Encapsulation of Bifidobacterium longum in alginate-dairy matrices and survival in simulated gastrointestinal conditions, refrigeration, cow milk and goat milk

Article

Accepted Version

It is advisable to refer to the publisher's version if you intend to cite from the work. See Guidance on citing.

www.reading.ac.uk/centaur

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

1 **Encapsulation of *Bifidobacterium longum* in alginate-dairy matrices and**

2 **survival in simulated gastrointestinal conditions, refrigeration, cow milk and**

3 **goat milk**

4

5 **Running title: Encapsulation of bifidobacteria in alginate-dairy based**

6 **matrices**

7 P.H.P. Prasanna [a, b*], D. Charalampopoulos [a]

8 [a] Department of Food and Nutritional Sciences, University of Reading, Whiteknights,

9 Reading, RG6 6AP, UK

10 [b] Department of Animal and Food Sciences, Faculty of Agriculture, Rajarata University of

11 Sri Lanka, Puliyankulama, Anuradhapura, Sri Lanka

12

13 [*]Corresponding author:

14 P.H.P. Prasanna

15 Department of Animal and Food Sciences, Faculty of Agriculture, Rajarata University of Sri

16 Lanka, Puliyankulama, Anuradhapura, Sri Lanka.

17 Tele:0094714458656

18 Fax:0094252221614

19 Email:phpprasanna@yahoo.com

20

21

22

23

Abstract

The aim of this study was to microencapsulate *Bifidobacterium longum* subsp. *infantis* CCUG 52486 using the extrusion method in a variety of matrices, namely sodium alginate (SA), sodium alginate-cow milk (SACM), sodium alginate-goat milk (SAGM) and sodium alginate-casein hydrolysate (SACH), and to evaluate the survival of free and encapsulated bacterial cells under different conditions. The encapsulation yield, size and surface morphology of the microcapsules were evaluated. The survival of microencapsulated bacterial cells and free bacterial cells were evaluated under simulated gastrointestinal conditions as well as in refrigeration, cow milk and goat milk during storage at 4 °C for 28 days. The average size of SACM capsules and SAGM capsules was 2.8±0.3 mm and 3.1±0.2 mm respectively. Goat milk and cow milk based matrices resulted in dense microcapsules which led to better performances in simulated gastrointestinal conditions than SA and SACH microcapsules. The bacterial cells encapsulated in SAGM showed the highest survival rate in cow milk (7.61 log cfu g^{-1}) and goat milk (8.10 log cfu g^{-1}) after the storage of 28 d. The cells encapsulated in SA and SACH and the free cells performed poorly under the simulated gastrointestinal conditions and in all different storage conditions. This study showed that SACM and SAGM are suitable to encapsulate *B. longum* subsp. *infantis* CCUG 52486 using the extrusion technique and more specifically, SAGM has a potential to be used as a new encapsulation material for encapsulating probiotic bacteria, resulting milk and goat milk-based products with higher probiotic cell concentrations during refrigerated storage.

Keywords: Encapsulation; *Bifidobacterium*; Cow milk; Goat milk; Survival, Refrigeration

1. Introduction

Bifidobacteria are a major group of probiotic microorganisms, which have been widely researched for their probiotic properties. Bifidobacteria are considered to exert many beneficial effects to the human host such as alleviation of lactose intolerance, reduction of serum cholesterol levels, synthesis of some vitamins, prevention of colonization of pathogens, modulation of the immune system, reduction of symptoms of irritable bowel disease, and prevention of diarrhoea (Shah, 2007; Xiao et al., 2003). They have been shown to be suitable for incorporation as a co-starter in different food products including dairy-based food formulations (Bunesova et al., 2015; Prasanna et al., 2014). The therapeutic concentration of probiotic bacteria in a product should be around 6 log CFU g−1 until the end of their shelf life (Donkor et al., 2006). In addition, bifidobacteria must endure the high acidic condition in the stomach and hydrolytic enzymes and bile salts in the small intestine prior to reaching the colon in large quantities, which is essential for effective permanent or transient colonization of bacteria (Song et al., 2013). Furthermore, most strains of bifidobacteria show poor growth and viability in milk and fermented milk products (Ranadheera et al., 2014).

In this context, microencapsulation has been widely researched to create a physical barrier protecting the bacteria from adverse conditions during production processes and digestion (Fritzen-Freire et al., 2012). There are many microencapsulation techniques which have been used with probiotics such as emulsion, extrusion, spray drying, freeze drying, coacervation, fluidized bed coating and phase separation (Rajam et al., 2012). Most of these techniques involve harsh processing conditions, which directly affect the viability and the performances of the encapsulated probiotic bacteria. However, the extrusion method involves mild conditions during probiotic encapsulation (Shi et al., 2013a). In this method, a hydrocolloid solution containing concentrated probiotic bacteria is dropped into a solidifying solution. Sodium alginate obtained from brown seaweed has been widely researched as an encapsulation material for probiotics. However, alginate cannot protect effectively probiotic bacteria from the highly acidic environment due to the porous structure of alginate beads, which supports the easy diffusion of acid and other materials inside (Rajam et al., 2012). Therefore, it is recommended to blend or coat alginate with other filler materials to overcome the above-mentioned disadvantages (Cook et al., 2013).

79 Many studies have reported the effectiveness of different alginate based matrices for
80 microencapsulation of probiotic, such as alginate-starch (Sultana et al., 2000), alginate-
81 chitosan (Chávarri et al., 2010; Krasaekoopt et al., 2004), alginate-gelatin (Li et al., 2009),
82 alginate-pectin (Sandoval-Castilla et al., 2010) and alginate-whey protein (Gbassi et al., 2009).
83 In addition, there has been a considerable interest in using dairy-based matrices to encapsulate
84 probiotic bacteria, since these materials contain lactose and proteins which can provide good
85 protection for cells during the handling and digestion process (Maciel et al., 2014). Milk and
86 milk proteins are used in many food formulations and are widely accepted by consumers due
87 to unique physicochemical properties. In the context of encapsulation, milk and milk proteins
88 have technological properties such as high buffering capacity, good emulsification properties
89 and the ability to make networks, even at low concentration (Würth et al., 2015). In addition,
90 it is reported that microcapsules containing dairy proteins can lead to higher bacterial survival
91 during digestion (Burgain et al., 2014). Furthermore, usage of milk based materials for
92 encapsulation of microorganisms would be suitable to be used in dairy-based food products
93 with improved physicochemical properties (Ranadheera et al., 2016). Therefore, there is a high
94 potential to use different milk types and milk based proteins with alginate to encapsulate,
95 protect and control the release of probiotic bacteria in the digestive tract (Özer et al., 2009;
96 Ranadheera et al., 2015).

97 However, there are few recorded reports on the effect of different alginate-dairy based matrices
98 on encapsulation of bifidobacteria. In addition, to the best of authors' knowledge goat milk has
99 not been used with alginate to encapsulate bifidobacteria using the extrusion technique.
100 Therefore, the aim of this study was to evaluate the survival of *Bifidobacterium longum* subsp.
101 *infantis* CCUG 52486 encapsulated in sodium alginate, sodium alginate-cow milk, sodium
102 alginate-goat milk and sodium alginate-casein hydrolysate in simulated gastrointestinal
103 conditions and during storage in cow milk, goat milk and refrigeration at 4 °C for 28 days. This
104 *Bifidobacterium* strain was selected as in our previous studies, it was shown to produce an
105 exopolysaccharide (EPS) in milk (Prasanna et al., 2012) and to improve the physicochemical
106 and rheological properties of low-fat set yoghurt (Prasanna et al., 2013). In addition, this strain
107 has been characterized as a probiotic strain (Gougoulias et al., 2008) and to have a high a high
108 angiotensin-I-converting enzyme (ACE) inhibitory activity in fermented milk (Gonzalez-
109 Gonzalez et al., 2011)

110

2. Materials and methods

2.1. Bacterial strain and growth conditions

B. longum subsp. *infantis* CCUG 52486 was obtained from the culture collection of the University of Göteborg in Sweden. The cell bank of microorganism was stored at −80 °C in Wilkins-Chalgren (WC) anaerobe broth (Oxoid, Hampshire, UK) containing 15% (v/v) glycerol. The frozen stock was initially propagated in Bifidobacteria Selective Medium (BSM) agar (Sigma-Aldrich, Dorset, UK) under anaerobic conditions at 37 °C for 72 h. Two successive cultures of bacteria were carried out in WC broth (Oxoid, UK) under anaerobic condition at 37 °C for 18 h. Subsequently, a cell aliquot of the preculture (1%, v/v) was used to inoculate 200 mL of WC broth (Oxoid, UK) and incubated at 37 °C for 18 h under anaerobic condition. Bacterial cells were harvested after by centrifugation at 10,000 rpm for 10 min at 4 °C. The pellet was washed with sterile phosphate buffered saline (PBS) (Oxoid, UK) and aseptically resuspended in 10 mL of PBS (Oxoid, UK) to prepare the concentrated cell suspension.

