Research Square

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Microencapsulation of probiotics by multiple emulsion using protein– polysaccharide blends: seawater stability and viability for aquaculture

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Abstract

The stability and viability of probiotic strains L. acidophilus and L. plantarum microencapsulated via double-emulsion ($W_1/O/W_2$) coupled with spray drying were characterized for shrimp larval culture. Three protein–polysaccharide blends (A: WPI/CMC/PEC; B: WPI/GA/MD; C: WPI/MD) were tested in a factorial design at 5, 8 and 10% total solids. Microcapsules were characterized by particle size, dissolution, buoyancy, and survival to the drying process. Two-way ANOVA revealed a significant blend × solids interaction ($p < 0.001$); blend B at 10% solids (B10) exhibited the lowest dissolution (4.5%, 95% bootstrap CI 4.10–4.90) and was re-encapsulated at two probiotic loads. Viability, quantified by plate counting and LIVE/DEAD fluorescence, showed close agreement (Lin's CCC = 0.997; paired permutation $p = 0.43$). After six months at 4°C, both formulations retained > 10^8 CFU g^{-1}. An indicative resampling-based projection suggested that the high-load formulations maintain > 10^8 CFU g^{-1} beyond the tested period, pending confirmation with additional timepoints. The system emerges as a robust, sustainable alternative to antibiotics for shrimp larviculture. Beyond aquaculture, this encapsulation approach illustrates how biopolymer design can safeguard sensitive bioactive microorganisms during processing and storage, with promising applications in functional foods and veterinary science.

1 Introduction

Probiotics are living microorganisms that, consumed in adequate amounts, confer health benefits on the host and improve the intestinal microbiota [1]. In aquaculture these benefits include modulation of the gastrointestinal tract, antagonism against pathogens, enhanced nutrient uptake and stimulation of the immune system [2–9]. Probiotics used in aquaculture include lactic acid bacteria (LAB) [10] that reduce pathogenic Vibrio [11–14] and strengthen the host immune response [2, 6–8, 15], improving survival and growth in penaeid shrimp larvae [16–17].

LAB, often anaerobic or microaerophilic, are sensitive to environmental oxygen. To maintain viability and protect them from oxidative stress, double emulsions are an effective vehicle for their incorporation into aquaculture feed [18–19]. A multiple emulsion ($W_1/O/W_2$) is an internal aqueous phase dispersed in an oil phase, itself dispersed in a continuous external aqueous phase [20], stabilized by biopolymers interacting through covalent or electrostatic forces [21]. Complex protein–polysaccharide matrices improve homogeneity and droplet stability [22–28]; the combination of whey protein isolate (WPI) with Arabic gum [23], maltodextrin [29], carboxymethyl cellulose (CMC) and pectin (PEC) [19, 27, 30–31] maintains the viability of encapsulated Lactobacillus [32–36]. Stable emulsions are converted into powder by spray drying, whose inlet–outlet temperatures, flow rate and pressure must be tuned to preserve probiotic survival [37–40].

Beyond mean values, a rigorous characterization of such systems benefits from resampling and simulation tools that quantify uncertainty and project performance: bootstrap confidence intervals, permutation tests for method agreement, and Monte Carlo shelf-life modelling have been used to describe probiotic viability and food stability under variability [41–43]. Probiotics also represent a

sustainable alternative to conventional antibiotics [26, 44–47]. Accordingly, the present study characterizes double-emulsion spray-dried microcapsules produced with canola oil and three biopolymer blends, quantifies survival to the drying process, and cross-validates viability by plate count and fluorescence. Because shrimp larvae feed by filtration, microencapsulated diets have been explored as substitutes for live microalgae [48], so the blends must confer buoyancy and low dissolution so that the microcapsules remain in the seawater column and protect the probiotics until ingestion. We hypothesized that the $W_1/O/W_2$ system with whey-protein/polysaccharide blends would provide microcapsule stability in the water column, demonstrable through buoyancy, dissolution and probiotic shelf-life, and that a resampling/Monte Carlo framework would provide a robust, probabilistic assessment of that stability.

It is important to emphasise that the present work is deliberately confined to the physicochemical and in vitro biological characterisation of the microcapsules. This screening phase is a necessary prerequisite for the rational selection of wall materials and probiotic loads, because the seawater stability, buoyancy and viability of the encapsulated cells must be optimised before undertaking costly and logistically complex larval-rearing trials. Establishing a robust formulation with well-characterised performance in seawater thus provides the essential foundation for subsequent in vivo validation of growth promotion and pathogen resistance.

