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ORIGINAL ARTICLE

Structural Change in Microbiota by a Probiotic Cocktail Enhances the Gut Barrier and Reduces Cancer via TLR2 Signaling in a Rat Model of Colon Cancer

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Abstract

Background Structural change in the gut microbiota is implicated in cancer. The beneficial modulation of the microbiota composition with probiotics and prebiotics prevents diseases.

Aim We investigated the effect of oligofructose–maltodextrin-enriched *Lactobacillus acidophilus*, *Bifidobacteria bifidum*, and *Bifidobacteria infantum* (LBB), on the gut microbiota composition and progression of colorectal cancer.

Methods Sprague Dawley rats were acclimatized, given ampicillin (75 mg/kg), and treated as follows; GCO: normal control; GPR: LBB only; GPC: LBB+ 1,2-dimethylhydrazine dihydrochloride (DMH); and GCA: DMH only (cancer control). 16S V4 Pyrosequencing for gut microbiota analysis, tumor studies, and the expression of MUC2, ZO-1, occludin, TLR2, TLR4, caspase 3, COX-2, and β-catenin were conducted at the end of experiment.

Results Probiotic LBB treatment altered the gut microbiota. The relative abundance of genera *Pseudomonas*, *Congregibacter*, *Clostridium*, *Candidactus* spp., *Phaeobacter*, *Escherichia*, *Helicobacter*, and *HTCC* was decreased ($P < 0.05$), but the genus *Lactobacillus* increased ($P < 0.05$), in LBB treatment than in cancer control. The altered gut microbiota was associated with decreased tumor incidence (80 % in GPC vs. 100 % in GCA, $P = 0.0001$), tumor volume (GPC 84.23 (42.75–188.4) mm^3 vs. GCA 243 (175.5–344.5) mm^3, $P < 0.0001$) and tumor multiplicity/count (GPC 2.92 ± 0.26 vs. GCA 6.27 ± 0.41; $P < 0.0001$). The expression of MUC2, ZO-1, occludin, and TLR2 was increased, but expression of TLR4, caspase 3, Cox-2, and β-catenin was decreased by LBB treatment than in cancer control GCA ($P < 0.05$).

Conclusion Administration of LBB modulates the gut microbiota and reduces colon cancer development by decreasing tumor incidence, multiplicity/count, and volume via enhanced TLR2-improved gut mucosa epithelial barrier integrity and suppression of apoptosis and inflammation.

Keywords Colorectal cancer · *Lactobacillus* · *Bifidobacteria* · Probiotics · Microbiota · Toll-like receptors (TLRs)

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Introduction

The gut microbiota plays an important role in human diseases. It is estimated that the human microbiota composition is about tenfold the number of human cells [1]. The gut

contains the highest concentration of microbiota, with close to 10^{14} bacteria and about 500 different species [2]. These bacteria are in a constant homeostasis involved in various functions including metabolic, synthetic, intestinal barrier, and immune homeostasis that maintains a healthy gut [1, 3–5]. The alteration of this complex homeostasis, termed dysbiosis, by genetic and environmental factors promotes various physiological functions like proliferation, angiogenesis, and apoptosis associated with human diseases [6]. However, gut bacterial dysbiosis has been linked with colorectal cancer (CRC) development [2].

Diet is an integral part of life, and dietary interventions are extensively studied in the prevention of CRC by mechanisms including reduction in activity of cancer-causing pathogenic bacteria, and anti-mutagenic and anti-carcinogenic properties [7]. Diet can influence the microbiota and its impact on human diseases. The dietary modulation of the microbiota via the use of prebiotics and probiotics in food products maintains a healthy gut and prevents diseases [8].

In a review by Compare and Nardone, the probiotics VSL#3, *Lactobacillus acidophilus*, *Bifidobacteria longum*, and *Lactobacillus gasseri*, ameliorated colonic carcinogenesis, inhibited preneoplastic lesions, and reduced tumor load and size [2]. The prebiotics inulin and fructo-oligosaccharide have been demonstrated to reduce the severity of dimethyl hydrazine-induced colon cancer in rats [2]. Consumption of inulin with *Bifidobacteria longum* and *Bifidobacteria lactis* with resistant starch was also able to decrease chemical-induced CRC and increase apoptotic response, respectively [2]. These findings suggest the influence of probiotics and prebiotics as anti-cancer therapeutic agents worth investigating to augment human life.

Cancer, however, is the second cause of mortality in most parts of the world. CRC ranks third and second cause of cancers in men and women, respectively [9]. Close to 80 % of CRC cases are sporadic [2] and preventable, accounting for an estimated 1.2 million annual cases worldwide [10]. Despite the availability of therapeutic treatments, patients with CRC are afflicted with other diseases at prognosis [11]. Chemotherapy, radiotherapy, and surgery are adjuvant treatments of CRC but vary in rates of local recurrence and survival [7]. Additionally, adverse side effects including immunosuppression, hair loss, diarrhea, fatigue, nausea, vomiting, and increased risk of infections and bleeding [7, 12] further complicate treatments of CRC. This calls for urgent search for alternative preventive and chemotherapeutic agents for the management of CRC.

Diet is a major important contributory risk factor for CRC, implying that CRC is potentially reducible via modification of risk factors for CRC [13]. However,

epidemiological evidence has linked diet to CRC via intestinal microbiota dysbiosis [14] and the composition, structure, and function of the gut microbiota [6]. Abundance of the bacteria *Bacteroides fragilis*, *Clostridium butyricum*, *Escherichia coli*, *Streptococcus bovis* and *Enterococcus* in the gut is related to pathogenic mechanisms such as DNA damage, reactive oxygen species production, increased cell proliferation, and activation of signaling pathways linked to CRC [2].

Therefore, beneficial modulation of the gut microbiota composition and metabolic activities through diet is a potential chemoprevention approach [8] that requires further studies. This further prompts more research into the use of probiotics and prebiotics as modulators of the gut microbiota to reduce the risk of CRC development. However, although probiotics and prebiotics have shown success in attenuating CRC and its concomitant effects [2], the benefits of interventional treatments are unknown.

