Vol. 13(5), pp. 77-90, 7 February, 2019
DOI: 10.5897/AJMR2018.9028
Article Number: DC192D160105
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Lactobacillus acidophilus and *Bifidobacteria* spp having antibacterial and antiviral effects on chronic HCV infection

Nanis G. Allam[1], Mohamed L. Salem[2], Hassan Elbatae[3] and Maii Moustafa Nabieh[1*]

[1]Department of Botany, Microbiology Section, Faculty of Science, Tanta University, Egypt.
[2]Department of Zoology, Immunology and Biotechnology Unit, Faculty of Science, Center of Excellence in Cancer Research Tanta University, Egypt.
[3]Hepatology, Tropical Diseases Medicine, Kafr Elsheikh University, Egypt.

Received 25 November, 2018; Accepted 11 January, 2019

Hepatitis C virus is a serious hepatic disease that could be developed into hepatocellular carcinoma. Previously, some probiotic strains showed a natural therapeutic activity against the fatty liver disorder. Therefore, it could make sense to evaluate the antiviral and antibacterial responses to probiotics *as Lactobacillus acidophilus* and *Bifidobacteria* spp. in patients with chronic hepatitis C virus. Twenty (20) patients with chronic hepatitis C (both gender in age 47± 5 years) were treated with capsule that contains probiotics (*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 by antibiotic sensitivity test, counts of leukocytes, $CD3^+$ T cells and $CD56^+$ natural killer cells by flowcytometry, DGAT1 by ELISA. Administration of probitics enhanced the treatment response rate to HCV treatments pegylated IFN-α and ribavirin by 25% and showed an antibacterial activity against five species of the most common infectious bacteria in chronic HCV patients which were identified by this study. $CD3^+$ cells counts and $CD56^+$ natural killer cells were increased. *L. acidophilus and Bifidobacterium* spp. can act as a supportive supplement with antiviral and antibacterial activities; we recommend Probiotics side by side with HCV treatments.

Key words: Hepatitis C virus, *Lactobacillus acidophilus* and *Bifidobacteria* spp., $CD3^+$, $CD56^+$, pegylated IFN-α and ribavirin.

INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of pathogenesis of liver disease including chronic hepatitis, fibrosis, cirrhosis, hepatocellular carcinoma, liver failure and death. Currently, no effective vaccine is available for

*Corresponding author. E-mail: Mainabieh00@gmail.com. Tel: + 01201606300.

HCV infection (Dore et al*.,* 2014). The most recent estimates of disease burden show about 185 million infections worldwide (Hanafiah et al*.,* 2013).

One of the most common HCV drug therapies was 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 (Lam et al*.,* 2014). Recently, Sovaldi®, alone or in combination with IFN and ribavirin, has also been introduced as a new treatment modality with promising antiviral effects (Heathcote and Main, 2005; Gane et al*.*, 2013).

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 (Gratz et al*.,* 2010).

Probiotics are defined as live microorganisms which are consumed as food or therapeutic supplements with beneficial effects on the health (Bermudez-Britet et al., 2012; Kechagia et al., 2013; Martin et al., 2013). For instance, Lactobacilli and befidobacteria are beneficial in the treatment of intestinal micro-flora disturbance by increasing gut permeability and re-establishment of the microbial equilibrium (Deye et al*.,* 2016; Bischoff et al*.,* 2014; Goldenberg et al*.*, 2013). With this regard, it was found that Lactobacilli and bifidobacteria provide antibacterial activity against common pathogenic bacteria such as *Escherichia coli*, *Staphylcoccus aureus* and *Salmonella typhimurium* ((Bali et al., 2011; Butel et al*.,* 2014), *Enterobacteriaceae* species, *Klebsiella* and *E. coli*. Indeed, these gram negative bacteria as well as gram-positive bacteria including *S. aureus* are the most common pathogenic bacteria in patients with chronic HCV, which is considered as secondary bacterial infections (Carrion et al*.,* 2009; Jalan et al*.,* 2014).

Triacylglycerol synthesis is catalyzed by acyl-CoA: diacylglycerol acyltransferase (DGAT) enzymes, DGAT1 and DGAT2 (Harris et al*.,* 2011). DGAT1, an enzyme involved in triglyceride synthesis and luminal LD maturation, targets core to lipid droplets LDs and bounded with NS5B in active HCV replication (Camus et al*.,* 2013). Its absence or inhibition leads to the inhibition of viral assembly and production (Herker et al*.,* 2010).

Besides their anti-bacterial activities, recent studies showed that both Lactobacilli and bifidobacteria have different immunomodulatory effects, including increasing phagocytosis NK cell activity (Yuan and Walker, 2004; Candore et al., 2008; Rask et al*.,* 2013), IgG and IgA synthesis (Stagg et al*.,* 2004; Tsai et al*.,* 2012), production of cytokines (Th1 or Th2) in both *in vivo* and *in vitro* systems, the development and maturation of mucosal and systemic NKT cells (Villamil et al*.,* 2002; Pagnini et al*.,* 2010). 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) (Doskali et al*.,* 2011). 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 in an induction of endogenous IFN-alpha/beta expression in hepatocytes and enhancement of anti-HCV cell-mediated immunity (Ye et al., 2009). Additionally, probiotic bacteria differentially activated dendretic cells (DCs) *in vitro* and induced $CD4^{+}$ T cells (Hart et al*.,* 2003). With these beneficial effects, probiotics have been recommended for use as biological safe product drug system by FDA (Degnan, 2008; Snydman, 2008).

This study aimed to enhance utilize *L.acidophilus* and *Bifidobacteria* spp. as a supportive supplement to the treatment strategy based on Pegylated IFN-α and ribavirin which provides antibacterial, antiviral effect, reduce DGAT1 and improving the immune response rate. Subsequently, increase the treatment response rate to HCV treatment.

MATERIALS AND METHODS

Patients

This study was adopted on 40 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 had an approval letter from the ethical committee, Faculty of Medicine, Tanta University, Egypt.

Treatment strategies involved in the study

L. acidophilus and *Bifidobacteria* spp combination (1.5 billion cells) were 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 within the host body to colonize in the gut, colon or the small intestine. The process may take days to months with the least reported being 2-3 weeks as mentioned by Swanson (2013). Three groups (A, B and C) were designed in this study. Group A included healthy volunteers as controls who took the capsule once a day for one month. Group B included chronic hepatitis C patients who took the capsule once a day for one month before treatment with pegylated IFN-α and ribavirin (single interferon injection weekly for 12 weeks). Group C of chronic hepatitis C patients was treated with only pegylated IFN-α and ribavirin (12 weeks). Patients were followed up clinically during the time course of the experiment.

