Original Research Article

Screening of pediococcusacidilactici strain one member of Lactic acid bacteria as probiotic: In vivo and vitro study

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

[1]Assistant 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, PurbaMedinipur, Haldia, West Bengal, India

Received: 03-12-2020 / Revised: 28-12-2020 / Accepted: 27-012-2021

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 LAB001 in presence of different concentration of bile salt Bile was observed. Salt Hydrolase (BSH) activity of LAB001 and UV-Vis spectroscopic analysis of deconjugated free bile acids was done. Test for cholesterol uptake by LAB001 and antibiotic resistance was noticed. **Results:** LAB001, M146 and M8 were more acid tolerant and were more bile tolerant than others. In anaerobic condition LAB001 can grow efficiently like aerobic condition. The bile salt deconjugation ability of LAB001 representing the highest BSH enzyme activity was calculated as the release of cholic acid and was 2.7 mM. Pure cholic acid and extracted free bile acid from *P. acidilactici* LAB001 showed a characteristic similarity in their absorption maxima. **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 LAB001presented may be partially ascribed to BSH activity in vitro.

Keywords: Probiotics, lactic acid bacteria, *Pediococcusacidilactici*, 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, 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 anticholesterolamic effect etc. 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)[7] 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

**Correspondence*
Dr.Kausik Maji
Professor & Head, Department of General Medicine, ICARE Institute of Medical Sciences and Research, Banbishnupur, PurbaMedinipur, Haldia, West Bengal, India.
E-mail: kausikm25@gmail.com

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*, *Bifidobacterium*, *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 isolates was checked at pH 1.5 and pH 2.5 following the method of Liong and Shah (2005)[12]. Isolates were grown in MRS broth for 24hrs 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 3hours after incubation at 37°C.Strains 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 each isolate and incubated for 3hrs at 37°C. Increases in absorbance at 620nm during this incubation period were used to compare growth of the cultures. The effect was compared with growth in MRS-Thio broth without oxgall.

Growth of LAB001 in anaerobic condition

Among the isolates LAB001 showed good acid and bile tolerance. As this strain was identified up to species level it was selected for further probiotic experiments. Growth of this strain in anaerobic condition was checked by adding 0.5% sodium thioglycolate in the MRS broth (Roth and Lively, 1955)[14]Lowering of pH after 24hrs at 37°C was compared with control MRS broth without any supplement. The anaerobic condition created by this chemical is comparable with an anaerobic chamber.

Growth of LAB001 in presence of different concentration of bile salt

Overnight culture of LAB001 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.

Effect of bile salt on bacteriocin production

The effect of bile salts on bacteriocin production was tested by inoculating 1% of overnight culture of LAB001 in MRS and MRS-Thio medium containing 0.3% oxgall and incubated for 24hrs at 37°C.Cell free supernatant was prepared by centrifuging at 5000 rpm for 10 min. The CFS was then tested against indicator lawn by agar well diffusion method.

Determination of time requirement for maximum deconjugation

LAB001 was grown on MRS-Thio medium supplemented with 0.2% sodium taurocholate for overnight. 15ml of this culture was taken and pH was adjusted to 7 with 1 N NaOH. It was centrifuged for 10 min at 12000 rpm to remove the cells. The supernatant fraction was adjusted to pH 1 with 1 N HCl. The free bile acids were extracted with 50ml of ethyl acetate three times. The extract was evaporated to dryness. The residue was dissolved in 15ml of 0.01N NaOH. The concentration of free bile salt in the sample was determined colorimetrically by following the protocol of Irvin *et al[15]* 1944.6ml of 16 N H_2SO_4 and 1ml of 1% furfuraldehyde was mixed with the dissolved residue. The mixture was heated to 65°C for 15 min in water bath and then cooled to room temperature slowly. 5ml glacial acetic acid was added to it, mixed properly and the absorbance was taken at 660nm. Uninoculated MRS-Thio medium with sodium taurocholate was taken as control.

The results were compared with the standard curve of cholic acid. All experiments were carried out with three replicates and the results were expressed as µ moles of cholic acid per ml.

Bile Salt Hydrolase (BSH) activity of LAB001

Qualitative bile salt hydrolase (BSH) activity of LAB001 was evaluated using the procedure described by du Toit*et al.*, 1998[16]. Sterile filter disks were impregnated in an overnight culture of LAB001 and placed on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid and 0.37 gm of $CaCl_2$/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.

UV-Vis spectroscopic analysis of deconjugated free bile acids

Extracted free bile acids after 24 hrs incubation was dissolved in 95% ethanol and absorption spectrum was analyzed in the range of 200-400nm by UV-Vis spectrophotometer (UV 1700, Pharmaspec, Shimadzu, Japan). Standard free bile acid i.e., cholic acid was dissolved in the same solvent and its absorption spectrum was compared as control.

Test for cholesterol uptake by LAB001

Cholesterol removal was studied according to a modified method of Gilliland *et al.*, 1985). LAB001 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 10min, 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 LAB001. 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

The antibiotic resistance of the isolate LAB001 was assessed by using antibiotic discs (Himedia Laboratories Pvt. Ltd., India) on MRS agar plates. A 10^6 cfu/ml of freshly grown LAB001 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 37^0C for 24h (Halami*et al.*, 1999)[18]. Three octadiscs (G-IV-Plus, Combi IV, Combi VII) containing 25 antibiotics with different concentration were taken for the experiment (Figure 1).

***In vivo* experiments**

***In vivo* persistence of LAB001 in the GIT of mice**

For *in vivo* experiments one month old Swiss Albino mice (female) was taken as experimental model. Group of ten animals received a

daily dose of $10^9$to 10^{10} cfu of live *Pediococcusacidilactici* LAB001 orally with water for three consecutive days. One group was fed normally (distilled water) to test as control. Fecal samples from both inoculated and uninoculated mice were collected daily and homogenized in MRS medium (100mg of feces/ml). Serial dilutions were made up to 10^{-8} and plated on streptomycin and vancomycin containing MRS plates and plates were incubated and enumerated after 2 days. All the experiments were performed in triplicate.

Effect of *Pediococcusacidilactici*LAB001on growth of Swiss Albino mice: Growth of both (n=10) control mice and bacteria fed mice (fed overnight grown 10^9 to 10^{10} live LAB001 cells) were checked by taking body weight before feeding and 7,15 and 30 days after feeding. Food consumption of each group was monitored daily. After the feeding period, the mice were fasted for 12 hours and then sacrificed.

