Journal of the Egyptian Society of Parasitology, Vol. 55, No. 1, April 2025
J. Egypt. Soc. Parasitol. (JESP), 55(1), 2025: 27 - 36
Online: 2090-2549

EFFECT OF PROBIOTICS IN MICE EXPERIMENTALLY INFECTED WITH CRYPTOSPORIDIOSIS AND ITS RELATION TO COLON CANCER

By

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Abstract

Probiotics are live microorganisms that offer health benefits. This study evaluated the cryptosporidiosis parasitic burden in experimentally infected mice received probiotics and its potential link to cancer. A total of six groups were included: Four were cryptosporidiosis-infected mice (30 mice each) and two control groups (10 mice each). The infected groups were immunocompetent mice without probiotics (G1), or with probiotics (G2), and immunocompromised mice without probiotics (G3) or with probiotics (G4). Cryptosporidiosis was diagnosed weeks 2 to 14 post-infection.

The results showed that in G1, medium parasitic burden was 50% at week 2 and gradually declined to 0% by week 10, but heavy burden started at 33.4% in week 2 and dropped to 0% by week 4. In G2, medium burden decreased from 50% at week 4 to 33.4% by week 14, whereas heavy burden, which began at 50% in week 2, peaked at 66.6% by week 14, with significant difference between them. For G3, medium burden was 66.6% at week 2 and declined to 50% by week 14, but heavy burden increased from 33.4% at week 2 to 50% at week 14. In G4, medium burden fluctuated, decreased from 16.7% at week 2 to 33.4% at week 14, but heavy burden declined from 83.3% at week 2 to 66.6% at week 14. However, the changes were insignificant. Regarding dysplastic changes, no dysplasia was observed in G1. In G2, four out of five (80%) dysplastic mice had a heavy parasitic burden. In G3, 66.6% (4/6) of dysplastic mice exhibited a heavy burden, but in G4, 78.6% (11/14) of dysplastic mice had a heavy burden, without significant differences

Keywords: Probiotics**,** Cryptosporidiosis, Experimental mice, Treatment.

Introduction

Cryptosporidiosis is a widespread protozoan disease caused by *Cryptosporidium* species of Phylum Apicomplexa (Rayen *et al,* 2021). More than 47 species and over 100 genotypes have been classified, but only *Cryptosporidium hominis* and *C. parvum* are zoonotic ones (Ježková *et al,* 2021). Cryptosporidiosis is a significant contributor to diarrheal diseases worldwide, as the second causes of moderate to severe diarrhea and malabsorption mainly among children (Bones *et al,* 2019). In Egypt, cryptosporidiosis prevalence ranged between 84% and 34% of immunocompromised and immunocompetent diarrheic children, with 10% of asymptomatic infections (Elsawey *et al,* 2020). Youssef *et al*. (2008) reviewed 61 cryptosporidiosis published Egyptian papers between 1985 and 2006, 19 of which reported diarrhea among immunocompetent individuals ranged from 0%-47% (median 9%, IQR 3-15%). Cryptosporidium spp. is primarily transmitted via ingestion of oocyst-contaminated food, water, or surfaces by fecal-oral route (Bouzid *et al,* 2013). Also, person-to-person transmission reported among household members, sexual partners, healthcare workers, and children in daycare centers and their caretakers, (Musher and Musher, 2004). Notably, *Cryptosporidium* oocysts exhibit exceptional resistance to environmental extremes and conventional water treatment methods, surviving at temperatures as low as -20°C and under high salinity conditions (Carey *et al,* 2004).

Beyond diarrhea, cryptosporidiosis was significantly higher among cancer colon patients reinforcing risk factor for developing the colorectal carcinogenesis cancer colon by its chronic inflammation, dysbiosis, and DNA damage (Abd El-Latif *et al,* 2023).

Clinical evidence has showed the presence

of *Cryptosporidium* DNA in colonic biopsies from patients newly diagnosed with adenocarcinoma, but only 7% of patients suffered from persistent gastrointestinal symptoms, but without neoplastic disease evidence of infection (Osman *et al,* 2017). Animal immunodeficient model demonstrated that *C. parvum* infection caused intraepithelial neoplasia, and invasive adenocarcinoma, with multiple parasite life stages identified in the affected intestinal tissues (Certad *et al,* 2010). Moreover, a significant statistical correlation was identified be-tween the parasitic burden in *Cryptosporidium* infection and the severity score of dysplastic lesions (Benamrouz *et al,* 2014).

