CHAPTER 9

Formulation of Probiotic Chocolates using Lyophilized Cells of *Lactobacillus rhamnosus* Fb

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Introduction

According to FAO/WHO (2001), "Probiotics are defined as live organisms that when administered in adequate amount confer health benefits to the host". The functional and physiological actions of probiotics is part of a very active field of research and so far, the main health benefits proposed to be associated with probiotics are the improvement of gastrointestinal function and immune system, control of lactose intolerance symptoms, reduction of blood cholesterol levels and prevention of carcinogenesis (Macfarlane and Cummings, 1999). To date many systems have been developed in order to deliver probiotics, including fermented and non-fermented dairy products, fruit juices, emulsions, breakfast cereals, cereal bars, ice creams, cheeses and their derivatives (Antunes *et al*., 2005; Cruz *et al*., 2009; dos Santos Leandro *et al*., 2013). The viability of bacteria in probiotic foods can be affected by harsh heat treatment, mechanical processing, storage temperature, physical state of the food matrix and the chemical microenvironment of the bacteria (Fu and Chen, 2011). A range of processes have been used to obtain dry probiotic formulations with prolonged shelf-life, the most common being freeze drying, vacuum drying and spray drying. Once successfully incorporated into a carrier matrix, probiotics should ideally retain their viability during storage, ultimately survive the low pH, digestive enzymes and bile salts of the human gastrointestinal tract, and reach the colon in order to confer their beneficial influences to the host. Dried preparations of live probiotic cultures are most convenient for long-term preservation and use in functional food applications. Freeze-drying is the most frequently used method for the production of probiotic-containing powders, although the exposure of bacterial cells to the attenuating effects of freezing and dehydration can lead to cell injury and decreased viability in many cases (Stanton *et al*., 2003). Freeze-drying is gentler than spray drying, allowing higher probiotic survival rates, but is more time-consuming and expensive, associated with high transport and storage costs of frozen concentrated cultures. Several factors have been identified as critical to microbial cell survival during drying and storage, including initial cell mass, growth conditions, the

composition of growth and drying media, and rehydration conditions. Approaches investigated for enhancing cell survival during the drying and storage of probiotics include the addition of osmo-, thermo-or cryo-protectants to the drying or growth media, micro-encapsulation and exploitation of the adaptive mechanisms of the living cells for survival under conditions of stress (Carvalho *et al*., 2004).

The focus of the present study was to evaluate the influence of freeze-drying process on the viability of bacterial cells alone and in combination with protective agents such as skim milk powder in combination with sugars. In addition, shelf-life and probiotic properties of the formulated combination was also evaluated. Subsequently with the aim to develop probiotic chocolate, the lyophilized cells were incorporated in commercially available dark and milk chocolate slabs and evaluated for the shelf-life, probiotic properties and antigenotoxic activity against 4-nitroquinoline-1-oxide and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG).

Materials and Methods

Bacterial strain and culture conditions

Lactobacillus rhamnosus Fb JX406746 isolated from infant feces was grown in MRS medium at 37°C. The culture was preserved in 10% skim milk at 4°C.

Preparation of frozen cells and lyophilized cells

The cells were harvested from 1 l of 24 h culture *L. rhamnosus* Fb growing in MRS medium by centrifugation (10000 rpm, 20 min, 4°C). The cell pellet was washed thrice with phosphate buffer saline (0.1 M, pH 7.0), again washed twice with phosphate buffer for the removal of medium components bound to the cells and the cells were separated by centrifugation. 500 µl concentrated cell pellet was distributed in Eppendroff tubes, stored-frozen for 18 h at -20°C, and lyophilized using Micromodulyo 0230 (ThermoScientific, USA). Viable count of frozen and lyophilized cells was determined using MRS medium. Probiotic properties of lyophilized cells were evaluated by the methods described below.

Influence of skim milk and sugars on the viability

Biomass mixed in equal proportion with (i) 10% skim milk, (ii) 10% skim milk containing 1% sugar (glucose, lactose or sucrose), was lyophilized as described above, and the viability of cells in the lyophilized biomass was determined at different time intervals using MRS medium.

