Evaluation of the role of combined prebiotic and probiotic supplements as prophylactic and therapeutic agents against experimental giardiasis

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ABSTRACT

Background: Giardiasis is considered as one of the most prevalent gastrointestinal parasitic infections worldwide. Most of the used anti-Giardia drugs may cause side effects and are often associated with clinical failure and drug resistance. Prebiotic and probiotic therapy has been posited as potential natural therapeutic agent for many parasitic diseases.

Objective: To evaluate the effect of combined prebiotic and probiotic therapy as a natural supplement and after addition to nitazoxanide (NTZ) in treatment of giardiasis in murine model.

Material and Methods: Ninety-six laboratory bred Swiss albino male mice were used. Mice were divided into three main groups that included 16 subgroups (6 mice each). Control group (A1-7) included control negative (non-infected non-treated), control positive (infected non-treated), and another five control subgroups (SGs) that received natural prebiotic and probiotic supplements and NTZ. Prophylactic group (B) included three SGs in which infected mice received prebiotic and probiotic separately and in combination for one week before infection and continued till the end of experiment. Infected treated group (C1-6) composed of six infected SGs that received treatment by prebiotic and probiotic separately, and combined prebiotic/probiotic, NTZ alone (full dose), and after addition of combined supplement to NTZ (half or full dose). Evaluation was achieved using parasitological, histopathological, immunohistochemical, and immunological parameters.

Results: There was significant reduction of Giardia cyst shedding in all prophylactic and infected treated SGs. The highest mean cyst reduction was observed in group C after addition of combined supplement to NTZ post infection (PI) and in group B receiving prophylactic combined supplement. Remarkable improvement of histopathological findings was observed especially in group C after receiving combined supplement in addition to NTZ, followed by other prophylactic and infected treated SGs. The results of immunohistochemical caspase 3 expression revealed that combined supplement was effective in preventing apoptotic cell death of intestinal cells that is induced by giardiasis especially after addition of combined supplement to NTZ in group C. Immunological assessment of interleukin-6 (IL-6) level showed significant increase in all treated SGs with boosted elevation in combined supplement after addition to NTZ in group C.

Conclusion: Combined prebiotic and probiotic products can be considered as good prophylactic and therapeutic agents when added to NTZ against giardiasis.

Keywords: caspase 3, giardiasis, interleukin 6, murine, nitazoxanide, prebiotic, probiotic.

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INTRODUCTION

Giardia is one of the most prevalent intestinal flagellated protozoans that causes diarrhea in both children and adults[1]. Giardiasis has a worldwide distribution with a higher incidence rate in developing countries than developed ones and a higher infection rate in children than adults[2]. In certain localities in Egypt, the prevalence of giardiasis is up to 30.2%, a fact that makes Egypt a hyper-endemic region according to the World Health Organization (WHO) criteria[3]. Giardiasis is transmitted via the fecal-oral route through direct or indirect ingestion of cysts. Symptoms of infection vary from asymptomatic to acute steatorrhea, nausea, epigastric pain, and weight loss[4]. Post-infection complications may arise after Giardia elimination in the form of stunting failure to thrive, chronic fatigue syndrome, irritable bowel syndrome, allergies and arthritis[5].

The host defense against giardiasis includes both innate and adaptive immune responses that act in synchrony to control the infection. Innate immune mechanisms are the first line of defense against
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Giardiasis. Adaptive immune mechanism requires mucosal humoral and cellular immune responses such as a balanced response of antigen-specific CD4+ T cells, the release of cytokines, including ILs 4 and 6, tumor necrosis factor (TNF)-a, and interferon (IFN)-c, and the production of specific IgA or IgG antibodies against parasite antigens. The release of cathepsin-like cysteine proteases allows this parasite to attenuate local inflammatory responses. One of the significant histopathological changes observed in giardiasis is a diffuse shortening of brush border microvilli. Villus atrophy and crypt hyperplasia was also reported. Giardiasis induces this enteric pathology by activation of caspases that are the key mechanism of enterocytes apoptosis.

