Sporopollenin exine microcapsules as potential intestinal delivery system of probiotics

Konstantinos Stamatopoulos, Vasiliki Kafourou, Hannah K. Batchelor and Spyros J. Konteles3**

Dr. K. S., V. K.
School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom
E-mail: stamatok@bham.ac.uk; skonteles@uniwa.gr

Prof. H. K. B.
Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, United Kingdom

Dr. S. J. K.
Department of Food Science and Technology, Faculty of Food Sciences, University of West Attica, Athens, 12243, Greece

Keywords: lactobacillus casei, sporopollenin, microencapsulation, probiotics, flow cytometry, mrs plate count, scanning electron microscope

Abstract

Despite several decades of research into encapsulation of bacteria, most of the proposed technologies are in the form of immobilized cultures. In this work, sporopollenin exine capsules (SECs) opened, using silica particles which act as pressing micro-probes, and loaded with *Lactobacillus casei* (*L. casei*) cells, are described for the first time. The proposed encapsulation provided ~30 times higher encapsulation yield (30.87%), compared to direct compression of SECs (0.99%). Encapsulated *L. casei* cells showed 1.21- and 2.25-folds higher viability compared to free cells, in in vitro simulated fasted and fed media representing the human GI tract, respectively. Encapsulated *L. casei* can proliferate inside the SECs, generating enough pressure to cause the SECs to burst and release the viable and metabolically active cells. The time course of the release in vitro was 12 ±2 h. The noticeable difference with the application of the SECs as a means of encapsulation is that the SECs may act as a bioreactor and provide time for the encapsulated cells to multiply thousands of times before being released, following the SEC's burst. The unique advantages of SECs alongside

the proposed encapsulation method, demonstrates the potential application of SECs as delivery system of probiotics to the distal part of the human GI tract.

1. Introduction

The sum of the microorganisms (microbiota) of the gastrointestinal (GI) tract - often also referred to as microbiome - is approximately 100 trillion (10^{11}–10^{12} cells mL^{-1}).[1] In recent years, it has become clear that the GI microbiota is a very important ecosystem of the human body, involved in the regulation of functions like metabolism, trophic, immunity and protection against biotic and abiotic pathogenic factors.[2] The importance of gut microbiota in human health was highlighted further when a multitude of health disorders was associated with profound changes in their composition (Dysbiosis). In light of these findings, the next stage of research was to look for ways to restore GI microbiota.[3] One of the most common strategies of gut microbiota restoration is the oral administration of a beneficial microorganism population targeting the distal intestine.

The microencapsulation technique is the main method of transferring beneficial bacteria into the small and large intestine.[4] In this method, the beneficial microorganisms are incorporated into carriers that protect them from the external adverse conditions following oral administration (low pH, bile salts) within the GI tract and allow them to release into the distal intestine.[5]

The main materials reported for the microencapsulation of bacteria are polymeric matrices, mainly alginates,[6] shellac,[7, 8] xanthan gum and carrageenan gum.[9] Recently, proteins have also been used for bacterial encapsulation like casein,[10] whey proteins[11] and soy protein,[12] that in some cases are coated with maltodextrins.[13] However, most of the proposed encapsulation technologies are in the form of immobilized cultures with significant impact on

probiotics' survivability during processing, storage and digestion due to poor mechanical and chemical resistance of the microencapsulation materials (e.g. alginate) used.[14]

Different triggering mechanisms have been implemented for controlled delivery of different molecules and cells including: pH,[15] microbial[16] and osmotic[17] resulting in degradation of the carrier due to either polymer solubilization and/or mechanical disruption of the microcapsule.

Recently, sporopollenin exine capsules (SECs), the outer part of the pollen shell, has attracted the interest of the scientific community as an alternative encapsulant, due to its good mechanical properties and its extremely high thermal and chemical resistance.[18] Despite several decades of research into pollen-based encapsulation methods, this has been mainly conducted on the encapsulation and controlled release of drugs,[19] dyes, oils[20] and proteins.[21, 22] Looking at the mechanical and chemical stability of this natural material, the utilization of SECs can be beneficial for the encapsulation of probiotics. This is because SECs could serve as natural hollow microcapsules that can potentially protect probiotics against adverse external conditions. Moreover, the elasticity associated with SECs [23] has the potential to facilitate controlled release of the probiotics, providing their proliferation inside SECs to sufficient degree to burst out of the SECs. However, there are several technical limitations to overcome. One of them is to open the SECs' aperture effectively without damaging their structure to allow entry of the probiotic cells. Another challenge is to assess the viability of the probiotics whilst they are inside the capsule, as fracturing the rigid capsule shell material to release the encapsulated bacteria will compromise their viability. Normally in the encapsulation methods where polymers are used, the release of the encapsulated bacteria is performed prior to viability assessment, after removing the coating and the polymeric matrix, based on the triggering mechanism (e.g. pH). Furthermore, the SECs need

85 to control release such that the encapsulated bacteria is released within the distal intestine,
86 enabling colonization, to demonstrate suitability as delivery system for probiotics.
87 This is the first report in which a method was developed to tackle these limitations and where
88 *Lactobacillus casei* - Gram positive, non-motile, rob-shape bacterium - was selected as
89 probiotic model. In this method: i) *Lycopodium clavatum* SECs were prepared and used as
90 they have shown chemical and morphological stability in in vitro[24-27] and in vivo[28, 29]
91 environment of human GI tract, ii) spherical silica particles were used, mixed with SECs, as
92 compression micro-probes to apply multiaxial compressing forces on SECs during tableting,
93 enabling opening of SECs' aperture, iii) ultrasonification was used to evacuate the air from
94 the opened SECs resulting in the suspended *L. casei* cells being sucked inside SECs as well
95 as to facilitate the closing of SECs' aperture, iv) flow cytometry and confocal imaging were
96 used to assess the viability and metabolic activity of *L. casei*, being inside the SECs,
97 following exposure to in vitro simulated human gastrointestinal tract and v) SEM images
98 were obtained at several time points to reveal the release mechanism of *L. casei* from SECs.
99 Unlike direct compression of SECs, this work shows that spherical silica particles of the same
100 size as SECs, act as compression micro-probes significantly improving the tableting
101 technique, applying multiaxial compressing force leading to effective opening of SECs
102 without damaging or permanently flattening SECs structure. In addition, a few minutes of
103 ultrasonification applied were enough to load SECs with *L. casei*. Furthermore, double
104 staining of *L. casei* with 5(6)-carboxyfluorescein diacetate (cFDA) and propidium iodide (PI)
105 provided a method to distinguish live, dead and injured bacteria inside the pollen, plus flow
106 cytometry and confocal imaging analysis without the a priori release of *L. casei* from the
107 SECs. This work highlights a unique characteristic of SECs, derived from their natural role to
108 protect pollen protoplasts until they are released, the ability to protect and allow *L. casei* to
109 grow within SECs, eventually generating enough pressure to cause the SECs to burst and

release the viable cells. SEM images show, that when SECs loaded with *L. casei* are spread onto a very nutritious medium, i.e. MRS agar, it takes 12 ±2h for *L. casei* to grow enough to cause SCEs to burst. This shows that even in the most idealistic environment, the time needed for *L. casei* to grow and release from SECs is within the appropriate timeframe to target the distal intestine as the transit time of the chyme from stomach to the terminal ileum is (4 – 6 h).[30] This result alongside the high viability of the microencapsulated *L. casei,* makes SECs an ideal intestinal delivery system of probiotics.

2. Results

2.1. Assessment of tableting techniques using SEM

One of the biggest challenges is to open SECs without collapsing their structure which would make them redundant for encapsulation. In this work three (3) different tableting methods were tested and their efficiency with regards to opening the SECs was evaluated in two ways: (a) visual examination of SEM images and (b) the %Encapsulation Yield (%EY). These two assessments had a complementary aim. By SEM microphotographs the SECs' evaluation was visual and qualitative, while the %EY quantitatively assessed their ability to encapsulate cells. **Figure 1**A shows a schematic of the different tableting techniques used within this work.

In the first tableting technique tested, SECs were directly compressed to a tablet (Figure 1Aa) using a 10 mm ø probe.[31] Figure 1Ba shows representative SEM images of SECs directly compressed to a tablet. Most of SECs were flat and only a very small number of them opened whilst retaining their original shape. In the second tableting technique, both SECs and untreated pollen grains were mixed in 1:1 ratio and external pressure was applied using again a 10 mm ø probe (Figure 1Ab). In this way the SECs were compressed between rigid micro-particles (i.e., untreated pollen shells) which acted as “micro pressing probes”. The SEM

images (Figure 1Bb) showed that by using this technique, the SECs did not flatten significantly and yet the number of them that had been opened was increased. However, this tableting technique has the disadvantage that it does not lead to an allergen-free sample since the untreated pollen particles still carry their allergenic proteins. However, these results are used as an indication of the shape and the size of an inert material that should be used to lead to an allergen free sample. Hence, a third technique was tested in which the SECs were mixed with spherical silica particles of the same size as the SECs (20 ø μm) at 2:1 ratio (Figure 1Ac). In addition, the size of the pressing probe was changed to 45 ø mm. With these latter changes, the number of flattened pollen shells was further reduced, and more opened SECs were obtained (Figure 1Bc). It needs to be noted, that other tableting techniques were also evaluated, using silica particles of different size and shape, however, without promising results (Figure S3, Supporting Information). In particular, a mix of SECs: silica particles (non-spherical 105 – 425 μm) in 2:1 ratio was used (Figure S3Aa) and the SEM images showed that the majority of the SECs were flattened and stuck onto the much larger silica particles (Figure S3Ba). The same outcome was observed irrespective of the SECs -to-silica particles ratio and compression force applied.

In addition, spherical silica particles (40-75 ø μm) were mixed with SECs with a SECs-to-silica ratio of 2:1 (Figure S3Ab). Considering a series of SEM microphotographs taken, it was clear that the use of spherical silica beads compared to the use of the non-spherical ones, led to an increased number of opened SECs, without resulting in them sticking on each other or on the silica beads (Figure S3Bb). However, spherical silica particles of similar to SECs diameter provided the most promising results (Figure S3C).

2.2. %Encapsulation Yield (%EY) of the tableting techniques

159 Following tableting and loading the SECs with *L. casei* cells, there should have been four
160 "cases" co-existing: (a) SECs that did not open, (b) SECs that did open but their structure was
161 practically destroyed, (c) those that did open but had no bacterial cells inside and (d) those
162 that did open and retained the *L. casei* cells inside them. From the above cases/populations, a,
163 b and c were lying within the region of empty SECs whereas d within the live region in the
164 FC plot (Figure 1C). The fraction of the total number of SECs (i.e. the total number of events
165 counted by FC) lying within the live, injured and dead regions was quantified and is the
166 numerator of %EY whereas the denominator is the number of the total events (Equation (1)).
167 Figure 1C shows the dot plots of FC analysis for each tableting technique; direct compression
168 of SECs (Figure 1Ca), mixed SECs: untreated pollen shells (Figure 1Cb) and mixed SECs:
169 silica particles-spherical 20 ø μm (Figure 1Cc). Figure 1D shows the %EY for the three SECs
170 tableting techniques tested; corresponding FC plots and %EY for the additional tableting
171 techniques explored can be found in Figure S3. The highest number of SECs that opened
172 suitably for encapsulation (30.87%) was obtained using spherical silica particles of 20 μm
173 (Sp20) and a 45 ø mm probe. The second highest %EY (14.65%) was obtained from the
174 tableting method where untreated pollen shells were mixed with SECs. The lowest %EY was
175 obtained by direct compression of SECs (0.99%) and using non-spherical silica particles
176 (2.02%), whereas spherical silica gel particles of 40-75 μm gave %EY of 6.15% (Fig. S3C);
177 sthe data used to calculate the %EY can be found in Table S1(Supplementary material).
178 Based on these findings, the best tableting technique, i.e. SECs:Sp20 and 45 ø mm press
179 probe, was used to prepare samples for the following stress tests.
180 A similar trend was observed with the plating method (MRS agar, Figure 1C and Figure
181 S3C). In particular, the highest number of colonies was obtained by using the SECs loaded
182 following the mixing with 20 μm spherical silica gel particles ($2.5x10^9$ cfu mL^{-1}). With
183 regards to other mixtures, SECs : untreated pollen mixture, SECs mixed with 40 – 75 μm

