RESEARCH ARTICLE

Impact of oral administration of Karish cheese on reduction of the risk of non-alcoholic fatty liver disease in rats fed high fructose diet

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Abstract: This study was carried out to evaluate the protective effect of two different types of Karish cheese, differing in starter culture used, against development of non-alcoholic fatty liver disease (NAFLD) in rat model. For that purpose, Wister rats were randomized into three groups; rats in the control group were fed high fructose (70%) diet while rats in experimental groups were fed the same diet mixed with 10 % of Karish cheese containing *Streptococcus thermophilus* NRRL-B- 41401 (group II) or Karish cheese containing *Lactobacillus acidophilus* NRRL-B-4495 and *Bifidobacterium longum* NRRL-B- 41409 with the same strain of *Streptococcus thermophilus* (group III). After five weeks of intervention, levels of plasma triglycerides, cholesterol, glucose, liver biomarkers (ALT and AST), interleukin (IL)-6, fecal enterobacteriaceae and hepatic index were significantly ($p < 0.05$) increased in rats in control group as compared to rats in both experimental groups. Also, levels of plasma liver markers, lipid profile, glucose and IL-6 were significantly lower in rats of group III than rats in group II. Furthermore, levels of plasma IL-10 were significantly increased in the experimental groups as compared to rats in control group. Results of this study indicated that eating probiotic Karish cheese decrease the risk of NAFLD through (I) blocking the inflammation process that associated with NAFLD, (II) enhancement of lipid profiles and (III) decreasing the levels of fecal enterobacteriaceae in rats. Indeed, extensive research on molecular mechanisms of health benefits of Karish cheese is still needed.

Key Words: Probiotication, diet, gut microbiota, fatty liver, Karish cheese, traditional foods

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as an accumulation of fat in triglycerides in the liver exceeding 5 to 10% of body weight in the absence of excessive alcohol consumption (Neuschwander-Tetri and Caldwell, 2003; Yan et al., 2007). Increased storage of fat in hepatocytes is associated with liver inflammation related non-alcoholic steatohepatitis (NASH). The risk of NAFLD rises in industrial as well as developing countries (Shoelson et al., 2007). NAFLD is multifactorial disease and the modern life style and diet like increased consumption of fast foods containing high levels of fats and fructose is associated with increasing the risk of obesity related NAFLD (Clark, 2006; Poutahidis et al., 2013). The cause of NAFLD pathogens is still remains unclear and it has limited pharmacological treatments (Byrne et al., 2009).

Changes in composition of gut microbiota seem to be associated with diet (given bacteria) and obesity. Animal and human studies suggest that gut microbiota plays an important role as a regulator of energy homeostasis (Musso et al., 2010) and it can produce significant amounts of various metabolites like acetate which induces *de novo* lipogensis system (Daubioul et al., 2002), and production of significant amounts of ethanol, as determined in the breath of obese mice (Cope et al., 2000) and in human plasma (Volynets et al., 2012). Lipopolysaccharides (LPS) are produced by intestinal *Enterobacteriaceae* and they act as an endotoxin and induce inflammation-related NASH (Miele et al., 2009).

Probiotics are live commensal microorganisms which, when consumed in adequate quantities, confer a health benefit to the host (FAO/WHO, 2001). Probiotics in either free or microencapsulated form may have beneficial effects in NAFLD by e.g. modulating the intestinal microbiota, producing different antibacterial substances, enhancing epithelial barrier function, reducing intestinal inflammation, and stimulating the immune system. However, the molecular mechanisms of probiotics are not fully understood (Iacono et al., 2011). Oral supplementation with probiotic *Lactobacillus* (*L.*) *acidophilus* CGMCC 2106 or *Bifidobacterium longum* CGMCC 2107 was shown to attenuate hepatic fat accumulation and reduce intestinal permeability in

