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Cellular injuries on spray-dried *Lactobacillus rhamnosus GG* and its stability during food storage

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Abstract

Purpose – This paper aims to evaluate the cellular injuries associated with spray-drying of *Lactobacillus rhamnosus GG* (*LGG*) in trehalose/monosodium glutamate (MSG) media by means of flow cytometry measurements; and also whether, and to what extent, the probiotic remain stable and viable in food formulations.

Design/methodology/approach – Spray-drying was applied in the production of trehalose-based preparations containing *LGG*. To gain more insights on the cellular damages that must have occurred during drying, flow cytometric analysis was applied in combination with carboxyfluorescein diacetate (cFDA) and PI stains. Spray-dried samples were observed by scanning electron microscopy (SEM). The storage stability of spray-dried *LGG* was monitored in food samples over a period of time.

Findings – It was observed that during spray-drying, 1.80×10^9 CFU/ml viable counts, which were equivalent of 68.8 per cent cells, were recovered in trehalose matrices but on incorporating 12.5 g/l MSG as a carrier component, survival rates were significantly improved. Density plot analysis showed a higher degree of membrane damage in cells spray-dried in trehalose without MSG. SEM revealed no difference in the shapes and surfaces of spray-dried samples. Evaluation of the recovery rates of *LGG*, initial count of $\sim 10^9$ CFU/ml or g, at storage time intervals revealed a minimum level of $\sim 10^5$ CFU/ml in apple juice after 12 days and $\sim 10^7$ CFU/g in chocolate beverages after ten weeks.

Originality/value – The potential contribution of MSG as a carrier component with trehalose in preventing higher losses during spray-drying and food storage is pointed out in this study.

Keywords Bacteria, Microbiology, Cytology, Food products

Paper type Research paper

Introduction

Probiotics-live micro-organisms which when administered in adequate amounts confer health benefits on the host (Saarela *et al.*, 2009) thus stimulating considerable interest in incorporating these into functional foods and pharmaceutical products. Although there is variability in the minimum recommended levels of probiotics in foods (Charteris *et al.*, 1998), an official standard requiring a minimum of at least 10^7 live micro-organisms/g or ml have been introduced by several food organizations (IDF, 1992; Ishibashi and Shimamura, 1993). Therefore, from a commercial point of view, an inexpensive method for large-scale production of cultures containing high levels of viable probiotic cells in a form suitable for product application is highly desirable (Gardiner *et al.*, 2000).

Spray-drying is one of the most useful techniques for preserving foods, agricultural products and pharmaceuticals. Biological materials, however, can be irreversibly

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damaged during drying as a result of osmotic stress and other dehydration-related stresses (Conrad *et al.*, 2000). To protect cells during drying, a wide range of protective compounds has been used (Meng *et al.*, 2008).

Most studies on probiotics only use plate counts to assess the viability of micro-organisms. Cells that are active but not culturable might contribute to many of the proposed health effects such as lactose conversion, production of antibacterial compounds, assimilation of cholesterol and anti-oxidants effects. Cells in this viable but not culturable state undergo metabolic changes leading to the production of cells that no longer actively form colonies on solid media, but retain other indicators of cell viability such as active membrane potential, maintenance of cellular integrity and the capacity for metabolic activity. However, for some probiotics effects the micro-organisms do not have to perform metabolic activities.

Probiotics formulated into foods encounter additional stress factors such as low pH and oxygen which impair cell viability. Prevention of the inactivation of cell membrane functions is necessary for probiotic survival during drying and in formulated foods.

Materials and methods

Test organism

Lactobacillus rhamnosus GG (*LGG*) was obtained from Valtion Teknillinen Tutkimuskeskus culture collection (Espoo, Finland). The cultures which were sent in freeze-dried form in glass ampoule were later stored as glass beads cultures (Roti$^{(R)}$-Store, Carl-Roth, Karlsruhe, D) in a −80°C freezer (U101, New Brunswick Scientific, Nürtingen, D) for long-term maintenance.

Preparation of samples

Cell suspensions were prepared as previously described by Sunny-Roberts and Knorr (2009). Sterilized trehalose or trehalose supplemented with 12.5 g/l monosodium glutamate (MSG) (Sigma-Aldrich) were used as carrier media for spray-drying process.

