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Protective Effect of Synbiotic Fermented Camel Milk on Non Alcoholic Fatty Liver Disease in Rats

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a universal epidemic that disperses among children with different ages' and adults. The current study aimed to evaluate the protective effects of fermented camel milk on progression of non-alcoholic fatty liver disease induced by a high fat diet and high fructose water in rat. Forty-two male rats were divided into seven different groups. The first group was given a standard diet to serve as a negative control group. The second group was fed on a high fat diet and high fructose in water (HFDHFr) to induce fatty liver disease, and served as a positive control group. The remaining five groups were fed on HFDHFr in addition to oral administration of camel milk (CM), fermented camel milk (FCM) containing nonencapsulated probiotic bacteria, FCM containing microencapsulated probiotic without prebiotic, FCM containing microencapsulated probiotic and 1% ginger extract or FCM containing microencapsulated probiotic and 10% beetroot extract, respectively. After 60 days, activity of liver enzymes, insulin resistance, lipid profile (TC, TG, HDL-C, and LDL-C), inflammation markers (TNF-α and NO) and oxidative stress (MDA, GSH and GSSG) were determined in serum of all rats' groups in addition to histopathological examination of liver tissue. Results revealed that oral administration of FCM containing microencapsulated probiotics with or without plant extract lowered the concentration of serum glucose, TNF-α, NO, TC, LDL-C, TG, MDA, GSSG, and the activity of liver enzymes. Furthermore, it significantly elevated HDL-C and GSH compared to positive control group. Moreover, histopathological examination showed that the group that was given FCM containing microencapsulated probiotics with beetroot extract was the nearest to negative control group followed by the group of FCM containing microencapsulated probiotics with ginger extract. It can be concluded that different tested treatments of FCM containing microencapsulated probiotics with plant extract reduced severity of fatty liver.

Key words: Nonalcoholic fatty liver diseases, Fermented camel milk, Synbiotic, Microencapsulated probiotic, Ginger extract, Beetroot extract, High fat diet high fructose in water..

Introduction

Negative changes of lifestyle *i.e.* fast food consumption and limiting of physical activity are serious factors that cause imbalance between energy intake and energy expenditure. This imbalance causes, metabolic syndrome which is associated with obesity, type II diabetes and fatty liver disease (Marchesini *et al.,* 2003; Couturier *et al.*, 2016). Fatty liver disease is one of the most dominant chronic liver diseases that refers to accumulation of fat within hepatic cells due to over consumption of alcohol or malnutrition (over consumption of carbohydrates, fats or combination of them).

Non-alcoholic fatty liver disease (NAFLD) is a universal epidemic that disperses among all different ages either children or adults. NAFLD includes a wide spectrum of liver injury starts with reversible stage called steatosis, which progresses to more serious conditions called steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma. NAFLD is associated with alterations of lipid

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metabolism, insulin resistance, secretion of proinflammatory cytokines and increased intestinal permeability. Furthermore, NAFLD may be a promoter for cardiovascular risk (Paolella *et al.,* 2014).

Prevention and treatment strategies of NAFLD include modification of lifestyle to reduce body weight through diet and physical activity, consuming healthy foods rich in antioxidants, lowering saturated fatty acids and carbohydrates intake as reviewed by McCarthy and Rinella (2011). No drugs can be used to treat NAFLD; only some clinical recommendations must be followed for management of NAFLD in addition to oral administration of some drugs like vitamins, antioxidants and improving insulin sensitivity.

Functional foods including fermented dairy products provide different healthy benefits in addition to their higher nutritional value. It could be an ideal choice to overcome metabolic syndrome diseases (Astrup 2014). Various clinical and animal studies confirmed healthy benefits of probiotic and/or prebiotic to alleviate severity of fatty liver features by lowering oxidative stress as well as improving insulin sensitivity. Hsieh *et al.,* (2013) approved efficiency of *Lactobacillus reuteri* GMNL-263 on suppressing steatosis and insulin resistance in rats fed on high fructose diet. Also, Famouri *et al.,* (2017) indicated that administration of a mixture of probiotic bacteria was effective in improving lipid profile and biochemical markers of NAFLD in obese children and adolescents with NAFLD. Moreover, Liang *et al.,* (2018) observed that administration of a mixture of probiotic strains (three Bifidobacterium strains and six Lactobacillus strains) in addition to galactooligosaccharide as prebiotic improved serum inflammatory markers, liver lipids and gut microbiota in rats fed on high fat diet to induce non-alcoholic fatty liver disease.

Plant extracts include numerous bioactive components such as ascorbic acid, phenolic substances, tocopherols, and carotenoids Tuorila & Gardello (2002). Medicinal properties of plants enable them to play a vital role in the prevention of NAFLD progression.

Previous studies mentioned various health benefits effects of beetroot extract. Betalain, the main component of beetroot extract, possesses a potent anti-inflammatory (Tesoriere, (2004)), antimicrobial, and antiviral effects (Strack *et al.,* (2003). Betalains possess other biological activities including hepatoprotective and anti-carcinogenic activities as reported by Račkauskienė, (2015). Beetroot extract has a high antioxidant activity due to its high betalain content, which inhibits heterocyclic amine formation and enhances free radical scavenging activities (Vulić *et al.,* (2014)).

Ginger is a common traditional medicine all over the world due to its therapeutic activities. Ginger extract possesses anti-microbial, anti-inflammatory and anti-oxidative effects (Rahmani *et al.,* (2014)). Gingerols, gingerol analogs and shogaols are active components found in ginger extract with a potent anti-inflammatory activity more than individual components (Lantz *et al.,* 2007).

