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# Effects of probiotics on methionine choline-deficient diet-induced steatohepatitis in rats

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***Background/aims:*** *Intestinal bacteria induce endogenous signals that play a pathogenic role in hepatic insulin resistance and non-alcoholic fatty liver disease. Probiotics could modulate the gut flora and could influence the gut-liver axis. We aimed to investigate the preventive effect of two probiotic mixtures on the methionine choline-deficient diet-induced non-alcoholic steatohepatitis model in rats.* ***Methods:*** *Two studies, short-term (2 weeks) and long-term (6 weeks), were carried out using 60 male Wistar rats. The 2-week study included six groups. Rats were fed with methionine choline-deficient diet or pair-fed control diet and were given a placebo or one of two probiotic mixtures (Pro-1 and Pro-2) by orogastric gavage. In the 6-week study, rats were allocated into four groups and were fed with methionine choline-deficient diet or pair-fed control diet and given a placebo or Pro-2. At the end of the 2- and 6-week periods, blood samples were obtained, the animals were sacrificed, and liver tissues were removed. Serum alanine aminotransferase activity was determined; histologic and immunohistochemical analysis was performed for steatosis, inflammation, protein expression of tumor necrosis factor-α, and apoptosis markers.* ***Results:*** *In both studies, methionine choline-deficient diet caused an elevation of serum alanine aminotransferase activity, which was slightly reduced by Pro-1 and Pro-2. In the 2- and 6-week studies, feeding with methionine choline-deficient diet resulted in steatosis and inflammation, but not fibrosis, in all rats. In the 2-week study, in rats fed with methionine choline-deficient diet and given Pro-1, steatosis and inflammation were present in 2 of 6 rats. In rats fed with methionine choline-deficient diet and given Pro-2, steatosis was detected in 3 of 6 rats, while inflammation was present in 2 of 6 rats. In the 6-week study, in rats fed with methionine choline-deficient diet and given Pro-2, steatosis and inflammation were present in 3 of 6 rat livers. In both the 2- and 6-week studies, methionine choline-deficient diet resulted in tumor necrosis factor-α, proapoptotic Bax, caspase 3, caspase 8, and anti-apoptotic Bcl-2 expression in all rat livers. Pro-1 and Pro-2 treatment influenced protein expression involved in apoptosis and tumor necrosis factor-α in varying degrees.* ***Conclusions:*** *Pro-1 and Pro-2 decrease methionine choline-deficient diet-induced steatohepatitis in rats. The preventive effect of probiotics may be due, in part, to modulation of apoptosis and their anti-inflammatory activity.*

**Key words:** Non-alcoholic fatty liver disease, methionine choline-deficient diet, probiotics

# Sıçanlarda metiyonin ve kolinden fakir diyetle oluşturulan steatohepatitte probiyotiklerin etkisi

***Amaç:*** *İntestinal bakteriler, hepatik insülin direnci ve non-alkolik yağlı karaciğer hastalığında patojenik rol oynayan endojen sinyalleri indüklerler. Probiyotikler barsak florasını düzenleyerek karaciğer-barsak ekseninde etki gösterebilirler. Bu çalışmada sıçan modellerinde metiyonin ve kolinden fakir diyet ile oluşturulan non-alkolik steatohepatitte iki probiyotik karışımının koruyucu etkisini araştırmayı amaçladık.* ***Yöntem ve Gereç:*** *Altmış adet erkek Wistar sıçan kullanılarak 2 ve 6 haftalık iki çalışma yapılmıştır. İki haftalık çalışma 6 gruptan oluşturulmuştur. Sıçanlar metiyonin ve kolinden fakir diyet ya da kontrol diyetle beslenmiş ve orogastrik sonda ile plasebo ya da iki probiyotik karışımı (Pro-1, Pro-2) verilmiştir. Altı haftalık çalışmada sıçanlar 4 gruba bölünerek metiyonin ve kolinden fakir diyet ya da kontrol diyet ile beslenmişler ve plasebo ya da Pro-2 verilmişlerdir. Çalışma sonunda sıçanlar kanları alınarak kurban edilmiş ve karaciğerleri alınmıştır. Serum alanin aminotransferazları saptanan hayvanlarda steatozis, inflamasyon, tümör nekroz faktörü-α ekspresyonu ve apopitozisin saptanması için histolojik ve immünhistokimyasal inceleme yapılmıştır.* ***Bulgular:*** *Her iki çalışmada da metiyonin ve kolinden fakir diyet serum alanin aminotransferaz seviyesinde, Pro-1 ve Pro-2 ile hafif düşürülebilen, bir artışa neden olmuştur. İki ve 6 haftalık çalışmalarda metiyonin ve kolinden fakir diyet tüm sıçanlarda steatozis ve inflamasyona neden olurken fibrozis oluşturmamıştır. İki haftalık çalışmada metiyonin ve kolinden fakir diyet ve Pro-1 ile beslenen 6 sıçanın 2'sinde steatozis ve inflamasyon görülmüştür. Metiyonin ve kolinden fakir diyet ve Pro-2 ile beslenen 6 sıçanın 3'ünde steatozis görülürken 2'sinde inflamasyon vardı. Altı haftalık çalışmada metiyonin ve kolinden fakir diyet ve Pro-2 ile beslenen 6 sıçanın 3'ünde steatozis ve inflamasyon vardı. Her iki çalışmada da metiyonin ve kolinden fakir diyet tüm sıçan karaciğerlerinde, tümör nekroz faktörü-α, proapopitotik Bax, caspase 3, caspase 8 ve anti-apopitotik Bcl-2 ekspresyonuna neden oldu.* ***Sonuç:*** *Pro-1 ve Pro-2, sıçanlarda metiyonin ve kolinden fakir diyet ile oluşturulan steatohepatiti azaltmaktadır. Probiyotiklerin koruyucu etkisi kısmen apopitoz modülasyonu ve antiinflamatuar etkilerine bağlı olabilir.*

**Anahtar kelimeler:** Nonalkolik yağlı karaciğer hastalığı, metiyonin ve kolinden fakir diyet, probiyotik

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) includes a spectrum of pathological hepatic changes ranging from steatosis, steatohepatitis, advanced fibrosis, and cirrhosis. The pathogenesis of NAFLD is uncertain. According to the 'two-hit' theory, liver fat accumulation and insulin resistance characterize the first hit and are responsible for the development of steatosis (1). Certain endogenous or exogenous toxins, oxidative stress and subsequent lipid peroxidation, together with the production of proinflammatory cytokines (e.g. tumor necrosis factor [TNF]-α), and hormones derived from adipose tissue initiate the second hit, which progresses from simple steatosis to non-alcoholic steatohepatitis (NASH) (2-6). It is suggested that most cryptogenic cirrhosis is the result of previously undiagnosed NASH (6,7).

