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Blastocystis ST-1 and ST-3 Mixed Infection Causes Increased Inflammatory Responses and NF-kappaB Protein Expression

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ABSTRACT

Background: *Blastocystis* sp. is one of the most common intestinal protozoa found in humans, but its pathogenesis is still unclear. One of the influencing factors is its genetic subtype. The subtypes that most commonly infect humans are subtypes 1–4. *Blastocystis* subtypes that are commonly found in Indonesia, especially in Padang City, are *Blastocystis* ST 1 and ST3.

Methods: This study was experimental with a post-test-only control group design. A total of 18 rats were randomly divided into 3 sample groups: control group, treatment group with *Blastocystis* ST-1 and ST-3 dose 10^4 and dose 10^5. The inflammatory response was assessed by histopathological and immunohistochemical examination of NF-kappaB protein. The Kruskal-Wallis test was used for ordinal histopathological scores, and one-way ANOVA (or Kruskal-Wallis if non-normal) was used for NF-kappaB expression to determine the significance of differences among the three groups.

Result: *Blastocystis* ST 1 and ST 3 mixed infection in rats caused mild-moderate inflammation in histopathological examination of intestinal tissue and increased NF-kappaB protein expression, especially in the dose 10^5 treatment group (14.45±5.51) compared to the dose 10^4 treatment group (8.24±2.72) and the control group (7.41±1.21). This difference was statistically significant ($P<0.05$).

Conclusion: This study concluded that *Blastocystis* ST-1 and ST-3 mixed infection can increase the inflammatory response in rats' intestinal tissue. This effect was particularly evident at higher doses 10^5.

INTRODUCTION

Blastocystis sp. is one of the most common intestinal protozoa found in humans. It is cosmopolitically distributed, with different prevalences in different countries. In general, the prevalence of *Blastocystis* sp is higher in developing countries than in developed countries. This is associated with poor hygiene and sanitation, low economic levels, exposure to animals, and consumption of drinking water contaminated with parasites [1,2].

Various epidemiological surveys of intestinal protozoa have found *Blastocystis* sp to be the most prevalent parasite. For example, a study conducted in five regions in Colombia found that *Blastocystis* sp. had the highest incidence of intestinal protozoa at 54.5%, followed by Giardia lamblia at 45.4% (3). Several similar studies in Iran also found the highest incidence of *Blastocystis* sp. compared to other intestinal protozoa [4,5].

Blastocystis sp was first discovered in 1911 by Allexeieff, but many things remain debated. One of them is the parasite's pathogenic potential. Some researchers claim that *Blastocystis* sp is a commensal microorganism or an opportunistic pathogen, while others believe that it is a true pathogen [6,7].

Based on *Blastocystis* sp. small subunit (SSU) rRNA gene analysis, at least 28 subtypes (ST) have been identified, and the subtypes that can infect humans are subtypes 1–9, with ST3 being the most dominant ST, followed by ST1 and ST2. The distribution of subtypes varies in different countries. The most prevalent ST in Iran is ST3, followed by ST1 and ST2 [8]. Kumara et al., [2] reported that the most common subtype in Malaysia was ST3 (54.7%). Rudzińska et al., [8] also reported that ST3 was dominant in Poland, the distribution of *Blastocystis* sp. subtypes in Indonesia differs in each region. Nofita et al., [9] report that the most common subtype in children on Sumba Island was ST1, followed by ST3 and ST2, while Yoshikawa et al., [10] reported that the most common ST in Jakarta City was ST3, followed by ST1, and in Padang City was ST1, followed by ST2.

Several experts believe the pathogenicity of *Blastocystis* sp. is related to its subtype. However, the studies that have been conducted show different results. Cakir et al., [11] reported that subtypes 1 and 2 are more pathogenic. Mohamed Find subtype 1 to be associated with colon cancer [7] and Hameed and Hassanin [12] successfully obtained protease as a virulent factor of *Blastocystis* subtype 3. Stensvold et al., [13] report *Blastocystis* subtype 4 caused acute diarrhoea in a patient in Denmark. *Blastocystis* subtype one was associated with the incidence of IBS in Indonesia [14].

