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Association of Enteric Protist *Blastocystis* spp. and Gut Microbiota with Hepatic Encephalopathy

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ABSTRACT

Background & Aims: Hepatic encephalopathy (HE) is a serious neuropsychiatric sequela emerging in the advanced stages of cirrhosis. The gut microbiota plays an important role in the development of HE. The aim of the study was to analyze the dynamic interplay between microbiota and *Blastocystis* in cirrhotic patients with or without encephalopathy.
Methods: The study was designed as cross-sectional study. A total of 37 patients from the Ankara city, admitted to the University Hospital within a 6-month period prior to enrolment into the study were included in the study. After the regular health checks, clinical histories, clinical examinations, and Psychometric HE Score (PHES) points, patients' MELD and CTP scores were recorded. The fecal microbiota configurations were characterized by targeting hypervariable regions V3 and V4 of the 16S rRNA gene using Illumina MiSeq System.
Results: *Blastocystis* spp. were detected in 21.6% (n = 8) of all cirrhotic patients. When those were analyzed by subgroups, four of them were subtype 2, three were subtype 3 and one was subtype 1. *Blastocystis* spp. were not found in any of the patients with HE; however, they were detected in 38.1% of the patients without HE. Also the increase in the bacterial diversity was observed along with the absence of *Blastocystis*. It was suggested that there was an inverse relationship between *Blastocystis* spp. and advanced stages of HE and the structure and composition of gut microbiota.
Conclusion: The absence of *Blastocystis* spp. is associated with the HE severity and dysbiosis in the gut microbiota.

Key words: Blastocystis – hepatic encephalopthy – minimal hepatic encephalopathy – gut microbiota – liver cirrhosis

Abbreviations: Bl: Blastocystis; CTP: Child-Turcotte-Pugh; DNA: deoxyribonucleic acid; HE: hepatic encephalopathy; IBD: inflammatory bowel disease; IBS: irritable bowel syndrome; MELD: Model for End Stage Liver Disease; MHE: Minimal hepatic encephalopathy; NASH: Non-alcoholic steatohepatitis; PBC: Primary Biliary Cirrhosis; PCR: Polymerase Chain Reaction; PHES: Psychometric Hepatic Encephalopathy Score; rRNA: ribosomal ribonucleic acid.

INTRODUCTION

Liver cirrhosis is a common consequence of chronic liver disease characterized by recurrent parenchymal damage [1]. Hepatic encephalopathy (HE) is a serious neuropsychiatric sequela emerging in the advanced stages of cirrhosis [2] and therefore considered to be a spectrum of the mental disorder [3]. The earliest phase of this spectrum is minimal hepatic encephalopathy (MHE), which may progress into coma and ultimately to death in the advanced stages [4]. While it is relatively easier to detect overt HE, tests are required for MHE [3]3. One of those tests is Psychometric Hepatic Encephalopathy Score (PHES) consisting of a series of tests (Number Connection Test-A, Number Connection Test-B, Digit Symbol Test, Serial Dotting Test, Line Drawing Test) [5]. Each test is scored between +1 and -3 standard deviations. Results are adjusted according to patients' age and level of education. In total, a score is determined between +6 and -18. Individuals scored between -4 and -6 are diagnosed with MHE [3].

New studies have determined the hepatic damage and predicted the complications of cirrhosis such as HE, as these play an important role in patients' mortality and morbidity. The

Child-Turcotte-Pugh (CTP) classification is the most widely used as being less complex and having a good predictive value [6]. It helps to predict complications in patients and also the response to invasive interventions [7].

Due to an increase in the need for liver transplants in recent years, the Model for End Stage Liver Disease (MELD) score has been developed to assess the short-term mortality in cirrhotic patients [8]. It is the best method to predict 3-month survival regardless of the etiology [8].

In healthy humans, nitrogenous metabolites generated by the gut bacteria from food, are transported by the portal vein to the liver, and metabolized through the urea cycle. Thus, one of the most important factors in the pathophysiology of HE is the gut microbiota [9]. The gut microbiota plays an important role in the development of HE by bacterial infections and the hyperdynamic circulatory state [9, 10]. In cirrhosis, reduced motility, decreased gastric acid secretion and pancreatobiliary secretions and portal hypertensive enteropathy affect the configuration of intestinal microbiota [10]. This alteration in gut microbiota is linked to HE [11].

Microbiota is a term used to describe the population of microorganisms living in the human body, especially in the gut and its communication with each other [11]. About 10^{14} bacterial cells live in the colon besides viruses and eukaryotic microorganisms [11]. Biochemical typing and culture have been used as gold standards to identify bacterial species; however, for the last two decades, culture-independent techniques have made enormous progress in the understanding of gut microbiota [11]. A class of these techniques depends on determining the sequence divergence of the small subunit ribosomal ribonucleic acid (16S rRNA) and can be used to profile diversity, qualitative and quantitative information on species and changes in the gut microbiota in health and disease [12, 13].

