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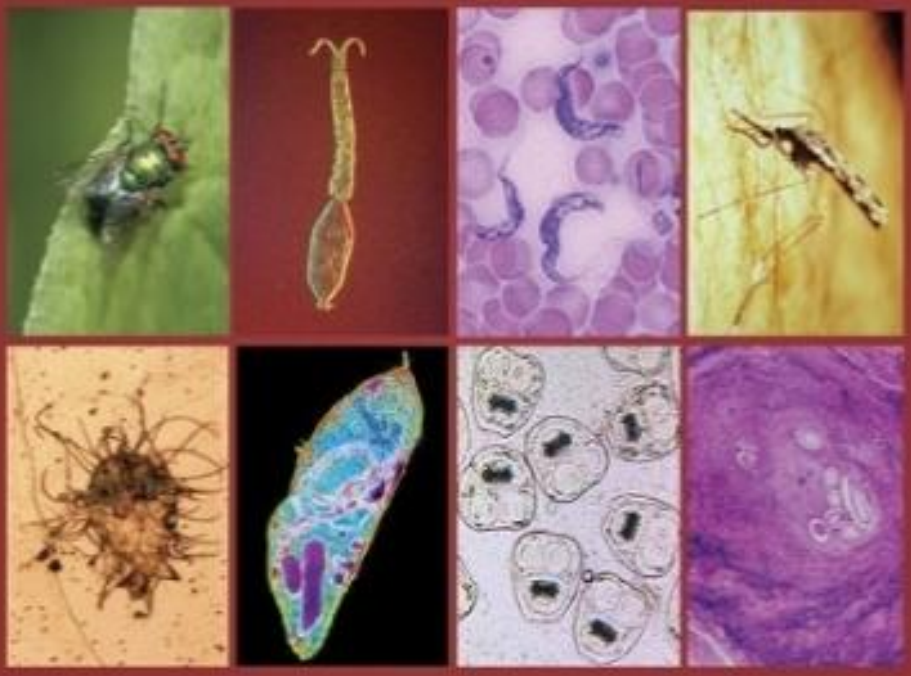

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Impact of *Entamoeba histolytica* on Lactase Activity and Intestinal Inflammation in Experimental Infected Mice

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ABSTRACT

Background and Objective: Lactose intolerance is one of the gastrointestinal disorders linked to *Entamoeba histolytica*, but the pathogenic factors that underlie this are still indistinct. This experimental study designed to determine the association between lactase enzyme activity and *Entamoeba* infection. **Methodology**: Forty mice were divided into 4 groups of 10 mice each that were used: uninfected mice (GI) (negative control); uninfected mice (GII) given lactose (positive control); *Entamoeba histolytica*-infected mice fed with lactose (GIII) and *Entamoeba histolytica* infected mice not receiving lactose (GIV). *Entamoeba complex* was identified by microscope and species differentiated molecularly. Lactase enzyme activity was assessed by spectrophotometry, and immunohistochemistry was used to examine and evaluate the expression of TNF-α and IL-1β in the intestine of each group. **Results**: *Entamoeba* complex cyst was detected microscopically**,** 10 selected samples were characterized and identified by molecular assay. *E. histolytica* was detected in 60% followed by *E. moshkovski* (30%) and *E. dispar* (1/10; 10%). Infected mice showed a significant decrease in lactase enzyme activity. The levels of TNF-α and IL-1β were elevated compared to mice that were non-infected ($P<0.001$). **Conclusion**: This study exposes a significant correlation between *Entamoeba histolytica* infection and decreased lactase enzyme activity, suggesting that parasitic invasion contributes to secondary lactose intolerance. The elevated intestinal expression of proinflammatory cytokines TNF-α and IL-1β in infected mice indicates that inflammation may play a crucial role in disrupting lactase function. Additional research is needed to explore the specific molecular pathways convoluted and to evaluate potential therapeutic interventions directing inflammation-mediated enzyme disruption in parasitic infections.

INTRODUCTION

Globally, intestinal parasite infections (IPIs) are a chief public health concern, but they are more prevalent in low- and middle-income nations, where they are connected with a high morbidity and mortality rate (Abayeneh & Amere, 2024). Every year, IPIs impact over 3.5 billion persons and result in over 450 million health issues (Belete *et al*., 2021). Gastrointestinal diseases are more frequently caused by protozoan infections than by helminths. *Cryptosporidium spp., Giardia intestinalis*, and *Entamoeba histolytica* are common intestinal protozoan parasites (Hemphill *et al.,* 2019 & Abdalal *et al*., 2024).

