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Enzymatic modification and adsorption of hydrophobic zein proteins on lactic acid bacteria stabilize Pickering emulsions

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ABSTRACT

The effect of enzymatic and physical modifications of the surface of two different strains from lactic acid bacteria, *Lactobacillus rhamnosus* (LGG) and *Lactobacillus delbruekii* subs. *lactis* ATCC 4797 (LBD), to stabilize medium-chain triglyceride (MCT) oil based Pickering emulsions were investigated. A section of cell wall degrading enzymes, lysozyme from chicken egg white and human, lysostaphin, mutanolysin from *Streptomyces globisporus* and proteinase k and the hydrophobic protein zein were used for enzymatic and physical surface modifications. Cell surface modifications were characterized by optical microscopy, scanning electron cryo-microscopy (Cryo-SEM), transmission electron microscopy (TEM), microbial adhesion to hexadecane (MATH) test and zeta potential measurements. The modified cell hydrophobicity in terms of MATH values were increased (around four times) by the enzymatic and physical modifications for LBD and LGG compared to the control. Emulsions stabilized by modified bacterial cells showed higher stability in comparison with unmodified samples, especially for the samples modified with chicken egg lysozyme. Confocal microscopy revealed that the modified bacterial cells were adsorbed at the interface between oil and water and preventing the oil particles from coalescence. Thus, modified bacterial cells can be used to formulate food-grade stable Pickering emulsions. Such Pickering emulsions can potentially be clean label alternatives to replace the conventional emulsion preparations.

1. Introduction

Lactic acid bacteria (LAB) are a renewable, biological, and nutritious source of micron-sized building blocks for soft materials. Such microorganisms are used as scaffolds and stabilizers of foams (Jiang et al., 2019, Heard et al., 2008), and emulsions (Dorobantu et al., 2004, Wongkongkatep et al., 2012, Firoozmand and Rousseau, 2016, Falco et al., 2017).

The key parameters governing the colloidal properties of LAB is the physicochemical properties of their cell wall. LAB (Gram-positive bacteria) has a dominantly hydrophilic nature due to presence of peptidoglycans, and polysaccharides (Delcour et al., 1999). There are different routes to alter the physicochemical properties of the cell wall of LAB such as changes in the fermentation conditions (Schär-Zammaretti et al., 2005, Deepika et al., 2012, Rosenberg and Rosenberg, 1985), adsorption of macromolecules such as polysaccharides, polyphenols, and proteins (Schär-Zammaretti and Ubbink, 2003, Morata et al., 2003, Falco et al.,

2017) to the surfaces of LAB, which is based on the electrostatic attraction between the oppositely charged macromolecules and bacterial cell surfaces. The hydrophobicity of LAB has also been increased by chemical modification of the bacterial cell wall using carboxylic acid anhydrides (Jiang et al., 2019) and acid chloride (Jiang et al., 2021).

Cell lysis enzymes, such as lysozyme, lysostaphin and proteinase K degrade the cell wall components and are used to release the intercellular materials such as DNA, RNA, protein, or organelles (Shehadul Islam et al., 2017, Lavigne et al., 2004, Salazar and Asenjo, 2007, Giovannoni et al., 2020). Such enzymes are used for different applications such as cancer diagnostics, drug screening, mRNA transcriptome determination, and analysis of the composition of specific proteins, lipids, and nucleic acids. The mechanism of lysozyme is the cleavage of $\beta(1-4)$ -linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues of the peptidoglycan layer (Syngai and Ahmed, 2019, Sharma et al., 2016, Salazar and Asenjo, 2007), whereas proteinase k leads to the hydrolysis and random breakdown of peptide

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chains in peptidoglycans (Amara, 2016, Shahriar et al., 2011, Saenger, 2013).

Pickering stabilization using lactic acid bacteria has been demonstrated in case of emulsions (Jiang et al., 2019, Jiang et al., 2021) and foam (Jiang et al., 2019) whereby the lactic acid bacteria was chemically modified using agents such as octenyl succinic anhydride. These studies demonstrated that lactic acid bacteria can be an active part in the structural formation of food if the surface properties can be changed so that they exhibit increased hydrophobicity. Lactic acid bacteria are highly structured renewable resources that can be produced by carbohydrates and nitrogen sources which do not possess functional properties with respect to emulsification. In this context, this process can be seen as an up-conversion of simple nutrients to ingredients having additional functional properties.

This paper presents a combination of enzymatic modification and adsorption of hydrophobic zein proteins to change the surface properties of two strains of *Lactobacillus*; *L. rhamnosus* (LGG) and *L. delbruekii* subsp. *lactis* ATCC 4797 (LBD). The modified bacteria were compared with unmodified bacteria to determine their ability to produce stabilized Pickering oil-in-water emulsions based on renewable resources.

2. Materials and methods

2.1. Materials and chemicals

Proteinase K from *Tritirachium album*, lysozyme from chicken egg white, lysostaphin from *Staphylococcus staphylolyticus*, human lysozyme, mutanolysin from *Streptomyces globisporus* ATCC 21553, zein from maize, potassium dihydrogen phosphate (KH₂PO₄), ethanol 96% and 4,6-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 Invitrogen, Carlsbad, USA. Medium-chain triglyceride (MCT) oil was purchased from AAK AB (publ.), Karlshamn, Sweden. Lyophilized *Lactobacillus rhamnosus* GG (LGG), LMG 18243, was obtained from Collections of Micro-organisms BCCM, Ghent, Belgium. Lyophilized *Lactobacillus delbruekii* subsp. *lactis* ATCC 4797 (Orla-Jensen, 1919) was kindly obtained from Finn K. Vogensen's collection (University of Copenhagen, personal communication). MRS broth (de Man, Rogosa and Sharpe), MRS agar and atmosphere generation system (AnaeroGen sachets) were acquired from Oxoid, Basingstoke, England. MilliQ water (18.2-MΩ-cm at 25 °C) was used in all the experiments.

2.2. Growth conditions and dry biomass of LAB

For growing the LAB, 100 µL of the frozen culture in 40 v/v% glycerol of LGG and LBD were inoculated into 10 ml MRS broth and incubated at 37 °C for 24 h. Then, 50 µL of the pre-cultures were inoculated into 10 ml MRS broth and incubated at 37 °C for 18–20 h. After growth, cells were harvested by centrifugation (4000 rpm, 5 min, and 25 °C), followed by two-time washing with MilliQ water. The collected cell pellets were used for further studies.

For the determination of dry biomass of LAB, 1 ml cell suspension in 3 ml MilliQ water was transferred to a pre-weighed aluminum boat. The cell suspension was put in an oven at 105 °C until a steady dry weight was gained. The determination of dry biomass was carried out in triplicate. The dry biomass was 28.10 ± 1.80 and 20.12 ± 1.89 mg for LGG and LBD, respectively.

2.3. Enzymatic and physical modifications of LAB

For enzymatic modification of LAB, the harvested cells were resuspended in MRS broth and then various enzymes (lysozyme from chicken egg white, lysostaphin, human lysozyme, mutanolysin from *Streptomyces globisporus* and proteinase k) were added in a specified amount. During the enzymatic degradation, samples were incubated at 37 °C while

mixing at 225 rpm using an orbital shaker for 15, 30 and 60 min. After enzymatic modification, the cells were harvested by centrifugation (4000 rpm, 5 min, 25 °C) and washed twice with MilliQ water.

For physical modification of LAB, 1 %wt zein protein was dissolved in 10 vol% aqueous ethanol and lyophilized overnight in an Edwards static freeze dryer (Buch & Holm A/S, UK) at one mbar. The unmodified cells and enzymatically-modified cells were resuspended in 2 ml of 0.1 % wt/vol freeze-dried zein protein solution in water. The samples were incubated for 30 min at 37 °C and 225 rpm using an orbital shaker. After adsorption of zein proteins, cells were harvested by centrifugation (4000 rpm, 5 min, 25 °C) and washed twice with MilliQ water. All the enzymatically- and physically-modified cell pellets were used for further studies.

