Efficient chemical hydrophobization of lactic acid bacteria – one-step formation of double emulsion

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Abstract

A novel concept of stabilizing multiple-phase food structure such as emulsion using solely the constitutional bacteria enables an all-natural food grade formulation and thus a clean label declaration. In this paper, we propose an efficient approach to hydrophobically modifying the surface of lactic acid bacteria *Lactobacillus rhamnosus* (LGG) using lauroyl ahloride (LC) in non-aqueous media. Compared to the unmodified bacteria, cell hydrophobicity was dramatically altered upon modification, according to the higher percentages of microbial adhesion to hexadecane (MATH) and water contact angles (WCA) of LC-modified bacteria. No evident changes were found in bacterial surface charge before and after LC modification. By using one-step homogenization, all the modified bacteria were able to generate stabile water-in-oil-in-water (W/O/W) double emulsions where bacteria were observed on oil-water interfaces of the primary and secondary droplets. Modification using high LC concentrations (10 and 20 w/w%) led to rapid autoaggregation of bacteria in aqueous solution. A long-term lethal effect of modification primarily come from lyophilization and no apparent impact was detected on the instantaneous culturability of modified bacteria.

Keywords

Efficient; Pickering stabilization; lactic acid bacteria; lauroyl chloride; lyophilization; double emulsion.

31 **1. Introduction**

32 Emulsions are one of the most important food structures, because the final products possess favorable
33 sensory properties such as texture, flavor and appearance. Due to the kinetic and thermodynamic
34 instability of emulsion, surfactants or emulsifiers are required to prevent destabilization during long time
35 storage (Dickinson, 2010b; Nushtaeva, 2016; Tavernier, Wijaya, Meeren, Dewettinck, & Patel, 2016). Single
36 W/O or O/W emulsions can be created using predominantly hydrophobic or hydrophilic stabilizers, while
37 the combination of stabilizers with complementary hydrophilic-lipophilic balance values can produce
38 double emulsions, where the compartmentalized primary dispersions contain even smaller droplets of a
39 different phase (Bhattacharjee, Chakraborty, & Mukhopadhyay, 2018; Dickinson, 2011, 2015).

40 Besides conventionally low molecular emulsifiers, emulsions can also be stabilized by adsorbed fine solid
41 particles, which is referred to as Pickering stabilization (Dickinson, 2010a; Murray, Durga, Yusoff, &
42 Stoyanov, 2011). Pickering stabilization is able to produce foams and emulsions with high stability as the
43 desorption is considered to be impossible due to much higher desorption energy than thermal energy
44 (Dickinson, 2015; Hua, Bevan, & Frechette, 2016; Jin et al., 2012). Microorganisms such as bacteria
45 (Dorobantu, Yeung, Foght, & Gray, 2004), yeasts (Firoozmand & Rousseau, 2015) and viruses (Kaur et al.,
46 2009; Russell et al., 2005) have exhibited their ability of stabilizing foams and emulsions. Although
47 applications were mainly reported in non-food area (Heard, Harvey, Johnson, Wells, & Angove, 2008),
48 recent efforts have been made to utilize food grade microorganisms for developing food foams and
49 emulsions (Firoozmand & Rousseau, 2015; Rayner et al., 2014).

50 Lactic acid bacteria, as important constitutional and nutritional components in dairy products,
51 demonstrate the potential to serve as also structural building blocks based on Pickering principles. While
52 certain strains showed their inherent surface activity as Pickering particles for emulsions and gels
53 (Dorobantu et al., 2004), most lactic acid bacteria as Gram positive bacteria, still exhibit a dominantly
54 hydrophilic nature attributed to large presence of peptidoglycan with a ratio of polysaccharides to

hydrocarbons (Boonaert & Rouxhet, 2000; Chapot-Chartier & Kulakauskas, 2014; Schär-Zammaretti & Ubbink, 2003). Hence, modification is necessary to alter their physiochemical properties towards suitable interfacial materials. Biologically, fermentation conditions like media composition (Schär-zammaretti et al., 2005), growth time (Rosenberg & Rosenberg, 1985) and temperature (Deepika, Karunakaran, Hurley, Biggs, & Charalampopoulos, 2012) can change the chemical composition and cell surface properties. Physical coating of bacteria with oppositely-charged chitosan (Wongkongkatep et al., 2012) and milk proteins (Falco, Geng, Cárdenas, & Risbo, 2017) was also capable of modifying the surface charge and cell hydrophobicity.

Chemical hydrophobization has been previously reported for polysaccharide nanoparticles like starches (Balic, Miljkovic, Ozsisli, & Simsek, 2017; Neelam, Vijay, & Lalit, 2012; Yusoff & Murray, 2011), celluloses (Jin et al., 2012) and chitosans (Fink, Höhne, Spange, & Simon, 2009) using carboxylic acid derivatives. The mechanism is that the hydrophobic chains of these chemicals covalently condense with hydroxyl or amine groups on the surface of polysaccharide particles through esterification or amidation (Ačkar et al., 2015; Cunha & Gandini, 2010). Considering the large presence of amino groups and hydroxyl groups in bacterial cell wall peptidoglycan and polysaccharides, their surface can be potentially modified with the similar principle. Yet, studies on the chemical modification of bacterial cell surface are very limited. Recently, the surface hydrophobicity of lactic acid bacteria *Lactobacillus acidophilus (La5) was increased* using octenyl succinic anhydride, and the modified bacteria were able to stabilize foam and emulsions (Jiang et al., 2019).

The present study aims to develop an efficient approach to chemically modifying the surface of lactic acid bacteria *Lactobacillus rhamnosus* (LGG) in a non-aqueous environment. The idea is that by using lauroyl chloride (LC), the hydrophilic bacteria can be hydrophobically modified through connecting the alkyl chains of LC to bacterial surface functional groups. Surface properties of the bacteria were evaluated using microbial adhesion to hexadecane (MATH), water contact angle (WCA) measurement, zeta potential measurement and the observation of bacterial aggregating behavior. Finally, unmodified and modified

78 bacteria are used for emulsion preparation and droplet size measurement, optical microscopy and confocal
79 microscopy were applied to characterize the produced emulsion.

80 2. Material and methods

81 *2.1. Materials and chemicals*

82 Lauroyl chloride (LC), glycerol, dimethyl sulfoxide (DMSO), hexadecane, sodium chloride (NaCl),
83 potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate
84 (KH_2PO_4), TWEEN® 80, sodium carbonate (Na_2CO_3) and 40,6-Diamidino-2-phenylindole (DAPI) were
85 purchased from Sigma-Aldrich, Steinheim, Germany. BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-
86 Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene) was bought from ThermoFisher Scientific, Molecular Probes,
87 Eugene, OR, USA. Medium-chain triglyceride (MCT) oil was a gift from AAK AB (publ.), Karlshamn, Sweden.
88 *Lactobacillus rhamnosus* GG (LGG) was bought from Collections of Micro-organisms BCCM, Ghent, Belgium.
89 MRS broth (de Man, Rogosa and Sharpe), MRS agar, and atmosphere generation system (AnaeroGen
90 sachets) were bought from Oxoid, Basingstoke, England. MRS broth and agar were sterilized in an autoclave
91 (115 °C, 15 min). All the chemicals were used as received. MilliQ water (18.2 MΩcm at 25 °C) was used in all
92 the experiments.

93 *2.2. Growth of bacteria and dry biomass determination*

94 First, 100 µL of a frozen stock solution of LGG was anaerobically propagated in 10 mL MRS broth at 37 °C,
95 for 24 h. Then, 250 µL of the preculture was anaerobically incubated in 50 mL MRS broth at 37 °C, for 24 h.
96 After growth, cells were harvested by centrifugation at 5000 × g for 5 min at 4 °C and washed twice with
97 sterile MilliQ water.