Drying process

The spray-drying of *L. rhamnosus GG* in the trehalose media was undertaken in a laboratory scale spray dryer (Büchi B-191, Flawil, Switzerland) as reported by Sunny-Roberts and Knorr (2009).

Determination of probiotic viability in spray-dried powders

Plate enumeration method

Viable cells in fresh and dried trehalose powders were enumerated by drop count technique on de Man, Rogosa and Sharpe (MRS) agar incubated at 37°C for 72 h.

Staining procedure and flow cytometric measurement

Double staining with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI). A 100 μl aliquot of rehydrated powder was mixed with 900 μl Ringer solution and centrifuged for ten minutes at 4,000 *g*. The pellet was resuspended in 100 μl PBS buffer (0.05 M and pH 7.0) and mixed together with 100 μl of 100 μM cFDA stock solution (Molecular Probes Inc., Leiden, The Netherlands), so that the concentration of cFDA in the reconstituted pellet suspension was 50 μM. The suspension was incubated at 37°C for ten minutes to allow intracellular enzymatic conversion of cFDA

into carboxyfluorescein (cF). After excessive cFDA was removed by centrifugation, 30 μM PI (Molecular Probes Inc., Leiden, The Netherlands) was added. The cell suspension was kept in an ice bath for ten minutes to allow labelling of the membrane-compromised cells prior to flow cytometric (FCM) measurement. FCM measurements were done as described by Ananta *et al.* (2005).

Electron microscopy

Spray-dried powders were mounted on stubs, dried with acetone by the CO_2 method using the CPD 030 Critical Point Dryer (BAL-TEC) followed by the coating of dried cells with gold (20 nm). Cells were examined and photographed with S-2700 Scanning Electron Microscope (HITACHI) operating at 20 kV. Micrographs were taken at various magnifications (Leverrier *et al.*, 2003).

Recovery rates

The stability of the spray-dried cells was monitored in a powdered chocolate dairy beverage stored at 25°C and apple juice (pasteurized 100 per cent apple juice without preservatives) stored at 4°C over a period of ten weeks and 14 days, respectively. Samples were mixed separately with spray-dried trehalose powders containing probiotic cells to a final concentration of 10^9 CFU/g or ml. The resultant samples were transferred into sterile plastic jars under aseptic conditions before storage. Samples were collected at intervals, serially diluted in Ringer's solution and plated on MRS agar plates. Survivors were enumerated after three to five days of anaerobic incubation at 37°C in an anaerobic jar.

Statistical analyses

Statistical analyses were performed using analysis of variance. Differences were considered as significant at $p < 0.05$. All statistical analyses were performed with Origin7 software package (OriginLab, Northampton, Massachusetts, USA).

Results and discussion

Survival rates during spray-drying in supplemented or non-supplemented trehalose

The spray-drying of *L. rhamnosus GG* in 20 per cent (w/w) trehalose resulted in 69 per cent survival rates which corresponded to viable counts of 1.8×10^9 CFU/ml. Survival was made possible by cell accumulation of trehalose from growth medium before drying processes (Sunny-Roberts and Knorr, 2009). Tymczyszyn *et al.* (2007) and Ferreira *et al.* (2005) demonstrated the preservation and survival of dehydrated bacteria after growing them in the presence of trehalose, sucrose or MSG.

The incorporation of 12.5 g/l MSG as a component of carrier media produced an increased survival, 80.8 per cent, which was significantly different ($p < 0.05$) from survival rates obtained when cells were dried only in trehalose media.

Besides, the carrier media, the final pH of the growth media of the cells might have influenced survival during spray-drying (Meng *et al.*, 2008). In this study, the final pH of the MRS growth medium at the time of harvest was ~3.8. Acid shock might have influenced the synthesis of heat shock proteins; thus improving bacterial resistances to drying. Similar reports were made in experiments with uncontrolled pH (Silva *et al.*, 2005; Meng *et al.*, 2008).

