Studies on Isolation, Characterization and Entrapment of Probiotic *Lactobacillus* from Milk and Milk Products

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Abstract

Lactobacilli strains were isolated from the milk of domestic animals for potential probiotic. Probiotic organisms such as *Lactobacillus* spp were isolated from different commercially prepared milk products such as curd, cheese, milk were isolation I, isolation II, and isolation III respectively analyzed and Lactic Acid Bacteria (LAB) were identified. The ability of *Lactobacillus* isolates in the rice fermentation of batter was also studied. Growth curve of *Lactobacillus* species isolated from milk products was identified by using bioreactor. Immobilization technique was also carried out to detect the efficiency of lactobacillus species as a probiotic, and also entrapped in different dilution. The food pathogens such as *Bacillus* spp, *Salmonella* spp, *Staphylococcus* spp, *E.coli,* were used for antimicrobial activity. *E.coli* was more susceptible than other organisms such as *Salmonella, Bacillus, Staphylococcus*. The other organisms showed least zone formation in different dilutions. These isolated LABs exhibited excellent probiotic characteristics, can be use in the protection and improvement of intestinal microbial flora and contribute health benefits to consumers.

Key Words: *Lactobacilli, Bacillus, Salmonella, Staphylococcus, E.col,* Probiotic organisms.

Introduction

The probiotics are living-health promoting microorganisms which will be incorporated into various kinds of foods. Lactic acid bacteria widely distributed in the nature and occurring indigenous microflora in raw milk that play an important role in many food and feed fermentations with increased shelf life. These *Lactobacilli* and *Bifidobacteria* live along with 600 species of microorganisms in the mouth and 400species in the small and large intestines. Fermented milks have been a part of the human diet since ancient times. Their efficacy in alleviating gastrointestinal disorders has been exploited in systems of traditional medicines world over. Lactic acid bacteria play an important role in preventing bacterial and fungal infections of the skin and gastrourinary tract. *Lactobacilli* have a protective role against vaginal infections. Improper function of the immune system that is related to decreased tolerance to indigenous microflora can lead to immunopathogenesis in chronic inflammatory bowel disease(Merger and Croitorou, 1998). Disruptions in intestinal permeability barriers can lead to translocation of bacteria into the blood stream (Wells *et al.,* 1988).

The mechanism of probiotic bacteria are postulated to influence the immune response, although genera in addition to *Lactobacilus* and *Bifidobacterium* have been evaluated as immune stimulators or "biological response modifiers" with varying degrees of success. Consumption of the probiotic milk did not result in a significant increase in receptor expression whereas consumption of regular milk did, suggesting that this strain may suppress a milk-induced immune inflammatory response (Trapp *et al.,* 1993). The ability of probiotic bacteria to influence colonization and activity of Helicobacter pylori, which is associated with chronic gastritis, peptic ulcers, and risk for gastric cancer has been evaluated (Marshall, 1994).

Similar to the intestinal tract, the urinogenital tract of women is highly colonized and susceptible to infection upon colonization disruption (Reid *et al.,* 1998). Several studies have correlated vaginal health with the presence of *Lactobacilli,* and specifically hydrogen peroxide producing *Lactobacilli.* Some clinical substantiation of the ability of probiotics to decrease recurrence of urogenital infection in women exists (Shalev *et al.,* 1996).

Studies on the effects of culture-containing dairy products or probiotic bacteria on cholesterol level have yielded equivocal results (Taylor and Williams, 1998). The effect of probiotic bacteria on reduction of serum cholesterol and mechanisms of any effects are unknown. One hypothesis suggests that some strains of *L.acidophilus* can assimilate the cholesterol molecule (Gilliland *et al.,* 1985). The benefits of probiotic as healthy ingredient could be enhanced if used in combination with other health promoting dietary strategies. Probiotics bacteria have always had a natural association with dairy foods. Dairy products containing probiotics provide not only viable bacteria, but also high-quality macronutrients and unique micronutrients found in fermented milk products (Meer *et al.,*1998). Probiotic bacteria have also been

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shown to preserve intestinal integrity and medicate the effects of inflammatory bowel disease irritable bowel syndrome, colitis, and alcoholic liver disease(Nanji, *et al.*, 1994).