2.2. Encapsulation of B. longum subsp. infantis CCUG 52486

Sterilized cow milk and sterilized goat milk were purchased from a local supermarket. Casein hydrolysate solution (2%, w/v, Sigma-Aldrich, UK) and sodium alginate solution (2%, w/v, low viscosity, Sigma-Aldrich, UK) were sterilized at 121 °C for 15 min. Three different alginate-dairy based microsphere formulations were prepared. They were SACM (sodium alginate/cow milk = 1.5/1, v/v), SAGM (sodium alginate/goat milk = 1.5/1, v/v) and SACH (sodium alginate/casein hydrolysate = 1.5/1, v/v); SA (sodium alginate) was used as the control. Each alginate-based formulation was mixed with the concentrated cell suspension at a ratio of 4:1 (alginate-based mixture solution: the concentrated cell suspension, v/v). In the case of free cells, 10 mL of the concentrated cell suspension was mixed with 40 mL of PBS (Oxoid, UK). The hydrocolloid-cell suspensions were dropped through a 21G needle into sterile 0.1 M CaCl$_2$ (Sigma-Aldrich, UK) under gentle stirring; the dropping height was 10 cm. Microcapsules were allowed to harden for 30 minutes and were then washed with sterile PBS (Oxoid, UK) and stored in sterilized plastic containers at 4 °C. The cell concentration encapsulated in the microcapsules was around 9 log cfu g^{-1}.

142 *2.3. Determination of encapsulation yield and size of alginate–milk microcapsules*

143 The encapsulation yield (EY) was determined using the following equation. EY = (Number of
144 cells released from microcapsules) / (Number of cells added to the respective alginate based
145 microsphere formulation) X 100. The size of different microcapsules was measured using a
146 vernier caliper. For this, 30 microcapsules were randomly selected from each microsphere
147 formulation to calculate the mean size.

148

149 *2.4. Determination of viability of free and encapsulated bacteria*

150 Samples of free *B. longum* subsp. *infantis* cells were serially diluted in PBS (Oxoid, UK) and
151 100 µL aliquots were plated on BSM agar (Sigma-Aldrich, UK) to enumerate the viable
152 bacterial counts. The plates were incubated under anaerobic conditions at 37 °C for 72 h. In
153 the case of encapsulated bacteria, the samples were completely dissolved in sterilized 50 mM
154 sodium citrate (Sigma-Aldrich, UK) solution at pH 7.5 before plating as described by Shi et al.
155 (2013a). For this, 1 g of the encapsulated bacteria was dissolved in 9 mL sodium citrate and
156 the samples were serially diluted in PBS (Oxoid, UK). Aliquots of 100 µL of the serially diluted
157 sample were plated on BSM agar (Sigma-Aldrich, UK) and after incubation, the viable cell
158 counts were enumerated.

159

160 *2.5. Survival of free and encapsulated bacteria in simulated gastrointestinal conditions*

161 Simulated gastric juice (SGJ) was prepared by dissolving 0.2% NaCl (w/v) in 0.08 M HCl, at
162 pH 2 as described by Sun and Griffiths (2000). The microcapsules (1 g) or the free cells (1 mL)
163 were added to glass tubes containing 9 mL of sterilized SGJ and placed in a water bath at 37
164 °C. Samples were taken at 0, 30, 60 and 120 min, during incubation. For the free cells, the
165 samples were taken and centrifuged at 10,000 rpm for 10 min, at 4 °C. The pellet was dissolved
166 in PBS (Oxoid, UK) and used for cell enumeration. In the case of microencapsulated bacterial
167 cells, the microcapsules were separated from the samples and dissolved in sodium citrate (50
168 mM) before plating. For enumeration, all samples were serially diluted in PBS (Oxoid, UK)
169 and viable cells were enumerated as described in Section 2.4.

170

171 Simulated intestinal juice (SIJ) was prepared as described by Chávarri et al. (2010). For this, 3
172 g of bile salt (Sigma-Aldrich, UK) were dissolved in 1 L of intestinal model solution (6.5 g/L

173 NaCl, 0.835 g/L KCl, 0.22 g/L $CaCl_2$ and 1.386 g/L $NaHCO_3$), at pH 7.5. Microcapsules (1 g)

174 or the free cells (1 mL) were added to glass tubes containing 9 mL of sterilized SIJ and placed

175 in a water bath at 37 ^{o}C. The sampling and enumeration of free and encapsulated *B. longum*

176 subsp. *infantis* CCUG 52486 were carried out as described previously.

177

178 *2.6. Survival of free and microencapsulated bacterial cells in refrigeration, cow milk and goat*

179 *milk during refrigerated storage*

180 In the case of refrigerated storage, microcapsules or free cells were stored (1 g for

181 microcapsules/ 1 mL for free cells in each portion) in sterilized centrifuge tubes (15 mL

182 capacity, polypropylene, Fisher Scientific, Loughborough, UK), at 4 ^{o}C for 28 days. In the case

183 of cow milk, 1 mL of the free cells or 1 g of the encapsulated bacteria was mixed with 10 mL

184 of sterilized cow milk in sterilized centrifuge tubes (15 mL capacity, polypropylene, Fisher

185 Scientific, UK). In the case of goat milk, 10 mL of sterilized goat milk in sterilized centrifuge

186 tubes (15 mL capacity, polypropylene, Fisher Scientific, UK) were mixed with 1 mL of the

187 free cells or 1 g of the encapsulated cells. The centrifuge tubes containing free and encapsulated

188 bacteria and inoculated milk samples were stored at 4 ^{o}C for 28 days. Afterwards, the samples

189 were collected on 0, 7, 14, 21 and 28 days and analyzed for the viability of cells as described

190 in Section 2.4.

191

192 *2.7. Scanning electron microscopic (SEM) analysis of surface of microcapsules*

193 The microcapsules were dehydrated sequentially in a series of ethanol solutions (30, 50, 70,

194 80, 90, and 100%). For this, the microcapsules were soaked for 15 minutes in each solution.

195 The dehydrated microcapsules were critical point dried using a critical point dryer (Balzers

196 CPD 030, Liechtenstein, Germany) with liquid carbon dioxide. The dried samples were fixed

197 to the SEM stubs with double-sided tape. Afterward, the microcapsules were gold coated using

198 an Edwards S150B sputter-coater for 2.5 min (Edwards, West Sussex, UK). The surface of

199 coated microcapsules was examined using a scanning electron microscope (FEI, Quanta 600

200 F, USA).

201

202 *2.8. Statistical analysis*

203 All the experiments were conducted in triplicate. Results of the size of microcapsules and

204 encapsulation efficiency were analyzed using one-way analysis of variance (ANOVA) with

205 Turkey's multiple comparison tests (SAS, version 9.2, SAS Institute Inc., Cary NC, USA).

206 Results of viable counts from simulated gastrointestinal conditions and from storage studies

207 were analyzed as a split-plot in time design using the General Linear Model (GLM) procedure

208 of SAS, version 9.2 (SAS Institute Inc., Cary NC, USA).