Publications in the past decade on gut microbiota have predominantly focused on the bacterial component of the community, which left gaps in our understanding of the role of other microorganisms including eukaryotic microorganisms and viruses [14]. One of the eukaryotic microorganisms in the gut microbiota is *Blastocystis*(Bl) [14]. *Blastocystis*, an anaerobic, single-celled stramenopile, is one of the most common intestinal protozoa in humans and animals [15]. There are four morphological forms described; vacuolar, granular, amoeboid and cystic [16-18]. It develops into vacuolar forms after the ingestion of cysts. Vacuolar forms may develop to amoeboid or granular forms in the large bowel [17]. At least 17 subtypes are determined by small subunit ribosomal RNA analysis [19]. Even though there is no consensus on *Blastocystis*' role in the gut microbiota the subtype ST1-4 was found to be prevalent in humans [19]. It is still controversial if *Blastocystis* is commensal or pathogen [17]. In the last 6-10 years, researches about subset of *Blastocystis* showed that it is capable of long-term host colonization, suggesting that *Blastocystis* is a common member of the healthy gut microbiota [14]. But the prevalence of *Blastocystis* varies widely from country to country: it is 0.5-1% in Japan [20] and 3.3% in Singapore [21], which are developed countries. In contrast, the prevalence is much higher in developing countries such as Brazil (40.9%) [22] and Egypt (33.3%) [23]. It is 11.6% in the healthy individuals in Turkey [24].

Although *in vitro* and genomic studies supporting the pathogenicity of *Blastocystis* spp. have been published [25-28], the relationship between *Blastocystis* spp. and human diseases is poorly understood [27, 29-31]. Different *Blastocystis* spp. can occupy the healthy human gut asymptomatically [14]. New technologies and studies focusing on analyzing bacterial communities are opening new horizons for the role *Blastocystis* plays in the intestine and its interkingdom interactions [31].

A recent study regarding the bacterial component of microbiota made by Andersen et al. (2015), found that individuals with the microbiota dominated by *Bacteroides* were less inclined to *Blastocystis* host than individuals with *Ruminococcus* and *Prevotella* enterotypes [31]. In that study, associations between enterotypes and *Blastocystis* were investigated by using a metagenomics approach [31]. The negative correlation detected between *Blastocystis* and the *Bacteroides* enterotype was able to point out a relationship between bacterial and parasite components of the gut microbiota [31]. On the other hand, this could also show that a low richness gut microbiota reduces *Blastocystis* [31]. Thus *Blastocystis* is positively correlated with species' richness and the *Bacteroides* enterotype is negatively correlated with richness [31].

The aim of our study was to analyze the dynamic interplay between microbiota and *Blastocystis* in cirrhotic patients with or without HE.

MATERIAL AND METHODS

Patients

A total of 37 patients (20 female, 17 male, median age 62 years) from the Ankara city, admitted to the University Hospital within a 6-month period prior to enrolment into the study (April-September 2014) were included in the study. After the regular health checks, clinical histories with data of recent travels, drugs used for chronic diseases, recent antibiotic use, gastrointestinal bleeding history, clinical examinations and Psychometric HE Score (PHES) points, MELD and CTP scores were recorded.

Stool specimens and microscopy

Stool samples collected from these patients were submitted to the laboratory for a fecal microscopy examination and a subsample of stools (500 mg) was stored immediately after collection and kept frozen at -20^0C until DNA extraction. Patients were grouped in two groups: with HE and without HE, based on the psychometric tests. Fresh stool specimens were examined immediately by performing formalin–ethyl acetate concentration techniques and iodine wet mounts, Trichrome-stained smears and modified acid-fast stains were performed in parallel [32]. Duplicate fresh samples from all patients were also cultivated in Jones's medium at 37^0C for 48–72 h. Cultures were examined for the presence of *Blastocystis* by light microscopy [33]. Other parasites were not observed.

DNA extraction, PCR and *Blastocystis* subtyping

From each frozen stool sample, 250 mg were used to extract genomic DNA using the QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

Genomic DNA was preserved (-80°C) until molecular analysis. The samples were verified for the presence of *Blastocystis* via PCR amplification of *Blastocystis*-specific SSU rRNA using the primers RD5 (5'-ATC TGG TTG ATC CTG CCAG T-3') and BhRDr (5'-GAG CTT TTT AAC TGC AAC AAC G-3') as recommended previously [34, 35]. PCR products were purified and sequenced by both strands using the dideoxy-terminal method (Macrogen, Korea). Sequences were edited in MEGA 4.0 and compared with reference sequences representing each ST in GenBank by BLAST queries.

