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Study the Therapeutic Effect of Grape-Flavored Whey Probiotic Beverage on Hypercholesterolemia induced in male laboratory rats

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Abstract:
The present study was conducted in the college of Veterinary Medicine/University of Basrah to evaluate the role of Grape-Flavored Whey Probiotic Beverage that prepared by mixing probiotic isolate (*L. acidophilus*) and whey in reducing the harmful effect of high cholesterol diet on laboratory rats. Therefore twenty four adult male rats weight 220-280 g were used and divided into four groups(6 for each). Control group (T1) received normal ration and drench with normal saline. The second, third and fourth groups(T2,T3 and T4 respectively) received high cholesterol diet for 30 day and then drenched by normal saline , whey, therapeutic syrup (5 ml) respectively for 30 days. Blood samples were collect and separate serum immediately for lipid profile (Tch,TG,LDL, VLDL and HDL) analysis. In addition , liver enzymes activity (ALT and AST) and renal function indicators were measured. The role of insulin, leptin and adiponectin also evaluated. The results revealed to the role of therapeutic syrup in reducing the values of lipid parameters (Tch, TG, LDL and VLDL) and improvement of LDL levels. The liver enzymes activities showed significant improvement due to administration of therapeutic syrup of probiotics while urea and creatinine appeared non- significant for all groups. Grape-Flavored Whey Probiotic Beverage administration revealed significant decrease in insulin, leptin and adiponestin levels compared with other hypercholesterolemic and control groups. Therefore, Grape-Flavored Whey Probiotic Beverage had ameliorative effect against the prognosis of high cholesterol diet on body physiology.

Keywords: Probiotic Beverage , Whey , Hypercholesterolemia

دراسة التأثير العلاجي لمشروب شرش بنكهة العنب والمدعم بالمعزز الحيوي على فرط كوليستيرول الدم المستحدث في ذكور الجرذان المختبرية.

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الخلاصة

أجريت هذه الدراسة في كلية الطب البيطري/جامعة البصرة لتقييم دور مشروب الشرش بنكهة العنب والمدعم بعزلة المعزز الحيوي (*L. acidophilus*) في تقليل التأثير الضار للنظام الغذائي عالي الكولسترول على جرذان المختبر. لذلك تم استخدام أربعة وعشرون من ذكور الجرذان البالغة تراوحت أوزانهم بين 220-280 غراماً وتم تقسيمهم إلى أربع مجموعات (6 لكل مجموعة). غُذيت المجموعة الضابطة (T1) بعليقة عادية وتم تجريعها بمحلول ملحي عادي . تم تغذية المجموعات الثانية والثالثة والرابعة (T2 وT3 وT4 على التوالي) بنظام غذائي عالي الكوليسترول لمدة 30 يوماً ثم تم تجريعها بالمحلول الملحي العادي والشرش والشراب العلاجي (5 مل) على التوالي لمدة 30 يوماً. تم جمع عينات الدم وتحليل مصل الدم على الفور لتحليل صورة الدهون (Tch، TG، LDL، VLDL وHDL). بالإضافة إلى ذلك ، تم قياس نشاط إنزيمات الكبد (ALT وAST) ومؤشرات وظائف الكلى. كما تم تقييم دور الأنسولين والليبتين والأديبونيكتين. كشفت النتائج عن دور الشراب العلاجي في خفض قيم معايير الدهون (Tch، TG، LDL و VLDL) وتحسين مستويات LDL. أظهرت فعالية إنزيمات الكبد تحسناً معنوياً نتيجة إعطاء شراب المعزز الحيوي العلاجي بينما لم تظهر تغيرات معنوية في مستويات اليوريا والكرياتينين في جميع المجموعات. اظهرت المجموعة التي تم تجريعها بمشروب الشرش بنكهة العنب والمدعم بالمعزز الحيوي انخفاضاً كبيراً في مستويات الأنسولين واللبتين والأديبونكتين مقارنة مع المجموعات الأخرى المصابة بارتفاع الكولسترول والمجموعة الضابطة. لذلك، كان مشروب الشرش بنكهة العنب والمدعم بالمعزز الحيوي تأثيراً محسناً ضد التأثيرات الضارة الناتجة عن اتباع نظام غذائي عالي الكوليسترول في فسيولوجيا الجسم.

