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All Bio-Based μ-Beads from Microalgae for Probiotics Delivery

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The food of the future aims to offer several essential qualities: i) environmental respect in sourcing raw materials; ii) sustainable transformation processes; iii) enrichment with probiotic microorganisms; iv) biocompatible natural matrices. These features both enhance the nutritional value of the food and improve its pharmacological and immunological properties. When probiotics are introduced into gut in adequate densities through diet, they symbiotically promote health by boosting immune defense, producing beneficial organic molecules, and providing essential metabolic pathways for better nutrient assimilation and biotransformation. A major challenge with probiotics is their low resistance to gastrointestinal (GI) transit due to pH and other adverse hydro-ionic conditions affecting their viability. Here, diatom microalgae (*Coscinodiscus granii*) is presented as a natural source of micro-pills, functionalized with biopolymers (Shellac and Chitosan) for enteric protection, with a loading value of 71 ± 7%, higher in comparison with the loading capacity tested for two other commercial polymers. Moreover, biosilica embedded and sealed with the enteric polymers best-protected probiotics under pH changes, and thermal and storage stresses by one-fold more than the control probiotics without or with the lone shielding polymers. These work outcomes describe envisaging silica hybrid microcarriers obtained from living microalgae, effectively protecting probiotics in an entirely biological formulation.

1. Introduction

The term *microbiota* defines the symbiotic polymicrobial community (viruses, bacteria, archaea, protozoa, yeasts, and molds) colonizing various organs and tissues of the mammalian body. Skin, oral cavity, digestive tract, and genital organs are heavily populated by microorganisms, and over 70% of them are mainly found in the gut. Specific diseases, an unbalanced diet, and daily stress agents can alter the delicate linkage between host and microbiota, leading to a medical condition called dysbiosis.[1] These negative outcomes can be treated recurring through the administration of probiotics, with preventive and curative effects. A living probiotic is an active microorganism administered as a food excipient, or drug, in adequate amounts, according to the World Health Organization, and subsequently in agreement with panels.[2] Living probiotic formulations should preserve the microbe's viability, especially during the storage processing and against thermal shock

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events.[3] Once implemented in sustainable foods, made of conventional foods enriched with alternative, additional natural matrices, probiotics should overcome the normal gastrointestinal transit, and then they will reach precise districts in order to trigger favorable effects on the consumers. Probiotic enrichment of food can be performed via food aging, fermentation, and maturation. The produced molecules and developed living microorganisms become so crucial and beneficial for the human body, leading to a positive and ready immune response of the host.[4]

Actually, for the probiotics, one of the main requirements involves the evaluation of their cell viability from production to oral administration, together with consumption and after the GI transit. Selection criteria, efficacy, safety issues, and GI resistance have attracted considerable attention as sustainable criteria for the food supplements of the future.[5] For instance, probiotics coming from nondairy matrices undergo treatments of food preservation with temperatures above 40 °C and storage cold conditions, strongly affecting the viability.[6] For this reason, several systems have been developed to preserve the viability of probiotics during long/short-term storage and improve their delivery. Among innovative strategies, encapsulation provides protection against adverse stressors.[7] Usually, the encapsulation consists of producing deposition layers or entrapping macro-matrices which contain and protect bacteria.[8] Materials exploited for encapsulation undergo specific manufacture procedures (nuzzling extrusion,[9] coacervation,[10] bead fluidification,[11] electrospraying, and spray-chilling[12]) to produce micro- to millimeter bead-like structures which physically entrap the microorganisms. So here a critical aspect is represented by the manufacture and the shaping of the micron-to-millimeter-containing structures. Without these technologies, materials often form amorphous macro-aggregates with low loading and delivery performances. However, most of these methods are time-consuming and expensive. More promising materials are those based on microparticles that contain probiotics, and are subsequently covered with carrier matrices with gastro-enteric protecting properties, based on polysaccharides and complex natural biopolymers, such as alginate,[13] carrageenan,[14] and chitosan.[15] Nevertheless, these materials again should be molded to produce semi-spherical or molten globule-containing structures.

In this paper, we overcome the step of producing the microencapsulating structures for probiotics by using the readily available, round-shaped, empty biosilica membrane directly extracted from the µ-sized *Coscinodiscus granii* diatom species. Interestingly, micro-sized cell empty structures obtained from bacteria, yeast, fungi, and nano-sized viruses, have been already reported in the literature, and ad hoc exploited as virtual spaces confining diverse organic or inorganic composites, or catalysts.[16] The cell inside, made of organic matter and water, is usually removed via cell dehydration, and the external membrane structures can be stabilized, or cross-linked, to form containing cavities or a reactive environment. For instance, empty natural structures from microorganisms can act as bioreactors for producing nanomaterials. These examples include virus[17] for metal nanoparticles synthesis, green microalgae,[18] like Spirulina (i.e., *Arthrospira platensis*), for the production of Pd@Ag core:shell nanoparticles, and model bacteria[19] for the synthesis of gold nanomaterials. Cell empty cages are presented also as promising materials for new-generation batteries. In fact, innovative Lithium–Sulfur battery cathodes based on bipolar microcapsules, obtained from empty *Staphylococcus aureus* cells, have been reported in literature enriching the world of functional biohybrids.[20] As external membranes, diatom frustules have been hugely used for producing materials, either in pristine forms or upon in vivo or in vitro functionalization with a plethora of functional molecules, resulting in hybrid microsystems with applications ranging from biomedicine[21,22] and drug delivery,[23] to photonics[24,25] and imaging.[26]