Among the forms of treatment for giardiasis, the use of chemotherapeutic drugs such as nitroimidazoles, furazolidone, paromomycin, benzimidazole compounds, NTZ as well as phototherapies are commonly highlighted. However, due to an increase in resistance to these compounds, there is an urgent need for the development of new therapeutic strategies to fight the pathogen more healthily and effectively. Therefore, natural interventions and improvement of the intestinal microbiota through probiotic administration may be an important supplement for treatment.

Table 1. Classification of mice into study groups and subgroups.

<table>
<thead>
<tr>
<th>Groups and subgroups</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control group (A)</strong></td>
<td>Non-infected non-treated.</td>
</tr>
<tr>
<td>A1</td>
<td>Infected non-treated.</td>
</tr>
<tr>
<td>A2</td>
<td>Non-infected, received prebiotic.</td>
</tr>
<tr>
<td>A3</td>
<td>Non-infected, received probiotic.</td>
</tr>
<tr>
<td>A4</td>
<td>Non-infected, received combined prebiotic/probiotic.</td>
</tr>
<tr>
<td>A5</td>
<td>Non-infected, received NTZ (full dose).</td>
</tr>
<tr>
<td>A6</td>
<td>Non-infected, received NTZ (half dose) + combined prebiotic/probiotic.</td>
</tr>
<tr>
<td><strong>Prophylactic group (B)</strong></td>
<td>Received prebiotic, then infected.</td>
</tr>
<tr>
<td>B1</td>
<td>Received probiotic, then infected.</td>
</tr>
<tr>
<td>B2</td>
<td>Received combined prebiotic/probiotic, then infected.</td>
</tr>
<tr>
<td><strong>Infected treated group (C)</strong></td>
<td>Infected, received prebiotic PI.</td>
</tr>
<tr>
<td>C1</td>
<td>Infected, received NTZ (full dose) + combined prebiotic/probiotic PI.</td>
</tr>
</tbody>
</table>

Therefore, the present study was designed to evaluate the effect of combined prebiotic and multi-strain probiotics against giardiasis as a natural prophylactic agent and therapeutic when added to NTZ.

**MATERIAL AND METHODS**

This case-control (experimental) study was conducted at Theodor Bilharz Research Institute (TBRI) and Parasitology Department, Faculty of Medicine for Girls (FMG), Al-Azhar University during the period from October 2019 to May 2021.

**Study design:** Mice were classified into three main groups: control (A), prophylactic (B), and infected treated (C). Groups were divided into 7, 3 and 6 SGs (6 mice each), respectively (Table 1). Animals infection was conducted on the 7th day, and were sacrificed using intraperitoneal anesthesia after 30 days PI. Parasitological, histopathological, immunohistochemical, and immunological parameters were used to achieve study objective.

**Experimental animals:** Laboratory bred Swiss albino male mice (No. = 96) weighing 20-25 g were divided as
6 mice for each of 16 SGs. Only parasite-free mice after stool examination were selected. The animals were provided and kept in TBRII Animal Producing Unit. They were kept also under specific pathogen-free conditions as well as lighting and temperature with free access to standard laboratory water and diet containing 24% protein, 4% fat and about 4-5% fiber.

**Mice infection**: Stool samples were collected from patients complaining of steatorrhea attending the outpatient clinics at Alzahraa and Abu-Elrish hospitals. Samples were microscopically examined by direct wet mount (with and without iodine)\(^\text{[22]}\) to obtain positive samples that were further processed by washing with saline and centrifugation for preparation of *Giardia* cysts inoculum. The latter was counted and adjusted to infect mice orally with 10\(^4\) *Giardia* cysts using orogastric gavage according to Dyab *et al*\(^\text{[22]}\). One week after infection, fecal pellets were collected daily and subjected to parasitological examination to ensure infection\(^\text{[23]}\).

