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Original article

In vivo evidence: Repression of mucosal immune responses in mice with colon cancer following sustained administration of *Streptococcus thermophiles*

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ABSTRACT

Probiotics have attracted considerable attention because of their ability to ameliorate disease and prevent cancer. In this study, we examined the immunomodulatory effects of a *Streptococcus thermophilus* probiotic on the intestinal mucosa azoxymethane-induced colon cancer. Sixty female mice were divided into four groups (n = 15 each). One group of untreated mice was used as a control (C group). Another mouse group was injected with azoxymethane once weekly for 8 weeks to induce colon cancer (CC group). Finally, two groups of mice were continuously treated twice per week from week 2 to 16 with either the *Lactobacillus plantarum* (Lac CC group) or *S. thermophilus* (Strep CC group) bacterial strain pre-and post-treatment as performed for the CC group. Remarkably, *Tlr2*, *Ifng*, *Il4*, *Il13*, *Il10*, and *Tp53* transcription were significantly downregulated in the Strep CC intestinal mucosa group. Additionally, IL2 expression was decreased significantly in the Strep CC mouse serum, whereas TNFα was remarkably elevated compared to that in the CC, Lac CC, and untreated groups. This study suggested that *Streptococcus thermophilus* did not interrupt or hinder colon cancer development in mice when administered as a prophylactic.

1. Introduction

Colon cancer (CC) or colorectal cancer (CRC) is one of the most prevalent cancers worldwide, with approximately 1.085 million cases annually followed by lung and breast cancers with 2.09 million and 2.08 million cases, respectively. The global rate of CRC is expected to reach approximately 2.2 million reported cases each year by 2030 (Arnold et al., 2017). CRC is more common among males than females and three to four times more prevalent in both sexes in developed than in developing countries (Shaukat et al., 2013; Smyth et al., 2018). Adenocarcinomas comprise the most common types of colorectal cancers; however, mucinous carcinomas and adenosquamous carcinomas are also common (Bray et al., 2013). CRC treatments have expanded beyond 5-fluorouracil-based chemotherapy to other tailored systemic treatment regimens, which include anti-angiogenic treatment, targeted therapy, and immunotherapy (Pallag et al., 2015; Wu, 2018)

Probiotics are useful nutritional supplements to reduce cancer risk, improve chemotherapy (Hadad et al., 2019) and radiotherapy outcomes, and reduce post-surgery side effects (Legesse Bedada et al., 2020). The efficacy of probiotics for preventing and treating different forms of cancer is influenced by factors such as the bacterial or fungal strains used, administered dose, and treatment duration (Legesse Bedada et al., 2020; El Hadad et al., 2019). Certain *Lactobacillus* strains have shown anti-mutagenic activity by binding carcinogenic heterocyclic amines (Górska et al., 2019).

Abbreviations: S, *Streptococcus*; *L*, *Lactobacillus*; AOM, Azoxymethane; TLR, Toll-Like receptor; IFNγ, interform gamma; TNFα, Tumor necrosis alpha; IL, interleukin; Th1, T helper lymphocyte; Treg, T regulatory lymphocyte.

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A study in rats showed that lactic acid bacteria (LAB) protected against CC development. Clinical experiments also indicated that several LAB strains are anti-carcinogenic, as they can suppress β-glucuronidase enzymes that produce carcinogenic compounds in the digestive tract (Iqbal et al., 2014).

Streptococcus thermophilus is a gram-positive bacterium with paired oval or short-chained cells, and it is a thermophilic and aerotolerant anaerobe. It is a member of the *salivarius* group, along with *Streptococcus salivarius* and *Streptococcus vestibularis* (Facklam, 2002; Gao et al., 2014). *Streptococcus thermophilus* has been identified as an LAB, and it is useful as a starter in dairy products, making it the second most common industrial LAB species after *Lactococcus lactis* (Iyer et al., 2010). Because it has been consumed by humans for decades without any evidence of causing disease, *S. thermophilus* is widely known as a safe *Streptococcus* species (Hols et al., 2005). The *S. thermophilus* genome is 1.8 Mb in length (Bai et al., 2016), making it one of the smallest genomes among LAB and *Streptococci* (Rasmussen et al., 2008; Delorme et al., 2010). *Streptococcus thermophilus* differs from its pathogenic ancestors because it has lost genes conferring pathogenicity and gained genes beneficial to survival in dairy products (Hols et al., 2005).

Several studies in humans and animals have demonstrated that *S. thermophilus* can benefit the host health, particularly in chronic gastric diseases (Uriot et al., 2017). *Streptococcus thermophilus* can stimulate the production of innate and adaptive mediators, including IL-12, IFNγ, TNFα (Kekkonen et al., 2008), and IL-6 (Latvala et al., 2008, 2011). This probiotic strain can also induce the production of anti-inflammatory IL-10 in macrophages (Junjua et al., 2016), and is recognized as an immune stimulator. Under certain conditions, it has similar probiotic proprieties to the very well-known available commercial probiotic strain of *Lactobacillus rhamnosus GG*. Because they synthesize lactic acid, both strains exert strong anti-proliferative effects on the HT-29 cancer cell line (Tarrah et al., 2018). Previous studies predicted that *S. thermophilus* can reduce the risk of CRC in mice. The tumor-suppressive activity of *S. thermophilus* is related to its production of β-galactosidase, which leads to the release of galactose that eliminates Hippo signaling and alters the Warburg effect (Li et al., 2020).

Streptococcus thermophilus and *Lactobacillus acidophilus* equally ameliorate the immune response in mice by activating murine immune cells, such as macrophages and lymphocytes, whether administered orally or intraperitoneally (Perdigon et al., 1987). *Streptococcus thermophilus* supernatant media can strengthen the intestinal mucosal capacity of colitis-prone C57BL/6 IL10-deficient mice infected with *Helicobacter* bacteria by activating the Th1 lymphocyte immune response (Ménard et al., 2005). Moreover, Wasilewska *et al.* confirmed the therapeutic effects of *S. thermophilus* on dextran sodium sulfate-induced colitis in mice. They observed immunomodulatory effects on all subsets of T helper cell activity and Treg response in mice suffering from colitis, as evidenced by improvement in colonic healing and regulation of T lymphocyte markers (Wasilewska et al., 2019).