2.4. Cultivability test of LAB

The drop-plating method was used to define the effect of enzymatic and physical treatments on the culture ability of bacteria. First, the unmodified and modified LGG and LBD samples were diluted with sterile PBS (pH 7.4). Then, 30 µL of diluted sample was distributed onto one-quarter of MRS agar plate. Finally, all the plates were incubated for 48 h in anaerobic condition at 37 °C. The count of culture-able bacteria in each suspension was presented with colony-forming units per millilitre (CFU/mL). All the data are showed as average ± standard deviation from triplicate examinations.

2.5. ξ potential measurements

Solutions and suspensions of the freeze-dried zein protein, modified and unmodified LGG and LBD cells were characterized with respect to zeta potential using a Zetasizer Nano ZSP (Malvern Instruments, Worcestershire, UK) at 25 ± 1 °C. Freeze-dried zein solution was prepared at zein protein concentration 0.1 % wt/vol in MilliQ water and was remained under vigorous stirring at room temperature overnight. The freeze-dried zein protein solution was centrifuged and filtered before use. The pellet was suspended in 10 ml MilliQ water. Then, the cell suspensions were diluted 5 times with MilliQ water prior to measurement. Each data point is shown as average ± standard deviation from triplicate examinations.

2.6. Microbial adhesion to hexadecane (MATH) method

The hydrophobicity of unmodified and modified LGG and LBD cells were determined via a modified MATH method described previously (Jiang et al., 2019). Briefly, pellets of unmodified and modified bacteria were suspended to an optical density of around 0.4 at 600 nm (A₀) in 10 mM KH₂PO₄ solution. 250 µL of each cell suspension was vortexed for 60 s (2 times 30 s) with 42 µL hexadecane using a IKA Vibrax VXR shaker. The suspension was let to stand for 15 min at room temperature to allow complete solvent separation. The suspensions were incubated at 4 °C for 10 min until the hexadecane phase transformed into a white solid mass. Then, the aqueous phase was mixed by the IKA Vibrax VXR shaker again for 30 s and the samples were kept on an ice bath. Finally, the aqueous phase was transferred to the 96-well plate to evaluate the optical density (A₁). The percentage of microbial adhesion to solvent was calculated by the following equation (1):

$$\%adhesion = \left(1 - \frac{A_1}{A_0}\right) \times 100 \quad (1)$$

Results were acquired from triplicated tests and the data are presented as average ± standard deviation.

2.7. Bacterial morphology

2.7.1. Optical microscopy

The suspension of unmodified and modified LGG and LBD cells were put on a microscope slide with a coverslip (0.17 mm thickness). The shape of the cells was observed using a light microscope (Olympus BX40, Buxton Pike, USA) with oil immersion objectives after 1 and 2 days after harvesting. The observation of samples was conducted at room temperature (25 ± 1 °C).

2.7.2. Scanning electron cryo-microscopy (Cryo-SEM)

Physically-modified LGG and LBD cells and the corresponding unmodified cells were sandwiched between 2×100 µm planchettes and cryopreserved by high-pressure freezing (HPM100, Leica). The sandwiched planchettes were mounted in a planchette holder (Leica) under liquid N₂ and transferred to a vitreous cryo transfer shuttle (VCT100, Leica). The samples were cracked for 1 min at -90 °C, and sputter-coated (6 nm) with Carbon/Platinum (MED020, Leica). Samples were investigated with an FEI Quanta 3D scanning electron microscope with an accelerating voltage of 2 kV.

2.7.3. Transmission electron microscopy (TEM)

Bacterial pellets were fixed with 2% v/v glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.2) and inserted in agarose, followed by washing three times in 0.15 M sodium phosphate buffer (pH 7.2). Samples were post fixed in 1% w/v OsO₄ with 0.05 M K₃Fe(CN)₆ in 0.12 M sodium phosphate buffer (pH 7.2) for 2 h. The dehydrated samples with a graded series of ethanol according to standard procedures were transferred to propylene oxide and inserted in Epon. Sections (approx. 60 nm thick) were made with Ultracut 7 (Leica, Vienna, Austria), and collected on copper nets with Formvar-supporting membranes. Sections were fixed using uranyl acetate and lead citrate and were examined using a Philips CM 100 Transmission EM (Philips, Eindhoven, The Netherlands) with an acceleration voltage of 80 kV. Digital images were captured with an OSIS Veleta digital slow-scan 2×2 k CCD camera using an ITEM software package.

2.8. Interfacial tension (IFT) measurement

The IFT measurements were performed between PBS (pure, containing modified and unmodified LGG and LBD cells) and MCT oil using a du Nöuy ring method with a force tensiometer (Sigma 703D Instruments, United Kingdom). Samples for measurement were prepared by suspending bacteria in PBS and using a compact digital rocker to produce a homogeneous dispersion.

2.9. Pickering emulsion preparation and stability

Pickering emulsions with unmodified and modified LGG and LBD pellets were prepared according to a previously described method (Jiang et al., 2019). In Pickering emulsion production, sterile PBS (pH of 7.4) and MCT oil were used as an aqueous phase and an oil phase, respectively. Bacteria were firstly suspended in PBS and the cell suspension was mixed with MCT oil phase on a volume/volume basis of either 40:60 or 60:40 by using an Ultra-Turrax homogenizer (T25 digital Ultra-Turrax®, IKA) at 24000 rpm for 60 s. Each sample was prepared in triplicate for more studies.

To evaluate the stability of Pickering emulsions over a two-week storage time at 4 °C, the mean volume weighted droplet diameter, $d_{4,3}$ and droplet size distribution were determined using a laser diffraction particle size analyzer (Mastersizer 3000, Malvern Instruments, Workshire, UK). The refractive index value of the emulsion was set to 1.47 and the absorption index was 0.01. The obscuration range was adjusted to be in the range of 8% to 15%.

2.10. Microstructure of LAB stabilized Pickering emulsions

The position status of LGG and LBD cells in Pickering emulsions were studied by Zeiss Cell Observer Spinning Disk with Yokogawa X1 module (Carl Zeiss Microscopy, New York, United States) with a Hamamatsu Orca Fusion sCMOS camera with 6.5 µm pixels. Prior to the Pickering emulsion preparation, a known amount of 5 mg/mL DAPI (4',6-Diamidino-2-phenylindole, 5 µL/mL) (Sigma-Aldrich, Steinheim, Germany) was added to the aqueous cell suspension to stain the bacteria. After 10 min incubation in darkness at room temperature with gentle shaking, the dyed aqueous suspension were mixed with MCT oil by using an Ultra-Turrax homogenizer (T25 digital Ultra-Turrax®, IKA) at 24000 rpm for 60 s. Then, 30 µL stained Pickering emulsion was transferred onto a large standard glass slide with a coverslip (0.15 mm thickness) applied to avoid evaporation and observed under 100X objective with oil. The excitation wavelength was 359 nm for DAPI. Images presented were the most demonstrative ones.

2.11. Statistical analysis

Significant differences ($p < 0.05$) were determined among samples using the statistical Minitab software version 16 (Minitab Inc. State College, PA, USA). Analysis of variance (one-way ANOVA with Fisher's multiple comparison test) were also assessed.

3. Results and discussion

3.1. Effect of enzymatic and physical modifications on the cultivability of bacterial cells

For enzymatic modification, the surface of two strains from lactic acid bacteria (LGG and LBD) were modified using enzymes such as lysozyme from chicken egg white and human, lysostaphin, mutanolysin from *S. globisporus*, and proteinase k. For physical modifications, the surfaces of the LGG and LBD cells were altered using zein solution (0.1 % wt/vol).

In order to assess the impact of physical and enzymatic modifications on the surface of LBD and LGG cells, the bacterial cultivability was measured using plate-counting method. The number of colony-forming units (CFU)/mL is given (Table 1). A statistical analysis of the data shows that no effect of any modification was identified in terms of bacterial cultivability.