FCM analysis of spray-dried bacterial cells

In order to clarify the cellular sites that were affected by spray-drying, FCM analysis was applied on fresh cultures or spray-dried cells. *LGG* was stained with both cFDA and PI. Basically this staining technique reveals the capability of viable cells in converting non-fluorescent cFDA into a membrane-impermeant fluorescent product, cF, which can be accumulated in cells' cytoplasm. Cells require a high degree of membrane integrity and functional cytoplasmic enzymes in order to retain this dye. Besides, cells with intact membranes exclude nucleic acid dyes (PI). PI enters cells with compromised membrane and binds to RNA or DNA thus forming a complex which emits red fluorescence when excited.

Bacterial populations were differentiated based on their fluorescence properties (Figure 1). The quadrants on the density plot were set in such a way that viable cells with intact membranes were in no. 4 where active cells showed high-green fluorescence and low-red fluorescence which signified accumulation of cF and exclusion of PI.

Control cells (in Ringer's solution) were encountered in no. 4 but as a result of thermal treatment (95°C, 15 minutes), the cells' membranes were compromised and cF retention ability was lost. Moreover, cells were not capable of excluding PI. Cells showed low-green fluorescence and high-red fluorescence and were gated in no. 1 (Figure 1(b)).

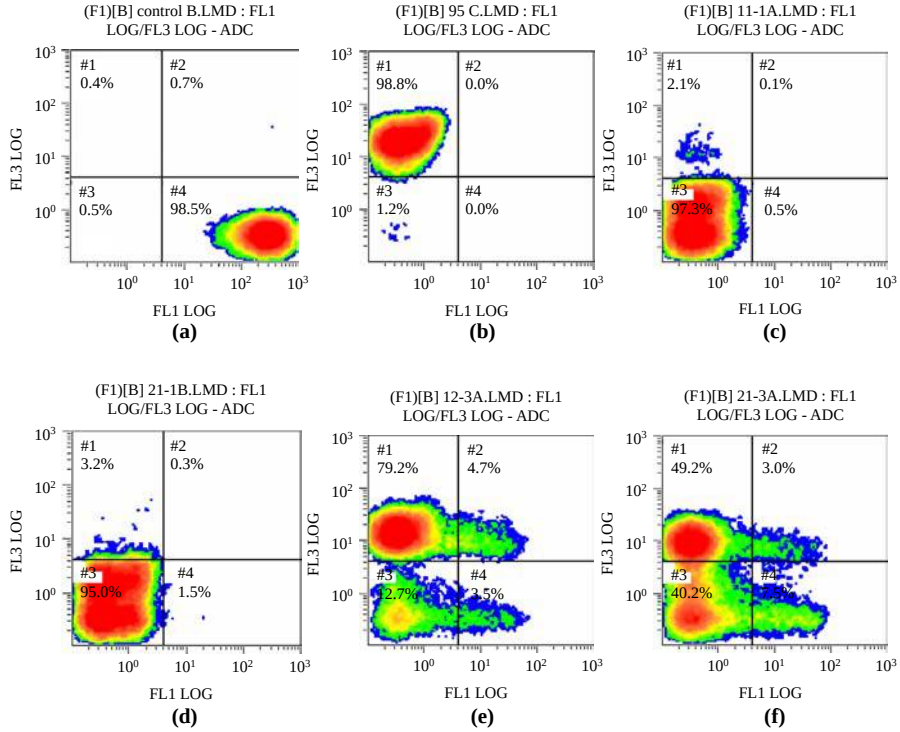


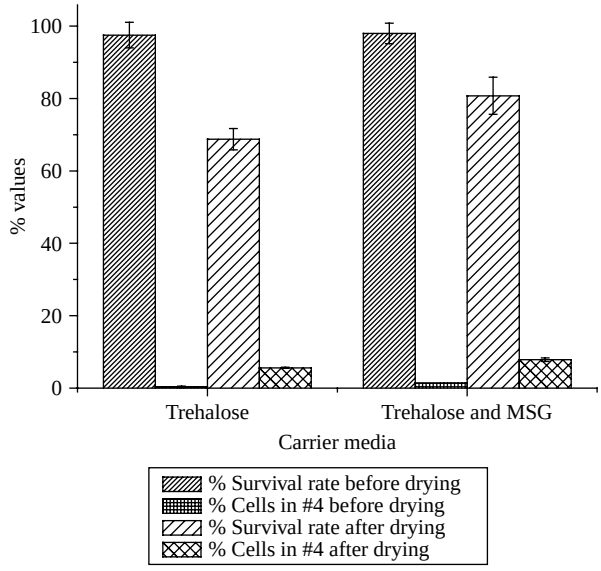
Figure 1.
Representative FCM density plot analysis of *L. rhamnosus GG* spray dried in 20 per cent (w/w) trehalose at 65-70°C air outlet temperature

