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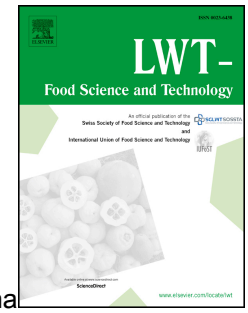

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Evaluation of the viability of free and encapsulated lactic acid bacteria using in vitro gastro intestinal model and post storage survivability studies of synbiotic microcapsules in dry food matrix

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Abstract

Gastro-intestinal tolerance is inevitable for probiotics to allow enough live cell arrival in colon for bestowing their health benefits. The efficiency of lactic acid bacteria (LAB) en route to colon was studied in an in vitro gastro-intestinal model. The effect of storage on bacterial and synbiotic microcapsules in dry food matrix was studied using *Pleurotus ostreatus* as the source of prebiotics. Cereal health mix from Tamil Nadu, Indian traditional dry food snack and universal health drink Malted health drink was used as the matrices to study the stability of microcapsules in dry formulations. LAB were checked in free and encapsulated forms for tolerance towards stress conditions and encapsulation was found to protect the bacteria and enhance their survival. There was 72% to 87% survivability of bacteria in synbiotic microcapsules after storage in dry food matrix. *L. bulgaricus* NCIM 2056 and *L. plantarum* NCIM 2083 showed highest level of survivability in the synbiotic microcapsules stored in all the dry food matrices studied. Shelf-stable (not requiring refrigeration) dry probiotic foods would be path breaking products in probiotics industry to overcome the disadvantages of liquid probiotic formulations. The present work is an initiative towards formulation of such product which can be replicated using probiotic LAB.

Key words: Encapsulation, *Pleurotus ostreatus*, synbiotic, dry food matrix, in vitro gastro intestinal model.

1. Introduction

There has been an explosion of health-based probiotic products since over a decade. The biological activity of probiotic bacteria owes to their ability of attachment to enterocytes thereby inhibiting the enteric pathogens from binding as a result of competitive exclusion. Probiotic bacteria are used in the food industry due to various beneficial properties including reduction of irritable bowel syndrome symptoms, immunomodulatory effects, and cholesterol reduction (FAO/WHO, 2006). Inclusion of probiotic bacteria in fermented products enhances their value as better therapeutic functional foods. It is necessary for all products having probiotic health claims

to have minimum 10^6 CFU/mL probiotic bacteria till expiry date, since minimum therapeutic level per day is considered to be 10^8–10^9 cells (Kailasapathy and Chin, 2000). Karimi et al. (2011) stated that probiotic products when consumed 100 g per day deliver about 10^9 viable cells into the intestine. Reports indicate poor survival of probiotics in food products as well as in the human gastro-intestinal system. Viability of probiotics in a product during consumption is important for their efficacy, as their survival is essential during processing and storage of food products (Mortazavian et al., 2012). Selecting better probiotic strains and providing them physical layering to enhance their survival, including the use of appropriate prebiotics and the optimal combination of probiotics and prebiotics (synbiotics), can increase the delivery of sufficient viable probiotics in functional food products to the consumers. Viability of probiotics in food matrix is affected by factors like pH, acidification during storage of fermented products, production of hydrogen peroxide, oxygen toxicity, processing and storage temperatures, rate and proportion of inoculation, micro-encapsulation, and stability during storage (Mortazavian et al., 2012). In order to act as probiotic in the gastrointestinal tract (GIT) and to exert their beneficial effect on the host it is essential for the bacteria to have protective mechanisms to withstand the low pH in the stomach, digestive enzymes, and bile in the small intestine (Argyri et al., 2012). In-vitro gastro intestinal model (one pot system) is preferably used to mimic this GIT micro-environment.

Microencapsulation is defined as a process in which the cells are retained within a membrane to reduce cell injury and death, producing particles in the nanometer (nanoencapsulation), micrometer (microencapsulation) or millimeter scale (Burgain et al., 2011). Encapsulation stabilises the probiotic cells, significantly enhancing their viability and stability in the production and handling of functional food as well as during their rehydration and lyophilisation. It also preserves the metabolic activity of probiotics in the gastrointestinal tract (Picot and Lacroix, 2004), and ensures viability during long-term storage (Zuidam and Nedovic, 2010). In addition, encapsulation improves and stabilises the sensory properties of the food as well as aids in the homogeneous distribution of probiotics throughout the product (Krasaekoopt et al., 2003). Mushrooms seem to be a potential candidate for prebiotics as it contains carbohydrates like chitin, hemicellulose, β and α-glucans, mannans, xylans, and galactans. Previous studies suggested that the polysaccharides from mushroom have immunomodulating properties like enhancement of lymphocyte proliferation and antibody production (Bao et al., 2001) as well as antitumor properties (Wasser, 2002) and help in cholesterol removal and prevention of obesity. Latest finding by Hearst et al. (2009) and Tsai et al. (2009) revealed antimicrobial and antioxidant properties of mushrooms, respectively. Other than its medicinal properties, edible mushrooms also show significant health improvement as they have low content of calories, sodium, fat, and cholesterol, while they are rich in protein, carbohydrate, fibre, vitamins, and minerals. These nutritional properties give mushrooms the potential to become a food supplement as well as a pharmaceutical agent. They are able to manipulate the composition of colonic microbiota in human gut by inhibition of exogenous pathogens (Rycroft et al., 2001), thus improving the host health (Roberfroid, 2002). Synytsya et al. (2009) showed that mushroom extracts were able to stimulate the growth of probiotics. The probiotic drinks generally contain live bacteria. They must also contain a source of nutrition for the bacteria to feed upon (Savini et al., 2010). Consequently, there is storage and shelf-life issue shortened for some live products. Furthermore, competition for nutrition between bacterial strains within a drink is another important complication. In addition, liquid probiotics all require refrigeration. On the other hand probiotic powders consist of

