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Research Article

Functional Characterization and Shelf-Life of Freeze-Dried Cells of *Lactobacillus rhamnosus* Fb in Carrier Media and Chocolate Formulations

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Abstract

Background and Objective: Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". The present study focused on the characterization of diverse formulations prepared with freeze-dried *Lactobacillus rhamnosus* Fb and evaluation of their shelf life along with their functionality and viability. **Materials and Methods:** Survival of *L. rhamnosus* Fb cells freeze-dried with or without carrier medium in simulated gastrointestinal conditions and during storage at 4°C was evaluated. Moreover, freeze-dried cells were added to the chocolate mixture. Formulated chocolates were evaluated for antigenotoxic activity against 4-NQO and MNNG using SOS-Chromotest. Statistical differences among the mean viability values were analysed by One-Way Analysis of Variance (ANOVA). **Results:** Experimental evidence implicated that the carrier medium during the freeze-drying process did not exert any significant influence on the viability of the cells, but during storage, help to preserve the viability and probiotic activity. The combination of skim milk with sucrose or lactose as a carrier medium helped in the retention of $\geq$80% viability during 4 months of storage. Freeze-dried cells (7.13 log CFU g^{-1}) mixed in chocolate, remained viable in simulated gastrointestinal conditions. New insight of the chocolate formulation containing *L. rhamnosus* Fb is the reduction of genotoxicity of food-borne mutagens (directing-acting carcinogens/mutagens) 4-nitroquinoline-1-oxide (81%) and N-methyl-N'-nitro-N-nitrosoguanidine (68%), evaluated using SOS-Chromotest. **Conclusion:** Hence, freeze-dried cells retained viability and functional activities, therefore, promoting the use of viable freeze-dried cells in the formulation of health-promoting indigenous functional food products.

Key words: Antigenotoxic activity, chocolate, freeze-drying, *Lactobacillus rhamnosus*, MNNG, 4-nitroquinoline-1-oxide, probiotics

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

International Scientific Association for Probiotics and Prebiotics (ISAPP) redefined probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[1]. Society is getting health conscious and there is the direct impact of diet on healthy lifestyle leading to increased demand for functional foods that could confer multiple health benefits beyond inherent basic nutrition. The efficient delivery of probiotics and functionality of probiotics to the targeted environment is an area of active research in the food industry. Probiotics associated with various health-promoting functions include antimicrobial activities against gastrointestinal pathogens, control of hypercholesterolemia, modulation of the immune system, prevention of carcinogenesis, improvement of gastrointestinal function, lactose digestion, reduction of colitis and inflammation[2]. Probiotics can be delivered either as fermented or non-fermented food products. Fermented products include curd, buttermilk, flavoured milk, cheese, butter, paneer etc., whereas non-fermented products fruit juices, emulsions, breakfast cereals, cereal bars, ice cream, chocolates, etc[3]. The potential probiotic strain should fulfil functional and safety properties to exert beneficial influences on the host[4]. Criteria of an ideal formulation of probiotics include retention of viability and probiotic functionality under the state of the food matrix and during food-processing and food-storage conditions. The freeze-drying process is one of the most common approaches to obtaining dry probiotic formulations with prolonged shelf life. Dried biomass has a longer shelf life and can be conveniently incorporated into various food preparations. Inclusion of Osmo-, thermo- or cryo-protectants or microencapsulated cells in probiotic formulations can enhance cell survival during processing and storage[5].

Functional foods are those that provide basic nutrition and at the same time promote health[6]. Colorectal cancer is a second major cause of cancer-related mortality worldwide[7]. Carcinogens are the main cause of colorectal cancer that is present in either food or the environment. Prevention of colorectal cancer can be achieved with the intake of antimutagens, nutritious healthy diet and formulation containing probiotic bacteria would help to reduce the load of carcinogens and provide protection from cancer.

Probiotics may exhibit antimutagenic and antigenotoxic activities by various mechanisms namely biotransformation of carcinogens, binding and inactivation, altering colonic metabolism, interaction with extracellular metabolites, stimulation of immune response, lowering the enzyme activity responsible for the conversion of procarcinogen to a carcinogen and production of anticarcinogenic enzymes[7]. Quantification of the beneficial effects of probiotic formulations with anticarcinogenic activity can justify their use as cancer prophylactics in humans. *Lactobacillus rhamnosus* Fb possesses functional activities and displays safety characteristics as per the WHO guidelines[8]. In addition, also removes acridine orange by binding[9].

