ISSN: 0067-2904

The Influence of *Entamoeba histolytica* Against Some Gut Microbiota in Children with Acute Amoebic

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Received: 31/1/2022 Accepted: 21/4/2022 Published: 30/1/2023

Abstract

This study aimed to evaluate the relationship between the microbiota number of some common bacterial species in children's gut with *Entamoeba histolytica* infection. To achieve that,49 samples of stool were collected, 29 from children with acute amebiasis and 20 from healthy children, children's ages ranging from 1 month to 12 years from both genders. Counting three types of bacteria *Lactobacillus* spp., *Bifidobacterium* spp. and *Escherichia coli*, using the standard curve obtained from Real-Time PCR was accomplished. The standard curve was made from decimal serial dilution of samples containing an unknown number of bacteria. The results showed that *E. histolytica* infection creates considerable changes in the intestinal microbiota numbers. *Lactobacillus* spp., *Escherichia coli* and *Bifidobacterium* spp. numbers were affected. The mean values of Lactobacillus spp. and Escherichia coli in the patient's group (children with amebiasis) were increased compared to the control group (healthy children). The mean value in the patient groups were (1947.4) and (430657.9), while in the control groups were (1400.16) and (193927.7) for *Lactobacillus* spp. and *Escherichia coli*, respectively. Whereas the mean value of *Bifidobacterium* spp. showed a significant decrease in the patient group (103.875) compared with the control group (166.75). In conclusion, this study show alteration in predominant gut bacteria in *E. histolytica* infected children.

Keywords: *Entamoeba histolytica*, amoebiasis, microbiota, real-time PCR, children.

تأثير *Entamoeba histolytica* في نمو بعض ميكروبات الأمعاء لدى الاطفال المصابين بالاميبا الحادة

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الخلاصة

هدفت هذه الدراسة إلى تقييم العلاقة بين عدد الميكروبات للأنواع البكتيرية الشائعة في أمعاء الأطفال المصابين بـ *E. histolytica*، لتحقيق ذلك.تم جمع 49 عينة من البراز ، 29 عينة من الأطفال المصابين بداء الزخارالاميبي الحاد و 20 عينة من الأطفال الأصحاء،تتراوح أعمار الأطفال بين شهر واحد و12 عاما من كلا الجنسين. تم احتساب ثلاثة أنواع من البكتيريا.*Lactobacillus* spp، *Bifidobacterium* spp. و *Escherichia coli*باستخدام qPCR بناءً على المنحنى القياسي الذي تم إنشاؤه من التخفيف العشري

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التسلسلي للعينات التي تحتوي على عدد غير معروف من البكتيريا.أظهرت نتيجة تفاعل البلمرة المتسلسل في الوقت الحقيقي (Real−Time PCR) أن الاصابة بـ *E. histolytica* تسبب تغيرات كبيرة في أعداد الجراثيم المعوية،فقد تأثر عدد بكتريا .*Lactobacillus* spp و *Escherichia coli*، إذ كان هنالك ارتفاع ملحوظ في قيمة المتوسط الحسابي في مجموعة المرضى مقارنة بمجموعة الأصحاء. وبلغت قيمة المتوسط الحسابي لمجموعة المرضى (1947.4) و (430657.9)، و لمجموعة الأصحاء (1400.16) و(193927.7) لبكتريا .*Lactobacillus* spp و *Escherichia coli*على التوالي.في حين انخفضت قيمة المتوسط الحسابي لعدد بكتريا .*Bifidobacterium* spp في مجموعة المرضى (103.875) مقارنة مع مجموعة الأصحاء (166.75). تم الاستلال من هذه الدراسة على وجود تغيير في بكتيريا الأمعاء السائدة في الأطفال المصابين بالزحار الأميبي الحاد .

