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Effect of Feeding Normal and High Cholesterol Diet Incorporated with Encapsulated and Non Encapsulated *Bifidobacterium bifidum* 235 and Prebiotics on Serum HDL-Cholesterol and LDL Cholesterol of S.D. Rats

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A B S T R A C T

The effect of supplementation of encapsulated *Bifidobacterium bifidum 235* with prebiotic in milk fat rich high cholesterol diet on the serum HDL and LDL cholesterol, the samples were collected on every 15 days interval. The LDL-Cholesterol (44.01%) of rats feed on high cholesterol diet supplemented with encapsulated synbiotic were significantly lower than all other groups, however there was significant increase in HDL- Cholesterol (4.55%) in rats fed with high cholesterol diet supplemented with encapsulated symbiotic. The reduction in LDL-Cholesterol (29.84%) was observed in rats feed with high cholesterol diet supplemented with encapsulated *Bifidobacterium bifidum* 235 which may be due to encapsulation that protected organisms from adverse conditions of gastro intestinal tract. The relatively lesser in LDL-Cholesterol and Increase in HDL-Cholesterol (4.97%) in rats fed with high cholesterol diet with non encapsulated synbiotic may be due to feeding of non-encapsulated synbiotic. In rats fed with high cholesterol diet with non encapsulated *Bifidobacterium bifidum 235* there was a least reduction in the LDL-Cholesterol (5.63%) and increase in HDL-Cholesterol (0.379%) when compared with fed only on control diet.

Introduction

Hyperlipidemia is a widely known key risk factor for cardiovascular diseases. High blood cholesterol and triacylglycerol levels are commonly considered important modulators and biomarkers of hyperlipidemic processes (Tsai *et al.*, 2009). Therefore, the management of these two parameters is necessary for cardiovascular health. Probiotic bacteria are defined by the World Health Organization (WHO) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" and are being examined for their efficacy in lowering total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) in humans (Jones *et al.*, 2012). Intestinal lactic acid bacterial (LAB) species with alleged health beneficial properties have been introduced as probiotics.

LAB species are important members of the normal intestinal microflora and showed beneficial effects in study of the molecular biology and genomics of *Lactobacillus* in immune function, anti-cancer, and antibiotic-associated diarrhea, travelers' diarrhea, pediatric diarrhea, inflammatory bowel disease and irritable bowel syndrome (Ljungh *et al.,* 2009).

Lactobacillus spp. occurs in the gastrointestinal ecosystem of humans, poultry, swine, and other animals. They are excellent probiotic microorganisms because of their activities in ameliorating enteric diseases, maintaining health, and inhibiting melanin synthesis (Chen *et al.,* 2013; Noohi *et al.,* 2014). *Lactobacillus reuteri* produces a broad-spectrum antimicrobial substance during fermentation of glycerol, which revealed that glycerol fermentation was associated with the production of beta-hydroxypropionic acid and trimethylene glycol (Talarico *et al.,* 1988). *L. reuteri* is used as a probiotic for chronic constipation. (Ojetti *et al.,* 2014), inhibits *Helicobacter pylori* load in humans (Holz *et al.,* 2014), and removes cholesterol (Jones*et al.,* 2012).

Previous studies used the hamster model to evaluate the hypolipidemic effect because it has many similarities with human fat-induced atherosclerotic disease. As for humans, hamsters are endowed with cholesterol ester transfer protein and all of the enzymatic pathways in lipoproteins and bile metabolism; atherosclerotic plaques develop in response to a fat diet in lesion-prone areas similar to humans (Sima *et al.,* 1990; Sullivan *et al.,* 1993; Stancu *et al.,* 2014).

L. reuteri 263 is a patented strain for improving the syndrome of diabetes (US 20110300117 A1) and renal fibrosis in diabetes (US 20120183504 A1), which is different from other strains such as *L. reuteri* L3 for preventing obesity in obese mice (Qiao *et al.,* 2015) or the *L. reuteri* LR6-fermented product for controlling hyperlipidemia in rats (Gilliland and Walker 1990). In addition, species of the same bacterial strains or even strains of the same species may feature different biological functions (Qiao *et al.,* 2015). Qiao *et al.,* demonstrated that *L. reuteri* L3 but not *L. reuteri* L10 had anti-inflammation and anti-obesity properties for obese mice (Qiao *et al.,* 2015). Because of the complexity of host-bacterial cross-talk and the importance of investigating specific bacterial strains, we conducted experiments to evaluate the therapeutic effectiveness of *L. reuteri* 263 supplementation on the regulation of hyperlipidemia in a dyslipidemic hamster model. We also examined the biochemical parameters and liver tissues by histopathology.

