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Research Article
Lactobacillus rhamnosus Enhances the Immunological Antitumor Effect of 5-Fluorouracil against Colon Cancer

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Abstract

Background and Objectives: 5-Fluorouracil (5-FU) is the most common anticancer therapeutic, even though its response rate as a single agent is usually less than 20%. *Lactobacillus rhamnosus* bacteria reduce the severity of gastrointestinal tract infections, with additional functions in cancer prevention. This study investigated the histological and immunological changes associated with the combination treatment of *L. rhamnosus* and 5-FU in mice with colon cancer. **Material and Methods:** Five groups of male mice were classified as follows; Group A: Mice injected with azoxymethane (AOM) to induce colon cancer, Group AL: Mice injected with AOM and orally administered *L. rhamnosus* alone, Group AF: Mice injected with AOM and administered 5-FU, Group AFL: Mice injected with AOM and treated with both *L. rhamnosus* and 5-FU and Group C: Untreated control mice. **Results:** A reduction in inflammatory features with a normal histological structure was observed in the colon of the AFL group compared to those in the other treated groups. The intestinal mucosa of the AFL group showed a significant downregulation in K-ras and Treg/IL-10 transcription levels. This downregulation was associated with an improvement in the innate and adaptive immune responses through increased TLR2 and Th1/IFNγ transcription. TNFα and IL-6 protein expression was significantly elevated in the serum of the AFL groups compared to levels in both the A and AF groups. **Conclusion:** This study provides evidence about the potential immunological influence of *L. rhamnosus* when used in combination with 5-FU as a novel colon cancer therapeutic strategy.

Key words: Colon cancer, *L. rhamnosus*, 5-fluorouracil, cytokines, antitumor immune response, probiotics

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Colon cancer or colorectal cancer (CRC) affects over 1 million people and accounts for an estimated half a million deaths worldwide[1]. The Saudi Cancer Registry (SCR) reported that CRC was the second most common malignancy among Saudis for all ages[2]. Diet interventions and natural bioactive supplements have now been extensively studied to reduce the risks of colon cancer as preventative measures[3].

The fluoropyrimidine 5-fluorouracil (5-FU) is a heterocyclic aromatic organic compound that is widely used as an anticancer therapeutic. Its structure is similar to that of the pyrimidine particles of DNA and RNA[4]. Anticancer drugs act by hindering fundamental biosynthetic processes or by integrating into macromolecules, such as DNA and RNA, to disrupt their normal functions. The 5-FU functions by directly blocking DNA replication while also inducing cytotoxicity through other mechanisms[5]. The 5-FU acts as a pivotal anticancer medication because of its broad-spectrum antitumor activity. Because 5-FU is highly compatible with other anticancer drugs. It is used to treat numerous types of malignancies[6]. The immune system is typically affected by 5-FU, as it selectively depletes immature myeloid cells (myeloid derived suppressor cells) that expand during tumor progression[7]. One study showed that 5-FU-mediated deficiency of myeloid-derived suppressor cells increased IFNγ production by tumor-specific $CD8^+$ T cells[5]. Another study showed that 5-FU treatment caused inflammatory infiltration of neutrophils and eosinophils, increased expression of IL-6 and TNFα and increased intestinal permeability in mice[8]. The 5-FU has been associated with intestinal damage, particularly intestinal stem cell apoptosis and intestinal mucositis[9]. These effects are correlated with increased levels of pro-inflammatory cytokines and neutrophil infiltration[10].

Probiotic bacteria, especially *Lactobacillus*, can directly invoke the host's immune responses by merging to pattern recognition receptors (PRR) displayed on different immune cells and many other tissues, including the intestinal epithelium[11]. These receptors facilitate the fundamental functions of the innate and adaptive immune responses via activation of naïve T cells, regulatory T cells (Treg) and antigen presenting cells (APCs), including dendritic cells (DCs) and macrophages[12]. An increase in the percentage of phagocytic leukocytes was observed after treatment with *L. rhamnosus* in mice infected with *E. coli*[13]; while a separate study showed that *L. rhamnosus* significantly increased IFNγ production[14,15]. Moreover, *L. rhamnosus* supplementation has been shown to have beneficial effects during chemotherapy, as cancer patients have reported less abdominal discomfort and chemotherapy dose reductions[16]. Although previous studies discussed the direct correlation of some probiotics and improvement of cancers, particularly colon cancer[17,18], it was previously unknown whether probiotics can boost the antitumor immune response while also enhancing the efficacy of chemotherapeutics. To answer this question, the present study evaluated the anticancer combination activity of *L. rhamnosus* bacteria and 5-FU in colon cancer. We examined cytokine and oncogenic gene expression signatures as well as histological alterations and found that combination treatment results in improved innate and adaptive immune responses and reduced inflammation in the colon.

MATERIALS AND METHODS

This study was carried out at the Applied Microbiology and Immunity Units at the Center King Fahed for Medical Research. The time schedule of this study was 8 months.

***L. rhamnosus* culture:** *L. rhamnosus* was cultured overnight in de Man Rogosa Sharpe Agar as previously described by Minelli and Benini[19]. The obtained *L. rhamnosus* bacterial pellets were suspended in 10% non-fat milk where the final concentration obtained was approximately equal to 10^9 Colony-Forming Units (CFU)[20].

