Accepted Manuscript

Encapsulation of *Lactobacillus plantarum* in porous maize starch

Haiteng Li, Van Thi Thuy Ho, Mark S. Turner, Sushil Dhital

PII: S0023-6438(16)30506-0

DOI: 10.1016/j.lwt.2016.08.019

Reference: YFSTL 5668

To appear in: *LWT - Food Science and Technology*

Received Date: 26 March 2016

Revised Date: 28 July 2016

Accepted Date: 13 August 2016

Please cite this article as: Li, H., Thuy Ho, V.T., Turner, M.S., Dhital, S., Encapsulation of *Lactobacillus plantarum* in porous maize starch, *LWT - Food Science and Technology* (2016), doi: 10.1016/j.lwt.2016.08.019.

Encapsulation of *Lactobacillus plantarum* in Porous Maize Starch

Haiteng Li[1], *Van Thi Thuy Ho*[1], *Mark S. Turner*[1,2] *and Sushil Dhital*[3]*

[1]School of Agriculture and Food Sciences, The University of Queensland, St Lucia, Qld 4072, Australia.

[2]Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Qld 4072, Australia.

[3]ARC Centre of Excellence in Plant Cell Walls, Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Qld 4072, Australia.

* Corresponding author.

Phone: +61 7 3346 7373; Fax: +61 7 3365 1177. Email address: s.dhital@uq.edu.au (S. Dhital)

Abstract

This study investigated the survival of probiotic *Lactobacillus plantarum* 299v microencapsulated in native maize starch or partially hydrolyzed maize starches after acid, bile and heat treatments. Scanning electron microscopy and confocal scanning laser microscopy confirmed that naturally present cavities and channels in native maize starch were enlarged by enzymatic hydrolysis allowing them to be filled with probiotics. The formulations using the modified starches had significantly higher initial viable cells compared to native starch after freeze-drying. Compared to free cells, the microencapsulated probiotic bacteria showed a significant improvement in acid tolerance. When comparing unmodified and modified starches, the enzymatic treatments did not significantly improve relative survival, but did result in significantly higher total probiotic numbers after exposure to acid (pH=2.0, 1 h), bile salt (3% w/v, 4 h) and heat (60 °C, 15min). These results demonstrate that porous maize starch granules allow for a high probiotic loading efficiency and provide enhanced protection to various stressful conditions compared to free cells.

Key words:

Probiotics, Prebiotics, Encapsulation, Modified Starch, *Lactobacillus plantarum*

1. Introduction

In recent years, probiotics are becoming more commonly incorporated into functional foods. Health-promoting microorganisms play an important role in promotion of the gastrointestinal tract (GIT) health (Kailasapathy & Chin, 2000). *Lactobacillus plantarum* 299v is added in many food products, mainly fermented milks, because of its recognized health properties, such as improvement of irritable bowel syndrome (Niedzielin, Kordecki, & Birkenfeld, 2001) and vascular endothelial function (Malik et al., 2015). However, the applications are limited by viability of probiotic cells, which is affected by processing and storage conditions and the environment in the GIT (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012). In order to confer a functional effect within the body, a probiotic food should contain an adequate number of viable bacteria ($> 10^7$ CFU g^{-1} of food) to exert a probiotic effect (Corona-Hernandez et al., 2013). Various carrier material and preparation techniques are used and investigated for encapsulation of probiotics. Food-grade polymers such as alginate, chitosan, pectin, carrageenan, whey, gelatin and lipids are extensively studied to immobilize bacteria (Anal & Singh, 2007). Extrusion and emulsion techniques are commonly applied to produce calcium alginate beads in which a particularly strong molecular network can be formed to entrap cells. Although alginate hydrogel beads were found to have positive effects in protection of probiotics in a gastric environment and during storage, other polymers should be incorporated to improve stability of alginate microcapsules, as the beads formed by alginate alone have relatively low mechanical stability and entrapment of probiotics is not stable in the presence of chelating agents (Krasaekoopt, Bhandari, & Deeth, 2003; Willaert & Baron, 1996).

Starch that is slowly digestible or resistant to pancreatic amylases has a prebiotic effect which is of great interest as it is known to promote the growth of intestinal microflora and subsequently induces health benefits within the body (Toppings & Clifton, 2001). Improvements in glycaemic control and bowel health are associated with the regular intake of fermentable dietary fibre (Nugent, 2005). Furthermore, selection of starch with smaller granule size, white in colour and bland flavour could impart attractive sensory characteristics for food applications.

Encapsulation is one of the best approaches to obtain a synbiotic effect of probiotic bacteria and enzyme resistant starch (Fuentes-Zaragoza et al., 2011). Wang, Brown, Evans, and Conway (1999) found that high-amylose maize starch enhanced the tolerance of *Bifidobacterium* to low pH and bile acids. The incorporation of starch within alginate gel beads has been widely employed to provide synergistic protection for probiotic bacteria (Chan et al., 2011; Homayouni, Azizi, Ehsani, Yarmand, & Razavi, 2008; Kailasapathy, 2006; Muthukumarasamy, Allan-Wojtas, & Holley, 2006; Sabikhi, Babu, Thompkinson, & Kapila, 2010; Sultana et al., 2000; Xing et al., 2014). However, knowledge on the use of porous granular starch as an encapsulating material is still in its infancy.

In order to obtain further functional properties and improve the performance as wall materials, starch granules can be modified into porous capsules which have industrial applications such as drug delivery, flavor entrapment etc. The presence of structure features like pores, channels and cavities in maize starch (Dhital, Shrestha, & Gidley, 2010) provides an expandable space which can be filled with bacteria after amylase digestion. The structures increase effective surface area, and facilitate a relatively higher enzymatic hydrolysis susceptibility compared to potato starch (Dhital et al., 2010). Maize starch is thus more applicable to be modified into porous capsules by

enzymatic digestion than potato starch. The target core material could be physically adsorbed in the pores and cavities without any covalent bonding, and the adsorbed molecules could be completely released in a sustained pattern (Wang, Yuan, & Yue, 2015). It is also worthy of note that partially hydrolyzed maize starch has been shown to remain slowly digestible like untreated native starch (Zhang, Ao, & Hamaker, 2006), which suggests that it can be used for targeted delivery to the large intestine. The process of enzyme digestion of maize starch resulted in a 3~4 times increase of the magnitude of specific surface area (Gao, Li, Bi, Mao, & Adhikari, 2013). The porous maize starch thus could provide an ideal internal surface for adherence of the probiotic bacteria during processing. However, further studies are required to understand the effect of microstructure of porous starch on properties of microcapsules containing probiotics. More accurate delivery in the digestive tract may be subsequently achieved by manipulating preparation of starch materials.

In this study, modified maize starches from different enzymatic treatments were employed as wall materials to encapsulate *L. plantarum* 299v. The morphologies of modified wall materials and probiotic starch capsules were characterized. Furthermore, stability of the probiotic strain in microcapsules was investigated by exposure to simple stimulated GIT conditions and under mild heat treatment.