Among different ruminants' milk, camel milk (CM) has a plenty of therapeutic properties such as hypocholesterolemic, antimicrobial, hypoglycemic, anticarcinogenic, antioxidant, immune-modulatory, antihypertensive and hepatoprotective effects; due to its unique composition as demonstrated by Ejtahed *et al.,* (2015). CM has a high concentration of vitamin C, insulin like protein and minerals (zinc, sodium, iron, magnesium, potassium and copper) but low in sugar, protein and cholesterol (Al Kanhal, 2010). Also, Korish & Arafah (2013) studied the effect of daily consumption of CM on high fat high cholesterol diet induced NAFLD in rat and indicated that CM is a natural choice to protect liver against NAFLD due to its anti-inflammatory and hypolipidimic effects. Moreover, Moslehishad *et al.,* (2013) determined the antioxidant activity of peptide released from fermented camel milk by *Lactobacillus rhamnosus* PTCC 1637 as well as fermented bovine milk and found that fermented camel milk exhibited more antioxidant activity than fermented bovine milk.

Additionally, our previous study indicated that microencapsulated probiotic bacteria with plant extract in chitosan coated beads as a double layer were the most tolerant to simulated gastrointestinal conditions, processing and storage of fermented camel milk at 4 ºC for 21 days (El-Abd *et al.,* 2018).

Consequently, the present study aimed to evaluate the effect of fermented camel milk containing microencapsulated probiotic bacteria combined with ginger or beetroot extract on non-alcoholic fatty liver disease in rats.

Materials and Methods

Animals

Adult male *Sprague-Dawley* rats, weighing 230±20g, were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR, Giza, Egypt). All animals received human care in compliance with the guidelines of the animal care and use committee of the NODCAR. Animals were kept in standard conditions and kept one week for adaptation before the beginning of the experiment. They were fed on a standard diet; and water was provided *ad libitum*.

Normal diet and non-alcoholic induction diet

Normal diet was prepared according to A.O.A.C. (2000), while high fat diet high fructose in water (HFDHFr) were used to induce non-alcoholic fatty liver in rats according to Charlton *et al.,* (2011).

Chemicals and kits

Methanol HPLC grade and Perchloric acid were purchased from Loba Co, India. DTNB; NADP; SSA Sulphosalsilic Acid; P- aminobenzyl glutamate and pyrogallol were purchased from TMMEDIA Co, India. GSH, GSSG; and 1,1,3,3- tetraethoxypropane were purchased from Sigma Aldrich USA. Potassium dichromate, monobasic potassium phosphate; nitrites and nitrate were obtained from Al Nasr chemicals, Abozabal (Qualybia- Egypt). All chemicals were of analytical grade.

Commercial kits from Bio-diagnostic Company (Egypt) were purchased to determine the following serum biochemical parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), total triglycerides (TG), high density lipoprotein cholesterol (HDL-C) levels and glucose concentration.

Preparation of bacterial starter cultures

Lyophilized yoghurt starter culture consisted of *lactobacillus delbrueckii subsp. bulgaricus* and *streptococcus salivarious subsp. thermophilus* were obtained from ChrHansen Lab., Copenhagen, Denmark. Lyophilized *Lactobacillus plantarum* B-4496 and *Bifidobacterium animalis* B-41405 were provided by Northern Regional Research Laboratory (NRRL), Agriculture Research Service, National Center for Agriculture, Peoria, Illinois, USA. *Lb.plantarum* B-4496 and *Bif. animalis* B-41405 were activated 3 times then enumerated in MRS broth medium under aerobic conditions; and MRS broth medium supplemented with 0.5% L-cystein HCL and 1% lithium chloride using the Gas Pak system for anaerobic conditions, respectively. Then incubated at 37°C/24 hrs. Microencapsulated probiotic bacteria coated with chitosan were prepared via extrusion microencapsulation technique according to El-Abd *et al.* (2018).

Production of fermented camel milk

Fresh camel milk was obtained from Animal Production Research Institute, Agriculture Research Center, Ministry of Agriculture. Then different treatments of fermented camel milk were produced as described by El-Abd *et al.,* (2018).

Animal experimental design

After 7 days for adaptation forty-two male rats were divided into seven different groups (6 rats each) as follows: The 1st group was fed on the normal diet to serve as a negative control group (C^-). The 2nd group was fed on HFDHFr to serve as a positive control group (C^+). The 3rd, 4th, 5th, 6th and 7th groups were fed on HFDHFr in addition to daily oral administration of camel milk (CM), fermented camel milk containing nonencapsulated probiotic bacteria (FCM+NP), fermented camel milk containing microencapsulated probiotic without prebiotic in chitosan coated beads (FCM+MP), fermented camel milk containing microencapsulated probiotic and 1% ginger extract in chitosan coated beads (FCM+ MPG) and fermented camel milk containing microencapsulated probiotic and

10% beetroot extract in chitosan coated beads (FCM+ MPB), respectively. Body weight was followed for each animal every week as well as at the end of the experiment. The experiment lasted 60 days.

Blood and tissues collection

Animals were scarified after 60 days of treatments, blood samples were collected from the retro-orbital venus plexus from all animals in test tubes. To separate serum, blood was centrifuged at 4000 rpm/15min. Liver tissues samples were removed at the time of sacrifice from rats for each group then washed with saline solution, weighted and immediately excised, preserved in 10% formalin until processing for histopathological examination.

Determination of biochemical parameters

Determination of liver enzymes

Separated serum was used for the determination of the activity of liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) according to Biodiagnostic kits procedure.

