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

Running title: Encapsulation of bifidobacteria in alginate-dairy based matrices

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

[a] Department of Food and Nutritional Sciences, University of Reading, Whiteknights, Reading, RG6 6AP, UK

[b] Department of Animal and Food Sciences, Faculty of Agriculture, Rajarata University of Sri Lanka, Puliyankulama, Anuradhapura, Sri Lanka

[*]Corresponding author:

P.H.P. Prasanna

Department of Animal and Food Sciences, Faculty of Agriculture, Rajarata University of Sri Lanka, Puliyankulama, Anuradhapura, Sri Lanka.

Tele:0094714458656

Fax:0094252221614

Email:phpprasanna@yahoo.com

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 ^{o}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).

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

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

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 ^{o}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 ^{o}C. The cell concentration encapsulated in the microcapsules was around 9 log cfu g^{-1}.

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

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

2.4. Determination of viability of free and encapsulated bacteria

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

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

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

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

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) or the free cells (1 mL) were added to glass tubes containing 9 mL of sterilized SIJ and placed in a water bath at 37 ^{o}C. The sampling and enumeration of free and encapsulated *B. longum* subsp. *infantis* CCUG 52486 were carried out as described previously.

2.6. Survival of free and microencapsulated bacterial cells in refrigeration, cow milk and goat milk during refrigerated storage

In the case of refrigerated storage, microcapsules or free cells were stored (1 g for microcapsules/ 1 mL for free cells in each portion) in sterilized centrifuge tubes (15 mL capacity, polypropylene, Fisher Scientific, Loughborough, UK), at 4 ^{o}C for 28 days. In the case of cow milk, 1 mL of the free cells or 1 g of the encapsulated bacteria was mixed with 10 mL of sterilized cow milk in sterilized centrifuge tubes (15 mL capacity, polypropylene, Fisher Scientific, UK). In the case of goat milk, 10 mL of sterilized goat milk in sterilized centrifuge tubes (15 mL capacity, polypropylene, Fisher Scientific, UK) were mixed with 1 mL of the free cells or 1 g of the encapsulated cells. The centrifuge tubes containing free and encapsulated bacteria and inoculated milk samples were stored at 4 ^{o}C for 28 days. Afterwards, the samples were collected on 0, 7, 14, 21 and 28 days and analyzed for the viability of cells as described in Section 2.4.

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

The microcapsules were dehydrated sequentially in a series of ethanol solutions (30, 50, 70, 80, 90, and 100%). For this, the microcapsules were soaked for 15 minutes in each solution. The dehydrated microcapsules were critical point dried using a critical point dryer (Balzers CPD 030, Liechtenstein, Germany) with liquid carbon dioxide. The dried samples were fixed to the SEM stubs with double-sided tape. Afterward, the microcapsules were gold coated using an Edwards S150B sputter-coater for 2.5 min (Edwards, West Sussex, UK). The surface of coated microcapsules was examined using a scanning electron microscope (FEI, Quanta 600 F, USA).

2.8. Statistical analysis

All the experiments were conducted in triplicate. Results of the size of microcapsules and encapsulation efficiency were analyzed using one-way analysis of variance (ANOVA) with

Turkey's multiple comparison tests (SAS, version 9.2, SAS Institute Inc., Cary NC, USA). Results of viable counts from simulated gastrointestinal conditions and from storage studies were analyzed as a split-plot in time design using the General Linear Model (GLM) procedure of SAS, version 9.2 (SAS Institute Inc., Cary NC, USA).

3. Results and discussion

3.1. Size, encapsulation yield and surface morphology of microcapsules

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

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

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

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

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

diffusion of bile salt into the microcapsules (Hansen et al., 2002; Lee and Heo, 2000). Similarly, Krasaekoopt et al. (2004) reported poor viability of *B. bifidum* ATCC 1994 capsulated in alginate matrices when exposed to bile salt solution.

