



Encapsulation of lactobacillus casei into calcium pectinate-chitosan beads for enteric delivery

Article

Accepted Version

Bepeyeva, A., de Barros, J. M. S., Albadran, H., Kakimov, A. K., Kakimova, Z. K., Charalampopoulos, D. and Khutoryanskiy, V. (2017) Encapsulation of lactobacillus casei into calcium pectinate-chitosan beads for enteric delivery. *Journal of Food Science*, 82 (12). pp. 2954-2959. ISSN 0022-1147 doi: <https://doi.org/10.1111/1750-3841.13974> Available at <http://centaur.reading.ac.uk/73925/>

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Published version at: <http://onlinelibrary.wiley.com/doi/10.1111/1750-3841.13974/full>

To link to this article DOI: <http://dx.doi.org/10.1111/1750-3841.13974>

Publisher: Wiley

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1 **Encapsulation of *Lactobacillus casei* into calcium pectinate-chitosan**
2 **beads for enteric delivery**

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20 **ABSTRACT**

21 Gel beads were prepared by extrusion of various types of pectin into 0.15 M calcium chloride. Size,
22 morphology, and textural properties of three types of beads were evaluated and it was established that
23 the use of 3 w/v % amidated pectin provides the optimal characteristics suitable for encapsulation of
24 live bacteria. *Lactobacillus casei* NCIMB 30185 (PXN37) was encapsulated into calcium pectinate gel
25 through the extrusion of a live bacteria dispersion in 3 w/v % pectin into a solution of calcium
26 chloride. The capsules were then additionally coated with chitosan. The viability of bacteria within
27 these capsules was studied under model gastrointestinal conditions in vitro (simulated gastric and
28 intestinal juices). It was established that pectin–chitosan capsules can provide protection to
29 *Lactobacillus casei* from the gastric acid and result in high levels of viable bacteria released in the
30 intestine.

31
32 **Practical Application:**

33 Encapsulation of *Lactobacillus casei* into calcium pectinate beads coated with chitosan provided
34 capsules capable of delivery live probiotic bacteria into the intestine.
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39 *Key words:*
40 Encapsulation
41 *Lactobacillus casei*
42 Pectin
43 Chitosan
44 Probiotic
45 Live bacteria
46

47 **1. Introduction**

48

49 Gastrointestinal delivery of live probiotic bacteria is considered as a promising approach to
50 improve the gut health. Probiotics provide some health benefits due to their ability to facilitate
51 digestion, produce vitamins, bust the immune system and prevent the growth of pathogenic bacteria
52 (Derrien and others 2015; Reid and others 2016).

53 Probiotics are typically found in some dairy products; however, their successful delivery to the gut
54 is often compromised because of the sensitivity of these bacteria to the acidic environment in the
55 stomach (Burgain and others 2011; Cook and others 2012). Encapsulation of live probiotics into
56 materials resistant to gastric acid is a viable strategy for their successful oral delivery. Previously, we
57 have reported the use of calcium alginate beads coated with chitosan for encapsulation and oral
58 delivery of *Bifidobacterium* species (Cook and others 2011; Cook and others 2013; Cook and others
59 2013; Nualkaekul and others 2013). It was established that the live bacteria encapsulated within
60 calcium alginate beads coated with chitosan may potentially survive the transit through the harsh
61 environment of the stomach and release high levels of live probiotic in the intestine. The protective
62 effect of chitosan coating was found to be due to its ability to delay acid diffusion into the capsules
63 (Cook and others 2013). It was also demonstrated that live probiotic bacteria encapsulated within
64 calcium alginate-chitosan show excellent survival in acidic juices (Nualkaekul and others 2013) and
65 during long storage in dry state (Albadran and others 2015).

66 Pectins is a group of anionic polysaccharides present in many fruits and vegetables and have a
67 wide range of food and pharmaceutical applications as gelling, thickening, and stabilizing agents
68 (Thakur, Singh, Handa & Rao, 1997). They consist of several types of carbohydrate repeating units,
69 including homogalacturonan (HG) and type I rhamnogalacturonan (RG-I). HG regions in pectins
70 consist of poly-galacturonic acid residues; some of these are partially methyl esterified. Pectins with a
71 high degree of methyl substitution (> 50%) are classified as high methyl ester pectins and those with <
72 50 % esterification are called low methyl ester pectins. Similarly to alginates, pectins are capable of
73 forming gels upon extrusion of their aqueous solutions into the media containing soluble calcium salts

74 (Morris et al, 2010). Pectin properties can also be modified by their reaction with ammonia under
75 alkaline conditions resulting in amidated pectins. Low methyl ester amidated pectins typically require
76 lower Ca^{2+} concentration to form gels and are more tolerant than low methyl ester pectin with respect
77 to excess calcium concentrations (Belitz and others 2004).