Effect of *Pediococcusacidilactici* LAB001 on cholesterol content of mice: A group of 10 Swiss albino mice (One month old) were fed *P. acidilactici* LAB001 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.

Toxicity test of liver

To check any toxic effect of LAB001 on mice liver toxicity test was performed by taking blood from liver. The most commonly performed blood tests, used to assess liver functions or liver injury is a simple blood test to determine the level of certain liver enzymes (proteins) in the blood. Under normal circumstances, these enzymes mostly reside within the cells of the liver. But when the liver is injured for any reason, these enzymes are spilled into the blood stream. Among the most sensitive and widely used liver enzymes are the aminotransferases. They include aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT). These enzymes are normally predominantly contained within liver cells and to a lesser degree in the muscle cells. If the liver is injured or damaged, they are released from liver and mixed into the blood, raising the AST and ALT enzyme blood levels and signaling liver disease or toxicity or damage.Serum Glutamic Pyruvate Transaminase (SGPT) and Serum Glutamate Oxaloacetate Transaminase (SGOT) were performed by using commercial kit of Span Diagnostic Ltd which follows Reitman and Frankel method (1975)[20] All the experiments were performed in triplicate and average was plotted.

Adherence of LAB001 to intestine of mice

Adherence of *Pediococcus acidilactici*LAB001 to the intestine of mice was checked by performing SEM study following standard protocol of Sarem-Damerdji*et al.* (1995)[21] with little modification. The jejunum portion of intestine of both LAB001 fed and non-fed control mice were cut aseptically, washed thoroughly with phosphate buffer saline (pH7). Then the samples were fixed with 2% glutaraldehyde in 20 mM Na-P buffer and 5% dimethyl sulfoxide (DMSO) mixture for 30 min. Washed with sterile water and they were dehydrated by passing through a series of alcohol grade from 30% to absolute alcohol, 10 min in every dilution. Then the samples were kept in iso-amyl acetate for 20 min. Then again in absolute alcohol and then coated with gold with ion sputter and observed under Scanning electron microscope (HITACHI-S530, Japan).

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. For this reason acid tolerance of the isolates were observed for 3 hrs. After 3hrs incubation period M146 and M8 showed slight increase in cell number, whereas the cell number was more or less same in LAB001 and rest strains (M147, M185 and M186) showed decrease in cell number (Figure 2). So we can say that LAB001, M146 and M8 are more acid tolerant than others.This result is comparable with the observation by Kabore*et al*., 2012, where a strain *Pediococcus acidilactici* L87 survived at pH 2.5, over a period of 4 hrs[22]

Bile salt tolerance study

According to Gilliland *et al*., (1985)[9], 0.3% bile tolerance is necessary for evaluation of bile-tolerant probiotic LAB. Though all the isolates survived the tested bile salt concentration (0.3%), the ability to grow in presence of bile salt varies among them (Figure 3). From the result it can be conclude that the strain LAB001, M186 and M8 were more bile tolerant than others. 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 (0.3%) (Hassanzadazar*et al*., 2012)[23]

Growth of LAB001 in anaerobic condition

In anaerobic condition LAB001 can grow efficiently like aerobic condition (Figure 4). The bacteria stay very less time in lag phase compared to aerobic growth. Less acid is produced during anaerobic condition. Ph lowers to 4.2 whereas in aerobic condition it is 3.4. This may be a reason for less bacteriocin production in anaerobic condition.

Growth of LAB001 in presence of different concentration of bile salt

From Figure 5, we found that LAB001 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%). This result is comparable to the observation by Ramirez-Chavarin*et al*., 2013[24]

Effect of bile salt on bacteriocin production

After growing 24hrs in presence of 0.3% oxgall, the CFS showed lowered activity against the indicator strain *Leuconostoc messenteroides* Ly. LAB001 produced 900AU/ml bacteriocin in presence of bile salt in comparison to 2000AU/ml in absence of bile salt. This result is supported by the observation Kheadr*et al.,* 2010[25] and Fernandez *et al*., 2013[26]

Determination of incubation time required for maximum deconjugation

Rapid deconjugation was found by LAB001. It started during 4 hrs of incubation (Figure 6) which corresponds to the logarithmic growth phase. Maximum release of cholic acid was found at 16 hrs (2.7 mM) *i.e*. at stationary phase. Then the concentration of released cholic acid lowers and reaches to 2.19 mM at 24 hrs. The result is consistent with the work reported earlier (Gopal *et al*., 1996)[27].The bile salt deconjugation ability of LAB001 representing the highest BSH enzyme activity was calculated as the release of cholic acid and was 2.7 mM. This amount can be compared with other LAB isolates showing BSH activity between the range of 2.03 ± 0.22 and 1.05 ± 0.25 mM (Pereira DI, *et al*., 2003)[28]

Bile Salt Hydrolase (BSH) activity of LAB001

The LAB isolate *P. acidilactici* LAB001 showed high BSH activity. It produced a large precipitation zone of about 22mm (Figure 7). White precipitation was also found around the paper disk containing LAB001. The activity obtained is greater than many probiotic bacterial strains including *Streptococcus* HJS-1 which produced a zone of 19 mm (Lim *et al*., 2004)[29]

UV-Vis spectroscopic analysis of deconjugated free bile acids

Pure cholic acid and extracted free bile acid from *P. acidilactici* LAB001 showed a characteristic similarity in their absorption maxima. The highest absorption maxima were around 202 nm to 204 nm (Figure 8). This indicates the deconjugation product is cholic acid.

Test for cholesterol uptake by LAB001

*P. acidilactici*LAB001 showed maximum cholesterol assimilation in presence of both sodium thioglycolate and oxbile (Figure 9). This observation is very similar to the observation by Tok and Aslim, 2010. Assimilation amount was not so much increased with incubation period. This observation is contradictory with the observation of Tok and Aslim, 2010[30]. According to them cholesterol assimilation increased at 48hrs incubation compared to 19 hrs incubation. The strain can assimilate about 2 mg/ml (1.88 mg/ml in 24 hrs and 1.98 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[31]. The amount is also high than *Lactobacillus delbrueckii* subsp. *bulgaricus* isolated from home-made yoghurt (strain B3 28.6µg/ml, strain B2 13.4µg/ml and strain A13 12.5µg/ml) by Tok and Aslim, 2010[30]. Again the amount is very less than two strains belonging to the species *Bifidobacterium. bifidum* (*B. bifidum* MB 107 and *B. bifidum* MB 109), which removed 81 and 50 mg of cholesterol per gram of biomass (Bordoni *et al*., 2013)[32]

Antibiotic resistance

Resistance of the probiotic strains to some antibiotics help in use for both preventive and therapeutic purposes in controlling intestinal infections. According to EI-Naggar, 2004[33], their resistance to antibiotics clarify their potential in minimizing the negative effects of antibiotic therapy on the host bacterial ecosystem. LAB001 showed resistance to different antibiotics like streptomycin, kanamycin, vancomycin, Amoxycillin, Norfloxacin, Methicillinetc (Table 1).

