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ORIGINAL ARTICLE

Gut–Liver Interactions in Patients with Nonalcoholic Fatty Liver Disease: Role of Intestinal Flora and Toll-like receptors

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ABSTRACT

Background: *There is a diverse community of micorganisms that supports the homeostasis of metabolic status and the balanced immune response in the host. Disturbed gut microbiome composition was found to be associated with multiple diseases in humans. Furthermore, there is an impaired balance between nutritional absorption and energy storage that is involved in the pathogenesis and progression of non-alcoholic fatty liver disease (NAFLD).* ***Objectives:*** *Investigate whether the gut bacterial microbiome could have a role in the inflammatory process leading to NAFLD development and decipher their effect on the immune response represented by Toll like receptor-4 levels.* ***Methodology****: One hundred and five adult participates were enrolled in the study, 70 patients diagnosed with NAFLD, they were further subdivided into 32 cases with pure steatosis (SS), and 38 cases with non alcoholic steatohepatitis (NASH), as well as 35 healthy subjects, all matched for age, gender. Full history and clinical examination, Body mass index (BMI), abdominal ultrasonography and liver biopsy were done. Liver function tests, lipid profile, blood sugar, insulin and C-peptide, fasting insulin, and serum levels of Toll like receptor (TLR-4) were measured. Fresh stool samples were homogenized and either used for immediate aerobic and anaerobic cultures of bacteria, or for measurement of bacterial flora levels using quantitative real time PCR of genes encoding 16S rRNA.* ***Results:*** *BMI, Fasting glucose, LDL Cholesterol, HOMA-IR, AST, ALT and GGT were significantly increased in NAFLD group when compared to control group. The serum levels of TLR-4 were significantly increased in NAFLD groups compared to normal control group, with SS having the lowest level than that detected in NASH group. Levels of bacteroids and lactobacilli were significantly increased in NAFLD groups compared to control group and its level was significantly increased in NASH compared to simple steatosis. Clostridia, bifidobacteria, and E.coli showed no significant difference among groups. The increased copy numbers of bacteroids as well as lactobacilli were positively correlated with BMI, ALT, AST, fasting glucose, triglycerides and, TLR-4 serum levels.* ***Conclusion:*** *Gut microbiota play an important role in NAFLD-related pathophysiology. Further large-scale studies are recommended to understand the mechanism of such effect and to evaluate possible therapeutic targets for prevention and control of NAFLD.*

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), is a form of chronic liver disease ranging in severity from simple fat infiltration (steatosis) to an advanced form, nonalcoholic steatohepatitis (NASH, steatosis with liver inflammation) with subsequent progression to fibrosis.[1] The relationship between gut flora and the development of NAFLD have been under study for almost the last decade. Uncontrolled growth of gut bacteria and translocation of gut microorganisms from the intestinal lumen predispose patients to multiple bacterial infections. [2]

The portal vein carries blood containing digested products and gut microbiota products to the liver.[3] Factors like increased gut permeability, and increased bacterial endotoxins, especially lipopolysaccharide (LPS) levels accompanied by activation of proinflammatory molecules are the main players involved in the development of alcoholic and NASH4. Bacterial LPS promote the development of NASH through production

of tumor necrosis factor (TNF)–α 5 and interleukin (IL)-1α. [6]

The human gut is home to around 100 trillion commensal organisms. This diverse community of organisms maintains the homeostasis of metabolic status and immune response in the host.[7] Disturbed gut bacterial environment was found to be associated with obesity and type 2 diabetes in animals and humans.[8] Some previous studies revealed that gut microbiota are associated with several human diseases, e.g., obesity, diabetes, autoimmune diseases, gastroenterological diseases, and liver diseases.[5]

In addition, changes in the gut bacteria can affect the absorption and storage of nutritional materials. There is an impaired balance between nutritional absorption and energy storage that is involved in the pathogenesis of NAFLD. [9] Bacterial flora produce short-chain fatty acids (SCFAs) through fermentation of complex carbohydrates in human colon, which consequentlymodulate the production of various inflammatory mediators, including TNF-α, IL–2, IL-6, and IL-10. [10]

The immune system is abnormally activated in patients with cirrhosis and ascites.[11] Bacterial translocation (BT) from the gut initiates a local inflammatory responseat the mesenteric lymph nodes through lymphocytes activation.[12] The process starts with increased reactive oxygen species (ROS) production in Kupffer cells and hepatocytes resulting in initiation of T-helper 1 (Th1) immune response and the production of tumor necrosis factor (TNF)-α by monocytes and promotes systemic inflammation. liver damage and mitochondrial dysfunction will follow which contribute to fat accumulation and development of NAFLD.[9]

