Growth Inhibitory Effects of Lactobacillus acidophilus, Lactobacillus reuteri, and Bifidobacterium bifidum Supernatant on Blastocystis Subtypes 1 and 3 Via the TLR4 Axis

Mohammad Gorgipour1*, Javid Sadraei1*, Majid Pirestani1, Ashraf Mohabati Mobarez2

1Department of Parasitology and Entomology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Abstract
Background: Blastocystis is a common protozoan parasite found in the human intestinal tract and is associated with several gastrointestinal symptoms as well as inflammatory conditions in the bowel. Probiotics are groups of beneficial microorganisms with a substantial impact on overall human health.

Objectives: This study aimed to investigate the growth inhibitory effects of Lactobacillus acidophilus, Lactobacillus reuteri, and Bifidobacterium bifidum on Blastocystis subtypes (ST) 1 and 3 through the evaluation of expression changes in Toll-like receptor 4 (TLR4) in Caco2 cell culture.

Materials and Methods: The parasite and Caco2 cells were cultured and maintained, and the supernatant of bacteria was prepared. The viability of parasites and cell cultures exposed to supernatants was measured separately by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, in comparison with metronidazole. In addition, TLR4 expression changes in cell cultures and co-cultures exposed to the supernatants were measured using real-time polymerase chain reaction (PCR).

Results: The mean viability of Caco2 cells exposed to the highest and lowest (100 vs. 10 µg/mL) concentrations of supernatants was in a similar range. The survival of Blastocystis ST1 and ST3 exposed to the 15, 25, and 35 µg/mL of probiotics supernatants significantly decreased during 24, 48, and 72 hours. Although not statistically significant, the findings indicated a decrease in TLR4 expression in Caco2 cells and an increase in gene expression in co-cultures exposed to the probiotic supernatants (15, 25, and 35 µg/mL).

Conclusion: This novel therapeutic field of study using probiotics compounds deserves further exploration to find unprecedented therapies against Blastocystis infection.

Keywords: Probiotics, Blastocystis, Growth inhibition, TLR4, Lactobacillus acidophilus

Background
Blastocystis sp. is a member of an extremely diverse group of heterotrophic/autotrophic protists or Stramenopiles and the only one being associated with human gastrointestinal infection.1 This anaerobic parasite has been known as the most common eukaryotic microorganism in human feces, and it has also been isolated from a wide range of animals.2 Morphologically, Blastocystis sp. demonstrates considerable polymorphism based on various factors such as metabolic and osmotic changes, and four major forms have been discovered so far: the vacuolar and cyst forms are regularly found in feces, whereas amoeboid and granular forms are common in symptomatic patients and old non-axenic cultures.3 In addition, the protozoan represents considerable genetic variations in the small subunit of the ribosomal RNA gene (SSU rDNA), so it is sorted into 22 distinct validated subtypes (STs), with zoonotic (ST1-ST8, ST12), anthropo-entomological (ST9), and animal (ST13-ST22) origins. In human populations, ST1-ST4, especially ST3, have been isolated. Subtypes differ regarding biology, immune response, drug resistance, pathogenicity, and their impact on gut microbiota.4,5 Fruits, vegetables, and/or water supplies contaminated with fecal matter of human or animal origin are the major sources of infection.6 According to the host’s immunity, hygienic conditions, and difficulty in diagnosis, the prevalence of the infection may vary among countries and regions, ranging from 30% to 76% in developed and developing countries, respectively.7,9 Although the commensal or pathogenic nature of Blastocystis sp. has been under debate for years,10 convincing evidence may indicate its pathogenic role in animal models and humans.10,11,12 Furthermore, controversy still exists on the association of Blastocystis sp. infection with irritable bowel syndrome and inflammatory bowel disease (IBD).14 Immunocompromised individuals are at higher risk of the infection, manifesting general gastrointestinal symptoms such as nausea, vomiting, abdominal pain, and diarrhea.7

