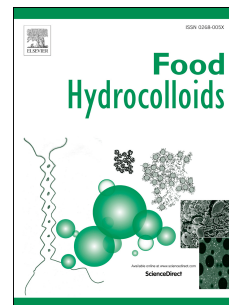


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Characterization of alginate-lactoferrin beads prepared by extrusion gelation method

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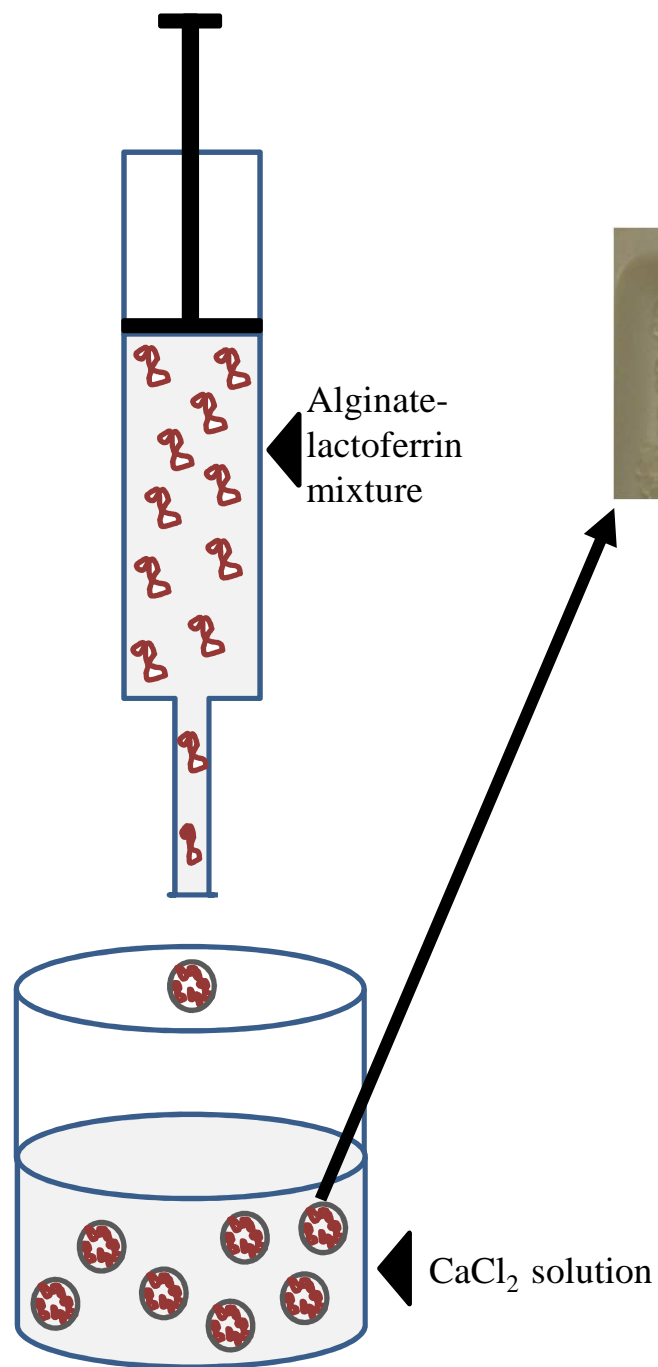
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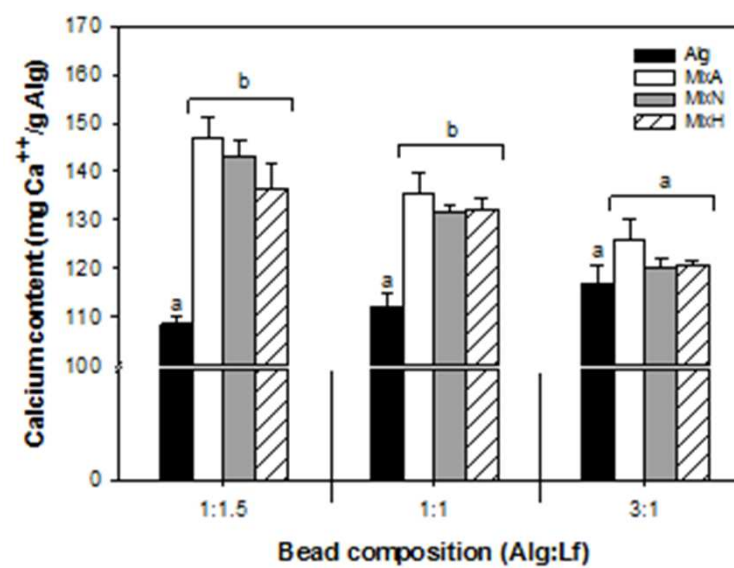
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Characterization of beads



Title Page Information

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1 Abstract

2 The potential use of lactoferrin (Lf) as a food ingredient is attracting increasing attention.
3 Encapsulation of Lf in alginate gel beads can potentially enhance its stability and safe
4 delivery. It is therefore important to understand the properties of alginate-Lf beads in order to
5 develop a successful encapsulation method. Three different forms (apo-, native- and holo-) of
6 bovine Lactoferrin (Lf) were encapsulated in alginate beads by the gel entrapment method
7 using calcium as the cross-linking ion. A minimum of 40% alginate was required in the
8 beading mixture to retain the form of a bead. Higher proportion of alginate reduced the water
9 holding capacity and calcium uptake by the beads and these effects were similar for all forms
10 of Lf. Longer cross-linking time led a greater amount of Lf and iron leaching from the beads
11 and this was most pronounced for holo-Lf compared to apo- and native-Lf. The elastic
12 modulus was affected by the composition (mixing ratio) of beads and not by the forms of Lf
13 with decreasing elasticity of the beads with increasing alginate content which was attributed
14 to the decreasing crosslinking density. The stability of the encapsulated Lf was evaluated
15 based on the amount leached into pH adjusted Millipore water and was affected by pH (4 and
16 7) for holo-Lf but not for apo- and native-Lf. The relative rate of Lf leaching at pH 4 for the
17 different forms of Lf was found not to directly correlate with the *pI* of the different forms of
18 Lf.

19 **Keywords:** Gel entrapment, bovine lactoferrin, alginate, electrostatic interaction, stability

20 1. Introduction

21 Lactoferrin (Lf) is an iron-binding glycoprotein (MW = 80 kDa) found in various biological
22 fluids of mammals (Marnila & Korhonen, 2009) and it has many health benefits to humans
23 and animals (Wakabayashi, Yamauchi, & Takase, 2006). Lf can bind iron with high affinity
24 ($K_D \sim 10^{-20}$ M) and can exist in either iron depleted (apo-) or in iron saturated (holo-) forms. In

25 nature (native-), it exists as a mixture of apo-, holo- (Steijns & van Hooijdonk, 2000) and also
26 monoferric Lf species saturated at either their N or C lobe (Brisson, Britten & Pouliot, 2007).
27 The iron binding ability along with its cationic nature is responsible for a diverse range of
28 physiological functions such as regulation of cellular growth and differentiation, intestinal
29 iron homeostasis, host defence against microbial infection and inflammation, regulation of
30 myelopoiesis, immunomodulatory and protection against cancer (Conneely & Ward, 2004;
31 Guo, Pan, Rowney & Hobman, 2007). These benefits enable Lf to be a potential supplement
32 in commercial food products (i.e. infant milk, supplemental tablet, yoghurt, skim milk, and
33 drinks). However, Lf can easily be denatured by heat treatment (Abe et al., 1991) and
34 processing condition including storage, freezing/thawing, and spray drying (Naidu, 2006)
35 thereby limiting its application. In addition, conditions such as pH, temperature and
36 conductivity can affect the functional properties of Lf and furthermore, Lf is susceptible to
37 degradation by proteolytic enzymes in the gastrointestinal tract of the human body (Onishi,
38 2011). Thus, a delivery system that protects Lf is required to be developed to deliver Lf as a
39 food component for maximum health benefit.

