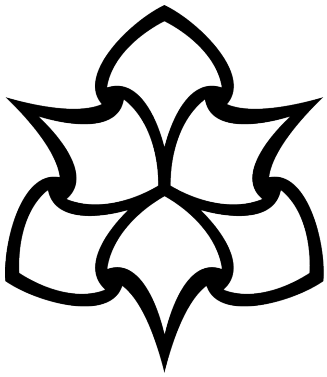

Efficient chemical hydrophobization of lactic acid bacteria – one-step formation of double emulsion

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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.

1. Introduction

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

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

Lactic acid bacteria, as important constitutional and nutritional components in dairy products, demonstrate the potential to serve as also structural building blocks based on Pickering principles. While certain strains showed their inherent surface activity as Pickering particles for emulsions and gels (Dorobantu et al., 2004), most lactic acid bacteria as Gram positive bacteria, still exhibit a dominantly 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

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

2. Material and methods

2.1. Materials and chemicals

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

2.2. Growth of bacteria and dry biomass determination

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

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

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

2.3. Lyophilization and LC modification of bacteria

In order to homogeneously disperse bacteria in oil, moisture was removed by lyophilizing bacteria prior to LC modification. After growing, the washed and collected bacteria were re-suspended in 5 mL sterile 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 w/w% KH_2PO4). The resulted cell suspensions were placed in a freezer set at -80 °C for 90 min. After the suspensions became completely frozen, the temperature of the lyophilizer (Edwards, Buch & Holm A/S, UK) was reduced to -50 °C and then the samples were lyophilized overnight.

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

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

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

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

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

2.5. *Water contact angle (WCA) measurement*

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

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

2.6. Surface tension and interfacial tension

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

2.7. Enumeration of viability

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

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

2.8. Zeta potential measurements

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

2.9. Emulsion preparation and characterization

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

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

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

2.10. Bright field microscopy

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

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

2.11. Confocal microscopy of bacteria-stabilized emulsions

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

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

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

3. Results

3.1. Culturability of modified bacteria

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

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

3.2. Surface properties of modified bacteria

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

3.2.1. Cell hydrophobicity

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

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

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

3.2.2. Surface tension, interfacial tension and surface charge

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

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

3.2.3. Bacterial autoaggregation

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

3.3. Emulsion stabilized by modified bacteria

3.3.1. Emulsion characterization

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

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

The stability of emulsion prepared with unmodified and LC-modified bacteria at 4 °C were investigated 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. Unmodified bacteria produced emulsion with lowest storage stability, where the mean droplet size dramatically increased to 135.4±6.3 μm after 3 weeks. Stability was found high for emulsion prepared using 3, 5 and 10 w/w% LC-modified bacteria, with the mean diameter staying steady over the whole observing period. Moreover, a moderate increase of droplet size was observed for both 1 and 20 w/w% LC-modified bacteria, demonstrating their relatively weaker Pickering stabilizing capacity compared to the other modified bacteria. Hence, neither too low or too high LC concentration were favorable for modifying bacteria towards good Pickering stabilizers.

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

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

3.3.2. Microstructure of bacteria stabilized emulsions

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

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

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

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

4. Discussion

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

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

The observed autoaggregation of LGG upon modification raises the discussion of colloidal stability of 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

