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Original Article

Lactobacillus and Bifidobacterium of Patients with Strongyloidiasis Compared with the Control Group

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ABSTRACT

In individuals with compromised immune systems, strongyloidiasis disease can lead to disseminated infections that can be fatal if diagnosis and treatment are delayed. The human gut is composed of numerous bacteria that play essential roles in the development of acquired immunity, and protection against pathogenic factors. This case-control study was conducted on individuals who were referred to the Diagnostic Laboratory of Strongyloidiasis in the School of Public Health, Tehran University of Medical Sciences. After DNA extraction from fecal samples, the 16SrRNA gene was examined using Real-time PCR. The levels of Lactobacillus acidophilus and Bifidobacterium bifidium were calculated in both groups (one group consisted of individuals suspected of strongyloidiasis, compared with the other group with no underlying disease). Finally, the collected data were analyzed. Out of 28 participants in this study, 16 (57%) were men and 12 (43%) were women, with ages ranging from 43 to 76 years. A statistically significant relationship was observed between underlying diseases, vegetable washing practices, and clinical symptoms of strongyloidiasis. DNA extraction from the fecal samples was performed using a DNA Extraction kit. The average level of L. acidophilus and B. bifidium were (4.07250±3.132533) 1012× and, (6.12857±3.519169) 1012× in the case group, respectively, which were lower compared to the control group.However, no significant association was found between the bacterial levels in the case and control groups and the incidence of strongyloidiasis ($p>0.05$), the control group had (7.04733± 6.542372) 1012×and (8.36643± 4.754185) 1012×, respectively. The odds ratio for L. acidophilus and B. bifidium were 1.13 and 1.14, respectively. It was observed that for each increase of 10 12 in the microliter of L.acidophilus and B. bifidium in the individual's intestines in areas endemic for strongyloidiasis, the chances of contracting this disease decreased by 13% and 14%, respectively. Future studies with a larger sample size, considering age, gender and other physiological factors related to strongyloidiasis, are suggested.

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1. Introduction

Strongyloidiasis is a disease caused by infection with *Strongyloides stercoralis* (*S.s*), a soil-transmitted helminths (STH) (1). This parasite is prevalent in tropical and subtropical regions (2), with an estimated 613 million people infected worldwide (3). Control and elimination strategies aimed at reducing the complications of strongyloidiasis, are among the main goals of the World Health Organization (WHO) through 2030 (4). This nematode is endemic in the northern and southern provinces of Iran (5). *S. stercoralis* has a unique life cycle that includes both free-living and parasitic stages (6). Most patients with strongyloidiasis are chronic carriers with on clinical symptoms (7). However, adult female worms reside deep in the intestinal crypts, where they lay eggs, potentially causing ileus paralysis. Larvae are dispersed throughout the ileum, causing mucosal damage and increasing mucus production, which can lead to edema and ultimately their atrophy. Symptoms include abdominal bloating, diarrhea, loss of appetite, malabsorption, steatorrhea (fatty stool), nausea, vomiting, and occasional constipation (7, 8).

In immunocompromised individuals, parasite burden significantly increases, leading to hyperinfection syndrome and disseminated disease, in such cases can even lead to the death of the host (9-11). The human intestine is composed of a large number of microorganisms, predominantly bacteria, forming a highly complex and diverse ecosystem with extensive genetic diversity. The collection of these microorganisms, along with their genomes in the gastrointestinal tract, is referred to as the gut microbiome, which varies based on geography regions, ethnicity, endemic and non-endemic regions of various diseases (12, 13). The gut microbiome plays a significant role in the functioning of both the acquired and innate immune system cells (14) and has a direct relationship with intestinal mucosal immunity (15).

Bacteria such as *Lactobacillus* and *Bifidobacterium* species are effective in improving the mucosal system of the digestive system and enhancing the host immune system (16). In recent years, the use of these bacteria ,as probiotics, have increased, and scientific advances in fields such as sequencing, metagenomics, and bioinformatics have provided a research platform for studying the role of the microbiome and controlling physiological systems, including the digestive system, immunity, and metabolism (16, 17). In this regard, it has been reported that the gut microbiome in untreated celiac patients has a significant reduction in *Lactobacillus* and *Bifidobacterium* compared to the control group (14).