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

Fig. 4 shows the viability of free and encapsulated *B. longum* subsp. *infantis* CCUG 52486 with different alginate-based matrices during the refrigerated storage at 4 ^{o}C. The cell concentration of free *B. longum* subsp. *infantis* CCUG 52486 decreased significantly ($p<0.05$) from 8.96 log cfu g^{-1} to 3.62 log cfu g^{-1}, indicating the inability of the free cells to maintain their viability under the refrigerated storage condition. The results further revealed that encapsulation could improve the viability of bacterial cells during refrigerated storage for 28 days. SA and SACH microcapsules showed higher cell viability than that of the free cells during the refrigerated storage. However, they were unable to maintain the viability of cells during the storage above the recommended count of 6 log cfu g^{-1}. Similarly, some studies reported that encapsulation of probiotic bacteria in sodium alginate could improve the storage stability of bacterial cells than that of the free cells (Chávarri et al., 2010; Krasaekoopt et al., 2004).

SACM and SAGM microcapsules gave better protection for the cells during the refrigerated storage and both materials were able to maintain the cell concentrations above 6 log cfu g^{-1} after 28 days of storage than SA and SACH. However, the final cell counts of these two microcapsules were not significantly different ($p<0.05$). This may be due to the denser surface morphology of alginate-dairy microcapsules [Fig.1 (B) and (C)], which can protect the encapsulated cells from adverse conditions of the environment. Similarly, some other alginate-based microcapsules have been shown to be effective to give better protection for probiotics during the refrigerated storage. Encapsulation of *Lactobacillus gasseri* and *B. bifidum* in chitosan-coated alginate microspheres was shown to be effective to maintain viability throughout the storage period at 4 ^{o}C for 28 days (Chávarri et al., 2010). In addition, Zou et al. (2011) showed that chitosan-coated alginate microspheres provided a better protection for the microencapsulated *B. bifidum* F-35 cells than that of the free cells during the storage at 4 ^{o}C for 1 month.

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

Table 2 shows the survival of free and encapsulated stored in cow milk at 4 °C for 28 days. The results indicated that encapsulation improved the survival of bacterial cells in cow milk during storage. The free cells showed poor storage stability in cow milk where the cell concentration was significantly ($p<0.05$) reduced from 8.65 log cfu mL^{-1} to 4.38 log cfu mL^{-1} within 28 days. SAGM microcapsules gave the best protection for the cells followed by SACM microcapsules. However, SA and SACH microcapsules could give a limited protection during the storage in cow milk. Fig. 5 shows the results of free and encapsulated bacterial counts in goat milk during storage at 4 °C for 28 days. There was a significant reduction ($p<0.05$) in the viability of free cells during the storage. However, the results revealed that encapsulation of *B. longum* subsp. *infantis* CCUG 52486 improved the survival of bacterial cells in goat milk during the storage period of 28 days. The highest survival of bacterial cells during the storage was observed with SAGM microcapsules followed by SACM microcapsules; where they maintained the viability of bacterial cells above 6 log cfu g^{-1} in goat milk during the storage period. Viable cell counts of SA and SACH microcapsules rapidly declined with the storage.

Poor viability of free cells in cow milk and goat milk is due to lack of availability of small peptides and free amino acids for their growth (Gomes et al., 1998; Martín-Diana et al., 2003). In this study, pure goat milk and cow milk were used to inoculate bacteria without any supplementation. Similarly, Hansen et al. (2002) observed poor viability of free *B. longum* Bb-46 cells in milk during storage at 4 °C for 16 days than that of encapsulated bacterial cells. The poor survival of bacterial cells encapsulated in SA and SACH is due to the fragile texture of walls of these microcapsules [Fig.1 (A) and (D)], which exposes bacterial cells to the external environment. The high survival rate observed with microencapsulated bacterial cells with SACM and SAGM [Fig.1 (B) and (C)] in cow milk and goat milk may be due to improved denser surface characteristics compared to SA and SACH. The modified structure of SACM and SASM could protect their content form the adverse external environments. There is no recognized published literature about the survival of bifidobacteria encapsulated using alginate-milk based matrices in goat milk during storage to compare with our results. However, some authors have reported that encapsulation can improve the viability of bifidobacteria in cow milk and cow milk-based products. Hansen et al. (2002) showed the effectiveness of alginate microcapsules to improve the viability of *B. longum* Bb-46 in cow milk during the storage at 4 °C for 16 days. In another study, *B. bifidum* encapsulated in alginate beads coated