Therefore, the liver-gut interaction is central to understand the pathophysiology of liver diseases, including non-alcoholic fatty liver disease (NAFLD) and hepatic encephalopathy. Current knowledge on the association between gut microbial composition and NASH is incomplete. Liver malfunction leads to altered genetic composition of gut microbial, and in the meantime specific bacterial species are present during liver pathogenesis, and the abundance of some bacteria is correlates with grade of liver disease, implying the term “gut-liver axis”.

Toll-like receptors (TLRs) constitute very important players in this microbiome balance. TLRs are trans membrane non-catalytic receptor proteins that prompt activation of innate and adaptive immune responses to microorganisms through recognition of conserved molecular patterns of organisms. In order to maintain immune tolerance to the luminal microorganisms the expression of TLRs by intestinal epithelial cells is normallyat its minimal. TLRs trigger a cascade of signal transduction with subsequent release of inflammatory cytokines and chemokines including TNF-α, IL-6, IL-10, TGF-β, CCL2, CCL5, and CXCL8. These released cytokines are associated with NASH progression and hence liver fibrosis.[13] Additionally, the gut bacteria provide a source of TLR ligands, and any compositional change can further increase the amount of TLR ligands delivered to the liver, which can stimulate liver cells to produce proinflammatory cytokines.[14] The current study aimed to investigate the possible role of gut bacteria in the development of non-alcoholic fatty liver and to find their relation to Toll like receptor-4 representing the innate immunity.

METHODOLOGY

Patients:

The present study was conducted on 70 patients (49 males and 21 females, age range 30- 52 years, all were attending the outpatient clinic of the National Liver Institute, from February 2016 to November 2016. NAFLD diagnosis was based on the ultrasonography changes, liver histopathologic features and laboratory findings. Thirty five (25 males and 10 females) with age ranged from 31-51 years were selected as a healthy control group. They were negative for serological markers of hepatitis B and C, with normal ultrasonography evaluation of their liver. Inclusion criteria: Negative markers for hepatitis B and C virus infection, absence of markers for autoimmune hepatitis, absence of alcohol consumption.

Exclusion criteria: Previously diagnosed diabetes mellitus, essential hypertension, those with features of early cirrhosis, autoimmune hepatitis, and patients received immunomodulators, immunosupressors or steroids, patients with inflammatory bowel disease, or receiving purgatives or antibiotics.

The protocol of the study was approved by ethical committee of NLI-Menoufia University and Al Azhar University, and a written consents were taken from the participants.

The following investigations were done to patients and control subjects:

1. Anthropometric examination including: Body mass index (BMI) was calculated as weight (in kilograms) divided by height squared (meters squared). Waist circumference was measured at the midpoint between the lower rib margin and the iliac crest.
2. All participants were subjected to complete medical and abdominal ultrasonography.
3. Liver biopsy to when possible to confirm patient diagnosis.
4. Venous blood was withdrawn from all participates following an overnight fast and collected in sterile vacutainer tubes, then centrifuged and the resulting serum was divided in aliquots that were kept at –20°C until assayed**.**

Liver function tests (ALT, AST, GGT, ALP, Albumin), as well as glucose level, lipid profile (triglycerides, total cholesterol, HDL-c and LDL-c) were measured using COBAS 6000 (Roche Diagnostics). Insulin and C peptide concentrations were measured on COBAS e 601 module (Electrochemiluminescence Technology, Roche Diagnostics- Germany). The degree of insulin resistance (IR) was calculated from the homeostasis model assessment (HOMA). HOMA index >3 is a criterion of insulin resistance**.** The HOMA index was calculated by the equation; Fasting plasma insulin (mIU/ml) **x** fasting plasma glucose (mmole/L)/ 22.5.

Fresh stool samples from all participants were immediately put into stool buffer until transported to laboratory, then homogenized by vortex and one part was used for immediate aerobic and anaerobic cultures of bacteria. Agar plates were incubated aerobically at 37°C for 24 hour to enumerate total aerobic bacteria or anaerobically for 48 hours. After bacterial growth, colony forming units (CFUs) was enumerated and expressed as the number by per gram of stool sample. Another part of homogenized stool sample was kept in aliquot and stored at –80°C for DNA extraction and molecular analysis.

