Original Research Article

Screening of a *Lactobacillus* species (LAB M8) as probiotic: *In vivo* and *In vitro* study

Sucheta Das[1], Kausik Maji[2*], Narayan Chandra Mandal[3], Sukanta Sen[4]

[1]Associate Professor, Department of Biotechnology, Haldia Institute of Technology, P.O HIT, HIT College Rd, Kshudiram Nagar, Haldia, West Bengal, India
[2]Professor & Head, Department of General Medicine, ICARE Institute of Medical Sciences and Research, Banbishnupur, Purba Medinipur, Haldia, West Bengal, India
[3]Professor, Department of Botany, VisvaBharati, Santiniketan, West Bengal, India
[4]Professor & Head, Department of Pharmacology, ICARE Institute of Medical Sciences and Research, Banbishnupur, Purba Medinipur, Haldia, West Bengal, India

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Abstract

Background: Probiotics are defined as "living microorganisms, which upon ingestion in certain numbers exert health benefits on the host beyond inherent basic nutrition". Because of many potential health-promoting benefits, there continues to be considerable interest in the use of probiotics as biotherapeutic agents. **Materials & Methods:** For bacteria to exert any probiotic effect they have to be able to survive both in stomach acid (pH 1.5) and bile acids (pH 2.5).Growth of LAB M8 in presence of different concentration of bile salt was observed. Bile Salt Hydrolase (BSH) activity of LAB M8 was checked. Test for cholesterol uptake by LAB M8 and antibiotic resistance was noticed. **Results:** LAB M8 was both acid tolerant and bile tolerant. In anaerobic condition LAB M8 can grow efficiently like aerobic condition. The bile salt deconjugation ability of LAB M8 was confirmed by observing the presence of zone of inhibition on plate assay. **Conclusion:** In conclusion, the probiotic strains isolated and characterized in this study have great potential as possible therapy for reducing cholesterol levels. The cholesterol-lowering effects of LAB M8 presented may be partially ascribed to BSH activity in vitro.

Keywords: Probiotics, lactic acid bacteria, *Lactobacillus sp.*, screening, bile-salt hydrolase, antibiotic resistance

Introduction

The use of foods derived from microbial activity goes back to the dawn of human civilization and fermented milks were probably the first foods to contain active micro-organisms. In 1965, the term 'probiotics' was first used by Lilly and Stillwell which represent 'substances secreted by one organism which stimulate the growth of another' [1,2].After nine years, Parker (1974) described probiotics as "organisms and substances which contribute to intestinal microbial balance"[3].Fuller (1992) proposed that probiotics were 'live microbial supplements which beneficially affects the host animal by improving its microbial balance'[4].The United Nations Food and Agriculture Organization and the World Health Organization (FAO/ WHO) in 2001, a consensus definition of probiotics was adopted as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[5].Upon ingestion the probiotic strain should show some beneficial effect on the host such as prevention of colonization of harmful microorganisms in intestine, alleviation of lactose intolerance, relief of constipation, antitumor or anti-carcinogenic effect, and improvement of growth rate and feed utilization of animals, improvement of balance of the intestinal microflora, maintaining a chronic and immunological balanced inflammatory response, maturation of immune system, and anti-cholesterolamic effect etc.

**Correspondence*
Dr. Kausik Maji

Professor & Head, Department of General Medicine, ICARE Institute of Medical Sciences and Research, Banbishnupur, Purba Medinipur, Haldia, West Bengal 721645, India
E-mail: dr.kausikmaji1970@gmail.com

