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Do oil-in-water (o/w) nano-emulsions have an effect on survival and growth of bacteria?

Hani El Kadri[1], Putu Virgina Partha Devanthi[1], Tim W Overton[1,2], Kostas Gkatzionis[1,2*]

1. School of Chemical Engineering, University of Birmingham, Birmingham, B152TT, United Kingdom

2. Institute of Microbiology & Infection, University of Birmingham, B152TT, Birmingham, United Kingdom

* Corresponding author.

E-mail address: k.gkatzionis@bham.ac.uk (K. Gkatzionis).

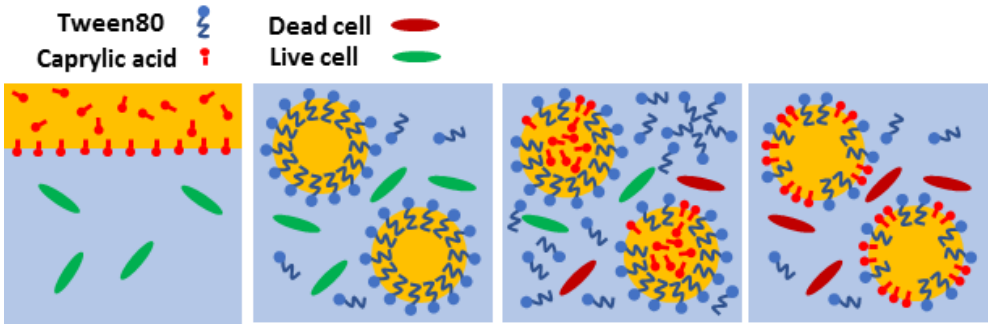
Abstract

Nano-emulsions (typically droplet diameter <1μm) are common in foods, and have been extensively reported to present antimicrobial activity, however, the mechanism is not well defined, and some studies reported no effect. A review of the literature was conducted and revealed strongly contradictory reports regarding the antimicrobial effect of nano-emulsions even in reference to similar microbial species and formulations. Following up, this study aimed to investigate the effect of nano-emulsions on four bacterial species (*Staphylococcus epidermidis, Bacillus cereus, Lactobacillus acidophilus* and five *Escherichia coli* strains) possessing different surface charge and hydrophobicity. Model oil-in-water (O/W) emulsions with different size of oil droplets were prepared with sunflower oil stabilised by polysorbate 80 (Tween80) emulsifier (hydrophilic), using high shear mixing followed by

ultrasonication. The viability of bacteria was monitored by culture, membrane integrity was assessed with flow cytometric analysis with propidium iodide (PI) staining and fluorescence microscopy monitored the spatial distribution of cells within the O/W emulsions. The stability of the nano-O/W emulsions in the presence of bacteria was assessed by monitoring the droplet size [D (4, 3)] and creaming height. In contrast to other reports the survival and growth of bacteria was not affected by the size of the oil droplets, no damage to the bacterial membrane was evident with flow cytometry and emulsion stability was not affected by the presence of bacteria during 7 days of storage. Furthermore, the antimicrobial activity of caprylic acid (CA) was compared between O/W coarse and nano-emulsions while varying the concentration of the hydrophilic surfactant Tween80. The activity of CA was similar in nano-emulsion and coarse emulsion; however, it was higher than in bulk oil and was reduced with increasing Tween80 concentration, suggesting that its efficacy is dictated by formulation rather than oil droplet size. The results demonstrated no enhanced antimicrobial activity due to nano-sized oil droplets and that conclusions on nano-emulsions should be taken with caution.

Keywords: Nano-emulsion; Antimicrobial Activity; Flow Cytometry; Bacterial Membrane Integrity; Caprylic Acid; Emulsion Stability

Graphical Abstract



1. Introduction

Nano-emulsions (typically with droplet diameter <1μm) gained popularity in food production due to improving food properties and formulations, for example, use of less fat and emulsifiers, increased emulsion stability and improved optical appearance, enhancement of taste and sensory perception of ingredients or masking of certain ingredients (Chaudhry and Castle, 2011). Nano-emulsion manufacturing requires more energy than emulsions with larger droplet sizes (Gupta *et al*., 2016) and they possess different physicochemical properties to coarse emulsions (McClements, 2010) due to their nano-sized droplets (Baglioni and Chelazzi, 2013) and increased interface. Nano-emulsions have shown antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria including *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus megaterium*, *Bacillus subtilis* and *Bacillus circulans* (Hamouda *et al*., 1999; Baker et al., 2000; Teixiera *et al*., 2007; Bharghava *et al*. 2015; Jo *et al*., 2015; Majeed *et al*., 2016; Lu *et al*., 2017). Furthermore, nano-emulsions were found to selectively disrupt the membrane of prokaryotic cells but not eukaryotic cells (Baker *et al*., 2000), which could expand their applications in managing safety and microbial growth in food through formulation. The antimicrobial effect of nano-emulsions has been attributed to their structure itself and the nano-sized droplets. When nano-emulsions are formed under high shearing forces (e.g. ultrasonication, high-pressure homogenisation or high-shear mixing) they acquire significant amount of energy as they are formed (Lee *et al*., 2010). The nano-droplets are thermodynamically driven to fuse with lipid-containing micro-organisms and the energy that was stored during formation of the nano-emulsion will be released to destabilise the membrane's lipid bilayer leading to

cell lysis and death (Hamouda et al., 1999; Hamouda and Baker, 2000; Myc *et al*., 2001; Hemmila *et al*., 2010).

However, after summarising and reviewing the literature discussing the antimicrobial activity of nano-emulsions (Table 1), there is evidence of controversy and no consistency of effect on the same species of bacteria. For example, two studies found no correlation between droplet size and antimicrobial activity (Buranasuksombat *et al*., 2011; Terjung *et al*., 2012). Buranasuksombat et al. (2011) found that nano-emulsions (<300 nm droplet size) made from soybean oil and the non-ionic surfactant Tween80 had no antimicrobial effects on *E. coli*, *S. typhimurium*, *L. monocytogenes*, *B. cereus* and *P. aeruginosa* after exposure for 30 minutes, unless the oil phase itself contained antimicrobial properties. Terjung *et al*. (2012) found that the antimicrobial properties of nano-emulsion (80 nm droplet size) made from Miglyol 812N and Tween80 were less effective in inhibiting growth of *E. coli* and *Listeria innocua* compared to coarse emulsion (3 µm). Therefore, more work is required to confirm with confidence antimicrobial activity of nano-emulsions, exclusive to structure and droplet size. In other cases (Table 1) the antimicrobial activity was investigated in nano-emulsions containing antimicrobial components which were either added in the formulation or were natural components of the oil; surprisingly, in many studies the controls in place were not appropriate for supporting the conclusions, and antimicrobial activity was attributed to nano-emulsion structure instead of the formulation and the antimicrobial component.

The aim of this study was to comprehensively assess the effect of a model O/W nano-emulsion on bacteria, specifically, microbial survival in minimal growth medium at ambient temperature (M9 medium at 25^{o}C), microbial growth in rich medium (30^{o}C), and cell membrane integrity by flow cytometric analysis. As the O/W

emulsion structure can be affected by the interaction of bacterial cell properties with the emulsion interface (Ly *et al*., 2006; Ly *et al*., 2008), the study included different Gram-negative and Gram-positive bacterial species and strains of varying surface charge, hydrophobicity and ability to form the protein adhesin curli. Finally, in order to investigate the effect of O/W emulsion structure, i.e. size of the oil droplets, in combination with antimicrobial components in formulation, caprylic acid (CA) was added in the oil phase. CA is an eight-carbon short-chain fatty acid found naturally in milk with well documented antimicrobial activity in bulk against various species (Nair *et al*., 2005; Annamali *et al*., 2000; Andrews *et al*., 2001), however, no study has yet assessed CA as part of an emulsion formulation. Since CA is minimally soluble in water and due to its fat solubility, it can be incorporated within the oil phase of O/W nano-emulsions, and highlights possible increases in antimicrobial activity due to increase in interface. Changes in the stability of O/W nano-emulsions in the presence of bacteria were monitored by measuring the droplet size and creaming height while fluorescence microscopy was employed to screen the localisation and distribution of bacteria within the emulsions.

2. Materials and Methods

2.1. Materials

The water-soluble emulsifier polysorbate 80 (Tween80), hexane 95% and caprylic acid (CA) ≥98% were purchased from Sigma-Aldrich (United Kingdom). Sunflower oil (food grade) was purchased from a local retailer (United Kingdom). Nucleic acid stains 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) and propidium iodide (PI) were purchased from Sigma-Aldrich (United Kingdom). Tryptic soy agar (Oxoid Ltd. CM0131), tryptic soy broth (Oxoid Ltd. CM0129), nutrient agar (Oxoid Ltd. CM0003), de Man, Rogosa and Sharpe (M.R.S) agar (OXOID CM0359) and broth (OXOID CM0361) were purchased from Fisher Scientific (United Kingdom).

2.2. Microbial cultures

Escherichia coli K-12 strains MG1655 (CGSC 6300), BW25113 (CGSC 7636), JM109 (NEB E4107), MC4100 (CGSC 6152) and its derivative PHL644 (MC4100 *malA-kan ompR234*) (Vidal *et al*. 1998) were maintained on tryptic soy agar at 4^{o}C. *Bacillus cereus* (NCTC 11143), and *Staphylococcus epidermidis* (NCIMB 10387) were maintained on nutrient agar at 4^{o}C. *Lactobacillus acidophilus (*ATCC 4356) was maintained on M.R.S agar at 4^{o}C. For obtaining cells in the exponential phase, cells were harvested by centrifugation (10,000 g, 10 minutes) and washed in *PBS* (phosphate buffered saline) solution twice. *E. coli*, *B. cereus*, and *S. epidermidis* cells were each transferred into 50 ml of tryptic soy broth, incubated at 37^{o}C for 24 hours shaking at 150 rpm and sub-cultured to 50 ml of tryptic soy broth for a further 2 hours (*E. coli*) or 4 hours (*B. cereus* and *S. epidermidis*). *L. acidophilus* cells were transferred into 50 ml of M.R.S broth, incubated at 37^{o}C for 42 hours and sub-cultured to 50 ml of M.R.S broth for a further 12 hours.

