

# Ramification of TNF- $\alpha$ -associated apoptosis on lactase enzyme activity due to *Blastocystis hominis* infection

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## Research Article

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# Abstract

*Blastocystis hominis* is a cosmopolitan protozoan that has been associated with several gastrointestinal disturbances involving lactose intolerance. However, the underlying pathogenic factors remain indistinct. 20 Swiss albino mice were utilized and assembled into four groups, each of five mice: group-I: received neither infection nor lactose (healthy control), group-II: received a single dose of 10000 cysts of *Blastocystis* and lactose diets in a dose of 12.5 g/ day/mouse for 7 consecutive days starting from day 14 p.i, group-III: non-infected mice with oral doses of lactose (12.5 g/day/mouse) for 7 consecutive days (positive control), group-IV: infected mice on lactose free diet (negative control). We investigated the histopathological changes using H&E stain. Also, lactase enzyme activity was measured using spectrophotometry and the production of TNF- $\alpha$  and apoptotic events were explored via immunohistochemistry and compared in the small intestine of all groups. The active inflammatory changes in the infected animals were moderate in the form of loss of villous architecture, increased ILC ( $P$ -value > 0.001) besides scattered forms of the parasite as compared to non-infected mice. There was a reduction in lactase enzyme activity p.i... The TNF- $\alpha$  levels were induced p.i. as compared to non-infected mice ( $P$ -value > 0.001). The expression of Bax protein was upgraded, while Bcl-2 expression decreased significantly with a reverse in Bax/Bcl2 ratio in infected animals. *Blastocystis* infection appears to humble lactase enzyme activity via the induction of apoptosis in the epithelial cells of the small intestinal brush border in a TNF- $\alpha$  associative pathway.

## Introduction

*Blastocystis* is a single-celled anaerobic and enteric parasite that was found to colonize small intestine and large intestine (Wang et al., 2004, Pavanelli et al., 2015 and Kumarasamy et al., 2017). Although, It is supported by strong clinical and scientific evidences that *Blastocystis* is pathogenic organism, the virulence factors, pathogenicity and other risk factors involved in disease manifestation are still obscure (Tan et al., 2010 and Scanlen, 2012).

Recently, *Blastocystis* infections are associated with a variety of gastrointestinal disorders and extraintestinal disorder; cutaneous and joint manifestation (Boorom et al., 2008 and Wawrzyniak et al., 2013). Clinical features of *Blastocystis* infection include abdominal pain, diarrhoea, nausea, anorexia, bloating, flatulence, lactose intolerance, constipation and weight loss (Tan et al., 2010) with significant association between *Blastocystis* and irritable bowel syndrome (IBS) (Tungtrongchitr et al., 2004, Yakoob et al., 2010, Jimenez-Gonzalez et al., 2012 and Poirier et al., 2012), *Blastocystis*-associated enteritis (Gallagher and Venglarcik 1985, Gorman et al., 1993, Zuckerman et al., 1994 and Rossignol et al., 2005), terminal ileitis (Tsang et al. 1989), and ulcerative colitis (Jeddy and Farrington, 1991, K k et al., 2019).

$\beta$ -glucosidases enzymes comprise lactase phlorizin hydrolase enzyme that catalyzes the digestion of lactose present in milk into glucose and galactose since early infancy. Lactase enzyme also termed the brush border enzyme that is produced by the enterocytes that line the luminal side of the brush border of the small intestine. Disorders in lactase enzyme lead to lactose intolerance, or incompetency in the

digestion of lactose resulting into its fermentation by intestinal microbiota causing gas production and abdominal discomfort (Daniels et al., 1981, Day et al., 2000). The relationship between microbial infections in the gastrointestinal tract and lactose intolerance has been speculated in several studies. In giardiasis infection carbohydrates is one of the most frequently mal-absorbed nutrients (Ament & Rubin, 1972, Ament et al., 1973, Singh et al., 2000). Additionally, rotavirus causes the destruction of the enterocytes lining the brush border of the intestinal mucosa and thus minimize the absorptive surface area and distress several digestive enzymes leading to osmotic diarrhoea primarily due to carbohydrate malabsorption (Graham et al., 2004). However, the hidden impact of blastocystosis on lactose intolerance has not been much explored.

TNF- $\alpha$  inflammatory cytokine is a protein in nature which is produced by the mesenchymal and epithelial cells for cell signaling to regulate the epithelial barrier in multiple ways, including mucus secretion, barrier permeability, proliferation/differentiation and wound healing (Günther et al., 2013). Additionally, if cells of immune system especially macrophages detect infection, they release TNF- $\alpha$  to alert other cells of immune system as well as cells of other tissue leading to inflammation (Lake et al., 1994).

Bax is a pro-apoptotic regulator while Bcl-2 is an anti-apoptotic. They are both essential in intrinsic cell death via mitochondria (Westphal et al., 2014). Yu et al. (2014) reported the incidence of apoptosis in intestines infected with *Trichinella spiralis* in murine models. Liu et al., (2020) reported that *Giardia duodenalis* triggers apoptosis in the epithelial cells lining the intestinal mucosa in a pathway dependent chiefly on reactive oxygen species. Buret et al. (2003) speculated that *Cryptosporidium andersoni* prompts apoptosis and interrupts tight junctions in the intestinal mucosa. In 1995, Wiley et al. characterized and identified the TNF dependent apoptosis. Also, Sundararajan et al. (2001) speculated that TNF- $\alpha$  triggers apoptosis through Bax-Bak interaction. However, in adenovirus infections, the virus possesses Bcl-2 homologue that blocks TNF- $\alpha$ -mediated apoptosis. In this context, in blastocystosis infection, the apoptotic events in the small intestinal tract are still not clear.

The present work is an experimental cross sectional study that manipulated murine models to reveal the pathological changes in the mucosa of the small intestine in *Blastocystis* induced infections. Additionally, the associative lactase enzyme activity and the role of intestinal epithelia as producers and targets for TNF- $\alpha$  cytokine in the apoptotic death were evaluated.

## Methodology And Material

### Recruitment of infective samples

Twenty -five patients attending Diagnostic and Research Unit of Parasitology (DRUP) at Kasr Al-Ainy school of medicine, the outpatient clinic of Abu El-Rish children hospital and the outpatient clinic of Theodor Bilharz Research Institute (TBRI) in the period from December 2019 to October 2020 were considered for the study. Two to three samples were collected from each patient. Patients with inflammatory bowel disease, gastrointestinal tumours were excluded as well as subjects who took antibiotics, proton pump inhibitors or non-steroidal anti-inflammatory drugs in the last 30 days. Subjects

with persistent gastrointestinal manifestations more than 6 months were also excluded. All samples were recruited in sterile clean plastic cups taking care that the specimens were not contaminated with water or urine then they were subjected to the routine macroscopic & microscopic examination.

### **Isolation of the parasite and culture conditions**

Positive stool samples containing *Blastocystis* were pooled together then emulsified in normal saline and strained through gauze into centrifuge tube. The tube was centrifuged at 2000 r.p.m. for 10 minutes, the supernatant was decanted. This process was repeated several times until the supernatant was totally clear.

The parasite was grown using Jones' media supplemented with 10% horse serum and antibiotic solution (Penicillin G and Streptomycin in a concentration of 0.1%). The pH was accustomed to pH 7 with Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, and NaCl prior to autoclaving for sterilization at 121°C. For culture inoculation, a stool portion was transferred aseptically into the culture tubes with a clean glass rod and mixed with the culture medium. Each tube was labelled with the patient's name and number, and the date and incubated at 37°C for 48 – 72 hrs. in the incubator.

