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Polymer and Crosslinker Content Influences Performance of Encapsulated Live Biotherapeutic Products

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Abstract

Introduction—Live biotherapeutic products (LBPs), or therapeutic microbes, are an emerging therapeutic modality for prevention and treatment of gastrointestinal diseases. Since LBPs are living, they are uniquely sensitive to external stresses (e.g., oxygen, acid) encountered during manufacturing, storage, and delivery. Here, we systematically evaluate how polymer and crosslinker concentration affects the performance of an encapsulated LBP toward developing a comprehensive framework for the characterization and optimization of LBP delivery systems.

Methods—We encapsulate a model LBP, *Lactobacillus casei* ATCC 393, in calcium chloride ($CaCl_2$)-crosslinked alginate beads, and evaluate how alginate and $CaCl_2$ concentrations influence LBP formulation performance, including: (i) encapsulation efficiency, (ii) shrinkage upon drying, (iii) survival upon lyophilization, (iv) acid resistance, (v) release, and (vi) metabolite secretion. Approaches from microbiology (e.g., colony forming unit enumeration), materials science (e.g., scanning electron microscopy), and pharmaceutical sciences (e.g., release assays) are employed.

Results—LBP-encapsulating alginate beads were systematically evaluated as a function of alginate and $CaCl_2$ concentrations. Specifically: (i) encapsulation efficiency of all formulations was $>50\%$, (ii) all alginate beads shrunk (after lyophilization) and recovered (after rehydration) similarly, (iii) at 10% alginate concentration, lower $CaCl_2$ concentration decreased survival upon lyophilization, (iv) 10% alginate improved acid resistance, (v) sustained release was enabled by increasing alginate and $CaCl_2$ concentrations, and (vi) encapsulation did not impair secretion of L-lactate as compared to free LBP.

Conclusions—This research demonstrates that polymer content and crosslinking extent modulate the performance of polymer-based LBP delivery systems, motivating research into the optimization of material properties for LBP delivery systems.

Keywords—Drug delivery, Polymer encapsulation, Probiotics, Alginate, Live biotherapeutic products.

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INTRODUCTION

Live biotherapeutic products (LBPs), or therapeutic microbes, include transplanted microbiota, feces-derived spores, rationally designed consortia, and engineered microbes that produce drugs locally.[46,49,50] LBPs are currently investigated in various clinical trials for the treatment of pathogenic infections, inflammatory diseases, and metabolic disorders[46]; recently, positive results from clinical trials of LBPs have been reported for treatment of recurrent *Clostridioides difficile* infections.[14] Uniquely, LBPs have the potential to: (i) serve as alternatives to antibiotics by circumventing current limitations of antibiotic therapies, such as displacing pathogens without risks of introducing antibiotic resistance,[35] or (ii) continuously produce biologics in situ[39] with engineered molecular machinery and potentially reduce the high dosing frequency required by biologics.[3] As such, LBPs have received considerable interest as a next-generation therapeutic.

Unlike other therapeutic modalities, LBPs are alive and face unique challenges in their development, formulation, manufacturing, and delivery. In particular, they are limited by: (i) manufacturing conditions that expose LBPs to oxygen,[43] heat,[9] and desiccation[36]; (ii) storage requirements such as ultra-low temperatures (e.g., -80 °C)[25]; (iii) delivery challenges such as acid and bile insults.[9] Toward addressing these issues and accelerating clinical translation of LBPs, efforts focus on developing a variety of delivery systems for LBPs.[28] One common approach to formulate LBPs is through polymer encapsulation.[28] Studies have reported encapsulation of LBPs in poly(vinyl alcohol),[38] alginate,[7] chitosan,[11] and gelatin.[2] The bulk polymeric matrix provides physical barriers to protect payloads from environmental stressors (e.g., oxygen, acid, bile) while providing additional functions, such as sustained release.[38] Another encapsulation strategy is to microscopically coat polymers onto individual microbes.[4,13,21] For instance, alternating alginate and chitosan layers have been used to decorate microbes to improve acid resistance and mucoadhesion.[4] While these examples describe promising benefits of polymer encapsulation toward formulating LBPs,[8] few studies have elucidated the quantitative relationship between material content (e.g., polymer and crosslinker concentrations in hydrogels) and LBP formulation performance (e.g., encapsulation efficiency, storage, acid resistance, release profiles). The motivation for investigating these relationships stems from a wide body of literature detailing the effects that material content can have on the formulation, delivery, and efficacy of other therapeutic modalities.[23] For example, encapsulated mammalian cells exhibited distinct cell viability, and *in vivo* outcomes in polymeric matrices with varying concentrations of polymer,[37] and crosslinker.[30] Tuning material content can vary the porosity, swelling, and degradation time of delivery systems, thus potentially modulating LBP encapsulation, stability, release profiles, and metabolism. Bridging this knowledge gap will improve understanding of LBP-material interactions and facilitate rational design of LBP delivery systems for different applications.

Here, we encapsulated a model LBP, *Lactobacillus casei* ATCC 393 (*L. casei* ATCC 393) in calcium chloride ($CaCl_2$)-crosslinked alginate beads with different polymer and crosslinker concentrations. Alginate is a naturally-derived polysaccharide material comprising glucuronic and mannuronic units, and generally recognized as safe (GRAS) [6]; as such, $CaCl_2$ crosslinked alginate has been a widely studied encapsulation material for LBPs [22]. *L. casei* ATCC 393 is a probiotic with preclinical evidence to mitigate diseases such as colon inflammation,[29,45] and produced through batch culture in this work. With a broad range of alginate (2%, 5%, and 10% wt vol^{-1}) and $CaCl_2$ (0.1 M, 0.5 M, and 1 M) concentrations (Table 1), we systematically evaluated the effects of polymer and crosslinker content on aspects of LBP formulation and performance, including: (i) encapsulation efficiency, (ii) shrinkage upon drying, (iii) survival upon lyophilization, (iv) acid resistance, (v) release, and (vi) secretion of L-lactate, a major metabolite of *L. casei* ATCC 393. Approaches from microbiology (e.g., colony forming unit enumeration), materials science (e.g., scanning electron microscopy), and pharmaceutical sciences (e.g., release assays) are employed toward offering a comprehensive framework to characterize LBP formulations. The results highlight that controlling polymer and crosslinker content modulates LBP formulations, thereby motivating research to optimize material properties of LBP delivery systems.