Toll Like Receptor-4 (TLR-4) assay:

TLR-4 concentrations were tested in patients' sera by a quantitative sandwich enzyme immunoassay technique using MyBioSource kit (California, San Diego (USA). Standard and samples, 100µl each were added to wells, then after incubation for 2 hours at 37°C, the liquid was aspirated (no washing) and 100µl of Biotin-antibody were added to each well. Plates were further incubated for 1 hour at 37°C followed by washing for 2-3 times. After washing, 100µl of HRP-avidin were added to each well followed by for 1 hour incubation at 37°C. The washing step was repeated, then 90µl of TMB Substrate was added to well and incubated for 30 minutes at 37°C in the dark. The optical density of each well was measured by a microplate reader set to 450 nm within 5 minutes. The intra-assay precision: CV%<8% and the sensitivity of the assay is less than 0.039 ng/ml.

detection of gut bacteria: *DNA extraction*:

Bacterial lysis was done by heating the homogenized stool sample to 95°C for 5 minutes, then DNA extraction and purification were done using a phenol/chloroform extraction method. In short, 100 mg of frozen feces was put in 750 µl of buffer containing of (200 mMNaCl, 100 mMTris [pH 8.0], 20 mM EDTA, 20 mg/ml lysozyme (Sigma-Aldrich) for bacterial lysis, then incubated for 30 minutes incubation at 37°C. Then 40 µl of 20% potassium proteinase and 85 µl of 10% SDS were added to the homogenate, and a second incubation was done at 65°C for 30 min followed by cooling on ice and centrifugation at high speed for 5 minutes. The supernatant was aspirated and transferred to 1.5 ml microfuge tube and further extraction was done for fecal DNA by phenol/chloroform/iso-amyl alcohol. The extracted DNA was precipitated by absolute ethanol at -20°C for 1 hour and suspended in DNase free H2O and then cleaned by cleaning solution and DNA concentrations were calculated.

Determination of quantitative PCR:

The quantitative PCR (qPCR) was done using a real-time ABI PRISM 7000 Sequence Detection System (Applied Biosystems, ABI). primers that amplify the genes encoding 16S rRNA from specific bacterial groups using the QuantiTect SYBR® Green PCR kit (Qiagen, Valencia, CA) were the ones used. Plates with 96-well was used, 1 × SYBR green qPCR Master Mix (Qiagen), 0.5 µM of each primer and 50 ng of purified fecal DNA in in a final volume of 25 µl. The PCR cycles were as follows: 15 min at 95°C, followed by 40 cycles of 95°C for 1 min, 30 s at the appropriate annealing temperature, and 72°C for 1 min. The amplification of bacterial groups was done by using the following primers:Escherichia coli:
Forward 5'GTTAATACCTTTGCTCATTGA-3',
reverse 5'ACCAGGGTATCTAATCCTGTT-3'
Bacteroides spp.:
Forward 5'ATAGCCTTTCGAAAGRAAGAT5-3',
Reverse 5'CCAGTATCAACTGCAATTTTA5'-3',
Lactobacillus spp.:
Forward 5'AGCAGTAGGGAATCTTCCA-3', Reverse 5'CACCGCTACACATGGAG-3', Bifidobacterium spp.: Forward 5'GGGTGGTAATGCCGGATG-3',
Reverse 5'TAAGCGATGGACTTTCACACC-3',
Clostridium spp.: Forward 5'CGGTACCTGACTAAGAAGC 3', Reverse 5'AGTTTYATTCTTGCGAACG-3'.

Total bacterial 16S rRNA in each sample was determined using universal 16S rRNA primers; forward, 5'-GTGSTGCAYGGYTGTCGTCA-3'; reverse, 5'-ACGTCRTCCMCACCT TCCTC-3') in fecal DNA from NAFLD patients and healthy controls.

Quantitative PCR standards were made by amplifying and cloning 16S rRNA target genes from a positive control strain by PCR method. Each sample DNA and standards were put in plates and the fluorescence signal originated from specific PCR products was given on melting curve of the PCR products. The concentrations of each bacterial group present in the NAFLD patients were expressed as a fold change as compared to healthy control group.