The focus of the present study was to evaluate the viability, functionality and shelf life of bacterial cells freeze-dried in the presence and absence of protective agents such as skim milk powder in combination with sugars. Subsequently, to develop chocolates with health-promoting Lactobacilli, the freeze-dried cells were incorporated in commercially available dark and milk chocolate slabs and evaluated for the functionality of cells, shelf-life and antigenotoxic activity against 4-nitroquinoline-1-oxide (4-NQO) and MNNG.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Biosciences, Saurashtra University, Rajkot, India from January to June, 2014.

Chemicals: The 4-NQO and MNNG used in the study were purchased from Lancaster (Alfa Aesar, CAS No. 56-57-5) and Sigma (St. Louis, MO, USA), respectively. Stock solutions of NQO (1 mg mL^{-1}) were prepared in dimethyl sulfoxide (Merck) while MNNG (1 mg mL^{-1}) in sterile distilled water and stored at 4°C. The o-nitrophenyl-β-D-galactopyranoside and p-nitrophenyl phosphate were obtained from Sigma (St. Louis, MO, USA).

Bacterial strains and culture conditions: *Lactobacillus rhamnosus* Fb JX406746 isolated from infant faeces as previously described by Pithva *et al.*[8] was maintained at -20°C in MRS medium (Himedia, Mumbai, India) with 10% glycerol. The frozen culture was grown on MRS agar plates and incubated at 37°C for 48 hrs. The activated culture was sub-cultured twice in the MRS medium. About 1 mL of activated culture was inoculated in a 50 mL medium and incubated at 37°C overnight. This was used to inoculate a 1000 mL MRS medium for biomass production.

Escherichia coli PQ37 (*sfiA::lacZ*) obtained from Institute Pasteur (Paris, France) for genotoxicity assay (SOS-Chromotest) was grown in Luria broth (Himedia, Mumbai, India) for 12-15 hrs at 37°C.

Preparation of frozen and freeze-dried cells: 24 hrs grown culture of *L. rhamnosus* Fb in 1000 mL of MRS medium was subjected to centrifugation (5000×g, 20 min, 4°C) for separation of cells. The cell pellet was washed twice with

phosphate buffer saline (0.1 mol L^{-1}, pH 7.0) and re-suspended in phosphate buffer (0.1 mol L^{-1}, pH 7.0) for the removal of medium components bound to the cells and centrifuged (5000×g, 20 min, 4°C). 500 µL concentrated cell pellet was distributed in Eppendorf tubes, stored-frozen for 18 hrs at -20°C and freeze-dried (freeze-dried) using MicroModulyo 0230 at -45°C, 10 hrs, 3.8×10^{-2} Torr (Thermo Scientific, USA). The viable cell count of frozen and freeze-dried cells was determined using an MRS medium. The functionality and survival of freeze-dried cells in simulated gastrointestinal conditions were evaluated by the methods described below.

Influence of carrier media on the viability of freeze-dried cells of *L. rhamnosus* Fb and shelf-life: Biomass mixed in equal proportion with (i) 10% (w/v) skim milk, (ii) 10% (w/v) skim milk containing 1% (w/v) sugar (glucose, lactose or sucrose), was freeze-dried as described above and the viability of cells in the freeze-dried biomass stored at 4°C was determined at 0, 2 and 4 months of intervals using MRS medium. A vial containing freeze-dried biomass was resuspended in phosphate buffer (0.1 mol L^{-1}, pH 7.0) and used to determine the retention of functionality and viability in gastrointestinal conditions.

Acid-bile tolerance and survival of freeze-dried cells under simulated gastro-intestinal fluid transit: 100 µL suspension of freeze-dried cells in phosphate buffer (*ca.* 10^8 CFU mL^{-1}) was inoculated in 3 mL MRS, MRS altered with bile salt (0.5-2%), pH (2, 3), NaCl (6%) and skim milk with phenol (0.4%) and incubated at 37°C for 4 hrs. 100 µL of culture from each of the tubes was taken, serially diluted, plated on MRS agar and incubated at 37°C for 48 hrs. Cell suspension prepared from freeze-dried biomass was exposed to simulated gastrointestinal fluids as described by Pithva *et al.*[8]. Simulated gastric and intestinal fluids were prepared as described by Charteris *et al.*[10].

