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Capsules as Mini-bioreactors: Effect of the Formulation on the Probiotic Metabolism and Confinement

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Encapsulation is a commonly used tool in the pharmaceutical and nutraceutical fields to protect the compound of interest from adverse environmental conditions (i.e., production process, gastrointestinal transit, immune defense) or to ensure a target or controlled release of an active principle. However, capsules can also be used as reactors, by which probiotics can carry out a fermentation with improved process performances. In the present work, hydrolyzed oatmeal capsules were developed as mini-bioreactors, segregating the probiotic strain in active fermentation and allowing the metabolite release. The aim was to study how composition can influence the ability to ferment and the confinement of microorganisms. Four different capsules formulations were studied varying $CaCl_2$ and alginate concentrations [(i) 0.5% alginate-0,1 M $CaCl_2$; (ii) 1% alginate-1 M $CaCl_2$; (iii) 1% alginate-3 M $CaCl_2$; (iv) 1% alginate-5 M $CaCl_2$]. The capsules were suspended in the same hydrolyzed oatmeal suspension used to produce them and left to ferment at 37°C for 24h. Microbiological and chemical analyses were carried out on both capsules and external liquids. An increase in the bacterial concentration of about 3 logs was recognized for all the first three formulations in the capsules, while a growth inhibition was observed for the (iv) formulation, the only formulation for which microorganism confinement was also noted. The highest lactic acid production (15.9 g/L) was observed for the (ii) formulation, while the lowest one was also recorded for formulation (iv). Optical analyses confirmed the different structural characteristics of the capsules.

1. Introduction

Encapsulation is a process by which a compound of interest is coated with a wall material or is trapped in an isolated environment, separated from the outside. Encapsulation has been widely used in the pharmaceutical field, where it can be used as a drug delivery system (Paulo and Santos 2017). However, encapsulation is increasingly involved also in food, cosmetic, and textile sectors, where it can be applied to preserve the active ingredient from the process production or during the storage, to increase nutraceuticals bioavailability, or to ensure a target (Reque and Brandelli 2021) or controlled release of the compound encapsulated (Casanova and Santos 2016). One of the main applications of encapsulation is linked to probiotic microorganisms: capsules are often used to protect bacteria during gastrointestinal transit and to release them in the target site (Rajam and Subramanian 2022). However, capsules can also be used as reactors, by which the performance of the reaction itself could be improved. For instance, immobilized microorganisms could enable a reaction faster than free microorganisms (Oliveira et al. 2011), or the reaction could be more efficient (Kurzbaum et al. 2020). Immobilization could impact microbial growth with faster fermentation due to shortening of the non-productive growth phase or with higher cell density than free cell fermentation; other possible effects of the immobilization are high cell productivities and high product yields (Ramakrishna and Prakasham 1999); immobilized bacteria can quickly be recovered and reused in fermentation processes (J. Duarte et al. 2013) and may allow obtaining

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continuous processes (Zhu 2007). One of the central aspects to consider when designing and developing capsules is the composition, which can influence the capsule's strength and porosity and consequently influence the ability to protect and segregate the encapsulated component (Di Natale et al. 2021). Furthermore, there is a need to choose food grade and GRAS materials if the capsules are intended for human consumption. Among materials, great attention can be paid to oat, which is a natural source of starch and ß-glucans, already used as encapsulating materials, as described respectively by Bennacef et al. (C Bennacef et al. 2021) and by Gani et al. (Gani et al. 2018). Oat is characterized by high antioxidant, anti-inflammatory, anti-allergenic, and anti-carcinogenic capacity (Bei, Q, Wu, Z., Chen 2020); furthermore, it can be considered a prebiotic and could be a suitable fermentation medium (Gallo et al. 2020).
In the present work, the capsules to be used as mini-bioreactors were produced by extrusion technique, using alginate, calcium chloride, and a hydrolyzed oatmeal suspension as coating materials. Different alginate capsule formulations were developed, varying the calcium chloride and the alginate concentrations. The encapsulated compound was *Lacticaseibacillus paracasei* CBA L74, previously described as a probiotic microorganism (Labruna et al. 2019). The encapsulated microorganism was then placed in a hydrolyzed oatmeal suspension, consisting of the same characteristics and concentration of the materials chosen for the encapsulation, and left to ferment for 24 h. To study the bacterial fermentation and the confinement ability of the capsules, both the capsules and the external suspensions were characterized from microbiological and chemical points of view. Microscopic imaging was also carried out. Although capsules have already been used as mini-bioreactors, this is the first case in which bacterial leakage during fermentation has also been studied, to the best of our knowledge. Therefore, the present work aimed to study how the formulation of capsules can impact the confinement and growth ability of the probiotic microorganism.