Determination of lipid profile

Serum total cholesterol (TC), total triglycerides (TG), high density lipoprotein cholesterol (HDL-C) levels were evaluated according to Biodiagnostic kits procedure. While Low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) were calculated according to Friedewald *et al.,* (1972) using the following equations:

$$\text{VLDL (mg/dl)} = \frac{\text{TG}}{5}$$

$$\text{LDL (mg/dl)} = \text{TC} - (\text{VLDL} + \text{HDL})$$

Determination of Insulin resistance

Glucose concentration was measured in serum according to Biodiagnostic kits procedure. While insulin was measured using ELISA Meso Scale Discovery A division of Meso Scale Diagnostics, LLC. 9238 Gaither Road Gaithersburg, MD 20877 USA according to Temple *et al.,* (1992). Insulin resistance was determined using the homeostasis model assessment (HOMA-IR) and calculated according to Okita *et al.,* (2013) using the following equation:

$$\text{HOMA} - \text{IR} = \frac{\text{fasting insulin (μIU/mL)} \times \text{fasting glucose (mmol/L)}}{22.5}$$

Determination of inflammatory markers

Serum TNF-α was assessed according to manufacturer's instructions using Rat TNF-α ELISA Kit, RayBio® (USA).

Serum Nitric Oxide (μmol/ ml) level was determined according to Papadoyannis *et al.,* (1999) by HPLC. Sodium nitrite and sodium nitrate were used for the reference standard preparation with stock concentration 1mg/ml. A standard mixture of nitrite and nitrate was used to determine the retention times and separation of the peaks. Nitrite and nitrate concentrations were equal in the mixture solution. The samples were analyzed on an Agilent HP 1100 series HPLC apparatus (USA). The analytical column was anion exchange PRP-X100 Hamilton, 150 x 4.1 mm, 10 μm. The mobile phase was a mixture of 0.1 M NaCl - methanol, at a volume ratio 45:55. The flow rate was 2 mL/min and wavelength was adjusted to 230 nm.

Determination of oxidative stress

Determination of serum MDA (nmol/ml) was performed by HPLC according to the method of Karatepe (2004), MDA standard was prepared by dissolving 25 µL 1,1,3,3 tetraethoxypropane (TEP) in 100 ml of water to give a 1 mM stock solution. Working standard was prepared by hydrolysis of 1 ml TEP stock solution in 50 ml 1% sulfuric acid and incubation for 2 h at room temperature. The resulting MDA standard of 20 nmol/ml was further diluted with 1% sulfuric acid to yield the final concentration of 1.25 nmol/ml to get the standard for the estimation of total MDA. The samples were analyzed on an Agilent HP 1100 series HPLC apparatus (USA). The analytical column was Supelcosil C18 (5 µm particle and 80 A^o pore size) (250 x 4.6 ID). Mobile phase consisted of 30 mmol KH_2PO_4 and methanol (65%-35%, H_3PO_4 by pH 4), and the mobile phase was adjusted at a 1.5 ml/ min flow rate, wavelength 250 nm.

Serum oxidized glutathione (GSSG) (µmol / ml) and reduced glutathione (GSH) (µmol / ml) were evaluated by HPLC using the method of Jayatilleke and Shaw (1993). Glutathione (oxidized and reduced) reference standards were purchased from Sigma Chemical Co. The standard was dissolved in 75% methanol in stock 1mg/ml and diluted before application to HPLC. The HPLC system of Agilent consisted of quaternary pump, a column oven, Rheodine injector and 20µl loop, UV variable wavelength detector. The report and chromatogram taken from Chemstation program was purchased from Agilent. Synerji RP Max column 3.9 at wavelength 210 nm with flow rate 2ml/min was used. Pot. Phosphate buffer - acetonitrile at PH 2.7 was used as an isocratic mobile phase.

Histopathological examination

Samples were taken from the liver of rats from all groups and embedded in 10% formalin solution for twenty four hours. Then specimens were cleared in xylol, embedded in paraffin and sectioned at 4 microns thickness. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stains for histopathological examination under the electric light microscope (Banchroft *et al*., 1996).

Statistical analysis

The values were expressed as the mean ± S.E. for each group. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test with $P<0.05$ being considered as statistically significant. Statistical analysis was conducted with SAS program (SAS, 1999).

Results and Discussion

Effect of camel milk with and without chitosan-coated beads containing different plant extract on

Final body weight, liver weight and liver index

Data in Table (1) illustrate the impact of feeding rats on High fat diet high fructose in water (HFDHFr) with and without different treatments of camel milk after 60 days compared to control groups. Concerning final body weight, significant differences were observed among all different groups. Final body weight of HFDHFr group showed a significant increase compared to control group. Additionally, HFDHFr group livers appeared enlarged and exhibited a significant increase in liver weight in comparison with control group. These results are in agreement with Charlton *et al.,* (2011) who compared the effects of different diets on some metabolic parameters, and observed that high fat diet with fructose caused significant increase in body and liver weights compared to control diet. Similarly, Masi *et al.,* (2017) investigated the effects of different diets on body weight and some biochemical parameters and found that feeding on combination of high fat diet with carbohydrate increased body weight compared to other diets. Also, Korish & Arafah (2013) found that feeding rat on high fat high cholesterol diet significantly increased body and liver weights compared to control

group fed on standard diet. Similarly, Hsieh *et al.,* (2013) studied the effect of administration of *Lb.reuteri* GMNL-263 on fatty liver features in rats fed on high fructose diet, and observed a significant increase in liver and body weight of positive control compared to negative control.

Oral administration of camel milk (CM) nonsignificantly decreased final body weight, however it significantly decreased liver weight compared to HFDHFr group. Fermented camel milk containing nonencapsulated probiotic (FCM+NP) caused a significant decrease in final body and liver weights compared to HFDHFr group. These results are in agreement with Ye *et al.,* (2017) who found that rats fed a high fat diet plus administration of *Lb. paracasei* Jlus66 caused a significantly reduction of body weight compared to control. Interestingly, no significant difference were observed between negative control group and groups received fermented camel milk containing microencapsulated probiotic with or without plant extract (FCM+MP, FCM+MPG, FCM+ MPB) in both final body weight and liver weight. These findings are in the same line of Liang *et al.,* (2018) who observed that feeding probiotics with prebiotic significantly declined body weight and liver weight of rats fed on high fat diet in comparison to control. Also, Ding *et al.,* (2017) found that oral administration of *Lb. plantarum* Lp3 with a high cholesterol diet significantly decreased the body weight of rats when compared with rats fed on a high cholesterol diet only. Additionally, no significant difference were found between normal group and *L. plantarum* group in body weight.