Table 1
Cultivability in terms of CFU/mL of unmodified and modified LBD and LGG bacteria.

Enzymatic Modification Samples	Log Originally Cell Density (CFU/ml)	Physical Modification Samples	Log Originally Cell Density (CFU/ml)
LBD	Control	Control	7.08 ± 0.04^A
	Proteinase K	Control/ Zein	7.08 ± 0.06^A
	Lysozyme (Egg chicken)	Proteinase K	7.08 ± 0.07^A
	Lysozyme (Human)	Proteinase K/ Zein	7.08 ± 0.07^A
	Lysostaphin	Lysozyme	7.08 ± 0.07^A
	Mutanolysin	Lysozyme/ Zein	7.09 ± 0.05^A
LGG	Control	Control	8.58 ± 0.01^A
	Proteinase K	Control/ Zein	8.58 ± 0.01^A
	Lysozyme (Egg chicken)	Proteinase K	8.59 ± 0.01^A
	Lysozyme (Human)	Proteinase K/ Zein	8.58 ± 0.01^A
	Lysostaphin	Lysozyme	8.58 ± 0.01^A
	Mutanolysin	Lysozyme/ Zein	8.58 ± 0.00^A

A: no significant difference at $p > 0.05$.

3.2. ζ -Potential measurements and microbial adhesion to hexadecane (MATH)

The effects of the modifications to the bacterial surface properties were evaluated by measurements of zeta potential and microbial adhesion to hexadecane (Figs. 1 and 2).

Zeta potentials of the bacterial surface before and after enzymatic modification for different treatment times were measured in MilliQ water. The zeta potential of unmodified LBD and LGG cells was in agreement with previous work (Agvei and Danquah, 2012, Doherty et al., 2010). The unmodified LGG was only slightly negatively charged with a zeta potential of -6.6 ± 1.1 whereas the surface of LBD was more negatively charged. For both strains, all enzymatic modifications generally led to more negative zeta potentials due to the alterations of surface chemical composition. The negative-charged surface exhibited by most LAB is mainly due to the large presence of teichoic acid, which consists of numerous phosphate groups, rendering the polyelectrolyte character of bacteria (Djeribi et al., 2013). Teichoic acids include wall teichoic acids, which covalently link to the peptidoglycan, and lipoteichoic acids, which anchor to the cell membrane. Enzymatic treatment using either lysozyme or proteinase K may lead to the breakdown of cell wall peptidoglycan, resulting in greater exposure of both kinds of teichoic acid. This could explain the more negative-charged surface possessed by two strains treated by enzymes.

Modified bacteria using various enzymes exhibited surface charge ranging from -23 to -35 mV and -6 to -11 mV for LBD and LGG, respectively. For both strains, chicken egg lysozyme treatment for 30 min gave the most negative zeta potential compared to other enzymes. For most enzymes, enzymatic treatment for 1-hour reduced the zeta potential compared to the unmodified cell zeta potential. The lowest level of the zeta potential for most enzymes was related to 1-hour treatment (Fig. 1).

The measurements of the cell surface hydrophobicity or hydrophilicity were carried out by "partitioning" the cells between aqueous and hexadecane phases. The non-polar solvent hexadecane is widely used in this assay to assess the hydrophobicity with the minimization of electrostatic effects between bacterial cells and hexadecane by performing the experiments in a high ionic strength solutions (10 mM KH_2PO_4) (Pelletier et al., 1997, Schär-Zammaretti and Ubbink, 2003). The percentages of unmodified LBD and LGG cells adhering to this non-polar solvent were 11.1 ± 3.5 and 6.3 ± 1.8 %, respectively. These results

demonstrated the hydrophilic surface of both LBD and LGG cells. As seen in Fig. 1, the treatment of up to 30 min with enzymes generally increased the hydrophobicity of LBD and LGG cells, except for LBD modified with lysostaphin enzyme, where the highest hydrophobicity value was reached already after 15 min (Fig. 1). The treatment for 1 h led to a decrease in MATH values compared to shorter treatment for all enzymes used in both strains except LGG modified with lysostaphin enzyme (Fig. 1). Therefore, in terms of the increase of bacterial surface hydrophobicity, the optimal treatment time for all enzymes on both strains was 30 min excluding the lysostaphin enzyme. The most effective enzymes were egg chicken lysozyme and proteinase K. Although the MATH value of LBD modified with human lysozyme enzyme was initially higher than LBD modified with proteinase K enzyme, the MATH value of these cells decreased sharply during the one-week storage time (data not shown), which may be as a consequence of slow reorganization of the cell wall. Therefore, egg chicken lysozyme and proteinase K enzymes for both strains with 30 min time treatment were selected to investigate further in combination with physical modifications using zein.

Zein is a hydrophobic protein showing only little solubility or dispersibility in water (Wang and Padua, 2010) and it was not possible to measure the zeta potential of zein in water due to the low solubility (data not shown). In order to modify the bacterial surfaces using the adsorption of zein, the aqueous solubility was improved. Briefly, after zein was freeze-dried from a 10 % ethanol solution it showed an improved solubility (see Supporting Information, Figure S1) and a strongly positive zeta potential of 51.8 ± 1.2 mV.

In general, the surface charges of zein-treated samples of LBD and LGG were less negative than the bacteria not treated with zein (Fig. 2) indicating some absorption of positive proteins to the negative bacterial surfaces. The surface charge effect of zein treatment was in general greater for LBD. This can be rationalized by the more negative zeta potential of LBD (-23 mV) compared to the almost neutral LGG (-6 mV) resulting in a potential increase in zein adsorption driven by electrostatic interactions between the zein and the bacteria.

Fig. 2 shows the effect of zein modification on the MATH values of LBD and LGG cells before and after modification. Fig. 2a demonstrated that the hydrophobicity of the LBD cells significantly increased after zein modification. The treatment involving zein was most efficient for LBD and gave MATH values in the range of 35% to 55%, higher than for treatments only involving enzymes. For LGG, the opposite situation was observed. Here the various enzyme treatments were found most efficient

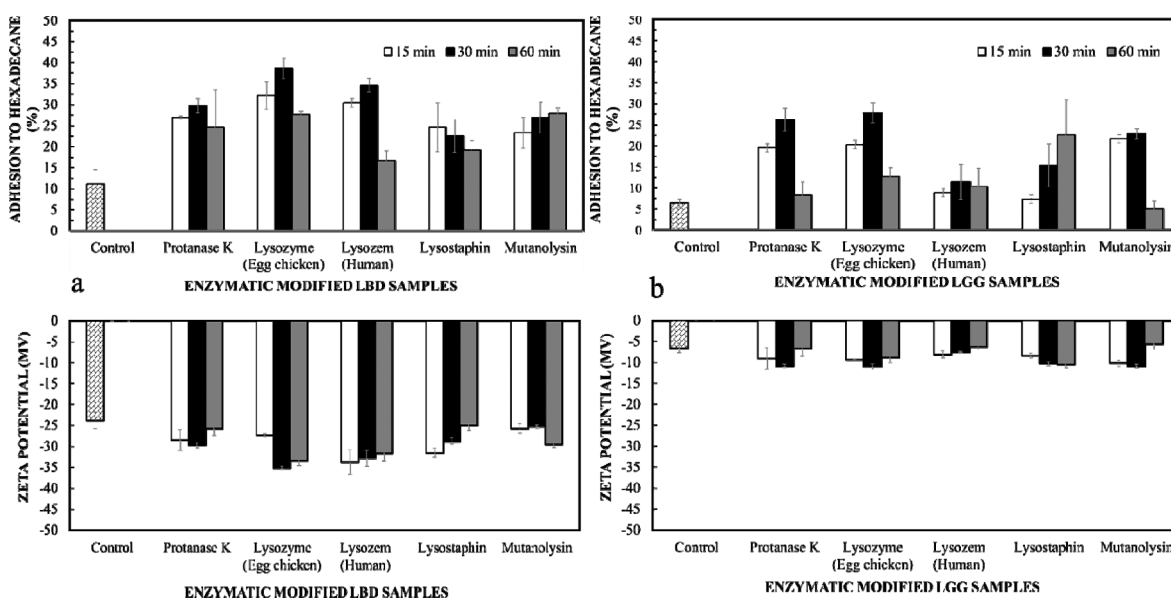


Fig. 1. Adhesion to hexadecane values and zeta potential of unmodified and modified (a) LBD and (b) LGG bacterial cells as a function of enzymatic modifications with different enzymes.

