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Probiotic properties of the riboflavin producing *Lactobacillus fermentum* strain isolated from yoghurt sample

S. Jayashree*, K. Jayaraman and G. Kalaichelvan

School of Biosciences and Technology, VIT University, Vellore-632 014, Tamil Nadu, India

**Corresponding Author, Email: sjayashree@vit.ac.in; Phone- 91-416-220- 2901; Fax- 91-416-224-3092*

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Abstract

The probiotic properties of the riboflavin producing lactic acid bacterium, *Lactobacillus fermentum* MTCC 8711 were evaluated. The strain was resistant to the acidic environment with the pH of 3.5 and more than 100% survivability of the cells was observed after 4 h. Similarly, 85% survivability was observed in the presence of 0.3% (w/v) bile salts. The strain exhibited β- galactosidase activity by blue colored colony formation in the MRS agar plates with X-gal and IPTG. Further, it was found to be a medium acidifier with a ΔpH value of 0.96 after 5 h of growth. The strain was also able to reduce the cholesterol up to 50% in the presence of cholesterol and bile salts. Since the strain possesses the basic properties of the probiotics and produces riboflavin, it could be considered as a better starter culture in the fermented food industry.

1. Introduction

'Probiotics' are live microorganisms, which impart health benefits to the host beyond inherent general nutrition (Guarner and Schaafsma, 1998). The probiotic bacteria include lactic acid bacteria (LAB), bifidobacteria, certain yeasts and bacilli. LAB are a major class of bacteria with probiotic properties, and the strains of *Lactobacillus acidophilus, Lb. johnsonii, Lb. casei, Lb. rhamnosus, Lb. gasseri,* and *Lb. reuteri* were reported for the probiotic preparations (Heller, 2001). They have been studied widely owing to their multifaceted application in various fermentations in the form of starter cultures.

Though the mechanism by which the probiotic bacteria act is largely not known, few of them could involve in the modification of the gut pH, competing for pathogen binding and receptor sites as well as for available nutrients and growth factors, stimulating immunomodulatory cells, and producing lactase (Parvez et al., 2006). The health benefits attributed by the probiotics include, boosting the immunity, reducing the duration of certain diarrheal diseases, treatment of gastro intestinal disorders including *Helicobacter pylori* infections, and inflammatory bowel diseases, such as Crohn's disease and irritable bowel syndrome, and reducing the development of atopic diseases in children (Senok et al., 2005).

The large consumption of probiotic by humans is in the form of dairy products containing LAB. LAB ferments various substrates like lactose, biogenic amines and allergenic compounds into short-chain fatty acids and other organic acids and gases along with other gut microflora (Gibson and Fuller, 2000). Probiotics are provided in products in one of following three basic ways: (i) as a culture concentrate added to a food (usually a dairy product) at medium levels, with little or no opportunity for culture growth; (ii) inoculated into a milk-based food (or dietary supplement) and allowed to grow to achieve high levels in a fermented food; (iii) as concentrated and dried cells packaged as dietary supplements such as powders, capsules, or tablets.

Fermented dairy products such as yoghurt, kefir, butter milk and cultured milk serve as a good source of probiotics. Among them, yoghurt is a staple food in Asian countries and has much been employed as a good source of probiotic. It is savored owing to its refreshing acidic and buttery taste, nutritive content and therapeutic properties which are preserved by the fermentation. Few health benefits harnessed by consuming yoghurt includes anti allergenic effect to milk protein, enhancing the bioavailability of other nutrients, breakdown of lactose to monosaccharide for improved digestibility boon for lactose intolerant people, increasing the calcium bioavailability, exerting antibiotic effect against gastrointestinal infections, stimulating the immunity, anticholesterol activity and anticancer effect (Otto,1988; Shahani and Chandan, 1979). Yoghurt exhibits antagonistic effect against a number of pathogenic and spoilage organisms both *in vivo* and *in vitro* (Hattingh and Viljoen, 2001). All these health benefits of yogurt are mainly attributed to the presence of LAB and to high calcium content (Banon, 1999). Few of the criteria based on which the probiotics are selected include, acid and bile stability, production of β-galactosidase enzyme, cholesterol assimilation, and

adherence to human intestinal cells (Gibson and Fuller, 2000). In this study, the probiotic properties of the riboflavin producing strain, *Lb. fermentum* MTCC 8711 was evaluated.