Materials and Methods

Probiotic organisms such as *Lactobacillus* spp were isolated from different commercially prepared milk products such as curd, cheese, milk were randomly collected in sterilized glass bottles. Milk products were serially diluted to 10^{-5}_10^{-6} using sterile distilled water and 0.1mL plated on to sterile de-Mann, Rogosa and Sharpe (MRS) agar. The MRS plates were maintained in microaerophilic condition and incubated at 37°C for 48h. After incubation well-isolated typical colonies were picked up and transferred to MRS broth and incubated at 37°C for 48h. The isolates were identified using standard morphological, cultural and biochemical reactions (Howells, 1992). The isolates were confirmed by motility test and various biochemical tests namely IMViC tests, starch utilisation and carbohydrate fermentation tests. Then the encapsulation of *lactobacilli* was formed by using sodium alginate. Approximately 50ml of milk was taken and it was pasteurized for few minutes. The *Lactobacillus* organism was formed in MRS media culture was inoculated in the pasteurized milk. It was kept at room temperature for 24 hrs.

The rice was weighed and soaked in water. The duration of soaking was noted and it was ground well to a smooth paste. *Lactobacillus* was inoculated into the batter. It was kept for fermentation at room temperature and the duration was noted (Fuller R., 1998). The MRS broth was prepared and sterilized. The sterilized broth was poured into the fermentor. The pH and temperature was maintained upto 6.5 and 25^0c respectively. After an hour the sample was collected from the sample collector and the reading was noted using the spectrophotometer. For every 1hour the sample was collected and the reading was recorded in the spectrophotometer for the observation of growth curve.

The organism which was formed in curd, cheese, milk are serially diluted and marked as sample1, sample2, sample3 respectively. The nutrient agar was sterilized and poured in petriplates for solidification. The food pathogens such as *Bacillus, Staphylococcus, E.coli, Salmonella* were diluted and 0.1ml of the dilutent was inoculated onto that plate. The whattmann filter paper was dipped in serially diluted sample and placed on the spreaded plate. The same procedure was repeated for the other sample. A control was maintained. This was incubated for 37^0c for 24hrs for zone formation (Danielsen and Wind., 2003).

Results

Based on isolation, enumeration, Grams staining, various Bio-chemical test and the organism was isolated from curd, cheese, milk products (ie) Isolation I, Isolation II, Isolation III, respectively and antimicrobial activity was checked. The growth of *Lactobacillus* isolates from substrates I, II, and III were serially diluted, and the growth was observed, Light Yellow colour and purple colour was observed in 10-1 dilutions, Dark yellow colour colony which appeared evenly in 10-2 dilution, Mild yellow colour colony which appeared evenly in 10-3 dilution and 10^{-4} dilution, and Fully yellow colour colony which appeared evenly in 10^{-5} and 10^{-6} dilutions (Table 1). The Enumeration of *Lactobacillus* isolates from different milk and milk products in which Isolates I, II, and III showed colonies. They were too numerous to count in 10^{-1} dilution and 10^{-2} dilutions, 30-40 colonies in 10^{-3} dilutions, 15-25 colonies in 10^{-4} dilutions, 10 colonies in 10^{-5} dilutions, Below 10 colonies in 10^{-6} dilutions (Table 2).

The colony characters of isolated organisms *Lactobacillus* was found to gram positive and appeared to have smooth, round, concave and translucent anaerobic organisms and it was observed in the culture plate in the form of long to very short rods often in chains. It was a non-motile organism (Table 3). The organisms were confirmed by different Bio-Chemical test. Isolate I, II, and III showed negative for Indole, Methylred, Voges proskauer, Catalase and positive for Citrate. In glucose and lactose Utilization no gas production occured and in starch hydrolysis no zone formation occurred (Table 4).