Cellular cholesterol homeostasis is very important for prevention of cardiovascular disease. Therefore decreasing serum cholesterol is very important to prevent the disease. HDL (High density lipoprotein)-cholesterol has been known to prevent arteriosclerosis by removing cholesterol from blood stream whereas LDL (Low density lipoprotein)-cholesterol fastens it by accumulating cholesterol in the blood vessel (Lee, 1997)[6].Several studies have indicated that consumption of certain cultured dairy products resulted in reduction of serum cholesterol. Mann and Spoerry (1974) found that serum cholesterol levels in men from a tribe of African Maasai warriors decreased after consumption of large amounts of milk fermented with a wild Lactobacillus strain[7].Larger amounts of free bile acids are excreted in feces as deconjugated bile salts are less soluble and less efficiently reabsorbed from the intestinal lumen than their conjugated counterparts. (Rodas, 1996)[8]Also, free bile salts are less efficient in the solubilization and absorption of lipids in the gut. Therefore, the deconjugation of bile acids by LAB bacteria could lead towards a reduction in serum cholesterol either by increasing the demand of cholesterol for de novo synthesis of bile acids which has lost through feces or by reducing cholesterol solubility and, thereby the absorption of cholesterol throughout the intestinal lumen. According to Gilliland *et al.* 1985[9], during the enterohepatic circulation the conjugated bile salts may be transformed by some intestinal bacteria through bile-salt hydrolase (BSH) enzyme and it split glycine or taurine from the steroid moiety, resulting in free (deconjugated) bile salts. BSH activity isobserved in some strains associated with the gastrointestinal tract (GIT), representing several species of *Lactobacillus*, *Enterococcus*, *Peptostreptococcus*, *Bifido bacterium*, *Clostridium*,and *Bacteroides*(Canzi *et al.*, 2000)[10] It has also been suggested that BSH activity should be a requirement in

the selection of probiotic organisms with cholesterol-lowering properties (Tahri *et al.*, 1997)[11]

Material &Methods

Acid Tolerance Study: For bacteria to exert any probiotic effect, they have to be able to survive both in stomach acid (pH 1.5)and bile acids (pH 2.5). So the survivability of the isolate LAB M8 was checked at pH 1.5 and pH 2.5 following the method of Liong and Shah (2005) [12]. Isolate was grown in MRS broth for 24 hrs at 37°C and centrifuged at 5000 rpm for 10min at 4°C. Pellets were washed three times in sterile saline (0.85% NaCl, pH 7). 1% of this solution was given as inoculums to MRS broth acidified with concentrated HCl to pH 1.5 and 2.5 and incubated at 37°C for 3 hrs. Non-acidified MRS broth was inoculated as same for control. OD values (600nm) and colony counts were taken at 0 hour,1.5 hours and 3 hours after incubation at 37°C.Strain that showed little or no reduction in cfu/ml, considered as acid tolerant.

Bile Salt Tolerance Study: This experiment was performed by following the method described by Walker and Gilliland, 1993[13]. MRS-Thiobroth, supplemented with 0.2% sodium thioglycolate and 0.3% oxgall, was inoculated with 1% overnight culture of LAB M8 and then incubated for 3hrs at 37°C. Increases in absorbance at 620nm during this incubation period were used to compare growth of the culture. The effect was compared with growth in MRS-Thio broth without oxgall.

Growth of LAB M8 in Anaerobic Condition: Growth of LAB M8 in anaerobic condition was checked by adding 0.5% sodium thioglycolate in the MRS broth (Roth and Lively, 1955) [14].The anaerobic condition created by this chemical is comparable with an anaerobic chamber.

Growth of LAB M8 in Presence of Different Concentration of Bile Salt: Overnight culture of LAB M8 was inoculated (1%) in to MRS broth containing 0.3%, 0.4%, 0.5%, 2% and 3% of oxgall (dehydrated fresh bile) and incubated for 12hrs at 37°C. Culture was monitored hourly for growth spectrophotometrically by taking optical density at 650 nm[15]

Bile Salt Hydrolase (BSH) Activity of LAB M8

Qualitative bile salt hydrolase(BSH) activity of LAB M8 was evaluated using the procedure described by du Toit *et al*., 1998[16]. Sterile filter disks were impregnated in an overnight culture of LAB M8 and placed on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid and 0.37 gm of CaCl2/L. The plates were then incubated anaerobically at 37°C for 72hrs, after which the diameters of the precipitation zones were measured. MRS agar plates without supplementation were used as controls. The strain was tested in duplicate.

