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Design of macrocapsules to improve bacterial viability and supplementation with a probiotic for young calves

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ABSTRACT

The gastrointestinal tract of calves is sterile at birth, and intestinal microorganisms are introduced from fecal, vaginal and environmental microbiota. The balance of the intestinal ecosystem of calves can be altered in farming systems due to separation from their mothers, feeding with milk replacers and elimination of the benefits of cows' milk, inadequate colostrum intake, stressful situations and use of antibiotics. Such practices may cause morbidity and mortality of young calves which can be related to economic losses. Periodic administration of a probiotic inoculum of bovine origin may favor establishment of a stable and balanced intestinal microbiota, which would improve the health of the calves. The viability and number of microorganisms inoculated is vital because the suggested minimum level (SML) of bacteria to produce beneficial effects is 10^6 CFU/ml. A technique that is currently being implemented to maintain the viability of probiotics is encapsulation, which consists of retaining the microorganisms within a porous gel matrix or within a semipermeable membrane containing a liquid core. In our study, we describe a new technique to produce alginate-starch macrocapsules, with the aim of producing probiotic macrocapsules to ensure bacterial viability during storage, and to facilitate administration of the inoculum to young calves with feed. To this end, we used the strain *Lactobacillus casei* DSPV 318 T, a probiotic inoculum of bovine origin, and it was evaluated by two formulations for conformation of the capsules: one of sodium alginate (10 g/l) and another of sodium alginate (5 g/l) + corn starch (5 g/l). These mixtures were dispersed into molds of 1 and 2 ml, placed at −20 °C, and, once frozen, submerged in a solution of $CaCl_2$ (0.1 M) for polymerization of alginate to maintain their shape and size. The capsules containing of 5 g/l of alginate +5 g/l of starch had the highest cellular count, and the incubation of the capsules in culture media for 9 h increased the bacterial concentration. Viability of cells was maintained at the SML for 2 mo by coating the capsules with chitosan and refrigerating at 4 °C. This was reflected in a final product with a high concentration of probiotic accessible for artificial rearing of calves, with a sufficiently long expiration time, and with a size similar to the feed starter pellet, which allowed it to be mixed homogeneously with the feed which was fed to the calves.

Abbreviations: SML, suggested minimum level; TCAI, trichloroacetic acid index.

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1. Introduction

The gastrointestinal tract of calves is sterile at birth, and intestinal microorganisms are introduced from fecal, vaginal and environmental microbiota. The impact of the intestinal microbiota is critical to host nutritional status and is of particular interest in farm animals that are reared in intensive systems (Rosmini et al., 2004) because the balance of the intestinal ecosystem can be altered by farming systems. This can be due to separation from their mothers, feeding with milk replacers and elimination of the benefits of cows' milk, inadequate colostrum intake, stressful situations and use of antibiotics. Such practices may cause morbidity and mortality of young calves which can be related to economic losses.

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). Administration of a probiotic inoculum of bovine origin may favor establishment of a stable and balanced intestinal microbiota which would improve calf health (Abe et al., 1995). To produce a beneficial effect, administration of the inoculum must be continuous because the inoculated strains leave the intestinal tract. The strain is selected taking into account its benefits for the host through *in vitro* and *in vivo* studies of its probiotic properties. The technological features of the strain were also evaluated, because they should demonstrate possible production of these cultures, and their stability and survival during storage (Dunne et al., 2001). The viability and number of microorganisms inoculated is vital because the suggested minimum level (SML) of bacteria to produce beneficial effects is 10^6 CFU/ml (Vinderola et al., 2000).

Encapsulation is currently being implemented to maintain the viability of probiotics. This consists of retaining the microorganisms within a porous gel matrix or within a semipermeable membrane containing a liquid core (Dembezynski and Jankowski, 2002). Coating increases survival of the cells by protecting them from the adverse effects of the surrounding environment (Doleyres and Lacroix, 2005), and protects bacteria from damage by subsequent processes such as drying of the microcapsules for storage at ambient temperatures (Champagne and Gardner, 2001). Microencapsulation provides advantages such as higher resistance to simulated gastric and intestinal conditions (Lian et al., 2003), biomass protection against possible contaminants (especially bacteriophages), and a decrease in production costs because of separation techniques such as centrifugation and filtration are not necessary to concentrate the bacteria in the culture medium (Dembezynski and Jankowski, 2002). Microcapsules also offer protection against oxygen for strict anaerobes.