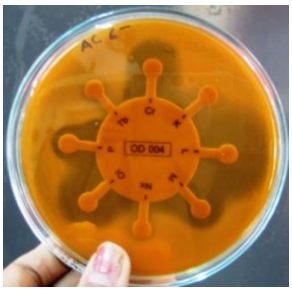


Fig. 1: Antibiotic disc on MRS agar plate against LAB001

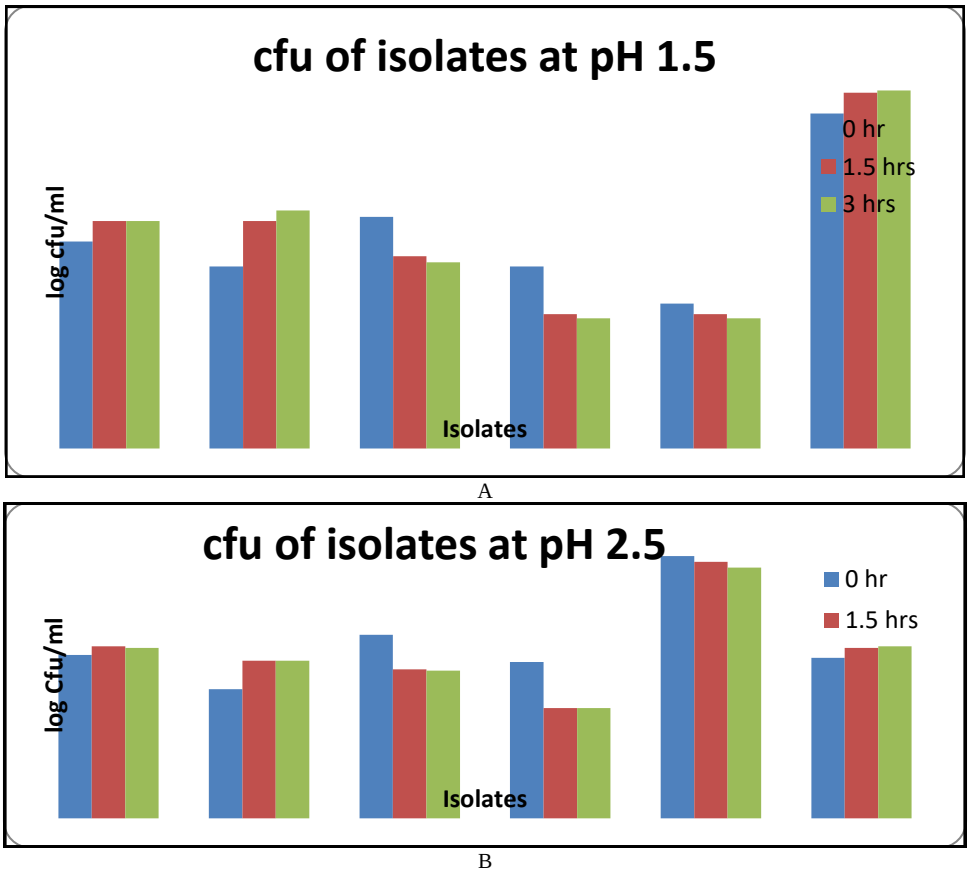


Fig. 2: Growth of LAB isolates at pH 1.5 (A) and at pH 2.5 (B)