The probiotic *Lactobacilus acidophilus*, *Bifidobacteria bifidum*, and *Bifidobacteria infantum* supplemented with oligofructose and maltodextrin (LBB) are commercially available probiotic. The effect of LBB on the gut microbiota and CRC, to the best of our knowledge, has not been investigated. Herein, we report the modulation of the gut microbiota by LBB using Illumina Miseq 2500 pyrosequencing and its subsequent prevention of colorectal cancer in a 1,2-dimethylhydrazine dihydrochloride (DMH)-induced colorectal cancer animal model via inhibition of Cox-2 expression and β-catenin.

Materials and Methods

Probiotic Preparation

LBB was obtained from Biostime Inc (Guangzhou, China). Hundred grams of the formula contains the following:

I.   *Lactobacilus acidophilus*: 6.4×10^{11} cfu
II. *Bifidobacteria spp* (*B. bifidum* and *B. infantum*): 1.9×10^{10} cfu and
III. Fructo-oligosaccharide and maltodextrin as supplement.

The dosage of the probiotic formula was calculated according to Meeh-Rubner conversion formula between human and rat [15] as follows:

d_{rat} (mg/kg) $= d_{human}$(mg/kg) $\times (k_{rat}/k_{human})$:
  where $k =$ conversion factor, $K_{rat} = 1$; $K_{human} = 0.16$.

The probiotic formula was administered orally at 0.9 g/kg body weight daily after conversion.

Colon Cancer Induction

Colorectal cancer was induced using the carcinogen 1,2-dimethylhydrazine dihydrochloride (DMH) as previously described with slight modification [16]. Briefly, DMH was freshly prepared before use (40 mg/kg body weight) in 1 mM EDTA–saline as vehicle solution. DMH was injected subcutaneously (SC) at the dorsal back weekly for 10 consecutive weeks after the pH of the solution was adjusted to between 6.35 and 7.0 with 1 nM NaOH.

Animal Experimental Procedure

This study was carried out under the approval of the Ethical and Research Committee of Dalian Medical University, China. Three-week-old forty (40) male SD rats were housed in plastic cages in four groups of ten with unlimited access to animal food and water under controlled conditions of humidity (44 ± 5 %), temperature (25 ± 2 °C), and 12-h:12-h light/dark cycles. After 1 week of acclimatization, animals were treated with ampicillin (75 mg/kg body weight) for 5 days and then treated for 23 weeks as follows: GCO (normal control, $N = 10$): basic animal chow and water daily; GPR (LBB only, $N = 10$): basic animal chow and water + LBB daily; GPC (LBB and DMH): basic animal chow and water daily + LBB daily + (DHM weekly for 10 consecutive weeks); and GCA (cancer control (DMH only)): basic animal chow and water + (DMH weekly for 10 consecutive weeks) (Fig. 1a).

The animals were euthanized by diethyl ether inhalation at the end of the 25th week of the experiment. Colons were surgically removed aseptically and opened longitudinally. Stool samples were removed and stored at −80 °C until DNA extraction for gut microbiota analysis. Tumors were counted, and measurements taken with calipers. The incidence, tumor multiplicity, and tumor volume were calculated. The incidence was defined as the proportion of rats with developed tumors. Tumor multiplicity was defined as total number of tumors divided by number of tumor bearing rats. Tumor volume was calculated as: Tumor volume = $A \times B^2 \times 0.5$, where A is the long diameter and B is the perpendicular short diameter [17]. The colon was then rinsed in cold PBS. Colon biopsies and tumors were excised, fixed in neutral buffered formalin, and processed by automatic tissue processor, and 5-μm-thick section cut for immunohistochemistry and H&E staining.

Immunohistochemistry

The colon tissue sections were deparaffinized, hydrated, and subjected to antigen retrieval in 10 mM citrate buffer (pH 6.0) at 100 °C for 20 min. The blocking of endogenous peroxidase activity and antigen detection with biotinylated secondary antibody and DAB were done using Splink detection kit (ZSGB-BIO, China) according to manufacturer's protocol. The incubation with Cox-2 antibody (1:100 dilutions) was done at 4 °C overnight. Four sections per group were stained. Cox-2 was expressed as mean intensity of Cox-2 staining, calculated as ratio of mean area of Cox-2 staining and mean integrated optical density using the Image Pro Plus 6 software.

DNA Extraction

Stool DNA was extracted from samples using Qiagen stool DNA extraction Kit (QIAGEN, China) according to manufacturer's instructions. Total genomic DNA was extracted using CTAB/SDS method. DNA quality was monitored on 1 % agarose gels and concentration adjusted to 1 ng/μl using sterile water.

Amplicon Generation

Bacteria 16S rRNA of the V4 distinct region was amplified from stool DNA extracted as template with the primers 515F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′. PCR were carried out in a 30-μl volume reaction, using 15 μl Phusion High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, and 10 ng DNA template. The PCR cycling conditions were: initial denaturation at 98 °C for 1 min, 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, elongation at 72 °C for 30 s, and finally 72 °C for 5 min.

PCR Product Quantification, Qualification, Mixing, and Purification

The PCR product obtained was run on 2 % agarose gels for detection. Samples with bright main strip between 400 and 450 bp were chosen, mixed in equidensity ratios, and purified with GenJET gel extraction kit (Thermo Scientific, Germany).

Library Preparation and Sequencing

Sequencing libraries were generated using NED Next Ultra DNA Library Prep Kit for Illumina (NED, USA) following manufacturer's recommendations, and index codes added. Sequence library quality was assessed on the Qubit@2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on Illumina MiSeq 2500 platform, and 250-bp paired-end reads generated.

