Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/3thcaw03

CHARACTERIZATION OF BIOPOLYMERIC ENCAPSULATION SYSTEM FOR IMPROVED SURVIVAL OF BIFIDOBACTERIUM LONGUM BL-101

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Received: 04-06-2024 Accepted: 08-07-2024 Published: 17-08-2024

ABSTRACT
Human like collagen and sodium alginate were used to prepare microspheres for the effective delivery of *Bifidobacterium longum* BL-101 in humans and animals. The microspheres were prepared through the method of extrusion. High survival rate of probiotics was detected after 21 days of storage, while the release rate of encapsulated *B. longum* BL-101 was also higher than non-encapsulated. The survival of probiotics is crucial to deliver benefits to host (either human or animal). It was observed that encapsulated *B. longum* BL-101 exhibited greater survival rates in simulated gastric and intestinal conditions in contrast with non-encapsulated probiotics. However, the encapsulation efficiency of human like collages and sodium alginate microspheres was 93.2±1.2% when 2.5 % HLC and 1.5 % sodium alginate were used.

Keywords: Microencapsulation, Human like collagen, *Bifidobacterium longum* BL-101, Sodium alginate, Survival in simulated gastrointestinal conditions.

INTRODUCTION
Bifidobacteria are Gram positive bacteria that exist in the human gastrointestinal tract (GIT). They are regarded as important commensals for promoting healthy GIT in humans and animals, despite the fact that they are not empirically dominating (Schell *et al.*, 2002). Due to their documented health benefits, bifidobacteria have been combined with various functional foods including cheeses, yogurt and other milk products (Su *et al.*, 2011). Due to the obligate anaerobic characteristics of bifidobacteria, their survival is not high when exposed to oxygen and low pH (pH 2) of gastric juice. Their low survival rate limited the usefulness in humans and animals, however, various techniques including microencapsulation could be helpful to stabilize bacteria in gastrointestinal conditions (Georgieva *et al.*, 2014).

The incorporation of probiotics in veterinary sciences has gained significant importance as an alternative to antibiotics to improve animal health and productivity (Anee *et al.*, 2021). *B. longum* BL-101 is considered a probiotic because of its ability to improve gut microbiota and hinder the pathogenic bacteria in several animal species. However, the compromised efficiency of these bacteria in harsh gastrointestinal conditions is addressed with microencapsulation. Several benefits in

veterinary sciences could be offered by incorporating microencapsulated *B. longum* BL-101. The protective encapsulation ensures the effective colonization and activity in the intestine of animals and humans. The use of this type of probiotic formulation could meet the demands of antibiotic-free animal products for health concerns and consumer preferences (Yang and Zhenlong, 2023).

In this era, microencapsulation has been widely used as it protects the probiotics from the harsh environment (low pH) of the intestine. The effective microencapsulation process should maintain the viability of probiotics during storage and deliver them in the colon to promote their colonization on the surfaces of mucosa. Various methods of microencapsulation are being widely used including freeze drying, extrusion, emulsion, spray chilling and spray drying. β-1,4-D-mannuronic acid is present in alginate along with α-1,4-L-glucuronic acid. As alginate is pH sensitive material, it is successfully used in microencapsulation of probiotics. A gel structure is formed by sodium alginate after replacement of sodium ion with calcium (bivalent cation). This gel structure of alginate is firm at low pH and release the probiotics under mild alkaline conditions. Due to this property, gel structure of alginate is used to produce a controlled release carrier in the form of microcapsules (Yoo *et al.*, 2006).

