Assessment of host biochemical factors and microbiota interactions and pathogenicity of *Blastocystis hominis* genotypes: A cross-sectional study

Original Article

Enas A El Saftawy[1,2], Marwa MI Ghallab[3], Tamer Haydara[4], Asmaa Ibrahim[5], Shimaa A Atta[6], Reham A Gad Allah[7], Marwa N Kamel[8], Salwa M Morsy[1,9]

Departments of Medical Parasitology, Faculty of Medicine[1-3], Cairo University[1], Armed Forces College of Medicine[2], Internal Medicine[4], Genetic Engineering[5], Immunology[6], and Clinical Chemistry[7], Kafr El-Sheikh University[3,4], Biotechnology Research Institute, Sadat City University[5], Theodor Bilharz Research Institute[6,7], Master in Quality Management, Arab Academy for Science, Technology, and Maritime Transport, Productivity and Quality Institute, Smart Village[8], and Modern University for Technology and Information[9], Egypt

ABSTRACT

Background: Although several clinical and scientific evidences confirmed *B. hominis* pathogenicity in irritable bowel syndrome (IBS), host biochemical risk factors involved in IBS manifestations are still ambiguous.
Objective: To investigate the interactions between *B. hominis* genotypes, host biochemical factors, and microbiota.
Patients and Methods: In a cross-sectional study, 167 stool samples from patients attending the Internal Medicine Department, of Kafr El-Sheikh University Hospital were examined. Using specific sequenced-tagged site (STS) primers, samples positive for *B. hominis* were subtyped. Reducing sugar and pH were assessed in *B. hominis*-positive samples. The lactate dehydrogenase (LDH) enzyme was measured in both *B. hominis* sole infections and blastocystosis coexisting with *G. intestinalis*. To determine the predominant co-existing organisms in blastocystosis infections, bacterial and fungal stool cultures were performed. Furthermore, laboratory-cultivated *B. hominis* was incubated *in vitro* with *Escherichia* (*Esch.*) *coli* and *Candida* non-*albicans* to assess possible interactions with models of normal microbiota.
Results: Of 167 cases, thirty-three (19.7%) were microscopically positive, and twenty seven (16.2%) were molecularly confirmed harboring *B. hominis*. Genotype 3 was solely detected with higher prevalence in summer and spring. There was a consistent chemical association of carbohydrate intolerance and acidic pH with genotype-3 *B. hominis* that seemed to augment IBS-like manifestations. Predominant overgrowth of *Esch. coli* in cultured stool samples was observed. Co-culture of *B. hominis* with *Esch. coli* and *C.* non-*albicans* augmented their growth whereas the parasite was suppressed. Coinfection of *B. hominis* with *G. intestinalis* showed a significant rise in LDH enzyme in stool samples compared with the presence of *B. hominis* alone.
Conclusion: Genotype-3 *B. hominis* is predominant and closely related to IBS-like manifestations. Blastocystosis appeared to be related to carbohydrate intolerance, fecal acidity, and enhanced effect on the growth of *Esch. coli* and *C.* non-*albicans*.

Keywords: *B. hominis*; *Candida*; *Escherichia coli*; genotype 3; lactate dehydrogenase; microbiota; reducing sugar.

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Corresponding Author: Salwa M.H. Morsy; **Tel.:** +20 1125337304**; Email:** smmorsy@kasralainy.edu.eg

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INTRODUCTION

Blastocystis spp. are single-celled anaerobic intestinal protozoa, and *B. hominis* is commonly detected in human stool samples. Blastocystosis is related to multiple gastrointestinal and extra-intestinal disorders. Abdominal pain, diarrhea, nausea, anorexia, abdominal distention, gas production, lactose intolerance, constipation, and loss of weight are common symptoms of blastocystosis. A noteworthy correlation exists between *Blastocystis* spp. and IBS, ulcerative colitis, terminal ileitis, and enteritis[1]. Accordingly, the possible interactions that occur between *Blastocystis* spp. and its microenvironment to produce these clinical manifestations are disputed. The virulence factors, pathogenicity, and other potential contributing factors to the presentation of the illness remain unknown[2].

