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Different stress tolerance of spray and freeze dried *Lactobacillus casei* Shirota microcapsules with different encapsulating agents

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Abstract In this study, the effects of encapsulation with maltodextrin and reconstituted skim milk (RSM) and their binary and ternary blends with gum arabic (GA) by spray and freeze drying methods on viability of probiotic *Lactobacillus casei* Shirota under different stress conditions were evaluated. All microcapsules showed high survival ratios (7.91–9.37 log cfu/g) after microencapsulation. The viability of microencapsulated cells was significantly higher than free cells when exposed to stress conditions. Spray dried microcapsules exposed to low pH showed small decrease in the viability of cells compared to freeze dried microcapsules, but freeze drying microcapsules showed higher protective effect at 85 and 90 °C. After exposure to 3% bile salt, almost 2.5 log decreases in the encapsulated cell counts were determined for both methods. The results indicated that using RSM:GA mixture as an encapsulating agent showed the higher cell protection against high temperature, acidic pH and bile salts.

Keywords Microencapsulation · Spray drying · Freeze drying · Probiotic · Stress conditions

Introduction

Probiotic microorganisms are defined as “live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host” (Guerin et al., 2017). They prevent gastrointestinal infections, diarrhea and inflammatory bowel diseases, decrease serum lipids and improve the immune system due to their anticarcinogenic, antibacterial and antimutagenic effects (Arslan et al., 2015). In order to exert these benefits, they must be present at least $10^{6\text{-}7}$ CFU of live microorganisms per g or mL at the moment of food consumption (Pinto et al., 2015; Rodrigues et al., 2011). However, the viability and bioactivity of probiotics sharply decrease during food processing, storage and consumption and passage through the stomach and intestinal system due to different stress conditions, such as simulated gastrointestinal conditionals, heat treatment and oxygen (Heidebach et al., 2012). In addition, other further disadvantages are the fact that probiotics generally used in fermented foods and lead to the deterioration of flavor and aroma of foods during storage due to bioactivity of living probiotic cells. Many studies showed that microencapsulation technique is a promising solution to these problems (Cook et al., 2012; Guerin et al., 2017; Liao et al., 2017). Microencapsulation is a useful technique that leads to stabilize and maintain of viability by protecting cells against harsh gastrointestinal environment, to deliver viable cells into foods, and also controlled release of them in the colon by enclosure of cells within the encapsulating agents (Ainsley Reid et al., 2005; Muthukumarasamy et al., 2006; O’Riordan et al., 2001). To provide these positive effects, it is convenient to convert the microcapsules into dry powder form by spray and freeze drying (Semyonov et al., 2010). The spray drying is a method commonly used in food industry, which provides

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some advantages such as low cost operation, produce large quantities of dried cells, having a single process unit for particle formation and drying and also suitable for produce of powders with particle size in the micrometer scale (Ghandi et al., 2012; Ilha et al., 2015, Pispan et al., 2013). Freeze drying is one of the most adequate methods for drying sensitive materials like microorganisms and also have some advantages such as less aroma loss during drying process and good dissolution properties of dried materials (Augustin and Hemar, 2009). Despite the advantages expressed for these methods, significant loss of probiotic viability can occur during the drying process and storage period due to changes in the physical state of membrane lipids or in the structure of sensitive proteins of bacterial cell caused by high inlet temperature and evaporation rates (Fritzen-Freire et al., 2013a; Ghandi et al., 2012; Semyonov et al., 2010). Hence, the use of biopolymer is very important to enhance of cell viability throughout stressful treatments during drying process and also storage period. Sugars (glucose, sucrose, oligosaccharide, glucose syrup i.e.), polysaccharides (starch and starch products, alginate, pectin, chitosan, cellulose derivatives, cyclodextrin, maltodextrin, dextrose i.e.), gums (arabic, guar, carrageenan i.e.), proteins (soy proteins, caseins, whey proteins, egg proteins, zein, gelatin or hydrolysates of these proteins i.e.) and their mixtures have been widely used for microencapsulation of probiotics (Augustin and Hemar, 2009; Cook et al., 2012; Estevinho et al., 2013; Heidebach et al., 2012). A variety of encapsulating agents including maltodextrin (MD), reconstituted skim milk (RSM), gum arabic (GA) have been evaluated in order to protect probiotic cells against the harsh conditions (Reyes et al., 2018b). The most commonly used encapsulating agents are MD and RSM due to their low cost and they showed prominent effect on improvement of cell survival during drying (Liao et al., 2017). However, MD has low emulsifying capacity and also it is caused to low volatile retention (Cano-Higuita et al., 2015). GA is a natural composite of proteins and polysaccharides, which is highly soluble and surface active material. It is mainly used in drying process due to its excellent emulsifier properties. However, its application in food industry is limited because of its high cost (Cano-Higuita et al., 2015; Reyes et al., 2018b). Besides carbohydrate-based wall materials, as a milk protein type, skim milk has a good amphiphilic property offering the necessary physicochemical and functional properties for microencapsulation of core materials (Gharsallaoui et al., 2007).