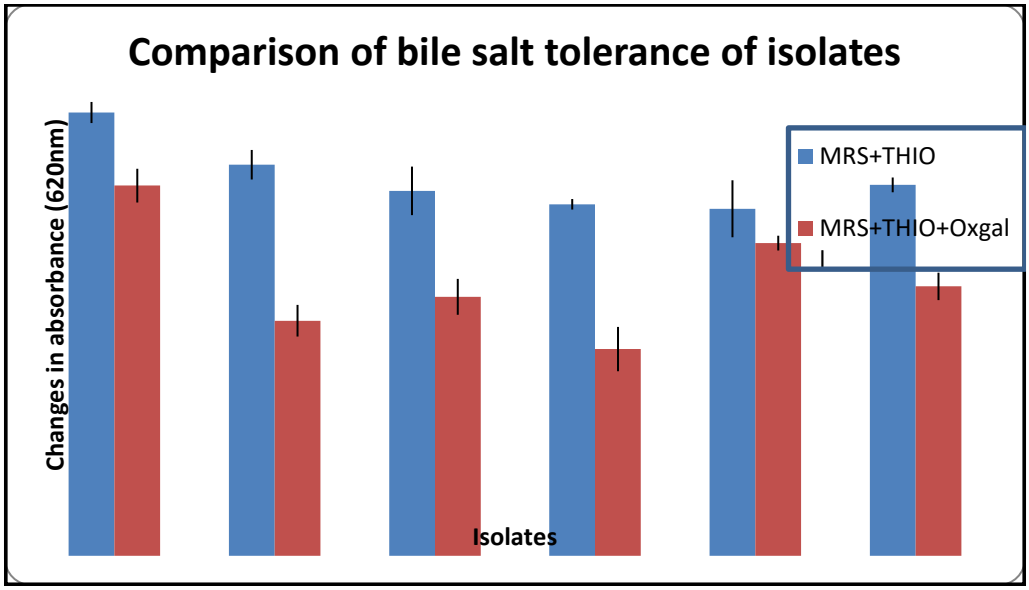


Fig. 3: Bile salt tolerance of LAB isolates

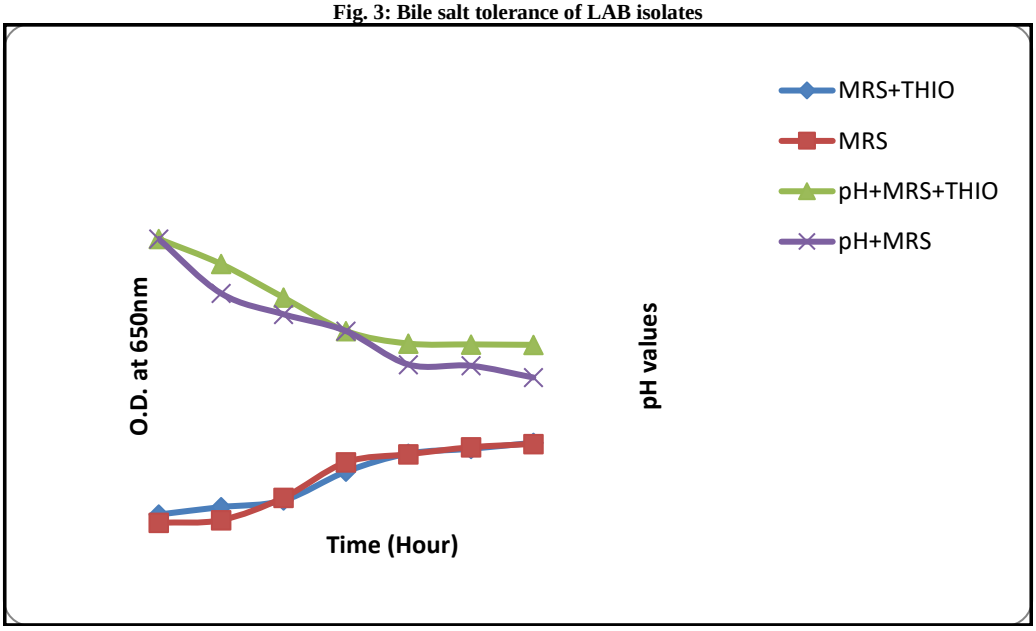


Fig. 4: Growth and culture pH of LAB001 in anaerobic condition

***In Vivo* experiments**

***In vivo* persistence of LAB001 in the GIT of mice**

On the selective antibiotic (vancomycin and streptomycin) containing MRS plates resistant bacteria were detected up to twelve days. From this experiment we can conclude that the bacteria *P. acidilactici* LAB001 can survive 12 days in the intestine of inoculated mice (**Figure 11**) as feeding was stopped after three days. This strain can survive within the gut of Swiss albino mice tolerating the low pH of stomach and intestine.