98 Dry weight of bacterial cells was determined following a standard method (Li & Mira de Orduña, 2010).
99 Briefly, the harvested cell pellets were re-suspended in 3 mL sterile MilliQ water and 1 mL cell suspension

100 was transferred to a pre-weighed sterile aluminum boat which was placed in a hot air oven at 105 °C. Then,
101 the total weight was measured regularly until a stable dry weight was obtained. Dry weight corresponding
102 to the original cell suspension was calculated accordingly. The procedure was carried out in triplicate. In the
103 end, the dry biomass corresponding to 250 µL preculture in 50 mL MRS broth was 78.0±1.7 mg.

104 ### *2.3. Lyophilization and LC modification of bacteria*

105 In order to homogeneously disperse bacteria in oil, moisture was removed by lyophilizing bacteria prior
106 to LC modification. After growing, the washed and collected bacteria were re-suspended in 5 mL sterile
107 phosphate buffered saline (PBS), pH 7.4 (0.8 w/w% NaCl, 0.02 w/w% KCl, 0.144 w/w% Na_2HPO4, and 0.024
108 w/w% KH_2PO4). The resulted cell suspensions were placed in a freezer set at -80 °C for 90 min. After the
109 suspensions became completely frozen, the temperature of the lyophilizer (Edwards, Buch & Holm A/S, UK)
110 was reduced to -50 °C and then the samples were lyophilized overnight.

111 The lyophilized bacteria were re-suspended in 10 mL MCT oil. The predetermined amount of Na_2CO_3
112 powder based on the full consumption of LC was added. Next, different amount (1, 3, 5, 10, 20 w/w% based
113 on the bacterial dry biomass) of LC was added into the bacterial oil suspension. The mixtures were stirred
114 for 3 h under room temperature. After the reaction, the bacterial oil suspensions were centrifuged at 5000
115 × g for 5 min at 4 °C and the upper oil supernatant was discarded. In order to wash away the excess oil,
116 TWEEN® 80 (5% in MilliQ water) was added and mixed homogenously with the cell pellets. This mixture was
117 sent to centrifugation at 5000 × g for 10 min at 4 °C and the turbid liquid part was discarded. Cell pellets left
118 in the bottom were washed twice with MilliQ water. The cell pellets were re-suspended in 5 mL PBS (pH
119 7.4) and incubated under 37 °C for 90 min to fully rehydrate. Before different investigations, the cells were
120 washed twice, and if necessary, bacterial suspensions were homogenized using an Ultra-Turrax
121 homogenizer (T25 digital Ultra-Turrax®, IKA) at 24000 rpm for 30 s, to break down the bacterial aggregates.

122 ### 2.4. *Microbial adhesion to hexadecane (MATH) method*

123 Characterization of bacterial hydrophobicity was carried out as described in previous reports (Pelletier et
124 al., 1997; Bellon-fontaine, Rault and Van Oss, 1996) with modifications for use at a smaller scale. Briefly,
125 unmodified and LC-modified bacteria pellets, respectively, were re-suspended in 10 mM KH_2PO_4 solution to
126 obtain an initial optical density (OD) of 0.8 at 600 nm (SpectraMax i3x, Molecular Devices LLC, USA), which
127 was measured by transferring 200 µL of cell suspension into a 96-well plate. An OD600 ~ 0.8 indicated an
128 approximate cell density of 10^8 CFU/mL. An aliquot of cell suspension (250 µL) was mixed with 42 µL
129 hexadecane in an Eppendorf tube. After 10 min incubation at room temperature, the mixtures were
130 vortexed at highest speed for 90s. After vortexing, mixtures were let to stand for 15 min at room
131 temperature to allow complete phase separation. Next, 200 µL of the lower aqueous phase was transferred
132 to the 96-well plate to measure OD values. The percentage of microbial adhesion to hexadecane was
133 calculated by the following equation (1):

134 $$\%\ \text{adhesion} = \left(1 - \frac{A1}{A0}\right) \times 100 \quad (1)$$

135 Where A_0 is the initial OD600 of the bacterial suspension and A_1 is the OD600 value of the lower aqueous
136 phase after phase separation. The MATH test was carried out for unmodified and LC-modified bacteria over
137 observed periods of 0 days, 1 week, 2 weeks, 3 weeks and 4 weeks, during which time, bacteria were
138 stored in PBS (pH 7.4) at 4 °C. All results were obtained from duplicated experiments and data are
139 presented as average ± standard deviation.

140 ### 2.5. *Water contact angle (WCA) measurement*

141 Bacterial cell hydrophobicity was directly evaluated by WCA measurements of bacterial lawns. First,
142 multiple layers of unmodified and LC-modified bacteria were deposited on 0.45 µm (pore size)
143 polyvinylidene difluoride membrane filters by drawing through the bacterial suspensions with negative
144 pressure. Filters with mounted bacterial layers were fixed onto glass slides and air-dried overnight, allowing

145 the formation of plateau contact angles (Bellon-Fontaine et al., 1996). The measurements were carried out
146 at room temperature by placing a sessile droplet of water on the prepared bacterial surface backlit using a
147 telecentric illuminator (Techspec, Edmund Optics, USA) fiber coupled to a white LED light source (3000 K).
148 Images were captured by a 5 megapixel monochrome CCD camera (Grasshopper 3, model: GS3-U3-50S5M-
149 C, Point Grey Research, Inc., Canada) connected to an objective with a 1.7× High Resolution 5Megapixel
150 Telecentric Lens (Techspec, Edmund Optics, USA). The contact angle of bacterial surface was analyzed using
151 ImageJ software. In each measurement, at least three filters were prepared.

152 ### *2.6. Surface tension and interfacial tension*

153 Surface tension of PBS, bacteria suspension in PBS and bacteria supernatant in PBS was measured based
154 on the Du Noüy ring method (Moran, Yeung, & Masliyah, 1999) using a surface tensiometer (Attension
155 Sigma 703D, Biolin Scentific, Finland). Bacteria with and without 10 w/w% LC modification were suspended
156 in 20 mL PBS and transferred into a glass Petri dish. For the measurement of bacterial supernatant, bacteria
157 were centrifuged at 5000 × g for 5 min at 4 °C, and the supernatant was transferred into the Petri dish.
158 Before each measurement, the ring was rinsed subsequently using distilled water and ethanol, then passed
159 through a Bunsen flame and left until cool. For interfacial tension measurement, after merging the ring into
160 PBS subphase, 15 mL MCT oil was gently added on top without disturbing the oil-water interface. All the
161 data are presented as average ± standard deviation from duplicated experiments.

162 ### *2.7. Enumeration of viability*

163 The effect of lyophilization and LC modification on cell culturability was investigated using drop-plating
164 method. The unmodified, LC-modified cell pellets, respectively, were suspended in 50 mL sterile PBS (pH
165 7.4). After preparing the dilutions, 30 µL of each dilution was evenly distributed in 5 to 6 drops on one
166 quarter of MRS agar plate in duplicates. When the bacterial cultures were well absorbed into the agar, all
167 the plates were incubated anaerobically at 37 °C for 48 h. After growth, only dilutions with the appeared

168 colony number between 30 and 300 were selected for counting. The count for culturable bacteria in each
169 suspension was expressed in colony forming units per milliliter (CFU/mL). The viability test was carried out
170 for both unmodified and LC-modified bacteria over periods of 0 days, 1 week and 2 weeks. All the data are
171 presented as average ± standard deviation from duplicated experiments.

172 ### *2.8. Zeta potential measurements*

173 Surface charge of unmodified and LC-modified bacteria was measured using a zeta sizer (Malvern
174 Zetasizer, Nano ZSP, UK) in a folded capillary cell. The background electrolyte solution was MilliQ water and
175 measurements were conducted at 25 °C. First, cell suspensions were diluted 10 times with MilliQ water and
176 around 1 mL of this suspension was injected into the capillary cell using a disposable syringe. The capillary
177 cell was rinsed subsequently with MilliQ water and sample before each measurement. This investigation
178 was also conducted over storage time (0 days, 1 week, 2 weeks, 3 weeks and 4 weeks) for unmodified and
179 LC-modified bacteria. All the data reported are the averages of duplicates and the results are presented as
180 average ± standard deviation.

