Effect of Probiotics Versus Silymarin on a Model of Non-Alcoholic Fatty Liver in Adult Male Albino Rats. A Histological Study

Samir N. Ibrahim, Nada G. Saied, Ghada G. Hamam and Heba M. Fawzy
Histology Department, Faculty of Medicine, Ain Shams University, Cairo Egypt

*E-mail: samram15@hotmail.com; nada12gamal@gmail.com; ghada.hamam@med.edu.asu.eg; hebafawzy@med.asu.edu.eg

INTRODUCTION
Non-alcoholic fatty liver disease (NAFLD) is defined as the accumulation of liver fat of >5% of liver weight with <10 gm of daily alcohol consumption. It is a major cause of abnormal liver functions worldwide. NAFLD includes a spectrum of pathologies ranging from simple hepatic steatosis (fatty liver), and non-alcoholic steatohepatitis (NASH) up to liver fibrosis and cirrhosis (Li et al., 2014). NAFLD is a rapidly spreading global public health problem. It affects between 10-24% of people worldwide (Qin and Tian 2010). Its prevalence in Egypt’s obese population is thought to be 22% for men and 48% for women (Kinnunen, Bastola, and Neupane 2021).

ARTICLE INFO
Received: 7/11/2023
Accepted: 13/12/2023
Available: 17/12/2023

Background: Non-alcoholic fatty liver disease is the most prevalent widespread liver disease that may progress to liver fibrosis and cirrhosis.
Aim: To compare the possible hepatoprotective effects of probiotics versus silymarin on a model of non-alcoholic fatty liver disease in adult male albino rats.
Materials and methods: Five equal groups were created by randomly dividing fifty adult albino male rats; group I (control). Rats in the remaining groups received a high-fat, high-fructose diet daily for eight weeks, then they were divided into: groups II in which the animals were sacrificed after 8 weeks and group III in which the animals were left without treatment for a further 4 weeks, groups IV and V were given high fat high fructose diet for 8 weeks then received oral probiotics and silymarin daily (respectively) for further four weeks, then they were sacrificed. Following the experiment, liver samples were exposed to hematoxylin and eosin, Masson’s trichrome, and immunohistochemical techniques including proliferating cell nuclear antigen (PCNA) and hepatocyte-paraffin-1. Histomorphometric studies were also done.
Results: A high-fat high-fructose diet resulted in liver vacuolations, a significant increase in collagen deposition, and PCNA expression, while Hepatocyte Paraffin-1 expression showed a significant decrease. Significant improvement of all histological aspects was noticed in probiotics and silymarin-treated groups. However, the silymarin-treated group showed a better effect compared to the probiotic-treated group.
Conclusion: administration of both probiotics and silymarin improved Non-alcoholic fatty liver disease. However, silymarin was superior to probiotics.

ABSTRACT

Keywords:
Non-alcoholic fatty liver, Probiotics, Silymarin, Hepatocyte paraffin 1, PCNA.

Citation: Egypt. Acad. J. Biolog. Sci. (D-Histology and histochemistry) Vol.15(2) pp133-151(2023)
DOI: 10.21608/EAJBSD.2023.330207
High dietary fructose intake causes dyslipidaemia, ectopic lipid deposition, and elevated hepatic insulin resistance (Seneff, Wainwright, and Mascitelli 2011). There is an increased opportunity of getting non-alcoholic fatty liver disease (NAFLD) if you drink beverages that have added sugar. Sugar's fructose rather than glucose may be the cause of the rise in plasma triglyceride contents caused by sugar-sweetened beverages (Bray 2013).

Probiotics are live microorganisms that provide health advantages when administered in adequate amounts. They are gram-positive, micro-aerophilic rod-shaped bacteria. Lactobacillus is a probiotic that is commonly found in many fermented food products. These days, a lot of chronic and systemic diseases in the field of medicine are treated by probiotics (Mohammed et al., 2020). The gut microbiota has been identified as the primary participant in the gut-liver cross-talk. A growing clinical and empirical data suggests that change of the gut-liver axis is implicated in the initiation and progression of NAFLD, (Federico et al., 2016). Probiotics have thus been suggested as a therapeutic method for the treatment and/or prevention of NAFLD (Kobyliak et al., 2016).

Silymarin is a blend of flavonolignans that is derived from milk thistle seeds. The hepatic protective impacts of silymarin have been extensively evident in both animal studies and clinical trials. It prevents the establishment of alcoholic steatohepatitis and provides protection against chemically induced liver injury and fibrosis induced by substances such as carbon tetrachloride and thioacetamide. (Marcolino Assis-Júnior et al., 2017).

This work was designed to compare the possible hepatoprotective effects of probiotics versus silymarin on a model of non-alcoholic fatty liver disease in adult male albino Wister rats by histological and immunohistochemical techniques.