209

210 3. Results and discussion

211 *3.1. Size, encapsulation yield and surface morphology of microcapsules*

212

213 Table *1* shows the size of the different microcapsules. The type of encapsulation material had

214 a significant influence ($p<0.05$) on the size of microcapsules. The largest microcapsules were

215 observed with SAGM while their sizes were not significantly different ($p>0.05$) with those of

216 SACM microcapsules. The smallest microcapsules in this study were observed with SA

217 though, the value was not significantly different with that of SACH. There is no published

218 literature to compare with the size of SAGM microcapsules, which have been prepared using

219 the extrusion technique. Our results showed that the addition of goat milk and cow milk to

220 sodium alginate resulted larger microcapsules than SA and SACH. This may be due to the

221 higher protein content of cow milk and goat milk which, can lead to higher total protein content

222 of SACM and SAGM. Similarly, Klemmer et al. (2011) and Shi et al. (2013a) reported that the

223 higher protein content in matrices could lead to larger microcapsules.

224

225 The type of encapsulating matrices had no significant ($p>0.05$) effect on the encapsulation

226 yield (

Table *1*) and the values ranged from 94.1% to 95.6%. Our results are in accordance with findings of Pan et al. (2013) who reported around 99% of the encapsulation efficiency of bacteria with alginate-skim milk. The results clearly showed that there was a very low loss of cell viability during the encapsulation which was due to the mild conditions used. In general, extrusion method is commonly used with hydrocolloids and reported to yield higher encapsulation yield (Krasaekoopt et al., 2003).

The surface morphology of the microcapsules was investigated using SEM micrographs. Fig.1 shows the surface of different microcapsules at a magnification of 10000. Porous microcapsules were observed with SA [Fig.1 (A)]. Furthermore, SA microcapsules had cracks on their surface and could not protect entrapped cells from adverse environmental conditions. Similarly, Li et al. (2009) reported porous structure for microcapsules produced using alginate. Modification of alginate with cow milk and goat milk resulted in the microcapsules (SACM, SAGM) with denser surface morphology [Fig.1 (B) and (C)]. In addition, these microcapsules did not have cracks that could ensure high protection for encapsulated cells from adverse conditions. SACH microcapsules showed irregular surface morphology [Fig.1 (D)] which could not give better protection for entrapped cells than that of SACM and SAGM microcapsules.

3.2. Survival of free and encapsulated B. longum subsp. infantis CCUG 52486 in simulated gastric juice

Microencapsulation provided a significant protection for the cells in simulated gastric juice (Fig. 2). The viable cell count of free *B. longum* subsp. *infantis* CCUG decreased significantly ($p<0.05$) within 90 min of the incubation period and the cell count of free cells dropped to an undetectable level ($< 10^1$ cfu mL^{-1}) after 120 min. This is because bifidobacteria are fastidious organisms which are sensitive to acidic environment leading to challenges in industrial applications. Similarly, Lee and Heo (2000) observed a rapid reduction of the cell viability of free *B. longum* KCTC 3128 within 30 min when exposed to a simulated gastric environment. The present study also demonstrated that sodium alginate itself could not protect *B. longum* subsp. *infantis* CCUG from the highly acidic environment for a long time. Alginate is a copolymer and composed of D-mannuronic and L-guluronic acids. This copolymer is not stable at low pH condition (Liserre et al., 2007). Dissolution and erosion of alginate occur at low pH

259 and lead for destruction of capsule structure. Our results are in accordance with findings of
260 Krasaekoopt et al. (2004) and who reported poor viability of bacterial cells microencapsulated
261 with alginate in simulated gastric juice. The results clearly showed that microencapsulation
262 with SACM and SAGM gave a better protection for the cells than SA and SACH. The viable
263 cell counts of SACM and SAGM microcapsules were 6.37 log cfu g^{-1} and 5.19 log cfu g^{-1}
264 respectively, after 120 min. The better protection observed in microencapsulated bacterial cells
265 by cow milk and goat milk based matrices may be due to the high buffering capacity of milk
266 proteins. In addition, milk proteins can interact with alginate and act as filling materials which
267 can seal the porous structure of alginate-milk based microcapsules (Kailasapathy, 2006). Our
268 results are in accordance with observations made in some other studies. Guérin et al. (2003)
269 reported that the encapsulated bifidobacteria in a mixed gel made of alginate, pectin and whey
270 proteins could survive better in simulated gastric juice at pH 2.5 due to buffering activities of
271 whey proteins.

272

273 *3.3. Survival of free and encapsulated bacterial cells in simulated intestinal juice*

274 The survival of free and encapsulated *B. longum* subsp. *infantis* CCUG 52486 in simulated
275 intestinal juice at 37 $^{\circ}$C for 2 h is presented in Fig. 3. Encapsulation gave a significant ($p<0.05$)
276 protection for bacterial cells in simulated intestinal juice. The viable count of free cells showed
277 a significant ($p<0.05$) decrease within 120 min. This may be due to the interaction of bile salt
278 with the free cells leading to lose of cell wall integrity. The loss of cell wall integrity may lead
279 to leakage of intercellular materials from the cells leading for death of cells (Bron et al., 2004).
280 Similarly, Clark and Martin (1994) reported a rapid decrease of the viability of free cells of *B.*
281 *adolescentis* in 2% bile salt solution at 37 $^{\circ}$C.

282

283 Milk based microcapsules (SACM and SAGM) were the most effective in protecting the cells
284 in simulated intestinal juice. It is due to milk ingredients, which can modify the textural
285 properties of alginate-milk based matrices [Fig.1 (B) and (C)], as the modified matrices resist
286 the diffusion of bile salt into the microcapsules. Similarly, alginate-milk based matrices were
287 shown to be effective in protection of *Lactobacillus bulgaricus* (Shi et al., 2013a; Shi et al.,
288 2013b) and *Enterococcus faecalis* (Shi et al., 2016) in simulated intestinal solution. SA and
289 SACH microcapsules provided a limited protection for bacterial cells during the incubation
290 period. This is due to the poor structure of those matrices [Fig.1 (A) and (D)], which can allow

291   diffusion of bile salt into the microcapsules (Hansen et al., 2002; Lee and Heo, 2000).

292   Similarly, Krasaekoopt et al. (2004) reported poor viability of *B. bifidum* ATCC 1994

293   capsulated in alginate matrices when exposed to bile salt solution.

294

295   *3.4. Stability of free and encapsulated bacteria cells under refrigerated condition*

296   Fig. 4 shows the viability of free and encapsulated *B. longum* subsp. *infantis* CCUG 52486

297   with different alginate-based matrices during the refrigerated storage at 4 $^{\circ}$C. The cell

298   concentration of free *B. longum* subsp. *infantis* CCUG 52486 decreased significantly (*p<0.05*)

299   from 8.96 log cfu g^{-1} to 3.62 log cfu g^{-1}, indicating the inability of the free cells to maintain

300   their viability under the refrigerated storage condition. The results further revealed that

301   encapsulation could improve the viability of bacterial cells during refrigerated storage for 28

302   days. SA and SACH microcapsules showed higher cell viability than that of the free cells

303   during the refrigerated storage. However, they were unable to maintain the viability of cells

304   during the storage above the recommended count of 6 log cfu g^{-1}. Similarly, some studies

305   reported that encapsulation of probiotic bacteria in sodium alginate could improve the storage

306   stability of bacterial cells than that of the free cells (Chávarri et al., 2010; Krasaekoopt et al.,

307   2004).

308

309   SACM and SAGM microcapsules gave better protection for the cells during the refrigerated

310   storage and both materials were able to maintain the cell concentrations above 6 log cfu g^{-1}

311   after 28 days of storage than SA and SACH. However, the final cell counts of these two

312   microcapsules were not significantly different (*p<0.05*). This may be due to the denser surface

313   morphology of alginate-dairy microcapsules [Fig.1 (B) and (C)], which can protect the

314   encapsulated cells from adverse conditions of the environment. Similarly, some other alginate-

315   based microcapsules have been shown to be effective to give better protection for probiotics

316   during the refrigerated storage. Encapsulation of *Lactobacillus gasseri* and *B. bifidum* in

317   chitosan-coated alginate microspheres was shown to be effective to maintain viability

318   throughout the storage period at 4 $^{\circ}$C for 28 days (Chávarri et al., 2010). In addition, Zou et al.

