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**Evaluation of Effect of Omega -3 Fatty Acids And
Probiotics In Rats With Nonalcoholic Fatty Liver Disease
(NAFLD) Induced By Special Diet**

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Abstract:

Nonalcoholic fatty liver disease (NAFLD) is defined as the excessive accumulation of triglycerides in $\geq 5\%$ of hepatocytes in the absence of significant alcohol consumption. This study aimed to investigate the dietary protective effect of omega-3 and probiotics on liver fatness and functions after induction of fatty liver in rats through feeding high fructose diet. This study included thirty (30) adult male Wister albino rats weighing 140 ± 10 , they were divided in to 5 groups: **Group (1)** included control negative rats, **Group (2)** included control positive rats, **Group (3)** included diseased rats treated with omega 3, **Group (4)** included diseased rats treated with probiotics, **Group (5)** included diseased rats treated with both omega 3 and probiotics. The results showed the highest improvement in lipid profile, liver functions and liver histopathology in group treated with both omega 3 and probiotics followed by group treated with probiotics and finally followed by group treated with omega 3 with a slight variations between group treated with probiotics and group treated with omega 3. From the previous results it can conclude that co-administration of omega-3 fatty acids and probiotics daily for 4 weeks to rats with NAFLD can significantly reduce liver fat, improve serum lipids, metabolic profile, and reduce pathological changes in fatty liver.

Key Words: liver functions, liver histopathology flaxseeds oil ,Glucose

Introduction:

Liver is the largest internal organ in the human body. The liver has multiple functions, it plays a core role in the maintenance of systemic lipid homeostasis (Nicetto *et al.*, 2019). It makes many of the chemicals required by the body to function normally, it breaks down and detoxifies substances in the body, and it also acts as a storage unit. It regulates most chemical levels in the blood and excretes a product called bile (Guido *et al.*, 2019). Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, both in adults and in children (Eslamparast *et al.*, 2013).

Omega-3 fatty acids are essential fatty acids because they are not synthesized by the body and must be obtained through diet or supplementation. Omega-3 (n-3) fatty acids are poly unsaturated fatty acids (PUFA) (Maggie and Covington, 2010). Omega-3 has many health benefits as attenuation of NAFLD, play role in brain health and neurological Functions, cardiovascular health and eye health (Georgiou and Prokopiou, 2016).

Probiotics is live microorganisms that, when administered in adequate amounts had health benefit on the host (Dailey *et al.*, 2020). Many data from animal experiments have indicated that modulating gut microbiota with probiotics preparations has a beneficial effect on NAFLD (Sharma *et al.*, 2013). Probiotics have many health benefits as improvement of NAFLD, improve eczema, help to control high blood pressure and reduce serum cholesterol levels (Kumar *et al.*, 2012).

Aim of the Work:

The present study aims to study the effect of omega 3 fatty acids and probiotics on NAFLD

Materials and Methods

1. Animals and diet

Adult male wistar albino rats were used in the present study weighting 140 ± 10 . They were purchased from the animal house of Mahalla Hepatology Teaching Hospital . All animals were allowed to acclimatize for a week on the normal diet before grouping.

All animals except the normal group were given 20% fructose in drinking water for two weeks in order to induce NAFLD (Tetri *et al.*, 2008) . The high fructose fed groups received fructose (20%) in drinking

water for a period of 2 weeks to induce fatty liver and elevate liver enzymes. Treatment was carried out by each drug separately (Omega 3, probiotics, and in combination) for 4 weeks from the fourth week until the end of the sixth week. Omega 3 was given as flaxseeds oil from local market in menoufia, it was given in dose of 1.7ml/kg of rat body weight orally (**Waniet al., 2015**). Probiotics were given as yoghurt fortified by probiotics live strain at a dose of 140 mg/kg (**Kobyliaket al., 2017**)

Group 1: Is control negative rats (healthy rats)

Group 2: Is control positive rats (diseased rats)

Group 3: Diseased rats that treated with flaxseeds oil 1.7 ml/kg of B.W orally for four weeks (omega 3).

Group 4: Diseased rats that treated with live probiotics strain 140 mg/kg powder in diet for four weeks.

Group 5: Diseased rats that treated with flaxseeds oil 1.7ml/kg BW orally and live probiotics strain 140 mg/kg powder in diet for four weeks.

