ORIGINAL ARTICLE

Internal Medicine Section

The **Synergistic Hypocholesterolaemic** and **Immunomodulatory** effect of two probiotic strains *in vivo*

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ABSTRACT

Probiotics are known to show beneficial effects to the host. But the effect varies with the species and the strain of the probiotics. The present study was designed to study the functional relationship of two probiotic strains.

Lactobacillus delbruckii 405 and *Lactobacillus casei subsp. casei 17* were tested in vivo separately and in a 1:1 combination (10^9cells /ml) for their hypocholesterolaemic and immunomodulatory potential in swiss albino mice.

Two sets of experimental groups were made. In the experimental group 1, the effects of *Lactobacillus spp.* alone or in combination was assessed for hypocholesterolaemic activity and in the experimental group 2, the effects of the probiotic alone or in combination were observed on immune response.

The Development of Antibody Titers, the Delayed Type Hypersensitivity test, the Nitroblue Tetrazolium Reduction test, the Inducible Nitric Oxide Synthase test and the Bactericidal Activity test were the tests which were employed to assess the functions of different immunocytes.

The data of the tests were statistically analyzed by using one-way ANOVA, followed by Turkey's Multiple Range Test which was applied for post hoc analysis.
Results and Conclusion: It was concluded that the two *Lactobacilli* strains acted synergistically *in vivo*, as the effect was more profound in the lowering of cholesterol levels and in the augmentation of the immune system.

Key Word: Hypocholesterolaemic -Immunomodulatory-Lactobacillus delbruckii-Lactobacillus casei

INTRODUTION

Cholesterol is an important basic block for the body tissues. Elevated blood cholesterol is a well known major risk factor for coronary heart diseases which are a main cause of death in Canada, U.S and many other countries around the world [1].

With the emergence of a more health conscious society, probiotic food products have gained attention from consumers and producers as an alternative safe source of medicines or neutraceuticals [2].

In this respect, the ingestion of probiotic lactic acid bacteria might be a natural way to decrease the serum cholesterol in humans [3].

However, amongst the lowering of cholesterol levels, probiotics consumption is also associated with the modulation of the immune system [4].

The possibility of the probiotics showing an immunomodulatory and a metabolic effect is due to their property of adhering to the intestinal surface and the subsequent colonization of the human gastrointestinal tract [5].

Previous experiments and clinical studies have revealed that probiotics are immunostimulatory as well as hypercholesteraemic. Moreover, it was found that there was an indirect correlation between the immune system and cholesterol levels [6].

Recently, there has been much interest in lactic acid bacteria due to their 'generally recognized as safe and beneficial effects' (GRAS) in health, which include anticholesterol and anticarcinogenic properties and the stimulation of the immune system [7], [5], [8].

Moreover, the beneficial effects of the probiotic strains vary not only at the species level, but at the strain level too. Hence, the main objective of this work was to study the potential effect of two sp. i.e. *Lactobacillus casei subsp. casei* and *Lactobacillus delbrueckii* alone and in combination on the immune system and on the cholesterol levels in mice.

2. MATERIALS AND METHODS:

2.1. Strains of microorganisms:

Strains of *Lactobacillus delbrueckii 405* (Strain A) and *Lactobacillus casei subsp. Casei 17* (Strain B) were procured from the NDRI (National Dairy Research Institute), Karnal, Haryana. The cultures which were so obtained were given two revival cycles in the de Man–Rogosa–Sharpe broth (MRS broth) at 37 °C.

The bacterial cultures were grown and maintained for further use. Further, Sample C was prepared by the combination of Strain A *(Lactobacillus delbrueckii 405)* and Strain B (*Lactobacillus casei subsp. Casei 17*) in a 1:1 ratio.

2.2. Animals:

Swiss albino male mice (18-22g) which were maintained on a standard laboratory diet (Kisan Feeds Ltd., Mumbai, India) and water ad libitum were employed in the present study. The animals were divided into respective groups each of a maximum of six animals, were housed individually in the departmental animal house and were exposed to 12 hr cycles of light and darkness. Two experimental set ups were made.

