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RESEARCH ARTICLE

Development and evaluation of intestinal targeted mucoadhesive microspheres of *Bacillus coagulans*

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Abstract

Background: Intestinal targeted mucoadhesive microsphere of probiotics may provide numerous associated health benefits.

Aim: To develop mucoadhesive microspheres that will deliver viable probiotic cells into gut protectively against harsh environmental conditions of stomach for extended period.

Materials and methods: Core mucoadhesive microspheres of *Bacillus coagulans* were prepared using hypromellose, following coacervation and phase separation technique and were then coated with hypromellose phthalate to achieve their site-specific release. Microspheres were evaluated for percent yield, entrapment efficiency, surface morphology, particle size and size distribution, flow property, swelling property, mucoadhesion property by the *in vitro* wash-off and the *ex vivo* mucoadhesive strength tests, *in vitro* release profile and release kinetic, *in vivo* probiotic activity, and stability. The values for kinetic constant and regression coefficient of model-dependent approaches and the difference factor, the similarity factor, and the Rescigno index of model-independent approaches were determined for accessing and comparing *in vitro* performance.

Results: Microsphere formulation batches have percent yield value between 56.26% and 69.13% and entrapment efficiency value between 66.95% and 77.89%. Microspheres were coarser with spherical shape having mean particle size from 28.03 to 48.31 µm. *In vitro B. coagulans* release profile follows zero-order kinetics and depends on the grade of hypromellose and the *B. coagulans*-to-hypromellose ratio. Experimental microspheres rendered adequate stability to *B. coagulans* at room temperature.

Conclusion: Microspheres had delivered *B. coagulans* in simulated intestinal condition following zero-order kinetics, protectively in simulated gastric condition, exhibiting appreciable mucoadhesion in intestinal condition, which could be useful to achieve site-specific delivery for extended period.

Keywords: Probiotics, *Bacillus coagulans*, mucoadhesive, microspheres, extended release

Introduction

Numerous health benefits associated with the intake of probiotic bacteria has created a big market of probiotic foods worldwide[1]. Reported health benefits of probiotics are: suppressing undesirable microorganism growth in the colon and in the gut, control of serum cholesterol level, reduction in the colon cancer risk, immune system stimulation, improved lactose utilization, and control of food-associated allergic inflammation[1–6]. Observed therapeutic benefits are partly associated with the ability of probiotics to secrete a bacteriocin, coagulin, which is active against broad spectrum of enteric microbes[5]. To act, probiotics must arrive in intestine alive and in sufficient numbers, which is suggested at 10^6–10^7 colony-forming unit or cfu[7]. As like other probiotic strains, *Bacillus coagulans* (mislabeled as *Lactobacillus sporogenes*) also suffers with wide variation in the actual content with respect to labeled claim[5]. Loss of cell viability within the delivery system results from the freeze-drying operation of probiotics during initial manufacturing, the processing conditions during formulation, and the storage requirements during shipment and

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storage *vis-à-vis* after consumption; their viability in the gastrointestinal (GI) tract is adversely affected by various hydrolytic enzymes, acidic conditions of stomach, and bile salts[5,7–13]. Nowadays, microencapsulation technologies had been attracted considerable attention due to their ability to reduce the cell loss during storage and shipment and to improve and maintain the active cells arriving in the intestine[7,8,11,13]. All these approaches have varying degree of success, as maintenance of high numbers of viable cells in probiotic formulation throughout shelf-life of product and during GI transit is a challenge, and the costs of operation remain a concern. Decreased performance and success of microspheres is owing to their short gastric retention time (GRT), a physiological limitation, which can be improved by coupling mucoadhesion characteristics to microspheres through developing mucoadhesive microspheres, which will in turn prolong GRT thereby enhancing bioavailability[14–16]. Excellent mucoadhesive property of hypromellose (hydroxypropyl methylcellulose) is conferring their utility in the preparation of mucoadhesive microspheres[15,16]. Hypromellose (i.e. Methocel) and hypromellose phthalate possesses aqueous solubility, are compatible with *B. coagulans*, and are safe for human oral consumption[17–19].

In context of above principles, a strong need was felt to develop a delivery system that will deliver *B. coagulans* into gut with increased efficiency and performance. In the present investigation, it is attempted to prepare *B. coagulans*-loaded mucoadhesive microspheres for delivery of viable *B. coagulans* into gut for extended period of time, protectively against harsh acidic conditions of stomach, using the several grades of hypromellose as mucoadhesive polymer and the hypromellose phthalate as enteric coating polymer.

Materials and methods

Materials

Freeze-dried powder of *B. coagulans* was kindly donated by Glenmark Pharmaceuticals Ltd. (Sinnar, Nasik, India). Grades of Methocel and hypromellose phthalate were gifted by Indoco Remedies Ltd. (Mumbai, India). Glucose yeast extract (GYE) agar media and other analytical grade laboratory chemicals were purchased from HiMedia Lab. Pvt. Ltd. (Mumbai, India).

In-house specifications compliance study of *B. coagulans*

Identification tests (description, microscopic examination, qualitative test for lactic acid production), viable *B. coagulans* spore count, lactic acid producing capacity estimation, loss on drying value determination, and the absence of contaminants study were done, as per the method of analysis (MOA) provided by the manufacturer.

