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In-vitro digestion of different forms of bovine lactoferrin encapsulated in alginate micro-gel particles

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Encapsulation

Lactoferin (Lf)   Alginate (Alg)   Lf-Alg mixture

Micro-gel particles

CaCl₂

***In-vitro* digestion**

SGF

SIF

SDS-PAGE Gels
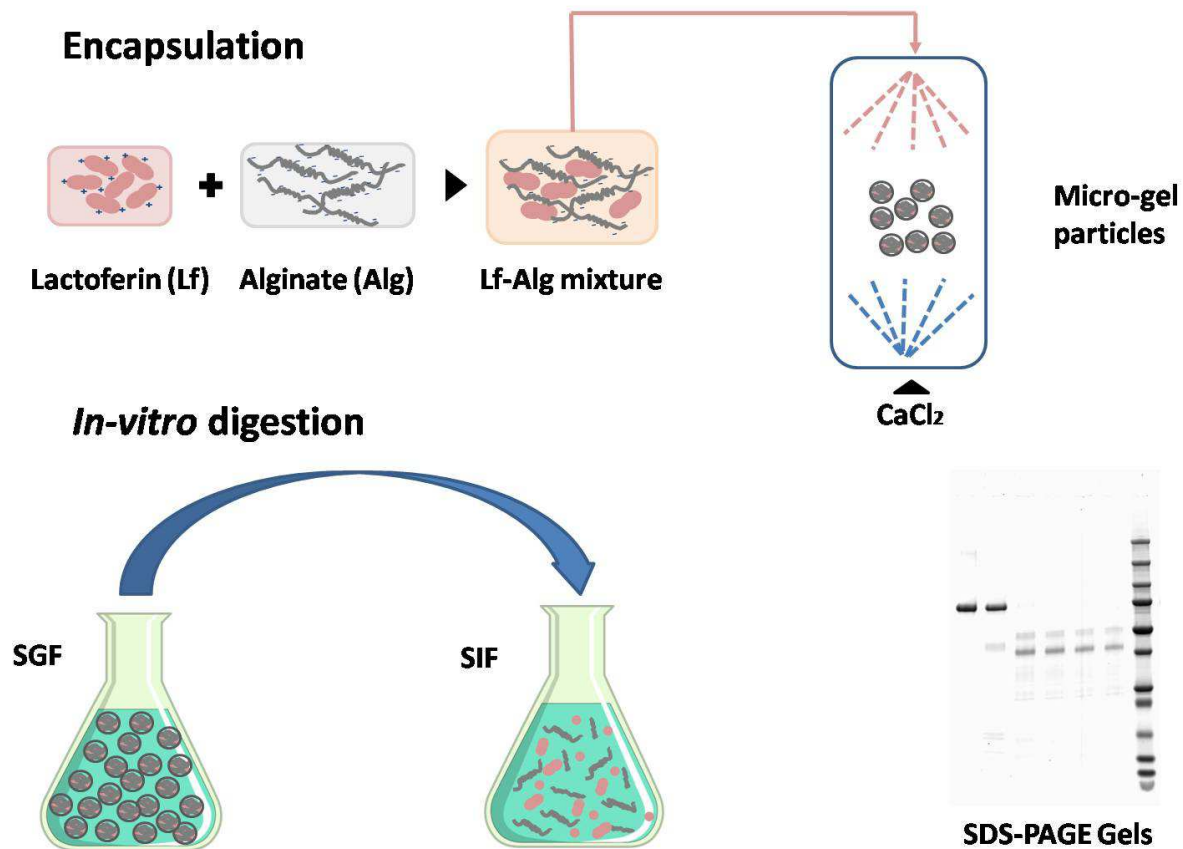
Title Page Information

Title: *In-vitro* digestion of different forms of bovine lactoferrin encapsulated in alginate micro-gel particles

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1 **Abstract**

2 Encapsulation of three forms of lactoferrin (Lf) (apo-, native- and holo-) was undertaken using the

3 novel impinging aerosol technique (Progel). The micro-gel particles were produced from a 2%

4 (w/w) solution of Lf and alginate (at equal mixing ratio) using 0.1 M $CaCl_2$ as the cross-linking

5 solution. An encapsulation efficiency of 68 − 88% was achieved based on the total amount of Lf

6 entrapped in alginate micro-gel matrix. Increasing the $CaCl_2$ concentration to 0.2 M reduced the

7 encapsulation efficiency. An *in-vitro* digestion study conducted in simulated gastric fluid (SGF) and

8 intestinal fluid (SIF) used pepsin and pancreatin (porcine) enzymes, respectively. Lf encapsulated

9 micro-gel particles were able to retain significantly higher amount (76 − 89%) of Lf (apo- and

10 native- forms) when digested in the SGF for 2 hours as compared to their corresponding un-

11 encapsulated pure Lf (41 − 58%). The effect of encapsulation on digestibility in SGF of holo-Lf

12 was minimal. Digestion of all forms of Lf, pure or encapsulated, in the SIF was very rapid. Within

13 10 min, apo- and native-Lf were completely digested, while holo-Lf, exhibited some resistance as

14 less than 5% remained after 10 min. This study showed that encapsulating apo- and native-Lf in

15 alginate micro-gel particles can provide protection from the action of pepsin in the SGF and allow

16 their releases in the SIF.

17 **Keywords:** Lactoferrin, alginate, micro-gel particles, *in-vitro* digestion, simulated gastric fluid,

18 simulated intestinal fluid.

19 **1. Introduction**

20 The possibility of supplementing different food products other than baby formula with lactoferrin

21 (Lf) has generated much attention in recent years because of its ability to exert many health

22 beneficial effects for humans. Antimicrobial, anti-inflammatory, immunomodulatory and anti-

23 carcinogenic effects are a few of the claimed health benefits of Lf (Legrand et al., 2008). These

24 physiological effects of Lf are attributed by its strongly cationic nature (Brock, 2002) with or

25 without the conjunction of its ability to bind iron with high affinity (K_D~ 10^{-20} M) (Moore,

Anderson, Groom, Haridas, & Baker, 1997). In addition, Lf can act as an iron carrier because of its iron binding ability and this has enabled its use as nutritional iron supplement (Steijns, 2001). Greater bioavailability of iron from Lf as compared to inorganic iron has been reported by several researchers (Ueno, Ueda, Morita, Kakehi, & Kobayashi, 2012; Hu et al., 2008).

To enable the use of Lf as a food ingredient, apart from optimising the processing conditions, it is required that it can withstand the harsh gastrointestinal conditions to reach the site of digestion and absorbance, the small intestine in its bioactive state (Lönnerdal, & Kelleher, 2009). Researches in the past have shown that oral delivery of Lf leads to diminished effects due to its breakdown by gastric conditions (Steijns, Brummer, Troost, & Saris, 2001; Eriksen et al., 2010). Different forms of Lf, iron-free (apo-), iron-saturated (holo-) or native-Lf (composed of a mixture of apo- and holo-Lf) (Steijns, & van Hooijdonk, 2000) and/or monoferric Lf (iron bound either in N or C lobe) (Brisson, Britten, & Pouliot, 2007) possess different physico-chemical properties (Bokkhim, Bansal, Grøndahl, & Bhandari, 2013). The differences in their molecular conformation and other properties can lead to difference in their resistance towards the harsh processing and gastrointestinal conditions. Research has shown that iron saturated holo-Lf is less susceptible to the gastric degradation (Steijns et al., 2001; Brock, Arzabe, Lampreave, & Piñeiro, 1976) and thermal denaturation (Sánchez et al., 1992; Conesa et al., 2008) compared to apo-Lf.

In the food sector, microencapsulation has been in use for more than 75 years to entrap in a matrix or coat sensitive compounds such as vitamins, antioxidants, flavours, bioactives, enzymes, peptides, proteins and microbial cells (Pegg, & Shahidi, 2007; Millqvist-Fureby, 2009; Gombotz, & Wee, 1998; Ding, & Shah, 2007). Various matrix materials such as starches, sugars, cellulose, hydrocolloids, lipids, and proteins have been used (Zuidam, & Shimoni, 2010). Encapsulation offers immobilization, protection, controlled release, structure and functionalization for sensitive compounds (Poncelet, 2006). Amongst these different microencapsulation materials, alginate gel particles have been reported to enhance the stability against processing and gastric conditions (pH

51 and proteolytic enzymes) for a number of water insoluble and micro-particulate core compounds

52 (Rayment et al., 2009; Brownlee, Seal, Wilcox, Dettmar, & Person, 2009).

53 Alginate is a natural polysaccharide, composed of unbranched binary copolymers of $(1\rightarrow 4)$ linked

54 β-D mannuronic acid (M) and α-L-guluronic acid (G) residues of widely varying composition and

55 sequence (Draget, 2009). Because of its biocompatibility, safety and ability to form gel particles

56 under mild conditions in the presence of calcium ions, it has been extensively used for

57 encapsulation and immobilization of sensitive active ingredients for food applications (Martinsen,

58 Storrø, & Skjåk-Bræk, 1992). The non-toxic nature yet synergistic effect of calcium on humans and

59 animals has enabled its use as the most favourable cross-linking ion in alginate based delivery

60 system (Draget & Taylor, 2011). *In-vitro* studies have shown that alginates retard the actions of a

61 range of digestive enzymes by limiting the availability of the enzyme to the substrates (Brownlee et

62 al., 2009). Andresen, Skipnes, Smidsrød, Ostgaard and Hemmer (1977) reported that calcium

63 treated alginate forms gel networks characterized by a pore size between 5 and 150 nm and allows

64 the diffusion of water soluble components with molecular weight as high as 300 kDa, in and out of

65 the calcium alginate gel network (Tanaka, Matsumura, & Veliky, 1984; Pothakamury, & Barbosa-

66 Cánovas, 1995). Degradation of alginate gel networks in the presence of chelating agents (eg.

67 citrates and phosphates) can also lead to release of encapsulated macromolecules such as proteins

68 (Gombotz & Wee, 1998). Furthermore, alginate is an anionic polysaccharide and therefore

69 electrostatic interactions (Draget, 2009) can occur in the presence of charged polymers (eg. cationic

70 proteins such as Lf) leading to a sustained release of macromolecules from the gel particles (Wells,

71 & Sheardown, 2007; Bokkhim, Bansal, Grøndahl, & Bhandari, 2014). Research has shown that

72 electrostatic as well as other intermolecular interactions occur between Lf and alginate and that the

73 extent of interactions is affected by the form of Lf (Peinado, Lesmes, Andrés, & McClements,

74 2010; Bokkhim, Bansal, Grøndahl, & Bhandari, 2015). These interactions minimise the loss of

75 entrapped Lf by diffusion, lower at pH 4 compared to pH 7 for native- and holo-Lf, thus ensuring

76 the stability of Lf within the alginate gel matrix (Bokkhim et al., 2014). The release of encapsulated

77 bioactive compounds from the alginate gel particles is governed by either diffusion or dissolution of

78 gel particles or a combination of both (Kuang, Oliveira, & Crean, 2010). In the human intestine, the

79 presence of chelating agents such as lactate, citrates and phosphates (Coppi, Iannuccelli, Leo,

80 Bernabei, & Cameroni, 2001) and other cations such as sodium ions (Gombotz & Wee, 1998) play

81 an important role in the destabilization of cross-linked alginate gel networks by removing the

82 calcium ions. *In-vitro* studies conducted on alginate gel particles has reported that the gel particles

83 were resistant to the gastric conditions while disintegrating in the intestinal conditions (Rayment et

84 al., 2009) which render them as a potential vehicle for controlled delivery.

85 The objective of this study is to encapsulate Lf in alginate micro-gel particles using a locally

86 developed impinging aerosol technique (Bhandari, 2009) in order to develop Lf encapsulated

87 micro-gel particles with enhanced technological properties for their potential use in food

88 formulations. *In-vitro* stability and release of Lf from the micro-gel particles were evaluated in

89 simulated gastric and intestinal fluids in the presence of proteolytic enzymes pepsin and pancreatin,

90 respectively.