Human-like collagen (HLC) is being used as hemostatic material, a novel biomaterial for blood vessel tissues, skin tissues, functional foods and artificial bones. It is generally expressed as recombinant *E. coli* BL21 as it contains cDNA of HLC. HLC is a fibrous protein that is water soluble and is a constituent of connective tissues. HLC participates in forming, supporting and protecting various bones and tissues. Other globular proteins require denatured treatment but HLC does not require one and could easily be dissolved into the alginate matrix (Su *et al.*, 2011). HLC is virus-free, biocompatible, and has shown excellent performance. The interaction of certain HLC groups with carboxyl groups of alginates is responsible for the easy integration of HLC into the alginate matrix. The incorporation of probiotics in veterinary sciences has gained significant importance as an alternative to antibiotics to improve animal health and productivity (Anee *et al.*, 2021). *B. longum* BL-101 is considered a probiotic because of its ability to improve gut microbiota and hinder the pathogenic bacteria in several animal species. However, the compromised efficiency of these bacteria in harsh gastrointestinal conditions is addressed with microencapsulation. Several benefits in veterinary sciences could be offered by incorporating microencapsulated *B. longum* BL-101. The protective encapsulation ensures the effective colonization and activity in the intestine of animals and humans. The use of this type of probiotic formulation could meet the demands of antibiotic-free animal products for health concerns and consumer preferences (Yang and Zhenlong, 2023).

The current work aims to determine the interaction of sodium alginate and human-like collagen for effective encapsulation to enhance the survival and stability of *Bifidobacterium longum* BL-101 through gastrointestinal conditions in humans as well as in animals. Various studies were conducted on the microencapsulation of bacteria but a fibrous protein like HLC was not used before that is easily soluble and provide protection to bacteria. For the preparation of microcapsules, the extrusion method was used. Furthermore, the physicochemical properties of microcapsules were also investigated including morphology of microcapsules, storage stability, release study, survival of *B. longum* BL-101 through simulated gastric fluid and simulated intestinal fluid along with encapsulation efficiency of microcapsules.

MATERIALS AND METHODS

Materials

Sodium alginate and HLC were bought from Sigma Chemical Co, located in USA. *Bifidobacterium longum* BL-101 was attained from the local market of Faisalabad, Pakistan. Pepsin and pancreatin were purchased from Sigma Aldrich (St. Louis, MO, USA). While MRS broth (De Man Rogosa and Sharpe) and MRS agar were obtained from Difco, Sparks, USA. Additionally, all other reagents and chemicals of analytical grade were obtained from Sigma Aldrich (St. Louis, MO, USA).

Activation and microencapsulation of *B. longum* BL-101

B. longum BL-101 were activated before encapsulation (Su *et al*., 2011). Sodium alginate (1.5%w/v) and HLC (1, 1.5, 2, 2.5% w/v) were used for microencapsulation. Alginate and HLC solutions were mixed with 5 mL of *B. longum* strain suspension with the volume ratio of 4 to 1. Droplets were formed through a conventional needle by using a syringe pump with a plastic syringe (5mL). The pressure was adjusted at 415 mbar along with the frequency at 420 Hz. A high voltage unit positive electrode was connected with the needle to create the electrostatic potential and $CaCl_2$ (2 % w/v) solution was used as the gelling bath. Beads were kept in $CaCl_2$ solution (2 % w/v) for 30 minutes for hardening, washed with distilled water and frozen for 2 hours at -72°C.

Table 1: Combination of wall materials for microencapsulation

Treatment	HLC (%)	Sodium alginate (%)
E_o	-	-
E_1	1	1.5
E_2	1.5	1.5
E_3	2	1.5
E_4	2.5	1.5

Storage stability and release study of *B. longum* BL-101

The stability during storage of non-encapsulated and encapsulated *B. longum* BL-101 was assessed for 28 days (0, 7, 14, 21 and 28 days) at 4ºC (Shi *et al*., 2013). The in-vitro release of *B. longum* BL-101 was in SIF (pH 7.4) at 37ºC and viable cells were counted after specific time intervals (0, 30, 60, 90 and 120 minutes) (Xu *et al*., 2016).

Survival of free and encapsulated *B. longum* BL-101 in simulated gastric juice (SGJ) and bile salt solution

SGJ (pH 2.0) and bile salt solution (pH 7.4) were prepared according to standard protocols (Rajam *et al*., 2012). Survival of bacteria was evaluated after pre-determined time interval (60 and 120 minutes) for SGJ and (0, 40, 80 and 120 minutes) for bile salt solution.

Encapsulation efficiency

The sterile sodium citrate solution (9.0 mL of 2.0 % w/v) of pH 7.0 was used to disintegrate the beads (Gebara *et al.*, 2013). Standard equation was used to calculate the encapsulation efficiency (Annan *et al*., 2008).