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The apical plasma membrane of the intestine in mammalians is comprised of abundant microvilli exposed to brush border enzymes responsible for the terminal stages of digestion and breakdown of carbohydrates and proteins in the small intestine[3]. Since early infancy, β-glucosidases enzymes catalyze the assimilation of lactose present in milk and dairy products into glucose and galactose. Disruption of the intestinal brush border and lactase enzyme leads to lactose intolerance or maldigestion of lactose leading to its fermentation by the enteric microbiota resulting in gas production and abdominal bloating[4].

A previous study reported the relationship between giardiasis and lactose intolerance[5]. Additionally, rotavirus damages the epithelial lining of the enteric brush border reducing the absorptive surface and reducing numerous digestive enzymes that become altered and disrupted. As a result, this viral infection is associated with osmotic diarrhea that occurs predominantly due to carbohydrate malabsorption[6].

In 2008, Hussein *et al*.[7] determined the deep effect of the genetic subtype in *Blastocystis* spp. virulence. Studies on the SSU rRNA gene showed that *Blastocystis* spp. are 17 genotypes with distinctive host specificity and different clinical manifestations ranging from acute gastroenteritis, and chronic gastrointestinal manifestation with a duration exceeding two weeks, to an asymptomatic carrier[8]. A prior study deduced that *Blastocystis* subtypes 1 and 3 are more prevalent in IBS and is associated with sever manifestations. Thus, the pathogenicity of *Blastocystis* appeared to be genotype dependent[9].

The inhibitory effect exerted by *Blastocystis* on beneficial bacteria, for instance, *Bifidobacterium* and *Lactobacillus* was reported[10,11]. On the contrary, other studies deduced that *Blastocystis* is asymptomatic in most colonized subjects triggering the presence of a higher diversity of enteric commensals and healthy microbiota[12,13]. These differences appeared to be related to *Blastocystis* subtypes[14]. Besides, *Blastocystis* has been found to alter the physiological balance of the enteric microbiota causing dysbiosis. Controversly, *Blastocystis* (ST1 and ST3) were manipulated in fecal microbiota transplantation therapy in patients with persistent *Clostridium diffcile* infection to recur the normal balance of the gut microbiota[15]. Moreover, dysbiosis towards fungal growth was associated with manifestations like those reported in IBS[16] simulating symptoms of blastocystosis. In addition, the impact of blastocystosis on LDH enzymes is still not recognized. The latter is a stable enzyme present in the cytoplasm of all cells that increases rapidly coinciding with the damage to the plasma membrane[17]. We aimed in the current work to investigate the interactions between *Blastocystis* and host biochemical factors, e.g., reducing sugar and PH in stool and microbiota in a genotype-dependent manner.

PATIENTS AND METHODS

This cross-sectional study was conducted in the Medical Parasitology Department, Faculty of Medicine, Kafr El-Sheikh University, and Laboratory of Molecular Medical Parasitology (LMMP), Medical Parasitology Department, Faculty of Medicine, Cairo University during the period from October 2022 to July 2023.

Study design: The study was conducted on gastrointestinal symptomatic patients, and collected stool samples were subjected to microscopic examination and genotyping for *Blastocystis*-positive samples. To assess host-parasite interactions, reducing sugar, pH, and relation with the growth of bacteria and fungi were measured.

Patients: Inclusion criteria were the presence of gastrointestinal manifestations in the associated clinical sheet, patients who didn't receive any anti-parasitic treatment in the last 12 months, and who consented to grant the team of the study with stool samples on three alternate days. Patients were classified into age groups. The included *Blastocystis*-infected patients were coded, and their demographic data were recorded.

Stool examination: The collected stool samples were subjected to macroscopic examination to check for smell and consistency and detect the presence of mucus, blood, or worms. A direct wet smear (unstained and iodine stained) was performed to screen for *Blastocystis* spp. and to exclude the presence of other parasites. The intensity of infection [count/high-power field (HPF)] was determined[18]. Only high-intensity samples (>5/HPF) were included.