319   (2011) showed that chitosan-coated alginate microspheres provided a better protection for the

320   microencapsulated *B. bifidum* F-35 cells than that of the free cells during the storage at 4 $^{\circ}$C

321   for 1 month.

322

323   *3.5. Survival of free and encapsulated bacterial cells in cow milk and goat milk at 4 °C*

324   Table 2 shows the survival of free and encapsulated stored in cow milk at 4 °C for 28 days.

325   The results indicated that encapsulation improved the survival of bacterial cells in cow milk

326   during storage. The free cells showed poor storage stability in cow milk where the cell

327   concentration was significantly ($p<0.05$) reduced from 8.65 log cfu mL^{-1} to 4.38 log cfu mL^{-1}

328   within 28 days. SAGM microcapsules gave the best protection for the cells followed by SACM

329   microcapsules. However, SA and SACH microcapsules could give a limited protection during

330   the storage in cow milk. Fig. 5 shows the results of free and encapsulated bacterial counts in

331   goat milk during storage at 4 °C for 28 days. There was a significant reduction (*p<0.05*) in the

332   viability of free cells during the storage. However, the results revealed that encapsulation of *B.*

333   *longum* subsp. *infantis* CCUG 52486 improved the survival of bacterial cells in goat milk

334   during the storage period of 28 days. The highest survival of bacterial cells during the storage

335   was observed with SAGM microcapsules followed by SACM microcapsules; where they

336   maintained the viability of bacterial cells above 6 log cfu g^{-1} in goat milk during the storage

337   period. Viable cell counts of SA and SACH microcapsules rapidly declined with the storage.

338

339   Poor viability of free cells in cow milk and goat milk is due to lack of availability of small

340   peptides and free amino acids for their growth (Gomes et al., 1998; Martín-Diana et al., 2003).

341   In this study, pure goat milk and cow milk were used to inoculate bacteria without any

342   supplementation. Similarly, Hansen et al. (2002) observed poor viability of free *B. longum* Bb-

343   46 cells in milk during storage at 4 °C for 16 days than that of encapsulated bacterial cells. The

344   poor survival of bacterial cells encapsulated in SA and SACH is due to the fragile texture of

345   walls of these microcapsules [Fig.1 (A) and (D)], which exposes bacterial cells to the external

346   environment. The high survival rate observed with microencapsulated bacterial cells with

347   SACM and SAGM [Fig.1 (B) and (C)] in cow milk and goat milk may be due to improved

348   denser surface characteristics compared to SA and SACH. The modified structure of SACM

349   and SASM could protect their content form the adverse external environments. There is no

350   recognized published literature about the survival of bifidobacteria encapsulated using

351   alginate-milk based matrices in goat milk during storage to compare with our results. However,

352   some authors have reported that encapsulation can improve the viability of bifidobacteria in

353   cow milk and cow milk-based products. Hansen et al. (2002) showed the effectiveness of

354   alginate microcapsules to improve the viability of *B. longum* Bb-46 in cow milk during the

355   storage at 4 °C for 16 days. In another study, *B. bifidum* encapsulated in alginate beads coated

13

356 with chitosan was shown to have better survival than the free cell in yoghurt after the storage

357 at 4 °C for 4 weeks (Krasaekoopt et al., 2006). In addition Kailasapathy (2006) showed that the

358 alginate-starch encapsulated *B. lactis* had higher survival than the free cells in yoghurt at 4 °C

359 for 7 weeks.

360

361 The present study demonstrates that encapsulation of *B. longum* subsp. *infantis* CCUG 52486

362 in SACM and SAGM microcapsules beneficially influences the viability of bacterial cells in

363 cow milk and goat milk during the storage at 4 °C for 28 days. Therefore, microencapsulation

364 of bifidobacteria with SACM and SAGM could be used to enhance the growth of them in non-

365 fermented cow milk and goat milk based products. Further studies should be carried out to

366 evaluate the effect of encapsulation of bifidobacteria with SACM and SAGM microcapsules

367 in fermented milk-based products and other food systems.

368

369 **4. Conclusions**

370 The mixing of alginate with cow milk and goat milk resulted in microcapsules with denser

371 surface and the cells encapsulated in these matrices performed better in simulated

372 gastrointestinal conditions than the bacterial cells encapsulated in SA and SACH

373 microcapsules. Improved structural characteristics of SACM and SAGM microcapsules could

374 improve survival of encapsulated bacterial cells in cow milk, goat milk and refrigeration at 4

375 °C for 28 days compared to SA and SACH microcapsules. Overall, this study showed that

376 mixing of goat milk and cow milk with alginate improved the protection provided by modified

377 microcapsules and could be used to improve survival of probiotic bacteria in non-fermented

378 cow milk and goat milk based products.

379

384

385 **References**

386 Bron, P. A., Marco, M., Hoffer, S. M., Van Mullekom, E., De Vos, W. M., & Kleerebezem,
387   M. (2004). Genetic characterization of the bile salt response in *Lactobacillus plantarum*
388   and analysis of responsive promoters in vitro and in situ in the gastrointestinal tract.
389   *Journal of Bacteriology, 186*(23), 7829-7835.

390 Bunesova, V., Musilova, S., Geigerova, M., Pechar, R., & Rada, V. (2015). Comparison of
391   mupirocin-based media for selective enumeration of bifidobacteria in probiotic
392   supplements. *Journal of Microbiological Methods, 109*, 106-109.

393 Burgain, J., Scher, J., Lebeer, S., Vanderleyden, J., Cailliez-Grimal, C., Corgneau, M.,
394   Francius, G., & Gaiani, C. (2014). Significance of bacterial surface molecules
395   interactions with milk proteins to enhance microencapsulation of *Lactobacillus*
396   *rhamnosus* GG. *Food Hydrocolloids, 41*, 60-70.

397 Chávarri, M., Marañón, I., Ares, R., Ibáñez, F. C., Marzo, F., & del Carmen Villarán, M.
398   (2010). Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules
399   improves survival in simulated gastro-intestinal conditions. *International Journal of*
400   *Food Microbiology, 142*(1), 185-189.

401 Clark, P. A., & Martin, J. H. X. (1994). Selection of bifidobacteria for use as dietary adjuncts
402   in cultured dairy foods. III. Tolerance to simulated bile concentrations of human small
403   intestines. *Cultured Dairy Products Journal, 29*, 18-21.

404 Cook, M. T., Tzortzis, G., Khutoryanskiy, V. V., & Charalampopoulos, D. (2013). Layer-by-
405   layer coating of alginate matrices with chitosan-alginate for the improved survival and
406   targeted delivery of probiotic bacteria after oral administration. *Journal of Materials*
407   *Chemistry B, 1*(1), 52-60.

408 Donkor, O., Henriksson, A., Vasiljevic, T., & Shah, N. (2006). Effect of acidification on the
409   activity of probiotics in yoghurt during cold storage. *International Dairy Journal,*
410   *16*(10), 1181-1189.

411 Fritzen-Freire, C. B., Prudêncio, E. S., Amboni, R. D. M. C., Pinto, S. S., Negrão-Murakami,
412   A. N., & Murakami, F. S. (2012). Microencapsulation of bifidobacteria by spray drying
413   in the presence of prebiotics. *Food Research International, 45*(1), 306-312.

414   Gbassi, G. K., Vandamme, T., Ennahar, S., & Marchioni, E. (2009). Microencapsulation of
415   *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins.
416   *International Journal of Food Microbiology, 129*(1), 103-105.

417   Gomes, A. M. P., Malcata, F. X., & Klaver, F. A. M. (1998). Growth enhancement of
418   *Bifidobacterium lactis* Bo and *Lactobacillus acidophilus* Ki by milk hydrolyzates.
419   *Journal of Dairy Science, 81*(11), 2817-2825.

420   Gonzalez-Gonzalez, C. R., Tuohy, K. M., & Jauregi, P. (2011). Production of angiotensin-I-
421   converting enzyme (ACE) inhibitory activity in milk fermented with probiotic strains:
422   effects of calcium, pH and peptides on the ACE-inhibitory activity. *International Dairy*
423   *Journal, 21*(9), 615-622.