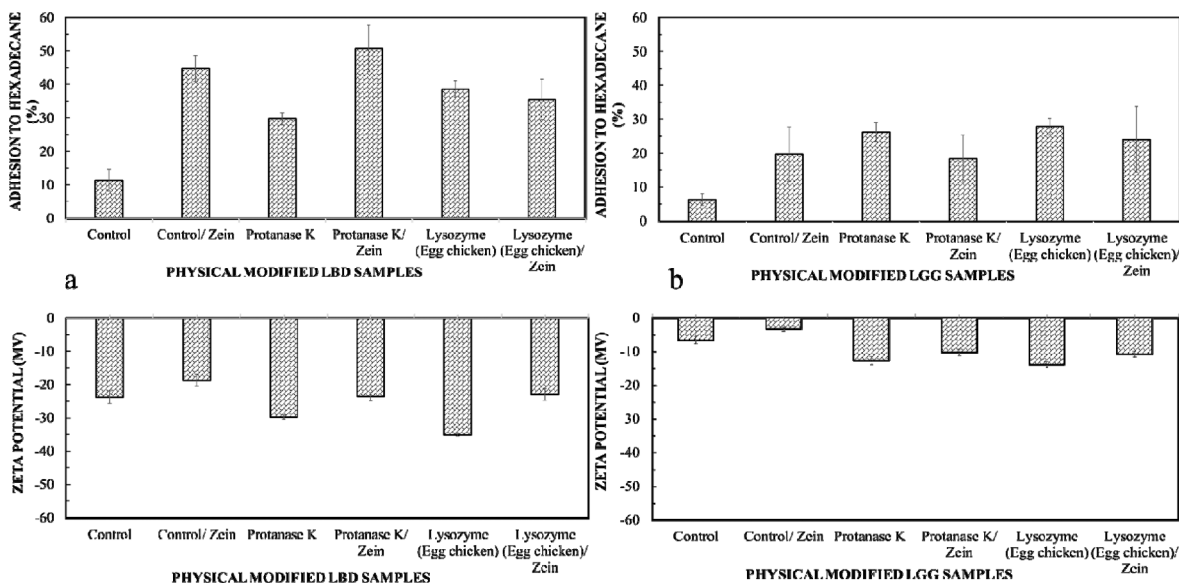


Fig. 2. Zeta potential and adhesion to hexadecane values of unmodified and modified (a) LBD and (b) LGG cells as a function of physical modifications with zein protein.

whereas adsorption by zein did not further increase the observed hydrophobicity (Fig. 2b).

The observation of zein induced changes of MATH values of LBD and

LGG was consistent with the observation of zeta potential, where the adsorption of zein was seemingly driven by electrostatic interactions whilst only a small effect was observed for the almost electroneutral

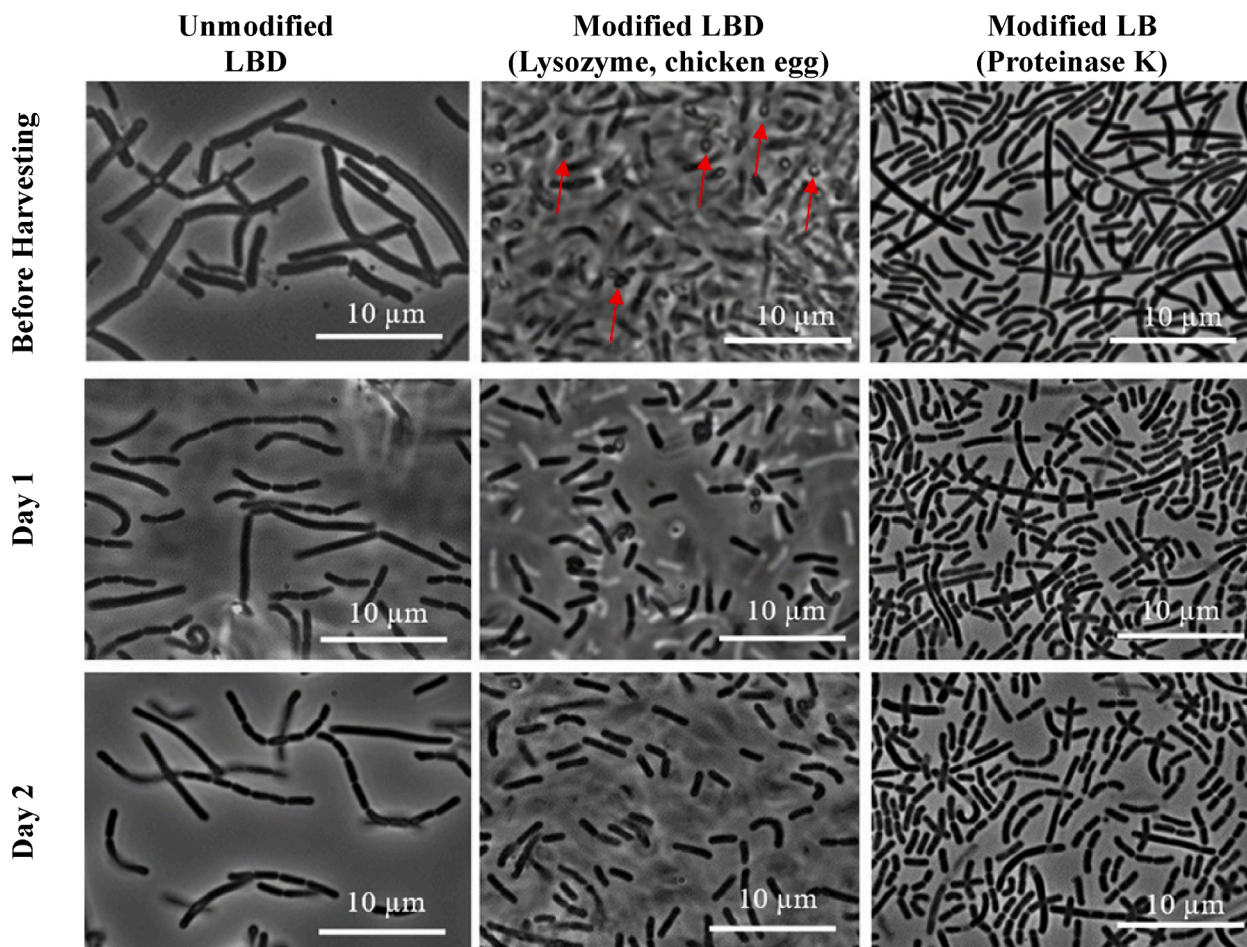


Fig. 3. Optical microscopy images of unmodified and modified LBD cells during storage time at higher magnification (100x) with oil; the red arrow indicates the protoplasts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LGG. In comparison, the MATH values for the modified LBD/zein, the modified proteinase K/zein and the modified lysozyme from egg chicken/zein samples reached 54%, 50% and 44%, respectively.

3.3. Bacterial morphology

The microstructure of unmodified and modified LBD (Figs. 3 and 5) and LGG (Figs. 4 and 6) cells were detected using optical microscopy, transmission electron microscopy (TEM), and cryo scanning electron microscopy (Cryo-SEM) at different magnifications. From optical micrographs of LBD (Fig. 3) and LGG (Fig. 4), it was seen that cells modified by proteinase K enzyme maintained rod-shaped morphology and often appeared as chains of several bacteria. This indicates that there is no major change in shape through modification by the enzyme proteinase K during their storage for 2 days as compared to unmodified cells.

