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Understanding and controlling the release mechanism of *Escherichia coli* in double $W_1/O/W_2$ emulsion globules in the presence of NaCl in the W_2 phase

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Abstract

Destabilising the structure of double emulsions can be useful for controlling release of substances at the desirable time. Release mechanisms of encapsulated compounds in double emulsions have been described; however, our understanding of bacterial release is limited and the presence of bacteria may affect the emulsion and vice versa. In this work, the stability and release properties of double $W_1/O/W_2$ emulsions were studied with or without the presence of GFP-tagged *Escherichia coli* (*E. coli*-GFP) in the inner aqueous phase (W_1) as well as the impact of altering the osmotic balance by adding NaCl in W_2. Double $W_1/O/W_2$ emulsion preparation and *E. coli*-GFP encapsulation was achieved using a two-step homogenisation process and structure was changed by altering the concentration of hydrophilic surfactant (Tween80, 0.5% to 10%) and W_1 (20% and 40%). The release of *E. coli*-GFP was monitored by culture and observed using fluorescence microscopy. The release of *E. coli*-GFP was significantly ($P<0.05$) increased when the osmotic balance was altered and the concentration of W_1 was high and Tween80 was low. In contrast, no release of *E. coli*-GFP occurred during osmotic balance alteration when the concentration of W_1 was low and Tween80 was high. Bacterial release occurred due to oil globule bursting independent of diffusion mechanisms. Changing the structure of the emulsion can be used for controlling bacterial release in double emulsions which occurs due to the bursting of the oil globules.

1) Introduction

Water-in-oil-in-water ($W_1/O/W_2$) emulsions are common types of double emulsion (DE) formed when a water in oil (W_1/O) emulsion is dispersed as oil globules in a continuous aqueous phase (W_2).[1] There has been increasing interest in $W_1/O/W_2$ emulsions due to their ability to entrap and protect hydrophilic substances and control their release from the W_1 to W_2 phases finding various applications in food,[2] pharmaceutics[3] and cosmetics.[4]

Among these, there are exciting advancements in $W_1/O/W_2$ emulsion applications for encapsulation of microbial species, for example, *Lactobacillus* spp. probiotics were protected from cytotoxic gastric juice,[5,6] bile salts,[7] prolonged storage at low temperatures[8] and during cheese manufacturing and melting[9]. Emulsions have also been used as analysis tools for *Escherichia coli* cultivation, genetic activation and quorum sensing.[10] A fluorescence-based method for rapid detection of bacteriophages was developed by encapsulating *E. coli* and lytic phages in $W_1/O/W_2$ emulsion.[11] More recently $W_1/O/W_2$ emulsions have been used as 3D microenvironments for the containment and growth of bacterial biofilms.[12] However, there is a lack of understanding of the mechanism by which bacteria are released from the W_1 phase of $W_1/O/W_2$ emulsions. In the case of chemical substances release was found to vary between diffusion-dependent transfer through the oil phase[13,3] and/or oil globule bursting.[14]

Furthermore, bacteria were found to affect the stability of single emulsions whilst the structure of emulsions can affect bacterial growth. This depends on the emulsion system's structure and composition as well as microbial cell properties such as metabolic activity,[15] planktonic cells versus colony formation and rate of growth,[16,17] surface charge,[18-20] and hydrophobicity.[21] Depending on the species and strain, bacteria with opposite surface charge to the emulsion droplet will destabilise the structure of the emulsion and tend to aggregate around the droplets.[17] *Escherichia coli* can metabolise glucose and grow within water droplets of W/O emulsions and excrete products that diffuse through the oil phase and into surrounding water droplets creating an osmotic mismatch leading to water flux and droplet shrinkage at rates depending on the strain, species and number of the microbial cells.[15] As opposed to growing in planktonic form, Gram negative and Gram positive bacteria were constrained to grow as colonies in O/W emulsions when the oil phase concentration was increased[22] and the oil droplet size was decreased.[23] However, studies investigating the effect of bacteria on stability of $W_1/O/W_2$ emulsions and the effect of $W_1/O/W_2$ emulsion structure on bacterial growth and viability are lacking.

Stable $W_1/O/W_2$ emulsions require a balance between the Laplace and osmotic pressures.[24] The flux of water from W_1 to W_2 due to a higher concentration gradient in W_2 could destabilise the $W_1/O/W_2$ emulsions as the globules shrink and/or collapse.[25, 26] However such destabilisation might be desired in certain applications

to control release of substances from the W_1 phase. This instability, if controlled could allow for the release of entrapped hydrophilic substances at the proper time. Thus oil globules have to be stable enough to contain the materials entrapped within their inner W_1 phase but at the same time susceptible to physiochemical changes so that they breakdown to deliver the entrapped hydrophilic substances to the outer W_2 phase.[14]

Release of substances from W_1 to W_2 in $W_1/O/W_2$ emulsions can occur by two general mechanisms; breaking of the oil globules or transport through the oil phase without breaking of the oil globules. Many studies investigated the release of compounds from $W_1/O/W_2$ emulsion globules such as NaCl,[27] drugs[3] and hormones.[28] Several mechanisms have been suggested for the movement of solutes or compounds in between the W_1 and W_2 phases and these include surfactant-facilitated transport through reverse micelles,[29] hydrated surfactant and/or diffusion through thin surfactant lamellae.[30] Tedajo *et al.* (2005) has shown that by diluting the $W_1/O/W_2$ emulsion in a hypo-osmotic solution oil globule bursting occurs leading to the release of antiseptics.[31] In another study it was reported that NaCl released into W_2 due to oil globule bursting was shown to increase with $W_1/O/W_2$ emulsion formulations containing higher amounts of W_1.[14] However, these studies investigated the release mechanism by oil globule bursting due to swelling which occurs when the osmotic pressure in W_2 is higher than W_1 created by the presence of NaCl in W_2.

The stability and release properties of $W_1/O/W_2$ emulsions can be controlled by altering the oil/water ratio, osmotic balance between the two aqueous phases, and the type and/or concentration of the emulsifiers.[14] However, no study has yet investigated how insoluble particles such as bacteria can be delivered from the inner to the outer phase of $W_1/O/W_2$ emulsions. In this study we microscopically visualised the mechanism by which release of bacteria occurs and investigated the effect of the structure of double emulsions on bacterial release facilitated by the presence of NaCl in W_2. Also the amount of inner W_1 phase and hydrophilic surfactant in W_2 were changed and their effect on $W_1/O/W_2$ emulsion stability in the presence of GFP-tagged *E. coli* was analysed. Furthermore, we investigated the effects of encapsulation, release and chemistry of emulsion on *E. coli*.

2) Experimental

2.1. Materials

The water soluble emulsifier polysorbate 80 (Tween80) was purchased from Sigma-Aldrich (United Kingdom). The oil soluble emulsifier polyglycerol polyricinoleate (PGPR) was provided by Danisco (Denmark). Sunflower oil (food grade) was purchased from a local retailer (United Kingdom). Sodium chloride (NaCl) 99% was

purchased from Sigma-Aldrich (Germany). The two stains 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) and propidium iodide (PI) were purchased from Sigma-Aldrich (United Kingdom). Nucleopore black polycarbonate membrane (13mm diameter; 2.0 µm pore size) and stainless steel 13mm Swinney filter holder was purchased from Millipore (United Kingdom). Tryptic soy agar (Oxoid Ltd. CM0131) and broth (Oxoid Ltd. CM0129) broth were purchased from Fisher Scientific (United Kingdom).

2.2. Microbial cultures

Escherichia coli strain K-12, MG1655 (ATCC 47076) and its derivative *E. coli* SCC1 (MG1655 $P_{A1/04/03}$-*gfp*mut3*, expressing green fluorescent protein (GFP) from the chromosome and subsequently referred to as *E. coli*-GFP)[32] were maintained on tryptic soy agar petri dishes at 4^{o}C. Cells were 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. For obtaining cells in the exponential phase cells were harvested by centrifugation (10,000 g, 10 minutes) and washed in de-ionised water twice. Cells were re-suspended in 10 ml of de-ionised water and used for encapsulation.

2.3. Preparation of $W_1/O/W_2$ emulsions

$W_1/O/W_2$ emulsions were prepared using a high shear mixer homogeniser (Silverson L5M) at room temperature using a two-step emulsification process (*Figure* S1). In the first step primary W_1/O emulsions were made. An oil phase was prepared by dissolving 2 wt% PGPR in sunflower oil. The inner aqueous phase (W_1) consisting of de-ionised water was emulsified (120 seconds) into the oil phase (W_1:O phase ratio of 20:80 or 40:60). For microbial-encapsulation de-ionised water containing the washed bacteria ($2x10^8$ CFU/ml) was used as W_1. The rotational speeds for homogenising the different formulations of primary W_1/O and $W_1/O/W_2$ emulsions are shown in *Table* S1. In the second step $W_1/O/W_2$ emulsion was made. The outer aqueous phase (W_2) was prepared by dissolving Tween80 (0.5%, 1%, 5% or 10 wt%) in de-ionised water at 60^{o}C for approximately 15 minutes. The previously prepared primary W_1/O emulsion was emulsified (60 seconds) into W_2 to form the $W_1/O/W_2$ emulsion (W_1/O:W_2 ratio of 20:80). Finally, 20 ml of the double $W_1/O/W_2$ emulsion was replaced by either de-ionised water or NaCl solution (final concentration of 0.02 M, 0.04 M, 0.085 M or 0.17 M) to alter the osmotic balance and the whole 100 ml of $W_1/O/W_2$ emulsion was transferred into sterile 500 ml conical flask and incubated at 25^{o}C over 1 hour or a 6 hour period shaking at 100 rpm to ensure homogenised mixing.