**MATERIALS AND METHODS**

1. **Drugs and Chemicals:**

   **Probiotics:** Lactobacillus plantarum subspecies plantarum DSA 20174 was prepared and purchased from the labs of the National Research Centre, Cairo, Egypt. Probiotics were given daily to rats by oral gavage before providing the standard chow diet and tap water at a dose of 1 mL/day. The concentration of probiotics was $10^9$ Colony-forming units (CFU)/mL/100 gm of body weight (Elshaer et al., 2019); (Mohammed et al., 2020).

   **Silymarin:**

   It was provided by CHEMICAL INDUSTRIES DEVELOPMENT GIZA -Under the License of MEDA Pharma GmbH &Co. KG- Germany. Silymarin capsules were dissolved in physiological saline. Silymarin was given daily to rats by oral gavage (50 mg/kg/day) (Mohamed et al., 2018).

2. **Induction of NAFLD:**

   NAFLD induction was accomplished through the administration of a high-fat, high-fructose (HFHF) diet daily for eight weeks. According to (Feillet-Coudray et al., 2019), the HFHF diet consisted of 35.5% fat, 32.4% carbohydrates and 18% proteins, in 100 grams of dry food with a total calorific value of 521 kcal/100 grams of dry food.

3. **Animals:**

   Fifty adult male albino Wistar rats were included in this study with an average weight of 150-200 gm and aged about eight weeks. The study animals were kept in pristine plastic cages covered in mesh wire. Throughout the trial, they had unrestricted access to tap water and a diet consisting of conventional rat chow. Suitable amounts of light, temperature, and humidity were provided for the animals’ care.

4. **Experimental Design:**

   Following the seven-day acclimatization period, five groups of rats were randomly assigned, 10 rats each: Group I (control group): rats were provided with a standard chow diet for the period of the experiment (12 weeks). Group II (NAFLD group): rats were fed HFHF diet daily for eight weeks from the beginning of the experiment, then they were sacrificed. Group III (untreated NAFLD): rats were fed HFHF diet daily for eight weeks, then they were given 1 ml physiological saline by oral gavage daily for a further four weeks during which animals were provided with a
standard chow diet, and then they were sacrificed. Group IV (NAFLD treated with Probiotics): rats were fed HFHF diet daily for eight weeks, then they were given probiotics daily by oral gavage for a further four weeks. Animals were fed a standard chow diet during the last 4 weeks (Elshaer et al., 2019) (Mohammed et al., 2020) then they were sacrificed. Group V (NAFLD treated with Silymarin): rats were fed HFHF diet daily for eight weeks, then were given silymarin daily by oral gavage for a further four weeks during which animals were provided with standard chow diet (Mohamed et al., 2018) and then they were sacrificed.

5. Sample Collection and Preparation of Tissues:

At the end of the experiment (8th, and 12th week), Rats underwent cervical dislocation as a means of sacrifice after injection of 40 mg/kg body weight of thiopental sodium phosphate intraperitoneally. (El-Kashef and Abdelrahman 2020). Through an anterior abdominal incision, the liver was dissected. The right lobe of the liver was collected from all animals. They were promptly fixed in 10% formalin for five days, then they were dehydrated in ascending grades of alcohol, cleared in xylene, and then they were embedded in paraffin. Serial sections of 5 µm thickness were cut and sections were then subjected to staining with Hematoxylin and Eosin stain (H&E) and Masson's trichrome stain for demonstration of collagen fibers. Paraffin sections were cut on positively charged slides and were subjected to immunohistochemical study for proliferating cell nuclear antigen (PCNA), and hepatocyte paraffin-1 (Hep Par-1). PCNA is an endogenous nuclear protein used in rat and human tissues to recognise cells that are replicating. The brown nuclear reaction was the indicator of a positive PCNA immunohistochemical method reaction. A portion of the skin's epidermis was stained to serve as the positive control. The PCNA kit was bought from Lab Vision in California, USA, and was diluted 1:200 for a duration of thirty minutes. A monoclonal antibody called hepatocyte paraffin-1 binds to an epitope on the membrane of hepatocellular mitochondria and produces the characteristic granular cytoplasmic pattern. Hep Par-1 positive immunoreactivity is shown as variable degrees of brown cytoplasmic staining. The bile ducts or any other nonparenchymal cells do not show any staining. Staining a portion of the control liver served as the positive control. Calbiochem Biotechnology, San Diego, CA, USA, provided us with ready-to-use Hep Par-1 antibodies (Dako FLEX, Clone OCH1E5) used for 30 minutes (Suvarna, Layton, and Bancroft 2018).

For microscopic examination, a microscope (Leica, DM2500) was used, using a Canon EOS 1100D Digital SLR camera at 10 (ocular) × 10 and 40 (object lens) magnification.