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Gut microbiota (bacteria, archaea, viruses, fungi, and protists) constitute a well-organized microbial community with a substantial impact on overall human health, metabolism, and immunity. Metagenomics studies have revealed that Blastocystis may indirectly promote a healthy gut state through increasing bacterial diversity while decreasing Bacteroides community-type population, which is strongly associated with inflammation, obesity, celiac disease, and colorectal cancer. On the other hand, a dysbiotic state may be induced directly or indirectly during Blastocystis colonization in the human gut, leading to a significant decrease in some beneficial bacteria, including Bifidobacterium and Lactobacillus. The members of Bifidobacterium (phylum: Actinobacteria) are non-pathogenic, gram-positive, and saccharolytic microorganisms naturally colonizing the human colon during the first days of life. Based on the species/strain, Bifidobacteria exhibit multiple beneficial functions, including intestinal microbial homeostasis, the production of a number of bioactive substances via the bioconversion process, vitamin production, and the control of pro-carcinogenic characteristics within the microbiota. Lactic acid bacteria are other health-promoting microorganisms in the human gut, being represented as gram-positive cocci or rods producing lactic acid during carbohydrate fermentation. The lactic acid bacteria group includes several genera such as Lactococcus, Lactobacillus, Leuconostoc, Enterococcus, Pediococcus, and Streptococcus, which facilitate lactose digestion, improve immune functions, and prevent a diarrheic state. In the food industry, probiotic products contain such good bacteria, providing a healthier state to the gut microbiota as well as metabolic and immune functions.

Toll-like receptors (TLRs) are a conserved family of pattern recognition receptors capable of detecting pathogen-associated molecular patterns. They consist of membrane-associated (TLRs 1, 2, 4, 5, 6, and 10) and intracellular (TLRs 3, 7, 8, and 9) receptors, recruiting adaptor proteins and subsequent signal transduction pathways for the suppression or upregulation of inflammatory genes. Some TLRs are abundantly expressed in the colonic mucosa, including TLR2, TLR4, and TLR5. TLR4 detects bacterial lipopolysaccharides and is associated with gut inflammatory diseases.

The current study was performed to assess the growth inhibitory effects of Lactobacillus acidophilus, L. reuteri, and Bifidobacterium bifidum on Blastocystis ST1 and ST3 through the evaluation of expression changes in TLR4 in Caco2 cell culture.

**Materials and Methods**

**Parasite Culture and Maintenance**

This study used two prevalent Blastocystis subtypes, ST1 and ST3, stored in the Department of Parasitology, Tarbiat Modares University, Tehran, Iran. For parasite culture, DMEM/F12 (GIBCO, Grand Island, NY) culture medium supplemented with ceftriaxone (100 µL/mL) and erythromycin (25 µL/mL) was utilized. To this end, 750 µL of media + bovine serum albumin (BSA) (GIBCO, Grand Island, NY) (4.5:0.5) and 100 µL of parasite samples were incubated in a culture flask at 37 °C, and subsequent culture passages were done after every 48-72 hours.

**In Vitro Experiments**

**Probiotic Bacteria Supernatant and Caco2 Cells**

To this end, 2 × 10⁴ Caco2 cells were enumerated and added to the wells of a 96-well culture plate in triplicate. About 200 µL of complete medium were also added...
to each well and incubated for 24 hours at 37 °C under 5% CO₂. The next day, 100 µL of complete medium and 100 µL of probiotic bacteria supernatant (10, 25, 40, 55, 70, 85, and 100 µg/mL) were added to the wells as triplicate. Additionally, 100 µL of complete medium and 100 µL of metronidazole (1.25, 2.5, 5, 10, 20, 40, and 80 µg/mL) were added to separate wells as triplicate. After 24, 48, and 72 hours of incubation, the supernatant was discarded, and 180 µL of complete medium and 20 µL of prepared 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide were added to each well, covered with an aluminum foil, and incubated for 3-5 hours at 37°C under 5% CO₂. Subsequently, the supernatant was discarded and 200 µL of dimethyl sulfoxide was added to the wells, making a homogenate through pipetting. Finally, the light absorbance of the wells was read in 570 nm and 630 nm (control) wavelengths using an enzyme-linked immunosorbent assay reader device.

**Probiotic Bacteria Supernatant and Blastocystis Species**

Initially, the parasite of both subtypes was enumerated using methylene blue stain and a Neubauer chamber. Then, 10 × 10⁴ parasites were added to a sterile microtube. In the next step, triplicate experiments were performed using different concentrations of probiotic bacteria supernatants (15, 25, and 35 µg/mL) and metronidazole (5, 10, 20, and 40 µg/mL) along with controls. Next, 24, 48, and 72 hours after exposing Blastocystis parasites to bacterial supernatant and metronidazole, 50 µL of microtub content was mixed with 50 µL methylene blue in a new microtube, and the parasite survival was evaluated.