2.4. Microscopic observation of the $W_1/O/W_2$ emulsions

Video time-lapse for observation of oil globule bursting and tracking of E. coli-GFP

For video microscopy the sample was placed on a microscope slide and the video was recorded under objective lens 40x magnification with a Moticam 10 camera via Motic Images Plus video acquisition software at 17fps.

Fluorescent and optical imaging of the $W_1/O/W_2$ emulsions and E. coli-GFP

The double $W_1/O/W_2$ emulsions with or without *E. coli*-GFP were observed using optical and fluorescent microscopy (Zeiss Axioplan) at room temperature. For optical microscopy imaging the sample was placed on a microscope slide and the image was acquired under objective lens 10x magnification with a digital colour camera system Motic Moticam 10 using a 10 megapixel CMOC camera via Motic Images Plus video acquisition software. For fluorescent microscopy imaging the sample was placed on a microscope slide and gently covered with a cover slip. The image was 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 light source used to excite the GFP was a mercury arc lamp and the emission was observed at 509 nm. Micrographs were overlaid using analysis software (ImageJ).

2.5. Measuring encapsulation efficiency (%) and quantifying of the release of *E. coli*-GFP during emulsification and storage.

Due to the differences in density between the W_2 phase and the oil globules creaming or phase separation occurs when $W_1/O/W_2$ emulsion is un-mixed and it partitions into a serum phase (W_2 phase) and a cream layer (containing oil globules).[33] The number of *E. coli* cells in the serum phase was measured immediately after preparation and as a function of storage time. Five millilitre sample of $W_1/O/W_2$ emulsion was collected and the serum phase was removed using a syringe. *E. coli* cell counts were made using serial dilutions in PBS (phosphate buffered saline) buffer solution and plating on tryptic soy agar using the Miles & Misra technique.[34] Colony forming units per millilitre (CFU/ml) were then calculated. As the serum phase contains no oil globules only unencapsulated (or those released when oil globules burst) viable bacteria were able to grow to colony forming units which could be counted. The encapsulation efficiency and release of *E. coli*-GFP were calculated by the following equations:

Encapsulation efficiency = $((N_0 - N) / N_0) \times 100\%$

Release = (Log_{10} N - Log_{10} N_T)

Where N is the number of unencapsulated viable bacterial cells immediately after forming the $W_1/O/W_2$ emulsion, N_0 displays the free viable bacterial cells before encapsulation and N_T displays the unencapsulated viable bacterial cells after incubation period.

2.6. Characterisation of $W_1/O/W_2$ emulsion stability.

Measurement of oil globule size of double $W_1/O/W_2$ emulsions

The particle size distribution of oil globules in the $W_1/O/W_2$ emulsion 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 = 633 nm). The dispersion unit stirring speed was kept at 2000 rpm and the measurement range was 0.02–2000 µm. 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%. 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.

Observation of phase separation

The cream height fraction of the $W_1/O/W_2$ emulsion was measured immediately after preparation and as a function of storage time. One millilitre of $W_1/O/W_2$ emulsion was collected by a 1 ml graduated syringe and left standing upright. 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:

$$H_{Cream} = \frac{H_{Double\ emulsion} - H_{Creamed\ phase}}{H_{Double\ emulsion}} \times 100\%$$

2.7. Determining the viability and health of *E. coli* (MG1655) after encapsulation and release using epifluorescence microscopy

E. coli MG1655 in serum phase or de-ionised water (control) was stained by adding DAPI (4µl/ml) and PI (4µl/ml) and incubated in the dark for 30 minutes. A nucleopore

black polycarbonate membrane filter with 0.2µm pore size and 13mm diameter was mounted shiny side, uppermost on a membrane Swinnex filter holder (Millipore) and the stained solution was passed through the membrane filter by injecting slowly with a syringe. The membrane filter was placed shiny side uppermost on a glass slide and a drop of immersion oil added to its surface and gently covered with a cover slip. The cells were then counted under objective lens of 100x magnification with a fluorescent microscope (Zeiss Axiolab) equipped with a mercury arc lamp and the emission was observed at 461 nm (DAPI) and 645 nm (PI). For each filter, 10 microscope (0.1 mm x 0.1 mm) fields were randomly selected and all cells within each field were counted and the average number of cells per field was calculated.[35] The serum phase volume was adjusted to yield a minimum of 400 total cell counts in 10 counting fields. The total number of *E. coli* cells from each sample of serum phase was calculated by multiplying the total number of fields with the average number of cells per field.

2.8. Statistical analysis

Each experiment was conducted 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

3.1. Primary W_1/O emulsion characterization

The stability of $W_1/O/W_2$ emulsions can be influenced by the size of W_1 droplets.[36] In this study we ensured that the homogenisation conditions were as such that the average mean size distribution of W_1 droplets was comparable (3-4µm; *Figure* S1) in all primary W_1/O formulations. Figure S2 shows optical images of the different W_1/O formulations and *E. coli*-GFP was successfully encapsulated within the W_1 droplets of the primary W_1/O emulsion (*Figure* S4).

3.2. Encapsulation efficiency (%) of *E. coli*-GFP cells in $W_1/O/W_2$ emulsions.

The bacterial counts for *E. coli*-GFP before and after the encapsulation process were used to calculate the encapsulation efficiency from different $W_1/O/W_2$ emulsions. The

results indicated successful entrapment of viable bacteria cells (>99.9%) in all the prepared $W_1/O/W_2$ emulsions (*Table* S2).

3.3. Microscopic observation of oil globule bursting and E. coli-GFP release

Using video-microscopy we were able to observe the bursting mechanism responsible for the release of *E. coli*-GFP from the oil globules after adding salt in W_2. Moreover, the oil globule bursting phenomenon was a reproducible observation and occurred for the majority of the oil globules while the inner W_1 water phase was still present. For all the double emulsion formulations when no NaCl was added to the W_2 phase the oil globules did not burst and the W_1 droplets encapsulating *E. coli*-GFP remained within the oil globules. Figure 2a and 2b show snapshots taken after adding NaCl to W_2 of $W_1/O/W_2$ emulsion made with 40% W_1 and 1% Tween80 (see supporting information, *Video* S1 and S2 respectively). The membrane of the oil globule ruptures and W_1 droplets encapsulating *E. coli*-GFP cells are released into W_2. Figure 2c shows snapshots taken after adding NaCl to W_2 of $W_1/O/W_2$ emulsion made with 20% W_1 and 5% Tween80 (see supporting information, *Video* S3). No oil globule bursting occurred and loss of W_1 droplets was observed and *E. coli*-GFP remained within the oil phase of the oil globules. Figure 1d shows snapshots taken after adding NaCl to W_2 of $W_1/O/W_2$ emulsion made with 40% W_1 and 1% Tween80 (see supporting information, *Video* S4). A coalescence event between two oil globules containing *E. coli*-GFP cells that remains within the newly coalesced oil globule. These results suggest that the release of *E. coli*-GFP into W_2 occurs due to the rupturing of the oil globule's interfacial film and seems to be independent of diffusion.

3.4. Influence of NaCl on release of E. coli-GFP

To understand the effect of varying the structure of $W_1/O/W_2$ emulsion on release of bacteria with and without NaCl in W_2 the release of *E. coli*-GFP over time was quantified (*Figure* 2, 3 and 4). Also the $W_1/O/W_2$ emulsion structure and the localisation of *E. coli*-GFP within the $W_1/O/W_2$ emulsion were microscopically visualised (*Figure* 5 and 6).

With the formulation containing 40% W_1 and stabilised with 1% Tween80 (*Figure* 2) the release of *E. coli*-GFP was significantly ($P<0.05$) increased with NaCl compared to without NaCl in W_2 but remained significantly similar with increasing NaCl concentrations. This suggests that the release of *E. coli*-GFP was not concentration dependent.

At 1% Tween80 and regardless of W_1 concentration the release of *E. coli*-GFP was significantly ($P<0.05$) higher with NaCl compared to without NaCl in W_2 (*Figure* 4)

showing that destabilising the emulsion by altering the osmotic balance affects the release *E. coli*-GFP. During no osmotic balance alteration the oil globules showed no loss of W_1 droplets (*Figure* 5A) and *E. coli*-GFP cells remained within W_1 (*Figure* 6A). However, after adding NaCl in W_2 the oil globules showed loss of W_1 droplets (*Figure* 5B) and *E. coli*-GFP cells were present in the oil phase of the oil globules and also in the W_2 phase (*Figure* 6B). After adding NaCl to W_1 the release of *E. coli*-GFP was significantly ($P<0.05$) higher at low concentrations of Tween80 (0.5% and 1%) compared to high concentrations of Tween80 (5% and 10%) (*Figure* 3 and 4). Interestingly, there was no significant difference in release of *E. coli*-GFP at 20% W_1 and 5% Tween80 with NaCl compared to without NaCl in W_2 (*Figure* 4) whilst the oil globules showed loss of W_1 droplets (*Figure* 5C) and *E. coli*-GFP cells were present in the oil phase of the oil globules (*Figure* 6C). This suggests that the release of *E. coli*-GFP is affected by the concentration of Tween80 during osmotic balance alteration.

