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Research Article

Investigation of a Probiotic Yeast as a Cholesterol Lowering Agent on Rats Fed on a High Cholesterol Enriched Diet [1]

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Abstract

Probiotic yeast, *Cryptococcus humicola* M5-2 strain, which has high cholesterol assimilation feature and isolated from traditionally produced cheese, was investigated *in vivo*. This study was conducted to determine cholesterol assimilation ability of *C. humicola* M5-2 in vivo and specify the effect of the strain on serum total cholesterol, HDL/LDL cholesterol and triglycerides levels in rats fed on cholesterol-enriched diet. *C. humicola* had the ability to assimilate cholesterol at the rate of 73.33% in media. The strain was used with two different concentrations in animal feed (high and low doses containing 2% and 0.1% lyophilized strains, respectively). When the treatment groups were compared, low dose feeding group had the positive results in terms of testing values. According to the results of serum analysis, triglyceride and total cholesterol level were decreased by 25% and 1.34% respectively. Especially, decreasing the percentage rate of triglyceride has not obtained in other *in vivo* studies. It is thought that health promoting effect will be possible when the obtained isolate is consumed with fermented foods.

Keywords: *Cryptococcus humicola, Cholesterol, Probiotics, Animal experiment*

Probiyotik Mayanın Yüksek Kolesterol İçeren Diyetle Beslenen Sıçanlarda Kolesterolü Düşürücü Ajan Olarak Kullanılabilirliğinin Araştırılması

Özet

Yüksek kolesterol asimilasyon yeteneğine sahip probiyotik maya *Cryptococcus humicola* M5-2 suşu, geleneksel olarak üretilmiş bir peynirden izole edilmiş ve *in vivo* ortamda incelenmiştir. Çalışmada, *C. humicola* M5-2 suşunun in vivo ortamda kolesterolü asimile etme yeteneği incelenmiş ve kolesterolce zengin diyetle beslenen sıçanlarda bu suş ile beslemenin serum toplam kolesterol, HDL/LDL kolesterol ve trigliserit seviyelerine etkisi belirlenmiştir. *C. humicola*' nın besiyeri içersindeki kolesterolü asimile etme oranı %73.33'tür. Hayvanların beslenmesinde suş iki farklı dozda kullanılmıştır (yüksek ve düşük doz, sırasıyla %2 ve %0.1 liyofilize suş içermektedir). Deneme grupları karşılaştırıldığında, düşük doz besleme grubu daha olumlu sonuçlar göstermiştir. Serum analiz sonucuna göre trigliserit ve toplam kolesterol düzeyi sırasıyla %25 ve %1.34 oranında düşme göstermiştir. Özellikle trigliserit düzeyindeki düşme oranı çoğu *in vivo* çalışmada rastlanmamıştır. Elde edilen suşun fermente gıdalar ile beraber tüketiminin sağlık üzerine iyileştirici etkiler yaratacağı düşünülmektedir.

Anahtar sözcükler: *Cryptococcus humicola, Kolesterol, Probiyotikler, Hayvan denemesi*

INTRODUCTION

Today, the drugs, which can reduce the lipid ratio, are standard tools for cholesterol therapy. However, this situation disturbs patients who use cholesterol drugs along the rest of life because of the negative effects. It is estimated that some patients who take cholesterol lowering prescription drugs and follow a low-fat or low-

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cholesterol diet, do not achieve adequate reductions in their cholesterol levels [1]. Patients have sought to decrease their blood cholesterol levels with naturally.

Yeasts are widely present in dairy products and some yeast strains are used in cheese microbiology and development of new products because of its probiotic features. Yeasts, which are used technologically for producing fermented foods, have beneficial effects on human health. When the features of yeast types are investigated, it's noticed that *Cryptococcus* species are capsulated yeasts and widely found in air, soil, pigeon droppings and foods such as cheese, milk, beans and wine [2]. Topcu [3] isolated and identified yeast strains from raw milk and cheese samples and found especially *Candida*, *Geotrichum*, *Trichosporon*, *Cryptococcus*, and *Saccharomyces* genus in cheese samples. Yeast can be isolated from many types of cheese as a natural contaminate and they can be found commonly in the environment microflora of dairy plants. So, these microorganisms also found as a natural contaminates in raw milk, air, operating equipment, brine and water. Zottola et al.[4] stated that mozzarella cheese was contaminated with *Cryptococcus humicola* from hands of workers. In raw milk samples, genetically defined dairy origin yeast strains were observed and *Cryptococcus humicola* strains found [5]. It is indicated that *Cryptococcus* yeast types were found in raw milk and also in pasteurized milk as a secondary contaminate. In investigated ninety raw milk samples the ratio of *Cryptococcus* was 14% [6]. In a study *Cryptococcus humicola* was found in white cheese samples at the rate of 5.34% [7]. *Candida* spp., *Pichia* spp., *Rhodotorula* spp., *Kluyveromyces* spp. and *Cryptococcus* spp. were the most dominant types of yeasts which grow in white cheese surface and found that of strains, ratio of *Cryptococcus* was 10% [8]. There are also *Cryptococcus* strains in kefir grains which have positive effects for human health [9].

