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Probiotic potential of lyophilized *Lactobacillus plantarum* GP

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Abstract

Purpose: Freeze drying of *Lactobacillus plantarum* GP in the presence of wall materials to achieve improved survival and retention of probiotic functionality during storage.

Methods: *L. plantarum* cells were lyophilized in the presence of inulin, fructooligosaccharides, lactulose, and/or skim milk. The lyophilized vials were stored at 8–10 °C up to 6 months and cells from these vials were evaluated for their probiotic functionality.

Results: *L. plantarum* GP freeze dried in the presence of wall material lactulose displayed viability of 98 ± 2.8% promising survival rate in the stress conditions of human digestive tract. The freeze dried cells of Lactobacilli retained the ability to adhere intestinal mucin layer, form biofilm, inhibit food spoilage and enteropathogens, produce β-galactosidase, bile salt hydrolase and γ-amino butyric acid, remove cholesterol, and scavenge DPPH radical.

Conclusion: Lyophilized cells of *L. plantarum* GP retained all the functional characteristics without any significant loss during storage, which prompts to incorporate prebiotics for the development of stable functional food products.

Keywords: *Lactobacillus plantrum*, Synbiotics, Prebiotics, Antioxidative, Cholesterol reduction

Introduction

Probiotics are redefined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (Hill et al. 2014). The rising interest and increasing demand by consumers for probiotics containing foods stimulated research related to this field since the year of 2000 (Jankovic et al. 2010). The strains characterized and used as probiotics belong to the group lactic acid bacteria (LAB). Several products including yogurt, cheese, ice cream, cereals, beverage powders, fruit juices, capsules, and chocolates have been developed and used as carriers for probiotic strains (Pavunc et al. 2011; Granato et al. 2015; Chavan et al. 2018; Konar et al. 2018). The dose and the viability of probiotic strains are important criteria for probiotic efficacy since the health benefits strongly depend on the viability of probiotic microorganisms. It has been suggested that a probiotic product should contain a minimum of 7 log cfu/g of viable cells at the time of consumption to confer health benefits (WHO 2006). However, a report has revealed lower survival rate of probiotics in traditional dairy products (de Vos et al. 2010). The poor survival of probiotics in probiotic products is attributed to processing conditions and environmental stress during storage such as pH and temperature variation as well as toxicity of oxygen and UV light. An adequate number of probiotic cells should retain the viability and functional properties during the storage and passage through stomach (Anal and Singh 2007).

The viability of probiotics may be increased by using prebiotics as prebiotics are resistant to gastric acidity, digestive enzymes of intestine, and environmental stress. Prebiotics have the potential in improving the stability and viability of LAB during processing, storage, and gastrointestinal tract transit (Burgain et al. 2011; Heidebach et al. 2012). Moreover, prebiotics also serve as fermentable carbohydrates for probiotic microorganisms in the colon (Gibson 1999). Hence, the combination of probiotic and prebiotics known as "synbiotics" is preferred

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as it selectively enhances the viable number of probiotic organisms (Gibson 1999; Gonzalez et al. 2011; Rodrigues et al. 2011). The selection of compounds is challenging as the variety of materials available in the market is huge. According to the guidelines (EAFUS 1998), the wall material should be of food grade as well as effective in protecting the probiotics. Compounds like sugar and skim milk also protect LAB during passage through the GIT and storage (Carvalho et al. 2004; Malik 1988). Besides this, material and lyophilization process should be compatible with the specific probiotic (de Vos et al. 2010). There are several methods, but not without limitations, like emulsification, coacervation, spray drying, and spray cooling to obtain dried viable cells which can be used in food products. Freeze drying therefore is a widely used method to produce dried viable LAB (Meng et al. 2008; Fonseca et al. 2015).

The objective of this work was to lyophilize the probiotic strain *Lactobacillus plantarum* GP isolated from grapes and to evaluate the changes in the probiotic efficacy upon lyophilization in the presence of different wall materials. The strain was selected on the basis of probiotic efficacy tested by various screening tests earlier (Shekh et al. 2016). Inulin, fructooligosaccharides and galactooligosaccharide (lactulose), skim milk powder, and sucrose were studied as wall materials to evaluate their influence on the stability and viability of probiotics during lyophilization and storage. The "synbiotics" were prepared by freeze drying and tested for survival in simulated oro-gastro-intestinal fluids, mucin adherence, biofilm formation ability, antibacterial activity, cholesterol removal, and antioxidant activity. The abovementioned probiotic properties were evaluated after the preparation of synbiotics and subsequently during the storage period of 6 months.

Materials and methods

Culture conditions and chemicals

Lactobacillus plantarum GP KF479387 (*L. plantarum* GP) isolated from grapes was grown on De Man Rogosa and Sharpe (MRS, pH 6.5) agar plates at 37 °C for 48 h. Isolated colony was inoculated in 5-mL MRS medium and subcultured twice in the MRS medium. One milliliter of activated culture was inoculated in 50-mL MRS medium, incubated for 18 h at 37 °C, and used for inoculating 1-L MRS medium for biomass production. Pure culture in MRS medium with 10% (v/v) glycerol was preserved at − 20 °C. *Lactobacillus rhamnosus* was used as reference strain throughout the study and was grown in identical cultural conditions as *L. plantarum* GP. The test microorganisms *Escherichia coli* MTCC 1697, *Enterobacter aerogenes* MTCC 111, *Salmonella typhi* MTCC 98, *Serratia marcescens* MTCC 97, *Shigella* sp., *Pseudomonas aeruginosa* MTCC 2587, *Proteus vulgaris, Klebsiella pneumoniae,* and *Bacillus* spp., were grown at 37 °C in static condition in 10-mL nutrient broth test tubes. *Staphylococcus aureus* MTCC 1144 was grown in test tube at 37 °C for 18 h in 10-mL Brain Heart Infusion broth for antimicrobial assay.

Lactulose was purchased from Sigma (USA). Fructooligosaccharides, inulin, and all other chemicals, if not indicated otherwise, were purchased from Himedia (India).