Probiotics was defined as live microorganisms that confer health benefits when consumed in adequate amounts, play a pivotal role in modulating gut microbiota (Macfarlane *et al,* 2004). They have been widely investigated as a potential strategy for managing gastrointestinal infections, either as standalone interventions or as adjuncts to conventional antimicrobial treatment (Guitard *et al,* 2006; Goyal *et al,* 2011; Lantier *et al,* 2014). Despite probiotics are widely explored as adjunct therapies for gastrointestinal infections, their role in cryptosporidiosis was controversial, with both beneficial and adverse effects (Pickerd *et al,* 2004; Goyal *et al,* 2013). However, conflicting findings suggested that probiotics could exacerbate the cryptosporidiosis infection by demonstrating increased oocyst shedding in probiotic-treated animals (Oliveira *et al,* 2016; Robertson *et al,* 2019). Moreover, certain studies on humans reported adverse outcomes in cryptosporidiosis patients after probiotic administration (Salazar-Lindo *et al,* 2004; Sindhu *et al,* 2014). Efforts to develop cryptosporidiosis vaccine were limited by insufficient understanding of the immune responses mediating protection (Helmy and Hafez, 2022). While probiotics have also, been extensively studied for their potential role in modulating cancer cell proliferation and apoptosis, their precise impact on tumor development remains an area of the active research (Śliżewska *et al,* 2020).

This study aimed to evaluate the impact of prolonged probiotic administration on *Cryptosporidium* burden of infection in experimentally infected mice and to explore its potential role in colorectal cancer development.

Materials and Methods

This study utilized 140 male Swiss Albino mice, each four weeks old and weighing between 15 and 20 grams. The mice were sourced from the National Research Center, Cairo, Egypt, and housed in the Animal Facility of the Faculty of Medicine, Suez Canal University, where all experiments were done.

The mice were kept in well-ventilated insect-proof cages and fed a standard hard-pellet diet (Benamrouz *et al,* 2014). Prior to the experiment study, intestinal parasites were ruled out through three consecutive stool analyses over three days (Garcia and Hyman, 2016). The Institutional Review Board has approved the experiments according to its animal ethical guidelines with ethical approval number 5103.

Cryptosporidium parvum oocyst source and preparation: *C. parvum* oocysts were procured from Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Oocysts were originally isolated from Holstein-Friesian calves infected naturally, with *Cryptosporidium* being the only identified pathogen. Subtype IIa A15G2R1 was selected because it is considered the prevailing subtype in many countries (Abdou *et al,* 2013; Imam *et al,* 2022). Oocysts were maintained in phosphate-buffered saline (PBS, pH 7.2) containing antibiotics (penicillin, streptomycin, gentamicin, and amphotericin B) and 0.01% Tween 20, and then stored at 4°C until use (Sayed *et al,* 2016).

Experimental design: Mice were randomly assigned to six groups: G1: Infected immunocompetent without probiotics treatment. G2: Infected immunocompetent treated with probiotics. G3: Infected immunocompromised, without probiotics treatment. G4: Infected immunocompromised, and probiotics treated. G5: Non-infected immunocompetent,

and probiotics treated. G6: Non-infected control (negative control). Each cage contained up to six mice. They were closely monitored for significant weight loss, severe diarrhea, or disruptions to daily activities, which were considered clinical endpoints (Immam *et al*, 2022).

Experimental infection and probiotic administration: *C. parvum* oocysts were administered orally using a sterile tuberculin syringe equipped with a polyethylene tube (Abdou *et al*, 2013). Mice in Gs 1, 2, 3, & 4 received 0.3ml of PBS containing 9×10^3 *C. parvum* oocysts via intra-esophageal inoculation (Oliveria and Widmer, 2018). Probiotics were administered to Gs 2, 4, & 5 using Kyo-Dophilus Multi 9 Probiotic tablets, which contained *Lactobacillus* species (*L. gasseri KS-13, L. rhamnosus*) and *Bifidobacterium* spec ies (*B. bifidum G9-1, B. longum MM-2, B. infantis,* and *B. breve*), along with other compatible bacterial species. This probiotic supplement was purchased from Wakunaga of America Company, USA. Probiotics were given in drinking water at a concentration of one tablet per 500ml, beginning one day prior to infection and continuing throughout the experiment (Guerin-Danan *et al*, 2021).

