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Effect of different coating materials on the biological characteristics and stability of microencapsulated *Lactobacillus acidophilus*

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Abstract

Effect of different coating materials on the biological characteristics and stability of microencapsulated *Lactobacillus acidophilus* was investigated. Results indicated that the surface and microstructure of microencapsulation was significant affected by the type of coating material. The complex carrier could provide protection for *L. acidophilus* cells against the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Cell survivals remain the counts with 2.1 and 3.72 logarithmic cycle reduction found in microencapsulated *L. acidophilus* with complex wall materials and for free cells after exposure to SIF for 180 min, respectively. Furthermore, at the high temperatures evaluated, the higher cell survival rate in microencapsulation embedded with the complex materials was found than that for free cells and that with other materials. Cells counts were reduced to 8.16, 7.17, 6.42 cfu·mL^{-1} and 5.86, 4.29, 2.32 log cfu·mL^{-1} for microencapsulation with complex materials and free cells at 50, 60 or 70 °C for 20 min, respectively. Its stability was also improved compared to free cells at refrigerated temperatures. For the cells release from microcapsules, the counts were increased with prolonged the incubation time. Moreover, the survival rate of cells in microencapsulation was better than that for free cells at bile salt concentration. Results showed that for improving protection against deleterious factors, the complex materials might be the better one for the preparation of microencapsulation.

Keywords: *Lactobacillus acidophilus*, Porous starch, Coating materials, Microbiological characteristics, stability

1 Introduction

Lactobacillus acidophilus as one of the important probiotic microorganisms could confer beneficial effects on the human gastrointestinal tract, which have become increasingly popular all over the word in recent years.[1-2] However, the live cell count of probitics including *L. acidophilus* is commanded at least 10^6-10^7CFU/g of product at the end of storage and at the time of consumption.[2-6] More importantly, the high survival numbers of bacteria was not observed easily during the processing and application of dairy products.[4-6] On the other hand, the high survival rate of probiotics is critical during processing and for its application in products.[6] This is because that it needs to remain higher viable activity during the time of pass through the stomach and intestine since the viability and activity of probiotics are needed at the site of action.[2, 7-9] But, many factors have been reported to influence the viability of *L. acidophilus* cell, such as the type and concentration of coating materials, hydrogen peroxide production, oxygen toxicity, stability in dried or frozen form, storage temperature, microenvironment pH and bile concentration, and the high temperature of dairy processing.[10-12] Therefore, in order to increase the survival of probitics cell, further research is need to be investigated. In recent years, microencapsulation is recognized as the useful technology to maintain the higher viability and stability of cells during its storage and application in foods.[7-9]

Microencapsulation could provide a better barrier and protection for free cells against harsh environmental conditions such as heat treatment, pH and freezing.[2, 7, 9] It was used as one important technology and has been investigated for improving their

viability by many other researchers [13-16]. As reported Picot and Lacroix, compared to the free cells, microencapsulated *B. breve* with whey protein as the coating material significantly improved the viable count of bacteria cell.[4] On the other hand, the type of coating materials is critical for the preparation of microencapsulation, which could affect its microbiological characteristics. [14, 17-20] Carbohydrates such as hydrolyzed starches, porous starches and starches alginate are the most common carrier materials for the probiotic cells. [18, 20-24] Porous starch could form strong complexes with alginates, which is stable in the presence of Ca^{2+} chelators and reduce the porosity of the gel.[22] Microencapsulation of cardamom oleoresin was prepared by spray drying using modified starch as wall material.[21] As reported by Xing *et al.*, porous starch with the optimum concentration mixed with sodium alginate as a carrier can be considered as an innovative technology to improve the stability of *L. acidophilus*.[2] On the other hand, the protective agent including mannitol, glycerol and sodium alginate in the preparation solution might provide the better function for the survival of cell in the microencapsulation.[2, 18] Furthermore, the investigation conducted by Mokarram *et al.* was evaluated the influence of alginate coating on survivability of probiotic bacteria in simulated gastric and intestinal juice.[23] More importantly, the biological characteristics and stability of cells in microencapsulation under different conditions are quite important for its application, which was significant influenced by the type of wall materials. However, none papers have been found to report that the effect of different coating materials on the biological characteristics and stability of *L. acidophilus* in the microencapsulation.

Therefore, the objective of this study was to understand the effect of different coating materials on the biological characteristics and stability of microencapsulated *L. acidophilus*. Morphological observation and thermo gravimetry/derivative thermo gravimetry was conducted. The influence of different coated materials on the survival rate of cells was also evaluated during exposure to artificial gastrointestinal fluid and storage at different temperature. The release of cells in simulated colonic pH juice was analysis. Moreover, the cell survival exposure to the bile salt solution was evaluated in order to understand its application property.

2. Materials and methods

2.1. Materials

L. acidophilus (CICC 6075) used as the active core material was purchased from China Center of Industrial Culture Collection (Beijing, China). Porous starch was purchased from Liaoning Lida Bio-Technology Co. Ltd. (Jinzhou, China). Sodium alginate (240±20 mPa·s) was purchased from Chengdu Ruifeng Lier Technology Co. Ltd. (Chengdu, China). All the other chemicals used were of analytical grade.

2.2 Preparation of microencapsulation

L. acidophilus in the freeze-dried ampoule was activated according to the method reported by Liserre *et al.* and Xing *et al.* with some modifications.[2, 25] *L. acidophilus* was activated in chalk litmus milk at 37°C for 24 h, and the cultures were maintained in a refrigerator (7±1°C). The culture was reactivated in MRS broth by transferring three times and the cells were harvested by centrifugation (3000*g*) at 4°C for 10 min and then washed twice with 0.85% (w/v) NaCl solution. The pellet was resuspended

111 in the saline solution to obtain a suspension with cell concentration of 10^9-10^{10}
112 $CFU \cdot mL^{-1}$.

113 *L. acidophilus* as core material was encapsulated in different coating material as
114 described by Sheu *et al.*, Mandal *et al.*, Liserre *et al.* and Xing *et al.* with some
115 modifications.[2, 6, 25] 50mL of *L. acidophilus* cell suspension with 10^9-10^{10} $CFU \cdot mL^{-1}$
116 was transferred into a sterilized beaker, which was added mannitol (10%), glycerol
117 (10%), porous starch (10%) and complex wall material (porous starch (10%)+
118 mannitol (3%) + glycerol (2%)) as the wall materials, respectively. Then, this solution
119 was shocked by ultrasonic wave for 20 min in order to the free cell coated by wall
120 material. The pH values of this solution were adjusted to 6.0 using NaOH and HCl
121 solutions. After absorption, sodium alginate (1.5%) and Tween 80 (0.2%) was added
122 dropwisely into the solutions with stirring. The obtained uniformly emulsion was
123 obtained by adding 0.1M calcium chloride (100mL) dropwisely for hardening of
124 microcapsules. The microcapsules were harvested by centrifuging at 4°C with
125 500*g*×10 min and washed twice with distilled water in order to float the residual
126 liquid of calcium chloride, mannitol and/or glycerol on the surface of
127 microencapsulation. Furthermore, the residual bacteria on the surface were also
128 washed twice with the sterilized saline solution (0.85%). The beads were separated by
129 filtration using filter paper and precooled in a refrigerator (-40°C) for 4 h. Then the
130 obtained material was freeze-dried at -58°C for 72 h using the freeze dried machines
131 (lyolab3000, Heto-Holten Co., Denmark) under 5mmHg. The frozen state was
132 maintained throughout the freeze-drying procedure. The obtained microencapsulation

transferred to a sterile Petrdish and stored in a refrigerator (7 °C).

2.3 Enumeration of *L. acidophilus* cells

The viable numbers of *L. acidophilus* were counted on MRS agar according to Grosso and Fávaro-Trindade, Pedroso *et al.* and Xing *et al.* with some modifications. [2, 26, 27] MRS agars was supplemented with lithium chloride (0.1%), L-cysteine (0.05%) and aniline blue (0.01%) for enumeration of *L. acidophilus* cell. Serial dilutions were prepared with a 2% sodium citrate solution. The plates were incubated with the anaerobic system at 37 °C for 72 h.

2.4 Morphological observation and thermogravimetric analysis

The morphology of microcapsules with different coating material was carried with SEM (scanning electron microscope). The microcapsules on a piece of adhesive paper were coated with gold with a vacuum sputtering coater before observing with SEM (JSM 6390 LV, Jeol, Tokyo, Japan) at an accelerating voltage of 15 kV.[2, 7, 28]

Thermo gravimetry/derivative thermo gravimetry (TGA/DrTGA) of microencapsulation with different coating materials were analysis using a TG/DTA-6300 thermobalance (Shimadzu, model DTA-6300, Kyoto, Japan).[29] The sample was placed in alumina pans and heated from 30 ℃ to 350 ℃ at a rate of 10 ℃ min^{-1}under a dynamic synthetic N_2 atmosphere (100 $mL \cdot min^{-1}$). The equipment was preliminarily calibrated with a standard reference of calcium oxalate.

