



# *Improving survival of probiotic bacteria using bacterial poly- $\gamma$ -glutamic acid*

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1 **Improving survival of probiotic bacteria using bacterial Poly- $\gamma$ -glutamic acid**

2  
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5  
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1       **ABSTRACT**

2       A major hurdle in producing a useful probiotic food product is bacterial survival  
3       during storage and ingestion. The aim of this study was to test the effect of  $\gamma$ -  
4       PGA immobilisation on the survival of probiotic bacteria when stored in acidic  
5       fruit juice. Fruit juices provide an alternative means of probiotic delivery,  
6       especially to lactose intolerant individuals. In addition, the survival of  $\gamma$ -PGA-  
7       immobilised cells in simulated gastric juice was also assessed. *Bifidobacteria*  
8       strains (*B. longum*, *B. breve*), immobilised on 2.5 %  $\gamma$ -PGA, survived  
9       significantly better ( $P < 0.05$ ) in orange and pomegranate juice for 39 and 11  
10      days respectively, compared to free cells. However, cells survived significantly  
11      better ( $P < 0.05$ ) when stored in orange juice compared to pomegranate juice.  
12      Moreover, both strains, when protected with 2.5 %  $\gamma$ -PGA, survived in simulated  
13      gastric juice (pH 2.0) with a marginal reduction ( $<0.47$  log CFU/ml) or no  
14      significant reduction in viable cells after four hours, whereas free cells died  
15      within two hours. In conclusion, this research indicates that  $\gamma$ -PGA can be used  
16      to protect *Bifidobacteria* cells in fruit juice, and could also help improve the  
17      survival of cells as they pass through the harsh conditions of the gastrointestinal  
18      tract (GIT). Following our previous report on the use of  $\gamma$ -PGA as a  
19      cryoprotectant for probiotic bacteria, this research further suggests that  $\gamma$ -PGA  
20      could be used to improve probiotic survival during the various stages of  
21      preparation, storage and ingestion of probiotic cells.

22  
23      Keywords: Probiotics,  $\gamma$ -PGA, *Bifidobacteria*, fruit juice, simulated gastric juice.

## 1       **1. INTRODUCTION**

2       The gastrointestinal tract (GIT) of humans is inhabited by a vast community of  
3       microbes representing well over 400 different microbial species, comprising  $10^{14}$   
4       bacterial cells which exceeds the number of eukaryotic cells in the human body  
5       by a factor of 10 (Cani and Delzenne, 2009; Turnbaugh et al., 2007). For proper  
6       functioning of the GIT, it is important to maintain a balance of the different  
7       organisms. This appears to be difficult due to various factors such as eating  
8       patterns, improper use of antibiotics and stress, all of which tend to favour the  
9       rise of pathogenic microorganisms and a decrease in beneficial microbes (Fooks  
10      and Gibson, 2002; McFarland and Dublin, 2008). Therefore, to maintain balanced  
11      microbial consortia in the GIT, beneficial organisms are often administered to  
12      individuals as ‘probiotic organisms’. Probiotic organisms are defined as live  
13      organisms that confer health benefits to the host when administered in adequate  
14      amount (Reid et al., 2003). Most of the studied probiotic organisms include  
15      different strains of *Lactobacillus* and *Bifidobacteria* (Theodorakopoulou et al.,  
16      2013). There are numerous health and nutritional benefits of probiotic organisms  
17      including the promotion of healthy digestive system, prevention of urinary tract  
18      infection, prevention of cancer and promotion of immune response (Allaker and  
19      Douglas, 2009; Bertazzoni et al., 2013; Patel and Goyal, 2013; Prakash et al.,  
20      2011).

21  
22      Although there is a lot of evidence in the literature on the benefits of probiotics,  
23      there remains an uncertainty about the usefulness of these bacteria. This  
24      uncertainty often stems from lack of reproducibility of the data, a possible  
25      consequence of insufficient/inconsistent numbers of live bacteria reaching the

