Research Article
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DETERMINATION OF VIABILITY OF *PEDIOCOCCUS SPP*. GS4 AFTER STORAGE INTO HARD GELATIN CAPSULE AND ITS SURVIVAL UNDER *IN VITRO* SIMULATED GASTROINTESTINAL CONDITION

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Received on: 15/01/12 Revised on: 11/02/12 Accepted on: 18/03/12

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ABSTRACT
Probiotics are regarded as panacea for modern life. Aim of the study was to formulate the capsule comprising probiotic strain, *Pediococcus* spp. GS4 isolated from fermented food, *Khadi* and to examine its viability with and without excipients by exposing to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) at different time intervals. The empty hard gelatin capsule was filled with FDPP [Freeze dried powder of *Pediococcus* spp. GS4] aseptically adding excipients (lactose, ascorbic acid and inulin) as Test and without excipients as Control. For stability studies both Test and Control capsules were stored at 4 ±1°C for 28 days. *In vitro* viability of probiotic cells were studied using SGF and SIF respectively. The optimal composition of effective formula was found to be composed of 6% lactose, 2.5% ascorbic acid and 2% inulin which could protect maximum viability of cells. After 28 days, the viability of Test was improved by 3.73 logs (CFU/ml) as compared to Control at 4 ±1° C ($P < 0.05$). Significant difference was observed between Test and Control when incubated sequentially in SGF (pH 2.5; 45 min and 90 min) and SIF (pH 6.8; 150 min and 210 min). Hence, *in vitro* test showed that combination of suitable excipients have significant effect on the survival of *Pediococcus* spp. GS4 when exposed to gastrointestinal conditions (SGF, SIF).
KEYWORDS: *Pediococcus* spp. GS4, excipients, simulated gastric fluid (SGF), simulated intestinal fluid (SIF), capsule, survivability

INTRODUCTION

Probiotic is a term that means "for life", more precisely "live microorganisms that beneficially affects the host beyond improving the intestinal microbial balance"[1]. Presently probiotics are defined as "live microorganisms which when administered in adequate amount confer a health benefit on the host"[2]. Probiotics, especially *Lactobacillus* and *Bifidobacterium* have been suggested to be associated with treatment of lactose intolerance, prevention and cure of bacterial infection, enhancement of immune response by modulation of cytokine gene expression, prevention of urogenital infections by colonization resistance, production of hydrogen peroxide as well as bio surfactants, treatment of hypercholesterolemia by assimilation of cholesterol within bacterial cells[3, 5]. Research showed that the antagonistic effect of *Pediococcus* against the pathogenic microbes like *Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogens* and *Staphylococcus aureus* resembles to their intrinsic properties primarily due to secretion of lactic acid and bacteriocin[6, 7]. *Pediococcus* spp. GS4 isolated from the well known Indian fermented food *Khadi*. It has similar biochemical characteristics like other Lactic acid bacteria (LAB) (acid tolerant, bile salt tolerant, lactic acid homofermenter, beta- galactosidase producer, Vancomycin resistant, assimilate cholesterol, anti-oxidative) and exerts its antimicrobial effect by secreting bacteriocin like compounds known as *Pediocin* and thus it is generally regarded as safe (GRAS)[8, 11]. Thus, this excellent probiotic strain has been selected for delivery and studied to develop suitable formulation(s) for beneficial bacteriotherapy which may exert beneficiary effects by production of inhibitory substance, blocking of adhesion sites, competition for nutrients and stimulation of immunity[12].

Prebiotics are non-digestible food ingredients that stimulate the growth and/or activity of bacteria in the digestive system in ways claimed to be beneficial to health. Prebiotics such as inulin (polysaccharides) acts as protective agent and emulsifier in probiotic formulations[13]. It has been reported that milk sugar such as lactose exerts protective effect on lactic acid bacteria (LAB) during storage[14]. Ascorbic acid was used as antioxidant agent to inhibit the oxidation of membrane lipids of probiotic strains and thus acts as protective agent and maintain the survivability of the LAB [14, 15].

Probiotic effect can only be acquired if sufficient numbers of viable bacteria survive passage through the stomach and can be delivered to the site of action. Although ample information is available promoting the beneficial effects of probiotic, formulating probiotic cells in viable forms is still the challenging task[16, 17]. These challenges include short shelf life even when stored at low temperature, safety and functional aspects, survival of the probiotic strain till it reaches the site of action and effect of food matrices on sustainability of probiotic in gastrointestinal tract [18, 19].