Preparation of cell suspension of lyophilized biomass

Vial containing lyophilized biomass was resuspended in phosphate buffer (0.1 M, pH 7.0), and used to determine the viability and probiotic properties.

Evaluation of acid-bile tolerance of lyophilized cells

Lyophilized cells were resuspended in phosphate buffer and 100 µl (*ca*. 10^8 cfu/ml) was used to inoculate MRS and modified MRS media with bile salt (0.5-2%), pH (2, 3), NaCl (4-6%) and skim milk containing phenol (0.4%) and incubated at 37°C. After 4 h of incubation, 0.1 ml aliquots from the tubes were serially diluted, plated on MRS agar and incubated at 37°C for 48 h. After 24 h of incubation, 0.1 ml aliquots from above media was re-inoculated in MRS medium and incubated (37°C for 24 h) for the evaluation of tolerance.

Survival of lyophilized cells under simulated gastro-intestinal fluid transit

Viability during simulated gastric and intestinal fluid transit was evaluated as described by Charteris *et al*. (1998a). 100 µl of cell suspension prepared from lyophilized biomass was mixed with 1 ml of simulated gastric fluid (SGF) and incubated for 2 h at 37°C. The cells pelleted by centrifugation were resuspended in 1 ml of simulated intestinal fluid (SIF) and incubated at 37°C for 3 h. SGF- and SGF-SIF-treated cells were serially diluted, and plated on MRS agar for the determination of cell viability. The control constituted of cells treated with phosphate buffer instead of SGF and SIF. The SGF and SIF fluids were prepared as described by (Pithva *et al*., 2014).

Formulation of probiotic chocolate using lyophilized cells of L. rhamnosus Fb

Dark and milk chocolates (75:25) g were held in boiling water bath (20-25 min) and mixed as it melted completely. The mixture was cooled to around 60°C before lyophilized cells of *L. rhamnosus Fb* (10^6 cfu/g) were added to the chocolate mixture and mixed properly. Chocolate mixture was then poured in mould and held at 4°C for 30-40 min, wrapped with aluminum foil, and stored at 4°C. The nutritional values of the dark and milk chocolates are given below: Dark chocolate - carbohydrate g: Protein: Total fat: Energy – 63.29: 3.2:28.01: 518.1 Kcal/100 g; Milk chocolate - carbohydrate g: Protein: Total fat: Energy – 61.90: 6.74:27.82: 524.9 Kcal/100 g.

Antigenotoxic activity of formulated chocolates against 4-NQO and MNNG

1 g of melted chocolate was suspended in 10 ml phosphate buffer, mixed with 10 µg 4-NQO and MNNG, and incubated at 37°C. After 30 min, 3 and 24 h of incubation, 0.1 ml of sample was used to determine the genotoxicity using SOS-Chromotest as described by Quilldrate *et al*. (1985).

Results

Viability of cells after freezing and lyophilization

Freezing did not alter the viability of the cells, while lyophilization caused 16% reduction in the initial count. Differences in the cell viability of lyophilized cells and along with carrier medium *i.e*., skim milk and sugars is shown in Fig. 1. The cells lyophilized with skim milk+sucrose exhibited higher survival followed by skim milk+lactose, skim milk, skim milk+glucose after 4 months of storage at 4°C.