2 Materials and methods

2.1 Materials, strains and controls

Whey protein isolate (WPI, 95–97%; Bipro Davisco, USA), Arabic gum (AG; Metropolitan Autonomous University), maltodextrin (MD) and CMC (Aqualon, Hercules, USA) and citrus pectin (PEC; Danisco Mexicana, Mexico) were used as encapsulating materials, with canola oil as oil phase (Grindsted PGPR and Panodan SDK as emulsifiers; Danisco, Mexico). Canola oil was selected for its high mono/polyunsaturated fatty-acid content, oxidative stability and neutral flavour; no other oils were evaluated. The medium was MRS (Difco, USA) with inulin [49] and honey (5%) [50] as prebiotics. *Lactobacillus plantarum* and *Lactobacillus acidophilus* (donated by Dra. Lorena Pedraza, Iberoamerican University) were selected as LAB widely reported as effective shrimp probiotics through Vibrio antagonism and immunomodulation [2, 14].

2.2 Probiotic growth and multiple emulsion

Lactobacillus acidophilus and *Lactobacillus plantarum* were activated separately in MRS broth (37°C, 24 h, anaerobic) and grown to 1×10^{10} CFU mL^{-1}; a 50:50 mixture formed the inoculum for the internal aqueous phase (W_1). The primary emulsion (W_1/O, canola oil with 10% Grindsted:Panodan 6:4) was homogenized (Ultraturrax T50, 7 600 rpm, 10 min) [23] and re-emulsified (5 200 rpm, 5 min) into the biopolymer blends at 5, 8 and 10% solids (external phase W_2). Emulsions were spray-dried (Mobile Minor Niro-atomizer) at inlet 170 ± 5°C, outlet 80 ± 5°C, 25 mL min^{-1}, 3 bar [24], within the range reported for LAB in protein–polysaccharide matrices [32–33, 37].

2.3 Viability, drying survival and fluorescence assay

Inoculum concentration was verified before encapsulation by plate count. Microcapsules (0.1 g) were hydrated in 0.9 mL saline (24 h); serial dilutions were plated on MRS agar (37°C, 24 h) and expressed as CFU g^{-1} of microcapsule (NOM-092-SSA1-1994) at 0, 2 and 6 months (4°C). Survival to the spray-drying process was calculated as the viable concentration recovered immediately after drying relative to the initial load (×100); this represents survival to the process, not encapsulation efficiency in the strict sense, which would require separating encapsulated from surface/free cells. Viability was also determined with a LIVE/DEAD BacLight kit (L-13152) in a BIO-TEK Synergy HT reader at excitation 485 nm and emission 530 nm (green, SYTO 9; intact membranes) and 630 nm (red, propidium iodide; damaged membranes); viability was obtained from the green/red ratio calibrated against standard curves of known live:dead proportions [33, 51–52]. Because the inoculum was a 50:50 mixture of the two strains and neither enumeration method discriminates between species, all counts refer to the combined *Lactobacillus acidophilus*/ *Lactobacillus. plantarum* consortium.

Survival to the spray-drying process (S) was calculated for each batch as the ratio of the viable count recovered immediately after drying (N_d) to the initial viable load (N_0):

$$S\ (\%) = 100 \times N_d / N_0 \quad (2.1)$$

2.4 Characterization of microcapsules

Morphology was observed by SEM (Jeol JSM-6030LV) and the volume-mean particle size with a Malvern 2600 analyzer (the full size distribution was not determined). Buoyancy (0.1 g on 10 mL artificial seawater, 28°C, transmittance at 395 nm) was fitted to a first-order decay model [53]. Dissolution (0.3 g in seawater, 30 ppt, pH 8, 28°C) was measured at 15, 30, 60, 120, 180 and 240 min. All determinations were in triplicate; the protein:polysaccharide ratio was 2:1 (Table 1).

Table 1
Proposed formulations for the microencapsulation of probiotics

Code	Encapsulating materials	Protein:polysaccharide	Total solids (%)	Surfactant W/O (%)
A	WPI/CMC/PEC	66:17:17	10 / 8 / 5	10
B	WPI/GA/MD	66:17:17	10 / 8 / 5	10
C	WPI/MD	66:34	10 / 8 / 5	10

WPI whey protein isolate, CMC carboxymethyl cellulose, PEC pectin, GA gum Arabic, MD maltodextrin

2.5 Statistical, resampling and Monte Carlo analysis

All experiments were performed with three independent batches per formulation (n = 3 experimental units); each batch was a separate emulsification and spray-drying run, and the three batches — not repeated measurements of a single batch — are the replicates used for every statistical analysis. Data