1 intestine. The benefits of probiotic bacteria are largely dependent on their ability  
2 to survive, colonize and multiply in the host (Anal and Singh, 2007; Burgain et  
3 al., 2011). It has been determined that a food with health claims from the  
4 addition of probiotics must contain at least  $10^6$  CFU/ml of viable bacteria  
5 (Kailasapathy and Chin, 2000). Probiotics are usually administered or added to  
6 food products as dry cultures prepared mainly by freeze drying, as they are easier  
7 to handle when freeze dried. However, the procedures used to prepare freeze  
8 dried probiotic bacteria are detrimental to cell structure and viability (Saarela et  
9 al., 2005). Furthermore, the nature of the food or drink product into which the  
10 probiotic microorganism is introduced is generally limited to milk based products  
11 which maintain a weak acidic environment tolerated by the microorganisms.  
12 Even under these conditions, shelf life is still restricted. In addition, there is a  
13 considerable loss in viability of probiotic bacteria when they encounter the very  
14 acidic conditions of the stomach and high bile concentration in the small  
15 intestine. The mechanism of probiotic response to bile salt and stimulated gastric  
16 juice has previously been described (Sánchez et al., 2007; Sanchez et al., 2005).  
17 Therefore, there remains a need to improve the viability of probiotic  
18 microorganisms as they pass through the freeze drying, storage and ingestion  
19 processes.

20  
21 Also, for individuals with lactose intolerance or milk protein allergy, it would be  
22 useful to broaden the range of products into which probiotic bacteria could be  
23 introduced, for example, fruit juices. Although fruit juices provide a less suitable  
24 environment for survival of probiotic bacteria (Saarela et al., 2006), which is  
25 most likely due to the low pH values of fruit juices compared to dairy products,

1 the innovation of suitable technologies to improve bacterial survival in fruit juice  
2 will be a beneficial discovery. This will not only bring about an alternative to  
3 probiotic delivery to lactose intolerant individuals, but it may also help the  
4 bacteria to endure the harsh acidic conditions of the stomach, thus improving the  
5 survival of ingested probiotics.

6  
7 Immobilisation and encapsulation of probiotic bacteria in different materials are  
8 present technologies used to improve the survival of probiotic cells during  
9 storage and ingestion. Although both terms (immobilisation and encapsulation)  
10 are often used interchangeably in literature, the latter refers to the formation of a  
11 continuous coating around an inner matrix wholly contained within the wall of  
12 the capsule, while the former involves trapping of the cells either within or  
13 throughout the matrix. In the former, a small part of the cell may be exposed at  
14 the surface while in the latter the cells are completely surrounded within a  
15 capsule (Kailasapathy, 2002; Vidhyalakshmi et al., 2009). Methods used for the  
16 immobilisation of probiotic cells include spray drying, freeze drying, extrusion  
17 and phase separation (Kailasapathy, 2002; Lozinsky et al., 2003; Vidhyalakshmi  
18 et al., 2009). Commonly used immobilisation materials include alginate, xanthan,  
19 carrageenan and phthalate cellulose (Doleyres and Lacroix, 2005). Although  
20 these materials have been shown to be useful in protecting cells during different  
21 stages of probiotic food preparation and ingestion, there remains the need to  
22 develop new materials which can protect cells not only during preparation,  
23 storage or ingestion but also throughout the process from preparation of the  
24 probiotic food product to its safe delivery to the host.

1 We have earlier shown that immobilising probiotic bacteria in poly- $\gamma$ -glutamic  
2 acid ( $\gamma$ -PGA) produced from a non-toxic microbial species of *Bacillus* can  
3 improve the survival of these organisms during freeze drying (Bhat et al., 2013).  
4 To continue with our investigation, this research was designed to study the  
5 protective effect of  $\gamma$ -PGA on probiotic bacterial survival in two types of fruit  
6 juices (orange and pomegranate juice) and in simulated gastric juice. Two  
7 *Bifidobacterium* strains were selected as model probiotic microorganisms for this  
8 study, owing to their known susceptibility to low pH and various stress  
9 conditions (Russell et al., 2011).

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## 2. MATERIAL AND METHODS

### 2.1 $\gamma$ -PGA

The sodium salt of  $\gamma$ -PGA (molecular weight – 257,000 Da) was produced using *B. subtilis* natto (ATCC 15245), following previously described methods (Kedia et al., 2010).

### 2.2 *Bacterial strains*

The probiotic bacterial strains used in this investigation were: *Bifidobacterium longum* NCIMB 8809 and *Bifidobacterium breve* NCIMB 8807. Strains were obtained from NCIMB as stock cultures and freeze-dried before storage at -20 °C. Prior to their use, the cultures were revived aseptically and grown anaerobically on Bifidobacteria Selective Medium (BSM) Agar at 37 °C in jars using an atmosphere generation system (Oxoid Anaerogen™) and an indicator strip.