الكلمات المفتاحية: مشروبات بروبيوتيك، مصل اللبن، فرط كوليستيرول الدم.

Introduction

Functional foods are ingredients that enhance body health by modulating physiological responses or reducing the risk of diseases in addition to providing essential nutrition. The general category of functional foods includes processed foods or fortified with health promoting additives, like vitamin-enriched products. (Priyanka & Anjali, 2017).Probiotic drinks are one of the functional food additives, typically dairy-based beverages with a consistency similar to milk which consumed for more digestive health (Shukla *et al.*,2013) .

Probiotics are living and non-pathogenic microbial supplements which is administrate in adequate quantities, probiotics have several roles in the body, They influence the host organism positively by improving gut health and enhancing intestinal mucosal integrity and suppress potentially pathogenic microorganisms by competing with them for nutrients as well as space for gut adherence (Khare & Gaur, 2020). Therefore, referred to it as "good microorganisms" and can be found in the form of oral consumer products such as dietary supplements, chocolates, yogurts, and many others (Harper *et al.*,2018). Lactic acid bacteria, especially lactobacilli, are the most widely used microorganisms (Wu *et al.*,2018) that demonstrated to have beneficial effects on obesity and related metabolic complications, including hypertension, distorted lipid profiles, and higher immune protection (Lollo *et al.*, 2015; Sperry *et al.*, 2018). Hypercholesterolaemia is one of the metabolic disorder plays a critical role in the development of atherosclerosis and cardiovascular diseases which led to death hundreds of people yearly, There are some evidences stated that probiotics could affect hypercholesterolemia via reducing cholesterol levels by different mechanisms (Hassan *et al.*,2019). Probiotics had also been considered as a potential intervention for the management of metabolic disorders such as dyslipidemia, which is caused by unusually high levels of serum cholesterol and triglycerides (TGs) (Palaniyandi *et al.* ,2019).

Numerous pharmacological and non-pharmacological methods have been used to decrease blood cholesterol. Various drugs have been developed to treat hypercholesterolemia, and the most common drugs include atorvastatin and rosuvastatin (statin drugs). However, prolonged usage of these drugs causes severe side effects (Anandharaj *et al.*,2020).

Recently, various scientists reported that the ingestion of several fermented products with probiotic bacteria decreases the serum cholesterol level (Anandharaj *et al.*,2020). However, several probiotics studies have been reported to have a hypocholesterolemic effect in animal models

(Damodharan *et al.*, 2015; 2016) and in human clinical trials (Ryan *et al.*,2015; Costabile *et al.*,2017). while Priyanka & Anjali, (2017) found that addition of probiotics to whey will enhance the properties and benefits on several health directions, as well as addition of some flavors or juices gives it better sensory properties (Shukla *et al.*,2013 ; Priyanka & Anjali,2017). Therefore, The current study designed to study the role of grape-flavored whey probiotic beverages in reducing harmful effect of high cholesterol levels on induced hypercholestermic male laboratory rats.

Materials and Methods

Preparation of Grape-Flavored Whey Probiotic Beverage :

Grape-Flavored Whey Probiotic Beverage was prepared according to Idan *et al.*(2021) by using raw milk, industrial grape juice, rennet and Probiotic isolate (*L. acidophilus*) was used from (GREENMADE / Finland). Whey was obtained by adding rennet enzymatic coagulant to the raw milk (0.7 mL/L of milk). Grape-flavored whey probiotic beverage prepared by used 20 % of grape juice and complete with whey to sterilized at 121° C for 15 minute in an autoclave steam sterilizer. Then probiotic isolate (*L. acidophilus*) (10^8 cfu/ml) had been added to incubate under anaerobic conditions in a shaker incubator at 37°C with a vibration motion of 55 rpm for 48 h. the prepared beverage was then stored at 4°C refrigerated temperature Until use.