91 **2. Materials and Methods**

92 **2.1. Materials**

93 Bovine lactoferrin (NatraFerrin), with iron saturation levels of approximately 1% (apo-) and 13%

94 (native-) were provided by MG Nutritionals®, Burnswick, Australia. Sodium alginate (PE 12001-

95 13.8 EN), GRINDSTED® Alginate FD 155 (M/G ratio 1.5; molecular mass 140 kDa) was from

96 Danisco Australia Pty. Ltd., Sydney, Australia. Calcium chloride dihydrate (99%), bile salts (from

97 ox gall; BL038-25G), sodium chloride and tri-sodium citrate dehydrate were purchased from Chem-

98 supply Pty. Ltd, SA, Australia. Pepsin from porcine gastric mucosa (*P6887*; 3200-4500 units/mg

99 protein), pancreatin from porcine pancreas (P-7545; Activity equivalent to 8×U.S.P. specification),

100 bis (2-hydroxymethyl) iminotris-[hydroxymethyl] methane) (bis-tris) (purity > 98%), monobasic

101 potassium phosphate, sodium hydroxide, sodium acetate trihydrate, Trizma® base, sodium

102 bicarbonate and glycine were purchased from Sigma Aldrich Co., Castle Hill, Australia (purity >

103 99%). Acetic acid (99%), hydrochloric acid (concentration ~ 31.5%) and methanol (99.8%) were

104 from Labtek Pty. Ltd., Brendale, Australia. Sodium dodecyl sulphate (SDS) was from Amresco,

105 Solon, USA and glycerol was from Ajax Finechem Pty. Ltd., Taren Point, Australia. The dyes,

106 bromophenol blue and Coomassie brilliant blue G-250, Mini-PROTEAN® TGX™ Gels (4 – 20%,

107 15 wells comb, 15 μL) were from BIO-RAD, Gladesville, Australia. Cellulose acetate membrane

108 filter (Ø=47 mm, pore size=0.45μm) was purchased from Advantec®, Toyo Roshi Kaisha, Ltd.,

109 Japan. All chemicals, unless otherwise stated, were of analytical grade. Millipore water was used

110 for all experiments. Iron saturated holo-Lf (99.7%) was prepared according to the method described

111 by Bokkhim, Tran, Bansal, Grøndahl and Bhandari (2014). 1% (w/v) solution of native-Lf was

112 prepared in 10 mM Tris-Cl buffer containing 75 mM NaCl, pH adjusted to 7.2 with HCl solution.

113 Calculated volume of fresh ferric nitrilotriacetic acid (FeNTA) solution [9.9 mM ferric nitrate and

114 8.5 mM nitrilotriacetic acid, pH adjusted to 7.0 with solid sodium bicarbonate] was added to the Lf

115 solution to achieve a molar ratio Lf:iron of 1:2; incubated at room temperature for an hour and

116 finally dialysed against Millipore water for 48 hours with three changes of water. The dialysed iron

117 saturated Lf solution was freeze dried prior to use in the study.

118 **2.2. Encapsulation of Lf in alginate micro-gel particles**

119 Two percent solids by weight solutions of sodium alginate (Alg) and the three forms of Lf (apo-,

120 native- & holo-) were prepared separately in Millipore water. To dissolve sodium alginate, water at

121 40 ℃ was used. The solutions were prepared by mixing for 2 hours at 600 rpm using a high shear

122 homogenizer (IKA ® RW 20 digital, USA) and allowed to stand at room temperature for another 2

123 hours. Subsequently the alginate and the Lf solution were mixed at equal ratio (Alg:Lf = 1:1) and

124 left standing overnight to remove any trapped air.

125 Micro-encapsulated Lf-alginate particles were prepared using the impinging aerosol technique

126 (Progel microencapsulating device, Bhandari, 2009) (Fig. 1). This continuous micro-gel forming

127 device was previously researched to encapsulate probiotics and pharmaceutical products (Sohail,

128 Turner, Coombes, Bostrom, & Bhandari, 2011; Hariyadi et al., 2012). The Lf-alginate mixture was

129 introduced from a nozzle into a close upright chamber at an air pressure of 500 kPa. A solution of

130 calcium chloride (0.1 M) was introduced from another nozzle fitted at the bottom of the device at an

131 air pressure of 200 kPa. The cascading fine droplets of the Lf-alginate mixture came in contact with

132 the uprising fine mist of calcium chloride inside the device, thus creating gelled particles instantly.

133 The micro-gel particles were collected from the bottom outlet along with the calcium chloride

134 solution and allowed to cure in the cross-linking solution for 30 minutes. The time interval of 30

135 minutes for cross-linking of 2% Lf-alginate beads (Lf:Alg = 1:1) was adapted based on the study

136 conducted by Bokkhim et al., 2014. After curing, the product was collected using vacuum filtration

137 with a filter paper (Advantec, Quantitative Filter Paper, Grade no. 3, Japan). The product was

138 washed twice with Millipore water to remove excess calcium, and then frozen at -18 °C and freeze-

139 dried (Christ, ALPHA 1-4 LSC, Osterode, Germany) under the standard condition; ice condenser

140 temperature of - 60 ± 5 ℃, shelf temperature of 10 ± 5 ℃ and vacuum of $0.021 - 0.040$ mbar for

141 72 hours. To study the effect of the calcium content in the cross-linking solution, micro-gel particles

142 of similar composition were produced using 0.2M $CaCl_2$. Control blank gel particles were prepared

143 from 2% alginate alone. The freeze dried micro-gel particles were stored in an air-tight aluminium

144 foil bag in a freezer (-18 □C) until future characterization. Sample names are outlined in Table 1.

145 The product recovery and encapsulation efficiency after freeze drying of the gel micro-particles

146 were calculated from equations 1 and 2, respectively.

147 $$\text{Product recovery} = \frac{Weight\ of\ freeze-dried\ micro-gel\ particles}{Weight\ of\ total\ solids\ in\ Lf-alginate\ mixture} \times 100\% \tag{1}$$

148 $$\text{Encapsulation efficiency} = \frac{Weight\ of\ protein\ in\ freeze-dried\ micro-gel\ particles}{Weight\ of\ total\ protein\ in\ Lf-alginate\ mixture} \times 100\% \tag{2}$$

149 Figure 1

150 **2.3. Characterization of Lf encapsulated alginate micro-gel particles**

151 2.3.1. Calcium and protein content

152 The micro-gel particles were characterized for their calcium and protein contents. The analyses

153 were conducted on freeze dried micro-gel particles. The calcium content of the micro-gel particles

154 was determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Varian

155 Vista Pro Radial ICP-OES system, Melbourne, Australia) after digesting the micro-gel particles in

156 nitric:perchloric acid (5:1). The calcium values were expressed per unit mass of alginate after

157 deducting the protein from the total mass. The protein content was analyzed following the

158 combustion protocol of Dumas method (Rayment, & Higginson, 1992) and the values are expressed

159 in percentage of dry weight.

160 2.3.2. Particle size measurement

161 The particle size of the freshly prepared (non-freeze dried) micro-gel particles encapsulating native-

162 Lf were measured using a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). This

163 method is based on laser diffraction by suspended particles in distilled water, at laser obscuration of

164 $\geq 15\%$ and laser intensity $\geq 75\%$. The results are expressed in volume weighted mean, D (4,3). The

165 freshly prepared micro-gel particles were collected after filtration and washed with Millipore water.

166 These washed micro-gel particles were re-suspended in Millipore water prior to particle size

167 measurement. The particle size of freeze dried micro-gel particles after rehydration was also

168 measured using the same method. All measurement were conducted at room temperature (22 ± 2

169 ºC).

170 **2.4. *In-vitro* digestion of different forms of Lf**

171 The protocol for *in-vitro* digestion of Lf or encapsulated Lf in micro-gel particles was developed

172 after comparative study of similar *in-vitro* digestion protocols used for different proteins. Dupont et

173 al. (2010) for food proteins, Mandalari et al. (2008) for almond protein, Eriksen et al. (2010) for

174 caprine whey proteins and Almaas et al. (2006) for caprine whey proteins including bovine Lf.

175 These protocols used a starting protein concentration of 25 – 50 mg protein/mL for gastric

176 processing. In this study, taking into account, the amount of calcium which is also ingested along

177 with the encapsulated Lf through micro-gel particles, a protein concentration of 25 mg Lf/mL was

178 used for gastric processing.

179 2.4.1. Simulated gastric digestion

180 The three different forms of Lf (apo-, native- and holo-) were digested in simulated gastric fluid

181 (SGF) (0.2% NaCl solution in Millipore water, pH adjusted to 2.0 with 1 M HCl, 4500 U

182 pepsin/mL). To 125 mg of Lf, 5 mL of SGF was added to achieve 180 U pepsin/mg Lf. The Lf

183 samples were incubated at 37 ℃ under constant horizontal shaking (100 strokes/min) (Julabo, SW-

184 22, GmbH, Germany). After 30, 60, 90 and 120 minutes, 100 μL of the digested sample was

185 removed and diluted with 2.4 mL of 0.1 M sodium bicarbonate solution (pH ~ 8.2) to achieve 0.1%

186 Lf. The high pH was used to reduce the activity of the pepsin enzyme. This diluted digested sample

187 was used immediately to prepare samples for SDS-PAGE gel electrophoresis (described below).

188 2.4.2. Simulated intestinal digestion

189 To study the effect of pancreatin on the different forms of Lf (apo-, native- & holo-), *in-vitro*

190 digestion of Lf was done in simulated intestinal fluid (SIF) prepared according to US Pharmacopeia

191 with slight modification in pH. 50 mg Lf was dissolved in 2 mL Millipore water. Then 2 mL of pre-

192 warmed SIF (37 ℃; 0.68% monobasic potassium phosphate; 0.5% bile salts; 1.0% pancreatin; pH

193 8.5) was added, pH was adjusted to 7.5 and incubated at 37 ℃ under constant horizontal shaking

194 (100 strokes/min). After 10, 20, 30 and 60 minutes, 100 μL of the sample was removed and diluted

195 with 1.15 mL of Millipore water to achieve 0.1% Lf. This diluted digested sample was used for

196 SDS-PAGE gel electrophoresis immediately.

197 **2.5. *In-vitro* digestion of encapsulated Lf**

198 2.5.1. Simulated gastric digestion

199 The micro-gel particles were digested in SGF. To 250 mg of micro-gel particles (equivalent to 125

200 mg Lf), 5 mL of pre-warmed (37 ℃) SGF was added (180 U pepsin/mg Lf). Samples were

201 incubated in a water bath under constant shaking (37 ℃, 100 horizontal strokes/min) for a set length

202 of time. After 30, 60, 90 and 120 minutes, the samples were filtered through cellulose acetate

203 membrane filter (0.45 μm) under vacuum and washed with Millipore water. The gel particles were

204 collected and dissolved in 12.5 mL of 0.1 M sodium citrate solution under constant shaking at 37

205 ℃. The activity of the pepsin enzyme was reduced because of high pH of sodium citrate (~ 8.4).

206 Once completely dissolved, 1 mL of the digested sample was further diluted with 9 mL of Millipore

207 water to achieve 0.1% Lf content. This diluted mixture was the base sample for SDS-PAGE gel

208 electrophoresis. As a control sample in SDS-PAGE gel electrophoresis, micro-gel particles which

209 had not been exposed to SGF were dissolved in 0.1 M sodium citrate solution (0.5% Lf). After

210 complete dissolution of the micro-gel particles, 1 mL of this solution was diluted with 4 mL of

211 Millipore water (0.1% Lf).

212 2.5.2. Simulated intestinal digestion

213 For *in-vitro* intestinal digestion, initial digestion of the encapsulated micro-gel particles (100 mg) in

214 SGF (2 mL) was conducted according to Section 2.5.1. for 2 h at 37 ℃. Then, 2 mL of pre-warmed

215 (37 ℃) SIF was added. The pH was adjusted to 7.5 with 1 M NaOH (~ 60 μL). The entire sample

216 was incubated at 37 ℃ under constant shaking (100 horizontal strokes/min) for a set interval of time

217 (10, 20, 30 & 60 min). At the end of the set time interval, the digested sample was diluted with 46

218 mL of Millipore water to achieve 0.1% Lf. This diluted sample was instantly used for SDS-PAGE

219 gel electrophoresis. The samples from the SGF digestion (digested for 2 h) were used as controls in

220 the SDS-PAGE gel electrophoresis.