Capability to grow in the presence of prebiotics

The growth of *Lactobacillus plantarum* GP and *L. rhamnosus* GG was screened on various prebiotics including inulin, fructooligosaccharides, and lactulose by agar plate assay as described by Kaplan and Hutkins (2000). Ten microliters of cell suspension prepared according to Shekh et al. (2016) from 18-h *Lactobacillus* cultures was spotted on MRS agar medium containing the prebiotics as carbon source (1% w/v) and 300-mg/L bromocresol purple as a color indicator and incubated at 37 °C for 48 h. A color change around the developing colonies from purple to yellow was recorded.

Preparation of lyophilized cells

The cells were harvested from 1 L of 24 h culture of *L. plantarum* GP and *L. rhamnosus* GG grown (at 37 °C in static condition) in MRS medium by centrifugation (5000×*g*, 20 min, 4 °C). Cell pellet (1 g) was washed thrice with distilled water (D/W) to remove medium components and mixed with 1 g of wall material (1:1, cells:prebiotics/sucrose). 0.5 g of skim milk was added to another set of cryovials as cryoprotective agent in addition to 0.5 g of wall materials (2:1:1; cells:prebiotics/sucrose:skim milk; Table 1). Control contained cells without any wall material or skim milk. 300-μL aqueous solution (20×10^9 cfu) from the abovementioned

Table 1 Viability (%) of *Lactobacillus plantarum* GP and *Lactobacillus rhamnosus* GG cells lyophilized in the presence of wall materials

Wall materials	Viability (%)	
	L. plantarum GP	*L. rhamnosus* GG
Control	75 ± 1.5	58 ± 3.8
Fructooligosaccharide	90 ± 0.9	77 ± 0.6
Inulin	90 ± 2.1	76 ± 2.0
Lactulose	98 ± 0.4*	60 ± 1.0
Sucrose	95 ± 0.6*	75 ± 3.1
Fructooligosaccharide + skim milk	83 ± 4.3	80 ± 1.4
Inulin + skim milk	91 ± 1.1	74 ± 2.0
Lactulose + skim milk	90 ± 2.5	89 ± 2.6*
Sucrose + skim milk	83 ± 1.5	89 ± 0.9*
Skim milk	68 ± 3.6	75 ± 1.2

*values significantly different ($P < 0.05$)

Eppendorf was distributed in cryovials, frozen at − 20 °C for 18 h, and lyophilized using Micromodulyo 0230 (ThermoScientific, USA; − 50 °C, 8 h, 3.8 × 10^{-2} Torr). Lyophilized vials stored up to 6 months at 8–10 °C were evaluated for their probiotic properties and lyophilized cells with different wall materials were observed by scanning electron microscopy.

Enumeration of bacteria and percentage of viability

Lyophilized cells were uniformly suspended in 300 μL of phosphate buffer saline (PBS–pH 7, 0.1 M; 0.85% NaCl) according to Rajam et al. (2012). Aliquots from the samples (0.1 mL) diluted in N-saline (0.85% NaCl) were pour plated on MRS agar and incubated at 37 °C for 48 h. All the enumerations were performed in duplicate and plates containing 20 to 350 colonies were considered for determining viable cell count (log cfu/mL). The number of viable cells from the vials before (N_0) and after lyophilization (N) was evaluated and expressed as viability (%): $(N/N_0) \times 100$

Scanning electron microscopy of lyophilized cells

Lyophilized powders of Lactobacilli were fixed to a sample slide using conductivity adhesive tape and subjected to 10 mA process current for 2 min (Sputter Coater, SC7620, Emitech) to achieve gold coating. The morphology of gold-coated samples was observed by scanning electron microscopy at 10.0 kv (Zeiss, EVO-18).

Probiotic properties

Survival of lyophilized cells in oro-gastro-intestinal fluids

Simulated saliva (SS), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared as in our previous reports (Pithva et al. 2014; Shekh et al. 2016) to mimic the transit of lyophilized cells in human oral and gastrointestinal cavities. Fifteen microliters of cell suspension (10^9 cells) prepared from lyophilized vials as above was incubated in 1-mL SS, SGF, and SIF for 10, 120, and 180 min respectively. The viable number of cells after each fluid transit, i.e., SS, SS-SGF, and SS-SGF-SIF was measured by plating appropriate dilution on molten MRS agar and cell viability was expressed as log cfu/mL.

Mucin adherence

The adhesive capacity of lyophilized cells to intestinal mucin layer was evaluated according to Dhanani and Bagchi (2013) in 96-well microtiter plate coated with porcine stomach type III mucin (Sigma). Briefly, 300 μL of mucin (0.5 mg/mL) in sterile Dulbecco's PBS (Sigma) was added in microtiter plate well and held at 4 °C overnight for mucin coating. Wells were washed thrice with PBS to remove unbound mucin. Fifteen microliters of cell suspension was then added to the well and final volume was adjusted to 200 μL with PBS and held at 37 °C to allow adherence of cells to mucin layer for 90 min. Unbound cells were withdrawn and wells were washed five times with PBS. Adhered cells were extracted using 300 μL of Triton X-100 (0.05% v/v, prepared in sterile PBS) for 20 min at 37 °C. The adhered cells were counted by plating appropriate dilution on MRS agar.

Biofilm formation

Sterile 96-well round bottom microtiter plate was filled with 200-μL MRS broth per well. Fifteen microliters cell suspension was added to the well and incubated at 37 °C for 72 h without agitation. Unbound bacterial cells were removed by washing plates thrice with sterile PBS and surface attached bacterial cells were stained with 0.1% (w/v) crystal violet in isopropanol-methanol-PBS (1:1:18) for 30 min (Watnick and Kolter 1999; Nilsson et al. 2008). Excess dye was removed by washing wells three times with PBS. The residual dye bound to the surface adhered cells was extracted with 200 μL of dimethyl sulphoxide and estimated by measuring A_{570} using microtiter plate reader (BioTek, μQuant, USA). The amount of crystal violet (μg) bound to the surface of microtiter plate wells was determined using the standard curve of crystal violet.

Antibacterial activity

The antibacterial action of *Lactobacillus* cells against food spoiling and/or human pathogens was determined by spot inoculation method (Schillinger and Lucke 1989) with minor modification. In brief, 2 μL of cell suspension was spotted on MRS agar plate and incubated for 24 h at 37 °C. Further, 100 μL of test pathogen was mixed with 10 mL of nutrient or BHI agar (1%) and overlaid on previously spot inoculated MRS agar plates with Lactobacilli cells and incubated at 37 °C for 24 h.