### 2.3 *Media*

BSM (a selective and differential media for *Bifidobacteria*) agar/broth were used for growth and enumeration of *Bifidobacteria* in simulated gastric juice (SGJ) experiment (Nualkaekul et al., 2011). BSM broth/agar were purchased and prepared according to the manufacturer's protocol (Sigma-Aldrich, U. K.). Phosphate buffered saline (PBS) obtained from Sigma-Aldrich was prepared by dissolving 1 tablet in 200 ml following manufacturer's instructions.

SGJ contained 2 g/l NaCl, 3.2 g/l pepsin powder and 20 ml of 1M HCl (British Pharmacopoeia Commission, 1993). The components were diluted in 900 ml

1 deionised water and after pH adjustment (pH 2.0), the volume was topped up to 1  
2 l. pH was adjusted using 1 M HCl or 1 M NaOH. This model solution was  
3 sterilized by passage through a sterile 0.2  $\mu\text{m}$  syringe filter.  
4

#### 5 ***2.4 Preparation of $\gamma$ -PGA-immobilised cells***

6 Freeze dried  $\gamma$ -PGA protected cells were prepared as described previously (Bhat  
7 et al., 2013). Cells were initially grown in BSM and recovered as pellets by  
8 centrifugation, and then washed with PBS and suspended in 10 ml suspension of  
9 10% w/v  $\gamma$ -PGA. The cell/ $\gamma$ -PGA suspension was then incubated at room  
10 temperature for 1 hr and then at -80 °C for 24 hrs. Subsequently, the samples  
11 were freeze dried (Edwards Freeze Dryer, Modulyo, UK) at - 40 °C and 5 mbar  
12 for 48 hrs (Bhat et al., 2013).  
13

#### 14 ***2.5 Cell survival in fruit juices***

15 Fresh samples of commercially available orange juice (Tropicana orange juice,  
16 Pure Premium Smooth No Bits – 100% freshly squeezed orange juice) and  
17 pomegranate juice (POM Wonderful 100% Pomegranate juice) were purchased from  
18 a local supermarket and used for the tests. No microbial growth was observed in  
19 overnight cultures of each juice at 37 °C under anaerobic conditions in BSM. The  
20 dry powder of  $\gamma$ -PGA-immobilised cells was suspended in 1 ml PBS and this  
21 suspension was added to 40 ml of the respective juice to give a final  
22 concentration of  $\gamma$ -PGA coated cells in fruit juice of 2.5 % (v/v). As control, non-  
23 immobilised free cells were inoculated in BSM broth under the aforementioned  
24 conditions and the cell pellets were obtained after centrifugation and washing  
25 with PBS. The cell pellets were suspended in 1 ml PBS and these cell

1 suspensions (immobilised samples and control) were added to each juice and  
2 stored at 4 °C. Samples were collected at day 0, 2, 4, 6, 8, 11, 13, 20, 28 and 39  
3 of storage for orange juice and at day 0, 2, 4, 6, 8, 11 and 13 for pomegranate  
4 juice. After appropriate dilution, 0.1ml aliquots from the dilutions were plated on  
5 BSM agar and incubated anaerobically at 37 °C for 48 hrs to assess the  
6 concentration (expressed as CFU/ml) of the free and immobilised probiotic cells  
7 in the respective fruit juice.

### 8 9 ***2.6 Organic acid concentration of fruit juice***

10 Organic acid analysis was performed using an Agilent 1100 HPLC system to  
11 assess any changes in the organoleptic composition of the fruit juice. A Prevail  
12 Organic Acid 5µm column with a UV detector was used for citric acid, malic  
13 acid, lactic acid, acetic acid and ascorbic acid analysis. A 25 mM KH<sub>2</sub>PO<sub>4</sub>  
14 solution (pH 2.5 adjusted with phosphoric acid) was used as the eluent. Before  
15 loading, the samples were filtered using 0.45 µm filters and diluted 10 fold with  
16 deionised water. Standard curves were prepared using known concentrations of  
17 the chemicals to be analysed.

### 18 19 ***2.7 Probiotic survival in simulated gastric juice***

20 The survival of the two *Bifidobacterium* strains (free and immobilised on γ-PGA)  
21 in SGJ was monitored for 4 hrs.

