Formulation and evaluation of *Bacillus coagulans*-loaded hypromellose mucoadhesive microspheres

Sk Md Athar Alli
Department of Pharmaceutical Technology, Jadavpur University, Kolkata, West Bengal, India

Abstract: Development of a novel delivery system has been attempted to deliver viable probiotic cells into the gut for a prolonged period of time while maintaining high numbers of viable cells within the formulation throughout the shelf-life of the product and during the gastrointestinal transit. Core mucoadhesive microspheres of *Bacillus coagulans* were developed employing several grades of hypromellose, a mucoadhesive polymer, following coacervation and phase separation technique and were subsequently enteric-coated with hypromellose phthalate. Microspheres were evaluated for percent yield; entrapment efficiency; in vitro swelling; surface morphology; particle size, size distribution, and zeta potential; flow property, mucoadhesion property by the ex vivo mucoadhesive strength test and the in vitro wash off test; in vitro release profile and release kinetic; in vivo probiotic activity; and stability. The values for the kinetic constant and regression coefficient of model-dependent approaches and the difference factor ($f_1$), the similarity factor ($f_2$), and the Rescigno index ($\xi_1$ and $\xi_2$) of model independent approaches were determined for comparing in vitro dissolution profiles. Freeze dried *B. coagulans* cells were successfully formulated as enteric-coated mucoadhesive microspheres with satisfactory physical structure and yield. The viability of *B. coagulans* was maintained in the simulated gastric conditions and during processing; in simulated intestinal conditions exhibiting mucoadhesion, and controlling and extending the viable cell release following zero-order; and was satisfactorily stable at room temperature. Test results depict statistically significant effects of the hypromellose grade and their concentration on the performance and release profile of formulations.

Keywords: probiotics, *B. coagulans*, mucoadhesive, microspheres, extended-release

Introduction

Reported health benefits of probiotics such as suppressing the growth of undesirable microorganisms in the colon and in the small intestine, controlling serum cholesterol level, reducing the risk of colon cancer, stimulating the immune system, improving lactose utilization, and controlling the allergic inflammation associated with food allergy has created a large market of probiotic foods worldwide.1–7 The therapeutic benefit of probiotics is associated with its ability to secrete a bacteriocin, coagulin, which is active against a broad spectrum of enteric microbes.7 To be therapeutically active, probiotics must arrive in the intestine alive and in sufficient numbers which is suggested at $10^6$–$10^7$ colony-forming units (cfu). B. coagulans, commonly mislabeled as *Lactobacillus sporogenes*, also suffers with wide variation between the actual content and the labeled claim of viable spores, as in other probiotic strains.7,8 Reduction in cell viability within the dosage form results from the freeze-drying operation of probiotics during the initial manufacturing, the processing conditions during formulation, and noncompliance to
storage requirements during transportation and storage.\textsuperscript{7–12} After their consumption, the hydrolytic enzymes, the acidic conditions of stomach, and the bile salts in the gastrointestinal (GI) tract also adversely affect the viability of probiotics.\textsuperscript{7,12–15} Various approaches such as the addition of different growth promoters, manipulation of fermentation and storage conditions of the food carriers, careful selection of the culture organisms according to their interrelationships and acid-bile resistance, and employing microencapsulation technology have been investigated to improve the amount of viable cells arriving in the intestine.\textsuperscript{7,13–15} However, all of these approaches have varying degrees of success, as maintenance of high numbers of viable cells within the product throughout its shelf-life and during GI transit is a challenge. Amongst all the above approaches, microencapsulation technologies have attracted considerable attention but their short residence time at the site of action/absorption limits their performance. Coupling mucoadhesion characteristics to microspheres through developing mucoadhesive microspheres in turn will enhance their residence time and specifically can target drug(s) to the site of action/absorption and thereby enhance performance.\textsuperscript{16,17} Hypromellose has been used as a polymer in the preparation of mucoadhesive microspheres due to its favorable mucoadhesive properties, and is safe for human oral consumption.\textsuperscript{16–18} Hypromellose and hypromellose phthalate are compatible with \textit{B. coagulans}\textsuperscript{19} and possess aqueous solubility.\textsuperscript{20}

In the context of the above principles, there is a need to develop a dosage form that will deliver \textit{B. coagulans} into the gut for a prolonged period of time with adequate stability during storage and GI transit. Thus, the present investigation attempted to prepare \textit{B. coagulans} loaded mucoadhesive microspheres, using several grades of hypromellose and subsequently enteric-coated with hypromellose phthalate, in order to deliver viable \textit{B. coagulans} into the gut for an extended period of time protecting against the harsh conditions of the GI tract and having an adequate shelf-life.