Statistical Analysis

Data has been collected and entered to the computer using SPSS (Statistical Package for Social Science) program for statistical analysis, (version 17; Inc., Chicago. IL). Data was entered numerical or categorical, as appropriate. Quantitative data was shown as mean, and SD, while qualitative data has been expressed as frequency and percent. Chi- square test was used to measure association between qualitative variables. Student t-test has been used to compare mean and SD of 2 sets of quantitative normally distributed data, while Mann Whitney test was used when this data

is not normally distributed. One way analysis of Variance (ANOVA) test will be used for comparison between three or more groups having quantitative normally distributed data and LSD post hoc test was done, while Kruskal-Wallis test will be used when this data is not normally distributed. Spearman's correlation has been used to study correlation between two variables having not normally distributed data. P-value was considered statistically significant when it is less than 0.05.

RESULTS

NAFLD patients were further classified as 32 cases with pure steatosis (SS), and 38 cases with non alcoholic steatohepatitis (NASH) by the criteria proposed by *Brunt et al.*[15]. The demographic and laboratory data are summarized in Table 1. The age and genders distribution was not statistically different between groups. Patients with SS and NASH had higher BMI and waist circumference when compared to health controls (HC) despite that all selected patients were non-obese (P<0.001 in both)(Table 1).

Clinical, metabolic, and biochemical markers among studied groups

Transaminases (ALT, AST), Triglycerides, fasting glucose, insulin, Alp, and GGT were significantly higher in NASH compared to SS and controls (P value: 0.001, 0.002, 0.001, 0.0001, and 0.001 respectively). HOMA-IR was higher in patients with NASH compared to SS (p =0.001). No differences were found in cholesterol levels between groups. All patients had normal liver synthetic function as determined by albumin levels (table 2).

In addition, the patients with either SS or NASH had lower levels of high-density lipoprotein cholesterol (p <0.001, and <0.01 respectively) compared with those of controls (table 2). TLR-4 levels were significantly increased in patients with NAFLD compared to control group. This increase was mainly attributed to higher levels of TLR-4 in NASH group than SS group, which alone did not show any significant difference from controls.

Bacterial populations in stool samples

Patients with SS and NASH had significantly higher fecal Bacteroides levels (2.5 and 3 fold respectively, p< 0.01) and lactobacilli (5 and 6 folds respectively, p < 0.001) compared to those with controls as depicted in (fig. 1). On the other hand, there were no differences between the groups regarding levels of bifidobacteria, clostridia, or E. coli.

Moreover, The levels of aerobic bacteria in fecal samples from NAFLD patients were significantly lower compared to those from healthy controls (2.8×10^7 vs. 7.2×10^8 CFUs/g feces, P = 0.001) for SS and (3×10^7 vs. 7.2×10^8 CFUs/g feces, P = 0.002) for NASH (fig. 2 C, 2D). The levels of anaerobic bacteria in fecal samples from NAFLD patients were significantly higher than healthy controls (3.4×10^9 vs. 2×10^8 CFUs/g feces, P = 0.01) for SS and (2.9×10^9 vs. 2×10^8CFUs/g feces, P = 0.014) for NASH. (fig. 2A, 2B)

In the current study, we measured fecal microorganisms changes in NASH patients along with its relationship to clinical and laboratory parameters and Toll-like receptor-4. Looking for potential relationships between BMI and bacteria counts, we found no statistically significant correlations between BMI, and fecal, Clostridia, bifidobacteria, or E. coli (p > 0.05) (Table 4) (Fig.3).

Interestingly, there was a statistically significant positive association between BMI and fecal Bacteroidetes counts (r= 0.55, $p < 0.01$) (Table 4)(Fig. 3). Significant correlations with ALT (r = 0.662, P <0.0001), AST (r = 0.637, P <0.001), triglycerides (r = 0.457 $p < 0.001$), LDL (r = 0.217 $p < 0.03$), and TLR-4 (r=0.779 p<0.001) were found for Bacteroidetes. For Lactobacilli, it showed positive correlation with BMI (r = 0.62, $p < 0.01$). There was also significant correlations between Lactobacilli counts and ALT (r = 0.689, p <0.001), AST (r = 0.73, P <0.001), Triglycerides (r = 0.467p < 0.001), LDL (r = 0.294, p < 0.03), HOMA (r = 0.2, p < 0.04), and TLR4 (r = .779 p < 0.001) (table 4) (fig. 3). Clostridia, Bifidobacteria, and E.coli showed no significant association with these markers (table 4) (fig. 3). Our findings suggest the role of intestinal flora in NAFLD and its potential as a marker to differentiate among individuals with or without NAFLD.