Furthermore, the choose of an appropriate method and encapsulating agents is of crucial importance for microencapsulation of probiotic bacteria due to the viability of encapsulated cells depends on microencapsulation method and/or encapsulating agent when exposed to different stress conditions such as high temperature, acidic and bile conditions (De Castro-Cislaghi et al., 2012). The present study investigated the influence of different encapsulating agents such as maltodextrin (MD) and reconstituted skim milk (RSM) and their binary and ternary blends with gum arabic (GA) on the viability of spray and freeze dried *Lactobacillus casei Shirota* microcapsules under different stress conditions.

Materials and methods

Materials

The bacterial strain used in this study was freeze dried culture of *Lactobacillus casei* Shirota (isolated from Yakult, a commercially available drink) obtained from the Food Control Laboratory Directorate, Erciyes University (Kayseri, Turkey). The encapsulating agents used were reconstitute skim milk (RSM; Pınar Dairy Co., İzmir, Turkey), maltodextrin (MD) of 13–17 dextrose equivalents and gum arabic (GA) (Sigma–Aldrich, Steinheim, Germany). All other reagents were of analytical grade.

Culture and sample preparation

L. casei Shirota was activated in de Man Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) supplemented with filter sterilized 0.5 g/l L–cysteine (Sigma–Aldrich, Steinheim, Germany) to create a more favorable environment and incubated at 37 °C for 16 h. An aliquot of culture was transferred and re-cultured in 50 mL of MRS broth under the same conditions for 24 h to obtain a cell density of about $10^{9\text{-}10}$ cfu/mL. The reactivated cultures were harvested by centrifugation at $2000\times g$ for 10 min at 4 °C using a refrigerated centrifuge (Nuve–Bench Top Centrifuge, NF 1200R, Ankara, Turkey) and bacterial pellet was washed three times with sterile 0.1% peptone solution.

The feed solutions for drying experiments were prepared by mixing the bacterial pellet with 30% (w/v) total solid containing RSM, MD and GA as previously described by Gul (2017). The encapsulating agents were dispersed in distilled water at room temperature and mixed until complete dissolution. Microcapsules were produced with the following encapsulating agent matrix formulations: pure maltodextrin (MD); 50% maltodextrin + 50% gum arabic (1:1 w/w, MD:GA); 50% maltodextrin + 50% reconstitute skim milk (1:1 w/w, MD:RSM); pure reconstitute skim milk (RSM); 50% reconstitute skim milk + 50% gum arabic (1:1 w/w, RSM:GA); and 33.3% maltodextrin + 33.3% reconstitute skim milk + 33.3% gum arabic (1:1:1 w/w/w, MD:RSM:GA). All the encapsulating media were heat treated at 80 °C for 30 min and then cooled to room temperature. The harvested culture was added into the encapsulating solutions to obtain a desired core-to-wall ratio of 1:1.5 (v/v) and then microencapsulated by spray and freeze drying methods.

Microencapsulation by spray and freeze drying

Microencapsulation by spray drying was performed with a laboratory scale spray dryer (Buchi Mini Spray Dryer B–290, Buchi Labortechnik, Flawil, Switzerland) with a peristaltic pump in the following conditions: inlet air temperature of 160 °C; suspension feed flow rate of 30% (9 mL/min); drying air flow rate of 601 L/h; and aspirator rate of 35 m^3/h, as described by Gul (2017). Outlet air temperature was recorded between 64 and 68 °C from the digital outlet temperature display on the spray dryer. The microcapsules containing probiotic bacteria were collected from the base of the cyclone and placed into sterile high density polyethylene (HDPE) bottles with screw–on lids and stored at 4 °C.

For freeze drying, encapsulating material solutions containing probiotic bacteria was placed into plastic plates with a layer of 1 cm thick and samples were frozen at − 40 °C for 24 h in a freezer (Arçelik, İstanbul, Turkey). Then frozen samples were introduced into a laboratory-scale freeze dryer (VirTis BenchTop ‘K’ Manifold Freeze Dryer, SP Scientific, Warminster, PA, USA) operating at 50–60 mTorr condenser pressure and − 85 °C drying temperature for 28 h. The dried samples were ground and kept into sterile HDPE bottles with screw-on lids and stored at 4 °C (Moayyedi et al., 2018).

Survival of free and microencapsulated cell under heat treatments

The resistance of free and microencapsulated *L. casei* Shirota to heat was determined according to the method of Sabikhi et al. (2010), with slight modifications. One mL of free cells suspension (∼ 10 log cfu/mL) and 0.1 g of the microencapsulated culture were transferred into test tubes containing 10 mL of peptone water (0.1 g 100 mL^{-1}) preheated to 72, 85 or 90 °C and the tubes maintained in a thermostatic water-bath (Nuve, Ankara, Turkey) previously cited conditions for 1 min. After the heat treatments, the tubes were cooled to room temperature in chilled water and cell counts were immediately performed.

Survival of free and microencapsulated cell under acidic conditions

The resistance of free and microencapsulated *L. casei* Shirota to acidic conditions was evaluated according to Iyer and Kailasapathy (2005), with some modifications. One mL of free cell and 0.1 g of the microencapsulated culture were added to 10 mL of a sterile aqueous solution of 0.2% (w/v) NaCl previously adjusted to pH 2.0, 3.0 and 4.0 with 0.1 or 1 N HCl and vortexed for 20 s for complete dispersion of the microparticles and free cells. The samples were incubated at 37 °C for 180 min and taken at 30, 60, 120 and 180 min for cell enumeration.