### **Animals**

Twenty laboratory bred male Swiss albino mice aged 3-4weeks age were supplied by the European Country Farms in Egypt and were affirmed in TBRI. Throughout the experiment, the mice were kept under standard experimental circumstances in a 12 h light/dark cycle and were kept on a standard diet containing 24% protein, 4% fat and about 4-5% fiber and water in the biological unit of TBRI under a temperature of 24°C. Periodic veterinary inspections were performed to approve that all animals were clear of common murine pathogens. Hygienic disposal was performed to remove mice waste and the dead animals.

### **Experimental design**

Sample size was calculated by: Statistical analysis of ANOVA test as (according to G power analysis (v.9.3.1)  $\alpha=0.05$ , sample size effect =0.4,  $1-\beta=0.5$ , number of group 4, one way, total sample size =20, sample size =5 mice (**Arifin and Zahiruddin, 2017**). Group I (healthy control group) received neither infection nor lactose. Group II was infected by *Blastocystis* and received lactose diet. Group III were non-infected mice and received lactose diet (positive control). Group IV was infected with *Blastocystis* without lactose intake (negative control).

### **Induction of infection**

Experimental mice were inoculated via intra oesophageal catheter with 10<sup>4</sup> cysts from *Blastocystis* culture (**Moe et al., 1997**) suspension in sterile saline. Weekly post-infection (p.i.), faecal samples from mice's rectum were collected and subjected to parasitological examination by direct wet mount technique to detect *Blastocystis* infection.

## Lactose administration

Fourteen days p.i., *Blastocystis* was detected in stool with more than 8 cysts in the field indicating heavy infection of mice when lactose diet was initiated to group II and group III for 7 consecutive days (Silvia et al., 2002). Extrapolation of animal dose was calculated using the following formula (Nair and Jacob, 2016):

$$\text{Animal dose} = 12.5 \times \text{human dose}$$

$$\text{Human dose} = 0.5 \text{ gm lactose} \times \text{body weight (kg)} \text{ dissolved in } 100 \text{ ml distilled water}$$

$$\text{Animal dose} = 12.5 \times 50 = 625 \text{ mg}; \text{ whereas dose per animal (20mg)} = 12.5 \text{ gm}$$

Twenty-one days post infection all mice were sacrificed by rapid decapitation of all groups by a well-trained laboratory technician. Mice were fastened overnight to minimize intestinal contents and parts of the small intestine were prepared for detection of lactase enzyme activity. They were stained with Haematoxylin and Eosin to detect any histopathologic or structural abnormalities in addition to immunostaining for detection of inflammatory (TNF- $\alpha$ ) and apoptotic biomarkers (Bax and Bcl-2) in situ in collected tissue of the intestine.

## Bioassay of lactase enzyme activity

Lactase activity was used as a biomarker of *Blastocystis hominis* induced mucosal damage. The activity of the enzyme was evaluated and defined as unites/mg of tissue protein.

## Sample preparation/extraction

Small intestinal tissue samples were accurately weighed; PBS (0.01 M, pH7~7.4) was added to the sample according to the ratio of weight (g): volume (mL) = 1:9; tissue samples were mechanically homogenized in ice water bath; all samples were then centrifuged at 3500 rpm for 10 min. Total protein concentration was investigated in the supernatant of homogenized tissue samples using NANODROP<sup>®</sup> 2000C spectrophotometer and the supernatant was stored at – 20 °C.

The enzymatic reaction was carried out according to the lactase assay kit (Cat. no. E-BC-K131-S, Elabscience Biotechnology Inc, USA). Briefly, 5.5 mmol/L glucose standard solution was prepared together with the blank, samples and control. The substrate solution was added, mixed thoroughly and incubated at 37 °C for 20 min. Afterwards, the stop solution was added and fully mixed, then all mixtures were centrifuged at 4000 r.p.m for 10 min. The chromogenic agent was added to supernatant of each mixture, mixed well and incubated for 10 min. Each sample was measured in triplicate at 505 nm using spectrophotometer.

The amount of lactase in 1 mg of protein that hydrolyzes 1 nmol of lactose per minute at 37°C and pH 6.0 was defined as 1 unit according to the following formula:

$$\text{Lactase activity (U/mg prot)} = \frac{OD_{\text{Sample}} - OD_{\text{Control}}}{OD_{\text{Standard}} - OD_{\text{Blank}}} \times \text{Concentration of standard (5.55 nmo/L) / Reaction time (20 min.)} \\ / \text{Protein concentration of sample (mg } \frac{\text{prot}}{\text{mL}}) \times 1000$$

## Histopathological examination

The excised segments of the small intestine were cut both transversely and longitudinally, labelled and numbered then fixed in formalin 10%. Then the samples were impregnated in molten paraffin for 6 hours. After that samples were sectioned by microtome at 5 µm thickness (**Drury and Wallington, 1980**) and stained with Hematoxylin & Eosin stain (H&E).

## Immunohistochemical staining

Detection of inflammatory biomarker (TNF) and apoptotic biomarkers (Bax and Bcl-2) was performed in situ in the intestine. Tissue sections were deparaffinised, dehydrated in absolute ethanol for 15 seconds, and incubated sequentially in 2% (v/v) H<sub>2</sub>O<sub>2</sub> in methanol for 30 to 45 seconds, 95% ethanol for 20 seconds, 70% ethanol for 20 seconds, distilled H<sub>2</sub>O for 1 minute, and PBS (120 mmol/L NaCl, 11.5 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 31.3 mmol/L KH<sub>2</sub>PO<sub>4</sub>, pH 7.4 to 7.6) for 5 minutes. Then the tissue sections were heated by microwaving in acidic buffer then washed twice in PBS for 5 minutes.

Tissue sections were pre-blocked for 30 to 45 minutes in Tris sodium potassium (TNK) solution (100 mmol/L Tris, pH 7.6 to 7.8; 550 mmol/L NaCl; 10 mmol/L KCl) containing 2% (w/v) bovine serum albumin, 0.1% Triton X-100, and 1% normal goat serum. We applied the following anti-rodent primary monoclonal antibodies: anti-Bax (1:1000 to 1:2000 v/v), anti-Bcl-2 (1:800 to 1:1500 v/v), and anti-TNF-α. Then the tissue sections were incubated overnight at room temperature. After washing with PBS, tissue sections were incubated for 1 hour with 2.8 pg/ml of biotinylated goat anti-rabbit antibody in the same buffer containing 0.5% normal mouse serum. Tissue sections were then washed and incubated for 30 to 45 minutes with an avidin-biotin complex reagent containing horseradish peroxidase (Vector) in TNK. Colour development was achieved by incubation for 10 minutes with a solution containing 3, 3'-diaminobenzidine and 0.1% (v/v) H<sub>2</sub>O<sub>2</sub> in TNK buffer. Counterstaining was performed using hematoxylin. Slides were photographed with a light microscope equipped with a 35-mm camera with Ektar 100 film (**Krajewski et al., 1994**). Median density, intensity, area percentage were measured and morphological analysis was carried using different magnification powers. Immunostained sections were evaluated in a blind fashion, with the help of a pathologist. Immunopositivity was scored from grade 1 to grade 4 by increasing the extent of immunostaining in 10 fields (1: 25%; 2: 25%–50%, 3: 50%–75%; 4: 75%–100%) and through the addition of the staining intensity grade: 1 (mild), 2 (moderate), and 3 (strong) immunoreactivity; the final grades were expressed as 1 (mild or weak), 2 (moderate), and 3 (strong).

