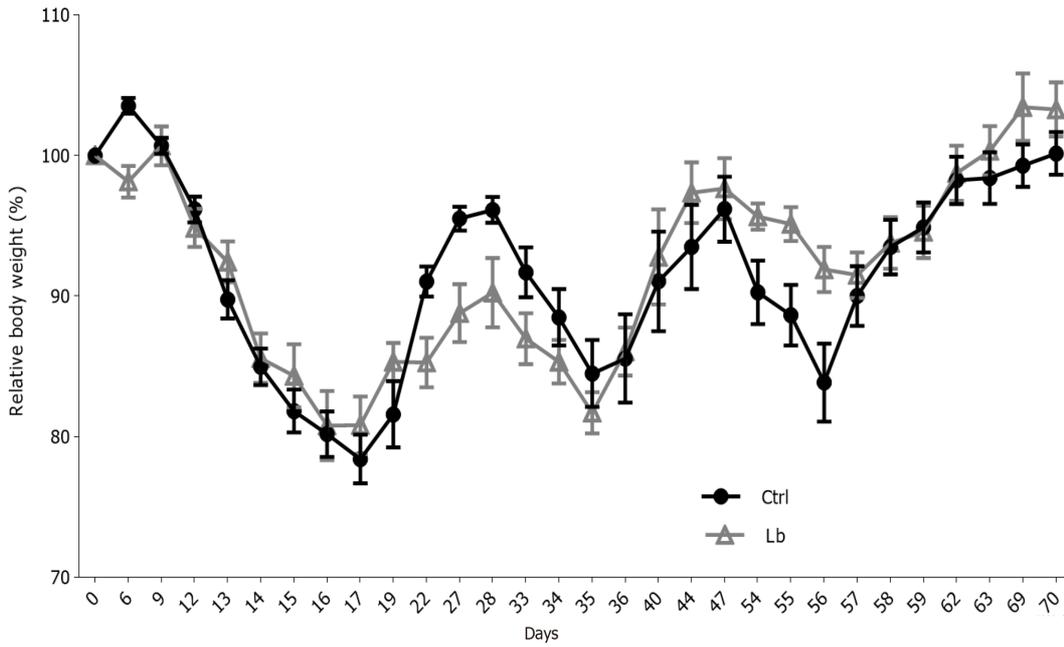
Regarding the cytokines measured in tumor tissues, we observed a pattern similar to that found in the inflamed colon (Figures 3 and 4). We observed a negative regulation of all analyzed cytokines, including IL-6, in mice treated with the probiotic (Figure 4), and an increase in IFN- $\gamma$  levels in this group (Figure 4).

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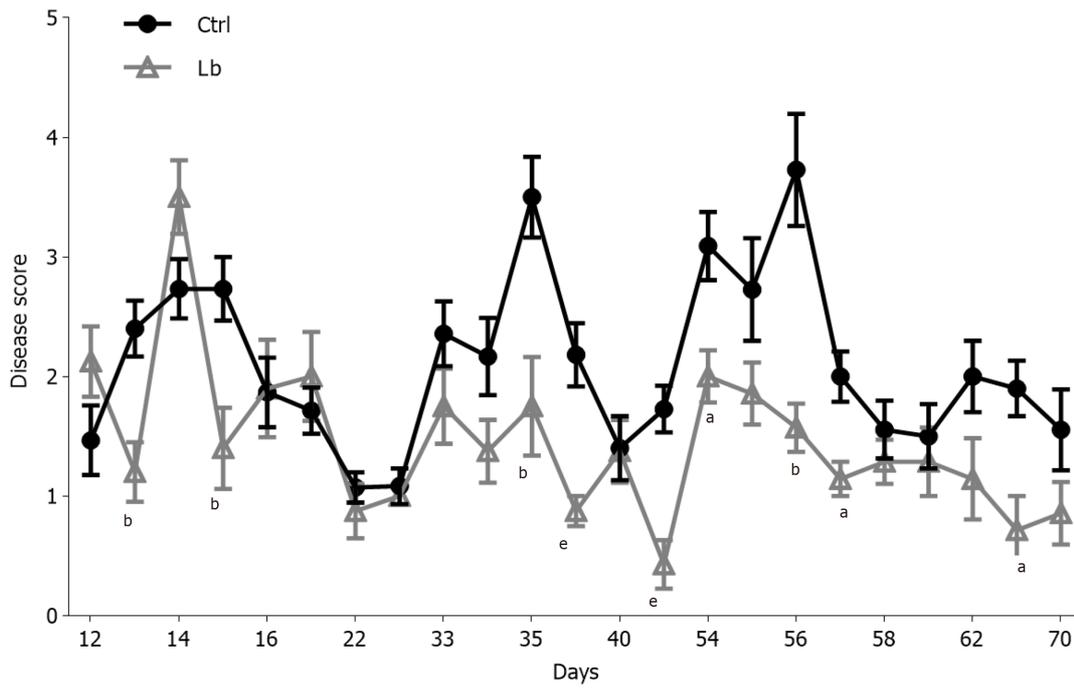
## DISCUSSION