40 Alginate is a natural polysaccharide produced by brown algae (*Phaeophyceae*) and bacteria
41 (*Azobacter vinelandii*). It is composed of unbranched binary copolymers of (1→4) linked β -
42 D-mannuronic acid (M) and α -L-guluronic acid (G) residues of widely varying composition
43 and sequence (Draget, 2009). It has the ability to retain water and form viscous solutions,
44 stabilize aqueous mixtures and form gels in the presence of divalent cations. Based on these
45 biophysical properties, alginate has been classified as a food additive and has been used in
46 food preparations as thickeners, emulsifiers and gelling and stabilizing agents (Brownlee,
47 Seal, Wilcox, Dettmar, & Pearson, 2009). Because of its unique gelling properties under mild
48 and non-toxic conditions, the use of alginate has been extended to biotechnological and

49 biomedical applications such as encapsulation and immobilization of enzymes and live cells
50 (Donati & Paoletti, 2009; Martinsen, Storrø, & Skjåk-Bræk, 1992).

51 Alginates possess ion exchange property due to the presence of the carboxylic groups in both
52 the M and G residues which have been shown to interact with cationic protein molecules
53 electrostatically. Examples of reported electrostatic interactions between the anionic alginate
54 and cationic proteins includes the proteins lysozyme and chymotrypsin (Wells & Sheardown,
55 2007) and Lf (Peinado, Lesmes, Andrés, & McClements, 2010). Such electrostatic
56 interactions will affect the release of proteins from the alginate gel system. Furthermore,
57 increased charge neutralization by positively charged components will cause a reduction in
58 intramolecular repulsion between individual alginate chains such that the system will be able
59 to adapt a tighter, more compact configuration and thereby affect diffusional pathways
60 (Stockwell, Davis, & Walker, 1986).

61 An alginate gel can be prepared by a simple diffusion method where a crosslinking ion is
62 allowed to diffuse from a large outer reservoir into an alginate solution forming a single gel
63 bead entrapping the active agent. Diffusion setting produces inhomogeneous alginate gels due
64 to the formation of a sharp gelling zone moving from the surface towards the centre of the gel
65 (Draget, 2009; Draget, Smidsrød, & Skjåk-Bræk, 2005; Draget & Taylor, 2011). An
66 extrusion technique is used for the formation of gel particles by the diffusion method (Desai
67 & Park, 2005) and is useful for encapsulation of heat labile active agents as they are
68 completely surrounded by wall material (Pegg & Shahidi, 2007). The gelling process is
69 influenced by several factors such as alginate concentration, molecular mass and the M/G
70 sequence of the alginate, the ratio between gelling and non-gelling ions and the presence of
71 complexing agents (eg. phosphates and citrates). In addition, gelation of alginate in a mixed
72 system, where charged polymers such as proteins interact electrostatically under favourable
73 conditions may lead to alterations in mechanical properties of the gel beads (Draget, 2009).

74 Pore size and pore size distribution in alginate gels are of importance as they determine the
75 diffusion properties. Anderson, Skipnes and Smidsrød (1977) reported that calcium-treated
76 alginate forms networks characterized by a pore size between 5 and 150 nm. Diffusion of
77 small molecules is not strongly affected by the alginate gel pore size, but the diffusion of
78 larger molecules, such as proteins, is somewhat restricted, although proteins with molecular
79 weight as high as 300 kDa are able to diffuse through the gel beads with a rate that depends
80 on their molecular size (Tanaka, Matsumura, & Veliky, 1984; Donati & Paoletti, 2009). The
81 M/G ratio of the alginate also affects the diffusivity of proteins in the gel matrix (Amsden &
82 Turner, 1999). Diffusion within the gel depends on porosity, however, since the gel matrix is
83 negatively charged, the influence of electrostatic forces between the matrix and the ionic
84 substrates must also be considered (Martinsen et al., 1992).

85 In this study, the gel encapsulation behaviour of different (apo-, native- and holo-) forms of
86 bovine Lf in calcium alginate beads was studied. The physico-chemical properties of Lf are
87 affected by its form (Bokkhim, Bansal, Grøndahl & Bhandari, 2013) and the technological
88 applications of Lf therefore, are likely to also be affected by the form of Lf used. This
89 warrants for a detailed study of the different forms of Lf. In addition, the effect of alginate-Lf
90 mixing ratio and crosslinking time on the efficiency of encapsulation and physical and
91 mechanical properties as well as stability of the beads were investigated.

92 **2. Materials and Methods**

93 **2.1. Materials**

94 Native- and apo-bovine lactoferrin (NatraFerrin) with 13 and 0.9% iron saturation
95 respectively were provided by MG Nutritionals®, Australia. Sodium alginate (PE 12001-13.8
96 EN, GRINDSTED® Alginate FD 155, M/G ratio 1.5) was donated by Danisco Australia Pty.
97 Ltd., Australia. The molecular mass of this alginate as determined by U-tube viscometry

98 using the appropriate Mark-Houwink constant was found to be 140 kDa (Vold, Kristiansen &
99 Christensen, 2006; Vold, Kristiansen & Christensen, 2007). Calcium chloride dihydrate
100 (99%) and sodium azide (99.5%) were purchased from Chem-supply Pty. Ltd. and Sigma
101 Aldrich Co., Australia, respectively. Millipore water was used in the preparation of all
102 samples. Holo-Lf was prepared in the laboratory according to the method of Bokkhim,
103 Bansal, Grøndahl and Bhandari (2013).

104 **2.2. Fabrication of beads**

105 Stock solutions (2, 3, 4 & 5%) by weight of alginate (Alg) and of Lf (apo-, native- and holo-)
106 were prepared in Millipore water. Alginate was dissolved in Millipore water using a high
107 shear homogeniser (IKA ® RW 20 digital, USA) at 600 rpm for 30 minutes. The alginate
108 solution was then heated at 40 °C in a water bath for 90 minutes to remove any trapped air
109 bubbles and then allowed to cool to room temperature. The Lf samples were dissolved in
110 Millipore water under constant stirring using a magnetic stirrer for 2 hours at room
111 temperature.

