



Volume 8, Issue 1, January 2019, www.ijfans.com e-ISSN: 2320-7876

**INTERNATIONAL JOURNAL OF FOOD AND
NUTRITIONAL SCIENCES**

IMPACT FACTOR ~ 1.021



Official Journal of IIFANS

Research Paper

Open Access

ASSESSMENT OF POTENTIALITY OF BIFIDOBACTERIA ANIMALIS SUBSP LACTIS. (HBM-NIN-06) ISOLATED FROM HUMAN BREAST MILK AND ITS USE IN THE DEVELOPMENT OF PROBIOTIC CURD**G Madhavi¹, M Shiva Prakash^{2*}, G Sumalatha¹, K B Chathyushya³, R Hemalatha⁴, Archana Giri⁵ and M Alimelu⁶****Corresponding Author: M Shiva Prakash, ✉ drmspnin@gmail.com*Received on: 31st October, 2018Accepted on: 21st November, 2018

Bifido's are widely used in the treatment of many diseases both in humans and animals in addition to their incorporation in aquatic feeds etc, therefore keeping this in view we have made an attempt to isolate this probiotic micro flora of human origin which may have colonization in human intestine and prevent or protect from common diseases viz.diarrhoea, irritable bowel syndrome, constipation, etc., including immunomodulation effects of both mucosal and systemic immunity. The *Bifidobacterium animalis sub sps lactis* (HBM-NIN-06) was isolated and identified from human breast milk by morphology, Gram's staining and biochemical tests, etc. This was further confirmed for Genus and Species by molecular methods (PCR) using specific primers. This Bifido had good probiotic potentials such as resistance to pH, bile salt tolerance at 0.3%, resistance to number of routinely prescribed antibiotics in addition this has shown to have anti-pathogenic effects on some food borne pathogens. Based on these observations we have developed a probiotic curd using starter cultures of *Lactobacillus bulgaricus sub sps delbruki* (UBLB-38) and *Streptococcus thermophilus* (UBST-50) with incorporation of the above isolated *Bifidobacterium animalis* (HBM-NIN-06). This Probiotic curd was assessed for its sensory evaluation on human volunteers and was found to have 90% acceptability and their viability retained upon storage at 4-8 °C for about one month Therefore the *Bifidobacterium animalis subsp lactis* which is of human origin and from Indian women may have immense health benefits therefore we have developed a food supplement which may be valuable for therapeutical application on human health.

Keywords: Human breastmilk, Bifidobacterium, Lactobacillusbulgaricus, Streptococcus thermophilus, Probiotic dahi

INTRODUCTION

Human breast milk contains attractive probiotic organisms, i.e., *Lactobacilli* and *Bifidobacterium* which are having

health benefits on the host. The microflora of human origin, have easy adaptation on dairy substrates and considered to be safe for infants. The probiotic curd (Indian dahi) is a

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popular fermented product of India which plays an important role as a food supplement along with regular diet. This is consumed by people all over India with a minimum 50-100 grams at a time or per day. This curd is traditionally viewed as a natural healing food supplement which has proteins, carbohydrates, fats, vitamins and minerals that are easily digestible. It is also designated as a functional food with probiotics that boosts natural as well as acquired immunity (This is used for treating various diseases like inflammatory and Irritable Bowl Syndrome (IBS), diabetes, hypercholesterolemia and cancer (Shadnough and Shaker, 2013)), etc. Based on this available information we have made an attempt to isolate and identify Bifido's from human breast milk and study their probiotic potential for their use in the development of probiotic curd for human consumption.

MATERIALS AND METHODS

The Human Breast milk samples of about 3-5 ml were collected under strict aseptic conditions from 20 lactating mothers at Niloufer Hospital, Osmania Medical College, Hyderabad, India. Out of 20 samples we could isolate five (5) Bifido's using standard microbiological, biochemical tests and confirmed the Genus and species with PCR and sequencing, etc. (Shiva Prakash and Madhavi, 2018).

Probiotic Properties of Isolates

Among these 5 strains, i.e., *Bifidobacterium animalis subs lactis* (HBM-NIN-06) was randomly selected and studied in detail for their probiotic potentials for its subsequent use in the development of probiotic curd. The probiotic properties include resistance to acid (pH 2, 3, 4), tolerance to Bile salts at (0.3%), antibiotic resistant/sensitivity against common antibiotics and its anti-pathogenic effect on various food borne pathogens tested.

Acid Resistant

The isolated Bifido colonies were inoculated into Bifido broth and incubated at 37 °C for 24 hrs under anaerobic conditions. After incubation the cells were harvested by centrifugation for 10 min at 5000 rpm at 4 °C. The pellet were washed once in phosphate-saline buffer (PBS at pH 7.2) and resuspended in PBS (pH3) which consists of 0.25% L-cysteine. The above incubated culture was plated on Bifidobacterium agar and appropriate dilutions were done and plates incubated at 37 °C under anaerobic conditions for 48 h.