Formulation of chocolate using freeze-dried cells of *L. rhamnosus* Fb: Dark and milk chocolates (75:25) g were melted in a boiling water bath (20-25 min) and mixed, cooled to around 45°C before freeze-dried cells of *L. rhamnosus* Fb (ca. 10^7 CFU g^{-1}) were added to the chocolate mixture and stirred properly. The chocolate mixture was then poured into the mould and held at 4°C for 30-40 min. The chocolates were manually de-moulded, wrapped with aluminium foil and stored at 8°C. Retention of functionality and survival of formulated chocolate in simulated gastrointestinal conditions were evaluated with the methods described above for the freeze-dried biomass. The nutritional values of the dark and milk chocolates are given below:

- Dark chocolate-carbohydrate: Protein: Total fat: Energy-63.29:3.2:28.01:518.1 Kcal 100 g^{-1}
- Milk chocolate-carbohydrate: Protein: Total fat: Energy-61.90:6.74:27.82:524.9 Kcal 100 g^{-1}
- Control chocolate was prepared without the addition of bacterial cells

Evaluation of cell viability in chocolate during *in vitro* simulated gastrointestinal conditions: 1 g of chocolate was melted in the water bath at 50°C and mixed in phosphate buffer to set the final volume to 10 mL. 100 µL aliquots from the tubes were serially diluted using sterile phosphate buffer. Diluted samples were plated on MRS agar and incubated at 37°C for 48 hrs. The cell viability in chocolate was determined at 0, 2 and 4 months during refrigerated storage. Survival of freeze-dried cells was evaluated in simulated gastrointestinal fluids as described by Pithva *et al.*[8] to determine the changes in the ability of freeze-dried cells, incorporated in chocolate formulation, to survive gastrointestinal conditions.

Antigenotoxic activity of chocolates containing freeze-dried cells of *L. rhamnosus* Fb against 4-NQO and MNNG: A total of 1 g of chocolate (stored for 3 days) was melted in the water bath at 50°C and mixed in phosphate buffer to set 10 mL final volume, incubated with 4-NQO and MNNG (10 µg mL^{-1}) at 37°C. After 30 min, 3 and 24 hrs of incubation, a 0.1 mL sample was used to determine the genotoxicity using SOS-Chromotest as described by Quillardet and Hofnung[11]. Chocolate without bacteria served as a negative control.

Statistical analysis: Viability values were represented as mean and standard deviations. Statistical differences among the mean viability values were analysed by One-Way Analysis of Variance (ANOVA) using Microsoft Excel 2010. The p-values of <0.05 were considered significant.

RESULTS

Viability of cells after freezing and freeze-drying (Fig. 1a-e): Freezing did not alter the viability of the *L. rhamnosus* Fb cells, but freeze-drying reduced the initial cell count by 16% (Fig. 1a). Comparison of bacterial counts in the presence of various carrier media indicated that skim milk with sucrose provided greater protection to the cells as the viable bacterial count after 4 months of storage at 4°C (Fig. 1e) was 88% of the original which was 80% in skim milk with lactose (Fig. 1d), 77% in skim milk (Fig. 1b) and 69% in skim milk with glucose (Fig. 1c).