The liver continuously receives blood from the gut via the portal system; thus, there is a close relationship between the gut and liver (8). One of the endogenous factors that contributes to the pathogenesis of NAFLD may be the intestinal bacterial flora (9-13). Animal studies have demonstrated that intestinal bacteria induce endogenous signals that play a pathogenic role in hepatic insulin resistance (13), and intestinal bacterial overgrowth plays a significant role in the pathogenesis of both alcoholic fatty liver disease and NAFLD (10-12). Bacterial overgrowth and translocation result in endotoxemia that directly stimulates hepatic Kupffer cells to produce TNF-α (14,15).

Probiotics could modulate the gut flora and could influence the gut-liver axis. Furthermore, many data indicate that probiotics may promote intestinal mucosal barrier function and mucosal recovery during a pathological condition. *Lactobacillus* GG has been shown to improve alcohol-induced gut leakiness and to blunt alcohol-induced oxidative stress and inflammation in both the intestine and liver (16). A two-organism probiotic combination has been reported to maintain and enhance the phosphorylation of tight junction proteins including tight junction protein 1 and occludin (17). Further, probiotics induce mucin gene expression and the facilitation of mucus layer integrity (18). In addition, the administration of *Bifidobacterium lactis* (19) and *Lactobacillus johnsonii* La1 (20) reduces the incidence of bacterial translocation.

Some experimental data indicate that probiotics prevent or alleviate liver steatosis and inflammation. Treatment with oral probiotic VSL#3, consisting of eight probiotic strains, has been reported to significantly improve the high fat diet-induced hepatic NKT cell depletion, insulin resistance and hepatic steatosis (21). In another study, *Lactobacillus* GG (16) and heat-killed *Lactobacillus brevis* SBC8803 (22) have been shown to ameliorate ethanol-induced liver injury and fatty liver. In contrast, VSL#3 has failed to prevent methionine choline-deficient diet (MCDD)-induced liver steatosis or inflammation, but to ameliorate MCDD-induced liver fibrosis (23).

We aimed to test the beneficial effects of two different probiotic mixtures for the MCDD-induced NASH model in rats. Some data propose that hepatocyte apoptosis may be a key component in disease progression to NASH (24). In this study, we also evaluated the immunohistochemical markers involved in hepatocyte apoptosis.

## MATERIALS AND METHODS

### Animals

Male Wistar albino rats (n=60) were fed with MCDD or pair-fed control diet and water *ad libitum* and kept in cages at 22±2°C with a 12-hour (h) dark–light cycle before and during experiments. Animals were allowed to acclimatize to their new conditions for one week prior to the commencement of the study. Experiments were approved by the Suleyman Demirel University, Animal Ethical Committee. During this experimental study, we acted according to the principles of the Guide for the Care and Use of Laboratory Animals (25).

### Animal Diets

To induce fatty liver, rats were fed with MCDD (C1070, Altromin, Germany). The control groups were fed with control diet (C1000, Altromin, Germany).

### Probiotic Microorganisms

Two probiotic mixtures were used in this study. The probiotics were prepared by the Suleyman Demirel University Faculty of Engineering and Architecture, Food Engineering Department.

*Pro-1* consisted of 13 bacterial strains that were isolated from the healthy human stool samples. They had the ability to resist the low pH and bile salts (26). These strains were *Lactobacillus fermentum* (BB16-75, AK2-8, AK5-22, AK6-26), *Lactobacillus plantarum* (AA17-73, AK7-28, AK8-31B) and *Enterococcus faecium* (AB6-21, AB16-68,

AK4-120, AK7-31, BK9-40, BK13-54). *Pro-2* consisted of six bacterial strains (*Enterococcus faecium* BK10-47 and *Lactobacillus plantarum* AB7-35, AC3-16, AC21-101, AB16-65, BK10-48). Lipid reduction of these strains was determined as 50.3-58.2% (27). These strains were also isolated from human feces. Molecular identification of all strains was made by 16S rRNA analyses (26). Each strain was grown in MRS broth at 37°C for 24 h until the cell number reached $10^9$ cfu/ml. The cells were centrifuged at 5000 x g for 10 minutes (min) at 20°C and then the cell pellets were washed twice in phosphate-buffered saline (PBS) solution, pH 7.4. Finally, the pellets were diluted in 10% sterile reconstituted skim milk to $1.3x10^{10}$ cfu/ml and $0.6 \times 10^{10}$ cfu/ml for Pro-1 and Pro-2, respectively.

## Study Design

Two studies, short-term (2 weeks) and long-term (6 weeks) were carried out.

The 2-week study included six groups. Thirty-six rats were allocated into six groups of six animals each. Groups 1, 2, and 3 were fed by MCDD and gavaged daily with 0.2 ml of placebo (skim milk), Pro-1 or Pro-2. Groups 4, 5, and 6 were fed with control diet and received placebo, Pro-1 or Pro-2, respectively, at the same doses. All rats were weighed at the same time, and food and water were monitored daily throughout the study periods. Control-diet groups were pair-fed to match the food intake of the MCDD-fed groups. Rats had free access to water.

In the 6-week study, 24 rats were allocated into four groups. Groups 1 and 2 were fed with MCDD and received 0.2 ml/day of placebo or Pro-2 (0.2 ml/day), respectively. Groups 3 and 4 were fed with control diet and received placebo or Pro-2, respectively, at the same doses.

At the end of the 2- and 6-week periods, blood samples were obtained, the animals were sacrificed by 100 ml/kg ketamine hydrochloride (Ketalar®, Parke-Davis, Eczacibası, İstanbul, Turkey) and 25 mg/kg xylazine hydrochloride (Rompun®, Bayer, Germany) injection, and liver tissues were removed.

## Biochemical Analysis

Blood samples were placed into jelly biochemistry tubes and centrifuged for 4 min at 4000 g to separate the serum. The alanine aminotransferase (ALT) activity was studied immediately using a spectrophotometric method on the Abbott Aeroset autoanalyzer using compatible commercial kits from Abbott.