After adding NaCl in W_2 the release of *E. coli*-GFP was significantly ($P<0.05$) higher at 1% Tween80 when W_1 was 40% compared to 20% W_1 (*Figure* 4). Furthermore, after adding NaCl in W_2 the release of *E. coli*-GFP was significantly ($P<0.05$) higher at 1% Tween80 when W_1 was 40% compared to 20% W_1 after 2 hours and then becomes non significant after 4 and 6 hours (*Figure* 4). These results indicate that the amount of W_1 affects the release of *E. coli*-GFP with or without osmotic balance alteration.

3.5. Changes in oil globule size

To understand how the presence of NaCl in W_2 affected the stability of the oil globules within different formulations of $W_1/O/W_2$ emulsions with or without *E. coli*-GFP, we measured the change in oil globule size (D (4, 3)) over time (*Figure* 7 and 8 and *Table* 1).

The presence of bacteria in W_1 had no effect on the D (4, 3) during the incubation period. There was no significant difference in the D (4, 3) with NaCl compared to without NaCl in W_2. After adding NaCl to W_2 the D (4, 3) significantly ($p<0.05$) decreased (*Figure* 7 and 8 and *Table* 1). When the concentration of NaCl in W_2 was low (0.02 M and 0.04 M) the reduction in D (4, 3) was significantly ($P<0.05$) smaller compared to when the concentration of NaCl in W_2 was high (0.085 M and 0.17 M) (*Figure* 7).

3.6. Changes in creaming behaviour

To understand how the presence of NaCl in W_2 affects the creaming of $W_1/O/W_2$ emulsions with or without bacteria we measured the change in percentage of loss of cream layer over time (*Figure* 7, 8, 9A and 9B).

The loss of cream layer thickness was significantly ($P<0.05$) increased at 40% W_1 and 1% Tween80 with NaCl compared to without NaCl in W_2 (*Figure* 7, 8, 9A and 9B) but was significantly similar over the varying concentrations of NaCl (*Figure* 4). After adding NaCl to W_2 the loss of creaming thickness was significantly ($P<0.05$) higher when the concentration of Tween80 was low (0.5% and 1%) compared to when high (5% and 10%) (*Figure* 8, 9A and 9B).

The presence of bacteria in W_1 had no effect on the cream layer thickness during the incubation period. When no NaCl was added in W_2 and at 20% or 40% W_1 there was no significant difference in loss of cream layer at 1% Tween80 compared to 5% Tween80 (*Figure* 9A and 9B). At 1% Tween80 the loss of cream layer was significantly ($P<0.05$) higher with NaCl compared to without NaCl in W_2 regardless of W_1 concentration. Moreover, the loss of cream layer was significantly ($P<0.05$) higher at 1% Tween80 and 40% W_1 compared to 20% W_1. Interestingly there was no significant difference in loss of cream layer at 20% W_1 and 5% Tween80 with NaCl compared to without NaCl in W_2.

3.7. Effect of encapsulation and release on E. coli viability

To understand the effect of $W_1/O/W_2$ emulsion structure and the release mechanism on *E. coli* we studied the viability of bacteria by plating and microscopic assessment of bacterial cells. There was no significant difference in the number of *E. coli* cells obtained from the plating method compared to microscopic enumeration (*Figure* 10A). However, after release into the W_2 phase, 84% of *E. coli* cells were PI positive (*Figure* 10B) even though the bacterial cells were viable suggesting that PI positive cells were not dead.

4) Discussion and conclusion

As expected, the presence of NaCl in the W_2 phase caused the oil globules to become significantly ($P<0.05$) smaller in size as NaCl creates an osmotic pressure gradient between the two aqueous phases forcing water to be transported from W_1 to W_2 resulting in oil globule shrinkage.[37,38] As water migrates from W_1 to W_2 the change in oil globule size diminishes because of the reduction in the concentration gradient. Also the reduction in D (4, 3) significantly ($P<0.05$) increased with higher concentration of NaCl (0.085 M and 0.17 M) compared to at lower concentration of NaCl (0.02 M and 0.04 M). The presence of 0.085 M and 0.17 M NaCl in W_2 create an osmotic pressure of 21 atm and 42.1 atm respectively whereas with 0.02 M and 0.04 M NaCl in W_2 create an osmotic pressure of only 5.26 atm and 10.5 atm respectively. Also the reduction in oil globule size was significantly smaller with 5% Tween80 compared to with 1% and 0.5% Tween80 regardless of W_1 concentration.

Excess Tween80 in the W_2 phase increases the rate of water transport from W_1 to W_2 through mixed reverse micelles and/or surfactant hydration mechanism.[39] Furthermore, the lifetime of W_1 droplets within the oil globules could last from several months to few minutes depending on the concentration of Tween80.[27]

Osmotic pressure difference that leads to the collapse of the oil globules was first observed when $W_1/O/W_2$ emulsion entrapping a drug was delivered *in vivo.*[40] This immediate collapse of $W_1/O/W_2$ globules was believed to occur due to the higher osmotic pressure of body fluids compared to the inner phase causing shrinking and/or bursting of the oil globules.[40] Since salt in W_2 create an osmotic pressure gradient which draws water from W_1 to W_2 and bacteria are hydrophilic we hypothesised that bacterial cells will move from the oil phase to W_2. We therefore wanted to investigate whether it is the bursting or shrinkage of the oil globules being responsible for the release of bacteria and subsequently try to control this mechanism by changing the structure of the $W_1/O/W_2$ emulsion.

In the video-microscopy results we observed that after adding NaCl in the W_2 phase of the formulation containing low concentration of Tween80 and high amount of the W_1 phase the oil globule bursts releasing W_1 droplets and *E. coli*-GFP into W_2 (see supporting information, *Video* S1 and S2). NaCl can interact with Tween80 and weaken the interfacial membrane of the oil globule.[41,26] Opawale and Burgess (1998) demonstrated that the effect of NaCl on the interfacial elasticity (relates to interfacial film strength) was surfactant specific.[41] The authors showed that for the multiple emulsion formulations that they studied, the interfacial elasticity was reduced with increasing concentrations of NaCl. In another study by Jiao et al. (2002) NaCl not only resulted in water loss due to reverse osmosis as did sodium salicylate when added to W_2 but caused a significant reduction in the interfacial elasticity of the film probably due to unfavourable interactions between NaCl and Tween80 and/or salting out.[26] Interestingly, after adding NaCl in the W_2 phase of the formulation containing high concentration of Tween80 and low amount of W_1 phase *E. coli*-GFP cells remain within the oil globule despite the loss in W_1 droplets (see supporting information, *Video* S3). The solubilisation of *E. coli*-GFP cells within the oil phase was probably due to the presence of PGPR that adsorbs at the bacterial lipid membrane due to its lipophilic nature. It was also observed that the W_1 droplets did not immediately disappear upon release. The W_1 droplets were delivered into the external phase after the oil globule bursts and these droplets persisted in W_2 confirmed by a small peak observed during particle size distribution measurements (*Figure* S5, S6 and S7) and microscopic observation of the serum phase (*Figure* S8). A similar observation was reported by Jiao et al. (2002) showing that upon applying force using a coverslip the more stable $W_1/O/W_2$ emulsion globules form structures that had a “dimpled” appearance as a result of W_1 droplets being pushed to the edge of the oil globule and these were in contact with the continuous phase being separated by a very thin bio-molecular film with no apparent Becke line.[26] However,

more investigation is needed to understand the nature of these W_1 droplets that persist within the W_2 phase.