Regarding to liver index, significant differences were found between HFDHFr group and groups received fermented camel milk (FCM+NP, FCM+MP, FCM+MPG, FCM+ MPB). These results are in harmony with Korish & Arafah (2013) who indicated that the highest liver weight as well as liver index were observed in rats fed on high fat high cholesterol diet. Moreover, no significant difference was observed between control group and different groups that received fermented camel milk either containing microencapsulated probiotic bacteria with or without plant extract and that was in accordance with previous findings observed by Liang *et al.,* (2018). Also, Hamad *et al.,* (2011) observed nonsignificant difference in liver index between control and all treatment groups that received whey protein products as a trails to alleviate nonalcoholic fatty liver severity.

Table 1: Effect of camel milk with and without chitosan-coated beads containing different plant extract on final body weight, liver weight and liver index

Groups	Parameters		
	Final B.W (g)	**Liver weight (g)**	**Liver index (%)**
Control (C^-)	280 ± 7.07 ^{c}	9.03 ± 0.25 ^{d}	3.23 ± 0.07 ^{bc}
HFDHFr (C^+)	386 ± 14.35 ^{a}	15.85 ± 0.50 ^{a}	4.15 ± 0.27 ^{a}
CM	374 ± 18.33 ^{a}	13.75 ± 0.73 ^{b}	3.71 ± 0.24 ^{ab}
FCM+NP	326 ± 10.29 ^{b}	11.29 ± 0.57 ^{c}	3.48 ± 0.22 ^{bc}
FCM+MP	306 ± 9.27 ^{bc}	9.62 ± 0.32 ^{d}	3.17 ± 0.20 ^{bc}
FCM+MPG	294 ± 6.00 ^{bc}	9.07 ± 0.43 ^{d}	3.09 ± 0.18 ^{bc}
FCM+ MPB	300 ± 9.48 ^{bc}	8.98 ± 0.55 ^{d}	3.00 ± 0.18 ^{c}

Different superscripts letters within the same column (a, b, c ……) are significantly different (P<0.05)
Control (C^-): rats fed on standard diet. **HFDHFr (C^+):** rats fed on high fat diet + high fructose in water**. CM:** rats fed on HFDHFr + camel milk**. FCM+NP:** rats fed on HFDHFr + fermented camel milk (FCM) containing nonencapsulated probiotic. **FCM+MP:** rats fed on HFDHFr + FCM containing microencapsulated probiotic without prebiotic**. FCM+MPG:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with ginger extract**. FCM+MPB:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with beetroot extract.

Insulin resistance and inflammatory markers

Data in Table (2) show the level of serum glucose, insulin, TNF-α and NO as well as HOMA-IR score after feeding rats on HFDHFr with and without oral administration of different camel milk treatments for 60 days compared to control group.

Feeding rat on HFDHFr significantly increased serum glucose levels, insulin concentration and HOMA-IR level as well as elevated inflammatory markers for both α-TNF and NO in comparison to control group. These findings are in agreement with Raubenheimer *et al.,* (2006), Charlton *et al.,* (2011) who illustrated that feeding rodents on HFD increased insulin and glucose levels leading to insulin resistant when compared to negative control group. Also, Hui *et al.,* (2004) demonstrated that

NAFLD is associated with increased proinflammatory cytokines TNF- α leading to increased IR, since lowering TNF- α signaling improves IR.

Although oral administration of CM with HFDHFr showed significant reduction of glucose level but, nonsignificant reduction of insulin levels were found in comparison to HFDHFr group. In contrast, rat groups that received oral administration of different treatments of fermented camel milk either containing microencapsulated on nonencapsulated probiotic significantly lowered serum glucose and insulin levels which led to a reduction in HOMA-IR levels compared to HFDHFr group. Furthermore, no significant differences of serum glucose and insulin levels were observed between FCM+ MP, FCM+ MPG, FCM+ MPB and control group.

Additionally, the groups that were fed on HFDHFr and receiving fermented camel milk containing nonencapsulated probiotic or microencapsulated probiotic with or without plant extract exhibited a significant improvement of insulin resistance compared to HFDHFr group. No significant differences of HOMA-IR scores were observed between FCM+ NP, FCM+ MPB, FCM+ MPG and control group; these treatments were able to restore HOMA-IR to normal values of negative control group. These results are in agreement with Hsieh *et al.,* (2013) who determined some biochemical parameters to assess the effect of administration of *Lactobacillus reuteri* GMNL-263 in rats fed on high fructose diet. They found that *Lactobacillus reuteri* GMNL-263 induced a significant reduction in insulin resistance and inflammatory cytokines TNF- α compared to positive control group. Also, Lee *et al.,* (2018) reported that oral administration of *Lactobacillus plantarum* Ln4 to mice fed on a high fat diet significantly reduced weight gain, lipid parameters and improved glucose uptake in their adipocytes.

