Iranian Journal of Microbiology
Volume 16 Number 4 (August 2024) 434-442

ORIGINAL ARTICLE

Immunomodulatory effects of live and UV-killed *Bacillus subtilis* natto on inflammatory response in human colorectal adenocarcinoma cell line *in vitro*

Parisa Abedi Elkhichi[1], Masoumeh Aslanimehr[1*], Amir Javadi[2], Abbas Yadegar[3,4*]

[1]Medical Microbiology Research Center, Qazvin University of Medical Sciences, Qazvin, Iran
[2]Department of Statistics, Qazvin University of Medical Sciences, Qazvin, Iran
[3]Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[4]Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Received: February 2024**, Accepted:** July 2024

ABSTRACT

Background and Objectives: Colorectal cancer (CRC) is a heterogeneous disease of the colon or rectum arising from adenoma precursors and serrated polyps. Recently, probiotics have been proposed as an effective and potential therapeutic approach for CRC prevention and treatment. Probiotics have been shown to alleviate inflammation by restoring the integrity of the mucosal barrier and impeding cancer progression.

Materials and Methods: In this study, we aimed to investigate the immunomodulatory effects of live and UV-killed *Bacillus subtilis* natto on the inflammatory response in CRC. Caco-2 cells were exposed to various concentrations of live and UV-killed *B. subtilis* natto, and cell viability was assessed using MTT assay. Gene expression analysis of IL-10, TGF-β, TLR2 and TLR4 was performed using RT-qPCR.

Results: Our findings showed that both live and UV-killed *B. subtilis* natto caused significant reduction in inflammatory response by decreasing the gene expression of TLR2 and TLR4, and enhancing the gene expression of IL-10 and TGF-β in Caco-2 cells as compared to control group.

Conclusion: The results of this study suggest that live and UV-killed *B. subtilis* natto may hold potential as a therapeutic supplement for modulating inflammation in CRC.

Keywords: *Bacillus subtilis*; Probiotics; Gene expression; Colorectal neoplasms; Immunomodulation

[*]Corresponding authors: Masoumeh Aslanimehr, Ph.D, Medical Microbiology Research Center, Qazvin University of Medical Sciences, Qazvin, Iran. Tel: +98-28-33336001 Fax: +98-28-3359996 Email: maslanimehr@qums.ac.ir

Abbas Yadegar, Ph.D, Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran; Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Tel: +98-21-22432518 Fax: +98-21-22432527 Email: a.yadegar@sbmu.ac.ir

INTRODUCTION

Colorectal cancer is characterized by uncontrolled cell division and survival of abnormal cells, specifically in the colon or rectum (1). CRC is one of the leading causes of cancer-related deaths globally, ranking third in men and second in women as the most prevalent form of cancer (2, 3). The development of CRC is influenced by multiple factors, such as change in cytokine concentration, dysregulation of signaling pathways, host immune response, dietary factors, and more recently, the emerging role of gut microbiota (4, 5). Altered gut microbiota disrupts the cellular and molecular processes of colonocytes through various mechanisms and release of different metabolites and structural components that interact with pathogen-associated molecular pattern (PAMP) and microbe-associated molecular pattern (MAMP) receptors such as toll-like receptors (TLRs) (6). This disorder leads to increased expression of cytokines and activation of oncogenic signaling pathways, resulting in the induction of inflammation and progression of CRC (7). Currently, surgery, chemotherapy, immunotherapy, and radiation therapy are utilized for the treatment and reduction of inflammation in CRC (8, 9). These treatments mainly lead to the occurrence of side effects such as mucositis, diarrhea, vomiting, and weight loss (10, 11). One promising approach is the administration of probiotic bacteria, which can be used as a supplement alongside treatment to enhance patient outcomes (12). Based on recent studies, probiotics are live microorganisms that aid in the treatment of CRC by regulating the immune system, improving digestive function, and modulating microbiota metabolism (13, 14). Most probiotics are members of the normal intestinal microbiota, and the majority of them belong to the group of lactic acid bacteria (LAB) such as *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Bifidobacterium* and *Bacillus* (13). Non-pathogenic *Bacillus* species, such as *Bacillus subtilis* natto as a traditional probiotic strain, exhibit enhanced survival and stability due to their capability to form spores (15). *B. subtilis* natto is a Gram-positive bacterium that is commonly found in fermented soybeans, especially in Chinese cuisine (16). This probiotic strain exhibits modulatory effects on gut microbiota and various signaling pathways such as TGF-β cascade, and can improve intestinal barrier function and integrity by inducing the production of anti-inflammatory and regulatory cytokines, particularly IL-10 (17, 18).

The potential risks associated with the use of live probiotics in high-risk individuals, such as immunocompromised patients, have become a significant concern in this field (19). In recent years, there has been an increasing attraction toward the use of non-living bacterial supplements as alternative products to reduce the potential risks associated with live probiotic strains (20). Based on scientific findings from clinical observations, UV-killed probiotics still retain the ability to exert anti-inflammatory and immunomodulatory activities (21).