2. Materials and Methods

2.1 Strain and Feedstock

Lacticaseibacillus paracasei CBA L74 (patented and provided by Heinz Italia S.p.A), a Gram-positive homo-fermentative and facultative anaerobic bacteria, was used as the probiotic microorganism. It was stored at −80 ◦C and revitalized in 9 mL of animal-free broth (AFB) (20 g/L Bacto Yeast Extract (BD Biosciences, Milan, Italy), 0.5 g/L $MgSO_4$ (Sigma-Aldrich, Milan, Italy), 50 g/L glucose (Sigma-Aldrich), 0.5 g/L citric acid (Sigma-Aldrich))

by incubation at 37 ˚C for 24 h. The bacterial load reached after revitalization was approximately 10^8 CFU/mL. Oat flour (Le Farine Magiche, Lo Conte Group) was purchased in a local market. A hydrolyzed oatmeal suspension (15% w/v in water) with 1% w/v glucose, was preliminary treated with 0.054% w/v amylase (E-BLAAM, Megazyme, Ireland) to avoid gelatinization during the sterilization treatment, carried out at 134°C for 40 min (Lentini et al. 2022). The hydrolyzed oatmeal suspension was used both for developing the capsules and for the external suspension in which capsules were fermented.

2.2 Encapsulation and Fermentation

Encapsulation was carried out by direct extrusion, according to Sáez-Orviz et al. (2021).
Four different formulations were developed (Table 1). To the hydrolyzed oatmeal suspension, 5 g/L (0.5% w/v) or 10 g/L (1% w/v) sodium alginate (Sigma-Aldrich) was added and mixed until homogeneous. Then, a volume of revitalized inoculum was added to have a concentration of approximately 10^6 CFU/mL in the oat suspension. The mixture was dropped into a $CaCl_2$ solution (0.1, 1, 3, or 5 M, Sigma-Aldrich) from a distance of 10 cm, using a peristaltic pump with a flow rate set at 5 mL/ min. The capsules were left in the $CaCl_2$ solution for 20 min at room temperature with gentle agitation. After this time, the capsules were collected and washed in distilled water. The capsules, obtained as described, were put in a hydrolyzed oatmeal suspension. The ratio between the volume used to produce the capsules and the external suspension in which they were suspended was set at 1:3. The fermentation was carried out in a 50 ml centrifuge tube for 24 hours at 37°C, using a wheel as a stirring system.

Table 1: Formulations of the capsules tested.

Formulation	*(i)*	*(ii)*	*(iii)*	*(iv)*
	0.5% alginate 0.1 M $CaCl_2$	1% alginate 1 M $CaCl_2$	1% alginate 3 M $CaCl_2$	1% alginate 5 M $CaCl_2$

2.3 Microbiological and Chemical Analysis

Samples were collected at the beginning (T0) and at the end of the fermentation (T24). The capsules and the external suspension were analyzed for bacterial concentration (to obtain information on the fermentation

performances but also on the confinement potential of the capsule) and for lactic acid production. The capsules were preliminary broken in 1% w/w sodium citrate solution at pH 6. Microbiological analyses were carried out by spread plate method, after serial dilution, on MRS agar plate (Oxoid, Basingstoke, UK) to check the *lactobacill*i concentration, but also on MacConkey agar (Oxoid, Basingstoke, UK) and Gelatin Peptone Bios Agar (Biolife, Milan, Italy), to exclude the presence of contaminants. All plates were incubated at 37 °C for 24 h. The lactic acid concentration was determined by high-performance liquid chromatography (HPLC), Agilent Technologies 1100, equipped with an Agilent Synergi Hydro-RP C18 column (250 mm × 4.6 mm and a pore size of 4 µm) with a visible/UV detector. The mobile phase consisted of 0.27% KH_2PO_4 aqueous solution at a pH=1,5 modified with H_3PO_4 with a column temperature of 60 °C and a 1 mL/min flow rate. The detection was set at 210 nm.