221 **2.6. SDS-PAGE gel electrophoresis**

222 The amount of Lf remaining undigested in the SGF and SIF after the set length of time was

223 determined by gel electrophoresis (SDS-PAGE) using 4 – 20% precast polyacrylamide gels under

224 reducing conditions. 100 μL of each sample (0.1% Lf) described in Sections 2.4.or 2.5. was added

225 to 200 μL of SDS-loading buffer (70 mM Tris-Cl, pH 6.8; 26% glycerol; 2.11% SDS and 0.01%

226 bromophenol blue dye). Finally, 5 μL of β-mercaptoethanol was added to each sample.

227 Subsequently it was heated at 95 ℃ for 5 minutes. The dilution of Lf samples (0.1% Lf), mixing

228 with loading buffer (1:2) and heating (95 ℃) were carried out continuously with very short time

229 lapse in-between to minimize further digestion by the enzymes pepsin and pancreatin. These

230 samples were kept frozen until loading onto the SDS-PAGE gels.

231 The frozen samples were thawed, vortexed and 5 μL was loaded into the wells of a SDS-PAGE gel.

232 Electrophoresis was conducted at 200 V for 47 minutes in a Mini-PROTEAN tetra cell system.

233 Following this, the SDS-PAGE gel was dipped in a fixative solution (20% acetic acid in 40%

234 methanol) for 5 minutes, drained and stained overnight under constant shaking (160 rpm) (IKA®

235 KS 130B, GmbH& Co. KG, Germany) at room temperature (22 ± 2 ℃) with Coomossie brilliant

236 blue R-250 solution containing 34% methanol. The SDS-PAGE gel was de-stained in de-staining

237 solution (1% acetic acid) for 24 hours with 2 changes. Scanning of SDS-PAGE gel was done with

238 Gel Densitiometer (GS-800 Calibrated Densitiometer, UMAX Technologies, Model UTA−2100XL,

239 Taiwan). The amount of intact Lf was normalized based on the relative quantity of control Lf

240 sample in lane T0 using Quantity One® software.

241 **2.7. Stability of micro-gel particles**

242 Micro-gel particle stability and integrity during *in-vitro* digestion was observed by recording

243 microscope images using an optical microscope (Prism Optical PRO 2300T, Scientific instrument,

244 Brisbane, Australia). Images were recorded using the software TSView7 under an eye piece Plan

245 achromat 10/0.25 at different time intervals during *in-vitro* gastric and intestinal digestion. The

246 particle size distribution of the micro-gel particles during *in-vitro* digestion was also analyzed using

247 Mastersizer 2000 as described above.

248 **2.8. Statistical analysis**

249   Results are presented as mean ± SD of triplicate experiments where applicable. For other

250   experiments, the number is indicated by n. The significance of differences between the values

251   (where applicable) were analyzed by MiniTab 16 software using Analysis of Variance (ANOVA)

252   with Tukey's HSD post hoc test at family error rate 5 at 95% confidence level.

3. Results and discussion

3.1. Encapsulation of Lf in alginate micro-gel particles

255   The encapsulation of Lf in alginate micro-gel particles using the Progel microencapsulating device

256   gave the highest product recovery for the combination of a 2% Lf-alginate mixture (1:1) with 0.1 M

257   CaCl$_2$ as the cross-linking solution for native-Lf (86 ± 8%). The actual product recovery of the

258   micro-gel particles containing apo-Lf and holo-Lf are not included here. During the atomization of

259   the Lf-alginate solution with apo- and holo-Lf, it was observed that the micro-gel particle production

260   was non-homogenous leading to a wide distribution of the particle size. In addition, in some

261   instances aggregation of particles were observed. The difference in behavior of the different forms of

262   Lf might be due to the differences in the viscosity of the mixtures. The viscosities of Lf-alginate

263   mixtures with apo- (721 ± 38 mPa s) and holo-Lf (514 ± 14 mPa s) were significantly lower than

264   that with native-Lf (1297 ± 36 mPa s) (Bokkhim et al., 2015). In order to be able to compare the

265   micro-gel particles with different forms of Lf, the same composition has to be used for all Lf-

266   alginate mixtures. Thus we limited the encapsulation study to the mixing ratio of 1:1 and total solids

267   content of 2%. In addition, from our previous study (Bokkhim et al., 2015), within the 2% total

268   solids content of Lf-alginate mixture, changing the mixing ratio alone was not able to increase the

269   viscosity of the mixtures with apo- and holo-Lf to the required level for improved encapsulation.

270   Increasing the concentration of calcium in the cross-linking solution to 0.2 M improved the micro-

271   gel particle formation process for Lf-alginate mixtures containing apo- and holo-Lf however, the Lf

272   entrapment efficiency was affected concomitantly as discussed below. The colors of the gel particles

273   were imparted by the colors of Lf used, and the difference in color of gel particles was very distinct

274   in their freeze-dried powdered form (Fig. 2).

275   The colors of the freeze-dried powders of the micro-gel particles made using 0.2 M $CaCl_2$ solution

276   appeared lighter than the freeze-dried powders of the micro-gel particles made from 0.1 M $CaCl_2$

277   solution in agreement with the observed lower encapsulation efficiency. The loss of Lf in the filtrate

278   solution which showed very light pinkish taint was also observed. Kim (1990) has shown that the

279   use of higher calcium ion concentration during cross-linking of alginate causes a rapid shrinking of

280   the alginate gel leading to formation of water cavities within the gelled layer of the particles due to

281   rapid release of bound water from the alginate network. In agreement with this, studies have shown

282   that the formation of a compact gel results when using high calcium ion concentrations and this is

283   associated with possible collapse of some junction zones leading to increased pore sizes (Donati, &

284   Paoletti, 2009) and formation of inhomogeneous gel structure which can affect the permeability

285   (Skjåk-Bræk, Grasdalen, & Smidsrød, 1989; Bellich, Borgogna, Cok, & Cesàro, 2011). This

286   ultimately will cause greater diffusion of Lf during micro-gel particle formation. In order to fully

287   elucidate the effect of the physico-chemical properties of Lf on the gelation process using the Progel

288   micro-encapsulating device, further investigations would be required, especially with regards to

289   calcium ion concentration and to optimize the encapsulation process for apo- and holo-Lf.

290   Figure 2.

291   **3.2. Characterization of Lf-alginate micro-gel particles**

292   *3.2.1. Calcium and protein content*

293   The calcium and protein content of the Lf-alginate micro-gel particles are presented in Table 1.

294   Apart from the micro-gel particles having apo-Lf (0.1 M $CaCl_2$), the calcium content of all other gel

295   particles were not significantly different. Increasing the calcium concentration (0.2 M) in the cross-

296   linking solution did not affect the calcium uptake by the micro-gel particles. The reason for higher

297   calcium uptake by the micro-gel particles having apo-Lf (0.1 M $CaCl_2$) is not very clearly

298 understood. The control alginate micro-gel particles (2%) showed no significant difference in

299 calcium content of Lf-alginate micro-gel particles produced using solution of CaCl$_2$ at 0.1 M (81 $\pm$ 7

300 mg Ca^{2+}/g alginate) and 0.2 M (85 $\pm$ 9 mg Ca^{2+}/g alginate). This indicates that the calcium content of

301 the washed micro-gel particles fabricated by the impinging aerosol technique using a cross-linking

302 time of 30 minutes is not affected by the calcium concentration of the cross-linking solution. This

303 might be related to the size of the gel particles, since it will take a short time for calcium to diffuse

304 into these micron-sized particles.

305 The protein content of the micro-gel particles was significantly higher when lower calcium

306 concentration (0.1 M) was used in the cross-linking solution. This illustrates the importance of

307 gelation rate to retain the core material. When using a high calcium concentration (0.2 M) in the

308 cross-linking mist, very rapid formation of densely cross-linked (Jao, Ho, & Chen, 2010) gel

309 particles could lead to excessive leaching of the Lf.

310 Table 1

311 *3.2.2. Particle size measurement*

312 The particle size of the micro-gel particles encapsulating native-Lf was measured using a

313 Mastersizer 2000. The particle size expressed as volume weighted mean D (4,3), of fresh micro-gel

314 particles prior to washing were significantly smaller (40 $\pm$ 1 μm) (P < 0.05) than the micro-gel

315 particles after washing (70 $\pm$ 8 μm). This could be due to osmotic swelling during washing with

316 Millipore water in the absence of calcium. The particle sizes of rehydrated freeze-dried micro-gel

317 particles in Millipore water (at 22 $\pm$ 2 ºC) were not significantly different (66 $\pm$ 3 μm) from that of

318 freshly washed micro-gel particles. Thus, the shape and size of the micro-gel particles were not

319 affected by freeze-drying which is based on the rapid sublimation of frozen water from the frozen

320 alginate gel particles. Microscopic pictures of unwashed, washed and rehydrated freeze-dried micro-

321 gel particles are presented in Figure 3 (A, B & C). Freeze-drying helped to create a porous gel

322 structure without significant collapse of primary micro-gel particles which recovered the original

323 shape and size when rehydrated. A similar result has been reported by Smrdel, Bogataj and Mrhar

324 (2008) for freeze-dried alginate particles. Furthermore, it has been reported that freeze drying of a

325 hydrocolloid gel produces stable solid cellular structures. The porous nature of such cellular

326 structures has enabled its use as carrier materials for drugs and other bioactive compounds enabling

327 their controlled release (Nussinovitch, A., 2005).

328 Figure 3 (A, B & C).

329 **3.3. *In-vitro* digestion of encapsulated Lf**

330 *3.3.1. Simulated gastric digestion*

331 The SDS-PAGE gel of apo-, native- and holo-Lf after 2 h *in-vitro* digestion in SGF (180 U

332 pepsin/mg Lf) is presented in Figure 4 (A) and that of Lf encapsulated in alginate micro-gel

333 particles in Figure 4 (B). In both SDS-PAGE gel (4 A & B), the major band in each lane

334 corresponding   to 75 kDa is the Lf. The lanes T0 represent the control samples, pure Lf at time 0 in

335 the SDS-PAGE gel (4 A) and encapsulated Lf at time 0 in SDS-PAGE gel (4 B). Their

336 corresponding amounts based on densitiometric analysis of the 75 kDa bands are taken as 100% to

337 normalize the relative amount of Lf in other lanes. These lanes showing several minor bands at

338 lower molecular mass could be due to the breakdown of Lf in the reducing conditions during

339 sample preparation for SDS-PAGE gel electrophoresis. *In-vitro* digestion of apo- and native-Lf

340 produced major bands at the vicinity of 50 kDa and 15 kDa whereas holo-Lf produced major bands

341 at 37 kDa but only minor bands at 50 kDa (Figure 4 A). Similar bands were seen but at lower

342 intensity for encapsulated Lf (Figure 4 B). This showed that the action of pepsin on Lf does not

343 always produce fragments of similar molecular mass with different forms of Lf. SDS-PAGE was

344 not able to detect pepsin at the level of concentration used in the experiment.

345 Comparative densitiometric analysis of the Lf and encapsulated Lf are presented in Figure 5.

346 Among the samples of pure Lf, holo-Lf was more resistant towards pepsin digestion as compared to

347 apo- and native-Lf. No significant difference between the values of undigested holo-Lf was noted

348 for different time intervals, even after 2 h in SGF where $96 \pm 0.2\%$ holo-Lf remained intact. Apo-

349 and native-Lf were more prone to pepsin digestion in the initial 30 min, but their concentrations

350 remained the same thereafter in the SGF. The result showed that only $54 \pm 6\%$ of apo- and $57 \pm 6\%$

351 of native-Lf remained after 30 min in the SGF. Almaas, Holm, Langsrud, Flengsrud and Vegarud

352 (2006) also reported similar trend where digestion of Lf from caprine whey by human gastric juice

353 occurred within the initial $22 - 30$ min and with no observable reaction thereafter. These values are

354 in agreement with an *in-vivo* digestion study of bovine Lf by Steijns, Brummer, Troost and Saris

355 (2001), where 62% of apo-Lf and 79% of holo-Lf remained after 30 min. Iron saturated Lf has been

356 reported to be more resistant to proteolysis than the corresponding apo-Lf (Brock et al., 1976;

357 Brines, & Brock, 1983). It has been reported that the compact molecular conformation due to the

358 binding of iron to the Lf, reduces its sensitivity to proteolysis (Sánchez et al., 1992).