In present study, two trials have been conducted to formulate the probiotic capsule and the viability has been evaluated by exposing the capsule content to SGF and SIF successively up to 28 days.

MATERIALS AND METHODS

Materials

All the media and components including lactose, inulin, ascorbic acid, MRS broth and agar were procured from Himedia, India. All chemicals were of reagent grade.

Microbial growth curve analysis

The probiotic potential of the LAB strain was previously isolated and characterized from Indian fermented food *Khadi* and identified as *Pediococcus* spp. GS4 by 16S rRNA sequencing (Accession number in Gen- Bank: NCBI HM044322)[8]. The *Pediococcus* spp. GS4 strain was maintained at -20 ^{0}C in Man Rogosa Sharpe (MRS) broth supplemented with 20% (v/v) glycerol.

For growth curve analysis, the stock culture was rejuvenated over three sub-culturing followed by inoculation (2% v/v) into MRS broth (100 ml) and incubation at 37 ^{0}C[20, 21]. At regular 4 h interval, an aliquot of 1 ml sample was serially diluted (1:10) using normal saline solution (0.9%) and was plated onto MRS agar media. Simultaneously, optical density (OD_{600}) of grown culture was determined using UV-Spectrophotometer (Shimadzu, Japan) up to 24 h. The viable cells were determined as colony forming unit (CFU/ml) by plate count method.

Preparation of *Pediococcus* spp. GS4 powder

The *Pediococcus* spp. GS4 was likely sub-cultured and 2% (v/v) were inoculated into fresh MRS broth and incubated at 37 ^{0}C for 12 h (Figure 1). Thus cells in early stationary phase were harvested by centrifugation at 10,000 rpm for 10 min and washed thrice and resuspended in PBS (phosphate buffer saline, pH7.2). Each resuspension was transferred into sterile petridish (13mm), frozen at −85 ^{0}C and freeze-dried for 18 h in a freeze-dryer (Thermo Fischer Micro Modulyo Freeze Dryer, USA) [16, 22]. Thus, the freeze dried *Pediococcus* powder (FDPP) was stored in sterile Eppendorf tube at 4 ± 1^{0}C for future use.

Procedure for Capsule filling

FDPP (2g) and the mixture of excipients comprising 6% lactose, 2.5% ascorbic acid and 2% inulin were blended aseptically and were considered as Test and without excipients as Control. Formula mixture was divided into 100 mg and filled in empty hard gelatin capsules. All capsules were stored at temperature of 4 ± 1 ^{0}C.

Estimation of viability

Viability of cells in Test during storage was determined by a plate count method at regular intervals of time up to 28 days. The determination of number of CFU/ml was performed in duplicate. The powder into the capsule was rehydrated in 1 ml of PBS and suspensions were serially diluted in sterile normal saline solution and colonies were enumerated after incubation of plates at 37 ^{0}C for 24 h. Results were expressed as log CFU/ml, and performed in duplicates.

Survival and enumeration of *Pediococcus* spp. GS4 with excipients in simulated gastric and intestinal conditions

The survival rates of Test were studied under simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) conditions. SGF and SIF were prepared following USP XXX method and performed according to the method reported earlier with brief modifications [13].

SGF was prepared by dissolving 0.2% NaCl (w/v) in distilled water and adjusting the pH at 2.5 with a 1N HCl solution. The solution was filter sterilized (0.45µ).

SIF was prepared by dissolving 0.68g of potassium dihydrogen phosphate (KH_2PO_4) in 25 ml of distilled water (solution A). And 7 ml of 0.2 M sodium hydroxide (NaOH) was made up to 50 ml with distilled water (solution B). Both solutions were mixed and were made up to 100 ml with distilled water and adjusted to pH 6.8.

The capsule was rehydrated in 1 ml of PBS and added to Erlenmeyer flask (50 ml) containing 6 ml of SGF (pH 2.5), incubated at 37 ^{0}C , with constant agitation at 100 r/min in shaker water bath, for 90 min. In the next step, the pH of sample was increased by adding SIF and pH was adjusted to 6.8 with 1N NaOH. Samples were incubated at 37 ^{0}C through 120 min to 210 min under constant agitation. SGF and SIF tests were performed in duplicate. Aliquots of 1 ml were removed after interval of 0, 45, 90, 150, 210 min and serially diluted with normal saline solution. The cell suspension was then enumerated by spread plate method. Plates were incubated for 24 h at 37 ^{0}C. The colonies formed were counted with the aid of colony counter (Servewell Instrument Pvt. Ltd., India). Same procedure was followed for Control. Results were calculated as log CFU/ml.

