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Gut microbiota characterization in Egyptian patients with hepatocellular carcinoma post-chronic hepatitis C virus genotype 4 infection

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ABSTRACT

This study aimed to evaluate the possible role of gut dysbiosis in the development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C virus (HCV) infection. This study was carried out on 400 individuals categorized into three groups: group I included 100 patients with HCC; group II included 200 chronic HCV-infected patients; and group III included 100 healthy control subjects. The included patients were evaluated using Child–Pugh, tumor (T), nodes (N), and metastases (M) (TNM), and Barcelona Clinic Liver Cancer (BCLC) scoring systems. In addition to routine investigations (complete blood counts, complete liver function tests, international normalized ratio, HCV antibodies, and hepatitis B surface antigen), polymerase chain reaction of stool microbiota, HCV infection, and alpha-fetoprotein were assessed for all patients. The results revealed no significant difference between HCC patients and control patients ($p > 0.05$) regarding *Bacteroides fragilis* and *Akkermansia muciniphila*. *Faecalibacterium prausnitzii* and *Bifidobacterium* were detected in fewer patients with HCC (51% and 43%, resp.) compared with healthy controls (70% and 76%, resp.) ($p < 0.05$). *Lactobacillus* and *Escherichia coli* were detected in more patients with HCC (80% and 81%, resp.) compared with healthy controls (36% and 58%, resp.), $p < 0.001$. No significant differences were detected between gut microbiota and HCC progression with respect to Child–Pugh or TNM scores. However, *B. fragilis* was detected in stool isolates from 66.7% of BCLC stage IV patients compared with 10.7% of BCLC stage I patients. Thus, characteristic patterns of *Bifidobacterium*, *Lactobacillus*, and *E. coli* species in patients with chronic HCV and HCC were detected. Therapies targeting altered gut microbiota may be beneficial in reducing the risk of HCC in patients with HCV infection, as altered gut microbiota is a predisposing factor for liver disease progression.

INTRODUCTION

The World Health Organization has reported that hepatocellular carcinoma (HCC) was the 5th most common cancer worldwide and the 2nd most predominant cause of death related to cancer in 2012 (World Health Organization, 2012). HCC is the most common liver cancer and may result from chronic hepatitis B virus infection, post-hepatitis C virus (HCV) liver cirrhosis, smoking, excess drinking of alcohol, and aflatoxin intake (American Cancer Society, 2016).

Viral hepatitis is the 7th leading cause of death worldwide (Stanaway *et al*., 2016). Approximately 50% of deaths related to chronic HCV infection that led to liver cirrhosis progressed to HCC development (Lavanchy, 2011; Mohd Hanafiah *et al*., 2013). HCV infection is a public health problem in Egypt, where it has the highest prevalence among nations according to the WHO (WHO, 2016). It is known that post-viral hepatitis HCC is mainly a result of hepatocellular inflammation, oxidative stress, abnormal signaling pathways with activation of oncogenes, and integration of viral DNA into the host genome (Sukowati *et al*., 2016; Tokino *et al*., 1991).

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The intestinal barriers that prevent the passage of microbes and their metabolites across the mucosa include the intact intestinal mucosal barrier, the mucus layer, secretion of immunoglobulin A, and associated lymphoid tissues (Peterson and Artis, 2014). A close relationship exists between the gut microbiome and the liver, mainly due to their anatomical positions and the flow of nutrients and blood supply from the gut via the portal vein. The liver is the most vulnerable organ to gut-derived toxins, dangerous metabolites, bacteria, and their metabolites (Miele *et al*., 2013; Schnabl and Brenner, 2014). Growth and alteration in gut microbiota cause damage to the intestinal wall, promoting the transmission of bacteria and metabolites that elicit systemic endotoxemia and disease in distant organs, including the liver (Qin *et al*., 2010; Yu and Schwabe, 2017).