**Quantitative Evaluation of Toll-like Receptor 4 Expression Using Real-Time Polymerase Chain Reaction RNA Extraction**

The RNA extraction was done using a RiboEX kit (GeneAll, South Korea) on ice, as follows: 1 mL of TRIzol reagent was added to each sample and incubated for 5 minutes. Then, 200 µL chloroform was added, vortexed (15 sec), and incubated for 2-3 minutes, and the samples were centrifuged at 12,000 × g for 15 minutes at 4 °C. The aqueous phase containing RNA was transferred to a new sterile microtube, and 10 µg/mL glycogen was added and thoroughly mixed. The obtained RNA was pelleted by mixing with 400 µL cold isopropyl alcohol, the microtubes were then incubated overnight, and centrifugation was done at 12,000 × g for 40 minutes at 4 °C. The RNA pellet was eluted with cold 75% ethanol at 12,000 × g for 10 minutes at 4 °C and was left to be dried. The RNA pellet was finally re-suspended in RNase-free water.

**Complementary DNA (cDNA) Synthesis**

A commercial kit (Easy cDNA synthesis, Pars Tous, Iran) was used for cDNA synthesis. In brief, a mixture of RNA, 10 µL of Buffer-MIX (2X), and 2 µL of reverse transcriptase was prepared and reached a volume of 20 µL using diethylpyrocarbonate-treated water. Subsequently, the mixture was vortexed and kept at room temperature for 10 minutes then incubated for 60 minutes at 47 °C in a thermocycler device to activate the reverse transcriptase enzyme. The reaction was terminated at 85 °C for 5 minutes, and the final mixture was maintained on ice or at 4 °C (short-time) or at -80 °C (long-time).

**Real-Time Polymerase Chain Reaction**

Initially, the experimental groups were sorted as follows: (1) TLR4 gene expression in Caco2 cells was exposed to *L. acidophilus* supernatant in comparison with control, (2) TLR4 gene expression in Caco2 cells was exposed to *L. reuteri* supernatant in comparison with control, (3) TLR4 gene expression in Caco2 cells was exposed to *B. bifidum* supernatant in comparison with control, (4) TLR4 gene expression in Caco2 cells was exposed to Blastocystis ST1 and ST3 in comparison with control, (5) TLR4 gene expression in Caco2 cells along with Blastocystis ST1 was exposed to *L. acidophilus* in comparison with control, (6) TLR4 gene expression in Caco2 cells along with Blastocystis ST1 was exposed to *L. reuteri* in comparison with control, (7) TLR4 gene expression in Caco2 cells along with Blastocystis ST1 was exposed to *B. bifidum* in comparison with control, (8) TLR4 gene expression in Caco2 cells along with Blastocystis ST3 was exposed to *L. reuteri* in comparison with control, (9) TLR4 gene expression in Caco2 cells along with Blastocystis ST3 was exposed to *L. acidophilus* in comparison with control, (10) TLR4 gene expression in Caco2 cells along with Blastocystis ST3 was exposed to *B. bifidum* in comparison with control, and (11) TLR4 gene expression was evaluated in Caco2 cells.

In this study, an SYBR-green-based real-time polymerase chain reaction (real-time PCR) assay was performed. For each reaction (20 µL of total volume), 10 µL of RealQ Plus 2X Master Mix Green, 0.5 µL of F primer (5'-AAGCCGAAAGGTGATTGTTG-3'), 0.5 µL of R primer (5'-CTGAGCAGGGTCTTCTCCAC-3'), 7 µL of distilled water, and 2 µL of synthesized cDNA were thoroughly mixed, then put onto the Rotor-Gene Q device (Qiagen company). The thermal conditions were as follows: one cycle of initial denaturation (95 °C, 10 minutes), followed by 40 cycles of denaturation (94 °C, 15 seconds), annealing (61 °C, 30 seconds), and extension (72 °C, 30 seconds). The Rotor-Gene Q series software was used for analyzing real-time PCR results, cycle threshold (CT), and melting-curve diagram. Furthermore, the Pfaffl formula was used to evaluate the supernatant effects on the changes in the expression of TLR4 in Caco2 cells as fold-change.

**Statistical Analysis**

Statistical analysis was conducted by a two-way ANOVA.
test followed by Tukey’s multiple comparison analysis. All analyses were conducted using GraphPad Prism software version 8, and statistical significance was set at a 95% confidence level for all tests ($P < 0.05$).