6. Histomorphometric Study and Statistical Analysis:

Histomorphometric analysis was performed on animals in each group. It was conducted using the Leica QWin V.3 program, an image analyzer installed on a computer. The computer was linked to a Leica DM2500 microscope (Wetzlar, Germany). Measurements were obtained from three distinct slides per animal across all groups. Five chosen non-overlapping fields were scrutinized from each slide to determine the mean area percentage of collagen, mean area percentage of PCNA, and mean optical density of Hep par 1 (X20).

Every study's morphometric data was gathered and then statistically examined. The Statistical Package for the Social Sciences (SPSS) statistical tool version 21 (IBM Inc., Chicago, Illinois, USA) was used to calculate the mean value and the standard deviation (SD) of the measured parameters in each group. Comparison between studied groups was done using One-way analysis of variance (ANOVA). LSD post-hoc test was done to detect significance between groups. Values were shown as mean ± SD. The significance of the data was determined
by probability of chance (P-value) where; P value < 0.05 was considered significant and p>0.05 was considered non-significant.

**Ethical Consideration:**

The animal experiment was conducted at the Research Centre Institute (MASRI) of Ain Shams University Faculty of Medicine. The study received approval from the Research Ethics Committee of the Faculty of Medicine, Ain Shams University (FMASU R303/2023). It was conducted in accordance with the guidelines set forth by the International Council on Harmonization (ICH) and the Islamic Organization for Medical Science (IOMS), as well as adhering to the regulations outlined by the US Office for Human Research Protections and the US Code of Federal Regulations. The study is covered by Federal Wide Assurance No. FWA 00017585.

**RESULTS**

1. **Histological and Immunohistochemical Results:**

Examination of the control’s H&E-stained sections revealed the normal structure of the classical liver’s lobules, which included a central vein and peripheral portal tracts. Bile ducts and branches of the hepatic artery and portal vein were observed within the portal tracts. Blood sinusoids were observed between the branching and anastomosing cords of hepatocytes that emerged from the central vein. Hepatocytes were seen to have central spherical open-face nuclei and acidophilic granular cytoplasm (Fig. 1A).

Analysing liver sections stained with H&E in rats belonging to group II (that received HFHF diet daily for eight weeks), showed marked affection of the liver structure. The parenchyma of the liver appeared distorted. Most of the hepatocytes were seen vacuolated. Ballooned hepatocytes were seen containing large cytoplasmic vacuoles and peripheral deeply stained flattened nuclei. Mallory bodies were seen in most hepatocytes as deep eosinophilic intracytoplasmic structures. Karyorrhectic nuclei as well as shrunken deeply stained nuclei were noticed in some hepatocytes. Obliterated blood sinusoids and mononuclear inflammatory cells in the portal tract were frequently observed (Fig. 1B).

Although an apparent decreased number of vacuolated hepatocytes was noticed in group III as compared to group II, most cells were seen with shrunken darkly stained nuclei. Moreover, Mallory bodies and karyorrhectic nuclei were still frequently noticed in most hepatocytes (Fig. 1C). In group IV, vacuolated hepatocytes and Mallory bodies were still noticed in some hepatocytes (Fig. 1D). While group V showed marked improvement of the structure of hepatocytes compared to groups III and IV. Most hepatocytes were seen with acidophilic cytoplasm and rounded open-face nuclei. Mallory bodies were occasionally noticed in a few hepatocytes. (Fig. 1E).

When examining Masson’s trichrome-stained sections from the control group, it was observed that there were only small quantities of collagen fibers present surrounding the central vein, within the portal tracts, and between hepatocytes. (Fig. 2A). In group II, an apparent increased amount of collagen fibers was noticed around most portal tracts and in-between hepatocytes (Fig. 2B). In group III, an apparent decreased amount of collagen fibers was seen around most portal tracts and in-between hepatocytes (Fig. 2C) compared to group II. In group IV moderate amount of collagen fibers was observed (Fig. 2D), while group V was nearly similar to the control group with an apparent decrease of perisinusoidal fibrosis (Fig. 2E).

A review of the control group revealed a small number of hepatocytes had a positive PCNA nuclear immune response (Fig. 3A). In group II and group III an observable rise in quantity and intensity of positive PCNA immune reactions was noticed in the nuclei of many hepatocytes. Positive PCNA reaction was also observed in the cytoplasm of some hepatocytes (Fig. 3B, 3C respectively). A moderate positive PCNA immune nuclear reaction was observed in some of the group IV hepatocytes. (Fig. 3D), while group V was comparable to the control group (Fig. 3E).