## Statistical analysis of data

The collected data were carefully revised, coded, tabulated and introduced to a personal computer using "Microsoft Office Excel Software" program (2010) for windows. The pre-coded data were then transferred to the Statistical Package of Social Science Software program, version 23 (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.) to be statistically analysed. Data were presented using range, mean, standard deviation (S.D.) for quantitative variables. Descriptive statistics were done in the form of the mean and the standard deviation (+/- S.D.) for the parametric numerical data. For the analytical statistics, paired t-test was used to assess the statistical significance among the experimental groups, *P*-value was considered significant at < 0.001. The results were represented in tables and graphs.

## Results

Infected animals either on the lactose diet (group II) or on lactose free diet (group IV) refused feeding and showed weight loss, diarrheic stool, lethargy and signs of depressions than that observed in the control non infected groups (group I and group II). One mouse died from group IV throughout the course of the experiment. The burden of parasite shedding in the stool of group II and group IV showed a significant increase 14 days p.i., *P*-value >0.001.

Histopathological examination of tissue sections from the small intestine of mice 21 days p.i. showed significant hyperplasia in the number of goblet cells in group II and group-IV as compared to non-infected groups (*P*-value > 0.001). Sections of the small intestine from infected mice showed moderate active inflammation in the form of shortening and broadening of the villi, edema of lamina propria, and moderate inflammatory cellular infiltrates. There was significant increase (*P*-value > 0.001) in the intraepithelial lymphocytic count in both infected animals on lactose diet (group II) and those infected but on lactose free diet (group IV) as compared with group I and group III. Scattered vacuolar forms of the parasite were also present in the mucosa of the infected mice, figure (1).

The overall concentrations of proteins revealed insignificant variation among different groups (*P*-value < 0.001). The concentrations and activity of Lactase enzyme in the small intestine showed significant reduction in the infected groups than in non-infected groups (*P*-value >0.001). However, the mean values of lactase enzyme activity in group II ( $4.593 \pm 0.775$ ) were higher than group IV ( $2.484 \pm 0.908$ ), figure (2). Mean values are presented in tables (1, 2, and 3).

**Table (1):** Concentrations of proteins in the tissue samples of small intestine of mice 21 days p.i.

<b>Groups</b>	Protein concentration (mgprot /ml)	<b>Mean +/-S.D.</b>	<b>P- value</b>
<b>Group I</b>	30.72	31.15 ± 1.045	< 0.001
	32.81		
	31.80		
	30.32		
	30.12		
<b>Group II</b>	24.12	25.91 ± 7.226	< 0.001
	35.21		
	25.27		
	16.31		
	28.64		
<b>Group III</b>	17.05	27.58 ± 8.955	< 0.001
	28.68		
	34.66		
	28.58		
	28.93		
<b>Group IV</b>	39.96	32.51±13.076	< 0.001
	36.95		
	27.10		
	13.35		
	45.20		

**Table (2):** Lactase enzyme concentrations in the small intestine of mice 21 days p.i.

<b>P- value</b>	<b>Mean +/-S.D.</b>	<b>Spectrophotometry Measurements</b>	<b>Groups</b>
>0.001	0.343 ±0.005	0.339 0.342 0.348 0.341 0.339	<b>Group I</b>
>0.001	0.170 ± 0.047	0.156 0.234 0.196 0.124 0.173	<b>Group II</b>
>0.001	0.490 ±0.324	0.184 0.456 0.829 0.457 0.465	<b>Group III</b>
>0.001	0.149 ±0.063	0.155 0.133 0.117 0.047 0.209	<b>Group IV</b>

**Table (3):** Measurements of lactase enzyme activity in the small intestine of mice 21 days p.i.

<b>P- value</b>	<b>Mean +/-S.D.</b>	<b>Lactase activity (U/mg prot.)</b>	<b>Groups</b>
>0.001	8.780 ± 0.263	8.97	<b>Group I</b>
		8.48	
		8.89	
		7.89	
		7.82	
>0.001	4.593 ± 0.775	4.59	<b>Group II</b>
		5.143	
		5.81	
		5.026	
		4.40	
>0.001	13.883 ±6.388	7.97	<b>Group III</b>
		13.288	
		20.69	
		13.37	
		14.12	
>0.001	2.484± 0.908	2.75	<b>Group IV</b>
		2.43	
		2.792	
		0.944	
		3.508	

### **Tumour necrosis factor alpha (TNF-α) immune staining**

In group-I and group-III, TNF-α was positive in small intestine of three of the 10 normal mice (3/10, 30%). The expression of TNF-α was mild and limited to superficial epithelial cells in lamina propria of the small intestine. These positive cells were few in number and largely confined to the subepithelial zone and to some degree in submucosa. In group II and group IV, TNF-α was positive in small intestine of six out of 10 infected mice (6/10, 60%). Among the positive sections, the expression of TNF-α was strong in 4 tissue samples and moderate in 2 samples, figure (3). Expression of TNF-α was cytoplasmic and extracellular, widely distributed in superficial and basal epithelial cells along the brush border of the small intestinal villi.

### **Apoptotic biomarker (Bax)**

In group I and group III, Bax protein was positive in the small intestine of four non-infected animals out of 10 mice (4/10, 40%). Among the positive sections, the expression of Bax was mild in 3 tissue samples (3/4, 75%) and moderate in 1 sample (1/4, 25%). Bax expression was mostly located at the free edge of the surface epithelial cells of the villi, especially at the tips in addition to the lower part or the base of the crypts. In group II and group IV, the expression of Bax protein was significantly detected in six mice out of 10 infected animals (6/10, 60%) as compared to non-infected groups ( $P$ -value  $>0.001$ ). Among positive sections, the expression of Bax was strong in 4 tissue samples (4/6, 66.6%) and moderate in 2 samples (2/6, 33.3%). Bax expression was widely distributed in the cytoplasm of the superficial and basal epithelial cells along the brush border of the villi of the small intestine, figure 4.

### **Anti-apoptotic biomarker (Bcl2)**

In group I and group III, Bcl2 protein was positive in small intestine of five of the 10 normal mice (5/10, 50%). Among the positive sections, the expression of Bcl2 was mild in one tissue sample (1/5, 20%), moderate in three samples (3/5, 60%) and strong in one tissue sample (1/11, 20%). Bcl-2 protein was mostly in the cytosol and nuclear envelope of epithelial cells in the lower part or base of crypts in normal small intestine. Expression of Bcl2 protein was also observed in the surface epithelial cells of the villi and at the lower part or base of the crypts in some cases. In group II and group IV, Bcl2 protein was positive in small intestine of two out of 10 infected mice (2/10, 20%) where the expression of Bcl2 was mild with  $P$ -value  $>0.001$  as compared to non-infected mice. Bcl2 expression was mostly in the cytoplasm and nuclear envelope of the epithelial cells of the small intestinal villi.

In regard to the extent of immunostaining and the grade of staining intensity, Bax/Bcl-2 ratio showed significant up-regulation in infected groups whereas in normal groups it was significantly down-regulated ( $P$ -value  $<0.001$ ).

## **Discussion**

In this study, detection of *Blastocystis* infection in the mucosa of the small intestine supports that *Blastocystis* can colonize small intestine as well as colon of the host. Similar to our results, Pavanelli et al. (2015) showed the distribution of vacuolar forms of *Blastocystis* in orally infected mice that involved the small intestine (duodenum), cecum and large intestine (proximal colon). As well, Wang et al. (2014) detected the DNA of *Blastocystis* within the contents of duodenum, jejunum and ileum in 36% (10/28) of experimentally infected pigs. Likewise, Defaye et al. (2018) reported that *Blastocystis* vacuolar forms were observed from duodenum to distal colon mainly localized in the lumen and in close contact with epithelial cells.