Bile Salt Tolerance

The Bifido cultures were incubated overnight at 37 °C for 24 hrs and centrifuge at 5000 rpm at 4 °C for 10 mins. The pellet was washed and cells were suspended in the PBS which contain 0.3% of bile was incubated for 4 hrs at 37 °C. The viable colonies were enumerated at every hour by using colony counter.

Antibiotic Resistance

The antibiotics sensitivity was carried with ampicillin (10 mcg), vancomycin (30 mcg), chloramphenicol (25 mcg), erythromycin (15 mcg), penicillin (1 unit), cephalothin (30 mcg), clindamycin (2 mcg), and oxacilin (1 mcg) Streptomycin (10 mcg), sulphatrid (300 mcg), and tetracycline (25 mcg). The antibiotic susceptibility of isolated Bifido bacteria was assessed using antibiotic discs by diffusion method (supplied by Hi-media) on Bifido bacterium agar plates. A 100 µl suspension of freshly grown Bifido bacterial cultures was spread on Bifido agar plates, placed with antibiotic discs on the surface of agar and incubated at 37 °C for 24 hrs.

Anti-Pathogenic Effect

For this test, spot or lawn method was used. The indicator pathogens were inoculated into nutrient broth and incubated at 37 °C for 16-18 hrs. These were over night incubated with cultures plated with indicator organisms like *E.coli* (MTCC443), *Pseudomonas* (MTCC741), *Bacillus cereus* (MTCC44), *Staphylococcus aureus* (MTCC96) (strains were obtained from MTCC Chandigarh, India) and incubated further for obtaining. Measurement of diameter of zone. The zone of inhibition was measured using a calibrated scale and bigger than 1 mm was considered to have antimicrobial activity (Fleming et al., 1985).

Development of Probiotic Curd

About 100 ml of cow's raw milk was procured from locally available Gowshala (dairy farm) and pasteurized. This was cooled to 40 °C for culturing of *Lactobacillus bulgaricus* (UBLB-38) and *Streptococcus thermophilus* (UBST-50) obtained from Unique Biotech Pvt. Ltd. Hyderabad at free of cost along with our identified Human breast milk isolate *Bifidobacterium animalis subs lactis* (HBM-NIN-06) under strict sterile and aseptic conditions. All samples were incubated at 37 °C for 5-6 hours for activation of culture to set the curd. After which these were confirmed for the presence of all the three probiotics. The developed probiotic curd was assessed for their sensory evaluation and viability

checked upon storage at 4 to 8 °C at various time periods, i.e., 7, 14, 21 and 30 days.

Sensory Analysis of the Developed Probiotic Curd

The sensory evaluation was carried out by trained panel of assessors on 9-point hedonic scale (Amerine *et al.*, 1965). Samples were served in plastic cups at temperature of 20 °C as recommended for sensory evaluation of fermented dairy products (IDF, 1997). Evaluation was based on the score of color, taste, flavor, consistency and overall acceptability. Each panel list scores were reflected on a hedonic scale of 1 to 9 where 1 = dislike extremely and 9 = like extremely. These were also checked and found to have the international standard FIL/IDF as described that the probiotic products should be contain a minimum of 10⁶ viable probiotic bacteria per gram of product at the time of consumption for claiming of health and functional aspects (Samona and Robinson, 1991; and Roy, 2005). The counts of viable bacteria upon storage retained about 10⁶ up to a period of 14 days thereafter declined with respect to all the probiotics used in the developed curd.

RESULTS AND DISCUSSION

We could achieve the isolation of *Bifidobacteria* despite for their presence of relatively low numbers in the Human Breast milk samples obtained. However we have confirmed about five (5) Bifido's by microbiological, biochemical and molecular techniques. Among these one of them was randomly selected and labeled as HBM-NIN-06. This *Bifidobacterium animalis subsps lactis* was evaluated for their probiotic properties. Our results on acid resistant (pH) and bile salt tolerance (3%) observed are in agreement with the earlier reports of Gueimonde *et al.* (2007) and Fernandez (2013) who also reported similar results. Further these results were in conformity with other investigators (Lankaputra and Shah, 1995; and Mishra, 2001). This Bifido was showing resistant to antibiotics like vancomycin and sensitive to gentamycin, erythromycin and chloramphenicol (Table 1). This information may be important as it is known that the probiotic microflora harbor transferable antibiotic/resistance genes and protect pathogenic disease causing bacteria in humans.