RESULTS AND DISCUSSION

Fabrication and Characterization of Alginate Beads

One common approach to fabricate crosslinked alginate is to introduce solubilized alginate into a solution of $CaCl_2$ crosslinker.[26] Here, we applied this technique to encapsulate *L. casei* ATCC 393 in alginate beads. Solubilized alginate with suspended *L.*

TABLE 1. Formulations with different concentrations of alginate and $CaCl_2$

Formulation #	F1	F2	F3	F4	F5
Alginate (wt vol^{-1})	2%	5%	10%	10%	10%
$CaCl_2$	1 M	1 M	1 M	0.5 M	0.1 M

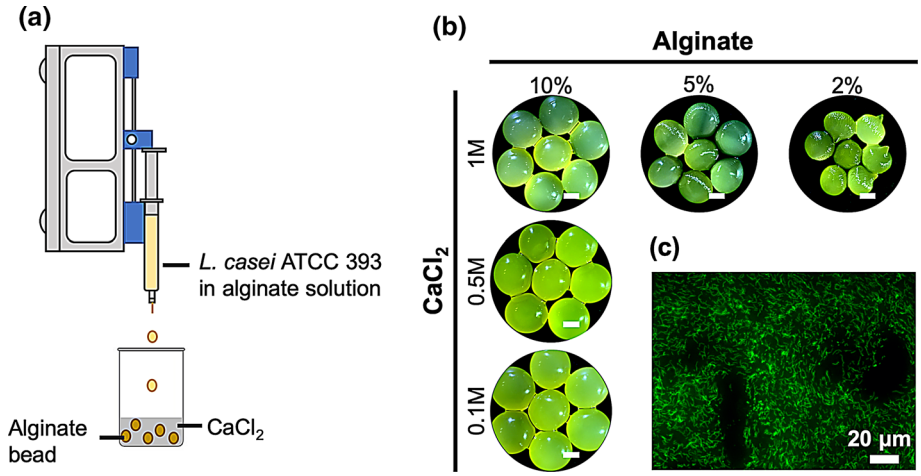

FIGURE 1. Fabrication and characterization of alginate beads. (a) Schematic of alginate bead fabrication. (b) Brightfield images of all groups of alginate beads encapsulating fluorescein-labeled *L. casei* ATCC 393. Scale bar = 1 mm. (c) Fluorescence imaging of the cross-section of an alginate bead (5% alginate, 1 M $CaCl_2$) encapsulating fluorescein-labeled *L. casei* ATCC 393.

casei ATCC 393 were loaded into syringes and added dropwise into $CaCl_2$ solution by a syringe pump (Fig. 1a). To comprehensively capture the effects of polymer and crosslinker content on LBP formulations, we investigated: (i) 2%, 5%, and 10% wt vol^{-1} alginate at 1 M $CaCl_2$, and (ii) 0.1 M, 0.5 M, and 1 M $CaCl_2$ at 10% wt vol^{-1} alginate collectively. These broad ranges of alginate and $CaCl_2$ concentrations have been described to influence aspects of alginate beads including material morphology, degradation, and substance diffusion,[33,48] thus potentially leading to distinct performance of LBP-encapsulating formulations. Fluorescein was conjugated onto the surface of *L. casei* ATCC 393 through NHS-mediated reactions to visualize encapsulation. *L. casei* ATCC 393 was successfully encapsulated into alginate beads as demonstrated by brightfield imaging of the macroscopic alginate beads (Fig. 1b) and fluorescence imaging of microscopic cross-sections (Fig. 1c). Beads with 10% alginate concentration appeared more spherical in shape as compared to counterparts fabricated with lower alginate concentrations (Fig. 1b). Detailed morphology of unencapsulated *L. casei* ATCC 393 (Fig. 2a) and *L. casei* ATCC 393-encapsulating alginate beads (Fig. 2b) were examined through scanning electron microscopy (SEM). *L. casei* ATCC 393-encapsulating alginate beads were dehydrated through treatment of gradient ethanol solutions followed by vacuum drying. Beads at 2% alginate exhibited irregular shape and looser polymer matrices with identifiable pores as compared to counterparts at higher alginate concentrations (Fig. 2b), indicating alginate concentrations regulated matrix porosity. *L. casei* ATCC 393 was individually embedded in all groups of alginate beads with clear boundaries between microbes and the encapsulating matrix (Fig. 2b), suggesting that the encapsulation did not involve strong interactions between cell walls and crosslinked alginate. The encapsulation efficiency (EE) across all the groups was above 50% (Fig. 3a). While the EE of 5% alginate group was significantly different from 2% and 10% alginate groups at 1 M $CaCl_2$, there were no monotonic correlations between EE and concentrations of alginate or $CaCl_2$ on a per-bead basis. Notably, colony forming unit (CFU) loading of *L. casei* ATCC 393 across all groups was statistically insignificant when starting with the same *L. casei* ATCC 393 concentration in the alginate suspensions (Fig. 3b). These results highlighted that alginate and $CaCl_2$ concentrations did not affect loading of *L. casei* ATCC 393.

Desiccation of formulations is a commoly used approach for long term preservation of microbes.[36] Among existing drying techniques to remove water in the formulations,[36] lyophilization is widely used to dry and formulate LBPs, including several investigational products in clinical trials[46]; as such, we have investigated how polymer and crosslinker content influences the lyophilization process. Lyophilization removes water in the LBP formulations through systematic freezing and drying steps. These processes have been reported to drastically impact morphology of hydrogels, including size and shape, which reflects their structural features and dictates performance upon rehydration such as swelling.[15,17] As such, we examined morphological changes of *L. casei* ATCC 393-free alginate beads through lyophilization and rehydration. All groups of alginate beads exhibited shrunk size, irregular shape, and wrinkled texture after lyophilization (Fig. 4a). After fabrication, and prior to