16S Library preparation

The fecal microbiota configurations of 37 patients were characterized by targeting hypervariable regions V3 and V4 of the 16S rRNA gen using the Illumina MiSeq System. The 16S rRNA Metagenomic Sequencing Library Protocol of the Illumina MiSeq System was followed to prepare the library. Primers were designed by adding Illumina adapter overhang nucleotide sequences to the two universal primer pair Forward (5'-CCTACGGGNGGCWGCAG-3' and Reverse (5'-GACTACHVGGGTATCTAATCC-3'). These primers (162S Amplicon F 5'-TCGTCGGCAGCGTCA GATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 16S rRNA Amplicon R5'-GTCTCGTGGGCTCGGAGATG TGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') were obtained from Integrated DNA Technologies (IDT) (Iowa, US).

DNA concentrations of 37 samples were measured in SpectraMax i3 platform (Molecular Devices, Sunnyvale, CA, US). 12.5 ng/µl template DNA was used for the amplification step of 16S rRNA V3 and V4 region in a total volume of 10 µl PCR reaction. For each sample, indices from the Nextera XT Index kit (FC-131-1001 or FC-131-1002) were added to the 5 µl of the volume. Biospeedy One DNA Polymerase (Bioeksen, Istanbul, Turkey) was used for all PCR reactions. For all other steps, Illumina protocol was strictly followed. In total, 20,562,042 high quality reads were obtained from 37 samples.

Bioinformatics analyses

Quality filtering and Amplicon assembly of 16S reads

After acquiring 37 16S rRNA amplicon sequencing samples in fast format from the MiSeq system, these samples were subjected to read quality filtering, dereplication, and amplicon assembly steps.

Maximum expected error filtering was applied to the reads assuming at most five expected errors in a read, following the procedure suggested by UPARSE[36] program. Overlapping amplicon reads pairs were merged using semi-global pairwise alignment (i.e. modified Needleman-Wunsch algorithm) and consensus amplicons were generated by selecting the base with higher Phred score. Dereplication and filtering out redundant reads were performed using UPARSE pipeline and final high-quality 16S sequences were obtained. This preconditioning step resulted in filtering out a total of 36% of the generated sequences.

OTU clustering and taxonomy assignment

OTU clustering was performed using a 97% similarity threshold following the procedure of the UPARSE system. After determining the clusters and eliminating the singletons, each cluster centroid (median) was taken as the taxonomic representative and used for being assigned to clade levels. RDP classifier [37] was used to classify the operational taxonomic units to bacterial taxa with a 95% confidence setting and taxonomic abundance profiles were generated for each sample. About 81.9% (SD. 4.9%) of the amplicons were assigned to known taxa successfully. A total of 217 genera and 74 families were identified. To observe the sampling depth sufficiency and metagenome coverage, rarefication curves were generated from species accumulation curves using subsampling without replacement (Fig. 1).

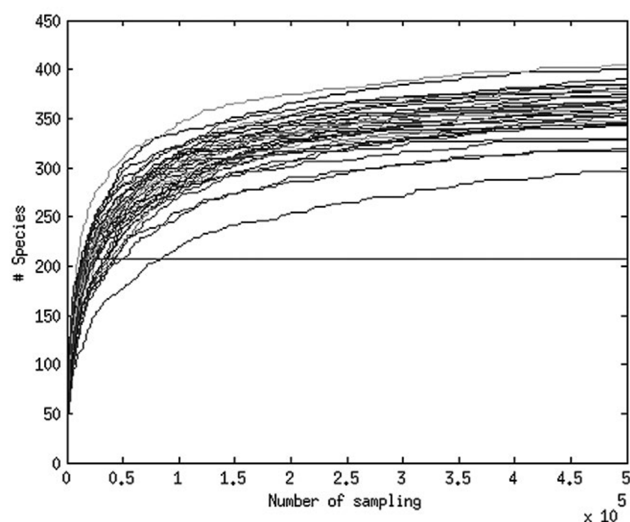


Fig. 1. Rarefaction curves of the sequenced 37 microbiome samples

Comparative analysis of metagenome samples

In order to capture the statistically meaningful microbiota composition differences, three main groups were compared in pairwise fashion (Table I). The tests were conducted at the clade levels of genus and family. Differentially abundant microbiota components were determined using LEfSe analysis [38].

Table I. Patients groups according to Blastocystis spp. growth and the presence of hepatic encephalopathy

Groups	*Blastocystis* spp. growth	Hepatic Encephalopathy
Group 1	*Blastocystis* spp. positive	negative
Group 2	*Blastocystis* spp. negative	negative
Group 3	*Blastocystis* spp. negative	positive

Ethics statement

This study was approved by the Research Ethics Committee of Gazi University (date: 12/03/2014 and reference number 253) and written informed consent was obtained from all participants prior to the study. All the methods used in the study were carried out in accordance with the approved guidelines and Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects.

RESULTS

The mean age of the patients was 59.1 ± 13 (19-87) and 54.1% (n = 20) were female. The etiology of cirrhosis is shown in Fig. 2.