When this immobilization technology is applied to bacteria added to food for humans, the disadvantage of producing a pearl size small enough to be imperceptible to the palate must be overcome. However, animal probiotic formulations, such as capsules, pills and granules (O'Mahony et al., 2009; Soto et al., 2009), have the most appropriate size for the animal to be inoculated. Production of macrocapsules of a size similar to that of the feed starter pellet may allow bacterial preservation, and may facilitate its administration to calves with feed.

Our aim was to produce probiotic macrocapsules to ensure bacterial viability during storage and to facilitate administration of the inoculum to young calves with their feed.

2. Materials and methods

2.1. Microorganism

The strain *Lactobacillus casei* DSPV 318 T, a probiotic inoculum of bovine origin, was used (Frizzo et al., 2010a,b; Soto et al., 2009). Its 16S rDNA gen sequence accession number in Genbank is FJ787305.

2.2. Biomass production

The culture medium consisted of skim milk powder (100 g/l), glucose (20 g/l), Mg (0.2 g/l), Mn (0.05 g/l) and casein hydrolysate (50 ml/l; prepared in the laboratory with 100 g/l skim milk and by digestion with neutrasa for 118 min with a final TCAI of 54.8). Sterilized medium was inoculated at 10 ml/l with an overnight culture of *L. casei* DSPV 318 T and incubated at 37 °C for 10 h. After incubation, EDTA (0.5 M) was added in a ratio 1/1 (mol of EDTA/mol of Ca^{2+} in milk, respectively) to obtain a clarified culture (Yanes et al., 2002).

2.3. Production of capsules

Two sterile solutions, one of sodium alginate (20 g/l, Aldrich™, catalog number 180947, St. Louis, MO, USA) and another of sodium alginate (10 g/l) + corn starch (10 g/l, food grade, Glutal S.A. Esperanza, Santa Fe, Argentina), were prepared (Bajpai and Sharma, 2004; Muthukumarasamy et al., 2006; Homayouni et al., 2008). Each solution was mixed with an aliquot of clarified culture in a proportion of 1:1 (Champagne and Gardner, 2001). These cultures reached final concentrations of 10 g/l of alginate and 5 g/l of alginate + 5 g/l of starch, were dispersed into molds (Doleyres and Lacroix, 2005) of 1 and 2 ml and then placed at −20 °C for 8 h. Once frozen, the molds were submerged in boiling water for 3 s to separate the capsules from the mold walls. The cap of the mold was removed and the capsules were placed into a 0.1 M $CaCl_2$ solution (Bajpai and Sharma, 2004; Muthukumarasamy et al., 2006). The capsules were left to stand for 1 h in this solution for polymerization of alginate, maintaining their shape and size, and then they were recovered in a sterile Buchner filter (Fig. 1). Bacterial viability was determined by the different types of capsules as they were dissolved by placing them in a sodium citrate solution (10 g/l, 1 capsule/50 ml) until disintegration (Champagne and Gardner, 2001). Serial decimal dilutions (*i.e.*, successive 1/10 dilutions,