In relation to Hepatocyte paraffin-1 (HepPar-1), the control group showed hepatocytes with a pronounced granular cytoplasmic response (Fig. 4A). In group II, hepatocytes displayed vacuolation without any Hep Par-1 immune response, while other hepatocytes exhibited a diminished granular reaction (Fig. 4B). In group III, areas of negative Hep Par-1 immune reaction were noticed. Conversely, some hepatocytes...
exhibited a reduced Hep Par 1 reaction (Fig. 4C). Group IV showed a dense granular cytoplasmic reaction in most areas of hepatic lobules. Other areas were seen with negative cytoplasmic reaction (Fig. 4D). In contrast, in group V, the majority of hepatocytes displayed a dense granular cytoplasmic immune reaction to Hep Par-1, which closely resembled that of the control group (Fig. 4E).

2 Statistical Results:

Statistical analysis using one-way ANOVA test revealed a notable (P<0.05) elevation in the mean area percentage of collagen fibers in groups II and III when compared to the control group. While probiotic and silymarin treatments (group IV and V respectively) showed a significant decrease compared to the untreated NAFLD group (group III). On the other hand, the silymarin group (group V) showed a significant decrease in the mean area percentage of collagen fibers compared to the probiotic group (group IV) (Table 1).

One-way ANOVA test showed the mean area percentage of PCNA reaction in groups II and III was significantly higher (p<0.05) than in the control group. Meanwhile, treatment with probiotics and silymarin (groups IV and V respectively) showed a significant decrease in the mean area percentage of PCNA reaction compared to the untreated group (group III). Non-significant change was noticed between groups IV and V (Table 1).

The one-way ANOVA test demonstrated a significant (p<0.05) reduction in the mean optical density of Hep Par 1 in groups II and III when compared to the control group. Treatment with probiotics and silymarin showed a significant increase in the mean optical density of Hep Par 1 compared to group III. Conversely, the silymarin group (group V) exhibited a notable increase in the mean optical density of Hep Par 1 compared to the probiotic group. (Table 1).

Table 1: showing the mean area percentage of collagen, the mean area percentage of PCNA and the mean optical density of Hep Par 1 in different groups:

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean area percentage of</td>
<td>10.1±2.3</td>
<td>52.2±5.7</td>
<td>36.5±4.27</td>
<td>26.7±2.4</td>
<td>12.2±1.15</td>
</tr>
<tr>
<td>collagen fibers (%)</td>
<td></td>
<td>(*▲∆#)</td>
<td>(*)∆#</td>
<td>(#■∆)</td>
<td>(■∆▲)</td>
</tr>
<tr>
<td>Mean area percentage of</td>
<td>4.7±1.6</td>
<td>30.2±3.5</td>
<td>28.13±4.7</td>
<td>8.3±2.1</td>
<td>5.08±1.22</td>
</tr>
<tr>
<td>PCNA (%)</td>
<td></td>
<td>(*▲#)</td>
<td>(*)∆#</td>
<td>(#∆)</td>
<td>(■∆)</td>
</tr>
<tr>
<td>Mean optical density of</td>
<td>79.1±5.3</td>
<td>53.1±3.8</td>
<td>56.5±3.02</td>
<td>66.45±3.04</td>
<td>78.2±2.05</td>
</tr>
<tr>
<td>Hep par 1</td>
<td></td>
<td>(*▲#)</td>
<td>(*)∆#</td>
<td>(#∆)</td>
<td>(■∆▲)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.
* Significant difference from group I.
■ Significant difference from group II.
∆Significant difference from group III.
▲ Significant difference from group IV.
# Significant difference from group V.
**Fig 1:** Photomicrographs of liver sections from portal and periportal areas of different groups. [A] Control group (group I), [B] NAFLD group (group II); [C] untreated NAFLD (group III); [D] NAFLD treated with probiotics (group IV); [E] NAFLD treated with Silymarin (group V). Hepatic sinusoids (S), hepatocytes with intracellular vacuoles (↑); ballooned hepatocytes with peripheral flattened nuclei (F), Mallory body (curved arrow), karyorrhectic nuclei (elbow arrow), shrunken deeply stained nuclei (▲), mononuclear inflammatory cells in the portal tract (*). **H&E X 400 (scale bar: 50 μm).**
Effect of Probiotics Versus Silymarin on a Model of Non-Alcoholic Fatty Liver in Adult Male Albino Rats.

Fig. 2: Photomicrographs of liver sections from different groups. [A] Control group (group I), [B] NAFLD group (group II); [C] untreated NAFLD (group III); [D] NAFLD treated with probiotics (group IV); [E] NAFLD treated with Silymarin (group V). Collagen fibers between hepatocytes (↑); and in the portal tract (▲). Masson’s trichrome stain X 400 (scale bar: 50 μm)
Fig. 3: Photomicrographs of liver sections from different groups. [A] Control group (group I), [B] NAFLD group (group II); [C] untreated NAFLD (group III); [D] NAFLD treated with probiotics (group IV); [E] NAFLD treated with Silymarin (group V). Hepatocytes with dense positive PCNA nuclear reaction (↑); hepatocytes with nuclear and cytoplasmic reaction (▲). Anti-PCNA antibody X100 (scale bar: 200 μm); Insets X400 (scale bar: 50 μm).
Effect of Probiotics Versus Silymarin on a Model of Non-Alcoholic Fatty Liver in Adult Male Albino Rats.