1. Introduction

Entamoeba histolytica is an intestinal protozoan parasite, and the causative agent of invasive amoebiasis remains a significant cause of morbidity and mortality, especially among children. They transmit through the consumption of fecally contaminated food and water [1; 2]. Amebiasis can be asymptomatic or cause colitis and extra-intestinal disease, particularly liver abscess [3]. On the other hand, the human gastrointestinal tract is a highly complex ecosystem with an extensive microbial community (Gut Microbiota). The microbiota is the ecological community of commensal microbes, which reside normally inside the human body, while the microbiome is the microbiota's whole [4]. These organisms form a symbiotic relationship that influences reach the entire host organism [5]. At birth, the entire intestinal tract of an infant is sterile, but it is colonized quickly by microbes. These microbes are highly variable in early infancy [6]. *Escherichia coli* is gram -negative, facultatively anaerobic, non-sporulating, rod-shaped, and a member of the Enterobacteriaceae family. It is one of the earliest gut colonizers, and most popular in the intestinal tract [7; 8]. Within hours after birth, it colonizes the gastrointestinal system of human babies and establishes a symbiotic connection with its human host, then coexists harmoniously as a commensal for decades [7]. *Lactobacillus* spp. are gram-positive, rod-shaped bacteria, mostly non-motile, catalase-negative, non-spore-forming, anaerobes, and members of the lactic acid bacterial group [9]**.** This species constitutes an essential part of a normal human bacterial flora commonly found in the gastrointestinal tract and mouth [9; 10]. The initial microbial inoculum is delivered to an infant through exposure to a birth canal in vaginally delivered infants and orally to infants fed mother's milk. Thus, the pioneer community in the gut is a bloom of *Lactobacilli* from the mother's vaginal tract and breast milk [11]. *Bifidobacteria* spp. are gram-positive, strictly anaerobic, and polymorphic rod-shaped belonging to the Actinobacteria phylum that comprises over 45 species [12]. After depleted oxygen by facultative anaerobes, *Bifidobacteria* populations are the most common genera in a healthy infant gut [13]. They predominate in newborn gut microbiota, particularly in infants with breastfeeding, where they can account for as much as 90% of a total bacterium in this environment [13; 14].

During the first three years of life, the development of the gut microbiome is affected by maternal and neonatal exposures, including mode of delivery, antibiotic exposure, hygiene, and feeding pattern (breast versus formula feeding) [15]. This gut microbiota is integrally linked to long-term child health and plays a role in metabolic, nutritional, and immunological processes [16]. It actually contributes to human physiology through food digestion and vitamin B and K production [17]. When parasites enter a body via an oral-fecal pathway, they interact directly with an intestine's commensal microbiota, forming a complex interacting system. These parasites can significantly impact the gut microbiota balance, influencing microbiota protection and colonizing this organ. *E. histolytica* can interact with the host

bacterial microbiota and benefit from there, as colonic microbiota breaks down complex carbohydrates into glycans that can serve as a nutrient source for *E. histolytica*. Due to only bacteria with the correct recognition molecules ingested by a parasite, this interaction is very selective [18]. At the same time, bacterial microbiota can impede gut colonization by parasites and resistance to infections of parasitic at mucosal sites or prevent their persistence in case of colonization [4;19;20]. The differences in interactions between parasites and bacteria may indicate variations in the clinical significance of *E. histolytica* [21]. Studies have noted that infection with *E. histolytica* directly affects microbiota and leads to dysbiosis characterized by significantly fewer *Eubacterium* and *Lactobacillus* while rising species of *Bifidobacterium* in samples of the stool as compared with healthy controls [22]. Iebba and his team described the *E. histolytica* significant alterations of predominant gut bacteria and depletion of some genera (e.g., *Bacteroide*s, *Eubacterium*, *Lactobacillus*) in the gut of infected patients [19]. This study aimed to assess the effects of *Entamoeba histolytica* infection on the count of *Lactobacillus* spp., *Bifidobacterium* spp. and *Escherichia coli* microbiota.