Samples collection and duration of study

Blood and urine samples were collected from the patient groups in

the time range from October 2013 to April 2014. Ten samples were collected before and after capsule 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 groups B and C.

Reagents and materials

The following media were used. 1) Nutrient agar (Thomas et al*.,* 1977) pH was adjusted to 5.0); 2) Mannitol salt agar (Koch, 1942) (Final pH: 7.4 ± 0.2 at 25°C); 3) MacConky agar (pH 7.4 ± 0.2 at 25°C). Gram stain (crystal violet, gram's iodine solution, acetone/ethanol (50:50 v: v), 0.1% basic fuchsin solution). VITEK 2 system (Funke, 1998), (bioMerieux Inc., Hazelwood, MO) VITEK 2 Cassette Loaded with Cards and Suspension Tubes being loaded Into the Automatic Transport System.

Antibiotics used for antibacterial sensitivity test (Barry, 1979) 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 (Stackebrandt, 1992) was purchased from Sigma (Sigma Scientific Services Co., Cairo, Egypt).

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

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

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) (Bièche, 1998). 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 *Lactobacillus acidophilus* and *Bifidobacteria* spp.

Isolation of bacteria from urine samples was performed on nutrient agar inoculated with 100 µl of each urine sample and incubated at 37°C for 18-24 h. Investigation of the isolated bacteria by Gram reaction was performed, eventually pure subculture of gram positive and gram negative were cultured in 20 mL of sterilized Macconky and Mannitol media, respectively, at 37°C for 18-24 h. 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, the numbers of the infected patients were recorded before and after the capsule administration in the three groups.

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 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; the identification cards were placed in slots that moved to the optical system where readings were observed each 15 min. Then 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 h at 37°C. Inhibition zones diameters were recorded in mm according to the Clinical and Laboratory Standards Institute (CLSI, 2013).

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 $MgCl_2$. Amplification started with initial denaturation for 5 min at 94°C, followed by 25 cycles of denaturation for 30 s at 94°C , annealing temperature of primers for 30 s at 50°C, and extension for 1 min at 72°C . The last extension for 15 min at 72°C was used. Submarine agarose gel electrophoresis in 1.2% agarose gel pre-staining 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 (Imran and Abd-Al-Kareem, 2016). The QIAGEN gel extraction kit serves as a gel purified for the amplified PCR product. GATC Company using ABI 3730xl DNA sequence used 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 at 4°C in the dark for 20 min, and then RBCs were lysed by adding BD FACS or ACK lysing solution (1x) for 15 min then centrifugation at 1250 rpm for 5 min. Then, the supernatant containing 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 (Givan et al., 2011).

White blood cells

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

Determination of DGAT1 enzyme level

Enzyme-linked Immunosorbent Assay Kit for Diacylglycerol-O-

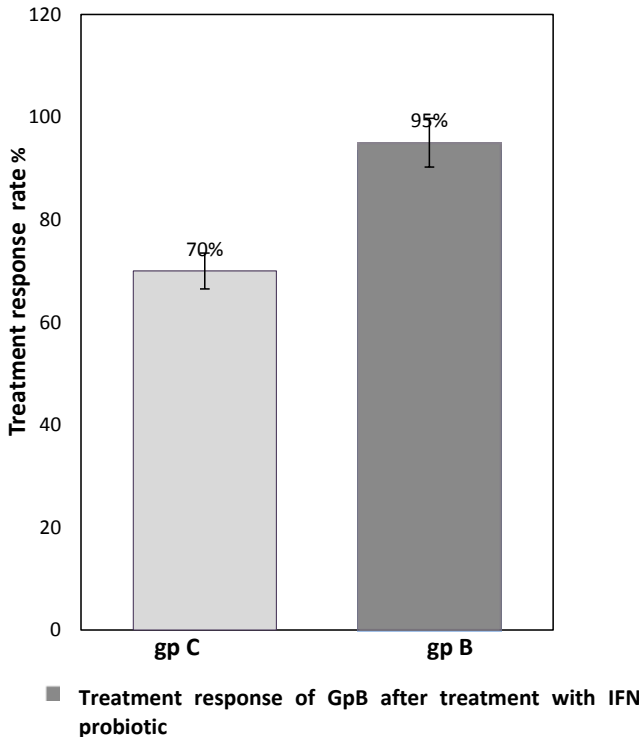


Figure 1. Treatment response rate of chronich HCV patients to IFN+ribavirin treatment in presence of probiotic administration in group B and in its abence in group C.

Acyltransferase Homolog 1 (DGAT1) .Organism Species: Homo sapiens (Human). This assay has high sensitivity and excellent specificity for detection of DGA T1.

Statistical analysis

The clinical data were recorded for 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" was used to test significance of the differences between means *P≤ 0.05, **P≤ 0.01.

RESULTS

The antiviral effect of probiotics by HCV PCR assays result

The antiviral effect of *L. acidophilus* and *Bifidobacteria* spp. capsule was determined by HCV PCR assay for three groups (Group A, B and C). The outcome data illustrated that *L. acidophilus* and *Bifidobacteria* spp 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 compared to the control group A which was negative (Figure 1; Table 1A,B).

Antibacterial activity of treatment with *Lactobacillus acidophilus* and *Bifidobacteria* spp.

The secondary infectious bacteria in patients with chronic HCV (groups B and C) were compared to that in the group A of healthy people which revealed no bacterial growth. 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 by 60% after probiotic supplementation. On the other hand, group C did not show any enhancement as it is nearly the same percentage of both the number of patients and the bacterial load after as well as before any treatments.

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

Table 1A. Quantitative estimation of HCV PCR before and after the treatment strategy with only IFN-α and ribavirin.

Patient (GpC)	Age	Sex	PCR IU/ML (Before)	PCR IU/ML (After)
1	45	F	428.847	Nil
2	50	M	997.631	Nil
3	50	M	1.060.807	Nil
4	53	M	29.217	Nil
5	48	M	500.000	Not responded
6	50	M	250.593	Nil
7	48	F	160.559	Nil
8	53	F	301.279	Nil
9	50	M	629.462	Nil
10	54	F	292.170	Nil
11	45	M	159.332	Nil
12	46	M	2.705.847	Nil
13	55	M	70.244	Not responded
14	53	F	428.897	Not responded
15	54	M	214.933	Not responded
6	49	M	2.921.707	Not responded
17	43	M	539.887	Not responded
18	40	M	233.345	Nil
19	45	M	144.876	Nil
20	40	M	233.98	Nil

M=Male, F=Female, Nil=too high response rate to revealed no HCV RNA observed (negative result), non-responded = high viral load ≥ the initial load. it's shown that the response rate to IFN-α and ribavirin treatment about 70%.