The potential impact of *B. subtilis* natto as a supplement in improving CRC remains unclear. Therefore, this study aimed to examine the effects of live and UV-killed *B. subtilis* natto on the expression levels of anti-inflammatory cytokines (TGF-β, IL-10), and TLR2 and TLR4 genes by quantitative real-time PCR (RT-qPCR) using human colon cancer cell line Caco-2 *in vitro.*

MATERIALS AND METHODS

Bacterial culture and growth conditions. *B. subtilis* natto (ATCC 7059) was obtained from World Intellectual Resource Co. (Taiwan). The strain was cultured on brain heart infusion agar (BHI, Merck, Germany) incubated under aerobic conditions for 24 h at 37°C. Colonies grown on BHI agar were then transferred to 200 ml of basal medium (BHI, Merck, Germany). The cultures were incubated at 37°C with gentle shaking at 150 rpm for 24 h under aerobic conditions.

Preparation of UV-killed *B. subtilis* natto. For preparation UV-killed *B. subtilis* natto, the bacterial suspension was centrifuged at 12,000 × g for 30 min at 4°C. The sedimented bacteria were then washed twice with sterile phosphate-buffered saline (PBS, pH 7) (Gibco, Darmstadt, Germany) and resuspended. The PBS suspension was distributed into several petri dishes and exposed to UV light at a wavelength of 254 nm for 2 h. This duration was achieved by exposing the petri dishes to UV light by 4 cycles of 30 minutes in each cycle. The UV-killed bacterial suspension was stored at -80°C for further analysis.

Caco-2 cell culture. The human colorectal epithelial adenocarcinoma cell line (Caco-2; ATCC HTB-37) was obtained from the Research Institute for Gas-

troenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (H-DMEM, Sigma, USA) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, USA), 2 mM L-glutamine, and 100 U/ml of penicillin and 100 µg/ml of streptomycin (Sigma-Aldrich, USA). Cells were incubated at 37°C under 5% CO_2 humidified in a 25 cm^2 culture flask (Cell Culture Flask, SPL, Korea). After reaching the 80% confluency, the cells were washed with sterile PBS (pH 7) and detached using 0.25% trypsin-EDTA (Gibco, USA). Caco-2 cells treated with 100 ng/ml lipopolysaccharide (LPS) obtained from *Escherichia coli* 0111: B4 (Sigma-Aldrich, St. Louis, Missouri, USA) and untreated cells served as control.

Cell viability assay. To measure the viability of Caco-2 cells, we used MTT assay. Briefly, Caco-2 cells were seeded in 96-well plates and treated with live and UV-killed *B. subtilis* natto at multiplicity of infection (MOI) of 10, 50, and 100 for 3, 6, 12, and 24 h time points. Based on results reported in previous studies and preliminary pilot experiments in our laboratory, MOI concentrations and time periods were selected for subsequent cell culture experiments (22, 23). After treatment, we added MTT solution (2 mg/ml) and incubated the plate at 37°C for 4 h. Afterward, the culture medium was replenished with fresh culture medium containing 10% MTT solution (0.5 mg/ml in PBS) (Sigma Aldrich, St. Louis, MO, USA), and the plate was incubated for 4 h at 37°C. The supernatant was eliminated and solubilized MTT-formazan in 200 µl of DMSO was added to each well. Finally, we read the absorbance of the samples using the ELISA reader device (ELX808, Biotek) at the wavelength of 570 nm and 650 nm. We calculated the percentage of cell survival using the following formula: Cell viability (%) = (X-A/Y-A) × 100, where "X" is the absorbance of treated cells, "Y" is the absorbance of untreated cells, and "A" is the absorbance of blank sample.

RNA extraction and cDNA synthesis. Total RNA was extracted from Caco-2 cells using the pure RNA mini kit (Parstous, Iran) following the manufacturer's instructions. The quantification and purity of the total RNA samples were assessed using agarose gel electrophoresis and a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA). To perform the reverse transcription of RNA to cDNA, a synthesis kit (Parstous, Iran) was employed, adhering to the manufacturer's protocols.

Gene expression analysis by RT-qPCR. To measure the mRNA expression level of TLR2, TLR4, TGF-β and IL-10, RT-qPCR analysis was performed with the SYBR Green qPCR Mastermix kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, the synthesized cDNA was amplified using RealQ Plus 2x Master Mix Green (Ampliqon, Odense, Denmark) and analyzed using the Rotor-Gene® Q (Qiagen, Hilden, Germany) real-time system. Primers used in this study are listed in (Table 1). Each amplification protocol consisted of an initial denaturation at 95°C for 10 min, followed by denaturation at 95°C for 20 s, annealing at 58-63°C for 30 s, and extension at 72°C for 20 s. The relative expression level was determined using $2^{-\Delta\Delta CT}$ method, and the β-actin housekeeping gene was served as the reference gene.

Statistical analysis. The statistical analysis was performed utilizing GraphPad Prism software (version 9, GraphPad Software, USA). To assess the statistical significance between groups, a one-way analysis of variance (ANOVA) was conducted. The data presented are the means obtained from a minimum of three independent experiments, and the error bars represent the standard error of the mean (SEM). Statistical significance was determined at the following levels: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

RESULTS

Effects of live and UV-killed *B. subtilis* natto on viability of Caco-2 cells. Viability of Caco-2 cells was evaluated following exposure to different concentrations of live and UV-killed *B. subtilis* natto (MOI 1, 10 and 100) using MTT assay. Overall, no statistically significant decrease was observed in the viability of Caco-2 cells upon treatment with different MOIs of both live and UV-killed *B. subtilis* natto after 24 h of treatment as compared to untreated cells. Based on viability results, we applied the highest concentration of live and UV-killed *B. subtilis* natto (MOI 100) for further cell culture experiments, which had no significant effect on the viability of Caco-2 cells (Fig. 1).