**Drugs and regimen dose**
- Commercial probiotic capsules were purchased from New Rhythm Comp. (New York, USA). They are composed of 25 billion multi-strain culture forming units (CFU/capsule). Each capsule is formulated with 20 of the most human probiotic strains (*L. acidophilus, L. rhamnosus, L. crispatus, L. plantarum, L. paracasei, L. bulgaricus, L. reuteri, L. casei, L. salivarius, L. helveticus, L. gasseri, Bifidobacterium (B.) lactis, B. bifidum, B. longum, B. breve, B. adolescentis, B. infantis, Leuconostoc mesenteroides, Lactococcus lactis, Streptococcus thermophiles*). Every capsule was emptied in 2.5 ml distilled water (DW) and was given in a dose of one billion CFU/mouse/day\(^\text{[23]}\). Each mouse received 0.1 ml/day, i.e. one billion CFU/day. Mice of SGs A4, A5, A7, B2, and B3 received the probiotic dose daily from the first day till the end the experiment (37 days). In infected treated SGs (C2, C3, C5, and C6); mice received the probiotic dose daily from the 12\(^\text{th}\) day PI till the end of the experiment (22 days).

- Commercial prebiotic (Inulin) pure powder was purchased from Bulk Food Warehouse (Burlington, Canada). It was prepared daily by dissolving 200 mg inulin powder in 10 ml DW, and given in a dose of 2 mg/0.1 ml/mouse/day\(^\text{[19]}\). Mice of SGs A3, A5, A7, B2, and B3 received the prebiotic dose daily from the first day till the end the experiment (37 days). In infected treated SGs (C1, C3, C5, and C6); mice received the prebiotic dose daily from the 12\(^\text{th}\) day PI till the end of the experiment (22 days).

- Nitazoxanide was obtained from Utopia Pharmaceuticals (Cairo, Egypt). It was given orally as a suspension form at a full dose of 100 mg/kg body weight and a half dose of 50 mg/kg body weight for three consecutive days only\(^\text{[24]}\). Each tablet (500 mg) was dissolved in 10 ml DW. Mice treated with full NTZ dose received 2 mg/0.04 ml/day, and those treated with half dose received 1 mg/0.02 ml/day. It was given either alone or in addition to combined prebiotic and probiotic.

- Doses were dispensed by an oral tube fitted to an insulin syringe (100 units/ml), and given as 10 units for probiotic and prebiotic administration. For full and half doses of NTZ, 4 and 2 units were given respectively.

**Parasitological examination**: After administration of treatment, fecal pellets were collected at 12\(^\text{th}\), 17\(^\text{th}\), 19\(^\text{th}\), and 30\(^\text{th}\) days PI\(^\text{[23]}\) and subjected to parasitological examination using the direct wet mount to count cyst number\(^\text{[22]}\) expressed per gram feces\(^\text{[26]}\). The percentage of reduction in parasite count = \(\frac{(a-b)}{a} \times 100\), where a = mean number of parasites in the control group, and b = mean number of parasites in the treated group.

**Histopathological examination**: After sacrifice of mice, segments of about one cm long from upper part of the small intestine were cut off and immediately fixed in 10% formalin then processed for paraffin embedding. All histopathological sections of 4 \(\mu\)m thickness were stained with Hematoxylin & Eosin stain (H&E)\(^\text{[27]}\).

**Caspase 3 immunohistochemistry**\(^\text{[15,28]}\): Four \(\mu\)m thickness of paraffin-embedded sections were used for immunohistochemical staining, using both polyclonal rabbit anti-active caspase-3 as primary antibody and biotinylated goat anti–rabbit antibody as secondary antibody (DAKO, Carpinteria, California, USA). Standard positive and negative control sections were used for each assay. The represented fields of each section were randomly selected and interpreted in a blinded manner. The staining for caspase 3 was considered positive in the cytoplasm of intestinal epithelium. The intensity of staining and percentage of positive cells were estimated, then caspase 3 expression in intestinal tissue was evaluated according to H score as follows: (-): negative, (+): low or mild staining, (++): moderate staining, (+++): strong staining.