112 The first series of beads were extruded by mixing alginate and Lf solutions of equal
113 concentration at equal mixing ratio (1:1) to achieve the final solid contents of 2%, 3%, 4%
114 and 5% in the mixture. Mixing was done at 600 rpm for 20 minutes. No attempts were made
115 to adjust the pH or the ionic concentration of the mixtures. 3 mL of the mixtures were
116 extruded into 18 mL of 0.1 M calcium chloride (CaCl_2) solution using 25G $^{5/8}$ " (internal
117 diameter ~ 0.26 mm) stainless steel PrecisionGlide® needles (Becton Dickinson and Co.,
118 USA) under constant stirring with a magnetic stirrer. The beads were allowed to crosslink in
119 the CaCl_2 solution for 30 minutes, removed from the crosslinking solution and subsequently
120 washed three times with Millipore water and finally drained on a sieve.

121 The second series of beads were extruded as above but using 30G^{1/2}" (internal diameter ~
122 0.16 mm) stainless steel PrecisionGlide® needles.

123 The third series of beads were extruded by mixing alginate (2%) and Lf (2%) solutions in
124 different ratios of Alg:Lf, specifically in the ratios 1:3, 1:1.5, 1:1, 1.5:1 and 3:1 using 30G^{1/2}"
125 PrecisionGlide® needles.

126 The term mixture should be understood as an alginate-Lf mixture and will be denoted as Mix
127 in the captions for figures. In addition, alginate-Lf mixtures having apo-, native- and holo-Lf
128 will be denoted by MixA, MixN and MixH, respectively. All the samples were prepared on
129 weight percentage.

130 **2.3. Characterization of beads**

131 2.3.1. Bead size

132 The extruded beads were measured for their diameter (mm). 20 beads of same composition
133 were lined in contact in a straight row and the total length measured using a calibrated scale.
134 The total length was divided by the number of beads to achieve the diameter of a single bead.

135 2.3.2. Water holding capacity

136 The water content (expressed as g water/g alginate) was determined as a measure of the water
137 holding capacity of the beads. The weight of alginate or the alginate-Lf mixture used for
138 extrusion as well as the weight of the extruded beads after draining was measured. The water
139 content was calculated from equation 1.

$$140 \quad \text{Water content} = (\text{Wt. of wet beads} - \text{Wt. of total dry matter}) / \text{Wt. of alginate} \quad (1)$$

141 Where Wt. of total dry matter = Lactoferrin and alginate in the beads.

142 2.3.3. Bead composition

143 The Lf content (%) of the beads was determined from the initial amount of Lf present in the
144 alginate-Lf mixture and the amount of Lf which had leached out into the cross-linking CaCl_2
145 solution during bead formation. The absorbance at a wavelength of 280 nm was measured
146 using UV-Visible Spectrometry (UV-Visible Spectrophotometer, Pharmacia, Ultrospec III,
147 LKB, England) and the concentration of Lf determined based on standard curves which were
148 plotted for the different forms of bovine Lf. This was measured at two time-points: at 30 and
149 60 minutes of cross-linking to determine the effect of cross-linking time on the amount of Lf
150 leaching from the beads.

151 The iron content (%) of the beads was determined based on the iron content measured in the
152 crosslinking CaCl_2 solution by Inductively Coupled Plasma Optical Emission Spectroscopy
153 (ICP-OES) (Varian Vista Pro Radial ICP-OES system, Melbourne, Australia).

154 The calcium content (%) of the beads was quantified by ICP-OES measurement and used as a
155 measure of the calcium uptake by the beads during their formation. The beads were freeze
156 dried (Christ, ALPHA 1-4 LSC, Osterode, Germany) for 72 hours, acid digested and analysed
157 by ICP-OES.

158 **2.4. Properties of beads**

159 **2.4.1. Mechanical property (Young's Modulus)**

160 To investigate the mechanical property in terms of elastic (Young's) modulus of the extruded
161 beads, the beads (2 %) with mixing ratios (Alg:Lf) of 1:1.5, 1:1 and 1.5:1 were used. This
162 range of mixing ratios was selected as a mixing ratio of 3:1 produced soft beads which
163 disintegrated on handling. After extrusion, the beads were stored submerged in Millipore
164 water for 24 hours prior to compression testing. A TA.XT Texture Analyser (Stable Micro
165 Systems, UK) with TA10 Cylinder ($D = 12.7$ mm, $L = 35$ mm) was used for compression
166 analysis. A single bead was compressed with a test speed of 0.1 mm/sec with a trigger force

167 of 5 g. The bead was compressed to 20 % of its diameter. The Young's modulus was
168 obtained from the gradient of the stress-strain curve. The gradient was calculated from the
169 linear part of the slope (15 – 40 % of total strain).

170 2.4.2. Stability

171 The amount of Lf leaching from the beads (Alg:Lf = 1:1) into Millipore water at pH 4 and 7
172 (22 ± 2 °C) for 6 weeks were determined as a measure of bead stability. 2 grams of beads
173 were placed in 20 mL of pH-adjusted water. 0.02 % of sodium azide was used as preservative
174 in the beading mixture as well as in pH-adjusted Millipore water. The stability test of the
175 beads was conducted under constant shaking at 240 rpm (IKA® KS 130 basic, GMBH & Co.
176 KG, Germany). The amount of Lf leaching into the pH-adjusted water was determined at an
177 interval of 1 week, using UV-visible spectrometry at 280 nm (Aitken & Learmonth, 1996)
178 and corresponding Lf standard curves.

179 2.5. Statistical analysis

180 For experiments with three or more replicates, significance of differences between the values
181 was analysed by Analysis of Variance (ANOVA) with Tukey's HSD post hoc test, family
182 error rate 5 at 95% confidence level using MiniTab 16. The number of experiments is
183 indicated by 'n' in the figures.

184 3. Results and discussion

185 3.1. Characterisation of beads from alginate-Lf mixtures with equal mixing ratios

186 Beads were extruded using two different sized needles ($25G^{5/8}$ " and $30G^{1/2}$ "") from alginate-Lf
187 beading mixtures, containing different forms of Lf, at equal mixing ratios. The concentration
188 of CaCl₂ in the cross-linking bath and time for cross-linking were kept constant at 0.1 M and
189 30 min, respectively.

190 With a bigger diameter needle (25G^{5/8}"), an effect of solid matter content of the beading
191 mixture on the size (bead diameter) was noticed only at and beyond 4% total solids (Fig. 1 A
192 & B). In addition, total solids content above 3% in alginate and 4% in alginate-Lf mixtures
193 led to bead deformation (tailing), which is a very common phenomenon with viscous alginate
194 solutions (Fundueanu, Nastruzzi, Carpov, Desbrieres, & Rinaudo, 1999). This shows that
195 alginate beads were more prone to tailing than the alginate-Lf beads when compared beads of
196 same total solid content. The diameter of the beads were 2.5 ± 0.2 mm, 2.6 ± 0.3 mm and 3.1
197 ± 0.3 mm for 2%, 3% and 4% solid contents, respectively. A value for 5% solid content
198 beads could not be obtained due to the irregular shape of the beads (Fig. 1 A & B).