Deconjugation of sodium taurocholate and sodium glycocholate

Ten milliliters volumes of MRS broth supplemented with 0.2% (w/v) sodium glycocholate (Sigma) and 0.2% (w/v) sodium taurocholate (Sigma) were inoculated with 15-μL cell suspension from each lyophilized capsule. Tubes were incubated aerobically at 37 °C for 19 h and analyzed for the release of free cholic acid in the medium according to Irvin et al. (1944) as modified by Walker and Gilliland (1993). Briefly, 10-mL culture of each strain after the incubation period was adjusted to pH 7.0 with 1 M NaOH and centrifuged (10,000×*g*, 4 °C, 10 min). The supernatant was transferred into a clean test tube, pH adjusted to 1.0 with 10 M HCl, and centrifuged (10,000×*g*, 4 °C, 10 min). One milliliter of supernatant was mixed with 2 mL of ethyl acetate, vortexed for 1 min, and then the phases were allowed to separate.

Two milliliters of the ethyl acetate layer was transferred to a clean test tube and evaporated to dryness at 60 °C in a water bath. The residues were immediately dissolved in 1 mL of 10 mM NaOH and mixed with 1 mL of 1% (w/v) furfuraldehyde (Sigma) and 1 mL of 8 M H_2SO_4. The reaction mixture was vortexed for 1 min and held at 65 °C in a water bath for 10 min. The test tubes were cooled to room temperature, 2 mL of glacial acetic acid was added, the mixture was vortexed for 1 min, and A_{660} was measured. The amount of cholic acid released was determined using cholic acid standard.

Cholesterol removal assay

Ten-milliliter MRS broth containing cholesterol (100 μg/mL) was inoculated with 15 μL of cell suspension prepared from lyophilized cells, incubated at 37 °C for 24 h, centrifuged (10,000×*g*, 4 °C, 10 min), and cholesterol concentration in the supernatant was determined according to Gilliland et al. (1985). 0.5 mL of supernatant and 3 mL of 95% ethanol were mixed and vortexed for 1 min. Two milliliters of 50% (w/v) KOH was added to the tubes, vortexed for 1 min, held at 65 °C in water bath for 10 min, cooled to room temperature, followed by the addition of 5-mL hexane and 3-mL distilled water, vortexed for 1 min, and the phases were allowed to separate. 2.5 mL of hexane layer was transferred to a clean test tube and evaporated to dryness at 60 °C in a water bath. The residues were then dissolved in 4-mL *o*-phthalaldehyde reagent, vortexed for 1 min, and held at room temperature for 10 min. Two milliliters of concentrated sulfuric acid was added to the mixture and vortexed for 1 min. A_{550} was measured to determine cholesterol using cholesterol standard. MRS with cholesterol (100 μg/mL) was used as a control. Percentage of cholesterol removed was calculated as $(A) = [(A_b - A_c)/A_b] \times 100$, where A_b = absorbance of blank and A_c = absorbance of cell supernatant.

β-galactosidase activity

Fifteen microliters of cell suspension (10^9 cells) prepared from lyophilized vials was mixed with 2.7 mL of permeabilization buffer containing g/L of 16.1 $Na_2HPO_4{\cdot}2H_2O$, 5.5 $NaH_2PO_4{\cdot}2H_2O$, 0.75 KCl, 0.25 $MgSO_4{\cdot}7H_2O$, 1.0 sodium dodecyl sulfate, 2.7 mL β-mercaptoethanol, and held at 37 °C for 30 min. After incubation, 0.6 mL of *o*-nitrophenyl-β-D-galactopyranosidase (4 mg/mL) was mixed and after the appearance of yellow color the reaction was terminated by adding 2 mL of chilled 1 M Na_2CO_3 solution. A_{420} was measured and the activity in the presence of lactose (Miller's unit) was determined using the formula $((A_c - A_b/t) \times 1000)$, where A_c is absorbance of lyophilized cells, A_b is absorbance of blank, and t is time in min.

DPPH scavenging activity

The DPPH radical scavenging activity of lyophilized cells was evaluated by the method of Shimada et al. (1992) with minor modifications. Fifteen microliters of cell suspension and 1.0 mL of freshly prepared 0.2 mM DPPH solution (in methanol) were mixed and allowed to react for 30 min in dark. PBS was used as blank and DPPH scavenging ability was expressed as percentage of scavenging ability: $(A_b–A_t/A_b)\times100$ by measuring decrease in A_{517}, where A_t = absorbance of test and A_b = absorbance of blank.

GABA production

Lactobacillus strains were grown in MRS medium containing monosodium glutamate (5% w/v) at 37 °C for 72 h. The cell-free supernatant that was obtained upon centrifugation (10,000×*g*, 4 °C, 10 min) was passed through a syringe filter (0.45 μm). GABA present in cell-free supernatant was separated and identified by TLC on activated silica gel plates (Silica gel 60 F_{254}, Merck) as in the method of Lee et al. (2010).

Statistical analysis

All experiments were performed in triplicate and repeated at least once and the data of representative experiment are presented as mean ± SD of the data. Statistical significance (*) and analysis of variance (ANOVA) were determined using Microsoft Excel 2010 at $P < 0.05$.

Results

Prebiotic utilization by agar plate assay

Lactobacillus plantarum GP utilized the three prebiotics as indicated by the appearance of yellow zone against a purple background on MRS agar plates indicating acid production. *Lactobacillus rhamnosus* GG did not utilize any of the prebiotics used in the study.

Viability (%) after lyophilization

The percentage of viability of *L. plantarum* GP and *L. rhamnosus* GG with or without wall materials is shown in Table 1. GP displayed higher viability with prebiotic lactulose and sugar sucrose by ≥ 95%. GG lyophilized with lactulose and sucrose in combination with skim milk exhibited 89% viability which was 58% in control. Scanning electron micrograph of GP (Fig. 1i, ii) with lactulose and sucrose indicated coating of cells with smooth matrix of wall materials. The rough and porous freeze-dried powders obtained by lyophilizing GG with skim milk and sucrose + skim milk (Fig. 1iii, iv) showed the presence of long filamentous cellular structures with matrices in scanning electron micrographs.