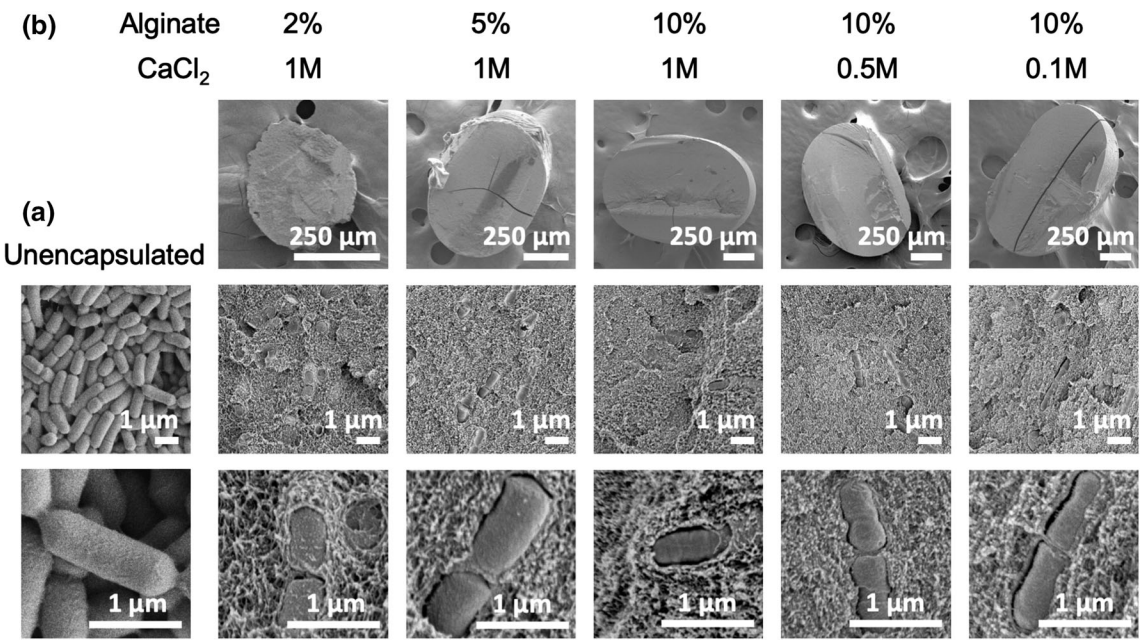

FIGURE 2. SEM images of *L. casei* ATCC 393-encapsulating alginate beads. (a) Unencapsulated *L. casei* ATCC 393. (b) *L. casei* ATCC 393 encapsulated in alginate beads with different alginate and $CaCl_2$ concentrations.

lyophilization, higher alginate concentrations led to formation of beads with larger size (Fig. 4b). The alginate-dependent size differences were mostly dictated by the size of droplets, which was governed by the liquid viscosity, surface tension, and gravity.[26] While varying $CaCl_2$ concentrations led to minimal differences in bead size at 10% alginate before lyophilization (Fig. 4a and 4b), these differences were more pronounced after lyophilization. Specifically, after lyophilization, smaller bead size demonstrated a trend with higher $CaCl_2$ concentrations, at the same alginate concentrations, during formulation (Fig. 4b). This could be explained by higher $CaCl_2$ concentrations providing more ion-crosslinked points in the matrix, eliciting stronger structural constraints toward bead shrinkage after water removal. Overall, alginate beads maintained structural integrity (Fig. 4a) and exhibited $< 50\%$ shrinkage of their original diameter after lyophilization (Fig. 4c), indicating these alginate beads possessed rigid structures. As lyophilized hydrogels may rehydrate upon administration into physiological fluids, we sought to evaluate the rehydration of lyophilized alginate beads. We incubated all groups of lyophilized alginate beads in pure water at room temperature, and observed rehydration and swelling after 30 min incubation (Fig. 4a). All rehydrated alginate beads were at least $> 60\%$ of their original, pre-lyophilized, diameter (Fig. 4d).

Lyophilization involves freezing and drying steps that can lead to microbe damage and subsequent viability loss of LBPs.[41] As such, lyoprotectants such as sugars, amino acids and proteins are often incorporated into the LBP formulations to mitigate these stresses.[36] To investigate how encapsulation with different alginate and $CaCl_2$ concentrations influenced LBP survival through lyophilization, we used skim milk as a model lyoprotectant. Skim milk contains lactose and whey proteins, and has been widely used to preserve LBP viability during lyophilization by stabilizing microbe structures.[5] *L. casei* ATCC 393-encapsulating alginate beads were pre-soaked in skim milk for 30 min at room temperature and subsequently removed from skim milk for lyophilization. Encapsulated *L. casei* ATCC 393 in most alginate bead formulations exhibited similar survival as compared to the unencapsulated control in the presence of skim milk (Fig. 5a), suggesting that crosslinked alginate allowed diffusion of skim milk into the polymer matrices and subsequent interaction of *L. casei* ATCC 393 for improving survival. Notably, beads formulated at the lowest $CaCl_2$ concentration exhibited decreased survival during lyophilization at 10% alginate (Fig. 5a). Towards explaining this, we analyzed water content of *L. casei* ATCC 393-encapsulating, skim milk-soaked, alginate beads by calculating weight loss before and after lyophilization on a per-bead basis (Fig. 5b and 5c). Higher water content in alginate beads may both increase ice formation and extend exposure to stresses during the water removal process, thus resulting in more microbe death during the lyophilization process.[1,27,31,32] We observed that alginate beads with decreased $CaCl_2$ concentrations con-

tained higher water content (Fig. 5c), which was weakly correlated to lower survival of *L. casei* ATCC 393 after lyophilization (Fig. 5a). The higher water content in alginate beads with lower $CaCl_2$ concentrations at 10% alginate was due to the increasing swelling capacity in the presence of lower crosslinking density and thus less matrix constraints.[18] While the relationship between water content of alginate beads and the survival of encapsulated *L. casei* ATCC 393 were not linear and require additional studies, this could potentially be explained by other confounding formulation differences, such as excipient distribution and material properties (e.g., stiffness) of alginate beads, which may play a role during lyophilization. Interestingly, unencapsulated *L. casei* ATCC 393 exhibited higher survival compared to encapsulated groups in the absence of lyoprotectants, indicating that $CaCl_2$-crosslinked alginate alone did not improve survival upon lyophilization (Fig. 5a). Combined together, these results highlight that polymer encapsulation is compatible with lyophilization approaches for formulating LBPs, while critical evaluation of polymer and crosslinker concentrations is needed to optimize morphology and LBP storage for developing polymer-based delivery systems.