are reported as mean with 95% bootstrap confidence intervals (CIs). Dissolution and particle size (Tables 2, 3) were analysed by two-way ANOVA (blend × solids or blend × concentration) with the interaction term and Tukey's HSD ($\alpha = 0.05$) [54] on arcsine-square-root–transformed percentages; exact p-values and partial eta-squared (η^2_p) are reported. Normality (Shapiro–Wilk) and homogeneity of variances (Levene) were examined but, given n = 3, treated as indicative only. Viability during storage was expressed as $\log_{10}$ CFU g^{-1} and analysed by two-way ANOVA (treatment × storage time) on the batch-level counts (n = 3 batches per formulation); the enumeration method was not treated as a replicate but compared separately. *Resampling and Monte Carlo.* Ninety-five-percent CIs were obtained by non-parametric percentile bootstrap (5000 resamples, fixed seed) [41]. Agreement between plate-count (PC) and fluorescence (F) enumeration was assessed by the Bland–Altman method, Lin's concordance correlation coefficient (CCC) and a two-sided paired permutation test (5000 sign permutations) [43]. As an indicative projection, a first-order log-linear decay model was fitted to the $\log_{10}$ counts of each formulation and its parameter uncertainty propagated by resampling (5000 iterations) to estimate the probability of retaining > 10^8 CFU g^{-1} up to 12 months [42]; because only three storage points were available, projections beyond the tested range assume monophasic decay and are interpreted as indicative. Given n = 3, F statistics are interpreted together with effect sizes and bootstrap CIs rather than in isolation. Resampling and simulation were implemented in Python 3.11 (NumPy 1.26; random seed 2026), with the analysis script and raw data available in the Zenodo repository (see Data availability); ANOVA in Statistica v10.

Table 2
Dissolution (%) after 4 h in seawater

Total solids	A (WPI/CMC/PEC)	B (WPI/GA/MD)	C (WPI/MD)
10%	5.8 ± 0.32^a	4.5 ± 0.23^a	12.4 ± 0.67^b
8%	7.7 ± 0.44^a	12.8 ± 0.72^b	13.2 ± 0.72^b
5%	9.9 ± 0.49^a	15.2 ± 0.84^b	22.6 ± 1.22^c

Mean ± SE (n = 3 independent batches per treatment). Different superscript letters within a row indicate significant differences between blends at the same solids level (Tukey's HSD, $p < 0.05$). Two-way ANOVA: blend, solids and interaction all significant ($p < 0.001$).

Table 3
Buoyancy, size, dissolution and drying survival of the final microcapsules

Formulation	CFU g^{-1}	Size (µm)	Dissolution (%)	Buoyancy (min)	Drying survival (%)
A1	10^{10}	36.2 ± 1.04^{b}	9.40 ± 0.52^{b}	500 ± 20	38.0 ± 1.7
A2	10^{9}	28.6 ± 0.84^{a}	10.7 ± 0.61^{b}	500 ± 10	37.0 ± 3.0
B1	10^{10}	34.8 ± 1.01^{b}	4.5 ± 0.29^{a}	1000 ± 50	30.7 ± 0.4
B2	10^{9}	28.2 ± 0.78^{a}	4.8 ± 0.29^{a}	1000 ± 20	37.2 ± 2.5

Mean ± SE (n = 3 independent batches). Different superscript letters within a column indicate significant differences (Tukey's HSD, $p < 0.05$). Drying survival = viable cells recovered after spray drying / initial load × 100 (survival to the process, not encapsulation efficiency).

For the indicative shelf-life projection, a first-order log-linear decay model was fitted to the $\log_{10}$ counts of each formulation, where N(t) is the viable count at storage time t, N_0 the initial count and k the inactivation rate constant:

$$\log_{10} N(t) = \log_{10} N_0 - k\,t \quad (2.2)$$

The authors used a large language model (Claude, Anthropic) to assist with statistical-analysis scripting, restructuring of the manuscript and language editing. All data, analyses and interpretations were verified and approved by the authors, who take full responsibility for the content of the article.

3 Results

Spray drying yielded microcapsules of spherical morphology, without cracks or surface craters (Fig. 1), corroborating structurally stable microcapsules with high resistance to droplet coalescence [55–56].

Dissolution differed significantly among treatments. Two-way ANOVA confirmed significant effects of blend ($F_{2,18} = 110.8$, $p < 0.001$, $\eta^2_p = 0.925$) and total solids ($F_{2,18} = 110.5$, $p < 0.001$, $\eta^2_p = 0.925$) and a significant blend × solids interaction ($F_{4,18} = 16.4$, $p < 0.001$, $\eta^2_p = 0.785$). The interaction explains the reversal in ranking across solids levels: at 10% solids blend B gave the lowest mean dissolution (B10 4.5%, 95% bootstrap CI 4.10–4.90), not significantly different from blend A but well below blend C, whereas at 8 and 5% blend A was the most stable (Table 2). Dissolution decreased with increasing solids in every blend (blend A: slope − 0.81, $R^2 = 0.99$).

The two most stable blends were re-encapsulated at two probiotic loads (10^{10} and 10^{9} CFU g^{-1}). Dissolution was affected by blend ($F_{1,8} = 144.6$, $p < 0.001$) but not significantly by probiotic load ($F_{1,8}$ =

3.17, p = 0.11): A1 and A2 did not differ significantly (9.4 vs 10.7%) and B1 and B2 were equivalent (Table 3). Particle size was governed by load ($F_{1,8}$ = 59.0, p < 0.001) rather than blend ($F_{1,8}$ = 0.95, p = 0.36): the 10^{10} formulations were larger (34.8–36.2 μm) than the 10^{9} ones (28.2–28.6 μm). Buoyancy, now determined in triplicate, reached 500 ± 20 min (blend A) and 1000 ± 50 min (blend B), well above the 240-min feeding interval [53].