Mice and experiment design: Fifty male 8 week old Swiss mice were obtained and held under standard conditions at the Center of King Fahad for Medical Research at King Abdul-Aziz University in Jeddah, Kingdom of Saudi Arabia. Mice were randomized into 5 groups, n = 10 mice per group. Group C mice were untreated and used as the negative control group. Group A mice received a total of 4 intraperitoneal (IP) injections of 10 mg kg^{-1} azoxymethane (AOM) over the course of 4 weeks (week 0-4) to induce colon cancer tumorigenesis (positive control group) and left untreated till week 11. Group AL mice received AOM injection in a similar fashion as group A and were treated with 10^9 CFU *L. rhamnosus* as a suspension in 2.0-2.5 mL of 10% non fat milk through oral gavage. Group AL mice were treated 3 times a week till the end of the experiment, with treatment starting 2 weeks before the 1st AOM injection. Group AF mice received AOM injection in a similar fashion as group A, followed by

injection with 50 mg kg^{-1} 5-FU once a week for 2 weeks, at weeks 8 and 10 after the 1st AOM injection. The last group, group AFL, included mice receiving 10^9 CFU *L. rhamnosus* pre and post-injection with AOM (in a similar fashion as the AL group) and treated with 5-FU once a week for 2 weeks, at weeks 8 and 10 after the 1st AOM injection. About 5 mice from each group (C, A, AL, AF and AFL) were sacrificed at week 9 and the remaining mice were sacrificed at week 11. Expression analysis were performed on samples from week 9 or 11 (Supplemental Table 1 for experimental timeline). All intestinal and blood serum samples were stored in -80°C until use.

Histological study: Mouse colon biopsies were dissected and fixed in 10% paraformaldehyde solution for 1 h. Approximately 6 µm thick murine colon sections were stained with hematoxylin and eosin (H and E) for light microscopy analysis[21].

Evaluation of murine serum expression of TNFα and IL-6 cytokines: TNFα and IL-6 screenings were performed in mice sera using the Capture Elisa kit (Thermofisher, Cat No.; BMS607/3 and BMS603/2, respectively) according to the manufacturer's instructions. Absorbance was measured at 450 nm and the concentrations of TNFα and IL-6 were determined using standard curves constructed from respective immunoglobulin standards.

Evaluation of murine intestinal gene expression regulation: The RNA later (QIAGEN, Cat NO.76106) was used to protect RNA in colon biopsies from all treatment groups: C, A, AL, AF and AFL. Biopsies were later subjected to total RNA according to the manufacturer's guidelines. All purified RNA samples were aliquoted and stored in -80°C until use.

Verso SYBR Green 1-Step qRT-PCR Kit Master Mix reagents (Thermo Scientific Cat No. AB-4108/C) were utilized to amplify and quantitate the previously obtained RNA samples. Each amplification reaction mixture for qRT-PCR was performed as previously described[22], by using a set of specific primers for each target gene (Supplemental Table 2). The mRNA relative ratio quantification of target genes was systematically calculated according to the $2^{-\Delta\Delta Ct}$ method. The calibrator sample employed was the same mouse sample performed in all runs[23].

Statistical analysis: Statistical evaluation of all groups was performed using Megastat software. One-way ANOVA parametric tests were performed for the ELISA results and the relative ratio of gene expressions. p-value <0.05 was deemed significant.

RESULTS

Effects of *L. rhamnosus* and 5-FU treatment on serum expression of TNFα and IL-6: To evaluate the effects on inflammation, cytokine levels of TNFα and IL-6 were examined. Combination treatment caused a reduction in TNFα levels compared to those of group AL ($p = 0.0042$), although there were no significant differences when compared with the levels in group A ($p>0.05$) (Fig. 1a). However, combination treatment resulted in a significant increase in TNFα compared to that of groups A and AF

Supplemental Table 1: Duration of the present experiment and the starting of each treatment administration

	Experiment duration (weeks)													
Main experiment process	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11
Starting experiment	√													
Lactobacillus administration	√	√	√	√	√	√	√	√	√	√	√	√	√	√
AOM injection			√	√	√	√	√							
5-FU											√		√	
Scarification time												√		√
End of the experiment														√

Supplemental Table 2: Pairs of primers were used for gene expressions of TLR2, IFNγ and IL-10 genes extracted from mice mucosal intestine using β-actin as a housekeeping reference gene

Genes	Polarity	Primer sequence (5'---'3)	Primer length	Nucleotide positions	Reference genes
IL-10	Sense	AGAGAAGCATGGCCCAGAAATC	22	336-357	NM010548
	Antisense	TCATGGCCTTGTAGACACCTTG	22	521-542	
TLR2	Sense	AACCTCAGACAAAGCGTCAAATC	23	797-816	NM011905
	Antisense	ACCAAGATCCAGAAGAGCCAAA	22	993-974	
IFNγ	Sense	GGCCATCAGCAACAACATAAGCGT	24	321 -344	NM008337
	Antisense	TGGGTTGTTGACCTCAAACTTGGC	24	415-438	
β-actin	Sense	TATTGGCAACGAGCGG	16	856-871	NM007393
	Antisense	CGGATGTCAACGTCAC	16	978-963	

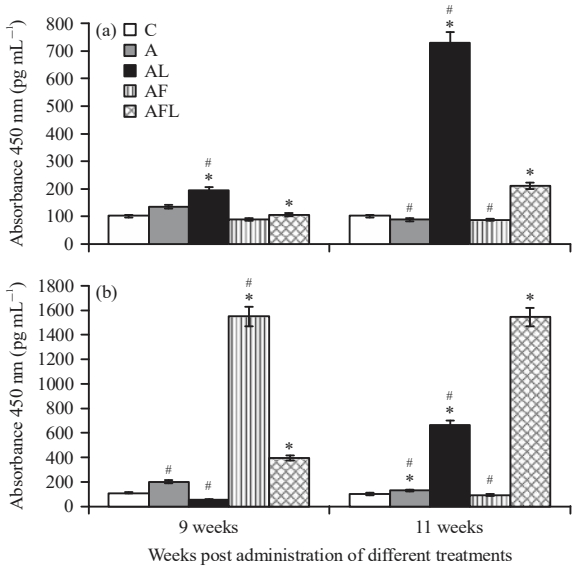

Fig. 1(a-b): Evaluation of (a) TNFα and (b) IL-6 levels obtained from the serum of different treated and untreated groups

*Comparison between controls and treated groups, #Comparison between AFL and the other treated groups, p<0.05 was determined to be significant as determined by the analysis of variance, the comparison was performed using one-factor ANOVA test

(p = 0.000). Further, TNFα expression in the untreated group at the 11th week was significantly lower than that of group AFL (p = 0.0001) (Fig. 1a).