2. Biological parameters:

1. Feed intake (FI).
2. Body weight gain (B.W.G) at one week intervals.
3. Feed efficiency ratio (FER).
4. Organs will be weighed and biological markers will be calculated.
5. Blood sampling and serum preparation.

3. Biochemical investigations:

Serum samples were analyzed for:

Lipids profile:

- Determination of serum total cholesterol according to the colorimetric method described by **Thomas, (1992)**.
- Determination of serum triglycerides (TG) determined by enzymatic method using kits according to the **Young and pestaner (1975) and Fossati and Prencipe , (1982)**.
- Determination of high density lipoprotein (*HDL*) according to the method described by (**Fredewaid 1972**) and (**Grodon and Amer 1977**)..
- Calculation of low density lipoprotein cholesterol (LDL-c) was calculated in mg/dl according to (**Lee and Nieman, 1996**) .

Liver functions:

- Determination of serum alanine aminotransferase (ALT) according to the method of **Bergmeyer, (1980)**.
- Determination of serum asparatate Amino Trans ferase (AST) according to the method of **Hafkenscheid, (1979)**.
- Determination of serum total protein as described by **Sonnenwirth and Jaret, (1980)**.
- Determination of serum Albumin level according to **Doumaset al.,(1971)** modified by **Spencer and Price, (1977)**.
- Activity of Serum alkaline phosphatase (ALP) will be estimated.
- Determination of serum bilirubin level .

4. Histopathological examination

The liver was prepared for histopathological examination, stained with hematoxylin and eosin (H&E) and then examined under low and high power microscope and representative photographs will be taken.

Results and Discussion:

The results of the current study were presented as the follows:

1-Weight gain:

The mean weight of control +ve group in the 4th week was 213.6±1.2 g while in group treated with both omega 3 and probiotics was 188.4±0.45 g which was better than the weight of rats treated with omega 3 alone (195.6±2.8 g) and also better than the weight of rats treated with probiotics alone (191.8±1.14g).

These results were in agreement with **Kobyliaket al., (2017)** who detected a significant difference in weight of studied different rat groups , the weight of rats in the group treated with both omega 3 and probiotics showed the nearest weight to the control negative group.

Table (1): Effect of feeding on weight gain (gram) of different rats groups

Weight of rats in grams	Control - ^{ve}	Control + ^{ve}	OMEGA 3	Probiotics	Mix group	P value of K test
Weight 1st week Mean ±SD	180±0	185±0	181±0.31	182±0.54	182±0.5	P <0.001**
Weight 2ndwk Mean ±SD	182.4±0.54	190.8±0.83	183.6±0.54	168.6±0.89	185.2±0.83	P <0.001**
Weight 3rd wk. Mean ±SD	185.2±0.83	198.4±1.14	187.8±0.44	191.6±1.14	186.4±0.54	P <0.001**
Weight 4th wk. Mean ±SD	187.4±0.54	213.6±1.2	195.6±2.8	191.8±1.14	188.4±0.45	P <0.001**

K: Kruskal-wallis, ******Highly significant p value and **SD:** standard deviation

2-Organs weight:

Table (2) showed a significant difference between the studied groups regarding the liver weight ($P \leq 0.05$). The mean liver weight in mix group that were treated with both omega 3 and probiotics was 3.39 ± 0.52 g that revealed better result than single use of omega 3 (mean 3.52 ± 0.4 g) or probiotics (mean 3.42 ± 0.52 g).

Table (2): Effect of feeding on some organs weight of different rats

Weight of some rat's organs in grams.	Control _{-ve}	Control _{+ve}	OMEGA 3	Probiotics	Mix group	P value of K test
Liver wt. (g) Mean \pm SD	3.38 ± 0.43	4.7 ± 0.9	3.52 ± 0.4	3.42 ± 0.52	3.39 ± 0.52	0.007*
Heart wt.(g) Mean \pm SD	0.44 ± 0.04	0.66 ± 0.08	0.52 ± 0.05	0.56 ± 0.09	0.50 ± 0.089	0.189
Rt kidney wt.(g) Mean \pm SD	0.58 ± 0.08	0.62 ± 0.45	0.7 ± 0.01	0.7 ± 0.01	0.7 ± 0.01	0.116
Lt kidney wt.(g) Mean \pm SD	0.64 ± 0.05	0.68 ± 0.04	0.72 ± 0.04	0.64 ± 0.05	0.64 ± 0.05	0.11
Rt testis wt.(g) Mean \pm SD	1.1 ± 0.16	0.98 ± 0.04	0.98 ± 0.04	0.98 ± 0.04	0.98 ± 0.04	0.23
Lt testis wt.(g) Mean \pm SD	1.12 ± 0.16	0.96 ± 0.5	0.96 ± 0.05	0.96 ± 0.05	0.96 ± 0.05	0.33

