Available Online at

http://www.ijcpa.in

IJCPA, 2015; 2(3): 142-146

International Journal of CHEMICAL AND PHARMACEUTICAL ANALYSIS

eISSN: 2348-0726 ; pISSN : 2395-2466

Research Article

PROBIOTIC BACTERIAL DNA AS AUGMENTER OF HYPOCHOLESTEREMIC ACTIVITY - AN EXPERIMENTAL STUDY

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Received: 30 July 2014 / Revised: 15 April 2015 / Accepted: 17 April 2015 / Online publication: 1 July 2015

ABSTRACT

Elevated blood cholesterol is a well known major risk factor for coronary heart diseases. In this respect, the ingestion of probiotic lactic acid bacteria might be a natural way to decrease the serum cholesterol in humans. Recent studies show that bacterial DNA containing immunostimulatory "CpG motifs" interact with Toll-like receptor 9 to initiate an immunostimulatory cascade that culminates in the maturation, differentiation and/or proliferation of multiple cell types leading to beneficial health effects. The present study was designed to compare the hypocholesteremic effect of probiotic bacteria and its isolated DNA's in swiss albino mice. In the experimental design, animals were divided into VII groups such as Untreated control, Positive control, Drug control (Atorvastatin ,100mg/kg, b.wt.),LB 405 (10^9cells day^{-1} $mouse^{-1}$ as oral dose) ,DNA LB 405 (75µg mL^{-1} $mouse^{-1}$), LB 405 + Atorvastatin, DNA LB 405 + Atorvastatin .The analysis of the results revealed that the DNA of probiotic strain alone or in the combination with standard drug reduced the total cholesterol levels 1.3 and 1.9 times more than probiotic alone or in combination with standard drug. It is concluded that DNA of probiotic is better and safer alternative therapeutic agent than the probiotics alone especially in those subjects where probiotics cannot be given.

Keywords: *Lactobacillus delbrueckii* – CpG DNA- Hypocholesteremic- Atorvastatin

1. INTRODUCTION

Coronary heart disease (CHD) is currently a leading cause of death worldwide. Although there are multiple risk factors for CHD, hypercholesterolemia remains a major determining factor for this pathology. Plenty of findings suggest that probiotics could remove cholesterol via various mechanisms and may be promising candidates for use as a dietary adjunct to serum cholesterol *in vivo* [1,2,3,4].

Recently, some bacterial cell components such as peptidoglycans, lipoteichoic acid, secrete soluble substances [5,6] and genomic DNA reportedly play role in immunomodulation responses but primary component is yet to be identified [7].

The immunostimulatory effect by bacterial DNA were defined to be dependent upon short sequences of CpG dinucleotides which differ from that found in eukaryotic [8, 9]. Only in bacterial DNA, unmethylated CpG motifs can be found [10]. In eukaryotic DNA, CpG-containing sequences occur at a much lower frequency than in bacterial DNA [11, 12] and they appear to be underrepresented in eukaryotic genomes; a phenomenon known as "CpG suppression" and when it is present, the

cytosine is methylated [13], which prevents their immune stimulatory effects [14, 8].

It has been evidenced that Bacterial DNA and immunostimulatory CpG-ODNs activate Antigen Presenting Cells (APCs) such as macrophages and dendritic cells. Cell activation occurs upon DNA endosomal uptake, resulting within minutes in activation of the Stress Kinase pathway and NF-kB. As a consequence, APCs produce cytokines including IL-12, IL-6 and IL-1 and upregulate coreceptor molecules [15].

The purpose of current study was to compare *in vivo*, anti cholesterolemic activity of probiotic viable bacteria's with their isolated genomic DNA.

2. MATERIALS AND METHODS

2.1 Bacterial strain and culture condition

The strain of *Lactobacillus delbrueckii* 405 (LB 405), *Lactobacillus brevis 403* (LB 403), *Bifidobacterium bifidium* BD4 234 (Bif 234) was procured from National Dairy Research Institute, Karnal, Haryana. The cultures so obtained were given two revival cycles in de Man–Rogosa–Sharpe broth (MRS broth) at 37 °C. Bacterial cultures were grown and maintained for further use. For genomic DNA preparation, cells were grown in the corresponding medium containing 1 to 1.5 % glycine to facilitate cell lysis[16].

