



# Ultrastructural changes and IgA modulatory effect of commercial prebiotic and probiotic in murine giardiasis

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**Abstract** Giardiasis, a parasitic infection of the gastrointestinal tract, is prevalent worldwide. The integrity of the intestinal epithelial barrier plays an important defensive role in giardiasis, and as Oral supplementation with prebiotics and probiotics is known to reinforce the intestinal barrier in many gastrointestinal diseases, this study assessed the effects of prebiotic and probiotic supplementation in giardiasis and compared the results with those obtained after nitazoxanide therapy. Swiss albino male lab-bred mice (n = 50) were divided into three major groups; Group I (control group), i.e., negative (noninfected nontreated) and positive controls (infected nontreated); Group II (preventive group), in which mice were provided prebiotic, probiotic, or a combination for 7 days before of infection, and Group III (therapy group), in which mice were administered prebiotic, probiotic, combined supplements and nitazoxanide from day 12 post-infection. The assessment was achieved through *Giardia* cyst count, histopathological examination and ultrastructure study. Also, Serological and immunohistochemical parameters were done to evaluate the modulation of IgA levels. Oral supplementation with prebiotic and

probiotic, either before or after infection (in preventive or therapy groups respectively) resulted in a significant reduction in *Giardia* cyst shedding. Remarkable histological and ultrastructure improvement in the intestinal changes, along with a significant increase in the serological and immunohistochemical IgA levels, were seen in mice provided combined supplements and nitazoxanide (in therapy group). Thus, our results indicate that combined prebiotic and probiotic supplementation has promising anti-*Giardia* activity and that it can restore intestinal structures and modulate IgA response, apart from providing synergistic effects when added to nitazoxanide.

**Keyword** Giardiasis · IgA · Immunohistochemical · Prebiotic · Probiotic · Serological · Ultrastructure

## Introduction

Giardiasis is the most prevalent protozoal illness in humans (Helmy et al. 2017). It can affect children, adults, and those who are malnourished or immune-compromised (Goyal et al. 2011), and is seen in both advanced and developing countries, albeit with a higher incidence in the latter (Zajackowski et al. 2018). In Egypt, the prevalence of giardiasis among children had risen to 27.3% (Bayoumy et al. 2010). The duodenum and jejunum are typical habitats of *G. duodenalis*, and hence, the effects of increased intestinal permeability due to *Giardia* infection include intestinal barrier dysfunction, host lymphocyte activation, and brush border microvilli shortening with or without villous atrophy (Troeger et al. 2007). Giardiasis may be asymptomatic or can cause acute and/or chronic clinical disease. The most common symptoms are steatorrhea, intermittent diarrhea, malabsorption, and weight loss, but some may develop

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post-infection (PI) or extraintestinal complications (Minetti et al. 2016). Multiple chemotherapeutic options are available, including nitazoxanide (NTZ), a less toxic choice that is approved by the FDA for the treatment of giardiasis in humans (Di Santo and Ehrisman 2013). Interestingly, NTZ can be used for the treatment of metronidazole-resistant giardiasis (Galeh et al. 2016). As parasitic resistance to NTZ has been reported by *in vitro* and *in vivo* experiments (Matadamas-Martínez et al. 2020) and in clinical isolates (Reyes-Vivas et al. 2014), there is an urgent need to develop alternative or complementary natural interventions against giardiasis (Abd-Elhamid et al. 2021).

Even though *Giardia* infection is usually self-limiting in immunocompetent persons, probably due to the effective host immune response against infection, resistant or chronic giardiasis can develop despite a healthy immune system (Faubert 2000). Parasite clearance in giardiasis requires a complex interaction between innate and adaptive immune responses. (Lopez-Romero et al. 2015) with gut barrier and its integrity being one of the essential components of the innate response. Similarly, many studies have reported that IgA antibodies against giardiasis are also crucial for protection and parasite clearance (Solaymani-Mohammadi et al. 2010). Hjollo et al. (2018) have reported that IgA targets *Giardia* variant surface proteins; Thus, patients with a dominant IgA deficiency will have a higher risk of acquiring chronic giardiasis (Oksenhendler et al. 2008). Nonetheless, the actual mechanisms of action of IgA antibodies in giardiasis is not fully understood and may involve immobilization or detachment of trophozoites from the intestinal epithelium or the mucus layer, rather than direct killing (Eckmann 2003). Further, serum IgA from the infected patient had been demonstrated to have a direct cytotoxic effect on the parasites *in vitro* (Langford et al. 2002), and human saliva from infected persons, as well as mother's milk, may contain anti-*Giardia* secretory IgA (sIgA) antibodies (Tellez et al. 2003; El-Gebaly et al. 2012). Similarly, Hjollo et al. (2018) have reported significantly high levels of serum and salivary IgA in Egyptian children with giardiasis. Furthermore, *Giardia*-infected mice that received excretory/secretory products from the parasite showed higher serum IgA levels (Jimenez et al. 2014) while IgA-deficient mice were unable to clear *G. muris* or *G. lamblia* infections (Langford et al. 2002). Congruently, chronic infection with *G. muris* may developed due to failure of sIgA to enter the intestinal lumen in mice with a deficient poly-Ig receptor (pIgR) (Davids et al. 2006).

Gut microbiota can significantly regulate both the mucosal and systemic immunity in the host (Benyacoub et al. 2005), and prebiotics and probiotics are examples of functional foods that, if consumed regularly, can facilitate the treatment and/or prevention of many diseases by boosting the host immunity and gut health (Markowiak and Zewska 2017). Prebiotics are a non-digestible food component that

support probiotic action while probiotics are live *microorganisms*. When consumed in adequate amounts, these supplements can provide a health benefit by altering microbial balance and immune homeostasis, modifying host immune response, regulating inflammatory cytokine levels, and maintaining epithelial integrity by enhancing sIgA production (Davani-Davari et al. 2019; Amreen 2020). Rose et al. (2021) have suggested that probiotic and prebiotic supplementation represent a useful preventive and therapeutic strategy for gastrointestinal diseases characterized by increased intestinal permeability because they improve intestinal barrier function by upregulating the Toll like receptors (TLR). Interestingly, administration of either prebiotics or probiotics in healthy and malnourished *Giardia*-infected mice led to systemic and mucosal IgA secretion against giardiasis (Goyal and Shukla 2013; Shukla et al. 2016, 2019; Mazroue et al. 2020).

Thus, the current study in *Giardia*-infected mice was designed to assess the effects of combined prebiotic and probiotic supplementation, with or without NTZ, on intestinal mucosal integrity and IgA levels.

## Materials and methods

This experimental study was performed at Theodor Bilharz Research Institute (TBRI) and Parasitology Department, Faculty of Medicine for Girls (FMG), Al-Azhar University.

### Preparation of parasite inoculum

*Giardia* cysts were obtained from stool samples of patients attending with diarrhea presenting to the outpatient clinics of Alzahraa hospitals. Collected samples were directly examined by wet mount under a microscope to ensure the presence of *Giardia* cysts and to exclude the presence of other parasites. Next, stool samples were processed, filtered, and concentrated by multiple rounds of centrifugation supernatant removal until the sample was totally clear. The parasite inoculum was counted and adjusted such that  $10^4$  *Giardia* cysts were administered to each mouse (Garcia 2007; Dyab et al. 2016).