Induction of Immunosuppression: To induce immunosuppression, the DEXA (Dexamethasone®) was given as 8mg/2ml ampoules purchased from Amriya Pharmaceuticals Co. Mice received an initial dose of 125µg/day for seven days starting one day before infection, and achieved immunosuppression within five days. A maintenance dose of 100 µg was given every other day to the end of experimental study (Al-khaliq *et al*, 2021).

Confirmation of infection: Mice underwent clinical assessment at 7 days post-infection (P.I.), with stool samples analyzed using Sheather's cover-slip flotation method and modified Ziehl-Neelsen staining to confirm infection (Guitard *et al*, 2006).

To monitor disease progression, six mice were sacrificed at five time points: 2nd week (early acute infection), 4th week (late acute infection), 8th week (chronic infection), 10th week (early complicated infection), and 14th week (late complicated infection). Infection was confirmed by the senior author's evaluation, but absence of infection was established based on three consecutive negative samples collected over three successive days.

Assessment of Endogenous Parasitic Stages and Dysplasia via Histopathology: Ileocecal tissues were harvested from six sacrificed mice per infected group at each time point to evaluate parasitic burden and dysplastic changes in the epithelial cells. Euthanasia was performed using ether before tissue collection. Two control mice were euthanized at each corresponding time point. The cecum was excised, sectioned into 1.0-1.5 cm segments, and fixed in 10% neutral-buffered formalin for 24 hours before processing for histopathological examination (Sheehan and Harpchiak, 1973).

Parasitic burden analysis: Hematoxylin-eosin-stained slides were microscopically examined. Since *Cryptosporidium* colonization was often patchy, the average of 10 microscope high-power fields (HPF) was assessed/mouse (Fayer *et al*, 1990). Slides were inspected for infection intensity and tissue pathology.

Cryptosporidium colonization scores (Rasmussen and Healey, 1992): 0: no parasite, 1+: moderate parasite in up to 50% of tissue, & 2+: extensive parasite in more than 50% of tissue.

Endogenous parasitic intensity was determined by counting parasites within 10 villus crypt units (Haeley *et al*, 1995), and parasite density per single villous crypt unit was recorded for each mouse and group (Abdou *et al*, 2013). A medium parasitic burden was defined as ≤50 parasites, while a heavy burden was >50%.

Dysplastic changes assessment: Dysplastic alterations within the epithelial surface were identified and reported for the presence or absence of dysplasia (Zhang YingDi *et al*, 2018).

Statistical analysis: Data were collected, computerized, and statistically analyzed by

using the statistical program (SPSS) Statistical Package for Social Science Version 23.0 (SPSS Inc., Chicago, Illinois, USA). The descriptive and analytical data included the frequencies and percentage. The P-value was considered significant at 0.05 or less and highly below 0.001.

Results

The data were given in tables (1, 2 & 3) and figures (1, 2, 3 & 4).

Table 1: *Cryptosporidium* burden in ileocecal tissue duration among probiotics-untreated (G1) and probiotics-treated (G4) immunocompetent mice

Groups \ Duration		2 weeks		4 weeks		8 weeks		10 weeks		14 weeks		P value
		No.	%	No.	%	No.	%	No.	%	No.	%	
G1	Medium (≤50%)	4	66.6	3	50	1	16.7	0	0	0	0	0.0355. Significant p < .05.
	Heavy (>50%)	2	33.4	0	0	0	0	0	0	0	0	
	Total	6	100	3+3*	50+50*	1+5*	16.7+83.3*	0	0	0	0	
G2	Medium (≤50%)	3	50	4	66.6	2	33.4	3	50	2	33.4	
	Heavy (>50%)	3	50	2	33.4	4	66.6	3	50	4	66.6	
	Total	6	100	6	100	6	100	6	100	6	100	

*Negative

Table 2: *Cryptosporidium* burden in ileocecal tissue duration among probiotics-untreated (G3) & probiotics-treated (G4) immunocompromised mice