with chitosan was shown to have better survival than the free cell in yoghurt after the storage at 4 ^{o}C for 4 weeks (Krasaekoopt et al., 2006). In addition Kailasapathy (2006) showed that the alginate-starch encapsulated *B. lactis* had higher survival than the free cells in yoghurt at 4 ^{o}C for 7 weeks.

The present study demonstrates that encapsulation of *B. longum* subsp. *infantis* CCUG 52486 in SACM and SAGM microcapsules beneficially influences the viability of bacterial cells in cow milk and goat milk during the storage at 4 ^{o}C for 28 days. Therefore, microencapsulation of bifidobacteria with SACM and SAGM could be used to enhance the growth of them in non-fermented cow milk and goat milk based products. Further studies should be carried out to evaluate the effect of encapsulation of bifidobacteria with SACM and SAGM microcapsules in fermented milk-based products and other food systems.

4. Conclusions

The mixing of alginate with cow milk and goat milk resulted in microcapsules with denser surface and the cells encapsulated in these matrices performed better in simulated gastrointestinal conditions than the bacterial cells encapsulated in SA and SACH microcapsules. Improved structural characteristics of SACM and SAGM microcapsules could improve survival of encapsulated bacterial cells in cow milk, goat milk and refrigeration at 4 ^{o}C for 28 days compared to SA and SACH microcapsules. Overall, this study showed that mixing of goat milk and cow milk with alginate improved the protection provided by modified microcapsules and could be used to improve survival of probiotic bacteria in non-fermented cow milk and goat milk based products.

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Figure captions

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

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

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

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

570 Fig. 5. Changes in the viable counts free and encapsulated bacteria in goat milk at 4 ºC 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.

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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).

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594 Table 2. Changes in the viability of free and encapsulated *B. longum* subsp. *infantis* CCUG
595 52486 in cow milk at 4 ºC 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*.