Fig. 1 Animal model and tumor studies **a** diagrammatic representation of animal model, **b** topographical view of representative opened colon for tumor studies; GCO and GPR shows no tumors, and GPC and GCA revealed visible tumors (*white arrowed*). #GCO, normal control; GPR, probiotic LBB only; GPC, probiotic LBB and cancer; and GCA, cancer control
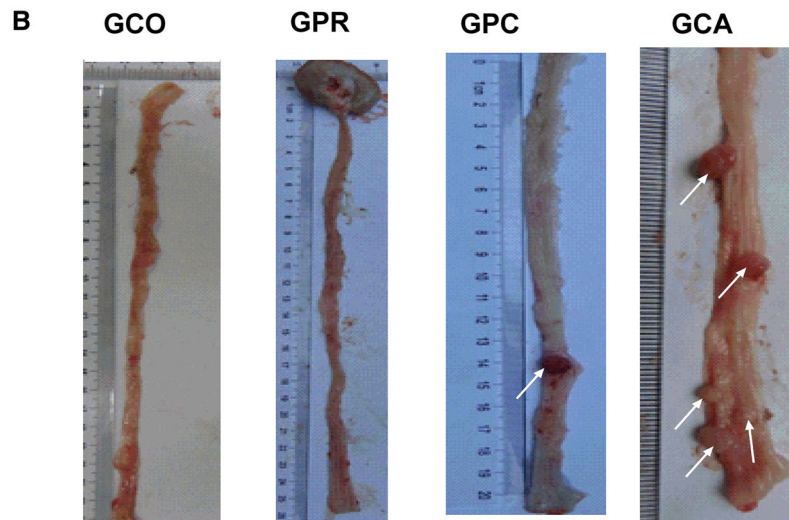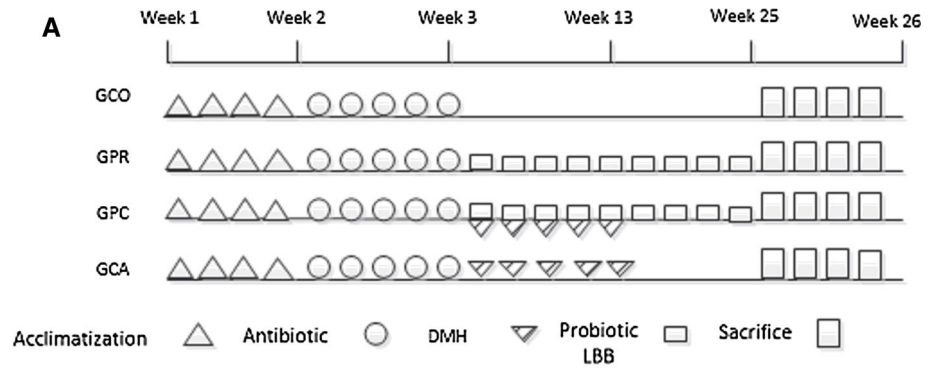
Data Analysis

Paired-end reads were assigned, truncated, and merged using FLASH [18] to generate raw tags. High-quality clean tags from the raw tags were obtained [19] and compared with Gold reference database to detect and remove chimeric sequences [20] using QIIME [21] and UCHIME [22] to obtain effective tags. UPARSE software [23] was used to analyze the effective tags, and tags with ≥97 % similarity were assigned to the same operational taxonomic unit (OTU). The Green Gene database [24] was used based on RDP classifier [25] algorithm to annotate taxonomic information for each OTU sequence.

Phylogenetic relationship analysis of OTUs was conducted using MUSCLE software [26]. The in-house Perl scripts of QIIME were used to analyze alpha-(within samples), beta-(among samples) diversity and Goods coverage (sequencing depth). The metrics, Chao 1, observed species, and Shannon index, were employed to compute alpha diversity from the OTUs. Rarefaction curves for sequencing depth were generated based on the above three metrics. The phylogenetic measures of beta diversity, the weighted and unweighted unifrac, were calculated with QIIME. The weighted and unweighted unifrac distance matrices were used for principal coordinate analysis (PCoA) to visualize the transformed matrix from a complex multidimensional data and unweighted pair group method with arithmetic means (UPGMA) hierarchical clustering to interpret the distance matrix.

Real-Time Comparative Quantitative PCR (qRT-PCR)

Total RNA was isolated from tissues using TRIzol reagent (Takara Bio, Japan) according to the methods of Lee et al. [27] and reverse-transcribed into cDNA using transgene all-in-one first-strand cDNA synthesis superMix for qPCR following manufacturer's protocol. qRT-PCR for mucin 2 (MUC2), zonula occludens (ZO-1), occludin, Toll-like receptors (TLR2 and TLR4), caspase 3, cyclooxygenase-2 (COX-2), and β-catenin was performed with SYBR Premix Ex Tag II (Takara Bio, Japan) in a 20-µl reaction volume: 10 µl SYBR Premix, 0.8 µl each of forward and reverse primers (0.4 µM), 2 µl of cDNA (<100 ng), and 6.4 µl Rnase-free water. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 30 s; 45 cycles of

95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min; and an extra melt curve cycle of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. The primers used are given in supplementary Table 1. β-actin was used as endogenous gene control. The expression of MUC2, ZO-1, occludin, TLR2, TLR4, caspase 3, COX-2, and β-catenin was quantified relative to the expression of β-actin as endogenous control and GCO as calibrator using the comparative Ct method. Results are presented as relative quantity (dR).

Statistical Analysis

Normality of the data was checked using the D'Agostino–Pearson Omnibus normality test. The data were expressed as mean ± SEM (symmetric) or as median (interquartile range) for asymmetric data. To estimate the differences in tumor volume, the Mann–Whitney test was used and unpaired t test for tumor multiplicity/count. Fisher's exact test was employed to analyze the difference in tumor incidence. ANOVA followed by Turkey's post hoc analysis was used to analyze differences in microbiota abundance at various levels. A P value of <0.05 was considered significant. The Mann–Whitney test, unpaired t test, Fisher's exact test, and ANOVA were performed using GraphPad Prism version 6.0 for windows, GraphPad software, La Jolla, California, USA, www.GraphPad.com.

Results

Probiotic LBB Reduces Tumor Incidence, Tumor Multiplicity/Count, and Tumor Volume

At the end of the 25th week of the experiment, animals were killed and tumors counted. No tumors were observed in GCO and GPR (Fig. 1a). All animals (100 %) in GCA developed tumors (Fig. 1b). In GPC, 80 % (12/15) developed tumors ($P < 0.0001$). Tumor multiplicity/count (GPC 2.92 ± 0.26 vs. GCA 6.27 ± 0.41; $P < 0.0001$) and tumor volume (GPC 84.25 (42.75–188.4) mm^3 vs. GCA 243 (175.5–344.5) mm^3; $P = 0.0073$) were significantly reduced in GPC compared with GCA as shown in Table 1. Histological examination of a normal colon and tumor is shown in supplementary figure 1. Administration of probiotic LBB lessened the tumor incidence, tumor multiplicity/count, and tumor volume, as shown above, compared with the cancer control.