So far our results showed that the release of *E. coli*-GFP is due to oil globule bursting independent to diffusion mechanism. In this study we found that at low concentration of Tween80 the release of *E. coli*-GFP was significantly ($P<0.05$) increased. The stabilizing effects of hydrophilic surfactants is increased when their concentration is higher in W_2 as they form multilayer arrangement of interdigitated surfactant chains that "wrap" the oil globules[42] and strengthen the interfacial film.[43] An increase in the amount of Tween80 adsorbed at the O/W_2 interface produces more condensed interfacial films, able to resist hole creation and propagation that leads to film rupture.[44] It has been reported that interfacial processes control the transport of water in $W_1/O/W_2$ emulsions rather than bulk diffusion.[43] When the W_1 droplets and oil globule interfaces are not physically in contact the spontaneous emulsification and reverse micellisation will be controlling the transport of water between the W_1 and W_2 phases with transport rates independent of NaCl concentration in W_2.[38] However, in the presence of NaCl in W_2 and when the W_1 droplet and oil globule interfaces are physically in contact the surfactant molecule hydrates at one interface, diffuse through the oil phase to dehydrate at the other interface that is in contact with the phase of higher solute concentration.[45] Moreover, during that process the amount of surfactant molecules at the globule's interface is reduced and this could lead to the interfacial film becoming more susceptible to rupture. At 1% Tween80 the amount of surfactant molecules in the W_2 is not sufficient to adequately replace the migrating surfactant molecules desorbing from the interface. In contrast at 5% Tween80 the amount of Tween80 in W_2 is sufficient to replace the migrating surfactant molecules from the interface and therefore prevents or slows down the rupture of the interfacial film. Also since more W_1 droplets exist within the oil globule at 40% W_1 compared to 20% W_1 the rate of surfactant hydration mechanism is increased and therefore more droplets would burst leading to the release of *E. coli*-GFP into W_2. Therefore, we believe that NaCl caused release of the *E. coli*-GFP cells through destructive interactions on the interfacial film of the oil globules. However, we cannot dismiss other mechanisms that in addition to globule bursting may have been responsible for the release of *E. coli*-GFP. For example, the presence of NaCl in W_2 screens the electrostatic repulsion between the oil globules,[39] which leads to the increase in coalescence events between the oil globules. Moreover, during this process the release of *E. coli*-GFP to W_2 phase is possible. However, we observed some coalescence events between the oil globules when NaCl was present in W_2 but this process did not lead to the release of *E. coli*-GFP as they remained within the oil globule after coalescence. Another possibility for the release of *E. coli*-GFP would be the increase in coalescence of W_1 droplets with the oil globule interface. However, this release mechanism was shown to increase with increasing amounts of Tween80[39] which if was occurring in our study would have resulted in increased release of *E. coli*-GFP at high concentration of Tween80 but that would be in contrast to what we observed

in this study. Further investigation is required to confirm if such mechanism was associated with the release of *E. coli*-GFP from the oil globules along with the bursting mechanism that we observed after adding NaCl in the W_2 phase.

When $W_1/O/W_2$ emulsions are created some of the lipophilic surfactant molecules move to the external O/W_2 interface and influence the stability of multiple emulsion oil globules.[46] The movement from W_1/O interface and adsorption of PGPR at the O/W_2 interface has been documented in $W_1/O/W_2$ emulsions.[47] The lipophilic and hydrophilic surfactants at the O/W_2 interface can interfere with each other's stabilizing performance affecting the interfacial film strength. Jiao et al. (2002) demonstrated that $W_1/O/W_2$ emulsions formed with Tween80 and Span80 or Span83 had high interfacial film strength but when formed with Tween80 and Span85 or Span60 the interfacial film strength was low.[26] The authors concluded that due to Span85's bulky alkyl chain tail which prevents interactions with Tween80 at the O/W_2 interface led to a decrease in interfacial film strength and that due Span60's higher hydrophilic-lipophilic balance (HLB) value (4.7) an unstable $W_1/O/W_2$ emulsion was formed. However, the interaction of PGPR with Tween surfactants at the O/W_2 interface and its effects on interfacial film strength is not yet well understood. Moreover, PGPR can compete with the hydrophilic surfactant at the O/W_2 interface leading to oil globule structural instability.[48] Assuming that PGPR may interact with Tween80 we believe that at low concentration of Tween80 (1% wt) in W_2 more PGPR molecules adsorbed at the O/W_2 interface which may have resulted in reduced interfacial film strength and/or unfavourable interactions with Tween80 eventually weakening the interfacial film making it more susceptible to rupture. However, further studies are required to test if this hypothesis is true.

We found that at high concentration of W_1 the release of *E. coli*-GFP increased. Even though the concentration of PGPR was kept at 2% in this study the amount of PGPR required for full surface coverage W_1 droplets interface is higher in $W_1/O/W_2$ emulsion containing 40% W_1 compared to 20% W_1. This will result in less amount of excess PGPR in the oil phase at 40% W_1 than at 20% W_1. Jiao et al. (2002) demonstrated that an increase in oil phase viscosity due to increase in the concentration of Span80 results in less deformation of double emulsion oil globules upon applying force (coverslip).[26] Also the presence of excess lipophilic surfactant in the oil phase was found to increase the visco-elasticity of the interfacial film of the oil globule.[49] Furthermore, according to the Mooney equation[50,51] higher W_1 fraction increases the viscosity of the oil globule and this in turn leads to its destabilisation and increases the likelihood of rupture of the W_1 droplets and oil globules.[52] However, this increase in viscosity has an opposite effect on emulsion stability than does the viscosity increase due to increased lipophilic surfactant in the oil phase.[52]

So far our results showed that the release of *E. coli*-GFP follows a similar pattern as changes in loss of cream layer independent to changes in D (4, 3). We believe that the cream layer thickness was related to the amount of oil globules in the emulsion and the increase in loss of cream layer thickness indicates loss of oil globules. The

presence of *E. coli*-GFP within the $W_1/O/W_2$ emulsion globules had no effect on the transport of water and/or the bursting of the oil globules. Since bacteria are living organisms and they secrete waste products a change in osmotic pressure inside the oil globules is expected.[15] However, with the bacterial cell concentration and the conditions that were used in this study *E. coli*-GFP showed no significant difference compared to without *E. coli*-GFP on the change in D (4, 3) or creaming stability. We also found no effect in viability of *E. coli* cells after being released from the $W_1/O/W_2$ emulsion. However, there was a significant increase in PI positive *E. coli* cells after release compared to control. Non-ionic surfactants can enhance the fluidity of the bacterial membrane and hence increase its permeability.[53] Since PI is a membrane integrity indicator[54] and in this study the number of PI stained cells do not correlate with cell viability data it is most probable that the surfactants increased the fluidity and permeability of the bacterial membrane allowing PI to permeate and bind to nucleic acids. Also NaCl may affect the bacterial membrane. In a recent study by Ghandi and Shah (2015) it was shown that 5% NaCl had a damaging effect on membrane integrity after 1 hour allowing PI to penetrate bacterial cells.[55] Therefore upon release into the W_2 phase *E. coli* cells were exposed to NaCl and this could have compromised membrane integrity allowing PI to permeate and stain the cells.

In this study the mechanism by which bacterial cells are released from $W_1/O/W_2$ emulsions has been demonstrated for the first time. Using video-microscopy it was shown that the release of bacterial cells was due to oil globule bursting independent to diffusion. This release mechanism has been demonstrated to be modulated by modifying the structure of the $W_1/O/W_2$ emulsion. The release of *E. coli*-GFP was higher at low concentrations of Tween80 and high volumes of W_1 after adding NaCl in the W_2 phase. Moreover, when the concentration of Tween80 was high and the volume of W_1 was low no release occurred after adding NaCl in the W_2 phase. The release of *E. coli*-GFP was facilitated by the addition of NaCl probably due to interfacial processes. Therefore, when formulating $W_1/O/W_2$ emulsions for release applications it is important to take into account the role of the structure and stability of the oil globule's interface. Although in this study we have shown the mechanism by which bacteria is released from $W_1/O/W_2$ emulsions after adding NaCl in the W_2 phase further investigation is required to understand the effect of NaCl on the interfacial film of the oil globule for example measuring the visco-elastic properties of the different formulations investigated. Also it is important to note that the amount of encapsulated *E. coli*-GFP can vary from globule to globule and this, added to differences in globule sizes, results in some globules releasing more bacteria than others. However, in this study the overall behaviour of the $W_1/O/W_2$ emulsion can be obtained as an average of the bulk properties for these experiments. Moreover, to better understand the release mechanism it would be ideal to further investigate the bursting phenomenon in a more homogenous system in terms of globule size distribution and number of encapsulated bacteria. Understanding how release can be controlled allows different $W_1/O/W_2$ emulsion formulations for various industrial applications. For example, a fermentation process can contain a secondary bacterial

species within the oil globules that can be released in a controlled manner over time. This can reduce the risk of contamination associated with introducing the secondary bacterial species after starting the fermentation process. Also by changing the structure of $W_1/O/W_2$ emulsions the oil globules encapsulating pH sensitive drugs or probiotics can be made more resistant to bursting when ingested or injected *in vitro*.