When modifying cells of both LGG and LBD with chicken egg lysozyme the morphology changed dramatically. The chicken egg lysozyme can hydrolyze the 1,4- β -glycosidic bonds between N-acetyl-glucosamine and N-acetyl-muramic acid in peptidoglycans, which leads to more severe degradation of the cell wall. Immediately after modification (before harvesting) protoplasts were observed as spherical and non-rod shaped cells. Briefly, protoplasts are bacteria with fully degraded cell wall, which cannot maintain their original shape. The protoplasts disappeared over the next two days due to action of the osmotic pressure of storage in pure water. For the modification using lysozyme, the chains broke up into individual bacteria. The remaining cells after two days therefore constituted modified cells that were intact with respect to formation of protoplasts.

TEM of thin sections of LGG and LBD cells stained with uranyl acetate and lead citrate demonstrated the cytoplasm (marked as Cy), plasma membrane (PM) and cell wall. The cell wall was divided into regions of different electron contrast, marked as CW_a and CW_b (Fig. 5a and 6a). The CW_b layer appeared “fluffy” and light grey whereas the inner CW_a layers was observed to be more compact and dark grey. Enzymatic treatment using lysozyme and proteinase K caused thinning of the inner layer of CW_a or loss of some parts. Also, the “fluffy” layer of CW_b expanded from 49 nm in unmodified LBD cell to 76 nm after treatments with lysozyme and proteinase K enzymes. Similar results were observed for LGG cells, where CW_b increased 1.8-fold and 2.4-fold, after treatments with lysozyme and proteinase K enzymes, respectively.

Cryo-SEM micrograph of LBD and LGG cells with and without zein particles are shown in Fig. 5b and 6b, respectively. The unmodified bacteria appear naked and smooth, whereas, for zein-treated bacteria, aggregates of zein were associated with the bacterial cells. The association of zein to bacterial cells was stronger for LBD, where the electrostatic attraction was also stronger.

3.4. Interfacial activity of bacterial cells

The modified bacteria were to be utilized as Pickering stabilizers of emulsions. Therefore, the effects of bacteria or excreted bacterial components on the oil and PBS buffer interfacial tensions were investigated. Briefly, suspensions of washed bacteria in contact with oil were agitated to allow bacteria to adsorb onto the interphase, and subsequently, the interfacial tension was measured using the du Noüy ring technique. Alternatively, bacteria were separated from buffer solutions prior to measurement of interfacial tension in order to evaluate the effect of

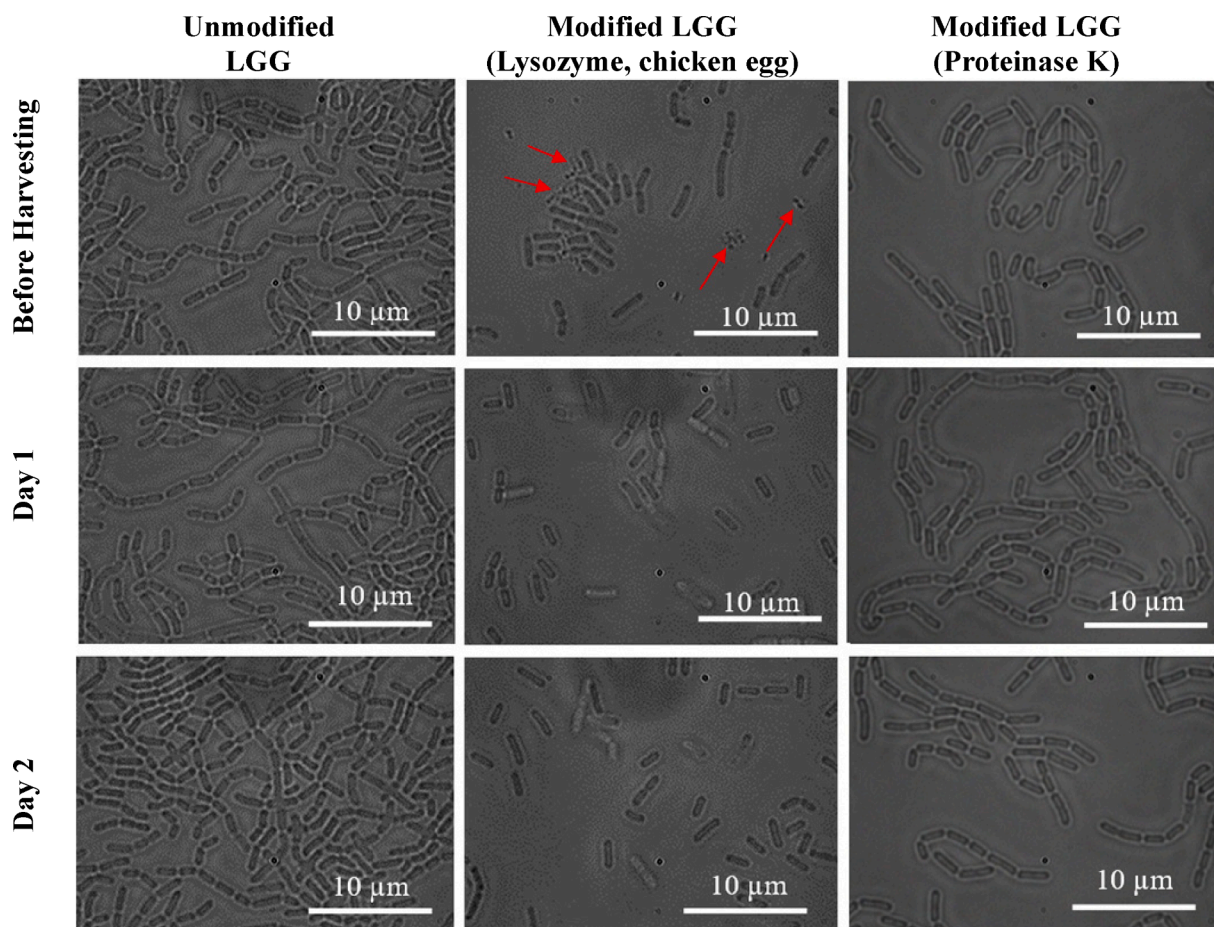


Fig. 4. Optical microscopy images of unmodified and modified LGG cells during storage time at higher magnification (100 \times) with oil immersion; the red arrow indicates the protoplasts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