Probiotic LBB Decreased the Complexity of Microbial Species Diversity

Summary of the sequencing data is presented in Fig. 2a. The complexity of the species diversity among the groups was estimated using Shannon index (community diversity) and Chao 1 (community richness). Chao 1 index (Fig. 2b) was higher in GCA than in GPR ($P = 0.0410$). The Shannon index (Fig. 2c) was also statistically higher in GCA compared to GPR ($P = 0.0035$). The results indicate a very diverse and rich microbiota species in GCA but a decreased in GPR. On analysis of the observed species, estimate of the amount of unique OTUs within the groups and the unique species in GCA were higher compared to GCO ($P = 0.004$), GPR ($P = <0.0001$), and GPC (0.0401) (Fig. 2d), indicating a unique diverse microbiota in the GCA.

To determine the depth of capture of data in the study, the sequencing depth and OTUs recovery were analyzed using the rarefaction curve analysis. The rarefaction estimates were plateauing at the highest number of OTUs analyzed, indicating that the OTUs recovered are substantial and representative of the samples and groups studied (not shown).

Treatment with Probiotic LBB Altered the Gut Microbiota

To analyze the gut microbiota structure associated with the different groups, the weighted unifrac distance matrix was calculated based on OTUs of each group and interpreted using the UPGMA. Also, principal coordinates analysis (PCoA) based on the unweighted unifrac distance matrix was used. The results indicated difference in the gut microbiota as shown by principal coordinates analysis (Fig. 3a) and the UPGMA of the weighted unifrac distance of the relative abundance of gut microbiota at phylum level (Fig. 3b).

The results of PCoA revealed altered microbiota structure between GCA, GCO, and GPR. The microbiota of GPC, however, clustered both with GCA and with GPR and GCO as seen from PC1 and PC2 (46.62 and 8.89 % of explained variance, respectively) (Fig. 3a).

The UPGMA showed microbiota structure is similar between GCO, GPC, and GPR and clustered separately from GCA. The microbiota of GPR and GPC, though similar to GCO, clustered away from GCO, revealing differences in microbiota structure within this cluster (Fig. 3b).

Probiotic LBB Decreased Relatively the Abundance of Pathogenic Bacteria

To investigate for the abundance of various bacteria in GPR, GPC, GCA, and GCO, the relative abundance of the bacteria annotation based on their representative OTUs in the various groups at different levels was compared.

We observed significant differences in the composition of microbiota between the groups. The dominant

Table 1 Probiotic LBB reduces tumor incidence, multiplicity/count, and volume

|	GCO (n = 15)	GPR (n = 15)	GPC (n = 15)	GCA (n = 15)	P value
Tumor incidence (%)	0 (0)	0 (0)	12 (80)[a]	10 (100)[a]	<0.0001
Tumor multiplicity/count (mean ± SEM)	0	0	35/12 (2.92 ± 0.26)[b]	94/15 (6.27 ± 0.41)[b]	<0.0001
Tumor volume median (mm^3) (P25–75)	0	0	84.25[c] (42.75–188.4)	243[c] (175.5–344.5)	0.0073

GCO, normal control; GPR, probiotic LBB only; GPC, probiotic LBB and cancer; and GCA, cancer control

[a] Fisher's exact test (GCA vs. GPC)

[b] Unpaired *t* test (GCA vs. GPC)

[c] Mann–Whitney test (GCA vs. GPC)

Fig. 2 Probiotic LBB treatment alters community characteristics **a** graph of pyrosequencing characteristics, **b** Chao 1 index, measure of community richness, reveals rich bacteria population in GCA compared to GPR, **c** Shannon diversity index, estimate of community diversity, shows diverse bacteria in GCA compared to GPR, and **d** observed species, measure of the amount of unique species in each group, shows a unique bacteria structure in GCA compared with GPC, GPR, and GCO. #GCO, normal control; GPR, probiotic LBB only; GPC, probiotic LBB and cancer; and GCA, cancer control
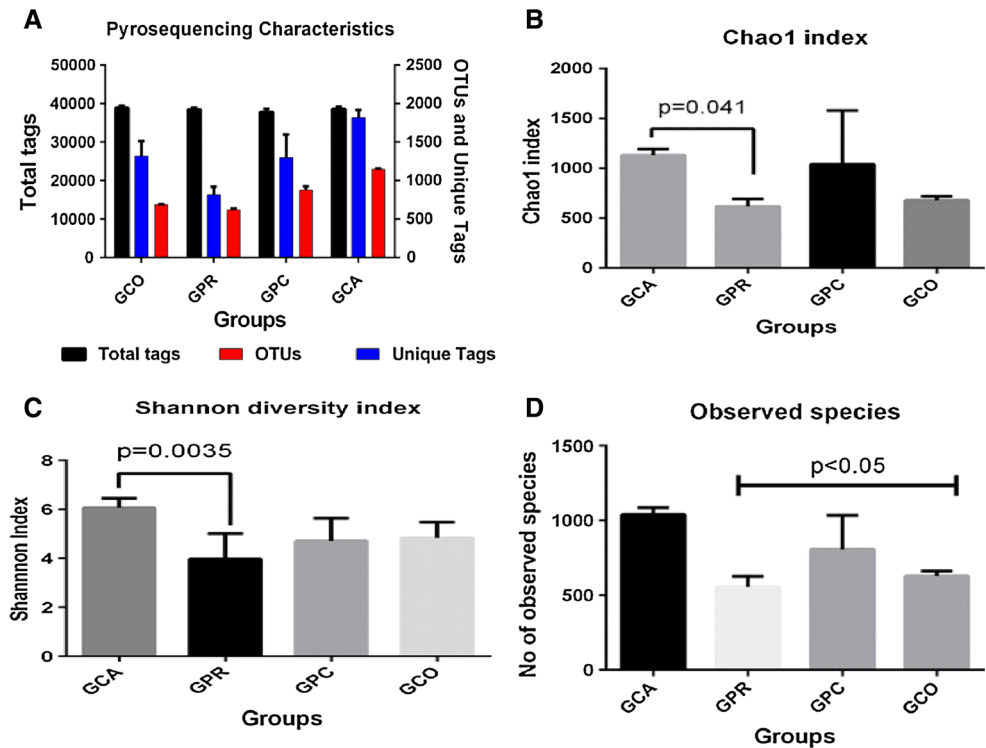
sequences, >1 % of total bacteria composition, obtained belong to the 4 phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Tenericutes* (Fig. 4a).