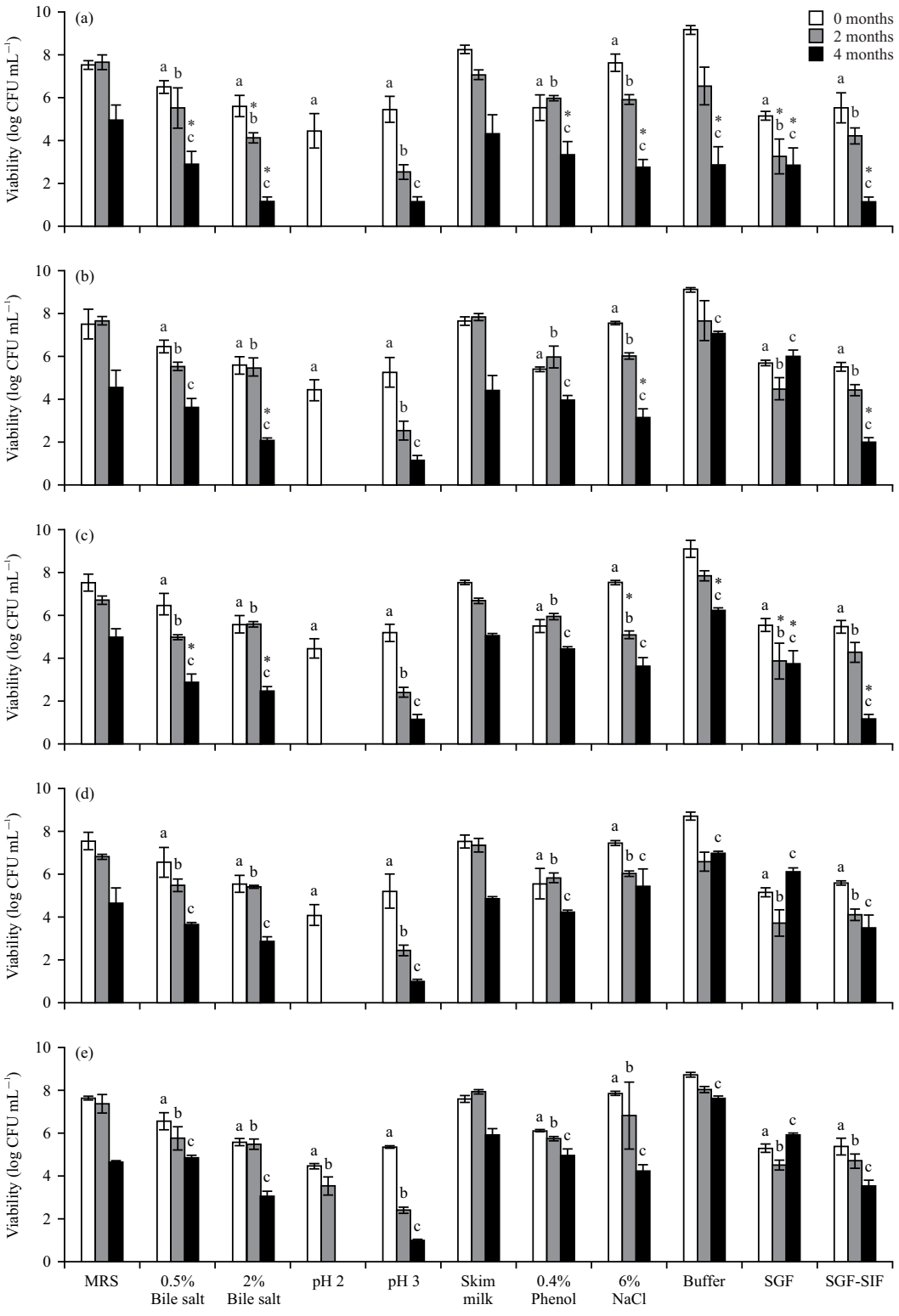

Fig. 1(a-e): Viability (log CFU mL^{-1}) of different groups under simulated gastrointestinal conditions determined after 0, 2 and 4 months of storage at 4°C, (a) Freeze-dried *L. rhamnosus* Fb cells without carrier media and with (b) Skim milk, (c) Skim milk with glucose, (d) Skim milk with lactose and (e) Skim milk with sucrose

Error bars represent standard deviation (n = 3). The statistical difference among cells viability with or without carrier media, 'a, b, c' indicates no significant difference ($p>0.05$) between cell counts determined after 0, 2 and 4 months, respectively while 'a, b, c' with (*) indicates significantly ($p<0.05$) lower count

Survival of freeze-dried cells in simulated gastro-intestinal conditions: Cells freeze-dried with or without carrier medium did not show a significant difference in their tolerance to acid, bile, NaCl, phenol and simulated gastrointestinal fluids (0 day) ($p>0.05$). However, a substantial difference was observed in the survival of cells stored for 2 and 4 months at 4°C when exposed for 4 hrs to 2% bile salt, pH 3 and simulated intestinal fluid. Cells freeze-dried with skim milk and skim milk containing sugars exhibited significantly ($p<0.05$) higher survival after exposure to bile salt, phenol and simulated gastrointestinal fluid in comparison to cells freeze-dried without carrier medium. Cells freeze-dried with skim milk and skim milk with lactose or sucrose after 4 months of storage exhibited significantly ($p<0.05$) higher survival in the presence of 0.5 and 2% bile salt in comparison to cells freeze-dried without carrier medium (Fig. 1d, e). However, no significant ($p>0.05$) difference was observed between the cells freeze-dried with or without carrier media in their survival at pH 3. No viable cell was detected upon prolonged exposure for 4 hrs to pH 2 in MRS medium after storage of 2 and 4 months except cells freeze-dried with sucrose. The cells freeze-dried with skim milk containing lactose and sucrose displayed significantly higher survival in 6% NaCl than the cells without carrier medium ($p<0.05$).