Preparation of mucoadhesive microspheres

Core mucoadhesive microspheres of hypromellose, loaded with *B. coagulans*, were prepared following coacervation and phase separation technique[20]. Five grams of hypromellose was dissolved in 200 mL of cold de-ionized (DI) water (4 ± 2°C) followed by addition of 200 mg Tween-80 with stirring. Resultant solution was filtered aseptically using 0.45 µm PVDF filter membrane (Millipore). *B. coagulans* was dispersed in the above solution, under stirring at 500 ± 25 revolutions per minute (rpm) with a mechanical stirrer (Remi, Mumbai, India), followed by gradually raising the temperature up to 30 ± 2°C and stirring was continued for 30 min. Twenty-five milliliter of acetone was added drop wise with stirring at 300 ± 25 rpm and stirred for 10 min. Microspheres thus obtained were filtered aseptically with 10-µm nylon filter (Millipore; NY10) followed by washing thrice with sterile water for injection (30 ± 2°C) and were kept in desiccator for 24 h. Entire process was carried out aseptically on bench of a horizontal laminar flow clean air work station (Klenzaids Bioclean Devices (P) Ltd., 1500048-24-24, Mumbai, India). All formulation batches were prepared in triplicate following the above method so as to verify reproducibility and their compositions were summarized in Table 1.

Coating of core microspheres

Ten percent w/w hypromellose phthalate (HP-50) solution was prepared by dissolving it in phosphate buffer pH 6.8[21]. To 200 mL of above solution 4 g of polyethylene

Table 1. The formulation formula and the percent yield value, the entrapment efficiency value, and the mean particle size value of formulation batches.

Formulation batch	Grade of Methocel used	Ratio of *B. coagulans* to Methocel	Percent yield (w/w)[a]	Entrapment efficiency (% w/w)[a]	Mean particle size (µm)[a]
F1	E5 Premium LV	1:1	66.43 ± 0.71	76.53 ± 0.83	28.19 ± 0.23
F2		1:2	68.01 ± 1.29	77.89 ± 1.16	28.03 ± 0.25
F3		1:3	69.13 ± 1.12	76.36 ± 1.06	28.33 ± 0.24
F4	E15 Premium LV	1:1	63.19 ± 1.24	71.64 ± 1.31	35.19 ± 0.91
F5		1:2	64.48 ± 1.19	71.09 ± 1.13	35.50 ± 0.85
F6		1:3	65.37 ± 1.01	70.91 ± 1.09	36.03 ± 0.76
F7	E10M Premium CR	1:1	56.26 ± 1.06	66.95 ± 1.14	47.93 ± 0.12
F8		1:2	57.43 ± 1.08	67.57 ± 1.25	48.21 ± 0.18
F9		1:3	58.86 ± 1.17	67.06 ± 2.31	48.31 ± 2.15

[a]Data are presented as mean value ± SD, $n = 3$.

glycol 200 (PEG-200) and 200 mg of Tween-80 were added with stirring, and then the solution was filtered aseptically using 0.45-µm PVDF filter membrane. Prepared core microspheres were dispersed in the above solution with stirring at 300 ± 25 rpm followed by drop wise addition of 40 mL of propan-2-ol and stirring was continued for 30 min. The coated microspheres were filtered and washed thrice with sterile water for injection (30 ± 2°C). Coated microspheres were kept in a desiccator for 24 h, then transferred aseptically into a sterile glass vial, sealed hermetically, and were stored in refrigerator for further manipulation. Entire process was carried out aseptically on bench of horizontal laminar flow clean air workstation.

Percent yield

Coated microspheres from each formulation batch were weighed and respective percent yield value was calculated using the following formula:

$$\text{Percent yield} = \frac{W_1 \text{ (Weight of microspheres recovered)}}{W_2\left[\text{Weight}\left\{\begin{array}{c}\text{drug (viable cell +}\\ \text{nonviable cell)+polymer}\end{array}\right\}\right]} \times 100$$

Calibration curve of *B. coagulans*

Standard concentrations of dispersions containing *B. coagulans* were prepared with the sterile simulated gastric fluid TS and the sterile simulated intestinal fluid TS separately and respective absorbance was measured at 600 nm with respect to respective blank, using UV/vis spectroscopic method (Shimadzu, UV-1700, Japan) so as to get calibration curve that directly relates optical density (OD) value to cell concentration and was the best way to obtain immediate result of number of cells (viable plus nonviable) present in the sample[22]. The method was validated for linearity, accuracy, and precision. OD value reveals roughly the total number of cells (viable plus nonviable) in the sample, helps in determining dilution factor for performing viable spore count but fails to reveal cfu an unit for expressing number of viable cells.

Viable *B. coagulans* spore count

Number of viable spores in sample was counted by the following procedures[17].

Dilution and heat treatment

One gram, alternately 1 mL solution, of sample containing *B. coagulans* was transferred aseptically into a pre-sterilized 10-mL volumetric flask containing 5.0 mL of sterile saline TS then sonicated for 10 min and diluted to 10 mL with sterile saline TS. One milliliter of above suspension was diluted to 10 mL with sterile saline TS in an autoclaved test tube (25 mm by 150 mm size), mixed thoroughly, and was serially diluted till a suitable dilution was achieved (~100 cell/mL). The final dilution tube was kept in a water bath at 70°C for 30 min followed by immediate cooling to about 45°C. Saline TS[21], simulated gastric fluid TS[21], and simulated intestinal fluid TS[21] contains inorganic salts but no carbon source, thus *B. coagulans* cells will not proliferate in this media but will remain in a state of stasis until they were plated on media containing carbon source.