### Experimental animals

Laboratory-bred, male, Swiss albino mice, 4–5 weeks old and weighing 20–25 g were provided by and maintained in the TBRI Animal Producing Unit. Animals were housed with unrestricted access to food and water and maintained according to laboratory standards. Gram's and Lugol's iodine were used to ensure that the supplied water was free of any bacterial or parasite (Tiwari et al. 2009). Pathogen-free mice were selected after stool examination and infected

**Table 1** The mean count of *Giardia* cysts in SG.I B and all SGs of Groups II and III on the 20th, and 30th days PI

SGs	20th day PI		30th day PI			
	Mean $\pm$ SD	ANOVA		Mean $\pm$ SD	ANOVA	
		F sample	P value		F sample	P value
SG.I B	15,246 $\pm$ 680.3	153.5	0.000***	1500 $\pm$ 557	31.3	0.000***
SG.II A	6499 $\pm$ 1389			617 $\pm$ 104.1		
SG.II B	4553 $\pm$ 1091			413 $\pm$ 102.6		
SG.II C	1160 $\pm$ 295			60 $\pm$ 36.1		
SG.III A	8267 $\pm$ 642.9			857 $\pm$ 51.3		
SG.III B	6767 $\pm$ 681			640 $\pm$ 115.3		
SG.III C	2367 $\pm$ 723.4			85 $\pm$ 5		
SG.III D	1667 $\pm$ 1155			92 $\pm$ 10.4		
SG.III E	383.3 $\pm$ 76.4			Zero		

*SG.IB* infected non treated, *SG.II A* received prebiotic prior to infection, *SG.II B* received probiotic prior to infection, *SG.II C* received combined prebiotic and probiotic prior to infection, *SG.III A* received prebiotic PI, *SG.III B* received probiotic PI, *SG.III C* received combined prebiotic and probiotic PI, *SG.III D* received NTZ PI, *SG.III E* received NTZ + combined prebiotic and probiotic PI

\*\*\* $P < 0.001$  very highly significant

orally by *Giardia* cysts. Treatment was started at the peak of cyst shedding, i.e., day 12 PI. Animals were sacrificed on 30th day PI when a decline in cyst shedding was observed in all groups. Animals were administered an anesthetic—anti-coagulant solution (500 mg/kg thiopental and 100 units/ml heparin) by intraperitoneal injection prior to sacrifice (Liang et al. 1987). Parasitological, histopathological, ultrastructural studies were conducted, and serological and immunohistochemical parameters were analyzed, as described below.

### Animal grouping

Mice (n = 50) were divided into:

*Group I Control group*, consisting of 10 mice that were further divided into 2 subgroups (SG):

*SG.I A* Noninfected nontreated mice (n = 5; negative control).

*SG.I B* Infected but nontreated mice (n = 5; positive control).

*Group II Preventive group*, n = 15 animals that were divided into 3 SGs:

*SG.II A* Mice receiving prebiotic prior to infection (n = 5).

*SG.II B* Mice receiving probiotic prior to infection (n = 5).

*SG.II C* Mice provided both prebiotic and probiotic prior to infection (n = 5).

*Group III Therapy group*, consisting of 25 mice, which were divided into 5 SGs:

*SG.III A* Mice provided prebiotic PI (n = 5).

*SG.III B* Mice provided probiotic PI (n = 5).

*SG.III C* Mice provided combined prebiotic and probiotic PI ((n = 5).

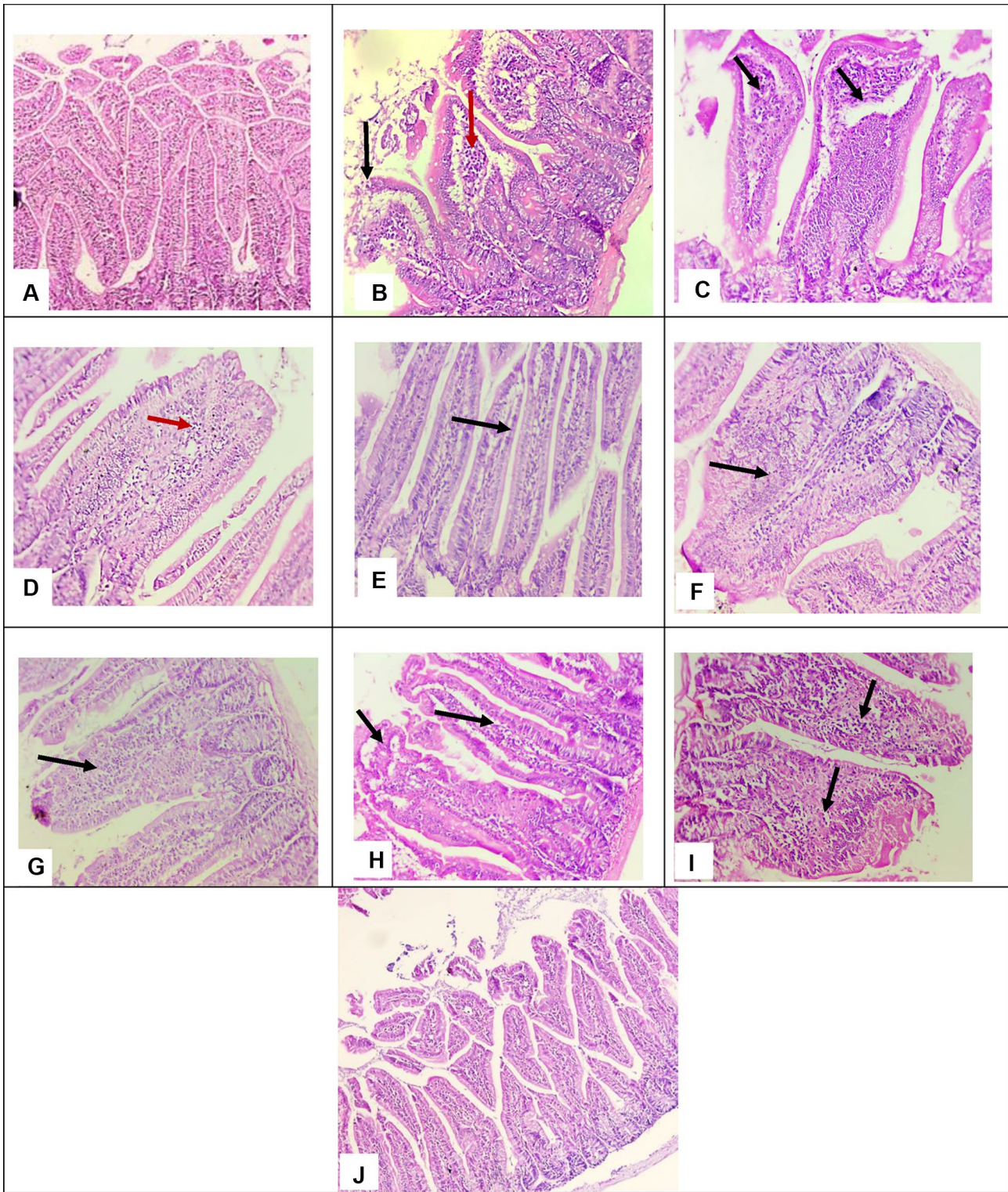
*SG.III D* Mice administered NTZ PI (n = 5).

*SG.III E* Mice administered NTZ + combined prebiotic and probiotic PI (n = 5).

