L.ACIDOPHILLUS AND BIFIDOBACTERIUM SPP CONTRIBUTE IN THE TREATMENT OF CHRONIC HEPATITIS C PATIENTS

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Abstract- Hepatitis C virus is a major leading cause of Liver pathogenesis. Probiotics showed a natural therapeutic activity against liver disorders. The antiviral and the antibacterial responses to L. acidophilus and Bifidobacteria spp in patients with chronic hepatitis C virus is a recommended study. Patients with chronic hepatitis C were treated with probiotics capsule that contains L. acidophilus and Bifidobacterium spp. They administered one capsule per day for a month before HCV treatment, blood and urine samples were collected before and after the given treatment and they were processed for a quantitative estimation of HCV by PCR, identification of bacteria by VITEK2 system and 16S r RNA gene sequencing assay, moreover, the estimation of antibacterial activity of probiotics, as well as counts of leukocytes, $CD3^+$ T cells and $CD56^+$ natural killer cells. Administration of Probiotics capsule enhanced the treatment response rate to HCV treatments as IFN-α and ribavirin by 25%. It had antibacterial activity against five species of the most common bacterial infections also It increased $CD3^+$ cells counts and $CD56^+$ natural killer cells in chronic HCV patients. Administration of probiotics as a capsule before HCV treatment can act as a supportive supplement with antiviral and antibacterial activities.

Index Terms — $CD3^+$; $CD56^+$; Hepatitis C virus; Lactobacillus acidophilus; Bifidobacterium bifidum.

I. INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of pathogenesis of liver disease that including chronic hepatitis, fibrosis, cirrhosis, hepatocellular cancer, liver failure and death. Currently, no effective vaccine is available for HCV infection [1]. The most recent estimates of disease burden shows about185 million infections worldwide [2]. One of the most common HCV drug therapy is pegylated interferon (IFN) and ribavirin combination therapy but it was limited to about 55% of patients with a lower efficacy and associated with severe side effects [3]. Recently, Sovaldi®, alone or in combination with IFN and ribavirin, has also been introduced as a new treatment modality with promising antiviral effects [4, 5]. However, exploring new therapeutic protocol especially those that can improve the existing ones and lower their toxicity can have significant impact on the anti-viral responses. One of the promising modalities is natural products as well as probiotics that have potential anti-toxic effects on liver functions [6].

Probiotics are defined as live microorganisms which are consumed as food or therapeutic supplements with beneficial effects on the health [7]. For instance, Lactobacilli and befidobacteria are beneficial in the treatment of intestinal micro-ora disturbance by increasing gut permeability [8-10]. Moreover, probiotics produce antimicrobial metabolites such as lactic, acetic, organic acids, hydrogen peroxide, diacetyl and bacteriocins, resulting in reducing the pH and inhibiting growth of pathogenic bacteria and re-establishment the microbial equilibrium [11-13] With this regard, it was found that Lactobacilli and bifidobacteria provides antibacterial activity against common pathogenic bacteria such as Escherichia coli, Staphylcoccus aureus and Salmonella typhimurium [14], Enterobacteriaceae species, Klebsiella and E. coli. Indeed, these gram negative bacteria as well as gram-positive bacteria including Staphylococcus aureus are the most common pathogenic bacteria in patients with chronic HCV which is considered as secondary bacterial infections[15-17].

Beside their anti-bacterial activities, recent studies showed that both Lactobacilli and bifidobacteria have different immunomodulatory effects, including increasing phagocytosis NK cell activity (18, 19), IgG and IgA synthesis [20,21], production of cytokines (Th1 or Th2) in both in vivo and in vitro systems, the development and maturation of mucosal and systemic NKT cells [22, 23] The cytotoxic effects of natural killer (NK; $CD3^-CD56^{dim}$) cells against virally infected cells caused inhibition of HCV replication as well as their cytotoxicity against hepatocellular carcinoma (HCC) [24]. The effects of probiotics on these cells are paramount significance; especially these NK cells can activate multiple elements of janus kinase/signal transducer and transcription (JAK/STAT) pathway, resulting an induction of endogenous IFN-alpha/beta expression in hepatocytes and enhancement of anti-HCV cell-mediated immunity [25]. Additionally, probiotic bacteria differentially activated dendretic cells (DCs) in vitro and induced $CD4^+$ T cells [26]. With these beneficial effects, probiotics have been recommended for use as biological product drug system by FDA [27].