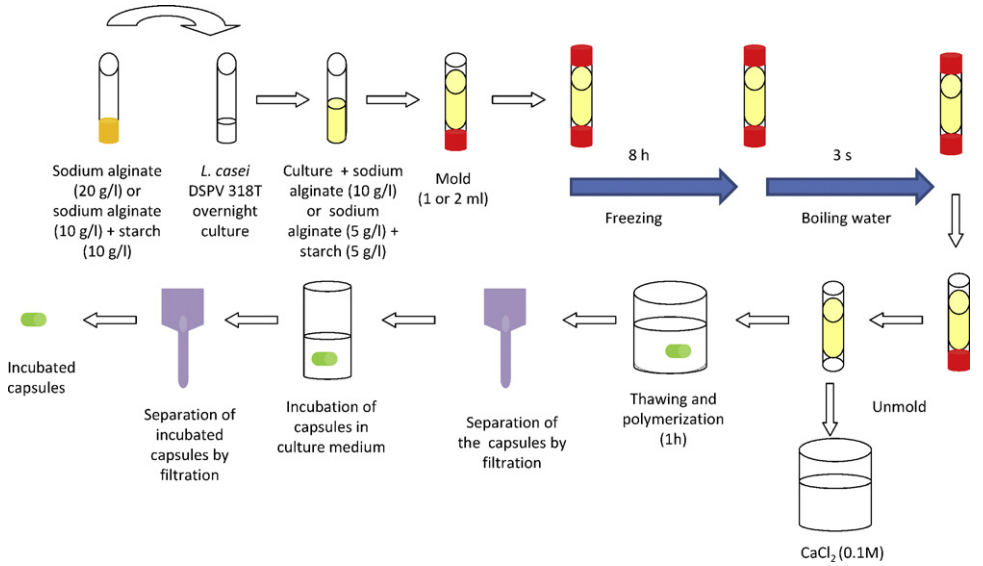

Fig. 1. Schematic representation of the manufacturing procedure of macrocapsules for dairy calves.

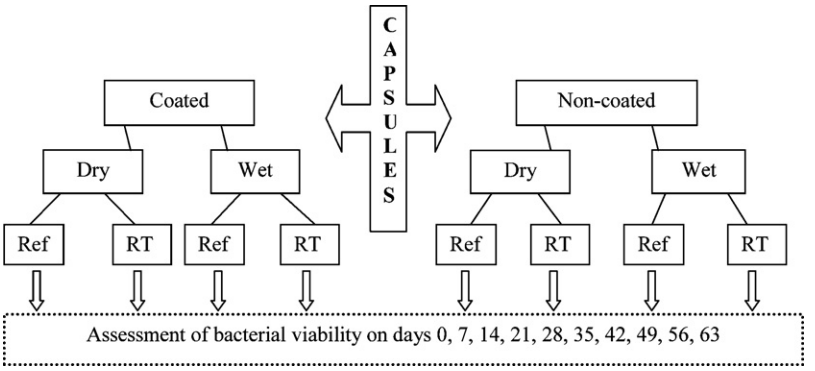

Fig. 2. Experimental design for conservation of the capsules in different temperature conditions. [Ref: capsules stored under refrigeration (*i.e.*, 4 °C); RT: capsules stored at room temperature (*i.e.*, 18 °C)].

each made by adding one part of capsule disintegration solution to 9 parts of Ringer 1/4 solution) were then made, spread in MRS agar Petri dishes and incubated at 37 °C for 48 h in aerobic conditions. Determinations were in triplicate.

2.4. Incubation of capsules in culture medium

To increase bacterial biomass inside the capsules, they were incubated in the same culture medium used for biomass production (Fig. 1). Bacterial growth was determined either at 9 or 18 h of incubation by the methodology described in Section 2.3.

2.5. Coating and storage of capsules

The conditions for the production of the capsules were: alginate (5 g/l) + starch (5 g/l) with a size of 2 ml, and then incubated for 9 h in aerobic conditions. In addition, the capsules were either coated or not with a polymer polycation (chitosan, Aldrich™, catalog number 448869, St. Louis, MO, USA). The chitosan solution was prepared at a concentration of 4 g/l and this solution was prepared with water acidified with glacial acetic acid (4.4 ml/l). This solution was adjusted to pH 5.7–6.0 with NaOH, and then sterilized in an autoclave for 15 min (Krasaekoopt et al., 2006). Capsules were kept in this solution for 15 min. Half of the capsules, both coated and not-coated, were dried with air in a laminar flow chamber for 8 h to constant weight, and these were called dry capsules, while capsules not exposed to drying were called wet capsules. The capsules were stored at different conditions (room temperature (*i.e.*, 18 °C) and refrigeration (*i.e.*, 4 °C)) and bacterial viability was determined at various times in the experimental design detailed in Fig. 2. For viability determinations, capsules coated with chitosan were dispersed in a sodium citrate solution (10 g/l, 1 capsule/50 ml) using a Stomacher (Seward Biomaster™,

Table 1
Counting of *L. casei* DSPV 318 T bacteria in capsules incubated in supplemented milk.