2. Materials and Methods

Microorganism

A riboflavin producing lactic acid bacterium was isolated from the yoghurt sample of the Vellore district, Tamil Nadu, India. By biochemical characterization and 16S rDNA sequencing, it was identified as *Lactobacillus fermentum* and the strain has been deposited in Microbial Type Culture Collection and Gene Bank, Chandigarh, India with the accession number of MTCC 8711. Previously, we have reported the characteristic features and the production of riboflavin by this strain (Jayashree et al., 2010). In this study, the probiotic properties of this strain were examined.

Acid tolerance

Acid tolerance of the strain was evaluated by viable count method as described by Gililand et al. (1984). One ml of the culture grown in the MRS broth for three generations having an A_{600} value of 0.28 at was inoculated in 9 ml of sterile MRS broth whose pH was adjusted to 3.5 with 5 N HCl. The culture was incubated at 37°C for 4 h under static conditions. One ml of samples collected at 0 h and after 4 h was serially diluted with sterile saline, in order to neutralize the acidity, and plated onto MRS agar plates. The plates were incubated at 37°C for 24 h and the CFU were calculated using colony counter (Serve well instruments, Banglore, india). The survivability of the culture after the exposure to low pH for 4 h was considered as the criteria for acid tolerance. The percentage survivability was calculated as follows:

% survivability = (log CFU 4 h / log CFU 0 h) ×100

Bile tolerance

The bile tolerance of the strain was evaluated by the method of Gililand et al. (2004). This was done by cultivating the cells on MRS broth with 0.3% (w/v) bile salts mixture (HiMedia, Mumbai, India) at 37°C for 24 h. The viability was checked by spreading of 100 µl of overnight grown cultures of appropriate dilutions onto MRS agar. Percentage survivability of the strains to 0.3% bile salts was calculated using the formula given below:

% survivability = (log CFU 24 h / log CFU 0 h) ×100

β-galactosidase activity

The β-galactosidase activity of the strain was performed by the method described by Karasova et al. (2002). The organism was plated onto MRS agar containing 0.01% X-gal (HiMedia, Mumbai, India) and 0.1 mM IPTG (HiMedia, Mumbai, India) as inducer and incubated for 24 h at 37°C. The β-galactosidase production of the strain was indicated by formation of the blue colored colonies.

Acidifying activity

Acidification was measured by the change in pH (ΔpH) according to the method described by Ayad et al. (2004). Five ml of MRS broth was inoculated with 1% of overnight grown culture and incubated at 37°C. The pH was measured every one hour for 24 h using a pH-meter (Thermon Electron Corporation, Beverly, USA). The acidification values were expressed as ΔpH, which is calculated as follows:

$\Delta pH = pH_t - pH_0$, where pH_t is the pH of the culture at time t and pH_0 is the pH at 0 h. The criteria for the classification of the strain as fast, medium or slow acidifier was based on the ΔpH value of 0.4 achieved after 3, 3-5 and > 5 h, respectively.

Cholesterol assimilation assay

Cholesterol assimilation property was determined by the method described by Searcy and Bergquist (1960). *Lb. fermentum* MTCC 8711 was grown in MRS broth supplemented with 0.3% bile salt mixture. 10 mg of cholesterol dissolved in 500 µl of ethanol was added to 100 ml of MRS broth with bile salt. The cultures were grown for 24 h at 37°C and the cells were removed by centrifugation at 8000 rpm for 10 min at 4°C. The spent broth was collected and the cholesterol level was estimated. The uninoculated broth was considered as control. To the 1 ml of spent broth, 3 ml of 95% ethanol followed by 2 ml of 50% potassium hydroxide were added. The contents were mixed well after the addition of each component. The tubes were heated for 10 min at 60°C in a water bath. After cooling, 5 ml of hexane was dispensed to all tubes and vortexed for 5 min at 20-second interval. Then 3 ml of water was added and mixed thoroughly. Tubes were allowed to stand for 15 min at 30°C to permit phase separation. 2.5 ml of hexane layer was transferred to a fresh test tube and allowed to dry completely. 1.5 ml of ferric chloride reagent was added to each test tube and allowed to stand for 10 min. One ml of concentrated sulphuric acid was added along the sides of the tube. The mixture was vortexed and allowed to stand for 45 min at 30°C. The absorbance was measured at 540 nm in spectrophotometer (Model UV-1700, Shimadzu, Tokyo, Japan). The concentration of cholesterol was determined using cholesterol standard graph. The percentage assimilation was calculated using the following formula:

$$\text{Percentage \% assimilation} = \frac{\text{Conc. of cholesterol in control - Conc. of cholesterol in sample}}{\text{Conc. of cholesterol in control}}$$