181 ### *2.9. Emulsion preparation and characterization*

182 Emulsion preparation was carried out according to a previous report (Rayner, Sjöö, Timgren, & Dejmek,
183 2012). The continuous phase was PBS with pH of 7.4, and MCT oil was used as the dispersed phase. Briefly,
184 pellets of unmodified and LC-modified bacteria were first suspended in 3 mL PBS (pH 7.4), and then 3 mL
185 MCT oil was added to the bacterial suspension. This was followed by mixing the two phases using an Ultra-
186 Turrax homogenizer (T25 digital Ultra-Turrax®, IKA, USA) at 22000 rpm for 60 s. In the final emulsions, the
187 water-to-oil ratio was 1:1 and the concentration of bacteria was around 1.3 w/w%. Each sample was
188 prepared in duplicates for further investigations.

189 Emulsions prepared with unmodified and modified bacteria was investigated over storage time in terms
190 of the mean droplet diameter over volume ($d_{4,3}$), the mean droplet diameter over surface ($d_{3,2}$) and droplet

191 size distribution using a laser diffraction particle size analyzer (Mastersizer 3000, Malvern Instruments,
192 Workshire, UK) at 25 °C. The obscuration range was set between 8 % and 15 %. The refractive index value
193 of the emulsion was set to 1.47 and the absorption index was set to 0.01. All the data reported are the
194 average of duplicates and the results are presented as average ± standard deviation.

195 ### *2.10. Bright field microscopy*

196 Bright field microscopy was used to study both the bacterial autoaggregation behavior as well as the
197 emulsion droplets produced using unmodified and modified bacteria. All the images were captured by a
198 Cool Snap RS Photometrics camera (Roper Scientific, Tucson, AZ, USA) connected to Zeiss Axioskop
199 microscope (Carl Zeiss, Goet- tingen, Germany), and processed with ImageJ software.

200 To investigate the aggregation behavior of bacteria, the washed bacteria before and after LC modification
201 were collected and re-suspended in 50 mL MilliQ water. After homogenization using vortex for 30 s, 10 µL
202 cell suspension was transferred onto a microscopic slide within 5 min after sample preparation and
203 observed under 10X objective. For the observation of emulsion droplet size, emulsion produced with
204 unmodified or modified bacteria were diluted 5 times using PBS (pH 7.4) in Eppendorf tubes. After gently
205 inverting the Eppendorf tube for a few times, 10 µL diluted emulsion was taken out and transferred onto a
206 microscopic slide. The observation was conducted under 10X objective without using the cover slide.
207 Images illustrated were the most representative ones.

208 ### *2.11. Confocal microscopy of bacteria-stabilized emulsions*

209 A confocal laser scanning microscope (Point Scanning Confocal and 2-photon microscope SP5-X MP UV,
210 Leica Microsystems, Germany) embedded with fluorescence lamp was used to investigate the
211 microstructure of emulsion produced with 10 w/w% LC-modified bacteria. The oil phase was stained with
212 BODIPY, with the excitation wavelength of 493 nm, while bacteria were stained with DAPI, excited with 358

213 nm. The images, showing the blue emission of DAPI and bright green emission of BODIPY, were processed
214 by Leica Microsystems LAS AL lite software.

215 Before emulsion preparation, 5 µL BODIPY solution in DMSO (1 mg/mL) and 15 µL DAPI solution in MilliQ
216 water (5 mg/mL) was added into 3 mL MCT oil and 3 mL bacterial suspension, respectively. This was
217 followed by a 10-min incubation in darkness at room temperature. The emulsion was prepared by mixing
218 the two stained phases using the Ultra-Turrax homogenizer at 22000 rpm for 60 s. An aliquot of the stained
219 emulsion (100 µL) was diluted 5 times with PBS (pH 7.4) in an Eppendorf tube and 50 µL of diluted emulsion
220 was transferred onto a welled slide or a standard microscope slide, covered with a coverslip (0.17 mm
221 thickness) for observation. All the samples were observed using 63X oil-immersion objective within 10 min
222 after sample preparation to avoid moisture evaporation. The scan mode was set to XYZ scanning performed
223 in an average of 6 lines. Resolution of the final images was 0.21 µm/0.21 µm/0.65 µm for X, Y, Z dimension,
224 respectively.

225 **3. Results**

226 ***3.1. Culturability of modified bacteria***

227 The effect of lyophilization and LC modification on bacterial culturability was investigated using plate
228 counting method (Figure 1). An instantaneously negative effect of lyophilization on culturability was not
229 observed for unmodified bacteria based on the unchanged culturability before and after lyophilization
230 (Figure 1A). Likewise, no obvious decrease in culturability was observed for bacteria immediately after LC
231 modification regardless of LC concentration (Figure 1B). A similar observation was also found in cell
232 membrane permeability right after modification (see Fig. S1) that the membrane intactness of bacteria with
233 and without LC modification was damaged to more or less the same degree. Nevertheless, a reduction of 2
234 log and 1.5 log in bacterial culturability over storage was observed during the first and the second week,
235 respectively. It was therefore inferred that the impact of LC modification alone on bacterial culturability

236 might be negligible compared to that of lyophilization and this time-hidden effect could be related to the
237 physical damage of cell membrane.

238 ### *3.2. Surface properties of modified bacteria*

239 LGG was modified using different LC concentrations towards higher cell hydrophobicity, where the LC
240 dosage was based on the dry biomass of bacteria. Besides, Ultra-turrax homogenization was applied before
241 MATH and zeta potential measurement, because firmly-aggregatedbacteria were observed when modifying
242 with high LC concentrations.

243 #### 3.2.1. Cell hydrophobicity

244 Bacterial adhesion to the non-polar solvent hexadecane reflects the hydrophobic/hydrophilic nature of
245 cell surface (Mortensen, Gori, Jespersen, & Arneborg, 2005) and the procedure was carried out in solution
246 with high ionic strength (10 mM KH_2PO_4) to minimize the electrostatic interactions (Rosenberg, 1984).
247 Figure 2A shows the stability of unmodified and LC-modified bacteria in terms of MATH after storing in PBS
248 buffer at 4°C for 4 weeks. The overall cell hydrophobicity remained virtually unchanged over one month.
249 This showed that the increase of cell hydrophobicity by LC modification was stable over time and hydrolysis
250 of the formed amide and ester bonds was not a severe problem. In addition, small increases in the
251 adhesion of 1 and 3w/w% LC-modified bacteria after one-week storage was observed.

252 Furthermore, WCAs of bacteria were measured immediately after modification as a complementary
253 assay to MATH. As seen from Figure 2B, unmodified bacteria, despite of the high presence of
254 polysaccharides on cell wall, possessed a WCA of 59.7°, which resulted in a weak adhesion (12.2%) to
255 hexadecane, indicating their intrinsically hydrophilic nature. However, the predominantly hydrophilic
256 surface of unmodified LGG was altered by LC modification, where the modification by 20 w/w% LC resulted
257 in the most hydrophobic bacteria, with a WCA rising up to 101.6°. Moreover, a good correlation between
258 the two methods was found in bacteria modified using LC concentrations below 5 w/w%, whereas for high

259 LC concentrations, the adhesion gradually approached to saturation while a continuous increase of WCA
260 was still observed.

261 3.2.2. Surface tension, interfacial tension and surface charge

262 Surface tension and interfacial tension of unmodified and 10 w/w% LC-modified bacterial suspension and
263 supernatant in PBS were measured (*Figure 3*A). Briefly, modified bacteria as well as bacteria-free
264 supernatant from suspensions of modified bacteria were able to lower the surface tension of PBS buffer
265 from 68% to 49%, indicating that some low-molecular surface-active components was associated with
266 modified bacteria. Interestingly, sample with 10 w/w% LC dosage but without bacteria encountered a
267 similar reduction in surface tension from 68% to 52%, indicating that this surface tension lowering effect
268 could be mainly due to the leftover lauroyl acid produced in LC modification process. However, the same
269 effect was not observed for the interfacial tension between MCT oil and aqueous with or without modified
270 bacteria.