Disruptions in the gut microbiota composition have been observed in gastrointestinal and systemic diseases such as autoimmune and allergic diseases, obesity, diabetes, and multiple myeloma (18-21). Intestinal worm parasites are harmful to human health due to nutritional competition with the host; therefore, worm infections can have broad effects on the host gut microbiome (22-24).

Recent studies confirm the hypothesis that infections with *Ascaris* spp, *Trichuris trichiura*, and hookworms in the gastrointestinal tract may play a positive or negative role in gut homeostasis by modulating the gut microbiome (25, 26). In recent years, scattered studies in endemic areas of strongyloidiasis worldwide have reported that the gut microbiome in patients with strongyloidiasis differs compared to healthy groups (27). Additionally, changes in the gut microbiome of strongyloidiasis patients before and after treatment have also been reported (28).

The changes in microbiota in patients with strongyloidiasis raise the hypothesis that alterations, especially bacteria such as *Lactobacillus* and *Bifidobacterium*, which are effective in maintaining the gut immune system (14), may have an impact on the conversion of chronic forms of strongyloidiasis to acute forms or can be utilized as probiotic agents in the treatment of patients and reduction of gastrointestinal symptoms and complications. However, studies in this area are very limited and apart from a few studies on other profiles of the gut microbiota (27-29), no study has yet addressed this question in Iran. Therefore, the present study was designed to investigate the levels of *Lactobacillus* and *Bifidobacterium* in the gut of patients with strongyloidiasis compared with the control group (non-strongyloidiasis) for the first time in Iran.

2. Materials and Methods

2.1. Ethical Approval

The project was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1402.178 All procedures were conducted in accordance with . We received written signed consent from all study participants.

2.2. Study Participants and Sample Collection

A case-control study was conducted between 2023-2024. The case group consisted of individuals suspected of strongyloidiasis who were referred to the Diagnostic

Laboratory of Strongyloidiasis at the School of Public Health, Tehran University of Medical Sciences. The control group comprised volunteers who were matched in terms of age and gender with the case group with no underlying disease or digestive problems. A total of 28 people participated in this study, which was categorized into strongyloidiasis (n=14) and non- strongyloidiasis (n=14) groups.

Initialverbal consent was obtained from individuals in both groups, followed by the completion of a questionnaire containing demographic information and clinical symptoms. Subsequently , three fecal samples were collected from each participant (or one sample in case of non-cooperation), and examined using parasitological methods, including direct smear, formalin-ether concentration, and agar plate culture. Differentiation of *S. stercoralis* from other intestinal nematodes was performed based on the morphological characteristics of the larva (5). All fecal specimens, upon arrival at the microbiology laboratory, were transferred to a freezer and kept at −80°C.

2.3. Molecular methods

DNA extraction from the fecal samples was performed using the Vira Gene Total DNA Extraction kit (Cat. No: VTO-2050), following the manufacturer's protocol. The investigation of the 16SrRNA gene for *L. acidophilus* and *B. bifidum* was conducted using primers (Table 1). *L. acidophilus* ATCC 4356, *B. bifidum* ATCC 29521 were used in this study as reference strains.

2.4. Real-Time PCR

PCR reactions were performed using the following reaction mixture: 10µL of 5 × 5X Real Time PCR master mix (High Rox amplicon),1 µM of each primer, 2 µL of DNA template, and 6 µL high pure water in a final volume of 20 µL.

Amplification and detection were performed using an ABI Step One real-time PCR machine (Applied Bio Systems, Foster City, CA). The amplification program consisted of a holding stage step at 95°C for 30 seconds, followed by 40 cycles of 30 seconds at 94°C, and a combined annealing/extension step at 62°C for 30 seconds. Finally, the cycling stage is at 72°C for 60 seconds.

To assess the bacterial load in the samples, a standard curve was prepared using 0.5 McFarland (pure culture) of *L. acidophilus* and *B. bifidum*, and then the standard curve was plotted. Subsequently, serial dilutions of standard DNA strains of *L. acidophilus* and *B. bifidum* were prepared, and their OD 260/280 was measured using NanoDrop (Thermo Scientific, USA). Then, the results were read, and for Real-time PCR, 100 ng/µl of sample DNA from both case and control groups was used. After calculating the copy numbers of DNA present in the samples and preparing a series of consecutive dilutions from each of the prepared dilutions, 2 µl of each dilution was used in Real-time PCR reaction.