In the present study, we evaluated the *in vivo* antitumor activity of *S. thermophilus*. Immunological, and molecular investigations of the mouse intestinal mucosa were evaluated during and after CC induction. We validated our results by comparing the same parameters in untreated (negative) control, positive control, and mice dosed with *L. plantarum*.

2. Materials and methods

2.1. Bacterial strains

Two bacterial strains, *L. plantarum ATCC = 8014* and *S. thermophilus ATCC = BAA-250,* were obtained from the Egyptian Laboratory for Microbiology. Both obtained strains were grown in sterile de Man, Rogosa and Sharpe Agar (BioLab, Naples, Italy) and incubated at 37 °C (pH 6.8) for 24 h. Bacterial cultures were performed twice. Each bacterium strain was transferred individually into de Man, Rogosa and Sharpe Agar broth medium (BioLab model No. EMRB20500-M) and incubated at 37 °C for 24 h. The cultures were then centrifuged at 5000 × *g* for 10 min at 4 °C. The cell pellets were washed twice with sterile phosphate-buffered saline (Cat no. MT21040CV; Thermo Fisher Scientific, Waltham, MA, USA) at pH 7.4 before centrifugation at 5000 × *g* for 10 min at 40 °C. Finally, the cell pellets were re-suspended in two different tubes containing 10% nonfat milk and phosphate-buffered saline. The final concentration obtained for *S. thermophilus* equaled 10^8 cells/mL, whereas that of *L. plantarum* was 10^9 cells/mL. The number of cells was valued using 1 mL of 10% nonfat milk. Bacteria were serially diluted 1:10 nonfat milk, and absorption was measured with a spectrophotometer at 600 nm (Spectro23, Labomed, Inc., Los Angeles, CA, USA) (Minelli and Benini, 2008; Xia et al., 2011; Stofilova et al., 2017).

2.2. Mouse model and CC induction

Sixty female Swiss mice (6–8 weeks old) weighing approximately 25–30 g were obtained from King Fahad Research Center and maintained in the animal research facility at King Abdulaziz University, Faculty of Science. Mice were maintained under normal laboratory conditions: standard room temperature 27 ± 2 °C with a 12-h light/12-h dark cycle and given a laboratory pelleted formula diet and water *ad libitum*. The Institutional Animal Care and Use Committee (IACUC No. 2019/4320656/2) of King Abdulaziz University, Faculty of Science approved all animal protocols.

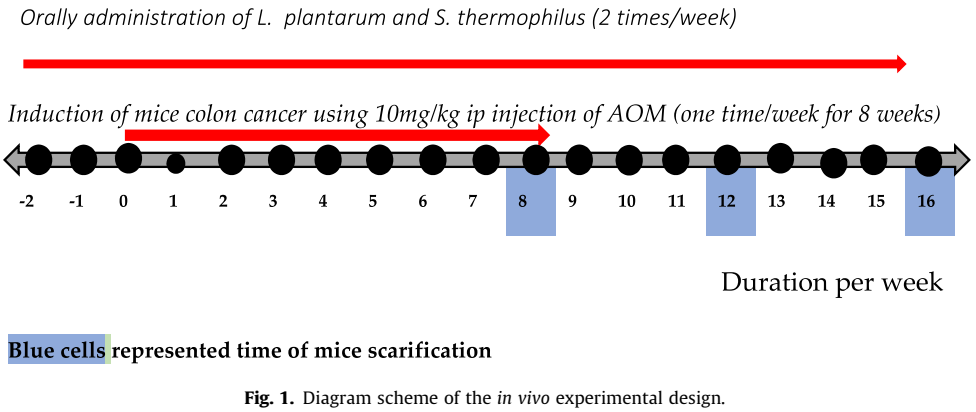


Fig. 1. Diagram scheme of the *in vivo* experimental design.

Azoxymethane (AOM) (Cat No. A5486, Sigma-Aldrich, St. Louis, MO, USA) is commonly used to induce CC in mammals. After dissolving 50 mg of AOM in 0.9% NaCl, a 10 mg/kg dose of AOM was injected into the mice intraperitoneally (IP) to induce CC as described by Bissahoyo et al. (2005).

2.3. In vivo experimental design

All mice were randomly categorized into four main groups (n = 15/group), with no exclusion criteria, thus ensuring that the median of each animal group was similar to those of prior experiments. The groups were as follows: mice used as a healthy control group (Group C, negative control); mice injected IP with 10 mg/kg AOM once per week for 8 weeks starting from week 0 until week 8 (group CC); mice orally administered 10^8 CFU *S. thermophilus* suspended in 10% nonfat milk twice weekly by gavage pre/post the weekly IP injection as in group CC (positive control); and mice continually administered *L. plantarum* twice weekly suspended in nonfat milk (from weeks 2 to 16) by gavage pre/post-treatment with AOM as in group CC. For the experiment, each set of three mice was housed in individual standard plastic mouse cages. Cages were filled with aspen chip bedding substrate (Tapvei, Harjumaa, Estonia) and had an enriched environment. Five mice from each group were anesthetized and sacrificed (Jaykaran and Kantharia, 2011) at weeks 8, 12, and 16 from CC induction. All efforts were made to minimize animal suffering during the experiment steps. Mouse