Dysbiosis is the loss of beneficial microorganisms and overgrowth of harmful microbes, changing the overall microbial profile (Petersen and Round, 2014). Dysbiosis of gut microbiota favors chronic hepatic inflammation with subsequent carcinogenesis, which can be elicited by the interaction between the liver, abnormal gut microbiota, and their metabolites together with the immune system via macrophages and Kupffer cells that secrete inflammatory cytokines, IL-8, tumor necrosis factor α, and interleukin 1 beta (Brandi *et al*., 2017; Chassaing *et al*., 2014; Rivera *et al*., 2007).

Logically, dysbiosis of gut microbiota augments the pathophysiology of viral hepatitis by inducing chronic inflammation of the liver with subsequent hepatic HCC (Fattovich *et al*., 2004). Mechanisms by which viral hepatitis induces disturbance of gut microbiota are unknown; however, maintaining gut homeostasis can prevent viral hepatitis-induced hepatic disease progression and HCC development. Therefore, in this study, we explored changes in gut microbiota between healthy volunteers, patients with chronic HCV infection, and patients with HCV-induced HCC.

PATIENTS AND METHODS

Study design and participants

This cross-sectional cohort study was carried out on 400 individuals with average body volume recruited from the Internal Medicine Department and Tropical Medicine and Gastroenterology Department of two dedicated centers (Helwan University Hospital and South Valley University Hospital) in Egypt, in collaboration with the Medical Biochemistry Department of the Faculty of Medicine, South Valley University, Qena, Egypt. The study period lasted from January 2017 to January 2020. The individuals were divided clinically into three groups. Group I consisted of 100 patients with HCC on top of liver cirrhosis caused by chronic HCV infection. The diagnosis of HCC was made according to American Association for the Study of Liver Diseases (AASLD) guidelines, based on clinical, laboratory, and imaging data and liver biopsy when required (Marrero *et al*., 2018). Group II consisted of 200 chronic HCV-infected patients. All patients were infected with HCV genotype 4. Group III consisted of 100 healthy control subjects negative for hepatitis markers and with normal abdominal ultrasounds. Patients below 18 years of age who underwent antibiotic therapy within the preceding 2 weeks, those who had autoimmune hepatitis, human immunodeficiency virus, or hepatitis B virus coinfection, those who reported excessive alcohol intake, and those with other etiologies of hepatic affection were excluded from the study. The study protocol was approved by the Qena Faculty of Medicine Ethical Committee, and written informed consent was obtained from each subject.

Clinical assessments

Complete medical histories were taken, and full clinical examinations and laboratory and radiological investigations were performed on each included subject. Serum alpha-fetoprotein (AFP) determination and multislice abdominal computed tomography (MSCT) were performed for patients with suspected HCC. For cirrhotic patients with hepatic focal lesions of >1 cm on abdominal ultrasound and AFP >20 ng/ml, MSCT imaging was performed. If typical features of HCC were not observed but HCC was still suspected, the liver was biopsied to confirm the diagnosis per AASLD guidelines. All patients with HCC were evaluated using a Child–Pugh score (Pugh *et al*., 1973), and tumor staging was assessed using tumor (T), nodes (N), and metastases (M) (TNM) and Barcelona Clinic Liver Cancer (BCLC) scoring systems (Llovet *et al*., 1999; Vauthey *et al*., 2002).

Investigatory workup

Blood samples and biochemical and molecular assays

We obtained 10 ml blood samples from each included subject under sterile conditions and transported them to the laboratory for routine laboratory investigations, including complete blood counts using Sysmex, serum creatinine, complete liver function tests [alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and total bilirubin] using an autoanalyzer (Dialab 450 system), prothrombin time, prothrombin concentration, and international normalized ratio (INR). HCV genotype estimation and HCV polymerase chain reaction (PCR) assessment were performed using reverse transcription PCR (RocheCOBAS TaqMan HCV assay version 2.0, lower limit of detection 15 IU/ml). hepatitis B surface antigen estimation and HCV antibody detection were performed using the automated MiniVidas immunoassay system (Biomerieux, Marcy l'Etoile, France). AFP assessment was also performed for patients with suspected HCC (Hassan *et al*., 2018).