2. Materials and methods

2.1 Preparation of probiotic culture

A probiotic strain *L. plantarum* 299v was obtained from a commercial probiotic capsule (IBS Support, Ethical Nutrients, Brisbane Australia) and confirmed as the correct species using 16s

rDNA sequencing (Sreekumar, Al-Attabi, Deeth, & Turner, 2009). The strain was grown in de Man, Rogosa and Sharpe (MRS; Oxoid Ltd, UK) broth at 37 °C for 24 h and then harvested by centrifugation at 4400 × *g* for 10 min. The cell pellet was washed twice and resuspended with sterile 0.2 M sodium phosphate buffer (pH=6.3) to obtain concentrated (approximately 10^{10} CFU mL^{-1}) probiotic organisms.

2.2 Preparation of porous starch

Pancreatic α-amylase (PA [A6255 Sigma]), pancreatin (P [P-1750 Sigma]), fungal α-amylase (FA [10065 Sigma]) were purchased from Sigma-Aldrich, USA. The three enzymes and two treatment times (30 min and 120 min) were applied to modify native maize starch (22.2% amylose, Penford Australia Ltd., Australia). The starch slurry (5% w/v) was prepared with PBS (Sigma-Aldrich, USA) buffer and enzymes (0.5 unit per mg of starch) was mixed with the starch suspension. A control was prepared without addition of enzymes. The samples, in 50 mL Falcon tubes, were continuously stirred with a magnetic stirrer bar at 250 × *g* during the incubation time in a water bath maintained at 37 °C. After 30 and 120 min, porous starches were harvested by centrifugation of the tubes at 4000 × *g* for 5 min. The residue was washed three times with excess of ethanol to remove soluble sugars and the residual enzymes. Finally, starch sediments were transferred to petri plates and vacuum-oven dried overnight at 40 °C. Two batches were prepared for each treatment. These wall materials after preparation are referred to as P30, P120, PA30, PA120, FA30, FA120 and Native, respectively. The degree of hydrolysis of starch as measured by released maltose was 18-22% for 30 min and 36-41% for 120 minute hydrolysis. The degree of hydrolysis was however not significantly different among enzymes at each time point.

2.3 Encapsulation of *L. plantarum* cells

L. plantarum cells were encapsulated in the prepared maize starches according to the method described by Lahtinen, Ouwehand, Salminen, Forssell, and Myllärinen (2007) with slight modifications. The bacterial culture (6 mL) was transferred into sterile tubes containing 2.0 g starch and stirred at 600 × *g* for 3 h using an orbital shaker (Labtek, Australia). Then the mixture was allowed to settle for 2 h. The supernatant was carefully pipetted off. The sediment was placed in a petri plate and pre-cooled in a freezer (-20 °C) for 4 h before being freeze-dried overnight. For the coating material, gelatinized starch was prepared by heating native starch (2% w/v) in water for 15 min on a hotplate until it formed a gel. After cooling to room temperature, the gel solution was gently mixed with the freeze-dried powder (3 mL g^{-1} starch). Gelatinised starch coated porous starch granules was recrystallized at -20 °C overnight followed by freeze-drying for 24 h. After that, the microcapsules were collected and maintained in sterile 10 mL tubes at 4 °C prior to testing. The encapsulation process was conducted in duplicate, separately using two batches of prepared starches.

2.4 Determination of viable bacteria

The number of viable *L. plantarum* was counted by the spread-plate technique on MRS agar. The microcapsules (0.10 g) were first added into 0.9 mL peptone water (0.1% w/v) containing pancreatin (0.5 unit / mg starch). The pancreatin was added to hydrolyze the starch releasing the encapsulated bacteria. The plates were rotated on an orbital shaker (Labtek, Australia) at 600 × *g* for 15 min. Serial dilutions were made with peptone water (0.1% w/v) and 0.1 ml samples from each of three consecutive dilutions were spread onto MRS agar. The agar plates were incubated at

37 °C for 36 h under anaerobic conditions generated by AnaeroGen 3.5 L (Oxoid Ltd). Colony forming units (CFU) were enumerated and recorded for plates on which 15-300 colonies can be viewed. The loss in bacteria viability was calculated as follows:

Reduction of viable bacteria = log N_0 – log N, where, N_0 and N are viable count (CFU g^{-1} or CFU mL^{-1}) before and after treatments (acid, bile and heat), respectively.

2.5 Resistance to acid and bile salt

Acid and bile salt stress survival experiments of microencapsulated and free probiotic bacteria were carried out in accordance with the method of Ding and Shah (2007). Free bacterial suspension (9.46 log CFU mL^{-1}) was used as a control. For acid stress, MRS broth was modified to pH 2.0 using 5.0 M HCl before sterilization by autoclaving at 121 °C for 15 min. The acidified broth (0.90 mL) was added into a test tube (2 mL) containing 0.10 g microcapsules or 0.10 mL free cell suspension. After incubation at 37 °C for 1 h and 2 h, 1 M NaOH was added to neutralize the acid. For bile salt stress, MRS broth containing 3 % (w/v) Oxgall bile salt (Chem-Supply, Australia) was adjusted to pH 5.8 using 5.0 M HCl. The bile salt solution (0.90 mL) was added into a test tube (2 mL) containing 0.10 g microcapsules or 0.10 mL free cell suspension and incubated at 37 °C for 2 h and 4 h separately. For determination of viable bacteria, microcapsules were centrifuged from broths (10000 × *g*, 5 min) and bacteria were released from starches as described in Section 2.4. For free bacteria, subsequent serial dilutions were vortexed for 30 s individually to disperse bacteria clusters which were formed during incubation. Enumeration of viable cells was carried out by the method described in Section 2.4. For each batch of microcapsules, duplicate tests were performed separately.

2.6 Resistance to heat

Evaluation of the stability of microencapsulated and free *L. plantarum* under mild heat treatment was carried out according to Mandal, Puniya, and Singh (2006). Before exposure to a dry block heater (60 °C), 0.10 g microcapsules or 0.10 mL free cells suspension was inoculated into 0.90 mL sodium phosphate buffer (pH=6.3) in a plastic test tube (2 mL). After 15 min incubation, the tubes were cooled to room temperature (23±2 °C) and the total number of viable cells was detected as described in Section 2.4.

2.7 Confocal laser scanning microscopy (CLSM)

CLSM (LSM 700, Carl Zeiss, Germany) was used to visualize the distribution of probiotic bacteria using a LIVE/DEAD BacLight kit L7012 (Molecular Probes^{TM}, Oregon, USA). Bacterial materials were stained following the manufacturer's protocol. Dry powder (~ 10 μg) was gently mixed with 10 μl of staining mixture (SYTO 9 and propidium iodide fluorescent dyes) on a microscope slide and covered with a square coverslip. After staining for 30 min in darkness, the samples were observed with 488 nm excitation wavelength and × 63 magnification objective (oil immersion).