184 spherical gel particles, SECs mixed with non-spherical silica gel particles and direct
185 compression of the SECs gave $1.7x10^7$, $1.9x10^6$, $5.0x10^5$, and $2x10^4$ (cfu mL^{-1}), respectively.
186
187 **2.3. In vitro stress test of free and encapsulated *L. casei* in simulated media of the**
188 **human GI tract**
189 After assessing the performance of the different tableting techniques, the best one was used to
190 produce more samples for the stress tests. Double staining was applied to assess viability
191 (cFDA & PI) as well as the metabolic status (cFDA) of live and injured bacteria during the
192 stress tests. MRS agar plating was also used alongside flow cytometry and confocal
193 microscopy to fully assess the protective nature of the SECs.
194
195 *2.3.1 Fed state*
196 FC analysis (**Figure 2**A) showed that the number of events within the live region was similar,
197 after the sequential exposure in $FeSGF_e$, $FeSGF_m$ and $FeSGF_I$ (i.e. for the first 4.5 h) for the
198 encapsulated bacteria (8,171 ±222 initial, 7,911 ±313 @1.5h, 7,670 ±256 @3h and 8,020
199 ±166 @4.5h). This was also confirmed by the number of colonies grown on MRS plates, 8.3
200 ± 0.11 (Initial), 8.3 ± 0.10 (@1.5h), 8.3 ± 0.06 (@3h) and 8.2 ± 0.07 (@4.5h) logcfu mL^{-1}, or
201 as a fraction of the initial population, 1, 1, 0.98 normalized logcfu mL^{-1} (**Figure 3**A),
202 respectively. FC and MRS plate count method showed a similar trend for the free bacteria
203 throughout the simulation of the GI tract environment exposure, except from the 4.5h time
204 point. At 4.5h, the FC analysis showed a significant fraction of the free *L. casei* to be within
205 the region of injured cells, although, MRS plate count showed no significant change of the
206 number of colonies formed. Nevertheless, the FC data after the 4.5h timepoint showed again
207 similar trend with MRS plate count. Specifically, with the FC method (Figure 2B), the
208 population of free bacteria was found to be 19,345 ±475 (initial), 18,623 ±278 (1.5h), 19,111

±151 (3h) and 19,261 ±317 (4.5h) whereas the corresponding logcfu mL^{-1} was found to be, 9.3 ±0.02 (initial), 9.2 ±0.4 (1.5h), 9.2 ±0.12 (@3h) and 9.1 ±0.07 (@4.5h) logcfu mL^{-1} or 0.99, 0.99 and 0.98 normalized logcfu mL^{-1} (Figure 3B, fed state), respectively. The next step was the sequential exposure of the samples in the three different simulated intestinal fluids ($FeSSI_e$, $FeSSI_m$ and $FeSSI_l$). In the early phase that lasted 40 min, the population of both encapsulated and free *L. casei* was decreased, compared to the previous stage (i.e., in stomach), by 1.0 and 1.4 log cycles, respectively. The sharpest reduction of free cells, by approx. 3 log cycles, was observed in the middle phase ($FeSSI_m$), which also lasted 40 min, while under the same conditions the reduction of the encapsulated *L. casei* population was statistically non-significant ($p > 0.05$) compared to the previous stage (i.e. $FeSSI_e$ media). As regards the last phase of the simulated intestinal fluid ($FeSSI_l$), the encapsulated *L. casei* population was further decreased by approx. 0.6 log cycles, while the free cell population by 0.2 log cycles. Finally, both free and encapsulated *L. casei* were exposed to the fed simulated colon (FeSC) media for 4.0 h. MRS plate counts for both encapsulated and free *L. casei* remained constant with respect to the populations at $FeSSI_l$. However, FC measurements showed a gradual reduction of live free bacteria that was not reflected in the plate counting method, implying that the injured bacteria were still culturable. At the end of the *in vitro* stress test, there was a total drop of 2 and 4.5 folds for encapsulated and free bacteria, respectively (Figure 3A and B, fed state). Similarly, FC showed a reduction of the events in the live region 16 (8,159 events initially → 482 @13h) and 47 (19,337 events initially → 411 @13h) fold reduction for encapsulated and free bacteria, respectively. This means that 2.25 and 2.94-folds more live bacteria, encapsulated in SECs, survived after the stress test, based on the plate count and FC methods, respectively. This can be clearly seen in Figure 3D, in which encapsulated and free bacteria were compared after normalization of the actual logcfu mL^{-1} values.

234

235 *2.3.2 Fasted state*

236 Unlike the fed state, a sharp decrease in the viability of both encapsulated and free bacteria
237 was observed in the fasted stomach media FaSGF (Figure 4A and B). In particular, 3.5 folds
238 decrease in the viability of *L. casei* was observed for encapsulated (8.3 (Initial) to 2.4 (@2h)
239 logcfu mL^{-1}) and 4.6 folds for the free *L. casei* cells (9.3 (initial) to 2.02(@2h) logcfu mL^{-1}).
240 When the samples were exposed for about 5.7 hours in simulated intestinal fluid (FaSSIF),
241 where the pH was increased from 1.6 to 6.5 and the bile salt concentration increased from
242 0.08 mM to 3 mM, the population of the encapsulated cells was decreased, but this drop was
243 statistically non-significantly (p>0.05), while the population of the free bacteria decreased by
244 approx. 0.5 log cycles (p<0.05).
245 In the last step, when the samples were transferred to the colonic media (FaSC), where pH
246 increased from 6.5 to 7.8 and bile salt concentration decreased from 3mM to 0.150 mM, the
247 cell population, both of encapsulated and free bacteria, remained practically unchanged.
248 However, encapsulated bacteria showed again higher viability in all simulated media
249 compared to free (Figure 4C). In particular, at the end of the stress test there were 1.21 folds
250 more live bacteria encapsulated in SECs (5.65 logcfu mL^{-1} @13h) compared to free (4.67
251 logcfu mL^{-1} @13h) as shown in Figure 4C.

252

253 **2.4. Release of *L. casei* cells from SECs**

254 SEM images of encapsulated *L. casei* cells, spread on MRS agar, were captured at several
255 time intervals (Figure 4). After an hour of anaerobic incubation at 37 ºC, no individual or
256 proliferated (i.e. colonies) of *L. casei* cells were observed on MRS agar. At 6 h some SECs
257 were opened and *L. casei* cells appeared on and around those SECs. At 12 h more SECs were
258 opened (burst) releasing thousands of *L. casei* cells on the MRS agar. What was noticed was

that the wall of the burst SECs was more curved especially close to the aperture (zoom in image @12 h, Figure 5), implying that the elastic SEC was deformed before burst. At 24 h, the entire surface of the MRS agar plate and SECs was covered by *L. casei* cells. These results showed that the encapsulated *L. casei* cells can proliferate inside SECs and can mechanically disrupt the SEC (burst), leading to their release.

3. Discussion

One of the main objectives of this work was to study whether it is possible to use sporopollenin exine capsules (allergen free pollen) as a carrier of beneficial bacteria, which would provide protection and allow them to thrive before their release in the distal intestine. A prerequisite of this goal was to effectively open the SECs, enabling sufficient load with bacteria in high numbers for adequate colonization of the distal part of the human GI tract. Previous studies have shown changes to the external morphology of SECs as a result of chemical treatment in specie-dependent manner;[24-26] in this study the combination of using NaOH as a pretreatment and the *L. calvatum* as pollen specie model, ensuring no changes on the external morphology of SECs as shown in Figure S1Ba. Opening the sporopollenin exine capsules by compression without damaging the integrity of their structure is crucial for successful encapsulation of the bacteria. This is because an elastic structure, like the SEC,[23] can be deformed without opening the aperture which is the "gate" for the bacteria to enter (and exit) the SEC. Previous studies have shown that sporopollenin exine can fracture (crack) after compression. However, due to the elastic properties of the wall, the sporopollenin exine can revert to its almost intact-appearance after removing pressure, although the cracks are still present.[23] These big crack openings are not suitable for controlled diffusion of the biorelevant media, as this normally takes place through the 15 – 20 nm pores on the intact sporopollenin exine,[32] as well as for the controlled release of the bacteria.

284 The results of this work showed that direct compression of a single empty flexible structure,
285 like a sporopollenin exine capsule, surrounded by other deformable particles, like in the case
286 of using only SECs for tableting, led to permanently flattened SECs (Figure 1Ba). This is
287 because deformable particles, like the SECs, absorb the compressive force and hence,
288 reaching a five tones (t) pressure force (see methods section) requires great displacement of
289 the press die probe, leaving only a very small gap between the probe and the base. This leads
290 to excessive compression and to permanently flattened SECs. This was also the case when a
291 pressing probe of 45 ø mm was used (data not shown). When a mixture of SECs and
292 untreated pollen shells was used, there was no excessive compression of the SECs (Figure
293 1Bb). This is probably due to the untreated pollen particles acting as small compressing
294 probes, pressing the untreated pollen particles from different directions (i.e. multiaxial),
295 leading to a more uniformly distributed pressure around the SECs and hence the successful
296 opening of the aperture. Moreover, the untreated pollen grains as more rigid particles, helped
297 to reach 5 t pressure force at a smaller displacement of the probe, hence excessive
298 compression was not needed / obtained at 5 t. In order to make an allergen-free sample, the
299 untreated pollen particles should be replaced with an inert material, like silica. Silica was
300 chosen as inert material extensively used in bio-applications and in food industry.[33] In the
301 case of using large rigid particles, like when non-spherical silica was used in this work, the
302 displacement is smaller for the probe, but the SECs were extensively compressed between the
303 non-spherical silica particles, leading again to the same results as with using only SECs. This
304 probably happened because the smaller particles, i.e. SECs, were compressed on the much
305 larger surface area of the silica particles. Thus, big silica particles do not act as micro-
306 pressing probes like the untreated pollen shells, but as a common die press. Hence, the SECs-
307 to-silica contact surface ratio should be considered in properly compressing the SECs in order
308 to open their aperture. Next, spherical silica particles of the size of 40-75 μm were used and

309 the tableting technique was improved compared to the non-spherical silica particles but still,
310 using untreated pollen was proven to be better. Thus, spherical silica particles of the same
311 size with SECs (20 μm) were used and significantly higher number of sporopollenin exine
312 capsules opened, as also confirmed by SEM (Figure 1Ac) as well as by FC, i.e. higher %EY
313 (Figure 1D). Another factor that helped to improve the tableting technique was the larger
314 diameter of the press die probe used, 45 ø mm whilst maintaining the same compression force
315 (5 t). In this case, the gap between the probe and the base was much smaller compared to
316 using a 10 ø mm press probe. Hence, the combination of rigid silica particles of the same size
317 with SECs and the larger probe helped to reach the 5 t pressure force at much smaller
318 displacement of the probe, and multiaxial compressing force applying on SECs. Further
319 reduction of the silica particles size, i.e. <20 μm, showed several technical limitations not
320 allowing to further explore the effect on the encapsulation efficiency (more technical details
321 can be found in the supplementary material).
322 Another challenge in this work was to monitor the metabolic state of the bacteria inside the
323 SECs. Usually, the viability of the encapsulated bacteria is assessed after their release from
324 the encapsulant. Thus, the bacteria are in a suspension, making plating, staining, imaging and
325 the corresponding analysis more straightforward. In this work, for the first time, the viability
326 of the bacteria, in particular *L. casei*, was assessed inside the SECs. The only way to release
327 the bacteria from the SECs was by using the bead-beating method (Figure S4). Hence, to
328 overcome this technical limitation, staining had to be performed after the encapsulation of the
329 bacteria. Moreover, flow cytometry needed to be adjusted to minimize the strong
330 autofluorescence of the sporopollenin (Figure S5). However, this reduced the FC sensitivity
331 and as the confocal images showed (Figure 2A), within a single SEC, live, dead and injured
332 *L. casei* cells can co-existed in different numbers. Moreover, in each SEC, one, tens, or
333 hundreds of cells may exist. Hence, the intensity of the emission in the green channel,

coming from the live cells inside the SEC, will not be strong enough in order for this particular SEC, with just one or few live cells, to appear within the live region. Thus, FC is likely to underestimate the true %EY. The plate count method could also underestimate the true population of live or injured but culturable *L. casei* cells. This is because colonies can be formed either from a SEC, which contains just one cell, or from a SEC that contains tens or hundreds of bacterial cells. Despite these technical limitations, FC and the plate count method could still provide qualitative and quantitative analysis of the viability of *L. casei* after exposure to *in vitro* simulated media of the human GI tract. It should be clear that with the proposed method, FC analysis differentiates the SECs according to which population of live, injured or dead is the most predominant within each SEC.