2.3. Bacterial-adhesion-to-hydrocarbon (BATH) test

The hydrophobicity of bacterial cell surfaces was evaluated as by bacterial-adhesion-to-hydrocarbon (BATH) according to the method proposed by Rosenberg *et al*. (1980). The optical density (*A*o) of bacterial cells (~10^9 CFU/ml) harvested in the exponential phase by centrifugation (10,000 g, 10 minutes) and washed twice in *PBS* and re-suspended in M9 medium was measured at 600nm. Four millilitres of the bacterial suspension were mixed with 1 ml hexane by vortexing for 2 minutes and then left to stand for 15 min to allow separation of layers, at which time the optical density at 600 nm (*A*t) was again measured by carefully removing a sample (1ml) from the aqueous phase. The percentage of bacterial adhesion to hexane was expressed by the difference of the absorbance of cell suspension before (*A*o) and after (*A*t) mixing with the solvent: (1−*A*t/*A*o) × 100. The percentage of bound cells was subsequently calculated by % adherence = (1-*At/A*o*)* x 100 where *A*o is the optical density measured at 600 nm of the bacterial suspension before mixing and *A*t is the absorbance after mixing. The mean percentage of partitioning of an organism into the hexane phase was calculated by using triplicate samples.

2.4. ζ-potential (zeta potential) measurements

For measuring the ζ-potential of bacteria, cells were harvested in exponential phase by centrifugation, washed twice in *PBS*, re-suspended and diluted in M9 medium to a density of 10^7 cells per ml. One millilitre of the samples was injected in a universal folded capillary cell (Model DTS 1070, Malvern Instruments Ltd, UK) equipped with platinum electrodes and a folded capillary, checking that all air bubbles were removed. The electrophoretic mobility (EM) at 150V of the suspended bacteria was then measured at 25^{o}C using Malvern ZetaSizer Nano ZS (Malvern Instruments Ltd,

UK), which uses the scattering of incident laser light to detect the bacteria at relative low magnification. The instrument was calibrated using the ζ-potential transfer standard (DTS1235) which has a ζ-potential of -42mV±4.2mV. The mobility of the bacteria under the applied voltage was converted to the ζ-potential using the Smoluchowski equation and reported as the average and standard deviation of measurements made on two freshly prepared samples, with three readings made per sample. For measuring the ζ-potential of single O/W emulsions, freshly made O/W emulsions were diluted 1:10 in M9 media and one millilitre of the diluted emulsions were then injected in a universal folded capillary cell and the ζ-potential was measured as previous.

2.5. Preparation of O/W emulsions

Coarse O/W emulsions were prepared using a high shear mixer homogeniser (Silverson L5M) at 25^{o}C. The continuous phase was prepared by dissolving Tween80 (8 wt%) in tryptic soy broth or M9 media at 60^{o}C for 15 minutes. Nano-emulsions were prepared by homogenising sunflower oil in the continuous phase at 5000 rpm for 60 seconds and the homogenised emulsions were sonicated with a probe sonicator (VCX 750 Sonics, USA) using a 22mm horn tip and operating at a frequency of 20 kHz and 750 watts for 4 minutes. Control coarse emulsions were prepared by homogenising sunflower oil in the continuous phase (ratio of 40:60 or 20:80) at 3000 rpm for 60 seconds. For the CA study, 0.5% CA was dissolved in the oil phase prior to homogenisation. For microbial viability studies, bacterial cells (~10^{8} CFU/ml) were washed twice and re-suspended in ten millilitres O/W emulsions (M9 media as continuous phase) or 6 ml M9 minimal growth medium (control) and incubated at 25^{o}C for 2 and 7 days on a rotator (Stuart SB3, UK) at 2 rpm to ensure

homogenised mixing. For growth studies, bacterial cells (~10^4 CFU/ml) were washed twice in PBS and re-suspended in ten millilitres of O/W emulsions (tryptic soy broth as continuous phase) or 6 ml of tryptic soy broth (control) and inoculated with and incubated at 30^{o}C over time on a rotator at 2 rpm. For the CA study, bacterial cells (~10^8 CFU/ml) were washed twice and re-suspended in ten millilitres O/W emulsions (M9 media as continuous phase) or 8 ml M9 minimal growth medium with 2 ml bulk oil with (0.5 or 1% CA) or without CA and incubated at 25^{o}C for 1, 8 and 24 hours on a rotator at 2 rpm.

2.6. Characterisation of emulsion stability during incubation

2.6.1. Measurement of oil globule size [D (4, 3)]

The particle size distribution of the oil globules was measured immediately after preparation and as a function of storage time using a laser diffraction particle size analyser (Malvern Mastersizer 2000, Malvern Instrument Ltd, Worcestershire, UK), equipped with a He-Ne laser (λ = 633nm). The dispersion unit stirring speed was kept at 2000 rpm and the measurement range was 0.02–2000µm. The optical parameters selected were: dispersed phase refractive index of n_D^{22} 1.39; oil globule absorbance of 0.01; and a dispersant liquid (distilled water) refractive index n_D^{22} 1.33; obscuration between 10% and 20%. Sample was added dropwise to the system until the obscuration was within an acceptable range. Particle size calculations were based on the Mie Scattering theory and the volume mean diameter values (D [4, 3]), and the percentage of volume corresponding to each observed population were calculated using the Mastersizer 2000 software.

2.6.2. Observation of phase separation

The cream height fraction of the micro emulsion was measured immediately after preparation and as a function of storage time. Five millilitres of O/W emulsion were transferred to a graduated 10ml centrifuge tube and left standing upright for 1 hour. The apparition of a cream layer was observed and the cream height fraction was visually measured at 1-hour from the time creaming started. The expression used for calculation of the creaming percentage height is as follows:

$$H_{Cream} = \frac{H_{Emulsion} - H_{Creamed\,phase}}{H_{Emulsion}} \text{ X } 100\%$$

2.7. Determination of bacterial cell viability and growth

Serial dilutions in *PBS* and plating on tryptic soy agar using the Miles & Misra technique (Miles *et al*., 1938) was conducted immediately after preparation and as a function of storage time to obtain bacterial cell counts as colony forming units per millilitre (CFU/ml).

2.8. Flow cytometric analysis of bacterial cells

Flow cytometric analysis was conducted immediately after preparation and as a function of storage time using a BD Accuri C6 flow cytometer (BD, Oxford, UK). From a 1 millilitre sample, the bacterial cells were harvested by centrifugation by centrifugation (10,000 g, 10 minutes) and washed twice and re-suspended in PBS. The bacterial cells were stained by adding PI (4µl/ml) and incubated in the dark for 30 minutes. Samples were excited using a 488nm solid state laser and particulate noise was eliminated using a Forward scatter height (FSC-H) threshold while 20,000 data points were collected at a maximum rate of 2500 events/s. Fluorescence was

detected using 670 LP filters corresponding to PI fluorescence. The data was analysed using CFlow (BD).

2.9. Fluorescent and optical imaging of bacteria in O/W emulsions

The O/W emulsions with bacteria were observed using optical and fluorescent microscopy (Zeiss Axioplan) at ambient temperature. The sample was stained by adding DAPI (4µl/ml) and incubated in the dark for 30 minutes. The stained sample was placed on a microscope slide and gently covered with a cover slip. The images were acquired under objective lens 100x magnification (oil immersion) with a digital camera system Axiocam ICm1 using a 1.4 megapixel monochrome CCD camera via AxioVision Software (Zeiss). The samples were observed at room temperature using a fluorescent microscope (Zeiss Axiolab) equipped with a mercury arc lamp and the emission was observed at 461nm (DAPI). Micrographs were overlaid using analysis software (ImageJ).

2.10. Statistical analysis

Each experiment was conducted at least in duplicate (N=2) and some cases in triplicate (N=3). The generated results were collected in Excel (Microsoft Corp.) for calculating means, standard deviations and error bars. For Student's *t*-test to compare two means or one-way analysis of variance (ANOVA) and the Tukey's HSD *post hoc* test to compare several means were used for checking whether there is significant difference among samples using IBM SPSS Statistics software version 21. Differences were considered significant at $P<0.05$.

3. Results and discussion

3.1. Effect of droplet size on the survival of bacteria

The O/W emulsion formulations were characterised in terms of oil droplet size [(D (4, 3)]. Two types of O/W emulsions with different D (4, 3) were achieved depending on the formulation: coarse emulsions (15-35μm) and nano-emulsions (170-650nm) (Fig. S1).

To understand the effect of droplet size on the survival of bacteria, the viability of bacteria in nano-emulsion was monitored and compared to coarse emulsion over time (Fig. 1). The M9 minimal growth medium used as continuous phase contains minimum nutrients that can sustain possible growth but lacks the presence of amino acids, therefore bacteria can grow in the exponential phase but slowly. As opposed to being in stationary phase (non-growing), bacteria in exponential phase of growth are more susceptible to stresses (Anderl *et al*., 2003; Matsuo *et al*., 2011) which allows for better detection any effects of nano-emulsion on bacterial survival. In this study, there was no significant difference observed in viability of different bacterial species in nano-emulsion compared to coarse emulsion and control M9 minimal growth medium, after 2 and 7 days. *E. coli* presented no difference in growth between nano- and coarse emulsions, and the effect was not strain dependent as no variation in responses was observed between different strains (Fig. 1 A-D). For *S. epidermidis* the reduction in counts observed was comparable between nano- and coarse emulsions. In the case of *L. acidophilus* the M9 minimal growth medium could not support its survival after day 2 and therefore this species was discontinued from this part of the study. Also, microscopic observation showed that all bacteria grew as planktonic cells and no clustering or colony formation was observed (Fig. 2). Since

colony formation in emulsion systems are associated with upregulation of stress genes (Prachaiyo and McLandsborough, 2003), it can be concluded that no such effects occurred in this study.