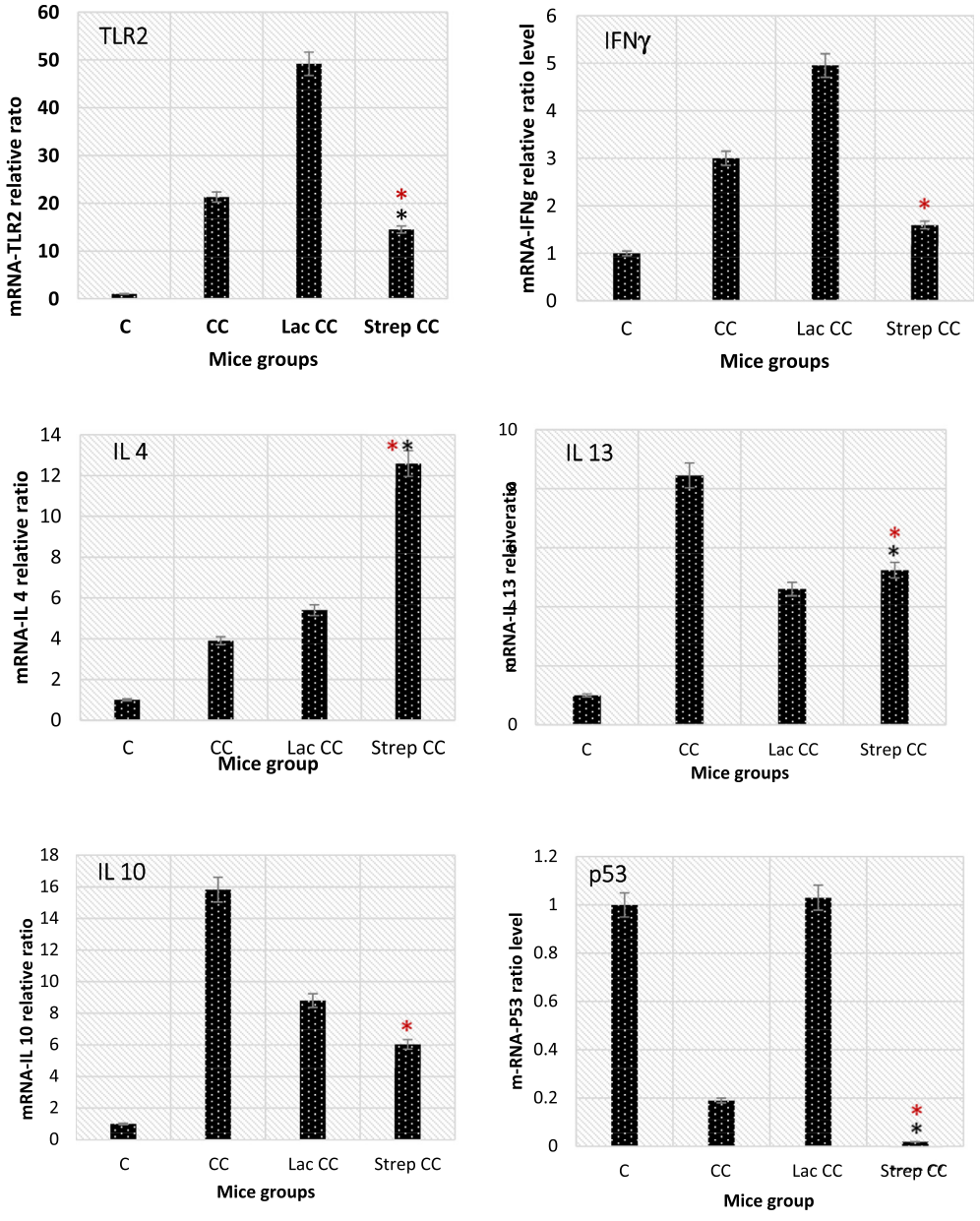


Fig. 2. Effects of Streptococcus thermophilus on the development after 8 weeks from the induction of mouse colon cancer. (A) Relative ratios of *TLR2*, *IFNγ*, *IL4*, *IL13*, *IL 10*, and *P53* transcription levels in mouse intestinal mucosa cells. (B) TNFα and IL2 levels in mouse sera. *Group C represents untreated, group CC represents mice injected with AOM, Lac CC mice administered with 10^9 CFU Lactobacillus strain and injected with AOM, and group Strep CC mice treated with 10^8 CFU Streptococcus strains and injected with AOM. Significance was set at P < 0.05 as determined by one-factor analysis of variance for comparison. (*) Comparison between controls and Strep CC group. (*) Comparison between Strep CC group and CC group. (#) Comparison between Strep CC group and Lac CC group. Every point represents the mean value of three separate tests, and vertical bars denote 5% around the mean.*

colon and blood sera were harvested from all treated and untreated groups and stored at − 80 °C for further examinations. (Fig. 1).

2.4. Intestinal mucosa RNA extraction and gene transcriptions

RNA was extracted from the colon of the untreated and treated mice groups using the RNeasy Maxi kit (Cat no. 75162, QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Pure eluted mouse intestinal RNA concentrations were calculated using a nano-drop instrument (DS-11, DeNovix. Wilmington, DE, USA) and subjected to amplification and quantification using Verso SYBR Green 1-Step qRT-PCR Master Mix (Cat no. AB-4104/C, Thermo Fisher Scientific) reagents. Supplementary Table 1 shows the specific primers used for amplification and relative ratio quantification of our target gene transcripts (*Tlr2*, *Ifng*, *Il4*, *Il13*, *Il10*, and *Tp53*) and the β-actin gene (ACTB), which was utilized as the housekeeping reference gene. Each PCR sample was adjusted to a final volume of 25 μL, and the concentration of each RNA sample was approximately 500 ng/total volume. The PCR profiles of the current target gene expression have previously been described in **El** Hadad et al., 2019.

2.5. Assay of total TNFα and IL2 in mouse blood sera by capture ELISA

TNFα and IL2 screening was performed using pre-coated microtiter polystyrene plates, mouse TNFα ELISA Kit (ab208348, Abcam, Cambridge, UK), and mouse IL2 ELISA Kit (ab46096, Abcam) as per the manufacturer's instructions.

2.6. Statistical methods

All data were statistically analyzed using MegaStat software (Version 10.2 release 2.1). One-way analysis of variance was used to determine the statistical significance of the variations within the untreated control group and significant differences between the treated and untreated groups. $P < 0.05$ was considered to indicate statistically significant differences.

3. Results

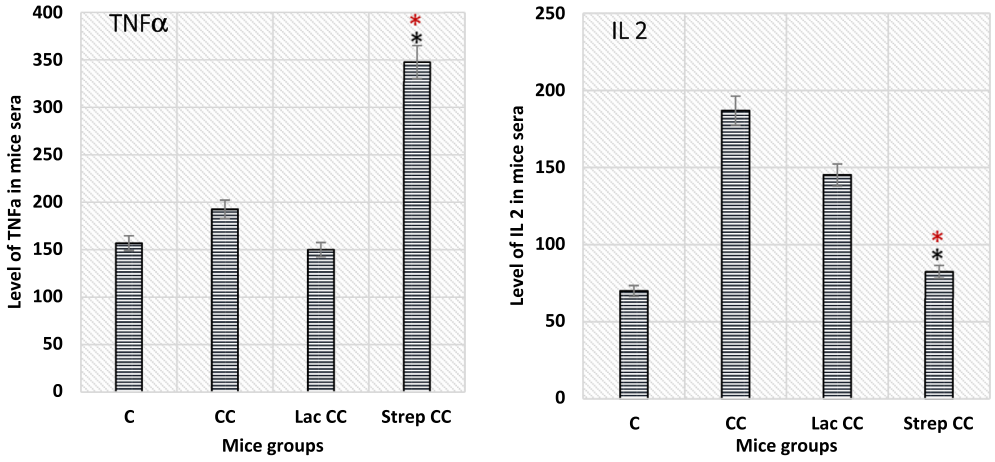
3.1. Effects of S. Thermophilus at 8 weeks from CC induction