## Histopathological and Immunohistochemical Assessment

Liver biopsy samples for histopathological assessment were fixed with 10% formalin for 72 h. Thereafter, the routine follow-up procedure was performed. Sections of 4 µm in thickness were obtained. Hematoxylin-eosin staining for routine analysis and Masson's trichrome staining for evaluation of fibrosis were performed and assessed histopathologically using light microscopy. Scoring was performed according to the scoring system of Sundaram et al. (28). Steatosis was scored between 0-4, inflammation between 0-4, and fibrosis between 0-4.

Histopathological changes in liver tissue, apoptosis and protein expression taking place in various stages of the apoptosis regulation were investigated using the immunohistochemical method. Tissue samples from each group were fixed in neutral formalin for 72 h and processed for paraffin embedding. Sections of 4-5 Ìm thickness were processed for polylysine microscope slides. For the immunohistochemical examination, slides were treated in a microwave oven in 0.01 M citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase activity was blocked by incubation for 20 min with 0.3% hydrogen peroxidase. Slides were stained with mouse monoclonal anti–caspase 3 antibody (rabbit polyclonal antibody Ab-4, 1 mg/ml; NeoMarkers, Fremont, CA, USA), caspase 8 (rabbit polyclonal antibody Ab-4, 1 mg/ml; NeoMarkers, Fremont, CA, USA), Bax (rabbit polyclonal antibody Ab-1, 3525; NeoMarkers, Fremont, CA, USA), Bcl-2 (rabbit polyclonal antibody Ab-1, 6837; NeoMarkers, Fremont, CA, USA) and TNF-α (mouse monoclonal antibody, sc-7317; Santa Cruz, CA, USA). Tissues from lymph node and breast were used for Bax and TNF-α immunostaining as negative controls. Tonsillary tissue was used for Bcl-2, caspase 3 and caspase 8 immunostaining as a negative control. No immunostaining was observed for these markers in the negative controls. Sections were incubated with the streptavidin–biotin peroxidase kit (Ultra Vision Large Volume Detection System Anti-polyvalent, HRP; LabVision, Fremont, CA, USA), and the reaction product was detected with diaminobenzidine. Finally, the sections were counter-stained with Mayer's hematoxylin, mounted with a mounting medium, and examined with the Olympus BH2 photo-light microscope.

### Statistical Analysis

All data are expressed as mean ± SEM. Significant difference in serum ALT concentrations between groups was determined by one way analysis of variance (ANOVA), followed by a *post hoc* Tukey's test when the analysis of variance suggested a significant difference between groups. Kruskal–Wallis analysis of variance was applied to assess differences in histological and immunohistological scores among the experimental groups. When the Kruskal-Wallis test indicated a significant difference, multiple comparisons were performed using the Mann-Whitney *U* test to determine which group differed from the others. The data were analyzed using the SPSS 11.0 statistical package program. A value of $p<0.05$ was considered significant.

## RESULTS

### Serum ALT Levels

Significantly elevated serum ALT levels were shown in rats fed MCDD compared with rats fed the control diet. In both studies, probiotic treatment showed an insignificant decrease in serum ALT levels of rats fed MCDD (Figures 1a, 1b).

### Histological and Immunohistochemical Evaluations

#### *Two-week study*

In the 2-week study, feeding with MCDD resulted in steatosis and inflammation, but not fibrosis, in all rats (Figure 2a). MCDD elicited TNF-α (Figure 2b), Bcl-2, Bax (Figure 2c), caspase 3 and caspase 8 expression in all rats with weak to strong immunohistochemical staining.

Pro-1 and Pro-2 treatment partly impeded steatohepatitis. Among the rats fed with MCDD and given Pro-1 (Group 2), steatosis and inflammation were present in 2 of 6 rats (Figure 2d). Among the rats fed with MCDD and given Pro-2 (Group 3), steatosis was detected in 3 of 6 rats, while inflammation was present in 2 of 6 rats (Figure 2g). However, only Pro-1 treatment significantly decreased scores of MCDD-induced steatosis and inflammation (Figure 3a).

In Group 2, TNF-α (Figure 2e) and Bax (Figure 2f) showed weak to strong immunohistochemical staining in all rats. Two of 6 rats were positive for Bcl-2, while 4 of 6 rats were positive for caspase 3 and caspase 8. In Group 3, 5 rats were positive for both caspase 8 and TNF-α (Figure 2h). Weak and strong staining was detected in all rats using Bax (Figure 2i). Only one rat stained positively with Bcl-2, while 4 rats were positive for caspase 3.

Steatosis and inflammation were not present in the rat groups fed with control diet (Groups 4, 5, 6) (Figures 2j, 2m, 2p). In addition, immunohistochemical staining did not detect TNF-α (Figures 2k, 2n, 2q), Bcl-2, Bax (Figures 2l, 2o, 2r), caspase 3, or caspase 8 in these groups.

#### *Six-week study*

In the 6-week study, histological analysis also revealed steatosis and inflammation, but not fibrosis, in all livers of rats fed with MCDD (Figure 4a). Steatosis scores were between 1 and 3. Weak to strong immunohistochemical staining was detected in all of the rats using Bcl-2, Bax, caspase 3, caspase 8, and TNF-α.