Test for Cholesterol Uptake by LAB M8: Cholesterol removal was studied according to a modified method of Gilliland *et al.*, 1985)[13]. LAB M8 was inoculated (1%) into 10 ml of MRS broth supplemented with 0.3% sodium thioglycollate, 0.3% oxgall and 1ml of cholesterol solution which was prepared in 60% ethanol (10mg/ ml). After incubation at 37°C for 24hrs, 1ml of culture was taken in a tube and centrifuged at 5000rpm for 10min. Rest of the culture was further incubated for 48hrs at 37°C and similarly pellet down by centrifugation. A modified colorimetric method as described by Rudel and Morris (1973)[17]was used to determine the amount of cholesterol in the uninoculated and spent broth. In this method 0.1ml supernatant of both 24 hrs and 48 hrs sample was mixed with 0.3ml of 33% KOH and 3ml of 95.5% ethanol, mixed thoroughly, and incubated in a 60°C water bath for 15 min. After the mixture was cooled to room temperature, 5ml of hexane was added to it. 3ml of distilled water was added to it and vortexed for 1min to ensure complete mixing. The mixture was allowed to stand for 15 min at room temperature. 1ml of hexane layer was pipette out into two tubes, and the solvent was evaporated under controlled condition. Then 2 ml of freshly prepared 0.05% (w/v) o- phthalaldehyde reagent was added to each tube and mixed properly. After 10 min, 1ml of concentrated H_2SO_4 was added and immediately mixed thoroughly.Absorbance at 550nm was measured with spectrophotometer. The control solution was assayed using the same procedure without LAB M8. Differences in the amount of cholesterol in the uninoculated control and in the spent broth samples were taken as amounts of cholesterol assimilated.

Antibiotic Resistance Study: The antibiotic resistance of the isolate LAB M8 was assessed by using antibiotic discs (Himedia Laboratories Pvt. Ltd.,India) on MRS agar plates. A 10^6cfu/ml of freshly grown LAB M8 was mixed with 5 ml of MRS soft agar (0.5% agar) and over layered on bottom layers of MRS agar. The antibiotic discs were placed on the surface of agar plates and the plates were kept at 4^0C for 1h for uniform diffusion, and then incubated at 37^0 C for 24h (Halami *et al.*, 1999)[18]

***In vivo* experiments**

Effect of *Lactobacillus LAB M8*on Cholesterol Content of Mice: A group of 10 Swiss albino mice (One month old) were fed *Lactobacillus* LAB M8 at a concentration 10^9 to 10^{10} per ml with distilled water. After feeding 30 days cholesterol content was measured from blood plasma. About 4ml of blood drawn aseptically from each mice, kept in sterile tubes in presence of an anticoagulant Triplex III. Then the samples were centrifuged and lipid profile of serum samples were performed by using a commercially available kit (SPAN) which follows Wybenga and Pileggi-one step method (1970) [19]. Concentration of total cholesterol, triglyceride, LDL and HDL were measured in both treated and control mice.

Results &Discussion

Acid Tolerance Study: Probiotic bacteria should be resistant to the enzymes of oral cavity such as lysozyme and should also have the ability to resist the digestion process in the stomach and intestinal tract. In stomach where pH is around 1.5, the foods stay for 90 min. After 3hrs incubation period LAB M8 showed slight increase in cell number both at pH 1.51 and pH 2.5 (Figure 1). So we can say that LAB M8 is acid tolerant[20]

Bile Salt Tolerance Study: According to Gilliland *et al*., (1985)[9], 0.3% bile tolerance is necessary for evaluation of bile-tolerant probiotic LAB.LAB M8 can survive the tested bile salt concentration(0.3%) in anaerobic condition(Figure 2).

This observation can be compared with some recent observations. Among 28 isolates tested for probiotics characters, only one isolate *Lactobacillus casei* could tolerate acid (2%) and bile salt (Hassanzadazar *et al*., 2012)[21]

Growth of LAB M8 in Anaerobic Condition: From Figure 3 it can be observed that in anaerobic condition LAB M8 can grow efficiently like aerobic condition.

