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Research Article

Anti-Cancer Effects of Probiotic *Lactobacillus acidophilus* for Colorectal Cancer Cell Line Caco-2 through Apoptosis Induction

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Abstract

Background: Colorectal cancer is one of the most common cancers worldwide. Probiotics are useful and non-pathogenic microorganisms in the gastrointestinal tract, which can show anticancer activity through the induction of apoptosis. This study aimed to evaluate the anti-proliferative effects of *Lactobacillus acidophilus* probiotic on the Caco-2 colorectal cancer cell line.

Methods: The supernatant (secreted metabolites) and bacterial extract of *L. acidophilus* probiotics were prepared and used as an anti-proliferative agent on the colorectal cancer cell line, Caco-2 *in vitro*. The effects of supernatant and extract of *L. acidophilus* were evaluated on the viability and proliferation of cancer cells using MTT assay. Moreover, morphological alterations of cancer cells treated with supernatant and extract of *L. acidophilus* were evaluated by an inverted phase-contrast microscope. The mRNA expression levels of apoptosis-related genes (SURVIVIN and SMAC) in treated cancer cells and untreated controls were evaluated using the Real-Time PCR method.

Results: The results showed that the supernatant and extract of *L. acidophilus* inhibited the viability and proliferation of cancer cells in a dose and time-dependent manner. Moreover, various morphological alterations were observed in the treated cancer cells, which are indicators of apoptosis induction. The mRNA expression of SURVIVIN and SMAC genes were significantly up-regulated and downregulated in the treated cancer cells, respectively.

Conclusion: The results of the present study suggested that the supernatant and extract of *L. acidophilus* could inhibit the viability and proliferation of colorectal cancer cell line, Caco-2 through induction of apoptosis, increase the survival rate of colon cancer patients.

Introduction

Colorectal cancer is one of the most common cancers and causes of death in the world.[1] Nowadays, chemotherapy followed by surgery is the most important method in the treatment of patients with colorectal cancer. However, the efficacy of colorectal cancer chemotherapy is limited, due to the resistance of colorectal cancer cells to chemotherapeutic drugs.[2] Therefore, the use of new therapeutic methods in the treatment of colorectal cancer has increased.[3,4] In recent years, many studies have been performed on the usability of probiotics in inhibition, management, and even treatment of various cancers, especially digestive system cancers.[5] Due to location and density of probiotic microorganisms in the gastrointestinal tract, colorectal cancer is the main target of probiotic therapy.[6]

Probiotics refer to harmless microorganisms that could have nutritional advantages. Also, probiotics provide health benefits when administered in adequate amounts.[7] So far, several effects of probiotics on gastrointestinal diseases have been reported.[8,9] Recently, several studies have been performed on the effects of *L. acidophilus* on various cancers.[10,11] Previous studies reported that *L. acidophilus* can cause cancer cell death through the induction of apoptosis.[9,12] Moreover, the evidence showed that probiotics play an important role in

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the regulation of cell proliferation and apoptosis.[12,13] In a recent study by Altonsy *et al.*[14] suggested that Lactobacillus genus induce the mitochondrial pathway of apoptosis in colorectal carcinoma cells. Therefore, probiotics can be considered as important anticancer agents, without any side effects.[15] However, a few studies have been performed on the anticancer effects of *L. acidophilus* probiotic and underlying mechanism of action. Therefore, in the present study, we investigated the effects of supernatant and extracts of *L. acidophilus* on the viability and proliferation of the Caco-2 colorectal cancer cell line and were elucidated the underlying mechanism of action.