Survival of free and microencapsulated cell under bile salt conditions

Determination the resistance of free and microencapsulated *L. casei* Shirota to the bile salt (Oxgall; Sigma Chemical Co., Saint Louis, MO, USA) conditions was conducted as; 0.1 g of microcapsule and 1 mL of free cell suspension were incubated at 37 °C for 180 min in sterile bile solutions (1, 2 and 3%, w/v, adjusted to pH 7.0 with 0.1 N NaOH). The samples were taken after 30, 60, 120 and 180 min incubation for cell viability (Iyer and Kailasapathy, 2005).

Enumeration of bacteria

The viability of the probiotic cell in solutions before and after treatments, the samples were serially diluted with peptone water (0.1%, w/v) and plated on MRS agar (Merck, Darmstadt, Germany). Colonies of probiotic bacteria were enumerated following incubation at 37 °C for 48 h under anaerobic conditions. Results were expressed as colony-forming units per milliliter or gram (CFU/mL or g).

Statistical analysis

All microcapsule productions and analysis were carried out at least triplicate and results were expressed as their mean values ± standard deviation (SD). The data analysis was carried out using the SPSS program for windows (version 21.0; SPSS Inc., Chicago, IL, USA). One–way analysis of variance (ANOVA) followed Duncan’s multiple-range test was used to determine significant differences ($P < 0.05$) amongst the means.

Results and discussion

Viability of microencapsulated *L. casei* Shirota after spray and freeze drying

The counts of microencapsulated *L. casei* Shirota after the spray and freeze drying processes are shown in Table 1. The initial count before spray and freeze drying was in the range of 9.39–9.53 log cfu/mL, and the viable cell

Table 1 Viability (log cfu/g) of *L. casei* Shirota microencapsulated with different encapsulating materials by spray and freeze drying techniques

Encapsulating agents	Viable cell after spray drying	Viable cell after freeze drying
MD	7.91 ± 0.15^{c}	8.71 ± 0.34^{b}
MD:GA	8.17 ± 0.08^{b}	9.12 ± 0.12^{a}
YST:MD	8.84 ± 0.13^{a}	9.21 ± 0.16^{a}
MD:GA:YST	8.39 ± 0.14^{b}	9.03 ± 0.33^{ab}
YST	8.96 ± 0.05^{a}	9.37 ± 0.06^{a}
YST:GA	9.09 ± 0.15^{a}	9.28 ± 0.11^{a}

Data are expressed as mean ± SD. Values followed by a different superscript letter in the same column are significantly different at $P < 0.05$

concentrations in all microcapsules were determined between 7.91 and 9.37 log cfu/g after the microencapsulation. The one-way ANOVA showed the significant effect of encapsulating agents and drying techniques on cells viability after microencapsulation ($p < 0.05$). While spray drying caused a loss in probiotic viability of between 0.43 and 1.62 log, only 0.02-0.69 log reduction in bacterial counts after the freeze drying was found. These results are in agreement with other studies that evaluate the viability of probiotic cells microencapsulated by spray and freeze drying methods (Arslan et al., 2015; Reyes et al., 2018a).

The results showed that higher viability was obtained when the cell were encapsulated in RSM and also MD microcapsules showed the lowest cell viability ($p < 0.05$). The utilization of GA in microencapsulation with RSM and MD significantly enhanced the cell viability during spray and freeze drying. Similar results obtained by Reyes et al., (2018a; 2018b) who stated that GA turned out to be best protective agent of cells after the drying process. GA is composed of a highly branched arrangement of simple sugars and also contains a protein component covalently bound within its molecular arrangement. The functional properties of GA is strongly related this fraction (Cano-Higuita et al., 2015). The numbers of viable cells in the microcapsules were fairly above 6–7 log cfu/g recommended to exert beneficial effects on the human host.

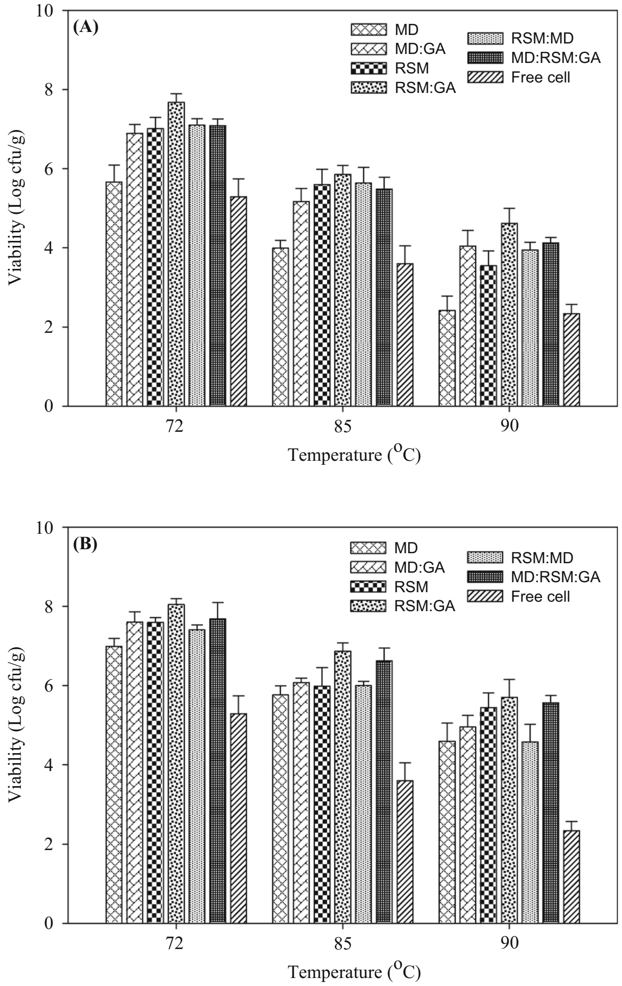

Fig. 1 Viability (log CFU/g) of *L. casei* Shirota free and microencapsulated with different encapsulating agents by spray (**A**) and freeze (**B**) drying after heatment