**Results**

**Effects of Probiotics Supernatants and Metronidazole on Caco2 Cells**

The toxicity of probiotics supernatants and metronidazole on Caco2 cells was evaluated using an MTT assay. In this regard, 10, 25, 40, 55, 70, 85, and 100 µg/mL of probiotics supernatants and 1.25, 2.5, 5, 10, 20, 40, and 80 µg/mL of metronidazole were examined in 24, 48, and 72 hours. The effect of *L. acidophilus* supernatant on the viability of Caco2 cells in the highest concentrations was 12.77% (24 hours), 9.5% (48 hours), and 8.81% (72 hours), respectively, and it was reported as 94.5% (24 hours), 94.27% (48 hours), and 99.6% (72 hours), respectively, in the lowest concentration. Moreover, the viability of Caco2 cells in the presence of the highest concentration (100 µg/mL) of *L. reuteri* was 11.67% (24 hours), 11% (48 hours), and 8.81% (72 hours), respectively, while the lowest concentration (10 µg/mL) resulted in 96.69% (24 hours), 90.75% (48 hours), and 100% (72 hours) viability, respectively. The effect of *B. bifidum* supernatant on the mean percentage of Caco2 cells in the presence of the highest concentration (100 µg/mL) of *B. bifidum* was 11.04% (24 hours), 11% (48 hours), and 9.54% (72 hours), while the lowest concentration (10 µg/mL) caused 87.81% (24 hours), 99.6% (48 hours), and 99.6% (72 hours) viability, respectively. Regarding the highest concentration of metronidazole (80 µg/mL), the mean percentage of viability was calculated to be 78% (24 hours), 74% (48 hours), and 63.5% (72 hours), respectively, whereas cellular viability in the presence of lowest drug concentration (1.25 µg/mL) was 96.5% (24 hours), 96.2% (48 hours), and 99.6% (72 hours), respectively. Figure 1 depicts the details of probiotics supernatants and their effects on Caco2 cells along with metronidazole.

**Effects of Probiotics Supernatants and Metronidazole on Blastocystis Subtype 1 and Subtype 3**

For this purpose, three concentration of bacterial supernatants (15, 25, and 35 µg/mL) and four concentration of metronidazole (5, 10, 20, and 40 µg/mL) were utilized. Based on the results derived from supernatants of all three examined probiotic bacteria, the survival of *Blastocystis* ST1 and ST3 significantly decreased during 24, 48, and 72 hours ($P < 0.0001$), as depicted in Figures 2-4. Moreover, Figure 5 presents the effects of metronidazole on *Blastocystis* ST1 and ST3.

**Toll-Like Receptor 4 Expression Using Real-time Polymerase Chain Reaction**

As seen in Figure 6, the gene expression of TLR4 in Caco2 cells exposed to *Blastocystis* ST1 and ST3 increased, though it was not statistically significant compared to the control ($P > 0.05$). Moreover, as observed in Figure 7, different concentrations of supernatants caused a decline in TLR4 gene expression in Caco2 cells, while it was not statistically significant compared to control ($P > 0.05$).

**Discussion**

The control of intestinal diseases and providing a healthy micromolecular milieu for the gut to function properly.
possibly result from the significant role of normal gut microbiota. Based on several recent studies, the gut microbiota can change the pattern of Blastocystis localization in the gut lumen. Metronidazole therapy of clinical Blastocystis infection, as the first-line drug, can render drug resistance to parasitic isolates, along with its lower efficacy and potential side effects. In the current study, we investigated the inhibitory effects of some important probiotic bacteria L. acidophilus, L. reuteri, and B. bifidum on in vitro Blastocystis growth through the determination of expression changes in the TLR4 in Caco2 cell culture.
Based on our results, a potent dose-dependent inhibitory effect of supernatant derived from the probiotic bacteria was observed against Blastocystis, highlighting the hypothesis that those people with stable gut microbiota and consuming probiotic-enriched food products are more tolerant to the colonization of the parasitic protozoa such as Blastocystis. On the other hand, a lack of such beneficial microorganisms in the large bowel due to an inappropriate regimen, antibiotic therapy, or some drugs such as proton pump inhibitors may render individuals susceptible to Blastocystis infection.27,28

Several studies have reported that Lactobacillus bacteria exert their protective and/or therapeutic effects through the production of antimicrobial compounds such as bacteriocins, decrease gut pH by stimulating LA-producing microflora, competitively bind to common pathogen-related receptors, and have nutritional competition with pathogenic agents.29 The fact that such probiotic bacterial microorganisms are capable of disrupting the life cycle of Blastocystis protozoan has opened new doors in the consumption of probiotics as a prophylactic treatment for blastocystosis or as an auxiliary nutritional regimen in combination with the standard drugs such as metronidazole.