Statistical analysis

All experiments were done in duplicate. Data were analysed by the one-way analysis of variance (ANOVA) of duplicate trials. P-value ($P < 0.05$) was considered statistically significant. Computations were done using Excel 2007 for Windows 7.

RESULTS AND DISCUSSION

Microbial growth curve analysis

Determination of pre stationary viable cells of a bacterium is pre-requisite to its shelf life study. Growth curve study revealed that the *Pediococcus* spp. GS4 reached to its pre stationary phase (optical density of 1.933 at 600nm) at the elapse of 12 h with 8.27 log CFU/ml viable cells (Figure 1). Importance of incorporation of bacteria at pre stationary phase is to increase shelf life and viability of the probiotics[21, 23]. The *Pediococcus* spp. GS4 showed 9.36 logs CFU/ml at 16th h and 6.90 logs CFU/ml at 24th h. The corresponding OD_{600} are 2.191 and 2.528 respectively (Figure 1).

Estimation of viability

The study result showed that there was significant sustainability of cells with excipients (Test) as compared to Control. The survivability of Test capsule showed 9.53 log CFU/ml viable count at the end of 1st day whereas 9.22 log CFU/ml at 14th day and 7.03 log CFU/ml at 28th day. This estimates the total loss of 2.5 log CFU/ml after 28 days since the inception of the study (Figure 2). Cell viability of Control was estimated to be 3.3 log CFU/ ml after at 28th day. Thus after 28 days the viability of Test was improved by 3.73 log (CFU/ml) as compared to control at 4 ±1 ºC ($P < 0.05$).

Significant difference in viability may attribute to emulsifying effect of inulin, cryoprotection of lactose and anti-oxidative activity of ascorbic acid. Result showed that the viability was not sustained with excipients rather declined gradually over the study period. This referred that none of the excipients used had effect on catabolic process and could not act as source of energy for *Pediococcus* spp. GS4. Moreover, cells in question were freeze-dried hence there was restriction of metabolic process. Freeze dried powder constitutes three states of

probiotic bacterial cells namely live or uninjured, sub lethally damaged and dead[21]. Properties of rehydration medium are also accountable for probiotic cell recovery. On rehydration the suspension of bacterial cells and excipients resulted into healing medium for injured cells. During this stage only lactose might play a role of dietary supplement to the uninjured probiotic cells.

Survival and enumeration of *Pediococcus* spp. GS4 with excipients in SGF and SIF conditions

The viability of probiotic strain was found declining while passing through simulated GI conditions. There was at least 4.88 log CFU/ml reduction in Test capsule after 90 min exposure to SGF on 1st day. Significant reduction in viable count was determined after passing through SIF for 210 min ($P < 0.05$) Figure 3[I]. On the other hand, reduction of 7.82 log CFU/ml was estimated after 210 min in case of control on 1st day of analysis. After 7 days of refrigerated storage, survivability of *Pediococcus* spp. GS4 was reduced considerably for all the samples studied as shown in Figure 3[II], [III], [IV] and [V]. This reduction in viable cell count may be due to autolysis.

The recommended dose for significant effects of probiotic was stipulated to be 10^9-10^{11} viable cells per dose. Use of suitable excipients and delivery system remain the choice for efficient formulation. However, our results indicated that protective effects of excipients on *Pediococcus* spp. GS4 in gastric and intestinal conditions could be achieved by proper selection and by determining their compatibility with each other. In a separate study, it was showed that optimum probiotic effect could be observed when these probiotic bacteria colonised in intestine by adhering to intestinal gut mucosa[19]. Again, the pharmacokinetic profiles of probiotics varied with the strain, its source of isolation, mode of delivery and formulation with respect to ingestion of food and fasting conditions, as reported elsewhere[5].