In this work, we present an easy and sustainable way to use the box-like silica structure of *Coscinodiscus granii* diatoms as porous containers for living probiotics. The process starts with inducing bacteria to enter across the micropores present on the nanostructured surface of diatoms by means of slight vacuum gradients. After this loading, a combination of the natural soft Shellac and Chitosan polymers was used as embedding polymer matrices sealing the microalgae shells.[27] Shellac (S) is an enteric polymer derived from the hardened secretion of the insect *Kerria lacca* found on trees in Asian countries,[28] already exploited for its gastro-protective resistance. Chitosan (CS) is a polymer with a cationic character found in the cell walls of some fungi and crustaceans, with applications in the fields of cosmetics, biotechnology, microbiology, and biomedicine. It is able to form stable gels once in contact with poly-anions, like polyphosphate, alginate,[29] or Xanthan gum. In this work, Chitosan is used in combination with Shellac for efficiently closing diatom-based microcapsules loaded with bacteria dispersions due to the formation of physically cross-linked hydrogels via ionic linkages between negative Shellac and positive Chitosan over silica shell surfaces. The paper shows intriguing results about the physical entrapment of probiotic bacteria directly into silica shells. Then, the combination of the abovementioned sealing polymers confers probiotics a certain resistance to harsh conditions, like simulated digestive solutions, storage, and thermal shocks, paving the way to an all-bio-based solution as bacterial supplements for future food.

2. Results and Discussion

The production of micro-beads (or pills) extracted from *Coscinodiscus granii* diatom cells and their functionalization involves different processes. After a simple extraction protocol, biosilica can be obtained integer from living giant diatom cells. The collected 3D boxes are induced to act as micro-containers for living probiotics using vacuum-assisted pumping, and an electrostatic co-precipitation of the biopolymers Chitosan and Shellac on the biosilica surface is performed to ensure the production of a fully bio-based, organic coating with pH-dependent tunable gate properties (see **Scheme 1**), aiming to a controlled release of the probiotics. The sequences of production of these systems are summarized in Scheme 1.

2.1. Diatoms Culture and Biosilica Purification

As reported in detail in the Experimental Section and Supporting information section (Diatoms culture and biosilica extraction), *Coscinodicus granii* diatoms (CCAP strain 1013/4) were cultured for 8 weeks and cells were then collected 24 h before the

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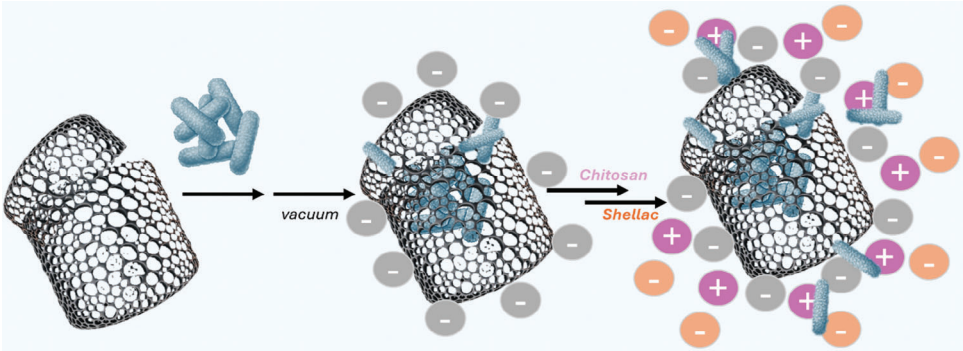

Scheme 1. Production of biosilica-based micro-containers for probiotics, from vacuum-assisted insertion of microorganisms to the electrostatic co-precipitation of the biopolymers, to ensure a tunable enclosing depending on the pH. Generalities about the scheme: biosilica with pores and microscopic opening points for probiotics; electrostatic elements given by the surface silanol moieties of the biosilica (negatively charged grey balls), the ammonium moiety from Chitosan (positively charged pink balls) and the carboxylate functions belonging to the acidic residues of Shellac (negatively charged orange balls).

cleaning procedures via overnight sedimentation in conic falcon. The starting point was the comparison of two extraction methods to produce 3D biosilica containers for probiotics loading: one method based on hard acid/oxidative treatment and the other one performed with detergent only. Scan electron microscopy (SEM) was performed to evaluate the efficiency of the two isolation methods. Comparing the two methods in **Figure 1** (a: acid-oxidative method; b: detergent method), only the SDS-based protocol led to the production of 3D box-like, empty substructures of biosilica, with entering points for probiotics easily visible in Figure 1bi,bii. On the contrary, the acid/oxidative cleaning protocol affords 2D, totally separated biosilica subparts which are not useful for probiotics upload (Figure 1ai,aii). The different content in organic matter was confirmed by using FTIR-ATR