Plating

GYE agar medium was liquefied and cooled to 45°C on a water bath. One milliliter of sample from the heat-treated final dilution tube was transferred aseptically into sterile Petri dish (six per sample) followed by pouring 15 mL of molten medium and mixing thoroughly. Plates were incubated in an inverted position at 40°C for 48 h after solidification.

Counting

Six plates were counted and the average count per plate was calculated. The number of cfu per unit (mL or g) of sample was calculated employing following equation:

$$\text{Number of cfu} = \frac{\left[\begin{array}{c}\text{Average number of colonies}\\ \text{counted per plate}\end{array}\right]}{[\text{Dilution factor}]}$$

Entrapment efficiency

Aseptically 250 mg of accurately weighed coated microspheres were kept with 10 mL of sterile simulated intestinal fluid TS in a hermetically sealed sterile glass vial at 4 ± 2°C for 24 h. Then solution was subjected for viable spore count (i.e. practical viable spore count value in cfu/g) and entrapment efficiency was calculated with the following equation:

$$\text{Percent entrapment efficiency} = \frac{\begin{array}{c}\text{Practical viable}\\ \text{spore count value}\end{array}}{\begin{array}{c}\text{Theoretical viable}\\ \text{spore count value}\end{array}} \times 100$$

Morphological examination

Coated microspheres were mounted on the aluminum stubs using double-sided adhesive tape. Then stubs were vacuum-coated with thin layer of gold and examined with Jeol JSM 5610 LV scanning electron microscope (SEM)[16,23].

Particle size and size distribution study

Suspension of coated microspheres, in *n*-hexane, subjected for particle size study using a Malvern 2600 Laser Diffraction Spectrometer and stirred magnetically during the study[24,25] and was expressed as the volume surface diameter.

Flow property study

Flow property of coated microspheres was determined from the result of study parameters namely Angle of repose (α), Carr's index (CI), and Hausner ratio (HR),

and all of these parameters were calculated employing equation given as below[21]. Angle of repose was determined by fixed funnel method and is calculated from the height (H) and the radius (R) of powder hip. Microspheres were filled in graduated cylinder and initial volume before tapping (V_0) was noted, then was tapped with tap density apparatus (Electrolab, ETD-1020, Mumbai, India) to a constant volume so as to get tapped volume (V_T).

$$\alpha = \tan^{-1}\,[H/R]$$

$$\text{CI} = [(V_0 - V_T)/V_0] \times 100$$

$$\text{HR} = V_0/V_T$$

In vitro swelling analysis

Initial diameter of coated microspheres was determined using a calibrated optical microscope (Labomed, CX RIII, Ambala, India) by wetting with simulated intestinal fluid TS on a glass slide and allowing to immerse in it. Final diameter of immersed microspheres was determined after 20 and 60 min[15]. Percent swelling was calculated with the following formula:

$$\text{Percent swelling} = \frac{(\text{Final diameter} - \text{Initial diameter})}{\text{Initial diameter}} \times 100$$

Mucoadhesion property analysis

Mucoadhesion property analysis of coated microspheres was carried out following institutional animal ethical committee guidelines and is performed by following tests:

In vitro wash-off test

In vitro wash-off test of coated microspheres was carried out to access their mucoadhesive property to the intestinal mucosa[26]. Freshly excised piece of intestinal mucosa (2 cm × 2 cm) from sheep were mounted onto glass slide (8 cm × 3 cm) with cyanoacrylate glue. Coated microspheres (100 numbers) were accurately counted and were spread onto the wet rinsed intestinal mucosa tissue and the prepared slide was hung onto one of the groves of a USP tablet disintegration test apparatus (Labindia Instruments Pvt. Ltd., DT 1000, Thane, Mumbai, India), with continuous oxygen supply. The apparatus was operated giving tissue specimen a regular up-and-down movement within the beaker of disintegration test apparatus, containing simulated intestinal fluid TS. The number of microspheres still adhering onto the tissue was counted at hourly intervals up to 12 h.

Ex vivo mucoadhesive strength determination

Coated microsphere suspension, in simulated intestinal fluid TS, was prepared and number of microsphere per milliliter (N_0) was determined by optical microscopy. One milliliter of above suspension was ingested to overnight fasted albino rats of either sex (in a group of three) using an oral feeding needle and were sacrificed at an interval of 0, 4, 8, and 12 h to isolate their stomach and intestine region. The stomach and the intestine regions were then cut opened longitudinally to count number of microspheres adhering to these regions (N_S). Percent adhesive strength was calculated using the following formula[15]:

$$\text{Percent adhesive strength} = \frac{N_S}{N_0} \times 100$$

In vitro release study

In vitro release profile study of *B. coagulans* from coated microspheres was done in a USP basket apparatus (Electrolab, TDT-06T, Mumbai, India) at 37 ± 0.5°C and 100 rpm containing 900 mL of sterile dissolution medium namely the simulated gastric fluid TS and the simulated intestinal fluid TS, and the basket was wrapped with 100 mesh nylon cloth containing about 600 mg of accurately weighed coated microspheres. Five milliliter of dissolution medium was withdrawn at predetermined time interval up to 18 h followed by immediate replacement with an equal volume of fresh dissolution medium. After suitable dilution, withdrawn samples were subjected for viable spore count and result was presented as percent viable *B. coagulans* cells released with respect to practical viable spore count value.