2.8 Morphology of porous starches

Scanning electron microscopy was used to monitor the morphological structure of starch after enzymatic treatment. The dried samples were thinly spread onto circular metal tubes covered with double sided carbon tape and coated with approximately 5 nm of platinum in an argon gas environment. The images were acquired using a Philips XL30 scanning electron microscope (Philips, Eindhoven, Netherlands) under an accelerating voltage of 5 kV.

2.9 Statistical analysis

For the each independent batch (n=2), duplicate samples were analysed. Average of duplicate tests for each batch was calculated prior to the analysis of significant differences ($p < 0.05$) using Tukey's simultaneous test in Minitab 17. All experimental data are expressed as mean ±S.D.

3. Results

3.1 Microstructure of the microcapsules

The microstructure of native and partially hydrolyzed maize starch was observed in scanning electron micrographs (Fig. 1). The images confirmed that the enzymatic action resulted in porous structures in the round and polygonal granules. The surface of native starch without amylase treatment (Fig. 1 B) remained relatively smooth and showed no visible holes, indicating the preparation for the control did not affect its morphology. Some tiny hollows and cracks on native granules are visible at higher magnification (Dhital et al., 2010). An increased size and numbers of pores were observed after enzymatic digestion (Fig. 1 C-H). Consistent with the degree of hydrolysis, surface pores and the pore sizes are visually similar among starches treated with amylase from various sources. Larger pores were formed by interconnection of holes which increased in number during enzyme treatment duration.

Confocal microscopy and nucleic acid staining technique were employed to observe the distribution of probiotic bacteria in starch materials. The porous starch granules clearly showed the 'inside out' hydrolysis pattern with the enlargement of cavities (Dhital et al., 2010) which entrapped the green-fluorescent bacteria in expanded cavities (Fig. 2 B and C), whereas unmodified starch only absorbed the bacteria on the surface (Crittenden et al., 2001) (Fig. 2 A).

The green fluorescent bacteria that had dimensions of 1 to 2 µm in length were stained by SYTO9 (green) but not by PI (red), which indicated that the bacterial membrane was not permeablized for the larger molecule of PI to pass through, suggesting that the bacteria were viable. Relatively higher cell loading can be observed in the modified starch despite the same bacterial suspension being used for the encapsulation process. As shown in Fig. 2 A, a small amount of bacteria adhered to the granule surface, which suggested that *L. plantarum* 299v is not a highly adhering strain for maize starch. Regarding the microstructure inside the starch granule under transmitted light, the 120-min hydrolyzed starch granule showed a relatively larger cavity than the 30-min treated granules. The observation is consistent with the SEM image (Fig. 1) where the starch granule cavities showed increased depth after digestion for a longer duration.

3.2 Viable cell numbers after microencapsulation process

The encapsulated cell numbers after the microencapsulation process were significantly improved by enzymatic modification (Table 1). Initial cell numbers were around 9 log CFU g^{-1} in all the modified starches compared to 7.77 log CFU g^{-1} for native starch. The result is consistent with the observation by CLSM that modified starch had a higher cell loading. However, there was no significant difference ($p > 0.05$) among the different porous starches.

3.3 Acid and bile salt tolerance of free and encapsulated *L. plantarum*

Microencapsulated bacteria showed greater acid resistance, with significantly less reduction of viability ($p < 0.05$) over the entire treatment period (Fig. 3 A). Free bacteria showed a rapid loss of viable cells, having 2.43 and 4.66 log CFU mL^{-1} reduction (from an initial viable count of 9.46 log

229 CFU mL^{-1}) at the end of 1-h and 2-h exposure to acid, respectively. By contrast, the loss of viable
230 bacteria after microencapsulation occurred at a relatively slow rate, with less than 2 log CFU g^{-1}
231 reduction by the end of incubation in the high acid environment. The viable counts of *L.*
232 *plantarum* encapsulated by modified starches remained more than 10^7 CFU g^{-1} after 2-h exposure
233 to acid (Table 1), which were significantly higher than free cells.
234 As for the microparticle prepared with native maize starch, the bacterial counts decreased from
235 7.77 to 6.69 and 6.15 log CFU g^{-1} at the end of 1 h and 2 h at pH 2.0, respectively (Table 1).
236 Compared to modified starches, native maize starch retained a significantly lower number of
237 viable cells after 1-h exposure to acid. However, log reductions of bacteria in unmodified and
238 modified starches were similar, which indicated that the enzymatic modification on starch
239 granules has little effect towards the acid resistance of *L. plantarum.*
240 The effect of the bile salt solution on the survivability of free and encapsulated *L. plantarum* was
241 also investigated (Fig. 3 B). There was no significant difference among the reduction of viable
242 counts of encapsulated or free *L. plantarum* in bile salt solution. Enzymatic modification did not
243 improve the protection effect of maize granules used as wall materials during exposure to bile salt
244 ($p > 0.05$). The reductions of free cells and bacteria encapsulated with native starch were 1.81 log
245 CFU mL^{-1} and 1.73 log CFU g^{-1} after 4-h exposure to bile salt, respectively, while the figures of
246 modified starches were less than 1.10 log CFU g^{-1}. Encapsulated cells prepared with native starch
247 granules decreased to 6.95 and 6.04 log CFU g^{-1} at the end of 2 h and 4 h incubation with bile salts,
248 respectively (Table 1). Compared to modified maize starch, native starch contained significantly
249 less viable bacteria after the 4-h period.

3.4 Stability of free and encapsulated *L. plantarum* under heat treatment

The reduction in cell viability of free and microencapsulated *L. plantarum* after the 60 °C heat treatment for 15 min is shown in Fig. 4. Encapsulation with maize starch granules (except unmodified starch and FA 30) combined with gelatinized starch coating significantly improved the heat tolerance of *L. plantarum* ($p < 0.05$). The enzymatic modification did not result in less log reduction after heat treatment compared with native starch ($p > 0.05$), but the viable cell counts of modified starches were significantly higher than unmodified maize starch after incubation at 60 ^{o}C for 15 min ($p < 0.05$) (Table 1). *L. plantarum* encapsulated in porous starches either from 30 min digestion or 120 min digestion showed similar survivability and therefore, the effect of digestion time was not significant ($p > 0.05$).

4. Discussion

Starch is not usually used alone for encapsulation, even though it is frequently reported as a supplementary component in microcapsule formulations. However, the current model of prebiotic–probiotic symbiosis was effective to study the effect of microstructure of porous starch on properties of the microcapsules. The presence of porous maize starch was essential for improving the recovery of encapsulated *L. plantarum* 299v formulations during the freeze-drying process. In addition, the viability remained relatively higher than the formulations using native starch after simulated digestion and heating treatments (Table 1). This could provide a robust delivery strategy for probiotics in food products and ultimately following ingestion.