### Experimental regimen of supplementation

*Probiotic* Commercially available product was purchased from New Rhythm Co. (New York, USA), which contained probiotic capsules containing 25 billion multi-strain culture forming units (CFU/capsule) of *B. lactis*, *B. bifidum*, *B. longum*, *B. breve*, *B. adolescentis*, *B. infantis*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, and *Streptococcus thermophiles*. One capsule was emptied and dissolved in 2.5 ml of distilled water and each mouse was orally administered one billion CFU/0.1 ml/day via an orogastric tube (Shukla et al. 2008; Shaaban et al. 2021). *Prebiotic* (inulin) was commercially purchased from BFW co. (Burlington, Canada) as a pure powder. To provide a dosage of 2 mg/0.1 ml/mouse/day of inulin, 200 mg of inulin powder was dissolved in 10 ml of distilled water. (Shukla et al. 2016; Shaaban et al. 2021). Mice in the preventive group (Group II) were provided the prebiotic and/or probiotic daily for seven days prior to infection till termination of the experiment. In the therapy group (Group III), prebiotic or probiotic supplements were given from the 12th day PI daily till termination of the experiment. *NTZ* was commercially purchased as a Nanazoxid





suspension (Utopia; Cairo/Egypt) and was given orally at a dose of 100 mg/kg/day from the 12th day PI for three consecutive days, either alone or combined with the pre- and probiotics (Abd El-Aziz et al. 2014; Shaaban et al. 2021; Hassan et al 2022).

**Giardia cyst count**

Stool samples from mice were collected daily to ensure infection following inoculation and to identify the peak timing of cyst shedding. As the peak was observed on day 12 PI, treatment was

**Fig. 1** Sections of jejunum of mice stained with H& E (A–J). **A** Control negative (SG.I A) displaying typical villous morphology with intact mucosal layer ( $\times 100$ ). **B** Control positive (SG.I B) displaying severe ulceration and villous atrophy (black arrow), villous core edema and excessive inflammatory cellular infiltration (red arrow) ( $\times 200$ ). **C** Prebiotic prior to infection (SG.II A) displaying moderate villous edema, atrophy, and excessive inflammatory cellular infiltration (arrow) ( $\times 200$ ). **D** Probiotic prior to infection (SG.II B) displaying mild villous shortening with expansion of villous core by mild inflammatory cellular infiltration (arrow) ( $\times 200$ ). **E** Combined supplement prior to infection (SG.II C) displaying showing mild villous expansion of villous core by mild inflammatory cellular infiltration (arrow) ( $\times 200$ ). **F** Prebiotic PI (SG.III A) displaying moderate villous shortening with expansion of villous core by edema and moderate inflammatory cellular infiltration (arrow) ( $\times 200$ ). **G** Probiotic PI (SG.III B) displaying moderate villous shortening and blunting with expansion of villous core by edema and moderate inflammatory cellular infiltration (arrow) ( $\times 200$ ). **H** Combined supplement PI (SG.III C) displaying mild villous shortening with expansion of villous core by mild inflammatory cellular infiltration ( $\times 200$ ). **I** NTZ PI (SG.III D) displaying moderate to severe villous shortening and blunting with expansion of villous core by excessive inflammatory cellular infiltration (arrow) ( $\times 200$ ). **J** Combined supplement+NTZ PI (SG.III E) displaying near normal villous pattern with intact mucosal layer ( $\times 100$ ) (Color figure online)

initiated at this time point. Treatment effect was assessed in fecal pellets collected on days 20 and 30 PI, and the pellets were microscopically visualized by direct wet mount after dissolution in 10% formalin saline. The number of cysts/gram feces was determined as described elsewhere (Garcia 2007; Shaaban et al. 2021).

### Histopathological examination

Sections of the jejunum, about 1 cm in lengths, were cut, fixed in 10% formalin, paraffin embedded, sectioned at a thickness of around 4  $\mu\text{m}$ , stained with hematoxylin and eosin (H&E) and examined under a light microscope (Ross and Pawlina 2016).

### Ultrastructure study (Stirling and Curry 2007)

Fragments of freshly dissected jejunal sections, about 1 mm in length, were immediately fixed in 2.5% glutaraldehyde and processed for electron microscopy. Specifically, samples were stained with uranyl acetate and lead citrate before visualization under a transmission electron microscope (TEM) (Stirling and Curry 2007).

### Determination of serum IgA antibody levels

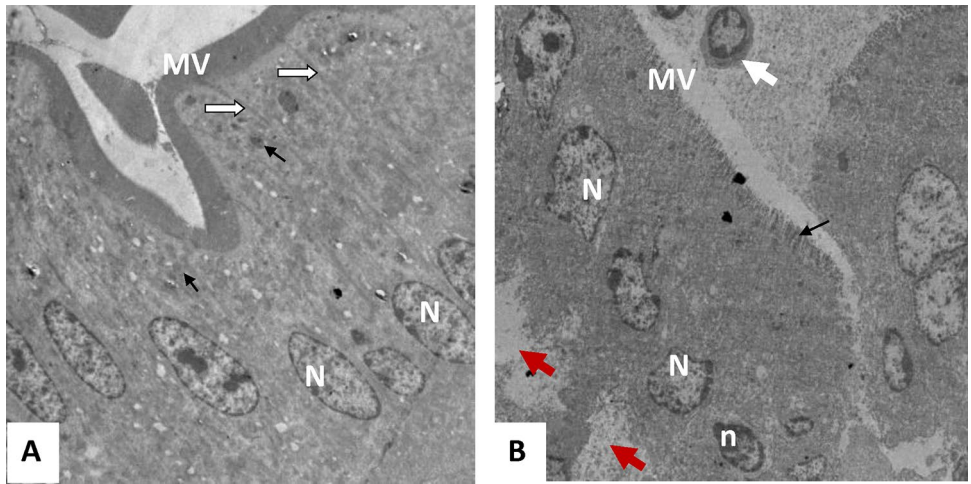
Blood samples were centrifuged at 3000 rpm for 5 min and sera were stored at  $-70\text{ }^{\circ}\text{C}$  until further evaluation of IgA antibody levels using an enzyme-linked immunosorbent assay (ELISA) kits (Sigma; USA), which depended on

reaction between IgA present in samples and the anti-Mouse IgA antibodies that were adsorbed to the surface of polystyrene microtiter wells. Briefly, 100  $\mu\text{L}$  of diluted samples and standards (Mouse IgA Calibrator) were added in duplicate into pre-designated wells and incubated at room temperature for 60 min. The plate was washed by diluted wash solution, then 100  $\mu\text{L}$  of diluted anti-IgA antibodies conjugated with horseradish peroxidase (HRP) were added to each well and incubated at room temperature for 30 min. Following another washing step, the amount of enzyme-bound in the complex is measured by the addition of 100  $\mu\text{L}$  a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB) and incubated in the dark at room temperature. After 10 min, 100  $\mu\text{L}$  of stop solution (0.3 M sulfuric acid) were added to each well. The quantity of bound enzyme varied proportionately with the concentration of IgA in the sample tested and absorbance measured at 450 nm. Steps of the procedure were done according to the manufacturer's instructions. The quantity of IgA in the test sample was interpolated from the standard curve constructed using standards and corrected for sample dilution (Goyal and Shukla 2013).