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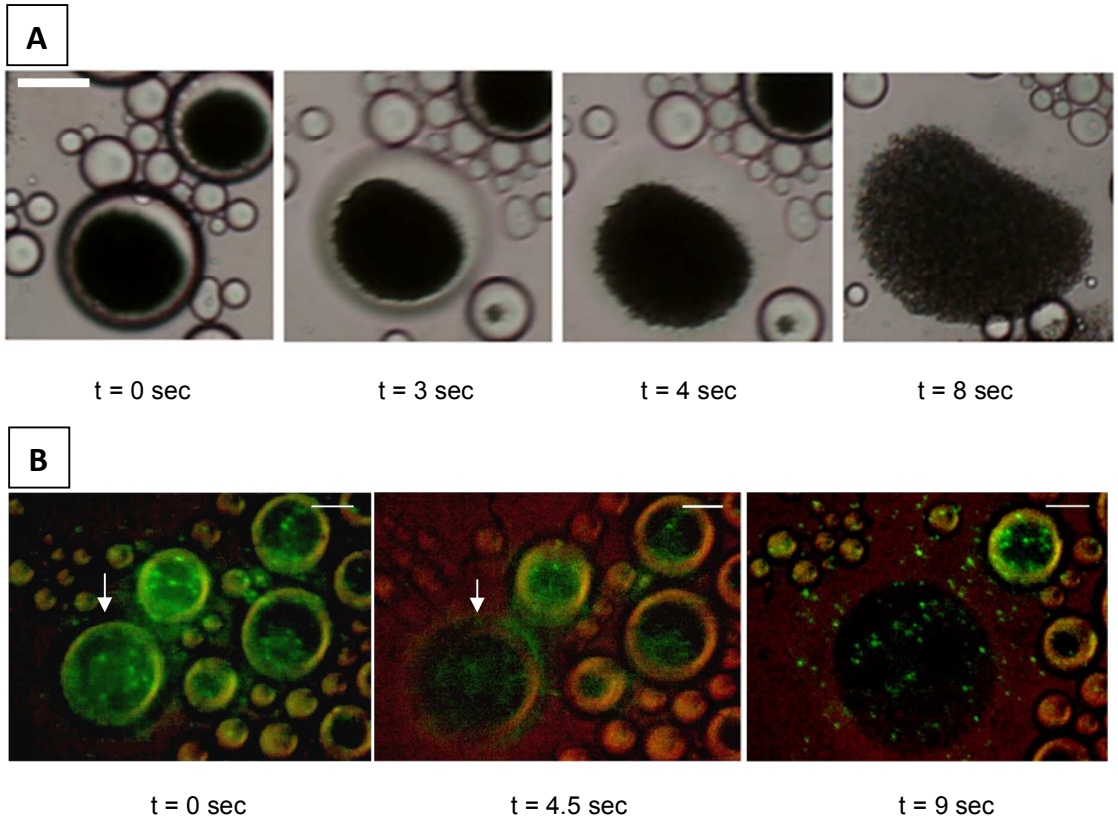
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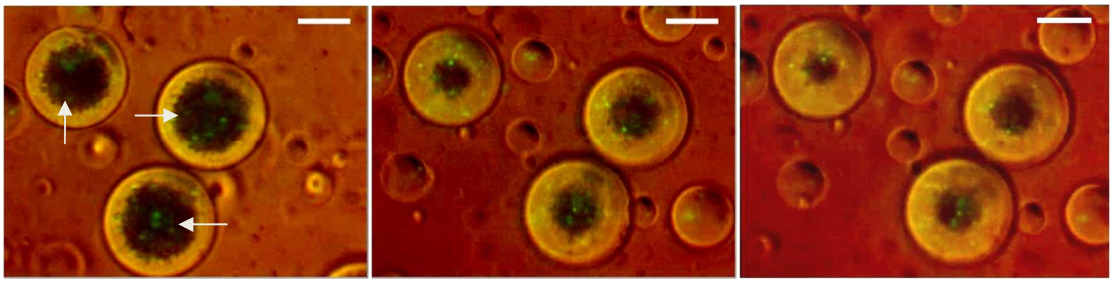

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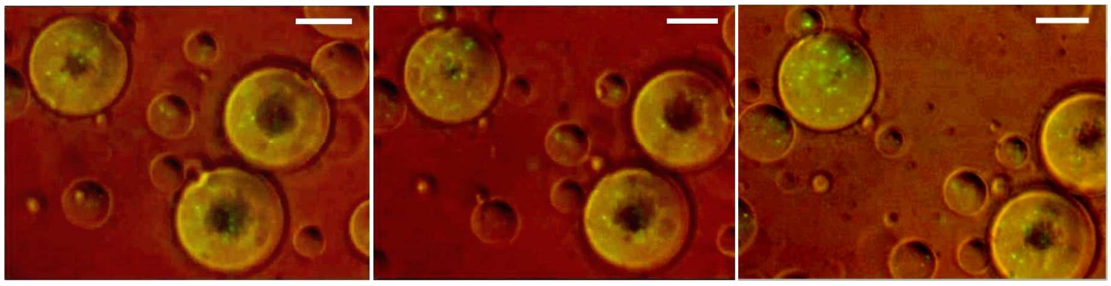

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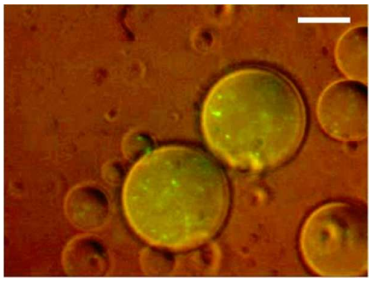

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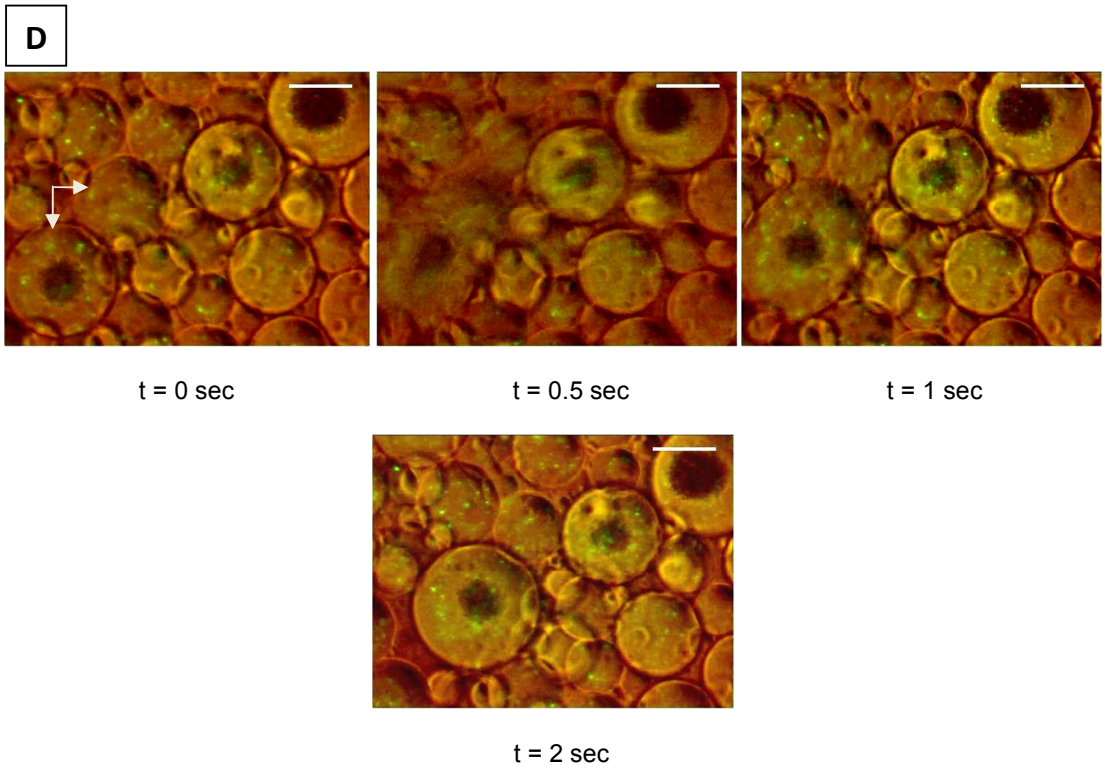

Figure 1. Optical and fluorescence snap shot images obtained from video-microscopy showing a scenario with oil globule bursting and release of W_1 droplets (A) or *E. coli*-GFP (B) after adding NaCl in W_2 and another scenario with no oil globule bursting and release of *E. coli*-GFP (C) after adding NaCl in W_2 and coalescence of oil globules with no release of *E. coli*-GFP (D) after adding NaCl in W_2. A) Optical image of oil globule bursting and release of the W_1 into W_2 phase. The $W_1/O/W_2$ emulsion was prepared with inner W_1 phase volume percentage of 40% containing bacteria and stabilized with 1% Tween80 containing 0.085 M NaCl in the W_2 phase. B) Fluorescent images of burst release of *E. coli*-GFP (green) from an oil globule (arrows) in a $W_1/O/W_2$ emulsion at different time points. The double emulsion was prepared with inner W_1 phase volume percentage of 40% containing bacteria and stabilized with 1% Tween80 containing 0.085 M NaCl in the W_2 phase. C) Fluorescent images of *E. coli*-GFP (green) within the oil globules during the loss of the W_1 droplets due to the presence of NaCl in the W_2 phase. The $W_1/O/W_2$ emulsion was prepared with inner W_1 phase volume percentage of 20% containing bacteria and stabilized with 5% Tween80 containing 0.085 M NaCl in the W_2 phase. The inner W_1 droplets can be observed as a dark mass within the oil globules (arrows). D) Fluorescent images of *E. coli*-GFP (green) within two oil globules (arrows) that are undergoing coalescence and during the loss of the W_1 droplets due to the presence of NaCl in the W_2 phase. The $W_1/O/W_2$ emulsion was prepared with inner W_1 phase volume percentage of 40% containing bacteria and stabilized with 1% Tween80 containing 0.085 M NaCl in the W_2 phase. Scale bar: 50μm.