Polymerase chain amplification of stool microbiota

Stool samples were collected from each participant and rapidly transported to the laboratory. At the laboratory, an aliquot was taken from stool in a 1.5 ml Eppendorf tube and stored at −80°C. DNA was extracted from the stored sample after thawing using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer`s instructions.

Positive control strains for PCR analysis included *Akkermansia muciniphila* (ATCC BAA-835), *Faecalibacterium prausnitzii* [ATCC (American type culture collection) 27766], *Bifidobacterium breve* (ATCC 15700), *Lactobacillus acidophilus* (ATCC 4356), *Bacteroides fragilis* (ATCC 25285), and *Escherichia coli* (ATCC 25922). These control strains were cultured according to the standard microbiological recommendations, and DNA from these strains was suspended in 1 ml phosphate buffer and extracted using the Qiagen extraction kit (Özkul *et al*., 2017).

The amplification of different bacterial species of microbiota was performed using the primers listed in Table 1. Amplification reactions were carried out on a total volume of 20 µl and consisted of 4 mM $MgCl_2$, 0.25 µM of each primer, and 2 µl of DNA template. Amplification involved an initial denaturation step at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at the specific annealing temperature (Table 1) for 5 seconds, and extension at 72°C for 10 seconds. The products of the amplified PCR were analyzed using gel electrophoresis (Özkul *et al*., 2017).

Abdominal multislice CT (HCC radiological hallmark)

The typical feature used for HCC diagnosis is arterial phase hyperintensity (washin), followed by portal venous washout to iso- or hypodensity (delayed phases) (European Association for the Study of the Liver; European Organisation for Research and Treatment of Cancer, 2012).

Statistical analysis

Data were analyzed using the Statistical Program for Social Science program version 24. Qualitative data are expressed as frequencies and percentages; quantitative data are expressed as means ± standard deviation. A chi-square test was used when comparing gut microbiota between groups. *p*-values of <0.05 were considered significant.

RESULTS

Demographic data of the study groups

This descriptive cross-sectional cohort study included 300 patients and 100 healthy controls. The first group included 100 patients with HCC, and their basic demographic and laboratory data are shown in Table 2. The second group included 200 patients with chronic HCV infection; their basic demographic data are shown in Table 3. The third group included 100 healthy volunteers whose gut microbiota were evaluated for comparison with patients from groups I (HCC) and II (HCV). Their demographic data are shown in Table 4.

Profile of gut microbiota among patients with HCC compared with the control group

Differences were detected between patients with HCC and controls ($p < 0.001$) in the levels of *Bifidobacterium*, which was detected in fewer patients with HCC (43%) compared with healthy controls (76%). However, *Lactobacillus* and *E. coli*

Table 2. Demographic, clinical, and routine laboratory data of HCC patients.

Variables	HCC group (*n* = 100)
Age (years) (Mean ± SD)	50.1 ± 5.5
Sex (*n*%)	
Male	76 (76%)
Female	24 (24%)
Smoking (*n*%)	44 (44%)
Diabetes mellitus (*n*%)	9 (9%)
Random blood glucose (mg/dl) (Mean ± SD)	137.2 ± 32.7
AFP (ng/ml) (Mean ± SD)	211.34±15.19
Complete blood count (Mean ± SD)	
Total leucocyte count ($\times 10^3$/cmm) (Mean ± SD)	6.9 ± 3.9
Hemoglobin (g/dl)	11.02 ± 1.0
Platelets ($\times 10^3$/cmm)	113.9 ± 37.6
Liver function tests (Mean ± SD)	
Albumin (g/dl) (Mean ± SD)	3.1 ± 0.5
Total bilirubin (mg/dl) (Mean ± SD)	1.98 ± 1.34
ALT (U/l) (Mean ± SD)	89.9 ± 9.18
AST (U/l) (Mean ± SD)	70.7 ± 9.57
INR (Mean ± SD)	1.5 ± 0.6
Renal function tests (Mean ± SD)	
Creatinine (mg/dl) (Mean ± SD)	1.09 ± 0.4
Scoring data of patients	
Child–Pugh class	
A	18 (18%)
B	38 (38%)
C	44 (44%)
TNM stage	
I	24 (24%)
II	40 (40%)
III (A–B)	28 (28%)
IV	8 (8%)
BCLC staging	
A	28 (28%)
B	26 (26%)
C	34 (34%)
D	12 (12%)

Table 1. Bacterial species, primers sequences, annealing and melting temperatures of PCR, and the result size of bp.