Viability of free and microencapsulated cells under heat treatments

The viability of free and microencapsulated cells exposed to 72, 85 and 90 °C is presented in Fig. 1. The viability of free cell after exposed to heat treatment at 72, 85 and 90 °C for 1 min was reduced as 5.24, 6.93 and 7.7 log, respectively. The survival of *L. casei* Shirota after heat treatment was remarkably protected by microencapsulation method ($p < 0.05$) and decrease of viability of spray and freeze dried microcapsules expose to high temperature was determined between 1.28–5.48 log and 1.23–4.63 log depends on encapsulating agents, respectively. These results agree with the findings of Ding and Shah (2007), Fritzen-Freire et al. (2013a) and Sabikhi et al. (2010) who reported that the highly significant differences between the reduction rates in the counts of the free and encapsulated cells were determined. Corcoran et al. (2008) stated that bacterial death could probably occur during excessive heat due to unfolding of the higher order structure of macromolecules, linkage between monomeric units and

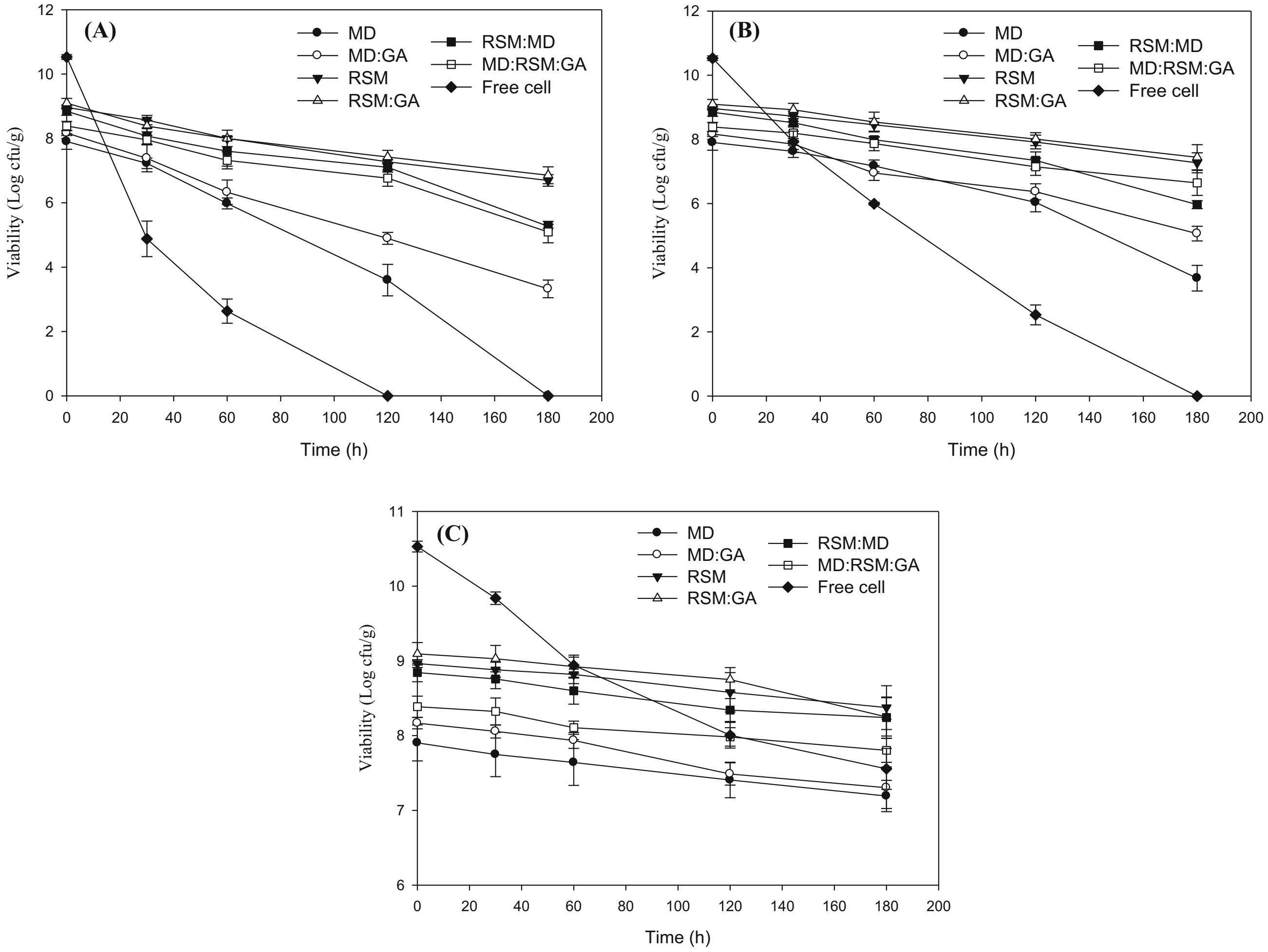

Fig. 2 Stability of free and microencapsulated *L. casei* Shirota by spray drying with different encapsulating agents, under in vitro acidic conditions at 37 °C, (**A**) pH 2.0; (**B**) pH 3.0; (**C**) pH 4.0