78 Previously, pectins were reported as materials for encapsulation of *Lactobacillus rhamnosus*
79 (Gerez and others 2012) and *Lactobacillus acidophilus* (Gebara and others 2013) with the subsequent
80 coating of these capsules with whey proteins in both cases. This coating provided a protective function
81 that ensured the effective transit of live bacteria through the gastrointestinal tract. Pectins were also
82 used for encapsulation of probiotics in combination with other materials such as alginates (Sandoval-
83 Castilla and others 2010), hyaluronic acid (Pliszcak and others 2011) and milk (Shi and others 2013).
84 Typically the use of alginates and pectins for encapsulation of probiotics give excellent encapsulation
85 efficiencies of 90-100 % (Shori, 2017).

86 In this work the ability of different pectins to form calcium gel capsules was evaluated and the
87 most promising materials were used for encapsulation of *Lactobacillus casei*. The gel capsules with
88 live probiotic were then coated with chitosan. In vitro experiments were also performed with the
89 encapsulated probiotic bacteria to establish the protective role of chitosan coating for the successful
90 transit of live bacteria through the gastrointestinal tract.

91

92 **2. Materials and methods**

93

94 2.1 Materials

95

96 Amidated pectin CU 020 (63 kDa, degrees of esterification and amidation are 28 % and 20 %, respectively),
97 low methyl ester pectin Classic CU 701 (54 kDa and degree of esterification 36 %), high methyl pectin
98 Classic CU-L 004/14 (67 kDa and degree of esterification 59 %) were obtained from HERBSTREITH & Fox KG
99 (Germany). Chitosan (103 kDa and degree of deacetylation 85.6%), and fluorescein isothiocyanate (FITC)
100 (isomer I) were purchased from Sigma-Aldrich (Gillingham, U.K.). Fluorescently-labelled chitosan was
101 synthesized according to the methodology described by our research group previously (Cook, 2011).
102 CaCl_2 was purchased from Fisher Scientific UK Limited. The microbiology media Man, Rogosa and Sharpe
103 (MRS) broth, agar, and phosphate-buffered saline (PBS) were purchased from Oxoid (Basingstoke, UK).
104 Pectin solutions and chitosan solution were purified by microfiltration (0.45 μm , Minisart filter,
105 Sartorius Stedim, Biotech). All other reagents and materials were sterilized in an autoclave (121°C,
106 15 min). *Lactobacillus casei* NCIMB 30185 (PXN 37) strain was received from Probiotics International Ltd
107 (Protexin) (Somerset, UK).

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2.2 Culture preparation

Lactobacillus casei NCIMB 30185 (1XN37) was spread onto MRS agar from a cell bank and was incubated at 37°C for 48 h. A colony was picked and inoculated into MRS broth (10 mL) and incubated at 37 °C for 24 h to get a bacterial suspension with an OD₆₀₀ of approximately 2.0. After growth the cells were separated by centrifugation for 10 min (3200 rpm, at 4°C), the supernatant was collected, the cell pellet was rinsed with 1 mL PBS and redispersed in 10 mL pectin solution to give approximately 6×10^9 CFU/mL.

2.3 Preparation of unloaded pectin capsules

Unloaded (without *L. casei*) pectin capsules were prepared using an extrusion method. 1, 2, and 3 w/v % pectin aqueous solutions (1 mL) were extruded with a syringe (0.8 mm diameter needle) and a pump (flow rate set at 2 mL min⁻¹) into 50 mL 0.15 M CaCl₂. Gel beads were formed upon contact with CaCl₂, and were left to harden for 30 min. The distance from the surface of the solution and the needle was 10 cm. After hardening, the pectin gel beads were removed from the solution *via* filtration. Chitosan coated capsules were prepared by dispersing pectin gel beads in 10 mL chitosan solution (0.4% w/v in 0.1 M acetic acid; pH set to 6 using 1M microfiltered NaOH,) under gentle stirring for 10 minutes and then subsequently were removed by filtration.