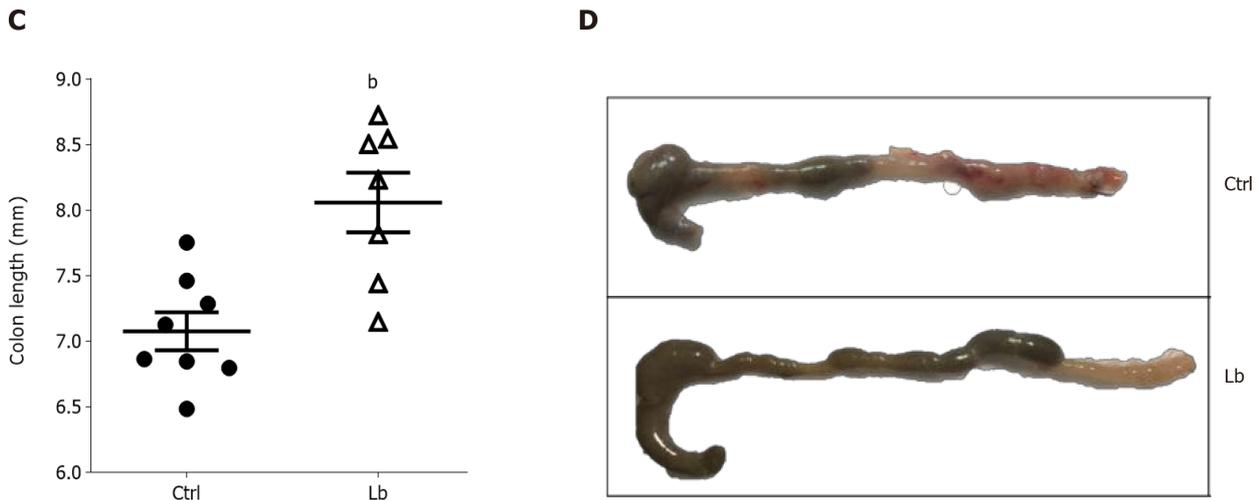
Recently, prebiotics and probiotics are being investigated as antitumor agents due to their capacity to modulate inflammatory responses. Studies have shown that

