

The effects of prebiotic, probiotic and synbiotic diets containing *Bacillus coagulans* and inulin on serum lipid profile in the rat

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Abstract

An *in vivo* trial was conducted to evaluate the effects of *Bacillus coagulans*, and inulin, either separately or in combination, on lipid profile using a rat model. Thirty-two male Wistar rats were randomly divided into four groups (n=8) and fed as follows: standard diet (control), standard diet with 5% w/w long chain inulin (prebiotic), standard diet with 10⁹ spores/day spores of *B. coagulans* by orogastric gavage (probiotic), and standard diet with 5% w/w long chain inulin and 10⁹ spores/day of *B. coagulans* (synbiotic). Rats were fed for 30 days. Serum samples were collected 10, 20 and 30 days following onset of treatment. Total, HDL and LDL cholesterol and triglycerides concentrations were analyzed. Results of this study showed that inulin potentially affected the lipid profile. An obvious decrease in serum total cholesterol and LDL-cholesterol of rats fed with inulin in symbiotic and prebiotic groups was seen in all sampling days. Inulin fed rats also demonstrated higher levels of HDL-cholesterol concentration; however this value in probiotic and control fed rats remains without significant change. According to the results of this study, *B. coagulans* did not contribute to any lipid profile changes after 30 days. Thus, further *in vitro* investigations on the characteristic of these bacteria could be useful to gain insights into understanding the treatment of probiotics in order to achieve the maximum beneficial effect.

Introduction

WHO has predicted that by 2030, cardiovascular diseases will be the most important cause of death, affecting approximately 23.6 million people around the world.¹ People

affected with hypercholesterolemia are at a three times higher risk of heart attack compared to those who have normal blood lipid profiles.² Pharmacological agents are able to reduce cholesterol levels effectively; however, they are expensive and the undesirable side effects have caused concerns about their therapeutic use. Therefore many investigations have been done to evaluate new approaches toward the identification of other dietary means of reducing blood cholesterol levels. These include dietary supplementation of probiotics and/or prebiotics. Probiotics are defined as *living microbial supplements that beneficially affect the host animals by improving its intestinal microbial balances*.³ Prebiotics are *indigestible fermented food substrates that selectively stimulate the growth, composition and activity of microflora in gastrointestinal tract and thus improve hosts' health and well-being*.⁴ When probiotics and prebiotics are used in combination they are known as *synbiotics*.

Micro-organisms used as a probiotic for human mainly belong to the *Lactobacillus* and *Bacillus spp.* *Bacillus* probiotics differ in many characteristics from those based on *Lactobacillus spp.* While *Lactobacillus* represents a normal resident gastrointestinal tract (GI) microflora of humans, *Bacillus* belongs only to the transitory GI bacteria.⁵ Most *Lactobacillus* probiotics are inactivated by bile and low gastric pH, whereas members of genus *Bacillus* are endospore forming bacteria that make it extremely heat-stable and resistant to adverse GI tract conditions and when germinate in GI tract, cause positive effects for the host. *B. coagulans* (reported incorrectly as *Lactobacillus sporogenes*)⁶ is a shelf stable bacteria that secretes L (+) lactic acid, short-chain fatty acids such as butyric acid and a bacteriocin called Coagulin, which has activity against a broad spectrum of enteric microbes.

Although many controversial studies have demonstrated the cholesterol-lowering effects of probiotics, prebiotics and synbiotics in animals and humans, there is also limited information on cholesterol-lowering effects of *B. coagulans* spores. Studies on the effects of *B. coagulans* on lipid profile have been limited to those who investigated the influence of administration of *B. coagulans* capsules (each containing 360 million spores) per day in hyperlipidemic patients for three months, they reported total serum cholesterol, LDL cholesterol and total cholesterol to HDL cholesterol, and LDL-cholesterol to HDL-cholesterol ratios were reduced significantly. They also found HDL-cholesterol was marginally increased.⁷ Panda *et al.*⁸ also reported this probiotic is able to reduce total cholesterol, VLDL and triglycerides in broiler chickens.

Considering our previous findings indicated significant changes in rat GI tract microbiota

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following administration of *B. coagulans* and inulin,⁹ this study was conducted to evaluate the *in vivo* effects of *B. coagulans* and inulin, separately and in combination on lipid profile using a rat model.

Materials and Methods

Preparation of spore suspension of probiotic bacteria

Lyophilized probiotic *B. coagulans* were donated by the Pardis Roshd Mehregan Company, Iran. It was grown aerobically in Nutrient Yeast extract Salt Medium (NYSM) agar¹⁰ at 37°C for 24 h. A single colony from the NYSM plate was inoculated into 500 mL of NYSM broth and incubated at 37°C with shaking at 250 rpm for 48 h. The bacterial suspension was pelleted three times by centrifugation at 3000×g for 20 min, and washed with sterile normal saline. Final pellet was re-suspended in 100 mL sterile normal saline. To determine the spore per ml of suspension, the solution was heated at 80°C for 15 min to kill the vegetative cells before appropriate serial dilution and plating in NYSM agar. Finally, the spore suspension was prepared at a concentration of 1×10⁹ spore/mL in sterile saline and kept in the refrigerator until use.