Table 1. Oligonucleotide primers used in RT-qPCR assays

Target gene	Primer designation	Oligonucleotide sequence (5′-3′)	Product size (bp)	Reference
TGF-β	TGFβ1-F	GCACAACTCCGGTGACATCAA	86	(20)
	TGFβ1-R	CAATTCCTGGCGATACCTCAG		
TLR2	hTLR2-F	TTATCCAGCACACGAATACACAG	160	(24)
	hTLR2-R	AGGCATCTGGTAGAGTCATCAA		
TLR4	hTLR4-F	AGACCTGTCCCTGAACCCTAT	147	(25)
	hTLR4-R	CGATGGACTTCTAAACCAGCCA		
IL-10	IL10-F	GTGGAGCAGGTGAAGAATGC	65	(26)
	IL10-R	TCACTCATGGCTTTGTAGATGC		
β-actin	ACTB-F	ATGTGGCCGAGGACTTTGATT	107	(27)
	ACTB-R	AGTGGGGTGGCTTTTAGGATG		

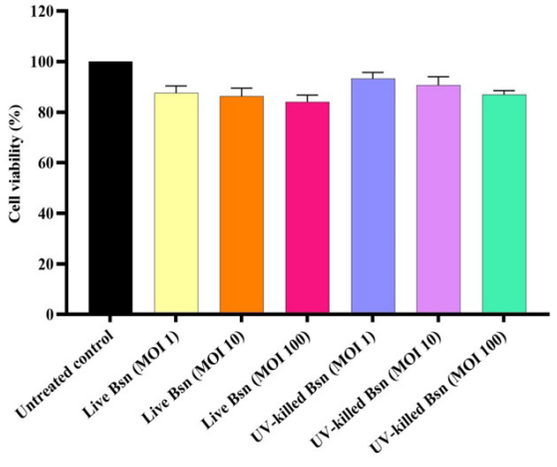

Fig. 1. Cell viability by MTT assay to determine the effects of live and UV-killed *B. subtilis* natto on Caco-2 cells. Different MOIs (MOI 1, 10, 100) of live and UV-killed *B. subtilis* natto were added to Caco-2 cell monolayer and incubated for 24 h. The data are expressed as the mean ± SEM of three independent experiments. The results are presented as the percentage of cell survival compared to control samples. MOI, multiplicity of infection; Bsn, *B. subtilis* natto.

***B. subtilis* natto significantly modulated the expression level of anti-inflammatory genes IL-10 and TGF-β.** To investigate the anti-inflammatory effects of live and UV-killed *B. subtilis* natto, gene expression levels of IL-10 and TGF-β was examined in Caco-2 cells. The results showed that both live and UV-killed *B. subtilis* natto increased the gene expression of IL-10 in Caco-2 cells after 24 h (Fig. 2A). However, this increase was found to be statistically significant only for live *B. subtilis* natto ($P < 0.01$). Furthermore, both live and UV-killed *B. subtilis* natto significantly upregulated the gene expression of TGF-β in Caco-2 cells after 24 h ($P < 0.001$, $P < 0.01$, respectively) (Fig. 2B).

***B. subtilis* natto significantly decreased TLR2 and TLR4 gene expression in treated Caco-2 cells.** In order to examine the mRNA expression levels of TLR2 and TLR4 genes, Caco-2 cells were treated with live and UV-killed *B. subtilis* natto for 24 h. As shown in (Fig. 3A), both live and UV-killed *B. subtilis* natto caused a significant reduction in TLR2 gene expression in Caco-2 cells compared to untreated cells ($P < 0.01$ and $P < 0.05$, respectively). Additionally, treatment with live and UV-killed *B. subtilis* natto resulted in a significant decrease in TLR4 expression in Caco-2 cells after 24 h ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 3B). Moreover, treatment of Caco-2 cells with LPS (100 ng/ml) significantly increased the gene expression of TLR2 and TLR4 as compared to untreated cells.

DISCUSSION

CRC is among the most prevalent life-threatening cancers and its mortality rate dramatically increases in patients aged over 50 years of age (28). A large body of evidence has established that CRC development has been associated with environmental and genetic factors such as physical inactivity, smoking, dietary choices, low fiber intake, alcohol consumption, and obesity (29, 30). Disturbance in the homeostasis of intestinal microbiota, characterized by an imbalance in their functional composition and meta-