Statistical analysis

The experiments were performed in triplicates and the results presented in the current study were expressed as the mean value ± standard deviation (SD) (Montgomery, 2008).

RESULTS

Storage stability of *B. longum* BL-101

The storage stability was determined to investigate the outcome of microencapsulation on the viability of probiotics during storage (4ºC) and the consequences are presented in (Figure 1). The outcomes declared that encapsulated bacteria depicted greater survival rate after 21 days of storage even at 4ºC as compared to non-encapsulated bacteria. The viability of free cells was rapidly decreased (from 9.70±0.49 to 4.25±0.03 log CFU/ml) after 21 days of storage. However, the viability count in all the encapsulated bacteria was almost at the suggested therapeutic level 10^7 log CFU/ml. The least drop in viability was detected in E_4 (from 8.43±0.24 to 8.21±0.16 log CFU/ml), followed by E_3 (from 8.52±0.19 to 8.09±0.11 log CFU/ml) after 21 days of storage.

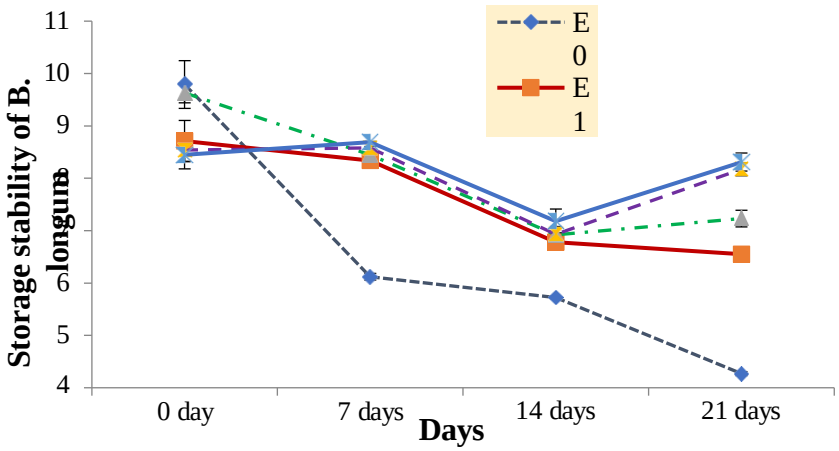

Figure 1. Storage stability of *B. longum* BL-10

Release study of *B. longum* BL-101

Another very important objective of encapsulation is the release of encapsulated bacteria in the intestine. For the provision of health benefits to the host, bacterial release should be made as soon as possible from the capsule. The release of *B. longum* BL-101 in SIF was observed (Figure 2) for 120 minutes and the release profile of various treatments was given in Figure 4. All the beads formulated by HLC and sodium alginate could release *B. longum* BL-101 to deliver benefits to the host. The release rate of bacteria was increased up to 10^8 log CFU/mL within 90 minutes and increased by 10^9 log CFU/ml in 120 minutes in the control group. Whereas the release rate of encapsulated *B. longum* BL-101 was lower and maximum release rate of up to 10^7 log CFU/ml after 120 minutes of incubation was observed. The best results for release rate were observed in E_3. As the HLC concentration was highest in E_3, more time was required for structural degradation in comparison with other treatment groups. It is concluded from these results that encapsulating material protects bacteria from adverse conditions and also influences the release profile of encapsulated *B. longum* BL-101.

