JOURNAL OF
AGRICULTURAL AND FOOD CHEMISTRY

Subscriber access provided by University of Manitoba Libraries

Agricultural and Environmental Chemistry

Development of novel microparticles for effective delivery of thymol and lauric acid to pig intestinal tract

Faith A Omonijo, Seung Il Kim, Tracy Guo, Qi Wang, Joshua Gong, Ludovic Lahaye, Jean-Christophe Bodin, Martin Nyachoti, Song Liu, and Chengbo Yang

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.8b02808 • Publication Date (Web): 24 Aug 2018

Downloaded from http://pubs.acs.org on August 27, 2018

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Published by American Chemical Society. Copyright © American Chemical Society. However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

1 **Running title: Microencapsulated essential oils and fatty acids for feed application**

2

3 **Title: Development of novel microparticles for effective delivery of thymol and lauric acid**
4 **to pig intestinal tract**

5
6 Faith A. Omonijo [1], Seungil Kim [2], Tracy Guo [4], Qi Wang [4], Joshua Gong [4], Ludovic Lahaye [5],
7 Jean-Christophe Bodin [5], Martin Nyachoti [1], Song Liu [2,3], and Chengbo Yang [1*]

8 *[1]Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada R3T 2N2;*
9 *[2]Biomedical Engineering, University of Manitoba, Winnipeg, MB, Canada R3T 2N2;*
10 *[3]Department of Biosystems Engineering, University of Manitoba, Winnipeg, MB, Canada R3T*
11 *2N2; [4]Guelph Research and Development Centre, Agriculture and Agri-Food Canada, 93 Stone*
12 *Road West, Guelph, ON, Canada N1G 5C9; [5]Jefo Nutrition Inc., Saint-Hyacinthe, Quebec,*
13 *Canada J2S 7B6*

14

15 * Corresponding author: Chengbo Yang, Ph.D., Assistant Professor

16 Livestock Nutrition/Nutritional Biochemistry

17 Department of Animal Science

18 University of Manitoba, Winnipeg, MB, R3T 2N2

19 Tel: +1.204.474.8188| Fax: +1.204.474.7628

20 Email: Chengbo.Yang@umanitoba.ca

21

22 **ABSTRACT**

23 Antibiotics have been widely supplemented in feeds at sub-therapeutic concentrations to prevent
24 post-weaning diarrhea and increase the overall productivity of pigs. However, the emergence of
25 antimicrobial-resistant bacteria worldwide has made it urgent to minimize the use of in-feed
26 antibiotics. The development of promising alternatives to in-feed antibiotics is crucial for
27 maintaining the suitability of swine production. Both medium-chain fatty acids (MCFA) and
28 essential oils exhibit great potential to post-weaning diarrhea; however; their direct inclusion has
29 compromised efficacy because of several factors including low stability, poor palatability and
30 low availability in the lower gut. Therefore, the objective of this study was to develop a
31 formulation of microparticles to deliver a model of essential oil (thymol) and MCFA (lauric
32 acid). The composite microparticles were produced by the incorporation of starch and alginate
33 through a melt-granulation process. The release of thymol and lauric acid from the microparticles
34 was *in vitro* determined using simulated salivary fluid (SSF), simulated gastric fluid (SGF) and
35 simulated intestinal fluid (SIF), consecutively. The microparticles prepared with 2% alginate
36 solution displayed a slow release of thymol and lauric acid in the SSF (21.2 ± 2.3%; 36 ± 1.1%),
37 SGF (73.7 ± 6.9%; 54.8 ± 1.7%) and SIF (99.1 ± 1.2%; 99.1 ± 0.6%), respectively, whereas, the
38 microparticles without alginate showed a rapid release of thymol and lauric acid from the SSF
39 (79.9 ± 11.8%; 84.9 ± 9.4%), SGF (92.5 ± 3.5%; 75.8 ± 5.9%) and SIF (93.3 ± 9.4%; 93.3 ±
40 4.6%), respectively. The thymol and lauric acid in the developed microparticles with or without
41 alginate both exhibited excellent stabilities (> 90%) during being stored at 4˚C for 12 weeks and
42 after being stored at room temperature for 2 weeks. These results evidenced that the approach
43 developed in the present study could be potentially employed to deliver thymol and lauric acid to

44 the lower gut of pigs, although, further *in vivo* investigations are necessary to validate the
45 efficacy of the microparticles.

46 **Keywords:** Encapsulation; Gut, Lauric acid, Microparticles, Slow release, Thymol

47 1. INTRODUCTION

48 Young animals are very vulnerable to diseases, and using antimicrobials is the most cost-
49 effective method to improve the health and productivity of food production animals raised with
50 conventional agricultural techniques (Looft et al., 2012; Yang et al., 2015). Although this
51 practice has been banned in Europe and other countries have also started to minimize the use of
52 antibiotics in the animal production, it still exists in major parts of the world (Hassan et al.,
53 2018). Therefore, replacing antibiotics with cost-effective alternatives remains crucial to ensure a
54 sustainable food animal production.

55 Essential oils are considered as valid candidates to replace antibiotics in the feed industry (Li et
56 al., 2012; Gong et al., 2014; Omonijo et al., 2018). Essential oils (e.g., thymol) are extracted
57 from plants and can promote growth performance and health in animals because of their
58 biological activities and antimicrobial activities (Si et al. 2006a; Edris, 2007; Del Nobile et al.
59 2008; Brenes et al., 2010; Puvaca et al., 2013; Rassu et al, 2014). With the identification and
60 characterization of bioactive components in plant extracts and significant progress in mechanistic
61 research with these components in food production animals, many research efforts have been
62 made to use essential oils substituting antibiotics within the animal production chain (Omonijo et
63 al., 2018). The rationales for using essential oils in animal feeds have relied on their abilities to
64 inhibit bacterial growth, reduce virulence through quorum-sensing disruption, and regulate innate
65 immunity of animals (Hassan et al., 2018). However, most essential oils have a high minimum
66 inhibitory concentration (MIC) that are unlikely accepted in the industry regarding cost-

67 efficiency, feed palatability and government regulation (Yang et al., 2015; Omonijo et al., 2018;
68 Hassan et al., 2018). Therefore, it is vital to ensure the delivery of essential oils to the target site
69 for increasing their efficacy.

70 Essential oils have very high volatility, and their bioactive compounds are readily degradable
71 when exposed to heat, oxygen, light, or during their interactions with other compounds, thus,
72 negatively affecting their biological activities and antimicrobial activities (Si et al., 2006a ;
73 Zhang et al., 2016a; Gonçalves et al., 2017). Additionally, several studies are demonstrating that
74 several essential oils including thymol and carvacrol have almost completely vanished in the
75 upper digestive tract of pigs (Michiels et al., 2008; Zhang et al., 2016a). Therefore, unprotected
76 essential oils can be significantly vanished at the manufacture, transportation, and storage of
77 feeds and as well as during delivery to the pig gut, thus hindering access to the distal part of pig
78 intestine (Omonijo et al., 2018). This serves as a major challenge to the use of essential oil in pig
79 feeds. Thus, it is crucial to establish a useful and practical delivery approach for using essential
80 oils in feeds.

81 Medium-chain fatty acids (MCFA) including lauric acid (C_{12}) and its ester derivatives also have
82 potential to substitute antibiotics in weaning piglets (Han et al., 2011; Zentek et al., 2012; 2013;
83 Hanczakowska et al., 2013; De Smet et al., 2016). Several studies indicated that MCFA could
84 inhibit *Salmonella* growth (Van Immerseel et al., 2004; Messens et al., 2010). Synergistic
85 antimicrobial activities between oregano oil and caprylic acid were observed with several strains
86 including *Salmonella* (Hulánková and Bořilová, 2011). Similarly, Vande Maele et al. (2016)
87 demonstrated in an *in vitro* study that a combination of lauric acid and cinnamaldehyde had
88 synergistic effects in inhibiting the growth of *Brachyspira hyodysenteriae* that causes swine
89 dysentery. The use of MCFA is popular both in the food and feed industries. However, some

90 MCFA and their ester derivatives can compromise feed palatability and acceptance and reduce
91 feed intake in pigs due to their unpleasant odors (Omonijo et al., 2018). Thus, it is also essential
92 to develop a useful and practical delivery approach for using MCFA in feeds.
93 Microencapsulation has been becoming one of the most popular and practical approaches to
94 mask the unpleasant taste/odor, and deliver bioactive compounds in food production animals
95 (Piva et al., 2007; Chitprasert et al., 2014). Ideal microencapsulation should not only stabilize
96 essential oils but also release them specifically in the targeted regions of the intestine (Chen et
97 al., 2017; Omonijo et al., 2018). Therefore, the objective of the present study was to develop a
98 formulation of microparticles containing both thymol and MCFA for effective delivery to pig
99 intestinal tract.