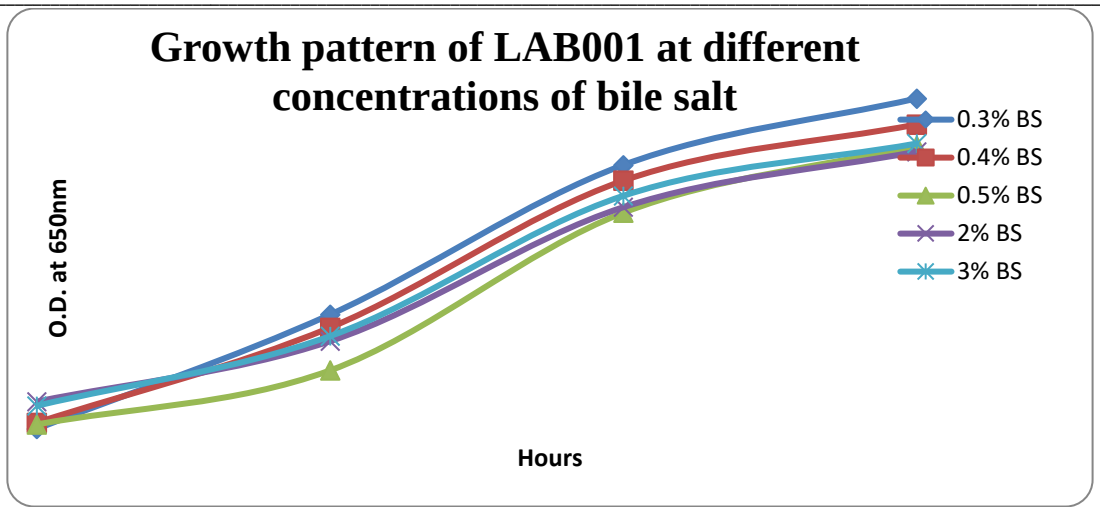


Fig. 5: Growth of LAB001 in presence of bile salt

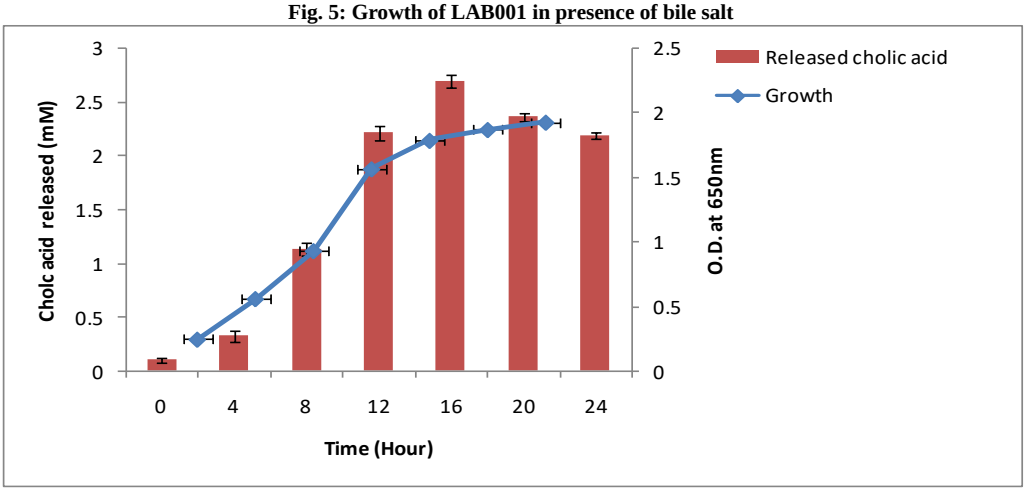
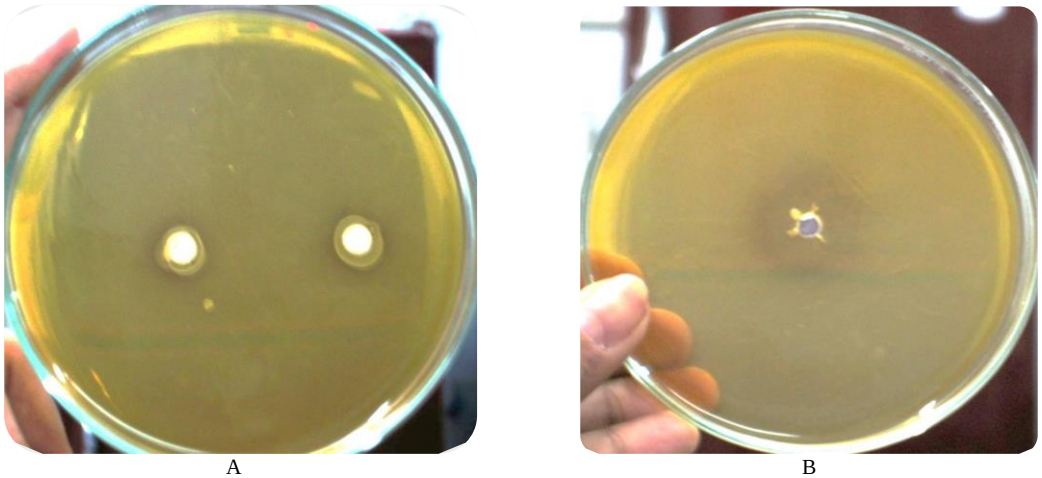
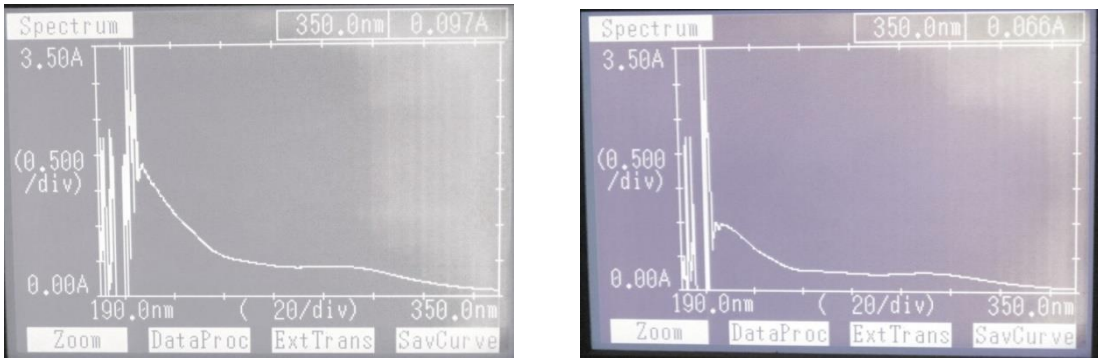


Fig. 6: Determination of incubation time required for maximum deconjugation



A B

Fig. 7: Detection of BSH activity. A: The white precipitates around paper disk containing LAB001 and the clearing of the medium are indicative of BSH activity. B: Clearing of medium by LAB001



A B

Fig. 8: UV-Vis spectroscopic analysis of deconjugated free bile acids. A: Cholic acid extracted after 24hrs and B: Standard cholic acid

Effect of *Pediococcusacidilactici*LAB001on growth of Swiss Albino mice

From Figure 10, we can conclude that *P. acidilactici* LAB001 did not show any adverse effect on the weight of the mice. All the mice appeared healthy throughout the feeding period. The growth was more or less similar like the growth of control mice even it was some better than control after 30 days. There were no differences in total food intake among the mice.

Effect of *Pediococcusacidilactici*LAB001 on cholesterol content of mice

From the results obtained (Figure 11, 12) 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 9.1%, whereas triglyceride 21.74% and LDL-cholesterol 10%. This result can be compared with the result of Xie*et al*., 2011[34] 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. The L.9-41-A treated rats achieved a maximal TCH reduction of5.3% and an LDL-C reduction of 32.9%; while the L.M1-16 treated rats achieved a TCH reduction of 12.5% and an LDL-C reduction of 17.3%.The two LAB strains did not demonstrate any significant influences on the HDL-C levels. However, serum TG level was significantly lower in the LAB-treated groups, specifically, the L.9-41-A treated group displayed a TG reduction of 16.9% and the L.M1-16 treated group displayed a TG reduction of 15.7%. This result can also be compared with An *et al*., 2011[35].

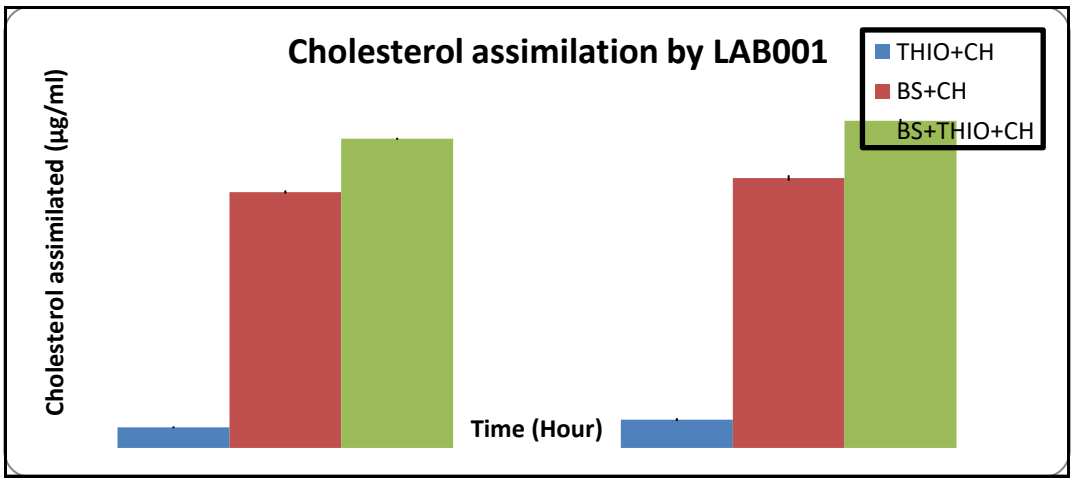


Fig. 9: Cholesterol uptake by LAB001

Table 1: Antibiotic sensitivity profile of LAB001

Antibiotics	Disc content (mcg)	Diameter of Inhibition Zone(mm) by LAB001	Interpretation (done following HiMedia protocol)
Penicillin G (P)	10 units	9	**Resistant (R)**
Tobramycin (Tb)	10	10	**R**
Cephaloridine (Cr)	30	16	Sensitive (S)
Kanamycin (K)	30	11	**R**
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	**R**
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	**R**