### Immunohistochemical assessment of IgA secreting plasma cells

Briefly, after sacrifice, jejunal segments of, about 1 cm long, were cut off, immediately fixed in 10% formalin, and then processed for paraffin embedding. Formalin-fixed paraffin-embedded (FFPE) tissue samples were sectioned at a thickness of 4  $\mu\text{m}$ , placed onto positively charged glass slides, deparaffinized, dehydrated, and subjected to steam heating for 25 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. Slides were incubated overnight in a humidity chamber with the primary antibody, namely, goat anti-mouse polyclonal IgA (Cat# I1890-20 J-HRP US Biological, Inc, USA; dilution 1:200). The secondary antibody (biotinylated anti-mouse IgG, DAKO, USA, dilution 1:500) was added at a concentration of 1  $\mu\text{g}/\text{mL}$  and incubated for 30 min at room temperature. Streptavidin–horseradish peroxidase and diaminobenzidine as chromogen were used for reaction readout. Phosphate-buffered saline (PBS) (pH 7.4) was used for washing as needed. Sections were counterstained with hematoxylin, dehydrated in a graded ethanol series, cleared in xylene, and a coverslip applied. Positive and negative control sections were used for each assay. IgA expression was evaluated using the qualitative H score wherein the number and intensity of stained cells were assessed on a scale that was scored as scanty (< 10% stained cells), mild (10–40%), moderate (40–60%) or marked (> 60%).

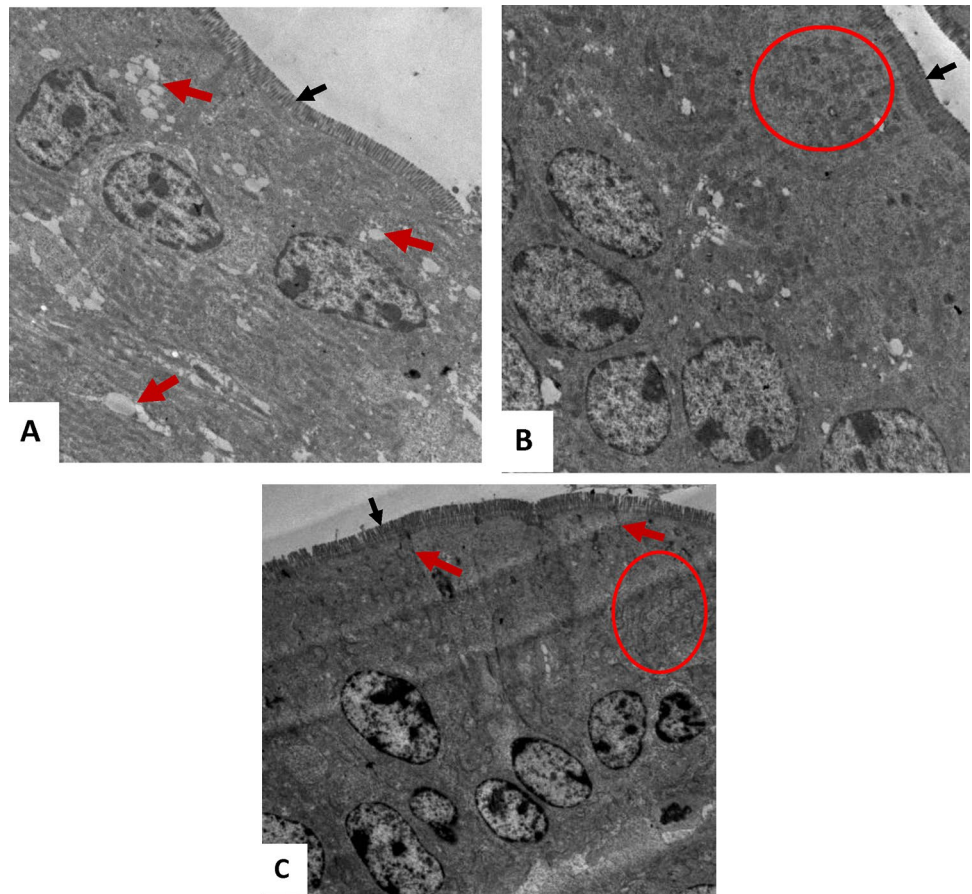




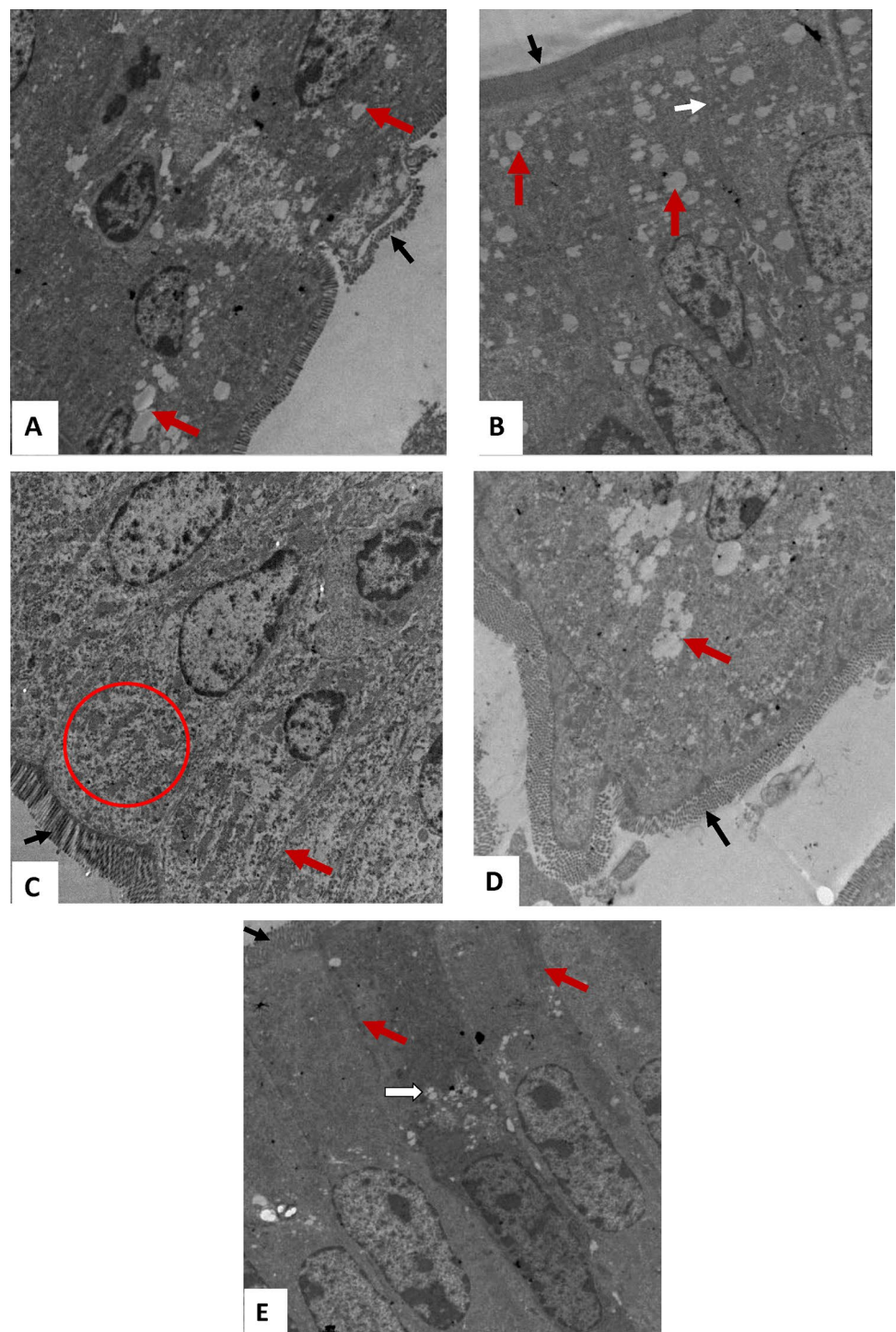
**Fig. 2** Electron photomicrographs of sections in enterocytes of mice jejunum from Group I: **A** Control negative (SG.I A) showing columnar cells with regular oval euchromatic nuclei (N), regular microvilli (MV) with brush border, normal mitochondria (black arrows) and prominent intercellular junctions (white arrows). **B** Control posi-

tive (SG.I B) showing irregular nuclei (N), the other contain small irregular shrunken condensed nucleus (n), loss of normal shape of the microvilli (MV) with tufts formation (black arrow), loss of intercellular junctions, intra-cellular spaces (edema) (red arrows) and lymphocytes near the intestinal lumen (white arrow) (Color figure online)