***Blastocystis* culture:** *Blastocystis*-positive stool samples were emulsified in sterile saline, and filtered through gauze into a centrifuge tube. For ten minutes, the tube was centrifuged at 2000 rpm; the supernatant was then decanted. Samples were repeatedly washed till the supernatant was entirely transparent. Horse serum (10%) and antibiotic solution (0.1% Streptomycin and Penicillin G) supplemented Jones' media used for culture. With the help of Na_2HPO_4, KH_2PO_4, and NaCl, the pH was adjusted to pH 7 before being autoclaved for sterilization at 121°C. To inoculate the culture, a stool portion (~10 gm) was aseptically transferred with a clean glass rod into the culture tubes and blended with the culture content[19,20].

***Blastocystis* genotyping:** Thirty-three stool samples that were confirmed microscopically for isolated blastocystosis were subjected to Genomic DNA extraction using QIAamp® DNA Mini Kit. The extracted DNA was amplified by PCR targeting specific SSU rDNA using the primers RD5 (5'-ATCTGGTTGATCCTGCCAGT-3`) and BhRDr (5'-GAGCTTTTTAACTGCAACAACG-3') to amplify the ~600 bp fragment that confirms blastocystosis[21]. The PCR

reaction mixture was prepared as 25 µl composed of 12.5 µl master mix, 1 µl of each primer, 0.1 µl Taq polymerase, 7.4 µl distilled water, and 3 µl of the extracted DNA. The cycling conditions were as follows: an initial denaturation for 4 min at 94°C followed by 35 cycles of denaturation for 60 sec at 94°C, annealing for 60 sec at 55°C and extension for 80 sec at 72°C. The final extension was done for 5 min at 72°C. The amplified products were visualized with 1.5% agarose gel electrophoresis after ethidium bromide staining[22].

To detect the subtype of *B. hominis*, a PCR reaction was performed using 7 sets of standardized subtype-specific STS primers[22]. Each set was used in a separate reaction (Table 1). The PCR reaction mixture was prepared in 25 µl composed of 12.5 µl master mix, 1 µl of each primer, 0.1 µl Taq polymerase, 8.4 µl distilled water, and 2 µl of the extracted DNA. The cycling conditions were as follows: an initial denaturation for 4 min at 94°C followed by 40 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 58°C and extension for 60 sec at 72°C. The final extension was performed for 5 minutes at 72°C[22].

Stool-reducing sugar: Approximately one volume of each fresh stool sample (within 2-4 h) was added to two volumes of water in a clean test tube. Two ml (15 drops) of Benedict's reagent ($CuSO_4$) were transferred to the test tube. The mixture was then heated for 3-5 min and observed for alteration in color or precipitate formation. The result was interpreted as follows: green color with no sediment corresponds to (traces) or 0.05-0.15% sugar in the solution; green color with sediment denotes (+) or 0.2-0.4%; olive-yellow color means (++) or 0.5 to 0.75%; orange color reflects (+++) or 1 to 1.65%; while red color is equivalent to (++++) or ≥ 2.0% (Fig. 1). No change in color reflected the absence of sugar in the stool sample[23].

Measurement of pH: The commercial urine test strips (Medi-Test combi 10R SGL, LOT no. 67111) were used to measure pH in the fresh stool samples. Readings were taken after 30 sec and compared with the color scale supplied with the kit. The test strips contain methyl red (3 µg) and bromothymol blue (10 µg) which are reacting substances that change in color between pH 5 and pH 9 (grading from orange to green to turquoise) (Fig. 1).

Evaluation of the extracted *Blastocystis*-genotype interactions with bacteria and fungi: To assess the overgrown microbiota in stool samples positive for *Blastocystis*, stool specimens were cultured in MacConkey agar, Selenite broth, *Salmonella-Shigella* agar (BBL) for Enterobacteriaceae, and Sabouraud's for fungi. In the case of weighty colony formation, supplementary biochemical reactions and serological tests were implemented. Moreover, we investigated (through *in vitro* cultivation) the impact of *Blastocystis* on *Esch. coli* growth and *Candida* non-*albicans* as models for normal gut bacteria, and fungi, respectively.