The pathogenic potential of *Blastocystis* ST1, ST4 and ST7 has been demonstrated based on in vitro studies with axenic cultures and animal studies with axenic isolates or purified cysts. However, this pathogenicity's molecular and cellular basis has yet to be fully elucidated. This has led to no precise therapeutic modalities to date. So far, the use of metronidazole, which is a commonly used antiprotozoa, is recommended. However, its effectiveness still needs to be determined; even in some studies, it has been shown that there is resistance to metronidazole [14–16].

This study will examine the effect of *Blastocystis* subtype 1&3 mixed infection on inflammatory response. *Blastocystis* subtypes 1&3 mixed infection were chosen in this study because no previous studies have examined the pathogenesis due to this mixed infection, and based on previous research, it was found that this subtype was the most common subtype found in Padang City. Meanwhile, the effect of *Blastocystis* subtype 1&3 mixed infection on the above has never been studied. Based on the description above, the researcher is interested in examining the effect of *Blastocystis* subtype 1&3 mixed infection inflammatory response in rats.

METHODS

Animal: Rats were first adapted for one week before being treated. Rats were placed in cages, one cage for one rat. In this study, Wistar rats aged 4 weeks and weighing 75 g/head were used. Cages were placed in an adequately ventilated room at a temperature of 25-26^{0}C and humidity of 40-60%, with a cycle of 12 hours of dark and 12 hours of light. Cages were cleaned daily. Food was given 20g/day/head, and water was given ad libitum. All rats were first subjected to direct faecal examination and culture to ensure no intestinal parasite infection. Eighteen rats were randomly divided into three groups. The first group as control was given PBS; the second group

was given a dose of 10^4 *Blastocystis* cysts/200 g BW orally, and the third group had a dose of 10^5 *Blastocystis* cysts/200 g BW orally. *Blastocystis* subtypes 1 and 3 inoculated into rat were obtained from faecal samples collected from elementary school students isolated from collection Parasitology Laboratory in Faculty of Medicine, Universitas Andalas.. Each sample was cultured with Jones Medium. *Blastocystis*-positive samples will then be subjected to PCR testing using STS primers to determine the subtype. The body weight of the rats was weighed once every two days until the day of termination. Termination was carried out on day 14. Before termination, faeces were examined to see *Blastocystis* infection.

Histopathology examination : After termination, colon and caecum tissues were taken and stored in 10% formalin. Paraffin blocks were then made, and hematoxylin and eosin (HE) staining was performed. Microscopic assessment was performed by taking images using an Olympus CX 33 microscope, 3.1 MB Sony Exmor Beta camera, and Betaview program at 100x (objective 10x) and 400x (objective 40x) magnification. Semiquantitative histological score assessment was based on the Barthel- Manja scoring (BM score) system by assessing the components of submucosal oedema, PMN leukocyte infiltration in the submucosa, goblet cells and surface epithelial integrity. Each component was scored on a scale of 0 to 3, where Score 0 indicates normal mucosal appearance with no significant inflammation (rather than absolute absence of immune cells, as resident immune cells are always present in healthy tissue), and Score 3 indicates severe abnormality [17].

Type and Research Design: This research is a pure experimental research using a post-test-only control group design.

Ethical Approval : This research verified from Research Ethics Committee, Faculty of Medicine, Universitas Andalas with number 92/UUN.16.2/KEP-FK/2023.

Data analysis: Univariate analysis was used to see the data distribution of each variable, which was then presented in the form of frequency distribution tables. To determine the difference in the mean expression of NF- kappaB and BM score, in each group, different statistical approaches were used depending on data type. Since the BM score (histopathological scoring) is ordinal data, the Kruskal-Wallis non-parametric test was used, followed by the Mann-Whitney U test with Bonferroni correction for post- hoc pairwise comparisons. For NF- kappaB protein expression (continuous data expressed as percentage of stained area), a one-way ANOVA test was conducted if the data were normally distributed and homogeneous; otherwise, a Kruskal-Wallis test was performed.

