

Bile tolerance, Bile Deconjugation and Cholesterol reducing properties of *Lactobacillus* strains isolated from traditional fermented foods

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ABSTRACT

Bile tolerance, bile deconjugation and cholesterol reducing properties of five strains of lactobacilli, viz. PD2, PC27, PSC6, PH5 and PFC21 were tested with a view to select culture for conducting *in vivo* feeding trial for hyperlipemic mice. Strain PD2 was found to be most bile tolerant, followed by PH5, PC27, PSC6 and PFC21. The strain PD2 released maximum amount of free cholic acid (2.37 mM) from sodium taurocholate. With respect to cholesterol assimilation, PH5 showed a maximum reduction of 76.85 %. Overall two strains among five gave a good performance in all these *in vitro* tests and hence, is recommended for *in vivo* feeding experiments.

Keywords: Lactic acid bacteria, Fermented food, probiotic, bile salt, cholesterol

Lactic acid bacteria are known among the beneficial group of microflora and their therapeutic effects are experienced since ages and scientifically validated. In the present era, their use as probiotics and functional foods ingredients has become common. Probiotics are defined as 'live microbial supplement that beneficially affect the host by improving its intestinal microbial balance' (Fuller, 1992). The best studied probiotic strains among the Lactobacilli, involves the species *Lactobacillus acidophilus*, *L. fermentum*,

L. plantarum, *L. brevis*, *L. rhamnosus*, *L. casei*, *L. paracasei*, *L. delbrueckii*, *L. reuteri*, *L. vaginalis*, *L. gastricus* and *L. salivarius*. Probiotics are generally believed to help host regulate the immune response, inhibit pathogenic bacteria, modulate gut flora, lower blood lipid concentration, etc. Among these functions, the hypocholesterolemic effect is of great significance for the maintenance of human cardiovascular health (FAO/WHO, 2002). While selecting the cultures for desirable trait like cholesterol lowering effect, features like host specificity, bile tolerance, bile salt hydrolase activity, bile deconjugation and cholesterol assimilation are required to be checked (Walker and Gilliland, 1993).

Elevated level of blood cholesterol is known to endorse atherosclerosis and facilitate the occurrence of myocardial infarction and stroke (Kaplan *et al.*, 1988). When the sum of the cholesterol synthesized and obtained from the diet exceeds the amount required for the synthesis of membranes, steroids and bile salts; accumulation of cholesterol in the blood vessels can occur, resulting in obstruction of the vessels. Heart failure from occluded coronary arteries is the one of the principal reason of fatality in industrialized societies (Ashar and Prajapati, 2001). Blood cholesterol reduction through dietary modification is preferred over drugs by physicians treating hyperlipemic patients, being a natural means without side effects. Increasing evidence suggests that selected members of the lactic acid bacteria, when consumed in sufficiently large amounts, exert therapeutic effects in humans as well as animals (Khedkar *et al.*, 1993). Different mechanisms has been advanced to explain cholesterol removal effect, including direct binding or assimilation of cholesterol (Gilliland *et al.*, 1985), enzymatic hydrolysis of conjugated bile salts (Corzo *et al.*, 1999) and co-precipitation of cholesterol with deconjugated bile salt (Klaver *et al.*, 1993) by probiotic lactic acid bacteria. Hence, the required culture that can grow well in presence of bile, deconjugate bile salts and assimilate cholesterol would be more useful in establishing and functioning well in the gastrointestinal tract.