In vitro release kinetic studies, statistical evaluation, and data fitting

In vitro drug dissolution profile from delivery system that characterized product more precisely than a single point dissolution test, under appropriate test condition, was described by different kinetic model where the dissolved amount of drug as a function of test time was studied. A mean value of three determinations, at each time point, was used to fit *in vitro* drug dissolution profile of all formulation batches to different kinetic models so as to find out best fit kinetic model and to determine their release exponents, whereas mean value of 12 determinations was used to estimate the factors of model-independent approach[27,28]. *In vitro* release kinetic studies, statistical evaluation, data fitting, nonlinear least square curve fitting, simulation, and plotting were performed using the Excel software 2007 (Microsoft Software Inc., USA) for determining parameters of each equation.

ANOVA-based procedures

Statistical analysis of *in vitro* release data and other data were performed using the one-way ANOVA at 5% level of significance ($P < 0.05$) using Microsoft excel 2007.

Model-dependent methods

Model-dependent approaches including zero-order, first-order, Higuchi square root, Hixson-Crowell and Weibull models, as described in Table 2, were applied considering amount of viable cell release as a function

Table 2. Mathematical models used to describe dissolution curves.

Zero order	$Q_1 = Q_0 + K_0 t$
First order	$\ln W_1 = \ln W_0 + Kt$
Hixson-Crowell	$Q_0^{1/3} - Q_1^{1/3} = K_s t$
Higuchi	$W_1 = K_H^{1/2}$
Weibull	$\mathrm{Log}[-\ln(1-m)] = b\log(t-T_i) - \log\alpha$

Q_0 is the initial amount of B. coagulans in the delivery system, Q^1 is the amount of B. coagulans in the delivery system at time t, W0 is the initial amount of B. coagulans in the delivery system, W1 is the amount of B. coagulans released in time t, K^0 is the zero-order proportionality constant, K is the first-order release rate constant, K^H is the Higuchi constant, K^s is a constant incorporating the surface–volume relation, m is the accumulated fraction of the B. coagulans in solution at time t, α is the time scale of the process, T^i is the location parameter that represents the lag time before the onset of the dissolution or release process, and b is the shape parameter.

of test time. Following plots namely cumulative percent viable cell release versus time (zero-order kinetic), log cumulative percent viable cell release versus time (first-order kinetic), cumulative percent viable cell release versus square root of time (Higuchi), cube root of percent viable cell remaining in matrix versus time (Hixson-Crowell cube root), and logarithm of amount of viable cell versus the logarithm of time (Weibull) were plotted.

Model-independent methods (pair-wise procedures)

Three factors of model-independent mathematical approach namely: f_1, f_2, and Rescigno index (ξ_i) were used to compare dissolution profiles, which help to assure similarity in product performance and signals bioequivalence. According to the nature of measurement, f_1 is described as the difference factor and f_2 is the similarity factor and were determined from cumulative data. Rescigno index (ξ_1 and ξ_2), a dimensional index, refers to area differences for non-cumulative data and to the difference between the dissolved amount of the test and the reference product in a given time interval. Factors f_1, f_2, and Rescigno index (ξ_i) were calculated from the following equations:

$$f_1 = \{[\sum_{t=1}^{n} |R_t - T_t|]/[\sum_{t=1}^{n} R_t]\} \times 100$$

$$f_2 = 50 \times \log\{[1+(1/n)\sum_{t=1}^{n}(R_t - T_t)^2]^{-0.5} \times 100 \text{ and}$$

$$\xi_i = [\{\sum_{t=1}^{n} |d_T(t) - d_R(t)|^i\}/\{\sum_{t=1}^{n} |d_T(t) - d_R(t)|^i\}]^{1/i}$$

where R_t is cumulative percent dissolved from the reference product and T_t is from the test product at each of the selected n time points of the test and the product, $d_T(t)$ is the test product dissolved amount and $d_R(t)$ is the reference product dissolved amount at each time point, and i is any positive integer number.

In vivo probiotic activity evaluation

In vivo probiotic activity evaluation of coated microspheres was done using mouse model enterococci stool colonization method[29] following institutional animal ethical committee guidelines. One milliliter of coated microspheres dispersion (10^2 cfu/mL), in simulated intestinal fluid TS, was orally fed to mouse (in group of six) using an oral feeding needle. The stools were collected periodically at 4 h interval up to 48 h and subjected for enterococci colonization density study.

Accelerated stability study of microspheres

Formulation batches microspheres were stored at several conditions of temperature and humidity (30 ± 2°C/65 ± 5% RH and 40 ± 2°C/75 ± 5% RH) in stability analysis chambers (Darwin Chambers Company, St. Louis, MO) and in refrigerator (2–8°C) for accelerated stability study[30]. The samples were subjected for viable *B. coagulans* cell content, color, and texture analysis at 2 weeks interval up to 12 weeks followed by 1 month interval up to 6 months, and results were compared with those of initial result (analysis result of samples prior to stability charging) and control samples kept at 2–8°C.

Results and discussion

Coacervation and phase separation technique described here appears to be a suitable method for preparation of the coated hypromellose microspheres loaded with *B. coagulans*. It is simple, less time-consuming and a two-step process, can maintain *B. coagulans* viability during processing as this method eliminates exposure of *B. coagulans* to high temperature, organic solvents, and mechanical stress.