Experimental Animals :

In the experiments, 24 adult male rats (*Rattus norvegicus*) were used, which obtained from the Animal House / College of Veterinary Medicine / University of Basrah with weights ranging from (240-280) g, and were distributed randomly on the basis of the weight of living mass into four groups evenly in plastic a stainless steel cages clamp. The studied rats were kept under controlled conditions (22 ± 3 °C temperature ; 40 – 45% moisture), and 12/12 hours light/ dark . all rats were acclimatized for two weeks in order to adapt them to the conditions of the experiment and leaving free access to water and food. The health of the rats was monitored throughout the trial period to keep the rats healthy until the end of the experiment.

Animal Diet :

Experimental animals were fed standard diets for rats AIN93 with the ingredients mentioned in Table (1) (Subcommittee of Laboratory Animal Nutrition, 1995) throughout the period of the experiment, as the ingredients were mixed and then made in very small pieces. They were then completely dried from moisture and placed in bags. Plastic and kept at (4-7) C.

Table 1: The rat's food ingredients

Quantities (g/kg)	**Treatments**			
	T1	**T2**	**T3**	**T4**
Skim Milk	200	200	200	**200**
Corn Starch	670	660	660	**660**
Vitamins and minerals	30	30	30	**30**
Corn oil	50	50	50	**50**
Cellulose	50	50	50	**50**
Cholesterol	0	10	10	**10**

Experimental design :

The experiment was carried out in the animal house - College of Veterinary Medicine / University of Basrah during the period of 26/6/2019 to 10/9/2019 including the acclimatization period.

The experimental animals divided into 4 groups (6 for each) as following:-

Control group T1: animals were fed on a standard diet and daily drench with 5 ml of normal saline solution(0.9%) for 60 days.

Cholesterol group T2: animals were fed a standard diet with added cholesterol (10 g / kg diet) for 30 days to induce hypercholesterolemia According to the AIN1993 standard rat diet (Subcommittee of Laboratory Animal Nutrition,1995). Then drench with 5 ml of normal saline 0.9 (Normal Saline) for 30 days.

Cholesterol + whey group T3: animals were fed a standard diet with cholesterol (10 g / kg diet) for 30 days to induce hypercholesterolemia, and then daily drench of whey (5 ml) for 30 days (Pan *et al.* , 2009).

Cholesterol + therapeutic syrup group T4: animals were fed a standard diet with added cholesterol (10 g / kg diet) for 30 days to induce hypercholesterolemia, and then daily drench with 5 ml of a therapeutic syrup for 30 days.

Blood Samples:

Blood samples were collected by drawing blood directly from the heart (heart puncture) after partial anesthesia with Dietheal ether by use of (10ml) disposable syringe. Blood sample (6ml) were placed in test tube (Gel/clot activator tube) which immediately centrifuged to separate serum for biochemical analysis.

Biochemical Blood Parameters:

Biochemical tests were performed at the end of the experiment to find out the changes resulting from the whey and therapeutic syrup drench on animals induced hypercholesterolemia. Serum total cholesterol, triglyceride ,HDL-c ,AST,ALT, urea and creatinine were measured by using especial kits for each parameters (Human Star company / German).While low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) calculated according to Friedewald, *et al.,* (1972).

Hormonal Assay:

The levels of hormones (insulin, leptin and adiponectin) in the blood serum of rats were measured by using kits for measuring these hormones according to the instructions of the company that supplied them (Bioassay Technology Laboratory) of Chinese origin.

Statistical analysis:

Data were analyzed using Complete Random Design (CRD) and using the ready-made statistical analysis program SPSS, Version 24 (2016). The averages were compared using the lowest significant difference LSD under a probability level ($p<0.05$).

Results

Table (2) shows effect of the therapeutic syrup on the characteristics of lipids and their transporters (lipoproteins) in the blood serum of hypercholesterolemic rats (mean ± standard error) .The results revealed a significant decrease ($p \leq 0.05$) in total cholesterol (Chol.), triglycerides(TG) , low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels in rats drench with therapeutic syrup (T4) compared to the second and third treatment. Also, this table appeared no significant differences between the fourth treatment ((T4) and the control (T1) for all above parameters.