100 2. MATERIALS AND METHODS

101 2.1. Materials

102 Thymol (≥ 98.5%), lauric acid (LA), palmitic acid (PA, C_{16}), stearic acid (SA, C_{18}), amylase,
103 sodium alginate (low viscosity), pepsin originated from porcine and pancreatin originated from
104 porcine were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Cornstarch was
105 purchased from Cargill (Cargill Inc., Minneapolis, MN, USA) and pre-gelatinized starch (1500)
106 from Coloran (West Point, PA, USA).

107 2.2. Selection of a suitable fatty acid

108 Three fatty acids including lauric acid, palmitic acid, and stearic acid were used in this
109 experiment because those have a melting point above a melting point (42˚C) of thymol and have
110 been used to deliver bioactive compounds (Ma et al., 2016; Pitigraisorn et al., 2017). The melting
111 points of lauric acid, palmitic acid, and stearic acid are 43˚C, 63˚C and 69˚C, respectively. Ten
112 grams of each fatty acid was mixed with 10 g of thymol, respectively. The mixtures were then

113 melted in a water bath at 70˚C. After melting, the mixtures were stirred for 30 min. The molten
114 mixture of each fatty acid with thymol was left to stay at 55˚C without stirring for 2 h before
115 placing at room temperature (23˚C) up to 6 h to allow for solidification.
116 To observe the crystal morphology of thymol, lauric acid, and their mixture, an emulsion of
117 thymol, lauric acid and the mixture of thymol and lauric acid (ratio 1:1) were prepared. Lauric
118 acid and thymol were melted at 70˚C individually or mixed at a ratio of 1:1, and then added into
119 the water at 10% with 1% tween 80 as a surfactant. The mixture was mixed using a Polytron
120 (PT10-35GT, Kinematica AG, Switzerland) for 2 min at 13,000 rpm to make an emulsion. Then,
121 three emulsions were stored at 4˚C overnight allowing the emulsions to crystallize. The crystal
122 morphology was examined under a microscope (Eclipse Ci, Nikon, Japan).
123

124 **2.3. The melting point of thymol, lauric acid, and their mixture**

125 Among the three fatty acids tested, lauric acid was selected for further study because its mixture
126 with thymol remained a homogeneous liquid at room temperature for 6 h. Before use, 1 g of
127 thymol and lauric acid each were kept at -80˚C for 30 min and then mixed by vortexing for 30
128 sec at 3,000 rpm. The mixture was kept in -80˚C for 3 h and then ground to a fine powder using a
129 grinder. The grinder was kept -20˚C for 3 h before use to avoid increasing temperature to higher
130 than the melting temperature of thymol and lauric acid. The melting temperature of the thymol,
131 lauric acid, and their mixture (50: 50 wt%) was measured by differential scanning calorimetry
132 (DSC). For the measurement, 12.1 mg thymol, 13.1 mg lauric acid, and 10.7 mg mixture were
133 weighed into individual Tzero Aluminum hermetic pans. The pan was placed in the chamber of
134 DSC (Q Series DSC, TA Instrument). The DSC was programmed as follow: 1) Equilibrate at

135 25˚C; 2) Jump to -10˚C; 3) Ramp 10˚C/min to 80˚C (1st run); 4) Cooling; 5) Equilibrate at -
136 10˚C; 6) Isothermal for 5 min; and 7) Ramp 10˚C/min to 80˚C (2nd run).

137 **2.4. Preparation of microparticles**

138 For preparing microparticles without adding 2% alginate solution, 5 g of lauric acid and 5 g of
139 thymol were weighed into a closed vial separately and melted at 70˚C in a water bath, mixed
140 together and stirred for 30 min. Thirty grams of cornstarch and 5 g of pre-gelatinized starch (a
141 ratio of 6:1) were weighed separately and then mixed in a container by hand shaking. The molten
142 thymol and lauric acid mixture was added into the starch mixture and then mixed by hand
143 stirring. Fifteen milliliters of distilled water (3 times of pre-gelatinized starch) was added to the
144 mixture. The containers were immediately placed into an ice-water bath for 1.5 h and kept in a
145 refrigerator (4˚C) overnight for solidification. The solid particles were then granulated into
146 micro-particles with a granulating machine (UAM Pharmag, Germany) at 90 rpm using a pore
147 size of 0.1 mm and dried at room temperature (23˚C) for 1 h before being stored in a refrigerator
148 (4˚C).

149 For preparing microparticles with alginate, a total of 0.3 g of alginate was weighed and dissolved
150 in 15 mL of distilled water to make a 2% (w/v) alginate solution. The same protocol described
151 above was used to make the microparticles except for replacing the 15 mL of water with the 2%
152 alginate solution.

153

154 **2.5. Morphology of microparticles**

155 The morphology of the microparticles produced with or without adding a 2% alginate solution
156 was determined with a light microscope (Axio Cam 105, Carl-Zeiss, Switzerland; Nikon eclipse,

157 Japan) at 10 × magnification and the Zen Image Software (2012) was used to determine the
158 surface diagram of the microparticles.

159 **2.6. *In vitro* release of thymol and lauric acid from the microparticles**

160 *In vitro* release of thymol and lauric acid from the microparticles was determined with simulated
161 digestive fluid using previously published procedures with some modifications (Minekus et al.
162 2014). The simulated salivary fluid (SSF) contained 15.1 mmol/L KCl, 13.6 mmol/L $NaHCO_3$,
163 3.7 mmol/L KH_2PO_4, 0.15 mmol/L $MgCl_2(H_2O)_6$ and 0.06 mmol/L $(NH_4)_2CO_3$. The simulated
164 gastric fluid (SGF) contained 47.2 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 6.9 mmol/L KCl, 0.9
165 mmol/L KH_2PO_4, 0.5 mmol/L $(NH_4)_2CO_3$ and 0.1 mmol/L $MgCl_2(H_2O)_6$. The simulated
166 intestinal fluid (SIF) contained 85 mmol/L $NaHCO_3$, 38.4 mmol/L NaCl, 6.8 mmol/L KCl, 0.8
167 mmol/L KH_2PO_4 and 0.33 mmol/L $MgCl_2(H_2O)_6$. The pH of SSF, SGF and SIF was adjusted
168 using HCl or NaOH to 7.0, 3.0 and 7.0, respectively. The final digestion mixtures of the
169 electrolyte solution for SSF, SGF and SIF contained 1.5, 0.15 and 0.6 mmol/L of $CaCl_2(H_2O)_2$,
170 respectively. Respective enzymes were also added to simulate digestion in pig digesta. Alpha-
171 amylase originated from human saliva was included in the SSF final digestion mixture at a
172 concentration of 75 U/mL. Pepsin originated from porcine gastric mucosa was added to the SGF
173 final digestion mixture at a concentration of 2000 U/mL and pancreatin originated from porcine
174 pancreas was added to the SIF final digestion mixture at a concentration of 100 U/mL.

175 Forty microparticle samples (each 0.5 g) were employed to mimic digestion within the mouth,
176 stomach and small intestine in pigs. Four samples were taken from each sampling point (0, 2, 30,
177 60, 90, 120, 150, 180, 210 and 240 min) with points between 0 to 2 min representing the
178 digestion in the mouth, 2 to 120 in the stomach and 120 to 240 min in the small intestine. All
179 simulated solutions were maintained at 37°C. The SSF was added to each of the samples at a

180 ratio of 1:1 and placed in the incubator with shaking (Innova TM. 4200, New Brunswick
181 Scientific, Edison/ NJ. USA) for 2 min. The pH was adjusted to 3 with 1M HCl before SGF was
182 added. At the end of the SGF stage, the pH was adjusted to 7 with 1 M NaOH followed by
183 addition of the SIF. To measure the concentration of thymol and lauric acid, 5 mL of oil
184 extraction solvent (hexane) was added to each of the supernatants, shaken (IKA Vibrax VXR
185 Basic, U.S.A) for 20 min and allowed to stay for 30 min. Each of the supernatant from each point
186 was diluted 10 times and the diluent was filtrated using a syringe-driven filter unit
187 (polyetrafluoroethylene, 0.22 nm) and further analyzed by gas chromatography (GC) following
188 the method explained below. Two replicates for each sample were used.
189 The column installed was SUPELCO WAX^{TM} 10 (fused silica capillary column; 60 m × 0.25
190 mm × 0.50 nm film thickness and the temperature limits from 35-280˚C). Thymol and lauric acid
191 were identified by comparing the retention time with the standard thymol and lauric acid and
192 their concentrations were calculated by comparing the total peak area of thymol and lauric acid
193 with the standard curve. Released thymol or lauric acid content = thymol or lauric acid
194 concentration in GC vial × 5 (volume of added hexane) × dilution times/thymol or lauric acid in
195 the dry samples × 100%.