Survival to the spray-drying process — viable cells recovered after drying relative to the initial load — ranged from 31 to 38% (A1 38.0 ± 1.7%, A2 37.0 ± 3.0%, B1 30.7 ± 0.4%, B2 37.2 ± 2.5%; n = 3 batches), consistent with values reported for spray-dried Lactobacillus in whey-protein matrices. Both encapsulated formulations retained more than 10^{8} CFU g^{-1} after six months, with a loss below one order of magnitude (two-way ANOVA on the batch-level $\log_{10}$ counts: significant effects of treatment ($F_{3,32}$ = 533, p < 0.001) and storage time ($F_{3,32}$ = 122, p < 0.001); Table 4, Fig. 2). Dissolution of the final formulations, with 95% bootstrap CIs, was 9.40% (8.50–10.30) for A1, 10.7% (9.60–11.70) for A2, 4.5% (4.00–5.00) for B1 and 4.8% (4.30–5.30) for B2.

Table 4
Probiotic concentration (CFU g^{-1}): initial, after drying and during storage at 4°C

Formula	Initial	Dry PC	Dry F	2 m PC	2 m F	6 m PC	6 m F
A1	2.2×10^{10c}	8.5×10^{9b}	8.0×10^{9b}	5.8×10^{9a}	6.2×10^{9a}	3.6×10^{9a}	3.8×10^{9a}
A2	1.5×10^{9c}	5.7×10^{8b}	6.0×10^{8b}	3.8×10^{8a}	4.1×10^{8a}	2.8×10^{8a}	2.5×10^{8a}
B1	2.5×10^{10b}	7.5×10^{9a}	7.4×10^{9a}	6.0×10^{9a}	5.0×10^{9a}	4.7×10^{9a}	3.9×10^{9a}
B2	1.5×10^{9c}	5.7×10^{8b}	6.0×10^{8b}	3.8×10^{8a}	4.1×10^{8a}	2.8×10^{8a}	2.6×10^{8a}

Two-way ANOVA (treatment × storage time) on batch-level $\log_{10}$ counts (n = 3 batches): treatment ($F_{3,32}$ = 533) and storage time ($F_{3,32}$ = 122) both significant (p < 0.001). Values are means of three independent batches. PC plate count, F fluorescence. Method agreement (separate analysis): bias 0.006 $\log_{10}$, 95% LoA − 0.08 to + 0.09, Lin's CCC 0.997, permutation p = 0.43. Initial = measured concentration; the 10^{10}/10^{9} in Table 3 are nominal targets.

The two enumeration methods agreed closely: mean bias (PC − F) 0.006 $\log_{10}$, 95% limits of agreement − 0.08 to + 0.09 $\log_{10}$, Lin's CCC 0.997, and the paired permutation test found no significant method bias (p = 0.43). Analysing the two enumeration methods separately, rather than pooling them as replicates, avoids underestimating the experimental error. The close agreement is consistent with the mild storage conditions (4°C), under which loss of culturability and loss of membrane integrity proceed together; the calibration of the green/red fluorescence ratio against known live:dead proportions (Section 2.3) supports this agreement, although under harsher stress a divergence attributable to viable-but-non-culturable cells would be expected. The fluorescence assay is therefore a rapid complement to plate counting under these conditions.

Within a conservative 12-month horizon, the resampling-based projection (Fig. 3) estimated that both high-load formulations remain above 10^{8} CFU g^{-1} with probability ≈ 1 under the fitted model, whereas the

low-load formulations approached the threshold (P = 0.75–0.82 at 12 months). Extrapolation of the log-linear model beyond the 6-month data (Fig. 3, shaded region) suggests longer stability for the high-load formulations, but this is indicative only and should be read as hypothesis-generating rather than predictive: probiotic decay is frequently biphasic, and additional storage timepoints are required to confirm any projection beyond the tested period.

4 Discussion

The three protein–polysaccharide blends behaved very differently in seawater, and the factorial design showed that the blend × solids interaction, rather than either factor alone, governed dissolution: at 10% solids the whey-protein/gum-arabic/maltodextrin blend (B10) gave the lowest dissolution and the highest buoyancy, whereas at lower solids the whey-protein/CMC/pectin blend (A) was more stable. Both re-encapsulated formulations survived spray drying (31–38%) and retained more than 10^8 CFU g^{-1} after six months at 4°C, and the two enumeration methods agreed closely (Lin's concordance correlation coefficient 0.997). Taken together, blend B at 10% solids emerges as the most promising vehicle, combining low dissolution, prolonged buoyancy above the 240-min feeding interval and a particle size compatible with the mouth gape of *Litopenaeus vannamei* mysis larvae.