These results are in contrast to many studies reporting nano-emulsions possessing antimicrobial activity against bacteria. However, in some of these studies the mechanism behind the antimicrobial effects of nano-emulsions have not been clearly justified mainly due to lack in use of proper controls. For example, TEOP and BCTP are the most commonly reported nano-emulsion formulations to possess antimicrobial activity against several species of micro-organisms including bacteria such as *E. coli*, *S. aureus* and *L. monocytogenes* (Hamouda *et al*., 1999; Teixeira *et al*., 2007; Buranasuksombat *et al*., 2011). BCTP is made of soybean oil containing the antimicrobial compound cetylpyridinium chloride (CPC) and stabilised with tri-*n*-butyl phosphate and Triton X-100 (emulsifier), while TEOP is made of ethyl oleate and stabilised with Tween80 and *n*-pentanol (co-emulsifier). In 2010, Ferriera *et al*. investigated the two nano-emulsion formulations and found that for the TEOP formulation, the antimicrobial effects were due to *n*-pentanol which sits at the O/W interface as no differences in reduction of bacterial counts were observed when the bacteria were treated with the TEOP formulation compared to a solution of *n*-pentanol with the same concentration. Moreover, for the BCTP formulation it was found that the antimicrobial effect was due to CPC (water soluble, cationic surface-active agent) and its efficacy was shown to be reduced when it was incorporated into the nano-emulsion compared to as a solution with the same concentration (Ferriera *et al*. 2010). The authors argued that controls were not included in studies reporting antimicrobial activity for the BCTP and TEOP formulations and that they could have evaluated the contributions of the different components of the emulsions for the

observed antimicrobial activity. Hamouda and Baker (2000) investigated the antimicrobial activity of two nano-emulsion formulations: 8N8 and W60C against *E. coli*, *Salmonella typhimurium* and *Vibrio cholera*. 8N8 is a water-in-oil nano-emulsion made of soybean oil containing CPC and stabilised with tri-z-butyl phosphate and Triton X-100 while W60C is a liposome made of soybean oil stabilised with Tween60, glycerol monooleate and refined soya sterols. Furthermore, both nano-emulsion formulations showed antimicrobial effects against all the bacteria; however, no testing of antimicrobial effects of the individual components of the nano-emulsion formulation was carried out. In this case, it is not possible to attribute such effects to high surface tensions of nano-sized droplets. Thus, the process is probably not mechanical, but rather chemical. Chang *et al*. (2012) studied the antimicrobial effects of thyme oil nano-emulsion on *Zygosaccharomyces bailii*. They found that nano-emulsions made with corn and MCT oil did not exhibit any antimicrobial effects unless mixed with thyme oil indicating that the latter rather than the size of the droplets was the reason behind its antimicrobial activity. More recently, Ghost *et al*. (2014) found that sesame oil nano-emulsion possessed antimicrobial activity against *S. aureus* only when the antimicrobial compound eugenol was present in the oil phase and no such effects were occurring in the absence of eugenol. Therefore, it could be concluded that the antimicrobial activity of nano-emulsions reported in several cases in the literature can only be attributed to the antimicrobial agents that they carry and no such activity can result from high surface tensions and cell wall diffusion of nano-sized droplets.

3.2. Effect of droplet size on bacterial injury

In many cases, antimicrobial treatments can affect bacterial cells, and although they remain alive, result in stressed and injured subpopulations, which cannot be detected with analysis by culture. In this study, the membrane integrity of the bacteria was assessed using flow cytometry combined with PI staining (Table 2) which is non-permeant but can penetrate cells with a compromised membrane and binds to double stranded DNA by intercalating between base pairs (Zhang *et al*., 2001). According to the flow cytometry data (Table 2; Fig. S2), there was no significant increase in percentage of PI positive cells observed after incubation in nano-emulsion compared to coarse emulsion and controls in M9 medium. These results confirm that the membrane integrity of the bacteria was not affected by the nano-sized droplets and are in contrast to studies that reported extensive damage to the membrane of bacteria after exposure to nano-emulsions. Extensive disintegration of the cell membrane, disruption to cell wall and lysis of *S. mutans* after exposure to soybean oil nano-emulsion containing CPC was observed using SEM (Karthikeyan *et al.*, 2011). Ghosh *et al*. (2013) found that exposure to basil oil nano-emulsions against *E. coli* led to deformation in bacterial membrane phospholipids (confirmed by FT-IR analysis) and stained positive with ethidium bromide (EtBr) which only stains the DNA of cells with a membrane that lost its structural integrity. Exposure to eucalyptus oil nano-emulsion led to damage of cell membrane of *S. aureus* observed using SEM (Sugumar *et al*., 2014). The exposure of *S. aureus*, *B. subtilis*, *E. coli* and *S. cerevisiae* to nano-emulsion made with D-limonene containing the antimicrobial nisin caused extensive membrane damage observed using SEM associated with release of cellular contents evident by leakage of the cytoplasmic content measured using UV absorbance (Zhang *et al*., 2014). In another study, exposure to oregano oil nano-emulsion led to disruption of the

bacterial membrane in *L. monocytogenes*, *S. typhimurium* and *E. coli* 0157:H7 observed using SEM (Bhargava *et al*., 2015). However, in all these studies, the antimicrobial activity of the nano-emulsion was compared to *PBS*, sterile water, or broth as control rather than being compared to the individual components of the nano-emulsion. In a study by Karthikeyan *et al*. (2012) reported that there were higher antimicrobial effects against biofilm and planktonic forms of *S. mutans*, *L. casei,* after a 1-minute exposure to soybean oil nano-emulsion containing CPC compared to CPC solution only more damage to the cells membrane was evident by increased fluorescence intensity of PI using fluorescence microscopy. However, no effects of the nano-emulsion without the incorporation of CPC was compared. CPC is water soluble and the lower antimicrobial activity with CPC solution would be expected since the concentration of CPC in the continuous phase of the nano-emulsion would be higher (due to the presence of the dispersed oil phase) thus bacterial cells will be exposed to a higher concentration of CPC in nano-emulsion compared to CPC solution. Although the study showed that nano-emulsion can damage the membrane of bacteria (Karthikeyan *et al*., 2012), it lacks use of full controls and conclusions should be interpreted with caution.

3.3. Effect of emulsion droplet size on cell growth

In order to investigate if nano-emulsions affect bacteria during growth and proliferation, viability was compared between O/W nano-emulsion and coarse emulsion made with tryptic soy broth as continuous phase (Fig. 3). Once again, there was no significant difference in growth of bacteria between nano- and coarse emulsion. Growth patterns were similar regardless of species and strains and comparable to tryptic soy broth (control). Also, bacteria grew as planktonic cells and no clustering or colony formation was observed as response to stress (Fig. 4).

Furthermore, the *E. coli* strain PHL644 which is a potent biofilm former that overexpress the protein adhesin curli (surface attachment structures) (Vidal *et* al., 1998; Perni *et al*., 2013) maintained its planktonic form within the nano-emulsion. In support to our results, Naïtali *et al*. (2009) found that the growth kinetics of *L. monocytoegenes* was not affected by incubation in nano-emulsions. In contrast, it was observed that as opposed to growing in planktonic form, *L. monocytogenes* were constrained to grow as colonies in O/W emulsions with higher oil phase concentrations (>80% *vs* 30 or 70%) and smaller droplet size (2µm *vs* 15 or 25µm) (Brocklehurst *et al*., 1995). The authors argued that in such emulsions the oil droplets were sufficiently close-packed and viscous to prevent the mobility of the bacteria forcing growth in colonies, therefore it was a response to space and not a biological response of cells to interaction with nano-sized droplets. Also, the growth rates of bacteria were reduced due to restricted diffusion of nutrients and oxygen or accumulation of waste but this only occurred at lower pH (5 *vs* 7). However, in this study the conditions were not similar as the oil phase concentration was 40% and the pH value of the emulsions during inoculation were around ~7.3.

3.4. Activity of antimicrobial caprylic acid in O/W emulsion of different droplet size

In order to investigate the effect of oil droplet size in O/W emulsions in combination with antimicrobial components, caprylic acid (CA) was added in the oil phase. Since CA is minimally soluble in water and soluble in fat, its effect should be affected by the surface area of the oil phase. The antimicrobial activity of O/W emulsions containing CA was investigated in varying concentration of Tween80 (Fig. 5).

Both nano- and coarse emulsions became antimicrobial by adding CA, as evidenced by survival of <2log CFU/ml after 8 hours. The antimicrobial activity of 0.5% CA was enhanced in emulsions (CA), and resulted to comparable bacterial reductions with 1% CA in bulk after 8 and 24 hours (Fig. 5). These results are in agreement with reports on higher activity of antimicrobial oils in emulsions compared to bulk form, including eucalyptus oil nano-emulsion against *B. cereus, S. aureus* and *E. coli* (Sugumar *et al*., 2013) thyme oil nano-emulsion against *E. coli* O157:H7, *L. monocytogenes* and S. *enteritidis* (Wu *et al*., 2014; Xue *et al*., 2015), *Thymus daenensis* essential oil against *E. coli* (Moghimi *et al*., 2016a), sage oil (*Saliva officinalis*) (Moghimi *et al*., 2016b) anise oil against *L. monocytogenes and E. coli* O157:H7 (Topuz *et al*., 2016), peppermint oil (PO) against *S. aureus* and *L. monocytogenes* (Liang *et al*., 2012). The EO's possess antimicrobial properties and the increasing surface area of the oil interface in these studies, enhances the activity on bacterial membrane compared to bulk form, without however any EO-emulsion synergistic effect being observed, and therefore nano-size globules in emulsion do not directly contribute to the activity. The CA molecule is oriented so that the carboxyl group protrudes into the aqueous phase, while the hydrocarbon tail is in the oil phase (Andersson *et al*., 2014). Therefore, it would be expected that the higher surface area in emulsion increases the amount of CA in contact with the bacterial membrane compared to bulk form. *S. epidermidis* was more susceptible than *E. coli* in bulk oil and 0.5% CA, and its viability significantly ($P<0.05$) decreased (~4-log CFU/ml and <2-log CFU/ml at 8 and 24h respectively). The increased susceptibility of *S. epidermidis* compared to *E. coli* in bulk oil containing 0.5% CA could be due to the lack of the outer membrane in Gram-positive bacteria which provides extra protection to the peptidoglycan cell wall in Gram-negative bacteria. These results

corroborate with studies reporting that Gram-positive bacteria are more sensitive to the antimicrobial effects of CA (Nair *et al*., 2005) and other fatty acids (Monk *et al*., 1996) than Gram-negative bacteria.