2. Materials and Methods

2. 1 Collection of specimens

A total of 78 stool specimens were collected from diarrheal children from hospitals in Baghdad city, from early September 2019 to the end of February 2020, and 20 samples from apparently healthy children Children's ages are ranged between 1month to 12 years from both genders, male and female.

2. 2 Extracting of DNA

DNA was extracted from patients and control stool samples, using QIAamp® Fast DNA Stool Mini Extraction Kit Qiagen / Germany, according to the company protocol.

2. 3 The primers

The primers used for the detection of normal flora *Lactobacillus* spp., *Bifidobacterium* spp. and *Escherichia coli* based on amplifying16S ribosomal RNA gene (16S rRNA) sequences of each bacterium utilizing qPCR primers were supplied by Macrogen Company/ Korea (Table1).

Table 1: The primers used in this study

Primer	Sequence	Annealing Temperature	PCR Product	Reference
Lac F	F 5`-AGCAGT AGGGAATCT TCCA-3`	60C°	345 bp	[23]
Lac R	R 5`-ATTYCACCGCTACACATG-3`			
Bif F	F 5`GCGTGCTTAACACATGCAAGTC-3`		125 bp	
Bif R	R 5`-CACCCGTTTCCAGGAGCTATT-3`			
Esc F	F 5`AGAAGCTTGCTCTTTGCTGA-3`		120 bp	
Esc R	R 5`-CTTTGGTCTTGCGACGTTAT-3`			

2. 4 Real-Time PCR

The quantitative PCR method which used in this study to detect *Lactobacillus* spp., *Bifidobacterium* spp., and *Escherichia coli* and their quantities based on amplifying 16S ribosomal RNA gene (16S rRNA) sequences of each bacterium by their specific primers after optimization of the primers annealing temperature. The PCR reactions were achieved in final volumes of 10µl; 5µl of SYBR Green PCR Master Mix (GoTaq® qPCR Master Mix, Promega, USA),0.5 µl of each primer and 2 µl of fecal DNA [23; 24;25]. Mic qPCR thermal

cycler (Bio Molecular System, Australia) was programmed as shown in Table 2. The results obtained by the software provided with the instrument.

After qPCR amplification, one sample with a high concentration from each bacterium amplifies by multiplex PCR 16S rRNA gene sequences, by specific primers [23]. The presence of amplification confirmed by agarose gel electrophoresis. Then, DNA concentration was detected using Quantus Fluorometer, by adding 1µl of DNA, 199µl of diluted Quant-fluor Dye. The mixture incubated at room temperature for 5min, then the values of DNA concentration was detected. The copy number was estimated according to the equation below [26], using templates of DNA to create standard curves for qPCR.

$m=[nbp]\ [1.096*10^{-21}\ g/bp]$

$m= g*10^{9}ng$

Copy Number = concentration/m

where: n= size of DNA (base pair) and m= mass of nucleotide

Table 2: Real-time PCR programfor16S rRNA gene detection of the studied bacteria

Steps	Temperature	Time (M:S)	No. of Cycle
Initial Denaturation	95ºC	05:00	1
Denaturation	95ºC	00:30	40
Annealing	60ºC	00:30 acquiring on Green	
Extension	72ºC	00:45	

2. 5 Construction of standard curves

The samples of known template copy numbers are serially diluted then amplified using a real-time qPCR to generate a standard curve (standard curve taken through threshold cycle (CT) versus linear regression of log concentration (copy/µl)). The standard curves, performed by plotting CT values against the logarithm of original template copy numbers, were designed for *Lactobacillus* spp., *Bifidobacterium* spp., and *Escherichia coli*. The concentration (copy/µl) of the sample was calculated using a standard curve produced by linear regression of plotted points. Lastly, the bacterial numbers of samples were determined.