probiotics that have been freeze-dried under low temperature and pressure without damaging the cells. This provides a suitable growth suspended state for the long-term storage of probiotic bacteria. Once moisture becomes available again after ingestion they rehydrate and subsequently, a proportion of cell start to divide again as before being freeze-dried. Researchers have found that the rehydrated probiotics are capable of effectively providing their respective benefits (Bohbot and Cardot, 2012). Whether or not refrigeration is required for probiotics is dependent upon the actual strains, some of which are heat and shelf stable and some of which are not. Hence, the present work was planned to study the efficiency of microencapsulation in protecting the LAB from acid, bile, and digestive enzymes. This work also aimed to evaluate the efficiency of encapsulation along with prebiotic molecules to increase their survivability during storage in dry food matrix.

2. Materials and methods

2.1. Chemicals

Pepsin, bile salt, and pancreatin solution were purchased from Sigma Aldrich, USA. All other chemicals used were analytical grade from Himedia, Mumbai, India.

2.2. Microorganisms

Four bacterial strains *Lactobacillus acidophilus* NCIM 2660, *Lactobacillus bulgaricus* NCIM 2056, *Lactobacillus fermentum* NCIM 2165 and, *Lactobacillus plantarum* NCIM 2083 were purchased from National Collection of Industrial Microorganisms (NCIM), Pune, India. Mushroom *Pleurotus ostreatus* was collected from Mr. Ashok, Ispat Mushroom House, Rourkela, Odisha, India.

2.3. In vitro gastro intestinal model

In vitro gastro intestinal model is custom made equipment assembled in Department of Fermentation and Biosynthesis, Faculty of Food Science and Nutrition, Poznan University of Life sciences, Poznan, Poland. It consisted of three main components (i) automatic pH controller (ii) acid (1M HCl solution) and alkali (1M $NaHCO_3$ solution) dispenser and (iii) water bath with magnetic stirrer. The digestion vessel is available with air tight cap with provisions for sampling tube, pH probe, thermometer, and provisions for adding acid/alkali solutions.

2.4. Evaluation of free bacterial cells for their tolerance in stomach and small intestinal conditions

Pepsin enzyme solution was prepared by dissolving 0.02 g pepsin (porcine gastric mucosa powder 800 – 2,500 units / mg protein) in 2 mL of 0.1 M HCl. Bile and pancreatin solution was prepared by dissolving 0.12 g of bile and 0.02 g of pancreatin in 10 mL of 0.1 M $NaHCO_3$ solution. Bacterial cells were grown for 48 h in MRS broth and then cells were harvested by centrifugation at 15,000 g for 15 minutes. Ten mL of saline was added to cell pellet and mixed thoroughly. The cell load was enumerated by serially diluting and plating appropriate dilutions on MRS agar plates. Nine mL of cell pellet suspension was added to 200 mL MRS broth in digestion vessel along with magnetic bead and mixed thoroughly on magnetic stirrer. The digestion unit was then kept in water bath. The pH was adjusted to 2.0 using the automatic pH controller and 2 mL pepsin was added to it. Two mL of spent broth was withdrawn from the digestion vessel after 2 h and

then pH of the digestion mixture was adjusted to 6.0. The cell load of the withdrawn sample was enumerated by plating undiluted as well as appropriate dilutions on MRS agar plates and incubation at 37°C for 48 h. Bile solution (10 mL) was added to the digestion vessel after 2 h and the pH was adjusted to 7.4. Again 2 mL of spent broth was withdrawn from the digestion vessel after 2 h and enumerated as earlier.

2.5. Evaluation of encapsulated bacterial cells for their tolerance in stomach and small intestinal conditions

Starch in $CaCl_2$ solution was prepared by dissolving 4 g starch in 0.61 g/100mL $CaCl_2$ solution. Sodium alginate solution (0.6 g/100mL), $CaCl_2$ solution (1.22 g/100mL) for encapsulation and tri sodium citrate solution (3 g/100mL) for dissolving the capsules to release the bacterial cells were prepared. Starch in $CaCl_2$ solution (30 mL) was added to cell pellet and mixed thoroughly. The cell load was enumerated by serially diluting and plating appropriate dilutions on MRS agar plates. Cell pellet suspension was taken in 31 gauge syringe and added drop by drop through the needle to the alginic acid solution with continuous mixing over magnetic stirrer. Beads formed were filtered and transferred to $CaCl_2$ solution and mixed on magnetic stirrer for 15 minutes. The beads were again filtered and stored in refrigerator. The mean diameter of beads was measured using ruler scale. The cell load of the digested microcapsules (digestion done as mentioned in subsection 2.4) was enumerated after dissolving the beads in 10 mL tri-sodium citrate solution followed by serial dilution and plating of appropriate dilutions on MRS agar plates and incubation at 37°C for 48 h. The encapsulation yield (EY), which is a combined measurement of the efficacy of entrapment and survival of viable cells during the microencapsulation procedure, was calculated as:

$$EY = (N / N_0) \times 100$$

Where N is the number of viable entrapped cells released from the microcapsules, and N_0 is the number of free cells added to the alginate mix during the encapsulation procedure (Chávarria et al., 2010; Sathyabama et al., 2014).