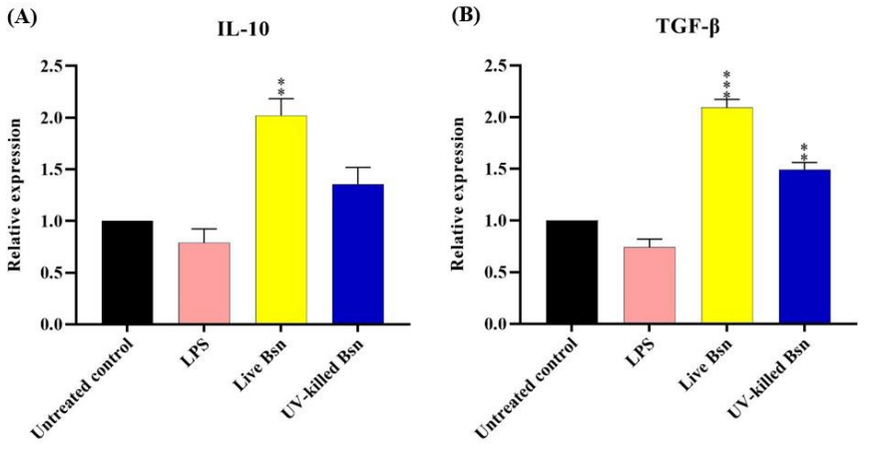

Fig. 2. Effects of live (MOI 100) and UV-killed (MOI 100) *B. subtilis* natto (Bsn) on the expression levels of anti-inflammatory genes IL-10 (A) and TGF-β (B) in Caco-2 cells by RT-qPCR. Expression data are normalized using β-actin as a reference gene. Caco-2 cells monolayers were treated with LPS for 24 h as a positive control. Data are shown as mean ± SEM from three independent experiments. **P < 0.01, ***P < 0.001 compared to the untreated control were considered statistically significant.

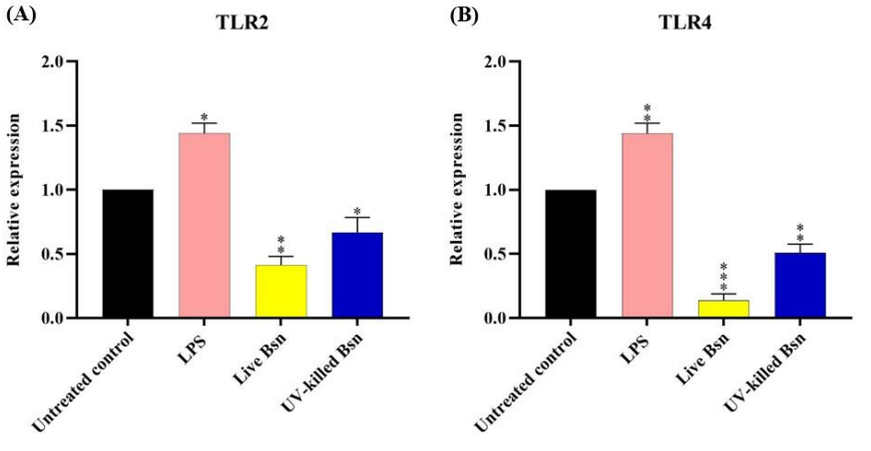

Fig. 3. Effects of live (MOI 100) and UV-killed (MOI 100) *B. subtilis* natto (Bsn) on TLR2 (A) and TLR4 (B) gene expression in Caco-2 cells after 24 h. Gene expression data are normalized using β-actin as the reference gene. Caco-2 cell monolayers were treated with LPS for 24 h as a positive control. Data are presented as the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 was considered statistically significant compared to the untreated control.

bolic activities, can initiate CRC (31). This disruption affects cell signaling pathways, including membrane receptor signaling cascades such as TGF-β and TLR pathways, and leads to the stimulation of inflammatory responses, resulting in the conversion of cellular proto-oncogenes into oncogenes (32, 33). Recently, there has been intense research focusing on preventive strategies and novel therapeutic approaches, such as probiotic consumption in individuals with a higher risk of gastrointestinal disorders and CRC (34). Probiotics can inhibit CRC progression through modulating the gut microbiota composition, suggesting that there is a potential connection between probiotics and CRC prevention (35). A study conducted by Verma et al. demonstrated that probiotic use can suppress the activity of certain oncogenic β-glucuronidase enzymes (36). Furthermore, several studies have demonstrated the potential role of specific probiotic strains, particularly *Lactobacillus acidophilus* in inhibiting the proliferation and survival of cancer cells through apoptosis (37-39). Saber et al. also reported that culture supernatant derived from the probiotic strain *Pichia kudriavzevii* enhances the expression of genes involved in apoptosis and

reduces the viability of colon cancer cells (40). These findings highlight the promising role of probiotics in modulation of oncogenic processes and their potential as a therapeutic intervention in CRC.

Although live probiotics have shown significant efficacy in treatment of gastrointestinal disorders and prevention of CRC, they may potentially cause complications in CRC patients with immunodeficiency (41). Live probiotic strains, when translocating from the gut to the bloodstream, may cause bacteremia in immunocompromised individuals. Therefore, the use of live probiotic bacteria may be associated with serious health problems particularly in immunocompromised patients (41). In a study by Kataria et al. it was demonstrated that the survival and viability of probiotics were not essential for exerting their beneficial and protective effects, as not all clinical benefits or functional mechanisms rely on live bacteria (42). To address this concern, in this study we applied UV-inactivated *B. subtilis* natto as a safer alternative supplement. This approach aimed to minimize potential side effects that may be associated with the use of live probiotic strains, especially in high-risk individuals. In addition, the protective effects of live *B. subtilis* natto probiotic on modulating inflammatory response and promoting the production of anti-inflammatory cytokines were investigated using Caco-2 cells. *B. subtilis* natto was first isolated by Sawamura in 1906 (43). This bacterium inhibits angiogenesis and tumor metastasis through the production of nattokinase, which has the potential to effectively prevent the development of cancer (44).