424   Gougoulias, C., Tuohy, K. M., & Gibson, G. R. (2008). Dietary-based gut flora modulation
425   against *Clostridium dificile* onset. *Food Science and Technology Bulletin: Functional*
426   *Foods, 4*(4), 31-41.

427   Guérin, D., Vuillemard, J. C., & Subirade, M. (2003). Protection of bifidobacteria encapsulated
428   in polysaccharide-protein gel beads against gastric juice and bile. *Journal of Food*
429   *Protection, 66*(11), 2076-2084.

430   Hansen, L. T., Allan-Wojtas, P. M., Jin, Y. L., & Paulson, A. T. (2002). Survival of Ca-alginate
431   microencapsulated *Bifidobacterium* spp. in milk and simulated gastrointestinal
432   conditions. *Food Microbiology, 19*(1), 35-45.

433   Kailasapathy, K. (2006). Survival of free and encapsulated probiotic bacteria and their effect
434   on the sensory properties of yoghurt. *LWT-Food Science and Technology, 39*(10),
435   1221-1227.

436   Klemmer, K. J., Korber, D. R., Low, N. H., & Nickerson, M. T. (2011). Pea protein-based
437   capsules for probiotic and prebiotic delivery. *International Journal of Food Science &*
438   *Technology, 46*(11), 2248-2256.

439   Krasaekoopt, W., Bhandari, B., & Deeth, H. (2003). Evaluation of encapsulation techniques of
440   probiotics for yoghurt. *International Dairy Journal, 13*(1), 3-13.

441   Krasaekoopt, W., Bhandari, B., & Deeth, H. (2004). The influence of coating materials on
442   some properties of alginate beads and survivability of microencapsulated probiotic
443   bacteria. *International Dairy Journal, 14*(8), 737-743.

444   Krasaekoopt, W., Bhandari, B., & Deeth, H. C. (2006). Survival of probiotics encapsulated in
445   chitosan-coated alginate beads in yoghurt from UHT-and conventionally treated milk
446   during storage. *LWT-Food Science and Technology, 39*(2), 177-183.

447   Lee, K. Y., & Heo, T. R. (2000). Survival of *Bifidobacterium longum* immobilized in calcium
448   alginate beads in simulated gastric juices and bile salt solution. *Applied and*
449   *Environmental Microbiology, 66*(2), 869-873.

450   Li, X. Y., Chen, X. G., Cha, D. S., Park, H. J., & Liu, C. S. (2009). Microencapsulation of a
451   probiotic bacteria with alginate-gelatin and its properties. *Journal of*
452   *Microencapsulation, 26*(4), 315-324.

453   Liserre, A. M., Ré, M. I., & Franco, B. D. G. M. (2007). Microencapsulation of
454   *Bifidobacterium animalis* subsp. *lactis* in modified alginate-chitosan beads and
455   evaluation of survival in simulated gastrointestinal conditions. *Food Biotechnology,*
456   *21*(1), 1-16.

457   Maciel, G. M., Chaves, K. S., Grosso, C. R. F., & Gigante, M. L. (2014). Microencapsulation
458   of *Lactobacillus acidophilus* La-5 by spray-drying using sweet whey and skim milk as
459   encapsulating materials. *Journal of Dairy Science, 97*(4), 1991-1998.

460   Martín-Diana, A. B., Janer, C., Peláez, C., & Requena, T. (2003). Development of a fermented
461   goat's milk containing probiotic bacteria. *International Dairy Journal, 13*(10), 827-833.

462   Özer, B., Kirmaci, H. A., Şenel, E., Atamer, M., & Hayaloğlu, A. (2009). Improving the
463   viability of *Bifidobacterium bifidum* BB-12 and *Lactobacillus acidophilus* LA-5 in
464   white-brined cheese by microencapsulation. *International Dairy Journal, 19*(1), 22-29.

465   Pan, L. X., Fang, X. J., Yu, Z., Xin, Y., Liu, X. Y., Shi, L. E., & Tang, Z. X. (2013).
466   Encapsulation in alginate–skim milk microspheres improves viability of *Lactobacillus*
467   *bulgaricus* in stimulated gastrointestinal conditions. *International Journal of Food*
468   *Sciences and Nutrition, 64*(3), 380-384.

469   Prasanna, P. H. P., Grandison, A. S., & Charalampopoulos, D. (2012). Effect of dairy-based
470   protein sources and temperature on growth, acidification and exopolysaccharide
471   production of *Bifidobacterium* strains in skim milk. *Food Research International,*
472   *47*(1), 6-12.

473   Prasanna, P. H. P., Grandison, A. S., & Charalampopoulos, D. (2013). Microbiological,
474   chemical and rheological properties of low fat set yoghurt produced with

exopolysaccharide (EPS) producing *Bifidobacterium* strains. *Food Research International, 51*(1), 15-22.

Prasanna, P. H. P., Grandison, A. S., & Charalampopoulos, D. (2014). Bifidobacteria in milk products: An overview of physiological and biochemical properties, exopolysaccharide production, selection criteria of milk products and health benefits. *Food Research International, 55*, 247-262.

Rajam, R., Karthik, P., Parthasarathi, S., Joseph, G., & Anandharamakrishnan, C. (2012). Effect of whey protein–alginate wall systems on survival of microencapsulated *Lactobacillus plantarum* in simulated gastrointestinal conditions. *Journal of Functional Foods, 4*(4), 891-898.

Ranadheera, C. S., Evans, C. A., Adams, M. C., & Baines, S. K. (2014). Effect of dairy probiotic combinations on *in vitro* gastrointestinal tolerance, intestinal epithelial cell adhesion and cytokine secretion. *Journal of Functional Foods, 8*, 18-25.

Ranadheera, C. S., Evans, C. A., Adams, M. C., & Baines, S. K. (2015). Microencapsulation of *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Propionibacterium jensenii* 702 by spray drying in goat's milk. *Small Ruminant Research, 123*(1), 155-159.

Ranadheera, C. S., Liyanaarachchi, W. S., Chandrapala, J., Dissanayake, M., & Vasiljevic, T. (2016). Utilizing unique properties of caseins and the casein micelle for delivery of sensitive food ingredients and bioactives. *Trends in Food Science & Technology, 57*(Part A), 178-187.

Sandoval-Castilla, O., Lobato-Calleros, C., García-Galindo, H., Alvarez-Ramírez, J., & Vernon-Carter, E. (2010). Textural properties of alginate–pectin beads and survivability of entrapped *Lb. casei* in simulated gastrointestinal conditions and in yoghurt. *Food Research International, 43*(1), 111-117.

Shah, N. P. (2007). Functional cultures and health benefits. *International Dairy Journal, 17*(11), 1262-1277.

Shi, L. E., Li, Z. H., Li, D. T., Xu, M., Chen, H. Y., Zhang, Z. L., & Tang, Z. X. (2013a). Encapsulation of probiotic *Lactobacillus bulgaricus* in alginate–milk microspheres and evaluation of the survival in simulated gastrointestinal conditions. *Journal of Food Engineering, 117*(1), 99-104.

506 Shi, L. E., Li, Z. H., Zhang, Z. L., Zhang, T. T., Yu, W. M., Zhou, M. L., & Tang, Z. X. (2013b).
507   Encapsulation of *Lactobacillus bulgaricus* in carrageenan-locust bean gum coated milk
508   microspheres with double layer structure. *LWT-Food Science and Technology, 54*(1),
509   147-151.

510 Shi, L. E., Zheng, W., Zhang, Y., & Tang, Z. X. (2016). Milk-alginate microspheres: Protection
511   and delivery of *Enterococcus faecalis* HZNU P2. *LWT-Food Science and Technology,*
512   *65*, 840-844.

513 Song, H., Yu, W., Gao, M., Liu, X., & Ma, X. (2013). Microencapsulated probiotics using
514   emulsification technique coupled with internal or external gelation process.
515   *Carbohydrate Polymers, 96*(1), 181-189.