**Immunological assessment**: Blood was collected after mice sacrifice and centrifuged at 3000 rpm for 5 min. Sera were separated and stored at -70°C until assessment. Boster immunoleader mouse enzyme-linked immunosorbent assay (ELISA) kit (Boster Biological Technology Co., Ltd.; California/USA) was used to quantify IL-6. Cytokine IL-6 level was calculated by comparing optical densities of tested sample to those of standard run in the same plate, values for standard curves and sample were calculated\(^\text{[29]}\).

**Statistical analysis**: Results were collected, tabulated and statistically analyzed using SPSS program version 16 for Microsoft Windows. Data were expressed as mean \(\pm\) standard deviation (SD) for quantitative
variables and percent for qualitative variables. Means were compared using ANOVA test and values were considered significant at $P \leq 0.05$.

**Ethical considerations:** The protocol of this study was approved by the ethical committee of Faculty of Medicine for Girls Al-Azhar University and TBRI. All animals in the current study were handled in compliance with the international valid guidelines for animal experimental ethics.

**RESULTS**

**Parasitological results:** It was observed that *Giardia* cysts shedding in feces of all infected mice increased gradually with highest peak at 12th day PI. In prophylactic (B) and infected treated (C) groups, there were significant reduction in the mean count of cyst shedding on all days (Tables 2, and 3). The percentage of reduction in *Giardia* cysts shedding was higher in single prophylactic administration (SGs: B2, and C2) than single probiotic administration (SGs: B1, and C1), and the highest was in combined prebiotic and probiotic (SGs: B3, and C3). It was observed that the administration of NTZ full and half doses combined with prebiotic and probiotic (SG: C5 and C6) had the highest percentage of reduction in *Giardia* cysts shedding at all days PI (Fig. 1).

**Histopathological results:** Histopathological examination of small intestine sections in the control non infected SGs (A1, A3, A4, A5, A6, and A7) that were represented in Fig. (2, a-c) showed intact villi with normal villus/crypt ratio and preserved Goblet cells. Control positive, infected non treated SG (A2) showed severe destructive pathological effect of *Giardia* (Fig. 2d) and *Giardia* trophozoites between and attached to villi (Fig. 2e).

Improvement of the histopathological changes with varying degrees were observed in all prophylactics (Fig. 3a-c) as well as infected treated SGs (Fig. 4 a-f). Remarkable improvement of histopathological findings was observed in SG C5 (Fig. 4e) followed by SG B3 (Fig. 3c) and SG C6 (Fig. 4f).

**Immunohistochemistry results:** Caspase-3 apoptotic activity showed strong expression in control positive (SG A2) (Fig. 5b). The peak of expression decreased with varying score in intestinal sections of all prophylactic and infected treated SGs (Fig. 5 c-k). No apoptotic expression of caspase-3 was observed in SG C5 treated with combined supplement in addition to NTZ (full dose) (Fig. 5j).

**Table 2.** The mean count and reduction% of *Giardia* cysts in all prophylactic SGs at the 17th, 19th, and 30th days PI.

<table>
<thead>
<tr>
<th>Prophylactic SGs</th>
<th>17th Days (PI)</th>
<th>19th Days (PI)</th>
<th>30th Days (PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Reduction %</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>A2</td>
<td>52000 ± 3535.5</td>
<td>--</td>
<td>24500 ± 707.1</td>
</tr>
<tr>
<td>B1</td>
<td>6875 ± 2651.7</td>
<td>87</td>
<td>4250 ± 1060.7</td>
</tr>
<tr>
<td>B2</td>
<td>5500 ± 707.1</td>
<td>89</td>
<td>3600 ± 548.5</td>
</tr>
<tr>
<td>B3</td>
<td>1500 ± 141.4</td>
<td>97</td>
<td>450 ± 212.1</td>
</tr>
</tbody>
</table>

Statistical analysis

ANOVA

*P* value

0.000*

0.000*

0.000*

A2: Infected non-treated; B1: Received prebiotic then infected; B2: Received probiotic then infected; B3: Received combined prebiotic/probiotic then infected. *Significant (*P*<0.05).