The predicted prevalence of *Entamoeba* infection in humans is as high as 50 million, leading to 2.237 million disability-adjusted life years (DALYs) and over 100,000 deaths per year (Fu *et al*.,2023). Human intestinal parasites include *Entamoeba histolytica*, *Entamoeba dispar, Entamoeba coli* and *Entamoeba moshkovskii* among other extensively spread species (Servián *et al*., 2024). *E. dispar* and *E. moshkovskii* are regarded as commensal and non-pathogenic, whereas *E. histolytica* is clearly listed as pathogenic. Because of its identical shape under microscopy, *E. histolytica* was mistakenly diagnosed (Hutagalung *et al*., 2024). *E. histolytica* infection takes place after the ingestion of cysts. Parasite excystation occurs in the small intestine and every single cyst produces eight trophozoites, that then pass to the large intestine, present both in the lumen and adhered to mucus and epithelial cells (Marie *et al*.,2014). Pathogenicity and immunity must be carefully balanced in order to establish an amoebic infection. Although several important phases in the process have been identified, such as mucosal layer disintegration, adhesion, injury, and diffusion to different organs, the exact mechanisms by which pathogenic amoebas enter host tissues remain unclear. Following amoebas' invasion of the tissues, the immune system fights the parasite (Uribe-Querol *et al*., 2020& Singh *et al.,* 2022).

Tumour necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) are among the pro-inflammatory cytokines that *E. histolytica* generates (Kissoon *et al.,* 2013& Moonah *et al.,* 2014). Activation of the inflammasome is one of the processes that leads to generation of cytokines (Mortimer *et al*., 2015). Abdel-Hafeez et al. institute a correlation between high TNF-α and the severity of the sickness and diarrhoea in patients infected with *E. histolytica*. In order to kill *E. histolytica*, TNF-α causes neutrophils and macrophages to create nitric oxide and reactive oxygen species; however, collateral tissue damage may also be caused by oxygen free radicals (Abdel-Hafeez *et al*., 2013).

Lactose is a disaccharide that is made up of two monosaccharides, galactose and glucose, merged by a β-1→4 bond. It takes a particular enzyme called lactase to hydrolyse this link, breaking down lactose into its integral parts so that glucose and galactose can be absorbed from the intestine. Some lactase-deficient people may develop lactose intolerance, which bases a variety of gastrointestinal (GI) symptoms, including bloating, diarrhoea, and abdominal discomfort, when they consume milk products (Trelis *et al*., 2019). Intestinal parasites may be a factor in the development of functional digestive diseases, according to new scientific research (Heine *et al*., 2017). Lactose malabsorption is frequently caused by parasite diseases such as giardiasis, cryptosporidiosis, and others (Chakarova *et al*., 2010).

Since lactose intolerance and *E. histolytica* infection are frequently accompanied by flatulence, abdominal pain, and diarrhoea, the current experimental study was carried out to assess the relationship between lactase enzyme activity and *Entamoeba* infection.

MATERIALS AND METHODS

Animals and Feed:

Forty male experimental mice aged 8–10 weeks and weighing 110–120 g, free of intestinal parasite infection, were supplied by the Theodor Bilharz Research Institute (TBRI). The study was approved by the animal ethical committee (CU. IACUC), number (CU. III. F.84.23). The animals were kept in clean wood-chip bedding and well-ventilated plastic cages in air-conditioned rooms (24±1°C). Regular cage cleanings and fresh bedding were constantly available; water was provided in special drinking bottles along with standard diet. All animal studies were conducted in accordance with internationally recognized

standards after receiving consent from TBRI's institutional ethics committee.

Grouping and Modelling:

The forty mice were split up into four groups, each consisting of ten mice. GI mice were non-infected (negative control), GII mice were non-infected given lactose, GIII mice were infected given lactose and GIV mice were infected with *Entamoeba* cysts without lactose intake (Fig. 1).