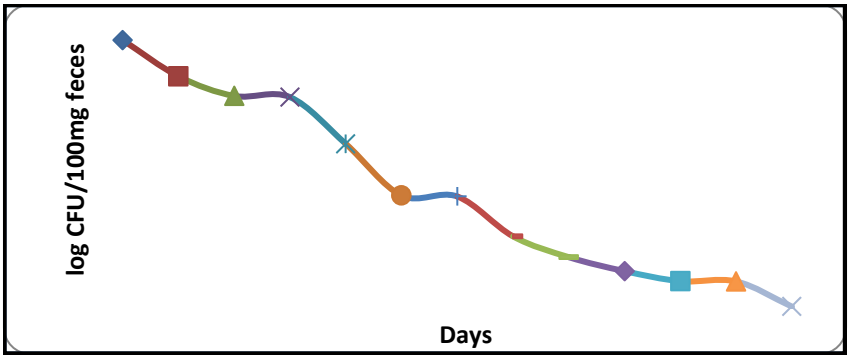


Fig. 10: Viable cell count of LAB001 from mice fecal sample

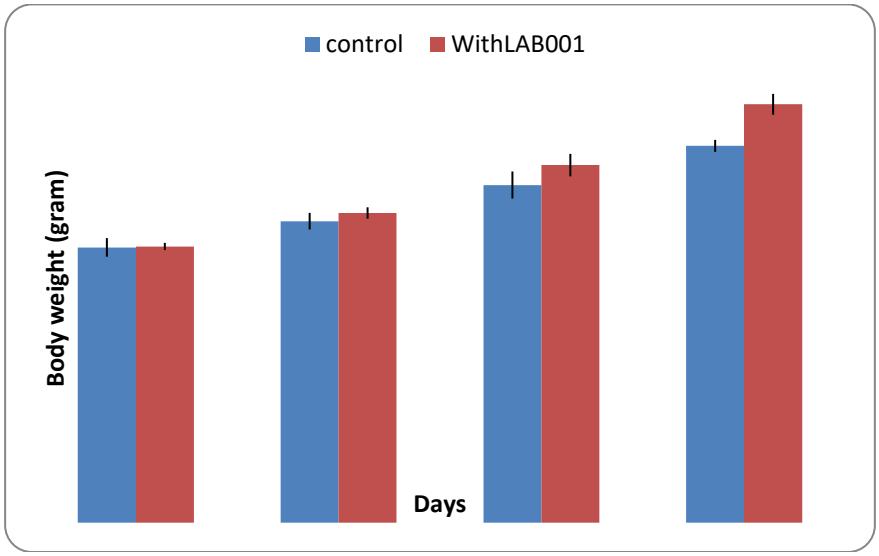


Fig. 11: Body weight of LAB001 fed and control mice

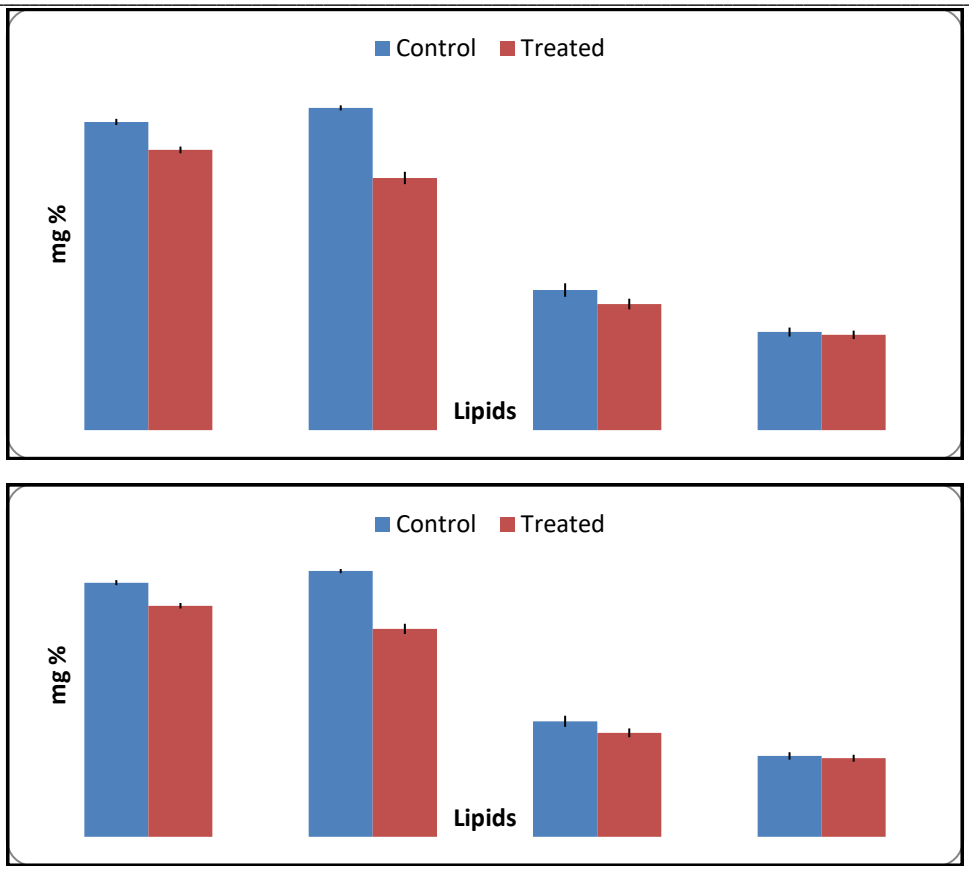


Fig 12: Effect of LAB001 on blood cholesterol level of mice

Toxicity test of liver

From the results of SGPT and SGOT (Figure 13) we can conclude there is no liver damage or no dysfunction of liver was observed after one month treatment with LAB001 as the concentration of enzymes were same as the control mice.

