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Original Article

Stability evaluation of freeze-dried *Lactobacillus paracasei* subsp. *tolerance* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in oral capsules

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Abstract

Freeze-drying is a common preservation technology in the pharmaceutical industry. Various studies have investigated the effect of different cryoprotectants on probiotics during freeze-drying. However, information on the effect of cryoprotectants on the stability of some Lactobacillus strains during freeze-drying seems scarce. Therefore, the aim of the present study was to establish production methods for preparation of oral capsule probiotics containing *Lactobacillus paracasei* subsp. *tolerance* and *Lactobacillus delbrueckii* subsp. *Bulgaricus.* It was also of interest to examine the effect of various formulations of cryoprotectant media containing skim milk, trehalose and sodium ascorbate on the survival rate of probiotic bacteria during freeze-drying at various storage temperatures. Without any cryoprotectant, few numbers of microorganisms survived. However, microorganisms tested maintained higher viability after freeze-drying in media containing at least one of the cryoprotectants. Use of skim milk in water resulted in an increased viability after lyophilization. Media with a combination of trehalose and skim milk maintained a higher percentage of live microorganisms, up to 82%. In general, bacteria retained a higher number of viable cells in capsules containing freeze-dried bacteria with sodium ascorbate after three months of storage. After this period, a marked decline was observed in all samples stored at 23°C compared to those stored at 4°C. The maximum survival rate (about 72-76%) was observed with media containing 6% skim milk, 8% trehalose and 4% sodium ascorbate.

Keywords: Probiotics; *Lactobacillus delbrueckii*; *Lactobacillus paracasei*; Freeze-drying

INTRODUCTION

Probiotics are living microorganisms which upon consumption in adequate quantities via ingestion confer beneficial effect on health beyond inherent basic nutrition (1).

Lactic acid bacteria and bifidobacteria are between the most common microorganisms used as probiotics. Mechanisms such as immunomodulation, growth inhibition of pathogens in the gastrointestinal and urogenital tract and improved intrinsic defensive mechanisms, which may be through production of hydrogen peroxide, organic acids, bacteriocins and the release of biosurfactants, are involved in the probiotic effect (2-5).

Because of their generally accepted benefits, probiotics during recent years have gained wide interest and represent an alternative to previous therapies (3). Freeze-drying is a commonly used technique for the production of dried powders of probiotics. In this process, probiotics are exposed to damage from the process conditions such as very low freezing temperatures and dehydration. Cells are first frozen to below the critical temperature of the formulation, and then dried by sublimation under high vacuum in two phases: primary drying, during which unbound

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water is removed and secondary drying, during which the bound water is removed (6,7). These stages can damage the constituents of the cell wall and lead to cell death. However, the presence of cryoprotectants in the drying medium increases the viability of cells after drying (8). It is important to optimize the production process of probiotic preparations in order to obtain a product with suitable properties and higher number of viable probiotic microorganisms. Among several probiotic preparations, there has been an increasing interest in the development of dried formulations (9-13).

Moreover, a variety of cryoprotectants have been used for lyophilisation of probiotics in order to increase the survival rate of microorganisms after freeze-drying. The role of cryoprotectants, such as skim milk powder, whey protein, trehalose, glycerol, betaine, adonitol, sucrose, glucose, lactose and polymers, have been investigated (14). It is technologically and economically reasonable to assess the influence of these compounds on the survival rate of probiotic bacteria and to verify a suitable combination which provides an effective medium for lyophilisation. However, limited data are available on the effect of various combinations of cryoprotectants on the stability of Lactobacillus strains during freeze-drying process. Furthermore, most studies have evaluated the survival of lactobacilli only during the freeze drying process and not during the storage of dosage forms. Therefore, the aim of this study was to prepare oral probiotic capsules and to evaluate the viability of two lactobacilli after freeze-drying and storage during a period of three months.