Table 2: Effect of camel milk with and without chitosan-coated beads containing different plant extract on insulin resistance and inflammatory markers

Groups	Parameters				
	Glucose (mmol/L)	**Insulin µIU/L**	**HOMA-IR score**	**TNF-α Pg/ml**	**NO (µmol/ml)**
Control (C^-)	4.25 ± 0.19 ^{d}	55.38 ± 1.41 ^{cd}	10.42 ± 0.35 ^{d}	44.19 ± 0.01 ^{e}	0.431 ± 0.01^{e}
HFDHFr (C^+)	12.95 ± 0.67 ^{a}	66.95 ± 2.62 ^{a}	38.26 ± 0.96 ^{a}	78.84 ± 0.01 ^{a}	0.751 ± 0.01 ^{a}
CM	9.29 ± 0.48 ^{b}	62.36 ± 2.26 ^{ab}	25.61 ± 0.76 ^{b}	73.32 ± 0.01 ^{b}	0.708 ± 0.01 ^{b}
FCMNP	5.48 ± 0.34 ^{c}	58.64 ± 0.62 ^{bc}	14.27 ± 0.88 ^{c}	59.59 ± 0.01 ^{c}	0.582 ± 0.01 ^{c}
FCM+MP	4.48 ± 0.25 ^{cd}	57.60 ± 1.15 ^{bcd}	11.45 ± 0.57 ^{d}	54.11 ± 0.01 ^{c}	0.544 ± 0.01 ^{c}
FCM+ MPG	4.14 ± 0.20 ^{d}	52.40 ± 1.14 ^{d}	9.61 ± 0.41 ^{d}	49.33 ± 0.01 ^{d}	0.488 ± 0.01 ^{d}
FCM+ MPB	4.29 ± 0.11 ^{d}	56.76 ± 2.76 ^{bcd}	10.82 ± 0.55 ^{d}	49.13 ± 0.01^{d}	0.470 ± 0.01 ^{d}

Different superscripts letters within the same column (a, b, c……) are significantly different (P<0.05)
Control (C^-): rats fed on standard diet. **HFDHFr (C^+):** rats fed on high fat diet + high fructose in water**. CM:** rats fed on HFDHFr + camel milk**. FCM+NP:** rats fed on HFDHFr + fermented camel milk (FCM) containing nonencapsulated probiotic. **FCM+MP:** rats fed on HFDHFr + FCM containing microencapsulated probiotic without prebiotic**. FCM+MPG:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with ginger extract**. FCM+MPB:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with beetroot extract.

Regarding to inflammatory markers, concentrations of both TNF-α and NO in serum of rats that received fermented camel milk containing microencapsulated probiotic (FCM+MP, FCM+MPG, FCM+MPB) or nonencapsulated probiotic (FCM+NP) were significantly less than HFDHFr group. It might be due to the anti-inflammatory effect of probiotic bacteria *Lactobacillus plantarum* B-4496 and *Bifidobacterium animalis* B-41405. Moreover, serum TNF-α and NO levels of rats received fermented camel milk containing microencapsulated probiotic with plant extract (ginger or beetroot) were near to negative control group; which may be attributed to the anti-inflammatory effect of ginger as reported by Sahebkar (2011). Our findings are in agreement with Fallah *et al.,* (2018) who examined the effects of fermented camel milk (FCM) on glycemic and inflammatory parameters in a double blind, randomized crossover trial, overweight/obese adolescents who received 250 ml of FCM / day for eight weeks compared to cow's yogurt and they found a significant decrease of TNF‐α levels by FCM consumption compared to cow's yogurt.

Also, Plaza-Diaz *et al.,* (2014) found that probiotic bacteria alleviated liver steatosis due to anti-inflammatory effect by decreasing concentration of TNF- α in serum of rats that received

Bifidobacterium breve CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036. Our results are in agreement with Li *et al.,* (2014) who indicated that fed rats on high fat diet to induce NAFLD strongly increased TNF-α in comparison with control group. On the other hand, administration of *L. plantarum* NCU116 to rats significantly reduced TNF-α level to level close to normal group.

Liver enzymes

Data in Table (3) show changes in the liver enzymes (ALT) and (AST), after feeding rats on HFDHFr with and without oral administration of different camel milk treatments for 60 days. Both of serum ALT and AST levels significantly increased in HFDHFr group compared to the negative control group which was in harmony with Charlton *et al.,* (2011) and Korish & Arafah (2013). However, camel milk (CM) significantly decreased the activity of serum ALT but it showed a nonsignificant reduction in serum AST levels compared to HFDHFr group.

However, oral administration of fermented camel milk using nonencapsulated probiotic (FCM+NP) as well as fermented camel milk using microencapsulated probiotic without prebiotic (FCM+MP) decreased significantly the activity of ALT and AST compared to HFDHFr group. Similarly, Famouri *et al.,* (2017) evaluated effects of consumption probiotic capsule containing *Lb.acidophilus* ATCC B3208, *Bif. lactis* DSMZ 32269, *Bif. bifidum* ATCC SD6576, *Lb. rhamnosus* DSMZ 21690 for 12 weeks on sonographic and biochemical parameters of 64 obese children with sonographic NAFLD and they found decrease in AST and ALT activities beside normal liver sonography. Also, Vajro *et al.,* (2011) observed a significant reduction of serum ALT of volunteers suffer from NAFLD after receiving *Lb. rhamnosus* GG for 8 weeks.

Interestingly, administration of fermented camel milk with chitosan coated beads containing either ginger (FCM+ MPG) or beetroot extract (FCM+ MPB) significantly decreased ALT and AST activities be nearly to the control group. Also, Malaguarnera *et al.,* (2012), Eslamparast *et al.,* (2014) demonstrated that using probiotics and prebiotic have reduced the activities of AST and ALT in patients with nonalcoholic fatty liver disease.