2.4 Production of probiotic containing pectin capsules

In order to produce probiotic containing capsules, the *L. casei* suspension was mixed with 3% amidated pectin solution (1:9 volume ratio) and the capsules were generated by extrusion and coated as described above. The viability and bacteria release studies are described in terms of quantity of extruded pectin (each batch of capsules corresponds to 1 mL of extruded pectin solution).

2.5 Morphology of the capsules prepared from grades of pectin

The morphology of the gel beads prepared using various types and different concentrations of pectin (1, 2, and 3 w/v % of amidated, low methyl ester and high methyl ester pectins) was evaluated with a light microscope (Leica DM2500). Gel beads were examined under $\times 1.6$ -2.5 magnification.

2.6 Mechanical properties and dimensions of gel beads

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143 The diameters of uncoated beads were determined with a light microscope (Leica DM2500) and
144 using an image analysis software (ImageJ). The compressive strength was determined using Texture
145 Analyser (Stable Microsystems, UK), with a P\1K steel probe at 1 mm s⁻¹ rate, with 0.01 N trigger
146 force.

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151 2.7 Viability of *Lactobacillus casei* in simulated gastric juice (SGJ)

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153 For non-encapsulated bacteria, *L. casei* was inoculated in MRS broth (10 mL) and the culture
154 incubated at 37°C for 24 hours. It was then separated by centrifugation for 10 min at 4 °C (3200 rpm);
155 the bacteria were collected and rinsed with PBS (1 mL). Following the incubation, a 1 mL aliquot was
156 taken to evaluate the initial cell concentration using the spread plating method. Cells were diluted with
157 PBS, and 0.1 mL of the appropriate dilution was spread onto MRS agar. Plates were incubated at 37
158 °C for 48 h and the colony forming units (CFU) were determined. To evaluate cell survival in gastric
159 conditions, SGJ (10 mL, pH 2.0) was added to cells and incubated at 37°C with shaking at 100 rpm.
160 After 15 min, 30 min, 60 min and 120 min intervals an aliquot (1 mL) of the SGJ was diluted in 9 mL
161 PBS and enumerated using spread plating method described above.

162 For encapsulated bacteria, 3 batches with *L. casei* were prepared from the same broth of cells.
163 Two of these batches were transferred into SGJ and incubated at 37 °C with shaking at 100 rpm. The
164 third batch was transferred into 100 mL of PBS and incubated for 1 h at 37 °C with shaking at 100
165 rpm. These batches were then homogenized in Seward stomach 400 circulator for 20 min at 230 rpm)
166 and enumerated to give the initial bacteria concentration. The two batches of beads in the SGJ were
167 removed after 1 and 2 h of incubation. These beads were placed into 100 mL of PBS and incubated for
168 1 h at 37 °C with shaking at 100 rpm. Then the beads were dissolved and enumerated using the
169 methods described above.

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171 2.8 Release of bacteria from capsules

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173 The release of bacteria from the capsules was studied during 120 min of incubation the
174 capsules in SGJ for 2 h (pH 2.0, 10 mL at 37°C with shaking at 100 rpm); then these were removed *via*
175 filtration and placed into simulated intestinal juice for 3 h (pH 7.2, 100 mL at 37°C with shaking at

176 100 rpm). Then this mixture was transferred into Seward stomach 400 circulator for 20 min (230 rpm)
177 and the bacteria counted by taking 1 mL aliquots. 1mL aliquots were also taken after 1 and 2 hours in
178 SGJ and after 1 and 2 hours in SIJ in order plot the bacteria release profiles. The enumeration of viable
179 bacteria was performed the spread plating method.

180

181 2.9 Determination of coat thickness using fluorescent microscopy

182

183 The thickness of the chitosan coat was determined by fluorescent microscopy using a Leica
184 DM2500 microscope. The lyophilized FITC-chitosan was dissolved in 0.1 M acetic acid to form 0.4%
185 (w/v) solution, with pH adjusted to 6.0 using 1 M NaOH. This was then used to coat pectin beads as
186 described in section 2.3. The bead images were taken using a Leica DM2500 fluorescent stereo-
187 microscope. The thickness of coating layer was determined using ImageJ.

188

189 2.10 Statistical analysis

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191 Statistical analysis was conducted with a two-tailed unpaired Student's t-test and $p < 0.05$ was
192 considered as significant.