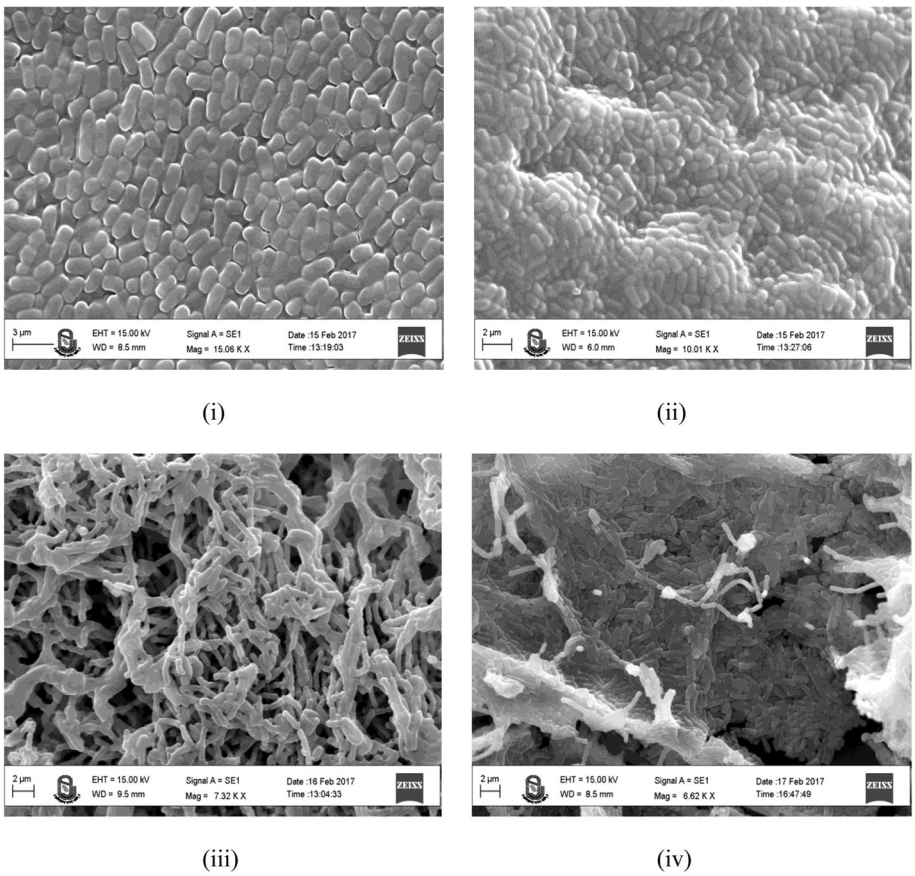

Fig. 1 Scanning electron micrographs of lyophilized cells of *Lactobacillus plantarum* GP with **i** lactulose, **ii** sucrose and *Lactobacillus rhamnosus* GG, **iii** lactulose + skim milk, and **iv** skim milk

Survival of lyophilized cells in oro-gastro-intestinal fluids

The survival of lyophilized cells of Lactobacilli after sequential exposure to simulated saliva (SS), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) was evaluated to determine their viability during oro-gastro-intestinal transit. The *Lactobacillus* strains stored for 2 months retained ≥ 88, 66, and 45% viability after subsequent exposure to SS, SS-SFG, and SS-SGF-SIF and an average reduction of 0.8, 2.0, and 3.2 log cycle was observed after exposure to SS, SS-SFG, and SS-SGF-SIF respectively. The lyophilized cells after 4 months of storage displayed ≥ 70, 45, and 30% viability during SS, SS-SGF, and SS-SGF-SIF transit respectively. The survival of lyophilized cells after 6 months of storage was ≥ 60, 44, and 28% in SS, SS-SGF, and SS-SGF-SIF respectively (Fig. 2). Among all the vials, GP lyophilized with lactulose displayed significantly higher survival rate after 2, 4, and 6 months storage period.

Mucin adherence

L. plantarum GP and *L. rhamnosus* GG cells lyophilized in the presence of prebiotic, sucrose, and/or skim milk exhibited different mucin adhesion capacity (Fig. 3). Lyophilized vials stored for 2 months retained significant mucin adhesion capacity (4.4 to 6.8 log cfu/well) in comparison with normally grown cells as reported previously (Shekh et al. 2016). Mucin adhesion capacity of lyophilized cells after 4 and 6 months of storage was in the range of 3.1 to 5.7 log cfu/well and 2.04 to 4.26 log cfu/well respectively.

Biofilm formation

The ability to form biofilm was considerably enhanced in *L. plantarum* GP and *L. rhamnosus* GG lyophilized with different wall materials as compared with cells lyophilized without any wall material. The experiment was carried out to determine the ability to form biofilm on the basis of crystal violet bound to biofilms produced by lyophilized cells after 2, 4, and 6 months storage (Fig. 4). The crystal violet binding was higher in GG + sucrose + skim milk ranging from 1.15, 0.81, and 0.39 μg after 2, 4, and 6 months of storage respectively.

Antimicrobial activity

Lyophilized cells exerted antimicrobial activity against selected test pathogens (Table 2). The extent of