The effect of Tween80 concentration on the antimicrobial activity of O/W emulsions containing CA was investigated (Fig. 5). Interestingly, the CA antimicrobial activity was evident for emulsions composed with 1% Tween80 but was not in samples with 8% Tween80. To ensure that these results were not due to differences in pH, the pH was measured after 24 hours and all the samples had ~pH 6-6.5 (results not shown). However, no differences between nano- and coarse emulsion were observed. The responses were comparable for *E. coli* (MG1655) and *S. epidermidis*, showing, overall, to be driven by formulation and not size of oil droplets. The concentration of Tween80 can affect the efficacy of antimicrobials within nano-emulsions (Donsi *et al*., 2011; Terjung *et al*., 2012). When the concentration of hydrophilic surfactants increases in the continuous phase they form multilayer arrangement of interdigitated surfactant chains that “wrap” the oil droplets (Tadros, 2013; El Kadri *et al*., 2015). Since Tween80 is a non-ionic surfactant that stabilises the emulsion by steric repulsion, its increase in concentration could prevent contact of CA with the bacterial membrane at the O/W interface.

Overall, the antimicrobial activity of CA was similar in nano- and coarse emulsions (Fig. 5). These results are in agreement with previous work on lemon myrtle in soybean oil nano-emulsion against *E. coli*, *L. monocytogenes, Salmonella typhimurium*, *P. aeruginosa* and *B. cereus* (Buranasuksombat *et al*., 2011) and cinnamaldahehyde against *E. coli* (Bilbao-Sainz *et al*., 2013), suggesting no synergistic effect. Therefore, the antimicrobial effect of nano-emulsions could be

considered a derivative of antimicrobials and their delivery through nano-sized droplets. However, a recent study reported the antimicrobial effect of anise oil (AO) nano-emulsion on *E. coli* and *L. monocytogenes* to be higher than AO coarse emulsion (Topuz *et al*., 2016) due to higher surface area. Donsi *et al*. (2011) found that O/W emulsions with smaller droplets have less antimicrobial effects compared to O/W emulsions with larger droplets due to mechanical stresses caused by the high-pressure homogenisation (HPH) process when forming nano-emulsions resulting in degradation of the antimicrobial agents such as phytophenols. In this study, it may be possible that the surface area provided by the coarse emulsion was enough to allow all the CA molecules to orient at the O/W interface comparably to nano-emulsion. Furthermore, the formation of nano-emulsion by ultrasonication generates heat which may affect the antimicrobial activity of CA. Pestana *et al*. (2015) showed that the amount of CA in milk samples was diminished after pasteurisation and ultra-high temperature (UHT) sterilisation.

3.5. Stability of nano-emulsions in the presence of bacteria

All O/W emulsions remained stable during the incubation period. The oil droplet size [(D (4, 3)] (Fig. 6; Table S1) and changes in creaming stability (data not shown) with or without bacteria in the continuous phase was monitored over time with no significant differences observed. Furthermore, there was no flocculation and aggregation of the oil droplets observed with any of the bacterial strains regardless of their surface characteristics. The most hydrophobic strains including *E. coli* (JM109), *S. epidermidis* and *B. cereus* (Table S2 and S3) resided within the continuous phase and did not aggregate around the oil droplets (Fig. 2 and 4). Similarly, the stability of O/W emulsions with CA was not affected by the presence of

bacteria (Fig. 6 and Fig. S1). Ly *et al*. (2006) found that the stability of O/W emulsions with bacteria was strain dependent and the negatively charged *Lactococcus lactis* (LLD16) provoked creaming, flocculation and aggregation by surrounding the positively charged oil globules whereas the positively charged *L. lactis* (LLD18) caused no such effects. In another study, it was shown that as opposed to the less negatively charged *E. coli* strain E21, the more negatively charged *E. coli* JM109 promoted faster creaming rates, coalescence and flocculation of O/W emulsions containing positively charged oil globules (Li *et al*., 2001). In this study, the oil globules in the nano-emulsion were stabilised by a non-ionic surfactant (Tween80), hence, the absolute magnitude of the droplet charge is very low (McClements, 2011; Tang *et al*., 2012). Since the bacterial membranes were found to be negatively charged (Table S2) and the oil droplets in all the O/W emulsions were less negatively charged (Table S3), thus they repel each other and bacterial cells will remain in the aqueous continuous phase. Therefore, the findings in this work on the antimicrobial activity over time, could not have been affected by changes in O/W emulsion stability.

4. Conclusion

The literature review identified controversy regarding the consistency and mechanism of antimicrobial activity reported for nano-emulsions. In this study reducing the size of oil droplets in O/W emulsions to the nano-scale had no direct effect on the viability and growth of bacteria when no antimicrobial agents were added and flow cytometry showed that the membrane integrity was intact. Controversy seems to come from studies suggesting that nano-emulsions possess

antimicrobial properties due to high surface tensions and cell wall diffusion of the nano-sized droplets, however, many of these studies were found to lack appropriate controls to test the action of individual components of the nano-emulsion or the action of the nano-emulsion without active ingredients. Therefore, some of the findings that attribute direct antimicrobial activity to nano-emulsions should be taken with caution, and further work is needed before concluding. In contrast, there is strong evidence that O/W nano-emulsions present higher antimicrobial activity due to higher interface; however, the case study based on CA did not show increased antimicrobial activity in nano- compared to coarse emulsion. Therefore, it is indicated that these responses should not always be expected and the antimicrobial effect of nano-emulsions depends on the antimicrobial agent and is affected by the formulation. Nano-emulsions remain an extremely promising asset in food formulation applications and they are known to promote stability, improve sensory perception, and enhance food functionality. In contrast, their manufacturing requires more energy. Therefore, their antimicrobial capability must be fully realised for assessing the benefits of application.

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Table 1. The antimicrobial activity of nano-emulsions correlated with their mean droplet size, ingredients (oil phase, stabilisers, antimicrobials) and the micro-organism it is tested against. The impact of the nano-emulsion is classified as positive (+) when the nano-sized droplets improves the antimicrobial activity with respect to the control, as negative (−) when it decreases the antimicrobial activity, and as neutral (+/−) when no significant change is observed or a significant change was observed only due to incorporation of antimicrobials and not because of nano-size droplets. The method of emulsification is mentioned as: HPH – High Pressure Homogenisation, HSH – High Shear Homogenisation, US – Ultrasonication, MFZ – Microfluidizer, CPI – Catastrophic Phase Inversion.

Emulsion type	Oil phase	Stabilisers	Continuous phase	Antimicrobials	Mean droplet size (nm)	Method of emulsification	Impact of nano-emulsion	Micro-organism	Controls	Author
O/W nano-emulsion	Thyme oil and corn oil (from 0 to 100% (w/w) corn oil 5% (w/w)	Tween80 and lauric arginate (LAE) or sodium dodecyl sulfate (SDS)	Buffer solution (10mM acetate, pH 4)	None	163nm	HPH	+	*Zygosaccharomyces bailli* *Saccharomyces cerevisiae* *Brettanomyces bruxellensis* *Brettanomyces naardenensis*	Buffer solution (10mM acetate, pH 4)	Ziaini *et al*. (2011)
O/W nano-emulsion	Soybean oil (%N.A.)	Ethylenediaminetetraacetic acid, glycerol, Tween20, and benzalkonium chloride	Saline solution 0.9% (w/w)	None	350nm	HSH	+	*P. aeruginosa*	Saline solution 0.9% (w/w)	Hemmila et al. (2010)
O/W nano-emulsion	Eucalyptus oil 16.66% (v/v)	Tween80	Water	None	17.1nm	US	+	*B. cereus* *S. aureus* *E. coli*	Bulk eucalyptus oil or water containing Tween80 16.66% (v/v)	Suguma r et al. (2013)
O/W nano-emulsion	Eucalyptus oil 16.66% (v/v)	Triton X-100	Water	None	3.8nm	US	+	*S. aureus*	Untreated sample	Suguma r et al. (2014)
O/W micro-emulsion	*Laurus nobilis* essential oil 15% (w/v)	Tween20 and ethanol	Water	None	10nm	N.A.	+	*Alternaria alternata*	Water	Xu et al. (2017)
O/W nano-emulsion	Soybean oil 25% (v/v)	Triton X-100	Water	CPC 1% (v/v)	100-800nm	MFZ	+	*Acinetobacter baumannii*	Ethanol 30% (v/v) or untreated	Hwang et al. (2013)

									sample	
O/W nano-emulsion	Anise oil 75% (w/w)	Alcolec PC75 (soy lecithin)	Water	None	117.2–275.7nm	HPH	+	*L. monocytogenes* *E. coli* O157:H7	Anise bulk oil or coarse emulsion	Topuz *et al*. (2016)
O/W nano-emulsion	Miglyol 812N 10% (w/w)	Tween80	Water	Carvacrol and eugenol (5, 15, 30 and 50 (w/w %))	80nm	HPH	-	*E. coli* C 600 *Listeria Innocua*	Coarse emulsion	Terjung *et al*. (2012)
O/W nano-emulsion	Pure peppermint oil, medium chain triglyceride (MCT), and their mixture at ratios of 1:5, 1:1, and 5:1 (v/v)	Modified starch	Water	None	184-228nm	HPH	-	*L. monocytogenes* *S. aureus*	Bulk peppermint oil, MCT nano-emulsion or untreated sample	Liang *et al*. (2012)
O/W nano-emulsion	Thyme oil and corn oil or MCT medium chain triglyceride (MCT) (from 0 to 100% (w/w)) (10% (w/w))	Tween 80	Aqueous buffer solution (5mM citrate buffer, pH 3.5)	None	160-196nm	HPH	+	*Zygosaccharomyces bailii*	Nano-emulsion with corn or MCT but no thyme oil	Chang *et al*. (2012)
O/W nano-emulsion	Citral oil 10% (w/w) Surfactants:	Span 85, Brij 97 and ethylene glycol	Water	None	28nm	US	+	*S. aureus* *E. coli* *Pseudomonas aeruginosa* *Enterococcus faecalis* *S. typhimurium* *L. monocytogenes*	Sulphadiazine	Lu *et al*. (2017)
O/W nano-emulsion	D-Limonene 5% (w/w) or a mixture of terpenes 5% (w/w)	Soy lecithin Solec Ip, Tween 20 and glycerol monooleate and CLEARGUM CO 01	Water	D-limonene and a mixture of terpenes extracted from *Melaleuca alternifolia* (0.1-10% (w/w)	74.4-156.6nm	HPH	+	*E. coli* *L. delbrueckii* *S. cerevisiae*	Sunflower oil with D-limonene (50:50) 10% (w/w) or Palm oil with a mixture of terpenes (50:50) 10% (w/w)	Donsì *et al*. (2011)