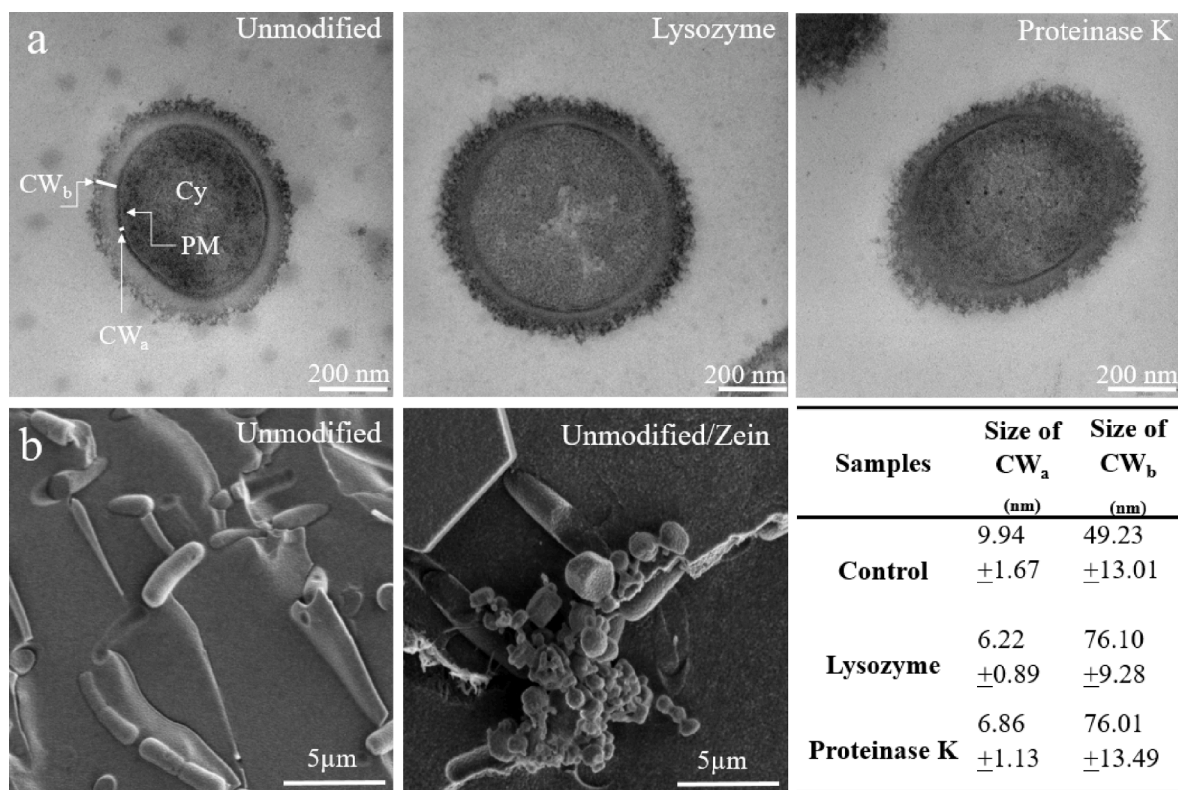


Fig. 5. EM images of unmodified and modified LBD cells (a) TEM (b) Cryo-SEM at higher magnification and table displaying size of cell wall of unmodified and modified LBD cells.

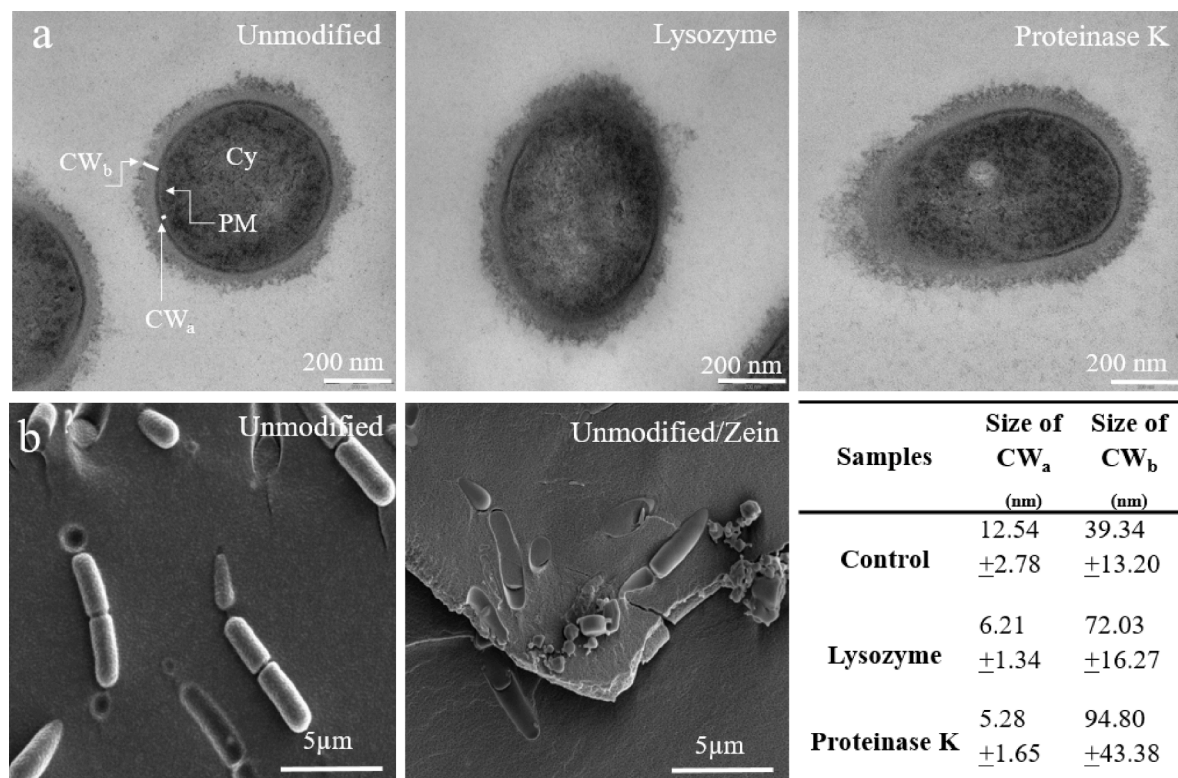


Fig. 6. EM images of unmodified and modified LGG cells (a) TEM (b) Cryo-SEM at higher magnification and table displaying size of cell wall of unmodified and modified LGG cells.

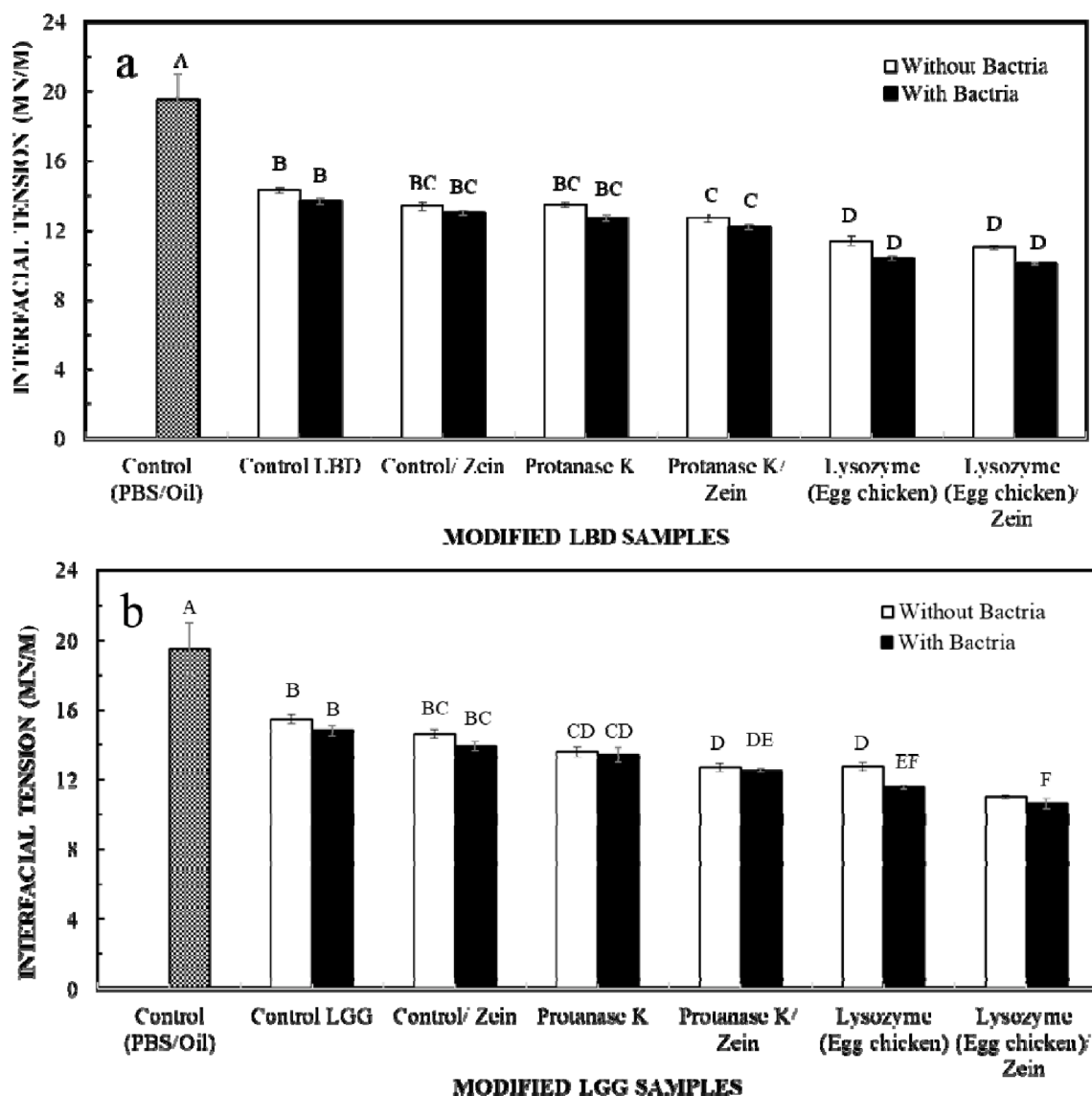


Fig. 7. Interfacial tension of unmodified and modified (a) LBD and (b) LGG cells. A-F: significant difference at $p < 0.05$.