2.5 Resistance to simulated gastric fluid and intestinal fluid

Resistance to the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was determined as described by Gbass *et al.* and Xing *et al.*. [2, 30] SGF consisted of 9

155 $g \cdot L^{-1}$ of sodium chloride and 3 $g \cdot L^{-1}$ of pepsin, and the pH was adjusted to 1.5 with
156 hydrochloric acid. SIF consisted of 9 $g \cdot L^{-1}$ of sodium chloride, 10 $g \cdot L^{-1}$ each of
157 pancreatin and trypsin and 3 $g \cdot L^{-1}$ of bile salts, and the pH was adjusted to 6.5 with
158 sodium hydroxide. Survey assays were conducted at 37 °C for 60, 120 and 180 min
159 after incubation of free and encapsulated cells in SGF and SIF, respectively.

160 **2.6 Stability of microcapsules under different temperature**

161 Stability of microencapsulated *L. acidophilus* under heat treatment (50, 60 or
162 70°C for 20min) and low temperature (4°C for 14 weeks) was investigated according
163 the method reported by Mandal *et al.* and Xing *et al.*.[2, 6] 1mL of the free cell
164 suspension or one gram of microcapsules (10^9-10^{10} $cfu \cdot mL^{-1}$) was transferred in test
165 tubes with 10mL of distilled water. The content was cooled to room temperature
166 (30 °C) after the different temperature treatment and the total number of viable cells
167 was enumerated as described as Section 2.3.

168 **2.7 Release of microencapsulated cells**

169 In vitro release of encapsulated *L. acidophilus* at simulated colonic pH juice was
170 investigated according to the method described by Rao *et al.*, Mokarram *et al.* and
171 Mandal *et al.* [6, 23, 31] 1 g of microcapsules was transferred into 10 mL simulated
172 colonic pH juice (0.1 M KH_2PO4, pH 7.4±0.2), followed by homogenization with a
173 magnetic stirrer for 10 min. Then, the mixed solution was incubated at 37 °C for 3 h.
174 The count of viable probiotic cells was carried out as described in Section 2.3 during
175 the incubation period.

176 **2.8 Survival of microencapsulated cells in bile salt solution**

Tolerance to simulated bile salt concentration simulated small intestine of human was carried out as the reported by Lee and Heo, Mandal *et al.* and Xing *et al.*. [2, 6, 32] Similar to low pH tolerance, 1 g of microcapsules or 1mL of the free cellsuspension (10^9-10^{10}cfu·mL^{-1}) were transferred in test tubes containing 10mL of 1% or 2% bile salt and incubated at 37°C. The viable cells of each bacterium were then enumerated using methods described in Section 2.3.[33]

2.9 Statistical analysis

The tests in this investigation were carried out in triplicate and the obtained test dates were analyzed using SPSS 13.0 software (SPSS Inc.). The results were expressed as mean ± S.D.. The one way analysis of variance procedure followed by Student-Newman-Keuls test was used to determine the significant difference (p<0.05) between treatment means.

3. Results and discussion

3.1 Morphological characterization

Morphological observation of microencapsulated *L. acidophilus* prepared with different coating materials was carried by SEM. The photomicrographs of microencapsulation with mannitol, glycerol, porous starch and complex wall material were shown in Fig.1A, 1B, 1C and1D, respectively. As can be seen in Fig.1A and1B, the SEM photos revealed that many *L. acidophilus* cells was found on the surface of wall materials. These photos indicated that the wall materials of mannitol and glycerol could provide the carrier action as the protective agent for free cells. However, the cell of *L. acidophilus* could not be embedded completely with mannitol and glycerol

during the processing period. Furthermore, the absence of mannitol and glycerol could provide the better protection as the competent of complex coating material for *L. acidophilus* cells. As shown in Fig.1C and1D, these photos revealed that, although the microparticles prepared with porous starch and complex wall materials containing porous starch were nearly spherical in shape, the walls of the particles were irregular. However, SEM photos in Fig.1C also revealed the absence of agglomeration was also observed among the microparticles of porous starch. On the other hand, for the microencapsulation prepared with the complex materials, these photos also revealed the absence of various sizes of micro-porous in the surface of modified starches confirming the formation of microcapsules and the protection provided for *L. acidophilus* cells. Moreover, the modified starches in the Fig.1D dispersed better than that in the Fig.1C, but some broken starch particles were also observed.

There results revealed that the application of these wall materials could sufficiently guarantee the integrity of free cell and provide the enough cell number for the application of microencapsulated *L. acidophilus*. For the preparation of microencapsulation, the choice of wall material is very important for its stability and application.[7, 20, 34] Similar results were observed by Krishnan *et al.* and Rodríguez-Huezo *et al.* .[21, 35] Moreover, the investigations about microencapsulated flavors also demonstrated the similar pattern of microcapsules prepared with modified starch as wall materials. [20, 21] The investigation of Krishnan *et al.* showed that microencapsulated cardamom oleoresin were broken and not complete, which used the modified starches as the coating material.[21] These results might be due to one

reason of the microstructure of biopolymer as coating materials could be influenced by the composition and competent of wall materials. This might be due to the micro-porous in the modified starch and the protective agent of mannitol, glycerol and sodium alginate as the carrier, as shown in Fig.1. The micro-porous in the starches could acts as the important protection carrier, and mannitol, glycerol and sodium alginate as the protection membrane adhered to the surface of porous starches, as shown in Fig. 1D. This combined action might be permitted the continued die loss and improving the stability of cells.[2, 18, 20] These results might be due to the result of widespread surface growth and cells released from the gel bead, which could lead to decreasing cell population in the beads and hence a higher cell release from a microcapsule resulted in lower cells number inside the beads.[36] In the present investigation, there were different cell loaded among different kinds of beads because they had a different type of coating material. In addition to differences in chemical characteristics, the capsule materials also possessed different physical properties.[28] Coating materials with more protection could result in a lower permeability for external and internal mass transfer and lead to a smaller amount of cell loaded. The complex materials as the coating showed less permeability for external and internal mass transfer compare to the other biopolymers. On the other hand, the interaction between the different coating materials and *L. acidophilus* could induce the change in structure and accordingly physical properties of biopolymers. Porous starch is one kind of modified starch and has been used as the carrier material successfully in the microencapsulation preparation. [4, 37] It is a denatured starch with suitable porous size

on the surface and excellent biocompatibility. This honeycomb structure of porous starch could improve the adhesive property and absorbability of the core materials. [4, 37, 38] In the prepared processing of microencapsulation, mannitol could be used as anti-sticking agent, filler and quality improver and improve the stability and storage property of particle products.[39] Glycerol has gained considerable attention because can be used as softeners, desiccant, lubricant and plasticizer for the prepared microencapsulation.[40] Moreover, glucose with the lower diffusion property was also reported in concentrated alginate gels due to decreased number and length of pores rather than decrease in pore diameter. [12, 41] Furthermore, alginate could also form a gel when in contact with calcium and multivalent cations. This crosslinked alginate matrix system could cause a faster degradation and release of active ingredients.[2, 6, 32] More importantly, the viscosity of prepared solutions during emulsification could also influence the interaction between different coating materials and the preparation of microencapsulation. During stirring in the preparation, the viscosity of these coating solutions was evaluated by the Digital Rotational Viscometer (NDJ-5S, Shanghai Jingxi Instrument Co., Ltd. Shanghai, China), which were about 3320 mPa·s, 2040 mPa·s, 12600 mPa·s and 12080 mPa·s for cmannitol, glycerol, porous starch and complex wall material as the coating materials, respectively. This interaction could provide the better function for the protection of the complex carrier for cell loaded. This starch complex capsule coating possesses an interphasic membrane and offers the possibility to encapsulate *L. acidophilus* without loss of viability. [2,5,6] SEM photos also indicated the suitability of porous starches complied with other materials as the

wall material for encapsulation of *L. acidophilus*. Furthermore, the stability and other characteristics of microencapsulation coated with different type of materials need to be investigated for it's extend application.