Bile salts are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum in the conjugated form in volumes ranging from 500 to 700 ml per day. Probiotic lactobacilli could resist the relevant physiological concentrations of human bile range from 0.1 to 0.3% (Dunne *et al.*, 2001). Bile salt is the major route of eliminating cholesterol from the body (Turley *et al.*, 1988), as well as one of the important pathways of cholesterol metabolism (Chen *et al.*, 1995). Most excreted conjugated bile

salts (about 97%) are reabsorbed from the small intestine and returned to the liver through the hepatic-portal circulation (Macdonald *et al.*, 1983). When they are deconjugated by bile salt hydrolase (BSH), the solubility and emulsifying capacity decrease. BSH catalyzes the hydrolysis of glycine and/or taurine conjugated bile salts into amino acids and free bile acids (Liong & Shah, 2005). Some of the free bile salts precipitate at the physiological pH of the intestinal lumen. Thus, in a steady-state situation, deconjugation of bile salts can reduce serum cholesterol levels by increasing the formation of new bile salts needed to replace those that have escaped the enterohepatic circulation (Reynier *et al.*, 1981). Gilliland *et al.*, (1985) explained this effect by an assimilation of cholesterol that occurred when the *Lactobacillus acidophilus* was growing anaerobically in the presence of bile salts.

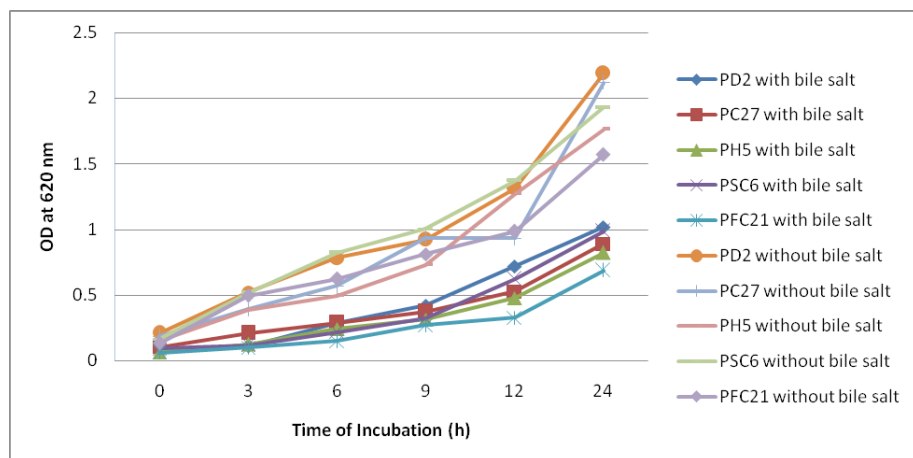


Fig. 1. Relative growth of different lactobacilli in presence and absence of bile salts.

The aim of this study was to compare various fermented food based isolates of lactobacilli for their tolerance to bile salt (sodium taurocholate), bile salt deconjugation ability, and cholesterol removal ability.

Materials and Methods

Five strains of lactobacilli PD2, PC27, PH5, PSC6 and PFC21 were isolated from dosa batter, curd, handva batter, soycurd and fermented cabbage, respectively. These cultures were maintained in 10 % reconstituted skim milk by weekly transfers. Before use in the study, they were activated by propagation in MRS broth by 2 daily transfers.

Bile Tolerance

Strains of lactobacilli were inoculated @ 2% in 100 ml of MRS and MRSB (MRS broth containing various concentrations of Sodium Taurocholate) and incubated at 37°C. Every hour, the growth was monitored by measuring the OD at 620 nm upto 24 h. A graph was plotted of A₆₂₀ nm versus incubation period and the cultures were compared for their bile tolerance based on the time required to increase A₆₂₀ nm by initial 0.3 units in MRS and MRSB (Gilliland and Walker, 1990).

Table 1. Bile tolerance amongst different strains of lactobacilli inoculated in control MRS broth

Lactobacilli Strains	Time taken to increase OD By 0.3 units (h)		Difference (h)
	MRS (Control)	MRSB (MRS broth + Sodium Taurocholate)	
PD2	3.1	6.7	3.6
PC27	3.2	8.3	5.1
PH5	3.9	8.9	5.0
PSC6	2.8	10.2	7.4
PFC21	4.2	11.8	7.6

Table 2. Deconjugation of sodium taurocholate by lactobacilli

Strains	Cholic Acid released
	Sodium Taurocholate ConcmmM
PD2	2.378±0.019
PC27	1.780±0.012
PSC6	1.271±0.020
PH5	2.019±0.020
PFC21	1.764±0.014