The objective of this study is to prepare lyophilized yeast culture which has probiotic properties. It is important that selected strain should have the same properties *in vivo* and *in vitro* conditions. In our previous study, yeasts were isolated and identified from several foods such as milk, cheese, yogurt, butter etc. and *in vitro* treatments about their cholesterol assimilation ratio and probiotic characteristics were researched (Research project: TUBITAK SBAG 111 S 513). The strain *Cryptococcus humicola* M5-2 was chosen because of its better probiotic characteristics and cholesterol assimilation ratio than the other examined yeast strains. In present study, we conducted to determine cholesterol assimilation ability of *Cryptococcus humicola* M5-2 *in vivo* and to specify the effect of feeding with the strain on total cholesterol, HDL/LDL cholesterol and serum triglycerides levels in rats fed cholesterol-enriched diet. In preclinical studies, experiments on animals before testing on humans are mandatory. For this reason, rats were preferred because of suitable tissue, size, anatomy, and phylogenetic properties.

MATERIAL and METHODS

Yeast Strain

Cryptococcus humicola M5-2 strain which was isolated from traditionally produced cheese was used in this study. The strain was identified with API ID 32 C test. *Cryptococcus humicola* was selected because of high cholesterol assimilation ratio (73.33%) in media. The other probiotic features of this strain were also determined. According to previous study, acid and bile tolerance of the strain was high; also the strain could survive for 90 min in artificial gastric fluid. Negative bile salt hydrolization was the other cause of this strain selection. For experimental inoculation of yeasts, the cultures of *Cryptococcus humicola* M5-2 were centrifuged at 8000 rpm for 10 min and pellets were washed with PBS-7.2 twice and lyophilized (VirTis benchtop-SLC). Finally, yeast intensities were determined by optical densitometer and $1x10^7$/mL living cells were used.

Animals

This study was approved by Local Ethics Committee of Animal Experiments of Süleyman Demirel University Hospital and performed following standard guidelines for the care and use of laboratory animals (No: B.30.2.SDÜ.0.05.06.00-65 Date: 04/10/2011). A total of 32 adult Wistar male rats (200-300 g weight) were housed under constant temperature (22±2°C) and humidity (60±5%), with 12-h dark/light cycles and allowed tap water and rat pellets *ad libitum* before and after the operation.

A pretreatment were applied to determine the highest lyophilized yeast amount before the main treatment. For this purpose, 2% lyophilized yeast were given to rats by oral gavage and effect was examined during a week. In preliminary test 3 rats were used. At the end of the week there was no negative situation in rats. Main animal treatment was carried out with 32 rats. All animals were fed on a basal diet for one week. After this adaptation period, the rats were divided randomly into 4 experimental groups of 8. The rats were housed in Euro type 4 cages. Each cage had 8 rats. Cages were cleaned 3 times a week.

Surgery

After the seven weeks feeding period, the rats were sacrificed by taking blood samples under anesthesia with 10% ketamine (Alpha, Alfas IBV) and 2% xylazine (Alfaz's, Alfas IBV). Blood samples were separated by centrifugation at 3.500 rpm for 8 min (Rotanta 460. Germany) and then serums were separated. Serums were stored at -80°C (Facis S.A. France) in medical biochemistry laboratory of Süleyman Demirel University until analysis.

Treatment

The rats were divided into four groups. One group

(negative control-Group 1) received a basal diet (cholesterol-free diet) throughout the experimental period of seven weeks and served as a negative control group. Second group (positive control-Group 2) fed on the basal diet with cholesterol and cholic acid added at a level of 1% (w/w) and 0.1% respectively (cholesterol-enriched diet). The third group (low dose lyophilized yeast supplemented-Group 3) fed on a cholesterol-enriched diet supplemented with lyophilized yeast at a level of 0.1%. The fourth group (high dose lyophilized yeast supplemented- Group 4) fed on a cholesterol-enriched diet supplemented with lyophilized yeast at a level of 2%. Cholesterol, cholic acid, and lyophilized yeast were dissolved in 2 mL water and given for all feeding period as a dose per day via oral gavage.