Groups \ Duration		2 weeks		4 weeks		8 weeks		10 weeks		14 weeks		P value
		No.	%	No.	%	No.	%	No.	%	No.	%	
G3	Medium (≤50%)	4	66.6	2	33.4	4	66.6	4	66.6	3	50	3.9543. .26644. Insignificant p < .05
	Heavy (>50%)	2	34.4	4	66.6	2	33.4	2	33.4	3	50	
	Total	6	100	6	100	6	100	6	100	6	100	
G4	Medium (≤50%)	1	16.7	4	66.6	2	33.4	3	50	2	33.4	
	Heavy (>50%)	5	83.3	2	33.4	4	66.6	3	50	4	66.6	
	Total	6	100	6	100	6	100	6	100	6	100	

Table 3: *Cryptosporidium* burden degree to dysplastic changes infection among G2, G3 &G4 mice.

Degree of burden	G2		G3		G4		P value
	No.	%	No.	%	No.	%	
Medium	1	20	2	33.4	3	21.4	0.826479, Insignificant p > 0.05.
Heavy	4	80	4	66.6	11	78.6	
Total	5	100	6	100	14	100	

Discussion

Generally, cryptosporidiosis opportunistic high risk and/or prolonged infection increased in patients with cellular and humoral immune deficiencies, such as HIV/AIDS, organ transplants, IgA deficiency, immunosuppressive drugs, and/or hypogammaglobulinemia (El-Bahnasawy *et al*, 2018). Besides, biotics-treated, and untreated immunocompetent mice. Those received probiotics exhbied a consistently higher parasitic load compared to untreated ones. This agreed with Guitard *et al*. (2006), they found that administeration of two different probiotic mixtures orally didn't significantly affect the intestinal cryptosporidiosis. Besides, Salazar-Lindo *et al*. (2004) in a clinical trial by didn't find marked effect of supplementing infant formula with *Lactobacillus GG* in cryptosporidiosis-associated diarrhea. But, others (Alak *et al*, 1999; Sanad *et al*, 2015; Gaber *et al*, 2022) reported a reduction in parasitic burd- extra-intestinal cryptosporidiosis to the biliary tract cause complications in sever HIV/AIDS patients, such as acalculous cholecystitis, pancreatitis, cholangitis, and/or stricture formations with a poor prognosis (Shirley *et al*, 2012).

In the present study, there was a significant difference in parasitic burden between proen after probiotic treatment of cryptosporidiosis infected immunocompetent mice. Ashraf and Shah (2014) reported that there was discrepancy in the probiotics enhances in the nonspecific cellular immune responses in the immunocompetent hosts by mechanisms, such as macrophage activation, stimulation of natural killer (NK) cells, antigen-specific cytotoxic T lymphocytes, and cytokine release in a strain-specific and dose-dependent way.

In the present study, probiotics treated immunocompromised mice exacerbated the parasitic burden. This agreed with Toro-Londono *et al*. (2019); Carey *et al*. (2021), and

Piazzesi *et al*. (2023), they found that chronic *C. parvum* infection in immunocompromised individuals played marked alterations in gut microbiota composition with persistent infection. In the immunodeficient mice, *Lact obacillus reuteri* caused the intestinal resistance to cryptosporidiosis (Alak *et al*, 1997), and reduced parasites in intestinal epithelium (Waters *et al*, 1999). Alak *et al*. (1999) provided more insight into this contradiction that *Lactobacillus* supplementation reduced *C. parvum* shedding in feces, but didn't suppress Th2 cytokine production linked to immunosuppression. Also, it didn't restore Th1 cytokines or IFN-γ, essential for parasites recovery. Besides, *Bifidobacterium infantis* inhibited chemokine CCL20 secretion in a dose-dependent manner (Sibartie *et al*, 2009), impairing the antimicrobial activity against *C. parvum* sporozoites (Guesdon *et al*, 2015; Laurent and Lacroix-Lamandé, 2017), as in the present study.