196 **2.7. Determining the stability of thymol and lauric acid in the microparticles**

197 The stability of thymol and lauric acid in the microparticles with or without alginate was
198 measured after being stored at room temperature (23˚C) for 2 weeks and during the storage at
199 4˚C for 12 weeks. The recovery rate of thymol and lauric acid were determined with the
200 procedure described as below. Samples were taken at different time points (1 week, 3 weeks, 6
201 weeks and 12 weeks) for analysis. Each sample (0.5g) was suspended in 15 mL of distilled water

202 containing Pancreatin (100 U/mL). The mixture was incubated and analyzed as described above.
203 Each of the samples was measured in triplicate.

204 3. RESULTS AND DISCUSSION

205 *3.1. Selection of a fatty acid*

206 There was no visible phase separation for all three mixtures at the molten state (Fig. 1A). After
207 being placed at room temperature (23˚C) for 6 h, the molten mixture of thymol and lauric acid
208 was still in a clear liquid state without having phase separation, however, the other two molten
209 mixtures (thymol / palmitic acid and thymol / stearic acid) solidified and formed a gel-like
210 mixture (Fig.1B).

211 These results are consistent with the DSC measurements. As shown in Fig. 2, the mixture of
212 lauric acid and thymol exhibited a single melting peak with a value of 30.6˚C, which is lower
213 than both that of thymol (52.8˚C) and lauric acid (47.4˚C). This suggested that the mixture of
214 lauric acid and thymol was in a eutectic solution, that is, a mixture of two or more pure
215 chemicals at certain ratios, in which the chemicals inhibit the crystallization process of one
216 another, resulting in a system having induced melting point depression (Washburn, 1924).

217 Once cooling the emulsions, thymol crystalized in irregular shapes (Fig. 3A), whereas lauric acid
218 crystalized in round shapes (Fig. 3B). The resulted mixture of the two crystalized into somewhat
219 ovular shaped particles without visible distinctions between the two individual components (Fig.
220 3C). This observation indicates that thymol and lauric acid co-crystalized together. Both results
221 from DSC and microscopy observation showed that thymol and lauric acid form a pair of a good
222 candidate for a formulation of antimicrobial microparticles for the following reasons. Firstly,
223 since lauric acid significantly reduced the melting point of thymol, it served as a liquid carrier for
224 thymol at room temperature for a period up to 6 h. This property provides an excellent

225 convenience for processing of thymol products such as in the present study. This is because
226 when at a liquid state, thymol and fatty acids can be easily mixed and better absorbed by the
227 starch granules which helps to ensure even distribution and better protection of the core
228 ingredients within the encapsulation matrix. Secondly, a combination of thymol with lauric acid
229 in one product may provide additional protective benefits to the animals. An *in vitro* study
230 demonstrated that lauric acid could effectively inhibit the growth of *Brachyspira hyodysenteriae*
231 with a MIC value less than 1.5 mM (Vande Maele et al., 2016). Dietary fats with a considerable
232 level of lauric acid and myristic acid increased broiler growth performance that may be related to
233 lauric acid's antimicrobial properties (Zeitz et al., 2015). Most recently there was a study
234 showing that lauric acid can reduce *Campylobacter* spp. in broiler meat (Zeiger et al., 2017).
235 Lauric acid's ester derivatives (e.g., monolaurin) are also known for their protective biological
236 activities as antimicrobial agents (Seleem et al., 2016). The exact mechanism of lauric acid anti-
237 microbial effect is still unclear. However, it is believed that some MCFA can damage the cell
238 membrane, therefore, causing bacterial death (Desbois et al. 2010). It has been believed that the
239 amphipathic structure of MCFA allows them to cause pores with a different size in the cell
240 membrane. MCFA also could cause bacteria death by reducing enzyme function, blocking
241 nutrient absorption and producing toxic compounds for bacteria (Desbois et al. 2010). Therefore,
242 in this study lauric acid is not only a suitable carrier for thymol but also a bioactive compound
243 with antimicrobial properties.

244 *3.2. Morphology of microparticles*

245 The compositions of microparticles with/without alginate include 66.22%/66.67% cornstarch,
246 11.03%/11.11% pre-gelatinized starch, 11.03%/11.11% thymol, 11.03%/11.11% lauric acid and
247 0.7%/0% alginate. The average particle sizes of the microparticles were 800 μm in diameter, and

248 this was similar to the average size of 890 μm for microparticles produced by Benavides et al.
249 (2016) through the method of ionic gelation of alginate. There is no difference in the average
250 particle size between the microparticles produced with or without alginate; however, the shapes
251 and surfaces of the two types of microparticles were different (Fig. 4). The microparticles with
252 alginate were mostly spherical with a relatively smooth surface, whereas those without alginate
253 had irregular shapes with rough edges and coarse surfaces.
254 Many kinds of polymers have been employed to encapsulate and deliver bioactive compounds in
255 both food and feed applications (Almeida et al., 2013; Zhang et al., 2016a; Chen et al., 2017).
256 For applications in animal feeds, it is better to use natural polymers that have been approved for
257 use in feeds. Starch is popularly used for microencapsulation because it is biodegradable, edible,
258 commonly available at low cost, nonallergic, easy to use and thermo-processable (Zhu, 2017).
259 Starch consists of both amylose and amylopectin (Tester et al., 2004; Udachan et al., 2012). Pre-
260 gelatinized starch has undergone processing under intense heat conditions by cooking, drying
261 and making into fine powder thus, leading to better solubility in water and being readily
262 solubilized at room temperature (Romano et al., 2018; Fiorda et al., 2015). The combined use of
263 cornstarch and pre-gelatinized starch in this study increases the water retentivity (Romano et al.
264 2018), thus promotes hydrogen bonding and the formation of the network in the encapsulation
265 matrix. As a natural polymer derived from brown seaweed, alginate is a linear and anionic
266 polysaccharide (Dragan, 2014). At room temperature, alginate is soluble in water allowing the
267 formation of gel without heating and cooling cycles, which make alginate as an attractive
268 microencapsulation material for feed applications (Benavides et al., 2016; Agüero et al., 2017).
269 The inclusion of alginate to the starch matrix improved the shape and surface properties. This

270 could be attributed to its remarkable crosslinking capability and excellent film-forming
271 properties.
272

273 *3.3. In vitro release profiles of thymol and lauric acid from the microparticles*

274 As shown in Fig. 5A, both thymol and lauric acid encapsulated in the microparticles with
275 alginate exhibited slow release profiles in the simulated gastrointestinal fluids. The cumulative
276 release (%) of thymol and lauric acid increased gradually to 21.2 ± 2.3 and 36.0 ± 1.1 in the SSF,
277 73.7 ± 6.9 and 36.8 ± 0.6 in SGF. Both thymol and lauric acid were completely released in the
278 SIF within 240 min. However, as shown in Fig. 5B, the microparticles produced without alginate
279 had a rapid release of thymol ($79.9 \pm 11.8\%$) and lauric acid ($80.8 \pm 5.9\%$) after incubation in the
280 SSF for 2 min. When the microparticles were placed in the SGF for 120 min, the cumulative
281 release rates reached $92.5 \pm 3.5\%$ and $75.8 \pm 5.9\%$ respectively for thymol and lauric acid. The
282 rest of thymol and lauric acid were released from both types of microparticles in less than 40
283 mins after they were placed in the SIF.