Determination of Cholesterol Level in Serum

Blood samples were collected from abdominal aorta, placed in sterile tubes, and vortexed (Labinco L 46, Netherlands). The obtained serum samples were analyzed for cholesterol, high density lipoprotein (HDL) cholesterol, and triglycerides. Kits were used for the analysis (Beckman Coulter AU 5800, USA). Low density lipoprotein (LDL) cholesterol levels were calculated by auto analyzer according to Fridewald formulation. Cholesterol levels were expressed in milligrams per deciliter (mg/dL).

Statistical Analyses

The generated data were analyzed by analysis of variance (ANOVA) and differences among mean values were treated with the Tukey's multiple comparison test. The statistical evaluation of the results was performed using the SPSS 17.0.0 (SPSS Inc., Chicago, IL).

RESULTS

There was no significant difference observed in point of rat's weight between the experimental groups during the seven weeks feeding period (P>0.05) *(Table 1)*. While the highest weight gain was observed in low dose yeast group, the lowest weight gain was observed in high dose yeast group.

Cholesterol supplementation to basal diet was increased the total cholesterol at a level 2.04 mg/dL. As seen in *Table 2*, there was no significant difference between the Group 2 and Group 4 in terms of the levels of total cholesterol and HDL cholesterol (P>0.05). High dose lyophilized yeast supplementation to diet had no effect on level of total cholesterol and HDL cholesterol, but it led to increase of LDL cholesterol level. In general, *in vitro* high assimilation rate was determined in the medium could not be observed *in vivo* because metabolism synthesizes cholesterol when needed. When *Table 2* was observed, feeding with low dose lyophilized yeast supplementation provide decreasing on triglyceride and total cholesterol level. Decline in the value of triglycerides was approximately 25% and reduction of total cholesterol level was 1.34%. These results could be considered as promising, because 1% reduction in cholesterol can reduce the risk of cardiovascular diseases for 2-3% [10]. In this respect, the selection of probiotic strains

Table 1. *Experimental animals weight*
Tablo 1. *Deney hayvanlarının ağırlıkları*

Days	Group 1	Group 2	Group 3	Group 4
1. day	269.75±34.59	277.13±33.15	271.88±16.74	258.63±40.32
2. week	271.88±34.93	279.13±34.10	273.63±16.85	261.00±39.80
3. week	287.13±36.38	281.63±33.73	275.88±16.87	263.75±39.73
4. week	290.25±35.76	285.63±33.76	280.38±17.37	267.38±39.26
5. week	294.13±35.87	283.43±32.66	284.13±17.50	271.38±39.24
6. week	300.38±37.14	290.29±32.89	290.00±17.49	276.75±38.76
7. week	329.38±33.32	327.71±25.74	336.88±19.36	303.75±43.65
Weight increase	22.11%	18.25%	23.91%	17.45%

Table 2. *Average level of triglyceride, total cholesterol, HDL cholesterol, LDL cholesterol*
Tablo 2. *Ortalama trigliserit, toplam kolesterol, HDL kolesterol ve LDL kolesterol değerleri*

Groups	Triglyceride (mg/dL)	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	LDL Cholesterol (mg/dL)
Group 1	44.88±7.74	38.25±5.12	23.75±5.06	4.71±2.06
Group 2	46.29±14.00	40.29±4.03	26.43±1.72	3.33±2.25
Group 3	34.75±11.63	39.75±5.52	27.50±3.89	6.00±1.29
Group 4	38.62±8.50	40.13±5.52	26.00±4.96	5.43±2.15

which have high percentage of cholesterol assimilation rates is important.

All organs of rats were investigated during the operation time. In Group 2, steatosis and caseation necrosis were observed around hearth. Rats were negative affected from high dose lyophilized yeast supplemented to diet (Group 4). In this group, the amount of blood of the rats was lower than in the other groups. Kidneys were larger than usual, color of liver was lighter. There was no observed any negative effect on Group 3 which was fed with low dose lyophilized yeast. When comparing the Group 3 and Group 4, feeding with high dose lyophilized yeast had negative effects on hematological values in parallel on organs.

DISCUSSION

Nowadays, probiotic yeasts can be delivered either in fermented foods or as lyophilized cultures administered orally. Several yeasts species, have been used in many probiotic preparations [11].