SD: Standard deviation and *: significant p value

3- liver functions:

Table (3) showed highly significant difference ($P \leq 0.001$) between the studied groups regarding ALT, AST and ALP enzymes' levels. The mean of ALT, AST and ALP enzymes in mix group (32.2 U/L, 33.2 U/L and 174 U/L, respectively) were lower than mean of liver enzymes in group treated with omega 3 alone (45 U/L, 43.2 U/L and 186.2 , respectively) and in group treated with probiotics alone (57.8 U/L, 56.4 U/L and 196 U/L, respectively). The results of this study showed improvement in liver functions and lipid profile in rats on omega 3 treatment where serum levels of liver enzymes were significantly lower in omega 3 group rats as compared to control positive groups. The mean of liver enzymes in group treated with probiotics were significantly lower as compared to control positive groups.

Wai-Sun Wong et al., (2015) reported that probiotics improved liver aminotransferase levels in patients with NAFLD.

Ma et al., (2013) they reported that meta-analysis study showed that probiotics significantly reduced ALT, AST and TNF- α which are all related to the process, development and consequences of NAFLD.

Table (3): Effect of feeding by omega 3 and probiotics on ALT, AST and ALP enzymes' levels of different rats groups

Level of different liver enzymes in	Control _{-ve}	Control _{+ve}	OMEGA 3	Probiotics	Mix group	P value of K test
ALT Mean \pm SD (U/L)	18.6 \pm 2.7	71.4 \pm 2.0	45 \pm 1.87	57.8 \pm 3.96	32.2 \pm 2.68	P \leq 0.001**
AST Mean \pm SD (U/L)	18.4 \pm 3.05	70.2 \pm 2.49	43.2 \pm 2.59	56.4 \pm 5.03	33.2 \pm 2.49	P \leq 0.001**
ALP Mean \pm SD (U/L)	157.6 \pm 4.28	221 \pm 5.34	186.2 \pm 2.8	196 \pm 4.53	174 \pm 4.36	P \leq 0.001**

** highly significant , **ALT**: Alanine transaminase , **AST**: Aspartate transaminases and **ALP**: Alkaline phosphatase

Table (4) showed highly significant difference (P \leq 0.001) between the studied groups regarding serum Albumin and bilirubin level. The mean of serum albumin level in control -ve group was 5.48 \pm 0.26 g/dl and in control +ve group was 2.18 \pm 0.16 g/dl . In mix group the mean of serum albumin level was 4.7 \pm 0.2 g/dl which was better than the mean serum albumin level in group treated with omega 3 alone (3.9 \pm 0.16 g/dl) and also better than mean serum level in group treated with probiotics alone (2.9 \pm 0.11 g/dl). The mean of serum bilirubin level in mix group was 0.66 \pm 0.03 mg/dl which is lower than the mean level in omega 3 group (0.85 \pm 0.04 mg/dl) and probiotics group (1.4 \pm 0.1 mg/dl)

Table (4): Effect of feeding on Albumin, bilirubin and total proteins of different rats groups.

Parametrs	Control _{-ve}	Control _{+ve}	OMEGA 3	Probiotics	Mix group	P value of K test
Albumin Mean \pm SD (gm/dl)	5.48 \pm 0.26	2.18 \pm 0.16	3.9 \pm 0.1	2.9 \pm 0.11	4.7 \pm 0.2	P <0.001**
Bilirubin Mean \pm SD (mg/dl)	0.404 \pm 0.0	1.56 \pm 0.3	0.85 \pm 0.04	1.4 \pm 0.1	0.66 \pm 0.03	P <0.001**
Total proteins Mean \pm SD (gm/dl)	6.62 \pm 0.19	3.9 \pm 0.16	5.5 \pm 0.08	4.52 \pm 0.15	6.08 \pm 0.08	P <0.001**

SD: standard deviation and **: Highly significant p value,

4- blood glucose and lipid profile:

Table (5) showed highly significant difference ($P \leq 0.001$) between the studied groups regarding blood glucose level and lipid profile level. The mean of blood glucose level in control –ve group was 94.6 ± 11.6 mg/dl , in control +ve group was 171.6 ± 6.3 mg/dl and in mix group was 130.2 ± 1.9 mg/dl , which was the lowest mean. Mean blood glucose level in group treated with omega 3 alone was 140.8 ± 1.9 mg/dl and in group treated with probiotics alone was 154 ± 2.92 mg/dl . The mean of serum cholesterol level in control –ve group was 162.6 ± 5.9 mg/dl , in control +ve group was 272.2 ± 8.1 mg/dl and in mix group was 190.8 ± 3.2 mg/dl which was significantly lower than the mean serum cholesterol level in group treated with omega 3 alone (219.6 ± 6.9 mg/dl) and also better than mean serum level in group treated with probiotics alone (254.6 ± 3.2 mg/dl).

The mean of triglyceride level (**TG**) in control –ve group was 151 ± 26.4 mg/dl, in control +ve group was 272.2 ± 8.11 mg/dl , in omega 3 group was 219.6 ± 6.9 mg/dl , in probiotics group was 254.6 ± 3.21 mg/dl and in mix group was 190.8 ± 3.19 mg/dl.

The mean of high density lipoprotein (**HDL**) in control –ve group was 45.6 ± 3.7 mg/dl , in control +ve group was 17.8 ± 2.38 mg/dl, in omega 3 group was 32.6 ± 2.1 mg/dl , in probiotics group was 24.8 ± 1.92 mg/dl and in mix group was 38.6 ± 1.14 mg/dl.

The mean of low density lipoprotein (**LDL**) in control –ve group was 95.2 ± 7.3 mg/dl, in control +ve group was 211.04 ± 8.1 mg/dl, in omega 3 group was 149.1 ± 5.4 mg/dl , in probiotics group was 191 ± 2.31 mg/dl and in mix group was 119.3 ± 3.7 mg/dl.

Table (5):Effect of feeding on glucose level and lipid profile of different rats groups

Parametrs	Control –ve	Control +ve	OMEGA 3	Probiotics	Mix group	P value of ANOVA test
Glucose Mean ±SD (mg/dl)	94.6±11.6	171.6±6.3	140.8±1.9	154±2.92	130.2±1.9	P <0.001**
Cholesterol Mean±SD (mg/dl)	162.6±5.9	272.2±8.1	219.6±6.9	254.6±3.2	190.8±3.2	P <0.001**
TG Mean±SD (mg/dl)	151±26.4	272.2±8.11	219.6±6.9	254.6±3.21	190.8±3.19	P <0.001**
HDL Mean±SD (mg/dl)	45.6±3.7	17.8±2.38	32.6±2.1	24.8±1.92	38.6±1.14	P <0.001**
LDL Mean±SD (mg/dl)	95.2±7.3	211.04±8.1	149.1±5.4	191±2.31	119.3±3.7	P <0.001**

** : high significant p value, **mg**: milligram per deciliter , **SD**: standard deviation , **TG**: triglyceride, **HDL**: high density lipoprotein and **LDL**: low density lipoprotein

However, on contrary of our study **Ma et al., (2013)** reported that level of HDL was significantly increased in the placebo treatment as compared with probiotic treatment, which was contrary to expectation, to explain this he added it is possible that the elevation in HDL requires long-term treatment or there are other mechanisms which have not been explored. Over several decades, more and more researchers confirmed that probiotics can lead to a decrease in serum cholesterol level in animals and humans.

Ma et al., (2013) concluded also that some studies did not report the positive effects of probiotics on reducing cholesterol in NAFLD/NASH patients, while the findings of the present study supported the reduction of cholesterol in NAFLD/NASH rats. From this study and many other studies, we can conclude that probiotics have positive effects in NAFLD/NASH.

Gilliland et al., (1990) in the early 1990s found that regular consumption of probiotics reduced cholesterol levels.

After dissection of rats, livers from the groups that were supplemented with Omega 3 and or probiotics had a similar gross appearance as those of the negative control animals. Livers in control positive group were yellow and greasy, suggesting fatty liver changes. There were a significant differences in liver weights between groups.