Table 1B. Quantitative estimation of HCV PCR before and after the treatment strategy including probiotic administration before IFN-α and ribavirin.

Patient's name (GpB)	Sex	Age	HCV PCR IU/ML (Before)	HCV PCR IU/ML (After)
1	F	38	23.030.498	Nil
2	M	43	1.005.317	Nil
3	M	45	1.016.250	Nil
4	M	44	1.581.138	Nil
5	M	56	1.787.737	Nil
6	M	50	804.704	Nil
7	M	48	199.35	Nil
8	F	53	214.933	Nil
9	M	50	367.821	Nil
10	M	45	138.772	Nil
11	M	50	193.035	Nil
12	F	48	7.684	Nil
13	M	45	13.561.362	Nil
14	M	55	23.207	Not responded
15	F	45	1.190.245	Nil
16	F	43	170.727	Nil
17	M	40	711.710	Nil
18	F	53	1.356.13	Nil
19	M	52	1.250.23	Nil
20	M	40	234.95	Nil

M=Male, F=Female, Nil=too high response rate to revealed no HCV RNA observed (negative result), non-responded = high viral load ≥ the initial load.

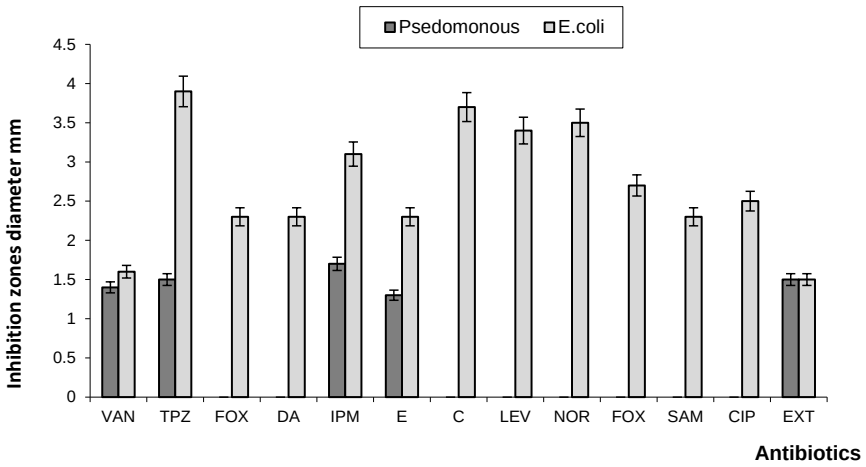


Figure 2. Inhibition zones diameters of different antibiotics that were used against *Escherichia coli* and *pseudomonas aeruginosa* and showed their resistance.

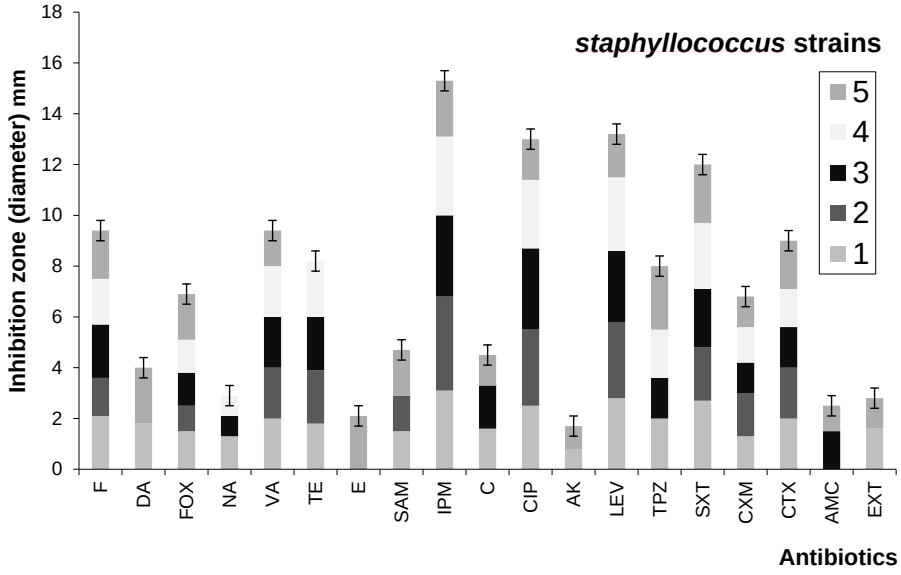


Figure 3. Inhibition zones diameters of different antibiotics against *Staphylococcus aureus strains*.

pathogens were identified in patients with chronic HCV as *S. lentus, S. aureus, Klebsiella pneumonia, P. aeruginosa* and *E. 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. *P. 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), chloramphenicol (C), amikacin (AK), piperacillin-tazobactam (TPZ), amoxicilin calvulinic acid (AMC) (Figures 2 and 3).

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.

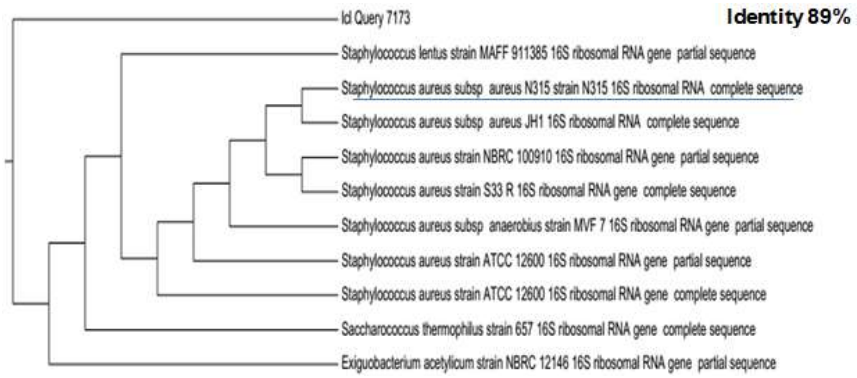


Figure 4. Phylogenetic tree of *staphylococcus aureus* by gene bank access and the highest identity was *staphylococcus aureus* N315 strain N315 16Sr ribosomal RNA.