Bacterial species	Primers sequences (Özkul *et al*.,2017)(5′-3′)	Annealing temperature (°C)	Melting temperature	Fragment size (bp)
Bifidobacterium spp.	CTCCTGGAAACGGGTGGGGTGTTCTTCCCGATATCTACA	56	90	550
B. fragilis group	ATAGCCTTTCGAAAGRAAGAT CCAGTATCAACTGCAATTTTA	50	86	495
Lactobacillus spp	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	55	86	341
A. muciniphila	CAGCACGTGAAGGTGGGGAC AM-1 CCTTGCGGTTGGCTTCAGAT AM-2	60	90	327
F. prausnitzii	GATGGCCTCGCGTCCGATTAG Fprau223F CCGAAGACCTTCTTCCTCC	60	88	199
E. coli	CATTGACGTTACCCGCAGAAGAAGCCTCTACGAGACTCAAGCTTGC	63	87	195

Table 3. Demographic, clinical, and routine laboratory data of chronic HCV infected patients.

Variables	Chronic HCV group (*n* = 200)
Age (years) (Mean ± SD)	53.1 ± 9.9
Sex (*n*%)	
Male	137 (68.5%)
Female	63 (31.5%)
Smoking (*n*%)	55 (27.5%)
Diabetes mellitus (*n*%)	35 (17.5%)
Random blood glucose (mg/dl) (Mean ± SD)	120.1 ± 46.2
Complete blood count (Mean ± SD)	
Total leucocyte count (×10^3/cmm) (Mean ± SD)	6.5 ± 4.01
Hemoglobin (g/dl)	12.7 ± 1.9
Platelets (×10^3/cmm)	201.8 ± 73.8
Liver function tests (Mean ± SD)	
Albumin (g/dl) (Mean ± SD)	3.4 ± 0.4
Total bilirubin (mg/dl) (Mean ± SD)	1.57 ± 1.3
ALT (U/l) (Mean ± SD)	39.1 ± 2.72
AST (U/l) (Mean ±SD)	38.1 ± 1.2
INR (Mean ± SD)	1.43 ± 0.4
Renal function tests (Mean ± SD)	
Creatinine (mg/dl) (Mean ±SD)	0.89 ± 0.2

Table 4. Demographic and routine laboratory data of healthy control subjects.

Variables	Control group (*n* = 100)
Age (years) (Mean ± SD)	49.9 ± 6.8
Sex (*n*%)	
Male	49 (49%)
Female	51 (51%)
Smoking (*n*%)	70 (70%)
Complete blood count (Mean ± SD)	
Total leucocyte count (×10^3/cmm) (Mean ± SD)	7.6 ± 2.3
Hemoglobin (g/dl)	13. 2 ± 1.7
Platelets (×10^3/cmm)	242.9 ± 92.2
Liver function tests (Mean ± SD)	
Albumin (g/dl) (Mean ± SD)	4.1 ± 0.5
Total bilirubin (mg/dl) (Mean ± SD)	0.82 ± 0.1
ALT (U/l) (Mean ± SD)	31.09 ± 4.1
AST (U/l) (Mean ± SD)	29.2 ± 4.4
INR (Mean ± SD)	1.1 ± 0.1
Renal function tests (Mean ± SD)	
Creatinine (mg/dl) (Mean ± SD)	0.81 ± 0.2

species were detected in more patients with HCC (80% and 81%) compared with healthy controls (36% and 58%, resp.). Statistically significant differences were also detected between patients with HCC and controls ($p < 0.05$) in *F. prausnitzii*, which was detected in fewer patients with HCC (51%) compared with healthy controls (70%). No statistically significant differences were detected between patients with HCC and controls ($p > 0.05$) regarding *B. fragilis* and *A. muciniphila* (Table 5).