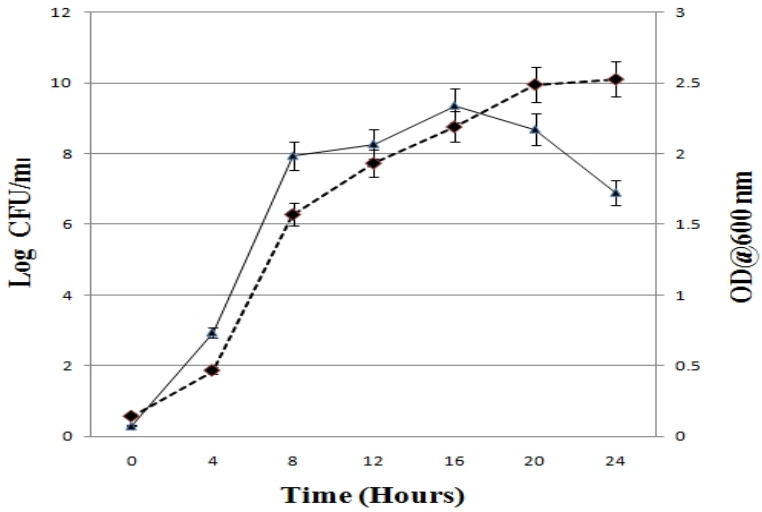

Figure 1: Growth curve of *Pediococcus* spp. GS4 at 37° C for 24h plotted against OD_{600} ( ) and Log CFU/ml ( ) with 4h interval of time

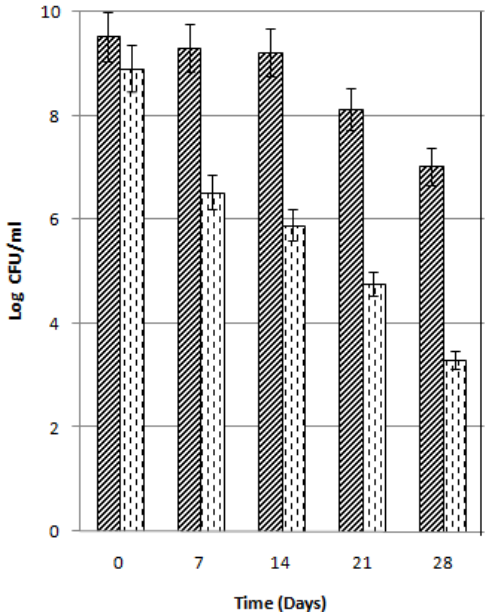

Figure 2: Survival of *Pediococcus* spp. GS4 over the period of 28 days stored at 4 ± 1° C, *Pediococcous* spp. GS4 with excipients ( ) and Control (without excipients) ( ).