Materials and methods

Materials

Freeze dried \textit{B. coagulans} (powder) and hypromellose phthalate (HP-50) were gift samples from Glenmark Pharmaceuticals Limited (Sinnar, Nasik, India). Grades of hypromellose (hydroxypropylmethylcellulose or HPMC) namely Methocel E5 Premium LV, Methocel E15 Premium LV, and Methocel K15M Premium were received from Indoco Remedies Limited (Rabale, Mumbai, India). Glucose Yeast Extract (GYE), agar media, and other analytical grade laboratory chemicals were purchased from HiMedia Laboratories Pvt Limited (Mumbai, India).

Methods

In-house compliance test of \textit{B. coagulans}

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

Preparation of mucoadhesive microspheres

Core \textit{B. coagulans} loaded mucoadhesive microspheres were prepared with hypromellose, employing coacervation and phase separation technique.\textsuperscript{16,17} Briefly, hypromellose (2.5 g) was dissolved in 100 mL of cold de-ionized (DI) water (4 ± 2°C). One hundred mg of tween-80 was dissolved in the above solution with stirring. Resultant solution was filtered aseptically using 0.45 µm PVDF filter membrane (Millipore, Bedford, MA). A calculated quantity of \textit{B. coagulans} was dispersed in the above solution, under stirring. The temperature of the resultant dispersion was raised gradually up to 30 ± 2°C with stirring at 500 ± 25 revolutions per minute (rpm) for 30 minutes. Acetone (25 mL) was added drop-wise with stirring at 300 ± 25 rpm for 10 minutes. Microspheres thus obtained were filtered aseptically with 10 µm nylon filter (NY10; Millipore), washed twice with sterile water for injection (30 ± 2°C) and then kept in a desiccator for 24 hours. The entire process was carried out aseptically on a bench of horizontal laminar flow clean air work station (1500048-24-24; Klenzaids Bioclean Devices (P) Ltd, Mumbai, India). All formulation batches were prepared in triplicate following the above method to verify reproducibility and their composition is summarized in Table 1.

Coating of microspheres

One hundred mL of hypromellose phthalate solution (10% w/w) was prepared by dissolving HP-50 in phosphate buffer pH-6.8\textsuperscript{21} with stirring. Two g of polyethylene glycol 200 (PEG-200) and 100 mg of tween-80 was added to it with stirring. The above solution was filtered aseptically using a 0.45 µm PVDF filter membrane. Core microspheres were dispersed in the above solution with stirring at 300 ± 25 rpm followed by drop-wise addition of 20 mL of propan-2-ol. Stirring was continued for 30 minutes. Coated microspheres, thus obtained, were filtered and washed thrice with sterile
Table 1  Formulation formula and percent yield value and entrapment efficiency value of all formulation batches

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Polymer used</th>
<th>B. coagulans to polymer ratio</th>
<th>Percent yield (w/w)*</th>
<th>Entrapment efficiency (% w/w)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Methocel E5</td>
<td>1:1</td>
<td>68.1 ± 3.12</td>
<td>85.36 ± 2.23</td>
</tr>
<tr>
<td>F2</td>
<td>Premium LV</td>
<td>1:2</td>
<td>66.6 ± 3.09</td>
<td>78.41 ± 1.76</td>
</tr>
<tr>
<td>F3</td>
<td>Premium</td>
<td>1:3</td>
<td>64.4 ± 3.18</td>
<td>72.22 ± 1.76</td>
</tr>
<tr>
<td>F4</td>
<td>Methocel E15</td>
<td>1:1</td>
<td>65.3 ± 3.59</td>
<td>78.00 ± 2.31</td>
</tr>
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<td>F5</td>
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<td>63.4 ± 3.67</td>
<td>74.92 ± 1.91</td>
</tr>
<tr>
<td>F6</td>
<td>Methocel K15M</td>
<td>1:3</td>
<td>62.1 ± 3.24</td>
<td>70.68 ± 1.79</td>
</tr>
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<td>F7</td>
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</tr>
<tr>
<td>F8</td>
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Note: *Data are presented as mean value ± SE, n = 3.