193

194 3. Results and discussion

195

196 3.1 Morphology, diameter and texture properties of beads

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198 The influence of pectin concentration on the formation of gel beads was initially investigated.
199 Three different types of pectin were studied as potential materials to form calcium pectinate gel
200 capsules (amidated pectin, low methyl pectin and high methyl pectin). It was observed that the
201 extrusion of these solutions into 0.15 M CaCl_2 results in the formation of gel beads of different shape
202 and it depends on the type of pectin used as well as the concentration of its solution (Fig. 1). More
203 spherical beads are formed upon increasing the polymer concentration, which is possibly related to an
204 increase in solution viscosity.

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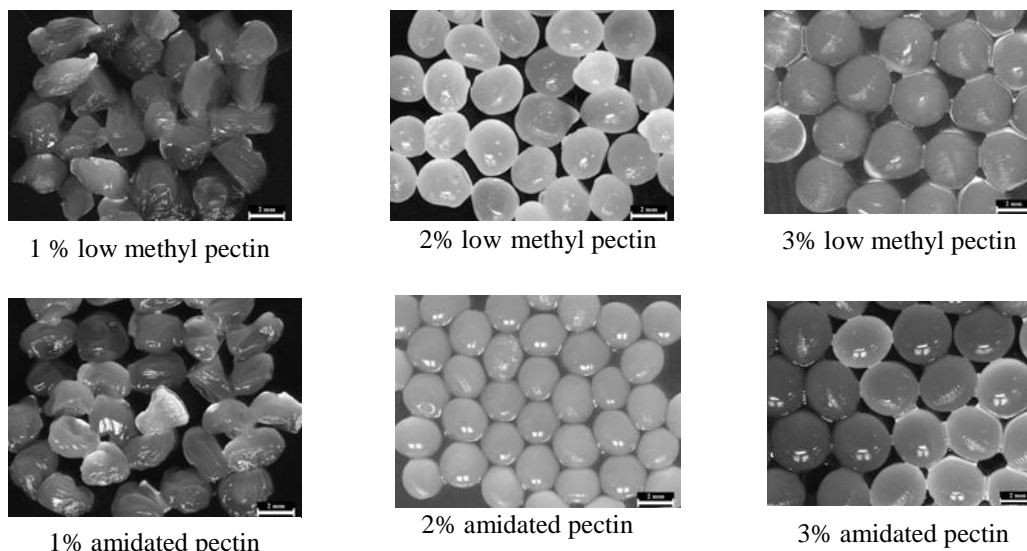
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219 Fig.1. Morphology of capsules prepared with different types and concentration of pectin. A 1.6-
220 2.5×magnification was used. The scale bar represents 2 mm.

221

222 It was noted that encapsulation with 1% (w/v) pectin does not give a spherical shape to the
223 capsules. Furthermore, when 1% (w/v) high methyl pectin was used it was observed that capsule
224 formation did not occur. This can be due to the fact that the gelling properties of pectin depend on the
225 degree of esterification (Soumya & Suvendu, 2012). It was previously reported that high methyl
226 pectins will form gel in the presence of sugars or other small molecules (e.g. polyols or monohydric
227 alcohols) (Oakenfull & Scott, 1984; Thakur, Singh & Handa, 1997), and at low pH (3.0–4.5).
228 Additionally, gelation of low methyl pectins takes place solely in the presence of divalent cations such
229 as calcium (Soumya & Suvendu, 2012).

230 Table 1 presents a summary of the key physicochemical characteristics (diameter and
231 compressive strength) of the gel beads from the different pectins used. Note that size and mechanical
232 strength were characterised only for spherical beads.

233

234

235

236 **Table 1.** Physical characteristics of pectin beads

Polymer	Concentration, % (w/v)	Compressive strength, N	Diameter of beads, mm	Comments
Low methyl pectin	1	-	-	Capsules are not spherical
High methyl pectin	1	-	-	Do not form capsules
Amidated pectin	1	-	-	Capsules are not spherical
Low methyl pectin	2	2.12±0.34	2.57±0.02	Spherical capsules

High methyl pectin	2	-	-	Do not form capsules
Amidated pectin	2	8.88±0.57	2.76±0.01	Spherical capsules
Low methyl pectin	3	3.32±0.29	3.47±0.03	Spherical capsules
Amidated pectin	3	9.37±0.56	3.50±0.05	Spherical capsules
High methyl pectin	3	-	-	Do not form capsules