284 The goal of a current delivery method is to release thymol and lauric acid at a low percentage in
285 the mouth and stomach but have a sustained release as it passes through the intestine (Piva et al.
286 2007). The fast release of thymol and lauric acid in SSF from the microparticles without alginate
287 is primarily due to the presence of alpha-amylase in the SSF, an enzyme that is known to digest
288 starch quickly. The excellent solubility of pre-gelatinized starch could also have contributed to
289 the fast release of the active components. The inclusion of alginate to the starch matrix markedly
290 reduced the release rate in the SSF. This is mainly due to the existences of carboxylic groups in
291 alginate molecules and calcium ions in the simulated digestive fluids. Calcium ions may form
292 crosslinks between carboxylic groups in addition to hydrogen bondings, leading to enhanced

293 networks of encapsulation matrix, therefore, retard the dissolution of starch molecules and slow
294 the release of thymol and lauric acid. The globular shaped and smooth surface of microparticles
295 with alginate would have a smaller specific surface area compared to the irregular shaped and
296 rough surface of microparticles without alginate. This may be another factor contributing to the
297 better release property of alginate containing microparticles. Notably, alginate also effectively
298 reduced the release of active components in the SGF which can be explained by the pH
299 sensitivity of alginate molecules. When it is under very acidic conditions (e.g., pH at stomach)
300 that are lower than its pKa, the carboxylic groups are not ionized and stay as COOH resulting in
301 an insoluble structure (Agüero et al., 2017). When pH is close to 7 which is similar to the
302 intestinal pH, the carboxylic groups became ionized (COO-) resulting in that the polymer chain
303 significantly expands and the hydrophilic alginate matrix enlarges (Agüero et al., 2017). In this
304 study, the results indeed demonstrated that alginate significantly decreased the release of thymol
305 and lauric acid in SGF and increased their release in the SIF. Many studies have shown that
306 alginate matrix prevented a quick release of active components in the acidic environment of the
307 stomach and allowed a prolonged release under the intestinal conditions (Zastre, 1997, Zhang et
308 al. 2016a). However, compounds that are highly soluble and have a low molecular weight cannot
309 be prevented from releasing in the mouth and stomach even though the granules matrix does not
310 erode or swell. The alginate-containing microparticles developed in this study need to be further
311 optimized to reduce the release rates in the SSF and SGF.

312 Although the release behavior of thymol and lauric acid from the microparticles provides
313 precious information, it is challenging to precisely demonstrate release behavior in pig gut
314 because of the complexity of gut physiological environments. This was supported by the study
315 indicating that the rate of release of encapsulated carvacrol in the pig stomach via *in vivo* studies

316 was 25% higher than the rate obtained from *in vitro* studies (Zhang et al., 2016a), which may be
317 due to the phenolic binding to other components such as fats and hydrophobic compounds
318 present in the diet (Lallès et al., 2009). Therefore, *in vivo* release behavior of the microparticles
319 has to be determined eventually in the gastrointestinal tract of pigs.

320

321 *3.4. The stability of microparticles with/without alginate during storage*

322 As shown in Fig. 6, thymol and lauric acid had good stabilities (> 95%) in both types of
323 microparticles with or without alginate after being stored at room temperature (23˚C) for 2
324 weeks. As shown in Fig. 7, thymol and lauric acid had good stabilities (> 90%) in both types of
325 microparticles with or without alginate after being stored at 4˚C for 12 weeks. Durante et al.
326 (2012) showed that the encapsulation of wheat bran oil into 2% (w/v) sodium alginate beads
327 significantly increased the stability of wheat bran oil at 4˚C. This was also found in the research
328 conducted by Otálora et al. (2016), that the encapsulation of betalain with calcium-alginate had
329 good stability when stored at low relative humidity.

330 Stability during storage is an essential factor that should be considered for a feed additive. Feed
331 additives have a 1-2 year shelf life under current industry practice. Our preliminary data
332 demonstrated that the current microparticles are stable during short-term storage. However, the
333 stability of long-term storage (e.g.1-2 year) must be further investigated. The inclusion of
334 antioxidants in the formula may be considered to enhance the stability of encapsulated thymol
335 and lauric acid. In conclusion, the formulation and method established in this study for the
336 encapsulation of thymol and lauric acid in microparticles are relatively simple and can be used as
337 a potential method to effectively deliver essential oils and MCFA to the pig intestinal tract. This
338 unique essential oil formula will be further optimized for better-controlled release though

339 investigating the physicochemical and molecular property of the microparticles. Retention of
340 encapsulated thymol and lauric acid during feed processing will be mimicked by the treatments
341 of steam for different time periods and validated in a real pelleting process. Further
342 investigations are needed to confirm the efficacy of the microparticles with *in vivo* studies.
343

344 **Notes**

345 The authors declare the following competing financial interest (s): Drs. C. Yang, S. Liu, and M.
346 Nyachoti have a patent application in process for the developed microparticles.
347

348 **ACKNOWLEDGMENTS**

349 This work was financially supported by the Natural Sciences and Engineering Council of Canada
350 (NSERC) CRD Grant (C. Yang, CRDPJ 503580-16), Manitoba Pork Council (C. Yang, 47370),
351 Jefo Nutrition Inc. (C. Yang, 47369), and the Start-Up Grant (C. Yang, 46561) and the Graduate
352 Enhancement of Tri-Council Stipends (GETS) program from the University of Manitoba. The
353 authors thank Natasha Brown and Bingqi Dong for their assistance in preparing the manuscript.
354 We also want to thank Philip Strange at AAFC for his help on the use of a microscope.
355

356 **REFERENCES**

357 1. Agüero L, Zaldivar-Silva D, Peña L, Dias ML. Alginate microparticles as oral colon drug
358 delivery device: A review. Carbohydr Polym. 2017, 168: 32-43.
359 2. Almeida AP, Rodríguez-Rojo S, Serra AT, Vila-Real H, Simplicio AL, Delgadilho I, da
360 Costa SB, da Costa LB, Nogueira ID, Duarte CM. Microencapsulation of oregano essential
361 oil in starch-based materials using supercritical fluid technology. Innov Food Sci Emerg
362 Technol. 2013, 20: 140-145.
363 3. Benavides S, Cortés P, Parada J, Franco W. Development of alginate microspheres
364 containing thyme essential oil using ionic gelation. Food Chem. 2016, 204: 77-83.
365 4. Brenes A, Roura E. Essential oils in poultry nutrition: Main effects and modes of action.
366 Anim Feed Sci Tech. 2010, 158: 1–14.
367 5. Chen J, Wang Q, Liu CM, Gong J. Issues deserve attention in encapsulating probiotics:
368 critical review of existing literatures. Crit Rev Food Sci Nutr. 2017, 57: 1228-1238.
369 6. Chitprasert P, Sutaphanit P. Holy basil (Ocimum sanctum Linn.) essential oil delivery to
370 swine gastrointestinal tract using gelatin microcapsules coated with aluminum
371 carboxymethyl cellulose and beeswax. J Agric Food Chem. 2014, 62: 12641-12648.
372 7. de Los Santos FS, Donoghue A, Venkitanarayanan K, Dirain M, Reyes-Herrera I, Blore P,
373 Donoghue DJ. Caprylic acid supplemented in feed reduces enteric Campylobacter jejuni
374 colonization in ten-day-old broiler chickens. Poult Sci. 2008, 87: 800-804.
375 8. De Smet S, Michiels J, Ovyn A, Dierick N, Laget M, Cools A, et al. Gut antibacterial effects
376 of C7 and C9 carboxylic acids in the diet of piglets. J Anim Sci. 2016, 94: 54e7.
377 9. Del Nobile MA, Conte A, Incoronato AL, Panza O. Antimicrobial efficacy and release
378 kinetics of thymol from zein films. J Food Eng. 2008, 89: 57-63.