Mechanistically, WPI forms amphiphilic films around the cells while Arabic gum raises the glass-transition temperature of the matrix, creating a thermal-shield effect that, with maltodextrin (which reduces hygroscopicity), preserved cell density and prevented lysis on dehydration [33, 57–58]. In blend A, the CMC–pectin network adds viscosity and mechanical strength: pectin forms a dense protective crust [59] and CMC minimizes shear damage at the nozzle [60].

Table 5 summarizes how the present system compares with previous encapsulation studies; unlike earlier reports, which target human gastrointestinal delivery, this work is oriented toward shrimp larval culture, where buoyancy and controlled dissolution in seawater are the governing requirements.

Table 5
Comparison of the present system with previous encapsulation studies

Study	Wall material	Microorganism	Method	Application
Shima et al. [36]	W/O/W emulsion	L. acidophilus	Double emulsion	Gastric protection (human)
Liang et al. [19]	WPC / pectin	L. rhamnosus	W/O/W	GI viability (human)
Rodríguez et al. [27]	Pectin–protein	L. reuteri	Double emulsion	GI delivery (human)
Altamirano-Ríos et al. [32–33]	WPI / gum Arabic	L. acidophilus	Spray drying	GI survival (human)
This study	WPI / GA / MD	L. acidophilus + L. plantarum	$W_1/O/W_2$ + spray drying	Shrimp larval culture

WPC whey protein concentrate, GI gastrointestinal. The present work is the only one oriented toward aquaculture (shrimp larval culture).

The drying survival obtained here (31–38%) lies within the range reported for spray-dried *Lactobacillus* in protein-based matrices, where survival commonly falls between 20 and 80% depending on strain, wall material and outlet temperature [37, 39]. The close agreement between plate counting and LIVE/DEAD fluorescence is consistent with the mild storage conditions used: at 4°C loss of culturability and loss of membrane integrity proceed together, so the fluorescence ratio tracks the viable count [51–52]; under harsher stress a divergence attributable to viable-but-non-culturable cells would be expected, and the fluorescence assay would then complement rather than replace plate counting. From an application standpoint, the buoyancy and slow dissolution of blend B are the properties that matter most for larviculture: because penaeid larvae feed by filtration, a microcapsule must remain suspended in the water column and release its contents gradually so that the probiotic is ingested rather than lost to the tank bottom [48, 53]. The 1000-min buoyancy of blend B, more than four-fold longer than the feeding interval, indicates that the capsules would remain available to the larvae between feedings.

While the in vivo efficacy of the selected formulations remains to be demonstrated, the rigorous physicochemical screening reported here serves precisely to de-risk those future biological trials. By identifying blend B (WPI/GA/MD, 10% solids) as the most stable in seawater, with favourable buoyancy and sustained viability, we provide a clear, evidence-based candidate for subsequent larval-rearing challenges. The absence of live-animal data in the present study is therefore not a shortcoming of the experimental design, but rather a deliberate sequential approach in which the formulation is optimised before proceeding to the more resource-intensive biological validation.

This study has several limitations that bound its conclusions and define the next steps. First, it is an *in vitro* characterization: the protective effect must ultimately be demonstrated *in vivo*, through larval-rearing trials measuring survival, growth and resistance to *Vibrio* challenge, which were beyond the

present scope. Second, although each formulation was produced as three independent batches, the sample size is modest and the storage study included only three time points, so the resampling-based shelf-life projection beyond six months is indicative rather than predictive and should be confirmed with additional time points. Third, non-encapsulated (free-cell) and single-emulsion controls were not included, so the incremental protection conferred by the double-emulsion architecture over simpler systems cannot be quantified here. Fourth, the particle-size distribution, water activity and residual moisture of the powders were not determined; these properties influence flowability, oxidation and long-term viability and should be characterized in future work. Finally, because the inoculum was a 50:50 mixture of the two strains and neither enumeration method discriminates between species, all counts refer to the combined *Lactobacillus acidophilus*/ *Lactobacillus plantarum* consortium rather than to each species individually. Addressing these points—particularly the *in vivo* evaluation—will determine whether the physicochemical advantages reported here translate into improved performance in shrimp larviculture.

5 Conclusion

Microcapsules produced with blend B (WPI/GA/MD, 10% solids) combine low dissolution (4.5–4.8% in 4 h, 95% bootstrap CI 4.00–5.00 for B1), high buoyancy (1000 min), a drying survival of ~ 31% and viability above 10^8 CFU g^{-1} after six months, with a particle size (28.2–34.8 µm) suitable for Mysis shrimp larvae. The factorial analysis identified the blend × solids interaction as the key determinant of stability; the plate-count/fluorescence concordance validates a rapid viability assay; and an indicative resampling-based projection suggests that the high-load formulations retain effective probiotic densities beyond the tested six months, to be confirmed with longer storage studies. The system is therefore a promising candidate for shrimp larval culture. The physicochemical foundation established here thus offers a ready-to-test candidate for the next stage of in vivo evaluation, which will ultimately determine its translational value for shrimp larviculture. This work was limited to an in-vitro physicochemical characterization: the particle-size distribution, water activity and residual moisture of the powders were not determined; non-encapsulated (free-cell) and single-emulsion controls, true encapsulation efficiency by independent methods, full release profiles and in-vivo retention and colonization in seawater were beyond its scope and should be addressed in future larval-rearing trials to confirm the protective effect and efficacy.

