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COMPARISON OF BIFIDOBACTERIUM AND LACTOBACILLUS SPECIES COUNT IN STOOL OF NON-ALCOHOLIC FATTY LIVER DISEASE GROUP WITH HEALTHY PERSON

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ABSTRACT

Background and Study Aims: Non-alcoholic fatty liver disease characterized by an increase of more than 5% to 10% of fat in the liver, is a chronic and multifactorial disorder. The aim of this study was to evaluate different species of Bifidobacterium and Lactobacillus, between patients with non-alcoholic fatty liver disease and healthy individuals.
Patients and Methods: From July to September 2014, 20 patients with histopathologically confirmed non-alcoholic fatty liver disease and 20 healthy individuals as controls were selected. Morphological and biochemical tests were used for identification of Bifidobacterium and Lactobacillus isolates from stools, and the number of Lactobacillus and Bifidobacterium spp. was performed using the pour plate assay.
Results: In the current study, total tested intestinal bacteria significantly differ between healthy individuals and patients with non-alcoholic fatty liver disease (Pv 0.030). The healthy group tended to have a higher frequency of L.fermentum, L.reuteri, L.salivaricus, B.longum, B.bifidum, and B.adolescentis count compared to non-alcoholic fatty liver diseases patients. In contrast, the L.acidophilus count, in non-alcoholic fatty liver disease patients, was higher than in the healthy group.
Conclusion: In conclusion this study shows that the imbalance in intestinal microbiota especially B.longum, L.fermentum, and B.reuteri may lead to an increased risk of non-alcoholic fatty liver disease.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a chronic and multifactorial disorder, which is strongly associated with insulin resistance.[1] NAFLD is characterized by an increase of more than 5% to 10% of fat in the liver. [2] The disease is rapidly growing in the world and has become a global health concern that occurs in all age groups and in both sexes.[1, 3] Its prevalence in adults is between 20% and 30% .[2]

In normal hosts, about 1014 microorganisms live in the human gut, of which roughly 99% include anaerobic bacteria.[4, 5] These microbiome lead to improved bowel function, food intake and metabolism of drugs.[6] Several factors such as age, diet, antibiotic treatment, health habits and infections affect the balance of the normal flora. Transfer of bacterial products, such as ethanol and LPS, from the intestinal lumen to mesenteric lymph circulatory system activates Kupffer cells in the liver, resulting in the production of pro-inflammatory cytokines by macrophages and increased production of free radicals.[7] Therefore, the

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liver is constantly exposed to LPS, which it derives from intestinal Gram-negative bacteria, thus, it helps the progression of inflammatory disease steatosis to NAFLD.[8]

Probiotics are live microorganisms that have beneficial effects on the human health.[9] The most important effects of probiotics are their place in the small intestine, their stimulation of the intestine, and their clearance of pathogens. This prevents the pathogens from sticking to the intestinal wall and inhibits the toxic effects of the toxins. In fact, the addition of prebiotics to the diet regimen is a non-drug factor for the treatment of chronic inflammatory diseases such as NAFLD.[10] The aim of the present study was to determine the difference between the number of Bifidobacterium spp. and Lactobacillus spp. in patients with NAFLD and healthy people.

Patients and Methods

Subjects

From July to September 2014, 20 biopsy– proven NAFLD patients and 20 healthy individuals, as controls, were selected. The liver biopsy is the gold standard method for diagnosis of NAFLD, however, the liver biochemical parameters alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was measured and ultra- sonography was also used. The exclusion criteria were use of antibiotics, the presence of severe infections, gastrointestinal disorders as constipation, diarrhea and abdominal pain 2 weeks prior to fecal sampling; diabetes mellitus, alcohol consumption, cholestatic diseases, triglycerides level more than 500 mg, chronic intestinal inflammatory diseases, heart failure, kidney disease, autoimmune diseases, hepatitis B and C, which was confirmed by the gastroenterologist in Imam Reza Hospital, Tabriz, Iran. Finally, the health status of the control group was confirmed. The study was approved by the Regional Ethics Committee, Tabriz University of Medical Sciences, Iran (No: 9361).

Bacteria strains

For each of the subjects, stool specimens were collected in sterile containers. Then, specimens were transferred quickly to the microbiology laboratory of Tabriz University of Medical Sciences. Serial dilution of fecal samples 1/10 with sterile physiological serum containing NaCl (0.85% m/v) enriched with 0.05% L-cysteine was prepared.[11]

In this study, 4 species of Lactobacillus (L.acidophilus, L.salivaricus, L.fermentumand and L. reuteri) and 3 Bifidobacterium species (B.Longum, B.bifidum and B.adolescentis) were evaluated in both groups. Diluted samples were cultured according to the standard pour plate method in MRS agar medium (Merck, Germany) and Bifidobacterium agar medium (Merck, Germany). All plates were placed in the anaerobic jar, under favorable conditions (0% = O2, 10% = CO2, 10% = H2) by Anoxomat (Mart, Lichtenvoorde, Nederland). The samples were incubated at 37°C for 72 hours. Identification of Lactobacillus was performed based on standard morphological methods. After morphological verification, biochemical tests, including sugar fermentation, catalase and methyl red test were performed.[12] Identification of Bifidobacterium genus with phenotypic testes were performed and fructose-6-phosphate phosphoketolase enzyme was used for the phenotypic confirmation of Bifidobacterium genus.[13] To identify the species of Bifidobacterium, sugar fermentation tests were also used.[11]

Statistical analysis

All the data was analyzed with SPSS software version 20.0 (SPSS Inc, Chicago, IL, USA). Comparative statistics were calculated using the two-tailed $\chi 2$ test and Fisher's exact test, and t-test when appropriate. A Pv of ≤ 0.05 was considered to be significant.