2.5. DNA Concentration and Copy Number Determination

Based on DNA concentration, copy numbers were calculated according to the following formula (30):

$$\text{Number of copies (molecules)} = \frac{X\text{ng} \times 6.0221 \times 10^{23}\text{molecules/mole}}{(N \times 660\text{ g/mole}) \times 1 \times 10^{9}\text{ng/g}}$$

Avogadro's number = 6.0221×10^{23}

X =DNA concentration is calculated according to Ct and standard curve.

N= length in base pair: *L. acidophilus* (1.95 bp) and *B. bifidum (*2.3bp)

Weight average of a base pair (g/mole) = 660

2.6. Data Analysis.

Data analysis was performed via Stata Version 17 and Fisher's exact T test and Mann-Whitney U test, finally determining the Odds Ratio. The significance level was considered at *p*-value <0 .05.

3. Results

16 males (57%) and 12 females (43%)participated in this study ,which was categorized into strongyloidiasis (n=14) and non-strongyloidiasis (n=14) groups based on their parasitological results. Their age ranged from 43 to 76 (mean= 65.36) years old. Based on parasitological methods, 14 individuals in the case group were positive for *S. stercoralis*.

In this study, 4 patients out of the case group had hyperinfection of strongyloidiasis. Participants in the study used tap water, regular water, and treated water for washing vegetables, and only 2 individuals out of all participants reported direct contact with soil, with one person mentioning contact with various animals.

Table 1.16S rRNA primers used to analyze *Lactobacillus acidiphilus* and *Bifid bacterium bifidum* in fecal samples.

Target bacteria	Primer	Oligonucleotide sequence (5′-3′)	Size (bp)	Product size(bp)	Reference
L.acidiphilus	Primer F	CCT TTC TAA GGA AGC GAA GGA T	22	129	*
	Primer R	ACG CTT GGT ATT CCA AAT CGC	21		
B. bifidum	Primer F	CCACATGATCGCATGTGATTG	21	185	**
	Primer R	CCGAAGGCTTGCTCCCAAA	19		

In the case group, 7 individuals (50%) had at least one underlying disease, among whom diabetes was observed in 4 patients (28.57%) out of 14 patients in the case group. Individuals with strongyloidiasis predominantly exhibited gastrointestinal, respiratory and dermatological symptoms. Additionally, none of the patients in this study had larval currents. By reviewing the medical records of individuals with strongyloidiasis, eosinophilia was observed in 7 patients' records (50%), ranging from 7% to 29% (mean: 12%). Statistical analysis of demographic information and strongyloidiasis can be found in Table 2.

However, a significant association was observed between the method of washing vegetables, clinical symptoms and underlying disease with strongyloidiasis ($p < 0.05$).

3.1. Molecular results

Real-time PCR test based on the 16SrRNA gene for *L. acidophilus* and *B. bifidum* was performed using the ABI 7500 Real-Time PCR system (USA). Initially, standard curves for the 16SrRNA A genes were plotted using primers specific to this study, based on standard cultures of *L. acidophilus* and *B. bifidum* (Tables 3 and 4) (Figures 1 and 2).

After conducting Real-time PCR tests, the Ct values for the case group for *L. acidophilus* ranged from 22.72 to 30.99 (with a mean of 26.65307 ± 2.513348), and for *B. bifidum* ranged from 20.16 to 29.16 (with a mean of 24.82079 ± 2.867510). In the control group, the Ct values for *L. acidophilus* ranged from 18.36 to 31.473 (with a mean of 25.93036 ± 4.141819) (Figures 3 and 4), and for *B. bifidum* ranged from 17.13 to 28.19 (with a mean of 22.93429 ± 3.853246) (Figures 5 and 6).