O/W nano-emulsion	Sunflower oil 8% (w/w)	Lecithin, pea proteins, sugar ester, and a combination of Tween20 and glycerol monooleate	Water	Carvacrol, D-limonene and cinnamaldehyde 2% (w/w)	170-240nm	HPH	+	*E. coli* *L. delbrueckii* *S. cerevisiae*	Water	Donsì *et al*. (2012)
O/W nano-emulsion	Soybean oil 25% v/v)	Triton X-100 10% (v/v)	Water	CPC 1% (w/v)	308nm Microfluidizer (M-110L, Microfluidics,Newton,MA) at 20,000 psi	MFZ	+	*S. mutans* (planktonic and biofilm)	Chlorhexidine digluconate 0.12% (v/v) or untreated sample	Karthikeyan *et al.* (2011)
O/W nano-emulsion	Soybean oil 25% (v/v)	Triton X-100 10% (v/v)	Water	CPC 1% (w/v)	308nm Microfluidizer (M-110L, Microfluidics,Newton,MA) at 20,000 psi for	MFZ	+	*S. mutans* (planktonic and biofilm) *L. casei* (planktonic and biofilm) *Actinomyces viscosus* *Candida albicans*	Chlorhexidine digluconate 0.12% (v/v) or untreated sample	Karthikeyan et al. (2012)
O/W nano-emulsion	**BCTP** Ethyl oleate 3% (v/v) **TEOP** Soybean oil 16% (v/v)	**BCTP** *n*-pentanol and Tween80 **TEOP** Tri-*n*-butyl phosphate 2% (v/v), and triton X-100 2% (v/v)	Water	None	Not mentioned	HSH	+	*S. typhimurium* *E. coli 0157:H7* *Pseudomonas aeruginosa* *S. aureus* *L. monocytogenes*	Untreated sample	Teixiera *et a*l. (2007)
W/O nano-emulsion	**X8W60PC** Oil 64% (w/w)	Three non-ionic detergents and solvent	Water	None	400–800nm	HSH	+	*Candida parapsilosis* *Fusarium oxysporum* *Candida albicans* *Candida tropicalis*	Untreated sample or bleach 6%	Myc et al. (2001)

								Microsporum gypseum, *Trichophyton mentagrophytes Trichophyton rubrum, and* *Aspergillus fumigatus*		
O/W nano-emulsion	Lemongrass oil 1% (v/v)	Tween80	Sodium alginate 1% (w/v)	None	4-35nm	MFZ	+	*E. coli*	Water	Salvia-Trujillo et al. (2015)
O/W nano-emulsion	*Thymus daenensis* oil 2% (w/w)	Tween80 and lecithin	Water	None	143nm	US	+	*E. coli*	Bulk *Thymus daenesis* oil or untreated sample	Moghimi et al. 2016a
O/W nano-emulsion	Sage oil (*Saliva officinalis*) 20% (w/w)	Tween80 and Span80	Water	None	222nm	US	+	*E. coli* *S. dysentery* *S. typhi*	Bulk sage oil or untreated sample	Moghimi et al. 2016b
O/W nano-emulsion	Soybean oil 25% (v/v)	Triton X-100 10% (v/v)	Water	CPC 1% (w/v)	168 nm	N.A.	+	*S. mutans* *L. casei*	Chlorhexidine gluconate 0.12% (v/v) or untreated sample	Lee et al. (2010)
W/O nano-emulsion	**BCTP** Soybean oil 80% (w/w) **BCTP 401** Soybean and peppermint oil 80% (w/w)	**BCTP** Tri-*n*-butyl phosphate and Triton X-100 **BCTP 401** Tri-n- butyl phosphate, Triton X-100, glycerol monosterate, refined soya sterols, Tween60	Water	CPC	400-800nm	N.A.	+	*B. cereus* spores *B. circulars* spores *B. megaterium* spores *B. subtilis* spores	Different dilutions of BCTP and BCTP 401 (1:10, 1:100 and 1:1000)	Hamouda et al. (1999)
W/O nano-emulsion	**8N8**	**8N8**	Water	**8N8**	400-800nm	N.A.	+	*E. coli*	Tris-EDTA	Hamoud

	Soybean oil 64% (w/w) **W60C** Soybean oil 20% (w/w)	Tri-*z*-butyl phosphate and Triton X-100 **W60C** Tween60, glycerol monooleate and refined soya sterols		None **W60C** CPC 1% (w/w)				*Vibrio cholerae* *S. typhimurium*	buffer solution	a & Baker (2000)
O/W nano-emulsion	Basil oil 6% (v/v)	Tween80	Water	None	29.3nm	US	+	*E. coli*	*PBS*	Ghosh et al. (2013)
O/W nano-emulsion	Mustard oil 6% (v/v)	Tween20	Water	None	18-430nm	Magnetic stirrer	+	*E. coli*	Untreated sample	Ghosh et al. (2012)
O/W nano-emulsion	Sesame oil 6% (v/v)	Tween20 or Tween80	Water	Euganol 1-6% (v/v)	20nm	US	+/-	*S. aureus*	*PBS* or sodium benzoate 0.3% (v/v)	Ghosh et al. (2014)
O/W nano-emulsion	**BCTP** Soybean oil 16% (v/v) **BCTP-CPC** Soybean oil 16% (v/v) **TEOP** Ethyl oleate 3% (v/v)	**BCTP** Tri-*n*-butyl phosphate, and Triton X-100 **BCTP-CPC** Tri-*n*-butyl phosphate, and Triton X-100 **TEOP** *n*-pentanol and Tween80	Water	**BCTP** Water **BCTP-CPC** CPC 0.25% (w/v) **TEOP** None	N.A.	US	+/-	*S. aureus* *E. coli* *L. monocytogenes*	Water, CPC solution 0.25% (w/v), tributyl phosphate solution, bulk soybean oil, Triton X-100, Tween80 or *n*-pentanol solution	Ferriera et al. (2010)
O/W nano-emulsion	**LMO** Lemon myrtle 5% (w/w) **SBO** Soybean oil 16% (w/w) **BCTP** Soybean oil 16% (w/w)	**LMO** Tween 80 **SBO** Tween80 **BCTP** Triton X-100, tributyl-*n*-phosphate	Water	None	97±2nm	MFZ	+/-	*E. coli* *L. monocytogenes* *S. Typhimurium* *P. aeruginosa* *B. cereus*	Coarse emulsion	Buranasuksombat et al. (2011)

O/W emulsion	Oregano oil 0.05 or 0.1% (w/w)	Tween80	Water	None	148nm	US	+	*L. monocytogenes* *S. Typhimurium* *E. coli* O157:H7	Water	Bhargha va et al. (2015)
O/W emulsion	Clove or canola oil 10% (v/v) and a mixture at ratios of 1:9, 3:7 and 5:5 10% (v/v)	Tween80 and modified starch	Water	None	151.3-203.9nm	HPH	+/-	*L. monocytogenes* *S. aureus* *E. coli*	Nano-emulsion with canola oil with no clove oil	Majeed et al. (2016)
O/W micro-emulsion	Micelles of Tween20 0.6% (w/w)	Tween20	Water	Trans-cinnamaldehyde 0.2% (w/w)	127nm	HPH	+	*S. Typhimurium* *S. aureus* *E. coli* O157:H7	Water or Watermelon juice	Jo et al. (2015)
O/W nano-emulsion	Grindsted Acetem 90-50K 10-15% (w/w)	Tween60	Water	Cinnamaldehyde 3-10% (w/w)	79±2nm	HPH	-	*L. monocytogenes* *E. coli* O157:H7	Nano-emulsion without cinnamaldehyde	Bilbao-Sainz et al. (2013)
O/W nano-emulsion	D-limonene 4% (w/w)	Propylene glycol and Tween80	Water	Nisin 0%, 0.5, 1.5 or 3.0% (w/w)	16.34nm-18.92nm	CPI	+	*S. aureus* *B. subtilis* *E. coli* *S. cerevisiae*	Nutrient (bacteria), YPD broth (yeast) or of kanamycin sulphate (50 lg/ml) in broth	Zhang et al. (2014)
O/W nano-emulsion	Thyme 1% (w/v)	Sodium caseinate and lecithin	Water	None	82.5-125.5nm	HSH	+	*E. coli O157:H7* *S. enterica serovar* Enteritidis *L. monocytogenes* Scott A	Bulk thyme oil or a mixture of water and milk	Xue et al. (2015)
O/W nano-emulsion	Hexane 10% (v/v)	Whey protein isolate	Water	Euganol 2% (v/v)	127-255 nm	HSH	-	*E. coli* O157:H7 *L. monocytogenes* Scott A	Untreated sample or eugenol (4.5 g/L) 2% reduced fat milk	Shah et al. (2013)
O/W nano-emulsion	Thyme oil 1% (w/w)	Propylene glycol and 1% sodium dodecyl sulfate	Water	None	279nm	HSH	+	*L. monocytogenes* Scott A *S. Enteritidis* *E. coli* O157:H7	Bulk thyme oil	Wu et al. (2014)