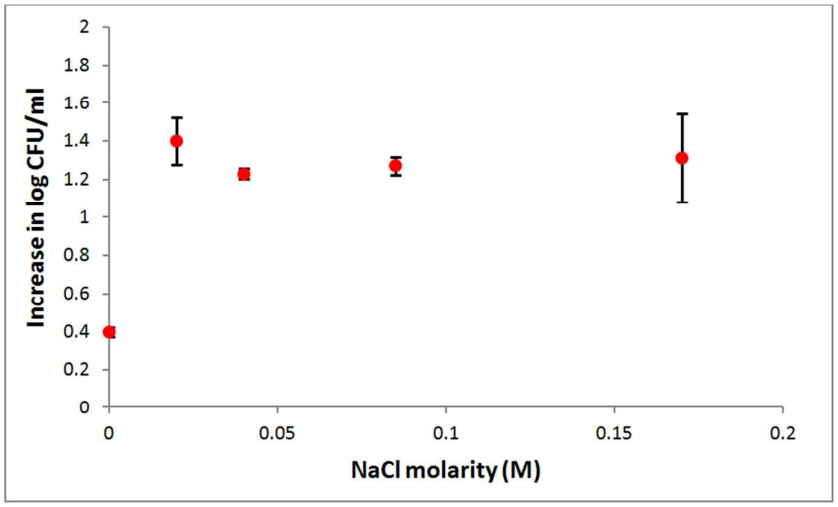

Figure 2. Amount of released bacteria in the outer W_2 phase of the $W_1/O/W_2$ emulsions after 1 hour incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with different inner W_1 phase volume percentage of 40% containing *E. coli*-GFP in the W_1 phase and stabilized with 1% Tween08 with or without 0.02 M, 0.04 M, 0.085 M or 0.17 M NaCl in the W_2 phase. Bars represent mean ± SEM taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

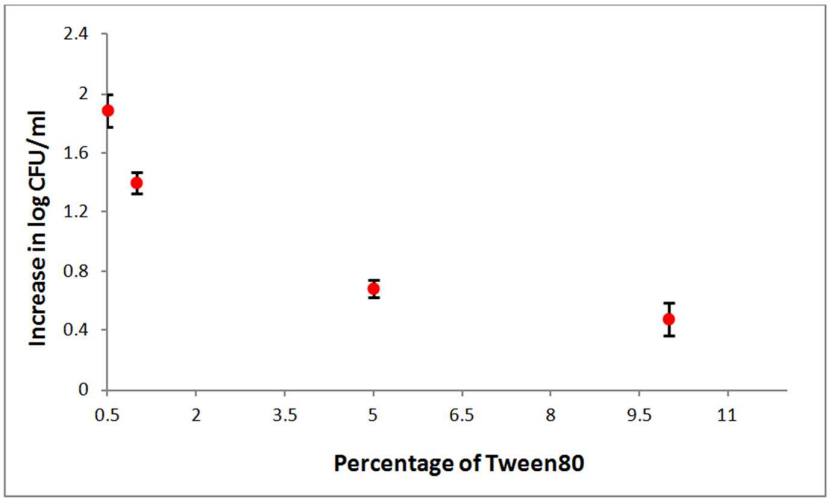

Figure 3. Amount of released bacteria in the outer W_2 phase of the $W_1/O/W_2$ emulsions after 1 hour incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with different inner W_1 phase volume percentage of 40% containing *E. coli*-GFP in the W_1 phase and stabilized with 0.5%, 1%, 5% or 10% Tween08 with 0.085 M NaCl in the W_2 phase. Bars represent mean ± SEM taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

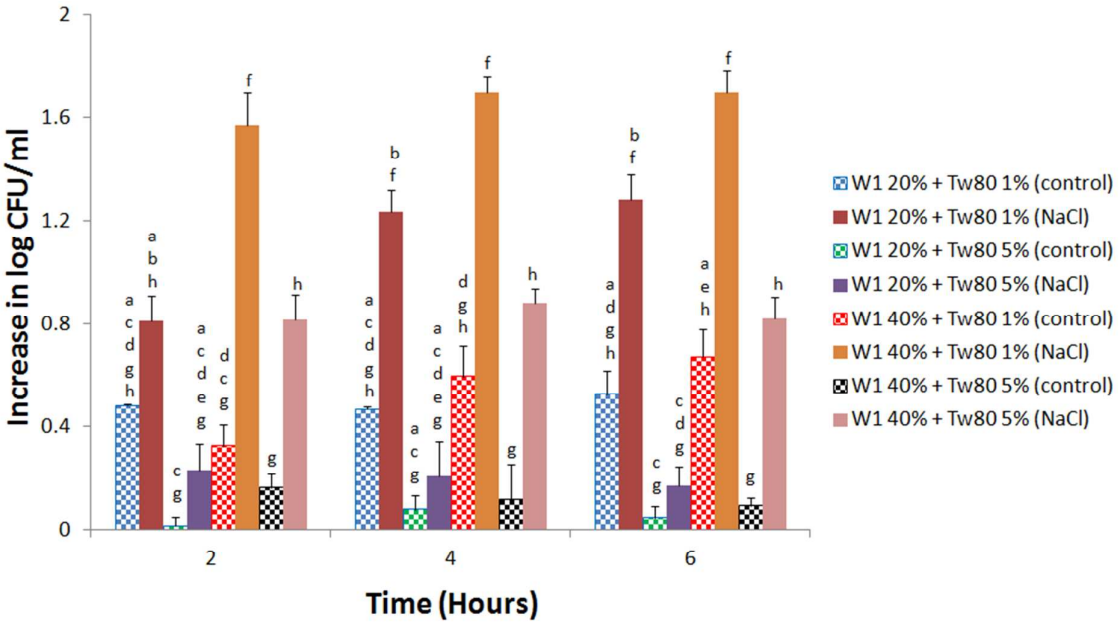

Figure 4. Amount of released bacteria in the outer W_2 phase of the $W_1/O/W_2$ emulsions after 2, 4 and 6 hours incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with different inner W_1 phase volume percentage of 20% or 40% containing *E. coli*-GFP in the W_1 phase and stabilized with 1% or 5% Tween08 with or without 0.085 M NaCl in W_2. 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.

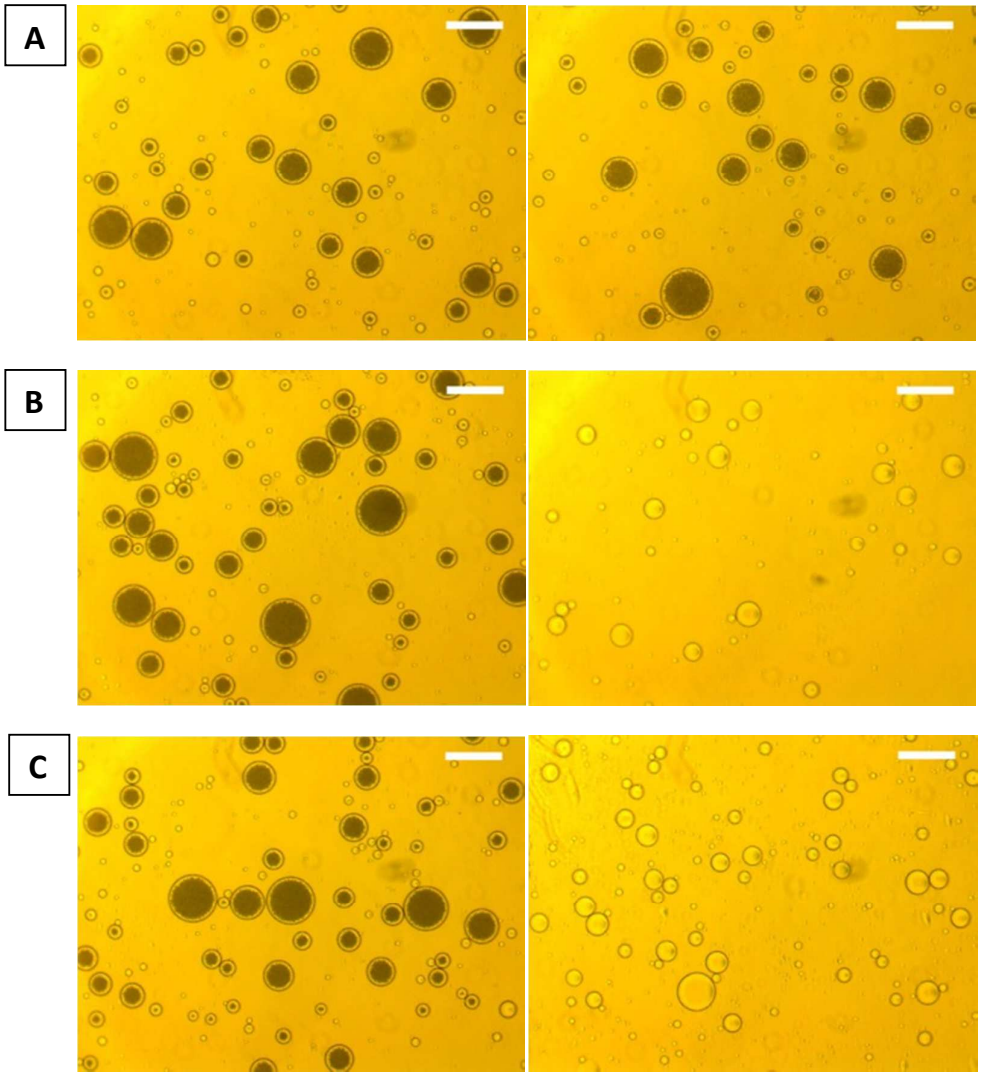

Figure 5. Optical microscopy images of $W_1/O/W_2$ emulsions at 0 (left) and 6 (right) hours. The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing *E. coli*-GFP and stabilized with 1% or 5% Tween80 with or without NaCl in W_2. The formulations were as follows: A) 20% W_1 and 1% Tween 80 with no NaCl in W_2; B) 20% W_1 and 1% Tween80 with 0.085 M NaCl in W_2; C) 20% W_1 and 5% Tween 80 with 0.085 M NaCl in W_2. Scale bar: 100μm.