Materials and Methods

Place of work

Department of Livestock Products Technology, College of Veterinary Science, Rajendranagar, Hyderabad-30.

Materials: Probiotic bacterial culture

The probiotic bacterial strain used in this study was pure freeze dried culture of *Bifidobacterium bifidum* 235 which was already characterized as probiotic in the laboratory of Department of Livestock Products Technology, College of Veterinary Science, Rajendranagar, Hyderabad.

Chemicals

Agar agar Type I, Tri ammonium citrate extra pure, Di potassium phosphate, Di potassium phosphate, Calcium chloride, D (+) Dextrose anhydrous, FOS (carbohydrate composition on % dry basis: 96.2% FOS and 3.8% of glucose, fructose, sucrose), *Lactobacillus* MRS agar,

Magnesium sulphate, Manganese sulphate, Polysorbate. MRS Agar was used for the enumeration of *Bifidobacterium bifidum 235.*

Equipments and instruments

Air Compressor, Refrigerated Centrifuge, Lyophiliser, pH meter, Electronic balance, Bacteriological Incubator, Laminar Flow, Peristaltic Pump, Magnetic stirrer with hot plate, Orbital Shaker Incubator, Vortex mixer Touch type, Kits for total cholesterol, from Transasia Bio-Medicals Ltd, Solan, India, Erba Mannheim semi-automatic serum analyser.

Methods: Culture activation and maintenance

B. bifidum 235strain was rehydrated in MRS broth and incubated for 24 h at 37^0C. Cells were then cultured in the same conditions for three successive transfers in MRS broth at 37^0C for 20-24 h. It was then properly activated and served as the inoculum. Then, it was cultured in MRS broth for production of freeze dried *B. bifidum* 235 using 5% inoculum respectively and incubated for 48 h at 37^0C and then the cells were harvested by centrifugation at 5000 rpm for 15 minutes at 4^0C and washed with 0.9% normal saline and lyophilised to get bacterial powder and stored at 4^0C.

Micro-encapsulation procedure

The micro-encapsulation of *B. bifidum* 235 using sodium alginate as coating material was carried out according to the method of Chen *et al.*, (2005), with some modification using micro-encapsulator. Solutions of sodium alginate (2%) containing approximately 10^6cfu/g of *B. bifidum* 235 with 0.1% by weight of commercial prebiotic FOS were atomized in 0.1 M calcium chloride, respectively. The atomization was achieved by forcing the sodium alginate solution through the micro-encapsulator device with the help of a peristaltic pump for 20 rpm and compressed air with 1MPa pressure. The solution of calcium chloride remained under constant magnetic stirring until the end of encapsulation. Alginate beads remained at rest for 30 minutes and were separated from the calcium chloride solution with sieves and washed with distilled water and dried at 40^0C for 48 h and alginate beads were stored at 4^0C.

Feed

Rat feed in the form of pellet (NIN standard feed) was procured by National Institute of Nutrition, Hyderabad, with the following formulation and specification: Composition of normal diet:

Wheat flour - 22.5%, Roasted Bengal gram flour - 60.0% Skim milk powder - 5.0 %, Casein - 4.0%, Refined sun flower oil - 4.0 %, Salt mixture - 4.0%, Vitamin mixture - 0.5%.

High fat diet composition: (NIN, Hyderabad)

Normal mice diet-750.0g, Dextrose monohydrate-75.0g, Sucrose-16.25g, Dextrin-16.25g, Ghee- 75.00g, Cholesterol - 12.50g, Sodium cholate - 5.0g, Cellulose - 12.50g, Mineral mix (AIN 93G) - 8.75g, Vitamin mix (AIN 93UX) - 2.5g, Choline chloride - 1.25g, Note: The total cholesterol content is 12.6 g/Kg of High fat diet.