On basics of these findings of their antiviral and antibacterial activities and their immunomodulatory effects, this study aimed to enhance utilize L.acidophillus and B.bifidum as a supportive adjuvant system to the conventional treatment strategy based on IFN-α and ribavirin.

II. MATERIALS AND METHODS

Patients

This study was adopted on selective patients (males and females) with chronic HCV who were diagnosed by a specialist physician that selected them according to a mean age of 47± 5 years and their positive results of HCV antibody antigen test and polymerase chain reaction (PCR) estimation which showed high viral load. The exclusion criteria included liver cirrhoses, HCC, hepatitis B virus (HBV), diabetes and/or renal impairment. Patients were recruited from the Virology Unit, El-Obour Hospital, Kafr El-sheikh, Egypt.

Ethical approval: This study has an approval letter from the ethical committee, Faculty of Medicine, Tanta University, Egypt.

Treatment strategies involved in the study

Lactobacillus acidophilus, Bifidobacterium bifidum combination (1.5 billion cells) was used in this study in the form of a capsule (Phillips'® Colon Health, Bayer Health Care, USA).The ingredients included potato starch, gelatin, and silicon dioxide. The probiotics with long term benefit takes longer to withstand the difficult conditions within the host body and colonize the gut, colon or the small intestine. The process may take days to months with the least reported being 2-3 weeks [28]. Two treatment strategies involved in this study. The first group (group A) included healthy volunteers as controls were administered probiotics capsule once a day for one month (4 weeks). The second group (group B) of chronic hepatitis C patients was administered probiotics capsule once a day for one month before treatment with IFN-α and ribavirin (single interferon injection weekly for 12 weeks). The third group (group C) of chronic hepatitis C patients were treated with only IFN-α and ribavirin (12 weeks). Patients were followed up clinically during the time course of experiment.

Samples collection:

Blood and urine samples were collected from the patient groups in the time range from October 2013 up to April 2014. Ten samples were collected before and after probiotics supplementation on (day 1, and day 30) from healthy volunteers as controls (group A). Twenty samples were collected before and after the given treatment strategies on day 1, 30 then day 120 in the patients group B and C.

Reagents and materials:

The following media were used. 1) Nutrient agar (29) PH was adjusted to 5.0). 2) Mannitol salt agar (30) (Final pH: 7.4 ± 0.2 at 25ºC). 3) MacConky agar (pH 7.4 ± 0.2 at 25ºC). Gram stain (Crystal violet, Gram's iodine solution, acetone/ethanol (50:50 v: v), 0.1% basic fuchsin solution).

- VITEK 2 system [31], (bioMerieux Inc., Hazelwood, MO) VITEK 2 Cassette Loaded with Cards and Suspension Tubes being loaded Into the Automatic Transport System.

-Antibodies used for antibacterial sensitivity test [32] included the following: nitrofurantion F, doxycyclin DA, OFloxacin OFX, Nalidexic acid NA, Vancomycine VA, Tetracycline TE, Erythromycin E, Amplicilin sulbactam SAM, Imipenem IPM, Amikacin AK, chloraphenicol C, Ciprofloxacin CIP, Levofloxacin LEV, Piperacillin-tazobactam TPZ, sulfamethoxazole SXT, Cefuroxime CXM, Cefataxime CTX, Amoxicilin calvulinic acid (AMC).

- The QIAGEN gel extraction kit for 16S rRNA gene sequencing (33) was purchased from Sigma (Sigma Scientific Services Co., Cairo, Egypt).

-Antibodies: Anti-human CD3 and CD56 monoclonal antibodies (BD Biosciences, SanJose, CA, USA) were used for immunophenotype analysis.

BD FACS lysing solution for RBCs lysis and PBS buffer solution for washing and suspension. EDTA tubes were used as anticoagulant tubes for blood collection.

Quantitative estimation of HCV RNA

Blood samples were used for RNA extraction. 20 µL of the extracted RNA was added to 30 µL of Master Mix in each 0.1 mL microtube and test was performed in automated instrument (HVD Auto Q server) [34]. A standard curve was automatically drawn with software using five quantification standard concentrations of HCV-RNA to analyze the viral RNA load.