237

238

239 As expected, it was found that with increasing the concentration of pectin, the size and
 240 mechanical strength of the capsules also increased. Amidated pectin also formed mechanically
 241 stronger capsules compared to low methyl ester pectin at the same polymer concentration. According
 242 to the literature (Chandramouli and others 2004; Sabikhi and others 2010; Hansen and others 2002)
 243 larger capsules typically provide better protection to cells towards adverse environmental conditions.
 244 Spheres with size ranges between 1 and 3 mm are preferably used in immobilised cell technology
 245 applications (Heidebach and others 2012). Anal and Singh (2007) also stated that the large size of
 246 bacteria (typically 1-4 μm) is a serious challenge for cell encapsulation. These dimensional
 247 characteristics limit cell loading in small capsules, and in the case that larger capsules they will have
 248 suboptimal textural and sensorial properties in food products. Muthukumarasamy et al. (2006)
 249 established that larger capsules (2-4 mm) improved the viability of *Lactobacillus reuteri* compared to
 250 smaller capsules (20-1000 μm). Chandramouli et al. (2004) also demonstrated that the viability of
 251 lactobacilli in simulated gastric conditions was greater for alginate capsules of larger size (200-1000
 252 μm). Due to their larger size and greater mechanical strength, amidated pectin (3% (w/v)) was selected
 253 for further work involving the encapsulation of *Lactobacillus casei*.

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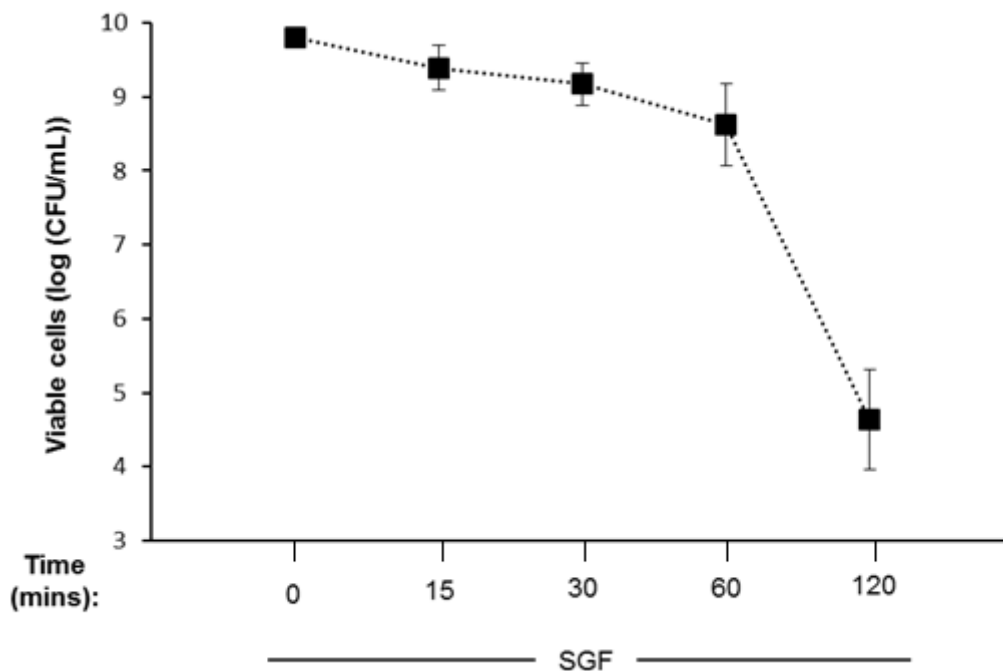
257 3.2 Viability of non-encapsulated *Lactobacillus casei* in SGJ

258

259 The viability of free *L. casei* in SGJ (pH 2.0) was evaluated to establish if these cells were acid
 260 resistant. After 60 minutes of SGJ exposure a reduction in cell viability of 1 log was observed (Fig. 2)
 261 and after 120 min even greater decrease in cell numbers of approximately 5 logs was seen, giving a
 262 final cell recovery of 10^5 CFU/mL. This indicates that *L. casei* is sensitive to the acidic environment in
 263 the stomach and there is a need for an encapsulation system to protect *L. casei* cells during its
 264 gastrointestinal delivery.

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269 Fig.2. Viability of free *Lactobacillus casei* in SGJ (pH 2.0), n=3.