2.6. Evaluation of temperature tolerance of free bacterial cells

One millilitre of overnight culture of *L. acidophilus* NCIM 2660 was transferred to each test tube containing 9 mL distilled water (1 tube for each time interval). These test tubes were exposed to different temperatures (60°C, 70°C, 80°C, and 90°C respectively) for 30 min at regular interval of 5 min in water bath. The viable cell load was enumerated by spreading on MRS agar and incubating at 37°C for 48 h. Same evaluation was conducted for the other three bacteria also. The experiment was repeated three times for all the four bacteria.

2.7. Evaluation of temperature tolerance of encapsulated bacterial cells

Tolerance of encapsulated *L. acidophilus* NCIM 2660 to temperature ranging from 100°C to 140°C was studied using distilled water as a suspending medium in oil bath. One g of the bacteria incorporated fresh microcapsules (10^{10} cells / g) was transferred in test tube containing 9 mL distilled water. After the heat treatment the content was cooled to room temperature and viable

cell load was enumerated as mentioned earlier in subsection 2.5. Same evaluation was conducted for the other three bacteria also. The experiment was repeated three times for all the four bacteria.

2.8. Preparation of *Pleurotus ostreatus* extract

P. ostreatus collected from Mr. Ashok, Ispat Mushroom House, Rourkela, Odisha, India was washed with distilled water to remove dirt and extraneous matters. After drying in oven at 70°C for 48 h it was ground to fine powder. First cold extraction was done with cold distilled water for 3 h. After drying the solid biomass it was washed twice with 80% ether. Protein precipitation of the solid biomass was done with Sevag reagent (chloroform: butanol in ratio 4:1) for 3 h. Then after air drying deionized water was added to the solid biomass to form a slurry (pH adjusted to 7) and was kept in hot water bath at 50°C for 10 h, followed by centrifugation at 5000 rpm for 20 min. Supernatant was collected and 3 parts absolute ethanol was added to it followed by air drying and re-suspension in deionized water. The extract was then lyophilized for 4 h and stored in air tight container until use.

2.9. Estimation of β-glucan in dried mushroom

β-glucan content of *Pleurotus ostreatus* was estimated using Megazyme β-glucan assay kit following the procedure given by the manufacturer.

A. Measurement of total glucan (α-glucan + β-glucan) plus D glucose in oligosaccharides, sucrose, and free D-glucose:

a) **Solubilisation and partial digestion of total glucan content from dried mushroom powder**

Mushroom was dried in the hot air oven at 90ºC overnight and powdered. 100 mg mushroom powder was taken in glass tube. 1.5 ml of concentrated HCl (37% v/v) was added to the tube. After proper vortexing it was placed in water bath at 30ºC for 45 min. Then 10 ml of distilled water was added to the tube and vortexed again. It was placed in a boiling water bath (~100ºC) for 2 h followed by cooling down at room temperature. Then 10 ml of 2 N KOH was added. All the content of the tube was transferred to 100 ml volumetric flasks. Sodium acetate buffer (200 mM, pH 5.0) was used to adjust the volume up to 100 ml. The sample was then centrifuged at 7000 rpm for 15 min.

b) **Measurement of total glucan plus D-glucose in oligosaccharides, sucrose and free D-glucose**

Supernatant (0.1 ml) was transferred in to a fresh test tube. Then 0.1 ml of a mixture of exo-1, 3-β-glucanase (20 U/ml) and β-glucosidase (4 U/ml) in 200 mM sodium acetate buffer (pH 5.0) was added to it and vortexed properly. It was then placed in water bath at 40ºC for 60 min. Then 3 ml of glucose oxidase/peroxidase mixture (GOPOD) was added to the tube and again placed in water bath at 40ºC for 20 min. Absorbance of the solution was recorded at 510 nm against reagent blank (reaction without supernatant) using UV visible spectrophotometer.

B. Measurement of α-glucan (phytoglycon and starch) plus D-glucose in sucrose, and free D-glucose:

a) **Solubilisation, hydrolysis and measurement of α-glucan**

Mushroom was dried in the hot air oven at 90ºC overnight and powdered. 100 mg of mushroom powder was taken in a glass tube. Two ml of 2 M KOH was added and the tube was kept on ice in a shaker at 150 rpm for 20 min for proper mixing and hydrolysis of the sample. Then 8 ml of 1.2 M sodium acetate buffer (pH 3.8) was added to the tube under continuous stirring. Immediately 0.2 ml of amyloglucosidase (1630 U/ml) plus invertase (500 U/ml) was added to the tube and properly vortexed. It was then placed in water bath at 40ºC for 30 min followed by centrifugation at 7500 rpm for 15 min.

b) Measurement of total glucan plus D-glucose in oligosaccharides, sucrose and free D-glucose

Supernatant (0.1 ml) was transferred into a fresh test tube. Then 0.1 ml of sodium acetate buffer (200mM, pH 5.0) and 3 ml of GOPOD reagent were added to the sample followed by incubation in water bath at 40ºC for 20 min. Absorbance of the solution was recorded as mentioned above in case of total glucan estimation.