Firmicutes was significantly enriched in GCO compared to GCA. The phyla *Bacteroidetes*, *Proteobacteria*, *Cyanobacteria*, *Planctomycetes*, and *Chlamydia* were increased significantly in GCA compared with GCO, GPR, and GPC. *Proteobacteria* bloomed significantly in GPC than in GPR. The abundance of *Armatimonadetes* was significantly higher in GCA than in GPR and GCO.

The relative abundance of the pathogenic family *Enterobacteriaceae*, *Pseudomonadaceae*, and *Clostridiaceae* and other family groups including *Alteromonadaceae*, *Wb1-P06*, *OM60*, Echinacea, and *Saprospiraceae* was enriched in GCA compared to GCO, GPR, and GPC. Also
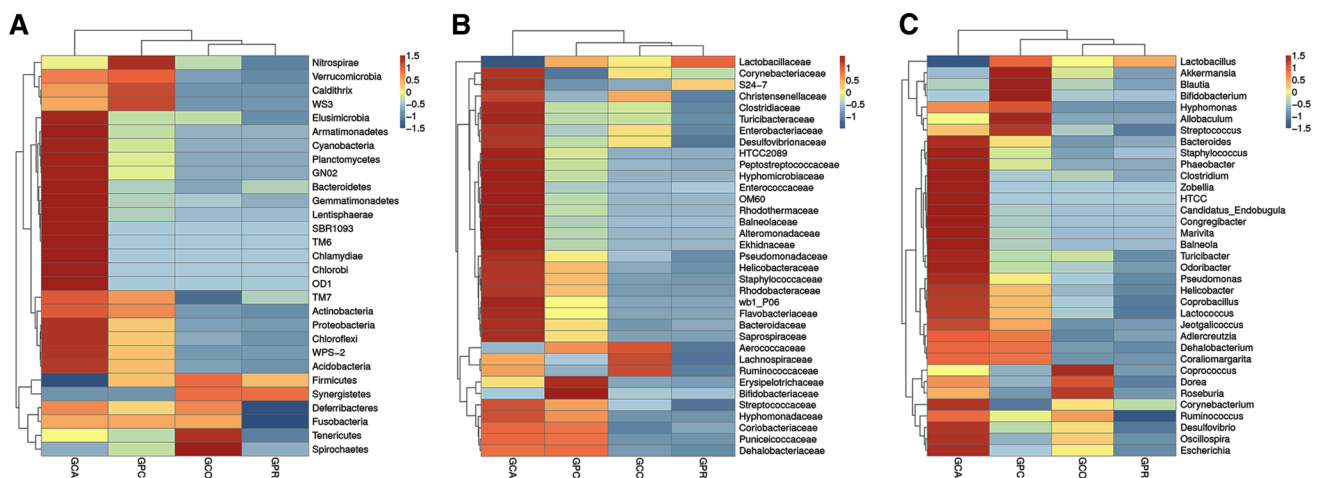
significantly increased families in GCA compared to GCO and GPR include *Helicobacteraceae*, *Hyphomonadaceae*, Coriobacteriaceae, Flavobacteriaceae, *Peptostreptococcaceae*, and *Rhodobacteraceae* (Fig. 4b).

The microbiotal composition was also different at genera level (Fig. 4c). About 141 genera were observed across all the groups. For genera that constitute more than 1 % of the total bacteria, *Lactobacillus*, a probiotic bacterium, was enriched in GPC compared to GCA and *Oscillospira* observed significantly increased in GCA compared to GPR. On the other hand, for genera with <1 % of the total bacterial composition, *Pseudomonas*, *Congregibacter*, *Candidactus Endobugula*, *Phaeobacter*, and *Clostridium* were enriched in GCA than in GCO, GPR, and GPC. Also, *Escherichia* was observed enriched in GCA compared to
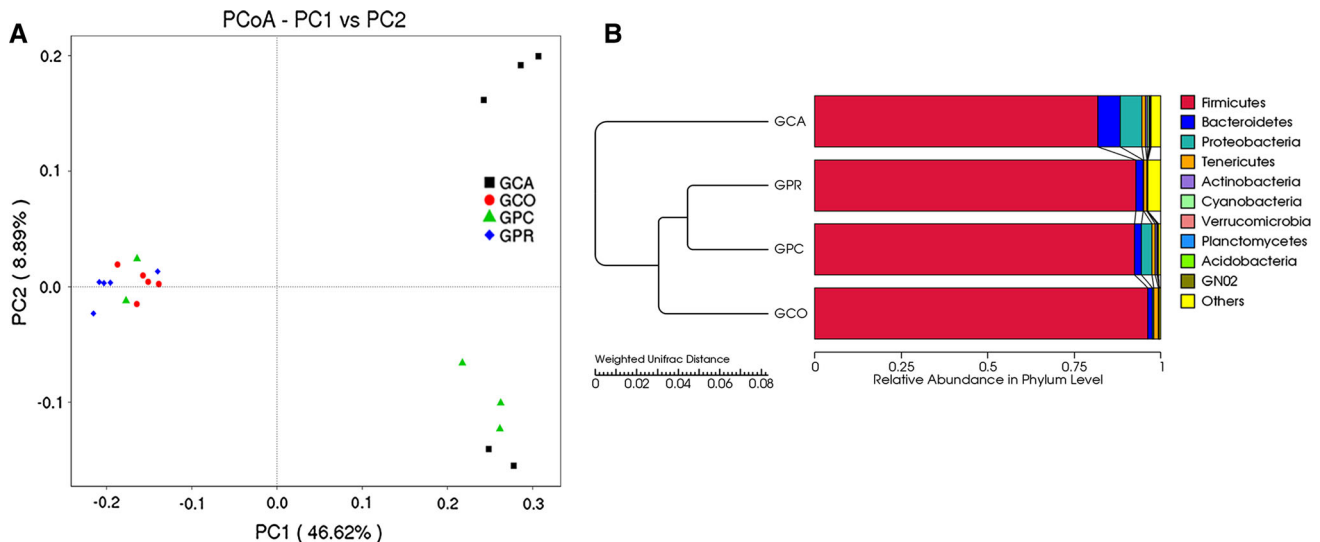
Fig. 3 Treatment with probiotic LBB alters the gut microbiota structure and composition **a** PCoA of unweighted unifrac distances, **b** UPGMA of the weighted unifrac distances of the relative abundance of bacteria at phylum level. #GCO, normal control; GPR, probiotic LBB only; GPC, probiotic LBB and cancer; and GCA, cancer control