	Bacterial counts (log CFU/g)	P	
		Treatment	Time × treatment
Capsule type			
Alginate	8.34	<0.001	0.004[a]
Alginate + starch	8.64		
Capsule size			
1 ml	8.51	0.086	0.094[b]
2 ml	8.46		

SEM: 0.003.
[a] Interaction capsule type × time.
[b] Interaction capsule size × time.

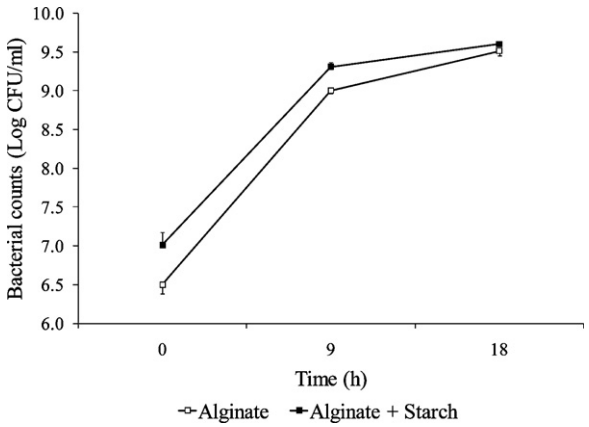

Fig. 3. Bacterial counts in alginate and alginate + starch capsules incubated at 0, 9 and 18 h (means ± SD). Alginate capsules: 8.34 log CFU/g; alginate + starch: 8.64 log CFU/g (effects, treatment (P<0.001) and interaction between treatment and period (P=0.004)).

Thetford, Norfolk, United Kingdom) and non-coated capsules were dissolved in a sodium citrate solution (10 g/l). Bacterial viability was then determined by the methodology described in Section 2.3.

2.6. Statistical analysis

Weight of the capsules were analyzed by one way ANOVA using Duncan's test.

The effect of the capsule type and size on bacterial growth was analyzed in a 2 (type: alginate and alginate + starch)×2 (size: 1 and 2 ml)×3 (time: 0, 9 and 18 h) and Duncan's test factorial design. Bacterial growth was analyzed by ANOVA for repeated measures, with time as repeated measures.

The effect of chitosan coverage (coated and non-coated), humidity (dry and wet) and storage temperature (refrigeration and room temperature) on the viability of lactic acid bacteria was analyzed by 2 (coverage: with or without chitosan)×2 (moisture: dry or wet)×2 (storage temperature: refrigeration or room temperature)×10 (time: 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 d) and Duncan's test factorial design. Viability data were analyzed by ANOVA for repeated measures, with time as repeated measures. For these analyses, SPSS 11.0 for Windows software was used with P<0.05 representing a significant difference between means.

3. Results

3.1. Production and incubation of capsules in culture medium

Capsules produced by addition of starch were similar in weight to capsules produced with sodium alginate only. Bacterial counts differed between both capsule types, showing the highest number with the alginate + starch capsules (P<0.001; Table 1). Bacterial growth within capsules presented a different behavior over time for both types of capsules (P=0.004; Table 1). At the time of formation of capsules, those made with alginate + starch had higher cell concentrations. After 9 h of incubation, bacterial counts increased 2.4 log CFU/g, and capsules made of alginate + starch continued having higher counts than alginate capsules. After 18 h, there was a further increase in bacterial counts and, although alginate + starch capsules continued having higher counts, alginate capsules increased more in proportion during this period (Fig. 3).

Table 2
Viability of encapsulated *L. casei* DSPV 318 T bacteria and stored under different conditions.

	Viability (log CFU/g)	P	
		Treatment	Time × treatment
Coating			
With	5.66	0.619	0.024[a]
Without	5.71		
Moisture			
Dry	5.79	0.076	0.147[b]
Wet	5.59		
Storage			
18 °C	4.18	<0.001	<0.001[c]
Temperature			
4 °C	7.19		

SEM: 0.364.
[a] Interaction coating × time.
[b] Interaction moisture × time.
[c] Interaction storage temperature × time.