Table 1. Sequence and expected PCR product size of standardized subtype-specific STS primers.

Subtype	STS primers	Sequence	Expected product size (bp)
1	SB83	**F:** 5'- GAAGGACTCTCTGACGZTGA-3` **R:** 5'-GTCCAAATGAAAGGCAGC-3`	351 bp
2	SB155	**F:** 5'-ATCAGCCTACAATCTCCTC-3` **R:** 5'-ATCGCCACTTCTCCAAT-3`	650 bp
3	SB227	**F:** 5'-TAGGATTTGGTGTTTGGAGA-3` **R:** 5'-TTAGAAGTGAAGGAGATGGAAG-3`	526 bp
4	SB332	**F:** 5'-GCATCCAGACTACTATCAACATT-3` **R:** 5'-CCATTTTCAGACAACCACTTA-3`	338 bp
5	SB340	**F:** 5'-TGTTCTTGTGTCTTCTCAGCTC-3` **R:** 5'-TTCTTTCACACTCCCGTCAT-3`	704 bp
6	SB336	**F:** 5'-GTGGGTAGAGGAAGGAAAACA-3` **R:** 5'-AGAACAAGTCGATGAAGTGAGAT-3`	317 bp
7	SB337	**F:** 5'-GTCTTTCCCTGTCTATTCTGCA-3` **R:** 5'-AATTCGGTCTGCTTCTTCTG-3`	487 bp

F: Forward; **R:** Reverse.

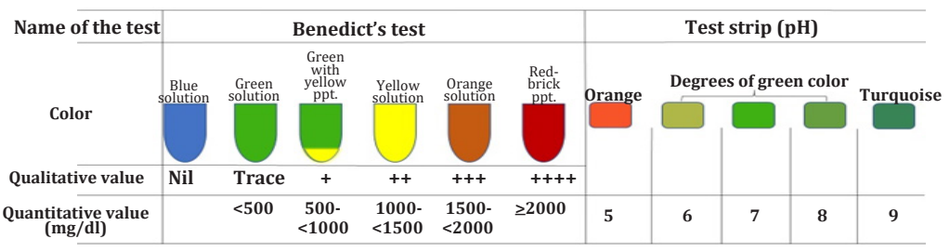

Fig. 1. Calorimetric interpretation of the results.

Preparation of *Esch. coli*: Isolated bacteria were collected by centrifugation (5525×g, 15 min) from tryptone soya broth after 48 h incubation. Sediments were washed 3 times with sterile phosphate-buffered saline (PBS) at pH 7. The pellet was suspended in a sterile Jones' medium[24]. The required optical density was accustomed in Jones' medium and the aliquots of the isolated bacteria were diluted to 1:104 with PBS. Fifty µl from the dilution was then spread on tryptone soy agar (TSA) plates. Plates were incubated at 37°C for 48 to 72 h and colonies were counted. The concentrations of bacterial suspensions were finally adjusted at 1×10^9 CFU/ml[24].

Preparation of *C.* non-*albicans*: The fungi were gathered from Sabouraud's broth (pH 5.8) after incubation for 6 days at 24.5°C. Sabouraud's broth is comprised of glucose (20 g/L) and mycological peptone (10 g/L). Isolates of *C.* non-*albicans* were centrifuged at $23 \times 10^2 \times g \times 10$ min. Sediments were washed 3 times in PBS. Fungi cells were counted by a hemocytometer and were accustomed to 1×10^9 CFU/ml[24].