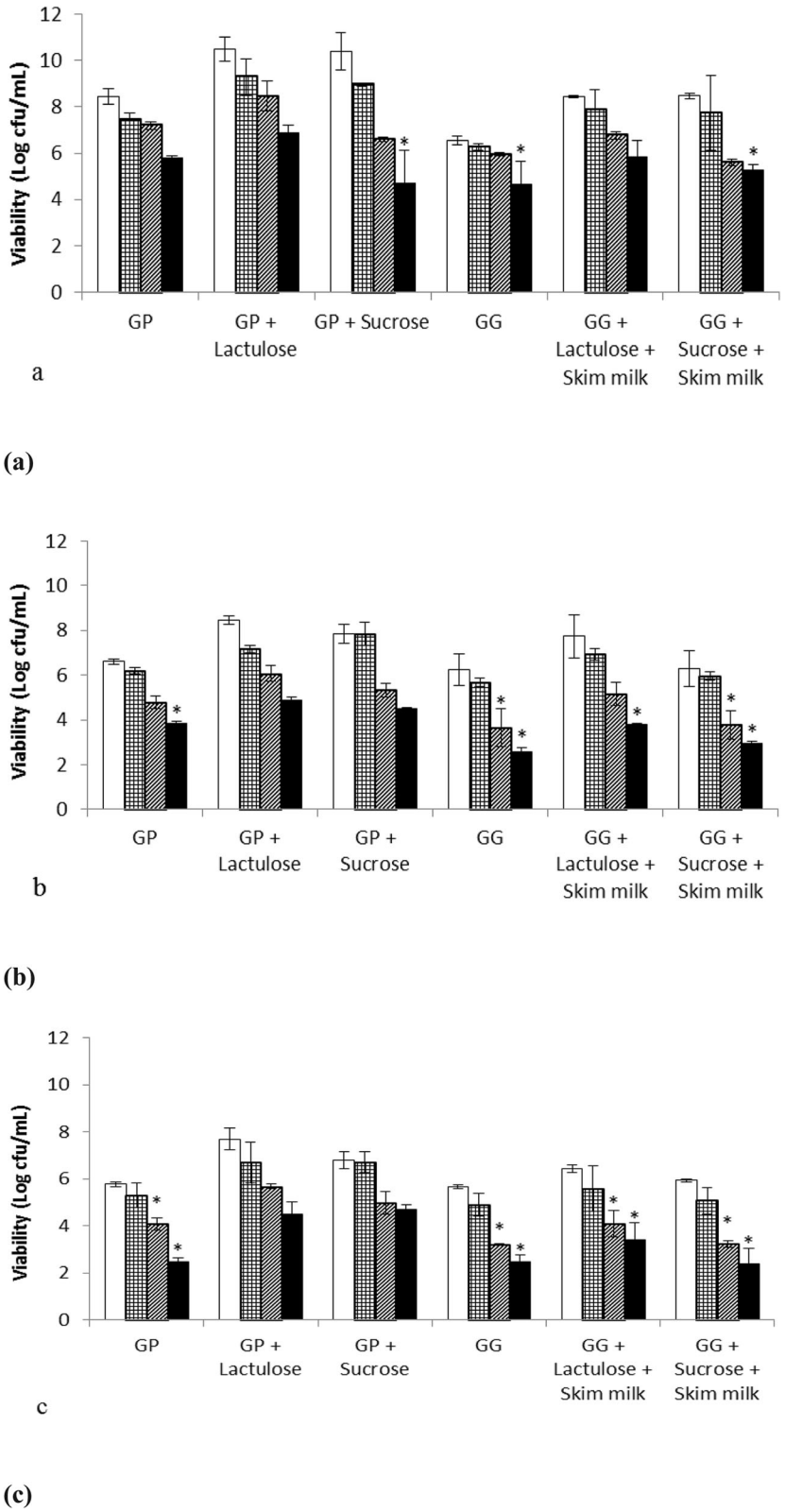

Fig. 2 Survival (log cfu/mL) of lyophilized *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* GP cells stored at 8–10 °C for **a** 2, **b** 4, and **c** 6 months (□, control) upon sequential exposure to SS (⊞), SS-SGF (▨), and SS-SGF-SIF (■) determined by viable count method on MRS medium. *significantly different ($P < 0.05$)

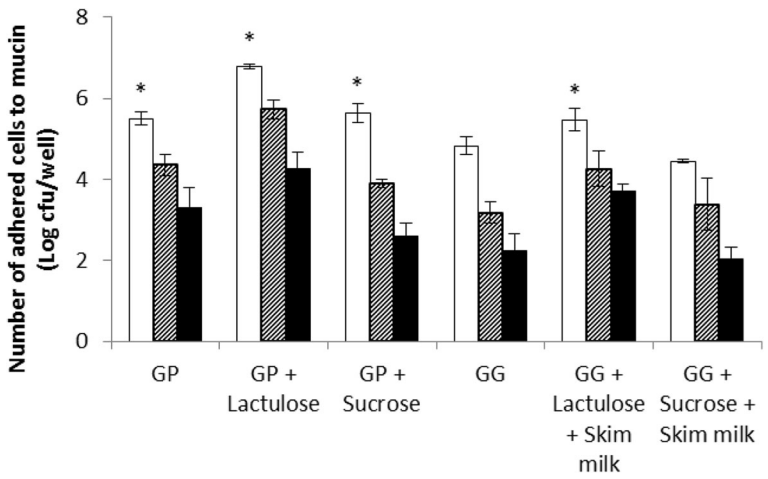

Fig. 3 Count of lyophilized bacteria (log cfu/well) stored at 8–10 °C for 2 (□), 4 (▨), and 6 months (■) to adhere to mucin in microtiter plate determined by viable count method using MRS medium after. *significantly different ($P < 0.05$)

antimicrobial activity varied with the test pathogen and the type of wall material used as protectant during lyophilization. The antimicrobial activity of lyophilized *L. plantarum* GP gradually decreased upon 2, 4, and 6 months of storage and was comparable with that of the reference strain GG.

Deconjugation of sodium taurocholate and sodium glycocholate

Deconjugation ability was determined from the amount of cholic acid released from sodium taurocholate (ST) and sodium glycocholate (SG). Cultures initiated with stored (up to 6 months) lyophilized *Lactobacillus* cells demonstrated both SG and ST deconjugation ability (Table 3), releasing varying amount of cholic acid (μmol/mL). The range for ST deconjugation was 0.09–0.54 μmol/mL, whereas SG deconjugation was in the range 0.31–1.66 μmol/mL. Among the lyophilized cells, GG + lactulose + skim milk released a significant amount of cholic acid (μmol/mL) from SG and ST.

Cholesterol removal assay

The cultures initiated with the stored lyophilized cells were observed to remove cholesterol to varying extent (44–72%). Cultures of GP lyophilized with sucrose and GG lyophilized without any wall material showed significantly higher cholesterol removal after 24 h of incubation in comparison with other cultures from lyophilized cells.