C. Estimation of β-glucan in mushroom extracts

β-glucan content = Total glucan content – α-glucan content

2.10. Preparation of synbiotic microcapsule and its utilization for formulation of dry functional food products

Microcapsules were prepared by extrusion method. Concentrated extract of *P. ostreatus* was mixed in 2.0% sodium alginate solution in 2:1 ratio and lyophilized bacteria was added to it followed by thorough mixing on magnetic stirrer for 10 min. The mixture was then filled in to a 31 gauge syringe and added drop wise to 0.075 mM cold $CaCl_2$ solution with slow stirring. The minute beads formed were filtered and immersed in 100 mM $CaCl_2$ solution for hardening and their size was measured. After 15 min the beads were filtered and lyophilized for about 4 hours at -45°C. These synbiotic capsules were then mixed with the dry food matrices in 1:1 ratio. The dry foods used were : a local Cereal health mix [Saga Food Products Pvt. Ltd., Chennai, India; Ingredients: cereals (82% - ragi, maize, millet, jowar, wheat, barley), pulses (12% - Fried gram, green gram), nuts (2% - groundnut, cashew nut & badam), 3% sago & 1% cardamom], a traditional fried Indian food snack [Haldiram, Nagpur, India; Ingredients: chick peas lentils (25%), vegetable oil, chick peas flour (10%), red lentils (8%), peanut (7%), potato, rice flakes (2%), green peas (1.5%) & wheat], and a universal Malted health drink [GlaxoSmithKline; Ingredients: Wheat flour (46%), malted barley (26%), dried whey (milk), sugar, calcium carbonate, dried skimmed milk, palm oil, salt, vitamin mix (vitamin A, C, D, E, B_1, B_2, B_5, B_6, B_{12}, niacin, folic acid, biotin), ferric pyrophosphate and zinc oxide]. Then the post storage survivability of bacteria in the different dry food matrices was studied as described in forthcoming subsections. Sensory analysis for changes in taste and flavour of the stored formulations was also done at regular intervals.

2.10.1. Evaluation of post storage survivability of bacteria in bacterial and synbiotic microcapsules incorporated in dry food matrix (Cereal health mix)

Cereal health mix was taken as the first model dry food. The lyophilized synbiotic microcapsules mixed in health powder (1:1 ratio) was stored in air tight container for 3 weeks at room temperature. Sampling was done at one week interval and viability of cells was checked after

digestion and dilution as mentioned earlier in subsection 2.5. Comparative analysis of post storage survivability of bacteria in microcapsules was done in presence and absence of *P. ostreatus* extract.

2.10.2. Evaluation of post storage survivability of synbiotic microcapsule in an Indian traditional dry food snack

Synbiotic microcapsule of *L. bulgaricus* NCIM 2056 and *L. plantarum* NCIM 2083 was added to dry food snack respectively in ratio 1:1 and the effect of storage (at room temperature in air tight container) on viability was studied for 2 weeks. Every week the stored beads were separated from the food matrix and dissolved in tri-sodium citrate (3%, pH 6). From the dissolved beads 200 µL was poured on MRS agar by pour plate method and incubated at 37°C for 48 h. The cells were enumerated.

2.10.3. Evaluation of post storage survivability of synbiotic microcapsule in dry health mix (Malted health drink)

One gram of synbiotic microcapsule of *L. bulgaricus* NCIM 2056 and *L. plantarum* NCIM 2083 was incorporated in the health mix (Malted health drink) in ratio 1:1 and the effect of storage (at room temperature in air tight container) on viability was studied after 2 weeks. The beads were separated from the food matrix after 2 weeks and dissolved in tri-sodium citrate (3%, pH 6). From the dissolved beads 200 µL was poured on MRS agar by pour plate method and incubated at 37°C for 48 h. The cells were enumerated.

2.11. Statistical Analysis

All the tests were performed in triplicate unless it is specified. Microsoft Excel 2010 was used for the calculation of standard deviation and Log values. SPSS (IBM Statistics) software version 19.0 was used for comparing the means through one-way ANOVA and mean differences were compared using Duncan's multiple range test.

3. Results and discussion

Probiotic microbes are proved to offer beneficial effects to human health by several researchers all over the world. Microbes are required to have some essential characters in order to be utilized as probiotics. One of the important characters is to resist the stress conditions of stomach (pH 2.0 and digestive enzyme pepsin) and small intestine (bile and pancreatic enzymes). To check the probiotic potential of our microbial isolates their ability to travel through stomach and small intestine was studied in the in vitro gastro intestinal model.