RESULTS

Effect of *Blastocystis* ST 1 and ST 3 mixed infection on intestinal histopathology

After inoculation, rats in the mixed- infection group (ST-1 and ST-3) showed a slight but statistically significant decrease in body weight compared to the control group ($p < 0.05$) starting from day 7 post-inoculation. Clinical observations revealed mild lethargy, ruffled fur, and reduced food intake in the infected groups. Stool examination revealed the presence of Blastocystis vacuolar forms beginning on day 3 post-inoculation, with PCR confirmation of ST-1 and ST-3 subtypes in all infected rats by day 7. The infection persisted up to day 14, indicating successful colonization. *Blastocystis* sp lives and colonizes in the colon and can cause inflammation and damage to the colonic epithelium. The degree of gut inflammation can be measured using the BM score. Figure 1 shows the histopathological picture of rats' intestinal mucosa, and Table 1 shows the Barthel Manja Score in the three groups of rats.

Photomicrograph of representative area showing mucosa (M), submucosa (S), and muscular layer (Mm). Control rats (panels a, d), rats with *Blastocystis* inoculation dose 10^4 (panels b, e), with *Blastocystis* inoculation dose 10^5 (panels c, f). Rats with *Blastocystis* inoculation treatment showed histological changes in the form of lamina propria oedema, with leukocyte infiltration ( )reduced goblet cells, damage to surface epithelial integrity in the form of desquamation ( ), and epithelial ulceration ( ).*Blastocystis* inoculation dose of 10^5 showed moderate damage in mucosal damage scoring. Hematoxylin Eosin. Scale; c; 100µm, f; 100µm.

Figure 1 shows a higher degree of inflammation in rats treated with dose 10^5 (K3) compared to rats treated with dose 10^4 (K2) and control group rats. There was oedema of the lamina propria, PMN cell infiltration, reduced number of goblet cells and epithelial damage in rats treated with

dose 10^5.

The BM score was used to assess the degree of inflammation in the intestinal tissue. The BM scores in each group of rats are shown in Table 1.

Based on Table 1, it can be seen that the degree of inflammation is higher in rats of the treatment group with a dose of 10^5 (K3), followed by rats of the treatment group with a dose of 10^4 (K2) and rats of the control group, with a mean score of 4.67 (moderate inflammation); 4.17 (moderate inflammation) and 2.33 (mild inflammation), respectively. This difference was statistically significant (P-value < 0.05).

Effect of *Blastocystis* ST 1 and ST 3 mixed infection on NF-κB protein expression

NF- κB protein expression was assessed by immunohistochemical examination (IHK). The results of the IHK examination in the three groups of rats can be seen in Figure 2. NF- κB expression in the rat intestine is seen in the membrane and cytoplasm of epithelial cells. The percentage of stained area was measured using the ImageJ program to determine the magnitude of expression. Based on Figure 2, there is an increase in NF-κB expression from the control group, the treatment group with a dose of 10^4 and the treatment group with a dose of 10^5, with mean expression values of 7.41±1.21%, 8.24±2.72%, and 14.45±5.51%, respectively. Representative images showed stained areas of approximately 8.63%, 9.37%, and 16.95%.

Representative photomicrographs showing NF- κB expression in intestinal tissue: control rats (panels a, d, g), rats inoculated with 10^4 dose *Blastocystis* inoculation (panels b, e, h), and rats inoculated with 10^5 dose Blastocystis inoculation (panels c, f, i). Expression measurement was performed by measuring the proportion of area (fraction of stained area) with the ImageJ program (ImageJ 1.49v software, National Institute of Health, Bethesda, MD, USA). The positively stained area is reported as a percentage area. Rats with *Blastocystis* inoculation treatment showed an increase in the fraction of NF-k B expression area, especially in the 10^5-dose treatment.