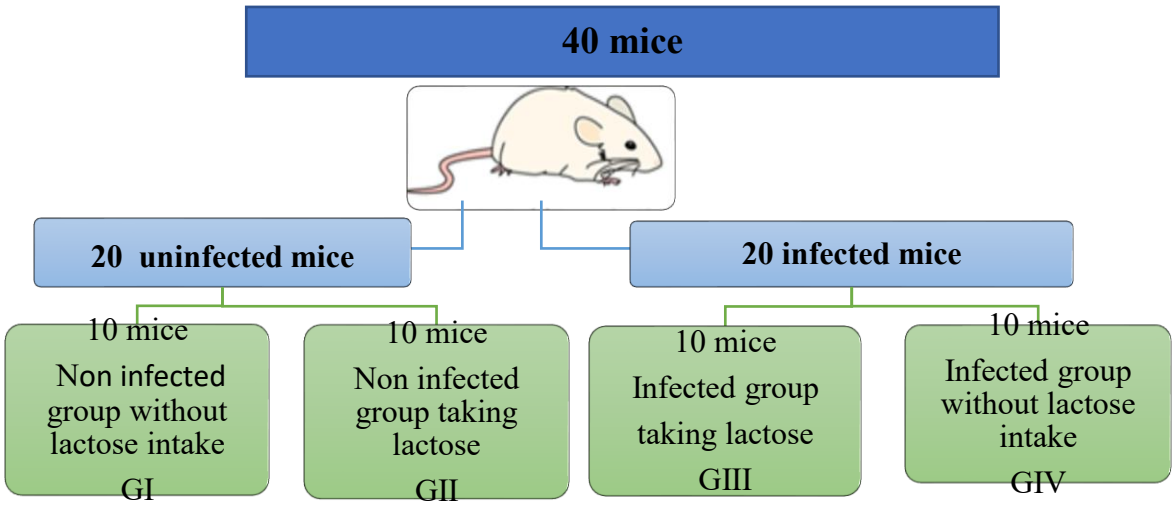

Fig. 1: Flow chart of experimental design and animal groups.

Infection Preparation and Induction:

Both macroscopical and microscopical analyses were performed on stool samples. Iodine staining and the saline wet mount procedure were both used to analyse each sample under a microscope. Then, in accordance with Khairnar and Parija, *Entamoeba* cysts were extracted from the positive samples (Khairnar and Parija, 2007). The stool sample was diluted with distilled water (1:10), filtered through four layers of gauze, and then two millilitres of the filtrate were mixed with two millilitres of PBS. Centrifugation for five minutes at room temperature at 2000 rpm/min. After discarding the precipitate, distilled water was used to dilute the filtrate (1:10). The mice were given *Entamoeba* cysts orally using diluted filtrate. An intraoesophageal catheter was used to inoculate experimental mice with 10^4 *Entamoeba* cysts. Faecal samples were collected every week after infection (p.i.) from mice and examined parasitologically using the direct wet mount to identify *Entamoeba*. Twenty-one days after infection, mice were decapitated after euthanization

Molecular Diagnostic Methods:

Genomic DNA Extraction:

According to the manufacturer's instructions, the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany, cat. no. 51604) was used to extract the genomic DNA of faecal samples that confirmed positive for *Entamoeba* complex. Until processing, the extracted DNA was retained at -20°C.

Molecular Identification of *Entamoeba complex:*

E. histolytica, E. dispar, and *E. moshkovskii* species were identified by amplifying the isolated DNA using multiplex PCR (mPCR), which targets the ssu-rRNA gene sequences (El-Badry *et al*.,2019). The multiple-PCR reaction worked best with a single reaction mixture that contained four primers (Table 1). PCR produced 167 bp, 753 bp, and 579 bp in the presence of *E. histolytica, E. dispar*, and *E. moshkovskii*, respectively (El-Badry *et al*., 2019). A UV light source was used to inspect and observe amplified DNA fragments on a 1.5% agarose gel stained with ethidium bromide.

Table 1: Primers sequences and PCR reaction conditions.

Primer name	Primer sequence	PCR Conditions
EntaF (common forward)	5′-ATG CAC GAG AGC GAA AGC AT-3′	35 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1min and 20 sec at 72
EhR	5′-GAT CTA GAA ACA ATG CTT CTC T-3′	
EdR	5′-CAC CAC TTA CTA TCC CTA CC-3′	
EmR	5′-TGA CCG GAG CCA GAG ACA T-3′	

Immunohistochemical Staining:

The distribution and immunohistochemistry reactivity of TNF-α and IL-1β were carried out in situ in the small and large intestine to accurately evaluate the immunological events. Several magnification powers were used to perform morphological analysis and evaluate the median density, intensity, and area percentage. With the assistance of a pathologist, immunostained sections were assessed in a blind manner. Immunopositivity was graded in 10 fields from grade 1 to grade 4 (Krajewski *et al*., 1994).