In contrast, table (2) referred to non- significant differences ($p \leq 0.05$) in the concentration of high density lipoprotein (HDL) in the blood serum of rats for the fourth treatment(T4) that animals treated with therapeutic syrup when compared to all studied treatments despite the improvement in their values but still non-significant with other studied groups.

Table (2) effect of the therapeutic syrup on the lipids profile and their transporters in the blood serum of hypercholesterolemic rats (mean ± standard error)

Teratments	characteristics of lipids and their transporters (mg/dl)				
	Chol.	TG	LDL	HDL	VLDL
T1	3.27±91.53 A	1.25±61.36 A	0.15±11.05 A	0.21±95.62 A	0.57±19.12 A
T2	7.11±142.16 B	8.34±36.34 B	5.11±78.39 B	0.51±137.16 C	0.21±27.43 C
T3	12.52±155.10 B	5.28±44.80 B	9.12±83.25 B	0.76±135.28 C	0.46±27.05 C
T4	5.23±86.53 A	4.09±49.50 AB	0.83±15.37 A	0.11±108.30 B	0.31±21.66 B
significant	*	*	*	*	*

*** different letters denote significance ($p \leq 0.05$)**

The effect of the therapeutic syrup on the liver enzymes activities (ALT & AST), urea and creatinine in the blood serum of hypercholesterolemic rats (Figure 1), referred to a significant decrease ($p < 0.05$) in the level of the ALT and AST enzyme activities for T4 in which the rats were dosed with the therapeutic syrup compared with the second and third experimental treatments T2 and T3, , respectively. While control treatment (T1) showed the lowest values ($p < 0.05$) for ALT and AST enzymes compared to all studied group.

Whereas, Urea and creatinine levels showed non- significant differences in their values among all studies groups.

The harmful effect of high cholesterol diet and administration of Grape-Flavored Whey Probiotic Beverage as therapeutic syrup on insulin, leptin and adeponectine hormones represented by figure (2). The effect of the therapeutic drink on the levels of the insulin hormone in the blood serum of rats induced with hypercholesterolemia showed significant differences ($P \leq 0.05$) among the treatments feeds on high cholesterol diet (T2, T3). This treatments recorded significant decrease ($p \leq 0.05$) in the level of the insulin hormone when compared with the control and T4 treatments. While the therapeutic drink showed a significant improvement in the level of the insulin hormone than the treatments that induced hypercholesterolemia (T2 and T3) and also higher than control treatment in value of insulin levels.