Fig. 4 Bacteria composition differs from probiotic LBB treatment and cancer control. The *color scale* indicates an increase or decrease in bacteria composition at phylum, family, and genus level. **a** Heatmap of relative abundance at phylum level; Firmicutes, Bacteroidetes, Proteobacteria, Cyanobacteria, Planctomycetes, Chlamydiae, and Armatimonadetes showed significant difference. **b** Heatmap of relative abundance of bacteria at family level; Enterobacteriaceae, Pseudomonadaceae, Clostridiaceae, Alteromonadaceae, Wb1-P06, OM60, Echinacea, Saprospiraceae,

Helicobacteraceae, Hyphomonadaceae, Coriobacteriaceae, Flavobacteriaceae, Peptostreptococcaceae and Rhodobacteraceae were significant, and **c** heatmap of relative abundance at genus level; Lactobacillus, Oscillospira, Pseudomonas, Congregibacter, Candidactus Endobugula, Phaeobacter, Clostridium, Escherichia, Helicobacter and Adlercreutzia showed significance. #GCO, normal control; GPR, probiotic LBB only; GPC, probiotic LBB and cancer; and GCA, cancer control

GPC and GPR but not in GCO. *Helicobacter* and *Adlercreutzia* increased significantly in GCA compared with GCO and GPR.

The genera *Pseudomonas*, *Escherichia*, and *Clostridium* and others are associated with pathogenic properties and are highly enriched in GCA than in GCO, GPC, and GPR. Hence, treatment with probiotic LBB modulates the gut microbiota via enhancing the growth of beneficial *Lactobacillus* and decreasing pathogenic bacteria.

Probiotic LBB Enhanced the Gut Mucosal and Intestinal Epithelial Barrier Integrity and Prevented Cancer via TLR2-Mediated Inhibition of Apoptosis and Inflammation

Gut mucosal integrity and intestinal epithelial barrier integrity were investigated by MUC2, ZO-1, and occludin. The mRNA level of MUC2, ZO-1, and occludin were suppressed in GCA but elevated in probiotic LBB

treatment in GPR and GPC (Fig. 5). Gut mucosal integrity (MUC2), adherence junction (ZO-1), and tight junction (occludin) are disrupted in GCA but enhanced in probiotic LBB treatment.

The interaction of gut microbiota with the gut wall pathogen recognition receptors (PRR) TLR2 and TLR4 was analyzed. The mRNA expression of TLR2 increased, but TLR4 decreased in probiotic LBB treatment in GPR and GPC than in GCA, correlating with gram-positive bacteria abundance in LBB treatment and elevated gram-negative pathogenic bacteria in GCA (Fig. 5). Downstream proteins of TLRs interaction affecting inflammation (COX-2) and apoptosis (caspase 3) as well as β-catenin were analyzed. COX-2, β-catenin, and apoptosis play a role in colon carcinogenesis, and their inhibition is promising effective targets in the prevention of colon cancer [28, 29]. Suppressed expression of caspase 3, COX-2, and β-catenin is observed in GPR and GPC compared to GCA ($P < 0.05$) as shown in Fig. 5 and supplementary figure 2 (COX-2 IHC), depicting decreased inflammation, apoptosis, and tumor progression.

The anticancer effect of probiotic LBB is mediated through TLR2-enhanced mucosal and intestinal epithelial barrier function and inhibition of apoptosis, inflammation, and β-catenin signaling pathways.

Discussion

Probiotics have been increasingly used worldwide to maintain a healthy gut and alleviate gastrointestinal diseases including cancer. To effectively study the beneficial and interventional chemopreventive effect of probiotic

LBB on the development of colorectal cancer, we employed the use of DMH-induced colorectal cancer animal model in our study. Colorectal cancer is a multistage process in humans. 1,2-Dimethylhydrazine dihydrochloride and its metabolite azoxymethane (AOM) have been effectively used in animal models for the studies of CRC and mimic the disease as it occurs in humans [30, 31]. These provide the effective platform for the interventional studies of the chemopreventive potential of probiotic LBB.

The data obtained in our study revealed LBB abated the development of colorectal cancer by reducing tumor incidence, tumor multiplicity, and tumor size. This result is in concordance with earlier findings of Walia et al. [32] and Singh et al. [33] using the probiotics *Lactobacillus* and *Bifidobacterium bifidum*, respectively. Also, the use of *Bifidobacteria* and oligofructans is reported to retard colon carcinogenesis [34] as confirmed in this report.

To further understand the effect of LBB in modulating the gut microbiota, we conducted pyrosequencing using the Illumina HiSeq 2500 platform on DNA extracted from stool taken at euthanasia. The results revealed changes in gut microbiota composition in LBB treatment from that of GCA and GCO. The phyla *Proteobacteria*, *Chlamydiae* and *Bacteroidetes*, among others, decreased in probiotic treatment groups but increased in GCA. *Proteobacteria* have been reported to be increased in experimental model of colitis, in patients with inflammatory bowel diseases and in colon cancer [16, 35]. Also, *Proteobacteria* possess type 3 secretory system and interact with intestinal cells modifying host cell proteins relevant in carcinogenesis [16, 36].