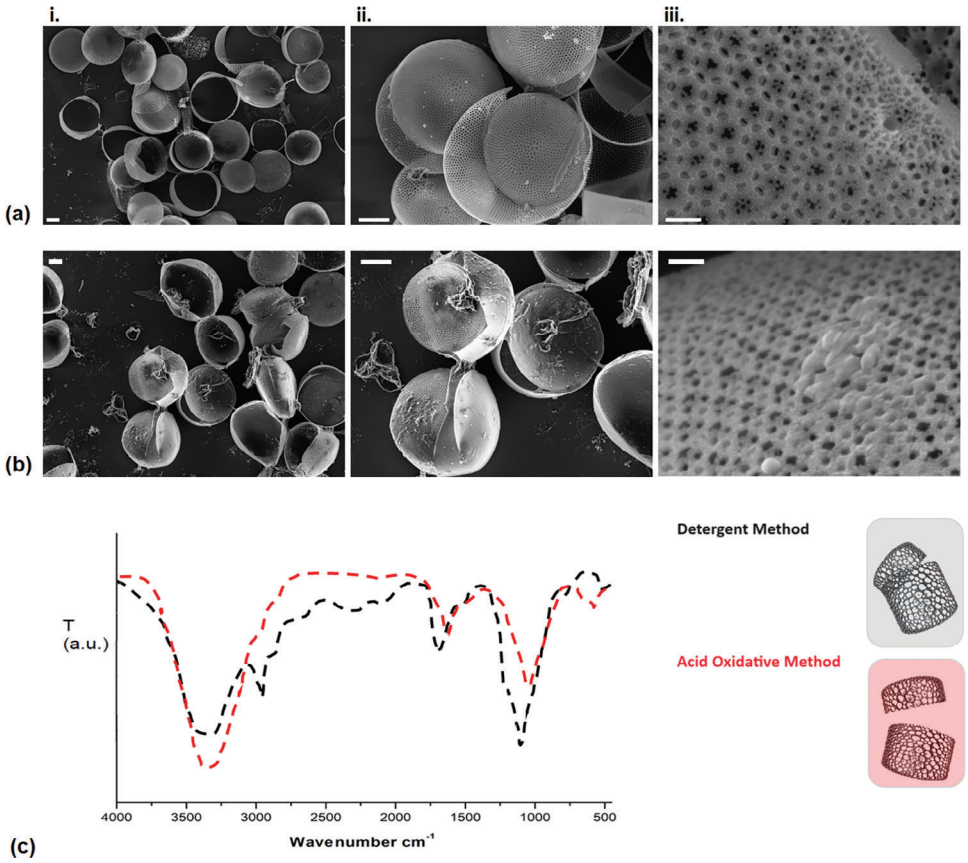

Figure 1. Scan electron microscopy pictures at 3 different magnification levels (i–iii.) of acid-oxidative a) and detergent-based b) extraction methods; FTIR-ATR spectra of biosilica extracted using acid-oxidative and detergent protocols c). Scale bars: i: 20 μm; ii: 20 μm; iii: 1 μm.

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spectroscopy. After SDS cleaning, —CH stretching signals set at 2994–2980 cm^{-1} are still visible in the spectrum, underlining the presence of residual organic material. The residual organic material is probably responsible for keeping the shell components (valves, girdle) still organized as empty box-like structures. High-resolution SEM microscopy pictures (Figure 1a,b iii) show that both methods of cleaning are not detrimental to the periodic disposition of pores in the silica lattice. This property is fundamental to enable probiotics exchange of chemicals with the external environment once microencapsulated.

2.2. Biosilica µ-Beads Preparation for Containing and Protecting Probiotics

The bare biosilica (without the bacterial load) is a versatile material capable of acting as a negatively charged surface which induces the precipitation of positively charged Chitosan and negatively charged Shellac via electrostatic layer-by-layer structure formation (Scheme S1, Supporting Information). Shellac was chosen as the material that can act as an enteric biopolymer, suitable for overcoming gastro-intestinal pH barriers, and sparking a thermo-protection for different biomedicine purposes, as reported in the literature.[30] This polymer is a natural resin, with low molecular weight, extracted from the lacquer insect. It is a biocompatible, gastro-, and heat-resistant polyester that is soluble at intestinal pH; it has already been used for intestinal delivery of probiotic microorganisms. In Figure S1b,c (Supporting Information), FTIR-ATR was used to investigate the presence of typical organic functional groups in lone biosilica, and related composites with Shellac and Shellac/Chitosan. The simultaneous presence of —CH stretching (2929, 2899 cm^{-1}), belonging to aleuritic, shelloic, and jalaric acid of Shellac, the —NH signal (3298 cm^{-1}) due to chitosan glucosamine, and —Si—O strong peak (1159 cm^{-1}) in biosilica, can be the major moieties found in the trifunctional composite and underlined by IR technique. Additionally, further investigation on surface area (BET, see Experimental Section) was performed on polymer-embedded biosilica, revealing a significative reduction in area values passing from bare biosilica to coated shells. In details, purified biosilica exhibits a surface area set at 21.59 $m^2\ g^{-1}$, which decreases to 16.08 $m^2\ g^{-1}$ for chitosan-decorated biosilica, till 11.44 $m^2\ g^{-1}$ once shells are double coated, first with chitosan and then with shellac.

UV–vis spectroscopy was exploited to study the degradability of the Shellac polymer exposed to different pH. This was possible by analyzing the dissolution of the intestinal pH using a range of 220–260 nm detection.[31] In this range, the natural special monomer shelloic acid, together with the jalaric acid, can be recorded and used to investigate the Shellac degradation, which is not hugely affected (at intestinal tract pH) by the presence of chitosan and shells. Shellac polymer solubilized in ethanol was used as a control for checking the maximal dissolution (48 h under stirring at 55 °C). With respect to the polymer subjected to model intestinal pH (7.8), the opening of the polymer structure and the related release of the monomers into solution appear less evident at model gastric pH (3).

The strategy for encapsulating bacteria into diatoms biosilica and then protecting this system with Chitosan and Shellac is based on a combination of vacuum entrapment and surface precipitation of the two polymers. This is due to the fact that the negatively charged biosilica surface induces the precipitation of positively charged Chitosan and negatively charged Shellac via electrostatic sandwich formation (as already discussed in Figure S1, Supporting Information). For the encapsulation experiments, a commercial preparation named SYNBIO®, composed of two gastro-resistant strains of probiotics: *Lactobacillus rhamnosus* IMC 501® and *Lactobacillus paracasei* IMC 502® has been chosen in 1:1 ratio. A culture medium (MRS Broth medium) was used to resuspend lyophilized cells of the probiotic mixture, which were twice incubated for 18 h at 37 °C to reach the exponential phase of growth. Once the exponential phase, 20 µL of this microbial suspension ($10^{7\text{-}10}$ cells mL^{-1}) were put in the fundus of conic vials. The biosilica preparation (**Figure 2a**) was then dropped on bacteria (20 µL, 270 shells/µL). The physical entrapment of the bacterial preparation in biosilica was performed by mixing these two suspensions and then performing vacuum pumping (see Experimental Section). Once diatom shells are loaded with bacteria dispersions, chitosan solution in acidic water and then a solution of pre-hydrolyzed Shellac were added. The choice of the ratio between the quantity of polymers and the density of silica shells has been extensively investigated in terms of probiotics loading efficiency. Each loading experiment was performed by labeling the bacteria with Cy3-NHS, producing a calibration curve (density of bacteria VS fluorescence intensity, Figure S2, Supporting Information), and comparing the fluorescence intensity of dispersions before and after the encapsulation process (Supporting Information). The investigation started by varying the amount of Chitosan necessary with a fixed quantity of Shellac to have a good loading % (see Figure S3, Supporting Information). Subsequently, with a constant Shellac/Chitosan ratio, we ascertained that a density of 270 shells μL^{-1} was enough not to decrease the loading capacity (see Figure S4a, Supporting Information). In the case of using 1800 and 5400 shells from *Coscinodiscus granii* (CG), there was a slight increase in loading capacity.

