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Cytokine profiling in mice suffering fatty liver pre administered with probiotics

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Abstract: The pro-inflammatory cytokines, as Tumor necrosis factor alpha (TNF-α) that being an endogenous pyrogen, that can cause apoptotic cell death, inflammation, and intervene the arrival of assortment cytokines such as Interleukin six (IL-6), Interleukin one beta (IL-1β) and Interleukin eight (IL-8) by activate macrophages. Dys-regulation, specifically excessive production of TNF-α has been detect in an assortment of many disorders like atherosclerosis, malignancy, and inflammatory bowel diseases. Probiotics are live bacteria, for example, *Lactobacilli* and *Bifidobacteria*; when consumed by people as adequate doses, these bacteria present a medical advantage on the host. This study estimated the efficacy and presumable action of two strains (*L. acidophilus* and *B. animalis*) to reduce fat accumulation in the liver of C57BL/6 male mice with diet-induced fats. Animals were divided into 4 groups: Group I; ten animals will receive normal diet for two weeks. Group II; ten animals will receive normal diet for the same period with probiotics. Group III; ten animals were subjected to high fatty diet; the high-fat diet contained 75% fat (butter) for the same period. Group IV: ten animals were subjected to high fatty diet for the same period with probiotics. TNF-α, IL-6 and IL-10 had been analyzed by enzyme-linked immunosorbent assay (ELISA), also lipogram and liver function tests were done. In this study the serum levels of IL-10 did not increase significantly in mice fed on high fatty diet in comparison with those on normal diet, while its level increase in animals which have probiotics introduced with fatty diet. In the present study the mean levels of IL-6 and TNFα, also liver enzymes and serum lipids were elevated in mice fed on high fatty diet in comparison with those on normal diet, while its level decreased in animals which had probiotics introduced with fatty diet.

keywords: Cytokines , probiotics , Fatty Liver , Steatosis , Interleukins

1.Introduction

Food and Agriculture Organization (FAO) and the World Health Association (WHA), defined probiotics as live bacteria which when managed in satisfactory doses give health advantages on the host.[(1)]

The intestinal bacterial population has a harmonious collaboration with all human body physiological functions. In this way, wholesome mediations might be valuable to certify with the treating of liver diseases by regulating gut microbiota. At the point when matured by intestinal bacteria, prebiotics induce the multiplication or action of probiotic microbes which help the host digestive system capacity and keep the multiplication of pathogenic organisms. [(2,3)]

The most widely recognized kinds of bacteria utilized as probiotics are *Lactobacilli* and *Bifidobacteria*, though different microbes and specific yeasts are likewise utilized. [(4)]

In spite of the fact that many in-vitro studies and animal models failed to demonstrate a probiotic impact, they can be utilized to describe a conceivable action of probiotic activity, decide the biosafety of probiotic bacterial strains or pass on other studies of probiotic bacterial strains. Consequently, in-vitro studies give the initial phase in assessing probiotics for nourishment utilize and ought to be pursue by double-blinded, randomized, placebo controlled clinical trials. Fitting target-specific in vitro thinks about that correspond with in vivo are prescribed. For instance in

vitro bile salts obstruction was appeared to correspond with gastric stability in vivo.[4]

NAFLD is emphatically connected to corpulence, with a revealed commonness more than 80% in large individual and just 16% in people with a typical BMI and without metabolic risk factors.[5]

NAFLD ranges from simple steatosis to inflammatory nonalcoholic steatohepatitis (NASH), with or without fibrosis. It is the most popular liver disease around the world, and has an expanding commonness. NASH, usually possible advances to deadly complications, for example, cirrhosis and hepatocellular carcinoma which is not a feature of simple steatosis.[6]

Cytokines are tiny proteins at first idea to be composition of the defense system, but have since been found to assume a considerably more extensive function in physiology. IL-6 initially recognized as a B-cell differentiation factor that comprises the B cells maturation into antibody-producing plasma cells.[7]

The pro-inflammatory cytokines, different studies have concentrated on TNF-α being an endogenous pyrogen, which can cause inflammation, apoptotic cell death, and intervene the arrival of assortment cytokines like IL-6, IL-8 and IL-1β by activate macrophages. Dys-regulation, specifically over production of TNF-α, has been found in an assortment of human diseases including malignancy, atherosclerosis and inflammatory bowel diseases.[8,9]

IL-10 is an acute anti-inflammatory cytokine that has pivotal, frequently and fundamental, function in prohibition inflammatory and auto immune diseases.[10,11]