Enumeration of bacteria in formulated chocolate: Survival of *L. rhamnosus* Fb cells in Chocolate formulation after 1 month storage at 4°C was 88% that decreased after 4 months to 72% (Fig. 2a). Moreover, formulated chocolate

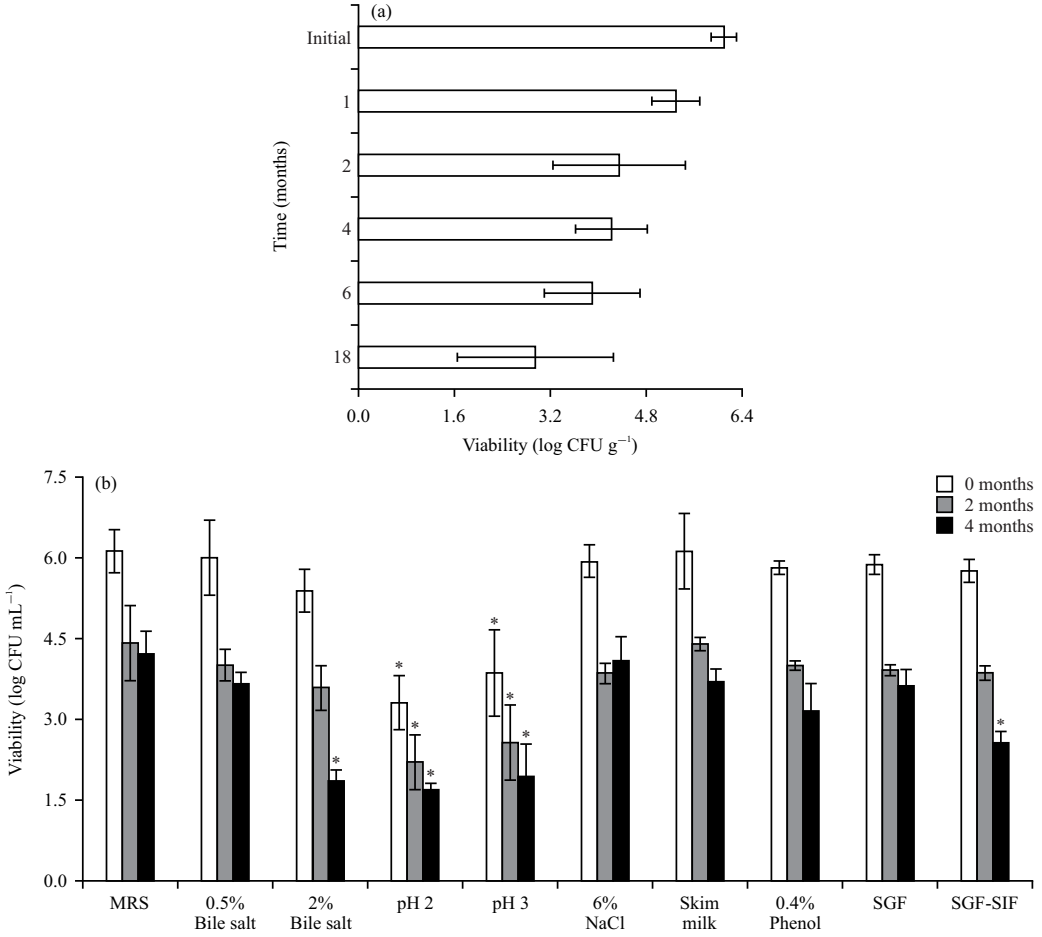

Fig. 2(a-b): Viability (log CFU g^{-1}) of *L. rhamnosus* Fb in formulated chocolate and under simulated gastrointestinal conditions at a different time interval during storage at 4°C, (a) Viability of freeze-dried *L. rhamnosus* Fb cells in formulated chocolate was determined at 0, 1, 2, 4, 6 and 18 months of storage at 4°C and (b) Viability of cells in the presence of bile salt, pH, NaCl, phenol and simulated gastric fluid (2 hrs) and simulated gastrointestinal fluid (2+3 hrs) after 0, 2 and 4 months of storage at 4°C