high fat treated rats (Xu et al., 2012). Wagnerberger et al (2013) demonstrated that *L. casei* Shirota treatment could attenuate the activation of the TLR4 signaling cascade and PPAR-γ activity in high fructose (30% fructose solution) treated C57BL/6 mice. Also, microencapsulated *L. fermentum* ATTC 11976 were able to reduce hepatic triglycerides and to decrease the expression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in golden Bio F1B Golden Syrian Hamsters fed methionine deficient/ choline (Bhathena et al., 2013). However, there is still need for developing traditional dairy foods to reduce the risk of NAFLD whereas the modifications of lifestyle and diet are not universally proven treatment (Anstee et al., 2011). Therefore, the present study aims at a clarification whether oral administration of Karish cheese, a traditional Egyptian soft cheese, manufactured from skimmed milk (Abou Donia, 1991), causes the modulation of plasma metabolic profile resulting in the reduction of the development of NAFLD in rats.

Materials and Methods

Propagation of cultures

Bifidobacterium (*B.*) *longum* NRRL- B- 41409, *Lactobacillus* (*L.*) *acidophilus* NRRL -B-4495 and *Streptococcus* (*S.*) *thermophilus* NRRL-B- 41401 were obtained from Northern Regional Research Laboratory (NRRL), Peoria, USA. *B. longum* was sub-cultured in Wilkins Chalgreen Broth (WCB, Oxoid Co., London, UK) at 37 °C for 24 h under anaerobic conditions using Anaerogen™compact sachets system (Oxoid, London, UK). *L. acidophilus* was sub-cultured in MRS while *S. thermophilus* was sub-cultured in M17 and incubated aerobically at 37 °C for 18 h.

Preparation of Karish cheese

Buffalo's skim milk was heated to 85 °C for 15 Sec. then was cooled to 42 °C and divided to two batches; first batch milk was inoculated with 2% (w/v) of *S. thermophilus* while the second batch was inoculated with 2% of *B. longum*, *L. acidophilus* and *S. thermophilus* (1:1:1, ABT mixed culture). Karish cheese was manufactured as described by Effat et al. (2001). The counts of *B. longum* (WCA, anaerobic, 37 °C for 72 h), *L. acidophilus* (MRS pH 5.8, aerobic, 37 °C for 72 h) and *S. thermophilus* (M17, aerobic, 37 °C for 48 h) in cheese samples were 7.85±0.42, 8.30±0.32 and 8.45±0.36 respectively.

Animals and feeding protocol

Male Wistar rats (Wistar Han IGS, Strain code: 273 Charles Rivers, California, USA),10 weeks old (250 g), were housed in micro-isolator plastic cages individually (n = 1/cage) and maintained on basal diet and water *ad libitum* for 2 weeks, until they reached 320 - 360 g, in ambient temperature and humidity with a 12 h light- dark cycle. Thereafter, rats were randomly allocated to three groups. Rats in the control group (n = 9) were accustomed to high fructose (70 %) diet (HFru). While rats in the experimental groups (n = 10) were accustomed to HFru diet mixed with 10 % Karish cheese containing *S. thermophiles* or ABT mixed cultures in a dosage as applied by Sharma et al. (2010) for five week of intervention. The composition of basal and high fructose diet was as previously described by Kawasaki et al. (2009). Animal care and experimentation performed in this study conformed to the guide for committee of scientific ethics at Agricultural Research Center, Giza, Egypt.

Blood collection

Rats were anaesthetized firstly using ketamine-xylazine anaesthesia (mixed in the ratio 4:1) with 0.25 mL/100 g body weight intra-peritoneally. At the end of experiment (after 5 weeks), blood samples (one mL) were collected from retinal vein into heparin tubes plasma and was immediately separated by centrifugation (4000 Xg for 10 min) and stored at -20°C until analysis.

Determination of biochemical parameters in plasma and blood samples

Plasma alanine transaminase (ALT), aspartate aminotransferase (AST), triglycerides, cholesterol (Thermo Fisher Scientific, Passau, Germany) and glucose (New Blood Sugar Test, Boehringer Mannheim, Germany) were determined using spectrophotometer (SHIMADZU, Tokyo, Japan). Interleukin (IL)-6 and 10 kits were measured by commercial ELISA kits (Genzyme Diagnostics, Cambridge, England).