199 Figure 1(A & B).

200 When a smaller diameter needle (30G^{1/2}"") was used for extrusion of the beads having similar
201 composition, the effect of total solids content on bead diameter (2.3 ± 0.3 mm) was negligible
202 and no tailing was observed (Fig. 2A). When different forms of Lf (native- and holo-) were
203 added to alginate, a change in bead colour was evident. While alginate beads are transparent
204 those containing Lf were translucent and represented the colour of the alginate and Lf
205 solutions from which they were produced. The colours of different forms of Lf powders were
206 different; apo-Lf appeared white while native-Lf appeared light pink and holo-Lf reddish
207 brown. They imparted similar colour to their respective beads. The beads with apo-Lf were
208 opaque (Fig. 2B). The colour intensity increased with the total Lf concentration in the
209 mixture (Fig. 2A). These visual pictures showed that Lf is retained by the beads. Because of
210 the uniformity in size, PrecisionGlide® needle (30G^{1/2}"") was used for extrusion of beads for
211 all further experiments.

212 Figure 2 (A & B).

213 From Figure 3, it can be seen that alginate is the responsible component in the mixture for the
214 water holding capacity of beads as adding Lf to the mixture did not significantly change the
215 water content, eg. the water content of alginate-Lf beads was not significantly different ($P >$
216 0.05) compared to that of alginate control beads. With lower alginate content (in alginate
217 control as well as in alginate-Lf beads), a higher water holding capacity of the beads was
218 observed. Such a trend has been widely reported (Yotsuyanagi, Ohkubo, Ohhashi, & Ikeda,
219 1986) and is related to a higher polymer density leading to partial collapse of the network and
220 a reduction in pore size (Donati & Paoletti, 2009). The water holding capacity of the beads
221 from mixtures having different forms of Lf was not significantly different ($P > 0.05$) at all
222 concentration levels. This indicates that all alginate-Lf beads would have similar pore size.

223 Figure 3.

224 **3.2. Characterisation of beads extruded from alginate-Lf mixtures with different mixing** 225 **ratios**

226 3.2.1. Bead composition

227 Beads extruded through 30G^{1/2}" needles from alginate-Lf beading mixtures (2%), containing
228 different forms of Lf, at different mixing ratios (Alg:Lf of 1:3, 1:1.5, 1:1 and 3:1) were used
229 in this section of the study. Evaluation of the amount Lf and iron leaching into the cross-
230 linking solution was done for all four mixing ratios. The reason for considering 2% total
231 solids in the mixtures was due to practical limitations because of viscosity increment.

232 3.2.1.1. *Effect of cross-linking time*

233 The amount of Lf leaching into the cross-linking solution during bead formation is
234 characterised by the Lf content of the beads (Supplementary Fig. S1). Depending on the bead
235 composition, the Lf content varied from 92–97 %. The Lf detected in the solution can be

236 attributed to Lf leaching from the gel beads into the bath solution during cross-linking
237 (George & Abraham, 2006) or rapid escape of Lf into the solution at the initial stage of bead
238 formation. When the cross-linking time was increased from 30 to 60 minutes, a larger amount
239 of Lf had leached (about 2%) into the curing bath (Supplementary Fig. S1). The additional
240 leaching of Lf after cross-linking is attributed to Lf (80 kDa) diffusing into the bath from the
241 cross-linked beads as research has shown that molecules with molecular weight as high as
242 300 kDa can diffuse from alginate gel beads into the water system (Tanaka et al., 1984;
243 Martinsen, Skjåk-Bræk, & Smidsrød, 1989; Donati & Paoletti, 2009). In addition, a trend was
244 noticed that with increasing amount of alginate in the Lf-alginate mixture (eg. from Alg:Lf of
245 1:3 to 3:1), the leaching of Lf from the beads was lower (eg. resulted in higher Lf content,
246 Supplementary Fig. S1). This trend is attributed to a higher concentration of alginate in the
247 bead leading to a denser matrix (eg. beads were all of the same diameter). For Lf-alginate
248 beads, a crosslinking time of 30 minutes was subsequently adapted for preparation of the
249 beads for further investigation.

250 3.2.1.2. Effect of different forms of Lf

251 The amount of different forms of Lf and iron that leaches into the cross-linking solution after
252 a cross-linking time of 30 min are presented in Figures 4 A and B, respectively. A clear trend
253 was seen for the effect of form of Lf in the alginate-Lf mixture; the Lf content of holo-Lf
254 being the lowest (Fig. 4 A). For all mixing ratios, the differences in Lf content of apo-Lf and
255 native-Lf were non-significant ($P > 0.05$) but were significantly higher ($P < 0.05$) than that of
256 holo-Lf. The iron content of the beads displayed the same trend. Iron retained by beads with
257 apo-Lf was significantly higher ($P < 0.05$) than that of holo-Lf, whereas beads with native-Lf
258 did not follow the trend (Fig. 4 B). It should, however, be noted that the determination of the
259 Lf content is considered more accurate due to the higher absorbance values measured. This
260 data indicate that the iron detected in the cross-linking solution is bound to the Lf. These

261 values were verified from iron analysis by ICP-OES and protein analysis by Dumas method
262 conducted on the freeze dried beads (data not shown). The differences in the molecular
263 structures of the different forms of Lf, in particular the structure of holo-Lf being more
264 compact compared to the structure of apo- and native-Lf (Sánchez et al., 1992; Brisson,
265 Britten, & Pouliot, 2007) and the differences in their physico-chemical properties (Bokkhim
266 et al., 2013) may be responsible for the differences observed.

267 Figure 4 (A & B).

268 **3.2.2. Water holding capacity**

269 The beads from section 3.2.1.2 were characterized for their water holding capacity except
270 beads produced from the mixing ratio (Alg:Lf) of 1:3 which were excluded as they
271 disintegrated during handling due to the low alginate content. Inclusion of any form of Lf
272 from 25 % (3:1) to 60 % (1:1.5) in the alginate-Lf mixture (total solids content of 2 %) did
273 not significantly affect the water holding capacity of the beads when compared to the alginate
274 control beads of similar alginate content (Fig. 5). However, the alginate content in the
275 mixture, affected the water held by the beads. Lower water content was observed for higher
276 alginate content, which followed a trend similar to beads extruded with same mixing ratio but
277 different solid contents (Fig. 3). This indicates that beads containing higher alginate content
278 have a denser matrix. Higher water holding capacity correlated with softer beads, and as
279 mentioned above the beads with 75 % Lf (1:3) disintegrated during bead handling. From this
280 experiment, it can be concluded that a minimum of 40 % alginate is required in the Lf-
281 alginate mixture (2 %) to form firm beads.