3.1. Evaluation of free bacterial cells for their tolerance in stomach and small intestinal conditions

Four bacterial isolates were checked in free form and three of them were found to be tolerating the stress conditions of stomach and small intestine. Free cell form of *L. bulgaricus* NCIM 2056 and *L. fermentum* NCIM 2156 showed moderate resistance to stress environment of gut. While *L. acidophilus* NCIM 2660 and *L. plantarum* NCIM 2083 showed negligible resistance. Those bacteria (Table 1) which were not tolerant to stress conditions of stomach (pH 2.0 and pepsin enzyme) however, after 4 h of digestion in conditions of small intestine (bile and pancreatin at pH

6.0) were found to grow in moderate amount. This shows that bacterial cells underwent dormant state due to acid shock in stomach and regained their growth when the pH reached to 6.0 in small intestine. Jin et al., (2012) reported that upon facing acid stress, the cells strengthen the cell wall integrity and change the cell membrane permeability to keep H^+ from entering followed by increased activity of F_0F_1-ATPases. The repair of acid-induced protein and DNA damage is an important response to acid stress in LABs. This could be a possible reason for revival of the cells observed by us.

3.2. Evaluation of encapsulated bacterial cells for their tolerance in stomach and small intestinal conditions

Encapsulated form of *L. acidophilus* NCIM 2660 was showing increased resistance as compared to free form. While *L. bulgaricus* NCIM 2056 and *L. fermentum* NCIM 2156 showed no increase in viability after encapsulation. This shows that encapsulation confers protection to bacteria against stress conditions depending on the type of bacteria. The bacterial load was seen to be more in the broth than in the microcapsules after 4 h digestion (Table 2) which shows that there was substantial release of bacterial cells from the microcapsules. Double encapsulation may be adopted in order to reduce this leakage and to provide better protection to the cells in accordance to Mokarram et al. (2009). According to Kim et al. (2008) encapsulated probiotic bacteria show greater tolerance during exposure to the in vitro gastrointestinal tract and thermal treatment as compared to their free form. However, sodium alginate gel presents porosity and sensibility to extreme pH, which can interfere both to the release and to protection of the compounds (Mortazavian and Sohrabvandi, 2007). Thus in order to overcome this obstacle and improve viability of microorganisms, inclusion of prebiotics in the capsule formulation can be considered (Chen, 2005). The post-acidification with encapsulated probiotic bacteria was slower compared to that with free form during storage period (Kailasapathy, 2006).

3.3. Evaluation of temperature tolerance of free bacterial cells

It was found that *L. acidophilus* NCIM 2660 and *L. fermentum* NCIM 2156 could tolerate upto 90°C for 1 min, while *L. bulgaricus* NCIM 2056 and *L. plantarum* NCIM 2083 were tolerant up to 100°C for 1 min (Fig. 1). All four of the organisms were able to survive up to 70°C for about 25 to 30 min. They also could tolerate upto 80°C for about 5 to 10 min. In LAB many heat shock proteins and proteases have been identified and found to be well conserved which allow them to withstand high temperature exposure (Prasad et al., 2003).

3.4. Evaluation of temperature tolerance of encapsulated bacterial cells

It was seen (Table 3) that encapsulation imparted increased temperature tolerance to *L. bulgaricus* NCIM 2056 and *L. plantarum* NCIM 2083 from 100°C (for 10 min) to 130°C (for 2 min). This increased temperature tolerance is bestowed by encapsulation (Ding and Shah, 2007) and could be exploited in spray drying techniques that can be employed for addition of viable load of probiotics to dry food matrix in powdered form.

3.5. β-glucan content in *P. ostreatus* mushroom

β-glucan (g/100 g) content of the mushroom was calculated to be 48% of its dry weight. This is in accordance to the values reported by Synytsya et al. (2008). β-glucan is considered as a potent

prebiotic fibre in mushrooms which can stimulate the growth of particular group of bacteria in colon. *P. ostreatus* contains a specific β-glucan called pleuran which is a well known prebiotic component.

3.6. Preparation of synbiotic microcapsule and its utilization for formulation of dry functional food products

Synbiotic microcapsules (containing both bacterial culture as well as mushroom extract) with average size ranging from 0.5 mm to 1 mm were prepared. There was no difference in the size of capsules with and without mushroom extract. Dry functional food products were formulated by adding the bacterial microcapsules and synbiotic microcapsules in different dry food matrices like cereal health mix, traditional food snack and malted health drink and were stored for further studies. No significant change was observed in the taste and flavor of the stored formulations (data is not given) as compared to the original food products (control). Study shows that incorporation of free and encapsulated bacteria do not substantially alter the overall sensory characteristics of food products and microencapsulation helps to enhance their survival during storage of food products (Kailasapathy, 2006).

3.6.1. Survivability study during storage of bacteria in bacterial microcapsules and synbiotic microcapsules incorporated in a local dry food matrix (Cereal health mix)

Survivability of bacteria in bacterial microcapsules (containing only bacterial culture without mushroom extract) and synbiotic microcapsules (containing both bacterial culture as well as mushroom extract) was studied after incorporation and storage in a local dry food matrix (Cereal health mix). There was 71% to 90% survivability of bacteria in microcapsules after 3 weeks of storage (Table 4). *L. acidophilus* NCIM 2660 showed highest survival closely followed by *L. bulgaricus* NCIM 2056 and *L. plantarum* NCIM 2083. However, *L. fermentum* NCIM 2156 showed least survival post storage. As can be clearly seen in Table 5, *L. bulgaricus* NCIM 2056 showed highest level of survivability (87%) in synbiotic microcapsules incorporated dry health mix stored for 3 weeks followed by *L. plantarum* NCIM 2083 (about 85%). Except for *L. acidophilus* NCIM 2660 rest were showing increased survivability in presence of prebiotic compounds of *P. ostreatus* as compared to in its absence. The reason behind this is unknown and needs detailed analysis. β-glucan, the non-digestible polysaccharides, of the *P. ostreatus* extract might be one of the potential prebiotic components responsible for the enhanced survivability of bacteria in case of synbiotic microcapsules. Arena et al., (2014) reported that β-glucans improved the growth rate of probiotic bacteria in unstressed conditions and enhanced the oro-gastrointestinal stress tolerance.