Enumeration of fecal enterobacteriaceae

Fecal samples of rats were freshly collected and immediately transferred to the anaerobic tube (Clinical Supply, Gifu, Japan) and weighted. Samples were examined for *Enterobacteriaceae* according to the methods of Mitsuoka (1992).

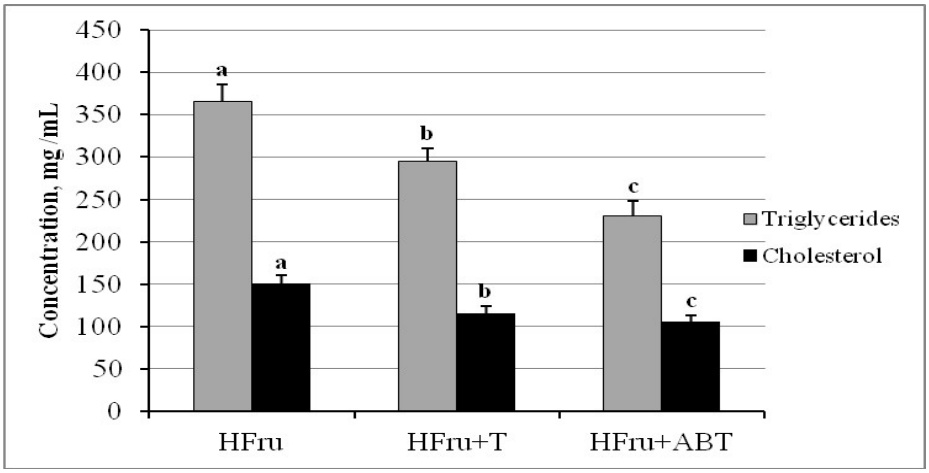
Statistical analysis

Results were analyzed statistically with SAS statics package (version 9.4, SAS Institute, Cary, NC). One – way ANOVA was used to assess the significance of differences between groups with $P < 0.05$ being considered significant. Data were expressed as mean ± standard error (SE). The Tukey test and one way analysis of variance were used for multiple comparisons between treatments.

Results and Discussion

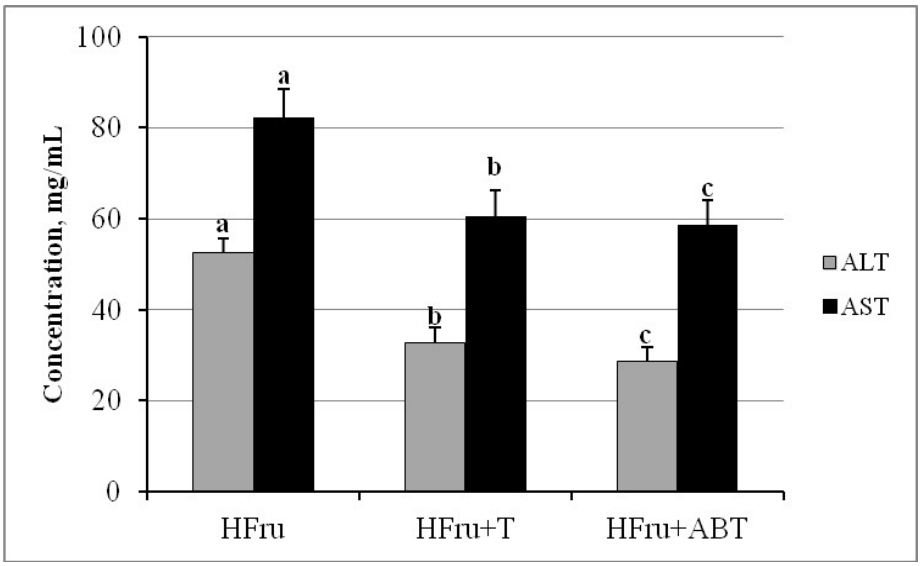
Increased level of dietary fructose, in fast foods, has been shown to be metabolized in the liver to triglycerides which lead to obesity, NAFLD and insulin resistance (Elliot et al., 2002; Tran et al., 2009). Several mechanisms have been suggested for the development of NAFLD by high fructose intake i.e. increased intestinal flora overgrowth, increased intestinal permeability,