359 Among the samples of encapsulated Lf, the digestion profile was not significantly different for the

360 different forms of Lf nor for different time intervals in the SGF. The micro-gel particles remained

361 intact throughout the *in-vitro* digestion in SGF for 2 h (Fig. 6 B) and a minimum of $76 \pm 9\%$ of the

362 encapsulated Lf remained undigested. This showed that encapsulating Lf, especially apo- and

363 native-Lf, in alginate micro-gel particles delays the action of pepsin by limiting its access to Lf

364 thereby leading to lower Lf degradation. The intermolecular interactions which occur between Lf

365 and alginate (Peinado, et al., 2010; Bokkhim et al., 2015; David-Birman, Mackie, & Lesmes, 2013)

366 could have played a role in making Lf less available for pepsin degradation. It should be noted that

367 during the gastric digestion, an increase in pH from 2.0 to 3.5 was observed for all types of micro-

368 gel particles. This increase in the pH will cause a lower activity of pepsin. Pepsin activity is

369 maximum at pH $1.5 - 2.5$ (Piper, & Fenton, 1965) and decreases by nearly 40% at pH 3.5

370 (Johnston, Dettmar, Bishwokarma, Lively, & Koufman, 2007). However, even at this reduced

371 activity, there is still a large excess of pepsin present (equivalent to 108 U/mg Lf). Some

372 encapsulated Lf is being digested by pepsin, which is possible as either the peripheral Lf diffuses

373 out of the particles (<0.1% in pH (2.0) adjusted Millipore water in 2 h) and become available to

374 pepsin degradation or pepsin being small in molecular size (~35 Da), can diffuse inside the particles

375 and act on the Lf, though at a slower rate.

376 Figure 4 (A & B).

377 Figure 5.

378 *3.3.2. Simulated intestinal digestion*

379 The *in-vitro* stability profile of the micro-gel particles through microscopic images is shown in

380 Figure 6 (A, B & C). From these images, it can be seen that the micro-gel particles remained intact

381 throughout the *in-vitro* digestion in the SGF for 2 h (Fig. 6 B) whereas the particles disintegrated in

382 the SIF (Fig. 6 C). The presence of phosphate salts and a higher pH (7.5) in the SIF could have led

383 to the dissolution of the micro-gel particles. High pH and the presence of salts (phosphates, sodium

384 etc.) have been attributed to the disintegration of alginate particles leading to burst release of

385 encapsulated proteins, thus exposing it to the proteolytic enzymes (George, & Abraham, 2006; Shi

386 et al., 2005).

387 Figure 6 (A, B & C).

388 Figure 7 (A) and 7 (B) show the SDS-PAGE gel of the different forms of Lf after 1 h *in-vitro*

389 digestion in SIF and of encapsulated Lf during successive *in-vitro* digestion in the SGF for 2 h

390 followed by 1 h in SIF, respectively. In both SDS-PAGE gels (7 A & B), Lf appeared as the major

391 bands in each lane corresponding to 75 kDa. The lanes T0 represent the control Lf samples without

392 any digestion for SDS-PAGE gel (7 A) and encapsulated Lf after 2 h *in-vitro* digestion in SGF for

393 SDS-PAGE gel (7 B). With pure Lf, *in-vitro* digestion of all Lf samples produced major bands at

394 the vicinity of 50 kDa and 37kDa with minor bands spread in-between 20 and 30 kDa. Some intact

395 holo-Lf was still detected after digestion in SIF for 1 h but the amount decreased with time.

396 Furthermore, with holo-Lf, the minor bands within the 20 – 30 kDa region were of higher intensity

397 compared to apo- and native-Lf. Encapsulated Lf also produced similar bands to pure Lf but with

398 additional minor bands below 20 kDa (Figure 7 B). Loading of the pancreatin in the SDS-PAGE

399   gels produced several bands, the most distinct at 50 kDa (amylase & lipase), four minor bands

400   mostly present around 25 kDa (trypsin, ribonuclease & protease) and two very faint bands in-

401   between the 10 – 15 kDa range (SDS-PAGE gel profile image not shown).

402   Comparative digestion profiles of pure Lf and encapsulated Lf by densitometry is given in Figure

403   8. The digestion of all forms of Lf by pancreatin was very rapid and after 10 mins both apo- and

404   native-Lf were completely digested. Holo-Lf was showed some resistance to pancreatin digestion

405   but the amount of holo-Lf remaining after 10 min was very low (< 5%). It has been reported that

406   bile salts aid in the hydrolysis of intact proteins during *in-vitro* duodenal digestion (Martos,

407   Contreras, Molina, & López-Fandiño, 2010). Brock, Arzabe, Lampreave and Piñeiro (1976) have

408   reported that holo-Lf is sensitive to trypsin digestion and that only 6% Lf remained intact after 3 h

409   digestion. The difference in survival time in our study can be attributed to the use of different

410   enzyme combination and protein to enzyme ratio.

411   The digestion pattern for encapsulated Lf in the micro-gel particles was not significantly different

412   from that of the corresponding Lf. This can be attributed to the low stability of the micro-gel

413   particles in SIF where rapid disintegration was observed. This would have caused the Lf to be

414   released into the digest making it prone to the action of pancreatin. Research has shown that intact

415   Lf from Lf-alginate nano-particles, which survived the gastric digestion beyond 40 min, was

416   subsequently digested in the duodenum.

417   Figure 7 (A & B).

418   Figure 8.

419   It has been shown that different concentrations of calcium in the cross-linking solution can give rise

420   to differences in the calcium gradient which is produced during the formation of gel particles. Such

421   different gelling zones affect the homogeneity of the particles (Donati & Paoletti, 2009). To

422   understand the effect of the calcium gradient of the alginate micro-gel particles on the digestibility

423   of encapsulated Lf, *in-vitro* gastric and intestinal digestion was conducted following the same

424   protocol for micro-gel particles but using 0.2 M CaCl$_2$. It was found that higher amounts of Lf was

425   digested by the pepsin during *in-vitro* digestion in the SGF as compared to gel particles produced

426   using 0.1 M CaCl$_2$ (data not shown). The change in porosity of the micro-gel particles could be a

427   contributing factor to this observation as it increases the accessibility of Lf to the action of pepsin

428   along with possibility of higher Lf leaching. The *in-vitro* simulated intestinal digestion profile was

429   similar to that observed for the 0.1 M CaCl$_2$ cross-linked micro-gel particles. The only difference in

430   the behavior of the 0.2 M CaCl$_2$ cross-linked micro-gel particles was an increased time for

431   disintegration in the SIF. Thus, longer time for disintegration of the micro-gel particles did not lead

432   to greater resistance to proteolytic enzymes during *in-vitro* digestion.

433   It was observed that the Lf and encapsulated Lf to some extent was digested in the SGF by pepsin

434   into smaller peptide fragments (seen in the SDS-PAGE gels at the 2 h time-point, Fig. 4 A & B).

435   Furthermore, peptide fragments were formed by pancreatin and were still present after 1 h of SIF

436   treatment (Fig. 7 B). Research has shown that the pepsin hydrolysates, especially lactoferricin and

437   lactoferrampin from Lf possess strong antimicrobial activity (Tomita et al., 2009; van der Kraan et

438   al., 2004). Almaas et al. (2006) reported that the digestion products of pepsin and trypsin of porcine

439   origin still conserve their antibacterial properties, though further degradation could lead to total loss

440   of activity. In addition, research has shown that the iron binding capacity of holo-Lf is unaffected

441   by proteolysis (Sánchez, Calvo, & Brock, 1992) by trypsin and chymotrypsin (Brines & Brock,

442   1983). Wakabayashi, Yamauchi and Takase (2006) has claimed that partially digested bovine Lf

443   peptides retain their biological activities and can exert various physiological effects similar to that

444   of intact Lf. The current study has demonstrated that the encapsulation of Lf in micro-gel particles

445   can delay its hydrolysis by pepsin in SGF, such that it enters the SIF where it encounter further

446   digestion by pancreatin releasing the peptides later in the digestion process as compared to un-

447   encapsulated Lf. The peptides originating from native- and holo-Lf remain in the SIF in

448   considerable amounts for more than 30 min. Further work will be required to confirm that the

449   digestion of encapsulated Lf maintains functional properties (antimicrobial and iron binding ability)

450   as predicted based on the previous work described.

451   **4. Conclusion**

452   The novel impinging aerosol technique (Progel) was successful at producing Lf-alginate micro-gel

453   particles with an encapsulation efficiency of higher than 68%. Calcium concentration of 0.1 M in

454   the cross-linking solution was found to be optimum to encapsulate a 2% mixture of Lf-alginate

455   (1:1) and increasing the calcium ion concentration to 0.2 M led to lower entrapment efficiency of Lf

456   by the micro-gel particles. The micro-gel particles had similar calcium content (except for apo-Lf)

457   regardless of the concentration of calcium in the cross-linking solution. The particle size of the Lf-

458   alginate micro-gel particles were not affected by freeze drying and rehydration. *In-vitro* studies

459   showed that encapsulated Lf (apo- and native-) were more resistant towards the action of pepsin in

460   the SGF as compared to their corresponding pure Lf, but the effect of encapsulation was not

461   significant for holo-Lf. The action of pepsin in SGF on Lf was more pronounced in the initial 30

462   minutes and the Lf concentration remained constant thereafter. The encapsulation of Lf did not

463   provide any significant delay in the digestion of Lf in the SIF. Holo-Lf was more resistant towards

464   the action of pancreatin in SIF, and the amount of intact holo-Lf remaining after the initial 10 min

465   was less than 5%. The findings of this research clearly demonstrate that encapsulation of Lf in

466   alginate micro-gel particles offers protection of apo- and native-Lf from pepsin, the enzyme of the

467   gastric juice. In the presence of salts and high pH, the alginate micro-gel particles dissolve to

468   release the Lf in SIF. Pancreatin partly digested the released Lf in SIF and the peptide fragments

469   produced survived the simulated intestinal condition for more than 30 min.

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Caption for table supplied:

Table 1

Calcium and protein content of freeze-dried micro-gel particlesprepared from 2% Lf-alginate

mixture (1:1) using three forms of Lf and two concentrations of $CaCl_2$ solutions.

Table:

Table 1

Sample name	Protein form	$[Ca^{2+}]$ (M)	Calcium content (mg/g alginate)	Protein content (%)
Alg 1	None	0.1	81 ± 7^B	-
Alg 2	None	0.2	85 ± 9^B	-
MA 1	Apo-Lf	0.1	104 ± 2^A	39.4 ± 0.5^A
MA 2	Apo-Lf	0.2	85 ± 2^B	20 ± 7^B
MN 1	Native-Lf	0.1	92 ± 2^{AB}	48 ± 2^A
MN 2	Native-Lf	0.2	87 ± 1^B	20 ± 1^B
MH 1	Holo-Lf	0.1	82 ± 2^B	48 ± 2^A
MH 2	Holo-Lf	0.2	82 ± 3^B	12.9 ± 0.3^B

Mean values of calcium content and protein content (vertical columns) that do not share a letter are significantly different at $P < 0.05$.