MATERIALS AND METHODS

Microorganisms

Lactobacillus paracasei subsp. *tolerance (*DSM 20258) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (DSM 20081) were selected as they are well known for their probiotic properties. Selected strains were subcultured in (de Man, Rogosa and Sharpe) MRS-broth (Merck, Germany) twice every 24 hours. Then, 20 ml of the culture was inoculated into 1 L of sterile MRS-broth and incubated at 37°C. Cells in the early stationary phase of growth were harvested by centrifugation (×6000 g, 10 min, 4°C), washed twice with sterile saline and resuspended in 100 ml of saline solution. This suspension was fractioned into ten flasks (10 ml), centrifuged and resuspended in distilled water with each cryoprotective medium. Suspensions were transferred into plates and frozen at -70°C. Combinations of cryoprotective media used in the present study are given in Table 1.

Freeze-drying

Frozen media were desiccated under vacuum for 24 h and freeze-dried powders were placed into capsules. Samples were obtained from each medium before and after freeze-drying and the numbers of viable cells were enumerated.

Storage

Capsules containing lyophilized powder were stored at two temperatures: 23°C and 4°C, for three months inside the desiccators containing silica-gel.

Table 1. Ingredients of cryoprotective media used for freeze drying of two probiotic lactobacilli.

Media	Skim milk (% w/v)	Trehalose (% w/v)	Sodium ascorbate (% w/v)
1	0	0	0
2	6	0	0
3	6	0	2
4	6	0	4
5	6	4	0
6	6	4	2
7	6	4	4
8	6	8	0
9	6	8	2
10	6	8	4

Enumeration of viable cells

The numbers of viable bacteria before and after lyophilisation and during storage were enumerated by a standard poured-plate method. Powders were suspended in sterile saline and serial dilutions of each sample were prepared.

All dilutions were poured into MRS-broth and plates were incubated at 37°C for 48 h. After the incubation time, colonies were enumerated and the mean number of bacteria was expressed as CFU/g. All experiments were repeated in duplicate.

Data analysis

The student *t*-test was used to measure statistical differences between means at 5% level of significance.

RESULTS

Survival of bacteria after freeze-drying

Survival rate was calculated as the total number of bacteria after freeze-drying divided by the total number of bacteria before freeze-drying. Tables 2 and 3, show the survival rate of bacteria after lyophilisation.

According to the data presented in the tables, when microorganisms were freeze-dried in water alone and in the absence of any cryoprotective substance, the survival rate was only 2-3% and the population of the bacteria decreased in a significant manner ($P<0.05$). The addition of 6% skim milk to the medium increased the viability of bacteria up to 20%. Microorganisms showed higher survival rate when trehalose was added to the cryoprotective

Table 2. Survival rate of *Lactobacillus paracasei* subsp. *tolerance* after freeze-drying and three months of storage at 23°C and 4°C (n=3).

Media	Before freeze-drying	After freeze-drying	Survival rate (%)	storage at 4°C	Survival rate (%)	storage at 23°C	Survival rate (%)
1	$(8.66\pm0.4)\times10^{11}$	$(2.6\pm0.4)\times10^{10}$	3	$(2.1\pm0.28)\times10^{6}$	0.008	$(1.31\pm0.08)\times10^{6}$	0.005
2	$(3.85\pm0.35)\times10^{11}$	$(7.7\pm0.18)\times10^{10}$	20	$(1.31\pm0.28)\times10^{10}$	17	$(6.9\pm0.04)\times10^{9}$	9
3	$(4.29\pm0.05)\times10^{11}$	$(8.16\pm0.33)\times10^{10}$	19	$(4.41\pm0.14)\times10^{10}$	54	$(7.34\pm0.28)\times10^{9}$	19
4	$(1.75\pm0.09)\times10^{11}$	$(3.33\pm0.04)\times10^{10}$	19	$(1.97\pm0.02)\times10^{10}$	59	$(9.99\pm0.14)\times10^{9}$	30
5	$(3.93\pm0.04)\times10^{11}$	$(2.44\pm0.04)\times10^{11}$	62	$(1.2\pm0.05)\times10^{11}$	49	$(5.12\pm0.28)\times10^{10}$	21
6	$(4.19\pm0.03)\times10^{11}$	$(2.66\pm0.04)\times10^{11}$	63	$(1.8\pm0.16)\times10^{11}$	68	$(6.86\pm0.07)\times10^{10}$	26
7	$(9.28\pm0.03)\times10^{10}$	$(5.85\pm0.28)\times10^{10}$	63	$(4.1\pm0.14)\times10^{10}$	70	$(1.63\pm0.07)\times10^{1}$	28
8	$(9.06\pm0.1)\times10^{10}$	$(7.07\pm0.08)\times10^{10}$	78	$(4.6\pm0.04)\times10^{10}$	65	$(1.97\pm0.02)\times10^{10}$	28
9	$(1.8\pm0.02)\times10^{11}$	$(1.5\pm0.02)\times10^{11}$	81	$(1.1\pm0.08)\times10^{11}$	73	$(4.95\pm0.07)\times10^{10}$	33
10	$(2.6\pm0.11)\times10^{11}$	$(2.23\pm0.04)\times10^{11}$	82	$(1.7\pm0.08)\times10^{11}$	76	$(8.25\pm0.05)\times10^{10}$	37