Materials and Methods

Probiotic materials

The standard strains of *L. acidophilus* (ATCC 4356) were purchased from Persian Type Culture Collection (PTCC) and were cultured on the de Man, Rogosa and Sharpe (MRS) agar medium (Merck, Germany). The obtained colonies were inoculated into MRS broth and incubated for 24 hours. The bacterial culture was sub-cultured in a fresh MRS medium and its absorbance was adjusted on 1 at 600 nm. The obtained bacterial culture was centrifuged and the supernatant was sterilized using a 0.22 µm syringe filter. The different concentrations of supernatant were prepared using Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco, United States America), containing 10% fetal bovine serum (FBS) (Gibco, United States America). Also, the same concentrations of the MRS medium were prepared and considered as negative controls. Moreover, the bacterial plate was resuspended by phosphate-buffered saline (PBS) and lysed using an ultrasonic bath (Siemens, Germany). The obtained bacterial lysates were sterilized using a 0.22 µm syringe filter (Sartorius, Germany). The different concentrations of bacterial extract were prepared using RPMI 1640 medium, containing 10% FBS.

Cell culture

The colorectal cancer cell line, Caco-2 was prepared from the Immunology Research Center (IRC), Tabriz University of Medical Sciences. The cancer cell culture was performed using RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin (100 units/ml-100 µg/ml) antibiotics (Gibco, United States America), and incubated in standard conditions at 37°C and 5% CO_2 (Memmert, Germany).

Cell viability assay

The cancer cells were seeded in a 96-well plate (1.5×10^4 cells/well) and incubated for 24 hours in standard conditions. The cancer cells were treated with different concentrations of supernatant (30%, 40%, 50%, 60%, 70%, and 80%) and extract (5%, 7.5%, 10%, 12.5%, and 15%) of *L. acidophilus* for 24, 48, 72, and 96 hours. The cancer cell viability was evaluated using the MTT assay (Gibco, United States America). The old medium was replaced with fresh medium containing MTT solution (5 mg/ml reagent in PBS) and incubated for 4 hours in standard conditions. Dimethyl sulfoxide (DMSO) was replaced with the previous medium and incubated for 30 minutes in standard conditions. The optical density (OD) of wells was measured at 570 nm and cell viability was evaluated by enzyme-linked immunosorbent assay (ELISA) reader (Bio Rad, United States America).

Morphological alterations assay

The cancer cells were seeded in a 6-well plate (2×10^5 cells/well) and incubated for 24 hours in standard conditions. The cancer cells were treated with different concentrations of supernatant (30%, 40%, and 50%) and extract (5%, 7.5%, and 10%) of *L. acidophilus* for 24, 48, 72, and 96 hours. The morphological alterations of treated cancer cells were monitored using an inverted phase-contrast microscope (Olympus, Japan).

Gene expression analysis

The cancer cells were seeded in a 6-well plate (2×10^5 cells/well) and incubated for 24 hours in standard conditions. The cancer cells were treated with different concentrations of supernatant (40%) and extract (7.5%) of *L. acidophilus* for 48 hours. The extraction of total RNA from treated cancer cells and untreated controls was performed using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, United States America). Finally, the solution was increased to a volume of 10 µL by adding 3 µL of Diethyl pyrocarbonate (DEPC) water. The quality and quantity of the extracted RNA were investigated by electrophoresis on 1% agarose gel and NanoDrop instrument (Thermo Scientific, United States America). Then cDNA synthesis was performed according to the manufacturer's instructions (Thermo Fisher, United States America). The changes in mRNA expression of *SURVIVIN* and *SMAC* genes were evaluated using quantitative real-time PCR (qRT-PCR) (Bio Rad, United States America). The primers sequences were: *SURVIVIN* (*BIRC5*) gene: F-CCCTTTCTCAAGGACCACCG and R-GTTCCTCTATGGGGTCGTCA; *SMAC* (*DIABLO*) gene: F-CAGAGGAGGAAGATGAAGTGTG and R-GCGGTTATAGAGGCCTGATCTG; *β-actin* (*ACTB*) gene: F-AGAGCTACGAGCTGCCTGAC and R-AGCACTGTGTTGGCGTACAG. The PCR reaction was performed in a 10 µl total volume: 5 µl PCR pre-Mix, 1 µl cDNA, 0.5 µl forward primer, and 0.5 µl reverse primers. The PCR program was as follows: initial denaturation (1 cycle in 94°C for 1 minute), denaturation (40 cycles in 94°C for 20 seconds), annealing (40 cycles in 59°C for 30 seconds), and extension (40 cycles in 72°C for 30 seconds). The *β-actin* gene was considered as an endogenous control.