Figure 1. Mean values of concentration of lipid profile in plasma of rats



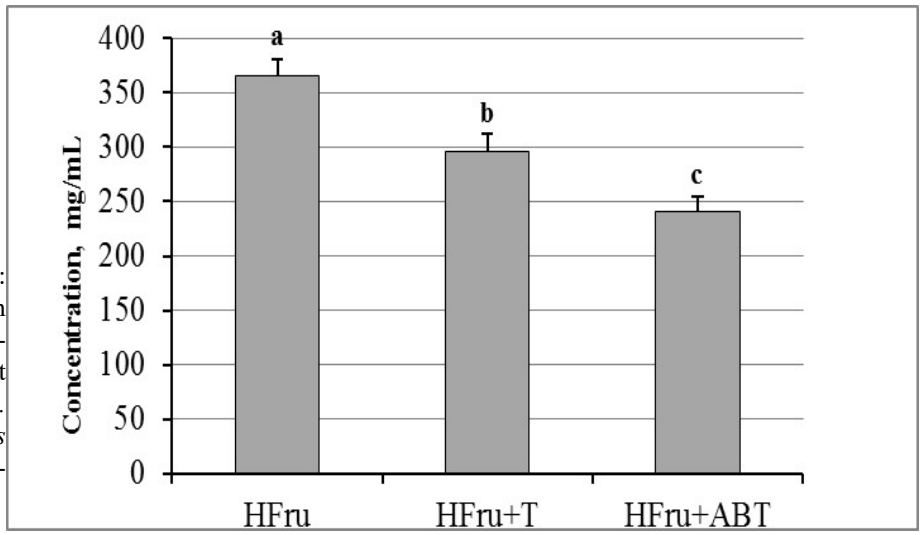
HFru: Rats fed high fructose diet, HFru+T: Rats fed high fructose diet mixed with Karish cheese made with *S. thermophilus* NRRL-B-41401, HFru+ABT: Rats fed high fructose diet mixed with Karish cheese made with *L. acidophilus* NRRL-B-4495, *S. thermophilus* NRRL-B-41401 and *B. longum* NRRL-B-41409.

Figure 2. Mean values of concentration of liver markers in plasma of rats



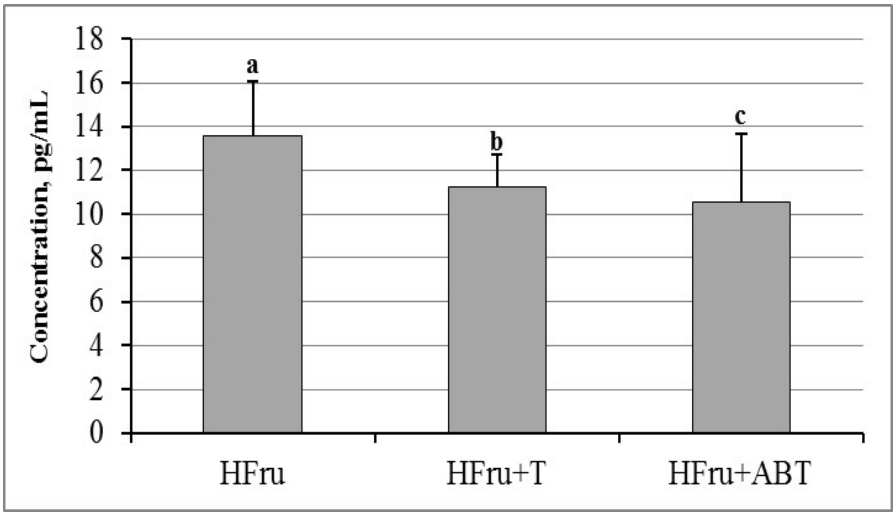
ALT: Alanine transaminase,AST:Aspartate aminotransferase,HFru: Rats fed high fructose diet, HFru+T: Rats fed high fructose diet mixed with Karish cheese made with *S. thermophilus* NRRL-B-41401, HFru+ABT: Rats fed high fructose diet mixed with Karish cheese made with *L. acidophilus* NRRL-B-4495, *S. thermophilus* NRRL-B-41401 and *B. longum* NRRL-B-41409.