**Table 3.** The mean count and reduction% of *Giardia* cysts in all infected treated SGs at the 17th, 19th, and 30th days PI.

<table>
<thead>
<tr>
<th>Infected treated SGs</th>
<th>17th Days (PI)</th>
<th>19th Days (PI)</th>
<th>30th Days (PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Reduction %</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>A2</td>
<td>52000 ± 3535.5</td>
<td>--</td>
<td>24500 ± 707.1</td>
</tr>
<tr>
<td>C1</td>
<td>13000 ± 4242.6</td>
<td>75</td>
<td>10500 ± 707.1</td>
</tr>
<tr>
<td>C2</td>
<td>7250 ± 1060.7</td>
<td>86</td>
<td>4250 ± 353.6</td>
</tr>
<tr>
<td>C3</td>
<td>2800 ± 1131.3</td>
<td>85</td>
<td>550 ± 424.2</td>
</tr>
<tr>
<td>C4</td>
<td>8500 ± 2121.3</td>
<td>84</td>
<td>150 ± 70.7</td>
</tr>
<tr>
<td>C5</td>
<td>80 ± 28.3</td>
<td>99.5</td>
<td>60 ± 56.6</td>
</tr>
<tr>
<td>C6</td>
<td>1200 ± 282.8</td>
<td>98</td>
<td>90 ± 14.1</td>
</tr>
</tbody>
</table>

Statistical analysis

ANOVA

*P* value

0.000*

0.000*

0.000*

A2: Infected non-treated; C1: Infected, received prebiotic PI; C2: Infected, received probiotic PI; C3: Infected, received combined prebiotic/probiotic PI; C4: Infected, received NTZ PI; C5: Infected, received NTZ (full dose) + combined prebiotic/probiotic PI; C6: Infected, received NTZ (half dose) + combined prebiotic/probiotic PI. *Significant (*P*<0.05).
Fig. 1. Percentage of reduction in *Giardia* cysts shedding in different prophylactic and infected treated SGs at 17th, 19th and 30th days PI. 

- **B1:** Prophylactic SG received prebiotic.
- **B2:** Prophylactic SG received probiotic.
- **B3:** Prophylactic SG received combined prebiotic + probiotic.
- **C1:** Treated SG received prebiotic PI.
- **C2:** Treated DG received probiotic PI.
- **C3:** Treated SG received combined prebiotic/probiotic PI.
- **C4:** Treated SG received NTZ (full dose) PI.
- **C5:** Treated SG received NTZ (full dose) + combined prebiotic/probiotic PI.
- **C6:** Treated SG received NTZ (half dose) + combined prebiotic/probiotic PI.

Fig. 2. Intestinal sections of mice in control group (A) stained with H&E. 

- **2a-2c:** SGs A1, A3, A4, A5, A6, and A7 showed intact villi with normal villus/crypt ratio (red lines) and preserved Goblet cells (red arrow) (x100, x200).
- **2d:** SG A2 showed marked villus shortening with expansion of villus core by excess inflammatory cellular infiltrate mainly of lymphocytes and plasma cells (black arrow), and focal villus epithelial necrosis with superficial ulceration (blue arrow) (x200).
- **2e:** SG A2 showed *Giardia* trophozoites (arrows) (x400).

Fig. 3. Intestinal sections of mice in prophylactic group stained with H&E. 