Survival of lyophilized cells in simulated gastro-intestinal conditions

The initial count of viable cells with the carrier media initial count ranged from 7.50 to 7.64 log cfu/ml (Fig. 2). No significant difference was observed among lyophilized cells with or without carrier medium exhibited similar tolerance to acid, bile, NaCl, phenol and simulated gastrointestinal fluids. After 4 months of storage lyophilized cells exhibited significantly less survival of cells when exposed to low pH, bile salt, and simulated gastrointestinal fluids. Substantial difference was observed in the survival of cells stored for 2 and 4 months at 4°C when exposed for 4 h to 2% bile salt, pH 3 and simulated intestinal fluid. Cells lyophilized with skim milk and skim milk containing sugars exhibited significantly (P<0.05) higher survival in the bile salt, phenol, simulated gastrointestinal fluid in comparison to cells without carrier medium. However, no significant (P<0.05) difference was observed among the cells lyophilized with or without carrier media in their survival pH 3, although pH tolerance ability of cells was reduced to 34 and 17% respectively during 2 and 4 months of storage. No viable cells could be detected upon prolong exposure for 4 h to pH 2 in MRS medium with successive storage of 2 and 4 months. There was strong correlation between the pH and exposure time of bacteria on the survival of bacteria and also storage period. Phenol tolerance ability did not alter during the storage. After 4 months of storage, the cells lyophilized with skim milk and skim milk+lactose, and sucrose exhibited significantly (P<0.05) higher survival in comparison to cells

without carrier medium. There was considerable reduction in the survival of bacteria in simulated intestinal fluid during the storage period of 4 months. However, cells lyophilized with skim milk+lactose and skim milk+sucrose showed significantly higher survival in comparison to cells lyophilized without carrier media and those lyophilized with skim milk.

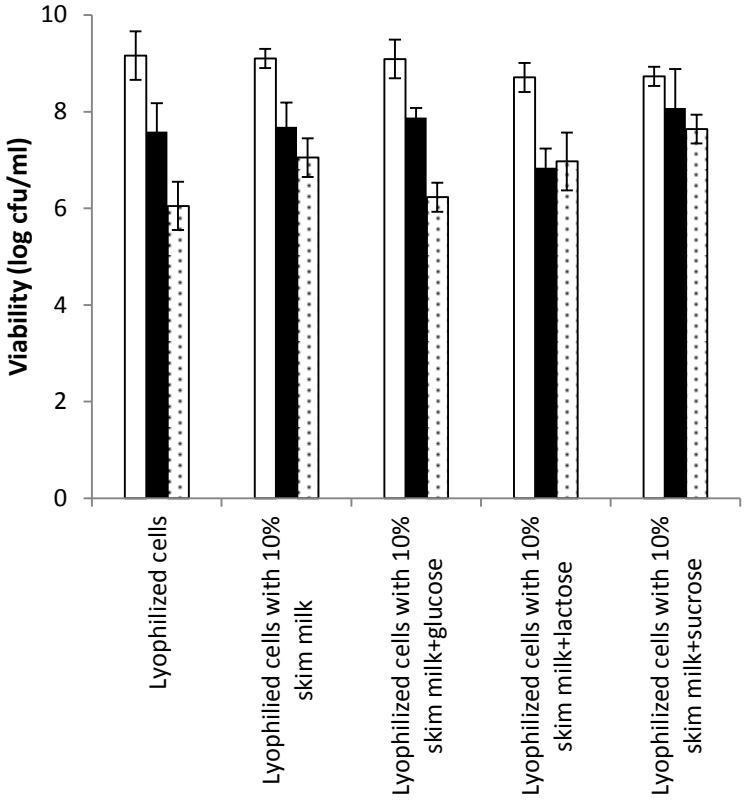

Fig. 1 Viability (log cfu/ml) of lyophilized cells and lyophilized with skim milk and glucose, lactose and sucrose after (□) initial count, (■) 2 months, (⧈) 4 months of storage at 4°C. Error bars represent standard deviation