Table 3: Effect of camel milk with and without chitosan-coated beads containing different plant extract on liver enzymes

Groups	ALT U/L	AST U/L
Control (C^-)	49.95 ± 2.87 ^{e}	61.60 ± 3.80 ^{d}
HFDHFr (C^+)	77.50 ± 0.88 ^{a}	106.08 ± 4.37 ^{a}
CM	72.80 ± 1.24 ^{b}	96.84 ± 4.15 ^{a}
FCMNP	60.13 ± 0.90 ^{c}	78.97 ± 3.38 ^{c}
FCM+MP	55.99 ± 0.95 ^{d}	73.08 ± 2.49 ^{bc}
FCM+ MPG	52.21 ± 0.69 ^{de}	69.07 ± 2.79 ^{bcd}
FCM+ MPB	50.34 ± 0.69 ^{e}	65.92 ± 2.56 ^{cd}

Different superscripts letters within the same column (a, b, c……) are significantly different ($P<0.05$)
Control (C^-): rats fed on standard diet. **HFDHFr (C^+):** rats fed on high fat diet + high fructose in water**. CM:** rats fed on HFDHFr + camel milk**. FCM+NP:** rats fed on HFDHFr + fermented camel milk (FCM) containing nonencapsulated probiotic. **FCM+MP:** rats fed on HFDHFr + FCM containing microencapsulated probiotic without prebiotic**. FCM+MPG:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with ginger extract**. FCM+MPB:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with beetroot extract.

Lipid profile

Data in Table (4) show lipid profile parameters of rats that were fed on HFDHFr with or without different treatments of camel milk for 60 days. Significant differences of serum TC, HDL-C, LDL-C, VLDL-C and TG levels were observed between HFDHFr group and the negative control group. HFDHFr group showed a significant increase in TC, LDL-C, VLDL-C and TG levels, while a significant decrease in HDL-C level compared to the control group. Similarly, Korish & Arafah (2013) and Charlton *et al.,* (2011) found significant alteration in lipid profile induced by high fat diet plus high carbohydrate as well as high fat diet plus high cholesterol in rodents.

Generally, camel milk group showed a significant decrease in serum TC, LDL-C, VLDL-C and TG levels and a significant elevation of HDL-C in comparison with HFDHFr group. These results are in agreement with Korish & Arafah (2013) who observed a significant improvement in lipid profile of serum rats fed on camel milk plus high fat high cholesterol diet compared to control group. Also, Garcia *et al.,* (2012) reported that camel milk contains the highest amount of phospholipids compared to human milk, cow milk, and mare milk. Phospholipids have important roles in lipid metabolism (digestion, absorption and transport) as demonstrated by Fave *et al.,* (2004).

Oral administration of fermented camel milk either containing nonencapsulated or microencapsulated probiotic significantly improved all lipid profile parameters when compared to HFDHFr group. Similarly, Ali *et al.,* (2013) found that feeding rats on cholesterol enriched diet plus fermented camel milk or fermented cow milk containing *Bifidobacterium* Bb-12 for 6 weeks resulted in a significant decrease in TC, LDL-C and VLDL- C levels in addition to elevation of serum HDL-C level compared to the control group. However, FCM milk + Bb-12 was more effective in lowering lipids in serum and livers than cow milk + Bb-12.

No significant differences were observed between negative control group and fermented camel milk containing chitosan coated beads either with ginger (FCM+MPG) or beetroot (FCM+MPB) extracts concerning to serum levels of TC, HDL-C and LDL-C. These findings are accordance with Jeun *et al.,* (2010) who examined the effect of administration of double-coated *L. plantarum* KCTC3928 on plasma cholesterol metabolism in mice, and stated that oral administration of double coated *L. plantarum* KCTC3928 exhibited hypocholesterolemic and hepatoprotective effects by increasing hepatic bile acid synthesis and fecal bile acid excretion.

Also, Famouri *et al.,* (2017) reported that administration of a mixture of probiotic bacteria was effective in improving lipid profile (significant reduction in serum TC, LDL-C, and TG) in obese children and adolescents with NAFLD. Finally, Schaamann *et al.,* (2001) stated that consumption of probiotic yoghurt containing *Bif. longum* and *Lb. acidophilus* reduced LDL-C level in hypercholesteremic women.

Table 4: Effect of camel milk with and without chitosan-coated beads containing different plant extract on lipid profile

Groups	Parameters				
	TC mg/dl	HDL-C mg/dl	LDL-C mg/dl	VLDL-C mg/dl	TG mg/dl
Control (C^-)	79.00 ± 1.97 ^{d}	36.20 ± 0.80 ^{a}	24.32 ± 2.08 ^{d}	18.47 ± 0.47 ^{f}	92.39 ± 2.38 ^{f}
HFDHFr (C^+)	183.94 ± 3.51 ^{a}	17.60 ± 1.00 ^{e}	124.85 ± 3.23 ^{a}	41.47 ± 0.76 ^{a}	207.37 ± 3.81 ^{a}
CM	128.05 ± 9.13 ^{b}	24.32 ± 1.27 ^{d}	71.09 ± 8.57 ^{b}	32.64 ± 1.47 ^{b}	163.22 ± 7.38 ^{b}
FCMNP	97.05 ± 3.32 ^{c}	29.06 ± 1.12 ^{c}	43.45 ± 3.85 ^{c}	24.53 ± 0.75 ^{c}	122.66 ± 3.76 ^{c}
FCM+MP	90.52 ± 2.15 ^{cd}	32.08 ± 1.38 ^{bc}	35.06 ± 1.33 ^{cd}	23.37 ± 0.67 ^{cd}	116.85 ± 3.39 ^{cd}
FCM+ MPG	84.73 ± 2.44 ^{cd}	35.40 ± 0.58 ^{a}	27.53 ± 3.17 ^{d}	21.80 ± 0.65 ^{de}	109.01 ± 3.28 ^{de}
FCM+ MPB	80.74 ± 2.03 ^{d}	34.52 ± 1.10 ^{ab}	25.57 ± 2.19 ^{d}	20.64 ± 0.54 ^{ef}	103.21 ± 2.72 ^{ef}

Different superscripts letters within the same column (a, b, c……) are significantly different (P<0.05)
Control (C^-): rats fed on standard diet. **HFDHFr (C^+):** rats fed on high fat diet + high fructose in water**. CM:** rats fed on HFDHFr + camel milk**. FCM+NP:** rats fed on HFDHFr + fermented camel milk (FCM) containing nonencapsulated probiotic. **FCM+MP:** rats fed on HFDHFr + FCM containing microencapsulated probiotic without prebiotic**. FCM+MPG:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with ginger extract**. FCM+MPB:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with beetroot extract.