Resistance to Simulated Gastrointestinal Challenges

As a major targeted site for LBP delivery are the intestines,[46] the upstream acidic environment in the stomach represents a considerable challenge that can decrease LBP viability and subsequently prevent their intestinal colonization.[8,9] Viability loss during gastric passage will lead to reduced exposure of LBP in the intestines, and suboptimal quantities of LBP has been attributed to compromised therapeutic outcomes in the clinic.[34] As such, it is critical to assess acid resistance of polymer-based systems toward LBP application in oral delivery. To mimic acid challenges, *L. casei* ATCC 393-encapsulating alginate beads with different alginate and $CaCl_2$ concentrations were subjected to simulated gastric fluid (SGF) at pH = 1.0 and 2.8 at 37 °C for up to 2 h. No viable *L. casei* ATCC 393 were detected for both the encapsulated and unencapsulated groups after incubation at pH = 1.0 for 1 h (Fig. 6a). However, all the encapsulated groups exhibited higher viability of *L. casei* ATCC 393 at 1 h and 2 h when incubated at pH = 2.8 compared to the unencapsulated control (Fig. 6b). Importantly, we observed that: (i) beads with 10% alginate demonstrated the highest protection against acid challenge at 2 h, regardless of $CaCl_2$ concentration, and (ii) lower alginate concentration decreased survival during acid challenge (Fig. 6b). These results could be explained by the denser polymer networks of the alginate beads that higher alginate concentrations provide, thereby likely reducing proton diffusion into the polymeric matrix. As a wide body of literature report complex macroscopic structures to improve acid resistance of delivery systems,[10,16,24] these findings highlight that simply tuning polymer concentration offers a facile approach to addressing acid challenges. The viability of encapsulated *L. casei* ATCC 393 remained comparable by changing the $CaCl_2$ concentration in the range of 0.1 M and 1 M at the fixed alginate concentration, suggesting the crosslinker concentrations did not result in detectable differences in acid resistance at 10% alginate.

In vitro Release

While no compelling evidence has yet to correlate LBP release profiles to *in vivo* therapeutic outcomes, we have previously demonstrated that slower release from an LBP depot resulted in a 1.5-fold increase of surface area coverage on ex vivo porcine intestines, as compared to bolus delivery given the same transit time.[38] Increases in surface area coverage of LBPs can

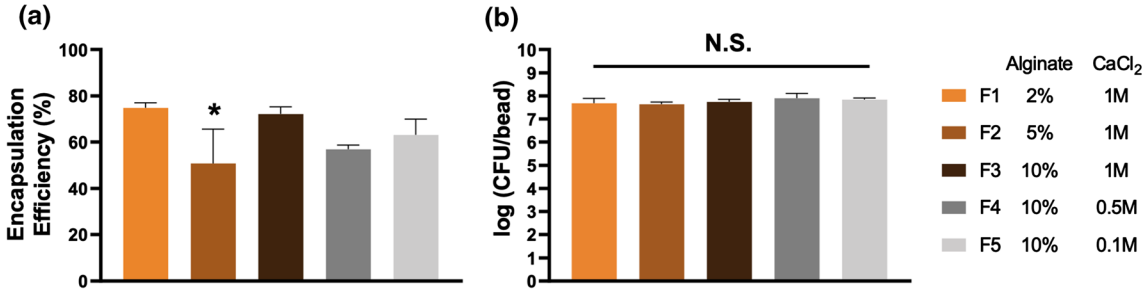

FIGURE 3. Encapsulation efficiency and CFU loading of *L. casei* ATCC 393 in alginate beads. (a) Encapsulation efficiency and (b) CFU loading of *L. casei* ATCC 393 in alginate beads with different alginate and $CaCl_2$ concentrations. Five alginate beads were analyzed in each of the three replicates (n = 3). Each error bar represents standard deviation. Statistical analysis was conducted using one-way ANOVA followed by post hoc Tukey's HSD test for pairwise comparison (statistical significance defined at $p < 0.05$). *: significantly different from F1 and F3. N.S.: not significant.