- **3a:** SG B1 showed moderate villus atrophy and blunting with expansion by moderate mononuclear inflammatory cell infiltrate (arrow) (x100).
- **3b 1-2:** SG B2 showed moderate widening of villi and expansion by mononuclear inflammatory cellular infiltrate (black arrow), crypt hyperplasia (red line) and increase mitotic activity (red arrows) (x100, 200x).
- **3c 1-2:** SG B3 showed mild villus shortening with expansion by edema, mild inflammatory cellular infiltrate (black arrow) and no crypt hyperplasia (x100, x200).
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Fig. 4. Intestinal sections of mice in infected treated group stained with H&E. 4a-1: SG C1 showed moderate villus atrophy and blunting with expansion by moderate mononuclear inflammatory cellular infiltrate and focal villus necrosis in villi (arrow) (x100, x200). 4b: SG C2 showed moderate villus shortening and blunting (arrow) with expansion of villus core by edema and moderate inflammatory cellular infiltrate (x100). 4c: SG C3 showed mild villus shortening with expansion by edema, mild inflammatory cellular infiltrate (arrow) (x100). 4d-1: SG C4 showed moderate villus shortening and blunting with expansion of villus core by edema, moderate inflammatory cellular infiltrate (red arrow) and crypt hyperplasia (red lines) (x100, x200). 4e: SG C5 showed intact villi, normal crypt/villus ratio with mild inflammatory cellular infiltration (arrow) (x100). 4f: SG C6 showed mild villus shortening with expansion by edema and inflammatory cellular infiltrate (arrow) (x100).

Fig. 5. Intestinal sections of mice (immunohistochemical staining for caspase-3). 5a: SG A1 showed negative expression (-) (x200). 5b: SG A2 showed brown cytoplasmic staining in intestinal epithelium, strong expression (+++) (arrows) (x400). 5c: SG B1 showed moderate expression (++) (arrows) (x200). 5d: SG B2 showed mild expression (+) (arrows) (x200). 5e: SG B3 showed mild expression (+) (arrows) (x200). 5f: SG C1 showed moderate expression (++) (arrows) (x400). 5g: SG C2 showed moderate expression (++) (arrows) (x400). 5h: SG C3 showed mild expression (+) (arrow) (x200). 5i: SG C4 showed moderate expression (++) (arrows) (x400). 5j: SG C5 showed negative expression (-) (x400). 5k: SG C6 showing mild expression (+) (arrow) (x200).

Immunological assessment of IL-6: The results showed significant increase in the mean level concentrations of IL-6 in all prophylactic (B) and infected treated (C) mice (Table 4).

Table 4. Comparison between mean±SD of serum IL-6 cytokine in different SGs.

<table>
<thead>
<tr>
<th>Study SGs</th>
<th>IL-6</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>ANOVA</td>
</tr>
<tr>
<td>A1</td>
<td>5.70 ± 0.57</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>16.10 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>16.85 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>18.75 ± 0.49</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>12.60 ± 0.71</td>
<td>254</td>
</tr>
<tr>
<td>C2</td>
<td>14.15 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>16.15 ± 0.92</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>16.65 ± 0.78</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>20.95 ± 0.92</td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td>19.15 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>10.35 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

A1: Non-infected non-treated; A2: Infected non-treated; B1: Received prebiotic then infected; B2: Received probiotic then infected; B3: Received combined prebiotic/probiotic then infected; C1: Infected, received prebiotic PI; C2: Infected, received probiotic PI; C3: Infected, received combined prebiotic/probiotic PI; C4: Infected, received NTZ (full dose) PI; C5: Infected, received NTZ (full dose) + combined prebiotic/probiotic PI; C6: Infected, received NTZ (half dose) + combined prebiotic/probiotic PI. *: Significant (P<0.05).
DISCUSSION

In the present study, prophylactic prebiotic and probiotic treatment (group B) prior to *Giardia* infection was done to evaluate the effect of infection if it happens in the presence of healthy intestinal flora. Shukla et al.\(^{[19]}\) reported that prebiotic inulin supplementation even to malnourished *Giardia*-infected mice reduced both cyst and trophozoite counts. The reduction in the severity of giardiasis after prebiotic supplementation was explained as due to increase of *Lactobacilli* survival and colonization in the gut. The results of probiotics alone as an anti-*Giardia* agent in the present study are in accordance with another report by Humen et al.\(^{[30]}\) who demonstrated that in animals receiving probiotic for seven days before *Giardia* inoculation, the intensity and time of infection were reduced. Similar results were reported\(^{[25]}\). Additionally, Shukla et al.\(^{[31]}\) reported that after supplementation by *L. casei*, the experimentally infected mice became *Giardia* free by day 30 PI.