2.2 Preparation of genomic DNA of bacterial strain

Genomic DNA was isolated and purified with several modifications [16].Briefly, an overnight culture (1.5 ml) was pelleted at 14000 rev min^{-1} (microcentrifuge) 25°C for 5 minutes and resuspended in 500µL EDTA (50mM^{-1}). 100 µL of 30$mgml^{-1}$ Lyosozyme was added to cell suspension and incubated for 60 minutes at 37°C. Cell lysis was achieved using NaOH/SDS solution (pH 12.5) and incubation 20 min at 37°C followed by 10 min incubation on ice. Protein removal was carried out with phenol followed by chloroform: isoamyl alcohol (24:1) extraction. DNA was precipitated by addition of isopropanol and washed with 70% ethanol to remove residual contamination. DNA was then resuspended in 20-30 µL of TE (Tris 10mM, EDTA 1mM pH 8.0).The concentration and purity of DNA were analyzed spectrophotometrically (Shimadzu, UV-1650 PC spectrometer) by measuring OD_{260}/OD_{280}.Only the DNA with OD_{260}/OD_{280} ratio ranging between 1.8 and 2.0 respectively was used. The quality of DNA was further analyzed on 1 % agarose gel (100V for 20-40 min) containing 0.5 μgm^{-1} ethidium bromide. The endotoxin level in the DNA preparation were <0.001 $ng\mu g^{-1}$ of DNA according to Limulius amebocyte lysate assay.

2.3. Mice

Swiss albino male mice (18-22gm) maintained on 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 minimum six animals, housed individually in the departmental animal house and were exposed to 12 hr cycle of light and dark. The experimental protocol was approved by Institutional Animal Ethical Committee (Registration No: 107/99/CP-CSEA-2010-40) were carried out as per the guidelines of committee for Purpose of Control and Supervision of Experimental on Animals (CPCSEA) Ministry of Environment and Forest, Government of India.

2.4 Induction of experimental Cholesteremia

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

2.5 Experimental animal design

Group I: **Untreated control** i.e. mice fed basal feed.

Group II: **Positive control** i.e. hypercholesteremic mice.

Group III: **Drug control** i.e. hypercholesteremic mice treated with Atorvastatin (10 mg/kg, b.wt.)

Group IV: **LB 405** i.e. Hypercholesteremic mice dosed with LB 405 (10^9cells day^{-1} $mouse^{-1}$) as oral dose

Group V: **DNA LB 405** i.e. Hypercholesteremic mice dosed with DNA of LB 405 in left tibialis anterior muscle [17] after 6 days at the rate of 75µg mL^{-1} $mouse^{-1}$.

Group VI: **LB 405 + Drug** i.e. Hypercholesteremic mice dosed with Atorvastatin (100mg/kg, b.wt) and LB 405 (10^9cells day^{-1} $mouse^{-1}$).

Group VII: **DNA LB 405 + Drug** i.e. Hypercholesteremic mice dosed with Atorvastatin (100mg/kg,b.wt) and DNA of LB 405 (75µg mL^{-1} $mouse^{1}$).

Animals received respective doses consecutively for 15 days. Blood samples were obtained from retro-orbital plexus on 16^{th} day. Serum was separated by centrifugation at 3000 rpm for 15 minutes.

2.6 Estimation of total serum cholesterol

Blood cholesterol level of animals was checked by using commercial diagnostic reagent kit manufactured by Span Diagnostic Ltd. India [18].

The concentration of cholesterol in mg/dl of the test samples was calculated as:

$$\% \text{ Cholesterol (mg/dl)} = \frac{\text{O.D. of Test (T)} \times 200}{\text{O.D. of Standard (S)}}$$

3. RESULTS AND DISCUSSION

3.1 Anti-Cholesteremic activity of DNA LB 405

The results (Table 1 & Fig. 1) revealed that treatment of hypercholesteremic animals with probiotic bacteria LB405, DNA and their combinations with standard drug i.e. Atorvastatin (LB 405 + Drug and DNA LB 405 + Drug) significantly ($p<0.05$) decreased the cholesterol level. Excess of fatty acids promote their conversion into cholesterol and triglycerides with concomitant increase in low density lipoprotein cholesterol [19] .The role of probiotics in reducing the cholesterol level is well known [20,21,22,23,24,25,26] .Moreover, the relationship between immune response and diabetes, cholesteremia and diabetes lead us to study the effect of DNA of probiotics on hypercholesteremic mice. But no study is available showing the effect of bacterial DNA or probiotic DNA on cholesterol level.