The Bifido HBM-NIN-06 showed the zone of inhibition of various food borne pathogens viz. *E.coli* (6 mm) *Staphylococcus aureus* (5 mm), *Bacillus cereus* (8 mm) and *Pseudomonas aeruginosa* (14 mm) indicating their role

Photograph 1: Showing the (A) Resistance at pH 2 for 3 hr, (B) Resistance to Bile Salt (0.3%) Breast Milk Isolate *Bifidobacterium Animalis subsp. Lactis*. (HBM-NIN-06)

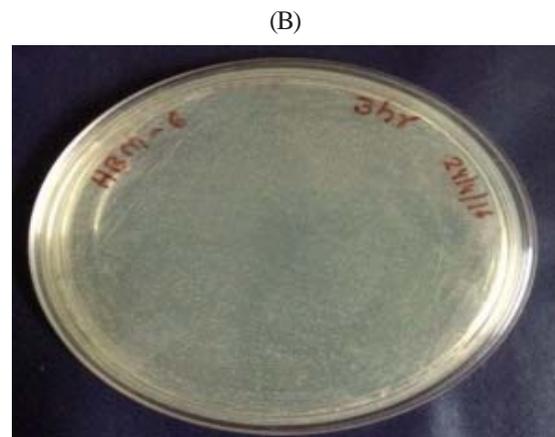
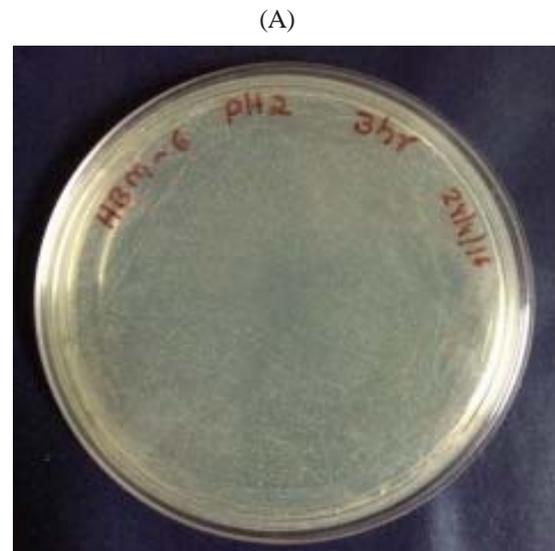


Table 1: Showing the Antibiotic Activity of the *Bifidobacterium* (Breast Milk Isolate)

Antibiotic	Conc (mcg)	Resistant/Sensitive
Ampicilin	10	Sensitive
Erythromycin	1	Resistant
Streptomycin	10	Resistant
Oxacilin	1	Resistant
Vancomycin	30	Sensitive
Chloramphenicol	25	Resistant
Clindamycin	2	Sensitive

Photograph 2: Showing the (C) Antibiotic Activity, (D) Diameter (mm) of the Inhibition Zones Produced by *Bifidobacterium Animalis subsp. Lactis*. (HBM-NIN-06) Against Various Food Borne Pathogenic Bacteria

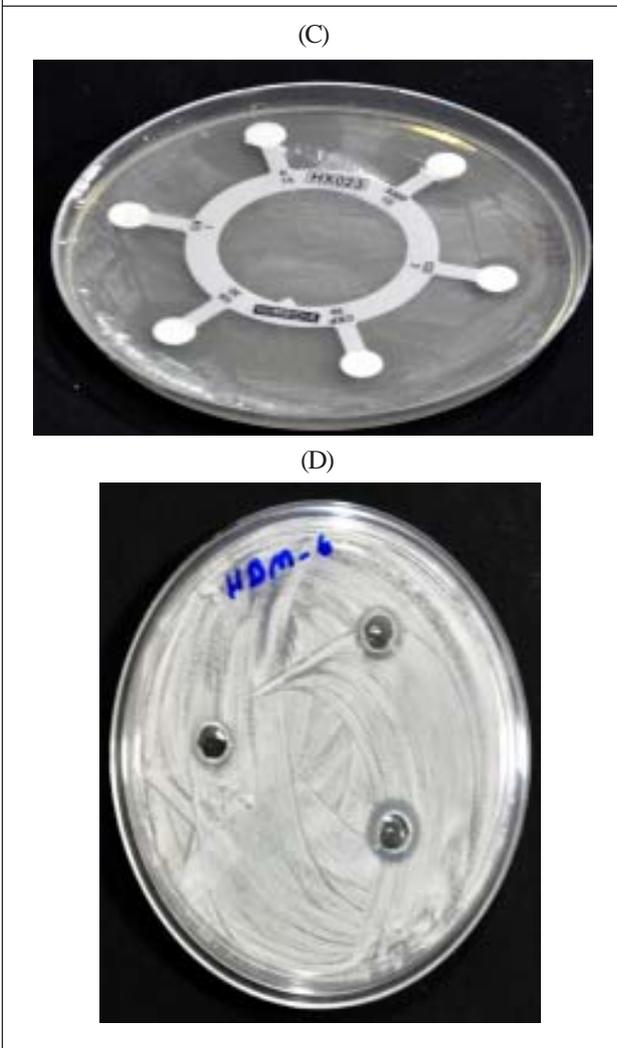


Table 2: Showing the *in Vitro* Antibacterial Activity of (Breast Milk Isolate) *Bifidobacterium Animalis Subsp. Lactis*. (HBM-NIN-06)

