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Studying the Effect of Human–Lactobacillus Ruteri on the Viability of Cryptosporidium Parvum

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Abstract

The goal of this study was to see how well Lactobacillus ruteri probiotic bacteria might infect Cryptosporidium parvum viability at various bacterial dilutions and statuses. Cryptosporidium was obtained from stool samples of diarrheal patients and diagnosed using parasitological methods, followed by molecular methods and cell line cultivation (Human adenocarcinoma cell). The treatment of Cryptosporidium Parvum by adding Lactobacillus ruteri dilutions and status were then incubated at incubation periods (24, 48, and 72 hr), detection of Cryptosporidium count was carried out microscopically, and the parasite's viability was determined by 3-(4,5 dimethylthiazol-2-yl)-2- Diphenyltetrazolium bromide(Mtt) technique. Lactobacillus ruteri can treat Cryptosporidium parvum by suppressing activity and lowering the count with extremely significant differences, according to the findings.

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Keywords: Cryptosporidium; Lactobacillus ruteri; Probiotic bacteria; MTT test.

1. Introduction

Intestinal protozoan infections continue to be a serious public health issue in tropical and subtropical regions of the world, particularly in rural populations, the high incidence of intestinal parasites in rural parts of developing countries is due to a lack of health knowledge, poor sanitation, and limited water availability [1]. Infectious disease in animals, particularly livestock, raises agricultural expenses and diminishes productivity [2]. C.parvum is a parasitic infection that causes diarrhea in both animals and humans all over the world, children and immunocompromised people are disproportionately affected by the disease in humans, and early infections have been linked to reduced growth [3]. Despite this huge impact on public health, there are presently no fully effective disease medicines or vaccinations available, the acute disease causes four million disability-adjusted life years each year, but chronic disease impacts, such as development stunting, are expected to cause another 7.85 million disabilities [4]. The life cycle of C, parvum is intricate, with several different shapes.; the infective stage of C, parvum is the oocyst, which is an environmentally resilient form that spreads by faecal_ oral transmission, ingesting oocysts causes the parasite to produce 4 motile forms, sporozoites, which invade epithelial cells lining the host's intestinal tract, the parasite initiates schizogony or merogony, or asexual replication, and the parasite begins sexual replication; these sexual forms combine to generate new infective oocysts, which are subsequently spread into the environment via the host's stool, once inside the epithelial cell, before forming a sexual commitment and beginning sexual replication, known as gametogony, these sexual shapes combine to make new infective oocysts stage, which is then released into the environment through the stool of the host [5]. Many uses and effects of L. reuteri have been seen L. reuteri can form antimicrobial particles, such as organic acids, and reuterin. As a result of its antimicrobial action, L. reuteri can inhibit the accumulation of pathogenic microorganisms and rearrengment the beneficial microbiota component in the host. Second, L. reuteri can use the host immune part, for instance, some L. reuteri strains can decrease the formation of pro-inflammatory cytokines while making control T cell growth and function, beside, bearing the capacity to strengthen the intestinal wall (6) Most studies use the effect of the probiotic intervention in the dealing with acute infectious diarrhoea. The well-regulated clinical experiment has seen that probiotics such as L reuteri, L casei, and Bifidobacterium lactis can decrease the time of acute rotavirus diarrhea, studies in immunodeficient mice have also theorized that treatment with probiotics can decrease cryptosporidium spp. burden in the intestinal epithelium cells during cryptosporidiosis of L. reuteri may reduce the microbial conversion from the intestinal lumen to the other tissues places (7).

Nomenclature			
C. Parvum	Cryptosporidium Parvum	M	Molarity
L. ruteri	Lactobacillus ruteri	Rpm	revolutions per minute
MTT	3-(4,5 dimethylthiazol-2-yl)-2- Diphenyltetrazolium bromide	MRS	De Man, Rogosa and Sharpe
PCR	Polymerase Chain Reaction	CCIF	cell-culture immunofluorescent

2. Materials and methods

Diarrheal stool samples were collected from people ranging in age from less than one year to 50 years old who visited hospitals and health care institutions, one hundred samples of human stool were examined by wet mount to investigate the presence of Cryptosporidium parvum. Non-bloody and bloody diarrhea stool samples were chosen at random for the study, stained by the modified acid-fast stain.