271 Zeta potential of bacterial surface before and after LC modification was measured in MilliQ water at
272 different storage time points. All the bacteria showed moderately negative net charges (*Figure 3*B),
273 indicating the presence of acidic groups such as phosphate groups in teichoic acid and carboxylate groups
274 in acidic polysaccharides (Djeribi, Boucherit, Bouchloukh, Zouaoui, & Latrache, 2013). After LC modification,
275 a slight increase in the net negative charges from approx. -11 to -14 mV was observed for all the modified
276 bacteria. However, this overall change was not obvious, suggesting the low occurrence of free amino
277 groups on bacterial surface compared to large amount of negative charges predominated by other
278 unreactive constituents such as teichoic acid. Besides, similar to MATH, an abrupt increase in negative
279 charges for bacteria modified with low LC concentrations (1, 3, 5 w/w%) was also observed after one week.
280 Otherwise, surface charges remained steady even after one month, indicating highly stable amide bonds
281 formed by LC and surface free amino groups.

282 3.2.3. Bacterial autoaggregation

283 Bacterial autoaggregation refers to the ability of bacteria to bind to themselves (Trunk, S. Khalil, & C. Leo,
284 2018). This behavior in aqueous media was microscopically observed for bacteria before and after LC
285 modification. Water suspensions of unmodified, 5, 10 and 20 w/w% LC modified bacteria are shown by
286 micrographs in *Figure 4*. Unlike unmodified bacteria, where almost no aggregates were observed, all the
287 modified bacteria showed their ability to autoaggregate in water to different degrees. For 5 w/w% LC
288 modified bacteria, a few cells started to form small and loose aggregates. By comparison, bacteria modified
289 using higher LC concentrations (10, 20 w/w%) exhibited their stronger ability to autoaggregate and form
290 compact clumps of large sizes, particularly for 20 w/w% LC modified bacteria. This significant change in
291 bacterial autoaggregation induced by LC modification could be related to the improved cell hydrophobicity.

292 ### *3.3. Emulsion stabilized by modified bacteria*

293 #### 3.3.1. Emulsion characterization

294 Modified and unmodified bacteria were subjected to prepare emulsions and all the LC-modified bacteria
295 produced water-in-oil-in-water double emulsions. The size distributions of bacteria-produced emulsions are
296 shown in *Figure 5*A. Unmodified bacteria produced emulsion droplets with a broader and larger size
297 distribution (from approx. 45 to 186 µm) compared to the emulsions made using modified bacteria, which
298 peaked at much smaller sizes (less than 75 µm). For modified bacteria, narrower spans and peaks with
299 lower sizes were obtained with the increase of LC concentration, demonstrating a positive correlation
300 between LC concentration and bacterial Pickering stabilizing ability. However, for the most modified
301 bacteria (20 w/w%), a broader span and peak of larger size (around 86 µm) was observed due to the
302 presence of large and compact aggregates. When keeping the same emulsion composition and mixing
303 conditions, extra high shear was required for 20 w/w% LC-modified bacteria to breakdown firmly-
304 aggregated bacteria. Moreover, small peaks at around 10 µm might be attributed to the un-adsorbed
305 bacteria, and in 20 w/w% LC-modified bacteria, this peak was followed by a plateau, indicating the
306 presence of diversely-sized bacterial aggregates.

307 Information regarding surface mean diameter $d_{3,2}$ and volume diameter $d_{4,3}$ as a function of LC
308 concentration is shown in *Figure 5*B. Since the presence of large particles tend to give a larger $d_{4,3}$, LC-
309 modified bacteria were less likely to produce big oil droplets resulted from droplet coalescence and
310 flocculation, compared to unmodified bacteria (101.9±7.6 µm). In contrast, $d_{3,2}$ can be more sensitive to the
311 number of smaller droplets and therefore an opposite trend was observed. Bacteria modified using 20
312 w/w% LC was an exception though, where both the $d_{4,3}$ (84.7±5.9 µm) and $d_{3,2}$ (57.6±2.9 µm) were high
313 compared to emulsions produced using other modified bacteria. This aligned with the broader size
314 distribution obtained for emulsion produced by 20 w/w% LC-modified bacteria, which pointed to the large
315 presence of bacterial aggregates, impeding their effective Pickering stabilization of oil droplets.

316 The stability of emulsion prepared with unmodified and LC-modified bacteria at 4 °C were investigated
317 over a period of up to 3 weeks and the mean diameter $d_{4,3}$ as a function of time is reported in *Figure 5*C.
318 Unmodified bacteria produced emulsion with lowest storage stability, where the mean droplet size
319 dramatically increased to 135.4±6.3 µm after 3 weeks. Stability was found high for emulsion prepared using
320 3, 5 and 10 w/w% LC-modified bacteria, with the mean diameter staying steady over the whole observing
321 period. Moreover, a moderate increase of droplet size was observed for both 1 and 20 w/w% LC-modified
322 bacteria, demonstrating their relatively weaker Pickering stabilizing capacity compared to the other
323 modified bacteria. Hence, neither too low or too high LC concentration were favorable for modifying
324 bacteria towards good Pickering stabilizers.

325 Moreover, emulsions produced with unmodified, 5, 10 and 20 w/w% LC modified bacteria were
326 microscopically observed. As shown in *Figure 6Figure 6*, all the bacteria were able to produce emulsion
327 droplets apparently containing smaller droplets within a few minutes after sample preparation. However,
328 the inner droplets formed by unmodified bacteria experienced rapid diffusion and coalescence and finally
329 disappeared, while the inner emulsions produced with modified bacteria kept stable even after one-month
330 storage (images not shown). Similar to droplet size measurement, 10 w/w% LC-modified bacteria created

331 emulsion of smaller and uniformly-distributed droplets. Moreover, reduced number of bacteria was found
332 in aqueous compartment between the oil droplets for 5 and 10 w/w% LC-modified bacteria compared to
333 unmodified bacteria. In addition, 10 and 20 w/w% LC-modified bacteria tended to form aggregates as also
334 confirmed by droplet size measurement.

335 3.3.2. Microstructure of bacteria stabilized emulsions

336 Only emulsion produced with 10 w/w% LC-modified bacteria was investigated in this experiment as the
337 emulsion droplets prepared using unmodified bacteria were not stable and fast coalescence was observed
338 upon addition of cover slide. Besides, the thickness of a single focal plane is too small to analyze the
339 coverage of bacteria, a z-stack series of images taken at different focal planes was therefore reconstructed
340 to access a relatively entire visualization of bacterial adsorption on oil droplets.

341 *Figure 7* showed CLSM images of outer and inner emulsion prepared with 10 w/w% LC-modified bacteria.
342 Sample was stained using DAPI and BODIPY solution to respectively highlight bacterial cells and MCT oil
343 droplets. However, BODIPY as a non-polar dye, is able to probe lipids, oil, membrane proteins and
344 therefore bacterial cells (Johnson, 2010). In *Figure 7*A, oil droplets highlighted in bright green fluorescence
345 were evenly-distributed in the aqueous phase and no droplet coalescence was observed during the whole
346 process. Furthermore, non-fluorescent dark droplets in small sizes were observed inside each oil droplet,
347 suggesting the existence of secondary W/O emulsion in the interior of outer droplets. The other channel
348 highlighting bacteria in blue fluorescence was shown in *Figure 7*B. Almost all the bacteria were found
349 residing at the water-oil interfaces and covering the entire oil droplets, with only very few bacteria present
350 in the aqueous compartment. Some bacteria were found on the edges of oil droplets connecting the
351 adjacent droplets and thereby playing a role in building the emulsion network.