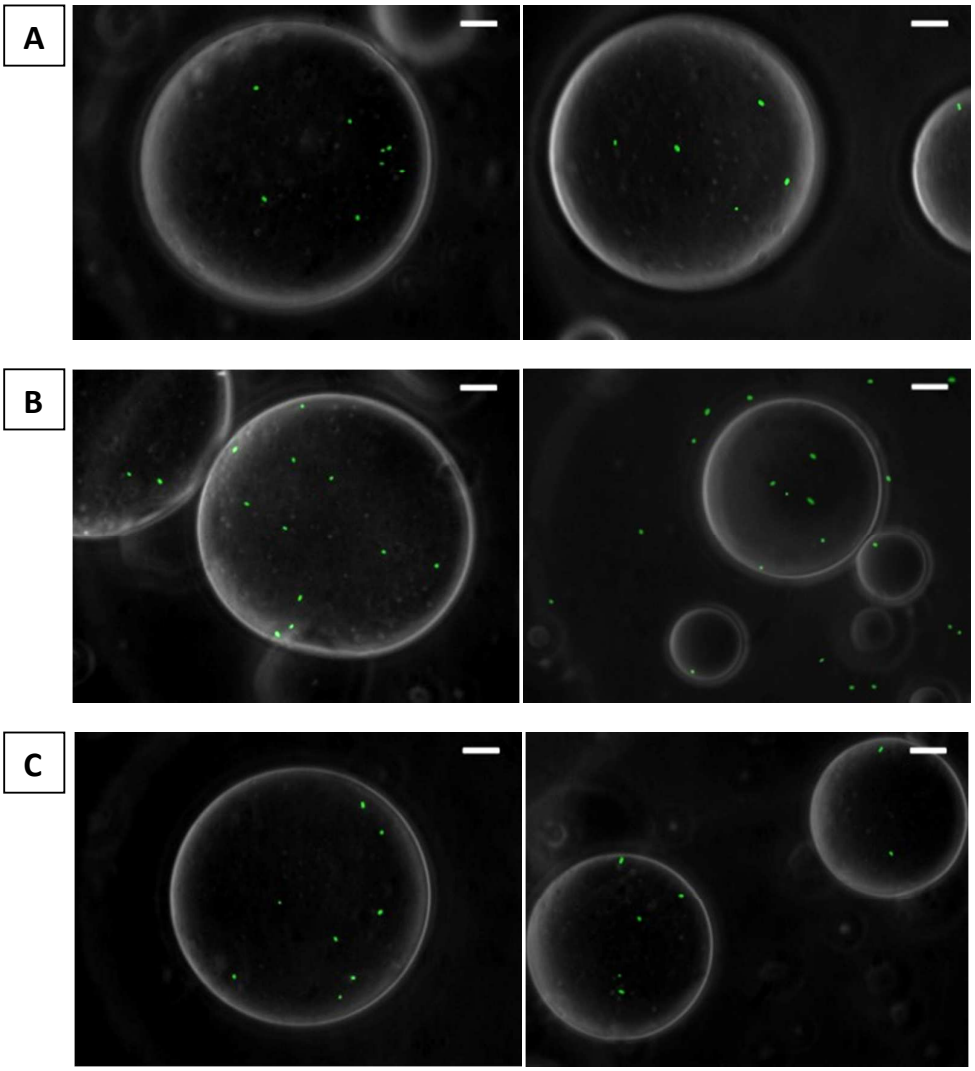

Figure 6. Photomicrographs composed from the optical and fluorescence images of *E.coli*-GFP within $W_1/O/W_2$ double emulsions at 0 (left) and 6 (right) hours. The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing bacteria and stabilized with 1% or 5% Tween80 with or without NaCl in W_2. The formulations were as follows: A) 20% W_1 and 1% Tween80 with no NaCl in W_2; B) 20% W_1 and 1% Tween80 with 0.085 M NaCl in W_2; C) 20% W_1 and 5% Tween80 with 0.085 M NaCl in W_2. W_1 droplets were not clearly visible under the microscope due to light being reflected from the surface of the oil globules. Scale bar: 10μm.

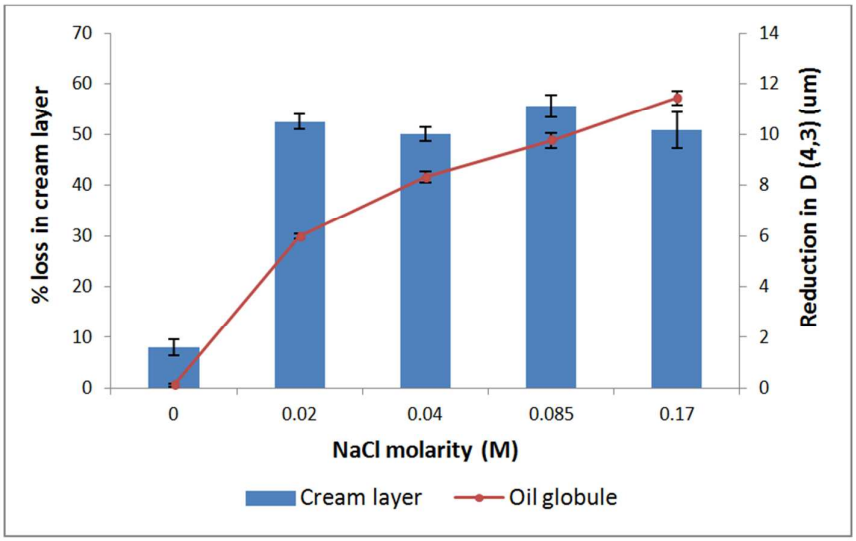

Figure 7. Reduction in mean oil globule diameter size (μm) and percentage loss in cream layer thickness of $W_1/O/W_2$ emulsions by light scattering [D (4, 3)] after 1 hour relative to hour 0 incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with 40% W_1 and stabilised with 1% Tween80 in the presence of bacteria without or with varying concentrations of NaCl (0.02 M, 0.04 M, 0.085 M or 0.17 M) in the W_2 phase. Results are taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

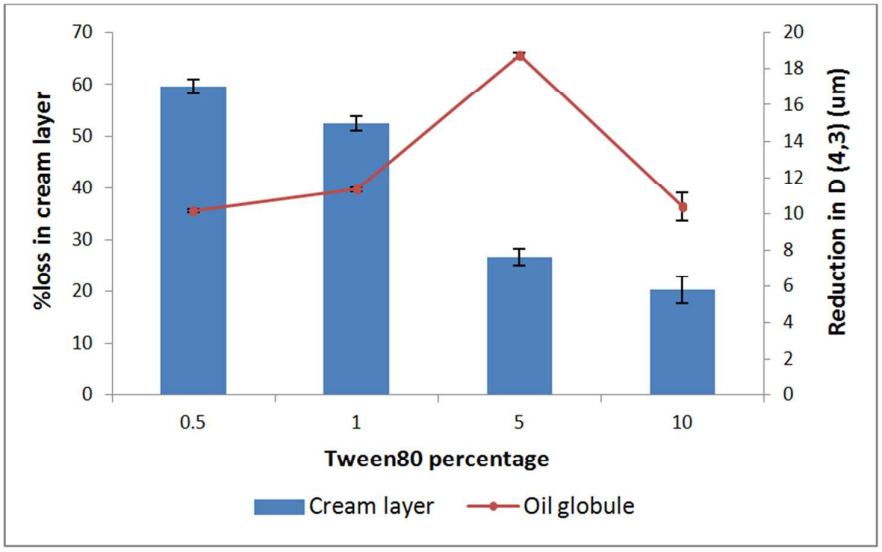

Figure 8. Reduction in mean oil globule diameter size (μm) and percentage loss in cream layer thickness of $W_1/O/W_2$ emulsions by light scattering [D (4, 3)] after 1 hour relative to hour 0 incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with 40% W_1 and stabilised with varying concentrations of Tween80 (0.5%, 1%, 5% or 10%) in the presence of bacteria with 0.085 M NaCl in the W_2 phase. Results are taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

Table 1. Change in mean oil globule diameter size (μm) of $W_1/O/W_2$ emulsions by light scattering [D (4, 3)] after 2, 4 and 6 hours relative to hour 0 incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with varying concentrations of Tween 80 and W_1 in the presence or absence of bacteria with or without 0.085 M NaCl in the W_2 phase. Results are taken from a minimum of 3 independent experiments.