To study the ability of Lactobacillus in the rice fermentation was studied by inoculating the bacteria in rice batter in 0 hrs incubation. It showed 400cm volume of rice batter with Lactobacillus and after 18hrs incubation it increased to 600cm volume of rice batter and it was observed with pores, slight convex and gas production (Table 5). The entrapment of *Lactobacillus* isolates analyzed by the immobilization of beads showed a number of colony appearance in different dilutions (Table 6).

S.NO	Substrate Used	Dilutions					
		10^{-1}	10^{-2}	10_{-3}	10^{-4}	10^{-5}	10^{-6}
1.	ISOLATE I	Light yellow colour inmiddle andpurple colour in surrounding	Dark yellow colour in middle	Mild yellow colour evenly	Mild yellow colour evenly	Fully yellow colour	Fully yellow colour Evenly
2.	ISOLATE II	Yellow colour inmiddle andpurple colour insurrounding	Fully Yellow Colour	Yellow Colour Evenly	Slightly purple on corner of the plate remaining are yellow	20% of purple colour and 80% yellow colour	Slightly yellow colour remaining are purple colour
3.	ISOLATE III	Slightly Yellow Colour Change In Plate	50% Of Slight Yellow Colour	30% of the plate is modified to yellow colour	Mild yellow colour is seenas some rounds	A Very Mild Yellow Colour	Mild Yellow Colour

Table 1. Growth of *Lactobacillus* spp isolates obtained from milk and different milk products.

S.No	Substrate Used	Dilutions					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1.	ISOLAT I	TNTC	TNTC	30-40 Colonies	15-25 Colonies	10 Colonies	Below 10
2.	ISOLATE II	TNTC	TNTC	20-30 Colonies	10-20 Colonies	20 Colonies	Below 10
3.	ISOLATE III	TNTC	72 Colonies	50 Colonies	30 Colonies	20 Colonies	Below 10

Table 2. Enumeration of *Lactobacillus* spp isolates from milk and different milk products (TNTC-Too Numerous to Count).

By using bioreactor growth curve was observed. Every 1hour the reading was noted by using spectrophotometer. In starting stage number of cells in spectrophotometer showed the reading of about 0.119nm and this was lag phase stage. After 2hour the number of cells in spectrophotometer reading was increased to 0.147nm and 4hours 0.525nm. This was in log phase. Then 5, 6, and 7hours incubation the spectrophotometer shows the reading as 1.559, 1.636, 1.654 respectively. It was the stationary phase. Finally in the 8hours incubation the growth showed the reading as 0.674nm and this was the decline phase (Table 7).

S.No	Substrate	Organism	Morphology	Observation	Motility
1.	ISOLAT I	*Lactobacillus spp.,*	Smooth, round, Anaerobic	Chains	Non-Motile
2.	ISOLATE II	*Lactobacillus spp.,*	Transulucent, anaerobic	Long to veryshort rods	Non-motile
3.	ISOLATE III	*Lactobacillus spp.,*	Smooth, concave Anaerobic	Long to veryshort rods often in chains	Non-motile

Table 3. Colony characters of *Lactobacillus.*spp

S.NO	Test	Substrate Used		
		ISOLATE I	ISOLATE II	ISOLATE III
1.	Indole	Negative	Negative	Negative
2.	Methyl Red	Negative	Negative	Negative
3.	Voges Proskauer	Negative	Negative	Negative
4.	Citrate	Positive	Positive	Positive
5.	Catalase	Negative	Negative	Negative
6.	Glucose	No Gas Production	No Gas Production	No Gas Production
7.	Lactose	No Gas Production	No Gas Production	No Gas Production
8.	Starch	No Zone Formation	No Zone Formation	No Zone Formation

Table 4. Bio-chemical tests for different organisms isolated from milk and milk products