2. 6 Statistical analysis

Data analyses were achieved with Excel application for Windows (version 2013) and SPSS (Statistical Package for the Social Sciences), version 25. Quantitative data presented as mean and standard error (Mean ±SE). Significant differences between mean were assessed by Mann-Whitney p-value. P-value ≤0.05 was considered statistically significant, and P-value ≤ 0.01 considered High statistically significant [27].

3. Results and Discussion

3. 1 Description of the study samples

In this study, 49 samples of the stool were analyzed and distributed; 29 were from children infected with *E. histolytica* (acute amebiasis) and 20 from apparently healthy children as a control. They were diagnosed microscopy at the Al- Al-Kadhimiya Hospital for Children and Central Teaching Hospital of Pediatrics in the medical city. Children's ages ranged between 1 month to 12 years from both genders, male and female (Table 3).

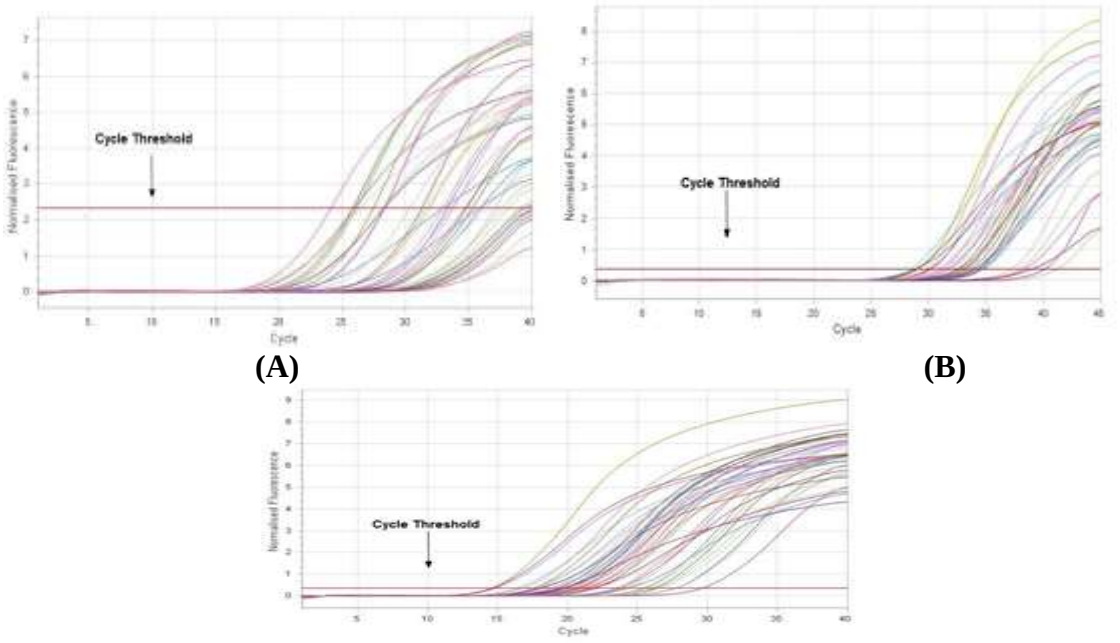
Table 3: Demographic Characteristic to Distribution of the study samples

The group		Patients (n=29) n (%)	Controls (n=20) n (%)
Age Range		1 month to 12 year	6 month to 12year
The gender	**Male**	19(65.5%)	8 (40.0%)
	Female	10(34.5%)	12 (60.0%)
Age Groups: <1-3 y		13(44.8%)	10 (50%)
4-6 y		11(37.9%)	6 (30%)
7-9y		3 (10.4%)	2 (10%)
10-12y		2 (6.9%)	2 (10%)

3. 2 Detection of intestinal microbiota

The DNA was extracted from 29 child stool samples with acute amoebic infection (examined by PCR) and 20 control samples healthy children), then subjected to real-time PCR analysis to assess the predominant gut flora that included: *Lactobacillus* spp., *Bifidobacterium* spp. and *E. coli* to the intention of qualification16S ribosomal RNA gene (16S rRNA) of each bacterium by their specific primers. The amplification specificity was checked by standard curve analysis, the results are shown below in Figures (1, A, B and C).