Bile deconjugation

Bile deconjugation ability was checked by the method of Irvin *et al.*, (1944), further modified by Walker and Gilliland (1993). Lactic isolates were grown in MRS broth containing 0.2% Sodium thioglycollate and 0.3% filter sterilized conjugated bile salt (Sodium Taurocholate) at 37°C under reduced O₂ condition in a gas pack jar for 24 h. After incubation, the broth

was adjusted to pH 7.0 using 1.0 N NaOH and then the volume was made to 25 ml with distilled water. The cells were removed by centrifugation at 5000 rpm for 20 min. Fifteen ml of the resultant supernatant was adjusted to pH 1.0 with 1 N HCl and the volume was increased to 24 ml with distilled water. Free cholic acid from 3 ml of this broth was extracted in 9 ml ethyl acetate. Three ml of ethyl acetate layer was separated in a fresh tube and dried at 60°C using Thermomixer (Eppendorf). The residues were dissolved in 1 ml of 1 N NaOH and 6 ml of 16 N sulphuric acid and 1 ml of 1 N NaOH furfuraldehyde to develop color. The tubes were heated for 15 min at 65°C and cooled to room temperature. The color development was stopped by adding 5 ml of glacial acetic acid in each tube. The absorbance was measured at 660 nm against reagent blank. The concentration of free cholic acid was calculated with the help of a standard curve and expressed as mM.

Table 3. Reduction in cholesterol content by different strains of lactobacilli

Lactobacilli Strain	% Removal of cholesterol	
	24 h	48 h
PD2	54.67	69.66
PC27	28.59	42.12
PSC6	13.65	22.68
PH5	52.34	76.85
PFC21	21.02	47.81

Cholesterol assimilating ability

Cholesterol assimilation by the culture was performed in 9 ml MRS broth containing 0.2% Sodium thioglycollate and 0.3% Sodium taurocholate to which 1 ml of filter sterilized human plasma as the source of cholesterol was aseptically added. The extraction of cholesterol remaining after 24 and 48 h of incubation was done as per Gilliland & Walker (1990) with slight modification. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C. Spent broth was collected and used for cholesterol assay. The estimation of the remaining cholesterol was performed as per Rudel and Morris (1973) with little variation. The test absorbance was read against reagent blank at 550 nm. The concentration of cholesterol was determined from the standard curve and expressed as µg/ml. the results were expressed in terms of percent reduction in cholesterol in spent broth as compared to uninoculated broth.

Results and Discussions

Bile Tolerance

The probiotic cultures are required to work under gastro intestinal conditions, and hence their tolerance to bile salts is desirable and as a prerequisite it should be tested in all the candidate strains. The result of relative bile salts tolerance by all 5 probiotic bacteria are shown in Table 1.

In all the cultures, presence of sodium taurocholate as bile salt definitely delayed the growth rate. The strain PD2, followed by PH5 was found to be most bile tolerant as the difference between growth rate in the presence and absence of 0.3% bile salt was very less. The strains PFC21 was found to be least bile tolerant. The growth curve of all five probiotic strains in the presence and absence of 0.3% Sodium Taurocholate are demonstrated in figure 1. In MRS broth with 0.3% bile, PD2 showed the best growth rate followed by PC27, PH5, PFC21 and PSC6.

The distinction in the bile tolerance amongst different strains of lactobacilli has been accounted by various researchers (Gilliland and Walker, 1990; Staley and Bush, 1984). Ashar & Prajapati (1998) tested four strains of lactobacilli and observed a similar variation with regard to growth in the normal MRS broth and that containing 0.3% bile salts. Khedkar *et al.*, (1993) also observed a similar result using three strains of *L.acidophilus*. Walker and Gilliland (1993) have found that the ability to grow in the presence of bile varied among 19 strains tested. Two strains namely ATCC 43121 and 251 grew well than the other strains, while the strain DKW-9 showed highest sensitivity to bile.