352 For the investigation of inner non-fluorescent droplets, a standard slide and a cover slide were applied to
353 press the primary droplets so that their coalescence could allow for better observation of inner droplets.
354 *Figure 7*C and D illustrated the stabilized secondary droplets stained by BODIPY and DAPI, respectively. The

355 greenish outer oil phase and non-fluorescent inner droplets confirmed the presence of the original W/O/W
356 double emulsion. The diameter of inner water droplets varied in the range from approx. 2 µm to 15 µm,
357 smaller than the primary oil droplets, which normally sized around 60 µm in diameter. Also, BODIPY and
358 DAPI staining showed completely-overlapped location of bacteria, either on the water-oil interfaces or
359 inside the inner water droplets.

360 **4. Discussion**

361 In the present work, an efficient scheme for modifying surface properties of lactic acid bacteria was
362 presented. The method was based on reaction with acid chlorides in non-aqueous media to ensure
363 solubility of the long chain acid chloride and avoid unwanted side reaction into free carboxylic acid. The
364 formed HCl was neutralized by suspended insoluble particles of $NaCO_3$. When remediated back into
365 aqueous solvent, the bacteria could be characterized and used for stabilization of emulsions. The
366 modification induced a strong response in terms of increase of water-air contact angle, adhesion to
367 hexadecane and autoaggregation, but did not pose any instantaneous effect on bacterial culturability other
368 than the effect of lyophilization.

369 Before modification, bacteria showed a fairly low adhesion to hexadecane (12.19%), which was
370 comparable to a previous study where 11.77% and 16.78% were reported as the adherence percentage to
371 hexadecane for two other *Lactobacillus rhamnosus* strains (Polak-Berecka, Wasko, Paduch, Skrzypek, &
372 Sroka-bartnicka, 2014). The most modified bacteria experienced a nearly five-time increase in their
373 adhesion to hexadecane from 12% to 70%. In a previous study, a scheme based on the acid-anhydrate
374 compound octenyl succinic anhydride (OSA) modification in aqueous medium resulted in bacterial adhesion
375 values on the lower side of 59% from 40% and the modification was detrimental to the viability as the high
376 dosing of OSA (Jiang et al., 2019).

377 The observed autoaggregation of LGG upon modification raises the discussion of colloidal stability of
378 bacteria in general. Seen in a simple DLVO picture, bacteria are micron-sized particles and fundamental

379 forces as described by Hamaker theory will set up attraction and result in aggregation (Kronberg, Holmberg,
380 & Lindman, 2014). Colloidal stability of suspensions of bacteria can then be a result of electrostatic, steric
381 or electrosteric repulsions. Here, the electrostatic attraction was unlikely to be the main cause of
382 autoaggregation, based on nearly unchanged surface charge after modification. More likely, stabilization
383 could be a result of steric repulsion caused by hydrophilic polymers such as cell wall cross-linked
384 polysaccharides, which were also the targets of chemical modification using acid chlorides. In this context, a
385 scenario was that upon modification, the polysaccharides lost compatibility with water and collapsed into a
386 more compact structure, which diminished the steric stabilization and thereby severe autoaggregation was
387 observed.

388 When comparing the results from MATH and WCA measurement for low degree of modification (below 5
389 w/w%), a good correlation was acquired in the overall trend, meaning that both methods functioned well
390 to generally quantify bacterial hydrophobicity. The hydrophilic surface of unmodified LGG was confirmed
391 by both a WCA of 59.7° and low MATH of 12.2%. These findings were in agreement with a previous study
392 that extremely hydrophilic bacteria such like L. casei 36 and L. casei 62 displayed a WCA lower than 30°,
393 which did not adhere to hexadecane at all (Hamadi & Latrache, 2008). Another work reported similar
394 findings that adhesion to hexadecane could only be observed when WCA was above 40° (van Loosdrecht,
395 Lyklema, Norde, Schraa, & Zehnder, 1987). For highly-modified bacteria, the WCAs concomitantly increased
396 by approx. 20% from 5 to 20 w/w% LC modification, whereas their adhesion to hexadecane gradually
397 approached to saturation and only 5% of the increase was observed. This discrepancy primarily comes from
398 the different measuring principle of MATH and WCA. It is worth remembering that MATH itself should be
399 understood as a dynamic Pickering adherence phenomenon and not an equilibrium of bacterial partitioning
400 between two phases (van Loosdrecht et al., 1987). Bacteria are of micron-scale size and the true
401 partitioning of hydrophilic and hydrophobic bacteria should be more or less exclusive to water and oil
402 respectively, in an abrupt manner (Binks, 2002; Hunter, Pugh, Franks, & Jameson, 2008; Levine, Bowen, &
403 Partridge, 1989). Therefore, as related to Pickering stabilization of hexadecane droplets, MATH could show

404 a low sensitivity when measuring highly hydrophobic bacteria due to the saturation effects of bacterial
405 binding at hexadecane droplets.

406 In contrast, measurement of WCA directly provides information of bacterial wettability. Theoretically, the
407 surface is considered hydrophilic if the WCA is less than 90° and hydrophobic if the WCA is greater than 90°
408 (Förch, Schönherr, & Jenkins, 2009). Bacteria modified with in particular 20 w/w% LC possessed a WCA of
409 101°, which is even higher than some intrinsically hydrophobic strains such as *L. acidophilus ATCC4356*
410 (WCA of 76°), where the anchoring of surface layer protein rendered their hydrophobic surface properties
411 (Van Der Mei, Van De Belt-Gritter, Pouwels, Martinez, & Busscher, 2003). Moreover, WCA measurement in
412 the present study was utilized as a complementary assay of MATH to confirm the enhancement of cell
413 hydrophobicity by LC modification, but still it should be noted that WCAs are in principle only able to
414 convey information on ability of bacteria to adhere on the water-air interface. Therefore, one could also in
415 the future investigate the three-phase contact angles involving various solvents such as oil, possibly by
416 analyzing the bacterial adsorption on oil droplets using confocal microscopy (Firoozmand & Rousseau,
417 2015) or interchanging the air phase with an oil phase in the present setup using optical observation of
418 macroscopic droplets.

419 In conventional lyophilization aiming to achieve long-term preservation of bacteria, cryoprotectants are
420 added in high concentration to ensure long-term culturability of bacteria. Cryoprotectants include sucrose
421 (Carvalho et al., 2003), lactose (Higl et al., 2007), skim milk (Saputra, Cahyanto, Rahayu, & Utami, 2015) or
422 L-cysteine (Hubálek, 2003), and large amount of such components would interfere with the modification
423 due to the presence of additional amino and hydroxyl groups. In our case, all the lyophilized bacteria
424 showed comparable culturability and degree of damage in cell membrane intactness in the initial day and
425 experienced a constant reduction of 2 logs in culturability over storage, regardless of LC modification.
426 Therefore, the time-dependent lethal effect of lyophilization could be mainly attributed to the deficient
427 protection during freezing process, where the physical damage of cell membrane structure occurred with

428 the formation of ice crystals (Volkert, Ananta, Luscher, & Knorr, 2008). Even so, viability was still
429 maintained to some extents after 1-week and 2-week storage according to the countable growth (6 and 4
430 logs CFU/mL, respectively). Additionally, changes in zeta potential and MATH might also reflect the self-
431 reorganization of cell wall by viable bacteria under the sub-optimal living conditions such as low
432 temperature and lack of nutrients (Oliver, 2005). Based on this, several interesting questions can be raised:
433 are the modified bacteria able to take nutrients from the ambient environment and reconstruct their cell
434 wall towards a more natural hydrophobicity? If yes, will these self-repaired bacteria desorb from the
435 interface and freely translocate into bulk phase considering such high emulsion creaminess? Can the viable
436 bacteria proliferate? If yes, to what degree do the daughter cells use the modified cell wall and maintain
437 the hydrophobicity of the original cells? With these questions, future studies can include the modification
438 by using small amount of cryoprotectants or alternative non-reactive cryoprotectants and try to investigate
439 the viability of modified bacteria present in Pickering emulsion to possibly find a region of both surface
440 modification and bacterial viability.