Indicator organisms/Food Borne Pathogens	Bifido Std. Diameter (mm)	HBM-NIN-06 Diameter (mm)
<i>E.coli</i>	4	6
<i>Staphylococcus aureus</i>	6	8
<i>Bacillus cerus</i>	3	5
<i>Pseudomonas aeruginosa</i>	10	14

Photograph 3: Showing Preparation of Probiotic Curd (*Lactobacillusbulgaricu* + *Streptococcus Thermophilus* and *Bifidobacterium Animalis subsp. Lactis*. (HBM-NIN-06)

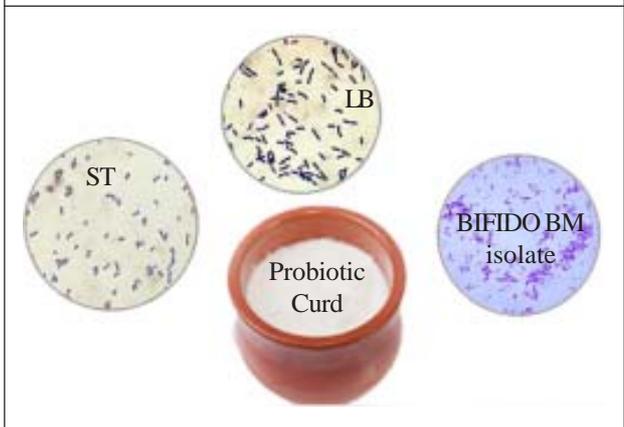


Table 3: Showing the Viability of the *Bifidobacterium Animalis subsp. Lactis*. (HBM-NIN-06) in the Developed Curd at Different Time Points Upon Storage at 4-8 °C

Probiotic Curd	0 Day (cfu/gm)	15 Day (cfu/gm)	30 Day (cfu/gm)
LB+ST+HBM-NIN-06	8×10^9	9.5×10^9	9.5×10^9
LB+ST+BifidoSTD	6×10^9	5.0×10^9	5×10^9

Note: This paper was presented and awarded from *International Journal of Food and Nutritional Science (IJFANS)* held during May 26-28th 2016 at JNU campus, New Delhi.

in production of biological antibiotics (ex. bacteriocin and acidiphilin) by this microflora. (Photograph-2 and Table 2)). However this needs to be further investigated for their nature of production which may be good substitute in place of antibiotics that are proven to be threat and dangerous that carry resistance to other pathogenic bacteria (Gibson and Wang, 1994).

The sensory evaluation of the developed product was found to have 90% acceptability by human voluntaries. The shelf life of the developed food supplement showed viability of all the probiotics up to a period of one month upon storage at 4 °C.

Viability of Bacterial Counts

The developed probiotic curd with starter cultures *Lactobacillus bulgaricus* and *Streptococcus thermophilus* with incorporation of our newly identified *Bifidobacterium animalis subsp lactis* (HBM-NIN-06) showed that the

bifidobacteria had a prolong the shelf-life probiotic curd during storage in comparison with standard probiotic curd (Table 3).

In this study, fermentation was limited in refrigerator but it was reported that bacteria cells have little or some fermentative activity even during storage at 6 °C (Nighswonger *et al.*, 1996). The Bifido's may more health benefits when consumed as reported in the literature and can also play a protective role against food borne pathogens during storage by competing for nutrients, producing organic acids and bacteriocins (biopreservation). Biopreservation is the extension of storage life and enhancing of safety of foods using the natural or controlled micro flora and/or their antimicrobial products was reported by Rodger (2001) and Alegre *et al.* (2011). In the present study the results of sensory evaluation of the developed product was more than 90% acceptability. The scores allocated for colour, flavour, taste and consistency showed that during the first 7 days of the storage period the developed Bifido curd (BM isolate) had the highest sensory acceptability compare with standard curd.

CONCLUSION

From this study it is understood that that the Human Breastmilk may be a good source for Bifido's that are useful in the development of dairy based food supplements for human consumption in the management of health especially for infants, malnourished children and other vulnerable individuals. However further studies are required for confirming their health benefits.

ACKNOWLEDGMENT

We hereby convey our sincere thanks to Indian Council of Medical Research (ICMR), New Delhi for providing Senior Research Fellowship to G Madhavi with (File No-3/1/29/2014-Nut). We also thanks to Dr. Ratna Sudha, Manager Director, Unique Biotech. Pvt. Ltd. Hyderabad, India for providing us the required probiotics at free of cost.

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