**Fig. 3** Electron micrographs of sections in enterocytes of mice jejunum from Group II (preventive group) (A–C): **A** Prebiotic (SG.II A) showing preserved structure of the microvilli although reduced in thickness (black arrow), intracytoplasmic vacuolization (red arrows) and loss of intercellular junctions. **B** Probiotic (SG.II B) showing preserved structure of the microvilli although reduced in thickness (black arrow), less prominent intercellular junctions, vacuolization of mitochondria (ellipse) and less vacuoles. **C** Prebiotic + probiotic (SG.II C) showing more prominent microvilli (black arrow) and intercellular junctions (red arrows) with abundant rough endoplasmic reticulum (rER) (ellipse) (Color figure online)



**Fig. 4** Electron micrographs of sections in enterocytes of mice jejunum from Group III (therapy group) mice (A–E) **A** prebiotic PI (SG.III A) showing less prominent microvilli with tuft formation (black arrow), interrupted intercellular junctions, increased intracytoplasmic vacuolization (red arrows). **B** Probiotic PI (SG.III B) showing preserved structure of the microvilli although reduced in thickness (black arrow), increased intracytoplasmic vacuolization (red arrows) and less prominent intercellular junctions (white arrow). **C** prebiotic + probiotic PI (SG.III C) showed more prominent microvilli (black arrow) and intercellular junctions (red arrows) with abundant mitochondria (ellipse). **D** NTZ PI (SG.III D) showed less prominent microvilli with tuft formation (black arrow), interrupted intercellular junctions and increased intracytoplasmic vacuolization (red arrow). **E** Probiotic + probiotic + NTZ PI (SG.III E) showing more preserved ultrastructure with prominent microvilli (black arrow), intercellular junctions (red arrows) and some intracytoplasmic vacuolization also seen (white arrows) (Color figure online)

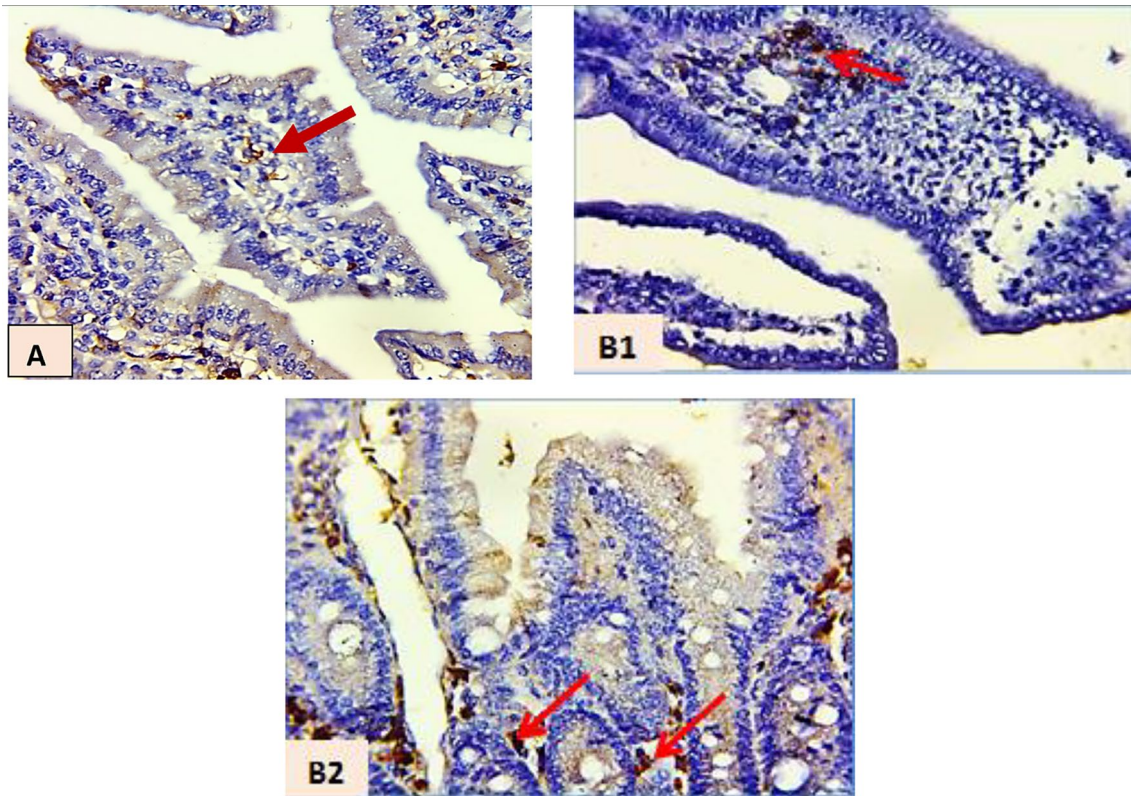


### Statistical analysis

Data collected were reviewed, coded, and statistically analyzed using the SPSS program (statistical package of social science; SPSS Inc., Chicago, IL, USA) version 16 for Microsoft Windows. Data are expressed as mean  $\pm$  standard deviation ( $\pm$  SD). The independent t-test was used to determine any significance in the differences between two

means while the Analysis of variance (ANOVA) test was used to determine the significance differences among more than two means. The level of significance set at a  $p$  value of  $<0.05$ . Hence; hence,  $P < 0.05^*$  was deemed significant,  $P < 0.01^{**}$  as highly significant, and  $P < 0.001^{***}$  as very highly significant.





**Fig. 5** Photomicrograph of jejunal sections of Group I (A, B) (Immunohistochemical staining for IgA) A (400X): Control negative (SG.I A) showing scanty IgA secretory plasma cells in the lamina

propria (arrows). B1 (400X) and B2 (400X): Control positive SG.I B mice showing mild IgA secretory plasma cells in the lamina propria (arrows)

**Table 2** Mean levels of serum IgA (ug/ml) in Group I (Control group)

SGs	Mean ± SD	
	SG.I A	SG.I B
IgA	62.65 ± 5.3	86.65 ± 2.9
P value	<0.05*	

SG.I A non infected non treated (control negative), SG.I B infected non treated (control positive)

\*P < 0.05 significant

**Table 3** Mean levels of serum IgA (ug/ml) in Group II (The preventive group)

SGs	Mean ± SD			
	SG.I A	SG.II A	SG.II B	SG.II C
IgA	62.65 ± 5.3	133.25 ± 1.06	151.2 ± 4.38	182.45 ± 3.89
P value	<0.01**			

SG.I A non infected non treated, SG.II A received prebiotic prior to infection, SG.II B received probiotic prior to infection, SG.II C received combined prebiotic and probiotic prior to infection