22  
23 The tests were carried out in an Electrotek Anaerobic Workstation, where a  
24 microaerophilic environment was maintained (10% CO<sub>2</sub> and 5% O<sub>2</sub>) at 37 °C.  
25 This would also test the effect of γ-PGA on the survival of bacteria when

1 exposed to some oxygen just before ingestion. The cell concentration was  
2 measured at 0 hrs, 1 hr, 2 hrs, 3 hrs and 4 hrs for  $\gamma$ -PGA-immobilised and free  
3 cell. This was done by making appropriate dilution of samples up to  $10^{-8}$  and  
4 plating them out on BSM agar. Plates were incubated anaerobically at 37 °C for  
5 48 hrs to assess the cell concentration at different incubation times in SGJ.

### 6 7 **2.8 Statistical analyses**

8 All experiments were performed in triplicate and results were analysed using  
9 Microsoft Excel 2010 and Graph Pad Prism 5. Two-factor ANOVA was used to  
10 analyse the differences in survival of *B. breve* and *B. longum* in either juices with  
11 respect to time while Student's T tests were used to compare the acid  
12 concentration in the juices before and after treatment with immobilized and non-  
13 immobilized probiotics. The Bonferroni multiple comparison test was used for  
14 non-parametric analysis of data to determine the difference between individual  
15 groups in a data set. A P. value  $\leq 0.05$  was considered to be statistically  
16 significant.

## 17 18 19 20 21 22 23 24 25 **3. RESULTS**

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### **3.1 $\gamma$ -PGA protection of probiotic cells in orange juice**

2.5 %  $\gamma$ -PGA-protected *B. longum* and *B. breve* were stored in orange juice for up to 39 days at 4 °C. The results are presented in figures 1 and 2 along with the control experiments with non-immobilised free cells. After 13 days of storage, a loss in viable cell concentration of around 4.0 log CFU/ml and 3.4 log CFU/ml was observed in free *B. breve* and *B. longum* cells, respectively. A complete loss in viability of free cells in orange juice was observed on day 20 of the experiment for both strains. In contrast,  $\gamma$ -PGA-immobilised *B. breve* and *B. longum* cells showed only around 0.5 log CFU/ml and 1.1 CFU/ml log reductions in viable cells, respectively, after 13 days of storage at 4 °C. On day 39, viable counts of around 6.6 log CFU/ml and 6.5 log CFU/ml were observed for immobilised *B. breve* and *B. longum* cells respectively, indicating a reduction of approximately 2.1 log CFU/ml and 3.2 log CFU/ml, respectively (Figures 1 and 2).

### **3.2 $\gamma$ -PGA protection of probiotic cells in pomegranate juice**

The results from the survival studies in pomegranate juice, carried out for up to 13 days of storage at 4 °C, are presented in Figures 3 and 4. A complete loss in viability for free *B. longum* cells was observed on day 6. In comparison, a complete loss in viability of free *B. breve* cells was observed on day 2. Another notable observation was a very low viable cell concentration of both free *B. longum* (~4.8 log CFU/ml) and *B. breve* (~5.0 log CFU/ml) cells on day 0 of the experiment, which indicates that a significant proportion of the cells died immediately after they were introduced into the juice. On the other hand, the

1 immobilised cells survived considerably better in the pomegranate juice than the  
2 free cells. Nevertheless, a loss in viable cell concentration of around 2.9 log  
3 CFU/ml and 2.1 log CFU/ml was observed for the  $\gamma$ -PGA-immobilised *B. longum*  
4 and *B. breve* cells after 2 days of storage, respectively. The cell concentration  
5 gradually declined until day 13 and day 11 with losses of around 5.5 log CFU/ml  
6 for *B. longum* and 4.0 log CFU/ml for *B. breve*, respectively. Both experiments  
7 were discontinued as the cell concentrations of both strains fell below the FAO  
8 recommended value of 6 – 7 log CFU/ml.

### 9 10 **3.3 pH and organic acid concentration of juices**

11 The pH of fresh orange juice, with no cells added was 3.91 (**Table 1**). This was  
12 reduced to 3.82 after 39 days of storage at 4 °C. The pH of the orange juice  
13 containing free *B. longum* or *B. breve* was reduced to 3.71 and 3.65, respectively,  
14 after 39 days of storage. On the other hand, the pH of the orange juice containing  
15  $\gamma$ -PGA immobilised *B. longum* or *B. breve* increased to 4.36 and 4.22,  
16 respectively, after 39 days storage (Table 1). For pomegranate juice, not much  
17 difference in the pH was observed between the fresh juice with no cells and juice  
18 containing free *Bifidobacterium* cells after 39 days of storage at 4 °C. However,  
19 pomegranate juice containing  $\gamma$ -PGA immobilised cells showed an increase in pH  
20 of around 0.85 compared to fresh juice, resulting in a pH of around 4.05, after 39  
21 days of storage.