Tlr2 transcription was significantly upregulated in the Strep CC mouse intestinal mucosa compared to that in the untreated control (P = 0.0042) and significantly downregulated compared to that in the Lac CC group (P = 0.0001). This receptor transcription was increased to a non-significant degree in the Strep CC group relative to that in the CC group (Fig. 2**A**). For *Th1* cytokine transcription, we observed non-significant downregulation in *IFNg* transcription in the Strep CC intestinal mucosa compared with in the C mouse group ($P > 0.05$); the increase was not significant compared to that in the CC group (P = 0.092). *Ifng* transcription significantly decreased in Strep CC mucosal cells compared to that in the Lac CC group (P = 0.0063) (Fig. 2**A**). Transcription levels of *Il4* were highly significantly increased in the mucosa of the Strep CC mouse group compared to those in the untreated, CC, and Lac CC mouse groups (P-values = 0.000, 0.025, and 0.0000, respectively) (Fig. 2**A**). After week 8, *Il13* transcription levels were highly significantly upregulated in the Strep CC mouse intestinal mucosa compared to that in the untreated control group (P = 0.003); however, it decreased significantly compared to that in the CC intestinal mucosa (P = 0.025). No significant alteration was observed in the *Il13* transcription levels in the Strep CC and Lac CC mouse groups ($P > 0.05$) (Fig. 2**A**). Transcription of *Il10*-mRNA post-exposure to *S. thermophilus* showed a non-significant increase in the mouse intestinal mucosa cells compared to that in the untreated group (P = 0.104); however, this anti-inflammatory cytokine decrease was not significant compared to that of the Lac CC group ($P > 0.05$). Additionally, *Il10*-mRNA transcription decreased significantly (P = 0.017) in mucosal cells of the Strep CC mice group after the same period compared with the CC group (P = 0.015) (Fig. 2**A**). *Tp53* tumor suppressor transcription was significantly inhibited in Strep CC mouse intestinal mucosa relative to that in the untreated group (P = 0.0013) and Lac CC group (P = 0.0011). The level of *Tp53* expression in the Strep CC intestinal mucosa group was similar to that in the CC group ($P > 0.05$) (Fig. 2**A**)

TNFα proinflammatory cytokine levels in the mouse sera collected from the Strep CC group revealed significant elevation compared to that in the untreated and Lac CC groups (P = 0.0424 and 0.048, respectively), despite a non-significant increase compared to that in the CC mice group ($P > 0.05$) (Fig. 2**B**). IL2 was highly significantly elevated in the sera of the Strep CC group compared to that in the sera of untreated control mice (P = 0.0072), whereas it was reduced significantly compared to that in the sera of CC and Lac CC mouse groups (P = 0.0005 and 0.0192, respectively) (Fig. 2**B**).

3.2. Effects of S. Thermophilus 12 weeks from CC induction

Histological sections of the intestinal mucosa from the positive control (CC group) showed enlargement of glandular elements



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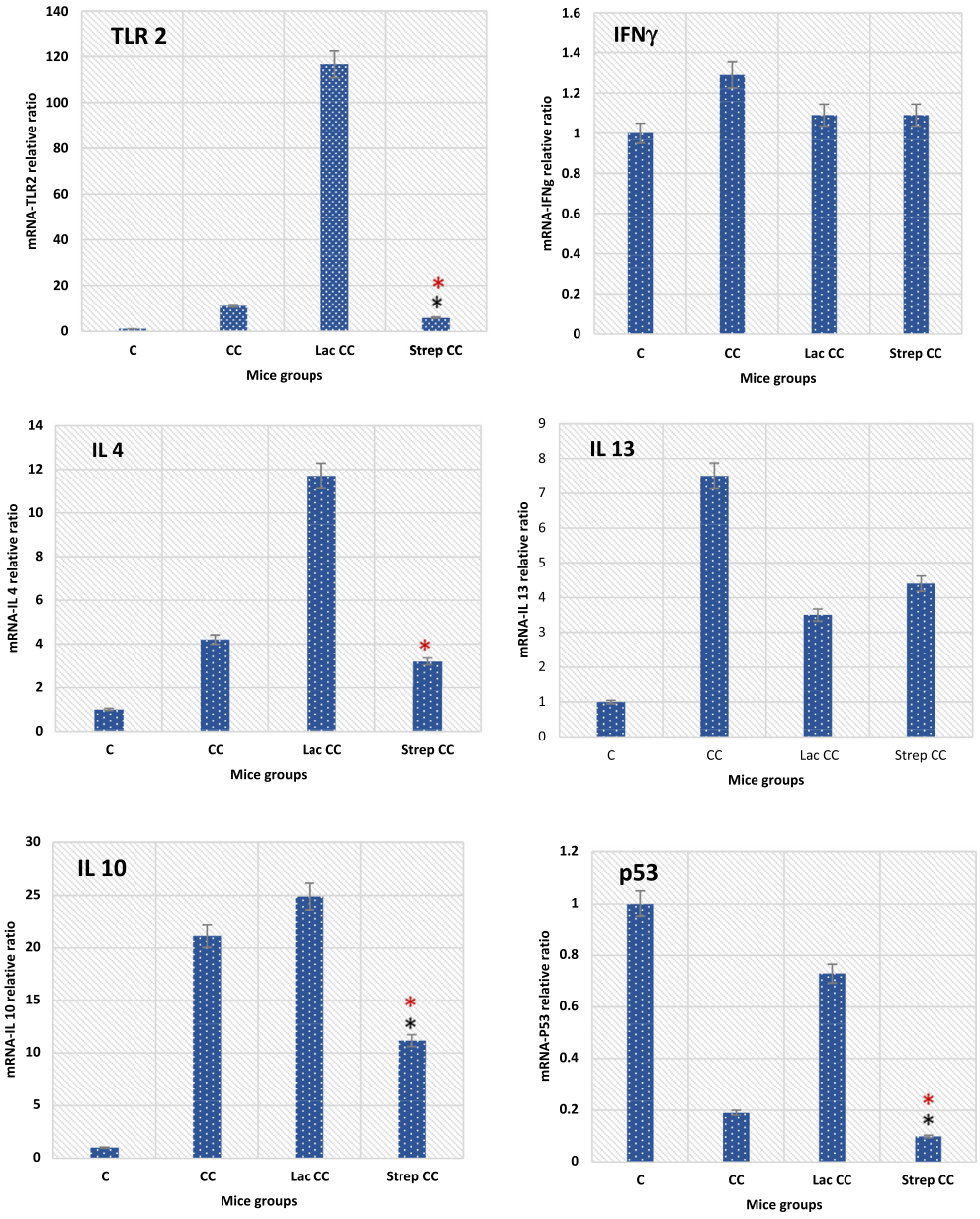