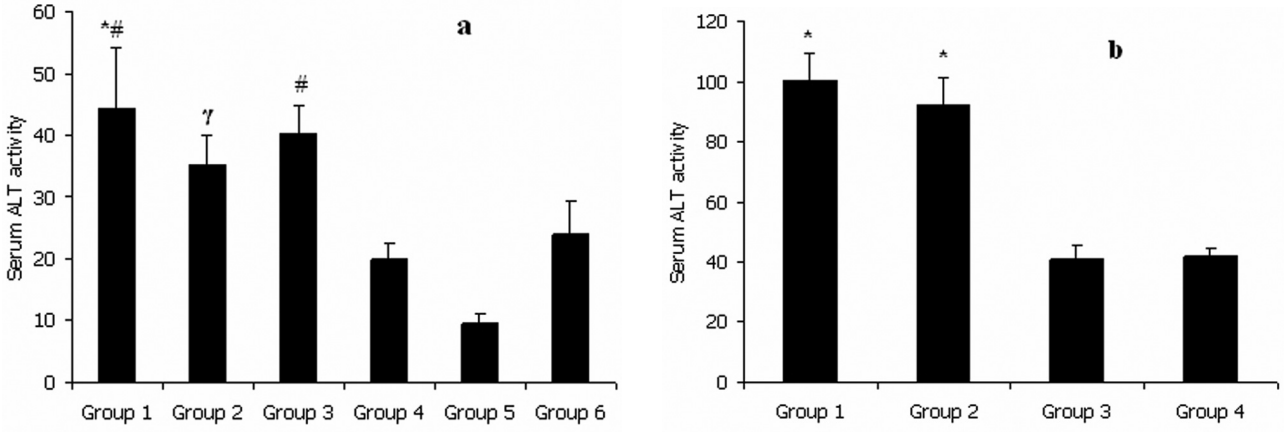


**Figure 1. a.** Serum ALT levels in the 2-week study. Group 1: MCDD+placebo, Group 2: MCDD+Pro-1, Group 3: MCDD+Pro-2, Group 4: Control diet+placebo, Group 5: Control diet+Pro-1, Group 6: Control diet+Pro-2. **According to Group 4, p<0.05; #According to Group 5, p<0.01; γAccording to Group 5, p<0.05.* **b.** Serum ALT levels in the 6-week study. Group 1: MCDD+placebo, Group 2: MCDD+Pro-2, Group 3: Control diet+placebo, Group 4: Control diet+Pro-2. **According to Groups 3 and 4, p<0.001.*

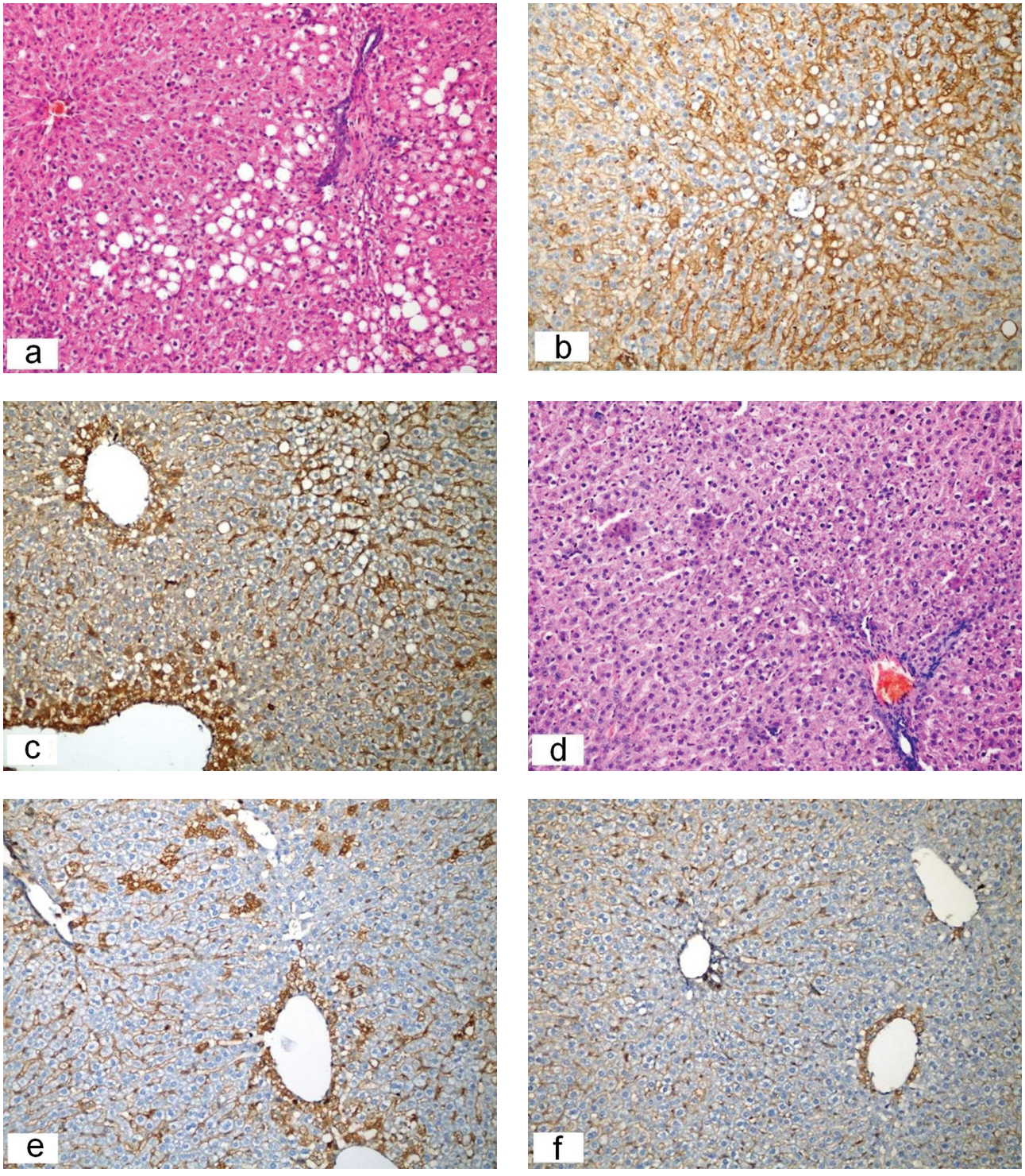


**Figure 2.** Immunohistochemical and histopathologic characteristics in Protocol I: Macrovesicular steatosis and inflammation in the rat liver in the CMDD group, H&E, x100 **(a)**. Diffuse and cytoplasmic TNF-α staining in hepatocytes in the CMDD group, x100 DAB **(b)**. Cytoplasmic Bax positivity in hepatocytes in the CMDD group, x100 DAB **(c)**. Mild inflammation in the rat liver in the CMDD+mixed probiotic group, H&E x100 **(d)**. Focal cytoplasmic staining with TNF-α in hepatocytes in the CMDD+mixed probiotic group, x100 DAB **(e)**. Weak cytoplasmic Bax positivity in hepatocytes in the CMDD+mixed probiotic group, x100 DAB **(f)**. Mild inflammation in the rat liver in the CMDD+LLP group, H&E x100 **(g)**. Weak cytoplasmic staining with TNF-α in hepatocytes in the CMDD+LLP group, x100 DAB **(h)**. Focal and weak cytoplasmic Bax positivity in hepatocytes in the CMDD+LLP group, x100 DAB **(i)**. Normal rat liver in the control, control+mixed probiotic and control+LLP groups, H&E x100 **(j, m, p)**. TNF-α negativity in the control, control+mixed probiotic and control+LLP groups, x100 DAB **(k, n, q)**. Bax negativity in the control, control+mixed probiotic and control+LLP groups, x100 DAB **(l, o, r)**.