Table 4 illustrated the results obtained from real-time PCR, the table, showing that of the 29 children (with acute amoebic infection) 20 (69%) were harbor *Lactobacillus* spp.,16(55%) *Bifidobacterium* spp., and 26 (90%) *E. coli*. While, of 20 healthy children 12 (60%) were found to harbor *Lactobacillus* spp., 16 (80%) *Bifidobacterium* spp. and18 (90%) *E. coli* It is possible to use real-time PCR to measure bacterial copy numbers in two ways, absolute and relative quantification. Absolute quantification was used in this study. One of the most accurate ways to estimate the number of bacterial cells in a sample is to use the standard curve approach, as described in the current and other previous studies. This agrees with a local study used to determine the number of bacteria [28], In which this method is fast, inexpensive and without any problems for safety. Furthermore, the methods are easy to implement and may monitor the bacterial copy number in a time-course study or a recombinant bioprocess [29].



(A) **(B)**

(C)

Figure 1: A- The presence of *Lactobacillus* spp. in patients and healthy children.
B- The presence of *Bifidobacterium* spp. in patients and healthy children.
C- The presence of *Escherichia coli* in patients and healthy children.

Table 4- Detection of the cycle threshold (CT) by RT-PCR in patient and control groups.

The groups	*Lactobacillus* Positive Ct	*Bifidobacterium* Positive Ct	*E. coli* Positive Ct
The patients n=29	20(69%)	16(55%)	26(90%)
The controls n=20	12(60%)	16(80%)	18(90%)
The total n=49	32(65%)	32(65%)	44(90%)

3. 3 Estimation of the bacterial numbers by the standard curve

The amplification specificity was checked by standard curve analysis, the results are shown previously in Figures (1-A, B, and C). The standard curves of patients and control samples contain 16S rRNA gene. The cycle threshold (CT) values are shown in the order listed below, from highest to lowest concentration, for *Lactobacillus* spp.: 7.00, 10.71, 14.81, 18.60, 22.73, 26.28, 30.10, 33.13 (Figure 2A), for *Bifidobacterium* spp.: 7.68, 11.44, 15.56, 19.37, 23.49, 27.05, 30.88, 33.90 (Figure 2B), and for *Escherichia coli*, 8.29, 12.10, 16.22, 20.04, 24.15, 27.64, 31.56, 34.55 (Figure 2C). Every standard curve gives the equation that uses to calculate the number of bacteria depending on threshold cycle (CT) values, which a device reads. The standard curve experiment's results and figures were calculated using a stool sample (200mg) for DNA extraction. Figure 3 (A, B, and C) represent the copy No. of each bacterium for patients and control groups.