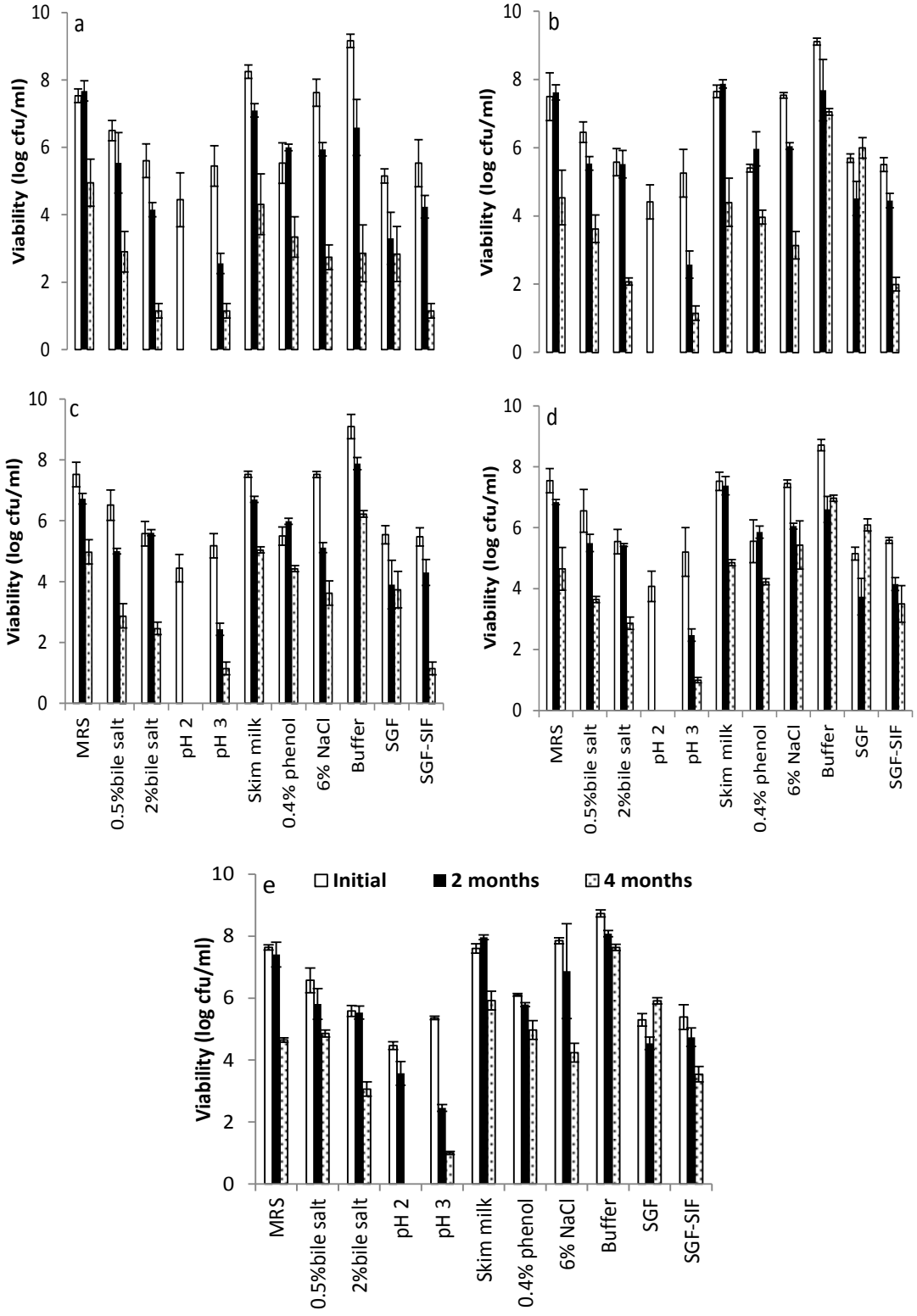

Fig. 2 Viability (log cfu/ml) of lyophilized cells (a), lyophilized with: cells+skim milk (b), cells+skim milk+glucose (c), cells+skim milk+lactose (d) and cells+skim milk+sucrose in the presence of bile salt (0.5-2%), pH (2-3), NaCl (6%), skim milk and skim milk+0.4% phenol and during simulated gastric fluid (2 h) and simulated gastrointestinal fluid (2+3 h) determined during the storage at 4°C using MRS medium. Error bars indicate the standard deviation

Survival of bacteria in formulated chocolate

Chocolates prepared with probiotic bacteria displayed high survival during 1 month storage at 4°C that gradually decreased to 48% after 18 months (Fig. 3a)