4.1 Preparation of starch materials

The choice of the type of starch is critical during the preparation of wall materials. We chose maize starch because it is slowly digestible and has the potential to deliver entrapped bacteria to the large colon. Limited amylase treatment can hydrolyze the starch in an 'inside out' pattern creating a void space allowing bacteria to diffuse into, as shown in Fig. 1, which is consistent with previous findings (Dhital et al., 2010; Dura, Błaszczak, & Rosell, 2014). In contrast, amylase action in potato starch lacking such structural features results in exo-corrosion followed by endo-corrosion (Dhital et al., 2010). Whilst the porous starch can also be obtained by other methods such as ultrasonic treatment (Luo et al., 2008) and microwave radiation (Luo, He, Fu, Luo, & Gao, 2006), these methods may not result in enlargement of cavities and channels sufficient for probiotic bacteria encapsulation as observed for amylase digested granules. Regarding the catalytic pattern of amylase, the relatively small granule size, a rough surface and the presence of surface pores and channels promote enzyme diffusion and adsorption leading to the rapid formation of widened pores, cavities and channels (Dhital et al., 2010). Additionally, adhesion of cells to starch granules was reported to vary among strains and starch types (Crittenden et al., 2001) and bacteria attached to a surface showed more resistance to hostile environments (Wang et al., 1999). Thus, a possible approach to increase bacterial survival is the selection of starch types for probiotic bacteria according to bacterial adherence, which is related to bacterial surface proteins that particularly bind to α-1,4-linked glucose saccharides (Crittenden et al., 2001). Encapsulation of the probiotics in the starch system can be obtained without addition of gelling agents or adjustment of pH, while the ionic polysaccharides in presence of appropriate salt or ionic condition could form gel/matrix that can entrap the bacteria. Thus, the porous maize

293 starch could be more processing friendly. A direct comparison of these different encapsulation
294 matrices would be interesting to determine in future work.
295 Considering the different enzymatic treatments, the changes in digestion time or amylase type did
296 not yield any difference in the performance of starch materials, though there was some difference
297 in microstructure between 120-min and 30-min hydrolyzed starch (Fig. 1). We found that the three
298 types of amylase (P, PA, FA) were each effective to create porous structure. On the other hand, the
299 digestion time may affect release properties of *L. plantarum*.
300 Considering the application point of view, the breakdown of the porous starch as wall material in
301 the digestive tract forms spaces for cell release. Thus, the release pattern of the encapsulated
302 bacteria in the starch system is associated with enzyme sensitivity of the prepared starches.
303 Shrestha et al. (2012) investigated changes of supramolecular/molecular structure of maize starch
304 during *in vitro* digestion and concluded that the granular architecture plays a major role in
305 controlling starch susceptibility to digestive enzymes. The characterization of structural features of
306 the modified starches is needed in future work to yield more plentiful information to predict the
307 release pattern of the starch encapsulation.

308 **4.2 Drying process**

309 Probiotic products are widely processed into a dried form which is stable during preservation and
310 convenient in handling. Freeze drying is frequently used in probiotic production procedures and
311 has a significant effect on the bacterial survival. The cellular membrane can be damaged by the
312 formation of intracellular ice crystals during the freezing process (Conrad, Miller, Cielenski, & de
313 Pablo, 2000) and the removal of water during the subsequent sublimation process. Cryoprotectant

agents have been employed to reduce the loss of viability due to the freeze-drying process (Capela, Hay, & Shah, 2006). Osmotic differences between bacteria and their external environment can be reduced when compatible cryoprotectant agents are used (Kets, Teunissen, & Bont, 1996). Sultana et al. (2000) demonstrated that incorporation of Hi-maize starch into an alginate formulation increased viability of encapsulated *Lactobacillus casei* in wet or freeze dried beads. Likewise, Etchepare et al. (2016) found that the initial probiotic count of alginate microcapsules containing Hi-maize (7.69 log CFU g^{-1}) was higher than the alginate microcapsules alone (6.65 log CFU g^{-1}) after freeze-drying. Akalın and Erişir (2008) added prebiotics including inulin and oligofructose into ice cream and found greater survival to freezing, thus indicating that prebiotics can have a positive effect on bacteria viability. In this work, porous starch resulted in higher recovery of encapsulated bacteria after the drying process (Table 1), suggesting that replacement of native starch with porous starch may further reduce the loss of viable cells during drying. Minor cold shocks prior to freeze-drying, to purposefully induce bacterial stress responses, could also be considered.

4.3 Viability test

The low survival rate of lactic acid bacteria under GIT conditions is a problem to ensure that sufficient quantities reach the large intestine to exhibit functionality. Stimulated gastrointestinal conditions have been widely applied to evaluate the effectiveness of encapsulation methods. There was a greater reduction of free *L. plantarum* under low acid environments than that encapsulated by starch matrix (Fig. 3 A). The bile salt reductions in plate counts tended to be less for bacteria encapsulated in porous starches than for free and native-encapsulated cells, but these differences

were not significant (Fig. 3 B). *L. plantarum* was more sensitive to acidity than bile salts, which is in agreement with previous reports (Ding & Shah, 2007; Liong & Shah, 2005). Bacterial viability is impaired during exposure to acids because energy consumption is increased to maintain a neutral intracellular pH level and metabolic reactions are inhibited (Cotter & Hill, 2003). Although the effect of acid and bile salts are investigated, the activity salivary amylase and pepsin in vivo, may affect the survivability of the probiotic cells. Thus, more detailed investigation in *vivo* or *in vitro* models is needed to elucidate the effect of oral-gastric conditions of cells viability.

Lactobacillus acidophilus LA1 entrapped by alginate and starch demonstrated better survival at low pH and high bile salt concentrations (Sabikhi et al., 2010). *L. acidophilus* CICC 6075 absorbed by porous starch and subsequently coated with alginate showed enhanced survival after incubation at pH 1.5 for 3 h (Xing et al., 2014). Cell viability during lyophilization and storage was significantly improved by filling starch into a Ca–alginate hydrogel (Chan et al., 2011). Physically modified resistant starch in combination with whey protein isolate in microencapsulated *L. rhamnosus* GG formulations also had a positive effect on bacterial survival in pH 3.5 citrate buffer (Ying et al., 2013).

However, there are conflicting studies which have reported that starch granules have no effect on stability of probiotic bacteria. The technology to encapsulate two *Bifidobacterium longum* strains with partially digested potato starch as wall material and amylose as coating material was found to result in similar survival rate as free form bacteria during storage (Lahtinen et al., 2007). O'Riordan, Andrews, Buckle, and Conway (2001) reported that octenyl succinated waxy maize starch did not affect viability of *Bifidobacterium* cells during exposure to ambient temperature

(19-24 °C) and acid stress. Thus, differences of the encapsulation techniques, bacteria strains and viability test procedures likely had impact on the results of previous attempts to increase probiotic survival. The acid and bile resistance are suggested to be strain-specific (Ding & Shah, 2007; Liong & Shah, 2005).