After a gentle rotation and mixing in the tube fundus, stable gels were formed due to the contact of bacteria in biosilica with the serially added positively charged Chitosan and negatively charged Shellac. In this case, the *sealed* microcapsules have been obtained due to the formation of physically cross-linked hydrogels via ionic linking (Figure 2b–d). Arrows in Figure 2 indicate the single bacteria cells. Moreover, Figure 2c shows the control samples of bacteria with neat polymers without diatom shells. The bacteria loading capacity of Shellac:Chitosan with biosilica was set at 71 ± 7%, higher than the loading capacity tested for other commercial 2 polymers: gelatin and hydroxypropyl methylcellulose (G, HPMC, Figure S5, Supporting Information). Moreover, as reported in Figure S4b (Supporting Information), the loading capacity strongly decreased to 44%, once the biosilica derived from the harsh cleaning methods was combined with the polymers instead of the silica box obtained from the soft purification protocol based on detergent. Related SEM pictures are reported in Figure S4c,d (Supporting Information).

In order to visualize bacteria cells during the experimental procedures, and to qualitatively test their viability, a fluorescein diacetate incubation and successive 2D fluorescence microscopy have been performed on all the proposed systems. Inset i. (Figure 2a) shows the auto-fluorescent biosilica shell, while ii. and iii. show

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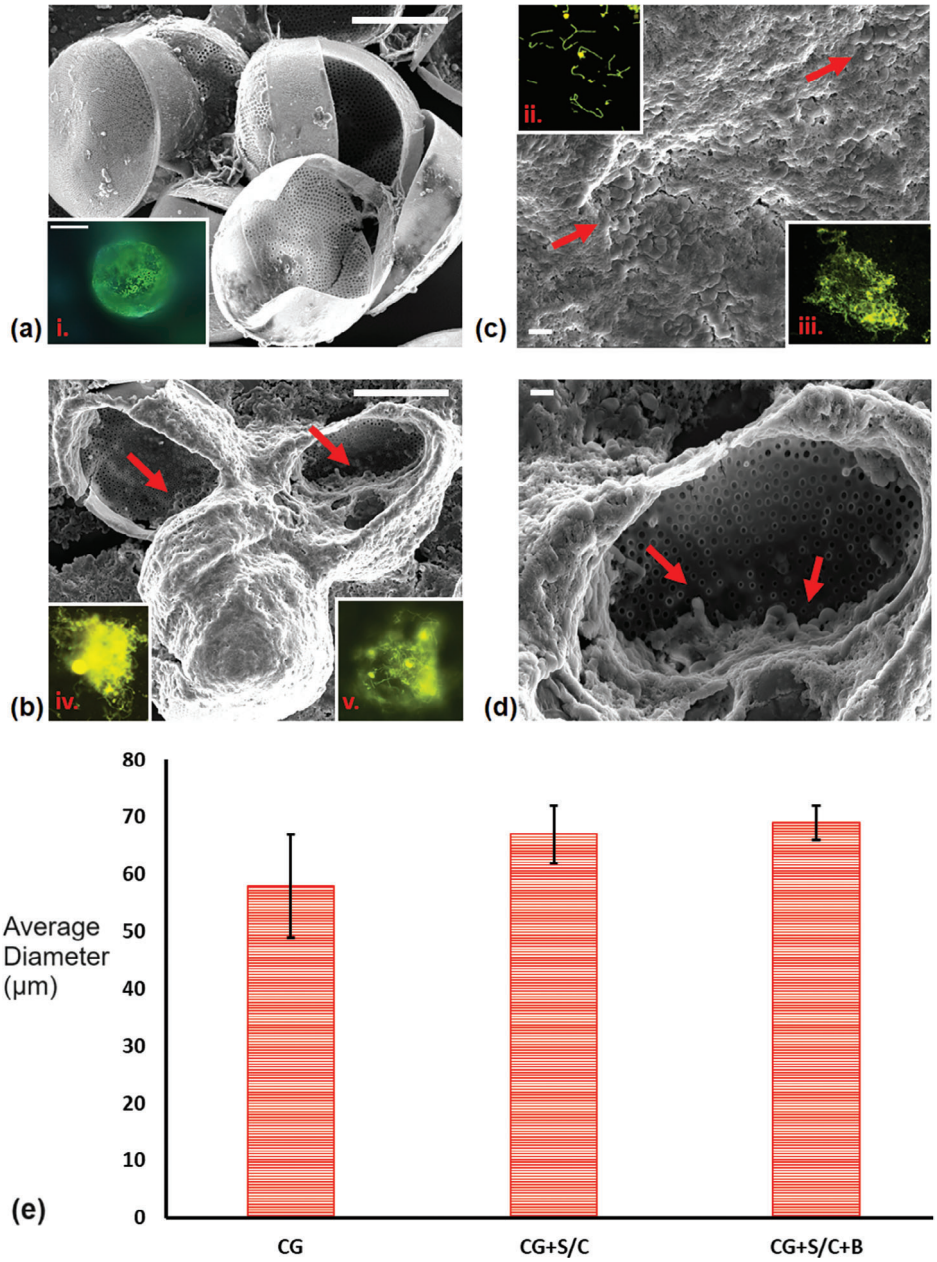