2.4 Optical Characterization

Freshly prepared capsules were morphologically characterized, after being preliminary cryo-sectioned (slices of 10 µm - Leica Biosystems Cryostat CM1520), by brightfield and fluorescence microscopy (The Leica DFC7000 T 2.8 MP Color Microscope Camera).

2.5 Statistical Analysis

Statistical analysis was performed using Prism 9®. Each test was carried out in triplicate, and mean values and standard deviations (n = 3) were calculated. Their statistical significance was evaluated by one-way ANOVA followed by Tukey's multiple comparisons test, accepting as significant only results with $p < 0.05$.

3. Results and Discussion

The bacterial growth and the lactic acid production observed on the capsules and the external suspension are discussed below.

3.1 Bacterial Growth

The performance of the fermentation and the potential ability to allow the confinement of the bacteria were evaluated by controlling the bacterial concentration both inside the capsules and in the external suspension at the beginning (T0) and at the end of the fermentation (T24). As can be seen in Figure 1A, an initial bacterial concentration of approximately 10^6 CFU/mL was achieved in all four capsule formulations, as expected. In three of the tested formulations (*i, ii, iii*), the capsules acted as mini-bioreactors, allowing fermentation, as can be seen from the bacterial concentrations found at T24, with a delta growth of 3 – 4 logs for all, without statistically significant differences (Figure 1B).

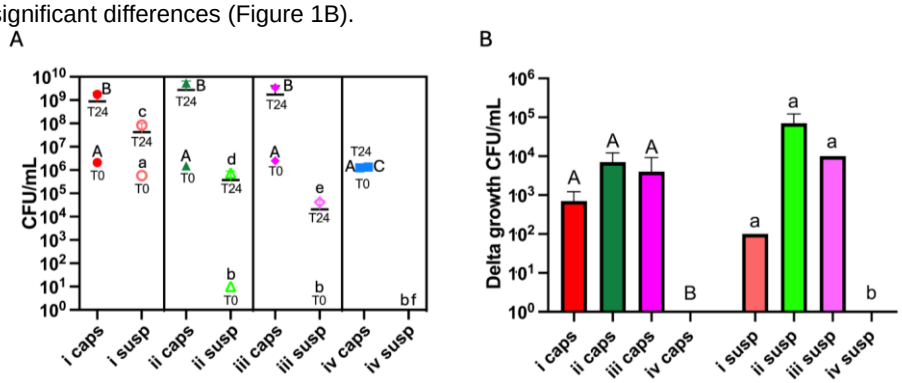

Figure 1. Bacterial concentrations (CFU/mL) evaluated inside the capsules and in the external suspension of the four formulations. A) Bacterial concentration (CFU/mL) measured at the beginning (T0) and at the end (T24) of fermentation for each capsule formulation (i, ii, iii, iv) and for the external suspensions in which they were suspended. B) Delta growth for all capsule formulations and related external suspension. Bars represent the standard deviation of three independent experiments. Different letters indicate significant differences (p < 0.01). Uppercase letters are used to identify the capsules, and lowercase letters are used for external suspensions.

In formulation *(iv)*, despite an initial bacterial concentration of 10^6 CFU/mL, no growth was observed, suggesting the capsule formulation is an essential factor influencing fermentation performance. This could be due to the high concentration of $CaCl_2$, which acts as a potential inhibitor of bacterial growth, as also demonstrated by Rajamma et al. (2021) (the higher the concentration of $CaCl_2$, the higher the growth inhibition rate of *Ralstonia pseudosolanacearum*) and by Bautista-Gallego et al. (2008) (the higher the $CaCl_2$ concentration, the higher the inhibition rate of *L. pentosus* and of *S. cerevisiae*).