Immunoregulatory mechanisms, including the regulatory cells, cytokines, and apoptosis, significantly contribute to the immune response control by deterring pathologic consequences in association with hyper-reactive immune functions; hence, most inflammatory disorders may arise when such regulatory mechanisms are lacking. An interesting hypothesis for the physiological function of the probiotic compounds focuses on their immunomodulatory capacity, so a great number of studies have emphasized this aspect of probiotic bacteria.30 Indeed, it has been found that some probiotic bacteria can modulate the in vitro expression of some of the pro-inflammatory and/or anti-inflammatory cytokines in a strain-dependent manner. For instance, L. sakei can induce interleukin-1 (IL-1), IL-8, and tumor necrosis factor α (TNF-α), whereas L. johnsonii instigates the expression of tissue growth factor in Caco2 cells.31

In the present study, the expression changes in the TLR4 gene were investigated in Caco2 cells exposed to the ST1 and ST3 of Blastocystis protozoan and supernatant of three probiotic bacteria, including L. acidophilus, L. reuteri, and B. bifidum. The results showed that Blastocystis can increase the expression of TLR4 molecule, though it was not statistically significant; on the contrary, a decrease in TLR4 expression was observed when Caco2 cells were exposed to the metabolites of the probiotic bacteria found within their supernatants. As a generally accepted concept, inappropriate responses of the mucosal immunity to the resident microbiota and other harmful antigens can result in the initiation and/or progression of IBD. Within the gut lumen, the host immune system along with millions of commensal microorganisms such as fungi, viruses, and bacteria maintain gut homeostasis.32,33 It has been recently found that TLR4 performs a central role in eliciting immune mechanisms towards the gut microbiota. The activation of the TLR4 signaling can lead to downstream cascades, being important in the initiation and progression of IBD.34 In sum, TLR4 initially detects pathogen-associated molecular patterns during inflammatory responses within the gut, then it employs myeloid differentiation factor 88 for subsequent nuclear factor kappa B (NF-κB) activation. The latter, in turn, leads to the production of different pro-inflammatory and inflammatory mediators. The NF-κB is an important transcription regulatory molecule that plays a critical role in the modulation of multiple cellular processes such as inflammation.35 Moreover, the activation of NF-κB can induce the production of pro-inflammatory cytokines such as tumor NF-α, IL-1β, and IL-12. In general, this signaling
pathway can induce the over-expression of inflammatory cytokines in the gut and subsequent stimulation toward IBD. It has been speculated that the metabolites produced by the probiotic bacteria can prevent the activation of NF-κB, hence decreasing the expression of TLR4 gene. One of the limitations of the present study was the use of bacterial supernatant instead of a direct study on antimicrobial peptides (bacteriocins) extracted from probiotic bacteria. It is suggested to confirm the results obtained in the mentioned specific protein samples in future studies. In future research, in vivo investigations in animal models are needed and necessary.

Conclusion
In sum, it is an emerging concept that probiotics can gain control of the growth of eukaryotic pathogens, and therapeutic approaches using probiotics can decrease the risk of parasitic infections or can be used as a complementary regimen along with traditional antiparasitic therapies. It is necessary to gain a better understanding of the underlying molecular mechanisms of the beneficial effects of probiotics in parasitic infections; hence, more extensive clinical research would broaden our knowledge of this issue. Future studies should emphasize the role of probiotics in the systemic immune responses as well as those in the gut membranes. In addition, future investigation on the impact of probiotics on parasitic infections such as Blastocystis sp. can be tested in transgenic and/or knockout mice for a better understanding of the underlying mechanisms.

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Authors’ Contribution
Conceptualization: Javid Sadraei,
Data curation: Mohammad Gorgipour,
Formal analysis: Mohammad Gorgipour, Majid Pirestani,
Funding acquisition: Javid Sadraei,
Investigation: Mohammad Gorgipour,
Methodology: Mohammad Gorgipour, Majid Pirestani, Ashraf Mohabati Mobarez,
Project administration: Javid Sadraei,
Resources: Javid Sadraei,
Software: Majid Pirestani,
Supervision: Javid Sadraei,
Validation: Javid Sadraei,
Visualization: Javid Sadraei, Majid Pirestani,
Writing—original draft: Mohammad Gorgipour, Javid Sadraei, Majid Pirestani, Ashraf Mohabati Mobarez,
Writing—review & editing: Mohammad Gorgipour, Javid Sadraei.

Competing Interests
The authors have no relevant financial or non-financial interests to disclose.

Data Availability Statement
The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval
All the samples were obtained and approved by the Institutional Health Research Ethics Committee of Tarbiat Modares University (IR.MODARES.REC.1397.139).

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