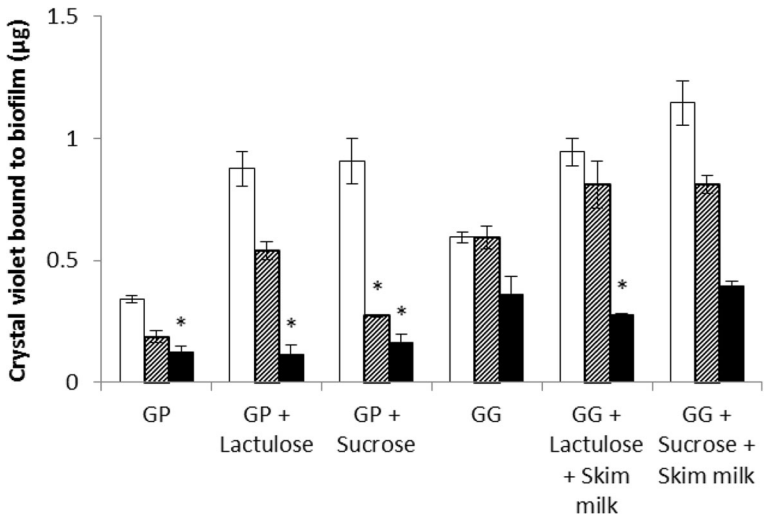

Fig. 4 Crystal violet bound (μg) to biofilms produced by *L. rhamnosus* GG and *L. plantarum* GP lyophilized cells stored at 8–10 °C for 2 (□), 4 (▨), and 6 months (■). *significantly different ($P < 0.05$)

Table 2 Antimicrobial activity exerted by lyophilized and stored (for 2, 4, and 6 months) *L. rhamnosus* GG and *L. plantarum* GP growing on MRS agar against Gram negative and Gram positive bacteria determined by spot overlay assay

Test strains	*Lactobacillus* strains + wall materials					
	GP	GP + lactulose	GP + sucrose	GG	GG + lactulose + skim milk	GG + lactulose + skim milk
	Zone of inhibition (mm)					
2 months						
E. coli	25 ± 0.3	30 ± 0.4	32 ± 0.7	25 ± 0.9	29 ± 1.4	33 ± 0.7
Ent. aerogenes	19 ± 2.1	27 ± 0	28 ± 0.2	19 ± 0.4	35 ± 2.4	38 ± 0.2
Sal. typhi	16 ± 0.4	24 ± 0.6	21 ± 0.7	25 ± 0.7	33 ± 0.2	33 ± 0.4
Shigella sp.*	25 ± 0.7	30 ± 4.2	31 ± 0.3	36 ± 0.7	27 ± 0.3	27 ± 0.4
*Pr. vulgaris**	18 ± 0.2	23 ± 1.4	22 ± 0.0	28 ± 2.1	33 ± 2.1	34 ± 1.1
*K. pneumoniae**	22 ± 0.3	26 ± 1.2	31 ± 0.3	27 ± 1.7	22 ± 0.1	2 ± 0.9
Staph. aureus	25 ± 0.4	22 ± 0.8	21 ± 0.6	24 ± 0.4	26 ± 0.4	25 ± 0.6
Sr. marcescens	25 ± 0.6	29 ± 2.1	33 ± 0.2	32 ± 0.2	31 ± 0.2	27 ± 1.6
Ps. aeruginosa	22 ± 0.8	24 ± 1.9	30 ± 0.6	29 ± 1.0	26 ± 0.4	25 ± 0.2
B. cereus	20 ± 0.2	21 ± 0.2	21 ± 0.4	21 ± 0.4	24 ± 1.0	24 ± 1.2
*B. megaterium**	19 ± 0.3	26 ± 2.8	32 ± 0.5	33 ± 0.8	34 ± 0.5	29 ± 1.2
B. subtilis	23 ± 0.4	24 ± 0	22 ± 0.6	23 ± 1.0	24 ± 0.6	20 ± 1.2
4 months						
E. coli	25 ± 0.7	25 ± 0.2	25 ± 0.5	21 ± 0.5	20 ± 0.5	24 ± 0.7
Ent. aerogenes	19 ± 0.7	19 ± 0.1	20 ± 0.0	17 ± 1.1	23 ± 0.5	24 ± 1.2
Sal. typhi	10 ± 0.2	22 ± 1.4	21 ± 0.1	16 ± 4.2	26 ± 1.4	27 ± 0.7
Shigella sp. *	24 ± 0.3	29 ± 0.7	22 ± 1.0	19 ± 0.9	27 ± 2.1	23 ± 2.1
*Pr. vulgaris**	15 ± 0.4	21 ± 1.4	19 ± 0.2	17 ± 0.3	25 ± 2.1	18 ± 0.8
*K. pneumoniae**	20 ± 0.6	25 ± 1.2	26 ± 0.8	20 ± 1.4	17 ± 0	19 ± 1.0
Staph. aureus	18 ± 0.4	18 ± 0.6	20 ± 0.2	20 ± 0.2	19 ± 0.2	17 ± 0.8
Sr. marcescens	24 ± 0.4	25 ± 0.2	26 ± 0.2	27 ± 0.2	29 ± 0.4	25 ± 1.9
Ps. aeruginosa	18 ± 1.0	20 ± 1.7	27 ± 0.8	25 ± 1.5	23 ± 0.7	23 ± 0.7
B. cereus	14 ± 1.4	16 ± 0.6	16 ± 0.4	16 ± 0.2	15 ± 0.2	17 ± 0.8
*B. megaterium**	15 ± 0.4	21 ± 3.1	26 ± 1.0	23 ± 0.4	23 ± 0.4	18 ± 2.5
B. subtilis	15 ± 0.6	18 ± 1.9	21 ± 0.8	15 ± 1.4	13 ± 0.6	14 ± 0.2
6 months						
E. coli	24 ± 0.4	24 ± 0.1	25 ± 1.4	14 ± 2.1	14 ± 3.5	20 ± 2.8
Ent. aerogenes	14 ± 0.4	16 ± 0.2	15 ± 0.2	14 ± 2.8	18 ± 0.7	18 ± 2.1
Sal. typhi	10 ± 0.4	20 ± 2.1	15 ± 0.3	11 ± 0.1	17 ± 2.8	18 ± 0.8
Shigella sp.*	17 ± 0.6	28 ± 4.2	22 ± 0.2	16 ± 0.4	23 ± 0.2	16 ± 0.6
*Pr. vulgaris**	13 ± 0.6	14 ± 2.1	15 ± 1.0	17 ± 4.9	16 ± 1.1	16 ± 2.8
*K. pneumoniae**	15 ± 0.8	18 ± 2.1	20 ± 1.0	16 ± 0.8	15 ± 0.4	16 ± 0.2
Staph. aureus	12 ± 0.5	14 ± 1.2	17 ± 0.3	15 ± 0.3	13 ± 0.1	12 ± 0.1
Sr. marcescens	16 ± 0.6	21 ± 1.4	23 ± 0.6	22 ± 0.2	22 ± 0.4	18 ± 1.0
Ps. aeruginosa	15 ± 0.6	17 ± 0.2	20 ± 0.8	19 ± 1.2	18 ± 0.8	17 ± 1.2
B. cereus	12 ± 1.0	11 ± 0.4	11 ± 0.4	11 ± 0.4	13 ± 0.4	15 ± 0.4
*B. megaterium**	13 ± 0.6	17 ± 0.8	22 ± 1.2	20 ± 2.1	16 ± 0.6	16 ± 0.6
B. subtilis	13 ± 0.8	17 ± 0.5	19 ± 0.1	15 ± 1.5	11 ± 0	10 ± 0.1