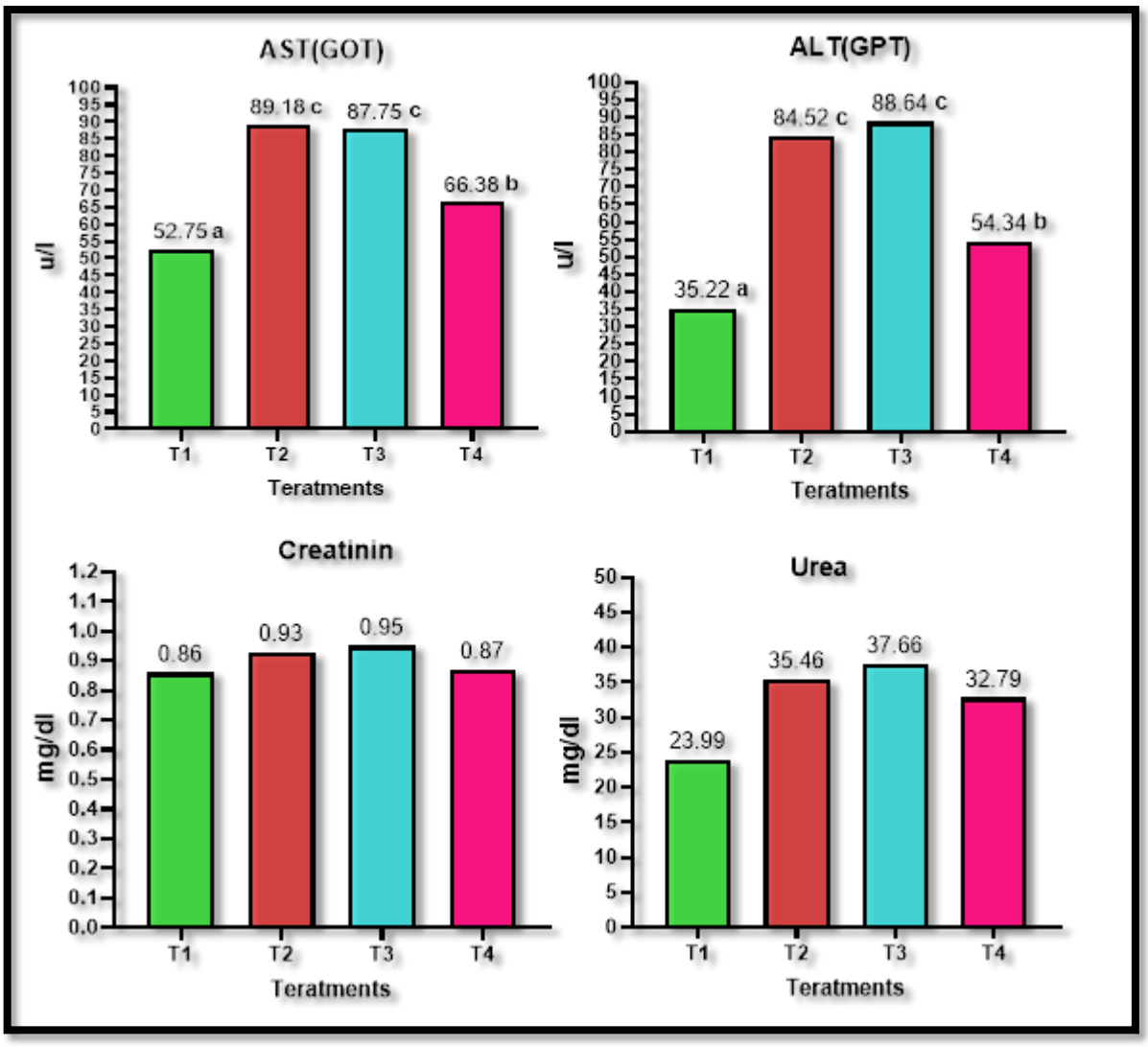

Figure (1) Effect of Therapeutic Syrup on Liver Enzymes activities, Urea and Creatinine in the Blood Serum of Hypercholesterolemic Rats.
*** different letters denote significance (p≤0.05)**

Also, the fourth treatment (T4), which induced hypercholesterolemia and dosed with a therapeutic drink were reveal significant difference in their values for leptin hormone when compared with control and high cholesterol diet treatments. The results showed a significant decrease in the level of the hormone leptin for T4 rats compared to T2 and T3 treatments. Whereas, the leptin level of the control treatment appeared significantly lower than the other studies treatments that intake high cholesterol in their diet.Also, results showed no significant differences between the levels of leptin hormone for T2 and T3 treatments despite intake of whey for the animals in T3.

The Figure also showed a significant difference ($p \leq 0.05$) among experimental treatments in the level of the adiponectin hormone. The fourth treatment (T4) outperformed in reducing the level of the adiponectin hormone compared with the second and third experimental treatments (T2 and T3) respectively. However, the hormone concentration remained significantly higher than the values of control treated animals. The results showed that there were no significant differences between the levels of the hormone adiponectin for the treatments (T2 and T3).