Growth of LAB M8 in Presence of Different Concentration of Bile Salt: From Figure 4, we found that LAB M8 can grow in presence of very high concentration of bile acid (3%) and its growth pattern was more or less same in all the salt concentrations (0.3%, 0.4%, 0.5%, 2% and 3%) tested. This result is comparable to the observation by Ramirez- Chavarin *et al*., 2013[22]

Bile Salt Hydrolase (BSH) Activity of LAB M8

The LAB isolate Lactobacillus LAB M8 showed high BSH activity. White precipitation was found around the paper disk containing LAB M8 (Figure 5). The activity can be compared with the result shown by Lim et al., 2004[23]

Test for Cholesterol Uptake by *Lactobacillus* LAB M8 (*in vitro* condition): LAB M8 maximum cholesterol assimilation in presence of both sodium thioglycolate and oxbile. The strain can assimilate about 2.3 mg/ml (1.92 mg/ml in 24 hrs and 2.28 mg/ml in 48hrs) from medium. This amount is more than *Bifidobacterium longum* SPM1207 (0.82 mg/ml), *Lactobacillus acidophilus* (LH) CBT (0.23 mg/ml) and *Enterococcus faecium* SPM1206 (0.44 mg/ml) observed by Lee *et al*., 2009[24]

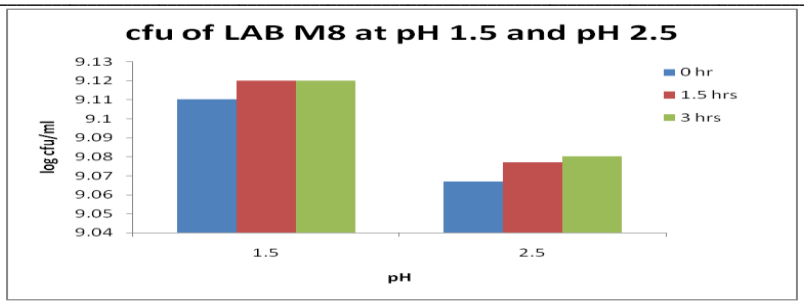

Fig 1: Growth of LAB M8 at pH 1.5 and at pH 2.5

Antibiotic Resistance

Resistance of the probiotic strains to some antibiotics (Figure 6) help in use for both preventive and therapeutic purposes in controlling intestinal infections. According to EI-Naggar, 2004, their resistances to antibiotics clarify their potential in minimizing the negative effects of antibiotic therapy on the host bacterial ecosystem[25]. LAB M8 showed resistance to different antibiotics like, vancomycin, Tobramycin, Norfloxacin, Methicillin etc (Table 1).