3.2 TG analysis of microencapsulated *L. acidophilus*

The thermogravimetric analysis was conducted in order to determine the thermal stability of complex materials as the carrier in the preparation of microencapsulation.[29] The weight loss process for many kinds of materials shows the relatively wide temperature range and indicates different thermogravimetric properties. [29, 42] The thermal behavior of microencapsulated *L. acidophilus* embedded with different materials can be observed in the TG curves shown in Fig.2. As shown in Fig.2a, the thermal behavior of microencapsulation with mannitol as the coating material showed that two representative stages were found in the thermogravimetric curves. The first mass loss of the TG curves refers to moisture loss (between 41 °C and 85.8 °C). The breakdown of the fructose chains in microcapsules and may have occurred between 249 °C and 305.6 °C. For thermal behavior of microencapsulation with glycerol, the result shown in Fig.2b indicated that three representative stages were found. The first mass loss of TG curves refers to moisture loss, which occurred at the temperature between 53.3 °C and 92.7 °C. The other two stages also indicated that the breakdown of the fructose chains in microcapsules might be occurred between 170.9 °C and 197.7 °C and between 271.3 °C and 307.6 °C. More important, the thermal behavior of microencapsulation with porous starch and complex wall materials were shown in Fig.2 c and 2d, respectively. Two representative stages were

found in the TG analysis curves. The first mass loss from TG curves referred to moisture loss, which were between 50.2°C and 115.3 °C for the microencapsulation prepared with porous starch. For microencapsulation coated with complex wall materials, the first stage of mass loss was observed not very clearly in the cruces of Fig.2d. It might be referred two stages about moisture loss, which was between 50.0°C and 111.3 °C, between 173.0°C and 193.9 °C, respectively. Furthermore, the breakdown of the fructose chains in microcapsules and may have occurred between 223.7 °C and 259.4 °C, between 233.8 °C and 271.8 °C for the microencapsulation coated with porous starch and complex wall materials, respectively. Above this temperature range, the second stage of mass loss corresponds to the decomposition process.

The thermogravimetric analysis in this investigation is one of the criteria needed in the designing and manufacturing of microencapsulation.[29] In the result of thermogravimetric analysis, TG curves (red color curve) express the relation between the reduction speeds of weight for the tested samples with the specified heating rate of tested temperature. [29, 43] It can know from the curve, the weight of the sample decreased most at the certain temperature, which indicates that this temperature might be the decomposition temperature of samples. DTG curves (blue color curve) indicate the function relationship between the quality change rate with time and temperature. [29, 44] It might indicated that a reaction is occurring between the different groups of coating materials with the formation of a new structure within the process of composites received. [29, 43, 44] Result above indicated that the thermal stability of

microencapsulation with different coating material is described as the limit temperature, which noted that the materials could be used with no damage to its usable properties as the carrier. According to Macêdo *et al.* (1997), in this step there can be occurrence of decomposition reactions in the constituents of the microcapsules, i.e., proteins and carbohydrates.[42] Moreover, Bohm *et al.* (2005) reported that thermal degradation of inulin has been described as being a consequence of the breakdown of the fructose chains and, as was noted in this study, the breakdown of the fructose chains in microcapsules with different coating material might be occurred between 227°C and 308 °C.[43] The analysis results also showed that more than one temperature were found at which the decomposition process occurred at the maximum rate for microencapsulation obtained from different carrier.[29] Moreover, with increasing the heating rate, the shift of decomposition process towards higher temperatures was also observed.[29, 43, 44] Two or three distinct areas of mass loss and two or three maxima on the DTG curves were observed from the course of change to TG and DTG curves of microencapsulation. This result indicated that it is a complex and heterogeneous, two-phase or three-stage process.[29] During this period, the first stage for thermal decomposition is connected to the degradation of hard segments and the second or third stages might be connected with the decomposition of soft segments.[29, 44] It was noted that the course of thermal decomposition of microencapsulation decomposition process depends on the structure and a type of the complex materials.[29, 44] However, further research is needed to be investigated in order to understand the deep mechanism on the different thermal behavior of microencapsulation coated with

different materials.

3.3 Resistance to the simulated gastric and intestinal fluids

In order to determinate the surviving rate of microencapsulated *L. acidophilus* passage through the stomach, the stability in SGF and SIF were need to be investigated. Effects of different coating material on the resistance to SGF and SIF were shown in Fig.3a and 3b, respectively. The initial population found in encapsulated *L. acidophilus* was about 10^9-10^{10} cfu·mL^{-1}. As shown in Fig.3a, after exposure to SGF for 180 min, survival counts of cell in the microencapsulation with mannitol, glycerol, porous starch and complex wall material as the carrier was remained 98.76%, 99.12%, 99.26% and 99.72% of the initial population found in encapsulated *L. acidophilus*, respectively. Moreover, for the free cell after exposure to SGF for 180 min, the survival number of *L. acidophilus* remains the count with 0.25 logarithmic cycle reduction. The higher survival rate of cells in microparticles indicated that the SGF and the exposure time were not significantly affected the survival of lactobacillus cells. On the other hand, after exposure to SIF for 60min and 180 min, cell survivals remain the counts with 2.24, 2.38, 2.19, 1.84 logarithmic cycle reduction and 3.03, 3.09, 2.66, 2.1 logarithmic cycle reduction from the initial population found in microencapsulated *L. acidophilus* with mannitol, glycerol, porous starch and complex wall material, respectively. Cell survivals remain the counts with 2.55 and 3.72 logarithmic cycle reduction for free cells as the control after exposure to SIF for 60min and 180 min, respectively. The counts of free cell were decrease significantly exposure to pH 1.5 for 180min. After 1 h of incubation, the survival of

353 microencapsulated cells was significantly lower in the microencapsulation with
354 mannitol and glycerol as the carriers compared to porous starch and complex wall
355 material beads, but it was still higher than free cells. Moreover, the highest visibility
356 of cell was observed in the microparticles with complex wall material after 180 min
357 exposure in the SIF.

358 For the application of microencapsulated bacteria cell, one of the major problems
359 is the low survival rate of *L. acidophilus* in gastric pH in the intestine system.[45]
360 Results showed that the microencapsulation used the complex material containing
361 porous starch as the carrier provided better protection for *L. acidophilus* against SGF
362 and SIF. The resistance of microencapsulated *L. acidophilus* cells was differing with
363 the different coating materials. The highest and lowest resistance to simulated gastric
364 fluids was found in the microencapsulation prepared with mannitol, glycerol, and
365 complex materials, respectively. However, no significant reduction in viable count
366 was observed in microcapsules with different coating materials as well free cells in
367 distilled water (pH 6.5) on incubation for up to 180 min. There was also no significant
368 difference in the viability of free cells in distilled water. In the present study, result
369 about the increase of resistance to simulated gastric and intestinal fluids was observed
370 compared to the survival of free cells.[23, 45-47] A large variation about the ability of *L.*
371 *acidophilus* to resist acid has been reported by other researchers.[20, 33, 48] According to
372 the investigation conducted by Corcoran *et al.*, they reported that the presence of
373 glucose could enhance the visibility of probiotic *lactobacilli* during the gastric
374 transit.[49] As reported by Krasaekoopt *et al.*, microencapsulated cells of *L. acidophilus*

375 in alginate beads survived better after sequential incubation in simulated gastric and
376 intestinal juices.[17] It was in agreement with Kim *et al.*, who reported that
377 microencapsulated *L. acidophilus* still maintained above 10^6 cfu·mL^{-1} at pH 1.5 after
378 2 h, but free cells *L. acidophilus* were completely destroyed after 1 h.[47] Chandramouli
379 *et al.* reported that a higher survival of *L. acidophilus* immobilized in alginate bead
380 was found in low pH environments.[50] The results reported by Chandramouli *et al.* and
381 Iyer *et al.* also indicated that microencapsulation could maintain the high viability in
382 gastro-intestinal conditions. [50, 17, 18, 32, 48-49, 51-53] According to the results reported by
383 Wang *et al.*, the stability of allicin microcapsules against pH was improved when
384 porous starch was used as the assisted wall material.[37] In their investigation, porous
385 starch with β-cyclodextrin could act as the cell carrier for many negative factors
386 including high temperature, acid and light; thus, cell stability might be improved.[2, 54]
387 These results indicated that the protection of *L. acidophilus* cells against SGF and SIF
388 might be explained by the combined function between the porous starch at the
389 appropriate concentration and mannitol, glycerol, sodium alginate at the protective
390 agent. The added coating afforded better protection to probiotic organisms compared
391 to uncoated microcapsules in the same time points.[53] This combined action could
392 provide protection of *L. acidophilus* cellular integrity and improve its stability.
393 Therefore, the probiotic cells loaded in the microencapsulation of starches could pass
394 through the gastric transit and be released in the vicinity of the site of action.