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

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

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

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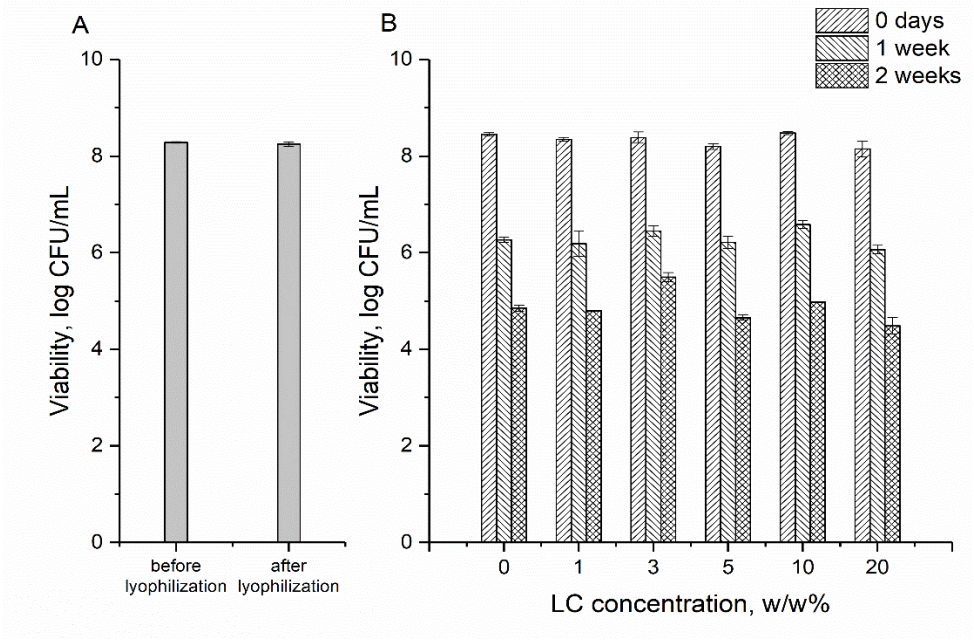
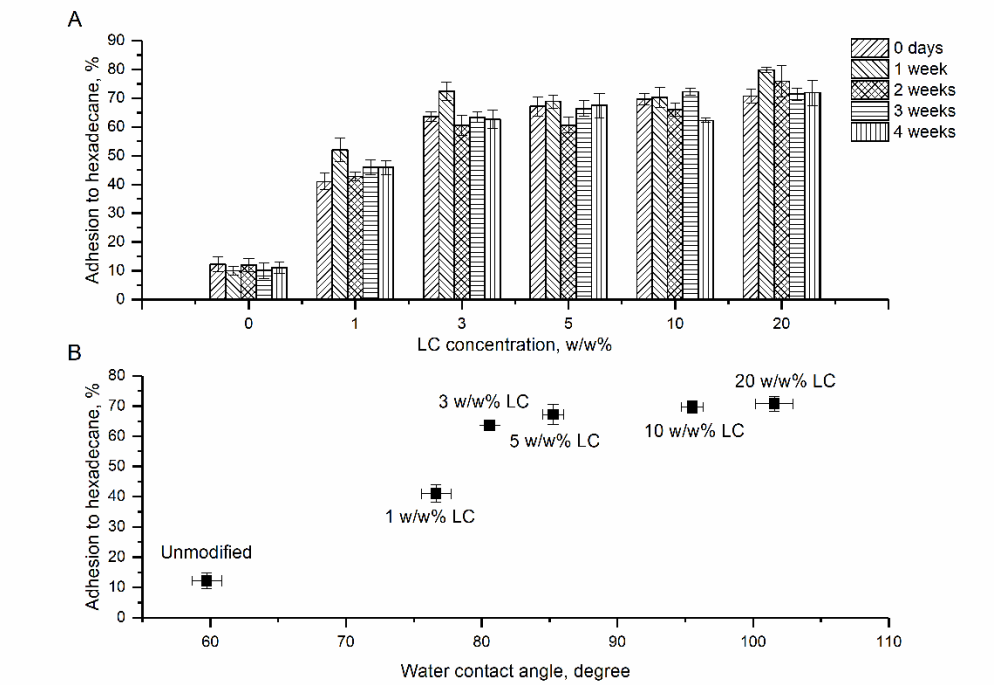