Our study demonstrated significant mean count of *Giardia* cysts reduction in both prophylactic (B) and infected treated (C) groups on the 17\(^{th}\), 19\(^{th}\) and 30\(^{th}\) days PI. Combined prebiotic and probiotic supplement in either of probiotic or infected treated groups, generally gave higher percentage of reduction than NTZ alone. The highest percentage of reduction was observed when combined prebiotic and probiotic was added to NTZ (full dose), followed by combined prebiotic and probiotic added to NTZ (half dose), then the prophylactic combined prebiotic and probiotic SG.

Amer et al.\(^{[32]}\) added that reduced parasite density both *in vitro* and *in vivo* occurred after administration of bacteriocins derived from newly isolated Egyptian strains of probiotic *Lactobacilli*. In a similar study, Mazroue et al.\(^{[35]}\) registered a significant reduction of the excreted *Giardia* cysts count in prophyballactic and infected treated probiotic SGs. It was postulated that the probiotic anti-*Giardia* effect may be due to interference with attachment of *Giardia* trophozoites to the mucosa, competition for nutrients and production of antimicrobial compounds\(^{[33]}\).

In a metanalysis study, Ventura et al.\(^{[34]}\) recorded the anti-giardial effect of different probiotic strains. Besides, Perrucci et al.\(^{[16]}\) stated that commercial multi-strain probiotic Slab51 containing 200 billion lactic acid bacteria showed higher antagonistic effects on *G. duodenalis* both *in vitro* and *ex vivo* intestinal tissue cultures. Similarly, Sand et al.\(^{[35]}\) reported a high therapeutic efficacy of potent commercial Acidopillus capsules containing 50 million live probiotic bacteria of five species against experimental giardiasis. In the present study, commercial multi-strain probiotics gave potent efficacy that may be explained by the synergistic effect of different probiotic strains used. The fact that prebiotics, probiotics, and symbiotics can be used as preventive and therapeutic agents for many human diseases was previously declared\(^{[36]}\). Their efficacy depends on the etiology of the disease and introducing the suitable probiotic strain to restore the balance of intestinal flora.

The significant reduction in cyst shedding after combined prebiotic and probiotic supplement in the present study agreed with the results of Shukla et al.\(^{[21]}\) who found that prophylactic administration of symbiotic (*L. casei* + inulin) to malnourished *Giardia*-infected mice led to increase body mass, small intestine mass, *Lactobacilli* counts, and reduced severity of giardiasis that was evident by decreased cyst and trophozoite counts. However, we recorded the highest reduction in cyst shedding after addition of NTZ to combined prebiotic and probiotic treatment, which implies that the most beneficial way to treat giardiasis might be through combination of nutritional interventions and chemotherapeutic agents\(^{[37]}\). This was confirmed by the combination of *Saccharomyces boulardii* with metronidazole to clear all the symptoms related to giardiasis\(^{[38]}\). Moreover, Shukla et al.\(^{[39]}\) observed the synergistic effect of co-administration of probiotic and albendazole, tinidazole, metronidazole, and NTZ to *Giardia*-infected mice, which resulted in reduced *Giardia* cysts shedding in feces and trophozoite numbers in the intestinal fluid. Prophyballactic and treated mice with both *L. casei* and metronidazole SGs showed the highest shedding reduction of *Giardia* cysts\(^{[35]}\). It is worth mentioning that NTZ was approved for the treatment of giardiasis in humans by the FDA\(^{[40]}\) and can be given as a potential alternative therapy for metronidazole-resistant giardiasis\(^{[41]}\). Accordingly, it was chosen as chemotherapeutic agent in the present study.