The average number of *L. acidophilus* and *B. bifidium* were (4.07250±3.132533)× 10^{12} and, (6.12857±3.519169) × 10^{12} in the case group respectively, which were lower than those in the control group, which had (7.04733± 6.542372)×10^{12} and (8.36643± 4.754185)×10^{12} respectively (Figure 3 and 4). However, with this sample size, various statistical analyses did not significant difference between the level of bacterial in the case and control groups and strongyloidiasis. In this study,

the level of *L. acidophilus* was in the 40 - 59 age group (7.09±6. 43)× 10^{12} higher than the 60 - 79 age group (4.51±5.41)× 10^{12}, and level of *B. bifidum* was in the 60 - 79 age group (5.3 (11.9-4.19)) × 10^{12} higher than the 40 - 59 age group (5.6 (10.9-3.9)) × 10^{12}, but no significant association was observed between age and level of bacterial with strongyloidiasis ($p > 0.05$). There were 16 male and 12 female participants. Examining the level of *L. acidophilus* was higher in males (5.9 ± 6.07) ×10^{12}than in females (3.9± 4.6) , ×10^{12} while the level of *B. bifidum* was higher in females (6.3 (12.5-5.2)) ×10^{12} than in males (4.9 (10.1-3.4)) ×10^{12}. However, no significant relationship was found between gender and level of bacteria ($p > 0.05$). Finally, the odds ratio were 1.13 for *L. acidophilus* and 1.14 for *B. bifidium.*

4. Discussion

The neglected intestinal nematode *S. stercoralis*, is the causative agent of strongyloidiasis (1). It can manifest in patients from asymptomatic carriage to hyperinfection and disseminated disease, depending on the host immune system (2).

Bacteria are the most important component of the gut microbiome, playing a crucial role in maintaining gut homeostasis and both innate and acquired immune responses against pathogens (15, 31). Recent studies have reported that helminthic infections in the gastrointestinal tract can lead to alterations in the gut microbiome (25). However, limited research has been conducted on the profiles of gut microbiota and strongyloidiasis in recent years worldwide (27, 28). Additionally, no studies have been conducted in this regard in Iran. Therefore, the present study aimed to investigate the levels of *Lactobacillus* and *Bifidobacterium* in the intestines of patients with strongy loidiasis compared to a control group (individuals without strongy loidiasis) based on 16SrRNA gene, for the first time in Iran.

In the current study, two groups were examined: the case group and the control group, each consisting of 14 individuals matched for gender and age. In this study, no significant relationship was found between occupation,

Table 2. Summary of the results that examined the relationship between demographic data and strongyloidiasis

Variable	Test	*P*-Value
Age	Fisher's	***p* >0.05**
Gender	Fisher's	***p* >0.05**
Underlying disease	Fisher's	***p* >0.05**
Diabetes	Fisher's	***p* < 0.05**
Clinical symptoms	Fisher's	***p* >0.05**
Washing vegetables	Fisher's	***p* < 0.05**
Contact with soil	Fisher's	***p* >0.05**
Contact with various animals	Fisher's	***p* >0.05**

Table 3. Standard Curve of 0.5 McFarland *L.acidophilus.*

ng/µl	CT			Mean
49/23	14/12	14/19	14/11	14/14
37/26	16/19	17/01	17/53	16/91
22/649	19/15	19/29	19/36	19/26667
11/364	24/36	24/45	24/5	24/43667
4/875	28/13	28/15	28/19	28/15667

Table 4. Standard Curve of 0.5 McFarland *B. bifidum.*

ng/µl	CT			Mean
51/873	13/23	13/36	13/37	13/32
40/218	15/64	15/62	15/55	15/60333
33/678	19/43	19/76	19/7	19/63
25/471	22/36	22/29	22/31	22/32
12/319	25/32	25/36	25/41	25/36333