***In vitro* cultivation:** *Blastocystis* was separately co-cultivated with these two organisms, *Esch. coli* and *C.* non-*albicans*, *in vitro*. *Blastocystis* extracted genotype and the isolated organisms were washed 3 times with pre-reduced sterile (PBS). The reduced PBS for co-culture guaranteed the presence of a low oxygen environment essential for the viability of *Blastocystis* cells rather than a simple PBS formulation that might result in bacterial overgrowth[10]. Next, 1×10^9 CFU/ml of each of the predominant bacteria isolates and fungi was co-cultured with 1×10^7 cells/ml of *Blastocystis* in one ml of PBS. Controls comprised merely organisms of *Blastocystis* (1×10^7 cells/ml), bacteria isolates (1×10^9 CFU/ml), and fungi (1×10^9 CFU/ml) suspended in 1 ml of PBS. After incubation for 24, 48, and 72 h at 37 °C, cells of *Blastocystis* (ST3) were counted using a hemocytometer. All experimented co-cultures were repetitively performed at least four times.

Assessment of LDH of the molecularly detected subtype (Kinetic UV method)[25]: To evaluate the damaging influence of the parasite on intestinal brush border, LDH was measured in *Blastocystis* sole infection (14 cases) and *Blastocystis* with *G. intestinalis* co-infection (14 cases). The LDH was determined by measuring the decrease in absorbance at 340 nm. The test is composed of 2 reagents; reagent 1 (R1 buffer) which includes 50 mmol/L phosphate buffer (pH 7.5), 3.0 mmol/L pyruvate, and 8.0 mmol/L sodium azide. Reagent 2 (R2 coenzyme) includes > 0.18 mmol/L NADH and sodium azide. Stool specimens were diluted and the ratio of direction decrease of sample to reagents ratio was 1: 50 at 37°C. Reading time was 1-3 min after zero adjustment against air reagent blank, yet the initial absorbance was read after 30 sec. The mean absorbance was recorded and the reference range was 1.00 - 2.5 AU[25].

Statistical methods: Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). Data was illustrated using frequencies (number of cases) and relative frequencies (percentages). For comparing categorical data, Chi-square (X^2) test was performed. Exact test was consumed instead when the expected frequency was less than 5 (Chan, 2003). Statistical significance is considered if P is less than 0.05.

Ethical considerations: Ethical approval was obtained from the Faculty of Medicine Kafr El-Sheikh University; Ethical Approval Committee number MKSU 50-11-23.

RESULTS

Microscopic examination and *Blastocystis* genotyping: Out of 167 stool samples, *Blastocystis* spp. was detected microscopically in 33 (19.7%) samples (Fig. 2A). Out of the total number examined 19.7% (33/167) were microscopically positive. Only 27/33 (81.9%) of microscopically positive samples proved positive by PCR. On genotyping of the PCR-positive samples, ST3 was the only detected genotype (Fig. 2B).

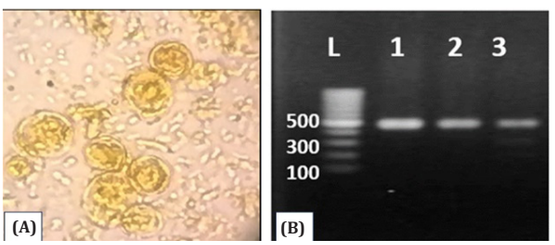

Fig. 2. (A) Clump of *Blastocystis* spp.; **(B)** Gel electrophoresis of amplified DNA products of the STS gene. **L:** 100 bp ladder. **Lanes 1-3:** positive samples of *Blastocystis* subtype 3 (526pb).

Among the molecularly positive *Blastocystis* samples, the highest prevalence was among the age group (20-29) years old (9 samples = 21.4%) followed by the age below 20 years old (8 samples; 19.5%). *Blastocystis* infections were neither detected in age groups 50-59 years old nor above 60 years old with no statistically significance difference between blastocystosis and age groups. Blastocystosis was more prevalent in females (16 samples; 20.3%) than in males (11 samples; 12.5%) with no statistical significance difference (Table 2). All molecularly positive patients were symptomatized with gastrointestinal manifestations such as diarrhea, abdominal pain, flatulence, and vomiting with no significant association observed (Table 2).