Notes: (a) Control cells (in Ringer's solution); (b) dead, membrane compromised cells following heat treatment at 95°C, 15 minutes; (c)cells in non-supplemented 20 per cent w/w trehalose prior to drying; (d) cells in trehalose supplemented with MSG prior to drying; (e) cells in non-supplemented 20 per cent w/w trehalose after drying; (f) cells in trehalose supplemented with MSG after drying

Analysis of the feed suspensions by FCM density plots revealed that more than 90 per cent control population in no. 4 moved to no. 3 (Figure 1(c) and (d)). The presence of such population in no. 3 indicated either a loss of esterase activity or cF extrusion (Ananta *et al.*, 2004). In previous studies, populations gated in no. 3 were reported to exhibit membrane permeabilization (Sunny-Roberts *et al.*, 2007), therefore, they could not retain cF but the penetration of PI into cells was not allowed. This signified that membrane integrity was still maintained as shown in Figure 2, where the survival rate of *LGG* on MRS plates was ~98 per cent which was significantly different from the percentage of cF stained cells (~1.4 per cent) in no. 4. The high survival rate could be as a result of the ability of cells in no. 3 to resume growth on MRS agar plates after a repair of transient permeabilized membranes.

Upon drying in non-supplemented 20 per cent (w/w) trehalose, more than half of the populations in no. 3 moved to no. 1 (Figure 1(e)) and they are referred to as "dead". Death was due to additional stress (oxidative, dehydration and osmotic) encountered in the course of spray-drying. FCM density plot analysis of cells dried in trehalose supplemented with MSG revealed high percentage of cells gated in no. 3 (Figure 1(f)). This difference was reflected in survival rates on MRS agar plates (Figure 2).

The gating of more cells in no. 4 (Figure 1(e) and (f)) was made possible as a result of the expression of stress metabolites (e.g. heat shock proteins) produced in the course of drying. These metabolites must have repaired the leaky membranes of some of the cells in no. 3 thus allowing the accumulation and enzymatic conversion of cFDA into cF resulting into cF fluorescence. However, the percentage of cF-stained population in no. 4 (<10 per cent) was significantly different ($p < 0.05$) from the survival rates on MRS agar plates (Figure 2). The retention of intracellular cF was not crucial in the maintenance of culturability (Bunthof *et al.*, 1999).



Notes: Results are means based on data from three drying experiments; error bars show SDs of the means

Figure 2.
The survival rates of *L. rhamnosus GG* spray dried in trehalose or trehalose supplemented with MSG at 65-70°C, by plating on MRS agar and the percentage of cF accumulating population in no. 4 of the flow cytometry density plots (Figure 1(e) and (f))

Scanning electron microscopy of spray-dried cells

The scanning electron microscopy (SEM) images of spray-dried *LGG* in skim milk powder and trehalose powder are as shown in Figure 3. Skim milk powder, the most commonly used drying medium, was used as a control sample. The survival rate of *LGG* in spray-dried skim milk was 75 and 69 per cent in non-supplemented trehalose.