3.6.2. Evaluation of survivability during storage of synbiotic microcapsule in an Indian traditional dry food snack

Since, *L. bulgaricus* NCIM 2056 (87%) and *L. plantarum* NCIM 2083 (85%) showed highest level of survivability in synbiotic microcapsules incorporated dry food matrix (Cereal health mix) stored for 3 weeks, these two were selected for further comparative analysis of post storage survivability in other dry food matrices in presence and absence of *P. ostreatus* extract. It was found that survivability of both *L. bulgaricus* NCIM 2056 and *L. plantarum* NCIM 2083 was higher in presence of *P. ostreatus* extract as compared to in its absence, after 2 weeks of storage of

the respective microcapsules in a traditional dry food snack (Table 6). Survivability of probiotic bacteria added in dry food matrix is essential and necessary to overcome the disadvantages of liquid based probiotic formulation. Shelf-stable (not requiring refrigeration) probiotic foods would be path breaking products in probiotics industry. The present work is an initiative towards formulation of such product. Further works are needed to evaluate the survivability of bacterial cells in dry food matrix beyond 2 weeks of storage period.

3.6.3. Evaluation of survivability during storage of synbiotic microcapsule in dry health drink (Malted health drink)

It was found that survivability of both *L. bulgaricus* NCIM 2056 and *L. plantarum* NCIM 2083 was higher in presence of *P. ostreatus* extract as compared to in its absence, after 2 weeks of storage of the respective synbiotic microcapsules in Malted health drink (Table 7).

4. Conclusion

The general aim of bacterial and synbiotic microencapsulation is to protect the bacteria in food products as well as en route colon since free cells usually loose viability in GIT stress conditions. It was observed that some of the bacterial strains underwent dormant state due to acid shock in stomach and regained their growth when the pH reached to 6.0 in small intestine. It can be concluded that encapsulation confers additional protection to bacteria against stress conditions of gut simulations as well as high temperature exposure. There is significant release of bacterial cells from the microcapsules and double encapsulation may be adopted in order to prevent this leakage. There was 72% to 87% survivability of the studied bacteria in synbiotic microcapsules i.e., in presence of *P. ostreatus* extract, wherein *L. bulgaricus* NCIM 2056 and *L. plantarum* NCIM 2083 showed highest level of survivability in all the dry food matrices studied after storage. This process may be employed in future for efficient production and commercialization of several dry shelf stable synbiotic functional foods by varying the sources of prebiotics, the dry food matrix used and the type of health promoting probiotic bacteria. Other edible mushrooms can also be utilized as source of prebiotics to find the best LAB growth enhancer.

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Table 1. Number of free bacterial cells surviving before and after digestion

Free cells	Time of digestion (hours)		
	0	2	4
	Log_{10} number of cells		
L. acidophilus NCIM 2660	9.0 ± 0.2^{a}	2.0 ± 0.1^{b} (22.2)	2.2 ± 0.1^{b} (24.4)
L. bulgaricus NCIM 2056	8.6 ± 0.2^{a}	0	3.3 ± 0.1^{b} (38.3)
L. fermentum NCIM 2156	9.1 ± 0.1^{a}	0	3.1 ± 0.1^{b} (34.0)
L. plantarum NCIM 2083	9.0 ± 0.1^{a}	0	0

Values represent mean ± standard deviation; n= 4 (duplicates of two dilutions).

Log_{10} number of cells is calculated per mL.

Values in parenthesis represent the percentage of original cells remaining i.e., surviving cells.

Superscript letters which are not similar are significantly different from each other ($P<0.05$).

Table 2. Number of surviving bacterial cells in microcapsules before and after digestion

Encapsulated cells	Time of digestion (hours)				
	0	4	EY (%)	2	4
	Log_{10} number of cells from microcapsules			Log_{10} number of cells from broth	
L. acidophilus NCIM 2660	8.6 ± 0.2^{a}	2.6 ± 0.5^{c} (30.2)	30.2	3.4 ± 0.1^{b} (39.5)	3.4 ± 0.1^{b} (40.0)
L. bulgaricus NCIM 2056	8.9 ± 0.2^{a}	3.3 ± 0.3c (37.0)	37	3.2 ± 0.1^{b} (36.0)	3.3 ± 0.1^{b} (37.0)
L. fermentum NCIM 2156	9.4 ± 0.1^{a}	3.1 ± 0.1c (32.9)	32.9	3.0 ± 0.1^{b} (32.0)	2.3 ± 0.1^{b} (24.4)
L. plantarum NCIM 2083	8.0 ± 0.1^{a}	0	0	0	0

Values represent mean ± standard deviation; n= 4 (duplicates of two dilutions).