Results

A total of 20 individuals in the control group (10 female and 10 male) and 20 NAFLDs (10 female and 10 male) were investigated in the study. The results of ALT and AST in NAFLDs group were significantly more than the control group (Pv ≤ 0.05) (Table 1).

In this study, 4 species of Lactobacillus (L.acidophilus, L.salivaricus, L.fermentumand and L. reuteri) and 3 Bifidobacterium species (B.Longum, B.bifidum and B.adolescentis) were identified in both groups (Table 2). In the current study, the total tested intestinal probiotic bacteria significantly differ between healthy individuals and patients with NAFLD (Pv 0.030). The total number of Lactobacillus ssp. in NAFLD patients was higher than in the healthy group, but there was no significant difference between the two groups (Pv 0.13). However, the difference between the total numbers of Bifidobacterium spp. in healthy individuals, compared to those with a NAFLD, is statistically significant (Pv 0.000) (Figure 1).

Comparison of the number of Lactobacillus and Bifidobacterium spp. showed that the count of L.fermentum, L. reuteri, L.salivaricus, B.longum, B. bifidum, and B. adolescentis, in healthy individuals, was higher compared to those with NAFLD. In contrast, the L. acidophilus count in NAFLD was higher than in the healthy group.

Discussion

The present study showed that there is a smaller amount of some species of the Lactobacillus and Bifidobacterium spp. in the intestinal flora of NAFLD patients, in contrast to the control group. However, despite the decline of some species of Lactobacillus spp. in patients with NAFLD (L.salivaricus, L.fermentum and L.reuteri**)** this reduction had no significant impact on the total amount of genera Lactobacillus (Pv 0.13). In contrast, reducing the number of B.longum, B.adolescentis, and B.bifidum in patients with NAFLDs had significant impact on the total number of Bifidobacterium genera and also the total intestinal tested bacteria (Pv ≤ 0.05).

Although the pathogenesis of NAFLD is not entirely clear, two theories have been proposed for this condition. The first hypothesis is that fat accumulation in the liver cause insulin resistance. The second theory states that oxidative stress and specifically lipid peroxidation leads to the production of pro-inflammatory cytokine such as TNF-α, adipokinaz and mitochondrial dysfunction. This simple steatosis then over time develops into non-alcoholic steatohepatitis (NASH).[14-17] However, a survey in a similar formation NAFLD has not been done. Nevertheless, a study has been conducted comparing type-1 diabetes in children with healthy children, and a significant increase in the number of bacterial genera such as Clostridium spp., Bacteroides spp., and Veillonella spp. has been observed. Also, a significant decrease in the number of bacterial genera such as Lactobacillus, Bifidobacterium, Blautiacoccoides/ Eubacteriumrectale and Prevotella spp. was reported in children with type-I diabetes. The finding could also confirm that diabetes is a risk factor of NAFLD development, also the bacterial flora in these patients was changed, which is similar to results in the present study.[18]

In a study conducted by Loguercio et al., the patients were classified into 4 groups (22 NAFLDs, 20 alcoholic fatty liver, 20 chronic hepatitis, and 16 cirrhosis patients), VSL # 3 was given for 3 months. In the NAFLD group and alcoholic fatty liver group, lipid peroxidation indicators improved significantly. Moreover, a reduction of cytokines (TNF -α, IL-6 and IL-10), in alcoholic fatty liver patients, was observed. This study showed that the imbalance in intestinal flora can be one of the causes of chronic liver diseases. Therefore, changes in the intestinal flora through probiotic agents can be an effective way to improve the treatment of chronic liver diseases.[19] In a study conducted by Xu et al., high-fat rats were divided into two groups. In their study, B.longum was added to the diet of rats in the first group and L.acidophilus to the second group. The results of this study suggest that, B.longum is more effective than L. acidophilus in reducing the accumulation of fat in the liver.[20] According to the studies on bacteria species, Bifidobacteria is the dominant in the faeces.[4] This suggests that this species of Bifidobacteria in the gut imbalance can be one of the causes of chronic liver injury, similar to NAFLD. Therefore, it is possible that the Bifidobacterium species is a key factor in the treatment of chronic liver diseases.[20] In another study conducted by Malaguarnera et al., 66 patients with NASH were randomly divided into two groups. The first group was prescribed lifestyle changes and for the second group, in addition to lifestyle changes, fructooligosaccharides (FOS) was administered in combination with B.longum. A significant reduction in serum levels of AST, TNF-α, C-reactive protein (CRP), homeostasis model assessment of insulin resistance (HOMA-IR), and endotoxin levels were reported in the group receiving B.longum bacteria and FOS along with lifestyle modification compared to those that only modified their lifestyle. It was found that NAFLD and NASH had significant improvement.[21,22] Evaluation of studies shows that despite conflicting results, there are similarities in some of the results with previous studies.