Stool-reducing sugar: All *Blastocystis*-positive stool samples were positive for reducing sugar (Fig. 3) with statistically significant differences compared to samples without parasitic infection (X^2= 0.00, P <0.001). Sugar (+) was detected in 80% of cases whereas the remaining 20% were (++) sugar.

Table 2. The rate of *Blastocystis* spp. in different age groups and in both sexes, the percentage of clinical data, and the seasonal variability of *Blastocystis* infection.

Variable	Category (No.)	PCR		Statistical analysis	
		Positive (n=27)	Negative (n=140)		
		Count (%)	Count (%)	X^2	*P* value
Age	< 20 (41)	8 (19.5)	33 (80.5)	0.017	0.207
	20-29 (42)	9 (21.4)	33 (78.5)		
	30-39 (40)	4 (10)	36 (90)		
	40-49 (24)	6 (25)	18 (75)		
	50-59 (11)	0 (0)	11 (100)		
	> 60 (9)	0 (0)	9 (100)		
Gender	Male (88)	11 (12.5)	77 (88.5)	0.067	0.174
	Female (79)	16 (20.3)	63 (79.7)		
Diarrhea	Yes (137)	26 (19.0)	111 (81.0)	0.021	0.051
	No (30)	1 (3.3)	29 (96.3)		
Abdominal pain	Yes (158)	27 (17.1)	131 (82.9)	0.169	0.357
	No (9)	0 (0)	9 (100)		
Flatulence	Yes (119)	20 (16.8)	99 (83.2)	0.176	0.724
	No (48)	7 (14.6)	41 (85.4)		
Vomiting	Yes (22)	2 (9.1)	20 (90.9)	0.175	0.535
	No (145)	25 (17.2)	120 (82.8)		

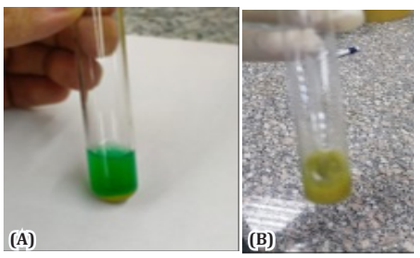

Fig. 3. Measurement of stool-reducing sugar. **(A)** Sugar content (+) using Benedict's test, whereas **(B)** showed (++) sugar.

Measurement of pH: All stool samples were acidic, pH with an average value of 5.5.

Evaluation of the extracted *Blastocystis*-genotype interactions with bacteria and fungi: In general, *Blastocystis* (ST3) positive samples showed an associated increase in the values of the enteric microbiota colony-forming unit per milliliter, predominantly for *Esch. coli*, compared with *Blastocystis*-negative controls. No other bacteria species were detected. Illustrative images of the *Esch. coli* colonies on MacConkey agar plates and positive tests for biochemical reactions are shown in Fig. (4A; B). When *Esch. coli* was co-cultivated with *Blastocystis* (ST3), the growth of parasite cells was inhibited, while bacteria attained high counts (P<0.03). Also, *C.* non-*albicans* exerted profound suppression of *Blastocystis* (ST3) cell counts and increased growth of fungi was detected compared to *Esch. coli* (P<0.001).

Enzymatic assessment of fecal LDH of the molecularly detected subtype: Overall, mean values of LDH in *Blastocystis* (ST3) sole infection and *Blastocystis* (ST3) - *G. intestinalis* co-infections were higher when compared with healthy controls (P=0.03). *Blastocystis* (ST3)-*G. intestinalis* co-infections exerted higher LDH values (P=0.02).

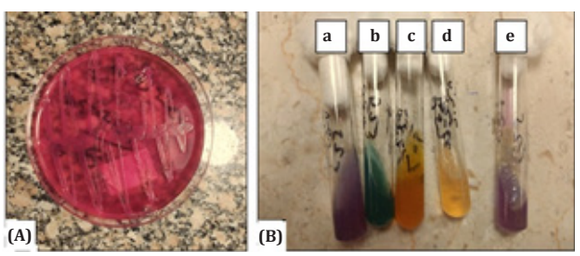

Fig. 4. Predominant growth of *Esch. coli* in *Blastocystis* positive fecal specimens **(A)** *Esch. coli* colonies on MacConkey agar plates. **(B)** Biochemical reactions specific to *Esch. coli*: **a.** Negative L-lysine; **b.** Negative citrate; **c.** Positive TSI (triple sugar iron agar) acid/acid, gas (positive); **d.** Negative urease; **e.** Positive indole (MIO),