Log_{10} number of cells is calculated per mL of dissolved beads.

Values in parenthesis represent the percentage of original cells remaining i.e., surviving cells.

EY = (N / No) × 100 {Where, EY is the encapsulation yield, N is the number of viable entrapped cells released from the microcapsules, and No is the number of free cells added to the alginate mix during encapsulation}.

Superscript letters which are not similar are significantly different from each other ($P<0.05$).

Table 3. Viable number of bacterial cells in microcapsules after exposure to different temperatures

Encapsulated bacteria	Initial count (Log_{10} number of cells)	Log_{10} number of cells after respective temperature exposure (tolerance time in min)				
		100°C	110°C	120°C	130°C	140°C
L. acidophilus NCIM 2660	9.1 ± 0.1	NG (1 min)	NG (1 min)	NG (1 min)	NG (1 min)	NG (1 min)
L. bulgaricus NCIM 2056	8.8 ± 0.1[a]	6.48 ± 0.1[a] (74.0) (10 min)	3.47 ± 0.2[a,b] (39.4) (1 min)	3.00 ± 0.1[a,b] (34.0) (1 min)	NG (1 min)	NG (1 min)
L. fermentum NCIM 2156	8.8 ± 0.1	NG (1 min)	NG (1 min)	NG (1 min)	NG (1 min)	NG (1 min)
L. plantarum NCIM 2083	7.5 ± 0.1[a]	7.00 ± 0.3[a] (93.3) (10 min)	4.90 ± 0.1[a] (65.3) (4 min)	4.20 ± 0.1[a] (56.0) (2 min)	3.70 ± 0.1[a] (49.3) (2 min)	NG (1 min)

NG - No Growth. Values in parenthesis without min represent the percentage of original cells remaining i.e., surviving cells.
Values in parenthesis with min represent the time (min) for which the encapsulated bacterial cells tolerate the respective temperature exposures.
Values represent mean ± standard deviation; n= 3.
Cells were counted from 20 number of microcapsules.
Log_{10} number of cells is calculated per mL of dissolved beads.
Superscript letters which are not similar are significantly different from each other ($P<0.05$).

Table 4. Survivability during storage of bacteria in microcapsules

Bacteria	Viability (weeks)		
	1	2	3
	Log_{10} number of cells (in microcapsules)		
L. acidophilus NCIM 2660	2.08 ± 0.2^{a}	1.92 ± 0.1^{b} (92.4)	1.88 ± 0.2^{c} (90.4)
L. bulgaricus NCIM 2056	2.16 ± 0.1^{a}	1.89 ± 0.1^{b} (87.4)	1.79 ± 0.3^{c} (82.8)
L. fermentum NCIM 2156	2.28 ± 0.3^{a}	1.81 ± 0.2^{b} (79.3)	1.62 ± 0.4^{c} (71.0)
L. plantarum NCIM 2083	2.27 ± 0.2^{a}	1.93 ± 0.3^{b} (85.3)	1.81 ± 0.2^{c} (81.4)

Cells were counted from 10 mg of microcapsules.

Values represent mean ± standard deviation; n= 4 (duplicates of two dilutions).

Values in parenthesis represent the percentage of original cells remaining i.e., surviving cells.

Log_{10} number of cells is calculated per mL of dissolved beads.

Superscript letters which are not similar are significantly different from each other ($P<0.05$).

Table 5. Survivability during storage of bacteria in synbiotic microcapsules incorporated in a dry cereal health mix

Bacteria	Viability (weeks)		
	1	2	3
	Log_{10} number of cells (in synbiotic microcapsules incorporated dry cereal health mix)		
L. acidophilus NCIM 2660	2.52 ± 0.2^{a}	2.21 ± 0.2^{b} (87.5)	1.89 ± 0.1^{c} (72.4)
L. bulgaricus NCIM 2056	2.51 ± 0.1^{a}	2.43 ± 0.1^{b} (97.0)	2.18 ± 0.1^{c} (87.0)
L. fermentum NCIM 2156	2.56 ± 0.3^{a}	2.13 ± 0.1^{b} (83.3)	1.91 ± 0.1^{c} (74.5)
L. plantarum NCIM 2083	2.64 ± 0.2^{a}	2.56 ± 0.2^{b} (97.1)	2.23 ± 0.2^{c} (84.6)

2:1 ratio of *P. ostreatus* extract: sodium alginate mixture was used.

Cells were counted from 10 mg of microcapsules.

Values represent mean ± standard deviation; n= 4 (duplicates of two dilutions).

Values in parenthesis represent the percentage of original cells remaining i.e., surviving cells.

Log_{10} number of cells is calculated per mL of dissolved beads.

Superscript letters which are not similar are significantly different from each other ($P<0.05$).