soluble components accompanying bacteria in absence of cells. Fig. 7 demonstrates that compared to pure oil/buffer systems, the interfacial tensions were lowered by components buffer solution previously in contact with washed bacteria. There was a non-significant tendency to a systematic further lowering of interfacial tension by the presence of bacterial cells. The interfacial tension was lowered mostly in the case of enzymatic modification and to a lesser extent by zein modification alone. Chicken egg lysozyme modification was most efficient and the interfacial tension was significantly ($P < 0.05$) lowered from 15 to 10 mN/m for both strains. This indicates that degradation products from enzymatic activity, despite the washing procedure, was surface active and accumulated at the oil water interface and this cell free effect should be taken into account when dealing with emulsions stabilized by bacteria. The systematic tendency of lowering effect of bacterial cells can be attributed to lowering of the interfacial free energy density by a Pickering effect.

3.5. Pickering stabilization by bacterial cells emulsion stability

Emulsions were produced using both the unmodified and modified LBD and LGG cells with 40 and 60 % MCT oil content and emulsion stability was evaluated for 2 weeks at 4 °C by measuring droplet size

distribution and mean diameter, $d_{4,3}$ as seen in Fig. 8.

Unmodified LBD cells were able to produce emulsions regardless of the oil content, while unmodified LGG cells were not able to produce the emulsion with 60% oil content. Emulsions with unmodified bacteria showed broad size distributions (Fig. 8 a-b) and the $d_{4,3}$ increased significantly over two weeks of storage, indicating droplet coalescence and emulsion instability. In contrast, emulsion stabilization using modified bacteria gave stable emulsions with no increase of $d_{4,3}$.

For these modified bacteria, size distributions peaking at smaller sizes with narrower spans were obtained, reflecting the efficiency of enzyme-modified bacteria to act as Pickering stabilizers. The overall droplet size of the emulsions was in the order of 50 to 100 μm . In the case of emulsions with 40% oil content, LGG and LBD modified by lysozyme, resulted in emulsion with smallest $d_{4,3}$ of 62 and 52 μm , respectively, and the best stability compared to other modified bacteria. When unmodified LGG and LBD was physically modified using zein the average droplet size was reduced and the greater effect in case of LBD was due to the greater hydrophobicity of LBD cells (Fig. 8a). The same effect was not seen for enzymatically-modified bacteria where zein did not give systematical improvements.

At 60% oil content, only Pickering emulsions using LGG modified bacteria by lysozyme and lysozyme/zein were produced, where

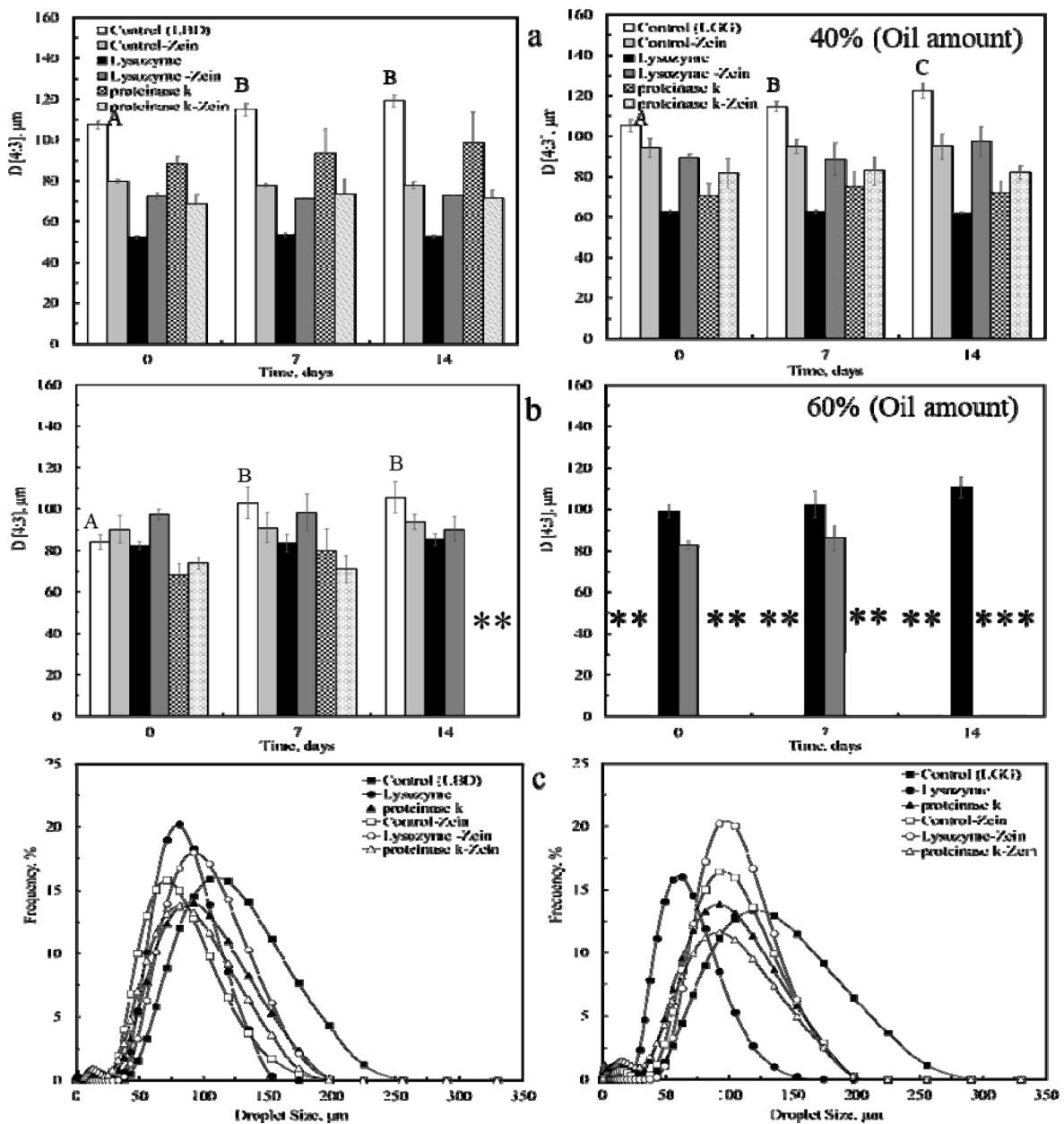


Fig. 8. Characterization of emulsions prepared with unmodified and modified LBD and LGG cells. a-b) Emulsion stability of emulsions against droplet coalescence displayed by changes in terms of mean $d_{4,3}$ values of oil droplets over time. c) Droplet size distribution immediately after emulsion preparation. An asterisk “*” indicates no emulsion stability. All modified samples showed A: not significant different at $p > 0.05$, all unmodified samples showed A-C: significant difference at $p < 0.05$.

Pickering emulsion with bacteria modified with only lysozyme was more stable and no significant difference in mean diameter was observed during the storage period (Fig. 8b). While in the corresponding systems with LBD cells, the stable Pickering emulsions during storage time were produced using all modified bacteria except bacteria modified by proteinase K and proteinase K/zein. Pickering emulsions by bacteria modified with proteinase K and proteinase K / zein showed the lowest size compared to other Pickering emulsions but were stable for only 1 week. This may be due to the effect of large amounts of oil on these modified bacteria, which breaks down the Pickering emulsions and sedimentation bacteria at the bottom of the tube after 1-week of storage time (Fig. 8b and see Supporting Information, Figure S3).