Total formulating processes of coated microspheres was carried out below 20°C in aqueous medium as the temperature above 20°C and the non-aqueous solvents adversely affects and decreases the viability of *B. coagulans*. Hypromellose is soluble in cold water, its solubility in water decreases with increase in temperature, and it is also insoluble in organic solvents like chloroform, dichloromethane, ether, acetone, and so on[19]. Hypromellose phthalate is insoluble in water and propan-2-ol and is soluble in aqueous alkali[19]. Due to above mentioned facts, to eliminate exposure of *B. coagulans* to non-aqueous solvents and to maintain viability of *B. coagulans* during processing, selection of the hypromellose as mucoadhesive polymer and the hypromellose phthalate as coating polymer is done. Tween-80 is incorporated in the formulation for homogeneously dispersing *B. coagulans* cells during the processing of core microsphere. PEG-200 is incorporated in the coating solution formulation to impart plasticity to the coat so as to prevent splitting and cracking of the coat.

In-house specifications compliance study of *B. coagulans*

Used *B. coagulans* spores had complied manufacturer's specifications, as specified in MOA.

Percent yield

Percent yield value of coated microsphere batches lies between 56.26% and 69.13% w/w, which was found to vary with variation in the grade of hypromellose but not with variation in the *B. coagulans*-to-hypromellose ratio (Table 1). Formulation containing Methocel E5 Premium LV (ME5) exhibited highest percent yield value. Grade of hypromellose affects the percent yield value in the order of ME5 > Methocel E15 Premium LV (ME15) > Methocel E10M Premium CR (ME10M).

Entrapment efficiency

The percent entrapment efficiency value of microspheres lies between 66.95% and 77.89% w/w that found to vary with variation in the grade of hypromellose but not with variation in the *B. coagulans*-to-hypromellose ratio (Table 1). Formulation containing ME5 has exhibited highest percent entrapment efficiency value. Grade of hypromellose affects entrapment efficiency that follows the order of ME5 > ME15 > ME10M.

Morphological examination

Coated mucoadhesive microspheres were coarser with spherical shape as evidenced from SEM photograph (Figure 1). Due to space constraint, SEM photographs of formulation F1, F4, and F7 were presented. Coarsest surface was observed with formulation containing ME5 with reference to ME15 and ME10M. Coarser surface texture in turn will improve the adhesion through stronger mechanical interactions[16].

Particle size and size distribution study

Mean particle size value of coated microsphere batches lies within the range of 28.03 to 48.31 μm (Table 1). Variation in the mean particle size value was observed with variation in the grade of hypromellose but not with variation in the *B. coagulans*-to-hypromellose ratio. Highest mean particle size value was observed with formulations containing ME10M. Grade of hypromellose affects mean particle size value in the order of ME10M > ME15 > ME5 (Table 1 and Figure 2).

Flow property study

Flow property of coated microsphere batches lies within very poor and very, very poor range.

In vitro swelling analysis

Percent swelling values, as a measure of *in vitro* swelling property, of coated microsphere batches ranges from 60.09% to 74.45%, at 60 min (Table 3). Variation in the percent swelling value was observed with variation in the grade of hypromellose and in the *B. coagulans*-to-hypromellose ratio. A decrease in percent swelling value was observed with increase in the *B. coagulans*-to-hypromellose ratio. Maximum swelling was observed with ME5, although grade of hypromellose affects percent swelling that follows the order of ME5 > ME15 > ME10M.