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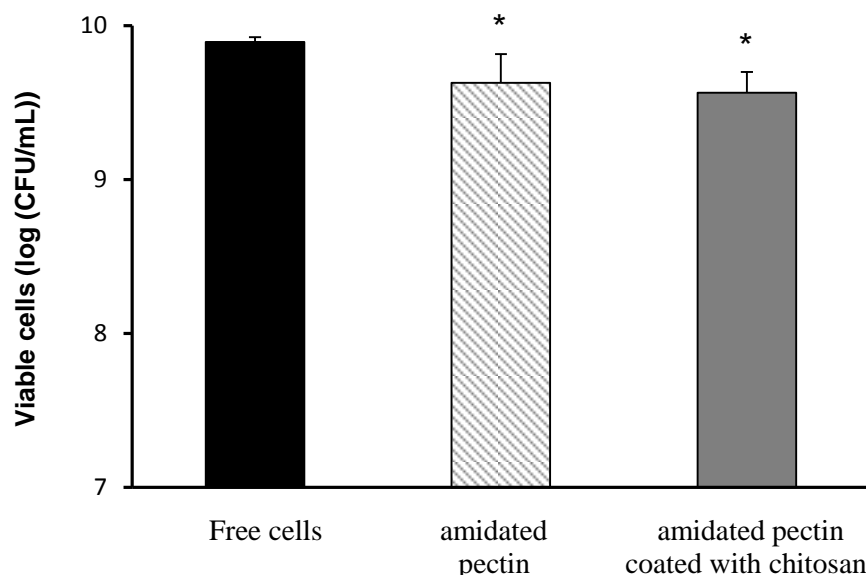
272 3.3 Viability of *Lactobacillus casei* during its encapsulation into calcium pectinate beads 273 and subsequent coating with chitosan

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275 The effect of encapsulation within calcium pectinate gel and the effect of coating with chitosan
276 on the viability of *L. casei* were investigated. It was established that the encapsulation process resulted
277 in approximately 0.3 log CFU/mL reduction in cell viability (Fig.3). However, there was no loss in
278 viability associated with the coating process. A similar small loss (~0.4 log CFU/mL) was previously
279 observed for the encapsulation of *B. breve* into calcium alginate beads (Cook and others 2011) and was
280 related to dissolution of the capsules rather than the encapsulation itself.

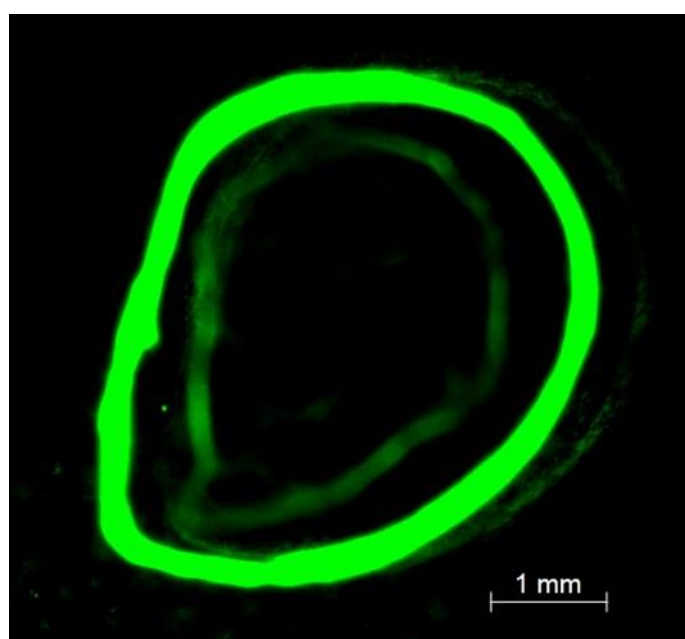
281 It is well known that chitosan has antimicrobial properties (Rabea and others 2003) and its direct
282 contact with live bacteria could potentially result in reduction of their viability. In order to establish
283 how deeply chitosan macromolecules can penetrate into calcium pectinate gel, experiments were
284 performed with FITC-chitosan using fluorescent microscopy. Fig.4 shows a fluorescent image of
285 calcium pectinate bead coated with FITC chitosan. The bright green band responsible for the
286 fluorescence of FITC-chitosan indicates that the depth of penetration of chitosan macromolecules into
287 calcium pectinate gel within 10 min exposure was around 0.236 ± 0.061 mm. This result indicates that

288 chitosan likely forms a coating on the surface of pectinate gels and does not penetrate deeply into
 289 calcium pectinate. However, this penetration of chitosan into pectinate gel is greater compared to its
 290 penetration into calcium alginate gels, reported in our previous publication (Cook and others 2011),
 291 where around 0.007 mm penetration depth was observed. The difference between permeability of these
 292 two gel materials presents some interest for further studies.