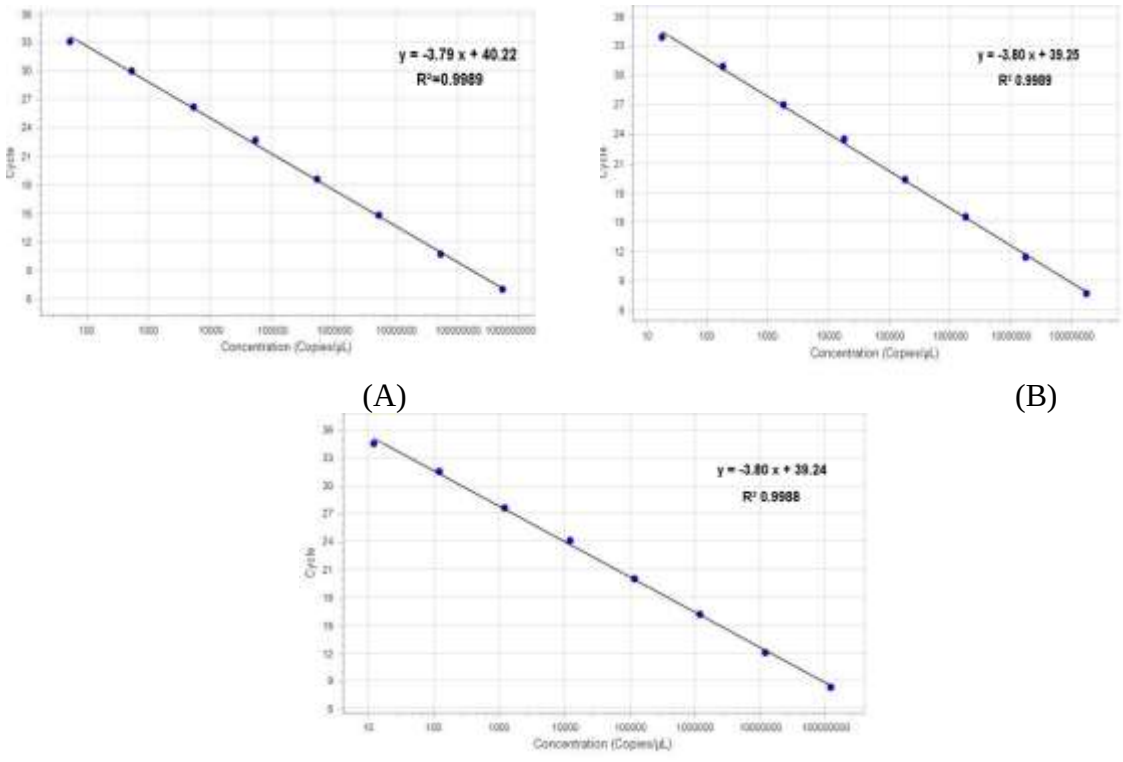


Figure 2: A-The construct of *Lactobacillus* spp**.** Standard curve of real-time PCR. The logarithm of their known beginning copy number (n) was plotted against s determined CT values for each set**. B -** The construct of *Bifidobacterium* spp**.** Standard curve of real-time PCR. The logarithm of their known beginning copy number (n) was plotted against s determined CT values for each set**. C-** The construct of *Escherichia coli* standard curve of real-time PCR. The logarithm of their known beginning copy number (n) was plotted against s determined CT values for each set**.**

3. 4 The relationship between intestinal microbiota numbers and *Entamoeba histolytica* infection

The results showed a difference in the mean values of *Lactobacillus* bacteria between the two samples groups (patients and control). There was high mean value for *Lactobacillus* spp. in the patients' group compared to the controls' group. The mean values representing the concentrations (copy No.) of bacteria for each group, were 1947.4 and 1400.16 respectively (Table 5). Burgess and his group's [18] reported that the parasitic survival was reduced by 71% when *Lactobacillus casei* co-cultured with *Entamoeba histolytica*, demonstrating that the gut microbiota can influence the progression of parasitic infection. That may clarify this higher level of *Lactobacillus* spp. in patients compared with healthy children that resulted in this study. While a study conducted in Delhi, India, [30] appearances *E. histolytica* selectively phagocytosed certain bacteria, including the *Lactobacillus* family, which benefit needed for gut health, cause dysbiosis of gut bacteria. Also, Iyer and others [31] found that the class of Bacilli (a primary intestinal bacteria class being phagocytized by this parasite), members of order *Lactobacillales* are taken up by *E. histolytica*.