\*\*P < 0.01 highly significant

## Results

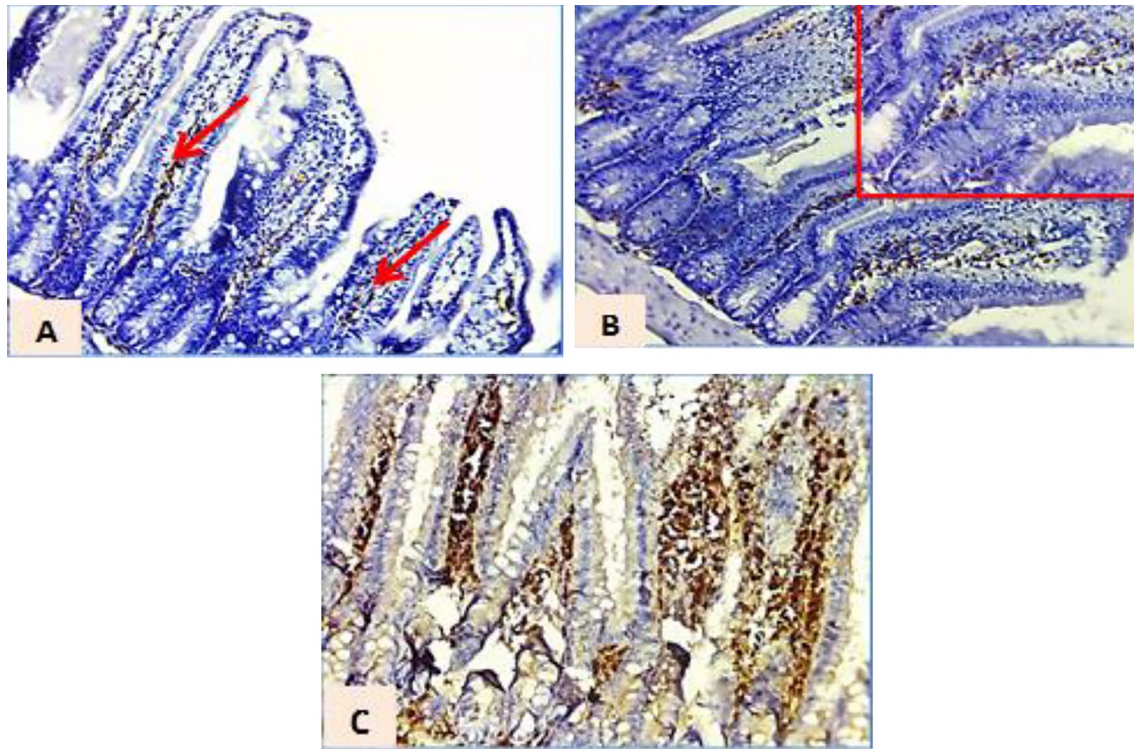
### Giardia cyst shedding count

Supplementation with pre- and probiotics before (Group II) and after infection (Group III) led to a very highly significant ( $p = 0.000$ ) reduction in *Giardia* cyst shedding on 20 and 30 days PI (Table 1). Prebiotic and probiotic supplementation prior to infection i.e., SG.II A, SG.II B and SG.II C (preventive group) led to a greater reduction in cyst shedding compared to supplementation after infection i.e., SG.III A, SG.III B, and SG.III C (in therapy group) (Table 1). The greatest reduction in cyst shedding was observed with the combination of NTZ and pre- and probiotic supplementation, i.e., in SG.III E (in therapy group) (Table 1).

### Histopathological evaluation

Jejunal sections showed normal villous structure in negative control animals (SGI.A; Fig. 1A), but marked histopathological changes in positive controls (SGI.B; Fig. 1B). Tissues from Groups II and III showed varying degrees of regeneration and





**Fig. 6** Photomicrograph of jejunal sections of Group II (preventive group) (A–C) (Immunohistochemical staining for IgA) **A** (200X): Prebiotic (SG.II A) showing moderate IgA secretory plasma cells in the lamina propria (arrows). **B** (200X, inset 400X): Probiotic (SG.II

**B**) showing moderate IgA secretory plasma cells in the lamina propria (arrows). **C** (200X): Prebiotic+probiotic (SG.II C) showing marked IgA secretory plasma cells in the lamina propria

**Table 4** Mean levels of serum IgA (ug/ml) in Group III (The Therapy group)

SGs	Mean $\pm$ SD					
	SG.I A	SG.III A	SG.III B	SG.III C	SG.III D	SG.III E
IgA	62.65 $\pm$ 5.3	112.4 $\pm$ 2.26	118.1 $\pm$ 1.27	173.9 $\pm$ 3.11	161.21 $\pm$ 2.81	197.45 $\pm$ 1.48
<i>P</i> value		<0.01**	<0.01**	<0.01**	<0.01**	<0.001**

*SG.I A* non infected non treated, *SG.III A* received prebiotic PI, *SG.III B* received prebiotic PI, *SG.III C* received combined prebiotic and probiotic PI, *SG.III D* received NTZ PI, *SG.III E* received NTZ+combined prebiotic and probiotic PI

\*\**P* < 0.01 highly significant

restoration of the villous epithelium (Fig. 1C–J). Prebiotic and probiotic supplementation led to greater improvement in Group II (preventive group) when administrated prior to infection (Fig. 1C–E). NTZ and combined supplements (SGIII.E; therapy group) showed prominent improvement with near normal villous architecture (Fig. 1J).

### Ultrastructure study

Transmission electron microscopy of jejunal enterocytes from Group I (control group) showed normal ultrastructure in SGI.A animals (Fig. 2A), but marked destruction in SGI.B animals (Fig. 2B). Samples from Groups II (preventive

group) and III (therapy group) showed varying degrees of improvement and regeneration of the columnar epithelium in Figs. 3A–C and 4A–E. When administrated prior to infection in Group II prebiotic and probiotic supplementation resulted in greater improvement (Fig. 3A–C) than their post infection administration (Fig. 4A–C). Congruently, combined supplements added to NTZ (SGIII.E; therapy group) showed marked improvement and regeneration of the microvilli on enterocytes (Fig. 4E).

### IgA serology

Mean serum IgA levels were significantly higher ( $p < 0.05$ ) in positive controls (SG.I A) compared to negative controls

**Table 5** Comparison between mean levels of serum IgA in groups (I, II, III)

IgA level (ug/ml)		ANOVA	
SGs		<i>F</i> sample	<i>P</i> value
Mean ± SD			
SG.II A 133.25 ± 1.06	SG.I A 62.65 ± 5.3	388.1	0.000***
SG.II B 151.2 ± 4.38	SG.I B 86.65 ± 2.9	299.5	0.000***
SG.II C 182.45 ± 3.89			
SG.III A 112.4 ± 2.26			
SG.III B 118.1 ± 1.27			
SG.III C 173.9 ± 3.11			
SG.III D 161.21 ± 2.81			
SG.III E 197.45 ± 1.48			

*SG.I A* non infected non treated, *SG.IB* infected non treated, *SG.II A* received prebiotic prior to infection, *SG.II B* received prebiotic prior to infection, *SG.II C* received combined prebiotic and probiotic prior to infection, *SG.III A* received prebiotic PI, *SG.III B* received probiotic PI, *SG.III C* received combined prebiotic and probiotic PI, *SG.III D* received NTZ PI, *SG.III E* received NTZ+ combined prebiotic and probiotic PI

\*\*\**P* < 0.001 very highly significant

(*SG.I B*) (Table 2). In Group II animals (preventive group), a highly significant increase (*p* < 0.01) in IgA levels was seen in all animals (Table 3). Similarly, IgA levels were significantly higher (*p* < 0.01) in Group III animals (therapy group; *SG.III A, B, C,* and *D*). Likewise, NTZ and combined supplementation (*SG.III E*) showed a very highly significant increase (*p* < 0.001) in serum IgA levels (Table 4). Compared to *SG.I (A or B)*, IgA levels were very highly significantly greater (*p* = 0.000) in all subgroups of Groups II and III. NTZ with combined supplementation in *SG.III E* elicited the highest mean IgA levels (Table 5).