TGF-β is a potent anti-inflammatory and pleiotropic cytokine that plays a crucial role in regulating various cellular processes, including proliferation, differentiation, apoptosis, and immune response (45). A study by Scott and colleagues have highlighted the significance of TGF-β as a key regulator of immune tolerance and homeostasis in the intestine, as its absence leads to increased inflammatory response (46). Therefore, investigating inflammatory responses and the expression of anti-inflammatory cytokines is an essential strategy for treating and preventing CRC. Our results demonstrated that live and UV-killed *B. subtilis* natto could induce the expression of anti-inflammatory cytokines (TGF-β and IL-10) in Caco-2 cells. In a study conducted by Xin et al., it was found that *B. subtilis* natto has the ability to enhance immune responses and stimulate the production of anti-inflammatory cytokines through the enhancement of phagocytic function in macrophages (47). In this context, Uesugi et al. demonstrated that *B. subtilis* natto can exert immunomodulatory effects which effectively mitigate inflammation by suppressing the production of inflammatory cytokines and upregulating the expression of anti-inflammatory cytokines such as IL-10 (17). In a study conducted by Fujii et al. it was observed that *B. subtilis* natto can induce antiviral and anti-inflammatory effects by promoting the production of IL-10 through M2 phenotype macrophages (48). Another study conducted by Magri et al. demonstrated that high content fructooligosaccharides produced by *B. subtilis* natto exhibit an antiproliferative effect on malignant epithelial cells (49). Consistent with previous research, the results of this study revealed that both live and UV-killed *B. subtilis* natto noticeably upregulated the expression of the anti-inflammatory cytokine IL-10 in Caco2 cells. These findings emphasize the potential role of probiotics in modulating the immune response and providing a protective effect against CRC.

TLRs are a diverse family of pattern recognition receptors (PRRs) that have an essential role in initiating the innate immune response. However, TLR dysregulation has been associated with the promotion of tumorigenesis (50). Studies have shown that TLR2 and TLR4 can contribute to angiogenesis and cancer progression by triggering a cascade of immune responses (51, 52). TLR2 is involved in the recognition of cell wall components and lipoproteins, while TLR4 acts as a receptor for LPS (53). The LPS of Gram-negative bacteria can stimulate transcription of nuclear factor kappa β (NF-kβ) by binding to host cell TLRs (54). In response to such stimuli, NF-kβ activates and phosphorylates the inhibitor IκB, leading to its ubiquitination and degradation (55). This activation process allows NF-kβ to translocate into the nucleus, where it initiates the transcription of inflammatory factors (56). The production of inflammatory agents significantly contributes to intestinal tissue damage and the progression of cancer (57). Notably, both TLR2 and TLR4 have been found to be increased in patients with CRC (53). In a recent study conducted by Sun et al. it was observed that *B. subtilis* natto exhibits a notable impact on mitigating disorders in intestinal barrier function by inhibiting TLR4 pathway and enhancing the functionality of the intestinal microbiota (58). Tobita reported that *B. subtilis* natto reduced the expression level of TLR4 in macrophage cell culture in response to Th1 cells

(59). In this regard, Keshavarz et al. demonstrated that heat-killed *Akkermansia muciniphila* might also affect the expression level TLR4 compared to control group (41). Moreover, Kuuge et al. investigated the administration of *L. acidophilus* in an animal model and showed that regulation of TLR2 and TLR4 genes can lead to reduction in tumor incidence (59). Recently, Nasiri et al. demonstrated that the expression level of TLR2 in Caco-2 cells treated with live or UV-killed *A. muciniphila* was significantly increased (60). In contrast, in the present study significant decrease was observed in the expression level of TLR2 and TLR4 in Caco-2 cells treated with live and UV-killed *B. subtilis* natto. This reduction in expression indicates anti-inflammatory potential of *B. subtilis* natto in Caco-2 cell line. Moreover, our results contrast with those of Shi et al. who demonstrated that the treatment of Caco-2 cells with pasteurized *A. muciniphila* led to a significantly increase in TLR2 expression, while TLR4 expression remained unaltered (61). They indicated that anti-inflammatory effects of *A. muciniphila* are related to the stimulation of TLR2, and not TLR4. The discrepancies in results might be due to differences in the type of probiotics, cell culture conditions, and models used in these studies. However, further research is required to clarify how UV-killed and live probiotics function as therapeutic supplements and how they stimulate the production of anti-inflammatory cytokines. Understanding the mechanisms of action and detailing the intracellular signaling pathways in cancer will help develop effective and preventive therapeutic strategies.