Antibiotic susceptibility test

Disk diffusion method described by Bauer et al. (1966) was followed for antibiotic susceptibility test. The bacterial culture of *Lb. fermentum* MTCC 8711 was inoculated into the MRS broth and incubated at 37 ºC for 12 h. Plates were made with Muller Hinton agar (Himedia, Mumbai, India) and allowed to solidify. A lawn culture was made on the plates using sterile swabs. The antibiotic discs of kanamycin, penicillin, vancomycin, ampicillin, streptomycin, bacitracin, trimethoprim, rifampicin (HiMedia, Mumbai, India) were placed in the plates. Agar plates with antibiotic disks were then incubated for 24 h. The diameters of the inhibition zones were measured using a ruler under a colony counter apparatus and the susceptibility pattern were recorded as per the manufacturer's instructions.

3. Results and Discussion

Acid tolerance

The probiotic LAB that are acquired through food consumption should sustain in the acidic conditions of the gastro intestinal tract in order to deliver its beneficial effects. The time required from entrance up to release from the stomach has been estimated to be approximately 90 min, wherein the further digestive processes requires longer residence time (Berrada, 1991). The acid tolerant capacity of the LAB differs from strain to strain (Ross et al., 2005). In this study, more than 100% of survivability of cells was observed after 4 h of exposure to the acid environment of pH 3.5. The log CFU value was increased from 5.85 to 6.90 after 4 h of incubation indicating that the strain is able to grow at pH 3.5. Similarly, Chou and Weimer (1999) had investigated and reported the acid tolerance of *Lactobacillus acidophilus* at pH 3.5.

Bile tolerance

To exploit a lactic acid bacterial strain as a probiotic, it is necessary to evaluate its ability to resist the effect of bile salts (Lee and Salminen, 1995). The exact mechanism for the bile salt resistance is not well understood but thought to be through the bile salt hydrolase (BSH) activity. More than 85% of the cells of *Lb. fermentum* MTCC 8711 were able to survive in the presence of the bile salt mixture concentration of 0.3% after 24 h of growth, exhibiting their bile tolerance capacity. The log CFU value was decreased from 7.80 to 6.84 with 87.69% of the survivability.

β-galactosidase activity

β -galactosidase is an enzyme that catalyzes the hydrolysis of the β -galactosides into monosaccharide sugars. The LAB possessing these enzymes can break down the galactose units of the milk and could make it simpler for the consumption of lactose intolerant people. Lactose intolerance is a wide spread disorder found in people who lack the enzyme lactase characterized by flatulence and bloating. Such people can tolerate lactose from yoghurt better than from milk (Sanders, 2000). Dairy products obtained by the probiotic organisms with β-galactosidase activity could be well suited for the lactose intolerant people. The strain *Lb. fermentum* MTCC 8711 possessed the β-galactosidase activity, which was determined by blue colored colony formation in MRS agar with 0.1% X-gal and 0.1 mM IPTG (Fig. 1).

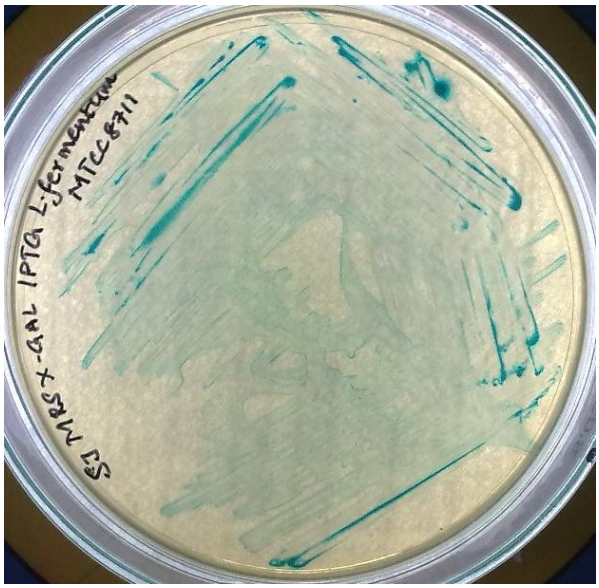

Fig. 1. The β-galactosidase activity of *Lb. fermentum* MTCC 8711 in MRS agar with 0.01% X-gal and 0.1 mM IPTG exhibiting the blue coloured colony formation.