DISCUSSION

Blastocystis is the utmost encountered protozoan in the stool of humans and several animals worldwide with no proven data on its pathogenic role yet[26]. In our study, *B. hominis* was detected in 19.7% and 16.2% by microscopic examination and PCR respectively. This percentage was in accordance with another study in Beni-Suef University Hospital, Egypt[27] in which the investigators documented a detection rate of 19.1%. However, our results differed from other studies in Egypt that reported higher detection rates of 41.7%[26], 35.7%[28], and 39%[29]. Conversely, a lower detection rate (8.1%) was reported by Salehi *et al*.[30] in Iran. Such differences may be due to the different geographical distribution of the study population and maybe also due to the use of different diagnostic techniques. There was no statistical significance between gender and blastocystosis which agrees with a prior study[23]. Age and gender variances in blastocystosis rates may be attributed to the accompanying risk factors and environmental circumstances rather than the individual's physiological properties[31]. The variable clinical presentations of the study population, included diarrhea, abdominal pain, flatulence, and vomiting with no significant differences. Likewise, no significant differences were recorded among various clinical symptoms of blastocystosis in a study conducted on immunocompromised Turkish patients[32].

All our patients with blastocystosis had chemically confirmed sugar malabsorption, acidic fecal pH, and classical semi-formed stools. In 2021, Basuony *et al*.[33] disclosed the hidden relationship between *B. hominis* and lactose intolerance. A previous study conducted by Ba´lint *et al*.[34] reported the existence of *B. hominis* in association with lactose intolerance in 6% of their cases where the manifestations of irritable bowel syndrome were recorded in 12%. Interestingly, a lactose-free diet was reported to reduce symptoms and parasite numbers in patients with blastocystosis[35]. Blastocystosis appeared to reduce the activity of lactase enzyme as a part of its immunological paradigm through the induction of TNF-α in the superficial epithelial cells of the lamina propria. Simultaneously, this was associated with increased apoptosis in the enteric epithelial cells and elevated BAX/BLC2 ratio[33]. *B. hominis* has been suggested to stimulate T cells, monocytes, macrophages, and natural killer cells through the upregulation of TNF-α, IFN-γ, and IL-12 that might be related to the absorption of *Blastocystis*-derived antigens *via* paracellular and transcellular pathways[9]. Mirza *et al*.[36] reported that both parasites and their lysates can damage the intestinal epithelial cells and degrade the tight junction proteins in the form of occludin and ZO1, thus increasing intestinal permeability. Parker *et al*.[37] demonstrated increased turnover of the epithelial cells coating the intestinal villi with an irreversible reduction in the length of the enteric villi as a pathological trait in blastocystosis.

In the current study, the predominant frequencies belonged to genotype 3. Similarly, a previous study conducted in Iran demonstrated the increased frequencies of genotype 3 (56.06%). However, they identified the existence of mixed infection of genotypes 3 and 4 in 42.88% of their cases[38]. Another study conducted in Makkah, Saudi Arabia identified high evidence of genotype 3 followed by genotype 1 and genotype 2. All three genotypes were associated with clinical symptoms[39]. In contrast to our results, a previous study predominantly defined genotype-1 in 65% followed by type-3 in 37%. Yakoob *et al*.[9] reported IBS in association with genotype 1 in 86% while in genotype 3 clinical manifestations were present in 47%. In 2019, El Saftawy *et al*.[40] proposed that the virulence of genotype 3 stands beyond the increased intensity of blastocystosis and the associated clinical manifestations. Two years later, another study conducted in Egypt demonstrated the close phylogenetic correlation between humans and animals in the isolates of genotype-3 hypothesizing the zoonotic transmission of the parasite and thus its epidemiological existence[29].