O/W nano-emulsion	Euganol 5-12.5% (w/w)	Tween20	Water	None	50–110nm	US	+	*Fusarium oxysporum f. sp. vasinfectum*	Untreated sample	Abd-Elsalam et al. (2015)
O/W micro-emulsion	Micelles 1, 2, 3, 5, 7.5, and 10.0% (w/v)	Surfynol 485W or SDS or Tween20 or CG20 contains LAE 10% (w/v)	Water	Eugenol or carvacrol 0.01% to 8.0% (w/v)	Not mentioned	Magnetic stirrer	+	*E. coli* O157:H7 *S. enterica* serotype	Water	Ruengvisesh et al. (2015)
O/W nano-emulsion	Sunflower oil 2-3% (w/w)	Tween20 and glycerol monooleate	Water	Carvacrol 2% (w/w), bergamot 3% (w/w), mandarin 3% (w/w) and lemon essential oils 3% (w/w)	133.4-176.4nm	HPH	+	*E. coli* O157:H7 *S. Typhimurium*	Untreated sample	Severino et al. (2015)
O/W nano-emulsion	Sunflower oil 2% (w/w)	Tween 20 and glycerol monooleate	Water	Mandarin essential oil 2% (w/w)	176.4 ± 14.5 nm	HPH	+	*L. innocua*	Untreated sample	Severino et al. (2014a)
O/W nano-emulsion	Sunflower oil 2-3% (w/w)	Tween 20 and glycerol monooleate	Water	Carvacrol 1% (w/w), bergamot 2% (w/w), mandarin 2% (w/w) and lemon essential oils 2% (w/w)	133.4-176.4nm	HPH	+	*L. monocytogenes* (5 strains)	Untreated sample	Severino et al. (2014b)
O/W nano-emulsion	Lemon, mandarin, oregano or clove essential oils 5% (w/w)	Glycerol monooleate or soy lecithin, whey protein isolate, pea proteins, Tween 20	Water	None	88-394nm	HPH	+	Endogenous flora of Rucola leaves	Untreated sample	Sessa et al. (2015)
O/W nano-emulsion	Lemongrass oil 0.5-4% (w/w)	Tween80 0.1, 0.5, 0.75 and 1% (w/w)	Water	None	56.5-87.6nm	HPH	+	*S. typhimurium* *E. coli* O157:H7	Untreated sample	Kim et al. (2013)
O/W nano-emulsion	Thyme and corn oil 10% (w/w)	Tween 80 or Tween and LAE	Water	None	<200nm	HPH	+	*Z. bailii*	Thyme or corn oil nano-emulsion with no LAE	Chang et al. (2015)

Table 2. Percentage of PI positive (dead) bacterial cells measured by flow cytometry at 0, 2 and 7-day incubation at 25°C. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 minimal growth medium) in the presence or absence of bacteria. Results are taken from a minimum of 2 independent experiments.

	Sample	Day 0	Day 1	Day 7
E. coli (MG1655)	M9 medium	1 ± 0^{a}	0.3 ± 0^{abc}	0.15 ± 0.07^{c}
	Coarse emulsion	1.15 ± 0.35^{ab}	0.65 ± 0.21^{ac}	0.2 ± 0^{c}
	Nano-emulsion	0.75 ± 0.21^{abc}	0.65 ± 0.21^{ac}	0.25 ± 0.07^{c}
E. coli (BW2115)	M9 medium	1.15 ± 0.07^{d}	1.2 ± 0^{d}	3.95 ± 0.21^{a}
	Coarse emulsion	0.8 ± 0.14^{d}	0.55 ± 0.07^{d}	5.45 ± 0.63^{b}
	Nano-emulsion	0.6 ± 0^{d}	0.6 ± 0.14^{d}	2.3 ± 0.14^{c}
E. coli (JM109)	M9 medium	2.2 ± 0^{a}	0.7 ± 0.28^{c}	0.4 ± 0^{c}
	Coarse emulsion	1.4 ± 0.14^{b}	1 ± 0.14^{bc}	0.55 ± 0.07^{c}
	Nano-emulsion	1.3 ± 0.14^{b}	0.45 ± 0.07^{c}	0.4 ± 0.14^{c}
E. coli (MC4100)	M9 medium	3.95 ± 0.35^{ab}	5.2 ± 1.27^{ab}	8.4 ± 0.28^{a}
	Coarse emulsion	3.55 ± 0.21^{ab}	3.2 ± 0.85^{ab}	7.95 ± 0.35^{a}
	Nano-emulsion	2.75 ± 0.49^{ab}	2.6 ± 0^{b}	6.55 ± 3.6^{ab}
E. coli (PHL644)	M9 medium	0.3 ± 0.14^{acde}	0.4 ± 0.14^{ade}	0.3 ± 0.14^{acde}
	Coarse emulsion	0.3 ± 0.14^{acde}	0.15 ± 0.07^{e}	0.3 ± 0.14^{acde}
	Nano-emulsion	0.15 ± 0.07^{de}	0.15 ± 0.07^{e}	0.15 ± 0.07^{de}
B. cereus	M9 medium	2.35 ± 0.07^{a}	8.1 ± 1.27^{b}	18.7 ± 6.93^{c}
	Coarse emulsion	2.7 ± 0^{a}	8.35 ± 1.06^{b}	22.1 ± 2^{cd}
	Nano-emulsion	2.95 ± 0.35^{a}	6.9 ± 0.42^{b}	30.75 ± 0.63^{d}
S. epidermidis	M9 medium	0.1 ± 0^{a}	0.1 ± 0^{a}	0 ± 0^{c}
	Coarse emulsion	0.05 ± 0.07^{b}	0.1 ± 0^{a}	0 ± 0^{c}
	Nano-emulsion	0.1 ± 0^{a}	0.1 ± 0^{a}	0 ± 0^{c}

The data was analysed with one-way ANOVA

[a] means ± standard deviation with different letters are significantly different

Figure captions

Figure 1. Changes in log CFU/ml of *E. coli (*MG1655) (A), *E. coli* (BW2115) (B), *E. coli* (JM109) (C), *E. coli* (MC4100) (D), *E. coli* (PHL644) (E), *B. cereus* (F) and *S. epidermidis* (G) within M9 minimal growth medium (control), nano-emulsion or coarse emulsion at day 0, 2, and 7 incubated at 25^{o}C. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 minimal growth medium). Bars represent mean ± SEM taken from a minimum of 2 independent experiments. The data was analysed with one-way ANOVA.

Figure 2. Photomicrographs composed from the optical and fluorescence images of *E. coli* (MG1655) (A), *S. epidermidis* (B) and *B. cereus* (C) within coarse emulsion and nano-emulsion at the end of the incubation period (7 days). The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase. Scale bar: 10µm.

Figure 3. Changes in log CFU/ml of *L. acidophilus* (A), *E. coli* (MG1655) (B), *E. coli* (BW2115) (C), *E. coli* (JM109) (D), *E. coli* (MC4100) (E), *E. coli* (PHL644) (F), *B. cereus* (G) and *S. epidermidis* (H) within broth (control), nano-emulsion or coarse emulsion over 24 or 48 hours relative to hour 0 incubation at 30^{o}C. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (tryptic soy broth). Bars represent mean ± SEM taken from a minimum of 2 independent experiments. The data was analysed with one-way ANOVA.

Figure 4. Photomicrographs composed from the optical and fluorescence images of *E. coli* (MG1655) (A), *S. epidermidis* (B), *B. cereus* (C) and *L. acidophilus* (D) within coarse emulsion and nano-emulsion the end of the incubation period. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase. Scale bar: 10µm.

Figure 5. Log CFU/ml of *E. coli* (MG1655) (A) and *S. epidermidis* (B) in bulk oil (control), nano-emulsion (NE), or coarse emulsion after 1, 8 and 24 hours incubated at 25^{o}C. The O/W emulsions were prepared with 20% oil phase containing no or 0.5% CA and stabilised with 1 or 8% Tween80 in M9 minimal growth medium (continuous phase). The bulk oil was prepared from 20% oil phase containing 0, 0.5 and 1% CA and M9 minimal growth medium. Bars represent mean ± SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data was analysed with one-way ANOVA. Abbreviations: NE, nano-emulsion; CA, caprylic acid.

Figure 6. The mean diameter size (µm) of the oil droplets by light scattering [D (4, 3)] of *E. coli* (MG1655) (A) and *S. epidermidis* (B) nano-emulsion (NE) or coarse emulsion at 0 and 24 hours incubated at 25^{o}C. The O/W emulsions were prepared with 20% oil phase containing 0.5% CA and stabilised with 1% or 8% Tween80 in M9 minimal growth medium (continuous phase) with or without bacteria. Abbreviations: NE, nano-emulsion; CA, caprylic acid.

Figure 1

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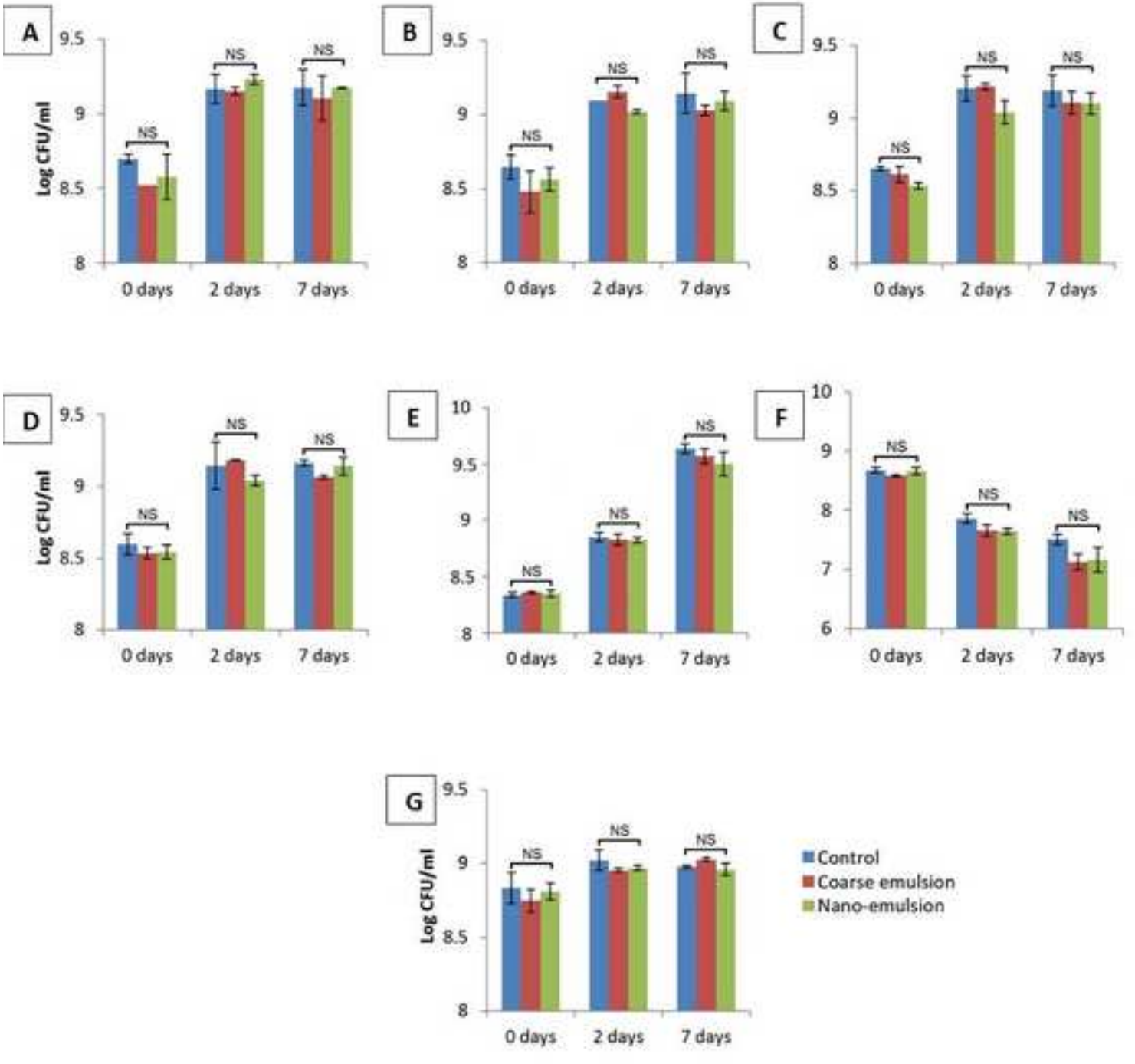