Figure 3. Mean values of concentration of glucose in plasma of rats



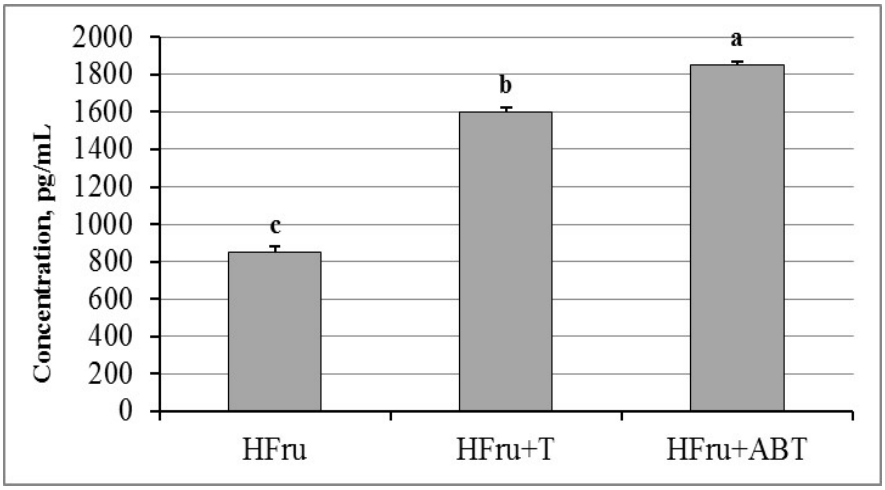
HFru: Rats fed high fructose diet, HFru+T: Rats fed high fructose diet mixed with Karish cheese made with *S. thermophilus* NRRL-B-41401, HFru+ABT: Rats fed high fructose diet mixed with Karish cheese made with *L. acidophilus* NRRL-B-4495, *S. thermophilus* NRRL-B-41401 and *B. longum* NRRL-B-41409.

Figure 4. Mean values of concentration of interleukin (IL)- 6 in plasma of rats



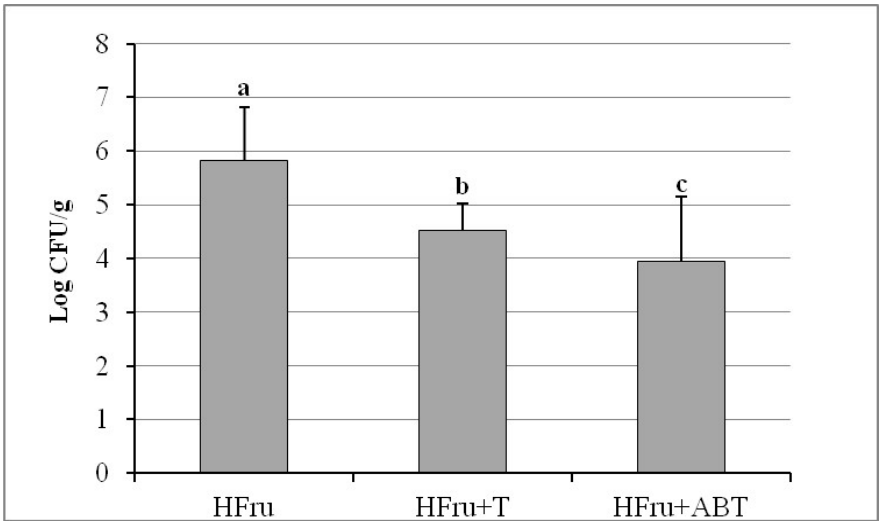
HFru: Rats fed high fructose diet, HFru+T: Rats fed high fructose diet mixed with Karish cheese made with *S. thermophilus* NRRL-B-41401, HFru+ABT: Rats fed high fructose diet mixed with Karish cheese made with *L. acidophilus* NRRL-B-4495, *S. thermophilus* NRRL-B-41401 and *B. longum* NRRL-B-41409