Lactose Administration and Sample Preparation:

After giving GII and GIII a lactose diet for seven consecutive days, *E. histolytica* was detected in the faeces 14 days after the infection, with more than eight cysts in the field, suggesting that the mice had a serious infection then lactose diets was administrated in a dose of 12.5 g/day/mouse for 7 consecutive days (Silvia *et al*., 2002 &Nair and Jacob, 2016). Twenty one days after infection, a competent lab operator promptly put an end to each batch of mice by decapitating them. Intestinal tissue was collected and preserved in PBS for the determination of lactase activity.

Phosphate buffered saline was added (1:9 g/ml) ratio. A centrifuge was used for 10 minutes at 3500 rpm after the tissue samples had been physically homogenised in a cold-water bath. Using a NANODROP® 2000C spectrophotometer, the total protein content of the supernatant of homogenised tissue samples was examined. At -20 ºC, the supernatant was then stored. Elabscience Biotechnology Inc., USA's lactase test kit (Cat. no. E-BC-K131-S) was used to perform the enzymatic reaction. In short, the blank, samples, and control were prepared along with a 5.5 mmol/L glucose standard solution. Following the addition, thorough mixing, and 20 minutes of incubation at 37 ºC, the stop solution was added, completely mixed, and all components were centrifuged for 10 minutes at 4000 rpm. After thoroughly mixing the chromogenic ingredient into each mixture's supernatant, it was incubated for ten minutes. A spectrophotometer was used to measure each sample in triplicate at 505 nm. According to the manufacturer's formula (Elabscience, 8th edition, Lactase Assay kit):

$$\textbf{Lactose activity (U/ mg protein)} = \frac{\frac{OD\ Sample - OD\ Control}{OD\ Standard - OD\ Blank} \times \frac{Concentration\ of\ standard\left(\frac{5.55nmo}{L}\right)}{Reaction\ time\ (20min.)}}{Protein\ concentration\ of\ sample(mg\ prot\ mL) \times 1000}$$

Statistical Analysis:

The statistical software SPSS version 28 was accustomed to analyse the data (Chicago, IL, USA). The baseline characteristics were displayed as a frequency (%) for all definite variables and as means (± standard deviation) for constant variables. To determine the statistical significance between the groups, a one-way ANOVA and a paired t-test were used. The definition of statistical significance was P-values < 0.05.

RESULTS

Identification of *Entamoeba* spp.:

Entamoeba complex cyst was detected microscopically (Fig. 2), among positive stool samples collected, 10 heavy infected samples were selected and

identified by molecular assay. As shown in Figure 3, the most common species detected were *E. histolytica* (6/10; 60%), *E. moshkovski* (3/10; 30%) and *E. dispar* (1/10; 10%).

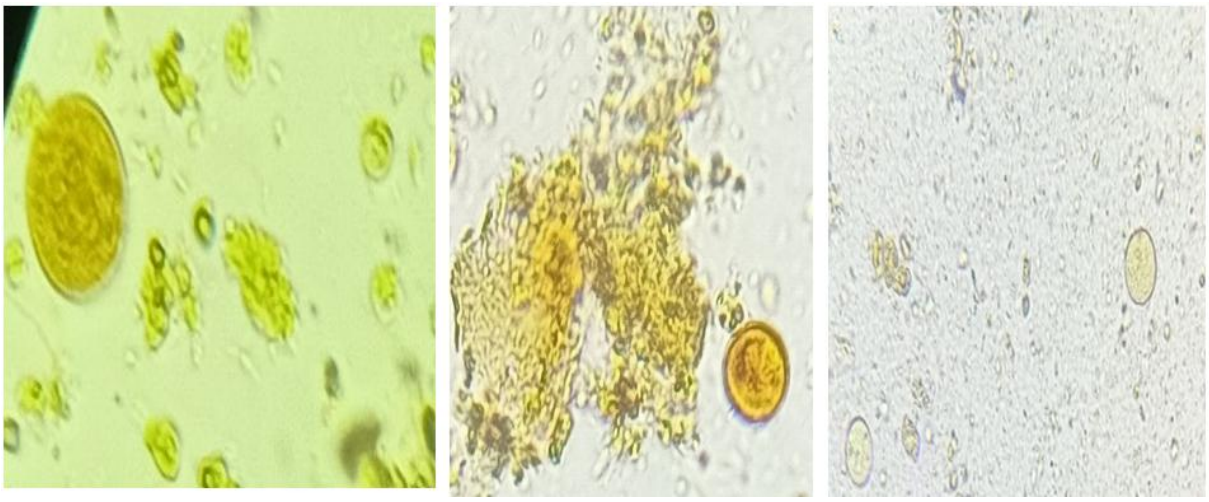

Fig. 2: Microscopic detection of *Entameoba histolytica* ysts using power lenses with 10x and 40x magnification.