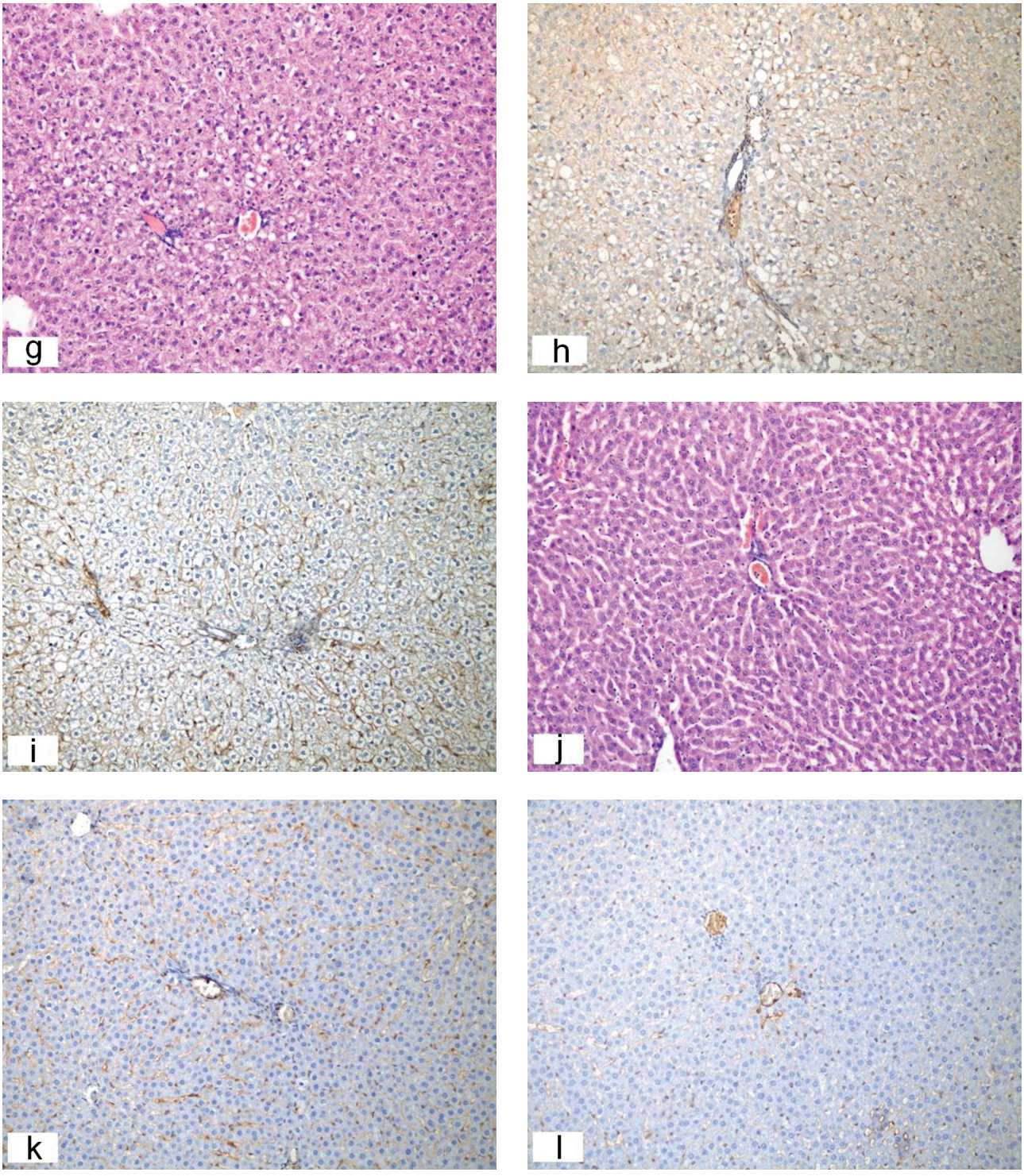


Figure 2 (cont.).

In rats fed with MCDD and given Pro-2 (Group 2), steatosis and inflammation were observed in only 3 (50%) rat livers. Steatosis scores were 1 and 2 (Figure 4b). Although histological scores of MCDD steatosis and inflammation reduced with Pro-2 treatment, it was not found to be statistically significant (Figure 3b). Weak to strong staining was present in all rats using Bax, Bcl-2, caspase 3, caspase 8, and TNF-α. In the rat groups fed with control diet and given placebo or Pro-2 (Groups 3 and 4), no steatosis or inflammation was detected (Figure 4c).

In both the 2- and 6-week studies, MCDD resulted in proapoptotic Bax, caspase 3, caspase 8, and anti-apoptotic Bcl-2 expression in all rat livers. Pro-1 treatment reduced the expression and staining intensity of Bax, caspase 3, caspase 8, and Bcl-2. Pro-2 did not influence the MCDD-induced caspase 8 expression in either the 2- or 6-week study. Pro-2 augmented the MCDD-induced Bax expression in the 6-week study. In both studies, Pro-1 and Pro-2 attenuated TNF-α expression to varying degrees (Tables 1, 2).