Table 1: Demographic and anthropometric data of studied groups.

Variables	*Controls n=30 Mean+SD*		*Simple steatosis n=32 Mean+SD*		*NASH n=38 Mean+SD*		*P-value*
Age (year)	39.66+5.71		41.84+5.97		41.07+6.46		0.367
BMI	24.46+1.61		31.68+3.08		32.21+3.12		<0.001
Waist (cm)	81.26+4.64		87.90+9.01		91.21+7.71		<0.001
	no	**%**	**no**	**%**	**no**	**%**	
Gender:							
-Male	20	66.7	23	71.9	26	68.4	
-Female	10	33.3	9	28.1	12	31.6	0.902

Table 2: Comparison between NAFLD and control groups regarding routine laboratory data.

Variables	*Controls n=30 Mean+SD*	*NAFLD n=70 Mean+SD*	*P-value*
HOMA	2.32+0.68	3.00+1.01	0.001
Triglyceride (mg/dl)	141.63+11.47	172.50+20.29	<0.001
Cholesterol (mg/dl)	190.53+17.34	196.51+19.58	0.151
HDL (mg/dl)	38.96+4.72	31.60+3.64	<0.001
LDL (mg/dl)	113.00+14.08	113.85+11.65	0.753
Fasting glucose(mg/dl)	84.23+7.42	93.58+7.89	<0.001
Insulin (mIU/ml)	6.54+1.16	93.58+1.32	<0.001
Albumin (g/dl)	4.44+0.36	3.91+0.29	<0.001
AST (U/L)	26.83+6.29	44.39+12.08	<0.001
ALT (U/L)	26.23+5.43	65.06+9.15	<0.001
GGT (U/L)	43.13+4.74	66.50+9.39	<0.001
ALP (U/L)	52.43+8.59	64.10+8.41	<0.001
TLR-4 (ng/ml)	3.45+0.62	5.42+2.23	<0.001

Table 3: Comparison between NAFLD subgroups and control groups regarding clinical and routine laboratory data of studied groups.

Variables	*Controls n=30 Mean+SD*	*Simple steatosis n=32 Mean+SD*	*NASH n=38 Mean+SD*	*P-value*	*Within groups p-value**
HOMA	2.32+0.68	3.50+1.04	2.58+0.78	<0.001	P1=<0.001 P2=0.210 P3=<0.001
Triglyceride (mg/dl)	141.63+11.47	178.09+19.51	167.78+19.97	<0.001	P1=<0.001 P2=<0.001 P3=0.017
Cholesterol (mg/dl)	190.53+17.34	198.75+19.67	194.63+19.56	0.239	P1=0.091 P2=0.379 P3=0.368
HDL **(U/L)**	38.96+4.72	30.75+3.31	32.31+3.79	<0.001	P1=<0.001 P2=<0.001 P3=0.103
LDL **(U/L)**	113.00+14.08	107.75+10.01	119.00+10.48	<0.001	P1=0.77 P2=<0.001 P3=<0.001
Fasting glucose (mg/dl)	84.23+7.42	93.21+8.01	93.89+7.89	<0.001	P1=<0.001 P2=<0.001 P3=0.719
Insulin (mIU/ml)	6.54+1.16	7.89+0.49	9.41+1.41	<0.001	P1=<0.001 P2=<0.001 P3=<0.001
Albumin (g/dl)	4.44+0.36	3.91+0.32	3.92+0.26	<0.001	P1=<0.001 P2=<0.001 P3=0.965
AST (U/L)	26.83+6.29	34.59+5.68	52.63+9.61	<0.001	P1=<0.001 P2=<0.001 P3=<0.001
ALT (U/L)	26.23+5.43	62.28+6.09	67.39+10.62	<0.001	P1=<0.001 P2=<0.001 P3=<0.001
GGT (U/L)	43.13+4.74	70.22+7.96	63.37+9.44	<0.001	P1=<0.001 P2=<0.001 P3=<0.001
ALP (U/L)	52.43+8.59	65.28+9.64	63.11+7.19	<0.001	P1=<0.001 P2=<0.001 P3=0.286
TLR-4 (ng/ml)	3.45+0.62	3.75+0.87	6.84+2.03	<0.001	P1=0.404 P2=<0.001 P3=<0.001

P1 between controls and simple steatosis, P2 between controls and NASH, P3 between simple steatosis and NASH.