Isolation of bacteria from patients with chronic HCV and antibacterial responses to L. acidophilus and B. bifidum

Isolation of bacteria from urine samples was performed on nutrient agar inoculated with 100 µl of each urine sample and incubated at 37ºC for 18-24 hours. Investigation of the isolated bacteria by Gram reaction was performed, eventually pure subculture of gram positive and gram negative were cultured on 20 mL of sterilized Macconky and Mannitol media, respectively, at 37ºC for 18-24 hours. The load of pathogenic bacteria was observed by detection of CFU/ml based on this equation CFU/mL = (no. of colonies x dilution factor) / volume of culture plate. Moreover, numbers of the infected patients before and after probiotic administration were recorded.

Identification of isolated bacteria:

VITEK 2 system:

This system provided 64 identification tests processed for identification of isolated bacterial strains while, BioMerieux VITEK® 2 system version: 06.01, it was used as lactase, alkalinization,

growth under inhibition conditions like oxidase, enzyme hydrolysis acidification test. Sufficient numbers of pure colonies were transferred into 3.0 mL of sterile saline, a special rack (cassette) of the suspended microorganism and the identification card were placed in slots that moved to the optical system where readings were observed each 15 minutes and the data were recorded.

Antibacterial sensitivity test (AST) (Kirby Bauer technique)

Sterile Petri plates with 20 mL of sterilized MacConkey and Mannitol agar were inoculated with 100 µL of each isolated bacteria, the antibiotics discs were placed on plates and incubated for 18-24 hours at 37ºC. Inhibition zones diameters were recorded in mm according to the Clinical and Laboratory Standards Institute (CLSI) [35].

16S r RNA gene sequences identification

PCR was performed in a thermal cycler (Bio-Rad MJ Research, Hercules, USA). The 50 µL reaction mixture consisted of, 5 µL of 10 × Taq buffer (100 mM Tris-HCl, 500 mM KCl pH 8.3),2.5 U of Taq DNA polymerase, 20 ng of genomic DNA, 200 µM dNTP, 10 p moles each universal primers, Forward primer CAAACAGGATTAGATACCCTG, reverse primer CGCGAAGAACCTTACC and 2.0 mM MgCl2. Amplification started with initial denaturation for 5 minutes at 94°C , followed by 25 cycles of denaturation for 30 seconds at 94°C , annealing temperature of primers for 30 seconds at 50°C, and extension for 1 minute at 72°C . The last extension for 15 minutes at 72°C was used. submarine agarose gel electrophoresis in 1.2% agarose gel with ethidium bromide at 8 V/cm was used for analyzing 5 µL of the amplified product, gel doc UV transilluminator was used for visualizing the PCR product. The QIAGEN gel extraction kit serves as a gel purified for the amplified PCR product. GATC Company using ABI 3730xl DNA sequence using forward and reverse primers for the sequencing of a total of 100 ng/µL concentration of 16S rRNA amplified product (Sigma Scientific Services Co., Cairo, Egypt).

Flow cytometric analysis

Fresh venous blood samples were collected on EDTA tubes and100 µl was stained by human mAbs in staining tubes using the recommended concentrations by the manufactures. The stained samples were incubated 4^{o}C in dark for 20 minutes, and then RBCs were lysed by adding BD FACS or ACK lysing solution (1x) for 15 minutes then centrifugation at 1250 rpm for 5 minutes. Then, the supernatant contained lysed RBCs was discarded and the cells were washed using PBS buffer solution. The cells were acquired on FACS Calibur or FACS Canto II (BD Biosciences, San Jose, CA, USA) and analyzed using. FACSDiva, Cell Quest (BD Biosciences) and Flow jo software (36).

White blood cells

The total number of leukocytes and differential lymphocytes, monocytes and neutrophils using fully automated instrument (CBC Swelab Alpha three part differential).

III. STATISTICAL ANALYSIS

The clinical data were recorded along the study and analyzed for each patient. The statistical presentation analysis of the present study was conducted using the mean, standard deviation, Chi-square test by software SPSS V.20. , Standard student "t test" test of significance of the differences between means *P≤ 0.05, **P≤ 0.01.