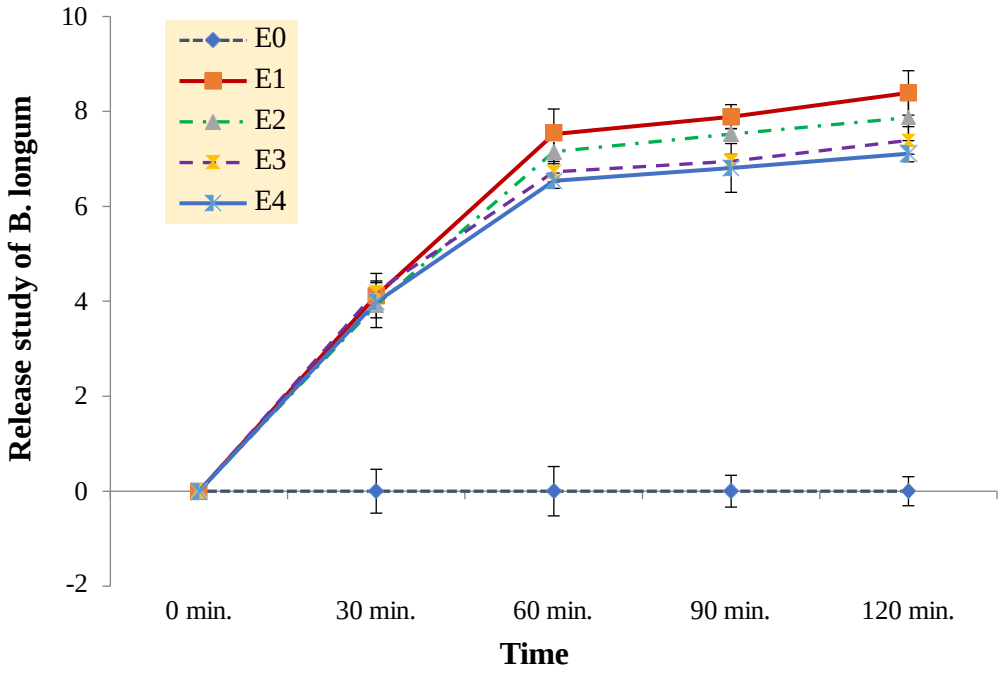

Figure 2. Release study of *B. longum* BL-101

Survival of free and encapsulated *B. longum* BL-101 in simulated gastric juice (SGJ)

Encapsulation with sodium alginate and HLC could meaningfully improve the survival of *B. longum* BL-101 in SGJ (Fig. 3). In SGJ, the viability of *B. longum* BL-101 was sustained for 30 minutes, after

that a rapid decline in viability was detected. Figure 5 declared that encapsulated *B. longum* presented resistance to SGJ in comparison with non-encapsulated cells. The maximum survival (6.30±0.07 log CFU/mL) was detected in E_4, followed by E_3 (6.14±0.04 log CFU/mL) after 120 minutes of incubation. While the maximum decline in viability was observed in E_o (1.85±0.05 log CFU/mL). It became clear from the study that SGF could easily enter into the free *B. longum*, while use of sodium alginate and HLC assisted in protection of *B. longum* as observed in E_2, E_3 and E_4.

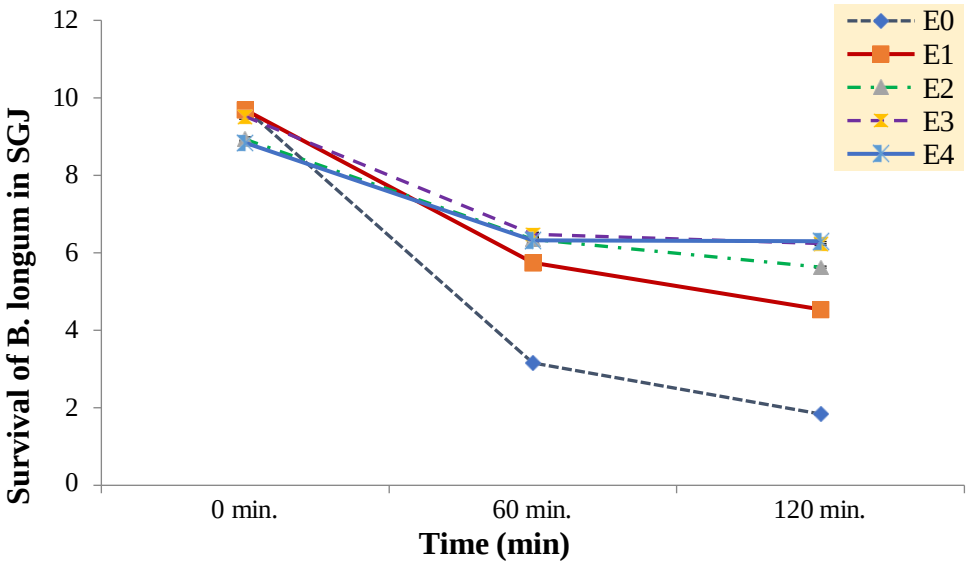

Figure 3. Survival of *B. longum* BL-101 in SGJ

Survival of free and encapsulated *B. longum* BL-101 in simulated intestinal fluid (SIF)