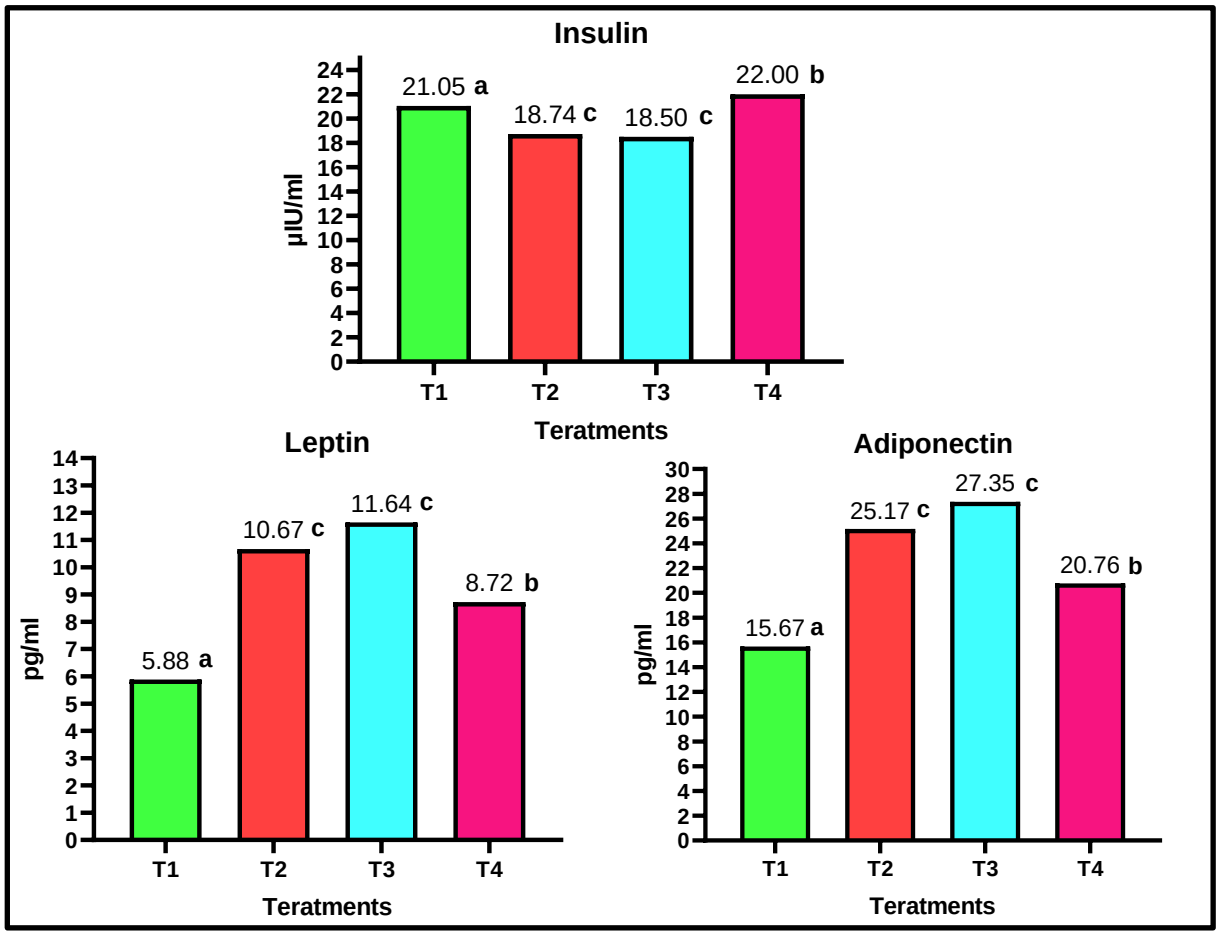

Figure (1) Effect of the therapeutic drink on the levels of insulin, leptin, and adiponectin in the blood serum of of hypercholesterolemic rats.
*** different letters denote significance (p≤0.05)**

Discussions

The results of the present study referred to decrease of lipid profile parameters (Tch,TG,LDL and VLDL) due to administration of Grape-Flavored Whey Probiotic Beverage (therapeutic syrup) in table 2. These results may be attributed to the effect of therapeutic syrup that administrated to rats and containing probiotic bacteria, which in turn produce hydroxyl menthyl glutarate compound through the fermentation process. The probiotic syrup would act to reduce cholesterol synthesis by inhibiting the enzyme Hydroxyl menthyl glutarate CoA-reductase (the limiting step for cholesterol synthesis) and possibly the bacteria's have the ability to convert cholesterol into unobservable coprostanol and cholestanol and excrete them in the faeces (Kumar *et al.*, 2012).

While Abdullah *et al*.,(2013) attributed the reduction in the cholesterol and triglyceride into role of the lactic acid bacteria in stimulating the lipase enzyme that responsible for the breakdown of triglycerides or due to the ability of bacteria to stimulate the secretion of bile acids from the gallbladder, by consume the cholesterol to synthesize bile acids and as a result, bacteria inhibit the absorption of cholesterol and triglycerides from the intestine and thus excrete them with waste products.