Temperature is a critical factor for the viability of probiotic bacteria. Inactivation of cells at high temperature can be related to many factors including fatty acid oxidation, DNA damage, protein denaturation and the formation of free radicals (Castro, Teixeira, & Kirby, 1997). The production of compounds including metabolic acids and bacteriocins are possibly responsible for cell death at high temperature (Fu & Chen, 2011). Compared to free cells, loss of viable cells at the high temperature can be reduced by the encapsulation method (Fig. 4). The result is consistent with the observation of Xing et al. (2014).

4.4 Microenvironment of porous starch

We aimed to immobilize the probiotic bacteria in starch matrices with the preservation of desired biological activity. The confocal micrographs showed that enlarged cavities of modified starch trapped the rod shaped bacteria (Fig. 2). The confinement of the cells to starch cavities or surfaces was hypothesized to facilitate the separation of cells from lethal agents in hostile environments. The changes in starch microstructure resulted in the improvement of initial viable bacteria (Table 1). The hollow structure may allow the microcapsule to contain more bacteria and therefore presumably to have more cells separated from bacterial culture. Bacteria entrapped inside porous structure possibly had less exposure to low temperature which subsequently triggers formation of ice crystals. Another possible reason is that the molecular mobility of water is depressed by porous

structures and thus cell dehydration could be decreased, because the adsorption capacity of maize starch was shown to increase after enzymatic modification (Gao et al., 2013; Wang et al., 2015). The starch matrices also offered effective protection against acidic solution (Fig.3 A), possibly by reducing exposure to H^+ ions in bulk solution. However, unmodified starch and modified starches offered similar protection to the probiotic bacteria under acid, bile and heat treatments, which may suggest that physical isolation within the porous structure is not sufficient to protect probiotic bacteria from the surrounding environments. The gelatinized starch solution (coating material) might not be able to sufficiently seal the pores of the porous starch.

5. Conclusion

In this work, we modified maize starch to obtain porous structures for the entrapment of probiotic bacteria. The results showed that enzymatic modification is an effective method to increase encapsulation yields when starch-based material is used for encapsulation and further ensures that the number of viable bacteria remain above the minimum dosage as requirement during food processing and digestion in the body. Major opportunities for enhancing synergistic properties between resistant starch and probiotics may come from rational selection of the kind of starches used for encapsulation in combination with accurate manipulation of preparation techniques.

Acknowledgements

The authors would like to thank Prof Mike Gidley for valuable suggestions and feedback on the manuscript.

References

399 Akalın, A. S., & Erişir, D. (2008). Effects of inulin and oligofructose on the rheological
400 characteristics and probiotic culture survival in low-fat probiotic ice cream. *Journal of*
401 *Food Science, 73*(4), M184-M188.
402 Anal, A. K., & Singh, H. (2007). Recent advances in microencapsulation of probiotics for
403 industrial applications and targeted delivery. *Trends in Food Science & Technology, 18*(5),
404 240-251.
405 Capela, P., Hay, T. K. C., & Shah, N. P. (2006). Effect of cryoprotectants, prebiotics and
406 microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt.
407 *Food Research International, 39*(2), 203-211.
408 Castro, H. P., Teixeira, P. M., & Kirby, R. (1997). Evidence of membrane damage in *Lactobacillus*
409 *bulgaricus* following freeze drying. *Journal of Applied Microbiology, 82*(1), 87-94.
410 Chan, E.-S., Wong, S.-L., Lee, J.-S., Lee, P.-P., Ti, T. B., Zhang, Z., . . . Yim, Z.-H. (2011). Effects
411 of starch filler on the physical properties of lyophilized calcium–alginate beads and the
412 viability of encapsulated cells. *Carbohydrate Polymers, 83*(1), 225-232.
413 Conrad, P. B., Miller, D. P., Cielenski, P. R., & de Pablo, J. J. (2000). Stabilization and
414 preservation of *Lactobacillus acidophilus* in saccharide matrices. *Cryobiology, 41*(1),
415 17-24.
416 Cook, M. T., Tzortzis, G., Charalampopoulos, D., & Khutoryanskiy, V. V. (2012).
417 Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled*
418 *Release, 162*(1), 56-67.
419 Corona‐Hernandez, R. I., Álvarez‐Parrilla, E., Lizardi‐Mendoza, J., Islas‐Rubio, A. R.,
420 Rosa, L. A., & Wall‐Medrano, A. (2013). Structural Stability and Viability of
421 Microencapsulated Probiotic Bacteria: A Review. *Comprehensive Reviews in Food Science*
422 *and Food Safety, 12*(6), 614-628.
423 Cotter, P. D., & Hill, C. (2003). Surviving the acid test: Responses of gram-positive bacteria to
424 low pH. *Microbiology and Molecular Biology Reviews, 67*(3), 429-429.
425 Crittenden, R., Laitila, A., Forssell, P., Mättö, J., Saarela, M., Mattila-Sandholm, T., & Myllärinen,
426 P. (2001). Adhesion of *Bifidobacteria* to granular starch and its implications in probiotic
427 technologies. *Applied and Environmental Microbiology, 67*(8), 3469-3475.
428 Dhital, S., Shrestha, A. K., & Gidley, M. J. (2010). Relationship between granule size and in vitro
429 digestibility of maize and potato starches. *Carbohydrate Polymers, 82*(2), 480-488.
430 Ding, W. K., & Shah, N. P. (2007). Acid, bile, and heat tolerance of free and microencapsulated
431 probiotic bacteria. *Journal of Food Science, 72*(9), M446-M450.
432 Dura, A., Błaszczak, W., & Rosell, C. M. (2014). Functionality of porous starch obtained by
433 amylase or amyloglucosidase treatments. *Carbohydrate Polymers, 101*(1), 837-845.
434 Etchepare, M. d. A., Raddatz, G. C., Cichoski, A. J., Flores, É. M. M., Barin, J. S., Queiroz Zepka,
435 L., . . . de Menezes, C. R. (2016). Effect of resistant starch (Hi-maize) on the survival of
436 *Lactobacillus acidophilus* microencapsulated with sodium alginate. *Journal of Functional*
437 *Foods, 21*, 321-329.
438 Fu, N., & Chen, X. D. (2011). Towards a maximal cell survival in convective thermal drying
439 processes. *Food Research International, 44*(5), 1127-1149.
440 Fuentes-Zaragoza, E., Sánchez-Zapata, E., Sendra, E., Sayas, E., Navarro, C., Fernández-Lõpez, J.,

& Pérez-Alvarez, J. A. (2011). Resistant starch as prebiotic: A review. *Starch - Stärke, 63*(7), 406-415.

Gao, F., Li, D., Bi, C.-h., Mao, Z.-h., & Adhikari, B. (2013). Application of various drying methods to produce enzymatically hydrolyzed porous starch granules. *Drying Technology, 31*(13-14), 1627-1634.

Homayouni, A., Azizi, A., Ehsani, M. R., Yarmand, M. S., & Razavi, S. H. (2008). Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. *Food Chemistry, 111*(1), 50-55.

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

Kailasapathy, K., & Chin, J. (2000). Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immunology and cell biology, 78*(1), 80-88.