Insufficiency or deviant idiom of IL-10 can upgrade inflammatory reaction to microbial test defy additionally cause expansion of inflammatory bowel diseases and auto immune diseases.[12]

In this way declined IL-10 expression or function can improve rescue of disease throughout an acute inflammation, but in addition amplify inflammation process, outcome in aggravated immunopathology and tissue impairment.[13,14]

Increased body fat ratio is considered as chronic inflammatory process that influences individuals regardless the age and race. Commonly, diseases identified with abundance fatty tissue have developed as the main sources of cardiovascular morbidity and mortality.[15]

People suffering from obesity have increased pervasiveness of hypertension, blood pressure dysfunction, cardiomegaly, atherosclerosis, and arterial calcification contrasted with ordinary body mass.[16,17]

In obese children and overweight, hemodynamic adjustments and atypical metabolic factors can be available until at extremely youthful period and are quietly active their way across chronic inflammatory pathogenesis.[18,19]

Recent proof proposes that instinctive fatty tissue is an inflammatory and metabolic gland that signs and balances the activity and digestion of the liver, brain, musculoskeletal system and cardiovascular system. [20,21]

The unbalanced generation of pro and anti-inflammatory cytokines produced from fat involved in the pathogenesis of NAFLD. [22]

Adjustment of endocrine, immune and inflammatory collaborations of adipose tissue may give fiction remedial (pharmacological) focuses for the remediation of NAFLD. [23,24]

Aim of the Work

The aim of this study is Assessment of probiotics role in amelioration to passivity effect of fatty liver in mice. Evaluation of the role of cytokines, either inflammatory or pro-inflammatory in the pathogenesis of fatty liver and the effect of probiotics on their serum levels, by immunoassay of IL-6, TNF-α a and IL-10 levels in mice with fatty liver.

Correlation between the effect of fatty diet on liver function tests in presence and absence of probiotic intake and study the effect of probiotics on the lipogram in mice ingesting high fatty diet.

2. Materials and Methods

Probiotics strains: Two lyophilized strains (*L. acidophilus* and *B. animalis*) obtained from Microbiologcal Resources Center (Cairo MIRCEN) were used in our study. The viability of the probiotic strains was confirmed by

observing their growth on Brain Heart Infusion broth (BHI) (Oxoid) under anaerobic conditions, using gas generating kits (Oxoid) within the anaerobic jar with catalyst. [25]

Animals: Age-matched (4-week-old), C57BL/6 male mice were used in all experiments. Forty mice were housed in steel cages. Probiotics were suspended in distilled water with bacterial viable count (10^8 – 10^9 CFU/mL); probiotic suspensions were administered orally mixed with their normal food for six times per week for two weeks.

Animals were divided into 4 groups: Group I; ten animals received normal diet for two weeks. Group II; ten animals received normal diet for the same period with probiotics. Group III; ten animals were subjected to high fatty diet; the high-fat diet contained 75% fat (butter) for the same period. Group IV: ten animals were subjected to high fatty diet for the same period with probiotics.

Liver function tests: Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT), were analyzed by kinetic method (Human, Germany).

Lipid profile: Serum levels of total cholesterol, Triglycerides (TG), High density Lipoprotein (HDL) and Low density Lipoprotein (LDL) were assayed by end point method (Human, Germany).

Immunoassay: TNF-α , IL-6 and IL-10 will measured by enzyme-linked immunosorbent assay (ELISA) (Elabscience Biotechnology Co.,Ltd, WuHan,China).

This ELISA Kit utilize Sandwich-ELISA technique. The micro ELISA plate showed in this kit already coated with cytokine specific antibodies.

Standards and samples were affixed to the appropriate wells assembled in the provided plate and were consolidated with the specific antibody.

Then a biotinylated specific antibody for the cytokine and Avidin-Horseradish Peroxidase (HRP) conjugate were affixed to each micro plate well respectively and incubated.

Excess amount were removed by washing. The substrate solution then affixed to each well. Only wells that contained the complex of cytokine, biotinylated detection antibody and avidin-HRP conjugate give blue color.

The enzyme-substrate reaction is determinate by adding sulphuric acid solution as a stopping reagent and the color turns yellow.

The optical density (OD) is measured spectrophotometrically by plate ELISA reader at a wave length of 450 nm. The OD value is positively proportional to the concentration of the cytokine in samples that can be calculated by plotting a standard curve that used as templet for cytokine concentration measuring.