In the present study, probiotic didn't show dysplasia in G1, but 80% of G2 mice with dysplastic changes exhibited a heavy parasitic burden. In G3, 66.6% of dysplastic mice had a heavy burden, but G4 showed 78.6% a heavy parasitic burden, without significant differences. This agreed with Certad *et al*. (2010) who, reported that *Cryptosporidium*-induced cellular transformation in SCID mice treated with dexamethasone with high doses (10^5-10^7oocysts) resulted in more severe neoplastic development compared to lower doses infected mice., with dysplasia and intraepithelial neoplasia high-grade in cecum as early as 46 days post-infection in immunohistochemical cellular proliferation three weeks post-infection (Lowe and Lin, 2000. *C. parvum* infection contributed to dysplastic changes by modulating apoptosis, a critical process in carcinogenesis (Meleet *et al*, 2004). Also, this agreed with Altounsy *et al*. (2010), who reported that *C. parvum* alternated the gut microbiome. Ras *et al*. (2015) found that in monkeys probiotics (*Lactobacillus rhamnosus*, linked *Bifidobacterium lactis*) induced mild apoptotic effects. Cryptosporidiosis was associated with microbial shifts linked to dysbiosis and inflammation (McKenney *et al*, 2017). Also, fungal microbiome was changed in horses post-infection (Wang *et al*, 2022). Besides, *Lactobacillus GG* promoted intestinal epithelial icell activity by pro- and anti-apoptotic pathways (Yan and Bolk, 2002).

Probiotics anticarcinogenic actions included modulation of the intestinal microbiota, suppression of cell proliferation and induction of apoptosis (Naeem *et al*, 2024). But, excess probiotic altered mechanisms (Zhao *et al*, 2016). Variations in microbiome composition influenced susceptibility to cryptosporidiosis pathology (Chappell *et al*, 2016).

Colorectal cancer development is a complex process with 70% of patients from the premalignant adenomas (Park *et al*, 2018).

Unregulated inflammatory cytokines production of TNF-α and IFN-γ enhanced apoptosis (Fan and Pedersen, 2021). *C. parvum* infection induced DNA damage in leucocytes (Atwa *et al*, 2020), which suppressed the IFN-γ secretion by NK cells, macrophages, T cells, and intestinal intraepithelial lymphocytes (Leav *et al*, 2005).

Besides, probiotic bacteria in vitro produce short-chain fatty acids (SCFAs), including butyrate and propionate direct inhibited the *C. parvum* growth (Keelaghan *et al*, 2022). But, cryptosporidiosis in goat kids and neonatal mice showed deplete butyrate-produced bacteria by increasing *Bacteroidetes* rates (Mammeri *et al*, 2020). Carey *et al*. (2021) found that infants with diarrhea exhibited lower *Megasphaera* levels of a SCFA-producing bacterium in evaluating cryptosporidiosis. Apoptotic changes induced by *C. parvum* infection as early as 24 hours P.I. in non-infected adjacent cells, potentially as a host strategy limiting its expansion (Chen *et al*, 2001; Liu *et al*, 2008). But, apoptosis in later infection facilitated parasite escaping from the host (McCole *et al*, 2000; Buret *et al*, 2003). While the apoptosis can help eliminating infected cells, excessive or uncontrolled apoptosis may promote dysplastic chang-

es, contributing to colorectal cancer development (Park *et al*, 2018).

Conclusion

The probiotic administration influences the *Cryptosporidium* burden and progression in infected mice, with potential implications for dysplasia and colorectal cancer development.

More study is ongoing to evaluate the probiotics strain-specific effects and interactions with host immune responses in cryptosporidiosis and will be published in due time elsewhere.

Authors' declaration: They declared that neither have conflicts of interest nor received any funds.

Authors' contribution: Authors equally shared in the practical and laboratory studies.

All shared in writing, reviewing paper and and approved manuscript publication.

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Explanation of figures

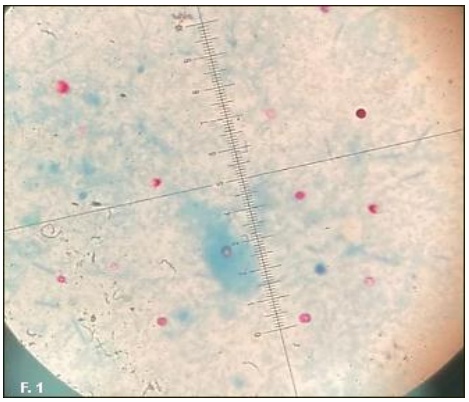
Fig. 1: Ocysts in stool samples obtained from severely infected mice stained with kinyoun (x1000).