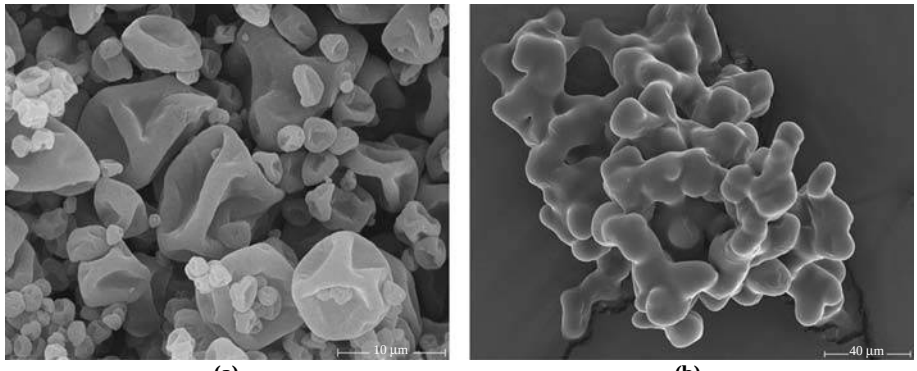
SEM of the spray-dried powders did not reveal that the probiotic cells were present. It was assumed that probiotics were encapsulated in the dried samples.

Gardiner *et al.* (2000) had similar observation and established the encapsulation of bacterial cells by confocal scanning laser microscopy technique. Skim milk had individual particles but with uniform appearances and surfaces (Figure 3(a)). The individuality of spray-dried particles served as protective compartments for spray-dried probiotics thus reducing cells' exposure to environmental stress. Non-supplemented trehalose appeared in an irregular form (Figure 3(b)), which must have exposed cells to stress impacts thus lowering viability. Thus, the reported differences in survival rates of the probiotic despite its being encapsulated could be related to the structural forms of powders. Supplemented trehalose had same structural form as non-supplemented trehalose (not shown), yet the former improved cells' survival rates due to anti-oxidant property.

Stability of spray-dried LGG in powdered chocolate dairy beverage and apple juice

Probiotic-containing spray-dried trehalose powders was mixed with a chocolate dairy powder or 100 per cent apple juice to a final concentration of $\sim 10^9$ CFU/g or ml. The addition of probiotics to food systems is a very effective means of restoring at least part of the initial value associated with microflora that are typically destroyed during treatments such as sterilization, pasteurization, disinfection, irradiation, washing and peeling (Suita-Cruce and Goulet, 2001).

There was a rapid decline in the viable counts of *LGG* (in non-supplemented trehalose) in dairy beverage powder over a period of eight weeks whereas survival rates were maintained at a higher level in beverage powder formulated with supplemented trehalose throughout the storage period (Table I). After ten weeks of storage, the viable counts in the latter remained constant at $\sim 10^7$ CFU/g (data not shown).



(a) **(b)**

Notes: (a) Skim milk powder; (b) trehalose powder spray dried at 65-70°C

Figure 3.
SEM images of a *L. rhamnosus GG*

Storage period (weeks)	Viable counts of *LGG* Non-supplemented trehalose[a] Concentrations (CFU/g)	Survival rates (%)	Supplemented trehalose (with MSG)[a] Concentrations (CFU/g)	Survival rates (%)
0	$4.40 \pm 0.29 \times 10^9$	100.0	$5.13 \pm 0.14 \times 10^9$	100.0
1	$6.75 \pm 0.30 \times 10^8$	15.3	$4.83 \pm 0.04 \times 10^9$	94.1
2	$1.50 \pm 0.10 \times 10^7$	0.3	$4.80 \pm 0.01 \times 10^9$	93.7
3	$6.23 \pm 0.25 \times 10^6$	0.1	$3.45 \pm 0.03 \times 10^9$	67.3
4	0[b]	0.0	$3.03 \pm 0.19 \times 10^9$	59.1
5	0	0.0	$2.63 \pm 0.06 \times 10^9$	51.2
6	0	0.0	$2.15 \pm 0.13 \times 10^9$	41.9
7	0	0.0	$7.64 \pm 0.20 \times 10^8$	14.9
8	0	0.0	$4.60 \pm 0.22 \times 10^7$	0.9

Notes: [a]Carrier media: initial bacterial concentration was $\sim 10^9$; [b] < 10 CFU/g; values are results of at least two independent experiments

Table I.
Stability of *L. rhamnosus GG* in a chocolate dairy beverage stored at 25°C

The composition of the chocolate powder includes cocoa, maltodextrin, sugar, salt, calcium carbonate and some vitamins. Besides, the water activity (0.35 ± 0.02) of this food product, it was assumed that the protective abilities of its components, maltodextrin and calcium (King and Su, 1993; Burin *et al.*, 2004), might have contributed to the probiotic survival and recovery rates. However, comparing these results in relation to supplementation factor, MSG played a significant role. A daily dose of at least 10^8 cells was proposed to elicit the health-promoting effect on consumers' health (Lourens-Hattingh and Viljoen, 2001). These high numbers achievable by consuming 100 g or ml of food containing a minimal level of 10^6 CFU/g or ml have been suggested to appropriately compensate for the possible loss in the numbers of probiotic organisms during passage through the stomach and intestine.