Pathology: The liver biopsy specimens were fixed in 10% neutral buffered/ formalin, then immersed in paraffin and sectioned by the microtome at 6µm thickness. The sectioned were stained with haematoxylin/eosin (HE) dyes for conventional histopathological evaluation.

Table (1): Inflammatory and pro-cytokines in different groups. P* (GI vs. GII), P** (GI vs GIII), P*** (GI vs GIV), P# (GII vs GIII), P## (GII vs GIV) andP### (GIII vs GIV).

	TNFα (pg/ml)	**IL6 (pg/ml)**	**IL10(pg/ml)**
	M±SD	M±SD	M±SD
GI	0.48±0.28	0.96±0.25	2.06±0.42
GII	0.48±0.08	0.52±0.29	1.28±0.29
GIII	1.36±0.35	34.0±12.9	7.36±8.41
GIV	0.88±0.55	13.6±6.40	24.3±9.23
P*	1.000	0.001	0.000
P**	0.000	0.000	0.001
P***	0.092	0.000	0.000
P#	0.000	0.000	0.000
P##	0.061	0.000	0.000
P###	0.023	0.000	0.001

3. Results:

Inflammatory and pro-cytokines in different groups: As shown in table (1), the mean levels of TNFα in group I (0.48±0.28pg/ml), show no significant difference ($P > 0.05$) in contrast to groups II and IV (0.48±0.08 &0.88±0.55 pg/ml), while the difference is highly significant when compared to group III (1.36±0.35pg/ml, $P < 0.05$). Also, it shows statistical significance when comparing it`s levels between groups II vs III and III vs IV while this significance is lost when comparing groups II vs IV.

The mean levels of IL-6 in group I (0.96±0.25pg/ml), were significantly different (P < 0.05) when contrast to groups II, III and IV (0.52±0.29, 34±12.9&13.6±6.4 pg/ml) respectively. This significant difference also, was observed among the four studied groups.

IL-10 shows mightily significant variation among the four groups (P < 0.05) with the following values (2.06±0.42,1.28±0.29, 7.36±8.41&24.3±9.23 pg/ml) respectively.

Table (2), shows the mean levels of liver enzymes assayed ± standard deviation with P values listed for statistical comparison between the different studied groups.

	ALT (IU/mL)	**AST (IU/mL)**	**GGT (IU/mL)**
	M±SD	M±SD	M±SD
GI	21.4±1.96	27.2±2.15	23.8±2.35
GII	22.6±3.57	25.00±2.58	23.4±3.57
GIII	143.2±38.4	139.0±55.3	55.8±29.4
GIV	76.00±45.2	64.8±32.7	45.8±12.5
P*	0.645	0.064	0.759
P**	0.000	0.000	0.009
P***	0.000	0.000	0.000
P#	0.000	0.000	0.004
P##	0.000	0.000	0.000
P###	0.010	0.010	0.543

Table (2): Liver enzymes in different groupsP* (GI vs GII), P** (GI vs GIII), P*** (GI vs GIV), P# (GII vs GIII), P## (GII vs GIV) andP### (GIII vs GIV)

The mean levels of ALT in group I (21.4±1.96IU/mL), show no significant difference (P > 0.05) compared to groups II (22.6±3.57IU/mL),While shows a highly significant with group III &IV (143.2±38.4 &76.0±45.2 IU/mL) , Also, it shows statistical significance between groups II vs III and II vs IV and III vs IV.

The mean levels of AST in in group I (27.2±2.15IU/mL), show no significant difference (P > 0.05) when compared to groups II (25.0±2.58IU/mL), but it was statistically significant when compared to groups III & IV (139.0±55.3 &64.8±32.7IU/mL) (P < 0.05). This significant difference also, observed between groups II vs III &II vs IV and III vs IV.

GGT shows a highly significant differences among the group I (23.8±2.35 IU/mL) (P < 0.05) with the group III and IV values (55.8±29.4,45.7±12.5 IU/mL) and between group II (23.4±3.57 IU/mL) vs III and II vs IV, while no significant (P >0.05) between group I vs II and III vs IV.

The mean levels of cholesterol in group I (183.6±8.17 mg/dL), shows a highly significant differences among groups II, III and IV (162.4±10.6, 303.6±52.8, 271.6±45.2 mg/dL) respectively (P<0.05) and between groups II vs III, II vs IV, While no significant (P> 0.05) between groups III vs IV, as shown in table (3).