2.3. Experiment 1: To study the cholesterol lowering activity of the probiotics

Cholesterolaemia was induced in mice by feeding them with 1 % cholesterol which was mixed in the normal diet. A total dose of 200mg/kg body wt was given over a period of 7 days.After seven days, the animals showing serum cholesterol levels above 120 mg/dl were selected for further experiments. The day on which the microbial diet was started, was considered as day 0.

The experimental animal design:
Group I: Untreated control i.e. mice which were fed the basal feed.
Group II: Positive control i.e. hypercholesterolaemic mice.
Group III: Drug control i.e. hypercholesterolaemic mice which were treated with Atorvastatin (100mg/kg, b.wt.)
Group IV: Hypercholesterolaemic mice which were dosed with Strain A
Group V: Hypercholesterolaemic mice which were dosed with Strain B
Group VI: Hypercholesterolaemic mice which were dosed with Sample C

2.3.1. Follow up of the experiment:
The animals received an oral dose of 100µL of either Strain A, Strain B or Sample C (10^9 cell/mouse) for 8 days consecutively. Blood samples were obtained from the retro-orbital plexus on the 4th and the 8th day of the oral dosing; the serum was separated by centrifugation at 3000 rpm for 15 minutes.

The blood cholesterol levels of the animals were checked by using a commercial diagnostic reagent kit which was manufactured by Span Diagnostics Ltd. India.

2.4 Experiment 2: To study the effect of probiotics on the immune response
2.4.1 Experimental animal design:
The animals were divided into six major groups:
Group I: Control group (not subjected to any treatment i.e. kept only on diet)
Group II: Antigen sensitized control (mice sensitized with Sheep Red Blood Cells (SRBC) and kept on normal diet)
Group III: Positive control (mice subjected to immune enhancer i.e. Levamisole (2.5 mg kg -1) with normal diet)
Group IV: Test group I (Strain A for 18 consecutive days at the rate of 10^9 cells / day/ mouse)
Group V: Test group II (Strain B for 18 consecutive days at the rate of 10^9 cells / day/ mouse)
Group VI: Test group III (Sample C for 18 consecutive days at the rate of 10^9 cells / day/ mouse)
2.4.2. Immunization:
Sheep blood was collected in Alsever's solution in the ratio 1:2 and was centrifuged at 400 × g for 10 min at 4 ° C. The erythrocyte pellet which was obtained was washed and suspended in PBS (0.1 M, pH 7.2) for further use [9]. All mice were antigenically challenged intraperitoneally with a single dose (100µl/ml of 1×10^7 cells/ml) of sheep red blood cells (SRBC).

2.5. Humoral Immune Response:
To assess the humoral immune response, blood was withdrawn from the retro-orbital plexus of all SRBC antigenically challenged animals on day 0 (pre-immunized), 8 and 13 (post immunization). The serum was separated and assayed by direct haemagglutination [10]. Titer was described as the highest dilution which was capable of visible agglutination. The results were expressed as the mean ± S.E.M. log titer of the individual animals.

2.6. Cell mediated immune response:
2.6.1. Delayed Type Hypersensitivity assay
Delayed Type Hypersensitivity response (DTH) was checked by the foot pad swelling method [11]. All SRBC primed groups were challenged intradermally on the 15th day with an SRBC suspension (1×10^7 /100µl saline) in the hind footpad. The control lateral paw was given an equal volume of saline.

The paw thickness was measured with a micro-caliper from the 24h interval up to 72hrs. The difference in the paw thickness as compared to the controls was taken as a measure of DTH and was expressed in mm. The results were expressed as the mean ± S.E.M. of the footpad thickness up to 72hrs.
2.6.2. Total lymphocyte isolation from the spleen:
The spleen was excised aseptically and the lymphocytes were isolated by teasing the tissue. The cells were centrifuged (400 × g for 10 min at 4 ° C) and lysed by using the ACK lyses solution (0.5M NH_4Cl, 10mM $KHCO_3$ and 0.1 mM disodium EDTA, pH 7.2). The lymphocytes which were obtained, were washed thrice in PBS, counted and adjusted to the desired concentration in RPMI for further use.