Moreover, by observing the bacterial concentration found at T0 in the suspension, it can be observed that the formulation is also a key factor for confinement: the formulation *(i)* did not prevent the bacterial escape, already starting from T0, when a presence of $5.8*10^5 \pm 3.5*10^5$ CFU/mL can be observed in the external suspension (Figure 1A). The increase in the molar concentration of $CaCl_2$, in formulations *(ii)* and *(iii)*, allowed respectively a reduction in bacterial escape and an initial retention of the bacteria, but this ability is lost during fermentation, considering the high presence of lactobacilli found at T24 in the external suspensions (*ii*: $7.4*10^5 \pm 7.9*10^4$ CFU/mL; *iii*: $4.1*10^4 \pm 4.6*10^3$ CFU/mL). Finally, bacterial confinement was obtained with formulation *(iv)*: the possible presence of bacteria was detected after 24 h of fermentation. However, as seen above, this formulation also prevents bacterial growth within the capsules and cannot be used as a mini-bioreactor.
In the three formulations in which internal growth was recorded, but confinement was not achieved, it is not possible to understand the exact dynamics of diffusion of the bacterial population: the CFU/mL obtained at T24 in the external suspensions could be due both to diffusion phenomena from inside the capsules to the external space, during fermentation, and to the active growth of the bacteria released at T0. A similar result was described by Li et al. (2017), who studied, among other things, the release of an engineered *E. coli* from alginate beads (2% w/w alginate extruded in 100 mM $CaCl_2$): they found a concentration of bacterial counts of approximately 10^5 CFU/mL after 6 hours of incubation at 37°C in the suspension. They hypothesized that the bacteria could originate from the leakage from the beads or from bacteria that were not initially removed in the suspension by washing. The limitation of the present work is linked to the need for more information on diffusion phenomena. In fact, as described, it is not possible to distinguish how much of the microbial concentration recognized in the external suspension is due to diffusion and how much to microbial growth. Studies are underway in this regard.

3.2 Lactic acid production

The lactic acid concentration was evaluated both inside the capsules and in the external suspensions at the beginning (T0) and at the end of the fermentation (T24). In all cases, as expected, lactic acid was absent at T0. However, after 24 h of fermentation, lactic acid concentration was strongly different between the capsule formulations, with the highest production in capsules with formulation (*ii*) (15.9 ± 1.8 g/L) and the lowest one in capsules with formulation *(iv)* (1.1 ± 0.1 g/L), as can be seen in Figure 2. The low production of lactic acid in the *(iv)* formulation can probably be attributed to the low bacterial growth observed, as previously described, but also to the high concentration (5 M) of $CaCl_2$ used to produce the capsules. A similar result was described by Yen and Lee (2010), who found that $CaCl_2$ concentrations greater than 1 M caused inhibition of lactic acid production by *Rhizopus Oryzae*.

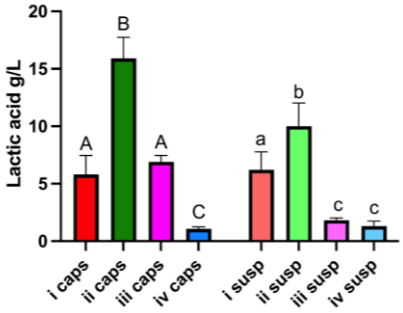

Figure 2. Lactic acid concentrations evaluated inside the capsules and in the external suspension of the four formulations. Lactic acid (g/L) produced during 24 h of fermentation, for each capsule formulation (i, ii, iii, iv) and for the external suspensions in which they were suspended. Bars represent the standard deviation of three independent experiments. Different letters indicate significant differences ($p < 0.05$). Uppercase letters are used to identify the capsules, and lowercase letters are used for external suspensions.

The lactic acid was also found in the external suspensions, and, also in this case, the highest concentration was found for the formulation *(ii)* (10.0 ± 2.0 g/L), while the lowest one for the formulation *(iv)* (1.3 ± 0.4 g/L). What is surprising is the concentration of lactic acid obtained with formulation *(ii)*, both in the capsules and in the external suspension. This large production can be explained by hypothesizing a threshold effect of the $CaCl_2$ concentrations, as also seen by Yen and Lee (2010) for $CaCl_2$ concentrations above 15%. However, contrary to what was observed by Yen and Lee, the greater production of lactic acid is not observed in formulation *(i)* (with the lower concentration of alginate and $CaCl_2$) but in formulation *(ii)*.
As for bacterial growth, also for lactic acid, it is not possible to distinguish, from this experiment, the lactic acid produced externally from that diffused by the capsules. The literature shows that the concentration of $CaCl_2$ used to produce capsules can impact the diffusion phenomena. Puguan and Kim (2015) found that the lower

the $CaCl_2$ concentration, the greater the release of low molecular weight molecules, and this is in accordance with the present study: although the lactic acid concentrations in the capsule made with formulation *(i)* and *(iii)* is statistically the same, the lactic acid concentration found in the external suspension of formulation *(i)* is higher than that measured in the external suspension of formulation *(iii)*. This may be due to increased lactic acid production *in situ*, due to the high concentration of bacteria found in the external suspension of the formulation (*i*). However, another possible cause could be the high permeability of the capsule to low molecular weight molecules, and this could suggest that the amount of lactic acid present in the external suspensions is also strongly supported by internal production.