Table (3): Lipid profile of different groups, P* (GI vs GII), P** (GI vs GIII), P*** (GI vs GIV), P# (GII vs GIII), P## (GII vs GIV) and P### (GIII vs GIV)

	T. Chol	**TG**	**HDL**	**LDL**
	M±SD	M±SD	M±SD	M±SD
GI	183.6 ±8.17	98.22 ±8.23	56.3± 2.67	98.7±1 2.6
GII	162.4 ±10.6	85.38 ±12.0	64.6± 3.56	80.7±9. 31
GIII	303.6 ±52.8	142± 18.9	73.4± 7.39	207.2± 44.9
GIV	271.6 ±45.2	133.1 ±31.4	69.8± 15.4	175.2± 51.3
P*	0.001	0.023	0.000	0.004
P**	0.000	0.000	0.000	0.000
P***	0.000	0.000	0.015	0.000
P#	0.000	0.000	0.004	0.000
P##	0.000	0.000	1.000	0.000
P###	0.288	0.288	0.288	0.288

Table (3) also shows the mean levels of triglycerides in group I (98.22±8.23 mg/dL), were significantly differ (P < 0.05) when contrasted to groups II, III and IV (85.38±12.0, 142.1±18.9&133.1±31.4 mg/dL) respectively. This significant difference also, observed when comparing groups II vs III and II vs IV, but show no significance (P >0.05) between groups III vs IV. HDL shows significant differences (P < 0.05) among group I (56.3±2.67 mg/dL) with groups II, III & IV (64.6±3.56, 73.4±7.39& 69.8±15.4mg/dL) respectively, and also between groups II vs III, while no significant divergence was observed between groups II vs IV and III vs IV.

While, LDL shows significant differences (P < 0.05) among group I (98.7±12.6 mg/dL) with group II, III, IV (80.7±9.31,207.2±44.9& 175.2±51.3mg/dL) respectively. and also show

between group II vs III, and II vs IV and while no significant between group III vs IV.

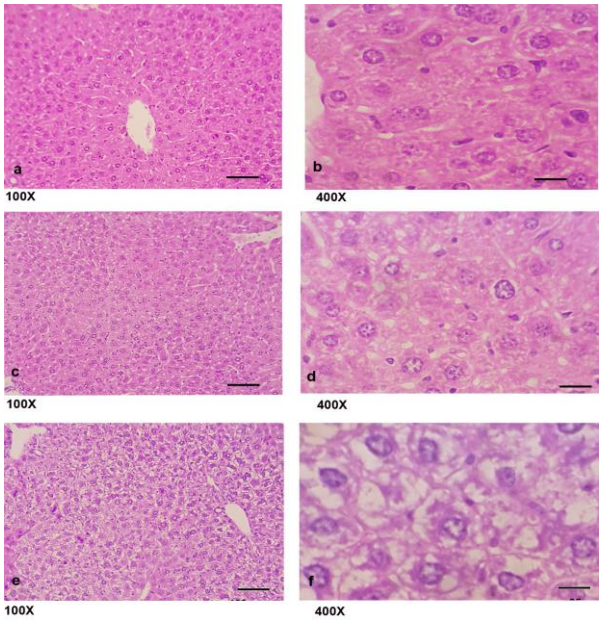

Fig (1): Group I, normal liver histology: Micrograph shows, the preserved lobular architecture with portal triad at periphery of hepatic and cords of hepatocytes irradiation from the central vein to the periphery (a, b). Group III, massive fatty liver degeneration: Micrograph shows, the altered hepatic architecture with normal portal tracts, some hepatocytes show small clear vacuoles (Micro vesicular). Other hepatocytes show large clear fat vacuoles pushing the nucleus to one side giving signet ring appearance (Macro vesicular) (c, d). Group IV: Mild fatty degeneration. Micrograph shows, the preserved hepatic architecture with normal portal tracts, some hepatocytes show small clear vacuoles (Micro vesicular). Rare hepatocytes show large clear fat vacuoles (e, f). Original magnification: × 100 with scale bar 100 μm and × 400 with scale bar 25 μm.

Discussion:

Probiotics are bacteria that provide metabolic help to the host when ingested in appropriate dose. The goal of probiotic ingestion is to create a symbiotic relationship between the human host and its flora.

The present study evaluates the possible action of two strains (*L. acidophilus* and *B. animalis*) to reduce fat accumulation in the liver of C57BL/6 male mice with fat rich diet.