In animal studies on cholesterol assimilation, generally direct impact of lactic acid bacteria or effect of the fermented products produced therefrom have been discussed. In line with these studies different results were obtained according to used strains. Several studies have shown the ability of probiotic microorganisms to lower cholesterol levels *in vivo*. Bertazzoni Minelli et al.[12] found lower serum cholesterol (3.73%-13.49%) and triglyceride (9.30%-40.23%) ratios and stated an increase on HDL cholesterol level in fats fed on fermented milk containing *Lactobacillus casei* compared to control group after 10 days. As a result of a 3-week feeding of pigs with a mixture of probiotic, levels of total cholesterol decreased from 3.25 mmol/L to 2.74 mmol/L and triglyceride levels were not change [13]. *Lactobacillus plantarum* addition to normal diet over 14 days reduced the levels of total serum cholesterol and triglyceride of mice at a ratio 7% and 10%, respectively. Nguyen et al.[14] Beena and Prasad [15] found lower serum cholesterol in rats fed on yoghurt containing *Bifidobacterium bifidum* (1.20 g/L) compared to a positive control (1.72 g/L) after 30 days. However, the administration of fermented milk containing bifidobacteria (10^9 cfu/g) to hypercholesterolaemic human subjects resulted in a decrease in the total cholesterol level from 3 to 1.50 g/L [16]. The mechanism(s) responsible for the cholesterol-lowering effect of probiotics remains unclear, but it has been suggested that the effect could be obtained through retarded cholesterol synthesis and increased degradation of cholesterol [17].

Diets supplemented with probiotics can also significantly reduce plasma triglycerides in broilers. In a study, broilers fed with addition of 0.5% *Saccharomyces cerevisiae* triglyceride and total cholesterol levels decreased at the rate of 22.67 and 9.95% respectively after 3 weeks [18]. Cholesterol level was significantly lower in broilers supplemented with thermotolerant probiotic yeast at different levels compared to control group [17]. Oral administration of probiotics has been shown to significantly reduce cholesterol levels by as much as 22 to 33% [19]. Further, decrease in cholesterol content of eggs of laying hens and broilers diets containing yeasts was reported by Yalçın et al.[20] and Yıldız et al.[21].

Seyidoğlu and Galip [22] and Seyidoğlu et al.[23] were conducted to evaluate the effect of *Saccharomyces cerevisiae* on the serum biochemical parameters in rabbits. The diets with the yeast reduced serum HDL cholesterol and triglycerides on the 90th feeding day. Similar to our findings serum cholesterol slightly decreased by the yeast. In a study performed by Güven and Güven [24] kefir grains was caused significant suppression in serum lipids on the rabbits fed with cholesterol supplemented diet.

Numbers of studies are quite a lot which is performed with bacteria *in vivo* conditions. Some of these studies show similarities with our study results [25,26]. Jin et al.[27] were determined serum cholesterol level of broilers fed with Lactobacillus cultures (*L. acidophilus, L. fermentum, L. crispatus* and *L. brevis*). In their study, culture supplementation to diets was in levels of 0.05%, 0.1% and 0.15% in the experimental groups and the decrease in the amount of serum cholesterol was found 1.56%, 17.18% and 8.59% respectively. Similar to our findings, high dose supplementation showed lower impact. In our study, the addition of 2% lyophilized yeast (high dose) did not affect the amount of serum cholesterol in rats and has created adverse effects on tissues and organs. It may cause some tissue damage by increasing doses so these results require further studies using different types and doses of yeasts. This situation showed how important was dose adjustments in this kind of work.

In conclusion, yeast supplementation has some beneficial effect by lowering cholesterol and triglycerides. In the present study, low dose lyophilized yeast supplemented diet (Group 3) showed more positive results. It was observed that the level of triglyceride and total cholesterol were reduced at the rate of 25% and 1.34%, respectively. Especially, reducing rate of triglyceride level was higher than many *in vivo* studies. Studies in recent years have shown that yeasts also play a significant role in the spontaneous fermentation of many indigenous food products. Usage of yeasts as a co-culture is performed by researchers because of the several promoting advantages of these organisms. They have a more diverse enzymatic profile that leads to the formation of aromatic substances, also their lipolytic and proteolytic activity is high and their interactions with starter cultures is favorable. It is therefore encouraged that additional efforts are placed on exploring the health beneficial effects of yeasts. According to these informations it was thought that when obtained isolates are consumed with fermented products, positive results will be demonstrated.

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