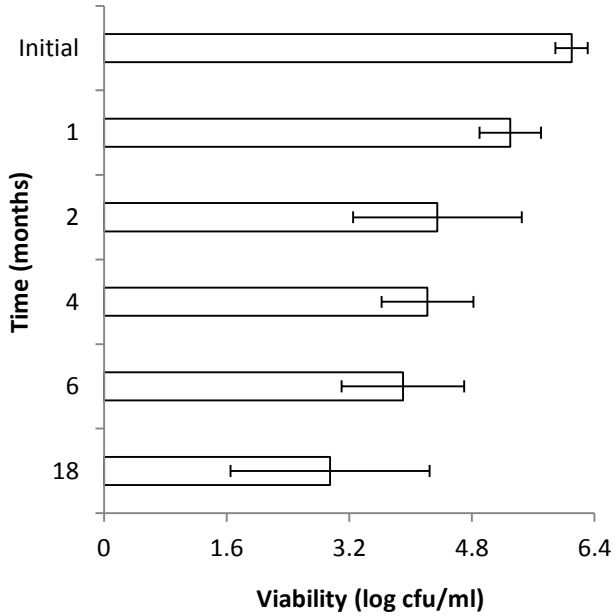

Fig. 3a Viability (log cfu/ml) of bacteria in formulated chocolate during storage (4°C) determined by viable count using MRS medium. Error bars represent standard deviation

Formulated chocolate was evaluated for the *in vitro* probiotic properties such as acid, bile, phenol and NaCl tolerance (Fig. 3b). There was no substantial difference in their abilities to tolerate NaCl, phenol and simulated gastric fluid, but after 4 months of storage tolerance of cells in the formulated chocolate to pH 2, 2% bile salt and simulated gastrointestinal fluid decreased significantly(P<0.05).

Antigenotoxic activity of formulated chocolate

Formulated chocolate exhibited 65-81 and 48-68% antigenotoxic activity against 4-NQO and MNNG respectively (Fig. 4). There was no significant (P<0.05) influence of incubation time on antigenotoxic activity of the formulated chocolate.