Declarations

Use of AI tools

A large language model (Research CLI) was used for statistical-analysis scripting, manuscript restructuring and language editing, as documented in the Methods. The authors verified and approved all outputs and take full responsibility for the content.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

Not applicable. The study did not involve experiments on live animals or humans; it comprised in vitro bacterial culture and physicochemical characterization of microcapsules.

Consent to participate / for publication

Not applicable.

Data availability

The complete batch-level raw data (Excel file) and a detailed step-by-step description of all statistical analyses (bootstrap, permutation tests, Monte Carlo projections, ANOVA) are openly available in Zenodo at https://doi.org/10.5281/zenodo.21748134. The analysis procedure document provides all formulas, random seed (2026) and computational steps necessary to fully reproduce the results reported in this paper.

Author Contribution

Conceptualization: I.Z. Hernández-Hernández, D. Voltolina Lobina, G. Cuzon, R. Pedroza-Islas, M.G. Gaxiola-Cortés; Methodology: I.Z. Hernández-Hernández, J.C. Espinoza-Tapia, R. Pedroza-Islas; Formal analysis and investigation: I.Z. Hernández-Hernández, J.C. Espinoza-Tapia; Software: J.C. Espinoza-Tapia; Writing – original draft preparation: I.Z. Hernández-Hernández; Writing – review and editing: J.C. Espinoza-Tapia, G. Cuzon, R. Pedroza-Islas, M.G. Gaxiola-Cortés; Resources: D. Voltolina Lobina, R. Pedroza-Islas, M.G. Gaxiola-Cortés; Supervision: D. Voltolina Lobina, R. Pedroza-Islas, M.G. Gaxiola-Cortés; Visualization: I.Z. Hernández-Hernández. Author D. Voltolina Lobina contributed to this work prior to his death and is included posthumously with the agreement of the co-authors.