441 Compared to the traditional production of double emulsions where a two-step emulsification process
442 using two distinctive stabilizers and two emulsification steps is required for the formation of primary and
443 secondary droplets respectively (Ruan, Zeng, Ren, & Yang, 2018; Thompson et al., 2015), double emulsion
444 was created in our study using LC-modified bacteria by a one-step mixing procedure. According to CLSM
445 micrographs, only a few bacteria adsorbed the inner droplets and the size of some droplets were even
446 smaller than a single cell, which is typically 3-5 µm in length (Passot et al., 2015). This possibly indicated the
447 presence of other surface-active compounds stabilizing the interfaces of the inner droplets. The idea of
448 such extra surface-active components is supported by the finding of a surface tension lowering effect from
449 68 to 49 mN/m for modified bacteria as the surface tension of a liquid can only be reduced upon the
450 presence of molecular surfactants and not be affected by solid particles (Brian & Chen, 1987; Harikrishnan,
451 Dhar, Agnihotri, Gedupudi, & Das, 2017). With also a lowering surface tension to 52 mN/m by native LC, we
452 speculated that the surface-active components primarily consisted of the leftover lauroyl acid from LC

453 modification. A further reduction in surface tension from 52 to 49 mN/m might be attributed to bio-
454 surfactants secreted by bacterial metabolism (C, Foght, J, Yeung, & Gray, 2004; Heard et al., 2008) and
455 dissociated cell wall components due to mechanical stresses. Moreover, the MCT oil could also be the
456 source of surface-active components in the form of hydrolysis products and that could explain the inability
457 of other components to lower interfacial tension further.

458 Indeed, a few works reported the joint stabilization of W/O/W double emulsion by Pickering particles and
459 a second low molecular surfactant. One-step preparation of Pickering double emulsion was previously
460 reported by using calcium phosphate (CP-CaP) particles and free fatty acids (Ruan et al., 2018). Other
461 examples include a two-step emulsification procedure based on particles like starch granules or karifin
462 nanoparticles as Pickering stabilizers for outer interface and inner water-soluble surfactants like
463 Polyglycerol polyricinoleate (PGPR) or Tween 80 (Matos, Timgren, Sjöö, Dejmek, & Rayner, 2013; Xiao, Lu,
464 & Huang, 2017). Seen in this perspective, the current one-step procedure is thought to be based on
465 hydrophobic low-molecular surfactants (mainly lauroyl acid) favoring W/O emulsions as well as micron-size
466 modified bacteria with a hydrophobicity and size that favors larger oil droplets suspended in water. In that
467 sense, the micron length scale of the bacteria is important as it defines the larger length scale of the oil
468 droplets and the ability to accommodate an inner small scale water phase.

469 **5. Conclusion**

470 Chemical modification of lactic acid bacteria LGG with LC was achieved efficiently in a non-aqueous
471 bacteria-friendly environment. Significant improvement in terms of cell hydrophobicity was demonstrated
472 by larger WCAs and higher bacterial adhesion to hexadecane, with the improved surface properties stable
473 over one month. Lyophilization posed a time-hidden effect on the bacterial culturability over two-week
474 storage while no instantaneous effect was observed after modification. Consequently, all the modified
475 bacteria were capable of creating stable W/O/W double emulsion by implementing a one-step
476 homogenization process and bacteria were microscopically observed residing at the oil-water interface of

477 both the primary and secondary emulsion droplets. Furthermore, bacteria modified using 10 and 20 w/w%
478 LC notably showed their ability to autoaggregate in aqueous solution. Therefore, a wide range of bacterial
479 hydrophobicity can be acquired by varying LC concentrations, which reveals possibilities for LC-modified
480 bacteria to serve different functionalities such like Pickering particles and potential fat replacers in food
481 structures.

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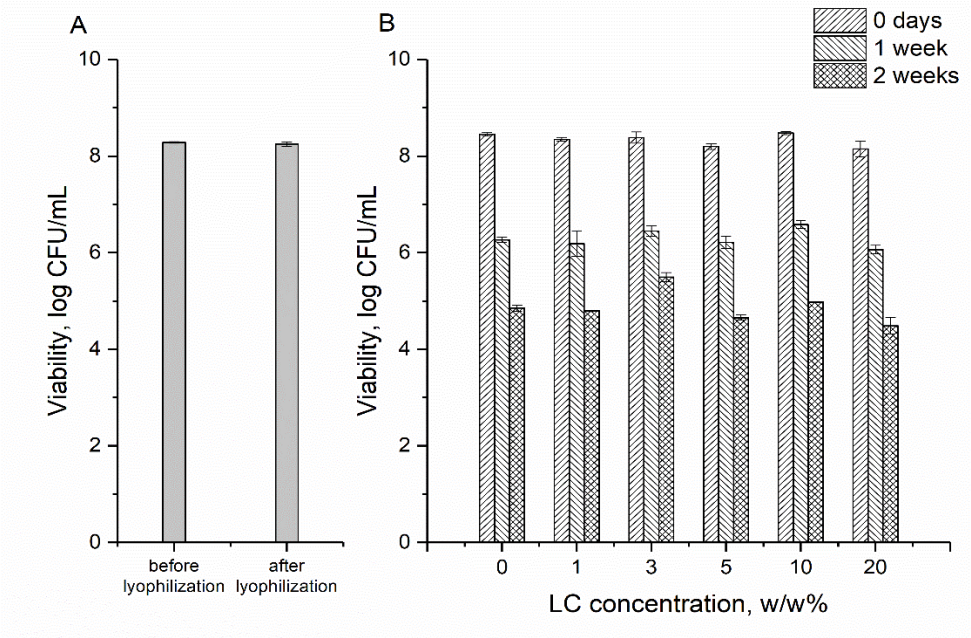

668

669 *Figure 1. Bacterial culturability (Log CFU/mL) before and after lyophilization without LC modification (A), and bacterial culturability*
670 *(Log CFU/mL) during 2-week storage as a function of LC modification (B) using plate counting method. Error bars represent the*
671 *standard deviation (n=2).*

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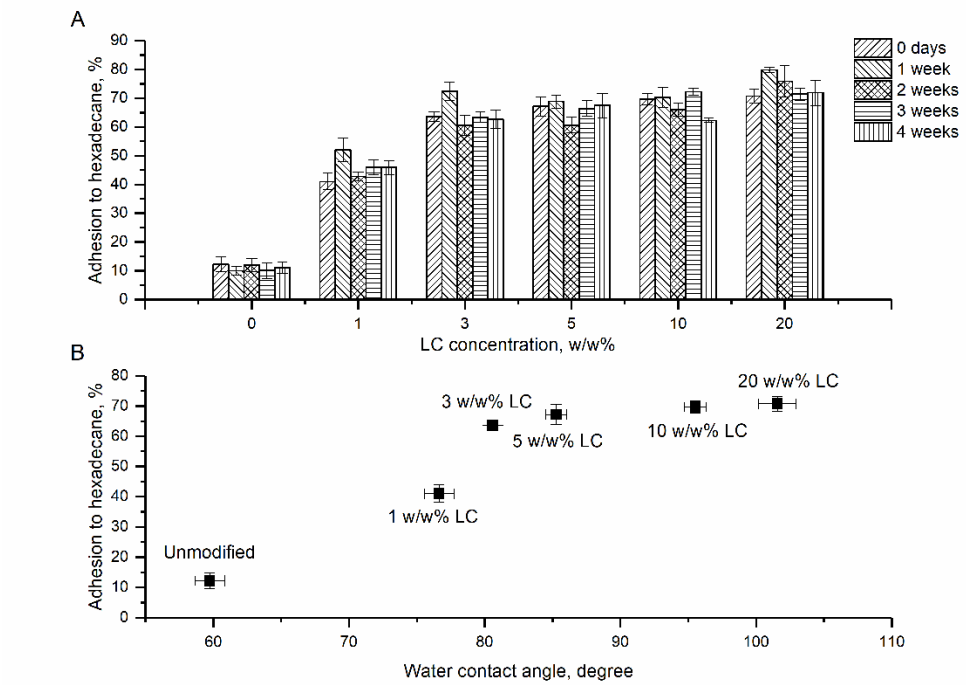