Percent yield study

Percent yield (w/w) of microspheres was calculated employing equation-1.

\[
\text{Percent yield} = \frac{W_1}{W_2} \times 100 \quad (1)
\]

Calibration curve of B. coagulans

Several standard concentrations of dispersions containing B. coagulans were prepared with the sterile simulated gastric fluid TS and the sterile simulated intestinal fluid TS separately. The respective absorbance optical density (OD) was measured at 600 nm with respect to their respective blank, employing UV/Vis spectroscopic method (UV-1700; Shimadzu, Kyoto, Japan), so as to get a calibration curve that relates the OD values to cell concentration. When a standard B. coagulans dispersion was analyzed repeatedly (n = 3), the mean error (accuracy) was found to be 0.98% and relative standard deviation (precision) was found to be 1.5%, indicating validity of the method for linearity, accuracy, and precision. The OD test is the best way to enumerate roughly the total number of cells (viable plus nonviable) present in the sample and helps to determine the dilution factor for performing viable spore count but fails to reveal cfu, the unit for expressing the number of viable cells. The simulated gastric fluid TS was liquefied and cooled to 45°C in a water bath. One mL of sample from the heat-treated final dilution tube was transferred into each sterile petri dish (six per sample) followed by pouring 15 mL of molten medium and mixing and then incubating in an inverted position at 40°C for 48 hours after solidification.

Counting

Six plates were counted and the average count per plate was calculated. The number of cfu per unit (mL or gram) of sample was calculated by employing equation-2.

B. coagulans-loaded hypromellose mucoadhesive microspheres

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Number of cfu
= \frac{\text{Average number of colonies counted per plate}}{\text{Dilution factor}} \quad (2)

Entrapment efficiency study
Two hundred mg of accurately weighed microspheres were transferred aseptically into a sterile glass vial containing 10 mL of sterile simulated intestinal fluid TS, sealed hermetically and kept at 4 ± 2°C for 24 hours. The dispersion was subjected to a viable B. coagulans spore count and the entrapment efficiency value was calculated using equation-3 and is reported in percent.

Entrapment efficiency = \frac{\text{Practical spore count}}{\text{Theoretical spore count}} \times 100 \quad (3)

In vitro swelling study
An in vitro swelling test of microspheres was conducted in simulated intestinal fluid TS. The size of dried microspheres and those after incubation in simulated intestinal fluid for 5 hours were measured using a calibrated optical microscope (CX III; Labomed, Ambala, India). Percent swelling value was determined from the diameter of microspheres at time t (Dt) and initial time t = 0 (D0) using equation-4.23

Percent swelling = \frac{[D_t - D_0]}{D_0} \times 100 \quad (4)

Morphological examination of microspheres
Coated microspheres were mounted on the aluminum stubs using double-sided adhesive tape. The stubs were then vacuum-coated with a thin layer of gold and examined with a scanning electron microscope (JSM 5610 LV; Jeol, Tokyo, Japan).16

Particle size, size distribution, and zeta potential study
The core and the coated microspheres were dispersed in DI water (pH 6.8) and sonicated for 2 minutes to get a homogenous dispersion (0.5% w/v). The resultant dispersions contained in small volume disposable zeta cells were subjected to particle size study using photon correlation spectroscopy with in-built Zetasizer (Nano ZS; Malvern Instruments, Worcestershire, UK) at 633 nm and 25 ± 0.1°C. Measured electrophoretic mobility (mm/s) is converted to zeta potential by in-built software based on the Helmholtz–Smoluchowski equation.24

\text{Percent adhesive strength} = \frac{N_v}{N_w} \times 100 \quad (8)