Regarding the changes in villous architectures, there was significant loss in the normal histological structure in infected mice; besides the significant proliferation of the intraepithelial lymphocytic counts, in the infected mice as compared to non-infected mice. Kumarasamy et al. (2017) reported the significant sloughing of the mucosal epithelium, reduction in the goblet cells, infiltration of polymorphonuclear neutrophils and monocytes in the intestinal mucosa. Also, Yao et al. (2005) demonstrated the presence of

the parasites in the whole gastrointestinal tract in the immunocompromised mice with severe edema, hyperemia and congestion in the tissues of jejunum, ileum, cecum and colon where the epithelial lining of the small intestine and colonic mucous membrane exhibited exfoliation, inflammatory cell infiltration in submucosa, and structural changes in glands. Furthermore, Camilleri et al. (2012) documented that biopsy analysis from *Blastocystis*-infected patients showed disorders in the integrity and permeability of the intestinal barrier. *Blastocystis* altered expression of tight junction (TJ) which allows the diffusion of luminal antigens and/or proteases to the submucosal compartments.

In the present study, there was a significant reduction in lactase enzyme activity in *Blastocystis* infected groups to its lowermost levels with a mean value  $2.484 \pm 0.908$ . This result of lactase enzyme activity was lower than normal groups with or without lactose diet and elucidated the relationship between *Blastocystis* infection and the reduction in the lactase enzyme activity which is commonly considered as the first-line of digestion for disaccharides. Previous studies reported the symptoms of lactose intolerance in 6% of patient and irritable bowel syndrome in 12% of *Blastocystis* infected patients (Bálint et al., 2014). Also, Yakoob et al (2011) demonstrated lactose intolerance in 21 % of patients with IBS, 15% in patients with chronic non-specific diarrhea and bloating in 50% of patients with IBS. Interestingly, Kain et al. (1987) reported that dietary management with lactose-free diet in patients with *B. hominis* infection was found to reduce either symptoms or parasite numbers in an epidemiological study.

In the current study, the effect of *B. hominis* infection on cell mediated response was investigated by immune staining of TNF- $\alpha$  in small intestine of all study groups. There was moderate to strong expression of the cytokine in the superficial epithelial cells in the lamina propria of small intestine in 60% of the infected mice (60%); whereas in normal groups, positive cells were largely confined to the subepithelial zone in only three mice. Iguchi et al. (2009) suggested that the intestinal colonization of *Blastocystis* parasites trigger the activation of T cells, monocytes/macrophages, and/or natural killer cells in local tissues through the upregulation of TNF- $\alpha$ , IFN- $\gamma$ , and IL-12. The same authors suggested the absorption of *Blastocystis*-derived molecules through the epithelial lining of the intestinal mucosa via paracellular and transcellular pathways. Additionally, Yakoob et al. (2014) reported the induction of Th1 immune responses and production of IFN- $\gamma$  and TNF- $\alpha$  from T and natural killer cells in *Blastocystis* infections. Similarly, Sarhan et al. (2019) reported the presence numerous immune positive inflammatory cells in the submucosa of an intestinal fold in *Blastocystis* infected mice. However, Jimenez et al. (2012) revealed the significant linkage disequilibrium in TNF- $\alpha$  expression in *Blastocystis* infection.

In the current study, expression of BAX protein was moderate to strong positive in 60% of the small where as in the normal groups the expression was confined to 40% of the cases and mostly located at the tips of the free edges of the surface epithelial cells of the villus. Additionally, the expression Bcl2 protein was mild to moderate in 20% of the infected mice as compared with non-infected mice where the expression of the protein was moderate to strong in 50% of the animals. Steck et al. (2011) and Mirza et al. (2012) reported that both parasite and parasite lysates have damaging effects on intestinal epithelial cells causing apoptosis and degradation of tight junction proteins occludin and ZO1, resulting in increased intestinal permeability. Similarly, Kumarasamy, et al., (2013) signified that *Blastocystis*-Ag stimulation

has caused various patterns of cytokine and apoptotic gene expressions with increase Th1 cytokines (e.g., IFN- $\gamma$  and TNF- $\alpha$ ) which represents the cellular immune responses. Furthermore, Puthia et al. (2006) reported that *Blastocystis ratti* induces apoptosis in the intestinal epithelial cells in a contact-independent manner. Besides, it rearranges F-actin protein, decreases transepithelial resistance, and increases epithelial permeability. Interestingly, Parker et al. (2019) demonstrated that in TNF-dependent intestinal inflammation the rate of apoptosis increases causing impairment in the turnover of the epithelial cells lining the intestinal villi and irreversible reduction in the length of the intestinal villi.

## Conclusion

*Blastocystis* infection was associated with reduced lactase enzyme activity. Immunity to *Blastocystis* infection involved the small intestinal mucosa in a TNF- $\alpha$  associative pattern. The rate of apoptosis despite being low in normal mucosa, Bax/Bcl2 ratio increased significantly in the mucosa of the small intestine of *Blastocystis* infected mice. Blastocystosis seems to trigger lactose intolerance with an associating induction to the expression of TNF- $\alpha$  and apoptotic proteins in the epithelial cells of the small intestine.

## Declarations

### Ethics statement

All procedures were performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and were approved by the institution accountable for animal ethics regarding care for animals and safe discarding of their waste products at TBRI and were approved by ethical committee of Kasr Al-Ainy school of Medicine, TBRI and the Cairo University of Institutional Animal Care and Use Committee (CU- IACUC) and was logged by CU/III/F/6/20.

## References

- Ament ME, Ochs HD, David DD: Structure and functions of the gastrointestinal tract in primary immunodeficiency syndrome. A study of 39 patients. *Medicine* 52:227-248, 1973.
- Ament ME, Rubin CE: Relation of giardiasis to abnormal intestinal structure and function in gastrointestinal immunodeficiency syndrome. *Gastroenterology* 62:216–226, 1972
- Arifin, W. N., & Zahiruddin, W. M. (2017). Sample size calculation in animal studies using resource equation approach. *The Malaysian journal of medical sciences: MJMS*, 24(5), 101.
- Bálint A, Dóczy I, Bereczki L, Gyulai R, Szűcs M, Farkas K, Urbán E, Nagy F, Szepes Z, Wittmann T (2014) Do not forget the stool examination!—cutaneous and gastrointestinal manifestations of *Blastocystis* infection. *Parasitol Res* 113(4):1585–1590
- Boorom, K. F., Smith, H., Nimri, L., Viscogliosi, E., Spanakos, G., Parkar, U., ... & Jones, M. S. (2008). Oh my aching gut: irritable bowel syndrome, Blastocystis, and asymptomatic infection. *Parasites &*

*Vectors*, 1(1), 1-16.