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Figures

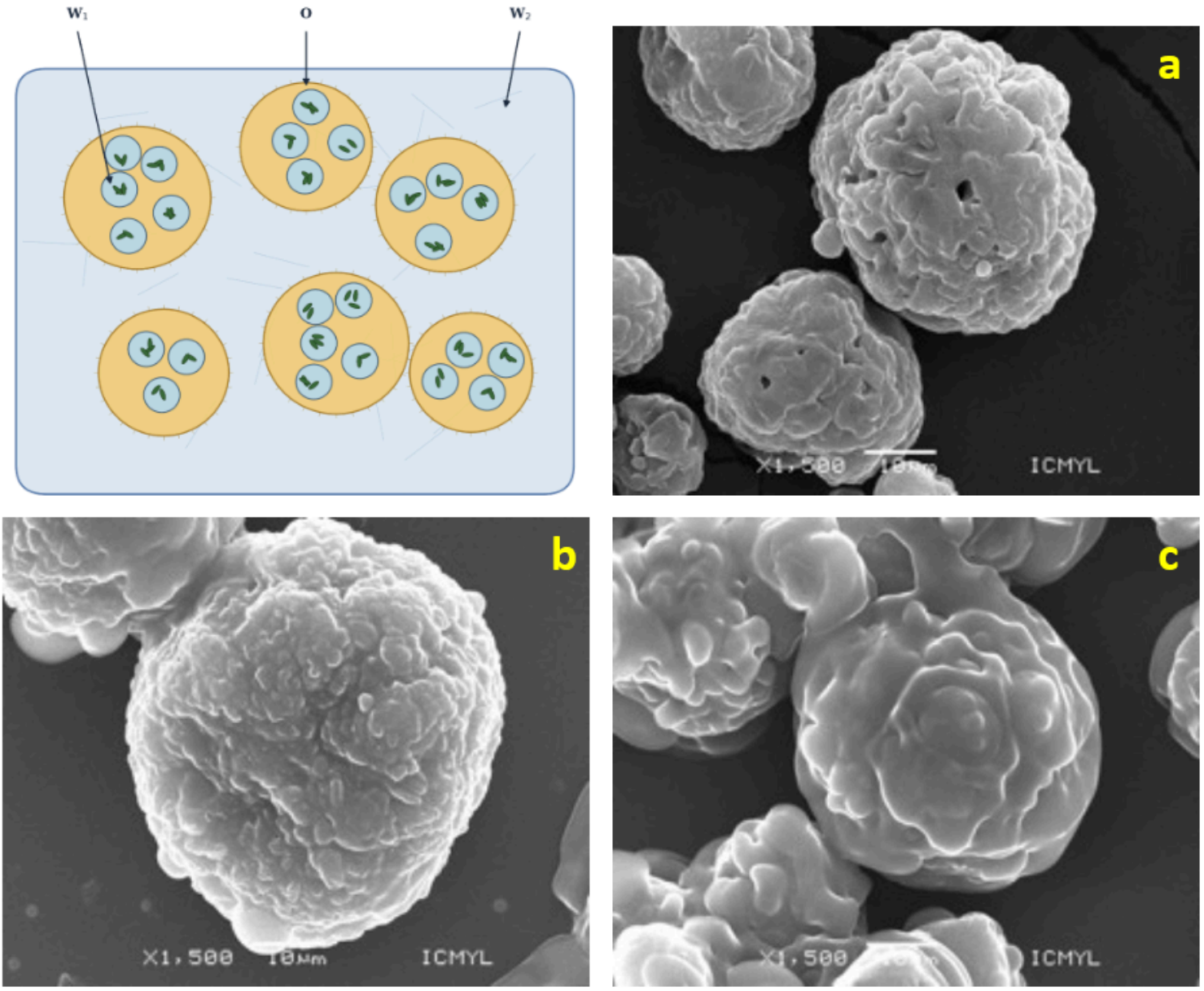


Figure 1

Schematic of the $W_1/O/W_2$ double emulsion and SEM micrographs of the microcapsules: a WPI/CMC/PEC, b WPI/GA/MD, c WPI/MD (scale bars within each micrograph)

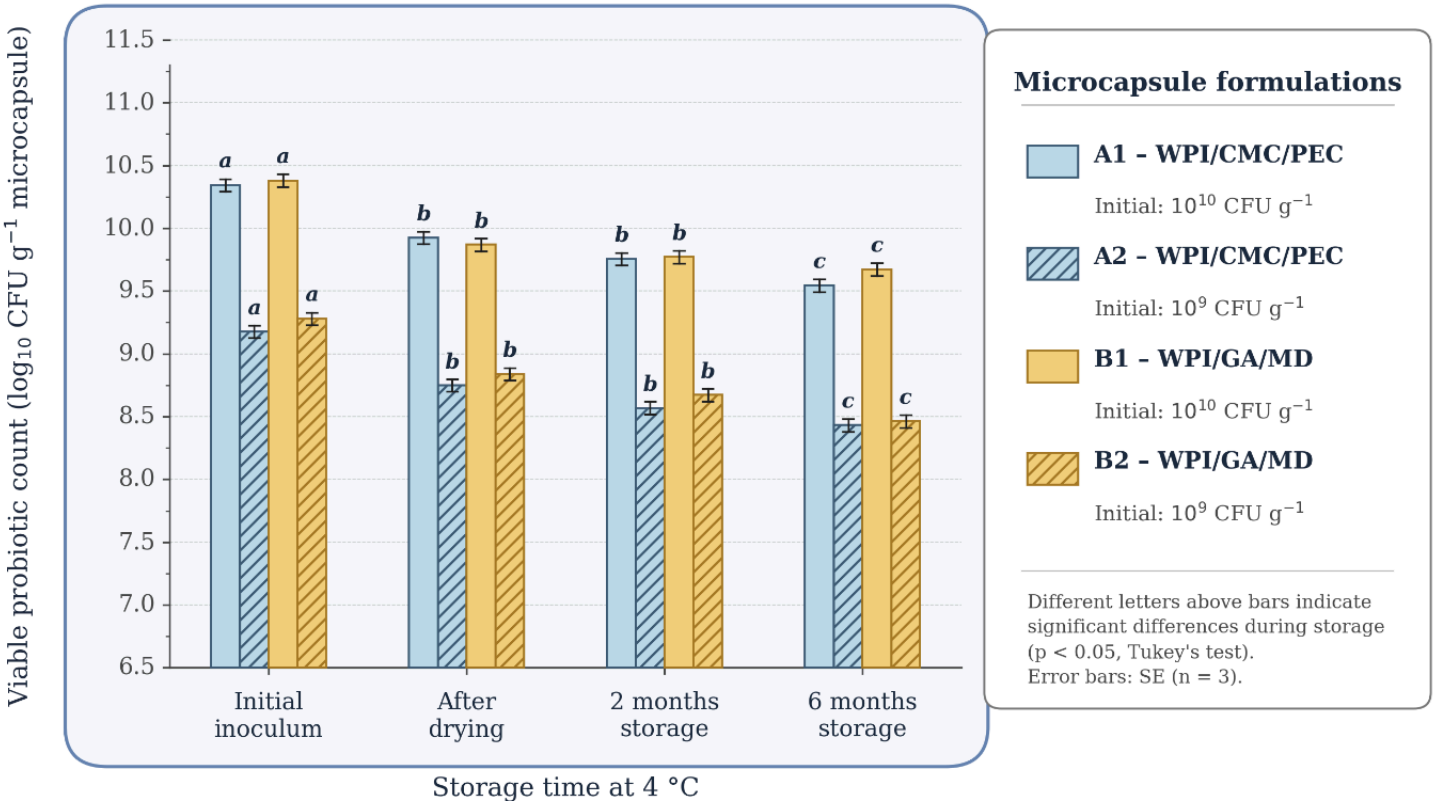


Figure 2

Viable probiotic counts (log_{10} CFU g^{-1}) of the four formulations during storage at 4 °C (0, 2, 6 months). Error bars: standard error (n = 3 independent batches)