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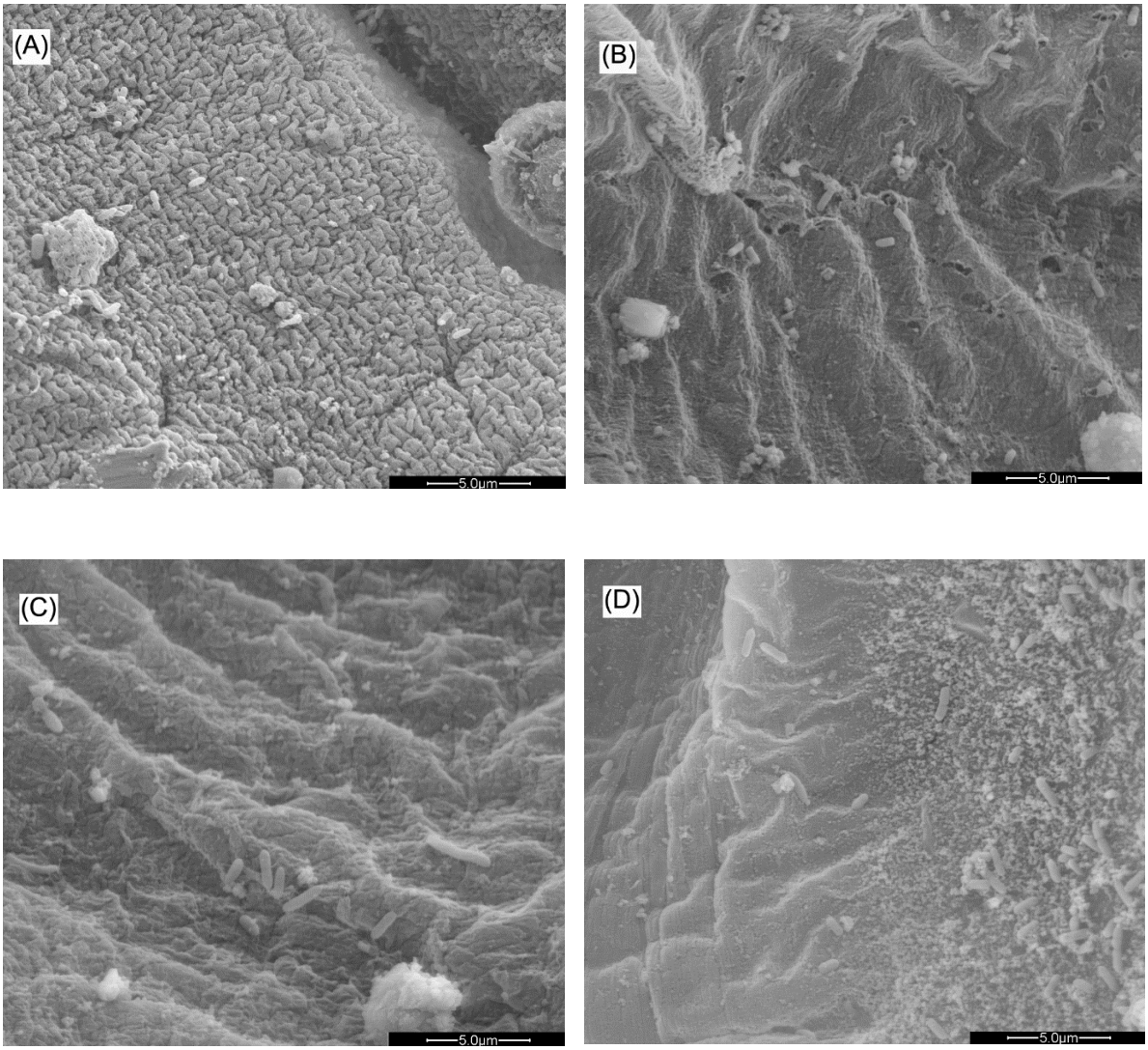