Kets, E., Teunissen, P., & Bont, J. d. (1996). Effect of compatible solutes on survival of lactic Acid bacteria subjected to drying. *Applied and Environmental Microbiology, 62*(1), 259-261.

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

Lahtinen, S. J., Ouwehand, A. C., Salminen, S. J., Forssell, P., & Myllärinen, P. (2007). Effect of starch- and lipid-based encapsulation on the culturability of two *Bifidobacterium longum* strains. *Letters in Applied Microbiology, 44*(5), 500-505.

Liong, M. T., & Shah, N. P. (2005). Acid and bile tolerance and cholesterol removal ability of *Lactobacilli* Strains. *Journal of Dairy Science, 88*(1), 55-66.

Luo, Z., Fu, X., He, X., Luo, F., Gao, Q., & Yu, S. (2008). Effect of ultrasonic treatment on the physicochemical properties of maize starches differing in amylose content. *Starch - Stärke, 60*(11), 646-653.

Luo, Z., He, X., Fu, X., Luo, F., & Gao, Q. (2006). Effect of microwave radiation on the physicochemical properties of normal maize, waxy maize and amylomaize V starches. *Starch - Stärke, 58*(9), 468-474.

Malik, M., Widlansky, M. E., Suboc, T., Coulliard, A., Su, J., Salzman, N. H., & Baker, J. E. (2015). Abstract 10898: The probiotic bacterium *Lactobacillus plantarum* 299v improves vascular endothelial function and decreases inflammatory biomarkers in men with established cardiovascular disease. *Circulation, 132*(Suppl 3), A10898.

Mandal, S., Puniya, A. K., & Singh, K. (2006). Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298. *International Dairy Journal, 16*(10), 1190-1195.

Muthukumarasamy, P., Allan‐Wojtas, P., & Holley, R. A. (2006). Stability of *Lactobacillus reuteri* in different types of microcapsules. *Journal of Food Science, 71*(1), M20-M24.

Niedzielin, K., Kordecki, H., & Birkenfeld, B. (2001). A controlled, double-blind, randomized study on the efficacy of *Lactobacillus plantarum* 299V in patients with irritable bowel syndrome. *European journal of Gastroenterology & Hepatology, 13*(10), 1143.

Nugent, A. P. (2005). Health properties of resistant starch. *Nutrition Bulletin, 30*(1), 27-54.

O'Riordan, K., Andrews, D., Buckle, K., & Conway, P. (2001). Evaluation of microencapsulation of a *Bifidobacterium* strain with starch as an approach to prolonging viability during storage. *Journal of Applied Microbiology, 91*(6), 1059.

Sabikhi, L., Babu, R., Thompkinson, D. K., & Kapila, S. (2010). Resistance of microencapsulated *Lactobacillus acidophilus* LA1 to processing treatments and simulated gut conditions. *Food and Bioprocess Technology, 3*(4), 586-593.

Shrestha, A. K., Blazek, J., Flanagan, B. M., Dhital, S., Larroque, O., Morell, M. K., . . . Gidley, M. J. (2012). Molecular, mesoscopic and microscopic structure evolution during amylase digestion of maize starch granules. *Carbohydrate Polymers*, 90(1), 23-33.

Sreekumar, R., Al-Attabi, Z., Deeth, H. C., & Turner, M. S. (2009). Volatile sulfur compounds produced by probiotic bacteria in the presence of cysteine or methionine. *Letters in Applied Microbiology, 48*(6), 777.

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

Topping, D. L., & Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological reviews, 81*(3), 1031-1064.

Wang, X., Brown, I. L., Evans, A. J., & Conway, P. L. (1999). The protective effects of high amylose maize (amylomaize) starch granules on the survival of *Bifidobacterium* spp. in the mouse intestinal tract. *Journal of Applied Microbiology, 87*(5), 631-639.

Wang, X., Yuan, Y., & Yue, T. (2015). The application of starch-based ingredients in flavor encapsulation. *Starch - Stärke, 67*(3-4), 225-236.

Willaert, R. G., & Baron, G. V. (1996). Gel entrapment and micro-encapsulation: Methods, applications and engineering principles. *Reviews in Chemical Engineering, 12*(1-2), 5-205.

Xing, Y., Xu, Q., Ma, Y., Che, Z., Cai, Y., & Jiang, L. (2014). Effect of porous starch concentrations on the microbiological characteristics of microencapsulated *Lactobacillus acidophilus*. *Food & Function, 5*(5), 972-983.

Ying, D., Schwander, S., Weerakkody, R., Sanguansri, L., Gantenbein-Demarchi, C., & Augustin, M. A. (2013). Microencapsulated *Lactobacillus rhamnosus* GG in whey protein and resistant starch matrices: Probiotic survival in fruit juice. *Journal of Functional Foods, 5*(1), 98-105.

Zhang, G., Ao, Z., & Hamaker, B. R. (2006). Slow digestion property of native cereal starches. *Biomacromolecules, 7*(11), 3252-3258.

519 **Table 1**

520 Viable bacteria count (log CFU g^{-1}) of encapsulated *L. plantarum* 299v after the
521 microencapsulation process, incubation in high acid (pH=2.0), bile salt (3% w/v) condition and
522 heat (60 ^{o}C) condition. * indicates statistically significant difference ($p < 0.05$) within the same
523 column. The viability data were from duplicate analysis of duplicate samples (n= 2).

		Acid		**Bile salt**		**Heat**
Wall materials	**Initial count**	1h	2h	2h	4h	15 min
Native	7.77±0.41*	6.69±0.17*	6.15±0.36	6.95±0.62*	6.04±0.47*	4.87±0.48*
PA30	9.11±0.14	8.34±0.37	7.55±0.85	8.34±0.06	8.13±0.49	6.40±0.13
PA120	9.06±0.13	8.29±0.26	7.29±0.96	8.37±0.05	8.00±0.30	6.60±0.21
P30	9.21±0.08	8.27±0.32	7.48±0.74	8.55±0.08	8.15±0.25	6.51±0.15
P120	8.94±0.05	8.23±0.14	7.68±0.37	8.46±0.10	7.99±0.42	6.50±0.08
FA30	8.95±0.16	7.99±0.08	7.12±0.03	8.32±0.16	7.98±0.58	6.19±0.27
FA120	8.96±0.37	8.05±0.23	7.25±0.29	8.28±0.14	7.97±0.51	6.43±0.15