22  
23 Fresh orange juice, without the presence of *Bifidobacterium* cells, contained  
24 around 425 mg/l of ascorbic acid and this concentration dropped to around 161  
25 mg/l after storage at 4 °C for 39 days (Table 2). Orange juice with free *B. longum*

1 and *B. breve* cells demonstrated a reduction in ascorbic acid concentration to  
2 around 79 mg/l and 173 mg/l, respectively, after 39 days of storage. Likewise,  
3 orange juice containing  $\gamma$ -PGA-immobilised *B. longum* or *B. breve* cells  
4 demonstrated a reduction in ascorbic acid concentration to around 71 mg/l and  
5 193 mg/l, respectively, after 39 days of storage at 4 °C (Table 2).

6  
7 The concentrations of citric acid and malic acid in the fresh orange juice were  
8 around 9.9 g/l and 3.8 g/l respectively. After 39 days of storage the difference in  
9 concentration of citric and malic acid in orange juice without *Bifidobacterium*  
10 cells was not significantly different when compared to that of fresh juice ( $P >$   
11 0.05). Similarly, in the orange juice with either free or  $\gamma$ -PGA immobilised *B.*  
12 *longum* or *B. breve* cells, there was no significant difference in the concentration  
13 of malic and citric acid compared to that of fresh juice ( $P > 0.05$ ) (Table 2).

14  
15 The concentrations of citric, malic and ascorbic acids in pomegranate juice stored  
16 at 4 °C for 39 days are shown in Table 3. Contrary to the observations in orange  
17 juice, no reduction in the ascorbic acid concentration was observed in the  
18 pomegranate juice with free and  $\gamma$ -PGA-immobilised cells (Table 3). The  
19 concentration of citric and malic acid in the pomegranate juice with free and  $\gamma$ -  
20 PGA-immobilised cells did not show any significant ( $P > 0.05$ ) reduction either  
21 (Table 3).

### 22 23 **3.4 Protection of probiotic cells in simulated gastric juice**

24 The effect of 2.5%  $\gamma$ -PGA immobilisation on the survival of *B. longum* and *B.*  
25 *breve* in simulated gastric juice (up to 4 hrs at pH 2) was investigated *in vitro* and

1 the data are presented in Figure 5. It can be seen that immobilisation protected  
2 the *Bifidobacterium* strains, as a marginal (~ 0.5 log CFU/ml) but not significant  
3 reduction in viable cell concentration after 4 hrs ( $P > 0.05$ ) was observed. In  
4 contrast, the free *B. longum* and *B. breve* cells showed a complete loss in  
5 viability within 2 hrs. Even after one hour in SGJ, the cell concentration of the  
6 free cells (around 4.0 – 4.2 log CFU/ml for both *Bifidobacterium* cells) was  
7 below the recommended FAO values ( $10^6 - 10^7$  CFU/ml) considered to be  
8 beneficial to the host.

#### 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 **4. DISCUSSION**

24 Fruit juices are good candidates for the incorporation of probiotic bacteria, since  
25 they are rich in nutrients (Ding and Shah, 2008) and often have oxygen



1 scavenging agents, such as ascorbic acid, which promote anaerobic conditions  
2 that are important for the survival of certain lactic acid bacteria and  
3 *Bifidobacteria*, commonly used as probiotics. In addition, fruit juices contain  
4 high amounts of sugars which could enhance the survival of the incorporated  
5 probiotic bacteria as they would serve as an energy source for maintenance of  
6 cellular activities (Delete). However, it is often seen that probiotic viability in  
7 fruit juices is hampered due to factors such as low pH and the presence of  
8 competing microorganisms (Granato et al., 2010).  $\gamma$ -PGA is a natural biopolymer  
9 that can be potentially incorporated into food as it is Generally Recognized As  
10 Safe (GRAS) by the FDA (Shyu and Sung, 2010) and has been shown from our  
11 previous research to have good cryoprotective properties for probiotic bacteria  
12 (Bhat et al., 2013). Among the various microorganisms used as probiotics,  
13 *Bifidobacteria* have attracted significant commercial interest over the years, as  
14 their beneficial health effects have been well documented, despite the fact that  
15 they are generally very sensitive to low pH conditions, either those in the food  
16 product or in the GIT (Cui et al., 2000). The aim of this work was to evaluate  
17 whether immobilisation of *Bifidobacterium* cells on  $\gamma$ -PGA, a GRAS polymer,  
18 can improve the survival of the cells in orange, pomegranate juice and simulated  
19 gastric juice.