Methods

Forty eight male *Sprague dawley* (S.D.) rats of uniform age and weight were procured from NIN, Hyderabad for the study. Feed and water was provided *ad libitum* throughout the experiment. Animals were housed in polypropylene cages in a well-ventilated animal house with 12h – 12h light – dark

cycles. Acclimatization period of 2 weeks was observed before the start of experiment. After an acclimatization period of 2 weeks, rats were randomly divided into 6 groups of 8 rats in each and serum samples were collected for total cholesterol estimation. Subsequently, group 1 was kept as normal control throughout the experimental period. Remaining 5 groups were kept on high cholesterol diet incorporated with encapsulated prebiotics and probiotics and non-encapsulated prebiotics and probiotics. The rats were provided with water for 24 h. Blood samples were collected and serum was separated for total cholesterol estimation. Experimental animal design: Six experimental diets were prepared as follow: Group 1: Negative control (high cholesterol diet) incorporated with ghee, Group 2: Positive control (normal diet), Group 3: Negative control supplemented with encapsulated *Bifidobacterium bifidum* 235 @ 10^6CFU/kg feed, Group: Negative control supplemented with non-encapsulated *Bifidobacterium bifidum* 235 @ 10^6CFU/kg feed. Group 5-Negative control diet supplemented with *Bifidobacterium bifidum* 235@10^6 CFU/kg feed and prebiotic @ 0.1% by weight, Group 6-Negative control supplemented with non-encapsulated *Bifidobacterium bifidum* 235 @ 10^6CFU/kg feed and prebiotic @ 0.1% by weight.

Blood collection

Blood collection was carried out at every 15 days interval for sero-biochemical analysis till the end of experiment (8 wks). Feed was withdrawn 12 h before the blood collection and blood was collected through retro-orbital plexus after ether anaesthesia into serum vacutainers and centrifuged at 3000 RPM for 15 min and serum was separated and stored at -20^{0}C till analysis. The sera samples were analyzed for the HDL cholesterol and LDL cholesterolon 1st, 15th, 30th, and 45thday.

Biochemical profile

Plasma was separated from the blood and used for HDL estimation.

Estimation of HDL – Cholesterol

The estimation of HDL- Cholesterol is done by using Phosphotungstic Acid method (Burstein *et al.*, 1970).

Mix well, incubate for 10 minutes at 37°C, or 12 minutes at 30°C. Read the absorbance of the standard and each at 505 nm or 505/670 nm for bichromatic analysers against reagent blank (Table 1).

Calculation

HDL Cholesterol = [(Absorbance of test / Absorbance of standard) X Concentration of Standard (md/dl) X Dilution factor]

Estimation of LDL- Cholesterol

LDL- Cholesterol is estimated by using Friedwald formula (1972),

LDL = TC – HDL- TG/5.0 (mg/dl)

TC - Total Cholesterol

HDL - High Density Lipoprotein

TG - Triglycerides

Results and Discussion

The mean HDL-cholesterol values are represented in Table 2. On the initial day the mean HDL-cholesterol values showed no significant difference. By the end of 45 days of feeding period there was a significant difference seen among the groups, the highest mean HDL-cholesterol was seen in the group II (73.12 mg/dl) rats and the lowest mean

HDL-cholesterol was seen in the group I (65.87 mg/dl) rats. There was an increase in the mean HDL-cholesterol of group III (67.25 mg/dl) rats in comparison with that on group IV (66.12mg/dl) rats. There was no significant difference seen in the mean HDL-cholesterol concentrations between group V (68.87 mg/dl) and group VI (69.15 mg/dl) rats. The effect on serum LDL-cholesterol is shown in the Table 3. On the initial day, there was no significant difference seen among all the groups.

The highest mean LDL cholesterol was seen in the group IV (25.38 mg/dl) rats and the lowest mean LDL cholesterol was seen in the group VI (23.11 mg/dl) rats. Among groups I (23.5mg/dl), II (23.45 mg/dl), III (25.05 mg/dl), and V (24.92 mg/dl) rats there was no significant difference. By the end of 45 days of feeding trail period the results showed significant difference, with the highest mean LDL- cholesterol was seen in the group I(35.92 mg/dl) rats which were fed on only high cholesterol diet, lowest mean LDL Cholesterol values were seen in the group II (19.50mg/dl) rats and which were fed on the normal diet. The mean LDL-cholesterol of group V (20.48 mg/dl) rats was reduced when compared with group VI (28.67 mg/dl), which were fed on high cholesterol diet along with encapsulated synbiotic and high cholesterol diet with non-encapsulated synbiotic respectively. The mean LDL-cholesterol of group III (25.18 mg/dl) rats was reduced compared to group IV (33.90 mg/dl) which were fed with high cholesterol diet along with encapsulated *Bifidobacterium bifidum 235* and high cholesterol diet with non-encapsulated *Bifidobacterium bifidum 235*. The effect of feeding high cholesterol diet supplemented with encapsulated pre and *Bifidobacterium bifidum 235,* is dealt here. The serum was analyzed for the following parameters, HDL-Cholesterol and LDL-Cholesterol. A reduction in serum LDL-cholesterol and increase in HDL-Cholesterol was observed, in group fed on the high cholesterol diet along with encapsulated synbiotic. The probable reason may be due to enhanced survivability of encapsulated probiotic and *Bifidobacterium bifidum 235* with prebiotic, withstanding exposure to the adverse conditions of gastro intestinal tract such as gastric acidity and bile reaction.