Fig. 4: Photomicrographs of liver sections from different groups. [A] Control group (group I); [B] NAFLD group (group II); [C] untreated NAFLD (group III); [D] NAFLD treated with probiotics (group IV); [E] NAFLD treated with Silymarin (group V). Hepatocytes with dense granular cytoplasmic reaction (↑); hepatocytes with decreased granular cytoplasmic reaction (▲); hepatocytes with negative reaction (Δ). Anti-hepatocyte paraffin-1 antibody X100 (scale bar: 200 μm); Inset X400.

DISCUSSION

NAFLD is a multisystemic illness frequently associated with obesity, insulin resistance, metabolic syndrome, and hyperlipidemia. (Targher, Tilg, and Byrne 2021). In the liver, NAFLD ranges from simple fat accumulation with or without different degrees of hepatic inflammation (steatohepatitis), to progressive fibrosis and finally to cirrhosis and end-stage liver disease (Chalasani et al., 2018).

The purpose of the current work was to compare the potential hepatoprotective effects of probiotics and silymarin on a model of non-alcoholic fatty liver disease in adult male albino Wister rats.

In the current study, the H&E-stained sections of groups II and III showed hepatocyte vacuolations in which hepatocytes were seen containing large vacuoles pushing the nucleus to the periphery. Similarly, (Tandra et al., 2011) reported that steatosis in NAFLD is usually seen as macrovesicular steatosis (large droplet steatosis) where a single, large vacuole of fat fills up the hepatocyte and displaces the nucleus to the periphery. They supposed that large
fat droplets are formed by the fusion of small droplets. (Awad et al. 2016) found that rats provided with a high-fat, high-cholesterol diet displayed localized hepatic necrosis and hepatocyte vacuolations.

These findings could be clarified by some authors who reported that the beginning and progression of NAFLD are characterized by pathological mechanisms termed the "multi-hit" model (Buzzetti, Pinzani, and Tsochatzis 2016). The initial hit in NAFLD is the failure of fatty acid metabolism, which causes insulin resistance and alters signaling transductions, making the hepatocytes vulnerable to the subsequent multiple hits (Duvnjak et al., 2007). (Yan et al., 2020) added that when the liver is overloaded with free fatty acids from dietary fats and carbohydrates, then, the fatty acid disposal mechanisms are exhausted, and fatty acids can either be transformed to lipotoxic lipids or destroyed by fatty acid B-oxidation and triglyceride released from the liver to serum. These lipotoxic lipids could lead to oxidative stress, endoplasmic reticulum stress, inflammation, and possibly cell death. They added that the evolution of NASH can also be influenced directly or indirectly by non-liver organs. Changes in the makeup of the gut microbiota or changes in intestinal lipid signaling during the progression of NAFLD can result in toxic microbiota products or even produce a leaky gut to release bacteria.

In the current study, groups II and III revealed the presence of ballooned hepatocytes that contained intracellular vacuoles as well as Mallory bodies. An apparent decreased number of vacuolated hepatocytes and Mallory bodies was noticed in groups IV and V (treatment with probiotics and silymarin respectively). According to a previous study, hepatocyte ballooning with pale or comparatively clear cytoplasm is a significant predictor of an increased risk of developing cirrhosis. Previously known as Mallory bodies, Mallory-Denk bodies are eosinophilic intracytoplasmic inclusions found inside the cytoplasm of hepatocytes that have ballooned. They consist of misfolded intermediate filaments, chaperone proteins, and heat shock proteins. The existence of these elements indicates less favorable outcomes, especially when accompanied by steatohepatitis and fibrosis. Hepatocyte ballooning and Mallory-Denk bodies are of major importance in the diagnosis of NASH (Brown and Kleiner 2016).

In our study, cellular infiltration of inflammatory cells was frequently seen in the portal tract of group II. Similarly, (Awad et al., 2016) and (Kleiner and Makhlof 2016) reported that inflammatory foci were more frequently observed around the portal areas in NAFLD. This could be illustrated by (Chen and Madak-Erdogan 2018) who reported that a high-fat diet changes gut microbiota composition and increases the concentration of gram-negative bacteria, which produces lipopolysaccharides that increase inflammatory response. Another explanation was made by (Zhao et al., 2019) who reported that HFHF causes damage to the intestinal epithelial barrier which increases endotoxin leakage. HFHF also increases the production of proinflammatory cytokines.