Figures

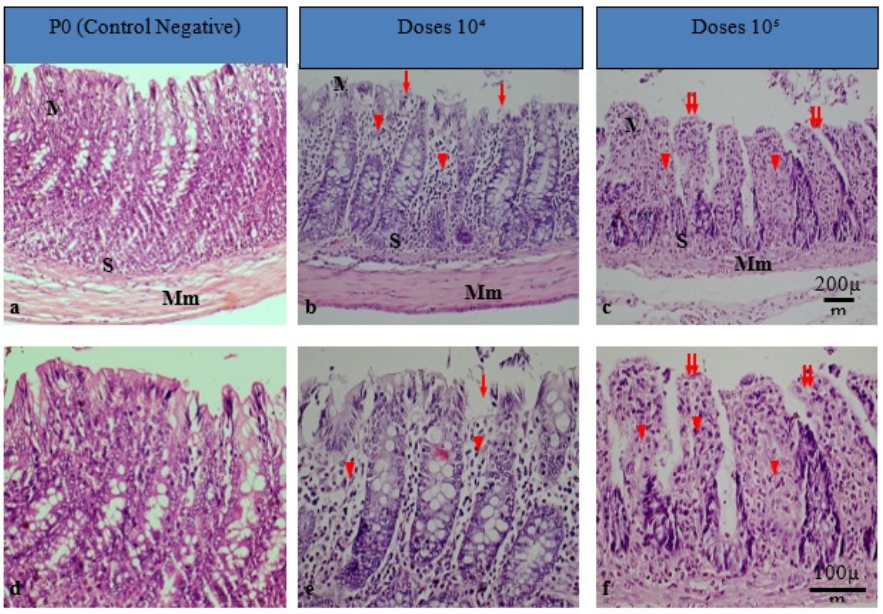

Figure 1: Histopathology of intestinal tissue in rats after inoculation with Blastocystis ST1 & ST3.

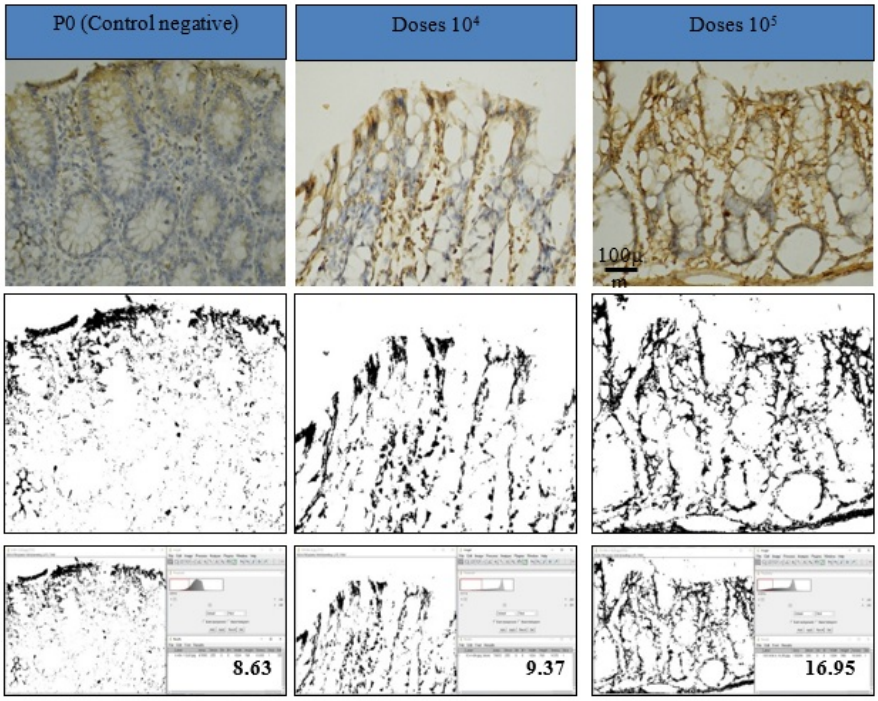

Figure 2: Expression of NF-κB protein in intestinal tissue of rats inoculated with Blastocystis ST1 & ST3.