Bile deconjugation ability

Bile salt deconjugation was determined by the amount of cholic acid released, which ranged from 2.37 to 1.27 mM. Strain PD2 released maximum amount (2.37 mM) of cholic acid from sodium taurocholate, followed by PH5, PC27, PFC21 and PSC6 after 24 h of growth at 37°C. In a related study by Yang *et al.*, (2008), all lactic acid bacterial strains could deconjugate sodium taurocholate by liberating cholic acid in the range of 0.98 mM (by *L. ruminis* La3) to 5.00 mM (by *L. plantarum* LS12). Our results also showed similarity with the results shown by Ashar & Prajapati (1998) and Liong & Shah (2005).

Deconjugation of bile salts release free bile acids which are less soluble and less likely to be absorbed by human intestinal lumen compared to

their conjugated counterparts, and are lost from the body through feces (Center *et al.*, 1990). This process could lead to a higher metabolism of cholesterol and, subsequently, the reduction of serum cholesterol (Reynier *et al.*, 1981). According to the theory of Klaver & Van der Meer (1993), Bile deconjugation will lead to drop the pH of fermentation media due to natural acid production by culture, cholesterol micelles destabilized and cholesterol co-precipitated with free bile acids. It was estimated that the highest bile salt Molarities throughout the small intestine is between 2.4 and 4.0 mM (Hofmann, 1977). With the high deconjugation activity by PD2 and PH5 followed by other strains towards sodium taurocholate at concentration that resemble the human bile, we postulate that these strains may deliver good *in vivo* deconjugation effects as observed from *in vitro* experiment.

In vitro cholesterol assimilation

Direct cholesterol assimilation in the intestine may be important in reducing the absorption of dietary cholesterol from the digestive system to blood. Cholesterol assimilation capacities vary with the strains of lactic acid bacteria.

The results of cholesterol assimilation by individual strains of lactobacilli after 24 and 48 h of incubation under reduced O₂ tension maintained in gas pack jar are presented in table 2. *In vitro* cholesterol reduction by test strains varied from 13.65 to 76.85 % within 48 h. Cultures PD2 and PH5 showed maximum reduction within 48 h. However, PFC21, probably being slow in growth showed only 21.02 % reduction in 24 h, but increased to 47.81 % in 48 h. PC27 showed consistent increase in cholesterol reduction reaching to 42.12 % in 48 h from 28.59 in 24 h. PSC6 showed the least reduction in cholesterol level both at 24 and 48 h of incubation.

Khedkar *et al.*, (1993) obtained a positive *in vitro* result with the strains of lactobacilli taking horse serum as the source of cholesterol. The same procedure was repeated by Ashar & Prajapati (1998) by taking human blood serum as a source of cholesterol during their research. Considering this fact we used human serum as the source of cholesterol. Other research workers have also reported such an *in vitro* reduction of cholesterol from broth medium during the growth of lactobacilli under reduced conditions (Walker and Gilliland, 1993; Staley and Bush, 1984 and Khedkar *et al.*, (1993). This was shown by the appearance of cholesterol in the cells during growth, which was associated with decrease in the concentrations of cholesterol in the growth medium. Ashar and Prajapati (1998) have

reported 25.3% assimilation at 48 h of incubation in culture I4. Danielson (1987) found that three strains of *L. acidophilus* could reduce cholesterol levels by 30-80% in MRS broth medium supplemented with 0.2 or 0.4% bile salts. Gilliland and Walker (1990) also monitored significant variations among cultures in the range of 27.5 to 103.9 µg/ml cholesterol assimilating ability in 12 cultures of *L. acidophilus* from human origin. Vujicic *et al.*, (1992) have found variations in cholesterol reduction by 6 kefir cultures by reporting 28-65 and 41-84% assimilation, respectively during 24 and 48 h of incubation by the test cultures. Based on the overall better performance, strain PD2 and PH5 could be recommended for further in vivo feeding trials.

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