destruction of the monomers. Sabikhi et al. (2010) also showed that encapsulated organisms were more resistant to heat shock, the viability of encapsulated cells reducing from 7.44 log to 6.25 log at 72 °C, 5.26 log at 85 °C, and 3.4 log at 90 °C, while the viable counts of free cells decreased below 1 log after heat treatments. On the other hand, Ilha et al. (2015) found that the survival of free and encapsulated *L. paracasei* FNU was similar after heat treatment at 65 °C in each time of incubation.

The viable cells encapsulated by spray drying technique was determined between 5.66 to 7.68, 3.99 to 5.85 and 2.41 to 4.62 log cfu/g when exposed to heat treatment at 72, 85 and 90 °C, respectively. The viability of cells microencapsulated with MD survived poorly for all heat treatment compared to other microcapsules and the protective effect of RSM:GA was found to be the highest ($p < 0.05$). On the other hand, utilization of GA in microencapsulation with other materials was enhanced the viability of *L. casei* Shirota during heat treatment ($p < 0.05$), however, all microcapsules except MD showed similar protective effect at 85 °C. The low protective effect of MD could be explained by the inability of the MD adequately replacing the water molecules due to its high molecular weight (Liao et al., 2017); hence, the maintenance of the structural and functional integrity of bacterial cellular membrane was less effective than other polymers when exposed to high temperature. The similar trend was found for freeze dried microcapsules during high temperature with a cell viability of between 4.57 and 8.04 log cfu/g. Furthermore, microencapsulation of *L. casei Shirota* with RSM:GA could provide good protection as in the case of spray dried microcapsules when expose to high temperature ($p < 0.05$).

Freeze dried microcapsules were found to show better preservative effect than spray dried microcapsules, which could be explained by the cellular damage of probiotic cells during spray drying because of high temperature (Ying et al., 2012) or by the particle size in the micrometer scale of microcapsules, increasing the capsule size