S.No	Hours	Rice with *Lactobaillus*	REMARKS
1.	0 hrs	400 CM	Pores, Gas Production.
2.	18 hrs	600CM	Slight Convex

Table 5. Test to study ability of *Llactobacillus* spp isolates in the rice fermentation of batter

Substrate used	Dilutions					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Immobilized Beads	Too numerous to count	To numerous to count	>50 colonies	<50 colonies	<20 colonies	<10 colonies

Table 6. Entrapment of *lactobacillus* spp isolates in alginate beads

S.No	Time	Incubation Period	Sample Absorbance (670nm)
1.	11.AM	1.00	0.119
2.	12.PM	2.00	0.147
3.	1.00PM	3.00	0.178
4.	2.00PM	4.00	0.524
5.	3.00PM	5.00	1.559
6.	4.00PM	6.00	1.636
7.	5.00PM	7.00	1.654
8.	6.00PM	8.00	0.674

Table 7. Growth curve of *lactobacillus* spp from milk and milk products

S.No	Organisms	Average (dia Mm) Dilutions					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1.	*E.coli*	11.2	10.4	8.8	7.4	6.0	5.4
2.	*Salmonella*	9.4	9	6.4	5.8	5.2	-
3.	*Bacillus*	9	8.2	8.8	7	6	-
4.	*Staphylococcus*	8	7.8	6.2	6	5.5	-

Table 8. Antimicrobial activity of *lactobacillus* spp isolates from isolates i

S.No	Organisms	Average (dia Mm) Dilutions					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1.	*E.coli*	11	10.5	8	7.8	6.2	5.4
2.	*Salmonella*	9	9	5.8	5.4	-	-
3.	*Bacillus*	9	8.2	7.5	7	6	-
4.	*Staphylococcus*	9	9	8.2	6.8	6.2	5.5

Table 9. Antimicrobial activity of *lactobacillus* spp isolates from isolates ii

S.No	Organisms	Average (DIA mm) Dilutions					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1.	*E.coli*	11.8	11.6	9.4	8.8	6.6	5.4
2.	*Salmonella*	9.4	9	8.2	6.4	5.8	5.8
3.	*Bacillus*	8.8	7.6	6.8	6.8	5.2	-
4.	*Staphylococcus*	9	8.6	7.1	5.9	5.2	-

Table 10. Antimicrobial activity of *lactobacillus* spp isolates from isolate iii

The different food pathogens such as *Bacillus*, *E.coli*, *Staphylococcus*, *Salmonella* were used for antimicrobial activity test. The *Lactobacillus* isolated from Isolate I, *E.coli* showed 11mm zone formation in 10^{-1} dilution, 10.5mm zone in 10^{-2} dilution, 8mm in 10^{-3} dilution, 7.8mm in 10^{-4}, 6.2mm in 10^{-5} and 5.4mm in 10^{-6} dilution. *Bacillus* showed 9mm in 10-1, 8.2mm in 10^{-2}, 7.5mm in 10^{-3}, 7mm in 10^{-4}, 6mm in 10^{-5} and No zone formation in10^{-6}. Staphylococcus showed 9mm in 10^{-2}, 9mm in 10^{-2}, 8.2mm in 10^{-3}, 6.8mm in 10^{-4}, 6.2mm in 10-5 and 5.5mm in 10^{-6} dilution. *Salmonella* showed 9mm in 10^{-1}, 9mm in 10^{-2}, 5.8mm in 10^{-3}, 5.4mm in 10^{-4}, and No zone formation was noticed in 10^{-5} and 10^{-6} dilution. (Table 8)