In vitro wash-off test
Freshly excised pieces of intestinal mucosa (2 cm × 2 cm) from sheep were mounted onto a glass slide (8 cm × 3 cm) with cyanoacrylate glue. Coated microspheres (100 numbers) were accurately counted and were spread onto wet rinsed intestinal mucosa tissue followed by hanging it onto one of the groves of a USP tablet disintegration test apparatus (DT 1000; Labindia Instruments Pvt Ltd, Mumbai, India), with continuous oxygen supply. The apparatus was operated giving tissue specimen regular up-and-down movements within the beaker of disintegration test apparatus, containing
In vitro release study
The in vitro release profile of *B. coagulans* from coated microspheres was studied using a USP basket apparatus (TDT-06T; Electrolab, 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*. About 500 mg of accurately weighed coated microspheres was placed in a basket (wrapped with 100 mesh nylon cloth) of the dissolution apparatus. Five mL of dissolution medium was withdrawn at a predetermined time interval up to 18 hours followed by immediate replacement with an equal volume of fresh dissolution medium. After suitable dilution samples were subjected for replacement with an equal volume of fresh dissolution medium. The model-independent mathematical approach is employed to compare the dissolution profile using three factors: *f*₁, equation-9; *f*₂, equation-10; and Rescigno index (*ξ*), equation-11. According to the nature of measurement, *f*₁ was described as the difference factor and *f*₂ as the similarity factor and are determined by employing cumulative data. The factor *f*₁ is proportional to the average difference between the two profiles and indicates the percent error between two curves over all time points, whereas factor *f*₂ is inversely proportional to the average squared difference between two profiles and is a logarithmic transformation of the sum-squared error of difference between the test *Tₙ* and reference products *Rᵢ*. Rescigno index (*ξ*₁ and *ξ*₂), a dimensional index, refer to area differences for noncumulative data and refer to the difference between the dissolved amount of the test and reference product in a given time interval.

\[
f₁ = \frac{\left[ \sum_{i=1}^{n} \left( R_i - T_i \right) \right]}{\left[ \sum_{i=1}^{n} R_i \right]} \times 100 \tag{9}
\]

\[
f₂ = 50 \times \log \left[ {1 + \left( 1/n \right) \sum_{i=1}^{n} \left( R_i - T_i \right)^2} \right]^{0.5} \times 100 \tag{10}
\]

\[
ξ₁ = \frac{\left[ \sum_{i=1}^{n} \left| d_T(t) - d_R(t) \right| \right]^{1/2}/\left[ \sum_{i=1}^{n} \left| d_T(t) - d_R(t) \right|^2 \right]^{1/2}} \tag{11}
\]

Note that *Rᵢ* is the cumulative percentage dissolved from the reference product; *Tᵢ* is from the test product at each of the selected time points, *n*, of the test and the product; *dᵢ* is the test product dissolved amount; *dᵢ* is the reference product dissolved amount at each time point; and *i* is any positive integer number.

Model-dependent methods
Model-dependent mathematical approaches including zero order, first order, Hixson–Crowell and Weibull model, as listed in Table 2, were applied considering the amount of viable cell release as a function of test time. The following plots were then plotted: cumulative percent viable cell release vs time (zero order kinetic model), log cumulative percent viable cell release vs time (first order kinetic model), cube root of percent drug remaining in matrix vs time (Hixson–Crowell cube root), and logarithm of the dissolved amount of drug vs the logarithm of time (Weibull model). Higuchi model was not considered as the prepared microspheres fail to comply to the assumptions of this model.
developmental processes of enteric-coated microsphere was carried out below 20°C in aqueous medium. Hypromellose is soluble in cold water and its solubility in water decreases with increase in temperature and is insoluble in organic solvents such as chloroform, dichloromethane, ether, and acetone. Hypromellose phthalate is soluble in aqueous alkali and is insoluble in water and propan-2-ol. Hypromellose as a mucoadhesive polymer and hypromellose phthalate as a coating polymer were selected due to their abovementioned properties.

In vivo probiotic activity evaluation

An in vivo probiotic activity evaluation of coated microspheres was performed using the mouse model Enterococci stool colonization method described by Donskey et al following institutional animal ethical committee guidelines. One mL of coated microspheres dispersion (10^2 cfu/mL), in simulated intestinal fluid TS, was orally fed to mice (in a group of six) using an oral feeding needle. The stools were collected periodically at six hour intervals up to 48 hours and subjected for enterococci colonization density study.

Accelerated stability study of microspheres

Following ICH guidelines, formulation batches of microspheres were stored at several conditions of temperature and humidity (30 ± 2°C/65 ± 5% RH and 40 ± 2°C/75 ± 5% RH) in a stability analysis chamber (Darwin Chambers Company, St Louis, MO) and in a refrigerator (2–8°C) for a period of 12 weeks. The samples were subjected for viable B. coagulans cell content, color, and texture analysis at 2-week intervals. These results were compared with those of the initial results (results of analysis of samples prior to stability charging) and control samples kept at 2–8°C. The absorption of any statistically significant decrease in viable B. coagulans cell content indicates product stability with the excipient and at the storage condition.