Survival of non-encapsulated and encapsulated *B. longum* BL-101 was observed during 120 minutes of exposure in bile salt. The procedure of encapsulation with sodium alginate and HLC could meaningfully improve the existence of *B. longum* BL-101 in bile salt (Fig. 4). The maximum survival (8.92±0.56 log CFU/ml) of *B. longum* BL-101 was detected in E_4, followed by E_3 (8.67±0.03 log CFU/mL) after 120 minutes of exposure. While the minimum survival was detected in E_o (5.21±0.31 log CFU/mL) in which non-encapsulated *B. longum* BL-101 were present. Figure 6 represented that all the encapsulated *B. longum* showed resistance to bile salt in comparison with non-encapsulated bacteria. It was unveiled from the outcomes that *B. longum* BL-101 was sensitive to bile salts but in all the encapsulated bacteria, the viable cell count was higher than 10^6 log CFU/ml which is the recommended level of bacteria for colonization in the intestine.

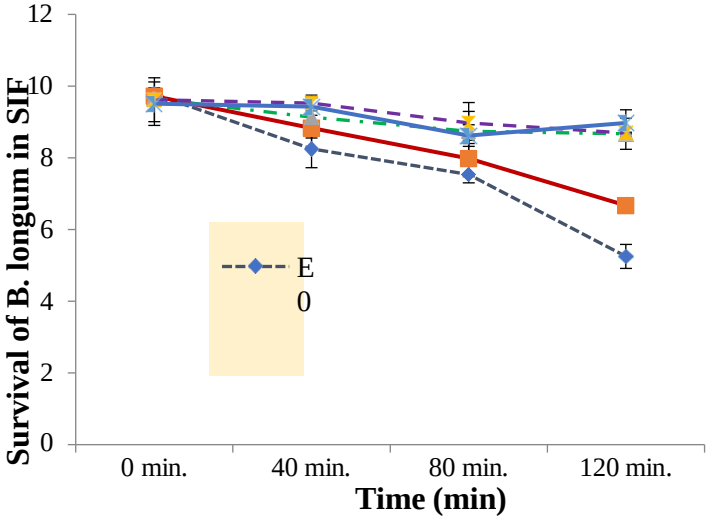

Figure 4. Survival of *B. longum* BL-101 in SIF

Encapsulation efficiency

Four different bead formulations were used and all presented encapsulation efficiency (EE) >85%. This efficiency is because of the wall materials; sodium alginate and HLC, which protect the bacterial cells. These wall materials protect the bacterial cell wall from disruption which increases the viability and encapsulation efficiency. The outcomes (Table 2) declared that there was a substantial alteration in the EE of various treatments and the highest encapsulation efficiency was observed in E_4 (92.8±1.1%), followed by E_3 (90.5±1.4%). All the formulations depicted high EE which shows the effectiveness of the encapsulation materials. Almost 10^7 CFU/g of viable cells were present in all the beads. It is advised that for colonization of probiotics, 10^7-10^9 CFU/g viable cells should be present.

Table. 4.2: Encapsulation efficiency

Treatment	Encapsulation efficiency (%)
E_1	85.4 ± 2.3
E_2	88.1 ± 1.7
E_3	90.5 ± 1.4
E_4	92.8 ± 1.1

Values are expressed as means ± SD

DISCUSSION

The storage stability of *B. longum* BL-101 was lowest in non-encapsulated group and increased with the increase in HLC content. Present results align with the literature as *B. longum* BL-05 were encapsulated with whey protein concentrate and the viability of non-encapsulated cells was lost after 28 days of storage in comparison with encapsulated cells (Yasmin *et al.*, 2019). *B. longum* BIOMA 5920 were encapsulated with sodium alginate and HLC, while number of non-encapsulated cells declined from 9.84 log CFU/ml to 5.97 log CFU/m (Su *et al*., 2011). *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* BB-12 were encapsulated with sodium alginate and pectin and the number of live bacteria after 30 days of storage in different treatments was meaningfully changed (Moghanjougi *et al.*, 2021). Similar results were presented for encapsulation of *B. longum* in alginate and dairy matrices (Prasanna and Charalampopoulos, 2018).