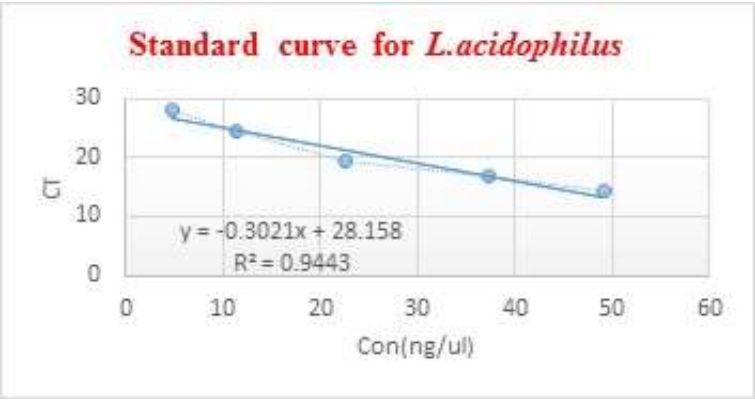

Figure 1. Standard Curve Graph of *L. acidophilus.*

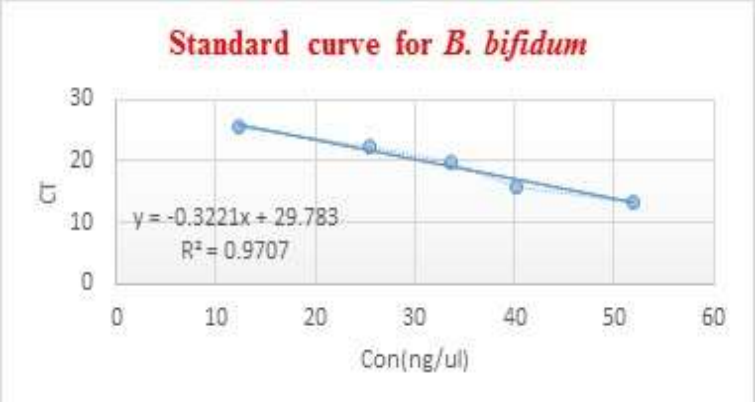

Figure 2. Standard Curve Graph of *B. bifidum.*

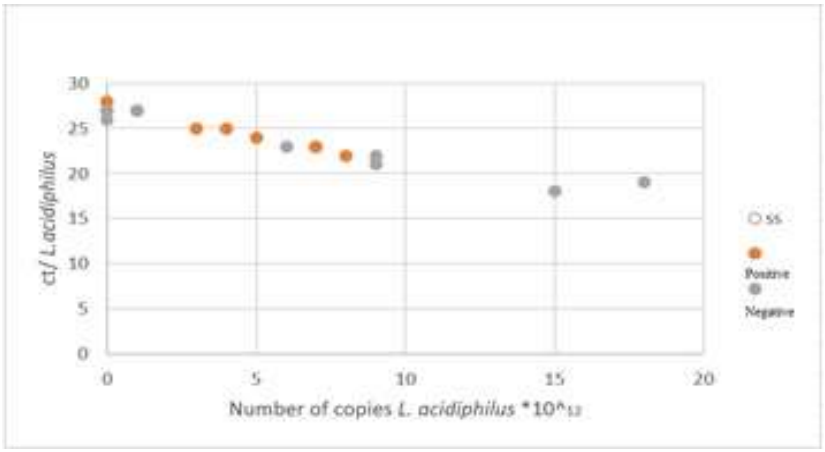

Figure 3. Comparison of *L. acidophilus* bacterial count and Ct values in the case and control groups in the present study.

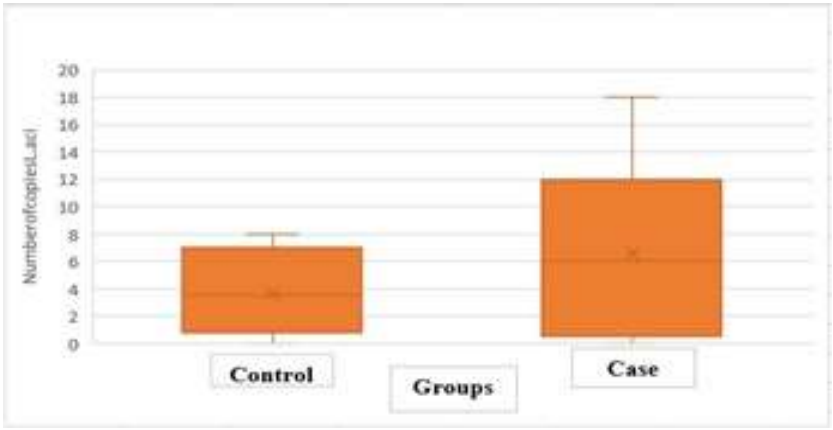

Figure 4. *Lactobacillus* quantified by Real time qPCR and expressed as copy number in patient and healthy volunteers.