The emulsions were formulated without any carbohydrates available as a nutritional source for the bacteria, and as expected, no signs of microbiological metabolism or growth which would influence the

emulsion stability was observed during the storage of the emulsions. Future investigations could involve a living system whereby the bacteria are able to proliferate whilst being surface active and functioning as Pickering stabilizers.

3.6. Microstructure of bacteria stabilized emulsions

Confocal microscopy images of Pickering emulsions produced using unmodified LBD bacteria and different combinations of chicken egg lysozyme proteinase K and zein is shown in Fig. 9. The Pickering emulsion droplets prepared using unmodified LGG and LGG/zein cells were not stable enough, and rapid coalescence was observed after adding a cover slide. The individual cells are stained using DAPI which can cross cell membranes and bind strongly to A-T rich regions of DNA (Kapuscinski, 1995). For all emulsions, oil droplets were covered with

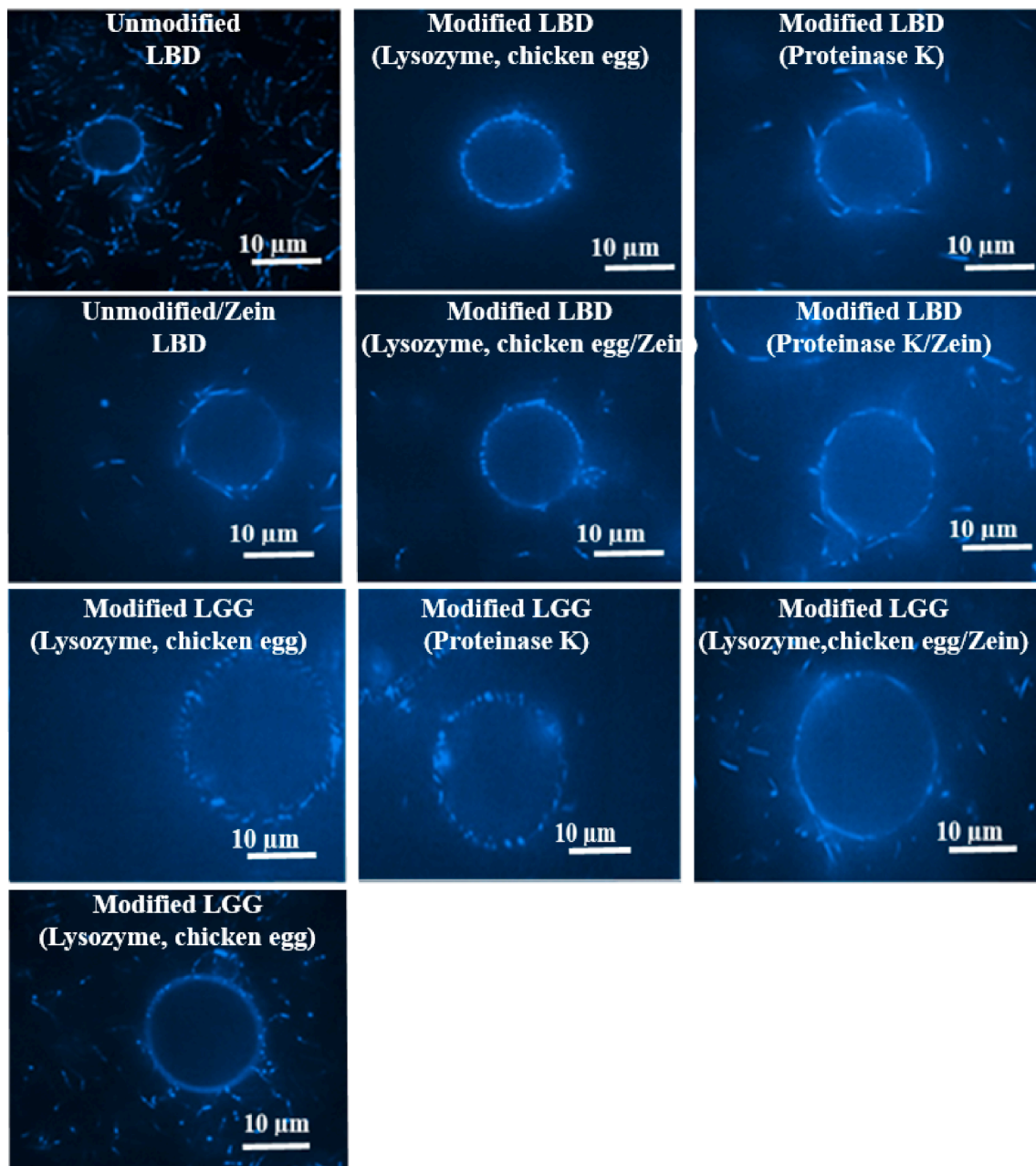


Fig. 9. CM micrographs of emulsion prepared using unmodified and modified LBD and LGG bacteria where the bacteria was highlighted using DAPI dyes (blue fluorescence).

bacterial cells and therefore the microstructure confirmed stabilization through a Pickering mechanism. Emulsions stabilized with modified bacteria, showed the lowest number of free bacterial cells not associated with droplet surfaces as compared to emulsions based on unmodified bacteria. Regardless of use of LGG or LBD, the modification with lysozyme treatment alone resulted in the most dense packing of bacterial cells at the water/oil interface and the lowest number of free bacteria cells present in the aqueous compartment of Pickering emulsions (Fig. 9). This indicates that Pickering stabilization was most efficient for the modified and hydrophobic bacterial variants and a bigger proportion of the bacteria was attached to interfacial rather being free.

4. Conclusions

We have demonstrated for the first time that LAB under enzymatic and physical treatments may be used as micron-sized Pickering-type

stabilizers to make and stabilize O/W emulsions. The microstructural evaluation showed that the LAB remained rod-shaped before and after modifications, and the protoplasts that were produced through the enzymatic modification with lysozyme chicken egg disappeared after approximately 2 days. Moreover, TEM and Cryo-SEM images confirmed the loss of the cell wall of LAB after enzymatic treatment and the attraction between the oppositely-charged zein and bacterial cell surfaces after physical treatment, respectively. In terms of the stabilization of Pickering emulsions, LAB modified by lysozyme from egg displaying higher hydrophobicity according to MATH, were able to more effectively cover the dispersed oil droplets and lead to more stable emulsions against droplet coalescence than bacteria modified with proteinase K during storage time. In addition, the confocal microscopic images illustrated that the zein particles attached much more to the surface of LBD bacteria than to LGG bacteria, which was due to the greater negative charge on the surface of LBD bacteria. This stronger adsorption of

zein proteins on LBD helped them adhere more to the oil-water interface and prohibit oil droplet coalescence via Pickering stabilization. Finally, this novel formulation to produce edible oil-in-water (O/W) Pickering emulsion can provide many possible food applications, such as the replacement of solid fat or synthetic surfactants, besides the improvement of emulsions with a 'natural' definition.

The droplet size of the emulsions was fairly large as a consequence of the relatively large micro-sized Pickering bacteria. Future work could focus of exploiting smaller-sized, surface-active bacterial fragments as stabilizers after a suitable mechanical treatment of the bacteria. Likewise, future work could focus on possible changes of functionality of Pickering bacteria induced by heat treatment (Pasteurization) or formulation of the bacteria into low-pH foods.

CRedit authorship contribution statement

Elhamalsadat Shekarforoush: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft. **Xiaoyi Jiang:** Conceptualization, Methodology, Writing – review & editing. **Musemma Kedir Muhammed:** Methodology, Writing – review & editing. **Kathryn A. Whitehead:** Writing – review & editing. **Nils Arneborg:** Writing – review & editing. **Jens Risbo:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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