The stability and viability of *LGG* in apple juice is as shown in Table II. In non-supplemented or supplemented trehalose there were reductions in viable counts reaching 2.25 and 4.34×10^7 CFU/ml, respectively, after eight days of storage.

Storage period (days)	Viable counts of *LGG* Non-supplemented trehalose[a] Concentrations (CFU/ml)	Survival rates (%)	Supplemented trehalose (with MSG)[a] Concentrations (CFU/ml)	Survival rates (%)
0	$1.75 \pm 0.01 \times 10^9$	100.0	$4.04 \pm 0.05 \times 10^9$	100.0
2	$6.41 \pm 0.12 \times 10^8$	36.6	$2.01 \pm 0.10 \times 10^9$	49.6
4	$6.15 \pm 0.23 \times 10^6$	0.4	$5.55 \pm 0.23 \times 10^8$	13.7
6	$5.92 \pm 0.07 \times 10^6$	0.3	$5.24 \pm 0.12 \times 10^8$	13.0
8	$2.25 \pm 0.05 \times 10^6$	0.1	$4.34 \pm 0.34 \times 10^7$	1.1
10	$3.07 \pm 0.28 \times 10^5$	0.0	$1.22 \pm 0.20 \times 10^7$	0.3
12	$6.05 \pm 0.22 \times 10^4$	0.0	$6.18 \pm 0.25 \times 10^5$	0.0

Notes: [a]Carrier media: initial bacterial concentration was $\sim 10^9$; values are results of at least two independent experiments

Table II.
Stability of *L. rhamnosus GG* in apple juice stored at 4°C

These decreases might be attributed to the antimicrobial activity associated with pH, other antimicrobial compounds and/or oxidative stress.

Saarela *et al.* (2009) achieved a better performance of acid stressed and freeze-dried *L. rhamnosus* cells in apple juice (pH 3.4-3.8) stored at 4°C. Although this apple juice had low pH as the apple juice (pH 3.2) in this study; the reported better performance of *LGG* by the aforementioned authors was attributed to fermentation at reduced pH before drying process. There must have been an induction of proteins synthesis during pH adjustment thus improving bacterial survival to a subsequent lethal acid challenge. Reid *et al.* (2007) reported the viability of microencapsulated *L. rhamnosus ROII* in frozen vegetable juice to be 33.4 per cent after two weeks, which was almost of same value (36.1 per cent) as the viable cells of spray-dried *LGG* (in supplemented trehalose) recovered from refrigerated apple juice after one week. The storage temperatures coupled with the method of probiotic preparation made it impossible to give a better comparison.

Comparing the results obtained in Tables I and II, the role of water activity (a_w) of food constituents (amongst other factors such as pH, food composition, etc.) on the level of viability and stability of added probiotics is considered as very important. One of the mechanisms that have been suggested to explain the protective effect of sugar matrix on biological systems is the ability of sugars to form a glassy structure in which biomolecules are embedded. When such dried glassy structure are exposed to increasing level of a_w there is a tendency of uptake of water and this can lead to lowering of the glass transition temperature (T_g), a parameter describing the glassy state of amorphous material. When the T_g reduces, the viability and/or stability of the embedded microbes reduces (Higl *et al.*, 2007). It can be assumed that the entrapment of *LGG* in a glassy state appeared to have a pronounced effect on absolute stability of the probiotic. Although MSG has been given a bad reputation as a suspicious additive, which many consumers believe gives allergies but many studies have found that it does not cause ill effects (McLaughlin, 2007). These present findings proved the suitability of MSG, when added to trehalose, as an effective anti-oxidant, which minimized oxidation of membrane lipids that might have impaired probiotic viability during food storage.

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