Molecular Identification of *Entamoeba* spp. in Stool Sample:

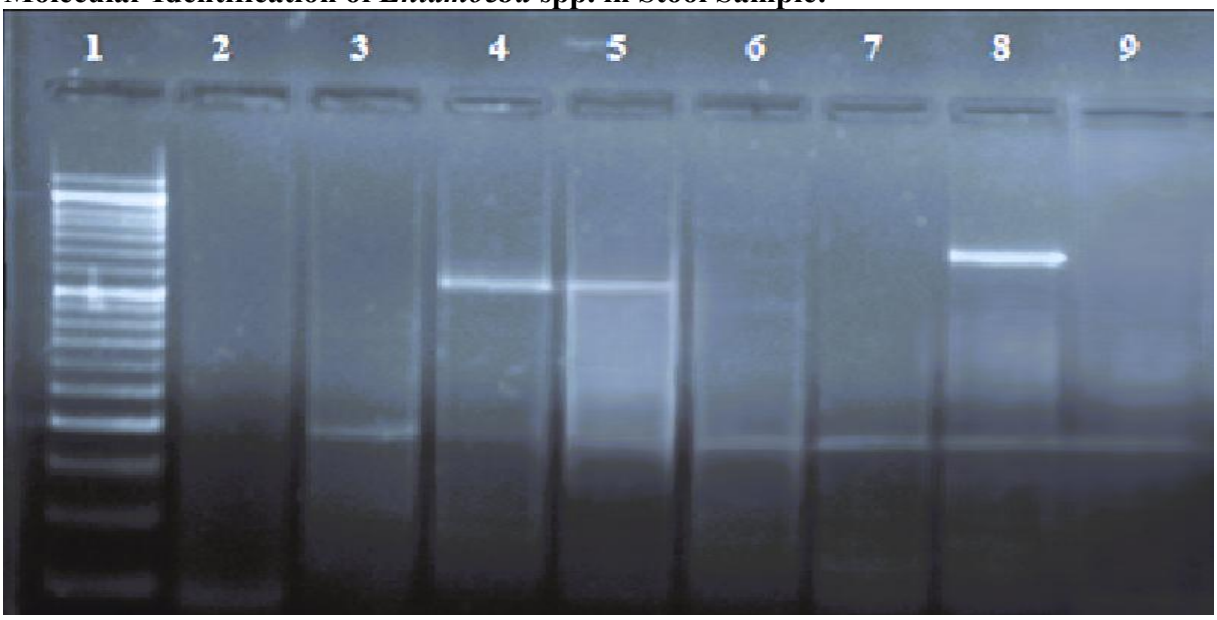

Fig. 3 Gel electrophoresis image of *Entamoeba* targeting SSUrNA; Lane 1: DNA ladder 50 bp; Lane 2: Negative control; Lane 3: Positive control of *E. histolytica* (166 bp); Lanes 4, 5: *E. moshkovski* (580 bp); Lanes 6, 7, 9 *E. histolytica* (166 bp); Lane 8: mixed infection of *E. dispar* (752 bp) and *E. histolytica* (166 bp).

Protein Concentration and Lactase Enzyme Activity:

The total protein concentrations showed no significant differences between the infected and non-infected groups ($P<0.05$). Compared to non-infected groups, the infected groups lactase enzyme activity was significantly lower ($P<0.001$) (Tables 2 & 3).

Table 2: Concentrations of proteins in the small and large intestinal tissue of mice 21 days p.i

Groups	**Protein concentration (mg protein /ml) Mean ± S.D**
GI	30.69±0.63
GII	27.41±5.87
GIII	35.19±12.18
GIV	26.43±6.81
P1= 0.059; *P2*=0.096; *P3*= 0.734; *P4*= 0.0645; *P5*= 0.065; *P6*= 0.086	

*Statistical significance *P*<0.05 * *P*1 between all studied groups; *P*2 GI vs. GII; *P*3 GI vs. GIII; P4 GI vs. GIV; *P5* GII vs. GIII; *P6* GII vs. GIV; *P7* GIII vs. GIV

Table 3: Measurements of lactase enzyme activity in the small and large intestine of mice 21 days p.i