Figure 5. Mean values of concentration of interleukin (IL)-10 in plasma of rats



HFru: Rats fed high fructose diet, HFru+T: Rats fed high fructose diet mixed with Karish cheese made with *S. thermophilus* NRRL-B-41401, HFru+ABT: Rats fed high fructose diet mixed with Karish cheese made with *L. acidophilus* NRRL-B-4495, *S. thermophilus* NRRL-B-41401 and *B. longum* NRRL-B-41409.

Figure 6. Mean values of count of total *Enterobacteriaceae* (Log CFU/g) in feces of rats



HFru: Rats fed high fructose diet, HFru+T: Rats fed high fructose diet mixed with Karish cheese made with *S. thermophilus* NRRL-B-41401, HFru+ABT: Rats fed high fructose diet mixed with Karish cheese made with *L. acidophilus* NRRL-B-4495, *S. thermophilus* NRRL-B-41401 and *B. longum* NRRL-B-41409.

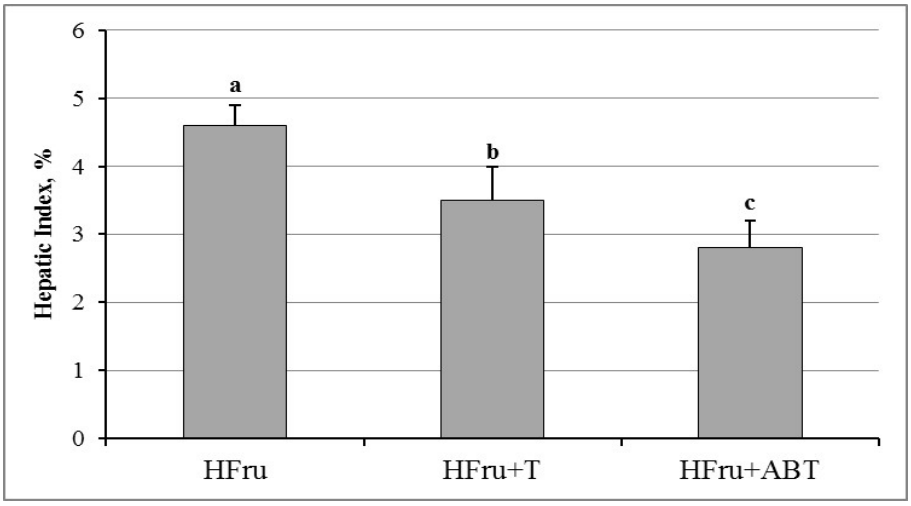


Figure 7. Mean values of Hepatic index (%) in rats

HFru: Rats fed high fructose diet, HFru+T: Rats fed high fructose diet mixed with Karish cheese made with *S. thermophilus* NRRL-B-41401, HFru+ABT: Rats fed high fructose diet mixed with Karish cheese made with *L. acidophilus* NRRL-B-4495, *S. thermophilus* NRRL-B-41401 and *B. longum* NRRL-B-41409.

translocation of bacterial endotoxin into portal plasma resulting in activation of Kupffer cells, increased proinflammatory cascade, excess of mitochondrial acetyl-CoA, stimulation of *de novo* lipogensis and inhibition of hepatic lipid β-oxidation (Spruss and Bergheim, 2005; Lim et al., 2010).