**A**

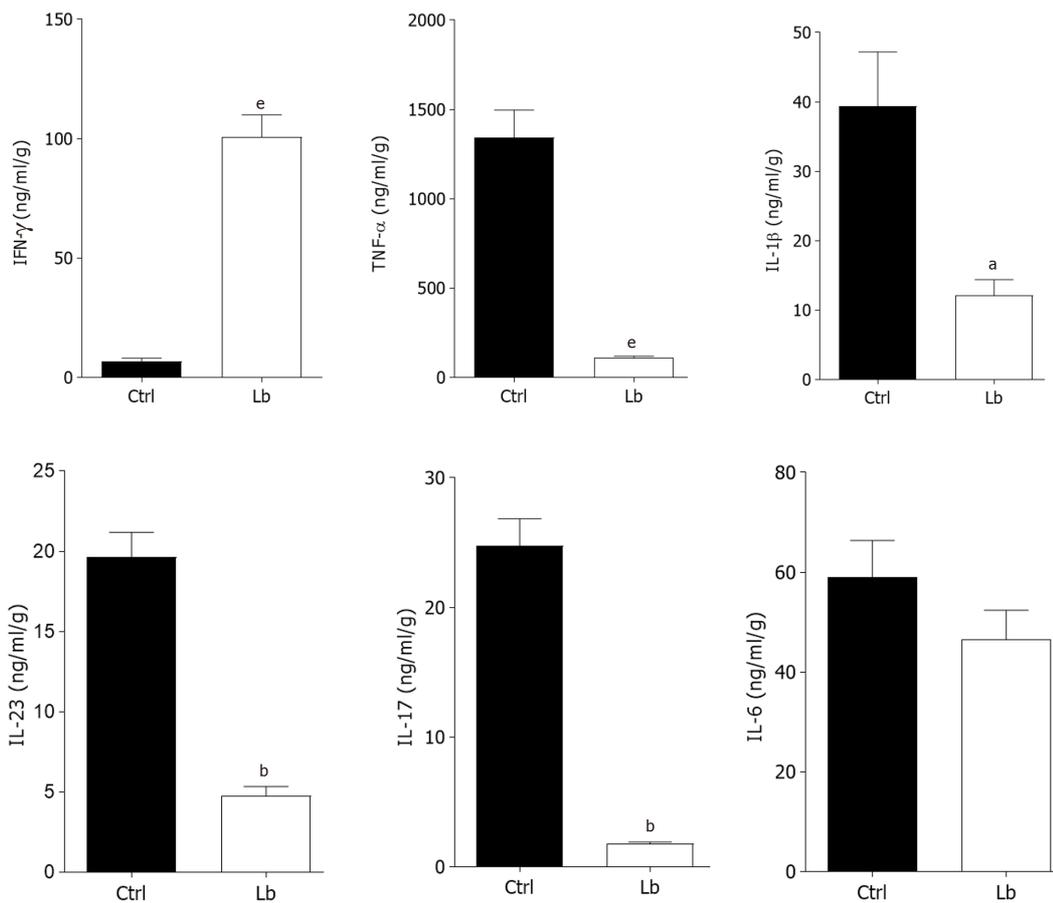


**B**



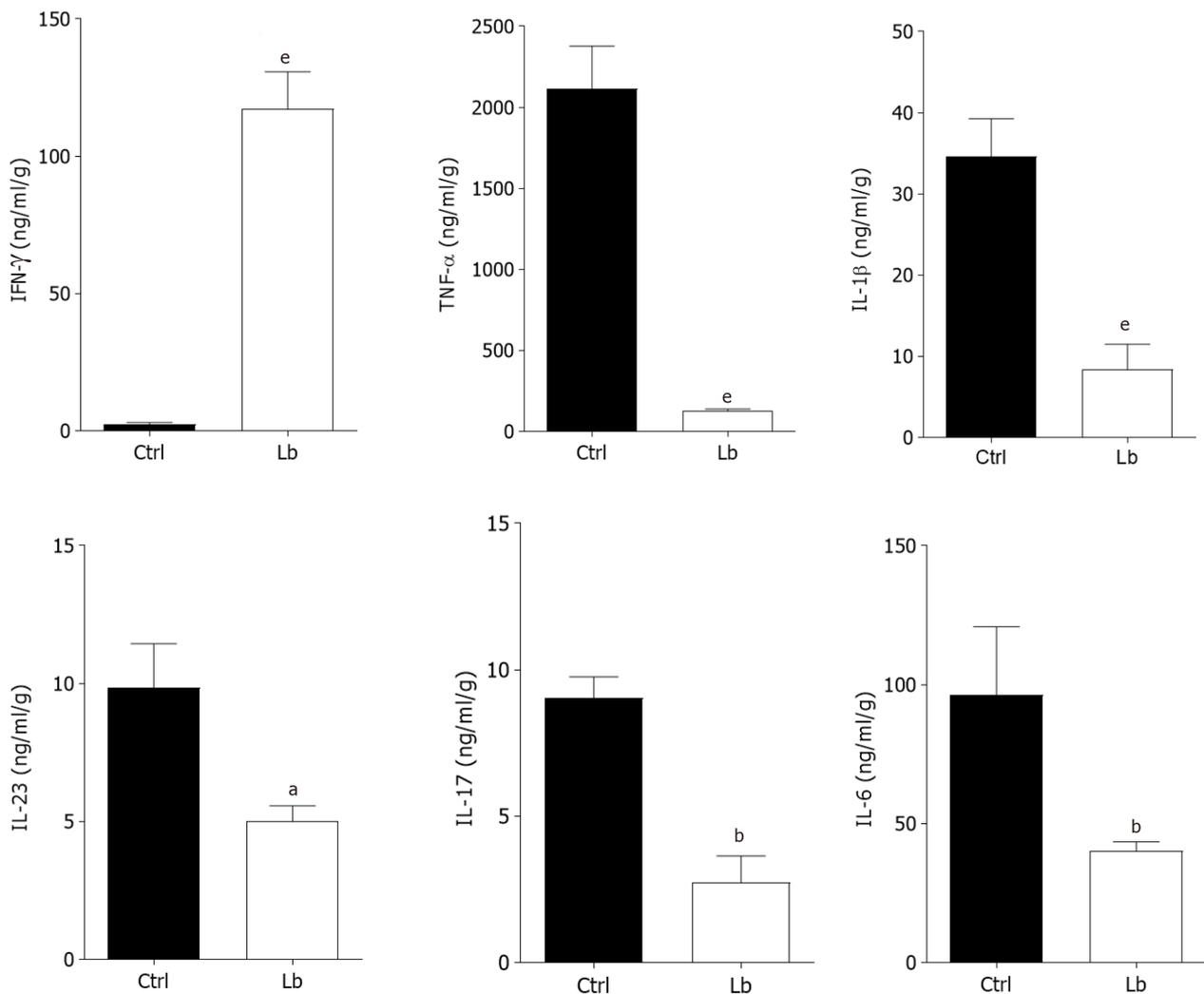


**Figure 2** *Lactobacillus bulgaricus* attenuates intestinal inflammation in azoxymethene/dextran sulfate sodium-exposed mice. A: Relative body weight of azoxymethene/dextran sulfate sodium-induced mice treated or not with probiotic; B: Disease score determined by a scoring system based on clinical signs, such as weight loss, humid perianal region, presence of diarrhea, blood in the stool or perianal region, hyporeactivity and piloerection; C: Colon length was determined using graduated images processed in ImageJ; D: Illustrative images of colon extension. Results are expressed as mean  $\pm$  EPM. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>e</sup> $P < 0.001$  vs controls. Ctrl: Controls; Lb: *Lactobacillus bulgaricus*.



**Figure 3** *Lactobacillus bulgaricus* regulates the production of intestinal proinflammatory cytokines in azoxymethene/dextran sulfate sodium-exposed mice. Segments of the colon which did not present tumors (inflamed colon) were homogenized and the levels of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-17, IFN- $\gamma$  e IL-23 per gram of tissue were determined by ELISA ( $n = 10$  mice per group). Results expressed as mean  $\pm$  SEM. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>e</sup> $P < 0.001$  vs controls. Ctrl: Controls; Lb: *Lactobacillus bulgaricus*.

probiotics may exert positive effects at different stages of colorectal carcinogenesis: Antimutagenic activity; inactivation of mutagens or carcinogens; reduction of