Figure 2. Scan electron microscopy pictures of biosilica a), biosilica loaded with bacteria and closed with polymers b), and related focus d), bacteria with polymers c). Scale bars: a, b: 30 μm; c, d: 2 μm. Fluorescence 2D microscopy pictures of biosilica i.), lone bacteria ii.), bacteria with polymers iii.), biosilica loaded with bacteria in the loaded state iv.) and empty state v.). Histograms of SEM plot regarding the average diameter size/frustule of bare soft-washed biosilica (CG), polymer-coated biosilica (CG+S/C), and loaded with bacteria first and polymer-coated after (CG + S/C + B).

lone bacteria cells and bacteria with Chitosan and Shellac precipitates. Insets iv. and v. have been inserted to visualize bacteria in shells closed with precipitated polymers, in loaded state and empty state respectively. Insets are related to SEM micrographic (Figure 2) showing lone box-like biosilica a), bacteria cells with Shellac and Chitosan (S/C) c) and enriched biosilica with S/C coatings, containing bacteria inside the micro-boxes. Figure 2d reports a focus on bacterial content in the sealed silica. To parametrize the SEM output obtained via imaging, a SEM plot regarding the average diameter size/frustule of bare soft-washed biosilica (CG), polymer-coated biosilica (CG+S/C), and loaded with bacteria first and polymer-coated after (CG+S/C+B) has been reported in Figure 2e, revealing a slight, not significative, increase in diameter size after the embedding with polymers and bacteria.

2.3. Simulated Gastro-Intestinal Transit

In the second phase of the experiment, we aimed to evaluate the ability of microcapsules to resist GI transit and subsequently deliver microbial cells at the intestinal level. The in vitro GI

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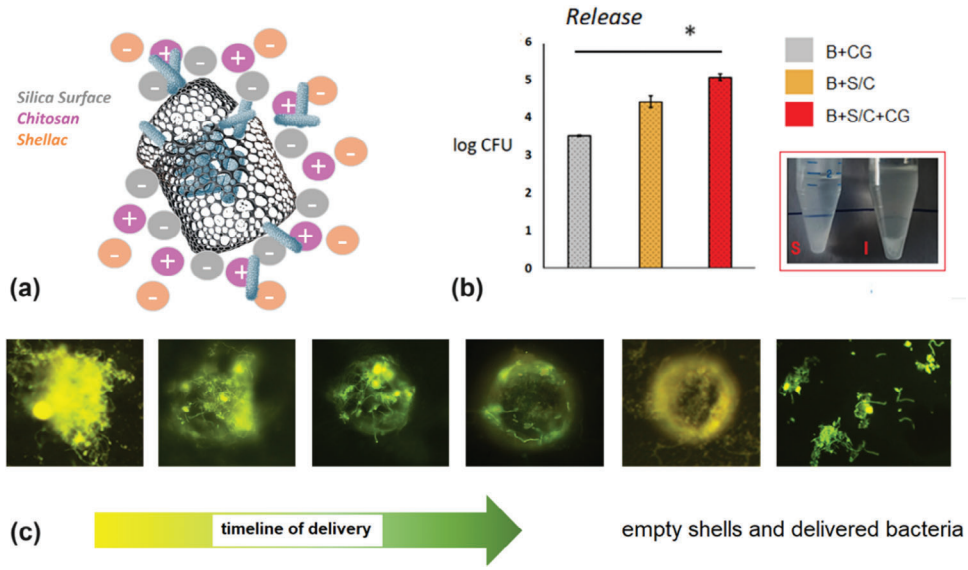

Figure 3. Scheme of the proposed final system for probiotics delivery a). Microbiological viability tests performed after simulated gastrointestinal transit b). Codes: B, bacteria; B + CG, bacteria in biosilica shells; B + S/C: bacteria precipitated with Shellac/Chitosan polymers; B + S/C + CG: bacteria entrapped in biosilica and closed with precipitated Shellac/Chitosan polymers; timeline of shots of the probiotics delivery from the loading to the emptying of the structures c). Anova online test was used for validation via p value < 0.05 (Anova Test).

simulation described by Fernàndez et al.[32] was applied with minor modifications, as reported in the Experimental Section.[33]

Starting from a suspension of microcapsules of the final schematized system (**Figure** 3a) containing 10^8 cells, we observed a higher release of probiotics at the simulated intestinal level from the combined effect of polymers and biosilica shells (B + S/C + CG) than from the composition-based on polymers exclusively (B + S/C). Noteworthy, both coating compositions allowed the bacteria to maintain a higher cell viability (CFU/) after exposure to GI simulating fluids than bacteria in biosilica shells (B + CG), although significance ($P < 0.05$) was solely found between B + S/C + CG and B + CG (Figure 3b). This result suggests the possibility of using these hybrid systems for the GI passage in pharmacological formulations (Figure 3a; inset: delivery of probiotics from biosilica microbeads makes the tested turbid suspension), despite further in vivo studies are needed to validate our probiotics vehicle as a delivery system targeting the colon specifically. The isolated and recorded single elements of this experiment are shown in Figure S6 (Supporting Information) after fluorescein diacetate staining, while the shots of probiotics-delivery, from the loading to the emptying of the structures, are shown in Figure 3c.