516 Sultana, K., Godward, G., Reynolds, N., Arumugaswamy, R., Peiris, P., & Kailasapathy, K.
517   (2000). Encapsulation of probiotic bacteria with alginate–starch and evaluation of
518   survival in simulated gastrointestinal conditions and in yoghurt. *International Journal*
519   *of Food Microbiology, 62*(1–2), 47-55.

520 Sun, W., & Griffiths, M. W. (2000). Survival of bifidobacteria in yogurt and simulated gastric
521   juice following immobilization in gellan–xanthan beads. *International Journal of Food*
522   *Microbiology, 61*(1), 17-25.

523 Würth, R., Hörmannsperger, G., Wilke, J., Foerst, P., Haller, D., & Kulozik, U. (2015).
524   Protective effect of milk protein based microencapsulation on bacterial survival in
525   simulated gastric juice versus the murine gastrointestinal system. *Journal of Functional*
526   *Foods, 15*, 116-125.

527 Xiao, J., Kondo, S., Takahashi, N., Miyaji, K., Oshida, K., Hiramatsu, A., Iwatsuki, K.,
528   Kokubo, S., & Hosono, A. (2003). Effects of milk products fermented by
529   *Bifidobacterium longum* on blood lipids in rats and healthy adult male volunteers.
530   *Journal of Dairy Science, 86*(7), 2452-2461.

531 Zou, Q., Zhao, J., Liu, X., Tian, F., Zhang, H., Zhang, H., & Chen, W. (2011).
532   Microencapsulation of *Bifidobacterium bifidum* F-35 in reinforced alginate
533   microspheres prepared by emulsification/internal gelation. *International Journal of*
534   *Food Science & Technology, 46*(8), 1672-1678.

535

536

537

538

539

540 **Figure captions**

541

542 Fig.1. Scanning electron micrographs showing the surface morphology of different
543 microcapsules. (A) SA, (B) SACM, (C) SAGM, (D) SACH (magnification 10000X). For
544 legend explanations see Table1.

545

546 Fig. 2. Survival of free and encapsulated *B. longum* subsp. *infantis* CCUG 52486 in simulated
547 gastric juice (pH 2) at 37 ^{o}C for 120 min. Vertical lines represent standard deviations.
548 [ABCDE]Means with different uppercase are significantly different ($p<0.05$) between each time,
549 for each type of alginate-dairy based microcapsule during the period of the analysis. [abcde]Means
550 with different lowercase are significantly different ($p<0.05$) between each type of alginate-
551 dairy based microcapsule, for a particular time of the analysis. For legend explanations see
552 Table1.

553

554 Fig. 3. Stability of free and encapsulated *B. longum* subsp. *infantis* CCUG 52486 in simulated
555 intestinal juice (pH 7.5) at 37 ^{o}C for 120 min. Vertical lines represent standard deviations.
556 [ABCDE]Means with different uppercase are significantly different ($p<0.05$) between each time,
557 for each type of alginate-dairy based microcapsule during the period of the analysis. [abcde]Means
558 with different lowercase are significantly different ($p<0.05$) between each type of alginate-
559 dairy based microcapsule, for a particular time of the analysis. For legend explanations see
560 Table1.

561

562 Fig. 4. Changes in the viable count of free and the encapsulated *B.*
563 *longum* subsp. *infantis* CCUG 52486 during refrigerated storage (4 ^{o}C) for 28 days. Vertical
564 lines represent standard deviations. [ABCDE]Means with different uppercase are significantly
565 different ($p<0.05$) between each time, for each type of alginate-dairy based microcapsule
566 during the storage. [abcd]Means with different lowercase are significantly different ($p<0.05$)
567 between each type of alginate-dairy based microcapsule, for a particular day of the storage
568 period. For legend explanations see Table1.

569

570   Fig. 5. Changes in the viable counts free and encapsulated bacteria in goat milk at 4 $^{\circ}$C for 28

571   days. Vertical lines represent standard deviations. [ABCDE]Means with different uppercase are

572   significantly different ($p<0.05$) between each time, for each type of alginate-dairy based

573   microcapsule during the storage. [abcd]Means with different lowercase are significantly different

574   ($p<0.05$) between each type of alginate-dairy based microcapsule, for a particular day of the

575   storage period. For legend explanations see Table1.

576

577

578

579

580

581

582

583

584

585

586   Table 1. Encapsulation yield and size of different microcapsules

Type of microcapsules	Size (mm)	Encapsulation yield (%)
SA	2.3 ± 0.4^b	95.6 ± 2.1^a
SACM	2.8 ± 0.3^a	94.9 ± 1.4^a
SAGM	3.1 ± 0.2^a	95.3 ± 1.6^a
SACH	2.4 ± 0.4^b	94.1 ± 2.7^a

587   [ab]Mean values (±standard deviation) within the same column not sharing a common superscript

588   differ significantly (P < 0.05). SA: microcapsules were prepared using alginate. SACM:

589   microcapsules were produced using alginate and cow milk at a ratio of 1.5:1 (v/v). SAGM:

590   microcapsules were produced using alginate and goat milk at a ratio of 1.5:1 (v/v). SACH:

591   microcapsules were prepared using alginate and casein hydrolysate at a ratio of 1.5:1 (v/v).

592

593

594   Table 2. Changes in the viability of free and encapsulated *B. longum* subsp. *infantis* CCUG

595   52486 in cow milk at 4 °C for 28 days.

Type of capsule	Period of storage (days)				
	0	7	14	21	28
SA (log cfu g^{-1})	$8.53 \pm 0.09^{A\,a}$	$8.05 \pm 0.09^{A\,b}$	$7.38 \pm 0.09^{B\,c}$	$6.84 \pm 0.40^{B\,c}$	$6.03 \pm 0.04^{C\,c}$
SACM (log cfu g^{-1})	$8.57 \pm 0.11^{A\,a}$	$8.42 \pm 0.05^{AB\,a}$	$8.25 \pm 0.07^{BC\,b}$	$8.13 \pm 0.11^{C\,b}$	$7.07 \pm 0.15^{D\,b}$
SAGM (log cfu g^{-1})	$8.63 \pm 0.31^{A\,a}$	$8.59 \pm 0.17^{A\,a}$	$8.54 \pm 0.03^{A\,a}$	$8.52 \pm 0.06^{A\,a}$	$7.61 \pm 0.24^{B\,a}$
SACH (log cfu g^{-1})	$8.49 \pm 0.03^{A\,a}$	$7.63 \pm 0.06^{B\,c}$	$6.93 \pm 0.18^{C\,d}$	$6.38 \pm 0.38^{C\,c}$	$5.50 \pm 0.05^{D\,d}$
Free Cells (log cfu mL^{-1})	$8.65 \pm 0.12^{A\,a}$	$7.13 \pm 0.16^{B\,d}$	$5.10 \pm 0.07^{C\,e}$	$4.83 \pm 0.10^{C\,d}$	$4.38 \pm 0.29^{D\,e}$

596   [ABCD]Means in the same row without common letter differ significantly (*p<0.05*) for each type

597   of microcapsules. [abcde]Means in the same column for each type of microcapsule without

598   common letter differ significantly (*p<0.05*) for a particular day of storage. Data are expressed