20  
21 In orange juice, free cells of both *Bifidobacterium* species, showed complete loss  
22 of viability within 20 days (Figure 1, 2) with the number of cells going below the  
23 FAO value of 6 – 7 log CFU/ml (Kailasapathy and Chin, 2000; Kurmann and  
24 Rasic, 1991) on day 11 (< 5 log CFU/ml). In contrast,  $\gamma$ -PGA-immobilised cells  
25 survived well for 39 days in orange juice with viable cell concentrations of 6.48

1 log CFU/ml and 6.61 log CFU/ml for *B. longum* and *B. breve* respectively. In  
2 pomegranate juice, there was a complete loss in viable cell concentration of free  
3 *B. longum* cells within 6 days, with cell concentration reducing immediately upon  
4 introduction into the juice. This result was similar to that of *B. breve*, however, a  
5 complete loss in viability was observed after only 2 days of introducing free cells  
6 into the juice. This shows that *B. longum* survived better in pomegranate juice  
7 compared to *B. breve*.  $\gamma$ -PGA immobilised cells survived significantly better than  
8 free cells in pomegranate juice although not to the same extent as those in orange  
9 juice.

10  
11 These results have clearly shown the bacterial protective ability of  $\gamma$ -PGA in fruit  
12 juices. Although the complete mechanism of protection within the juice is not  
13 fully understood, one possible explanation would be the physical entrapment of  
14 the cells to the polymer matrix. Also, it is possible that some of the  $\gamma$ -PGA may  
15 have solubilised over time in the juice, as a pH of  $\sim 4.0$  has previously been  
16 shown to result in over 50% disruption of the  $\alpha$ -helical conformation of  $\gamma$ -PGA  
17 (Ho et al., 2006), thus leading to a higher pH level in both orange and  
18 pomegranate juice with  $\gamma$ -PGA-immobilized cells compared to juice with free  
19 cells or without any cells (Table 1). This increased pH may have created a better  
20 environment for the survival of the cells. However as the pH of the juice with  $\gamma$ -  
21 PGA-immobilized cells remained below 4.5, the pH change of  $< 1$  may not  
22 completely account for the increased survival as previous research has shown  
23 complete loss in cell growth of most probiotic cell at pH between 4.5 and 5.5  
24 with cell death at pH of 4.1 or below (Shah and Dave, 2002). Thus, physical  
25 entrapment within the  $\gamma$ -PGA matrix as well as increase in the pH of the juice

1 may both play important roles in the protection of probiotic cells in fruit juice by  
2  $\gamma$ -PGA.

3  
4 *B. longum* encapsulated in Ca-alginate beads has previously been shown to  
5 survive 6 weeks in orange juice with a reduction in viable cell number of ~3.0  
6 log CFU/ml by the end of storage at 4 °C (Ding and Shah, 2008). In our  
7 experiment,  $\gamma$ -PGA immobilised cells showed a similar loss over a similar period.  
8 However, studies have shown that Ca-alginate is not effective in protecting cells  
9 in low pH conditions and that survival depends on various parameters such as  
10 alginate concentration and bead size (Anal and Singh, 2007; Chandramouli et al.,  
11 2004; Hansen et al., 2002; Krasaekoopt et al., 2004; Lee and Heo, 2000;  
12 Mortazavian et al., 2008). Contrarily, our experiment showed that  $\gamma$ -PGA is not  
13 only effective in protecting cells in juice but can also protect cells in low pH  
14 environments (Figure 5), thus making it a more desirable choice as a probiotic  
15 immobilisation material in fruit juices compared to Ca-alginate. In another study,  
16 *B. breve* protected with either polydextrose or a mixture of polydextrose and oat  
17 had a loss in viable cell number of ~ 6 Log CFU/ml within 20 days of incubation  
18 in a fruit juice with pH value of 3.8 (similar to that of orange juice used in this  
19 experiment) (Saarela et al., 2011). The researchers earlier showed a loss in viable  
20 cell number of ~ 5 Log CFU/ml of *Lactobacillus rhamnosus* protected with  
21 sucrose in apple juice for 4 weeks at 4 °C (Saarela et al., 2006). These results  
22 indicate that  $\gamma$ -PGA has more potential as an immobilisation material (with a cell  
23 loss of ~ 3 Log CFU/ml in fruit orange juice at 4 °C) for the protection of  
24 probiotic cells in juice when compared to polydextrose or sucrose.