2.4. Stability Test and Thermal Resistance

The most promising results were obtained in terms of resistance at 4 °C in the absence of dispersion buffer, so in a dehydrated state, (**Figure** 4a) and to heat treatment at 50 and 55 °C (Figure 4b). Both experiments were carried out to evaluate the

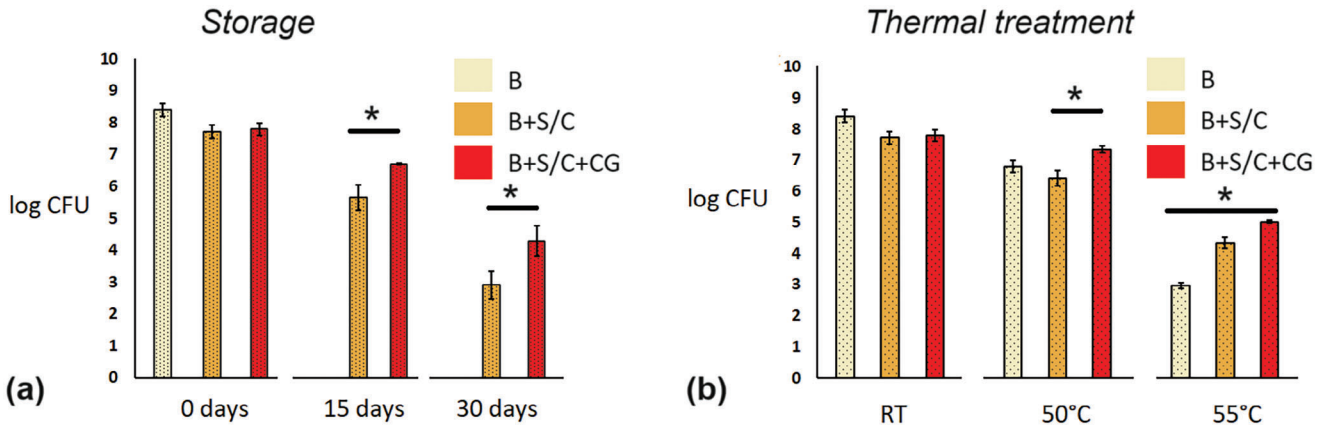

Figure 4. Microbiological viability tests were performed after low temperature and dehydrated state storage a) and thermal shock induction b). Codes: B, bacteria; B + S/C: bacteria precipitated with Shellac/Chitosan polymers; B + S/C + CG: bacteria entrapped in biosilica and closed with precipitated Shellac/Chitosan polymers. Anova online test was used for validation via p value < 0.05 (Anova Test).

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effect of storage on viability, including anomalies in thermal preservation (see Experimental Section). In these cases, biosilica (CG) becomes a protective factor increasing the ability, compared to polymers (S/C), of protection of microbes to resist both low temperature (and in the absence of buffer) and after high temperature treatment.

The system proposed represents an effective protocol of encapsulation and protection of living probiotics based for the first time on the micro-containing property of empty shells of *Coscinodiscus granii* diatoms. Together with the combination of polyester Shellac polymer with cationic chitosan, already known in the literature for their gate opening/closing effect due to pH variations, the system results a green, sustainable, and innovative formulation for probiotic loading/delivery. In terms of encapsulation efficiency, our biosilica-based system is similar and comparable to other alginate:chitosan, alginate:carrageenan, and alginate:caseinate co-precipitated beads,[34] in which the loading of probiotics is set ≈70–80%. However, these systems lack control of the bead's size and the overall statistical dispersity. To overcome these issues, the size of alginate microbeads can be well defined by applying time-consuming processing based on double aerosol[35] or spray nozzle extrusion.[36] In our case, nature governs the size of the silica shells, directly giving micro-pills ready to be fulfilled with microorganisms. Natural bio-silicification gives de facto diatom microalgae the possibility to produce highly reproducible and monodispersed silica shells. The resulting natural construct ensures thermal and dehydration protection of probiotics, using biosilica shells which act both as monodispersed empty beads and active surfaces for easily interacting with co-precipitating enteric co-polymers (negatively charged Shellac and positive Chitosan). A possible explanation of the observed protection effect against the extreme external conditions is the presence of the nano/micro-structured features which may act as micro-niches for probiotics keeping a good vitality due to a continuative condition of internal hydration and a non-direct contact with the polymers coating the silica. The latter, if used without the porous silica would likely be detrimental for probiotics, suffocating or cluttering cells. Moreover, the hard and porous siliceous material exhibits good thermal stability and isolation properties, helping the probiotics also to avoid thermal stress. Among the 2 proposed biopolymers, chitosan improves the biological aspects of our applications, and it is considered here an added value. As reported in literature, CS removes uremic toxins[37] from patients intestine with Chronic Kidney Disease (CKD) and Acute Kidney Injury (AKI), and it can be orally administered aiming to mitigate the retention of aromatic toxins produced by the gut flora and reaching an effective purification during a molecular approach for Renal Replacement Therapies (RRTs).[38]

3. Conclusion

The field of probiotics and related health effects daily increases. Several strains contribute, symbiotically working with the host, to increase and ameliorate the well-being of people. However, not all strains can nowadays be used as probiotics due to the low degree of resistance to gastrointestinal transit. Our adopted protocols showed promising results in terms of preserving a heterogenous microbial community from external stressors, using fully bio-based solutions from Nature. In particular, microalgae results are always highly attractive as a sustainable solution for the food industry, material science, and pharmaceutics, and finally for biofuel production. Given low costs for achieving a high rate of growth and confirming the resistance of public opinion to use artificial xeno-materials, especially for different biomedicine applications, we efficiently introduced microalgae shells here as µ-pills for carrying and delivering probiotics. The present study efficiently aimed to give strength to the innovative approaches preserving the viability of probiotic microbial cells. Further in vivo studies involving animal models or human volunteers are needed to validate this promising probiotics-vehicle as targeted delivery at the colon level, overcoming the limitation that our findings are based on simulated gastrointestinal transit only.