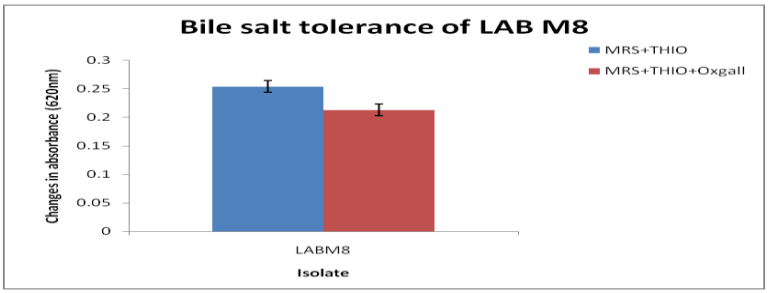

Fig 2: Bile salt tolerance (0.3%) of LAB M8 strain

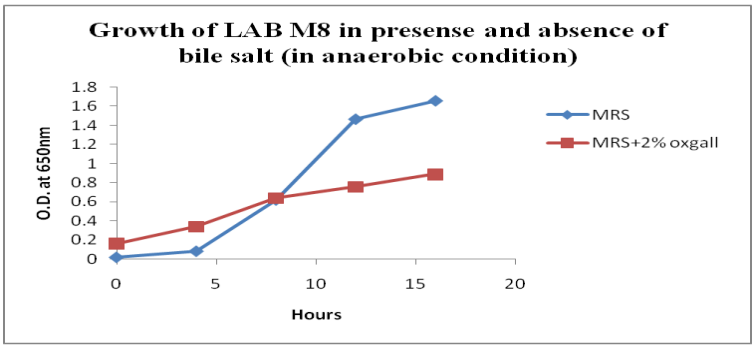

Fig 3: Growth of LAB M8 strain in anaerobic condition

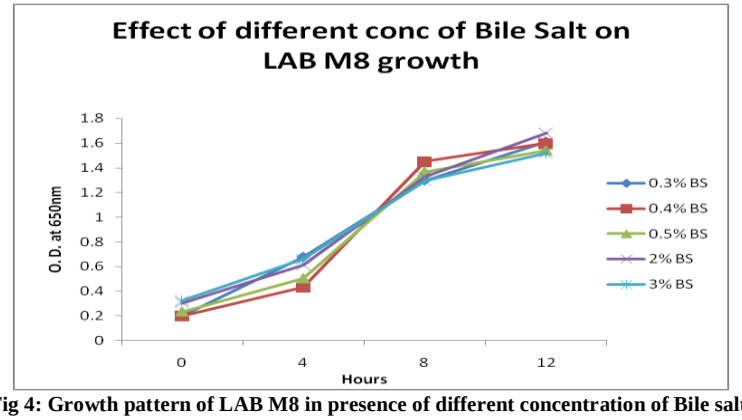

Fig 4: Growth pattern of LAB M8 in presence of different concentration of Bile salt

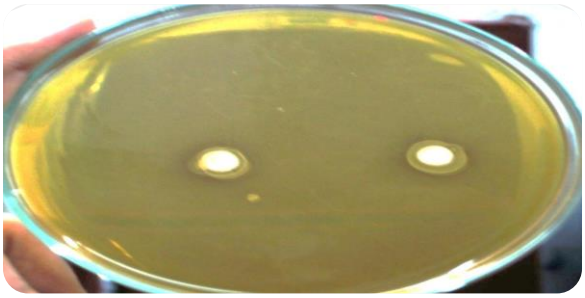

Fig 5: Detection of BSH activity. The white precipitates around paper disk containing LABM8 and the clearing of the medium are indicative of BSH activity

***In Vivo* experiments**

Effect of *Lactobacillus* LAB M8 on Cholesterol Content of Mice

From the results obtained (Figure 7) we found a little change in the concentration of LDL-cholesterol and no change in HDL- cholesterol compared to control mice. The concentration of total cholesterol and triglyceride lowers significantly in treated mice after one month feeding. Total cholesterol lowers 8.6%, whereas triglyceride 18.74% and LDL-cholesterol 8%. This result can be compared with the result of Xie *et al*., 2011[26]. They showed that feeding of two *Lactobacillus* species [2 mL (10^9 CFU/mL) daily of *L. plantarum* 9-41-A and *L. fermentum*M1-16 solutions respectively] lowers the blood cholesterol level significantly after six week of intra-gastrical application of the strains to male Sprague-Dawley rats[27].