*indicates clinical strains obtained from Government Hospital, Rajkot, India. Other strains obtained from MTCC (Microbial Type Culture Collection Centre) Chandigarh, India

Table 3 β-galactosidase, bile salt hydrolase, percentage of cholesterol reduction, and DPPH radical scavenging activities of lyophilized *L. rhamnosus* GG and *L. plantarum* GP stored for 6 months

Lactobacillus Strains + wall materials	β-galactosidase activity (Miller's unit)	Bile salt hydrolase activity (cholic acid releases)		Cholesterol removal (%)	DPPH radical scavenging activity (%)
		SG (μmol/mL)	ST (μmol/mL)		
GP	313 ± 0.2*	0.33 ± 0.08	0.35 ± 0.02	44 ± 1.9	58 ± 0.5
GP + lactulose	321 ± 0.3*	0.74 ± 0.13	0.46 ± 0.09	67 ± 2.7	62 ± 0.8
GP + sucrose	278 ± 0.8*	0.31 ± 0.14	0.14 ± 0.03	60 ± 1.5	77 ± 1.2*
GG	54 ± 0.8	0.83 ± 0.32	0.13 ± 0.01	58 ± 1.0	73 ± 0.9*
GG + lactulose + skim milk	80 ± 0.3	1.66 ± 0.04*	0.54 ± 0.11	72 ± 4.6*	53 ± 0.3
GG + sucrose + skim milk	143 ± 0.5	0.66 ± 0.2	0.09 ± 0.01	59 ± 3.0	57 ± 0.3

*values significantly different ($P < 0.05$)

β-galactosidase activity

Cultures of *L. plantarum* GP and *L. rhamnosus* GG initiated by lyophilized cells stored for 6 months produced β-galactosidase activity (≥ 54 Miller's unit) in the presence of lactose in MRS medium (Table 3). Cultures initiated with GP cells lyophilized with lactulose as wall material produced significantly higher β-galactosidase activity (321 Miller's unit) than the free GP cells grown in MRS with lactose (Shekh et al. 2016).

DPPH scavenging activity

L. plantarum GP lyophilized with sucrose exhibited strong DPPH scavenging activity (77 ± 1.2%) similar to the reference strain *L. rhamnosus* GG (73 ± 0.9%), whereas the scavenging ability of the GP and GG cells lyophilized in the presence of other wall materials was considerably lower (Table 3).

GABA production

L. plantarum GP with and without wall material produced GABA which was observed as intense spots on TLC plates.

Discussion

A probiotic microorganism used in formulations has to fulfill certain selection criteria for acceptance in the final probiotic product. These include probiotic efficacy, non-pathogenicity, and survival for prolonged period during processing, storage, and gastrointestinal tract transit. Prebiotics, skim milk, and sucrose were used in various combinations to enhance the survival of *Lactobacillus plantarum* GP cells during lyophilization and storage. The lyophilized vials without any wall material, included as control, showed significant reduction in viability after lyophilization. The survival rate during 6 months of storage was also poor in cells lyophilized without any wall material. Among the different combinations of cryoprotectants, GP retained significant viability (%) with lactulose and skim milk, whereas in the case of GG it was with lactulose + skim milk and sucrose + skim milk. The prolonged survival is due to the ability of a strain to ferment particular prebiotic and sugar and use them as fermentable carbon sources (Slavin 2013; McLaughlin et al. 2015). Lactose, lactose + skim milk, and ascorbic acids exerted similar cryoprotective effects during lyophilization (Zárate and Nader-Macias 2006; Montel Mendoza et al. 2014).

The functional and health beneficial aspects of probiotics may include survival, adhesion, and colonization during gastrointestinal tract transit, antimicrobial activity, and production of enzymes like β-galactosidase, bile salt hydrolase (BSH), and glutamate decarboxylase. We evaluated the survival of Lactobacilli after lyophilization in the presence of simulated GIT conditions and antimicrobial activity against food spoilage and GIT pathogens. The probiotics present in food products encounter various environmental conditions upon ingestion by the host and during GIT transit. Our experimental data substantiate the viability of lyophilized cells of *L. plantarum* GP and *L. rhamnosus* GG through a sequential exposure to simulated gastric and intestinal conditions. GP and GG cells lyophilized with wall materials had significantly higher survival rate and shelf life than the cells lyophilized without any wall material. Lactulose acts as a protectant and metabolized into low molecular weight acids (German et al. 2008) by Lactobacilli thereby stimulating the growth of acid-tolerant Lactobacilli (Collins et al. 2017). In this study, lyophilization of cells with lactulose significantly improved the viability of GP during the stress conditions of human GIT as compared with sucrose where survival of cells decreased upon storage. Rajam and Anandharamakrishnan (2015) reported the protective effects of fructooligosaccharides + whey protein isolate during simulated transit of *L. plantarum*.