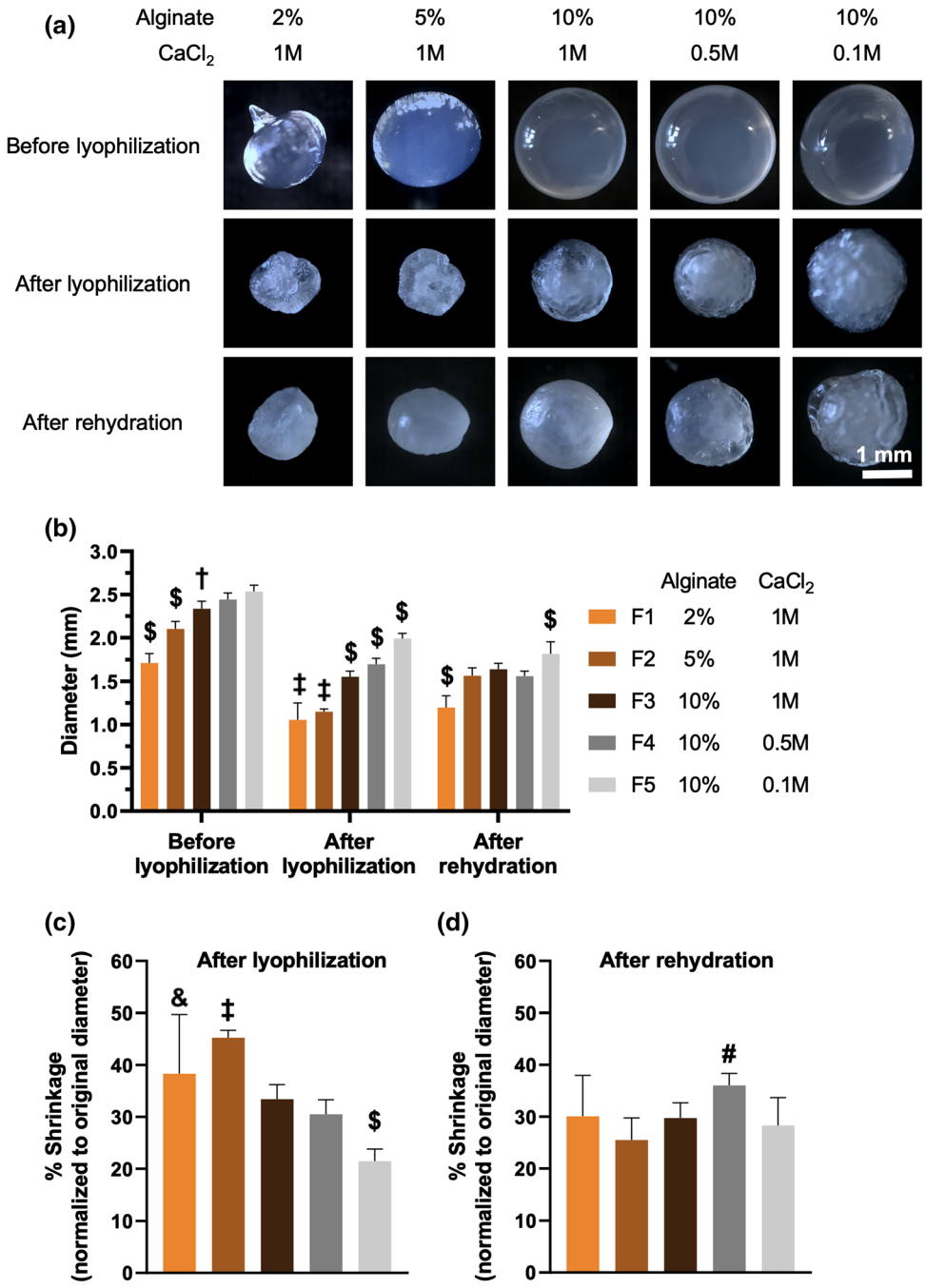

FIGURE 4. Size analysis of fresh, lyophilized, and rehydrated alginate beads. (a) Representative brightfield images of alginate beads before lyophilization, after lyophilization, and after rehydration in water for 30 min at room temperature. (b) Diameter of alginate beads throughout the lyophilization and rehydration process. (c) Shrinkage, relative to the original diameter, of alginate beads after lyophilization. (d) Shrinkage, relative to the original diameter, of alginate beads after rehydration. In (b–d), n = 10 beads. Each error bar represents standard deviation. Statistical analysis was conducted using one-way ANOVA followed by post hoc Tukey's HSD test for pairwise comparison (statistical significance defined at $p < 0.05$). $: significantly different from all other groups. †: significantly different from F1, F2, and F5. ‡: significantly different from F3, F4, and F5. &: significantly different from F4 and F5. #: significantly different from F2 and F5.

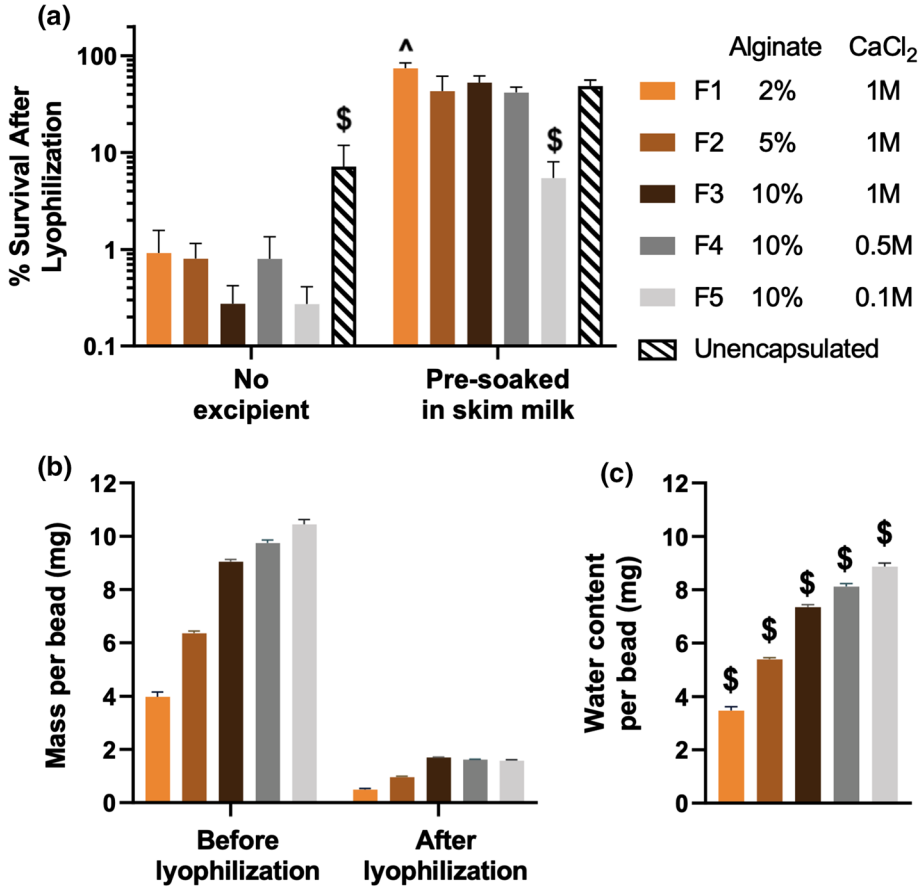

FIGURE 5. Viability and water content of lyophilized *L. casei* ATCC 393-encapsulated alginate beads. (a) Survival of encapsulated and unencapsulated *L. casei* ATCC 393 after lyophilization. Five representative beads were analyzed in each of the replicates (n = 3). (b) Mass of *L. casei* ATCC 393, skim milk-soaked, alginate beads before and after lyophilization. (c) Water content in *L. casei* ATCC 393, skim milk-soaked alginate beads. In (b) and (c), fifteen representative alginate beads were analyzed in each of the replicates (n = 3). Each error bar represents standard deviation. Statistical analysis was conducted using one-way ANOVA followed by post hoc Tukey's HSD test for pairwise comparison (statistical significance defined at $p < 0.05$). $: significantly different from all other groups. ^: significantly different from F2, F4, and F5.

potentially provide two distinct advantages *in vivo*: (i) increased interactions between LBPs and targeted surfaces (e.g., mucus, epithelium) may improve adhesion and subsequent colonization opportunities for LBPs; (ii) enhanced systemic absorption of LBP-secreted therapeutics through epithelial barriers. As such, there is a need to understand the effects of polymer and crosslinker concentrations on LBP release toward optimizing release profiles of LBP formulations. Alginate beads were incubated in simulated intestinal fluid (SIF) as a model release condition. The phosphate ions in the SIF can competitively bind with calcium ions in crosslinked alginate and accelerate bead dissolution. We found that higher alginate concentrations prolonged release of *L. casei* ATCC 393 from the alginate beads, leading to 3 distinct profiles with 75% cumulative release at 2 h, 4 h, and 6 h (Fig. 7). Similarly, we observed higher $CaCl_2$ concentrations also prolonged release (Fig. 7). Notably, F2, F3, and F4 exhibited biphasic release profiles (initial slow release followed by rapid release), implying dominance of bulk erosion during bead dissolution. The distinct release properties of LBP delivery systems may be useful in controlling LBP distribution at sites of interest *in vivo*. While many approaches exist to manipulate LBP release, such as modulating material choice and geometry,[38] these results highlighted that varying polymer and crosslinker concentrations is also capable of tuning LBP release profiles, thus offering new insights toward achieving controlled release of LBPs. Future efforts need to focus on elucidating how different release profiles impact LBP performance *in vivo*, which will pave the way for translating these polymer-encapsulated LBP delivery systems to clinical application.