3.3 Optical Characterization

Capsules were optical characterized by brightfield and fluorescence microscopy, as shown in Figure 3.

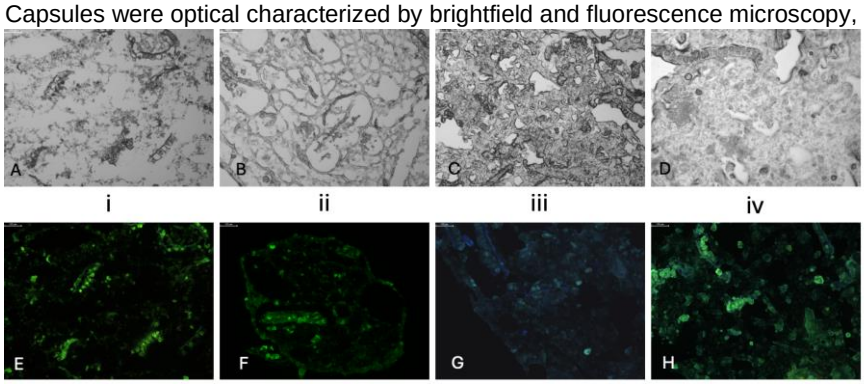

Figure 2Brightfield and fluorescence microscopy of the four capsules. A, B, C, D: brightfield images of the formulations (i, ii, iii, and iv), respectively. E, F, G, H: fluorescence images of the formulations (i, ii, iii and iv), respectively.

As it is possible to note, the higher the $CaCl_2$ concentration used, the denser the matrix of the capsules. The same result was highlighted by Chai et al. (Chai et al. 2004), who found an increase in the wall thickness of hollow alginate capsules by increasing the concentration of $CaCl_2$, and by Blandino, Maćas, and Cantero (Blandino, Maćas, and Cantero 2000), who also found reduced diffusion coefficient for capsules developed with increasing cationic concentration. However, in both cases, alginate capsules were developed by inverse gelation, and the results are not perfectly comparable to those obtained in the present work.

4. Conclusions

The alginate capsules developed by direct extrusion changing the alginate and the $CaCl_2$ concentration have been tested as mini-bioreactors. The bacterial growth and the lactic acid production during the fermentation of the encapsulated *L. paracasei* CBA L74 were used as markers of both the fermentation performances and the confinement ability of the capsules. As seen, the formulation strongly affects the proliferation, considering the inhibition effect found for high $CaCl_2$ concentration, but also the confinement. Surprisingly, the metabolism of microorganisms also seems affected by the formulation of the capsule. A possible explanation could be that formulation *(ii)* guarantees, with its mesh, a semi-confinement that can create an optimal environment for the growth and metabolism of the Lactobacillus. This semi-confinement is not observable neither in formulation *(i)*, which is too permeable, nor in formulations *(iii)* and *(iv)*, which are too impermeable and which also suffer from metabolism inhibition due to the high concentrations of $CaCl_2$. Further investigations could help us understand. From the present study, it emerges that capsules developed with 1% alginate 1M $CaCl_2$ acted as mini-bioreactors, ensuring high bacterial growth (T24 bacterial concentration: $5.5*10^8 \pm 2.7*10^9$ CFU/mL) and high lactic acid production (T24 lactic acid concentration: 15.9 $\pm$ 1.8 g/L), although did not allow bacterial confinement. The only formulation capable of confinement (*iv* formulation) did not function as a mini-bioreactor, as no microbial growth was observed, and this can be due to the high $CaCl_2$ concentration, acting as a potential bacterial inhibitor. In conclusion, the choice of the formulation of the capsules is a pivotal aspect, considering that both bacterial growth and the confinement ability are linked to it.

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