Preliminary results from two pilot non-randomized studies proposed that probiotics could be afforded easily, may promote classical liver function tests, and may reduce fatty liver infiltration. This probiotic combination has likewise shown possibility as a therapeutic formula for some metabolic disorders. [26]

In this study the levels of anti-inflammatory cytokines (IL-10) and Pro-inflammatory cytokines (IL-6 and TNFα), were measured using sandwich ELISA technique in the serum of the tested animals.

In present this study the serum values of IL-6 increased in mice feed on high fatty diet in comparison with those on normal diet, while its levels decreased in animals which have probiotics introduced with fatty diet. These findings lies in parallel with[26] who stated thatIL-6 values was mightily increased in NAFLD patients, mostly with advanced stages, contrasted to patient with hepatitis Band[27]Who measured IL-6 emerged as the most important cytokine in NAFLD patients and found that, levels of IL-6 increased selectively in NAFLD patients only. The levels of IL-6 were elevated in patients with advanced stages of NAFLD. They also concluded that, IL-6 markedly increased serum level and liver expression of IL-6 in NASH patients, which correlated with inflammation and fibrosis.

In the this study the serum levels of TNFαis significantly elevated in mice fed on high fatty diet when compared to those on normal diet, while its level decrease in animals which have probiotics introduced with fatty diet. It also lies in contrast with Kumar et al., (2012)' [26] who did not find a significant increase in TNFα to be with any important disease variables, while its levels were highly significant in NAFLD patients than the controls, this probably due to different study populations or the lack of organization for some factors that might have influenced its serum levels.

Furthermore, Eslamparast et al., (2014)**,** [28] stated that creation of pro-inflammatory cytokines, like tumor necrosis factor (TNF)-α, in NAFLD was decreased after supplementation with a symbiotic, which is a mixture of nine strains of probiotics and fructo-oligosaccharides, in a study with 52 adults over 28 weeks, showed that symbiotic supplementation demoralized decrease produce of TNF-α.

Conflict with many studies referred to a substantial determination, as they did not esteem the gut microbiota to assure the mechanism of action indicated. Moreover, these results are still debatable because identical studies did not detect respectable modifications in the rate of TNF-α following remediation with several probiotics. [29,30]

In another study, Seo et al., (2013), [31] concluded that healthy animals with highest serum TNF-α Levels had a much significant risk of increasing NAFLD.

In this study the anti-inflammatory IL-10 levels increased in mice treated with probiotics whatever had normal diet or fatty diet, otherwise it decreased in group fed on fatty diet in comparison with normal diet, not so far IL-10 was least significant in mice in the high fat diet group compared to those in the control group.

Regulatory T cells modulate their inflammation-suppression effect by secreting many certain cytokines, such as IL-10 that mightily inhibit a Th-1 controlling pro-inflammatory response and other cytokines like TGF-α which stimulate regulatory T cells. Specific probiotic strains modulate inflammation through their ability to effectiveness the secretion of these modulating cytokines. This effect has been proved in-vivo, in various experimental animal models.[32]

In our present study, the liver enzymes (ALT and AST) were significantly increased in groups which have fat in its diet, while their levels were normal in those animals consuming normal diet or fatty diet with probiotics. GGT level have no significant change in different groups.

ALT is the main liver enzyme that mostly increased by high aggregation of fat in the liver and can be used as a biomarker of NAFLD. [33]

Serum levels of liver enzymes especially GGT and ALT are proportionally correlated with fasting levels of insulin needed for keeping normal hepatic glycogen to glucose conversion. [34]

In this, study the level of lipid profile (cholesterol, triglyceride and LDL), increase in comparison between normal diet and fatty diet. Probiotics administration did not decrease their levels significantly while HDL level was slightly changed in between different groups.

These data were also consistent with Liang and Vaziri (2003), [35] as they demonstrated that, oral supply of probiotic efficiently decrease fat deposition in the liver, in spite of continuous consuming of a high-fat diet. Hepatic-fat reduction was referred to the redistribution of lipid depots in the body rather than intestinal malabsorption of fat. In the liver lipid (TG and total cholesterol) metabolism was mutated due to bacterial metabolism.

A slight reduction in hepatic neutral lipids was demonstrated in the cholesterol, TG and LDL may protect liver from damage. The excessed neutral lipids in the non-treated animal livers may cause evolution to severe steatosis stages. [36]

The data from our present study recommend further definitive controlled studies.

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