Antimicrobial activity of *Lactobacillus* isolates from Isolate II, *E.coli* showed 11.2mm in 10^{-1} dilution, 10.4mm in 10^{-2},8.8mm in 10^{-3},7.4mm in 10^{-4},6mm in 10^{-5}, 5.4mm in 10^{-6} dilution. *Bacillus* showed 9mm in 10-1,8.2mm in 10-2,8.8mm in 10^{-3},7mm in 10^{-4}, 6mm in 10^{-5}, and No zone formation was noticed in 10^{-6} dilution. *Staphylococcus* showed 8mm in 10^{-1},7.8mm in 10^{-2}, 6.2mm in 10^{-3}, 6mm in 10^{-4}, 5.5mm in 10^{-5}, No zone formation in 10^{-6} dilution. *Salmonella* showed 9.4mm in 10^{-1}, 9mm in 10^{-2}, 6.4mm in 10-3, 5.8mm in 10^{-4}, 5.2mm in 10^{-5}, No zone formation in 10^{-6} dilutions. (Table 9)

Antimicrobial activity of *Lactobacillus* isolates from Isolate III. *E.coli* showed 11.8mm in 10^{-1}, 11.6mm in 10^{-2}, 9.4mm in 10^{-3}, 8.8mm in 10^{-4}, 6.6mm in 10^{-5}, 5.4mm in 10^{-6} dilutions. *Salmonella* showed 9.4mm in 10^{-1}, 9mm in 10^{-2}, 8.2mm in 10^{-3}, 6.4mm in 10^{-4}, 5.8mm in 10^{-5}, and 10^{-6} dilutions. *Bacillus* showed 8.8mm in 10^{-1}, 7.6mm in 10^{-2}, 6.8mm in 10^{-3} and $10^{-4,}$ 5.2mm in 10^{-5} and No zone formation in 10-6 dilutions. *Staphylococcus* showed 9mm in 10^{-1}, 8.6mm in 10^{-2}, 7.1mm in $10^{-3,}$ 5.9mm in 10^{-4}, 5.2mm in 10^{-5}, No zone formation 10^{-6} dilutions (Table 10).

Discussion

Lactobacillus is the most widely encountered for probiotics because they display numerous antimicrobial activities. The beneficial role of lactic acid organisms in preserving integrity and health has been documented (Gilliland., 1990). Probiotics bacteria have been shown to improve the clinical outcome in many intestinal disease targets (Elmer *et al.*,1996). The influence of probiotic bacteria on the immune response has been thoroughly reviewed (Perdigon and Alvarez,1992; Perdigon *et al.*, 1995). Biological effects correlated with enhanced immune function, such as protection against viral and bacterial pathogens and tumor inhibition, have also been measured (Marteau and Rambaud, 1993). The ability of certain probiotic *Lactobacilli* and *Bifidobacteria* to enzymatically deconjugate bile acids has also been suggested to have a role in regulating cholesterol levels in humans (De Smet *et al.,* 1994). Lactic acid bacteria may improve intestinal mobility and relieve constipation, particularly in people who consume Probiotics. (Seki *et al.*, 1978). Probiotics may exert a beneficial effect on allergic reaction by improving mucosal barriers function. Probiotic consumption by young children may improve immune system development. Probiotics such as lactobacillus GG may be helpful in alleviating some of the symptoms of food allergies such as those associated with milk protein(Majamaa and Isolauri, 1997). According to the present study the *Lactobacillus* isolated from curd, milk and cheese did not produce H_2O_2 which was similar to the findings of Yuksekdag *et al.,* 2004. Growth of *L.acidophilus* will also reduce the milk redox potential and thereby stimulate growth of *Bifidobacterium.* However, *Bifidobacterium* can also be inhibited by a fast growing *Lactobacillus* strain. It was confirmed by Gomes and Malcata.,1999.

Conclusion

The ability of *Lactobacillus* isolates in the rice fermentation of batter was studied. Growth curve of species isolated from milk products identified by bioreactor. Immobilization technique was carried out to detect the efficiency of a probiotic, and also entrapped in different dilutions. *E.coli* was more susceptible than *Salmonella, Bacillus, Staphylococcus.* The other organisms showed least zone formation in different dilutions.It can be concluded in the present study that Lactic acid bacteria had an inhibitory effect against *E.coli and Bacillus* Spp was found to be less susceptible and least zone formation was formed.

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