CONCLUSION

Our findings indicated that *B. subtilis* natto, both in live and UV-killed forms, has the potential to modulate the expression of inflammatory cytokines and enhance anti-inflammatory response *in vitro*. We also proposed that UV-killed *B. subtilis* natto can be used as a safe alternative to live bacteria, and is able to modulate intestinal inflammation. These results could suggest that *B. subtilis* natto could be considered as a therapeutic supplement for individuals with CRC. Despite the promising data obtained in the present study, it is important to acknowledge the limitations. It is necessary to validate the gene expression data determined in this study through protein expression assays. Preclinical studies and *in vivo* experiments using appropriate animal models for colon cancer are required to corroborate the findings obtained in this work.

ACKNOWLEDGEMENTS

We would like to thank the staff of Foodborne and Waterborne Diseases Research Center in Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran, as well as Department of Microbiology and Medical Microbiology Research Center of Qazvin University of Medical Sciences, Qazvin, Iran. This study was supported financially Qazvin University of Medical Sciences, Qazvin, Iran. Also, the study was approved by the ethical committee of Qazvin Medical University (IR.QUMS.REC.1400.089).

REFERENCES

1. Ciardiello F, Ciardiello D, Martini G, Napolitano S, Tabernero J, Cervantes A. Clinical management of metastatic colorectal cancer in the era of precision medicine. *CA Cancer J Clin* 2022; 72: 372-401.
2. Onyoh EF, Hsu W-F, Chang L-C, Lee Y-C, Wu M-S, Chiu H-M. The rise of colorectal cancer in Asia: epidemiology, screening, and management. *Curr Gastroenterol Rep* 2019; 21: 36.
3. Fong W, Li Q, Yu J. Gut microbiota modulation: a novel strategy for prevention and treatment of colorectal cancer. *Oncogene* 2020; 39: 4925-4943.
4. Kim J, Lee HK. Potential role of the gut microbiome in colorectal cancer progression. *Front Immunol* 2022; 12: 807648.
5. Schmitt M, Greten FR. The inflammatory pathogenesis of colorectal cancer. *Nat Rev Immunol* 2021; 21: 653-667.
6. Yao P, Tan F, Gao H, Wang L, Yang T, Cheng Y. Effects of probiotics on Toll-like receptor expression in ulcerative colitis rats induced by 2, 4, 6-trinitro-benzene sulfonic acid. *Mol Med Rep* 2017; 15: 1973-1980.
7. Klampfer L. Cytokines, inflammation and colon cancer. *Curr Cancer Drug Targets* 2011; 11: 451-464.
8. Johdi NA, Sukor NF. Colorectal cancer immunotherapy: options and strategies. *Front Immunol* 2020; 11: 1624.
9. Abedi Elkhichi P, Dabiri H, Nazemalhosseini Mojarad E, Rezasoltani S, Asadzadeh Aghdaei H, Pouriran R, et al. Prevalence of *Helicobacter pylori* in patients with colorectal cancer. *Int J Mol Clin Microbiol* 2018; 8:

1001-1005.
10. Buccafusca G, Proserpio I, Tralongo AC, Rametta Giuliano S, Tralongo P. Early colorectal cancer: diagnosis, treatment and survivorship care. *Crit Rev Oncol Hematol* 2019; 136: 20-30.
11. Miknevicius P, Zulpaite R, Leber B, Strupas K, Stiegler P, Schemmer P. The impact of probiotics on intestinal mucositis during chemotherapy for colorectal cancer: a comprehensive review of animal studies. *Int J Mol Sci* 2021; 22: 9347.
12. Eslami M, Yousefi B, Kokhaei P, Hemati M, Nejad ZR, Arabkari V, et al. Importance of probiotics in the prevention and treatment of colorectal cancer. *J Cell Physiol* 2019; 234: 17127-17143.
13. Molska M, Reguła J. Potential mechanisms of probiotics action in the prevention and treatment of colorectal cancer. *Nutrients* 2019; 11: 2453.
14. Ding S, Hu C, Fang J, Liu G. The protective role of probiotics against colorectal cancer. *Oxid Med Cell Longev* 2020; 2020: 8884583.
15. Mazkour S, Shekarforoush SS, Basiri S. The effects of supplementation of *Bacillus subtilis* and *Bacillus coagulans* spores on the intestinal microflora and growth performance in rat. *Iran J Microbiol* 2019; 11: 260-266.
16. Ruiz Sella SR, Bueno T, de Oliveira AAB, Karp SG, Soccol CR. *Bacillus subtilis* natto as a potential probiotic in animal nutrition. *Crit Rev Biotechnol* 2021; 41: 355-369.
17. Uesugi T, Mori S, Miyanaga K, Yamamoto N. GroEL secreted from *Bacillus subtilis* natto exerted a crucial role for anti-inflammatory IL-10 induction in THP-1 cells. *Microorganisms* 2023; 11: 1281.
18. Azimirad M, Alebouyeh M, Naji T. Inhibition of lipopolysaccharide-induced interleukin 8 in human adenocarcinoma cell line HT-29 by spore probiotics: *B. coagulans* and *B. subtilis* (natto). *Probiotics Antimicrob Proteins* 2017; 9: 56-63.
19. Adams CA. The probiotic paradox: live and dead cells are biological response modifiers. *Nutr Res Rev* 2010; 23: 37-46.
20. Keshavarz Azizi Raftar S, Ashrafian F, Yadegar A, Lari A, Moradi HR, Shahriary A, et al. The protective effects of live and pasteurized *Akkermansia muciniphila* and its extracellular vesicles against HFD/CCl4-induced liver injury. *Microbiol Spectr* 2021; 9 (2): e0048421.
21. Angélica Garrido-Pereira M, Braga AL, Rocha AFd, Sampaio LA, Abreu PC. Effect of ultraviolet (UV) radiation on the abundance and respiration rates of probiotic bacteria. *Aquac Res* 2013; 44: 261-267.
22. Nabavi-Rad A, Jamshidizadeh S, Azizi M, Yadegar A, Robinson K, Monaghan TM, et al. The synergistic effect of levilactobacillus brevis IBRC-M10790 and vitamin D3 on *Helicobacter pylori*-induced inflammation. *Front Cell Infect Microbiol* 2023; 13: 1171469.
23. Paolillo R, Romano Carratelli C, Sorrentino S, Mazzola N, Rizzo A. Immunomodulatory effects of *Lactobacillus plantarum* on human colon cancer cells. *Int Immunopharmacol* 2009; 9: 1265-12671.
24. Wang S, Liu K, Seneviratne CJ, Li X, Cheung GS, Jin L, et al. Lipoteichoic acid from an *Enterococcus faecalis* clinical strain promotes TNF-α expression through the NF-κB and p38 MAPK signaling pathways in differentiated THP-1 macrophages. *Biomed Rep* 2015; 3: 697-702.
25. Li J, Lin S, Vanhoutte PM, Woo CW, Xu A. Akkermansia muciniphila protects against atherosclerosis by preventing metabolic endotoxemia-induced inflammation in Apoe−/− mice. *Circulation* 2016; 133: 2434-2446.
26. González-Domínguez É, Domínguez-Soto Á, Nieto C, Flores-Sevilla JL, Pacheco-Blanco M, Campos-Pena V, et al. Atypical activin A and IL-10 production impairs human CD16+ monocyte differentiation into anti-inflammatory macrophages. *J Immunol* 2016; 196: 1327-1337.
27. Ofinran O, Bose U, Hay D, Abdul S, Tufatelli C, Khan R. Selection of suitable reference genes for gene expression studies in normal human ovarian tissues, borderline ovarian tumours and ovarian cancer. *Mol Med Rep* 2016; 14: 5725-5731.
28. Xi Y, Xu P. Global colorectal cancer burden in 2020 and projections to 2040. *Transl Oncol* 2021; 14: 101174.
29. Rawla P, Sunkara T, Barsouk A. Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors. *Prz Gastroenterol* 2019; 14: 89-103.
30. Hua H, Sun Y, He X, Chen Y, Teng L, Lu C. Intestinal microbiota in colorectal adenoma-carcinoma sequence. *Front Med (Lausanne)* 2022; 9: 888340.
31. Dong J, Tai JW, Lu L-F. miRNA–Microbiota interaction in Gut Homeostasis and colorectal cancer. *Trends Cancer* 2019; 5: 666-669.
32. Gu S, Zaidi S, Hassan MI, Mohammad T, Malta TM, Noushmehr H, et al. Mutated CEACAMs disrupt transforming growth factor beta signaling and alter the intestinal microbiome to promote colorectal carcinogenesis. *Gastroenterology* 2020; 158: 238-252.
33. Kontomanolis EN, Koutras A, Syllaios A, Schizas D, Mastoraki A, Garmpis N, et al. Role of oncogenes and tumor-suppressor genes in carcinogenesis: a review. *Anticancer Res* 2020; 40: 6009-6015.
34. Drago L. Probiotics and colon cancer. *Microorganisms* 2019; 7: 66.
35. Kumar KS, Sastry N, Polaki H, Mishra V. Colon cancer prevention through probiotics: an overview. *J Cancer Sci Ther* 2015; 7: 81-92.
36. Verma A, Shukla G. Probiotics *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* suppresses