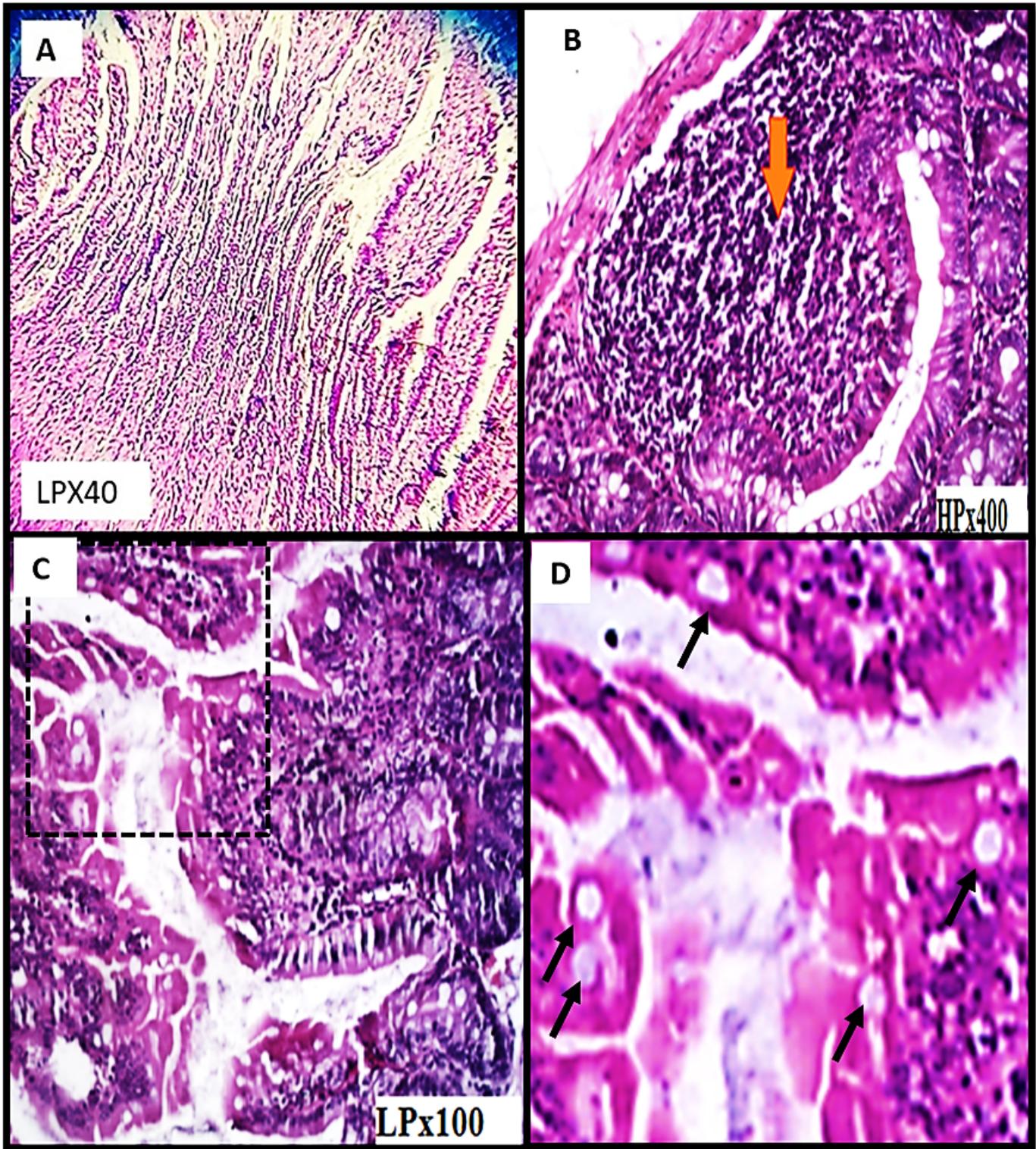
- Buret, A. G., Chin, A. C., & Scott, K. G. (2003). Infection of human and bovine epithelial cells with *Cryptosporidium andersoni* induces apoptosis and disrupts tight junctional ZO-1: effects of epidermal growth factor. *International journal for parasitology*, 33(12), 1363-1371.
- Camilleri, Á., Madsen, K., Spiller, R., Van Meerveld, B. G., & Verne, G. N. (2012). Intestinal barrier function in health and gastrointestinal disease. *Neurogastroenterology & Motility*, 24(6), 503-512.
- Daniels, L.B., Coyle, P.J., Chiao, Y.B., Glew, R.H. and Labow, R.S., 1981. Purification and characterization of a cytosolic broad specificity beta-glucosidase from human liver. *Journal of Biological Chemistry*, 256(24), pp.13004-13013.
- Day, A. J., Cañada, F. J., Díaz, J. C., Kroon, P. A., Mclauchlan, R., Faulds, C. B., ... & Williamson, G. (2000). Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS letters*, 468(2-3), 166-170.
- Defaye, M., Nourrisson, C., Baudu, E., Warwzyniak, I., Bonnin, V., Bonnet, M., & Poirier, P. (2018) Efficient and reproducible experimental infections of rats with *Blastocystis* spp. *Plos one*, 13(11), e0207669.
- Drury, R. A. B., & Wallington, E. A. (1980). Carleton's histological technique 5th ed. *New York: Churchill Livingstone*.
- Gallagher, P. G., & Venglarcik, J. S. (1985). *Blastocystis hominis* enteritis. *The Pediatric Infectious Disease Journal*, 4(5), 556.
- Graham, D. Y., Sackman, J. W., & Estes, M. K. (1984). Pathogenesis of rotavirus-induced diarrhea. *Digestive diseases and sciences*, 29(11), 1028-1035.
- Günther, C., Neumann, H., Neurath, M. F., & Becker, C. (2013). Apoptosis, necrosis and necroptosis: cell death regulation in the intestinal epithelium. *Gut*, 62(7), 1062-1071.
- Iguchi, A., Yoshikawa, H., Yamada, M., Kimata, I. and Arizono, N., (2009): Expression of interferon gamma and proinflammatory cytokines in the cecal mucosa of rats experimentally infected with *Blastocystis* sp. strain RN94-9. *Parasitology research*, 105(1), p.135.
- Jeddy, T. A., & Farrington, G. H. (1991). *Blastocystis hominis* complicating ulcerative colitis. *Journal of the Royal Society of Medicine*, 84(10), 623.
- Jimenez-Gonzalez DE, Martinez-Flores WA, Reyes-Gordillo J, Ramirez-Miranda ME, Arroyo-Escalante S, Romero-Valdovinos M, et al. (2012): *Blastocystis* infection is associated with irritable bowel syndrome in a Mexican patient population. *Parasitol Res* 110: 1269–1275. pmid:21870243.
- Jiménez-González, D.; Martínez-Flores, W.; Reyes-Gordillo, J.; Ramírez-Miranda, M.; Arroyo-Escalante, S.; Romero-Valdovinos, M.; Stark, D.; Souza-Saldivar, V.; Martínez- Hernández, F.; Flisser, A.; Olivo-Díaz, A. and Maravilla, P. (2012): *Blastocystis* infection is associated with irritable bowel syndrome in a Mexican patient population. *Parasitol. Res.*, 110: 1269-1275.
- Kain, K. C., Noble, M. A., Freeman, H. J., & Barteluk, R. L. (1987). Epidemiology and clinical features associated with *Blastocystis hominis* infection. *Diagnostic microbiology and infectious disease*, 8(4), 235-244.