2.6.3. The Nitroblue Tetrazolium Reduction assay:
The NBT reduction test was evaluated by employing the method which has been described [12]. Briefly, the lymphocyte suspension was incubated with NBT and the formazon which was formed, was extracted in dioxan. The reduction in NBT was measured spectrophotometrically at 520 nm (Shimadzu, UV-1650 PC) against dioxan as the blank. The results were expressed as the mean ± S.E.M. of the percentage dye which was reduced to formazon.

2.6.4. The Inducible Nitric Oxide Synthase activity:
The inducible nitric oxide synthase activity in the lymphocyte suspension was evaluated by a previously described procedure [12] by using arginine. The colour which was developed (indicating the presence of citrulline) was measured spectrophotometrically at 540nm against RPMI and the Griess reagent as blank and the results were expressed as the mean ± S.E.M. of the percentage enzyme which was produced.

2.6.5 Bactericidal activity:
The bactericidal activity was determined [13]. Briefly, the lymphocyte suspension was incubated with the bacterial suspension (Escherichia coli) at 37° C for 60 min. The lymphocytes were lysed with sterile distilled water which was spread on the agar plate and were incubated at 37 ° C for 24 h. The bacterial suspension was spread in the control plate. The number of colony forming units (CFU) which were developed in the control and the test plates were counted and the results were expressed as the mean ± S.E.M. of the bactericidal activity.

2.7. Statistical Analysis:
All the results were expressed as mean ± S.E.M. The data of the tests were statistically analyzed by using one-way ANOVA, followed by Turkey's Multiple Range Test which was applied for post hoc analysis. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

3. RESULTS:

3.1. Experiment 1: The cholesterol lowering activity of probiotics
Both the strains of probiotics when given individually reduced the serum cholesterol levels. In Group V which was treated with Strain B, there was a 27.96 % decrease in the cholesterol levels as compared to the Group IV mice which were treated with Strain A. The probiotics in the combination (Group VI) reduced the total serum cholesterol levels [25.37 % on 8th day of dosing] [Table/Fig1], which was even higher than the effect which was shown by the standard drug atorvastatin [11.49 % (Group III)].

Groups	-7th Day	0 Day	4th Day	8th Day
Untreated control i.e. mice fed basal feed	89.00 ± 0.22	89.43 ± 0.07	89.21 ± 0.05	88.9 ± 0.11
*Positive control i.e. hypercholesteremic mice	85.99 ± 0.50	138.6 ± 0.23	139.2 ± 0.23	139.2 ± 0.06
*Drug treated Hypercholesterolemic	85.15 ± 0.13	137.4 ± 0.23	132.7 ± 0.23	121.6 ± 1.49

*Hypercholesteremic mice dosed with Strain A	88.80 ± 0.08	130.1 ± 0.67	108.2 ± 0.38	99.9 ± 0.76
*Hypercholesterolemic mice dosed with Strain B	88.67 ± 0.03	134.1 ± 0.68	101.8 ± 0.49	96.6 ± 1.14
*Hypercholesterolemic mice dosed with Sample C	86.19 ± 0.97	133.2 ± 0.64	103.1 ± 0.26	99.4 ± 0.10

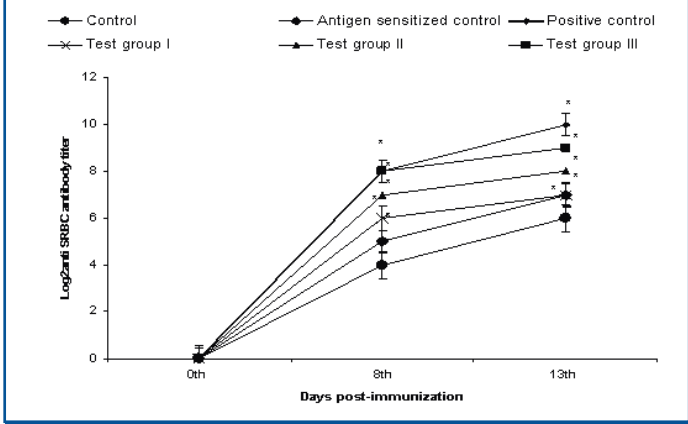
[Table/Fig 1]: Cholesterol lowering activity of probiotic

* Hypercholesterolemic groups fed on cholesterol diet from day -7 to 0.