At week 9, IL-6 levels were significantly higher in the AFL group than in the untreated group (p = 0.004) as well as in groups AL and A (p = 0.001 and 0.0344, respectively). However, the AFL group had significantly lower IL-6 levels than the AF group (p = 0.000) (Fig. 1b). At week 11, a significant elevation was detected in IL-6 levels in the serum of AFL group compared to those of the untreated group (p = 0.000), as well as those of group A, AL and AF (p = 0.000) (Fig. 1b).

Histological colon alterations in response to combination treatment with *L. rhamnosus* and 5-FU: Colon samples from the untreated group demonstrated normal crypts (glandular architecture) and goblet cells which show normal nuclei (Fig. 2a). Meanwhile, colon changes in the treated groups showed the following findings at week 9; group A had architecture distortion, hyperchromasia and goblet cell depletion (Fig. 2b). Group AL colon structure seemed more normal, with a decrease in both goblet cell number and inflammatory features (Fig. 2c). Less distorted architecture, an increase in goblet cell number and slightly decreased inflammation was observed in group AF (Fig. 2d). Interestingly, group AFL colon samples showed normal inflammation with hyperplastic changes, as well as goblet cell restoration (Fig. 2e, g).

Eleven weeks from the beginning of the experiment, colon mucosa in group A showed dysplasia, which can lead to tumor formation. Mucosa from this group also showed a villus architecture, stratification of the nuclei, hyperchromasia and a depletion in goblet cell numbers (Fig. 3a). Group AL showed an increased inflammatory response and evidence of hyperplasia (Fig. 3b). Group AF colon biopsy samples (Fig. 3c) showed a distorted architecture and a decreased inflammatory response with hyperplastic changes, with an increase in goblet cell number. Finally, group AFL showed a normal colon structure, with normal crypt and normal inflammation in a similar fashion as the untreated group (Fig. 3d).

Effects of *L. rhamnosus* and 5-FU treatment on the regulation of tumor gene expression: The mRNA profiles of the tumor suppressor P53 and oncogene K-ras were compared among the intestinal biopsies of each group. About 9 weeks after the 1st AOM injection (CRC induction), P53 levels were significantly lower in all the treated groups than in the untreated group (p = 0.000). The P53 levels were not significantly different in the intestinal mucosa of group AFL compared to those in the mucosa of groups A, AL and AF (p>0.05) (Fig. 4a). At week 11, p53 levels remained lower in groups A, AL, AF and AFL than in the untreated group (p = 0.000). However, P53 transcription levels in group AFL were not significantly higher than those of group AL and AF (p>0.05), which were significantly higher than those of group A (p = 0.000) (Fig. 4a).

Gene expression analysis at week 9 showed a significant increase in K-ras levels in the colons of all treated groups compared with levels in the untreated group (p = 0.000). Remarkably, K-ras expression was significantly lower in the AFL group than in both A and AF groups (p = 0.001 and 0.000, respectively), although it was not significantly lower than that of the AL group (p>0.05) (Fig. 4b). At 11 weeks, K-ras gene expression was still significantly upregulated in all treated groups compared to that in the untreated group (p = 0.000). Group AFL showed non-significant higher levels than groups AL and AF (p>0.05), although the levels were markedly decreased in comparison to group A K-ras levels (p = 0.000) (Fig. 4b).