1 A more recent experiment by Nualkaekul et al., showed that coating alginate  
2 bead encapsulated *B. longum* or *L. plantarum* with either chitosan, gelatin or  
3 glucomannan resulted in increased survival of the cells stored in either cranberry  
4 or pomegranate juice for 6 weeks at 4 °C (Nualkaekul et al., 2013). Depending on  
5 the coating material and the concentration used, they reported that a cell death of  
6 less than 1 Log CFU/ml could be achieved (Nualkaekul et al., 2013). Although  
7 alginate bead coating resulted in increased cell survival in pomegranate juice  
8 when compared to  $\gamma$ -PGA, the process of encapsulation is more demanding when  
9 compared to the freeze drying process used in the production of  $\gamma$ -PGA  
10 immobilised cells. Also, the cost of encapsulation and coating are higher when  
11 compared to the cost of preparation of freeze dried  $\gamma$ -PGA-immobilised cells.  
12 Furthermore, coating alginate beads caused an increased size (> 3.5mm) which is  
13 likely to affect the texture of juice considerably, compared to  $\gamma$ -PGA-  
14 immobilized cells. This change in the texture of the juice may be unpleasant to  
15 consumers and thus may lead to reduced acceptance (Nualkaekul et al., 2013).  
16 Therefore, immobilising cells using  $\gamma$ -PGA could be a more acceptable and less  
17 intensive/costly means of protecting probiotic cells in fruit juice compared to  
18 coated alginate beads. Additionally, combining  $\gamma$ -PGA with other polymers may  
19 also increase the survival of immobilised probiotic cells and should be  
20 considered as a means of improving the use of  $\gamma$ -PGA as an immobilisation agent  
21 in probiotic protection (Delete).

22  
23 Comparing the *Bifidobacteria* survival results obtained from orange juice to that  
24 of pomegranate juice, it is clear that *Bifidobacteria* survived better in orange  
25 juice, with very poor survival recorded for unprotected *Bifidobacteria* cells in

1 pomegranate juice. Also, *B. longum* survived significantly better ( $P < 0.05$ ) in  
2 pomegranate juice compared to *B. breve*. The poor survival of unprotected *B.*  
3 *longum* cells in pomegranate juice compared to orange juice has been  
4 demonstrated in other studies (Nualkaekul and Charalampopoulos, 2011;  
5 Nualkaekul et al., 2011), where *B. longum* viable cell concentration decreased by  
6  $\sim 8$  log CFU/ml and  $\sim 0.8$  log CFU/ml in pomegranate and orange juice  
7 respectively within 1 week of storage at 4 °C (Delete). The poor survival of  
8 *Bifidobacteria* in pomegranate juice compared to orange juice may be due to its  
9 higher acidity most likely due to the much higher level of citric and malic acid  
10 (Tables 2 and 3). This high acidity which is likely to have resulted in the sharp  
11 decrease in viability may also be responsible for the lack of oxidation of ascorbic  
12 acid by the cells. Thus, no reduction in the acid content was observed in  
13 pomegranate juice after 39 days compared to orange juice. The higher ascorbic  
14 acid concentration in orange juice (Table 2) may also aid *Bifidobacteria* survival.  
15 Ascorbic acid is an oxygen scavenger (Janjarasskul et al., 2013) and helps  
16 promote anaerobic conditions which are ideal for *Bifidobacteria* growth and  
17 survival. This could account for better survival of *Bifidobacteria* in orange juice  
18 as these cells are known to be very sensitive to oxygen. In addition, pomegranate  
19 juice contains a high amount of polyphenolic compounds which have antioxidant  
20 and anti-microbial properties (Al-Zoreky, 2009; Chidambara Murthy et al., 2002;  
21 Johanningsmeier and Harris, 2011).

22  
23 This experiment showed that orange juice has more potential as a vehicle for the  
24 delivery of probiotics compared to pomegranate juice. However, the addition of  
25 probiotics to pomegranate juice will help increase its present therapeutic

1 benefits, as it is known to contain several beneficial constituents including  
2 ellagic acid, punicic acid, flavonoids, anthocyanidins, anthocyanin, estrogenic  
3 flavonols and flavones (Devi et al., 2011). Thus, more research should be focused  
4 on the development of advanced technology to improve the survival of probiotics  
5 in pomegranate juice. One means of doing this could be combining  $\gamma$ -PGA with  
6 other biocompatible polymers such as polyglycerol sebacate (PGS) (Delete).