Fig. 3. Effects of Streptococcus thermophilus on the development after 12 weeks from the induction of mouse colon cancer. (A) Relative ratios of *TLR2*, *IFNγ*, *IL4*, *IL13*, *IL 10*, and *P53* transcription levels in mouse intestinal mucosa cells, (B) TNFα and IL2 in mouse sera. *Group C represents untreated mice, group CC represents mice injected with AOM, group Lac CC represents mice administered 10^9 CFU Lactobacillus strain and injected with AOM. Group Strep CC represents mice treated with 10^8 CFU Streptococcus strain and injected with AOM. Significance was set at P < 0.05 as determined by one-factor analysis of variance. (*) Comparison between controls and Strep CC group. (*) Comparison between Strep CC group and CC group. (#) Comparison between Strep CC group and Lac CC group. Each point represents the mean value of three separate tests, and vertical bars denote 5% around the mean.*

associated with a mild increase in nuclear staining (hyperchromatosis). The *Tlr2* transcription level showed similar expression to that detected at week 8, where it was significantly higher than that in the untreated mouse group (P = 0.039) and significantly lower than that in the Lac CC mouse group (P = 0.0000). Additionally, *Tlr2* transcription showed non-significant differences in the Strep CC group compared to that in the CC group (Fig. 3**A).** Further analysis of the *Ifng|Th1* transcription level showed non-significant diversity in the Strep CC mouse intestinal mucosa compared to those in the C, CC, and Lac CC mouse groups (P > 0.05) (Fig. 3**A).** Completing transcription level analysis of *Il4* verified a non-significant increase in the Strep CC mouse intestinal mucosa cells compared to that in the CC or untreated groups (P > 0.05) but was significantly decreased compared to those in the Lac CC mice group (P = 0.0081) (Fig. 3**A).** Additionally, the Strep CC mouse intestinal mucosa showed non-significant regulation of *Il13* expression compared to the Lac CC, CC, and untreated control groups (P > 0.05) (Fig. 3**A).** Mouse intestinal mucosa exposed continuously to *S. thermophilus* showed significant downregulation of the *Il10* transcription level compared to the untreated and Lac CC mouse groups (P = 0.0011 and 0.0208, respectively). Furthermore, *Il10* transcription was not significantly decreased in the Strep CC group compared to that in the CC mouse group (P > 0.05) (Fig. 3**A).** Continuous administration of *S. thermophilus* probiotics to mice bearing CC for 12 weeks significantly downregulated *Tp53* transcription in the intestinal mucosa compared to that in

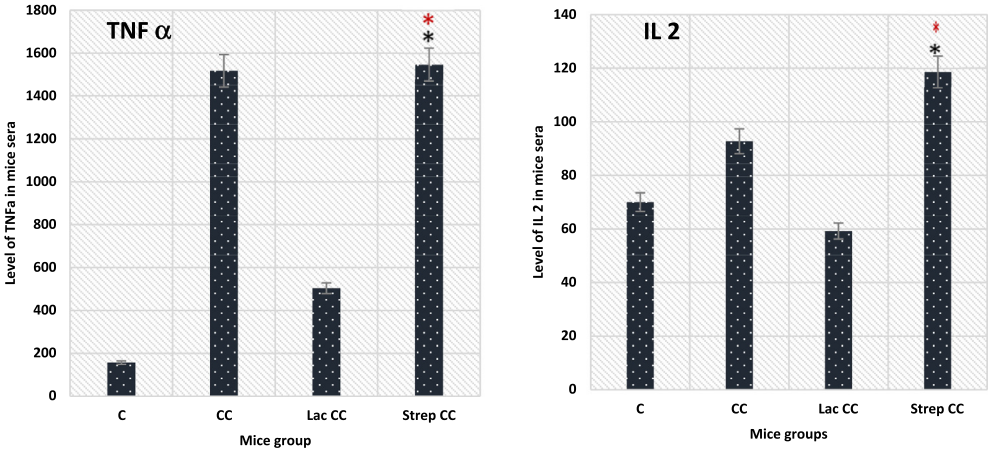

Fig. 3 (*continued*)

the untreated group (C group) and Lac CC group (P = 0.0038, and 0.0135, respectively). The level of *Tp53* expression of the Strep CC group remained similar to that in the CC group (Fig. 3**A**).

TNFα levels were significantly increased in the mouse sera collected from the Strep CC group compared to that in the sera from untreated and Lac CC groups (P = 0.000, and 0.000, respectively). This cytokine was increased non-significantly compared to that in the mouse sera of the CC groups (P > 0.05), and both levels were similar (Fig. 3**B**). Although the IL2 serum level of the Strep CC group increased significantly compared to that in the untreated and Lac CC groups (P = 0.0314 and 0.0131, respectively), it did not increase significantly compared to that in the CC group (P > 0.05). Additionally, this proinflammatory T cell cytokine showed no significant differences in the mouse sera of the Strep CC group compared to that in the sera of the CC group (P > 0.05) (Fig. 3**B**).