Acidifying property

Acidifying property of a LAB strain is very important in the fermented food industry for the coagulation of adventitious micro flora especially in the cheese preparation and is also used as adjunct cultures for their autolytic and amino peptidase activity (Ayad et al., 2004). The strain *Lb. fermentum* was found to be medium acidifier with ΔpH value of 0.96 after 5 h. The acidifying property of this strain is shown in Fig. 2.

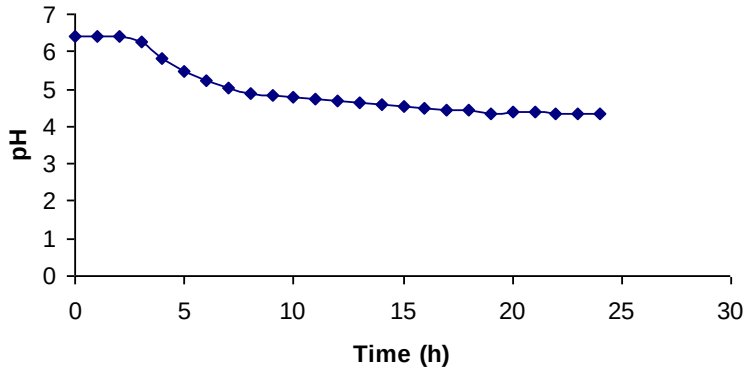

Fig. 2. Acidifying property of *Lactobacillus fermentum* MTCC 8711.

Cholesterol assimilation property

Elevated level of certain blood lipids like cholesterol is a greater risk for cardiovascular disease. Among the probiotic effects of the lactic acid bacteria (LAB), the assimilation of cholesterol is very important for reducing the absorption of dietary cholesterol from the digestive system into the blood (Hepner, 1979). Many studies have shown that the cholesterol removal would be related to the ability of the cultures to deconjugate bile salts (Gilliland and Walker, 1989). The application of *Lb. acidophillus* as probiotic has been shown to decrease the serum cholesterol levels in human and animals (Lee et al., 1992). Lim et al. (2004) have reported 57.0%, 64.4% and 58.6% hypo cholesterolemic effects by *Streptococcus* HJS-1, *Lactobacillus* HJL-37 and *Bifidobacterium* HJB-4 respectively. In the present study, *Lb. fermentum* MTCC 8711 showed up to 50% of reduction in cholesterol level in the MRS broth supplemented with cholesterol and 0.3% bile salts mixture. The probiotic properties of the strain *Lb. fermentum* MTCC 8711 is summarized in Table 1.

Table 1. The probiotic properties of *Lb. fermentum* MTCC 8711.

Probiotic properties	**Results**
Riboflavin production	Positive
β-galactosidase activity	Positive
Acid tolerance	>100%
Bile tolerance	>85%
Acidifying property	Medium acidifier (ΔpH = 0.96 after 5 h)
Cholesterol assimilation	Reduces the cholesterol up to 50%

Antibiotic susceptibility

Probiotic strains should possess resistance to commonly used antibiotics, in addition to their metabolic activities benefiting the well being of the host. The strain was resistant to chloramphenicol, rifampicin, trimethoprim and vancomycin. Since the strain is resistant to these antibiotics, it could be an efficient probiotic organism as it can persist during antimicrobial chemotherapy. However, these resistance patterns should be intrinsic and non transmissible. The transmission of antibiotic resistance to unrelated pathogenic bacteria in the gut is a major health concern, with obvious ramifications for the selection and safety of probiotic strains (Curragh and Collins, 1992). Studies on localization of these genes in the genome, i.e., whether these genes are coded in the chromosome or the transferrable plasmids, are under progress. The strain was sensitive to ampicillin, tetracycline, erythromycin and Kanamycin. Similarly, Zhou et al. (2005) have

reported that the probiotic organisms belong to *Lactobacillus* and *Bifidobacterium* were susceptible to β-lactam antibiotics such as penicillin G and ampicillin.

Conclusion

Probiotics impart health benefits at a lower cost and are utilized in the developing countries like India. The strain *Lb. fermentum* MTCC 8711 produces riboflavin, which is an essential vitamin. In this study, we have shown that the organism possess probiotic properties such as acid tolerance, bile tolerance, β-galactosidase activity, acidifying property and cholesterol assimilation efficiency. Therefore, this strain could be considered as better starter culture as it has the riboflavin producing ability and probiotic properties.