Results and discussion

The coacervation and phase separation technique described here appears to be a suitable method for the preparation of enteric-coated hypromellose microspheres loaded with B. coagulans. It is a two-step process, simple and less time consuming, and eliminates the exposure of B. coagulans to high temperatures, organic solvents, and mechanical stress, thereby maintaining their viability during processing. Temperatures above 20°C and nonaqueous solvents adversely affect and decrease the viability of B. coagulans, thus the

Table 2 Mathematical models used to describe dissolution curves

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero order</td>
<td>( Q_t = Q_i + K_t t )</td>
</tr>
<tr>
<td>First order</td>
<td>( \ln W_i = \ln W_0 + K_t t )</td>
</tr>
<tr>
<td>Hixson–Crowell</td>
<td>( Q_t = Q_i \left(1 - e^{-K_t t}\right) )</td>
</tr>
<tr>
<td>Weibull</td>
<td>( \log[\ln(1-m)] = b \log(t-T) - \log c )</td>
</tr>
</tbody>
</table>

Notes: Where \( Q_i \) is the initial amount of drug in the pharmaceutical dosage form, \( Q_t \) is the amount of drug in the pharmaceutical dosage form at time \( t \), \( W_i \) is initial amount of drug in the dosage form, \( W_t \) is the amount of drug released in time \( t \), and \( K \) is the zero order proportionality constant, and \( K_t \) is the first order release rate constant, \( K_0 \) is a constant incorporating the surface-volume relation, \( m \) is the accumulated fraction of the drug in solution at time \( t \), \( c \) is the time scale of the process, \( T \) is the location parameter represents the lag time before the onset of the dissolution or release process, and \( b \) is the shape parameter.
Table 3  Percent swelling, mean particle size, zeta potential, and percent adhesive strength values of microspheres of all formulation batches

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Percent swelling</th>
<th>Mean particle size (μm)</th>
<th>Zeta potential (mV) of uncoated microspheres</th>
<th>Percent adhesive strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.23 ± 0.61</td>
<td>26.61 ± 1.15</td>
<td>−15.86 ± 0.45</td>
<td>83.1 ± 1.23</td>
</tr>
<tr>
<td>F2</td>
<td>1.28 ± 0.56</td>
<td>27.03 ± 1.34</td>
<td>−15.61 ± 0.92</td>
<td>83.4 ± 1.35</td>
</tr>
<tr>
<td>F3</td>
<td>1.36 ± 0.59</td>
<td>27.13 ± 1.64</td>
<td>−16.01 ± 0.81</td>
<td>82.8 ± 1.37</td>
</tr>
<tr>
<td>F4</td>
<td>1.10 ± 0.50</td>
<td>33.45 ± 1.58</td>
<td>−16.17 ± 0.65</td>
<td>78.1 ± 1.17</td>
</tr>
<tr>
<td>F5</td>
<td>1.14 ± 0.54</td>
<td>34.24 ± 1.71</td>
<td>−16.06 ± 0.83</td>
<td>78.9 ± 1.24</td>
</tr>
<tr>
<td>F6</td>
<td>1.19 ± 0.59</td>
<td>32.26 ± 1.39</td>
<td>−15.97 ± 0.67</td>
<td>78.5 ± 1.41</td>
</tr>
<tr>
<td>F7</td>
<td>0.85 ± 0.34</td>
<td>48.40 ± 2.32</td>
<td>−13.67 ± 0.87</td>
<td>72.6 ± 1.36</td>
</tr>
<tr>
<td>F8</td>
<td>0.87 ± 0.46</td>
<td>48.95 ± 2.24</td>
<td>−13.45 ± 0.53</td>
<td>73.8 ± 1.16</td>
</tr>
<tr>
<td>F9</td>
<td>0.89 ± 0.41</td>
<td>49.01 ± 2.19</td>
<td>−13.28 ± 0.41</td>
<td>72.3 ± 1.14</td>
</tr>
</tbody>
</table>

Note: Data are presented as mean value ± SE, n = 3.