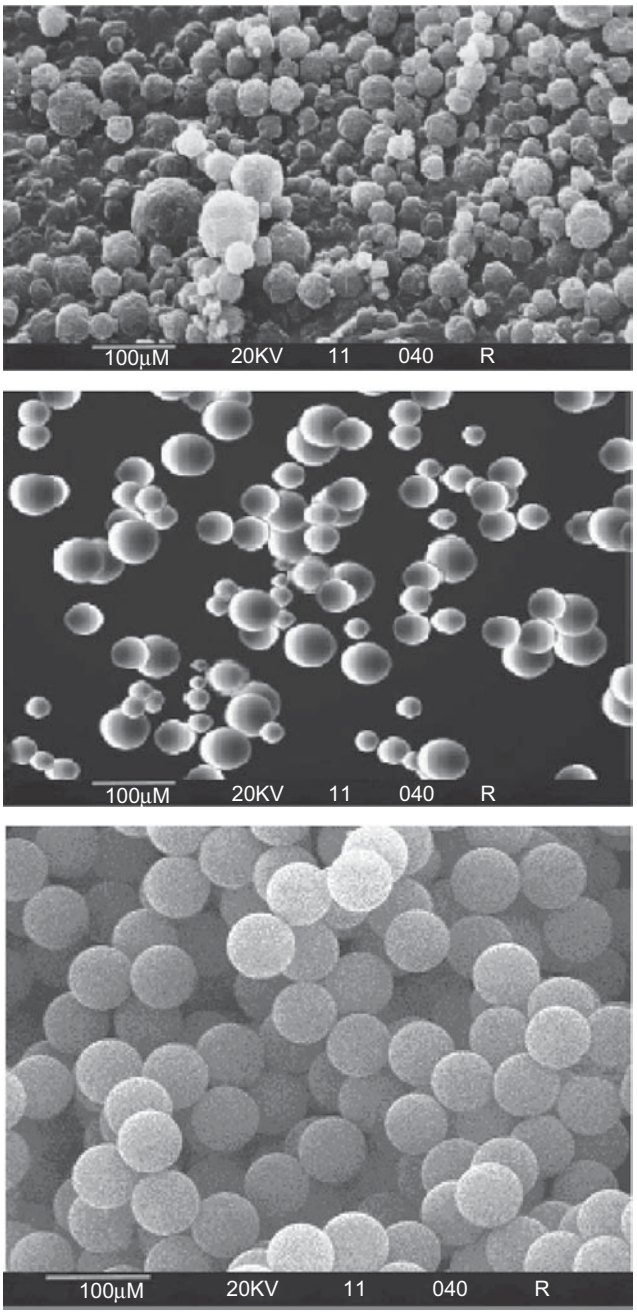

Figure 1. Scanning electron microscopy (SEM) photographs of microspheres from formulation batches F1, F4, and F7.

Mucoadhesion property analysis

The *in vitro* wash-off test, as a measure of mucoadhesive efficiency, result (Table 4) reveals that even after 12 h some of the coated microspheres were remained adhered to the intestinal mucosa, indicating that microspheres possesses strong mucoadhesion affinity for intestinal mucosa, and microspheres may retain in the intestinal tract for an extended period of time. *In vitro* wash-off test result also reveals that *B. coagulans*-

Table 4. Results of *in vitro* wash-off test of the microspheres from all formulation batches.

Formulation batch	Percent microspheres adhering to intestinal mucosa tissue at time points (h)						
	1 h	2 h	4 h	6 h	8 h	10 h	12 h
F1	89 ± 2.1	79 ± 2.1	62 ± 2.0	40 ± 1.5	32 ± 2.1	24 ± 2.0	16 ± 1.9
F2	86 ± 1.8	76 ± 2.4	61 ± 2.1	39 ± 1.9	29 ± 1.9	18 ± 2.2	13 ± 1.5
F3	85 ± 1.5	74 ± 2.0	58 ± 2.3	43 ± 2.1	30 ± 1.9	21 ± 1.7	12 ± 2.0
F4	71 ± 2.1	58 ± 1.2	41 ± 1.6	33 ± 1.7	22 ± 2.0	15 ± 1.9	09 ± 1.0
F5	72 ± 1.9	57 ± 2.3	39 ± 2.0	32 ± 1.5	24 ± 1.8	16 ± 1.8	11 ± 1.8
F6	73 ± 2.0	60 ± 1.8	45 ± 2.2	31 ± 1.9	23 ± 2.0	18 ± 1.9	12 ± 1.7
F7	62 ± 1.6	49 ± 2.0	34 ± 2.1	22 ± 1.9	16 ± 1.5	13 ± 1.2	09 ± 1.1
F8	63 ± 1.2	45 ± 1.0	33 ± 1.4	24 ± 1.8	18 ± 1.7	12 ± 1.1	08 ± 0.8
F9	60 ± 2.2	48 ± 1.1	32 ± 1.8	25 ± 1.6	19 ± 1.3	10 ± 1.5	07 ± 1.1

Data are presented as mean value ± SD, $n = 3$.

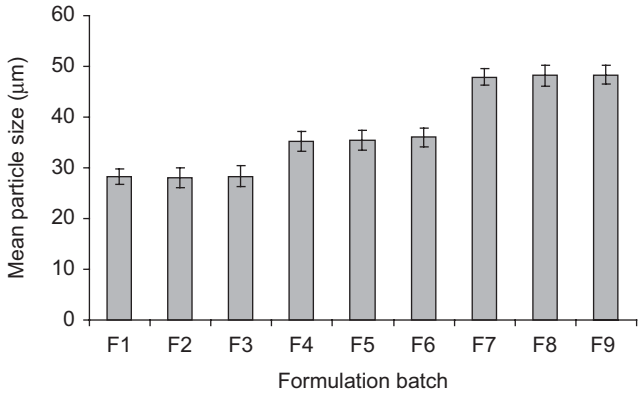

Figure 2. Histogram of mean particle size distribution of all formulation batches.

Table 3. The percent swelling value and the percent adhesive strength value of microspheres from all formulation batches.

Formulation batch	Percent swelling		Percent adhesive strength
	At 20 min	At 60 min	
F1	39.15 ± 0.59	74.45 ± 0.56	82.16 ± 1.23
F2	37.89 ± 0.41	68.79 ± 0.32	81.65 ± 1.35
F3	34.62 ± 0.32	61.63 ± 0.49	79.89 ± 1.37
F4	35.21 ± 0.50	69.93 ± 0.41	77.91 ± 1.17
F5	32.49 ± 0.64	66.19 ± 0.37	76.57 ± 1.24
F6	29.23 ± 0.59	63.14 ± 0.47	76.12 ± 1.41
F7	28.18 ± 0.34	67.34 ± 0.53	71.18 ± 1.36
F8	26.74 ± 0.46	63.67 ± 0.31	71.62 ± 1.16
F9	25.81 ± 0.81	60.09 ± 0.11	70.97 ± 1.14

Data are presented as mean value ± SD, $n = 3$.

to-hypromellose ratio possesses no significant effect on the mucoadhesion properties of the microspheres but it varies with variation in the grade of polymer. Grade of hypromellose effect on the mucoadhesion properties follows the order of ME5 > ME15 > ME10M. A similar trend was also observed with the percent adhesive strength value, a measure of *ex vivo* mucoadhesive strength, which ranges between 70.97% and 82.16% (Table 3). Highest percent mucoadhesion value was observed with ME5. Mucoadhesion property analysis results evidenced that the mucoadhesion property of coated microspheres varies with variation in the grade of hypromellose and not with the variation in *B. coagulans*-to-hypromellose ratio, also the microspheres possessing strong mucoadhesion affinity with the intestinal mucosa thus may retain in the intestinal tract for an extended period of time.