Tables

Group	Number of Samples	Barthel Manja Score						
		Submucosa oedema	PMN	Goblet cell	Epitel deskuamasi	Total score	Mean ±SD	P-value
K1	15	0	1	0	0	1	2.17 ± 0.753	0.007
	16	1	1	0	1	3		
	23	0	1	0	1	2		
	31	1	1	0	1	3		
	52	0	1	0	1	2		
	50	0	1	0	1	2		
K2	4	1	2	0	1	4	4.17 ± 0.983	
	5	1	2	0	1	4		
	7	1	1	0	1	3		
	8	0	2	1	1	4		
	9	1	2	2	1	6		
	10	0	2	1	1	4		
K3	1	1	2	2	3	8	4.67 ± 1.862	
	2	0	1	1	1	3		
	3	1	2	1	1	5		
	12	0	2	1	1	4		
	13	1	1	1	2	5		
	14	0	1	1	1	3		

Table 1: Histologic assessment of rat intestinal damage according to the Barthel scoring system.

DISCUSSION

Blastocystis ST 1 and ST 3 mixed infection

The coexistence of both may result in additive or synergistic effects on NF-κB activation, as observed in this study. The prevalence of ST1 & ST3 co-infection has been reported in Indonesia [9,10] and other countries, with varying proportions up to 15–30% in certain populations. Recent epidemiological studies also report the dominance of ST3 followed by ST1 in Southeast Asia and Latin America [2,3].

In this study, faecal cultures were performed on elementary school-aged children. Based on the

identification results by PCR examination, the faecal samples contained *Blastocystis* with subtypes ST1 and ST3. A Columbia study found that 43.55% of *Blastocystis* infections were caused by ST3, and ST1 caused 38.7%.

It was found that ST1 and ST3 subtypes are pathogenic, often found in adolescence, and are associated with the incidence of irritable bowel syndrome (IBS) [18]. *Blastocystis* subtypes often found in patients with IBS are ST 1, ST 3, and ST 4 [19]. It is also known that the most common subtype found in patients with colorectal cancer is ST 3, as much as 75%, followed by ST 1, as much as 16.7% [19].

Mixed infection with *Blastocystis* subtypes ST1 and ST3 has been increasingly reported in both humans and experimental animal models, and may be associated with more severe pathological outcomes compared to single- subtype infections. Studies have shown that ST1 is often associated with proinflammatory responses, while ST3 is more commonly found in asymptomatic individuals but can modulate host immunity depending on host factors and microbiota composition. The coexistence of ST1 and ST3 in one host may lead to additive or synergistic inflammatory effects, as observed in our study with increased NF-κB expression and histopathological damage, particularly in the group receiving the higher inoculation dose.

A study by Wawrzyniak et al., [20] indicated that different *Blastocystis* subtypes interact variably with host epithelial and immune cells, with ST1 known to induce IL-8 production in vitro, a key chemokine in neutrophil recruitment. Our results are consistent with these findings, demonstrating elevated histological inflammation and immune signaling. Mixed infection with ST1 and ST3 can cause a higher inflammatory response compared to single infection. ST1 is known to be pro-inflammatory with IL-8 induction [20], while ST3 is often found in asymptomatic individuals but can modulate host-dependent immunity [14].

Effect of *Blastocystis* ST 1 and ST3 Mixed Infection on Intestinal Histopathology Picture

In this study, histopathological changes induced by *Blastocystis* inoculation were predominantly localized to the mucosal layer of the intestine, with no evident extension into the deeper submucosa or muscularis propria. This limited inflammatory pattern may be attributed to both the inoculated dose and the stage of *Blastocystis* used. The vacuolar form often utilized in experimental infections is generally associated with epithelial adherence rather than deep tissue invasion. Our findings are consistent with those of Ajjampur et al., [14] and Tan [21], who also reported mucosal- restricted inflammation, goblet cell depletion, and epithelial disruption in models infected with ST1 or ST3 subtypes using doses ranging from 10^4 to 10^6 cysts/mL.