In the present study, periportal and—pericellular/perisinusoidal fibrosis was observed in Masson’s trichrome-stained sections in groups II and III with a significant rise in the mean area percentage of collagen fibers when compared to the control group. A similar finding was observed by (Kleiner and Makhlof 2016) who stated that fibrosis is a histological criterion that denotes chronicity and disease development. Regardless of age, it is frequently seen in >80% of patients with NASH even though it is not one of the diagnostic criteria for steatohepatitis. The distinctive fibrosis patterns in fatty liver disease include centrizonal fibrosis and pericellular/perisinusoidal fibrosis, which show the deposition of fibrous
tissue in the Disse space in conjunction with stellate cell activity. Periportal fibrosis and bridging fibrosis will be developed as the disease progresses.

According to some authors, hepatic stellate cells (HSCs) are important in mediating hepatic fibrosis, which may account for the periportal fibrosis seen in the current study. The HSCs transform from a dormant to a highly proliferative myofibroblast-like cell type upon hepatic damage. These activated HSCs will release large volumes of extracellular matrix (Khomich, Ivanov, and Bartosch 2019). Another explanation for increased collagen fibers in NAFLD groups of the present study was made by some investigators who reported that failure of fatty acid metabolism resulted in the expression of pro-fibrogenic factors, that lead to activation of the fibrogenic cascade (Subramanian et al., 2022). It was reported that TGF-B is regarded as the primary fibrogenic cytokine released by the activated HSCs (Friedman 2004). Previous study by other authors have indicated that liver fibrogenesis is triggered by hepatocyte injury, leading to the mobilization of inflammatory cells and platelets. This, in turn, activates Kupffer cells, which subsequently release cytokines and growth factors (Mostafa et al., 2017).

In the current study, rats of group II that received HFHF diet for 8 weeks, demonstrated a significant rise in the PCNA-positive response compared to the control group. Recognizing replicating cells in rodent and human tissues requires the use of an endogenous nuclear protein called PCNA. PCNA antigen-antibody complexes displayed different patterns of staining. (Elsaeer et al., 2019). Groups II and III in the present investigation displayed both cytoplasmic and nuclear PCNA responses. According to a report, the staining pattern of PCNA was classified according to the reaction’s intensity and cellular distribution. Hepatocytes in quiescence or in G0 showed no apparent staining. A light-brown nuclear staining that ranged from patchy to homogeneous was observed in GI hepatocytes. Nuclear staining was uniformly strong and ranged from brown to black in S-phase cells. Nuclear and cytoplasmic staining with a diffuse speckled brown colour was used to identify hepatocytes in the G2 phase. The cytoplasmic staining of mitotic hepatocytes was widespread and speckled brown. (Elsaeer et al., 2019)(Foley et al., 1993). This could explain the results of the present work of increased mitotic activity in group II as indicated by both nuclear and cytoplasmic PCNA reactions.

The observed significant rise in the mean area percentage of PCNA positive reaction in groups II and III of the current study could be due to increased mitotic activity as a compensatory mechanism of increased hepatocytes apoptosis following HFHF. Similarly, (Wree et al., 2011) stated that free fatty acids (FFAs) cause lipoapoptosis in hepatocytes which is a key characteristic of NAFLD. They reported that toxic FFAs could increase the sensitivity of hepatocytes to cytokine toxicity that leads to activation of the lysosomal pathway of cell death. They noticed increased apoptotic markers in liver biopsy samples in NAFLD. Furthermore, it was noted that FFAs can trigger the intrinsic apoptotic pathway, which causes caspase activation, cytochrome c release, and mitochondrial permeabilization(Malhi et al., 2006). Another explanation was made by some authors who reported that failure of fatty acid metabolism in NAFLD leads to mitochondrial fatty acid oxidation which causes oxidative stress, that leads to necrosis/apoptosis of hepatocytes (Duvnjak et al., 2007). (Xiao et al., 2013) noticed a significant increase in the phosphorylated p53 protein in NAFLD rats, indicating the activation of the master regulator of cellular death.

In control group of the current study, intense granular cytoplasmic Hep Par-1 reaction was observed in most hepatocytes. It was reported that hepatocytes in healthy tissues showed a
high level of Hep Par-1 expression. It is thought that the epitope that reacts with Hep Par-1 is a mitochondrial-associated antigen, likely present on the membrane of hepatocellular mitochondria (Lugli et al., 2004). In the current study, a significant decline in the mean optical density of Hep Par-1 was observed in group II compared to the control group. It was reported that in NAFLD, reactive oxygen species (ROS) are produced in excess amounts. Increased ROS could damage the mitochondrial membrane and mitochondrial DNA (Kleiner et al., 2005). This illustrates our finding of the significantly decreased expression of Hep Par-1 in group II compared to the control group. (Ping et al., 2020) added, injured hepatocyte mitochondria release molecular danger signals called mito-DAMPs which trigger a strong inflammatory response and directly activate the hepatic stellate cells, which in turn cause liver scarring.