Before discussing the viability results, it should be pointed out that the spherical silica particles could effectively be differentiated from the SECs in FS vs. SS plot (Supplementary Figure S5A). Hence, there was not any interference due to the presence of silica particles in the FC analysis.

The protective effect of encapsulation was evident in both fasted and fed state simulated media. In the fasted state, the significant reduction in viability of both encapsulated and free bacteria, occurred in the FaSGF stage due to the extremely low pH (1.6) while in the following stages of the test a further decrease in population was not as dramatic. The antibacterial action of low pH values is well known and well documented. However, it should be noted that the SECs were pre-hydrated due to the encapsulation procedure. Thus, the effect of low pH would be immediate, due to fast diffusion of the gastric media, compared to a dried sample (e.g. freeze dries encapsulated *L. casei*), where the hydration and hence the diffusion of the gastric media, is likely to be much slower. However, extra work needs to be conducted to confirm this.

In contrast, the elevated bile salt concentration in the fed simulated environment is expected to be the main reason lowering the viability in the media of the small intestine (population was constant in fed stomach for both free and encapsulated). Bile salts possess antimicrobial properties against *L. casei*.[34] González-Vázquez et al showed a two-fold reduction in the viability of *L. casei* after 4h exposure in 5.8 mM oxgall bile extract. In our work, a 4.5-fold reduction was observed after ~4h in *L. casei* population exposed to 4.5 – 10 mM bile salt extract in the fed simulated environment (see Table 1 for the composition of the simulated media in experimental section). The composition of the bile salt extract is another parameter affecting the viability of *L. casei*. Glycine conjugates have a stronger effect over strain survival than taurine conjugates.[34] González-Vázquez et al used oxgall bile salt extract, whereas porcine extract was used in our work. Porcine bile extract consists of 30% glycocholic acid, 40% taurocholic acid, 7% taurodeoxycholic acid, 15% glycodeoxycholic acid and 5% hyodeoxycholic acid.[35] Thus 45% of the bile acids in this work were glycine conjugates, whereas oxgall bile extract contains 30.4% glycine conjugates.[36]

Considering the high concentration of bile salts and the use of porcine bile extract, high in glycine conjugates, an only two-fold reduction in the viability of *L. casei*, shows the protective properties of sporopollenin against the harsh environment of the small intestine in the fed state. These protective properties are derived from the chemistry of the material of the pollen wall, i.e. sporopollenin. The sporopollenin surface possesses ionizable groups (phenols and carboxylic acids) that become increasingly negatively charged in pH >5.[37] Thus, SECs' wall surface will be negatively charged in the small intestine and colon under fasted and fed conditions. Significant fraction of the bile salts in porcine extract are glycine conjugated (45%) with pKa values 4 – 5 whereas another fraction of 47% is taurine conjugated bile salts with their pKa values likely to fall in the range of pH = -1.5 to +1.5.[38] Thus in this work the majority of the bile salts were ionized in simulated media of the small intestine and the colon.

Hence, it seems that negative electrostatic repulsion forces took place between SECs surface and the bile salts, which could explain the protective mechanism and hence the higher viability observed for the SECs encapsulated *L. casei*.

As mentioned above, colonies were formed on MRS agar plate. However, the number of *L. casei* cells present in each SEC that could form each of the observed colonies, is unknown. The colonies, appearing on the MRS agar, should be formed after proliferation and release from the SECs. If the SEC has a crack which has not been closed completely, most of the cells are likely to be washed out from the SECs cavity and separated from the encapsulated *L. casei* population after sequential transferring to each media and filtering before analysis. It is also likely the cells to be dead or at very low numbers, as the simulated media will diffuse freely inside the SECs. Thus, the high viability observed for the encapsulated *L. casei* should be the result of proper closure of the aperture of most of the SECs. This can be seen in Figure S6 where an SEM image of a cut SEC was captured immediately after the encapsulation of *L. casei*. In addition, existing literature demonstrates that *L. clavatum* can withstand the GI environment[28, 29]. Hence, the release of the bacteria from a closed capsule should be due to mechanical disruption (burst) of the SECs, caused by elevated pressure due to the proliferation of the bacteria encapsulated within it. In order to confirm this hypothesis, SEM images were taken at several time intervals after the encapsulation of *L. casei* (Figure 4). Indeed, pollen seemed to act as a vehicle that protected and allowed *L. casei* to grow inside it in such numbers that enough pressure could be generated, causing the SECs to burst (Figure 4 @12h). SECs loaded with *L. casei* spread on the very nutritious medium, i.e. MRS agar, takes 12 ±2 h for *L. casei* to growth enough to cause SCEs to burst. This shows that even in the most idealistic environment, the time needed for *L. casei* to grow and release from SECs is longer than the transit time of the chyme from stomach to the terminal ileum (4 – 6 h).[30] Moreover, the residence time of the fluids within the colon ranges 16 – 46 h,[39] ensuring

enough time for the encapsulated bacteria to potentially growth inside the SECs and be released within the colonic region. This result alongside the high viability of the microencapsulated *L. casei* makes SECs an ideal delivery system for probiotics. However, it should be pointed out that the release time may vary (12 ±2 h), and it depends on whether the SECs have properly closed or not as well as from the initial or the remaining number of live bacteria in SECs. In those cases the bacteria may release sooner @10h (Figure S7).

4. Conclusions

Several encapsulation means have been proposed as carriers of health promoting bacteria to the human distal intestine so that they can eventually settle and thrive there. This work shows a novel tableting technique to effectively open SECs to encapsulate bacteria. Once the *L. casei* cells are encapsulated, the SEC protects them when exposed to simulated environment of the human gastrointestinal tract.

The noticeable difference with the application of the SECs as a means of encapsulation is that the SECs may act as a bioreactor and provide time for the encapsulated cells to multiply thousands of times before being released, following the SEC's burst.

This unique feature of encapsulation within SECs, means that the initial loading of bacteria is not the main factor determining the efficacy, as even few encapsulated live bacteria within hours can be multiplied exponentially and give thousands of high vitality bacteria, ready to be released from SEC due to its inherited shell elasticity.

Further work is required to evaluate loading of alternative materials as well as the behavior of the encapsulated bacteria when released at the presence of other free bacterial populations, mimicking a more realistic distal intestine environment. Furthermore, a drying step after the encapsulation should be applied to further reduce the hydration rate of SECs and potentially increase their protective effect, especially in the fasted state.

5. Experimental Section

Chemicals

Lycopodium clavatum, hydrochloric acid, sodium hydroxide, potassium hydroxide, Tween 20, Glutaraldehyde solution, paraformaldehyde, 6-Carboxyfluorescein diacetate (cFDA, C5041), Propidium Iodine (PI), Phosphate-buffered saline (PBS) tablets and MRS (De Man, Rogosa, Sharpe) broth were purchased from Sigma. Ethanol absolute, acetone and orthophosphoric acid (85%, v/v) from Fisher Scientific. Centrifugal filter units (UFC30 SV00) were purchased from Merck Millipore.

Sporopollenin exine capsules preparation

Sporopollenin exine capsules (SECs) were prepared by removing the protoplast based on previous protocols published, with some modifications.[28, 31] Briefly, 60 g dry *Lycopodium clavatum* pollen was suspended into 450 mL aqueous solution of NaOH (2M) and the mixture was stirred under reflux for 12 h at room temperature. Following filtration, the collected pollen was diluted in ~900 mL hot distilled water, filtered again and sequentially washed with hot distilled water (5 x 300 mL) and hot ethanol (5 x 300 mL). Following overnight drying at 60ºC in a fume cupboard, pollen was stirred under reflux and smooth boiling (with glass beads) for 7 days in 450 mL orthophosphoric acid (85%, v/v). Then, the mixture was filtered again and the pollen was diluted in ~900mL hot distilled water and washed sequentially with hot distilled water (5 x 300mL), acetone (300mL), 2M hydrochloric acid (300mL), 2M sodium hydroxide (300mL), hot distilled water (5 x 300mL), acetone (300mL) and hot ethanol (300mL). Finally, SECs were dried at 60°C in an air-drying oven until constant weight was recorded and then were kept in a desiccator at 4°C until required for further experiments. The complete removal of the protoplast was confirmed using CHN elemental analysis (EA1110, CE Instruments) and a multiplication factor 6.25 was used to convert nitrogen (%) to protein (%).[32] Furthermore, scanning electron micrographs (SEM)

458 of SECs were taken to confirm protoplast removal and any damage of the SEC due to the
459 chemical treatment.
460 *Tableting and opening SECs*
461 SECs, untreated pollen grains and silica gel particles either of cubic shape or beads of various
462 sizes were available. Five different combinations were tested in order to open as many SECs
463 as possible using compression techniques (tableting): (a) solely SECs, (b) SECs mixed with
464 untreated pollen grains in 1:1 ratio, (c) SECs mixed with non-spherical silica gel particles
465 (105 - 425 μm) in 2:1 ratio (d) SECs mixed with silica gel beads of (40-75 ø μm in 2:1 ratio
466 and (e) SECs mixed with silica beads of diameter 20 μm in 2:1 ratio. A maximum of 5 t
467 external force was applied using a 10 or 45 ø mm probe. Samples of SECs prepared by all the
468 above-mentioned techniques, were studied using SEM.
469 *Preparation of L. casei cell suspension*
470 Stock cultures of *L. casei* (ATCC 393) maintained at -80°C in MRS broth with 30% glycerol
471 (v/v) were thawed and 100 μL streaked onto an MRS Agar plate which was incubated for 24
472 h at 37°C under anaerobic conditions (Oxoid AN0020C AnaeroGen Compact). After the
473 incubation, a colony was transferred into 20 mL of deaerated MRS broth and incubated again
474 for 24 h under anaerobic conditions at 37°C. After the second incubation, 200 μL of the broth
475 culture was transferred into deaerated MRS broth (20 mL) and incubated overnight under
476 anaerobic conditions at 37°C to obtain *L. casei* cells at the early exponential phase. Then the
477 culture was centrifuged for 10 min at 3900 rpm. The supernatant was decanted and the
478 culture was washed twice with deaerated PBS. Finally, the culture was re-suspended in 20
479 mL fresh PBS (approx. 109 cfu mL^{-1}) which was the "working" cell suspension.
480
481 *Encapsulation of L. casei cells in SECs*

The following protocol was applied in all tableting techniques (see section 2.3 Tableting and opening *SECs*) in triplicate, to evaluate their ability to be loaded with *L. casei* cells. First, 0.010 g of loose powder obtained from each tableting technique tablet was mixed with 1 mL of working *L. casei* suspension (approx. 10^9 cfu mL^{-1}) in Eppendorf vials and treated for 30 s in an ultrasound bath (60 W). Subsequently, from each sample the non-encapsulated bacteria were separated from the SECs by filtration. Each sample was centrifuged using filtration tubes (5 μm pore size, Dutscher Scientific) for 20 s at 12000 rpm and then washed with deaerated PBS. The SECs were retained on the filter while the precipitant was the non-encapsulated *L. casei* cells. The SECs were resuspended into 1 mL deaerated PBS, gently shaken and centrifuged again using filtration tubes. This procedure was repeated six times and each sample was examined under optical microscope, using methylene blue, to ensure that all the non-encapsulated bacteria had been efficiently washed off from the SECs. Finally, each sample was resuspended in 1 mL deaerated PBS and that comprised the working solution for the plate count method (MRS agar) and for flow cytometry analysis.