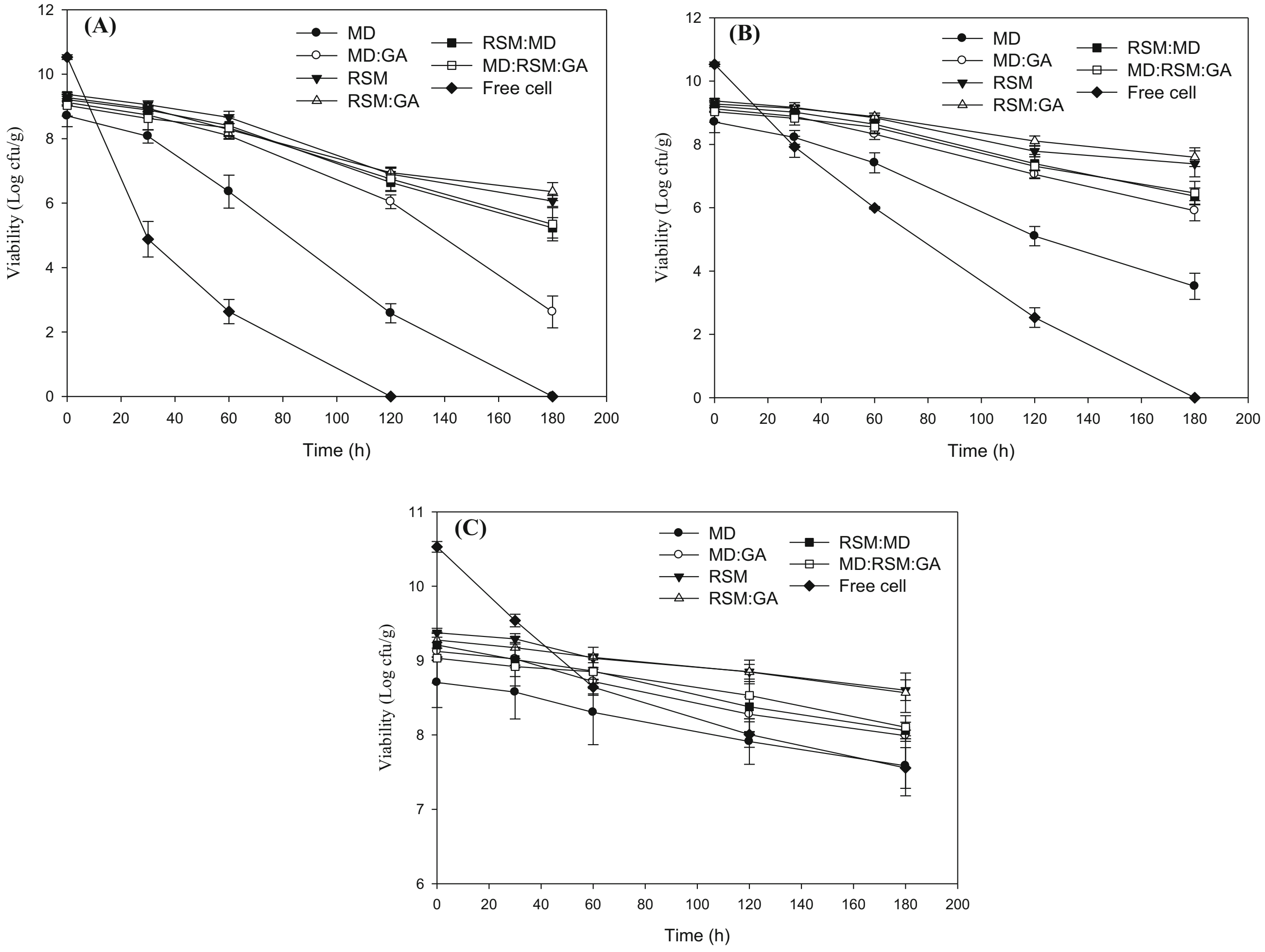

Fig. 3 Stability of free and microencapsulated *L. casei* Shirota by freeze drying with different encapsulating agents, under in vitro acidic conditions at 37 °C, (**A**) pH 2.0; (**B**) pH 3.0; (**C**) pH 4.0

increases the survival of probiotics in heat treatment (Mandal et al., 2006).

Viability of free and microencapsulated cells under acidic conditions

The viability of probiotic bacteria should maintain stable in the stomach until reach the intestinal tract. Due to the secretion of gastric juice in the stomach compartment, stomach has a pH of between 2.0 and 3.0. This acidic environment is the main hurdle for the viability of the probiotic microorganisms due to food remains in the stomach revolves around 3 h (De Castro-Cislaghi et al., 2012; Ilha et al., 2015). Therefore, the pH was selected as 2.0, 3.0 and 4.0 for exposure time as 3 h. Figures 2 and 3 show the viability of *L. casei* Shirota under acidic conditions at pH 2.0, 3.0 and 4.0 during incubation of 3 h for both of free cells and the cells microencapsulated with different encapsulating agents by spray and freeze drying. Initially there was an average of 10.53 log cfu/mL of viable probiotic bacteria and the viability of cells was not determined after 2 and 3 h of incubation at pH 2.0 and 3.0, respectively. At pH 4.0, there was a decrease of 2.97 log in the free cells. Microencapsulation of *L. casei* Shirota with different encapsulating agents by spray and freeze drying significantly improved the survival of probiotic bacteria after exposure to acidic conditions when compared with the free cells ($p < 0.05$). At pH 2.0, 2.24–7.9 log reduction was determined in microcapsules obtained by spray drying; however, 2.91–8.71 log reduction was determined in freeze dried capsules. The spray and freeze dried microcapsules produced with only MD showed lower protective effect and any probiotic cells could not detect after 3 h of incubation at pH 2.0. On the other hand, RSM:GA microcapsules were showed most protective effect and there was only a small decrease (2.24–2.92 log) in the cell counts under similar