7  
8 Apart from storage, another concern with regards to probiotic delivery is the  
9 survival of the bacteria during gastrointestinal tract transit where they encounter  
10 the gastric juice in the stomach as well as bile in the small intestine. A large  
11 reduction in viability of probiotic bacteria has been demonstrated in the stomach,  
12 a region of high acidity (Cui et al., 2000; Su et al., 2011; Vasiljevic and Shah,  
13 2008). This study also found that free *Bifidobacterium* cells (*B. longum* and *B.*  
14 *breve*) died immediately with a 5 log CFU/ml reduction in cell concentration  
15 within 1 hr of incubation in simulated gastric juice and had reduced to  
16 undetectable levels within 2 hrs. Since  $\gamma$ -PGA is stable in acidic environments,  
17 and disintegrates in weaker acidic or neutral environments (Ho et al., 2006), it is  
18 a potential vehicle for the delivery of viable probiotic cells through the stomach  
19 environment. Results obtained from our experiment indicated that both  $\gamma$ -PGA-  
20 immobilised *B. longum* and *B. breve* cells showed marginal or no loss in viable  
21 cell concentration over a 4 hrs period of incubation in simulated gastric juice.

22  
23 A comparison of  $\gamma$ -PGA protection of *Bifidobacterium* cells to other approaches  
24 such as alginate-human like collagen, alginate-poly-l-lysine and milk chocolate  
25 (Cui et al., 2000; Khalf et al., 2010; Possemiers et al., 2010; Su et al., 2011) in

1 simulated gastric juice (Table 4) shows that  $\gamma$ -PGA could offer better protection  
2 to probiotic cells in gastric juice. The protection is possibly due to the fact that  $\gamma$ -  
3 PGA remains structurally stable in a low pH environment and disintegrates as the  
4 pH increases to above 6 (Ho et al., 2006) thus providing protection to cells from  
5 the highly acidic environment. In a pH stability study (Ho et al., 2006), strong  
6 intra-molecular hydrogen bonding existed in  $\gamma$ -PGA at pH 2.0, as at that pH it  
7 formed a stable compact  $\alpha$ -helix conformation. When the pH was increased to  
8 4.09 about 50% of the insoluble  $\alpha$ -helix conformations transformed into a linear  
9 random-coil conformation. At pH 6.0, all the intra-molecular hydrogen bonding  
10 was disrupted and a complete change in conformation was seen from  $\alpha$ -helix to  
11 random-coils (Delete). This property of  $\gamma$ -PGA is possibly useful for protecting  
12 probiotic bacteria in highly acidic gastric juice environment (pH 2.0). Moreover,  
13 and importantly for the intestinal delivery of probiotics, as  $\gamma$ -PGA reaches the  
14 intestine (pH ~ 6.0 - 6.5) the polymer should disintegrate because of the  
15 disruption of hydrogen bonds (Ho et al., 2006) leading to the release of the  
16 bacteria into the target site.

## 21 **5. CONCLUSIONS**

22 This research has shown that  $\gamma$ -PGA can be successfully used to immobilise  
23 probiotic *Bifidobacterium* strains and improve their survival during storage in  
24 acidic fruit juices, such as orange and pomegranate juice. This offers the  
25 opportunity to use fruit juices as alternatives to dairy products for probiotic

1 delivery. This would be particularly beneficial for individuals who are lactose  
2 intolerant or are allergic to milk proteins, as dairy products are presently the  
3 major sources for probiotic delivery. In addition,  $\gamma$ -PGA can potentially protect  
4 *Bifidobacterium* strains against the acidic conditions of the stomach during  
5 gastrointestinal tract passage. This study, along with our previous work, suggests  
6 that  $\gamma$ -PGA could potentially be used as a single protective agent for probiotic  
7 bacteria during freeze drying, storage in fruit juices and during transit through  
8 the gastrointestinal tract.

9  
10 Future work must include the protection of probiotic cells by  $\gamma$ -PGA in bile salt  
11 as well as their release in the intestine and subsequent colonisation of the host.  
12 Thus  $\gamma$ -PGA could have the potential to facilitate the effective delivery of  
13 sufficient numbers of viable probiotic bacteria to the gastrointestinal target sites  
14 leading to successful colonisation, which is important for delivering beneficial  
15 health effects (Delete).

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