Animals and diets

Thirty-two male Wistar rats (200 ± 8.4 g) were provided by the Animal Centre of Razi Research Institute, Shiraz, Iran. Animals were randomly assigned to four dietary groups ($n=8/\text{group}$) and housed in groups of six rats per cage in a temperature controlled environment ($22 \pm 2^\circ\text{C}$) with $55 \pm 10\%$ relative humidity and controlled lighting (12 h light/dark cycle).

Rats were randomly divided into 4 groups and fed as follows: i) standard diet (control), ii) standard diet supplemented with 5% w/w long chain inulin (Sensus, Netherlands) (prebiotic), iii) standard diet with 10^9 spores/day *B. coagulans* (gavage 1ml of prepared spore suspension using a blunt ended needle) (probiotic), and iv) standard diet supplied with 5% w/w long chain inulin and 10^9 spores/day *B. coagulans* (synbiotic). The standard pellet feedstuff contained 14.5% protein, 4.7% ash,

51.2% starch, 4.3% sugar and 4% fat (3.2 kcal/g). Regarding micronutrients, the feedstuff contained 0.72% calcium, 0.6% phosphorus, 0.23% magnesium and 0.25% chloride among others. The inulin content in the rat diet was calculated based on food intake. The food intake of each rat with mean of 200 g body weight is 10 g/day, it means each rat received 0.5 g inulin/day. Food and distilled water were provided *ad libitum*.

To assimilate the experimental conditions, the control and prebiotic group were gavaged with 1 mL of sterile normal saline once a day.

Animal ethics

This experiment was accomplished under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986

were followed, regarding the standards in the protection of animals used for experimental purposes.

Experimental design and sampling

All animals were acclimatized for 2 weeks before the experimental session. Rats were fed the diets for 30 days. On day 10, 20 and 30 of trial, animals were anesthetized with Diethyl ether and 3 mL blood samples were collected from each animal through heart puncture and left to stand for 30 min at room temperature (20°C) for coagulation before being centrifuged at 750 g for 15 minutes. Serum samples were stored at -20°C until analysis.

Lipid components analysis

The analysis of the serum for total cholesterol was done using a commercial kit (Ziest Chem Diagnostics, Tehran, Iran) by a modified

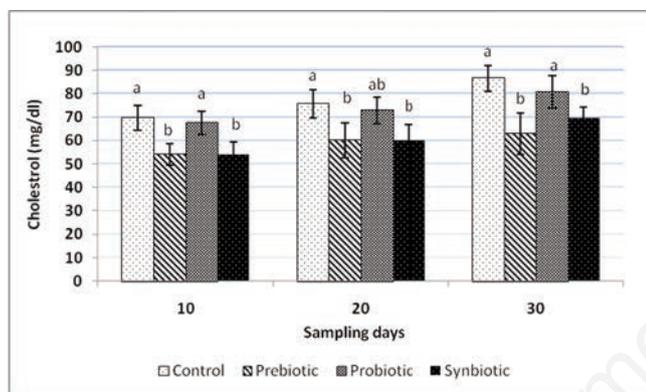


Figure 1. Effect of oral administration of *Bacillus coagulans* (probiotic diet) and inulin (prebiotic diet) separately and in combination (synbiotic diet) on serum total cholesterol levels in male Wistar rats. Values are expressed as mean for 8 animals. Bars represent standard deviation values. The different letters in the same sampling day indicate significant differences ($P < 0.05$)

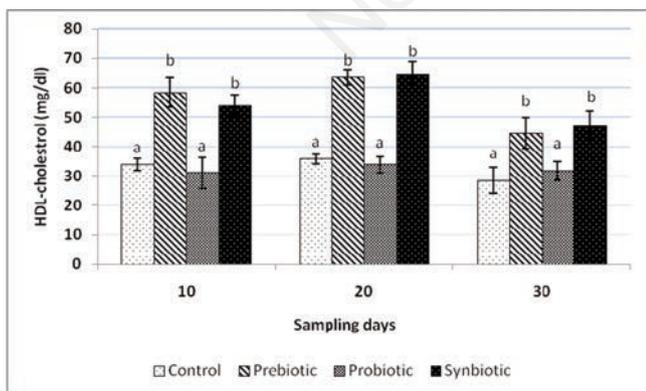


Figure 2. Effect of oral administration of *Bacillus coagulans* (probiotic diet) and inulin (prebiotic diet) separately and in combination (synbiotic diet) on serum triglycerides levels in male Wistar rats. Values are expressed as mean for 8 animals. Bars represent standard deviation values. The different letters in the same sampling day indicate significant differences ($P < 0.05$).