379 10. Desbois AP, Smith VJ. Antibacterial free fatty acids: activities, mechanisms of action and
380 biotechnological potential. Appl Environ Microbiol. 2010, 85: 1629-1642.
381 11. Dragan ES. Design and applications of interpenetrating polymer network hydrogels. A
382 review. Chem Engin J. 2014, 243: 572-590.
383 12. Durante M, Lenucci MS, Laddomada B, Mita G, Caretto S. Effects of sodium alginate bead
384 encapsulation on the storage stability of durum wheat (Triticum durum Desf.) bran oil
385 extracted by supercritical CO_2. J Agric Food Chem. 2012, 60: 10689-10695.
386 13. Edris AE. Pharmaceutical and therapeutic potentials of essential oils and their individual
387 volatile constituents: a review. Phytother Res. 2007, 21: 308-323.
388 14. Fiorda FA, Soares Jr MS, da Silva FA, de Moura CM, Grossmann MV. Physical quality of
389 snacks and technological properties of pre-gelatinized flours formulated with cassava starch
390 and dehydrated cassava bagasse as a function of extrusion variables. LWT-Food Sci Technol.
391 2015, 62: 1112-1119.
392 15. Gonçalves ND, de Lima Pena, F, Sartoratto A, Derlamelina C, Duarte MCT, Antunes AEC,
393 Prata AS. Encapsulated thyme (Thymus vulgaris) essential oil used as a natural preservative
394 in bakery product. Food Res Int. 2017, 96: 154-160.
395 16. Gong J, Yin F, Hou Y, Yin Y. Chinese herbs as alternatives to antibiotics in feed for swine
396 and poultry production: Potential and challenges in application. Can J Anim Sci. 2014, 94:
397 223–241.
398 17. Han YK, Hwang IH, Thacker PA. Use of a micro-encapsulated eucalyptus-medium chain
399 fatty acid product as an alternative to zinc oxide and antibiotics for weaned pigs. J Swine
400 Health Prod. 2011, 19: 34-43.

401 18. Hanczakowska E, Szewczyk A, Swiatkiewicz M, Okon K. Short-and medium-chain fatty
402 acids as a feed supplement for weaning and nursery pigs. Pol J Vet Sci. 2013, 16: 647-654.
403 19. Hassan YI, Lahaye L, Gong MM, Peng J, Gong J, Liu S, Gay C, Yang C. Innovative drugs,
404 chemicals, and enzymes within the animal production chain. Vet Res. 2018, 49: 71.
405 20. Hulánková R, Bořilová G. In vitro combined effect of oregano essential oil and caprylic acid
406 against Salmonella serovars, Escherichia coli O157: H7, Staphylococcus aureus and Listeria
407 monocytogenes. Acta Vet Brno. 2011, 80: 343-348.
408 21. Lallès JP, Bosi P, Janczyk P, Koopmans SJ, Torrallardona D. Impact of bioactive substances
409 on the gastrointestinal tract and performance of weaned piglets: a review. Animal. 2009, 3:
410 1625-1643.
411 22. Li SY, Ru YJ, Liu M, Xu B, Péron A, Shi XG. The effect of essential oils on performance,
412 immunity and gut microbial population in weaner pigs. Livest Sci. 2012, 145: 119–123.
413 23. Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, Sul WJ, Stedtfeld TM,
414 Chai B, Cole JR, Hashsham SA, Tiedje JM, Stanton TB. In-feed antibiotic effects on the
415 swine intestinal microbiome. Proc Natl Acad Sci USA. 2012, 109: 1691-6.
416 24. Ma YH, Wang Q, Gong J and Wu XY. Formulation of granules for site-specific delivery of
417 an antimicrobial essential oil to the animal intestinal tract. J Pharm Sci. 2016, 105: 1124-
418 1133.
419 25. Messens W, Goris J, Dierick N, Herman L and Heyndrickx M. Inhibition of Salmonella
420 typhimurium by medium-chain fatty acids in an in vitro simulation of the porcine cecum. Vet
421 Microbiol. 2010, 141:73-80.

ACS Paragon Plus Environment

422 26. Michiels J, Missotten J, Dierick N, Fremaut D, Maene P, ade Smet S. In vitro degradation
423 and in vivo passage kinetics of carvacrol, thymol, eugenol and trans-cinnamaldehyde along
424 the gastrointestinal tract of piglets. J Sci Food Agric. 2008, 88: 2371–2381.
425 27. Minekus M, Alminger M, Alvito P, Ballance S, Bohn TO, Bourlieu C, Carriere F, Boutrou R,
426 Corredig M, Dupont D, Dufour C, Egger L, Golding M, Karakaya S, Kirkhus B, Le Feunteun
427 S, Lesmes U, Macierzanka A, Mackie A, Marze S, McClements DJ, Ménard O, Recio I,
428 Santos CN, Singh RP, Vegarud GE, Wickham MS, Weitschies W, Brodkorb A. A
429 standardised static in vitro digestion method suitable for food–an international consensus.
430 Food Funct. 2014, 5: 1113-1124.
431 28. Omonijo FA, Ni L, Gong J, Wang Q, Lahaye L, Yang C. Essential oils as alternatives to
432 antibiotics in swine production. Anim Nutr. 2018, 4: 126-136.
433 29. Otálora MC, Carriazo JG, Iturriaga L, Osorio C, Nazareno MA. Encapsulating betalains from
434 Opuntia ficus-indica fruits by ionic gelation: Pigment chemical stability during storage of
435 beads. Food Chem. 2016, 202: 373-382.
436 30. Pitigraisorn P, Srichaisupakit K, Wongpadungkiat N, Wongsasulak S. Encapsulation of
437 Lactobacillus acidophilus in moist-heat-resistant multilayered microcapsules. J Food Engin.
438 2017, 192: 11-18.
439 31. Piva A， Pizzamiglio V, Morlacchini M, Tedeschi M, Piva G. Lipid microencapsulation
440 allows slow release of organic acids and natural identical flavors along the swine intestine. J
441 Anim Sci. 2007, 85: 486-493.
442 32. Puvaca N, Stanacev V, Glamocic D, Levicc J, Peric L, Stanacev V, Milic D. Beneficial
443 effects of phytoadditives in broiler nutrition. World's Poult Sci J. 2013, 69: 27–34.

444 33. Rassu G, Nieddu M, Bosi P, Trevisi P, Colombo M, Priori D, Manconi P, Giunchedi P,
445 Gavini E, Boatto G. Encapsulation and modified-release of thymol from oral microparticles
446 as adjuvant or substitute to current medications. Phytomed. 2014, 21: 1627-1632.
447 34. Romano N, Kanmani N, Ebrahimi M, Chong CM, Teh JC, Hoseinifar SH, Amin SN,
448 Kamarudin MS, Kumar V. Combination of dietary pre-gelatinized starch and
449 isomaltooligosaccharides improved pellet characteristics, subsequent feeding efficiencies and
450 physiological status in African catfish, Clarias gariepinus, juveniles. Aquaculture. 2018, 484:
451 293-302.
452 35. Rossi R, Pastorelli G, Cannata S, Corino C. Recent advances in the use of fatty acids as
453 supplements in pig diets: a review. Anim Feed Sci Technol. 2010, 162: 1-11.
454 36. Seleem D, Chen E, Benso B, Pardi V, Murata RM. In vitro evaluation of antifungal activity
455 of monolaurin against Candida albicans biofilms. PeerJ. 2016, 4: e2148.
456 37. Si W, Gong J, Chanas C, Cui S, Yu H, Caballero C, Friendship RM. In vitro assessment of
457 antimicrobial activity of carvacrol, thymol and cinnamaldehyde towards Salmonella serotype
458 Typhimurium DT104: Effects of pig diets and emulsification in hydrocolloids. J Appl
459 Microbiol. 2006a, 101: 1282–1291.
460 38. Si W, Gong J, Tsao R, Zhou T, Yu H, Poppe C, Johnson R, Du Z. Antimicrobial activity of
461 essential oils and structurally related synthetic food additives towards selected pathogenic
462 and beneficial gut bacteria. J Appl Microbiol. 2006b, 100: 296-305.
463 39. Tester RF, Karkalas J, Qi X. Starch—composition, fine structure and architecture. J Cereal
464 Sci. 2004, 39: 151-165.
465 40. Udachan IS, Sahu AK, Hend FM. Extraction and characterization of sorghum (Sorghum
466 bicolor L. Moench) starch. Int Food Res J. 2012, 19: 315-319.