Statistical analysis

The experiments in this study were repeated three times and the obtained data from three independent experiments were presented as the mean ± standard deviation (SD). The statistical analysis was performed using the one-way

analysis of variance (ANOVA), Student's t-test, and Tukey's post hoc test using Graph-Pad Prism (version 7.0). The p-values < 0.05 were considered as significant.

Results

Cancer cell viability

The results of the anti-proliferative effects of supernatant and extract of *L. acidophilus* showed in a dose and time-dependent manner. The viability of the treated cancer cells was significantly decreased in high concentrations of *L. acidophilus* supernatant. However, the anti-proliferative activity of *L. acidophilus* extract was significantly more than *L. acidophilus* supernatant. The 40% concentration of *L. acidophilus* supernatant leads to 50% colorectal cancer cells death after 48 hours, which considered as half-maximal inhibition concentration (IC_{50}) . At the same time, the IC_{50} of *L. acidophilus* extract was 7.5% (Figure 1).

Morphological alterations

We observed various morphological alterations in colorectal cancer cells treated with *L. acidophilus*, which can cause programmed cell death. The morphological alterations include fragmented nuclei, membrane damage,

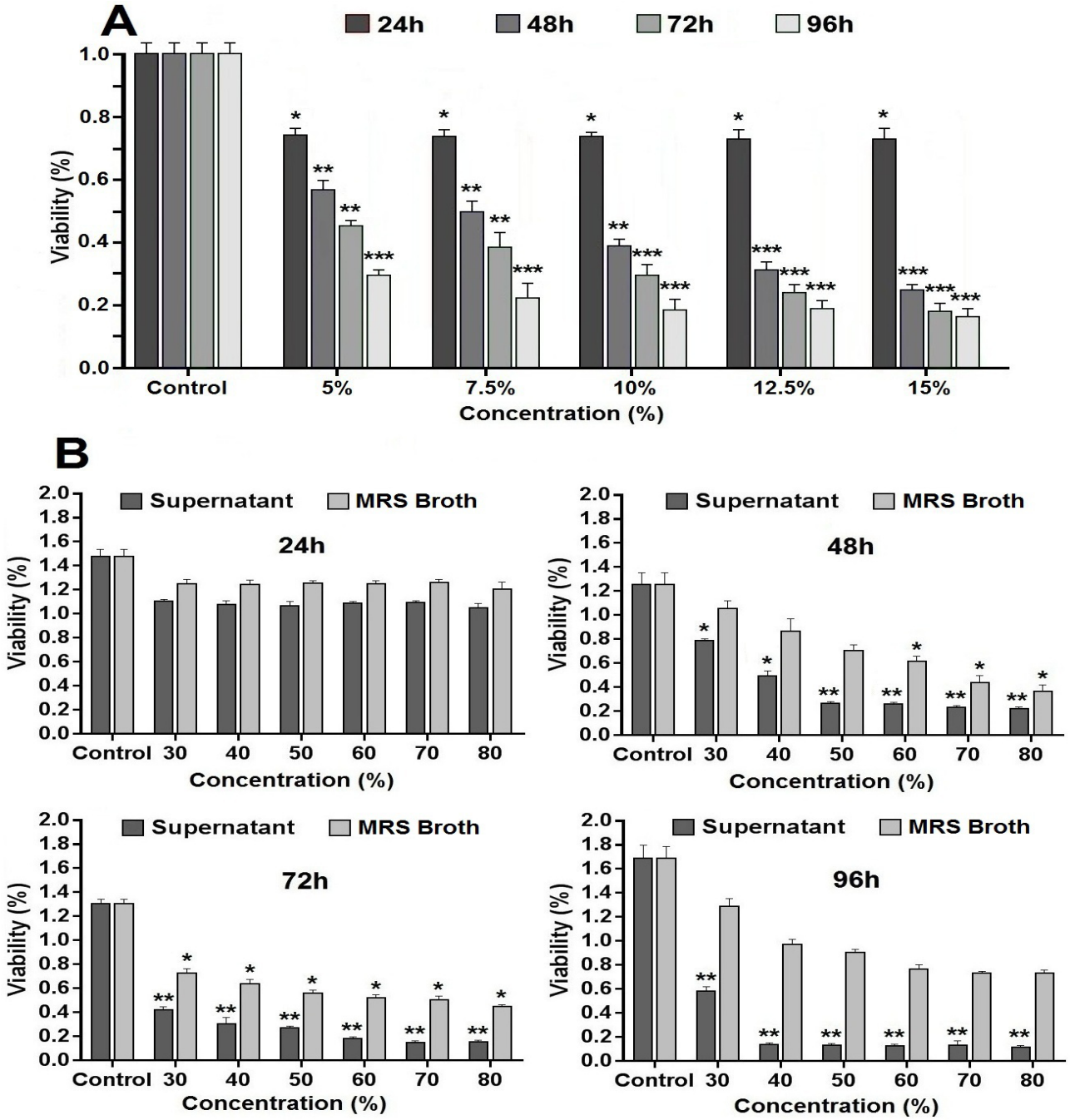

Figure 1. The viability of colorectal cancer cells treated with different concentration of *L. acidophilus* probiotic extract (A) and supernatant (B) for 24, 48, 72 and 96 hours (*p<0.01 and **p<0.001).

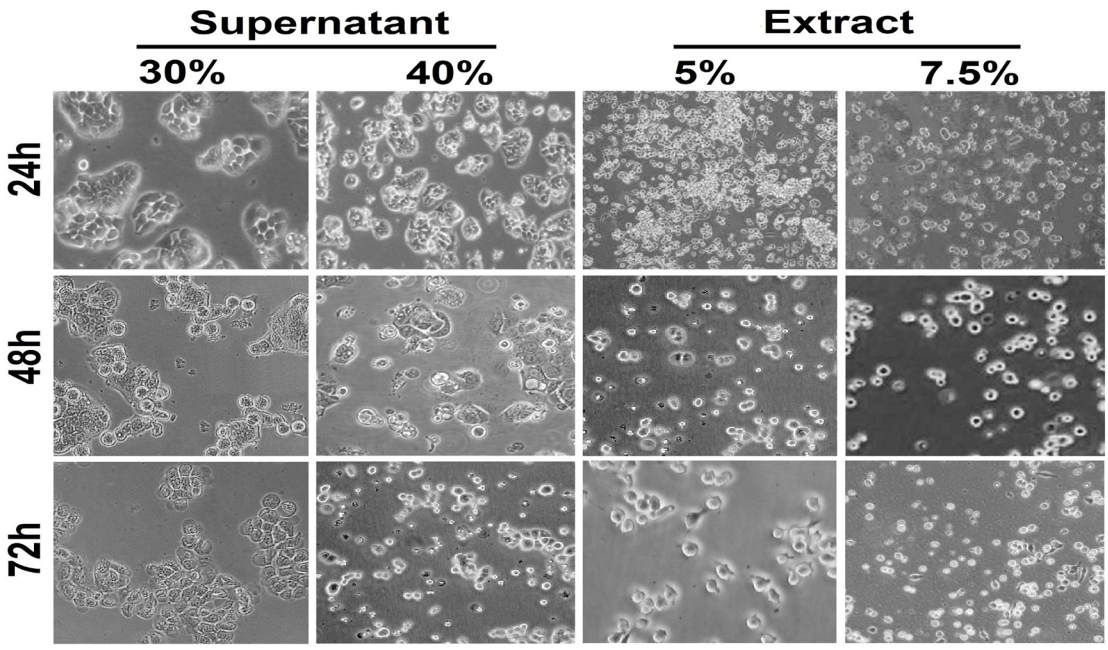

Figure 2. Morphological alteration of colorectal cancer cells treated with *L. acidophilus* probiotic supernatant (30%, 40%, and 50%) and extract (5%, 7.5%, and 10%) for 24, 48, 72 and 96 hours.

cell shrinkage, and decreased cell size, which are indicators of programmed cell death. The observed morphological alterations were in a concentration and time-dependent manner. Moreover, these alterations in treated colorectal cancer cells with *L. acidophilus* extract were more significant than *L. acidophilus* supernatant (Figure 2).