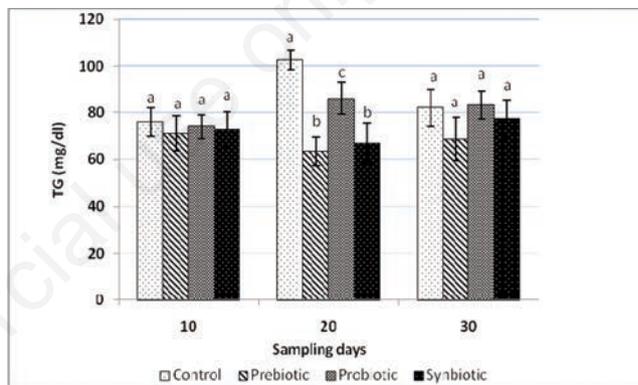


Figure 3. Effect of oral administration of *Bacillus coagulans* (probiotic diet) and inulin (prebiotic diet) separately and in combination (synbiotic diet) on serum HDL-cholesterol levels in male Wistar rats. Values are expressed as mean for 8 animals. Bars represent standard deviation values. The different letters in the same sampling day indicate significant differences ($P < 0.05$).

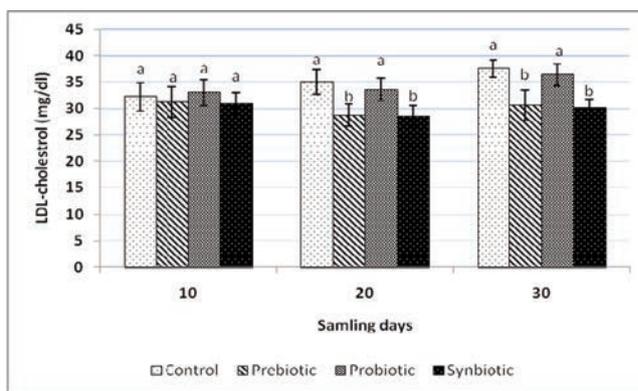


Figure 4. Effect of oral administration of *Bacillus coagulans* (probiotic diet) and inulin (prebiotic diet) separately and in combination (synbiotic diet) on serum LDL-cholesterol levels in male Wistar rats. Values are expressed as mean for 8 animals. Bars represent standard deviation values. The different letters in the same sampling day indicate significant differences ($P < 0.05$).

Abell-Kendall/Levey-Brodie (A-K) method and the measurement of serum triglyceride was accomplished based on the enzymatic procedure by a commercial kit (Ziest Chem Diagnostics, Tehran, Iran).

Lipoproteins including HDL-cholesterol (mg/dL), and LDL-cholesterol (mg/dL) were analyzed by quantitative enzymatic colorimetric method using test kits supplied by STAN-BIO Laboratories, Boerne, TX, USA. All reactions were measured using Digital UV/VIS Spectrophotometer (CE 292, series 2, Cecil Instruments, Cambridge, England).

Statistical analysis

In order to determine the difference among treatments, an Analysis of Variance (ANOVA) was used and when differences were detected, a Duncan's multiple comparison test was used to differentiate the treatment means. The analysis was carried out using SPSS (version 19, SPSS Inc) at a significance level of 0.05.

Results

The effect of different diets supplementation on serum total cholesterol is shown in Figure 1. Supplementation with inulin caused significant decrease in serum total cholesterol of rats, on prebiotic and synbiotic diets after 10, 20 and 30 days of treatment initiation.

On day 10, there were no significant differences in triglycerides values between treatments and control (Figure 2). By day 20, serum triglycerides concentrations in control, probiotic, prebiotic and synbiotic groups were 102.6, 86.1, 63.2 and 66.9 mg/dL, respectively, which shows inulin has a potential role in triglycerides decrease ($P < 0.001$). On day 30, lower serum triglycerides ($P = 0.23$) was seen in prebiotic and synbiotic groups compared to probiotic and control but it was not significant.

On day 10, 20 and 30 of trial HDL-cholesterol level increased significantly ($P < 0.001$) in serum of rats fed with inulin, in prebiotic and synbiotic groups, while rats fed by only *B. coagulans* remained without significant change (Figure 3).

Changes in LDL-cholesterol level during 30 days treatment with experimental diets are shown in Figure 4. Treatment with different diets appeared to have no effect on the LDL cholesterol concentration in rats by day 10. However, supplementation with prebiotic and synbiotic diets significantly lowered LDL-cholesterol concentration over 20 and 30 days; rats on the probiotic and control diets showed increased in LDL-cholesterol values.