5-Histopathological study:

Histopathological picture in rats on omega 3 (group 3) showed micro vesicular steatosis and also rats treated with probiotics (group 4) showed microvesicularsteatosis(**photo 2**) while positive control rats have macro and microvesicularsteatosis (**photo 1**). Rats on omega 3 and probiotics (group 5) showed microvesicularsteatosis with less steatosis(**photo 3**) than group 2 and group 3. This mean that the best improvement regarding histopathological changes in the liver noticed after combined treatment with omega 3 and probiotics but still not reaching the normal histology of the liver.

Kobyliaket al., (2017)reported that rats fed with a high fat diet combined with n-3 PUFAs supplementation were protected against severe NAFLD development.

Moreover, **Di Minnoet al., (2012)** reported that n-3 PUFAs supplementation improves hepatic steatosis in obese animals by modifying the genetic expression of key enzymes. n-3 PUFAs are

emerging as a potential treatment of liver steatosis. They cannot be synthesized by the human body and, thus, must be derived from exogenous sources (fish oil, flax seeds, etc.).

Boyrazet *al.*,(2015) concluded in more detail, n-3 PUFAs are potent activators of up-regulates several genes involved in the stimulation of fatty acid oxidation and improved insulin sensitivity and down-regulates pro-inflammatory genes, such as TNF- α and IL-6.

El-Kader and El-Den Ashmawy, (2015)reported that n-3 PUFAs stimulates the transcription of several lipogenic and glycolytic genes Moreover n-3 PUFAs can inhibit hepatic glycolysis and lipogenesis.

Regarding effect of probiotics on fatty liver this study showed improvement in liver function ,lipid profile and histopathological picture of the liver in rats on probiotics treatment where serum levels of liver enzymes, cholesterol and triglycerides were significantly lower in these rats compared to positive controls. Level of HDL was also significantly higher in these rats compared to positive controls.

It was observed in this study an improvement in the liver enzymes and lipid Profile by using combination of both omega3 and probiotics in mix group and this improvement was more than using omega3 or probiotics separately.

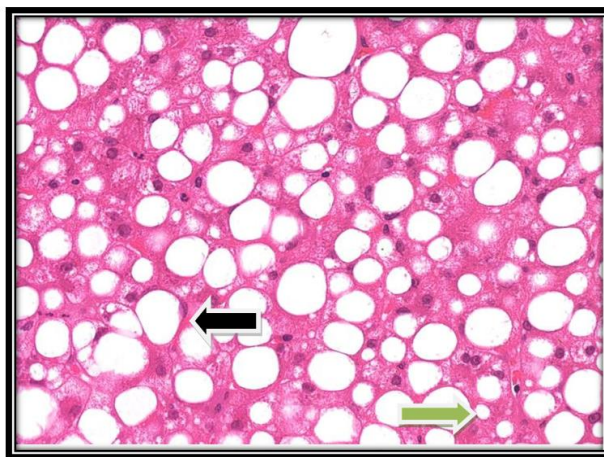


Photo (1): Mixed Micro-vesicular steatosis (green arrow) and Macro-vesicular steatosis with signet ring appearance of the cell (black arrow) in control positive group (H&E 400).

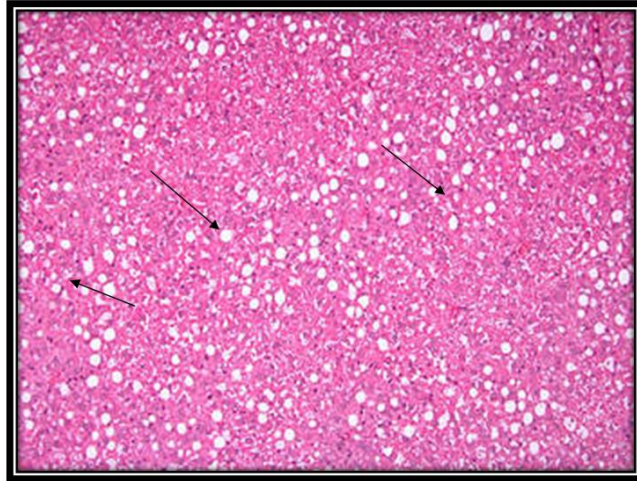


Photo (2): Micro-vesicular stasis seen in group treated with omega 3 alone or probiotics alone (black arrows) (H&E 100)