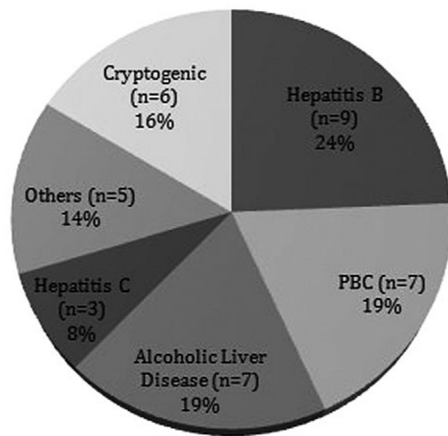


Fig. 2. Etiology of liver cirrhosis in the patients included in the study. PBC: Primary Biliary Cirrhosis, Others (5) consist of non-alcoholic steatohepatitis (2), Budd-Chiari syndrome (1) and autoimmune hepatitis (2).

Blastocystis spp. were detected in 21.6% (n=8) of all cirrhotic patients. When *Blastocystis* spp. were analyzed by subgroups, four of them were subtype 2, three were subtype 3 and one was subtype 1. *Blastocystis* spp. was not found in any of the patients with HE (none of 16 patients with either clinical or MHE); however, *Blastocystis* spp. were detected in 8 of 21 (38.1%) patients without HE (p = 0.006).

In none of the CTP class C patients, *Blastocystis* spp. were detected while 7 (36.8%) of the CTP A patients with mild cirrhosis and 1 (9.1%) of the CTP B patients were infected.

Likewise, when the patients were classified according to MELD score, *Blastocystis* spp. growth was 33.3% in the patients with score ≤ 8 and 6.7% in the patients with score 8-20. There was no growth in patients with score ≥20 (Table II).

The patients with encephalopathy were divided into subgroups as overt and minimal. The analyses were made according to these subgroups. There was a statistically significant difference between these groups and patients without encephalopathy for *Blastocystis* spp. growth (p=0.005 Pearson Chi-Square) (Table III). Also there was an inverse correlation between the presence of *Blastocystis* spp. and HE (Kendall's correlation test, τ=-0.458, p=0.006) (Table IV). Decreasing trend in the frequency of *Blastocystis* spp. detection was observed with cirrhotic stage progressing (τ= -0.366, p=0.021) (Table IV).

A percentage of 24.3% of the cirrhotic patients (n=9) use oral lactulose in order to reduce the effects of hyperammonemia. Lactulose use was significantly higher in the patients with a high CTP score (p=0.013) and MELD score (p=0.0001) than expected. Similarly, lactulose use was also significantly higher in the patients with HE (p=0.024). While there is such a relationship, there was not a significant relationship according to lactulose use between patients with or without *Blastocystis* spp. growth (p=0.649).

Profiles of bacteria populations of the cirrhotic patients are given in Fig. 3 when sorted according to the presence of HE.

These bacteria populations were determined according to density and diversity among three groups (Fig. 4). Increase in

Table II. Growth of *Blastocystis* spp. in patients classified according to MELD Score

MELD score	n	%	*Blastocystis* spp. growth
≤8	21	56.8	33.3 %
8-20	15	40.5	6.7 %
≥20	1	2.7	0 %

Table III. Relationship of hepatic encephalopathy and *Blastocystis* spp. infection in cirrhotic patients

Hepatic encephalopathy	*Blastocystis* spp. (-) n (%)	*Blastocystis* spp. (+) n (%)
Absent	13 (61.9%)	8 (38.1%)
Minimal	8 (100%)	0
Overt	8 100%)	0