599   as mean ± standard deviation. For legend explanations see
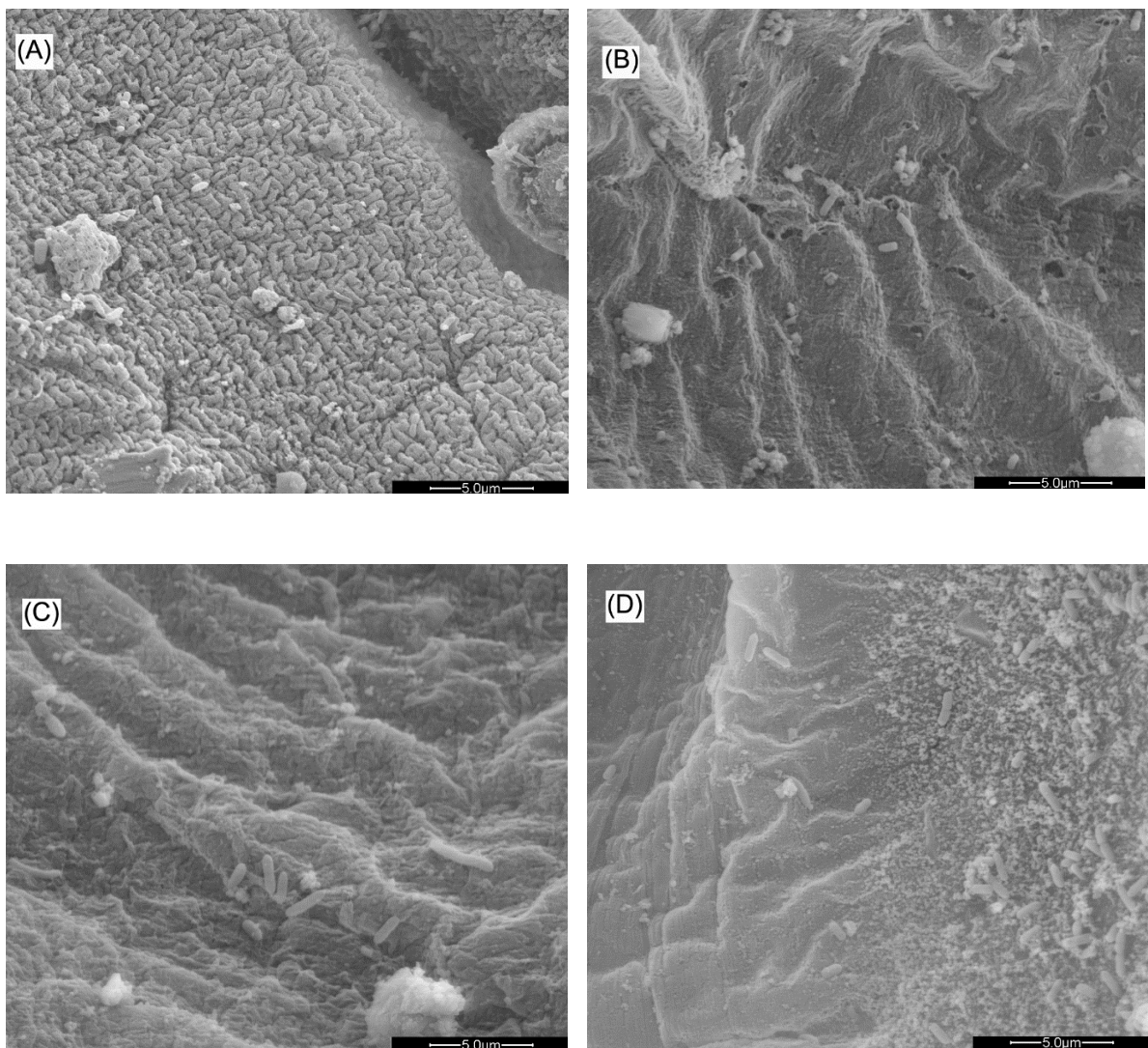
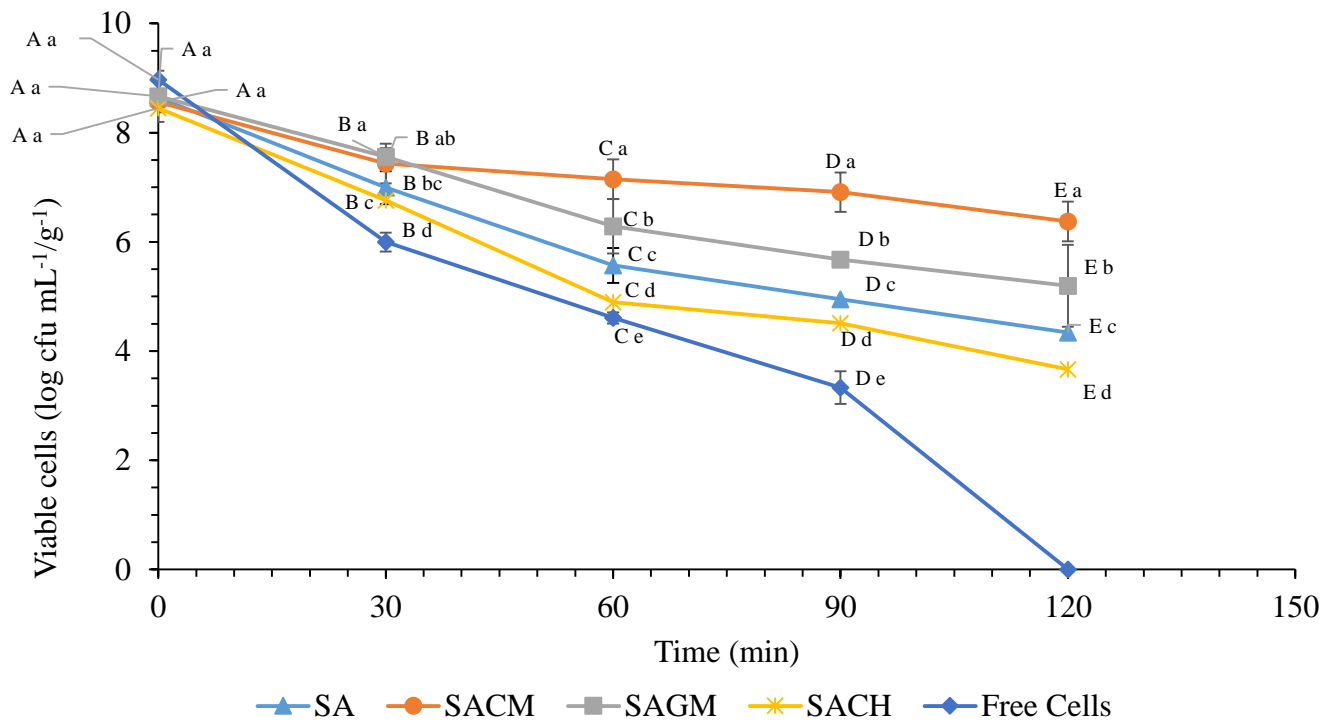600   Table *1*.

601

602

603

604

605

606

607

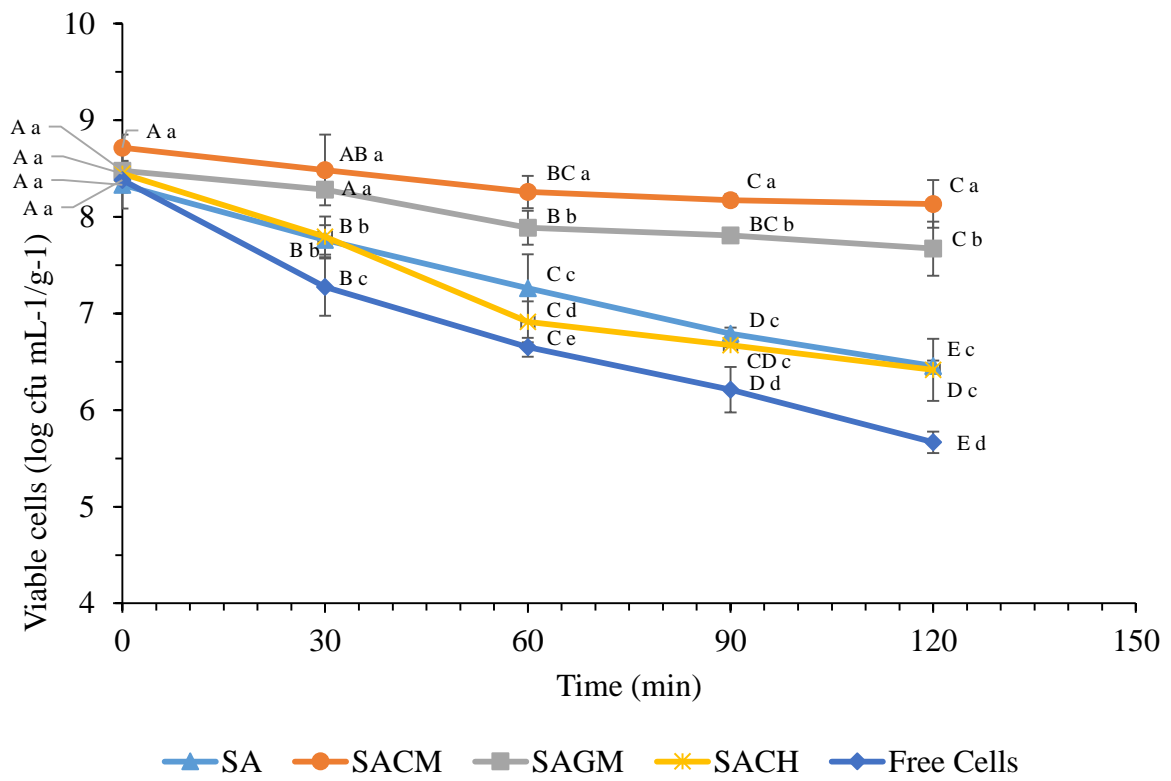608   Fig.1.