Staining of L. casei cells

By the use of appropriate staining techniques, confocal microscopy and flow cytometry, the *L.casei* bacteria were differentiated and enumerated - living, compromised membrane (injured) and dead cells, either encapsulated or non-encapsulated, depending on the experimentation protocol. Specifically, two dyes with complementary functions were used: Propidium Iodine (PI) was used to stain dead/injured cells, while Carboxyfluorescein diacetate (cFDA) was used to stain metabolically active live/injured cells.[40]

PI staining: The working solution of PI was 1 mg mL^{-1} in distilled water and the staining carried out as follows: 5 μL PI were added to 995 μL of the working solution of encapsulated or free bacteria. The mixture was incubated for 15 min at 37°C in a water bath. In order to

enhance the diffusion of the dye in the encapsulated cells, the sample was ultrasonicated for 5 s (60 W).

cFDA staining: The dye working solution was 1 mM in PBS. 10 μL were added to 990 μL of the working solution of encapsulated or free bacteria. The solution was incubated for 15 min at 37^{o}C in a water bath and was also ultrasonicated as described above.

Then, all the samples were washed three times with deaerated PBS, fixed with 4% (v/v) paraformaldehyde solution for 1 h at 4^{o}C and then washed twice with deaerated PBS. Between the washing steps, the samples were centrifuged at 12000 rpm for 10 s. The same steps, in terms of staining and fixation, were followed for blank samples, i.e., SECs and the silica spheres.

Dual staining: Initially three types of *L. casei* cells were prepared: (a) fresh 8-hour incubated culture, (b) heat killed (75^{o}C/ 15 min) culture and (c) various mixtures of (a) and (b). Dual staining trials were performed according to the above described protocols in order to achieve the fine tuning needed for the flow cytometry and for microscopy. The PI was used first, followed by cFDA.

Tableting evaluation by calculating the %Encapsulation Yield (%EY)

Encapsulation Yield (%EY) (Equation 1) was determined as the ratio of the number of SECs loaded with *L. casei* in relation to the total number of SECs.

This ratio was used as an index to assess the ability of SECs, produced by different tableting techniques, to be loaded *with L. casei* cells.

$$\%EY = \frac{N_{\mathrm{SECs}:L.casei}}{N_{T_{SECs}}} \quad (1)$$

where $N_{\mathrm{SECs}:L.casei}$ is the number of SECs loaded with L. casei and $N_{T_{\mathrm{SECs}}}$ total number of SECs. The number of SECs loaded with *L. casei* accounts for all the cells, i.e. living, dead

and injured, presented initially in SECs. Quantitative analysis was carried out by flow cytometry.

Monitoring L. casei viability by flow cytometry and MRS agar

The encapsulated and free populations of *L. casei*, were counted using two techniques: (a) the standard technique of plating on agar and (b) by flow cytometry (FC). Initially 0.01 g of each SECs sample were suspended into 1 mL deaerated PBS and then serially and decimally diluted (100 μL to 900 μL PBS) with the same solution. The diluted suspensions were then used for analyses by plating on agar and flow-cytometry.

Flow Cytometry: Samples were analyzed by a Cyan ADP flow cytometer (Beckman Coulter) in order to assess the viability of the encapsulated bacteria. The set-up of the instrument was carried-out as follows: For minimizing the interference of the strong autofluorescence of the SEC[41] and to separate the signal coming from the silica spheres, SECs and silica beads were first injected, separately and simultaneously at 2:1 ratio into the flow cytometer instrument. Finally, the voltage (mV) for Side Scattering (SS), green (FL1) and red (FL3) channel was set to 596, 420 and 633, respectively, whereas amp gain for the Forward Scattering (FS) was set to 60. The flow rate of samples was set to 20 μL min^{-1} and the sample concentration was adjusted to keep the count lower than 1,000 events s^{-1}. Deaerated PBS was used as sheath fluid. From the decimal diluted suspension that had been made, samples were taken, sequentially stained with PI and cFDA and sorted as follows: The sort gates were defined on an FL1-versus-FL3 dot plot of cFDA- and PI-stained cells. The sorter was set to single-cell mode and to stop after 2 min, which corresponded to an acquisition of 25,000 to 40,000 events. The analysis of the flow cytometry data was performed using open-source software (Flowing Software 2.5.1). Details of the optimization of the flow cytometry can be found in Figure S5.

Plating method: 100 μL of each dilution were taken and plated by spreading onto MRS agar medium and were then incubated under anaerobic conditions (Oxoid AN0020C AnaeroGen Compact) for 48h at 37ºC. In addition, comparison of the viability of free and encapsulated bacteria was performed by plate count method after normalizing cfu mL^{-1}, i.e. cfu $mL^{-1}{}_{i}$ / cfu $mL^{-1}{}_{initial}$, where i is the number of cfu mL^{-1} at each time point. This is because the initial cfu $mL^{-1}{}_{initial}$ was different for the encapsulated and the free bacteria.

Microscopy studies

Scanning Electron Microscopy (SEM): Scanning Electron Microscopy (JEOL 6060, Japan) was used to obtain images of SECs, derived from the different tableting techniques tested. The samples were freeze dried overnight and then were platinum coated (10 kV beam) using an auto coater (JEOL Smart Coater, Japan). Images were captured at different magnifications.

Confocal laser scanning microscopy analysis (CLSM). CLSM analysis was performed to confirm: (a) the absence of the protoplast after the chemical treatment of the pollen, (b) the encapsulation of the bacterial cells and to also (c) evaluate the impact of the simulated media of the human GI tract on the viability of the bacterial cells. A Leica TCS SP2 (UK) confocal microscope equipped with a DMRE2 inverted microscope, AOBS spectral detector and six laser lines (405/476/488/514/546/647 nm) was employed to carry out the CLSM analysis. Capturing conditions were set as follows: laser excitation lines 514 nm and 647 nm with an HCX FL Plan 100x 1.25 objective lens. The iris was tuned according to sample conditions, and all images were captured within the middle region of the SECs. The remaining settings were fixed for all samples, for which at least three images were obtained. Image processing was performed with Lass X software (Leica, UK).

In vitro stress test of free and encapsulated in SECs L. casei bacteria in simulated media of the human GI tract.

The survival of free as well as SECs encapsulated *L. casei* bacteria was tested under two conditions of the gastrointestinal (GI) tract: (a) Fasted and (b) Fed. The first one corresponds to the conditions of the GIT without food while the second one with food. The compositions of the biorelevant media are given in Table 1.

Fasted state: 0.01 g of loose powder, that contained approx. 10^8 cfu mL^{-1} *L. casei* cells (SECs encapsulated bacteria) and free *L. casei* cell pellet (10^9 cfu $mL^{-}1$) were added separately to 1 mL Fasted state Simulated Gastric Fluid (FaSGF). Following incubation (37ºC for 2h) the mixtures were centrifuged at 12000 rpm/ 20 s. The supernatants were decanted and the pellets of the free bacteria as well as of the SECs were resuspended into 1 mL Fasted state Simulated Intestine Fluid (FaSIF), respectively. The mixtures were incubated at 37ºC for 5 h and the above described procedure was repeated and then the precipitants were resuspended in 1 mL Fasted state Simulated Large Intestine Fluid (FaSLIF) and incubated at 37 ºC for 8h. During all the previously described sequential centrifugation and reconstitution steps samples at several time points (10 μL each) were taken, resuspended into 1 mL deaerated PBS and accordingly analyzed by confocal microscopy, flow cytometry and agar plating (MRS agar).

Fed state: Unlike the fasted state, in the fed state three different biorelevant media were used for the gastric and upper intestinal simulated region of the GI tract, which reflects the time-depended changes in composition upon food digestion. Thus, the free as well as the SECs encapsulated *L. casei* were sequentially exposed to early, middle and late gastric ($FeSGF_e$, $FeSGF_m$ and $FeSGF_l$) and small intestinal ($FeSSI_e$, $FeSSI_m$ and $FeSSI_l$) media for 1.5 h in each media. The incubation time in colonic media (large intestine) was 4 h in total at 37ºC and samples were taken every 2 h. Concerning the experimentation steps, these were identical to the ones followed for the fasted state (i.e. incubation, centrifugation, resuspension).

Release of L. casei cells from SECs incubated on MRS agar

The purpose of these experiments was to understand how the bacteria are released from SECs. SECs loaded with *L. casei* and spread within a very small area of the MRS agar plate and stored @37 ºC under anaerobic conditions. Then, the sample was cut several times at specific time intervals (1, 6, 10 and 24h) and prepared for SEM as described above. So, the SEM images are coming from the same sample, i.e. no different batch of tablet, encapsulated *L. casei* to capture SEM images at the different time intervals. The process was repeated for three tablets and representative SEM images are reported.

Statistical analysis

One-way ANOVA was employed for statistical analysis in Sigmaplot (v. 12.5). All the experiments were performed in triplicate. Values of $p < 0.05$ were considered as statistically significant.

Supporting Information

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

Acknowledgements

The authors would like to thank Mr. Athanasios Karabotsos (Department of Conservation of Antiquities and Works of Art, School of Applied Arts & Culture, University of West Attica) for his work on SEM images of sporopollenin exine capsules.

Received: ((will be filled in by the editorial staff))
Revised: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

References

1. Cox, L. M.; Weiner, H. L., *Neurotherapeutics* **2018,** *15* (1), 135-145. DOI 10.1007/s13311-017-0598-8.
2. Vargason, A. M.; Santhosh, S.; Anselmo, A. C., *Small* **2020,** *16* (25), 2001705. DOI 10.1002/smll.202001705.