4. Experimental Section

Materials and Equipment: Wax-free Shellac, Chitosan, bi-distilled water, Tris-hydrochloride, and MRS Agar Medium were purchased from Sigma Aldrich. Bacterium consortium SYNBIO® was purchased by SACCO Srl. *Coscinodiscus granii* was purchased from the CCAP culture collection. FTIR-ATR (Fourier Transformed Infrared-Attenuated Total Reflectance) spectra of bare and functionalized substrates were performed by Perkin Elmer Spectrum Two Spectrophotometer equipped with A 2 × 2 mm diamond crystal (4000–400 cm^{-1} range, with a 2 cm^{-1} resolution). UV–vis Shimadzu 2401 PC spectrophotometer was used to record the absorption spectra of the different solutions of Shellac in ethanol and degradation samples. An Axiomat, Zeiss microscope (Oberkochen, Germany) with all active filters was used to observe stained bacteria with and without encapsulating composites. Scan Electron Microscopy imaging was possible with an external service. The analyses of the surface area/mass of samples (40–45 mg) were carried out via Brunauer–Emmett–Teller (BET) investigation, using a 30% N_2/70% He gas mixture on a Pulse Chemisorb 2750 (Micromeritics) instrument equipped with a Thermal Conductivity Detector (TCD).

Diatoms Culture and Biosilica Extraction: *Coscinodicus granii* diatoms (CCAP strain 1013/4) were cultured in polystyrene flasks (250 mL) in a static vertical incubator, in the presence of a sterile sodium metasilicate enriched medium composed of an f/2 Guillard solution with nutrients and seawater. The diatom culture was monitored to check the cell's viability by controlling of cells and chloroplast's integrity and luminescence over time. After 8 weeks of the inoculum, cells were collected 24 h before the cleaning procedures via overnight sedimentation in conic falcon, and 2 extraction methods were compared to produce biosilica containers for probiotics loading: one based on hard acid/oxidative treatment (50 µL of H_2SO_4 and 100 µL H_2O_2, 70 °C 2 h) and the other one performed with detergent (2% SDS, EDTA 100 mM, 55 °C 2 h). Both treatments were carried out on a high-density pellet (1 mL, 150–300 cells μL^{-1}) of cultured diatoms. Scan electron microscopy (SEM) was performed to evaluate the efficiency of the two isolation methods.[39]

Culture Methods for the Probiotic Cells: The probiotic mixture, named SYNBIO® (Sacco s.r.l., Cadorago, Italy) in the form of lyophilized cells and packaged in bags was resuspended into tubes containing 10 mL of MRS broth medium (De Man, Rogosa, and Sharpe; Oxoid Ltd., Basingstoke, Hampshire, England, UK) previously sterilized. Each bag of the commercial probiotics mixture SYNBIO® contains 10^9 living cells of two probiotic strains in ratio 1:1, specifically *Lactobacillus rhamnosus* IMC 501® (recently taxonomically reassigned to *Lacticaseibacillus*) and *Lactobacillus paracasei* IMC 502® (reassigned to *Lacticaseibacillus*). After the inoculum, samples were incubated for 24 h at 37 °C. A second inoculum (4%) was then carried out in tubes containing 10 mL of sterilized MRS to obtain the microbial exponential phase of growth (10^9 CFU mL^{-1}) after 18 h at 37 °C, verified by spectrophotometer OD (wavelength 620 nm). Before passing to further experiments, the broth culture was centrifuged (10 min × 10,000 *rpm*) and the resulting pellet, washed twice with saline solution (NaCl 0.9 g L^{-1}), was

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extra-processed. The same medium (MRS), added with agar (12 g L^{-1}), was used to evaluate by plating the viable cell counts (Log_{10} CFU mL^{-1}) after the simulated GI transit, storage, and thermic experiments. Differences were assessed by plate counts of serial dilutions incubated at 37 °C for 48 h according to the manufacturer's instructions.

Microscopic Determination and Calibration Curve Defined on Bacteria Stained with Cy3-NHS: A volume of 1 mL of bacteria dispersions (10^9 cells/mL) in Tris:HCl 25 mM pH 8.1 was incubated with 2 µL of Cy3-NHS in DMSO (500 µM). A 2 h of staining reaction was performed under stirring (200 rpm) at room temperature. Then 3 cycles of washing in Tris:HCl 25 mM pH 7.2 and centrifugation (6000 rpm; 15′) were performed to purify the bacteria pellets. Cy3-NHS staining was checked using 2D fluorescence microscopies, and a calibration curve was designed correlating 6 cell densities and fluorescence intensity recorded by exciting at 521 nm and recording at 567 nm (Figure S2, Supporting Information). For the microscopic determination of fluorescence recorded for Cy3-NHS stained bacteria, 2D fluorescence micrographs were recorded using a TRITC-cube filter in dichroic:emission filtering system.

General Procedure for Bacteria Encapsulation: The physical entrapment of the bacterial preparation in biosilica was performed by incubating for 30 min at room temperature these two suspensions and then performing vacuum pumping (3 times). Once diatoms shells are loaded with bacteria dispersions, chitosan solution in acidic water (best volume: 7 µL in Tris:HCl 10 mM, pH 6; conc. of chitosan: 5 mg mL^{-1}) and then a solution of pre-hydrolyzed Shellac (best volume: 7 µL in carbonate potassium buffer, pH 8.5; conc. of shellac: 40 mg mL^{-1}) were added. The choice of the ratio between the quantity of polymers and density of silica shells has been extensively investigated in terms of probiotics loading efficiency, as reported.