668

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).*

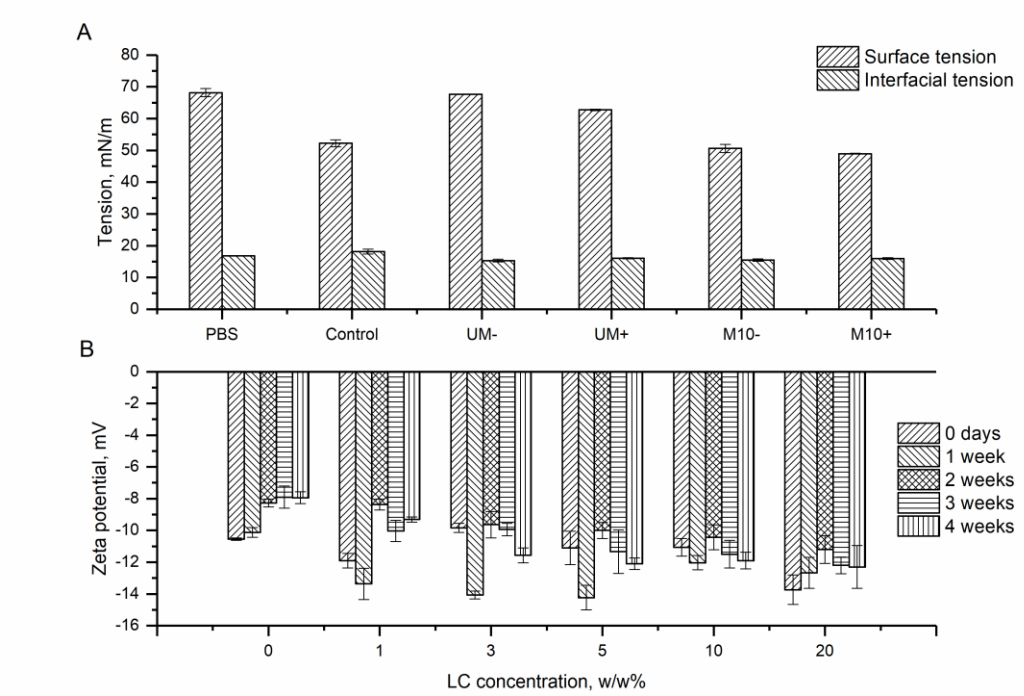
672



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).*