**Figure 4** *Lactobacillus bulgaricus* regulates the production of tumors proinflammatory cytokines in azoxymethene/dextran sulfate sodium-exposed mice. Tumor tissues were homogenized and the concentration of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-17, IFN- $\gamma$  e IL-23 per gram of tissue (colon) were determined by ELISA ( $n = 10$  mice per group). Results expressed as mean  $\pm$  SEM. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>e</sup> $P < 0.001$  vs controls. Ctrl: Controls; Lb: *Lactobacillus bulgaricus*.

intestinal pH; immunomodulatory effects; intestinal microbiota modulation; regulation of apoptosis and cell differentiation; and tyrosine kinase signaling pathway inhibition<sup>[21]</sup>. In addition, among probiotics, the genera *Lactobacillus* has been reported to exert immuno-regulatory effects, including modulation of innate immune responses and promotion of humoral and cellular immunity<sup>[22]</sup>, suppression of pathogens and restoration of gut microbiota homeostasis<sup>[23]</sup> and improvement of IBD<sup>[24]</sup>. In the present study, we used an experimental model of CAC to investigate the effects of the probiotic *L. bulgaricus* on colon carcinogenesis. We showed that *L. bulgaricus* negatively regulated tumor progression, resulting in an expressive reduction of total tumor volume and mean size of tumors. Furthermore, the probiotic also attenuated the clinical signs of intestinal inflammation inducing a decrease in intestinal and tumor levels of IL-6, TNF- $\alpha$ , IL-17, IL-23 and IL-1 $\beta$ .

Similarly, it has been recently observed that *L. salivary* and *L. fermentum* reduced the proliferation of colon cells in sporadic CRC<sup>[25,26]</sup>. Given that cell proliferation defines the speed of cancer development<sup>[27]</sup>, probiotics capable of modulating cell proliferation are of great interest to prevent tumor growth and/or metastasis.

Our results also demonstrated that the probiotic *L. bulgaricus* attenuated intestinal inflammation by decreasing intestinal and tumor levels of IL-6, TNF- $\alpha$ , IL-17, IL-23 and IL-1 $\beta$ . Finally, we also demonstrated an increase in IFN- $\gamma$  levels in animals treated with *L. bulgaricus*. Due to their involvement in the pathogenesis of IBDs and CAC, the development of strategies that target the inflammatory cytokines IL-6, TNF- $\alpha$ , IL-17, IL-23 and IL-1 $\beta$  is of potential interest in the therapeutic field. In a clinical trial with CRC patients a significant reduction in the blood levels of the proinflammatory

cytokines TNF- $\alpha$ , IL-12, IL-1 $\beta$ , IL-6, IL-17 and IL-22, was observed after six months of a mix of probiotics consumption<sup>[28]</sup>.

The cytokine IL-1 $\beta$  is found at high levels in several types of cancers and in CRC its expression is increased throughout the tumor progression<sup>[29]</sup>. IL-1 $\beta$  activates Wnt pathway in colon cancer cells promoting their growth and invasion<sup>[30]</sup>. TNF- $\alpha$  is an important inflammatory mediator whose effects have been implicated in several cellular events, such as cell proliferation, differentiation and cell death<sup>[31]</sup>. Anti-TNF therapies have been successfully used in IBD patients which confirms the crucial role of this cytokine in IBD and CAC development<sup>[32]</sup>. Increased expression of TNF- $\alpha$  promotes cancer development through both leukocytic and nonhematopoietic cell TNFR1 expression in colonic tissue has been reported in studies using CAC model induced by AOM and DSS<sup>[33,34]</sup>.