Oxidative stress markers

The effect of feeding rats on HFDHFr plus oral administration of camel milk, nonencapsulated probiotic, microencapsulated probiotic with or without plant extract on oxidative stress markers are shown in Table (5). HFDHFr group exhibited a significant elevation of oxidative stress markers (MDA and GSSG) in addition to significant reduction of the serum level of GSH in comparison with the negative control group. It might be due to the feeding of high fat diet that increased free radicals resulting in increased oxidative stress that plays a key role in NAFLD progression. Additionally, production of reactive oxygen species generates lipid peroxides, thus caused a damage in hepatic membranes, proteins, and DNA as reported by **Li *et al.,* (2014)** who found

a higher MDA content and a lower content of GSH in serum of rats with NAFLD fed on a high fat diet, compared to control group.

Although, camel milk decreased significantly malondialdehyde (MDA) but it showed no significant changes of GSSG and GSH compared to HFDHFr group. In contrast, Oral administration of fermented camel milk containing nonencapsulated probiotic or microencapsulated probiotic without plant extract significantly improved serum levels of MDA, GSSG and GSH compared to HFDHFr. Moreover, no significant differences were observed between the groups that were received fermented camel milk containing microencapsulated probiotic with plant extract (either ginger or beetroot) and the negative control group.

Our findings are in agreement with Zhao *et al.,* (2018) who examined the ability of *Lactobacillus plantarum* CCFM10 and RS15-3 to fight oxidative stress in mice and observed that both strains significantly reduced the changes occurred in MDA and GSH levels. Also, Valenlia *et al.,* (2018) examined the effects of administration *Lactobacillus plantarum* and inulin for eight weeks on insulin and oxidative markers in rats fed on high-fat diet plus streptozotocin. They found a significant reduction in MDA levels in the diabetes + *Lb. plantarum*, diabetes + inulin, and diabetes + *Lb. plantarum* + inulin groups.

Table 5: Effect of camel milk with and without chitosan-coated beads containing different plant extract on oxidative stress markers

Groups	Parameters		
	MDA (nmol/ml)	**GSH (µmol/ml)**	**GSSG (µmol/ml)**
Control (C^-)	2.11 ± 0.060 ^{e}	5.33 ± 0.14 ^{a}	0.323 ± 0.01 ^{d}
HFDHFr (C^+)	3.77 ± 0.10 ^{a}	3.02 ± 0.09 ^{d}	0.560 ± 0.02 ^{a}
CM	3.51 ± 0.11 ^{b}	3.19 ± 0.09 ^{d}	0.529 ± 0.01 ^{a}
FCMNP	2.85 ± 0.06 ^{c}	3.99 ± 0.10 ^{c}	0.436 ± 0.01 ^{b}
FCM+MP	2.59 ± 0.05 ^{d}	4.59 ± 0.10 ^{b}	0.393 ± 0.08 ^{bc}
FCM+ MPG	2.35 ± 0.09 ^{de}	4.98 ± 0.16 ^{a}	0.345 ± 0.09 ^{cd}
FCM+ MPB	2.28 ± 0.07 ^{e}	5.26 ± 0.13 ^{a}	0.360 ± 0.01 ^{cd}

Different superscripts letters within the same column (a, b, c……) are significantly different ($P<0.05$)
Control (C^-): rats fed on standard diet. **HFDHFr (C^+):** rats fed on high fat diet + high fructose in water**. CM:** rats fed on HFDHFr + camel milk**. FCM+NP:** rats fed on HFDHFr + fermented camel milk (FCM) containing nonencapsulated probiotic. **FCM+MP:** rats fed on HFDHFr + FCM containing microencapsulated probiotic without prebiotic**. FCM+MPG:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with ginger extract**. FCM+MPB:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with beetroot extract.

Results of Histological Examination

Histopathological examination of liver tissue from rats fed on HFDHFr plus oral administration of camel milk with or without microencapsulated probiotic in addition to control groups are represented in Fig (1). Microscopic examination of liver of control rats (C^-) revealed normal histological structure for central veins, portal areas and hepatic parenchymal cells**.** Regarding the examination of various liver sections of HFDHFr rats (C^+) showed vascular congestion, wide spread vacuolar degeneration (fatty change) with fat cyst formation and hepatocellular necrosis. The vacuolar degeneration appeared as microvesicular and macrovesicular types, signet ring appearance and scattered necrotic cells as well as congested hepatic sinusoids**.** The livers of HFDHFr group that was treated with CM showed congestion of the central vein and hepatic sinusoids, marked vacuolar degeneration and necrosis of the hepatic cells**.** The liver of HFDHFr rats that treated with FCM+NP showed hepatocellular vacuolar degeneration as a groups of cells or in scattered hepatocytes and necrotic cells**.** Some livers showed cellular swelling with granular and vacuolar degeneration**.** Liver of HFDHFr rats that was treated with FCM+MP showed a degree of vacuolar degeneration of some hepatic cells and necrotic changes. Liver of HFDHFr group that was treated with FCM+MPG showed a moderate degree of fatty change among the hepatic cells with few scattered necrotic cells**.** Liver of HFDHFr rats group that was treated with FCM+MPB showed good protection with normal appearance of the hepatic parenchymal cells with no obvious fatty change**,** only few cells was observed with vacuolated cytoplasm**.**