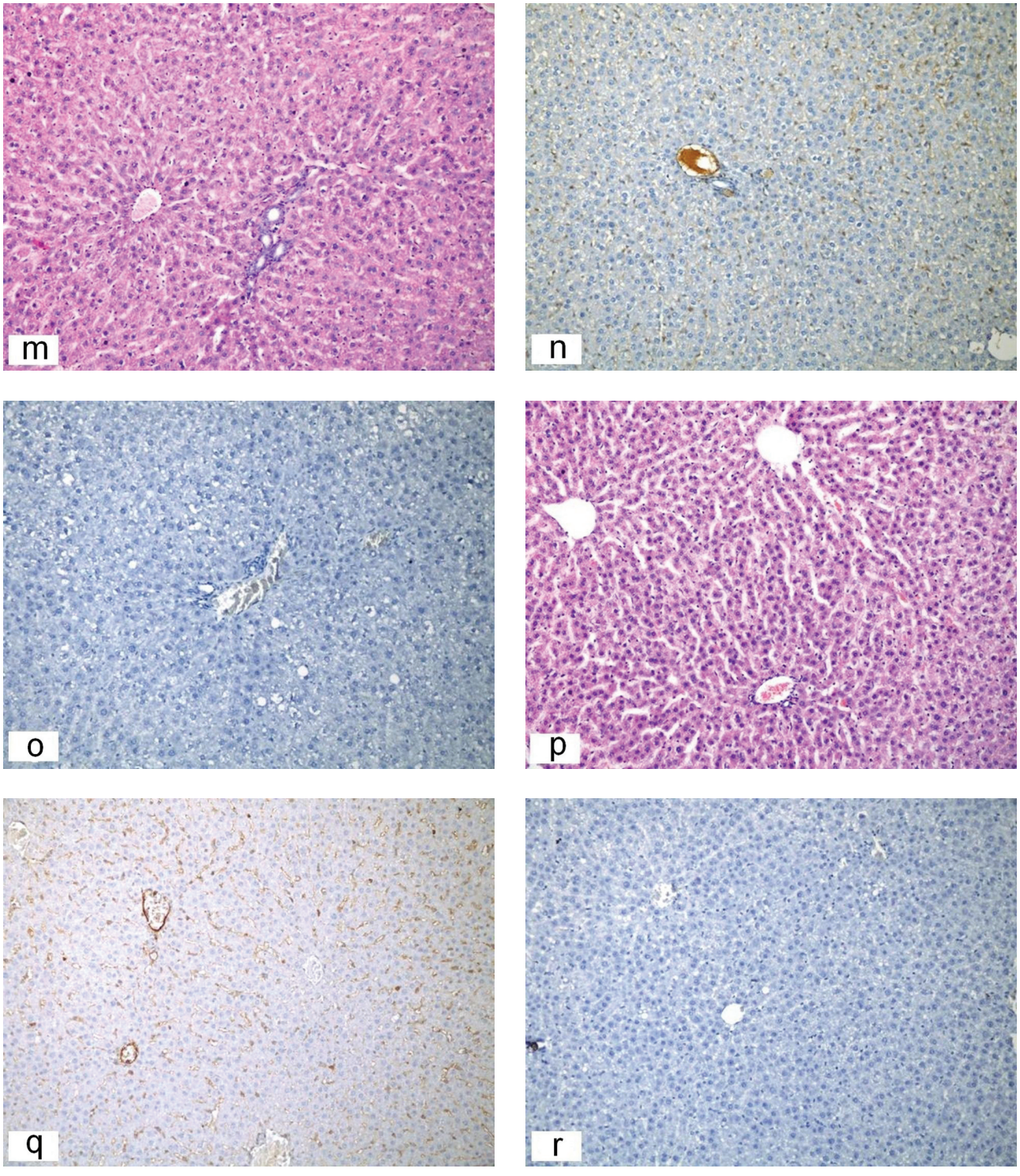


Figure 2 (cont.).

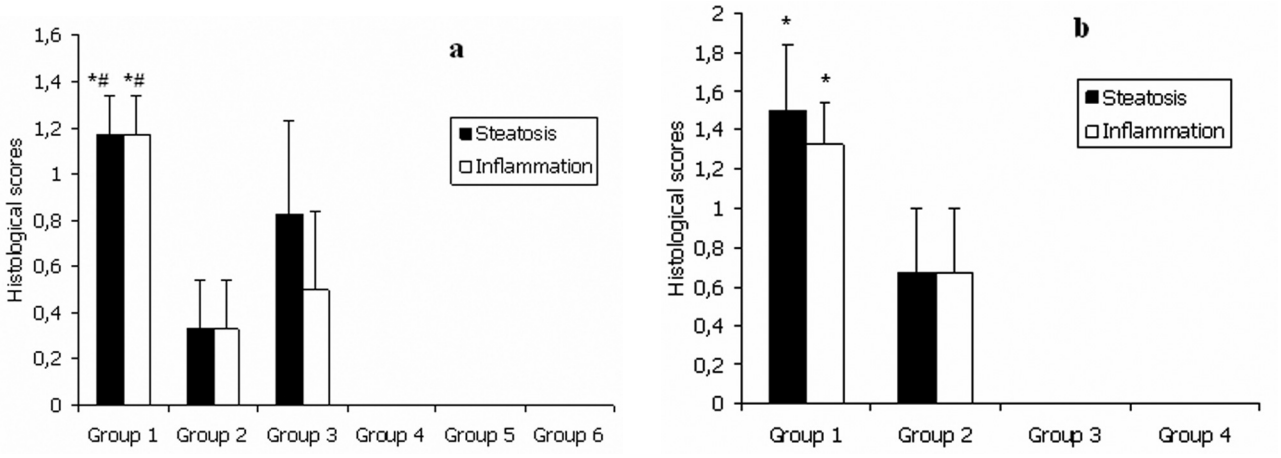


**Figure 3.** Histologic steatosis and inflammation scores of livers in the 2-week study. Group 1: MCDD+placebo, Group 2: MCDD+Pro-1, Group 3: MCDD+Pro-2, Group 4: Control diet+placebo, Group 5: Control diet+Pro-1, Group 6: Control diet+Pro-2. **According to Group 2, p=0.018; #According to Groups 4, 5, 6, p=0.001.* **b.** Histologic steatosis and inflammation scores of livers in the 6-week study. Group 1: MCDD+placebo, Group 2: MCDD+Pro-2, Group 3: Control diet+placebo, Group 4: Control diet+Pro-2. **According to Groups 3 and 4, p=0.002.*

**Table 1.** Immunohistochemical protein expression of apoptotic markers and TNF-α in the 2- week study

| Groups† | Bax | Bcl-2 | Caspase 3 | Caspase 8 | TNF-α |
|---|---|---|---|---|---|
| 1 | 1.83+1.17* | 1.33+0.33*# | 1.50+0.22* | 1.17+0.31* | 2.0+0.0* |
| 2 | 1.67+0.21* | 0.50+0.34 | 1.00+0.37# | 0.83+0.31# | 1.33+0.21* |
| 3 | 1.50+0.22* | 0.17+0.17 | 0.83+0.31# | 1.17+0.31* | 1.50+0.34* |
| 4 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 |
| | *According to Groups 4, 5, 6: p<0.01 | *According to Group 3: p=0.019; #According to Groups 4, 5, 6: p<0.01 | *According to Groups 4, 5, 6: p<0.01; #According to Groups 4, 5, 6: p<0.05 | *According to Groups 4, 5, 6: p<0.01; #According to Groups 4, 5, 6: p<0.05 | *According to Groups 4, 5, 6: p<0.01 |