Table 4: Correlation data of biochemical data and risk factors with altered bacterial counts

Variables		*Bacteroids*	*Lactobacillus*
BMI	**P value**	<0.001	<0.001
	r	0.553	0.625
Triglycerides	**P value**	<0.001	<0.001
	r	0.457	0.467
HOMA	**P value**	0.067	0.041
	r	0.184	0.205
ALT	**P value**	<0001	<0.001
	r	0.662	0.689
AST	**P value**	<0.001	<0.001
	r	0.737	0.730
LDL	**P value**	0.030	0.003
	r	0.217	0.294
TLR-4	**P value**	<0.001	<0.001
	r	0.779	0.804
Aerobic bacteria	**P value**	<0.001	<0.001
	r	-0.469	-0.510
Anaerobic bacteria	**P value**	0.647	0.023
	r	0.046	0.227

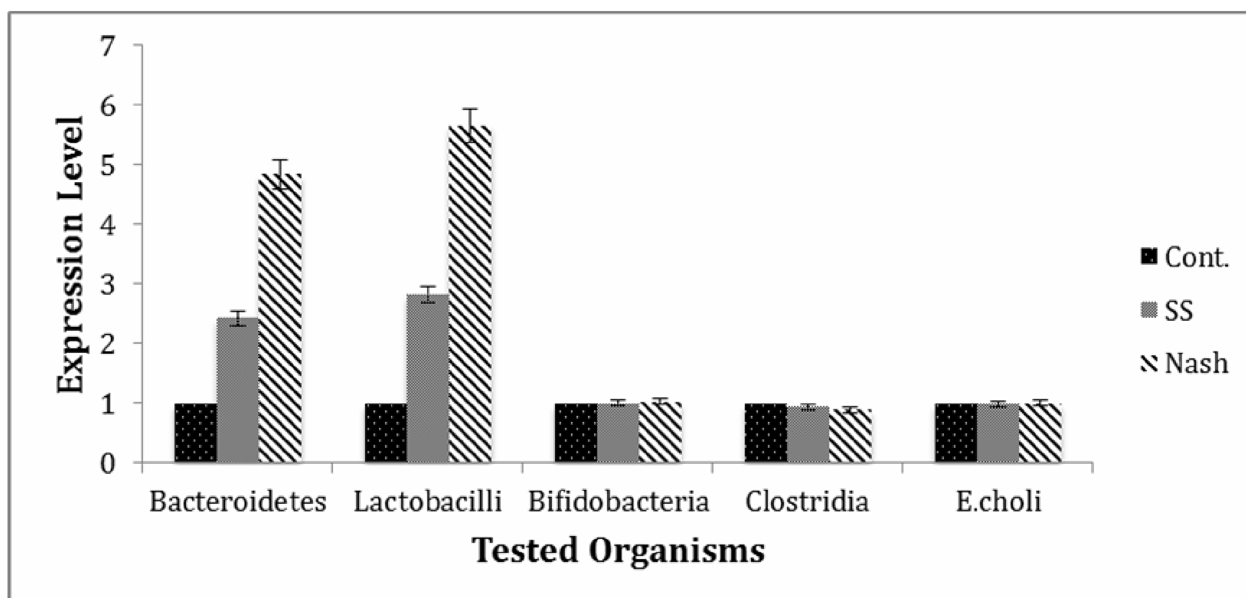

Fig. 1: Altered levels of gut microorganisms in patients with NAFLD

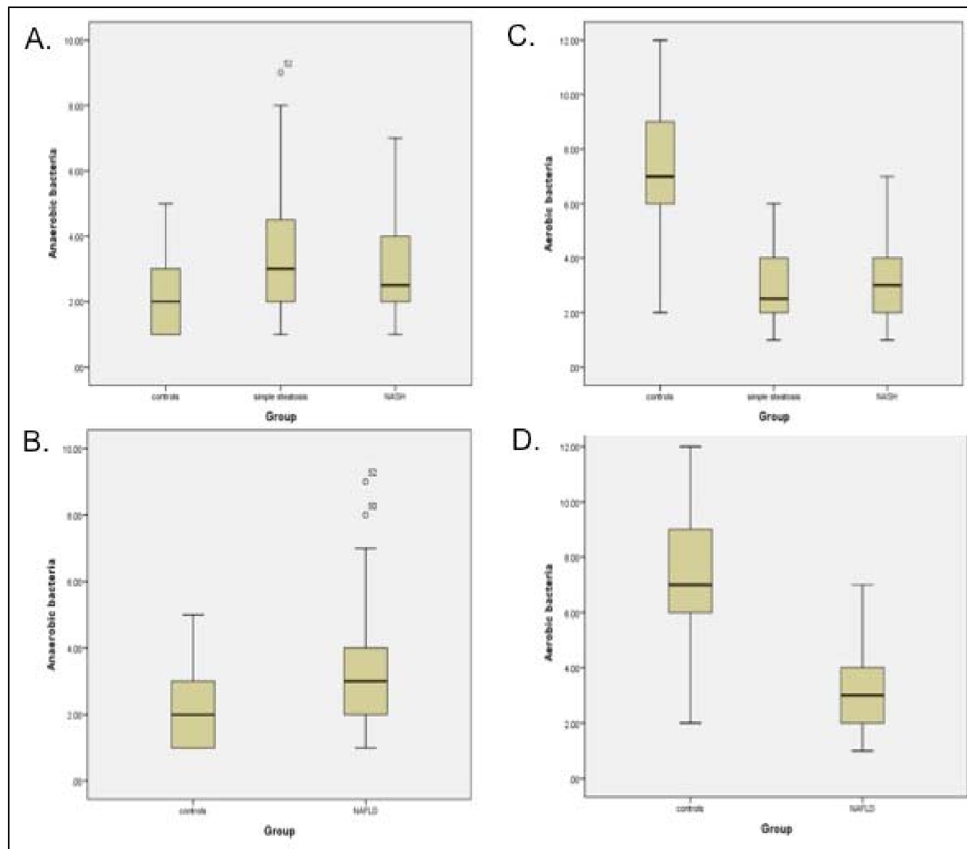

Fig. 2: Levels of aerobic and anaerobic bacteria among studied groups