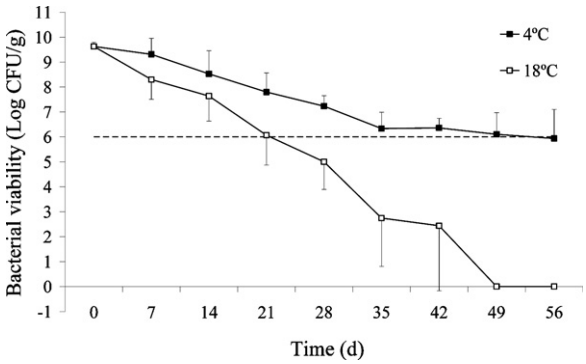

Fig. 4. Bacterial viability in capsules stored in different temperature conditions: 4 °C and 18 °C during 63 d (means ± SD). 4 °C: 7.19 log CFU/g; 18 °C: 4.18 log CFU/g (treatment (P<0.001) and interaction between treatment and period (P<0.001)). The dotted line represents the suggested minimum level.

The size of the capsule did not influence bacterial counts (Table 1), and bacterial growth over time was similar in different size capsules.

3.2. Coating and storage of capsules

During the 63 d of the experiment, bacterial viability within the capsules decreased (P<0.001). The analyzed factors moisture, coating and storage temperature showed different effects on bacteria viability.

The capsules that were subjected to drying lost between 86 and 92% of their initial weight due to water loss, and tended (P=0.076; Table 2) to have higher bacterial concentrations than the wet ones.

Storage temperature influenced maintenance of bacterial viability. Capsules which were refrigerated maintained more bacterial viability than those stored at 18 °C (P<0.001; Table 2). There was also an effect of storage temperature on the viability of encapsulated bacteria over time (P<0.001; Table 2). While the viability of bacteria decreased over the study period, the loss of viability was less pronounced at 4 °C than at 18 °C (Fig. 4).

There was no direct effect of capsule coating on viability of bacteria (Table 2), but there was a differential behavior over time (P=0.024). Until day 28 the viability of the capsules was similar but, from day 35 to 42 inclusive, coated capsules decreased viability relative to non-coated which maintained more stable values. In counts made on day 49, non-coated capsules cell viability declined sharply, with values similar to those of coated capsules. This was maintained until day 63, when the viability of the coated capsules remained constant from day 42, and non-coated ones decreased bacterial counts (Fig. 5).

In contrast, there was a tendency (P=0.061) to maintain higher viability in a combined effect between storage temperature and capsule coating. Analysis over time of the interaction between these 2 factors showed a difference (P<0.001, Fig. 6). At 4 °C, all capsules kept bacteria alive until the end of the experiment, but only those coated with chitosan kept bacterial counts over the SML throughout the evaluation period. In addition, non-coated capsules maintained at 4 °C kept the SML until day 42 only (Fig. 6). At 18 °C, viable bacteria had counts above the detection limit of 1.70 log CFU/g only until day 42.

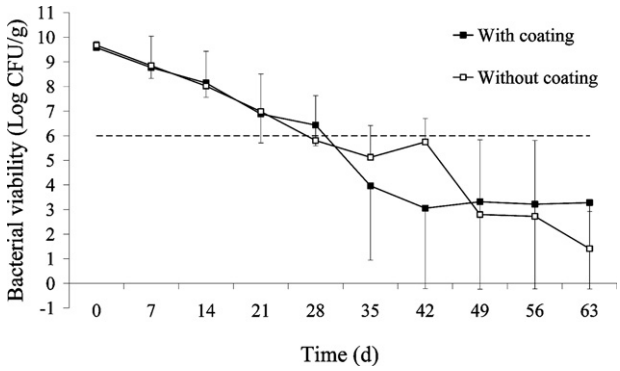

Fig. 5. Bacterial viability in capsules with and without coating during 63 d (means ± SD). With coating 5.66 log CFU/g; Without coating 5.71 log CFU/g (treatment (P=0.619) and interaction between treatment and period (P=0.024)). The dotted line represents the suggested minimum level.