Table 6. Survivability during storage of bacteria in synbiotic microcapsules in traditional dry food snack: effect of storage time on survivability of *L. plantarum* NCIM 2083 and *L. bulgaricus* NCIM 2056

Bacteria	Microcapsules / Synbiotic microcapsules	viability (weeks)		
		0	1	2
L. plantarum (NCIM 2083)	Microcapsules without prebiotics	4.0 ± 0.3^{a}	2.02 ± 0.1^{b} (50.5)	1.70 ± 0.3^{c} (42.5)
	Synbiotic microcapsules with *P. ostreatus* extract	4.0 ± 0.1^{a}	3.00 ± 0.5^{b} (75.0)	2.47 ± 0.2^{c} (62.0)
L. bulgaricus (NCIM 2056)	Microcapsules without prebiotics	4.0 ± 0.4^{a}	1.54 ± 0.5^{b} (38.5)	0.84 ± 0.1^{c} (21.0)
	Synbiotic microcapsules with *P. ostreatus* extract	4.0 ± 0.2^{a}	2.35 ± 0.1^{b} (59.0)	1.77 ± 0.2^{c} (44.0)

2:1 ratio of *P. ostreatus* extract: sodium alginate mixture was used.

Cells were counted from 10 mg of microcapsules.

Values represent mean ± standard deviation; n= 4 (duplicates of two dilutions).

Values in parenthesis represent the percentage of original cells remaining i.e., surviving cells.

Log_{10} number of cells is calculated per mL of dissolved beads.

Superscript letters which are not similar are significantly different from each other ($P<0.05$).

Table 7. Survivability during storage of bacteria in synbiotic microcapsules in a dry malted health drink: effect of storage time on survivability of *L. plantarum* NCIM 2083 and *L. bulgaricus* NCIM 2056

Bacteria	Microcapsules / Synbiotic microcapsules	viability (weeks)		
		0	1	2
L. plantarum (NCIM 2083)	Microcapsules without prebiotics	5.0 ± 0.5^{a}	2.3 ± 0.4^{b} (46.0)	1.7 ± 0.3^{c} (34.0)
	Synbiotic microcapsules with *P. ostreatus* extract	5.0 ± 0.1^{a}	3.8 ± 0.3^{b} (76.0)	2.5 ± 0.2^{c} (50.0)
L. bulgaricus (NCIM 2056)	Microcapsules without prebiotics	4.0 ± 0.4^{a}	2.3 ± 0.6^{b} (57.5)	1.2 ± 0.1^{c} (30.0)
	Synbiotic microcapsules with *P. ostreatus* extract	4.0 ± 0.2^{a}	2.8 ± 0.3^{b} (70.0)	1.9 ± 0.6^{c} (47.5)

2:1 ratio of *P. ostreatus* extract: sodium alginate mixture was used.

Cells were counted from 10 mg of microcapsules.

Values represent mean ± standard deviation; n= 4 (duplicates of two dilutions).

Values in parenthesis represent the percentage of original cells remaining i.e., surviving cells.

Log_{10} number of cells is calculated per mL of dissolved beads.

Superscript letters which are not similar are significantly different from each other ($P<0.05$).

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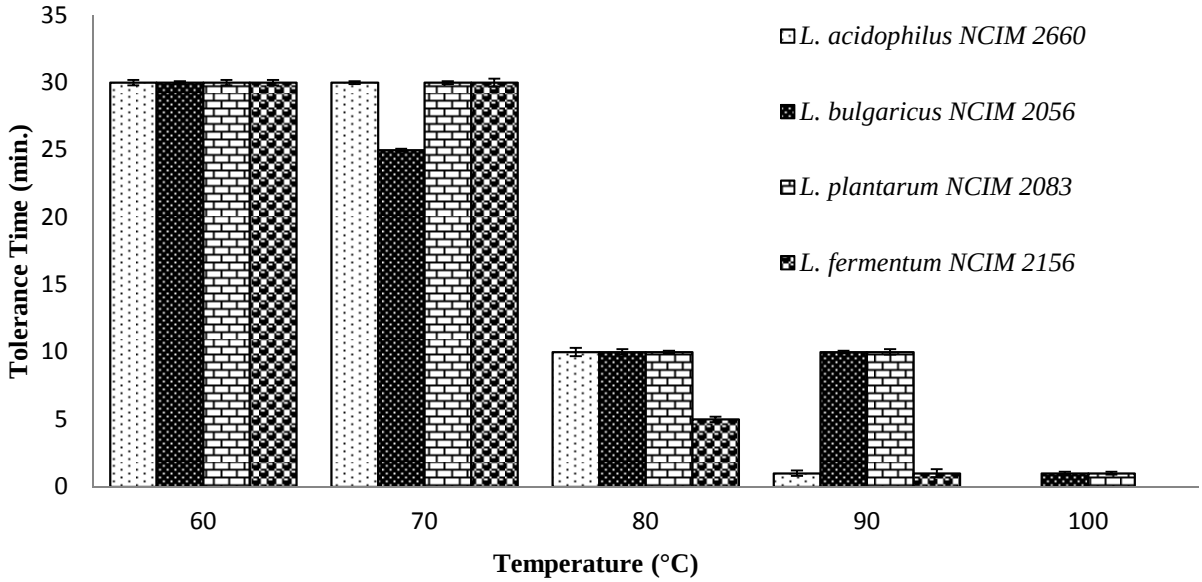

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567 Fig.1. Tolerance of free bacterial cells towards increasing temperature exposure

Highlights:

- LAB undergoes dormancy in gastric and revives in small intestinal conditions.
- *Pleurotus ostreatus* extract enhances LAB survivability when encapsulated together.
- Encapsulation protects LAB from gastrointestinal and high temperature stress.
- Dry shelf stable health foods with synbiotic microcapsules are proposed.