While polymer encapsulation enables acid protection and controlled release, it may also impact critical substance exchange for encapsulated LBPs, such as

nutrient uptake and metabolite secretion. Notably, metabolites are widely recognized as important mediators of LBPs that modulate microbiome and host physiology.[42] L-lactate, one major metabolite of *L. casei* ATCC 393, has been reported to render anti-inflammatory effects in mammalian intestines.[19] As such, we sought to quantify the effects of polymer encapsulation on L-lactate secretion of encapsulated *L. casei* ATCC 393. We immersed approximately 10^8 CFU encapsulated *L. casei* ATCC 393 in DeMan-Rogosa-Sharpe (MRS), a standard growth media for lactobacilli, at 37 °C for 1 h (Fig. 8a). To rule out the confounding L-lactate secreted by free *L. casei* ATCC 393 that were released from the alginate beads during incubation, we also examined viability of *L. casei* ATCC 393 in the supernatant (Fig. 8b). Quantitatively, nearly 100% of viable *L. casei* ATCC 393 remained encapsulated in the alginate beads at 1 h (Fig. 8c), implying that the L-lactate secreted by free *L. casei* ATCC 393 was negligible. We then quantified the L-lactate content in the alginate beads and supernatant (Fig. 8d). Over 70% of total L-lactate was distributed in the supernatant across all groups (Fig. 8e), suggesting crosslinked alginate allowed diffusion of metabolites into the external microenvironment. To account for the variance of CFU when comparing secreted L-lactate content across different groups, L-lactate in the supernatant was normalized to the total CFU in the corresponding group. No significant difference of L-lactate secretion was observed across groups of encapsulated and unencapsulated *L. casei* ATCC 393 (Figure 8f), highlighting that key metabolic activities, and secretion of metabolites, were not influenced by polymer and crosslinker concentrations. These results indicated that encapsulated *L. casei* ATCC 393 retained the capability of modulating the surrounding environment through metabolite secretion. Considering that L-lactate is a small molecule, this study will inspire future work on biologic-secreting LBPs, as biologics are larger in size and thus their release into the microenvironment from microbial hosts within alginate beads will be more likely to be impacted by polymer matrices as compared to small molecules. In addition, emerging efforts use encapsulation strategies to achieve biocontainment (preventing escape into external environment and avoiding safety concerns) of genetically engineered microbes while still allowing substance diffusion to support functions of these microbes.[44] As such, our study will motivate evaluation of material content as an important parameter in designing delivery systems that aim to accomplish biocontainment and secretion-related functions of encapsulated microbes simultaneously.

CONCLUSION

In summary, by tuning alginate and $CaCl_2$ concentrations, we demonstrate that polymer and crosslinker content modulates performance of polymer-based LBP delivery systems (Table 2). While alginate has been used for encapsulating probiotics preclinically for decades,[10] alginate-encapsulated LBPs are not currently approved for clinical use; existing LBPs in clinical trials are typically suspensions or lyophilized powders.[46] Although alginate delivery systems have been widely studied and even advanced into the clinic for other living therapeutic modalities, for example alginate encapsulation of mammalian cells (e.g., islet beta-cells) for transplantation,[40] systematic characterization of alginate-encapsulated LBPs has yet to be performed. Importantly, unlike widely studied alginate delivery systems for mammalian cell transplantation into subcutaneous tissues, encapsulated LBPs are

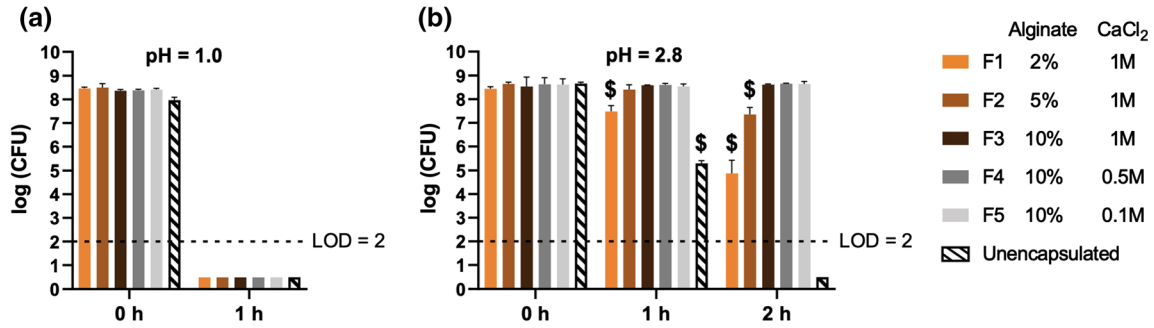

FIGURE 6. Acid challenge of *L. casei* ATCC 393 in simulated gastric fluid (SGF). (a) Viability of *L. casei* ATCC 393 at pH = 1.0. (b) Viability of *L. casei* ATCC 393 at pH = 2.8. The unencapsulated group was prepared by directly suspending *L. casei* ATCC 393 in 1 mL of either solution. Five representative alginate beads were analyzed in each of the replicates (n = 3). Each error bar represents standard deviation. At each time point, statistical analysis was conducted using one-way ANOVA followed by post hoc Tukey's HSD test for pairwise comparison (statistical significance defined at $p < 0.05$). $: significantly different from all other groups. LOD: limit of detection.

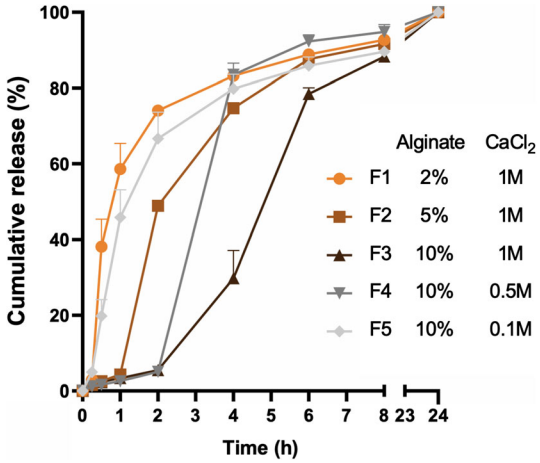

FIGURE 7. Tunable release of *L. casei* ATCC 393 from alginate beads in simulated intestinal fluid at 37 °C. Five representative alginate beads were analyzed in each of the replicates (n = 3). Each error bar represents standard deviation.

predominately designed for oral delivery.[28] As such, compared to challenges (e.g., immunological response, fibrosis) facing the subcutaneous administered alginate systems for mammalian cell transplantation,[12] the unique microenvironment and distinct delivery requirements of the GI tract may lead to LBP-specific opportunities for clinical translation of alginate systems.[20] Therefore, evaluating and describing the effects of alginate and crosslinker content on encapsulated LBPs may support the eventual rational design of clinically relevant LBP formulations in future applications. Additionally, considering ongoing research of other polymer-based LBP formulations (e.g., gelatin)[2] and strategies (e.g., microbial surface modification),[4,47] efforts to provide a comprehensive framework toward characterizing performance of LBP formulations may motivate future research into how material properties influence encapsulated LBPs in different formulations.