In the present study with cessation of HFHF diet for four weeks in group III, a slight improvement was noticed in H&E stained sections as compared to group II. (Xiao et al., 2013) reported that treatment of NAFLD includes pharmacological therapy and lifestyle strategies (such as weight loss, dietary changes, and physical activity). They added that the most well-known methods for managing NAFLD include diet change and weight loss. Although numerous clinical studies have demonstrated the therapeutic efficacy of lifestyle treatments in slowing the course of NAFLD, the fundamental mechanisms underlying these therapies remain largely unclear.

Probiotics were administered in the current investigation for four weeks after HFHF diet in group IV, causing a significant improvement in liver architecture (as shown by H&E and Masson’s trichrome stain) compared to group II. This finding aligned with (Briskey et al., 2016), Who found that mice fed HFD with probiotics supplementation showed 60% steatosis, while those fed HFD alone showed 85% steatosis. In addition, (Liang et al., 2018) added that in early human and animal studies, probiotics were found to treat HFD-induced NAFLD and its related lipid metabolic problems.

Some authors have suggested that the beneficial effects of probiotics observed in this study may be explained by the following: probiotics administration maintains the integrity of the intestinal epithelial barrier, so probiotics might partially counteract the negative effects of HFD. This theory was inspired by research that demonstrated the preservation of transepithelial permeability in cells subjected to injury by probiotics administration (Kocot et al., 2022).

A similar explanation for the role of probiotics in the treatment of NAFLD was described by some authors who reported that probiotic treatment may have slowed the advancement of NAFLD by preserving the physical barrier's integrity in the gut. The decrease in hepatic triglyceride levels observed in the animals given an HFD and probiotic supplements offers support to this theory. They hypothesized that this may be due to a reduction in the translocation of pathogens and their byproduct over the intestinal epithelial barrier in response to changes in bacterial distribution caused by HFDs (Peterson and Artis 2014).

Probiotics have been shown to have the strain-specific potential for the treatment of NAFLD in previous animal studies. The most beneficial impacts on NAFLD are linked to Lactobacillus and Bifidobacterium strains (Ritze et al., 2014). Administration of Lactobacillus resulted in improvement of insulin sensitivity, decreased lipopolysaccharide absorption, reduction of serum cholesterol level, reduction of liver fat accumulation, prevention of lipid peroxidation, activation of nuclear factor kappa B, and subsequent attenuation of steatosis. This occurs in various animal models of diet- and genetically-determined obesity (Savcheniuk et al., 2014).
In the present work treatment of NAFLD with silymarin resulted in improvement of hepatic steatosis as observed in H&E-stained sections compared to both groups II&III as well as probiotic treated group. This could be explained by some authors who reported that the main hepatoprotective mechanism of silymarin is its capacity to scavenge free radicals. It increases the amount of glutathione in the cells, which inhibits lipid peroxidation, and increases membrane stability (Polyak et al., 2010).

It was reported that the positive effects of silymarin on liver cells include antioxidative effects, direct and indirect impacts on fibrosis, as well as metabolic pathway regulation. Silymarin inhibits the generation of free radicals and nitric oxide in the presence of oxidative stress. It also increases the content of adenosine triphosphate and reinstates the baseline expression levels of antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase. (Surai 2015).

In the present study, periportal fibrosis was significantly reduced in sections of group V compared to both groups II and III as well as a probiotic group. This was in accordance with (Kim et al., 2012) who revealed that silymarin may have protective effects in diet-induced NASH by inhibiting HSCs activation and interfering with tumor necrosis factor-alpha (TNF-α)’s function as an inflammatory cytokine. Silymarin also had an antifibrogenic effect through the reduction of α1-procollagen mRNA expression in isolated HSCs. It was reported that silymarin decreases the deposition of collagen fibers because it prevents HSCs from becoming myofibroblasts, which are responsible for the deposition of collagen fibers that cause cirrhosis (Polyak et al., 2010).

In the current work, the administration of silymarin in group V led to a significant reduction in PCNA expression in hepatocytes compared to groups II and III. According to (Cengiz et al., 2015), silymarin decreases caspase activity and inhibits the growth of apoptotic cells. According to this opinion, it was suggested that decreased apoptosis of hepatocytes could lead to a decrease in the need for proliferation and mitosis with a subsequent decrease in PCNA reaction as observed in group V of the current study.

In the current study, the mean optical density of Hep Par-1 was significantly increased in sections of group V compared to NAFLD as well as in the probiotics-treated group. According to a study conducted by (Baldini et al., 2020), Silymarin was identified to enhance the mitochondrial electron transport chain under conditions of oxidative stress, simultaneously preserving the integrity of the mitochondrial respiratory chain. This results in less electron leakage and a decrease in the activity of ROS-producing enzymes in the mitochondria. According to this opinion, the preservation of mitochondrial structure with silymarin administration might be the cause of restoration of the mean optical density of Hep Par 1 reaction in group V of the present study.