the grade of hypromellose. The highest mean particle size value was observed with formulations containing Methocel K15M Premium. The effect of the grade of hypromellose on the mean particle size value follows the order of Methocel K15M Premium > Methocel E15 Premium LV > Methocel E5 Premium LV (Figure 2 and Table 3). Variation in B. coagulans-to-hypromellose ratio does not influence the mean particle size value of formulation batches. The zeta potential value (Table 3) of core microspheres was negative as expected due to the methoxy group of hypromellose. Core microspheres prepared with the Methocel E15 Premium LV and the Methocel E5 Premium LV possessed nearly the same zeta potential value as both have the same ratio of methoxy and hydroxypropoxy groups, and are both higher than that prepared with Methocel K15M Premium. This may be due to an increase in the methoxy content of hypromellose polymer. A nearly constant negative zeta potential value (−7.66 ± 0.87) was observed with coated microspheres of all formulation batches, which is lower than that of uncoated microspheres, confirms the presence of hypromellose phthalate on the surface of microspheres. The zeta potential report of uncoated microspheres from formulation-F1 is presented in Figure 3. The flow properties of formulation batches lie between the passable and very poor range.

The percent adhesive strength value of all formulation batches, as a measure of ex vivo mucoadhesive strength test, lies between 72.3% and 83.1%, and an increase in B. coagulans-to-hypromellose ratio does not affect it (Table 3). The highest percent adhesive strength value was observed with Methocel E5 Premium LV and the effect of the grade of hypromellose on percent adhesive strength value follows the order of Methocel E5 Premium LV > Methocel E15 Premium LV > Methocel K15M Premium. The in vitro wash-off test results (Table 4), as a measure of mucoadhesive efficiency, reveals that even after 12 hours, some of the microspheres remained adhered to the intestinal mucosa indicating that microspheres possess strong mucoadhesion affinity for the intestinal mucosa. The above experimental results suggest that mucoadhesion properties of microspheres vary with differences in the grade of polymer and does not vary with differences in the B. coagulans-to-hypromellose ratio. Microspheres possessing strong mucoadhesion affinity
for intestinal mucosa may thus be retained in the intestinal tract for an extended period of time.

In simulated gastric fluid \( TS \), the amount of \( B.\ coagulans \) released from the coated microsphere system is negligible, while in simulated intestinal fluid \( TS \), \( B.\ coagulans \) release is almost regulated and extended within the investigational period of 18 hours (Figure 4). This indicates that enteric-coating of microspheres has been competently protecting the viability of \( B.\ coagulans \) in gastric pH, preventing the release of \( B.\ coagulans \) in gastric pH, and releasing \( B.\ coagulans \) in the intestinal pH.

The model independent (pair wise approach) release exponents \((f_1, f_2, \xi_1\) and \(\xi_2\)) values are listed in Table 5. Normally, regulatory authorities have set a public standard of \( f_1 \) values between 1 and 15, and \( f_2 \) values between 50 and 100 to indicate similarity between two dissolution profiles, while the \( \xi_1 \) and \( \xi_2 \) indices always present values between 0 and 1 inclusive, where an index value of 0 denotes two identical release profiles, and a value of 1 denotes when the drug from either the test or the reference is not released at all.\(^{26,27}\) Data from Table 5 reveal that, for all formulation pairs, the \( \xi_1 \) values lie between 0.06 and 0.24, and \( \xi_2 \) values lies between 0.14 and 0.32 (ie, above 0 and below 1), and the \( f_1 \) value lies between 16.85 and 42.60 (ie, above 15), and for almost all formulation pairs, the \( f_2 \) value lies between 27.54 and 50.00 (ie, below 50) indicating dissimilarity in product performance of the formulation batches when compared to each other, ie, intrapolymer (having the same grade of hypromellose but differing from each other by \( B.\ coagulans \)-to-hypromellose ratio) and interpolymer (having the same

### Table 4 Results of in vitro wash-off test of all formulation batches

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Percent microspheres adhering to tissue at time points (in hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>F1</td>
<td>62 ± 1.6</td>
</tr>
<tr>
<td>F2</td>
<td>63 ± 1.2</td>
</tr>
<tr>
<td>F3</td>
<td>69 ± 2.2</td>
</tr>
<tr>
<td>F4</td>
<td>71 ± 2.1</td>
</tr>
<tr>
<td>F5</td>
<td>72 ± 1.9</td>
</tr>
<tr>
<td>F6</td>
<td>73 ± 2.0</td>
</tr>
<tr>
<td>F7</td>
<td>89 ± 2.1</td>
</tr>
<tr>
<td>F8</td>
<td>86 ± 1.8</td>
</tr>
<tr>
<td>F9</td>
<td>85 ± 1.5</td>
</tr>
</tbody>
</table>