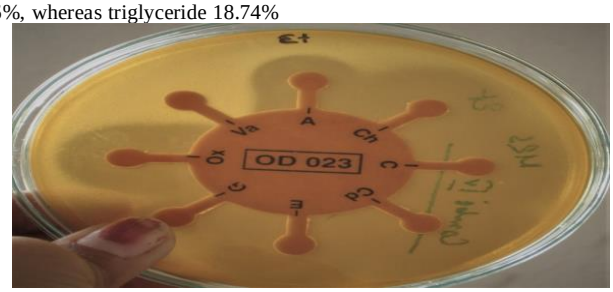

Fig 6: Antibiotic disc on MRS agar plate against LAB M8

Table 1: Antibiotic sensitivity profile of LAB M8

Antibiotics	Disc content (mcg)	Diameter of Inhibition Zone(mm) by LAB M8	Interpretation (done following HiMedia protocol)
Penicillin G (P)	10 units	9	Sensitive (S)
Tobramycin (Tb)	10	10	Resistant(R)
Cephaloridine (Cr)	30	16	S
Kanamycin(K)	30	11	S
Linomycin (L)	2	26.5	S
Methicillin (M)	5	9	R
Norfloxacin (Nx)	10	11	R
Oleandomycin (Ol)	15	22	S
Amoxycillin (Am)	10	0	S
Tetracyclin (T)	10	27	S
Penicillin (P)	2 units	25	S
Cloxacillin (Cx)	5	0	R
Erythromycin (E)	15	29	S
Co-Trimoxazole (Co)	25	0	R
Penicillin V(Pv)	3	22	S
Cephalexin (Cp)	30	18	R
Clindamycin (Cd)	2	27	S
Chloramphenicol (C)	30	21	S
Cephalothin (Ch)	30	19	S
Ampicillin (A)	10	14	Intermediate
Vancomycin (Va)	30	0	R
Oxacillin (Ox)	1	17.5	S
Gentamicin (G)	10	17	S
Streptomycin (S)	10	0	S

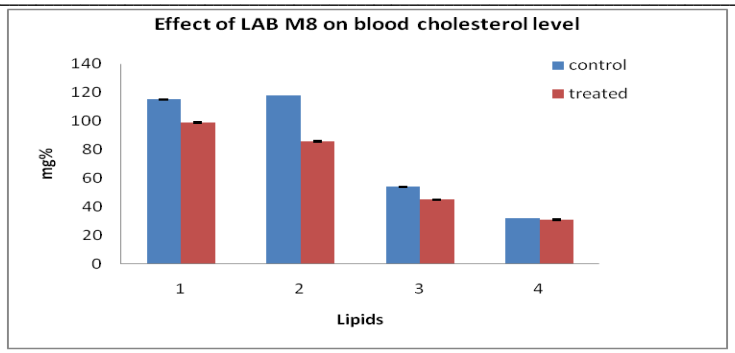

Fig 7:Effect of LAB M8 on blood cholesterol level of mice (1: Cholesterol, 2: Triglyceride, 3: LDL, 4: HDL)

Several mechanisms for cholesterol removal by probiotics have been proposed, such as deconjugation of bile salts by bile-salt hydrolase (BSH), assimilation of cholesterol into bacterial cell membranes, production of short-chain fatty acids (SCFAs) during the growth of probiotics, and cholesterol conversion into coprostanol[28]. Numerous clinical studies have concluded that BSH-active probiotic bacteria, or products containing them, are efficient in lowering total and low- density lipoprotein cholesterol. However, the mechanisms of action of BSH-active probiotic bacteria need to be further supported [28,29].Numerous clinical studies have concluded that BSH-active probiotic bacteria, or products containing them, are efficient in lowering total and low-density lipoprotein cholesterol. There is also the need for a meta-analysis to provide better information regarding the therapeutic use of BSH-active probiotic bacteria. The future of BSH-active probiotic bacteria most likely lies as a combination therapy with already existing treatment options[30].

Conclusion

Nearly all bifidobacteria species and strains have bile salt hydrolase activity, whereas this activity can only be found in selected species of lactobacilli. A strong correlation can be observed between the habitat of a genus or species and the presence of bile salt hydrolase activity. Most often bile salt hydrolase activity is found in strains that have been isolated from the intestines or from feces from mammals--an environment rich in conjugated and unconjugated bile acids. Lactobacilli with BSH activity have the ability to survive and colonize the lower small intestine where the enterohepatic cycle takes place. Therefore, BSH activity is considered an important colonization factor and an essential criterion for the selection of probiotic isolates with cholesterol-lowering properties. In conclusion, the probiotic strains isolated and characterized (*Lactobacillus* M8) in this study have great potential as possible therapy for reducing cholesterol levels. The cholesterol-lowering effects of LAB M8 presented may be partially ascribed to BSH activity *in vitro*.

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