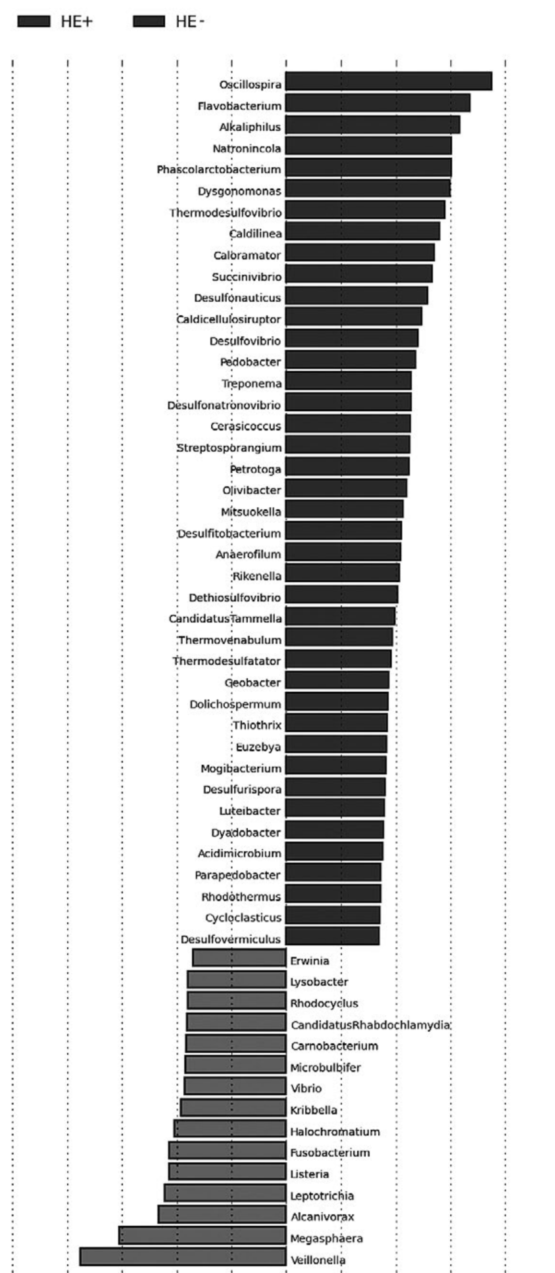


Fig. 3. Changes of some bacteria genera in cirrhotic patients with or without encephalopathy sorted based on linear discriminant analysis scores.

Table IV. Correlation between the specific groups.

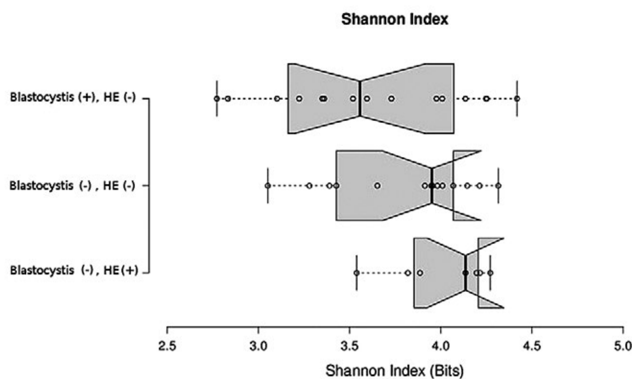
			HE	Blastocystis	CTP groups	MELD groups
Kendall‘s tau_b	HE	Correlation Coefficient	1.000	-0.458**	0.608**	0.454**
		Sig. (2-tailed)	.	0.006	0.000	0.006
		N	37	37	37	37
	Blastocystis	Correlation Coefficient	-0.458**	1.000	-0.366*	-0.322
		Sig. (2-tailed)	0.006	.	0.021	0.050
		N	37	37	37	37
	CTP groups	Correlation Coefficient	0.608**	-0.366*	1.000	0.574**
		Sig. (2-tailed)	0.000	0.021	.	0.000
		N	37	37	37	37
	MELD groups	Correlation Coefficient	0.454**	-0.322	0.574**	1.000
		Sig. (2-tailed)	0.006	0.050	0.000	.
		N	37	37	37	37

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed). HE: hepatic encephalopathy.

the bacterial diversity was observed along with the absence of *Blastocystis*. This connection was not statistically significant but a trend could be seen. Lack of statistical significance is probably due to the low number of participants. This relationship could be detected in larger studies.

The groups were compared according to the two major phyla (*Firmicutes* and *Bacteroidetes*). The analysis showed that the rate of *Bacteroidetes* was higher when *Blastocystis* was absent and encephalopathy was arising (Kruskal-Wallis, p=0.03). On the other hand, there was no significant change in the *Firmicutes* (Kruskal-Wallis, p=0.11) (Fig. 5).

There was no correlation of *Enterobactericeae* with CTP and MELD scores (r = 0.1036, p-value= 0.547 and r = 0.1654, p= 0.33 respectively). However, we found that *Enterobactericeae* was enriched in HE patient samples (p= 0.0058). We observed a negative correlation of *Ruminococcaceae* with CTP and MELD (r = -0.3927, p= 0.018 and r = -0.3844, p= 0.02 respectively). At the same time, this relationship was also observed in *Ruminococaceae* population between the analyzed groups (p= 0.0391, Kruskal-Wallis).



Fi. 4. Bacterial density and diversity between the groups in Shannon index.