The cholesterol-lowering activity of lactic acid bacteria has not yet been worked out completely, probiotics may alter serum cholesterol by two possible mechanisms: (1) directly binding dietary cholesterol into the small intestine before cholesterol can be absorbed into the body (Gilliland and walker, 1990; Noh *et al.,* 1997; Pereira and Gibson, 2002) and (2) bile salt deconjugation by bile salt hydrolase to produce free bileacids (Gilliland and Speck, 1997; Park *et al.,* 2007; Liong and Shah, 2005). Free bile acids thus formed by the deconjugation of conjugated bile salts are less soluble and are lesslikely to be reabsorbed by the intestinal lumen compared to bile salts, and are lost from the human body through faeces (Center, 1993; Begley *et al.,* 2006). This could lead to continuous excretion of cholesterol and, subsequently, may result in the reduction of serum cholesterol (Reynier *et al.,* 1981).

The serum samples of rats at the end of at 6th week showed significant difference in the lipid profiles between the of normal and high cholesterol diets. The LDL-cholesterol (44.01%) of group V rats were significantly lower than all other groups, however there was significant in HDL-cholesterol (4.55%) was significantly higher in group V rats when compared with all the other groups. This may be due to feeding of high cholesterol diet with encapsulated synbiotic. Liong *et al.,* (2007), reported that feeding of synbiotic containing *L. acidophilus* ATCC 4962, fructooligosaccharides, inulin, and mannitol decreased plasma LDL-Cholesterol.

Table.1 Assay procedure

Pipette into tubes marked	Blank	Standard	Test
Working Reagent	1000µl	1000µl	1000µl
Distilled Water	50µl	--	--
HDL Standard	--	50µl	--
Test	--	--	50µl

Table.2 Effect of feeding normal and high cholesterol diet incorporated with encapsulated and non-encapsulated *Bifidobacterium bifidum* 235 and prebiotics on serum HDL Cholesterol of S.D. rats

GROUP	TREATMENTS	Serum HDL Cholesterol (g/dl)			
		1st Day	15th Day	30th Day	45th Day
GROUP I	High Cholesterol Diet(NC)	$34.62^{a}\pm1.0$	$42.75^{d}\pm0.8$	$52.37^{d}\pm1.3$	$65.87^{d}\pm1.1$
GROUP II	Normal diet	$33.75^{ab}\pm1.0$	$53.25^{a}\pm0.7$	$61.00^{a}\pm0.7$	$73.12^{a}\pm0.8$
GROUP III	NC+encapsulated *B.bifidum 235*	$33.75^{ab}\pm1.2$	$49.25^{b}\pm0.4$	$54.37^{c}\pm1.3$	$67.25^{c}\pm0.9$
GROUP IV	NC+Non encapsulated *B.bifidum 235*	$32.87^{b}\pm1.4$	$48.5^{bc}\pm0.9$	$57.12^{b}\pm0.6$	$66.12^{d}\pm0.9$
GROUP V	NC+encapsulated prebiotic +*B.bifidum 235*	$32.87^{b}\pm1.3$	$49.12^{b}\pm1.1$	$56.25^{b}\pm1.8$	$68.87^{b}\pm0.6$
GROUP VI	NC+Non encapsulated prebiotic +*B.bifidum 235*	$33.62^{ab}\pm1.9$	$47.87^{c}\pm0.3$	$54.37^{c}\pm1.0$	$69.15^{b}\pm0.9$

**abcd Means with different superscripts in the same column differ significantly, ($p<0.05$); means are obtained at every 15days interval

Table.3 Effect of feeding normal and high cholesterol diet incorporated with encapsulated and non-encapsulated *Bifidobacterium bifidum* 235 and prebiotics on serum LDL Cholesterol of S.D. rats