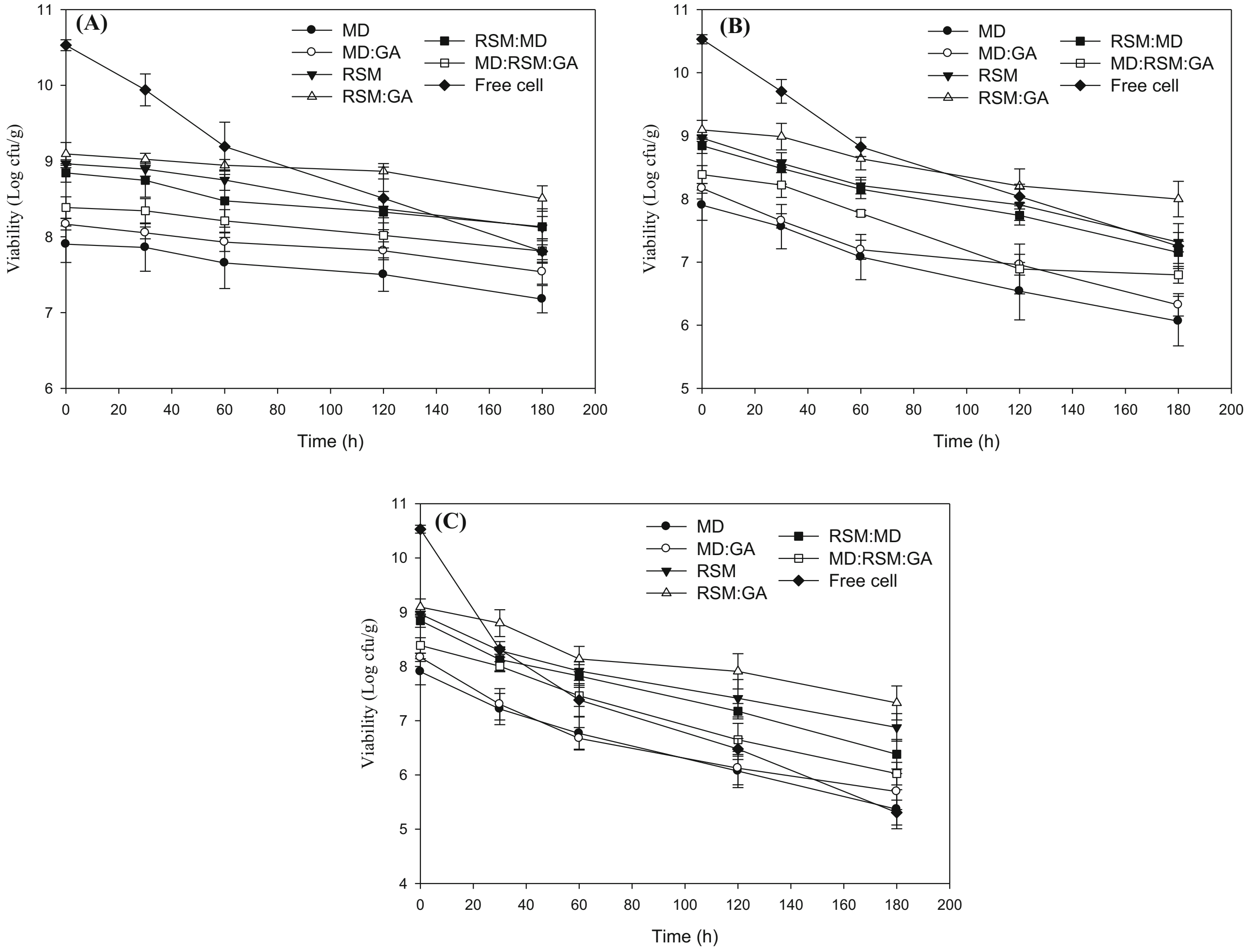

Fig. 4 Stability of free and microencapsulated *L. casei* Shirota by spray drying with different encapsulating agents, in bile (**A**) 1%; (**B**) 2%; (**C**) 3%

conditions. At pH 3.0, there was a decrease ranged from 1.65 to 4.22 log and 1.68 to 5.19 log in spray and freeze dried microcapsules, respectively. The RSM:GA and MD were found to be obtaining the highest and lowest cell viability of microencapsulated *L. casei* Shirota when exposed to pH 3.0 during 3 h of incubation. There was a small decrease in the counts of microencapsulated cells (0.58 to 1.15 log) depends on encapsulation technique and materials when exposed to pH 4.0 for 3 h. The current results agree with the findings of Fritzen-Freire et al. (2013a) who reported that a decrease of approximately 2 log was determined in the number of encapsulated cells after 3 h of incubation at pH 2.0, Ilha et al. (2015) also stated that the encapsulated cells survived well with an average loss of only 1 and 0.3 log after exposure to pH 2.0 and 3.0, respectively compared to free cells with an average loss of 4.25 log. Tee et al. (2014) stated that only one log cycle reduction of encapsulated probiotic cell number was observed after 2 h of incubation at pH 2.0. On the other hand, Brinques and Ayub (2011) found that the viability of free and microencapsulated *L. plantarum* was drastically reduced under acidic conditions, with no significant differences between free and microencapsulated cells.

It can be argued that a small decrease in the viability of *L. casei* Shirota microencapsulated with RSM:GA determined under acidic conditions. According to Arslan et al. (2015), the viability of cells encapsulated with proteins was obtained higher than the cells encapsulated with polysaccharides, which attributed by the protein based wall materials have decreased the acid effect on the microcapsule core material because of they have less polar group like hydroxyl than polysaccharides. In addition, GA mainly consists of two different polymers, glycoprotein and polysaccharide. The addition of GA to the suspending medium prior to spray drying lead to increase the viability of *L. paracasei* during gastric transit (Desmond et al., 2002). However, Lian et al. (2003) noted that RSM