To date, this is the first report on the effects of Bifidobacterium and Lactobacillus spp. on NAFLD in Iran. Here, solely Bifidobacterium and Lactobacillus spp. were studied, but stool include many different microbiota. Thus it would be very advisable for other normal flora to be studied, in order to determine the normal flora effects on NAFLD. We also suggest molecular detection, because some probiotics are fastidious or non-cultivable, which may help in identifying the pathogenesis and in designing a program to control the disease. Since the importance of NAFLD is increasing around the world, NAFLD management is one of the main problems of human health. It seems that implementing Bifidobacterium species into diet may be an important factor in the prevention of NAFLD or its treatment strategy. We recommend conducting more studies concerning this issue and particularly conducting in vivo and clinical trial searches before the administration of probiotics in the treatment of NAFLD.

In conclusion, the decrease in Bifidobacterium genus, especially B.longum followed by B.bifidum, and B.adolescentis might lead to an imbalance of the normal intestinal flora, and may increase the risk of NAFLD.

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Table 1. Demographic and laboratory data of subjects

Parameter	**Control (n =20)**	**NAFLD (n = 20)**	**Pv**
Sex (M/F)	10/10	10/10	≤1.00
Age (years)	54 ± 12	53 ± 11	≤0.849
AST (U/L)	22 .2 ± 5.1	60.2 ± 9.7	≤0.050
ALT (U/L)	21.9 ± 5.8	28.8 ± 7.5	≤0.012

Table 2. Comparison of bacterial count between control and NAFLD groups

Bacterial count	**Control (cfu/gr)** ± SD	**NAFLD (cfu/gr)** ± SD	**Pv**
L. acidophilus	2.2×1011 ± 0.81×1011	7×1011 ± 6.5×1011	0.102

L.salivaricus	3.3×1010 ± 1.8×1010	1.1×1010 ± 1.0×1010	0.072
L.fermentum	3.9×1010 ± 1.5×1010	6.5×106 ± 4.4×106	0.000
L.reuteri	1.8×109 ± 1.1×109	4.5×106 ± 4×106	0.001
B.longum	22×1011 ± 8.1×1011	2.7×1011 ± 1.2×1011	0.000
B. bifidum	4.2×1010 ± 2.6×1010	2.9×1010 ± 1.6×1010	0.408
B. adolescentis	1.0×109 ± 0.9×109	0.25×109 ± 0.24×109	0.134
Total Lactobacillus spp.	2.9×1011 ± 1.0×1011	7.1×1011 ± 6.5×1011	0.131
Total Bifidobacterium spp.	22×1011 ± 8.2×1011	3×1011 ± 1.3×1011	0.000
Total	25×1011 ± 8.8×1011	10×1011 ± 6.6×1011	0.030

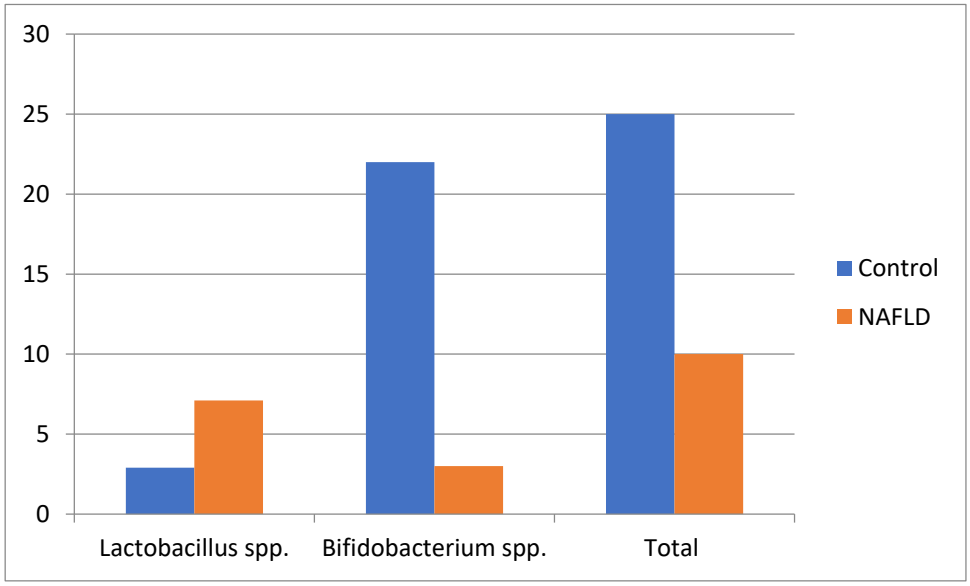

Figure 1. Total count of the Lactobacillus and Bifidobacterium spp. in control and NAFLD groups