2.1. Concentration Method for Cryptosporidium purification

Samples of stool containing oocysts were mixed in a sterile container with ten times its volume of water, this solution was passed through a three-layer sterile gas, filtered to remove excess material, then centrifuged at four hundred rpm for ten min, and the precipitate was mixed again with ten times its volume of water and centrifuged at 400 rpm for 5 min, five ml water was added to the resulting precipitate, the obtained solution was slowly added to 3 ml of 0.85 mM sucrose, the solution is centrifuged at 600 rpm for 10 min, the accumulated that containing parasite slowly transferred to another container, the solution washed two times with water, antibiotics are added and kept in distilled water at 4 °C for one week [7].

2.2. Cell line

At 37 °C in a 5% CO2 atmosphere, ATCC (CCL 244) human adenocoarcinoma cells (HCT-8) were grown to confluence in RPMI 1640 media supplemented with 4.5mM glucose, 1.5mM sodium bicarbonate, 100mM sodium pyruvate, 1 m HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, 1 percent l-glutamine, and 10% fetal bovine serum, the toxicity of supernatant and nutritious broth to a human colorectal carcinoma cell line initiated from an adult male[HCT-8] was tested using the tell titer 96t AQueous nonradioactive cell proliferation assay kit., 100 ml solution of 104 HCT-8 cells in supplementary RPMI 1640[8] was placed in a 96-well plate[8].

2.2.1. Procedure of Cultivation

The method of producing c.parvum infections in cell cultures using c.parvum oocysts and purification of the sporozoites, complete oocysts, combinations of integrity oocysts, excysted oocysts, free sporozoites, and purification sporozoites are used to supplement in vitro cultivation, even without special treatment, excystation will frequently happen in the cell culture medium quickly after feeding into host cells, although oocyst age, excystation efficiency, or sporozoite intact post purification may have a detrimental impact on complete infection, even without special treatment, excystation can happen in the cell culture medium quickly after feeding onto host cells. traditionally, oocysts were suspended in a buffered salt solution composed of 0.25 ٪ trypsin and 0.75 ٪ sodium taurocholate, the best to use 0.75 ٪ synthetic sodium taurocholate alone in DMEM (Dulbecco's Modified Eagle Medium) basal medium unless the oocyst origin is old (>6 months), incubating at 37 °C for 10 to 15 min (as a trigger) or 45 to 60 min (for total sporozoite generation), the bleach has been wiped away (or neutralized with 0.1 ٪ sodium thiosulfate), and the oocysts are inoculated onto the majority of oocyst making handled and kept in the presence of 2.5 ٪ K2Cr2O7 if aseptic procedures are employed during the post-purification processing of the samples, they rarely contain live microbial contamination [9].

2.2.2. Ethical Approval

Consent from the patient's parents was taken.

3. Preparation of Probiotic Bacteria

3.1. L. reuteri preparation

L. reuteri (ATCC1655) was obtained from AL-Kufa University and cultivated in MRS-agar culture medium at 37 ° C for 18 hours under microaerophilic conditions. 12 percent was cultivated in nutrient medium with 5% glycerol for storage at -70 ° C after the formation of tiny or milky colonies and full growth. It should be highlighted that confirmatory and diagnostic tests such as warm gammosis (for long bacilli, gram-positive, without spores), Albert staining, and others should be used at all stages to ensure that the bacteria are lactobacilli (for bacilli with grains). For a comprehensive diagnosis, the catalase test was utilized [10].

4. Treatment

Probiotic bacteria (L. ruteri) were used to treat Cryptosporidium parvum in dilutions (1:1, 1:2, and 1:5), while duplicate RPMI control wells received an additional 100 ml of supplemented RPMI 1640. 100 mL of diluted water was poured into duplicate test wells (1:1, 1:2, 1:5 in supplemented RPMI 1640). The efficacy of bacterial MRS-Agar culture media, a topical solution containing L. ruteri bacteria in three dilutions 1:1,1:2, and 1:5 on human adenocarcinoma cells were assessed. To the wells of the plate, 100 l of culture medium containing (105× 1) cells were added, followed by 100µl of bacterial MRS culture media [11].