Captions for Figures supplied:

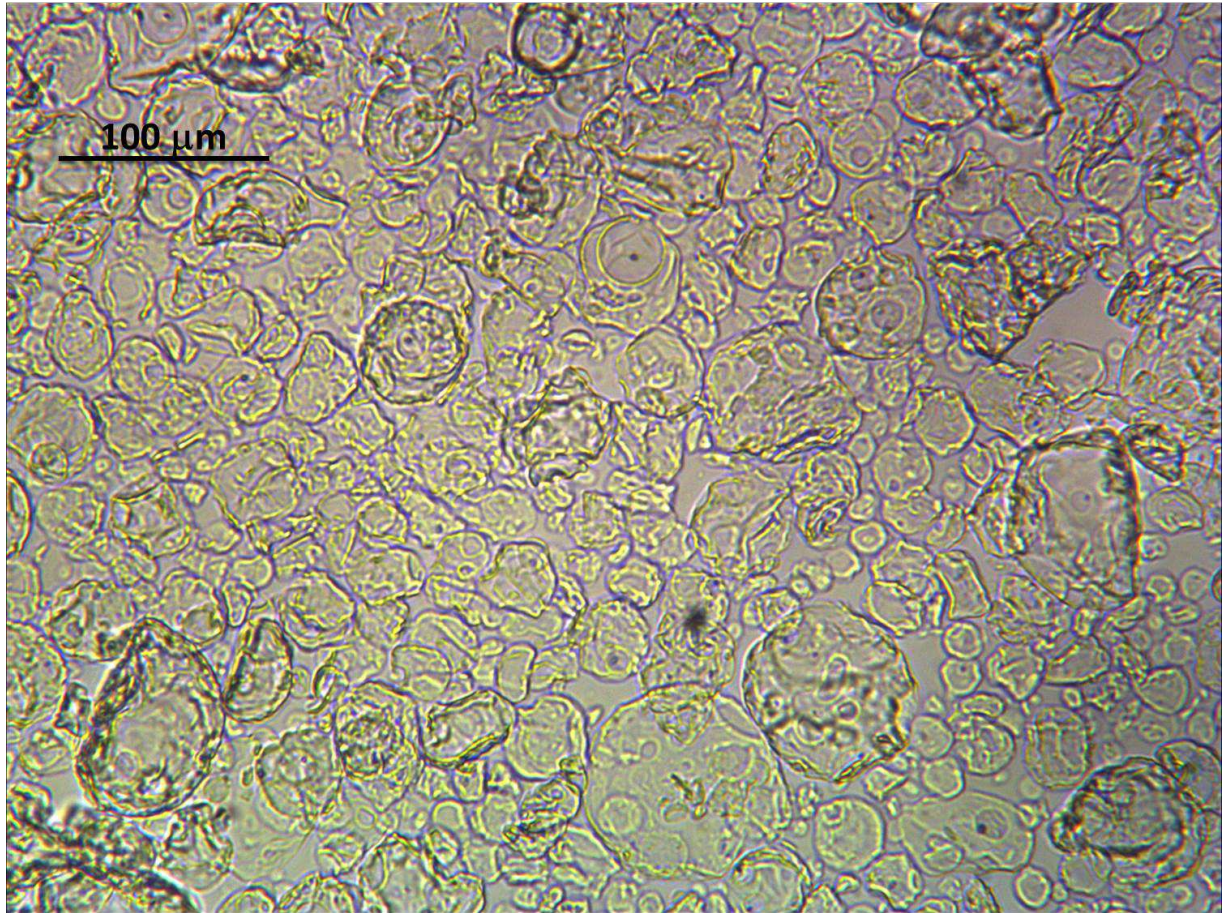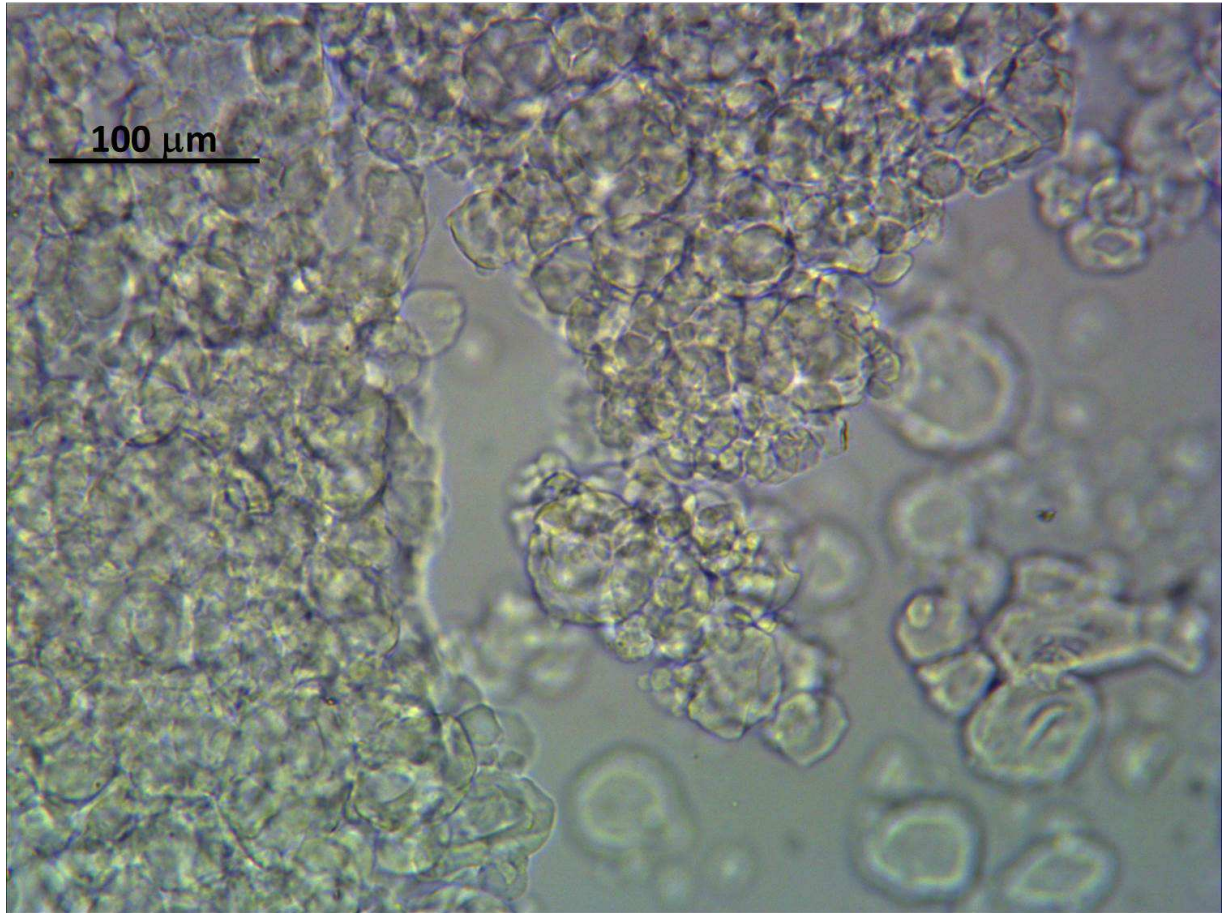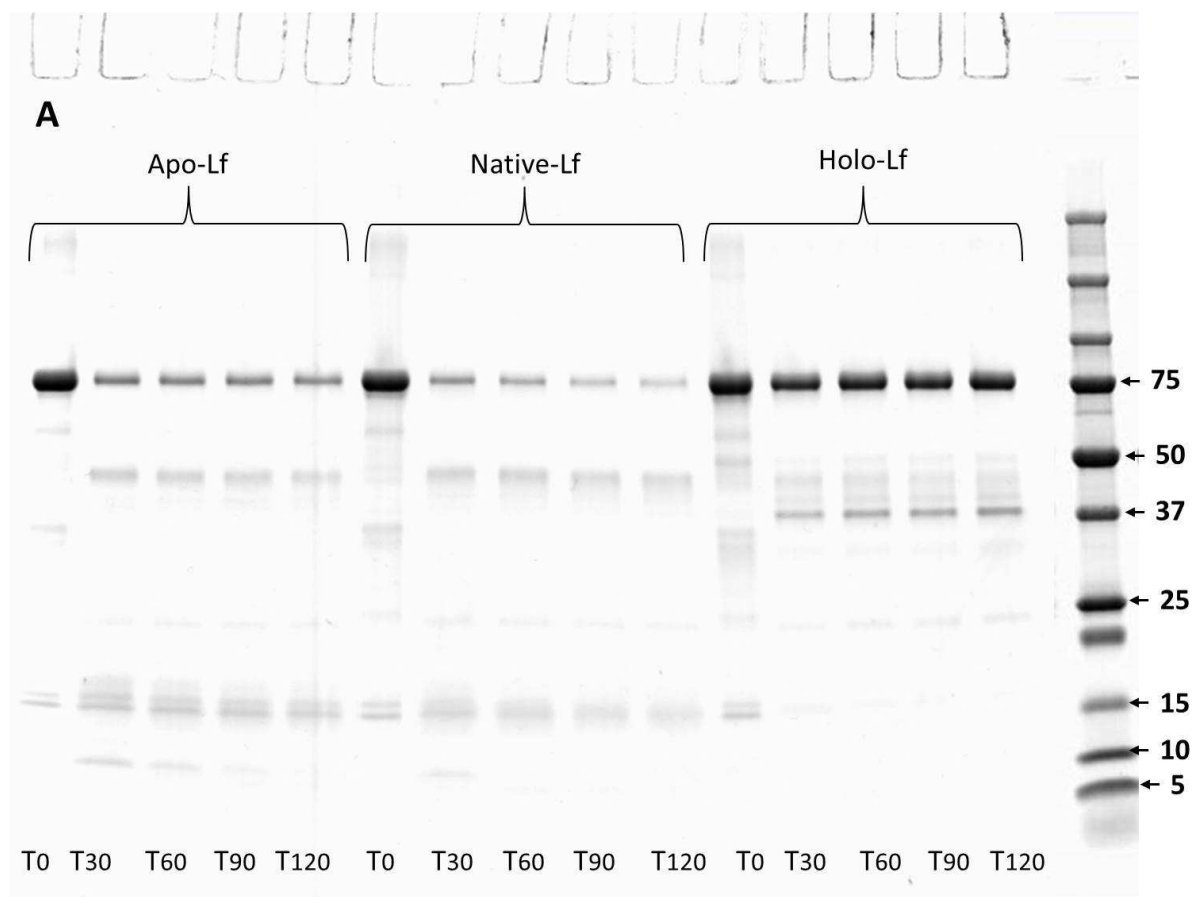| Figure	Caption	Remarks/Format
Fig. 1	Schematic diagram showing the novel Impinging aerosol apparatus (Adapted from Ching et al., 2015).	TIFF
Fig. 2	Pictures of freeze-dried Lf and Lf-alginate micro-gel particles (Top row: Three forms of Lf; middle row: 2% Lf-alginate micro-gel particles formed using 0.1 M $CaCl_2$; bottom row: 2% Lf-alginate micro-gel particles formed using 0.2 M $CaCl_2$). M denotes the Lf-alginate mixture and A, N & H represent apo-, native- &holo-Lf, respectively. 1 & 2 indicate the calcium chloride concentration used in the cross-linking solution, 0.1M and 0.2M, respectively).	JPEG
Fig. 3	Microscopic pictures of MN 1 gel particles A) As prepared, B) Washed & C) Freeze-dried & rehydrated in Millipore water.	JPEG
Fig. 4	SDS-PAGE profile of Lf after 2 h *in-vitro* digestion in SGF of (A) apo-, native- and holo-Lf and (B) Lf from micro-gel particles MA 1, MN 1 & MH 1 at different time intervals (T in min). T0 represents the control sample in each group. The last lane is in each gel is the molecular marker (kDa).	JPEG
Fig. 5	Digestion profile of Lf (band at 75 kDa) based on densitiometric values after *in-vitro* digestion in SGF for 2 h. The bars across groups that do not share a letter is significantly different at $P < 0.05$ (n = 2).	TIFF
Fig. 6	Microscopic pictures of freeze-dried micro-gel particles (MN 1) during *in-vitro* digestion at (A) Time 0 (SGF), (B) Time 2 h (SGF) & (C) Time 4 h (2 h in SGF + 2 h in SIF).	JPEG
Fig. 7	SDS-PAGE profile of Lf after *in-vitro* digestion of (A) apo-, native- and holo-Lf in SIF for 1 h and (B) Lf from micro-gel particles MA 1, MN 1 & MH 1 in SGF for 2 h and subsequent digestion in SIF for 1 h at different time intervals (T in min). T0 represents the control sample in each group. The last lane in each gel is the molecular marker (kDa).	JPEG
Fig. 8	Digestion profile of Lf based on densitiometric values after *in-vitro* digestion in SIF. For pure Lf, digestion was done in SIF for 1 hr and for the micro-gel particles MA 1, MN 1 & MH 1, digestion was done in SGF for 2 h with subsequent digestion in SIF for 1 h. The bars across groups that do not share a letter is significantly different at $P < 0.05$ (n = 2).	TIFF

Sodium alginate + core material

Compressed air

Atomising nozzles

Encapsulation chamber

Compressed air

Final product (Calcium alginate beads)