DMH-induced procarcinogenic fecal enzymes and preneoplastic aberrant crypt foci in early colon carcinogenesis in Sprague Dawley rats. *Nutr Cancer* 2013; 65: 84-91.
37. Kim B-K, Yoon Y-S, Ryu Y, Chung M-J. Probiotic-derived p8 protein induce apoptosis via regulation of RNF152 in colorectal cancer cells. *Am J Cancer Res* 2021; 11: 746-759.
38. Yue Y, Wang S, Shi J, Xie Q, Li N, Guan J, et al. Effects of *Lactobacillus acidophilus* KLDS1. 0901 on proliferation and apoptosis of colon cancer cells. *Front Microbiol* 2022; 12: 788040.
39. Isazadeh A, Hajazimian S, Shadman B, Safaei S, Bedoustani AB, Chavoshi R, et al. Anti-cancer effects of probiotic *Lactobacillus acidophilus* for colorectal cancer cell line caco-2 through apoptosis induction. *Pharm Sci* 2021; 27: 262-267.
40. Saber A, Alipour B, Faghfoori Z, Mousavi Jam A, Yari Khosroushahi A. Secretion metabolites of probiotic yeast, *Pichia kudriavzevii* AS-12, induces apoptosis pathways in human colorectal cancer cell lines. *Nutr Res* 2017; 41: 36-46.
41. Keshavarz Azizi Raftar S, Abdollahiyan S, Azimirad M, Yadegar A, Vaziri F, Moshiri A, et al. The anti-fibrotic effects of heat-killed *Akkermansia muciniphila* MucT on liver fibrosis markers and activation of hepatic stellate cells. *Probiotics Antimicrob Proteins* 2021; 13: 776-787.
42. Kataria J, Li N, Wynn JL, Neu J. Probiotic microbes: do they need to be alive to be beneficial? *Nutr Rev* 2009; 67: 546-550.
43. Nagai T. Bacteriophages of *Bacillus subtilis* (natto) and their contamination in natto factories. Intech Open; 2012. https://doi.org/10.5772/33555
44. Zhang B, Chai J, He L, Dusanbieke M, Gong A. Nattokinase produced by natto fermentation with *Bacillus subtilis* inhibits breast cancer growth. *Int J Clin Exp Med* 2019; 12: 13380-13387.
45. Batlle E, Massagué J. Transforming growth factor-β signaling in immunity and cancer. *Immunity* 2019; 50: 924-940.
46. Daniel SG, Ball CL, Besselsen DG, Doetschman T, Hurwitz BL. Functional changes in the gut microbiome contribute to transforming growth factor β-deficient colon cancer. *mSystems* 2017; 2(5): e00065-17.
47. Xu X, Huang Q, Mao Y, Cui Z, Li Y, Huang Y, et al. Immunomodulatory effects of *Bacillus subtilis* (natto) B4 spores on murine macrophages. *Microbiol Immunol* 2012; 56: 817-824.
48. Fujii K, Kubo Y, Noguchi T, Tobita K. Effects of *Bacillus subtilis* natto strains on antiviral responses in Resiquimod-Stimulated human M1-Phenotype macrophages. *Foods* 2023; 12: 313.
49. Magri A, Oliveira M, Baldo C, Tischer C, Sartori D, Mantovani MS, et al. Production of fructooligosaccharides by *Bacillus subtilis* natto CCT7712 and their antiproliferative potential. *J Appl Microbiol* 2020; 128: 1414-1426.
50. Beilmann-Lehtonen I, Böckelman C, Mustonen H, Koskensalo S, Hagström J, Haglund C. The prognostic role of tissue TLR2 and TLR4 in colorectal cancer. *Virchows Arch* 2020; 477: 705-715.
51. Li T-T, Ogino S, Qian ZR. Toll-like receptor signaling in colorectal cancer: carcinogenesis to cancer therapy. *World J Gastroenterol* 2014; 20: 17699-17708.
52. Pastille E, Faßnacht T, Adamczyk A, Ngo Thi Phuong N, Buer J, Westendorf AM. Inhibition of TLR4 signaling impedes tumor growth in colitis-associated colon cancer. *Front Immunol* 2021; 12: 669747.
53. Meng S, Li Y, Zang X, Jiang Z, Ning H, Li J. Effect of TLR2 on the proliferation of inflammation-related colorectal cancer and sporadic colorectal cancer. *Cancer Cell Int* 2020; 20: 95.
54. Li Y, Yang S, Lun J, Gao J, Gao X, Gong Z, et al. Inhibitory effects of the *Lactobacillus rhamnosus* GG effector protein HM0539 on inflammatory response through the TLR4/MyD88/NF-κB axis. *Front Immunol* 2020; 11: 551449.
55. Li M, Liu P, Wang B, Zhou J, Yang J. Inhibition of nuclear factor Kappa B as a therapeutic target for lung cancer. *Altern Ther Health Med* 2022; 28: 44-51.
56. Sharma RK, Otsuka M, Gaba G, Mehta S. Inhibitors of transcription factor nuclear factor-kappa beta (NF-κβ)-DNA binding. *RSC Adv* 2013; 3: 1282-1296.
57. Slattery ML, Mullany LE, Sakoda L, Samowitz WS, Wolff RK, Stevens JR, et al. The NF-κB signalling pathway in colorectal cancer: associations between dysregulated gene and miRNA expression. *J Cancer Res Clin Oncol* 2018; 144: 269-283.
58. Sun R, Niu H, Sun M, Miao X, Jin X, Xu X, et al. Effects of *Bacillus subtilis* natto JLCC513 on gut microbiota and intestinal barrier function in obese rats. *J Appl Microbiol* 2022; 133: 3634-3644.
59. Tobita K, Meguro R. *Bacillus subtilis* BN strain promotes Th1 response via Toll-like receptor 2 in polarized mouse M1 macrophage. *J Food Biochem* 2022; 46(2): e14046.
60. Nasiri G, Azimirad M, Goudarzi H, Amirkamali S, Yadegar A, Ghalavand Z, et al. The inhibitory effects of live and UV-killed Akkermansia muciniphila and its derivatives on cytotoxicity and inflammatory response induced by *Clostridioides difficile* RT001 *in vitro*. *Int Microbiol* 2024; 27: 393-409.
61. Shi M, Yue Y, Ma C, Dong L, Chen F. Pasteurized Akkermansia muciniphila ameliorate the LPS-induced intestinal barrier dysfunction via modulating AMPK and NF-κB through TLR2 in Caco-2 cells. *Nutrients* 2022; 14: 764.