3.3. Effects of S. Thermophilus after 16 weeks of mouse CC induction

The *Tlr2* transcription level was increased significantly in the intestinal mucosa of the Strep CC mouse group compared to that in the untreated mice group (P = 0.0369), whereas this decrease was not significant compared to those of the Lac CC mouse intestinal mucosa (P > 0.05). The *Tlr2* mRNA level showed highly significant downregulation in the Strep CC group compared to that in the CC mouse group (P = 0.0020) (Fig. 4**A**). Regarding *Ifng* transcription levels, the Strep CC mouse intestinal mucosa exhibited non-significant differences compared to that in the C and Lac CC groups (P > 0.05). The *Ifng*-mRNA transcription level showed a highly significant decrease in the intestinal mucosa of the Strep CC mouse group compared to that of the CC group (P = 0.001) (Fig. 4**A**). *Il4* transcription in the Strep CC intestinal mucosa was similar to those detected in either the untreated control or the Lac CC group (P > 0.05). Furthermore, *Il4* transcription was highly significantly decreased in the Strep CC mouse group compared to that in the CC mice group (P = 0.0173) (Fig. 4**A**). The *Il13* transcription level was elevated to a non-significant degree in the mouse intestinal mucosa of the Strep CC mouse group compared to that in the untreated and Lac CC groups (P > 0.05). The Strep CC mouse group showed an extremely significant decrease in *Il13* transcription compared to the CC group (P = 0.0199) (Fig. 4**A**). After 16 weeks of continuous *S. thermophilus* administration, Strep CC mucosa cells still showed very highly significant elevation of *Il10* transcription compared to the untreated and Lac CC mouse groups (P = 0.003 and 0.0056, respectively). No significant differences were observed in the expression of these cytokines in the intestinal mucosa of the Strep CC group relative to that in the CC group (P > 0.05) (Fig. 4**A**). *Tp53* expression was reduced significantly in mouse intestine cells extracted from the Strep CC group compared to those from either the untreated or CC group (P = 0.0011 and 0.0002, respectively). The Strep CC group showed a non-significant increase in this tumor suppressor gene compared to the Lac CC group (Fig. 4**A**).

TNFα levels were significantly elevated in the mouse blood sera of the Strep CC group compared to that in the untreated, CC, and Lac CC groups, with each group showing P-values of 0.0000 (Fig. 4**B**). Although the serum IL2 level was elevated to a non-significant degree in the Strep CC mouse group compared to that in the Lac CC group and reached the same level (P > 0.05), the decrease in this T cell cytokine was not significant in the Strep CC group sera compared to those in either untreated or CC group sera (P > 0.05) (Fig. 4**B**).

4. Discussion

The impact of the microbiota on cancer progression has attracted considerable attention since the first local bacterial injection to treat sarcomas in the late 19th century (Raj et al., 2018). Probiotics are important constituents in many traditional foods, which have been successfully used to cure lethal intestinal disorders (Patel and Goyal, 2013; Terpou et al., 2019), and play a role in preventing and altering cancer growth (Louis et al., 2014). As with many other tumors, dietary habits, lifestyle, and alterations in the gut microflora play a key role in CRC pathogenesis (Wang et al., 2012; Louis et al., 2014). We evaluated the safety of *S. thermophilus* as an antitumor agent with anti-inflammatory properties in mice with chemically induced CC.

TLR2 is a unity of the pattern-recognition receptors (Beutler, 2009), and probiotics mainly act as general immune enhancers **(El** Hadad et al., 2019). TLR2 facilitates the recognition of commensal or pathogenic organisms, playing a pivotal role in the immunomodulatory effect of probiotics (Llewellyn and Foey, 2017). Mice in the Strep CC group showed an early significant increase in *Tlr2* transcription starting from weeks 8 to 16 after CC induction compared to the untreated control mice. These results contrast those in the mouse mucosa exposed to *L. plantarum* for the same periods, in which *Tlr2* transcription decreased significantly. Notably, in our study, Strep CC *Tlr2* transcription in the mouse intestinal mucosa showed non-significant improvement compared to that in the CC group at weeks 8 and 12. The same receptor was highly significantly downregulated in the Strep CC group compared to that in the CC mouse group. The main membrane structural constituent of both studied probiotics is peptidoglycan (Bernard et al., 2011; Layec et al., 2009). Our results suggest