GROUP	TREATMENTS	Serum LDL Cholesterol (g/dl)			
		1st day	15th day	30th day	45th day
GROUP I	High Cholesterol Diet(NC)	$23.5^{ab}\pm1.6$	$33.32^{a}\pm1.1$	$38.40^{a}\pm1.7$	$35.92^{a}\pm1.0$
GROUP II	Normal diet	$23.45^{ab}\pm1.2$	$13.82^{d}\pm1.3$	$20.75^{e}\pm1.2$	$19.50^{e}\pm1.4$
GROUP III	NC+encapsulated *B.bifidum 235*	$25.05^{ab}\pm1.3$	$24.33^{c}\pm1.1$	$28.43^{b}\pm1.5$	$25.18^{d}\pm1.0$
GROUP IV	NC+Non encapsulated *B.bifidum 235*	$25.38^{a}\pm1.6$	$27.75^{b}\pm0.7$	$28.75^{b}\pm1.0$	$33.90^{b}\pm1.3$
GROUP V	NC+encapsulated prebiotic +*B.bifidum 235*	$24.92^{ab}\pm1.8$	$23.35^{c}\pm1.0$	$24.32^{d}\pm1.1$	$20.48^{e}\pm0.7$
GROUP VI	NC+Non encapsulated prebiotic +*B.bifidum 235*	$23.11^{b}\pm1.8$	$26.45^{b}\pm1.9$	$26.90^{c}\pm1.5$	$28.67^{c}\pm1.2$

**abcdef Means with different superscripts in the same column differ significantly, ($p<0.05$); means are obtained at every 15days interval

Schaafsm and Guarner, (1998) also observed that daily consumption of 375 ml synbiotic milk in humans (containing of 10^7-10^8 CFU/g of *Lactobacillus acidophilus* and 2.5% (g/100 g) of fructooligosaccharides) resulted in a significant decline in LDL-cholesterol and LDL/HDL ratio of 5.4% and 5.3% respectively.

A reduction in LDL-cholesterol (29.84%) and an increase in HDL-cholesterol (2.09%) were seen in the group III rats. This may be attributed due to feeding of high cholesterol diet with encapsulated *Bifidobacterium bifidum 235*.These results are in agreement with Kumar *et al.,* (2011), who observed that the plasma LDL-cholesterol values obtained from high cholesterol diet supplemented with micro encapsulated *L.plantarum* Lp91 were significantly lower than the hypercholesterolemic control group, and the HDL-cholesterol values were higher than the control group.

The reduction of LDL- cholesterol (20.85%) and increase in HDL-cholesterol (4.97%) in group VI rats was better when compared to group IV, may be due to feeding on high cholesterol diet with non-encapsulated synbiotic. Similar results were observed by with Chen *et al.,* (2010) who reported that feeding of soybean oligosaccharides to 50 wistar rats @ 450mg/kg BW /day for 45 days showed a reduction in LDL-cholesterol by 43.0%, and an increase in HDL-cholesterol by 81.9%. The reduction was higher probably due to addition of high concentration of prebiotic sugars. In group IV rats which were on high cholesterol diet with non-encapsulated *Bifidobacterium bifidum 235*, there was a reduction in the LDL-cholesterol (5.63%) and increase in HDL-Cholesterol (0.379%) when compared with group II fed with only high cholesterol diet. Similar results were observed by Kumar *et al.,* (2011) who states that *L. plantarum* strains could reduce plasma LDL-cholesterol and increase HDL-cholesterol in rats fed a diet high in cholesterol. Jeun *et al.,* (2010) also reported that administration of 10^9 CFU/ml of *L.plantarum* KCTC 3928 for 4 weeks in hypercholestermia induced rats resulted in reduction in LDL-Cholesterol by 42%, and increase in HDL-Cholesterol by 35%. Abd El-Gawada *et al.,* (2005), also reported that the feeding of *Bifidobacterium longum* Bb-46 in 48 male albino hypercholesterolemic rats @ of 0.07% (w/v) for 35 days showed a reduction of LDL-cholesterol (56.3%). Xiao *et al.,* (2003) also reported a study in 32 human subjects fed with *B.longum* BL1 @ 10^8 CFU/g for 4 weeks showed reduction in serum LDL-Cholesterol and increase in HDL-cholesterol.

Probiotics are claimed to have beneficial effects on health. However, only few well-performed studies have looked at clearly defined health effects such as serum cholesterol concentrations. Hyper cholesterolemia is strongly associated with coronary heart disease and arteriosclerosis and decreasing serum cholesterol is an important treatment option. From this study it may be concluded that feeding of encapsulated *Bifidobacterium bifidum 235* and prebiotic has shown increase in the serum HDL Cholesterol and decrease in LDL Cholesterol probably encapsulation with prebiotic has enhanced survivability of *Bifidobacterium bifidum 235* with prebiotic which helped in enhancing survival during exposure to the adverse conditions of gastro intestinal tract such as gastric acidity and bile reaction.

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