Researchers have associated *Chlamydia* infections with epithelial cells disruption, DNA damage, enhanced
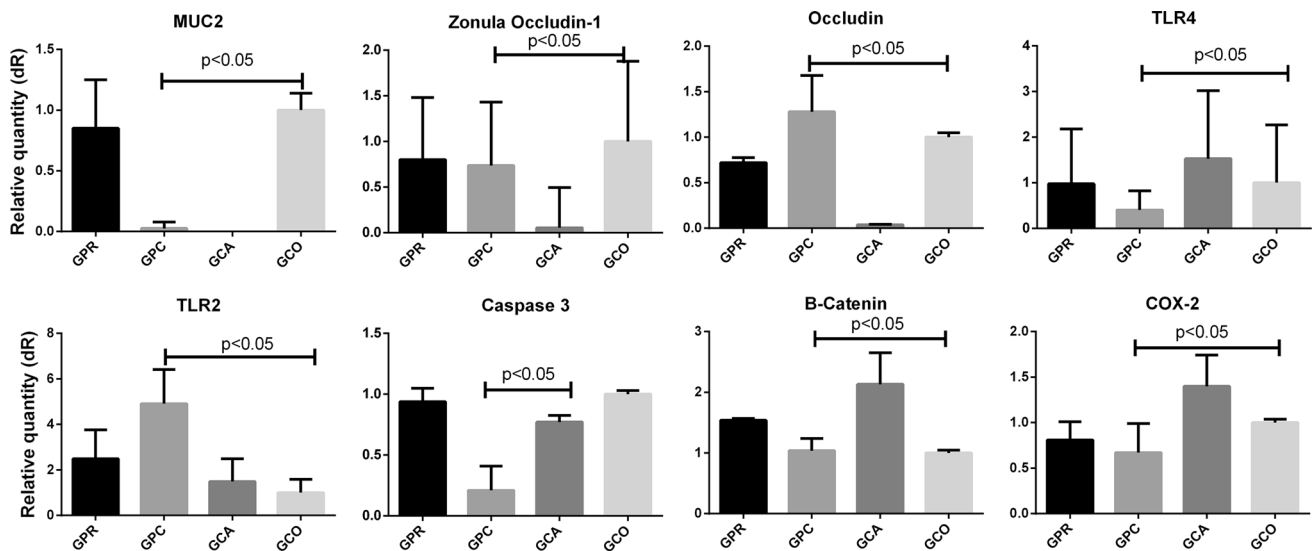
Fig. 5 Relative expression of mRNA levels of MUC2, ZO-1, occludin, TLR2, TLR4, caspase 3, β-catenin, and COX-2. Probiotic LBB treatment enhanced epithelial barrier integrity, inhibited apoptosis, and ameliorated colon cancer. #GCO, normal control; GPR, probiotic LBB only; GPC, probiotic LBB and cancer; and GCA, cancer control

oncogenic signaling, increased cell proliferation and reduction in apoptosis. Additionally, chronic persistent inflammation, a hallmark of cancers, following *Chlamydia* infections is associated with increased cell division, malfunction in DNA repair, oxidative stress, increased expression of prostaglandins and cytokines, inflammatory pathway stimulation and cancer [37–41]. These and other factors reveal the possible contribution of *Chlamydia* infections to cancer development (CRC). This impact was suppressed by treatment with LBB in our study.

The phylum *Bacteroidetes* has both probiotic and colitogenic properties [16]. The genus *Bacteroides* of this phylum has been associated with inflammation and CRC, especially *B. fragilis* [6, 42]. Though not detected in our study, *B. fragilis* toxins metalloprotease and fragilysin are associated with induction of colonic tumors and proliferation by the Wnt/B-catenin pathway, respectively [43]. *B. fragilis* has the propensity to modulate the gut microbiota to promote mucosal immune response, damage to epithelial cells and subsequently promoting colorectal adenomas and cancer [44]. Also, *B. fragilis* is involved in activation of NF-kb induction of inflammatory mediators resulting in inflammation and ultimately carcinogenesis [45, 46]. Additionally, *B. fragilis* activation of Stat3, and induction of IL-17 and DNA damage by genotoxin enhances tumorigenesis [47, 48]. Similar events could be the drive in tumorigenesis in our cancer control group. However, intervention with LBB treatment ameliorated this effect and subsequently reduced colon cancer.

The relative abundance of the pathogenic family *Enterobacteriaceae*, *Helicobacteraceae*, *Clostridiaceae*, and *Pseudomonadaceae* is aplenty in GCA. Genera of bacteria associated with these families including *Escherichia*, *Helicobacter*, *Clostridium* and *Pseudomonas* was found increased in GCA but decreased by LBB treatment.

Probiotic *Bifidobacteria* is reported to inhibit colon cancer progression by suppressing growth of the pathogenic bacteria *Escherichia coli* and *Clostridium*, and also via lowering of intestinal pH [7]. Moreover, a decrease in pathogenic bacteria modulates bacterial enzymes such as beta-glucosidase capable of converting carcinogens to inactive forms [7]. Aside modulating the gut microbiota to inhibit colon cancer, *Bifidobacteria* may interact with P450 s of the liver via its metabolites and subsequently inactivate carcinogens, bind and eliminate carcinogens via feces limiting its absorption into the intestines [7].

The Genera *Pseudomonas* and *Escherichia* harbor the gene *SpeC* for ornithine decarboxylase (ODC) activity [49, 50]. *Bifidobacteria bifidum* of probiotic LBB is reported to have inhibited carcinogen-induced cell proliferation by inhibition of ornithine decarboxylase. ODC is linked with polyamines biosynthesis that plays a role in cell proliferation, differentiation and macromolecular synthesis

associated with increasing adenoma and carcinomas of the colon [7].

Also, *Pseudomonas* cytotoxins *ExoV* and *ExoS* are associated with intracellular membrane destruction, promotion of Rac1 inactivation and induction of necrosis and apoptosis via the type 3 secretory system promoting carcinogenesis [36]. Colorectal cancer initiating lesions is associated with *Escherichia coli*-induced DNA damage especially by its enzyme colibactin. *Escherichia coli* polyketide synthase found common in CRC and IBD encodes for this enzyme colibactin [44]. Virulence factors of *Escherichia coli* that could drive carcinogenesis include pore formation by hemolysin E, bacterial attachment to mammalian cells via AIDA-1 adhesion like protein, cytotoxicity to eukaryotic cells by cytolethal toxin, cytolethal necrotizing factors, and invasion of epithelial cells via intimins and invasins [51, 52]. These factors are involved in microvilli destruction, cytoskeletal rearrangement and host cytoskeletal proteins aggregation associated with colonic hyperplasia [52]. Given that there is reduced abundance of *Pseudomonas* and *Escherichia* in probiotic LBB treatment, and its subsequent reduction in colon cancer, inhibition of metabolic and pathogenic activities of these pathogens could be a probable mechanism of LBB anti-tumor effect.

Host metabolism and immunity are essential to all living cells but are affected by secondary bile acids, such as deoxycholic acid, which contribute to CRC development [53]. However, some clades of *Clostridium* are capable of metabolizing secondary bile acids and might explain the contribution of this phylum to cancer in our study [44] but suppressed by the administration of probiotic LBB.

As for *Helicobacter pylori*, its infection is been linked with gastric carcinoma and lymphoma [54], and also increased risk of colorectal adenoma and adenocarcinoma [55]. Increased abundance of *Helicobacter* in GCA as found in our study might have contributed to colon carcinogenesis but abated by the use of probiotic LBB.