677



678

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

684

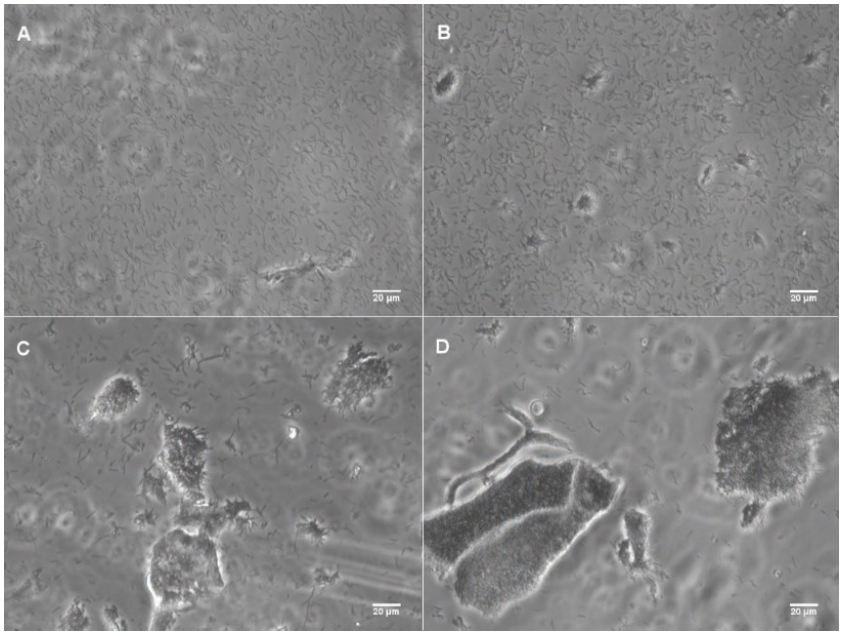
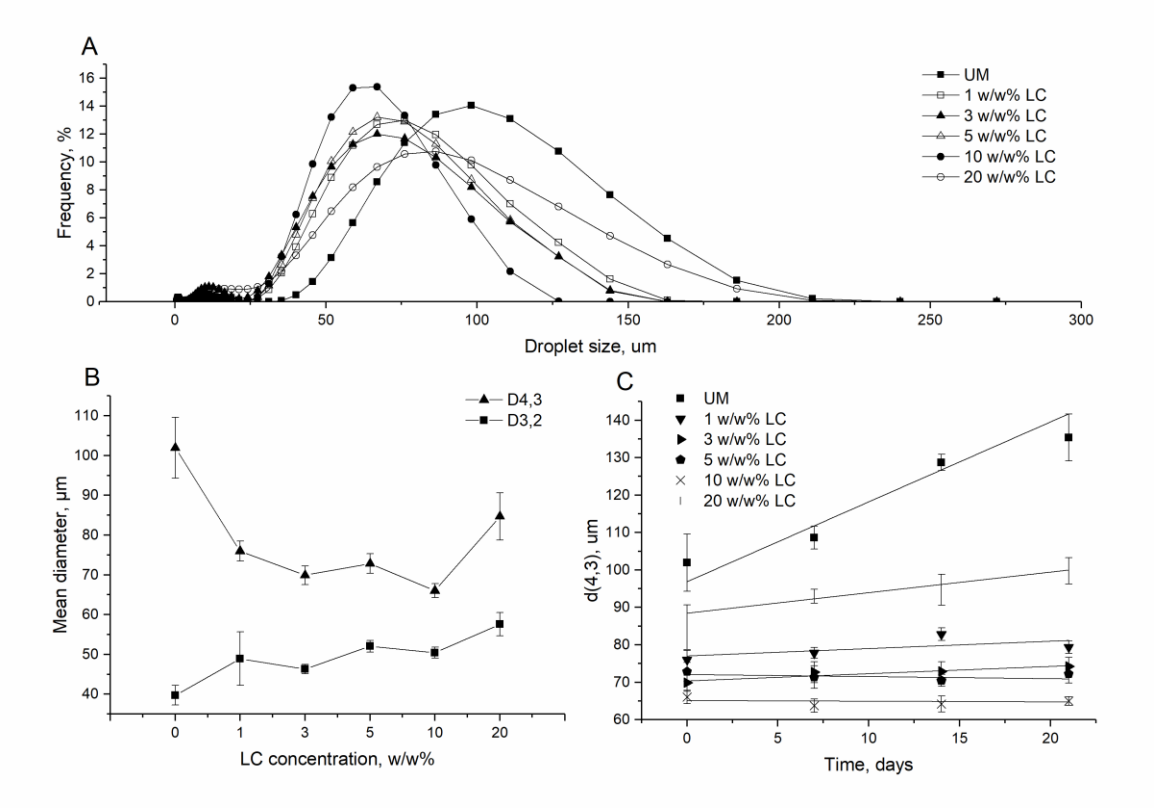