282 Figure 5.

283 3.2.3. Calcium uptake by the beads

284 The beads from section 3.2.1.2 (except for the mixing ration (Alg:Lf) of 1:3) were
285 characterized with regards to the amount of calcium uptake during bead formation and the
286 data for different compositions is shown in Figure 6. The calcium uptake by the beads having
287 different forms of Lf, calculated on the basis of unit mass of alginate, was not significantly
288 affected by the forms of Lf used in beading mixture but was significantly different from that
289 of control alginate beads at mixing ratios 1:1.5 and 1:1. However, the difference diminished
290 as the alginate content increased to 75% (3:1). This showed that addition of Lf to alginate,
291 thereby decreasing the alginate content in the mixture, increases the calcium uptake by the
292 alginate component during bead formation at lower alginate content (below 75%) . On the
293 other hand, the calcium uptake by the alginate control beads having different final
294 concentration was not significantly different ($P > 0.05$). An explanation for these observations
295 is that the presence of protein in the alginate-Lf mixture delays the formation of a sharp
296 gelling zone during gelation allowing calcium to diffuse relatively fast towards the centre of
297 the bead, ultimately leading to a higher amount of calcium participation in crosslinking. The
298 amount of calcium (mg) per unit mass of alginate (g) ranged from $109 \pm 2 - 117 \pm 4$ for the
299 controls alginate beads to $137 \pm 5 - 147 \pm 5$ mg/g for mixtures (1:1.5). These results are very
300 similar to the values of 160 ± 30 mg/g for beads prepared from a 2 % sodium alginate (M/G
301 ratio 1.5) in 0.27 M CaCl_2 reported by Tan et al. (2008).

302 Figure 6.

303 3.3. Properties of beads

304 3.3.1. Mechanical property of extruded beads

305 Beads containing native-Lf described in sections 3.2.2 and 3.2.3 formed from solutions with
306 mixing ratios (Alg:Lf) of 1:1.5 and 1:1 as well as with a mixing ratio of 1.5:1 were

307 characterised for their compressibility. As it was found that the water holding capacity and
308 the calcium content of the beads were not significantly affected by the forms of Lf used, only
309 native-Lf encapsulated in alginate was included in this experiment. The mechanical property,
310 specifically the elastic modulus of the extruded beads is presented in Figure 7. It is evident
311 that alginate beads show no significant difference in the elasticity of the gel beads when the
312 concentration of alginate is increased from 0.8 to 1.0 % but the gel elasticity increases
313 significantly when the alginate content is increased to 1.2 %. This agrees with the previous
314 results by Martinsen, Skjåk-Bræk, & Smidsrød (1989) and considering that the beads showed
315 the same level of calcium uptake (Fig. 6), it is attributed to the difference in their water
316 holding capacity (Fig. 5). On the other hand, among the beads having native-Lf in their
317 compositions, the elasticity of beads with 60% Lf (Alg:Lf = 1:1.5) were not significantly
318 different from that of beads with 50% Lf (1:1) but significantly different ($P < 0.05$) from that
319 of beads with 40% Lf (1.5:1). The form of Lf showed no significant difference in the
320 elasticity of the beads (results not shown). This trend of decreasing elasticity of the beads
321 with increasing alginate content for the alginate-Lf beads correlates with the higher
322 crosslinking density (eg. higher calcium uptake) seen for the 1:1.5 Alg:Lf beads (Fig. 6) and
323 is consistent with previous reports (Martinsen et al., 1989). Furthermore, it can be seen that
324 the alginate-Lf beads (1:1) are not significantly different than the control beads (1% alginate)
325 yet by increasing or decreasing either Lf or alginate in the mixing ratio by more than 20%,
326 the elasticity of the beads was significantly altered.

327 Figure 7.

328 3.3.2. Stability of extruded beads

329 Beads described in sections 3.2.2 and 3.2.3 formed from solutions with a single mixing ratio
330 (Alg:Lf) of 1:1 were characterised for their stability with respect to the amount of Lf leaching

331 into pH-adjusted water at two different pH values. The pH values were chosen so as to ensure
332 that only diffusion process would take place. At lower pH (< 4.0), alginate gels become
333 unstable due to proton catalysed hydrolysis (Draget, 2009) and breaks down into lower
334 molecular components (Gombotz & Wee, 1998). This will ultimately affect the crosslinking
335 and stability of the beads and thereby the mechanism of release of Lf. The choice of a single
336 mixing ratio was based on the Lf content following a similar trend for beads with different
337 ratios (see Fig. 4). The cumulative leaching of Lf from the alginate-Lf beads against time
338 (days) at pH 4 and 7 is shown in Figure 8 A and B, respectively, and is expressed in
339 percentage based on the initial Lf content of the beads at day 0. No significant effect of pH (4
340 and 7) was observed on the leaching of apo- and native-Lf from their respective beads, but the
341 leaching of holo-Lf was significantly ($P < 0.05$) affected by pH and was higher at pH 7 than
342 at pH 4. Leaching of a higher amount of holo-Lf at pH 7 as compared to pH 4 might be due to
343 higher swelling ratio of alginate beads at neutral pH (Shi, Alves & Mano, 2006). At the same
344 time, it has been reported that holo-Lf has a more compact molecular conformation as
345 compared to apo- and native-Lf (Sánchez et al., 1992; Brisson et al., 2007). Thus, the
346 combination of the gel network size and the protein size might have contributed to leaching of
347 higher amounts of holo-Lf at pH 7. Similar trends with respect to pH have been reported by
348 Shi, Alves and Mano (2006) for the drug indomethacin and by Huguet and Dellacherie (1996)
349 for bovine serum albumin. On the other hand, the amount of apo-Lf leaching from the beads
350 was significantly higher than that of native- and holo-Lf at pH 4. It has been reported that a
351 protein with a low pI may be released more rapidly from the alginate matrix (Gombotz &
352 Wee, 1998). In our past research (Bokkhim et al., 2013), it was found that apo-Lf
353 demonstrates a net charge of zero in pH range of 5.5-6.5, which is lower than the actual pI
354 (8.5-9.5) of Lf. This means that while native- and holo-Lf have an overall positive charge at
355 both pH values used in the current stability study, apo-Lf will have a positive charge only at

356 pH 4. It thus appears that the relative rates of leaching at pH 4 are not solely related to the
357 charge of the different forms of Lf. However, our previous study also concluded that apo-Lf
358 has different physicochemical properties as well as iron binding capacity compared to native-
359 and holo-Lf and it thus appears that the differences in Lf leaching for the different forms of Lf
360 is related to more subtle differences between the proteins. The maximum amount of apo-Lf
361 which had leached from the beads in 6 weeks did not exceed 9 % of the initial Lf content in
362 the beads and lower amounts were observed for the other forms of Lf. This indicates a high
363 overall stability of Lf within the alginate gel matrix.

364 Figure 8 (A & B).

365 **4. Conclusion**

366 Lf (apo-, native- and holo-) can be encapsulated in alginate beads by the extrusion gel
367 entrapment method. Alginate in the alginate-Lf mixture was found to be the major
368 contributing factor in determining the water holding capacity of the beads, and it was
369 inversely proportional to the alginate content in the mixture. It was found that a minimum of
370 40% alginate was necessary in the alginate-Lf mixture to form firm beads. At low alginate
371 content, the presence of Lf in the mixture led to an increase in calcium uptake by the beads.
372 The leaching of holo-Lf from alginate-Lf mixture during gelation was found to be higher than
373 apo- and native-Lf and correlate with the leaching of iron. The gel strength of the beads could
374 be significantly altered by increasing either alginate or Lf content by 20% in the mixture. The
375 cumulative leaching of apo-Lf from the beads into pH-adjusted water was less than 9% in 42
376 days and was higher than that of native- and holo-Lf at both pH values. This indicates a high
377 overall stability of Lf within the alginate gel matrix. Apart from leaching of Lf during
378 gelation and upon immersing the beads in pH-adjusted water, the form of Lf in the mixture
379 did not generate significant differences in the properties of the beads. The findings of this
380 study clearly showed that Lf can be encapsulated efficiently in alginate beads using gel

381 entrapment method to produce beads with desired properties and can be used as the basis for
382 commercial scale up.