MATERIALS AND METHODS

Materials

Sodium alginate and ethanol were purchased from Sigma-Aldrich (Missouri, USA). Calcium chloride ($CaCl_2$), sodium hydroxide, sucrose, glutaraldehyde, phosphate-buffered saline (PBS), acetone, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Massachusetts, USA). *Lactobacillus casei* ATCC 393 (*L. casei* ATCC 393), NHS-fluorescein, and DeMan-Rogosa-Sharpe (MRS) broth were purchased from Thermo Scientific (California, USA). MRS agar and skim milk powder were purchased from Becton, Dickinson and Company (New Jersey, USA). Simulated gastric fluid, enzyme-free simulated intestinal fluid (SIF), LB broth, and agar were purchased from VWR (Pennsylvania, USA).

Methods

Bacteria Growth and Quantification

L. casei ATCC 393 was inoculated from glycerol stocks into autoclaved MRS broth and incubated overnight statically at 37 °C. Before use, bacteria were collected *via* centrifugation at 4000 rpm for 10 min at room temperature and washed once in sterile water. OD_{600} values were determined by GENESYS 30 visible spectrophotometer (Thermo Scientific, California, USA) with bacteria-free media subtracted. To quantify bacterial viability, aqueous samples were serially diluted, and drop-plated (10 μL) on MRS agar. Colony forming units (CFUs) were enumerated after incubation of agar plates at 37 °C for 48–60 h. To obtain each replicate of per-bead viability, five alginate beads were first homogenized in 1 mL sodium citrate (0.055 M); viability in the homogenized suspension was quantified as described above and divided by five as the per-bead viability.

Fabrication of Alginate Beads

L. casei ATCC 393 (0.3–1 $\times$ 10^{10} CFU/mL, final concentration) was added into alginate solutions (2%, 5%, and 10% wt vol^{-1}) followed by vortexing to obtain homogenous suspensions. Then the suspensions were introduced dropwise into 0.1 M, 0.5 M or 1 M $CaCl_2$ solutions (stirred at 60 rpm) at the speed of 10 mL/h through a 27-gauge needle, powered by a syringe pump (Fisher Scientific, Massachusetts, USA). After hardening in the $CaCl_2$ solutions for 20 min, alginate beads were collected with cell strainers (40 μm mesh filters) and briefly washed in sterile water once before further experiments. To encapsulate fluorescein-labeled *L. casei* ATCC 393 in the alginate beads, bacteria were first incubated in 1 mg/mL NHS-fluorescein in DMSO-PBS ($Vol_{(DMSO)}$: $Vol_{(PBS)}$ = 1:9) solution at room temperature for 1 h. Then the bacteria were pelleted through centrifugation and washed in PBS three times to remove free NHS-fluorescein before addition into alginate solutions.

Characterization of Alginate Beads

For fluorescence imaging, alginate beads (5% wt vol^{-1} alginate, 1 M $CaCl_2$) encapsulating fluorescein-labeled *L. casei* ATCC 393 were incubated in 10% vol vol^{-1} formaldehyde overnight at room temperature and then dehydrated in 30% wt vol^{-1} sucrose solution

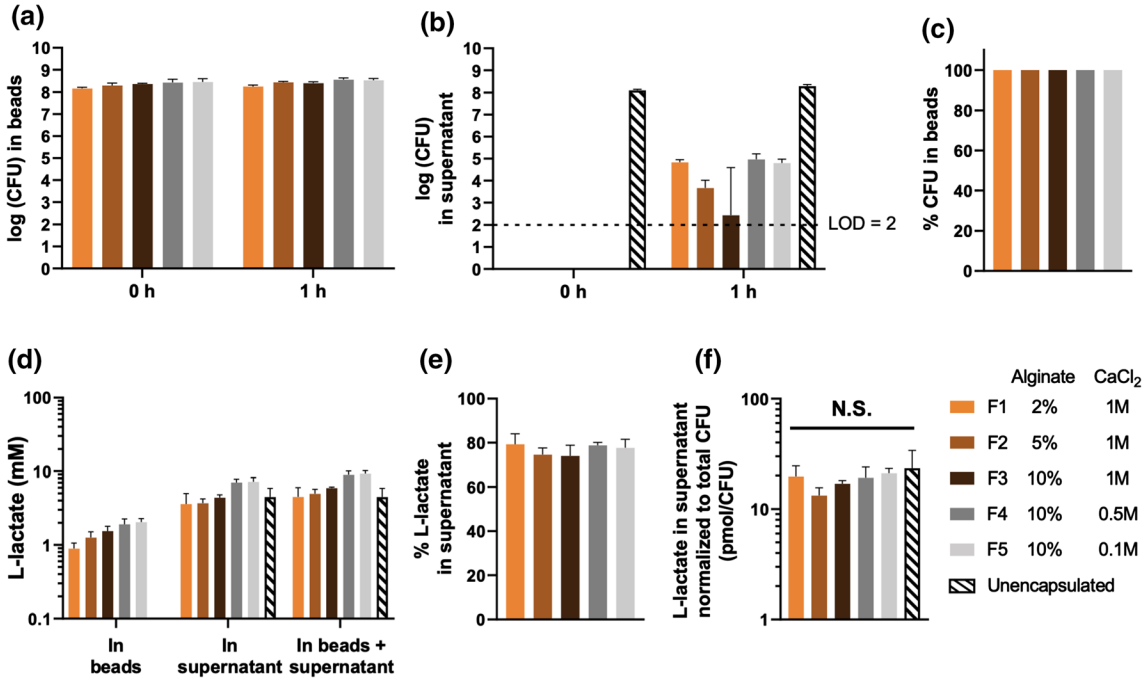

FIGURE 8. L-lactate secretion from *L. casei* ATCC 393-encapsulating alginate beads. (a) Viability of *L. casei* ATCC 393 in the alginate beads. (b) Viability of *L. casei* ATCC 393 in the supernatant. (c) Percentage of viable *L. casei* ATCC 393 in the alginate beads as compared to the summation of CFU in the alginate beads and supernatant at 1 h. (d) L-lactate content in the alginate beads and supernatant at 1 h. (e) Percentage of L-lactate in the supernatant as compared to the summation of L-lactate in the alginate beads and supernatant. (f) L-lactate content in the supernatant normalized to the summation of CFU in the alginate beads and supernatant. Five representative alginate beads were analyzed in each of the replicates (n = 3). Statistical analysis was conducted using one-way ANOVA (statistical significance defined at $p < 0.05$). N.S.: not significant. LOD: limit of detection.