LB 405 alone (Group IV) and DNA LB 405 (Group V) resulted in 26.11 % and 32.49 % decrease in cholesterol level respectively. Treatment with DNA LB 405 alone and standard drug resulted in 32.49 % and 11.29 % decrease in cholesterol level respectively as compared to untreated control. Whereas, when given in combination with drug Atorvastatin, the DNA LB 405 (Group VII) showed 50.43 % decrease in cholesterol in hypercholesteremic animals. The present study revealed that the DNA of probiotic strain alone or in the combination with standard drug reduced the total cholesterol levels 1.3 and 1.9 times more than LB 405 alone or in combination with standard drug. Similar to present study, the removal of cholesterol by 12 *lactobacillus* strains *in vitro* was investigated [27, 28]. The *lactobacillus* strains also showed differences in their ability to remove cholesterol from the growth medium (26.74-85.41%). Significant ($P<0.05$) correlations were observed between cholesterol removal and deconjugation of sodium taurocholate. *In vivo* studies, proved the cholesterol lowering activity of probiotics using single and multiple strains revealed that combination of probiotics reduced the total cholesterol levels 25-29% as compared to the 11% only by the standard drug treatment [29,30,31].

The analysis of the results revealed that the DNA of probiotic strain alone or in the combination with standard drug reduced the total cholesterol levels 1.3 and 1.9 times more than LB 405 alone (Group IV) or in combination with standard drug (Group VI) i.e.

DNA LB 405 + Drug (Group VII) > DNA LB 405 (Group V) > LB 405 + Drug (Group VI) > LB 405 (Group IV) > Drug control (Group III).

The CpG oligonucleotide is an immunostimulatory sequence present primarily in bacteria [32, 33, 34] and the results of present study using probiotic bacterial DNA might explain one of the mechanisms by which bacterial infections can inhibit the development of cholesterol in mice.

Table 1 Effect of LB 405, DNA LB 405 and their combinations with standard drug on serum cholesterol level.

Groups	Days			% Reduction from 0 day
	-7th	0th	16th	
Untreated control	89.00 ± 0.22	89.43 ± 0.07	88.9 ± 0.11	
Positive control	85.99 ± 0.05	135.6 ± 0.23	159.2 ±0.06 [a]	
Drug control	86.80 ± 0.08	130.1 ± 0.67	116.9 ± 0.76 [a]	11.29 %
LB 405	87.12 ± 3.99	138.6 ± 0.81	109.9 ±2.91 [a,b]	26.11%
DNA LB 405	86.91 ± 1.00	137.01 ± 1.97	103.41 ± 1.34 [a,b]	32.49 %
LB 405 + Drug	86.14 ± 2.34	136.56 ± 1.29	100.50 ± 2.91 [a,b]	35.88%
DNA LB 405 + Drug	89.15 ± 0.12	137.8 ± 0.25	91.6 ± 1.99 [a,b]	50.33 %

The results are presented as Mean ± S.E.M (n=6) [a] $p<0.001$ as compared with untreated control. [b] $p<0.05$ as compared to Drug control group.

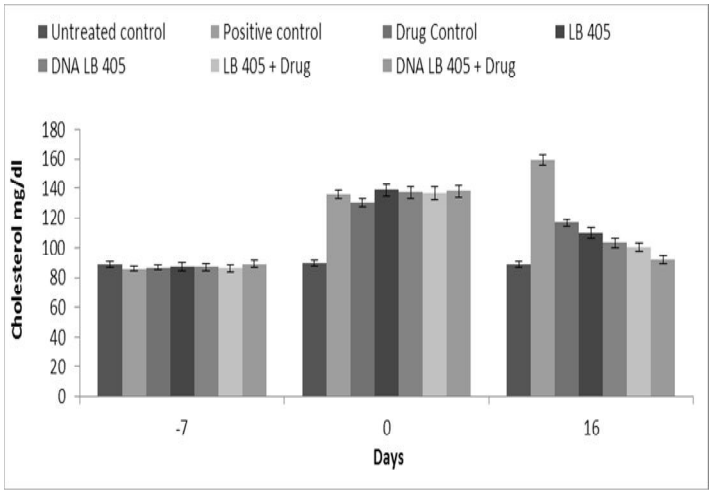

Fig. 1: Influence of LB 405, DNA LB 405 and their combination with standard drug on the cholesteremic mice. The results are presented as Mean ± SEM (n = 6).

4. CONCLUSION

It is concluded that genomic DNA of probiotics should be exploited as a potent anticholesterolemic biotherapeutic agent.

5. ACKNOWLEDGEMENT

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

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