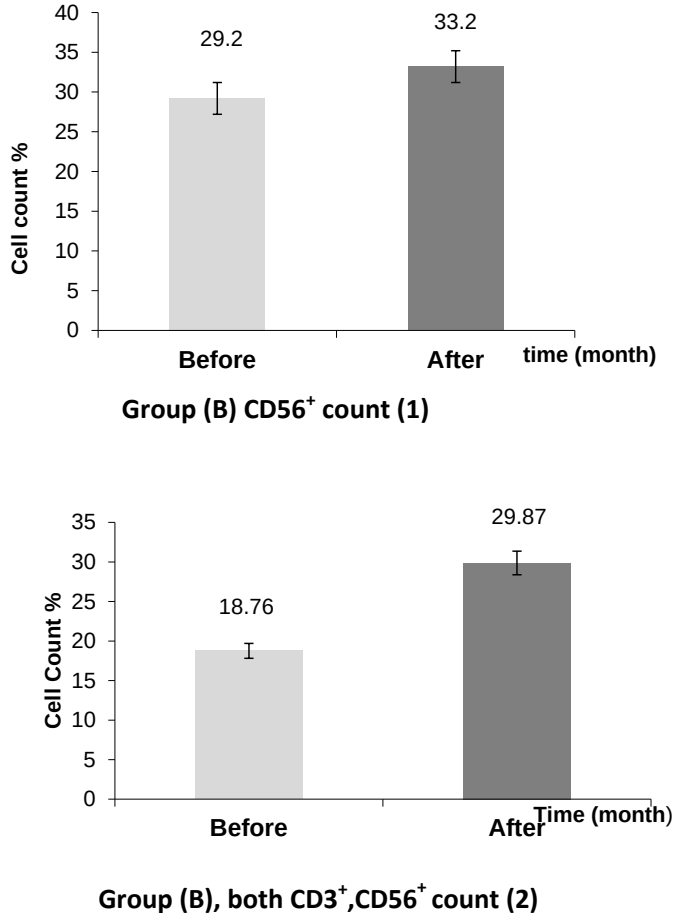


Figure 5. CD56 $^{+}$ and CD3 $^{+}$ cells count % in Group B of patients with hepatitis C before and after oral administration of probiotics.

Analysis of CD3^{+} and CD56^{+} cells counts

Treatment with *L. acidophilus* and *Bifidobacteria* spp. resulted in significant enhancement in the numbers of CD56^{+} NK cells (P values = 0.001) and CD3^{+} T cells (Figures 5 and 6; Table 2).

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 did not undergo this test as they did not take probiotics (Figure 7; Tables 3, 4).

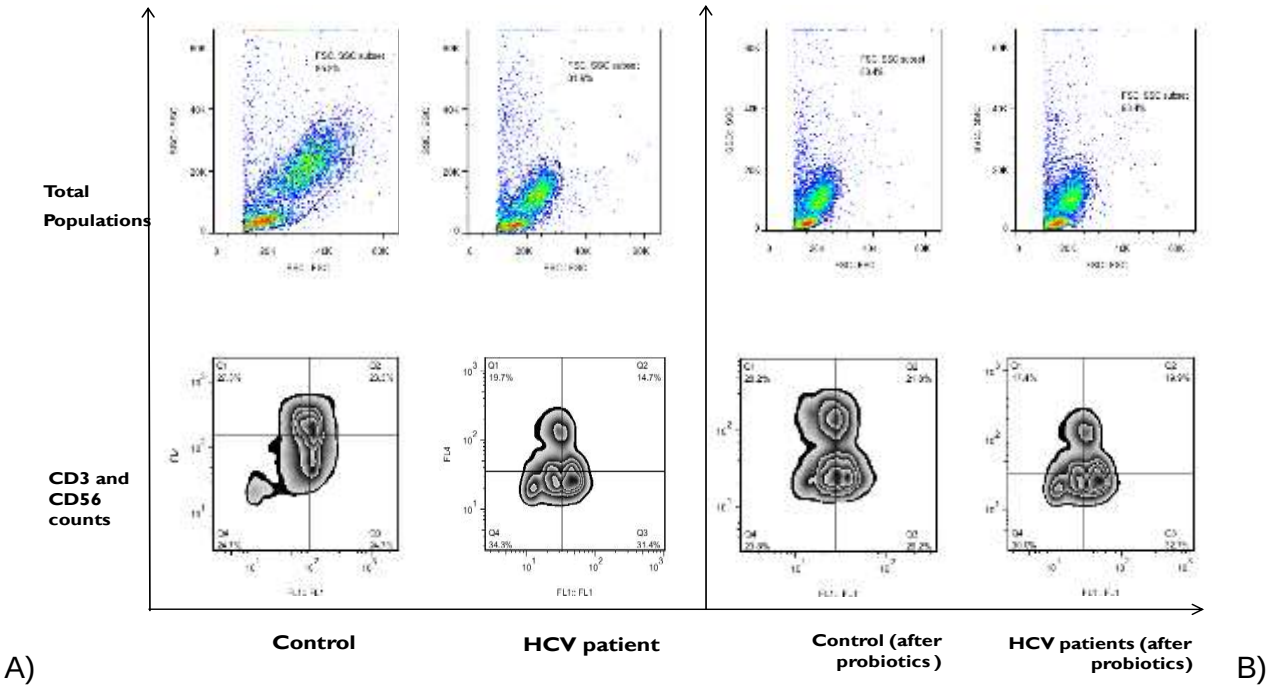
White blood cells

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 did not show observed change after probiotics administration. This parameter was not measured in group C as it did not take probiotics (Figure 8).

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 strengthening immune defenses against both bacterial and viral infections.