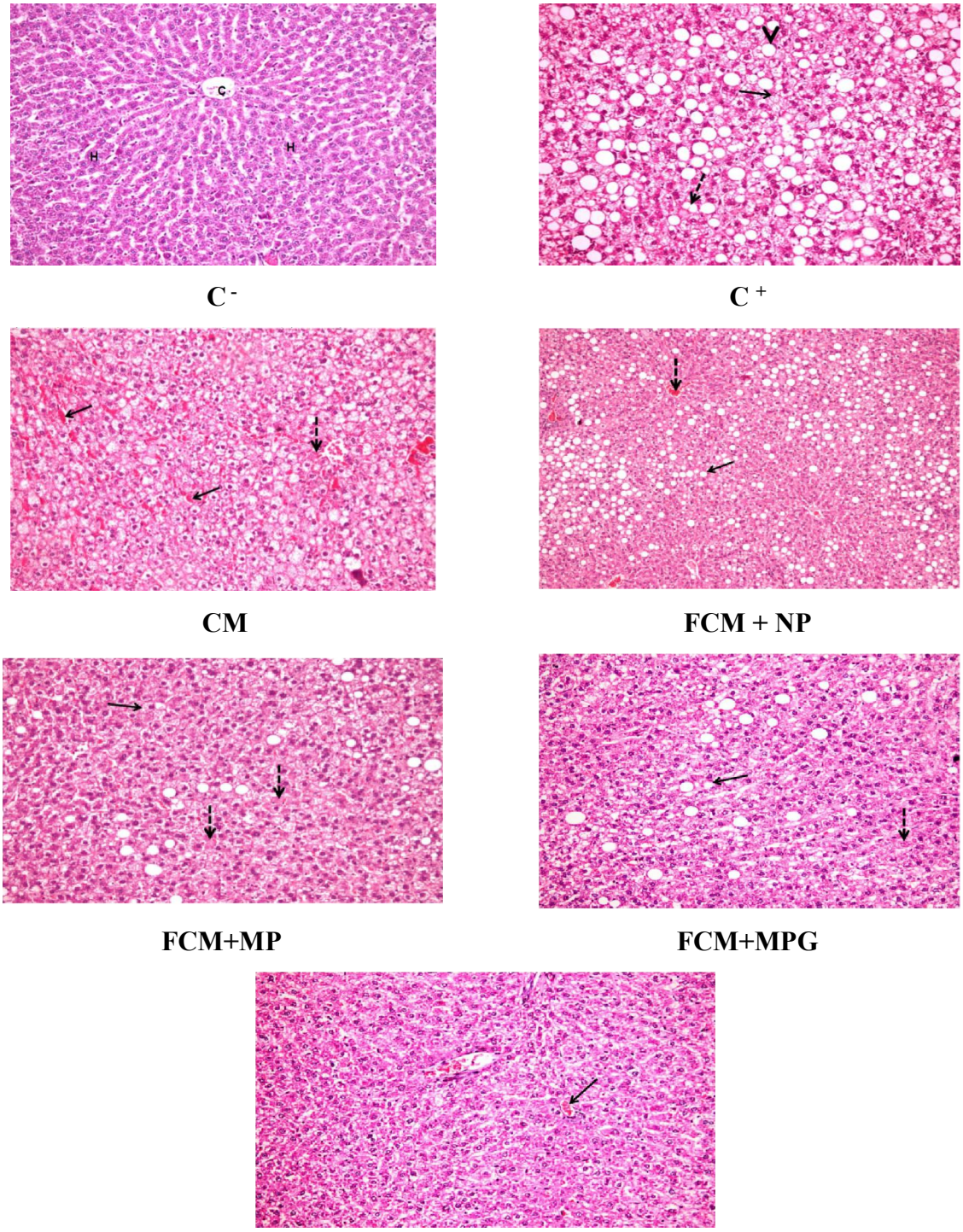

Fig. 1: Effect of camel milk with and without chitosan-coated beads containing different plant extract on histopathological examination of liver tissue for all groups (H&E, X200).
Control (C^-): rats fed on standard diet. **HFDHFr (C^+):** rats fed on high fat diet + high fructose in water**. CM:** rats fed on HFDHFr + camel milk**. FCM+NP:** rats fed on HFDHFr + fermented camel milk (FCM) containing nonencapsulated probiotic. **FCM+MP:** rats fed on HFDHFr + FCM containing microencapsulated probiotic without prebiotic**. FCM+MPG:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with ginger extract**. FCM+MPB:** rats fed on HFDHFr + FCM containing microencapsulated probiotic with beetroot extract.

Finally, it could be noticed that the obtained histopathological results for HFDHFr group showed the most prominent alteration of the hepatic tissue. While, examination of the other treated

groups showed that the FCM+MPB was the best in achieving protection of the hepatic tissue against the HFDHFr followed by FCM+MPG & FCM+MP which were in the same level of protection and finally FCM+NP. Whereas, treatment by CM showed the least improvement.

Conclusion

High fat diet plus high fructose in water induced nonalcoholic fatty liver with alteration in lipid profile, liver enzymes, oxidative stress and increased inflammatory cytokines in rats. Oral administration of camel milk caused a mild amelioration for previous parameters compared to HFDHFr group but still with strong gape compared to negative control group. In contrast, daily administration of fermented camel milk containing nonencapsulated probiotic or microencapsulated probiotics with or without plant extract lowered liver enzymes activity, proinflammatory cytokines and oxidative stress markers as well as enhanced insulin sensitivity, lipid profile and antioxidants parameters in serum rats. Furthermore, oral administration of fermented camel milk containing microencapsulated probiotic with plant extract either ginger or beetroot showed markedly amelioration of NAFLD compared to HFDHFr group. Moreover, the histological examination showed that fermented camel milk containing microencapsulated probiotics with beetroot extract had the highest protective effect followed by fermented camel milk containing microencapsulated probiotics with ginger extract. Finally, the obtained findings exhibited the efficiency of fermented camel milk containing microencapsulated probiotic with plant extract to protect liver during 60 days of feeding rats on high fat diet plus high fructose in water in addition to prevent nonalcoholic fatty liver progression.

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