These results were in agreement with the findings of (Nasser *et al.* 2020) when they used milk fortified with lactic acid bacteria to reduce the cholesterol concentration in the blood serum of male rats. And referred to its effect on cholesterol concentration in rat blood serum. Also agreement with Jiang *et al.* (2019) whom reducing serum cholesterol levels of hypercholesterolemic rats by drench with the probiotic (*L. reuteri* A9) in group and (*L. mucosae* A13) in other group. Thakkar *et al.,* (2020) study the effect of Lactobacillus probiotic bacteria isolated from fermented food on reducing triglyceride concentration. It also agreed with Gazal (2020) who investigate the effect of probiotics in lowering serum triglycerides mice by fed synergistic cheese fortified with a mixture of Lactobacillus probiotics (Bifidobacterium). Palaniyandi *et al*., (2019) showed that some strains of Lactobacillus acidophilus make it possible for cholesterol to be bound to intestine's lumen and as a result decrease its absorption and this increase utilization of cellular cholesterol and enhanced LDL-C uptake to decrease the total serum cholesterol and other lipid profile and permit for HDL to increase in blood serum for get more cholesterol and return back to liver (Ma *et al*., 2019) . other explanation discovered the reduction in lipid profile indices into the decreased of acylCoA:cholesterol acyltransferase (ACAT) activity(the up regulator of the LDL receptors in the liver) is associated with decreased secretion of VLDL level and then LDL receptors became available to bind with LDL-C and then metabolized.((Abdullah *et al*., 2013).

These results are in agreement with those of Wang *et al.* (2019) whom study the effect of lactobacillus in lowering LDL and VLDL lipoprotein concentration in rat serum. It also agreed with the findings of (Nasser *et al.* 2020) in reducing the concentration of LDL and VLDL lipoprotein in the blood serum of rats that were dosed with milk fortified with probiotic bacteria. This study also agreed with the findings of Hong *et al.* (2015) on the effect of fermented whey syrup, Using lactic acid bacteria (*Lactobacillus plantarum* DKL 121) in reducing LDL and VLDL lipoprotein concentrations of rat blood serum.

The improvement of HDL levels are agreement with the Ding *et al.* (2017) in raising the concentration of HDL in the blood of rats dosed with (Traditional fermented Tibetan yak milk) fortified with probiotic bacteria. These also consistent with the findings of Ayed and Hamada (2017). In raising the level of HDL cholesterol in the blood of male rats fed with screening milk fermented with probiotic bacteria for each of the treatments in which the isolates were used: *Lactobacillus acidophilus, Bifidobacterium bifidum* and their mixture.

Measurement of liver enzymes activities are considered as one of the most important indicators of the liver and other organs healthy. According to Ioannou,(2006) mentioned that the elevated serum ALT levels in the absence of viral hepatitis and alcoholism has been reported to lead to a higher risk of cardiovascular disease with the risk greater in women. The decrease in enzyme concentrations during the experiment shows the positive role for the therapeutic syrup containing the probiotics. The anti-oxidative effect of probiotics acted as protective to preserve the damage in the hepatocyte that occurs due to high cholesterol diet (Wang *et al.*, 2019). therefore, be able to preserved the liver cells from being destroyed although the high dose of cholesterol is exposed to animals. This is what the study of Al-Hamdani (2019) found about a decrease in liver enzymes due to the effect of types of prebiotics. Asemi *et al.* (2017) recorded the beneficial effect of probiotics and their ability to regulate liver enzymes in pregnant women. Also, these results are in agreement with what was found in the study of Gazal (2020) in reducing levels of liver enzymes. ALT and AST in serum of mice fed synergistic cheese.