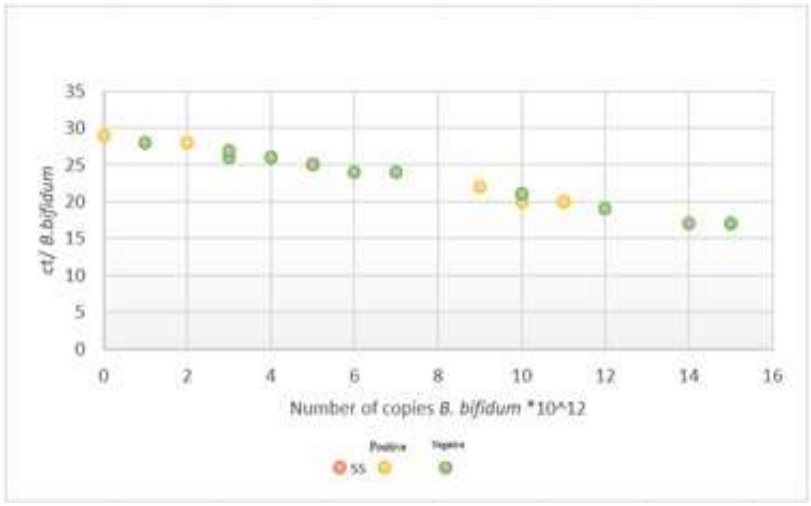

Figure 5. Comparison of *B. bifidum* bacterial count and Ct values in the case and control groups in the present study.

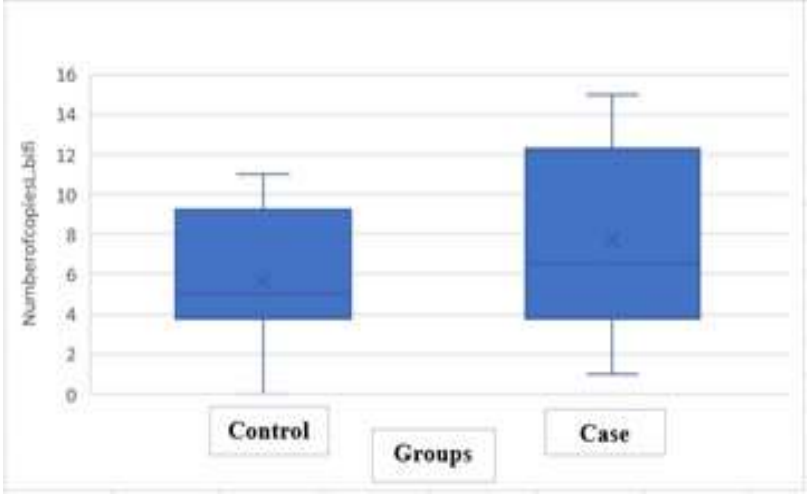

Figure 6. *B. bifidum* quantified by Real time qPCR and expressed as copy number in patient and healthy volunteers.

direct contact with soil, contact with various animals, and strongyloidiasis (p>0.05). However, a significant association was observed between the method of washing vegetables and the incidence of strongyloidiasis (p< 0.05). Individuals with strongyloidiasis predominantly exhibited gastrointestinal, respiratory and dermatological symptoms, and a significant correlation was found between clinical symptoms and strongyloidiasis in this study (p < 0.05). Furthermore, none of the patients in this study reported larval currents.

Based on this study, recent studies in Iran have reported that patients with strongyloidiasis present with at least one clinical symptom, including gastrointestinal, dermatological, or respiratory manifestations. The prevalence of clinical symptoms in some studies is consistent with our findings, encompassing gastrointestinal, respiratory, and dermatological symptoms (32).

Sometimes, contrary to our study, dermatological symptoms have been reported more frequently than respiratory symptoms (33). However, in these studies, no larval currents have been observed in any patients, which is consistent with our findings. In the present study, eosinophilia was observed in the medical records of 7 patients (50%), ranging from 7% to 29% (with a mean of 12%), which is consistent with previous studies conducted (32, 33).