3. Ashwar, B. A.; Gani, A.; Gani, A.; Shah, A.; Masoodi, F. A., *Food Chemistry* **2018,** *239*, 287-294. DOI https://doi.org/10.1016/j.foodchem.2017.06.110.
4. Krunić, T. Ž.; Obradović, N. S.; Rakin, M. B., *Food Chemistry* **2019,** *293*, 74-82. DOI https://doi.org/10.1016/j.foodchem.2019.04.062.
5. Cook, M. T.; Tzortzis, G.; Charalampopoulos, D.; Khutoryanskiy, V. V., *Journal of Controlled Release* **2012,** *162* (1), 56-67. DOI https://doi.org/10.1016/j.jconrel.2012.06.003.
6. Cook, M. T.; Tzortzis, G.; Charalampopoulos, D.; Khutoryanskiy, V. V., *Biomacromolecules* **2011,** *12* (7), 2834-40. DOI 10.1021/bm200576h.
7. Wu, B.; Yang, C.; Li, B.; Feng, L.; Hai, M.; Zhao, C.-X.; Chen, D.; Liu, K.; Weitz, D. A., *Small* **2020,** *16* (30), 2002716. DOI https://doi.org/10.1002/smll.202002716.
8. Hamad, S. A.; Stoyanov, S. D.; Paunov, V. N., *Soft Matter* **2012,** *8* (18), 5069-5077. DOI 10.1039/C2SM07488E.
9. Mirzaei, H.; Pourjafar, H.; Homayouni, A., *Food Chemistry* **2012,** *132* (4), 1966-1970. DOI https://doi.org/10.1016/j.foodchem.2011.12.033.
10. Ding, W. K.; Shah, N. P., *Journal of Food Science* **2009,** *74* (2), M100-M107. DOI 10.1111/j.1750-3841.2009.01067.x.
11. Burgain, J.; Gaiani, C.; Cailliez-Grimal, C.; Jeandel, C.; Scher, J., *Innovative Food Science & Emerging Technologies* **2013,** *19*, 233-242. DOI https://doi.org/10.1016/j.ifset.2013.04.012.
12. Smilkov, K.; Petreska Ivanovska, T.; Petrushevska Tozi, L.; Petkovska, R.; Hadjieva, J.; Popovski, E.; Stafilov, T.; Grozdanov, A.; Mladenovska, K., *J Microencapsul* **2014,** *31* (2), 166-75. DOI 10.3109/02652048.2013.824511.
13. Hadzieva, J.; Mladenovska, K.; Crcarevska, M. S.; Dodov, M. G.; Dimchevska, S.; Geškovski, N.; Grozdanov, A.; Popovski, E.; Petruševski, G.; Chachorovska, M.; Ivanovska, T. P.; Petruševska-Tozi, L.; Ugarkovic, S.; Goracinova, K., *Food technology and biotechnology* **2017,** *55* (2), 173-186. DOI 10.17113/ftb.55.02.17.4991.
14. Samedi, L.; Charles, A. L., *Foods* **2019,** *8* (5). DOI 10.3390/foods8050175.
15. Jia, H.; Kerr, L. L., *Journal of Applied Polymer Science* **2015,** *132* (7). DOI 10.1002/app.41570.
16. Sinha, V. R.; Kumria, R., *European Journal of Pharmaceutical Sciences* **2003,** *18* (1), 3-18. DOI https://doi.org/10.1016/S0928-0987(02)00221-X.
17. Zhang, W.; Qu, L.; Pei, H.; Qin, Z.; Didier, J.; Wu, Z.; Bobe, F.; Ingber, D. E.; Weitz, D. A., *Small* **2019,** *15* (42), 1903087. DOI 10.1002/smll.201903087.
18. Chen, J.; Wang, Q.; Liu, C.-M.; Gong, J., *Critical Reviews in Food Science and Nutrition* **2017,** *57* (6), 1228-1238. DOI 10.1080/10408398.2014.977991.
19. Park, J. H.; Seo, J.; Jackman, J. A.; Cho, N.-J., *Scientific Reports* **2016,** *6* (1), 28017. DOI 10.1038/srep28017.
20. Mundargi, R. C.; Tan, E.-L.; Seo, J.; Cho, N.-J., *Journal of Industrial and Engineering Chemistry* **2016,** *36*, 102-108. DOI https://doi.org/10.1016/j.jiec.2016.01.022.
21. Barrier, S.; Diego-Taboada, A.; Thomasson, M. J.; Madden, L.; Pointon, J. C.; Wadhawan, J. D.; Beckett, S. T.; Atkin, S. L.; Mackenzie, G., *Journal of Materials Chemistry* **2011,** *21* (4), 975-981. DOI 10.1039/C0JM02246B.
22. Sudareva, N.; Suvorova, O.; Saprykina, N.; Vilesov, A.; Bel'tiukov, P.; Petunov, S.; Radilov, A., *Journal of Materials Chemistry B* **2017,** *5* (37), 7711-7720. DOI 10.1039/C7TB01681F.
23. Rowley, J. R.; Skvarla, J. J., *Grana* **2000,** *39*, 1 - 7.
24. Gonzalez-Cruz, P.; Uddin, M. J.; Atwe, S. U.; Abidi, N.; Gill, H. S., *ACS Biomaterials Science & Engineering* **2018,** *4* (7), 2319-2329. DOI 10.1021/acsbiomaterials.8b00304.

25. Uddin, M. J.; Liyanage, S.; Abidi, N.; Gill, H. S., *Journal of Pharmaceutical Sciences* **2018,** *107* (12), 3047-3059. DOI https://doi.org/10.1016/j.xphs.2018.07.028.
26. Becherini, S.; Mitmoen, M.; Tran, C. D., *ACS Applied Materials & Interfaces* **2019,** *11* (47), 44708-44721. DOI 10.1021/acsami.9b15530.
27. Fan, T.-F.; Potroz, M. G.; Tan, E.-L.; Ibrahim, M. S.; Miyako, E.; Cho, N.-J., *Scientific Reports* **2019,** *9* (1), 9626. DOI 10.1038/s41598-019-46131-w.
28. Atwe, S. U.; Ma, Y.; Gill, H. S., *J Control Release* **2014,** *194*, 45-52. DOI 10.1016/j.jconrel.2014.08.010.
29. Jorde, W.; Linskens, H. F., *Allergy* **1974,** *29* (3), 165-175. DOI 10.1111/j.1398-9995.1974.tb01646.x.
30. Maurer, A. H., *J Nucl Med* **2015,** *56* (9), 1395-400. DOI 10.2967/jnumed.113.134551.
31. Hamad, S. A.; Dyab, A. F. K.; Stoyanov, S. D.; Paunov, V. N., *Journal of Materials Chemistry* **2011,** *21* (44), 18018-18023. DOI 10.1039/C1JM13719K.
32. Mundargi, R. C.; Potroz, M. G.; Park, J. H.; Seo, J.; Tan, E.-L.; Lee, J. H.; Cho, N.-J., *Scientific Reports* **2016,** *6* (1), 19960. DOI 10.1038/srep19960.
33. Jaganathan, H.; Godin, B., *Advanced Drug Delivery Reviews* **2012,** *64* (15), 1800-1819. DOI https://doi.org/10.1016/j.addr.2012.05.008.
34. González-Vázquez, R.; Azaola-Espinosa, A.; Mayorga-Reyes, L.; Reyes-Nava, L. A.; Shah, N. P.; Rivera-Espinoza, Y., *Probiotics and Antimicrobial Proteins* **2015,** *7* (4), 242-248. DOI 10.1007/s12602-015-9202-x.
35. Okoli, A. S.; Raftery, M. J.; Mendz, G. L., *Proteome Science* **2012,** *10* (1), 27. DOI 10.1186/1477-5956-10-27.
36. Hu, P.-L.; Yuan, Y.-H.; Yue, T.-L.; Guo, C.-F., *PLOS ONE* **2018,** *13* (3), e0192964. DOI 10.1371/journal.pone.0192964.
37. Binks, B. P.; Boa, A. N.; Kibble, M. A.; Mackenzie, G.; Rocher, A., *Soft Matter* **2011,** *7* (8), 4017-4024. DOI 10.1039/C0SM01516D.
38. Carey, M. C., *Hepatology* **1984,** *4* (S2), 66S-71S. DOI 10.1002/hep.1840040812.
39. Hammer, J.; Pruckmayer, M.; Bergmann, H.; Kletter, K.; Gangl, A., *Gut* **1997,** *41* (5), 658-663. DOI 10.1136/gut.41.5.658.
40. Amor, K. B.; Breeuwer, P.; Verbaarschot, P.; Rombouts, F. M.; Akkermans, A. D. L.; De Vos, W. M.; Abee, T., *Applied and environmental microbiology* **2002,** *68* (11), 5209-5216. DOI 10.1128/aem.68.11.5209-5216.2002.
41. Mitsumoto, K.; Yabusaki, K.; Aoyagi, H., *Journal of Bioscience and Bioengineering* **2009,** *107* (1), 90-94. DOI https://doi.org/10.1016/j.jbiosc.2008.10.001.
42. Vertzoni, N. F. a. M., *The Open Drug Delivery Journal* **2010,** *4*, 2-13.
43. Vertzoni, M.; Diakidou, A.; Chatzilias, M.; Söderlind, E.; Abrahamsson, B.; Dressman, J. B.; Reppas, C., *Pharmaceutical Research* **2010,** *27* (10), 2187-2196. DOI 10.1007/s11095-010-0223-6.

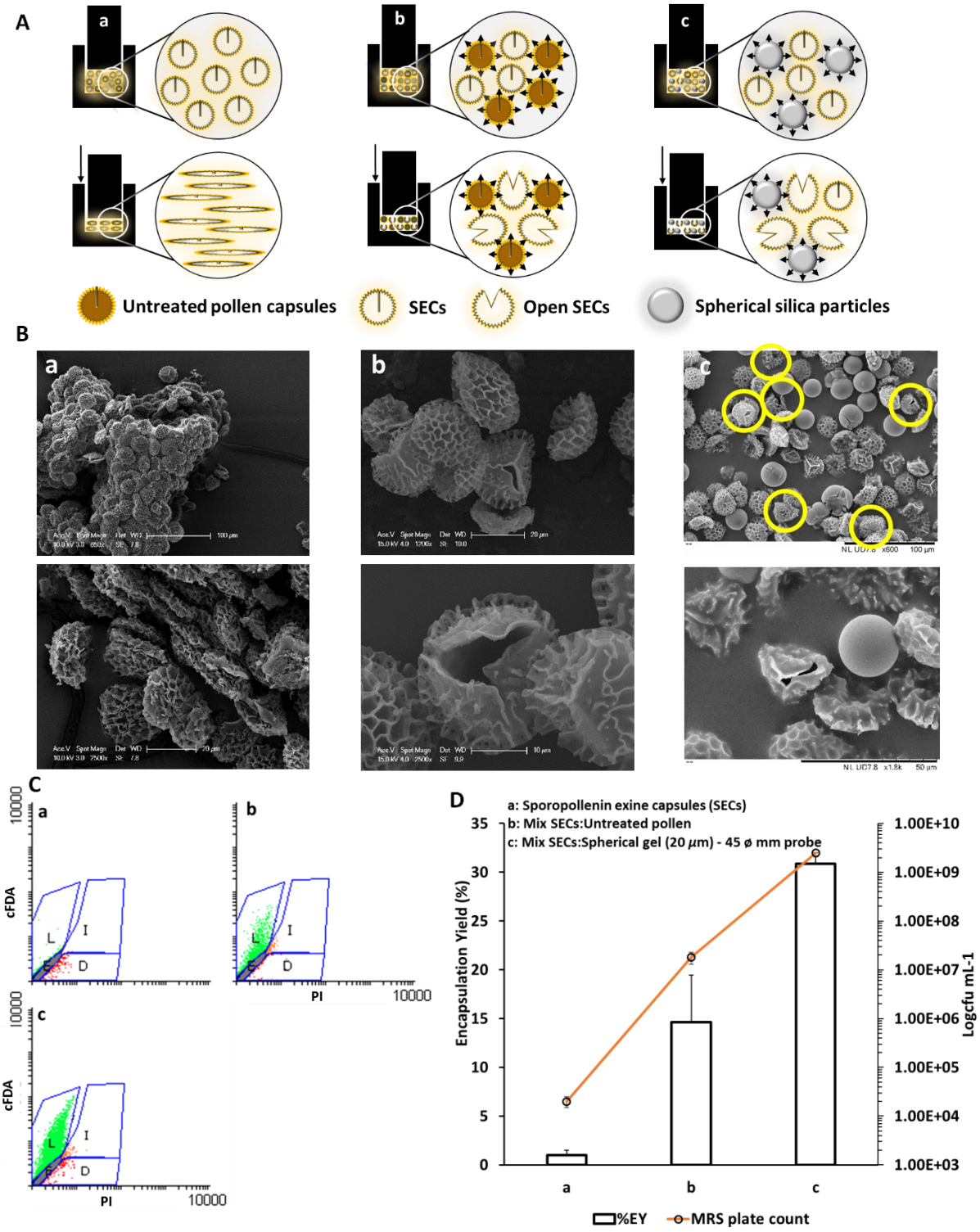


Figure 1. Results of different tableting techniques applied to open Sporopollenin Exine Capsules (SECs). A) Schematic of the tableting techniques. B) SEM images obtained for each tableting technique. C) Dot plots of flow cytometry (FC) analysis of each tableting technique conducted after loading SECs with *L. casei* cells stained with cFDA. D) Evaluation of the performance of the tableting techniques based on %Encapsulation Yield (%EY) and MRS plate count. Small letters, (a) direct compression of SECs, (b) mix of SECs: untreated pollen shells mixture at 1:1 ratio, (c) mix of SECs: spherical silica particles. Capital letter in

FC dot plots, L: Live, I: Injured, D: Dead and E: SECs. A 10 ø mm pressing probe used in (a) and (b) whereas in (c) a 45 ø mm pressing probe used.