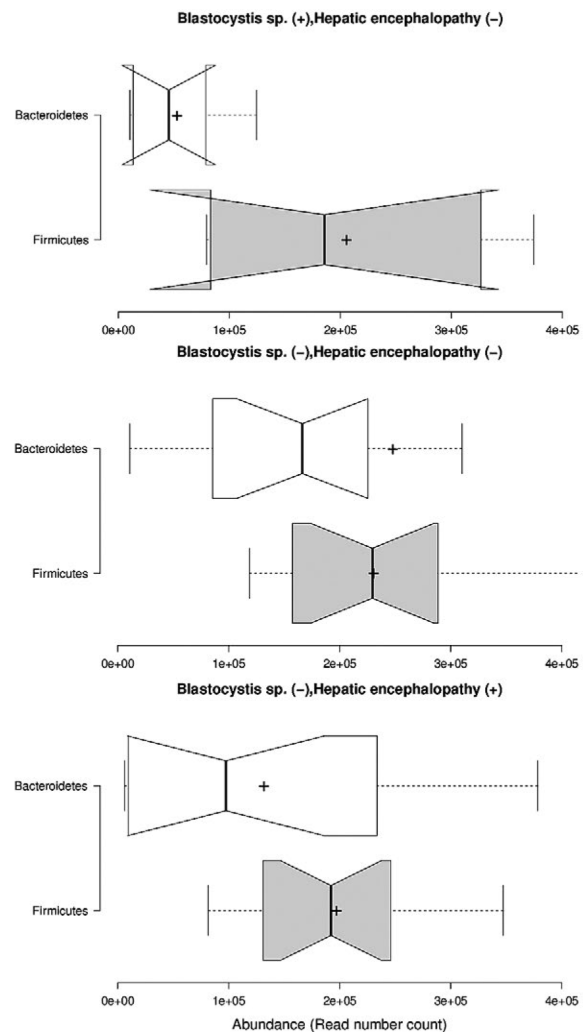


Fig. 5. Changes of *Bacteroidetes* and *Firmicutes* populations between the groups.

When bacterial abundance profiles were compared among the groups (Fig. 6), *Alkaliphilus* and *Flavobacterium* populations were found to be higher in Group 1. Conversely, *Veillonella* and *Streptococcus* populations were lower in Group 1.

Lachnospiraceae population, especially *Fecalibacterium*, was evaluated; analysis showed that there is no significant difference between the groups (Kruskal-Wallis, p= 0.0937).

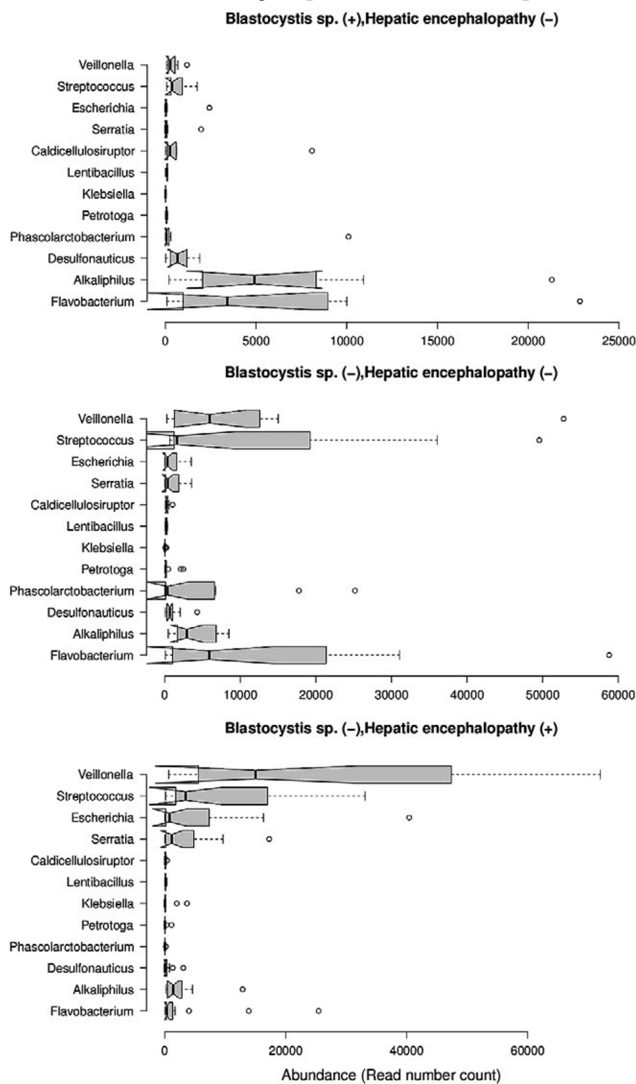


Fig. 6. Differences of bacterial populations between the groups.

These bacteria populations are considered as a part of healthy microbiota. These results support that the patients' microbiotal environments were affected at a similar rate by the underlying disease.

We found that there were no differences in *Lachnospiraceae* abundance between the groups (r= 0.1668, p= 0.33 for CTP; r= 0.3133, p= 0.0628 for MELD). *Ruminococaceae* had a negative correlation with CTP score, MELD score, HE and the presence of *Blastocystis* spp. (r= -0.3927, p= 0.018 for CTP; r= -0.3844, p= 0.02 for MELD) (Fig. 7); *Clostridiales Cluster* XIV also had a weak negative correlation with CTP score (r= -0.3114, p= 0.0345) and MELD score (r= -0.2284, p= 0.018) (Fig. 7).

Although there was no significant difference of *Lactobacillus* between the groups (Fig. 7), a negative correlation between *Lactobacillus* and *Blastocystis* spp can be observed. However, there was not direct relationship between lactulose administration and *Lactobacillus* population (p= 0.11).