467 41. Van Immerseel F, De Buck J, Boyen F, Bohez L, Pasmans F, Volf J, Sevcik M, Rychlik I,
468 Haesebrouck F and Ducatelle R. Medium-chain fatty acids decrease colonization and
469 invasion through hilA suppression shortly after infection of chickens with Salmonella
470 enterica serovar Enteritidis. Appl Environ Microbiol. 2004, 70:3582-3587.
471 42. Vande Maele L, Heyndrickx M, Maes D, De Pauw N, Mahu M, Verlinden M, Haesebrouck
472 F, Martel A, Pasmans F, Boyen F. In vitro susceptibility of Brachyspira hyodysebteruae to
473 organic acids and essential oil components. J Vet Med Sci. 2016, 78: 325-328.
474 43. Washburn EW. Melting and freezing points of pure substances and of eutectic mixtures. Ind.
475 Eng. Chem. 1924, 16: 275-275.
476 44. Yang CB, Chowdhury MAK, Hou Y, Gong J. Phytogenic compounds as alternatives to in-
477 feed antibiotics: potentials and challenges in application. Pathogens. 2015, 4: 137-156.
478 45. Zastre JA. Evaluation of calcium alginate beads as a prolonged release delivery system for an
479 orally active iron chelator. PhD thesis at the University of Manitoba.1997.
480 46. Zeiger K, Popp J, Becker A, Hankel J, Visscher C, Klein G, Meemken D. Lauric acid as feed
481 additive - An approach to reducing Campylobacter spp. in broiler meat. PLoS One. 2017, 12:
482 e0175693.
483 47. Zeitz JO, Fennhoff J, Kluge H, Stangl GI, Eder K. Effects of dietary fats rich in lauric and
484 myristic acid on performance, intestinal morphology, gut microbes, and meat quality in
485 broilers. Poult Sci. 2015, 94: 2404-2413.
486 48. Zentek J, Buchheit-Renko S, Ferrara F, Vahjen W, Van Kessel A, Pieper R. Nutritional and
487 physiological role of medium-chain triglycerides and medium-chain fatty acids in piglets.
488 Anim Health Res Rev. 2011, 12: 83-93.

489 49. Zentek J, Buchheit-Renko S, Männer K, Pieper R, Vahjen W. Intestinal concentrations of
490 free and encapsulated dietary medium-chain fatty acids and effects on gastric microbial
491 ecology and bacterial metabolic products in the digestive tract of piglets. Arch Anim Nutr.
492 2012, 66: 14-26.
493 50. Zentek J, Ferrara F, Pieper R, Tedin L, Meyer W, Vahjen W. Effects of dietary combinations
494 of organic acids and medium chain fatty acids on the gastrointestinal microbial ecology and
495 bacterial metabolites in the digestive tract of weaning piglets. J Anim Sci 2013, 91: 3200-
496 3210.
497 51. Zhang Y, Wang QC, Yu H, Zhu J, de Lange K, Yin Y, Wang Q, Gong J. Evaluation of
498 alginate-whey protein microcapsules for intestinal delivery of lipophilic compounds in pigs. J
499 Sci Food Agric. 2016a, 96: 2674-2681.
500 52. Zhang Z, Zhang R, Zou L, McClements DJ. Protein encapsulation in alginate hydrogel
501 beads: Effect of pH on microgel stability, protein retention and protein release. Food
502 Hydrocol. 2016b, 58: 308-315.
503 53. Zhu F. Encapsulation and delivery of food ingredients using starch-based systems. Food
504 Chem. 2017, 229: 542-552.

505 **Figure legends**

506 **Fig. 1**. A) Pictures showing the molten mixture of thymol and fatty acids at 0 min at room
507 temperature (23°C). B) Pictures showing the molten mixture of thymol and fatty acids at 6 h at
508 room temperature (23°C). FA1- mixture of thymol and lauric acid, FA2 – mixture of thymol and
509 palmitic acid; FA3 – mixture of thymol and stearic acid.

510

511 **Fig. 2.** Differential scanning calorimetry (DSC) of (A)Thymol, (B) Lauric acid, and (C) Mixture
512 of thymol and lauric acid (50: 50wt%). The second run with heating rate 10 °C/min from -10°C
513 to 80°C.

514

515 **Fig. 3.** Morphology of crystals of thymol (A) and lauric acid (B) and a mixture of thymol and
516 lauric acid (C) after crystallization. The measuring bar in the pictures were 1µm.

517

518 **Fig. 4.** Morphology and surface diagram of the microparticles of lauric acid and thymol with and
519 without 2% alginate observed with a light microscope. (A) Morphology of microparticles with
520 alginate; (B) Morphology of microparticles without alginate; (C) Surface diagram of
521 microparticles with alginate and (D) Surface diagram of microparticles without 2% alginate.

522

523 **Fig. 5.** *In vitro* release profile of thymol and lauric acid from the microparticles with (A) and
524 without (B) alginate using simulated fluids (SSF - simulated salivary fluid, SGF - simulated
525 gastric fluid and SIF - simulated intestinal fluid). (Mean ± SD, n = 4).

526

527 **Fig. 6.** Stability of the microparticles of: (A) thymol in the microparticles with alginate, (B)
528 lauric acid in the microparticles with alginate, (C) thymol in the microparticles without alginate
529 and (D) lauric acid in the microparticles without alginate. Samples were stored at room
530 temperature (23˚C) for 2 weeks. (Mean ± SD, n = 4).

531 **Fig. 7.** Stability of the microparticles of thymol and lauric acid with (A) and without (B) alginate
532 stored at 4˚C for 12 weeks. (Mean ± SD, n = 4).