Discussion

This feeding trial was conducted to investigate the effect of *B. coagulans* and inulin, separately and in combination on serum lipid profile using rats as a model. Administration of inulin in rats on prebiotic and synbiotic diets showed significantly ($P < 0.05$) lower total cholesterol level compared to probiotic and control rats in day 10, 20 and 30. Significant changes in triglyceride concentration are limited to day 20, when rats on prebiotic and synbiotic diets showed lower levels of triglyceride concentrations, while on day 10 and 30 no significant differences between groups were observed.

According to the results of this study, inulin played an important role in changing lipid profile effectively. An obvious decrease in the serum total cholesterol and LDL-cholesterol of rats fed with inulin in synbiotic and prebiotic groups was seen in all sampling days. Inulin fed rats also demonstrated higher levels of HDL-cholesterol concentration; however this value in probiotic and control fed rats showed no significant change.

There are two suggested mechanisms that have been attributed to hypocholesterolemic effect of prebiotics such as inulin; decreased cholesterol absorption by enhancing cholesterol excretion via feces and the selective fermentation by intestinal bacterial microflora causing production of short-chain fatty acids (SCFAs).¹¹

Results of this study concerning hypocholesterolemic effect of inulin are in agreement with several studies conducted *in vivo* trials. Kim and Shin¹² reported administration of inulin in hypercholesterolemic rats for 4-weeks decreased serum LDL-cholesterol with increased serum HDL-cholesterol levels ($P < 0.05$) compared to the control.¹² Causey *et al.*¹³ showed consuming 20 g/day inulin for 3 weeks caused significant decrease in serum triglycerides in twelve subjects.¹³ Similarly, in another study involving eight healthy volunteers with a daily consumption of 10 g of inulin for 3 weeks, significant decrease in plasma triacylglycerides concentrations compared to the placebo was observed.¹⁴ In another study by Brighenti *et al.*,¹⁵ significant ($P < 0.05$) reduction in plasma total cholesterol and triacylglycerols of twelve healthy rats was seen following 12-week consumption of 50 g of a rice-based ready-to-eat cereal containing 18% inulin.¹⁵ According to the results of this study, *B. coagulans* did not contribute to any lipid profile changes after 30 days. Although many studies have demonstrated cholesterol-lowering effects of probiotics in both animals and humans, debatable results have also been reported concerning inability of a particular strain of probiotic bacteria to improve lipid profile. A study by Hatakka *et al.*¹⁶ reported that

administration of *L. rhamnosus* LC705 (10¹⁰ CFU/g per capsule; two capsules daily) did not influence blood lipid profiles in thirty-eight men after a 4-week treatment.¹⁶ Simons *et al.*¹⁷ and Lewis and Burmeister¹⁸ also refuted effect of *L. fermentum*, (2×10⁹ CFU per capsule; four capsules daily) and *L. acidophilus* on human lipid profiles, respectively.

Several possible mechanisms for hypocholesterolemic effects of probiotics are: incorporation of cholesterol into the cellular membranes by growing cells and deconjugation of bile via bile salt hydrolase. Once deconjugated, bile acids are less soluble and absorbed by the intestines, leading to increasing their rates of excretion in the feces. Cholesterol is used to synthesize new bile acids in a homeostatic response, resulting in lowering of serum cholesterol.¹⁹⁻²¹

Ooi and Liang²⁰ in a review article attributed these controversial findings to various factors such as different strains of probiotics, administration dosage, analytical accuracy of lipid analyses, duration of treatment period, clinical characteristic of subjects, inadequate sample sizes, and lack of suitable controls or placebo groups.²

Considering the mentioned reasons hypothesized by Ooi and Liang,²⁰ normolipidemic condition of used rat model in the current study may be a reason for failure of these probiotic bacteria in changing lipid profile.²⁰ In addition to all of the above, the feeding period of 30 days may not be sufficient to observe a significant change in lipid profile.

However, the strains of bacteria used as a cholesterol lowering agent must be bile tolerant, have the ability to deconjugate bile acids, and bind cholesterol. In addition, the ability of particular strain of probiotic bacteria to attach permanently to the gut wall and hence continuous supply might be necessary to exert the effects.^{21,22} The results of our previous study indicated that these bacteria are not able to colonize the intestine and are quickly eliminated in feces.⁹ As such, daily consumption of probiotic products is necessary for any long-term effect on metabolism.

Conclusions

Prebiotic inulin significantly reduced the serum total cholesterol, LDL-cholesterol and increased the HDL cholesterol of rats during the treatment period. But no influence of probiotic *B. coagulans* on lipid profile was observed. In order to justify the cholesterol-lowering effect of *B. coagulans*, *in vitro* studies proposed mechanisms of action are necessary. Thus, further investigations on the characteristic of these bacteria could be useful to gain insights into understanding the treatment of

probiotics in order to achieve the maximum beneficial effect.

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