DISCUSSION

In this study we investigated the prevalence of *Blastocystis* spp. infection in cirrhotic patients and its correlation with HE and gut microbiota. The prevalence of *Blastocystis* spp. in all cirrhotic patients was 21.6%. Interestingly, Doğruman-Al et al. (2010) reported that the prevalence of *Blastocystis* spp. among healthy individuals in Ankara, Turkey was 11.6% [24]. In terms of subtypes of *Blastocystis* spp., subtype 2 was the most prevalent among the cohort in this study, followed by subtype 3. On the other hand, in 2008, Doğruman-Al et al. published a study about *Blastocystis* subtypes and their relationship with gastrointestinal symptoms [39]. It was found that subtype 3 was most common between symptomatic and asymptomatic groups, the second dominant genotype was subtype 1 in the symptomatic group and subtype 2 in the asymptomatic group [39]. It was suggested that subtype 1 is linked to elevated pathogenicity but subtype 2 is non-pathogenic [39]. As compared to our study, the domination of subtype 2 (mentioned as non-pathogenic) could play a key role in the configuration of the microbiota and development of HE in cirrhotic patients.

The relationship between *Blastocystis* and the bacterial component of microbiota has been investigated in a few cases, especially in the study by Nourisson et al. [40]. In this study, patients with Irritable Bowel Syndrome (IBS) were compared with controls. There was a significant difference between *Blastocystis* carriage and a negative correlation between *Bifidobacteria* and *Blastocystis* presence [40]. Also, *Faecalibacterium prausnitzii*, which is an indicator of the healthy gut [41], was found less often both in the *Blastocystis*-positive and IBS-C groups [40]. It was suggested that the decrease in *Blastocystis* presence could be related to the inflammatory environment. Change in microbiota, which has a key role in the protection against pathogens, could influence the presence of *Blastocystis* in the IBS patients. These data suggest that *Blastocystis* might have an impact on gut microbiota [42, 43]. Additionally, *Blastocystis* may cause changes in the composition of the gut microbiota by interacting with it [40]. Also, Verma et al. reported that the amount of the major genera of the gut microbiota was reduced after it was infected by *Entamoeba histolytica* [44].

In our study, we observed that the prevalence of *Blastocystis* spp. decreased as cirrhosis progressed. In cirrhotic patients, oral lactulose and antibiotic use increase with the progression of CTP stage and HE. It may reduce the incidence of *Blastocystis* spp. in gut microbiota by affecting the microbiota directly or indirectly. However, in our study there were no patients using antibiotics. Also, there was no significant relationship between the presence of *Blastocystis* spp. and oral lactulose use (p=0.379). Additionally, an increase in the bacterial diversity was observed along with the absence of *Blastocystis*. However, this was not statistically significant, probably due to the low number of participants. But this trend showed the effect of *Blastocystis* spp. on the microbiota. This knowledge contrasts with a recent study made by Audebert et al. [45],

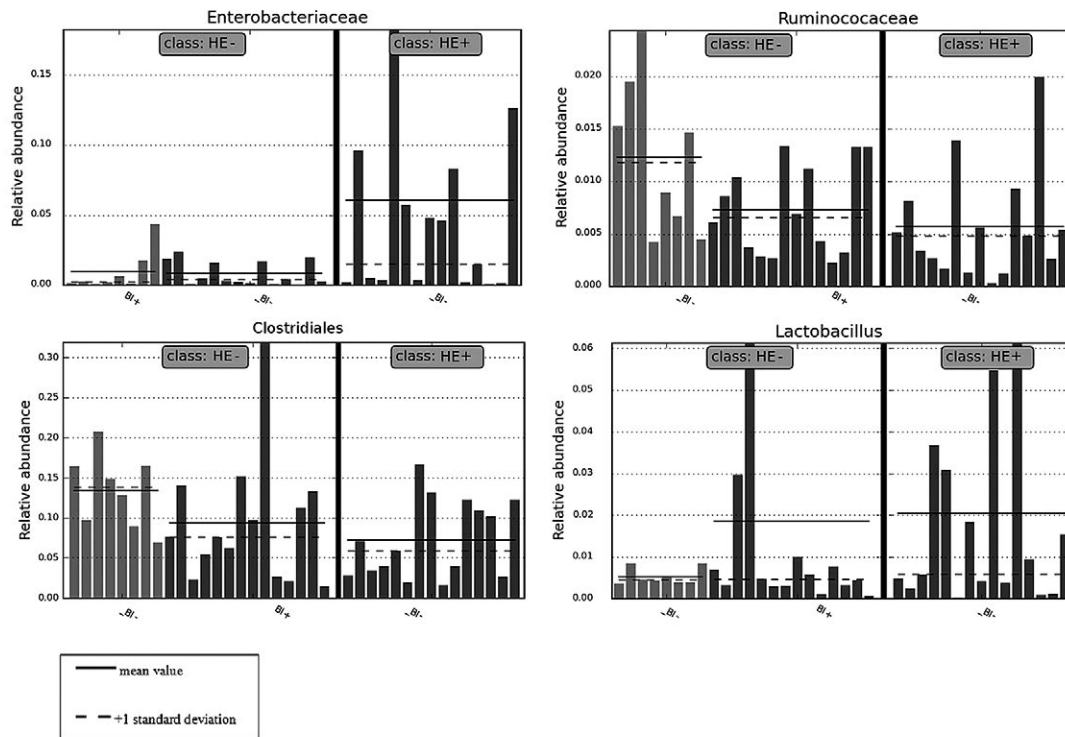


Fig. 7. Population changes of *Enterobacteriaceae*, *Ruminococacaeae*, *Clostridiales Cluster* XIV and *Lactobacillus* between the groups.