293 Fig.3. Viability of *Lactobacillus casei* during the encapsulation process: a) free cells, b) cells
 294 encapsulated into 3% calcium pectinate, c) cells encapsulated into 3% calcium pectinate with chitosan
 295 coating. Amidated pectin was used in these experiments (n=5). p values denoted by * ($p < 0.05$),
 296 signifies statistical difference when compared to free cells.
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Fig.4. Fluorescent microscopy image showing penetration of FITC-chitosan (green) into calcium pectinate capsule.

3.4 Viability of encapsulated *Lactobacillus casei* in SGJ (pH 2.0)

Previous studies (Gebara and others 2013; Chotiko and others, 2016) indicated the usefulness of pectin-based materials for encapsulation of various probiotics and highlighted the need for using protective coatings to improve bacteria viability.

Calcium pectinate beads prepared from 3% amidated pectin without and with chitosan coating were evaluated under simulated gastric conditions (2 hours at pH 2.0) to study the effect of encapsulation on the protection to probiotics from the low pH in the stomach. After 120 min of exposure to acidic pH, coated capsules showed very high viability of bacteria without any significant ($p>0.05$) drop in the levels of live cells for up to 120 min. The viability of cells residing within the uncoated beads dropped to less than 7 logs CFU/mL within 60 mins and then to less than 4 logs CFU/mL within 120 mins. Crucially, after 2 hours in simulated gastric conditions the coated capsules showed no loss in cell viability, resulting in a cell recovery of 9.6 logs CFU/mL, which proves that a pectin chitosan coated system effectively protects the cells in a very acidic environment. This also suggests that chitosan coating alone is responsible for total acid protection. The protective effect observed due to chitosan, a basic polysaccharide, is likely to be because of the ability of this polysaccharide to neutralize H^+ ions penetrating into the beads, i.e. chitosan coat acts as a buffering layer preventing acid ingress (Cook et al. 2013). Moreover, it was reported by us previously (Cook et al, 2011) that chitosan-coated alginate beads offered weaker protection compared to chitosan coated pectinate. However, to better understand the protection mechanism, the interaction between pectin and chitosan has to be studied further.



328

329 Fig.5. Viability of encapsulated *Lactobacillus casei* cells in calcium pectinate beads with and without
 330 chitosan coating. Calcium pectinate beads were prepared using 3% amidated pectin.

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333 3.5 Release of encapsulated bacteria in simulated intestinal juice (SIJ)