Profile of gut microbiota among patients with chronic HCV infection compared with the control group

A statistically significant difference was detected between patients with HCV and controls ($p < 0.001$) regarding *Bifidobacterium* and *F. prausnitzii*, which were detected in fewer patients in the HCV group (44% and 38%) compared with healthy controls (76% and 70%, resp.). On the other hand, *Lactobacillus* was detected in more patients in the HCV group (62%) compared with healthy controls (36%). Statistically significant differences were detected between patients with HCV and controls ($p < 0.05$) of *E. coli* which was detected in more patients with HCV (70%) compared with healthy controls (58%). However, no statistically significant differences were found between patients with HCV and healthy volunteers ($p > 0.05$) in *B. fragilis* and *A. muciniphila* species (Table 6).

Comparison between patients with HCC and chronic HCV infection

Statistically significant differences were detected between the HCC and HCV groups ($p < 0.001$) regarding *F. prausnitzii* in the gut microbiota, which was detected in a greater number in patients with HCC (51%) than in patients with HCV (38%). *Lactobacillus* and *E. coli* were also detected in more patients in the HCC group (80% and 81%) than those in the HCV group (62% and 70%, resp.). No statistically significant differences were detected between patients with HCC and HCV ($p > 0.05$) regarding *Bifidobacterium*, *B. fragilis*, and *A. muciniphila* (Table 7).

Correlation between gut microbiota and clinical scores in the HCC group

No significant correlations could be detected between gut microbiota and either Child–Pugh grade or TNM stage. A correlation was detected, however, between the presence of *B. fragilis* and BCLC staging, with the highest detection in stage IV patients (66.7%) (Table 8).

DISCUSSION

The gut and the liver are physiologically and anatomically connected, known as the gut–liver axis. Gut microbiota and their metabolites affect both hepatocytes and stromal cells (hepatic stellate and Kupffer cells) (Ohtani and Kawada, 2019). Intestinal dysbiosis is also observed in different chronic hepatic diseases, which has caused concern on the role of the gut microbiota in the development and progression of hepatic diseases and aggravation of related complications (Sandler *et al*., 2011; Konturek *et al*., 2018). Few studies have evaluated the role of gut dysbiosis in patients with viral hepatitis. Therefore, in this study, we sought to compare changes that occur in the gut microbiota of patients with chronic hepatitis C, HCC, and normal healthy controls.

The different species of the genus *Bifidobacterium* are reported to have beneficial health effects, including the regulation

Table 5. Comparisons between HCC patients and control as regards studied gut micro-biota.

Gut micro-biota	HCC patients(N = 100)	Control (*n* = 100)	X^2	*p*-value
Bifidobacterium (n%)	43 (43%)	76 (76%)	22.6	<0.001 (HS)
B. fragilis (n%)	41(41%)	34 (34%)	1.04	0.307 (NS)
Lactobacillus (n%)	80 (80%)	36 (36%)	39.7	<0.001 (HS)
A. muciniphila (n%)	46 (46%)	49 (49%)	0.18	0.671 (NS)
F. prausnitzii (n%)	51 (51%)	70 (70%)	7.6	0.006 (S)
E. coli (n%)	81 (81%)	58 (58%)	12.5	<0.001 (HS)

X^2 = Chi-square test.
S = *p*-value < 0.05 is considered significant.
HS = *p*-value < 0.001 is considered highly significant.
NS = *p*-value > 0.05 is considered non-significant.

Table 6. Comparison between chronic HCV infected patients and control as regards studied gut micro-biota.