The normal intestinal architecture demonstrated in our histopathological sections of control non-infected non treated mice was similarly recorded in the supplemented and NTZ control SGs. In reverse the control positive infected mice revealed marked histopathological changes which agreed with several researches reporting marked villus shortening, necrosis and ulceration with excess inflammatory cellular infiltrates as a destructive effect of *Giardia*\(^{[8,13,15,19,35]}\).

It was also observed that supplementation of either prebiotic or probiotic to infected mice helped in partial improvement of the histopathological changes which was better in prophylactic (B) than infected treated (C) groups and more specifically in probiotic treated SGs (B2, C2). These results agreed with the previous observation\(^{[15]}\) of reduced mucosal damage in microvilli with prebiotic supplementation either prior to or simultaneously with experimental
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Giardia infection. Also, many authors reported that administration of probiotics to Giardia infected mice reduced atrophy of villi and cellular infiltration\[8,13,15,20\]. In the present study, remarkable improvement in the histopathological changes with mild villus atrophy was noticed with combined prebiotic and probiotic supplementation either in prophylactic (B3) or infected treated mice (C3). Similar results were observed after addition of combined supplement to NTZ (half dose) (C6). While addition of combined supplement to NTZ (full dose) (C5) helped in restoring the normal mucosal architecture with normal villus to crypt ratio, although mild inflammation in the lamina propria was still noticed.

Shukla et al.[21] observed that supplementation of symbiotic (L. casei + inulin) to malnourished-Giardia infected mice helped in better improvement of the affected mucosal layers. Restoring nearly normal villus architecture in Giardia-infected mice after addition of probiotic to chemotherapeutic agents was confirmed after co-administration of probiotic and albendazole\[20\], and also in mice treated with both L. casei and metronidazole\[13\].

Concerning the immunohistochemical results of intestinal sections, caspase-3 apoptotic activity expression was strong in control positive infected non-treated (A2) mice which agreed with previous observations\[16,20\]. Prebiotic and probiotic supplement in the present study, reduced apoptotic caspase-3 expression with varying scores in all prophylactic and infected treated SGs. Reduction in the peak of caspase-3 expression was documented in ex vivo intestinal tissue cultures after 18 h of incubation with probiotic Slab51\[20\]. On the other hand, in the present study treatment with combined supplement in addition to full dose NTZ (C5) revealed no apoptotic expression of caspase-3 which agreed with the corresponding histopathological result of the same SG (C5) in our present study. This proves that combinations of these supplements with specific treatment are promising for enterocytes protection against giardiasis.

Some cytokines, such as IL-6, IL-12 and IL-17, can activate both innate and adaptive pathways\[19,45\]. IL-6 released by mast cells, dendritic cells or T cells, is a key regulator of B-cell maturation and promotes antibody class switching to produce IgA\[16,48\]. It was recorded that IL-6-deficient mice were unable to eradicate Giardia and had altered intestinal cytokine responses despite having normal levels of IgA\[46,47\]. Our present study demonstrated significant increase in serum IL-6 level in all prophylactic and infected treated groups. The highest IL-6 level was observed in full dose NTZ combined with prebiotic and probiotic (C5), followed by half dose NTZ combined with prebiotic and probiotic (C6), then prophylactic combined prebiotic and probiotic (B3). The results of the present study concur with those of research that revealed modulation of both non-specific and specific immune responses. This modulation was manifested by increased nitric oxide, secretory anti-Giardia IgA and IgG antibody and anti-inflammatory cytokines (ILs 6, and 10) levels, after separate supplementation of either prebiotic inulin\[19\] or probiotic L. casei\[13,44,49\], and also with combined supplements\[21\] either prior to or simultaneously with Giardia infection to nourished or malnourished mice.

In conclusion combined probiotic and probiotics (multi strains) have demonstrated a significant potential supplement for medication of giardiasis. Hence, combined products can be considered as successful prophylactic and therapeutic agents when added to NTZ for treatment of giardiasis.

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