Groups	**Lactase activity (U/mg protein) Mean ± S.D**
GI	9.12±0.44
GII	14.78±4.03
GIII	2.46±0.84
GIV	5.08±0.69
P1= 0.000**; *P2*= 0.0003**; *P3*< 0.0001**; *P4*< 0.0001**; *P5*< 0.0001**; *P6*= < 0.0001**	

P<0.01** highly significant difference; *P*<0.05 * significant difference; *P*1 between all studied groups; *P*2 GI vs. GII; *P*3 GI vs. GIII; *P4* GII vs. GIII; *P5* GII vs. GIV; *P6* GIII vs. GIV

Immunostaining of TNF-α and IL-1β

TNF-α and IL-1β levels in the intestine were 60.0% in infected groups compared to 30.0% in normal mice. Only superficial epithelial cells in the intestine's lamina propria showed moderate production of TNF-α and IL-1β **(**Figs. 4 & 5).

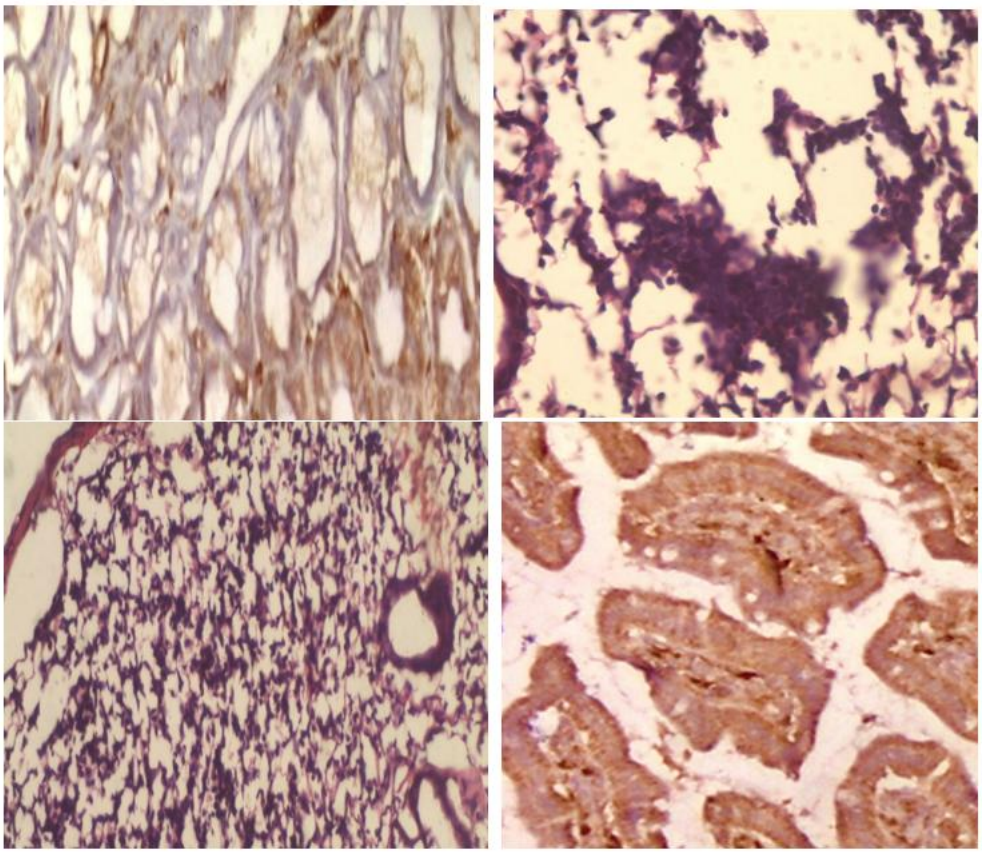

Fig. 4: Tumor necrosis factor (TNF-α) immunoreactive cells in *Entameba histolytica* infected intestinal tissue of mice. TNF-α expression was mainly cytoplasmic and extracellular with moderate to severe dense infiltrate of brown stained TNF-α positive cells in lamina propria and submucosa of the intestine.