673

674 *Figure 2. Bacterial hydrophobicity by MATH test and WCA measurement. A) Stability of unmodified and LC-modified bacteria in*
675 *terms of MATH as a function of storage time (one month) and B) Relationship between WCA and MATH of unmodified and LC-*
676 *modified bacteria immediately after modification. Error bars represent the standard deviation (n=2).*

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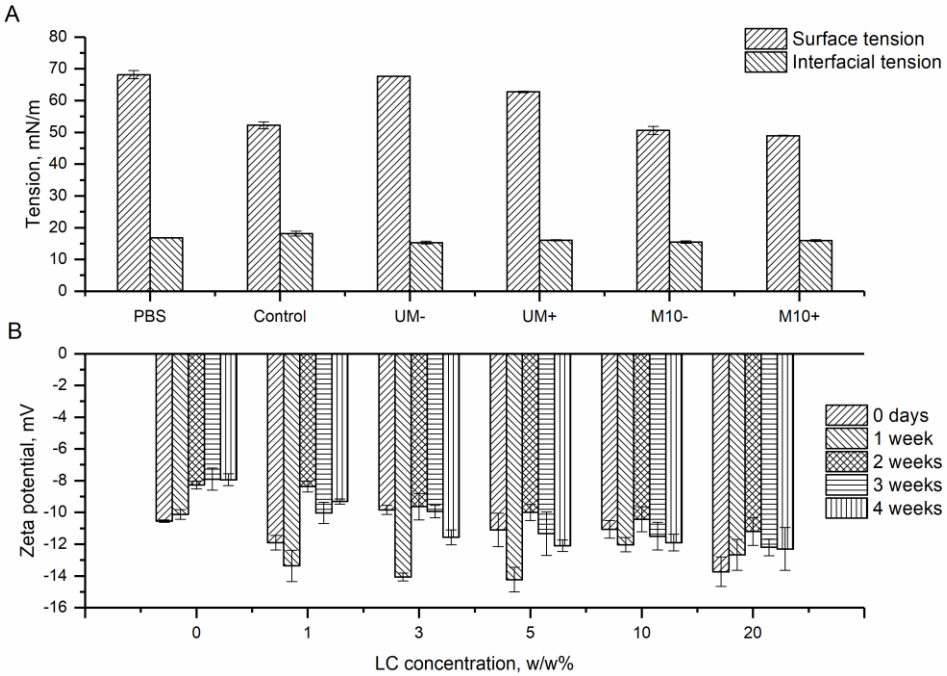
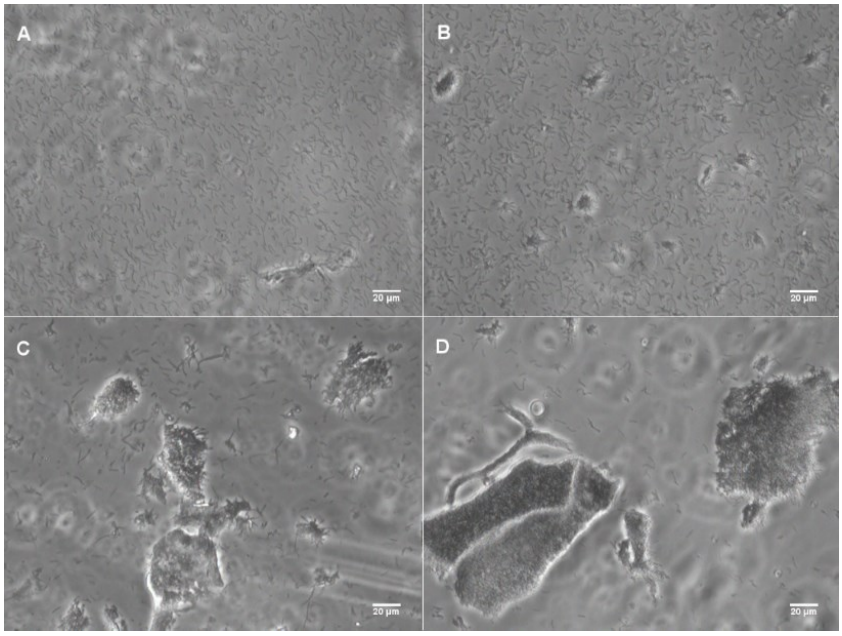


Figure 3. Surface tension and interfacial tension and stability in zeta potential stability of unmodified and LC-modified bacteria. A) Surface tension and interfacial tension with MCT oil of PBS (pH 7.4), controlled LC-dosed PBS, unmodified bacterial suspension (UM+) and supernatant (UM-) and 10 w/w% LC-modified bacterial suspension (M10+) and supernatant (M10-). B) Stability of unmodified and LC-modified bacteria in terms of zeta potential measured in MilliQ water as a function of storage time (one month). Error bars represent the standard deviation (n=2).

685 

686 *Figure 4. Bright field microscopy images of unmodified (A) and 5 (B), 10 (C), 20 (D) w/w% LC-modified bacterial suspensions in*
687 *water. More severe bacterial autoaggregation was observed with the increase of LC concentration. Scale bars represent 20 µm.*

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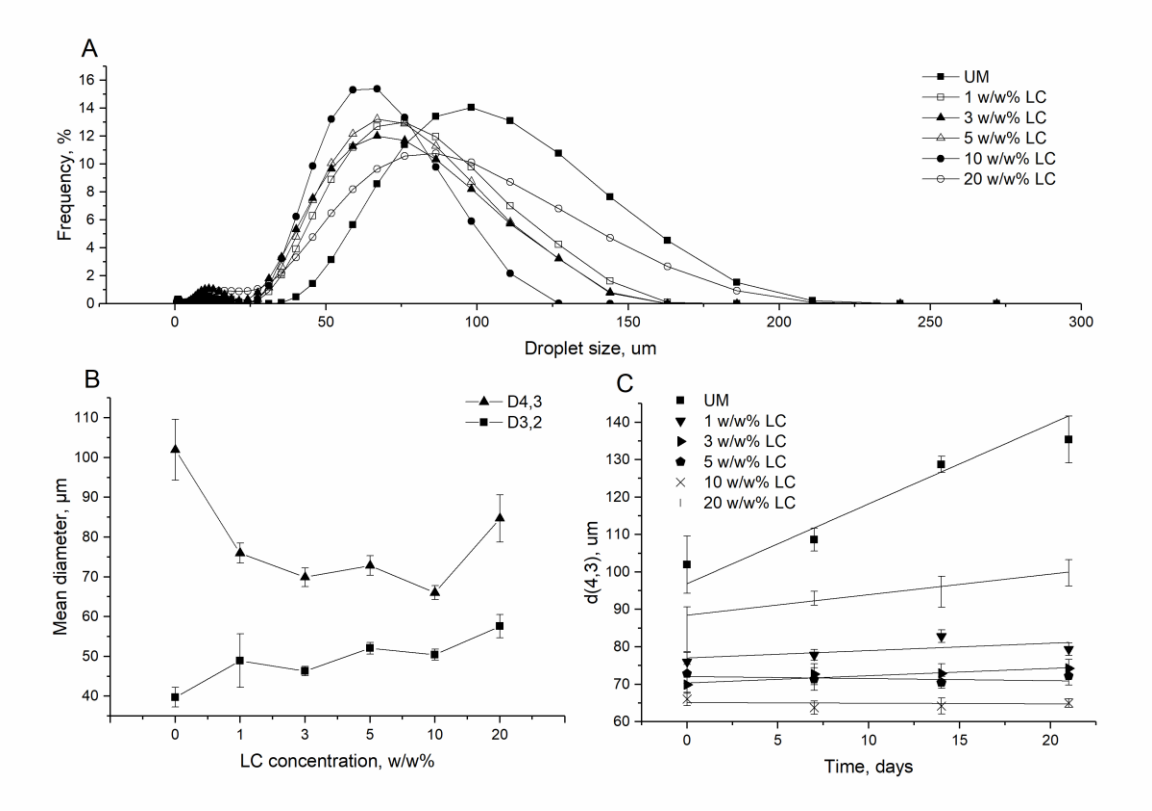

Figure 5. Characterization of emulsions prepared using unmodified and LC-modified bacteria. A) Droplet size distribution immediately after emulsion preparation. B) Surface mean diameter d3,2 and volume diameter d4,3 of oil droplets immediately after emulsion preparation. C) Storage stability of emulsions in terms of d4,3 values as a function of time (3 weeks). Error bars represent the standard deviation (n=2).