- Kök, M., Çekin, Y., Çekin, A. H., Uyar, S., Harmandar, F., & Şahintürk, Y. (2019). The role of Blastocystis hominis in the activation of ulcerative colitis. *The Turkish Journal of Gastroenterology*, 30(1), 40.
- Krajewski, S., Krajewska, M., Shabaik, A., Wang, H. G., Irie, S., Fong, L., & Reed, J. C. (1994). Immunohistochemical analysis of in vivo patterns of Bcl-X expression. *Cancer Research*, 54(21), 5501-5507.
- Kumarasamy, V., Kuppusamy, U. R., Jayalakshmi, P., Samudi, C., Ragavan, N. D., & Kumar, S. (2017). Exacerbation of colon carcinogenesis by Blastocystis sp. *PloS one*, 12(8), e0183097.
- Kumarasamy, V., Kuppusamy, U. R., Samudi, C., & Kumar, S. (2013). Blastocystis sp. subtype 3 triggers higher proliferation of human colorectal cancer cells, HCT116. *Parasitology research*, 112(10), 3551-3555.
- Lake, F. R., Noble, P. W., Henson, P. M., & Riches, D. W. (1994). Functional switching of macrophage responses to tumor necrosis factor-alpha (TNF alpha) by interferons. Implications for the pleiotropic activities of TNF alpha. *The Journal of clinical investigation*, 93(4), 1661-1669.
- Liu, L., Fang, R., Wei, Z., Wu, J., Li, X., & Li, W. (2020). Giardia duodenalis Induces Apoptosis in Intestinal Epithelial Cells via Reactive Oxygen Species-Mediated Mitochondrial Pathway In Vitro. *Pathogens*, 9(9), 693.
- Mirza, H., Wu, Z., Teo, J. D. & Tan, K. S. Statin pleiotropy prevents rho kinase-mediated intestinal epithelial barrier compromise induced by Blastocystis cysteine proteases. *Cell Microbiol*14, 1474–84 (2012).
- **Moe, K. T., Singh, M., Howe, J., Ho, L. C., Tan, S. W., Chen, X. Q. & Yap, E. H. (1997):** Experimental Blastocystis hominis infection in laboratory mice. *Parasitology research*, 83(4), 319-321.
- Nair, A. B., & Jacob, S. (2016). A simple practice guide for dose conversion between animals and human. *Journal of basic and clinical pharmacy*, 7(2), 27.
- O'Gorman, M. A., Orenstein, S. R., Proujansky, R., Wadowsky, R. M., Putnam, P. E., & Kocoshis, S. A. (1993). Prevalence and characteristics of Blastocystis hominis infection in children. *Clinical pediatrics*, 32(2), 91-96.
- Parker, A., Vaux, L., Patterson, A. M., Modasia, A., Muraro, D., Fletcher, A. G., ... & Pin, C. (2019). Elevated apoptosis impairs epithelial cell turnover and shortens villi in TNF-driven intestinal inflammation. *Cell death & disease*, 10(2), 1-13.
- Pavanelli, M. F., Kaneshima, E. N., Uda, C. F., Colli, C. M., Falavigna-Guilherm, A. L., & Gomes, M. L. (2015). Pathogenicity of Blastocystis sp. to the gastrointestinal tract of mice: relationship between inoculum size and period of infection. *Revista do Instituto de Medicina Tropical de São Paulo*, 57(6), 467-472.
- Poirier, P., Wawrzyniak, I., Vivarès, C.P., Delbac, F. and El Alaoui, H., (2012): New insights into Blastocystis spp.: a potential link with irritable bowel syndrome. *PLoS Pathog*, 8(3), p.e1002545.
- Puthia, M. K., Sio, S. W., Lu, J. & Tan, K. S. Blastocystis ratti induces contact-independent apoptosis, F-actin rearrangement, and barrier function disruption in IEC-6 cells. *Infect Immun*74, 4114–23 (2006).

- Rossignol, J. F., Kabil, S. M., Said, M., Samir, H., & Younis, A. M. (2005). Effect of nitazoxanide in persistent diarrhea and enteritis associated with *Blastocystis hominis*. *Clinical Gastroenterology and Hepatology*, 3(10), 987-991.
- Sarhan, R., Saad, G., Ezz Eldin, H., & Hetta, M. (2019) : A novel impact of *Boswellia serrata* on *Blastocystis* spp. infected mice. *Parasitologists United Journal*, 12(2), 116-122.
- Scanlan, P. (2012): *Blastocystis*: past pitfalls and future perspectives. *Trends Parasitol.*, 28: 327-334.
- Singh, K. D., Bhasin, D. K., Rana, S. V., Vaiphei, K., Katyal, R., Vinayak, V. K., & Singh, K. (2000). Effect of *Giardia lamblia* on duodenal disaccharidase levels in humans. *Tropical gastroenterology: official journal of the Digestive Diseases Foundation*, 21(4), 174-176.
- Steck N, Mueller K, Schemann M, Haller D (2011) Bacterial proteases in IBD and IBS. *Gut*. E-pub ahead of print 7 September 2011. doi:10.1136/gutjnl-2011-300775.
- Sundararajan, R., Cuconati, A., Nelson, D., & White, E. (2001). Tumor necrosis factor- $\alpha$  induces Bax-Bak interaction and apoptosis, which is inhibited by adenovirus E1B 19K. *Journal of Biological Chemistry*, 276(48), 45120-45127.
- Tan, K., Mirza, H., Teo, J., Wu, B. and Macary, P. (2010): Current views on the clinical relevance of *Blastocystis* spp. *Curr. Infect. Dis. Rep.*, 12: 28–35.
- Tsang, T. K., Levin, B. S., & Morse, S. R. (1989). Terminal ileitis associated with *Blastocystis hominis* infection. *American Journal of Gastroenterology (Springer Nature)*, 84(7).
- Tungtrongchitr A, Manatsathit S, Kositchaiwat C, Ongrotchanakun J, Munkong N, Chinabutr P, Leelakusolvong S, Chaicumpa W. (2004): *Blastocystis hominis* infection in irritable bowel syndrome patients. *Southeast Asian journal of tropical medicine and public health*. 2004 Sep 4;35:705-10.
- Wang, L. (2004): Changing patterns in intestinal parasitic infections among Southeast Asian laborers in Taiwan. *Parasitol. Res.*, 92: 18-21.
- Wang, W., Owen, H., Traub, R. J., Cuttell, L., Inpankaew, T., & Bielefeldt-Ohmann, H. (2014). Molecular epidemiology of *Blastocystis* in pigs and their in-contact humans in Southeast Queensland, Australia, and Cambodia. *Veterinary parasitology*, 203(3-4), 264-269.
- Wawrzyniak, I.; Poirier, P.; Viscogliosi, E.; Dionigia, M.; Texier, C.; Delbac, F. and El Alaoui, H. (2013): *Blastocystis*, an unrecognized parasite: an overview of pathogenesis and diagnosis. *Ther. Adv. Infect. Dis.*, 1(5): 167-178.
- Westphal, D., Kluck, R. M., & Dewson, G. (2014). Building blocks of the apoptotic pore: how Bax and Bak are activated and oligomerize during apoptosis. *Cell Death & Differentiation*, 21(2), 196-205.
- Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., ... & Goodwin, R. G. (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*, 3(6), 673-682.
- Yakoob J, Jafri W, Beg MA, Abbas Z, Naz S, Islam M, et al. (2010): Irritable bowel syndrome: is it associated with genotypes of *Blastocystis hominis*. *Parasitol Res* 106: 1033–1038. pmid:20177906.

- Yakoob,, Abbas, Z., Khan, R., Hamid, S., Awan, S., & Jafri, W. (2011) :Small intestinal bacterial overgrowth and lactose intolerance contribute to irritable bowel syndrome symptomatology in Pakistan. *Saudi journal of gastroenterology : official journal of the Saudi Gastroenterology Association*, 17(6), 371–375. <https://doi.org/10.4103/1319-3767.87176>.
- Yakoob, J., Abbas, Z., Usman, M. W., Sultana, A., Islam, M., Awan, S., ... & Jafri, W. (2014) Cytokine changes in colonic mucosa associated with Blastocystis spp. subtypes 1 and 3 in diarrhoea-predominant irritable bowel syndrome. *Parasitology*, 141(7), 957.
- Yao FR, Qiao JY, Zhao Y, et al.(2005): Experimental infection of mice with Blastocystis hominis ,Chinese Journal of Parasitology & Parasitic Diseases.23(6):444-448.
- Yu, Y. R., Deng, M. J., Lu, W. W., Zhang, J. S., Jia, M. Z., Huang, J., & Qi, Y. F. (2014). Endoplasmic reticulum stress-mediated apoptosis is activated in intestines of mice with Trichinella spiralis infection. *Experimental parasitology*, 145, 1-6.
- Zuckerman, M. J., Watts, M. T., Ho, H. O. I., & Meriano, F. V. (1994). Blastocystis hominis infection and intestinal injury. *The American journal of the medical sciences*, 308(2), 96-101.