†Group 1: MCDD+placebo, 2: MCDD+Pro-1, 3: MCDD+Pro-2, 4: Control diet+placebo, 5: Control diet+Pro-1, 6: Control diet+Pro-2. *Values are expressed mean±S.E.M.*

**Table 2.** Immunohistochemical protein expression of apoptotic markers and TNF-α in the 6- week study

| Groups† | Bax | Bcl-2 | Caspase 3 | Caspase 8 | TNF-α |
|---|---|---|---|---|---|
| 1 | 1.83+1.67* | 1.83+1.67* | 1.67+0.21* | 1.17+0.31* | 1.50+0.22* |
| 2 | 2.0+0* | 1.67+0.21* | 1.33+0.21* | 1.17+0.17* | 1.33+0.21* |
| 3 | 0.33+0.21 | 0 | 0 | 0 | 0 |
| 4 | 0.33+0.21 | 0.33+0.21 | 0 | 0 | 0 |
| | *According to Groups 3, 4: p<0.01 | *According to Groups 3, 4: p<0.01 | *According to Groups 3, 4: p<0.01 | *According to Groups 3, 4: p<0.01 | *According to Groups 3, 4: p<0.01 |

†Group 1: MCDD+placebo, 2: MCDD+Pro-2, 3: Control diet+placebo, 4: Control diet+Pro-2. *Values are expressed mean±S.E.M.*

## DISCUSSION

Intestinal bacteria have been suggested to contribute to the pathogenesis of NAFLD via increased endogenous production of ethanol and direct activation of inflammatory cytokines in luminal epithelial cells and non-parenchymal liver cells, causing the release of lipopolysaccharide (6). Further, intestinal bacteria induce endogenous signals that play a pathogenic role in hepatic insulin resistan-

ce (13). Accumulated data also suggest that quantitative and qualitative differences in gut microbiota exist between lean and obese and between diabetic and non-diabetic individuals (29).

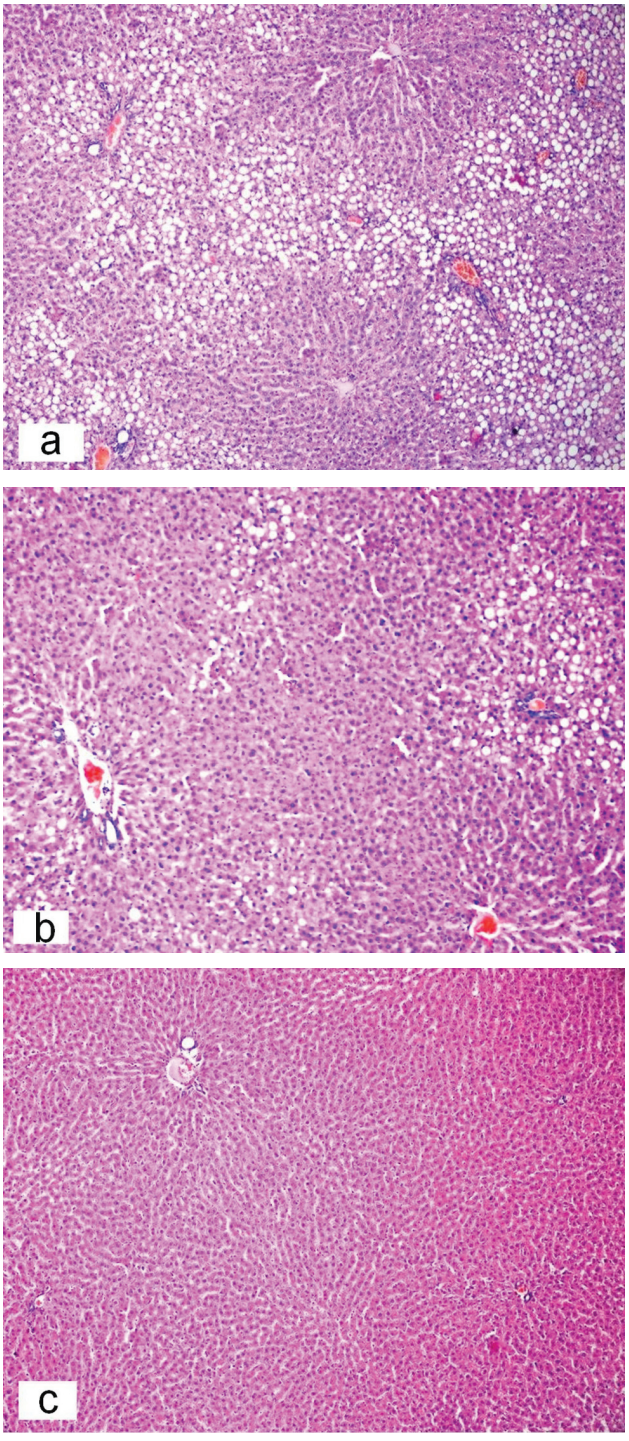


**Figure 4.** Histopathologic characteristics in Protocol II: Marked macrovesicular steatosis in the rat liver in the CMDD group, H&E x100 (a). Moderate steatosis in the rat liver in the CMDD+ LLP group, H&E x100 (b). Normal rat liver in the control group, H&E x100 (c).

Modulation of the enteric microflora by probiotics or synbiotics has been observed to enhance intestinal barrier function (16-18,30-33), reduce bacterial translocation (19,20,34-36) and reduce circulating endotoxin levels (37).

Some data also support the concept that intestinal bacteria induce endogenous signals that play a pathogenic role in hepatic insulin resistance in NAFLD and that probiotics improve high fat diet-induced steatosis and insulin resistance. TNF-α has long been known to play an important role in the pathogenesis of insulin resistance (38,39). It is now evident that stimuli that cause insulin resistance also promote the production of proinflammatory cytokines, such as TNF-α, and thus may incite an inflammatory response that damages the liver (6). TNF-α activates stress-related kinases, such as Jun N-terminal kinase (JNK) and inhibitor of nuclear factor κB kinases β (IKK-β). Activation of these kinases has been proven to be essential for insulin resistance, because insulin resistance cannot occur when kinase activation is abrogated experimentally (39-42). In a controlled study, when ob/ob mice were fed a high-fat diet, treatment with VSL#3 reduced the activity of JNK and decreased the DNA binding activity of nuclear factor κB, the target of IKK-β (13). In another study, young male Sprague-Dawley rats fed with the high-fat diet and treated with VSL#3 had significantly lower liver TNF-α levels than in the high-fat diet group (43).