Gut micro-biota	HCV patients (*n* = 200)	Control (*n* = 100)	X^2	*p*-value
Bifidobacterium (n%)	88 (44%)	76 (76%)	27.5	<0.001 (HS)
B. fragilis (n%)	82 (41%)	34 (34%)	1.37	0.241 (NS)
Lactobacillus (n%)	124 (62%)	36 (36%)	18.1	<0.001 (HS)
A. muciniphila (n%)	83 (42%)	49 (49%)	1.52	0.217 (NS)
F. prausnitzii (n%)	75 (38%)	70 (70%)	28.2	<0.001 (HS)
E. coli (n%)	140 (70%)	58 (58%)	4.27	0.339 (S)

X^2 = Chi-square test.
S = *p*-value < 0.05 is considered significant.
HS = *p*-value < 0.001 is considered highly significant.
NS = *p*-value > 0.05 is considered non-significant.

Table 7. Comparison between HCC patients and HCV patients as regards gut micro-biota.

Gut micro-biota	HCC patients (*n* = 100)	HCV (*n* = 200)	X^2	*p*-value
Bifidobacterium (n%)	43 (43%)	88 (44%)	0.027	0.869 (NS)
B. fragilis (n%)	41(41%)	82 (41%)	0.0	1.0 (NS)
Lactobacillus (n%)	80 (80%)	124 (62%)	9.9	0.002 (S)
A. muciniphila (n%)	46 (46%)	83 (42%)	0.55	0.458 (NS)
F. prausnitzii (n%)	51 (51%)	75 (38%)	4.9	0.026 (S)
E. coli (n%)	81 (81%)	140 (70%)	4.2	0.041 (S)

X^2 = Chi-square test.
S = *p*-value < 0.05 is considered significant.
NS = *p*-value > 0.05 is considered non-significant.

of homeostasis of other intestinal microbes, the suppression of pathogens and harmful bacteria that colonize the gut mucosa, the modification of local and systemic immune responses, the inhibition of procarcinogen enzymatic activities of the gut microbiota, and the mending of the gut mucosal barrier by lowering the levels of lipopolysaccharides (Mayo and van Sinderen, 2010; Pinzone *et al*., 2012). In our study, less than half of patients with HCC and chronic HCV infection had detectable *Bifidobacterium* in their stool samples, compared with 76% of healthy volunteers. This finding agrees with Yu *et al*. (2010), who found that decreased *Bifidobacterium* species led to the accumulation of lipopolysaccharides, which act as pathological mediators of inflammation-associated HCC. In addition, impairment of gut wall integrity due to the absence of *Bifidobacterium* may allow translocation of pathogenic bacteria and endotoxins, which can lead to chronic hepatic inflammation with a subsequent risk of the development of HCC.

It is known that *Lactobacillus* species are important for human health, as they can decrease gastrointestinal pH to protect the host against invasion by pathogens (Martín *et al*., 2013). On the other hand, *Lactobacilli* may also be pathogenic in susceptible patients via several mechanisms, including the ability of some strains to bind to the intestinal wall and translocate into the bloodstream, leading to bacteremia. Also, some can adhere to collagen in the extracellular matrix and produce glycosidase, an enzyme that contributes to the damage of affected tissues (Apostolou *et al*., 2001; Harty *et al*., 1994; Oakey *et al*., 1995). In this study, most patients with HCC and chronic HCV infection had greater amounts of *Lactobacillus* in their stool than healthy controls, with more detected in patients with HCC than in chronic

Table 8. Correlation between the studied gut- microbiota and different scores in the HCC group.