Conclusions:
Probiotic and silymarin administration both improved NAFLD but silymarin exceeded probiotics.

Conflicts of interest/Competing interests: The authors have no conflicting financial or competing interests.

Acknowledgements: The authors are thankful to the members of Ain Shams University Faculty of Medicine’s Research Center Institute (MASRI).

REFERENCES


El-Kashef, Dalia H., and Rehab S. Abdelrahman. 2020. “Montelukast Ameliorates Concanavalin A-Induced Autoimmune Hepatitis in Mice via Inhibiting TNF-α/JNK Signaling Pathway.” *Toxicology and Applied Pharmacology*


Kleiner, David E., and Hala R. Makhlouf. 2016. “Histology of...
NAFLD and NASH in Adults and Children.” Clinical Liver Disease; 20(2): 293–312.


Li, Chuan, Shao Ping Nie, Ke Xue Zhu, Qiao Ding, Chang Li, Tao Xiong, and Ming Yong Xie. 2014. “Lactobacillus Plantarum NCU116 Improves Liver Function, Oxidative Stress and Lipid Metabolism in Rats with High Fat Diet Induced Non-Alcoholic Fatty Liver Disease.” Food & Function 5(12):3216–23. doi: 10.1039/C4FO00549J.


Mohammed, Safaa K., Yosra M. Magdy, Dalia AA El-Waseef, Enas S.


Surai, Peter F. 2015. “Silymarin as a Natural Antioxidant: An Overview of the Current Evidence and Perspectives.”


Zhao, Ze-Hua, Feng-Zhi Xin, Yaqian Xue, Zhimin Hu, Yamei Han, Fengguang Ma, Da Zhou, Xiao-Lin Liu, Aoyuan Cui, Zhengshuai Liu, Yuxiao Liu, Jing Gao, Qin Pan, Yu Li, and Jian-Gao Fan. 2019. “Experimental & Molecular Medicine Indole-3-Propionic Acid Inhibits Gut Dysbiosis and Endotoxin Leakage to Attenuate Steatohepatitis in Rats.” Experimental & Molecular Medicine. doi: 10.1038/s12276-019-0304-5.
Effect of Probiotics Versus Silymarin on a Model of Non-Alcoholic Fatty Liver in Adult Male Albino Rats.

ARABIC SUMMARY

دراسة نسيجية مقارنة بين تأثير البروبيوتيك مقابل سيليمارين على نموذج للكبد الدهني غير الكحولي في ذكور الجرذان البيضاء.

سمير نيروز إبراهيم, ندا جمال سعيد, غادة جلال همام, هبه محمد فوزي
قسم الهستولوجيا وبiology الحياتية كلية الطب - جامعة عين شمس

المقدمة: مرض الكبد الدهني غير الكحولي هو أكثر أمراض الكبد انتشارا والتي قد تتطور إلى تليف أو تشمع الكبد.

الهدف: المقارنة بين التأثيرات الوقائية للكبد المحتملة للبروبيوتيك مقابل سيليمارين على نموذج لمرض الكبد الدهني غير الكحولي في ذكور الجرذان البالغة.

المؤلفون: تم تقسيم خمسين من ذكور الجرذان البيضاء البالغة عشوائيا إلى خمس مجموعات متساوية.

المجموعة الأولى (الضابطة): تلقت الفئران في المجموعات المتبقية نظاما غذائيا عالي الدهون عالي الفركتوز يوميا لمدة ثمانية أسابيع ، ثم تم تقسيمها إلى: المجموعتان الثانية والثالثة التي تم فيها التضخيم بالفئران بعد ثمانية أسابيع و 12 أسبوعا (على التوالي) ، أعطيت المجموعتين الرابعة والخامسة البروبيوتيك عن طريق الفم وسيليمارين يوميا (على التوالي) لمدة أربعة أسابيع أخرى ، ثم تم التضحية بها. بعد التجربة ، تعرضت عينات الكبد للهيماتوكسيلين والإيوزين ، وثلاثي الألوان لاماسون ، والتقنيات الكيميائية المناعية بما في ذلك المستضد النووي للخلايا المتكاثرة (PCNA).

النتائج: أدى النظام الغذائي عالي الدهون والفركتوز إلى خراب الكبد، وزيادة واضحة في تعبير خلايا الكبد البارافين-1 وزيادة واضحة في المستضد النووي للخلايا المتكاثرة. لوحظ تحسن كبير في جميع الجوانب النسيجية في البروبيوتيك والجماعات المعالجة بالسيليمارين. ومع ذلك ، أظهرت المجموعة المعالجة سيليمارين تأثيراً أفضل مقارنة بالمجموعة المعالجة بالبروبيوتيك.

الاستنتاج: تناول كل من البروبيوتيك وسيليمارين تحسن مرض الكبد الدهني غير الكحولي. ومع ذلك ، كان سيليمارين متفوقاً على البروبيوتيك.