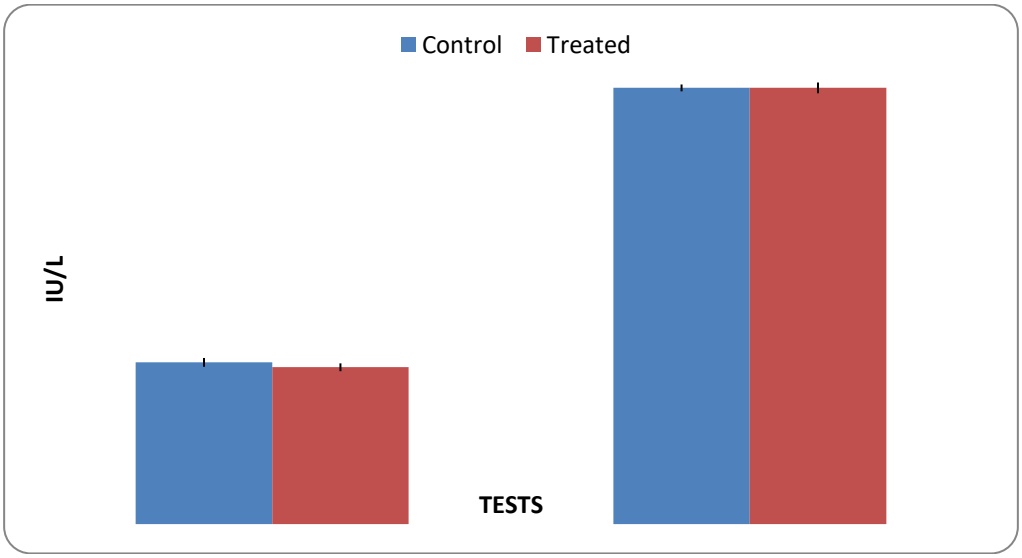
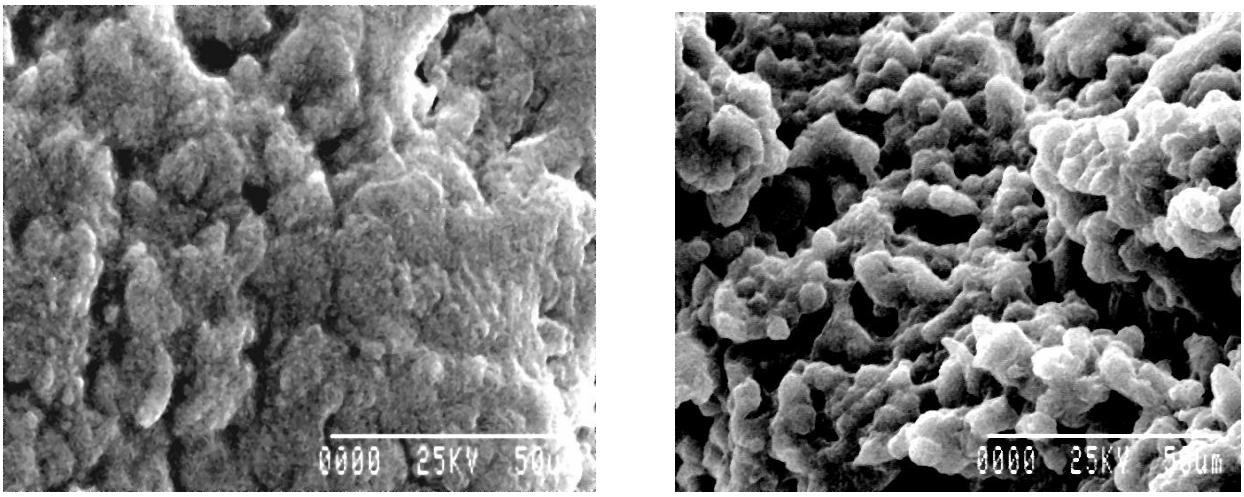


Fig. 13: Liver toxicity tests

Adherence of LAB001 to intestine of mice

SEM analysis of *P. acidilactici* LAB001 treated and untreated mice (Figure 14) intestine showed greater colonization property. There are several reports which showed the adherence property of probiotics LAB strains into epithelial cell lines.



A B

Figure 14: Scanning electron micrograph of *P.acidilactici* LAB001 treated (B) and untreated (A) mice intestine

It has been proposed that BSHs facilitate incorporation of cholesterol or bile into bacterial membranes[36,37].This incorporation may increase the tensile strength of the membranes or may change their fluidity or charge. Cell surface modifications that may result from BSH activity could potentially offer protection against perturbation of the structure and integrity of bacterial membranes by the immune system, and such resistance mechanisms may be important in establishing persistent infections. Such a function may strongly select for commensals possessing BSH enzymes while mitigating against BSH-negative pathogens or other transients.

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[38]. 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[39]. 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.

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 in this study have great potential as possible therapy for reducing cholesterol levels. The cholesterol-lowering effects of LAB001 presented may be partially ascribed to BSH activity in vitro.

References

1. Lilly DM, Stillwell RH. Growth promoting factors produced by probiotics. Science. 1965; 147**:**747-8.
2. Gupta V, Garg R. Probiotics. Indian J Med Microbiol. 2009; 27:202-9.
3. Parker RB. Probiotics, the other half of the antibiotic story. Anim. Nutr. Health. 1974; 29:4-8.
4. Fuller R. Probiotics in man and animals. J ApplBacteriol. 1989; 66(5):365-78.
5. Food and Agricultural Organization of the United Nations and World Health Organization. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. World Health Organization [online], 2001.
6. Lee YW. Effect of fermented milk on the blood cholesterol level of Korean. J FdHyg Safety 1997; 12:83–95.
7. Mann GV, Spoerry A. Studies of a surfactant and cholesteremia in the Maasai. Am. J. Clin. Nutr. 1974; 27:464 469.
8. De Rodas, Gilliland BZSE, Maxwell CV. Hypocholesterolemic action of Lactobacillus acidophilus ATCC 43121 and calcium in swine with hypercholesterolemia induced by diet. J. Dairy Sci. 1996; 79:2121–2128.
9. Gilliland SE, Nelson CR, Maxwell C. Assimilation of cholesterol by Lactobacillus acidophilus. Appl. Environ. Microbiol. 1985; 49:377–381.
10. Canzi E, Zanchi R, Camaschella P, Cresci A, Greppi GF, Orpianesi C, Serrantoni M, Ferrari A. Modulation by lactic acid bacteria of the intestinal ecosystem and plasma cholesterol in rabbits fed a casein diet. Nutr Res. 2000; 20:1329-1340.
11. Tahri K, Grill JP, Schneider F. Involvement of trihydroxyconjugated bile salts in cholesterol assimilation by bifidobacteria. Curr.Microbiol. 1997; 34:79–84.