IV. RESULTS

The antiviral effect of probiotics by HCV PCR assays result

The antiviral effect of probiotics was determined by HCV PCR assay for two groups (Group B and C). The outcome data illustrated that probiotics increased the number of responded patients to the IFN-α and ribavirin treatment about 95 % in group B compared to their number in group C about 70 % which was treated with only IFN-α and ribavirin treatment (Figure 1)

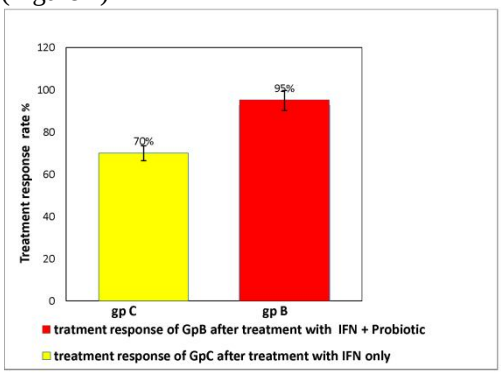


Figure1.Treatment response rate of chronich HCV patients to IFN+ribavirin treatment in presence of probiotic administration in group B and in its abence in group C.

Antibacterial activity of treatment with L. acidophilus and B. bifidum

The secondary bacterial infections in patients with chronic HCV (gp B, C) were compared to that in healthy people (gp A). It was found that, the number of patients and the load of isolated bacteria before oral administration of the probiotic capsules were higher than after administration. The loads of Gram positive and negative bacteria were reduced to 43.2% and 76%, respectively in urine samples as a response to probiotic administration in group B; the number of infected patients was reduced 60% after probiotic supplementation. On the other hand, group C didn't show any enhancement as it's nearly the same percentage of both the number of patients and the bacterial load as well as before any treatments, the

group A of healthy people revealed no bacterial growth.

Identification of Isolated bacteria using VITEK 2 system

The microscopic investigation of Gram reaction revealed presence of G+ve isolates in cocci form especially Staphylococci form while G-ve isolates were bacilli. They were cultured on selective media for further biochemical identification by VITEK 2 system. Five main bacterial pathogens were identified in patients with chronic HCV as Staphylococcus lentus, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa and Eschirechia coli.

Antibacterial sensitivity of the isolated bacteria

It was found that the investigated antibiotics against isolated bacterial species revealed different inhibition zones with variable degrees of resistance. Pseudomonas aeruginosa was resistant against the following antibiotics: Ofloxacin (OFX), Nalidixic acid (NA), chloramphenicol (C), Levofloxacin (LEV), Norfloxacin (NOR), Furantoin (F), and Ampilicillin+sulbactam (SAM). Moreover, among 5 isolated S. aureus , isolate number 2 was the most resistant against doxycyclin (DA), Nalidexic acid (NA) , Erythromycin (E) , chloraphenicol (C) , Amikacin (AK) , Piperacillin-tazobactam (TPZ), Amoxicilin calvulinic acid (AMC) (Figure 2,3).

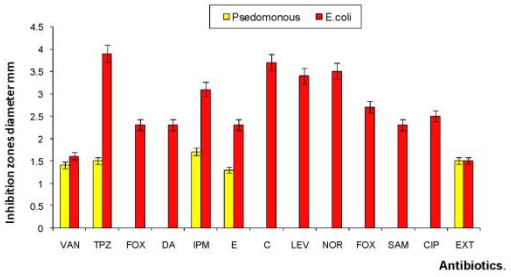


Figure 2.Inhibition zones diameters of different antibiotics that were used against *Escherichia coli* and *pseudomonas aeruginosa* and showed their resistance.

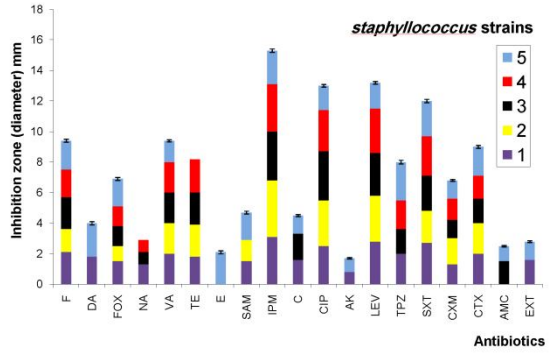


Figure 3.Inhibition zones diameters of different antibiotics against *Staphylococcus aureus strains.*

16S rRNA bacterial identification

The 16S rRNA gene sequence proved that S. aureus number 2 (highly antibiotic resistant) is subsp. strain N315 16S ribosomal RNA with identity about 89%, and the phylogenetic tree was constructed as illustrated in Figure 4.