533

534

535

536

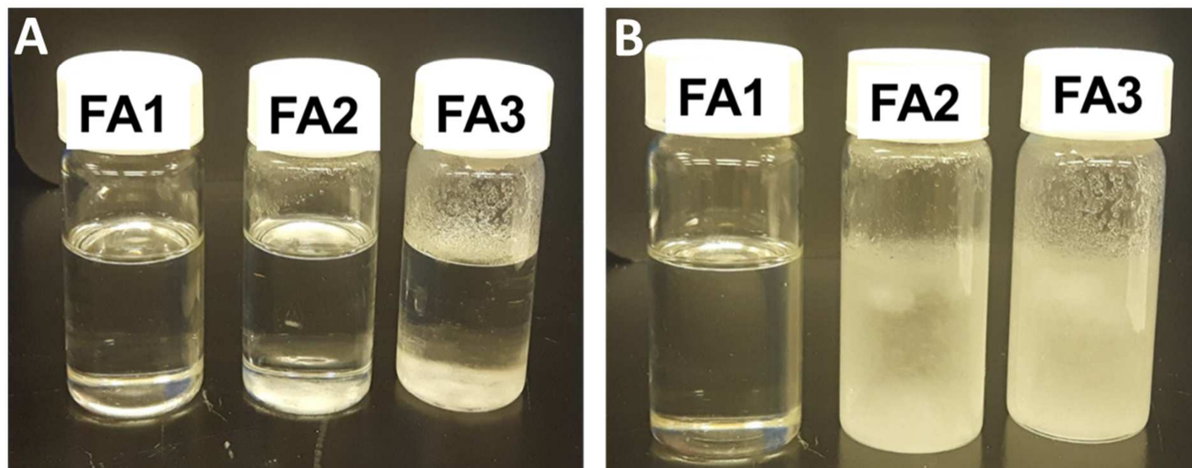
537

538

539

540 ***Fig. 1. Omonijo et al. (2018)***

541



542

543

544

545

546

547

548

549

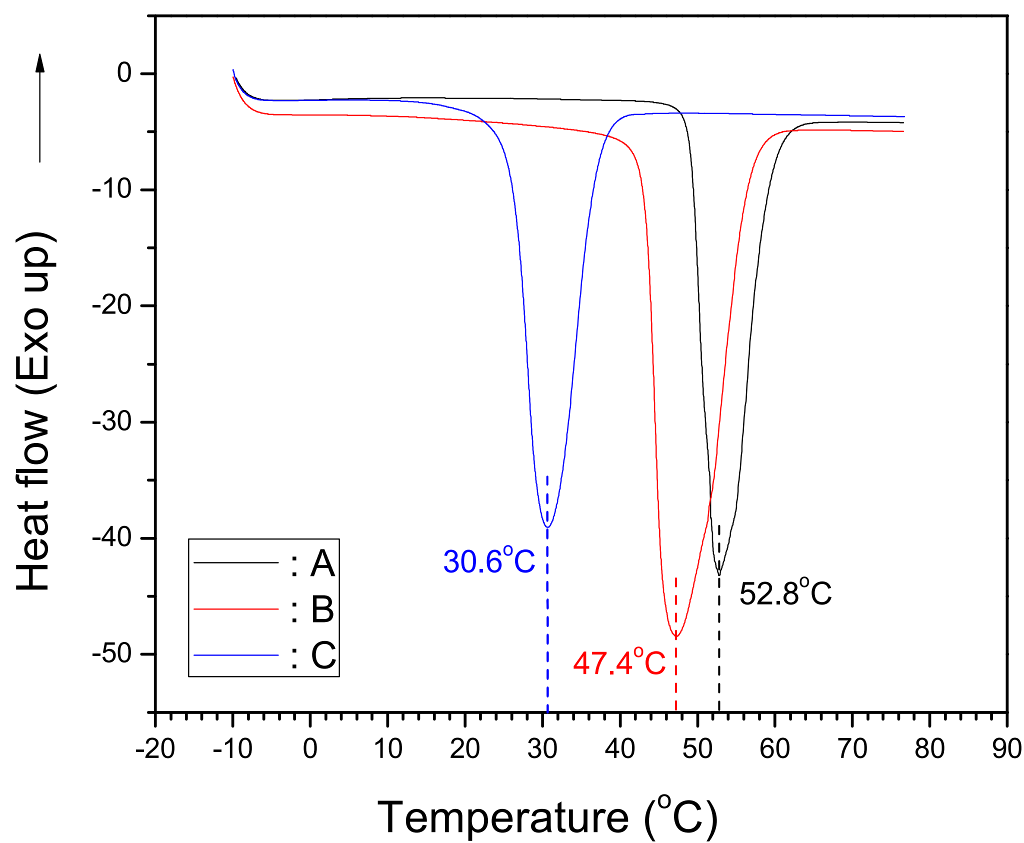
550

551

552

553 *Fig. 2. Omonijo et al. (2018)*

554



555
556

557

558

559

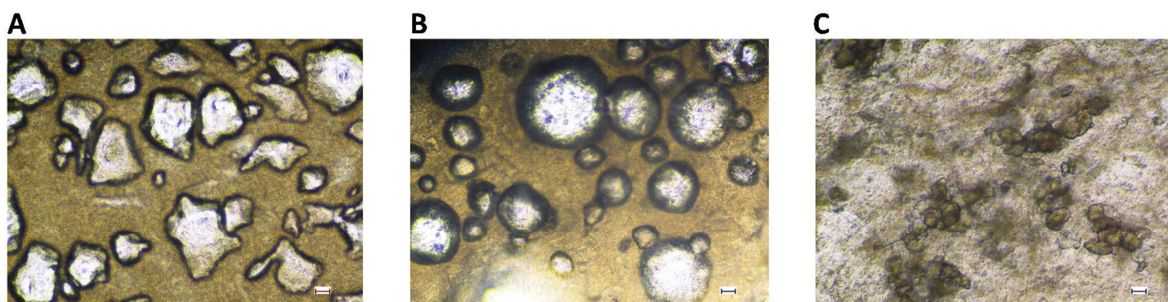
560

561

562

563 ***Fig. 3. Omonijo et al. (2018)***

564



565

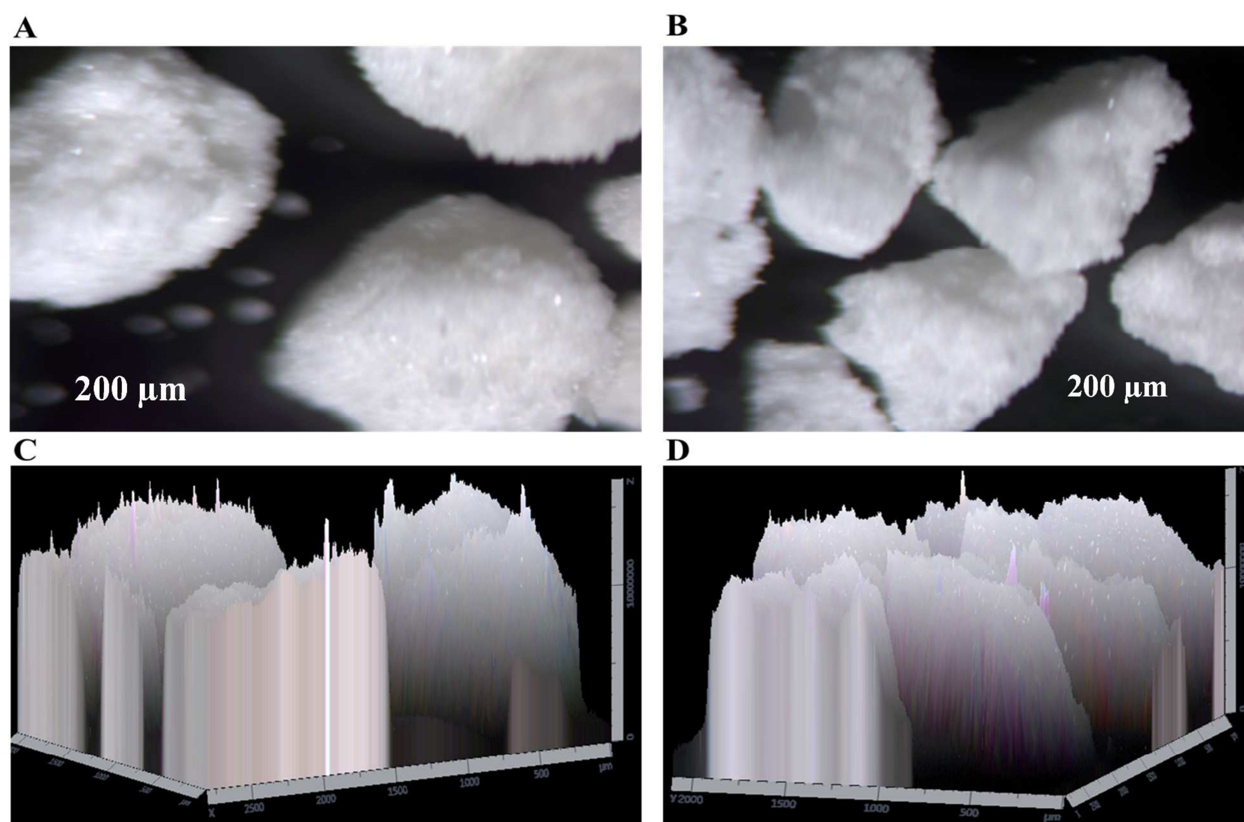
566

567

568

569 ***Fig. 4. Omonijo et al. (2018)***

570



571

572