Overabundance of the probiotic genera *Lactobacillus* was observed in LBB treatment groups compared with GCA. Also observed was the bloom of *Oscillospira* in GCA than LBB treated groups. *Lactobacillus acidophilus* prevents carcinogen-induced DNA damage mediated via release of inflammatory and regulatory cytokines by gut immune cells in the colon [56]. The combinational use of the probiotics *Lactobacillus rhamnosus* and *Bifidobacteria lactis* as well as the prebiotics oligofructose and inulin altered and reduced colon cancer biomarkers, reduced colonic cell proliferation and necrosis, and prevented colon cancer [57]. Similar effect could be exerted by treatment with LBB in this study to reduce colon cancer.

Increased presence of *Oscillospira* was observed in animals fed with high-fat diet [58], risk factor for both

diabetes and CRC. Diabetes in itself is a risk factor for CRC [59]. *Oscillospira* is reported to promote the pathogenesis of type 1 diabetes and as an early impairment in the gut that might lead to obesity [58, 60]. These findings place *Oscillospira* abundance associated with colon cancer as found in our studies but lessened by probiotic LBB.

The gut mucosal barrier protects the epithelium from chemical and mechanical damage and also acting as a lubricant for intestinal motility [61, 62]. The intestinal epithelial barrier, on the other hand, acts as a gatekeeper to substances in the lumen, controls host defense, and maintains immune homeostasis [62]. We observed that in GCA, the gut mucosa and intestinal epithelial cell barrier integrity via MUC2, ZO-1, and occludin degraded significantly compared with LBB treatment in GPR and GPC. The loss of MUC2, ZO-1, and occludin and hence intestinal mucosa and epithelial barrier integrity has been reported in cancer and inflammatory diseases of the gastrointestinal tract [61, 63]. Also, MUC2 deficiency in mice is associated with tumorigenesis and larger tumor development [61]. The abundance of the bacteria *H. pylori*, *E. coli*, and *Pseudomonas* is reported to degrade mucus and disrupt epithelial tight junctions through breakage of mucus disulfide bonds, protease activity, activation of myosin light chain kinase, and Rho GTpases, resulting in actomyosin and perijunctional actin contraction [64]. On the other hand, the enhanced secretion of MUC2, tight junction function, and intestinal epithelial cell barrier function via activation of ZO-1 and occludin has been reported in the use of *Lactobacillus spp*, *Bifidobacteria spp*, and other probiotic strains as confirmed in this study [63–65].

The pattern recognition receptors on the gut wall, including TLR2 and TLR4, play an essential role in microbial recognition, induction of antimicrobial genes and in the maintenance of host immune and intestinal homeostasis [62]. The increased expression of TLR2 and decreased expression of TLR4 are observed in probiotic LBB treatment in GPR and GPC. However, in GCA, the converse expression of TLR2 and TLR4 was prominent. TLR4 overexpression is reported in colon cancer and is associated with tumor progression [2]. On the other hand, the down-regulation or blockage of TLR4 expression resulted in decrease in tumor growth, incidence, and multiplicity [2]. With TLR2, its deficiency is reported to induce tumor development, tight junction disruption, and exacerbation of intestinal inflammation [62, 66]. The expression of TLR2, however, preserves tight junction barrier assembly, promotes goblet cell mucin secretion, suppresses mucosal inflammation, and induces anti-inflammatory response [66–68] corroborating with the findings in our study.

Inflammation and apoptosis are key targets in cancer prevention strategy. The effect of LBB on apoptosis, inflammation, and tumor progression was assessed via caspase 3, COX-2 and β-catenin activity, respectively. Probiotic LBB treatment reduced the expression of caspase 3, COX-2, and β-catenin which are elevated in cancer control GCA. COX-2 is overly elevated in inflammatory diseases and cancer [69] and also a known promoter of colon carcinogenesis [69, 70]. The pathogenic bacteria *Escherichia*, *Pseudomonas*, *Chlamydia*, and *Helicobacter* are reported to induce COX-2 expression [71–74]. Additionally, increased expression of TLR4 is reportedly required for induction of COX-2 in colitis injury. *Lactobacillus and* other probiotics, on the other hand, have been reported to have inhibitory effect on Cox-2 expression [32, 69, 70, 75]. Hence, the observed increased expression of Cox-2 in cancer control associated with the pathogenic bacteria and elevated TLR4, and the decreased expression in probiotic LBB treatment associated with *Lactobacillus* substantiates our findings. β-Catenin activation is a necessary step in colon carcinogenesis [76] and is increased in cancer control GCA compared to probiotic LBB treatment. The pathogenic bacteria *Escherichia*, *Pseudomonas*, and *Helicobacter* possess the type 3 secretory system involved in the activation of the β-catenin signaling and promoting epithelial cell proliferation and mucosal hyperplasia [76, 77]. These bacteria could be involved in the increase in cancer progression in GCA but decrease in probiotic LBB in GPC. Also, dysplasia and enhanced β-catenin expression are reported in TLR2 deficient mice [66] and corroborate with the findings of decreased TLR2 and increased β-catenin expression in GCA. Finally, treatment with probiotic LBB in cancer decreases apoptosis via reduced expression of caspase 3 compared to cancer control. The inhibition of apoptosis by *Lactobacillus* and *Bifidobacteria* strains, as well as other probiotics like VSL#3, has been reported [65]. Also, the expression of TLR2 as in LBB treatment protects against apoptosis [68]. On the other hand, TLR2 deficiency in mice, as occurred in cancer control GCA, resulted in increased apoptosis of intestinal epithelial cells [67]. Taken together, these reports reinforce the observed decreased apoptosis following probiotic LBB treatment in GPC associated with enhanced expression of TLR2.

In summary, oligofructose–maltodextrin-enriched *Lactobacillus acidophilus*, *Bifidobacteria bifidum*, and *Bifidobacteria infantis* is a potential agent for reduction in colon cancer. LBB modulates the gut microbiota by decreasing the pathogenic bacteria *Escherichia*, *Pseudomonas*, *Helicobacter*, *Chlamydia*, among others, and increasing the beneficial probiotic bacteria *Lactobacillus* associated with reduction in colon cancer development decreasing tumor incidence, multiplicity, and growth. The anticancer effect of LBB correlated with increased TLR2 signaling, enhanced intestinal mucosal and epithelial

barrier function, inhibition of apoptosis, inflammation, and β-catenin signaling pathway.

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