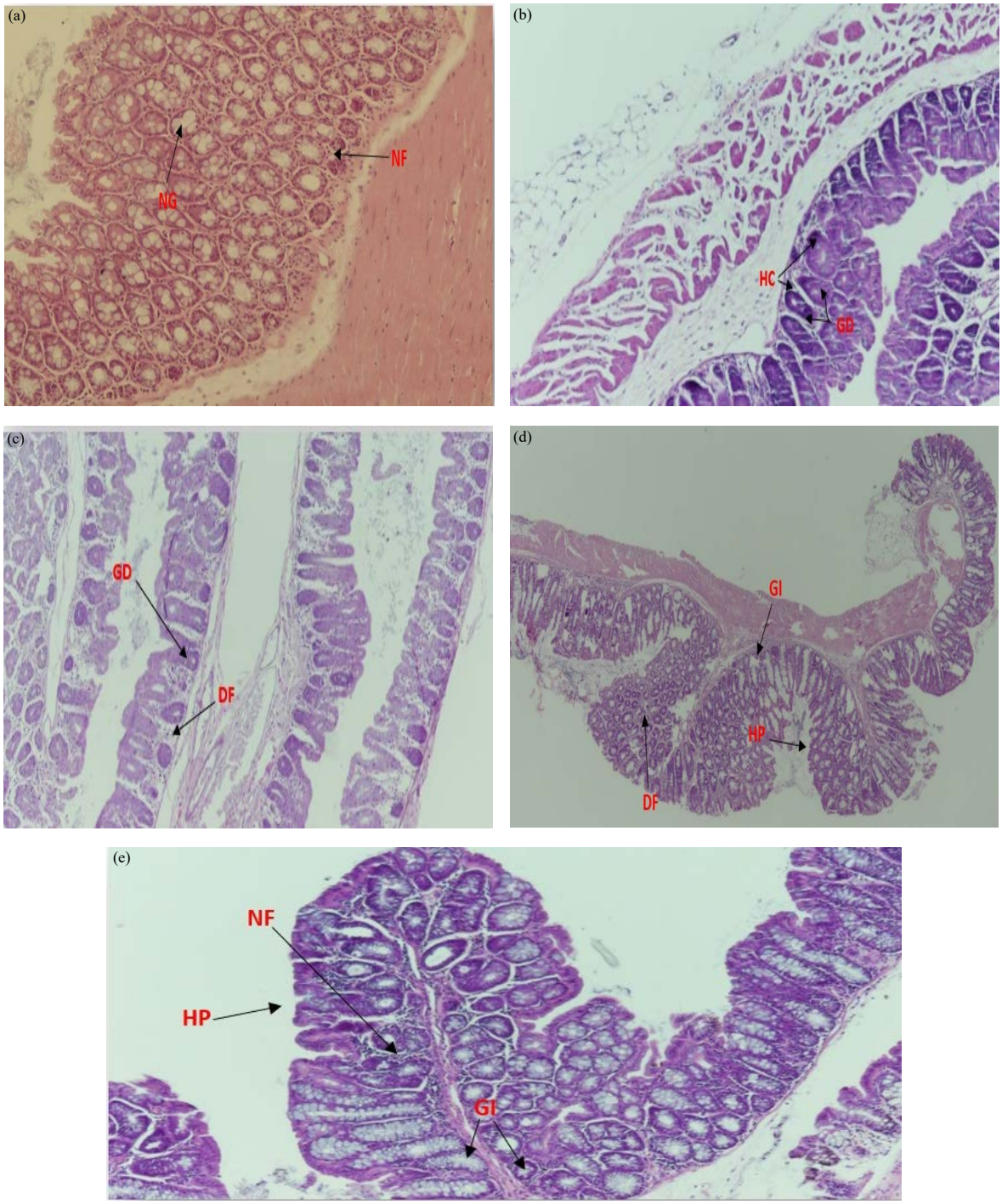

Fig. 2(a-e): Histological findings in the colon of different groups' tissue after 9 weeks from the first AOM injection (magnification 4x), (a) Colon samples from the untreated group C showed normal crypt (glandular architecture), normal inflammation (NF), normal goblet cells (NG) and normal nuclei, (b) Group A colon samples showed architecture distortion, hyperchromasia (HC) and goblet cell depletion (GD), (c) Colon samples from the group AL showed normal architecture, goblet cell depletion (GD) and decreased inflammation (DF), (d) Group AF colon samples showed an increase in the goblet cell number (GI), hyperplastic changes (HP) and slightly decreased inflammation (DF) and (e) Group AFL colon samples showed goblet cell restoration (GI), normal inflammation (NF) and hyperplastic changes (HP)
Sections stained with H and E dye

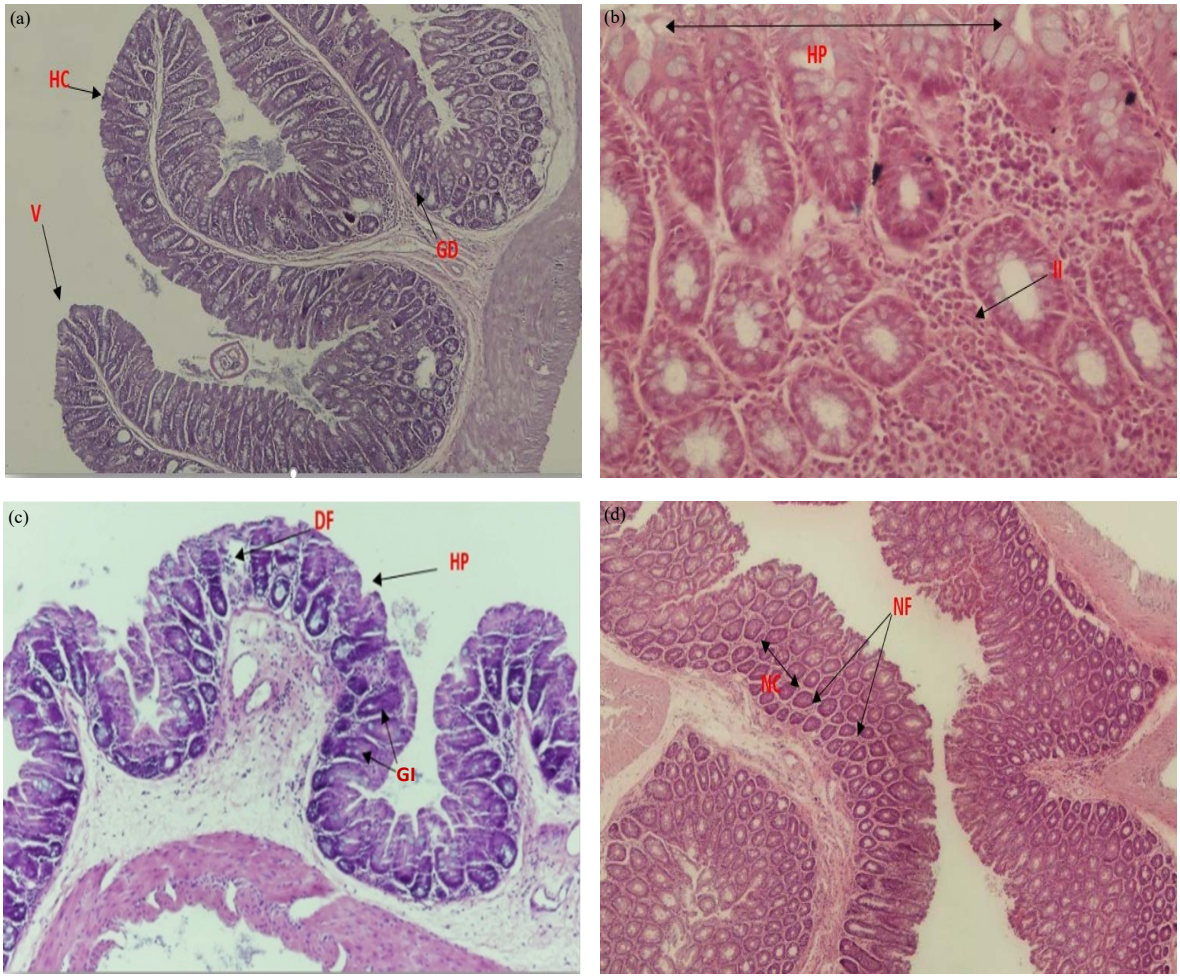

Fig. 3(a-d): Histological findings in colon tissue 11 weeks after the first AOM injection. (a) Group A showed dysplastic changes, villous architecture (V), stratification of the nuclei, hyperchromasia (HC) and goblet cell depletion (GD), (b) Group AL showed an increase in inflammatory features (II) and some hyperplastic changes (HP), (c) Group AF group showed a decrease in inflammation (DF), hyperplastic changes (HP), increased number of goblet cells (GI) and more distorted architecture and (d) Group AFL appeared normal, with normal crypt (NC) and normal inflammation (NF)
Section stained with H and E dye