Figure 2

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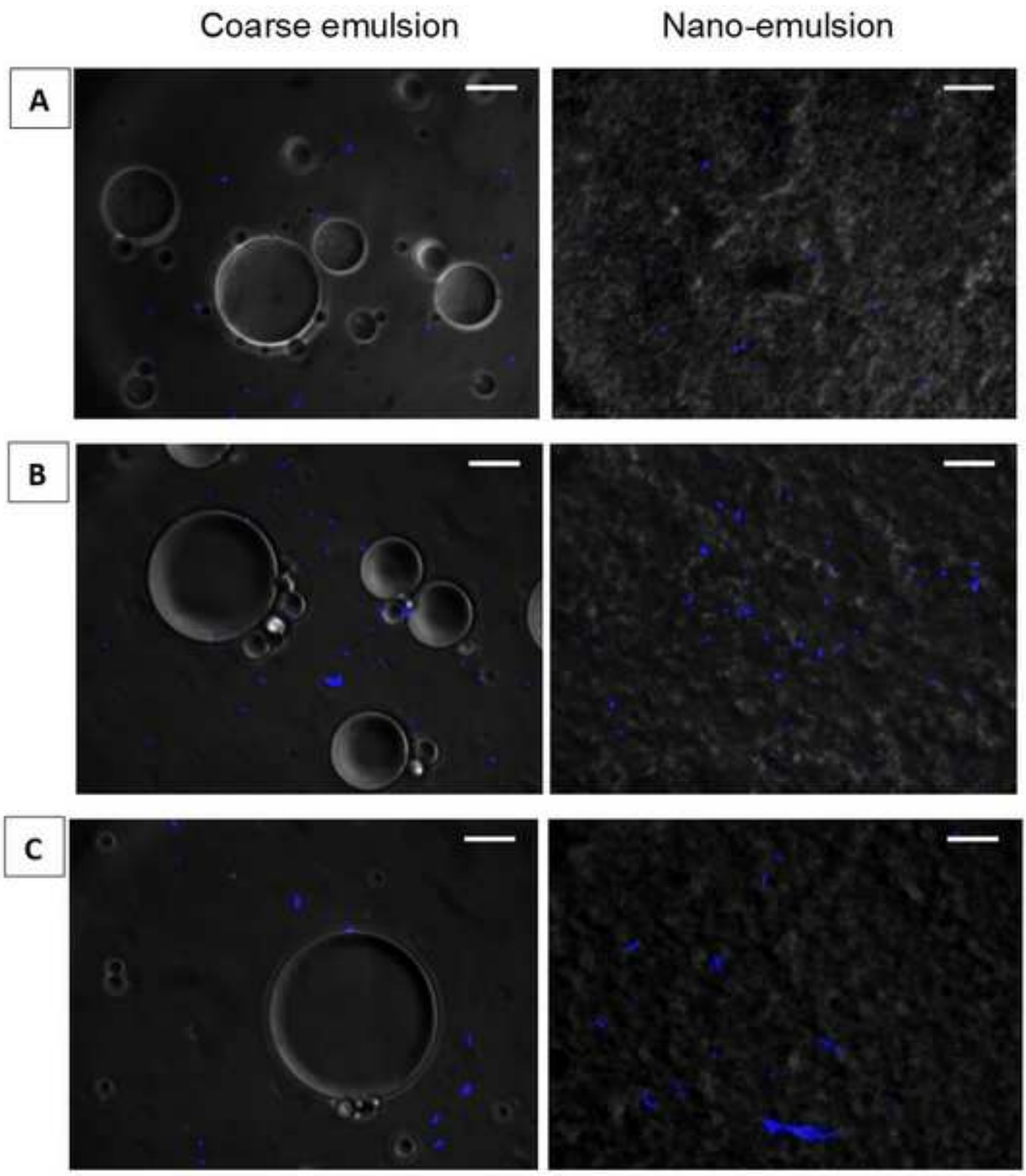

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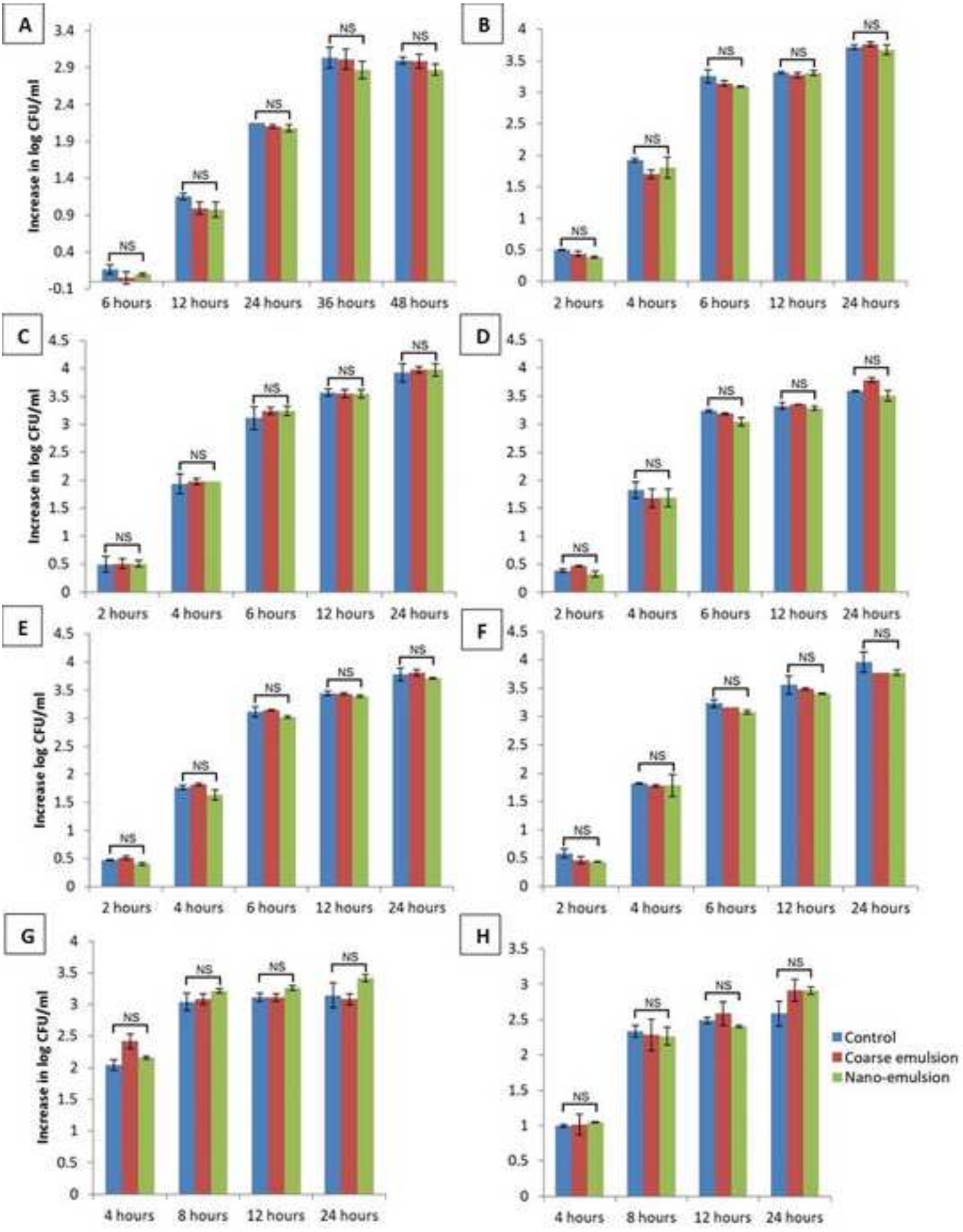