395 **3.4 Stability of microencapsulation under different temperature**

396 The loss of activity could occur during the microcapsules under the heat treatment

and cold storage conditions. Therefore, the effects of different coating materials on the stability of *L. acidophilus* microcapsules throughout storage at different temperatures were shown in Fig. 4 and 5. As shown in Fig.4a, free cells in distilled water were drastically reduced to 5.86, 4.29 and 2.32 log cfu·mL^{-1} on heat treatments at 50, 60 or 70 °C for 20min, respectively. Microencapsulated *L. acidophilus* with different coating materials showed higher survival for a period of up to 20 min of storage at these high temperatures than that for free cells. Cells counts in the microencapsulation were drastically reduced from 9.10 log cfu·mL^{-1} (in distilled water) to 7.35, 6.37, and 5.53 log cfu·mL^{-1} for mannitol, 7.73, 6.69 and 5.81 cfu·mL^{-1} for glycerol, 7.98, 6.94 and 5.85 log cfu·mL^{-1} for porous starch, 8.16, 7.17 and 6.42 cfu·mL^{-1} for complex materials on heat treatments at 50, 60 or 70 °C for 20min, respectively. Microencapsulation with complex wall materials improved the stability of *L. acidophilus* and presented a logarithmic cycle reduction of 2.69 (70°C), 1.99 (60°C) and 0.98 log cfu·g^{-1} (50°C) during the storage period, respectively. Further, the survival of *lactobacilli* was found to decrease with increasing the treated time. On the other hand, Figure 5 shows the stability of free and encapsulated probiotic bacteria during 12 weeks of storage in the refrigerator at 4 °C. The viability of microencapsulated cells showed different stability between microcapsules with different coating materials in the same storage conditions. After 12 weeks, the survival of *L. acidophilus* in microcapsule mannitol, glycerol, porous starch and complex materials decreased to 7.30, 7.46, 7.76 and 7.86 log cfu·g^{-1}, respectively. However, the numbers of free cells decreased from 9.08 to 4.12 log cfu·g^{-1}

419 respectively after 12 week, and low survival counts was noted at the end of storage.
420 The decrease rate was significantly different between the microencapsulated with
421 different material, which was also significantly influenced by the storage time.

422 Results showed that the immobilized cells in the micro-porous of modified
423 starches as complex materials showed the lowest loss of viability of cells and good
424 stability at the end of the time under the different temperature conditions. Improving
425 the stability of *L. acidophilus* cells with complex wall materials during storage could
426 reduce the loss of cells to the medium during its application. As reported by
427 Krasaekoopt *et al.*, it confirmed that starch mixed with cationic polymers could
428 improve the stability of the microcapsules.[18] They also reported that the stability of
429 cell could increase with decreasing the treated temperature and low temperatures
430 could prevent active ingredient exposure and promote a longer shelf life of
431 microcapsules. It is well known that heat treatment could influence the survival of
432 lactic acid bacteria. [47, 54] This observation on the survival of *L. acidophilus* at high
433 temperatures was agrees with the findings of Jeffery *et al.* and Kim *et al.*. [47, 54]
434 Moreover, *L. acidophilus* might die quickly during storage at the refrigerated
435 temperature. Therefore, one of the main aims of the present study was to check the
436 viability of microencapsulated *L. acidophilus* over a period of time under refrigeration.
437 Several investigations also showed that the survival of microencapsulated bacteria
438 was higher than that of free cells during the storage time. [18, 48] Koo *et al.* reported that
439 *L. bulgaricus* loaded in chitosan coated alginate showed higher stability than free cell
440 culture.[55] Furthermore, the results of Medina and Jordano demonstrated that the viable

cells of *L. acidophilus* decreased rapidly during the storage time under refrigeration at 7°C, especially between days 10 and 17.[56] For the microencapsulation products stored under refrigeration, one of the main focuses should be on the minimum viable level, which required for the bacterium to be beneficial to health. Results of this study indicated a great variability after the storage at 4°C of 12 weeks in the survival ability of microencapsulated *L. acidophilus* with the complex wall materials. Inactivation of cells in the microparticles can be related to many factors, such as fatty acid oxidation, DNA damage and the formation of free radicals under the high temperature.[57] Release of cells during the heat conditions might be due to the collapse of beads. More importantly, survival mechanisms exhibited by bacteria are generally referred to as the adaptive stress response. [58] The higher viability of microencapsulated cells towards heat treatments could be explained by this theory. However, probiotic cells were injured and killed with increasing the osmotic pressure.[58] On the other hand, the variability is also highly dependent on the kinds of capsule materials. The complex wall materials including porous starch is a substance that is accumulated within the cells to reduce the osmotic difference with the external environment or a substance that surrounds cells to improve heat and cold tolerance.[59] The wall materials of mannitol and glycerol might not be able to protect cells from injury completely, which could induce the reduction in probiotic viability. It is interesting to note that the mixtures of porous starch, mannitol and glycerol as the capsule materials showed the best viability and had better viability than the sole wall material. Therefore, our results suggested that the complex materials containing porous starches used as wall

material had a positive effect on the protection of *L. acidophilus* during the heated and refrigerated period. For this reason, changes in the population of viable bacteria during the expected shelf-life of product should be known to some extent and taken as a basis for selection of coating material.

3.5 Release of microencapsulated cells in simulated colonic pH juice

An *in vitro* system was utilized in order to determine the effect of the acidic pH of the stomach on the survival of encapsulated probiotics. Release of cells from microencapsulation in simulated colonic pH juice 37 °C was investigated and the results are shown in Fig. 6. The released cell counts were between 8.34 and 8.76 log cfu·g^{-1} at the end of the storage period upon immediate exposure to the solution of simulated colonic pH. With an increased incubation time, the release of cells was increased. After 1.0 h and 2.0 h in the immediate medium, the released cell counts from microencapsulation with mannitol, glycerol, porous starch and complex materials as the wall material were 7.32, 6.91, 6.74, 6.46 log cfu·g^{-1}and 8.76, 8.63, 8.53, 8.34 log cfu·g^{-1}, respectively. Results also indicated that the total counts of cells released from microencapsulation prepared with different coating materials increased significantly before the period of the first 1.5 h. During this period, the type of wall materials affected the released cell counts. However, after the exposure time of 1.5 h, no significant change on the count of released cells was observed. As shown in Fig.6, free cell of *L. acidophilus* were sensitive to the acidic environment and the ingestion of unprotected cell might result in reduced viability of 5 log reduction after 2 h.

The release of *L. acidophilus* cells from microcapsules is essential for growth and

colonization of probiotics in the vicinity of the site of action.[6] The effect of different coating materials on the release of *L. acidophilus* needed to be investigated.[60] This study was conducted to compare the released performance of probiotic Lactobacillus from microencapsulation with different coating materials in simulated colonic pH juice during storage at 37 °C. The above result also indicated that the type and structure of coating materials also affected the released cell counts from microencapsulation at the first period of 1.5 h. However, there was no significant change on the count of released cells among microencapsulation with different coating material at the end of the exposure time. This demonstrated that no effect of different coating material on the cells release was observed after exposure to the medium solution for 2.5 h. This action could promote a longer shelf life of the microcapsules and provide a better release performance of active cells in the microcapsules. The result obtained in this investigation was consisted with that of Rao *et al.*, who reported that the microencapsulated *B. pseudolongum* resisted the simulated gastric and intestinal juices up to 60 min.[31] This implies that the different coating materials could control the release of cell from microencapsulation during the first period after inoculation. These results also indicate that strain release from the product will depend on many factors such as pH, presence of preservatives and the structure of different coating materials.[2, 61] Zhang *et al.* have shown that the survival ability of *L. acidophilus* was significantly affected when subjected to low pH.[62] Frank and Hassan reported that neutralized juices inhibited probiotic bacteria. This indicated that acid injury was responsible for the inhibitory effect. As reported by

Vinderola *et al.*, the survival of *B. bifidum* and *L. acidophilus* during refrigerated storage at 5°C in milk.[60] Their results also proved that the product acidity has a major impact on the microbial viability during its shelf-life.[60] In order to be successful candidates for functional food applications, the release characteristic of active cells in the intestine is one of the aims of microencapsulation.[4, 60, 61] Picot and Lacroix also reported the progressive release of viable cells from whey protein-based microcapsules in simulated intestinal conditions.[4] This indicated that the coating of complex beads provided the best protection in simulated gastric juice because the protected interspace forms in the double layer membrane and as a result, the diffusion of gastric juice into the beads may be limited.[6,23,31] This microstructure will protect cells from interacting with the gastric juice.[63] Microencapsulated cells survived better than that for free cells after sequential incubation in simulated gastric and intestinal juices, and the complex coating enhanced the survivability of cells more than other coating materials. Porous starch with a honeycomb structure can provide the stability release characteristics of cells from the network formed with porous starch and sodium alginate.[37] Porous capsules suitable for immobilizing *L. acidophilus cells* were coated with sodium alginate, which served both to position the microorganisms in the capsule pores and to form spaces for cell release.[37, 38, 64] These results allow us to conclude that microencapsulation prepared with the complex materials as the wall material could provide satisfactory release properties of *L. acidophilus* cells.