Survival rate was expressed as the log of colony forming units and was reported before freeze-drying, after freeze-drying and after three months storage at 23 and 4°C in the presence of cryoprotective media. Data are expressed as mean ± SD.

Table 3. Survival rate of *Lactobacillus delbrueckii* subsp. *bulgaricus* after freeze-drying and three months storage at 23°C and 4°C (n=3).

Media	Before freeze-drying	After freeze-drying	Survival rate (%)	storage at 4°C	Survival rate (%)	storage at 23°C	Survival rate (%)
1	$(1.91\pm0.5)\times10^{10}$	$(3.83\pm0.12)\times10^{8}$	2	$(2.3\pm0.28)\times10^{4}$	0.01	$(1.91\pm0.11)\times10^{4}$	0.01
2	$(1.39\pm0.04)\times10^{10}$	$(2.33\pm0.07)\times10^{9}$	18	$(4.9\pm0.3)\times10^{8}$	21	$(2.79\pm0.72)\times10^{8}$	12
3	$(1.38\pm0.12)\times10^{8}$	$(2.5\pm0.6)\times10^{9}$	18	$(1.2\pm0.09)\times10^{9}$	48	$(3.75\pm0.82)\times10^{8}$	15
4	$(3.47\pm0.46)\times10^{10}$	$(5.9\pm0.9)\times10^{9}$	17	$(3.2\pm0.7)\times10^{9}$	54	$(9.44\pm0.08)\times10^{8}$	16
5	$(1.17\pm0.11)\times10^{10}$	$(6.8\pm0.14)\times10^{9}$	58	$(2.4\pm0.04)\times10^{9}$	35	$(6.12\pm0.18)\times10^{8}$	9
6	$(6.25\pm0.26)\times10^{9}$	$(3.5\pm0.31)\times10^{9}$	56	$(2.3\pm0.08)\times10^{9}$	65	$(7\pm0.08)\times10^{8}$	20
7	$(1.72\pm0.04)\times10^{10}$	$(1\pm0.14)\times10^{9}$	58	$(6.7\pm0.26)\times10^{8}$	67	$(2.4\pm0.08)\times10^{8}$	24
8	$(3.23\pm0.04)\times10^{10}$	$(2.33\pm0.07)\times10^{9}$	72	$(1.4\pm0.05)\times10^{9}$	60	$(8.36\pm0.31)\times10^{9}$	20
9	$(8.36\pm0.16)\times10^{9}$	$(6.02\pm0.25)\times10^{9}$	72	$(2.5\pm0.07)\times10^{9}$	68	$(1.98\pm0.09)\times10^{9}$	33
10	$(1.47\pm0.02)\times10^{10}$	$(1.09\pm0.3)\times10^{10}$	74	$(7.9\pm0.3)\times10^{9}$	72	$(3.81\pm0.11)\times10^{9}$	35

Survival rate was expressed as the log of colony forming units and was reported before freeze-drying, after freeze-drying and after three months storage at 23 and 4°C in the presence of cryoprotective media. Data are expressed as mean ± SD.

media. Media containing 8% trehalose showed greater effect than that containing 4%. A survival percentage of about 17-19% was found in media containing sodium ascorbate and skim milk, indicating that sodium ascorbate at a concentration of 2% (w/v) did not exhibit a good protective effect during freeze-drying (P<0.05). Lyophilisation of microorganisms with 8% trehalose and 2% sodium ascorbate was similar when 8% trehalose and 4% sodium ascorbate were incorporated into the media.