In vitro release study

In vitro release profile study reveals that *B. coagulans* release from the microspheres in simulated gastric condition was negligible, although in simulated intestinal condition the release was almost regulated and extended (Figure 3). Enteric coating of microspheres had prevented the release of *B. coagulans* in gastric pH but releasing *B. coagulans* in intestinal pH, and hence can result in site-specific delivery of *B. coagulans* to the gut while preventing the viability loss of *B. coagulans* in the delivery system at gastric pH.

The values for kinetic constant and release exponent of model-dependent approaches (zero-order and Weibull) are listed in Table 5. The mechanism of *B. coagulans* release from the microspheres follows zero-order kinetics, since the plot of the cumulative percent viable *B. coagulans* cell release versus time were found to be linear, and have highest regression coefficient (r^2) value in comparison with that of first-order, Higuchi, Hixson-Crowell, and Weibull model when compared within intra-formulation batches. For all formulation batches zero-order kinetic constant value ranges from 2.4013 to 5.7619, whereas zero-order r^2 value ranges from 0.9856 to 0.9985. Study of shape parameter values of Weibull model from Table 5, for all formulation batches, reveals that the curve is sigmoid or S-shaped with upward curvature followed by a turning point, as $\beta > 1$[28], whereas the location parameter (T_d) value that characterizes the time interval necessary to dissolve or release 63.2% of the drug present in the delivery system[28] ranges from 9.299 to 30.08 h; and the r^2 value ranges from 0.915 to 0.981.

The model-independent approaches factors (f_1, f_2, ξ_1, and ξ_2) values listed in Table 6 reveal that for all formulation pairs the ξ_1 values lies between 0.0607 and 0.24 (i.e. above 0 and below 1), ξ_2 values lies between 0.1422 and 0.3154 (i.e. above 0 and below 1), f_1 value lies between 17.84 and 41.41 (i.e. above 15), and f_2 value lies between 28.34 and 62.81 indicating dissimilarity in product performance[27,28]

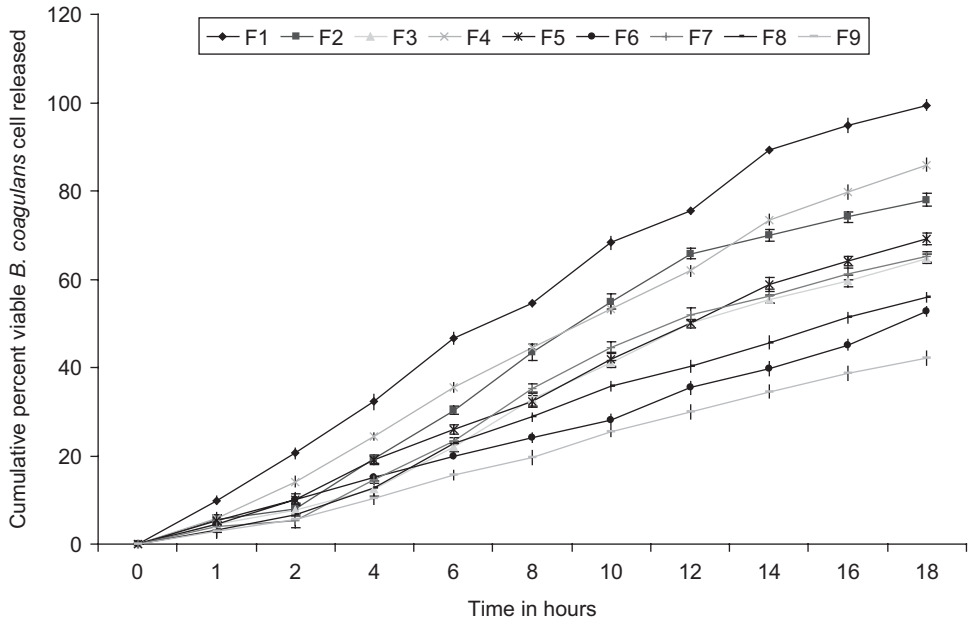

Figure 3. Comparative *in vitro* release profile of *B. coagulans* from coated microspheres of all formulation batches, in the simulated intestinal fluid TS, following zero-order kinetics.

Table 5. Linearization of *B. coagulans* dissolution profile using model-dependent approaches, that is, the zero order and the Weibull.

