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

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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 malted health drink were 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%–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.

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 & Chin, 2000). Karimi, Mortazavian, and Da Cruz (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, Mohammadi, & Sohrabvandi, 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

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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, Gaiani, Linder, & Scher, 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 & Lacroix, 2004), and ensures viability during long-term storage (Zuidam & 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, Bhandari, & Deeth, 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, Liu, Fang, & Li, 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, Jones, Gibson, & Rastall, 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 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 & 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 of LAB 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, Institute of Food Technology of Plant Origin, 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 (1 M HCl solution) and alkali (1 M $NaHCO_3$ 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 800–2500 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 15000 *g* for 15 min. 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 when the pH was 6.0 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/100 mL $CaCl_2$ solution. Sodium alginate solution (0.6 g/100 mL), $CaCl_2$ solution (1.22 g/100 mL) for encapsulation and tri sodium citrate solution (3 g/100 mL) 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