12. Liong MT, Shah NP. Acid and bile tolerance and cholesterol removal ability of Lactobacilli strains. J. Dairy Sci. 2005; 88:55–66.
13. Walker DR, Gilliland SE. Relationship among bile tolerance, bile salt deconjugation and assimilation of cholesterol by Lactobacillus acidophilus. J. Dairy Sci. 1993; 76:956-961.
14. Roth NG, Lively DH. Germination of spores of certain aerobic bacilli under anaerobic conditions. J Bacteriol. 1956; 71(2):162–166.
15. Irvin JL, Johnston CG, Kopala J. A photometric method of the determination of cholates in bile and blood. J. Biol. Chem. 1944; 153:439-457.
16. du Toit M, Franz CMAP, Dicks LMT, Schillinger U, Haberer P, Warlies B et al. Characterization and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content. Int. J. Food Microbiol. 1998; 40:93-104.
17. Rudel LL, Morris MD. Determination of cholesterol using o-phthalaldehyde. J. Lipid Res. 1973; 14:364-366.
18. Halami PM, Chandrashekar A, Joseph R. Characterization of bacteriocinogenic strains of lactic acid bacteria in fowl and fish intestines and mushroom. Food Biotechnol. 1999; 13(2):121-136.
19. Wybenga DR, Pileggi VJ, Dirstine PH, Di Giorgio. Direct manual determination of serum total cholesterol with a single stable reagent. Clin Chem. 1970; 16:980–984.
20. Reitman S, Frankel S. Invitro determination of transaminase activity in serum. Am. J. Clin. Path. 1975; 28:56-58.
21. Sarem-Damerdji L, Sarem F, Marchel L, Nicolas JP. In vitro colonization ability of human colon mucosa by exogenous Lactobacillus strains. FEMS Microbiol. Lett. 1995; 131:133-137.
22. Kabore D,Sawadogo-Lingani H, Mamoudou HD, Diawara B, Jacobsen M. Acid resistance, bile tolerance and antimicrobial properties of dominant lactic acid bacteria isolated from traditional “maari” baobab seeds fermented condiment. Afr. J. of Biotechnol. 2012; 11.5:1197-1206.
23. Hassanzadazar H, Ehsani A, Mardani K, Hesari J. Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. Vet. Res. Forum. 2012; 3(5):181-185.
24. Ramirez-Chavarin ML, Wacher C, Eslava-Campos CA, Perez-Chabela ML. Probiotic potential of thermotolerant lactic acid bacteria strains isolated from cooked meat products. Int. Food Res. J. 2013; 20(2):991-1000.
25. Kheadr E, Zihler A, Dabour N, Lacroix C, Le Blay G, Fliss I. Study of the physicochemical and biological stability of pediocin PA-1 in the upper gastrointestinal tract conditions using a dynamic in vitro model. J. of Appl. Microbiol. 2010; 109(1):54–64.
26. Fernandez B, Hammami R, Savard P, Jean J, Fliss I. Pediococcusacidilactici UL5 and Lactococcuslactis ATCC 11454 are able to survive and express their bacteriocin genes under simulated gastrointestinal conditions. J Appl Microbiol. 2014; 116(3):677-88.
27. Gopal A, Shah NP, Roginski H. Bile tolerance, taurocholate deconjugation, and cholesterol removal by Lactobacillus acidophilus and Bifidobacterium spp. Milchwissenschaft. 1996; 51:619-623.
28. Pereira DI, McCartney AL, Gibson GR. An in vitro study of the probiotic potential of a bile-salt-hydrolyzing Lactobacillus fermentum strain, and determination of its cholesterol-lowering properties. Appl Environ Microbiol. 2003; 69(8):4743-52.
29. Lim HJ, Kim SY, Lee WK. Isolation of cholesterol lowering lactic acid bacteria from human intestine for probiotics use. J. Vet. Sci. 2004; 5:391-395.
30. Tok E,Aslim B. Cholesterol removal by some lactic acid bacteria that can be used as probiotics.Microbiol.Immunol. 2010; 54:257-264.
31. Lee DK, Jang S, Baek EH, Kim MJ, Lee KS, Shin HS et al. Lactic acid bacteria affect serum cholesterol levels, harmful fecal enzyme activity, and fecal water content. Lipids in Health and Disease. 2009; 8:21-28.
32. Bordoni A, Amaretti A, Leonardi A, Boschetti E, Danesi F, Matteuzzi D et al. Cholesterol-lowering probiotics: in vitro selection and in vivo testing of bifidobacteria. Appl. Microbiol. andBiotechnol. 2013; 97(18):8273-8281.
33. El-Naggar MYM. Comparative study of probiotic cultures to control the growth of Escherichia coli O157:H7 and Salmonella typhimurium. Asian Network for Scientific Information Biotechnol. 2004; 3(2):173-180.
34. Xie N, Cui Y, Yin Y, Zhao X, Yang J, Wang Z et al. Effects of two Lactobacillus strains on lipid metabolism and intestinal microflora in rats fed a high-cholesterol diet. BMC Complement Altern Med. 2011;11:53.
35. An HM, Park SY, Lee DK, Kim JR, Cha MK, Lee SW et al. Antiobesity and lipid-lowering effects of Bifidobacterium spp. in high fat diet-induced obese rats. Lipids in Health and Disease 2011; 10:116-126.
36. Taranto MP, F Sesma, AP Ruiz Holgado, G. Font de Valdez. Bile salt hydrolase plays a key role on cholesterol removal by *Lactobacillusreuteri*. Biotechnol. Lett. 1997; 19:845–847.
37. Taranto MP, Fernandez MurgaML, G Lorca, G. Font de Valdez. Bile salts and cholesterol induce changes in the lipid cell membraneof *Lactobacillus reuteri*. J. Appl. Microbiol. 2003; 95:86–91.
38. Tsai CC, Lin PP, Hsieh YM, Zhang ZY, Wu HC, Huang CC. Cholesterol-lowering potentials of lactic acid bacteria based on bilesalt hydrolase activity and effect of potent strains on cholesterol metabolism in vitro and in vivo. Scientific World Journal. 2014; 2014:690752.
39. Jones ML, Tomaro-Duchesneau C, Martoni CJ, Prakash S. Cholesterol lowering with bile salt hydrolase-active probiotic bacteria, mechanism of action, clinical evidence, and future direction for heart health applications. Expert Opin Biol Ther. 2013; 13(5):631-42.

Conflict of Interest: Nil
Source of support:Nil