Inhibition of DGAT1 reduces HCV viral production

The outcome data illustrated that the concentration of DGAT1 enzyme was reduced as a result of probiotics capsule administration inside the same group B (chronic HCV patients) from 528 up to 373 ng/ mml (Table 5). More evidence was observed after three months of the treatment protocols in group (B) DGAT1 concentration was about 373 ng /mml lesser compared to group (C)



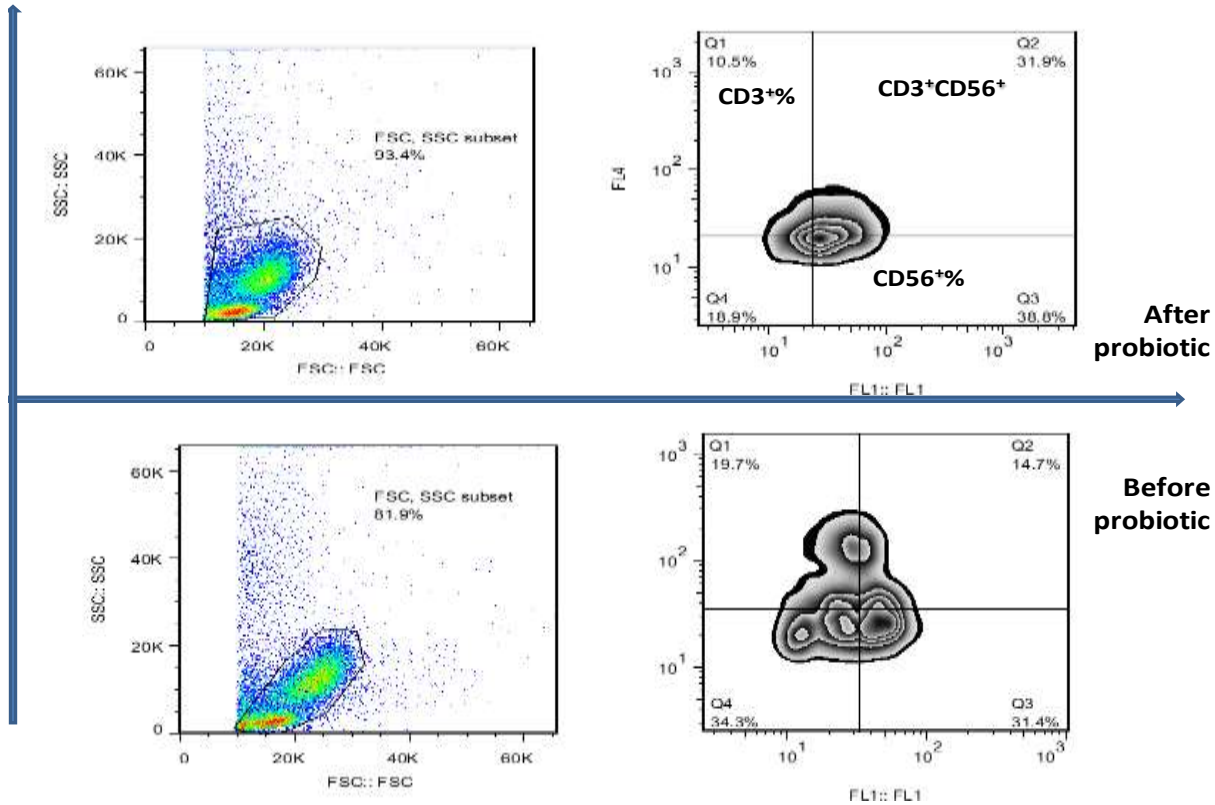


Figure 6. A, B. Analysis of CD3+ (FL4) cells count and CD56+ (FL1) cells count and total populations before and after administration of probiotics capsule by using FLOWJOW software.

who treated only with pegylated IFN and it was 1038 ng/mml higher with a significance P. value = 0.05*.

On the other hand, the presented study revealed the correlation between the level of DGAT1 and HCV infection which was determined in group (C) of patients treated with only pegylated IFN-α and ribavirin without

Table 2A, 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*.

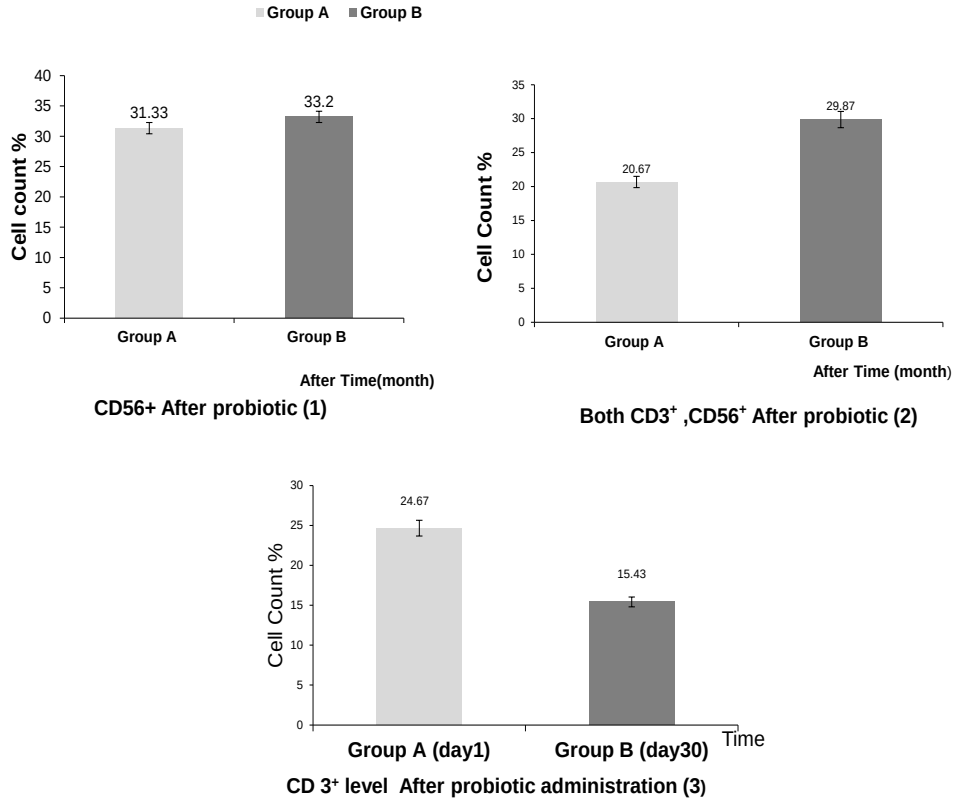


Figure 7. CD56+ cells (1), CD3+ CD56+ cells (2) and CD3+ cells (3) in group B compared to control group A oral administration or probiotics capsule.

Table 3. Populations of $CD3^+$ and $CD56^+$ cells in both healthy individuals (group A) and patients with chronic HCV (group B) before any treatments.