3.6 Survival rate of microencapsulated cells in bile salt solutions

The effect of different coating materials on the survival rate of *L. acidophilus* in

529 microencapsulation exposure to the solutions of 1% and 2% bile salt was investigated.
530 As the results shown in Fig. 7a, free cells decreased from 9.10 to 6.59 log cfu·mL^{-1}
531 and from 9.13 to 4.85 log cfu·mL^{-1} after exposure to 1% and 2% bile salt solutions for
532 12 h, respectively. However, according to the results shown in Fig. 6b-e, survival rate
533 of cell in microencapsulation with different coating materials improved firstly and
534 then reduced the viability of cells at similar bile salt concentrations.
535 Microencapsulated cells embedded with mannitol and glycerol decreased from 9.1 to
536 7.63 log cfu·g^{-1}, from 9.14 to 6.60 log cfu·g^{-1} and from 9.10 to 7.85 log cfu·g^{-1}, from
537 9.12 to 7.10 log cfu·g^{-1} on exposure to 1% and 2% bile salt after 12 h, respectively.
538 However, after exposure to 2% bile salt for 12 h, microencapsulation with porous
539 starch and the complex material decreased from 9.14 to 6.95 log cfu·g^{-1} and from 9.12
540 to 7.10 log cfu·g^{-1}, respectively. This indicated that the viability of *L. acidophilus*
541 decreased proportionately with concentration and time of exposure to bile salt and that
542 a highest survival of cells among these microparticles was obtained on encapsulation
543 in the complex wall material containing porous starch.

544 The survival of microencapsulated *L. acidophilus* was better at high bile salt
545 concentration than that for free cells. The obtained results indicated that the protection
546 of *L. acidophilus* cells against the bile salt solution might be explained by the
547 combined function between porous starch and sodium alginate in the preparation
548 solution. According to the investigation conducted by Trindade and Grosso, the
549 immobilization of *L. acidophilus* in alginate beads was not effective in protecting the
550 cells from 2% and 4% bile salt.[65] Chandramouli *et al.* reported that encapsulation of *L.*

551 *acidophilus* in alginate significantly increased the viability in 1% bile salt.[50] As
552 reported by Murata *et al.*, the chitosan coating provides the best protection in bile salt
553 solution because an ion exchange reaction takes place when the beads absorb bile
554 salt.[63] This was consisted with that of Koo *et al.* and Yu *et al.*, who reported that
555 *L.casei* entrapped in alginate beads containing chitosan had higher viability than in
556 alginate without chitosan.[55] Bile salt solutions were always used to determine whether
557 coating materials would increase survival of cells in this environment. This is because
558 that bile tolerance is often used as a criterion for probiotic strain selection, which is
559 similar to that of the digestive system. As reported by Sultana *et al.*,
560 microencapsulated *L. acidophilus* decreased by two log cycles compared to the initial
561 cell count in 1% and 2% bile salt solutions.[52] Moreover, the survival rate decreased
562 proportionately with the time exposed to bile salt solutions.[32] The combined action
563 could provide protection of the *L. acidophilus* cellular integrity and improve its
564 stability. This might be due to the shell and net structures that were formed with
565 porous starch and sodium alginate, which could act as a physical and permeable
566 barrier for negative factors. [2, 37, 38, 64] A similar result was also reported by Corcoran *et*
567 *al.*, in their investigation, the presence of glucose could also enhance the visibility of
568 probiotic lactobacilli during gastric transit.[51] The protection afforded was dependent
569 to some extent on the type and chemical characteristics of the media.[2, 6, 66] The
570 polysaccharide-containing matrix with micropores provided more protection to the
571 probiotic when porous starch were used in combination with sodium alginate for the
572 preparation of microencapsulated *L. acidophilus*. This point might be due to the

interplay between the role of porous starch in protecting probiotics, which could provide the enough space and maintain the integrity of cell membranes.[66-67] Therefore, our results suggested that the complex material containing porous starches and alginate beads used as the wall materials have a positive effect on the resistance of *L. acidophilus* to bile salt solution.

4. Conclusions

New methods to produce applicable coating materials appear therefore as an important task. This investigation was conducted about effect of different coating materials on the biological characteristics and stability of microencapsulated *L. acidophilus*. Indeed, the type of coating material significantly affected the surface and microstructure of microencapsulation. The complex materials as the coating could provide the better protection for probiotics cells against the passage through gastric and intestinal fluids. It showed a higher survival rate of *L. acidophilus* with the different coating materials at the different temperatures evaluated. With increased incubation time, the release of cells was increased and there was no significant change indicating no effect of different coating materials on the cells release from microcapsules. The viability and stability of cells in the microencapsulation at refrigerated storage temperatures was also improved. These results domenstarted that the complex wall material containing porous starch might be the better one for the preparation of microencapsulated *L. acidophilus* in order to increasing protect against environmental deleterious factors.

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818 Fig.1. Morphology of microencapsulated *L. acidophilus* different coating materials
819 observed by SEM (the different porous starch concentrations (A: mannitol (10%), B:
820 glycerol (10%), C: porous starch, D: complex wall material; (a) 500×, (b) 3000×).
821
822 Fig.2. TG/DTG analysis of microencapsulated *L. acidophilus* with different coating
823 materials ((a): mannitol (10%), (b): glycerol (10%), (c): porous starch, (d): complex
824 wall material).
825
826 Fig.3. Effect of pH on viable counts of free and microencapsulated *L. acidophilus*
827 with different coating materials (log CFU mL^{-1} for free cells and log CFU g^{-1} for
828 microencapsulated cells; (a) 6.5 and (b) pH 1.5). Mean bars with different letters (a–c)
829 in the same coating material with different incubation time differ significantly
830 ($p<0.05$). Mean bars with different letters (p-t) at the same incubation time with
831 different coating material differ significantly ($p<0.05$).
832
833 Fig.4. Effect of heat treatments on viable counts of microencapsulated *L. acidophilus*
834 (log CFU g^{-1}) with different coating materials ((a): mannitol (10%), (b): glycerol
835 (10%), (c): porous starch, (d): complex wall material). Mean bars with different letters
836 (a-c) at the same temperature for different heating times differ significantly ($p<0.05$).
837 Mean bars with different letters (p-r) for same heating time at different temperatures
838 differ significantly ($p < 0.05$).
839 Fig.5. Effect of low temperature on viable counts of free cells and microencapsulated

840 *L. acidophilus* (log CFU g^{-1}) with different coating materials ((a): free cells; (b):
841 microencapsulated *L. acidophilus* with different coating materials). Mean bars with
842 different letters (a-g) with same coating material at the different incubation time differ
843 significantly ($p<0.05$). Mean bars with different letters (p-r) at the same incubation
844 time for different coating materials differ significantly ($p<0.05$).
845
846 Fig.6. Effect of bile salt on viable counts of microencapsulated *L. acidophilus* (log
847 CFU g^{-1}) with different coating materials ((a): mannitol (10%), (b): glycerol (10%),
848 (c): porous starch, (d): complex wall material). Mean bars with different letters (a-c)
849 in the same bile salt concentration at different incubation times differ significantly
850 ($p<0.05$). Mean bars with different letters (p-r) at the same incubation time in
851 different bile salt concentrations differ significantly ($p<0.05$).
852
853 Fig.7. Effect of different coating materials on the release characteristics of
854 microencapsulated *L. acidophilus* (log CFU g^{-1}). Mean bars with different letters (a-e)
855 in the same coating materials at different incubation times differ significantly
856 ($p<0.05$). Mean bars with different letters (p-r) at the same incubation time in
857 different coating material differ significantly ($p<0.05$).
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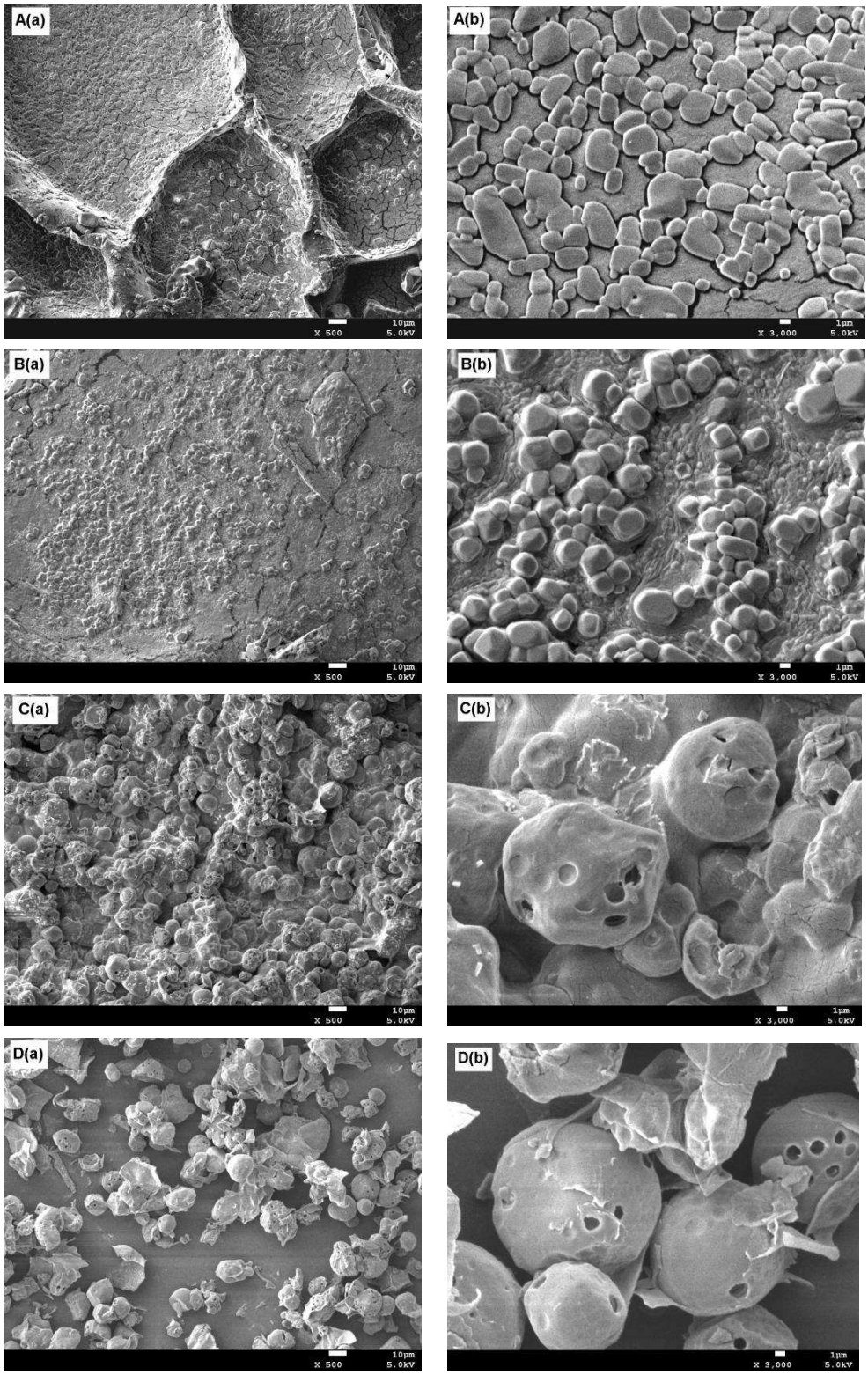
862 **Figure 1**