Variables	BMI	Triglycerides	Fasting Glucose	ALT	AST	GGT	TOLL R
Bacteroids							
Bifidobacteria							
Clostridium							
Lactobacillus							
E.coli							

Positive correlation **P<No correlation =0.01**

Fig. 3: Correlation matrix of bacteriological data with the laboratory data

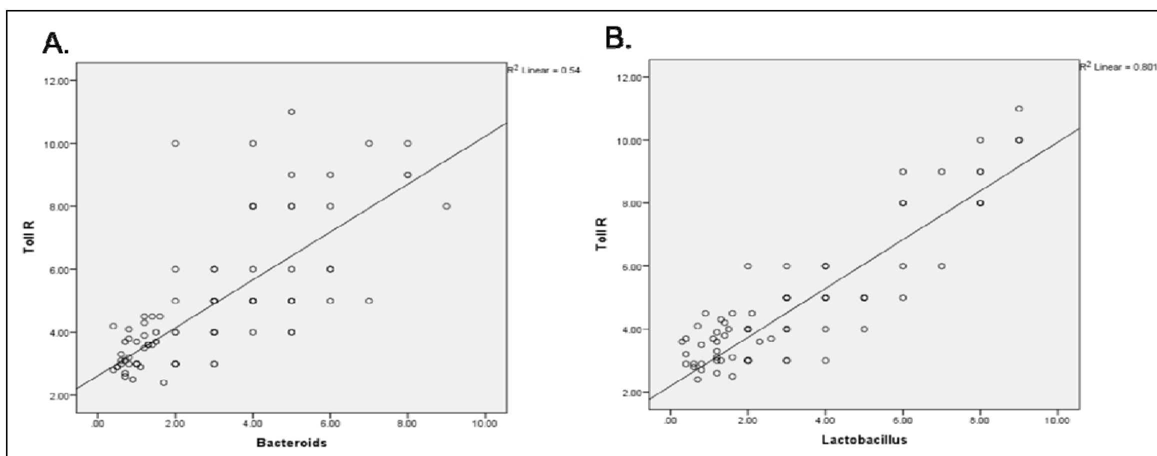

Fig. 4: Correlation study of TLR-4 levels with the abundance of a) Bacteroids and, b) Lactobacilli

DISCUSSION

The exact pathogenesis of NAFLD is not entirely understood. Some liver intrinsic factors include altered hepatic glucose metabolism, insulin resistance, and altered lipid metabolism.[16] Other factors such as bacterial overgrowth, immune dysfunction, alteration of the luminal factors, and altered intestinal permeability are involved in the pathogenesis of NASH and its subsequent complications.[9]