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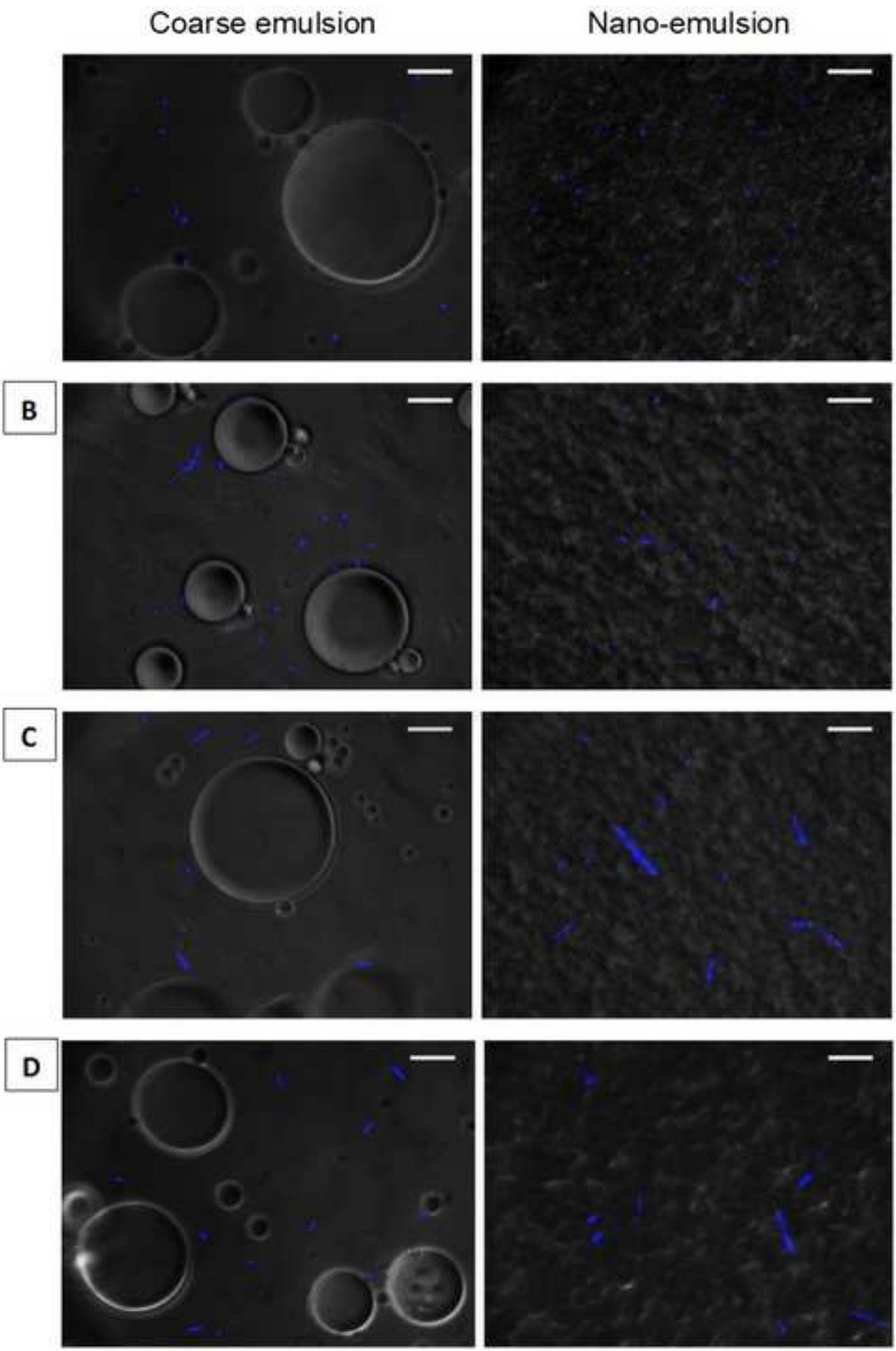

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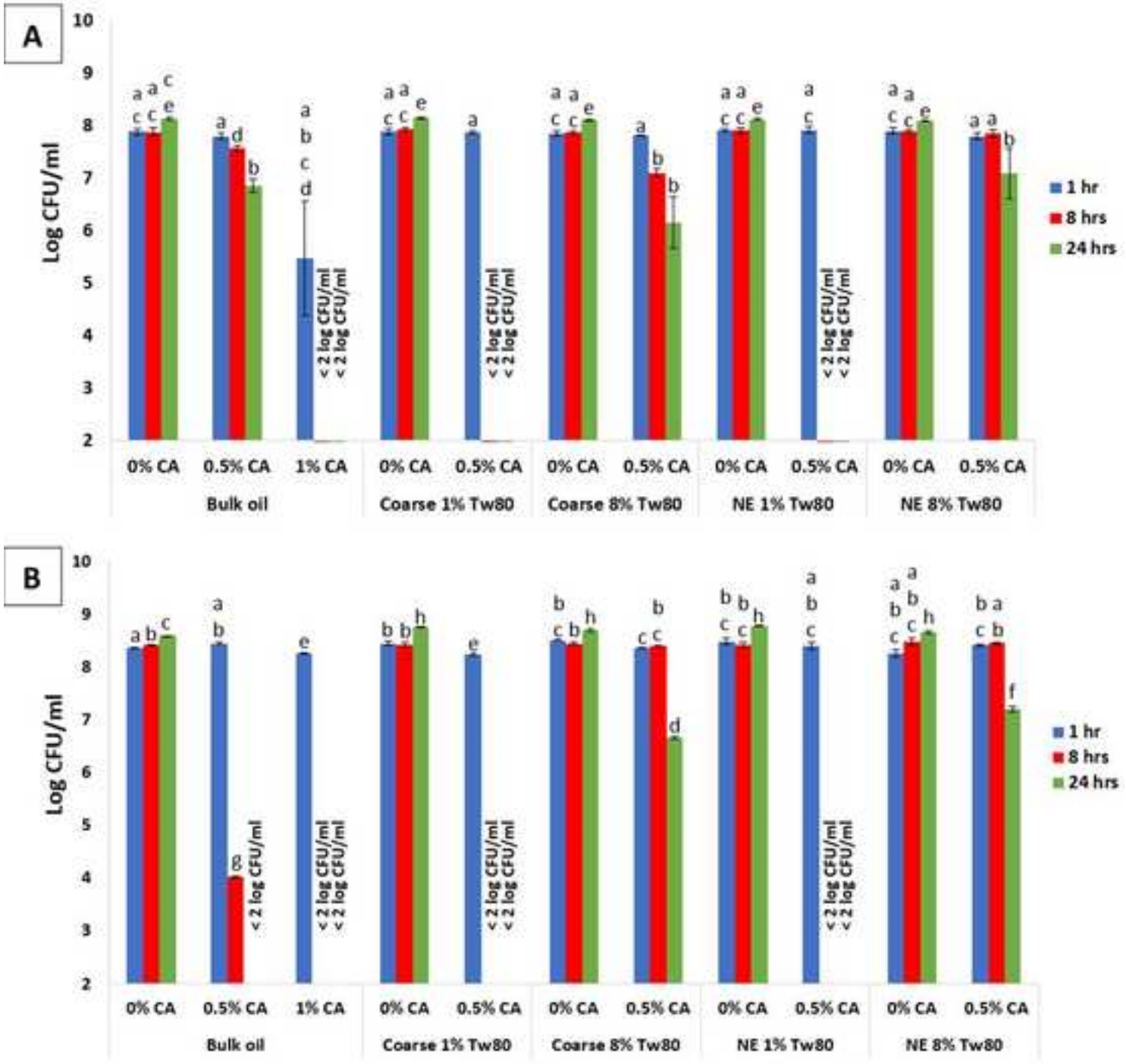

Figure 6

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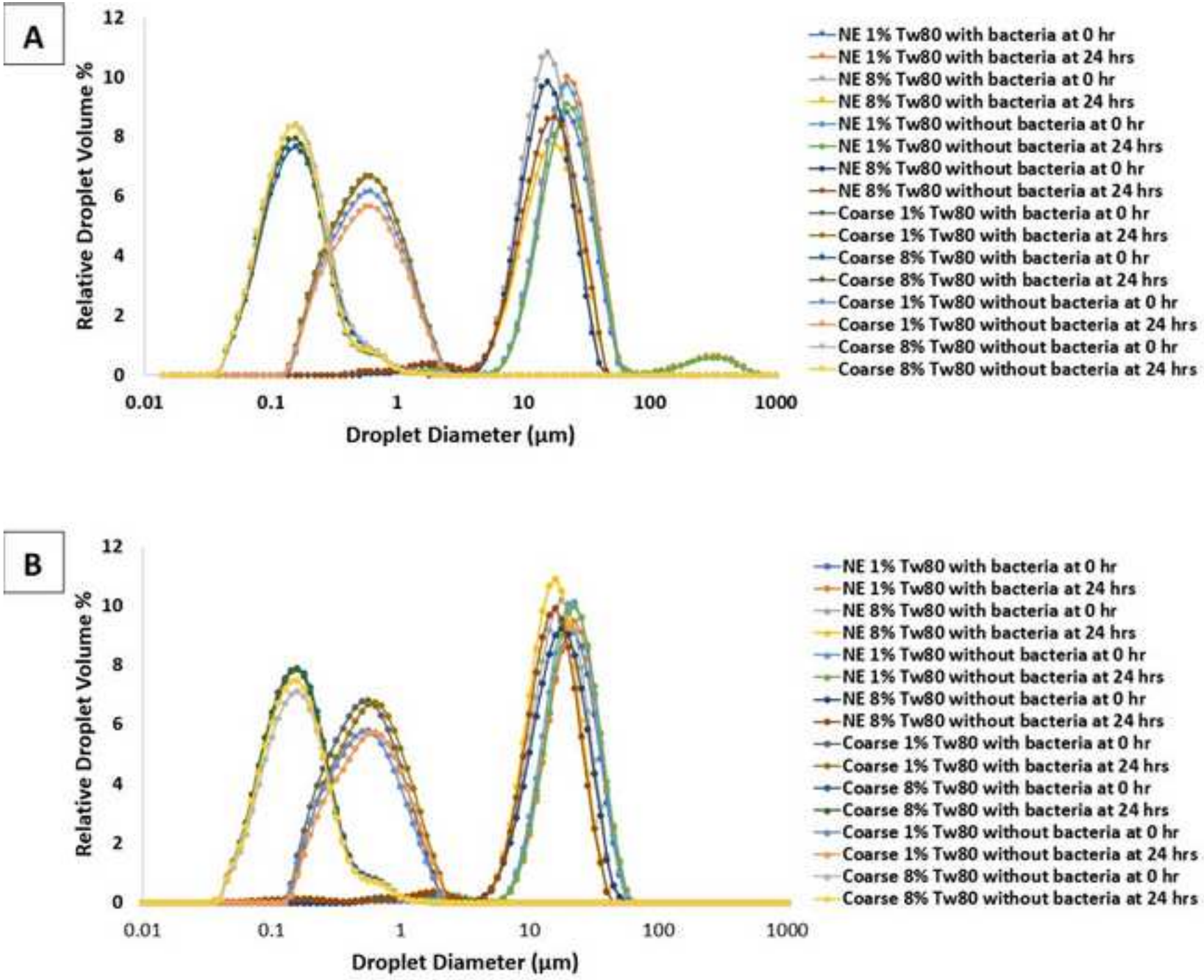

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Graphical Abstract

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