3.2. Experiment 2: The effect of probiotics on the immune response

3.2.1. Humoral Immune Response:

Test group III (Sample C) had a significantly higher antibody titer as compared to the Test group I (Strain A) and the Test group II (Strain B), as depicted in [Table/Fig 2].The anti SRBC antibody titer of Test group III was double than that shown by the Test group II and it was four times higher than that shown by the Test group I on the13th day.



[Table/Fig 2]: Effect of different groups on production of anti-SRBC antibody titer on pre-immunization (0th day) and post-immunization (8th and 13th day). * p< 0.05 in comparison to sensitized control

3.2.2. Cell mediated immune response:

3.2.2.1 Delayed type hypersensitivity:

The effect of the combination on T-cell response was studied by using the delayed type hypersensitivity response. In the untreated control group, no rise in the footpad thickness was observed. However, a combination of the two strains (Test group III) showed a significant (p<0.05) elicitation of the T-cell response, as was evident by an increase in the foot pad thickness as compared to those in the antigen sensitized control group and in the individual strains treated groups IV and V. The maximum effect was observed in Sample C after 48 hours by using a combination of the two strains of probiotics. [Table/Fig 3]

Animal groups	Footpad thickness (mm)			
	Time period (h) after SRBC challenge			
	0	24	48	72
Control	1.67 ± 0.01	1.67± 0.01	1.67 ± 0.01	1.67 ±0.01
Antigen sensitized (untreated)	1.68 ± 0.01	1.71 ± 0.01*	1.70 ± 0.02*	1.67 ± 0.01
Positive control	1.69 ± 0.01	1.86 ± 0.02**	1.99 ± 0.02	1.81 ± 0.03
Test group I	1.69 ± 0.01	1.74 ± 0.01	1.72 ± 0.01	1.70 ± 0.02
Test group II	1.67 ± 0.01	1.72 ± 0.02	1.70 ± 0.02	1.68 ± 0.01
Test group III	1.68 ± 0.01	1.80 ± 0.03**	1.88 ± 0.02**	1.80 ± 0.03

[Table/Fig 3]: Delayed Type Hypersensitivity response

The results are represented as mean ± S.E.M (n=6)
* p<0.001 in comparison to untreated control.
** P < 0.05 in comparison to sensitized control

3.2.2.2. iNOS activity:

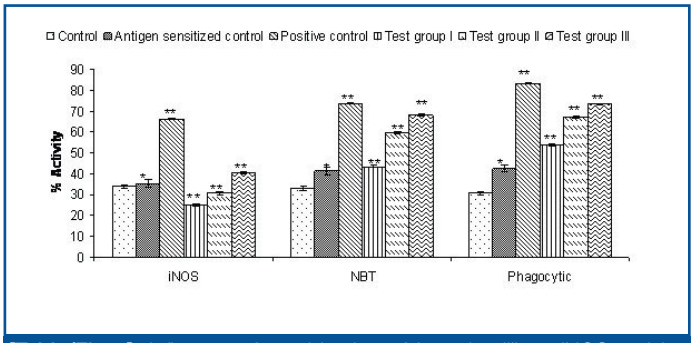
Fig.2 indicates the *Lactobacilli* modulated cell mediated immune response. In the Test group III (Sample C), where the animals received a mixture of the two strains, the iNOS activity was found to be 70 % higher than that in the Test group I (Strain A) and 33.41 % higher than that in the Test group II (Strain B).

3.2.2.3. NBT reduction:

Lactobacilli, in combination and alone, significantly increased the NBT reduction as compared to the controls [Table/Fig 4]. As with the iNOS activity, the NBT reduction activity of the combination (Test group III) was the highest among all the three groups. It was calculated to be 15.9 % higher than that in the Test group I and in the Test group II respectively.

3.2.2.4. Bactericidal activity:

The effect of the test materials on bactericidal activity was studied in terms of the number of colony forming units (CFU).The treatment of the animals with test group III (Sample C) reduced the number of colonies and thus enhanced the bactericidal activity as compared to Test group I and II, where the strains of *Lactobacilli* were given alone [Table/Fig 2].