4.1. Enumeration of oocys

Before and after the treatment of parasite, oocysts of C. parvum were counted at three time intervals of 24 hr, 48 hr, and 72 hr, the clering steps of this procedure were not necessary because nearly pure de ionized water was used to perform fluorescent marking and complete enumeration as explaining in (EPA Method 1622) which describes as a procedures for analysis of Cryptosporidium in water samples, by concentration, immunomagnetic separation, which needed clearing, immunomagnetic separation of the oocysts from the substance distribution, and then counted of the aim microorganisms based on the finding of the immunofluorescence test, 4',6-diamidino-2-phenylindole (DAPI) staining results, and differential water samples were vacuum filtered onto a membrane filter with a pore size of 0.22 mm, after which the filter was treated with 330 mL of immunofluoresence antibody suspension, and oocyst retuned were detected (enumeration by comparison to hemacytometer counts of the oocyst stock solution), the results obtained using this oocyst counting method varied from 79 ٪ to 94 ٪using epifluorescence microscopy at 400x using a Nikon Microscope equipped with mercury vapor light, digital camera, and picture analysis system [12].

4.2. MTT Protocol for Viability of Cryptosporidium parvum

After an extra hour of incubation at 37°C, 5% CO2, [mean absorbance of test wells–mean absorbance of RPMI control wells] /mean absorbance of RPMI control wells] was used to enumerate the percent availability of c.parvum.* Test treatments were detected non-active in wells with more than 85percent active cells, in the cell culture immunofluorescent experiment, the large amount of each test and control that was deemed non-toxic to HCT-8 cell one layers (which was a 1:4 dilution) was studied, each 1:4 diluted test supernatant had a pH of 5.4, whereas the 1:5 diluted MRS broths control had a pH of 6.3 [13].

5. Results

One hundred samples from patients who suffered from diarrhea for Cryptosporidium detection, beside 20 samples for healthy control, the results showed, that positive samples were (35 %).

Table 1 the mean and standard error of cell supernatant of L. ruteri treated Cryptosporidium with different times from incubation (24 hour, 48 hour, and 72 hour), the parasite microscopically counted, the results show the supernatant status of L. ruteri can decrease the count of Cryptosporidium with high significant differences, 24hr (Mean±S.E=5033.3±33.33), 48 hr (Mean±S.E= 2116.7±60.09) and 72 hr (Mean±S.E=306.67±6.67) respectively in comparison with the control group.

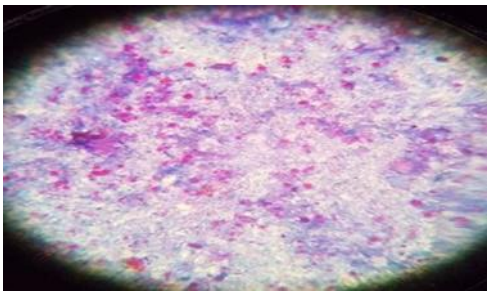

Fig 1. Cryptosporidium by y modified ziehl-Neelsen Stain (100x)

Table 1 Effect of L. ruteri supernatant on the count of Cryptosporidium parvum by using microscopic technique.

Supernatant bacteria	Cell count (Mean+S.E)	ANOVA test (p-value)
Control	14200±152.75	P=0.001 Sig *(P value ≤ 0.05)
24 hr.	5033.3±33.33	
48 hr.	2116.7±60.09	
72 hr.	306.67±6.67	

Pellet L. ruteri in a treatment of Cryptosporidium with different incubation periods (24 hour, 48 hour, and 72 hour) and count effecting determined in the Table 2, showed the pellet L. ruteri decreased the count of Cryptosporidium with high significant differences, in 24hr (Mean±S.E=5100±57.74), 48 hr(Mean±S.E= 1033.3±33.33) and 72 hr (Mean±S.E=240±5.77) respectively, also with high significant value at p≤0.001 in comparison with control wells.

Using a dead L. acidophilus in the treatment of Cryptosporidium parvum and determination count affected (Table 3) with declined the mean and standard error of with different times from incubation (24 hour, 48 hour, and 72 hour), the results showed the dead L. ruteri could decrease the count of Cryptosporidium parvum without significant differences in comparison with control group p=0.21

(Mean±S.E=16200±152.75), also with no significant value with different incubation period, it was 24hr (Mean±S.E=7766.7±3333.83), after 48 hr (Mean±S.E=8433.3±233.33) and after 72 hr Mean±S.E=4926.7±37.12).