**Note:** Data are presented as mean value ± SE, \( n = 3 \).
The mechanism of *B. coagulans* release from the microspheres follows the zero-order kinetic model, since the plot of cumulative percent viable cell release versus time were found to be linear, and have a highest regression coefficient ($r^2$) value in comparison to that of the first order, Hixson–Crowell, and Weibull model when compared with intraformulation batches. For all formulation batches, the zero-order kinetic model $r^2$ value lies between 0.9820 and 0.9978, while the zero-order kinetic constant value lies between 2.3708 and 5.5517. In Weibull model, the shape parameter characterizes the curve as either exponential ($\beta = 1$, case-1), sigmoid or S-shaped with upward curvature followed by a turning point ($\beta > 1$, case-2), or parabolic with a higher initial slope, and after that consistent with the exponential ($\beta < 1$, case-3), while the location parameter ($T_d$) characterizes the time interval necessary to dissolve or release 63.2% of the drug present in the pharmaceutical dosage form, determined by employing relation $\alpha = (T_d)^{\beta}$ from the shape parameter ($\beta$) and scale parameter ($\alpha$). Study of shape parameter values of the Weibull model from Table 6 reveal that the curve is sigmoid or S-shaped with upward curvature followed by a turning point ($\beta > 1$), while the $T_d$ value of all formulation batches lies between 9.31 and 30.06.

A plot showing the viable *B. coagulans* release profile following the zero-order kinetic model of all formulation batches is shown in Figure 4, and reveals that an increase in the *B. coagulans*-to-polymer ratio significantly decreases the rate of viable *B. coagulans* release from microspheres while variation in the grade of hypromellose influences the rate of viable *B. coagulans* release from microspheres following...
the order of Methocel E5 Premium LV > Methocel E15 Premium LV > Methocel K15M Premium.

In vivo probiotic activity evaluation showed that oral administration of coated microspheres of *B. coagulans* from all formulation batches resulted in statistically significant reductions in the density of enterococci colonization in the stool of Albino mice from 24 hours to 36 hours. Satisfactory reductions in the density of enterococci colonization in the all formulation batches resulted in statistically significant administration of coated microspheres of Methocel K15M Premium.

Prepared in vivo probiotic activity in the mouse model showed that the prepared *B. coagulans* loaded microspheres have potential for follow-up studies to verify its potentiality for human use.

The stability results reveal that prepared *B. coagulans* loaded microspheres exhibit adequate stability at the storage condition of 30 ± 2°C/65 ± 5% RH, as no statistically significant decreases in viable *B. coagulans* cell content were observed, and also signifies that the *B. coagulans* cells are 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 was observed, indicating product instability at this storage condition.

Formulation F1 (containing *B. coagulans*-to-Methocel E5 Premium LV with ratio of 1:1) was found to be superior to the other prototype formulations with respect to product performance, as it exhibited the highest entrapment efficiency, highest mucoadhesion efficiency, the ability to protect the viability of *B. coagulans* cells during storage and GI transit, and releases viable *B. coagulans* cells in the gut for an extended period of time, as shown via zero-order kinetics.

### Conclusion

Experimental results suggest that enteric-coated hypromellose microspheres loaded with *B. coagulans* could be prepared by the conventional cocaverection and phase separation method, and have the potential to deliver viable *B. coagulans* cells into the gut for an extended period of time, while maintaining the viability of *B. coagulans* during storage and GI transit, and could be viewed as an alternative to conventional dosage forms. However, extensive in vivo studies are required to establish the use of mucoadhesive microspheres as an alternative to conventional dosage forms of *B. coagulans*.

### Acknowledgments

The author wishes to thank Glenmark Pharmaceuticals Limited, Sinnar, Nasik, India, for the sample of freeze dried *B. coagulans* and hypromellose phthalate (HP-50). Words are insufficient to express my gratitude to Indoco Remedies Limited, Rabale, Mumbai, India, for samples of used grades of methocel. The author will remain indebted to Jadavpur University, Kolkata, India, for support to complete this work.

### Disclosure

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

### References