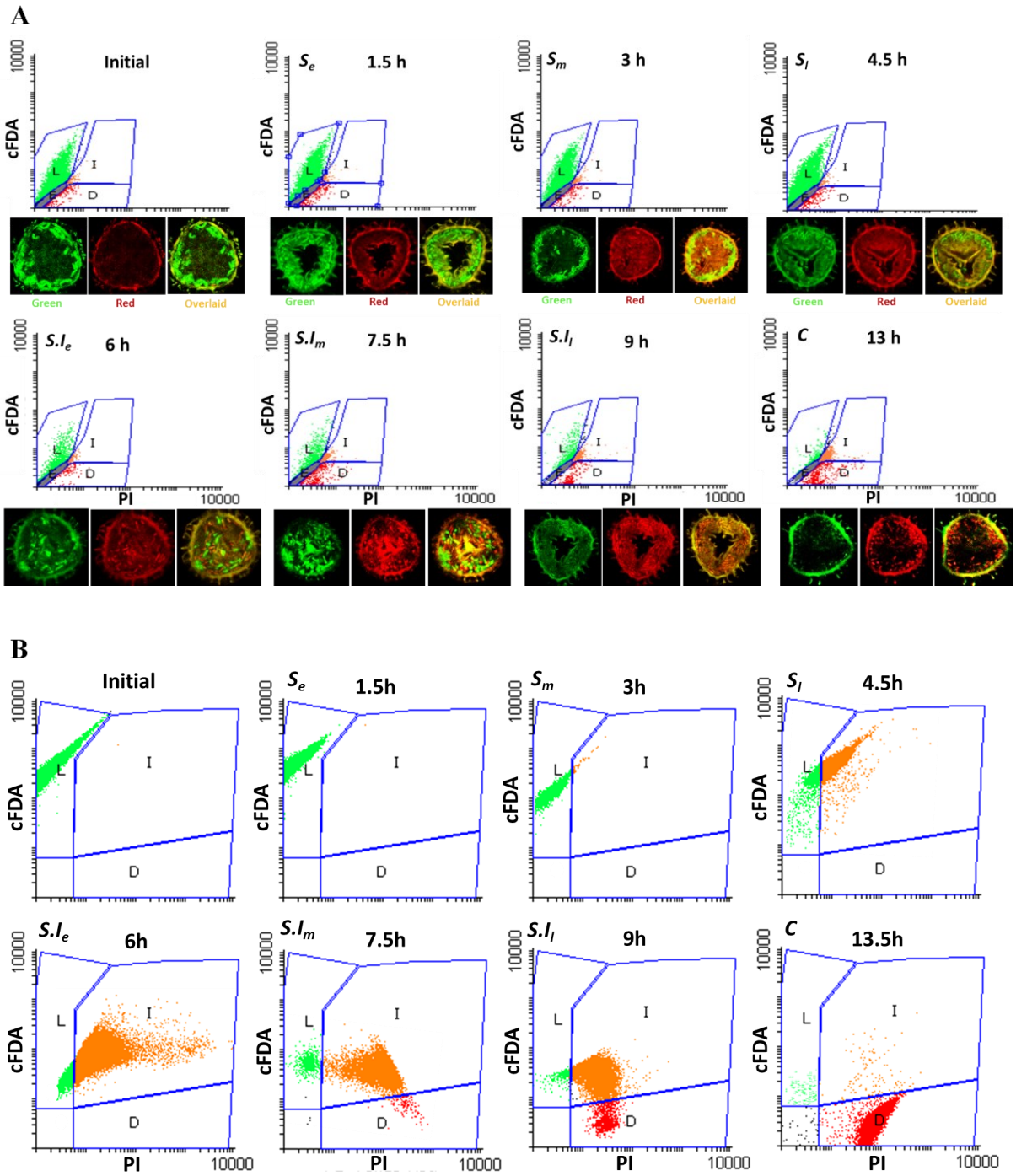


Figure 2. Flow Cytometry analysis of the viability of encapsulated (A) and free (B) *L. casei* in fed state. Representative confocal images at each time point of the stress test are provided for encapsulated *L. casei*; S: stomach, S.I: small intestine and C: colon; small letters: e, m and l, denote the early, middle and late stage of the in vitro stress test in stomach and small intestine as reported in Table 1. The capital letters within the dot plots denote the different regions: L: Live, I: Injured and D: Dead region.

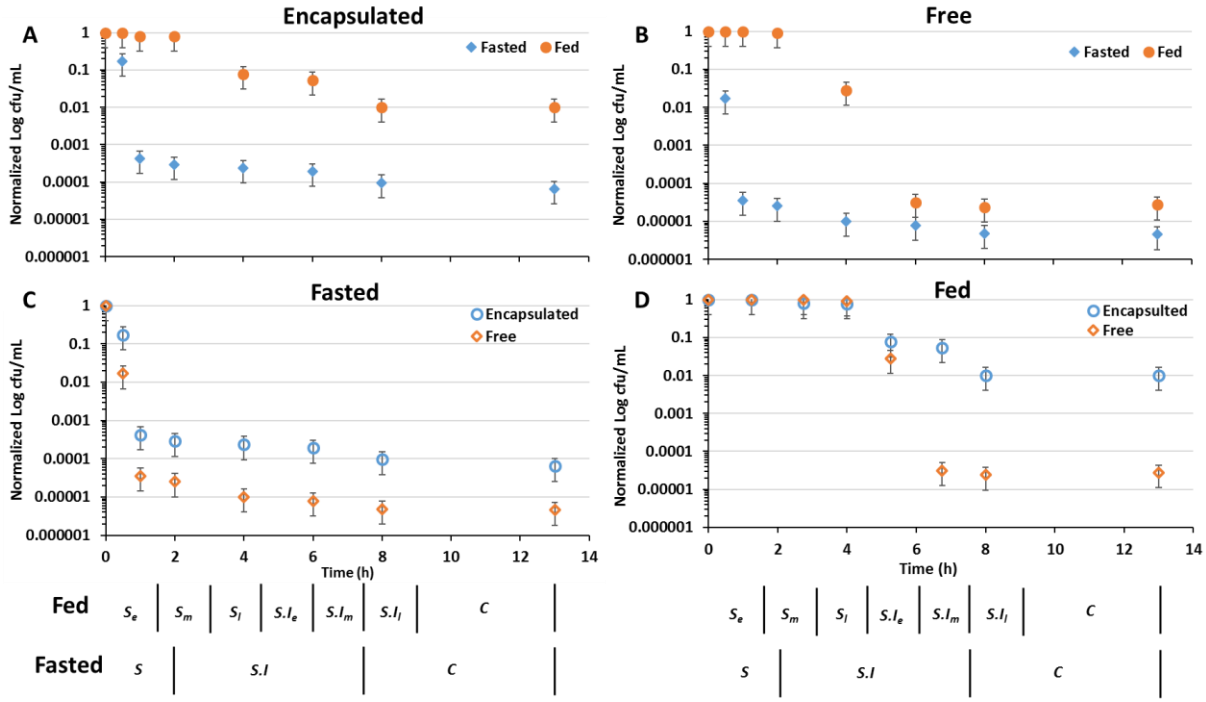


Figure 3. Plots of viability of *L. casei* cells with plate count method. Encapsulated (A) and free (B) *L. casei* cells in fasted and fed simulated media, with comparison of the viability of the encapsulated and free *L.casei* in fasted (C) and fed state (D). Data presented is an average of triplicate measurements with standard deviation (n = 3). S: stomach, S.I: small intestine and C: colon; small letters: e, m and l, denotes the early, middle and late stage of the in vitro stress test in stomach and small intestine in fed state as reported in Table 1.

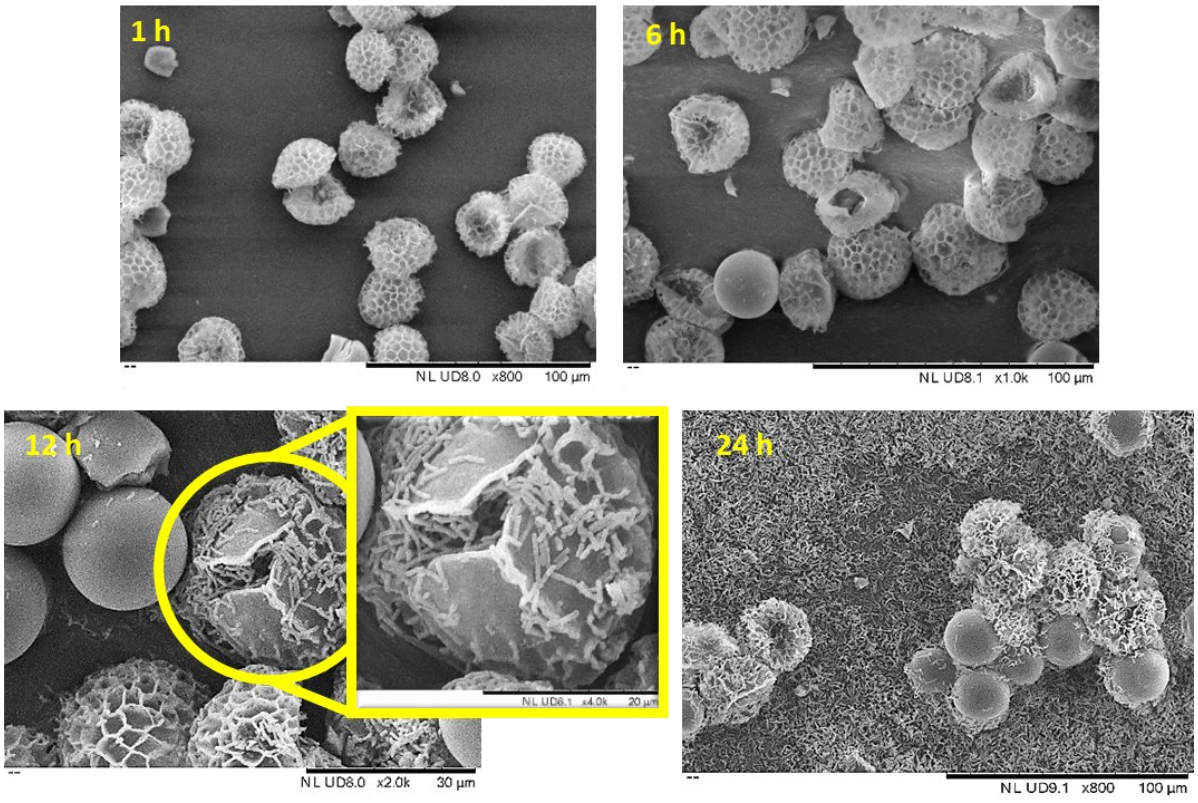


Figure 4. Evolution of the release of the encapsulated *L. casei* cells from the SECs. Representative SEM images of encapsulated *L. casei* after incubation at 37 ºC for 1, 6, 12 and 24 h.

Table 1: Composition of the media used to simulate the fasted and fed state of stomach, small and large intestine

Composition	Gastric[a]				Small intestine[a]				Large intestine[b]	
	Fasted	Fed			Fasted	Fed			Fasted	Fed
		Early	Middle	Late		Early	Middle	Late		
Bile salts, (mM)[c]	0.08				3	10	7.5	4.5	0.150	0.6
Lecithin, (mM)	0.02				0.2	3	2	0.5		
Pepsin, (mg mL^{-1})	0.1				-	-	-	-		
NaCl, (mM)	34.2	148	237	122.6	68.6	145.2	122.8	51		
CH_3COOH, (mM)			17.12							
CH_3COONa, (mM)			29.75							
Tris, (g L^{-1})									5.5	3.7
H_3PO_4, (mM)				5.5						
NaH_2PO_4, (mM)				32						
Milk/buffer		1:0	1:1	1:3						
NaOH, (M)					0.035	0.052	0.065	0.07	0.5	
Maleic acid, (g L^{-1})					19.1	28.6	44	58	8.8	3.5
Sodium oleate, (mM)						40	30	0.8		
Palmitic acid, (mM)									0.1	0.2
Glucose (g L^{-1})									0	14
pH	1.6	6.4	5.0	3.0	6.5	6.5	5.8	5.4	7.8	6.0

a)[42]; b)[43]; c)Porcine bile salt extract

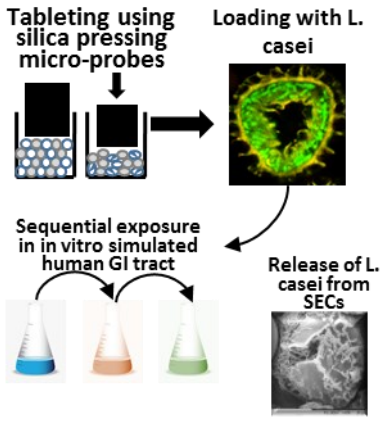
ToC text

In this work, a novel tableting technique uses silica particles as pressing micro-probes enabling the use of Sporopollenin Exine Capsules (SECs) as delivery system of probiotics. Encapsulated *L. casei* cells in SECs show high viability in in vitro simulated human GI tract environment. *L. casei* cells grow inside the SECs, generating pressure and causing SECs to burst and release viable cells.