524

525

Figure Captions

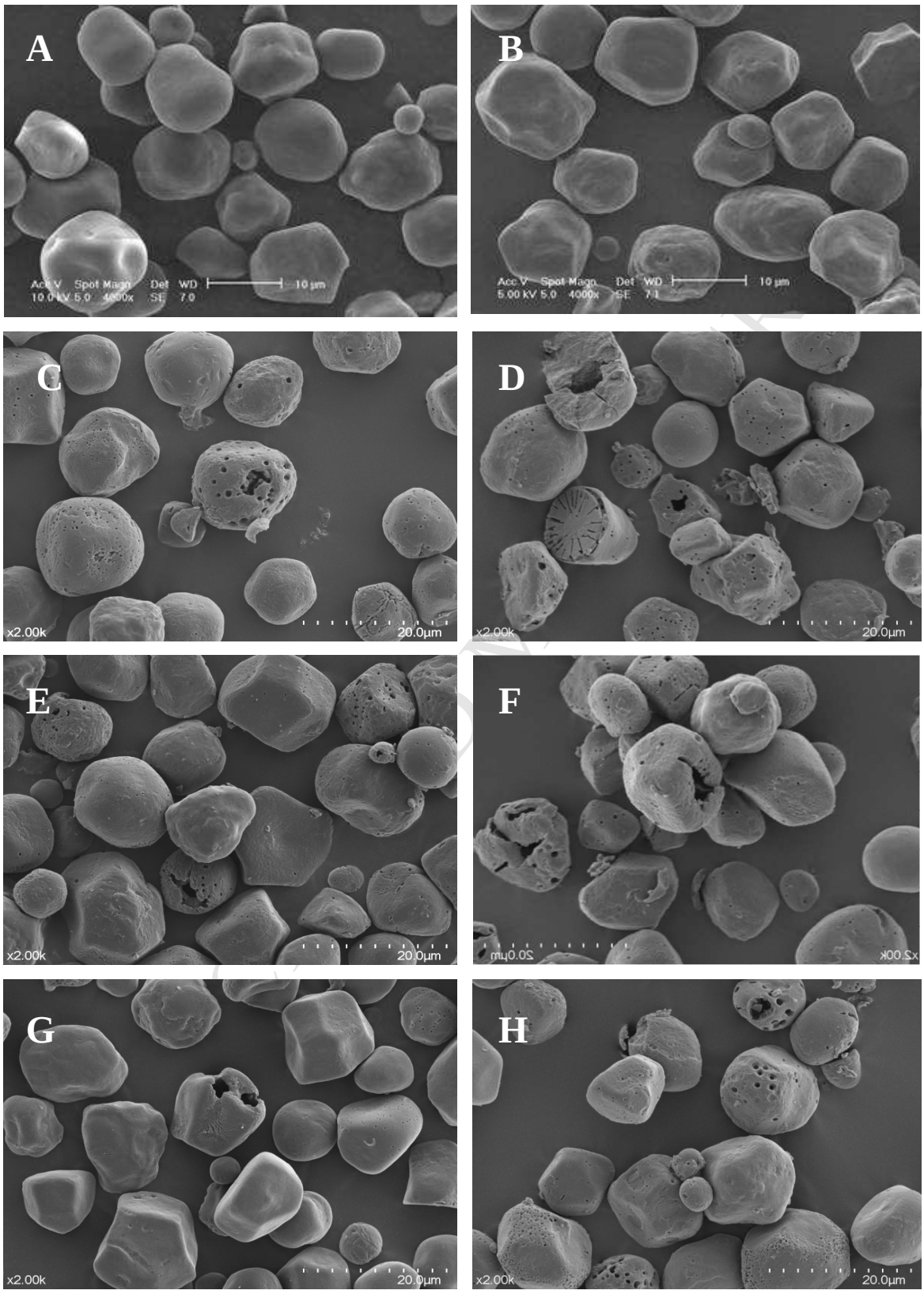
Fig. 1. Scanning electron micrographs of starch granules before (A) and after enzymatic digestion: control (B), pancreatic α-amylase (C and D), pancreatin (E and F), and fungal α-amylase (G and H); 30-min digestion (C, E and G), and 120-min digestion (D, F and H).

Fig. 2. CLSM images of (A) unmodified starch with cells attached to the granule surface, (B) 30-min hydrolyzed microcapsule and (C) 120-min hydrolyzed microcapsule with cells trapped inside (arrow shows large pore opening).

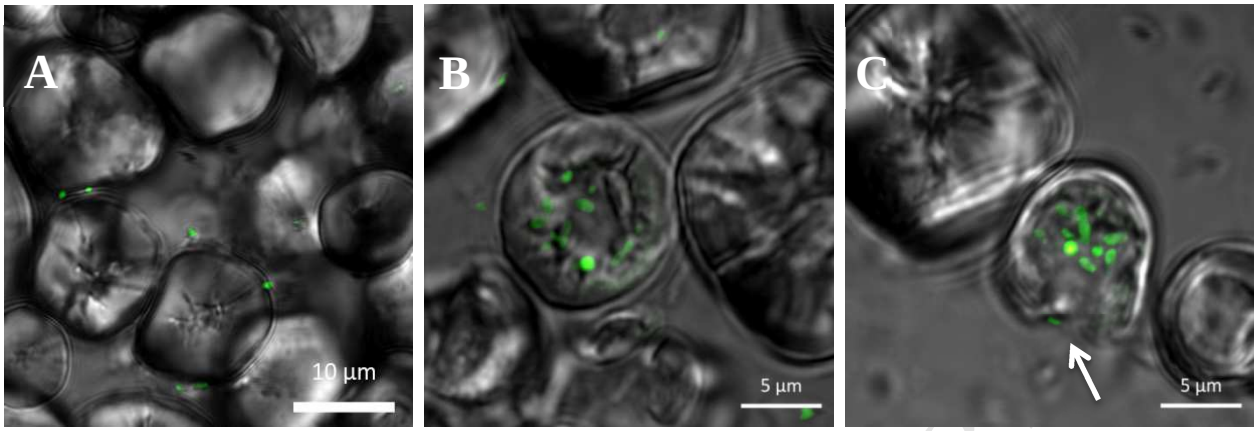
Fig. 3. Reduction of viable count of free or microencapsulated *L. plantarum* 299v during incubation (37 °C) in (A) high acid (pH=2.0) and (B) bile salt (3% w/v) conditions. Mean and standard errors were calculated using data of duplicate analysis of duplicate samples (n= 2). * indicates statistically significant difference ($p < 0.05$) at the same time point.

Fig. 4. Reduction of viable count of free or microencapsulated *L. plantarum* 299v after exposure to heat (60 °C) in sodium phosphate buffer (pH=6.3) for 15 min. Mean bars with different letters (a-b) are significantly different ($p < 0.05$). Mean and standard errors were calculated using data of duplicate analysis of duplicate samples (n= 2).

Fig. 1.