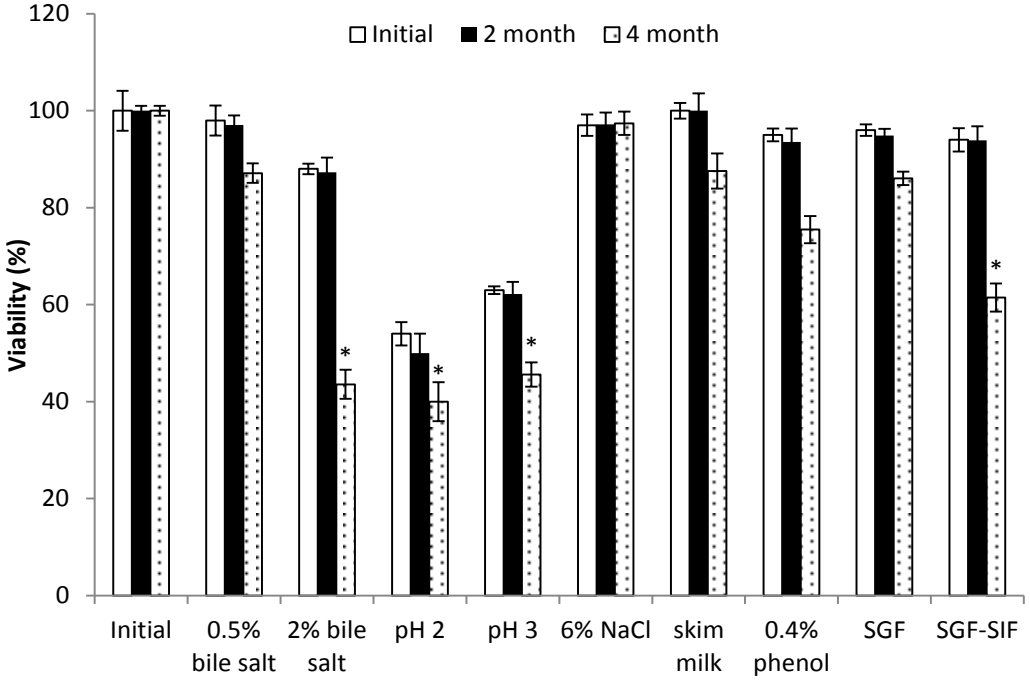

Fig. 3b Viability (%) of *L. rhamnosus* Fb in formulated chocolate in the presence of bile salt (0.5, 2%), pH (2, 3), NaCl (6%), skim milk and phenol (0.4%), simulated gastric fluid (2 h) and simulated gastrointestinal fluid (2+3 h). Viability was determined on MRS medium using viable count method. Error bars represent standard deviation, *significantly different than the initial count (P<0.05)

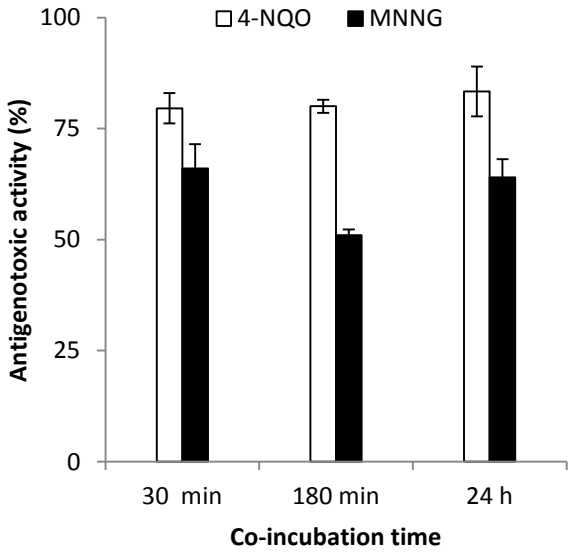

Fig. 4 Antigenotoxic activity of formulated chocolate against 4-NQO (□) and MNNG (■) determined by SOS-Chromotest at an interval of co-incubation time (30 and 180 min and 24 h) and error bars represent standard deviation.

Discussion

The industrial use of lactobacilli as starter cultures for the food industry depends on the concentration and preservation technologies employed, which are required to guarantee long-term delivery of stable cultures in terms of viability and functional activity (Carvalho *et al*., 2004). Freeze-drying is the most frequently used method for the production of probiotic-containing powders, although the exposure of bacterial cells to the diminishing effects of freezing and dehydration can lead to the cell injury and decreased viability in many cases. The present study describes the influence of freeze drying process on the viability of the cells with or without carrier media, *in vitro* probiotic properties and shelf life of cells. In addition, probiotic properties of formulated chocolate and its antigenotoxic activity against potent carcinogens 4-nitroquinoline-1-oxide and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine were also evaluated.

Development of an efficient formulation of live bacterial cells is a major challenge in the commercialization of probiotic products. Probiotic formulations should retain predetermined dose of live bacterial cells during storage. Freeze-drying is a more convenient process and such cells can be used conveniently as a functional ingredient in a variety of probiotic food formulations. Most LAB cultures of commercial interest for the dairy industry, skim milk powder is selected as drying medium because it (i) prevents cellular injury by stabilizing the cell membrance constituents (Castro *et al*., 1996; Selmer-Olsen *et al*., 1999), (ii) creates a porous structure in the freeze-dried product that makes rehydration easier and (iii) contains proteins that provide a protective coating for the cells (Abadias *et al*., 2001). Various sugars such as glucose, fructose, lactose, mannose and sucrose have been tested for their protective effect during drying and subsequent storage (Leslie *et al*., 1995; Linders *et al*., 1997 a, b; Carvalho *et al*., 2003).