The adhesion to mammalian epithelial cells is an essential attribute for probiotic bacteria in functional foods to survive and colonize the gastrointestinal tract to exert claimed probiotic benefits. *L. plantarum* GP and *L. rhamnosus* GG displayed a significant number of log cfu/well adhered to mucus layer during storage of 6

months. The wall materials provide protection to the lyophilized cells of Lactobacilli during freeze drying and storage that resulted in significant mucin adhesion capacity. Zárate and Nader-Macias (2006) reported similar restored mucin adhesion capacity of lyophilized cells of Lactobacilli after the first subculturing. Prebiotics and sucrose also improve the biofilm formation capacity of cultures from lyophilized cells by altering their cell surface properties according to Lebeer et al. (2007), where biofilm formation capacity of GG increased by 1.5 fold in the presence of inulin.

The microbial contamination of food can have many undesirable consequences ranging from spoilage to food-borne illness. The use of LAB with potential antimicrobial activity is a promising alternative treatment for such problems. *Lactobacillus* strains exhibit broad spectrum antimicrobial activity against Gram positive and Gram negative major food spoiling organisms and gastrointestinal pathogens. *Lactobacillus* strains show varying degree of strain specific antibacterial activity against tested pathogens. Strain specific and growth phase dependent production of antimicrobial substances has also been reported previously (Zárate and Nader-Macias 2006; Boricha et al. 2019). *Lactobacillus* strains freeze dried with lactose, skim milk, and ascorbic acid exerted antimicrobial action as the antimicrobials production was not affected by lyophilization (Zárate and Nader-Macias 2006). Lyophilized vials of *L. plantarum* GP stored up to 6 months retained antimicrobial activity against selected food spoiling organisms and human GIT pathogens. The lyophilized cells of *L. plantarum* GP can be incorporated in a food product to prevent food spoilage and infection by the opportunistic GIT pathogens.

The elevated levels of serum cholesterol are considered as one of the major factors associated with coronary heart diseases (Prema et al. 2010) because of improper food habits. Therefore, maintaining lower serum cholesterol is required to prevent cardiovascular diseases. Diets with probiotic strains are one of the promising strategies for controlling serum cholesterol level. BSH produced by Lactobacilli catalyses the hydrolysis of glycine- or taurine-conjugated bile salts into the amino acids and free bile acids (Corzo and Gilliland 1999). Deconjugated bile salts are less soluble than conjugated bile salts, resulting in lower absorption in the intestinal lumen, leading to their elimination in the feces. Since free bile acids are extracted from the body, the synthesis of new bile salts from cholesterol can reduce the total cholesterol concentration in the body. *Lactobacillus* GG and GP mediated deconjugation of bile salts was significantly higher (0.09–1.66 μmol/ml cholic acid) and therefore in recent years, interest has increased to use bile salt hydrolysis to influence the cholesterol metabolism of humans. Moreover, cultures initiated with lyophilized cells efficiently removed cholesterol from the medium (44–72%). Miremadi et al. (2014) reported a similar cholesterol removal activity by normally grown cells of Lactobacilli and Bifidobacteria. Several mechanisms proposed include assimilation of cholesterol (Pereira and Gibson 2002; Wang et al. 2014), surface binding (Liong and Shah 2005), incorporation into cellular membranes (Lye et al. 2010a), co-precipitation with deconjugated bile (Liong and Shah 2006), enzymatic deconjugation of bile acids by BSH (Lambert et al. 2008), conversion of cholesterol into coprostanol (Lye et al. 2010b), and production of short-chain fatty acids by probiotics (De Preter et al. 2007).

The deficiency of β-galactosidase is the cause of lactose intolerance as this enzyme hydrolyses lactose into galactose and glucose. Most fermented foods, especially fermented milk, improve lactose digestion. The probiotic organisms utilize lactose and convert it into short-chain fatty acid which is beneficial for the host by preventing colonic disease (Pessione et al. 2015). *L. plantarum* GP produced a significant amount of β-galactosidase in the medium with lactose even after lyophilization. Thus, the products fermented with β-galactosidase producer GP serve as a source for β-galactosidase and play a vital role for the treatment of lactose intolerance.

Increasing scientific evidence suggests that oxidative stress is involved in the pathogenesis of various disorders and diseases, such as alcohol-induced liver injury, non-alcoholic fatty liver disease, aging, and cancer (Beckman and Ames 1998; Cederbaum et al. 2009; Nobili et al. 2010; Yu and Kim 2014). Oxidative stress is a result of an imbalance between the production and elimination of reactive oxygen species (ROS) and free radicals, which are primarily removed by the endogenous antioxidant defense system (Halliwell and Gutteridge 1985). Consumption of antioxidants, which can quench free radicals and ROS, is beneficial to human health. Synthetic antioxidants are effective in slowing oxidation but pose concerns in regard to the safety and toxicity of the antioxidants (Sies 1993; Velioglu et al. 1998). DPPH is usually used to determine the proton-radical scavenging action of the antioxidant. DPPH possesses a proton free radical and shows a characteristic absorption at 517 nm wavelength. When DPPH was encountered with proton-radical scavengers, the purple color of DPPH solution faded rapidly. *Lactobacillus* strains have a role as antioxidative agents by scavenging different free radicals like hydroxyl, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), Fe^{2+}, and superoxide anion radicals (Shekh et al. 2016, unpublished data).

GABA is a non-protein amino acid produced by α-decarboxylation of glutamate by the action of enzyme glutamate decarboxylase. It acts as a major inhibitory

neurotransmitter in the mammalian central nervous system (Schousboe and Waagepetersen 2007), having hypotensive, tranquilizing, and diuretic effects, and can prevent diabetes (Hayakawa et al. 2004; Li and Cao 2010). Therefore, GABA producing vials of *L. plantarum* GP can be included in fermented foods to acquire relief from the abovementioned conditions.

Conclusion

Lactobacillus plantarum GP possesses significant viability during the storage and exposure to simulated oro-gastro-intestinal fluids when formulated with the wall material lactulose, sucrose, and skim milk. The lyophilized cells of the strain GP display promising antimicrobial action towards food spoilage and pathogenic organisms. These synbiotics are functional probiotics having ability to adhere mucin layer, produce biofilms, and improve in lactose intolerance and hypercholesterolemia. Additionally, these synbiotics act as antioxidants and produce BSH and GABA up to 6 months of storage. Therefore, synbiotics prepared by lyophilization can be used to formulate probiotic food products to improve overall human health.

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