Konstantinos Stamatopoulos*, Vasiliki Kafourou, Hannah K. Batchelor and Spyros J. Konteles3*

Sporopollenin exine microcapsules as potential intestinal delivery system of probiotics

ToC figure



Supporting Information

Sporopollenin exine microcapsules as potential intestinal delivery system of probiotics

Konstantinos Stamatopoulos, Vasiliki Kafourou, Hannah K. Batchelor and Spyros J. Konteles3**

Exploring the use of silica particles sizes <20 μm to improve encapsulation efficiency

Smaller particle sizes (5 & 10 μm) were used but their use was impractical for several reasons:

a) Silica of that size was very hydroscopic and regardless of the mixing ratio with pollen the final product had a paste-like consistency making it very challenging to form tablets from this mixture.

b) However, we proceeded with encapsulation and we noticed that even 14 washing steps of silica-pollen mixture with distilled water to remove the free bacteria weren't enough and there were still free bacteria. This had also implications in flow cytometry analysis, as the free bacteria adhered on silica particles and/or pollen were stained altering the analysis.

c) There were also implications in filtering process as we had to use Eppendorf with filter of smaller pore size (4 μm for 5 μm silica particles) resulting in blocking the filter. We used 15000 rpm instead of 12000 rpm or 12000 rpm for longer period during centrifugation with no success. So, we didn't further investigate silica particles of smaller size.

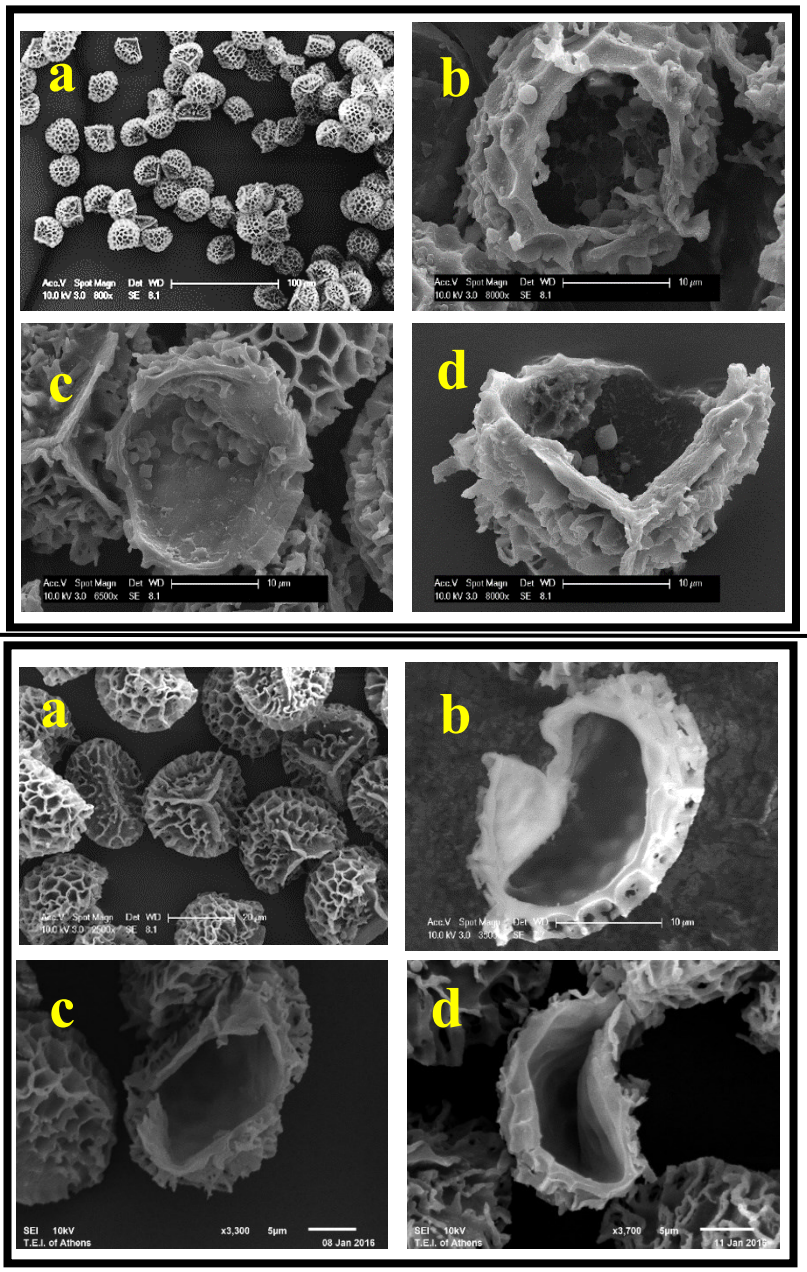


Figure S1. SEM images of chemically untreated (A) and treated pollen (B). Images with small 'a' letter show the external appearance of the SECs before (Aa) and after (Ba) the chemical treatment, whereas images with small 'b, c and d' show the broken SECs to assess the chemical treatment and the complete remove of the protoplast.

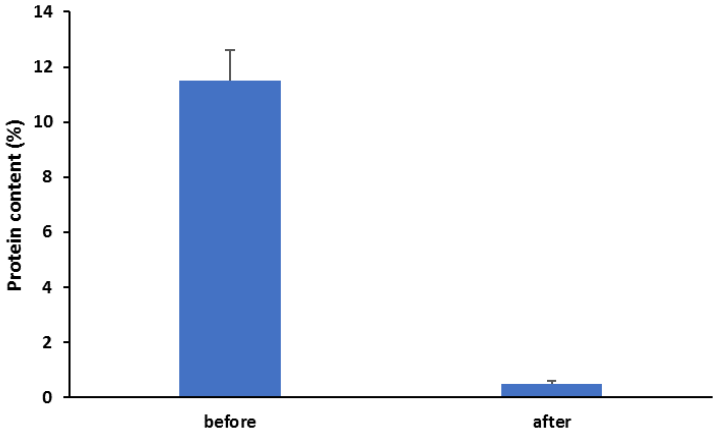


Figure S2. Protein content in pollen capsules before and after chemical treatment. The protein content was obtained from CHN elemental analysis data. Data presented is an average of triplicate measurements with standard deviation (n = 3).

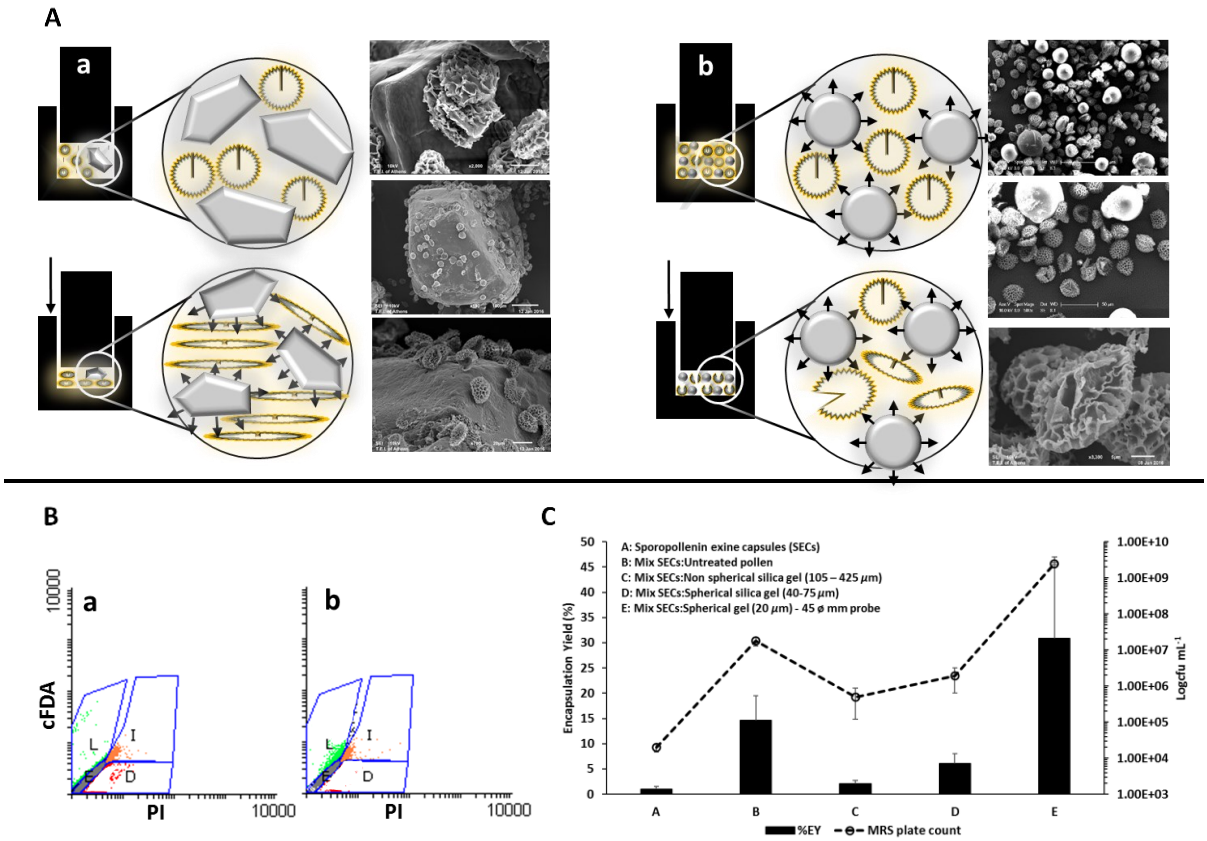


Figure S3. Performance of the additional tableting techniques tested to open Sporopollenin Exine Capsules (SECs). A) Schematic of the tableting techniques and the corresponding SEM images obtained for each tableting technique. B) Dot plots of flow cytometry (FC) analysis of each tableting technique conducted after loading SECs with *L. casei* cells stained with cFDA. C) Evaluation and comparison of the performance of all the tableting techniques based on %Encapsulation Yield (%EY) and MRS plate count. Small letters, (a) mix of SECs: silica particles (non-spherical 105 – 425 μm) in 2:1 ratio, (b) mix of SECs: silica particles (spherical 40-75 ø μm) in 2:1 ratio. Capital letter in FC dot plots, L: Live, I: Injured, D: Dead and E: SECs. A 10 ø mm pressing probe used in (a) and (b).

Table S1. %Encapsulation Yield (%EY) determined by Flow cytometry analysis of different tableting techniques. Events in the live region were accounted for the calculations.