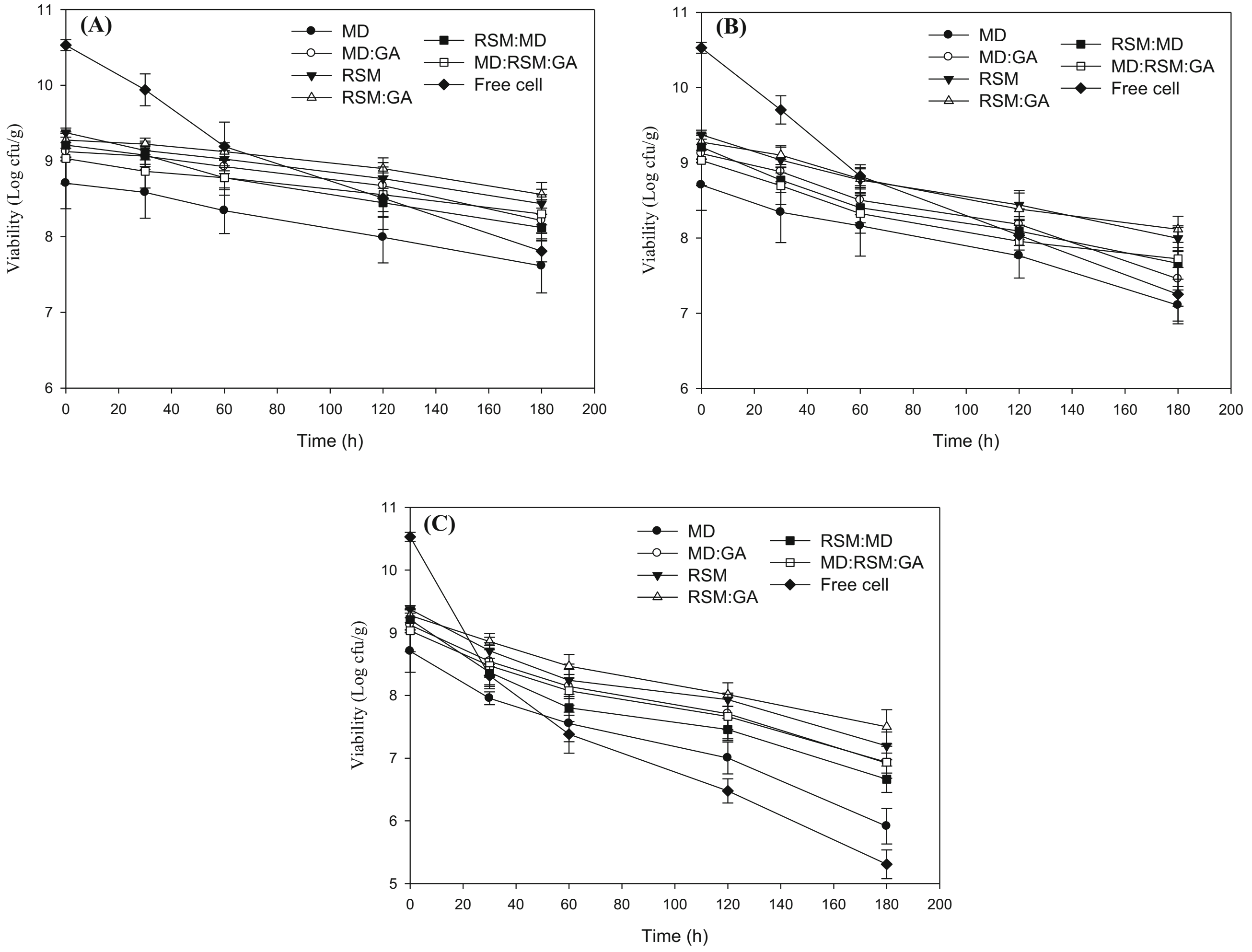

Fig. 5 Stability of free and microencapsulated *L. casei* Shirota by freeze drying with different encapsulating agents, in bile (**A**) 1%; (**B**) 2%; (**C**) 3%

microcapsules showed less protective effects on the viability of cells than GA, gelatin and starch after incubation at pH 2.0.

Viability of free and microencapsulated cells under bile salts

The effect of the bile salt on the viability of free and microencapsulated *L. casei* Shirota by spray and freeze drying techniques with different encapsulating agents is shown in Figs. 4 and 5. The viability of free cells in 1, 2 and 3% bile solutions decreased 2.72, 3.27 and 5.22 log after 3 h of incubation, respectively. Microencapsulated cells remained viable in the presence of 1% bile salts, showing only reduction of cell viability between 0.57 and 1.09 log depends on encapsulating agents and drying methods, and the viability in the RSM:GA and RSM:MD:GA microcapsules was found stable during incubation under same condition. When expose to 2% bile, the microencapsulated cells showed decrease of between 1.09 and 1.84 log, and RSM:GA microcapsules obtained both of spray and freeze dried methods had more protective effect on the cell viability than others ($p < 0.05$). The log reduction in the number of microencapsulated cells in the 3% bile salts was determined below approximately 3 log. Microencapsulation of *L. casei* Shirota using RSM:GA showed best protective effect against bile salts. Although the decrease of viability changed depend on encapsulating agents expose to 1% bile, there was no statistical difference between the groups ($p > 0.05$). Whereas, microcapsules of probiotic cells obtained encapsulating agents containing RSM well protect cells in comparison to others in 2 and 3% bile concentrations. The microcapsules, indicate a similar trend that was found under high temperature and acidic conditions, showed the largest decreases during incubation in bile salts ($p < 0.05$). The results of this study concur with other similar study reported by Fritzen-Freire et al. (2013b), Ilha et al. (2015) and Sabikhi et al. (2010) who stated that microencapsulation of probiotic bacteria by drying technologies enhanced the resistance of cells to presence of bile salts compared to free probiotic cells. However, De Castro-Cislaghi et al. (2012) concluded that the free cells did not show any decrease in viability when expose to 1% bile salts, but microcapsules showed decrease of 4.13 log after 24 h of incubation. Similar results were found by Favaro-Trindade and Grosso (2002) who stated that the spray drying process did not alter the tolerance of *L. acidophilus* and *Bifidobacterium lactis* to the presence of bile salts.

The present study showed that *L. casei* Shirota was very sensitive to stress conditions especially high temperature and acidic pH. Microencapsulation of *L. casei* Shirota with RSM and MD, and their blends with GA as binary and ternary by spray and freeze drying techniques was significantly enhanced the survival of probiotic bacteria against the stress conditions. Despite microencapsulation by freeze drying more effective than spray drying to protect probiotic cells, the viability of all microcapsules (except MD microcapsules) remained > 5 log cfu/g after exposure to stress conditions studied in this present work. Moreover, RSM:GA binary blend was a promising encapsulating agent at all stress conditions for *L. casei* Shirota.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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