who compared the bacterial diversity between *Blastocystis*-free and *Blastocystis*-colonized patients and found a higher bacterial diversity in *Blastocystis*-colonized patients. Sampling populations might be the reason of this difference. In our study, all the patients were cirrhotic. Our data suggests that the change in the bacterial diversity and the presence of *Blastocystis* could play a key role in the pathophysiology of HE and there is a need for further studies regarding this topic.

Dysbiosis has an important role in the pathogenesis of HE. Qin et al. observed that the MELD score was linked positively with *Enterobacteriaceae* and negatively with *Ruminococcaceae* [46]. In our study, we could not find a correlation between *Enterobactericeae* with CTP and MELD scores. However, there was a strong connection between *Enterobactericeae* and HE (p= 0.0058). In accordance with Qin's study, we observed the negative correlation of *Ruminococcaceae* with CTP and MELD. At the same time, this relationship was also observed in *Ruminococacaeae* population between the analyzed groups.

Populations of the other bacterial species were compared between the groups. *Alkaliphilus* and *Flavobacterium* population were higher in Group 1; on the other hand, the populations were becoming lower in Group 3. Conversely, when *Veillonella* and *Streptococcus* population were lower in Group 1, and higher in Group 2. The presence of *Blastocystis* spp. was variable between groups as correlated with these bacterial changes. Group 2 located between Group 1 and 3 was observed as a transition period.

Animal models are playing an important role in gathering information about the connection between HE, gut microbiota and *Blastocystis* spp. When considering changes in the gut microbiota and the high prevalence of *Blastocystis* spp. in IBS patients, *Blastocystis* spp. may be used as an indicator of the dysbiosis [40].

This hypothesis is supported by Scanlan & Stensvold [30]. *Blastocystis* was more common in patients with *Prevotella* and *Ruminococcus* enterotypes, on the other hand, it was seen rarely in patients with *Bacteroides* enterotypes [31]. This relationship is not very clear, but likely a reduction in the bacterial variety in microbiota in association with an increase in a few bacterial species is a common situation in IBD patients [47, 48].

Bacterial taxa such as *Lachnospiraceae*, *Ruminococcaceaea* and *Clostridiales Cluster* XIV (consisting of some genera and species such as *Fecaelibacterium*) are often seen as the healthy microbiota [41, 49, 50]. Sokol et al. determined *Fecalibacterium* as an anti-inflammatory bacterium and with a potential to be a probiotic [41].

In our study, *Lachnospiraceae* population, especially *Fecalibacterium*, was evaluated. The analysis showed that there was no significant difference between the groups. This result may have occurred because of the sampling group. The sampling group consisted of cirrhotic patients and some of them had HE Therefore, the microbiotal environment was not healthy. This information also supports that the *Fecalibacterium* population does not directly affect the presence of encephalopathy. However, there will be more accurate data when compared to healthy individuals.

Ruminococacaeae had a negative correlation with CTP score, MELD score, HE and the presence of *Blastocystis* spp.; *Clostridiales Cluster* XIV also had a weak negative correlation with CTP score and MELD score.

Lactobacillus is used sometimes as a probiotic. And it has been suggested that it reduces the strength of potentially harmful bacteria [51]. Lactulose administration increases the autochthonous bacteria such as *Lactobacilllaceae* [51]. Although there was not a significant difference of *Lactobacillus* between the groups, it was seen as a negative correlation

between *Lactobacillus* and *Blastocystis* spp. However, there was not a direct relationship between lactulose administration and *Lactobacillus* population.

CONCLUSION

Because the major source of hyperammonia is the gut microbiota, there is a relationship between the gut microbiota and cirrhotic patients with HE. Our data suggests an inverse relationship between *Blastocystis* spp. and advanced stages of HE and that the structure and composition of gut microbiota significantly shifts. Thus, absence of *Blastocystis* spp. was associated with HE severity and dysbiosis in the gut microbiota. Further research is warranted to elucidate the potential role of this protist in HE.

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Authors' contributions: Y.S. planned the study, designed the patient groups, sent samples to the laboratory, wrote the article. D.I. planned the study and wrote the article. D.F. examined microscopically the stool samples, isolated DNAs and edited the article. N.U. made the statistical analysis. U.D. made the Metagenomics analysis. S.F. collected the stool samples and isolated the DNAs. Y.S. edited the English typing.

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