	DE formulations	2 hours	4 hours	6 hours
No *E. coli*-GFP	20% W_1, 1% Tw80, no NaCl	1±0.98[a]	2.58±2.7[a]	2.23±1.7[a]
	20% W_1, 1% Tw80, 0.085 M NaCl	14.3±4.65[b]	16.7±4.26[b]	15.26±1.8[b]
	20% W_1, 5% Tw80, no NaCl	0.16±1.23[a]	1±1.25[a]	-0.03±1.3[a]
	20% W_1, 5% Tw80, 0.085 M NaCl	28.5±9.1[c]	28.5±9.3[c]	28.2±10.3[c]
	40% W_1, 1% Tw80, no NaCl	0.5±0.46[a]	0.7±0.59[a]	0.78±0.26[a]
	40% W_1, 1% Tw80, 0.085 M NaCl	22.7±1.35[c]	22.9±0.8[c]	25.3±0.8[c]
	40% W_1, 5% Tw80, no NaCl	1.2±0.6[a]	1.27±0.6[a]	1.99±1.14[a]
	40% W_1, 5% Tw80, 0.085 M NaCl	43.6±2.5[d]	44.2±2.93[d]	43.6±2.54[d]
With *E. coli*-GFP	20% W_1, 1% Tw80, no NaCl	2.4±1.26[a]	3.6±0.64[a]	2.55±2.2[a]
	20% W_1, 1% Tw80, 0.085 M NaCl	14.5±2.65[b]	18.9±3.1[b]	18.3±5.4[b]
	20% W_1, 5% Tw80, no NaCl	1.19±0.33[a]	1±0.39[a]	-1.77±3.3[a]
	20% W_1, 5% Tw80, 0.085 M NaCl	30.7±5.1[c]	30.8±4.3[c]	30.2±3.8[c]
	40% W_1, 1% Tw80, no NaCl	1.37±0.73[a]	1.77±0.96[a]	3.6±1.55[a]
	40% W_1, 1% Tw80, 0.085 M NaCl	21±1[c]	22.4±0.64[c]	24.8±1.4[c]
	40% W_1, 5% Tw80, no NaCl	0.13±0.17[a]	1±0.2[a]	1.1±0.37[a]
	40% W_1, 5% Tw80, 0.085 M NaCl	39.1±1.96[d]	41.6±2.35[d]	41±2.1[d]

The data was analysed with one-way ANOVA.

[a, b, c, d] means ± standard deviation with different letters are significantly different

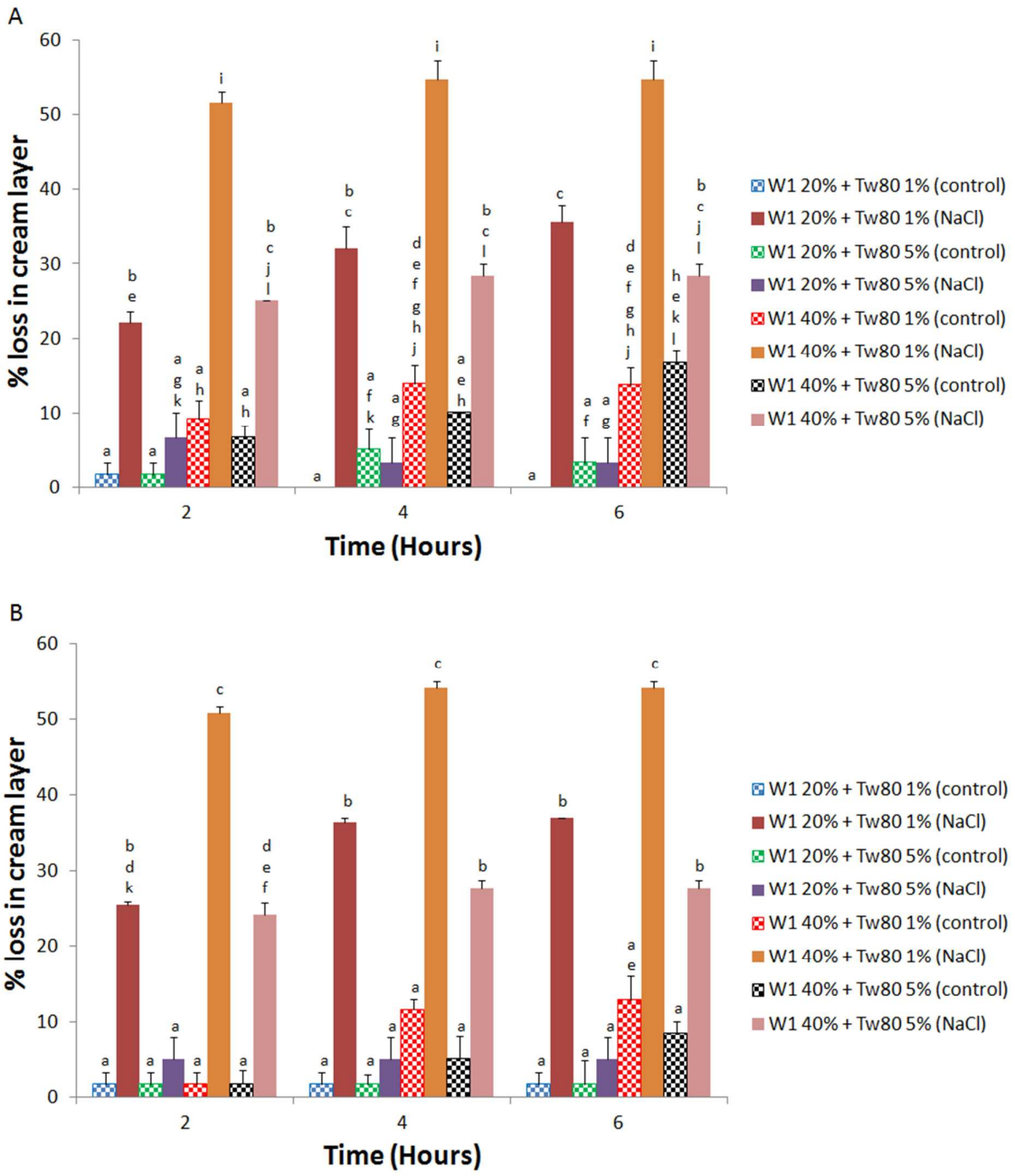

Figure 9. Percentage of cream layer loss of $W_1/O/W_2$ emulsions after 2, 4 and 6 hours incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with different inner-phase (W_1) volume percentage of 20% or 40% with (A) or without (B) *E. coli*-GFP in the W_1 phase and stabilized with 1% or 5% Tween80 with or without 0.085 M NaCl in the W_2 phase. 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.

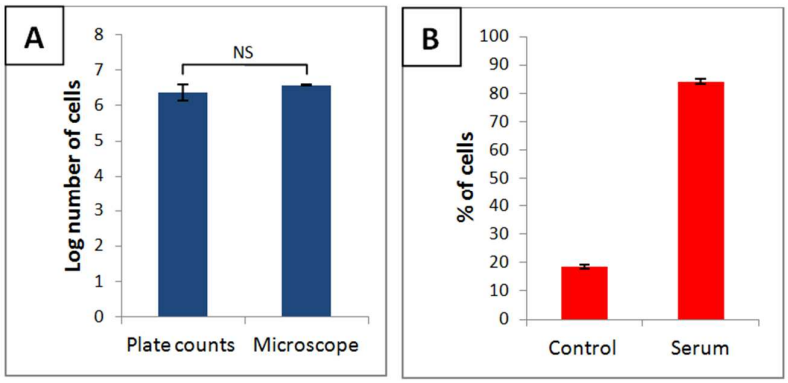

Figure 10. Log number of released *E. coli* cells in serum phase quantified by plate counting and microscopic enumeration (A) and percentage of PI positive *E. coli* cells in serum phase and control (B). The $W_1/O/W_2$ emulsion was made with 40% W_1 and stabilized with 1% Tween80 with 0.085 M NaCl in W_2. Bars represent mean ± SEM taken from a minimum of 3 independent experiments. The data was analysed with Student's *t*-Test.

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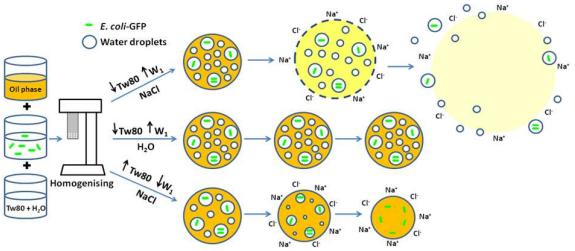
Graphical Abstract



Highlights

- The results suggest that release of bacteria from $W_1/O/W_2$ emulsion can be controlled by varying the formulation.
- Release occurs due to oil globule bursting independent to diffusion.