Figure 4. Bright field microscopy images of unmodified (A) and 5 (B), 10 (C), 20 (D) w/w% LC-modified bacterial suspensions in water. More severe bacterial autoaggregation was observed with the increase of LC concentration. Scale bars represent 20 µm.



689

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

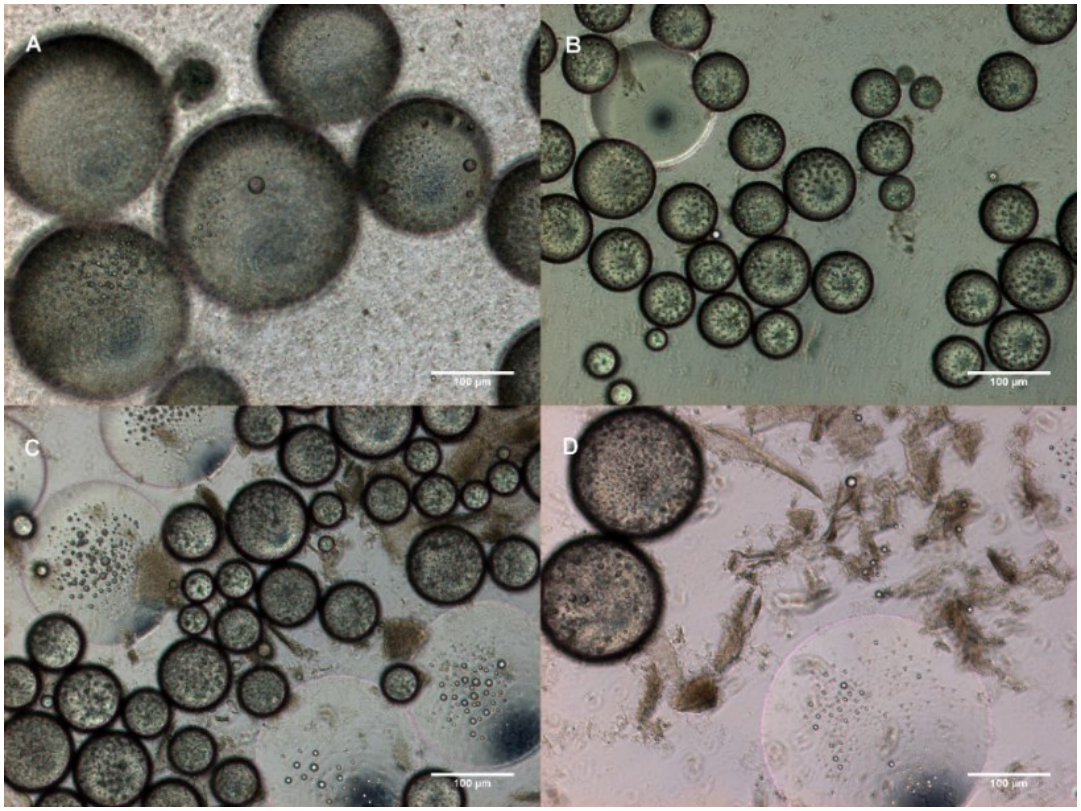


Figure 6. Optical microscopy images of emulsion oil droplets produced using unmodified, 5, 10 and 20 w/w% LC-modified bacteria. Smaller droplets were found for emulsion prepared with 5 (B) and 10 (C) w/w% LC-modified bacteria than unmodified bacteria (A). Relatively large emulsion droplets were produced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

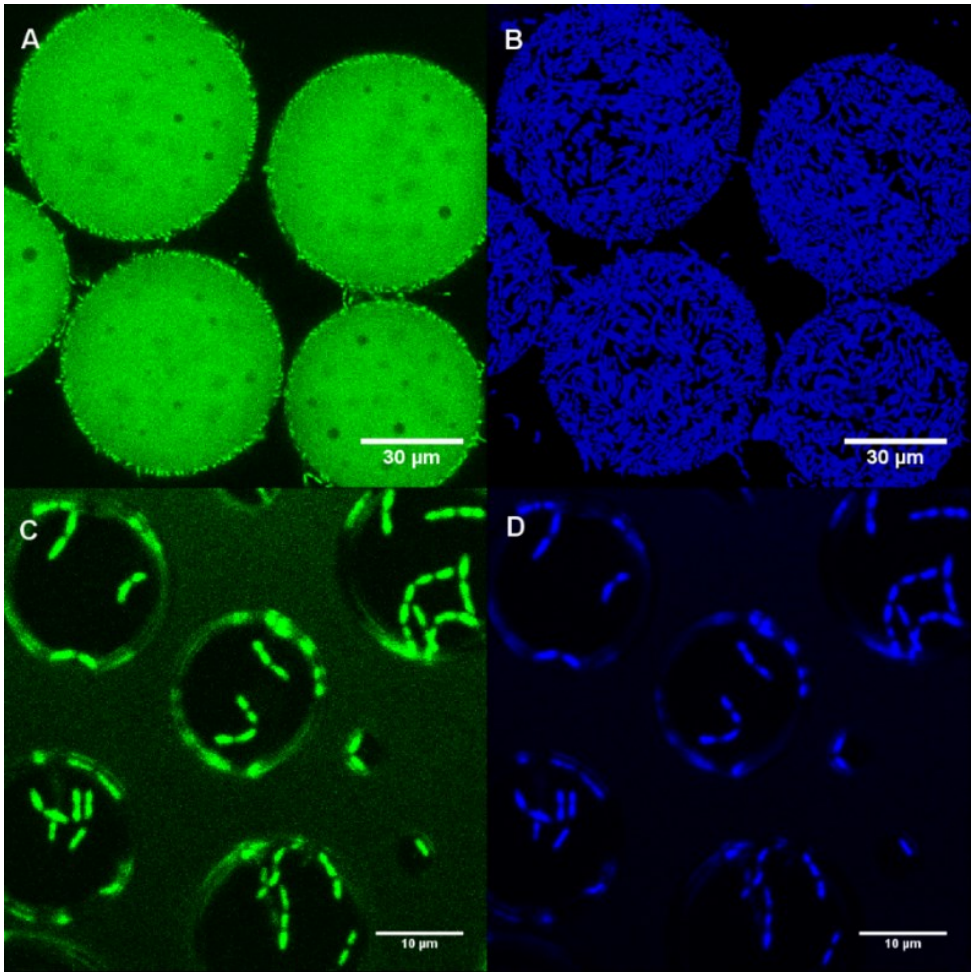


Figure 7. CLSM images of double emulsion prepared using 10 w/w% LC-modified bacteria. The organic phase (MCT oil) was stained using BODIPY (seen in panel A and C as green fluorescence) and bacteria were highlighted using DAPI (seen in panel B and D as blue fluorescence). The primary o/w emulsion (A) 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.