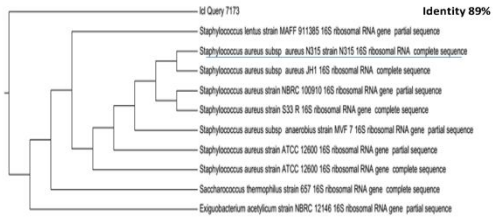
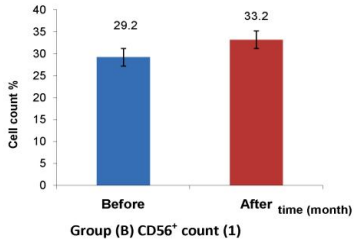


Figure 4. Phylogenetic tree of *staphylococcus aureus* by gene bank access and the highest identity was *staphylococcus aureus* N315 strain N315 16Sr ribosomal RNA

Analysis of $CD3^+$ and $CD56^+$ cells counts

Treatment with L. acidophilus and B. bifidum resulted in significant enhancement in the numbers of $CD56^+$ NK cells (P values = 0.001) and $CD3^+$ T cells (Figures 5, 6; table 1)



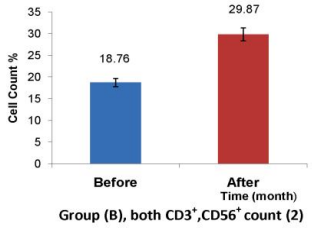


Figure 5.$CD56^+$ and $CD3^+$ cells count % in Group B of patients with hepatitis C before and after oral administration of probiotics .

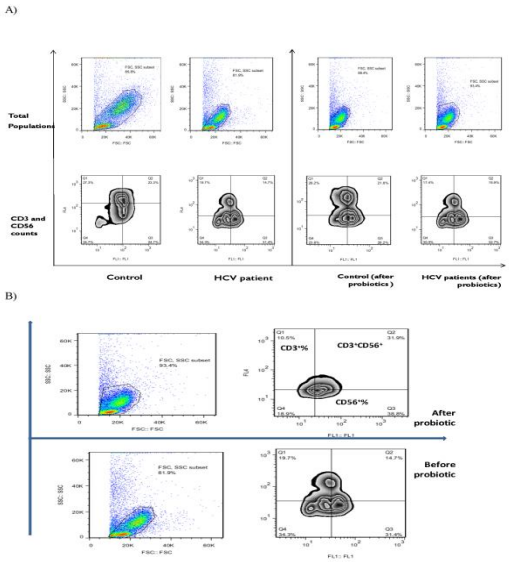


Fig. 6 A,B FlowJO analysis of $CD3^+$ (FL4) cells count %, CD56+ cells count (FL1) and total populations before and after probiotic capsule administration one per day for month before IFN-α.

Table (2):- A, B, C, the immune response in patients with chronic hepatitis c virus before and after oral administration of probiotics capsule, group (B).

(A):- $CD3^+$cells populations

CD 3^+ cells % Gp B	Before	After
Range	12 – 25	11 – 24
Mean ± SD	18.53 ± 4.26	15.43 ± 3.58
t. test	4.659	
P. value	0.040*	

(B):- $CD56^+$ cells populations

CD 56^+ cells % Gp B	Before	After
Range	22 – 38	27 – 39
Mean ± SD	29.2 ± 4.06	33.2 ± 3.84
t. test	7.692	
P. value	0.010*	

(C):- both $CD3^+$ $CD56^+$ cells populations before and after probiotic capsule

Both $CD3^+$ and $CD56^+$ cells % gpB	Before	After
Range	10 – 29	16 – 38
Mean ± SD	18.76 ± 5.43	29.87 ± 5.77
t. test	29.470	
P. value	0.001*	

P.value= 0.01-0.05 showed significant result, gpB = chronic HCV patients. As its shown probiotics increased the populations of $CD3^+$ (A)=0.04* and $CD56^+$(B)=0.01* and both (C) with high significant value = 0.001*.

It was found that the counts of $CD3^+$ and $CD56^+$ cells in healthy individuals group A were more than group B before oral administration of probiotics. $CD3^+$ and $CD56^+$cells counts increased after probiotic capsule administration in both groups A and B. In group B, their production slightly increased than group A after probiotic administration, but level of $CD3^+$ cells in Group A was more than Group B, group C didn't undergo this test as they didn't take probiotics (Figure 7; Table 2).