A: populations of $CD3^+$ in healthy individuals and patients with chronic HCV		
CD 3 $^+$ cells %	**Group A**	**Group B**
Range	18 – 27	12 – 25
Mean ± SD	23.0 ± 4.58	18.53 ± 4.26
t. test	2.699	
P. value	0.120	

B: Populations of $CD56^+$ in healthy individuals and in chronic HCV patients		
CD 56^+ cells %	**Group A**	**Group B**
Range	24 – 36	22 – 38
Mean ± SD	30 ± 6.11	29.0 ± 4.06
t. test	0.002	
P. value	0.962	

A. Mean ± SD showed slightly decreasing of CD 3^+ cells % in group B (chronic HCV patients) than group A (healthy individuals) before any treatment. **B.** Mean ± SD showed slightly decreasing of CD 56^+ cells % in group B (chronic HCV patients) than group A (healthy individuals) before any treatment.

Table 4A, 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 probiotics capsule administration 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*	

(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	

(C):- $CD3^+$ $CD56^+$ cells populations after probiotic capsule administration for a month		
Both $CD3^+$ $CD56^+$ cells % After	**Group A**	**Group B**
Range	19 – 22	16 – 38
Mean ± SD	20.67 ± 1.53	29.87 ± 5.77
t. test	7.197	
P. value	0.016	

A: 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:** 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:** 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.

probiotics capsule, while the concentration of DGAT1 enzyme increased from 624 up to 1038 ng/mml. DGAT1 concentration in group (A) was 302 ng/mml less than its concentration in group (B) which was 528 ng/mml (Table

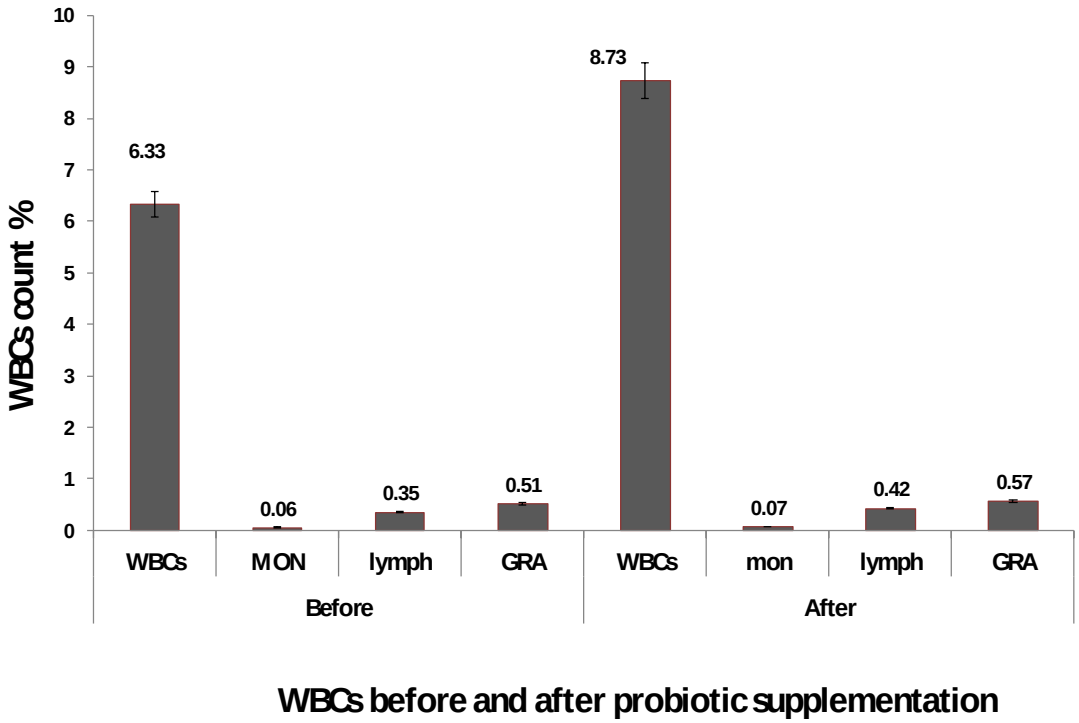


Figure 8. Leukocytes (WBCs) monocytes, lymphocytes, granulocytes count before and after the administration of probiotics capsule.

Table 5. Concentration of DGAT1 enzyme in healthy individuals (gpA) compared to its concentration in patients with chronic hepatitis C (gp B) before any treatments.

DGAT1 (Before any treatments)	gp A	gp B
Range	68.9 – 1011	42.3 – 1819
Mean ± SD	302.1 ± 264.2	528.4 ± 438.7
t. test	2.214	
P. value	0.148	

P. value= 0.01-0.05 showed significant result, Mean ± SD showed the mean and standard error, gpA = healthy individuals, gpB = chronic HCV patients, according to the mean data, it increased in gp B.

Table 6. Concentration of DGAT1 enzyme after probiotics administration and IFN-α injection treatment protocol in group (B) compared to its concentration in group (C) after IFN-α injection only.

DGAT1 After	gp B	gp C
Range	126.2 – 1027.0	172.7 – 2162
Mean ± SD	373.63 ± 262.99	1038.1 ± 806.03
t. test	9.471	
P. value	0.005*	

P. value= 0.01-0.05 showed significant result, Mean ± SD showed the mean and standard error, gpc = chronic HCV patients (only IFN), gpB = chronic HCV patients (probiotics plus IFN), as shown it increased with a significance P. value = 0.05*.

6).

DISCUSSION

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 were treated with *L. acidophilus* and *Bifidobacteria* spp. 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 (Servin, 2004). 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 *Bifidobacteria* spp. 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 *S. typhimurium, E. coli* and *S. aureus* that proved what Hor and Liong (2014) and Hütt (2006) noted. 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 (Garcia-Valdecasas et al*.,* 2009; Kawano et al*.,* 2015) 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. (2009); (Sikorska and Smoragiewicz, 2013) 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 et al. (2012), Ibrahim et al*.* (2010) explained the 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 immuno-compromised 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 (Ibrahim et al., 2010). Due to 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 included G +ve or G-ve bacteria which was an evidence. Moreover, treatment with probiotics capsule (one per day for a month before IFN-α) resulted in improving 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 (Ye et al., 2009), including phagocytosis, NK cell activity, IgG, IgA) (Chen and Morgan, 2006) 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 (Tsai et al*.,* 2012). Other studies showed that probiotics, including, *Lactobacillus* and *Bifidobacterium*, increased expression level of the activation molecule CD69 on $CD4^+$ (Th) and $CD3^+CD56^+$ (NKT cells) (Takeda et al., 2013; Kim et al., 2008).