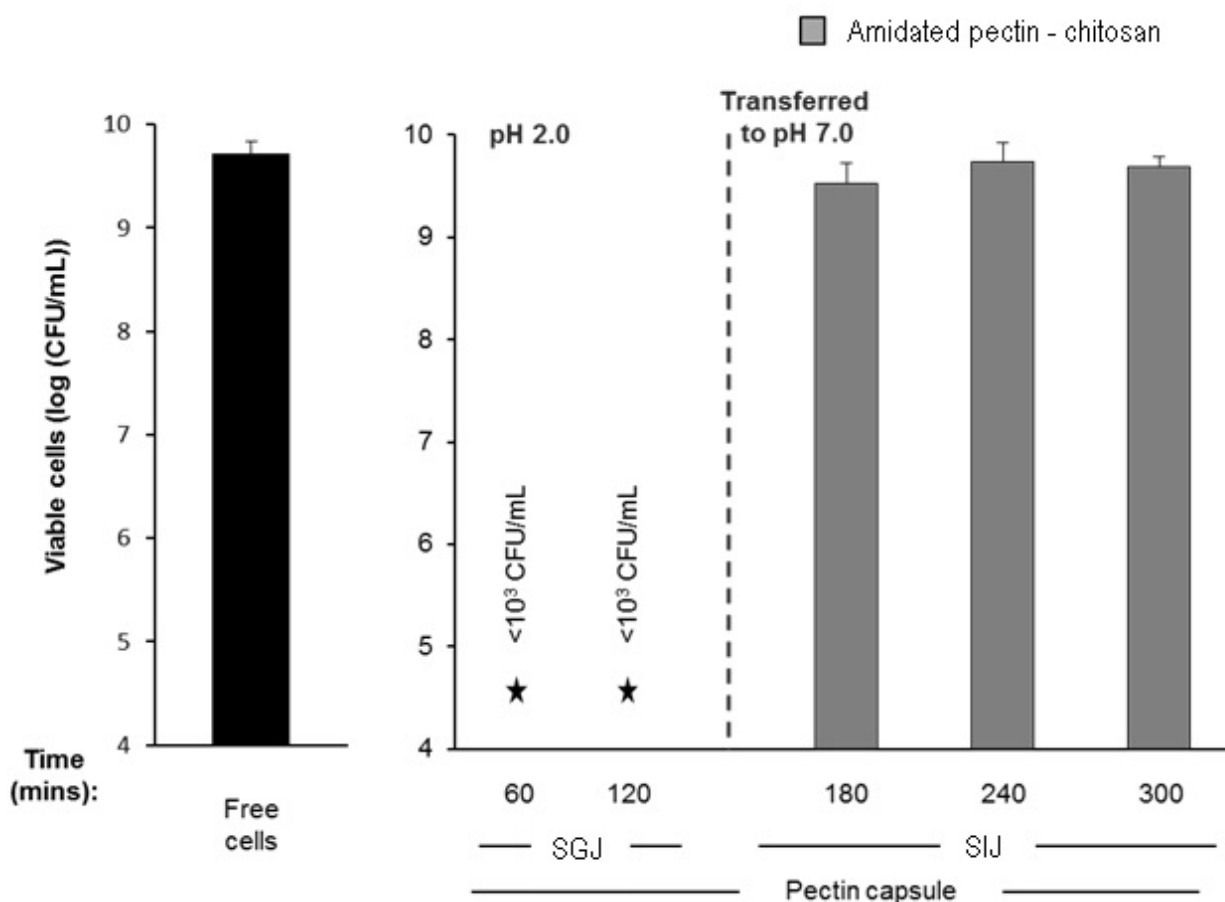
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335 In order to be effective as enteric delivery system, the capsules must preserve the viability of the
 336 probiotic cells during their transit through the stomach and deliver these cells alive and in high
 337 numbers in the intestine (Suita-Cruz & Goulet, 2001).

338 As the produced pectin-chitosan coated capsules provided good results in the acid challenge test,
 339 their ability to deliver cells to the intestine was investigated. In this *in vitro* experiment,
 340 gastrointestinal passage was simulated by exposing the capsules to low pH in the stomach during 2
 341 hours, followed by exposure to the high pH encountered in the intestine for 3 hours. The main aim of
 342 this experiment was to understand the release profile of the bacteria in the GI tract. It was observed
 343 that no cells were recovered during gastric transit, but crucially after 1 hour of transferring to SIJ a
 344 quick release effect was seen and a complete cell recovery was achieved (Fig.6). Disintegration of the
 345 pectinate capsules and release of bacteria in SIJ observed in this study is in accordance with the
 346 literature for similar systems. For example, Gebara et al (2013) have also reported complete
 347 disintegration of pectin microparticles coated with whey protein at pH 7.0. This disintegration is
 348 related to the dissociation of pectin-chitosan polyelectrolyte complex coating (Birch et al, 2014) as
 349 well as calcium pectinate gel (Günter and others 2016) under these pH conditions.

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353 Fig.6. Release of *Lactobacillus casei* after exposure to SGJ (2 h) and SIJ (3 h), n=4.

354

355

4. Conclusion

356

357 Hydrated calcium pectinate capsules containing live *L. casei* were produced using the extrusion
 358 technique and were subsequently coated with chitosan. Pectin capsules without chitosan coat were
 359 found to provide limited protection to *L. casei* in simulated gastric juice. Coating with chitosan
 360 effectively protected the bacterial cells from the acid in the simulated gastric juice and intestinal juice,
 361 suggesting that these capsules are suitable for gastrointestinal delivery of viable cells. In the future, the
 362 evaluation of sensory characteristics of these capsules will be of interest.

363

364

Acknowledgements

365 This work was funded by the grant from the Ministry of Education and Science, Republic of
 366 Kazakhstan under the project "Developing immunostimulating dairy products with encapsulated
 367 synbiotics" (grant number 0115PK01199). The authors also acknowledge Shakarim State University of
 368 Semey (Kazakhstan) for providing additional funding to support this project.

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