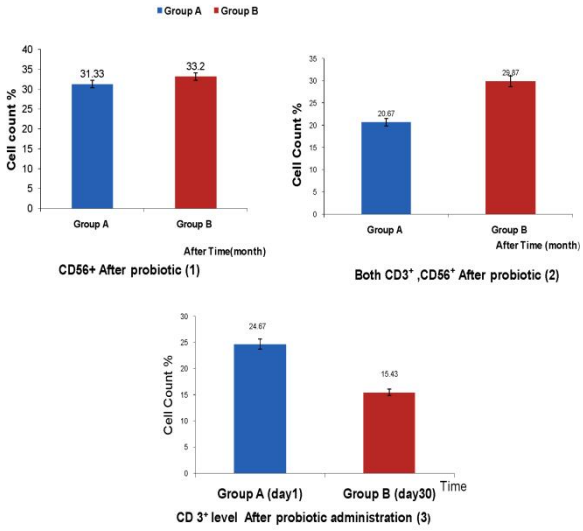


Figure 7. $CD56^+$cells (1), $CD3^+$ $CD56^+$ cells (2) and $CD3^+$ cells (3) in Group (B) of patients with hepatitis C compared to Control group (A) (Healthy people) after oral taking of probiotic capsule

Table (2):- A, B, C: - the immune response in healthy people (group A) compared with its response in patients with chronic hepatitis c virus (group B) after oral administration of probiotics capsule.

(A):- $CD3^+$ cells populations after administration of probiotics capsule for a month.

CD 3^+ cells After	Group A	Group B
Range	21 – 28	11 – 24
Mean ± SD	24.67 ± 3.51	15.43 ± 3.58
t. test	16.708	
P. value	0.001*	

P.value= 0.01-0.05 showed significant result, gpA = healthy individuals, gpB = chronic HCV patients. As its shown probiotics increased the populations of CD3+ with high significant value = 0.001*.

(B):- $CD56^+$ cells populations after probiotics capsule administration for a month.

$CD56^+$cells After	Group A	Group B
Range	26 – 35	27 – 39
Mean ± SD	31.33 ± 4.73	33.2 ± 3.84
t. test	0.555	
P. value	0.467	

P.value= 0.01-0.05 showed significant result, gpA = healthy individuals, gpB = chronic HCV patients. As shown, probiotics increased the populations of CD56+ cells with significant p.value 0.46.

(C):- $CD3^+$ $CD56^+$ cells populations after probiotic capsule administration for a month.

$CD3^+$ $CD56^+$cells % After	Group A	Group B
Range	19 – 22	16 – 38
Mean ± SD	20.67 ± 1.5	29.87 ± 5.77
t. test	7.197	
P. value	0.016	

P.value= 0.01-0.05 showed significant result, gpA = healthy individuals, gpB = chronic HCV patients. As shown, probiotics increased the populations of $CD3^+$ and $CD56^+$ cells with 0.016 significant values.

White blood cells: The differential count of Leucocytes (WBCs), including monocytes , lymphocytes and granulocytes, increased after oral administration of the probiotics capsules in group B by 8.7 %, while total WBCs count before probiotics capsule administration was 6.33% compared to group A (healthy individuals) controls as they didn't show observed change after probiotics administration, this parameter wasn't measured in group C as it didn't take probiotics (Figure 8).

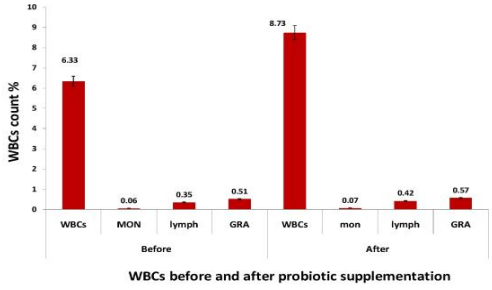


Figure 8. Leukocytes (WBCs) monocytes, lymphocytes, granulocytes count before and after the administration of probiotics capsule.

Subsequently, probiotics enhanced the immune responses by increasing the immune cells that produce T lymphocytes ($CD3^+$), natural killer cells ($CD56^+$), Monocytes, Basophiles and eosinophiles thus strengthened immune defenses against both bacterial and viral infections.