Additionally, probiotics are considered by FDA as biological products which are safe (Doskali et al., 2011; Snydman*,* 2008) with protecting and supporting the liver and the immune function (Gratz et al*.,* 2010). As a result of this clinical study, the level of DGAT1 after probiotic capsule and treatment by IFN-α injection group (B) was less than its level after treatment by IFN-α injection only group(c) with P value = 0.005* significance. DGAT1 enzyme level catalyzes the fat droplet formation, where probiotics decrease the fat droplet formation that in agree with (Hung et al., 2017) and DGAT1 enzyme which involved in lipoprotein coat structure of HCV particle (Blaising and Pécheur, 2013) and as a result the virus could not replicate or escape from the immunity defense.

Conclusion

In conclusion, oral administration of *L. acidophilus* and *Bifidobacteria* spp. before treatment with IFN-α and ribavirin associated with significant antiviral activity, and antibacterial effects result in enhancement of the immune status and antibacterial metabolites.

Recommendation and Future work

This study recommends prescription of probiotic side by

side twithHCV treatment due to the antiviral and antibacterial effects of probiotics. Moreover it enhanced the immune response against the viral and bacterial infection. It is recommended to work on other probiotic species and study its role in the treatment of HCV infection.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

REFERENCES

Bali V, Panesar PS, Bera MB (2011). Isolation, screening and evaluation of antimicrobial activity of potential bacteriocin producing lactic acid bacteria isolate. Microbiology Journal 1: 113-119.

Barry AL (1979). Methods of measuring zones of inhibition with the Bauer-Kirby disk susceptibility test. Journal of Clinical Microbiology 10(6):885-889.

Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, Gómez-Llorente C, Gil A (2012). Probiotic mechanisms of action. Annals of Nutrition and Metabolism 61(2):160-174.

Bièche I, Olivi M, Champème MH, Vidaud D, Lidereau R, Vidaud M (1998). Novel approach to quantitative polymerase chain reaction using real-time detection: application to the detection of gene amplification in breast cancer. International Journal of Cancer 78(5):661-666.

Bischoff G, Barbara G, Buurman W, Ockhuizen T, Schulzke D, Serino M, Tilg H, Watson A, Wells M (2014). Intestinal permeability--a new target for disease prevention and therapy. BMC Gastroenterology 14:189.

Blaising J, Pécheur EI (2013). "Lipids A Key for Hepatitis C Virus Entry and a Potential Target for Antiviral Strategies." Biochimie. 10.1016/j.biochi.2012.07.016.

Butel MJ (2014). Probiotics, Gut Microbiota and Health. Medecine et Maladies Infectieuses 44(1):1-8

Camus G, Herker E, Modi AA, Haas JT, Ramage HR, Farese RV, Ott M (2013). Diacylglycerol acyltransferase-1 localizes hepatitis C virus NS5A protein to lipid droplets and enhances NS5A interaction with the viral capsid core. Journal of Biological Chemistry 288(14):9915-9923.

Candore G, Balistreri CR, Colonna-Romano G, Grimaldi MP, Lio D, Listi' F, Scola L, Vasto S, Caruso C (2008). Immunosenescence and anti-immunosenescence therapies: the case of probiotics. Rejuvenation Research 11(2):425-432.

Carrion JA, Martinez-Bauer E, Crespo G, Ramirez S, Perez-del-Pulgar S, Garcia-Valdecasas JC, Navasa M, Forns X (2009). Antiviral therapy increases the risk of bacterial infections in HCV-infected cirrhotic patients awaiting liver transplantation: A retrospective study. Journal of Hepatology 50(4):719-728.

Chen SL, Morgan TR (2006). The natural history of hepatitis C virus (HCV) infection. International Journal of Medical Sciences 3(2):47-52.

CLSI (2013). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement, M100-S22.

Deye N, Vincent F, Michel P, Ehrmann S, Da Silva D, Piagnerelli M, Kimmoun A, Hamzaoui O, Lacherade JC, de Jonghe B, Brouard F (2016). Changes in cardiac arrest patients' temperature management after the 2013 "TTM" trial: results from an international survey. Annals of Intensive Care 6(1):4.

Dore GJ, Ward J, Thursz M (2014). Hepatitis C disease burden and strategies to manage the burden (Guest Editors Mark Thursz, Gregory Dore and John Ward). Journal of Viral Hepatitis 21 Suppl 1:1-4.

Doskali M, Tanaka Y, Ohira M, Ishiyama K, Tashiro H, Chayama K, Ohdan H (2011). Possibility of adoptive immunotherapy with peripheral blood-derived $CD3^{-}CD56+$ and CD3+CD56+ cells for inducing antihepatocellular carcinoma and antihepatitis C virus activity. Journal of Immunotherapy 34(2):129-138.

Degnan FH (2008). The US Food and Drug Administration and probiotics: regulatory categorization. Clinical Infectious Diseases 46 Suppl 2:S133-136.

Funke G (1998). Evaluation of the VITEK 2 system for the rapid identification of medically relevant Gram-negative rods. Journal of Clinical Microbiology 36(7):1948-1952.

Gane EJ, Stedman CA, Hyland RH, Ding X, Svarovskaia E, Symonds WT, Hindes RG, Berrey MM (2013). Nucleotide polymerase inhibitor sofosbuvir plus ribavirin for hepatitis C. The New England Journal of Medicine 368(1):34-44.

Givan AL (2011). Flow cytometry: an introduction. Methods in Molecular Biology 699:1-29

Goldenberg JZ, Ma SS, Saxton JD, Martzen MR, Vandvik PO, Thorlund K, Guyatt GH, Johnston BC (2013). Probiotics for the Prevention of Clostridium Difficile-Associated Diarrhea in Adults and Children. Cochrane Database of Systematic Reviews. /10.1002/14651858.CD006095.pub4.

Gratz SW, Mykkanen H, El-Nezami HS (2010). Probiotics and gut health: A special focus on liver diseases. World Journal of Gastroenterology 16(4):403-410.

Harris CA, Haas JT, Streeper RS, Stone SJ, Kumari M, Yang K, Han X, Brownell N, Gross RW, Zechner R, Farese RV (2011). DGAT enzymes are required for triacylglycerol synthesis and lipid droplets in adipocytes. Journal of Lipid Research 52(4):657-667.