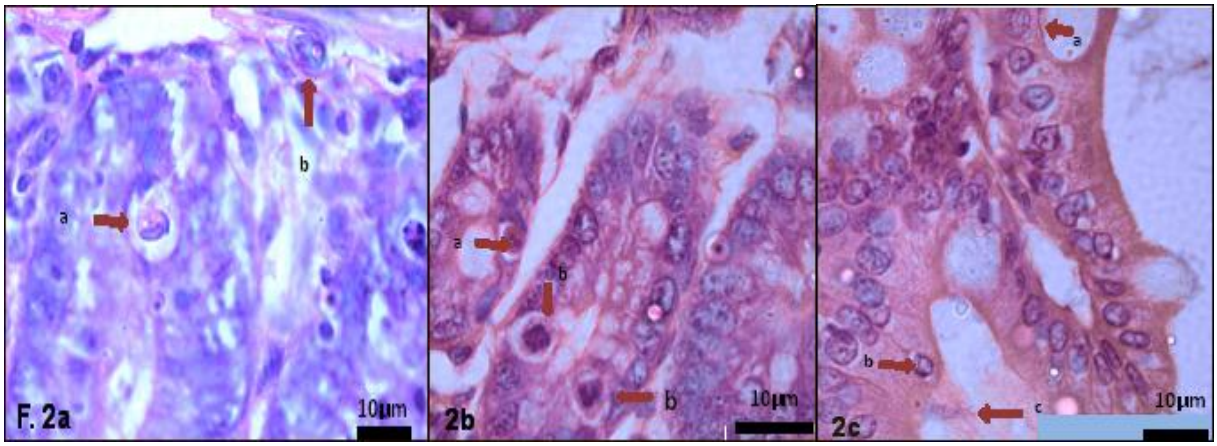
Fig.2A: Histopathology ileocecal tissues of *Cryptosporidium*-infected developmental stages mouse: arrows: (a) meront type II, (b) microgamont B: arrows: (a) trophozoite, (b) macrogamont (H&E, 1000 x), 7 C: arrows: (a) meront type I, (b) oocyst, (c)sporozoite (H&E, 1000 x).

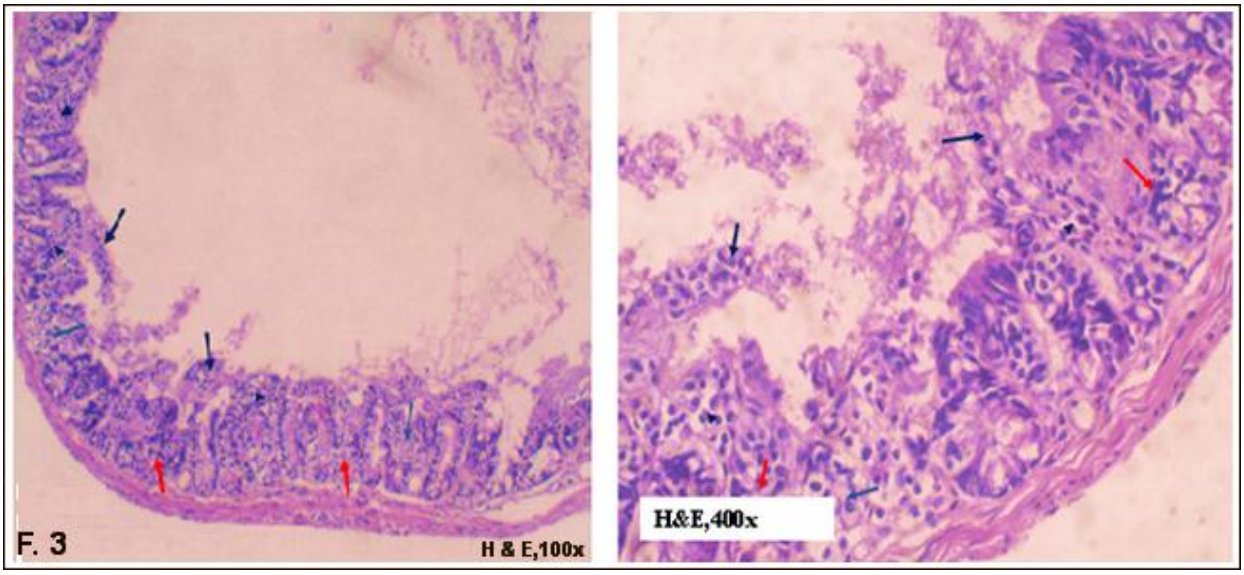
Fig. 3: Different inflammatory changes inside intestinal cecal tissues of probiotics-treated *Cryptosporidium*-infected mice. Colonic mucosal damage with irregularity of surface epithelium and degenerated epithelial cells falling in lumen (Black arrows) and increase in chronic lympho-palsmacytic infiltrate in lamina propria (Arrowheads), Areas of complete drop out glands (Blue arrows). Some inflammatory cells attacked crypts (Red arrows), Inflammation (2), Inflamed area/extent (2), Crypt damage (1) Involvement % (3). Score= 8