Potential mechanisms by which prebiotics may improve insulin resistance include modulating energy metabolism, controlling obesity, and increasing the production of GLP-1 (Glucagon-like peptide 1) and GLP-2 (Glucagon-like peptide 2) by modifying pancreatic and plasma insulin secretion, beta cell mass, and their functions. GLP-2 also enhances insulin sensitivity in the liver, fat, and muscle (Gargari *et al.*, 2013). Probiotics have also been found

effective in improving insulin resistance by reducing the concentration of endotoxins, increasing the pH of the stool, and also by reducing the production and absorption of enterotoxins (Eslamparast *et al.*, 2014). Thus, modified microorganisms in the intestine can be effective in improving blood sugar status through the use of probiotics and prebiotics.

Most previous studies showed that treatment with prebiotics or probiotics can control blood sugar or reduce insulin in the blood. Daubioul *et al*. (2005) observed a significant decrease in insulin concentration after oligofructose administration among non-alcoholic fatty liver patients after 4 weeks. In another study, the effect of probiotic supplementation on improving glycemic parameters was shown to significantly decrease glucose and insulin levels during fasting compared to the placebo group (Rajkumar *et al*., 2015).

Leptin, an anti-obesity hormone produced by adipose tissue, to regulate body weight by controlling food intake and energy expenditure (Friedman, 2002). In mice fed high in fat, Lee *et al*. (2006) confirmed. Lactobacillus rhamnosus PL60 showed a decrease in the level of the hormone leptin and an anti-obesity effect due to the production of conjugated linoleic acid.

The results above are agreement with the findings of Behrouz *et al.* (2017) on the effect of taking probiotics and prebiotics (for 12 weeks) in lowering the serum level of leptin in patients with non-alcoholic fatty liver disease. Moreover, serum leptin concentration was reduced by Lactobacillus gasseri SBT205 in rats. Zucker rats are lean in body and the reason for this has been attributed to the low volume of adipocytes in the bodies of these rats (Hamad et al., 2008). Another study also indicated that the level of leptin decreased due to a mixture of Bifidobacteria strains (*B. pseudocatenulatum* SPM 1204, *B. longum* SPM1205 and *B. longum* SPM 1207) in obese rats (An *et al*., 2012).

The reduction in the adiponectin hormone levels due to administration of Grape-Flavored Whey Probiotic Beverage (therapeutic syrup) are in agreement with the results of the Sabico *et al*. (2019) study in lowering the serum level of the hormone adiponectin in patients with type 2 diabetes who took probiotic supplements for 6 months. Several studies have also shown that probiotic treatment improves the level of adiponectin or the gene expression of adiponectin. One of the studies compared normal flora (NMF) mice and one free of germs (GF), and the study revealed that gene expression of adiponectin (Adipoq) was up-regulated in groups of germ-free mice treated with lactic acid bacteria (Nerstedt *et al.*, 2007). Moreover, Higurashi *et al*. (2007) indicated that feeding with the use of probiotic cheese can prevent the accumulation of fat in the abdominal area and maintain the concentration of the hormone adiponectin in the serum of mice fed on a diet with a high caloric content, while one study found that the strain of Lactobacillus plantarum NO.14 Exercises a white lipid-lowering effect in mice fed high in lipids with no change in adiponectin (Takemura *et al*., 2010). While Kadooka *et al*. (2010) used *L. gasseri* SBT2055 probiotic bacteria to regulate abdominal obesity in obese adults, as the probiotic treatment included a significant reduction in the visceral and subcutaneous fat areas in the abdomen from baseline and a significant increase in the molecular weight of adiponectin in their serum. Moreover, a large-scale clinical study by Luoto *et al*. (2012) that pregnant women who took a mixture of probiotic bacteria (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis*) had a higher concentration of adiponectin compared to the placebo group, which was inversely associated with maternal weight gain during pregnancy.

It is now more accepted that leptin can regulate food intake and energy expenditure through the effect of the hypothalamus, and adiponectin can enhance the oxidation of tissue fat, leading to lower levels of fatty acids and triglyceride content in tissues associated with insulin sensitivity (Bacchetta *et al.*, 2009).

Conclusions:

Grape-Flavored Whey Probiotic Beverage (therapeutic syrup) prepared by probiotic isolate (*L. acidophilus*) , whey and grape juice had reduction effect on harmful lipid accumulation in the serum or in the body by regulating of insulin, leptin and adiponectin hormones levels.

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