Up-regulation of IL-17 has also been reported in colitis and colorectal tumors<sup>[35]</sup>. The differentiation of Th17 cells may occur in the presence of different combinations of the cytokines TGF- $\beta$ , IL-6, IL-1 $\beta$  and/or IL-23, while its maintenance requires only IL-23 and/or IL-1 $\beta$ <sup>[36]</sup>. Although we have not attempted to elucidate the molecular mechanisms that mediate the inhibitory role of *L. bulgaricus* in the regulation of IL-17 and IL-23 in our study, we hypothesize that the reduced expression of TNF- $\alpha$  is involved. In fact, previous studies have clearly shown that NF- $\kappa$ B, a critical mediator of TNF- $\alpha$  signaling, regulates the transcription of the IL-23p19 gene<sup>[37]</sup>. A recent finding showed that the probiotics *Bifidobacterium breve* and *Lactobacillus rhamnosus* GG inhibit LPS-induced expression of IL-23 in intestinal cells cultured in a condition of histone acetylation inhibition and increased DNA methylation<sup>[38]</sup>. This finding might provide another potential mechanism for the *L. bulgaricus*-mediated negative regulation of IL-23.

Only a few trials in IBD patients have examined the composition of intestinal microbiota before and after supplementation therapy, so the effect (if any) of administering probiotics to the resident microbiota is not fully understood. However, it has been suggested that probiotics can change the intestinal ecosystem by generating an ecological environment that is unfavorable to the growth of noxious species, increasing the number of *Lactobacillus* and *Bifidobacteria* and stabilizing the intestinal microbiota<sup>[39,40]</sup>.

Dysregulation of gut microbiota has been associated with increased inflammation and the administration of probiotics have been reported to prevent chronic inflammatory diseases<sup>[41]</sup>. In recent years there has been growing interest in the possible application of probiotics as a part of combination therapy with conventional treatment of cancer<sup>[41-43]</sup>. However, studies investigating probiotics effects in patients with CRC are still very limited. For clinical application in humans many other studies, mainly randomized controlled trials would be needed to better evaluate the dosage, duration of the intervention and host physiology for confirm these findings<sup>[41]</sup>.

Several researches have indicated that the use of probiotics might improve beneficial microbiota, induce the release of antimicrobials and anticarcinogenic agents that help to remove carcinogens, and modulate immune responses that decrease intestinal inflammation in CRC patients<sup>[41,44,45]</sup>. Here, we have shown that *L. bulgaricus* inhibited CAC *via* a negative regulation of intestinal inflammation. Although a deeper characterization of the molecular mechanisms underlying *L. bulgaricus* anti-inflammatory activity, the strength of our findings indicates a relevant and evidenced phenotypic pattern, which may be important in IBD field to prevent inflammation-associated tumorigenesis. To the best of our knowledge this is the first study to investigate and provide promising evidences of a preventive effect of the probiotic *L. bulgaricus* in cancer development in an experimental model of CAC.

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## CONCLUSION

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In conclusion, our results show an anti-inflammatory and antitumor role of *L. bulgaricus* in colitis-associated carcinogenesis which may play an important role in prevention and treatment of CAC in the future. However, although the antitumor effects of this probiotic are promising, the mechanisms by which they occur still need to be better elucidated. Nevertheless, our study is extremely important as regards to the potential use of *L. bulgaricus* as a new therapeutic agent that can mediate and/or regulate the progression of CAC.

## ARTICLE HIGHLIGHTS

**Research background**

Intestinal inflammatory disorders are associated with the infiltration of immune cells and the proinflammatory release of cytokines that play a critical role in the onset and progression of colitis-associated cancer (CAC). Recent studies suggested that the intestinal microbiota has an essential role in carcinogenesis. Probiotic supplementation is an alternative means of favorably modulating the intestinal microbiota. Currently, it has become increasingly evident that intestinal microbiota plays a crucial role in the pathogenesis of inflammatory bowel diseases (IBD) and colorectal cancer. Moreover, increasing evidence suggests that probiotics prevent inflammation and carcinogenesis and several bacteria strains have been used for the prevention and treatment of the infectious colitis, IBD. Thus, probiotic modulation of intestinal microbiota has emerged as a potential chemo-preventive agent.

**Research motivation**

Although supplementation with probiotics have been reported to prevent CAC, little is known about the administration of strains of *Lactobacillus bulgaricus* (*L. bulgaricus*), as well as their impact on neoplastic changes in the intestinal mucosa. Our study may contribute to address the gaps in the literature of how this probiotic, dose and supplementation time used for this experimental model impact on colitis, serum cytokines and neoplastic development.