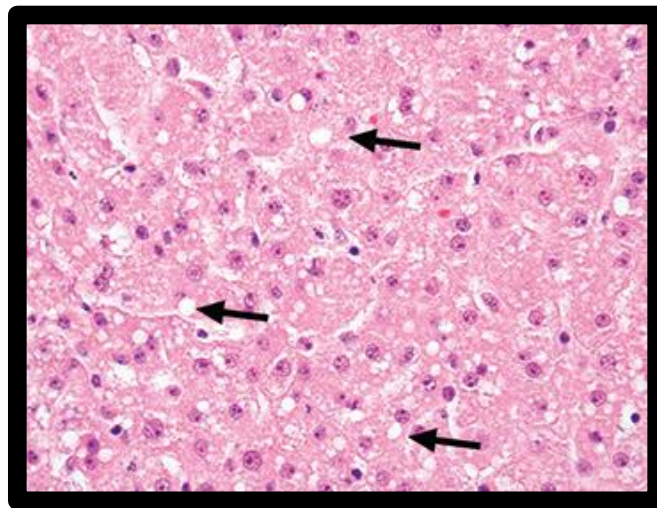


Photo (3): Micro-vesicular stasis seen in mixed group group treated with omega 3 and probiotics (black arrows) (H&E 200).

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تقييم تأثير اوميجا-3 والبروبيوتيك على الفئران المصابه بمرض الكبد الدهني
الغير كحولي الناتج من تناول وجبات خاصه

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استاذ التغذية وعلوم الاطعمه المتفرغ وعميد كلية الاقتصاد المنزلي سابقا جامعه المنوفيه*

زميل التغذية بمستشفى كبد المحلهاالتعليمي**

ماجستير فى الاقتصاد المنزلى تخصص التغذية وعلوم الاطعمة كلية الاقتصاد المنزلى، جامعة المنوفية ، شبين الكوم مصر.***

تهدف الدراسة الى تقييم التأثير الوقائي والغذائي لأوميجا 3 والبروبيوتيك على وظائف الكبد الدهنى غير كحولى المعروف بتراكم المفرط للدهون الثلاثيه بنسبه اكثر من 5% من الخلايا الكبديه . وقد اشتملت هذه الدراسة على ثلاثون من ذكور الفئران البيضاء تتراوح اوزانهم 140 جرام \pm 10 جرام، تم تقسيمهم إلى 5 مجموعات كل مجموعه 6 فئران : المجموعة (1) المجموعه الكنترول السالبه وتم تغذيتها بوجبات صحيه لضمان الزيادة الطبيعیه في الوزن ،الاربع مجموعات الاخرى تم تغذيتها بوجبات عاليه الفركتوز لمده اربع اسابيع لاحداث الكبد الدهنى ، وقسمت الى المجموعه (2) التى شملت المجموعه الكنترول الموجبه وهذه المجموعه تم تغذيتها على وجبه عاليه الفركتوز طول فتره الدراسه، المجموعه (3) شملت المجموعه التى عولجت ب أوميجا 3 وشملت المجموعه (4) الفئران التى عولجت بالبروبيوتيك ، وشملت المجموعه (5) الفئران التى عولجت بكل من أوميجا 3 والبروبيوتيك. أظهرت النتائج أعلى تحسن في مستوى الدهون الكلى و الكوليسترول وانخفاض فى الدهون الثلاثيه و الكوليسترول والدهون المنخفضة الكثافة (LDL) وتحسن فى وظائف الكبد و الأنسجة الكبدية في المجموعه التى عولجت بكل من أوميجا 3 والبروبيوتيك تليها المجموعه التى عولجت بالبروبيوتيك ثم أعقبها المجموعه المعالجه بالأوميجا 3 مع اختلاف طفيف بين المجموعه المعالجه بالبروبيوتيك والمجموعه المعالجه ب أوميجا 3. وتوصلت الدراسات أن المعالجه المشتركة باحماض أوميجا 3 والبروبيوتيك معا يومياً لمدة 4 أسابيع للفئران المصابه ب(NAFLD) يمكن أن تخفض بشكل كبير مستوى دهون الكبد ، وتحسن مستوى الدهون في الدم وتقليل التغيرات المرضية في الكبد الدهني .

الكلمات الكاشفة: وظائف الكبد_ تشريح الكبد_ زيت بذور الكتان_ الجلوكوز