608 Fig.1.

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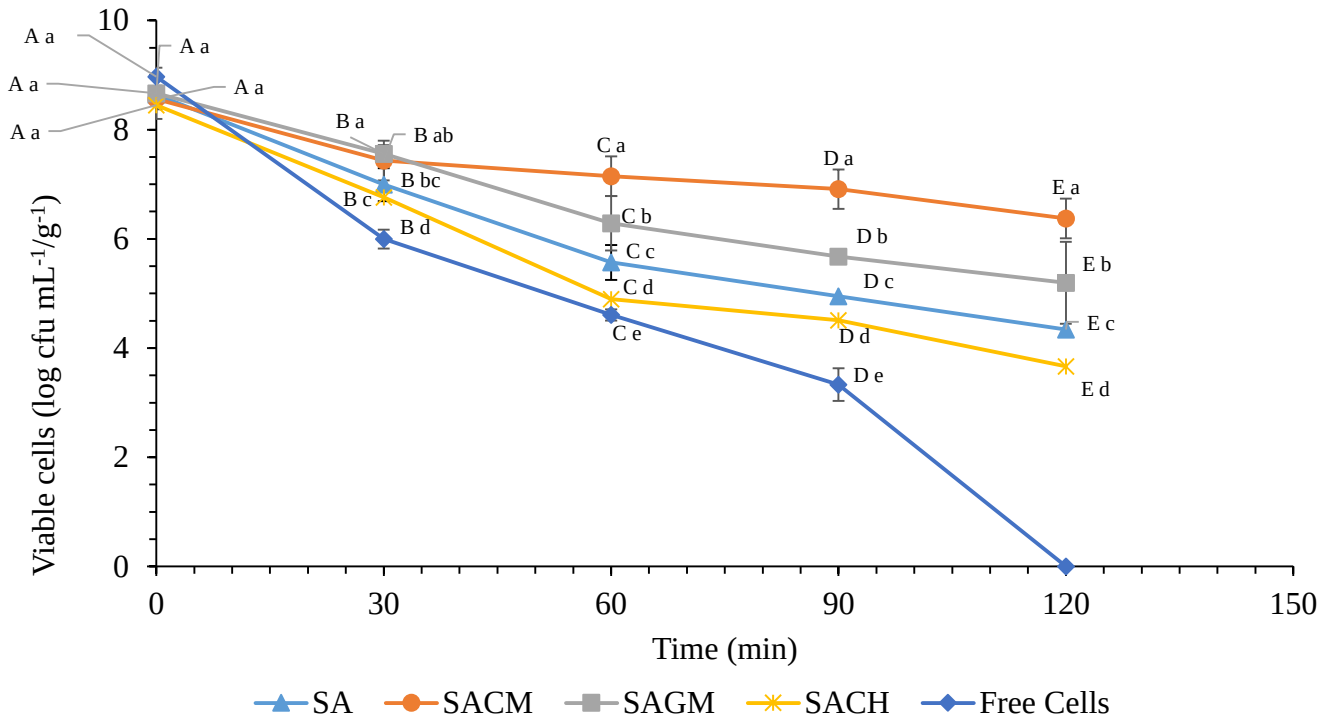
A a
A a
A a
A a
B a
B ab
B bc
B c
B d
C a
C b
C c
C d
C e
D a
D b
D c
D d
D e
E a
E b
E c
E d
Viable cells (log cfu mL-1/g-1)
Time (min)
0
30
60
90
120
150
2
4
6
8
10
SA
SACM
SAGM
SACH
Free Cells

Fig. 2.

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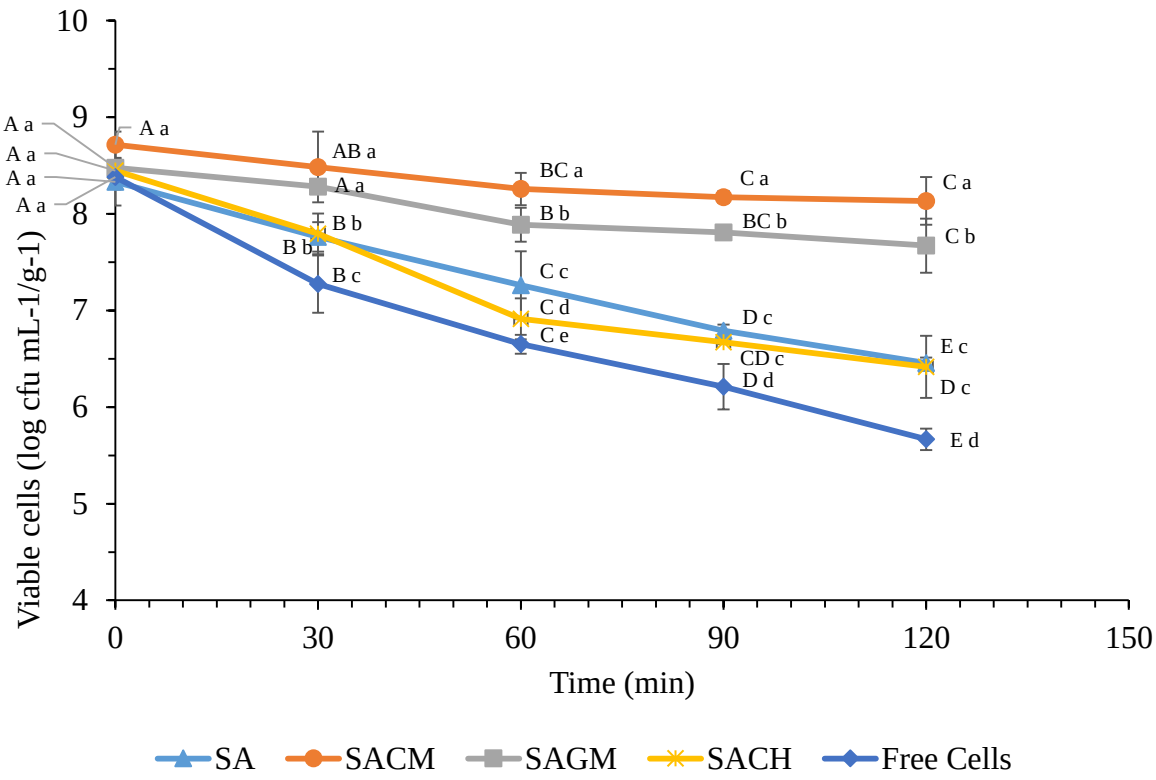