## Figures



**Figure 1**

Histological images of the murine intestinal biopsies stained with H&E: (A) Tissue section from a healthy normal control. (B) Lymphocytic aggregation (arrow) 21 days p.i. with *Blastocystis hominis*. (B) Vacuolar forms of *Blastocystis hominis* in the intestinal mucosa. (C) Scattered parasites in the mucosa cropped and magnified from B (arrows).

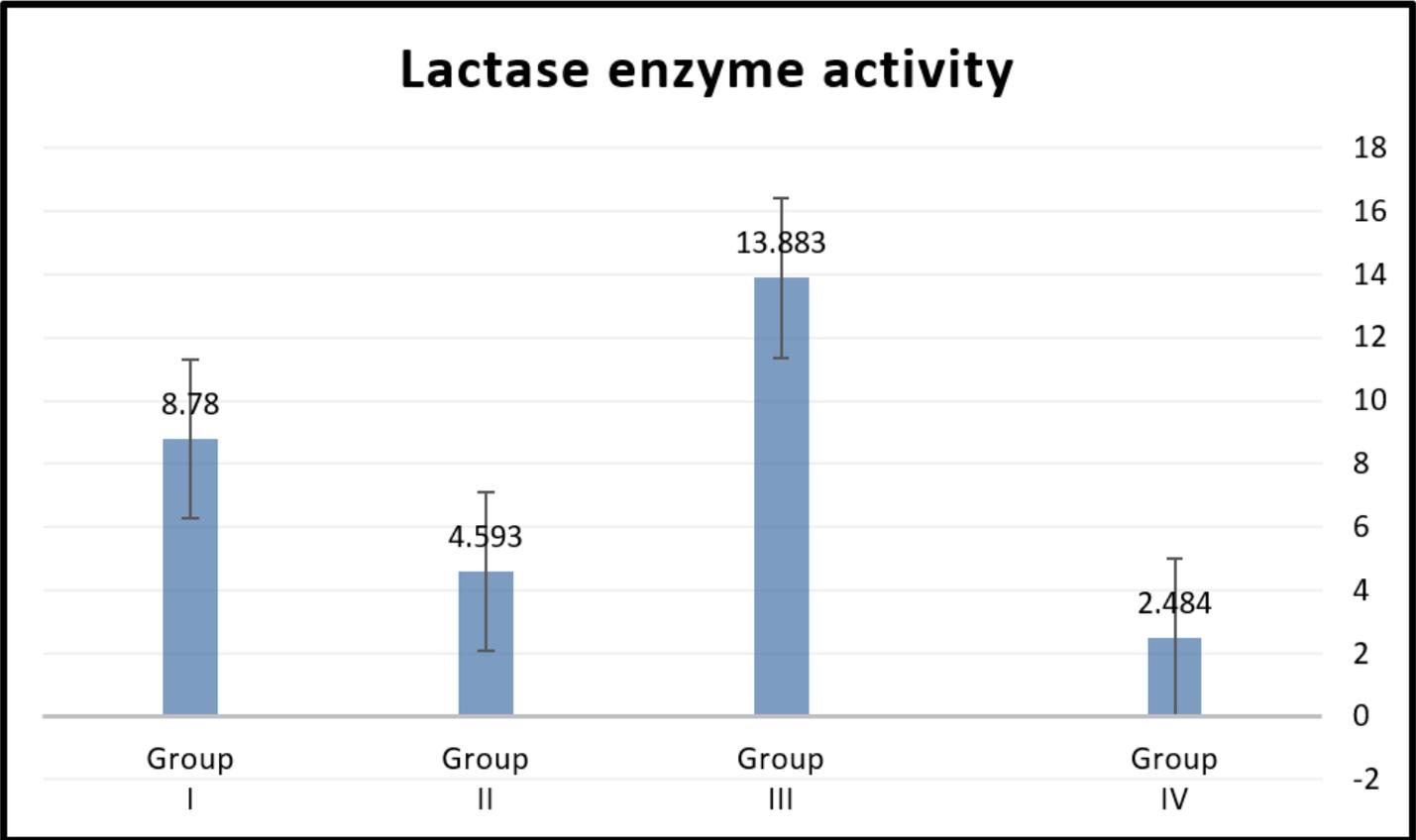
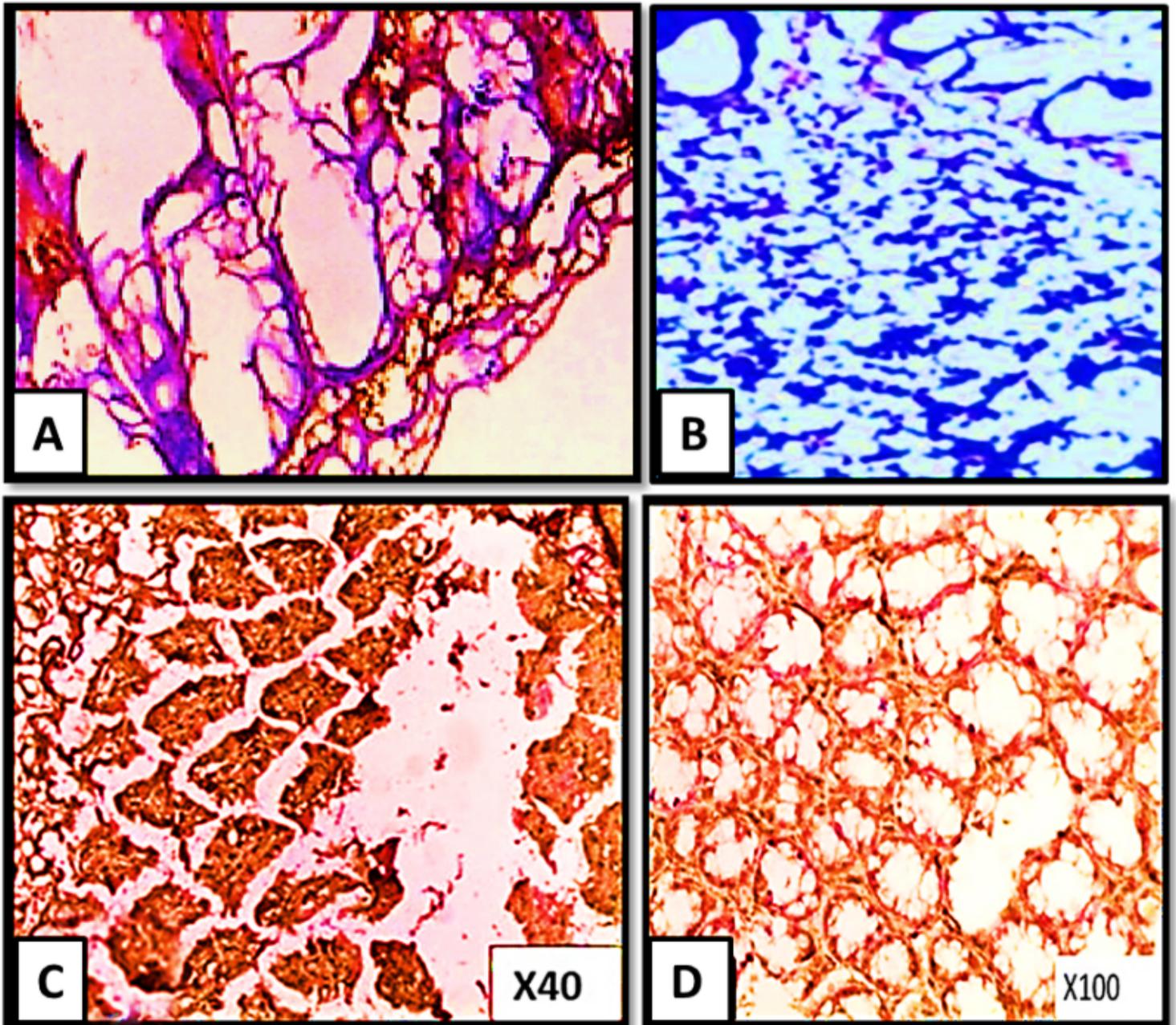