### Immunohistochemistry for IgA secreting plasma cells

Immunohistochemistry of jejunal sections from negative control animals (*SG.I A*) showed scanty IgA secreting plasma cells in the lamina propria (Fig. 5A), while there were a few more plasma cells in positive control mice (*SG.I B*); Fig. 5(B1, B2). Such cells appear brown colored in the lamina propria. Group II (preventive group) animals showed the presence of a moderate number of IgA secretory plasma cells (Fig. 6A, B) while there was a marked increase in IgA secretory plasma cells upon combined supplementation in *SG.II C* (Fig. 6C). In Group III (therapy group), a few IgA secreting plasma cells were seen in the prebiotic (*SG.*

*III A*), and the probiotic (*SG.III B*) subgroups, respectively (Fig. 7A, B). However, a moderate number of IgA secreting plasma cells were seen in the NTZ (*SG.III D*) group (Fig. 7D). A marked increase in IgA secreting plasma cells was seen with combined supplements in (*SG.III C*; Fig. 6C) and when added to NTZ in the *SG.III E* (Fig. 7E).

### Discussion

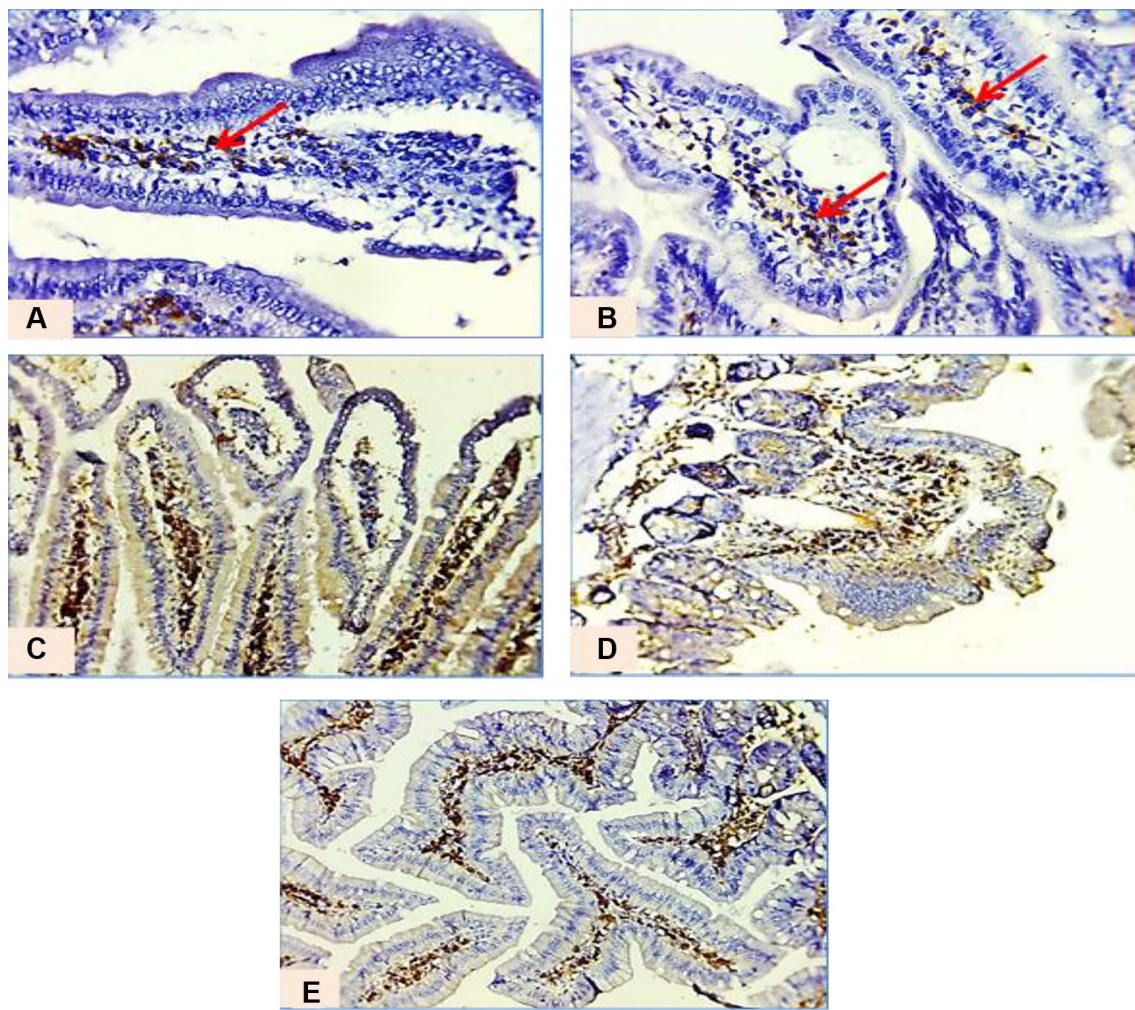
Giardiasis can affect children, the malnourished, and immune-compromised patients, and this study aimed to evaluate the impact of commercially available prebiotics and probiotics as alternative natural or synergistic supplements to NTZ. NTZ is an antiprotozoal chemotherapeutic drug against giardiasis and we evaluated the effects of these natural supplements on restoration of gut morphology and IgA modulation because these factors play an important role in pathogen (Goyal et al. 2011).

Our results show a significant reduction in *Giardia* cyst shedding after prebiotic and probiotic supplementation. These results are in agreement with those from previous studies of prebiotic supplementation to either healthy or malnourished *Giardia*-infected mice, which have reported a reduction in the severity of giardiasis and in cyst and trophozoite counts (Shukla et al. 2016; Shaaban et al. 2021). Similar results, viz., reduction in the intensity and duration of *Giardia* infection, have been reported after probiotic supplementation, either prior to or simultaneously with the infection (Shukla et al. 2008; Goyal and Shukla 2013; Ventura et al. 2018; Mazroue et al. 2020; Shaaban et al. 2021; Dashti and Zarebavani 2021).

Combined prebiotic and probiotic administration prior to infection (*SG.II C*) resulted in a greater reduction in cyst shedding compared to individual supplementation, and these observations also concur with those from previous studies (Shukla et al. 2019; Shaaban et al. 2021). This synergistic anti-*Giardia* effect can be explained by the fact that prebiotics can potentiate physiological and systemic actions of some probiotic strains (Collins et al., 2018). Congruently, while the effects of NTZ alone on cyst shedding reduction were significant, combined supplementation further boosted this decrease, and these results closely conform to data reported previously (Shaaban et al. 2021).