Error bars represent standard deviation (n = 3), and *Significantly decreased in comparison to control ($p<0.05$)

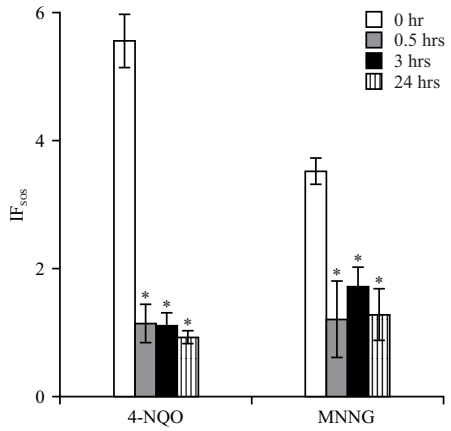

Fig. 3: SOS-induction factor (IF_{SOS}) of 4-NQO and MNNG after co-incubation with chocolate containing freeze-dried *L. rhamnosus* Fb cells, evaluated by SOS-Chromotest
Error bars indicate standard deviation (n = 3) and *Significantly reduced in comparison to control ($p<0.05$)

exhibited no substantial difference in the ability of *L. rhamnosus* Fb to tolerate NaCl, phenol and survival in the simulated gastric fluid after 2 months of storage. However, their tolerance to pH 2, bile salt (2%) and simulated gastrointestinal fluid decreased significantly ($p<0.05$) after 4 months of storage (Fig. 2b).

Antigenotoxic activity of chocolate containing *L. rhamnosus* Fb: Genotoxicity of 4-NQO and MNNG monitored using SOS-induction factor (IF_{SOS}), decreased upon co-incubation with chocolate containing beneficial bacterial cells. Initial IF_{SOS} of 4-NQO was 5.57 which subsequently decreased to 1.14 and 1.11 following 30 and 180 min of incubation with chocolate. Initial IF_{SOS} of MNNG was 3.53 decreased to 1.28 after 24 hrs of incubation with chocolate-containing bacteria (Fig. 3).

DISCUSSION

Functional attributes of *Lactobacillus* are mainly associated with their viability, it is of utmost importance to ensure minimal loss in the viability of cells during storage of probiotic-based formulation and gastrointestinal transit. Therefore, it is a major challenge to develop competent formulations containing live bacterial cells and the commercialization of health-promoting bio-based food products. Therefore, the study was initiated to develop an efficient formulation containing freeze-dried cells of *L. rhamnosus* Fb. The present study showed freeze-dried cells of *L. rhamnosus* Fb retained viability after freeze-drying and with carrier media significantly higher indicates can be used expediently in various food formulations. To minimize cellular injury during freeze-drying, skim milk powder and sugars were used as carrier media as skim milk is widely used in the dairy and food industry. Similarly, Leslie *et al.*[12] and Linders *et al.*[13] reported various sugars such as glucose, fructose, lactose, mannose and sucrose to protect cells during drying and subsequent storage.

The GIT passage, involves exposure to gastric acidity, bile juice and digestive enzymes pose a major survival obstacle for ingested bacteria before they reach the intestine in viable form[10]. In this respect, retention of cellular viability and functionality during gastrointestinal transit, apart from the freeze-drying process and storage, is also critical for the potential *Lactobacillus* strains to exert their beneficial influences on the host. Ability to tolerate digestive stresses is one of the important properties of the successful incorporation of *Lactobacillus* cells into functional foods. Therefore, freeze-dried cells with or without carrier media were evaluated for their survival in gastrointestinal conditions namely acid, bile, phenol and NaCl. Survival of cells decreased significantly upon simulated gastrointestinal transit after 4 months of storage except with the cells freeze-dried in the presence of skim milk with sucrose or lactose indicating protection provided by the skim milk and sugars. Linders *et al.*[13] also studied sucrose for its ability to minimize the dehydration inactivation of *L. plantarum* cells during fluidized bed drying. A strong correlation was observed between cell survival and the pH, exposure time and storage. Phenol tolerance of cells did not alter during storage. Despite the viability reduction during gastrointestinal transit, the viable cell number was still high enough to exert the impact of this product on human health. Saarela *et al.*[14] mentioned in the study that survival of *L. rhamnosus* E800 in simulated gastric digestion differs as per the carrier used for freeze-drying. Therefore, individual strain in actual conditions of the food should be evaluated for gastric acid tolerance and its significant test for designing new functional food products[15]. Moreover, many studies have reported that the food matrix can have a significant effect on the survival of bacteria during gastric transit. Cheese exerted additional protection on probiotic bacteria upon exposure to gastric juice[16]. The immediate environment has a direct influence on the viability of the cells, for instance, the presence of fermentable sugars in an acidic environment can be beneficial in maintaining the viability of some species of lactobacilli[17].