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629 Fig. 3.

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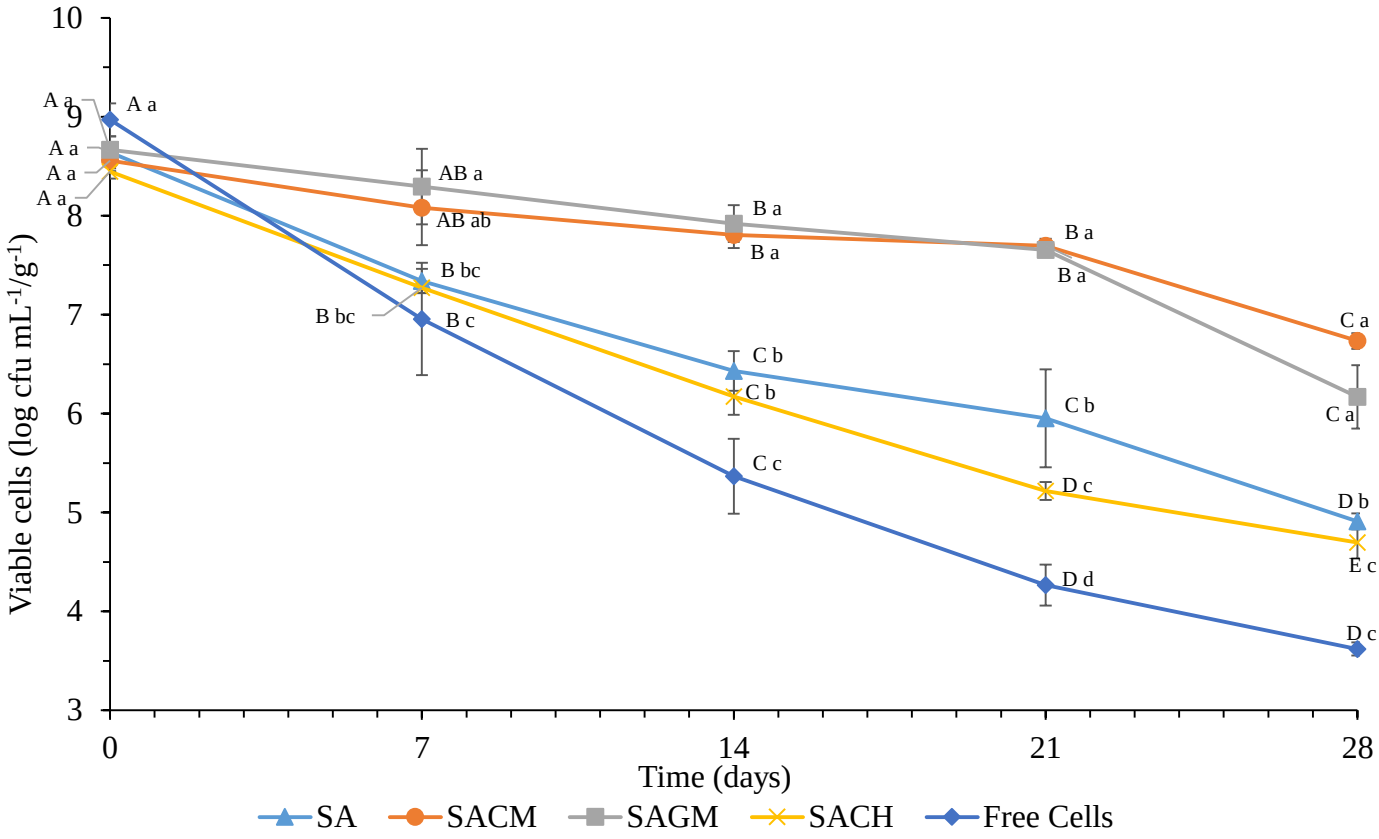