[Table/Fig 4]: Influence of combination of Lactobacilli on iNOS activity, NBT reduction and bactericidal activity. The results are expressed as mean ± S.E.M (n=6).*p<0.001 in comparison to untreated control; ** p<0.05 in comparison to sensitized control

4. DISCUSSION:

In the present study, the effect of probiotics in the form of a combination was studied on cholesterol levels and on the immune response. The results revealed that the two probiotic strains in the combination reduced the total cholesterol levels (25% -29%) as compared to 11% only by the standard drug treatment. This shows that probiotics are more effective cholesterol reducers than the standard drug. But there was hardly any difference in the reduction of the cholesterol levels when the probiotics were given individually or in a combination. Many previous studies have already been carried out and they have shown that probiotics can reduce the cholesterol levels [14], [1], [7].

In vitro studies which were done in the past have evaluated a number of mechanisms which were proposed for the cholesterol lowering effects of probiotics and prebiotics. One of the proposed mechanisms includes the enzymatic deconjugation of the bile acids by the bile-salt hydrolase of the probiotics. Bile, a water-soluble end

product of cholesterol in the liver, is stored and concentrated in the gallbladder, and is released into the duodenum upon the ingestion of food [15]. It consists of cholesterol, phospholipids, conjugated bile acids, bile pigments and electrolytes. Once they are deconjugated, the bile acids are less soluble and are absorbed by the intestines, thus leading to their elimination in the faeces. Cholesterol is used to synthesize new bile acids in a homeostatic response, thus resulting in the lowering of the serum cholesterol levels [15].

Most of the *in vivo* trials which were conducted thus far have focused heavily on verifying the hypocholesterolaemic effects of the probiotics, rather than the mechanisms which were involved. However, lactic acid bacteria with active bile salt hydrolyse (BSH) or products containing them are suggested to lower the cholesterol levels through their interaction with the host bile salt metabolism. *Lactobacilli* with BSH activity have an advantage in surviving and colonizing the lower small intestine where the enterohepatic cycle takes place and therefore, BHS activity may be considered as an important colonization factor [16].

Levamisole is a well-known stimulant of B cells, T cells, monocytes and macrophages. Hence, a comparative study of Levamisole was used as a positive control while studying the effect of probiotics on the immune response. In the experiment, the animals were sensitized with SRBC, as the SRBC mediated immune response is a highly sensitive indicator of immunological integrity and requires a coordinated interaction of various immune system cells. Our study is a mirror image of this coordination between the various immune system cells [17].

The Anti SRBC antibody titer development, the Delayed Type Hypersensitivity test, the Nitroblue Tetrazolium Reduction test, the Inducible Nitric Oxide Synthase test and the Bactericidal activity test were the tests which were employed to assess the functions of different immunocytes. The maximum antibody titer was in the group receiving a combination of the two strains of *Lactobacillus* (Test group III) than those receiving a single strain (Test group I and Test group II). The Delayed Type Hypersensitivity test, the Nitroblue Tetrazolium Reduction test, the Inducible Nitric Oxide Synthase test and the Bactericidal activity test showed a similar pattern i.e. these activities were maximum in the Test group III as compared to the Test groups I and II.The NBT reduction test is an indirect marker of the oxygen dependent bactericidal activity of the phagocytes and the metabolic activity of the granulocytes or the monocytes [18], [19].

The present results indicate that a combination of the probiotics is capable of stimulating the immune function of macrophages, as evidenced by an increase in NBT reduction and in the bactericidal activity in all the treated groups. The functional ability of the macrophages was evident from the increased expression of iNOS that oxidizes L-arginine to citrulline and nitric oxide. The iNOS activity is correlated to the bactericidal activity of the macrophages and has been documented as a measure of the immunomodulatory potential [20].

5. CONCLUSION:

It was concluded that probiotics, in combination, can augment the effect of individual probiotic strains as immunomodulators as well as hypocholesterolaemic agents and in future, they need to be exploited in the formulations of neutraceuticals.

6. ACKNOWLEDGEMENT

The financial aid to the department in the form of a FIST grant for the purchase of instruments by DST, Govt. of India, is fully acknowledged.

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DECLARATION ON COMPETING INTERESTS: No competing Interests
Date of Submission: **Oct 22, 2010**
Peer Review Completion: **Dec 04, 2010**
Date of Acceptance: **Jan 11, 2011**
Date of Final Publication: **Apr 11, 2011**