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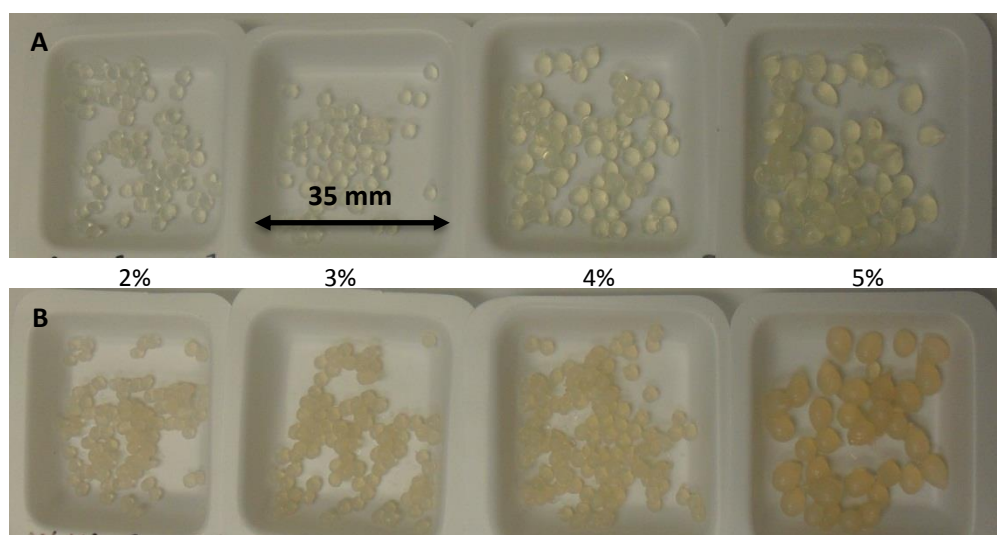
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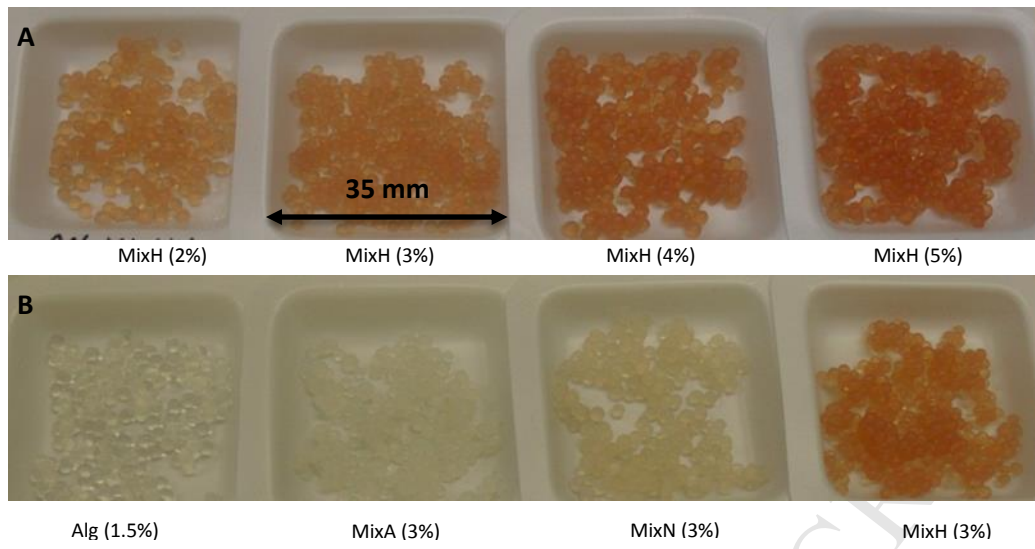
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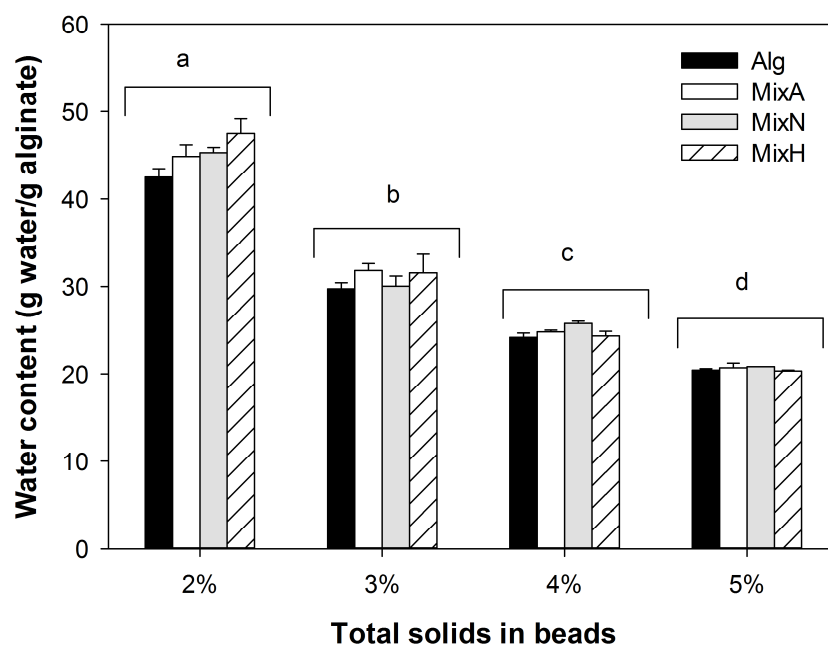
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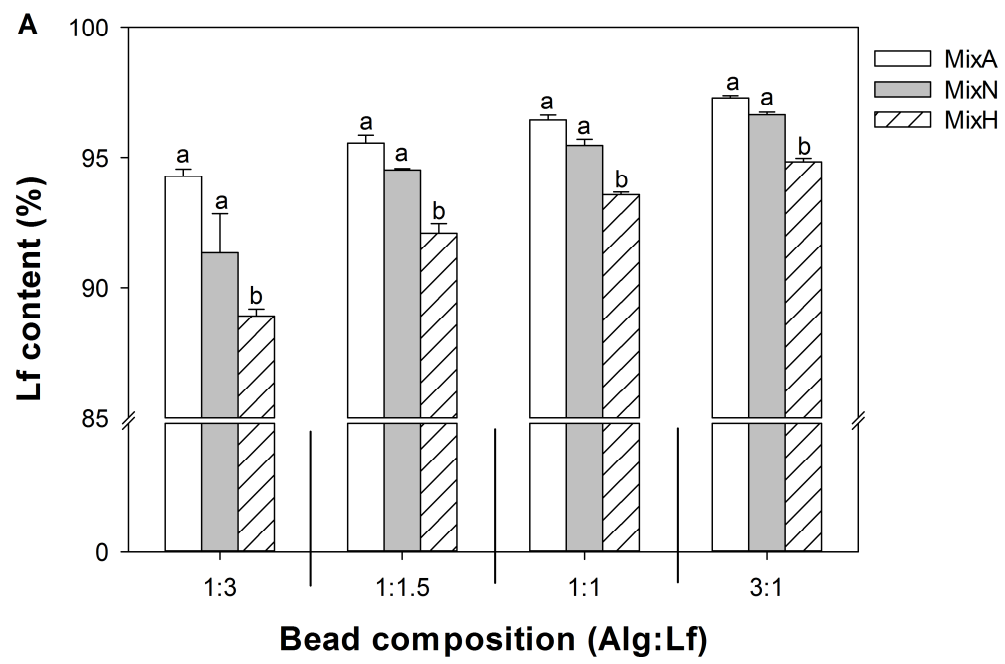
Captions for figures supplied:

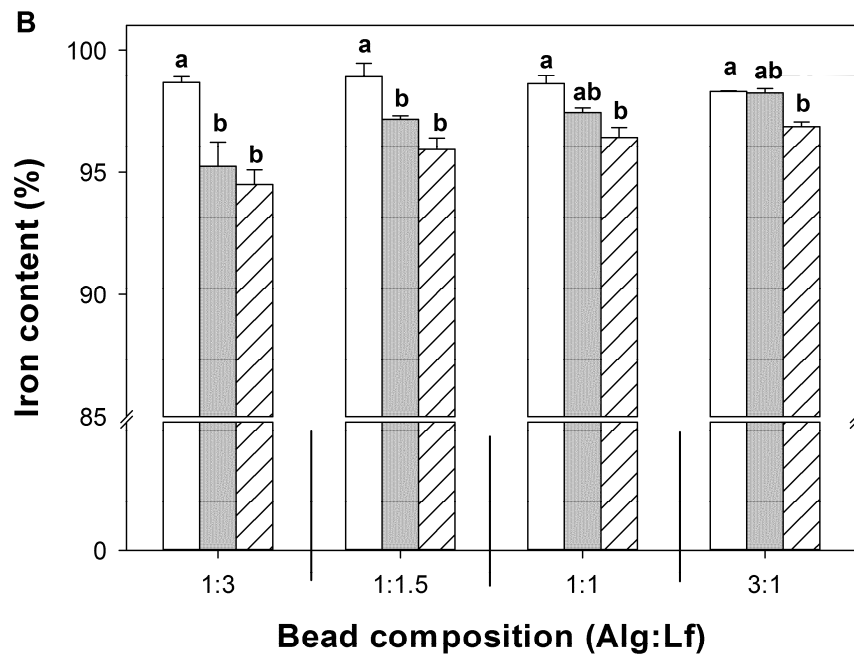
Figure	Caption	Remarks/Format
Fig. 1	Photographs of beads extruded through PrecisionGlide® 25G ^{5/8} " needles. (A) Alg and (B) MixN (1:1) with different total solid contents.	PDF
Fig. 2	Photographs of beads extruded through PrecisionGlide® 30G ^{1/2} " needles. (A) Beads extruded from MixH (1:1) with different total solids content in the mixture. (B) Beads extruded from alginate-Lf mixtures (total solids content, 3%; 1:1) having different forms of Lf. Alginate beads (1.5%) were extruded as control.	PDF
Fig. 3	Water holding capacity of beads extruded from alginate-Lf mixtures (1:1). Alginate beads having total alginate content of 1, 1.5, 2 & 2.5% were extruded as controls. The collective bars for each group that do not share a letter is significantly different at $P < 0.05$ (n=3).	TIFF
Fig. 4	(A) Amount of Lf remaining and (B) Iron remaining in beads prepared from mixtures (2%) having different forms of Lf (apo-, native- & holo-) and alginate in different ratios. The bars within the same column that did not share a letter are significantly different ($P < 0.05$) (n=3).	TIFF
Fig. 5	Water holding capacity of beads prepared from mixtures (2%) with different composition. Alginate beads (0.8, 1.0 & 1.5%) were extruded as control (n=3).	TIFF
Fig. 6	Calcium uptake of beads extruded from different composition mixtures (2%). Alginate beads (0.8, 1.0 & 1.5%) were extruded as control. The collective bars for each group that do not share a letter are significantly different at ($P < 0.05$) (n=3).	TIFF
Fig. 7	Mechanical property (Young's Modulus) of alginate-Lf beads (2%) having different mixing ratios. Alginate control beads (0.8, 1.0 & 1.2%) were extruded accordingly (n=6).	TIFF
Fig. 8	Cumulative leaching of Lf (%) from Lf-alginate beads (1:1) in Millipore water at different pH. (A) pH 4 and (B) pH 7. MixA (—●—), MixN (·····), MixH (—▼—).	TIFF