Many studies have described the destructive effects of *Giardia* infection on intestinal epithelial cells and we similarly show villous distortion, atrophy, edema and sloughing of the upper tips of some villi with excess inflammatory cell infiltration (Shukla et al. 2008; Shukla et al. 2013; Shukla et al. 2019; Mazroue et al. 2020; Shaaban et al. 2021). Nevertheless, while NTZ therapy led to histopathological improvement, combined prebiotic and probiotic supplementation led to greater improvement than NTZ alone and it





**Fig. 7** Photomicrograph of jejunal sections of Group III (therapy group) (A–E) (Immunohistochemical staining for IgA) **A** (400X): Prebiotic PI (SG.III A) showing mild IgA secretory plasma cells in the lamina propria (arrows). **B** (400X): Probiotic PI (SG.III B) showing mild IgA secretory plasma cells in the lamina propria (arrows).

**C** (200X): Prebiotic+probiotic PI (SG.III C) showing marked IgA secretory plasma cells in the lamina propria. **D** (200X): NTZ PI (SG. III D) showing moderate IgA secretory plasma cells in the lamina propria. **E** (200X): Prebiotic+probiotic+NTZ PI (SG.III E) showing marked IgA secretory plasma cells in the lamina propria

nearly restored normal villous structure when administrated with combined supplementation. This improvement in gut morphology can be attributed to the protective ability of the combination of prebiotic and probiotics in maintaining intestinal epithelial cell morphology, as described by Shukla et al. (2019) and Shaaban et al. (2021). Specifically, prebiotics and probiotics, when provided together, positively regulate epithelial cell growth and differentiation (Vyas and Ranganathan 2009).

Ultrastructure analysis of jejunal enterocytes corroborated histological observations, and our TEM results. Hence, positive controls showed massive damage and distortion in microvillous enterocytes including loss of microvilli, interrupted of intercellular junctions, edema and extrusion of cellular contents into the lumen, that are in agreement with

data from scanning electron microscopy reported previously (Shukla et al. 2019). Interestingly, we demonstrate that prebiotic and probiotic supplementation, either individually or in combination, showed more improvement in microvilli morphology than NTZ alone. Additionally, combined supplementation with NTZ demonstrated marked regeneration from ultrastructural damage. These observations are in agreement with those of Shukla et al. (2011) who noticed rapid regeneration of the atrophied epithelium in *Giardia*-infected mice upon supplementation with the probiotic *Lactobacillus casei*. The healing effects of combined supplementation described here are also similar to those reported by Shukla et al. (2019); specifically, that administration of probiotic *Lactobacillus casei* and the prebiotic inulin

resulted in greater improvement in microvilli morphology in *Giardia*-infected mice.

Serum IgA levels showed a highly significant increase in Groups II and III, and IgA levels were higher upon supplementation prior to infection rather than after infection, irrespective of whether the commercial prebiotic and probiotic provided separately or together. These results agree with those reported by Hardy et al. (2013), Goyal and Shukla (2013), Shukla et al. (2016), Hajare (2017), Mazroue et al (2020) and Dashti and Zarebavani (2021) who describe a significant increase in serum anti-*Giardia* IgA levels after prebiotic (inulin) or probiotic supplementation.

Elevated serum IgA levels upon combined supplementation have also been reported previously by Shukla et al. (2019), who state that use of a synbiotic (*Lactobacillus casei* + inulin) boosted the levels of anti-*Giardia* IgA antibodies and other cytokines (e.g., IL-6 and IL-10) when administered prophylactically, i.e., prior to infection, to malnourished mice. We also report increased IgA levels upon PI combined supplementation as a therapeutic. In fact, IL-6 is considered to be a key regulator of B-cell maturation, which helps in IgA production by promoting antibody class switching (Scheller et al. 2011). Hence, NTZ treatment in the SG.III D group resulted in elevated IgA levels, which can be connected to a previously reported increase in IL-6 levels after NTZ treatment in mice (Shaaban et al. 2021). The observed increase in IgA levels upon NTZ + combined supplementation closely conforms to results from a previous study (Shaaban et al. 2021). Thus, some authors have suggested that probiotics or combined supplements (prebiotic + probiotic), when added to antiprotozoal drugs, can be therapeutically more effective against giardiasis through enhancing the immune response (Ventura et al. 2007; Mazroue et al. 2020; Shaaban et al. 2021).

Immunohistochemistry of jejunal intestinal sections from negative control mice showed scanty IgA secreting plasma cells in the lamina propria. Further, only a few IgA secreting plasma cells were seen in infected positive control animals. Similarly, that others have stated that specific anti-*Giardia* secretory IgA (sIgA) can be detected in *Giardia*-infected patients (Rodríguez et al. (2004); Toma and Al-Hadraawy (2009). Further, there was scanty increase in IgA secreting plasma cells in an infected nontreated mouse model of giardiasis (Mazroue et al., 2020).

Prebiotic (SG.II A) and probiotic (SG.II B) supplementation in the preventive group, and NTZ therapy (SG.III D) led to the presence of a moderate number of IgA secreting plasma cells, while prebiotic (SG.III A) and probiotic (SG.III B) supplementation in the therapy group resulted in fewer IgA secreting plasma cells. Shukla et al. (2016) have measured the levels of anti-*Giardia* IgA and IgG antibodies in the intestinal fluids of malnourished mice and have stated that prebiotic supplementation in giardiasis resulted in a

significant increase in sIgA and IgG levels. Benyacoub et al. (2005), and Goyal and Shukla (2013) have also reported that oral feeding of probiotic prior to or simultaneously with *Giardia* infection resulted in a significant increase in the levels of specific sIgA antibody, IgA + cells, and CD4 + T lymphocytes. In contrast, Mazroue et al. (2020) have demonstrated the presence of a moderate number of IgA cells in the lamina propria with a marked increase in IgA cells in noninfected control and *Giardia*-infected mice respectively after probiotic supplementation. Thus, probiotics play a critical role in IgA modulation to help clear parasitic infections and reduce the severity of clinical presentations and PI complications in infected patients (Raheem et al. 2021; Fekete et al. 2021). Furthermore, combined prebiotic and probiotic supplementation, either before infection (group II) or when added to NTZ (group III) showed a marked increase in IgA secreting plasma cells. A similar result has been reported by Shukla et al. (2019). Likewise, addition of probiotic to metronidazole showed a marked increase in IgA cells in intestinal sections (Mazroue et al. 2020).

## Conclusions

In summary, this study provides insights into ultrastructural changes occurring during healing after combined prebiotic and probiotics supplementation. These results corroborated histopathological observations, indicating that natural supplements have a pivotal role in infection resolution and pathological sequelae. Furthermore, serum IgA and immunohistochemistry results illustrate that combined supplementation can promote anti-*Giardia* immunomodulatory properties. Thus, based on our results, it can be concluded that combined supplementation with pre- and probiotics increases the efficacy and therapeutic potential of NTZ against giardiasis. Hence, oral administration of combined prebiotic and probiotic may be recommended for protection against *Giardia* infection.

**Author contributions** HZR, study planning, samples collection, experimental work support, data analysis of all parameters, and manuscript writing. SDEA, histopathological and immunohistochemical data analysis. HFI, immunological data analysis and manuscript text correction. SGA, histopathological and ultrastructural data studies assessment.

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**Data availability** This article contains all the data produced or analyzed throughout the study.

**Declarations**



**Conflict of interest** No author claims to have any conflict of interest.

**Ethical approval** Compliance with ethical standards the ethical committee of the national institution (Faculty of Medicine for Girls Al-Azhar University and TBRI) and the current international standards for animal handling were followed in the conduct of this study. The Faculty of Medicine for Girls at Al-Azhar University's ethics committee gave the Protocol their approval (RHDIRB/2018122001, Protocol No. 563/2021).

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