Numerous functional foods consumed as a part of a regular diet confer consumers with well-known benefits of lactic acid bacteria. To develop a functional food using *L. rhamnosus* Fb, chocolate was selected as a delivery medium for the health-promoting constituents of foods. According to various reports Boylston *et al.*[18], Aragon-Alegro *et al.*[19], Terpou *et al.*[20] the count of beneficial bacteria in the foodstuff should be approximate 10^6 CFU g^{-1} and to obtain a beneficial effect, daily ingestion of 10^8-10^9 CFU for an individual has been advocated. Karimi *et al.*[21] reported that ~100 g/day of functional food products should be consumed to deliver about 10^9 viable cells into the intestine on a regular daily basis. The study emphasizes that it is desirable to enrich chocolate with higher numbers of freeze-dried bacterial cells and cells freeze-dried in a carrier medium such as skim milk would increase the survival of these bacteria in chocolate and the ability to survive in gastrointestinal conditions that can contribute enhanced benefits of chocolate containing viable beneficial bacterial cells on human health.

Similarly, Nebesny *et al.*[22] reported dark chocolates supplemented with *L. casei* and *L. paracasei* and sweeteners (sucrose, isomalt and aspartame) retained 10^6-10^7 CFU g^{-1} viable cells after 12 months of storage at 4 and 18°C. Similar to this report[22], *L. rhamnosus* Fb is not able to grow in chocolate indicating they neither assimilate chocolate ingredients nor secreted metabolites. Aragon-Alegro *et al.*[19] mentioned that chocolate mousse is an efficient vehicle for the delivery of *L. paracasei* LBC 82 strain. Possemiers *et al.*[23] stated bacteria and chocolate are an effective blend for the delivery of probiotics. They reported that coating chocolates with probiotics are one of the best strategies to safeguard the cells through delivery from gastrointestinal stress conditions.

These experimental evidence indicates that directly mixing freeze-dried bacterial cells in chocolate is perhaps a more effective way for their efficient delivery as it also protects gastrointestinal hurdles. Technologically, it is an easy and simple process of making chocolate with health-promoting *L. rhamnosus* Fb cells as it requires neither additional equipment nor the construction of a specific design by its manufacturers in the food industry.

The chocolate containing *Lactobacillus* cells demonstrated the ability to counteract potent carcinogens such as 4-NQO (81%) and MNNG (68%). This is the first report describing the antigenotoxicity potential of chocolates supplemented with indigenous *Lactobacillus* cells, it opens a new dimension in the food industry. In addition, such chocolate provides beneficial influences and protective effects in the form of the most-liking food among people.

CONCLUSION

The present study emphasised the importance of freeze-drying as a convenient method for bio-based formulations and the mixing of cells in chocolate matrices is considered a simple and affordable technique for the delivery of viable cells. This study following the main selection criteria proposed by FAO/WHO for probiotics demonstrated that *L. rhamnosus* Fb adequately fulfils technological criteria such as freezing, freeze-drying and storage and is capable of successful simulated gastrointestinal transit. In addition, carrier media such as skim milk, sucrose and lactose are effective in terms of improving shelf life. Chocolate supplemented with *L. rhamnosus* Fb is novel and promising as it exhibits tolerance to gastrointestinal conditions and possesses additional DNA bioprotective activity against carcinogens 4-NQO and MNNG. This can be considered a chocolate formulation with *Lactobacillus* functionality and more apparent health-promoting properties over the conventional chocolate.

SIGNIFICANCE STATEMENT

The study reveals freeze-drying is a convenient method for the preparation of bio-based health-promoting products. Freeze-drying of *Lactobacillus* cells in the presence of carrier media such as skim milk and sugars provide protection and extends its viability and functionality in gastrointestinal conditions. Chocolate containing viable cells of *L. rhamnosus* Fb exhibit antigenotoxic activity against potent carcinogens 4-NQO and MNNG. These findings promote the use of viable freeze-dried cells in the formulation of health-promoting indigenous functional food products.

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