Effects of *L. rhamnosus* and 5-FU treatment on the regulation of immunological gene expression: TLR2 transcription levels were significantly enhanced in the AFL mice mucosal intestine compared to untreated, AL and AF group ($p = 0.0006$, 0.0008 and 0.006, respectively). However, TLR2 levels were significantly lower compared to that of group A ($p = 0.0000$) at week 9 (Fig. 5a). At 11 weeks, the significant decrease of this receptor was still observed in AFL mucosa compared to that of the untreated group and group A ($p = 0.000$ and 0.0014, respectively), though it showed non-significant differences compared to levels of both AL and AF groups ($p>0.05$) (Fig. 5a).

Regarding the IFNγ gene expression at week 9, the intestinal mucosa of the AFL group showed a significant increase in the level of IFNγ expression compared to that of the untreated group ($p = 0.0009$). Although no significant differences were reported in IFNγ transcription level in the intestinal mucosa of the AFL group in comparison with that of either group A or AF ($p>0.05$), the expression was significantly reduced compared to group AL mucosal expression ($p = 0.0031$) (Fig. 5b). Observations after 11 weeks reported a significant upregulation of IFNγ transcription in the mucosa of AFL groups in comparison to the untreated group levels ($p = 0.000$). Although the level of this target

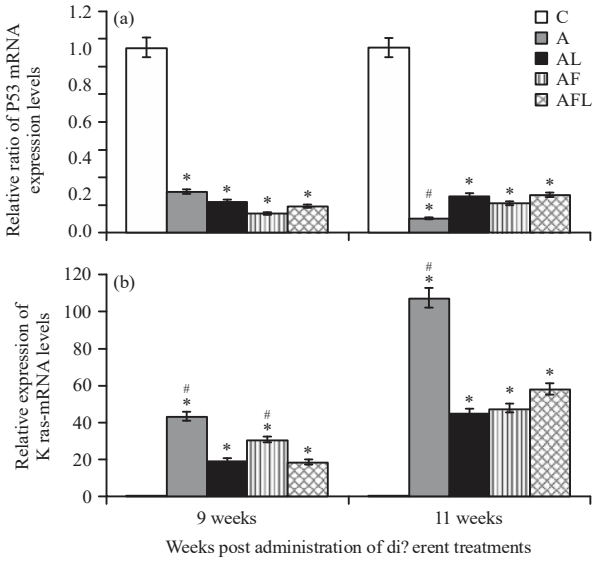

Fig. 4(a-b): Relative ratio of total RNA isolated from intestines. Tumor gene expression levels were analyzed via qRT-PCR and normalized to β-actin, (a) P53 transcription and (b) K-ras transcription

*Comparison between controls and treated groups, #Comparison between AFL and the other treated groups, the total concentration of RNA samples for each test were approximately 700 ng, $p<0.05$ was considered to be significant as determined by analysis of variance Comparisons were performed using one-factor ANOVA test

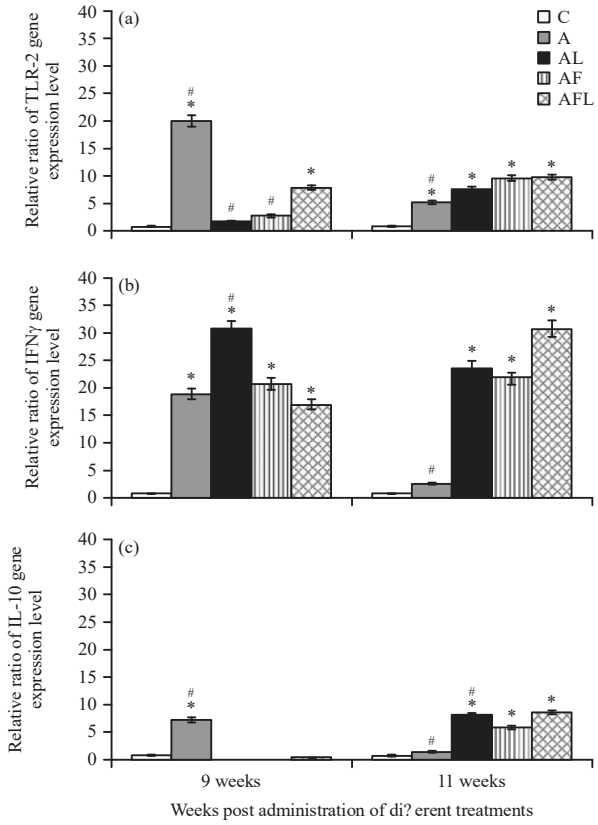

Fig. 5(a-c): Relative ratio of total RNA isolated from the intestinal mucosa. Cytokine transcription levels were analyzed via qRT-PCR and normalized to β-actin, (a) TLR-2 gene expression, (b) IFNγ gene expression and (c) IL-10 gene expression

*Comparison between controls and treated groups, #Comparison between the AFL and the other treated groups, the total concentration of RNA samples for each test was approximately 700 ng, $p<0.05$ was considered to be significant as determined by analysis of variance, the comparison was made using one-factor ANOVA test

cytokine was not significantly upregulated in the intestinal mucosa of the AFL group compared to that of groups AL and AF ($p>0.05$), it was significantly higher than group *A* levels (Fig. 5b).