In the present study, the level of bacterial in the case groups was as follows: *L. acidophilus* and *B. bifidum* were calculated to be (4.07250 ± 3.132532) $\times 10^{12}$ and (6.12857 ± 3.519169) $\times 10^{12}$, respectively. These counts were lower than those in the control group, which were (7.04733 ± 6.542372)$\times 10^{12}$ and (8.36643 ± 4.754185)$\times 10^{12}$ respectively. However, with this sample size, no significant association was found between the level of bacterial in the case and control groups and the incidence of strongyloidiasis (p > 0.05). In our study, the counts of *L. acidophilus* and *B. bifidum* in different age groups within the case and control groups showed that, despite the higher count of *L. acidophilus* in the age 40 - 59 years compared to 60 _ 79 years and the higher count of *B. bifidum* in the age group of 60 - 79 years compared to 40 to 59 years, no significant association was found between age and bacterial counts with the incidence of strongyloidiasis (p > 0.05). Additionally, despite the higher count of *L. acidophilus* in men compared to women and the higher count of *B. bifidum* in women compared to men, no significant association was found between gender and bacterial counts in this study (p > 0.05).

Furthermore, by examining the 16SrRNA gene of the gut microbiome in individuals infected with soil-transmitted helminths during treatment with a single dose of albendazole (400 mg), a reduction in the gut microbiome of patients was observed 10 to 14 days after treatment. The results of this study suggested the possibility of using probiotic supplements as an adjunct therapy to enhance the effectiveness of albendazole (34). Additionally, in a study on children in a rural area in Thailand infected with soil-transmitted helminths such as *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms, the gut microbiome was examined before and after treatment using the V4 region of the 16SrRNA gene. Significant alpha diversity in the bacterial microbiome was not observed, but beta diversity, including an increase in *Akkermansia muciniphila* and *Bacteroides corprophilus*, and a decrease in *Bifidobacterium adolescentis*, was reported in these individuals (25).

By examining the 16SrRNA gene of the gut microbiome in individuals positive for *S. stercoralis* in northern Thailand before and after treatment, an increase in alpha diversity of the gut microbiota and a decrease in beta diversity in individuals positive for *S. stercoralis* compared to *S. stercoralis*-negative individuals were reported. In this study, individuals' positive for *S. stercoralis* showed increased levels of fecal amino acids, while those negative for *S. stercoralis* showed increased levels of short-chain fatty acids in feces (27, 35). Additionally, by investigating the effect of chronic strongyloidiasis infection on the gut microbiome of 42 volunteers (divided into two groups of patients and healthy individuals) based on the 16SrRNA gene, it was reported that *Ruminococcus* torques was more abundant in patients, suggesting that this increase may enhance the patient's ability to expel the parasite effectively. According to this study, chronic infection with *S. stercoralis* alters the proteomic composition of the host gut bacteria (28).

However, it should be noted that *Bifidobacterium* and *Lactobacilli* species have been identified as the best microbial options for enhancing the immune system in various studies (15). In the present study, the level of *L. acidophilus* compared to *B. bifidum* showed a greater

difference between the case and control groups. However, ultimately, due to the low sample size, no significant relationship was observed between the level of bacterial and susceptibility to strongyloidiasis.

It was observed that for every increase of 10^{12} bacteria per microliter of *L. acidophilus* and *B. bifidum* in the intestines of individuals in endemic areas of strongyloidiasis, the chance of developing strongyloidiasis decreased by 13% and 14%, respectively. However, for a more comprehensive investigation of the relationship between the levels of *L. acidophilus* and *B. bifidum* in the gut of strongyloidiasis patients (taking into account their gender and age), a larger sample size from various geographical regions in different age groups will be required in future studies.

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Authors' Contribution

A. K: carried out the laboratory experiments, the prepared the draft of the manuscript. E.B.K: revision of the work, Editing of the manuscript. S. B. J: contributed to the conceptualization of the study. N.F: participated in data analysis and interpretation. E.D: Editing of the manuscript. Z. F. K*: article writing and study designed.

Ethics

This study was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1402.178). All stages of research were conducted following the Declaration of Helsinki. Written informed consent was obtained from the patient for publication of this case report.

Conflict of Interest

The authors declare that they have no competing interests.

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Data Availability

All data generated are included in the current article.

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