Table 2 Effect of L. ruteri pellet on the count of Cryptosporidium parvum using microscopic technique

pellet bacteria	Cell count (Mean+S.E)	ANOVA test (p-value)
Control	14033±33.33	P=0.001 Sig *(P value ≤ 0.05)
24 hr.	5100±57.74	
48 hr.	1033.3±33.33	
72 hr.	240±5.77	

Table 3 Effect of dead L. ruteri on the count of Cryptosporidium parvum using microscopic technique

Dead bacteria	Cell count (Mean+S.E)	ANOVA test (p-value)
Control	16200±152.75	P=0.21 Sig *(P value ≤ 0.05)
24 hr.	7766.7±3333.83	
48 hr.	8433.3±233.33	
72 hr.	4926.7±37.12	

Table 4 showed the mean and standard error of viability for using different dilutions of L. ruteri (1:1, 1: 2, and 1:5) on the viability of Cryptosporidium by using of MTT technique. With high significant values for all dilutions (0.001) with a P value ≤0.05, it was (Mean±S.E=11.01±0.29), (Mean±S.E=15.57±0.35) and Mean±S.E=30.34±1.80) respectively, also the results showed there was a high significant value compared with control group Mean±S.E=99.63±0.32).

Table 4 Illustrated the effect of L. ruteri dilutions (1:1, 1: 2, and 1:5) on the viability of Cryptosporidium parvum compared with control by using of MTT technique

Control &dilution	Viability (%) Mean+S.E	T-test (p-value)
Control	99.63±0.32	P=0.001
1:1	11.01±0.29	High Sig
1:2	15.57±0.35	*(P value ≤ 0.05)
1:5	30.34±1.80	

The mean and standard error of using supernatant L. ruteri in treatment of Cryptosporidium parvum with different times from incubation (24 hr, 48 hr, and 72 hr) by using MTT technique that showed in the Table 5, the results illustrated the supernatant L. ruteri can inhibit the activity of Cryptosporidium parvum with high significant differences at P value ≤ 0.001 after 24 hr,48 hr and 72 hr (Mean±S.E=42.08±0.64, 32.59±1.01and 12.65±0.41) respectively, also with high significant value as compared with control in very high significant value also.

Table 5 Effect of L. ruteri supernatant on the viability of Cryptosporidium according to different times of incubation time by MTT technique

Supernatant	Percentage (Mean+S.E)	T-test (p-value)
Control	99.63±0.32	P=0.001 High Sig *(P value ≤ 0.05)
24 hr.	42.08±0.64	
48 hr.	32.59±1.01	
72 hr.	12.65±0.41	

Pellet L. acidophilus in the Table 6 used in the treatment Cryptosporidium parvum and determination of the activity by MTT technique, the mean and standard error (Mean±S.E=52.53±0.54, 31.83±0.38 and 9.17±0.17) (24 hr, 48 hr, and 72 hr) respectively, results showed the pellet status of L. ruteri can inhibit the Cryptosporidium parvum with high significant value at P value ≤ 0.001, comparison with control in (Mean±S.E=99.63±0.32).

Table 6: different times of incubation of L. ruteri pellet and its effects on the viability of Cryptosporidium parvum by using of MTT technique

pellet bacteria	Percentage (Mean+S.E)	ANOVA test (p-value)
Control	99.63±0.32	P=0.001
24 hr.	52.53±0.54	High Sig
48 hr.	31.83±0.38	*(P value ≤ 0.05)
72 hr.	9.17±0.17	

Using a dead L. ruteri in the treatment of Cryptosporidium parvum and determination of its viability was shown in Table 7 declined the mean and standard error of different times of incubation (24 hour, 48 hour, and 72 hour). The results showed that dead L. acidophilus could inhibit Cryptosporidium with highly significant differences in comparison with the control group (Mean±S.E=99.63±0.32), also high significant value with different incubation period, 24hr (Mean±S.E=62.45±0.75), 48 hr (Mean±S.E=49.57±0.55) and 72 hr Mean±S.E=35.29±1.09).