IL-10 mucosal gene expression was not significantly different in the AFL group in comparison with the untreated group ($p>0.05$) 9 weeks after CRC induction. The mucosa of the AFL group showed a sharp decrease in IL-10 expression compared to group A expression ($p = 0.000$), although it was not significantly higher compared to that of group AL and AF ($p>0.05$) (Fig. 5c). Eleven weeks post-CRC induction, intestinal mucosa of the AFL group reported an extremely significant upregulation in IL-10 transcription compared to both control and group A expression ($p = 0.000$). However, the levels were not significantly different compared to those of the AL group ($p>0.05$), although they were significantly higher than those of the AF group ($p = 0.001$) (Fig. 5c).

DISCUSSION

Because 5-FU can impair the immune system, identifying ways to boost immune response and enhance 5-FU efficacy is of clinical interest. The human gut microbiota has a notable influence on enhancing the function of the immune system[24-26] and is indirectly involved in preventing cancer development[27]. Pro-inflammatory cytokines and chronic inflammation are known critical factors in carcinogenesis and tumor prevention[28-29]. The present work confirmed the predominant effect of probiotics on the tumor immune responses during treatment with 5-FU, particularly in cytokine expression.

IL-6 is mainly secreted in response to tissue damage, pathogens, chronic inflammation induction and autoimmune disease[30]. It has been demonstrated that IL-6 favors the clonal expansion of IgA B lymphocytes[22] and is significantly increased in preclinical models and in cancer patients during chemotherapy treatment[31-33]. Elevated IL-6

levels in the serum of the present AFL group compared to those in all other groups is in agreement with previous studies that demonstrated the capability of *L. rhamnosus* in increasing some proinflammatory cytokines[34], especially IL-6.

Colons of the AFL group appeared normal in structure, with normal crypt structure and normal inflammation. However, our findings of increased inflammation and hyperplasia in the colons of the AL group are in contradiction to the protective properties of probiotics, especially *Lactobacillus* bacteria against colon cancer[35,36]. Interestingly, these results suggest that the combination of *L. rhamnosus* with 5-FU reduced all the histological alterations associated with colon tumor formation.

Evaluating gene expression of the tumor suppressor P53 and the oncogene K-ras was used to determine probiotic effects on boosting the anti-cancer response of 5-FU. The results indicated that a combination of 5-FU and a probiotic did not significantly alter P53 or K-ras levels compared with single agents, although the combination did result in a non-significant reduction in K-ras expression. One previous study showed the combination of *L. rhamnosus* with celecoxib when administrated one week prior to colon cancer induction via 1,2-dimethylhydrazine, results in K-ras downregulation and P53 upregulation on CRC-bearing mice[37]. Though our results did not show a synergistic effect on gene expression of P53 or K-ras, it is possible that other time points or different dosages of *L. rhamnosus* would show significant differences.

TLR-2 is located on several immune cells including macrophages, DCs and regulatory T cells[38,39], where its main function is recognizing lipoproteins and peptidoglycans of different pathogens[40]. In this study, an early significant upregulation of TLR-2 transcription was detected in the intestinal mucosa of the AFL group compared to expression in the untreated, AL and AF group. The present results confirmed the capability of this combination on stimulation of TLR2 transcription more than *L. rhamnosus* as a single agent[41,42]. Upregulated TLR-2 can lead to activation of macrophages and DC cells in the Peyer's patches of the intestines[43], which regularly contribute to the anti-tumor immune response by presenting tumor antigens to Th1 lymphocytes cells[44-47]. This receptor can also stimulate the proliferation and the differentiation of Tc lymphocytes and provide important signals to produce critical cytotoxic cytokines including IFNγ[47-50] and IL-4. In the current study, although all treated groups showed an immediate significant upregulation in IFNγ transcription compared to that in the untreated group, group AL showed an earlier increase in this cytokine compared to the AFL group. Nonetheless, a significant upregulation of this cytokine was observed in the AFL group compared to that in the A group. The current IFNγ transcription data are in agreement with many previous studies indicating the improvement of *L. rhamnosus* or 5-FU individually on either or both Th and Tc lymphocytes[47,51,52]. The observed increase in IL 10 transcription was observed in both the AL and AFL group compared to that in the AF group. These results suggest that *L. rhamnosus* alone or combined with 5-FU shifts the immune response to decrease the inflammatory process. Because chronic inflammation has been identified as a contributor to the development of tumor formation of many types of tumors[53,54], our results suggest that *L. rhamnosus* administration is a potential therapeutic strategy to boost the antitumor immune response.

CONCLUSION

This study illustrated the positive immunomodulatory effect of the combination of *L. rhamnosus* and 5-FU on colon cancer. This combination suppressed pathological tumorigenesis, as the histological structure of the colon mucosa was similar to the normal structure. The results also show that administration of *L. rhamnosus* in combination with 5-FU results in increased IL-6 expression and decreased K-ras expression. Collectively, this study introduced a new theory on the combination of probiotics with 5-FU, which may be an effective therapeutic strategy for colon cancer.

SIGNIFICANCE STATEMENT

This study found that probiotics can boost the antitumor immune response which can be beneficial for colon cancer treatment. This study will help researchers to uncover the critical areas of probiotic administration on immunological endpoints that many researchers were not able to previously explore. Thus, this work points to a new theory on probiotics as cancer therapeutics.

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REFERENCES

1. Boyle, P. and M.J. Langman, 2000. ABC of colorectal cancer: Epidemiology. Br. Med. J., Vol. 321. 10.1136/sbmj.0012452.
2. Mosli, M.H. and M.S. Al-Ahwal, 2012. Colorectal cancer in the Kingdom of Saudi Arabia: Need for screening. Asian Pac. J. Cancer Prevent., 13: 3809-3813.