The results obtained were similar to several previous studies, where there was exfoliation of the intestinal epithelium, inflammatory cell infiltration in the submucosa, hyperplasia of goblet cells, and *Blastocystis* infiltration in all layers of the colon in the immunosuppressed mice group [22]. It was also found that colonic epithelial damage decreased goblet cells and leukocyte infiltration in the lamina propria in mice inoculated with *Blastocystis* ST 7 [14]. The same results were also reported in histopathological changes in the intestinal tissue of mice infected with *Blastocystis* ST 1. Changes occur in the infiltration of inflammatory cells in the lamina propria, mucosal oedema erosion and ulcers on the epithelium [23]. However, changes in the histopathological picture of the rat intestinal mucosa due to mixed infection with *Blastocystis* ST 1 and ST 3 have not been published.

An assessment is made using the Barthel Manja score to see the degree of inflammation in this intestinal tissue. The mean score in the treatment group given *Blastocystis* dose 10^5 was 4.67 ± 1.862, and in the treatment group given *Blastocystis* dose 10^4 was 4.17 ± 0.983. This illustrates that mild-moderate inflammation has occurred in the intestinal tissue of these rats. Meanwhile, the untreated rats only experienced mild inflammation, with a mean score of 2.33. Based on the Barthel Manja score, there was a significant difference between the control and groups with treatment doses of 10^4 and 10^5 ($p<0.05$). Interestingly, mild inflammatory changes such as occasional leukocyte infiltration and slight epithelial alteration were also observed in the intestinal tissues of untreated (control) rats. These findings may be attributed to several factors. First, low-level background inflammation is not uncommon in laboratory rodents and may result from environmental stressors, changes in microbiota composition, or dietary factors [24]. Second, subclinical infections or exposure to opportunistic microorganisms present in the gut microbiota, even under standard laboratory conditions, can lead to localized immune activation

[25]. Lastly, technical handling or procedural stress during experimental setup may induce physiological stress responses that indirectly affect intestinal immune status, including mild mucosal inflammation.

Despite this baseline inflammation, the extent and severity of tissue changes were significantly higher in the Blastocystis-inoculated groups, as demonstrated by increased NF-κB expression and more pronounced histopathological alterations. This supports the interpretation that the observed inflammation in treated groups was specifically induced by Blastocystis exposure, beyond the physiological baseline present in controls.

The persistent mild-moderate inflammation in intestinal tissue infected with *Blastocystis* sp is one of the factors causing Inflammatory bowel disease (IBS). *Blastocystis* sp is estimated to secrete twenty-two types of proteases, including 20 cysteine proteases, one serine protease, and one aspartic protease. These proteases are involved in paracellular permeability, inflammation and hypersensitivity. These proteases can cause mucus disruption, further triggering inflammatory and allergic responses caused by chronic exposure to luminal antigens. In addition, proteases secreted by intestinal parasites and bacteria can also target receptors on the surface of intestinal cells. Type 2 protease-activated receptors induce inflammation and tight junction (TJ) disruption, often seen in IBS. In this study, there was no change in the clinical appearance of the rats. This is because the inflammation was moderate, and the infection time was short.

Effect of *Blastocystis* ST 1 and ST 3 mixed infection on NF-κB protein expression

Nuclear Factor Kappa-B (NF-κB) has a vital role in the regulation of the immune system at all stages, such as primary and secondary development of lymphoid tissue, hematopoiesis and recognition of Danger associated molecular patterns (DAMPs) or pathogen- associated molecular patterns (PAMPs). It regulates the effector mechanisms of the immune system. NF-κB has long been considered a prototypical proinflammatory signalling pathway. Studies have shown that tissue proinflammatory cytokine and chemokine production depend on NF-κB. NF-κB controls various genes involved in the inflammatory process, resulting in increased expression of NF-κB in various inflammatory diseases, such as inflammatory bowel disease (IBD) [26].

In this study, an immunohistochemical test was performed to see the expression of NF-κB protein in rat intestinal tissue. The group of rats treated with *Blastocystis* doses 10^4 and 10^5 showed an increase in NF-κB expression, while in the control group, there was baseline NF-κB expression. Statistical tests showed a significant difference between the dose 10^5 treatment group, the dose 10^4 treatment group ($p=0.024$), and the control group ($p=0.011$). This shows that there is an increase in inflammatory response in the group of rats with treatment, and there is an effect of increasing the dose on NF-κB expression. This aligns with the histopathological examination results, which showed a mild-moderate inflammatory response in the treated rats.