Huge numbers of bacterial species constitute the gut bacterial flora, and they all display an unbelievably wide range of metabolic functions. Our studies revealed that the altered microbiota associated with the presence of NAFLD significantly correlated with liver biochemical markers. *Bacteroidetes* represent the dominant bacteria in our bodies, accounting for 99% of the whole microbiome.[17]

In this study, PCR with 16S rRNA-based specific primers was used as the most sensitive and rapid method for detection of bacterial flora. Since the conventional PCR methods does not give the quantitative detection of the target bacteria, using species-specific primers in real-time PCR can quantify the bacteria through measurement of SYBR Green I fluorescence which determine the amount of PCR products in each cycle done.

Our results showed that *Bacteroidetes were increased in NAFLD group compared to controls. In agreement* with our results, higher levels of *Bacteroidetes* were reported by Zhu *et al.* in obese patients compared with healthy controls[7]. *On the contrary*, reports by Mouzaki*et al.*, have shown lower *Bacteroidetes*l evels in NASH patients than in patients with simple steatosis and controls.[18], [19] The decreased levels of *Bacteroidetes* has also been demonstrated in patients with high BMI previously.[20]

Bacteria such as *Lactobacillus* and *Bifidobacterium, are* probiotic bacteria have beneficial effects that are carried out through anti-inflammatory actions leading to stabilization of the intestinal barrier. Consequently they constitute a barrier against liver damage.[21] A number of studies, which were done, previously demonstrated that the probiotic bacteria ameliorate features of NAFLD in humans[22] and in mouse models.[23]

A significant number of studies support the important role of the gut flora imbalance in the pathogenesis of metabolic disturbance in NAFLD.[24] According to accumulating data, patients with NASH and NAFLD have different fecal microbiome composition than healthy controls. A probable explanation of the contradicting reports is that, different clinical and laboratory make up of the studied groups could affect the microbiome population differently. Still future studies are needed to establish the clinical relevance of our findings to the pathogenesis of NAFLD. In this study we found a significant correlation between the bacteroids and lactobacilli on one side and well-known clinical and biochemical data associated with of NAFLD on the other side, such as higher blood ALT, AST, GGT, BMI, and glucose levels. This finding suggests that gut microbiome imbalance is involved in the progression of NAFLD. Wang et al[3] reported similar results to our findings. They showed that NAFLD associated with microbiome dysbiosis is also accompanied by higher blood biochemical indices.

For this reason and based on the other mentioned reports showing that gut flora play a significant role in the development of alcoholic liver disease and hepatocarcinogenesis., trials using probiotics was performed to investigate their role in the prevention or treatment of liver diseases[18].

In our study we found that TLR-4 levels correlated positively with bacteroidetes and lactobacilli counts (R=0.22, and 0.29 respectively). Similarly, TLRs role was examined in mice knockout models by researchers who discovered that, more severe diet-induced NASH resulted from influx of intestinally derived toll-like receptor-4 (TLR-4) and toll-like receptor[9] (TLR-9) agonists into the portal circulating activated tumor necrosis factor alpha (TNFα) in the liver. For all, understanding the mechanistics of TLR-4 role in human NASH would be the next research requirement.

Antibiotic treatment with ciprofloxacin and metronidazole in transgenic mice lacking inflammosome that developed worsened NASH, reduced the severity of disease and abolished transmission of the phenotype confirming that gut microbiota drove NASH progression in this model. These findings are clinically relevant, as human studies have demonstrated that NASH patients usually have greater endotoxemia and higher liver TNFα levels than patients with simple hepatic steatosis.[7]

In addition, administration of norfloxacin failed to influence alanine aminotransferase levels, or markers of endotoxin release when tested in non-diabetic patients with NAFLD.[25] Modifying the microbiota might have a beneficial effect on some pathological conditions, and complications of liver disease could potentially be reduced by altering the microbiota.

In conclusion, it has become obvious that gut microbiota play an important role in NAFLD-related pathophysiology, but the biggest challenge is to understand the mechanisms by which they affect NAFLD. Studying these mechanisms will help identify new therapeutic targets that can improve the outcome in affected patients. In addition, probiotics and prebiotics administration can help correct the imbalance in gut microflora, a theory that might represent an important therapeutic strategy in the treatment of NAFLD.

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