The release rate of *B. longum* BL-101 was affected with microencapsulation and highest in non-encapsulated group while decreased in groups in which microencapsulated probiotics were present. *B. longum* BIOMA 5920 were encapsulated with sodium alginate and HLC. Maximum number of bacteria was released within the first 30 minutes followed by gradual decline in the number of released bacteria (Su *et al*., 2011). *Lactobacillus bulgaricus* was microencapsulated with carrageenan-locust bean gum and maximum bacteria were released in first 45 minutes (Shi *et al*., 2013). Effect of inulin-alginate encapsulated beads on the viability of *Lactobacillus plantarum* was determined (Wang *et al*., 2016). Concentrate of whey protein and delivery system based on pectin-alginate was developed for better endurance of *B. longum* BL-05 (Yasmin *et al*., 2019). It was revealed that 70% probiotics were released during first 60 minutes after their exposure to SIF. The release was because of the swelling and erosion of polymer hydrogel when exposed to SIF. The release of probiotics from protein-based beads was faster due to the existence of protein digestible enzymes in the intestine which helps in collapsing the polymer matrix (Doherty *et al*., 2011).

Survival rate of *B. longum* BL-101 in simulated gastric juice was increased with microencapsulation. *B. longum* was encapsulated with alginate-dairy matrices and encapsulation protected the probiotics while passing through the harsh environment of the intestine (Prasanna and Charalampopoulos, 2018). The encapsulated probiotics experienced better protection than the free cells after 60 minutes of incubation (Chang *et al*., 2021). Extra protection for *Bifidobacterium bifidum* F-35 was provided by the alginate-pectin blend due to the formation of stronger gel at low pH (Zou *et al.*, 2011). The survival rate of *B. longum* encapsulated with calcium alginate beads was greater than the non-encapsulated cells in SGJ (Lee and Heo, 2000).

Survival of *B. longum* BL-101 in intestinal fluid was increased in microencapsulated groups. *B. longum* LMG 13197 was encapsulated in the vegetal and vegetal-inulin matrix. Number of non-encapsulated bacteria was declined after exposure to SIF with a loss of 5.890 log units after 6 hours of exposure (Amakiri and Mapitsi, 2016). Supercritical CO_2 interpolymer complexes were developed to improve the survival of *B. longum* Bb-46 in SGF and there was a minor reduction in viable count because of microencapsulation (Thantsha *et al.*, 2009). Pectin gave better protection to *B. longum* BL-05 as compared to whey protein concentrate in microencapsulation (Yasmin *et al*., 2019). Capsule was fractured upon exposure to intestinal fluid and after 120 minutes, there was a rise in the release of bacteria (Kataria *et al.*, 2018).

Microencapsulation of *L. plantarum* with alginate, fenugreek and psyllium was conducted and the efficiency was increased by 99% (Haghshenas *et al.*, 2015). While microencapsulation of *L. acidophillus* with sodium alginate and calcium sources enhanced the yield. Encapsulation efficiency was also increased with the increased concentration of HLC (Su *et al.*, 2011).

CONCLUSION

Microspheres of human like collagen and sodium alginate were prepared with the method of extrusion. These materials formed intermolecular hydrogen bonding and improved stability of beads. Different formulations were used for beads preparation and the best results were presented by the treatment group in which 1.5 % sodium alginate and 2.5 % human like collagen were used. The best treatment group also gave the higher encapsulation efficiency and greater survival of *B. longum* BL-101 through the gastrointestinal conditions. Microspheres prepared from alginate and HLC have the potential to be used as a delivery system of bioactive compounds' oral administration in both humans and animals.

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