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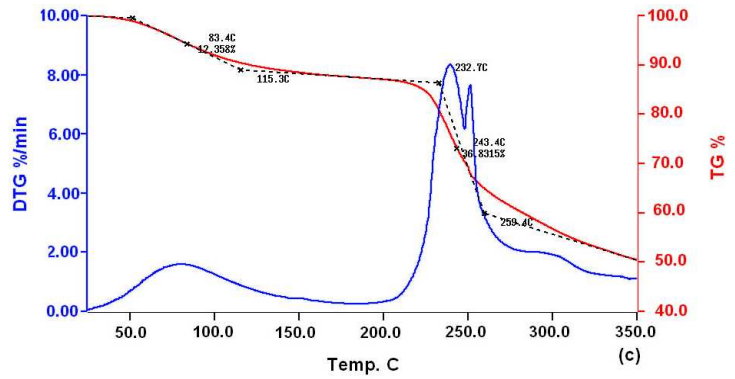
868 **Figure 2**



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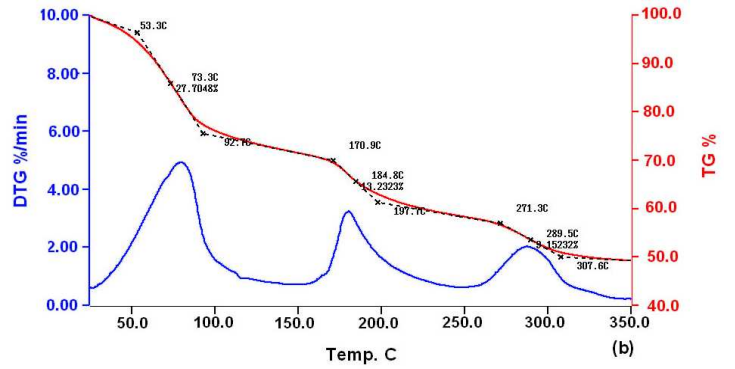

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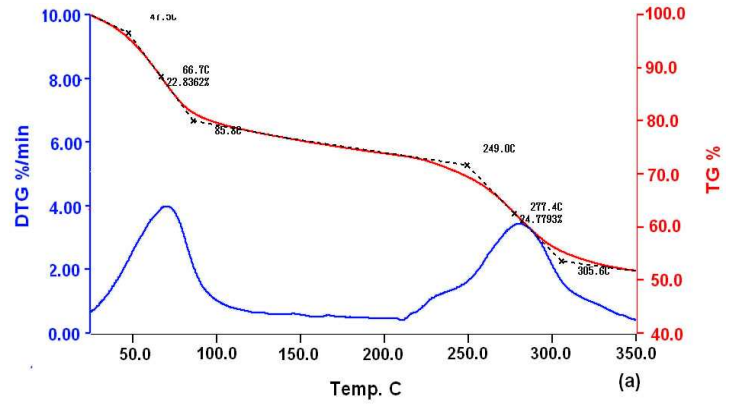

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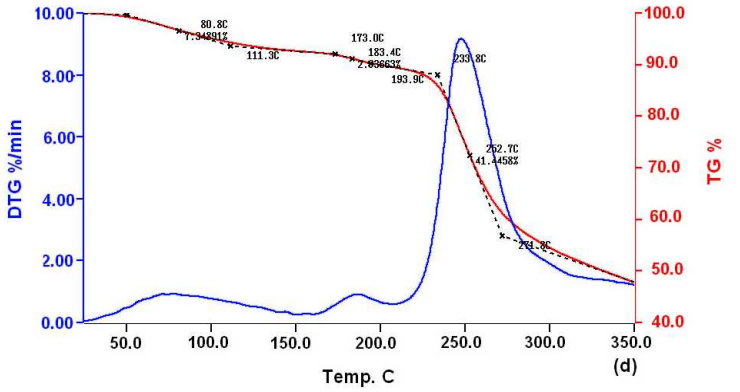

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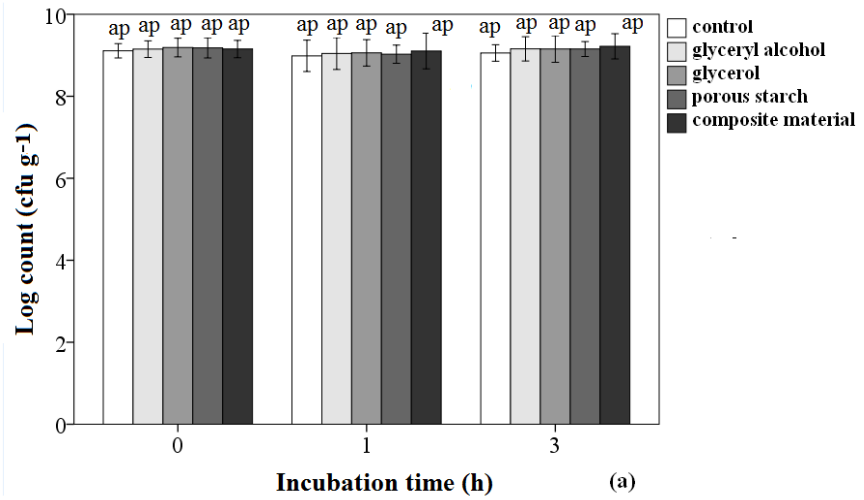
873 **Figure 3**



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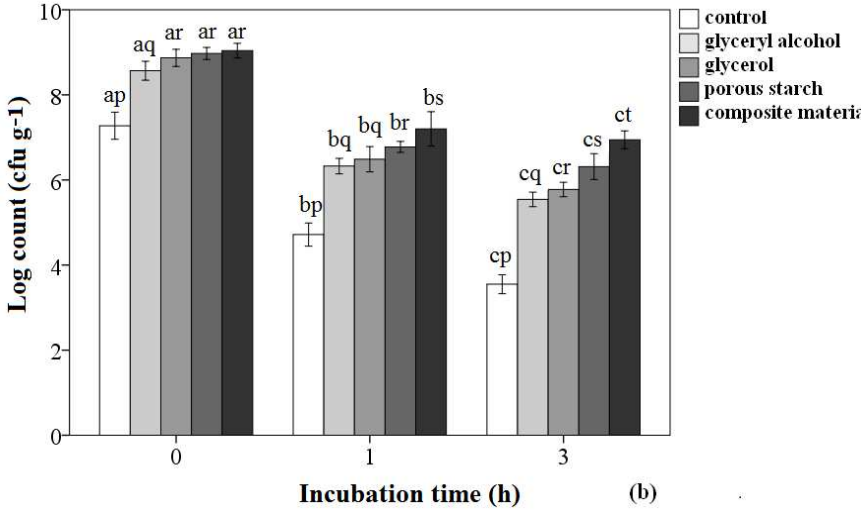