Calcium chloride feed

A

Apo-Lf   Native-Lf   Holo-Lf

75
50
37
25
15
10
5

T0 T30 T60 T90 T120   T0 T30 T60 T90 T120   T0 T30 T60 T90 T120

B

MA 1　　　　MN 1　　　　MH 1

← 75

← 50

← 37
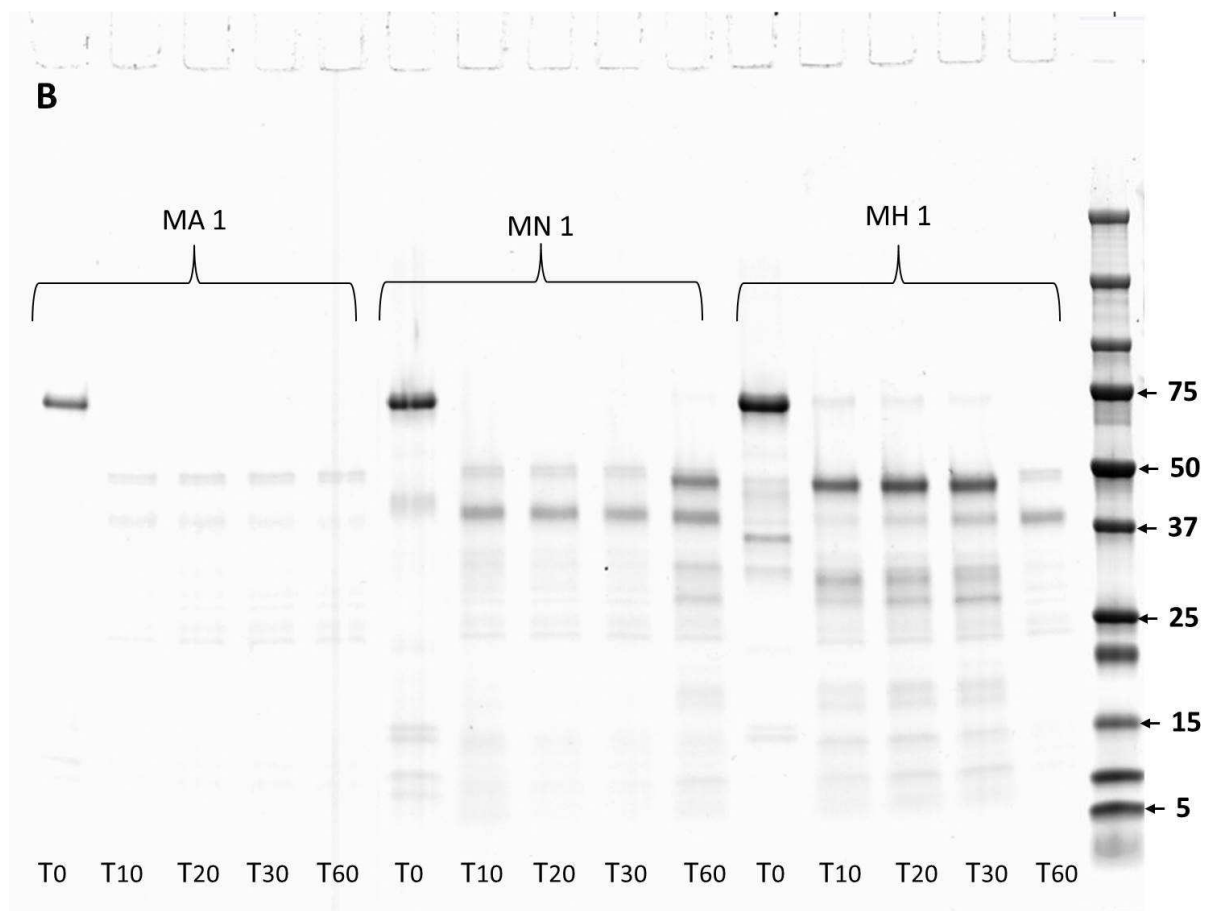
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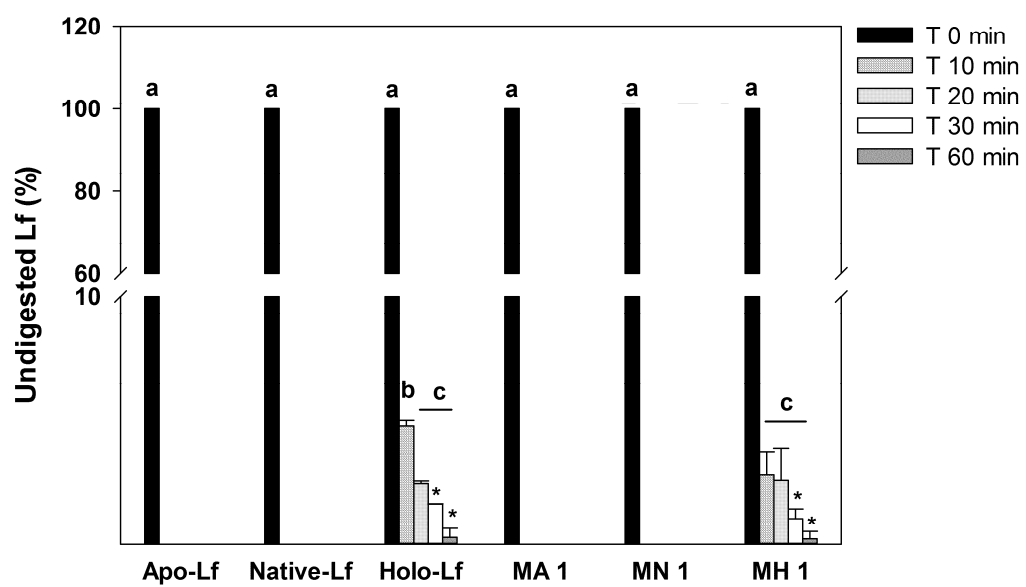← 15