Storage of freeze-dried bacteria

After three months storage of lyophilised powders of bacteria, live cells in each cryoprotective media were enumerated. Results are shown in Tables 2 and 3. At the end of this period, the microbial population declined less than 1 log scale in capsules stored at 23°C or 4°C. The reduction rate of the initial population of probiotics was significant in all media when stored at 23°C (P<0.05). As a general result, sodium ascorbate acted as a protective substance and improved the survival of bacteria. The combination of 8% trehalose and 6% skim milk exhibited a lower cryoprotective effect than the combination of 6% skim milk, 4% trehalose and 4% sodium ascorbate. This data shows that increasing the concentration of trehalose from 4% to 8% without sodium ascorbate increased the survival rate after freeze-drying but not upon storage. The two lactobacilli under study conserved higher viability with a higher concentration of sodium ascorbate. A survival percentage of about 76% was found in capsules containing 6% skim milk, 4% sodium ascorbate and 8% trehalose in the case of *L. Paracasei* and 72% in the case of *L. Delbrueckii*. Therefore, this medium was the most effective amongst the tested cryoprotective combinations.

DISCUSSION

The aim of this study was to investigate the feasibility of preparation of oral probiotic capsules and to evaluate the effect of various combinations of cryoprotective agents on the survival rate of bacteria after freeze-drying and subsequent storage. The cryoprotectants tested increased the survival of two lactobacilli immediately after freeze-drying and upon storage.

Trehalose showed a higher protective effect for both lactobacilli. The highest survival rate after lyophilisation was observed with media containing skim milk and trehalose. This is in agreement with findings of previous studies (1,17). Zayed and coworkers observed that trehalose alone or in combination with skim milk and/or sucrose greatly enhanced the survival of *Lactobacillus salivarius* upon freez drying and storage (1). Furthermore, Giulio and coworkers examined capability of cryoprotective sugars on the protection of various strains of lactic acid bacteria during freeze drying and concluded that trehalose was the most effective one in term of bacterial viability (17). Siaterlis and coworkers, however, found better protection for sucrose at 5 and 10%(w/v) concentration than trehalose and sorbitol when they studied the effect of cryoprotectants on survival of two probiotic lactobacilli during freeze drying (18).

Recently, Li and coworkers proved that freeze drying can destroy membrane structure and change cell viability and activity of lactate dehydrogenase in *Lactobacillus reuteri.* They also observed that membrane intactness and fluidity were increased when they used trehalose or skim milk as cryoprotectant during freeze drying process (19). Disaccharides such as trehalose and sucrose are able to prevent membrane damage and maintain the structure of proteins and biomolecules, limit the intracellular mobility of vital structures and maintain the function of these structures during the freezing stage (20,21). In our study, the increased protective effect of trehalose in the presence of skim milk may be due to a higher total solid content of the medium.

At the end of the storage period, microorganisms retained a high number of viable cells in capsules containing sodium ascorbate and trehalose. Sodium ascorbate was found to favour the stability of bacteria during storage and therefore should be considered for the production of lyophilized cultures. The protective effect of sodium ascorbate have also

been reported in previous studies (15,16). Kurtman showed that stability of *Lactobacillus acidophilus* depended on the water activity of the lyophilised powder, the level of oxygen and the concentration of sodium ascorbate (15). Zarate and Nader reported that probiotics survived in a high number at the end of storage in powders lyophilised in the presence of ascorbic acid. They showed that capsules with lactobacilli suspended in milk, lactose and ascorbic acid provided a longer shelf life (16). Although the effectiveness of the combination of three selected cryoprotectants (skim milk, sodium ascorbate and trehalose) at different concentrations was investigated in the present study, further studies are needed to select better cryoprotective exipients and delivery systems and to optimize the lyophilisation conditions to obtain a preparation with the desired activities.

CONCLUSION

The three cryoprotectants added to the microbial suspensions before drying increased the viability of microorganisms after freeze-drying and storage. The combination of trehalose, sodium ascorbate and skim milk in the concentration of 8%(w/v), 4%(w/v) and 6%(w/v), respectively was found to be the most effective cryoprotectant medium.

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