Loading % of Stained Bacteria: Constant Quantity of Shellac, Variable Quantity of Chitosan: Dispersions of bacteria were put in the fundus of the conic falcon (20 µL, 10^9 cells mL^{-1}) and incubated in contact with Chitosan first and Shellac as a second step. After gentle rotation and mixing in the tube fundus, a stable gel was formed due to the contact of negatively charged bacteria, and the serial added positively charged Chitosan and negatively charged Shellac. In the case of the determination of loading %, bacteria were pre-stained as first reported, and incubated with pre-hydrolyzed Shellac solution alone (B+S; 7 µL in carbonate potassium buffer, pH 8.5; conc. of shellac: 40 mg mL^{-1}) and Chitosan solution alone (B+C; 7 µL in Tris:HCl 10 mM, pH 6; conc. of chitosan: 5 mg mL^{-1}) as controls, while samples were prepared with constant quantity of bacteria and Shellac and varying the Chitosan volumes added (B+S/C 1: 1.5 µL of chitosan solution; B+S/C 2: 3.5 µL of chitosan solution; B+S/C 3: 7 µL of chitosan solution; B+S/C 4: 14 µL of chitosan solution). After 30′ of incubation, all the samples were suspended in 2 mL of Tris:HCl 10 mM, pH 6.8, and after 1 h of sedimentation (gravity brings down the bacteria embedded in polymer matrices) the supernatants were analyzed with spectrofluorimetry (exc. 521 nm; em. 567 nm). The emission intensities were interpolated to obtain the number of bacteria cells and data were converted in % of loading (using 100% as the total amount of starting bacteria). The experiments were then continued using the solution B+S/C 3: 7 µL of chitosan solution and the same volume of Shellac.

Loading % of Stained Bacteria: Constant Ratio of Shellac and Chitosan, Variable Density of Shells: The protocol resulted slightly divergent due to the introduction of biosilica. Dispersions of bacteria were put in the fundus of the conic falcon (20 µL, 10^9 cells mL^{-1}) and the biosilica preparation at different densities was then dropped on bacteria (20 µL). The physical entrapment in biosilica was performed incubating for 30′ the suspensions and then performing 3 cycles of vacuum pumping. Then the incubation with the previously tested Chitosan and Shellac solutions, at the chosen ratio of volumes (7+7 µL), was performed. And incubated in contact with Chitosan first and Shellac as a second step. Again after 30′ of incubation, all the samples were suspended in 2 mL of Tris:HCl 10 mM, pH 6.8, and after 1 h of sedimentation (gravity brings down the bacteria embedded in polymer matrices) the supernatants were analysed with spectrofluorimetry (exc. 521 nm; em. 567 nm). With the introduction of shells into the polymer matrices the percentage of loading has been again calculated as mentioned before, so the emission intensities were interpolated for obtaining the number of bacteria cells, and data were converted in % of loading (using 100% as the total amount of starting bacteria). Actually, the introduction of shells generally improved the loading percentage. The experiments were then continued using 5400 shells in the samples, so 270 shells μL^{-1}.

Loading % of Stained Bacteria: Comparison with Other Polymers Used for Probiotics Loading: Here the protocol of producing hybrids based on biosilica entrapping bacteria and 2 other polymers used for probiotics loading is presented as a check. As reported before, again dispersions of bacteria were put in the fundus of conic falcon (20 µL, 10^9 cells mL^{-1}) and the biosilica preparation at a constant density (20 µL; 270 shells μL^{-1}) was then dropped on bacteria (20 µL). The physical entrapment in biosilica was performed by incubating for 30′ the suspensions and then performing 3 cycles of vacuum pumping. Then the incubation with hydroxypropyl methylcellulose (HPMG) and gelatin (Gel) (7+7 µL; 40 mg mL^{-1} in ethanol:water solutions, 1:9) was performed. Again after 30′ of incubation, all the samples were suspended in 2 mL of Tris:HCl 10 mM, pH 6.8, and after 1 h of sedimentation (gravity brings down the bacteria embedded in polymer matrices) the supernatants were analyzed with spectrofluorimetry (exc. 521 nm; em. 567 nm). Applying the same conceptual principle of calculation as previously reported, the use of shells in combination with the other 2 polymers did not bring a sufficient loading percentage.

Simulated Gastrointestinal Transit: A suspension of microcapsules containing 10^8 cells, was incubated with 2 mL of gastric solution (pH 3.0, 3 h at 37 °C) containing NaCl (125 mM), KCl (7 mM), NaHCO3 (45 mM), and pepsin (3 g L^{-1}) (Sigma-Aldrich Co., St. Louis, MO, USA). After simulated gastric fluid exposure, microcapsules were added to 2 mL simulated intestinal fluid (pH 8.0, 3 h at 37 °C) containing pancreatin and Oxgall bile salt (Sigma-Aldrich Co.) (0.1% and 0.15% w/v, respectively). Peristalsis was simulated by keeping samples under stirring conditions (150 × *g*). At the end of the intestinal phase, the viability of delivered cells was evaluated by plate counts in MRS agar medium as described above.

Stability Test and Thermal Resistance: Free (B) and encapsulated (B+S/C and B+S/C+CG) probiotics were subjected to storage (+4 °C) until 30 days and heat-tolerance (at 50 and 55 °C) treatments. Thereafter, probiotic cells (encapsulated and free) were added in test tubes containing saline solution (NaCl 0.9 g L^{-1}) and, according to tenfold serial dilutions, plated on MRS agar to assess the residual cell viability (Log_{10} CFU mL^{-1}).

Statistical Analysis: Plate counts were performed as biological triplicate and expressed as means ± standard deviation (±SD). Differences were assessed by one-way ANOVA with *p*-values (p) < 0.05 indicating a statistically significant difference.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords

biosilica micro-pills, chitosan, enteric polymers, probiotic microorganisms, shellac

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