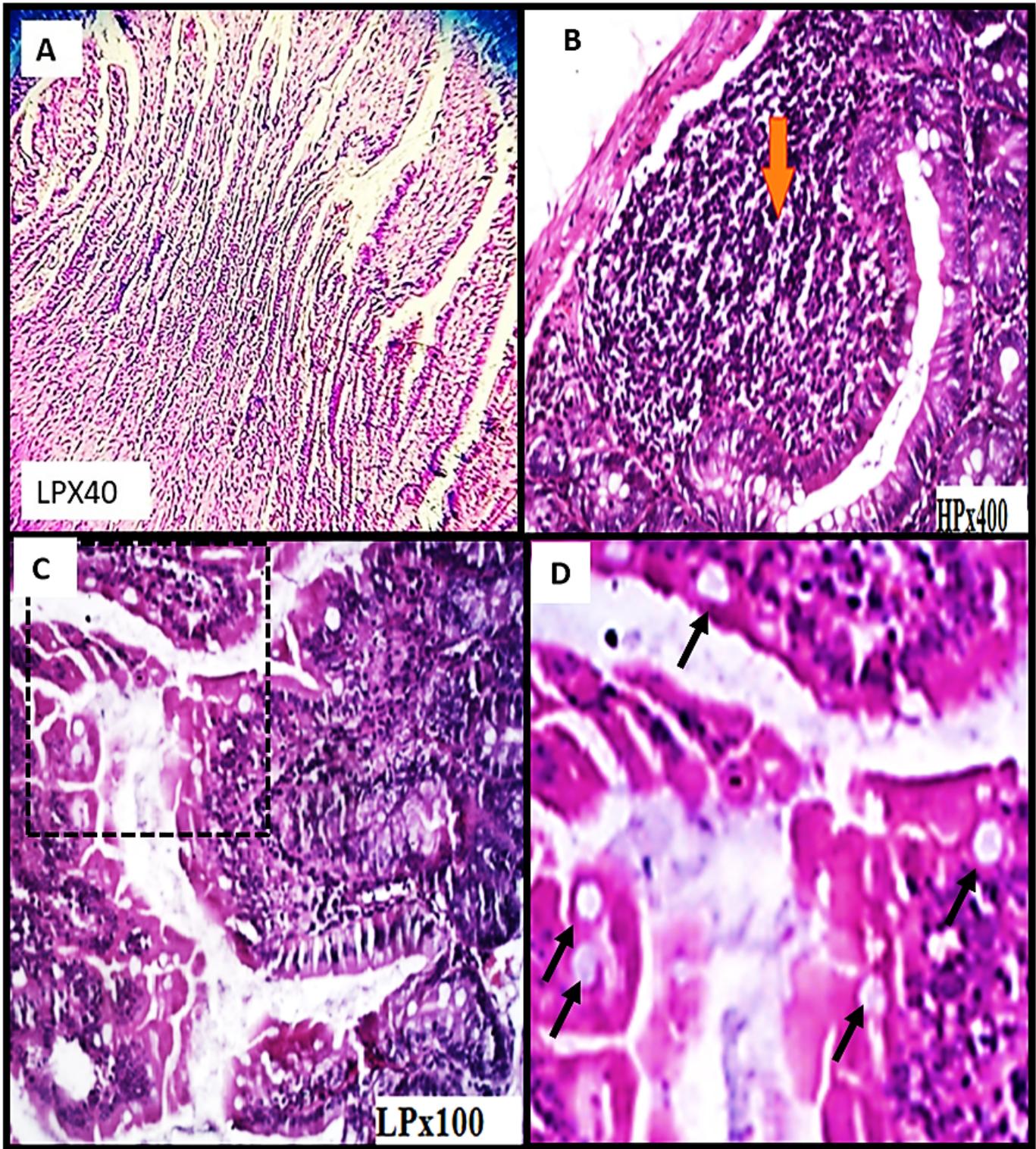
Figure 2

Chart shows comparison of lactase enzyme activity between different groups



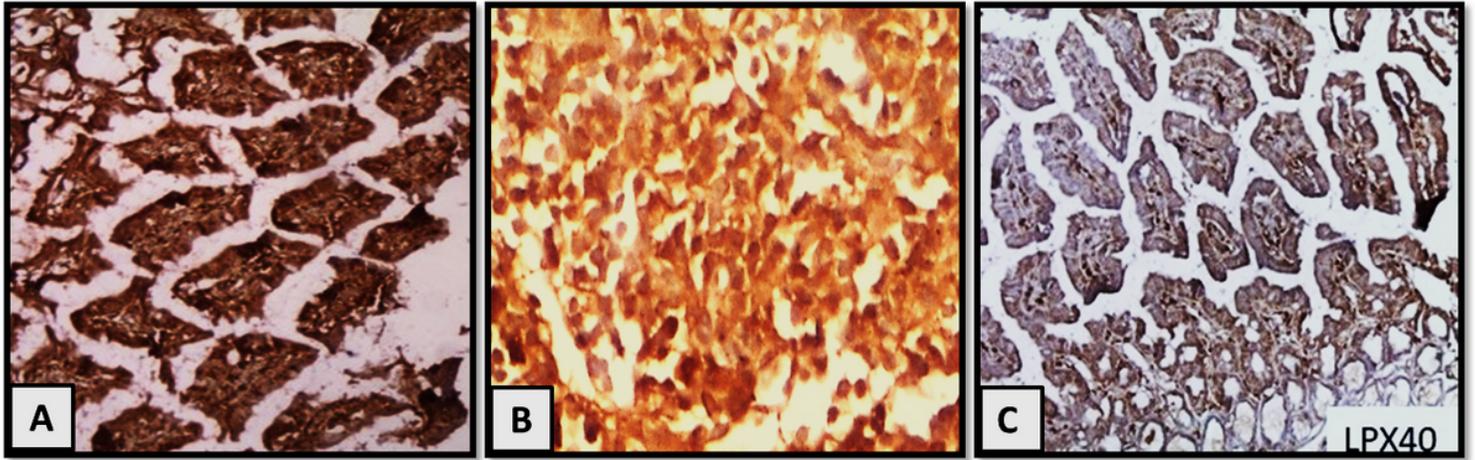
**Figure 3**

Microphotograph showing the expression of TNF- $\alpha$  in the small intestine of mice: Mild expression of TNF- $\alpha$  in healthy control (A). Intense expression of TNF- $\alpha$  in infected mice 21 days p.i.. (B,C), (D) Control slide: primary immune staining was omitted; magnification, 200 X.



**Figure 4**

Photomicrograph shows immune reactivity for Bax. (A, B) Bax expression in superficial and basal epithelial cells along the brush border of the small intestinal villi, magnification x40 and x100 respectively. (C, D) BAX positive cells are brown in color and expressed in the cytoplasm, magnification x200.



**Figure 5**

Photomicrograph shows immune reactivity for Bcl-2. (A), (B) Strong Bcl-2 expression along the brush border of the small intestinal villi in non-infected mice, magnification x200. (C) Expression of Bcl-2 in *Blastocystis hominis* infected mice 21 p.i., magnification x40.