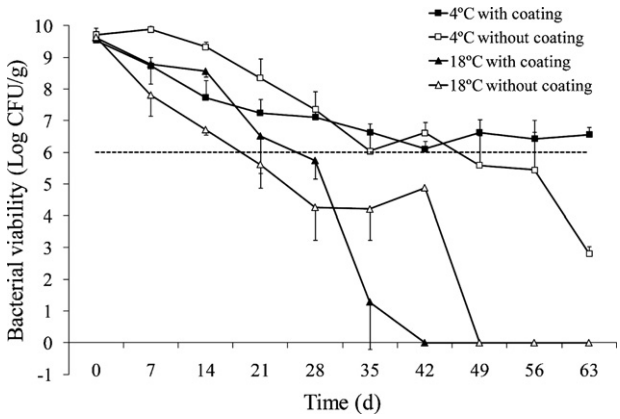

Fig. 6. Bacterial viability in capsules with and without coating storage at 4 °C and 18 °C during 63 d (means ± SD). 4 °C with coating 7.27 log CFU/g; 4 °C without coating 7.11 log CFU/g; 18 °C with coating 4.05 log CFU/g; 18 °C without coating 4.31 log CFU/g (interaction between treatments (storage temperature and coating P=0.061) and interaction between treatments and period (P<0.001)). The dotted line represents the suggested minimum level.

4. Discussion

Alginate is the material most widely used to form the matrix of probiotic capsules (Chandramouli et al., 2004; Truelstrup Hansen et al., 2002) because of its ability to form a gel in the presence of multivalent cations regardless of temperature to create an alginate immobilization matrix available to living cells. However production of alginate capsules with addition of starch has advantages, as replacement of half of the alginate with starch increased the bacterial concentration and decreased the cost of production because starch is less expensive than alginate. These two formulations were those that best resisted the gastric conditions studied by Muthukumarasamy et al. (2006), which is attributed to the protective nature of the polymer networks generated during encapsulation.

Production of high populations of bacteria in alginate beads is possible by incubating the capsules in a nutrient medium (Champagne et al., 2000). Incubation of capsules in culture medium increased bacterial concentration up to 2 log CFU/g of capsule. By studying the concentration of microorganisms in dry capsules, we found that there were higher bacterial counts than in wet capsules, which may be because water loss reduces the volume of the capsule causing an increase in the number of bacteria per gram of capsule. In contrast, drying had no adverse effects on cell viability over time.

The capsules have a kind of peripheral "skin" which increases thickness of the cover materials (Krasaekoopt et al., 2004). This increase in the external thickness of the capsules allowed coated capsules to maintain bacterial viability for longer than non-coated ones, possibly due to better protection of bacteria against harmful environmental factors. In addition, this coverage may protect bacteria during passage through the gastrointestinal tract, increasing their viability until they reach the site where they impart their probiotic effect.

Methods of freeze or spray-drying of probiotics added to food are not the best option for viability preservation because direct contact of microorganisms with the product diminishes its bacterial counts. Encapsulation is an alternative to solve this problem (Muthukumarasamy et al., 2006), and has the additional advantage of the protection provided by the capsule to gastric conditions (Picot and Lacroix, 2004). Due to the macrocapsules having less surface contact than microcapsules per

unit weight, they would protect bacteria against gastric conditions more efficiently. This is related to the physical barrier, and the increased distance of the bacteria with the external environment (Lee and Heo, 2000; Muthukumarasamy et al., 2006).

Environmental sensitivity of some probiotic strains frequently limits their practical use in non-refrigerated food and pharmaceutical supplements. In this sense, encapsulation may improve the viability of the strains during storage (Crittenden et al., 2006). In our study, coated and dry capsules kept their SML for 21 d at 18 °C. Obtaining a final product with a size similar to a feed starter pellet would allow it to be mixed homogeneously with the feed fed to the calf, thereby maintaining the bacterial viability necessary to exercise their probiotic effect during that period. Another possibility is to maintain probiotic capsules separate from the feed starter under refrigeration. In this way, probiotics may have an expiration time of at least 2 mo, and may be combined with the starter at the time it is being fed to the calves. This option also allows addition of capsules to milk or milk replacers for those calves that do not eat starter.

5. Conclusions

We describe a new technique to generate alginate/starch macrocapsules, a tool to create a product with a high probiotic concentration and an expiration time sufficiently long to implement in artificial rearing of calves which may be incorporated into both liquid and solid feeds. In the near future, production of capsules capable of maintaining viability of probiotic bacteria for animals should be directed to using methodologies that generate larger capsules, as this will allow inclusion of larger quantities of microorganisms and reduce negative effects of an adverse environment during storage and transit across biological barriers of the host.

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