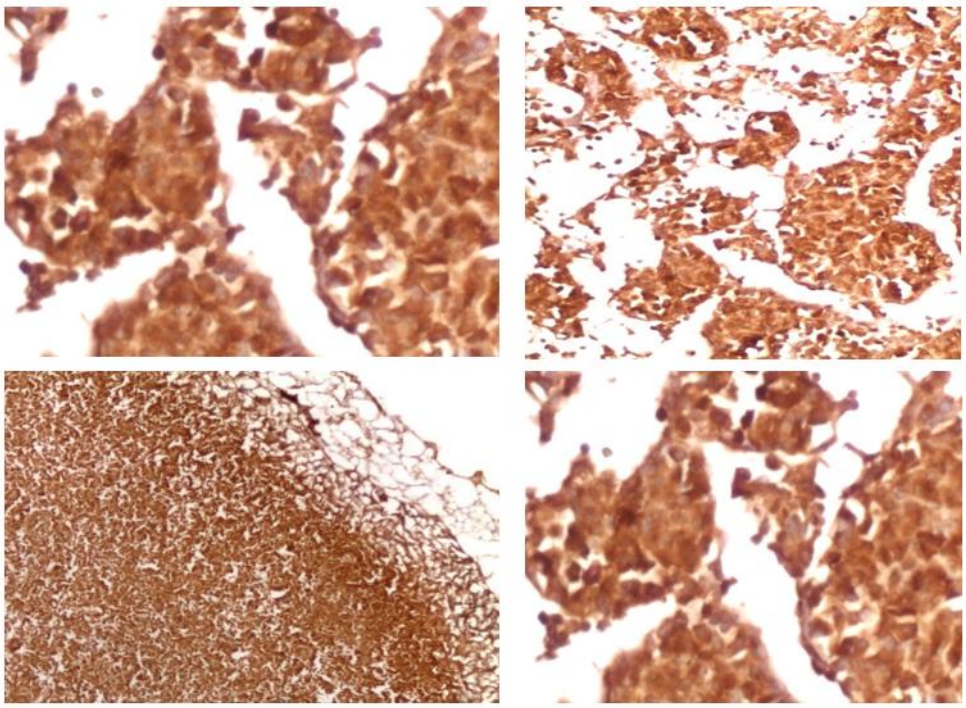

Fig. 5: Immunohistochemistry in *Entameba histolytica* infected intestinal tissue. Interleukin 1 beta expression in superficial and basal epithelial cells along infected mucosal glands in colon and small intestinal villi of the brush border respectively**.** Interleukin 1 beta positive cells are brown in color and expressed in the cytoplasm.

DISCUSSION

About 33% to 75% of the world's inhabitants complains of lactose intolerance (LI), which is caused by a lactase deficiency. The prevalence varies according to the region (Szilagyi *et al.*, 2016 & Ratajczak *et al.,* 2021& Kempinski *et al*., 2024). Lactose intolerance can have a variety of causes. The amount of lactase enzyme often decreases gradually after weaning from breastfeeding in cases with primary lactose intolerance. For the majority of land animals, this is typical, and the loss is irreversible. Inflammatory bowel disease, coeliac disease, rotavirus, giardiasis and other parasite infections, AIDS, and cystic fibrosis are among the illnesses that cause secondary lactose intolerance. Depending on the cause, secondary lactose intolerance might last for a long period (Szilagyi and Ishayek, 2018). Since there are few studies discussing lactose malabsorption in *E. histolytica* infections, the current experimental study was carried out to assess the relationship between lactase enzyme activity and *E. histolytica* infection.

The actual distinction between *E. histolytica* from *E. moshkovskii* and *E. dispar* is vital for the estimation of the true prevalence and appropriate therapeutic treatment of infection in various geographic areas (Hasan *et al*., 2024). In order to distinguish between *Entamoeba* species that have the same morphology in both trophozoite and cyst stages, the polymerase chain reaction was used. Infection was confirmed by PCR in this study, which revealed *E. histolytica* 60%, *E. moshkovski* 30%, and *E. dispar* 10%. This finding is reliable with Sri-Hidajati et al, who found that no samples tested positive for *E. dispar*, while 58% of positive samples tested positive for *E. histolytica* and 17% for *E. moshkovskii* (Sri-Hidajati *et al*., 2018).

In the current study, lactase enzyme activity in *E. histolytica* infected groups was significantly reduced to the lowest levels with a mean value (2.46±0.84) in comparison to non-infected group with mean value (14.78±4.03). This result of lactase enzyme activity was lower than noninfected groups with or without lactose diet and clarified the relation-ship between *E. histolytica* infection and the decrease in the lactase enzyme activity which is generally considered as the first-route of disaccharides digestion. These go with Rana et al who conducted a study using hydrogen breath tests in India and reported that among 41 individuals shedding *E. histolytica* cysts, 78% (32/41**)** showed lactose malabsorption, compared to 42.5% in controls with a statistically significant difference (Rana *et al*., 2004).