Many previous studies have reported an increase in inflammatory response in various *Blastocystis* subtypes by seeing an increase in proinflammatory cytokines. *Blastocystis* ST 7 was reported to cause an increase in IL-6, IL-1 and TNFα. *Blastocystis* ST 1 increased the production of interleukin 8 (IL-8) and Granulocyte-Macrophage Colony-Stimulating Factor (G-M-CSF) in human colon cancer cells. Several animal studies in rats and mice have also reported upregulation of proinflammatory gene expression and intense infiltration of proinflammatory cells in the colon. A recent study also showed significantly higher levels of IL-17 and IL-23 in *Blastocystis*-infected mice. However, not many studies have looked directly at NF-κB protein expression. An in-vitro study on HT-29 and T-84 colonic epithelial cells reported *Blastocystis* ST4 was able to inhibit LPS-mediated NF-κB activation. At the same time, ST7 enhanced the effect of LPS-mediated NF-κB activation [27,28]. However, it should be noted that these results were only observed in an in vitro system, which has various limitations, such as the difficulty of creating an anaerobic atmosphere that matches the parasite's native environment, isolates obtained from culture are generally vacuolar forms, which may not be the forms associated with adhesion and pathogenesis in vivo [15].

These studies demonstrate the pathogenic potential of *Blastocystis* despite the long-standing controversy over whether it is an intestinal pathogen. Differences in virulence have been attributed to different intestinal parasite subtypes, which are supported by studies that revealed

variations in cysteine protease activity between subtypes [29]. No previous studies have examined NF-κB expression in experimental animals inoculated with *Blastocystis* ST1 and ST3.

Blastocystis sp infection causes intestinal symptoms such as abdominal pain, nausea, vomiting and diarrhoea. In addition, *Blastocystis* sp infection can cause functional Irritable Bowel Syndrome (IBS), abdominal discomfort and changes in defecation frequency. This can occur because *Blastocystis* sp causes epithelial barrier dysfunction, which is regulated by tight junctions. Proteases that modulate the intestinal epithelium will induce damage to the tight junction and cause barrier dysfunction or leakage [30]. In in-vivo studies, blastocysts will attach to the surface of the intestinal mucosa and increase mucosal permeability by producing cysteine protease, degrading IgA, inducing the secretion of inflammatory cytokines such as IL-8 and causing apoptosis in host cells. *Blastocystis* will persist in the human intestine for long periods without causing gastrointestinal symptoms [31].

Blastocystis ST3 invades the gastrointestinal tract by stimulating the production of cysteine protease, which increases IL-8 in the intestinal mucosa. Based on colonoscopy examination results, 53.3% found gastrointestinal epithelial changes such as colitis and ileitis. In ST1 infection, 4.4% had abnormalities, and 2.7% had abnormal histopathology [32,33].

This study found that mixed infection with *Blastocystis* ST 1 and ST 3 at the dose of 10^5 caused inflammation up to mucosal area of the rats's colon as showed through histopathology examination and expression of NF-κB in the colonic tissue. There was an effect of the dose of *Blastocystis* given with the changes that occurred. Changes were more pronounced in the group of rats given *Blastocystis* dose 10^5.

As presented in various literature, many factors influence the onset of clinical symptoms in *Blastocystis* sp infection. In addition to the influence of the parasite's genetic subtype and virulence, the host response and the gut microbiota also influence the onset of clinical symptoms. Recent studies have discussed the relationship between *Blastocystis* infection and the gut microbiota regarding the appearance of various intestinal disorders.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

Nofita conducted experiments and carried out research procedures in the laboratory, N. Irawati contributed to editing and finalizing the manuscript, N. Suharti was responsible for language editing and data processing, Hasmiwati contributed to the monitoring of experimental animals, E. Darwin and Tofrizal were responsible for histopathological anatomical interpretation, H. Ali and A.W Murni determined the research design and conducted data analysis.

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