Apoptosis-related genes expression

The expression of apoptosis-related genes showed that supernatant and extract of *L. acidophilus* significantly increased mRNA expression of the *SMAC* gene in colorectal cancer cells (Figure 3A). In contrast, mRNA expression of the *SURVIVIN* gene was significantly decreased (Figure 3B). The regulation of apoptosis-related genes in colorectal cancer cells by the extract was more profound than supernatant treatments.

Discussion

One of the important pathological processes in colorectal cancer is the inhibition of apoptosis, which is caused by the suppression of pre-apoptotic genes and/or induction of anti-apoptotic genes.[16] The uncontrolled cell proliferation and apoptosis resistance are two main characteristics of cancer cells.[17] Therefore, various compounds that induce apoptosis in cancer cells, can be considered as an anticancer agent.[18] The evidence indicates that at least 50% of human cancers are caused by inappropriate diet. Therefore, various foodstuffs such as probiotics and their effects on cancer cells have been widely evaluated. Previous studies suggested that probiotics have anticancer activity through the induction of apoptosis.[18,19]

In the present study, we evaluated the effect of *L. acidophilus* probiotic on the cell viability, morphological alterations, and expression of apoptosis-related genes in colorectal cancer cells Caco-2. The results showed that

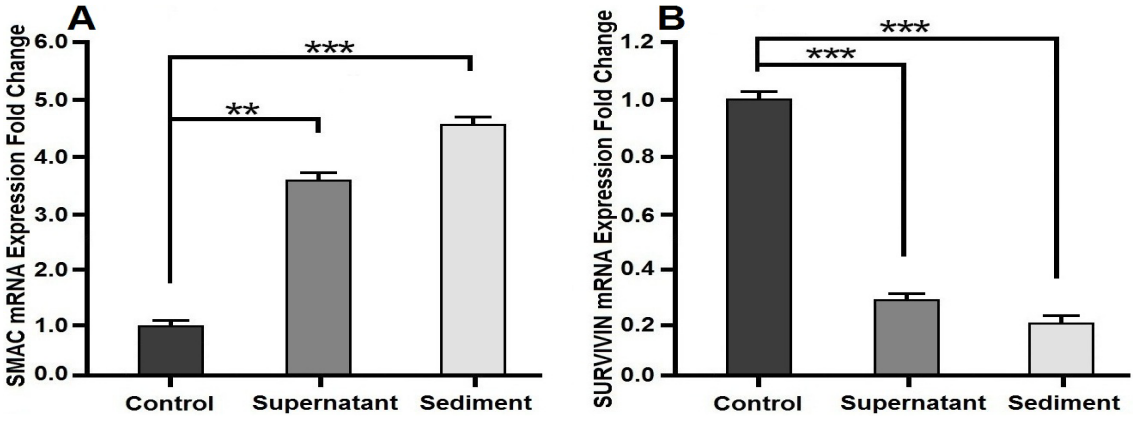

Figure 3. The mRNA expression of *SMAC* (A) and *SURVININ* (B) genes in colorectal cancer cells treated with *L. acidophilus* probiotic supernatant (40%) and extract (7.5%) for 48 hours (**p<0.001 and ***p<0.0001).