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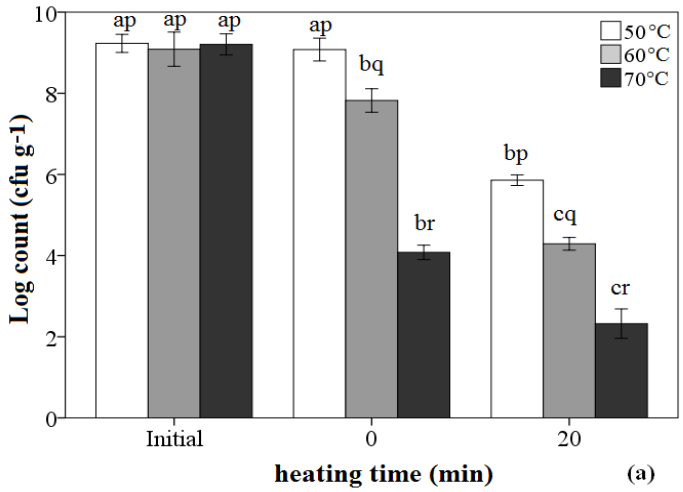
883 **Figure 4**



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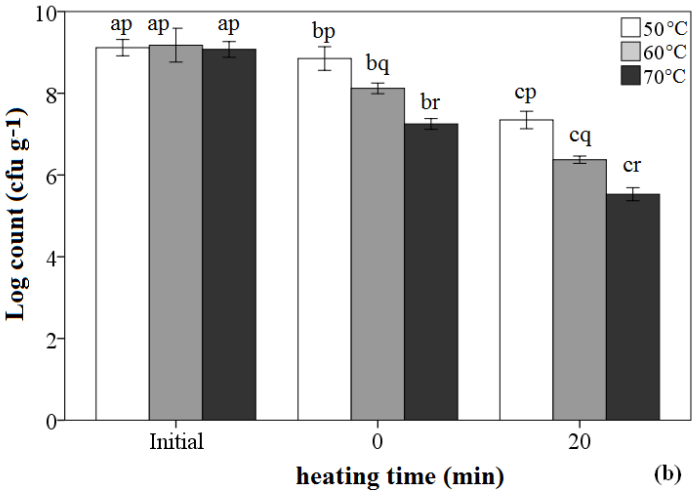

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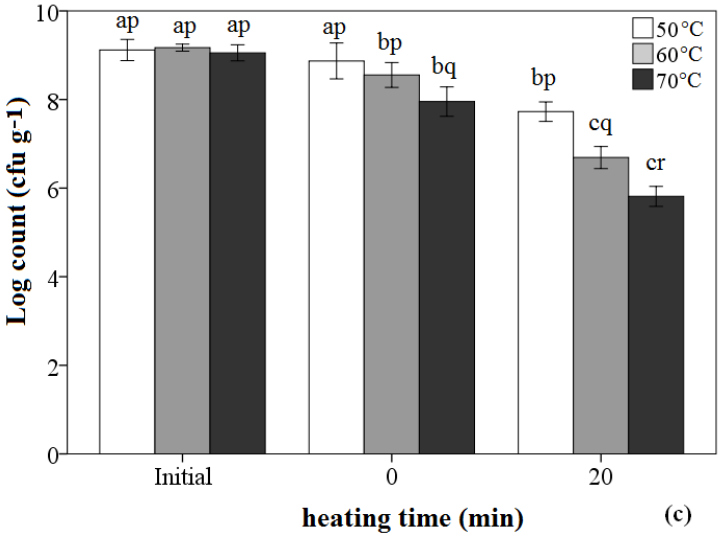

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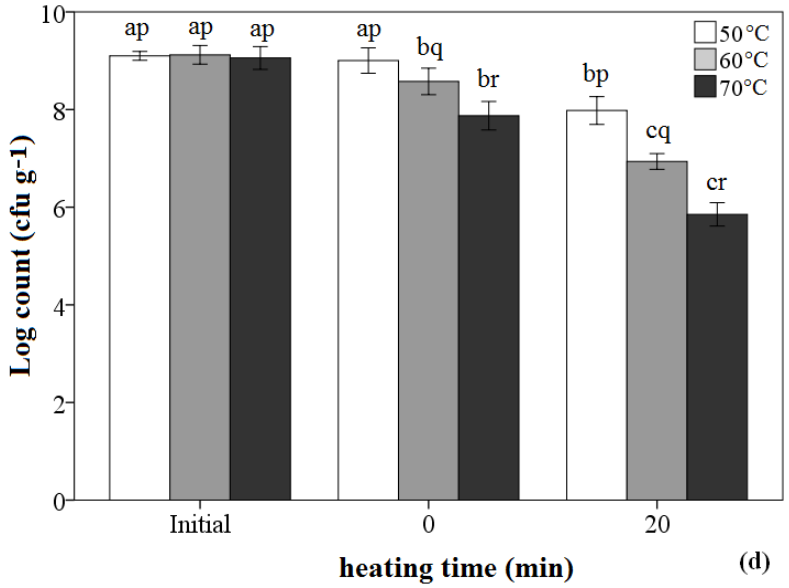

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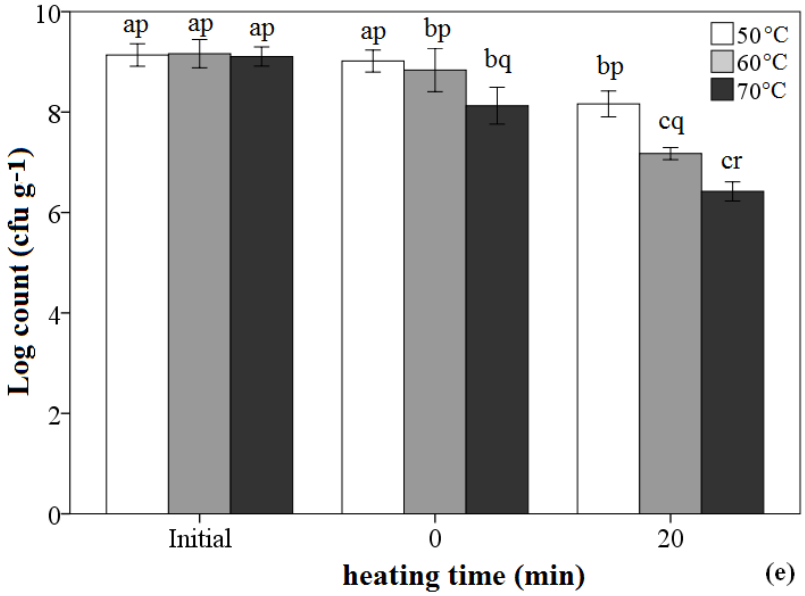

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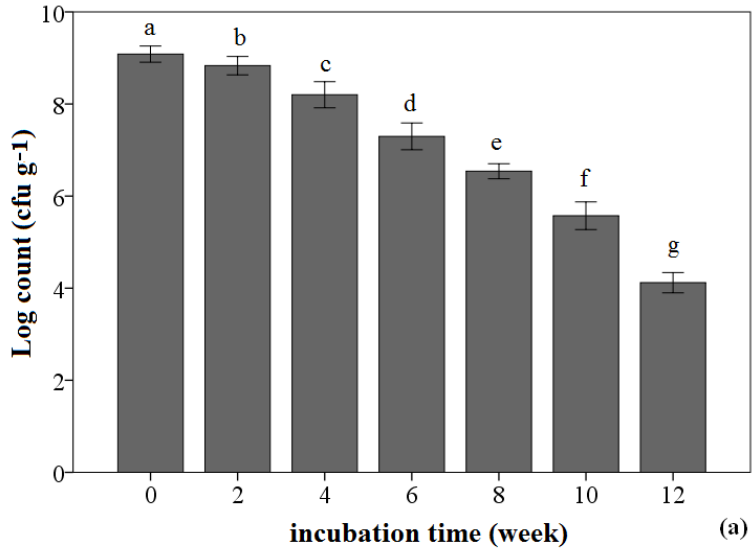
896 **Figure 5**



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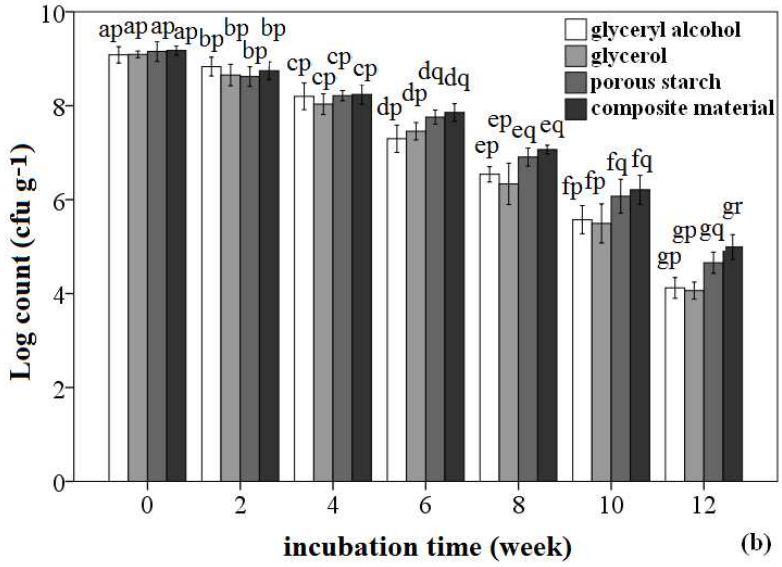