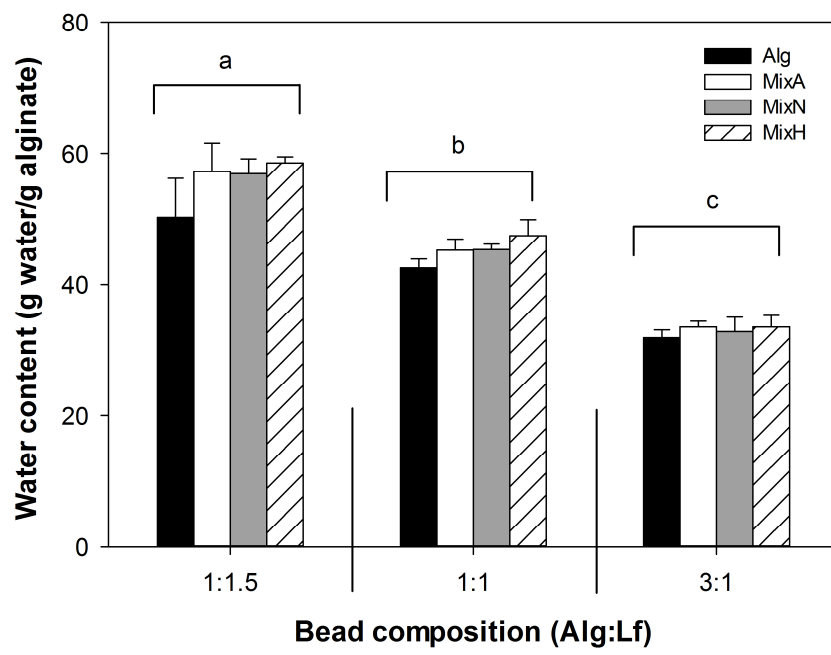


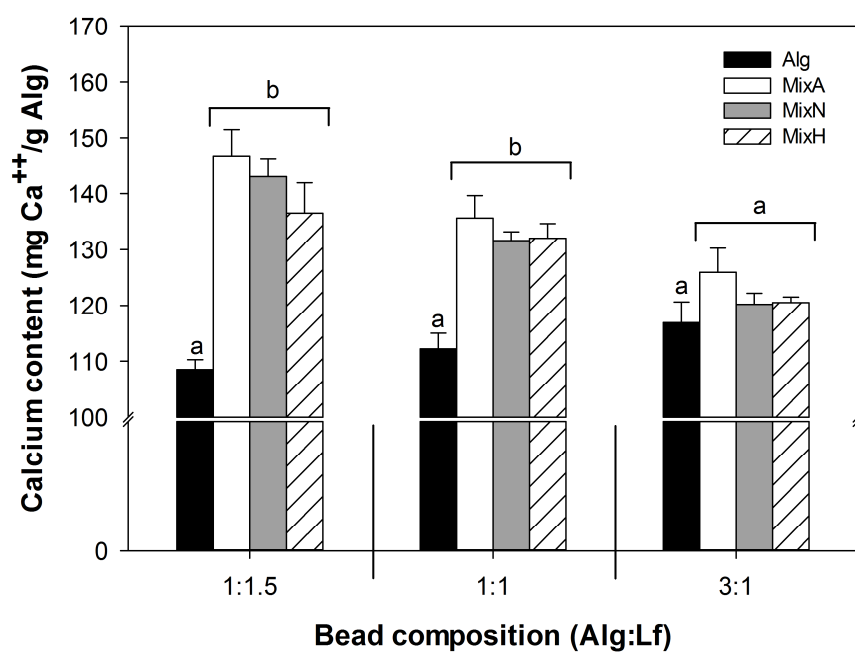


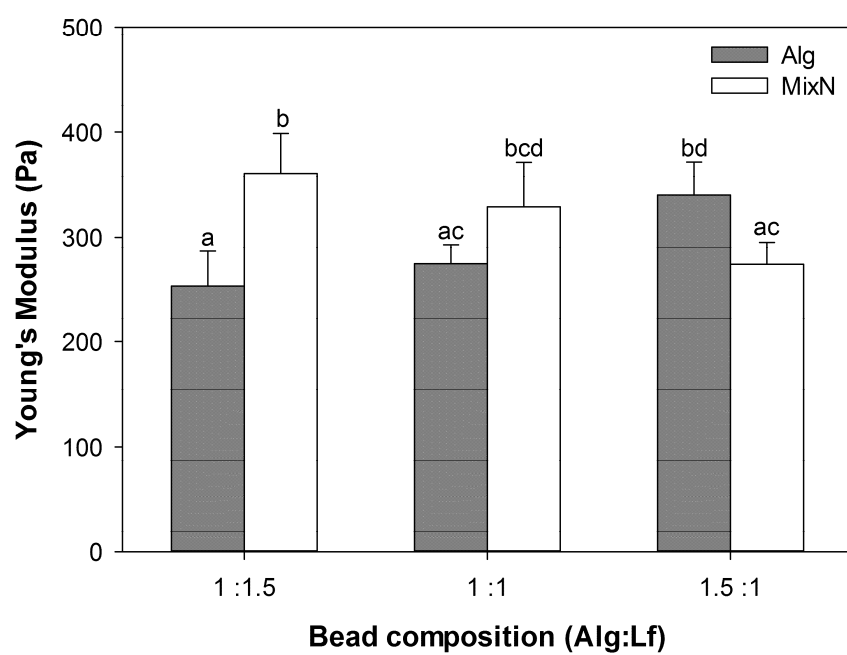


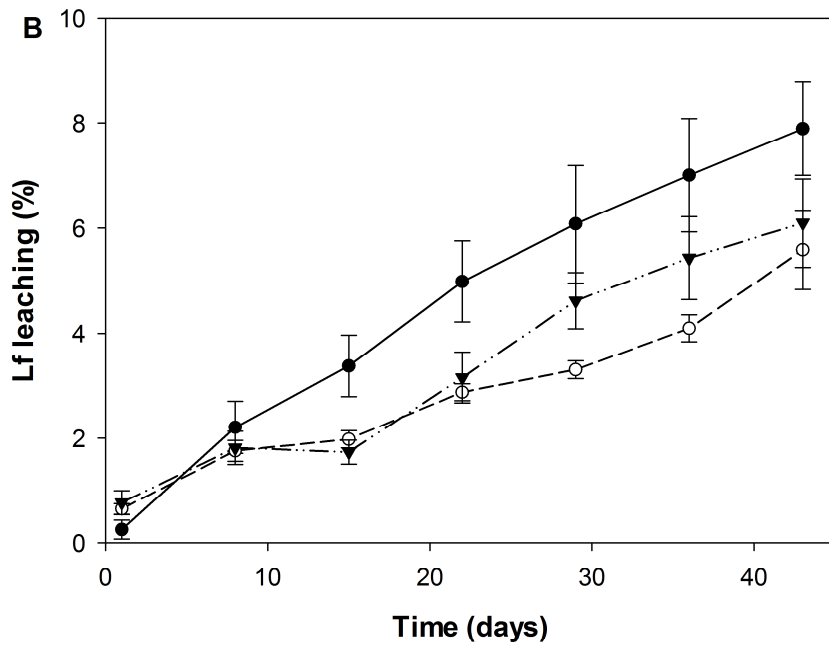
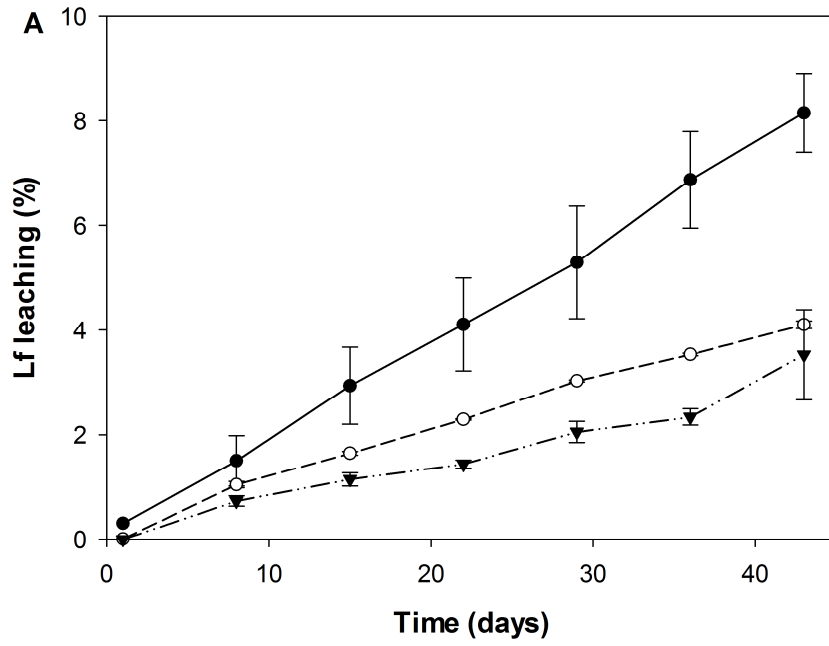












Captions for Supplementary figure supplied:

Figure	Caption	Remarks/Format
Fig. S1	Amount of Lf remaining in the alginate-Lf beads (MixN; total solids 2%) at different mixing ratios, cross-linked for 30 and 60 minutes based on the amount of Lf leached into the cross-linking solution (n=1).	TIFF