Clinically, acute diarrheal disease runs a self-limiting course. Consequently, the value of *B. hominis* as a cause in diarrheal individuals is questionable. In the current study, *Esch. coli* yielded positive overgrowth in samples infected by *B. hominis* (ST3), and interestingly *in vitro* co-culture showed the inhibitory effect of *Esch. coli* on *B. hominis* proliferation. Thus, stool cultures in patients with blastocystosis may not reveal the presence of pathogenic bacteria while exerting dysbiosis which can be significant in some clinical conditions, particularly in immunocompromised subjects[41]. This may indicate the effect of using empirical antibiotics prior to obtaining culture causing additional dysbiosis in the gut microbiota in patients with blastocystosis.

Our current results may be attributed to the competition between *Esch. coli* and *B. hominis* for the metabolism of lactic acid[24]. *Esch. coli* are gram-negative, facultative anaerobe and lactose fermenting bacteria that produce hydrogen sulfide. Park *et al*.[42] reported that *Esch. coli* synthesizes lactose operon for lactose transportation and α-1,2-fucosyltransferase for lactose solubility. Another explanation is that *Esch. coli* yields the production of endotoxins, for example, lipopolysaccharides which could be phagocytosed by the parasite causing its destruction. Hence, it is worth demonstrating the impact of gut commensals on the proliferation of the parasite. In contrast to our results, Lepczyńska and Dzika[24] demonstrated that the counts of *B. hominis* cells significantly increased

starting from day 2 of co-incubation after the addition of *Esch. coli in vitro*.

There is scarce research concerning the *Candida*-protozoa interactions, whereas the bulk of research demonstrated the interactions between the enteric bacteria and fungi. Our study manipulated the interface between *B. hominis* cells (ST3) and *C.* non-*albicans in vitro* to evaluate the susceptibility to blastocystosis in patients colonized by *C.* non-*albicans* as one of the natural enteric microbiota. In the current study, *C.* non-*albicans* prohibited the growth of *B. hominis* cells profoundly and this finding might be attributed to the competition between the *B. hominis* cells and *Candida* for nutrition and the colonized enteric space[43]. Regarding the triggered acidity of stool specimens infected with *B. hominis* (ST3), Sherrington *et al*.[44] demonstrated the high adaptability of *Candida* to any possible alterations in the enteric pH. Lepczyńska and Dzika[24] assumed that *Candida* inhibits the growth of pathogenic protozoa but to a small degree and that the toxins produced by *Candida* do not affect the proliferation of the protozoa despite being destructive to the bacteria and intestinal brush border.

In the current study, *B. hominis* exhibited an overall increase in the LDH levels either solely or when co-existing with giardiasis. Additionally, *Blastocystis*-*Giardia* coinfections revealed the highest values. Kumar *et al*.[17] determined that LDH increases with different forms of cellular damage involving apoptosis and necrosis. Basuony *et al*.[33] reported that blastocystosis triggers apoptosis via TNF-α and increases apoptotic biomarkers in the intestinal brush borders. Besides, *G. intestinalis* was reported to trigger cellular apoptosis through the production of reactive oxygen species, mitochondria-mediated pathways, and caspases[45]. Therefore, there were elevated levels of LDH in blastocystosis that were augmented by giardiasis.

To conclude, our data underlined the disregarded association between *B. hominis* and carbohydrate intolerance and fecal acidity. Infection with *B. hominis* was observed to be closely related to IBS-like manifestations with the dominatingly isolated genotype-3. However, this effect is always overlooked by the apparent association between the carbohydrate dietary component and the upsurge of the gastrointestinal manifestations without complete recognition of the associated pathogenic pattern of the parasite. It is suggested that *B. hominis* has an enhancing impact on the growth of *Esch. coli* and C. non-*albicans*; yet the same pathogens do not exert the same proliferative effect on *B. hominis*. Blastocystosis appeared to exert damaging effects on the cells of the intestinal brush border especially when co-existing with *G. intestinalis* thus increasing levels of LDH.

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