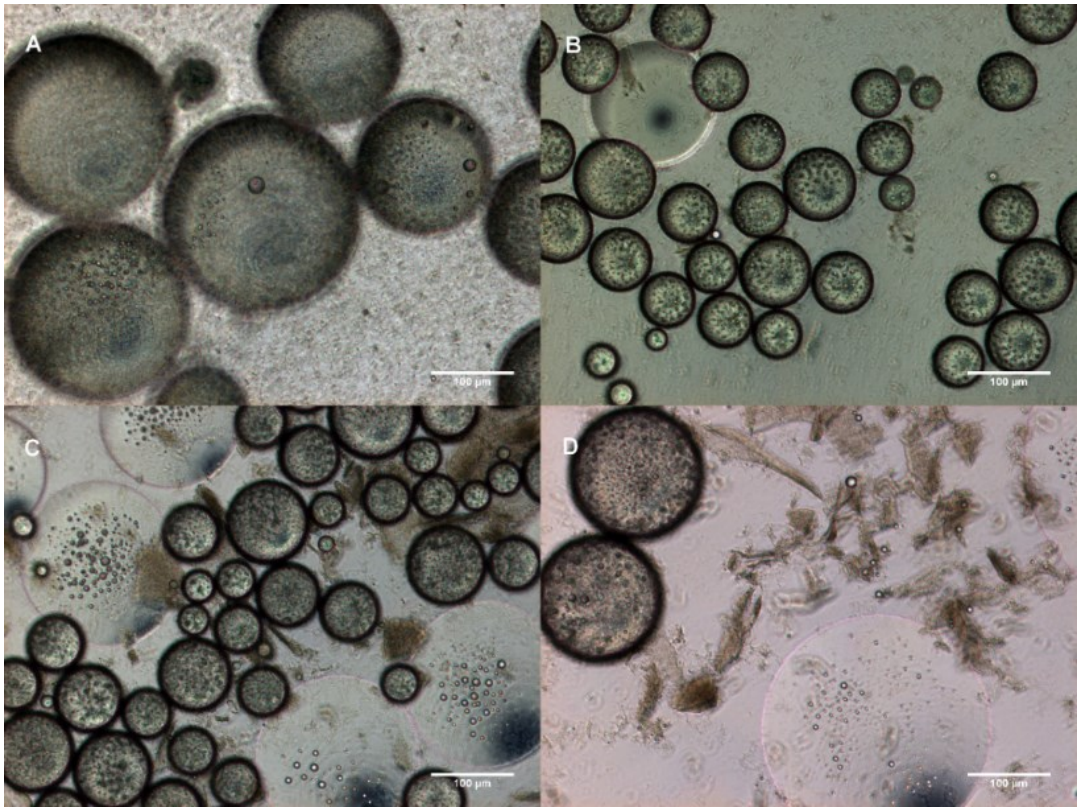

Figure 6. Optical microscopy images of emulsion oil droplets produced using unmodified, 5, 10 and 20 w/w% LC-modified bacteria. Smaller droplets were found for emulsion prepared with 5 (B) and 10 (C) w/w% LC-modified bacteria than unmodified bacteria (A). Relatively large emulsion droplets were produced by 20 w/w% LC-modified bacteria due to the occurrence of severe bacterial clumping (D). Scale bars represent 100 µm.

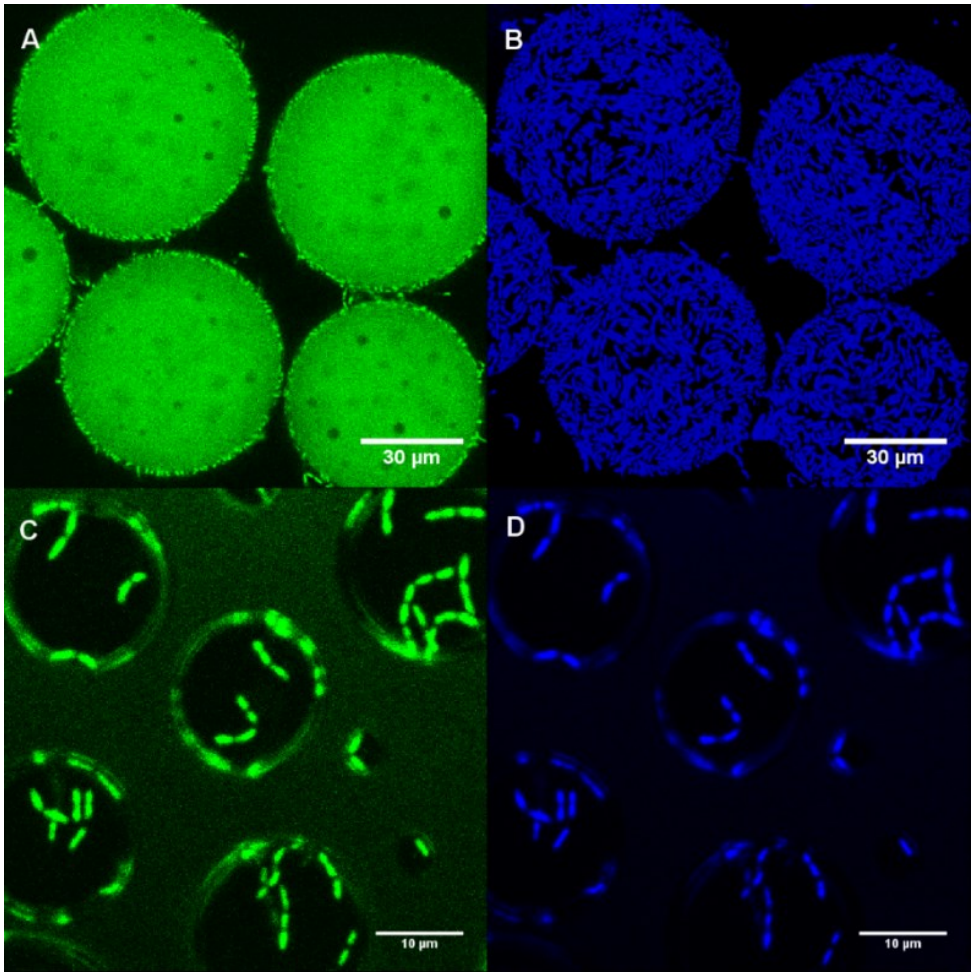

Figure 7. CLSM images of double emulsion prepared using 10 w/w% LC-modified bacteria. The organic phase (MCT oil) was stained using BODIPY (seen in panel A and C as green fluorescence) and bacteria were highlighted using DAPI (seen in panel B and D as blue fluorescence). The primary o/w emulsion (A) and secondary w/o emulsion (C) were indicated by the fluorescent oil phase respectively. Bacteria were found adsorbing on the interfaces of primary oil droplets (B) and the secondary water droplets, with a few bacteria distributing inside the inner water droplets (D). Panel A and C showed the maximum intensity projections of a z-stack series of 30 image planes with an average of 6 lines for each plane, where a total thickness of 19.47 µm of the sample was scanned through. Scale bars represent 30 µm for A, B and 10 µm for C, D.