The present study demonstrates that our probiotic combinations (Pro-1 and Pro-2) decreased the incidence of steatohepatitis in at least 50% of the rats in both the short- and long-term studies. Based on the histological scores, Pro-1 caused a statistically significant decrease in MCDD-induced steatohepatitis. Pro-2, which consists of bacteria, previously determined *in vitro* to lower lipid content in the media, may not be suitable for the MCDD-steatohepatitis model, but could be expected to elicit a stronger preventive effect on the high-fat diet steatosis model. Methionine and choline are precursors of phosphatidylcholine, which is an essential substrate for very low density lipoproteins (VLDL) synthesis (44). It has been found that MCDD downregulates stearoyl-coenzyme A desaturase-1, a key enzyme in triglyceride synthesis, with minimal upregulation of ,-oxidation genes in wild-type mice (45). In insulin-resistant db/db and insulin-sensitive db/m mice, the MCDD has been reported to reduce triglyceride secretion and dow-

nregulate genes involved in triglyceride synthesis (46). Therefore, increased fatty acid uptake and decreased VLDL secretion represent two important mechanisms by which the MCDD promotes intrahepatic lipid accumulation in this model. (46). Tahan et al. (47) found that MCDD feeding caused glucose intolerance in both the short-term (4 weeks) and long-term (12 weeks) studies in male Wistar rats, indicating that MCDD induces insulin resistance.

Velayudham et al. (23) reported that VSL#3 failed to prevent MCDD-induced liver steatosis or inflammation. MCDD, even in the presence of VSL#3, induced upregulation of serum endotoxin and expression of the Toll-like receptor 4 signaling components and nuclear factor ÎB activation. However, in that study, VSL#3 treatment was found to ameliorate MCDD-induced liver fibrosis and resulted in diminished accumulation of collagen and ·-smooth muscle actin (23). It is well known that different probiotic strains may demonstrate opposite effects. It has been reported that heat-inactivated *Lactobacillus plantarum* NCIMB8826 leads to strong interleukin (IL)-10 production, an anti-inflammatory cytokine, while *Lactococcus lactis* MG1363 stimulates TNF-α, IF-gamma and IL-12 production, proinflammatory cytokines, by human peripheral blood mononuclear cells (48). Further, lactobacillus strains with apparently similar properties *in vitro* may have distinct patterns of colonization and may induce heterogeneous immune responses *in vivo* (49).

Some probiotic strains have also been reported to have beneficial effects on alcoholic steatohepatitis by influencing various mechanisms. *Lactobacillus* GG probiotic gavage has significantly ameliorated alcoholic steatohepatitis in rats (16). This improvement has been found to be associated with reduced markers of intestinal and liver oxidative stress, inflammation and preserved gut barrier function. Orally administered heat-killed *Lactobacillus brevis* SBC8803 has ameliorated ethanol-induced liver injury and fatty liver, suppressed the overexpression of TNF-α, sterol regulatory element-binding protein (SREBP)-1 and SREBP-2 mRNA in the liver, and upregulated the expression of heat shock protein 25 mRNA in the small intestine (22). Authors have speculated that the inhibition of TNF-α and SREBPs upregulation by *L. brevis* is due to the inhibition of gut-derived endotoxin migration into the liver through the enhancement of intestinal barrier function by the induction of cytoprotective heat shock proteins (22). In mice treated with MCDD, TNF-α protein levels have been reported to increase by 2.2-fold in the liver compared with mice fed the control diet (50). Our findings support the previous reports that MCDD induces TNF-α expression. Pro-1 and Pro-2 decreased TNF-α expressions in varying degrees; however, the 2-week study showed that Pro-1 was more potent than Pro-2. A possible mechanism for the beneficial effect of our probiotic combinations may be due to their anti-inflammatory activities.

Apoptosis is a programmed cell death process that is predominantly regulated by the family of caspase proteases; however, there are additional caspase-independent cell death mechanisms as well (51). Pathologically increased hepatocyte apoptosis is an important mechanism contributing to inflammation and fibrosis of the liver (52). It has been suggested that hepatocyte apoptosis may be a key component of the 'second hit' in disease progression of NASH (24). In addition to their role in apoptosis, caspases also regulate inflammatory cytokines (53). In Wistar rats, MCDD feeding has been reported to increase the number of TUNEL-positive cells significantly (54). Further, the apoptotic index has been found to correlate well with serum ALT levels and inflammatory activity (54). In Db/Db mice, Anstee et al. (55) found that MCDD significantly increased apoptosis, which was reduced by VX-166 (a caspase inhibitor) treatment. VX-166 did not reduce steatosis but reduced histological inflammation, serum ALT levels and oxidative stress (55). On the other hand, MCDD has been reported to induce the level of phosphorylated STAT3 and Bcl-2 protein, both anti-apoptotic pathways, in the liver (50).

In our study, MCDD resulted in both: proapoptotic, via Bax, caspase 3 and caspase 8, and anti-apoptotic, via Bcl-2, expression in all rat livers, supporting previous studies. Pro-1 treatment reduced the expression rate and staining intensity of Bax, caspase 3, caspase 8, and Bcl-2. Pro-2 did not influence the MCDD-induced caspase 8 expression in either the 2- or 6-week study. Pro-2 did augment the MCDD-induced proapoptotic Bax expression in the 6-week study. We also observed that Pro-2 significantly suppressed the anti-apoptotic Bcl-2 expression in the 2-week study (Table 1). These findings could explain the decreased effectiveness of Pro-2 compared to Pro-1 in preventing steatohepatitis.

In conclusion, the probiotic combinations, Pro-1 and Pro-2, reduce MCDD-induced steatohepatitis in rats. The preventive effect of Pro-1 may be due, at least, to its suppressive effect on MCDD-induced proapoptotic protein expression and also to its anti-inflammatory effect. Pro-2, which consists of *in vitro* lipid-lowering bacteria, may have a more desired effect in other experimental models, e.g. high fat diet-induced steatosis.

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