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References

Ayad, E. H. E., Nashat, S., El-Sadek, N., Metwaly, H. and ElSoda, M. 2004. Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. Food Microbiol. 21: 715-725.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C., Turk, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45: 493– 496.

Berrada, N., Lemeland, J. F., Laroch, G., Thouvenot, P., and piaia, M. 1991. *Bifidobacterium* from fermented milks: Survival during gastric transit. J. Dairy Sci. 74: 409-413.

Chou, L. S., and Weimer, B. 1999. Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. J. Dairy Sci. 82: 23–31.

Curragh, H. J., and Collins, M. A. 1992. High levels of spontaneous drug resistance in *Lactobacillus*. J. Appl. Bacteriol. 73: 31– 36.

Desobry-Banon, S., Vetier, N., Hardy, J. 1999. Health benefits of yogurt consumption. A review. Int. J. Food Properties. 2: 1-12.

Gibson, G.R., R. Fuller. 2000. Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and probiotics for human use. J. Nutr. 130: 391S-395S.

Gilliland, S. E. and H. S. Kim. 1984. Effect of viable starter culture bacteria in yogurt on lactose utilization in humans. J. Dairy Sci. 67: 1-6.

Gilliland, S. E. and Walker, D. K. 1989. Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypo cholesteremic effect in humans. J. Dairy Sci. 73: 905–911.

Guarner, F., Schaafsma, G. J. 1998. Probiotics. Int. J. Food Microbiol. 39: 237-238.

Heller, K. J. 2001. Probiotic bacteria in fermented foods: product characteristics and starter organism. Am. J Clin. Nutr. 73: 374S–9S

Hepner, G., Fried, R., Jeor, S., Fusetti, L., and Morin, R. 1979 Hypocholesterolemic effect of yogurt and milk. Am. J Clin. Nutr. 32: 19–24.

Jayashree, S., Jayaraman, K., and Kalaichelvan, G. (2010). Isolation, screening and characterization of riboflavin producing lactic acid bacteria from Katpadi, Vellore district. Recent Res. Sci. Technol. 2: 83-88.

Karasova, P., Spiwok, V., Mala, S., Kralova, B. and Russell, N. J. 2002. Beta-galactosidase activity in psychotrophic micro organisms and their potential use in food industry. Czech J. Food Sci. 20: 43-47.

Lee, Y. W., Roh, W. S., Kim, J. G. 1992**.** Benefits of fermented milk in rats fed by hyper cholesterolemic diet (II). Korean J Food Hyg. 7: 123-135.

Lee, Y-K. and Salminen, S. 1995. The coming age of probiotics. Trends Food Sci. Technol. 6: 241-245.

Lim, H. J., Kim, S. Y., Lee, W. K. 2004. Isolation of cholesterol-lowering lactic acid bacteria from human intestine for probiotic use. J. Vet. Sci. 5(4): 391–395.

Lourens-Hattingh, A and Viljoen, B. C. 2001. Yogurt as probiotic carrier food. Int. Dairy J. 11: 1-17.

Otto, A. 1988. An unfinished masterpiece. Dairy Foods. 89: 32.

Parvez, S., Malik, K.A., Ah Kang, S., and Kim, H.Y. (2006) Probiotics and their fermented food products are beneficial for health. *J. Appl. Microbiol.* 100, 1171–1185.

Ross, R. P., Desmond, C., Fitzgerald, G. F. and Staton, C. 2005. Overcoming the technological hurdles in the development of probiotic food. J. Appl. Microbiol. 98: 1410-1417.

Sanders, M. E. 2000. Considerations for use of probiotic bacteria to modulate human health. J. Nutr. 130: 384S-390S.

Searchy and bergquist. L. M. 1960. New color reaction for the quantification of serum cholesterol. Clin. Chim. Acta. 5: 192-199.

Senok, A. C., Ismaeel, A. Y., and Botta, G. A. 2005. Probiotics: facts and myths. Clin. Microbiol. Infect. 11: 958–966.

Shahani, K. M. and Chandan, R. C. 1979. Nutritional and healthful aspects of cultured and culture containing dairy foods. J. Dairy Sci. 62:1685-1694.

Zhou, J. S., Pillidge, C. J., Gopal, P. K., Gill, H. S. 2005. Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. Int. J. Food Microbiol. 98: 211 – 217.