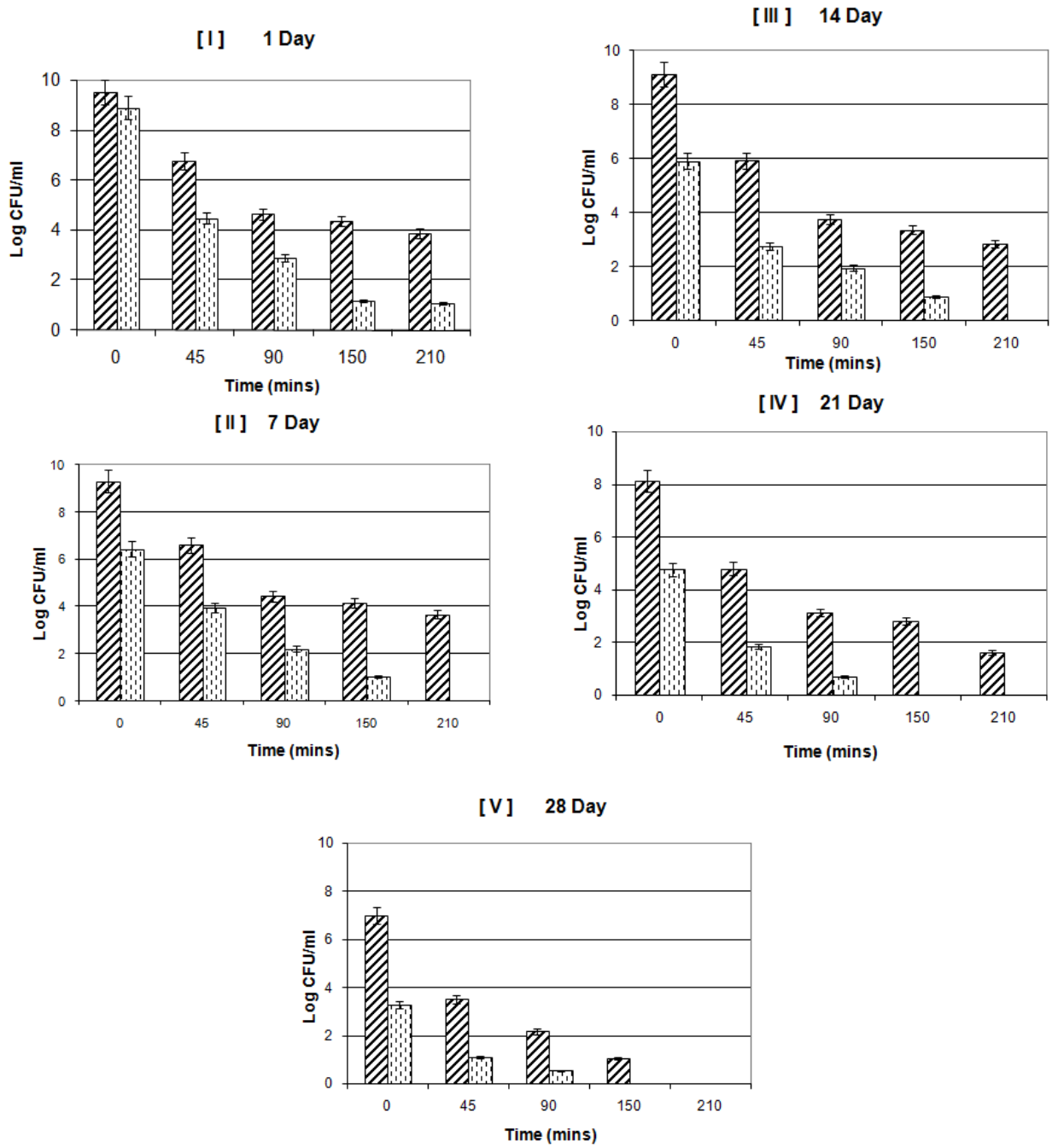

Figure 3: Survival of *Pediococcus* spp. GS4 with excipients (▨) and Control (without excipients) (▦) during storage of 1, 7, 14, 21 and 28 days (I, II, III, IV and V respectively) in SGF and SIF for 45, 90, 150, 210 mins respectively.

Like some related studies, our results reconcile with and showed that probiotic strain with excipients persuade better survival than control in SGF and SIF because of protective activity of lactose and inulin assigned to prevention of injurious eutectic freezing of cell fluids by trapping salts in a highly viscous or glass-like phase[13]. Anti-oxidative effect of ascorbic acid seems to be good protection. Studies showed that ascorbic acid inhibit the membrane lipid oxidation which in turn increases the survival of cells and their storage in dried state[14]. In case of control as there was no protective agents and cells were not able to recover hence lead to decrease in viable cell count gradually since 14th day. The viability was completely lost at the end of 28 days. This reduction in viable cells may be due to susceptibility of young cell culture to freeze drying[18]. Three parameters accounted for product stability including (i) moisture content of freeze dried powder, (ii) storage conditions and (iii) type of bacteria among several others[24]. To date very few probiotic formulation are patented and approved for safe human use[25]. Hence formulation of probiotic drug is a challenging task.

In conclusion, our study indicates that freeze dried strain of *Pediococcus* spp. GS4 has more *in vitro* tolerance to SGF when it is delivered to site of action with excipients. Inulin, lactose and ascorbic acid altogether have protective effect and improve the viability of *Pediococcus* spp. GS4. Additionally frozen storage conditions recommended for the improvement of the viability of the cells. Our study indicates that probiotic strain with

excipients secured and maintained its probiotic properties during the storage of 28 days. Further studies to determine the viability of *Pediococcus* spp. GS4 with other excipients, improvement in shelf life, and retention of probiotic properties and formulation of new delivery system are in progress. We believe that this formulation may have industrial as well as therapeutic benefits in future.

ACKNOWLEDGMENT

The authors acknowledge VIT University, Vellore, India for providing the laboratory facilities and the funds for carrying out the research work.