573

574

575

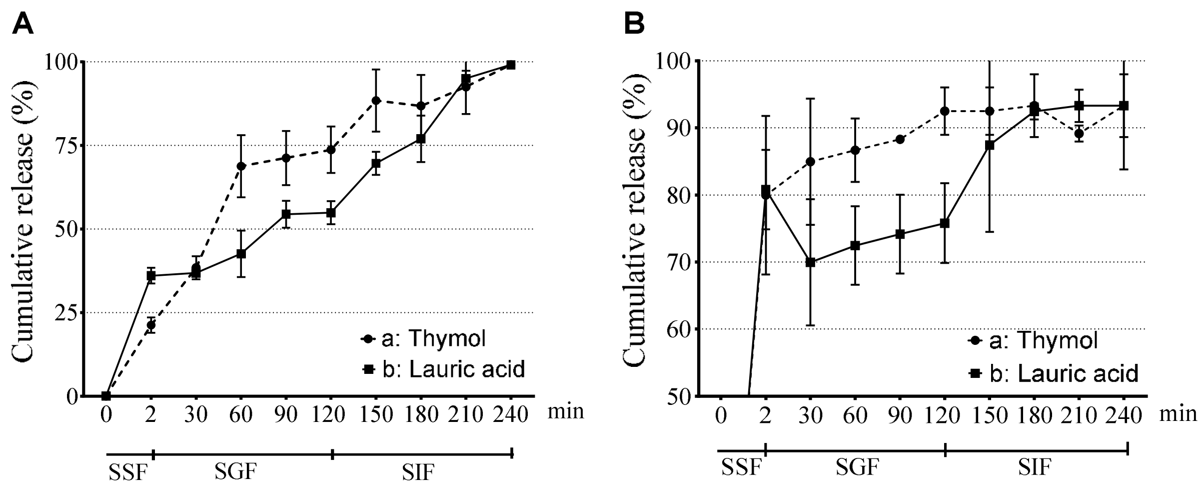
576

577

578

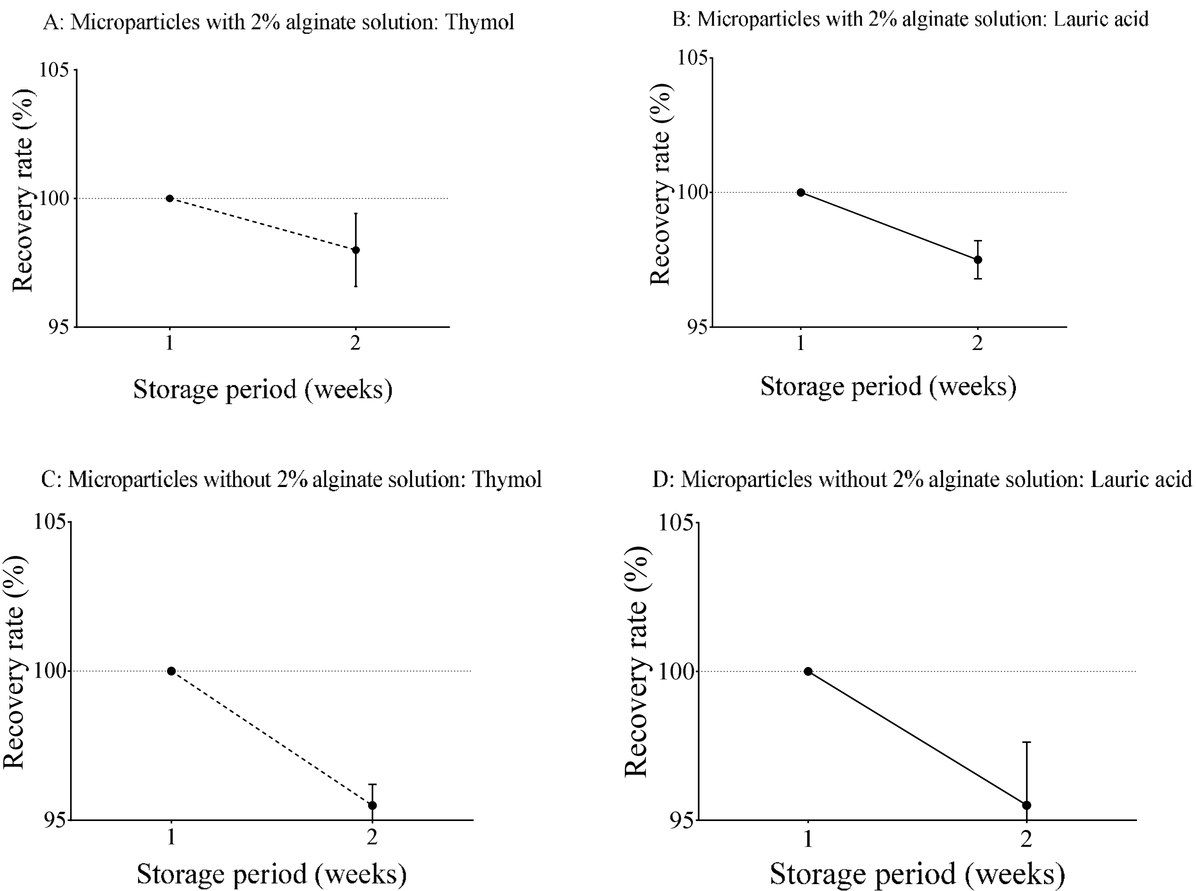
579

580 ***Fig. 5. Omonijo et al. (2018)***



581
582
583
584
585
586
587
588
589
590
591
592
593
594
595

596 *Fig. 6. Omonijo et al. (2018)*



597

598

599

600

601

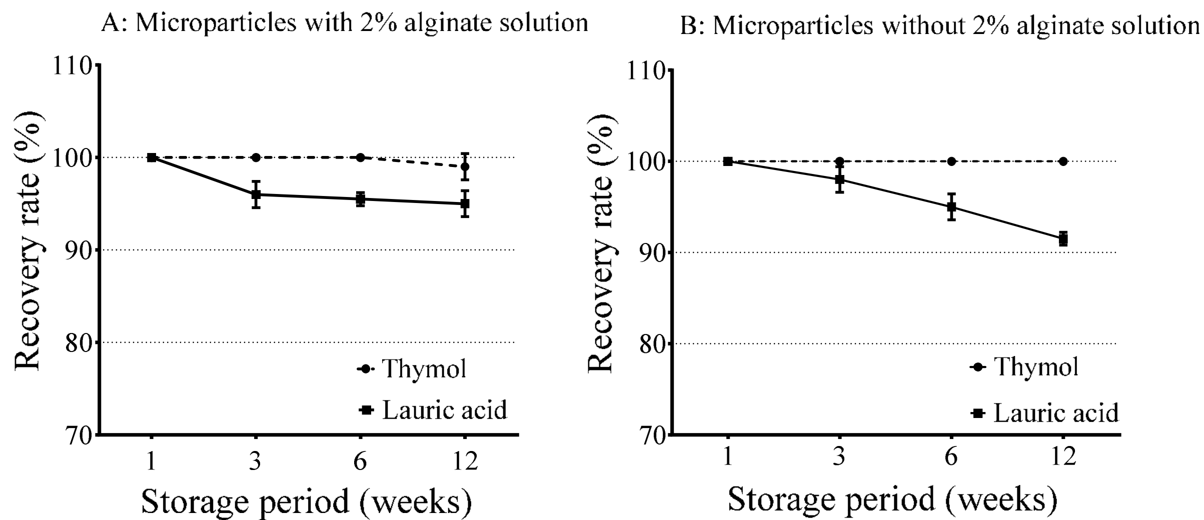
602

603

604

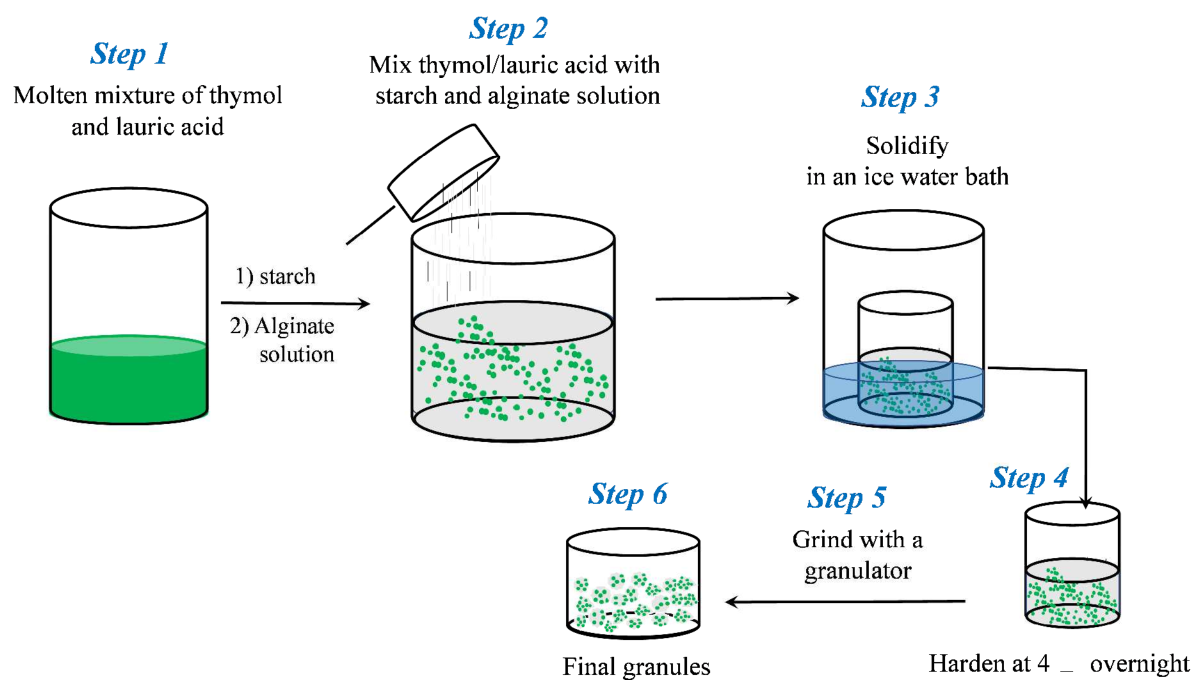
605

606 ***Fig. 7. Omonijo et al. (2018)***



607
608
609
610
611
612
613
614
615
616
617
618
619
620
621

622 TOC Graphic



623