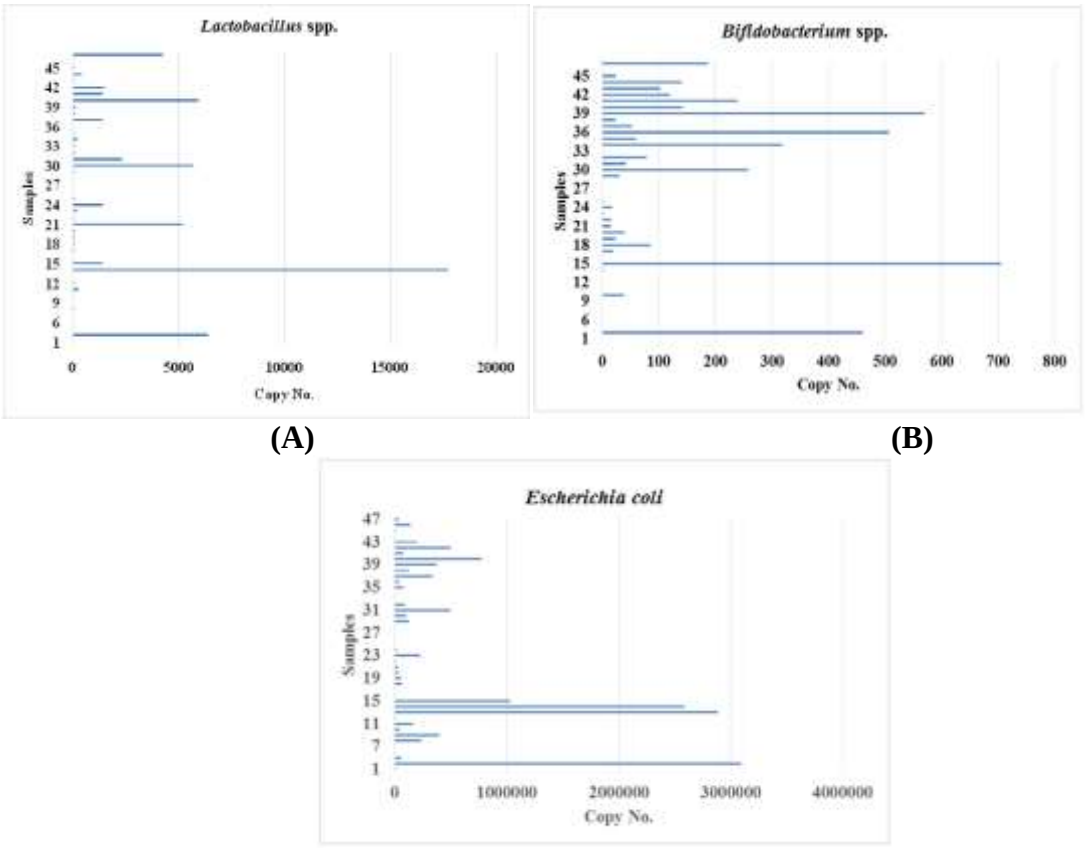


Figure 3: A-The concentrations (copy No.) of *Lactobacillus* spp. bacteria in patients and control groups, B-The concentrations (copy No.) of *Bifidobacterium* spp. bacteria in patients and control groups, C- The concentrations **(**copy No.) of *Escherichia coli* bacteria in patients and control groups.

The increase in *Lactobacillus* spp. numbers could be due to the possibility of a link between these bacteria and resistance to ameba infection [18]. Perhaps another explanation is that *E. histolytica* causes significant changes in the structure of the human host's gut microbiota populations [5]. At the same time, gut microbiota represents a factor that may strongly interfere with the pathophysiology of parasitic infections [32].

As for, *Bifidobacterium* bacteria the results reached there is a significant difference in the number of *Bifidobacterium* bacteria in the patient's group as compared with the control group, means values were 103.875 and 166.75 respectively (Table 5), which explains the low *Bifidobacterium* bacteria mean in the patients.