3. Van Guelpen, B., J. Hultdin, I. Johansson, G. Hallmans and R. Stenling *et al*., 2006. Low folate levels may protect against colorectal cancer. Gut, 55: 1461-1466.
4. Zhang, L., N. Li, R. Caicedo and J. Neu, 2005. Alive and dead *Lactobacillus rhamnosus* GG decrease tumor necrosis factor-α-induced interleukin-8 production in Caco-2 cells. J. Nut., 135: 1752-1756.
5. Longley, D.B., D.P. Harkin and P.G. Johnston, 2003. 5-fluorouracil: Mechanisms of action and clinical strategies. Nat. Rev. Cancer, 3: 330-338.
6. Miura, K., M. Kinouchi, K. Ishida, W. Fujibuchi and T. Naitoh *et al*., 2010. 5-fu metabolism in cancer and orally-administrable 5-fu drugs. Cancers, 2: 1717-1730.
7. Ghiringhelli, F., M. Bruchard and L. Apetoh, 2013. Immune effects of 5-fluorouracil: Ambivalence matters. OncoImmunology, Vol. 2. 10.4161/onci.23139.
8. Ferreira, T.M., A.J. Leonel, M.A. Melo, R.R. Santos and D.C. Cara *et al*., 2012. Oral supplementation of butyrate reduces mucositis and intestinal permeability associated with 5 fluorouracil administration. Lipids, 47: 669-678.
9. Justino, P.F., L.F. Melo, A.F. Nogueira, J.V. Costa and L.M. Silva *et al*., 2014. Treatment with *Saccharomyces boulardii* reduces the inflammation and dysfunction of the gastrointestinal tract in 5-fluorouracil-induced intestinal mucositis in mice. Br. J. Nutr., 111: 1611-1621.
10. Soares, P.M.G., J.M.S.C. Mota, A.S. Gomes, R.B. Oliveira and A.M.S. Assreuy *et al*., 2008. Gastrointestinal dysmotility in 5-fluorouracil-induced intestinal mucositis outlasts inflammatory process resolution. Cancer Chemother. Pharmacol., 63: 91-98.
11. Kawai, T. and S. Akira, 2010. The role of pattern-recognition receptors in innate immunity: Update on toll-like receptors. Nat. Immunol., 11: 373-384.
12. Rescigno, M., 2010. Intestinal dendritic cells. Adv. Immunol., 107: 109-138.
13. Shu, Q. and H.S. Gill, 2002. Immune protection mediated by the probiotic *Lactobacillus rhamnosus* HN001 against *Escherichia coli* O157:H7 infection in mice. FEMS Immunol. Med. Microbiol., 34: 59-64.
14. Gill, H.S. and K.J. Rutherfurd, 2001. Viability and dose-response studies on the effects of the immunoenhancing lactic acid bacterium *Lactobacillus rhamnosus* in mice. Br. J. Nutr., 86: 285-289.
15. Borchers, A.T., C. Selmi, F.J. Meyers, C.L. Keen and M.E. Gershwin, 2009. Probiotics and immunity. J. Gastroenterol., 44: 26-46.
16. Osterlund, P., T. Ruotsalainen, R. Korpela, M. Saxelin and A. Ollus *et al*., 2007. *Lactobacillus* supplementation for diarrhoea related to chemotherapy of colorectal cancer: A randomised study. Br. J. Cancer, 97: 1028-1034.
17. Tiptiri-Kourpeti, A., K. Spyridopoulou, V. Santarmaki, G. Aindelis and E. Tompoulidou *et al*., 2016. *Lactobacillus casei* exerts anti-proliferative effects accompanied by apoptotic cell death and up-regulation of TRAIL in colon carcinoma cells. Plos One, Vol. 11. 10.1371/journal.pone.0147960.
18. Ding, C., W. Tang, X. Fan and G. Wu, 2018. Intestinal microbiota: A novel perspective in colorectal cancer biotherapeutics. OncoTargets Ther., 11: 4797-4810.
19. Minelli, E.B. and A. Benini, 2008. Relationship between number of bacteria and their probiotic effects. Microb. Ecol. Health Dis., 20: 180-183.
20. Stofilova, J., T. Langerholc, C. Botta, P. Treven and L. Gradisnik *et al*., 2017. Cytokine production *in vitro* and in rat model of colitis in response to *Lactobacillus plantarum* LS/07. Biomed. Pharmacother., 94: 1176-1185.
21. Rabah, S., S. El Hadad and F. Albani, 2013. Histological changes of mice lungs after daily exposure to different concentration of Incense smoke. Life Sci. J., 10: 552-560.
22. Elbanna, K., S. El Hadad, A. Assaeedi, A. Aldahlawi, M. Khider and A. Alhebshi, 2018. *In vitro* and *in vivo* evidences for innate immune stimulators lactic acid bacterial starters isolated from fermented camel dairy products. Scient. Rep., Vol. 8. 10.1038/s41598-018-31006-3.
23. Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25: 402-408.
24. Gibson, G.R., 2008. Prebiotics as gut microflora management tools. J. Clin. Gastroenterol., 42: S75-S79.
25. Schrezenmeir, J. and M. de Vrese, 2001. Probiotics, prebiotics and synbiotics-approaching a definition. Am. J. Clin. Nutr., 73: 361S-364S.
26. Urbanska, A.M., J. Bhathena, C. Martoni and S. Prakash, 2009. Estimation of the potential antitumor activity of microencapsulated *Lactobacillus acidophilus* yogurt formulation in the attenuation of tumorigenesis in Apc (Min/+) mice. Digest. Dis. Sci., 54: 264-273.
27. McNaught, C.E., N.P. Woodcock, A.D.G. Anderson and J. MacFie, 2005. A prospective randomised trial of probiotics in critically ill patients. Clin. Nutr., 24: 211-219.
28. Borruel, N., M. Carol, F. Casellas, M. Antolin and F. de Lara *et al*., 2002. Increased mucosal tumour necrosis factor α production in Crohn's disease can be downregulated *ex vivo* by probiotic bacteria. Gut, 51: 659-664.
29. Dinarello, C.A., 2010. Why not treat human cancer with interleukin-1 blockade? Cancer Metastasis Rev., 29: 317-329.
30. Tanaka, T., M. Narazaki and T. Kishimoto, 2014. IL-6 in inflammation, immunity and disease. Cold Spring Harbor Perspect. Biol., Vol. 6. 10.1101/cshperspect.a016295.