	Kinetic constant and release exponents of zero-order and Weibull model					
	Zero order		Weibull			
Formulation batch	K_0	r^2	r^2	α (Scale parameter)	β (Shape parameter)	T_d (Location parameter)
F1	5.7619	0.9985	0.920 ± 0.35	72.34 ± 0.529	1.289 ± 0.083	27.69
F2	4.6741	0.9893	0.915 ± 0.36	54.31 ± 0.456	1.174 ± 0.072	30.08
F3	3.8074	0.9882	0.959 ± 0.25	40.21 ± 0.302	1.233 ± 0.089	19.98
F4	4.8092	0.9968	0.955 ± 0.27	55.98 ± 0.379	1.333 ± 0.091	20.47
F5	3.8901	0.9953	0.972 ± 0.21	43.51 ± 0.289	1.378 ± 0.083	15.42
F6	2.8001	0.9931	0.981 ± 0.17	31.72 ± 0.246	1.549 ± 0.077	9.299
F7	3. 9870	0.9856	0.926 ± 0.34	42.00 ± 0.374	1.102 ± 0.081	29.66
F8	3.1912	0.9903	0.947 ± 0.29	35.39 ± 0.325	1.197 ± 0.069	19.67
F9	2.4013	0.9974	0.969 ± 0.22	25.92 ± 0.242	1.311 ± 0.092	11.98

K_0 is the zero-order proportionality constant and r^2 is the regression coefficient.

of the formulation batches when compared in pair with each other, that is, intra-polymer wise (having same grade of hypromellose but differs from each other by *B. coagulans*-to-hypromellose ratio) and inter-polymer wise (having same ratio of *B. coagulans*-to-hypromellose but differs from each other by grade of hypromellose).

A plot of viable *B. coagulans* release profile following zero-order kinetic model of all formulation batches shown in Figure 3 reveals that the viable *B. coagulans* release rate from microspheres had decreased with an increase in viable *B. coagulans*-to-hypromellose ratio although the grade of hypromellose decreases the rate of viable *B. coagulans* release from microspheres by the order of ME10M > ME15 > ME5.

In vivo probiotic activity evaluation

In vivo probiotic activity evaluation result depicts that oral administration of enteric coated mucoadhesive microspheres of *B. coagulans*, from all formulation batches, had resulted in statistically significant decrease in density of enterococci colonization in the stool of mouse up to the time periods that ranges between 24 and 36 h.

Accelerated stability study

Accelerated stability study result reveals that the prepared *B. coagulans*-loaded microspheres exhibit adequate stability at the storage condition of 30 ± 2°C/65 ± 5% RH, as the change in color and texture and the statistically significant decrease in viable *B. coagulans* cell content with respect to practical viable spore count value was not observed, and also reveals that the *B. coagulans* cells were compatible with the excipients used in the development of microspheres. At the storage condition of 40 ± 2°C/75 ± 5% RH, statistically significant decrease in viable *B. coagulans* cell content with respect to practical

Table 6. Mean value of dissimilarity factor (f_1), similarity factor (f_2), and two indices of Rescigno (ξ_1 and ξ_2).

Formulation pair	f_1	f_2	ξ_1	ξ_2
F1 vs. F2	25.01	40.06	0.0729	0.2126
F1 vs. F3	41.41	28.34	0.1383	0.2290
F2 vs. F3	23.96	44.45	0.1046	0.1861
F4 vs. F5	22.08	46.57	0.1643	0.2751
F4 vs. F6	40.69	30.78	0.1014	0.2626
F5 vs. F6	29.23	44.41	0.1345	0.2362
F7 vs. F8	17.84	55.95	0.0607	0.2614
F7 vs. F9	37.89	38.79	0.2110	0.2733
F8 vs. F9	25.13	52.13	0.2400	0.3033
F1 vs. F4	22.02	42.31	0.2091	0.2742
F1 vs. F7	40.09	29.06	0.1120	0.2117
F4 vs. F7	25.01	43.12	0.2128	0.3154
F2 vs. F5	20.21	48.09	0.1091	0.1422
F2 vs. F8	34.56	37.11	0.1418	0.1818
F5 vs. F8	20.00	54.01	0.1212	0.1691
F3 vs. F6	23.91	48.93	0.2147	0.3027
F3 vs. F9	36.01	41.63	0.0752	0.2012
F6 vs. F9	17.91	62.81	0.0941	0.2264

viable spore count value was observed, indicating product instability at this storage condition.

Study of performed experimental results reveals that intestinal targeted mucoadhesive microspheres of *B. coagulans* could be prepared successfully with reproducibility and stability, using hypromellose as the mucoadhesive and release controlling polymer and hypromellose phthalate as enteric coating polymer following coacervation and phase separation technique. To be specific, formulation F1 (containing *B. coagulans*-to-ME5 with ratio of 1:1) was found to be the most suitable formulation and is superior to other prototypes under development with regards to physicochemical evaluation parameters value and product performances. Satisfactory *in vitro* performance of product in the simulated gastric condition and in the simulated intestinal condition with a release profile that is controlled and extended following zero-order kinetic, an attribute highly desirable for any controlled and extended release formulation, is a desired achievement.

Conclusion

Freeze-dried *B. coagulans* cells can be successfully formulated as intestinal retentive-targeted mucoadhesive microspheres with satisfactory physicochemical evaluation parameters value, maintaining viability of *B. coagulans* in the simulated gastric condition and during processing, in simulated intestinal condition exhibiting mucoadhesion and controlling as well as extending the viable cell release following zero-order kinetic and having satisfactory stability at room temperature. Elicited satisfactory *in vivo* probiotic activity in the mouse model warrants the potentiality for commencing follow-up studies of *B. coagulans*-loaded intestinal targeted mucoadhesive microspheres for human use, as an alternative to conventional delivery systems.

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Declaration of interest

No conflicts of interest were declared in relation to this article.

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