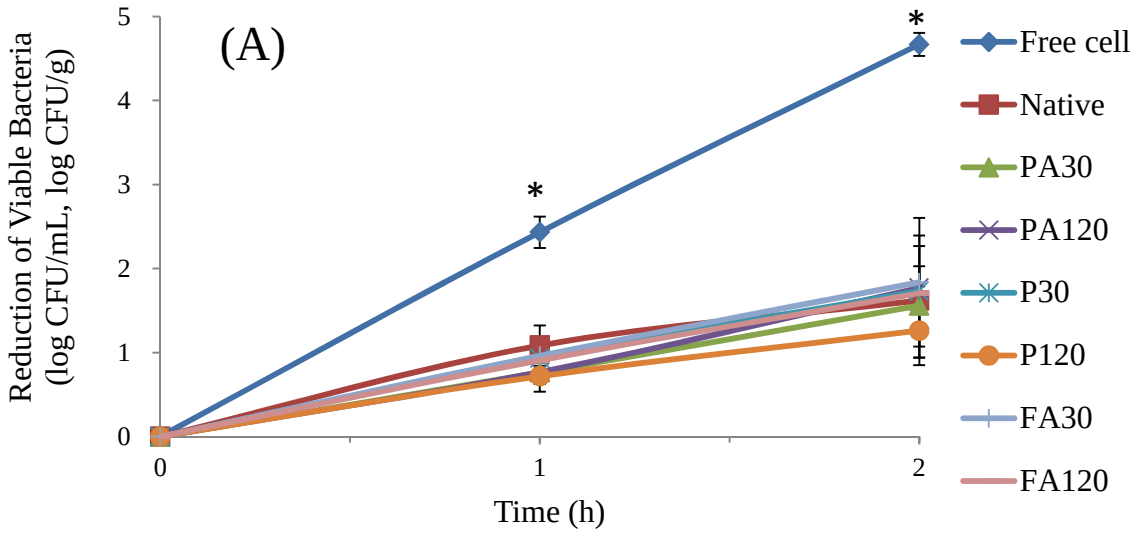


552 **Fig. 2.**

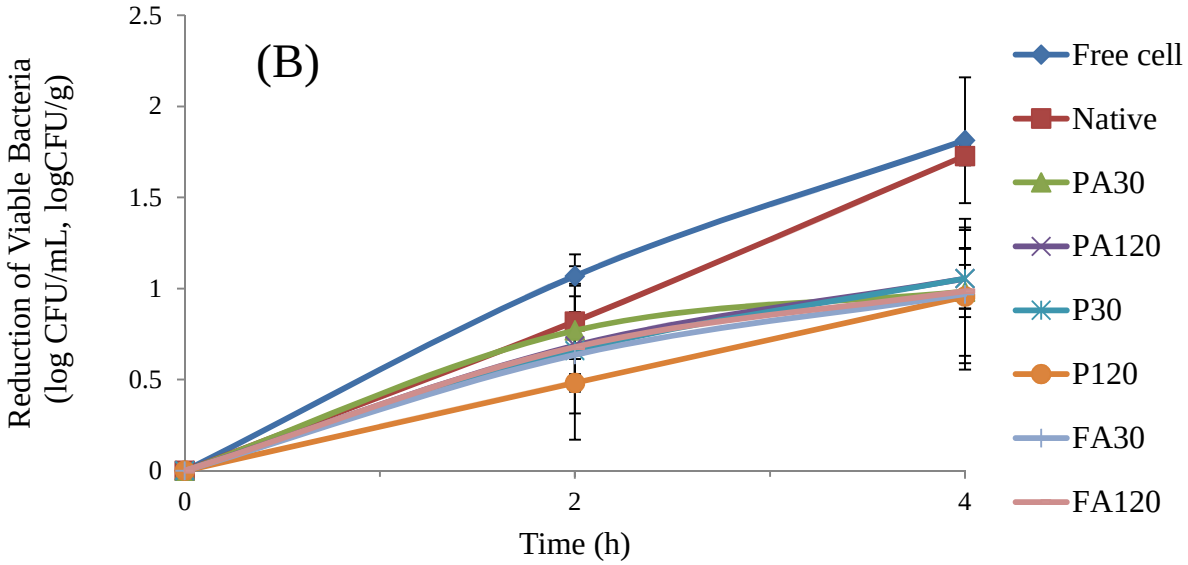


553
554

555 **Fig. 3.**



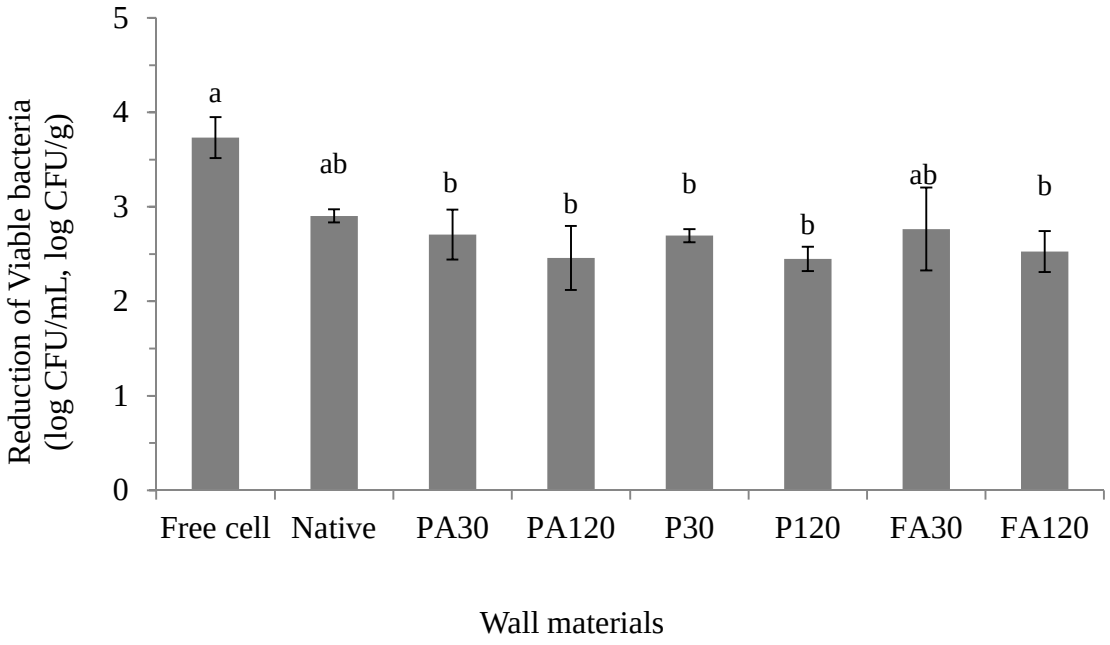
556



557

558

559 **Fig. 4.**



560

561

Encapsulation of *Lactobacillus plantarum* in Porous Maize Starch

*Haiteng Li[1], Van Thi Thuy Ho[1], Mark S. Turner[1,2] and Sushil Dhital[3]**

[1]School of Agriculture and Food Sciences, The University of Queensland, St Lucia, Qld 4072, Australia.

[2]Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Qld 4072, Australia.

[3]ARC Centre of Excellence in Plant Cell Walls, Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, Qld 4072, Australia

* Corresponding author.

Phone: +61 7 3346 7373; Fax: +61 7 3365 1177. Email address: s.dhital@uq.edu.au (S. Dhital)

Highlights

- Amylase creates ample void space in maize starch allowing bacteria to diffuse into
- Porous starch allows for a high probiotic loading efficiency
- Porous starch offers enhanced protection to hostile conditions
- The viable counts in porous starch remain higher than native starch