After five weeks of intervention, levels of triglycerides (365.51±20.12 mg/mL), cholesterol (150.52±9.32 mg/mL), ALT (52.50±3.20 mg/mL), AST (82.35±6.12 mg/mL), glucose (362.15±15.01 mg/mL), and IL-6 (13.56±2.51 pg/mL) were significantly ($P < 0.05$) increased in rats fed high fructose diet (control group) as shown in Figs. (1, 2, 3 and 4) respectively. These results are in the same line of results obtained by Kawasaki et al. (2009) who reported that feeding Wister rats with 70% fructose resulted in increased levels of plasma triglycerides, cholesterol, ALT and AST and induced liver steatohepatitis after five weeks of intervention.

On the other hand, the percentage of reduction in levels of triglycerides, cholesterol, ALT, AST, glucose and IL-6 was 19.2, 23.0, 26.5, 21.0, 17.1 % respectively for rats in Group (II); rats fed high fructose diet supplemented with *S. thermophilus*. However, it was 37.0, 30.0, 29.0, 34.1, and 22.0 % respectively for rats in Group (III); rats fed high fructose diet supplemented with ABT mixed culture. Different research groups reported that supplementation with probiotics could decrease the pathogenesis of NAFLD through reduction levels of plasma triglycerides, cholesterol, ALT, AST and glucose either in animal models or human (Ma et al., 2008 ; Esposito et al., 2009; Xu et al., 2012 and Hsieh et al., 2013). Levels of plasma IL-10 were significantly increased with 53.1 % and 54.0 % in both experimental groups compared to control group (Figure 5). This result supports results obtained by Poutahidis et al. (2013) who reported that eating yoghurt containing *L. reuteri* could decrease levels of IL-6 and enhance secretion of IL-10.

Counts of total fecal enterobacteriaceae were significantly ($P < 0.05$) decreased in both experimental groups as compared to control group as shown in Fig 6. This result indicates that modulation of fecal enterobacteriaceae might be due to ability of different lactic acid and bifidobacteria to produce different antimicrobial substances (Tagg and Dierksen, 2003; Candela et al., 2005; Collado et al., 2007; Gabrielli et al., 2009 and El Dieb et al., 2010) thus increasing their colonization and predominance in the gut. Probiotic *B. animalis* subsp. *lactis*, *L. paracasei* and *L. rhamnosus* could alter different groups of gut microbiota in mice fed high fat diet (Wang et al., 2015).

Hepatic index (HI) % (relative liver weight) shown in Figure 7, which is a simple tool reflecting fatty liver in humans (Lee et al., 2010) and animal models (Hamed et al., 2011; Endo et al., 2013), was significantly decreased in rats fed Karish cheese supplemented with either *S. thermophilus* or ABT cultures by 16.6 % and 39.1 % respectively. This finding might suggest a trend toward reduction of the development of NAFLD. Hsieh et al. (2013) found that oral administration of *L. reuteri* GMNL 263 could decrease the liver weight or rats fed high fructose diet. Finally, data presented in this investigation illustrate that supplementation of Karish cheese with mixed probiotic cultures has more health impact toward reduction of the development of NAFLD than using a single strain.

Conclusions

Development of traditional dairy foods by supplementation with probiotics (probiotication)may be one of the useful strategies for reduction of the risk of NAFLD. In this investigation, supplementation of Karish cheese with *L. acidophilus* and *B. longum* are found superior to ameliorate the risk of NAFLD and this effect may attribute to block the inflammation process associated with NAFLD, enhancement of plasma lipid profiles and decreasing the levels of fecal enterobacteriaceae which represent a main source for LPS. Finally, this study provides

evidences that tested *L. acidophilus* and *B. longum* may be a promising therapeutic agent in treating NAFLD. This presents the first study of its kind in health benefits of probiotic Karish cheese toward reduction of the risk of NAFLD.

Acknowledgments

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