REFERENCES

1. Fuller R. A Review: Probiotics in man and animals. J Appl Bacteriol 1989; 66:365-378.
2. FAO/WHO, Health and Nutritional properties of probiotics in food including powder milk with live Lactic Acid Bacteria, Report from joint food and Agriculture Organization (FAO) of the United Nations/World Health Organization (WHO) expert consultations, Argentina, 2001; 1-34.
3. Garai-Ibabe G, Teresa DM, Irastorza A et al. Naturally occurring 2-substituted (1,3)-b-D-glucan producing *Lactobacillus suebicus* and *Pediococcus parvulus* strains with potential utility in the production of functional foods. Bioresource Technology, 2010; 101: 9254–9263.
4. Reid G, Bruce AW, Smeianov V. The role of lactobacilli in preventing urogenital and intestinal infections. Intl Dairy J 1998; 8: 555–562.
5. Parvez S, Malik K A, Kang S, Kim HY. Probiotics and their fermented food products are beneficial for health. J Appl Microbiol 2006; 100: 1171–1185.
6. Daeschel MA, Klaenhammer TR. Association of a 13.6-megadalton plasmid in *Pediococcus pentosaceus* with bacteriocin activity. Appl Environ Microbiol 1985; 50:1538-1541.
7. Lee SH, Lillehoj HS, Dalloul RA et al. Influence of Pediococcus-Based Probiotic on Coccidiosis in Broiler Chickens. Poultry Sci 2007; 86:63–66.
8. Gowri Sukumar and Asit Ranjan Ghosh. *Pediococcus* spp. – A potential probiotic isolated from *Khadi* (an Indian fermented food) and identified by 16S rDNA sequence analysis. Afr J Food Sci 2010; 4(9): 597 – 602.
9. Gowri Sukumar and Asit Ranjan Ghosh. Study of the probiotic potential of lactic acid bacteria isolated from a variety of Indian fermented food. J Phar Res 2010; 3(9): 10.
10. Shruthy VV, Pavithra M, Gowri S et al. Probiotic Potential Among Lactic Acid Bacteria Isolated From Curd. Int J Res Ayur & Pharm 2011; 2(2):602-609.
11. Sukumar G , Ghosh AR. Anti-Oxidative Potential of probiotic Bacteria from Indian Fermented Food. Intl J Res Ayur & Pharm 2011; 2(3):983-986.
12. Wohlgemuth S, Loh G, Blaut M. Recent developments and perspectives in the investigation of probiotic effects. Int J Med Microbiol 2010; 300**:**3–10.
13. Buriti FCA, Cardarelli HR, Filisetti TM et al., Synbiotic potential of fresh cream cheese supplemented with inulin and Lactobacillus paracasei in co-culture with Streptococcus thermophilus. Food Chemistry 2007; 104: 1605–1610.
14. Zarate G, Nader-Macias ME. Viability and biological properties of probiotic vaginal lactobacilli after lyophilization and refrigerated storage into gelatin capsules. Process Biochem 2006; 41:1779–1785.
15. Castro HP, Teixeira PM, Kirby R. Storage of lyophilized cultures of Lactobacillus bulgaricus under different relative humidities and atmospheres. Appl Microbiol Biotechn 1995; 44:172–176.
16. Chan ES, Zhang Z. Bioencapsulation by compression coating of probiotic bacteria for their protection in an acidic medium. Process Biochem 2005; 40:3346–3351.
17. Klayraung S, Viernstein H, Okonogi S. Development of tablets containing probiotics: Effects of formulation and processing parameters on bacterial viability. Intl Journal of Pharmacy, 2009; 370:54–60.
18. Saarela M, Mogensen G, Fonden G et al. Probiotic bacteria: safety, functional and technological properties. J Biotech 2000; 84:197–215.
19. Saxelin M, Lassig A, Karjalainen H et al. Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt or cheese. Intl J Food Microbiol 2010; 144: 293–300.
20. Zwietering MH, Jongenburger I, Rombouts FM et al. Modeling of the bacterial growth curve. Appl Environ Microbiol 1990; 56:1875-1881.
21. Fatemeh S, Mustafa1 S, Arbakariya A et al. Optimization of a cryoprotective medium and survival of freeze-dried *Bifidobacterium infantis* 20088 throughout storage, rehydration and gastrointestinal tract transit for infant formula probiotic applications. Afr J Microbiol & Res 2011; 5(21): 3373-3384.
22. Zayed G, Roos Y. Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze drying and storage. Process Biochem 2004; 39:1081–1086.
23. Corcoran BM, Stanton C, Fitzgerald GF, Ross RP. Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice. Microbiology 2007; 153:291–299.
24. Wirjantoro TI, Phianmongkhol A. The Viability of Lactic Acid Bacteria and *Bifidobacterium bifidum* in Yoghurt Powder During Storage. J Natl Sci 2009; 8(1): 95-104.
25. Watson BF, Watson TS, inventors; Renew Life, Incorporations. Probiotic Formulation. US patent 5164183; 5310555; 5322836; 553.1989.

Source of support: Nil, Conflict of interest: None Declared