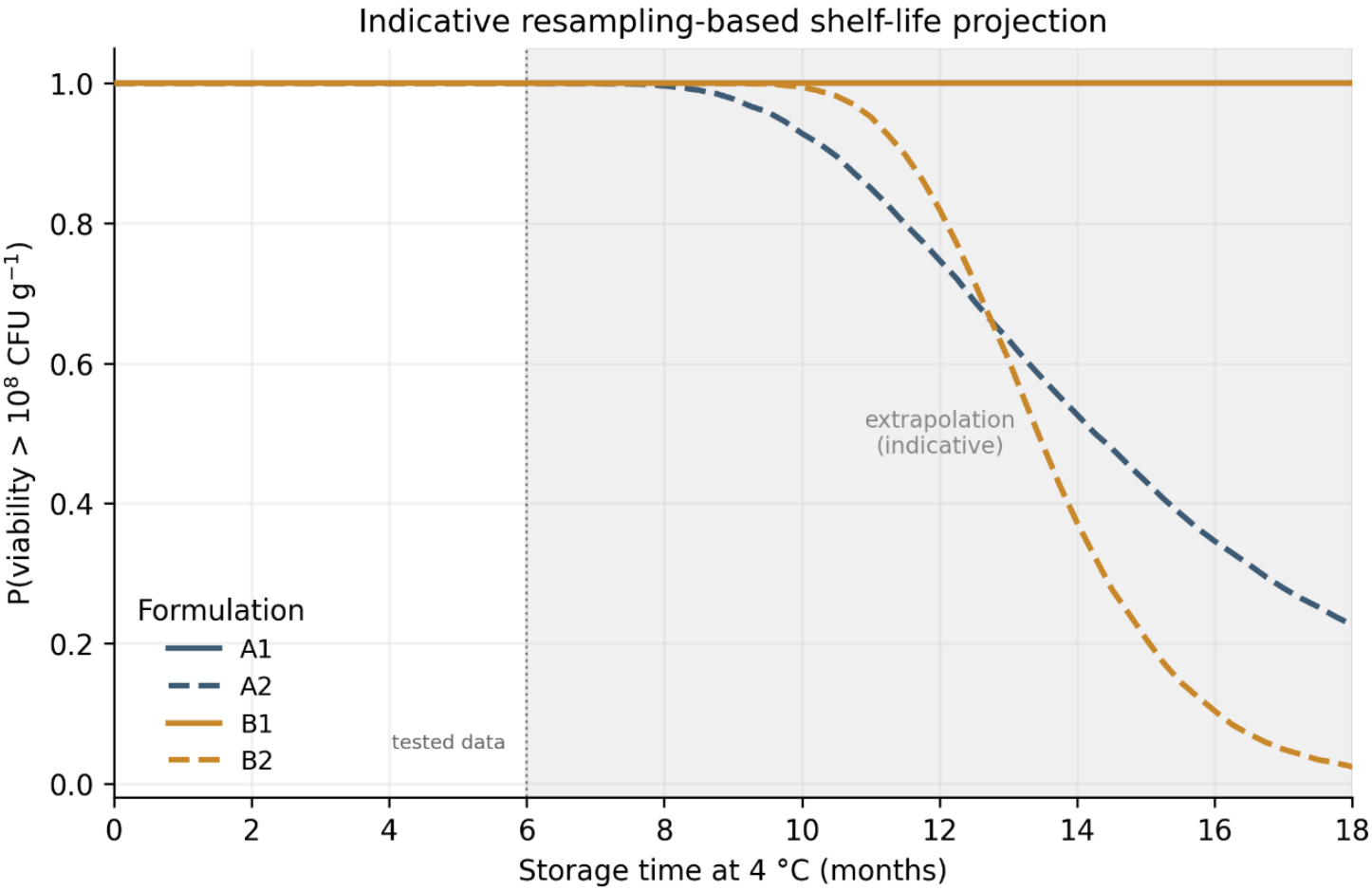


Figure 3

Indicative resampling-based shelf-life projection (5000 iterations): probability of retaining >10^8 CFU g^{-1} as a function of storage time at 4 °C, obtained by propagating the uncertainty of a first-order log-linear decay model. The shaded area denotes extrapolation beyond the 6-month experimental data and is indicative only

Supplementary Files

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- SupplementaryDataset.xlsx