supernatant and extract of *L. acidophilus* could cause inhibition of colorectal cancer cells growth in a concentration and time-dependent manner. Moreover, supernatant and extract of *L. acidophilus* can cause morphological alterations that indicate programmed cell death. Many studies have investigated the anticancer effects of probiotics, especially lactobacilli, on colorectal cancer cells.[18-20] In a study, Soltan Dallal *et al.*[18] reported that the supernatant and extract of *L. acidophilus* decreased cell proliferation, as well as increased cell apoptosis and necrosis in colorectal cancer cells. In the other study, Nami *et al.*[19] showed that *L. acidophilus* induces apoptosis in cervical, gastric, breast, and colorectal cancer cells.Also, Baldwin *et al.*[20] reported that the combination of *L. acidophilus* and *L. casei* extracts induces apoptosis in colorectal cancer cells. However, the exact mechanisms of cancer cell death in the presence of probiotics remain unknown. Therefore, we investigated the role of apoptosis in the anticancer activity of probiotics.

Recently, the identification of underlying mechanisms of probiotics effects against cancer cells is one of the main objectives in previous studies.[8,21] In a study by Kim *et al.*[8] reported that external polysaccharides, derived from *L. acidophilus* probiotic, increased colorectal cancer cell death through induction of apoptosis. In another study, Taverniti *et al.*[21] reported that the cell wall and peptidoglycan derived from *L. bulgaricus* and *L. casei* decreased the viability of colorectal cancer cells.Therefore, the anticancer activity of lactobacilli can be due to polysaccharides and peptidoglycans. In the present study, we demonstrated that the anti-proliferative activity of *L. acidophilus* extract against colorectal cancer cells was significantly more than *L. acidophilus* supernatant. However, the *L. acidophilus* supernatant consists of various enzymes, proteins, and toxins that significantly decrease the viability of colorectal cancer cells. Hence, the presence of polysaccharides, peptidoglycans, proteins, enzymes, toxins, and other components in supernatant and extract of *L. acidophilus* may be involved in the induction of apoptosis in cancer cells.

So far, limited studies have been conducted on the effects of probiotics on the regulation of apoptosis-related genes.[22,23] In a study, Asoudeh-Fard *et al.*[22] reported that *L. Plantarum* down-regulated expression of MAPK gene, as well as up-regulated expression of PTEN gene in oral cancer cells, which cause to induction of apoptosis. In another study by Tukenmez *et al.*[23] reported that external polysaccharides of lactobacilli down-regulates expression of *BCL-2* and *SURVIVIN* genes, as well as up-regulates expression of *Cas3*, *Cas9*, and *BAX* genes in colorectal cancer cells, which cause initiation of apoptosis.Previous studies reported that the expression of *SMAC* and *SURVIVIN* genes are associated with decreased and increased viability of colorectal cancer cells, respectively.[4,24] Moreover, our study showed that the supernatant and extract of *L. acidophilus* significantly increased *SMAC* gene expression levels, as well as decreased *SURVIVIN* gene expression levels in colorectal cancer cells. Therefore, various components in supernatant and extract of *L. acidophilus* increase colorectal cancer cell death through the regulation of apoptosis-related gene expression.

Conclusion

In conclusion, we suggested that the effects of *L. acidophilus* probiotics are not limited to stimulation of the immune system, but also prevent the viability and proliferation of colorectal cancer cells. The results of this study indicated that *L. acidophilus* probiotic directly interacts with cancer cells and indirectly inhibits cell proliferation by the release of various metabolites. According to obtained results in the present study and the low-grade nature of the colorectal cancer cell line Caco-2, we suggest that the use of *L. acidophilus* probiotic may be a promising tool to prevent the incidence of colorectal cancer. However, further studies are required to identify other possible signaling pathways and mechanisms involved in the anticancer effects of the *L. acidophilus* probiotic.

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Author Contributions

MN and BB: Designed the study concept, DS: Analyzed data and prepared the manuscript. AI, SH, BS, SS, and ABB: Conducted experimental studies and drafted the manuscript. RC and MM: Involved in drafting the manuscript and preparing the final revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflict of Interests

The authors declare that there are no conflicts of interest.

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