Variables	Child staging				*p*-value
	Child A (*n* = 18)	Child B (*n* = 38)	Child C (*n* = 44)		
Bifidobacterium (*n*%)	7 (38.9%)	13 (34.2%)	23 (52.3%)		0.239 (NS)
B. fragilis (*n*%)	6 (33.3%)	12 (31.6%)	23 (52.3%)		0.126 (NS)
Lactobacillus (*n*%)	12 (66.7%)	29 (76.3%)	39 (88.6%)		0.112 (NS)
A. muciniphila (*n*%)	10 (55.6%)	18 (47.4%)	18 (40.9%)		0.563 (NS)
F. prausnitzii (*n*%)	7 (38.9%)	20 (52.6%)	24 (54.5%)		0.517 (NS)
E. coli (*n*%)	14 (77.8%)	31 (81.6%)	36 (81.8%)		0.928 (NS)
TNM staging					
	TNM I (*n* = 24)	TNM II (*n* = 40)	TNM III (*n* = 28)	TNM IV (*n* = 8)	
Bifidobacterium (*n*%)	9 (37.5%)	18 (45%)	12 (42.9%)	4 (50%)	0.914 (NS)
B. fragilis (*n*%)	9 (37.5%)	16 (40%)	12 (42.9%)	4 (50%)	0.931 (NS)
Lactobacillus (*n*%)	18 (75%)	34 (85%)	21 (75%)	7 (87.5%)	0.633 (NS)
A. muciniphila (*n*%)	14 (58.3%)	15 (37.5%)	15 (53.6%)	2 (25%)	0.195 (NS)
F. prausnitzii (*n*%)	12 (50%)	21 (52.5%)	13 (46.4%)	5 (62.5%)	0.872 (NS)
E. coli (*n*%)	21 (87.5%)	34 (85%)	20 (71.4%)	6 (75%)	0.403 (NS)
BCLC staging					
	BCLC I (*n* = 28)	BCLC II (*n* = 26)	BCLC III (*n* = 34)	BCLC IV (*n* = 12)	
Bifidobacterium (*n*%)	9 (32.1%)	9 (34.6%)	19 (55.9%)	6 (50%)	0.201
B. fragilis (*n*%)	3 (10.7%)	13 (50%)	17 (50%)	8 (66.7%)	0.001**
Lactobacillus (*n*%)	21(75%)	22 (84.6%)	27 (79.4%)	10 (83.3%)	0.832
A. muciniphila (*n*%)	21 (75%)	22 (84.6%)	27 (79.4%)	10 (83.3%)	0.832
F. prausnitzii (*n*%)	12 (42.9%)	15 (57.7%)	20 (58.8%)	4 (33.3%)	0.316
E. coli (*n*%)	24 (85.7%)	20 (76.9%)	29 (85.3%)	8 (66.7%)	0.441

X^2 = Chi-square test.
HS = *p*-value < 0.001 is considered highly significant.
NS = *p*-value > 0.05 is considered non-significant.

HCV-infected patients. This agrees with Sherid *et al*. (2016) who reported the involvement of *Lactobacillus* in the development of bacteremia and liver abscesses. On the contrary, Zhang *et al*. (2012) reported that disturbance of gut microbiota homeostasis with decreased *Lactobacilli* led to the damage of mucosa, endotoxemia, systemic inflammation, and tumor formation.

In healthy individuals, *F. prausnitzii* represented >5% of the gut flora. This species plays an important role in improving the immune system, as it has anti-inflammatory activities such as promoting IL-10 secretion and inhibiting IL-12 and interferon-γ expression (Fukui, 2019; Miquel *et al*., 2013). In this study, *F. prausnitzii* was detected in about half of the patients with HCC and 38% of the patients with chronic HCV infection compared with 70% of healthy controls. This agrees with Munukka *et al*. (2017) who found mice treated with *F. prausnitzii* had improved hepatic ALT and AST levels and decreased adipose tissue inflammation. Also, in a study on patients with HCC, Liu *et al*. (2019) found that HCC is caused by different factors that decreased levels of *Faecalibacterium,* which resulted in reduction in the levels of anti-inflammatory short-chain fatty acids.

The family *Enterobacteriaceae* includes medically relevant species such as *Salmonella*, *E. coli*, *Yersinia pestis*, *Klebsiella*, *Shigella*, *Proteus*, *Enterobacter*, *Serratia*, and *Citrobacter*. Many members of species of this family are normally present in humans as gut microbiota. Some *Enterobacteria* are pathogens because they produce endotoxins; when released into the bloodstream, they can cause a systemic inflammatory and vasodilatory response (Brenner *et al*., 2005; Paterson and Doi, 2017). In this study, *E. coli* was found in stool samples of about 80% of patients with HCC and 70% of patients with chronic HCV infection. This finding agrees with many studies that reported that increased *Enterobacteriaceae* was linked to the progression of liver cirrhosis and development of cirrhosis-related complications (Bajaj *et al*., 2014; Chen *et al*., 2011; Lax *et al*., 2012; Sanduzzi Zamparelli *et al*., 2017).