Table 5: The concentrations of intestinal microbiota in patients and control groups by real-time PCR

Concentrations (Copy No.) of the bacteria						
Intestinal microbiota	***Lactobacillus* spp.**		***Bifidobacterium* spp.**		***Escherichia coli***	
The groups	**Patients**	**Controls**	**Patients**	**Controls**	**Patients**	**Controls**
No.	20	12	16	16	26	18
Mean	1947.4	1400.16	103.87	166.75	430657.9	193927.7
S.E.	950.09	538.50	48.88	43.51	180515.80	51404.749
p-value	0.6		0.001*		0.29	

S.E.= Standard Error of Mean.
P-value≤0.05

In other words, the number of *Bifidobacterium* spp. was affected by *Entamoeba histolytica* infection, which may be due to the members of *Bifidobacterium* bacteria phagocytosed by *E. histolytica* [31]. The trophozoite stage of *Entamoeba* feeds on intestinal commensal bacteria because of the nutritional demands of the parasite in the colon, and bacterial phagocytized in the colon is considered a triggering mechanism of *E. histolytica* invasiveness [18]. Accordingly, the presence of *E. histolytica* causes significant alterations in the structure of human gut microbiota communities [22;33]. That causes gut bacterial dysbiosis, allows the parasite to multiply in the human intestinal lumen, which is necessary for boosting the infectious process because *Bifidobacterium* species are common inhabitants and have a health-promoting role [30].

Another reason that may lead to a decrease in *Bifidobacterium* bacteria mean in *E. histolytica* infected children, is that the patients have taken antibiotics. O'Sullivan and his team demonstrated that the culturable *Bifidobacterium* spp. population in fecal samples from the antibiotic-treated patient decreased when compared with the untreated group [34;35]**.** Rouhani [36] suggests *Bifidobacterium* spp. phagocytosis by *E. histolytica* leads to decrease bacterial number during amebiasis.

On the other hand, Verma *et al*. [30] found a significant increase in *Bifidobacterium* bacteria in children infected with *E. histolytica* compared to healthy control individuals, using quantitative PCR quantitative to determine the absolute amount of 16S rRNA in samples. Figure (3.4B) showed the concentrations **(**copy No.) of each bacterial group.

Escherichia coli numbers exhibited differences between the patients' group and control group (Table 4). The mean value of *E. coli* was higher in the patients' group (430657.9) than the controls (193927.7), but the differ was not significant. Shaulov and his group'(Shaulov *et al.*, 2018) [37] found that *E. coli* confers increased resistance against oxidative stress to the parasite, hence the number of bacteria increased during amebiasis.

Labruyère and others [38] have demonstrated that gut microbiota presence reduces infection by the parasite. They found that trophozoites isolated from amoebiasis patients lost

virulence progressively in axenic cultures. While, a study conducted in Sydney, Australia [30], this study revealed that the interactions of amoebae with a variety of gram-negative gut bacteria, such as *Escherichia coli* strains, could be responsible for the increased amoebic virulence. The current study showed that the numbers of *Escherichia coli* were a little higher in the patients with amebiasis. These absolute numbers provide an indication that there is a strong interaction between the composition of the intestinal microbiota and protozoan parasites during amebiasis [4;20]. Also, previous studies showed significant changes in the predominant gut microbiota in patients infected with *E. histolytica* [22; 39]

4. Conclusions

The occurrence of *E. histolytica* induces considerable changes in the intestinal microbiota populations in the patient children. Where, the number of *Lactobacillus* spp. and *Escherichia coli* was increased in children with amebiasis, compared to healthy children, while the number of *Bifidobacterium* spp. was decreased in infected children. In which the gut microbiota can influence the progression of parasitic infection. That can explain the clinical variation in parasitic infections and why only around 10% of *E. histolytica* infected people develop intestinal amebiasis.

5. Acknowledgment

The authors appreciate the laboratory staff of Al- Al-Kadhimiya Hospital for Children and Central Teaching Hospital of Pediatrics in the medical city for his assistance in sample collection, also they give the deepest gratitude to the College of Science at the University of Baghdad for the lab. Accessories to complete the work and also for approval this work through Ethical Approval Committee in University headquarters.

6. Ethical Clearance

The research ethical committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq.

7. Conflict of interest

The authors declare that they have no conflict of interest.

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