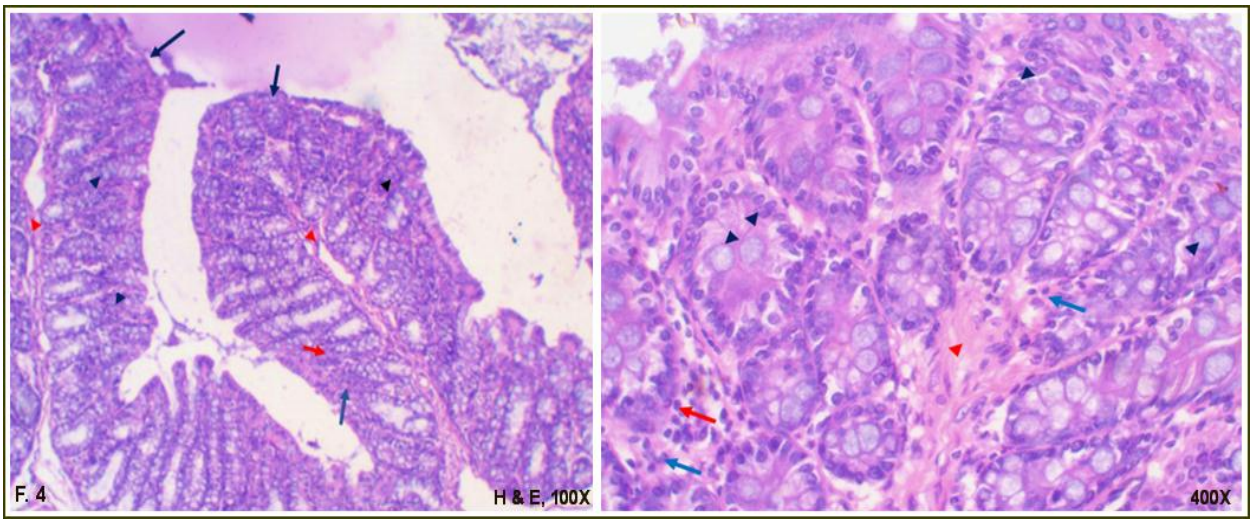
Fig.4: Dysplastic changes in ileocecal tissues of *Cryptosporidium*-infected mice. Papillary proliferation of mucosal epithelium forming adenoma-like configuration (Black arrows), with fibro-vascular cores (Red arrowheads), with epithelial dysplastic changes (Black arrowheads); enlarged hyperchromatic nuclei, nuclear stratification and dysplastic goblet cells. An increase in chronic lymphopalsmacytic infiltrate in lamina propria (Blue arrows). Some inflammatory cells attacked crypts (Red arrows) (H&E, 100x).

Fig.4: Dysplastic changes in ileocecal tissues of *Cryptosporidium*-infected mice. Papillary proliferation of mucosal epithelium forming adenoma-like configuration (Black arrows), with fibro-vascular cores (Red arrowheads), with epithelial dysplastic changes (Black arrowheads); enlarged hyperchromatic nuclei, nuclear stratification and dysplastic goblet cells. An increase in chronic lympho-palsmacytic infiltrate in lamina propria (Blue arrows). Some inflammatory cells are attacking crypts (Red arrows)






F. 3
H & E,100x
H&E,400x


F. 4
H & E, 100X
400X