Hart A, Stagg A, Lammers K, Rizzello F, Gionchetti P, Campieri M, Knight S, Kamm M (2003). Probiotic Bacteria induce maturation and modulate cytokine production by human dendritic cells. Digestive Disease Week Abstracts and Itinerary Planner 2003:1246.

Heathcote J, Main J (2005). Treatment of hepatitis C. Journal of Viral Hepatitis 12(3):223-235.

Herker E, Harris C, Hernandez C, Carpentier A, Kaehlcke K, Rosenberg AR, Farese Jr RV, Ott M (2010). Efficient hepatitis C virus particle formation requires diacylglycerol acyltransferase-1. Nature Medicine 16(11):1295-1298.

Hor YY, Liong MT (2014). Use of extracellular extracts of lactic acid bacteria and bifidobacteria for the inhibition of dermatological pathogen *Staphylococcus aureus*. Dermatologica Sinica pp. 1-7.

Hung YH, Carreiro AL, Buhman KK (2017). Dgat1 and Dgat2 regulate enterocyte triacylglycerol distribution and alter proteins associated with cytoplasmic lipid droplets in response to dietary fat. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids 1862(6):600-614.

Hütt P (2006). Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. Journal of Applied Microbiology 100(6):1324-1332.

Ibrahim F, Ruvio S, Granlund L, Salminen S, Viitanen M, Ouwehand AC (2010). Probiotics and immunosenescence: cheese as a carrier. FEMS Immunology and Medical Microbiology 59(1):53-59.

Imran ZK, Abd Al-Karrem Z (2016). Evaluation of natural cloning of azole-resistant genes CDR1, CDR2, MDR and ERG11 between clinical and soil isolates of *Candida albicans* based on gene expression. International Journal of PharmTech Research 9(11):229-236.

Jacobs DM, Gaudier E, Duynhoven JV, Vaughan EE (2009). Non-digestible food ingredients, colonic microbiota and the impact on gut health and immunity: a role for metabolomics. Current Drug Metabolism 10(1):41-54.

Jalan R, Fernandez J, Wiest R, Schnabl B, Moreau R, Angeli P, Stadlbauer V, Gustot T, Bernardi M, Canton R, Albillos A (2014). Bacterial infections in cirrhosis: a position statement based on the EASL Special Conference (2013). Journal of Hepatology 60(6):1310-1324.

Kawano A, Ogawa E, Furusyo N, Nakamuta M, Kajiwara E, Dohmen K, Nomura H, Takahashi K, Satoh T, Azuma K, Tanabe Y (2015). Bacterial infection as an adverse effect of telaprevir-based triple therapy for chronic hepatitis C infection. Internal Medicine 54(6):567-572.

Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou K, Skarmoutsou N, Fakiri EM (2013). Health benefits of probiotics: a review. ISRN Nutrition 2013:481651.

Koch FE (1942). Mannitol salt agar (MSA) Zentr. Bakt. Labt. Orig.; 149:122.

Kim JY, Choi YO, Ji GE (2008). Effect of oral probiotics (*Bifidobacterium lactis* AD011 and *Lactobacillus acidophilus* AD031) administration on ovalbumin-induced food allergy mouse model. Journal of Microbiology and Biotechnology 18(8):1393-1400.

Lam B, Henry L, Younossi Z (2014). Sofosbuvir (Sovaldi) for the treatment of hepatitis C. Expert Review of Clinical Pharmacology 7(5):555-566.

Hanafiah KM, Groeger J, Flaxman AD, Wiersma ST (2013). Global epidemiology of hepatitis C virus infection: New estimates of age-specific antibody to HCV seroprevalence. Hepatology 57(4):1333-1342.

Martín R, Miquel S, Ulmer J, Kechaou N, Langella P, Bermúdez-Humarán LG (2013). Role of commensal and probiotic bacteria in human health: a focus on inflammatory bowel disease. Microbial Cell Factories 12:71.

Rask C, Adlerberth I, Berggren A., Ahren IL, Wold AE (2013). Differential effect on cell-mediated immunity in human volunteers after intake of different lactobacilli. Clin Exp Immunol 172(2):321-332

Reis JA, Paula AT, Casarotti SN, Penna ALB (2012). Lactic acid bacteria antimicrobial compounds: Characteristics and applications. Food Engineering Reviews 4:124-140.

Swanson L (2013). How long does it take for Probiotics to Work? Long term benefits of probiotics, probiotic 101. https://probiotics101.probacto.com/how-long-does-it-take-for-probiotics-to-work/

Stagg AJ, Hart AL, Knight SC, Kamm MA (2004). "Microbial-gut interactions in health and disease. Interactions between dendritic cells and bacteria in the regulation of intestinal immunity." Best Practice and Research Clinical Gastroenterology 18(2):255-270.

Stackebrandt E, Liesack W, Witt D, (1992). Ribosomal RNA and rDNA sequence analyses. Gene 115(1-2):255-260.

Sikorska H, Smoragiewicz W (2013). Role of probiotics in the prevention and treatment of meticillin-resistant *Staphylococcus aureus* infections. International Journal of Antimicrobial Agents 42(6):475-481.

Servin AL (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiology Reviews 28(4):405-440.

Snydman DR (2008). The safety of probiotics. Clinical Infectious Diseases 46(Supplement_2):S104-S111.

Takeda S, Kawahara S, HIDAKA M, Yoshida H, Watanabe W, Takeshita M, Kikuchi Y, Bumbein D, Muguruma M, Kurokawa M (2013). Effects of oral administration of probiotics from Mongolian dairy products on the Th1 immune response in mice. Bioscience, Biotechnology and Biochemistry 77(7):1372-1378.

Tsai YT, Cheng PC, Pan TM (2012). The immunomodulatory effects of lactic acid bacteria for improving immune functions and benefits. Appl Microbiol Biotechnol 96(4):853-862

Thomas CJ, McMeekin TA, Balis C (1977). Retention of bacteria in liquid films at agar surfaces. Applied and Environmental Microbiology 34(4).

Villamil L, Tafalla C, Figueras A, Novoa B (2002). Evaluation of immunomodulatory effects of lactic acid bacteria in turbot (*Scophthalmus maximus*). Clinical and Diagnostic Laboratory Immunology 9(6):1318-1323.

Yuan Q, Walker WA (2004). Innate immunity of the gut: mucosal defense in health and disease. Journal of Pediatric Gastroenterology and Nutrition 38(5):463-473.

Ye L (2009). CD56+ T cells inhibit hepatitis C virus replication in human hepatocytes. Hepatology 49(3):753-762.