for 24 h. Next, alginate beads were frozen in optimal cutting temperature compound (Sakura Tissue-Tek) on dry ice and stored at -80 °C overnight. Samples were sliced into pieces with 3 μm in thickness and imaged with a fluorescence microscope. To obtain scanning electron microscopy (SEM) images, alginate beads were first fixed in glutaraldehyde (2.5% vol vol^{-1}) for 1 h at room temperature followed by treatment with each of the gradient ethanol solutions (50%, 70%, 90%, and 100% vol vol^{-1} in water) for 10 min at room temperature. Then alginate beads were mounted onto a SEM stub with adhesive carbon tape and stored in a vacuum desiccator before imaging. Alginate beads were sectioned by a razor blade to expose cross-sections. Plain *L. casei* ATCC 393 was prepared by resuspending glutaraldehyde-treated and dehydrated *L. casei* ATCC 393 in acetone and loading an aliquot on a stub until complete solvent evaporation.

Encapsulation Efficiency (EE)

0.5 mL (V) alginate solution containing fluorescein-labeled *L. casei* ATCC 393 was used for bead fabrication as described above and the number (N) of alginate beads fabricated with the 0.5 mL liquid was recorded. Then five alginate beads were incubated in 1 mL (V_1) sodium citrate (0.055 M) for 15 min followed by manual homogenization. The bacterial concentration in the homogenized sodium citrate solution (C_1) and the original bacterial concentration in alginate solution (C) were indicated by the fluorescence signal on a plate reader at excitation/emission = 487 nm/528 nm, respectively. EE = $(C_1V_1N)/(5CV) \times 100\%$.

Lyophilization

For morphology analysis, *L. casei* ATCC 393-free alginate beads were snap frozen in liquid nitrogen and then dried on a benchtop lyophilizer overnight. Lyophilized alginate beads were rehydrated in water for 30 min at room temperature. Diameter of alginate beads, calculated as the average of three measurements in orthogonal directions, was measured by a digital caliper (World Precision Instruments, Florida, USA). To evaluate survival upon lyophilization, *L. casei* ATCC 393-encapsulating alginate beads were immersed in skim milk (12% wt vol^{-1}) for 30 min at room temperature, then separated from skim milk, and lyophilized as described above. Alginate beads without incubation in skim milk were lyophilized for compar-

TABLE 2. Effects of alginate and $CaCl_2$ concentrations on performance of *L. casei* ATCC 393-encapsulating alginate beads

	Alginate (2%, 5%, and 10% wt vol^{-1})	$CaCl_2$ (0.1 M, 0.5 M, and 1 M)
Size	Higher alginate concentrations led to larger size	Lower $CaCl_2$ concentrations led to larger size
Shape	Higher alginate concentrations led to more spherical shape	Comparably spherical
Encapsulation efficiency	> 50%, comparable across all groups evaluated	
CFU Loading	Comparable across all groups evaluated	
Post-lyophilization/ rehydration morphology	Comparable across all groups evaluated: (i) < 50% shrinkage of original diameter post lyophilization, (ii) > 60% of original diameter post rehydration at 30 min, (iii) irregular shape post lyophilization and rehydration	
Survival upon lyophilization	Comparable	Lower $CaCl_2$ concentrations decreased LBP survival
Acid resistance	(i) comparable at pH = 1.0, (ii) higher alginate concentrations improved LBP survival at pH = 2.8	Comparable
Release	Higher alginate concentrations slowed release	Higher $CaCl_2$ concentrations slowed release
L-lactate secretion	Comparable across all groups evaluated	

ison. Unencapsulated *L. casei* ATCC 393 were directly lyophilized in water or skim milk (12% wt vol^{-1}). *L. casei* ATCC 393 in pre-lyophilized formulations were considered as 100% viability to calculate post-lyophilization survival. Water content of *L. casei* ATCC 393-encapsulating, skim milk-soaked alginate beads was calculated as the weight loss before and after lyophilization on a per-bead basis.

Acid Challenge

Five alginate beads of each group were incubated in 1 mL SGF (pH = 1.0 or pH = 2.8, pH adjusted with 1 M sodium hydroxide) at 37 °C. At indicated time points, alginate beads were removed from SGF and the viability was quantified as described above.

Controlled Release

Five alginate beads encapsulating fluorescein-labeled *L. casei* ATCC 393 were incubated in 5 mL filter-sterilized SIF at 37 °C under rotation. At indicated time points, the release media containing alginate solids was filtered through a cell strainer (40 μm) and the collected solids in the strainer were transferred into 5 mL fresh SIF for continuous release study. 200 μL of the filtrate was read on a plate reader (excitation/emission = 487 nm/528 nm) to quantify bacterial release.

L-Lactate Measurement

Five alginate beads of each group were incubated in 1 mL MRS at 37 °C. At indicated time points, MRS was removed and centrifuged at 8000 rpm for 1 min to pellet down released bacteria. L-lactate in the supernatant was quantified with an EnzyChrom™ Lactate Assay Kit (BioAssay Systems, California, USA) according to the instruction manual. To quantify the L-lactate within alginate beads, alginate beads were isolated and homogenized in 1 mL sodium citrate (0.055 M). Then the suspension was pelleted at 8000 rpm for 1 min and L-lactate in the supernatant was quantified as described above. Unencapsulated *L. casei* ATCC 393 in MRS was used as control.

Statistical Analysis

Experiments were run in triplicate and data were presented as mean ± SD unless otherwise noted. Parametric one-way ANOVA and post hoc Tukey's HSD test were used to evaluate significant differences, as indicated in relevant figure legends. $\alpha = 0.05$. P values less than 0.05 were considered significantly different. All statistical analysis was performed in Prism (version 8.4.3, Graphpad Software, LLC).

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CONFLICT OF INTEREST

KQ., Y.H., and A.C.A. declare that they have no conflict of interest.

ETHICAL APPROVAL

No animal studies were carried out by the authors for this article. No human studies were carried out by the authors for this article.

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