DISCUSION

In this work, we performed a study providing a principle and clinical guidance that aimed to reduce HCV severity in a representative group of Egyptian patients with chronic HCV infection (where HCV genotype 4 was dominant). The patients we treated with probioics a month before starting the conventional therapy with IFN-α and ribavirin which was the available therapy at the time of study. However the benefits of probiotics were the main objective of this study and developing new treatments which are various latterly. It was assessed the probiotic treatments on three main parameters, including the viral titer, the secondary bacterial infections, as well as the count of immune cells and their differential numbers and phenotypes. It was found that treatment with L. acidophilus and B. bifidum induced antiviral and antibacterial activities and increased the immune cell numbers. These pilot studies indicate that probiotics can be used as an adjuvant system during conventional HCV therapy.

The outcome data in vivo demonstrated that the most common secondary bacterial infections associated with chronic HCV patients were (S.lentus, S. aureus, K. pneumonia, P. aeruginosa and E.coli) from the investigated HCV patients in the current study showed resistance against common used antibiotics especially P. aeruginosa and strain no 2 S. aureus. The bacterial load and the numbers of the infected patients with bacteria remarkably reduced in response to oral administration probiotic capsules. This is in line with prior studies which detected antibacterial activity of Lactobacilli and bifidobacteria in the laboratory against common disease pathogens such as Salmonella typhimurium, E.coli and S. aureus that proofed what said by Hor, Y.Y. and Liong, M.T [37-39]. The identification with VITEK 2 system as a precise automated technique was used to identify the isolated pathogens into P. aeuroginosa, E. coli, S. lentus, S. aureus, K. pneumonia. This is consistent with prior studies [15-17] which showed that E. coli, Klebsiella, and Enterobacteriaceae species were the most common pathogenic bacteria in patients with chronic HCV infection. As expected, the group of healthy people A showed no bacterial growth on both of media. In the same connection, Jacobs et al. [40] illustrated and explained the mechanism of the antibacterial activity of Lactobacillus strains. The activity was correlated to production of metabolites such as lactic and acetic acid and that reduced the pH. In the same context, Reis [41], Ibrahim [42] explained activity of Probiotic cell free supernatant by presence of acetic acid, lactic acid, organic acids, hydrogen peroxide, diacetyl, phenols and bacteriocins using GC-Mass.

It has been established that HCV patients are immunocompromised since viral infection causes several symptoms by time related to severity of the infection and progression of the disease. Subsequently, the immunocompromised patients develop fibroses followed by cirrhosis and end up by HCC. Throughout all these stages several biochemical changes have been noticed including decreasing of albumin, increasing of liver enzymes, liver dysfunction, immune dysfunction, diarrhea, respiration and digestion difficulties [43]. As a result of these dysfunction several other side effects emerge which are related to secondary bacterial infection.

Another objective of this study was to investigate whether treating with probiotics can also enhance the immune responses through increasing the counts of immune cells $CD3^+$ and $CD56^+$. Also it was to investigate the antibacterial effects of probiotics on the load of pathogenic bacteria that including G +ve

or G-ve bacteria which was an evidence [44]. Moreover, treatment with probiotics capsule (one per day for a month before IFN-α) resulted in improvement the treatment response rate of chronic HCV patients that reflected by PCR assay result. These data suggest that probiotic can be used as a supportive therapy to the treatment of HCV infection whether it based on IFN-α treatment only or with sovaldi and IFN-α protocol. With this regard, it has been found in preclinical and clinical studies that administration of probiotics enhanced the innate and adaptive immune defenses [25], including phagocytosis, NK cell activity, IgG, IgA) [44] as well as induction of Th1 and Th2 both in vivo and in vitro systems, while maintaining the balance between the strength of the Th2-like response, and Th1/like response. In line with these studies on the effect of exogenous probiotics, endogenous probiotics (gut microbiota) have been reported to control systemic NKT cells [21].Other studies showed that probiotics, including, lactobacillus and bifidobacterium, increased the expression level of the activation molecule CD69 on $CD4^+$ (Th) and $CD3^+CD56^+$ (NKT cells) [45,46]. Additionally, probiotics are considered by FDA as biological products [27] with protecting and supporting the liver and immune function [6]. In Conclusion, oral administration of pobiotics before treatment with IFN-α and ribavirin associated with significant antiviral activity, and antibacterial effects through enhancement of immune status and their antibacterial metabolites.

DISCLOSURE

The authors declare that they have no conflict of interest.

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