Bacteroides is the most predominant bacteria in colon, with *B. fragilis* being the most abundant among all *Bacteroides* species (Ahmed *et al*., 2018). *Bacteroides,* together with other gut commensal bacteria, provides the human body with energy through carbohydrate fermentation, producing a pool of volatile fatty acids that are absorbed by the colon (Lopez *et al*., 1996). *Akkermansia muciniphila* is one gut microbiome known to have an anti-inflammatory effect in humans by improving hepatic inflammation and protecting liver cells against damage via an immune cell-mediated mechanism. Also, this species is believed to have a role in cancer response to immunotherapy (Routy *et al*., 2018; van Passel *et al*., 2011; Wu *et al*., 2017). In our study, we found no significant difference between patients with HCC and chronically HCV-

infected patients in comparison with the control group regarding stool isolates of *B. fragilis*. However, Chen *et al*. (2011) found a significant decrease in *Bacteroides* levels in patients with liver cirrhosis-related complications. On the other hand, Xie *et al*. (2016) reported a marked increase in *Bacteroides* species levels in a mouse model of HCC development post-nonalcoholic steatohepatitis.

Also, in this study, no significant difference was detected between HCC patients and chronic HCV patients compared with the control group regarding the stool isolate of *A. muciniphila*. *Akkermansia muciniphila* was detected in the stool of less than half of patients with HCC and HCV. To some extent, different experimental studies on animal models are in accordance with our results. They have demonstrated that the presence of gut *A. muciniphila* can enhance the anticancer effect of T-cell-based immunotherapies (Matson *et al*., 2018; Routy *et al*., 2018; Sivan *et al*., 2015; Vétizou *et al*., 2015).

Though many staging systems for HCC are used worldwide, no system is considered superior in evaluating suitable treatment and patient prognosis (Wildi *et al*., 2004). Child–Pugh and TNM have a better predictive ability for overall survival than BCLC (Sirivatanauksorn and Tovikkai, 2011). However, TNM fails to evaluate patient prognosis accurately because it only evaluates tumor extension, and BCLC has demonstrated better prognostic ability than the TNM staging system (Cillo *et al*., 2006). Therefore, in this study, we used different systems to evaluate patients with HCC. No significant differences were detected between gut microbiota and HCC progression with respect to Child–Pugh or TNM scoring systems. However, a significant difference was detected between the number of positive stool isolates of *B. fragilis* and the BCLC staging system; *B. fragilis* was isolated from 66.7% of patients with BCLC stage IV compared with 10.7% of patients with BCLC stage I. This agrees with Guoxiang *et al.* who found a marked increase in *Bacteroides* species along with increased lipopolysaccharide levels correlated with the progression of liver disease from steatosis, leading to HCC development (Xie *et al*., 2016).

CONCLUSIONS

A characteristic pattern of *Bifidobacterium*, *Lactobacillus*, and *E. coli* species was detected in patients with chronic HCV and HCC. Dysbiosis may, therefore, be associated with the progression of liver disease and hepatic carcinogenesis. It may also suggest that a probiotic can help prevent the progression of liver disease. However, the role of dysbiosis in the development of liver disease and hepatic carcinogenesis is under debate. Further studies are needed in a large cohort of patients to derive additional meaning from these results.

DECLARATION

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AUTHOR'S CONTRIBUTION

Study concept and design: KM, HAO, HA, MHH, and AMMS. Patient selection and clinical assessments: HAO and AMMS. Biochemical and molecular analysis of stool and blood samples: KM, HA, and MHH. Statistical analysis: KM, HAO, HA, MHH, and AMMS. Literature search: KM, HAO, HA, MHH, and AMMS. All authors revised and approved the final manuscript version.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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GRAPHICAL ABSTRACT