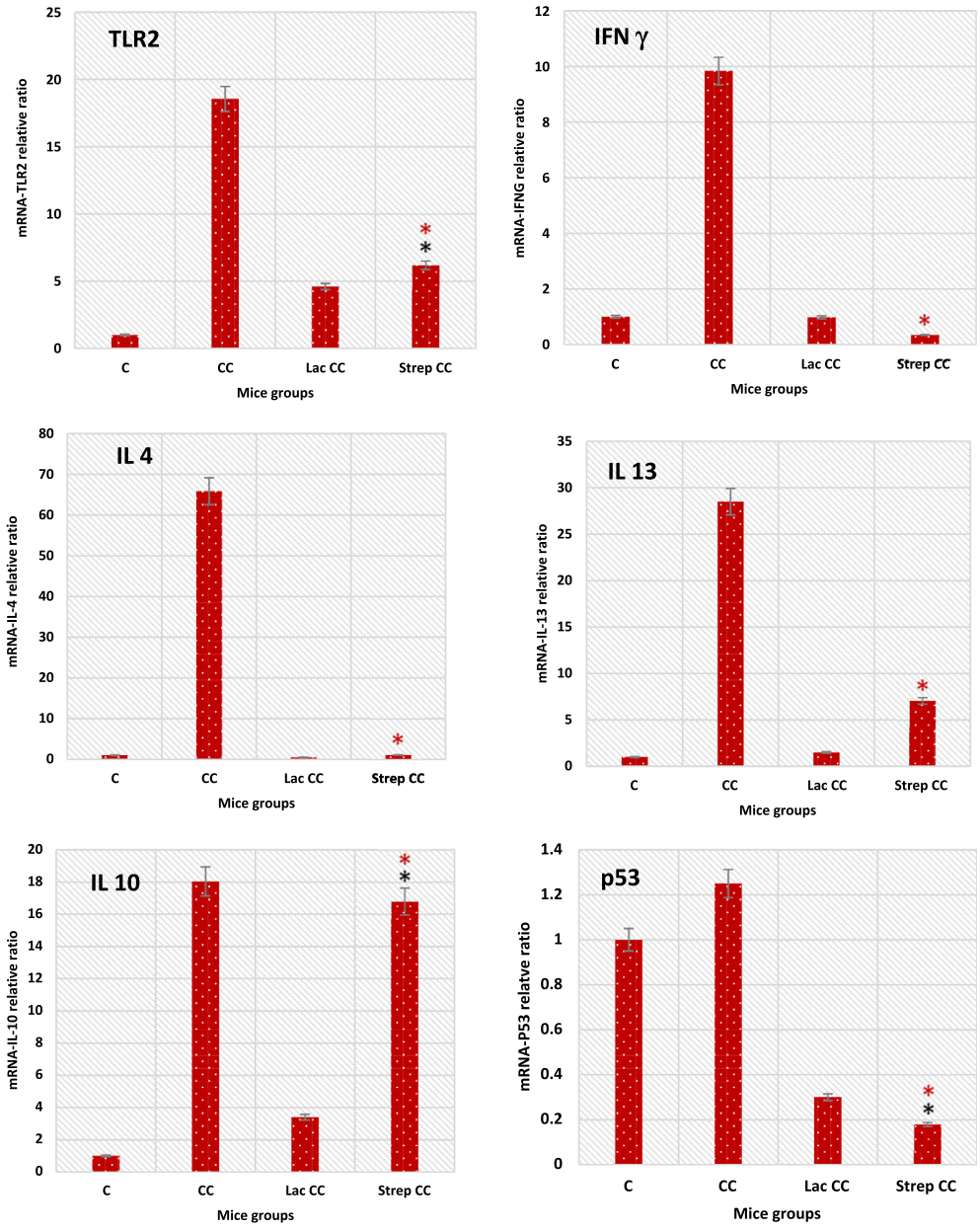

Fig. 4. Effects of Streptococcus thermophilus on the development after 16 weeks from the induction of mouse colon cancer. (A) Relative ratios of *TLR2*, *IFNγ*, *IL4*, *IL13*, *IL10*, and *P53* transcription levels in mouse intestinal mucosa cells, (B) TNFα and IL2 in mouse sera. *Group C represents untreated, group CC mice injected with AOM, group Lac CC mice administered* 10^9 *CFU Lactobacillus strain and injected with AOM. Group Strep CC represents mice treated with* 10^8 *CFU Streptococcus strain and injected with AOM. Significance was set at P < 0.05 as determined by one-factor analysis of variance. (*) Comparison between controls and Strep CC group. (*) Comparison between Strep CC group and CC group. (#) Comparison between Strep CC group and Lac CC group. Each point represents the mean value of three separate tests, and vertical bars denote 5% around the mean.*

that *Streptococcus* has a smaller impact on the expression of *Tlr2*, an innate immunity mediator, than *Lactobacillus* during CC induction in mice. This peptidoglycan component interacts with TLR2 and TLR4 to stimulate several cytokine-secreting immune cells (Haller et al., 2002). Additionally, our *Tlr2* transcription results coincided with the species and strain-specificity hypothesis of *Tlr2* interactions, wherein it is important to select specific strains for promoting specific immune effects (Ren et al., 2016) that instantly affect epithelial cell activation (Yan, and Polk, 2011**).**

TLRs augment inflammatory reactions, which are considered the most vital innate (Roberts-Thomson et al., 2011), and adaptive immune mediators (Naglik 2014). Oral probiotic regimens are significantly correlated with cytokine stimulation in the intestinal mucosa, serum, or even in remote mucosal sites (Villena et al., 2012). For the past three decades, IFNγ has been considered an immunological antitumor agent. It plays a key role in combating the escape of the tumor oncogenic machinery from the body's tumor surveillance (Schreiber et al., 2011). Generally, Strep CC/*Ifng* transcription was non-significantly downregulated compared to that in the untreated, Lac CC, and CC mouse groups in all experiment periods, except for a significant decline compared to that in the Lac CC (week 8) and CC mouse groups (week 16). Notably, *Ifng* transcription shows significant tumor inhibitory activity, although its extent of growth inhibition is inconsistent (Kortylewski et al., 2004).

Continuous administration of the *Streptococcus* strain failed to activate Th1 by stimulating *Ifng* transcription in mouse intestinal mucosa during CC induction. Growth inhibition is dependent on STAT1 activation (Kortylewski et al., 2004). Notably, the STAT1 signal is typically stimulated by a decreased concentration of IFNγ,

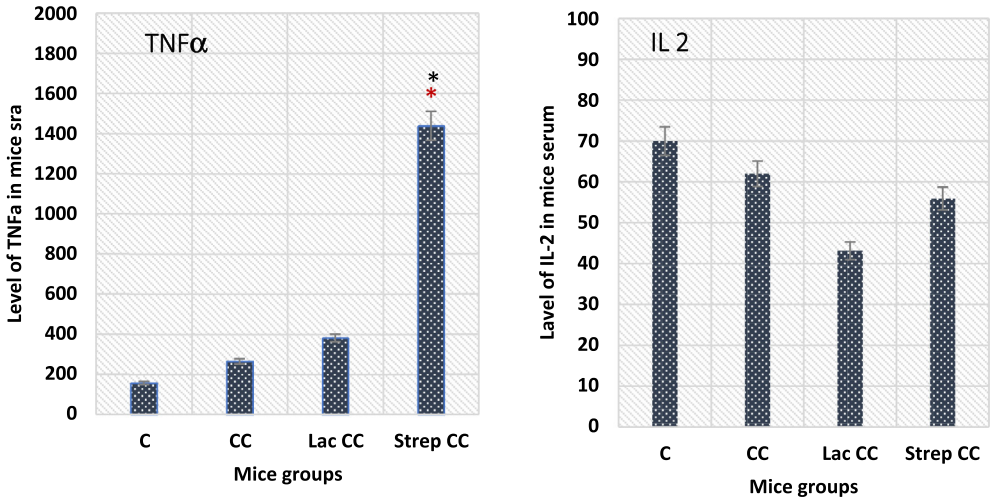

Fig. 4 (*continued*)

but growth inhibition was only apparent at a considerably higher concentration (Ramana et al., 2001). IL10 is categorized as a Th2 CD4 cytokine that affects the proliferation of Th1 CD4 T cells and their secretions, specifically IFNγ (Ejrnaes et al., 2006). Notably, IL10 decreases inflammation by inhibiting proinflammatory cytokine expression from T cells and macrophages (Ouyang et al., 2011). It may also suppress antitumor activity by suppressing tumor-promoting inflammation and activation of cytotoxic CD8 T cells (Oft, 2014).