Tableting technique	Events in Live region			Total events			%EY	
	Counts	Mean	SD	Counts	Mean	SD	Mean	SD
Empty pollen (Hamad et al 2011)	190 575 224	330	174	30105 28931 40023	33019.67	6093	1.00	0.53
Mix empty:non empty pollen	8103 6521 3594 4088	5577	1832	30105 28931 40023 43340	35599.75	7168	15.66	20.14
Non-spherical silica gel	602 944 834 323	676	238	26111 33568 39687 34566	33483	5598	2.02	16.72
Spherical silica gel (40-75 µm)	1501 3539 2006 2571	2404	757	35679 41567 40123 39123	39123	2506	6.15	6.40
Spherical silica gel (20 µm)	10949 9700 21578 4760	11747	6130	40642 30851 42666 38033	38048	5159	30.87	13.56

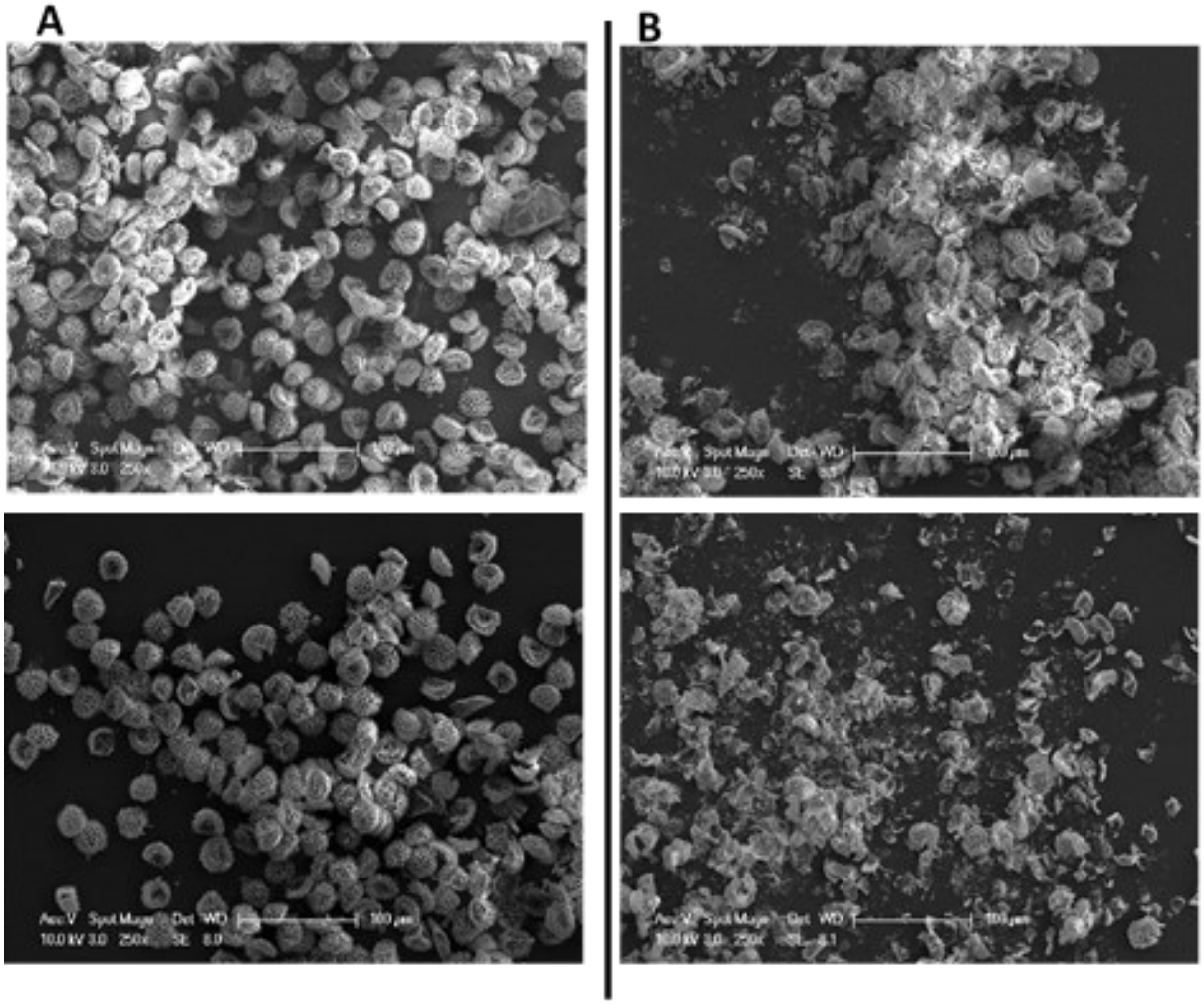


Figure S4. SEM images of empty pollen shells after bead-beating at 2800 rpm of 30 s (A) and 1 min (B). BEADBUG™ 3 POSITION BEAD HOMOGENIZER, Benchmarkscientific, was used, with 1.5mm beads.

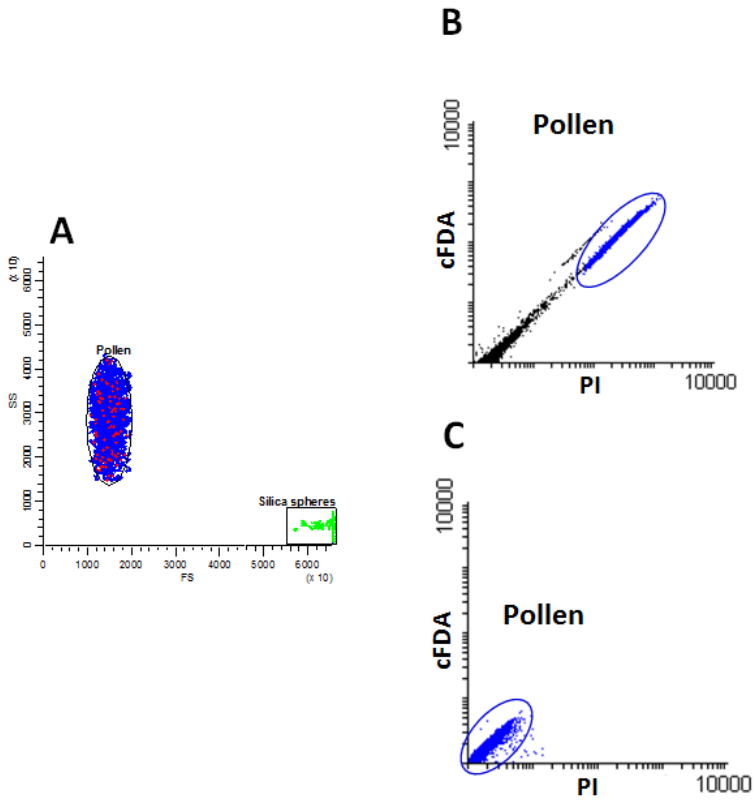


Figure S5. Flow cytometry of empty pollen capsules. Forward and side scatter of empty pollen (A), biparametric flow analysis, based on green (cFDA) and red (PI) fluorescence emission before (B) and after (C) adjusting FL1 FL3 sort gates to reduce pollen's autofluorescence.

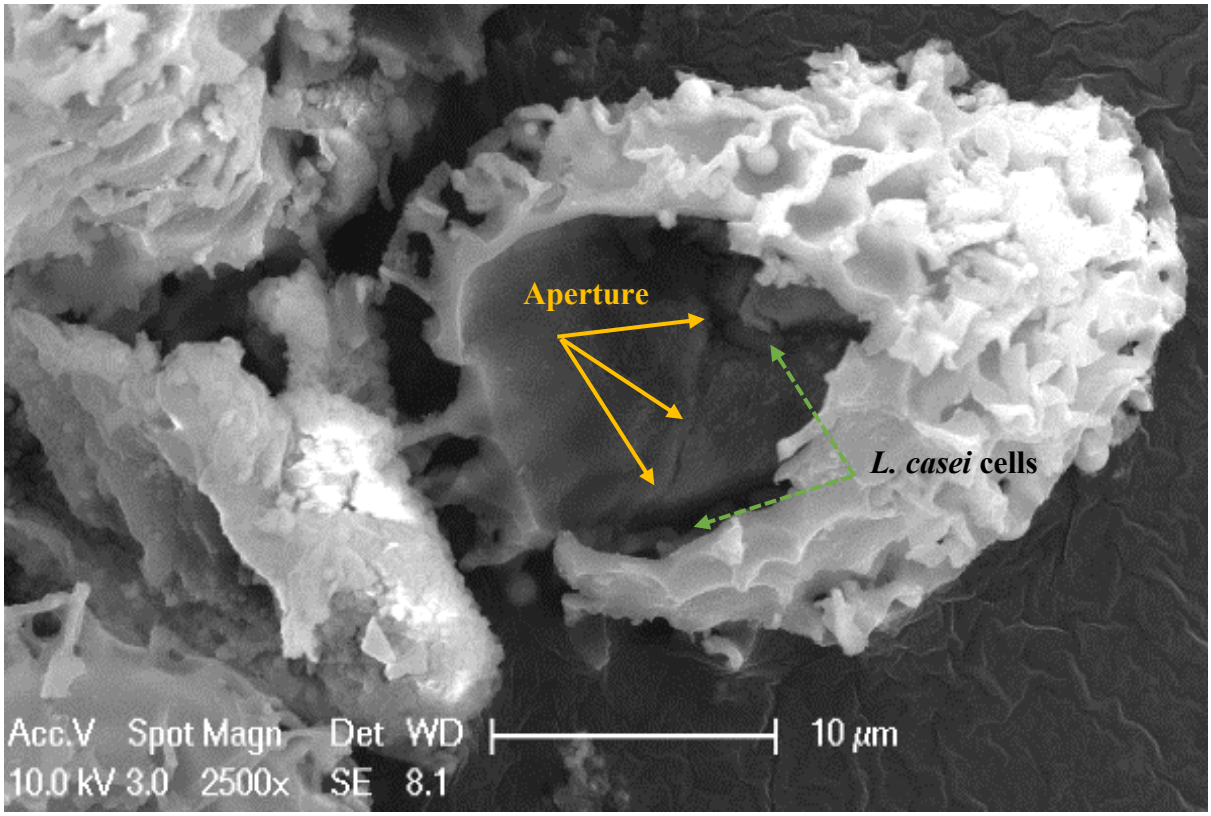


Figure S6. SEM image of cut pollen after encapsulation of *L. casei* cells. Image shows the closed aperture of the pollen (orange arrow) and the *L. casei* cells inside the pollen capsule (dotted green arrows). The sample was frozen with liquid nitrogen and the pollen was subsequently cracked open by pestle and mortar.

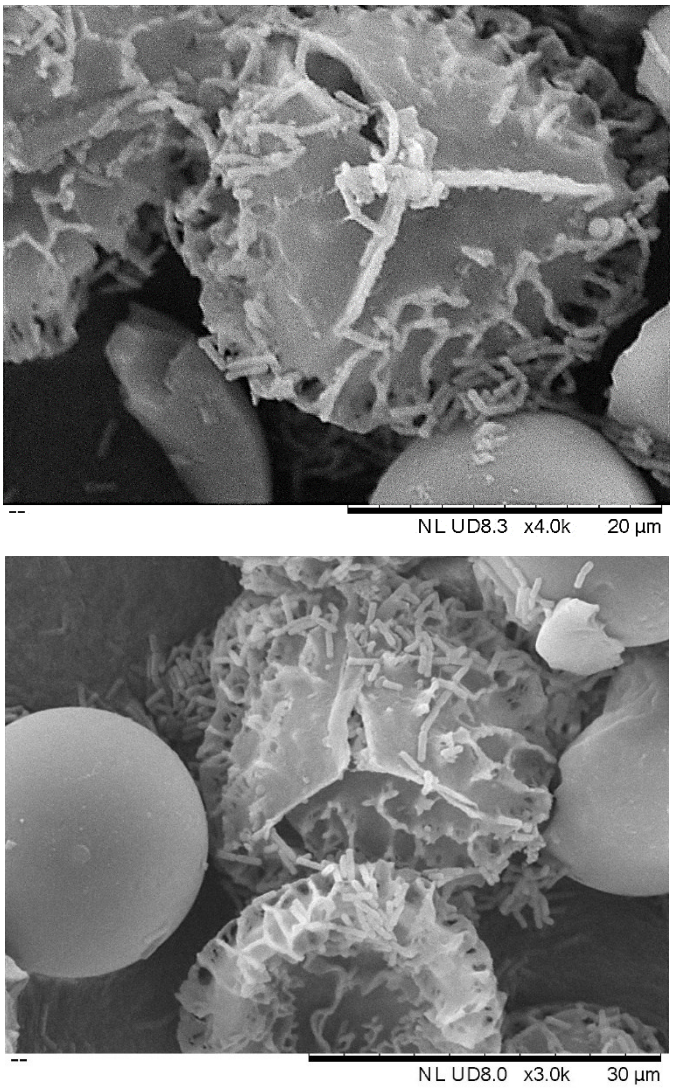


Figure S7. Representative SEM images of encapsulated *L. casei* after incubation at 37 ºC for 10 h.