In this context, skim milk and sugars glucose, lactose and sucrose were used as carrier media during the freeze-drying process. Lyophilization caused the 16% reduction in viable count of cells; carrier medium provided the protection against loss of cell viability during the storage period of 4 months. Skim milk+sucrose, skim milk+lactose proven to be effective in comparison to skim milk, skim milk+glucose, and free cells. On the contrary to the previous reports the sugars that are metabolized were significantly less effective than those that are not metabolizable. G-Allegria *et*

al. (2004) reported *L. plantarum* strains originating from different sources to survive over 90% after freezing and lyophilization.

Gastrointestinal passage, involves exposure to stomach acid, bile salts and enzymes, and represents a major survival hurdle for probiotic bacteria, before they reach the intestine in viable form. In this respect, culture viability during freeze-drying process, storage (shelf life) and retain their probiotic properties during gastrointestinal exposure to exert their beneficial influences on the host. It is not surprising, therefore, acid and bile tolerance are among the criteria for selection of probiotic strains. Ability to tolerate digestive stresses is one of the important properties for the successful incorporation of probiotics into functional foods. Survival of *L. rhamnosus* Fb was significantly higher in simulated gastric fluid with carrier media skim milk and various sugars in comparison to cells lyophilized without carrier medium. Survival of bacteria intensely decreased upon simulated gastrointestinal transit during storage period of 4 months except with skim milk+sucrose and skim milk+lactose indicates the protection provided by the skim milk and sugars. Saarela *et al*. (2006) reported different survival of *L. rhamnosus* E800 exposed to simulated gastric digestion, depending on the carrier used for freeze-drying. Therefore, the evaluation of gastric acid tolerance of each strain in the particular conditions of the food that will serve as vehicle represents an important test to be considered during the design of new probiotic products (Forssten *et al*., 2011). In addition, several studies have demonstrated that the food matrix can have a significant effect on the survival of bacteria during gastric transit. Cheddar cheese was shown to elicit a protective effect on probiotic bacteria upon exposure to gastric juice when compared with yoghurt (Gardiner, 2000). The composition of the environment can also influence microbial viability; for example, the presence of fermentable sugars in the acidic environment can help to maintain the viability of some species of lactobacilli (Corcoran, 2004).

Numerous functional foods are consumed as part of a normal diet and they provide consumers with well-documented benefits of probiotic bacteria. With the aim of developing functional food, using probiotic *L. rhamnosus* Fb, chocolate was selected as a delivery medium for the probiotic application. According to various reports, the count of probiotic cells in foodstuff should be approximate 10^6 cfu/g and in order to obtain a beneficial effect, a daily ingestion of 10^8-10^9 cfu for each. Possemiers *et al*. (2010) have reported bacteria and chocolate to be a successful combination for

probiotic delivery. They have showed that coating chocolates with probiotics as an excellent solution to protect them from environmental stress conditions and for optimal delivery. Our experimental evidences indicate that directly mixing lyophilized bacterial cells in chocolate is proven to be excellent medium another way for their efficient delivery and provides protection against gastrointestinal hurdles. Technologically, it is easy and simple process of making probiotic chocolates. Moreover, the chocolate demonstrated ability to counteract potent carcinogens such as 4-NQO and MNNG. This is the first report describes the antigenotoxic potential of probiotic chocolates; these could open the new dimension for the research to study the mechanisms of antigenotoxicity. In addition, such chocolate provides beneficial influences and protective effects in the form of most liking foods among people.

In conclusion, following the main selection criteria proposed by FAO/WHO for probiotics, *L. rhamnosus* Fb is able to adequately support different technological conditions such as frozen storage, and salts and able to overcome simulated gastrointestinal transit. In addition, carrier media such as skim milk, sucrose, and lactose are effective in terms of storage stability. Chocolates prepared with *L. rhamnosus* Fb exhibited tolerance to gastrointestinal conditions as well as antigenotoxic activities against carcinogens 4-NQO and MNNG. *L. rhamnosus* Fb possessing additional DNA bioprotective activity can be regarded as a potential probiotic for the formulation of new probiotic foods.