Continuous administration of *S. thermophilus* caused non-significant alterations in *Il10* transcription in the mouse intestinal mucosa compared to that in the untreated, Lac CC (at week 8), and CC mouse groups (at weeks 12 and 16). *Il10* transcription was downregulated significantly compared to in the CC (at week 8), untreated, and Lac CC groups (at week 12), and increased significantly compared to that in the untreated and Lac CC groups (at week 16). The late increase in Strep CC/*Il10* transcription may be related to the significant upregulation of Treg and Th1-mediated IL12 (Ozdemir, 2010). Furthermore, this upregulation in Strep CC/*Il10* transcription suppresses MHC class II expression in antigen-presenting cells by promoting alternative macrophage activation (Nakamura et al., 2015) but may also be essential for stimulation of T cytotoxic cells (Oft, 2014). Further analysis of Th2 cytokines IL4 and IL13 showed that they regulate immune responses and the immune microenvironment, not only under normal physiological conditions but also in cancer, where they are structurally and functionally related (Suzuki et al., 2015). Previous studies showed that probiotics can stimulate Th2′s anticancer immune surveillance by generating IL4 and IL13 cytokines (Coussen et al., 2013; de Araújo et al., 2017). These previous studies conflict with the current *Il4* and *Il13* transcription results from the Strep CC mouse mucosa. In the current study, Strep CC *Il4* transcription in the intestinal mucosa showed early elevation compared to all other untreated and treated mice groups, but this increase was not significant compared to those in the untreated and CC mouse groups, and was significantly decreased compared to that in Lac CC after 12 and 16 weeks from CC induction. Blocking of Strep CC *Il4* transcription may affect tumor inhibition, leading to increased proliferation of CC cells (Mager et al., 2016). In the present study, Strep CC *Il13* transcription increased significantly after 8 weeks compared to that in the untreated mouse intestinal mucosa, before it decreased significantly after 12 and 16 weeks compared to that in the CC mouse group. Both current probiotics caused non-significant differences in *Il13* transcription in mouse intestinal mucosa following CC induction. Additionally, IL4Rα and IL13Rα1 chain receptors of IL4 and IL13 cytokines are considered as functional receptors in cancer cells and may act as biomarkers of tumor aggressiveness (Suzuki et al., 2015).

TNFα influences the pathogenesis of intestinal inflammation disease (Ruder et al., 2019). Several probiotics known to be immune stimulators can induce systemic proinflammatory cytokines such as TNFα (Castillo et al., 2011). Most probiotics interact with immune cells at the mucosal interface, stimulating the generation of proinflammatory cytokines, including TNFα (Lee et al., 1993) and activating lymphocyte cells (Borruel et al., 2002). Several studies showed that TNFα was decreased significantly after using probiotic strains on patients with intestinal inflammatory diseases (Breese et al., 1994; Reimund et al., 1996; Woywodt et al., 1999; Plaza-Díaz et al., 2017), particularly CRC (Lenoir et al., 2016). The present *Tnfa* transcription increased significantly in the Strep CC mouse sera compared to that in the untreated and Lac CC groups throughout the experiment. The Strep-*TNFα* level did not increase significantly in the mouse sera at weeks 8 and 12 but showed significant elevation after 16 weeks compared to that in the CC mouse group. Indeed, low concentrations improve CC prognosis by increasing TROP-2 and ERK1/2 signaling transcription (Zhang et al., 2019).

IL2 functions as a T cell growth factor (Zhu et al., 2010), modulator of T helper cell differentiation (Yu et al., 2009), promoter of Treg cell development (Cheng et al., 2011), and augmenter of cytolytic activity of natural killer and lymphokine-activated killer cells (Liao et al., 2013). At week 8, the IL2 level showed a significant decrease in the Strep CC mouse serum compared to that in the Lac CC and CC groups, whereas at week 12, this increase was significant compared to that in the Lac CC group and not significant compared to that in the CC group. Additionally, at week 16, IL2 in the Strep CC sera group showed non-significant differences compared to that in all other groups. This result confirmed that *Lactobacillus* probiotics elevated proinflammatory cytokines to develop an extra efficient immune response against cancer (Yazdi et al., 2012), whereas *S. thermophilus* did not.

Mutations in nucleotide sequences and epigenetic variations in normal colon epithelium lead to the development of CC. The loss of genomic stability generates an agreeable environment for tumor suppressor gene alterations. *Tp53* transcription showed unrestricted inhibition in Strep CC intestinal mucosa compared to that in the untreated and Lac CC mouse groups at 8, 12, and 16 weeks after CC induction. Additionally, the same tumor suppressor gene transcription of the Strep CC intestinal mucosa showed a non-significant difference compared to that of the CC mice group in

all experiment periods. Remarkably, inactivation of the tumor suppressor gene *Tp53* is one of the most critical determinants of CRC tumor induction and progression (Kinzler and Vogelstein, 1996). The exceptional inhibition of *Tp53* transcription in the Strep CC group indicates that *S. thermophilus* cannot protect against CC (Kumar et al., 2010a; Kumar et al., 2010b).

5. Conclusions

The extent of immunomodulatory effects of *S. thermophilus* is controversial and varies between studies and strains being evaluated. We demonstrated the negative immunomodulatory impacts of *S. thermophilus* on the mouse intestine, a part of the mucosal immunity, during CC induction using AOM. At the molecular and immunological levels, *S. thermophilus* significantly repressed all studied immunological markers except for *Tnfa,* which appeared to increase the rate of CC progression compared to that in the *L. plantarum* group or AOM-injected group. We observed weak antitumor activity of *S. thermophilus* probiotics during CC progression. Our results suggest that not all probiotics have equivalent activity in cancer prophylaxis. Finally, further studies should be performed to determine the mechanism of *S. thermophilus* bacteria on CC by determining its effects at the epigenetic level.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

Conceived the idea of this paper: SLH. Conceived and designed the experiments: SLH, AD and MAS. Animal treatment and sacrifice supervision: MAS, JA, FB, and SH. Performed the experiments: MAS. Analyzed the data: SLH, AD and MAS. Wrote and edited the paper: SLH, MAS, JA, and SH. Reviewed the paper: SLH, AD, FB, and HA. All authors have read and approved the manuscript.

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Consent for publication

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article and its additional files.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.04.090.

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