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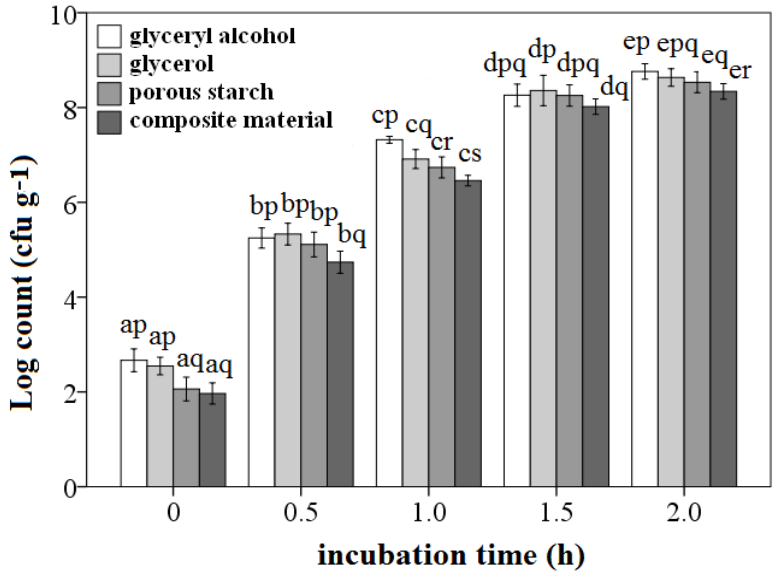
905 **Figure 6**



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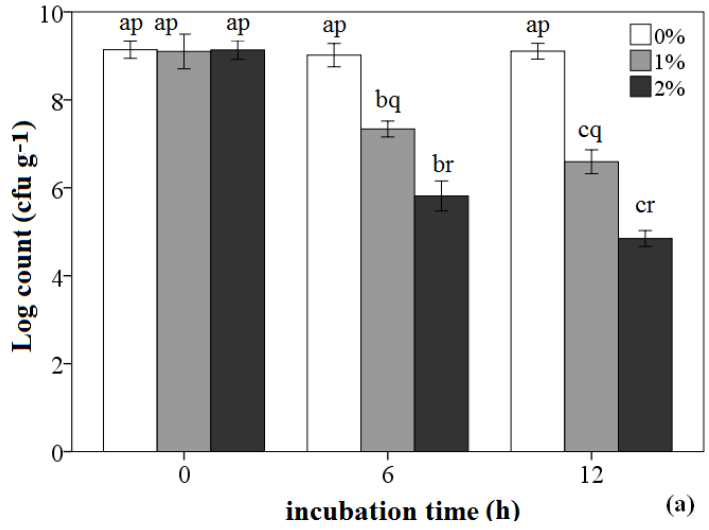
921 **Figure 7**



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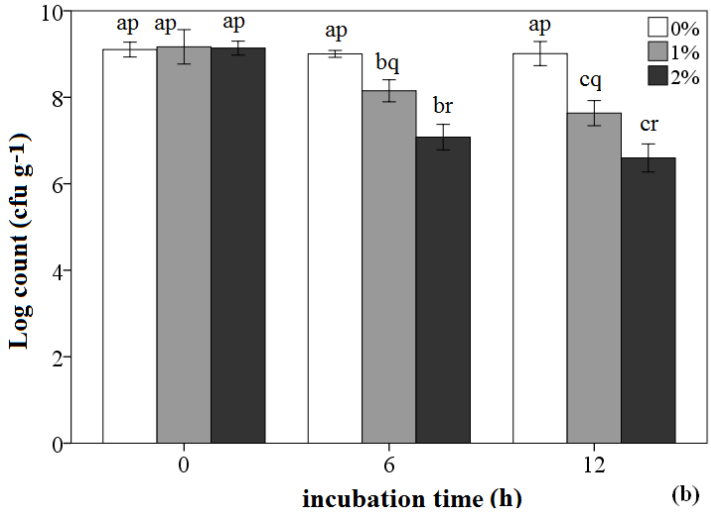

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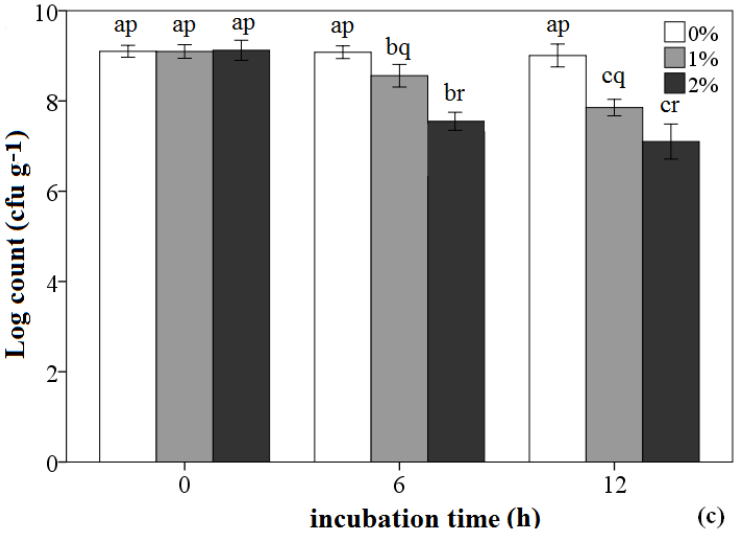

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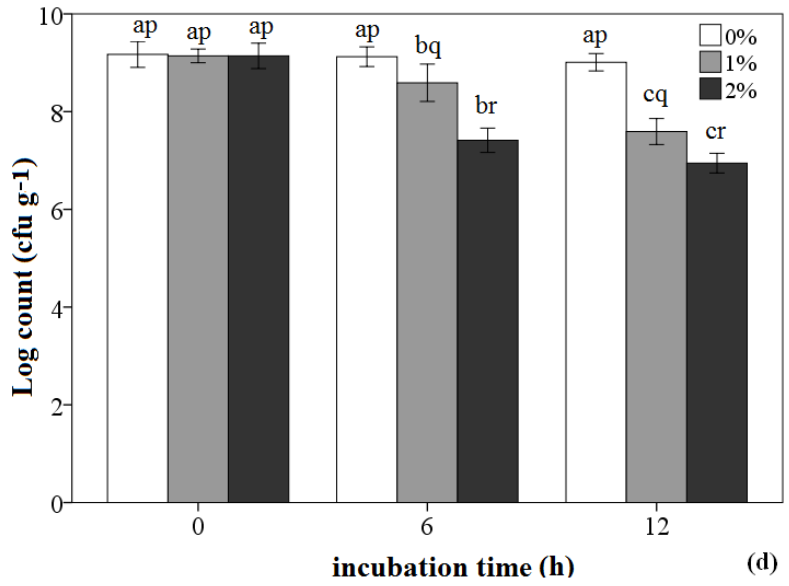

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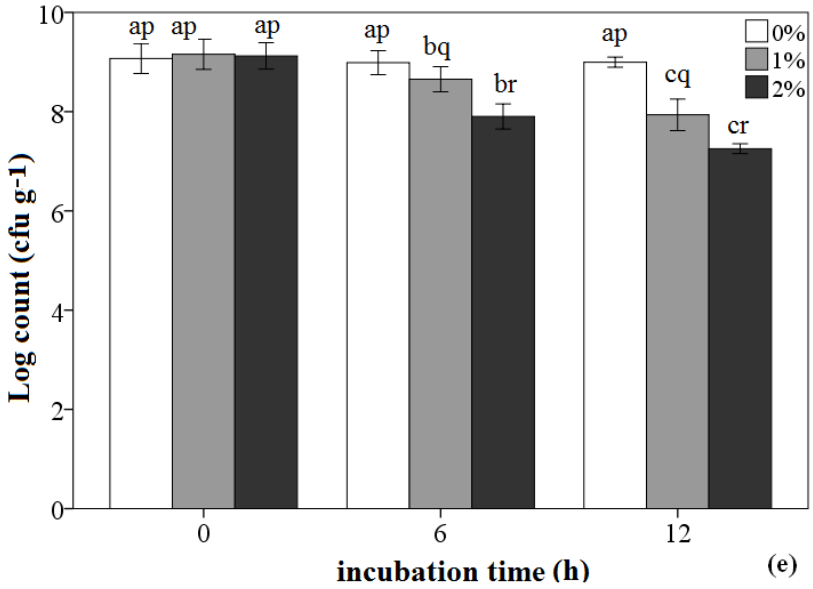

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