Pro-inflammatory cytokines for instance IL-1β and TNFα are produced in response to intestinal tissue damage caused by *E. histolytica*. These pro-inflammatory cytokines are essential for the protection against amebiasis and are associated with susceptibility to infection (Uddin *et al*., 2022). Overzealous pro-inflammatory reactions may be harmful and harm host tissue (Peterson *et al.*,2010 & Noor *et al*.,2017). TNF-α and IL-1β immunohistochemical identifying in the intestine of each study group was used to examine the impact of *E. histolytica* infection on cell-mediated response. Sixty percent of the infected mice had moderate to strong cytokine expression in the superficial epithelial cells of the intestine's lamina propria, while only 30 % of the not infected groups had positive cells that were mostly restricted to the subepithelial zone.

The TNF-α is a pro-inflammatory cytokine, mostly produced by macrophages. It can lead to tissue inflammation via the activation of macrophages and neutrophils, also up-regulation of further pro-inflammatory mediators. In addition, it can upsurge cell permeability, producing impairment of barrier function and edema development. The TNF-α shows a vital role in mucosal inflammation, and is raised in the gastrointestinal inflammatory colitis. For instance, this cytokine is

recognized to be of important prominence in the pathogenesis of inflammatory bowel disease (IBD) (Rojas-Cartagena et al., 2005). In the present study, immunohistochemical examination of TNF-α showed moderate to strong cytokine expression in the superficial epithelial cells of the intestine's lamina propria in 60% of infected mice. This means that *E. histolytica* infection increase production and expression of TNF-α in intestinal mucosa. This goes with Peterson et al. who reported that higher levels of TNF-α were linked with increased risk of first and recurrent episodes of *E. histolytica*-related diarrheal in infected children (Peterson *et al*.,2010).

Interleukin-1β (IL-1β) is effective proinflammatory cytokine that plays a central role in the innate immune response to infection and tissue injury. As a member of the IL-1 family, IL-1β is made as an inactive originator (pro–IL-1β) and requires enzymatic cleavage by inflammatory caspases primarily caspase1.Its release is typically mediated by inflammasome complexes, which detect a wide range of pathogenic and endogenous danger signals .Upon activation, IL-1β initiates a cascade of immune responses, including fever, leukocyte recruitment, and the amplification of cytokine networks. These responses, while essential for pathogen clearance, can also contribute to immunopathology when dysregulated (Guo *et al*., 2015).

In the context of parasitic infections, IL-1β has emerged as a critical mediator of host defense and inflammation. During *Entamoeba histolytica* infection, IL-1β is rapidly secreted by macrophages and epithelial cells in response to direct contact with live trophozoites. This contact activates caspase-1 and caspase-4 pathways via parasite surface lectins (Gal/GalNAc) and cysteine proteases (EhCP-A5), which engage host receptors such as α5β1 integrin, leading to gasdermin D–dependent pore formation and IL-1β secretion (Hou *et al*.,2023).

Many studies stated that *Entamoeba histolytica* triggers IL-1β release in macrophages through caspase-4/1 activation, engaging the Gal/GalNAc lectin and EhCP-A5 via $\alpha_5\beta_1$-integrin, and involving gasdermin D and stated that direct contact between live trophozoites and macrophages induces rapid IL-1β and IL-18 secretion (plus IL-1α, FGF-2, IP-10) (Quach *et al*.,2019& Mortimer *et al.*,2014).

Conclusion

This study reveals a significant relationship between *Entamoeba histolytica* infection and decreased lactase enzyme activity, suggesting that parasitic invasion contributes to secondary lactose intolerance. The elevated intestinal expression of proinflammatory cytokines TNF-α and IL-1β in infected mice indicates that inflammation may play a key role in disrupting lactase function. More research is needed to explore the specific molecular pathways convoluted and to evaluate potential therapeutic interventions directing inflammation-mediated enzyme disruption in parasitic infections.

Declarations:

Ethical approval: The study was approved by the animal ethical committee (CU. IACUC), number (CU. III. F.84.23).

Competing interests: The authors pronounce that they have no conflict of interest that affects this study.

Availability of Data and Materials: All datasets analysed and described during the present study are available from the corresponding author upon reasonable request.

Authors contributions: Gehad A. Basuony and Noura A. Ragab ; Methodology, Software, Formal analysis, Investigation, Resources, Visualization, and Writing-original draft. Gehad A. Basuony, Mohamed A. Shemis and Noura A. Ragab: Conceptualization, Data curation, Writing, review and editing, and Supervision. All authors have read and agreed to the published version of the manuscript.

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