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633 Fig. 4.

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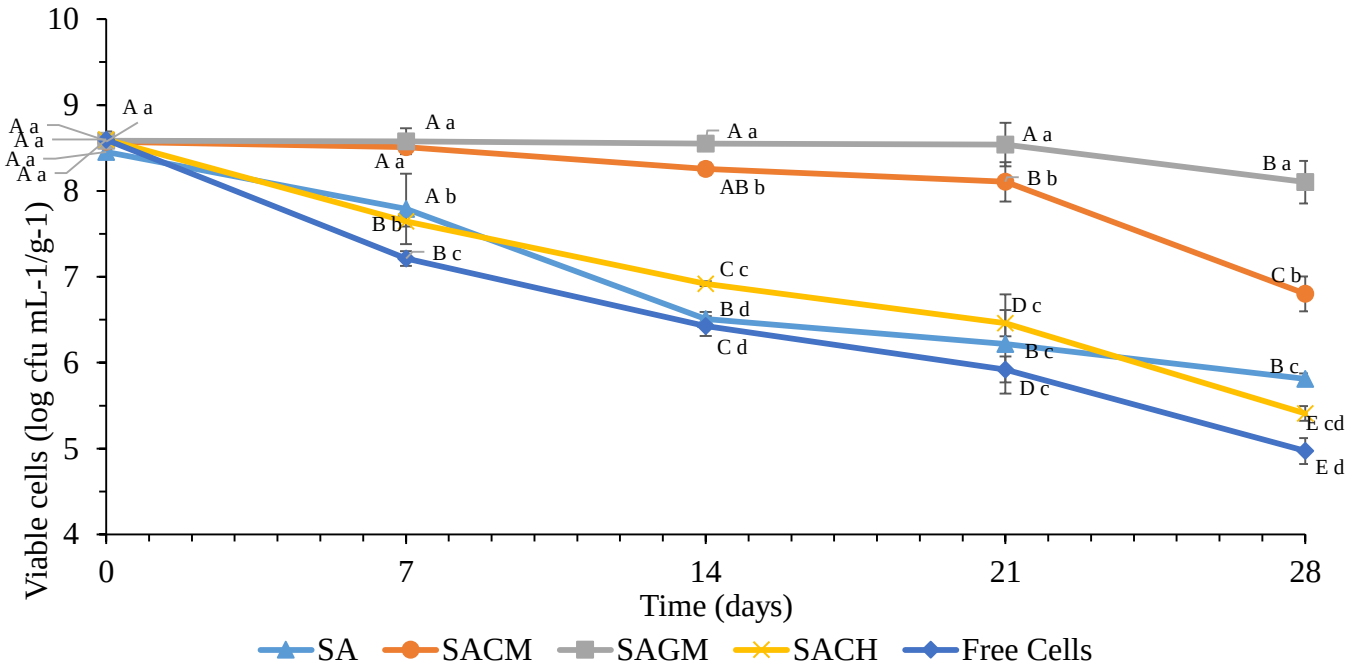
Viable cells (log cfu mL-1/g-1)
Time (days)
0
7
14
21
28
4
5
6
7
8
9
10
A a
A a
A a
A a
A a
A a
A a
A a
A b
B b
B c
B a
AB b
B b
C b
C c
B d
C d
D c
B c
D c
B c
E cd
E d
SA
SACM
SAGM
SACH
Free Cells

Fig. 5.