Table 7 The effect of dead L. ruteri on the viability of Cryptosporidium according to different times of incubation time by using of MTT technique

dead bacteria	Percentage (Mean+S.E)	ANOVA test (p-value)
Control	99.63±0.32	P=0.001
24 hr.	62.45±0.75	High Sig
48 hr.	49.57±0.55	*(P value ≤ 0.05)
72 hr.	35.29±1.09	

6. Discussion

The main problem with cryptosporidiosis is that there are no effective treatment or prevention options for this important zoonotic disease, the most well-documented benefit of probiotic intervention is the treatment of sever diarrheal infection. The best controlled clinical experiment has shown that bacteria like L reuteri can reduce the duration of acute diarrhea (14). The treatment with probiotics bacteria has also been shown to reduce Cryptosporidium parvum burden in the intestinal epithelium cells during Cryptosporidium parvum infection in immune-deficient mice [15]. The current study's findings were in agreement with Qi C. in china 2021, who found that non-diluted (concentrated), (1:2), and (1:5) dilutions of L. ruteri suspension significantly decreased the percentage of active C. parvum oocysts when compared with controls [16]. There were no marked differences in the percent decrease in oocyst activity between the L. acidophilus and L. reuteri supernatants, probiotic bacteria makes increased the immune system and compete with infectious microorganism indirectly, prompting them to escape from the gut [17]. Probiotics alone or in combination with Nita-zoxanide may be beneficial because the second does not reject autoinfection while the making does so by rejecting cysts from penetrating epithelial cells [18]. In addition, there was an agreement with Mathipa study in 2017 in south Africa that discussed the logic of treating Cryptosporidium in vivo, in immunosuppressed mice, and the efficacy of L. reuteri as a probiotic for the inhibition of C. parvum infection was evaluated. , following daily L. reuteri inoculation (108 cfu/day) for 10 days, mice were challenged with 6.5 x 106 C. parvum oocysts and inoculated L. reuteri throughout the research, mice that were not supplemented developed long-term cryptosporidium infection and inhibited large amounts of oocysts in their stool, but mice who were supplemented with L. reuteri and challenged with C. parvum had parasite accumulation inhibited from the gut epithelium [19]. The current study found that L. ruteri in three states (supernatant, pellet, and high spirits) inhibited Cryptosporidium viability and microscopic number in high spirits, which could be because L. reuteri forms wide types of antimicrobial reagents called reuterin during fermentation, which inhibits Cryptosporidium except in the case of bacteria in their dead form, which could be due to the low bacterial ability to produce Cryptosporidium parvum killer substances and this agreed with Al-Alousi of Iraq in 2018 [20]. The creation of targeted probiotic editing methods to decrease the severity of cryptosporidium infection may be made possible by identifying specific pathways that affect pathogeninc virulence in response to probiotics or food [21]. Methods to detect metabolite changes will be necessary to supplement the knowledge provided from 16S amplicon sequencing, finally, the noticed side toward facultative anaerobes suggests that pathogenic alterations in the human and mice intestines in reaction to enteric infections are similar, boosting the use of the rodent cryptosporidiosis model [22].

The treatment of acute infectious diarrhoea is the most well-documented benefit of probiotic bacteria intervention; probiotics such as L. rhamnosus GG, L. reuteri, L. casei, and B. lactis have been shown to decrease the duration of severe rotavirus diarrhea in well-controlled clinical trials, in immunocompromised animals, probiotic bacteria treatment has been found to reduce Cryptosporidium parvum burden in the intestinal epithelium during cryptosporidium infection, in immunocompetent patients, cryptosporidiosis usually clears up in two to four weeks. The symptoms had improved within 10 days of starting probiotic medication, diarrhoea, gastrointestinal pain, and school absences were all reduced as a result of chronic diarrhea, this is the first time probiotics have been used to treat c. parvum in human, according to the

present study, with rapid clinical improvement and infection eradication, this non-toxic treatment has the potential to help people [26]. In the disappearance of uses of drugs to regulate cryptosporidiosis, a search for remodeling treatments is needed in most cases, while, there is no information on how perturbation of the probiotic, whether introduced by diet, probiotics, antiprotozoa, or prebiotics, affects entero pathogens, some think cryptosporidium , knowing specific procedure affecting pathogen pathogenicity in response to the diet may facilitate the development of aimed microbiota editing measures to mitigate the acute case of cryptosporidiosis [23].

7. Conclusions

Count of C. Parvum cultivated in cell line (Human adenocarcinoma cell) decreased with high significant differences when treated by L. ruteri in dilutions of (1:1, 1:2, and 1:3) by microscopic counting, MTT method, L. ruteri in dilutions of (1:1, 1:2, and 1:3) and through incubation period of (24, 48, 72) hr. had the ability to inhibit the intestinal parasites C. Parvum with high significant differences, different status (supernatant, pellet, and dead) of L. ruteri with three incubated period (24, 48, 72) hr. had the capacity to inhibit C. Parvum with high significant differences.

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