**Research objectives**

The purpose of this study is to investigate the effect of the probiotic *L. bulgaricus* during the development of an experimental model of CAC. Overall, this study intends to strengthen data from preclinical studies, encouraging clinical trials to investigate their role in preventing colitis and CAC in humans.

**Research methods**

We used an experimental model of CAC. For mice treatment,  $1 \times 10^9$  CFU were diluted in 200  $\mu$ L of PBS and orally given to each mouse, 3 times a week during all experimental period. Prior to tumor induction, C57BL/6 mice were randomly distributed in 2 groups ( $n = 10$ ) and treated with PBS (control group) or *L. bulgaricus* (Lb group) by gavage (0.2 mL/mouse) for one week. For CAC induction, mice were intraperitoneally (i.p.) injected with a single dose (10 mg/kg in 300  $\mu$ L solution) of azoxymethene (Sigma-Aldrich), followed by 3 cycles of one week of 2.5% dextran sulfate sodium (DSS) diluted in drinking water intercalated for 2 wk of normal water. Mice were euthanized 12th week after CAC induction. Intestinal inflammation *in vivo*, or disease score, was determined by scoring clinical signs. The severity of intestinal inflammation was assessed by measuring the length of the entire large intestine. Also, the dimensions of the colorectal tumors were measured with pachymeter and the volumes were calculated by the formula:  $(\text{width})^2 \times \text{length}/2$ . For histological analysis, distal colon parts were fixed in 4% p-formaldehyde in phosphate-buffered formalin and unblocked in paraffin. Tissue sections (4.0  $\mu$ m) were prepared from the paraffin-embedded tissue blocks, stained with hematoxylin and eosin and evaluated in a blinded fashion by an experienced pathologist. Cytokines levels were determined from colon and/or tumor samples by ELISA. Statistical analyses were performed using GraphPad Prism version 6.0. A 2-tailed *P* value < 0.05 was considered to be statistically significant.

**Research results**

We have shown that *L. bulgaricus* treatment inhibited the total tumor volume and mean size of tumors. Although we did not observe differences in body weight loss between control and *L. bulgaricus*-treated, we found differences in clinical signals in *L. bulgaricus*-treated mice, which showed a lower clinical score on the 13th and 15th days after tumor initiation. In addition to the attenuation of intestinal inflammation score, we observed that the treatment with *L. bulgaricus* reduced the DSS-induced shortening of the colon. In segments of the large intestine that did not present tumors (inflamed colon) we also observed a reduction of at least 2-fold in the levels of the cytokines TNF- $\alpha$ , IL-1 $\beta$ , IL-23 and IL-17 in *L. bulgaricus*-treated mice in comparison to controls. In contrast, increased concentrations of IFN- $\gamma$  were also observed in Lb group. Regarding the cytokines measured in tumor tissues, we observed a pattern similar to that found in the inflamed colon with a negative regulation of proinflammatory cytokines in mice

treated with the probiotic and an increase in IFN- $\gamma$  levels in this group. Overall, these findings highlight the protective effect of *L. bulgaricus* in the regulation of gut inflammation and preventing CAC development. Thus, further clinical trials are needed to confirm these preclinical insights.

### Research conclusions

We found an anti-inflammatory role and consequent antitumor effect of *L. bulgaricus* on CAC that may be used as a promising tool for the prevention and treatment of CAC. In summary, *L. bulgaricus* treatment during colitis-associated colorectal carcinogenesis model may be responsible for anti-inflammatory and antitumor role by lowering proinflammatory cytokine expression.

### Research perspectives

The present study has shown that *L. bulgaricus* inhibited CAC via a negative regulation of intestinal inflammation. Hence, has demonstrates promising evidence on *L. bulgaricus* probiotic has a preventive potential in CAC development. Therefore, clinical trials are needed to confirm this hypothesis and increase the therapeutic arsenal against CAC.

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