31. Galdeano, C.M., A. de Moreno de LeBlanc, G. Vinderola, M.E.B. Bonet and G. Perdigon, 2007. Proposed model: Mechanisms of immunomodulation induced by probiotic bacteria. Clin. Vaccine Immunol., 14: 485-492.
32. Weymann, K.B., L.J. Wood, X. Zhu and D.L. Marks, 2014. A role for orexin in cytotoxic chemotherapy-induced fatigue. Brain Behav. Immun., 37: 84-94.
33. Wood, L.J., L.M. Nail, A. Gilster, K.A. Winters and C.R. Elsea, 2006. Cancer chemotherapy-related symptoms: Evidence to suggest a role for proinflammatory cytokines. Oncol. Nurs. Forum, 33: 535-542.
34. Wang, X.S., L.A. Williams, S. Krishnan, Z. Liao and P. Liu *et al*., 2012. Serum sTNF-R1, IL-6 and the development of fatigue in patients with gastrointestinal cancer undergoing chemoradiation therapy. Brain Behav. Immun., 26: 699-705.
35. Dong, H., I. Rowland and P. Yaqoob, 2012. Comparative effects of six probiotic strains on immune function *in vitro*. Br. J. Nutr., 108: 459-470.
36. Danenberg, P.V., B. Gustavsson, P. Johnston, P. Lindberg and R. Moser *et al*., 2016. Folates as adjuvants to anticancer agents: Chemical rationale and mechanism of action. Crit. Rev. Oncol./Hematol., 106: 118-131.
37. Femia, A.P., C. Luceri, P. Dolara, A. Giannini and A. Biggeri *et al*., 2002. Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* on azoxymethane-induced colon carcinogenesis in rats. Carcinogenesis, 23: 1953-1960.
38. Walia, S., R. Kamal, D.K. Dhawan and S.S. Kanwar, 2018. Chemoprevention by probiotics during 1, 2-dimethylhydrazine-induced colon carcinogenesis in rats. Digest. Dis. Sci., 63: 900-909.
39. Sharaf, L.K., M. Sharma, D. Chandel and G. Shukla, 2018. Prophylactic intervention of probiotics (*L. acidophilus*, *L. rhamnosus* GG) and celecoxib modulate Bax-mediated apoptosis in 1, 2-dimethylhydrazine-induced experimental colon carcinogenesis. BMC Cancer, Vol. 18. 10.1186/s12885-018-4999-9.
40. Michallet, M.C., E. Meylan, M.A. Ermolaeva, J. Vazquez and M. Rebsamen *et al*., 2008. TRADD protein is an essential component of the RIG-like helicase antiviral pathway. Immunity, 28: 651-661.
41. Albiger, B., S. Dahlberg, B. Henriques Normark and S. Normark, 2007. Role of the innate immune system in host defence against bacterial infections: Focus on the Toll like receptors. J. Int. Med., 261: 511-528.
42. Hirschfeld, M., J.J. Weis, V. Toshchakov, C.A. Salkowski and M.J. Cody *et al*., 2001. Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect. Immun., 69: 1477-1482.
43. Ouwehand, A.C., P.V. Kirjavainen, M.M. Gronlund, E. Isolauri and S.J. Salminen, 1999. Adhesion of probiotic micro-organisms to intestinal mucus. Int. Dairy J., 9: 623-630.
44. Creagh, E.M. and L.A. O'Neill, 2006. TLRs, NLRs and RLRs: A trinity of pathogen sensors that co-operate in innate immunity. Trends Immunol., 27: 352-357.
45. Liu, G., L. Zhang and Y. Zhao, 2010. Modulation of immune responses through direct activation of toll like receptors to T cells. Clin. Exp. Immunol., 160: 168-175.
46. Grivennikov, S.I., F.R. Greten and M. Karin, 2010. Immunity, inflammation and cancer. Cell, 140: 883-899.
47. Qian, B.Z. and J.W. Pollard, 2010. Macrophage diversity enhances tumor progression and metastasis. Cell, 141: 39-51.
48. Gallois, A. and N. Bhardwaj, 2013. Dendritic cell-targeted approaches to modulate immune dysfunction in the tumor microenvironment. Front. Immunol., Vol. 4. 10.3389/fimmu.2013.00436.
49. Gerrard, T.L., D.J. Cohen and A.M. Kaplan, 1981. Human neutrophil-mediated cytotoxicity to tumor cells. J. Nat. Cancer Inst., 66: 483-488.
50. Munegowda, M.A., Y. Deng, S.J. Mulligan and Jim Xiang, 2011. Th17 and Th17-stimulated $CD8^+$ T cells play a distinct role in Th17-induced preventive and therapeutic antitumor immunity. Cancer Immunol., Immunother., Vol. 60. 10.1007/s00262-011-1054-y.
51. Sun, Q., R.L. Burton and K.G. Lucas, 2002. Cytokine production and cytolytic mechanism of $CD4^+$ cytotoxic T lymphocytes in *ex vivo* expanded therapeutic Epstein-Barr virus-specific T-cell cultures. Blood, 99: 3302-3309.
52. Mittendorf, E.A., G. Alatrash, N. Qiao, Y. Wu and P. Sukhumalchandra *et al*., 2012. Validating FAO aquacrop using landsat images and regional crop information. Cancer Res., 721: 3153-3162.
53. Huang, L., J.S.C. Chiau, M.L. Cheng, W.T. Chan and C.B. Jiang *et al*., 2019. SCID/NOD mice model for 5-FU induced intestinal mucositis: Safety and effects of probiotics as therapy. Pediatr. Neonatol., 60: 252-260.
54. Aggarwal, B.B., R.V. Vijayalekshmi and B. Sung, 2009. Targeting inflammatory pathways for prevention and therapy of cancer: Short-term friend, long-term foe. Clin. Cancer Res., 15: 425-430.