609

610

611

612

613

614

Fig. 2.

617

618

619

620

621

622

623

624

625

626

627

Fig. 3.

628

629

630

631

632

Fig. 4.
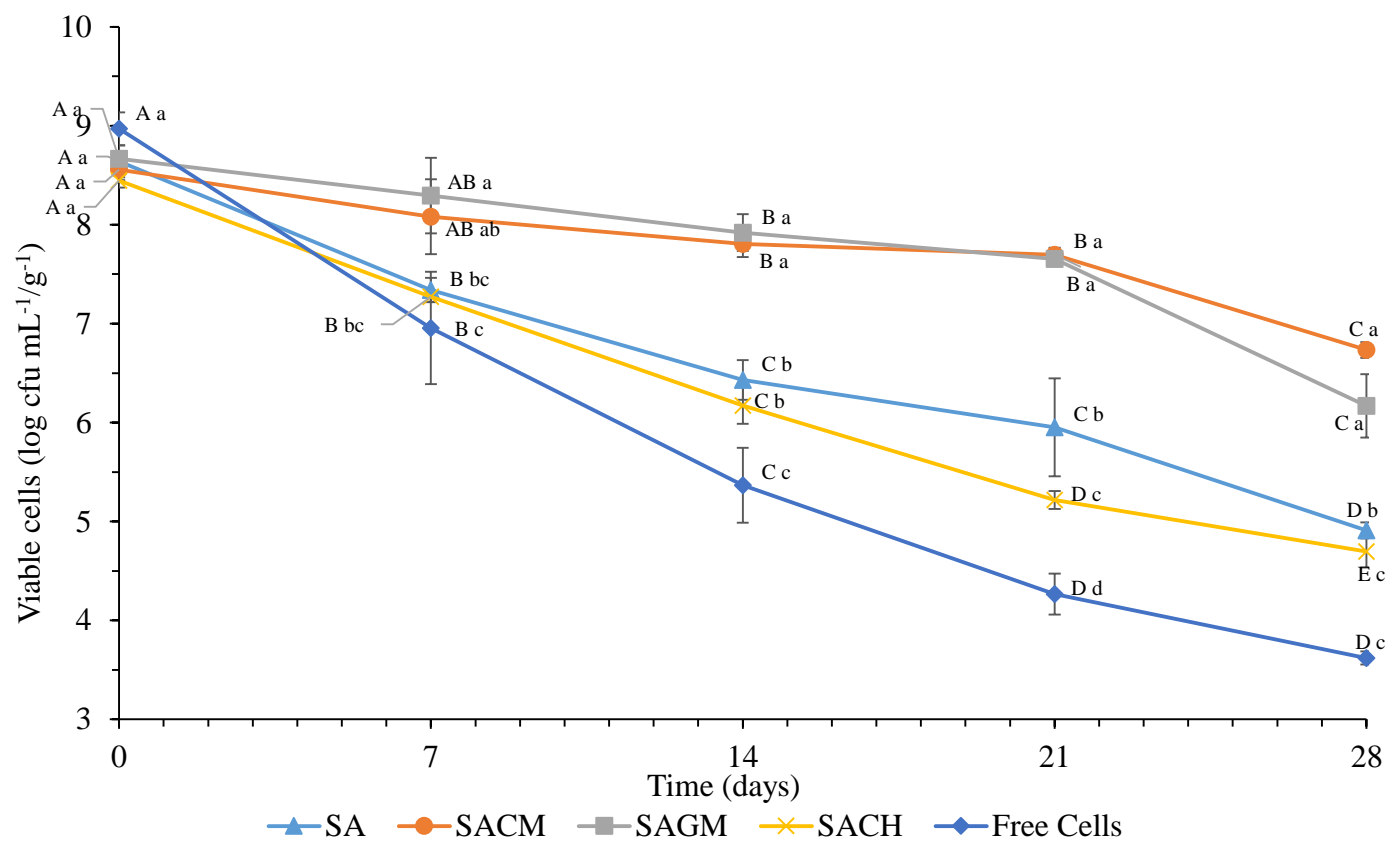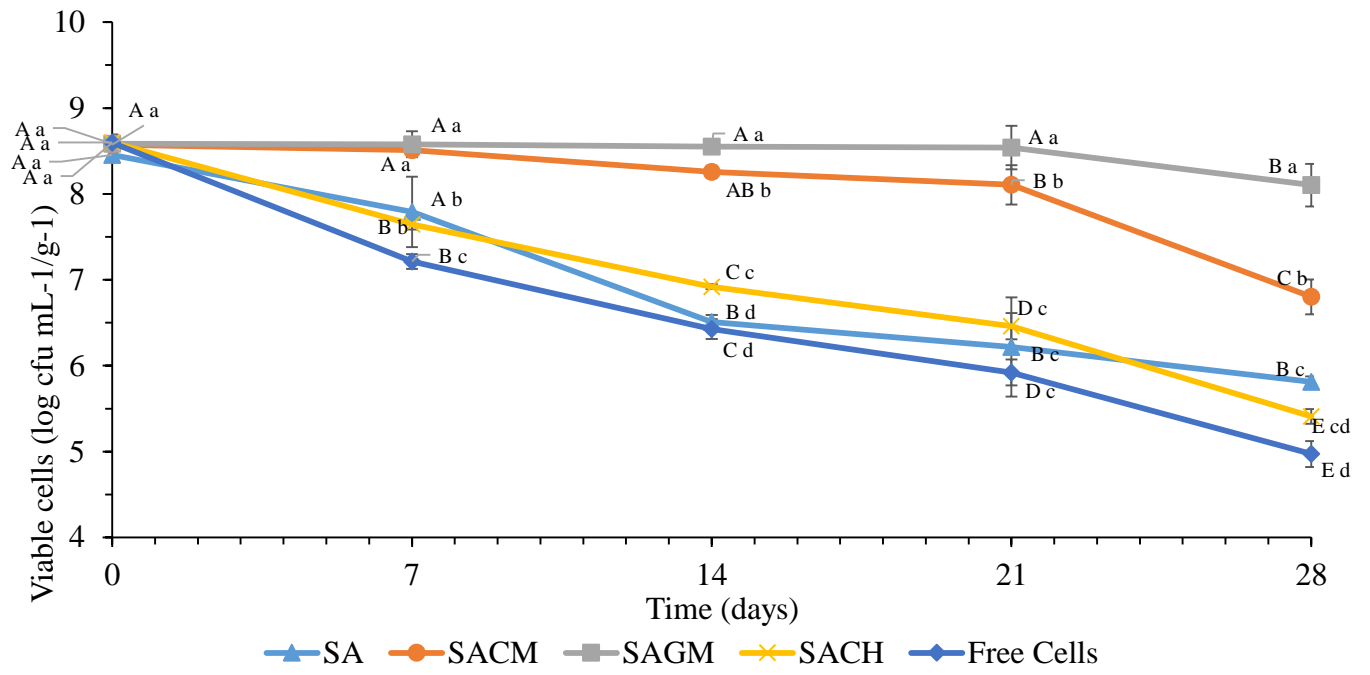
633

634

635

27

636

637   Fig. 5.

638

639

640

641

642