← 5

T0　T30　T60　T90　T120　T0　T30　T60　T90　T120　T0　T30　T60　T90　T120
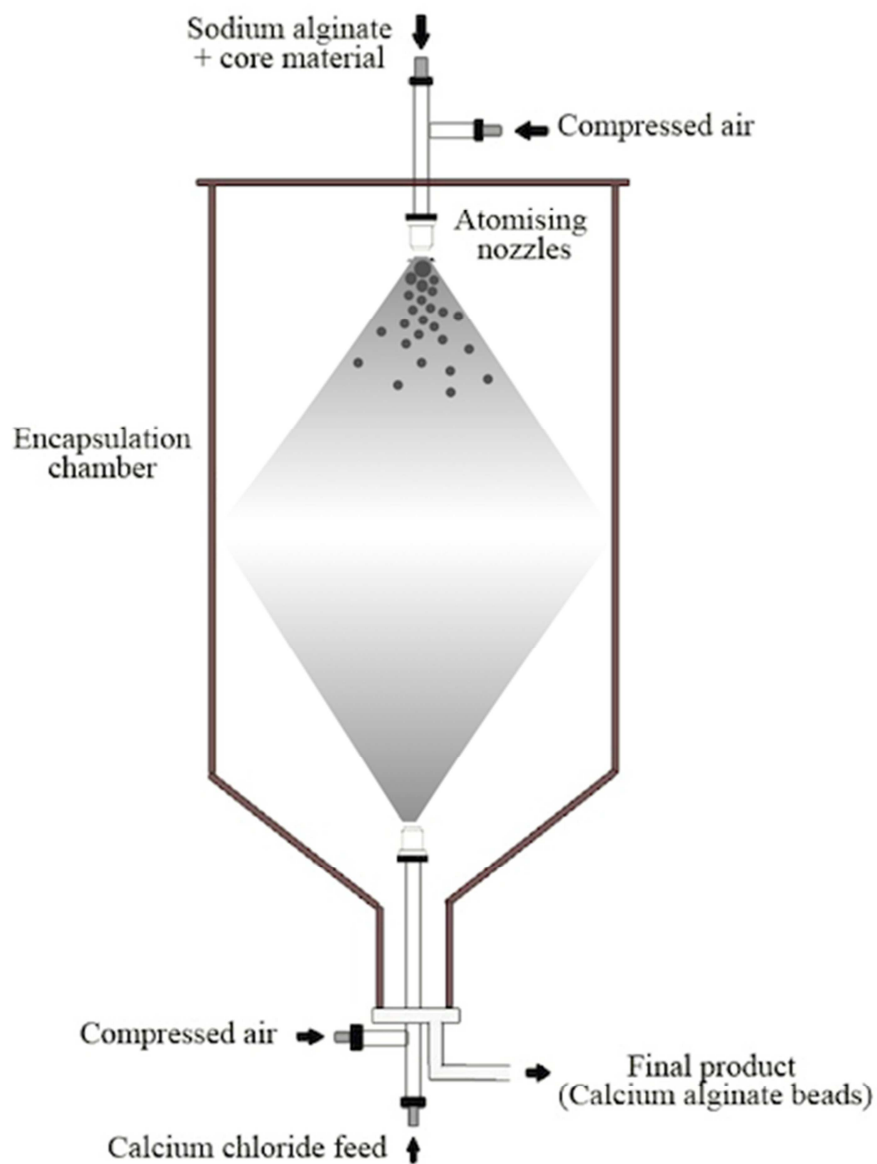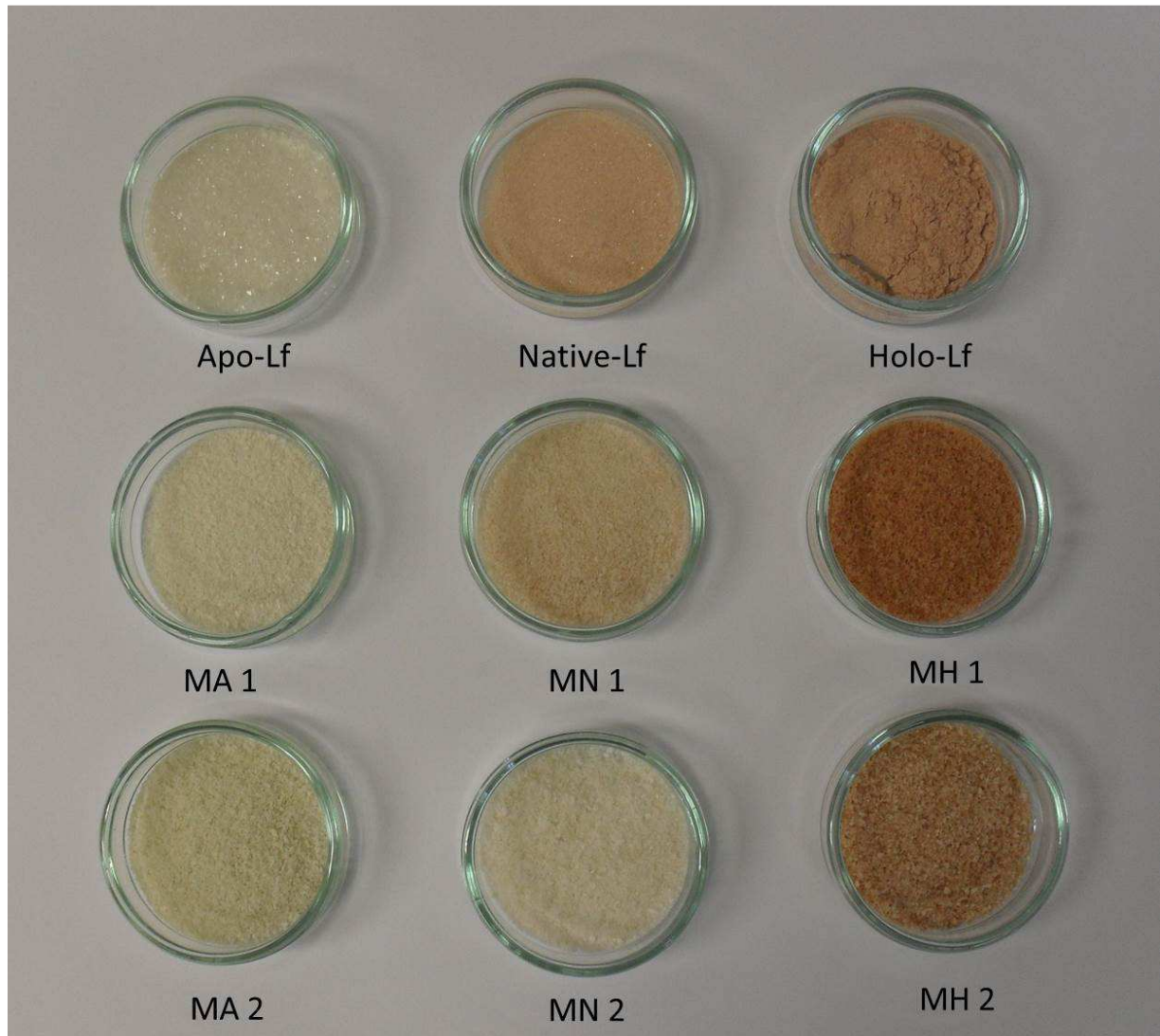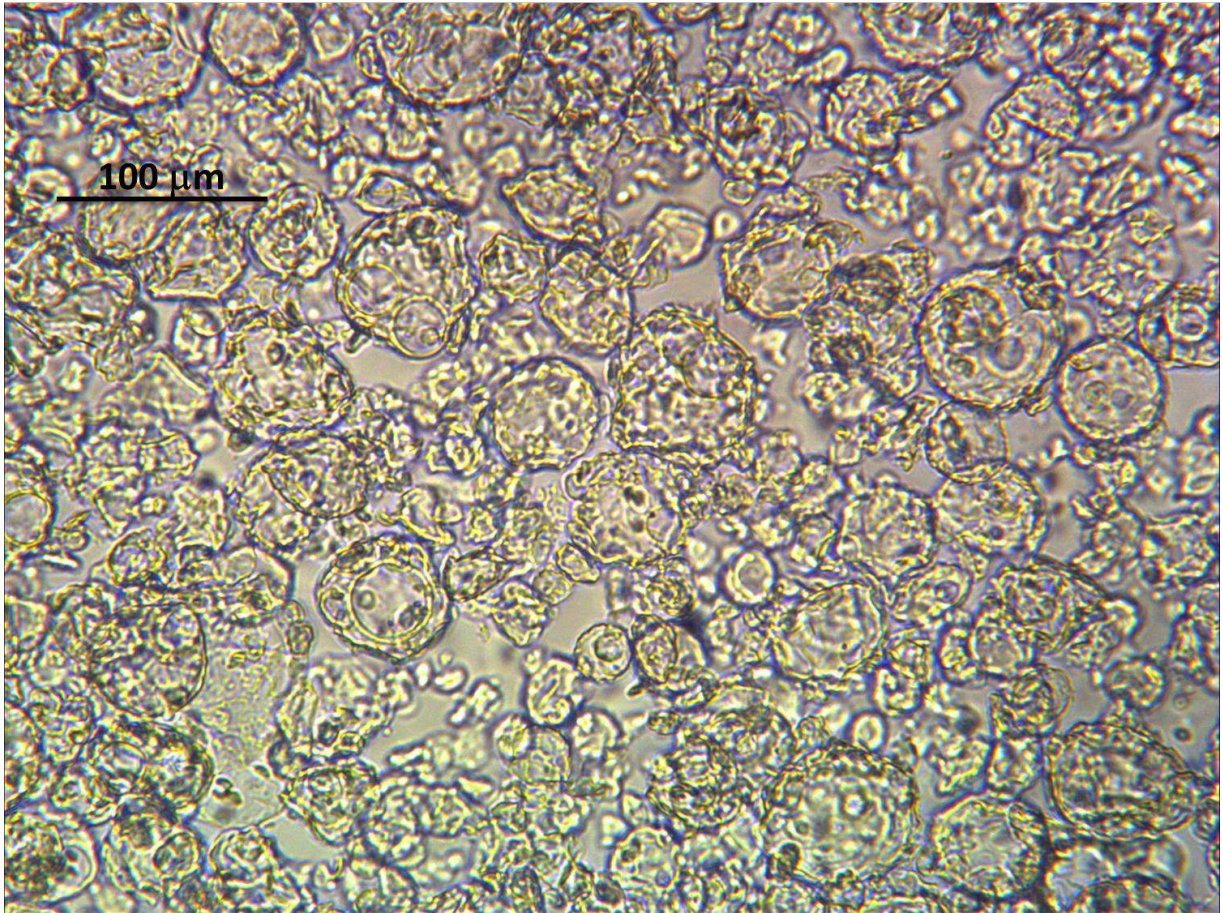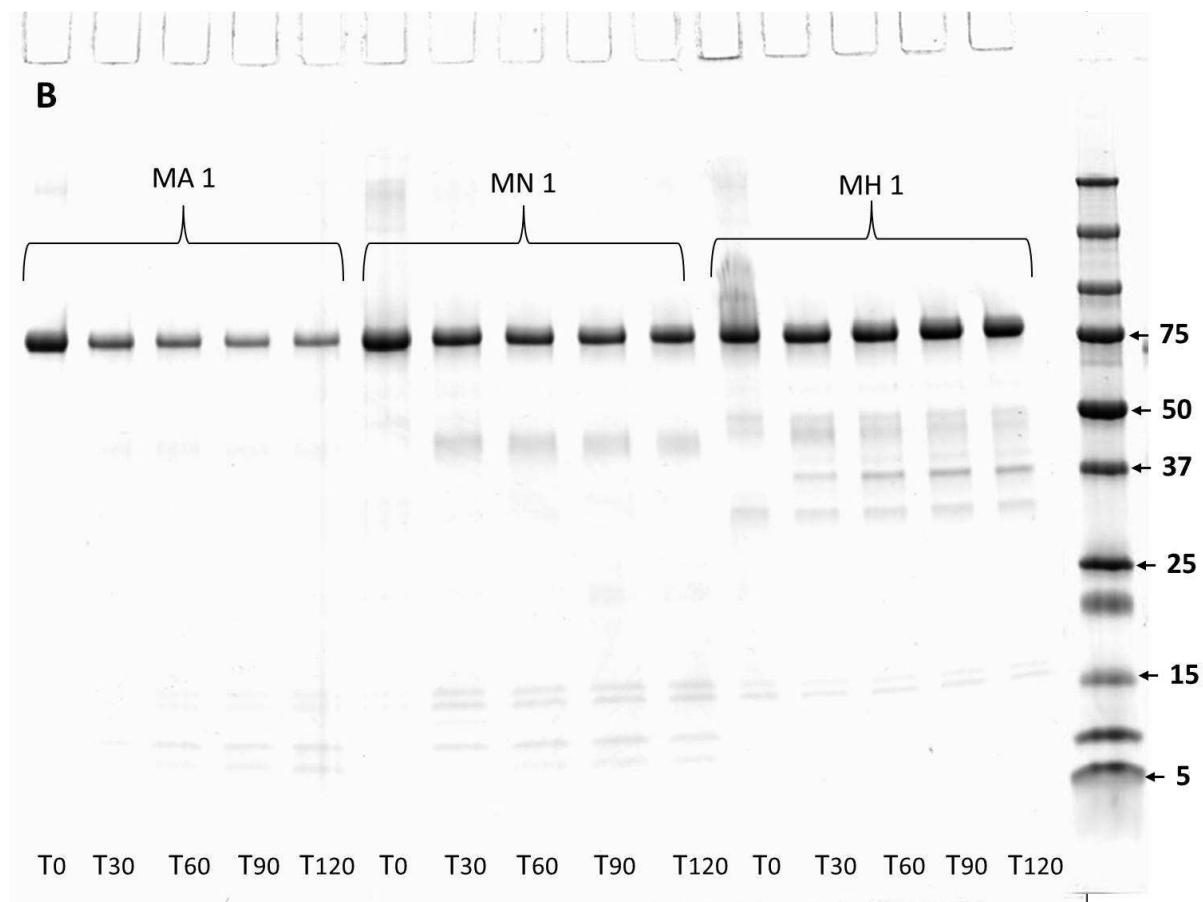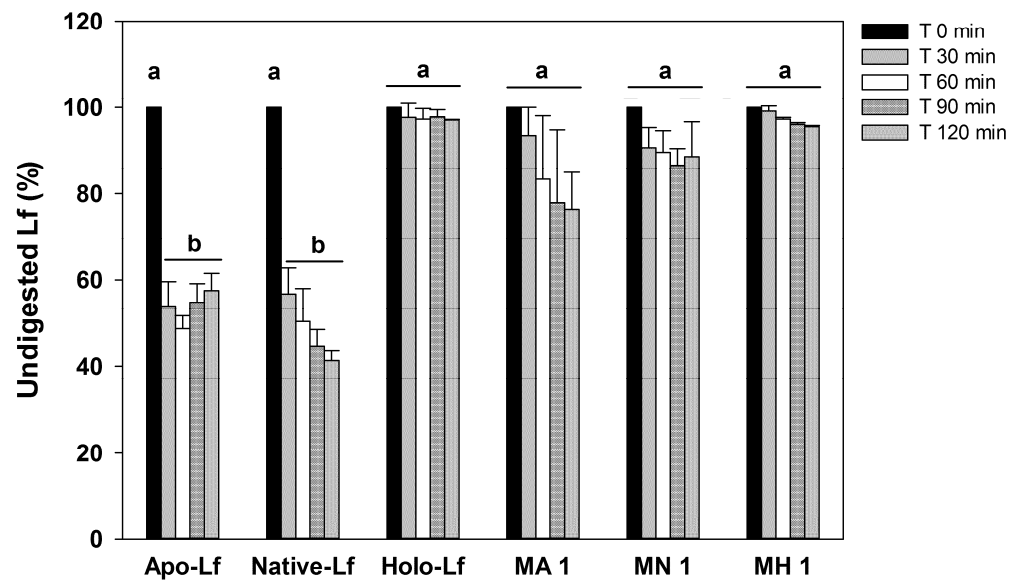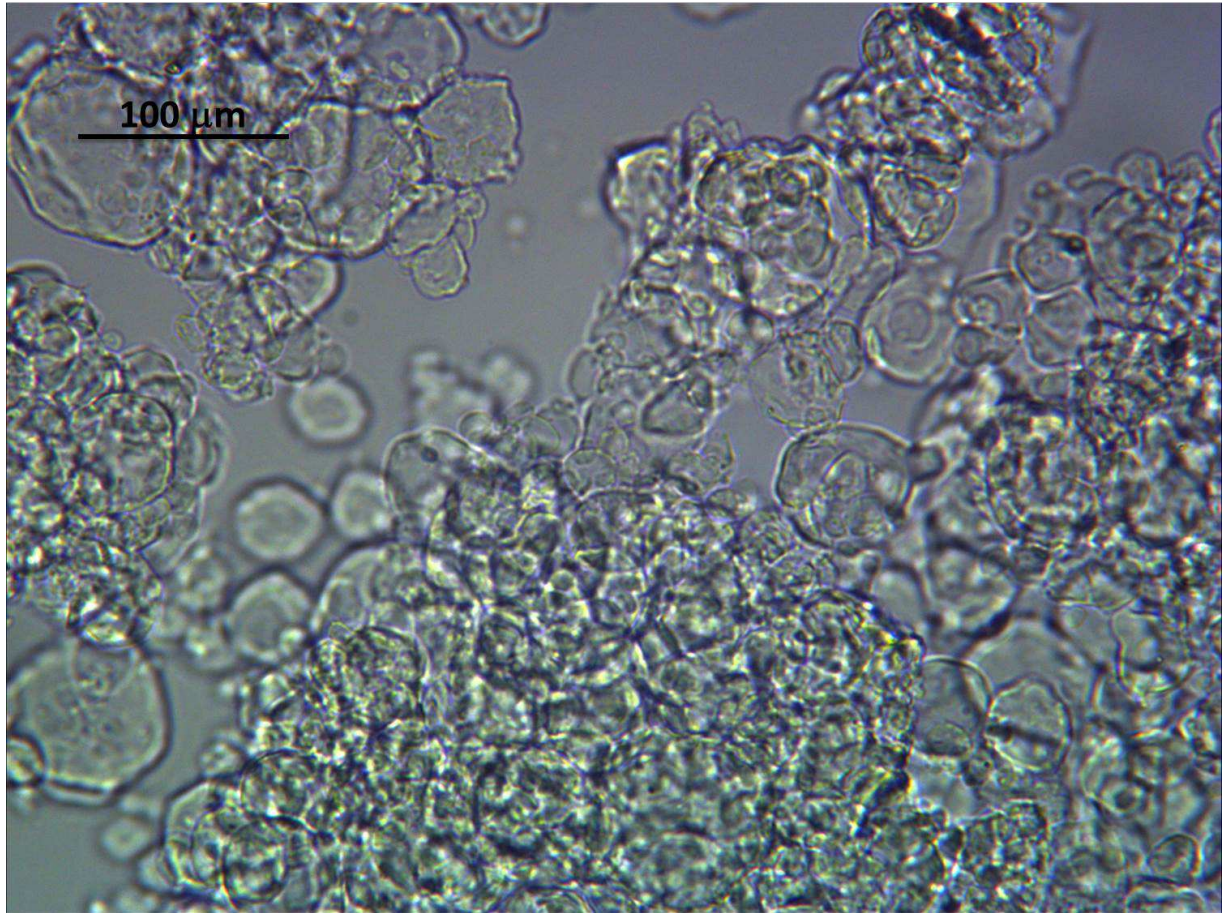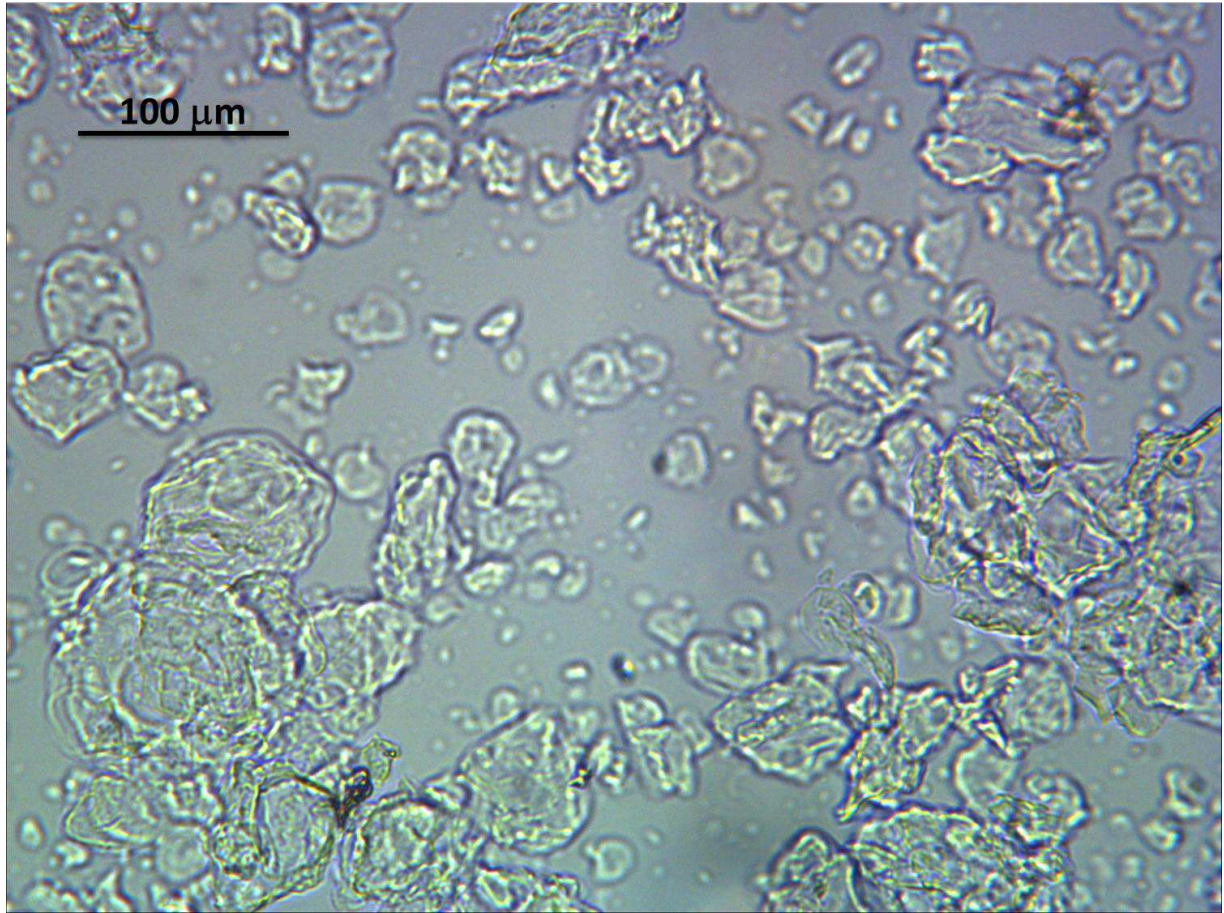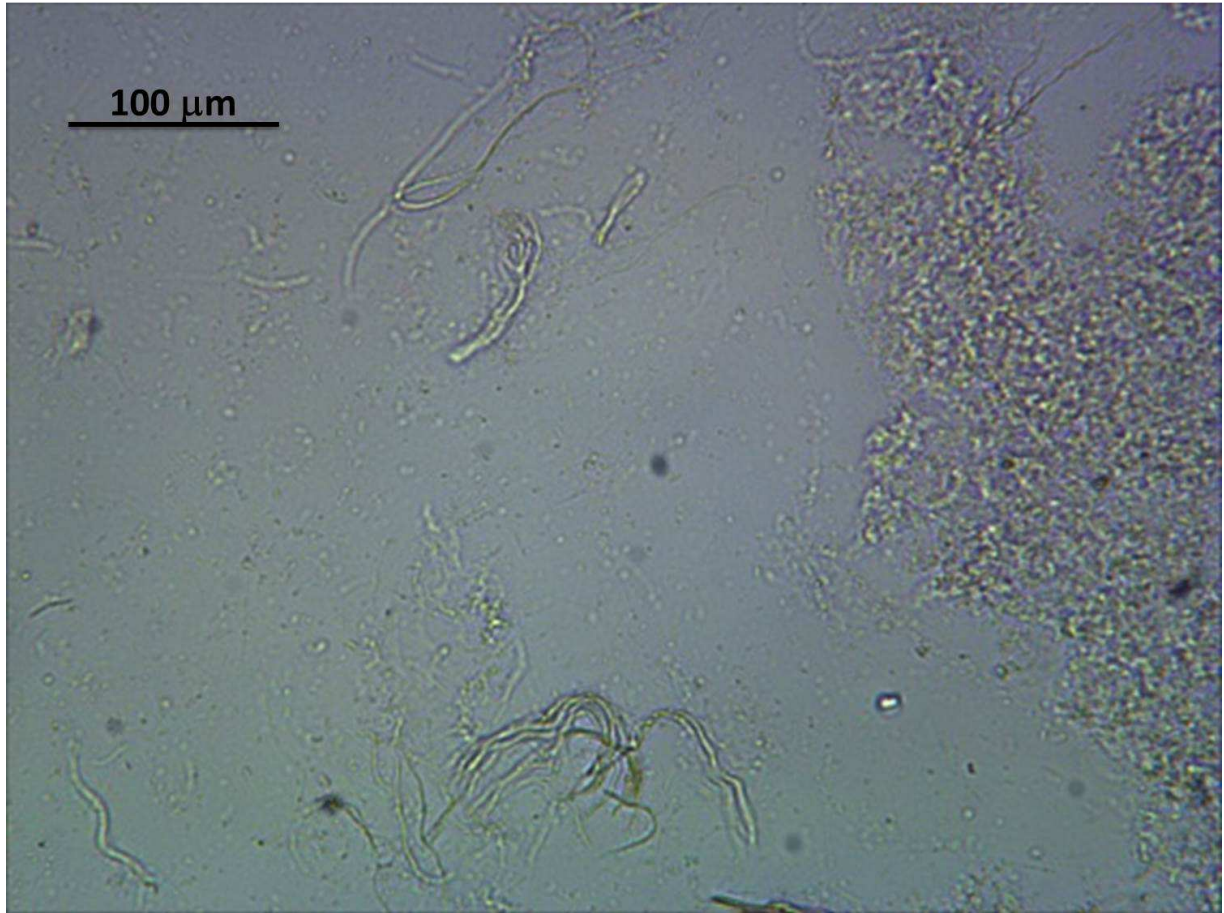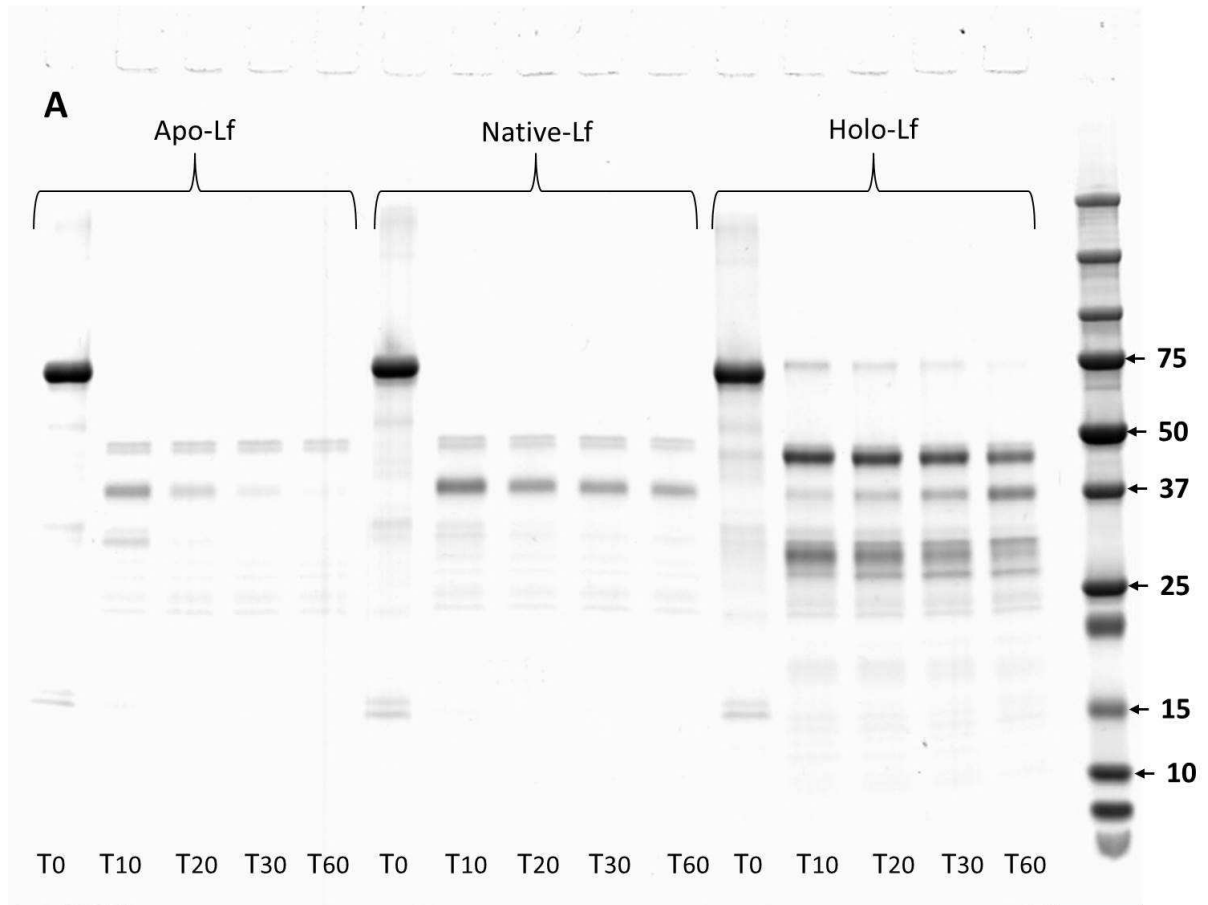
Highlights:

1. Resistance to enzymes and acid in the environment is affected by the forms of Lactoferrin (Lf).

2. Encapsulating Lf in alginate micro-gel particles can provide protection from enzymatic and acidic action.

3. Encapsulated Lf is released in the simulated intestinal fluid by dissolution of the micro-gel particles.

4. Holo-Lf is resistant to the action of enzyme and acid without encapsulation.